Regulation of Expression of Signal Transduction Cascade Elements by G-protein Coupled Receptors

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Abstract

It is now known that external stimuli such as hormones, neurotransmitters, growth factors, light and drugs initiate their biological actions by interacting with receptor macromolecules which are located in the cell membranes. Many transmembrane receptors are coupled to specific effector molecules via G proteins. These trimeric 'receptor-G protein-effector' systems play a primary role in cellular signalling.

Molecular cloning techniques have revealed the presence of cDNA species encoding many receptor isoforms or splice variants as well as many G protein subunits. In this study, cDNA species either the long or the short isoforms of the rat TRH receptor were expressed stably in Rat 1 fibroblasts, and clones expressing specific binding of [^{3}H]TRH were detected and expanded. Clones expressing each of these receptors at levels up to 1 pmol/mg of membrane protein were selected for analysis. Reverse transcriptase-PCR on RNA isolated from these clones confirmed that each clone expressed the expected splice variant. Both receptor splice variants bound [^{3}H]TRH with an approximate K_d of 80 nM.

In the presence of TRH, both receptor subtypes were able to stimulate inositol phosphate generation in a pertussis toxin-insensitive manner with similar EC_{50} values. However, despite reports that TRH receptors can also interact with the G proteins G_s and G_i2, neither receptor splice variant was able to modulate adenylyl cyclase activity in either a positive or negative manner. These data indicate that the long and short isoforms of the rat TRH receptor have similar affinities for TRH and display similar abilities to interact with the G_q-like G proteins, but show no ability to regulate adenylyl cyclase in this genetic background.

Although downregulation of signal transducer, G protein α subunits, is a

well-documented phenomenon the mechanism has been unclear. Maintained exposure of Rat 1 fibroblasts transfected to express the molecularly defined $\alpha_{1A/D}$, α_{1B} , and α_{1C} adrenergic receptors resulted in a large downregulation of receptors and also a marked downregulation of cellular levels of both of the phosphoinositidase C-linked G proteins, $G_q\alpha$ and $G_{11}\alpha$ when exposed to the α_1 adrenergic agonist phenylephrine.

In order to examine the mechanism of phenylephrine-induced downregulation of $G_q\alpha$ and $G_{11}\alpha$, pulse-chase ^{35}S-amino acid labelling experiments were performed with each of the $\alpha_{1A/D}$, α_{1B} , and α_{1C} adrenergic receptor expressing cell lines. The rate of degradation of $G_q\alpha$ and $G_{11}\alpha$, which was adequately modeled by a monoexponential with half-life between 33 and 40h in each of the cell lines in the absence of agonist, was accelerated about 4 fold in the presence of phenylephrine. Each of the $\alpha_{1A/D}$, α_{1B} , and α_{1C} adrenergic receptor expressing cell lines prelabelled with *myo*-[^{3}H]inositol were shown to generate high levels of inositol phosphates when exposed to phenylephrine. Additionally, the degree of $G_q\alpha$ and $G_{11}\alpha$ downregulation in Rat 1 fibroblasts transfected to express either the wild type hamster α_{1B} adrenergic receptor or a constitutively active mutant (CAM) form of this receptor was examined. The sustained presence of phenylephrine resulted in substantially greater downregulation in cells expressing the CAMα_{1B} adrenergic receptor compared to the wild type at all concentrations of agonist. The enhanced capacity of agonist to stimulate second messenger production at the CAMα_{1B} adrenergic receptor and to regulate cellular levels of its associated G proteins by stimulating their rate of degradation is indicative of an enhanced stoichiometry of coupling of this form of the receptor to $G_q\alpha$ and $G_{11}\alpha$.

Treatment of cells expressing the CAMα_{1B} adrenergic receptor with α_1 adrenergic antagonists phentolamine, HV-723, corynanthine, YM-12617, 5-methyl urapidil and WB4101 resulted in upregulation (2-3 fold in 24h) in levels of this

receptor. Pretreatment of the CAMα_{1B} adrenergic receptor expressing cells with phentolamine caused greater maximal output (about 2-fold) from the $G_q\alpha$ and $G_{11}\alpha$ to the phosphoinositidase C pathway as well as phospholipase D pathway than the untreated cells upon addition of phenylephrine. However, the potency of phenylephrine was unchanged. By using this system in which the increased receptor levels in response to treatment with phentolamine caused an increase in the basal level of phospholipase D activity, screening of potential inverse agonists was performed. All of the above named α_1 antagonists appeared to be capable of acting as inverse agonists at the CAMα_{1B} adrenergic receptor.

