MOLECULAR STUDIES ON THE CONTROL OF THE EXPRESSION OF THE NPY-Y1 RECEPTOR GENE

A dissertation submitted for the degree of Doctor of Philosophy in the University of Glasgow

by

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October, 1997

PREFACE

This dissertation is the result of my own work and includes nothing which is the result of work done in collaboration.

Some of the work presented in chapter 3, 4, and 5 has been previously submit for publication.

Juan Carlos Bournat

October, 1997

Dedicated to my wife Carolina for all her love and support

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Professor Janet M. Allen, for providing me with the opportunity to learn from her experience, for her advice and support, without which this thesis would not have been possible.

I would like to acknowledge the University of Glasgow and the Overseas Research Scheme (ORS) for studentship support.

I would like to acknowledge to Professor Dan Larhammar and Dr. Ingrid Lundlell (Upssala University) for provide me with the rat NPY-Y1 promoter clone.

I would like to thank to Dr. Mark V. Rogers (Cellular Bioassay Design Group, Lead Discovery Unit, Glaxo-Wellcome) for provide me with a mutant PC12-*ras* negative cell line (PC12 asn17-w7).

I also wish to thank Dr. Robert Strang for his essential support. In addition, I also owe a great deal to Professor Lindsay for his help. Many thanks to Isabel MacGregor (Office of International Program) for her financial support.

To my friends and colleagues in the laboratory over the years, especially Terry for their superb technical support, Anne, Paddy, Alirio, Joe, Ian, David, Margaret, Emma: heaps to thank for rallying round. Your great company and collective wisdom have helped to make this time enjoyable as productive, both in and outwith the lab.

I am very grateful to my family for their very real support and encouragement throughout the various degrees, and everything else, both past and present.

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ABBREVIATIONS

A	adenosine
AA	arachidonic acid
ACh	acetylcholine
ADP	adenosine diphosphate
AP-1	activation protein 1
AP-2	activation protein 2
aPKC	atypical PKC
ATG	methionine codon
ATP	adenosine 5'-triphosphate
BDNF	brain derived neurotrophic factor
bp	base pair
BSA	bovine serum albumin
C	cytosine
°C	degree celsius
Ca^{++}	calcium
CaLB	calcium binding site
CaM	calcium/calmodulin
CaMK II	calcium/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
CAT	chloramphenicol acetyl transferase
CBP	CREB binding protein
CC	calphostin C
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
CHO	chinese hamster ovary
Ci	Curie
CNS	central nervous system
cPKC	conventional PKC
cpm	counts per minute
CRE	cAMP response element
CREB	cAMP responsive binding element protein
Csk	carboxy terminal *src* kinase
Da	Dalton

DA	dopamine
DAG	diacylglycerol
DBC	dybutiryl cyclic AMP
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DRG	dorsal root ganglion
DTT	dithiothreitol
EC_{50}	enzyme concentration
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ERE	estrogen receptor element
ERKs	extracellular regulated kinases
FGF	fibroblast growth factor
FFAs	cis unsatured fatty acid
G	guanosine
g	gram
g	gravitational field
GABA	gamma-aminobutyric acid
GAP	GTPase activating protein
GAP-43	growth associated protein-43
GAPs	GTPase activating proteins
GDIs	GDP dissociation inhibitors
GDP	guanosin diphosphate
GNRF	guanine nucleotide releasing factors
GPCRs	G protein coupled receptor
GRE	glucocorticoid responsive element
GSK-3	glycogen synthase kinase-3
GTP	guanosine triphosphate
GTPase	guanosine triphosphate phosphatase
GRB-2	growth factor receptor bound protein-2
h	hour
H-89	N-[2-((3-(4-bromophenyl)-2-propenyl)-amino)-ethyl] isoquinolinesulfonamide
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IC_{50}	inhibitory concentration
ICV	intracerebroventricular

IEG	immediate early gene
IP_3	1,4,5-inositol trisphosphate
i.u.	international unit
K^+	potassium
kbp	kilobase pair(s)
KCl	potassium chloride
K_D	dissociation constant
KDa	kilodaltons
K_m	Michaelis constant
l	litre
LDCV	large dense core vesicles
LPA	lyso-phosphatidic acid
LRE	late responsive gene
M	molar
m	meter
MAP	microtubule associated protein
MAPK	mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MEK	MAPK or ERK kinase
MEKK	MAPK or ERK kinase kinase
mM	millimolar
mol	mole
MOPS	morpholinepropanesulfonic acid
mRNA	messenger ribonucleic acid
MWM	molecular weight markers
Na^{++}	sodium
NA	noradrenaline
NGF	nerve growth factor
nPKC	novel PKC
NPY	neuropeptide Y
NPY-Y1	Y1 neuropeptide receptor
OPR	open reading frame
$p75^{NTR}$	low affinity NGF receptor
$p140^{trk}$	high affinity NGF receptor
$p21^{ras}$	ras protein

$p90^{rsk}$	ribosomal S6 kinase
PA	phosphatidic acid
PACAP	Pituitary adenylyl cyclase activating peptide
PACAP-IR	PACAP immunoreactivity
PAGE	polyacrilamide gel electrophoresis
PBS	phosphate buffered saline
PC	phosphatidyl choline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PH	pleckstrin homology domain
PI	phosphatidyl inositol
PIP	phosphatidyl inositol 4 phosphate
PIP2	phosphatidyl inositol 4,5 bisphosphate
PI-PLC	phosphatidyl inositol-phospholipase C
PI-3-K	phosphatidyl inositol-3 kinase
PKA	cyclic AMP dependent protein kinase
PKB	protein kinase B
PKC	calcium-phospholipid dependent protein kinase
PLA_2	phospholipase A_2
PLC	phospholipase C
PLC-γ1	phospholipase C-γ1
PMA	phorbol-12-myristate-13 acetate
PNMT	phenylethanolamine-N-methyltransferase
PNS	peripheral nervous system
PP	pancreatic polypeptide
PPH	phosphatidic acid phosphohydrolase
PS	phosphatidyl serine
PTB	protein tyrosine binding domains
PTKs	protein tyrosine kinases
PTyr	phosphotyrosine
PTX	pertussis toxin
PVN	paraventricular nucleus
PYK-2	proline-rich tyrosine kinase 2
PYY	peptide YY
RACK	receptor activated PKC
Raf	raf kinase

RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
RTKs	receptor tyrosine kinases
RT-PCR	reverse transcription polymerase chain reaction
s	second
S	svedberg units
SA	sympatho-adrenal
SCG	superior cervical ganglion
SDS	sodium dodecyl sulfate
shc	src homology collagen
SH2	src homology 2 domain
SH3	src homology 3 domain
SIF	small intensely fluorescent cells
S1P	spyngosine-1-phosphate
SR	sarcoplasmic reticulum
SRE	serum responsive element
SRF	serum responsive factor
SSC	sodium chloride sodium citrate buffer
SSV	Small synaptic vesicles
T	thymidine
TCF	ternary complex factor
T_D	(50%) dissociation temperature
TE	Tris/EDTA buffer
TEMED	N,N,N',N'- tetramethylethyl/enediamine
TGF	transforming growth factor
TH	tyrosine hydroxylase
TM	transmembrane
TnC	troponin C
TNF	tumor necrosis factor
TPA	12-0-tetradecanoylphorbol-13-acetate
TRE	TPA response element
Tris	tris(hydroxymethyl)aminomethane
Trk	tropomyosin receptor kinase
tRNA	transfer RNA
UT	untranslated

UV	ultraviolet
V	volts
VIP	vasoactive intestinal peptide
VIP-IR	VIP-like immunoreactivity
Vmax	maximal rate
v/v	volume per volume
w/v	weight per volume

ABSTRACT

MOLECULAR STUDIES ON THE CONTROL OF THE EXPRESSION OF THE NPY-Y1 RECEPTOR GENE

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The cDNA that encodes for the rat NPY-Y1 receptor has been reported (Eva at al., 1990) and predicts a classical G-protein coupled receptor. This receptor has been linked to important physiological process in the nervous system (Grundemar and Hakanson, 1994).

In this thesis the gene that encodes for the promoter of the rat NPY-Y1 receptor was obtained by PCR subcloning.

(i) This region contains several consensus sequences for transcription factors: a "TATA-like" box, a "CAAT-like" box, an Sp1 site, an AP1 site, two CRE elements, a non-palindromic ERE and four non-palindromic GRE.

(ii) This region has been attached to the reporter function, luciferase, so that the expression of this enzyme has been placed under the control of the promoter region of the NPY-Y1 receptor (pY1-LUC).

(iii) The pY1-LUC has been transiently transfected into PC12 cells, a rat phaeochromocytoma cell line (Greene and Tischler, 1976), GT1-7 cells, a mouse hypothalamic cell line (Mellon et al., 1990) and RINm5f cells, a rat β-pancreatic cell line (Pollak et al., 1993) which express constitutive levels of the NPY-Y1 receptor. It is thus possible to measure the basal transcriptional activity of this gene and study the factors which affect its transcriptional levels in these cells.

(iv) Differentiation of the cells into an adrenal chromaffin-like phenotype with dexamethasone and in a neuronal-like phenotype with NGF or PACAP increased the transcriptional activity of the NPY-Y1 gene in PC12 cells. Moreover, activation of PKA with DBC or forskolin, and activation of PKC with DH1 or PMA also increased the expression of this gene in PC12 cells.

(v) The transcriptional response to NGF was dependent on *trk* A receptor and PKC activation but independent of *ras* -MAPK activation. Moreover, the effect of PACAP-38 was dependent in both PKA and PKC activation, but is also independent in *ras* -MAPK activation.

Thus, the transcription of the NPY-Y1 receptor its under tight regulation by the glucocorticoid receptor, PKA and PKC signalling in a sympatho-adrenal model system, PC12 cells.

CHAPTER 1

GENERAL INTRODUCTION

1.1 THE NEURAL CREST CELLS

Generation of neuronal diversity in the nervous system remains one of the most complex and compelling puzzles in developmental biology. This neural development programme involves the co-ordination of numerous molecular events including cell birth and differentiation, cell migration and survival, direct neurite (axon and dendrite) outgrowth and growth cone targeting and finally synaptogenesis and selective cell death (Nichols, 1994). Differentiation is a complex process that begins early in embryonic development with the determination of cells in the ectoderm to become neuronal precursors; thus the cells begin to divide (proliferation stage) and then migrate after the neuronal cells have become post-mitotic. Neuronal differentiation culminates in the acquisition of a specific morphology and function that distinguishes this specific cell type (Nichols, 1994).

One of the best characterised systems of neural differentiation and development is the neural crest and its derivatives (Landis and Patterson, 1981; Anderson, 1989; Sieber-Blum, 1990). Epithelial cells that line the wall of the neural tube, the neuroepithelium, generate all the neurons and glial cells of the central nervous system (CNS) and peripheral nervous system (PNS). Other cells within this region give rise to a specialised group of migratory cells called the neural crest. Neural crest cells emerge from the dorsal region of the neural tube and most of these cells migrate to many peripheral locations where they coalesce. The ultimate result of neural crest cell migration and differentiation is a surprising variety of cell types which includes neurons and cells of the autonomic and sensory nervous system.

The ultimate fate of these cells is critically controlled by the local environment through which they migrate. Restrictions in cell fate appear to occur during the process of cell migration since the developmental options of neural crest cells appear to be gradually restricted by changes in cellular environment as they migrate in the periphery of neural crest cells, cells of the sympatho-adrenal (SA) lineage, give rise to the sympathetic nervous system and the adrenal medulla (Anderson, 1993; Bronner-Fraser, 1994). This lineage comprises the major catecholaminergic descendents of the neural crest which are the sympathetic neurons, chromaffin cells and another group of cells found in sympathetic ganglia known as small intensely fluorescent (SIF) cells (Anderson, 1993). Precursors of these distinct cell classes can be isolated from the embryonic adrenal medulla or sympathetic ganglia. When grown in culture, it is possible to control the fate of these immature cells by varying culture conditions. It has been demonstrated that the differentiation of adrenal progenitor cells into chromaffin cells is dependent upon the presence of glucocorticoid hormones; indeed, these cells are exposed to high levels of such hormones upon migration to the adrenal gland since adrenal cortex synthesises high levels of glucocorticoids.

Neural crest cells destined to form sympathetic ganglia can become either: (i) SIF cells, which process may depend on the availability of low levels of glucocorticoids; or (ii) sympathetic neurons, which have been demonstrated to require nerve growth factor (NGF). It has also been shown that as progenitor cells differentiate into sympathetic neurons, they gradually lose sensitivity to glucocorticoids and acquire dependence on NGF for survival. Evidence thus suggests that a pluripotent SA progenitor exists which is capable of differentiation to a variety of cell types and that the nature of the differentiation response is dependent on local environmental signals (Anderson, 1993).

Adrenal chromaffin cells are considered to be following stimulation, a modified form of postganglionic sympathetic neuron. Chromaffin cells release their transmitter stores to the circulation while postganglionic sympathetic neurons release transmitter locally to sympathetically-innervated target organs. Both cell types are innervated by cholinergic preganglionic sympathetic cells. Furthermore, these two cell types are both catecholaminergic (Langley and Grant, 1995). Thus, even though chromaffin cells exhibit a distinct morphology from sympathetic cells the common heritage of chromaffin cells and sympathetic neurons is reflected in certain functional similarities in these cell types. In addition, precursors of the SA lineage express peptidergic potential and this expression is more prolific, in terms of amount and diversity of peptides, than in the adult. Both neuropeptide (NPY) and vasoactive intestinal peptide (VIP) are expressed in embryonic superior cervical ganglion (SCG) (Tyrrell and Landis, 1994).

1.2 PC12 CELLS

PC12 is a clonal cell line established from a solid tumour of an adrenal gland (phaeochromocytoma) of an irradiated New England Deaconess Hospital white rat by subcutaneous passage (Warren and Chute, 1972; DeLellis et al., 1973). Under resting conditions, PC12 cells proliferate and display a number of ultrastructural and biochemical characteristics of immature adrenal medullary chromaffin cells which constitute their non-neoplasic counterpart (Tischler and Greene, 1975; Greene and Tischler, 1976). They have morphological features that more closely resemble those of chromaffin cells found in the late embryonic rat adrenal glands than those of adults. The cells have a round shape, tend to clump and protrude short processes. PC12 cells synthesise catecholamines, and stores them in both large dense core vesicles (LDCVs) and small synaptic-like vesicles (SSVs) (Greene and Rein, 1977a). PC12 cells release both dopamine (DA) and noradrenaline (NA) in a Ca^{++}-dependent mechanism in response to depolarising concentrations of K^{+} or to nicotinic cholinergic stimulation and in response to veratridine, a voltage-gated Na^{++} channel activator (Greene and Rein, 1977a; Green and Rein, 1978; Ritchie, 1979).

PC12 cells, unlike many normal chromaffin cells or other phaeochromocytomas, lack phenylethanolamine-N-methyl transferase (PNMT) and thus do not synthesise adrenaline, and cannot be induced to do so by high concentrations of corticosteroids. Chromaffin cells also contain large amounts of opiate-like peptides but PC12 cells do not (Guroff, 1985). Another characteristic of PC12 cells which makes them different from normal chromaffin cells is that they have a saturable, high affinity uptake mechanism for noradrenaline (NA) which is characteristic of sympathetic neurons.

PC12 cells have biochemical markers not found in chromaffin cells or other phaeochromocytomas such as acetylcholine (ACh), endorphins, enkephalin, γ-aminobutyric acid (GABA), somatostatin, substance P and receptors for ACh, substance P and GABA (Greene and Rein, 1977b). Intracellular recordings has shown that PC12 cells cannot generate action potentials in response to chemical or electrical stimulation in the absence of NGF, although they do possess voltage-gated Na^{++} channels (Dichter et al., 1977). Both chromaffin cells and phaeochromocytomas are excitable in the absence of NGF (Greene and Tischler, 1982).

1.3 NERVE GROWTH FACTOR (NGF)

Growth factors in the nervous system include secreted and cell-bound proteins as well as steroid hormones. The established action of growth factors in the developing and adult nervous system is as retrograde, or target-derived signals (Curtis and DiStefano, 1994). Apart from their classical growth and survival activities, these factors are also used by neuronal targets to regulate the type and number of neurons that innervate them (Patterson and Nawa, 1993). Influences on phenotype include the nature of neurotransmitter and neuropeptide produced and the synaptic input received by the dendrites of the neuron (Landis, 1990). Differentiation factors can affect neuronal gene expression as well as neuronal phenotype without changing neuronal growth or survival. Thus, depending on the responsive cell population, the same factor can act both as a differentiation factor or as a growth factor (Landis, 1990; Patterson and Nawa, 1993).

NGF was the first neurotrophic agent to be identified and serves as the prototype for this class of polypeptide growth factors (Thoenen and Barde, 1980; Thoenen and Edgar, 1985; Thoenen, 1991). NGF is required for the maintenance of specific neuronal cell types, such as sympathetic postganglionic neurons. Response of explanted sympathetic neurons to this target-derived agent includes anabolic alterations, induction of neuronal morphology and increased survival of the cells (Levi-Montalcini et al., 1987). Thus, markers of sympathetic neuronal differentiation are initiated by NGF.

The biological activity of NGF can be divided into three: (i) trophic, (ii) tropic and (iii) differentiative. In relation to the trophic actions, *in vivo* experiments have revealed that developing sensory and sympathetic neurones depend on NGF for their survival. Sympathetic neurons continue to depend on NGF during maturation, whereas sensory neurons do not, although they seem to require NGF for normal biochemical and morphological homeostasis (Johnson et al., 1986). It has also been established that certain central neurons also respond to NGF, in particular the basal forebrain cholinergic system of rodent and human brains (Hefti et al., 1986; Whittemore and Seiger, 1987). The tropic role of NGF is exemplified by the induction of neuronal sprouting of NGF-responsive neurons towards a source of NGF in culture (Gundersen and Barrett, 1979).

NGF is the best characterised member of a family of "neurotrophins" that includes brain derived neurotrophic factor (BDNF) (Barde et al., 1982; Barde, 1988; 1989), neurotrophin-3 (NT-3) (Maisonpierre et al., 1990; Rosenthal et. al, 1990), neurotrophin-4/5 (NT-4/5) (Berkemeir et al., 1991; Hallbook et al., 1991) and neurotrophin-6 (NT-6) (McDonald and Chao, 1995). These molecules promote the differentiation, growth and survival of discrete, and sometimes overlapping neuronal cells through specific receptor tyrosine kinases (RTKs) similar to those used by "traditional" growth factors such as Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF) and Platelet-derived Growth Factor (PDGF).

Neurotrophin receptors are encoded by a gene family, designated *trk* (tropomyosin receptor kinase), which includes three related loci: *trk A*, *trk B* and *trk C* (Chao, 1992 a,b; Ebendal, 1992; Roback et al., 1992; Chao and Hempstead, 1995). Neurotrophins are synthesised and released from target organs of neurotrophin-responsive nerve cells and exert their actions by binding to specific RTKs on the cell surface of responsive cells which lead to activation of specific intracellular signalling mechanisms (See NGF signalling pathway) and internalisation of the neurotrophin/receptor complex (desensitisation) and retrograde transport to the neuronal cell body.

NGF is required for differentiation and survival of sympathetic neurons and some sensory neurons in PNS and of selective basal forebrain cholinergic neurons in CNS. It also acts on cells of the adrenal medulla and on a number of tumour lines (Barde, 1988; 1989). Other cytokines known to influence neuronal gene expression include insulin and insulin-like growth factors (IGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and transforming growth factor (TGF) (Walicke, 1989; Patterson and Nawa, 1993; Silos-Santiago et al., 1995).

1.3.1 NGF BIOCHEMISTRY

A major contribution in the route to study NGF was the discovery that submaxillary glands of the male mouse produce and secrete large amounts of NGF (Cohen, 1960). The presence of large amounts of NGF in these tissues provided the basis for its purification and determination of amino acid sequence. Antibodies able to block biological activity were also developed using NGF from mouse submaxillary glands (Angeletti et al., 1973).

NGF can be isolated as a 7 S, high-molecular weight (130 kDa) complex consisting of three subunits: α, β and γ (Thoenen and Barde, 1980; Fahnestock, 1991; Bradshaw et al., 1993). a and g subunits are thought to be accessory proteins not responsible for the growth-promoting actions of NGF while the 2.5S β subunit has been demonstrated to confer the neurotrophic responses associated with NGF in responsive cells.

The primary structure of NGF shows a high degree of conservation among different species. β–NGF is synthesised as a glycosylated precursor which is processed at amino- and carboxy-terminals to the mature protein of molecular weight 13.2 kDa. 2.5S β-NGF is present in the 7S complex as a noncovalently linked dimer of molecular weight 26 kDa. This dimer is composed of two identical units each of 118 amino acids; both monomers and dimers are biologically active. Three intrachain disulphide bonds are present in each monomer and disruption of these bonds completely abolishes biological activity of NGF. The crystal structure of NGF has been resolved to 2.3 angstroms (McDonald et al., 1991). The protein function is a dimer which each monomer is a 130 kDa polypeptide complex containing three subunits (α, β, γ) stabilised by zinc ions (Bradshaw et al., 1993).

1.3.2 NGF RECEPTORS

The distribution of specific binding sites for NGF defines its responsiveness in the target tissues (Sutter et al., 1991; Meakin and Shooter, 1992; Raffioni and Bradshaw, 1993; Barbacid, 1995). In PNS, NGF can bind to specific surface receptors of sympathetic neurons and subsets of dorsal root ganglion (DRG) cells, the latter being a subgroup of sensory neurons. NGF receptors are also found on neural cell precursors such as chromaffin cell precursors, neuroepithelial stem cells and sensory ganglia precursor cells. NGF receptors are also present in neuroblastoma cells and pheochromocytoma cells, such as PC12 cells.

When NGF binds to its receptor on the cell membrane of the target cells, it triggers several signal transduction mechanisms involved in coordinating the multiple changes which lead to the response of PC12 cells to NGF. NGF interacts with two cell surface receptors in PC12 cells

and primary neuron cultures : (i) $p75^{NTR}$, a low affinity receptor (Kd = 10^{-8}-10^{-9} M), is a cell surface glycoprotein of 75-80 kDa with a single cytoplasmic domain and lacks structural features known to mediate signal transduction in other receptors (Berg et al., 1991a). (ii) $p140^{trk}$, a high affinity receptor (Kd = 10^{-10}-10^{-11} M), is a tyrosine kinase receptor which either alone or in combination with $p75^{NTR}$ binds NGF. This high affinity receptor mediates most of the known effects of NGF and is the product of the *trk A* proto-oncogene ($p140^{trk}$) (Hempstead et al., 1991; Klein et al., 1991a; Martin-Zanca et al., 1991).

1.3.2.1 LOW AFFINITY RECEPTOR ($p75^{NTR}$)

The $p75^{NTR}$ low affinity receptor is a type I integral membrane protein of 400 amino acids comprising a single transmembrane spanning region with four negatively-charged, cysteine-rich extracellular regions and a unique cytoplasmic domain which is conserved between species; a G protein binding site has been identified (Johnson et al., 1986; Sutter et al., 1991; Meakin and Shooter, 1992). $p75^{NTR}$ is a member of a superfamily of cell surface proteins which includes the receptor for tumour necrosis factor (TNF). Interestingly, some of these receptors mediate cell death, suggesting that $p75^{NTR}$ may play a role in determining neuronal cell survival. The $p75^{NTR}$ has a wide distribution compared with *trk* receptors and is expressed on a variety of cell types including Schwann cells, motor neurons and cerebellar Purkinje cells (Meakin and Shooter, 1992).

In contrast to the rather restricted binding of neurotrophins to their respective *trk* receptor, all neurotrophins bind this low affinity receptor ($p75^{NTR}$). This receptor has been implicated in phospholipase A_2 (PLA_2) activation (MacEwan, 1996) which itself is linked to the activation of the sphingomyelin cycle which results in the production of ceramides (Dobrowsky et al., 1995). Thus, $p75^{NTR}$ has been suggested to play a role in apoptosis since ceramides have been implicated in this process (Chao, 1995) and introduction of $p75^{NTR}$ into immortalised neuronal cells induces this process (Rabizadeh et al., 1993).

1.3.2.2 HIGH AFFINITY RECEPTOR ($p140^{trk}$)

The $p140^{trk}$ receptor shares a number of features with members of the RTKs family; they have an extracellular binding domain, a transmembrane domain and a cytoplasmic tail with tyrosine kinase activity. The $p140^{trk}$ receptor consists of 790 aminoacids, has a molecular weight of 135 kDa and is heavily glycosylated. Immunoglobulin G-C2 (IgG-C2) domains, and leucine/cysteine-rich repeats have been identified in the extracellular domain and the cytoplasmatic tail has a consensus tyrosine kinase domain (Sutter et al., 1991; Meakin and Shooter, 1992).

Modification of Lys-32 residue on the β-NGF protein together with either Lys-34 or Glu-35 results in loss of binding to $p75^{NTR}$ without affecting binding to $p140^{trk}$ (Ibañez et al., 1992). This modified form of NGF is still able to bind $p140^{trk}$ with high affinity and retain biological activity as determined by neurite outgrowth in explanted chick embryonic sympathetic ganglia. Others have reported evidence that expression of $p140^{trk}$ is sufficient to mediate mitogenic properties of NGF in heterologous cell systems, such as mouse fibroblast NIH 3T3 cells line (Cordon-Cardo et al., 1991). Thus $p140^{trk}$ interacts with overlapping but distinct populations of intracellular target proteins in different cell types to produce proliferative or differentiative responses (Hempstead et al., 1992).

The $p140^{trk}$ receptor mediates NGF-responsiveness in neuroblastoma and PC12 cells (Kaplan et al., 1991a,b ; Klein et al., 1991). Overexpression of $p140^{trk}$ accelerates NGF-induced differentiation pathways in PC12 cells, including enhanced NGF-dependent $p140^{trk}$ autophosphorylation and phosphorylation of phospholipase C-γ1 (PLC-γ1), phosphoinositol-3 kinase (PI-3-K) and mitogen-activated protein kinase 1 (MAPK-1) (Hempstead et al., 1992; Saltiel and Decker, 1994; Stephens et al., 1994). The PC12-nnr5 cell subline, which express $p75^{NTR}$, but lacks measurable levels of $p140^{trk}$ receptor, fails to respond to physiological levels of NGF but introduction of $p140^{trk}$ receptor into these cells restores the NGF-dependent induction of neurite outgrowth (Loeb and Greene, 1993).

The $p140^{trk}$ high-affinity NGF receptor (*trkA*) belongs to a family of tyrosine kinase receptors which includes *trk B* (Klein et al., 1989, 1991b, 1992) and *trk C* (Lamballe et al., 1991) receptors. These *trk* receptors, however, are the products of separate genes and interact with specific neurotrophins (Chao, 1992 a, b; Ebendal, 1992; Roback et al., 1992; Chao and Hempstead, 1995). The *trk A* receptor preferentially interacts with NGF, the *trk B* receptor interacts with BDNF and NT-4/5, and *trk C* receptor interacts with NT-3.

The identification of $p140^{trk}$ as an NGF receptor was made after it was noted that the distribution of $p140^{trk}$, first identified as an oncogene fusion protein with tropomyosin (tropomyosin receptor kinase or *trk*) was restricted to a targets of NGF action in neural crest-derived sensory neurons (Martin-Zanca et al., 1990). Indeed, $p140^{trk}$ receptor proved to be an high affinity NGF receptor whose tyrosine kinase activity and autophosphorylation is stimulated upon binding of NGF. *Trk A* receptor expression is restricted during development to those cell types defined to be NGF-responsive, including sensory and sympathetic neurons in PNS and a subsets of cholinergic neurons of the basal forebrain of CNS (Martin-Zanca et al., 1990). PC12 cells do not respond to BDNF and NT-3 because they lack *trk B* and *trk C* receptors.

1.3.3 NGF ACTIONS ON NEURAL CREST CELLS

1.3.3.1 NGF ACTIONS ON ADRENAL CHROMAFFIN CELLS

Adrenal chromaffin cells, from which PC12 cells derive, develop from neural crest cells that migrate ventrally from the apex of the neural tube to the dorsal aorta (Anderson, 1989, 1993). This SA cell type gives rise to sympathetic neurons, SIF cells and chromaffin cells. SA progenitor cells that migrate to the adrenal gland encounter high concentration of glucocorticoids, hormones which are synthesised in the adrenal cortex. The high level of glucocorticoids induce the SA cells to adopt a chromaffin phenotype by two different mechanisms. First, glucocorticoids upregulate genes whose products are abundant in chromaffin cells such as PNMT (Bohn et al., 1981) and second, inhibit neuronal differentiation induced by NGF (Unsicker et al., 1978; Anderson, 1993). This inhibition is reciprocal as NGF can inhibit the ability of glucocorticoids to upregulate PNMT (Stein et al., 1988). This antagonism probably reflects events related to the differentiation process of cells from the SA lineage (Seeley et al., 1984).

Adrenal chromaffin cells are responsive to NGF at a defined stage in their ontology which is distinct from that of sympathetic neurons (Anderson, 1993). Neural crest cells retain plasticity as late as the neonatal stage, as shown by the ability of NGF to transform neonatal chromaffin cells into sympathetic neurons (Aloe and Levi-Montelcini, 1979; Douple et al., 1985). This NGF dependent conversion is antagonised by dexamethasone, a synthetic glucocorticoid. Adrenal chromaffin cells appear to respond to NGF in two temporal components (Lillien and Claude, 1985; Tischler et al., 1993). Early effects include an increase in proliferation and neuritogenesis. More delayed effects, seen after two weeks of NGF treatment include withdrawal from the mitotic cell cycle and development of more mature neuronal cell characteristics, as seen in NGF-treated PC12 and sympathetic neurons. Functional aspects modified upon treatment with NGF of adrenal chromaffin cells include the induction of tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) activities (Acheson et al., 1984; Acheson and Thoenen, 1987).

1.3.3.2 NGF ACTIONS ON SYMPATHETIC NEURONS

NGF effects are restricted to specific cell types in CNS and PNS (Barde, 1989; Martin-Zanca et al., 1990). Primary sensory neurons are well known to be NGF-responsive. Recent evidence supports a role for NGF in CNS in a subset of cholinergic neurons of the forebrain. NGF has also a profound and universal influence on sympathetic neurons.

NGF acts as a survival factor for specific neurons during defined periods of their ontogenesis; the administration of NGF antibodies in the early post-natal period leads to the complete destruction of sympathetic ganglia (Levi-Montalcini and Angeletti, 1968). As a corollary, it has been demonstrated that exogenous NGF can reduce the level of naturally-occurring neuronal death in sympathetic ganglia (Oppenheim et al., 1982). That existing developing neurons compete with each other for NGF for their survival is a generally accepted aspect in the model of nervous system development. This hypothesis is supported by the fact that NGF mRNA exists in target tissues of sympathetic neurons at very low and therefore limiting levels. In addition, a link between levels of NGF and density of sympathetic innervation, has been demonstrated (Barde, 1989).

NGF is thus described as a target-derived molecule that controls neuronal survival at the time of target innervation (Shelton and Reichardt, 1984; Heumann and Schwab, 1985). Recent data from both NGF and NGF receptor knockout mice confirm previous findings (Smeyne et al., 1994; Snider, 1994). Both knockout phenotypes have dramatically depleted populations of DRG neurons by birth and both have virtually absent sympathetic ganglia by 10 days of age; most animals die within the first month of life.

In addition to effects on survival and general growth of sympathetic neurons, NGF influences differentiation of these cells at both morphological and functional levels during defined periods of ontogenesis (Thoenen and Barde, 1980). Morphological effects include: (i) enhanced transformation of neuroblast into differentiated neurons; (ii) heightened development of Golgi cisternae; (iii) enhanced formation and ramification of neurofilaments and microtubules; and (iv) promotion of fibre elongation as well as directional effects on neurite growth.

Differentiation and development of the sympathetic nervous system by NGF has been extensively studied using a variety of cellular models. *In vivo* , NGF is an absolute requirement for survival of sympathetic neurons. Primary culture of sympathetic neurons has allowed for the characterisation of many properties and response of these cells. Significant inherent disadvantages, however, in their use in dissecting the mechanism of NGF action lie in the fact that these cells require NGF for their survival and cannot be isolated in an NGF-naive state. The creation of an NGF-naive cell line, PC12, which responds to NGF by differentiating to a sympathetic neuron-like phenotype but does not require the neurotrophin for survival, has proved an invaluable tool in the study of neuronal differentiation (Tischler and Greene, 1975; Greene and Tischler, 1976). Thus, the initial effects of NGF can be studied, in contrast to sympathetic neurons which, by necessity, will have been exposed to NGF before explantation. PC12 cells can be also used to study NGF dependent regeneration of neurites as PC12 cells previously treated with NGF will regenerate neurites only in the presence of NGF (Greene, 1977).

1.3.3.3 NGF ACTIONS ON PC12 CELLS

The discovery that an adrenal chromaffin cell line derived from a rat phaeochromocytoma responds to NGF by extending processes, provided a replicating cell model to study NGF induced differentiation (Tischler and Greene, 1975). As mentioned before, it is thought that these NGF effects are representative of the events that occur during neuronal differentiation, particularly for cells derived from the neural crest (Greene and Tischler, 1976, Tischler and Greene, 1978). Thus, PC12 cells represent a model for the pluripotent progenitor SA cell; the differentiation response of which seems not to be restricted to either neural or non-neural types (Landis and Patterson, 1981; Anderson, 1989; Sieber-Blum, 1990).

The actions of NGF on PC12 include the dramatic changes in morphological and functional characteristics very similar to those seen in sympathetic neuronal differentiation by NGF (Greene and Tischler, 1976; Guroff, 1985; Halegoua et al., 1991). The overall effect of NGF on PC12 cells is to convert them from a population of replicating adrenal chromaffin-like cells to a population of non-replicating post-ganglionic sympathetic neuron-like cells. PC12 cells flatten down, decrease proliferative rate (cease mitosis), undergo cell hypertrophy, increase cell-substratum adhesiveness and extend long, branching neurites with prominent growth cones (Guroff, 1985).

NGF causes PC12 cells to undergo a major reorganisation of their metabolic process. The differentiation process comprises a multiplicity of rapid and transient changes as well as delayed and prolonged alterations. Thus, NGF-mediated differentiation of the PC12 cells can be separated into two types of events: (i) early events (generally occuring in minutes and transcriptional-independent) (ii) delayed events (generally occuring after 24-48 hours and transcriptional-dependent) (Burnstein and Greene, 1978; Greene, 1984).

1.3.3.3.1 Rapid Changes to PC12 Cells

NGF has rapid effects on PC12 morphology, second messenger synthesis and transcription of immediate early genes (IEGs). Membrane structural changes, including ruffling of lamellopodia, take place in minutes (Connolly et al., 1979, 1981, 1984). The cells become more adhesive to the culture dish, as well as to each other. NGF also triggers an increase in transport across the plasma membrane (Greene and Tischler, 1982). NGF increases calcium efflux and sodium influx by the stimulation of the activity of the Na^+-, K^+-pump (Greene and Tischler, 1982). The rate of amino acid uptake increases between 15 minutes and 24 hours after NGF treatment. These experiments, however, showed no increase in the uptake of nucleosides or noradrenaline (McGuire and Greene, 1979).

Within minutes of adding NGF to PC12 cells, it is possible to observe changes in the pattern of protein phosphorylation (Halegoua and Patrick, 1980) and breakdown of phosphatidyl inositol (PI) (Contreras and Guroff, 1987). Changes in phosphorylation have been correlated with either enhanced or reduced kinase activities as well as changes in phosphoprotein phosphatase activities (Halegoua et al., 1991).

Most of the phosphorylation changes described occur on serine or threonine residues, but phosphorylation on tyrosine residues has also been described. Many phosphorylation events are rapid but in some instances can occur over a broad time range after NGF addition. Among those proteins phosphorylated are TH, ribosomal protein S6, histones H1a and H3, non-histone chromosomal high mobility group protein HMG17, microtubule associated protein (MAP) 1,2, other MAPs which include chartins, a neuronal intermediate filament protein, called peripherin and synapsin (Halegoua and Patrick, 1980). Conversely, decreased phosphorylation of histone H2a and other proteins has been reported following NGF treatment. The correlation of phosphorylation changes with altered cellular activity has been demonstrated in some cases. For example, increased phosphorylation of TH as a result of NGF treatment of PC12 cells leads to activation of the enzyme and increased catecholamine synthesis (Zigmond et al., 1989).

Rapid changes to IEG expression also occurs within minutes after NGF treatment. Early responsive genes are characterized by mRNAs detectable within minutes of NGF stimulation. Their mRNAs normally have a relatively short half-live and their introduction is characteristically not dependent upon ongoing protein synthesis. PC12 cells transiently express a number of IEGs such as *c-fos, c-jun, jun-B* and *c-myc*. (Greenberg et al., 1985; Kruijer et al., 1985; Wu et al., 1989). The products of these IEGs are involved in mediating late events in the response to NGF, since many of these genes encode transcription factors which control the expression of late response genes (Sheng and Greenberg, 1990; Morgan and Curran, 1991). Induction of these genes by NGF has been implicated in the more delayed gene transcription-dependent actions of NGF in PC12 cells (McMahon and Monroe, 1992). Nicotine and potassium depolarisation can also induce expression of *c-fos* and *junB*, in NGF-differentiated PC12 cells (Greenberg et al., 1986).

1.3.3.3.2 Long-Term Changes to PC12 Cells

NGF also results in late long-term, translation-dependent alterations in PC12 cells. Maximal accumulation of mRNAs of the late responsive genes occurs only after several hours of NGF treatment. Their induction can be blocked by pre-treatment with agents that inhibit translation such as cycloheximide, indicating a requirement for on-going protein synthesis.

Less rapid responses of PC12 cells to NGF include changes in the expression of several genes, such as neuropeptides, neurotransmitter synthesis enzymes, cytoskeletal and adhesion molecules, cell surface receptors and components of cell signalling machinery (Garrels and Shubert, 1979; Guroff, 1985; Halegoua et al., 1991). Moreover, the presence of increased cellular concentrations of RNA and protein upon NGF-stimulation of PC12 cells has been suggested to influence quantitative aspects of process growth (Gunning et al., 1981).

Amongst the proteins induced by NGF are neuronal enzymes required for acetylcholine and catecholamine biosynthesis, such as choline acetyltransferase (CAT), acetylcholinesterase and TH (Schubert et al., 1977; Goodman and Herschhman, 1978; Lucas et al., 1980). Also up-regulated at the level of protein synthesis are the NGF-inducible large external glycoprotein (NILE) (McGuire et al., 1978), neurofilament protein subunits (Lee and Page, 1984), growth associated protein (GAP-43) (Basi et al., 1987; Costello et al., 1990) and MAPs such as chartin, Tau proteins and tubulin (Black et al., 1986). It has been suggested these microtubule and neurofilamental proteins are involved in the organisation of cytoskeletal proteins during growth of axons and have been implicated in neurite formation (McGuire et al., 1978; Drubin et al., 1985; Lee, 1985; Black et al., 1986). NGF also induces the synthesis of neuron-specific enolase, synaptophysin, SCG-10, Thy-1 and VGF (Lee and Page, 1984; Guroff, 1985).

NGF induces the synthesis and storage of neurotransmitters, such as acetylcholine and catecholamines (DA and NA) and increases responsiveness to ACh (Dichter et al., 1977; Levi and Alema, 1991). Other NGF-inducible proteins with important functional consequences include proteins involved in transmitter release, such as synapsin I and Ca^{++}-channels (Garber et al., 1989). PC12 cells also exhibit other characteristics associated with sympathetic neurones which include the acquisition of excitable Na^{++}-based action potentials (Dichter et al., 1977; O'Lague et al., 1980, 1985; Rudy et al., 1982, 1987). This functional change has been correlated with induction of voltage-sensitive type II sodium channels (Mandel et al., 1988; Kalman, et al., 1990; Pollock et al., 1990; D'Arcangelo and Halegoua, 1993; Fanger et al., 1993).

NGF treatment is initially mitogenic to PC12 cells but these cells cease to divide after 3-5 days (Burstein and Greene, 1982; Boonstra et al., 1983; Peunoova and Enikolopov,1995). The mitogenic effect of NGF relates to its ability to act in PC12 cells in S and G2 phases to permit progression through the cell cycle before differentiation is initiated from G1 (Rudkin et al., 1989). In addition, DNA synthesis also increases, which has contributed to the proposal that NGF can also act as a mitogen in PC12 cells (Burstein and Greene, 1982). Inhibition of cell cycle progression occurs during programmed events like senescence, programmed cell death, differentiation and neuronal cell development (Ross, 1996). Most neurons undergo their final

division during embryogenesis and differentiation proceeds from arrest in G1 phase. Unlike other growth factors, NGF does not support multiple rounds of division of PC12 cells. As with other inducers of differentiation, such as activators of adenylyl cyclase and cAMP analogues, NGF causes growth arrest and accumulation of PC12 cells in the G_1 phase of the cell cycle and this arrest is necessary before initiation of differentiation processes can occur (Rudkin et al., 1989).

These changes are followed by outgrowth of neurite-like processes after several days (Greene and Tischler, 1976). By 24 hours, NGF-treated PC12 cells become increasingly neuronal in morphology, producing neurites that elongate an average of 30-50 μm/day. These neurites are long, thin (approximately 1 μm in diameter), branched and vesiculated and contain varicosities and prominent growth cones (Luckenbill-Edds et al., 1979). Synaptic-like vesicles (20-60 nm) form in clusters at the ends of these varicosities (Roger-Jacobs and Stevens, 1986). Structural characterisation of growth cones has led to the identification of microspikes, filopodia, actin and microtubules within these structures. Growth cones also appear to have components of transmitter release machinery: catecholamine-containing LDCVs, SSVs and Ca^{++}-channels. Finally, these neurites are capable of forming functional synapses, as demonstrated in co-culture with rat skeletal muscle cells, with which they form cholinergic synapses (Schubert et al., 1977).

The NGF-induced phenotype is reversible meaning that removal of NGF leads to loss of differentiated traits and resumption of cell division (Greene and Tischler, 1982). Thus, 24 hours after withdrawal of NGF most cells lose their processes and after three days most cells resume multiplication (Greene and Tischler, 1982). Priming was observed in experiments where cells exposed to an initial pre-treatment with NGF exhibited enhanced differentiation when re-exposed to NGF. This model suggests that a transcription-dependent slow accumulation of a set of proteins is a prerequisite for morphological differentiation. The accumulation of proteins primes the cells for rapid, transcription-independent, neurite outgrowth in the further presence of NGF.

PC12 cells offer a window on a period in the natural history of sympathetic neurons that was previously unavailable. It is, in fact, largely due to the substantial work of NGF action on PC12 cells that a model has been developed of NGF differentiation in which a variety of cellular control mechanisms are co-ordinately recruited for this process. The NGF response is initiated by binding of NGF to its receptors resulting in the stimulation of multiple second messenger pathways, the activation of which causes a series of post-translational modifications to specific proteins leading to immediate changes in cellular phenotype as well as eliciting gene expression changes resulting in the more delayed alterations.

1.4 NGF SIGNALLING PATHWAYS

1.4.1 PROTEIN PHOSPHORYLATION AND NEURONAL FUNCTION

Phosphorylation is the major language used within the cells to communicate information from the environment. Covalent modification by phosphorylation serves to alter the structure and function of proteins involved in cellular structure, metabolism, cell growth and differentiation. Thus, it is not surprising that 30% of eukaryotic proteins are estimated to be phosphoproteins. It is estimated that there are over a thousand kinases, so that over 1% of the genes would be predicted to encode kinases. These kinases differ in their regulation, substrate-specificity and cellular localisation. Kinases with specificity for one substrate exist; other kinases, such as the second messenger-regulated kinases, cAMP-dependent protein kinase (PKA), Ca^{++}/ phospholipid-dependent protein kinase (PKC) and Ca^{++}/calmodulin-dependent protein kinase (CaM-K) phosphorylate a variety of substrates (Walaas and Greengard, 1991).

The eukaryotic protein kinases constitute one of the largest superfamilies of homologous proteins that are related by virtue of their catalytic domain. Most related protein kinases are specific for either serine/threonine and/or tyrosine phosphorylation. The common structure of these kinases is reflected in the conserved structure of the kinase domain: a core of 30-32 kDa comprised of 12 subdomains that is conserved throughout phylogeny (Hanks et al., 1988).

Phosphorylation of protein kinases is now recognised to be essential for their regulation. For many kinases, this phosphorylation occurs on a loop termed the "activation loop" near the entrance to the catalytic core. Phosphorylation of this loop is thought to correctly align residues involved in catalysis. Kinases regulated by phosphorylation of their activation loop include PKA, PKC (Edwards and Newton, 1997) and CaM-K. Mutation of this activation loop, typically to glutamate, mimics the effect of the phosphate in allowing constitutive activation of the kinase (Newton, 1995). The activation of kinases by phosphorylation results in tight regulation of the kinase cascade within the cell. In most cases, kinases are held in an inactive state because an autoinhibitory domain occupies the substrate-binding cavity. These autoinhibitory domains are called "pseudosubstrates" because they contain a stretch of amino acids similar to those found in phosphorylation motifs of substrates. These autoinhibitory domains can be on a separate polypeptide, as in the case of the regulatory subunit of PKA, or be part of the same polypeptide as the kinase core, as in the PKC. Importantly, binding of cofactors to the regulatory domain causes a conformational change that releases the pseudosubstrate and allows access of substrates to the active site (Newton, 1995).

The affinity of kinases for their substrates is dictated by residues surrounding the phosphorylation site of the substrate, so that "consensus" sequences can be defined. It is important to note that factors such as the secondary and tertiary structure of the protein, and accessibility of the sequence, also play major roles in determining whether a site will be phosphorylated (Newton, 1995). With the myriad of kinase families and isozymes within kinases families, it has been difficult to dissect the contribution of specific kinases in specific signalling pathways. To this end, recent advances using antisense DNA, expression of dominant negative mutant kinases or the use of cell-permeant activators or inhibitors has allowed much headway in understanding how signals are transduced. Inhibitors of kinases fall into two classes: those that inhibit the chemistry (e.g. competitively bind to the active site), and those that inhibit the regulation of the kinase (e.g. by binding to the regulatory domain and preventing the binding of cofactor) (Newton, 1995).

In the nervous system, reversible phosphorylation is involved in regulating processes as diverse as biosynthesis of neurotransmitter, activity of ion channels and cell differentiation (Walaas and Greengard, 1991). Phosphorylation can affect a protein's activity by changing its allosteric conformation (Sprang et al., 1988) or by inducing electrostatic repulsion on its surface (Hurley et al., 1990). These properties can be reversed by dephosphorylation of the protein by changing its properties back its original state. Reversible phosphorylation provides a flexible method of controlling protein activity. Some substrates can be phosphorylated by different protein kinases in different or the same region of the molecule, and each phosphorylation event can change the properties of the protein in a particular way as a substrate for other protein kinases (Hunter, 1995).

Protein phosphorylation is central to the process involved in regulation of gene transcription. Many transcription factors are activated by phosphorylation thus increasing transcription of specific genes. Phosphorylation of transcription factors can affect their activity at three different levels: (i) they can be prevented from entering the nucleus and coming in to contact with their target sequences, (ii) the affinity for their target sequence can be modified by phosphorylation and (iii) their ability to interact with the transcriptional machinery can be altered (Hunter and Karim, 1992).

1.4.2 NGF-MEDIATED TYROSINE PHOSPHORYLATION

NGF is internalised after binding to its receptor on the cell surface, but internalisation is not involved in its action on its target cells (Heumann et al., 1981). Therefore, a mechanism able to transmit the signal must exist for the target cells to respond to NGF. Binding of NGF to PC12

cells results in activation of several protein kinase cascade systems through the intracellular tyrosine protein kinase domain of the high affinity NGF receptor (Halegoua et al., 1991).

Protein tyrosine phosphorylation plays a role in regulating cellular processes including differentiation, proliferation and tumorigenesis (Brewster et al., 1993). Phosphorylation at tyrosine residues occurs as a cellular response to a variety of stimuli including cytokines, hormones and growth factors. The initial event triggered by these stimuli is a process of activation, dimerization and autophosphorylation of RTKs. This in turn initiates an intracellular cascade which involves the phosphorylation of several proteins. The balance of tyrosine phosphorylation is controlled by the opposing activities of protein tyrosine kinases and protein tyrosine phosphatases (Walton et al., 1993).

The binding of NGF to its receptors and their activation result in stimulation of a number of cell signalling systems in chromaffin cells, sympathetic neurons and PC12 cells (Grimes et al., 1993; Szeberenyi and Erhardt, 1994; Greene and Kaplan, 1995). Indeed, work using PC12 cells has facilitated the identification of the involvement of these pathways. Second messenger systems activated by NGF activation include the MAPK, PLC-γ and PI-3-K signalling pathways (Hempstead et al., 1992; Saltiel and Decker, 1994; Stephens et al., 1994).

Ligand binding to the extracellular domain of the receptor produces a conformational change in the cytosolic domain causing adjacent sections to phosphorylate each other and thereby enhance the kinase activity towards other substrates (Cantley et al., 1991). Activation of *trk A* is followed by receptor dimerization or oligomerization and trans-autophosphorylation of five tyrosine residues on *trk A* : Tyr-490, Tyr-670, Tyr-674, Tyr-675 and Tyr-785 (Loeb et al., 1992, 1994). These phosphotyrosine residues act as docking sites for downstream effector substrates by specifically binding these substrates through a non-catalytic region of 100-110 amino acid residues called *src* -homology 2 (SH2) domains (Sonyang and Cantley, 1995) or protein tyrosine binding (PTB) domains (Pawson, 1995). SH2 is a domain that recognises peptide motifs bearing phosphotyrosine (pTyr) on receptors and cytoplasmic proteins. *trkA* has multiple auto-phosphorylation sites, each of which is relatively specific for a particular SH2 protein. Two of these phosphotyrosines Tyr-490 and Tyr-785 bind two proteins *src* -homology/collagen (Shc) and PLC-γ1, respectively (Kaplan and Stephens, 1994; Loeb et al., 1994; Stephens et al., 1994b). Two other proteins, p85 subunit of PI-3-K (Obermeir et al., 1993) and MAPK-1 (Loeb et al., 1992) also form complexes with tyrosine phosphorylated *trkA* in PC12 cells.

The binding of substrates to *trk A* enhances their specific activity either through receptor-induced tyrosine phosphorylation or alterations of conformation, thereby initiating a cascade of signalling events that ultimately leads to neuronal differentiation. NGF stimulates the

phosphorylation on tyrosine of PLC-γ1 (Vetter et al., 1991), Shc (Ohmichi et al., 1994) and the p85 regulatory subunit of PI-3-K (Soltoff et al., 1992). The activities of PLC-γ1 and PI-3-K are also stimulated in response to NGF (Kim et al., 1991; Soltoff et al., 1992). PLC-γ1 tyrosine phosphorylation directly increases its catalytic activity. However, the regulatory subunit p85 of the PI-3-K does not possess a catalytic domain and its phosphorylation does not result in changes of activity. This protein serves as adaptor molecules to couple the activated receptor to other intermediates. Such proteins contain an additional *src* -homology 3 (SH3) domain.

SH3 domains are composed of approximately 60 amino acid residues containing a proline-rich sequence of 10 amino acids. NGF also stimulates an increase in the $p21^{ras}$ protein bound to GTP mediated by adaptor molecules containing SH2 and SH3 domains, which are capable of binding to guanine nucleotide release factors (GNRFs) and stimulates the association with the MAPK-1 increasing its serine/threonine activity (Loeb et al., 1992). Recently it has been shown that SH3 domains within proteins may also be involved in other aspects of function, particularly the targeting of molecules to specific subcellular locations. Indeed a number of cytoskeletal proteins, for example a-spectrin, contain SH3 domains, consistent with the idea that the SH3 domain is involved in membrane localization (Pawson, 1995).

Thus, NGF exert its actions in PC12 cells through multiple protein tyrosine phosphorylation events. These initial phosphorylation events triggered by NGF are followed by activation of several protein serine/threonine kinases that include members of PKC, Raf, MAPKK, MAPK and ribosomal S6 kinase families ($p90^{rsk}$) or tyrosine kinases, such as $pp60^{src}$ kinase (Cantley et al., 1991). Several PC12 cellular proteins undergo increased phosphorylation including soluble proteins (tyrosine hydroxylase, ribosomal S6 protein), nuclear proteins (histone H3, H17, HMG17) and cytoskeletal proteins (MAP 1.2, peripherin, chartins, β-tubulin) (Halegoua and Patrick, 1980).

1.4.3 SECOND MESSENGER SYSTEMS IN NGF SIGNALLING

1.4.3.1 *src* KINASE ($pp60^{c\text{-}src}$)

$pp60^{c\text{-}src}$ belongs to a family of cytosolic protein tyrosine kinase which have been proposed to play a role in signal transduction (Cooper and Howell, 1993). The $pp60^{c\text{-}src}$ family has five conserved regions of homology. At the amino terminal region of the protein, there is an extreme myristoylation signal responsible for the addition of myristic acid required for membrane localisation (Croos et al., 1985). Adjacent to this region are the SH2 and SH3 regions, the kinase domain and the noncatalytic tail (Cooper and Howell, 1993).

An inhibitory phosphorylation site, Tyr-527, in the non-catalytic carboxy-terminal tail has been identified and is conserved in the *src* family members (Cooper and Howell, 1993). A specific carboxy-terminal *src* kinase (Csk) has been isolated which phosphorylates pp60$^{c\text{-}src}$ on Tyr-527, resulting in inhibition of activity. A tyrosine phosphorylation site (Tyr-416) is also present in the catalytic domain and autophosphorylation within this domain stimulates kinase activity. pp60$^{c\text{-}src}$ is activated by growth factors and other cellular activators even though they lack extracellular domains. In some cases this activation may be mediated by G proteins and/or PKC activity (Cantley et al., 1991), although in other cases pp60$^{c\text{-}src}$ has been shown to associate directly with the cytosolic domain of RTKs. pp60$^{c\text{-}src}$ activates Shc, the universal tyrosine kinase substrate adaptor protein in the ternary complex, activating p21^{ras} (Hashimoto et al., 1994). The mechanisms of pp60$^{c\text{-}src}$ activation are still unclear. In the inactivated state, the carboxy-terminal phosphorylated tyrosine (Tyr-527) binds to the SH2 domain. Following receptor autophosphorylation, the receptor tyrosine kinase phosphopeptide sequence has higher affinity for the SH2 domain of pp60$^{c\text{-}src}$ and displaces the carboxy-terminal inhibitory phosphotyrosine, initiating an allosteric change in the molecule and catalysing autophosphorylation at Tyr-416. Since phosphorylation is essential for carboxy-terminal-SH2 interactions, it is likely that a receptor-associated protein tyrosine phosphatase is also involved (Cooper and Howell, 1993). Once pp60$^{c\text{-}src}$ is activated, its SH3 domain plays a role in the recognition of proline-rich regions of the substrates.

pp60$^{c\text{-}src}$ involvement in NGF signalling is demonstrated by v-*src* mimicking NGF induction of differentiation and neurite outgrowth and other neuronal characteristics in PC12 cells (Thomas et al., 1991). Microinjection of pp60$^{c\text{-}src}$ has shown that this protein is required for transduction of signals for phenotypic change in PC12 cells. Furthermore, sequential p21^{ras} and pp60$^{c\text{-}src}$ actions are required for NGF-induced signals to be transduced (Kremer et al., 1991; D'Arcangelo and Halegoua, 1993). In addition, microinjection of anti-*src* antibodies into PC12 cells prevents NGF-induced but not p21^{ras} induced neurite outgrowth, while anti-*ras* antibodies block both NGF and pp60$^{c\text{-}src}$ effects, indicating that pp60$^{c\text{-}src}$ activation lies upstream of p21^{ras} in the cascade of events mediating NGF effects (Kremer et al., 1991).

1.4.3.2 MAP KINASE CASCADE

The MAP kinase cascade has been extensively characterised in yeasts and mammals (Cobb and Goldsmith, 1995; Hunter, 1995; Marshall, 1995a). MAP kinase cascades are part of a three kinase pathway consisting of a MAP kinase kinase kinase (MAPKKK), a MAP kinase activator (MAPKK), and a MAP kinase (MAPK) which act sequentially (Cobb and Goldsmith, 1995). MAPKK can be activated by one or two MAPKKK but reports strongly suggest that the MAP kinases are activated only by dedicated MAPK activators. In this cascade, activated

MAPKs translocate to the nucleus and activate transcription by phosphorylation of transcription factors (Crowley et al., 1994).

Proteins of the MAPK cascade are involved in development and differentiation, proliferation and oncogenesis (Cobb and Goldsmith, 1995; Hunter, 1995; Marshall, 1995b). Oncogene products are involved in every step of the MAPK cascade of events involved in mediating the response of PC12 cells to NGF. Oncogenes encode for proteins that are either growth factors, growth factor receptors, transducers of growth factor responses or transcription factors (Cantley et al., 1991). The transducers of the growth factor responses include several members of the MAPK cascade. Thus, overexpression of the $p140^{trk}$ and point mutations in $p21^{ras}$ can render these upstream components transforming. At the level of the kinase cascade itself, *raf-1* and *mos* are oncogenic proteins (Seger and Krebs, 1995).

1.4.3.2.1 $p21^{ras}$ protein

The best understood means of activating the MAPK pathway is that used by RTKs which transduce their signals via a $p21^{ras}$-dependent pathway (Marshall, 1995a) (Figure 1.1). $p21^{ras}$ belongs to a superfamily of small GTP binding proteins consisting of some 60 distinct mammalian proteins. The first members to be discovered were $p21^{v\text{-}Ha\text{-}ras}$ and $p21^{v\text{-}Ki\text{-}ras}$, the 21 kDa proteins encoded by the oncogenes of the Harvey and Kirsten rat sarcoma viruses. These proteins are responsible for the transforming ability of the retroviruses. A third, closely related transforming protein, $p21^{N\text{-}ras}$, was later discovered as an oncogene product overexpressed in a neuroblastoma (Marshall, 1995b).

$p21^{ras}$ plays a critical role in mediating the mitogenic and differentiating actions of tyrosine kinase-coupled receptors (Downward, 1990; Bourne et al., 1991). $p21^{ras}$ is a plasma membrane associated protein which is activated upon binding to GTP and becomes inactive after hydrolysis of GTP to GDP. GTPase activating protein (GAP) is a 120 kDa protein that interacts with $p21^{ras}$ with low affinity to stimulate the otherwise slow intrinsic hydrolysis of GTP on normal $p21^{ras}$ (Trahey and McCormick, 1987). The release of bound GDP is stimulated by guanine nucleotide releasing proteins (GNRPs), such as Sos, Vav and Ras-GEF which promotes its replacement by GTP, therefore activating $p21^{ras}$ (Marshall, 1995b). GNRPs possess proline-rich regions which are probable motifs for protein-protein interactions involving SH3 domains. These exchange factors interact with adapter proteins, such as Ash, Grb-2, Nck and Shc via the SH3 domains and these adapter proteins, in turn, interact with the phosphorylated tyrosine residues of activated RTKs via SH2 domains. In this way, the receptors are coupled to downstream signalling mechanisms through $p21^{ras}$ which can then result in the activation of IEGs and subsequent gene expression (Marshall, 1995b).

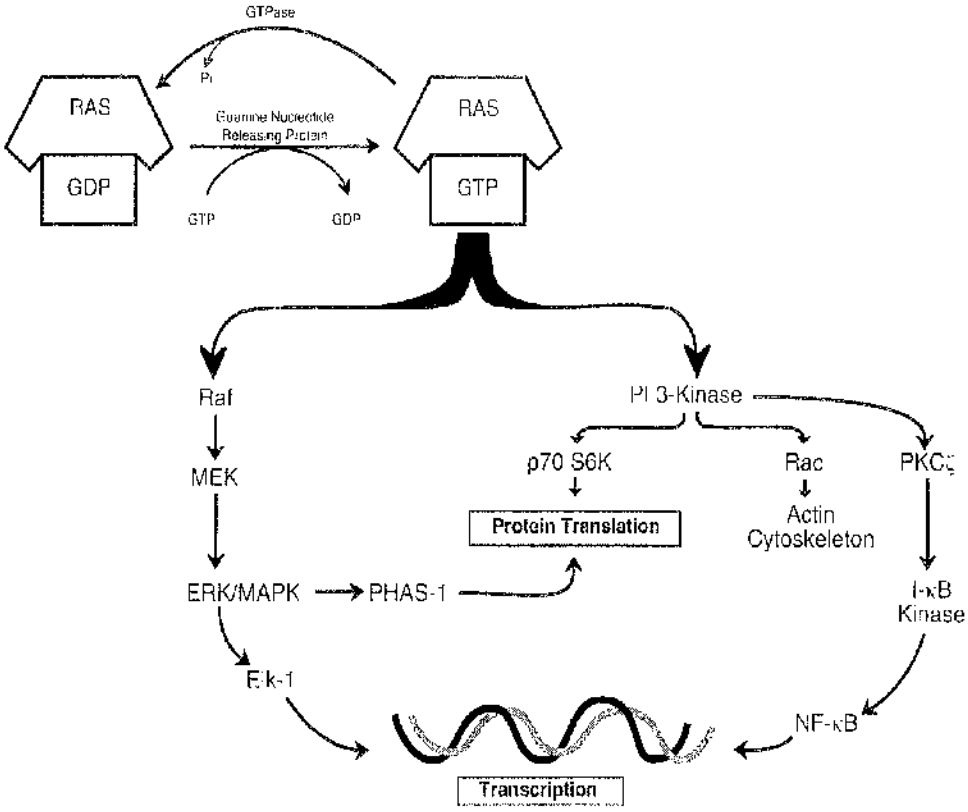


FIGURE 1.1 AN OVERVIEW OF RAS SIGNALLING
(Taken 1998 Signal Transduction Calbiochem catalog)

It has been demonstrated that NGF, FGF and interleukin-6 induce accumulation of the GTP-bound active form of p21^{ras} in PC12 cells (Qui and Green, 1991, 1992; Nakafuku et al., 1992; Ng and Shooter, 1993). Binding of NGF to the high affinity receptor results in autophosphorylation of p140^{trk} on tyrosine residues within 5 minutes and activation of p21^{ras} (Ras-GTP) within 20 minutes after NGF treatment by induced increase in GNRPs activity and reduced GAP activity. Thus, these factors, each of which can cause differentiation of PC12 cells induce increased levels of p21^{ras} activity.

RTKs use adapter proteins to couple p21^{ras} to autophosphorylated receptors. In PC12 cells, there is evidence to suggest that the Shc-Grb2-Sos ternary complex performs this role (Hashimoto et al., 1994). Autophosphorylation of *trk A* following binding of NGF and the resulting phosphotyrosine domains serve as docking sites to recruitment of two adapter proteins, Shc and growth factor receptor bound protein-2 (Grb-2). SH2 domains, which are present in both Shc and Grb-2, mediate high affinity binding to specific pTyr residues. The SH2-homology domains of Shc bind directly to the*trk A* receptor and the SH2-homology domains of Grb-2 then bind to Shc already phosphorylated on tyrosine residues (Hashimoto et al., 1994). In addition to its carboxy-terminal SH2 domain, Shc has a second structurally unrelated protein tyrosine binding (PTB) domain at its amino-terminus, which also dictates receptor-Shc interactions.

SH3 domains bind with high affinity to proline-rich regions of proteins. The SH3 domain of Grb-2 mediates its binding to the carboxy-terminus of Sos-1. The Grb-2 proline-rich SH3 domain-binding sites facilitate the recruitment of Sos-1 to the membrane in proximity to the membrane-bound isoprenylated p21^{ras}. This recruitment of Sos-1 to the cytoplasmic surface of the membrane activates p21^{ras} by catalyzing GDP for GTP exchange (Hashimoto et al., 1994). Other components of the ternary complex, including Sos-1 and Ras-GAP, contain pleckstrin-homology (PH) domains, which have been shown to bind to both Gbg subunits and phospholipids (Pawson, 1995). Thus, Grb-2 is an adaptor protein that bridges Shc to Sos-1 and then activates p21^{ras} (Kaplan and Stephens, 1994).

Thus, Sos-1 a member of the GNRPs family enhances the rate of GDP-GTP exchange on p21^{ras}, leading to p21^{ras} activation. The GTP-bound form of p21^{ras} then binds *raf* protein kinase, thereby targeting it to the membrane where *c-raf* activity is increased (Vojtek et al., 1993). The direct binding of *raf* to p21^{ras} *in vivo* leads to translocation of the kinase to the plasma membrane. Activated *raf* kinase is therefore the first component of the now committed MAPK module and activation of the other kinases in the module follows, leading to the activation of the MAPK cascade. Activation of MAPKs causes hyperphosphorylation of Sos-1 and results in its dissociation from Grb-2 and hence to the inactivation of p21^{ras} (Kaplan and Stephens, 1994).

Biochemical and genetic evidence indicates that $p21^{ras}$ functions downstream of the *trk A* receptor and *src* -related protein tyrosine kinases and upstream of the serine/threonine kinase *c-raf* (Kaplan and Stephens, 1994). Introduction of the oncogenic form of $p21^{ras}$ into NGF-responsive cells by microinjection of the protein, transfection with the gene or infection with a virus carrying the gene can mimic the NGF-mediated differentiation effects, including neurite outgrowth (Bar-Sagi and Feramisco, 1985; Brightman et al., 1990; Kremer et al., 1991). Furthermore, microinjection of anti-*ras* antibodies or transfection with dominant negative interfering variants of $p21^{ras}$ (Asn-17) block NGF-induced neurite outgrowth in PC12 cells (Hagag et al., 1986; Kremer et al., 1991). Indeed, expression of a dominant inhibitory mutant of $p21^{ras}$ is able to block the activation of *raf-1*, MAPKs and $p90^{rsk}$ by NGF and phorbol ester, while expression of an activated $p21^{ras}$ variant leads to tyrosine phosphorylation and increase of MAPK activity (Thomas et al., 1992). These results support that $p21^{ras}$ is central to NGF-induced intracellular signalling pathways in PC12 cells (Szeberenyi et al., 1990; Robbins et al., 1992).

The $p21^{ras}$ pathway, however, only appears to be responsible for some of the responses of PC12 cells to NGF (Borasio et al., 1993). Tyrosine phosphorylation of *trk A* or PLC-γ1 were not altered by the inhibition of $p21^{ras}$ activity (Thomas et al., 1992). $p21^{ras}$ may not required for some of the early responses of cells to NGF (shape change, elaboration of short neurites). Futhermore, expression of the constitutively activated form of $p21^{ras}$, *raf-1* or mitogen-activated protein kinase/ERK kinase (MEK-1) in PC12 cells cannot mimic the complete neuronal differentiation pattern seen with NGF (Crowley et al., 1994) nor induce the appareance of the differentiation-associated gene Thy-1 or of Na^{++}-channel genes, an ultimate marker of acquisition of neuronal phenotype (D'arcangelo and Halegoua, 1993; Fanger et al., 1993). Thus, other targets of NGF action might act in novel intracellular signalling pathways in PC12 cells.

$p21^{ras}$ has been shown to interact *in vivo* and *in vitro* with *raf* kinase (Moodie et al., 1993; Warne et al., 1993). This interaction is dependent on $p21^{ras}$ being in the GTP bound (activated) state (Vojtek et al., 1993). Microinjection experiments with monoclonal anti-*ras* antibody inhibited *raf* signalling and provided direct evidence that *raf* kinase was downstream of $p21^{ras}$ in PC12 cells (Smith et al., 1986). Expression of activated forms of $p21^{ras}$ in PC12 cells is necessary and sufficient to activate both *raf-1* and *B-raf* as well as the MAPKs (Troppmair et al., 1992; Wood et al., 1992) and dominant inhibitory $p21^{ras}$ blocks NGF-induced hyperphosphorylation and activation of *raf-1* and MAPKs activation (Robbins et al., 1992).

1.4.3.2.2 *raf* Kinase (MAPKKK)

The MAPKKK are the first components in the signalling by MAPK modules and are divided into two groups, the *raf* kinases and the MEK kinases. The proto-oncogene *raf-1* is well known to code for a cytosolic serine/threonine kinase of the $pp60^{c\text{-}src}$ family that consists of an amino-terminal negative regulatory domain containing a conserved region 1 (CR1) which includes a *ras* -binding domain (RBD) and a cysteine-rich domain (CRD), a serine/threonine rich domain (CR2) and a carboxy-terminal kinase domain (CR3) (Warne et al., 1993; Avruch et al., 1994; Morrison and Cutler, 1997). This combination of functional domains is similar to that present in the PKC family of serine/threonine kinases.

Three isozymes of raf protein have been identified in mammalian tissues: (i) *A-raf*, (ii) *B-raf* and (iii) *raf-1* (also known as *c-raf*) (Daum et al., 1994). *raf-1* protein is the best characterised member of this family, because of its ubiquitous tissue distribution and its occurrence in many cells lines (Morrison and Cutler, 1997). *raf-1* gets activated when it is translocated to the cell membrane after association with $p21^{ras}$ by a process that has not been fully elucidated but has been reported to involve the members of the 14-3-3 proteins, a well-conserved family of proteins present in mammalian cells as well as flies, yeast, and plants that has been implicated in biochemical processes such as inhibition of PKC, activation of tyrosine and tryptophan hydroxylases, and stimulation of exocytosis from adrenal chromaffin cells (Aitken, 1995).

At the cell surface membrane, *raf-1* may become fully activated by either autophosphorylation or by the action of either serine/threonine- or tyrosine-specific protein kinases (Daum et al., 1994). *raf-1* is phosphorylated at serine residues following stimulation of the NGF receptor. *raf-1* also becomes phosphorylated on tyrosine residues by members of the *src*- family of kinases, and this phosphorylation is essential for its activation (Fabian et al., 1993). Certain isoforms of PKC have been demonstrated to phosphorylate *raf-1* (Kolch et al., 1993). In fibroblasts, PKC-δ activates the MAPK pathway in a manner independent of $p21^{ras}$ and dependent on *raf-1* phosphorylation (Ueda et al., 1996). Thus, PKC activation may act synergistically with NGF-induced activation of *raf-1* to increase *raf-1* activity and subsequent MAPK activation. In contrast, the observations that activation of PKA inhibits *raf-1* activity have suggested that PKA phosphorylation plays a role in suppressing *raf-1* activity. The activation of PKA by cAMP has been implicated in the negative regulation of MAPK cascade in Rat-1 cells and PC12 cells expressing PDGF receptor (Cook and McCormick, 1993; Wu et al., 1993; Vaillancourt et al., 1994). PKA may inhibit the activity of *raf-1* at two different levels, by regulating the affinity for *ras* -GTP binding through phosphorylation of *raf-1* at Ser-43 and by regulation of its catalytic activity through phosphorylation of other residues (Hafner et al., 1994).

B-Raf protein, has a much more restricted distribution, being limited to brain, spinal cord, ovary and testis; reminiscent, of the distribution of the $p140^{trk}$ receptor. *B-raf* protein can be expressed as either a 68- or 95- kDa polypeptide. Treatment of PC12 cells with NGF does not alter the levels of *B-raf* mRNA, but does induce rapid and transient phosphorylation of *B-raf*. *B-raf* is phosphorylated exclusively on serine residues; no tyrosine phosphorylation is observed (Oshima et al., 1991; Jaiswal et al., 1994; Traverse and Cohen, 1994). Concomitant with the increase in serine phosphorylation, NGF treatment also increased the serine/threonine kinase activity of *B-raf* within 1-2 minutes (Oshima et al., 1991). Recently, it has been shown that cAMP can activate the MAPK cascade in PC12 cells through a *B-raf* dependent pathway and activation of the small G protein *rap-1* (Vossler et al., 1997). Thus, in *B-raf* expressing cells, such as PC12 cells, activation of *B-raf* provides a mechanism for tissue-specific regulation of cell growth and differentiation via MAPK cascade.

1.4.3.2.3 MAPK kinase (MAPKK)

The MAPKs are phosphorylated at both threonine and tyrosine residues for a "dual specificity" enzyme, unique to each MAPK isoform termed MAP kinase kinase (MAPKK), also known as MAP/*Erk* kinase (MEK) following treatment with various growth factors (Boulton et al., 1991; Gomez and Cohen, 1991; Ahn et al., 1992; Crews et al., 1992). These kinases phosphorylate TEY, TPY, or TGY motifs on each MAPK isotype (Cano and Mahadave, 1995). MEK-1 and MEK-2 phosphorylate MAPK-1 and MAPK-2 respectively (Kyriakis et al., 1992; Dent et al., 1992). MEKs are highly specific kinases that phosphorylate only MAPKs. Other kinases related to MAPK-1 and MAPK-2, despite possessing activating phosphorylation sites, are poor *in vitro* substrates for MEK-1 (Lin et al., 1995). MEK-1 and MEK-2 are "dual specificity" kinases activated by *raf-1* through phosphorylation on two serine residues within the catalytic domain, Ser-217 and Ser-221 as well as tyrosine residues (Boulton et al., 1991).

Constitutively activated mutants of MEK-1 stimulate PC12 cells to undergo neuronal differentiation and dominant negative mutants of MEK-1 inhibit growth factor-induced PC12 differentiation (Crowley et al., 1994). This indicates that activation of MEKs is necessary and sufficient for PC12 differentiation. MEK activity is increased by NGF very rapidly (within 30 seconds) and this activation always precedes the development of MAPK activity in PC12 cells (Gomez and Cohen, 1991; Traverse et al., 1992). The time course of MEK activity correlates with the time course of tyrosine phosphorylation of MAPKs in PC12 cells. Sustained activation of MEKs is observed after NGF binding to the PC12 cells whereas MEK is only transiently activated by EGF. This suggests that alterations in MEK activity dictate the sustained activation of MAPKs by NGF and transient activation of MAPKs by EGF (Traverse et al., 1992).

1.4.3.2.4 Mitogen-Activated Protein Kinases (MAPK)

Many cytokine receptors, RTKs and heterotrimeric G-protein coupled receptors (GPCRs) activate a class of intracellular protein serine/threonine kinases termed MAPKs, also know as extracellular-signal regulated kinases (ERKs) (Anderson and Maller, 1990; Gomez and Cohen, 1991; Pelech and Sanghera, 1992). These are considered to represent switch molecules as they can convert signals from receptor-associated tyrosine kinases into serine/threonine phosphorylation (Crews and Erikson, 1993). p42 (MAPK-2) and p44 (MAPK-1) kinase isoforms are the most well-studied members of the MAP kinase family and have been implicated in induction of cell cycle re-entry, differentiation, migration and apoptosis. They are activated when phosphorylated on both the threonine and the tyrosine residue (Sturgill et al., 1988; Anderson and Maller, 1990) within the threonine-glutamine-tyrosine (TEY) motif in domain VIII (Lloyd and Wooten, 1992; Peraldi et al., 1993; Cano and Mahadevan, 1995).

A mechanism which gives the MAPK cascade specificity is the kinetics of MAPK activation. MAPK activation is associated with both cell proliferation and differentiation and it is the duration of MAPK activation that determines whether a stimulus elicits proliferation or differentiation. In PC12 cells, sustained activation of this pathway leads to differentiation while transient activation leads to proliferation (Qiu and Green, 1992; Traverse et al., 1992; Marshall, 1995). In particular, the NGF stimulated MAPK activity is exceptionally robust in PC12 cells (Tsao et al., 1990). From a low basal level, MAPK activity is increased at least 10 fold and largely sustained for at least 90 minutes. The plateau phase of MAPK activation by NGF is particularly important since NGF, and bFGF, which induce neurite outgrowth in PC12 cells, cause prolonged MAPK phosphorylation, whereas EGF, which does not induce neurite outgrowth in PC12 cells causes only transient MAPK phosphorylation (Traverse et al., 1992).

Since EGF mediates its proliferative effects via the MAPK cascade but this activation is of a more transient nature than that elicited by NGF, it has been proposed that a minimum threshold of MAPK activation is required to elicit activation of the differentiation response (Marshall, 1995). Specifically, transient activation is believed to be involved in the proliferative response induced by mitogenic growth factors while sustained MAPK activation is implicated in the differentiation response by such factors as NGF and bFGF. There are potentially many different pathways for receptors to signal transient, versus sustained MAPK activation: differences in ligand concentration and receptor number, rate of internalization and down-regulation of receptors and differential usage of signalling pathway downstream of receptor (Marshall, 1995).

Stimulation with NGF causes a translocation of MAPKs from the cytosol to the nucleus. Nuclear translocation of MAPKs does not occur after stimulation with EGF. These findings suggest that the sustained activation of the MAPKs is required for entry to the nucleus, thereby initiating gene transcription events required for neuronal differentiation of PC12 cells. Thus, quantitative differences in MAPK activation are translated into a qualitative difference in transcription factor activation, indicating that the cellular response is determined by which transcription factors are present in the cell. Thus, sustained activation of MAPK is just a final switch determined by previous events that set which MAPK transcription factors are present in the cell (Marshall, 1995).

Following prolonged activation, MAPK translocates into the nucleus and phosphorylates nuclear targets such as c-*myc, elk-1, c-jun* and ATF-2. Translocation of both MAPK-1 and MAPK-2 kinases has been shown to occur after mitogen stimulation (Sanghera et al., 1992) and MAPK-2 translocation has been shown to occur after exposure of PC12 cells to NGF (Traverse et al., 1992; Nguyen et al., 1993). MAPKs phosphorylate the transcription factor *elk-1* which leads to an increase in the formation of the ternary complex that binds the serum response element (SRE) (Gille et al., 1992). Thus, these kinases phosphorylate *elk-1* at a cluster of serines/threonines residues at its carboxy-terminus (particularly Ser-383) critical for transcriptional activation (Marais et al., 1993).

Substrates of MAPKs can be also found on the cell surface and in the cytoplasm. On the cell surface, the NGF receptor, the EGF receptor and Sos-1 all serve as substrates for MAPKs. In the cytoplasm, one group of protein kinases that have been identified as MAPK substrates can be divided into two subgroups based on their location within the MAPK signalling pathway. The first subgroup includes the protein kinases that lead to MAPK activation, such as the MAPKKK (*raf-1*, MEKK-1) (Anderson et al.,1991; Lee et al.,1992) and the MAPKK (Matsuda et al., 1993). This suggests that the MAPK cascade might be regulated by MAPK itself (Anderson et al., 1991; Lee et al., 1992). The second subgroup are the protein kinases that are downstream of MAPK. The ribosomal S6 kinase II ($p90^{rsk}$) is phosphorylated and activated by MAPK (Sturgill et al., 1988). Another substrate is the MAPKAPK-2 which phosphorylates glycogen synthase kinase-3 (GSK-3) (Stokoe et al., 1992).

MAPK also phosphorylates cytoskeletal proteins, such as *Tau* and MAP 1, 2 and 4 proteins (Drewes et al., 1992). This phosphorylation regulates cytoskeletal rearrangements and cellular morphology. Cytoplasmic PLA_2 is another MAPK substrate and its phosphorylation on Ser-505 causes an increase in enzymatic activity resulting in increased arachidonic acid release and the formation of lysophospholipids from membrane phospholipids (Lin et al., 1993). Thus, MAPKs mediate several actions of growth factors and cytokines on translation, transcription, cytoskeletal function and cell signalling.

Certain dual specificity phosphatases selectively inactivate MAPK by dephosphorylating the two sites that are required to activate them, threonine and tyrosine residues (Hunter, 1995). *In vitro* experiments have demonstrated that these phosphatases are highly specific for MAPKs (Alessi et al., 1993; Zheng and Guan, 1993). The genes encoding these phosphatases are themselves inducible by growth factors and other stimuli that activate the MAPK. A number of genes have been cloned in different laboratories and include: 3CH134, CL100, HVH1, PAC-1, and *erp-1* (Alessi et al., 1993; Zheng and Guan, 1993).

1.4.3.3 MAPK SIGNALLING TO NUCLEUS

Intracellular mechanisms mediating the effect of growth factors on the nucleus may involve modification of proteins already present in the cytosol by processes such as enzymatic cleavage or phosphorylation (Hunter and Karin, 1992). Some effects, however, may require the synthesis of new proteins which in turn regulate expression of other genes. Based on the events required for a gene to be influenced by an extracellular factor, it is possible to identify two major groups of genes: (i) immediate early responsive genes (IEGs) in which specific transcription factors required for their transcription are already present in the cytoplasm and are activated directly, shortly after the binding of the ligand to its receptor, (ii) late responsive genes (LRGs) which are activated by transcription factors which required synthesis *de novo* . These transcription factors are usually codified by a subset of the IEGs, and their effects thus depend on fully active translation machinery.

Several intracellular mechanisms seem to be involved in transmitting the NGF signal to the nucleus. The intracellular organisation and hierarchy of these second messenger systems remains to be fully solved. Although activation of different second messenger systems can reproduce some of the observed responses of PC12 cells to NGF, it is becoming evident that a complex pattern of multiple interconnected intracellular pathways is responsible for the observed effects of NGF in these cells.

1.4.3.3.1 AP-1 TRANSCRIPTION FACTOR

A class of cellular genes, IEGs, has been identified whose transcription is rapidly and transiently activated. Several members of the IEG family encode putative transcription factors, critical to the cell response to growth factors. *c-fos* and *c-jun* proto-oncogenes are the most extensively studied of these genes (Sheng and Greenberg, 1990).

c-jun , encodes a DNA-binding protein that is present in the transcription factor activation protein 1 (AP-1). *c-jun* binds to a TPA-responsive element (TRE) (5'-TGAGTCA-3') in a

complex with the product of the *c-fos* proto-oncogene. As a heterodimer, Fos and Jun proteins bind to the AP-1 site with high affinity by interaction of their leucine zipper domains and thereby regulate transcription of genes. The Jun family members bind to DNA either as homodimers or as heterodimers with members of the Fos and activating transcription factor/cAMP responsive element binding protein (ATF/CREB). The Fos family bind to DNA as heterodimers with the Jun family and with selected members of the ATF/CREB family (Sheng and Greenberg, 1990).

AP-1 was first identified as a transcription factor extracted from HeLa cells that bound selectively to *cis* -elements of SV40 and human metallothionein IIA genes (Bohmann et al., 1987). AP-1 was found to be a complex of related proteins encoded by different genes. This complex recognises and binds the consensus DNA sequence (TRE element) which has been shown to confer phorbol ester inducibility to the associated genes. AP-1 can function as an intermediate in signal transduction mediated by cell surface receptors, including growth factor receptors (Curran and Franza, 1988; Morgan and Curran, 1991).

The AP-1 family also includes other members belong either to the Fos (*c-fos* , *fosB* , *fra1* , *fra2*) and Jun (*junB* and *junD*) (Curran and Franza, 1988). A functional DNA binding domain seems to require the formation of dimers between members of the AP-1 family. This dimer formation is dependent on a structural motif of leucine residues repeated every 7 amino acids capable of forming a bimolecular coiled structure know as a "leucine zipper" (Kerppola and Curran, 1991). The DNA binding region is a basic amino acid rich region immediately adjacent to the leucine zipper motif. It is thought that DNA binding is required but not sufficient for AP-1 induced transcriptional activation. Induction of *c-jun* by phorbol esters is thought to result from a positive autoregulatory mechanism and to involve dephosphorylation of a preexisting *c-jun* at inhibitory phosphorylation sites next to its DNA-binding domain (Boyle et al.,1991). Comparisons between cellular and viral Jun protein suggest that a regulatory domain present in the carboxy-terminal region of *c-jun* is responsible for the significantly lower transcriptional activity of *c-jun* compared with *v-jun* . This putative repressor domain may also play a role in the signal dependent induction of *c-jun* activity (Bohmann and Tjian, 1989).

These rapid changes activate gene expression, through modification and activation of pre-existing transcription factors (Fu and Zhang, 1993). NGF produces a complete rearrangement of gene transcription and translation machinery by activation of multiple second messenger systems. NGF activates a number of genes in PC12 cells. Several of these are activated within 15 minutes and activation is transient. These include IEGs, such as c-*fos* , NGFI-A, NGFI-B, NGFI-C, *c-jun* and *jun* B genes (Sheng and Greenberg, 1990). Other genes, including c-*myc*, are more slowly activated over 1-2 h. Still other late responsive genes are slowly and persistently activated by NGF over several hours or days (Halegoua et al., 1991).

The *c-fos* mRNA induction is observed after 5 minutes of NGF addition and peaks within 15 to 30 minutes (Greenberg et al., 1985). This rise has been reported to be an early event in the NGF induced differentiation of PC12 cells and indeed is thought to be required to mediate some of the late responses to NGF (Halegoua et al., 1991). The proto-oncogene *c-fos* , is one of these IEG and its transient induction is an important step in evoking a normal response to NGF. Other factors known to induce *c-fos* in PC12 cells include fibroblast growth factor (FGF), interleukin 6 (IL-6), nicotine agonists, depolarising stimuli and cAMP analogues (Morgan and Curran, 1991).

1.4.3.3.2 AP1 ACTIVATION

In most cell types, members of the *c-fos* and *c-jun* gene families are expressed at relatively low levels, but they are induced transiently by a wide range of extracellular stimuli (Sheng and Greenberg, 1990). *c-fos* has become a model gene for the study of inducible expression because *c-fos* activation occurs in a wide range of cell types in response to a diverse set of agents including specific polypeptide growth factors, serum, phorbol esters, neurotransmitters and agents that elevate intracellular Ca^{++} and cAMP (Angel and Karim, 1991).

Although the *c-fos* and *c-jun* genes exhibit cell type, stimulus and temporal specificity of the expression, a relatively large subset of these gene families can be co-expressed in any given situation. Therefore, mechanisms exist that permit the recognition and assembly of specific dimeric complexes with the appropriate DNA regulatory element (Sheen and Greenberg, 1990). The role of *c-fos* and *c-jun* in gene transcription is complex and may be regulated in several ways including association with different dimerization partners, interaction with other transcription factors, effects on DNA topology, and reduction/oxidation of a conserved cysteine residue in the DNA binding domain. In addition, phosphorylation of *c-fos* and *c-jun* by several protein kinases is affected by dimerization and binding to DNA (Abate et al., 1993).

1.4.3.3.2.1 *c-fos* activation

Activation of AP-1 transcription factor by NGF and PKC, has been well documented (Sheen and Greenberg, 1990). For instance, *c-fos* activation via the PKC pathway has been shown to be regulated by a serum responsive element (SRE) (Treisman, 1990, 1995). SRE is a significant component of the c-*fos* promoter that contributes to the mitogen-stimulated expression of the c-*fos* protein. The trans-acting factors that bind to this element to form a ternary complex include serum response factor ($p67^{SRF}$) and ternary complex factor ($p62^{TCF}$) (Treisman, 1990, 1995). Characterisation of $p62^{TCF}$ protein indicates that this factor is represented by *elk-1* , a member of the *ets* (E-twenty-six) gene family (Treisman, 1990).

Member of the *Ets* gene family have a conserved DNA binding domain and regulate transcriptional initiation from a variety of cellular and viral genes and enhancer elements by binding to specific DNA sequences on the SRE (Macleod et al., 1992).

The carboxy-terminal activation domain of *elk-1* is phosphorylated by MAPKs (Marais et al., 1993). *Elk-1* is phosphorylated by MAPKs at a cluster of serine and threonine motifs at its carboxy-terminus, particularly at Ser-383/Ser-389. These phosphorylation events are critical for transcriptional activation of *elk-1*. $p90^{rsk}$ phosphorylates SRF at Ser-103. This phosphorylation facilitates the formation of the ternary complex factor (TCF) composed by *elk-1*, SRF and SRE, and stimulates its ability to activate transcription, without affecting its DNA binding properties (Edwards, 1994). $p90^{rsk}$ phosphorylates *c-fos* on the trans-repression domain at the carboxy-terminal region of *c-fos* (Ser-362) in close proximity to another MAPK site (Ser-374). These cooperative phosphorylations by two enzymes may play a role in the inactivation of *c-fos* , raising the posibility that the MAPK cascade not only activates AP-1 but may also participate in repression (Edwards, 1994). $p90^{rsk}$ phosphorylates glycogen synthase kinase-3 (GSK-3) on Ser-9 that leads to its inactivation. Because GSK-3 kinase seems to play a role in the negative regulation of *c-jun* , the transient activation of GSK-3 kinase would enable rapid activation of the AP-1 activity that is normally repressed by this kinase.

1.4.3.3.2.2 *c-jun* activation

c-jun is a component of the transcription factor AP-1 that binds and activates transcription at AP-1/TRE elements. The *c-jun* promoter is somewhat simpler, and operates through one major *cis* -element, the *c-jun* TRE (Angel et al., 1988). This *c-jun* TRE sequence differs from the consensus TRE sequence by a 1-base pair insertion and due to this change is more efficiently recognized by *c-jun*/ATF-2 heterodimers (Karim, 1995). ATF is a family of at least ten different transcription factors encoded either by different genes or generated by differential splicing. These factors form homodimers or heterodimers with other members of the ATF family or Jun families which can bind to both the AP-1 or CRE/TRE responsive element (Karim, 1995).

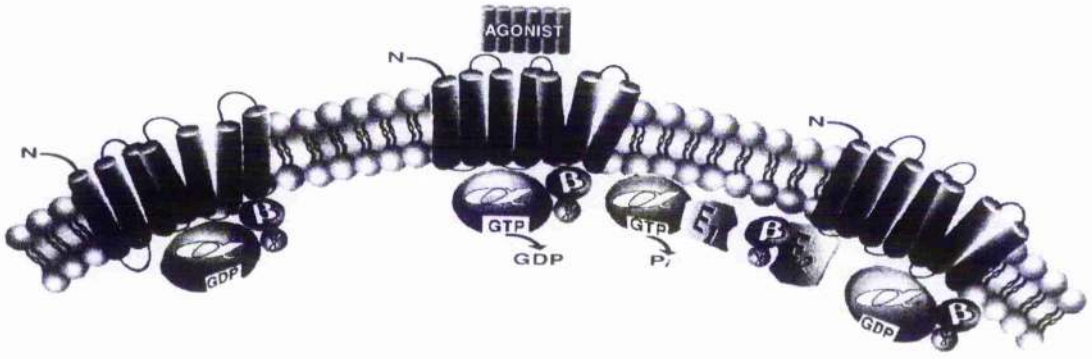
Unlike *c-fos* , both *c-jun* and ATF-2 are constitutively present in the cell. Like the *c-fos* SRE, the *c-jun* TRE is constitutively occupied *in vivo* (Karim, 1995). Following exposures to stimuli, such as inflammatory cytokines, UV irradiation, osmotic stress, both *c-jun* and ATF-2 are rapidly phosphorylated and these phosphorylation stimulates their ability to activate transcription, thereby leading to induction of the gene expression (Karim, 1995). Phosphorylation of *c-jun* at Ser-63 and Ser-73, located within its transactivation domain, potentiates its ability to activate transcription as either homodimer or a heterodimer with *c-fos* . Furthermore, mutation of Ser-63 and/or Ser-73 renders *c-jun* nonresponsive to mitogenic and

stress-induced signalling pathways. Phosphorylation at these sites also may potentiate *c-jun* transcriptional activity through recruitment of CREB binding protein (CBP). CBP connected the phosphorylated activation domains of CREB or *c-jun* to the basal transcriptional machinery (Karim, 1995). Thus, MAPK and $p90^{rsk}$ kinase activation by growth factors, such as NGF, leads to *c-fos* induction, which upon translocation to the nucleus combines with pre-existing *c-jun* proteins to form AP-1 heterodimers that are more stable than those formed by *c-jun* proteins alone (Edwards, 1994). However, another part of the increase in AP-1 activity is due to *c-jun* and ATF-2 phosphorylation (Karim, 1995).

1.5 G-PROTEIN COUPLED RECEPTORS (GPCRs)

All biological systems have the ability to process and respond to enormous amounts of information. Much of this information is provided to individual cells in the form of changes in concentration of hormones, growth factors and neuromodulators. Several families of cell surface receptors have been characterized that are coupled to different mechanisms of signal transduction, one of these is coupled to heterotrimeric G-proteins, the G-protein coupled receptors (GPCRs) (Simon et al., 1991). Comparison of the deduced amino acid sequences of GPCRs reveals a similar secondary structure: a single polypeptide chain containing an amino-terminal region, six relatively conserved hydrophobic domains plus a seventh region of lower hydrophobicity spanning the membrane (seven transmembrane region), and a carboxy terminal region (Savarese and Fraser, 1992).

The amino-terminal sequence of most GPCRs contains putative sites for N-linked glycosylation (Asn-Xaa-Ser/Thr) and is presumed to be located extracellularly. The membrane-spanning regions of GPCRs contains acidic amino acids (aspartate and glutamate) and the putative second and third extracellular domains contain cysteine residues (disulphide bond) involved in ligand binding. Many neurotransmitters have a positive charge centre and many peptide hormones contain basic amino acids indispensable for biological activity. This raises the possibility that ligand binding may, in part, involve acidic amino acids. Conserved transmembrane aspartate residues and cytoplasmic domains (third cytoplasmic domain) are also involved in receptor activation by conformational change that facilitates receptor-G-protein interactions and G-protein activation (Savarese and Fraser, 1992). The carboxy-terminal sequence contains putative phosphorylation sites and is also involved in homologous or heterologous desensitization and receptor down-regulation (Savarese and Fraser, 1992). Thus, strictly conserved amino acid residues may play a role in maintaining the structure of the receptor, perhaps by determining protein folding, whereas those residues conserved among major classes may play a role in defining their unique functional properties (Savarese and Fraser, 1992).



E_1 Effectors
cGMP-PDE
Adenylyl Cyclase
PLC-β
Na^+/K^+ Exchange
Ca^{2+} Channels

E_2 Effectors
PLC-β
I_kAch GIRK1/CIRK$^+$
Adenylyl Cyclase
β-Adrenergic Receptor Kinase

FIGURE 1.2 AN OVERVIEW OF G-PROTEIN SIGNALLING
(Taken from 1998 Signal Transduction Calbiochem catalog)

1.5.1 G-PROTEINS

Signal transduction G-proteins occur in two forms, (i) "small G proteins" that are generally found as single polypeptides composed of about 200 amino acids associated with growth factor receptors, such as RTKs and (ii) heterotrimeric G-proteins that are made up of α (36-52 kDa), β (35-36 kDa), and γ (8-10 kDa) subunits and are associated with GPCRs (Simon et al., 1991). The small G-proteins, such as the members of the $p21^{ras}$ family, function in regulating cell growth, protein secretion and intracellular vesicular trafficking. The heterotrimeric G-proteins are generally associated with signal transduction by GPCRs.

Signal transduction by GPCRs is initiated by ligand binding which stabilizes an alternate conformational form of the receptor and thus transmits information across the cell membrane. The ligand-bound receptor initiates two processes: one leads to desensitization, and occurs through receptor modification, and the other is a signal-generating process that begins with the activation of the heterotrimeric G-protein (Simon et al., 1991).

Interaction of the G-protein with the activated receptor promotes the exchange of guanosine diphosphate (GDP), bound to the α subunit, for guanosine triphosphate (GTP) and the subsequent dissociation of the α-GTP complex from the bg heterodimer. The α subunit with GTP bound and the free βγ subunit may interact with effector proteins that further amplify the signal. Such effectors include ion channels and enzymes, such as adenylyl cyclases, guanylyl cyclases, phospholipases, and phosphodiesterases, that generate regulatory molecules or second messengers (Rens-Domiano and Hamm, 1995) (Figure 1.2).

Low molecular weight second messengers, such as cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP) or intracellular Ca^{++} in turn, generate dramatic changes including selective protein phosphorylation, activation of gene transcription, cytoskeletal reorganization, secretion and membrane depolarization (Simon et al., 1991). Termination of the signal occurs when GTP bound by the a subunit of the G protein is hydrolyzed to GDP. The a subunit exhibits an intrinsic but slow GTPase activity. Hydrolysis of bound GTP leads to reassociation of the α subunit with the βγ complex and stops further effector enzyme activation.

1.5.1.1 Gα SUBUNITS

Four families of a subunits of G-proteins have been identified: Gs, Gi, Gq and G_{12} (Rens-Domiano and Hamm, 1995). The Gs family includes Gs and Golf, and mediates the hormonal stimulation of adenylyl cyclase and closing of Ca^{++} channels. The Gi family includes Gi, Gt,

Go, Ggust and Gz. Gi is generally involved in the inhibition of adenylyl cyclase and opening of K^+ channels. Gt mediates the stimulation of cGMP phosphodiesterase and Go, primarily a neuronal G-protein, mediates Ca^{++} channel closure and inhibition of phosphoinositol (PI) turnover. Gq are generally coupled to the activation of phospholipase and stimulation of PI turnover. G12 and G13 compose the fourth class of G-proteins and their effectors systems are unclear (Rens-Domiano and Hamm, 1995).

The α subunit consist of two domains: (i) a GTPase domain that contains the guanine nucleotide-binding pocket as well as sites for binding receptors, effectors and βγ subunit, and (ii) a helical domain. The amino-terminus appears to be an important site of interaction with Gβγ. The carboxy-terminus has an important role in defining the specificity of G-protein receptor interactions and is also involved in receptor desensitization (Rens-Damiano and Hamm, 1995).

The functional role of specific α subunits is not obvious from their structural classification. Activated Gi subunits lower intracellular cAMP levels. However, Go has been implicated in increasing PI turnover. GTP dependent signalling pathways resistent to pertussis toxin (PTX) have also been described; all of these lack the cysteine residues four amino acids from the carboxy-terminal end that is the target for PTX-mediated adenosine diphosphate (ADP) ribosylation. They include two novel classes: G11 and Gq involved in PTX-resistant coupling to PLC activation (Rens-Damiano and Hamm, 1995).

1.5.1.2 Gβγ SUBUNITS

During the signalling process, βγ subunits act to coordinate cellular responses. βγ subunit increases the affinity of the a subunits for GDP and can facilitate deactivation of α subunits, activities which promote the inactive state of the G-protein. However, they also have several additional functions, these include: (i) localizating a subunits in the membrane, (ii) stabilising the interaction of a subunits with receptors in a functional complex, (iii) modulating the effects of activated a subunits (Neer, 1994, 1995).

Thus, βγ subunits act as a helper protein that facilitates activation-deactivation, localization and stability of α-subunits. However, bg subunits directly can be activated effector enzymes, such as β-adrenergic receptor kinase, PLA2, PLCβ2, types II and IV adenylyl cyclases, phosducin, muscarinic K^+ channels, PH domain-containing tyrosine kinases, PI-3-K and N-type Ca^{++} channels (Sternweis, 1994). The βγ subunit may also act through $p21^{ras}$ to activate MAPK pathway (Crespo et al., 1994).