Contents

Chapter 1: Introduction

List of Figures

List of Tables

Abbreviations

The abbreviations used in this thesis are as set out in "Instructions to Authors" Biochemical Journal [1985] 225, 1-26 with the following additions :

AC	Adenylyl cyclase
ADP	Adenosine 5'-diphosphate
ARF	ADP ribosylation factor
ATP	Adenosine 5'-triphosphate
βARK	β-adrenergic receptor kinase
B_{max}	Maximal binding capacity
BSA	Bovine serum albumin
CAM	Constitutively active mutant
cAMP	Adenosine 3', 5'-cyclic monophosphate
CPM	Counts per minute
CTX	Cholera toxin
DAG	sn-1, 2-diacylglycerol
DMEM	Dulbeccos' modified Eagles' medium
DMSO	Dimethylsulphoxide
DPM	Disintegrations per minute
EC_{50}	Median effective dose
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraactic acid
EGF	Epidermal growth factor
EGTA	Ethyleneglycol-bis (β-aminoethyl ehter) N, N, N', N'-tetraacetic acid

FCS	Foetal calf serum
GAP	GTPase activating protein
GDP	Guanosine 5'-diphosphate
Gpp[NH]p	Guanylyl 5'-[βγ imido] diphosphate
G protein	Guanine nucleotide binding protein
GRK	G protein coupled receptor kinase
GTPγS	Guanosine 5'-[3-o-thio]triphosphate
GTP	Guanosine 5'-triphosphate
h	Hours
HEPES	4-[2-Hydroxyethyl]-1-piperazine-N'-2-ethane-sulphonic acid
IP_3	D-*myo*-inositol-1, 4, 5-trisphsophate
Kd	Equilibrium dissociation constant; representing theconcentration of a ligand that half-maximally occupies the receptor at equilibrium
kDa	Kilodaltons
LPA	Lysophosphatidic acid
MAPK	Mitogen activated protein kinase
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NP-40	Nonidet P-40 detergent
PBS	Phosphate buffered saline
PKA	Protein kinase A
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PIC	Phosphinositidase C
PLD	phospholipase D

PMSF	Phenylmethylsulphonyl fluoride
PTX	Pertussis toxin
R	Receptor
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS-PAGE	Sodium dodecylsulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SV40	simian virus 40
TEMED	N, N, N', N' tetramethylethylenediamine
TCA	trichloroacetic acid
TRH	Thyrotropin-releasing hormone
Tris	Tris (hydroxymethyl) aminomethane
WT	Wild type

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Chapter 1. Introduction

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1.1. Historical perspectives of cellular signalling

It is now apparent that external stimuli including hormones, neurotransmitters, growth factors, light and most drugs initiate their biological actions by interacting with receptor macromolecules which are located in the plasma membranes of cells. Many transmembrane receptors are coupled to specific effector molecules via intermediary coupling guanine nucleotide binding proteins (G proteins) and it is known that these trimeric "receptor-G protein-effector" systems play a major role in defining the ability of cells to respond to and process information from external stimuli as shown in **Figure 1.1**.

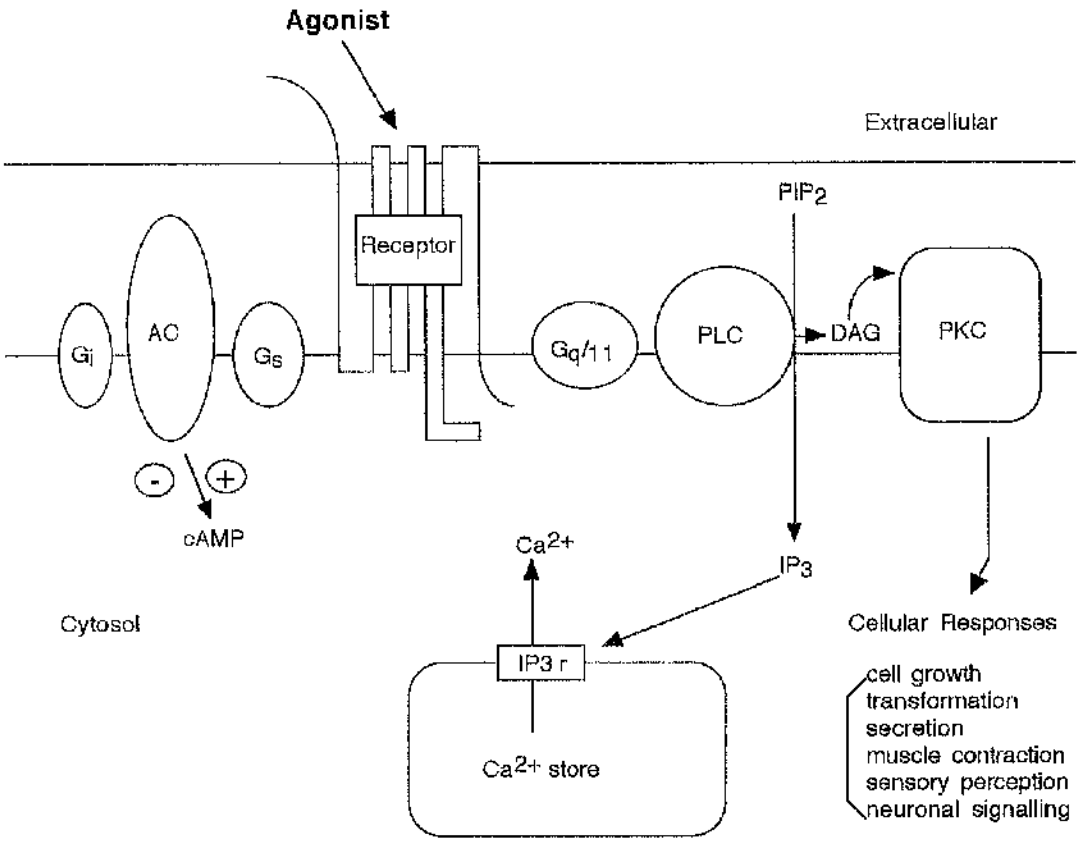
Two signalling systems are primarily responsible for the production and amplification of intracellular signals resulting from specific extracellular stimuli. These are the receptor tyrosine kinases (RTKs) and the G-protein-coupled receptors (GPCRs) which will mainly be discussed in this introduction.