1.5.1.3 G-PROTEINS AS MITOGENS

Our current understanding of the process regulating cell growth, division, and differentiation has been derived from the study of transforming viral oncogenes and their cellular homologues, principally on the role of receptor and non-receptor tyrosine kinases as regulators of low molecular weight G-proteins, such as $p21^{ras}$ and its relatives. The low molecular weight G-proteins, like their heterotrimeric counterparts, are signalling intermediates whose activation is achieved by exchange of GDP for GTP, but unlike heterotrimeric G-proteins, the low molecular weight G-proteins lack the intrinsic ability to catalyze GDP release and GTP hydrolysis and are therefore dependent on exogenous proteins, guanine-nucleotide releasing proteins (GNRPs), GDP-dissociation inhibitors (GDIs) and GTPase activating protein (GAPs) for this regulation (Simon et al., 1991).

Several ligands that signal via GPCRs have been shown to elicit mitogenic responses, including stimulation of DNA synthesis, expression of nuclear oncogenes and cell proliferation (Van Biesen et al., 1996). Thus, the potential of GPCRs to act as proto-oncogenes has been suggested, indicating that GPCRs could play a important role in tumour induction or progression. Activation of MAPK cascade by GPCRs signalling via PTX-sensitive and insensitive G-proteins has been reported (Van Biesen et al., 1996). GPCRs agonists, including angiotensin II, somatostatin, endothelin-1, interleukin-8, bombesin, oxytocin and vasopressin induce MAPK activation. The MAPK activation by GPCRs coupled to PTX-insensitive G-proteins occurs via two distinct signal pathways: (i) Gs-dependent and/or (ii) Gq-dependent (Van Biesen et al., 1996).

The role of Gs in regulating cell growth is complex and apparently cell type-specific. cAMP was the first second messenger to be identified, and its role in regulating physiological processes is well established. Hormone receptors increase intracellular cAMP by increasing the amount of the free a subunit of the GTP binding protein which activates adenylyl cyclase (Simon et al., 1991). cAMP inhibits the proliferation of many cell lines in the early Go to G1 phase as well as mid-G1 phase and G2. In contrast, in some cells, such as Swiss 3T3 cells, cAMP is a mitogenic messenger and promotes the G1 to S phase transition in the cell cycle. Lack of forskolin-mediated MAPK-1/2 inhibition in COS-7 and Swiss 3T3 cells and an activating role for cAMP on MAPK activation in PC12 cells (Frödin et al.,1994; Young et al.,1994) have been observed, suggesting a cAMP sensitive pathway in these cells. Furthermore, a constitutively activated Gs mutant and a cAMP analog increased MAPK-1 activity in COS-7 cells (Faure et al., 1994). Thus, these results indicate that Gs-coupled receptors stimulate the MAPK pathway by activating adenylyl cyclase and raising intracellular cAMP in these cell lines.

Gq activates PLC-β, resulting in the conversion of PI-4,5-P2 (PIP2) to inositol triphosphate (IP3) and diacylglycerol (DAG) (Simon et al., 1991). An activated Gq mutant and activation of PLCβ2 stimulate MAPKs (Hawes et al., 1995). Activation of MAPK by the PTX-insensitive Gq/11 has been reported in both *ras* -dependent (Eguchi et al., 1996) and *ras* -independent manners (Ueda et al., 1996). Treatment of the PC12 cells with tumor-promoting phorbol esters that directly activate PKC results in the activation of MAPK in a *ras* -independent manner. Futhermore, the down-regulation of PKC activity in CHO and COS-7 cells by chronic exposure to phorbol esters blocks Gq-mediated mitogenic signals (Hawes et al., 1995). Gq also activates a series of intracellular signals, which include Ca^{++}/calmodulin and PKC activation of specific non-RTKs, such as PYK2, which in turn mediates *ras* -dependent MAPK activation of Shc and Shc-Grb2-Sos complex formation. These observations define a Gq- and Ca^{++} -mediated mitogenic pathway that requires the same intermediates as the RTK-mediated pathways (Van Biesen et al., 1996).

MAPK-1/2 activation via GPCRs coupled to PTX-sensitive pathway occurs via Gβγ subunits. Gβγ subunits are capable of transmitting mitogenic signals in COS-7 cells and these signalling pathways appear to require the activity of RTKs and not PI turnover, supporting the hypothesis that Gi-receptors activate mitogenesis by elevating intracellular level of tyrosine phosphorylation of the RTKs and/or Shc-Grb2 adapter protein via a non-RTK, such as *src* -family kinases. Protein tyrosine phosphatases such as SHP2 may mediate dephosphorylation of the carboxy-terminal regulatory tyrosine residue of c-*src* family kinases, thereby stabilizing the kinase in the active state. Thus, GPCRs and RTKs can induce $p21^{ras}$ activation via convergent pathways (Van Biesen et al., 1996).

1.5.2 GPCR SUBTYPES

GPCRs can be further classified into three subfamilies: (i) rhodopsin/β adrenergic, (ii) secretin/vasoactive intestinal peptide (VIP) and (iii) metabotropic glutamate (mGlu) receptor families (Strader et al., 1995). The secretin/VIP family includes receptors for the peptides calcitonin, secretin, glucagon, glucagon-like peptide I, gastric inhibitory peptide, parathyroid hormone, vasoactive intestinal peptide (VIP), pituitary adenylyl cyclase activating peptide (PACAP) and growth hormone releasing hormone (Strader et al., 1995). These receptors share 25-50% amino acid identity among themselves and little primary sequence homology with the rhodopsin/b-adrenergic and metabotropic glutamate receptor families.

The secretin/VIP family of GPCRs all contain an amino-terminal hydrophobic domain, which is presumed to serve as signal sequence. Within this amino-terminal hydrophilic domain are a series of conserved cysteines presumed to play a role in determining the tertiary structure of

this region. The sequence conservation in the amino-terminal region of these receptors suggest a role in mediating receptor-ligand interactions. All members of the secretin/VIP subfamily are coupled to Gs and upon activation of intracellular cAMP (Strader et al., 1995). In addition to coupling to Gs, many of these receptors (e.g. calcitonin, glucagon, PACAP, VIP) have been reported to activate other G-proteins, leading to increases in intracellular Ca^{++} levels and PI hydrolysis. Thus, it appears that the potential to signal through multiple second messenger pathways may be a hallmark of this receptor subfamily (Strader et al., 1995).

1.6 PITUITARY ADENYLYL CYCLASE ACTIVATING POLYPEPTIDE (PACAP)

PACAP is a polypeptide discovered as a consequence of a search for candidate novel hypothalamic-releasing hormones (Arimura, 1992a; Arimura and Shioda, 1995). The search strategy centred around the observations that releasing hormones, such as growth hormone-releasing hormone, luteinizing hormone releasing-hormone and corticotropin releasing hormone, increase cAMP as a common response parameter. The search for a candidate hormone that increases cAMP in pituitary cells resulted in the isolation of two substances from ovine hypothalamus which are now known as PACAP-27 and PACAP-38 (Miyata et al., 1989).

1.6.1 PACAP BIOCHEMISTRY

PACAP is synthesised from a single gene as a precursor protein of 176 amino acids (human) and 175 amino acids (rat), with a molecular weight of approximately 19.5 kDa. The amino acid sequence corresponding to PACAP-38 is located between His-132 and Lys-169, which are flanked by sequences recognised in the post-translational proteolytic processing of various peptide hormone precursors (Steiner et al., 1980). Contained within the PACAP-38 sequence is another consensus sequence for proteolytic processing which likely yields PACAP-27. Several lines of evidence support the hypothesis that PACAP-27 and PACAP-38 are generated from the same precursor polypeptide (Arimura, 1992a). PACAP has been shown to belong to the same polypeptide family as vasoactive intestinal peptide (VIP). Indeed, the amino-terminal 28 amino acid residues of PACAP-38 show 68% homology with vasoactive intestinal peptide (VIP) (Arimura, 1992a).

The cDNA for rat PACAP-38 was cloned and shown to be identical at the amino acid level to both human and ovine peptide (Kimura et al., 1990; Ogi et al., 1990). The human genomic clone has been isolated and reveals PACAP to be a gene of five exons and four introns (Hosoya et al., 1992). PACAP-38 is encoded, however, by the single exon (number five); thus, PACAP-27 and PACAP-38 are not generated by alternative splicing. Southern blot

analysis, even under low-stringency conditions, indicating that the PACAP-38 gene is probably unique (Arimura, 1992a).

Interestingly, the 5'-flanking promoter region of the human PACAP gene contains no apparent TATA, CAAT or GC boxes, which are sequences known to be required for accurate initiation of transcription. Several cyclic AMP responsive elements (CREs) and a TPA response element (TRE) have, however, been identified in the 5'-flanking region of the gene which may mediate transcriptional regulation by cAMP/PKA or PKC-dependent pathways (Ohkubo et al., 1994). PACAP expression can be induced by cAMP or phosphodiesterase inhibitors in IMR32 cells, a PACAP producing neuroblastoma cell line, and therefore it is possible that autocrine stimulation of PACAP expression occurs (Ohkubo et al., 1994). The PACAP gene is localised to chromosome 18p11 which has been associated with holoprosencephaly, or failure of the forebrain to divide into hemispheres; this finding provides supporting evidence for a role of PACAP in brain development (Arimura and Shioda, 1995).

1.6.2 PACAP DISTRIBUTION

A variety of studies have indicated that PACAP distribution in neurons is similar among mammalian species and has pleiotropic distribution (Arimura and Shioda, 1995). PACAP has been found to be abundant in CNS as well as in PNS such as adrenal gland (Arimura et al., 1991). The rat brain contains high levels in basal forebrain, hypothalamus and brainstem. Moderate concentrations of PACAP were found in cerebral cortex, hippocampus, thalamus, amygdala and locus coeruleus, brainstem, posterior pituitary and spinal cord, where many PACAP-immunoreactive (PACAP-IR) fibres and terminals were detected. PACAP-IR nerve cell bodies have also been demonstrated in the dorsal root and trigeminal ganglia (Arimura, 1992a; Arimura and Shioda, 1995).

In the periphery, PACAP-IR nerve fibres occur in several organs, including the blood and lymphatic vascular systems, adrenal gland, skin, digestive, respiratory, urinary, and reproductive organs and the eye (Arimura and Shioda, 1995). PACAP and VIP have been found to coexist in nerve cell bodies and fibres in the gut of chickens and humans and in fibres in the gastric mucosa of mice, as well as in neurons in the human ovarian cortex. PACAP-IR has also been identified in both endocrine and exocrine parts of rat and mouse pancreas. In all other regions of the gut, many PACAP-IR and VIP-IR cell bodies and fibres were distinct. The demonstration of PACAP-IR in peripheral lymphoid tissues such as mesenteric lymph nodes (Gaytan et al., 1994) suggests that PACAP may have a potential role in immune system communications and thus may serve to integrate communication between neuroendocrine and immune systems (Arimura and Shioda, 1995).

1.6.3 PACAP RECEPTORS

Both PACAP and VIP have been demonstrated to target cells via cell-surface receptors which have been identified to belong to the subfamily of secretin/glucagon, seven-transmembrane spanning region, GPCRs superfamily (Strader et al., 1995). Several receptors specific for PACAP or VIP have now been identified pharmacologically and the cDNAs encoding these receptors have been characterised. These defined receptors are distinct with regard to primary amino acid sequence and ligand affinity (Ishihara et al., 1992; Spengler et al., 1993).

Two distinct PACAP receptors, type I and type II, have been defined on the basis of pharmacology, signalling activation and distribution (Harmar and Lutz, 1994; Journot et al., 1994; Arimura and Shioda, 1995). The PACAP type I receptor has a high affinity for PACAP but much reduced affinity for other members of the secretin/glucagon/VIP subfamily, while the PACAP type II receptor, also known as the VIP type I receptor, can bind PACAP and VIP with similar affinities (Couvineau et al., 1994; Arimura and Shioda, 1995). Two isoforms for the type I receptor, designated type Ia and type Ib, on the basis of differential affinities for PACAP-27 and PACAP-38 have been proposed (Arimura, 1992b).

1.6.3.1 PACAP TYPE I RECEPTOR

The cloning of the rat and human PACAP type I receptors have been reported (Hashimoto et al., 1993; Hosoya et al., 1993; Morrow et al., 1993; Pisegna and Wank, 1993; Spengler et al., 1993). The PACAP type I receptor is encoded by an mRNA of between 7.5 and 9.5 kb and belongs to the GPCR superfamily with seven conserved transmembrane domains, containing many of the motifs expected for a G protein-coupled receptor. While the amino-terminal domains of the secretin/glucagon/VIP receptor family are highly divergent, substantial similarity at specific positions (including six cysteine residues, postulated to confer general conformation of the extracellular binding domain) is noted (Mayo, 1991). The PACAP type I receptor is most similar to that of the VIP type I receptor, with 51% homology. Homologies with other secretin/glucagon/VIP receptors lie between 47% for secretin receptor and 37% for parathyroid hormone and gastric inhibitory peptide.

Rat and human PACAP type I receptors share 92.5% amino acid homology and similar pharmacology (Ogi et al., 1993); PACAP-27 (IC_{50} = 0.5-1.3 nM) and PACAP-38 (IC_{50} = 0.2-1.3 nM) are very much more potent than VIP (IC_{50} > 1μM) in displacing the binding of [^{125}I] PACAP-27 (Arimura and Shioda, 1995). As for the VIP type I receptor, the PACAP type I receptor is coupled positively to adenylyl cyclase and cAMP production. The relative effectiveness of these compounds in stimulating adenylyl cyclase activity for the PACAP type I

receptor, however, is markedly different; half-maximal stimulation of cAMP production is achieved at 0.1 nM for PACAP-27, 0.4 nM for PACAP-38 or greater than 1000 nM for VIP (Journot et al., 1994).

The array of receptor types available to PACAP is further extended by the existence of a number of splice variants for the PACAP type I receptor (Spengler et al., 1993). Unlike many GPCRs previously described, the PACAP type I receptor mRNA can be alternatively spliced resulting in the generation of multiple receptor variants. Variation resulting from alternate splicing of the PACAP type I receptor mRNA is localised to the third intracellular loop of the receptor, an area known to be required for the molecular coupling of receptors of this class with G-proteins and thus with the effector molecules (Savarese and Fraser, 1992).

Differential splice variants are generated by insertion in the third intracellular loop of either one and/or two cassettes of 28 amino acids ("Hip" and "Hop") (Spengler et al., 1993). In addition, two variants of the Hop cassette exist; in the Hop-2 variant, the Ser-349 which is found within the Hop-1 species is deleted. Thus, the primary gene transcript of PACAP type I receptor is capable of alternative processing of these exon cassettes to yield mature mRNA containing any of the Hip, Hop-1 or Hop-2 insertions. Additionally, the generation of concatamers of the cassettes Hip or Hop during differential splicing results in mRNA containing both Hip-Hop1 or Hip-Hop2 (Journot et al., 1994). Indeed, as many as five separate splice variants have been identified which are generated by the alternative splicing of an 84 base pair exon which encode the third intracellular loop of this receptor. This region of the receptor has previously been implicated in coupling with accessory signalling machinery, such as G-proteins (Savarese and Fraser, 1992).

The existence of multiple variants of the receptor at this site may reflect a mechanism for the coupling of PACAP to multiple signalling pathways. GPCRs have been directly and/or indirectly linked with multiple signalling pathways, including adenylyl cyclase, PLC, MAPK and PI-3-K (Hepler and Gilman, 1992; Exton, 1994; Neer, 1994; Gudermann et al., 1996; Van biesen et al., 1996). Furthermore, it has been demonstrated that the profile of expression of splice variants is tissue-dependent as well as perhaps developmentally regulated (Spengler et al., 1993). Thus, ligands signalling through the PACAP type I receptor are potentially capable of signalling through a wide variety of pathways; moreover, the pathway of choice in any given instance, may be dependent upon the tissue involved and its physiological and/or developmental status.

The PACAP type I receptor has been localised to a variety of tissues on the basis of autoradiographic, binding strategies and/or *in situ* hybridisation (Arimura and Shioda, 1995). The PACAP type I receptor has a widespread distribution in CNS with highest levels in the

olfactory bulb, the dentate gyrus of the hippocampus and cerebellum, and significant concentrations in hypothalamus, thalamus, brain stem, cerebral cortex, basal ganglia and anterior and intermediate lobes of the pituitary which is consistent with its role in the regulation of pituitary functions. At the periphery, the PACAP type I receptor is predominantly found in the adrenal gland and testis (Cauvin et al., 1991; Lam et al., 1990).

1.6.3.2 PACAP TYPE II RECEPTOR

The existence of a PACAP type II distinct from type I was initially characterised on the basis of pharmacology by virtue of its similar affinities for PACAP or VIP (Harmar and Lutz, 1994). These peptides compete for a single binding site in lung, liver, intestine, ovary, thymus and splenocytes but these receptor sites are negligible in hypothalamus and pituitary. The distribution of type II PACAP receptor is shared with that for VIP receptor type 1, which has also been demonstrated to exhibit similar affinities for PACAP and VIP. Thus, there is current consensus that the PACAP type II receptor is identical to the VIP type I receptor since their distribution, pharmacology and putative intracellular signalling coupling mechanisms appear to be the same.

1.6.4 PACAP-VIP BIOLOGICAL ACTIONS

PACAP has been shown to affect a wide variety of physiological processes in a large number of tissues and organs; these actions appear to be mediated via cAMP and PI turnover induced by activation of PACAP receptors (Arimura and Shioda, 1995). Specifically, PACAP has been demonstrated to serve as neurotransmitter and neuromodulator (Guo and Wakade, 1994), pituitary releasing hormone (Miyata et al., 1989), vasorelaxant dilator (Arimura, 1992a), secretagogue (Yada et al., 1994), mediator of neuroimmune communication (Gaytan et al., 1994) and to play a role in reproductive activity (McArdle, 1994).

The high concentration of PACAP-38 in the adrenal gland in rat and other animals suggests an important functional role in adrenal chromaffin cells. PACAP-38 has been localised to adrenaline-containing cells of the adrenal medulla; no co-existence with vasoactive intestinal peptide (VIP) has been noted. Moreover, PACAP-IR fibres have also been identified in the adrenal medulla (Tabarin et al., 1994). The PACAP type I receptor has been identified by autoradiography (Shivers et al., 1991) and PACAP type I receptor mRNA is found in adrenal medulla tissue (Spengler et al., 1993).

A role for VIP in trans-synaptic regulation of the TH activity has been implied by the finding that VIP-IR is present in cells of the intermediolateral column (IML) (Sasek and Zigmond, 1989) and adrenal medulla (Yoshikawa et al., 1990). VIP is also present in ACh positive nerve

terminals of sympathetic ganglia and SIF cells (Lars-Gösta et al., 1993). VIP has been reported to be as potent as ACh in evoking the secretion of catecholamines in the adrenal gland (Malhotra et al., 1987). VIP increases TH activity within SCG (Schwarzchild and Zigmond, 1989) and adrenal gland (Malhotra and Wakade, 1987; Malhotra et al., 1989). VIP also increased the activity of existing pools of TH (Roskoski et al., 1989) and induced the expression of the TH gene in PC12 cells (Wessels-Reiker et al., 1991). Thus, trans-synaptic activation of TH in postganglionic sympathetic nerves via non cholinergic neuromodulators, such as VIP and PACAP present in pre-ganglionic nerve terminals is implicated in the restoration of synaptic stores of catecholamines. VIP and PACAP also stimulated transcriptional activation of the NPY gene in a PKA-dependent manner in PC12 cells (Colbert et al., 1994).

PACAP induces adenylyl cyclase activity and elevates cytosolic Ca^{++} levels in adrenal tissue (Watanabe et al., 1992). Other cells of SA lineage are well known to be responsive to VIP in micromolar amounts but PACAP stimulates cAMP production more potently than VIP in these cases, generally at nanomolar concentrations. Moreover, both PACAP and VIP, at 0.1-1 nM and 0.1-1 μM, respectively, stimulate the phosphorylation of TH, the rate-limiting enzyme in the synthesis of catecholamines in PC12 cells (Strong et al., 1992). PACAP has been demonstrated to modulate catecholamine and NPY expression in PC12 cells (Colbert et al., 1994) and in rat superior cervical ganglion (SCG) (May and Braas, 1995). Stimulation of the splanchnic nerve induces PACAP levels in adrenal perfusates (Wakade et al., 1992). Thus, like VIP, PACAP would appear to function as a physiological noncholinergic neurotransmitter in the rat SA system (Watanabe et al., 1995).

In addition to these actions, PACAP and VIP are involved in differentiation, neural growth and survival of specific rat neuronal cells, such as PC12 cells (Deutsch and Sun, 1992; Colbert et al., 1994; Okumura et al., 1994; Hernandez et al., 1995), sympathetic neuroblast (Dicicco-Blom and Deutsch, 1992), corticotrope cell line (Braas et al., 1994) and neonatal chromaffin cells (Wolf and Krieglstein, 1995). The state of differentiation and secretory profile of mammalian cells of SA lineage are altered by the neuroendocrine peptide VIP but typically at micromolar or higher concentrations (Colbert et al., 1994; Okumura et al., 1994). Both, PACAP-27 and PACAP-38 are much more potent activators (typically at nanomolar concentrations) than VIP on signal transduction in cells of SA lineage (Deutsch and Sun, 1992; Colbert et al., 1994; Hernandez et al., 1995).

A trophic role for VIP through cAMP has been suggested using cultured sympathetic neuroblast (Pincus et al., 1990). Incubation of the PC12 cells with micromolar concentrations of VIP induces neurite extension (Colbert et al., 1994; Okumura et al., 1994). In response to VIP treatment over several days, PC12 cells appeared to take on a neuronal morphology. Thus

the cells flatten down and extend long, slender neurites with prominent growth cones (Colbert et al., 1994; Okumura et al., 1994). However, a lack of neurotrophic effect of VIP on PC12 cells over similar time courses even when comparable concentrations of the peptide were added to cell cultures under similar conditions to those described (Deutsch and Sun, 1992; Hernandez et al., 1995). The basis for these contradictory findings remains unclear but may again reflect inherent variability in the PC12 cell population in different laboratories.

The effects of PACAP on morphological differentiation and growth of PC12 cells are the same as those seen upon treatment with VIP. Several other reports describe the morphological effects of PACAP on this pheochromocytoma-derived cell line (Deutsch and Sun, 1992; Frödin et al., 1994; Hernandez et al., 1995). Both the 27- and 38-amino acids forms of PACAP stimulate adenylyl cyclase and elevate cAMP levels in PC12 cells with a EC_{50} at nanomolar levels (Deutsch and Sun, 1992). PACAP-38 additionally is a potent activator of the PI turnover in PC12 cells, elevating the content of inositol phosphates by 8-fold ($EC_{50} = 7 \times 10^{-9}$ M) and is an effective inducer of neuronal morphology, whereas PACAP-27 is 200-fold less potent in activating the PI turnover and much less active in promoting neurite outgrowth (Deutsch and Sun, 1992). Moreover, the concentration of PACAP required to elicit a comparable differentiation response to that of VIP in PC12 cells, is a thousand times lower. The pharmacology of these differences mirrors the peptide binding distinctiveness of the PACAP type I and PACAP type II/VIP type I receptors (Arimura, 1992b; Arimura and Shioda, 1995). Similar findings have been reported by a number of laboratories (Watanabe et al., 1990; Colbert et al., 1994; Okumura et al., 1994). The differential sensitivity of PC12 cells to PACAP and VIP may reflect signalling of these peptides through the PACAP type I receptor rather than the PACAP type II/VIP type I receptor.

The PACAP type I receptor is known to be coupled to cAMP production as well as PI turnover (Spengler et al., 1993; Harmar and Lutz, 1994; Journot et al., 1994). The precise molecular coupling machinery of the receptor to these pathways remains to be elucidated but is likely to involve the variable third intracellular loop which is the site of previously described alternate splicing. The Hop cassette encodes a consensus motif for phosphorylation by PKC and may thus possibly contribute to the specific fine-tuned regulation of PACAP receptor function (Harmar and Lutz, 1994; Journot et al., 1994; Spengler et al., 1993). The presence of the Hip cassette has been reported to impair adenylyl cyclase stimulation and abolish PLC stimulation (Spengler et al., 1993). Expression of the Hip-Hop concatamer has been demonstrated to elicit intracellular signalling responses that are intermediate between those of Hop and Hip isoforms. In the adrenal gland, the Hop variant has been reported to be almost exclusively expressed; no concatamer is detectable in this tissue (Spengler et al., 1993).

The PACAP type I receptor containing the Hip cassette identified in PC12 cells plays a prominent role in VIP- or PACAP-induced neuronal differentiation in these cells. There is evidence that PACAP acts through the MAPK cascade in PC12 cells (Frödin et al., 1994; Young et al., 1994; Barrie et al., 1997). The mechanisms involved in such potential signalling pathway capability are not yet resolved but recruitment of molecules along these pathways also may well be influenced by the upstream PACAP receptor splice variants available.

The change to a neuronal pattern of morphology observed in PC12 cells following the addition of PACAP or VIP suggests that these peptides may play a trophic role in the nervous system, in addition to the neurotransmitter function of PACAP and VIP in postganglionic sympathetic nerves. Thus, within preganglionic sympathetic nerves, the trophic role of these peptides may be their primary action in the developing nervous system, whereas their regulation of sympathetic nerve function may occur later once the specialised enviroment is achieved by high steroid input to the adrenal medulla and the establishment of NGF dependence in the sympathetic nervous system.

That such classical neuromodulators may have the novel capability to act as trans-synaptic or autocrine neurotrophins upon cells derived from the neural crest is supported by several lines of evidence. Firstly, VIP and PACAP-IR have been identified in cells or afferent fibres of sympathetic ganglia and chromaffin cells and, in some cases, in these cells themselves and thus may act through classical synaptic transmission mechanisms to mediate unorthodox action (Sasek and Zigmond, 1989; Tabarin et al., 1994; Arimura and Shioda, 1995). Moreover, the time of expression initiation *in vivo* of these peptides co-incides with innervation and differentiation schedules in the ontology of sympathetic development; VIP expression appears one day after functional connections with preganglionic neurons are established (Tyrrell and Landis, 1994). Thus, it has been proposed that VIP and PACAP may serve as trophic agents in the development of SA cells at a time prior to, or overlapping with, that identified for the classic NGF action.

1.6.5 PACAP-VIP SIGNALLING PATHWAY

PACAP-27 and PACAP-38 increase adenylyl cyclase activity and elevate intracellular cAMP levels in PC12 cells at nanomolar concentrations. PACAP-38 is a potent activator of PLC and PI turnover in PC12 cells and is an effective inducer of neuronal morphology, whereas PACAP-27 is much less potent in activating PI turnover and in promoting neurite outgrowth (Deutsch and Sun, 1992; Hernandez et al., 1995). Thus, PACAP-38 but not PACAP-27, results in a concentration-dependent increase in neurite outgrowth in PC12 cells. PACAP-38 result in a rapid but transient activation of MAPK-1 (Frödin et al., 1994; Young et al., 1994;

Barrie et al., 1997) consistent with the binding to the PACAP type I receptor and the biological potency of PACAP-38 in PC12 cells and chromaffin cells.

Forskolin, an adenylyl cyclase activator acted non-additively with PACAP-38 on MAPK-1 activation indicating cAMP involvement in the PACAP-38 response in PC12 cells (Frödin et al., 1994). PACAP-38 stimulation of MAPK-1 is inhibited following PKC downregulation with phorbol esters, suggesting PKC is also involved in the PACAP-38 response (Frödin et al., 1994). PACAP-38 potentiates the NGF-stimulated neurite outgrowth with a concominant potentiation of NGF-stimulated MAPK-1 activation (Frödin et al., 1994). Taken together these observations with PACAP-38 in PC12 cells, suggest that the synergistic stimulation of MAPKs by cAMP/PKA and activators of PKC occur upstream in the MAPK cascade and this stimulation appear to be involved in the neuronal differentiation of PC12 cells (Barrie et al., 1997).

1.7 cAMP IN SIGNALLING PATHWAY

1.7.1 ADENYLYL CYCLASE

Adenylyl cyclases comprise a heterogeneous multigene family, members of which are variously regulated by the Gα and Gβγ subunit of G-proteins, by Ca^{++} and by protein kinases (Mons and Cooper, 1994). All eight isoforms share a commom double motif with six membrane-spanning domains, and two large cytoplasmic segments. Based on sequence similaraties, the isoforms have been classified into three subfamilies : (i) the type I-like group (types I, II and VIII) are stimulated by Ca^{++} and Gs but not by Gβγ or PKC, (ii) the type II-like group (types II, IV and VII) are stimulated by Gs and Gβγ but not by Ca^{++} and PKC, (iii) the type V-like group (types V and VI) are stimulated by Gs but not by Gβγ or PKC and are inhibited by Ca^{++} (Mons and Cooper, 1994). Functional properties are shared within these subfamilies, although individual isoforms display unique responses. Broadly speaking, each adenylyl cyclase can be regulated by a subunits of a G-protein, although type I, in particular, is quite refractory to stimulation by Gs and the type II-like group is refractory to inhibition by Gi. However, individual isoforms show a range of responses to factors such as Ca^{++}, PKC and bg subunits of G-proteins (Mons and Cooper, 1994).

1.7.2 cAMP-DEPENDENT PROTEIN KINASE (PKA)

cAMP is one of the most ubiquitous regulatory molecules in biological systems. It controls a great variety of metabolic processes. Its role in regulating transcriptional activity is central to the role it plays in many systems. Several lines of evidence implicate the activation of PKA as an intermediate in the transcriptional activity of cAMP. Transfection of the somatostatin gene

cAMP responsive element (CRE) in PC12 cells deficient in type II cAMP dependent protein kinase showed that this element cannot respond to cAMP in these cells (Montminty et al., 1986). Furthermore, transfection of a DNA encoding an inhibitor of the catalytic domain of PKA blocks induction of genes which are upregulated by cAMP (Grove et al., 1987). This protein kinase inhibitor (PKI) is a 77 amino acid (8 kDa) protein which binds and inhibits the PKA catalytic subunit in a potent (Ki = 0.098 nM) and highly specific manner.

Phosphodiesterase hydrolysis of cAMP leads to a reassociation of the catalytic and regulatory subunits regenerating the inactive holoenzyme and leading to cessation of cAMP effects in the cells (Walaas and Greengard, 1991). Five major classes of phosphodiesterases have been reported: (i) type I (CaM-dependent), (ii) type II (cGMP-stimulated), (iii) type III (cGMP-inhibited), (iv) type IV (cAMP-specific) and (v) type V (cGMP-specific). These isozymes exhibit sequence homology in their catalytic domain and are differentially expressed and regulated in different cells.

PKA consist of tetramers composed of two regulatory subunits and two catalytic subunits that, in the absence of cAMP, bind together to form an inactive complex. Molecular genetic studies have shown that there are multiple isoforms of catalytic and regulatory subunits which have distinct properties and tissue distribution (Walaas and Greengard, 1991). PKA is found in two classes or isoforms which differ in their regulatory subunits. PKA type I is soluble and widely expressed, whereas the PKA II isoforms, PKAIIα and PKAIIβ, are membrane anchored and are particularly abundant in neuroendocrine cells, such as PC12 cells. The regulatory units of PKA have two cooperative binding sites for cAMP which induce them to dissociate from the catalytic subunits, making them active. Thus, the free catalytic subunit translocates to the nucleus and phosphorylates different transcription factors (Cassano et al., 1996).

Activation of PKA by cAMP causes phosphorylation of transcription factors which are responsible for the transcriptional activity of cAMP. Transcription factors under control of PKA include the activator protein 2 (AP-2) and CREB (Roesler et al., 1988). The mechanisms by which phosphorylation of these transcription factors affects their transcriptional activity is not completely clear. Although phosphorylation does not seems to affect binding affinity of AP-2 or CREB to their DNA consensus sites, it is clear that phosphorylation is important for the increase in transcriptional activity suggesting that other mechanisms are involved in mediating cAMP-induced transcription (Roesler et al., 1988).

1.7.3 cAMP RESPONSIVE ELEMENT (CRE)

cAMP functions to coordinate diverse metabolic processes ranging from the breakdown of glycogen to the synthesis of a number of specific enzymes. The mechanisms by which cAMP

exerts these diverse effects are pleiotropic in nature altering numerous steps in a metabolic pathway. cAMP is also responsible for a very marked induction of gene expression in eukaryotes (Roesler et al., 1988). The promoter-regulatory regions of these genes have been isolated and CRE characterized by functional analysis. There are two classes of genes which are transcriptionally induced by cAMP: (i) Group 1 genes are those which are rapidly regulated by cAMP, usually within minutes and in a cycloheximide-insensitive manner, (ii) Group 2 genes are those where the transcription is increased only after several hours of cAMP treatment and in a cycloheximide-sensitive manner. Since transcription of the genes in Group 1 seems to be insensitive to cycloheximide, in turn their responsiveness to cAMP most likely involves the rapid modification of a pre-existing protein to a more active form (Roesler et al., 1988).

There are two classes of *cis* -acting elements identified in the cAMP-regulated genes of Group 1. The 8-bp palindromic sequence T(G/T)ACGTCA, originally termed a CRE, lie generally within the first 150 bp of the 5'-flanking region, and therefore available evidence supports a dual role for the CRE as both a basal and an inducible transcriptional enhancer element (Roesler et al., 1988). Another regulatory consensus sequence CCCCNGGG, is bound by the AP-2 and can be induced by treatment with either forskolin, which raises the concentration of cAMP and phorbol esters, which activates PKC. Considerable variation occurs on this palindromic consensus sequence as comparison of AP-2 binding sequences across different genes show variability in all 8 base pairs. This sequence also can act as a basal enhancer, increasing the rate of gene transcription in the absence of any hormonal stimulation (Roesler et al., 1988).

1.7.4 cAMP RESPONSIVE BINDING PROTEIN (CREB)

Using DNA affinity chromatography a protein has been purified with a molecular mass of 43-kDa wich bound specifically to the CRE (Montminy and Bilezikjian, 1987). CREB is a transcription factor which binds the CRE and activates transcription in response to a variety of extracellular signals including neurotransmitters, hormones, membrane depolarization and growth and neurotrophic factors. CREB protein is ubiquitious being distributed in a variety of tissues and its phosphorylation by PKA does not seem to increase the amount of CRE binding activity but is essential for transcriptional activation (Yamamoto et al., 1988; Mellon et al., 1989). In addition, phosphorylated CREB can turn on transcription of the gene for another transcriptional activator, the proto-oncogene *c-fos* (Edwards, 1994). Thus, it is conceivable that these two transcription factors, AP-1 and CREB work cooperativily for the regulation of gene transcription in response to the same stimuli.

Thus, cAMP alters the transcriptional activities of these proteins independent of their binding to regulatory elements in the target gene. In the case of cAMP-regulated genes, transcription factors such as CREB appear to bind to the CRE sites in a cAMP-independent manner, and

thus resemble basal transcription factors, which maintain a basal rate of gene transcription, which is dependent of their concentration in a specific tissue. Moreover, unlike transcription factors whose activity is regulated, at least in part, by subcellular localization, nuclear expression of CREB is constitutive (Roesler et al., 1988). Rapid changes in the levels of cAMP activate PKA, resulting in the translocation of its catalytic subunit into the nucleus, leading to a PKA-mediated modification of the CREB proteins, already bound to their respective elements in the promoter-regulatory region of the gene. This modification in the binding protein alters transcription levels of these genes (Roesler et al., 1988).

Phosphorylation of CREB by PKA appears to be necessary and sufficient for the activation of transcription by cAMP (Yamamoto et al., 1988). In either of two forms of CREB (CREB-327 and CREB-341) that result from alternative splicing, a mutation of the PKA phosphorylation site Ser-119/Ser-133 to an alanine or aspartic acid residue prevented activation by PKA *in vitro* and abolished the cAMP control of transcription *in vivo* . A phosphate group in the transactivating region, within a domain termed the P-box (a 46 amino-acid sequence, residues 92-137 of CREB) is therefore essential for a transcriptionally active protein (Fiol et al., 1994). This domain of CREB is serine rich and includes consensus phosphorylation sites for casein kinase II, CaM-K II, PKC and the well established PKA site (Dash et al., 1991; Sheng et al., 1991).

1.8 PHOSPHOLIPASE SIGNALLING PATHWAY

1.8.1 PHOSPHOLIPASES C (PLC)

Many growth factors, hormones and neurotransmitters effect intracellular regulation through activation of PI-PLC enzymes which hydrolyse the plasma membrane phospholipid, PI-4,5-bisphosphate (PIP_2) and results in the production of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), which both can serve as second messengers. IP_3 regulates the Ca^{++} release of intracellular stores, while Ca^{++} and DAG are activators of PKC (Rhee and Choi, 1992; Divecha and Irvine, 1995; Lee and Rhee, 1995).

Three isotypes of PI-PLC have been identified in mammalian tissues either through purification of proteins or isolation of cDNAs that code for homologous proteins : PLC-β (4 isoforms), PLC-γ (2 isoforms) and PLC-δ (4 isoforms) (Lee and Rhee, 1995). The overall similarity is low except in two regions: X= 170 amino acids (60% homology) and Y= 260 amino acids (40% homology). All PLCs have an amino terminal region of 300 amino acids before the X domain and a PH homology domain (100 amino acids) which is found in many signalling molecules as it is involved in complex formation recruitment to the plasma membrane (Toker and Cantley, 1997). PLC-β and PLC-δ have only 50-70 amino acids between X and Y. The

PLC-γ family have 400 amino acids between X and Y and this region contains two SH2 and one SH3 domains involved in protein-protein interactions (Pawson, 1995).

The PLC-β isoform has been shown to be activated by a subunit of the heterotrimeric G-proteins which are members of the $Ga_{q/11}$ subunit class (Rens-Domiano and Hamm, 1995). The activation of PI-PLCs by G-protein is either sensitive or insensitive to inhibition by PTX depending on the cell type. Screening of cDNA libraries under low stringency conditions has resulted in the cloning of four subtypes of PLC-β: PLC-β1, PLC-β2, PLC-β3 and PLC-β4 (Smrcka and Sternweis, 1993; Lee and Ree, 1995). PLC-βs do not possess the SH2 domain, but all four subtypes are activated by purified $Ga_{q/11}$ subunits with the following efficacy: PLC-β3 ≥ PLC-β1 >> PLC-β2/PLC-β4 (Smrcka and Sternweis, 1993).

PI-PLC subtypes are activated by βγ subunits, generally in a PTX-sensitive manner. βγ subunits stimulate PLC-β3 ≥ PLC-β2 >> PLC-β1. βγ subunits do not stimulate PLC-β4 (Smrcka and Sternweis, 1993; Sternweis, 1994). βγ complexes required prenylation of the carboxy-terminal CAAX motif of the γ subunit to regulate PLC-β indicating a possible role for bg complexes in PLC-β membrane recruitment/association. Mutational analysis suggests that the carboxy-terminal region of PLC-βs is the site of regulation by $Ga_{q/11}$ subunits and βγ subunits interact with the PLC-βs at the amino-terminal domain which contains the PH homology domain upstream of the X region (Smrcka and Sternweis, 1993; Sternweis, 1994). Thus, Gα and βγ subunits may act in a mutually reinforcing manner to activate PLC-βs consistent with the separate binding sites for $G\alpha_{q/11}$ and βγ subunits on PLC-β.

The most of the receptor-coupled PIP2 hydrolysis occurs at the cell membrane and is responsible for transducing many of the early events associated with cell activation. However, PIP2 also binds a number of proteins including profilin (actin monomer sequestering protein) and gelsolin (actin filament protein) and this binding favours actin polymerisation and inhibits PIP2 hydrolysis by all the PI-PLCs. Receptor activation of the PI-PLCs, PIP2 hydrolysis and subsequent Ca^{++} mobilisation leads to actin depolymerisation, increases cell motility and changes in cell structure. PIP2 is also hydrolysed in the nucleus in response to cell stimulation with mitogens and this correlates with a corresponding increase of DAG which precedes translocation to the nucleus and activation of PKC (Divecha and Irvine, 1995; Lee and Rhee, 1995).

The hydrolysis of PIP2 by receptor activated PLC is considered to be an important signal transduction mechanism for regulating the effect of extracellular signals such as growth factors, hormones and neurotransmitters (Lee and Rhee, 1995, Spiegel et al., 1997). In the unstimulated cell, more than 95% of PLC is present in the cytosol. Binding of NGF to $p140^{trk}$ receptor, phosphorylates crosswise several tyrosine residues in the receptor carboxyl tail and

thereby binds the SH2 domains of the PLC-γ molecule. PLC-γ is phosphorylated on tyrosine residues (771, 783, 1254) and is activated (Vetter et al., 1991). It is then thought that PLC dissociates from the receptor, binds to the membrane cytoskeleton and thus brings the catalytic domains of the enzyme to the plasma membrane where they hydrolyse inositol phospholipids (Rhee, 1991). As this phosphorylation has been shown to induce hydrolytic activity of PLC-γ by the NGF receptor, it has been proposed that activation of PLC-γ may constitute a link between NGF-dependent activation of $p140^{trk}$ and the stimulation of intracellular second messenger pathways (Lee and Rhee, 1995; Spiegel et al., 1997).

Among the metabolic changes in NGF-treated PC12 cells that have been suggested to result from elevations of PLC-γ1 activity are changes in ion fluxes and intracellular pH, cytoskeletal rearrangements and induction of cellular genes (Stephens et al., 1994). An important role of PLC-γ1 in NGF signalling events is the stimulation of certain gene transcriptional events. Mutation of the site in *trkA* responsible for inducing the tyrosine phosphorylation, receptor association and activation of PLC-γ1 results in selective loss of the NGF-mediated increases of peripherin in PC12 cells, a gene codifying an intermediate filament protein (Loeb et al., 1994).

Upon stimulation of cell surface receptors, DAG is immediately produced from from PIP_2, as a result of PLC activation. This DAG molecule is rapidly converted to phosphatidic acid (PA) by the action of DAG kinase, and further to PI, PIP and PIP_2 (English et al., 1996). The level of DAG, however, often increases again with a relatively slow onset, and persists for minutes or hours. This sustained elevation of DAG is frequently observed in response to various long-acting signals such as cytokines, growth factors and phorbol esters. Sustained elevation of DAG levels for several hours is a prerequisite essential for long-term cellular responses such as growth and differentiation, although it is not known whether continuous PKC activation is needed during the cell cycle. This second wave of DAG results from the hydrolysis of phosphatidylcholine (PC) by PLA_2, PLC and PLD (Boarder, 1994; Morris et al., 1996). The DAG produced from PC hydrolysis is slowly degraded by DAG lipase.

1.8.2 PHOSPHOLIPASE D (PLD)

PLD is a phosphodiesterase that catalyses the hydrolysis of glycerophospholipids to yield PA or its analogues. The mammalian PLD also catalyses the transphosphatidylation reaction (base exchange reaction) and incorporates choline, ethanolamine and serine into phospholipids (Boarder, 1994; Morris et al., 1996). PLD generates a phosphatidyl moiety which uses water as a nucleophilic acceptor. This base exchange reaction represents a minor pathway for the synthesis of most phospholipids. There must be a phosphatidyl ≈ enzyme intermediate, preserving the high energy phosphate bond. This phosphoric group is transferred preferentially to primary alcohols such as methanol, ethanol and n-butanol in aqueous medium to produce

PA. Non-cytotoxic concentrations of butanol inhibit the PLD-catalysed production of PA by diverting the product to phosphatidyl-butanol, but do not affect the production of PA formed by the sequential actions of PLC and DAG kinase.

PLD hydrolyses PC and cleaves the phosphodiester bond distal to the glycerol backbone, resulting in the formation of choline and PA. PA and its metabolic product lysophosphatidic acid (LPA) are proposed to serve as a second messenger or mediator for mitogenic signals in certain cell types (English et al., 1996). This acidic lipid is suggested to be implicated in intracellular Ca^{++} mobilisation, direct activation of PKC and has also been shown to stimulate the GTP-bound $p21^{ras}$, PLCg1, PLD and PIP-kinase. In addition, the hydrolysis of the steroyl arachinodil species of PA by PLA2 releases arachidonic acid (AA), another proposed second messenger (English et al., 1996).

PA produced from PC is hydrolysed to DAG by the action of phosphatidic acid phosphohydrolase (PPII), thereby activating PKC. Several lines of evidence show, at a later phase of cellular response, PLD plays a major role in the production of DAG from PC to prolong PKC activation. PLD activation is also involved in mitogenesis in some cell lines (Boarder et al., 1994). For example, PC12 cells divide in culture and show PLD responses to purinoceptor-mediated agonists and bradykinin, while adrenal chromaffin cells do not divide and exhibit no PLD responses. PLD enzymes may play a role in intracellular protein trafficking (vesicle transport and exocitosis) and mitogenic signalling (Boarder et al., 1994).

Receptors are coupled to PLD by events that are either downstream of PLC activation or are independent of PLC (Boarder et al., 1994). The PLC-independent mechanisms have been described as those either directly coupling to a G-protein or those linked to a tyrosine protein kinase. However, the most commonly reported form of linkage of receptors to PLD involves an elevated intracellular Ca^{++} concentration and activation of PKC. This is consistent with the initial activation of PLC and consequent hydrolysis of PIP2 (Boarder et al, 1994). PLD receptors are also direct coupling to PLD via G-protein. However, in many cases there has been no adequate distinction between receptors that have a direct G-protein linkage and receptors that are dependent on G-proteins but via an intermediate PLC intermediate step. The stimulation of PLD by tyrosine kinase mechanisms involves either receptors or non-receptors of the intrinsic tyrosine kinase type or GPCRs where the tyrosine kinase activity is downstream of PLC. Agonists that enhance tyrosine phosphorylation via GPCRs stimulate both Ca^{++}- and PKC- dependent activation of tyrosine kinases, phosphorylating both soluble and membrane bound proteins in association with PLD activation (Boarder et al, 1994).

PLD activity is stimulated by PKC activators such as phorbol esters and DAG analogues, sometimes synergistically with Ca^{++} ionophore, such as ionomycin (Boarder et al, 1994). It is

also suggested that tyrosine phosphorylation takes part in the regulation of PLD, since pervanadate, a tyrosine phosphatase inhibitor, enhances PLD activity, whereas tyrosine kinase inhibitors prevent enzyme activation. In addition, an endogenous membrane constituent, sphingosine, stimulates both mitogenesis and PLD (Spiegel and Milstein, 1996). Sphingosine-1-phosphate (SPP), a metabolic product of sphingosine, is mitogenic and stimulates PLD activity. It elevates PA and intracellular Ca^{++} without the hydrolysis of PIP_2, and in some cases may provide the link in the chain between intrinsic tyrosine kinase receptors and PLD activation (Spiegel and Milstein, 1996).

1.9 PI-3-KINASE SIGNALLING PATHWAY

Activation of PI-3-K is a common response to stimulation of cells by numerous ligands, including those that signal through cytokine receptors, GPCRs and RTKs (Toker and Cantley, 1997). The activation of PI-3-K by RTKs is mediated by the SH2 domains of the 85-kDa regulatory subunit (p85) and 110-kDa catalytic subunit (p110) (Kaplan et al., 1987). In addition, PI-3-K activity increases in response to ligands of GPCRs (Stephens et al., 1991). A PI-3-K isotype, p110γ is activated *in vitro* by bg subunits of G-proteins and is sensitive to inhibition by wortmannin (Stoyanov et al., 1995). A potential PH domain is located near its amino terminus. This novel PI-3-Kγ is activated by direct binding to a 101-kDa regulatory subunit (p101) (Stephens et al., 1997).

In vitro , PI-3-K enzyme can phosphorylate PI, PI-4-P or PI-4,5-P_2 to produce PI-3-P, PI-3,4-P_2 and PI-3,4,5-P_3 respectively (Toker and Cantley, 1997). PI-3-P is present in unstimulated cells and does not significantly increase in response to cell activators, however, PI-3,4-P_2 and PI-3,4,5-P_3 are normally absent in unstimulated cells and appear within seconds to minutes of cell activation. The physiologically important product is PI-3,4,5-P_3 which is formed by the action of the kinase on PI-4,5-P_2. PI-3,4,5-P_3 has been shown to bind specifically to the SH2 domains of *c-src* and the p85 regulatory subunit of PI-3-K (Toker and Cantley, 1997). The *in vivo* relevance of this result is supported by the observation that inhibition of PI-3,4,5-P_3 production results in enhanced association of the p85 subunit of PI-3-K with tyrosine phosphorylated insulin receptor (Toker and Cantley, 1997). These results suggests that PI-3,4,5-P_3 binds directly to the SH2 domains of p85 and causes dissociation of PI-3-K from tyrosine phosphorylated proteins. Similar interaction between PI-3,4,5-P_3 and the *c-src* SH2 domain might activate *c-src* by displacing the interaction between the *c-src* SH2 domain and its tyrosine-phosphorylated carboxy-terminus. This *c-src* activation also correlates with the recruitment and binding of PI-3-K to the plasma membrane and *in vivo* PI-3-K activation (Varticovsky et al., 1991).

PI-3,4-P2 and PI-3,4,5-P3 also may bind to the amino-terminal PH domain of a serine/threonine kinase Akt/PKB (Tower and Cantley, 1997). This binding promotes dimerization and kinase autophosphorylation, resulting in activation of the protein kinase activity (Burgering and Coffer, 1995; Franke et al., 1995). Akt/PKB phosphorylate $p70^{S6K}$ (Boudewijn et al., 1995) and this latter kinase is implicated in mitotic proliferative events since phosphorylation of ribosomal protein S6 and increased protein synthesis during mitogenesis and meiotic maturation have been demonstrated to be correlated (Chou and Blenis, 1995).

Thus PH domains are a common structural motif used as universal membrane adapter and bind with high affinity to inositol phosphates such as PI-4,5-bisphosphate (PIP2) and PI-3,4,5-triphosphate (PIP3) (Pawson, 1995). This motif is found in over 100 proteins, including GNRFs such as Sos-1, GAPs proteins such as *ras* -GAP, PLC-δ1, cytoskeletal proteins, PI-3-K and protein tyrosine kinases (PTKs) (Tower and Cantley, 1997).

In PC12 cells, NGF-dependent phosphorylation of p85 regulatory subunit of PI-3-K by $pp140^{trk}$ is weak (Stephens and Kaplan, 1994). However, association of a phosphotyrosine-containing protein with molecular masses of 110 kDa with PI-3-K is detected in PC12 cells. Elevation of PI-3,4-P2 and PI-3,4,5-P3 levels has been observed several minutes after NGF stimulation, returning to near normal levels after 10 minutes (Kimura et al., 1994). This response is totally inhibited by wortmannin. Moreover, NGF-induced neurite elongation of PC12 cells is suppressed by wortmannin (Kimura et al., 1994).

Most mitogenic stimuli activate both $p21^{ras}$ and PI-3-K. This could reflect the fact that there are common components, such as tyrosine kinases, in the upstream regulatory pathways for both proteins. It could also be the result of a system in which $p21^{ras}$ is capable, directly or indirectly, of regulating PI-3-K. The association of PI-3-K and $p21^{ras}$ is direct (Rodriguez-Viciana et al., 1994) and a dominant negative $p21^{ras}$ mutant inhibits the ability of NGF to elevate PI-3 phophorylated lipids levels in PC12 cells *in vivo*. Furthermore, $p21^{ras}$ coexpressed with p85 and p110 causes the elevation of cellular 3-phosphorylated phosphoinositides in CHO cells (Rodriguez-Viciana et al., 1994).

The physiological role of 3-phosphorylated phosphoinositides is not well known, but PI-3,4,5-P3 potently and selectively activated the Ca^{++}-independent PKC isoforms (especially ε and η) and the phorbol ester/DAG-insensitive PKC-ζ but was less effective or selective in activating conventional PKC (cPKC) (Nakanishi et al., 1993). Many of the agents that stimulate PI-3,4-P2 and PI-3,4,5-P3 production *in vivo* also stimulate production of DAG. Since PKC-ε and PKC-η can be activated by either DAG or the phosphoinositides *in vitro* , some redundancy in the PI-3-K pathway and the PLC pathways is expected.

In growth factor stimulated cells, PI-3,4-P_2 and PI-3,4,5-P_3 levels remain elevated much longer than Ca^{++} levels, indicating that this pathway may cause a much more prolonged activation of PKCs than the PLCγ pathway. In addition, there is circumstantial evidence that PKC isoforms may mediate mitogenic effects of the PI-3-K pathway. PKC-ζ has also been reported to be critical for mitogenic signal transduction in fibroblasts (Berra et al., 1993) and a dominant negative PKC-z subspecies blocked NF-kB activation (Diaz-Meco et al., 1994). It is therefore apparent that the novel and atypical PKC isoforms are signalling downstream of certain mitogenic pathways, such as the PI-3-K pathway. However, it is clear that this mitogenic signal ultimately depends on the cell types studied and the pattern of expression of PKC isoforms.

1.10 CALCIUM-PHOSPHOLIPID DEPENDENT PROTEIN KINASE C (PKC) SIGNALLING PATHWAY

One of the earliest products of inositol phospholipid hydrolysis by PLCs and PLDs is DAG which remains membrane-associated, and initiates the activation of PKCs (Nishizuka, 1986, 1988, 1995). PKCs constitute a family of enzymes transiently activated by Ca^{++}, DAG and phosphatidylserine (PS). PKCs are involved in mediating responses as diverse as modulating ion conductance mediated by phosphorylating membrane proteins such as ion channels, pumps and ion exchange proteins, cell proliferation and differentiation (Nishizuka, 1986, 1988, 1995). It has been shown that PKC is the cellular receptor for tumour-promoting phorbol esters (Niedel et al., 1983) which induce expression of several proto-oncogenes such as *c-fos* (Sigmund et al., 1990). The PKC family of enzymes transduces the myriad of signals promoting lipid hydrolysis. The prevalence of this enzyme family in signalling is exemplified by the diverse transduction mechanisms that result in the generation of the PKC activator DAG. Signals that stimulate members of the large family of GPCRs, RTKs or non-RTKs can cause DAG production, either rapidly by activation of PLCs or more slowly and long lasting by activation of PLD to yield PA and then DAG. In addition, *cis* -unsaturated fatty acid (FFAs) generation by PLA2 activation modulates PKC activity (Nishizuka, 1995).

The members of the PKC family respond differently to various combinations of lipids including several phospholipids, particularly DAG, FFA, lyso-PC, PS and many other lipid metabolites in membranes, and hence the patterns of activation of the PKC isoforms may vary in extent, duration, and intracellular localisation. In fact the PKC isoforms show distinct tissue distribution and specific intracellular localisation (Nishizuka, 1995). In most tissues, PKC is present mainly in the soluble fraction in its inactive form. After activation, PKC translocates to the plasma membrane. Recently, intracellular PKC receptors (RACK) have been identified which bind activated PKC in the presence of Ca^{++} and PS (Mochly-Rosen et al., 1991).

Receptor-mediated activation of PKC is transient; DAG disappears from the cell membrane shortly after its formation either due to further degradation into AA, or to its conversion back to inositol phospholipids. Although the activity of PKC itself is transient, its consequence within the cell could potentially be long lasting, depending on the half life of specific phosphorylated substrates (Nishizuka, 1986, 1988, 1995).

1.10.1 PKC ENZYMATIC ACTIVITY

PKC uses ATP as substrate, with a Km for ATP in the low μM range (Newton, 1995). PKC typically phosphorylates serine or threonine residues in basic sequences but displays significantly less specificity than PKA. PKC have a wide range of targets, including members of the MAPK pathway (Newton, 1995).

The function of PKC is regulated by two equally important mechanisms. First, the enzyme is rendered catalytically competent by phosphorylation which regulates the active site and subcellular localisation of the enzyme. Second, binding of ligands or second messengers promotes the PKC membrane association and removes the pseudosubstrate from the substrate-binding site. Regulation of two independent mechanisms may provide exquisite fine-tuning for this family of enzymes, ensuring low basal activity in the midst of complex intracellular pathways (Newton, 1995).

PKC can be targeted for inhibition by taking advantage of either the catalytic or regulatory domains. The enzyme comprises a single polypeptide with the amino- terminal half containing the pseudosubstrate and cofactor binding sites, and the carboxy-terminal half containing the kinase core. The function of the protein can be inhibited by molecules such as calphostin C (CC) which binds the regulatory domain and competes with cofactor binding, or by synthetic peptides based on its pseudosubstrate which block the substrate binding cavity (Newton, 1995).

1.10.2 PKC STRUCTURE

Members of the PKC family are a single polypeptide, comprised of : (i) an amino-terminal regulatory region (approximately 20-40 kDa) and (ii) a carboxy-terminal catalytic region (approximately 45 KDa). The amino-terminal half of the regulatory domain of cPKC contains two conserved regions, C1 and C2, that play a role in the regulation of enzyme activity of PKC (Newton, 1995).

The C1 domain is about 150 amino acids and contains a pseudosubstrate domain and two tandem repeats of a cys-rich region (zinc butterfly domains). The pseudosubstrate domain is a conserved sequence of amino acids that resembles a consensus phosphorylation site in PKC substrates. This site interacts with the substrate-binding pocket in the catalytic domain and represses PKC activity. The cofactor binding reduces the affinity of this interaction. The two tandem repeats of cysteine-rich, zinc finger-like motifs that forms the phospholipid-dependent and DAG/phorbol ester binding site are found in other signal transducers such as DAG kinases, Vav, a GNRF protein, which is known to be involved in the activation of p21*ras* protein and n-chimaerin, a GAP protein, which inactivates another small G-protein, *rac* (Newton, 1995).

The C2 domain (CalB) contains 50 amino acids and is the recognition site for acidic phospholipids and the Ca^{++} -binding site. The CalB domains are also found in cPLA2, PLC-γ and GAPs. The binding of intracellular Ca^{++} to the C2 region of the cPKC isoenzymes causes the translocation of these enzymes to the plasma membrane where they are activated following binding to DAG and PS. This binding is mediated by RACK proteins which act to facilitate intracellular docking that mediates the association and activation of signal transducer containing CalB domains to subcellular locations (Mochly-Rosen et al., 1991).

The carboxy-terminal half of the PKC contains two additional highly conserved regions, C3 and C4 domains. The C3 and C4 domains form the ATP- and substrate-binding lobes of the PKC. These domain halves are separated by a hinge region that becomes proteolytically labile when the enzyme is membrane-bound. The proteolytically generated kinase domain (protein kinase M), freed of inhibition of the pseudosubstrate, is constitutively active (Newton, 1995).

In the current model, inactive PKC is folded with the catalytic domain masked by the pseudosubstrate domain and the RACK binding site (CaLB) masked by interaction of a pseudoRACK sequence with the PKC carboxy-terminal region. Interaction with membrane lipids and RACK1 causes conformational change and activation of PKC (Newton, 1995). Activation makes PKC more proteolytically sensitive, probably by unfolding the molecule. Thus, downregulation is caused by increased degradation in the absence of transcription or translation. Chronic activation by phorbol esters, such as PMA results in PKC isoform downregulation at different rates. Atypical PKCs, such as PKC-ζ are not downregulated or responsive to PMA (Newton, 1995).

Phorbol esters have been an important tool in studying the functions of PKC. They have a structure very similar to DAG and are able to activate PKC both *in vivo* and *in vitro* by increasing the affinity of the enzyme for Ca^{++}. Phorbol esters are very stable molecules that induce a prolonged association of PKC with the membrane thereby inducing the degradation of

PKC with the subsequent disappearance of PKC activity from the cell (Bazzi and Nelsestuen, 1988). This phorbol ester-induced depletion of PKC activity may interfere with the feedback control mechanisms of PKC and it has been suggested that this action may be involved in the uncontrolled cell proliferation reported for phorbol esters (Wilkinson and Hallam, 1994).

1.10.3 PKC SUBTYPES

PKC exist as a growing family of serine/threonine kinases with a wide range of physiological functions (Nishizuka, 1995). To date, 12 members have been described and subdivided into three groups: (i) classical or conventional PKCs (cPKC) comprising α, βI, βII and γ (activated by Ca^{++}, phospholipid and DAG), (ii) new or novel PKCs (nPKC) comprising δ, ε, η (L), θ and $\propto$ (insensitive to Ca^{++}) and (iii) atypical PKCs (aPKC) comprising ι/λ and ζ, a subgroup of PKC isoforms whose activity is not affected by phorbol ester or the natural activator DAG (Nishizuka, 1995). All the known members of the PKC family require PS for activity. Other lipids such as phosphatidylethanolamine (PE) exert cooperativity but phosphatidylcholine (PC) and sphingomyelin inhibit PKC activity.

DAG activates the cPKC isoforms in such a way that it increases greatly their apparent affinity for Ca^{++} in the micromolar range (Nishizuka, 1995). However, these Ca^{++} concentrations are still above the physiological range. *cis* -unsaturated fatty acids, such as AA together with DAG, further increase the affinity of cPKC isoforms for Ca^{++}, thereby causing full enzyme activation at the basal level of intracellular Ca^{++}. For activation of cPKC isoforms, increase in the Ca^{++} concentration and phospholipid degradation are hence interrelated, and sometimes complementary to each other.

The nPKC isoforms, which lack the C2 region, do not require Ca^{++} for their activation, but the δ, ε, and η (L) isoforms are activated by PI-3,4,5-P3, a product of the PI-3-K reaction. The PKC-δ and PKC-ε isoforms are phosphorylated at tyrosine residues and this phosphorylation is enhanced by treatment of the cells with phorbol esters (Nishizuka, 1995).

The aPKC isoforms, also lack the C2 region and the C1 domain contains only one cys-rich motif. The PKC-ζ isoform is dependent on PS for its catalytic activity and is activated by *cis* -unsaturated fatty acids, such as AA as well as PI-3,4-P2 and PI-3,4,5-P3. This isoform does not respond to DAG or phorbol esters. The signal pathway leading to the activation of the aPKC isoforms is relatively unknown, but the overexpression of this group of enzymes enhances the transcriptional activation of a set of *cis* -acting elements in response to serum and other growth factors, but not phorbol esters.

1.10.4 PKC AND THE MAPK PATHWAY

There is strong evidence that PKC activation is an early and important event in NGF-induced intracellular signalling. Direct measurement of PKC activity in PC12 cells indicate that NGF treatment of these cells produces a 4 to 5-fold increase in PKC activity after 30 to 60 minutes, returning to control levels within 7 hours, even in the continuous presence of NGF (Hama et al., 1986; Heasley and Johson, 1989; Kondratyev et al., 1990; Ohmichi et al., 1993 a; Wooten et al., 1994). Phorbol ester stimulation of PKC induces GTP loading of cellular p21^{ras} (Downward et al., 1990; Wood et al., 1992) and phosphorylation of *raf-1* (Morrison et al., 1988), MAPKs (Chen et al., 1991b; Wood et al., 1992), p90^{RSK} (Chung et al., 1991; Chen et al., 1991 a, b; Wood et al., 1992) in PC12 cells.

PC12 cells express multiple PKC isoforms: α, β, γ, δ, ε and ζ (Wooten et al., 1992) and NGF treatment of PC12 cells results in translocation as well as activation of PKC-δ and PKC-ζ isoforms (Ohmichi et al., 1993, Wooten et al., 1994). More important, in cells deficient of phorbol ester/DAG-sensitive isoforms (α, β, γ, δ and ε) NGF translocates the phorbol ester/DAG-insensitive isoform PKC-ζ (Wooten et al., 1994) and removal of PKC-ζ from cells deficient in the PMA-sensitive isoform inhibits NGF-induced neurite outgrowth (Coleman and Wooten, 1994).

In other systems, PKC-ζ operates in a cooperative fashion with p21^{ras} and integrates signals from both PC-PLC and PI-3-K pathways (Berra et al., 1993; Nakanishi et al., 1993). PKC-ε and PKC-η (Toker et al., 1994) and PKC-ζ (Nakanishi et al., 1993) are activated *in vitro* and *in vivo* by PI-3,4-P2 and PI-3,4,5-P3. Wortmannin can also block activation of *raf-1* and MAPKs in fibroblasts. Because phorbol esters can activate *raf-1 in vivo* , and PKCs can phosphorylate *raf-1 in vitro* (Morrison et al., 1988), it could be argued that wortmannin is blocking PKC-dependent activation of *raf-1* .

PKC is a common effector molecule activated by receptor ligand interaction that leads to MAPK pathway activation (Kazlauskas and Cooper, 1988; L'Allemain et al., 1991). PKC-α has been shown to activate MAPK via p21^{ras} in PC12 cells (Robbins et al., 1992; Wood et al., 1992; Crespo et al., 1994) following exposure to phorbol esters. PKC-a may also directly phosphorylate *raf-1* at Ser-499 (Kolch et al., 1993). Recently it has been reported that a newly discovered protein tyrosine kinase, proline-rich tyrosine kinase 2 (PYK-2), acts as a switch that relays messages from PKCs and increased cytoplasmic Ca^{++} to the adapter molecule Shc thus activating p21^{ras}--mediated MAPK activation (Lev et al., 1995). Constitutively active mutants of PKC-d activate the MEK/MAPK pathway in a manner independent of p21^{ras} and dependent of *c-raf* (Ueda et al., 1996).

1.10.5 PKC SIGNALLING TO NUCLEUS

PKCs as well as MAPKs, plays a pivotal role in cellular regulation. For example CHO cells overexpressing the PKC-α and PKC-δ isoforms exhibit prolonged activation of MAPKs upon treatment with phorbol esters and arrest of the cell division at the G2/M phase (Watanabe et al., 1992). It has also been suggested that phorbol esters-induced myeloid differentiation is mediated by PKC-α and PKC-δ isoforms (Mischak et al., 1993). Thus, these observations suggest that the PKC-α and PKC-δ isoforms distributed widely in all cell types and tissues have the potential to control nuclear functions, probably at specific points of the cell cycle. However, unusually sustained activation of the PKC signalling by phorbol esters may lead to unregulated cell proliferation. Well-coordinated regulation of PKC activation may be important for normal cell functions. In addition, mechanisms of down-regulation of MAPKs are probably involved in the effects of sustained activation of the PKC (Nebreda, 1994; Ueki et al., 1994).

The PKC signalling pathway often converges with other signalling pathways at the point of nuclear protein transcription factors that are indispensable for gene transcription (Nishizuka, 1995). For example, AP1 activity is related to phorbol esters and this gene activation may be integrated with the synergistic action of the Ca^{++} and PKC signalling pathways.

1.11 CALCIUM SIGNALLING PATHWAY

The divalent cation Ca^{++} is used by cells as a ubiquitous second messenger to control many cellular processes including muscle contraction, secretion, metabolism, neuronal excitability, cell proliferation and cell death (Clapham, 1995). The cytosolic level of Ca^{++} in resting cells is kept low (10-100 nM) but stimulation results in the level increasing to 50 to 1000 nM. The cell has access to two sources of Ca^{++}: (i) entry from the external medium by plasma membrane Ca^{++} channels (voltage-operated channels, receptor-operated channels and store-operated channels), and (ii) release from internal stores (IP_3 and ryanodine receptors) (Furuichi and Mikoshiba, 1995). These Ca^{++} ON mechanisms are balanced by Ca^{++} pumps which constitute the OFF mechanisms responsible for removing the Ca^{++} signal. The surface membrane has a Na^{+}/Ca^{++}-exchanger (found mainly in excitable cells) and the ubiquitous plasma membrane Ca^{++}-ATPase. The latter is regulated by calmodulin, acidic phospholipids, PKA and PKC. The internal stores have a endoplasmic/sarcoplasmic reticulum (ER/SR) Ca^{++}-ATPase (Simpson et al., 1995).

Both the cytoplasm and the lumen of the ER/SR have proteins capable of buffering Ca^{++}. As Ca^{++} is pumped into the lumen of the ER/SR, it is buffered by storage proteins such as calsequestrin and calreticulin which have a low affinity (Kd in the mM range) but a high

capacity (approximately 50 Ca^{++} ions bound/molecule). When Ca^{++} enters the cytosol it is rapidly buffered by proteins such as calbindin, calretinin and parvalbumin. More than 90% of the Ca^{++} entering the cell is bound to these buffers with the remainder representing the stimulus-evoked elevation of Ca^{++} responsible for activating the Ca^{++} sensors and activation of the Ca^{++}-mediated signalling pathways (Simpson et al., 1995).

Most of the intracellular effects of Ca^{++} are mediated by two groups of proteins, the annexins and the EF-hand proteins. The annexins represent a heterogeneous family that share a common property of interacting with membranes in a Ca^{++}-dependent manner. Since they have a low affinity for Ca^{++} their action seems to be restricted to domains near membranes where Ca^{++} channels create localised high elevations of Ca^{++}. Annexins have been implicated in the control of PLA2, cytoskeletal reorganisation, vesicle movement and some may function as Ca^{++} channels (Simpson et al., 1995).

The EF hand proteins such as calmodulin (CaM) and troponin C (TnC), which have a characterised Ca^{++}-binding domain between two helices (named after the E and F a-helices of parvalbumin), are the major Ca^{++} sensors. TnC functions in cardiac and skeletal muscle whereas CaM has a much more general role (Simpson et al., 1995). CaM exerts its effects by stimulating a multifunctional CaM-dependent proteins, such as CaM-dependent kinases or CaM-dependent protein phosphatase known as calcineurin (PP2C).

CaM-dependent systems are activated in response to receptors that lead to an increase in cytoplasmic Ca^{++}, either by stimulating influx from extracellular sources or from intracellular stores. GPCRs or RTKs potentially stimulate CaM-dependent processes by stimulation of the PI turnover through PLC activation, production of IP_3 and release of intracellular Ca^{++} or by increasing the activity of plasma membrane Ca^{++}-channels (Simpson et al., 1995). Alternatively, GPCRs or RTKs could change the level of expression of CaM or CaM-binding proteins (CaM-BPs).

CaM-K II is a large multimeric enzyme (630 kDa) composed of two related subunits with 9 of α (50 kDa) and 3 of β (60 kDa) respectively, and like other kinases undergoes an autophosphorylation that seems to be an intramolecular process. CaM-K II have a relative broad substrate specificity with respect to endogenous proteins in the nervous system. CaM-K II is detected in PC12 cells and the phosphorylation of the enzyme is increased by ionophore (A23187) treatment (Tokumitsu et al., 1990). A specific inhibitor of CAM-K II, KN-62, blocked the phosphorylation of CAM-K II dose dependently in PC12 cells (Tokumitsu et al., 1990).

1.12 NEUROPEPTIDE Y (NPY)

Over the last 20 to 30 years, there has been a radical change in the concept of neurotransmission. Until then, neurotransmission was thought to use small compounds including a number of modified amino acids such as adrenaline, noradrenaline, GABA and serotonin, together with unrelated compounds such as acetylcholine. The discovery that a number of peptides were co-localised with these "classical" neurotransmitters within the same neurone and, furthermore, were co-released following nerve stimulation, challenged the prevailing concepts and formulated the concept of "peptidergic neurons" (Hokfelt, 1991).

At the same time, neuropeptides have also challenged the classification of chemical messengers either as endocrine hormones, acting at distance through release into the blood stream, or as neurotransmitters, acting locally in the synapse. Peptide neurotransmitters are synthesised in the cell body and stored in synaptic vesicles, which are transported along the cytoskeleton of the axon to the synapse. At the synapse they await the electrical impulse, which causes Ca^{++} ions to enter the cell as a cue for the release of neurotransmitter. About 50 different neurotransmitters are known today. One of the most abundant peptide neurotransmitters in the human brain is neuropeptide Y (NPY) (Tatemoto, et al., 1982).

1.12.1 NPY DISCOVERY

NPY belongs to a family of regulatory peptides which also includes pancreatic polypeptide (PP) and peptide YY (PYY). NPY was discovered using a chemical method to trace polypeptides with an amide structure at their carboxyl termini (Tatemoto and Mutt, 1978). This post-translational modification is characteristic of many other biologically active peptide such as oxytocin, secretin, substance P, vasopressin and VIP although these were isolated on the basis of their biological activity rather than structure.

Sequence analysis revealed that NPY comprised 36 amino acids and had a tyrosine residue at its amino-terminus and tyrosine amide residue at its carboxy-terminus. Since the biological functions of these peptides were not established at the time of their isolation, their were named according to a convention to reflect the chemical structure of the amino- and carboxy-terminal amino acid using the international single letter code (Y represents tyrosine). As NPY and PYY have identical amino- and carboxy-terminal amino acids, the neural origin of NPY was used to differentiate between the two peptides, as PYY had been purified from porcine gut (Tatemoto and Mutt, 1980).

NPY constitutes one of the most highly conserved neuroendocrine peptides known (Larhammar, 1996a). The predicted rat and human mature NPY amino acid sequences are identical and differ from the porcine isolate by a single amino acid substitution at position 17, where the leucine residue in porcine NPY is replaced by a methionine residue (Larhammar, 1993, 1996a). It has been determined that NPY displays a high degree of homology within vertebrates showing a 92% sequence homology between the ray *Torpedo marmorata* and mammals, which separated more than 400 million years ago (Blomqvist et al., 1992).

1.12.2 NPY STRUCTURE

NPY family of peptides consist of 36 amino acids and have an amidated carboxy-terminal tyrosine residue. The tertiary structure of this family of peptides has been referred to as the member of the "PP-fold structure" and was modelled by using the avian pancreatic polypeptide crystal structure determined by X-ray crystallography (Blundell and Wood, 1982; Glover et al., 1983). The model shows that NPY has a tight, compact tertiary structure characterised as a hairpin-like structure where the amino and carboxy parts are stabilised by the hydrophobic moieties of a proline helix (residues 1-9) and an amphiphilic α-helix (residues 14-30) joined by a tight β-turn. This fold is followed by the carboxy terminal-tail of six amino acids which project away from the α-helix (Allen et al., 1987a). This structure predicts that the amino- and carboxy-termini of NPY are spatially closely related. In fact, it has been shown that this proximity is essential for biological activity as the epitope that binds to the NPY-Y1 receptor is composed of residues present in the combined amino- and carboxy-termini of the molecule (Washlestedt et al., 1990).

1.12.3 NPY DISTRIBUTION

1.12.3.1 NPY IN CENTRAL NERVOUS SYSTEM

NPY is the most abundant neuropeptide in the rat brain (Allen et al., 1983). Neurons containing NPY-IR are abundant in many areas of CNS and spinal cord with the exception of the cerebellum. Broadly, two basic types of neurons have been found to be immunoreactive for NPY. The first of these are the majority and constitute "interneurons". These cells give rise to short axons and to local connections. The second type is the long-projecting neuron, axons project a long distance from the cell body and innervate structures in other regions of the brain. Within the CNS, NPY is co-localised with a wide variety of other neurotransmitters. The pattern of co-localisation depends of the anatomical site of the NPY-containing neurons. In addition, NPY appears very early in the developing brain and can be transient in nature (Foster et al., 1989).

The distribution of NPY has mainly been studied in the rat brain, where it is especially concentrated in brain areas associated with the limbic system, including cortical areas and the hippocampus, hypothalamus, amygdala and cortex (Allen et al., 1983). High concentrations of NPY are found in the hippocampus where it is localised in interneurons in the hippocampal formation. NPY co-exists with somatostatin and possibly with GABA in the hippocamppus. The influence of NPY is largely inhibitory, acting at presynaptic nerve terminals to reduce the influx of Ca^{++} ions through voltage-dependent channels. Thus, NPY controls the excitatory throughput within the hippocampus.

High levels on NPY are also found in the arcuate nucleus and median eminence of the hypothalamus and from adrenergic and noradrenergic several brain stem nuclei nerve terminals found in the suprachiasmatic nuclei (Allen et al., 1983). Some of these nerve terminals can be found in association with the capillaries that form the hypothalamo-pituitary portal system providing support for the notion that NPY is secreted locally into this portal system to affect anterior pituitary functions. In CNS, NPY is particularly concentrated in the brain stem nuclei, particularly in the noradrenergic A1 and A4 nuclei and the adrenergic C1, C2 and C3 nuclei (Allen et al., 1983).

1.12.3.2 NPY IN PERIPHERAL NERVOUS SYSTEM

NPY is found exclusively in cells that have migrated from the neural crest. In the periphery, NPY is present in peripheral post-ganglionic sympathetic nerves supplying blood vessels in many tissues, such as nasal mucosa, submandibular gland, heart, kidney, lungs, pancreas, uterus and urinary bladder. It is also present in enteric and cardiac nerve endings. However, NPY is not exclusively localised in this branch of the autonomic nervous system, a small proportion of NPY has been traced to a subset of parasympathetic nerves (Grundemar and Hakanson, 1994).

The adrenal medulla of different species of mammals contains high concentrations of NPY where it is co-localised with catecholamines. In the adrenal medulla and in sympathetic nerve terminals, NPY is present only in the electron dense vesicles (Fried et al., 1985). NPY is frequently found in a number of a rodent and human cell lines derived from neural crest tumours such as ganglioneuroblastomas and phaeochromocytomas (Allen et al., 1987b).

The distribution of NPY and its related peptides in numerous nerve terminals and cell bodies suggests a wide-ranging role in somatic, sensory and cognitive brain functions as well as in endocrine regulation. In many neurons, NPY is co-localised and co-released with other neurotransmitters such as noradrenaline, somatostatin and GABA which also suggests a modulatory role for NPY (Grundemar and Hakanson, 1994).

1.12.4 EFFECTS OF THE NPY FAMILY OF PEPTIDES

1.12.4.1 NPY ACTIONS IN CENTRAL NERVOUS SYSTEM

Even at the time of its discovery, it was clear that NPY was present in high concentrations in the brain (Tatemoto et al., 1982). Indeed, its concentration in certain regions of the brain exceeds those of cholecystokinin and somatostatin, which had previously been considered the most abundant neuropeptides in the human brain (Adrian et al., 1983).

In CNS, NPY has been associated with modulation of autonomic functions such as regulatory roles in most of the areas where it has been localised. It has been associated with central regulation of blood pressure (Fuxe et al., 1983), circadian rhythm (Albers and Ferris, 1985), feeding behaviour (Tempel et al., 1988), memory processing (Flood et al., 1989) and control of neuroendocrine functions (Kerkerian et al., 1986). Additional reported effects of NPY include regulation of anxiety, release of pituitary hormones, thermogenesis and energy balance as well as electrolyte homeostasis (Grundemar and Håkanson, 1994).

Many effects of NPY in CNS can be attributed to inhibition of neurotransmitter release. NPY inhibits excitatory synaptic transmission in CA1 neurons of the rat hippocampus by suppression of glutamate release via inhibition of Ca^{++} currents. NPY also inhibits excitatory and inhibitory synaptic potential in 5-hydroxytryptamine containing cells of dorsal raphe nucleus and it reduces spontaneous firing rate in the locus coeruleus (Grundemar and Håkanson, 1994).

The study of the effects of NPY in the CNS has been approached by injecting the peptide or peptide analogues into discrete areas of the brain. Peripherally the physiological effects have been studied by administering NPY intravenously or to tissue preparations. Many of the effects of NPY have been observed primarily in experimental animals. However, caution has to be exercised when applying these effects to humans as demonstrated by differing distribution of receptor subtypes in rodents and humans (Lundell et al., 1996; Dumont et al., 1997).

1.12.4.2 NPY ACTIONS IN PERIPHERAL NERVOUS SYSTEM

In many different preparations, NPY has been shown to be a vasoconstrictor. Intravenous NPY causes a sustained rise in blood pressure in many different species including man. Use of a number of isolated vascular preparations either *in vitro* or *in vivo* has shown this vasoconstrictor action of NPY. This is a particularly prominent feature in the coronary and renal vasculature. The response to NPY on vascular smooth muscle is characteristically

described as slow in onset and prolonged (Lundberg and Tatemoto, 1982). In addition to the vasoconstrictor action of NPY, the peptide also plays a role in potentiating the action of other vasoconstrictors (Ekblad et al., 1984a). These observations have suggested that the physiological role of NPY may be more to modulate the action of catecholamines on vascular smooth muscle cells rather than a direct effect.

At the sympathetic neuroeffector junction, NPY acts both pre-synaptically and post-synaptically (Wahlestedt et al., 1986). In this way, NPY mimics catecholamine actions and, in a similar fashion, the action of NPY on its two receptor subtypes (NPY-Y1 and NPY-Y2) mimics that for catecholamine stimulation of the α1- and α2-adrenoreceptor. A large number of binding studies have demonstrated the presence of NPY/PYY receptors in vascular smooth muscle. The predominant receptor mediating peripheral vasoconstriction is the NPY-Y1 receptor, since this effect is evoked by NPY-Y1 receptor agonists (Wahlestedts et al., 1990). The recent emergence of specific antagonists BIBP 3226 (Rudolf et al., 1994) and SR120819A (Serradeil-Le Gal et al., 1994) made it possible to confirm that NPY-Y1 receptors are implicated in the vasoconstrictor action of NPY (Malmström and Lundberg, 1995; Serradeil-Le Gal, 1997). The presynaptic effects of NPY at the sympathetic neuroeffector junction are mediated through the NPY-Y2 receptor. This action of NPY on the NPY-Y2 receptor mimics activation of the a2-adrenoreceptor. NPY acts presynaptically to inhibit release of catecholamines and NPY itself into the synapse. Postsynaptic NPY-Y2 receptors may also mediate a direct effect on vasoconstriction (Grundemar and Håkanson, 1993).

In the adrenal gland, NPY is present in chromaffin cells were it is co-stored with noradrenaline in large electron dense secretory vesicles (Lundberg et al., 1982a; Ekblad et al., 1984a). In the adrenal medulla, NPY co-exists with a complex mixture of adrenaline and noradrenaline and neuropeptides. Neuropeptides present in these vesicles include calcitonin gene related peptide and enkephalin. After stimulation of the splanchnic nerve innervating the adrenal medulla, these components are released and transported to their target organs through the general circulation.

In the adrenal medulla, NPY-IR can be depleted by pretreatment with reserpine for 24 hours, which increases firing of sympathetic neurones (Lundberg et al., 1985; Allen et al., 1986 b). It has been shown that in the adrenal medulla, NPY mRNA expression can be regulated by preganglionic nerve activity. Insulin-induced hypoglycaemia produced an increase in splanchnic nerve activity causing a significant rise in NPY mRNA in the rat adrenal medulla (Fischer-Colbrie et al., 1988). This effect on NPY mRNA is dependent on nerve activity as it could be prevented by bilateral transection of the splanchnic nerve and by pretreatment with chlorisondamine (Schalling et al., 1991).

1.13 NPY RECEPTORS

Physiological studies with different ligands as well as *in situ* hybridisation with radiolabelled ligands have revealed the existence of different NPY receptor subtypes located in different organs and brain regions (Dumont et al., 1997; Grundemar, 1997). Elucidation of the functions of the NPY family of peptides requires understanding of the various receptor subtypes the peptides act upon. NPY receptors are sensitive to pertussis toxin, which ADP-ribosylates, and thereby inactivates, some G-proteins including the three forms of G_i and Go (Larhammar et al., 1993a). The second messenger responses evoked by activation of NPY receptors are inhibition of forskolin-induced adenylyl cyclase activity and elevation of intracellular Ca^{++} levels. NPY receptors may be coupled to mobilisation of Ca^{++} from intracellular stores via inositol phosphate dependent and independent mechanisms (Michel, 1991).

1.13.1 CHARACTERISATION OF NPY RECEPTOR SUBTYPES

The existence of many subtypes of NPY receptors has been demonstrated by functional and binding studies after administration of a range of peptide analogues to cells, tissue and organ preparations. Early physiological studies (Wahlestedt et al., 1986; 1990) predicted two different subtypes of receptors, NPY-Y1 and NPY-Y2. When using preparations of sympathetic neuro-effector junctions three different actions of NPY could be distinguished: (i) a direct postjunctional response of vasoconstriction, (ii) a postjunctional enhancement of noradrenaline-evoked vasoconstriction and finally (iii) a presynaptic suppression of noradrenaline release. By employing the full length peptide or carboxy-terminal parts of NPY or PYY molecules the two different subtypes of receptors were defined (Wahlestedt et al., 1986; 1990).

The postjunctional response requires the full length NPY peptide and is defined as the NPY-Y1 receptor. The NPY-Y1 receptor also binds NPY and PYY with similar affinity and can also be activated by [Leu^{31}-Pro^{34}]-NPY analogues where the amino acids 31 and 34 of NPY had been substituted for the corresponding amino acids from PP (Fuhlendorff et al., 1990). The prejunctional response could be evoked by amino terminally truncated peptides, NPY_{2-36} and NPY_{13-36}, and is defined as the NPY-Y2 receptor. The NPY-Y2 receptor binds both NPY and PYY with high affinity (Wahlestedt et al., 1986; 1990). To the date, six different receptors subtypes have been demonstrated to bind NPY. These NPY receptors are all G-protein coupled receptors (Wahlestedt and Reis, 1993; Gehlert, 1994; Larhammar, 1996a; Grundemar, 1997; Larhammar, 1997).

1.13.1.1 NPY-Y1 RECEPTORS

NPY-Y1 receptor sites are particularly concentrated in the cortical areas of the brain, in the cerebral cortex and olfactory nucleus and mixed populations of NPY-Y1 and NPY-Y2 receptors are found in various central nuclei (Dumont et al., 1997). In CNS, the NPY-Y1 receptor has been linked with different physiological processes, including stimulation of feeding behaviour (Kalra et al., 1990), stimulation of luteinising hormone-releasing hormone release (McDonald, 1990; McDonald and Koening, 1993) and anxiolytic-sedative effect (Wahlested et al., 1993). In PNS, NPY-Y1 receptor is found predominantly at the sympathetic postjunctional sites in blood vessels, where it mediates the contractile response to NPY of vascular smooth muscle cells, both directly and indirectly by potentiating the action of other pressor agents, such as noradrenaline and adrenaline (Wahlestedt et al., 1986; 1990).

The NPY-Y1 receptor has been identified in a number of cell lines, in particular it has been shown to be present on a number of human neuroblastoma cells (Sheikh et al., 1989). These cell lines include human erythro-leukaemia cells (HEL) and human neuroblastoma cell line (MC-IXC). The NPY-Y1 receptor has been reported to be present on a rat pheochromocytoma cell line : PC12 cells (Schwartz et al., 1987; DiMaggio et al., 1994).

Studies of solubilised NPY-Y1 receptors from neuroblastoma (MC-IXC) cells indicate that the receptor is a glycoprotein with a molecular weight of approximately 70 kDa (Wan and Lau, 1993). It has been suggested that the NPY-Y1 receptor binds to both the amino- and carboxy-termini of NPY peptide. The most notable feature of the NPY-Y1 receptor is the loss of agonist potency when only one or two amino-terminal amino acid residues are eliminated or substituted. By contrast, substitutions in the carboxy-terminus have been introduced without loss of potency, although the carboxy-terminal amide tyrosine residue is essential for biological activity. On the basis of results of studies using NPY analogues, it has been suggested that the hairpin loop is not essential for binding but it promotes binding of the NPY-Y1 receptor by bringing the amino- and carboxy-termini of NPY molecule in close apposition (Allen et al., 1987a).

1.13.1.2 NPY-Y2 RECEPTORS

The majority of NPY receptors in CNS are NPY-Y2 in nature with high densities seen in the hippocampal formation. Activation of NPY-Y2 receptors inhibits the release of glutamate from terminals that synapse onto rat hippocampal CA1 neurons. In PNS, the NPY-Y2 receptor is predominantly pre-junctional, principally in sympathetic, parasympathetic and sensory C-fibres (Grundemar and Håkanson, 1994). In these fibres the NPY effect is predominantly supression of transmitter release. Others effects associated with activation of NPY-Y2 receptors include:

vasoconstriction (certain blood vessels), antisecretory effect (intestine), inhibition of lipolysis (lipocytes), enhanced memory retention (hippocampus), inhibition of consummatory behaviour (hypothalamus) and suppression of noradrenaline release (locus coeruleus) (Grundemar and Håkanson, 1994).

Like the NPY-Y1 receptor, there are several human neuroblastoma cell lines that harbour NPY-Y2 receptors, including SMS-MSN, SMS-KAN, CHP-234 and SK-N-BE2 (Sheikh et al., 1989). Some of these cell lines have been utilised as *in vitro* models for NPY-Y2 agonist assays. The NPY-Y2 receptor recognises a great variety of carboxy-terminal NPY/PYY fragments (Grundemar et al., 1993). NPY-Y2 receptor binding has also been found in PC12 cells (DiMaggio et al., 1994).

1.13.1.3 NPY-Y3 RECEPTORS

A third receptor subtype NPY-Y3, was also demonstrated in early studies, which selectively bound NPY and NPY analogues but not PYY (Wahlestedt et al., 1992; Grundemar et al., 1993). When NPY analogues are injected into the nucleus tractus solitarium of the brainstem of rats, bradycardia, hypotension and inhibition of glutamate effects are observed. In addition bilateral stimulation of NPY-Y3 receptors in the nucleus tractus solitarium results in elevated blood pressure and attenuated baroceptor reflex. These central cardiovascular effects are mediated by NPY-Y3 receptors in the nucleus tractus solitarium brainstem (Grundemar and Håkanson, 1994).

NPY-Y3 receptors have been observed on rat cardiac membrane (Balasubramaniam et al., 1990) and on bovine adrenal chromaffin cells (Wahlestedt et al., 1992). In adrenal medulla, NPY-Y3 receptors suppress the nicotine-stimulated release of catecholamines, such as adrenaline. In adrenal cortex, NPY-Y3 receptors mediate the release of aldosterone (Grundemar and Håkanson, 1994).

1.13.1.4 PP-Y4 RECEPTORS

PP is released from pancreatic endocrine cells. PP receptors have been characterised by binding or functional assays in liver, intestine, spinal cord, adrenal gland, brainstem nuclei and PC12 cells (Grundemar, 1997; Larhammar, 1997). PP exerts effects on pancreatic exocrine secretion, gall bladder contraction, gastrointestinal motility, gastric acid secretion and corticosterone secretion. PYY is also expressed in PP cells and in somatostatin cells as well as in endocrine cells of the large intestine. PYY has similar actions to PP and both are released into the circulation in response to meal.

1.13.1.5 NPY-Y5 RECEPTORS

NPY is a powerful stimulant of food intake and is proposed to activate a specific hypothalamic ""NPY Y1-like feeding receptor" (Stanley et al., 1986; 1992). This has been suggested by results that show that the specific NPY-Y1 receptor analogue [Pro^{34}]-NPY is a potent stimulator of feeding (Stanley et al., 1992). However, this NPY-Y1 receptor analogue, is only able to give approximately half of the feeding stimulation of NPY (O'Shea et al., 1997). The specific NPY-Y2 recognising analogue, NPY2-36, is equally active as NPY when injected into the hypothalamus of rats (Kalra et al., 1991). In addition, PYY is a potent stimulator of feeding *in vivo* whereas neither rat PP nor NPY13-36 elicit feeding. Taken together, this pharmacological profile to different NPY analogues suggests a separate distinct NPY receptor to mediate feeding.

The novel fragment [Pro^{34}]-NPY3-36 is as effective at stimulating food intake as the classical NPY-Y1 analogue [Pro^{34}]-NPY. This new analogue bound to the NPY-Y1 receptor with only 1/20th of the affinity of NPY and failed to inhibit adenylyl cyclase through this receptor. In addition, the NPY-Y1 receptor antagonist BIBP-3226, which does not bind NPY-Y2, PP-Y4 and NPY-Y5 receptors, significantly reduced NPY induced feeding. These results suggested that the feeding effect of icv NPY involves a novel receptor and that is functionally distinct from the recognised receptor subtypes (O'Shea et al., 1997).

1.13.2 CLONING OF NPY RECEPTORS

1.13.2.1 NPY-Y1 RECEPTORS

Molecular and physiological characterisation of receptors requires access to molecular clones that can be used for functional expression in cell lines and for design of specific DNA and RNA probes. Several of the above described receptors have now been cloned. Previous studies had described the molecular cloning of the NPY-Y1 receptor cDNA from rat (Eva et al., 1990; Krause et al., 1992), mouse (Eva et al., 1992; Nakamura et al., 1995), human (Herzog et al., 1992; Larhammar et al., 1992) and *Xenopus* (Blomqvist et al., 1995).

1.13.2.2 NPY-Y2 RECEPTORS

Expression cloning allowed the isolation of the human NPY-Y2 receptor (Gerald et al., 1995; Rose et al., 1995; Gehlert et al., 1996a; Rimland et al., 1996). This receptor displays only 31% and 33% amino acid identity to the NPY-Y1 and PP1-Y4 receptors. The mouse orthologue was subsequently cloned by homology screening (Nakamura et al., 1996).

The human NPY-Y2 receptor is 381 amino acids in length and has a molecular mass of approximately 42 kDa. There is a single N-linked glycosylation site in the amino-terminal region and a cysteine in the carboxy-terminal region that may be involved in palmytoylation (Rose et al., 1995). Northern blot analysis of poly(A)$^+$ RNA from human brain regions revealed a 4.4 kb hybridising band. Brain regions expressing relatively high levels of mRNA encoding for NPY-Y2 receptor include the amygdala and hippocampus. Moderate levels were found in cerebral cortex. Lower levels were seen in the caudate, subthalamic nucleus and medulla. Very low levels were seen in the substantia nigra, thalamus and putamen. No hybridisation could be seen in the cerebellum and spinal cord (Gehlert et al., 1996a). Peripheral tissues displayed no detectable hybridisation. This suggest that either another form of the NPY-Y2 receptor is found in the periphery or that mRNA transcript is present at levels undetectable by Northern Blot analysis (Gerald et al., 1995; Rose et al., 1995; Gehlert et al., 1996a).

1.13.2.3 NPY-Y3 RECEPTORS

A bovine receptor clone, LCR1, was proposed to correspond to the NPY-Y3 receptor (Rimland et al., 1991), however the human orthologue of LCR1 exhibited no specific binding or functional response to NPY (Herzog et al., 1993a; Jazin et al., 1993a) and this receptor is no more similar to the NPY-Y1 receptor than another GPCR. Recently, the LCR1 receptor which is expressed in T helper cells, has been found to correspond to fusin, the CXCR-4 receptor which serves as an entry cofactor for the HIV virus (Feng et al., 1996).

1.14.2.4 PP-Y4 RECEPTORS

A high affinity human PP1-Y4 receptor with 53% amino acid identity in the transmembrane domains to the human NPY-Y1 receptor was cloned by several groups (Bard et al., 1995; Lundell et al., 1995; Gehlert, et al., 1996 b; Yan et al., 1996). Mouse (Gregor et al., 1996 b) and rat (Lundell et al., 1996; Yan et al., 1996) PP1-Y4 receptors have also been cloned.

The human PP1-Y4 receptor encodes a predicted protein of 375 amino acids with a molecular mass of 41 kDa. The human PP1-Y4 receptor has subnanomolar affinity for PP and nanomolar affinity for PYY and NPY, raising the possibility that it may be shared by all three pancreatic polypeptides. Tissue distribution studies in humans and mice suggest potential roles for PP1-Y4 in gastrointestinal tract, heart, prostate, as well as in neural and endocrine signalling (Gehlert et al., 1996b).

The primary amino acid sequence of the rat PP1-Y4 receptor is only 75% similar to the human PP1-Y4 receptor. Both human and rat PP1-Y4 receptors bind PP with high affinity, but the rat PP1-Y4 receptor has substantially lower affinity for both PYY and NPY. Thus, PP is the

endogenous ligand at the rat PP-Y4 receptor (Lundell et al., 1996). The distribution of PP1-Y4 receptor mRNAs is also found to differ between human and rat. The human PP1-Y4 receptor mRNA is abundantly expressed in colon, intestine and pancreas. It agrees well with the sites of PP and PYY synthesis and with the involvement of PP and PYY in regulating gastrointestinal functions (Lundell et al., 1995). In the rat, PP-Y4 receptor mRNA are detected in testis, lung, colon, while none was detected in small intestine and stomach (Lundell et al., 1996). In addition, PP have a prominent role in the CNS and control of gastrointestinal functions by acting on receptors in the rat brainstem. Thus, the sequence, the tissue distribution, and the binding profile of the PP1-Y4 receptor differ between rat and humans (Lundell et al., 1995; 1996).

A novel member of this family was recently cloned and named PP2-Y4 (Gregor et al., 1996a). The coding region of the mouse PP2-Y4 gene reveals no introns and predicts a receptor of 371 amino acids. Percent identities of the mouse PP2-Y4 receptor to mouse NPY-Y1, mouse PP1-Y4 and human NPY-Y2 receptors are 53%, 42% and 31%, respectively (Gregor et al., 1996a). The human PP2-Y4 cDNA and gene sequences display a single base deletion in the coding region, resulting in a frameshift mutation that predicts a truncated receptor of 290 amino acids. This suggests that the human PP2-Y4 is either functionally inactive or its has acquired a PP-independent function (Gregor et al., 1996a).

1.13.2.5 NPY-Y5 RECEPTORS

An additional receptor, NPY-Y5, referred to as the "feeding receptor" was also isolated using expression cloning from rat (Gerald et al., 1996; Hu et al., 1996) and human (Gerald et al., 1996). This receptor shows a binding profile that is consistent with that of the proposed "feeding receptor" in that it binds NPY, [Leu^{31},Pro^{34}]-NPY and NPY2-36 with similar affinities and is expressed in regions of the hypothalamus which mediate food intake: arcuate nucleus, lateral hypothalamus and paraventricular nucleus. However, recent studies indicate that the pharmacological profile for the expressed receptor is not identical that observed for the feeding response (O'Shea et al., 1997).

The rat cDNA encodes a 456 amino-acid protein with less than 33% overall amino acid identity to known NPY-type receptors. The human homologue has 88% amino acid identity to the rat receptor. The mRNA is found in the CNS, including the paraventricular nucleus of the hypothalamus but not in peripheral tissues. Southern blot analysis indicates both rat and human genomes contain a single NPY-Y5 receptor gene. The human NPY-Y1 and NPY-Y5 receptor genes map, in opposite orientation, to the same locus on chromosome 4q (Herzog et al., 1997).

1.13.2.6 NPY-Y6 RECEPTORS

Concomitantly yet another receptor, independently also called NPY-Y5, later renamed NPY-Y6, with 56% amino acid identity to the rat NPY-Y1 receptor, 47% overall identity to the rat PP1-Y4 receptor and only 33 % identity to the rat NPY-Y2 receptor was cloned in mouse with low stringency homology screenings using a NPY-Y1 probe (Weinberg et al., 1996).

The murine NPY-Y6 gene encodes a predicted 371 amino acid polypeptide. Like the NPY-Y5 receptor it exhibits a pharmacological profile that is similar to that described for the "feeding receptor" and has hypothalamic expression, including anterior hypothalamus, bed nucleus stria terminalis, the suprachiasmatic nucleus and the ventromedial nucleus with no localisation apparent elsewhere in the brain and with significant mRNA expression only within the kidney (Weinberg et al., 1996). The orthologue of the mouse NPY-Y6 receptor has been cloned in human, rabbit and primates (Matsumoto et al., 1996). In the primates species the predicted receptor is truncated after TM6 by a frameshift mutation although its mRNA is abundantly expressed in heart and skeletal muscle, suggesting a novel function of the human gene. Southern blot analysis indicate that the human genome contains a single copy of this gene on the chromosome 5 (Matsumoto et al., 1996).

1.14 MOLECULAR BIOLOGY OF NPY-Y1 RECEPTOR

1.14.1 NPY-Y1 RECEPTOR cDNA CLONES

The cDNA that encodes the NPY-Y1 receptor has been defined although it was originally reported as an "orphan" seven transmembrane domain receptor belonging to the typical GPCR family (Eva et al., 1990). By predicted sequence analysis, the receptor resembled the tachykinin family of receptors, possessing within the putative transmembrane domain 33% and 31% amino acid homology with neuromedin K and substance K receptors, respectively. However, when transiently expressed, it failed to bind any known tachykinin (Eva et al., 1990). It has subsequently been redefined as the NPY-Y1 receptor as the expressed protein displays all the features of the NPY-Y1 receptor binding (Herzog et al., 1992; Larhammar et al., 1992).

1.14.1.1 RAT NPY-Y1 RECEPTOR

The rat clone, named FC5, which specified an open reading frame of 349 amino acids (34.4 kDa) was obtained from a forebrain cDNA library (Eva et al., 1990). The predicted polypeptide contains three potential N-glycosylation sites in the amino-terminal extracellular domain. Other

consensus sequences for post-translational modifications include four target amino acids sites for protein kinases within the third intracellular loop and the intracellular located carboxyl terminus. There are two potential phosphorylation sites for PKC at residues Thr-257 and Ser-362, two sites for tyrosine kinase at Tyr-252 and Tyr-346 and one site for PKA at Ser-375. The presence of these sites suggest that the receptor may be regulated by phosphorylation (Eva et al., 1990).

Northern blot analysis found a prominent mRNA species of approximately 4 Kb in rat cerebral cortex, hippocampus, hypothalamic nucleus and thalamic nuclei. No signal was observed in peripheral tissues (Eva et al., 1990). An expression vector carrying the NPY-Y1 receptor cDNA was transiently expressed in human embryonic kidney 293 cells (Krause et al., 1992). Inhibition of luteinising hormone-stimulated adenylyl cyclase and Ca^{++} mobilisation in 293 cells was reported for NPY-stimulated cells and might reflect receptor coupling to a Gi protein.

1.14.1.2 HUMAN NPY-Y1 RECEPTOR

The human NPY-Y1 receptor was cloned from a fetal library (Herzog et al., 1992; Larhammar et al., 1992). This receptor consists of 384 amino acids and show 94% identity to that deduced from the rat FC5 clone. It is notable that the sequence DRY (Asp-Arg-Tyr), which follows TM3 in most receptors belonging to the GPCR superfamily, reads ERH (Glu-Arg-His) in the NPY-Y1 sequences of both human and rat. Several threonine and serine residues, candidates for phosphorylation are found in the carboxy-terminal sequence (Herzog et al., 1992; Larhammar et al., 1992).

Northern blot analysis using a human neuroblastoma cell line, SK-N-MC, revealed a single 3.5-Kb mRNA species. Southern hybridisation to human genomic DNA suggest that the genome contains a single NPY-Y1 receptor gene. The cDNA of the human NPY-Y1 receptor was transferred to an expression vector and used to transfect COS-1 cells. In COS-1 cells NPY and PYY accelerated Ca^{++} influx and inhibited forskolin-stimulated cAMP accumulation (Larhammar et al., 1992).

1.14.1.3 MOUSE NPY-Y1 RECEPTOR

The mouse NPY-Y1α receptor was cloned from a bone marrow cDNA library (Nakamura et al., 1995). The polypeptide encoded by this cDNA is 93% homologous to the human NPY-Y1 receptor. The cytoplasmic tail of this receptor contains 6 serine residues and 4 threonine residues, as possible phosphate acceptors. Furthermore, there are four N-linked glycosylation sites in the amino-terminal domain and the extracellular loops. Another isoform NPY-Y1β is identical to the NPY-Y1α receptor from the 5'-untranslated region to the third extracellular

region, but is completely different in the seventh transmembrane, the cytoplasmic tail and 3'-untranslated region (Nakamura et al., 1995).

Southern blot analysis of the mouse genomic DNA indicated that the two NPY receptor isoforms originate from a single gene (Nakamura et al., 1995). The mouse NPY-Y1β receptor is produced by RNA splicing of the third intron, located downstream from position 908 of the NPY-Y1α receptor. Sequences around the putative junctions fit well with the proposed consensus sequences for RNA splicing. Despite the lack of seventh transmembrane domain and cytoplasmic tail, the NPY-Y1β receptor shows ligand binding characteristics identical to those of the NPY-Y1α receptor. This suggest the extracellular loops rather than the seventh transmembrane domain plays a critical role for the ligand binding.

The two NPY receptor mouse isoforms were expressed in chinese hamster ovary (CHO) cells. The binding of NPY to the NPY-Y1a receptor results in a significant increase of intracellular Ca^{++} and the inhibition of forskolin-stimulated cAMP accumulation (Nakamura et al., 1995). A transient generation of IP3 evoked by NPY and NPY-induced intracellular Ca^{++} increase was markedly suppressed by treatment with U-73122, a PLC inhibitor. When the CHO cells were treated with PTX, the intracellular Ca^{++} increases and inhibition of forskolin-induced cAMP production were completely abolished (Nakamura et al., 1995). This results suggest the NPY-Y1a receptor couples to PTX-sensitive G-proteins, probably Gi/Go.

NPY activated MAPK in CHO cells and this activation was completely abolished by PTX treatment (Nakamura et al., 1995). Pre-treatment of the cells with wortmannin inhibited the MAPK activation in response to NPY. Treatment with wortmannin, did not affect intracellular Ca^{++} mobilisation (Nakamura et al., 1995). In addition, the MAPK activity was not inhibited by treatment with BAPTA/AM, an intracellular Ca^{++} chelator, while it completely abolished the NPY-induced intracellular Ca^{++} increase. These observations suggest that the NPY-Y1α receptor mediates MAPK activation via a Ca^{++}-independent but a wortmannin-sensitive pathway. In contrast, NPY-evoked cell responses such as intracellular Ca^{++} increase, inhibition of forskolin-induced cAMP production or MAPK activation was not detected expressing the NPY-Y1β receptor in CHO cells. These later results suggest that the cytoplasmic tail of the NPY-Y1α receptor may contribute to G-protein activation (Nakamura et al., 1995).

Gene expression analysis by Northern blots and RT-PCR indicate that the NPY-Y1α receptor is highly expressed in the brain, heart, kidney, spleen, skeletal muscle and lung, whereas the other isoform, the NPY-Y1β receptor is expressed in mouse embryonic developmental stage (7 and 11 days), bone marrow cells and several hematopoietic cell lines (Nakamura et al., 1995).

These results suggest that the NPY-Y1β receptor is an embryonic and a bone marrow form of the NPY-Y1 receptor, which decrease in expression during development and differentiation.

1.14.1.4 *Xenopus* NPY-Y1 RECEPTOR

The *Xenopus* NPY-Y1 receptor shares 81% amino acid sequence identity with the human NPY-Y1 receptor in the transmembrane spanning regions I to VII (Blomqvist et al., 1995). The extracellular amino-terminal region, transmembrane IV and second extracellular loop contain several replacements suggesting that these portions have limited direct interactions with the peptide ligand. A conserved aromatic residue in the third intracellular loop is conserved in the mammalian and *Xenopus* NPY-Y1 receptors in the sequence (YTKIFLRLKRRN). This sequence is important in G-protein coupling to the PI pathway. Another conserved sequence of the NPY-Y1 receptor family is the ERH motif in the beginning of the second extracellular loop (Blomqvist et al., 1995).

1.14.2 NPY RECEPTOR GENOMIC CLONES

1.14.2.1 MOUSE NPY-Y1 RECEPTOR

The mouse gene for the NPY-Y1 receptor was characterised by DNA sequencing and gene expression studies (Eva et al., 1992). The mouse NPY-Y1 receptor shares 98% amino acid homology with the rat NPY-Y1 receptor. It comprises three exons with a 6.4 Kb intron in the 5'-untranslated region and a 80 bp internal intronic sequence immediately after transmembrane region V. The mouse NPY-Y1 receptor also contain an alternate exon 4 located 15 kb downstream of exon 3 (Nakamura et al., 1995). The 6.4 Kb intron is flanked at the 5'-flanking region by 84 bp matching the rat cDNA sequence (Eva et al., 1992). Primer extension analysis indicates the presence of several transcription initiation sites all located closely to the end of the 5'-untranslated region of the DNA.

The 5'-flanking region of this gene lacks canonical TATA or CCAAT consensus sequences in the proximity of the multiple transcription initiation sites, but contains consensus sequences for various transcriptions factors including an Sp1 binding site (GGGGCG), a CRE binding site (CGTCA), three sequences related to the AP-1 motif (TGAGTCA), three non-palindromic estrogen receptor elements (ERE), two in the form of TGACC and one in the form of GGTCA, five non-palindromic glucocorticoid receptor elements (GRE) (AGGACT) and one non-palindromic sequence (GGGATTTCAC) which bind to the transcription factor NF-κB (Eva et al., 1992).

A 1,300 bp genomic fragment of the 5'-flanking region drives the expression of the lacZ reporter gene in NG108-15 cells, a mouse-rat hybrid neuroblastoma glioma cell line and primary cultures of cortical neurons from newborn rat brain which express the NPY-Y1 receptor but not in glial and human embryonic kidney 293 cells which do not constitutively express this receptor, suggesting a restricted tissue specificity of the isolated promoter sequence (Eva et al., 1992). Analysis of this 1.3 kb genomic fragment with a reporter gene assay suggested the presence of a 181-bp cell type specific core and upstream of this region two positive and two negative regulatory regions (Musso et al. 1997). One of the positive regulatory sequence includes a ERE binding site (GGTCA) and a GRE binding site (AGGACT) and the another positive regulatory sequence includes an AP-1/GRE binding site (Musso et al., 1997).

Sequence analysis of the NPY-Y1 receptor promoter identified two decameric sequences corresponding to consensus binding sites for NF-κB/Rel proteins (Musso et al., 1997). NF-κB is an important regulator of genes activated during the immune response. Gel shift analysis indicated that a 29-bp oligonucleotide comprising the two putative κB sites, specifically binds κB-related complexes in nuclear extracts from rat brain areas, NG108-15 cells, and the murine T cell clone A.E7. In nuclear extracts from A.E7 and NG108-15 cells, this NF-κB sequence specifically binds an additional complex. Transient transfection studies demonstrated that this NF-κB sequence acts as an enhancer element, inferring its potential role in regulation of the NPY-Y1 receptor gene expression (Musso et al., 1997).

1.14.2.2 HUMAN NPY-Y1 RECEPTOR

A 14 Kb region of genomic DNA encoding the human NPY-Y1 receptor gene was cloned and the human gene localised to chromosome 4q (31.3-32) (Herzog et al., 1993b). The NPY-Y1 receptor gene is divided into three exons. The first 80 nucleotides of the 5'-untranslated sequence of the human NPY-Y1 receptor mRNA are separated by a 6 Kb intron from the second exon. The second intron (97 bp) contains an in-frame stop codon and is located after the fifth transmembrane domain (Herzog et al., 1993b). The nucleotide sequences of the two introns adjoining the splice junctions are consistent with the recognised splicing consensus sequence GT/AC.

In the 5'-flanking region a potential transcription start site was found 210 nucleotides upstream of the initiation methionine codon. A potential polyadenylation site (ATTAAA) was found which is consistent with the size (3.5 Kb) of the NPY-Y1 receptor mRNA in Northern blot analysis of placenta and kidney (Herzog et al., 1993b). Several cDNA clones encoding the human NPY-Y1 receptor have been isolated that contain different sequences at their 5'-ends (Ball et al., 1995). Analysis of NPY-Y1 transcripts in the SK-N-MC neuroblastoma cell line

shows that the at least three forms of the NPY-Y1 receptor are generated by alternate exon splicing. The exons are found 6.4 (1A), 18.4 (1B) and 23.9 (1C) Kb upstream of exon 2 (Ball et al., 1995).

The exon 1A (hippocampus cDNA clone) was determined to be 80 bp, the exon 1B (testis cDNA clone) was 106 bp and the exon 1C (SK-N-MC cDNA clone) was 109 bp (Ball et al., 1995). The promoter A lacks a typical TATA and CCAAT box, however it contains a CRE binding site (CGACGTCA), an AP1 binding site (TGAATCA) and an octamer-binding (Oct-1) site (ATTTGCAT). Promoter B also lacks canonical TATA and CCAAT motifs and has a high G+C content (approximately 70%) in the 200 bp preceding the transcription start site. It contain two AP-2 binding sites (CCGCGGGC), a GRE binding site (TGTTCT), an AP-1 motif (TGAATAA) and a recognition site for NF-κB. The promoter C has a potential response element for a glucocorticoid receptor; this promoter also lacks a TATA and/or CCAAT box (Ball et al., 1995).

Alternative splicing of sequences within the 5'-untranslated region can regulate receptor expression at the translational level. For example, the presence of a open reading frame within the 5'-untranslated region can result in the inefficient initiation of translation and suppression of protein expression. The human NPY-Y1 receptor gene contains a short open reading frame in the 5'-untranslated region situated on exon 1C; however, no start codons are present in either exon 1A or 1B. The differential use of these exons may be a mechanism for translational regulation of the NPY-Y1 receptor protein in a cell specific manner (Ball et al., 1995).

Little is known about the transcriptional regulation of the NPY-Y1 receptor gene. Administration of glucocorticoids to rats causes an up-regulation of NPY-Y1 receptor expression in the arcuate nucleus, although it is not known if this is a direct effect of the glucocorticoids acting on the NPY-Y1 receptor gene promoters. Moreover, in the human gene, there are potential binding sites for the glucocorticoid receptor in promoters A, B and C (Ball et al., 1995). The molecular mechanisms responsible for regulation of human NPY-Y1 receptor expression are unknown; however, they may result from alteration of the transcriptional regulatory pathway.

Undifferentiated PC12 cells express the NPY-Y1 receptor subtype and this receptor can be detected by binding studies in cells differentiated by either dexamethasone or NGF (DiMaggio et al., 1994). The NPY-Y2 receptor, in contrast, could only be found by binding analysis in cells differentiated by NGF. Thus, no detectable binding of NPY-Y2 receptor-selective agonist, NPY13-36 could be measured in undifferentiated or dexamethasone differentiated PC12 cells (DiMaggio et al., 1994). This data suggests differential expression of NPY receptor subtypes. However, in this study, no measurements were made of the number of receptors

following the different agents. Therefore, it is unclear whether differentiation agents affect NPY-Y1 receptor numbers and gene expression.

1.14.2.3 HUMAN NPY-2 RECEPTOR

The human NPY-Y2 receptor gene spans 9 kb of genomic sequence and is encoded on two exons (Ammar et al., 1996). As in the NPY-Y1 receptor gene, the 5'-untranslated region of the NPY-Y2 receptor is interrupted by an intervening sequence (aproximately 4.5 Kb), however the NPY-Y2 receptor gene does not contain an intron analogous to that present in the open reading frame of the NPY-Y1 receptor (Herzog et al., 1993b). RT-PCR studies indicate that the transcription begins in the region upstream of position -1270, most likely within the adjacent 200 bp of sequence. Thus, the estimated length of the 5'-untranslated region is the 1.3-1.4 Kb and the total length of the mRNA is approximately 4.5 kb, which is the estimated size of the single NPY-Y2 receptor mRNA detected in Northern blot analysis (Gerald et al., 1995; Rose et al., 1995). The NPY-Y2 receptor gene maps to the human chromosome 4q31, the same region containing the NPY-Y1 receptor (Herzog et al., 1993b) and NPY-Y5 (Herzog et al., 1997), suggesting that these subtypes may have arisen by gene duplication despite their structural difference. In addition, the NPY-Y5 receptor gene also is present in the same region, in the reverse orientation (Gerald et al., 1996; Herzog et al., 1997). Southern blot analysis of human genomic DNA indicates that the gene encoding the NPY-Y2 receptor is single copy and distinct from that encoding the NPY-Y1 receptor.

1.14.2.4 HUMAN NPY-5 RECEPTOR

The human NPY-Y1 and NPY-Y5 receptor genes are transcribed in opposite directions from a common promoter region on chromosome 4q31-32 (Herzog et al., 1997). One of the alternately spliced 5'-exons of the NPY-Y1 receptor gene (exon 1C) is also an integral part of the large third intracellular loop of the NPY-Y5 receptor (Gerald et al., 1996).

The close proximity of the two NPY receptors genes suggests that they have envolved from a duplication event with the small intron interrupting the open reading frame of the NPY-Y1 receptor gene converted into a functional sequence within the NPY-Y5 gene, while the reverse complementary sequence was utilised as an alternatively spliced 5'-exon for the NPY-Y1 receptor gene (exon 1C). Thus, transcriptional activation of the NPY-Y1 exon 1C would have an inhibitory effect on NPY-Y5 gene expression. However, such a mechanism may represent only one aspect of a regulatory interaction between these two receptors.

Analysis of different cDNA clones demonstrates that the NPY-Y5 clone also uses at least two alternatively spliced exons, encoding part of the 5'-untranslated region, which are controlled by

different promoters. NPY-Y5 exon 1A and NPY-Y5 exon 1B are in close proximity to exon 1B of the NPY-Y1 receptor suggesting the possibility of a co-regulation of transcription of both genes through a common promoter region. As both NPY-Y1 and NPY-Y5 receptors are thought to play an important role in the regulation of food intake, co-ordinate expression of their specific genes may be important in the modulation of NPY activity.

1.15 OBJECTIVES

The NPY-Y1 receptor has been linked to important physiological process in the nervous system (Grundemar and Hakanson, 1994). Understanding the molecular mechanisms controlling NPY-Y1 receptor gene expression will provide clues, not only about the numerous systems where this receptor plays a regulatory role, but also about the role of NPY receptors in general on the overall organization of the nervous system.

In this thesis the transcriptional control of the NPY-Y1 receptor gene will be studied using the luciferase reporter system in a model of sympatho-adrenal system, PC12 cells, which constitutively express this neuropeptide receptor (DiMaggio et al., 1994). This model will be useful in understanding the function of different activators and inhibitors of specific intracellular signalling pathways and the molecular mechanisms involved in the basal and induced transcriptional activation of this neuropeptide receptor gene by different factors, such as dexamethasone, NGF and PACAP. These factors are well known to affect the levels of expression of other late responsive genes in PC12 cells (See Introduction) using the putative transcription factor sites, such as AP1, CRE and GRE present in the promoter region of the NPY-Y1 receptor gene. Morover, specific deletions of these transcription factor sites wil be made to understand its specific role in the transcriptional control of this gene. Lastly, the post-transcriptional control of this gene will be measure using Northern blot, RPA or RT-PCR to determine the expression of the specific mRNA of the NPY-Y1 receptor gene in PC12 cells and compare with results obtaining the reporter assay systems.

CHAPTER 2

MATERIALS AND METHODS

2.1 GENERAL LABORATORY SUPPLIES AND PROCEDURES

2.1.1 GENERAL STOCK CHEMICALS AND REAGENTS

General stock chemicals were of the best quality commercially available (analytical or molecular biology grade) and with exception of those listed below were from BDH Chemicals Ltd, Poole, Dorset, U.K.

Ammonium acetate, bromophenol blue, n-butanol, caesium chloride, chloroform, denhardt's solution, dextran sulphate, diethylpyrocarbonate (DEPC), EDTA, ethidium bromide, formaldehyde, formamide, glycerol, guanidine thiocyanate, isoamyl alcohol, isopropanol, N-laurylsarcosine, orange G, phenol, sodium acetate, sodium citrate, polyvinilpyrollidone (PVN), TEMED, Trizma acetate were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K.

Ammonium persulphate, acrylamide, boric acid, N,N'-methylene *bis* -acrylamide, sodium dodecyl sulphate (SDS) were from Fisons Scientific Equipment, Loughborough, Leicestershire, U.K.

Low melting point (LMP), Nusieve and Seakem GTG agarose were supplied by FMC Bioproducts, Rockland, USA.

Filter paper was obtained from Whatman International, Maidstone, Kent, U.K.

2.1.2 MOLECULAR BIOLOGY ENZYMES AND KITS

Molecular biology enzymes with exception of those listed below were from Boehringer Mannheim Ltd., East Sussex, UK or Promega Corp., Madison, USA.

Avr II and *Eco* NI were from New England Biolabs, Hertfordshire, UK.

Dynazyme polymerase was from Finnzymes Oy, Flow Instruments, UK.

Taq DNA Polymerase, DOTAP and DOSPER liposomal transfection reagent were from Boehringer Mannheim Ltd., East Sussex, UK.

Thermosequenase™ cycle sequencing kit and Redivue™ [$a^{35}S$]-dATP, [$a^{32}P$]-UTP and [g-^{32}P]-ATP were from Amersham LIFE SCIENCE Inc., Cleveland, USA.

GeneAmp® RNA-PCR kit was obtained from Perkin-Elmer (Roche Molecular Systems), New Jersey, USA.

Bio-Rad Protein Assay Dye reagent Concentrate and AG® 501-X8 Bio-Rex® MSZ 501 (D) were from Bio-Rad Laboratories Ltd, Hertfordshire, UK.

USBioclean™ MP Kit and shrimp alkaline phosphatase were from United States Biochemical, Cleveland, USA.

Polaroid Type 667 Black and White Instant Pack Film and DS-34 Camera System were from Polaroid Corp., Cambridge, USA.

Biomax MR™ film was from Eastman Kodak Company, USA.

Wizard™ Maxipreps DNA Purification system and Wizard DNA Clean-Up system, AMV Reverse Transcriptase Primer Extension System, Riboprobe® *in vitro* Transcription System, Cell Culture Lysis Reagent, Beetle Luciferin, Luciferase Assay System, Dual luciferase™ Reporter System and Luciferase Reporter Vectors were from Promega Corp., Madison, USA.

Ribonuclease Protection Assay (RPA II™) kit was from Ambion, Austin, Texas.

PKC Assay kit, Lipofectin® and Lipofectamine® Reagent were from Life Technologies Inc., Paisley, Scotland.

Ligator Kit was from R&D System Europe Ltd., Abingdon, U.K.

2.1.3 BACTERIAL AND CELL CULTURE REAGENTS

All the tissue culture reagents were of the best quality commercially available and with exception of those listed below were from Life Technologies Inc., Paisley, Scotland.

Ampicillin, rat collagen type I, poly-D-Lysine, calcium chloride, HEPES buffer, insulin, transferrin, glutamine, putrescine, L-thyroxin, selenium, tri-iodo-thyroxin was from Sigma (London) Chemical Co., Poole, Dorset, U.K.

Bacto-Agar, Bacto-Tryptone, Bacto-Yeast extract and LB medium were obtained from Difco Laboratories, Detroit, USA.

BSA patho-o-cyte 4 was from ICN-Flow Biomedicals, High-Wycombr, UK.

G-418 and H-89 were from Alexis Biochemical, Nottingham, UK.

PD98059 was from New England Biolabs , Hertfordshire, UK.

Calphostin C, dexamethasone, 17-b estradiol, progesterone, NGF, PACAP-38, DBC, forskolin, ionomycin, PMA, DII1, K252a, KN-62, wortmannin and LY 294002 were from Calbiochem-Novabiochem, Nottingham, U.K.

Compositions of all solutions and buffers used are listed in Appendix I when not specified in the text. Equipment is specified in the text. Water for general laboratory use was single-distilled or deionized. Solutions were sterilised by autoclaving to high temperature (121°C) with high pressure (15 lb/in^2) for 20 minutes in an autoclave or by filtration through 0.22 μm filter units (Millex-GV, Millipore).

In addition, general laboratory precautions and procedures were followed:

Toxic chemicals: many solvents such as chloroform, butanol, isoamyl alcohol and phenol are toxic. Other reagents such as ethidium bromide, DEPC and formamide are potential carcinogenic.

Radioactivity: ^{32}P and ^{35}S were used with appropriate shielding, stored and discarded in the appropriate radioactive area.

RNase contamination: RNase contamination was minimised in the laboratory. When working with solutions and equipment used for RNA analysis, gloves were worn at all times to prevent nucleases contamination. Sterile, disposable plasticware was used whenever possible and plastic disposable pipette tips and microcentrifuge tubes were autoclaved prior to use. All glassware and other bakeable equipment, were baked at 270°C for at least eight hours. Separate chemical stocks were designated for RNA use only. Solutions for RNA use were treated overnight with 0.05-0.1% diethylpyrocarbonate (DEPC), a non-specific inhibitor of ribonuclease, and then autoclaved to destroy any remaining DEPC. Dilutions of stock chemicals and enzyme reaction buffers were made with DEPC-treated water.

TABLE 2.1 BACTERIAL STRAINS AND PLASMIDS

Strain/Plasmid	Genotype	Source/Reference
Escherichia coli JM109	F' [*tra* D36,pro A^+B^+, *Lac* Iq, lacZΔM15] *rec* A1, *sup* E44, *end* A1, *hsd* R17 (r_k^-,m_k^+), *gyr* A96, *thi* -1, el4$^-$ (mcrA$^-$) *rel* A1Δ (*lac -pro* A^+B^+)	Promega Corporation, Madison, USA.
Escherichia coli TG1	F' [*tra* D36,pro A^+B^+, *Lac* Iq, lacZΔM15] *sup* E44, *sup* EΔ (hsd M-mcrB) 5 (r_k^- m_k, McrB) *thi* -1 rel A1Δ (*lac- pro* A^+B^+)	Amershan, Cleveland, USA.
pBluescript SK$^-$ (2961 bp)	General cloning plasmid	Stratagene, La Jolla, USA.
pGL3-Basic (4818 bp)	Promoter-less firefly luciferase reporter vector	Promega Corporation, Madison, USA.
pGL3-Control (5256 bp)	SV40-driven firefly luciferase reporter vector	Promega Corporation, Madison, USA.
pRL-CMV (4079 bp)	CMV-driven *Renilla* luciferase reporter vector	Promega Corporation, Madison, USA.
pRL-SV40 (3705 bp)	SV40-driven *Renilla* luciferase reporter vector	Promega Corporation, Madison, USA.
p TAg (3816 bp)	TA-cloning vector	R&D Systems, Abingdon, U.K.
pT7-RNA 18S human	Antisense control template	Ambion, Austin, Texas.

For all *Escherichia coli* strains, the genes listed above signify that the bacterium carries a mutated allele. The genes listed above as being present on the F' episome, however, represent the wild-type alleles.

2.3 MAMMALIAN CELL CULTURE

All cell lines were maintained at 37°C in a water-saturated atmosphere of 95% air and 6.5% CO_2 in tissue culture-designated incubators (Napco, model 5410). Cell culture was performed in laminar flow tissue culture hoods (Microflow, Biological Safety Cabinet). Solutions, materials and reagents used for cell culture were sterilised either by autoclaving to high temperature (121°C) with high pressure (15 lb/in^2) for 20 minutes or by filtration through 0.22 μm filter units (Millipore, Millex-GV).

2.3.1 PREPARATION OF TISSUE CULTURE PLATES

2.3.1.1 PREPARATION OF COLLAGEN-COATED TISSUE CULTURE PLATES

Tissue culture plates (Nunc) were coated with collagen to promote cell adhesion of PC12 wild type cells. Plates were briefly incubated with 0.25 mg/ml type I rat tail collagen in 0.5 M acetic acid (See Appendix I) at room temperature in a laminar flood cabinet (Microflow, Biological Safety Cabinet). After the collagen solution was recovered for reuse, plates were washed once with 80% ethanol and then allowed to dry for two to three hours in a tissue culture hood. Plates were then replaced in their original storage plastic bags and sealed with adhesive tape. Plates prepared in this way were stored for up to a month at room temperature before use.

2.3.1.2 PREPARATION OF POLY-D-LYSINE TISSUE CULTURE PLATES

Tissue culture plates (Nunc) were coated with poly-D-lysine to promote cell adhesion of PC12asn17-W7 cells. Add 5 mg poly-D-lysine to 30 ml sterile tissue culture water. Aliquot into 1 ml fractions and store at -20°C until required. Dilute 1ml aliquot into 10 ml tissue culture sterile water. Add to the plate and ensure that the bottom of the plate has an even covering in the laminar flood cabinet (Microflow, Biological Safety Cabinet). Leave for 30 minutes in the hood. Aspirate liquid and wash culture plate once with tissue culture sterile water. Following the plates were treated with rat tail collagen as a mentioned before.

2.3.1.3 DEXTRAN COATED CHARCOAL STRIPPED SERUM

Foetal calf serum (FCS) was dialysed against Hank's Modified Buffer (Appendix I) over 48 hours at 4°C and transferred to a glass container and heat inactivated for 45 minutes at 56°C. Serum was cooled to 4°C and added to a pellet of dextran coated charcoal (Appendix I). This

solution was allowed to stir at 4°C for 30 minutes before centrifuguing at 12,000 g x 30 minutes at 4°C. The supernant was filter sterilised through 0.22 µm filters (Millipore).

2.3.2 CULTURE CONDITIONS OF THE CELLS

2.3.2.1 PC12 PARENTAL CELL LINE

PC12 cells were a gift of Dr X. Brakefield, Boston University, Boston. PC12 cells were grown on collagen-coated polystyrene tissue culture plates (Nunc) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% foetal calf serum (FCS), 10% heat-inactivated horse serum (HS), 2 mM glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin. PC12 cells were routinely maintained on 100 mm diameter collagen-coated plates and subcultured every third day at a plating density of approximately 1-5 x 10^3 cells/cm^2. As the PC12 adherent cells were only weakly attached, cells were dislodged and resuspended by vigorous pipetting.

2.3.2.2 PC12asn17-W7 CELL LINE

PC12asn17-W7 cells (PC12 cells stable transfected with a plasmid [pRSVN17] containing an inhibitory mutation of the human c-Ha-*ras* gene) (Buensuceso, 1995) were maintained using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with SATO mix (Bottenstein, 1985) containing (in mg/L): progesterone, 0.062; putrescine, 16.1; thyroxin, 0.4; selenium, 0.039; apo-transferrin (human) 100; insulin (bovine pancreas), 10; tri-iodo-thyronine, 0.377. The medium was also supplemented with 1% BSA Path-o-cyte 4, 2% fetal calf serum (FCS), 2 mM glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin, 800 µg/ml geneticin (G418; neomycin) and NGF (40 ng/ml). PC12asn17-W7 cultures were maintained by routine passage in poly-D-lysine coated plates followed by air-dried rat tail collagen and recovered by incubation for 2-3 minutes with 0.05% trypsin, after resuspension in DMEM complete serum solution.

2.3.2.3 COS-7 CELL LINE

COS-7 cells were routinely grown on tissue culture plates (Nunc) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% calf serum (CS), 2 mM glutamine, 100 units/ml penicillin and 100 mg/ml streptomycin. Cells were routinely maintained on 100 mm diameter plates and recovered every third day by incubation for 2-3 minutes with 0.05% trypsin, after resuspension in DMEM complete serum solution. Cells were cultured in 60 mm plates for most transfection experiments.

2.3.2.4 RINm5f CELL LINE

RINm5f cells were a gift of Dr. Steve Ashcroft, Nuffield Department of Clinical Biochemistry, John Radcliffe Hospital, Oxford. RINm5f cells were routinely grown on tissue culture plates (Nunc) in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS), 2 mM glutamine, 100 units/ml penicillin and 100 mg/ml streptomycin. RINm5f cells were routinely maintained on 100 mm diameter tissue culture plates and recovered every third day by incubation for 2-3 minutes with 0.05% trypsin, after resuspension in RPMI complete serum solution.

2.3.2.5 GT1-7 CELL LINE

GT1-7 cells were a gift Professor Pamela Mellon, University of California, San Diego. GT1-7 cells were routinely grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% foetal calf serum (FCS), 5% horse serum (HS), 2mM glutamine, 100 units/ml penicillin and 100 mg/ml streptomycin. Cells were routinely maintained on 100 mm diameter plates and recovered every third day by incubation for 2-3 minutes with 0.05% trypsin, after resuspension in DMEM complete serum solution. Cells were cultured in 60 mm plates for most transfection experiments.

2.3.3 CELL QUANTIFICATION

Total cell counts were determined using a hemocytometer (Neubauer improved, model BS748). With the cover-slip in place, 15 µl of cells suspended in PBS (1X) solution were transferred to the hemocytometer. Cells were counted in the middle and four corner squares and included those on top and left touching middle line of the perimeter of each square. The average number of cells per millilitre was calculated to be the average count per square multiplied both by the dilution factor and 10^4.

2.3.4 MICROSCOPY OBSERVATIONS

Routine visualisation of the cells in culture was accomplished at 10-times magnification with a inverted microscopy (Nikon, model TMS). Cell quantification in the hemocytometer was accomplished at 40-times magnification with a contrast phase microscope (Nikon, model Labophot-2 phase).

2.4 CELLS TRANSFECTIONS

2.4.1 CALCIUM-PHOSPHATE METHOD

Solutions (See Appendix I):
10 µg plasmid DNA
Tris-HCl (10 mM) pH 7.4/ EDTA (0.1 mM) pH 8.0 (TE buffer)
$CaCl_2$ (0.5 M)
2X Hepes buffered saline (2X HBS) pH 7.05 (pH is critical)
Serum free DMEM. Complete DMEM

PC12 cells (50-70% confluent plates) were transfected using the calcium phosphate co-precipitation procedure. All the experimental conditions were performed in duplicate. Cells were plated the day before transfection in 60 mm plates. 10 µg of plasmid DNA to be transfected was brought to a final volume of 125 µl with a sterile solution containing TE buffer or double distilled sterile water. This DNA solution was mixed with an equal volume of 0.5 M $CaCl_2$. While vortexing thoroughly, the DNA-CaCl2 solution was added drop by drop to 250 µl Hepes Buffered Saline (2X HBS), pH 7.05 in a sterile 10 ml tube. The pH of the 2X HBS is critical (range 7.05-7.12).The mixture was let to stand for 30 to 60 minutes to allow the precipitate to form. Meanwhile, the medium was removed from the PC12 cells and replaced by 3 ml of serum-free DMEM medium pre-warmed to 37°C. After 30-60 minutes the DNA was dropped onto the cells and incubated overnight (14-20 hours). The following day, the cells were placed in pre-warmed complete DMEM medium and treated with the different agents (See PC12 cells treatment). In the case of the inhibitors the cells were incubated with these compounds for 60 minutes before any subsequents treatments. After this the cells were incubated for a further 48 hours before harvesting for measurement of luciferase activity (See Luciferase Reporter Assay).

2.4.2 DEAE-DEXTRAN METHOD

Solutions (See Appendix I):
DEAE-Dextran (10 mg/ml) in PBS (1X) filter sterile
Chloroquine (100 mM) in PBS (1X)
Tris-HCl (10 mM) pH 7.5/ EDTA (0.1mM) pH 8.0
DMEM, NU serum (10% v/v) plus usual additions
COS medium
10% DMSO

COS-7 cells need to be split the day before to achieve about 50% confluency. In a sterile 1.5 ml eppendorf tube, 10 µg of plasmid DNA in 250 µl TE buffer was mixed with 50 µl of DEAE-Dextran (See Appendix I). The mix was incubated at room temperature for 30 minutes. In the meantime, DMEM medium supplemented with NU medium (See Appendix I) and chloroquine (100 µM) was warmed to 37°C. DMEM medium was removed from the COS-7 cells, and then the prewarm NU medium was added to the cells. The DNA mix was added drop by drop and incubated at 37°C for up to 4 hours. Thus, the COS-7 cells were incubated at room temperature for 2 minutes with 10% DMSO in PBS (1X) (DMSO shock). The cells were then washed once with sterile PBS (1X) and the placed in COS cell complete medium.

2.4.3 LIPOSOME-MEDIATED METHOD

PC12 asn17-W7 cells (*ras* -negative cell line), RINm 5f cells (rat insulinome cell line) and GT1-7 cells (mouse hypothalamic cell line) were transfected using the liposome reagent (DOTAP/DOSPER) (Boehringer Mannheim) or (LIPOFECTIN®/LIPOFECTAMINE®) (Life Technologies) following the company instructions. However, the process have been optimised for liposome concentration, DNA concentration and incubation time.

DOTAP method use 2.5 µg of DNA (in 100 µl HEPES Buffer 1X) and 30 µl of DOTAP (in 100 µl HEPES Buffer 1X). DOSPER method uses 1 µg of DNA (in 50 µl HEPES Buffer 1X) and 5 µl of DOSPER (in 50 µl HEPES Buffer 1X). Mix DNA and liposome solutions and incubate 10-15 minutes and room temperature. LIPOFECTIN method use 2 µg DNA (in 100 µl serum-free medium) and 20 µl LIPOFECTIN (in 100 µl serum-free medium). LIPOFECTAMINE method use 1 µg DNA (in 100 µl serum-free medium) and 10 µl of LIPOFECTAMINE (in 100 µl serum-free medium). Mix DNA and liposome solutions and incubate 10-15 minutes at room temperature. Thus is then added dropwise the DNA/liposome complex to the cultures (in complete serum medium) and incubate overnight at 37°C in a cell culture incubator (See culture conditions of the cells).

2.5 CELL TREATMENTS

The different cells lines were treated for up to two days with various activators or inhibitors compounds to induce cell differentiation or proliferation. Compounds were added directly into the complete serum medium immediately after cells had been transfected (by overnight incubation). The inhibitors compounds were added to the cells one hour before treatment. Duplicate sets of cells were plated at a density of approximately 1-2 x 10^6 in 60 mm plates. Control duplicate plates were prepared for the vehicle alone in a concentration up to 0.1% .

TABLE 2.2 PHARMACOLOGICAL ACTIVATOR COMPOUNDS

COMPOUND	STOCK CONC.	WORK CONC.	EFFECT
Dexamethasone, (synthetic)	1 mM *	1 µM	Glucocorticoid receptor
17-β Estradiol	1 mM *	1 µM	Estrogen receptor
Progesterone	1 mM *	1 µM	Progesterone receptor
Nerve Growth Factor(mouse 2.5S)	20 mg/ml **	100 ng/ml	NGF receptor
PACAP-38 (ovine)	5 µM ***	5 nM	PACAP receptor
Forskolin (*Coleus forskohlii*)	10 mM ****	10 µM	Adenylyl Cyclase
Dibutyryl-cAMP (synthetic)	30 mM *****	1 mM	Protein Kinase A
DH1 (synthetic)	1 mM ****	1 nM	Protein Kinase C
PMA (synthetic)	1 mM ****	1 nM	Protein Kinase C
Ionomycin (*S. conglobatus*)	10 mM ****	10 µM	Ca^{++} ionophore

Solubility of the different compounds (Store at -70°C):

* Compound soluble in ethanol

** Compound soluble in serum free DMEM medium

*** Compound soluble in 5 % acetic acid/1 % BSA

**** Compound soluble in anhydrous dimethyl sulfoxide (DMSO)

***** Compound soluble in sterile water or PBS (1X)

TABLA 2.3 PHARMACOLOGICAL INHIBITOR COMPOUNDS

COMPOUND	STOCK CONC.	WORK CONC.	EFFECT
H-89 (synthetic)	10 mM *****	10 µM	Protein Kinase A (Ki = 48 nM)
Calphostin C (*C.cladosporoides*)	1 mM ****	0.5 µM	Protein Kinase C (IC50 = 50 nM)
Genistein (synthetic)	50 mM *	50 µM	Tyrosine Kinase (IC50 = 25 µM)
K-252a (*Nocardiopsis sp*)	200 µM ****	200 nM	$p140^{trk}$ Tyrosine Kinase Receptor
KN-62 (synthetic)	10 mM ****	10 µM	CaM Kinase II (Ki = 900 nM)
LY 294002 (synthetic)	10 mM	10 µM	PI-3 Kinase (IC50 = 1.4 µM)
Wortmannin (*T. wortmannin*)	10 mM ****	10 µM	PI-3 kinase (IC50 = 5 nM)
Sodium Orthovanadate	100 mM *****	50 µM	Protein tyrosine phosphatase
PD 98059 (Synthetic)	20 mM ****	40 µM	MEK-1 Kinase (IC50 = 2 µM)

Solubility of the different compounds (Store at -70°C) :

* Compound soluble in ethanol

** Compound soluble in DMEM medium

*** Compound soluble in 5% (v/v) acetic acid/1% (w/v) BSA

**** Compound soluble in anhydrous dimethyl sulfoxide (DMSO)

***** Compound soluble in sterile water or PBS (1X)

Calphostin C requires light for activation.

Na_3VO_4 was activated by depolymerisation (See Appendix I)).

2.6 BACTERIAL CELL CULTURE

All bacterial work was performed at the laboratory bench using *Escherichia coli*.. Solutions, media, glassware and plastic ware were sterilised by autoclaving. The bench was swabbed with ethanol prior to use and a bunsen burner flame maintained aseptic conditions in the area and sterilised tools used, such as glass spreading devices were dipped in ethanol prior to flaming. Bacterial cultures were grown in liquid medium in a spinning wheel (Napco E series, model 301) or with shaking (flasks) at 37°C or on solid medium at 37°C in an incubator (Gallemkap). Bacteria in liquid or solid media were stored at 4°C for 4-6 weeks.

2.6.1 PREPARATION OF BACTERIAL MEDIUM

2.6.1.1 LIQUID MEDIUM (LB)

Premixed powdered Luria-Bertani medium (LB) consists of Bacto-tryptone (10 g/l); Bacto-yeast extract (5 g/l) and NaCl (10 g/l) formulated to have a final pH of 7.5. 20 g of LB premix powder (Difco) were dissolved per litre in distilled water and autoclaved at 121°C, 15 lb/in^2 for 20 minutes. The medium was then allowed to cool to about 50°C before addition of antibiotics (See antibiotic supplements). The medium plus antibiotics was stored at 4°C.

2.6.1.2 SOLID MEDIUM (AGAR)

The LB agar premix powder (Difco) was prepared in a similar way to the liquid medium. Thirty-two grams of powder were added per litre of distilled water and the mixture was autoclaved at 121°C, 15 lb/in^2 for 20 minutes as instructed by the manufacturer. The medium was then allowed to cool to about 50°C before addition of antibiotics (See antibiotic supplements). After adding antibiotics, the medium was poured into 100 mm plastic bacteria plates using about 30 ml of medium per plate. The agar was allowed to solidify at room temperature and the plastic plates were dried at 37°C opened and inverted for 30 minutes. Once the plates were dry, they were either used directly to plate transformed bacteria or stored at 4°C in their original plastic storage sleeves for up to 4-6 weeks.

2.6.1.3 MINIMUM MEDIUM (M9)

Bacteria strains containing a F' episome, such as JM109 and TG1 were grown on minimal plates (M9). To make a M9 minimum medium, 0.5 g NaCl; 1.0 g NH_4Cl; 3.0 g KH_2PO_4; 6.0g Na_2HPO_4 were dissolved in 900 ml of distilled water and the mixture was autoclaved at 121°C, 15 lb/in^2 for 20 minutes. After cooling to 50°C, filter sterized 0.1 ml $CaCl_2$ (1M); 2.0

ml $MgSO_4$ (1M) and 10.0 ml glucose (20%) were added to the medium and the volume was adjusted to 1 liter. This medium was stored at 4°C. A solution of thiamine-HCl (1M) was prepared in distilled water and filter-sterilized, and stored at -20°C. In order to prepare the minimal medium plates, 1.7 g agar was dissolved in 100 ml water and allowed to cool to about 50°C. At this point 0.12 ml of thiamine solution and 1.2 ml of M9 medium were added to the agar solution and this mix was poured into 100 mm plastic bacteria plates using about 30 ml of medium per plate.

2.6.1.4 TYM MEDIUM

To make competentent cells, bacteria were grown in TYM broth medium. To make TYM medium Bacto-tryptone (20 g/l), bacto-yeast extract (5 g/l), NaCl (5.8 g/l) and MgCl2 (0.01 M) were added to distilled water and autoclaved at 121°C, 15 lb/in^2 for 20 minutes. TYM medium was stored at room temperature.

2.6.1.5 ANTIBIOTIC SUPPLEMENTS

Ampicillin was added to the medium to a final concentration of 100 µg/ml. A stock solution of 100 mg/ml (ampicillin) was made in distilled water and sterilised by filtration (0.22 µm filters, Millipore). Ampicillin stock solution was then aliquoted and store at -20°C.

2.6.2 COMPETENT BACTERIA PREPARATION

2.6.2.1 COMPETENT BACTERIA

The desired *Escherichia coli* strain (usually JM109 or TG1) was streaked on to an M9 minimum medium agar bacterial plate and allowed to grow overnight at 37°C. The next day, a single colony was selected and transferred into 5 ml of TYM broth medium (see Appendix I) and allowed to growth in a spinning wheel at 37°C for 2 to 3 hours. This bacterial culture was then transferred to a 250 ml conical flask (erlenmeyer) containing 100 ml TYM broth medium and allowed to grow until the absorbance (optical density) of the suspension was about 0.5 at a wavelength of 550 nm (Beckman, model DU640B). Bacteria were cooled in an iced water bath for 10 minutes in order to achieve a quick drop in the temperature.

The bacterial suspension was then centrifuged in sterilised polypropylene tubes at 2,600 x g for 10 minutes at 4°C (Beckman, model Avanti J25). The resultant bacterial pellet was drained and resuspended in 30 to 40 ml per 100 ml of TfbI solution (see Appendix I), and incubated on ice for 5 minutes. The cells were centrifuged again for 10 minutes at 2,600 x g at 4°C (Beckman, model Avanti J25). The pellet was resuspended in 4 ml of TfbII solution (See Appendix I) per

100 ml of initial culture. The competent cells were aliquoted into 0.1 ml aliquots in polypropylene sterile microcentrifuge tubes, rapidly frozen in liquid nitrogen and stored at -70°C.

2.6.2.2 BACTERIA TRANSFORMATION

A 100 µl of competent cell was thawed on ice for each sample of plasmid DNA to be transformed. 10-100 ng of plasmid DNA was added to the suspension of competent bacteria and after mixing, incubated on ice for 15-30 minutes. The bacteria/DNA mixture were then "heat -shocked" by incubating for 1 minute at 42°C or 5 minutes at 37°C, chilled in ice and then made up to 400 µl with LB. Expression of antibiotic resistance was allowed for incubation for 1 hour at 37°C. Transformed bacteria were selected by plating 100-200 µl of this mixture on agar LB plates containing 100 µg/ml ampicillin.

Control transformation reactions using circular, non-recombinant plasmid vector were performed alongside experimental samples to assess transformation frequency. To test these cells 1 µl of 1 ng/ml or 1 pg/ml of plasmid DNA were transformed into 100 µl cells. The competence achieved was generally 10^7 colonies/µg of plasmid. Reactions containing only water or linear plasmid were performed as a negative control of the transformation.

2.7 NUCLEIC ACID ISOLATION

2.7.1 TOTAL RNA ISOLATION

Solutions (See Appendix I):
Guanidium thiocyanate (4M)
Sodium citrate pII7.0 (25 mM)
Sarcosyl (0.5%)
β-mercaptoethanol (100 mM)
Sodium acetate pH 4.0 (2 M)
Phenol (saturated with Tris pII 4.5)
Chloroform: isoamylalcohol (24:1)
Isopropanol (100%)

Cells were washed on the culture plate once with 2 ml phosphate-buffered saline solution (PBS) and harvested in 1 ml of PBS. Cells were subsequently kept on ice during total RNA extraction. Cells were pelleted by centrifugation at 8,000 x g at 4°C for few seconds and the resulting pellet was dissolved in 600 µl of the guanidinium isothiocyanate solution by pippeting

up and down. Cell homogenates were then acidified with 0.1 volume of 2 M sodium acetate (pH 4) and extracted with one volume of acid-saturated phenol (pH 4.5) in the presence of 0.2 volumes of chloroform: isoamyl alcohol (24:1 v/v). The mix was keep in ice for 15 minutes and then was centrifuged for 20 minutes at 8,000 x g at 4°C.

The aqueous phase, containing the RNA, was recovered and total RNA was precipitated by incubating for 60 minutes at -20°C adding one volume of 100% isopropanol. RNA was pelleted by centrifugation at 12,000 x g for 30 minutes at 4°C. After decanting the supernatant, the pellet was dissolved in 300 µl of guanidinium isothiocyanate solution and RNA was reprecipitated by adding one volume of 100% isopropanol. After centrifugation, the final pellet was dissolved in DEPC-treated water. RNA samples were stored as aqueous solutions at -70°C or as isopropanol precipitates at -20°C.

2.7.2 DNA (PLASMID) ISOLATION

2.7.2.1 PLASMID MINIPREP

Solutions (See Appendix I):
Tris-HCl (50 mM), pH 8.0, EDTA (10 mM), 100 µg/ml RNase A (Solution I)
NaOH (0.2 M); SDS (1%) (Solution II)
potassium acetate (1.32 M), pH 4.8 (Solution III).
phenol:chloroform:isomyl alcohol (24 : 24 : 1)
NaOAc (3 M), pH 7.0
ethanol (100%)
ethanol (70%)

Individuals colonies were picked from agar plates using sterile plastic disposable pipette tips and placed into 5 ml of LB medium with ampicillin contained in a 30 ml sterile glass test-tube. Bacteria were cultured at 37°C under constant agitation to saturation for 18 hours (New Brunswick Scientific, model G25). 1.5 ml of the bacteria suspension was poured into an microcentrifuge tube and the bacteria pelleted by centrifugation for one minute at 12,000 x g at room temperature. The supernatant was removed using an aspirator.

The bacterial pellet was resuspended in 100 µl of cell resuspension solution (solution I). Bacteria were lysed by addition of 200 µl of cell lysis solution (solution II). The samples were mixed by inversion and incubated in ice for 5 minutes. The lysates were neutralised by addition of 100 µl of a ice-cold neutralisation solution (solution III). Following mixing by inversion, the tubes were centrifuged for 5 minutes at 12,000 x g at room temperature and the supernatant poured into a microcentrifuge tube. An equal volume of phenol (pH 7.5) was added to the

supernatant, vortexed and then centrifuged for 2 minutes at 12,000 x g at room temperature to separate the phases. The aqueous phase was removed to a new microcentrifuge tube.

The plasmid DNA was precipitated in ethanol. To do this, one tenth volume of 3 M NaOAc (pH 7.0) and 2 volumes of 100% ethanol were added and the DNA was precipitated by centrifugation for 5 minutes at 12,000 x g at 4°C. The pellet was rinsed twice with 70% ethanol, air dried and dissolved in 100 µl of TE buffer (See Appendix I).

2.7.2.2 PLASMID MAXIPREP

CsCl Method

solutions (See Appendix I):
Tris-HCl (50 mM), pH 8.0, EDTA (10 mM), 100 µg/ml RNase A (Solution I)
NaOH (0.2 M); SDS (1%) (Solution II)
potassium acetate (1.32 M), pH 4.8 (Solution III).
isopropanol (100 %)
Tris-HCl (50 mM), pH 8.0/EDTA (10 mM) (TE buffer)
4.0 g CsCl plus 250 µl EtBr
n-butanol (equilibrated with 1 M NaCl)
NH4OAc (7.5 M)
ethanol (100%)
ethanol (70%)

Typically, one litre of bacterial medium was prepared. Bacteria were plated the night before on LB agar plates with antibiotics and single colonies were picked as for miniprep. The selected bacterial clone was grown for 2 to 4 hours in 5 ml of LB medium and then added to a 2 litre flask containing 1 litre of LB medium containing 100 µg/ml ampicillin and allowed to grow overnight at 37°C in a shaker incubator (200-250 rotations per minute) (New Brunswick Scientific, model G25). The bacterial suspension was centrifuged in 250 ml centrifuge bottle for 5 minutes at 4°C and the supernatants discarded. The bacterial pellets were resuspended in a total of 20 ml of solution I (See Appendix I), 40 ml of solution II (See Appendix I) was then added and mixed gently without shaking. 20 ml of ice-cold solution III (See Appendix I) was then added and the suspension shaken 5 times vigorously.

Following centrifugation at 12,000 x g for 10 minutes at 4°C (Beckman, model Avanti J25), the supernatant was filtered through two layers of cheese cloth gauze into a clean 500 ml centrifuge bottle. An equal volume of isopropanol was then added to each bottle and the mix allowed to stand on ice for 15 minutes. The suspension was centrifugued at 12,000 x g for 10

minutes at 4°C, after pouring off the supernatant, the pellet was washed twice with 70% ethanol. The pellet was then dissolved in a final volume of 3.5 ml TE buffer per initial litre. This solution was used to dissolve 4.0 g of ceasium chloride (CsCl). 250 µl of ethidium bromide (10 mg/ml) was then added to the DNA solution. The supernatant was transferred to a quick seal tube (Beckman) and centrifuged for 18 hours at 55,000 rpm at 18°C (Beckman, model Optima TL).

After centrifugation, two horizontal bands were usually observed, the upper band is bacterial chromosomal DNA, the lower band is the plasmid DNA. RNA migrate to the botton of the tube. The lower band was removed using a syringe and needle and placed into an butanol resistant 10 ml plastic tube. Ethidium bromide was extracted by addition of 5 to 6 ml of butanol saturated with 1M NaCl. After shaking vigorously, the two phases were allowed to separate by standing the tube for several minutes. The upper butanol phase was removed and the DNA extracted a second and/or third time by addition of a further 5 to 6 ml of salt saturated butanol.

After removing all the butanol, the DNA was precipitated by adding 0.36 volume of 7.5 M NH_4OAc (pH 7.0) and 2.5 volume of 100% ethanol. The DNA was pelleted by centrifuging for 15-30 minutes at 12,000 x g at 4°C (Beckman, model Avanti J25). The final pellet was washed with 70% ethanol, air dried and resuspended in TE buffer, usually 1 ml for each initial litre of bacterial culture depending on the DNA yield. These high quality plasmid DNA were routinely used for transfection experiments.

QUIAGEN OR WIZARD MAXIPREP METHOD

Large-scale plasmid preparations were performed using two kits: Quiagen plasmid midi kit (Qiagen) and Wizard Maxipreps DNA Purification System (Promega) exactly as instructed in the manufacturer protocols. This plasmid DNA was routinely used for subcloning, sequencing and other enzymatic reactions.

2.8 NUCLEIC ACID MANIPULATION

2.8.1 NUCLEIC ACID GENERAL METHODS

2.8.1.1 PHENOL/CHLOROFORM EXTRACTION

Protein and salts were removed from solutions of DNA or RNA by extraction with phenol/chloroform. One volume of phenol (equilibrated to pH 7.5 for DNA or equilibrated to

pH 4.5 for RNA) was added to the nucleic acid solution, vortexed thoroughly and each sample was spun in a microcentrifuge at 12,000 x g for 5 minutes at room temperature (4°C for RNA samples). The upper aqueous layer was transferred to fresh microcentrifuge tubes and an equal volume of phenol : chloroform : isoamyl alcohol (24 : 24 : 1) was added to the mixture, mixed by vortex and then was centrifuged at 12,000 x g for 5 minutes at room temperature (4°C for RNA samples). The upper aqueous phase was removed and transferred to a clean tube taking care to avoid contamination with the protein-aqueous interphase.

2.8.1.2 ALCOHOL PRECIPITATION OF NUCLEIC ACID

DNA or RNA was precipitated by the addition of monovalent Na^+ cations to 0.3M (sodium acetate 3M stock solution) or $NH4^+$ to 0.5 M (ammonium acetate 7.5 M stock solution) and the subsequent addition of 2.0 volumes (for DNA), 2.5 volumes (for RNA) or 3 volumes (for oligonucleotides) of ethanol or 1 volume of isopropanol. Precipitation was allowed to proceed at -20°C for overnight incubation or -70°C for 10-15 minutes. When the amount or the size of nucleic acid to be precipitated was small, 10 µg of linear polyacrylamide or 10 µg of tRNA carrier was added to the tube to facilitate precipitation. Oligonucleotides were precipitated in the presence of 10 mM $MgCl_2$ to optimise recovery. The DNA or RNA samples were centrifuged at 12,000 x g for 30 minutes at 4°C. After removal of the supernatants, the pellets were air-dried and resuspend in nuclease-free distilled water or TE buffer.

2.8.1.3 QUANTIFICATION OF NUCLEIC ACID

DNA or RNA was quantified by spectrophotometry measurements (Beckman, model DU-640B) at 260 nm. Contamination by carbohydrates was determinated at 230 nm and by proteins at 280 nm. For double stranded DNA quantification, 1 OD unit (260 nm) represented 50 mg/ml, for RNA quantification 1 OD unit (260 nm) represented 40 mg/ml and for single stranded DNA 1 OD unit (260 nM) represent 33 mg/ml. Quantification of radioactive label incorporation was performed by liquid scintillation counting (Beckman, model LS 6500).

2.8.2 NUCLEIC ACID MODIFICATION

2.8.2.1 RESTRICTION ENDONUCLEASE REACTION

DNA was digested with restriction endonuclease enzymes using conditions and buffers as suggested and supplied for the manufacturers (Boehringer Manheim or Promega). Typically an enzyme would be provided with a 10X reaction buffer and DNA would be digested in a 10-20 µl volume containing 1 unit enzyme/µg of DNA for 1 hour at 37°C. The reaction was

terminated by incubation at 65°C for 15 minutes or phenol/chloroform extraction and ethanol precipitation.

2.8.2.2 PHOSPHORYLATION REACTION

T4 polynucleotide kinase was used to transfer the γ-phosphate group of ATP to the 5'-hydroxyl terminus of oligonucleotides (primer extension assays) or DNA fragments. In a typical reaction, 10 pmol of oligonucleotide or DNA ends were phosphorylated for one hour in a 20 µl reaction volume containing ATP (1mM), 2 µl kinase buffer (10X) (see Appendix I) and 10 units of enzyme. The reaction was terminated by incubation at 65°C for 15 minutes.

2.8.2.3 DEPH0SPHORYLATION REACTION

Shrimp alkaline phosphatase (SAP) was used to remove the terminal 5'-phosphate group from plasmid DNA. Reactions were carried out for 30 minutes at 37°C in a reaction volume of 20 µl containing: 1-5 µg DNA, 2 µl phosphatase buffer (10X) (See Appendix I) and 5 units of enzyme. The reaction was terminated by heating to 65°C for 15 minutes.

2.8.2.4 LIGATION REACTION

T4 DNA ligase was used to catalyse the formation of a phosphodiester bond between adjacent 3'-hydroxyl groups and 5'-phosphate termini in DNA. Ligations were carried out in a total volume of 20 µl with a 1:3 molar ratio of plasmid: insert DNA. Typically a ligation reaction was set up in a 20 µl volume containing: 100-500 ng of DNA, ATP (2 mM), ligase buffer (1X) (without ATP), 10 units of T4 DNA ligase and made up to 20 µl with distilled water. After chilling on ice, the reaction was allowed to proceed overnight to 15°C. The reaction was terminated by heating to 65°C for 15 minutes.

2.8.2.5 DNA POLYMERIZATION (FILLING-IN) REACTION

Large fragment (Klenow) of the DNA polymerase I was used to convert the 5'-protruding end to a blunt end for subcloning purposes. The conversion were carried out in a 20 µl reaction containing: 1-5 µg DNA previosly cut with the specific restriction endonuclease enzyme, 2 µl polymerase buffer (10X), 2 µl DTT (10 mM), 2 µl dNTPs mix (0.5 mM each) and 1-5 units DNA Klenow-polymerase I. The reaction was incubated for 15 minutes at 37°C and the DNA polymerase I was inactivated by heating to 65°C for 15 minutes or by phenol/chloroform extraction and ethanol precipitation.

2.8.3 NUCLEIC ACID SIZE SEPARATION

2.8.3.1 AGAROSE ELECTROPHORESIS

2.8.3.1.1 RNA Formaldehyde Gel Electrophoresis

Solutions (See Appendix I):
MOPS buffer (10X)
formamide deionized (100%)
formaldehyde (37%)
RNA loading buffer (10X)
iodoacetamide (1 M)
ethidium bromide (10 mg/ml)
DEPC-treated water

RNA samples (in 15 µl DEPC water) were denatured at 65°C for 15 minutes in RNA sample buffer (See Appendix I) in a final volume of 45 µl. Formaldehyde-denatured RNA was the mixed with 5 µl of RNA loading buffer (10X) and resolved on 1% agarose gels (15 cm x 10 cm x 1 cm) containing MOPS (1X) running buffer, formaldehyde (1.5%), 0.1 M iodoacetamide and 0.5 µg/ml ethidium bromide.

Equal amounts of denatured RNA (20-50 µg) measured spectrophotometrically (Beckman, model DU-640) were loaded in each lane of the agarose gel. Electrophoresis was carried out at a voltage constant (10 V/cm) in MOPS buffer (1X) containing 0.5 µg/ml ethidium bromide for 2-3 hours at room temperature. After electrophoresis, gels were visualized by ultraviolet light at 254 nm to assess RNA integrity and loading precision. These gels were used for RNA blotting for hybridisation with specific DNA probes (Northern blot assay) (See Nucleic acid immobilization).

2.8.3.1.2 DNA Gel Electrophoresis

Solutions (Appendix I):
TAE buffer (50X)
ethidium bromide (10 mg/ml)
DNA loading buffer (10X)

DNA samples to be run in agarose gels were first mixed with an appropriate volume of 10X DNA loading buffer (See Appendix I). Samples were typically run in a 1 to 2 % agarose

prepared in 1X TAE buffer containing ethidium bromide (0.5 µg/ml) and dissolved by heating in a microwave oven.

Gels were cast on 5 cm x 7 cm glass plates with teflon well combs. Electrophoresis was performed in a horizontal system for 30-60 minutes a 10 V/cm at room temperature in 1 X TAE buffer, 0.5 µg/ml ethidium bromide and molecular weight markers : (i) λ *Hind* III/*Eco* RI-digested (Sigma) or (ii) 1 Kb Ladder (Life Technologies) were run alongside the samples. Gels were then photographed under ultraviolet illumination at 254 nm in a transilluminator (Fotodyne, model Foto/Prep I) using Polaroid 667 B/W film and a camera (Polaroid, model DS-34)

2.8.3.1.3 Oligonucleotide Gel Electrophoresis

Oligonucleotide quality was determined by electrophoresis in 5% Nusieve agarose (FMC products) containing 0.5 µg/ml of ethidium bromide at voltage constant (5 V/cm) for 1 hour. Typically 400 pmoles of oligonucleotides were first mixed with an appropriate volume of 10X DNA loading buffer (See Appendix I).

2.8.3.2 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Solutions (Appendix I):
Acrylamide:*bis* -acrylamide solution (40%)
TBE buffer (10X)
ammonium persulfate (10 %)
TEMED
Urea

2.8.3.2.1 PAGE Gel for Ribonuclease Protection Assay (RPA)

Denaturing PAGE gels for ribonuclease protection assays (RPA) contained 4% acrylamide and 7 M urea (See Appendix I). The gel was run in a vertical electrophoresis system (BRL) at 250 V for 2-3 hours and the results were visualized by autoradiography (overnight incubation at -70°C with intensifying screen).

2.8.3.2.2 PAGE Gel for Primer Extension Assay (PEA)

Denaturing PAGE gels for primer extension assays contained 8% acrylamide and 7 M urea (See Appendix I). The gel was run in a vertical system at 250 V for 2-3 hours and the results were visualized by autoradiography (overnight incubation at -70°C with intensifying screen).

2.8.3.2.3 DNA Sequencing Gel Electrophoresis

The sequencing PAGE gels contained 8% acrylamide and 7 M urea (See Appendix I). The sequencing gel was run using a sequencing apparatus at 60 W constant power. Gels were fixed in 10% acetic acid, 10% methanol for 30 minutes following electrophoresis and were vacuum dried onto a sheet of Whatman 3MM paper using a gel drier (Savant, model S6D4050). The dried gel was overnight autoradiographed at room temperature using a Biomax MS film (Eastman-Kodak).

2.8.4 NUCLEIC ACID PURIFICATION

2.8.4.1 AGAROSE DNA GEL PURIFICATION

2.8.4.1.1 Low Melting Point (LMP) Agarose

Low melting point agarose gels were used to purify DNA fragments. These gels (0.75-1%) were run in TAE buffer containing ethidium bromide (0.5 µg/ml) at 5 V/cm at room temperature. Whilst viewing on a UV transiluminator (Fotodyne, Foto/Prep I) at 300-360 nm to minimise damage to the DNA, the required band was excised using a clean scalpel. The gel slice was melted in a equal volume of TAE buffer at 65°C for 5-10 minutes and an aliquot of this solution, such that the final concentration of agarose did not exceed 0.2% was used for enzymatic reactions, such as ligation, restriction or PCR reactions.

2.8.4.1.2 USBioclean Purification Kit

Following electrophoresis, DNA was extracted from 1X TAE-agarose gels by binding to glass powder in high salt concentration using the USBioclean Kit (US Biochemicals). DNA was electrophoresed in 1X TAE-buffered agarose gels containing ethidium bromide (0.5 µg/ml). DNA was visualised under UV illumination at 300-360 nm to minimise damage to the DNA. The band of interest was excised from the gel with a clean scalpel blade and the gel slice was transferred to a microcentrifuge tube.

DNA was extracted exactly as instructed in the manufacturer protocols (United States Biochemical). The DNA yield was determined spectrophotometrically and the integrity of the purified DNA was assesed by electrophoresis of an aliquot of the sample in ethidium-containing agarose gels. This plasmid DNA was routinely used for restriction, ligation and other enzymatic reactions.

2.8.4.2 SOLUTION DNA PURIFICATION

Solutions (See Appendix I):
Isopropanol (80%)
Prewarmed (65-70 ºC) distilled water or TE buffer

Concentration of DNA samples (between 50 and 500 µl), removal of small nucleotides from PCR reactions and purification of DNA from proteins or salts were made using the Wizard DNA Clean-Up System (Promega) according to the manufacturer instruction. This plasmid DNA was routinely used for restriction, ligation and other enzymatic reactions.

2.8.5 NUCLEIC ACID IMMOBILISATION

2.8.5.1 RNA TRANSFER TO MEMBRANE

Solutions (See Appendix I):
SSC buffer (10X)
Water (DEPC-treated)

Transfer of size-separated, gel-electrophoresed RNA to nylon, charge modified membranes was made by capillary action for subsequent hybridisation analysis. After agarose gel electrophoresis and photography in the UV trasnsiluminator (See RNA formaldehide gel electrophoresis), the gel was then laid up upside down on several layers of filter paper (Whatmann, 3 MM) soaked in SSC buffer (10X); these layers were overlaid on a filter paper wick extending from a tray filled with SSC buffer (10X).

A piece of nylon membrane (Dupont, Genescreen), cut to the size of the gel and nicked in one corner for orientation purpose, was prewet in DEPC water and laid on top of the gel surface such that all the bubbles were excluded between the gel and membrane. Three layers of solution-saturated filter paper (Whatmann, 3MM) were placed on top of the membrane to produce the capillary force needed to transfer the RNA. These layers were placed avoiding trapping of bubbles and then a 4-6 cm pile of dry absorbent paper was placed on top. A glass plate added weight to ensure contact of dry paper with the wet filter paper. Transfer was carried out overnight. The next day, the efficiency of the transfer was checked by examining the agarose gel in a UV transiluminator for the presence of rRNAs. The RNA was cross-linked to the nylon membrane by exposure of the blot for 30 seconds to each side on top of a UV transiluminator at 254 nm. The nylon membrane was stored at room temperature before hybridization.

2.9 NUCLEIC ACID SYNTHESIS

2.9.1 OLIGONUCLEOTIDE DESIGN AND SYNTHESIS

The following oligonucleotides were designated, specific for (See Appendix II):
(i) the open reading frame of the rat NPY-Y1 receptor gene (2010-2011),
(ii) the 5'-flanking region of the rat NPY-Y1 receptor gene (2023-2024),
(iii) the 3'-flanking region of the rat NPY-Y1 receptor gene (2486-2487).

These oligonucleotides were synthesized commercially by Dr. V. Math, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Science, University of Glasgow on an Applied Biosystems Model 280A DNA synthesiser using phosphoramidate technology. Oligonucleotides were supplied in 35% NH_4OH and were purified by ethanol precipitation. They were dissolved in distilled water. The concentration of the purified oligonucleotide was determined by measuring the absorbance at 260 nm. Typically 20 µl of oligonucleotide solution was diluted in 3 ml of water and the absorbance measured in a 1 cm quartz cuvette, assuming that the OD of 1.0 corresponds to a concentration of 33 µg/ml of single stranded DNA.

The following oligonucleotides were designated specific for (See Appendix II):
(i) the open reading frame of the luciferase gene (T1886 and Y7464),
ii) the 5'-flanking region of the NPY-Y1 receptor gene (Y7465 to Y7468),
(iii) specific deletions of the 3'-flanking region of the rat NPY-Y1 receptor gene (T5707-T5708),
(iv) specific deletions of the promoter region of the rat NPY-Y1 receptor gene, including AP1 site (V5339-V5340) and progressive deletions of the rat NPY-Y1 promoter region (V7613 to V7617),
(v) the open reading frame of the rat cyclophilin gene (Y7462 to Y7463).

These oligonucleotides were synthesized commercially by Oswel DNA Service (University of Southampton) and were dissolved in sterile water. These oligonucleotides were previously quantified and were ready to use.

All the oligonucleotides were designated using the *prime* program (GCG software, University of Wisconsin) and checked for complementary hybridisation with the DNA data bank using the *blast* program (National Institute of Health, Bethesda, Maryland). The analysis of the sequence of the NPY-Y1 receptor promoter for binding sites was made using the WEB Signal Scan 4.05 program (University of Minnesota).

TABLE 2.4 OLIGONUCLEOTIDES

OLIGONUCLEOTIDE	SOURCE	USE
2010-2011	University of Glasgow	PCR open reading frame of NPY-Y1 receptor
2024-2025	University of Glasgow	PCR promoter region of the NPY-Y1 receptor gene
2486-2487	University of Glasgow	RT-PCR 3'-coding region of NPY-Y1 receptor mRNA
T7507-T7508	Oswel	RT-PCR deletion 3'-coding of NPY-Y1 receptor mRNA
T 1886	Oswel	Reverse primer for RPA assay luciferase mRNA
Y7464-Y7467	Oswel	RT-PCR for luciferase mRNA
V5339-V5340	Oswel	PCR deletion of the AP-1 site
V7613-V7617	Oswel	PCR progressive deletions of NPY-Y1 promoter region
Y7642-Y7643	Oswel	RT-PCR rat cyclophilin mRNA

Complete oligodeoxynucleotides sequences are contained in Appendix II.

2.9.2 cRNA SYNTHESIS BY *IN VITRO* TRANSCRIPTION

Large amounts of unlabelled cRNA were generated by *in vitro* transcription for use as internal control for quantification of the NPY-Y1 receptor mRNA by competitive RT-PCR using as a DNA template a deletion clone generated by RT-PCR (See DNA cloning).

The synthesis was made in a final volume of 100 µl containing: 20 µl transcription buffer (5X) (See Appendix I), 10 µl of DTT (100 mM), 20 µl rNTPs mix (25 mM each ribonucleotide), 100 units of recombinant RNasin® ribonuclease inhibitor, 5-10 µg of linear DNA template (See DNA cloning), 2 µl T7 RNA polymerase (15-20 U/µl). The *in vitro* reaction was incubated at 37°C for 60-120 minutes and the DNA template was removed by incubation with RQ1 RNase-free DNase. The *in vitro* reaction was extracted with phenol/chloroform several times and precipitated at -70°C with ammonium acetate and ethanol (See removal of unincorporated nucleotides). After the removal of the DNA template and unincorporated nucleotides, the RNA concentration was determined by UV absorbance. The DNase-treated transcript was examined in agarose gels loaded with 1-2 µl of cRNA denaturated in 18-20 µl of a RNA sample buffer (See Appendix I) and 2-5 µl of RNA loading buffer (See Appendix I). The sample was heat for 5-10 minutes at 65-70°C prior to the loading and the gel was run under standard conditions of DNA samples (0.5X TBE or 1X TAE buffer).

2.9.3 cDNA SYNTHESIS

2.9.3.1 PRIMER EXTENSION (AMV RT)

Analysis of the transcriptional initiation site of the 5'-flanking (promoter) region of the rat NPY-Y1 receptor gene present in the pY1-LUC in PC12 cells was made using the primer extension assay system kit (Promega). Typically a radiolabelled oligonucleotide (See 5'-labeling of oligonucleotides) was annealed to a cRNA control sample (actinomycin D) or a total RNA PC12 sample previosly transfected with the pY1-LUC vector. The reaction was incubated at 60°C for 30 minutes and then the reaction was placed at room temperature to cool for 10 minutes.

A "master" reverse transcriptase (RT) was prepared which included per tube, 5 µl of AMV primer extension (2X) buffer (See Appendix I), 1.4 µl sodium pyrophosphatase (40 mM), 5 units of (AMV) reverse transcriptase and nuclease free water to a final volume of 9 µl. Immediatly aliquoted into each reaction tube which contained annealed primer/RNA. The RT-PCR reaction was incubated at 41-42°C for 30 minutes and 20 µl of RNA loading buffer (See Appendix I) was added to each tube. The tubes were heated at 90°C for 10 minutes and the

samples were loaded directly onto a denaturing polyacrylamide gel (See PAGE gel for PEA). The remainder of the samples was stored at -20°C.

2.9.3.2 RT-PCR (MMLV RT)

Gene expression analysis of NPY-Y1 receptor in PC12 cells was performed by reverse transcription of the mRNA using the Moloney murine leukemia virus (MMLV) reverse transcriptase present in the GeneAmp© RNA-PCR kit (Perkin-Elmer).

Typically a 20 µl reaction was set up containing: 4 µl of MgCl2 (25 mM), 2 µl of PCR Buffer II (10X) (See Appendix I), 8 µl of a dNTPs mix (10 mM each), 1 µl of RNase inhibitor (20 U/µl), 1 µl of MuLV reverse transcriptase (50 U/µl), 1 µl of random hexamers (50 mM) or oligodT (50 mM) and 0.5-1 µg of total RNA from PC12 cells transfected with the pY1-LUC. Total RNA was previously incubated for 15 minutes at 37°C with RQ1 RNase-free DNAse (1u/µg plasmid) for remove the DNA template, extracted with phenol/chloroform and ethanol precipitated. RT-reactions were incubated at room temperature for 10 minutes and incubated at 42°C for 15 minutes. RT-reactions were terminated by incubation at 99°C for 5 minutes and cooling at 4°C before procceding with the PCR reaction .

2.9.4 DNA AMPLIFICATION (PCR)

2.9.4.1 cDNA AMPLIFICATION BY RT-PCR

Gene expression analysis of the rat NPY-Y1 receptor were performed by reverse transcription (See cDNA synthesis) and amplification of the cDNA using the GeneAmp© RNA-PCR Kit (Perkin-Elmer) following the manufacturer instruction. Control positive reactions were included in the kit and control negative reaction included no reverse transcriptase enzyme and total RNA for cells (e.g. COS-7 cells) which do not constitutively express the rat NPY-Y1 receptor .

2.9.4.2 DNA AMPLIFICATION BY PCR

Polymerase chain reactions (PCR) were run in a termocycler (Stratagene, model RoboCycler® 40 temperature cycler). DNA used as template for the PCR reaction was previously purificated by phenol/chlorophorm extraction and ethanol precipitation or using the Wizard DNA Clean-Up System (Promega). PCR reactions were carried using eppendorf tubes (500 µl) in a final volume of 100 µl of reaction.

2.9.4.2.1 Dynazyme DNA Polymerase

DNA was amplified using the thermostable DNA polymerase Dynazyme (Finnzymes Oy, Flowgen Instruments) according to the manufacturer instructions. Unlike many other thermostable DNA polymerases which result in amplified molecules with a single, 3' A-overhang, amplification with Dynazyme results in blunt ends products. This feature of amplification was exploited during subcloning of amplified cDNA fragments into plasmid (described below).

A typical reaction (100 µl) consisted: 10 µl of Dynazyme polymerase buffer (10X) (See Appendix I); 0.2 µM of each primer; 1-5 ng of DNA template; 1 µl of all four dNTPs (25 mM); 2.5 units of Dynazyme polymerase and nuclease-free distilled water up to a final volume of 100 µl. Further, 100 µl of mineral oil (DNAse free) was layered over the top of the reaction to reduce evaporation. The reaction profile was set up in a similar way to the Taq DNA polymerase.

2.9.4.2.2 Taq DNA Polymerase

Reactions were set up as follow: 10 µl of Taq polymerase buffer (See Appendix I); 8 µl of $MgCl_2$ (25 mM); 0.2 µM of each primer; 1-5 ng of DNA template; 1 µl of all four dNTPs (25 mM); 2.5 units of Taq polymerase (Boehringer Mannheim or Promega Corp.) and distilled water up to a final volume of 100 µl. Further 100 µl of mineral oil (DNAse free) was layered over the top of the reaction to reduce evaporation. Reaction mixtures containing only one primer or no target DNA were used as a control negative of the PCR reaction.

TABLE 2.5 PCR REACTION CONDITIONS

melting step	annealing step	polymerisation step	cycles
2 minutes (94°C)			1 cycle
1 minute (94°C)	1 minute (40-60°C)	1 minute (72°C)	30-40 cycles
		7 minutes (72°C)	1 cycle

The exact annealing temperature used was dependent upon the calculated melting temperatures (Tm) of the primers and the desired stringency of the PCR reaction. The exact times of the various cycle parameters were also varied in order to optimise PCR conditions. On completion of the cycles, the samples were cooled immediatly to 4°C and 10 µl of the PCR reaction were mixed with the DNA loading buffer (See Appendix I) and visualised on a 1-2% agarose/TAE

gel. The samples were then cleaned with phenol/chloroform extraction or using the Wizard DNA Clean-Up System kit (Promega) and stored at -20°C for late use.

2.10 DNA CLONING

2.10.1 SUBCLONING OF PCR PRODUCTS

2.10.1.1 DIRECT CLONING

2.10.1.1.1 Blunt-end Cloning

PCR products generated using the thermostable DNAzyme DNA polymerase, which generates blunt-end DNA products were cloned into the EcoRV site (blunt-end) of the polylinker region of the pBluescribe SK+ multifuctional vector (Stratagene). Using this method was cloned the open reading frame of the rat NPY-Y1 receptor gene from a cDNA library of RINm5f cell line (See Chapter 7. Cloning of the ORF of the NPY-Y1 receptor).

2.10.1.1.2 TA Cloning

The PCR products generated using the thermostable Taq DNA polymerase, which generates DNA products with 3'-A overhangs were cloned in the T-overhangs of the pTAg cloning vector using the LigATor kit (R&D System) following the manufacturer instructions. Using this method were cloned the deletions of the AP-1 site and the progressive deletions of the 5'-flanking (promoter) region of the rat NPY-Y1 receptor gene (See Chapter 6. Deletions of the NPY-Y1 promoter region), as well as a fragment of the 3'-flanking region of the NPY-Y1 receptor gene and its deletions. This deletion clones were used in an attempt to made a competitive RT-PCR for study the NPY-Y1 receptor gene expression in PC12 cells (See Chapter 7. RT-PCR detection of the NPY-Y1 receptor mRNA).

2.10.1.1 INDIRECT CLONING

PCR products generated using both thermostable DNA polymerases (DNAzyme or Taq) were indirectly cloned into the polylinker region of the pBluescribe SK+ multifunctional vector (Stratagene) after modification with specific restriction endonuclease digestion. The restriction enzymes were present at the 5'-region of the PCR primers or in the DNA amplified. Using this indirect method was cloned the 5'-flanking (promoter) region of the NPY-Y1 receptor gene in pBluescribe SK+ (See Chapter 6. Cloning of the NPY-Y1 promoter region).

2.10.2 SUBCLONING OF DNA PRODUCTS

PCR fragments cloned in pBluescribe SK+ (Stratagene) or pTAg (R&D Systems) vectors were further subcloned into the polylinker region of specific vectors, such as pGL3-Basic (luciferase reporter vector) (Promega) using specific restriction enzymes. This method was used to subclone in a bidirectional way the 5'-flanking region of the NPY-Y1 receptor gene to create the pY1-LUC fusion gene (See Chapter 6. Cloning of the NPY-Y1 promoter region). Furthermore, specific deletion of the CRE binding element and the AP1-GRE binding element present in the promoter region of the NPY-Y1 receptor gene were deleted using specific restriction enzymes present in both sides of these sequences (See Chapter 6. Deletions of the NPY-Y1 promoter region).

2.11 DNA SEQUENCING

2.11.1 THERMOSEQUENASE™ CYCLE METHOD

ThermoSequenase™ cycle sequencing kit (Amershan) was used for double stranded sequencing of the deletion clones using the dideoxy chain termination method and PCR reaction. Template plasmid DNA was prepared using Quiagen (Qiagen Inc.) or Wizard Maxiprep System (Promega) kits following the manufacturer instructions.

2.11.1.1 LABELING STEP

This protocol was used to 3'-dNTP internal label cycle of oligonucleotides. For each set of four sequencing lanes a single labeling reaction is run, containing in a volume of 17.5 µl: 1 µl M13 universal cycle primer (0.5 pmol/µl), 2 µl sequencing reaction buffer (10X) (See Appendix I), 5 µl DNA (100 ng), 1 µl 7-deaza-dGTP cycle mix, 1 µl dCTP cycle mix, 0.5 µl [α-^{35}S] dATP (10 µCi/µl, 1000 Ci/mmol), 2 µl Thermo Sequenase™. Positive control reactions containing 5 µl of pUC18 The reaction was overlayed with 15 µl of mineral oil and run with the following parameters:

TABLE 2.6 LABELLING STEP

melting step	annealing step	polymerisation step	cycles
2 minutes (95°C)			1 cycle
15 seconds (95°C)	30 seconds (60°C)		50-60 cycles

2.11.1.2 TERMINATION STEP

For each labelling reaction, 4 tubes were set up such that each tubes labelled G, A, T, C contained either 4 µl of ddGTP, ddATP, ddTTP and ddCTP termination mix, respectivily. Once the labelling reaction was complete, 3.5 µl of labelling reaction was removed and transfer to the termination tubes, mixed by pipeting up and down several times and overlayed with 10 µl of mineral oil. The termination reaction was run following the parameters:

TABLE 2.7 TERMINATION STEP

melting step	annealing step	polymerisation step	cycles
2 minutes (95°C)			1 cycle
30 seconds (95°C)	30 seconds (60°C)	120 seconds (72°C)	50-60 cycles

Termination reaction was finish with 4 µl of stop solution (See Appendix I) and the samples were store at -20°C. The samples were boiled to 70°C for 2-10 minutes before loading 3 µl onto a 8% polyacrylamide gel (See DNA sequencing gel).

2.12 NUCLEIC ACID LABELLING

2.12.1 DNA PROBES

2.12.1.1 5'-END LABELLING

Oligonucleotides probes were labelled using T4 polynucleotide kinase. The labelling reactions were set up in a 10 µl reaction containing: oligonucleotides in distillate water (8-10 pmoles), 1 µl T4 PNK buffer (10 X) (See Appendix I), 5 µl [γ-^{32}P] ATP (at 3,000 Ci/mmol, 10 mCi/ml), 1 µl T4 PNK (8-10 units), nuclease free water to a final volume of 10 µl. The reaction was incubated at 37°C for 30 minutes and terminated by heat inactivation at 90°C for 2 minutes then briefly spun in a microcentrifuge.

Unincorporated labeled dNTPs were removed by selective precipitation of the labeled DNA (See removal of unincorporated label). The percent of incorporation of the specific end-labelled nucleotide was determined by scintillation counter using filter-binding assay (See determination of the percent of incorporation) and the final concentration was adjusted by addition of nuclease-free water. The probe was stored in TE buffer or distilled water at -20°C for further use in the primer extension assays.

2.12.1.2 RANDOM-PRIMER LABELLING

^{32}P-labelled DNA fragments used for blot hybridisation were prepared by random-primed DNA synthesis using a High Prime kit (Boehringer Mannheim). In this method a mixture of random hexamers is used to prime DNA synthesis *in vitro* from any linear double stranded DNA template. A full length double stranded rat NPY-Y1 receptor cDNA cloned in pBluescribe SK$^+$ was used as a probe to analyse NPY-Y1 receptor mRNA expression in PC12 cells.

The NPY-Y1 cDNA was released from the vector pBluescribe SK$^+$ by digestion with *Bam* HI-*Hind* III restriction enzymes (See Cloning NPY-Y1 receptor) followed by electrophoresis in a 1% low melting point agarose gel. The DNA to be labelled was visualized by UV and the agarose containing the fragment (1.3 kb) corresponding to the open reading frame of the NPY-Y1 receptor was excised from the gel. The agarose plug was placed into a eppendorf tube containing 20 µl of TE buffer and the agarose was melted at 95°C for 2 minutes to separate the two cDNA strands, after which the sample was kept at 37°C until was required.

In a typical reaction, approximately 100-500 ng of linearized DNA in 20 µl of TE buffer was strand-separated by boiling for ten minutes. 5 µl of this DNA was then snap-cooled on ice and 4 µl High Prime buffer solution (5X) (See Appendix I), 5 units Klenow-fragment of the DNA polymerase I, 2 µl dNTP mix (0.125 mM dATP, dTTP, dGTP), 50 µCi [α^{32}P]dCTP (3000 Ci/mmol, 10 mCi/ml, Amersham) and distilled water up to 20 µl of final volume was added. The reaction was incubated for 15-30 minutes at 37°C. The reaction was terminated by heating at 95°C for 2 minutes and susequently chilling in an ice bath. EDTA was added to a final concentration of 20 mM.

Labelled DNA was dissolved in 50 µl of TE buffer and an aliquot of this mixture was used to determine the percent of incorporation by liquid scintillation spectrophotometry (See determination of the percent incorporation). DNA was routinely labelled to a specific activity of approximately 1 X 10^9 dpm/µg. The unincorporated nucleotides were removed by ethanol precipitation at -20°C following synthesis (See removal of unincorporated nucleotides). The labelled DNA was strand-separated by boiling for 10 minutes immediately prior to use in hybridisation of Northern blots.

12.2.2 RNA PROBES

2.12.2.1 *IN VITRO* TRANSCRIPTION LABELLING

^{32}P-labelled cRNA fragments used for ribonuclease protection assays (RPA) were prepared using a Riboprobe® *in vitro* transcription system (Promega). Antisense control cRNA was

provided for the RPA II kit (Ambion) and consisted of a linearized pT7 RNA 18S (Ambion) containing a 80 bp human RNA 18S gene fragment in the antisense orientation under the transcriptional control of T7 promoter. The size of the transcript is 114 nucleotides when transcribed with T7 RNA polymerase.

In a typical reaction 100-500 ng of a linearised template DNA in distilled water or TE buffer was added to a tube containing: 4 µl transcription optimised buffer (5X) (See Appendix I), 2 µl of DTT (100 mM), 4 µl of ATP, CTP, GTP (2.5 mM each), 2.4 µl of UTP (100 µM), 50 µCi [α^{32}P]UTP (500 Ci/mmol, Amersham), 20 units of recombinant RNasin® ribonuclease inhibitor, 15-20 units of T7 RNA polymerase and nuclease-free water up to a volume of 20 µl. The reaction was incubate for 60 minutes at 37°C. DNA template was removed by digestion with RQ1 RNAse-free DNAse (1U/µg of DNA) at 37°C for 15 minutes. The reaction was extracted with phenol/chloroform several times, and selectivily precipitated with ammonium acetate and ethanol for incubation at -20°C for 30 minutes.

2.12.3 REMOVAL OF UNINCORPORATED NUCLEOTIDES

Unincorporated labelled dNTPs were removed by selective precipitation of the DNA or RNA. The DNA or RNA labelled sample was precipitated with 0.5 volume of ammonium acetate (7.5 M) and 2 volumes (DNA) or 2.5 volumes (RNA) of ethanol. The mixture was centrifugued at 12,000 x g for 15 minutes and the pellet was resuspend in 100 µl of ammonium acetate (2M) and 200 µl of ethanol and incubated at -20°C for 30 minutes. This solution was centrifuged at 12,000 x g for 5 minutes at 4°C, the supernatant was removed and the DNA or RNA pellet was resuspend in 10-20 µl of TE buffer (DNA) or DEPC-treated water (RNA) for be used as a probe in Northern blot or ribonuclease protection assay.

2.12.4 DETERMINATION OF PERCENT OF INCORPORATION

2.12.4.1 DNA PERCENT INCORPORATION (FILTER-BINDING ASSAY)

The level of the incorporation of the radiolabel into the DNA sample was determined by spotting 1 µl of the diluted sample 1:100 in 0.2 M EDTA (pH 8.0) before and after the removal of the unincorporated nucleotides onto two filters (Whatman, DE-81) and the filters were air-dried. Each filter was placed in a scintillation vial and then were counted in a scintillation counter (Beckman, model LS6500).

2.12.4.2 RNA PERCENT INCORPORATION (TCA PRECIPITATION)

The labelling reaction mixture was diluted 1:10 in water and 1 µl of the diluted sample was spotted onto a glass-fiber filter (Whatman, GF). The filter was air-dried and keept aside to determine total cpm. Other 1 µl of the dilution sample was added to 100 µl of carrier nucleic acid (tRNA at 1 µg/ml), mixed and then was added 0.5 ml of ice-cold 5% TCA. The mixture was incubated on ice for 5 minutes. The precipitated RNA was collected by vacuum filtration onto a glass-fiber pre-wet with 5% TCA and washed twice with 5 ml of ice-cold 5% TCA. The glass-fiber filters were rinsed with 2 ml of acetone and air-dried. Each filter was placed in a scintillation vial and there were counted in a scintillation counter (Beckman, model LS6500).

2.13 NUCLEIC ACID HYBRIDISATION

2.13.1 NORTHERN BLOT HYBRIDISATION

Northern blots were analysed by hybridisation with radioactively-labelled cDNA (random primer) or cRNA (*in vitro* transcription) fragments. Sheared, strand-separated salmon sperm DNA or poly A DNA was used to block non-specific sites on blots during both prehybridisation and hybridisation.

2.13.1.1 cDNA PROBES

Solutions (See Appendix I):
0.77 M Na_2PO_4 (pH 7.2)
SDS 20 %
PolyA (2 µg/µl)
Samon sperm DNA (100 µg/ml)

For hybridisation with cDNA probes, filters were prehybridised for at least 30-60 minutes at 55°C in 20-30 ml of 0.77 M phosphate buffer (pH 7.2) containing 6.6% SDS and 100 µg poly A DNA in plastic bags in a hybridisation oven (Techne Ltd., model HB-2D). The probe was boiled for 10 minutes and added directly to the hybridization bag. After addition of the labelled probe, hybridisation was allowed to proceed overnight at 55°C. The next day filters were washed once in 2 x SSC/1% SDS at room temperature for 30 seconds and then for a further ten minutes at 55°C. Washed filters were laid on wash-solution-saturated filter paper and sealed into plastic bags.

2.13.1.2 cRNA PROBES

Solutions (See Appendix I):
deionized formamide (100 %)
SSC (20X)
Denhardt's solution (50X)
Salmon sperm DNA (100 µg/ml)

For hybridisation with cRNA probes, filters were prehybridised for 1-2 hours in 5 to 10 ml/100 cm^2 of hybridisation solution containing formamide (50%), SSC (5X), Denhard's solution (5X), SDS (1%), 50 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA and 100 µg/ml of salmon sperm DNA at 55°C in plastic bags or in a hybridisation oven (Techne corp., model HB-2D). Labelled cRNA probe was then added to the blots and hybridisation allowed to proceed overnight at the same temperature. Blots were washed twice for 15 minutes in 0.2 X SSC/0.1% SDS at room temperature and then twice for 15 minutes in 1 X SSC/0.1 % SDS at 55°C and the blot was prepared for exposure as described above.

2.13.2 SIGNAL DETECTION

Blots were appeased to film X-OMAT-AR (Estman-Kodak) with or without intensifying screen and allowed to expose at -70°C. Film was developed using conventional photographic technology. Alternatively, blots were exposed to phosphorimager (PI) screens with cassettes and the resulting images captured from the screens to a database using the bio-imaging analyser (Fuji Photo Film Co. Ltd, Fujix BAS1000 MacBAS). Hardcopy images of the data were obtained using Adobe Photoshop software (Adobe Systems Incorporated).

2.13.3 REHYBRIDISATION

Blots were stripped of radioactive probe for rehybridisation by incubating blots three times in boiled 0.1 x SSC/0.1 % SDS until the solution reached room temperature. Stripped blots, laid upon stripping solution-saturated filter paper in sealed hybridisation bags, could be stored at -20°C before rehybridisation .

2.14 RIBONUCLEASE PROTECTION ASSAY

Ribonuclease Protection Assay (RPA) is an sensitive procedure for detection and quantification of mRNA in a complex sample mixture of total cellular RNA. The ribonuclease protection assay was performed using the RPA II™ kit (Ambion).

2.14.1 HYBRIDISATION OF PROBE AND SAMPLE RNA

Solutions (Appendix I):
Ammonium acetate (5 M)
Ethanol (100%)
Hybridization buffer (solution A)
RNase digestion buffer (solution B)
RNase Inactivation/Precipitation mixture (solution D)
RNA Loading buffer (solution E)
Probe elution buffer (solution F)
RNase mixture (solution R)

For each experimental tube the labelled probe (2-8 x 10^4 cpm) was mixed with the sample RNA (10-50 µg) in a 1.5 ml microfuge tube. 1 negative control tube was set up for every probe to be used containing 10 µg of yeast RNA.The probe was co-precipitated with the sample RNAs by adjusting the concentration of ammonium acetate to 0.5 M and adding 2.5 volumes of ethanol. The mixture was incubated at -20°C for 15 minutes and then centrifugued at 12,000 x g for 15 minutes at 4°C. The supernatant was removed and the samples air-dried for 5 minutes at room temperature. The pellet was resuspended in 20 µl solution A (hybridisation buffer) by thoroughly vortexing and then were centrifugued briefly. All the tubes were heated at 95°C for 3-4 minutes, re-vortexed and re-centrifuged briefly. The tubes were incubated at 45°C overnight to permit hybridisation of the probe and complementary mRNA in the sample RNA. The required hybridisation time was extended because the low abundance of target RNA (NPY-Y1 receptor) in the PC12 sample.

2.14.2 RNase DIGESTION OF HYBRIDISED PROBE AND SAMPLE RNAs

Working dilutions of solution R (concentrated RNase A/RNase T1 mixture) were prepared in solution B (RNase digestion buffer). The standard dilution was 1:100 (2 µl of solution R in 200 µl of solution B). 200 µl of diluted RNase solution (RNase digestion buffer and RNases) was added to all the experimental tubes, and to the control negative tube (yeast RNA). The tubes were vortex and centrifugued briefly. The mixture was incubated for 30 minutes at 37°C and then 300 µl of solution D (RNAse inactivation/precipitation mixture) was added to each tube. The RNA was precipited for incubation at -20°C for at least 15 minutes. The tubes were centrifugued at 12,000 x g for 15 minutes at 4°C and each pellet was dissolved in 8 µl of

solution E (gel loading buffer for denaturing gels) and all the samples were heated for 3-4 minutes at 95°C and run in a denaturing polyacrylamide gel (PAGE gel).

2.14.3 SEPARATION AND DETECTION OF PROTECTED FRAGMENTS

A denaturing PAGE gel suitable for separation of protected fragments of expected size (typically 4% polyacrylamide plus 7 M urea) was prepared (See denaturing PAGE for ribonuclease protection assay) and run at approximately 250 volts for about 1-2 hours in TBE (1X) buffer. The gel was covered with plastic wrap, and exposed to X-ray film X-OMAT (Estman-Kodak) at -70°C with an intensifying screen.

2.15 LUCIFERASE REPORTER ASSAY

Transfected PC12 cells were washed and harvested in PBS (1X). Luciferase activity was measured using the protocol and Luciferase Assay system kit (Promega). The amount of total protein in each sample was determined spectrophotometrically using the Bradford Bio-Rad procedure. The amount of the luciferase activity was determined using a luminometer (Turner, model TD-20e).

2.15.1 GENERAL CONDITIONS

Light intensity is a measure of the rate of catalysis by luciferase, and is therefore dependent upon temperature. The temperature optimum for luciferase activity is approximately room temperature (20-25°C). The sample to be assayed and the Luciferase Assay Reagent were fully equilibrated to room temperature before the measurements. Luciferase activity is stable for several hours at room temperature (See Chapter 3. Transfection of PC12 cells).

2.15.2 INSTRUMENT CONSIDERATIONS

Luminometers provide the most convenient, versatile and sensitive means of quantifying light emission from the luciferase reaction. The most sensitive luminometers utilise photomultiplier tubes to detect photons (Turner, model 20e). The photomultiplier luminometers have two important properties which affect performance: the background signal developed by the photomultiplier tube is temperature-dependent, therefore is important to ensure that they are at ambient temperature (19-23°C) and the sensitivity of photomultipliers is wave-length dependent. The photomultiplier tubes requires 1-3 seconds to stabilize after the sample is introduced. Therefore an initial delay of at least 3 seconds was allowed and then the luminiscence was measured for 15 seconds.

The linear range of the light detection method was determinated (See Chapter 3. Transfection of PC12 cells) because luminometers usually experience signal saturation at high light intensities. A standard curve of light units vs. relative enzyme concentration was maked using a serial dilutions of luciferase (cell culture extract) in cell culture lysis (1X) with 1mg/ml BSA (See Chapter 3. Transfection PC12 cells). The addition of BSA is necessary to ensure that luciferase is not lost from solution by adsorption on to container surfaces.

2.15.3 STANDARD LUCIFERASE ASSAY

2.15.3.1 pGL3 LUCIFERASE REPORTER VECTORS

pGL3 Luciferase Reporter Vectors (Promega) were used for a quantitative analysis of factors that regulate mammalian gene expression. The Luciferase reporter vectors carry a modified coding region for firefly (*Photinus pyralis*) luciferase that has been optimised to monitor transcriptional activity in transfected eukaryotic cells. The assay of this genetic reporter is rapid, sensitive and quantitative.

pGL3 Luciferase Reporter Vectors contain a high copy number prokaryotic ColE1 origin of replication for maintenance in *E. coli*,, an ampicillin-resistance gene for selection, and a filamentous phage origin of replication (f1 origin) for single-stranded DNA production.pGL3 vectors carries a modified luciferase gene (*luc*+) which contains multiple new features which were made to increase luciferase expression, improve *in vivo* vector stability, and provide greater flexibility in genetic manipulations (Promega).