Since the first "second messenger" hypothesis for hormone action in cellular signalling based on the observation that the actions of adrenaline on canine liver could be mimicked by the heat stable factor, cAMP, it has been proposed that adrenaline bound to specific cell surface receptors could cause a conformational change in the catalytic moiety of adenylyl cyclase resulting in an activation of the enzyme [Rall *et al.*, 1957; Sutherland *et al.*, 1962]. The requirement of GTP in glucagon-mediated regulation of adenylyl cyclase [Rodbell *et al.*, 1971] , the effect of GTP in the enhanced dissociation of glucagon from its receptor [Rodbell *et al.*, 1971; Harwood *et al.*, 1973] and the action of non-hydrolysable analogues of GTP in the activation of adenylyl cyclase without any agonist stimulation [Schramm & Rodbell, 1975] suggested that a

Figure 1.1. Schematic diagram for transmembrane signal transduction

General signalling pathways for the generation of second messengers are shown. Upon stimulation by external stimuli (agonist) G protein-coupled receptors are activated and transmit the signal to the effector enzymes via G proteins. G_s and G_i represent stimulatory and inhibitory Gα subunits which stimulate and inhibit adenylyl cyclase activity, respectively. $G_q/G_{11}\alpha$ subunits activate phophoinositidase C (PIC) which hydrolyses phosphatidyl-4,5-bisphosphate (PIP_2) and generates two second messengers, diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). DAG and Ca^{2+}, which is released from an internal Ca^{2+} store, the endoplasmic reticulum (ER), stimulate protein kinase C (PKC) which in turn causes many cellular responses including cell growth, transformation, secretion, muscle contraction, sensory perception, and neuronal signalling.

Figure 1.1.


Agonist
Extracellular
Receptor
PIP2
Gi
AC
Gs
Gq/11
PLC
DAG
PKC
-
+
cAMP
Ca2+
IP3
Cytosol
IP3 r
Cellular Responses
Ca2+ store
cell growth
transformation
secretion
muscle contraction
sensory perception
neuronal signalling

guanine nucleotide binding site was invovled in the regulation of adenylyl cyclase and the role of ligand-bound receptor was to facilitate the guanine nucleotide mediated activation of the enzyme.

The more specific role of GTP in regulating receptor-effector interactions was demonstrated by high affinity GTPase activity associated with β-adrenergic stimulation of adenylyl cyclase, and GTP hydrolysis appeared to terminate the activation of the enzyme [Casel & Selinger, 1976]. Therefore, two states, an active GTP-bound state and an inactive GDP-bound state were suggested, and receptor activation was believed to cause the exchange of GDP for GTP. The ability to reconstitute this activity in membranes of the murine S49 lymphoma mutant, cyc⁻, which is deficient in adenylyl cyclase activity [Ross *et al.*, 1978] lead to the purification of this element which is known as G_s for its stimulatory effect on adenylyl cyclase [Northup *et al.*, 1980].