2.15.3.1.1 pGL3-Basic Vector

pGL3-Basic vector (Promega) lacks eukaryotic promoter and enhancer sequences allowing maximum flexibility in cloning putative regulatory sequences. Expression of the luciferase activity in cells transfected with this plasmid depends on insertion and proper orientation of a functional promoter upstream of the luciferase gene. This plasmid was used to clone the promoter region of the NPY-Y1 receptor gene.

2.15.3.1.2 pGL3-Control Vector

pGL3-Control vector (Promega) contains a simian virus 40 (SV40) early promoter/enhancer sequences resulting in a strong expression of luciferase in many types of mammalian cells. This plasmid was used to monitor transfection efficiency in PC12 cells.

2.15.3.2 LUCIFERASE ASSAY METHOD

Solutions (See Appendix I):
Luciferase Assay Substrate
Luciferase Assay Buffer
Cell Culture Lysis Reagent (5X)
PBS (1X)

Constructs were transfected into mammalian cells and after 48 hours, luciferase activity was measured. The Luciferase Assay Reagent was prepared adding 10 ml of luciferase assay buffer (Promega) to a vial containing 7 mg of lyophilised luciferase assay substrate (Promega). To avoid exposure to multiple freeze/thaw cycles, the reconstituted luciferase reagent were placed into working aliquots (1ml) and stored at -70ºC.

The standard luciferase assay (Promega) was made following the manufacturer instructions : 4 volumenes of PBS (1X) were added to 1 volume of Cell Culture Lysis (5X) Reagent (CCLR) to produce a 1X stock. This diluted Lysis Reagent (1X) and a aliquot of Luciferase Reagent was equilibrated to room temperature. The growth medium was removed from the cells. The cells were washed once in PBS buffer (1X) and then resuspended in 1.5 ml of PBS Buffer (1X). The cells were centrifuged at 12,000 x g for 5 seconds in a microcentrifuge and the pellet was resuspended in 100-200 µl of CCLR (1X) (per 60 mm culture dish) and incubated a room temperature for 10-15 minutes. The mixture was spun briefly (5 seconds) in a microcentrifuge to pellet large debris and the supernatant was placed in a new tube. The cell extract was stored indefinitely at -70ºC.

At this point, a 5-10 µl aliquot was used for protein quatification using the Bradford method (See Total Protein Quantification). Up to 20 µl of cell extract equilibrated were mixed with 80 µl of Luciferase Assay Reagent equilibrated at room temperature in disposable polypropylene cuvettes (8 x 50 mm) (Promega). The mixture was vortex briefly (few seconds) and the light produced was measured for a period of 15 seconds in a lumimometer (Turner, model 20e).

2.15.4 DUAL LUCIFERASE ASSAY

2.15.4.1 pRL LUCIFERASE REPORTER VECTORS

The pRL family of *Renilla* luciferase (*Rluc*) control reporter vectors contain the cDNA of the luciferase from a anthozoan coelenterate*Renilla reniformis* (sea pansy). Expression of (*Rluc*) was drived by one of two promoter elements (CMV or SV40), as described below. These vectors were used as internal control of the transfection efficiences in PC12 cells.

2.15.4.1.1 pRL-CMV

pRL-CMV vector contains the cytomegalovirus (CMV) immediate-early enhancer/promoter region which provides constitutive expression of *Renilla* luciferase in many types of mammaliam cells, including PC12 cells.

2.15.4.1.2 pRL-SV40

pRL-SV40 vector contains the simian virus 40 (SV40) early enhancer/promoter region and contains the SV40 origin of replication, which allows transient, episomal replication in cells expressing the SV40 large T antigen, such as COS-1 and COS-7 cells. This vector also provides constitutive expression of *Renilla* luciferase in many types of mammaliam cells, including PC12 cells.

2.15.4.2 DUAL-LUCIFERASE™ REPORTER METHOD

Dual-luciferase™ reporter assay system (Promega) and the pRL vectors (Promega) were used to provides an internal control value (*Renilla* luciferase) to which the expression of the experimental firefly luciferase reporter gene may be normalized.

Solutions (Appendix I):
Luciferase Assay Buffer II
Luciferase Assay Substrate
Stop & Glo™ Buffer
Stop & Glo™ Substrate
Stop & Glo™ Substrate Solvent
Passive Lysis Buffer (5X)

Quantification of the luminiscent signal from each of the two luciferase reporter enzymes was performed immediately following lysate preparation. The firefly luciferase reporter assay was initiated by adding an aliquot of lysate to the Luciferase Assay Reagent II (Promega). Quenching of firefly luminiscence and concominant activation of *Renilla* luciferase was accomplished by adding Stop & Glo™ reagent (Promega) to the sample tube immediatly after quantification of the firefly luciferase reaction. The passive lysis buffer (PLB) (Promega) is designated to provide optimum performance and stability of the firefly and *Renilla* luciferase reporter enzymes. PLB was supplied as a 5X concentrate and was diluted in a 1X working concentration with PBS (1X).

The Luciferase Reagent II (Promega) was prepared by resuspending the lyophilized Luciferase Assay Substrate (Promega) in 10 ml of the Luciferase Assay Buffer II (Promega). This reagent was placed in aliquots (1 ml) and stored at -70°C. The Stop & Glo™ substrate (50X) was prepared by transfering 200 µl of the Stop & Glo™ substrate solvent (Promega) into the vial containing the dried Stop & Glo™ substrate (Promega). This reagent was stored at -70°C. The Stop & Glo™ reagent was prepared fresh by adding 20 µl of Stop & Glo™ substrate (Promega) to 1ml of Stop & Glo™ buffer (Promega). This reagent was stored at -70°C.

The assays for firefly luciferase activity and *Renilla* luciferase activity were performed sequentially using one reaction tube. The growth medium was removed from the cells and the cells were washed once in PBS buffer (1X) and resuspend in 1.5 ml of PBS Buffer (1X). The cells were centrifuged at 12,000 x g for 5 seconds in a microcentrifuge and the pellet was resuspended in 100-200 µl of passive lysis (1X) Reagent (per 60 mm culture dish) and incubated a room temperature for 10-15 minutes. The mixture was spun briefly (5 seconds) in a microcentrifuge to pellet large debris and the supernatant was placed in a new tube. The cell extract was stored at -70°C. At this point, a 5-10 µl aliquot was used for protein quantification using the Bradford method (See Total Protein Quantification). Up to 20 µl of cell extract equilibrated was mixed with 80 µl of Luciferase Assay Reagent II equilibrated at room temperature in polypropylene cuvettes (8 x 50 mm, Promega). The mixture was vortexed for few seconds and the reaction was placed in a luminometer (Turner, model 20e). The light produced was measured for a period of 15 seconds with a 3 seconds delay. Thus, 80 µl of Stop & Glo™ were addedd to mesure the *Renilla* luciferase activity for a period of 15 seconds with a 3 seconds delay in a lumimometer.

2.16 Ca^{++}- PHOSPHOLIPID DEPENDENT PROTEIN KINASE (PKC) ASSAY

The PKC Assay System kit (Life Technologies) is base on the phosphorylation of a synthetic peptide from myelin basic protein (Ac-MBP 4-14) which is acetylated in the amino-terminal glutamine in order to maintain its stability. PKC specificity is confirmed by using the PKC pseudosubstrate inhibitor peptide PKC (19-36) which acts a potent inhibitor for this substrate, as well other physiological relevant substrates such as myosin light chain kinase (MLCK) and peptides derived from glycogen synthase.

2.16.1 CELL EXTRACT PREPARATION

Solutions (See Appendix I):
Buffer A (extraction buffer)
PBS (1X)

Total cellular PKC activity was measured using the PKC assay kit (Life Technologies, Inc). PC12 cells were plated in 60 mm tissue culture plates and incubated overnight. Pharmacological activator compounds (NGF and PMA) were added directly to the cells from suitable stocks (See Cell treatments). After incubation, cells were washed once with 2 ml PBS (1X) and then resuspended in 1.5 ml of PBS buffer (1X). The cells were centrifuged at 12,000 x g for 5 seconds in a microcentrifuge and the pellet was resuspended into 500 µl of ice cold buffer A (See Appendix I). PC12 cells were homogenised by 10-15 strokes on ice pre-cooled dounce homogenizer, always maintaining the tubes on ice. At this point, a 10 µl aliquot was saved for protein determination using the Bradford micromethod (See 'Total Protein Quantification). The samples were incubated on ice for 30 minutes. The cell debris was removed by centrifugation for 2 minutes at 12,000 x g at 4°C and supernatants were saved in clean eppendorf tubes.

2.16.2 PARTIAL PURIFICATION OF PKC ACTIVITY

Solution (See Appendix I):
Buffer B (DEAE wash buffer)
Buffer C (DEAE elution buffer)

PKC was then partially purified from these cell extracts by DEAE cellulose ion-exchange chromatography. 0.25 g of DEAE cellulose (Whatman, DE-52) was resuspended in 1 ml buffer B (Appendix I). The DEAE-cellulose was poured into 5 ml plastic columns (1 column per cell extract) and washed with 2 ml of buffer B (DEAE wash buffer). Once the buffer level had reached the surface of the resin, cell extracts were loaded onto the columns and once the extract had entered the resin, these were washed with 5 ml of buffer B. Samples were eluted with 5 ml of buffer C (Appendix I). Eluents were recovered in fresh tubes and kept on ice.

2.16.3 PKC ACTIVITY ASSAY

Solutions (See Appendix I):
Buffer C (DEAE elution buffer)
Lipid mixture (10X)
Inhibitor solution (5X)
[γ-^{32}P]ATP/substrate solution (5X)

PKC activity for each plate of cells were assayed in duplicate. Incorporation of ^{32}P-ATP into a specific PKC substrate was measured using [γ-^{32}-P]ATP (6000 Ci/mmol) (Amersham) as a donor. The substrate consisted of an acetylated synthetic peptide from myelin basic protein

(MBP). 5 to 25 µl of cell extract was adjusted to a final volume of 25 µl with elution buffer C and mixed with 5 µl of 10X lipid mixture (Appendix I) and 10 µl water. Control samples containing the PKC inhibitor, PKC (19-36), which is based on the pseudosubstrate region common to the α-, β- and γ- isozymes of PKC were prepared in the same way but substituting the lipid mixture for 10 µl of 5X inhibitor solution (See Appendix I) and 5 µl water. Samples were incubate a room temperature for 20 minutes to allow inhibitor to bind and then mixed with 10 µl 5X [γ-^{32}P]ATP/substrate solution (Appendix I). These were incubated for 5 minutes at 30°C, and the reaction stopped by spotting 25 µl from each sample on to phosphocellulose paper discs. All the samples were spotted in phosphocellulose discs (Whatman, DE-51) and this discs were washed twice in 500 ml of phosphoric acid (1%) in a rocker for 3-5 minutes. The sheet was then washed twice with 500 ml of distilled water for 2-3 minutes each time. Each phophocellulose disc was punched out into scintillation vials. 5 ml of scintillation fluid was added to each vial and samples were counted in a β-counter for 3 minutes (Beckman, LS6500).

2.16.4 TOTAL PROTEIN QUANTIFICATION

The BioRad Protein Assay Dye Reagent Concentrate (BioRad) containing an acidic solution of Coomasie Brilliant Blue G-250 was used to determine total protein concentrations. Typically, 5-10 µl of cells extract (Bradford method) was added to a mix of 200 µl of the dye reagent (5X) (See Appendix I) and 800 µl of distilled water for each sample in a eppendorf tube and vortexed immediately avoiding excess foaming. After 5 minutes but before of 45-60 minutes, sample absorbance was assessed in a spectrophotometer at 595 nm (Beckman, model DU-640B). "Blank" control reaction contained lysis buffer and dye concentrated reagent. All the measurement were made in triplicate.

CHAPTER 3

GENERAL ASPECTS OF THE TRANSCRIPTIONAL ACTIVATION OF THE NPY-Y1 RECEPTOR GENE IN PC12 CELLS

3.1 INTRODUCTION

3.1.1 REPORTER ASSAY SYSTEMS

Genetic reporter systems have contributed to the study of eukaryotic gene expression and regulation. Although reporter genes have played a significant role in numerous applications, they are most frequently used as indicators of transcriptional activity in cells. Typically, a reporter gene is joined to a promoter sequence in an expression vector that is transferred into the cells. Following the transfer, the cells are assayed for the presence of the reporter, this can be achieved by directly measuring the amount of reporter mRNA, the reporter protein itself or the enzymatic activity of the reporter protein. An ideal reporter gene is not endogenously expressed in the cell type of interest, and is amenable to assays that are sensitive, quantitative, rapid, easy and reproducible (Groskreutz and Schenborn, 1996).

One popular reporter system uses bioluminescence. The luciferase enzyme used most frequently for this reporter gene technology is derived from the cloned sequence of the *luciferase* gene obtained from the North American firefly *Photinus pyralis* (Groskreutz and Schenborn, 1996). The gene encoding firefly luciferase is highly effective for this purpose because the luciferase assay is extremely sensitive (sensitivity to 10^{-20} moles of luciferase), high degree of linearity (at least 8 orders of magnitude), rapid and easy to perform (cell lysis and assays in 5 minutes), flexible (can be used with a luminometer or scintillation counter), safe (no radioactivity) and relatively inexpensive (Wood, 1991).

Renilla luciferase, a 36kDa monomeric protein, is composed of 3% carbohydrate when purified from its natural source, the sea pansy *Renilla reniformis* (Wood, 1991). However, like firefly luciferase, post-translational modification is not required for activity and the enzyme may function as a genetic reporter immediately following translation. Since the firefly and *Renilla* luciferases are of distinct evolutionary origins, they have dissimilar enzyme structures and substrate requirements. These differences make it possible to discriminate between their respective bioluminescent reactions. Thus, using the Dual-Luciferase™ Reporter Assay System, the luminescence from firefly luciferase ("experimental" reporter) may be quenched while simultaneously activating the luminescent of *Renilla* luciferase ("control" reporter).

The use of dual reporter refers to the simultaneous expression and measurement of two individual reporter enzymes within a single system. Typically, the "experimental" reporter reflects the effect of the specific experimental conditions on the level of gene expression, while the activity of the co-transfected "control" reporter provides an internal control by which each value within the experimental set can be normalised. The control reporter gene is usually driven

by a strong, constitutive promoter and is co-transfected with "experimental" vectors. The experimental regulatory sequences are linked to a different reporter gene so that the relative activities of the two reporters gene products can be assayed individually. Thus, a "control" gene vector can be used to normalise for transfection efficiency or cell lysate recovery between treatments or transfections experiments. Normalising the activity of the experimental reporter to the activity of the internal control effectively eliminates inherent variabilities that can undermine experimental accuracy.

Reporter vectors allow functional identification and characterisation of cis-acting transcriptional elements (promoter and enhancers) because expression of the reporter is correlated with the transcriptional activity of the reporter gene. For these types of the studies, promoter regions are cloned upstream of the reporter gene and enhancer elements are cloned upstream or downstream from the gene. The fusion gene is introduced into cultured cells by transfection methods or into a germ cell to produce transgenic organisms. Using reporter gene technology, promoter and enhancer have been characterized that regulate cell-, tissue- and developmentally-defined gene expression (Groskreutz and Schenborn, 1996).

3.1.2 TRANSFECTION METHODS

The ability to introduce recombinant DNA into heterologous cellular systems has increased the knowledge of mechanisms governing regulation of gene expression. Initially, these studies relied on the creation of clonal cell lines that contained the promoter of interest (Wigler et al., 1978). This approach was time-consuming and the activity of the promoter could be greatly affected by its chromosomal location. This was later replaced by systems in which the DNA is located in plasmid vectors and transiently introduced into the cells (Gorman et al., 1982). This approach has been used to study transcriptional activity of many genes providing a suitable system to manipulate gene structure and expression.

Chemical (calcium phosphate, DEAE-dextran, liposomes), electrical (electroporation) and physical (microinjection) methods are used to introduced nucleic acids in a process referred to as transfection. An ideal method to transfer genes or macromolecules of interest into eukaryotic cells exhibit the following features: (i) high efficiency of transfection, (ii) low toxicity, (iii) reproducibility and (iv) suitability for *in vivo* and *in vitro* applications.

3.1.3 EUKARYOTIC TRANSCRIPTION REGULATION

In eukaryotes, three RNA polymerase complexes play important roles in transcription. RNA polymerase I transcribes ribosomal RNAs (rRNAs), RNA polymerase II transcribes protein-encoding genes (mRNAs), and RNA polymerase III transcribes transfer RNA genes (tRNAs).

The RNA polymerase II basal transcription complex consist of RNA polymerase II, which itself is a multi-subunit complex, together with several other protein factors (or general transcription factors) such as TBP (TATA-Binding Protein) and TFIIB. This mega-complex associates with DNA sequences in the promoter region in close proximity to the transcription initiation site preceding the gene coding region (McKnight and Yamamoto, 1992).

Other regulatory sequences, in addition to the promoter sequences which associate with the RNA polymerase II mega-complex, can influence the transcriptional activity of the genes. The region of DNA upstream from the transcription initiation site should be expected to be rich in *cis* -acting elements that comprise promoters, enhancers and silencers that regulate transcription directed by RNA polymerase II. The *cis* -acting sequences are recognised by sequence specific DNA binding proteins that act in *trans* to either activate or repress the rate of transcription of a particular gene. These specific transcription factors may act individually or in combination to modify basal transcription activity (McKnight and Yamamoto, 1992).

Over the last few years DNA consensus sequences that are recognised by *trans* -activating factors have been defined. Although the presence of such consensus sequences on the gene does not constitute, itself, evidence that particular *trans* -activating factors are functionally involved in the regulation of the gene, their presence provides clues about the mechanisms of control of the expression of a particular gene. Several DNA-binding proteins possess characteristics structural motifs which define classes or families of related transcription factors. Other features in addition to DNA sequences play a role in the binding of transcription factors to DNA. An additional level of transcriptional regulation is provided by protein:protein interaction, which contributes to the binding specificity of some groups of transcription factors. Lastly, the activities of some factors can be controlled by other enzymes such as kinases, phosphatases, proteases or other modifying enzymes which regulates the protein structural state and affects its DNA-binding ability.

3.1.4 PROMOTER REGION OF THE RAT NPY-Y1 GENE

The region of the rat NPY-Y1 receptor gene that lies upstream to the open reading frame has been attached to the reporter function, luciferase, so that the expression of this enzyme has been placed under the control of this 5'-flanking region of the rat NPY-Y1 receptor gene. In Figure 3.1, the partial sequence of the promoter region of the rat NPY-Y1 receptor gene spanning the sequences -600 bp to the +160 to the putative transcription start site is shown indicating consensus sequences for various transcription factors.

1 GCTACTTTGG CTTTTTAAAG GACTTCTTTTT GTTCTCTTA GACTGGGGGA

GRE GRE

51 AGTAACTTCT CGGTACTGGC GTGCGGAATAG TTGAGCGTCA CAAGAGCAG

CRE

101 CGGCAAACCC ATTAGCGGAG GCTTTGCGGTG AAGTGCATC CATCCCCCTT

151 CCCGTAAATA AGCTCTCCCC TTCATTCTTGA CTAGGGCTT AAAGCTCGGA

201 CTCTCGGAGG AAAAGGACTT CGCCACAAGAT GGCACTGAC CTGCAGACAA

GRE ERE

251 AGGAAATAGC GGTGGCATTG ACTTTTAAAAC AGGCTTGTA AATGCGCCTT

301 CCCTGAGTCA AGGACTAGTG GGGTGAGAGCT CTGTGTGTT CGGGTCTGAA

AP1 GRE

351 AGTGTGGCTT ACGTTTATCT GCTTTTCTTTC CAGAATTCC TAGGCAACGC

401 ACATCCGAGG TGTGCATCCG AGGTGTTGGGA GGAGGTTGT GGGGGAAACC

451 TTGGGTGATC CTCGTGGGGT GCCGTCACATG ATCCTGGAT GAGGTGGAGT

CRE

501 TCGGCTTTAA GGGGGGCGTCT CTTCTATCTTC CTCAATCTT TAGGGTTTGA

Sp1 CAAT

551 GCAGGAGAAA TACCAACAGA TCTTTCTTCCC TCC*AGTGACACTCGTCCCG*

TATA +1

601 *CTTCAACACA GGCGAACAGA CGGATTCTTTA AAAGA GTAT TCAGTTCAAG*

EXON I EXON II

651 *GGAACGAAGA ATTGAGAATT ATTTTTGGTGA ATGGATTCA AATAAATGGA*

701 *ATAAGAGACA GCTGAAGATC TGATCCATTTC GAAGAACCC TAACAGTCCG*

751 *TTTGTCTAAT CCATAACAAC AAAACGTAAAA A ATGAATTC*

MET

FIGURE 3.1 SEQUENCE OF RAT NPY-Y1 GENE

Sequence of 790 bp of the 5'-flanking region of rat NPY-Y1 receptor gene (Gene Bank accesion number: X95507; Lundell and Larhammar, 1996). **CRE** (cAMP responsive element), **GRE** (glucocorticoid responsive element), **ERE** (estrogen reponsive element), **AP1** (AP1 responsive site), **Sp1** (Sp1 responsive site), **CAAT** (CAAT-like box), **TATA** (TATA-like box), **+1** (putative transcription start site), **MET** (methionine).

The promoter region of the NPY-Y1 receptor gene upstream to the open reading frame was aligned to the presence of consensus sequences (WEB Signal Scan 4.05 program, University of Minnesota). This gene contains in this region several consensus sequences for transcription factors (Figure 3.2): an AT-rich "TATA-like" box (AAATA) is located at -20 bp (using as a reference the putative transcription start site) and there is a proposed "CAAT" box (CAATCT) at -45 bp, a GC rich Sp1 site (GGGGCG) at -73 bp, one AP-1 site (TGAGTCA) at -273 bp, two partial cAMP response elements (CRE) (CGTCA) at -113 bp and -493 bp, one non-palindromic esteroid responsive element (ERE) (TGACC) at -350 bp and four non-palindromic glucocorticoid response elements (GRE): three in the form of AGGACT at -230 bp, -373 bp and -553 bp and one in the form of TGTTCT at -540 bp.

This chapter deals primarily with the establishment of a reporter function assay to study the transcriptional regulation of the rat NPY-Y1 receptor gene using the enzyme luciferase. The fusion gene (pY1-LUC) has been expressed in PC12 cells, a clonal cell line derived from a rat pheochromocytoma (Greene and Tischler, 1976), which expresses constitutive levels of the NPY-Y1 receptor (DiMaggio et al., 1994).

3.2 RESULTS

3.2.1 TRANSFECTION OF PC12 CELLS

In order to establish the optimal conditions for PC12 cells transfection, one method was initially tested: calcium phosphate co-precipitation. Previous results in this laboratory using a different reporter vector reveal that calcium phosphate was the optimal method of introducing DNA into PC12 cells (Balbi, 1994). This procedure was therefore adopted and used to study the efficiency in the transfection of the luciferase reporter vector. In order to obtain information about the transfection efficiency, PC12 cells were initially transformed with one construct: pGL3-Control (Promega), in which the luciferase transcription is driven by an SV40 early enhancer/promoter. This is a strong viral promoter/enhancer active in many types of cells, including PC12 cells. This construct was used to optimise and standardise the conditions necessary to PC12 cells transfection and obtain measurable levels of luciferase enzyme activity.

Optimal conditions were found empirically and these were used routinely for all subsequent experiments. When transforming cells with DNA constructs containing the luciferase gene, several parameters can affect the levels of luciferase activity obtained in cell extracts, such as amount and quality of DNA added to the cells, the time the cells were incubated with the DNA or the use of compounds (DMSO, glycerol) which improve the transfection efficiency. All the experiments were performed in duplicate and the results are shown as the mean ± standard deviation of at least three experiments.

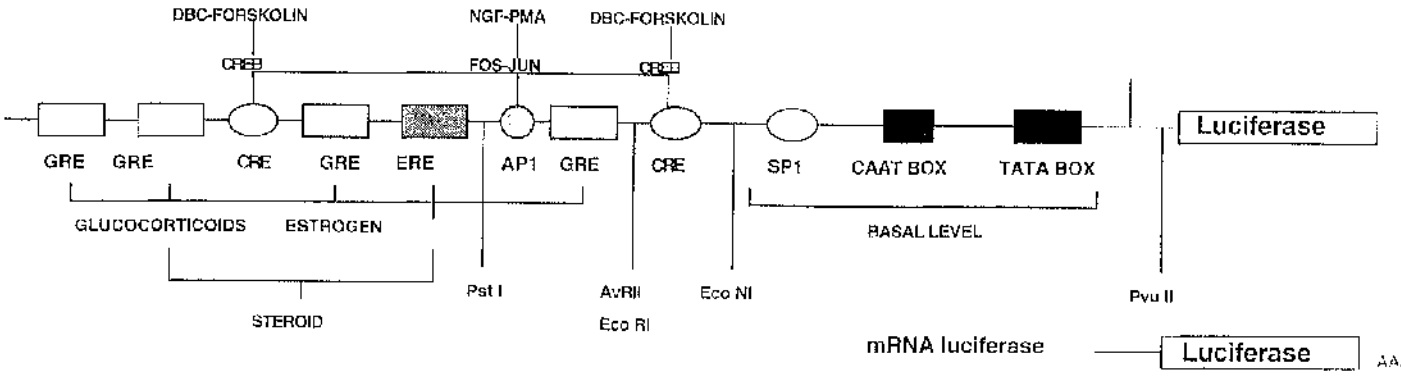


FIGURE 3.2 RESTRICTION ENZYME AND PUTATIVE BINDING SITES OF RAT NPY-Y1 PROMOTER

Restriction enzymes sites (***Avr* II**, ***Eco* RI**, ***Eco* NI**, ***Pst* I**, ***Pvu* II**) and putative binding sites in the promoter region of rat NPY-Y1 receptor gene cloned in the luciferase reporter vector (pY1-LUC). **CRE** (cAMP responsive element), **GRE** (glucocorticoid responsive element), **ERE** (estrogen reponsive element), **AP1** (AP1 responsive site), **Sp1** (Sp1 responsive site), **CAAT** (CAAT-like box), **TATA** (TATA-like box), **+1** (trascription initiation site). **LUC** (Luciferase open reading frame).

3.2.1.1 DNA QUALITY

The success of a particular gene transfer experiment depends largely on the quality of the DNA preparation. Indeed, the requirement for highly purified form I (covalently closed circular supercoiled) plasmid molecules to ensure both consistency and optimal levels of gene expression in "transient" assays is well documented. Therefore, in order to ensure homogeneity on DNA preparations, DNA used in these experiments was prepared using the large scale maxiprep alkaline-lysis method and purified by ultracentrifugation through caesium chloride (CsCl) gradient (See Chapter 2. DNA plasmid isolation).

3.2.1.2 DNA CONCENTRATION

The amount of DNA is a parameter known to affect the efficiency of the transfection. Initially, different concentrations of pGL3-Control reporter vector were tested for the transfection experiments (Figure 3-3). Maximum luciferase activity was obtained using 10 µg of control plasmid, increasing amounts of plasmid did not increase significantly the levels of luciferase activity. In the following experiments, 10 µg of plasmid DNA was therefore chosen for transfection in PC12 cells.

3.2.1.3 TIME POINT OF HARVESTING

Using 10 µg of pGL3-Control, the maximum levels of luciferase activity were detected in PC12 cell extracts after 2 days of transfection (Figure 3-4). Cell extracts of non-transformed PC12 cells showed no detectable luciferase activity as compared with cells transformed with pGL3-Control during each day (data not shown). This is an absolute requirement of the reporter assay method as it important that the only source of luciferase activity be derived from the plasmid DNA transformed into the PC12 cells.

3.2.1.4 LINEARITY OF THE ASSAY

A PC12 cell extract was diluted over a 100 fold range and the luciferase activity was measured (Figure 3-5). These results demonstrated that the luciferase activity was linear over this range of dilution, which means it is possible detect small variations in the levels of luciferase activity over this range of concentration of firefly luciferase enzyme in PC12 cells.

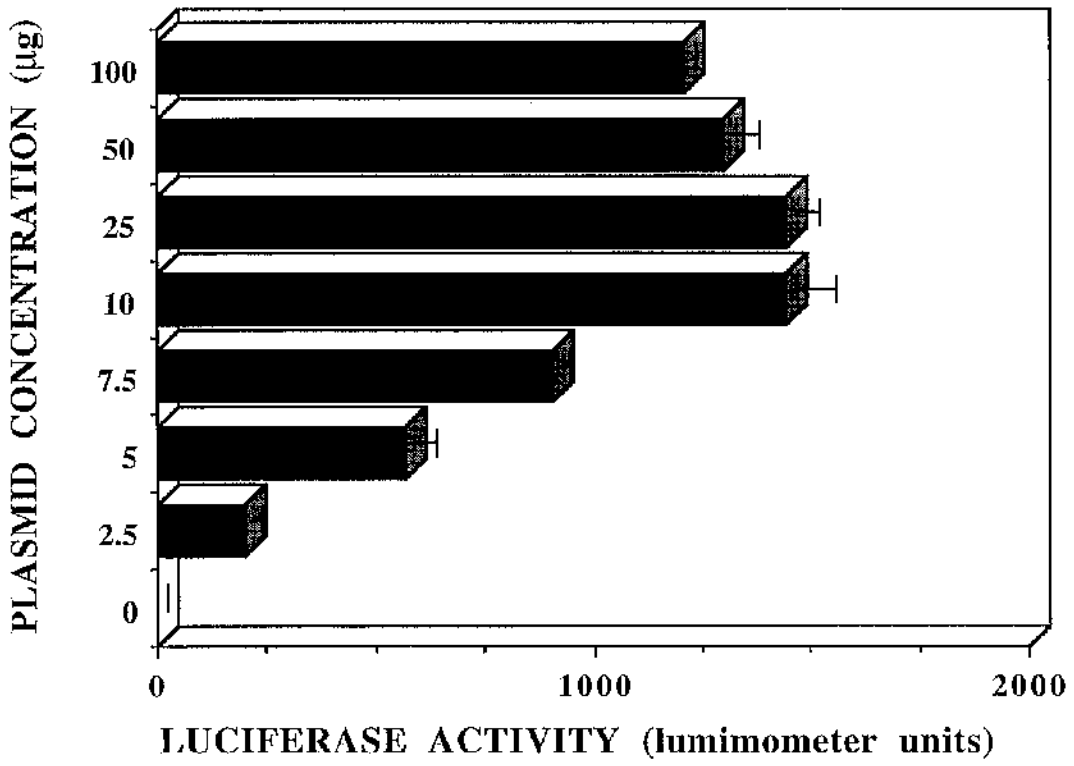


FIGURE 3.3 PLASMID CONCENTRATION EFFECT

Concentration effect on luciferase expression of pGL3-SV40 (Promega). PC12 cells were transfected with different concentrations of the pGL3-SV40 and 48 hours after transfection the cells were collected for luciferase assay (Promega). Each experiment represents the absolute luciferase activity of transfected cells compared to corresponding untransfected cells. Values are mean ± SEM from 3 or more transfections experiments, each performed in duplicate with plasmids from at least two different preparations.

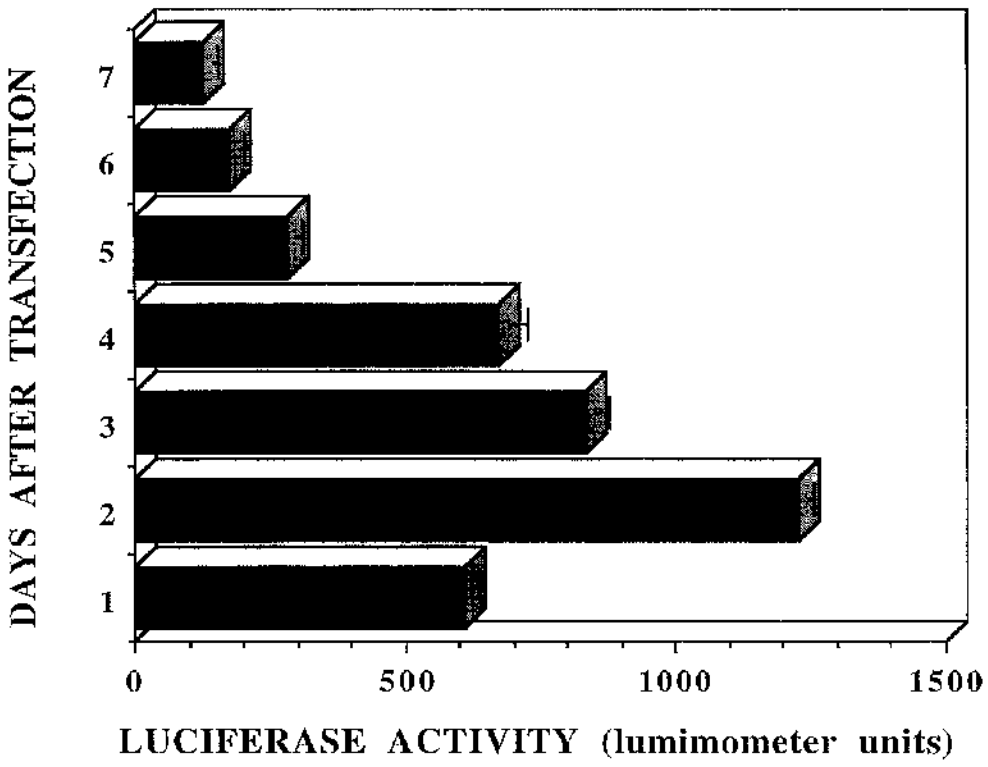


FIGURE 3.4 TIME COURSE EFFECT

Time course effect on luciferase expression of pGL3-SV40 (Promega). PC12 cells were transfected with pGL3-SV40 (10 μg) and then collected at the indicate times for luciferase assay (Promega). Each experiment represents the absolute luciferase activity of transfected cells compared to corresponding untransfected cells. Values are mean ± SEM from 3 or more transfections experiments, each performed in duplicate with plasmids from at least two different preparations.

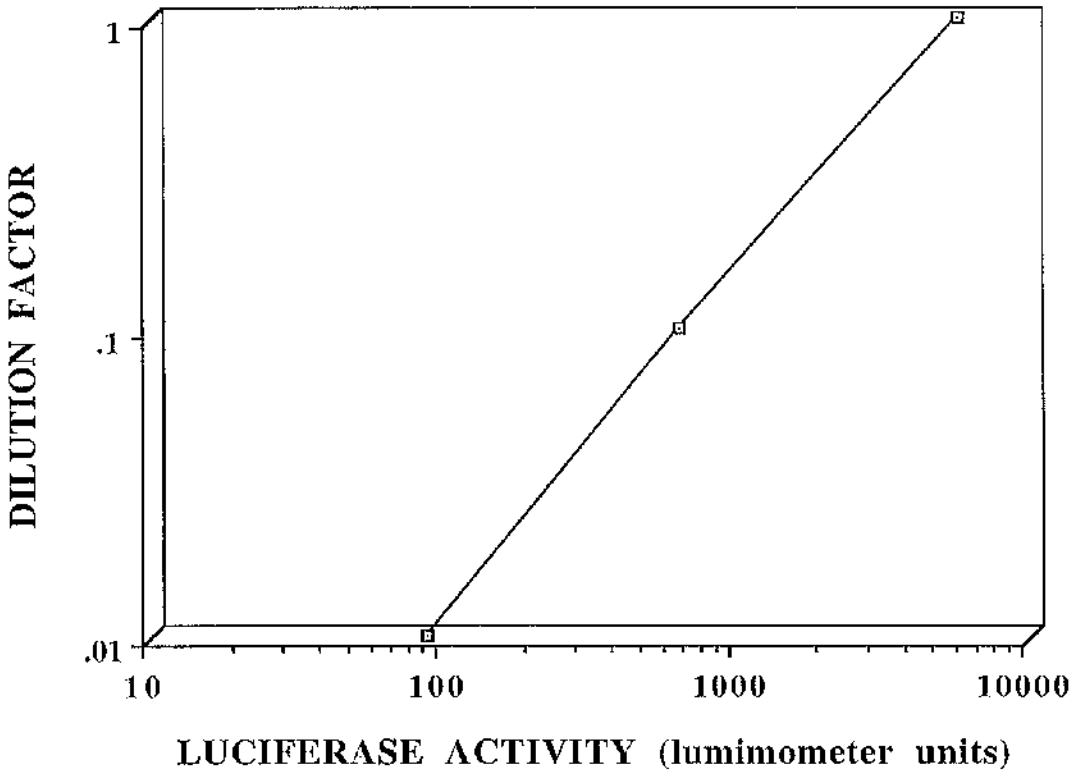


FIGURE 3.5 LINEAR RESPONSE LUCIFERASE ASSAY

Linear response of luciferase assay from cell extracts of COS-7 cells transfected with pGL3-SV40 (1 μg) and collected 48 hours after transfection for luciferase assay (Promega). Each experiment represents the absolute luciferase activity of transfected cells compared to corresponding untransfected cells. Values are mean from 3 or more transfections experiments, each performed in duplicate with plasmids from at least two different preparations.

3.2.1.5 REPRODUCIBILITY OF THE ASSAY

Different aliquots of the same PC12 cell extracts were assayed for luciferase activity under identical conditions. Similar levels of luciferase were obtained with the different aliquots (data not shown).These results demonstrated that the luciferase reporter assay is highly reproducible.

3.2.1.6 THERMAL STABILITY OF THE LUCIFERASE ENZYME

A cell extract was frozen and thawed several times and the luciferase activity was measured. Similar levels of luciferase were obtained with the different assays (data not shown). These results demonstrated that luciferase is a stable enzyme.

3.2.2 DESIGN AND CONSTRUCTION OF THE pY1-LUC FUSION GENE

The transcriptional activity of the rat NPY-Y1 receptor gene was measured using a fusion gene reporter system, the luciferase assay (Promega) in which a fragment of approximately 760 bp of the 5'-flanking region of this gene was synthesized by polymerase chain reaction (PCR) from a rat hypothalamic clone (pRHT35) obtained from Professor Dan Larhammar, Uppsala University, Department of Medical Genetics (Figure 3-6). This clone was selected in a screening of a commercial rat hypothalamic library and has on the 5'-side of the open reading frame of the NPY-Y1 gene approximately 600 bp of the promoter region of the gene.

3.2.2.1 OLIGONUCLEOTIDES FOR AMPLIFICATION OF THE PROMOTER

The oligonucleotides 2023-2024 (Appendix II) were used to amplify by PCR the rat NPY-Y1 promoter from a rat hypothalamic clone (Figure 3-7A). The 5'-region of the reverse primer (2023) was modified to include a *Bam* HI site to be used for subcloning this promoter in the pGL3-Basic reporter vector (Promega). This primer anneals to the 5'-UT region (at the exon II) of the rat NPY-Y1 gene (-36 to -57 from the first ATG). Thus, this PCR fragment excludes the first ATG codon (methionine) but includes the putative cap site and the potential start transcription sites of the rat NPY-Y1 receptor gene. The forward primer (2024) was also modifed at the 5'-region to include a *Bam* HI site. This primer bind at the 5'-flanking promoter region of the NPY-Y1 gene (-795 to -774 from the first ATG). Thus, these primers (2023-2024) were used to amplify by PCR 600 bp of the rat NPY-Y1 promoter region (Figure 3.7B). PCR fragment (760 bp) (Figure 3-7B) of the promoter of the NPY-Y1 gene generated using the primers (2023-2024) was cloned in the *Bam* HI site of the pBluescribe SK$^+$ vector (Stratagene) and then subcloned in the *Bgl* II site of the pGL3-Basic vector (Promega) in order to study the factors involved in the transcriptional control of the NPY-Y1 gene.

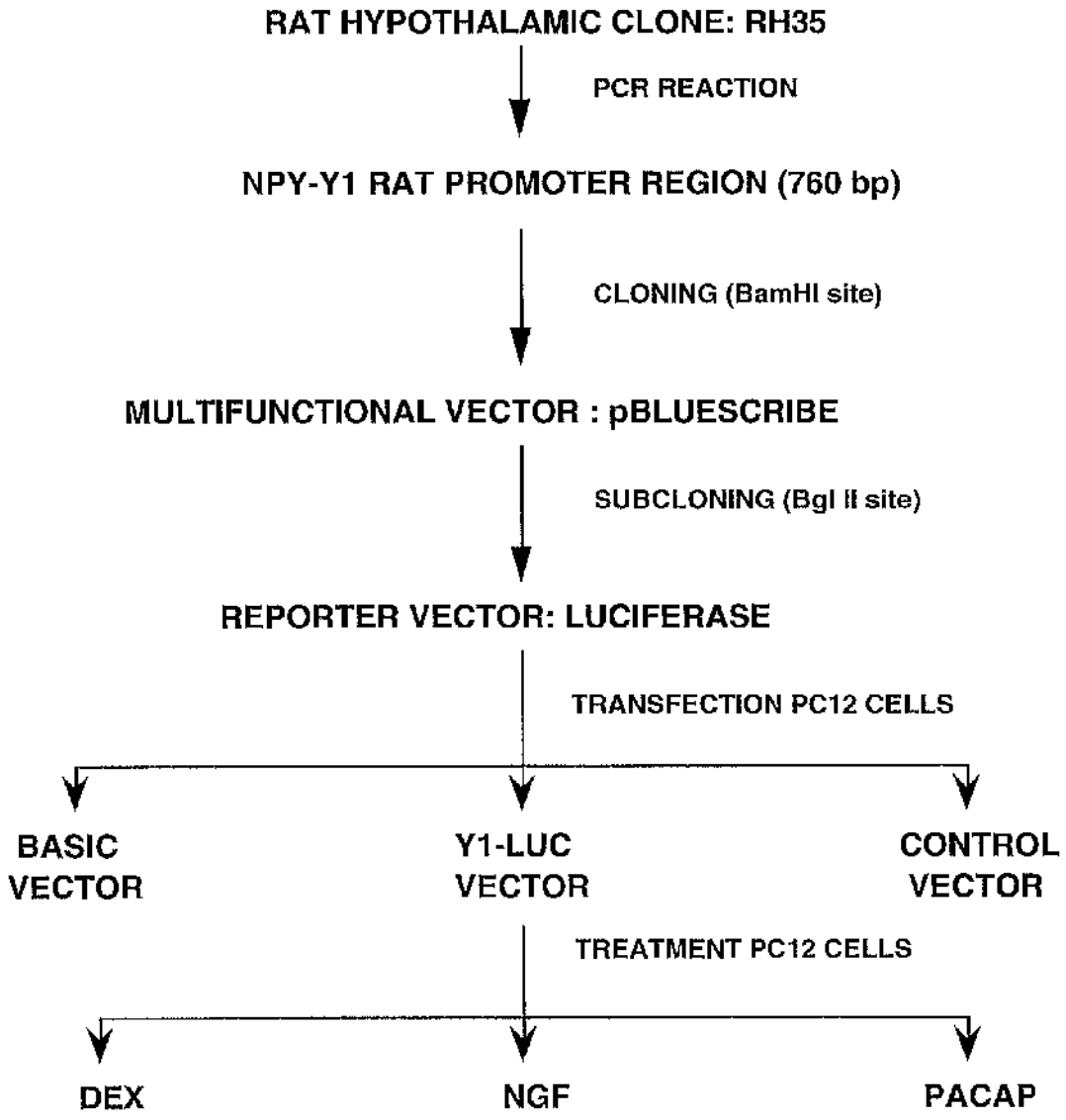


FIGURE 3.6 STRATEGY FOR CLONING THE PROMOTER REGION OF THE NPY-Y1 RECEPTOR GENE

Methodological strategy used for cloning the 5'-flanking region of the rat NPY Y1 receptor gene into the reporter vector pGL3-Basic (Promega)

3.2.2.2 SUBCLONING OF THE PROMOTER OF THE NPY-Y1 GENE

The promoter of the NPY-Y1 gene was amplified by PCR using rat hypothalamic DNA clone (Figure 3-7A). A PCR fragment of the predicted molecular weight, approximately 760 bp (Figure 3-7B) was generated using both primers (2023-2024). PCR product containing approximately 600 bp of the NPY-Y1 promoter was extracted with phenol/chloroform and the DNA precipitated by addition of 2 volumes of ethanol and overnight incubation at -20°C. This PCR fragment was digested with *Bam* HI (only present at the 5'-region of the primers) and extracted again with phenol/chloroform and ethanol precipitated. In parallel, the pBluescribe SK+ (Stratagene) was also digested with *Bam HI* and the enzyme was then inactivated by heating the reaction for 15 minutes at 65°C. The free ends of the vector were dephosphorylated with shrimp alkaline phosphatase (SAP) and the enzyme was inactivated by heating for 15 minutes at 65°C.

PCR product (760 bp) was ligated in a bidirectional manner into the *Bam* HI site of the polylinker region of the pBluescribe SK+ (Stratagene) (Figure 3.7 C). 10 µl of each ligation reaction were transformed into competent JM109 or TG1 bacteria. Positive clones were used to miniprep extractions and the plasmids were cut with *Hind* III-*Pst* I to determine the orientation of the cloning (Figure 3.7 D). One positive clone (pBS-Y1 gene) containing the expected fragment was used to large scale plasmid purification using the Wizard Maxiprep kit (Promega). This fusion gene (pBS-Y1gene) was used to subcloning the promoter region of the NPY-Y1 receptor gene into the *Bgl II* restriction site of the polylinker region of the reporter vector pGL3-Basic (Promega). This clone (pBS-Y1gene) was also used to delete one of the CRE sites of this promoter by restriction enzyme digestion (See Chapter 6. Mutagenesis studies of the promoter region of the rat NPY-Y1 receptor gene).

Following, the pBS-Y1 gene was digested with *Bam* HI, the DNA separated by size in a agarose gel and the insert was purified from the gel using the USBioclean kit (US Biochemicals). In parallel, the pGL3-Basic vector (Promega) was digested with *Bgl* II and the enzyme was inactivated by heating for 15 minutes at 65°C. The free ends of the vector were dephosphorylated by treatment with SAP and the enzyme was also inactivated by heating for 15 minutes at 65°C. The insert (760 bp) containing the promoter of the NPY-Y1 gene was ligated into the *Bgl* II site of pGL3-Basic (Promega). Positive clones were cut with different restriction enzymes to determined the orientation of the insert (Figure 3-7 F). One clone (pY1-LUC) with the insert in the correct orientation was used to transfect and develop the luciferase reporter assay in PC12 cells (Figure 3.7 F).

FIGURE 3.7 SUBCLONING OF THE PROMOTER OF NPY-Y1 RECEPTOR GENE

A. The 5'-flanking region of the NPY-Y1 receptor gene was amplified by PCR from a rat hypothalamic clone (**pRH35**).

B. Agarose gel showing a expected 760 bp fragment of the NPY-Y1 receptor gene.

Lane 1: MWM (λ x *Hind* III-*Eco* RI). **Lane 2**: NPY-Y1 promoter (760 bp).

C. PCR product was cloned into *Bam* HI site of the pBluescribe-SK$^+$ (**pBS-Y1gene**).

D. Agarose gel showing the orientation of NPY-Y1 promoter cloning.

Lane 1: MWM (λ x *Hind* III-*Eco* RI) **Lane 2**: pBS-SK$^+$ x *Hind* III-*Pst* I.

Lane 3: pBS-Y1gene x *Hind* III-*Pst* I. **Lane 4**: pBS-Y1gene x *Hind* III-*Pst* I.

E. PCR product was subcloned into the *Bgl* II site of the pGL3-Basic (**pY1-LUC**).

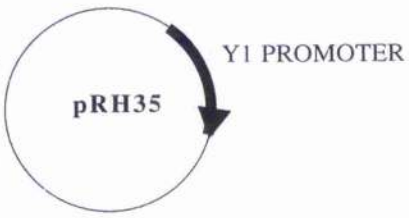
F. Agarose gel showing the restriction map of NPY-Y1 promoter.

Lane 1: MWM (λ x *Hind* III-*Eco* RI). **Lane 2**: pY1-LUC uncut

Lane 3: pY1-LUC x *Hind* III-*Kpn* I. **Lane 4**: pY1-LUC x *Hind* III-*Mlu* II.

Lane 5: pY1-LUC x *Hind* III- *Pst* I **Lane 6:** pY1-LUC x *Hind* III- *Pst* I

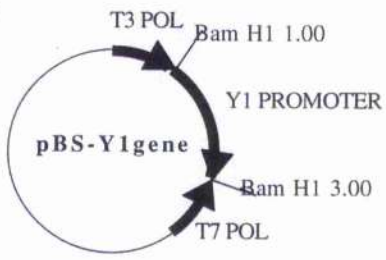
A


Y1 PROMOTER
pRH35

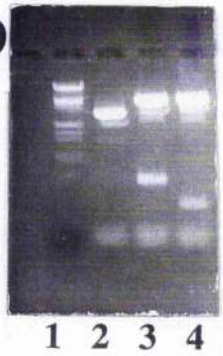
B


1 2

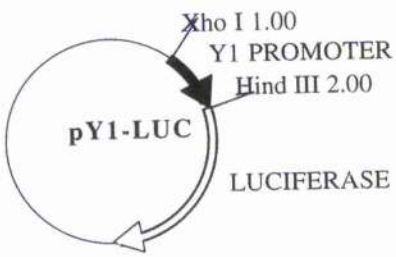
C


T3 POL
Bam H1 1.00
Y1 PROMOTER
pBS-Y1gene
Bam H1 3.00
T7 POL

D


1 2 3 4

E


Xho I 1.00
Y1 PROMOTER
Hind III 2.00
pY1-LUC
LUCIFERASE

F


1 2 3 4 5 6

3.2.3 BASAL LEVELS OF LUCIFERASE ACTIVITY OF THE pY1-LUC IN PC12 CELLS

To determine whether the NPY-Y1 receptor genomic fragment upstream to the open reading frame contains promoter sequences, transfection experiments were completed in PC12 cells. Expression levels of luciferase were compared for this construct (pY1-LUC) in unstimulated PC12 cells to the levels achieved after transfection of either the promoterless plasmid, pGL3-Basic, or to the maximal levels of luciferase obtained after transfection of the plasmid pGL3-Control, in which the luciferase transcription is driven by the SV40 promoter/enhancer.

Very low levels of luciferase activity were observed in PC12 cells transfected with the pGL3-Basic indicating that the vector itself contained no sequences that could activate gene expression (Figure 3-8). Maximal activity was achieved with a control plasmid, pGL3-Control (Figure 3-8). Transfection of the fusion gene Y1-LUC, in which the luciferase was placed under the control of the promoter region of the rat NPY-Y1 gene, resulted in slightly higher levels of luciferase activity compared to the promoterless plasmid but were considerably less than the maximal activity obtained with the control vector (Figure 3-8). These results indicate a constitutive level of NPY-Y1 transcription in PC12 cells in keeping with the observation that these cells express NPY-Y1 receptors on their cell surface (Di Maggio et al., 1994). Use of pGL3-Control indicates that PC12 cells are capable of produce high levels of luciferase and that maximal levels are well above of the levels measured for the NPY-Y1 promoter. The luciferase assay is therefore able to report factors that increase or decrease transcriptional activity in PC12 cells.

Moreover, this promoter region is active in other cells lines such as GT1-7, an mouse hypothalamic cell line (Mellon et al., 1990) and RINm5f, an rat pancreatic cell line (Polak et al., 1990) which also express this neuropeptide receptor. In these cell lines the pY1-LUC also results in slightly higher levels of luciferase activity compared to the promoterless vector (pGL3-Basic) but considerable less that the activity obtained with the control vector (pGL3-Control), indicating that the luciferase is a good reporter system to study mechanisms of transcriptional control of this gene in this cells. This results open the possibility of more detailed studies in two different systems: a hypothalamic neurosecretory neuron (GT1-7) which control reproductive function by producing GnRH and a β-pancreatic secretory cell (RINm5f) which control energy homeostasis by producing insulin.

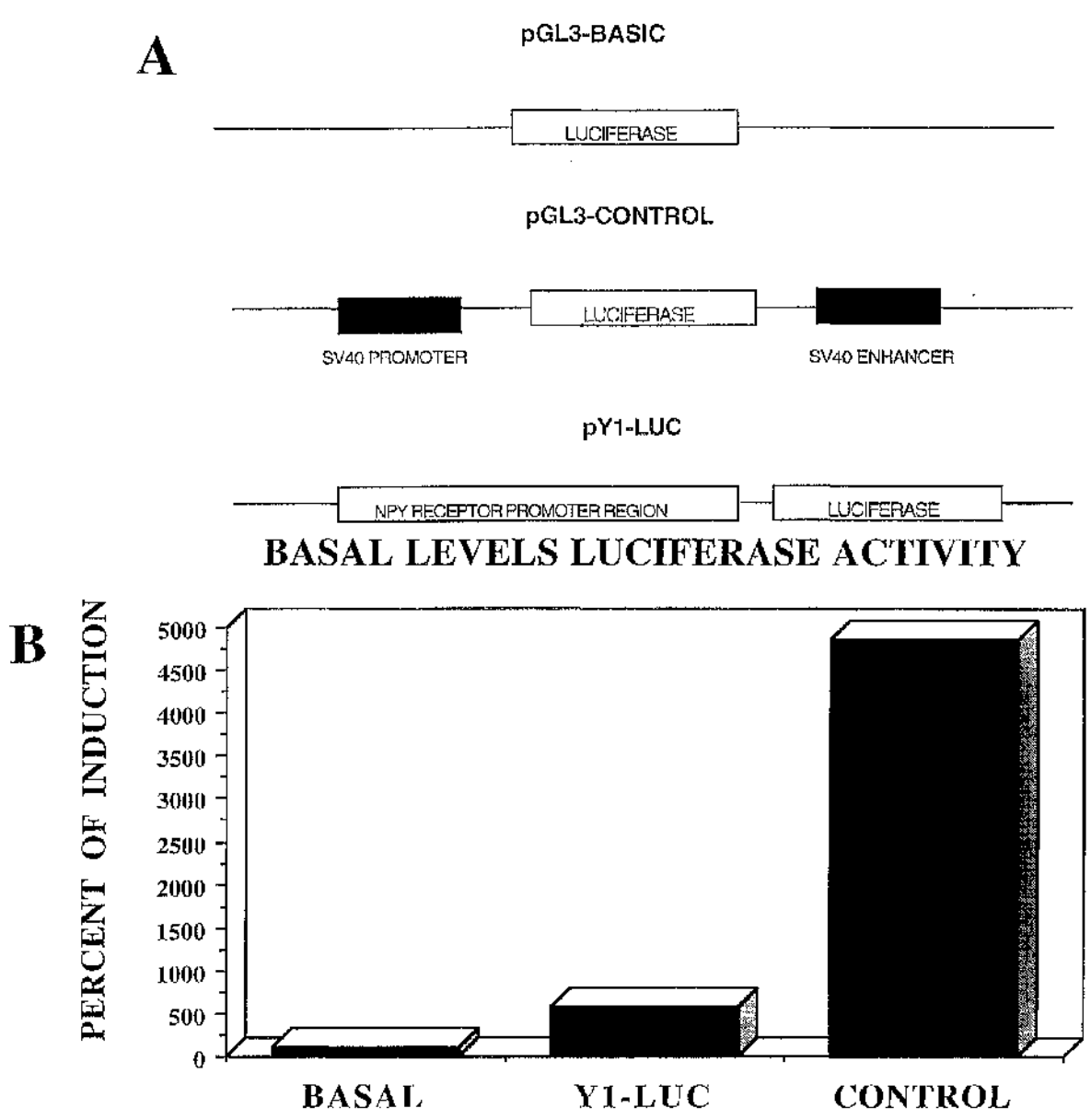


FIGURE 3.8 BASAL LEVELS OF LUCIFERASE ACTIVITY IN PC12 CELLS

A. Diagramatic structure of promoterless reporter vector (pGL3-Basic), NPY-Y1 promoter-luciferase fusion gene (pY1-LUC) and SV40 reporter vector (pGL3-Control).

B. Basal luciferase activity levels of transfected PC12 cells with **BASAL** (pGL3-Basic, 10 µg), **Y1-LUC** (pY1-LUC, 10 µg) and **CONTROL** (pGL3-Control, 10 µg). The cells were collected 48 hours after transfection for luciferase assay (Promega). Each experiment represents the absolute luciferase activity of transfected cells compared to corresponding untransfected cells. Values are mean ± SEM from 3 or more transfections experiments, each performed in duplicate with plasmids from at least two different preparations.

Experiments in an african green monkey kidney cell line, COS-7 cells, that does not constitutively express NPY-Y1 receptors showed that the expression of the luciferase gene driven for the rat NPY-Y1 promoter was similar that the obtained using the pGL3-Basic (data not shown). However, these cells were able to make active luciferase enzyme as the pGL3-Control was functional (data not shown). Taken together, this data suggests a restricted tissue-specific expression of this neuropeptide receptor consistent with the known distribution.

3.2.4 TRANSCRIPTIONAL ACTIVATION OF THE RAT NPY-Y1 GENE IN PC12 CELLS

Once the calcium phosphate transfection was adjusted to provide reproducible and detectable luciferase activity in PC12 cells, subsequent experiments were designed to study factors affecting the NPY-Y1 promoter activity. In all these experiments non-transformed PC12 cells showed no detectable luciferase activity and the pGL3-Basic promoterless vector was unresponsive to the different treatments (data not shown). All the experiments were made in duplicate and the results are the mean ± standard deviation of at least three independent experiments. Untreated PC12 cells were grown in the presence of vehicle (up to 0.1%) in DMEM complete medium.

The transcription of the NPY-Y1 gene was increased by various pharmacological agents in PC12 cells (Figure 3-9):

(i) Dexamethasone (1 μM), a synthetic glucocorticoid, increased luciferase activity 2.79 ± 0.21 fold over basal levels.

(ii) Nerve Growth Factor (NGF) (100 ng/ml), a neurotrophic factor, increased luciferase activity 2.76 ± 0.18 fold over basal levels.

(iii) Pituitary Adenylyl Cyclase Activating Polypeptide (PACAP) (5 nM), a polypeptide, increased luciferase activity 3.01 ± 0.18 fold over basal levels.

(iv) Forskolin (30 μM), an activator of adenylyl cyclase, increased luciferase activity 3.48 ± 0.14 fold over basal levels.

(v) Dibutyryl cyclic AMP (DBC) (1 mM), an analogue of cAMP, increased luciferase activity 2.83 ± 0.25 fold over basal levels.

(vi) Phorbol-12-miristate-13 acetate (PMA) (1 nM), an phorbol ester, increased luciferase activity 1.81 ± 0.16 fold over basal levels.

(vii) DH1 (1 nM), an synthetic activator of PKC, increased luciferase activity 3.20 ± 0.2fold over basal levels.

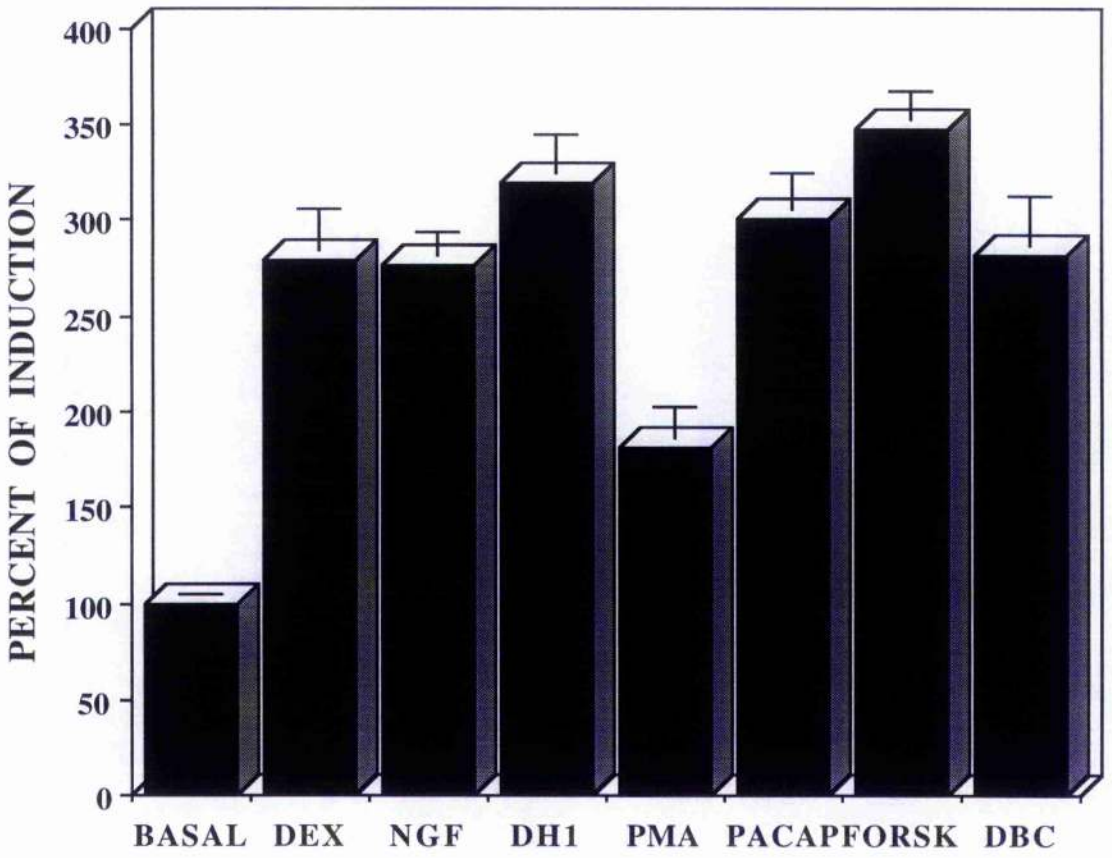


FIGURE 3.9 TRANSCRIPTIONAL ACTIVATION OF THE NPY-Y1 RECEPTOR GENE

Effect of different agents in the transcriptional activity of the NPY-Y1 receptor gene. PC12 cells were transfected with pY1-LUC (10 μg). 20 hours after transfection the cells were treated with **DEX** (dexamethasone, 1 μM), **NGF** (nerve growth factor, 100 ng/ml), **PACAP** (pituitary activating adenylyl cyclase polypeptide, 5 nM), **FORSK** (forskolin, 10 μM), **DBC** (dibutyryl cyclic AMP, 1 mM), **DH1** (1 nM) or **PMA** (phorbol-12-myristate-13-acetate, 1 nM) for 48 hours and then the cells were collected for luciferase assay (Promega). Each experiment represents the percent of luciferase activity in treated cells compared with that the percent (100%) in the corresponding control cells (0.1% vehicle only). Values are mean ± SEM from 3 or more independent transfections experiments, each performed in duplicate with plasmids from at least two different preparations.

Other pharmacological agents, such as the steroids 17-β estradiol (1 μM) and progesterone (1μM), and a calcium ionophore, ionomycin (10 μM) did not increase the luciferase activity over basal levels in PC12 cells (data not shown). However, in preliminary results in GT1-7 cells, the transcription of this neuropeptide receptor is induced under steroid, 17-β estradiol (1μM) and progesterone (1 μM) treatment (data not shown).

NPY-Y1 receptor gene was unresponsive to NGF (100 ng/ml) and PACAP (5 nM) activation in COS-7 cells, a monkey kidney SV40-transformed cell line (data not shown). Taken together, these results showed that the mechanisms which control the transcription of this neuropeptide receptor gene are cell- or tissue-dependent consistent with the known distribution of the this neuropeptide receptor gene in rat (Eva et al., 1990).

Additionally, when reporter functions are used to study gene regulation, it is important to confirm that differences in the levels of reporter activity reflect differences in transcriptional activity of the gene and are not a result of influences on post-transcriptional effects, such as mRNA stability. Previous experiments demonstrated that pGL3-Control, had high levels of luciferase activity in PC12 cells (Figure 3-8) and this viral promoter should be unresponsive to exogenous agents in these cells.

pGL3-Control was used indirectly to analyse for effects on mRNA stability because assay changes in luciferase activity can only result from post-transcriptional effects. Results showed that pGL3-Control driven luciferase activity was unresponsive to the different pharmacological agents used to increase pY1-LUC-driven luciferase activity in PC12 cells (data not shown). This indicates that the differences observed in the luciferase activity using this different pharmacological agents were primarily due to differences in the transcriptional activity of the NPY-Y1 receptor gene.

3.2.5 DUAL LUCIFERASE™ REPORTER ASSAY SYSTEM

Dual-Luciferase™ Reporter (DLR) Assay System (Promega) combines the speed and sensitivity of two luciferase reporter enzymes into an integrated, single-tube, dual-reporter assay format. DLR assay system provides sequential quantification of both firefly (*Photinus pyralis*) and sea pansy (*Renilla reniformis*) luciferase in cell lysates. Quantitating the luminescent signal from each of the two luciferase reporter enzymes may be performed immediately following lysate preparation, without the need for dividing samples or performing additional treatments.

pRL-CMV vector contains the cytomegalovirus (CMV) immediate early enhancer/promoter region, which provides constitutive expression of *Renilla* luciferase in PC12 cells (Figure 3-10). pRL-SV40 vector contains the SV40 promoter/ enhancer, and also provides with a strong expression of *Renilla* luciferase in PC12 cells (Figure 3-10). To help ensure independent genetic expression between experimental and control reporter genes, it is necessary to perform preliminary co-transfection experiments to optimise both the amount of vector DNA and the ratio of co-reporter vectors added to the transfection mix.

The extreme sensitivity of both firefly and *Renilla* luciferase assays, and the large linear range of luminiscence, allows accurate measurement of even vastly different experimental and control luminescence values. Therefore, it is possible to add relatively small quantities of a pRL vector to provide low-level, constitutive expression of *Renilla* luciferase activity. Ratios of 10 : 1 for experimental vector (pY1-LUC) (10 μg): control vector (pRL-CMV or pRL-SV40) (1 μg) result in measurable and no saturable levels of both luciferase (firefly and sea pansy) in PC12 cells (Figure 3-10). These results shown that the *Renilla* luciferase is co-expressed from both vectors, pRL-CMV and pRL-SV40 with the firefly luciferase from pY1-LUC and both pRL reporter vectors can be used as internal control for determining transfection efficiencies in PC12 cells.

PC12 cells transfected with pRL-CMV (1 μg) were treated with different activator compounds for 48 hours in a DMEM complete medium. Dexamethasone (1 μM) and NGF (100 ng/ml) increased the *Renilla* luciferase activity over basal levels, 2.4 and 2.9 fold respectively (Figure 3-11) indicating that this reporter vector is not suitable to be used as an internal control in PC12 cells treated with these agents. Additionally, PC12 cells treated with PACAP (5 nM) and Forskolin (10 μM) also increase the *Renilla* luciferase activity in comparison to basal levels, 1.6 and 2.3, respectivily (Figure 3-11), and therefore the pRL-CMV cannot be used as internal control in PC12 cells treated with these compounds.

PC12 cells transfected with pRL-SV40 (1 μg) were treated with different agents for 48 hours in a DMEM complete medium. Dexamethasone (1μM) and NGF (100 ng/ml) increased the *Renilla* luciferase activity, 3.7 and 2.1 fold over basal levels, respectivily (Figure 3-12). However, PC12 cells treated with PACAP (5 nM) and Forskolin (10 μM) did not increase the *Renilla* luciferase activity in comparison to the basal levels in PC12 cells (Figure 3-12) and for this reason, the pRL-SV40 can be used as internal control in PC12 cells treated with PACAP and other PKA activators, such as forskolin (See Chapter 5).

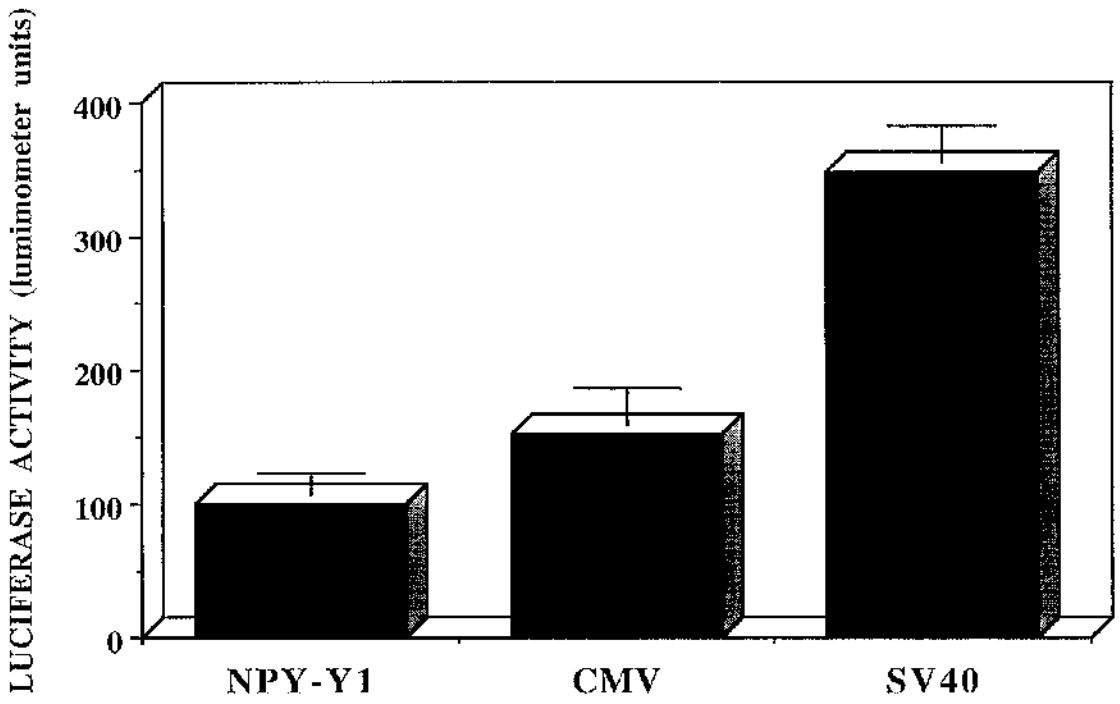


FIGURE 3.10 CO-EXPRESSION OF FIREFLY AND *RENILLA* LUCIFERASE IN PC12 CELLS

Co-expression effect of firefly luciferase directed by the NPY-Y1 promoter (pY1-LUC) and *Renilla* luciferase directed by the cytomegalovirus (CMV) promoter (pRL-CMV, Promega) or the simian virus 40 (SV40) promoter (pRL-SV40, Promega). PC12 cells were co-transfected with **NPY-Y1** (pY1-LUC, 10 μg) and **CMV** (pRL-CMV, 1 μg) or **SV40** (pRL-SV40, 1 μg) and 48 hours transfection the cells were collected for Dual-luciferase assay (Promega). Each experiment represents the luciferase activity of transfected cells compared to corresponding untransfected cells. Values are mean ± SEM from 3 or more transfections experiments, each performed in duplicate with plasmids from at least two different preparations.

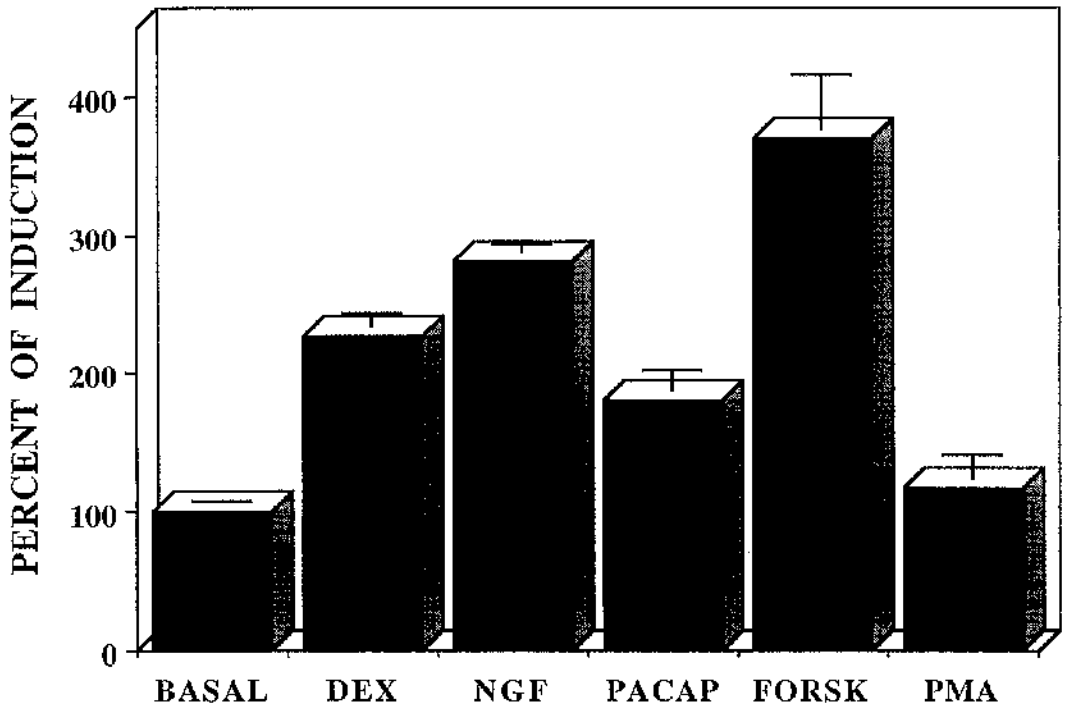


FIGURE 3.11 TRANSCRIPTIONAL ACTIVATION OF THE CMV-DIRECTED *RENILLA* LUCIFERASE IN PC12 CELLS

Effect of different agents in the transcriptional activation of the *Renilla* luciferase gene directed by the CMV immediate early enhancer/promoter. PC12 cells were transfected with pRL-CMV (1 μg). 20 hours after transfection the cells were treated with **DEX** (dexamethasone, 1 μg), **NGF** (nerve growth factor, 100 ng/ml), **PACAP** (pituitary activating adenylyl cyclase polypeptide, 5 nM), **FORSK** (Forskolin, 10 μM) or **PMA** (phorbol-12-myristate-13-acetate, 1 nM) for 48 hours when the cells were collected for Dual-luciferase assay (Promega). Each experiment represents the percent of luciferase activity in treated cells normalized to 100% for the control cells (0.1% vehicle only). Values are mean ± SEM from 3 or more transfections experiments, each performed in duplicate with plasmids from at least two different preparations.

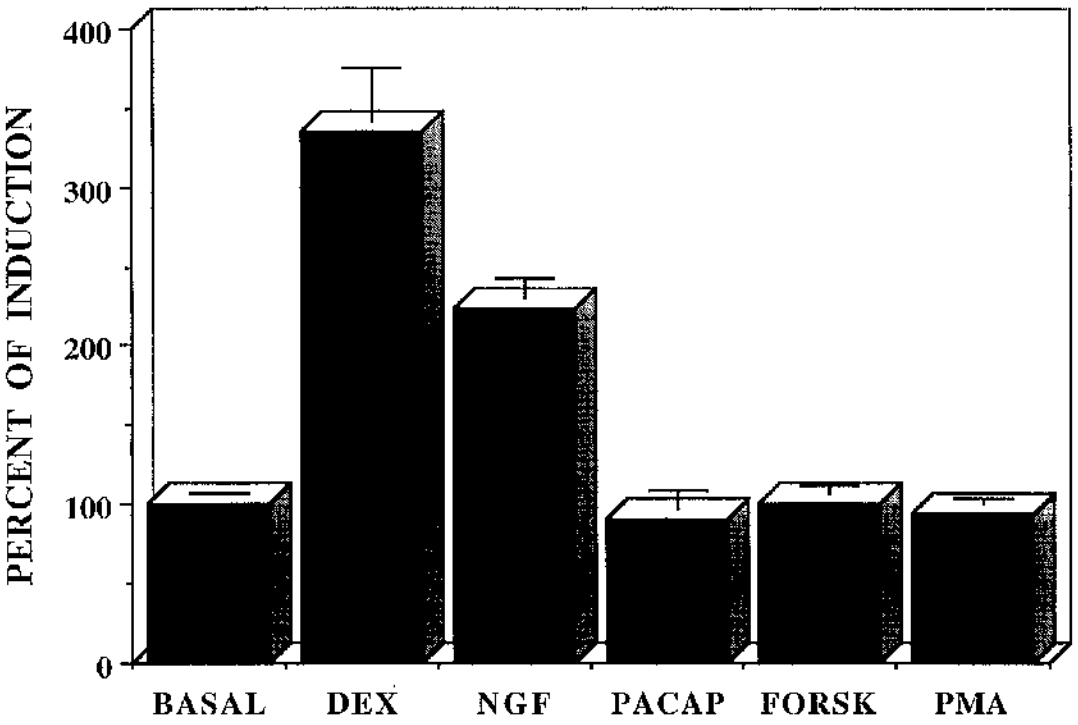


FIGURE 3.12 TRANSCRIPTIONAL ACTIVATION OF THE SV40-DIRECTED *RENILLA* LUCIFERASE IN PC12 CELLS

Effect of different agents on the transcriptional activation of the *Renilla* luciferase gene directed by the SV40 early enhancer/promoter. PC12 cells were transfected with pRL-CMV (1 μg). 20 hours after transfection the cells were treated with **DEX** (dexamethasone, 1 μg), **NGF** (nerve growth factor, 100 ng/ml), **PACAP** (pituitary activating adenylyl cyclase polipeptide, 5 nM), **FORSK** (Forskolin, 10 μM) or **PMA** (phorbol-12-myristate-13-acetate, 1 nM) for 48 hours when the cells were collected for Dual-luciferase assay (Promega). Each experiment represents the percent of luciferase activity in treated cells normalized to 100% for the control cells (0.1% vehicle only). Values are mean ± SEM from 3 or more transfections experiments, each performed in duplicate with plasmids from at least two different preparations.

3.2.6 TRANSCRIPTION INITIATION SITE OF THE NPY-Y1 GENE

3.2.6.1 PRIMER EXTENSION ANALYSIS

In order to obtain information about the location of the transcription initiation site in the promoter region of the NPY-Y1 receptor gene present in the pY1-LUC fusion gene, primer extension analysis were carried out using an 25-mer antisense oligonucleotide (T1886) (See Appendix II) corresponding to the nucleotides 107 to 83 to the ORF of the luciferase gene (Figure 3-13).

In this reaction, 10 pmol of antisense oligonucleotide (T1886) (Appendix II) was end-labelled using the AMV reverse transcriptase primer extension system kit (Promega) following the manufacturer instructions (See Chapter 2. Primer extension assay). The primer extension product of the luciferase mRNA was expected to be approximately 300 bases in length (taking as a start transcription site the putative cap site of the rat NPY-Y1 promoter) (Figure 3-12). As a positive control 10 pmol of control primer was used which anneals to 25 fmol of an *in vitro* transcribed product (1.2 kb kanamycin positive control cRNA) supplied with the primer extension system kit (Promega). Primer extension product of the control RNA is 87 bases in length. As a negative control RNase-free water replaced the control RNA was used. As DNA markers a end-labelled ϕX174 *Hinf* I DNA supplied with the primer extension system kit was used.

Initially, the primer extension reaction was carried out using 10 μg of total RNA from PC12 cells transfected with 10 μg of pY1-LUC fusion gene. The aliquots were taken after 4 hours, 12 hours and 24 hours of transfection. The transfection efficiency was monitored following the luciferase activity of an aliquot of the transfected PC12 cells (data not shown). In addition, the integrity of the total RNA was determined using a RNA-formaldehyde denaturing gel (data not shown). As seen in the figure 3-14A, the PAGE-gel of the primer extension products reveal a band of 87 bases in length corresponding to a positive control product, however the expected band of the luciferase mRNA did not appear. Further experiments, increasing the amounts of total RNA (up to 50 μg) and/or reducing the stringency of the hybridization conditions (from 58°C to 35°C) did not reveal the primer extension product for the luciferase mRNA (data not shown). For this reason was used a more sensitive approach to study the transcription initiation site of the NPY-Y1 gene : RT-PCR amplification.

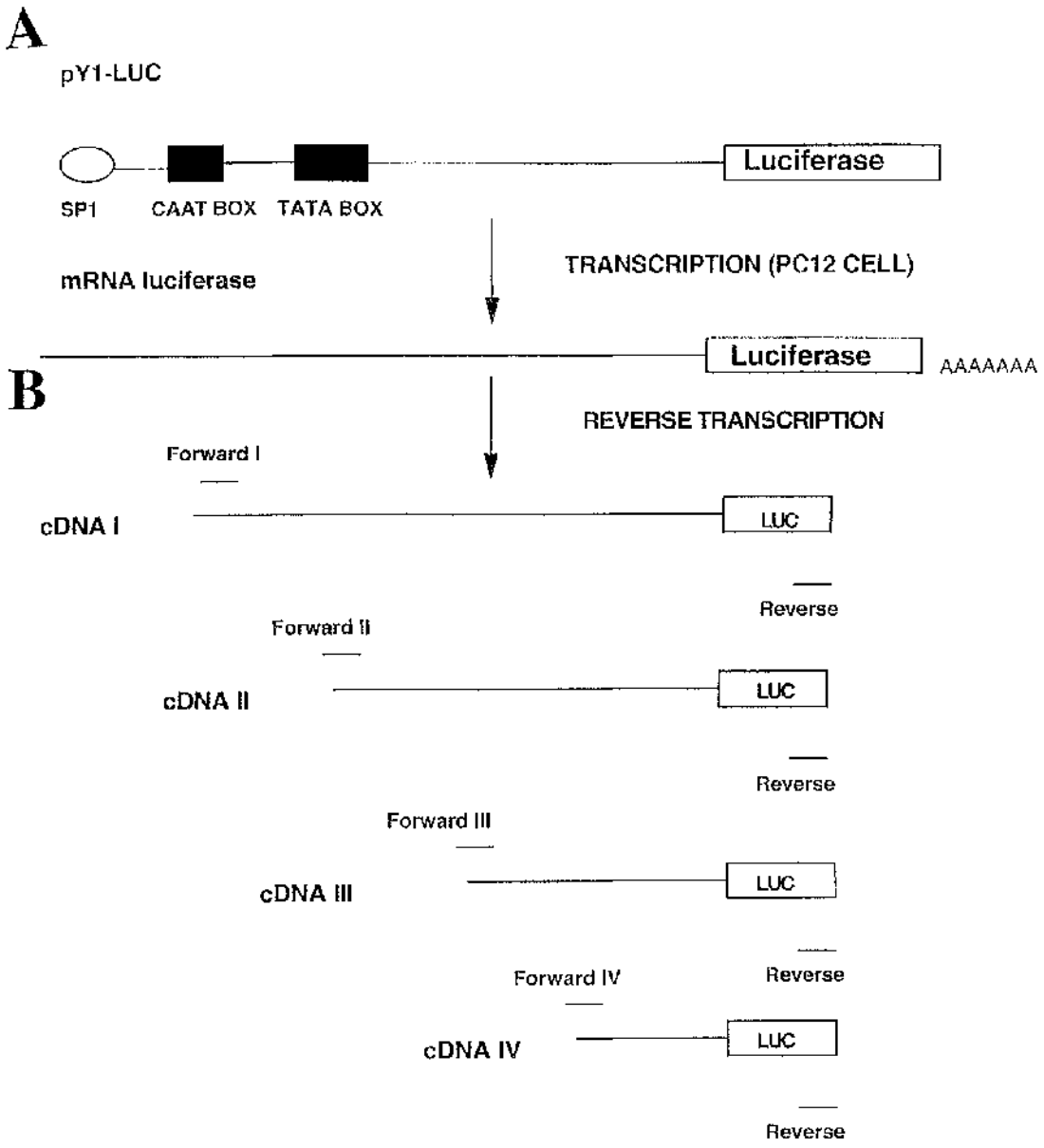


FIGURE 3.13 TRANSCRIPTION INITIATION SITE OF THE NPY-Y1 RECEPTOR GENE IN PC12 CELLS

A. Diagramatic structure of the region closer to the putative transcription start site of the promoter of the NPY-Y1 receptor gene (pY1-LUC). Putative binding sites for transcription factors and annealing sites of the primers are presented.

B. Schematic size of expected PCR products are showed. **TATA** (TATA-like box), **CAAT** (CAAT-like box) and **Sp1** (Sp1 responsive element). **+1** (putative start transcription site).

3.2.6.2 RT-PCR ANALYSIS

To determine the approximate location of the transcription start site in the NPY-Y1 receptor gene present in the pY1-LUC fusion gene, RT-PCR was used to amplify the 5'-end of the luciferase transcript. First strand cDNAs were synthesized using 1 µg of total RNA from PC12 cells transfected for 1 day with pY1-LUC (10 µg) (See Chapter 2. Cell transfection). Total RNA was incubated for 15 minutes at 37 °C with RQ1 RNase-free DNAse (1u/µg plasmid) to remove the DNA template before the RT reaction, extracted twice with acid phenol/chloroform and ethanol precipitated. The transfection efficiency was monitored by measuring the luciferase activity in an aliquot of the transfected PC12 cells (data not shown). In addition, the integrity of the total RNA extracted from PC12 cells was determined using a RNA-formaldehyde denaturing gel (data not shown).

For the RT reaction a 15-mer antisense oligonucleotide (Y7464) was used (Appendix II). This primer anneals to residues 97 to 83 of the open reading frame of the luciferase gene present in the pY1-LUC (Figure 3-13B). Amplifications were performed using primers corresponding to upstream sequences (see below), and the products were analyzed by agarose gel electrophoresis (Figure 3-14B). As a negative control RT-PCR reactions were performed using total RNA extracted from PC12 cells and RT-PCR reactions in which the reverse transcriptase enzyme (MMLV) was omited (to discard genomic or plasmid contamination). These RT-PCR reactions showed no detectable products (data not shown), indicating that the RT-PCR products obtained were derived from the luciferase mRNA specifically transcribed for the pY1-LUC fusion gene.

PCR products of the expected sizes were amplified using the following primers (Appendix II):

(i) Y7465 corresponding to residues -35 to -20 (342 bp PCR product)
(ii) Y7466 corresponding to residues -19 to -5 (327 bp PCR product)
(iii) Y7467 corresponding to residues +2 to +17 (300 bp PCR product)

These results suggests that the transcription of the NPY-Y1 receptor gene begins upstream to the putative transcription start site (cap site). However more precise location of the transcription start site requires the design of upstream forward primers and further RT-PCR experiments.

FIGURE 3.14A PRIMER EXTENSION ASSAY OF THE NPY-Y1 PROMOTER

Primer extension assay assay of luciferase mRNA under the control of NPY-Y1 promoter (pY1-LUC). Total RNA (10 μg) of PC12 cells was extracted after 4 hours, 12 hours and 24 hours of transfection. As a negative control RNAse free water was used and kanamycin cRNA (primer extension assay kit, Promega) was used as a positive control. PAGE gel. Run 250 Volts x 2 hours. Autoradiograph exposure time: overnight without intensifying screen (70°C).

Lane 1: MWM (ϕX174 x *Hinf* I)

Lane 2: RNAse free water (negative control)

Lane 3: cRNA kanamycin (positive control)

Lane 4: mRNA luciferase (PC12, 4 hours)

Lane 5: mRNA luciferase (PC12, 12 hours)

Lane 6: mRNA luciferase (PC12, 24 hours)

FIGURE 3.14B RT-PCR AMPLIFICATION OF THE NPY-Y1 PROMOTER

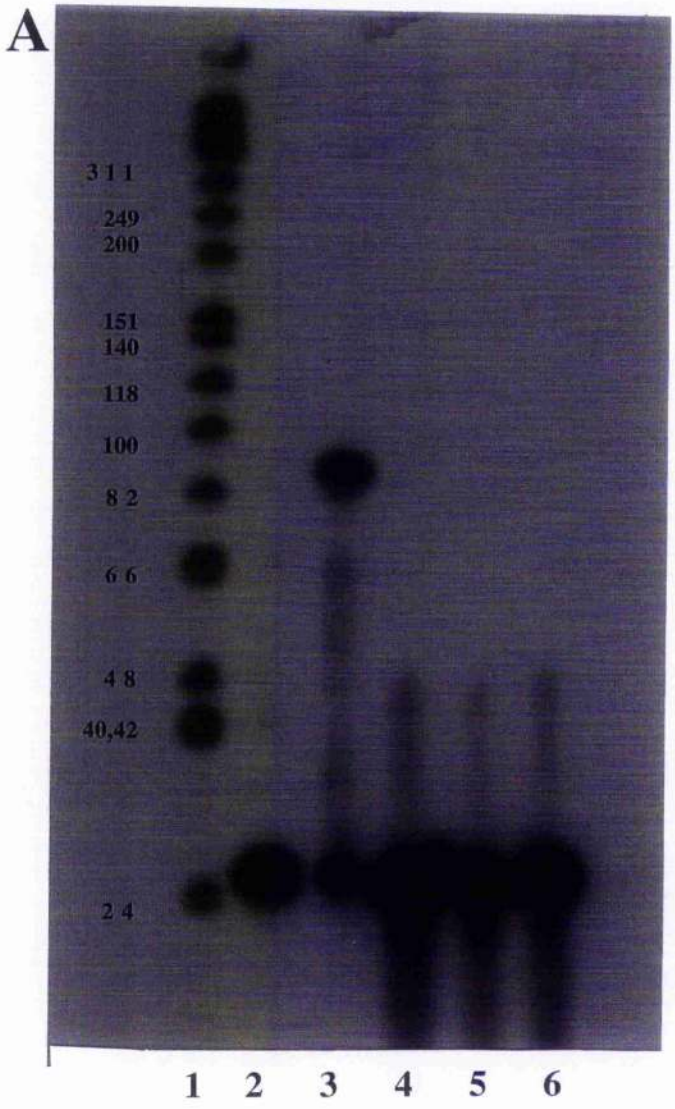
Agarose gel electrophoresis of RT-PCR products of luciferase mRNA under the control of the NPY-Y1 promoter (pY1-LUC). PC12 cells were transfected with pY1-LUC (10 μg) and 24 hours after transfection the cells were collected for total RNA extraction. Total RNA (1 μg) was amplified by RT-PCR and products of the expected size were obtained:

Lane 1: MWM (1 Kb ladder)

Lane 2: RT-PCR product (342 bp) (primers Y7464-Y7465)

Lane 3: RT-PCR product (327 bp) (primers Y7464-Y7466)

Lane 4: RT-PCR product (300 bp) (primers Y7464-Y7467)


A
311
249
200
151
140
118
100
82
66
48
40,42
24
1 2 3 4 5 6


B
1 2 3 4

3.3 DISCUSSION

3.3.1 LUCIFERASE REPORTER ASSAY IN PC12 CELLS

The nervous system contains diverse populations of cells which interconnect in precise manners. The identity of these cells is determined by their ability to transcribe a particular set of genes. The factors participating in NPY-Y1 receptor gene expression have to satisfy unique criteria since NPY-Y1 receptor is distributed in a variety of cell populations throughout the central and peripheral nervous system (See Chapter 1, NPY receptors). These experiments represent the first characterisation of a rat NPY-Y1 gene transcriptional activity using reporter functions (luciferase enzyme) in a pheochromocytoma cell line: PC12 cells.

The use of reporter functions offers several advantages for the study of regulation of gene transcription. Once the 5'-flanking region of the gene has been cloned, it is possible to delete, substitute or alter specific sequences and study how these modifications affect the normal response of the gene. Several reporter genes have been defined; one of these is the gene for the firefly enzyme, luciferase. This gene is not present in mammalian cells, therefore, any measurable activity is derived from the transfected DNA (Wood, 1991). It is readily translated into protein in eukaryotic cells and, therefore, levels of luciferase activity relate directly with levels of gene transcription (Wood, 1991).

Several methods can be used to transfect PC12 cells. Previous results in this laboratory revealed that the $CaPO_4$ co-precipitation was the optimal method to introducing DNA into this cell line (Balbi, 1994). However, optimal transfection conditions were found empirically using the pGL3-Control vector (Promega), in which the luciferase transcription is driven by a SV40 early promoter/enhancer. Using this reporter vector it was found that 10 μg of CsCl-derived plasmid DNA (type I form), overnight (12-16 hours) incubation with the transfection mix (calcium phosphate plus DNA) and harvesting the cells two days (48 hours) after transfection resulted in maximum levels of luciferase activity. These conditions were used routinely for all subsequent transfections.

In addition it was found that the luciferase activity was: (i) linear over the range of dilution (1: 5 to 1: 20) used in the assay, (ii) highly reproducible and (iii) resistant to repeat cycles of freeze-thawing. Finally it was found that cell extracts of non-transformed PC12 cells showed no detectable luciferase activity indicating that the only source of luciferase was derived from the plasmid transfected into the PC12 cells.

Firefly luminiscence has been the most extensively studied bioluminiscence system (Wood, 1991). The light-producing reaction requires the enzyme (E) luciferase, the substrate (LH_2) luciferin, Mg^{++}, ATP and molecular oxygen (O_2). The initial reaction is the rapid conversion, in the presence of Mg^{++} and ATP, of luciferin to luciferyl adenylate, which, in the presence of luciferase, combines with molecular oxygen to give an oxyluciferyl adenylate-enzyme complex in the excited state :

$$LH_2 \text{ (luciferin)} + E \text{ (enzyme)} + ATP \xrightarrow{Mg^{++}} E : LH_2 : AMP + PP$$

After light emission, the ground-state complex dissociates to form enzyme, AMP, oxyluciferin, and carbon dioxide (CO_2), the last being derived from the carboxyl group of luciferin :

$$E : LH_2 : AMP + O_2 \longrightarrow E + L = O \text{ (oxyluciferin)} + CO_2 + AMP + light \quad (hn \text{ max., } 562 \text{ nm})$$

The reaction catalysed by firefly luciferase [*Photynus pyralis* luciferin: oxygen 4-oxydoreductase (decarboxilating, ATP-hydrolysing) EC 1.13.12.7] is the mono-oxidative decarboxylation of beetle D-luciferin, utilising ATP and O_2 as co-substrates with concomitant production of a photon of green light. The oxidation occurs from an enzyme intermediate, luciferyl AMP. However, coenzyme A (CoA) is also substrate of luciferase, and in the presence of CoA, oxidation occurs from luciferyl-CoA with more favourable total kinetics, as it produces constant light intensities which gradually decreases after 60 seconds with greater sensitivity. The basis of the extended "glow" reaction of the enhanced firefly luciferase assay is that energy, provided by splitting of ATP, is released from the reaction in the form of light, which can be measured using a luminometer or standard liquid scintillation counters. Because light output is linearly proportional to the amount of luciferase, luminiscence correlates directly to the expression of the luciferase reporter gene in transfected cells or cell lysates.

Luciferase is a monomeric protein of 60.7 kDa that does not require post-translational processing for enzymatic activity (Wood, 1991). Thus, it can function as a genetic reporter immediately upon translation. Light production by luciferase enzyme has the highest quantum efficiency known of any bioluminiscent reaction and allows for greater enzymatic turnover of luciferase, resulting in greater light intensity that is nearly constant for measurements of up to several minutes (Wood, 1991).

Luciferase allow 100-1000 times greater sensitivity that is expected from the CAT assay. However, compared to CAT, the firefly luciferase protein has a shorter half-life in transfected mammalian cells, making the luciferase reporter suited for transient assays and short-lived effects. An added advantage is that the luciferase assay results can be obtained in minutes compared to hours, or even days, for the radioactive CAT assay. In addition, the linear range of the firefly luciferase assay extends over 8 orders of magnitude of firefly concentration (Wood, 1991).

The luciferase assay is a good reporter system for PC12 cells. Very low levels of luciferase activity was achieved with the promoterless plasmid (pGL3-Basic) indicating that this vector contained no sequences that could activate gene expression in PC12 cells. Maximum levels was achieved with the control vector (pGL3-Control) indicating that PC12 cells were capable of producing high levels of active luciferase enzyme. Finally, slightly higher levels of luciferase activity was achieved with the fusion gene pY1-LUC (in which the luciferase was placed under the control of the promoter region of the rat NPY-Y1 gene) compared to the promoterless vector but considerably less that the activity obtained with the control vector. This is in keeping with previous results that indicate that these cells have low constitutive levels of NPY-Y1 gene expression (DiMaggio et al., 1994).

3.3.2 RAT NPY-Y1 RECEPTOR PROMOTER REGION

Previous to the initiation of this project, a construct (plasmid RHT 35) containing the promoter region of the NPY-Y1 gene was cloned from a rat hypothalamic library (see above). This construct contains 1.3 Kb of the rat NPY-Y1 gene, including approximately 600 bp of the promoter and 700 bp of the transcriptional region of the gene, including 230 bp of the 5'-UT region (approximately 80 bp of the exon I and 150 bp of the exon II), and including 470 bp of the ORF. This clone has also correctly spliced away intron 1 (Prof. Larhammar, personal communication) (Figure 3-15).

The promoter region of the rat NPY-Y1 receptor gene contains several consensus sequences that constitute putative binding sites for different transcription factors (Figure 3.1), including :
(i) an AT-rich "TATA-like" box (AAATA) located at -20 bp
(ii) a putative "CAAT" box (CAATCT) located at -45 bp,
(iii) a GC rich Sp1 site (GGGGCG) located at -73 bp.
iv) one AP-1 site (TGAGTCA) located at -237 bp.
v) two cAMP response elements (CRE) (CGTCA) located at -113 bp and -493 bp.
vi) one non-palindromic steroid responsive element (ERE) (TGACC) at -350 bp.
vii) four non-palindromic glucocorticoid response elements (GRE): three in the form of AGGACT located at -230 bp, -373 bp and -553 bp and one in the form of TGTTCT at -540 bp

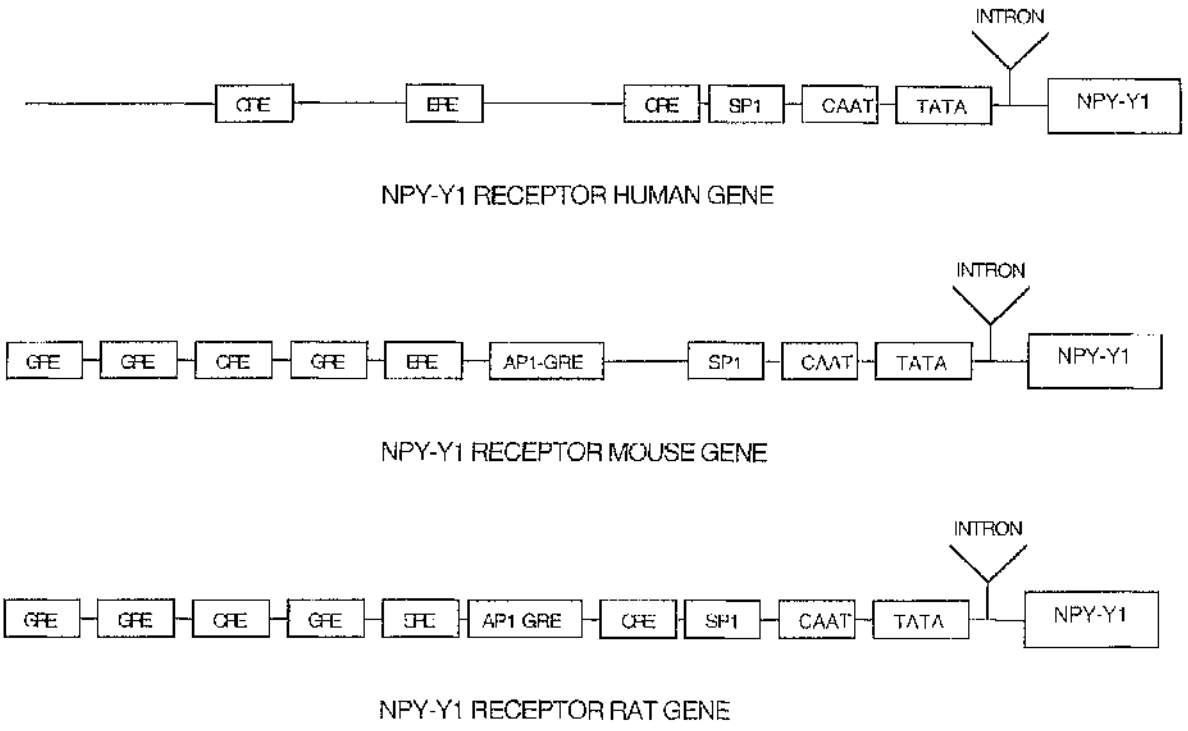


FIGURE 3.15 PUTATIVE BINDING SITES OF HUMAN, MOUSE AND RAT NPY-Y1 RECEPTOR GENES

Comparison of the putative binding sites present in the 5'-flanking promoter region of the human, mouse and rat NPY-Y1 receptor genes. **CRE** (cAMP responsive element), **GRE** (glucocorticoid responsive element), **ERE** (estrogen reponsive element), **AP1** (AP1 responsive site), **Sp1** (Sp1 responsive site), **CAAT** (CAAT-like box), **TATA** (TATA-like box), **NPY-Y1** (NPY-Y1 open reading frame).

3.3.2.1 BASAL RESPONSIVE ELEMENTS

These consensus sequences for transcription factors are located using as a reference the putative transcription cap site of the human NPY-Y1 promoter (Herzog et al.,1993). A sequence comparisons between the mouse, rat and human promoters of the NPY-Y1 gene shows a high degree of homology in the region close to the putative start site, including a highly conserved (AAATA) "TATA-like" box and the (CAATCT) "CAAT-like" box (Figure 3-15). These elements are likely to be involved in the control of the basal levels of transcriptional activity of this gene in PC12 cells. The "TATA-like" box is conserved in mouse, rat and human NPY-Y1 genes (Figure 3.15). The TATA-box is the binding site for TFIID (TATA-Binding protein), a transcription factor involved in the formation of an active complex *in vitro* capable of initiating RNA synthesis by RNA polymerases (McKnight and Yamamoto, 1992). However, preliminary results using RT-PCR (Figures 3-14B) suggest that the transcription start site in this gene is upstream of the putative cap site. Further RT-PCR studies will be necessary to obtain more precise location of the transcription start site of this gene.

The (GGGGCG) GC-box rich constitutes the putative Sp1 binding site. This DNA element is only conserved in mouse and rat NPY-Y1 gene (Figure 3.15) and constitutes another element that may contribute to the basal activity of the NPY-Y1 gene. The Sp1 binding site has been reported to be non-inducible but is an enhancer of constitutive expression of several genes, specially housekeeping genes (Kadonaga et al., 1987; Minth and Dixon, 1990).

3.3.3.2 AP-1 RESPONSIVE ELEMENT

The promoter region of the NPY-Y1 gene has a complete AP-1 site (TGAGTCA) at -273 bp of the putative transcriptional start site. This element is only conserved in mouse and rat NPY-Y1 gene (Figure 4-14). However the promoter region of the human NPY-Y1 gene has an AP-1 site at position -763 (Ball et al., 1995) which is shared with the mouse NPY-Y1 gene (Musso et al., 1997).

The AP-1 site present in this region constitutes the most likely element to mediate the NGF-PMA transcriptional activation of the NPY-Y1 receptor gene (Figure 3-9). This element constitutes a consensus binding site for the transcriptional factor AP-1, comprising dimers of the *c-fos* and *c-jun* family of proteins (Curran and Franza, 1988). Expression of these proteins is known to be activated by NGF and phorbol esters, in PC12 cells (Bartel et al., 1989). Although the mechanisms mediating control of gene expression differs from gene to gene, AP-1 mediated transcriptional control has been reported in PC12 cells (Machida et al., 1991).

3.3.3.3 CRE RESPONSIVE ELEMENT

The promoter region of the rat NPY-Y1 gene has two CRE sites (CGTCA). These sequences at position -113 (conserved in rat and human NPY-Y1 gene) and -493 (conserved in mouse, rat and human NPY-Y1 gene) were also found in the other neuropeptides gene, where it is essential for biological activity of the cAMP-regulating enhancement (Roesler et al., 1988). Diverse agents which increase the levels of cAMP in PC12 cells, including the peptide PACAP, an activator of adenylyl cyclase via GPCRs, forskolin, a direct activator of adenylyl cyclase and dibutyryl cAMP (DBC), an analog of cAMP increase the levels of luciferase activity in pY1-LUC transfected PC12 cells (Figure 3-9). Therefore, it is possible that the CRE were mediating the response of the NPY-Y1 promoter to PACAP and other activators of the PKA second messenger pathway in PC12 cells (See Chapter 5).

3.3.3.4 GRE RESPONSIVE ELEMENT

Several reverse complement GRE non-palindromic consensus sequences (AGGACT) were found at -230 bp, -373 bp and -553 bp (conserved in mouse, rat and human) and one (TGTTCT) at -540 bp (conserved only in rat) of the putative transcriptional start site (Figure 3-14). Interestingly each of the four elements in the 5'-flanking region of the NPY-Y1 gene do not comprise palindromic structures. However the close location of the GRE at -540 and -553 in the NPY-Y1 gene, may allow these two sites to act cooperativily with monomeric glucocorticoid receptors. It has been also reported that GRE without palindromic structure can form a functional complex with monomeric glucocorticoid receptor in murine mammary tumour virus (MMTV) and activate gene transcription (Chapelakis et al., 1990). The four GRE binding elements of the NPY-Y1 gene may explain the dexamethasone-inducibility of the NPY-Y1 gene transcription (Figure 3-9). Moreover, the GR has been found to repress AP-1 stimulated transcription (Lopes da Silva and Burbach, 1995). The NPY-Y1 promoter has an overlapping AP1-GRE consensus site and the interactions between these two factors may affect the NPY-Y1 gene transcription.

These DNA binding elements are the primary candidates involved in the transcriptional control of the NPY-Y1 receptor gene in PC12 cells, although it is not possible to rule out the presence of other less conspicuous but important regulatory elements. The transcriptional activity of this gene may be controlled by differential interactions of these proteins with the promoter. Further diversity could be generated by controlling the regulatory activity of these DNA binding proteins. In this way a combination of several transcription elements could result in a specific expression pattern observed for the NPY-Y1 receptor gene in the nervous system (See Chapter 1. NPY-Y1 receptors).

3.3.3 POST-TRANSCRIPTIONAL CONTROL OF THE LUCIFERASE GENE IN PC12 CELLS

It is important to confirm that the differences observed in the levels of luciferase activity using the activators compounds reflect differences in the transcriptional activity of NPY-Y1 receptor gene and are not result of post-transcriptional effects, such as mRNA stability. For this reason the pGL3-Control in which the luciferase activity is under the control of the SV40 early enhacer/promoter was used. Results showed that the levels of luciferase activity driven for the pGL3-Control (Promega) were not altered by the different activator compounds which are known to increase the luciferase activity driven for the pY1-LUC fusion gene in PC12 cells (data not shown). This result suggests that the differences in transcriptional activation by the different pharmacological compounds were due to a differences in the transcriptional activity of the NPY-Y1 receptor gene in PC12 cells.

3.3.4 DUAL-LUCIFERASE™ REPORTER ASSAY SYSTEM

A control reporter vector (pRL) and the Dual-Luciferase™ reporter assay system (Promega) was used in order to normalize for tranfection efficiency or cell lysate recovery between transfection experiments. Sources of variation in transfection efficiency include: quality of the DNA preparations, small changes in the pH of the transfection solutions or differences in the cell density or healthy, among others. Typically, pRL-CMV or pRL-SV40 were used as a control reporter gene in co-transfected experiments with the pY1-LUC vector. These control vectors were used because they are linked to a different reporter gene (*Renilla* luciferase) and were under the control of a strong constitutive viral promoters. Optimal conditions for the co-transfection of both plasmids were found empirically. This co-transfection experments indicate that the*Renilla* luciferase driven for these reporters vectors are active in PC12 cells and can be co-expressed with the firefly luciferase (Figure 3-10).

However, CMV immediate early enhancer/promoter present in the pRL-CMV vector was responsive to all the pharmacological activator compounds tested: dexamethasone (1μM), NGF (100 ng/ml), PACAP (5nM) and forskolin (10 μM) (Figure 3-11) which precluded its use as internal control. SV40 early enhancer/promoter present in the pRL-SV40 reporter vector was responsive to dexamethasone (1 μM) and NGF (100 ng/ml), but was unresponsive to PACAP (5 nM) and forskolin (10 μM) (Figure 3-12). These results suggest that this vector can be used as as internal control in PC12 cells treated with PACAP and other activators of the PKA signalling pathway, such as forskolin (See Chapter 5).

Dexamethasone and NGF-dependent activation of the *Renilla* luciferase activity driven by the pRL-SV40 vector could be a consecuence of post-transcriptional effects, such as increase of the mRNA stability of the *Renilla* luciferase enzyme in PC12 cells or due to intrinsic differences between the reporter vectors, such as non-specific response by sequences present in the pRL-SV40. Further experiments, including studies of the mRNA stability of the *Renilla* luciferase enzyme using specific inhibitors of the RNA synthesis, such a actynomicin D will be necessary to undertand this differences in the dexamethasone and NGF-dependent response of the SV40 early enhancer/promoter present in this two reporter vectors in PC12 cells. In addition, cloning the promoter region of the NPY-Y1 receptor gene in the promoterless pRL-null vector (Promega) and mesurements of the *Renilla* luciferase activity under dexamethasone (1μM) or NGF (100 ng/ml) treatment of PC12 cells may indicate the possible effect of the vector in the transcriptional activation of this gene.

3.3.5 TRANSCRIPTIONAL CONTROL OF NPY-Y1 IN OTHER CELL LINES

NPY-Y1 mRNA is highly expressed in several regions of the rat forebrain, including the hippocampus and hypotalamus (See Chapter 1. NPY receptors). 600 bp of the 5'-flanking region of the NPY-Y1 gene contains a functional promoter that is active in a neural crest-derived cell line: PC12 cells. In addition, this promoter is active in GT1-7, a mouse hypothalamic cell line (Mellon et al., 1990) and RINm5f, an rat pancreatic cell line (Polak et al., 1993) which also express this neuropeptide receptor (See Chapter 8) but not in a kidney-derived SV40-transformed cell line: COS-7 cells. These results suggest that the NPY-Y1 gene contains a cell-type specific core promoter consistent with the known distribution of the NPY-Y1 receptor gene.

Results obtained using GT1-7 and RINm5f cells open the possibility of more detailed studies of the transcriptional activation of the NPY-Y1 receptor gene in two different systems. GT1-7 cells are a model of hypothalamic GnRH neurons which control the hormonal cascade regulating mammalian reproduction (Mellon et al., 1990). Previous results have been shown that NPY is a peptide involved in the control of the reproductive function in mammals (See Chapter 1. Effects of NPY family of peptides). It will be important to know the effect of steroids, such as estrogen and progesterone in the control of the expression of this neuropeptide receptor. RINm5f cells are a model of a β-pancreatic secretory cell (RINm5f) which control energy homeostasis by producing insulin (Polak et al., 1993). NPY is involved in the insulin secretion in this cell line by binding to a NPY-Y1 receptor. It will be also important to known the effect of different agents which control the secretion of insulin in the expression of this neuropeptide receptor gene.

In preliminary results in GT1-7 cells the transcription of NPY-Y1 is induced by steroid (progesterone and estrogen) treatment. It is possible that the estrogen response element (ERE) (TGACC) located 350 nucleotides upstream to the putative transcription start site is the *cis* -element involved in this estrogen-induced response. In experiments in COS-7 cells the promoter of the NPY-Y1 gene was unresponsive to NGF and PACAP activation (data not shown). These results suggest that the mechanisms controlling the transcriptional activation of the NPY-Y1 gene are cell-type specic, and the differences are a consecuence of the expression and activation of a different subset of transcription factors.

3.4 SUMMARY

The major results from this chapter were:

1.- The promoter region of the rat NPY-Y1 receptor gene was cloned upstream to the luciferase reporter enzyme.

Approximately 600 bp of the 5'-flanking promoter region of the rat NPY-Y1 gene that lies upstream to the open reading frame has been attached to the reporter function, luciferase, such that the expression of this enzyme has been placed under the control of this promoter region .

2.- The promoter region of the rat NPY-Y1 receptor gene contain consensus sequences for transcription factors.

This gene contains in this region several consensus sequences for transcription factors : a AT-rich "TATA-like" box (AAATA) at -20 bp (using as a reference the putative transcription start site), a proposed "CAAT" box (CCATCT) at -45 bp, a GC rich Sp1 site (GGGGCG) at -73 bp, one AP-1 site (TGAGTCA) at -237 bp, two partial cyclic AMP response elements (CRE) (CGTCA) at -113 bp and -493 bp, one non-palindromic estrogen responsive element (ERE) (TGACC) at -350 bp and four non-palindromic glucocorticoid response elements (GRE): three (AGGACT) at -230 bp, -373 bp and -553 bp and one (TGTTCT) at -540 bp.

3.- Optimal conditions for transfection of the Y1-LUC were obtained empirically.

Optimal conditions for parameters which improve the transfection efficiency and can affect the levels of luciferase activity obtained in PC12 cells, such as amount and quality of DNA added to the cells , the time the cells were incubated with the DNA and conditions of the luciferase assay were found empirically and used in all the transfection experiments.

4.- The luciferase assay was a good reporter system in PC12 cells.

4.1- Very low levels of luciferase activity were obtained in PC12 cells transfected with the promoterless vector (pGL3-Basic) indicating that this plasmid contained no sequences that could activate gene expression in PC12 cells.

4.2- Maximal activity was achieved by transfecting PC12 cells with a control vector (pGL3-Control) in which the luciferase has been placed under the control of a SV40 early promoter/enhancer. Use of the control SV40-driven early promoter/enhancer indicates that PC12 cells are capable of producing high levels of active luciferase enzyme and that maximal levels of enzyme activity are well above the levels measured for the rat NPY-Y1 promoter, indicating that the luciferase assay is therefore able to report factors that increase or decrease transcriptional activity in PC12 cells.

4.3- Transfection of the fusion gene Y1-LUC (in which the luciferase was placed under the control of the 5'-flanking region of the rat NPY-Y1 receptor gene) resulted in slightly higher levels of luciferase activity compared to the promoterless plasmid but were considerably less than the maximal activity obtained with the control vector . These results indicate a constitutive level of transcription of the rat NPY-Y1 gene in PC12 cells in keeping with the observation that these cells express a few receptors on their cell surface (DiMaggio et al., 1994).

5.-Differents pharmacological agents increased the transcriptional activity of the rat NPY-Y1 promoter in PC12 cells.

PC12 cells were transformed with the fusion gene pY1-LUC were treated with different agents for 48 hours in a DMEM complete medium :

5.1- dexamethasone (1 μM), increased luciferase activity 2.79± 0.22 -fold over basal levels.

5.2- NGF (100 ng/ml), increased luciferase activity 2.76 ± 0.18-fold over basal levels,

5.3- Pituitary Adenylyl Cyclase Activating Polypeptide (PACAP) (5nM), increased luciferase activity 3.01 ± 0.18-fold over basal levels,

5.4- forskolin (10 μM), increased luciferase activity 3.48 ± 0.14-fold over basal levels,

5.5- Dibutyryl Cyclic AMP (DBC) (1 mM), increased luciferase activity 2.83 ± 0.25-fold over basal levels,

5.6- phorbol-12-miristate-13 acetate (PMA) (1 nM) increased luciferase activity 1.82 ± 0.16 -fold over basal levels, and

5.7- DH1 (1 nM), increased luciferase activity 3.20 ± 0.20-fold over basal levels

6.- The same pharmacological agents which increased the luciferase activity driven for the rat NPY-Y1 promoter in PC12 cells, did not increase the luciferase activity driven for pGL3-Basic or pGL3-Control vectors.

The levels of luciferase activity driven of the promoterless vector (pGL3-Basic) were unresponsive to the same concentrations of different pharmacological agents which increase the luciferase activity driven for the pY1-LUC fusion gene, indicating that the vector itself no contains sequences that will drive a specific response for these different activator compounds. Additionally, the SV40-driven levels of luciferase activity of the control vector (pGL3-Control) were also unresponsive to the same agents, indicating that the increases observed in the luciferase activity for the pY1-LUC in PC12 cells treated with this agents is a consecuence of the transcriptional activation of the NPY-Y1 gene and not a result of influences on post-transcriptional effects, such as mRNA stability.

7.- Rat NPY-Y1 receptor promoter drive the expression of the luciferase in GT1-7 cells and was responsive to steroids (estrogen and progesterone) (preliminary results).

In preliminary results in GT1-7 cells, a mouse hypothalamic neurosecretory cell line, the transcription of the NPY-Y1 receptor gene is induced by steroid (estrogen and progesterone) treatment.

8.- Rat NPY-Y1 receptor promoter did not drive the expression of the luciferase in COS-7 cells and was unresponsive to NGF and PACAP activation.

In COS-7 cells the expression of the luciferase driven for the rat NPY-Y1 promoter was similar to the basal levels of luciferase activity obtained using the promoterless vector (pGL3-Basic). However, these cells were able to make active luciferase enzyme as the control vector (pGL3-Control) results in high levels of luciferase enzyme. This data suggests a restricted cell- or tissue-specificity of the rat NPY-Y1 receptor gene consistent with the known NPY-Y1 receptor gene expression. In addition, in COS-7 cells the rat NPY-Y1 receptor gene was unresponsive to the NGF and PACAP transcriptional activation observed in PC12 cells. This results indicated that the NGF and PACAP response is a specific response of neural crest-derived cells, such as PC12 cells.

9.- pRL-CMV vector was not a good internal control in PC12 cells treated with the different pharmacological agents which increase the luciferase activity driven by the pY1-LUC fusion gene in PC12 cells.

pRL-CMV vector contains the cytomegalovirus (CMV) immediate early enhancer/promoter region, which provides constitutive expression of *Renilla* luciferase in PC12 cells and can be co-expressed with the firefly luciferase enzyme. This promoter was responsive to dexamethasone (1 μM) and NGF (100 ng/ml), increasing the levels of *Renilla* luciferase activity, indicating that this reporter vector is not suitable to be used as internal control in PC12 cells treated with these agents. In addition, PC12 cells treated with PACAP (5 nM) and Forskolin (10 μM) also increase the *Renilla* luciferase activity over basal levels, and therefore pRL-CMV cannot be used as internal control in PC12 cells treated with these agents which increase intracellular cAMP.

10.- pRL-SV40 vector was not a good internal control in PC12 cells treated with dexamethasone and NGF but was a good internal control in PC12 cells treated with PACAP and forskolin.

The pRL-SV40 vector contains the SV40 early promoter/enhancer, which provides constitutive expression of the*Renilla* luciferase in PC12 cells and can be co-expressed with the firefly luciferase enzyme. This promoter was responsive to dexamethasone (1μM) and NGF (100 ng/ml), indicating that this reporter vector is not suitable to be used as internal control in PC12 cells treated with these agents. However, PC12 cells treated with PACAP (5 nM) and Forskolin (10 μM) did not increase the *Renilla* luciferase activity in comparison to the basal levels in PC12 cells.Thus, pRL-SV40 can be used as internal control in PC12 cells treated with PACAP and other PKA activators, such as forskolin.

CHAPTER 4

NGF-INDUCED EFFECTS IN THE TRANSCRIPTIONAL ACTIVATION OF THE NPY-Y1 RECEPTOR GENE IN PC12 CELLS

4.1 INTRODUCTION

4.1.1 PROTEIN PHOSPHORYLATION

Phosphorylation is the major language used within the cells to communicate information from the environment. Covalent modification by phosphorylation serves to alter the structure and function of proteins involved in cellular structure, metabolism, cell growth, among many other functions. Thus, it is not surprising that 30% of eukaryotic proteins are estimated to be phosphoproteins. It is estimated that there are over a thousand kinases, so that over 1% of the genes would encode kinases. These kinases differ in their regulation, their substrate-specificity, and their cellular localization (Walaas and Greengard, 1991).

In the nervous system in particular, reversible phosphorylation is involved in processes as diverse as biosynthesis of neurotransmitter, activity of ion channels and cell differentiation. Protein phosphorylation is also central to the process involved in regulation of gene transcription. Many transcription factors are activated by phosphorylation thus increasing or decreasing transcription of specific genes (Hunter and Karim, 1992).

With the myriad of kinase families and isozymes within kinases families, it has been difficult to dissect the contribution of specific kinases in specific signalling pathways. To this end, recent advances using antisense DNA, expression of dominant negative mutant kinases or the use of cell-permeable activators or inhibitors has allowed much headway in understanding how different signals pathways are transduced.

4.1.2 NGF SIGNALLING PATHWAY

When NGF binds to its receptor on the cell membrane of target cells, it triggers several signal transduction mechanisms. Two different receptors have been proposed for NGF. A high affinity receptor comprising the *trk A* protooncogene ($p140^{trk}$) is a member of the tyrosine kinase family of transmembrane receptors (Martin-Zanca et al., 1991). This high-affinity receptor mediates most of the known biological effects of NGF. The second receptor is a cell surface glycoprotein of 75-80 kDa with a single transmembrane domain ($p75^{NTR}$). This low-affinity receptor lacks structural features in its cytoplasmic domain known to mediate signal transduction in other receptors (Berg et al., 1991).

Activation of *trk A* receptor is followed by receptor dimerization and trans-autophosphorylation of five tyrosine residues on *trk A* (Loeb et al., 1994). These phosphotyrosine residues act as docking sites for downstream effector substrates by specifically binding and activation of

intracellular proteins : PLC (PLCγ1), Shc, PI-3-K and MAPK-1 (Kaplan and Stephens, 1994). The binding to *trkA* molecules initiates a cascade of signalling events that leads to neuronal differentiation (Figure 4-1). These initial tyrosine phosphorylation events triggered by NGF are followed by activation of several protein serine/ threonine kinases that include members of the PKC, *raf* , MEK, MAPK and ribosomal S6 kinase families (Cantley et al., 1991) (See Chapter 1. NGF signalling pathway).

NGF was the first neurotrophic agent to be identified and now serves as the prototype for this class of polypeptide growth factors (Thoenen and Barde, 1980). NGF is required for the maintenance of specific neuronal crest-derived cell types which include sympathetic postganglionic neurons. Responses of explanted sympathetic neurons to this target-derived agent includes general anabolic alterations, induction of neuronal morphology and increased survival of the cells. Morphologically, the cells initiate neurite outgrowth and increased cell body size (Greene and Tiscler, 1992). Thus, classic markers of sympathetic neuronal differentiation which include specific morphological alterations as well as various anabolic responses are initiated by NGF.

4.1.3 PC12 CELL LINE

The clonal cell line, PC12 was established from an rat adrenal medullary tumour (phaeochromocytoma), which was passaged subcutaneously in New England Deaconess Hospital Sprage-Dowley rats (Warren and Chute, 1972; DeLellis et al., 1973). This cell line can be induced to undergo differentiation to a neuronal phenotype upon treatment with NGF (Tischler and Greene, 1975). PC12 cells have become a important model system to study the mechanisms involved in mediating NGF effects on target cells in areas such as neuronal development and nerve injury. It is thought that the effects of NGF on PC12 cells are representative of the events that occur during sympathetic neuronal differentiation, particularly for cells derived from the neural crest (Tischler and Greene, 1978).

In resting state, PC12 cells undergo mitosis and display ultrastructural and biochemical characteristics of immature adrenal medullary chromaffin cells which constitute their non-neoplasic counterpart, including cathecholamine synthesis, storage and release from both large, dense core granules and small synaptic-like vesicles (Greene and Rein, 1977a). In the presence of nanomolar concentrations of NGF, PC12 cells differentiate rapidly but reversibly into a phenotype which resembles mature postganglionic sympathetic neurons (Greene and Tischler, 1976). PC12 cells flatten down, decrease their proliferative rate (cease mitosis), undergo cell hypertrophy, increased cell-substratum adhesiveness and extend long, branching neurites with prominent growth cones (Guroff, 1985) (See Chapter 1. PC12 cells).

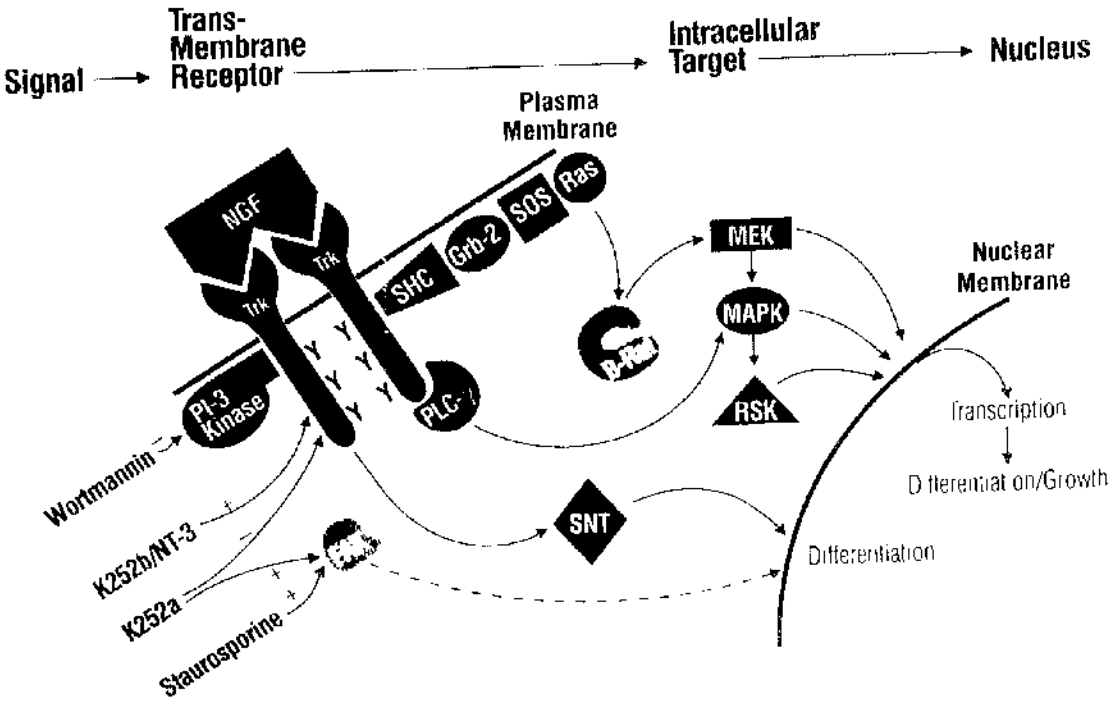


FIGURE 4.1 NGF SIGNALLING PATHWAY

Schematic NGF signal transduction pathways in PC12 cells (Taken from Signal Transduction of Neurotrophin Receptors. Neural Notes. Volume 1. Issue 1. Promega). Abbreviations: tyrosine residues (**Y**); stimulation signal (+); inhibitory signal (-); putative signalling (**dashed line**).

4.1.4 NEUROPEPTIDE Y-Y1 RECEPTOR

Neuropeptide Y (NPY) is a 36 amino acid polypeptide that is found widely distributed throughout the peripheral and central nervous system. NPY is found in high concentrations in a number of peripheral neurons, including those of the sympathetic nervous system (postganglionic neurons), particularly supplying cardiac structures and vascular tissue beds (Allen et al., 1986a). NPY is also found in a subpopulation of adrenal medullary chromaffin cells (Varndell et al., 1984).

The actions of NPY at a number of peripheral neuroeffector junctions have been particularly well studied (Whalestedt et al., 1986, 1990). NPY produces both pre- and postsynaptic effects. In virtually all the instances, the presynaptic effects of NPY are manifest as inhibition of neurotransmitter release. For example, NPY is a powerful inhibitor of noradrenaline (NA) release from sympathetic neurons. Postsynaptically, NPY is a well characterized vasoconstrictor and is able to enhance the effects of other vasopressor agents, such as NA. It is generally accepted that the postsynaptic actions of NPY are mediated through NPY-Y1 receptors, whereas the presynaptic actions are mediated through NPY-Y2 receptors (Wahlestedt et al., 1986, 1990).

High concentrations of NPY are found in PC12 cells (Allen et al., 1984). Undifferentiated PC12 cells contain NPY mRNA which is further translated to the prohormone and processed to the mature peptide. This NPY mRNA content in PC12 cells is increased markedly by NGF (Allen et al., 1987b, Balbi and Allen, 1994). Undifferentiated PC12 cells, also express NPY receptors of the Y1 subtype (Schwartz et al., 1987).

PC12 cells differentiated to a sympathetic-like neuronal phenotype by NGF are also responsive to the NPY-Y1 receptor-selective agonist [Pro^{34}]-NPY with inhibition of evoked cAMP and enhanced evoked catecholamine overflow (DiMaggio et al., 1994). However, PC12 cells treated with NGF are also responsive to NPY-Y2 receptor-selective agonist NPY_{13-36} with inhibited accumulation on evoked cAMP and inhibition catecolamine overflow (DiMaggio et al., 1994). Moreover, PC12 cells differentiated to a mature adrenal chromaffin-like cells with dexamethasone are responsive to [Pro^{34}]-NPY with inhibition of evoked cAMP and enhanced agonist evoked catecholamine overflow, but dexamethasone-differentiated PC12 cells were unresponsive to NPY_{13-36} (DiMaggio et al., 1994) Thus, in this model, sympathetic-like neurones express both NPY-Y1 and NPY-Y2 receptors. However, mature adrenal chromaffin-like cells express only functional NPY-Y1 receptors but not NPY-Y2 receptors.

Model systems are required in order to simplify the study of the factors and molecular mechanisms that affect the expression of NPY receptor genes. In the case of adrenal chromaffin cells and post-ganglionic sympathetic neurones, PC12 cells may be an adequate model to study the regulation of the NPY-Y1 receptor gene expression by NGF and other growth factors.

This chapter deals primarily with the characterisation of the molecular mechanisms involved in the transcriptional activation of the NPY-Y1 receptor gene in PC12 cells following NGF treatment. The luciferase reporter system was used to study the role of the cAMP dependent protein kinase (PKA), Ca^{++}/ phospholipid-dependent protein kinase (PKC), MAP kinase (MAPK) and PI-3 kinase (PI-3-K) signalling pathways in the NGF-mediated response in PC12 cells.

4.2 RESULTS

Due to variability between different PC12 cell sublines, and in order to obtain consistent and reproducible results, all the experiments were performed using PC12 cells from the same source. It is also well known that these cells can mutate and lose some or all their characteristics. In order to minimise these events, initial stocks of PC12 cells were replicated by a maximum of 10 passages, working cells were discarded and new vials of frozen cells thawed.

4.2.1 NGF CONCENTRATION EFFECTS IN THE TRANSCRIPTIONAL ACTIVATION OF THE NPY-Y1 GENE

In the previous chapter, PC12 cells were shown to respond to NGF by differentiating into a phenotype that resembles postganglionic sympathetic neurones. Cells transfected with the pY1-LUC fusion gene and treated with NGF (100 ng/ml) demonstrated a consistent 2.76 ± 0.18-fold increase in the measurable luciferase activity compared to the basal levels (Figure 3-9). This activation of the NPY-Y1 receptor gene in PC12 cells was also observed at low concentrations of NGF (Figure 4-2). Thus, the threshold concentration of the NGF induction of NPY-Y1 receptor gene was 10 ng/ml (Figure 4-2). Increasing concentrations above this threshold resulted in significant increases in the levels of the luciferase activity (Figure 4-2). These results show that the effect of NGF on the transcription of the NPY-Y1 gene in PC12 cells is concentration dependent. A value of 100 ng/ml of NGF was chosen for all subsequent experiments as it lies in the linear region of the concentration response, thus any variation of this response could easily detected.

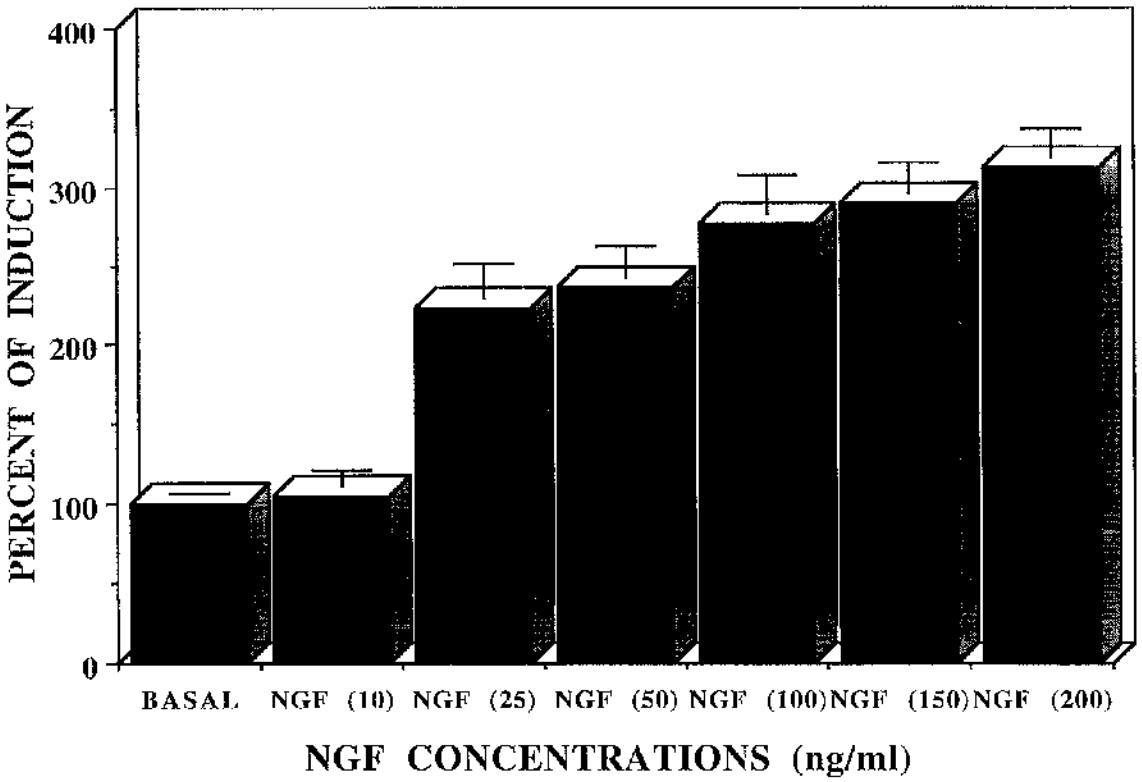


FIGURE 4.2 NGF CONCENTRATION EFFECT

Effect of a range of NGF concentration on the transcriptional activity of the NPY-Y1 receptor gene. PC12 cells were transfected with pY1-LUC (10 µg). 20 hours after transfection PC12 cells were treated with different **NGF** concentrations for 48 hours when the cells were collected for luciferase assay (Promega). Each experiment represents the percent of luciferase activity in NGF treated cells normalised to 100% for thecontrol cells (0.1% vehicle only). Values are mean ± SEM from 3 or more transfections experiments, each performed in duplicate with plasmids from at least two different preparations.

4.2.2 MECHANISMS INVOLVED IN THE NGF-INDUCED TRANSCRIPTIONAL ACTIVATION OF THE NPY-Y1 GENE

In order to study the role of different second messenger pathways in mediating the NGF activation of the NPY-Y1 receptor gene in PC12 cells, the effect of various inhibitors of specific signalling pathways were examined (Figure 4-1). For all these experiments, PC12 cells were transiently transformed with the pY1-LUC (10 μg) and the next day cells were preincubated for one hour with specific protein kinases inhibitors before addition of NGF (100 ng/ml). The cells were maintained with the inhibitors and neurotrophic stimuli for 48 hours and the relative levels of luciferase activity was measured in a luminometer.

All the experiments were performed in duplicate and the results are shown as the mean ± SEM of at least three independent experiments. Untreated PC12 cells were grown in the presence of vehicle (up to 0.1%) in DMEM complete medium.

4.2.2.1 EFFECT OF THE TYROSINE KINASE PATHWAY

4.2.2.1.1 *trk A* tyrosine kinase receptor inhibitor (K-252a)

Pretreatment of PC12 cells with K-252a (200 nM) completely blocked the 2.76 ± 0.18-fold NGF transcriptional activation of the NPY-Y1 gene (Figure 4-3). K-252a at this nM concentration is a specific inhibitor of *trk A* tyrosine kinase receptor and is known to block the biological effects of NGF in PC12 cells (Berg et al., 1992). The inhibitory effect of K-252a on the NGF induction of NPY-Y1 receptor gene transcription in PC12 cells was shown to be concentration dependent (Figure 4-3). However, K-252a (200 nM) had no effect on the maintenance of the basal levels of activity of the NPY-Y1 receptor gene in PC12 cells (Figure 4.3).

4.2.2.1.2 General tyrosine kinase and phosphatase inhibitors (Genistein and Vanadate)

Genistein is a synthetic general inhibitor of many different protein tyrosine kinases and is known to inhibit EGFR tyrosine kinase (IC_{50} = 2.6 μM) and $pp60^{v\text{-}src}$ (IC_{50} = 25 μM) by acting as a competitive inhibitor of ATP (Akiyama et al., 1987). Genistein (50 μM) resulted in a paradoxical effect on NPY-Y1 receptor gene expression as it increased, both the basal- and NGF-induced transcriptional activity of the NPY-Y1 receptor gene in PC12 cells (Figure 4-4).

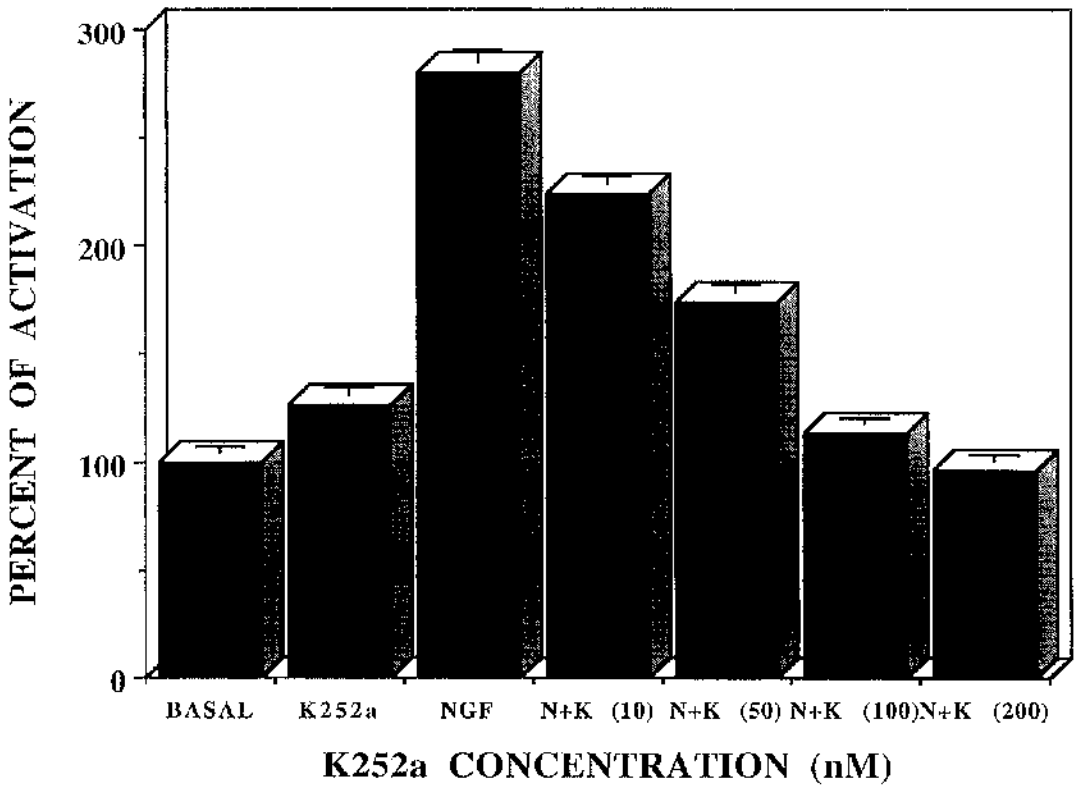


FIGURE 4.3 K-252a CONCENTRATION EFFECT ON THE NGF RESPONSE

Effect of a range of K-252a concentration on the NGF-dependent transcriptional activation of the NPY-Y1 receptor gene. PC12 cells were transfected with pY1-LUC (10 μg). 20 hours after transfection the PC12 cells were pretreated for 1 hour with different **K-252a** concentrations and then treated with **NGF** (100 ng/ml) for 48 hours when the cells were collected for luciferase assay (Promega). As a control experiment PC12 cells were treated with **K-252a** (200 nM) (basal effect) or **NGF** (100 ng/ml) alone for 48 hours. Each experiment represents the percent of luciferase activity in K-252a treated cells normalised (100%) for the control cells (0.1% vehicle only). Values are mean ± SEM from 3 or more transfections experiments, each performed in duplicate with plasmids from at least two different preparations.

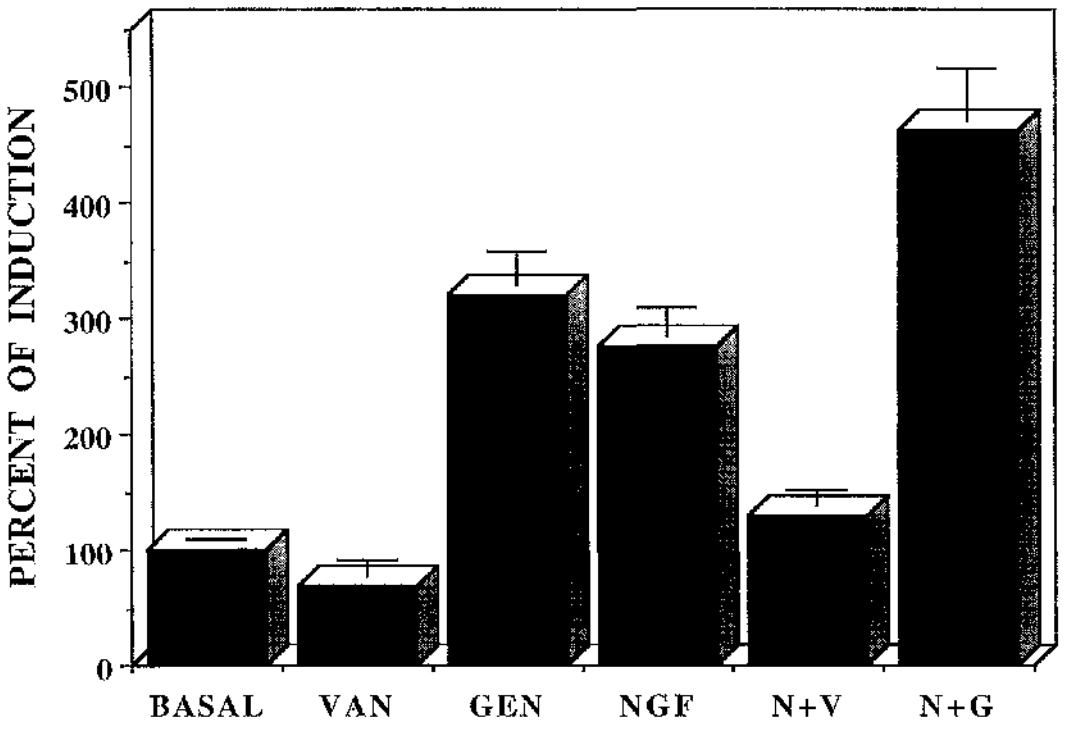


FIGURE 4.4 GENISTEIN AND VANADATE EFFECT ON THE NGF RESPONSE

GEN (genistein, 50 μM) and **VAN** (sodium orthovanadate, 30 μM) effect in the basal- and NGF-dependent transcriptional activity of the NPY-Y1 receptor gene. PC12 cells were transfected with pY1-LUC (10 μg). 20 hours after transfection the cells were pretreated for 1 hour with **GEN** (genistein, 50 μM) or **VAN** (sodium orthovanadate, 30 μM) and then the cells were treated with **NGF** (100 ng/ml) for 48 hours when the cells were collected for luciferase assay (Promega). As a control experiment PC12 cells were treated with **GEN** (genistein, 50 μM), **VAN** (sodium orthovanadate, 30 μM) (basal effects) or **NGF** (100 ng/ml) alone for 48 hours. Each experiment represents the percent of luciferase activity in treated cells normalised (100%) for the control cells (0.1% vehicle only). Values are mean ± SEM from 3 or more transfections experiments, each performed in duplicate with plasmids from at least two different preparations.

Sodium orthovanadate (Na_3VO_4) is a broad range inhibitor of protein tyrosine phosphatases (PTPases) (Gordon, 1991). Sodium orthovanadate (30 μM) reduced both the basal- and NGF-induced transcriptional activity of the NPY-Y1 receptor gene in PC12 cells (Figure 4-4). These effects of sodium orthovanadate (Na_3VO_4) and genistein on the basal- and NGF-induced transcriptional activity of the NPY-Y1 receptor gene indicate that is possible an intrinsic tyrosine kinase activity different to the associated with the *trk A* receptor inhibits the transcriptional activation of the NPY-Y1 receptor gene in PC12 cells. This specific tyrosine kinase activity remains to be elucidated.

4.2.2.2 EFFECT OF THE RAS-MAP KINASE PATHWAY

4.2.2.2.1 NGF effects in a ras-negative PC12 cell line

A stable mutant PC12 cell subline (PC12 asn17-W7) (Buensuceso, 1995) over-expressing a dominant negative human $p21^{ras}$ gene (encoding p21N17, a human c-Ha-*ras* gene with a point mutation in codon 17 resulting in a serine to asparagine substitution in the translated protein) (Feig and Cooper, 1988) was used to examine the role of the *ras* -MAP kinase pathway in mediating the effect of NGF on the transcriptional activation of the NPY-Y1 receptor gene. This cell line was a gift from Dr. Mark V. Rogers from Cellular Bioassay Design Group, Lead Discovery Unit, Glaxo-Wellcome, UK.

NGF (100 ng/ml) did not induce the known morphological changes in PC12asn17-W7 compared to PC12-wild type cells (data not shown). However, NGF (100 ng/ml) resulted in similar transcriptional activation of the NPY-Y1 receptor gene in this PC12-*ras* dominant negative cell line in comparison with the effect in the PC12-wild type cells (Figure 4-5). This result indicated that the $p21^{ras}$ protein is not involved in mediating the NGF-induced transcriptional activation of NPY-Y1 receptor gene in PC12 cells.

4.2.2.2.2 MEK-1 kinase inhibitor (PD 98059)

PD 98059 is a cell permeable and potent inhibitor of MEK-1 known to block the 4-fold increase in MAPK activity induced by NGF in PC12 cells ($IC_{50} = 2\mu M$) (Alessi et al., 1995). PD 98059 (20 μM) was shown to induce a increase in both the basal- and NGF-dependent transcriptional activation of the NPY-Y1 receptor gene in PC12 cells (Figure 4-6). This potent increase of the luciferase activity of the pY1-LUC mediated by PD 98059 (20 μM) suggests that MEK-1 kinase activity has an inhibitory action on both, the basal- and NGF-mediated transcriptional activation of the NPY-Y1 receptor gene in PC12 cells.

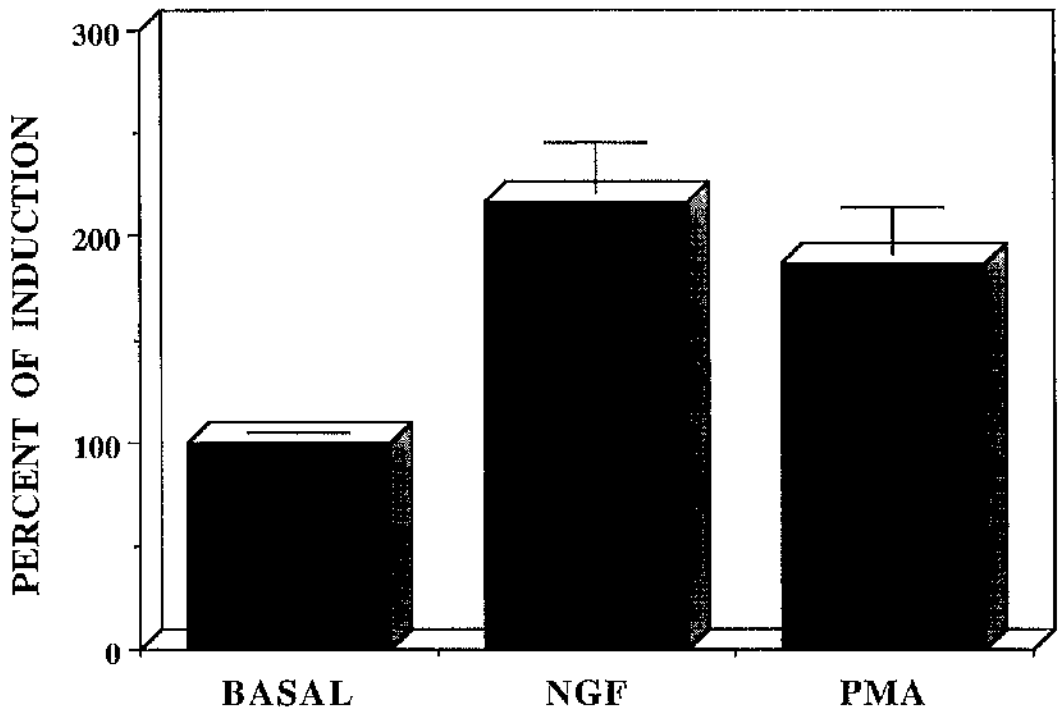


FIGURE 4.5 RAS EFFECT ON THE NGF RESPONSE

Effect of different agents on the transcription of the luciferase gene directed by the NPY-Y1 promoter gene in a PC12-*ras* negative cell line (Buensuceso, 1995). PC12 asn17-W7 were transfected with pY1-LUC (2.5 μg) using the liposome-mediated method. 20 hours after transfection the cells were treated with **NGF** (nerve growth factor, 100 ng/ml) or **PMA** (phorbol-12-myristate-13-acetate, 1 nM) for 48 hours when the cells were collected for luciferase assay (Promega). Each experiment represents the percent of luciferase activity in treated cells normalised (100%) for the control cells (0.1% vehicle only). Values are mean ± SEM from 3 or more transfections experiments, each performed in duplicate with plasmids from at least two different preparations.

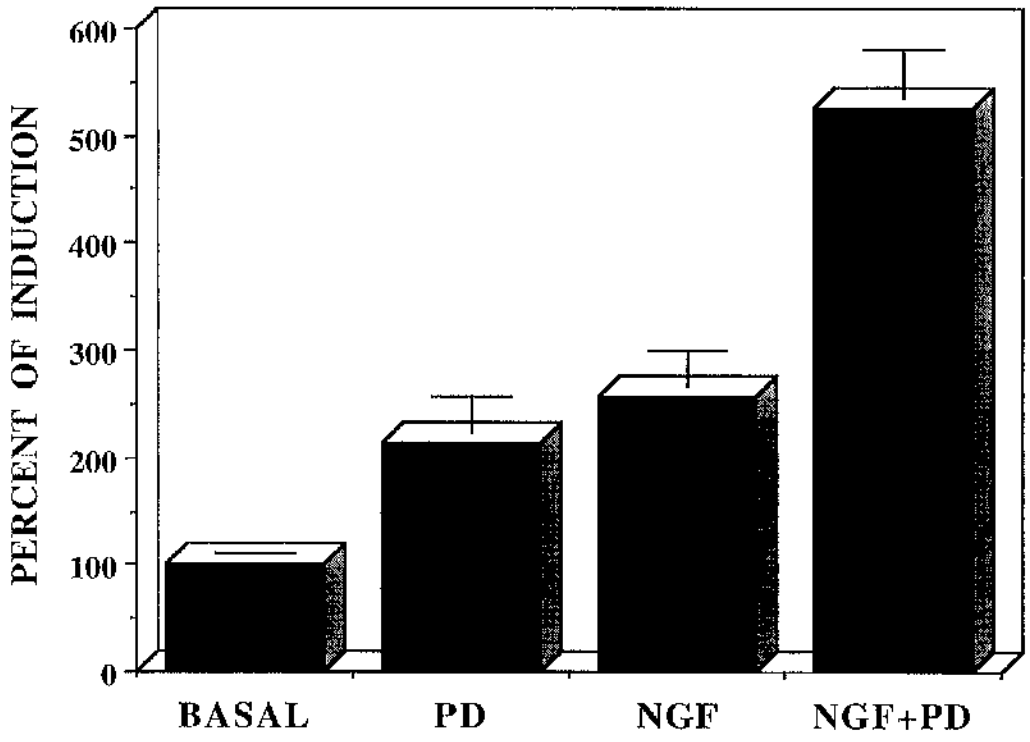


FIGURE 4.6 PD98059 EFFECT ON THE NGF RESPONSE

PD98059 effect on the basal- and NGF-dependent transcriptional activity of the NPY-Y1 receptor gene. PC12 cells were transfected with pY1-LUC (10 μg). 20 hours after transfection the cells were pretreated with **PD** (PD98059, 20 μM) for 1 hour and then the cells were treated with **NGF** (nerve growth factor, 100 ng/ml) for 48 hours when the cells were collected for luciferase assay (Promega). As a control experiment PC12 cells were treated with **PD** (PD98059, 20 uM) (basal effect) or **NGF** (nerve growth factor, 100 ng/ml) alone for 48 hours. Each experiment represents the percent of luciferase activity in PD98059 treated cells normalised (100%) for the control cells (0.1% vehicle only). Values are mean ± SEM from 3 or more transfections experiments, each performed in duplicate with plasmids from at least two different preparations.

4.2.2.3 EFFECT OF THE PKC PATHWAY

4.2.2.3.1 Protein kinase C inhibitor (Calphostin C)

Calphostin C was shown to block the effect of NGF on NPY-Y1 receptor gene transcription in PC12 cells in a concentration dependent fashion (Figure 4-7). Preincubation of PC12 cells with calphostin C (1 μM), a highly specific inhibitor of PKC (Ki = 50 nM) (Kobayashi et al., 1989) completely blocked the NGF-induced increase in transcriptional activity of the NPY-Y1 gene (Figure 4-7).

4.2.2.3.2 PMA effects on NPY-Y1 transcriptional activity

Phorbol diesters such as phorbol-12-myristate-13-acetate (PMA) activates PKC *in vitro* and *in vivo* even at nanomolar concentrations (Nishizuka, 1988). In the previous chapter, PMA (1 nM) was shown to produce a reproducible 1.82 ± 0.16-fold increase in the transcriptional activity of the NPY-Y1 receptor gene in PC12 cells (Figure 3-9). This 1.82 ± 0.16-fold transcriptional activation was shown not to be affected by increasing concentrations (1 nM to 10 μM) of the calcium ionophore ionomycin (data not shown) indicating that increases in intracellular calcium had no effect in mediating this PMA effect in PC12 cells. Furthermore, the effect of PMA on the transcriptional activation of the NPY-Y1 receptor gene was shown to be concentration dependent in PC12 cells (Figure 4-8). Increasing (in the high nM range) or decreasing (in the high pM range) the concentration of PMA resulted in a decrease of luciferase activity, indicating that the maximal transcriptional activation of the NPY-Y1 receptor gene in PC12 cells was obtained in the low nanomolar range of PMA, specifically between 1-10 nM (Figure 4-8).

Similar results were obtained using DH1, a synthetic PKC activator which binds PKC at the same site as phorbol esters. DH1 (1 nM) was shown to produced a 2.8 ± 0.10-fold increase in luciferase activity over basal levels (Figure 4-9). This increase is higher that the increase produced by PMA (1 nM), and is similar to the 2.76 ± 0.17-fold increase obtained by NGF-dependent activation of the NPY-Y1 gene. However, DH1 was shown to produce a similar concentration response effect on the transcriptional activity of the NPY-Y1 gene in PC12 cells in comparison that the concentration response obtained by PMA (Figure 4-8). Maximal luciferase activity was observed in the low nanomolar range between 1-10 nM and increasing concentrations of DH1 (high nM range) reduced the luciferase levels obtained using the pY1-LUC in PC12 cells (Figure 4-9).

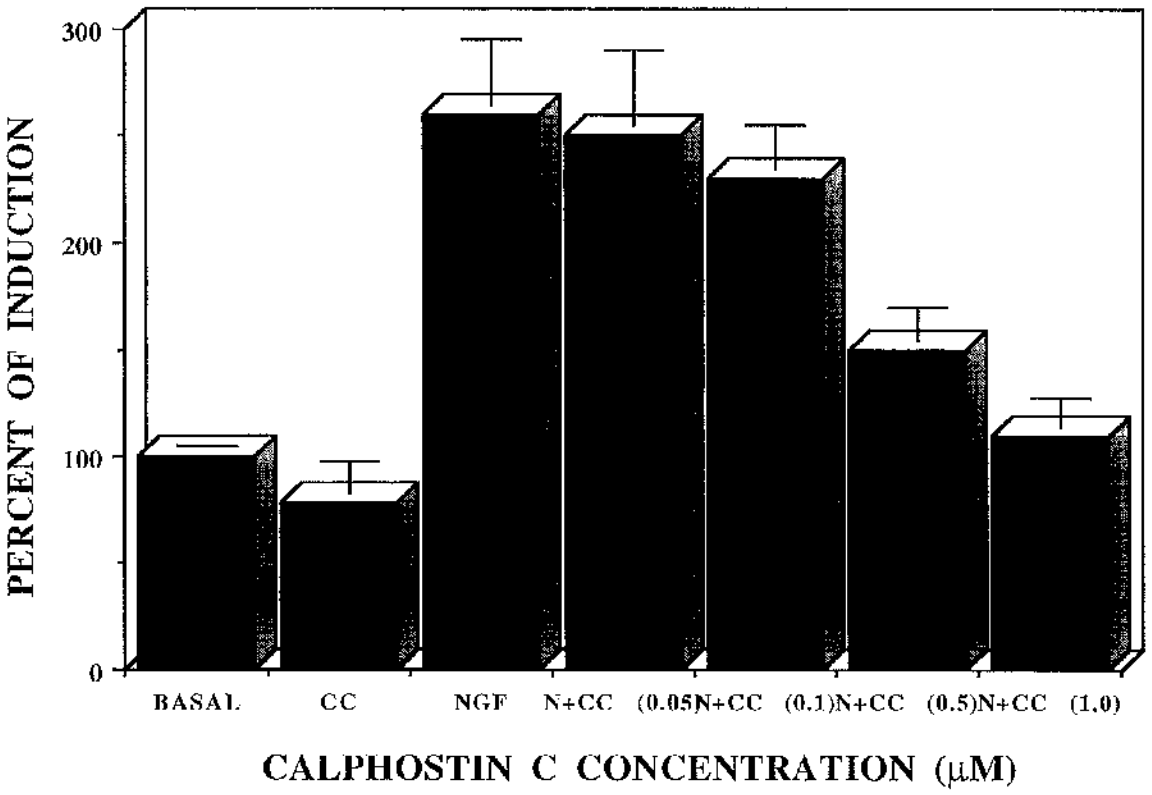


FIGURE 4.7 CALPHOSTIN C CONCENTRATION EFFECT ON THE NGF RESPONSE

Effect of a range of calphostin C concentration on the NGF-dependent transcriptional activity of the NPY-Y1 receptor gene. PC12 cells were transfected with pY1-LUC (10 μg). 20 hours after transfection the cells were pretreated with different concentrations of **CC** (Calphostin C) for 1 hour and then the cells were treated with **NGF** (nerve growth factor, 100 ng/ml) for 48 hours when the cells were collected for luciferase assay (Promega). As a control experiment PC12 cells were treated with **CC** (Calphostin C, 1 μM) (basal effect) or **NGF** (nerve growth factor, 100 ng/ml) alone for 48 hours. Each experiment represents the percent of luciferase activity in Calphostin C treated cells normalised (100%) for the control cells (0.1% vehicle only). Values are mean ± SEM from 3 or more transfections experiments, each performed in duplicate with plasmids from at least two different preparations with similar results.

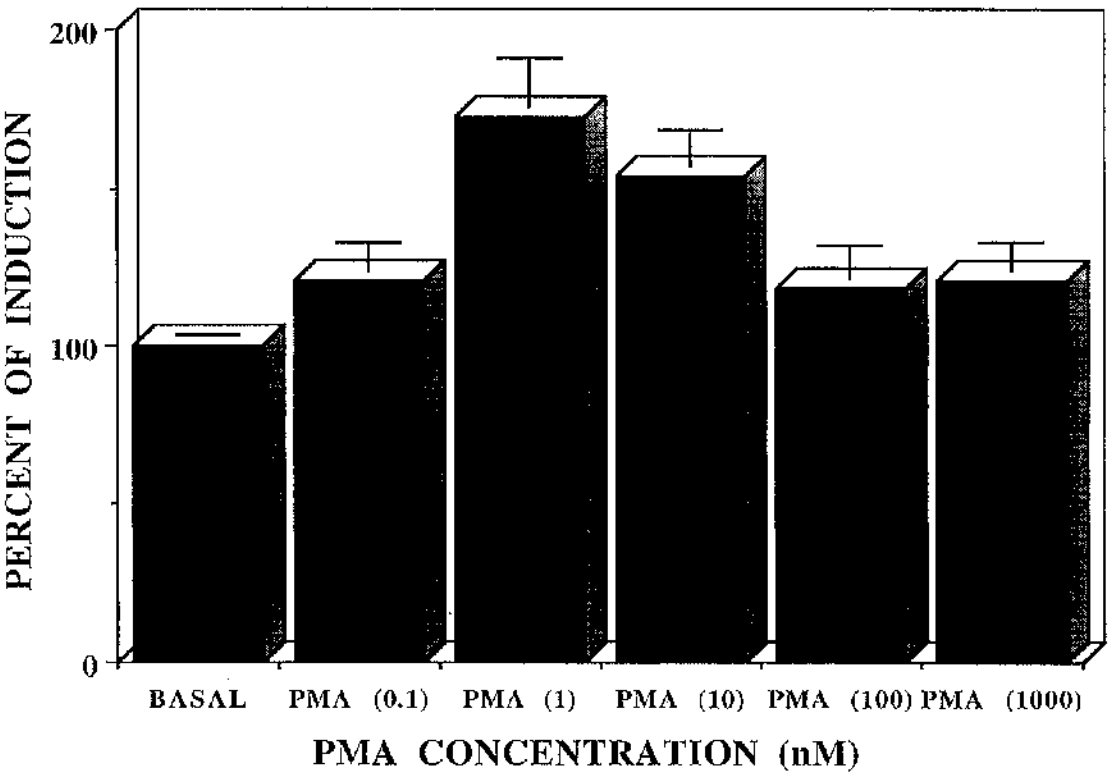


FIGURE 4.8 PMA CONCENTRATION EFFECT

Effect of a range of PMA concentration on the transcriptional activity of the NPY-Y1 receptor gene. PC12 cells were transfected with pY1-LUC (10 μg). 20 hours after transfection the cells were treated with different concentrations of **PMA** (phorbol-12-myristate-13-acetate) for 48 hours when the cells were collected for luciferase assay (Promega). Each experiment represent the percent of luciferase activity in PMA treated cells normalised (100%) for the control cells (0.1% vehicle only). Values are mean ± SEM from 3 or more transfections experiments, each performed in duplicate with plasmids from at least two different preparations.

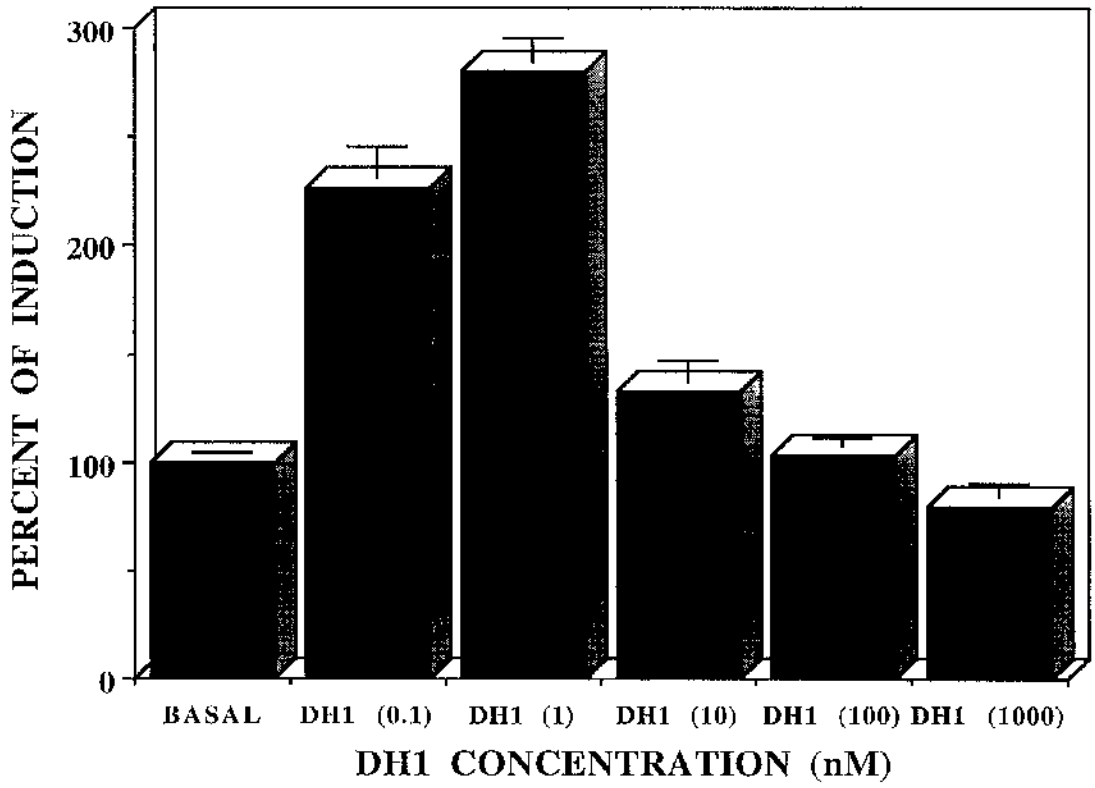


FIGURE 4.9 DH1 CONCENTRATION EFFECT

Effect of a range of DH1 concentration on the transcriptional activity of the NPY-Y1 receptor gene. PC12 cells were transfected with pY1-LUC (10 µg). 20 hours after transfection the cells were treated with different **DH1** concentrations for 48 hours when the cells were collected for luciferase assay (Promega). Each experiment represents the percent of luciferase activity in DH1 treated cells normalised (100%) for the control cells (0.1% vehicle only). Values are mean ± SEM from 3 or more transfections experiments, each performed in duplicate with plasmids from at least two different preparations.

Calphostin C (1 μM) resulted in a complete inhibition of both, the PMA- and DH1-mediated (Figure 4-10) transcriptional activation of the NPY-Y1 gene in PC12 cells. However, preincubation with H-89 (30 μM), K-252a (200 nM) and KN-62 (1μM) (See Chapter 2. Inhibitor compounds) did not affect the subsequent PMA- or DH1-dependent response of the NPY-Y1 receptor gene in PC12 cells (data not shown).

In addition, the response of the NPY-Y1 receptor gene to PMA (1nM) was identical in the PC12-ras negative cells compared to the PC12-wild type (Figure 4-5). These results indicated that DH1- and PMA-dependent increase of the transcriptional activity of the NPY-Y1 receptor gene in PC12 cells is primarily dependent in PKC activation and is independent on the activation of another intracellular signalling pathways, including PKA, CaM-K II and the*trkA*-receptor pathway.

4.2.2.3.3 NGF and PMA effects on PKC activity in PC12 cells

The previous results of this chapter, suggest a direct involvement of PKC in mediating the NGF and PMA transcriptional activation of the NPY-Y1 receptor gene in PC12 cells. In order to test whether NGF and PMA indeed increased PKC activity in these cells, PC12 cells were treated with NGF (100 ng/ml) and two concentrations of PMA (1nM and 1μM).

Direct measurement of PKC activity in PC12 cell extracts demonstrated a 2.0-fold increase following the addition of NGF (100 ng/ml) (Figure 4-11). This rise in PKC activity was observed within 1 hour following the addition of NGF (100 ng/ml). A similar increase (1.8-fold) in the PKC activity was observed using PMA (1nM) but this increase was completely suppressed using higher concentrations of PMA (1 μM) (Figure 4-11). This suppression of PKC activity is well documented as PMA is routinely used to downregulate PKC by long term incubations (Nishizuka, 1988).

4.2.2.4 EFFECT OF THE PHOSPHOLIPASE D PATHWAY

Pretreatment of PC12 cells with 1-butanol (0.3%) 1 hour before the cells were treated with NGF (100 nM) did not affect the NGF-induced increase in the transcriptional activation of the NPY-Y1 gene (data not shown).This preliminary result suggest that the putative supression of the PLD activity did not affect the luciferase activity of the pY1-LUC in PC12 cells.

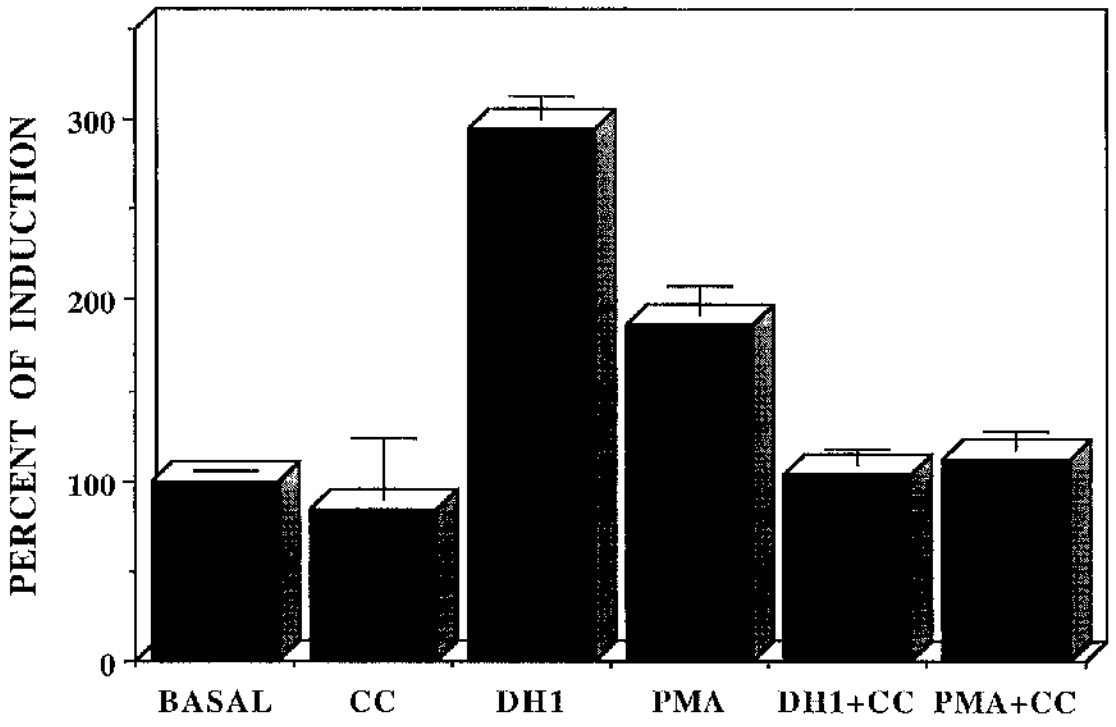


FIGURE 4.10 CALPHOSTIN C EFFECT ON THE DH1 OR PMA RESPONSE

Calphostin C effect on the DH1- and PMA-induced transcriptional activation of the NPY-Y1 receptor gene. PC12 cells were transfected with pY1-LUC (10 μg). 20 hours after transfection the cells were pretreated with **CC** (calphostin C, 1 μM) for 1 hour and then the cells were treated with **DH1** (1 nM) or **PMA** (phorbol-12-myristate-13-acetate, 1 nM) for 48 hours when the cells were collected for luciferase assay (Promega). As a control experiment the cells were treated with **CC** (Calphostin C, 1 μM), **DH1** (1 nM) or **PMA** (phorbol-12-myristate-13-acetate, 1 nM) alone. Each experiment represents the percent of luciferase activity in treated cells normalised (100%) for the control cells (0.1% vehicle only).Values are mean ± SEM from 3 or more transfections experiments, each performed in duplicate with plasmids from at least two different preparations.

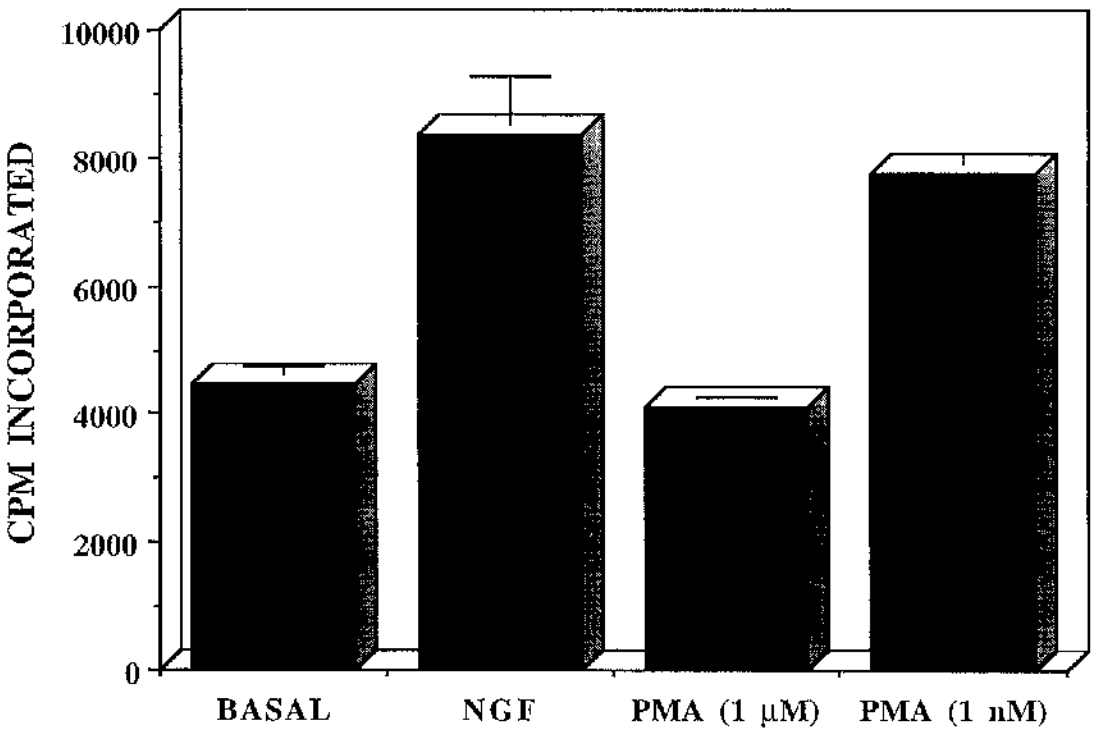


FIGURE 4.11 NGF AND PMA EFFECT ON THE PKC ACTIVITY IN PC12 CELLS

Effect of NGF and PMA stimulation on PKC activity in PC12 cells. PC12 cells were treated with **NGF** (nerve growth factor, 100 ng/ml) and two different **PMA** concentrations (phorbol-12-myristate-13-acetate, 1 nM or 1 μM) for 1 hour and then the cells were collected for measurement of the PKC activity (Life Technologies). Data shown are mean ± SEM of triplicate assays of one representative experiment performed two times with similar results.

4.2.2.5 EFFECT OF THE Ca^{++}/CALMODULIN KINASE II PATHWAY

KN-62 (1 μM), a specific inhibitor of a rat brain CaM-K II (Ki = 900 nM) (Tokumitsu et al., 1990) had no effect in the NGF-induced transcriptional activation of the NPY-Y1 receptor gene in PC12 cells. In addition, KN-62 (1 μM) did not alter the basal levels of luciferase activity of the pY1-LUC in PC12 cells. These results indicated that the CaM-K II activity has no effect on the maintenance of the basal- or NGF-induced transcriptional activation of the NPY-Y1 receptor gene in PC12 cells.

4.2.2.6 EFFECT OF THE PI-3-KINASE PATHWAY

Two selective and potent inhibitors of PI-3-K were examined. Wortmannin is a fungal metabolite known to be an irreversible, cell permeable and potent inhibitor of PI-3-K in purified preparations and cytosolic fractions (IC_{50} = 5 nM) (Nakanishi et al., 1992, 1994). LY 294002 is a synthetic, cell permeable and specific PI-3-K inhibitor that acts on the ATP binding site of the enzyme (IC_{50} = 1.4 μM, Ki = 1.6 μM).

Neither, Wortmannin (10 μM) or LY 294002 (10 μM) inhibited the basal- or NGF-induced transcriptional activation of NPY-Y1 receptor gene in PC12 cells (data not shown). These results indicating that the PI-3-K signalling pathway, which is know can be activated by NGF in PC12 cells (See Chapter 1. PI-3-kinase signalling pathway) had no effect on the maintenance of the basal levels or in the NGF-induced increase of the NPY-Y1 transcriptional activity of PC12 cells.

4.2.2.7 EFFECT OF THE PKA PATHWAY

Pretreatment of PC12 cells with H-89, dihydrochloride (30 μM), a synthetic cell-permeable and specific inhibitor of the cAMP-dependent protein kinase A (PKA) (Ki= 48 nM) (Chijiwa et al., 1990), had no effect on the NGF-stimulated transcriptional activation of the NPY-Y1 receptor gene in PC12 cells (data not shown).

However, H-89 (30 μM) reduced the basal levels of luciferase activity of the pY1-LUC fusion gene in PC12 cells (See Chapter 5). These results indicated that PKA signalling pathway is involved in the maintenance of the basal transcriptional activity of the NPY-Y1 receptor gene in PC12 cells but not in the NGF-induced response.

4.3 DISCUSSION

In this chapter, an attempt was made to study the intracellular mechanisms behind the transcriptional control by NGF of the NPY-Y1 receptor gene in PC12 cells using a luciferase reporter assay system (Promega). In particular the role of different intracellular pathways activated by NGF in PC12 cells, including the *ras* -MAPK, the PKC and the PI-3-K signalling pathways have been examined. Another pathways, such as the PKA and the CaM-K II pathway were also examined.

Effects of NGF on the transcriptional activation of several genes has been suggested to be mediated by activation of both PKA and PKC second messenger pathways as specific activators of both systems mimic and potentiate the effects of NGF in PC12 cells (Greene et al., 1984). Results presented in this chapter support the hypothesis that NGF-induced PKC activity mediates the transcriptional activation of NPY-Y1 receptor gene in a *ras* - and MEK1-independent manner in PC12 cells.

4.3.1 NGF CONCENTRATION EFFECTS ON THE TRANSCRIPTIONAL ACTIVATION OF THE NPY-Y1 GENE

NGF is the best characterized member of a family of "neurotrophins" that includes BDNF, NT-3 and NT-4/5 (Barde, 1989). These molecules promote the differentiation, growth and survival of distinct neuronal cell populations through specific RTKs. Neurotrophin receptors are encoded by a gene family, designated *trk* , which so far includes three related loci: *trkA* , *trkB* and *trkC* (Chao et al., 1992).

Previous studies have shown that NGF differentiated PC12 cells were responsive to a NPY-Y1 receptor selective agonist [Pro^{34}]-NPY (DiMaggio et al., 1994). The data presented in this chapter shows that NGF increased in a concentration dependent manner the transcriptional activity of the NPY-Y1 receptor gene in PC12 cells (Figure 4-1).

4.3.2 MECHANISMS INVOLVED IN THE NGF-INDUCED TRANSCRIPTIONAL ACTIVATION OF THE NPY-Y1 GENE

4.3.2.1 EFFECT OF TYROSINE KINASE PATHWAY

Protein tyrosine phosphorylation plays a role in regulating fundamental cellular processes including differentiation, proliferation and tumorigenesis (Brewster et al., 1993) Phosphorylation at tyrosine residues occurs as a cellular response to a variety of stimuli

including growth factors, hormones and cytokines. The initial events include activation, dimerization and autophosphorylation of RTKs. This in turn initiates intracellular signalling cascades involving the phosphorylation of several other key regulatory proteins. The balance of tyrosine phosphorylation is controlled by the opposing activities of protein tyrosine kinases and protein tyrosine phosphatases (Walton and Dixon, 1993)

4.3.2.1.1 *trk A* tyrosine kinase receptor inhibitor (K-252a)

Pretreatment of PC12 cells with K-252a, was shown to block in a concentration dependent manner the NGF-induced transcriptional activation of the NPY-Y1 receptor gene (Figure 4-3). However, K-252a (200 nM) had no effect in the maintenance of the basal levels of activity of the NPY-Y1 receptor gene in PC12 cells (Figure 4-3). This results suggest that the effect of NGF on the NPY-Y1 transcriptional activation is dependent on *trkA* receptor activation, but *trkA* receptor tyrosine kinase activity is not involved in the maintenance of the basal levels of activity of the NPY-Y1 gene in PC12 cells.

K-252a is a fungal alkaloid-like compound isolated from *Nocardiopsis sp.* and originally characterized as an inhibitor of PKC and cyclic nucleotide-dependent kinases. However, K-252a had no effect on the response of PC12 cells to DBC or PMA. Interestingly, K-252a in nanomolar quantities has been shown to be a specific inhibitor of NGF-induced biochemical responses and neurite outgrowth in PC12 cells (Berg et al., 1992). Virtually all of the known biochemical events induced by NGF in PC12 cells are inhibited by K-252a, including tyrosine phosphorylation of cellular proteins, increase in *c-fos* transcription (Lazarovichi et al., 1989), MAPK-2 activation (Tsao et al., 1990), $p21^{ras}$ activation (Rovelly et al., 1993) and MEK-1 activation (Jaiswal et al., 1993).

K-252a exerts its action upon NGF-specific signal transduction in PC12 cells by inhibiting *in vivo* and *in vitro* tyrosine phosphorylation and kinase activity of the *trkA* receptor, while having no effect on the EGF- and FGF-stimulated responses (Berg et al., 1992). However, K-252a has a paradoxical neurotrophic effect in some other cells lines without apparently altering *trk* activity or stimulating known *trk* substrates (Rasouly et al., 1992). K-252a treatment of PC12 cells in the absence of any growth factor stimulation, results in neuronal differentiation (data not shown). This neurotrophic effect in PC12 cells appears to occur through signal transduction pathways that are independent from those used by *trk* (Rasouly et al., 1992) and may involve $p60^{c\text{-}src}$ tyrosine kinase family members and a 145 kDa tyrosine phosphorylated protein, suggesting that *trkA* may be not be the only cellular target of K-252a (Rasouly et al., 1992).

4.3.2.1.2 General tyrosine kinase and phosphatase inhibitors (Genistein and Vanadate)

Genistein, a broad range tyrosine kinase inhibitor acts as a competitive inhibitor of ATP. Genistein (50 μM) has been shown to increase both the basal- and NGF-induced (Figure 4-4) transcriptional activity of the NPY-Y1 gene in PC12 cells. The effect of genistein (50 μM) on basal transcriptional activity of the NPY-Y1 receptor gene suggests that an intrinsic tyrosine kinase, different from the *trkA* receptor associated tyrosine kinase, is involved in regulating basal transcriptional activity of the NPY-Y1 receptor gene in PC12 cells.

$pp60^{c\text{-}src}$ has been implicated in the propagation of differentiation signals which involve the *ras* -dependent activation of MAPKs (Wood et al., 1992). $pp60^{c\text{-}src}$ is thought to act downstream of *trkA* in the NGF-induced signalling pathway in PC12 cells and there are two lines of evidence to support this view. Firstly, PC12 cells transfected with $pp60^{v\text{-}src}$ temperature-sensitive gene undergo phenotypic change and express neurites at the permissive temperature resembling NGF-induced phenotypic change (Rausch et al., 1989). Secondly, NGF-induced neurite outgrowth could be blocked by microinjection of monoclonal antibodies to either the SH2 domain of $pp60^{v\text{-}src}$ or the unique amino-terminal region of $pp60^{c\text{-}src}$ (Kremer et al., 1991). Moderate basal $pp60^{c\text{-}src}$ activity is observed in control PC12 cells as shown by autophosphorylation. This probably relates to the fact that basal $pp60^{c\text{-}src}$ activity is necessary for an intrinsic function as growth or inhibition of apoptosis (Rukenstein et al., 1991; Batistatou and Greene, 1991).

Moreover, sodium orthovanadate (30 μM), a broad range tyrosine phosphatase inhibitor (Gordon, 1991) decreased both the basal- and NGF-induced (Figure 4-4) transcriptional activity of the NPY-Y1 receptor gene in PC12 cells. Vanadate, which mimics the transition state of phosphate (Gordon, 1991) affects the activity of enzymes which form phosphoenzyme intermediates with enzyme-phosphate bonds and is a well-established inhibitor of protein tyrosine phosphatases. Vanadate treatment of intact cells results in accumulation of phosphotyrosine in many proteins and as consequence has been shown to effect mitogenic and differentiation signals in many different cell types (Gordon, 1991). Vanadate (30 μM) induced neurite outgrowth in PC12 cells (data not shown) by stimulation of tyrosine kinase activity following inhibition of specific tyrosine phosphatases. The vanadate-induced neurite outgrowth is not inhibited by the specific inhibitor of *trkA* tyrosine kinase receptor activity, K-252a, indicating there are fundamental differences in the tyrosine kinases involved in vanadate- and NGF-induced neuronal differentiation of PC12 cells (Buensuceso, 1995).

Effect of vanadate on pp60$^{c\text{-}src}$ is complex. pp60$^{c\text{-}src}$ tyrosine kinase activity is increased by vanadate indicating that the regulatory carboxy-terminal phosphorylation site (Tyr-527) which is known to supress pp60$^{c\text{-}src}$ activity (Cooper et al., 1986) is not phosphorylated by the C-terminal kinase (*csk*) which phosphorylate this site on this kinase (Okada et al., 1991). The prolonged nature of pp60$^{c\text{-}src}$ activation lasting more than 30 minutes in response to vanadate would be similar to the sequential *src-ras* actions in NGF signalling which has already been described (Kremer et al., 1991) and can explain the vanadate inhibitory effect in the basal- and NGF-induced transcriptional activity of the NPY-Y1 gene in PC12 cells.

Genistein is also known to inhibited the increase of *ras* -GTP induced by NGF and PMA (Nakafaku et al., 1992). In addition, MAPK activity can be induced by Na3VO4 independently from NGF (Tsao et al., 1990). Taken together, these results suggested that activation of a intrinsic tyrosine kinase activity may have a negative effect on NPY-Y1 transcriptional activity and in the effects of genistein and vanadate could be involved the *ras* -MAPK pathway.

4.3.2.2 EFFECTS OF THE RAS-MAP KINASE PATHWAY

4.3.2.2.1 NGF effects in a *ras* -negative PC12 cell line

A common pathway leading from cell surface growth factor receptors to MAPKs involves p21^{ras}, a member of a small GTP-binding proteins from the *ras* family (See Chapter 1. NGF signalling pathway). Treatment of the PC12 cells with NGF induces a rapid increase in tyrosine phosphorylation of multiple cellular proteins. Induction of tyrosine phosphorylation triggered by NGF in PC12 cells can be separated into: (i) *ras* -dependent and (ii) *ras* -independent events. *ras* -dependent events include the phosphorylation of MAPK, p90^{rsk} and *raf-1*. *ras* -independent events include NGF-induced activation of *trkA* , tyrosine phosphorylation of PLC-g1 (Thomas et al., 1992) and activation of the expression of neuronal specific genes, such as voltage-gated Na^{+} channel (Fanger et al., 1993).

Substitution of asparagine for serine at position 17 decreased the affinity of c-Ha-*ras* for GTP 20- to 40-fold without significantly affecting its affinity for GDP (Feig and Cooper, 1988). A stable mutant PC12 cell subline (PC12 asn17-W7) (Buensuceso, 1995) over-expressing this dominant inhibitory human p21^{ras} gene was used to examine the role of the *ras*-MAP kinase pathway in mediating the effect of NGF on the NPY-Y1 transcriptional activation. NGF (100 ng/ml) results in a similar transcriptional activation of the NPY-Y1 receptor gene in the PC12-*ras* negative cell line in comparison with the PC12-wild type cells (Figure 4-5). These results demonstrated that the NGF-induced transcriptional activation of the NPY-Y1 receptor gene is *ras* -independent in PC12 cells.

4.3.2.2.2 MEK-1 kinase inhibitor (PD 98059)

Many RTKs activate a class of intracellular protein serine/threonine kinases, MAPKs. In PC12 cells, two related MAPKs, MAPK-1 ($p44^{mapk}$) and MAPK-2 ($p42^{mapk}$) are phosphorylated and activated on tyrosine and threonine residues following NGF treatment (Tsao et al., 1990). MAPKs activation is associated with both cell proliferation and differentiation and it is the duration of MAPKs activation that determines whether a stimulus elicits proliferation or differentiation (Traverse et al., 1992).

PD 98059 is a small molecular weight non-competitive (with respect to ATP) *in vivo* and *in vitro* inhibitor of the upstream kinase activator of MAPKs, MEK-1 kinase (Alessi et al., 1995). PD 98059 binds to the inactive forms of MEK-1 and prevents both the activation and phosphorylation of MEK-1 *in vitro* by upstream activators such as *c-raf* or MEKK-1 (IC_{50} = 5-10 μM) and also inhibits the activation of MEK-2 by*c-raf* (IC_{50} = 50 μM) (Alessi et al., 1995).

PD 98059 selectively blocks growth factor-induced phosphorylation and activation of MAPKs *in vitro* and *in vivo* (Pang et al., 1995) and also blocks the 4-fold NGF-induced stimulation of MAPKs in PC12 cells with a half-maximal inhibition at approximately 2 μM and complete inhibition at 10-100 μM (Pang et al., 1995). PD 98059 completely blocks the tyrosine phosphorylation of MAPKs but does not affect the autophosphorylation of the*trkA* receptor, the phosphorylation of its substrate Shc and NGF-dependent activation of PI-3-K (Pang et al., 1995). PD 98059 completely blocks NGF-induced neurite formation in PC12 cells without altering cell viability indicating that the phosphorylation and activation of the MAPK pathway is required for NGF-induced neuronal differentiation in PC12 cells.

PD 98059 (20 μM) was shown to increase both, the basal- and NGF-dependent transcriptional activation of the NPY-Y1 receptor gene in PC12 cells (Figure 4-6). This paradoxical increase of the NPY-Y1 promoter activity after PD 98059 (20 μM) treatment suggests that basal- and NGF-dependent MEK-1 kinase activity have a negative effect in the transcriptional activation of the NPY-Y1 gene in PC12 cells.

4.3.2.3 EFFECT OF THE PKC PATHWAY

PI turnover has been implicated in the action of NGF in PC12 cells (Traynor, 1984; Contreras and Guroff, 1987). PI-PLC, the enzyme responsible for hydrolysis of inositol phospholipids is activated by *trk A* receptor through tyrosine phosphorylation (Vetter et al., 1991). However, IP3 and the intracellular mobilisation of Ca^{++} is not generated in response to NGF in PC12 cells (Chang et al., 1989). Moreover, the NGF stimulation of IP turnover occurs only in the

presence of other agonists and may merely reflect non-specific increases in phospholipid synthesis (See Chapter 1. NGF signalling pathway).

PKC activation is believed to be an early event after NGF stimulation in PC12 cells (Hama et al., 1986; Heasley and Johnson, 1989; Kondratyev et al., 1990; Ohmichi et al., 1993; Wooten et al., 1994). Staurosporine, a PKC inhibitor causes a concentration-dependent inhibition of c-*fos* induction by NGF, consistent with the finding that the c-*fos* gene regulation may be influenced by PKC (Sheng and Greenberg, 1990).

4.3.2.3.1 Protein kinase C inhibitor (Calphostin C)

PKC is a major mediator of signal transduction, and is activated *in vivo* by DAG and Ca^{++}. DAG activates PKC by decreasing its Km for calcium. PKC can also be activated by tumour promoting phorbol esters, such as PMA and this activation is a central point involved in a wide range of cellular responses (See Chapter 1. PKC signalling pathway).

Calphostin C is a microbial compound isolated from *Clodosporium cladosporioides* which is a cell permeable, potent and highly specific inhibitor of PKC (Ki = 50 nM) (Kobayashi et al., 1989). Calphostin C interacts with the regulatory domain of PKC by competing at the binding site of diacylglycerol and phorbol esters and does not compete with Ca^{++} or phospholipids (Kobayashi et al., 1989).

PKC inhibitory activity of calphostin C is reported to be light-dependent (Bruns et al., 1991). Calphostin C and others PKC inhibitors which contain a so-called "3-10 perylenequinone" must be illuminated in the presence of PKC in order for it to show inhibition of [^{3}H]-phorbol 12,13-dibutyrate binding or inhibitory activity against the enzyme (Bruns et al., 1991). This light requirement for the inhibitory effect of calphostin C was found only by chance. These "3-10 perylenequinone" compounds are not closely analogous, but they all contain numerous fused aromatic rings and absorb visible light. Thus, as a class they indicate that PKC has one or more binding domains which accept certain planar or nearly planar polycyclic aromatic compounds (Bruns et al., 1991)

Preincubation of PC12 cells with calphostin C completely blocked in a concentration dependent manner the increase in transcriptional activity of the NPY-Y1 gene induced by NGF (Figure 4-7). Calphostin C (1 μM) also reduced the basal levels of luciferase activity of the pY1-LUC fusion gene (Figure 4-7). These results showed that the PKC signalling pathway is involved in the basal- and NGF-dependent transcriptional activity of this neuropeptide receptor gene in PC12 cells.

4.3.2.3.2 PMA effects on NPY-Y1 transcriptional activity

Phorbol esters, the tumour-promoting activators of PKC, share certain biological activities of NGF in PC12 cells (Greenberg et al., 1985). Several key second messenger pathways known to co-ordinate the responses to NGF in PC12 cells have been shown to be activated by PKC. These includes activation of *raf-1* (Morrison et al., 1988), *B-raf* (Oshima et al., 1991), MEK (Jaiswal et al., 1993), MAPK (Chung et al., 1991) and $p90^{rsk}$ (Chen et al., 1991). PMA is able to produce only a modest increase in MEK activity (Jaiswal et al., 1993) and this correlates with the modest stimulation of MAPKs by phorbol esters in PC12 cells (Chung et al., 1991). However, PMA prevents the subsequent decline in MAPK phosphorylation and activity and this decline is accentuated by downregulation of PKC, suggesting that PKC may negatively regulate phosphatases that dephosphorylate and deactivate MAPK (Tsao et al., 1990). Although these results taken together do not directly prove that NGF action involves the stimulation of PKC, they suggest that active PKC occurs high up in the NGF signalling pathway and is necessary for the some of the NGF-dependent cascade of events in PC12 cells.

PMA (1nM) produced a 1.82 ± 0.16-fold increase in the transcription of the NPY-Y1 receptor gene in PC12 cells (Figure 3-9). Furthermore, the effect of PMA on the transcriptional activation of the NPY-Y1 receptor gene was shown to be concentration dependent in PC12 cells (Figure 4-8). Increasing (in the high nM range) or decreasing (in the high pM range) the concentration of PMA resulted in a significant decrease of luciferase activity, indicating that the maximal transcriptional activation of the NPY-Y1 promoter in PC12 cells was obtained in the low nanomolar range of PMA, specifically between 1-10 nM (Figure 4-8).

This 1.82 ± 0.16-fold PMA-mediated increase is relative low in comparison to the 2.76 ± 0.18-fold NGF-mediated increase and is not affected by co-incubation with increasing concentrations (1 nM to 10 μM) of the calcium ionophore ionomycin (data not shown). Ionophores, such as ionomycin are hydrophobic molecules that increase the membrane permeability of specific inorganic ions. They shield the charge of the ion to be transported, enabling it to penetrate the hydrophobic interior of the lipid bilayer. Ionomycin is natural compound isolated from *Streptomyces conglobatus* and is a highly specific divalent cation mobile ion carrier which binds Ca^{++} in a one-to-one steochiometry. Results presented here indicate that increases in cytosolic free intracellular Ca^{++} resulting from the addition of ionomycin did not affect the PMA-induced transcriptional activation of the NPY-Y1 receptor gene in PC12 cells. However, elevations of intracellular Ca^{++} and phorbol esters activation of PKC are known to act synergistically in some biological systems (Wilkimson and Hallam, 1994).

Similar results were obtained using DH1, a synthetic protein kinase activator which binds PKC at the same site as phorbol esters (Mastro and Grove, 1991). DH1 embody a modified 3,5-diaminobenzyl alcohol derivatives, which a potency sligtly weaker than those typical of diacylglycerols. DH1 (1 nM) was shown to produced a 2.8 ± 0.10-fold increase in luciferase activity over basal levels (Figure 4-9). DH1 also produce a similar concentration dependent response on the transcriptional levels of the NPY-Y1 receptor gene in PC12 cells compared to that obtained by PMA (Figure 4-9). Maximal luciferase activity was observed in the low nanomolar range between 1-10 nM and increasing concentrations of DH1 (high nM range) reduced the luciferase levels obtained using the pY1-LUC in PC12 cells (Figure 4-9).

Calphostin C (1 μM) results in a complete inhibition in both, the PMA (1nM)- and DH1 (1nM)-mediated (Figure 4-10) transcriptional activation of the NPY-Y1 gene in PC12 cells. However, preincubation with different pharmacological inhibitors (See Chapter 2. Inhibitor compounds): H-89 (30 μM), K-252a (200 nM) and KN-62 (1 μM) did not affect the subsequent response of the NPY-Y1 promoter to PMA (1 nM) or DH1 (1 nM) in PC12 cells (data not shown). These results indicate that the DH1- and PMA-mediated increase of the transcriptional activity of the NPY-Y1 gene in PC12 cells is dependent in PKC activation and is independent on the activation of other signalling pathways, including PKA, CaM-K II and *trkA* -receptor pathways.

4.3.2.3.3 NGF and PMA effects on PKC activity in PC12 cells

Direct measurement of PKC activity in PC12 cells treated for 1 hour with NGF (100 ng/ml) showed a 2.0-fold increase in comparison to basal levels (Figure 4-11). This increase in PKC activity is similar to the NGF-mediated transcriptional activation of the NPY-Y1 receptor gene in PC12 cells (Figure 4-2). Moreover, when PC12 cells were treated with PMA (1 nM), the response of PKC activity was comparable (1.8-fold increase) to that obtained with NGF (Figure 4-11). However, PC12 cells treated for 1 hour with PMA (1 μM) had a PKC activity similar to that obtained in PC12-untreated cells (basal levels). Previous results in this laboratory have shown that PKC activity was significantly lower than basal levels after 3 hours of treatment with PMA (0.4 μM) while levels returned only to basal values following NGF (50 ng/ml) treatment for 3 hours (Balbi and Allen, 1994). This result suggests that the PMA inhibitory effect on the transcriptional activation of NPY-Y1 gene in PC12 cells will be a consequence of a downregulation of the PKC.

The results obtained in this work and previous results in this laboratory make it necessary to be cautious in the interpretation of the effect of PMA on PKC activity in PC12 cells. It has been reported that short term treatment with PMA produces a transient activation of PKC (Nishizuka, 1988). However, phorbol esters have a secondary effect on PKC prolonging the

association of the protein with the cellular membrane. This prolonged association is thought to promote the degradation of the enzyme and its disappearance from the cells (Nishizuka, 1988). Moreover, persistent activators of PKC, such as phorbol esters, may act supraphysiologically and activate processes via PKC that are not normally driven by physiologically relevant activators. DAG and related analogues have only a transient existence in a cell, being rapidly metabolised by both DAG lipase and DAG kinase. By contrast phorbol esters are only poorly metabolised; the presence of PMA, has been detected in cells several hours after administration.

Stimulation with supramaximal concentrations of phorbol esters can also cause the phosphorylation of "non-specific" substrates. Furthermore, it would appear that the specificity of PKC for substrates in intact cells is less precise as the concentration of exogenously applied activator is increased. Phorbol esters also bind other receptors in the cell. For example, neuronal chimaerin binds phorbol esters with high affinity, sterospecificity and requirement for phospholipids enabling it to regulate $p21^{rac}$-GTPase activity (Wilkinson and Hallam, 1994). Together, this information raises the possibility that the effect of PMA on the transcriptional activation of the NPY-Y1 receptor gene may be a complex biphasic response to both short and long term effect of PMA on PKC activity on PC12 cells.

4.3.2.3.4 EFFECT OF THE PHOSPHOLIPASE D PATHWAY

DAG is a messenger generated either as a result of hydrolysis of PI by PLC (which also generates IP3, in turn releasing intracellular Ca^{++}) or via hydrolysis of PC by PLD. Diverse agents activates PLD in PC12 cells, which in turn hydrolyses PC to generate PA and choline (See Chapter 1. Phospholipase D). NGF also causes a rapid increase of DAG in PC12 cells, which peaks by about 2-3 minutes and declines to basal levels by 5 minutes. After its formation, DAG is rapidly phosphorylated to produce PA (Chang et al., 1989). However, there is a second wave of slow DAG production after 5 minutes of NGF treatment which suggest the activation of PLD (Chan et al., 1989).

PC12 cells were pretreated 1 hour with 1-butanol (0.3%) before the cells were treated with increasing concentrations of NGF (10-100 nM). 1-butanol (0.3%) inhibited the production of PA and in turn DAG in other cell systems, diverting the reaction to the production of phosphatidylbutanol (See Chapter 1. Phospholipase D). However, this treatment did not affect the NGF-induced increase in the transcriptional activation of the NPY-Y1 receptor gene in PC12 cells. These preliminary results sugests that PLD-activity is not involved in the NGF-induced increase of NPY-Y1 gene transcription in PC12 cells. However, direct measurements of PLD activity in PC12 cells treated with NGF are necesary for confirm this results.

4.3.3.5 EFFECT OF THE Ca^{++}/CALMODULIN KINASE II PATHWAY

KN-62 is a cell permeable and potent inhibitor of rat brain CaM-K II (Ki=900 nM) (Tokomitsu et al., 1990). KN-62 inhibits the CaM-dependent autophosphorylation of both, a (50 kDa) and b (60 kDa) subunits of CaM-K II. However, KN-62 did not inhibit the activity of already autophosphorylated (activated) CaM-K II (Tokomitsu et al., 1990). Kinetic analysis indicate that this inhibitory effect of KN-62 was competitive with respect to calmodulin but not ATP, however, KN-62 is not a calmodulin antagonists but a specific CaM-K II enzyme inhibitor. Thus, KN-62 affects the interaction between calmodulin and CaM-K II following inhibition of this kinase activity by directly binding to the calmodulin site of the enzyme (Tokomitsu et al., 1990).

KN-62 (1 μM) had no effect on the NGF-induced transcriptional activation of the NPY-Y1 receptor gene in PC12 cells. KN-62 (1 μM) also did not block the basal levels of luciferase activity of the pY1-LUC in PC12 cells. These results indicated that CaM-K II activity had no effect on the maintenance of the basal levels or in the NGF-induced transcriptional activity of the NPY-Y1 receptor gene in PC12 cells. This result directly supports others in showing that Ca^{++} signalling pathways itself are not involved in mediating NGF effects (See Chapter 1. Ca^{++} signalling pathway).

4.3.2.6 EFFECT OF THE PI-3-KINASE PATHWAY

NGF has been shown to stimulate the levels of PI-3,4-P_2 and PI-3,4,5-P_3 in PC12 cells, which reach a maximum after 3 minutes and decline after 10 minutes (Kimura et al., 1994). Pretreatment of PC12 cells with wortmannin (100 nM) for 30 minutes abolished the elevation of PI-3,4,5-P_3 level after NGF stimulation and completely inhibit PI-3-kinase activity (Kimura et al., 1994). Wortmannin did not affect the tyrosine phosphorylation of PLC-γ, Shc and MAPK. Transient inhibition of PI-3-K activity at the time of NGF stimulation had no effect on activation of *trkA* receptor tyrosine kinase, $p21^{ras}$ or neurite formation.However, continuous inhibition of PI-3-K blocked differentiation at the step just before neurite formation (Kimura et al., 1994) .

Wortmannin is a low-molecular weight cell-permeant hydrophobic compound with a sterol-type structure (Nakanishi et al., 1990,1992). This fungal metabolite isolated from *Talaromyces wortmannin* KY12420 is a potent inhibitor of PI-3-kinase. The inhibition is non-competitive with respect to phosphatidyl inositol or ATP and is irreversible (Nakanishi et al., 1990,1992). Wortmannin covalently binds the p110 catalytic subunit of PI-3-K in a region that is either within the active site or that functionally interacts with it. Wortmannin also inhibits other

kinases such as myosin light chain kinase (IC_{50} = 200 nM) and PI-4 kinase (IC_{50} = 300 nM) and also has been shown to block receptor-mediated PI-PLC, PLD and PLA2 in Swiss 3T3 cells (Nakanishi et al., 1990,1992) indicating that is not a specific inhibitor of PI-3-K. Inhibition of PI breakdown by wortmannin would certainly interfere with DAG formation and PKC activation and could, therefore, secondarily affect the PKC-dependent activation of PLD. Wortmannin also blocks $p70^{rsk}$ activation and in contrast to rapamycin does not inhibit PMA activation of $p70^{rsk}$ (Nakanishi et al., 1990,1992).

LY 294002 is a cell-permeable, potent and specific synthetic PI-3-K inhibitor that acts on the ATP-binding site of the enzyme (IC_{50} = 1.4 μM, Ki = 1.6 μM). Does not affect the activity of MAPK, PKC, $p90^{rsk}$ or $p60^{c\text{-}src}$ (Vlahos et al., 1994). Neither, LY 294002 (10 μM) or wortmannin (10 μM) inhibit the basal- or the NGF-induced increase in the transcriptional activation of NPY-Y1 receptor gene in PC12 cells. These results indicated that the PI-3-K signalling pathway had no effect on the maintenance of the transcriptional basal levels or the NGF-induced increase of the NPY-Y1 transcriptional activity in this cells.

4.3.2.7 EFFECT OF THE PKA PATHWAY

Evidence of the potential role of PKA in mediating NGF effects on target cell are contradictory. Early reports proposed that cAMP mediates the neurotrophic actions of NGF (Race and Wagner, 1985). NGF has been described to increased cAMP levels in PC12 cell after NGF exposure. PKA activity seems to be necessary for the NGF induced phosphorylation of TH, at least in some of the potential phosphorylation sites (Cremins et al., 1986). In addition, distinct cellular RNA and protein levels and morphology patterns have been identified to be induced by cAMP and NGF (Gunning et al., 1981). However, many other reports have refuted claims that NGF induces cAMP synthesis (Halegoua et al., 1991). PC12 cells containing a mutated regulatory subunit of PKA showed a comparable responses to parental cells in several aspects of the NGF induced differentiation such as neurite outgrowth and protein phosphorylation (Ginty et al., 1991). NGF induced activation of GAP-43, erg-1 and neurite development are all unaffected in these PKA-deficient cells suggesting that NGF is able to produce all these responses via PKA-independent mechanisms. However, PKA activity is necessary for the post-translational modification of Na^{++} channels in NGF treated PC12 cells (Ginty et al., 1992).

Moreover, H-89, dihydrochloride a synthetic isoquinolinesulfonamide, is a potent and selective inhibitor of PKA activity (Ki = 50 nM) (Chijiwa et al., 1990). Pretreatment of PC12 cells with H-89 (30 μM) led to a concentration-dependent inhibition of the forskolin-induced protein phosphorylation but the NGF-induced protein phosphorylation was not inhibited (Chijiwa et al., 1990).

Furthermore, early experiments showed that the NGF-induced priming of PC12 cells was different to the priming induced by DBC, an analogue of cAMP and activator of PKA. When PC12 cells were treated for several days with NGF and then replated in the presence of NGF, they regenerate their neurites within 24 hours. Instead, when PC12 cells were pre-treated with DBC and then replated in the presence of NGF they do not regenerate their neurites as rapidly (Greene, 1978). It is not clear whether NGF treatment of PC12 cells induces an increase in PKA activity in these cells. It has been suggested that NGF is able to affect cAMP metabolism, but by mechanisms independent of activation of adenylyl cyclase activity in PC12 cells as measured by conversion of [2- ^{3}H] adenine to [^{3}H] cAMP (Race and Wagner et al., 1985).

In concordance with these results, pretreatment of PC12 cells with H-89 (30 μM) had no effect on the NGF-stimulated transcriptional activation of the NPY-Y1 receptor gene in PC12 cells. However, H-89 (30 μM) reduced the basal levels of luciferase activity of the pY1-LUC fusion gene in PC12 cells (See Chapter 5), indicating that PKA is involved in the maintenance of the basal levels of transcriptional activity of the NPY-Y1 receptor gene in PC12 cells.

4.4 SUMMARY

1.- Effect of NGF on the promoter region of the NPY-Y1 receptor gene in PC12 cells is concentration dependent.

The threshold concentration of the NGF induction of NPY-Y1 receptor gene was 10 ng/ml. Increasing concentrations above this threshold resulted in significant increases in the levels of the luciferase activity.

2.- Effect of NGF on the NPY-Y1 transcriptional activation is dependent on *trkA* activation.

Pretreatment of the PC12 cells with K-252a, a specific inhibitor of *trkA* tyrosine kinase receptor completely blocked in a concentration dependent manner the NGF transcriptional activation of the NPY-Y1 receptor gene. However, K-252a had no effect on the maintenance of the basal levels of transcription of the NPY-Y1 receptor gene in PC12 cells.

3.- Effect of both, basal- and NGF-induced transcriptional activation of the NPY-Y1 gene is inhibited by a tyrosine kinase activity.

Genistein (50μM), a broad range inhibitor of tyrosine kinases increased both, the basal- and NGF-induced transcriptional activity of the NPY-Y1 receptor gene in PC12 cells. Sodium orthovanadate (Na_3VO_4) (30μM), a broad range inhibitor of protein tyrosine phosphatases

(PTPases) reduced both, the basal and NGF-induced transcriptional activity of the NPY-Y1 receptor gene in PC12 cells.

4.- Effect of NGF on the NPY-Y1 transcriptional activation is independent of p21^{ras} and is inhibited by MEK-1 activity.

4.1- NGF resulted in similar transcriptional activation of the NPY-Y1 receptor gene in a PC12-*ras* negative cell line, indicating that the p21^{ras} protein had no effect on the NGF-induced transcriptional activation of NPY-Y1 gene in PC12 cells.

4.2- PD 98059 (20 μM) resulted in a increase in both, basal- and NGF-dependent transcriptional activation of the NPY-Y1 receptor gene in PC12 cells. This PD 98059 (20 μM) increase of the NPY-Y1 transcriptional activity suggesting that NGF-dependent MEK-1 kinase activity, may inhibit the transcriptional activation of the NPY-Y1 receptor gene in PC12 cells.

5.- Effect of both, basal- and NGF transcriptional activation of the NPY-Y1 gene is dependent on PKC activation

5.1- Preincubation of PC12 cells with calphostin C decreased in a concentration dependent manner the basal-dependent and NGF-induced transcriptional activity of the NPY-Y1 gene.

5.2- DII1 and PMA, which are specific activators of PKC increased in a concentration-dependent manner the transcriptional activity of NPY-Y1 gene, and these activation was completely block by calphostin C (1 μM).

5.3- Direct measurement of PKC activity in PC12 cell extracts showed a increase following the addition of NGF. Similar increase in the PKC activity was observed using PMA (1nM) but not PMA (1 μM).

6.- Effect of NGF on the NPY-Y1 transcriptional activation is independent on PKA, CaM-K II , PI-3-K and PLD activation

6.1- Pretreatment of PC12 cells with H-89 (30 μM), a specific inhibitor of PKA had no effect in the NGF-stimulated transcriptional activation of the NPY-Y1 gene in PC12 cells. However, H-89 (30 μM) reduced the basal levels of transcriptional activity of this gene.

6.2- Pretreatment of PC12 cells with KN-62 (1 μM) a CaM-K II inhibitor, LY 294002 (10 μM) or wortmannin (10 μM), PI-3-K inhibitors had no effect in the basal- or NGF-induced transcriptional activation of the NPY-Y1 gene in PC12 cells.

6.3- Preliminary results indicated that pretreatment of PC12 cells with 1-butanol (0.3 %), which is known to inhibit PLD activity in other cells systems, had no effect in the basal- or NGF-induced transcriptional activation of the NPY-Y1 receptor gene in PC12 cells.

CHAPTER 5

PACAP-38 INDUCED EFFECTS IN THE TRANSCRIPTIONAL ACTIVATION OF THE NPY-Y1 RECEPTOR GENE IN PC12 CELLS

5.1 INTRODUCTION

5.1.1 PEPTIDERGIC REGULATION OF GENE EXPRESSION

Neuronal plasticity depends on the capacity of cells to make long-term adjustments to environmental stimuli. Neurons, in addition to the classical neurotransmitter, contain one or more peptides which have the potential role to acting as neurotransmitters.Neurons can produce a variety of peptides that are secreted from axons and dendrites and are collectively known as neuropeptides. These polypeptides are believed to serve as neurotransmitters and neuromodulators by their interaction with specific receptors after secretion and have been implicated in the control of behaviour as well as autonomic and motor functions. The co-existence and co-release of two or more messengers constitutes a fundamental principle in the nervous and endocrine system (Kupfermann, 1991). Co-transmission is a important signalling process which generates a more diverse set of responses by pre- or post-synaptic mechanisms.

In the autonomic nervous system, continuous low frequency stimulation of sympathetic fibers favour the preferential release of small classical neurotransmitter-containing vesicles while high-firing frequency stimulation in bursts causes the release of large vesicles containing peptides in addition to classical neurotransmitters (Bartfai et al., 1988). The response of post-ganglionic-sympathetic neurons in the sympathetic ganglia is regulated by interaction with multiple transmitters. Afferent fibres to the ganglia, such as pre-ganglionic sympathetic neurones (Krukoff, 1987), small intensily fluorescent cells (SIF) (Lars-Gösta et al., 1993) and primary sensory fibres (Quigg et al.,1990) store and release combinations of ACh, catecholamines and neuropeptides. PACAP and VIP are in pre-synaptic fibres of the sympathetic nervous system, including fibres innervating adrenal chromaffin cells. In PC12 cells, they exert a regulatory effect on the synthesis of tyrosine hydroxylase (TH) (Wessels-Reikeretal, 1991) and NPY (Colbert et al., 1994). Thus, trans-synaptic activation of TH and NPY synthesis in postganglionic sympathetic nerves via non cholinergic neuromoduladors, such as VIP and PACAP present in pre-ganglionic nerve terminals is implicated in the restoration of synaptic stores of cathecolamines and NPY.

Thus, the activity-dependent release of peptide neurotransmitters begins a cascade of events which culminates in post-synaptic changes in gene expression. Trans-synaptic activation requires, as its initial step, the binding of the peptide to its receptor. It is well established that interaction of catecholamines/peptides with their specific receptors leads to activation of two major intracellular signalling pathways: the PKA and the PKC pathways. Several receptor systems mediate postsynaptic changes in gene transcription through these second messenger

pathways, which are activated upon ligand binding. They link the neurotransmitter receptors to the molecular regulators of gene transcription (Armstrong and Montminty, 1993).

5.1.2 G-PROTEIN COUPLED RECEPTORS (GPCRs)

All biological systems have the ability to process and respond to enormous amount of information. Much of this information is provided to individual cells in the form of changes in concentration of hormones, growth factors and neuromodulators. Several families of cell surface receptors have been characterised that are coupled to different mechanisms of signal transduction; one of these are coupled to G-proteins (Simon et al., 1991). Signal transduction by G-proteins occur in two forms, the "small G proteins" that are found as single polypeptides and the heterotrimeric G-proteins that are made up of α, β, and γ subunits. Signal transduction by heterotrimeric G-proteins is initiated by ligand binding which stabilises an alternative conformational form of the receptor and thus transmits information across the cell membrane. The ligand-bound receptor initiates two processes: one leads to desensitisation and the other generates a signal that begins with the activation of the G-protein (Simon et al., 1991).

GPCRs can be further classified into three subfamilies, termed the rhodopsin/b-adrenergic, secretin/glucagon/VIP and metabotropic glutamate receptor families (Strader et al., 1995). The secretin/glucagon/VIP family includes receptors for the peptides secretin, glucagon, glucagon-like peptide I, parathyroid hormone, VIP, PACAP, calcitonin and growth hormone releasing hormone. These receptors share 25-50% amino acid identity among themselves and all contain an amino-terminal hydrophobic domain, which is presumed to served as signal sequence followed by a hydrophilic domain, preceding the seven transmembrane (TM) domains (Strader et al., 1995). All members of the secretin/glucagon/VIP subfamily of receptors are coupled to Gs and upon activation of intracellular cAMP (Strader et al., 1995). In addition to coupling to Gs, many of these receptors, such as glucagon, calcitonin, PACAP, VIP, among others, have been reported to activate other G-proteins, such as Gq, leading to increases in intracellular Ca^{++} levels and inositol phosphate hydrolysis. Thus, it appears that the potential to signal through multiple second messenger pathways may be a hallmark of this GPCR subfamily.

5.1.3 PACAP POLYPEPTIDE

PACAP was discovered as a consequence of a search for candidate novel hypothalamic-releasing hormones (Arimura, 1992a; Arimura and Shioda, 1995). The search strategy centred around the observations that known pituitary releasing hormones increase cAMP as a common response parameter in hypothalamic tissues. The search for a candidate hormone that increases cAMP in pituitary cell cultures resulted in the isolation of two substances from ovine

hypothalamus which are now known as PACAP-27 and PACAP-38 (Miyata et al., 1989). PACAP has a amino acid homology with VIP and has been localised by immunohistochemistry to CNS (e.g. hypothalamic area), gastrointestinal and respiratory tract, adrenal gland and testis (Arimura, 1992a). Receptors localised in CNS, pituitary, adrenal medulla and germ cells of the testis are highly specific for PACAP and not are shared with VIP (Arimura, 1992a).

They are two types of PACAP receptors on the basis of pharmacology and signalling activation and distribution (Harmar and Lutz, 1994; Journot et al., 1994; Arimura and Shioda, 1995). The type I receptor has a high affinity for PACAP but much reduced affinity for other members of the secretin/glucagon/VIP family while the type II receptor can bind PACAP and VIP with similar affinities (Arimura, 1992b). The PACAP type-I receptor (found in hypothalamus, brain stem, pituitary, adrenal gland and testes) specific for PACAP is coupled to adenylyl cyclase and PLC. The existence of a PACAP type II distinct from type I was characterised on the basis of pharmacology by nature of its similar affinities for VIP or PACAP (Harmar and Lutz, 1994). Thus, there is a consensus that the PACAP type II receptor is identical to the VIP type I receptor since their distribution, pharmacology and putative signalling coupling mechanisms appear to be the same (Harmar and Lutz, 1994).

PACAP is a hypophysiotrophic hormone, neurotransmitter, neuromodulator or vasoregulator that may play a multifunctional role in the CNS and PNS, with excitatory receptors linked to adenylyl cyclase (Tatsuno et al., 1991). The state of differentiation and secretory profile of mammalian cells of the SA neuronal lineage are altered by VIP but typically at 10^{-6} M or higher concentrations (Colbert et al., 1994; Okumura et al., 1994). However, the 27- and 38-amino acids forms of PACAP elevate cAMP levels and stimulate adenylyl cyclase in PC12 cells with an EC50 near 10^{-9} M (Deutsch and Sun, 1992). PACAP-38 is also a potent activator of the IP turnover in PC12 cells, elevating the content of IP by 8-fold ($EC_{50} = 7 \times 10^{-9}$ M) and is a effective inducer of neuronal morphology (Deutsch and Sun, 1992). In PC12 cells, PACAP has been shown to increase intracellular cytosolic Ca^{++} using two different mechanisms: release of intracellular stores and entry of external Ca^{++} (Hernandez et al., 1995) (Figure 5.1).

The change in neuronal pattern of morphology observed in PC12 cells following the addition of VIP or PACAP suggests that these peptides may play an important trophic role in CNS and PNS, in addition to the accepted neurotransmitter function in post-ganglionic sympathetic nerves. Thus, it is possible that, within pre-ganglionic sympathetic nerves, the trophic role of these peptides may be their primary action in the developing nervous system, whereas their regulation of sympathetic nerve function may occur latter once the specialised environment are achieved by steroid input to the adrenal medulla and the establishment of NGF dependence in the sympathetic nervous system.

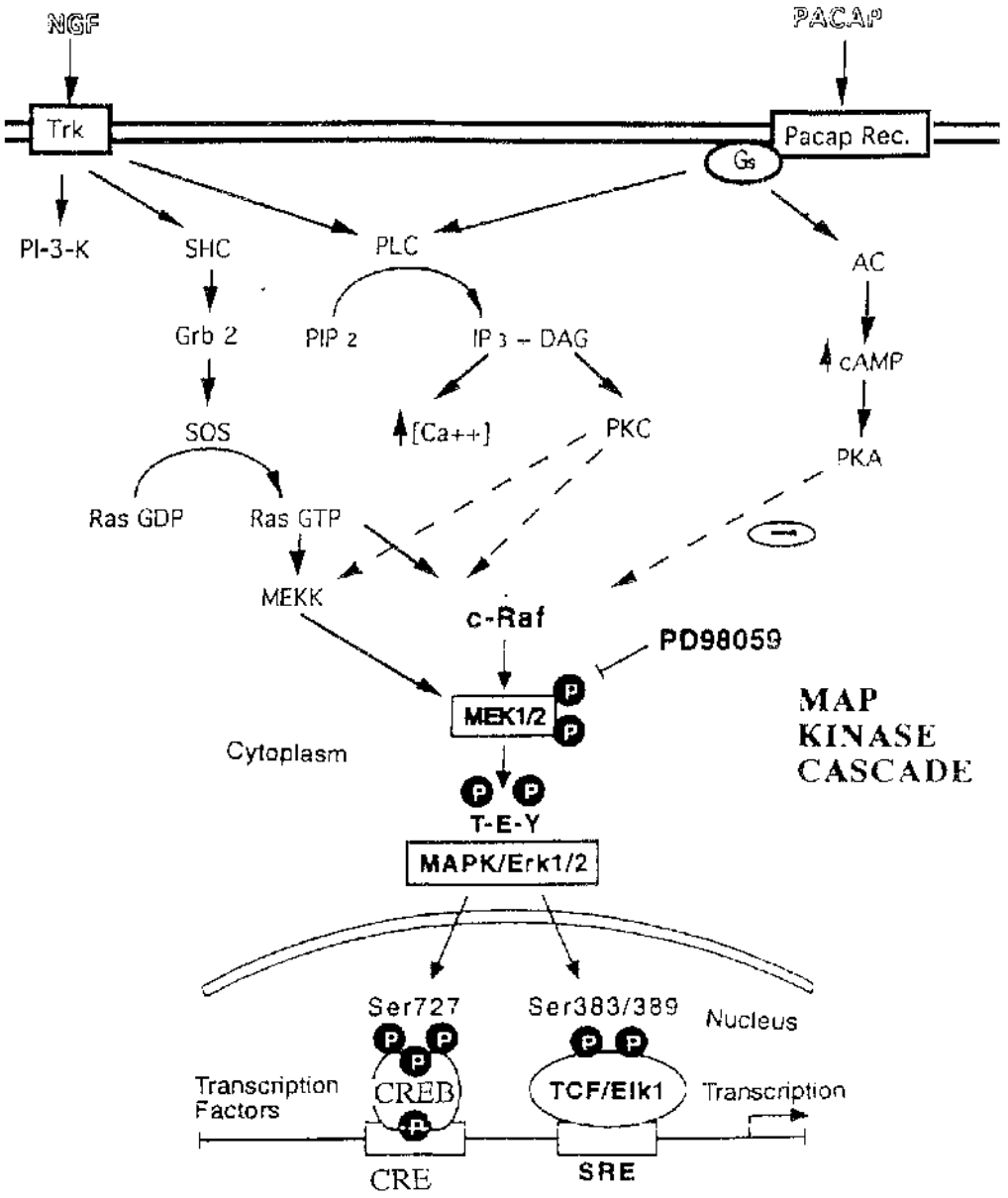


FIGURE 5.1 NGF-PACAP SIGNALLING PATHWAY

Schematic of the NGF-PACAP intracellular signaling pathways in PC12 cells.
NGF (nerve growth factor), **Trk** (p140^{trk} receptor). **PI-3-K** (phosphoinositol-3-kinase). **SHC** (*src* -homology collagen), **Grb2** (growth factor receptor bound protein-2). **SOS** (Son of sevenless protein). **MEKK** (Mitogen or extracellular-regulated kinase kinase). **Raf** (c-Raf kinase). **B-raf** (B-raf kinase). **MEK** (Mitogen or extracellular-regulated kinase). **ERK 1/2** (Extracellular signal-regulated kinase). **PACAP** (pituitary adenylyl cyclase activating polypeptide). **Gs** (Ga subunit protein). **AC** (adenylyl cyclase). **PKA** (protein kinase A). **PLC** (phospholipase C). **IP3** (inositol triphosphate). **DAG** (diacylglycerol). **Ca^{++}** (intracellular calcium). **PKC** (protein kinase C).

In this chapter, an attempt was made to further study the molecular mechanisms involved in the role of PKA, PKC and the *ras* -MAPK second messenger pathways in the PACAP-38 transcriptional activation of NPY-Y1 gene in PC12 cells by using the Dual-luciferase™ reporter system (Figure 5.1). The role of PACAP-38 was studied as there is evidence that this peptide increases NPY gene expression in PC12 cells (Colbert et al., 1994).

All the experiments were performed in duplicate and the results are shown as the mean ± SEM of at least three independent experiments. Untreated PC12 cells were grown in the presence of vehicle (up to 0.1%) in DMEM complete medium. Reporter vector, pRL-SV40 (Promega) was used as a internal control of the transfection efficiency in PC12 cells.

5.2 RESULTS

5.2.1 PACAP-38 INDUCE A CONCENTRATION DEPENDENT INCREASE IN THE NPY-Y1 TRANSCRIPTIONAL ACTIVITY

PC12 cells were shown to respond to PACAP-38 (5 nM) by differentiating into a neuronal-like phenotype (data not shown). PC12 cells co-transfected with the "experimental reporter vector" pY1-LUC (10 µg) fusion gene and the "control reporter vector" pRL-SV40 (1 µg), and treated with PACAP-38 (5 nM) for 48 hours resulted in a 2.79 ± 0.18-fold increase in the transcriptional activation of the NPY-Y1 gene compared to the basal levels (Figure 5-2). However, the levels of *Renilla* luciferase activity of PC12 cells treated with PACAP-38 (5 nM) were similar to the basal levels (data not shown). These results indicated that this reporter vector is a good internal control to discriminate between plate-to-plate transfection efficiencies in PC12 cells treated with PACAP-38 (5 nM).

PACAP-38-mediated increase of the transcriptional activation of the NPY-Y1 receptor gene in PC12 cells was concentration dependent (Figure 5-2). The threshold concentration of the PACAP-38 induction of NPY-Y1 receptor gene expression was 0.5 nM (Figure 5-2). Increasing concentrations above this threshold resulted in a increases in the levels of the luciferase activity. A value of 5 nM of PACAP was chosen as it lies in the linear region of the concentration response (Figure 5-2). Thus, any variation due to inhibition or stimulation of this PACAP-38 response could be easily detected.

These range of concentration (0.5-50 nM) of the PACAP-38 effect on the transcription of the NPY-Y1 gene is consistent with the binding affinity of the PACAP type I receptor and the biological potency of PACAP-38 in PC12 cells (Deutsch and Sun, 1992) suggesting receptor specificity and physiological relevance of the NPY-Y1 transcriptional stimulation by PACAP-38 in PC12 cells.

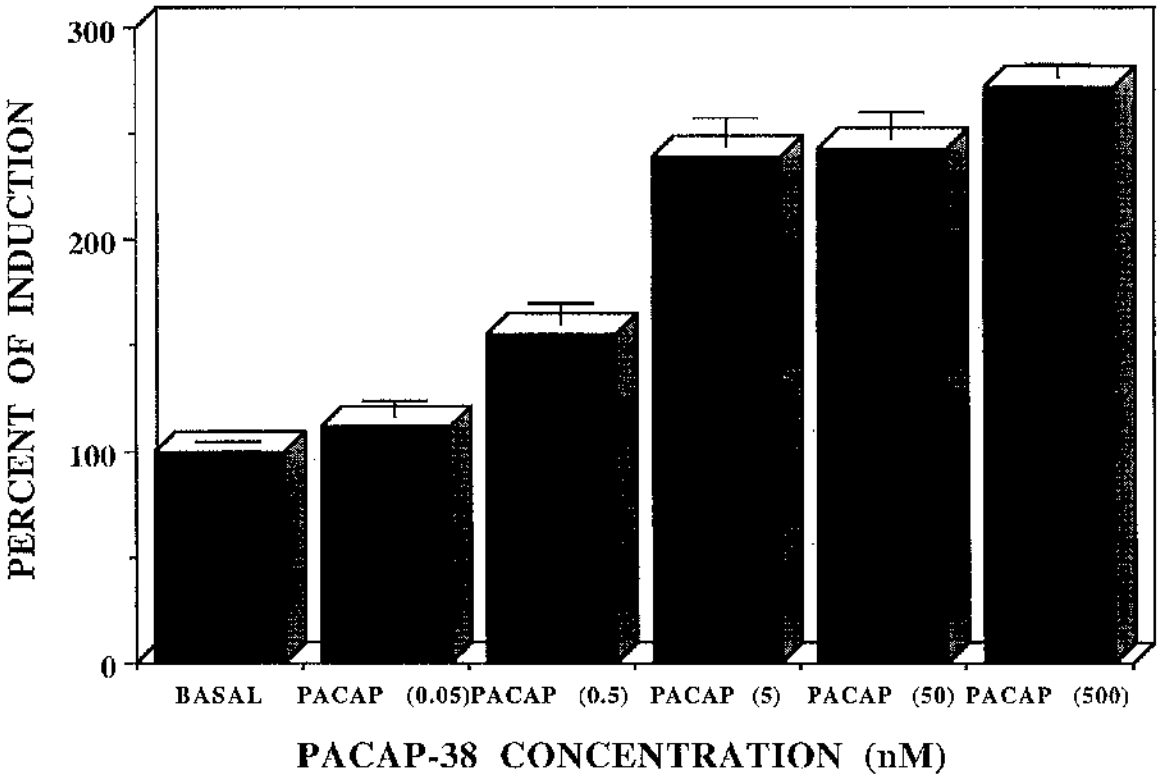


FIGURE 5.2 PACAP CONCENTRATION EFFECT

Effect of a range of PACAP-38 concentration on the transcriptional activation of the NPY-Y1 receptor gene. PC12 cells were co-transfected with pY1-LUC (10 μg) and pRL-SV40 (1 μg). 20 hours after transfection PC12 cells were treated with different **PACAP** (PACAP-38) concentrations for 48 hours when the cells were collected for Dual-luciferase assay (Promega). Each experiment represents the normalized luciferase activity (using the pRL-SV40 *Renilla* luciferase as internal control) in PACAP treated cells compared to that percent (100%) in the corresponding untreated cells (0.1% vehicle only). Values are mean ± SEM from 3 or more independent transfections experiments, each performed in duplicate with plasmids from at least two different preparations.

5.2.2 EFFECT OF THE PKA PATHWAY IN THE NPY-Y1 TRANSCRIPTIONAL ACTIVITY

5.2.2.1 EFFECT OF THE INCREASE OF INTRACELLULAR cAMP

Different pharmacological agents known to increase intracellular cAMP can activate the NPY-Y1 transcriptional activity in PC12 cells. Forskolin increases intracellular cAMP levels by direct binding and activation of adenylyl cyclase (EC_{50} = 10 μM) (Laurenza, 1989). This in turn increases the activity of PKA signalling pathway. Forskolin resulted in a concentration-dependent activation of the NPY-Y1 transcriptional activity in PC12 cells (Figure 5-3). The threshold of forskolin activation was 1 μM and a increasing concentrations above this threshold resulted in increases in the levels of luciferase activity (Figure 5-3). In the following experiments was used 10 μM of forskolin, as this concentration was in the linear range of transcriptional activation of the NPY-Y1 gene in PC12 cells and did not affect the levels of *Renilla* luciferase activity of the pRL-SV40 control vector (data not shown).

DBC permeates the cell membrane and is metabolised in the cell to generate the active cAMP analogue (Posternak and Weiman, 1974). DBC also results in a concentration-dependent increase in the NPY-Y1 transcriptional activation in PC12 cells (Figure 5-4). The threshold of DBC activation was 0.1 mM and increasing concentrations resulted in increases in the transcriptional activation of the NPY-Y1 gene (Figure 5-4). In the following experiments 1 mM DBC was used, as this concentration was in the linear range of pY1-LUC derived luciferase activity in PC12 cells and also did not increase or decrease the levels of *Renilla* luciferase activity of the pRL-SV40 control reporter vector (data not shown).

The levels of transcriptional activity of the NPY-Y1 receptor gene reached with forskolin (Figure 5-3) and DBC (Figure 5-4) were higher than the levels obtained with PACAP-38 (Figure 5-2). These results suggested that forskolin and DBC at these range of concentrations results in a more robust and/or long lasting activation of the cAMP-PKA pathway in PC12 cells than obtained with PACAP-38. Alternative, PACAP-38 can activate the Ca^{++} signalling pathway (Hernandez et al., 1995) and in consequence a CaM phosphodiesterase. Moreover, preliminary results indicated that forskolin (10 μM) and DBC (1 mM) act synergistically with PACAP-38 (5 nM) to increase the NPY-Y1 transcriptional activity in PC12 cells (data not shown). Taken together, these observations indicated that the activation of the cAMP-PKA pathway was involved in the PACAP-38 stimulation of the NPY-Y1 transcriptional activity in PC12 cells, as pharmacological agents which are well known to increase the intracellular levels of cAMP and in turn increase PKA activity in PC12 cells, mimic this PACAP-38 response.

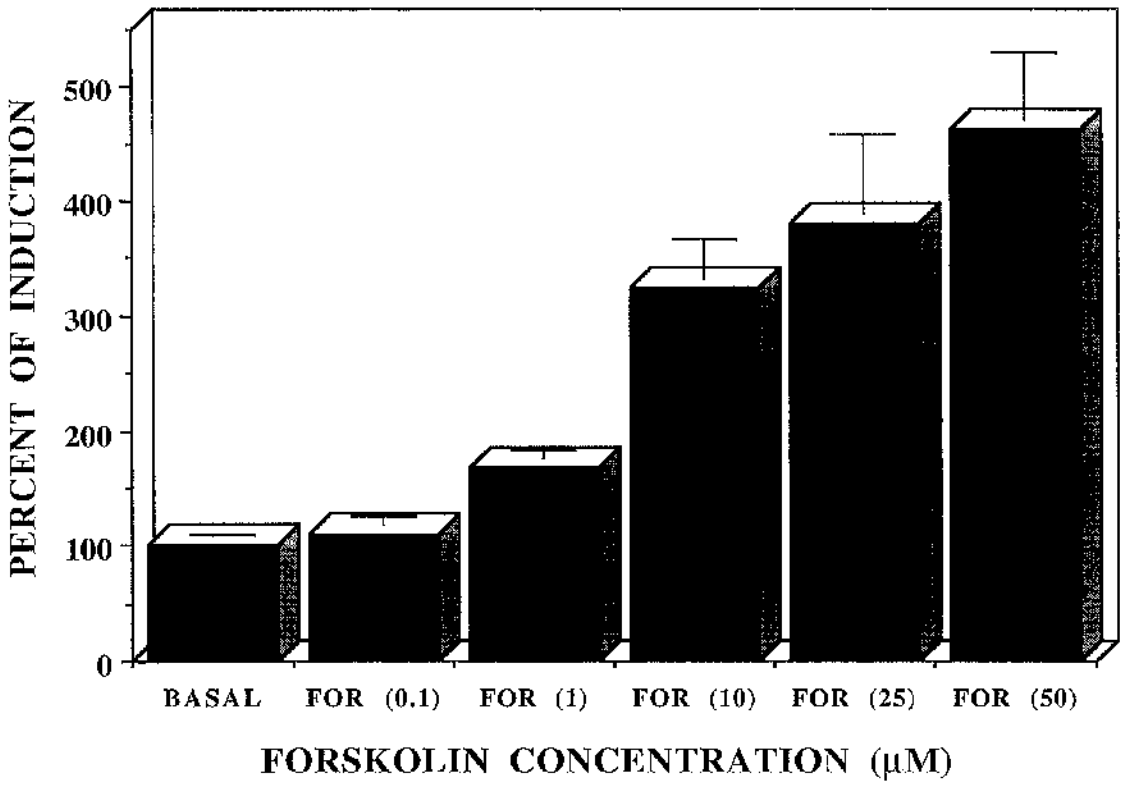


FIGURE 5.3 FORSKOLIN CONCENTRATION EFFECT

Effect of a range of forskolin concentration on the transcriptional activation of the NPY-Y1 receptor gene. PC12 cells were co-transfected with pY1-LUC (10 μg) and pRL-SV40 (1 μg). 20 hours after transfection PC12 cells were treated with different **FOR** (forskolin) concentrations for 48 hours when the cells were collected for Dual-luciferase assay (Promega). Each experiment represents the normalized luciferase activity (using the pRL-SV40 *Renilla* luciferase as internal control) in forskolin treated cells compared to that percent (100%) in the corresponding untreated cells (0.1% vehicle only). Values are mean ± SEM from 3 or more independent transfections experiments, each performed in duplicate with plasmids from at least two different preparations.

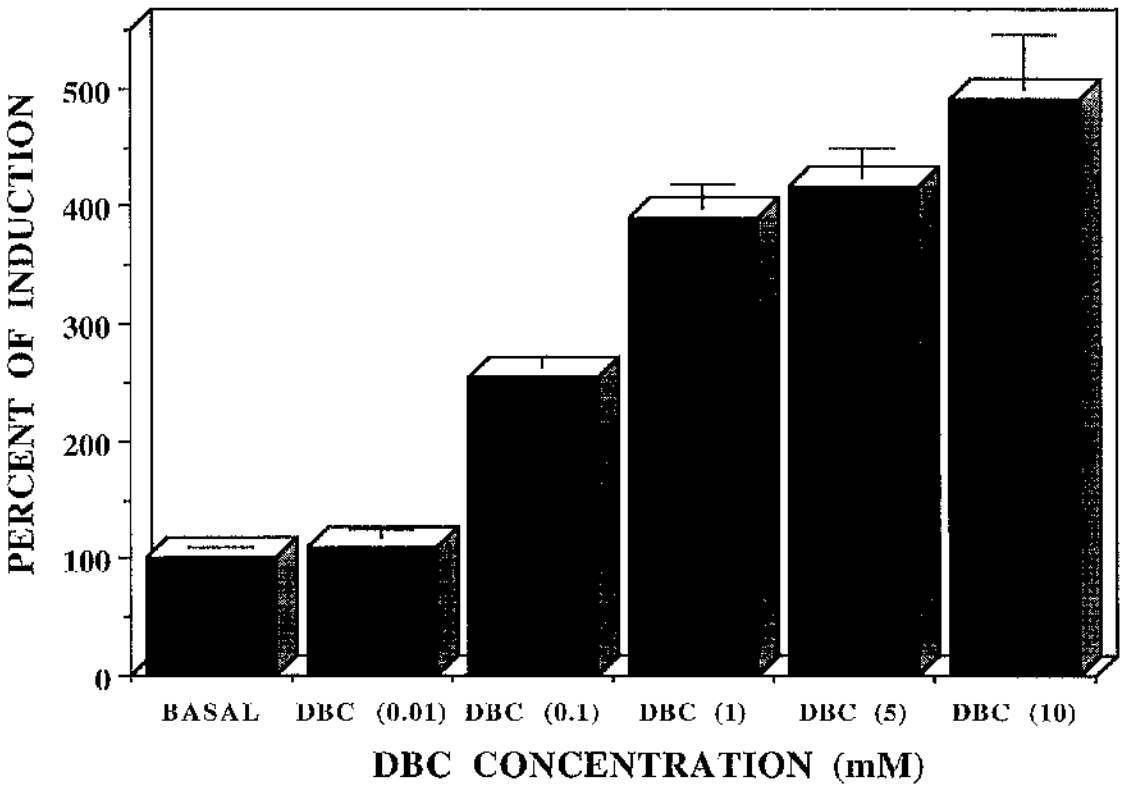


FIGURE 5.4 DBC CONCENTRATION EFFECT

Effect of a range of DBC concentration on the transcriptional activation of the NPY-Y1 receptor gene. PC12 cells were co-transfected with pY1-LUC (10 µg) and pRL-SV40 (1 µg). 20 hours after transfection PC12 cells were treated with different **DBC** (dibutyril cAMP) concentrations for 48 hours when the cells were collected for Dual-luciferase assay (Promega). Each experiment represents the normalized luciferase activity (using the pRL-SV40 *Renilla* luciferase as internal control) in DBC treated cells compared to that percent (100%) in the corresponding untreated cells (0.1% vehicle only). Values are mean ± SEM from 3 or more transfections experiments, each performed in duplicate with plasmids from at least two different preparations.

5.2.2.2 EFFECT OF PKA INHIBITOR (H-89)

In order to study the role of the PKA second messenger pathway in mediating the PACAP-38 activation of the NPY-Y1 receptor gene in PC12 cells, the effect of a specific inhibitor of this signalling pathway was examined. For all these experiments, PC12 cells were transiently co-transfected with pY1-LUC (10 µg) and pRL-SV40 (1 µg). The next day the cells were preincubated for one hour with H-89, a specific PKA inhibitor before addition of PACAP-38 (5 nM). PC12 cells were then preincubated with H-89 for 1 hour and then treated with PACAP-38 for 48 hours and the relative levels of firefly and sea pansy activity were measured in a luminometer using the Dual-Luciferase™ Reporter Assay kit (Promega). Both, H-89 (30 µM) and PACAP-38 (5 nM) did not increase the *Renilla* luciferase activity over basal levels (data not shown), indicating that the reporter vector pRL-SV40 was a good internal control for these experiments.

Pretreatment of PC12 cells with H-89, a potent and selective inhibitor of PKA (Ki = 50 nM) (Chijiwa et al., 1990) led to a concentration-dependent inhibition of the PACAP 38 (5 nM) induced increase in the NPY-Y1 transcriptional activity (Figure 5-5). Thus, the threshold concentration for H-89 inhibitory effect was 10 µM and 30 µM completely blocked this PACAP-38 response in PC12 cells. H-89 (30 µM) also reduced the basal levels of transcriptional activity of the NPY-Y1 receptor gene in PC12 cells (Figure 5-5), indicating that the PKA signalling pathway is also involved in the maintenance of the basal levels of transcription of the NPY-Y1 gene in PC12 cells. Moreover, DBC (1 mM) and forskolin (10 µM)-mediated transcriptional activation of the NPY-Y1 gene were completely blocked by H-89 (30 µM) in PC12 cells (data not shown).

These results using H-89 suggested that PC12 cells have an intrinsic PKA activity which is involved in the constitutive expression of the NPY-Y1 receptor gene in PC12 cells consistent with previous results which shown a constitutive binding of NPY-Y1 receptor agonist (DiMaggio et al., 1994). Moreover, the PKA second messenger pathway is also involved in the PACAP-38 (5 nM) mediated transcriptional activation of this gene. Finally, the H-89 (30 µM) block of the DBC and forskolin-mediated NPY-Y1 gene transcriptional activity confirms this previous result. Taken together, these results indicated that the cAMP-PKA second messenger pathway is involved in the transcriptional activity of this neuropeptide receptor gene in PC12 cells.

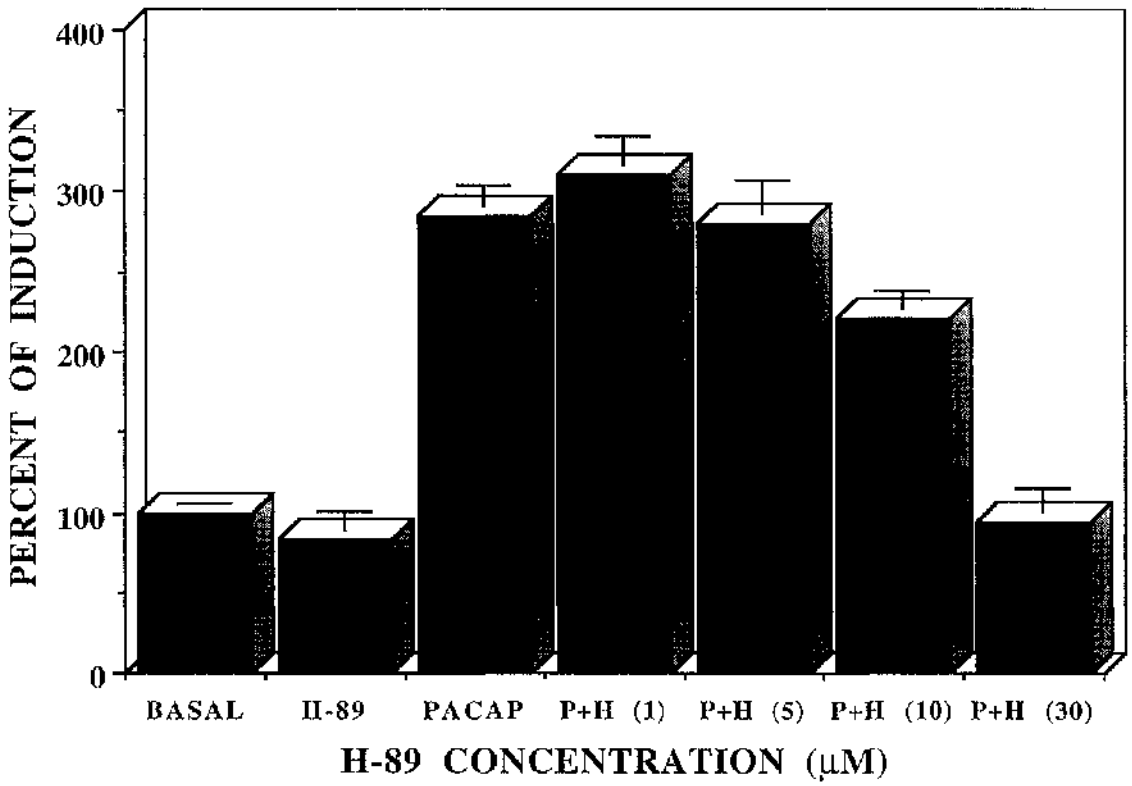


FIGURE 5.5 H-89 CONCENTRATION EFFECT ON THE PACAP RESPONSE

Effect of a range of H-89 concentration on the PACAP-38 induced transcriptional activation of the NPY-Y1 receptor gene. PC12 cells were co-transfected with pY1-LUC (10 μg) and pRL-SV40 (1 μg). 20 hours after transfection PC12 cells were pretreated for 1 hour with different **H-89** concentrations and then the cells were treated with **PACAP** (PACAP-38, 5 nM) (**P+H**) for 48 hours when the cells were collected for Dual-luciferase assay (Promega). As a control experiment the cells were treated with **H-89** (30 μM) and **PACAP** (PACAP-38, 5 nM) alone for 48 hours. Each experiment represents the normalized luciferase activity (using the pRL-SV40 *Renilla* luciferase as internal control) in treated cells compared to that percent (100%) in the corresponding untreated cells (0.1% vehicle only). Values are mean ± SEM from 3 or more transfections experiments, each performed in duplicate with plasmids from at least two different preparations.

5.2.3 EFFECT OF THE PKC PATHWAY IN THE NPY-Y1 TRANSCRIPTIONAL ACTIVITY

5.2.3.1 EFFECT OF PKC INHIBITOR (CALPHOSTIN C)

Preincubation of PC12 cells with calphostin C (1 μM), a highly specific inhibitor of PKC (Ki = 50 nM) (Kobayashi et al., 1989) reduced by 55% the PACAP-38 (313.3% ± 18.6%) mediated transcriptional activation of the NPY-Y1 receptor gene to 175% ± 7.07% (Figure 5-7). This concentration of calphostin C did not affect the levels of *Renilla* luciferase activity in comparison with the basal levels (data not shown). In the previous chapter calphostin C (1 μM) has also been shown to inhibit the basal levels of luciferase activity of the pY1-LUC in PC12 cells (Figure 4-7). These results suggested that the PKC second messenger pathway is involved in both, the basal- and the PACAP 38-induced transcriptional activity of the NPY-Y1 gene in PC12 cells.

5.2.4 EFFECT OF THE CaM KINASE II PATHWAY

KN-62 (1 μM), a specific inhibitor of a rat brain CaM-K II (Ki = 900 nM) (Tokumitsu et al., 1990) had no effect in the PACAP 38 (5 nM)-induced transcriptional activation of the NPY-Y1 gene in PC12 cells (data not shown). This result indicates that CaM-K II activity appear not be involved in the PACAP 38-induced transcriptional activation of the NPY-Y1 receptor gene in PC12 cells.

5.2.5 EFFECT OF THE RAS-MAP KINASE PATHWAY

PACAP-38, forskolin and DBC stimulate the MAPK activity in PC12 cells (Frödin et al., 1994; Young et al., 1994; Barrie et al., 1997). PACAP-38 (5 nM) is a efficient activator of MAPK, showing a rapid (5 minutes) and long lasting activity peak (Barrie et al., 1997). For this reason, the effect of the*ras* -MAPK pathway in the PACAP-38 mediated transcriptional activation of the NPY-Y1 gene was studied.

5.2.5.1 PACAP-38 EFFECTS IN A RAS-NEGATIVE PC12 CELL LINE

A stable mutant PC12 cell subline (PC12 asn17-W7) over-expressing a dominant inhibitory human $p21^{ras}$ gene (Feig and Cooper, 1988) was used to examine the role of the *ras* -MAP kinase pathway in mediating the effect of PACAP-38 on the transcriptional activation of the NPY-Y1 receptor gene. This cell line was transfected with the pY1-LUC fusion gene using the liposome-mediated method.

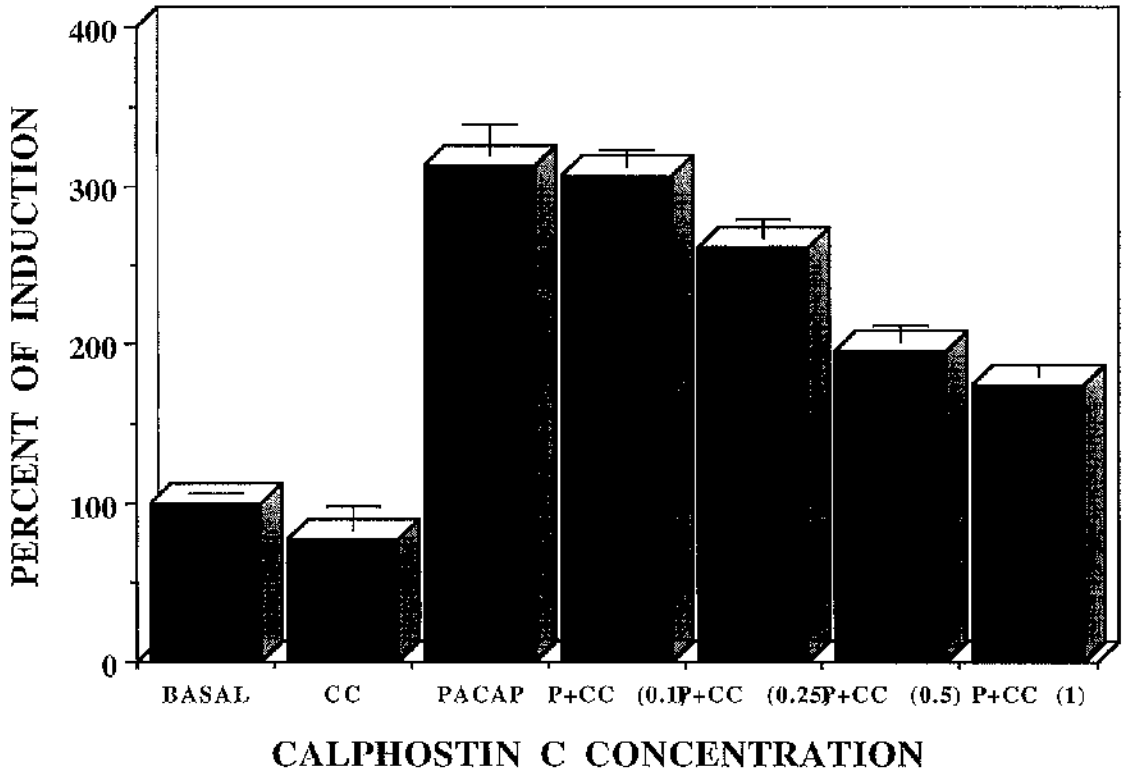


FIGURE 5.6 CALPHOSTIN C CONCENTRATION EFFECT ON THE PACAP RESPONSE

Calphostin C concentration effect in the PACAP-38 induced transcriptional activation of the NPY-Y1 receptor gene. PC12 cells were co-transfected with pY1-LUC (10 μg) and pRL-SV40 (1 μg). 20 hours after transfection PC12 cells were pretreated for 1 hour with different **CC** (Calphostin C) concentrations and then the cells were treated with **PACAP** (PACAP-38,5 nM) (**P + CC**) for 48 hours when the cells were collected for Dual-luciferase assay (Promega). As a control experiment the cells were treated with **CC** (Calphostin C, 1 μM) and **PACAP** (PACAP-38, 5 nM) alone for 48 hours.Each experiment represents the normalized luciferase activity (using the pRL-SV40 *Renilla* luciferase as internal control) in treated cells compared to that percent (100%) in the corresponding untreated cells (0.1% vehicle only). Values are mean ± SEM from 3 or more transfections experiments, each performed in duplicate with plasmids from at least two different preparations.

PACAP-38 (5 nM) induced similar morphological changes in PC12asn17-W7 compared to wild type cells (data not shown) and a similar increase of the MAPK activity (Barrie et al., 1997). PACAP-38 (5 nM) also resulted in a similar transcriptional activation of the NPY-Y1 receptor gene in the PC12-*ras* negative cell line in comparison to the PC12-wild type cells (Figure 5-7). This result indicated that the $p21^{ras}$ protein appear not be involved in the PACAP 38-induced transcriptional activation of NPY-Y1 receptor gene in PC12 cells.

5.2.5.2 EFFECT OF MEK-1 KINASE INHIBITOR (PD 98059)

PD 98059 is a inhibitor of MEK-1 known to block the increase in MAPK activity by NGF in PC12 cells (Alessi et al., 1995). In the previous chapter, PD 98059 (20 μM) resulted in a increase in both, the basal- and NGF-dependent (Figure 4-6) transcriptional activity of the NPY-Y1 gene in PC12 cells. Moreover, PD 98059 (20 μM) increased the PACAP-38 (5 nM) mediated transcriptional activation of the NPY-Y1 gene in PC12 cells (Figure 5-8). Taken together these results suggests that the increase of MEK-1 kinase activity mediated by PACAP-38 in PC12 cells has an inhibitory effect in the transcriptional activation of the NPY-Y1 gene.

5.3 DISCUSSION

In the present chapter has been demonstrated that the transcription of the NPY-Y1 gene increases in response to peptidergic stimulation (PACAP-38) and in response to several pharmacological agents known to increase cAMP and in turn PKA activity in PC12 cells. Furthermore, the role of both, the PKC and the *ras* -MAPK pathway in the PACAP-38 transcriptional activation of NPY-Y1 gene were studied using specific activators and inhibitors of each pathway, and a mutant PC12 *ras* -negative cell line (PC12asn17-W7).

5.3.1 PACAP-38 INDUCES A CONCENTRATION DEPENDENT INCREASE IN THE NPY-Y1 TRANSCRIPTIONAL ACTIVITY

PACAP induces adenylyl cyclase activity and elevates cytosolic Ca^{++} levels in adrenal tissue (Watanabe et al., 1995) and stimulation of the splanchnic nerve increases PACAP levels in adrenal perfusates (Wakade et al., 1992). PACAP has also been demonstrated to modulate catecholamine and NPY expression in rat SCG (May and Braas, 1995) and stimulate the phosphorylation of TH (Strong et al., 1992). Thus, like VIP, PACAP would appear to function as a physiological noncholinergic neurotransmitter in the rat SA system (Watanabe et al., 1995). Moreover, in other cells of this lineage, such as neonatal chromaffin cells (Wolf and Kriegistein, 1995) and PC12 cells (Deutsch and Sun, 1992; Colbert et al., 1994; Hernandez et al., 1995) which are well known to be responsive to VIP in micromolar amounts, PACAP stimulates neurite formation generally at more physiological (nanomolar) concentrations.

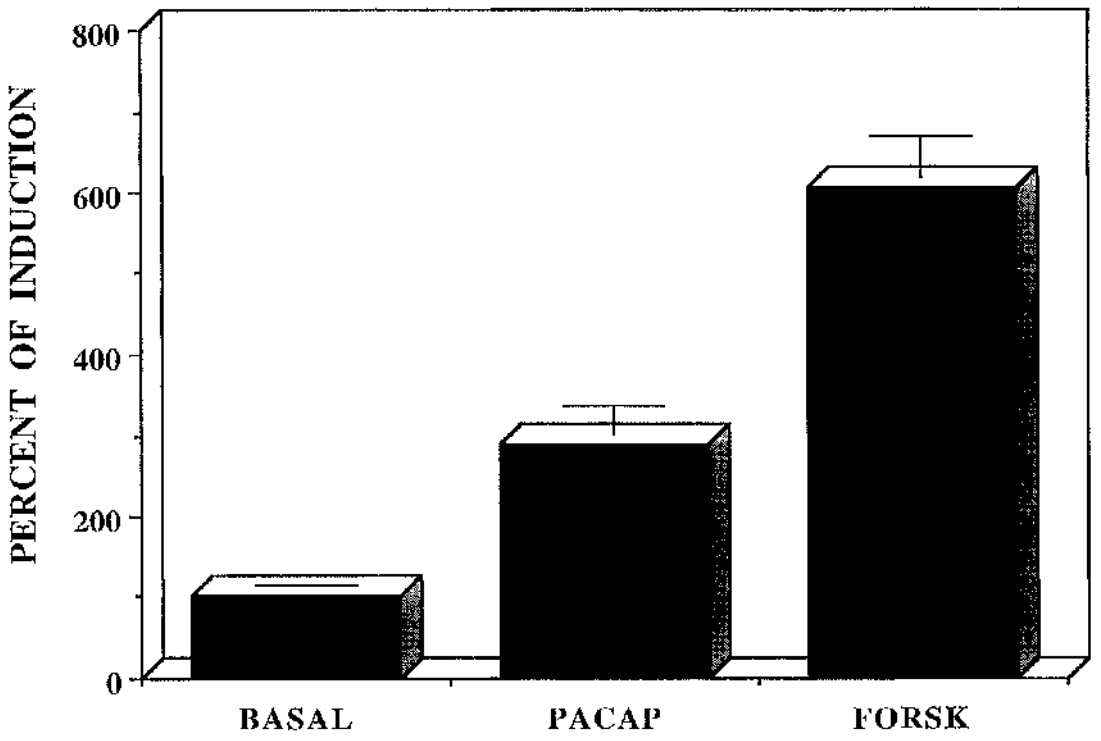


FIGURE 5.7 RAS EFFECT ON THE PACAP RESPONSE

Effect of PACAP and forskolin on the transcription of the luciferase gene directed by the promoter region of the NPY-Y1 receptor gene in a PC12-ras negative cell line (Buensuceso, 1995). PC12asn17-W7 cells were transfected with pY1-LUC (1-2.5 µg) using the liposome-mediated method. 20 hours after transfection the cells were treated with **PACAP** (PACAP-38, 5 nM) or **FORSK** (forskolin, 10 µM) for 48 hours and then the cells were collected for luciferase assay (Promega). Each experiment represents the luciferase activity of treated cells compared to that percent (100%) in the corresponding control cells (0.1% vehicle only). Values are mean ± SEM from 3 or more transfections experiments, each performed in duplicate with plasmids from at least two different preparations.

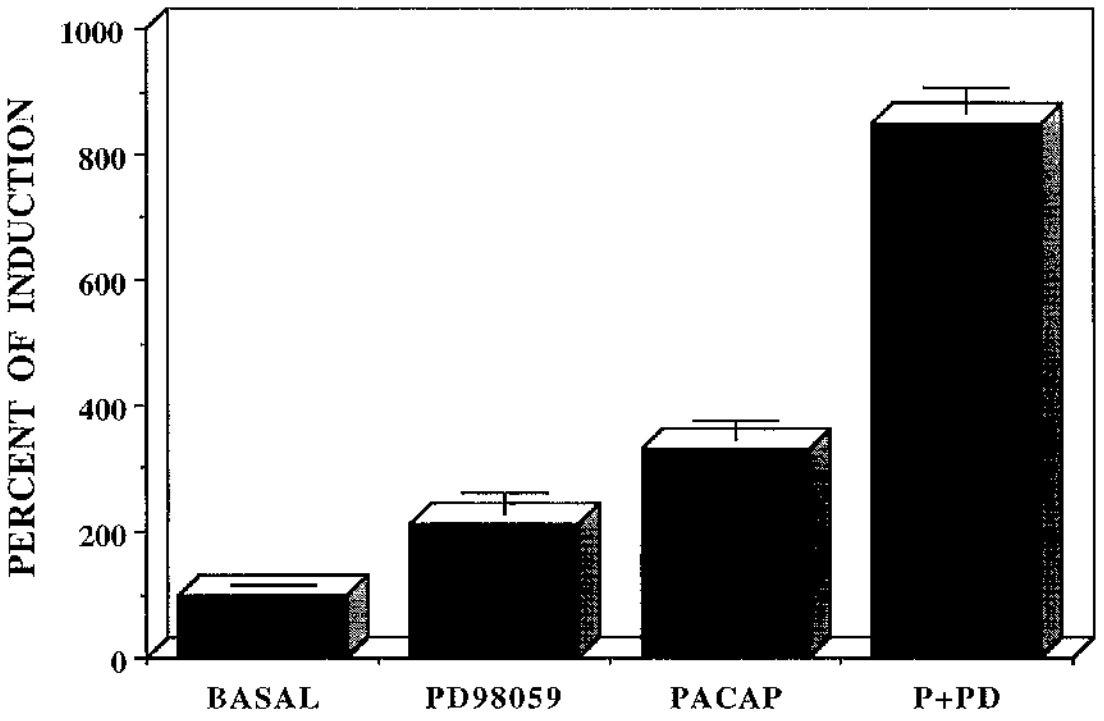


FIGURE 5.8 PD98059 EFFECT ON THE PACAP RESPONSE

PD98059 effect on the basal- and PACAP-dependent transcriptional activity of the NPY-Y1 receptor gene. PC12 cells were co-transfected with pY1-LUC (10 μg) and pRL-SV40 (1 μg). 20 hours after transfection PC12 cells were pretreated for 1 hour with **PD98059** (20 μM) and then the cells were treated with **PACAP** (PACAP-38, 5 nM) for 48 hours when the cells were collected for Dual-luciferase assay (Promega). As a control experiment the cells were treated with **PD98059** (20 μM) and **PACAP** (PACAP-38, 5 nM) alone for 48 hours. Each experiment represents the normalized luciferase activity (using the pRL-SV40 *Renilla* luciferase as internal control) in treated cells compared to that percent (100%) in the corresponding untreated cells (0.1% vehicle). Values are mean ± SEM from 3 or more transfections experiments, each performed in duplicate with plasmids from at least two different preparations.

PC12 cells were co-transfected with pY1-LUC and pRL-SV40, and treated for 48 hours with increasing concentrations of PACAP-38 (0.05 to 50 nM) (Figure 5-2). This experiment showed that PACAP-38 increased the transcriptional activity of the NPY-Y1 receptor gene in PC12 cells in a concentration-dependent fashion (Figure 5-2). The range of concentration of the PACAP 38-mediated increase (0.5 to 50 nM) was consistent with the binding affinity of the PACAP type I receptor and the biological potency of PACAP-38 in PC12 cells (Deutsch and Sun, 1992). These results suggested a receptor specificity and physiological relevance of the stimulation of the NPY-Y1 transcriptional activity by PACAP-38 in PC12 cells.

The 2.79 ± 0.18-fold increase in the transcriptional activity of the NPY-Y1 receptor gene mediated by PACAP-38 (5 nM) appear to be independent of variations on the transfection efficiencies as the levels of *Renilla* luciferase activity of PC12 cells co-transfected with the pRL-SV40 control vector and treated with PACAP 38 (5 nM) were similar to the levels of *Renilla* luciferase activity of untreated PC12 cells (data not shown). Previous results has been shown that the control reporter vector pRL-SV40 alone did not increase *Renilla* luciferase activity in response to PACAP-38 (5 nM) in PC12 cells (Figure 3-12). Thus, normalising the firefly luciferase activity of pY1-LUC to the sea pansy luciferase activity of pRL-SV40 eliminates inherent variabilities that can undermine experimental accuracy.

5.3.2 EFFECTS OF THE PKA PATHWAY IN THE NPY-Y1 TRANSCRIPTIONAL ACTIVITY

cAMP functions to coordinate diverse metabolic process by mechanisms which are pleiotropic in nature, altering numerous steps in the metabolic pathway simultaneously. Since the discovery of PKA, the mechanisms of action of a number of additional extracellular signals have been found to involve regulation of the formation of cAMP and thereby control of its target enzyme, PKA (See Chapter 1. cAMP in signalling pathway).

The biologically relevant effects of cAMP are mediated by activation of the PKA. PKA is a tetramer consisting of two regulatory subunits and two catalytic subunits. Upon binding of cAMP to the regulatory subunit, the holoenzyme dissociates to yield a regulatory subunit dimer and two free catalytic subunits which are available to phosphorylate cellular substrates. The active catalytic subunit (c-PKA) phosphorylate several cytoplasmatic substrates. In addition, a fraction of the c-PKA translocates to the nucleus where it phosphorylates nuclear transcription factors that are responsible for the activation of cAMP-induced genes (Roesler et al., 1988). PKA is found in two classes which differ in their regulatory subunits: PKA type I is soluble and widely expressed, whereas the PKA II isoforms, PKAIIα and PKAIIβ, are membrane anchored and particularly abundant in neuroendocrine cells, such as PC12 cells (Cassano et al., 1996).

PC12 cells, extend neurites in response not only to NGF but also to cAMP-enhancing agents, however these neurites appear to be different both in their length and in the mechanism by which they are formed. Elevation in intracellular levels of cAMP in PC12 cells also increases TH enzyme activity by both, PKA phosphorylation and by activating transcription and also increase phosphorylation of ribosomal S6 and histone H2b (Ginty et al., 1991). In addition, DBC and forskolin increase the expression of neuronal-specific genes, such as egr-1 (IEGs), VGF and GAP-43 (LRGs) in PC12 cells (Ginty et al., 1991; Okumura et al., 1994) (See Chapter 1. cAMP in signalling pathway).

5.3.2.1 EFFECT OF THE INCREASE OF INTRACELLULAR cAMP

Previous results have shown that PACAP-38 results in a increase in neurite density in PC12 cells (Deutsch and Sun, 1992; Colbert et al., 1994; Hernandez et al., 1995). This morphological change is mimicked using different pharmacological agents known to increase intracellular levels of cAMP in PC12 cells, such as forskolin (10 μM) or DBC (1 mM) (Hernandez et al., 1995). Morever, forskolin also stimulates NPY expression in PC12 cells (Higuchi et al., 1988) and rat brain cells (Magni and Barnea, 1992). VIP and PACAP-38 also increase the transcriptional activity of the NPY gene in PC12 cells (Colbert et al., 1994).

Results shown in the previous section of this chapter demonstrated that PACAP-38 increased in a concentration-dependent manner the NPY-Y1 transcriptional activity in PC12 cells (Figure 5-2). Forskolin is a diterpene isolated from the Indian plant *Coleus forskohlii* which activates adenylyl cyclase in membranes and intact cells (EC_{50} = 5-10 μM) (Laurenza, 1989). This activation of adenylyl cyclase by forskolin is rapid, reversible and does not require guanine nucleotides. The increase in cAMP in turn increases the activity of PKA (Laurenza, 1989). Forskolin also increased in a concentration-dependent fashion the NPY-Y1 transcriptional activity in PC12 cells (Figure 5-3). The range of concentration (10-30 μM) of the forskolin effect in the transcriptional activity of the NPY-Y1 gene was consistent with the known biological potency (eg. PKA activation) of this pharmacological agent in PC12 cells (Balbi and Allen, 1994).

Dibutyryl-cAMP (DBC) is a cell-permeable cAMP analogue that specifically activates PKA (Posternak and Weiman, 1974). DBC also resulted in a concentration-dependent transcriptional activation of the NPY-Y1 receptor gene in PC12 cells (Figure 5-4). Preliminary results indicate that the DBC (1 mM) and forskolin (10 μM) transcriptional activation of the NPY-Y1 gene was synergistic with PACAP-38 (5 nM) supporting a role of the cAMP-PKA pathway in this PACAP-38 mediated response in PC12 cells (data not shown).

However, the increase of NPY-Y1 transcriptional activity reached with forskolin and DBC was higher than the maximal levels obtained with PACAP-38, suggesting that both forskolin and DBC induced increases of the intracellular levels of cAMP and in turn activation of the PKA pathway in PC12 cells were in a more robust or/and long lasting way than the obtained by PACAP-38. Direct measurements of the PKA activity in PC12 cells following PACAP-38, forskolin and DBC treatment will be necessary to answer this hypothesis.

PACAP-38 has been shown to increase the intracellular levels of Ca^{++} in PC12 cells (Hernandez et al., 1995), which in turn could activate CaM-dependent enzymes such as the type I phosphodiesterase or calcineurin, a serine/ threonine phosphatase. cAMP is hydrolysed by phosphodiesterases in the cell, leading to cessation of the cAMP dependent biological effects. At least five major class of phosphodiesterases have been reported that differ in their kinetic properties (See cAMP in signalling pathway). The type I phosphodiesterases are CaM-dependent and selectively inhibited by 8-methoxymethyl-3-isobutyl-1-methylxanthine (IBMX). Further experiments with specific inhibitors of the CaM-dependent phosphatases or phosphodiesterases, will be necessary to determine the involvement of these enzymes in the PACAP-38 induced transcriptional activation of the NPY-Y1 gene in PC12 cells.

5.3.2.2 EFFECT OF PKA INHIBITOR (H-89)

H-89 is a potent and selective inhibitor of the PKA activity (Ki = 50 nM) in competitive fashion against ATP in PC12 cells (Chijiwa et al., 1990). Pretreatment of PC12 cells with H-89 (30 μM) results in a inhibition of the forskolin-induced protein phosphorylation, with no decrease in intracellular cAMP levels (Chijiwa et al., 1990) and also significantly inhibited the forskolin- and DBC-induced neurite outgrowth (Chijiwa et al., 1990).

H-89 had been shown to result in a concentration-dependent inhibition of the PACAP-38 (5 nM) mediated increase in the NPY-Y1 transcriptional activity in PC12 cells (Figure 5-5). H-89 (30 μM) results in a complete inhibition of this PACAP-38 (5 nM) response in PC12 cells. Moreover, this is the same concentration use for block the biological effects (eg. neurite formation) of forskolin and DBC in PC12 cells (Chijiwa et al., 1990). Furthermore, the DBC (1 mM) and forskolin (10 μM) mediated transcriptional activation of the NPY-Y1 gene were blocked by H-89 (30 μM) in PC12 cells (data not shown). Taken together, these results indicated a cAMP-PKA involvement in the PACAP-38 (5 nM) transcriptional activation of the NPY-Y1 receptor gene in PC12 cells.

5.3.3 EFFECTS OF THE PKC PATHWAY IN THE NPY-Y1 TRANSCRIPTIONAL ACTIVITY

VIP, when added at 10^{-6} M, is quite effective in promoting differentiation and survival of PC12 cells (Colbert et al., 1994; Okumura et al., 1994). VIP added to SA cells at 10^{-6} M not only activates adenylyl cyclase, but also has been found to activate the PI pathway (Malhotra et al., 1989). PACAP-38 but not PACAP-27 is also a potent activator of the inositol phospholipid signalling cascade in PC12 cells, elevating the content of inositol phosphates by 8-fold at 10^{-8} M ($EC_{50} = 7 \times 10^{-9}$ M) (Deutsch and Sun, 1992). PACAP-38 at 10^{-8} M is also an effective inducer of neuronal morphology in PC12 cells, whereas PACAP-27 is much less potent in promoting neurite outgrowth (Deutsch and Sun, 1992).

Speculatively, other signalling molecules stimulated as part of the PI cascade may also play a role in the PACAP-38 response in PC12 cells, such as PKC and CaM-K II. Pharmacological activators of PKC, like phorbol esters (PMA) and DH1 increased the NPY-Y1 transcriptional activity in a concentration-dependent manner (Figures 4-8 and 4-9). For this reason, the effect of the PKC signalling pathway in the PACAP38 mediated NPY-Y1 transcriptional activation in PC12 cells was studied.

5.3.3.1 EFFECT OF PKC INHIBITOR (CALPHOSTIN C)

Preincubation of the PC12 cells with calphostin C, a specific inhibitor of PKC (Ki = 50 nM) (Kobayashi et al., 1989) reduced the PACAP-38-mediated NPY-Y1 transcriptional activation in a concentration-dependent fashion (Figure 5-6). PACAP-38 (5 nM) response was markedly reduced (55%) following preincubation with calphostin C (1 μM) but the forskolin and DBC stimulation were not affected by calphostin C (1 μM) (data not shown), indicating that at this concentration, calphostin C selectively blocks the PKC-dependent transcriptional activation of the NPY-Y1 receptor gene in PC12 cells. These results suggested that PKC activity was also involved in the PACAP 38-mediated transcriptional activation of this neuropeptide receptor gene in PC12 cells. Taken together, these results suggests that both PKA and PKC pathways are involved in the PACAP-38 activation of the NPY-Y1 transcription in PC12 cells.

5.3.4 EFFECT OF THE CaM KINASE II PATHWAY

Previous studies suggested that the CaM-dependent and the cAMP-PKA signalling pathways could converge in their effects on some gene regulatory events in PC12 cells (Sheng et al., 1990). In addition, exposure of PC12 cells to PACAP-38 resulted in elevation of intracellular levels of Ca^{++} in PC12 cells (Hernandez et al., 1995). KN-62 is a cell-permeable and specific

inhibitor of CaM-K II in PC12 cells (Ki = 900 nM) (Tokumitsu et al., 1990). However, KN-62 (1 μM) had no effect in the PACAP 38-induced transcriptional activation of the NPY-Y1 receptor gene in PC12 cells (data not shown). This result indicated that CaM-K II activity has no effect on the PACAP 38-induced transcriptional activity of the NPY-Y1 receptor gene in this cell line.

5.3.5 EFFECT OF THE RAS-MAP KINASE PATHWAY

MAPKs are activated not only by stimulation of RTKs, but also by GPCRs (See Chapter 1, G-protein as mitogens). Different pharmacological agents known to increases cAMP, such as IBMX, cholera toxin, forskolin and DBC has been shown to stimulate MAPKs activity in PC12 cells (Frödin et al., 1994; Young et al., 1994). PACAP-38 has been shown to be an activator of MAPKs (Barrie et al., 1997). PACAP-38 stimulation of MAPKs is inhibited by PKC down-regulation and preincubation with calphostin C, a specific PKC inhibitor (Barrie et al., 1997). PMA also has been shown to promote a sustained activation of MAPK (Frödin et al., 1994; Young et al., 1994). These results suggests that MAPKs cascade may integrate and amplify signals originating from receptors employing both PKA- and PKC-dependent pathways, such as the PACAP type I receptor.

5.3.5.1 PACAP-38 EFFECTS IN A RAS-NEGATIVE PC12 CELL LINE

PACAP-38 (5 nM) results in a transcriptional activation of the NPY-Y1 receptor gene in the PC12-*ras* negative cell line in a similar manner to the PC12-wild type cells (Figure 5-7). This result indicated that the $p21^{ras}$ protein is not involved in mediating the PACAP 38-induced activation of NPY-Y1 receptor gene in PC12 cells.

5.3.5.2 EFFECT OF THE MEK-1 KINASE INHIBITOR (PD 98059)

PACAP-38 results in a activation of MEK-1 which was overall similar to that the observed with cAMP in combination with PMA (Frödin et al., 1994). PD 98059 has also been shown to inhibited the PACAP 38-mediated activation of the MAPKs in PC12 cells (Barrie et al., 1997). Results in the previous chapter has been shown that PD 98059 (20 μM) results in a increase in both the basal- and NGF-dependent (Figure 4-6) transcriptional activation of the NPY-Y1 gene in PC12 cells. PD 98059 (20 μM) also increase the PACAP 38-mediated transcriptional activity of the NPY-Y1 gene in PC12 cells (Figure 5-9). This results suggests that PACAP-38 mediated increase of MEK-1 kinase activity has an inhibitory effect in the transcriptional activation of the NPY-Y1 receptor gene in PC12 cells.

5.4 SUMMARY

1.- PACAP-38 induce a concentration dependent transcriptional activation of the NPY-Y1 gene in PC12 cells.

The threshold concentration of the PACAP 38 induction of NPY-Y1 receptor gene was 0.5 nM. Increasing concentrations above this threshold resulted in significant increases in the levels of the luciferase activity .

2.- PACAP-38 (5 nM) induce a transcriptional activation of NPY-Y1 gene in a PKA-dependent manner in PC12 cells.

2.1- Pharmacological agents known to increase cAMP, such as forskolin and DBC mimicked the PACAP-38 effect on the NPY-Y1 transcription.

2.1.1- Forskolin increased in concentration-dependent manner the NPY-Y1 transcriptional activity in PC12 cells. The threshold of forskolin activation was 1 μM and increasing concentrations increase the transcriptional activation of the NPY-Y1 receptor gene.

2.1.2- DBC increased in concentration-dependent manner the NPY-Y1 transcriptional activity in PC12 cells . The threshold of DBC activation was 0.1 mM and increasing concentrations increase the levels of luciferase activity.

2.2- A specific PKA inhibitor (H-89) significantly block the PACAP-38 (5 nM) activation of the NPY-Y1 transcription

Pretreatment of PC12 cells with H-89 led to a concentration-dependent inhibition of the PACAP 38-dependent increase in the NPY-Y1 transcriptional activity. H-89 (30 μM) completely reduced this PACAP-38 response in PC12 cells, indicating that in this transcriptional activation is involved PKA activity.

2.3- A specific PKA inhibitor (H-89) block the forskolin (10 μM) and DBC (1mM) activation of NPY-Y1 transcription.

DBC (1 mM) and forskolin (10 μM)-mediated transcriptional activation of the NPY-Y1 gene were blocked by H-89 (30 μM) in PC12 cells, indicating that in this forskolin and DBC-mediated response was involved PKA activity.

3.- PACAP-38 induce a transcriptional activation of the NPY-Y1 gene in a PKC-dependent manner in PC12 cells.

3.1- A specific PKC inhibitor (calphostin C) significantly inhibited the PACAP-38 (5 nM) activation of the NPY-Y1 transcription.

Preincubation of PC12 cells with calphostin C resulted in a concentration-dependent reduction on PACAP-38 (5 nM) mediated increase in the NPY-Y1 transcriptional activity. Calphostin C (1 μM) significantly reduced (55%) this PACAP-38 response indicating that in this transcriptional activation was involved PKC activity.

3.2- A specific PKC inhibitor (calphostin C) did not reduced the forskolin (10 μM) and DBC (1 mM) activation of the NPY-Y1 transcription.

Pretreatment of PC12 cells with calphostin C (500 nM) did not affect the forskolin (10 μM) and DBC (1 mM) mediated transcriptional activation of the NPY-Y1 gene, indicating that this forskolin and DBC response was independent of PKC activation (data not shown).

4.- PACAP-38 (5 nM) induced a activation of the NPY-Y1 transcription in a Ca^{++}/CaM kinase II independent manner in PC12 cells.

Pretreatment of the PC12 cells with KN-62 (1 μM) did not no affect the PACAP-38 (5 nM) induced transcriptional activation of the NPY-Y1 receptor gene in PC12 cells, indicating that this PACAP-38 response was independent of CaM-K II activity.

5.- PACAP-38 (5 nM) induced a activation of the NPY-Y1 transcription in a *ras* -independent manner in PC12 cells.

PACAP-38 (5 nM) results in similar transcriptional activation of the NPY-Y1 receptor gene in the PC12-*ras* negative cell line in comparison to the PC12-wild type PC12 cells, indicating that in this PACAP-38 response is not involved the $p21^{ras}$ protein.

6.- PACAP-38 (5 nM) induced activation of the NPY-Y1 transcription was inhibited by MEK-1 kinase activity.

Pretreatment of the PC12 cells with PD 98059 (20 μM) increase the PACAP-38 (5nM) mediated activation of the NPY-Y1 transcription (Figure 5-9), indicating that the PACAP-38 induced MEK-1 kinase activity has an inhibitory effect in the transcriptional activation of this neuropeptide receptor gene.

CHAPTER 6

MUTAGENESIS STUDIES OF THE PROMOTER REGION OF THE RAT NPY-Y1 RECEPTOR GENE

6.1 INTRODUCTION

6.1.1 MUTAGENESIS TECHNIQUES

Alteration of a DNA sequence by specific chemical, physical or enzymatic methods (mutagenesis) is a valuable technique in molecular biology. These techniques allow very precise structural genetic manipulations and are used in many applications, such as DNA sequencing, cloning, DNA structural analysis, protein expression studies and the study of structure-function relationship of cloned gene products. These techniques also offer the possibility to study in great detail the interaction between a particular gene and the machinery involved in regulating its expression. The effect of such manipulations can then be studied in suitable cell lines or even animal models. Mutagenesis techniques can be classified on the type of mutation introduced (deletion, insertion or substitution), as well as by the method (random or sequence-specific).

The location of functional elements within a DNA fragment are often determined by making a set of unidirectional deletions. This random method takes advantage of the unique properties of Exonuclease III (Exo III) which will specifically digest DNA from 5'-protruding or blunt-end restriction site, but not 3'-overhangs (Henikoff, 1987). Oligonucleotide or site-directed mutagenesis is another technique that enables the researcher to create very specific modifications of a cloned DNA (Kunkel, 1985). By using this technique the role of specific sequences in the regulatory regions of genes can be studied in detail by deleting, exchanging or adding sequences to the putative regulatory regions of a gene. In this chapter, another technique, the polymerase chain reaction (PCR) was used to make specific deletions of the promoter region of the NPY-Y1 gene.

PCR is a relative simple technique by which a DNA template is amplified many thousand- or million-fold quickly and reliably (Mullis and Faloona, 1987). PCR process is exquisitely sensitive. By amplifiying just a small portion of a nucleic acid target, this technique effectively isolates that portion from the rest of the nucleic acids. Using this technique, were created specific deletions in the promoter region of the NPY-Y1 gene that can be difficult to create using other techniques. One approach was made specific deletions of the putative DNA binding sites for transcription factors, such as the AP-1 and the GRE site (Figure 6-1). Another approach was made specific progressive deletions in this promoter region (Figure 6-2).

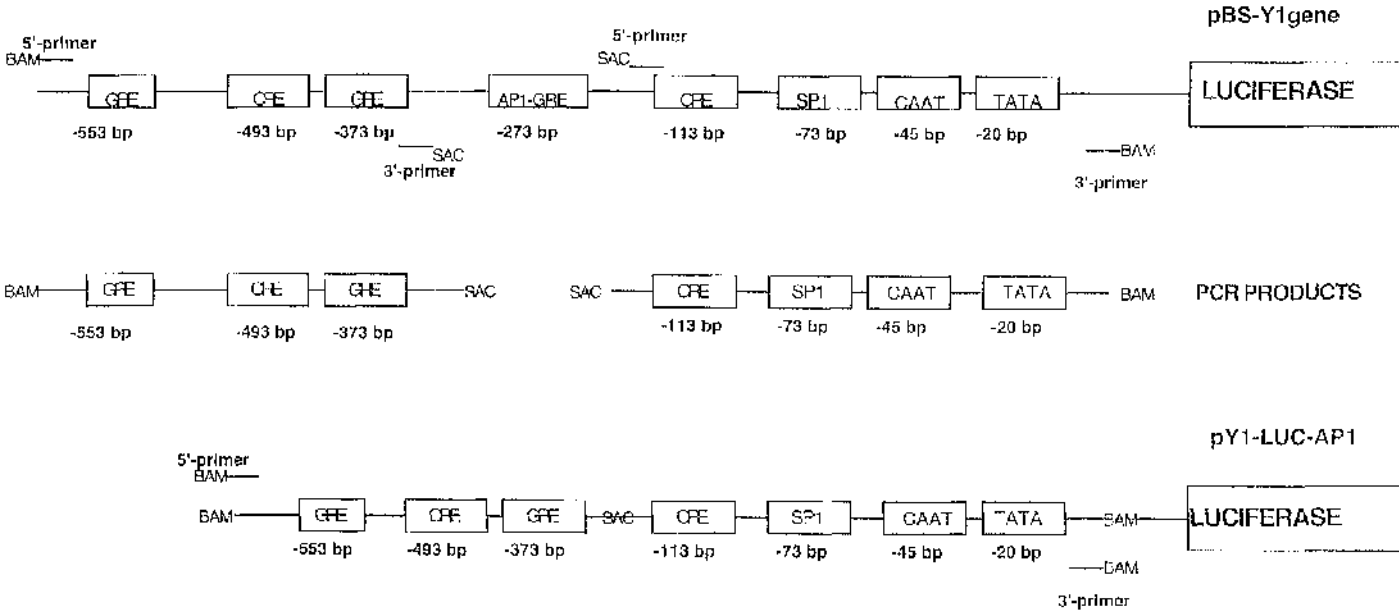


FIGURE 6.1 DELETION OF THE AP1 SITE OF THE PROMOTER OF THE NPY-Y1 GENE

Methodological approach used to delete a putative AP1 binding site of the promoter region of the rat NPY-Y1 receptor gene by PCR. **TATA** (TATA-like box), **CAAT** (CAAT-like box), **Sp1** (Sp1 responsive site), **CRE** (cAMP responsive element), **GRE** (glucocorticoid responsive element), **ERE** (estrogen reponsive element), **AP1-GRE** (AP1-GRE responsive element). **LUCIFERASE** (luciferase open reading frame). **5'-primer** (forward primer). **3'-primer** (reverse primer).

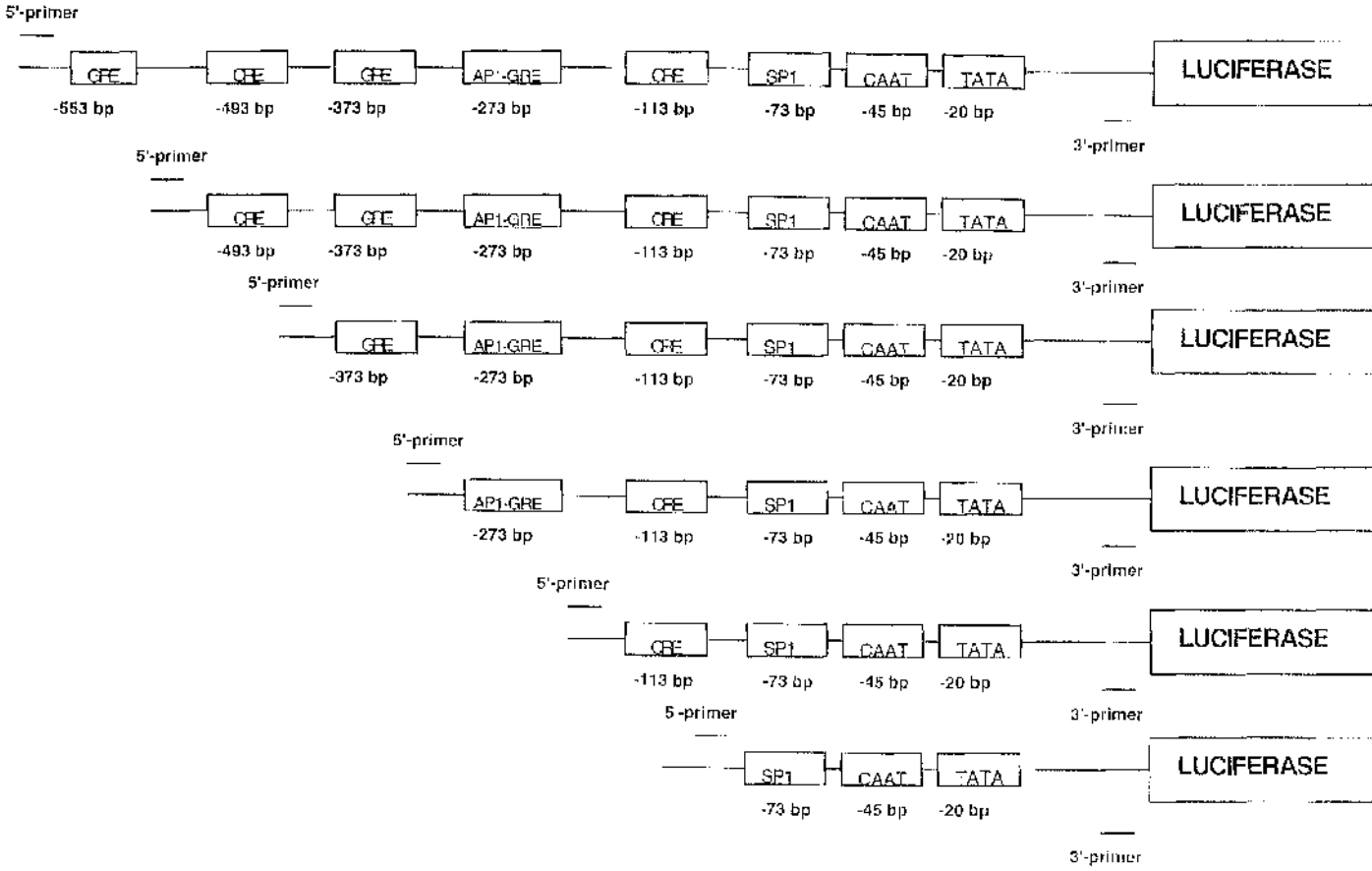


FIGURE 6.2 PROGRESSIVE DELETIONS OF THE PROMOTER OF THE NPY-Y1

Methodological approach to the progressive deletions of putative binding sites for transcription factors of the promoter region of the rat NPY-Y1 receptor gene by PCR. **TATA** (TATA-like box), **CAAT** (CAAT-like box), **Sp1** (Sp1 responsive site), **CRE** (c-AMP responsive element), **GRE** (glucocorticoid responsive element), **ERE** (estrogen reponsive element), **AP1-GRE** (AP1-GRE responsive element). **LUCIFERASE** (luciferase open reading frame). **5'-primer** (forward primer). **3'-primer** (reverse primer).

6.1.2 TRANSCRIPTION FACTORS

The transcription process is the essential first step in the conversion of the genetic information from the DNA into protein and this process is also the major point at which gene expression is regulated. In most cases, gene regulation is achieved by activating (or repressing) the transcription of particular genes. Once this has occured, all the other stages of gene expression (RNA processing, translation, post-translation) follow and the appropiate protein is synthesized in a cell-type specific or inducible manner. Both the basal process of transcription itself and its regulation are controlled by specific short DNA sequences in the gene, promoters or enhancers (McKnight and Yamamoto, 1992). These sequences act by binding specific proteins known as transcription factors, which then influence the rate of transcription of the gene.

Candidate regulatory regions are defined consensus sequences known to mediate the activity of transcription factors in other genes and systems. However, the fact that a gene possesses a consensus sequence does not necessarily constitute evidence that it is relevant to the mechanism being studied. For a consensus sequence to be defined as functional in the transcriptional regulation of a gene, several criteria must be met. Firstly, the relevant transcription factor must be shown to be present in the cell and that it is appropriately activated. A direct interaction between the DNA sequence and the transcription factor must also be demonstrated. Finally, it must have an effect on the transcriptional activity of the gene.

There are several possible approaches to demonstrate that a particular transcription factor is involved in the regulation of a particular gene. It is possible to block its biological activity by microinjecting specific antibodies into cells. In the case of transcription factors that are not constitutively present in the cell, but which require stimulation of gene transcription and translation, it is also possible to use antisense oligonucleotides to specifically block transcription and/or translation of the gene that encodes for it. Another approach is mutate the consensus sequences for transcription factors present within the gene and study how these modifications affect the transcriptional control of the gene. The use of reporter function systems has simplified the problem of defining elements within a gene responsible for transcriptional regulation. In combination with mutagenesis, it is possible to define a role for a particular sequence.

6.1.3 DELETIONS OF THE PROMOTER OF THE NPY-Y1 GENE

The promoter region of the rat NPY-Y1 receptor gene is under the control of the PKA signalling pathway as suggested in the previous chapter (See Chapter 5). This promoter region

contains two partial CRE sites which have been reported to mediate transcriptional control by cAMP in other systems (Roesler et al., 1988). These two partial CRE sites (CGTCA) are located at approximately -113 bp and -493 bp (using as a reference the putative transcription start site) in the NPY-Y1 receptor gene and constitute the primary candidates to be tested as possible mediators of transcriptional activation of the NPY-Y1 gene by factors which increases cAMP, such as PACAP-38, forskolin and DBC in PC12 cells (See Chapter 5). In order to delete the CRE binding site (CGTCA) located at -113 bp two restriction enzymes located on either side of this consensus sequence were used (Figure 6-3).

The promoter region of the NPY-Y1 gene contains a AP-1 consensus site (TGAGTCA) located at approximately -273 bp (using as a reference the putative transcription start site). Evidence, indicates that PKC mediates the NGF (See Chapter 4) and PACAP-38 (See Chapter 5) transcriptional activation of the NPY-Y1 receptor gene in PC12 cells. In particular, the activation of the AP-1 transcription factor by NGF and, specifically by PKC, has been well documented (See Chapter 1. NGF signalling pathway), and suggests that this site may be involved in the PKC-mediated transcriptional activation of the NPY-Y1 gene. In order to delete the AP-1 consensus site present in this promoter region, the PCR was used (Figure 6-1).

The promoter region of the NPY-Y1 receptor gene also contains GRE (AGGACT) consensus sequence overlapping with the AP1-site, located at approximately -230 bp (using as as reference the putative transcription start site). The putative interaction between two transcription factors can affect the gene transcription of the NPY-Y1 gene. This promoter region also contains one additional GRE site (AGGACT) located at approximately -373 bp and one imperfect palindromic GRE site located at approximately -540 bp (using as a reference the putative transcriptional start site). These GRE sites are the primary candidates to mediate the dexamethasone-mediated transcriptional activation of the NPY-Y1 gene in PC12 cells (See Chapter 3). In order to delete these GRE sites as well as other consensus sites, progressive deletions of the promoter of the NPY-Y1 gene were obtained by PCR (Figure 6-2).

This chapter describes the mutagenesis of the promoter region of the NPY-Y1 gene in order to deleted specific DNA consensus sequences for transcription factors, such as AP-1, CRE and GRE. These deletion constructs will be used to examine the role of these sequences in mediate the transcriptional control of this neuropeptide receptor gene by pharmacological agents, such as dexamethasone, NGF and PACAP, capable of activating several signalling pathways in PC12 cells (See Chapter 1), including nuclear hormone receptors, PKC and PKA pathways, among others (See Chapters 3, 4 and 5) or in other cell lines which expressing this receptor, such as RINm5f and GT1-7. The luciferase reporter assay developed in this thesis (See Chapter 3) provides a suitable system to test the effect of these mutations in the transcriptional control of the NPY-Y1 gene.

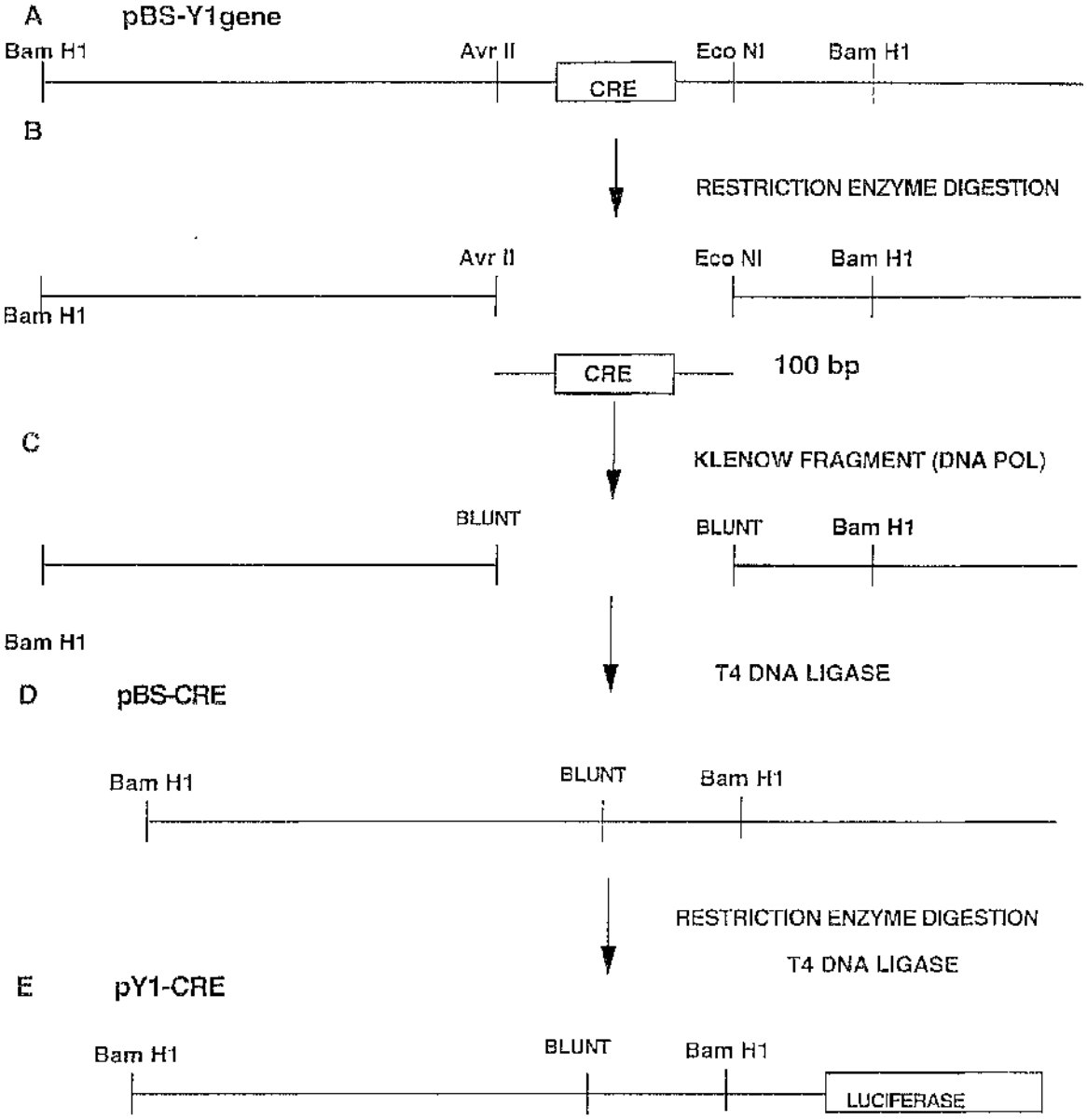


FIGURE 6.3 DELETION OF THE CRE SITE OF THE PROMOTER OF THE NPY-Y1 GENE

Methodological approach used to delete a putative CRE binding site of the promoter region of the rat NPY-Y1 receptor gene by restriction enzyme digestion.

A. The promoter region of the NPY-Y1 gene was digested with *Avr* II and *Eco* NI.

B. The two fragments were separated by agarose gel electrophoresis,

C. The plasmid ends were made blunt by the Klenow fragment DNA polymerase.

D. Both ends were ligated by T4 DNA ligase, transformed in bacteria and plasmid recovered for DNA sequencing.

E. The CRE-deleted NPY-Y1 promoter was subcloned into the pGL3-Basic (pY1-CRE).

6.2 RESULTS

6.2.1 OLIGONUCLEOTIDES FOR DELETION OF THE PROMOTER OF THE NPY-Y1 GENE BY PCR

6.2.1.1 DELETION OF THE AP-1 SITE

The oligonucleotides (V5339-V5340) (Appendix II) were used to deleted the putative AP-1 binding site of the promoter region of the NPY-Y1 gene (Figure 6-1). The internal forward primer (V5339) was modified at the 5'-region to add a *Sac* II restriction site. This restriction site is not present in the pTAg cloning vector (R&D Systems) and in the promoter region of the NPY-Y1 gene and was used for subcloning purposes (Figure 6-1). This primer (V5339) anneals to the nucleotides -265 to -246 from the putative start transcription site of the NPY-Y1 gene. The internal reverse primer (V5340) was also modified at the 5'-region to add a *Sac* II restriction site. This primer (V5340) anneals to the nucleotides -283 to -301 from the putative start transcription site of the NPY-Y1 receptor gene.

The forward primer (2024) and the internal reverse primer (V5340) were used to generate a PCR fragment of approximately 310 bp of the promoter region of the NPY-Y1 gene (Figure 6.4). The internal forward (V5339) and the reverse primer (2023) were used to generate another PCR fragment of approximately 430 bp of the promoter region of the NPY-Y1 gene (Figure 6-4). These two PCR fragments were cut with *Sac* II, the enzyme was inactivated by incubation at 65°C for 15 minutes and ligated to the pTAg-cloning vector (see Cloning PCR products).

6.2.1.2 Progressive Deletions of the Promoter of the NPY-Y1 Gene

Oligonucleotides (V7613 to V7617) (Appendix II) were used as forward primers to progressive delete the promoter region of the NPY-Y1 receptor gene by PCR using the original reverse primer (2023) and the pY1-LUC as template (Appendix II) (Figure 6-2). All of these oligonucleotides were modified at the 5'-region to add a *Bam* HI restriction site for subcloning purposes in the pGL3-Basic reporter vector (Promega).

These oligonucleotides anneals to the promoter region of the rat NPY-Y1 receptor gene at the following sequences (from the putative start transcription site) :

(i) the forward (V7613) primer was used to delete two GRE binding sites and anneals to the nucleotides -540 to -519 generating a PCR product of 700 bp,
(ii) the forward (V7614) primer was used to delete a CRE binding site and anneals to the nucleotides -420 to -399 generating a PCR product of 580 bp,
(iii) the forward (V7615) primer was used to delete a second GRE binding site and anneals to the nucleotides -310 to -289 generating a PCR fragment of 470 bp,
(iv) the forward (V7616) primer was used to delete a AP1-GRE binding site and anneals to the nucleotides -270 to -249 generating a PCR product of 430 bp,
(v) the forward (V7617) primer was used to delete a second CRE binding site and anneals to the nucleotides -110 to -89 generating a PCR product of 270 bp.

6.2.2 CLONING OF THE PCR PRODUCTS

6.2.2.1 CONSTRUCTION OF A FUSION GENE LACKING THE CRE SITE OF THE PROMOTER OF THE NPY-Y1 GENE

In order to delete one of the CRE consensus sites (CGTCA) located at approximately -113 bp from the putative cap site within the promoter region of the NPY-Y1 receptor gene two restriction enzymes located on either side of this consensus sequence were used (Figure 6-3). The promoter of the NPY-Y1 gene has a unique *Avr* II restriction site located 5' to this CRE site. A second unique *Eco* NI restriction site is located 3' to the CRE consensus site. These restriction enzymes sites are not present in the pBluescribe SK$^+$ (Stratagene) and are separated by approximately 100 bp (Figure 6-3).

The pBS-Y1 fusion gene (pBS-Y1gene) was cut with *Eco* NI, and the restriction enzyme was heat inactivated. Following the pBS-Y1 gene was cut with *Avr* II. The restriction reaction was extracted with phenol-chloroform and precipitated with ethanol (Figure 6-3A). 5'-overhangs were filling-in with the Klenow (large) fragment of the *E. coli* DNA polymerase I and excess of dNTPs (0.4 mM) (Figure 6-3B). Blunt-end fragments were ligated by overnight incubation with T4 DNA ligase and the ligation products were used to transformed JM109 or TG1 bacteria (Figure 6-3C). Several positive colonies were recovered, the plasmids were extracted and cut with *Bam* III. One positive clone (pBS-CRE) was obtained by size in a 2% agarose gel and used for large scale plasmid purification using the Wizard maxiprep system (Figure 6-3D). This clone (pBS-CRE) was further used to subclone in a bidirectional manner the CRE-deleted NPY-Y1 promoter region into the *Bgl* II site of polylinker of the pGL3-Basic vector (Promega).

pBS-CRE was cut with the restriction enzyme *Bam* III, DNA separated by size in a agarose gel and the insert was excised from the gel using a scalpel. DNA was purified using the USBioclean kit (United States Biochemical). In parallel, the pGL3-Basic vector was cut with the restriction enzyme *Bgl* II, extracted with phenol-chloroform and precipitated with ethanol. The free ends of the vector were dephosphorylated by treatment with shrimp alkaline phosphatase (SAP). This enzyme was heat inactivated before the overnight ligation. Ligation products were used to transformed JM109 or TG1 bacteria, and several positive clones were selected in LB plates with ampicillin, the plasmid extracted by miniprep extraction and cut with the *Hind* III-*Pst* I restriction enzymes for determine the orientation of the insert.

One positive clone (pY1-CRE) with the CRE-deleted promoter of the NPY-Y1 gene in the correct orientation was used to large scale plasmid purification (CsCl method). This clone (pY1-CRE) will be used for luciferase reporter analysis involving the factors in the cAMP-mediated transcriptional activation of the NPY-Y1 gene in PC12 cells.

6.2.2.2 CONSTRUCTION OF A FUSION GENE LACKING THE AP-1 CONSENSUS SITE OF THE PROMOTER OF THE NPY-Y1 GENE

In order to delete the AP-1 consensus site (TGAGTCA) present within the promoter of the NPY-Y1 gene, the PCR was used (Figure 6-1). NPY-Y1 promoter present in the pY1-LUC was amplified using the "original" forward primer (2024) (Appendix II) and a "internal" reverse primer (V5340) (Appendix II) located immediately upstream of the AP-1 site (Figure 6-4A). This pair of primers (2024-V5340) generated a PCR product (product I) of approximately 310 bp which is similar in molecular weight of a band seen in a 2 % agarose gel (Figure 6-4B). The "internal" forward primer (V5339) (Appendix II) located immediately downstream of the AP1-site and the "original" reverse primer (2023) (Appendix II) were also used to amplified a PCR product (product II) from the pY1-LUC as template. This pair of primers (V5339-2023) generated a second PCR product (product II) of approximately 430 bp, which is similar to a band seen in the 2 % agarose gel (Figure 6-4B).

The "original" forward primer (2024) and reverse primer (2023) were modified at the 5'-end to add a *Bam* HI restriction site not present in the promoter region of the NPY-Y1 receptor gene and used to subclone the ligated PCR products in a compatible *Bgl* II restriction site of the polylinker of the pGL3-Basic reporter vector (Promega). The "internal" reverse primer (V5340) and forward primer (V5339) were also modified at the 5'-end to add a*Sac* II restriction site not present in both, the promoter region of the NPY-Y1 receptor gene and the pTAg cloning vector (R&D Systems). This *Sac* II restriction site was used to ligate the two PCR products in a correct orientation for subsequent cloning in the pTAg vector (Figure 6-1).

Following PCR amplification, the two PCR products (products I and II) were digested with the *Sac* II restriction enzyme by overnight incubation, extracted with phenol-chloroform and ethanol precipitated. These PCR products were ligated by overnight incubation with T4 DNA ligase in the presence of the pTAg-cloning vector (R&D Systems). In theory, only the correct ligated products transformed in competent JM109 or TG1 bacteria results in positive colonies when where plated on agar plates containing ampicillin, because the linear DNA is only poor transformed in bacteria. The vector alone was used to transform the bacteria and check the integrity of the T-overhangs as a negative control. Plasmid DNA was isolated from positive colonies and cut with the *Sac II* restriction enzyme. This *Sac II* restriction site is no present in the pTAg vector (R&D Systems) and only plasmids with the correct ligated product can be linearised.

One positive clone (pTA-AP1) was amplified by large scale plasmid purification using the Wizard Maxipreps System (Promega) and the insert was verified by sequencing using the Thermosequenase™ cycle-sequencing kit (Amershan). This clone containing the AP1-deleted NPY-Y1 promoter region was subcloned into the *Bgl* II site of the pGL3-Basic vector (Promega).

pTA-AP1 was cut with the *Bam* HI restriction enzyme, and the DNA was separated by size in a agarose gel. A DNA band with the expected molecular weight (780 bp) containing the AP1-deleted promoter region of the NPY-Y1 gene was excised from the agarose gel with a scalpel and purified using the USBioclean kit (USB). In parallel, the pGL3-Basic was cut with the restriction enzyme *Bgl II* , extracted with phenol-chloroform and precipitated with ethanol. The free ends of the vector were dephosphorylated by treatment with SAP (USB). This enzyme was heat inactivated before overnight ligation. Positive colonies were isolated and the DNA plasmid was isolated and cut with *Hind* III-*Pst* I restriction enzymes to verify the orientation of the insert (data not shown).

One positive clone (pY1-AP1) with the AP1-deleted promoter region of the NPY-Y1 gene in the correct orientation was used to large scale plasmid purification using the CsCl method (See Chapter 2). This clone (pY1-AP1) will be used to analyse using the luciferase reporter function the effect of this putative binding site in the transcriptional activation of the NPY-Y1 receptor gene mediated by pharmacological agents which are known to increase the expression of this transcription factor in PC12 cells, such as NGF and PMA (see Chapter 4).

FIGURE 6.4 SUBCLONING THE AP1-DELETED AND THE PROGRESSIVE DELETED NPY-Y1 PROMOTER

Strategy used for subcloning the AP1-deleted promoter regions of the NPY-Y1 receptor gene into the pGL3-Basic reporter vector (Promega).

A. Two fragments of the promoter region of the NPY-Y1 receptor gene present in the pY1-LUC were amplified by PCR.

B. Agarose gel showing the two PCR products.

Lane 1: MWM (λ x*Hind* III-*Eco* RI). **Lane 2**: PCR product (NPY-Y1 promoter)

Lane 3: PCR product I (313 bp) **Lane 4**: PCR product II (429 bp)

C. PCR products were cut with *Sac* II and ligated into the pTAg-cloning vector (pTA-AP1). Insert was obtained by *Bam* HI digestion and subcloned into the pGL3-Basic reporter vector (pY1-AP1).

Strategy used for subcloning the progressive-deleted promoter regions of the NPY-Y1 receptor gene into the pGL3-Basic reporter vector (Promega).

D. Five deletion fragments of the promoter region of the NPY-Y1 receptor gene present in the pY1-LUC were amplified by PCR. Agarose gel showing the five PCR products.

Lane 1: MWM (λ x *Hind* III-*Eco* RI) . **Lane 2**: PCR deletion I (Clone A).

Lane 3: PCR deletion II (Clone B). **Lane 4**: PCR deletion III (Clone C).

Lane 5: PCR deletion IV (Clone D). **Lane 6**: PCR deletion V (Clone E).

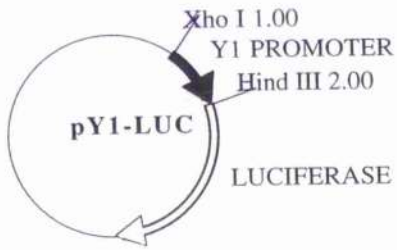
E. PCR products were ligated to the pTAg-cloning vector (R&D Systems). The expected inserts were obtained by *Bam* HI digestion and subcloned into the pGL3-Basic vector (Promega). Agarose gel of the restriction enzyme digestions.

Lane 1: MWM (λ x *Hind* III-*Eco* RI) . **Lane 2**: PCR deletion I (Clone A).

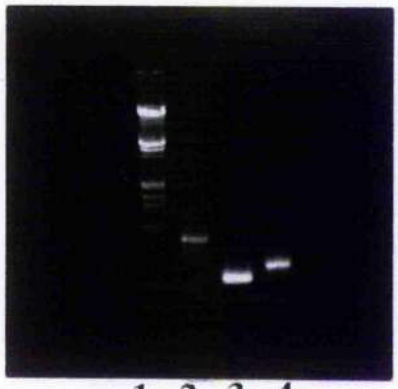
Lane 3: PCR deletion II (Clone B). **Lane 4**: PCR deletion III (Clone C).

Lane 5: PCR deletion IV (Clone D). **Lane 6**: PCR deletion V (Clone E).

A

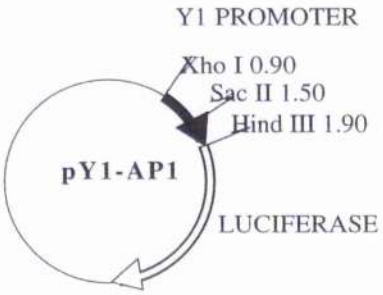


B

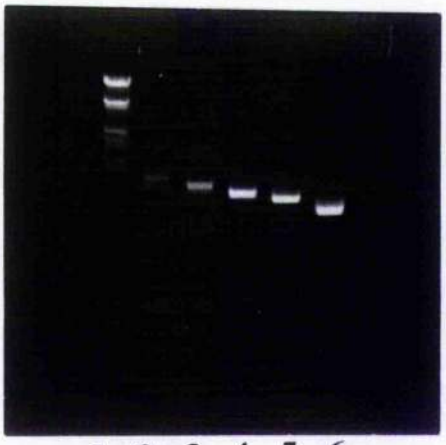


1 2 3 4

C



D



1 2 3 4 5 6

E

1 2 3 4 5 6

6.2.2.3 CONSTRUCTION OF PROGRESSIVE DELETIONS OF THE PROMOTER OF THE NPY-Y1 GENE

Progressive deletions of the 5'-flanking promoter region of the rat NPY-Y1 receptor gene present in the pY1-LUC were obtained using PCR (Figure 6-2). These deletion clones were amplified from the pY1-LUC as template using a set of different forward primers (V7613 to V7617) but only one reverse primer (2023) (Figure 6-2). The different forward primers were designed to result in progressive deletions of putative consensus sequences for a transcription factors present in the NPY-Y1 promoter (Figure 6-2).

Five diferent clones (A to E) containing progressive deletions of different putative binding sites (AP-1, CRE, GRE) for transcription factors present in the 5'-flanking promoter region of the NPY-Y1 receptor gene were obtained by PCR (Figure 6-4D):

(i) the first deletion clone (clone A) lacks approximately 60 bp which includes two non-palindromic GRE. These GRE sites may be involved in the dexamethasone-mediated transcriptional activation of the NPY-Y1 gene,
(ii) the second deletion clone (clone B) lacks approximately 120 bp which also includes a CRE binding site. This CRE site may be involved in the PACAP-38, forskolin and DBC-mediated transcriptional activation of the NPY-Y1 gene,
(iii) the third deletion clone (clone C) lacks approximately 180 bp which also includes a non-palindromic GRE . This second GRE site may be also involved in the dexamethasone-mediated transcriptional activation of the NPY-Y1 gene,
(iv) the fourth deletion clone (clone D) lacks approximately 290 bp which also includes a AP-1 consensus site and a non-palindromic GRE. This AP1 site may be involved in the NGF and PMA-mediated transcriptional activation of the NPY-Y1 gene,
(v) the fifth deletion clone (clone E) lacks approximately 390 bp which also includes a second CRE binding site. This second CRE site may be also involved in the PACAP-38, forskolin and DBC-mediated transcriptional activation of the NPY-Y1 gene. This clone E contains only the "basal" promoter sequence of the NPY-Y1 gene, including the Sp1, CAAT-like box and TATA-like box sites.

All the forward primers (V7613 to V7617) and the "original" reverse primer (2023) were modified at the 5'-end by adding a *Bam HI* restriction site not present in the 5'-flanking promoter region of the NPY-Y1 receptor gene (See oligonucleotide for deletion of the promoter of the NPY-Y1 gene). This *Bam HI* restriction site was further used for subcloning in a bidirectional manner the PCR products into the *Bgl II* restriction site of the polylinker of the pGL3-Basic vector (Promega).

PCR products for all the deletions (products A to E) were separated by size in a 2 % agarose gel (Figure 6-4D). The DNA products were excised from the gel with a scalpel and the DNA purified using the USBioclean kit (United States Biochemical). Following, these PCR products containing the different deletions of the promoter of the NPY-Y1 receptor gene were cloned directly into a pTAg-cloning vector (D&R Systems) by overnight incubation with T4 DNA ligase. Ligations products were used to transform competent JM101 or TG1 bacteria. Positive colonies were obtained and plasmid DNA was isolated from these colonies (miniprep extraction) and cut with *Bam* HI. Positive clones (A to E) containing DNA bands with the expected molecular weights were subsequently used for large scale plasmid purification using the Wizard Maxipreps System (Promega). The inserts containing the progressive deletions of the promoter of the NPY-Y1 gene were sequenced using a Thermosequenase™ cycle sequencing kit (Amershan).

These deletion clones (A to E) were cut with *Bam* HI and the inserts were separated by size in a 2 % agarose gel (Figure 4E). DNA products were excised from the gel with a scalpel and the DNA was purified using the USBioclean kit (USB). In parallalel, the pGL3-Basic vector was cut with *Bgl* II , extracted with phenol-chloroform and precipitated with ethanol. The free ends of the pGL3-vector were dephosphorylated by treatment with shrimp alkaline phosphatase (SAP). Inserts containing the progressive deletions of the promoter of the NPY-Y1 gene were subcloned into the *Bgl* II site of the pGL3-Basic vector (Promega). Several positive clones were obtained and cut with different restriction enzymes to verify the orientation of the insert. Positive clones were isolated and plasmid DNA was purified using the CsCl method (See Chapter 2).

These deletion clones (A to E) will be used for analysis of the luciferase reporter function in PC12 cells. In particular, the role of the putative binding sites for transcription factors in the levels of the basal and induced transcriptional activity of the NPY-Y1 receptor gene will be determine. These deletions clones will be useful to understand the molecular mechanisms involved in the transcriptional control of this neuropeptide receptor gene in PC12 cells.

6.3 DISCUSSION

In this chapter, putative consensus sequences mediating dexamethasone, NGF and PACAP regulation of NPY-Y1 receptor gene transcriptional activity were deleted from the 5'-flanking promoter region of this gene using standard deletion techniques, such as restriction enzymes digestion or PCR. Using these molecular biology techniques, several different constructs were made, each one with a defined deletion corresponding to consensus sites for transcription factors. The effect of these deletions on the transcriptional control of the NPY-Y1 receptor gene will be analysed using the luciferase reporter assay system described in the chapter 3.

6.3.1 PUTATIVE ROLE OF THE CRE SITES

All the cAMP-regulated genes are expressed in tissues which are responsive to hormones or regulatory factors, and their rate of transcription is rapidly altered by cAMP. The promoter-regulatory regions of several of these genes has been isolated and cAMP responsive elements (CRE) characterized by functional analysis (Roessler et al., 1988). There are two classes of genes which are transcriptionally induced by cAMP. Group 1 genes are those which are rapidly regulated by cAMP, usually within minutes in cycloheximide-insensitive manner, while the transcription of genes of group 2 are increased after several hours of cAMP treatment and are cycloheximide-sensitive.

There are two classes of *cis* -acting elements identified in the cAMP-regulated genes (Roesler et al., 1988). A 8-bp palindromic sequence T(G/T)ACGTCA, originally termed a CRE lies generally within the first 150 bp of the 5'-flanking region. Available evidence supports a dual role for this CRE as both a basal and an inducible transcriptional enhancer element. There are two partial CRE sites (CGTCA) located at -113 bp and -493 bp (using as a reference the putative transcription start site) in the NPY-Y1 promoter region (Figure 3-1). The other is the regulatory consensus sequence CCCCAGGC, termed Activator Protein 2 (AP-2) which can be induced by treatment with either forskolin or phorbol esters. However, this sequence is not found in the promoter of the rat NPY-Y1 gene.

A protein has been purified with a relative molecular mass of 43-kDa which binds specifically to the CRE site. This cAMP responsive element binding protein (CREB) is phosphorylated *in vitro* by the catalytic subunit of PKA and *in vivo* by treatment of the cells with forskolin (Roesler et al., 1988). However, CREB is ubiquitious distributed in a variety of tissues and its PKA phosphorylation does not seem to increase the amount of CRE binding activity but is essential for transcriptional activation. Thus, rapid changes in the intracellular levels of cAMP lead to a PKA-mediated modification of the transcriptional activation domains of CREB, already bound to their respective elements in the promoter-regulatory region of the gene (Riabowol et al., 1988; Mellon et al., 1989). This modification in the binding protein can alter transcription levels of a cAMP-regulated genes.In addition, phosphorylated CREB can turn on transcription of the gene for at least one other transcriptional activator, *c-fos* (Edwards, 1994), a member of the AP-1 protein, which in turn can contribute to activation of the NPY-Y1 receptor gene. Thus, it is conceivable that these two mechanisms work cooperativily for the CREB-mediated regulation of the NPY-Y1 gene transcription in response to same stimuli in PC12 cells.

NPY-Y1 receptor gene is under the control of cAMP as sugested in the previous chapter. The NPY-Y1 promoter contains two CRE sites (CGTCA) which have been reported to mediate transcriptional control by cAMP in several systems, including a group of neuropeptides genes such as somatostatin, proenkephalin and VIP (Roessler et al., 1988). Thus, these CRE sites constitutes the primary candidate to be tested as mediators of the cAMP-dependent transcriptional control of this gene. In order to define the region of the gene conferring responsiveness to known pharmacological agents which increase cAMP in PC12 cells , such as PACAP-38, forskolin and DBC, the construct pY1-CRE was made in which one of the CRE binding sites was deleted from the promoter region of the NPY-Y1 gene.

6.3.2 PUTATIVE ROLE OF THE AP-1 SITE

A class of cellular genes, termed immediate early genes (IEG), have been identified whose transcription is rapidly and transiently activated. Several members of the IEG family encode putative transcription factors, critical to the response of the cell to growth factors. *c-fos* and *c-jun* proto-oncogenes are the most extensively studied of these genes (Sheng and Greenberg 1990).*c-jun* , is a DNA-binding protein that binds to a phorbol-ester responsive element (5'-TGAGTCA-3') either as homodimers or as heterodimers. The *c-fos* family bind to DNA as heterodimers with the *c-jun* family and with selected members of the ATF/CRE-BP family (Sheng and Greenberg, 1990).

In most cell types, members of the *c-fos* and *c-jun* gene families are expressed at relatively low levels, but they are induced transiently by a wide range of extracellular stimuli (Sheng and Greenberg 1990). *c-fos* has become a model gene for the study of inducible expression because *c-fos* activation occurs in a wide range of cell types in response to a diverse set of agents including specific growth factors, phorbol esters, neurotransmitters and agents that elevate intracellular calcium or cAMP (Bartel et al., 1989).

It is well known that NGF and PMA transiently activate members of the AP-1 family such as *c-fos* and *c-jun* , and that this activation is at least partially dependent on PKC activity (Sigmund et al., 1990). AP-1 consensus sites are present in several genes regulated by NGF and PMA such as the gene for *c-fos* (Koening et al., 1989), transin (Machida et al., 1991) and NGFIA (DeFranco et al., 1993). Moreover, previous results, has been shown that the NGF-PMA mediated transcriptional activation of the NPY gene is dependent on PKC activity (Balbi and Allen, 1994). A specific deletion of the AP-1 site present in the promoter of this neuropeptide gene significantly reduced the NGF response whereas the response to PMA was completely abolished (Balbi, 1994).

Evidence presented in this thesis, using calphostin C (an specific inhibitor of PKC) and PMA, indicates that PKC is also involved in the NGF-dependent transcriptional activation of the NPY-Y1 receptor gene in PC12 cells (Chapter 4). In addition, the PACAP-38 mediated transcriptional activation of the NPY-Y1 gene also appears to be dependent on PKC activity (See Chapter 5). Therefore it is possible that the AP-1 site present in the promoter of this neuropeptide receptor gene may be involved in mediating this PKC-dependent response in PC12 cells. In order to evaluate the importance of the AP-1 site in the transcriptional activation of the NPY-Y1 receptor gene by NGF and pharmacological agents known to increase AP-1 activity in PC12 cells, such as phorbol esters, the construct pY1-AP1 in which the AP1 consensus site was deleted from the promoter region of the NPY-Y1 receptor gene was made. Transfection experiments using this deletion clone and also the clones with the progressive deletions of the promoter will be used to provide an understanding of the transcriptional control mechanisms of this neuropeptide receptor gene.

6.3.3 PUTATIVE ROLE OF THE GRE SITES

Nuclear receptors consists of proteins that mediate the actions of many important cell regulators. These includes the steroid hormones (oestrogens, progestins, androgens, glucocorticoids, mineralocorticoids, ecdysteroids, vitamin D), thyroid hormones and retinoids (Evans, 1988). In contrast to receptors for peptide hormones, which are located in the cell membrane, nuclear receptors are present within the cell. Each of these intracellular receptors contains a DNA binding domain, which shows a high degree of homology with other members of the family, a ligand-binding region showing some homology, and different variable regions. The DNA-binding domain contains two "zinc fingers" (looped structures involving chelated metal ions) that are responsible for reaction with DNA in the hormone responsive element (HRE) of target genes. Receptors for thyroid hormones and retinoic acid, appear to exist only in a DNA-binding state in the nucleus, but those for steroid hormones are present in an inactive form in the cytoplasm. Thus, ligand binding converts the native receptor to a biochemically functional state that can bind in the DNA and enhance gene transcription (Evans,1988).

Glucocorticoid receptor (GR), like other members of the steroid receptor superfamily consists of three structural domains, the amino-terminal domain, the DNA-binding domain and the hormone binding-domain (Beato, 1989). The amino-terminal domain is essential for full transcriptional activity and contains an acidic region that acts as transcriptional activator. The DNA binding domain, comprising approximately 75 amino acids, contains two zinc-fingers that confer DNA binding specificity on the receptor. It also contains a basic region involved in nuclear localization and possible interactions with DNA. The hormone-binding domain, upon binding of hormone promotes DNA binding and transcriptional activation. In the absence of the hormone, this domain supresses receptor activity (Beato, 1989).

The action of nuclear hormone receptors can be modulated through "cross-talk" with signal transduction cascades activated by external signals with other transcription factors (Lopes da Silva and Burbach, 1995). Heterodimerization with other promiscuous nuclear receptors such as retinoid-X-receptor (RXR) and non-nuclear receptor such as Jun/Fos (AP-1) proteins can activate or inhibit transcription. For example, the GR has been found to repress AP-1 stimulated transcription, through a segment of the amino-terminus region of this receptor. At the DNA level further interaction can result from sharing or overlapping of responsive elements with other nuclear hormone receptors or non-receptor factors. The NPY-Y1 promoter region has two overlapping consensus sequences, an AP1 and non-palindromic GRE (Figure 3-1). These interactions may affect the NPY-Y1 gene transcription positively or negatively.

The regulatory DNA sequences require for hormone-dependent activation of transcription have been termed hormone responsive elements (HREs) and are the specific high affinity DNA binding sites for the activated hormone-receptor complexes. GRE is a consensus 15 bp imperfect palindromic DNA sequence: 5'-AG(A/G)ACANNNTGTTCT-3' (Beato, 1989). The fact of GRE are palindromic structure is in agreement with the finding that GR bind as dimers to their respective response elements. In some genes, like the NPY-Y1 receptor, GREs are often clustered, forming hormone responsive units (HRUs) that behave as strong inducible enhancers and in some cases are formed by a combination of several imperfect HREs that are poorly activated separately but act synergistic fashion. The promoter region of the NPY-Y1 receptor gene contains four non-palindromic GRE sites. In order to study the relevance of these GREs in the dexamethasone-mediated activation of the NPY-Y1 gene, progressive deletions of this promoter were created. Further experiments will be necesary to understand the steroid receptor control of this neuropeptide receptor gene in PC12 cells.

6.4 SUMMARY

6.1 A specific deletion of a CRE site of the promoter of the NPY-Y1 gene was created using restriction enzymes.

The NPY-Y1 receptor gene is under the transcriptional control of the PKA signalling pathway in PC12 cells (Chapter 5). The promoter region of this gene has two CRE elements which are known to mediate the cAMP-PKA-dependent transcriptional control of other genes. In order to evaluate the role of one of these CRE sites, the construct pY1-CRE was created using restriction enzymes.

6.2 A specific deletion of a AP1-GRE site of the promoter NPY-Y1 gene was create using PCR.

NPY-Y1 receptor gene is under the transcriptional control of the PKC signalling pathway in PC12 cells (Chapter 4). The promoter region of this gene has an AP1 site which is known to mediate the PKC-dependent transcriptional control of other genes. Thus, in order to evaluate the role of this sequence in the NGF and PMA-dependent response of this gene, the construct pY1-AP1 was made using PCR. This clone has the luciferase gene under the control of a AP1-deleted version of the promoter region of this neuropeptide receptor gene.

6.3 Progressive deletions of a putative DNA binding sequences of the promoter of the NPY-Y1 gene were created using PCR.

NPY-Y1 receptor gene is under the transcriptional control of the glucocorticoid receptor signalling pathway in PC12 cells (Chapter 3). The promoter region of this gene has several non-palindromic sequences which are known to mediate the glucocorticoid-dependent transcriptional control of other genes. In order to evaluate the role of this consensus sequences in the dexamethasone-dependent response of this gene, five different deletion clones were made using PCR. These clones have the luciferase gene under the control of progressive deleted version of the promoter region of this neuropeptide gene.

CHAPTER 7

REGULATION OF THE mRNA LEVELS OF THE NPY-Y1 RECEPTOR GENE

7.1 INTRODUCTION

7.1.1 QUANTIFICATION OF mRNA LEVELS

Cells respond to environment changes and development processes by regulation of their genes. Gene regulation occurs at several levels: (i) transcription of a gene to a primary heteronuclear RNA (hRNA), (ii) processing of the primary transcript to messenger RNA (mRNA), (iii) stabilisation of the mRNA in the nucleus or cytoplasm, (iv) translation of the mRNA into a protein and (v) post-translational modifications of the protein, such as acylation, glycosylation and/or phosphorylation (Watson et al., 1992).

Control of gene transcription, the process in which gene DNA sequence serves as template for mRNA synthesis, plays a critical role in the multistep process that regulates gene expression. Gene transcription levels change in response to a wide variety of signals that occur during cell development, differentiation and physiological function. Changes in transcription levels may also occur in response to disease and other pathological factors. In turn, these changes in gene transcription levels cause variations in the steady-state levels of individual mRNAs.

One of the most studied gene regulation mechanism is the transcriptional control by which the level of transcription of the activated genes is modified or the transcription of the inactivated genes is induced. Although, the phenotypic changes involved in the transcriptional control can be considerable, alterations in the gene expression are in general moderate. Therefore, for the study of the changes in gene expression are necessary sensitive methods that permit the detection and/or quantitative analysis of specific mRNAs (Becker-Andre, 1991).

There are two principal approaches for detection and/or quantification of absolute or relative levels of the specific mRNAs in different cell, tissues or organs (Becker-Andre, 1991):

(i) Quantification by hybridisation with specific probe.
(ii) Quantification by PCR.

7.1.2 QUANTIFICATION BY HYBRIDISATION WITH A SPECIFIC PROBE

Traditionally, levels of individual mRNAs have been analysed by procedures such as, RNA dot/slot blots, Northern blots and Nuclease Protection Assays. The several methods that used this approach are based on molecular hybridisation using labelled probes. In these methods the mRNAs amount is proportional to the hybridisation signal intensity; the specificity is obtained

by the hybridisation condition and the sensitivity is consequence of the increase in the specific signal detection and the reduction of the background as a consequence of the non-specific binding of the labelled probes.

There are two principal hybridisation strategies:

(i) Hybridisation in mixture phase (e.g. RNA dot/slot blots, Northern blots), in which the mRNA in a pool are fixed irreversibly to a solid phase (nitrocellulose or nylon membranes) and a solution that contains labelled probe of nucleic acid (DNA or RNA) is hybridised to the immobilised mRNA. By using autoradiography or non-radioactive methods is possible detect and/or quantify the amounts of probe specifically hybridised, and these amounts are directly proportional to the amount of mRNA immobilised in the solid phase (membrane).

(ii) Hybridisation in solution (e.g. Nuclease protection assays). In these techniques the mRNAs are hybridised with a excess probe of DNA or RNA in the same phase (solution). The non-hybridised probe is specifically removed by digestion with nuclease S1 (probes of DNA) or RNAse A-RNAse T1 (probes of RNA). In this method the absolute concentration of the sequence of interest is calculated from the rate of hybridization of a small amount of a specific radioactive probe with a known quantity of purified cellular RNA. Thus, the concentration of the mRNA of interest can be estimated by autoradiography or scintillation counting from the amount of radioactivity that becomes resistant to nuclease treatment. This method offers the advantage of higher sensitivity than hybridisation in mixture phase and results less background.

7.1.2.1 QUANTIFICATION BY HYBRIDISATION IN MIXTURE PHASE

7.1.2.1.1 Northern Blots

Northern hybridizations is a method in which the size and amount of specific mRNA molecules in preparations of total or poly(dA) RNA are determined. RNA is separated according to size by electrophoresis through a denaturing (formaldehyde) agarose gel and is then transferred to activated cellulose, nitrocellulose or nylon membranes. RNA of interest is then located by hybridization with radiolabelled DNA (double stranded, single stranded or oligonucleotides) or RNA probes followed by autoradiography. The sensitivity with which RNA bound to the membranes can be detected is such that species of mRNA comprising no more than 0.001% of the mRNA can be rapidly identified and easily quantified.

7.1.2.2 QUANTIFICATION BY HYBRIDISATION IN SOLUTION

7.1.2.2.1 Ribonuclease Protection Assay

Ribonuclease protection assay (RPA) is an sensitive technique for the detection and quantitation of mRNA species in a complex sample mixture of total cellular RNA. For the RPA, a labelled RNA probe is synthesised that is complementary to the target RNA to be analysed. This is done by inserting the probe fragment into one of the common transcription vectors under the control of a bacteriophage promoter (either the T3, T7 or SP6 promoter) and using the corresponding T3, T7 or SP6 RNA polymerase to generate an antisense RNA transcript of high specific activity.

The labelled probe is then mixed with the sample RNA and incubated under conditions that favor hybridisation of complementary transcripts. After hybridisation, the mixture is treated with ribonuclease to degrade single-stranded, unhybridised probe. Labelled probe that hybridised to complementary RNA in the sample mixture will be protected from ribonuclease digestion, and can be separated by polyacrylamide gel electrophoresis (PAGE) and visualised by autoradiography. When the probe is present in molar excess over the target fragment, the intensity of the protected fragment will be directly proportional to the amount of complementary RNA in the sample mixture.

7.1.3 QUANTIFICATION BY PCR

One of the most used applications of PCR is RT-PCR that permit the qualitative and quantitative analysis of the levels of the expression of specific genes using both reverse transcription (conversion of mRNA to cDNA) and PCR (selective amplification of DNA). This PCR-based method has been termed as RT-PCR (Rappolee et al., 1991), RNA-PCR (Kawasaki, 1991), RNA phenotyping (Chelly et al., 1988) or message amplification phenotyping (MAPPing) (Brenner et al., 1989).

RT-PCR it is 1,000 to 10,000 times more sensitive than traditional techniques of RNA blotting (Byrne et al., 1988; Wang et al., 1989). This extraordinary sensitivity of RT-PCR has been used to amplify, clone and characterise mRNAs of short life and/or present in very little amounts and detection mRNAs of specific cells in a heterogeneous mixture of cells. For these reasons it can be used for diagnosis of genetic diseases, cancer and the study of the susceptibility of diseases (Gilliland et al., 1990; Wang et al., 1990; Siebert et al., 1992; Ferre et al., 1994). In the RT-PCR process the RNA is first isolated from cells or tissues and then used as a template for reverse transcription to complementary DNA (cDNA). The cDNA in turn is used as template for PCR, using primers designed to amplify a selected cDNA region. The

PCR product is analysed by agarose gel electrophoresis. The amplified cDNA is identified by size of the PCR product and this product can be further validated by restriction digestion, hybridization or nucleotide sequencing.

PCR is an exponential reaction in which small variations in amplification efficiency can yield large changes in the amount of products. In addition, later cycles of PCR exhibit the plateau effect, in which the rate of amplification slows and eventually levels off. These characteristics of PCR can make it difficult to obtain quantitative data. However, if specific conditions and proper controls are used, quantitative information about mRNA levels can be obtained. Careful kinetic analyses can be used to determine initial concentrations of mRNAs by linear regression analysis without internal controls (Singer-Sam et al., 1989). Nonetheless, it necessary to include internal controls to address the problem of tube-to-tube variation in amplification efficiency. Internal controls can be a endogenous mRNA or exogenous cRNA. In addition, exogenous standards can be designed with the same or different primer annealing sequences as their target molecules.

One advantage of using endogenous internal mRNA controls is that the yield of RNA and the efficiency of the reverse transcription can vary to some extent without loss of accuracy. However, preliminary studies must be performed to ensure that the levels of the endogenous control mRNA does not change during the experiment. This must be tested because many housekeeping genes whose expression may seen unrelated to the experimental conditions, are transcriptionally regulated by many different pharmacological agents. In addition, the data must be collected before the amplification reaction reaches the plateau phase. This can be difficult if the endogenous control gene is expressed at a different level than the target gene or if their relative amplification efficiencies differ greatly.

Exogenous internal standards that share the same primer annealing sequences with the target allow calculation of the absolute amount of target mRNA. A similar method, termed competitive PCR, circumvents many of the disadvantage of the other quantitative methods. Competitive PCR is accurate enough to discern differences in mRNA levels as small as 2- to 3-fold or smaller. This is comparable to the accuracy of quantitative methods that used either endogenous or exogenous internal standards in non-competitive experiments. Competitive PCR can be used to measure relative changes in mRNA levels as well, for example, in gene regulation studies. However two conditions must be met to use competitive PCR. First, the molar quantity of the competitor RNA or DNA must be known (usually this is not a problem because it can be measured by UV spectrophotometry). Second, the amplification efficiency of the competitor and target must be identical. This is often true because the standard and target possess the same primer binding sequences. If the standard is a RNA fragment, the efficiency of the reverse transcription must also be considered.

Perhaps the most important advantage of competitive PCR is that useful data can be obtained during the entire course of amplification, even after the reaction has reached the plateau phase. This is not the case for methods using internal standards. Homologous competitor fragments have the same amplification efficiency as their corresponding target but can form heteroduplexes which can complicate the measurement of PCR products. Heterologous competitor fragments, on the other hand, cannot form heteroduplexes, but their amplification efficiencies must be shown to be equal (or very similar to) that of the target.

7.2 RESULTS

7.2.1 TOTAL RNA ISOLATION

The use of high-quality RNA is critical for the success of gene expression analysis. To ensure optimal quantification of the mRNA levels of the NPY-Y1 receptor gene in PC12 cells, the quality of the all the total RNA preparations were assessed by examining a number of factors. Firstly, the absorbance at 260 nm (indicating the amount of RNA) was compared to that absorbance at 280 nm (indicating the contamination with proteins). Routinely the ratio 260/280 nm was about 1.8-2.0 indicating a high quality RNA (Chapter 2. Quantification of the nucleic acid). If the ratio fell outside this range, the RNA preparation was discarded. Secondly, the RNA was separated by size in a denaturing (formaldehyde) agarose gel electrophoresis to check that not be degraded by ribonucleases (See Chapter 2. Nucleic acid separation), by examining the intactness of the ribosomal (rRNA) bands 18 S and 28 S . If the RNA is intact, it will exhibit a sharp and distinct 28 S and 18 S rRNA bands, with the 28 S band about twice as intense as the 18 S band under UV light (Figure 7-3A).

The most common and successful methods for isolation pure, intact total RNA are modifications of the original guanidium thiocyanate method (Chirgwin et al, 1979). One method was used in the thesis (See Chapter 2. Total RNA isolation). This method was chosen because of its simplicity, speed, ability to handle many samples and consistent. In this method the cells were disrupted in high concentrations (4 M) of guanidium thiocyanate to rapidly inactivate ribonucleases. The resultating lysate was extracted with an acidic solution of chloroform: phenol: isoamyl alcohol (24 : 24 : 1) to remove proteins and DNA, and the total RNA was precipitated with isopropanol (Chomczynsky and Sacchi, 1987). The total RNA yield per 60 mm plate (containing approximately 1.8-2.0 x 10^6 cells) obtained by the acid phenol method was about 80-100 µg. This value was reproducible during the course of this thesis. Isolated total RNA was stored as an ethanol precipitate at -20°C for several months or in aqueous solution (DEPC-treated water) at -80°C for immediate use. Total RNA stored in ethanol remained in good conditions for several months as assessed by denaturing agarose gel electrophoresis. However, repeated freeze and thaw cycles of the RNA solution were avoid.

7.2.2 NORTHERN BLOT ANALYSIS

In order to study the relative expression of the NPY-Y1 receptor gene in PC12 cells, Northern blot analysis was performed using total RNA and random-primer radioactive-labelled probes complementary to the mRNA of this neuropeptide receptor. As a positive control of the Northern blot analysis a full length double stranded cDNA for open reading frame of the rat NPY gene was used. The NPY cDNA was released from the pGEM-3 vector (Promega) by digestion with *Eco* RI of the construct NPY-5 supplied by Professor Janet M. Allen (Allen et al., 1987) (See Chapter 2. Nucleic acid labelling).

7.2.2.1 OLIGONUCLEOTIDES FOR PROBE OF THE NPY-Y1 GENE

The oligonucleotides 2010-2011 (Appendix II) were used to amplify by PCR the ORF of the rat NPY-Y1 gene from a cDNA plasmid library of RINm5f cells supplied by Professor Janet M. Allen (Figure 7.1). Forward primer (2010) was modified at the 5'-region to include a *Hind* III restriction enzyme site to be used for subcloning purposes. This primer anneals to the 5'-UT region (at the exon II) of the rat NPY-Y1 gene, specifically at -54 to -30 bp from the first ATG. Reverse primer (2011) was modified at the 5'-region to include an *Xba* I restriction enzyme site to be used for subcloning purposes. This primer anneals to the 3'-UT region (at the exon III) of the rat NPY-Y1 receptor gene, specifically at 1284 to 1260 bp from the first ATG (Appendix II). This cDNA clone containing the ORF of the rat NPY-Y1 receptor (pBS-Y1) was further used as a probe by random-primer labelling for Northern blot assays.

7.2.2.2 CLONING OF THE ORF OF THE NPY-Y1 GENE

The ORF of the rat NPY-Y1 gene was amplified by PCR using the Dynazyme DNA polymerase from a cDNA plasmid library of RINm5f cells (rat insulinoma pancreatic cell line expressing the NPY-Y1 receptor) (Figure 7-2A). This PCR product (1.3 kb) (Figure 7-2B) containing the ORF of the rat NPY-Y1 gene was first extracted with phenol/chloroform and the DNA precipitated by addition of 2 volume of ethanol. Following the PCR product (1.3 kb) generated with the Dynazyme polymerase was phosphorylated with T4 polynucleotide kinase or digested with the restriction enzymes *Hind* III and *Xba* I. This DNA was extracted again with phenol/ chloroform and ethanol precipitated.

In parallel, the pBluescribe SK$^+$ (Stratagene) was digested with *EcoR* V and the enzyme was then inactivated by heating the reaction for 15 minutes at 65 °C. The free ends of the vector were dephosphorylated by treatment with SAP and the enzyme was also inactivated by heating for 15 minutes at 65 °C. As alternative method of cloning, the pBluescribe SK$^+$ was digested

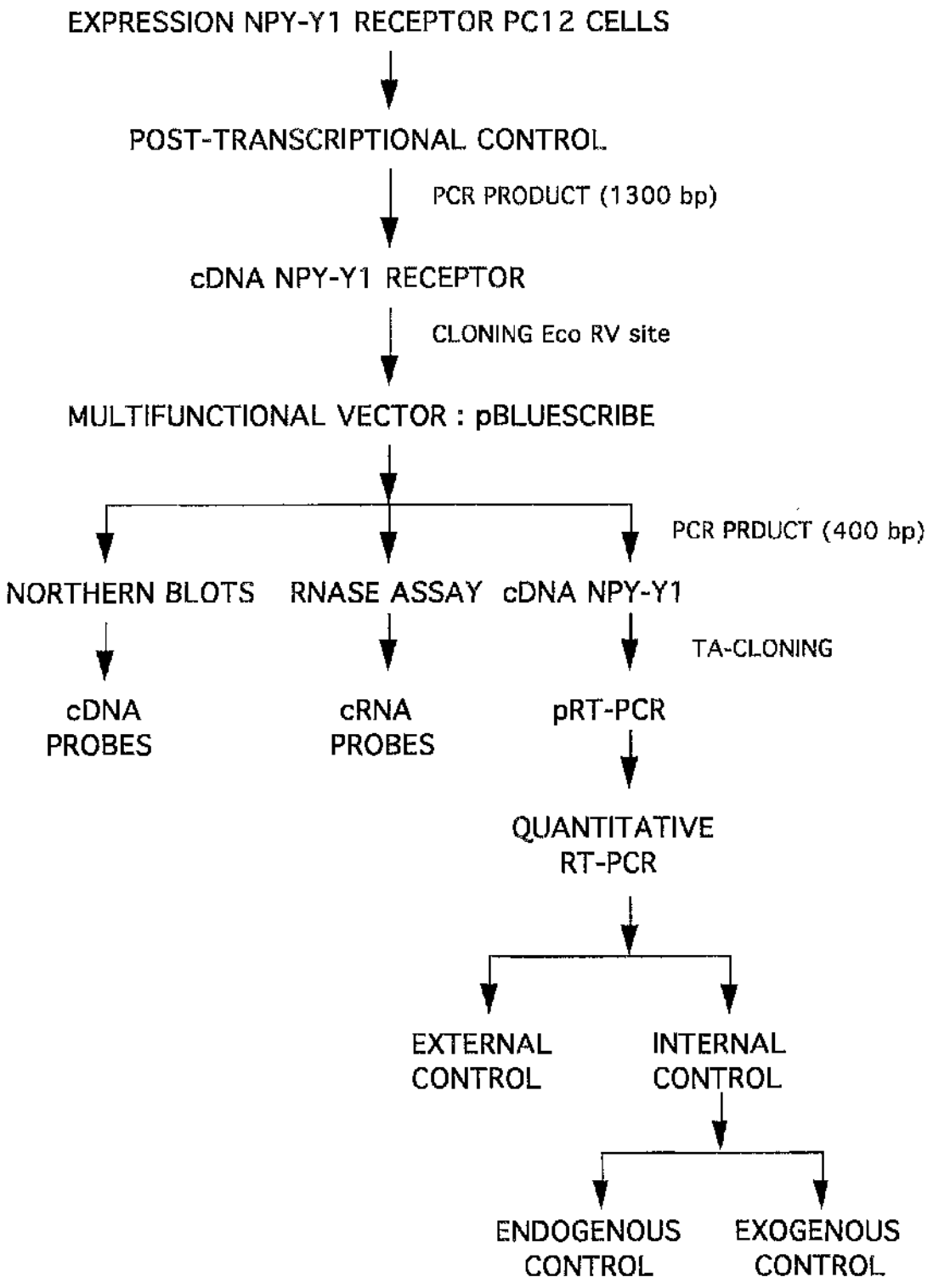


FIGURE 7.1 STRATEGY FOR QUANTIFICATION OF THE NPY-Y1 RECEPTOR GENE IN PC12 CELLS

Methodological strategy used for quantification of the NPY-Y1 receptor gene in PC12 cells

simultaneously with two restriction enzymes *Hind* III-*Xba* I (present in the 5'-end of the forward and reverse primers but not in the ORF of the NPY-Y1 gene) and these enzymes were also inactivated by heating the reaction for 15 minutes at 65 °C.

The PCR product (1.3 Kb) containing the ORF of the NPY-Y1 gene treated with T4 polynucleotide kinase was bidirectionally ligated into the *Eco R* V (blunt-end) site of the polylinker region of the pBluescribe SK+ previously treated with SAP phosphatase (Figure 7-2C). In addition, PCR product previously digested with *Hind* III-*Xba* I was also ligated into the *Hind* III-*Xba* I (sticky-end) sites of the pBluescribe SK+ (Figure 7-1). Ligation of these PCR products into the *EcoR* V or *Hind* III-*Xba* I sites of the pBluescribe SK+ was carried out by overnight incubation at 15 °C. A negative control ligation reaction was performed to test efficiency of the vector dephosphorylation (blunt-end cloning), using identical conditions but the PCR product was replaced by water. Positive control reaction was performed using 10-100 ng of the *Eco R* V-digested pBluescribe SK+ to test the efficiency of the ligation reaction. 10 µl of each ligation reaction were transformed into competent bacteria and transforming colonies selected on agar plates containing 100 µg/ml ampicillin.

A NPY-Y1 receptor clone (pBS-Y1) was generated from the *EcoR* V (blunt-end) cloning procedure and this insert was used as a probe for Northern blot assays (Figure 7-2C). Insert was obtained for the pBS-Y1 by digestion with *Hind* III-*Xba* I (Figure 7-2D) and purified in a 1% LMP agarose gel. 1.3 Kb insert was labelled by random-primer (See Chapter 2. Nucleic acid labelling). This clone was also used to generate cRNA antisense probes for RPA (See Chapter 2. Nucleic acid labelling). Orientation of the insert in the pBS-Y1 was determined by digestion with several restriction enzymes, includind *Pst* I and *Xho* I (Figure 7-2D) confirming that the insert was cloned in the opposite direction of the T7 promoter. The clone was digested with the restriction enzyme *Nde* I which generates a 5'-protruding end and results in a "run-off" transcripts (600 bp) from the insert sequence (Figure 7-2E). Linearized pBS-Y1 was extracted with phenol/chloroform before using the DNA for *in vitro* transcription (See Chapter 2. cRNA synthesis).

7.2.2.3 NORTHERN-BLOTS

For Northern blot analysis, equal amounts of the total RNA was loaded in each well of a denaturing (formaldehyde) agarose gel (See Chapter 2. Nucleic acid separation). This uniform loading was confirmed by comparing the intensity of the rat ribosomal RNA bands, 18 S (4.8 Kb) and 28 S (1.9 kb) from the different samples loaded side by side (Figure 7.3A).

FIGURE 7.2 CLONING NPY-Y1 RECEPTOR GENE

Strategy used for cloning the open reading frame of the NPY-Y1 receptor gene

A. RINm5f cDNA plasmid library was used to amplify by PCR a 1.3 Kb fragment of the ORF of the NPY-Y1 receptor gene.

B. Agarose gel showing a 1.3 Kb PCR from the RINm5f cDNA library.

Lane 1: MWM (λ x *Hind* III-*Eco* RI) **Lane 2**: PCR product (1.3 Kb)

C. The PCR product was cloned in the Eco RV of pBS-SK$^+$ (pBS-Y1)

D. Agarose gel showing a restriction map of the PCR insert of the pBS-Y1. Inserts were obtained by *Hind* III-*Xba* I restriction and used as a probe to Northern blot.

Lane 1: pBS-Y1 x *Xho* I **Lane 2**: pBS-Y1 x *Pst* I

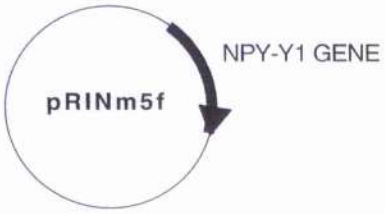
Lane 3: pBS-Y1 x *Hind* III-*Xba* I **Lane 4**: MWM (λ x *Hind* III-*Eco* RI)

E. pBS-Y1 was cut with Nde I and used to generated antisense cRNA probe to RPA. Agarose denaturing gel showing antisense cRNA of the NPY-Y1 receptor.

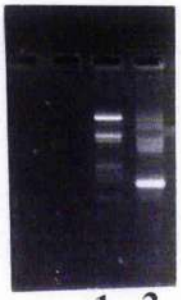
Lane 1: MWM (λ x *Hind* III-*Eco* RI) **Lane 3**: antisense cRNA mouse β-actin.

Lane 5: antisense cRNA NPY-Y1 receptor.

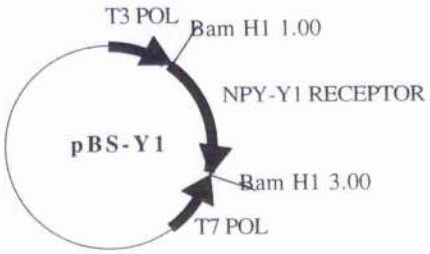
A


NPY-Y1 GENE
pRINm5f

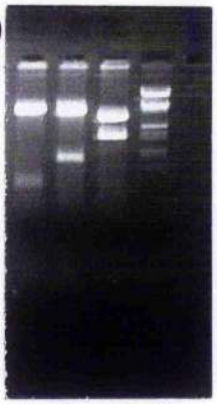
B


1 2

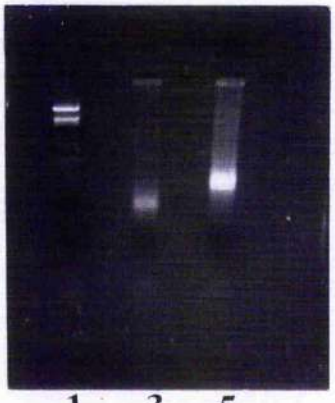
C


T3 POL
Bam H1 1.00
NPY-Y1 RECEPTOR
pBS-Y1
Bam H1 3.00
T7 POL

D


1 2 3 4

E


1 3 5

For Northern-blot analysis, total RNA was electrophoresed in the presence of ethidium bromide (See Chapter 2. Nucleic acid separation). This did not interfere with the performance of the assay and provide additional advantages as it was possible to verify equal loading of total RNA into different lanes of the gel, and visualization of the transfer of the total RNA to the membrane. A photograph of the gel under UV light was taken routinely to confirm the quality of the total RNA preparation (Figure 7-3A).

Autoradiographic results of this experiment was illustrated in the figure 7.3B. Up to 50 µg of total RNA of PC12 cells were loading in each lane and hybridised to a high-specificity cDNA probes for NPY-Y1 receptor (Figure 7.3 A) and NPY (positive control) (Figure 7-3 B). The rat NPY-Y1 receptor probe result in no signal after overnight incubation at high stringency conditions (Chapter 2. Nucleic acid hybridization). However, using the rat NPY probe the mRNA was detected in untreated PC12 cells and the treatment with NGF (100 ng/ml) for 48 hours resulted in a approximately 10-fold increase in the amount of NPY mRNA over basal levels. Similar level of NGF-induced activation was previously reported to this neuropeptide gene in PC12 cells (Allen et al., 1987; Balbi and Allen, 1994). For this reason the hibridization conditions were made less stringet, reducing the temperature of the incubation (45 °C) and/or increasing the SDS (2X) in the washing buffer (See Chapter 2. Nucleic acid hybridization), but in this case the rat NPY-Y1 receptor probe hybridised to the 28 S and 18 S ribosomal RNA (data not shown).

Since the dissociation temperatures of the NPY and NPY-Y1 receptor probes were comparable and specific activities generated for these labelled probes were similar, as quantitated by liquid scintillation counting, the different hybridisation signal likely reflects inherent differences in the abundance of the both mRNAs in PC12 cells. For this reason a more sensitive approach, ribonuclease protection assay (RPA) was used.

7.2.3 RIBONUCLEASE PROTECTION ASSAY

7.2.3.1 LABELLING OF THE DNA MARKERS

Dephosphorylated fragments of ϕX174 digested with *Hinf* I (250 ng) were used as a molecular weight markers (MWM) in the RPA. This DNA markers were 5'-end labelled with T4 polynucleotide kinase (See Chapter 2. Nucleic acid labelling) and were stored in 200 µl of nuclease-free water (DEPC-treated) at -20 °C. For accurate sizing of single stranded denatured DNA or RNA it is important that the DNA markers be denatured before loading them on the gel (See Chapter 2). The denaturation of the DNA markers for RPA was made as following: 1 µl of the phosphorylated markers was added to the RNA loading dye (Appendix II), heated at 90 °C for 10 minutes and immediately loaded on the denaturing PAGE (Figure 7.3C).

FIGURE 7.3 NORTHERN BLOT AND RIBONUCLEASE PROTECTION ASSAY

FIGURE 7.3A TOTAL RNA EXTRACTED FROM PC12 CELLS

Equal amounts of total RNA (10 μg) were extracted from unstimulated and NGF (100 ng/ml) stimulated PC12 cells for 48 hours. RNA samples were denatured and subsequently electrophoresed in a formaldehyde gel. Run 100 volts x 2 hours. 18S and 28S ribosomal RNAs (rRNA) are marked with arrows.

Lane 1: Total RNA of untreated PC12 cells.

Lane 2: Total RNA of NGF-treated cells.

FIGURE 7.3B NORTHERN BLOT ASSAY

Northern blot assay of the NPY mRNA in unstimulated and NGF (100 ng/ml) treated PC12 cells for 48 hours. Gel described before (Figure 7.3A) was blotted to nylon membranes and hybridization analysis with radioactively-labelled NPY cDNA probe was performed. Autoradiograph exposure time: overnight with intensifying screen (-70°C). Autoradiograph of the membrane was scanned using the phosphoimager scanning densitometer (Fuji, model BAS1000 MacBas). 18S and 28S ribosomal RNAs (rRNA) are marked with arrows.

Lane 1: Total RNA of untreated PC12 cells.

Lane 2: Total RNA of NGF-treated PC12 cells.

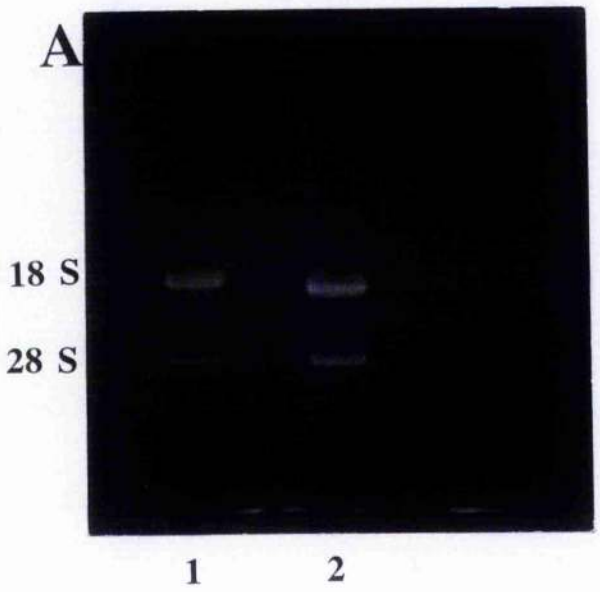
FIGURE 7.3C RIBONUCLEASE PROTECTION ASSAY

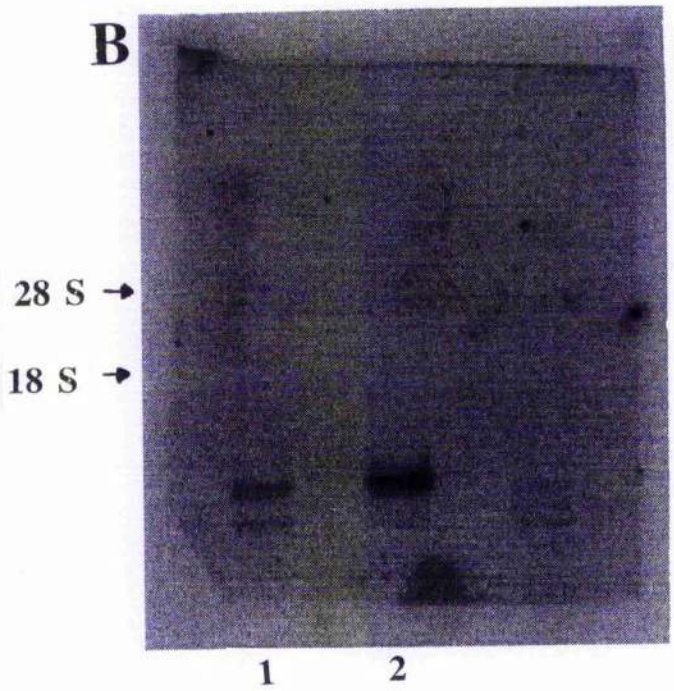
Ribonuclease protection assay (RPA) of RNA 18S and NPY-Y1 mRNAs in unstimulated PC12 cells using the human RNA 18S probe (protected fragment of 80 bases). As a negative control yeast RNA (10 μg) (RPA II kit, Ambion) was used and as a positive control antisense human RNA 18S probe was used. Total RNA (10 μg) of unstimulated PC12 cells was used. The samples were run at 250 Volts x 2 hours in a PAGE (4%) gel. Autoradiograph exposure time: overnight with intensifying screen (-70°C).

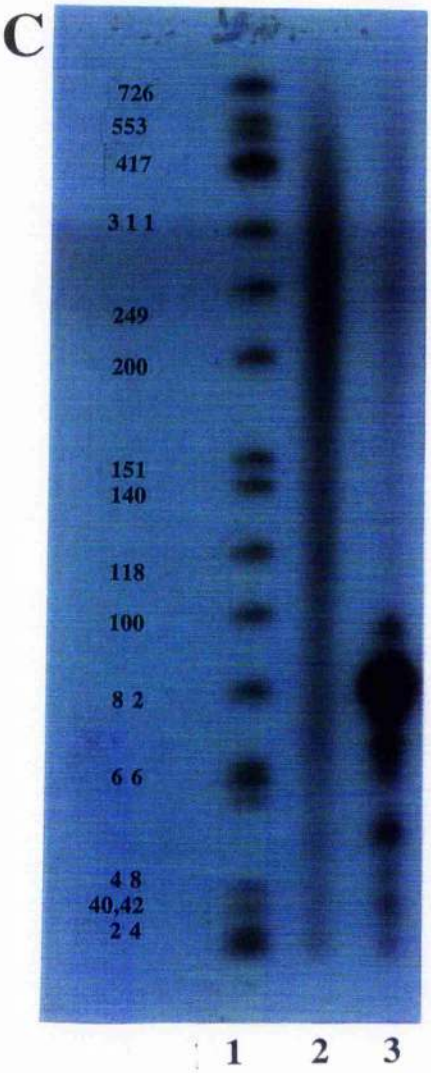
Lane 1: MWM (ϕX174 x *Hinf* I)

Lane 2: yeast total RNA (human RNA 18 S)

Lane 3: PC12 total RNA (human RNA 18 S + NPY-Y1 receptor probe)


A
18 S
28 S
1
2


B
28 S
18 S
1
2


C
726
553
417
311
249
200
151
140
118
100
82
66
48
40,42
24
1
2
3

7.2.3.2 LABELLING OF THE RNA PROBE

A preliminary requirement for a RPA is the synthesis of the labelled antisense RNA transcript which is used as hybridization probe for the detection of the mRNA of interest. For the synthesis of the cRNA antisense probe the pBS-Y1 was linearized with *Nde* I restriction enzyme (See Chapter 2. Nucleic acid labelling). This *Nde* I restriction site is present at the position 749 of the first ATG (methionine) in the open reading frame of the NPY Y1 receptor gene and results in a 5'-overhang ends. *In vitro* transcription reaction using T7 RNA polymerase generated a probe of approximately 600 nucleotides of full length transcript and 540 base protected fragment (Figure 7.2E). This size helps to maximize synthesis of full length transcripts and increase the sensitivity of the RPA assay.

In vitro transcription reaction using the Riboprobe Assay System (Promega) generated a RNA probe with high-specific activity (data not shown). This RNA radiolabelled probe not was gel purified because most of the transcript is present as a single band of the expected size, as assessed on a 2 % agarose gel (Figure 7.2E). Unincorporated ribonucleotides were removed for ethanol precipitation before the proportion of radiolabel incorporated into transcript was determinated by trichloroacetic acid (TCA) precipitation (See Chapter 2. Nucleic acid labelling). However, unincorporated nucleotides generally not cause problems, since they will not hybridize to the target mRNA and run off to the end of the gel when the protected fragments are separated. The probe was usually stored at -20 °C in DEPC-treated water.

As a negative control for the RPA, a yeast tRNA (10 µg) supplied with the RPA II *in vitro* transcription kit (Ambion) was used. As a positive control of the RPA a human RNA 18S antisense probe supplied with the RPA II *in vitro* transcription kit (Ambion) was used. This human RNA 18S probe is under the control of T7 RNA promoter. In the case of this human RNA 18S probe a 1/500 dilution of the RNAse solution was used to assess total RNA of PC12 cells and the yeast tRNA control tube was digested with the same reduced amount of RNAse.

7.2.3.3 HYBRIDIZATION, RNAse DIGESTION AND SEPARATION OF PROTECTED FRAGMENTS

As a negative control for the RPA one tube of yeast tRNA (10 µg) with the radiolabelled human RNA 18S antisense probe was set up (Figure 7-4). This tube was incubated overnight at 45°C with a diluted amount of RNase (1/500 dilution of the RNase solution). This reaction showed the expected result as no specific signal can be seen on the lane, although some smaller, smeared, heterogeneous material can be seen, which is presumably due to radiolytic decay of the human probe.

As a positive control for the RPA one tube of PC12 cells RNA (10 µg) with the radiolabelled human RNA 18S antisense probe and the NPY-Y1 receptor antisense probe was set up (See Chapter 2. Ribonuclease protection assay). This tube was incubated overnight a 45 °C and then treated with a diluted amount of RNAse (1/500 dilution of the RNAse solution). This lane showed the expected result as a band of 80 nucleotides can be seen corresponding to the human RNA 18S protected fragment (Figure 7.3C). Differences observed with the DNA markers were a consequence of the different relative mobilities of DNA and RNA fragments in 7M urea polyacrylamide gels, with RNA fragments running somewhat slower (approximately 10 %) than their DNA counterparts. This result confirms that the RNA is in optimal conditions and the human RNA 18S probe is over molar excess over the 18S mRNA.

The NPY-Y1 receptor antisense probe was also mixed with PC12 total RNA (10 µg). However, the expected fragment of approximately 540 nucleotides corresponding to the NPY-Y1 receptor mRNA was not observed. As an assessment of the probe quality, the yeast tRNA was mixed with the NPY-Y1 receptor antisense probe and incubated without RNAse. This result showed a band representing mainly the full-length probe (data not shown). Moreover, increasing the sensitivity of the assay, by increasing the concentrations of total RNA of the PC12 cells (up to 50 µg) or increasing the dilution of the RNAse solution (up to 1/50) did not result in the expected specific signal corresponding to the NPY-Y1 receptor mRNA (data not shown).

The results of the ribonuclease protection assay (RPA) experiments studies suggest that the expression of the NPY-Y1 receptor gene is very low in the PC12 cells. For this reason a more sensitive approach, reverse transcription-polymerase chain reaction (RT-PCR) was used.

7.2.4 RT-PCR DETECTION OF THE NPY-Y1 GENE

7.2.4.1 OLIGONUCLEOTIDES FOR QUANTITATIVE RT-PCR

7.2.4.1.1 Oligonucleotides for Positive Control

Oligonucleotides 2486-2487 (Appendix II) were used to amplify by RT-PCR a small fragment (approximately 400 bp) of the 3'-flanking region of the NPY-Y1 receptor gene (Figure 7-4A). Forward primer (2486) was designed to anneal to 3'-flanking region of the NPY-Y1 receptor gene, specifically to the nucleotides 871 to 888 from the first ATG. Reverse primer (2487) was designed to anneal to the 3'-UT region of the NPY-Y1 receptor gene, specifically to the nucleotides 1281 to 1260 from the first ATG.

PCR fragment of the NPY-Y1 receptor (approximately 400 bp) was amplified using PC12 cells total RNA (1 µg) and the Taq DNA polymerase (Figure 7-4A) and was subsequently cloned in the pTAg vector (R&D Systems). One positive clone (pRT-PCR) was further used to create a antisense cRNAs by *in vitro* transcription for use as positive control of the RT-PCR amplification of the mRNA of the NPY-Y1 receptor gene (Figure 7-4B). This small PCR fragment was also used for create by PCR a specific deletion of 100 nucleotides for be used as exogenous internal control for a competitive RT-PCR.

7.2.4.1.2 Oligonucleotides For Exogenous Control

Oligonucleotides (T7507-T7508) (Appendix II) were used to create a PCR deletion of approximately 100 bp in the small PCR fragment (400 bp) of the 3'-flanking region of the NPY-Y1 receptor cloned in the pTAg vector (pRT-PCR clone) for use as exogenous control in a competitive RT-PCR. The internal reverse primer (T7507) was modified to include at the 5'-flanking region a *Sac* II restriction site not present in the pTAg cloning vector or the PCR fragment (to be used for cloning purposes). This primer anneals to the nucleotides 941 to 923 (from the first ATG of the NPY-Y1 receptor gene). The internal forward primer (T7508) was also modified at the 5'-flanking region to include a *Sac* II restriction site. This primer anneals to the nucleotides 1151 to 1169 of the NPY-Y1 gene.

Forward primer (2486) and the "internal" reverse primer (T5707) were used to generate a PCR fragment of approximately 170 bp (product I) (Figure 7-6). The "internal" forward (T5708) and the reverse primer (2487) were used to synthesise another PCR fragment of approximately 130 bp (product II) (Figure 7-6). These two PCR fragments were ligated and re-amplified to generated a DNA fragment of approximately 300 bp containing a specific deletion of 100 bp of the 3'-flanking region of the NPY-Y1 gene (Figure 7-6). This PCR fragment (300 bp) was subsequently cloned into the pTAg vector (R&D Systems). One positive clone (pTA/ΔRT-PCR) was further used to make antisense cRNAs by *in vitro* transcription using the Riboprobe System kit (Figure 7-4E). This cRNAs were used to develop a competitive RT-PCR to study the NPY-Y1 receptor gene expression in PC12 cells.

7.2.4.1.3 Oligonucleotides For Endogenous Control (RT-PCR)

Oligonucleotides (Y7462-Y7463) (Appendix II) were used to detected by RT-PCR a small fragment (approximately 300 bp) of the open reading frame of the rat cyclophilin gene (Appendix III) for be used as endogenous control for a quantitative RT-PCR. The rat cyclophylin gene is constitutively expressed in several eukaryotic cell lines and expression is not altered by NGF (Fanger et al., 1993) which is known to increase the transcriptional activity of the NPY-Y1 receptor gene in PC12 cells .

Forward primer (Y7462) anneals to the nucleotides 232 to 256 from the first ATG of the rat cyclophilin gene. The reverse primer (Y7463) anneals to the nucleotides 534 to 513 from the first ATG of this gene. The RT-PCR reaction of total RNA of PC12 cells using these primers generated a DNA fragment of 300 bp (Figure 7-7). Thus, this DNA will be used as internal control in a quantitative RT-PCR (multiplex RT-PCR) of the NPY-Y1 gene in PC12 cells.

7.2.5 CLONING PCR PRODUCTS

7.2.5.1 CLONING A FRAGMENT OF ORF OF THE NPY-Y1 GENE

Oligonucleotides 2486-2487 (Appendix II) were used to amplify by RT-PCR a small fragment (approximately 400 bp) of the 3'-flanking region of the NPY-Y1 receptor gene (Figure 7-3A). This PCR fragment of the NPY-Y1 gene was amplified using PC12 cells total RNA as a template and the Taq DNA polymerase (Figure 7-4A). The PCR fragment generated was subsequently cloned into the pTAg vector (R&D Systems) using the manufacturer instructions. Positive colonies were obtained, plasmid was isolated from these colonies and digested with the restriction enzymes *Hind* III- *Bam* HI present in the polylinker region of the pTAg-cloning vector, at both sides of the cloning site. Positive clones were obtained with a insert of the expected size (approximately 400 bp) and digested with *Hinc* II to determine the orientation of the insert (data not shown). One positive clone (pRT-PCR) with the insert cloned in the opposite direction to the T7 RNA polymerase was used to large scale plasmid purification using the Wizard Maxipreps kit (Figure 7-4B). This clone was deleted in 100 bp to create antisense cRNAs by *in vitro* transcription for be use as internal control of the RT-PCR amplification of the NPY-Y1 receptor gene.

7.2.5.2 CLONING A DELETION FRAGMENT OF THE pRT-PCR

In order to delete 100 bp of the 3'-flanking region of the rat NPY-Y1 receptor gene present in the pTA/RT-PCR, the PCR reaction was used (Figure 7-4C). The pRT-PCR was amplified using the "original" forward primer (2486) and the "internal" reverse primer (T5707). This pair of primers generates a PCR fragment of 170 bp (product I) which is similar to the molecular weight of a band seen in the 2% agarose gel (Figure 7-4C). The "internal" forward (T5708) and the "original" reverse primer (2487) were used to create other PCR fragment of 130 bp (product II) which is similar to the molecular weight of a band seen in the same gel (Figure 7-4C).

FIGURE 7.4 CLONING OF A 3'-FRAGMENT OF THE NPY-Y1 GENE AND ITS DELETION

Cloning a PCR fragment (400 bp) of the 3'-flanking region of the NPY-Y1 gene.

A. A small fragment (400 bp) of the 3'-region of the NPY-Y1 receptor gene was amplified by RT-PCR using total RNA of unstimulated PC12 cells. As a negative control total RNA (1 μg) of COS-7 cells was used and cRNA IL-1α (GeneAmp kit, Perkin Elmer) was used as a positive control. Agarose gel showing a PCR product of the expected size (400 bp).

Lane 1: MWM (λ x *Hind* III- *Eco* RI). **Lane 2**: RT-PCR COS-cells

Lane 3: RT-PCR cRNA IL-1α (300 bp) **Lane 4**: RT-PCR PC12 cells (400 bp)

B. PCR fragment was ligated into the pTAg-cloning vector (pTA/RT-PCR). This clone was used to generate a cRNA external control for a quantitative RT-PCR.

Strategy used for the generation of a exogenous internal control for a quantification of NPY-Y1 mRNA in PC12 cells by competitive RT-PCR.

C. Two fragments of the 3'-region of the NPY-Y1 receptor gene were amplified by PCR using the pRT-PCR as template.

Agarose gel showing two PCR products with the expected size.

Lane 1: MWM (λ x*Hind* III-*Eco* RI).

Lane 2: PCR product I (170 bp).

Lane 3: PCR product II (130 bp).

D. PCR products were ligated and reamplified using the Taq DNA polymerase. Agarose gel showing a PCR product of the expected size (300 bp).

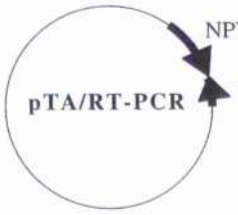
Lane 1: MWM (λ x*Hind* III-*Eco* RI). **Lane 3**: PCR product (300 bp).

E. PCR product was ligated into the pTAg cloning vector (pTA/RT-ΔPCR).

A

1 2 3 4

B



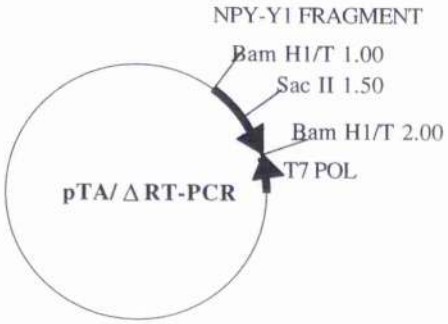
C

1 2 3

D

1 3

E



These two PCR fragments were digested by overnight incubation with the *Sac* II and ligated using T4 DNA ligase. The ligation reaction was reamplified using PCR and the "original" forward (2486) and reverse (2487) primers. In theory, only the correct ligated products can be re-amplified to generated a DNA fragment of approximately 300 bp containing a specific deletion of the 3'-flanking region of the NPY-Y1 gene present in the pRT-PCR clone (Figure 7-4D). This PCR fragment (300 bp) was subsequently cloned into the pTAg cloning vector (R&D Systems). Positive colonies were obtained, plasmid was isolated from these colonies and digested with *Sac* II. This restriction enzyme is not present in the pTAg cloning vector and only plasmids with the correct ligated product can be linearised. In addition, the orientation of the clone was verified using the *Hinc* II restriction enzyme, only present in the 5'-flanking region of the insert but not in the pTAg-cloning vector (data not shown). One positive clone (pTA/ΔRT-PCR), with the insert cloned in the opposite orientation of the T7 RNA polymerase was obtained and amplified by large scale plasmid purification using the Wizard Maxipreps kit (Figure 7-4E).

The deletion in this clone was further confirmed using one restriction site (*Acc* I) present in the 100 bp deleted region of the NPY-Y1 gene of the pRT-PCR but not in the pTAg-cloning vector. This restriction enzyme (*Acc* I) can cut once the pTA/RT-PCR but cannot cut the pTA/ΔRT-PCR (data not shown). However, the restriction enzyme *Sac* II cannot cut the pRT-PCR but cut once the pTA/ΔRT-PCR (data not shown). This clone was further used to synthesise antisense cRNAs by *in vitro* transcription using the Riboprobe System kit (Promega). This antisense cRNAs were used as internal control in a competitive RT-PCR to study the NPY-Y1 receptor gene expression in PC12 cells.

7.2.6 QUANTITATIVE RT-PCR OF THE NPY-Y1 GENE

7.2.6.1 cRNA SYNTHESIS (*IN VITRO* TRANSCRIPTION)

Antisense cRNAs for be used as internal control of the competitive RT-PCR were synthesized from the clone pTA/ΔRT-PCR using T7 RNA polymerase and the Riboprobe kit (Promega). This clone was linearized with the *Hind* III enzyme present in polylinker region of the pTAg vector, opposite to the T7 RNA polymerase. This DNA fragment was used as a template for a "*in vitro* " transcription reaction in order to synthesize a "run-off" transcripts of 300 bp to be used as internal control in a competitive RT-PCR reaction.

7.2.6.2 cDNA SYNTHESIS (REVERSE TRANSCRIPTION)

cDNA template was synthesized from a cRNA or total RNA of PC12 cells by reverse transcription (RT) using the random primers present in the GeneAmp RNA PCR kit (Perkin

Elmer-Cetus) following the manufacturer instructions (See Chapter 2. cDNA synthesis). As a positive control IL-1a cRNA was amplified by two specific primers DM151-DM152 which generated a 308-bp product. As negative control total RNA of COS-7 cells which do not express the NPY-Y1 gene was used.

7.2.6.3 DNA AMPLIFICATION (PCR)

In order to define the linear range of amplification of the NPY-Y1 gene in PC12 cells, a RT-PCR reaction was set-up. Total RNA (1 μg) of unstimulated PC12 cells was amplified using the "original" forward (2486) and reverse (2487) primers and different PCR cycles (10-40 cycles) (Figure 7-5A). These primers amplify a PCR product of 400 bp of the 3'-flanking region of the NPY-Y1 receptor gene. However this product can be detected only after 40 cycles of PCR amplification (Figure 7-5A). This result indicates that the mRNA for the NPY-Y1 gene is expressed at very low levels in PC12 cells, consistent with the previous negative results obtained with other less sensitive techniques, such as Northern blots and RPA. Thus, the next RT-PCR reactions were made using 35-40 cycles of amplification.

In the following experiment, the NPY-Y1 gene was amplified using total RNA of PC12 cells, as well as of other cells lines which express this neuropeptide receptor gene, RINm5f cells (a rat pancreatic cell line) (Polak et al., 1993). Preliminary results show that PC12 cells and RINm5f have similar levels of NPY-Y1 receptor expression (Figure 7-5B).

In the next experiment, the mRNA of the NPY-Y1 receptor gene was amplified from PC12 cells treated with different pharmacological agents which are known to increase the levels of transcription of this gene, such as dexamethasone, NGF, PACAP, forskolin and PMA (See Chapter 3). This showed that in comparison with the basal mRNA levels, the different aggents increased the NPY-Y1 receptor gene expression (Figure 7-5C). However, these relative results are difficult to quantitate without internal control, because PCR is an exponential reaction in which small differences in the efficiency of the amplification results in significant differences in the amount of the product. They are two approaches to be used as internal control for a quantitative RT-PCR: (i) endogenous control and (ii) exogenous (homogenous or heterogeneous) control.

7.2.6.3.1 RT-PCR with Endogenous Control

Endogenous control are in generally housekeeping genes whose expression is not altered under the different treatments and for this reason can be used as internal control of the RT-PCR reactions. In the case of PC12 cells several housekeeping genes can be used, such as tubulin and cyclophilin genes. Cyclophilin gene has the advantage that expression is known to be not

FIGURE 7.5 QUANTITATIVE RT-PCR OF THE NPY-Y1 GENE

7.5A LINEAR RANGE OF RT-PCR AMPLIFICATION

Agarose gel of RT-PCR products of NPY-Y1 receptor mRNA using different PCR cycles (kinetic analysis). Total RNA (1 μg) from unstimulated PC12 cells was used to amplified a 400 bp of the 3'-flanking region of the NPY-Y1 mRNA by RT-PCR with the indicated number of cycles. As a positive control a IL-1α cRNA (300 bp) was used.

Lane 1: MWM (λ x *Hind* III-*Eco* RI) **Lane 2**: RT-PCR IL-1α cRNA (40 cycles)
Lane 3: RT-PCR PC12 cells (10 cycles) **Lane 4**: RT-PCR PC12 cells (20 cycles)
Lane 5: RT-PCR PC12 cells (30 cycles) **Lane 6**: RT-PCR PC12 cells (40 cycles)

FIGURE 7.5B RT-PCR AMPLIFICATION NPY-Y1 mRNA FROM PC12 AND RINm5f CELLS

Agarose gel of RT-PCR products of NPY-Y1 receptor mRNA from RINm5f and PC12 cells. Total RNA (1 μg) from unstimulated RINm5f and PC12 cells was used to amplified a 400 bp fragment of the 3'-region of the NPY-Y1 mRNA by RT-PCR (40 cycles).Antisense NPY-Y1 cRNA (300 bp) and antisense NPY-Y1 cRNA (400 bp) were used as external control of the NPY-Y1 gene RT-PCR amplification.

Lane 1: MWM (λ x*Hind* III-*Eco* RI) **Lane 2**: NPY-Y1 cRNA (300 bp) (1 μg)
Lane 3: NPY-Y1 cRNA (400 bp) (1 μg) **Lane 4**: Total RNA RINm5f cells (1 μg)
Lane 5: Total RNA PC12 cells (1 μg)

FIGURE 7.5C RT-PCR AMPLIFICATION OF NPY-Y1 mRNA OF PC12 CELLS TREATED WITH DIFFERENT AGENTS

Agarose gel of RT-PCR amplification of a 400 bp fragment of the 3'-flanking region of the NPY-Y1 mRNA. Total RNA (1 μg) of unstimulated (basal) and PC12 cells treated with different agents : **DEX** (dexamethasone 1 μM), **NGF** (nerve growth factor 100 ng/ml), **PACAP** (PACAP-38, 5 nM), **FORSK** (forskolin, 10 μM), **PMA** (phorbol-12-myristate-13-acetate, 1 nM) for 48 hours was used for RT-PCR reaction (40 cycles). As a positive control a IL-1α cRNA (300 bp) was used. Total RNA (1μg) of unstimulated COS-7 cells was used as negative control. NPY-Y1 cRNA (400 bp) was used as external control.

Lane 1: MWM (λ x*Hind* III-*Eco* RI) **Lane 2**: IL-1α cRNA (10^6 copies)
Lane 3: RT-PCR COS-7 (1 μg) **Lane 4**: antisense NPY-Y1 cRNA (1 μg)
Lane 5: PC12 basal (1 μg) **Lane 6**: PC12 DEX (1μg)
Lane 7: PC12 NGF (1 μg) **Lane 8**: PC12 PACAP (1 μg)
Lane 9: PC12 FORSK (1 μg) **Lane 10**: PC12 PMA (1μg)

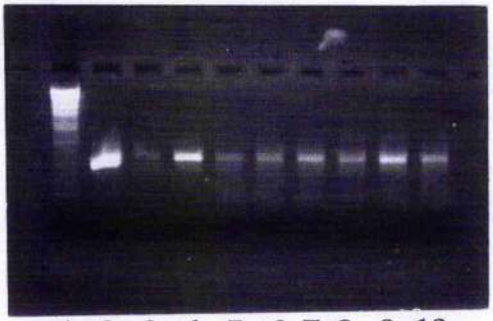
A

1 2 3 4 5 6

B

1 2 3 4 5

C



1 2 3 4 5 6 7 8 9 10

affected by NGF treatment of the PC12 cells (Fanger et al., 1993) and for this reason was choice as internal control for a quantitative RT-PCR. In order to obtain the relative levels of amplification of this gene under the different treatments, total RNA of untreated PC12 cells and cells treated for 48 hours with different agents, such as NGF, PACAP, forskolin and PMA were amplified using the forward (Y7462) and reverse (Y7463) primers of the rat cyclophilin gene. These primers synthesise a RT-PCR product of 300 bp after 40 cycles of amplification (Figure 7-6A). This result showed that the mRNA levels of this gene are not altered by these agents. For this reason this gene can be used as internal control for a quantitative RT-PCR.

The next step was try to define the conditions for co-amplification of the two mRNAs (cyclophilin and NPY-Y1 genes) in a one tube of RT-PCR reaction. This was difficult due to intrinsic differences in the levels of the expression of both genes in PC12 cells. Several attempts were made using constant amounts (20 pmol) of the primers for the NPY-Y1 receptor gene and different dilutions of the primers for the cyclophilin gene. However, after several attempts optimal conditions were defined for a narrow margin (between 1 to 5 pmoles) of the primers for the cyclophilin gene after 35 cycles of amplification (Figure 7-6B). Further co-experiments, including a analysis of the linear range of co-amplification of both messages under these conditions will be necessary to obtain valid results of this quantitative RT-PCR.

7.2.6.3.2 RT-PCR with Exogenous Control

Exogenous controls are genes that are added to the RT-PCR reaction and can be used as internal standard. The theory behind the use of exogenous gene sequences is similar to that described before for endogenous reference sequences. However, there is a significant advantage, as the amount of added standard is precisely known, it is possible to calculate the absolute amount of target mRNA present in the sample.

In order to design a competitive RT-PCR of the NPY-Y1 receptor gene in PC12 cells a exogenous internal standard was prepared. This exogenous control is a small cRNA fragment (300 bp) of the NPY-Y1 gene which can be amplified using the same forward (2486) and reverse (2487) primers used to detect of a 400 bp fragment of NPY-Y1 gene. The theory behind this indicates that the amplification occurs in a truly competitive fashion because the standard and the target sequences actually compete for the same primers and therefore, for amplification. Moreover, the two products can be distinguished by size in a agarose gel. Initially, optimal conditions for the amplification of the cRNA (internal standard) were defined (Figure 7-12). This result indicated that 1 µg of cRNA (internal control) results in a similar levels of amplification for 1 µg of total RNA of PC12 cells. However, further experiments will be necessary for generating a standard curve using dilution of the cRNA standard containing the same amount of sample mRNA (or total RNA), to quantitate the initial amount of target.

FIGURE 7.6 RT-PCR AMPLIFICATION OF THE RAT CYCLOPHILIN GENE

FIGURE 7.6A RT-PCR AMPLIFICATION OF RAT CYCLOPHILIN mRNA OF PC12 CELLS TREATED WITH DIFFERENT AGENTS

Agarose gel of RT-PCR amplification of a 300 bp fragment of the open reading frame of rat cyclophilin mRNA. Total RNA (1 μg) of unstimulated (basal) and PC12 cells treated with different agents : **DEX** (dexamethasone 1 μM), **NGF** (nerve growth factor 100 ng/ml), **PMA** (phorbol-12-myristate-13-acetate, 1 nM), **PACAP** (PACAP-38, 5 nM) and **FORSK** (forskolin, 10 μM) for 48 hours was used for the RT-PCR reaction (40 cycles).

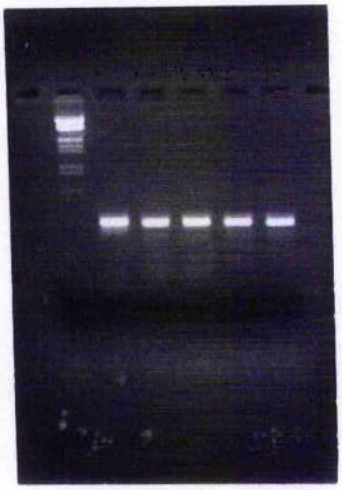
Lane 1: MWM (λ x*Hind* III-*Eco* RI) **Lane 2**: PC12-Basal (1μg)
Lane 3: PC12-NGF (1 μg) **Lane 4**: PC12-PACAP (1μg)
Lane 5: PC12-FORSK (1μg) **Lane 6:** PC12-PMA (1 μg)

FIGURE 7.6B RT-PCR CO-AMPLIFICATION OF CYCLOPHILIN AND NPY-Y1 mRNA OF PC12 CELLS TREATED WITH DIFFERENT AGENTS

Agarose gel of RT-PCR amplification of a 300 bp fragment of the open reading frame of rat cyclophilin mRNA and a 400 bp fragment of the 3'-flanking region of the NPY-Y1 mRNA. Total RNA (1 μg) of unstimulated (basal) and PC12 cells treated with different agents : **NGF** (nerve growth factor 100 ng/ml), **PMA** (phorbol-12-myristate-13-acetate, 1 nM), **PACAP** (PACAP-38, 5 nM) and **FORSK** (forskolin, 10 μM) for 48 hours was used for the RT-PCR reaction (40 cycles).

Lane 1: MWM (1 Kb ladder) **Lane 2**: PC12-BASAL (1μg)
Lane 3: PC12-NGF (1 μg) **Lane 4**: PC12-PMA (1μg)
Lane 5: PC12-PACAP (1μg) **Lane 6**: PC12-FORSK (1 μg)

A



1 2 3 4 5 6

B

1 2 3 4 5 6

7.3 DISCUSSION

Previous results (Chapters 3, 4 and 5) have shown that pharmacological agents, such as dexamethasone, NGF, PACAP, forskolin and PMA among others, known to alter the levels of the expression of several genes in PC12 cells, also increase the transcriptional activity of the NPY-Y1 receptor gene (Figure 3-10). However, it is important to remember that the amount of mRNA measured at any time is influenced not only by the rate of transcription of the gene but also by other post-transcriptional mechanisms, such as mRNA stability. Moreover, many factors that alter transcription of genes, also alter the stability of its mRNA, in turn increasing the pool of measurable RNA.

7.3.1 NORTHERN BLOT ANALYSIS

In this chapter an attempt was made to quantify the mRNA levels of the NPY-Y1 gene in PC12 cells. There are a number of different techniques for measuring mRNAs levels. The initial approach used was that of Northern blot analysis which is least sensitive technique but most robust. The general principle is that the total RNA is extracted from the cells or tissues, electrophoresed through denaturing agarose gel and transferred onto a blot. Specific mRNA is visualized by probing the blot with an antisense version of the mRNA radioactive or non-radioactive labelled to allow visualisation. Total RNA was extracted from the different cells used in this thesis. The quality of the total RNA was assayed by two different ways: first, the relative ratio 260/280 was generally 1.8-2.0 indicating that the levels of contamination with proteins are very low; second, the RNA was separated by size in a denaturing agarose gel and the presence of sharp and distinctive ribosomal 28 S and 18 S bands was regarded as an index of high quality non-degraded RNA (Figure 7-3A). Moreover, consistent yields of total RNA were obtained using this acid-phenol extraction method (Chomczynski et al., 1989) indicating that this is a reliable method of total RNA extraction. Northern were probed until radio labelled cDNA or cRNA probes derived from the cDNA of the NPY-Y1.

However, no signal was identified. As a consequence of this negative result, the same blot was probed for the NPY mRNA, which is known to be present in PC12 cells (Allen et al., 1987b). Using this neuropeptide cDNA probe a specific signal was obtained corresponding to the NPY mRNA (Figure 7-3B). This results indicated that the RNA was in good condition. The different hybridisation signal likely reflects inherent differences in the abundance of both mRNAs in PC12 cells.

7.3.2 RIBONUCLEASE PROTECTION ASSAY ANALYSIS

The RPA is a sensitive technique in which a synthetic RNA probe is hybridized in a complex sample mixture of RNA to the specific mRNA. After hybridization, the mixture is treated with ribonuclease to degrade single-stranded unhybridized probe. However, the labelled probe that hybridises to the mRNA is protected from ribonuclease digestion and can be separated by PAGE and visualized by autoradiography. For the RPA assay, the NPY-Y1 cDNA in pBS-Y1 was digested with *Nde* I to allow the synthesis of a cRNA antisense transcript by "*in vitro* " transcription. As a positive control in the RPA assay, the human RNA 18S probe was used. The expected site band for the protected fragment (80 nucleotides) of human RNA 18S probe was identified (Figure 7-3C). However, the expected protected fragment (540 nucleotides) for the NPY-Y1 probe was not observed. Increasing the sensitivity of the assay did not alter this negative result, indicating that the message of the NPY-Y1 receptor is very rare in PC12 cells.

7.3.3 RT-PCR ANALYSIS

RT-PCR permits the qualitative and quantitative analysis of the levels of the expression of specific genes. RT-PCR is 1,000 to 10,000 times more sensitive than traditional techniques of RNA blotting (Byrne et al., 1988; Wang et al., 1989). The extraordinary sensitivity of RT-PCR has been used to amplify, clone and characterise mRNAs of short life and/or present in very little amounts and detection mRNAs of specific cells in a heterogeneous mixture of cells (Gilliland et al., 1990). In the RT-PCR process, RNA is first isolated from cells or tissues and then used as a template for reverse transcription to cDNA. The cDNA in turn is used as template for PCR, using primers designed to amplify a selected cDNA region. The PCR product is analysed by agarose gel electrophoresis and identified by size. It can be further validated by restriction digestion, hybridization or nucleotide sequencing.

The extent of the expression of the genes under study can be estimated by knowing the amount of RNA used for the synthesis of cDNA, the amount of cDNA used for PCR and the number of PCR cycles needed to generate a visible band on an agarose gel. However, while RT-PCR is an extremely sensitive method of mRNA analysis, obtaining quantitative information with this technique can be difficult. This is due primarily to the fact that there are two sequential enzymatic reactions involved: the synthesis of DNA from the RNA template by reverse transcriptase and the specific amplification of the DNA by polymerase chain reaction (PCR). Both steps must be considered for quantification of mRNA levels.

The cDNA synthesis step will reflect the relative amount of mRNA in the cells or tissue only if two conditions are met: (i) a high proportion of the RNA is intact and (ii) cDNA synthesis is efficient for the mRNA under study. PCR products can be measured with ^{32}P-labelled or non-

isotopically dNTPs, primers or probes by blot hybridization or by densitometry of fluorescent-stained gels, biotin-streptavidin binding and antibody detection of digoxigenin.

The goal of quantitative PCR is to deduce, from the final amount of PCR product, either the initial number of target molecules (N_0) or the relative starting levels of target molecules among several samples. Thus, the first step in this process is to measure the amount of PCR product. Several methods are commonly used to quantify PCR products. The most straightforward approach is to measure the incorporation of labelled nucleotides or primers into PCR products resolved by gel electrophoresis. Other strategies for quantifying PCR products are based on hybridisation. The most common of these methods is to probe a Southern blot of the PCR product using a radioactively labelled probe designed to hybridise to specific, amplified sequences. Because the nucleic acid probes only hybridise to the corresponding amplified DNA sequences, this method offers the advantage of detecting only the correct PCR product. Non-specific products should not produce a signal. In this thesis the measurement of the ethidium bromide luminescence emanating from PCR products resolved by gel electrophoresis was the method of choice (Nakayama et al., 1992).

7.3.3.1 The exponential nature of the PCR

The PCR amplification step is a complex, exponential process and for this reason small variations in amplification efficiency of the reaction can drastically affect the yield of products. In addition, the efficiency of PCR decreases at the latter stages of amplification due to depletion of reaction components, diminished enzymatic activity and accumulation of products. Although PCR is an efficient technique, exponential amplification is not a limitless process. Kinetic analysis of PCR show two reaction phases: one exponential and other linear (Figure 7-15). The following factors may account for any observed plateau effect: the product accumulates to a concentration at which reassociation competes with primer annealing and extension, the molar ratio of polymerase to template falls below a critical value, inhibitors of polymerase activity, such as pyrophosphates, may accumulate, and one or more of the components (generally the DNA polymerase) necessary for the reaction become limiting .

The number of cycles needed to reach the plateau phase varies and this variability makes it difficult to predict precisely the time course of the reaction or the amount of product synthesised before plateau phase is reached. The uncertainties inherent in the plateau effect, as well as the exponential nature of PCR, contribute to the difficulty of performing quantitative PCR. Therefore any attempt to quantitate mRNA levels by RT-PCR must be limited to the analysis of products generated only during the exponential phase of the amplification. Under these conditions, RT-PCR can yield precise information about changes in mRNA levels.

In order to define the exponential phase of amplification of the NPY-Y1 gene in PC12 cells, 1 µg of total RNA of untreated PC12 cells was amplified using the forward (2486) and reverse (2487) primers and different PCR cycles (10-40 cycles) (Figure 7-7). This RT-PCR reaction generates a PCR product of 400 bp which can be detected only after 40 cycles of RT-PCR amplification (Figure 7-5A). This result indicates that the mRNA for the NPY-Y1 gene is expressed at very low levels in PC12 cells, which are consistent with the previous negative results obtained with other less sensitive techniques, such as Northern blots and ribonuclease protection assay (RPA). However more detailed RT-PCR studies, including the use of radiolabelled primers or hybridization probes will be necessary to know the exponential phase of amplification of the NPY-Y1 gene under these RT-PCR conditions.

In addition, the mRNA of the NPY-Y1 gene was amplified by RT-PCR from different cells lines which are known to express this neuropeptide receptor gene, such as RINm5f cells. This result showed that the levels of the NPY-Y1 mRNA expression are similar between the two cell lines:(Figure 7-5B). This preliminary results needs to be confirmed using an internal control to validate this differences. The mRNA levels of the NPY-Y1 receptor gene were analysed for PC12 cells treated with different agents which are known to increase the levels of transcription of this gene (See Chapter 3). This preliminary result showed that mRNA levels increased in comparison with basal levels of NPY-Y1 expression (Figure 7-5C).

By definition, PCR process is a chain reaction. The products from one cycle of amplification serve as substrates for the next. Therefore, the amount of product increases exponentially and not linearly, as in most enzymatic processes. Under ideal or theoretical conditions, the amount of product doubles during each cycle of the PCR reaction according to equation (1) : $N = N_0\ 2^n$, where N is the number of amplified molecules, N_0 is the number of molecules and n is the number of amplification cycles. This equation indicates a linear relationship between the number of amplified target molecules and the initial number of target molecules. The principal problem is that the amplification efficiency, that is, the fraction of the template replicated during each reaction cycle, is a crucial factor and experimentally is less than perfect. Thus, a PCR process is described by equation (2) : $N=N_0\,(1+E)^n$, where E is the amplification efficiency. Because of the exponential nature of PCR, a very small change in amplification efficiency, E, can yield dramatic differences in the amount of product, N, even if the initial number of target molecules, N_0, is the same. Several experimental factors may affect the efficiency of amplification, including: the sequence and length of the DNA being amplified, the sequence of the primers and impurities in the sample, among others.

Although these factors can be controlled, there are variations between tubes that can make precise quantification of the PCR products difficult such as small variations in the temperature of the thermal cycling apparatus. Unfortunately, tube-to-tube variation in amplification

efficiency can be both significant and unpredictable. They are two approaches to be used as internal control for a quantitative RT-PCR: (i) RT-PCR with endogenous control and (ii) RT-PCR with exogenous control.

7.3.3.2 Quantitative RT-PCR with Endogenous Control

Relative quantification of specific mRNA can be obtained comparing the relation between mRNA and a calibration curve using series dilutions of a synthetic cRNA, a cDNA or standard cells that contain a know number of copies of a target DNA (Michael et al., 1992; Winters et al., 1992). The kinetic analyses of PCR reactions using a target mRNA and a cRNA standard curve can reveal differences in the efficiency of amplification, due to variations in the efficiency of hybridisation of the primers, and, for this reason, is not possible to directly correlate the amount of target mRNA and the amount of the cRNA standard. For more accurate quantitation of mRNA levels, corrections for tube to tube variation in amplification efficiency are necessary. This is most commonly performed by inclusion of a internal standard.

An endogenous sequence or gene transcript that is normally present in the sample and is known to be present at constant levels throughout the series of samples to be compared, can be used as an internal standard in quantitative RT-PCR reactions (Chelly et al., 1988; Noonan et al., 1990; Abe et al., 1992; Kellog et al., 1992; Singer-Sam et al., 1992). Endogenous mRNA standards, typically house keeping genes or genes that are structurally or functionally related to the target mRNA, have been used to determine relative levels of specific mRNAs (Noonan et al., 1990; Murphy et al., 1990; Kinoshita et al., 1992). Ribosomal RNA genes are also used as an endogenous internal control for quantitation of mRNAs (Khan et al., 1992). These techniques avoid an absolute quantification of specific mRNA.

In this approach, the endogenous standard sequence is amplified using a second pair of gene-specific primers, either in two separate PCR reactions, or in the same reaction as the target sequence. This method is sometimes referred as "multiplex PCR" (Gaudette et al., 1991) or "differential PCR" (Frye et al., 1989). The ratio of the amount of PCR products generated by target and endogenous sequences in the samples is then determined and compared. The data from this type of experiment must be obtained before the amplification reactions reach the plateau phase. Moreover, the data must be collected either by a titration of the sequences to be amplified, or by kinetic analysis, to ensure that signals are derived only from the exponential phase of the amplification.

The relative initial amounts of a target sequence (N_{0t}) and the endogenous standard (N_{0s}) (i.e., the ratio N_{0t}/N_{0s}) can be determined from equation (4) : $N_{0t}/N_{0s} = N_t\ (1+E_s)^n / N_s\ (1+E_t)^n$ (The subscripts "t" and "s" refer to the target and standard sequences, respectively) where: N_{0t}

= initial number of target molecules, N_{0s} = initial number of standard molecules, N_t = number of amplified target molecules, N_s = number of amplified standard molecules, E_t = amplification efficiency of the target, E_s = amplification efficiency of the standard and n = number of amplification cycles. Values for E for the target and standard may be calculated from the slope of a graph of Log N as a function of cycle number (n). When the amplification efficiencies of the two reactions, target and standard, are identical, i.e., $E_t = E_s$, the analysis is greatly simplified (Santagatti et al., 1993).

However, even without a full mathematical analysis, and even in case where E_s does not equal E_t, it has been shown empirically that endogenous mRNAs can be used to normalise target mRNA levels between samples to be compared. Thus, instead of determining the ratio of the initial absolute amounts of target and standard using linear regression, the relative amounts of PCR products generated by the target and standard templates in different samples is simply compared. Although it has not been shown theoretically, it has been suggested that if the internal standard mRNA is expressed at the same level in two samples, the ratio of PCR products generated from the target and standard should indicate the relative level of expression of the target mRNA in those samples (Horikoshi et al., 1992; Neubauer et al., 1991). Furthermore, it may be true that if the target and standard are amplified in the same tube, tube to tube variations in amplification efficiency (due, for example, to pippeting error, sample impurities, or partially degraded RNA) may be minimised as well.

Perhaps the greatest advantage of using the expression of an endogenous sequence as an integral standard is that the reference mRNA and the target mRNA are usually processed together for the entire duration of the experiment, from RNA extraction through PCR amplification. This tends to minimise differences in RNA yield between samples, an important advantage, particularly for analysis of small tissue samples where the quantities of RNA are too small to measure by UV spectrophotometry. In addition, if the entire population of mRNA is converted to cDNA by the use of oligo(dT) primers or random hexamers, the overall efficiency of the cDNA synthesis also is somewhat normalised.

Not withstanding the advantages to this approach, several complications may arise when amplification of endogenous mRNAs is used for semi-quantitative analysis. For this method to be reliable, the level of expression of the reference standard must be the same in each sample to be compared and must not change as a result of the experimental treatment. Unfortunately, few if any genes are expressed strictly in this manner. This is even the case for many housekeeping genes, including b-actin. Therefore, the level of the mRNA used as the endogenous standard must be examined very carefully to ensure its constancy among all of the experimental conditions studied. In order to obtain the relative levels of amplification by RT-PCR of the rat cyclophilin gene (a housekeeping gene) in PC12 cells under the different treatments (NGF,

PACAP, forskolin and PMA), total RNA was amplified using the forward (Y7462) and reverse (Y7463) primers generating a product of 300 bp after 40 cycles of amplification (Figure 7-6A). Results showed that the mRNA levels of this gene was not altered by the agents known to increase the transcriptional activation of the NPY-Y1 gene. For this reason this gene can be used as internal control for a quantitative RT-PCR in PC12 cells using the above mentioned pharmacological agents.

Another challenge of this approach is to obtain values of N_t and N_s before the amplification reactions reach the plateau phase, especially when the relative levels of expression of the standard and target sequences differ greatly. In addition, the cyclophilin mRNA (internal control) may be present at a much higher level than the target transcript, and amplification of this control may approach plateau phase well in the advance of that of the target sequence. One solution to this problem involves simply waiting until later stages of the amplification before adding the primers for the endogenous standard. Other researchers used gene-specific primers to synthesise cDNA from the control and target mRNAs in separate tubes and then mixed dilutions of the control and target cDNAs before performing multiplex PCR. Interference is a frequently observed problem when more than one set of primers is used in the same PCR reaction. In general the amount of product generated (from either the target, the standard, or both) is often dramatically reduced when both sequences are amplified in a single reaction. In fact, primer pairs that function truly independently seem to be the exception rather than the rule.

For this reason, the next series of experiments were performed to try to define the conditions for a co-amplification of the two messages (cyclophilin and NPY-Y1 genes) in one tube of RT-PCR reaction. Different experiments were made using constant amounts (20 pmol) of the primers for the NPY-Y1 gene and several dilutions of the primers for the rat cyclophilin gene. After several failed experiments the optimal conditions were set-up for a narrow margin of dilution (between 1 to 5 pmoles) of the primers for the cyclophilin gene after 35 cycles of amplification (Figure 7-11). However, further co-amplification RT-PCR experiments, including an analysis of the exponential phase of co-amplification of both mRNAs using radiolabelled primers will be necessary to obtain valid results of this quantitative RT-PCR.

7.3.3.2 Quantitative RT-PCR With Exogenous Control

Exogenous sequence can also be used as internal RT-PCR control. In this approach, a synthetic cRNA control containing the same primer template sequences as the experimental target is added to the target sample and amplified simultaneously with the target transcript in a single RT-PCR reaction mixture. The theory behind use of exogenously added gene sequences as internal controls is similar to that described above for endogenous reference sequences. With both types of internal controls, the amount of amplified control can be quantified after the

experiment, and the change in the amount of control is proportional to the change in the amount of the target. However, there is a significant advantage in using an exogenously added sequence as the internal control; namely, the initial amount of control used in the RT-PCR reaction is precisely known. This makes it possible to calculate the absolute level of target mRNA present in the original sample.

Competitive PCR uses an exogenous template as an internal control and the amplification takes place in a competitive fashion because the control and target sequences compete for the same primers and, therefore, for amplification. A critically important requirement of this type of experiment is that the value of efficiency (E) be the same for both the target and control mRNAs. This can be accomplished by designing the control to contain the same primer binding sequence as the corresponding target mRNA. In many cases this is sufficient to make E_s equal to E_t (Becker-André and Hahlbrock, 1989; Gilliland et al., 1990).

As the control cRNA molecules and the target mRNA molecules are co-amplified in the same tube, changes in any of the variants that affect the efficiency of the RT-PCR reaction are identified and affect in equal proportion the amounts of amplified products of both RNA. For this reason it is not necessary to do kinetic studies of the efficiency of the reaction for each both of the RNA molecules by separated. In addition, in the competitive RT-PCR reaction the initial proportion of target mRNA molecules and control cRNA molecules stay constant during the amplification, and the quantification can be done in the linear phase (saturation) of the RT-PCR. The amounts produced by RT-PCR of both RNAs can be obtained using densitometric analysis in gels stained with ethidium bromide (Clementi et al., 1993; Ferre et al., 1994).

Although the variables that affect the efficiency of a RT-PCR reaction are carefully controlled, small differences in the rate of amplification of a target mRNA and cRNA control are magnified during the exponential phase of the PCR, and can underestimate or overestimate the amount of unknown mRNA. To avoid these, it is necessary use as internal control a cRNA molecule that has the same nucleotide sequence as the target mRNA and, the same set of primers should be used to amplify both RNAs in a same tube. Unfortunately, the products of the PCR can not be distinguished because they have the same sequence and size. For this reason, it is necessary to use a cRNA control, whose product can be distinguished from the target mRNA by size, hybridisation with a specific probe or use restriction enzymes (Becker-Andre et al., 1989; Gilliland et al., 1990). The cRNA standard can be made by an allelic variant (for example, a gene with a small deletion or insertion, as a fragment of genomic DNA that contain a small intron) (Becker-Andre et al., 1989) or a mutant cDNA (prepared by a change in a pair of bases by direct mutagenesis) which contain or lose a restriction enzyme site (Gilliland et al., 1990).

A exogenous internal control for a competitive RT-PCR of the NPY-Y1 receptor gene in PC12 cells was prepared. This exogenous control is a internal control cRNA of the 3'-flanking region of the NPY-Y1 receptor gene which can be amplified to generate a fragment of approximately 300 bp. This fragment was amplified using the same pair of primers (2486-2487) which were used to amplify a 400 bp fragment of the mRNA of this neuropeptide receptor gene. The two products can be separated by size in a 2% agarose gel. Initially, the optimal conditions for the amplification of the cRNA (internal standard) were obtained (Figure 7-5B). However, further experiments in which a dilution series of the cRNA control sequence and a constant amount of the total RNA of the PC12 cells are co-amplified will be necessary to determin the absolute amounts of this NPY-Y1 receptor gene in these cells. Quantification will be performed after competitive amplification of the entire series of reactions and is achieved by distinguishing the two PCR products from each tube by differences in size. An advantage of this method is that, because the ratio of target to standard is constant during the amplification, it is not necessary to obtain data before the plateau phase of the reaction.

Finally, in a competitive PCR, the competitor fragment takes the place of the standard. When the amplification efficiencies of the target and standard molecules are the same, equation (4) can be simplified to equation (5): $N_{0t}/N_{0s} = N_t/N_s = A_t/A_s$ where: N_{0t} = initial number of target molecules, N_{0s} = initial number of standard molecules, N_t = number of amplified target molecules, N_s = number of amplified standard molecules, A_t = amount of amplified target (in cpm or OD_{260} units), A_s = amount of amplified standard (in cpm or OD_{260} units). Thus, for any value of n, the initial ratio of target to standard is equal to the ratio of their amplification products (i.e., N_t/N_s or A_t/A_s). This has been demonstrated both theoretically (Nedelman et al., 1992) and empirically (Bouaboula et al., 1992). Therefore, if the standard and target sequences amplify with the same efficiency, the absolute initial amount of target mRNA, can be determined by allowing known amounts of internal cRNA molecules to compete with the target for primer binding.

7.4 SUMMARY

7.4.1 The complete open reading frame of the NPY-Y1 receptor gene was obtained by PCR from a RINm5f cDNA library.

A 1.3 kb fragment containing the ORF of the NPY-Y1 gene was cloned opposite to the T7 RNA polymerase of the pBluescribe SK+ vector. This clone was verified by specific restriction enzyme digestion and was used to synthesize cDNA probes for Northern blots or cRNA probes for RPA.

7.4.2 Northern blot assays fail to detect the signal of the NPY-Y1 receptor gene in PC12 cells.

7.4.2.1 High quality RNA was purified and quantified from PC12 cells. This total RNA was separated by size, blotted in nylon membranes and hybridized with two specific DNA probes.

7.4.2.2 A NPY probe results in detectable signal of this neuropeptide mRNA in unstimulated PC12 cells. Moreover, this signal was increased in PC12 cells treated with NGF consistent with previous results (Allen et al., 1987; Balbi and Allen, 1994). This result indicates that the total RNA extracted from the PC12 cells is in good conditions and the labelling reaction results in a probe with high specific activity.

7.4.2.3 A NPY-Y1 receptor probe fails to produce a detectable signal of this neuropeptide receptor mRNA in untreated PC12 cells under the same hybridization conditions used for the NPY probe. Moreover, increasing the amount of RNA or decreasing the stringency of the hybridization did not increase the specific signal. This results indicate that in comparison with the NPY, the NPY-Y1 receptor is expressed at very low levels in PC12 cells.

7.4.3 Ribonuclease protection assays (RPA) also fail to detect the signal of the NPY-Y1 receptor gene in PC12 cells

7.4.3.1 A human RNA 18S antisense probe results in a protected fragment of approximately 80 nucleotides in untreated PC12 cells. This result indicates that the total RNA extracted from the PC12 cells is in good conditions and the "*in vitro* " labelling reaction results in a probe with high specific activity.

7.4.3.2 A NPY-Y1 receptor antisense probe fails to protect a fragment of approximately 540 nucleotides in untreated PC12 cells. Moreover, increasing the sensitivity of the RPA did not increase the specific signal. This result confirms the previous Northen blot results that the PC12 cells express very low levels of the NPY-Y1 receptor mRNA.

7.4.4 RT-PCR detect the mRNA of the NPY-Y1 receptor gene in PC12 cells but only after 40 cycles of amplification.

7.4.4.1 mRNA of the NPY-Y1 receptor gene was detected after 40 cycles of RT-PCR amplification in PC12 cells. Moreover, similar results were obtained using RINm5f cells. This results indicate a very low levels of expression of the mRNA in theses cells.

7.4.4.2 Preliminary results indicate that different pharmacological agents known to increase the levels of transcription of this gene in PC12 cells also increase levels of NPY-Y1 gene expression. However, an internal control need to be used to confirm this results

7.4.5 A small fragment (300 bp) of the rat cyclophilin gene was amplified by RT-PCR to be used as endogenous control in a quantitative RT-PCR.

7.4.5.1 The levels of amplification of a fragment of 300 bp of a rat cyclophilin gene were not altered by different pharmacological agents known to increase the levels of the NPY-Y1 gene in PC12 cells. This result indicates that this mRNA is a good internal control for the RT-PCR quantification of NPY-Y1 receptor mRNA.

7.4.5.2 Preliminary results indicate that the co-amplification of both mRNAs, the NPY-Y1 receptor gene and the rat cyclophilin gene, were obtained but in a narrow range of primer concentrations, NPY-Y1 gene (20 pmoles) and rat cyclophilin (3-5 picomoles) after 40 cycles of RT-PCR amplification.

7.4.6 A small fragment (300 bp) of the 3'-flanking region of the NPY-Y1 receptor gene was amplified by RT-PCR to be used as exogenous control in a competitive RT-PCR.

7.4.6.1 Preliminary results indicated that a exogenous cRNA fragment (300 bp) synthesized by "*in vitro* " transcription can be amplified by the same primers used to amplified the mRNA of the NPY-Y1 receptor gene (Figure 7-12). This results indicate that this cRNA is a good internal control for a competitive RT-PCR.

CHAPTER 8

GENERAL DISCUSSION

8.1 RELEVANCE OF THE NPY-Y1 RECEPTOR

The role of neuropeptide receptors in the functional organisation of the nervous system is continuously expanding. Neuropeptide receptors are widely distributed throughout the central and peripheral nervous system and they have been found to play a major role in almost all systems where they have been identified. NPY receptors, in particular, are among the most abundant neuropeptide receptors in the nervous system (Larhammar et al., 1997).

In the CNS, the NPY-Y1 receptor has been linked with different physiological process, including anxyolitic-sedative effects, cardiovascular effects, inflammation and nociception and release of pituitary hormones, among others (Wahlestedt et al., 1990; Grundemar and Häkanson, 1994). In the PNS, the NPY-Y1 receptor is found at the sympathetic postsynaptic sites where it mediates the vascular smooth muscle contractile response to NPY (Wahlestedt et al., 1990; Grundemar and Häkanson, 1994). Understanding the molecular mechanisms controlling NPY-Y1 receptor gene expression will provide clues not only about the numerous systems where this receptor plays a regulatory role but also about the role of NPY receptors in general on the overall organisation of the nervous system.

The nervous system contains diverse populations of cells which interconnect in precise manners. The identity of these cells is determined by their ability to transcribe a particular set of genes. The factors participating in NPY-Y1 receptor gene expression have to satisfy unique criteria since this receptor is distributed in specific cell populations throughout the nervous system (Eva et al., 1990).

The PC12 cell line is a clonal cell line established from a rat adrenal medullary tumour. These cells have been widely used as an important model system to examine the process of neuronal differentiation induced by NGF (Greene and Tischler, 1976). PC12 cells have high concentrations of NPY (Allen et al., 1984) and also express NPY-Y1 receptors (Schwartz et al., 1990). Moreover, PC12 cells differentiated to a neuronal-like phenotype by NGF or to adrenal chromaffin-like cells with dexamethasone are responsive to selective NPY-Y1 agonists with inhibition of evoked cAMP and induced catecholamine overflow (DiMaggio et al., 1994). For this reason this cell line was used as a sympatho-adrenal (SA) model system to study the molecular mechanisms controlling expression of the NPY-Y1 receptor gene.

8.2 THE LUCIFERASE REPORTER SYSTEM

The experiments presented in this thesis represent the first characterisation of the molecular mechanisms involved in the transcriptional control of the NPY-Y1 receptor gene in PC12 cells.

Approximately 600 bp of the promoter region of the rat NPY-Y1 receptor gene that lies upstream to the open reading frame have been attached to the reporter function, luciferase, so the expression of this enzyme has been placed under the transcriptional control of the promoter of this neuropeptide receptor gene.

Optimal conditions for several parameters which improve the transfection efficiency and can affect levels of luciferase activity in PC12 cells were found empirically. These parameters include the amount and quality of the DNA added to the cells, the time that the cells were incubated with the DNA and general conditions of the luciferase assay, such as the time point of harvesting, among others. In addition, luciferase activity was found to be: (i) linear over the range of dilution (1 : 5 to 1 : 20) used in the assay, (ii) highly reproducible and (iii) resistant to repeat cycles of freeze-thawing. Finally, it was found that cell extracts of non-transformed PC12 cells showed no detectable luciferase activity indicating that the only source of luciferase activity was derived from the DNA (pY1-LUC) transfected into the cells.

The luciferase assay was found to be a good reporter system for PC12 cells. Very low levels of luciferase activity were achieved with the promoterless plasmid (pGL3-Basic) indicating that the vector itself contained no sequences that could activate gene expression in PC12 cells. Maximum levels were achieved with the control vector (pGL3-Control) in which the luciferase is under the control of a SV40 early enhancer/promoter, indicating that PC12 cells were able to produce high levels of active luciferase enzyme. Slightly higher levels of luciferase activity were measured in cells transfected with the fusion gene pY1-LUC compared to the promoterless vector. These results indicate that the luciferase assay was able to report factors that increase or decrease transcriptional activity in PC12 cells. These results also demonstrate a low constitutive basal level of transcription of this gene in PC12 cells in keeping with previous results that showed that these cells express NPY-Y1 receptors on their cell surface (DiMaggio et al., 1994).

Different agents known to cause the differentiation of PC12 cells, such as dexamethasone (to adrenal chromaffin cells) and NGF (to post-ganglionic sympathetic neurons) also increase the transcriptional activity of the NPY-Y1 receptor gene. However, it is important to confirm that the differences observed in the levels of luciferase activity using these different treatments, reflect differences in the transcriptional activity of the NPY-Y1 receptor gene and are not the result fn post-transcriptional effects, such as mRNA stability or non-specific effects of sequences present in the promoterless vector (pGL3-Basic). Luciferase activity was unaffected by these treatments when expressed from the promotorless plasmid (pGL3-Basic) or from SV40 promoter (pGL3-control).

This result indicates that the differences in the luciferase activity obtained with the pharmacological agents resulted from effects on the transcriptional activity of the NPY-Y1 receptor gene. However, further measurements of luciferase mRNA using specific inhibitors of the RNA polymerase, such as actinomycin D, will be necessary to determine that the expression of the luciferase enzyme is not affected by post-trancriptional mechanisms, such as mRNA stability, induced for these different treatments in PC12 cells.

Thus, this region of the NPY-Y1 receptor gene contains a functional promoter that is active and responsive to transcriptional activation in a neural crest-derived (sympathoadrenal) cell line: PC12 cells. Moreover, this promoter region is also active in other cells lines such as GT1-7, a mouse hypothalamic neurosecretory (GnRH) cell line (Mellon et al., 1990) and RINm5f, a rat b-pancreatic insulinomic cell line (Gazdar et al., 1980) which also express this neuropeptide receptor. In these cell lines, the pY1-LUC results in slightly higher levels of luciferase activity compared to the promoterless vector (pGL3-Basic) but considerable less than the activity obtained with the control vector (pGL3-Control), indicating that the luciferase is a also a good reporter assay system to study mechanisms of transcriptional control of the NPY-Y1 receptor gene in these cells. This promoter is inactive in COS-7 cells, a monkey kidney-derived SV-40 transformed cell line. NGF and PACAP did not increase the transcriptional activity of the NPY-Y1 receptor gene in these cells. Taken together, these results indicate that NPY-Y1 gene contains a cell-type specific core promoter consistent with its known distribution in rats (Eva et al., 1990).

A control reporter vector (pRL) and the Dual-Luciferase™ reporter assay system were used in order to normalize for transfection efficiencies. Sources of variation in transfection efficiency include: quality of the DNA preparations, small changes in the pH of the transfection solutions or differences in the density or healthy of the cells, among others. Typically, pRL-CMV or pRL-SV40 were used as control reporter genes in co-transfected experiments with the pY1-LUC vector. These control vectors were used because they are linked to a different reporter gene (*Renilla* luciferase) and were under the control of constitutive active promoters in PC12 cells. However, the CMV immediate early enhancer/promoter present in pRL-CMV was responsive to all the activator compounds tested: dexamethasone (1μM), NGF (100 ng/ml), PACAP (5 nM) and forskolin (10 μM) which precluded its use as internal control. The SV40 early enhancer/promoter region present in pRL-SV40 was responsive only to dexamethasone (1 μM) and NGF (100 ng/ml), but was unresponsive to PACAP (5 nM) and forskolin (10 μM). For this reason pRL-SV40 was used as internal control in PC12 cells treated with PACAP and other PKA activators, such as forskolin and DBC.

8.3 PROMOTER REGION OF THE RAT NPY-Y1 GENE

The promoter region of the rat NPY-Y1 receptor gene contains several consensus sequences that constitute putative binding sites for different transcription factors including : (i) an AT-rich "TATA-like" box (AAATA), (ii) a putative "CCAAT" box (TCAAT), (iii) a GC rich Sp1 binding site (GGGGCG), (iv) an AP-1 binding site (TGAGTCA), (v) two CRE binding sites (CGTCA), (vi) one ERE binding site (TGACC), (vii) three GRE binding sites (AGGACT) and (viii) one GRE binding site (TGTTCT).

8.3.1 BASAL REGION OF THE PROMOTER OF THE NPY-Y1 GENE

A sequence comparison between the promoter of the mouse, rat and human NPY-Y1 genes showed a high degree of homology in the region close to the putative start site, including a highly conserved "TATA-like" box (AAATA) and "CAAT-like" box (TCAAT). These elements are likely to be involved in the control of the basal levels of transcriptional activity of this gene in PC12 cells. However, preliminary results in this thesis using RT-PCR suggest that the transcription initiation site in the rat NPY-Y1 gene is upstream of the putative cap site. These results are similar to primer extension analysis which revealed several closely spaced sites for transcription initiation in the promoter of the mouse (Eva et al., 1992) and human (Ball et al., 1995) NPY-Y1 receptor genes. Further RT-PCR studies using upstream primers will be necessary to obtain a more precise location of the start transcription site of this gene.

The GC-box (GGGGCG) constitutes a putative Sp1 binding site. This site is conserved in the mouse and rat NPY-Y1 promoters and constitutes another element that may contribute to the basal transcriptional activity of this gene in PC12 cells. Sp1 is a ubiquitous transcription factor that may recruit TATA-box binding proteins or initiation complexes to the initiation site in promoters lacking consensus TATA-boxes. Sp1 has been reported to be non-inducible but is an enhancer of constitutive expression of several genes, specially housekeeping genes (Kadonaga et al., 1987). However, Sp1-like proteins has also been reported to be involved in the NGF-mediated transcriptional activation of the NPY gene (Minth and Dixon, 1990). Further deletions of this site will be important to know the relevance of Sp1 in the transcriptional activation of the NPY-Y1 receptor gene in PC12 cells.

8.3.2 AP-1 SITE OF THE PROMOTER OF THE NPY-Y1 GENE

The promoter of the rat NPY-Y1 gene has a complete AP-1 site (TGAGTCA). This element is also present in the promoter of mouse NPY-Y1 gene (Eva et al., 1992). Moreover, the promoter of human NPY-Y1 gene have another AP-1 site located upstream of this binding site

(Ball et al., 1995) shared with the mouse NPY-Y1 gene (Musso et al., 1997) and the mouse NPY-Y1 promoter also have another AP-1 site downstream of this binding site. This element constitutes a consensus DNA binding site for the transcriptional factor AP-1, comprising dimers of the *c-fos* and *c-jun* families of proteins (Curran and Franza, 1988). Expression of *c-fos* is known to be activated in PC12 cells by growth factors, such as NGF and by phorbol esters, such as PMA and this activation is at least partially dependent on PKC activity in this cells (Sigmund et al., 1990). *c-fos* activation via the PKC-dependent pathway has been shown to be regulated by a serum responsive element (SRE) (Treisman, 1995). SRE is the specific binding site for a nuclear protein, serum responsive factor (SRF), present in nuclear extracts of PC12 cells (Sheng and Greenberg, 1990). In addition, the PKC-mediated induction of *c-jun* transcriptional activity is thought to result from a positive autoregulatory mechanism and to involve dephosphorylation for an PKC-activated phosphatase at inhibitory phosphorylation site next to a carboxy-terminal DNA-binding domain of *c-jun* . Thus, transcriptional activation of *c-fos* and post-translational regulation of pre-existing of *c-jun* can induce AP-1 transcriptional activity, in turn increasing the expression of late responsive genes in PC12 cells.

The AP-1 site is the most like element to mediate the NGF- and PMA-dependent transcriptional activation of the NPY-Y1 receptor gene in PC12 cells. AP-1 consensus sites are present in several late responsive genes (LRG) regulated by NGF and PMA in PC12 cells, such as the gene for transin/stromelysin (Machida et al., 1989), neurotensin/neuromedin N (Kislauskis and Dobner, 1990), tyrosine hydroxylase (Gizan-Ginsberg and Ziff, 1990) and NPY (Balbi and Allen, 1994).

Previous results, have shown that the NGF and PMA mediated transcriptional activation of the NPY gene is dependent on PKC activity and a specific deletion of the AP-1 site significantly reduced the NGF response whereas the response to PMA was completely abolished (Balbi, 1994; Balbi and Allen, 1994). Moreover, in the neurotensin/neuromedin N gene, mutation of the AP-1 site reduced the transcriptional response to NGF (Kislauskis and Dobner, 1990). AP-1 sites are also involved in mediating NGF inducibility of the tyrosine hydroxylase (Gizan-Ginsberg and Ziff, 1990) and transin genes (Machida et al., 1989) in PC12 cells.

In order to evaluate the role of this AP-1 site in the control of the expression of the NPY-Y1 receptor gene in PC12 cells, a specific mutation of this site was obtained by PCR and cloned in the luciferase reporter vector (pY1-AP1). Thus the luciferase activity is under the control of this AP-1 deleted version of the promoter of the NPY-Y1 receptor gene. Further luciferase reporter assay experiments using this deletion fusion gene will be necessary to determine the relevance of this AP-1 mutation in the control of the basal- and NGF-dependent transcriptional activation of the NPY-Y1 receptor gene in PC12 cells.

8.3.2.1 NGF EFFECTS ON THE TRANSCRIPTION OF THE NPY-Y1 GENE

The mechanisms underlying the transcriptional effects of NGF have been the subject of intense research (Halegoua et al., 1991). While there has been a continuous input of information about the intracellular mechanisms activated during the early stages of NGF-induced differentiation, including transcription of immediate early genes (IEGs), such as *c-fos* , *c-myc* , NGF1-A, NGF1-B and NGF1-C (Halegoua et al., 1991) relatively little is known about the effect of NGF on late response genes (LRG). This group of late responsive genes include peripherin (Leonard et al., 1988), neurotrensin/neuromedin N (Kislauskis and Dobner, 1990), tyrosine hydroxylase (Gizan-Ginsberg and Ziff, 1990), transin/stromelysin (Machida et al., 1991), voltage-dependent sodium channel (Ginty et al., 1992), GAP-43 (Perrone-Bizzozero et al., 1993) and NPY (Balbi and Allen, 1994).

It is becoming apparent that the mechanisms of the regulation of this LRG by NGF are different from those of the regulation of IEG. The transcriptional induction of IEGs is transient and independent of protein synthesis while that of LRGs is usually long lasting (> 6 hours) and requires ongoing protein synthesis (Sheng and Greenberg, 1990). IEGs can regulate the expression of LRGs by virtue of their transcriptional regulatory activity and thereby act as mediators of long term changes in gene expression induced by external stimuli such as NGF (Morgan and Curran, 1991). Further experiments using cyclohexamide, a potent inhibitor of protein synthesis that interferes with mRNA translation at the ribosomes will be necessary to understand the role of the translation machinary and the synthesis *de novo* of transcription factors which are not normally present in non-differentiated cells in the activation of NPY-Y1 gene in PC12 cells.

Binding of NGF to its high affinity receptor, *trk A* , results in dimerization and trans-auto-phosphorylation of tyrosine residues in the cytoplasmic domain (Kaplan and Stephens, 1994). Four intracellular proteins are activated by the phosphorylated *trk A* receptor: PLCg-1 (Vetter et al., 1991), PI-3 kinase (Raffioni and Bradshaw, 1992), *shc* (Obermeir et al., 1993) and MAPK-1 (Loeb et al, 1994). This initial tyrosine phosphorylation events are followed by the activation of several protein kinases, that include members of the PKC, *raf-1* , MEKs, MAPKs and S6 kinases, which are involved in mediating the NGF effects in PC12 cells (Halegoua et al., 1991). Stimulation with NGF causes translocation of these kinases from the cytosol to the nucleus, which results in the activation of specific nuclear targets, such as the transcription factors *c-fos* , *c-jun* and *c-myc* , there by initiating specific gene transcription events required for mediating the NGF response in PC12 cells.

8.3.2.1.1 Role of Tyrosine Kinase Pathway

In chapter 4, the results from experiments aimed at characterising mechanisms involved in the NGF-induced transcriptional activation of the NPY-Y1 receptor gene were detailed. NGF results in a concentration-dependent increase in the transcriptional activation of this gene in PC12 cells. The concentration range (10-100 nM) of the NGF is consistent with the known biological potency of this neurotrophic factor in PC12 cells. The increase in transcriptional activity of the NPY-Y1 receptor gene is consistent with previous studies which have shown that NGF differentiated PC12 cells are responsive to the NPY-Y1 receptor selective agonists (DiMaggio et al., 1994).

This NGF-induced response of the NPY-Y1 receptor gene is dependent in *trk A* receptor activation. Pretreatment of PC12 cells with K-252a (200 nM), a specific inhibitor of the tyrosine kinase activity of the *trk A* receptor (Berg et al., 1992) completely blocked the NGF transcriptional activation of the NPY-Y1 gene but had no effect on basal levels in PC12 cells. It will be useful establish weather other neurotrophins such as BDNF, NT-3 and NT-4/5 affect the transcriptional activity of this promoter to understand the role of the low affinity $P75^{NTR}$ receptor in this response.

Basal- and NGF-dependent activation of transcriptional of NPY-Y1 receptor gene in PC12 cells appear to be negatively regulated by an intrinsic and NGF-induced tyrosine kinase. Genistein (50 μM) increased both the basal- and NGF-dependent transcriptional activity of the NPY-Y1 gene. Previous results have been shown that genistein (50 μM) inhibits $p60^{c\text{-}src}$ kinase activity (Akiyama et al., 1987). Moreover, genistein also inhibits the increase of *ras* -GTP induced by NGF in PC12 cells (Nakafuku et al., 1992). $p60^{c\text{-}src}$ activation appear occurs upstream of $p21^{ras}$, and this activation is required for the $p60^{c\text{-}src}$ induced neurite outgrowth in PC12 cells (Kremer et al., 1991). These possibly suggest that genistein inhibition of an intrinsic tyrosine kinase (possible $p60^{c\text{-}src}$ kinase) by the inhibition of $p21^{ras}$ activation, results in the basal- and NGF-dependent transcriptional activation of the NPY-Y1 receptor gene in PC12 cells. These results are consistent with the role of the *ras* -MAP kinase pathway in the transcriptional activation of this neuropeptide receptor gene in PC12 cells. However, further experiments will be necessary to confirm this hypothesis, including the use of specific antibodies against $p60^{c\text{-}src}$ or expression of a dominant negative mutant of this kinase to mimic the inhibitory effect of genistein. In addition, constitutive active mutants of this tyrosine kinase, will be useful to demonstrate the negative role of the $p60^{c\text{-}src}$ in the transcriptional activation of the NPY-Y1 gene in PC12 cells. Moreover, the effect of the $p21^{ras}$ activity in this genistein effect could be confirmed in a PC12-*ras* negative cell line (Buensuceso, 1995). Finally, the use of an inactive analogue of genistein, such as daidzein (Akiyama et al., 1987) will be important to confirm the specificity of this result.

The role of sodium orthovanadate in the reduction of basal- and NGF-dependent transcriptional activation of the NPY-Y1 gene could be more complex. Sodium orthovanadate induces differentiation and neurite outgrowth of PC12 cells by increasing tyrosine kinase activity. Sodium orthovanadate also increases $p60^{c\text{-}src}$ tyrosine kinase activity in PC12 cells (Buensuceso, 1995). Moreover, MAPK activity also can be induced by sodium orthovanadate (Tsao et al., 1990). This MAPK activation is independent of *trk A* tyrosine kinase activity, as it cannot be blocked by K-252a, it is dependent on $p21^{ras}$ activity, as a PC12-*ras* negative cell line did not exhibit MAPK activity or neurite outgrowth upon exposure to sodium orthovanadate (Buensuceso, 1995). Taken together, this results suggest that the negative effect of sodium orthovanadate on the transcription of the NPY-Y1 receptor gene involves the sequential activation of *src-ras*- MAPK in PC12 cells, following a model of sequential *src-ras* activation as proposed by NGF in PC12 cells (Kremer et al., 1991). However, will be important to demonstrate the role of the MAPK pathway in this sodium orthovanadate effect using a PC12-*ras* negative cell line or specific inhibitors of this pathway, such as PD98059.

8.3.2.1.2 Role of the ras-MAPK pathway

Previous results have been shown that $p21^{ras}$ is central to the NGF-induced signalling pathway in PC12 cells. NGF induces an accumulation of the GTP-bound active form of $p21^{ras}$ in PC12 cells (Qui and Green,1991). Moreover, introduction of constitutively active oncogenic form of $p21^{ras}$ into PC12 cells can mimic the NGF-mediated differentiation, including neurite outgrowth. In addition, microinjection of anti-*ras* antibodies or transfection with a dominant negative mutant of $p21^{ras}$ blocks NGF-dependent effects in PC12 cells (Kremer et al., 1991). Furthermore, expression of a dominant negative mutant $p21^{ras}$ also blocks the NGF-dependent activation of *raf-1* , MAPKs and RSKs in PC12 cells (Thomas et al., 1992; Wood et al., 1992).

However, the NGF-dependent transcriptional activation of the NPY-Y1 receptor gene is independent of the *ras* -MAPK pathway. NGF induced a similar activation of NPY-Y1 gene transcription in a PC12-*ras* negative cell line compared with a PC12-wild type cell line. This result indicates that the $p21^{ras}$ protein is not involved in this NGF-dependent effect and is consistent with previous results which have been shown that some of the NGF-dependent effects in PC12 cells are *ras* -independent. In particular, the NGF-dependent induction of the differentiation-associated gene Thy-1 (D'arcangelo and Halegoua, 1993) or a voltage-gated sodium channel, a late responsive gene (Fanger et al., 1993), are *ras* -independent in PC12 cells. Further experiments will be necessary to understand the role of the $p21^{ras}$ protein in the NGF-dependent transcriptional activation of the NPY-Y1 receptor gene in PC12 cells. For example, it will be useful to know if NGF can increase PKC activity and *c-fos* expression in

this PC12-*ras* negative cell line in similar way to that observed for the PC12-wild type. Moreover, it will be important to know the effect of block specific signalling pathways, such as PKC (using calphostin C), PI-3-K (using wortmannin) and *ras* -MAPK kinase (using PD 98059) in the transcriptional activation of the NPY-Y1 gene in this cell line. Finally, this PC12-*ras* negative cell line will be a useful model to understand the role of the *src-ras* activation in the transcription of this neuropeptide receptor gene, using genistein and sodium orthovanadate as mentioned before.

Pretreatment of the PC12-wild type cells with PD 98059 (20 μM), a MEK-1 kinase inhibitor blocks NGF-induced neuronal differentiation of PC12 cells (Alessi et al., 1995), this inhibitor paradoxically increased the basal transcriptional activation of the NPY-Y1 gene, indicating that intrinsic MEK-1 kinase activity may inhibit the transcription of this neuropeptide receptor gene in PC12 cells. It is tempting to speculate that the NGF activation of the MAPK pathway has a negative effect on the transcriptional activation of NPY-Y1 receptor gene in PC12 cells.

8.3.2.1.3 Role of the PKC pathway

There is evidence that PKC activation is an early and important event following NGF treatment of PC12 cells. NGF induces rapid hydrolysis of phosphoinositides and increases PKC activity in this cell line (Hama et al., 1986; Heasley and Johson, 1989: Balbi and Allen, 1994). PI-turnover has been implicated in the action of NGF in PC12 cells (Contreras and Guroff, 1987). This effect is likely mediated through phosphorylation and activation of PLC-γ1 by the tyrosine kinase activity of *trk A* (Vetter et al., 1991). Specific mutation of the site in the *trk A* receptor responsible for the activation of PLC-γ1 results in a selective loss of the NGF-mediated increase of a late responsive gene peripherin (Loeb et al., 1994).

NGF causes an increase in DAG production in PC12 cells, which peaks by about 2-3 minutes and declines to basal levels by 5 minutes (Chan et al., 1989). There is a second wave of slow and long lasting DAG production 5 minutes after NGF treatment which suggests activation of another phospholipids PLD (Chan et al., 1989). PLD hydrolyses PC to generate PA and choline. This PA is hydrolysed to DAG by the action of PAH enzyme, and this DAG may activate PKC. However, pretreatment of PC12 cells with butan-1-ol (0.3 % v/v), which diverts the production of PA to phosphatydilbutanol, and so blocks DAG production did not affect the NGF-induced transcriptional activation of the NPY-Y1 gene. These results indicate that PLD activity is not responsible for the NGF-mediated response in PC12 cells. Further experiments, including the use of inhibitors of phospholipases will be useful to understand the role of this enzymes in these NGF-mediated response.

PKC activation seems to be a necessary step for the transcriptional activation of the NPY-Y1 gene by NGF in PC12 cells as suggested by the complete inhibition following pretreatment of PC12 cells with calphostin C (1 μM), a specific inhibitor of PKC. This result is consisting with the calphostin C-mediated inhibition of the transcription of the NPY gene in PC12 cells (Balbi and Allen, 1994).

PMA have a similar structure to DAG and is able to activate PKC both *in vivo* and *in vitro* by increasing the affinity of the enzyme for Ca^{++} (Nishizuka, 1988). In PC12 cells, activation of PKC with PMA induces tyrosine phosphorylation. Moreover, PMA can mimic the NGF-induced phosphorylation of MAPKs and this strong and long-lasting activation is dependent on $p21^{ras}$ activity in PC12 cells and is completed inhibited by PKC down-regulation by long-term incubation with PMA (Thomas et al., 1992). However, phorbol esters do not promote neurite outgrowth in PC12 cells (Young et al., 1994), indicating perhaps PMA affect component(s) of the differentiation machinery.

DH-1 (1 nM) an synthetic activator of PKC which binds PKC at the same site as phorbol esters (Mastro and Grove, 1989) induces a similar increase in the transcriptional activation of the NPY-Y1 gene in comparison with that for NGF. DH-1 activation is completely block by calphostin C (1 μM), indicating that this response is dependent on PKC activation. PMA results in a relatively small but consistent increase in transcription of the NPY-Y1 receptor gene in PC12 cells. This effect was not altered by increasing intracellular Ca^{++} using the ionophore ionomycin, which is known to act synergistically with phorbol esters in the activation of PKC in some biological systems (Wilkimson and Hallan, 1994). However, this PMA-dependent response is completely blocked by calphostin C (1 μM).

PC12 cells treated with PMA (1 nM) for 1 hour resulted in an increase of PKC activity which was considerably higher than obtained using PMA (1 μM). The levels of PKC activity obtained with PMA (1 μM) are very similar to the basal levels of PKC activity. This result is consistent with previous experiments indicating that PKC activity is lower than basal levels after 3 hours of treatment with PMA (0.4 μM) (Hama et al., 1986; Balbi and Allen, 1994). PKC is down-regulated by long-term treatment with phorbol esters, which is mostly due to degradation of membrane-bound activated PKC by the proteolytic enzymes as calpain (Nishizuka,1988). The extent of downregulation is dependent on the cell type, isoform of the enzyme, the time of treatment and phorbol ester concentration (Nishizuka, 1988). PKC activation after NGF showed a different time course. Thus 1 hour after NGF treatment, PKC activity was equivalent to PMA (1 nM). However, PKC levels returned to basal values 3 hours after NGF treatment (Balbi, 1994).Taken together, these results suggests that the PMA-dependent fail to mimic the NGF-induced transcriptional activation of NPY-Y1 receptor gene in PC12 cells will be a consequence of a downregulation of PKC by long term incubation with PMA.

Thus, results obtained with PMA should be used carefully when attempting to understand the role of PKC in mediating the effects of NGF in PC12 cells. Together, this information raises the possibility that the effect of phorbol esters may be a complex response to both short and long term effect of PMA on the PKC activity on PC12 cells. Further experiments, including a time course of PKC activation upon DH1 (1 nM) and PMA (1 nM) treatment will be necessary to understand the differences observed with these two specific activators of PKC in the transcriptional activation of the NPY-Y1 receptor gene in PC12 cells. In addition, it will be important to know a time course of the transcriptional activation of this neuropeptide receptor gene with PMA (1 nM) and PMA (1 μM) in PC12 cells.

PC12 cells can be depleted of several PKC isoenzymes by treatment with high concentrations of phorbol esters for several hours (Roivainen et al., 1995). Previous studies have been shown that pretreatment of the PC12 cells with 100 nM PMA for 24 hours downregulates the β, δ and ε PKC isoforms. The conventional PKC-α is downregulated after treatment with 1 μM PMA for 24 hours, whereas the atypical PKC-ζ is resistant to downregulation by PMA (Rovainen et al., 1995). The role of PMA sensitive-PKC activity in the NGF-dependent increase in transcriptional activation of this gene in PC12 cells can be studied by depletion of these specific PKC isoforms by long term incubation with PMA (1 μM). Finally, it will be important to understand the role of the specific inhibitors, such as K-252a, calphostin C, genistein and sodium orthovanadate which are known to affect the NGF-dependent transcriptional activation of the NPY-Y1 receptor gene in the PKC activity and other PKC-dependent responses such as *c-fos* expression in both PC12-*ras* negative and PC12-wild-type cells.

Post-transcriptional mechanisms may be also involved in the effect of PMA in the luciferase activity in PC12 cells. Phorbol esters have been shown to desestabilise specific mRNAs. It has been suggested that the mechanisms involved in the selective elimination of mRNAs in early thymocytes involve PKC-mediated synthesis of sequence-specific proteins that degrade these mRNAs (Takahama and Singer, 1992). For this reason, time course experiments of luciferase mRNA levels, using specific inhibitors of RNA synthesis, such as actinomycin D will be necessary to understand the role of PMA in the stability of the luciferase mRNA in PC12 cells. Moreover, the use of the luciferase under the control of other known PMA-responsive promoter, such as NPY (Balbi and Allen, 1994), will be useful to understand the effect of post-transcriptional mechanisms in this PMA-dependent response. However, preliminary results using the pGL3-Basic and the pGL3-Control vector indicates that PMA (1 nM) after 48 hours of incubation had no effect in the levels of luciferase activity in PC12 cells, indicating that the increase of luciferase activity is dependent in the transcriptional activation of the NPY-Y1 gene, and is independent of non-specific sequences present in the vector or post-transcriptional effects such as luciferase mRNA stability.

8.3.2.1.4 Role of the PI-3-K pathway

NGF is able to activate PI-3-K and this in turn stimulates an increase in levels of PI-3,4-P2 and PI-3,4,5-P3 in PC12 cells, which reach a maximum after 3 minutes and decline after 10 minutes (Kimura et al., 1994). Pretreatment of PC12 cells with wortmannin (100 nM) for 30 minutes abolished the elevation of PI-3,4-P2 levels after NGF stimulation and completely inhibited PI-3-K activity (Kimura et al., 1994). These 3-inositol phosphorylated phospholipids are potent and selective activators of Ca^{++} independent PKC and phorbol ester insensitive PKC (Nakanishi et al., 1993). PC12 express multiple PKC isoforms, including conventional PKCs (α, β, γ), novel PKCs (δ, ε) and atypical PKC (ζ) (Wooten et al., 1992). NGF treatment of the PC12 cells result in translocation of the PKC-δ and ζ isoforms (Wooten et al., 1994).

However, neither wortmannin (10 μM) or LY 294002 (10 μM), another specific inhibitor of the PI-3-K inhibited the basal- or NGF-dependent transcriptional activation of the NPY-Y1 gene in PC12 cells, indicating that the NGF-induced PI-3-K activity has no role in mediating this response. Further experiments using constitutive active or dominant negative mutants of the different PKC isoforms, will be important to understand the role of these enzymes in the NGF-mediated response in the transcription of this neuropeptide receptor gene in PC12 cells.

8.3.2.1.5 Role of the PKA pathway

The role of PKA in mediating NGF effects on PC12 cells is controversial. Early reports proposed that cAMP mediates the neurotrophic actions of NGF. It has been suggested that NGF is able to affect cAMP metabolism, but by mechanisms independent of activation of adenylyl cyclase activity as measured by conversion of |2-^{3}H| adenine to |^{3}H| cAMP (Race and Wagner et al., 1985). NGF has been described to produce a transient increase in PKA activity in PC12 cells (Kniper et al., 1993) and this activity seems to be necessary for the NGF induced phosphorylation of tyrosine hydroxylase, at least at some of the potential phosphorylation sites (Cremins et al., 1986). PKA has also been shown to be necessary for the post-translational modification of sodium channels by NGF in PC12 cells (Ginty et al., 1992). Thus, it is generally believed that PKA may play a role in some but not all of the NGF actions in PC12 cells (Halegoua et al., 1991). However, many other reports have refuted claims that NGF signals through cAMP (Halegoua et al., 1991; Balbi and Allen, 1994). PC12 cells containing a mutated regulatory subunit of PKA showed comparable responses to parental cells in several aspects of the NGF induced differentiation such as neurite outgrowth and protein phosphorylation (Ginty et al., 1991). For example, the NGF induced expression of GAP-43, transcriptional activation of erg-1 and induction of ornitine decarboxylase activity are all unaffected in these PKA-deficient cells suggesting that NGF is able to produce all these responses via PKA-independent mechanisms (Ginty et al., 1991).

Previous experiments have shown that the NGF-induced priming of PC12 cells is different to the priming induced by DBC, an analogue of cAMP (Greene, 1978). When PC12 cells are treated for several days with NGF and then replated in the presence of NGF, the rapid regeneration of neurites was not observed within 24 hours. Instead, when PC12 cells were pre-treated with DBC and then replated in the presence of NGF, the rapid regeneration of their neurites was not observed. H-89, dihydrochloride a synthetic isoquinolinesulfonamide, is a potent and selective inhibitor of PKA activity (Chijiwa et al., 1990). Pretreatment of PC12 cells with H-89 (30 μM) led to a dose-dependent inhibition of the forskolin-induced protein phosphorylation and neurite outgrowth but the NGF-induced protein phosphorylation and neuronal differentiation were not inhibited (Chijiwa et al., 1990). Pretreatment of PC12 cells with H-89 (30 μM) had no effect on the NGF-stimulated transcriptional activation of the NPY-Y1 receptor gene in PC12 cells, indicating that this NGF effect is not dependent in PKA activation. However, H-89 (30 μM) reduced basal levels of transcriptional activity of the NPY-Y1 receptor gene in PC12 cells, indicating that PKA activity is involved in the basal transcription of this gene. Moreover, the NPY-Y1 promoter has two CRE elements and is under transcriptional control of pharmacological agents that increase cAMP, such as PACAP (5 nM), forskolin (10 μM) and DBC (1 mM) in PC12 cells.For this reason its important to know the role of these CRE sites site in the NGF-mediated transcriptional activation of the NPY-Y1 gene.Further experiments, including the use of the luciferase enzyme under the control of CRE-deleted versions of the NPY-Y1 promoter will be useful to understand the role of this transcription factor in the NGF-mediated transcriptional activity of this neuropeptide receptor gene in PC12 cells.

Previous studies have suggested that NGF may activate CREB independently of PKA activation (Hawley et al., 1992). In addition, this transcription factor can be phosphorylated and activated by cross-talk with other signalling pathways, such as PKC (Xie and Rohstein, 1995) and CaM-K II (Sheng et al., 1991). However, CaM-K II appears not involved in the NGF-mediated response, as KN-62 (1 μM) a specific inhibitor of the CaM kinase II activity in PC12 cells (Tokomitsu et al., 1990) failed to block NGF-induced transcriptional activation of the NPY-Y1 receptor in these cells. It will be important to know the role of the Ca^{++} signalling pathway in the transcriptional activation of this neuropeptide receptor gene: specifically the role of membrane depolarisation, induced either by potassium chloride or by neurotransmitters, which are known to increase the expression of *c-fos* (Sheng and Greenberg, 1990) and the phosphorylation and activation of CREB (Sheng et al., 1991). Further studies, including luciferase reporter assays using the AP-1 and CRE-deleted versions of the promoter of the NPY-Y1 receptor gene or transient expression of CREB will be necessary to understand the role of this Ca^{++} signalling pathway and cross-talk with other signalling pathways, such as the PKA and PKC in the transcriptional activation of this neuropeptide receptor gene in PC12 cells.

8.3.3 CRE SITES OF THE PROMOTER OF THE NPY-Y1 GENE

The promoter region of the rat NPY-Y1 receptor gene has two partial CRE sites (CGTCA). These sequences are the most likely elements to mediate the basal- and PACAP-dependent transcriptional activation of the NPY-Y1 receptor gene in PC12 cells. CRE sites are found in a group of rat neuropeptide genes, exemplified by somatostatin, proenkephalin and vasoactive intestinal peptide (VIP) whose expression is regulated by cAMP (Roesler et al., 1988). Different cAMP-regulated genes are expressed in PC12 cells, including the dopamine beta-hydroxylase and tyrosine hydroxylase genes, which are regulated in a co-ordinate fashion by PKA pathway in PC12 cells via CRE elements (Roesler et al., 1988).

The available evidence supports a dual role for the CRE site as both a basal and an inducible transcriptional enhancer element (Roesler et al., 1988). CREB appears to bind the CRE site in a cAMP-independent manner, and thus resembling a basal transcription factor. Moreover, unlike transcription factors whose activity is regulated, at least in part, by subcellular localisation, nuclear expression of CREB is constitutive in PC12 cells. Rapid changes in the intracellular levels of cAMP leads to PKA-mediated phosphorylation of the transcriptional activation domains of CREB, already bound to their respective CRE elements in the promoter-regulatory region of the gene. This phosphorylation is thought to be necessary and sufficient for the activation of transcription by cAMP (Roessler et al., 1988).

The activation domain of CREB is serine rich and includes consensus phosphorylation sites for different protein kinases, such as PKC, casein kinase II and CaM-K II (Fiol et al., 1994). Thus, CREB is involved in the cross-talk between different signalling pathways (Dash et al., 1991; Sheng et al., 1991). Both Ca^{++} and cAMP stimulation of PC12 cells results in the rapid phosphorylation of transcription factor CREB at Ser-133 (Sheng et al., 1991). These findings suggest that CREB is a convergence point for synaptic activity acting through PKA and impulse activity acting through CaM-K in PC12 cells. Together the two kinases are able to activate transcription in a synergistic fashion in PC12 cells. Further experiments will be necessary to understand the role of this cross-talk in the transcriptional activation of the NPY-Y1 gene.

The experiments described in the chapter 5 were designed to understand the role of trans-synaptic activity and in particular the relevance of peptides co-released from pre-synaptic nerve terminals in the control of the NPY-Y1 receptor gene expression in a model of post-synaptic cells, PC12 cell line. PACAP and VIP are present in pre-synaptic fibres of the sympathetic nervous system, including fibres innervating adrenal chromaffin cells (Yoshikawa et al., 1990) and are released after electrical stimulation (Malhotra et al., 1989). In PC12 cells, they exert a

regulatory effect on the synthesis of tyrosine hydroxylase (TH) (Wessels-Reiker et al, 1991) and NPY (Colbert et al., 1994). The fact that neuropeptides induce gene expression in PC12 cells constitutes evidence that neuromodulators other that classical neurotransmitters such as ACh may participate in trans-synaptic control of gene regulation. For this reason, it will be important to know the role of these peptides in the trans-synaptic control of the expression of a neuropeptide receptor, NPY-Y1. The activity-dependent release of peptide neurotransmitters begins a cascade of events which culminates in post-synaptic changes in gene expression. Trans-synaptic activation requires, as its initial step, the binding of the peptide to its receptor. It is well established that interaction of PACAP with their specific receptors leads to activation of two major intracellular signalling pathways: the PKA and the PLC pathways (Arimura, 1992b). These receptor system mediate post-synaptic changes in gene transcription through these second messenger pathways, which are activated upon ligand binding. They link the neurotransmitter receptors to molecular regulators of gene transcription (Armstrong and Montminy, 1993).

Diverse agents which increase the levels of intracellular cAMP in PC12 cells increased the transcriptional activity of the NPY-Y1 receptor gene in PC12 cells. These include PACAP-38, an activator of adenylyl cyclase via GPCRs, forskolin, and direct activator of adenylyl cyclase and DBC. In chapter 5, an attempt was made to further study the molecular mechanisms involved in the role of different signalling pathways activated by PACAP-38 in PC12 cells, such as the PKA, PKC and the *ras* -MAP kinase pathways (Deutsch and Sun, 1992; Colbert et al., 1994; Hernandez et al., 1995) in the transcriptional activation of NPY-Y1 receptor gene by using the Dual-luciferase™ reporter system and pRL-SV40 as internal control of transfection.

In order to evaluate the role of one of these CRE sites in the transcriptional control of the NPY-Y1 receptor gene in PC12 cells, a specific mutation was obtained by restriction enzyme digestion and cloned in the luciferase reporter vector (pY1-CRE). Further luciferase reporter assay experiments using this deletion clone will be necessary to determine the relevance of this mutation in the basal- and PACAP-dependent transcriptional activation of the NPY-Y1 gene.

8.3.3.1 PACAP EFFECT ON THE TRANSCRIPTION OF THE NPY-Y1 GENE

PACAP-38 stimulates adenylyl cyclase activity and elevates cAMP, and is a potent activator of the PI cascade at nanomolar concentrations in PC12 cells by specific binding to a type I receptor in PC12 cells (Deutsch and Sun, 1992). PACAP-38 has previously been shown to cause PC12 cells to adopt a neuronal morphology (Colbert et al., 1994; Deutsch and Sun, 1992; Hernandez et al., 1995). This PACAP-38 effect is mediated by the rapid but long lasting activation of MAPK through a *ras* -independent but PKC-dependent mechanism (Barrie and Allen, 1994).

PACAP-38 induced a concentration-dependent increase in the transcriptional activity of the NPY-Y1 receptor gene in PC12 cells. The concentration range (0.5-5 nM) of the PACAP-38 effect on the promoter region of the NPY-Y1 receptor gene is consistent with the binding affinity of the PACAP type I receptor and the biological potency of PACAP-38 in PC12 cells (Deutsch and Sun, 1992). However it will be important to know the effect of another related neuropeptide VIP on the transcriptional activity of the NPY-Y1 receptor gene, because its known that these two peptides have a similar distribution in rats (Arimura, 1992b)

The PACAP-38 transcriptional activation of the NPY-Y1 gene is not dependent in differences in transfection efficiency because PC12 cells were co-transfected with a control reporter vector, pRL-SV40, in which the activity of the *Renilla* luciferase enzyme is under the control of SV40 early enhancer/promoter. The levels of *Renilla* luciferase activity of PACAP-38 treated cells were similar to the basal levels (untreated cells), indicating that differences in luciferase activity upon PACAP-38 treatment are not related to variations in transfection efficiency. In addition, PACAP-38 (5 nM) did not increase the levels of luciferase activity of the promoterless vector (pGL3-Basic) and the control vector (pGL3-Control). These results indicate that non-specific sequences of the reporter vector or post-transcriptional mechanisms, such as mRNA stability are not involved in this PACAP-38 response.

8.3.3.1.1 Role of the PKA pathway

PACAP-38 is a potent activator of adenylyl cyclase and increase cAMP in PC12 cells with an EC50 of 1 nM (Deutsch and Sun, 1992). PACAP-38 (5 nM) also increased the transcriptional activity of NPY-Y1 receptor gene in PC12 cells. This transcriptional activation was mimicked by different pharmacological compounds known to increase intracellular cAMP, such as forskolin (10 μM), by direct binding and activation of adenylyl cyclase (Laurenza, 1989) and DBC (1 mM), a compound which permeates the cell membrane and is metabolised to generate cAMP (Posternak and Weiman, 1974). Both forskolin- and DBC-mediated transcriptional activation of the NPY-Y1 receptor gene were also found to be independent of transfection efficiency, because these agents did not increase the *Renilla* luciferase activity of the pRL-SV40 in co-transfection experiments in PC12 cells.

H-89 a potent and selective inhibitor of PKA in PC12 cells (Chijiwa et al., 1990; Balbi and Allen, 1994) led to a concentration-dependent reduction of the basal- and PACAP-38 induced transcriptional activation of the NPY-Y1 gene in PC12 cells. Moreover, H-89 (30 μM) completely blocked the forskolin (10 μM) and DBC (1 mM) dependent transcriptional activation of NPY-Y1 gene. This result is consistent with previous experiments which showed that PACAP-38 activates transcription of the NPY gene in PC12 cells, and that this activation is completely block by pretreatment with H-89 (Colbert et al., 1994). Taken together, this results

indicates that the PKA signalling pathway is mediating the transcriptional response of the NPY-Y1 receptor gene to PACAP-38, forskolin and DBC. H-89 (30 μM) also reduced the basal levels of transcriptional activity of the NPY-Y1 receptor gene in PC12 cells.

Taken together, these results indicate that the activation of the PKA pathway is involved in the basal- and PACAP-38 induced transcriptional activity of the NPY-Y1 receptor gene in PC12 cells. Further experiments will be necessary to confirm this results, such as the expression of a constitutively active catalytic PKA subunit or expression of the protein kinase inhibitor peptide (PKI). In addition, the role of the PKA pathway can be confirmed with the use of a clonal cell line deficient in PKA (Ginty et al., 1991).

8.3.3.1.2 Role of the PKC pathway

PACAP-38 is also a potent activator of the PI turnover in PC12 cells with a EC_{50} of 7×10^{-9} M (Deutscht and Sun, 1992; Hernandez et al., 1995). This effect appeared to be mediated by direct activation of a specific phospholipase-coupled to a G-protein. There are several splice variants of the PACAP type I receptor that demonstrate different abilities to activate adenylyl cyclase and PLC in PC12 cells (Sprengler et al., 1993).

Previous results has been shown that the PKC signalling pathway is involved in the basal- and NGF-dependent transcriptional activation of the NPY-Y1 receptor gene in PC12 cells. To evaluate the role of this pathway in the PACAP-38 response a specific inhibitor of PKC, calphostin C (Kobayashi et al., 1989) was used. Calphostin C (1 μM) results in a reduction of the PACAP-38 (5 nM) mediated transcriptional activation of the NPY-Y1 receptor gene in PC12 cells. However, calphostin C (500 nM) did not affect the forskolin (10 μM) or DBC (1 mM)-dependent transcriptional activation of this neuropeptide receptor gene, indicating that in these responses PKC activity was not involved.

Evidence for cross-talk between the cAMP system and PKC has been established in the literature. PKC-dependent phosphorylation and activation of adenylyl cyclase is well documented (Kawabe et al., 1994). Increases in intracellular cAMP levels have been shown to regulate PKC activity (Anderson and Breckon, 1991; Houslay, 1991). This interaction has been described as a monodirectional mode of signalling transduction whereby PKA may influence PKC in a positive fashion. Evidence is accumulating that phosphorylation of PKC may likely play a key role in regulation of PKC activity. Phosphorylation of PKC appears to influence positively the activity of the enzyme, resulting in a molecule that is constitutively active and independent of the regulation exerted by second messengers. For example, PKC-ζ is phosphorylated and activated by PKA in PC12 cells (Wooten et al., 1996).

PC12 cells are neuroendocrine cells in which several biological responses to extracellular stimuli are elicited via ion channel activation. Specifically other signalling pathways stimulated as part of the PI breakdown, such as the Ca^{++} signalling pathway may also play a role in the PACAP-38 response in PC12 cells. PACAP-38 results in a increase in the intracellular levels of Ca^{++} in PC12 cells (Hernandez et al., 1995). However, KN-62 (1 μM) a specific inhibitor of the CaM-K II (Tokumitsu et al., 1990) had no effect in the PACAP-38 induced transcriptional activation of the NPY-Y1 receptor gene, indicating that this kinase is not involved in this PACAP-38 response in PC12 cells. However, it is possible that other related CaM-dependent proteins, such as calcineurin (PP2B phosphatase) and/or phosphodiesterases are involved in a Ca^{++}-dependent signalling pathway. The type I phosphodiesterases, specific enzymes which hydrolyse cAMP leading to cessation of its biological effects, are CaM-dependent. The use of selective inhibitors of this phosphodiesterase, such as IBMX, may increase the PACAP-38 transcriptional activation of the NPY-Y1 receptor gene in PC12 cells.

8.3.3.1.3 Role of the *ras* -MAPK pathway

PACAP-38 has been shown to stimulate MAPK activity in PC12 cells (Frödin et al., 1994; Young et al., 1994; Barrie et al., 1997). This increase of MAPK activity is rapid (5 minutes) and long lasting (up to 60 minutes). Moreover, this MAPK stimulation is *ras* -independent and sensitive to PKC inhibition (Frödin et al., 1994; Barrie et al., 1997). In similar way, PACAP-38 (5 nM) has been shown to activate transcription of the NPY-Y1 receptor gene in a PC12-*ras* negative cell line. This result indicates that the $p21^{ras}$ protein is not required to mediate the response to PACAP-38. However, it will be important to know the role of specific signalling pathways activated by PACAP-38 in PC12 cells, such as the PKA and PKC pathways, in the transcriptional activation of the NPY-Y1 receptor gene in this PC12-*ras* negative cell line.

PD98059, is a potent inhibitor of MEK-1 known to block the increase in MAPK activity and neuronal differentiation by PACAP-38 in PC12 cells (Barrie et al., 1997). Pretreatment of the PC12 cells with PD98059 (20 μM) increased basal levels of transcription the NPY-Y1 gene. This paradoxical finding makes it difficult to interpret the role of MEK-kinase in mediating the response to PACAP.

8.3.4 GRE SITES OF THE PROMOTER OF THE NPY-Y1 GENE

The regulatory DNA sequences required for nuclear hormone-dependent activation of transcription have been termed hormone responsive elements (HREs) being the specific high affinity DNA binding sites for the activated nuclear hormone-receptor complexes. The glucocorticoid response element (GRE) is a consensus 15 bp imperfect palindromic DNA sequence: 5'-AG(A/G)AC(A/T)NNNTGTTCT-3' (Beato, 1989). Surprisingly, the GREs

always coincide with DNA sequences which are also required for androgen, progesterone and mineralocorticoid regulation of transcription. Thus, the nucleotide sequence for the progesterone responsive element (PRE), the androgen responsive element (ARE) and the mineralocorticoid responsive element (MRE) cannot be discriminated from the GRE consensus sequence.

Several reverse complement non-palindromic GRE sites (AGGACT) were found at -230 bp, -373 bp and -553 bp (conserved in mouse, rat and human NPY-Y1 genes) and one (TGTTCT) at -540 bp (conserved only in rat NPY-Y1 gene) of the putative transcriptional start site. Moreover, in the NPY-Y1 human gene there are additional potential binding sites for the glucocorticoid receptor (GR) in promoters A, B and C (Ball et al., 1995). These GRE sites in the promoter of the NPY-Y1 receptor gene are the most likely elements to mediate the glucocorticoid (dexamethasone) transcriptional activation of this gene in PC12 cells.

It has been reported that the palindromic structure of GRE sequence is required for the binding of the dimerised receptor to potentiate fully transcriptional activity (Chalepakis et al., 1990). Each of the four elements in the promoter of the NPY-Y1 gene do not comprise a palindromic structure. However, the close location of GREs at -540 and -553 in the NPY-Y1 gene, suggests that these sites may act co-operatively. Synergistic action of several imperfect GRE has been reported (Chalepakis et al., 1990). In some cases, this type of synergy has been shown to result from co-operative DNA binding of the nuclear hormone receptors and to depend on the distance separating the adjacent HREs, suggesting that protein-protein interactions are involved. For example, GREs without palindromic structure can form a functional complex with monomeric GR in MMTV and activate gene transcription (Chalepakis et al., 1990). In addition, dexamethasone increases the transcription of the rat NPY gene and three non-palindromic GRE are found far upstream of this gene (Misaki et al., 1992).

The NPY-Y1 promoter region also has two overlapping consensus sequences; the AP-1 and a non-palindromic GRE at -230 bp using as as reference the putative transcription start site. The putative interaction of these transcription factors by direct protein-protein association may affect the NPY-Y1 gene expression positively (transcriptionally active complex) or negatively (complex unable to bind the hormone responsive element) in PC12 cells (Lopes da Silva and Burbach, 1995). In order to understand the role of this AP1-GRE site, a specific deletion was made by PCR and cloned in the luciferase reporter vector (pY1-AP1). Further experiments will be necessary to understand the role of this site in the dexamethasone response on this NPY-Y1 receptor gene in PC12 cells. However, to understand the role of each DNA binding element in the transcriptional activation of this gene it will be necessary to make site-direct mutations of both the AP-1 and GRE site.

In addition, in order to study the relevance of the others GREs elements in the dexamethasone induced activation of the NPY-Y1 receptor gene, progressive deletions of the promoter were made by PCR and cloned in the luciferase reporter vector. Clone A lacks the two closer located GRE sites far upstream of the NPY-Y1 promoter. Clones C and D also lacks one of the non-palindromic GRE and the AP1-GRE site, respectively. Further experiments will be necessary to understand the role of each specific non-palindromic GRE element in the basal- and dexamethasone-dependent transcriptional activation of the NPY-Y1 receptor gene in PC12 cells.

8.3.4.1 DEXAMETHASONE EFFECTS ON THE TRANSCRIPTION OF THE NPY-Y1 GENE

Glucocorticoids positively regulate several genes governing neurotransmitter and neuropeptide biosynthesis, such as tyrosine hydroxylase, proenkephalin and NPY (Higuchi et al., 1988). During rat development glucocorticoids can direct the differentiation of bipotential neural crest cells toward the chromaffin cell phenotype. It has been demonstrated that differentiation of adrenal progenitor cells into chromaffin cells is dependent upon the presence of glucocorticoid hormones; indeed these cells are exposed to high levels of such hormones upon migration to the adrenal gland since the adrenal cortex synthesises high levels of glucocorticoids.

The majority of NPY-Y1 neurons in the hypothalamus and the brain contains a high density of nuclear glucocorticoid receptors (Härfstrand et al., 1989) and the NPY content of cultured rat hypothalamic neurons is increased with glucocorticoids (Corder et al., 1990). However, little is known about the transcriptional activation of the NPY-Y1 receptor gene. Administration of glucocorticoids to rats causes an up-regulation of NPY-Y1 receptor expression in the arcuate nucleus. Moreover, in the mouse adrenocortical cell line Y-1, dexamethasone increases the density of NPY-Y1 receptors (Weng et al., 1995). In keeping with these observations dexamethasone (1 μM) increased the transcription of the NPY-Y1 receptor gene in PC12 cells grown in a charcoal/dextran treated serum to remove endogenous steroids in the DMEM medium. This result is consistent with previous findings that show that dexamethasone treated cells are responsive to the NPY-Y1 agonist (DiMaggio et al., 1994).

Preliminary results indicated that sex steroids, such as estrogen (1 μM) and progesterone (1μM) did not increase the transcription of this gene in PC12, indicating that the glucocorticoid specificity of this response reflects a GR-mediated response or that PC12 cells lack estrogen receptor (ER) or progesterone receptor (PR). However, estrogen (1 μM) and progesterone (1 μM) induced the transcriptional activation of the NPY-Y1 receptor gene in a mouse hypothalamic cell line, GT1-7 (Mellon et al., 1990). The progesterone receptor (PR) can bind the same GREs present in the promoter of the NPY-Y1 gene, however, the estrogen receptor

(ER) can bind a non-palindromic ERE (TGACC) present at -350 bp of the transcription start site. The role of this ERE element in the estrogen mediated activation of the NPY-Y1 receptor gene in the GT1-7 cells can be analysed using the deletion clone C_1 will lacks this responsive element region. However, specific deletion of this element using site-direct mutagenesis will be important to understand the role of estrogen in the transcriptional activation of this neurosecretory (GnRH) cell line.

8.4 REGULATION OF THE NPY-Y1 mRNA IN PC12 CELLS

In chapter 7 an attempt was made to quantify the NPY-Y1 mRNA in PC12 cells. The amount of mRNA measured at any time is dependent not only by the rate of transcription of the gene but also by post-transcriptional mechanisms such as the stability of the mRNA. mRNA stabilisation is a economical and energetically favourable way to increase protein synthesis by making use of pre-existing mRNA. Under conditions of intense neuronal activity, an increase in the half-life of the NPY-Y1 mRNA would allow for increased production and subsequent replenishment of the NPY-Y1 receptor stores. This increase the complexity of regulation, allows for more diverse responses to signals effecting the overall level of NPY-Y1 receptor production in PC12 cells.

There are several methods of measuring specific mRNAs, including Northern blot analysis, ribonuclease protection assay (RPA) and quantitative RT-PCR. These are listed in order of sensitivity and in degree of complexity. Northern blots and RPA failed to detect the NPY-Y1 mRNA in PC12 cells, indicating that this mRNA is expressed at very low levels in these cells. One alternative will be used polyA^+ mRNA instead of total RNA, but this approach is time consuming. For this reason, selective amplification of the NPY-Y1 mRNA by quantitative RT-PCR was the approach chosen.

Using 1 µg of total RNA of PC12 cells the NPY-Y1 receptor mRNA was detected after 40 cycles of RT-PCR amplification. This result is consistent with previous findings using Northern blots and RPA, indicating that the NPY-Y1 mRNA is expressed at very low levels in PC12 cells. Moreover, similar results results were obtained RINm5f cells. Taken together, this results indicates a very low levels of constitutive expression of this neuropeptide receptor gene in these cell lines.

by treatment with agents known to increase the levels of transcription of the NPY-Y1 gene in PC12 cells, such as NGF, PACAP, forskolin and PMA. This result is consistent with previous findings which show that the cyclophylin gene is constitutively expressed in PC12 cells and unaffected by NGF and forskolin and PMA treatment (Machida et al., 1989). Results indicate that co-amplification of both the NPY-Y1 receptor gene and the cyclophilin gene is possible but that a narrow ratio of NPY-Y1 primers (20 pmol) to cyclophilin primers (2-5 pmol) is required. Further experiments will be necessary to found the linear range of co-amplification of both messages in PC12 cells. As exogenous control an homogeneus cRNA fragment (300 bp) was used to allow amplification using the same primers used to amplified the mRNA of the NPY-Y1 receptor gene was used. However, further co-amplification experiments will be necessary to set-up the conditions for used this internal control in a competitive RT-PCR.

8.5 SUMMARY

The major achievement reported in this thesis was the subcloning of 600 bp of the promoter region of the rat NPY-Y1 receptor gene in the luciferase reporter vector (pY1-LUC). This fusion gene was further transiently transfected in PC12 cells and was shown to produce low levels of luciferase activity consistent with previous findings that indicate that PC12 cells express constitutive levels of the NPY-Y1 gene (DiMaggio et al. 1994). Further, the effect of different factors in the transcriptional control of this neuropeptide receptor gene was obtained. Dexamethasone, NGF and PACAP increased the transcriptional activity of this gene in PC12 cells, indicating that the NPY-Y1 receptor expression is under tight regulation in this cell line.

The effect of NGF in the transcriptional activation of the NPY-Y1 receptor gene in PC12 cells was dependent in *trk* A receptor and PKC activation, but is independent in the activation of the *ras* -MAP kinase, PI-3-K and CaM-K II intracellular signalling pathways. Moreover, the effect of PACAP-38 was dependent in PKA and PKC activation but is also independent on the *ras* -MAP kinase pathway. In addition, activation of both pathways using DBC, forskolin (PKA) or DH1, PMA (PKC) also mimic the increase in the transcriptional activity of the NPY-Y1 gene obtained with NGF and PACAP. In conclusion, both the PKA and PKC intracellular signalling pathways are involved in the transcriptional control of this neuropeptide receptor gene in PC12 cells. For understanding the role of the putative transcription factor sites present in the promoter region of the NPY-Y1 receptor gene, specific deletion of these sites were made using standard molecular biology techniqes. Further experiments using these specific mutants will be necessary to assess the role of the AP1, CRE and GRE sites in the dexamethasone, NGF and PACAP transcriptional activation of the NPY-Y1 gene.Northern blots, RPA and RT-PCR experiments indicated that the NPY-Y1 gene was expressed at very low levels in PC12 cells. Further experiments using competitive RT-PCR will be necessary to know the effect of dexamethasone, NGF and PACAP in the expression of NPY-Y1 gene in PC12 cells.

APPENDIX I

MAMMALIAN CELL CULTURE

RAT TAIL COLLAGEN

25 mg rat type I collagen in 100 ml 0.5 M acetic acid (4.3 ml glacial acetic acid/120 ml water). Filter sterilise the acetic acid and dissolve the collagen in the sterile acetic acid solution. Aliquot in 10 ml fractions and store at 4°C.

POLY-D-LYSINE

5 mg poly-D-lysine (70,000-150,000 MW) in 30 ml sterile tissue culture water. Aliquot in 1ml fractions and store at -20°C.

DMEM

Dissolve powder in 1 litre deionized water. Add 3.7 g sodium bicarbonate/litre medium and 110 mg sodium pyruvate/litre medium. Adjust pH to between 7.2-7.35 with HCl. Filter sterile in 400 ml aliquots and store 4°C. Test sterility by placing 3 ml of medium in the incubator for 48 hours.

RPMI 1640

Dissolve powder in 1 litre deionized water. Add 2.0 g sodium bicarbonate/litre medium. Adjust pH to 6.8-7.0 with HCl. Filter sterile in 400 ml aliquots and store at 4°C. Test sterility by placing 3 ml of medium in the incubator for 48 hours.

GLUTAMINE (0.2 M)

Glutamine (200 mM) in 100 ml water. Stir and heat to 50°C to dissolve. Filter sterilise in 5 ml aliquots and freeze with the lids loose. Store at -20°C.

PENICILLIN/STREPTOMYCIN

Penicilin 10,000 units/ml (100 X), streptomycin 10,000 mg/ml (100 X). Aliquot in 10 ml fractions and store at -20°C.

TRYPSIN/EDTA (10X)

Trypsin (0.05%), EDTA (0.005 M) in PBS (1X). Filter sterilise in 10 ml aliquots. Store at -20 °C. Dilute 1/10 trypsin/EDTA (1X) in sterile PBS (1X).

PBS (20X)

NaCl (137 mM), KCl (2.7 mM), $Na_2HPO_4.2H_2O$ (8 mM), KH_2PO_4 (147 mM) in distilled water. Sterilise by autoclave and store at room temperature in 500 ml aliquots.

HANK'S MODIFFIED BUFFER

$CaCl_2$ (1.3 mM), KCl (5.4 mM) $MgCl_2$ (0.5 mM), $MgSO_4$ (0.5 mM), NaCl (137 mM), $NaCO_3$ (4 mM), NaH_2PO_4 (0.4 mM) in distilled water.

DEXTRAN COATED CHARCOAL

Charcoal (0.25% w/v), Dextran (0.025% w/v) in PBS (1X). Store in 25 ml aliquots at 4°C.

MAMMALIAN CELL TRANSFECTION

CaCl2 (2.5 M)

CaCl2 (2.5 M) in deionized water. Filter sterile and store in 10 ml aliquots at -20°C. Dilute 1/5 (0.5 M) in sterile PBS (1X) before using for transfection.

HEPES BUFFER (2X)

NaCl (280 mM), HEPES (50 M), Na2HPO4(1.5 mM) in deionized water. Adjust to pH 7.05-7.1 with NaOH. Filter sterile and store in 50 ml aliquots at -20°C.

TE BUFFER (TRIS 10 mM/EDTA 0.1 mM)

Tris (10mM) pH 7.4 and EDTA (0.1 mM) in sterile deionized water. Filter sterile and store in 10 ml aliquots at room temperature.

DEAE-DEXTRAN (10 mg/ml)

DEAE-Dextran (10 mg/ml) in PBS (1X). Filter sterilise and store in 1ml aliquots at 4°C.

CHLOROQUINE (100 mM)

Chloroquine (100 mM) in 10 ml of PBS (1X).Filter sterilise and store in 10 ml aliquots at 4°C.

DMSO (10% v/v)

23.5 ml PBS (20X) plus 27 ml DMSO in 400 ml sterile water. Store in 500 ml aliquots at room temperature.

HEPES BUFFER (1X)

NaCl (150 mM), HEPES (20 mM) in deionized water. Adjust to pH 7.4 with NaOH. Filter sterile and store in 50 ml aliquots at -20°C.

BACTERIA CELL CULTURE

LB MEDIUM

Bacto-tryptone (1% w/v), bacto-yeast extract (0.5% w/v) and NaCl (1% w/v) in distilled water. Sterilise by autoclaving and store in 500 ml aliquots at room temperature.

M9 MINIMUM MEDIUM

M9 solution: NaCl (0.05% w/v), NH_4Cl (0.1% w/v), KH_2PO_4 (0.3% w/v), Na_2HPO_4 (0.6% w/v) in distilled water. Sterilise by autoclaving. After cooling at 50°C add$CaCl_2$ (1 M), $MgSO_4$ (1 M) and glucose (20% v/v). Store in 500 ml aliquots at 4°C. thiamine-HCl solution (1 M) in distilled water. Filter sterile and store in 1 ml aliquots at -20°C.
To make M9 plates prepare 1.7 agar (1.7 % w/v) in 100 ml distiled water. Sterilise by autoclaving. After cooling at 50°C add 0.12 ml thiamine solution (1M) and 1.2 ml M9 solution. Pour in the plastic plates.

TYM MEDIUM

Bacto-tryptone (2 w/v %), Bacto-yeast extract (0.5% w/), NaCl (0.1 M) and $MgCl_2$ (0.01 M) in 100 ml of distilled water. Sterilise by autoclaving and store in 500 ml aliquots at room temperature

COMPETENT BACTERIA SOLUTION (Tfb I)

KOAc (30 mM), $MnCl_2$ (50 mM), KCl (100 mM), $CaCl_2$ (10 mM), glycerol (15% v/v), EDTA (0.2 mM) in distilled water. Filter sterilise and store in 500 ml aliquots at 4°C.

COMPETENT BACTERIA SOLUTION (Tfb II)

Na-MOPS pH7 (10 mM), CaCl2 (75 mM), KCl (10 mM), glycerol (15 % v/v) in distilled water. Filter sterilise and store in 500 ml aliquots at 4 °C.

TOTAL RNA EXTRACTION

DEPC TREATED WATER

Add DEPC (0.1 % v/v) to distilled water and stirred for 1-2 hours. Autoclave the DEPC water and store in 1000 ml aliquots at room temperature.

GUANIDIUM THYOCIANATE BUFFER

Guanidinum thiocyanate (4M), sodium citrate pH 7.0 (25 mM), sarcosyl (0.5% w/v) in DEPC-treated water. Store in 50 ml aliquots at 4°C for up to six months. Just before use add 7.2 µl/ml b-mercaptoethanol (100 mM). Store in 10 ml aliquots at 4°C for up to three months.

SODIUM ACETATE (2 M)

Sodium acetate (2 M) in 100 ml of DEPC treated water. Adjust the pH to 4.0 with acetic acid glacial. Store in 50 ml aliquots at 4°C.

PLASMID DNA EXTRACTION

SOLUTION I (Resuspension solution)

Tris (50mM) pH 7.4, EDTA (0.1 mM) (pH 8.0) in distilled water. Store in 100 ml aliquots at room temperature. Add RNAse A to 100 mg/ml final concentration.

SOLUTION II (Lysis solution)

NaOH (0.2 M), SDS (1% w/v) in distilled water. Store in 100 ml aliquots at 4°C.

SOLUTION III (Neutralization solution)

potassium acetate (1.32 M) pH 4.8 in distilled water. Store in 100 ml aliquots at 4°C.

NUCLEIC ACID SOLUTIONS

AMMONIUM ACETATE (7.5 M)

Ammonium acetate (7.5 M) in distilled water. Adjust pH 7.0 with acetic acid glacial. Store in 100 ml aliquots at 4°C.

SODIUM ACETATE (3 M)

Sodium acetate (3 M) in distilled water. Adjust pH 7.0 with acetic acid glacial. Store in 100 ml aliquots at 4°C.

$MgCl_2$ (1 M)

$MgCl_2$ (1 M) in distilled water. Store in 100 ml aliquots at 4°C.

ETHIDIUM BROMIDE (10 mg/ml)

Ethidium bromide (10 mg/ml) in TE buffer. Store away from light at room temperature.

IODOACETAMIDE (0.5M)

Iodoacetamide (0.5 M) dissolved in ethanol. Store away of the light at 4°C.

DNA LOADING BUFFER

Glycerol (50% v/v), TAE buffer (1X) and bromophenol blue (1% v/v) in ditilled water. Store in 1 ml aliquots at -20°C.

cDNA LOADING BUFFER

Formamide (98% v/v), EDTA (10 mM), xylene cyanol (0.1% w/v) and bromophenol blue (0.1% w/v) in distilled water.Store in 1 ml aliquots at -20°C.

RNA LOADING BUFFER

Glycerol (50% v/v), EDTA (1mM) and bromophenol blue (0.4% w/v) in distilled water. Store in 1 ml aliquots at -20°C.

RNA SAMPLE BUFFER

Deionized formamide (10% v/v), formaldehyde (10% v/v) and MOPS 1X in DEPC treated water. Store in 1 ml aliquots at -20°C.

RESTRICTION ENZYME BUFFERS

LOW SALT BUFFER (10 X)

Tris-HCl (10mM) pH 7.5, $MgCl_2$ (10mM), DTT (1mM) in distilled water.

MEDIUM SALT BUFFER (10 X)

NaCl (50mM), Tris-Cl (10mM) pH 7.5, $MgCl_2$ (10mM), DTT (1mM) in distilled water.

HIGH SALT BUFFER (10 X)

NaCl (100mM), Tris-Cl (10mM) pH 7.5, $MgCl_2$ (10mM), DTT (1mM) in distilled water.

DNA MODIFYING ENZYME BUFFERS

T4 POLYNUCLEOTIDE KINASE BUFFER (10 X)

Tris-HCl (500 mM) pH 7.4, $MgCl_2$ (100 mM), DTT (100 mM), glycerol (40% v/v) in distilled water. Store at -20°C.

SAP BUFFER (10 X)

Tris-HCl (200 mM) pH 8.0, $MgCl_2$ (100 mM) in distilled water.Store in 1 ml aliquots at -20°C.

T4 DNA LIGASE BUFFER (10 X)

Tris-HCl (500 mM) pH 7.5, $MgCl_2$ (100 mM), DTT (200 mM), ATP (10 mM) in distilled water.Store in 1 ml aliquots at -20°C.

DNA POLYMERASE I BUFFER (10 X)

Tris-HCl (500 mM) pH 8.0, $MgCl_2$ (100 mM), DTT (1 mM), BSA (500 µg/ml) in distilled water.Store 1 ml aliquots at -20°C.

AMV REVERSE TRANSCRIPTASE (5 X)

Tris-HCl (250 mM) pH 8.3, KCl (250 mM), $MgCl_2$ (50 mM), DTT (50 mM) and spermidine (2.5 mM) in distilled water. Store in 1 ml aliquots at -20°C.

AMV PRIMER EXTENSION (2X)

Tris-HCl (100 mM) pH 8.3, KCl (100 mM), $MgCl_2$ (20 mM), DTT (20 mM), dNTP (2 mM), spermidine (1mM) in distilled water. Store in 1 ml aliquots at -20°C.

DNAZYME DNA POLYMERASE (10X)

Tris-HCl (100 mM) pH 8.3, KCl (100 mM), EGTA (7.5 mM)and Triton X-100 (0.1% w/v)in distilled water. Store in 1 ml aliquots at -20°C.

TAQ DNA POLYMERASE (10X)

Tris-HCl (10 mM) pH 8.3, KCl (50 mM) in distilled water. Store in 1 ml aliquots at -20°C.

TRANSCRIPTION OPTIMIZED BUFFER (5X)

Tris-HCl (200 mM) pH 7.5, NaCl (50 mM), MgCl2 (30 mM) and spermidine (10 mM) in distilled water.Store in 1 ml aliquots at -20°C.

NUCLEIC ACID SEPARATION

TAE BUFFER (50X)

Tris base (2 M), sodium acetate.(1 M), EDTA (50 mM) in distilled water. Store at 4°C.

TBE BUFFER (20 X)

Tris base (1 M), borid acid (1 M), EDTA (20 mM) in distilled water. Store at 4°C.

MOPS BUFFER (10X)

MOPS (0.2 M), 20 ml 0.5 M EDTA. $2H_20$ (10 mM), 16.6 ml 3 M sodium acetate (in DEPC treated water. Adjust at pII 7.0 with NaOH. Store at 4°C.

GLYCEROL TOLERANT BUFFER (20X)

Tris base (216 gm), Taurine (gm), EDTA (4 gm) in 1000 ml of deionized water. Store at room temperature.

NORTHERN BLOT ASSAYS

FORMALDEHYDE (deionized)

Add 5 g Dowex XG8 to 100 ml formamide and stir at room temperature for 1 hour. Filter twice through Whatman N1 paper. Store in 1 ml aliquots at -70°C.

SSC BUFFER (20X)

NaCl (3 M) and Na3 citrate (0.3 M) in DEPC treated water. Adjust at pII 7.0 with NaOH 10N. Store in 500 ml aliquots 4°C.

HYBRIDIZATION SOLUTION (DNA PROBES)

Buffer phoosphate (0.77 M) pH 7.2, SDS (6.6% w/v) and polyA DNA (100 µg/ml) in distilled water. Store in 100 ml aliquots at room temperature.

DENHARDT'S SOLUTION

Ficoll (Type 400) (2% w/v), polyvinyl polyrrolidone (2% w/v) and BSA (2% w/v) in DEPC treated water. Sterilize by filtration and store at -20°C.

HYBRIDIZATION SOLUTION (RNA PROBES)

Formamide deionized (50% v/v), SSC (5X), dextran sulfate (10% w/v), Denhardt's solution (5X), DTT (1 mM), SDS (1% w/v) and salmon DNA (100 µg/ml) in DEPC treated water. Store at room temperature.

RIBONUCLEASE PROTECTION ASSAYS

SOLUTION A

Formamide deionized (80% v/v), sodium citrate (100 mM) pH 6.4, sodium acetate (300 mM) pH 6.4 and EDTA (1 mM). Store at -20°C.

SOLUTION F

Ammonium acetate (0.5 M0, EDTA (1 mM) and SDS (0.2% w/v) in DEPC trated water. Store at room temperature. Store at -20°C.

SOLUTION R

RNAse A (250 units/ml) and RNAse T1 (10,000 unit/ml) DEPC treated water. Store at -20°C.

LUCIFERASE ASSAYS

LUCIFERASE ASSAY REAGENT

Tricine (20 mM), $(MgCO_3)_4Mg(OH)_2$ (1.7 mM), $MgSO_4$ (2.67 mM), EDTA (0.1 mM), DTT (33.3 mM), coenzyme A (270 µM), ATP (530 µM) pH 7.8 in distilled water.

CELL CULTURE LYSIS BUFFER (1X)

Tris-phosphate (25 mM) pH 7.8, DTT (2 mM), glycerol (1% w/v) and Triton X-100 (1% w/v) in distilled water.

PKC ASSAY SYSTEM

BUFFER A (EXTRACTION BUFFER)

Tris-HCl (20 mM) pH 7.5, EDTA (0.5 mM), EGTA (0.5 mM), Triton X-100 ().5 % w/v). aprotinin (25 µg/ml) and leupeptin (25 µg/ml) in distilled water.

BUFFER B (DEAE WASH BUFFER)

Tris-HCl (20 mM) pH 7.5, EDTA (0.5 mM), EGTA (0.5 mM) in distilled water.

BUFFER C (ELUTION BUFFER)

Tris-HCl (20 mM) pH 7.5, EDTA (0.5 mM), EGTA (0.5 mM), β-mercaptoethanol (10 mM), NaCl (0.2 M) in distilled water.

APPENDIX II

SYNTHETIC OLIGONUCLEOTIDES

RAT NPY-Y1 RECEPTOR (open reading frame)

Sequence 2010 (forward primer)
5'-TTC GGA GCT TCC TAA CAG TCC GTT-3'

Sequence 2011 (reverse primer)
5'-GGA TGT TCT AGA CTT TCC AAA CCT-3'

RAT NPY-Y1 RECEPTOR (5'-promoter region)

Sequence 2024 (forward primer)
5'-GGC ACG AGG ATC CTT TGG CTT-3'

Sequence 2023 (reverse primer)
5'-CTG TTA GGA TCC TTC GAA ATG G-3'

RAT NPY-Y1 RECEPTOR (3'-flanking region)

Sequence 2486 (forward primer)
5'-ATC ATT GCC ACC TGC AAC-3'

Sequence 2487 (reverse primer)
5'-TGT CCT GGA CTT TCC AAA C-3'

RAT NPY-Y1 RECEPTOR (deletion 3'-flanking region)

Sequence T7507 (internal reverse)
5'-GTC ACC GCG GTC GTC TCG AGA CCG GAA G-3'

Sequence T7508 (internal forward)
5'-GTC ACC GCG GTC TGA AGC TGC TGA GAA C-3'

RAT NPY-Y1 RECEPTOR (deletion AP-1 site)

Sequence V5339 (internal forward)
5'-GTC ACC GCG GGT GAG AGC TCT GTG TGG-3'

Sequence V5340 (internal reverse)
5'-GTC ACC GCG GGG AAG GCG CAT TTA CAA-3'

RAT NPY-Y1 RECEPTOR (progressive promoter deletions)

Sequence V7613 (forward primer-clone A)
5'-AAC TTC TCG GAT CCG GCG TGC-3'

Sequence V7614 (forward primer-clone B)
5'-CTT GAC TAG GGA TCC AAG CTC-3'

Sequence V7615 (forward primer-clone C)
5'-CAG GAT CCT AAA TGC GCC TTC-3'

Sequence V7616 (forward primer-clone D)
5'-TTC GGA TCC GAC AGT GTG GGC-3'

Sequence V7617 (forward primer-clone E)
5'-CAT GAT CCT GGA TCC GGT GGA-3'

FIREFLY LUCIFERASE GENE (open reading frame)

Sequence T1886 (reverse primer RPA)
5'-TAT CTC TTC ATA GCC TTA TGC AGT T-3'

FIREFLY LUCIFERASE GENE (open reading frame)

Sequence Y7464 (reverse primer RT-PCR)
5'-TAG CCT TAT GCA GTT-3'

Sequence Y7465 (forward primer RT-PCR)
5'-GGA GAA ATA CCA ACA-3'

Sequence Y7466 (forward primer RT-PCR)
5'-GAT CTT TCT TCC CTC-3'

Sequence Y7467 (forward primer RT-PCR)
5'-CAG TGA CAC TCG TCC-3'

RAT CYCLOPHILIN GENE (open reading frame)

Sequence Y7462 (forward primer)
5'-GGT GAC TTC ACA CGC CAT AAT G-3'

Sequence Y7463 (reverse primer)
5'-GAG TTG TCC ACA GTC GGA GAT G-3'

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