By the 1980's, it was known that the G protein-mediated receptor signalling cascade could be controlled by turning on and off the signal [Gilman, 1984]. The receptor was coupled to a GDP liganded G protein in the resting state and agonist activation of the receptor caused a conformational change in both proteins leading to the exchange of GDP for GTP which promoted dissociation of the G protein from its trimeric form into a free active α and a βγ complex. The GTP-bound α subunit could then interact with the final executor effector system before its intrinsic GTPase activity hydrolysed the GTP to GDP promoting reformation of the holomeric protein and reassociation with the receptor causing deactivation of the cascade [Gilman, 1984; Gilman, 1987].

Many variants of the three (α, β, and γ) subunits of G proteins have been identified by the advent of molecular cloning techniques. The functional roles for individual G proteins has been revealed, and it is now known that both the GTP-binding α subunit and the βγ subunit complex are able to influence the activity of effector systems including adenylyl cyclase, phosphoinositidase C, phospholipase A_2,

phospholipase D, various ion channels and MAP Kinase activity independently or simultaneously, either synergistically or in opposition [Hepler & Gilman, 1992; Clapham & Neer, 1993; Sternweis, 1994; Inglese *et al.*, 1995].

More recently, the studies of crystal stucture of $G_i1\alpha$ revealed that crystals of the GDP-bound $G_i1\alpha$ monomers were linked, head-to-tail, in endless polymers [Mixon *et al.*, 1995]. Determination of the crystal structure of transducin [Noel *et al.*, 1993] and of the transducin $\beta\gamma$ dimer [Sondek *et al.*, 1996] has allowed the mechanism of the nucleotide-dependent engagement of the α and $\beta\gamma$ subunits that regulate their interaction with receptor and effector molecules to be understood at the molecular level. The interaction involves two distinct interfaces and alters the conformation of the α subunits but not of the $\beta\gamma$ subunits. The sites for post-translational modification on $G_t\alpha$ and $G_t\gamma$ combined with the receptor-binding region of $G_t\alpha$ suggested a plausible orientation with respect to the membrane surface and an activated heptahelical receptor, rhodopsin [Lambright *et al.*, 1996].

1.2. G protein-coupled receptors (GPCRs)

The first step in the transfer of signals from the outside to the inside of the cell is the binding of a hormone, neurotransmitter or growth factor to a transmembrane receptor on the plasma membrane. The superfamily of G protein-coupled receptors, with hundreds of members, is known to recognize a vast array of substances, including protein and peptide hormones, nucleotide and amino acid neuromodulators, biogenic amine neurotransmitters, lipid autacoids, sugar and alkaloid tastants, volatile organic odorants, calcium ions, and photons of visible and ultraviolet light.

The effect of guanine nucleotides on ligand binding to a receptor was first noted by Rodbell and coworkers [1971], with the demonstration that the affinity of glucagon binding to the plasma membrane of rat liver, was decreased by the presence of guanine

Table 1.1. G protein-coupled receptors

Numerous hormones and neurotrasmitters are linked to a number of interwoven second messenger cascades via various receptor subtypes and G proteins. Abbreviations: ACTH, adrenocorticotropin; CRF, corticotropin-releasing factor; FSH, follicle stimulating hormone; GHRH, growth hormone -releasing hormone; hCG, human chorionic gonadotropin; LH, leuteinizing hormone; MSH, melanocyte stimulating hormone; $PGE_{1\&2}$, prostagladin E_1 & E_1; PHI_2, prostagladin I_2; PTH, parathyroid hormone; 5-HT, 5-hydroxy-tryptamine; TSH, thyroid stimulating hormone; TRH, thyrotropin-releasing hormone; VIP, vasoactive intestinal peptide; CCK, cholecystokinin ; fMLP, fMet-Leu-Phe; NAF, nerve-activating factor; GnRH, gonadotropin-releasing hormone; IgE, immunoglobulin E; TXA_2, thromboxane A_2; GABA, γ–amino-butyric acid.

* denotes the existence of subtypes of the receptor.

Table 1.1

Effectors	Receptors	
Adenylyl cyclase stimulation	ACTH	LH*
	Adenosine A_2	MSH
	Adrenergic β_1, β_2	Olfactory*
	Calcitonin	PGE_1, PGE_2
	CRF	PGI_2
	Dopamine D_1	PTH*
	FSH	Secretin
	GHRH	5-HT_2
	Glucagon G_2	Taste
	hCG*	TSH*
	Histamine H_2	Vasopressin V_2
		VIP
Adenylyl cyclase inhibition	Adenosine A_1	Opioid δ, γ, μ
	Adrenergic α_{2A}, α_{2B}	PAF
	Angiotensin II	PGE_1, PGE_2
	Bradykinin*	Thrombin*
	Dopamine D_2	5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}
	Histamine H_3	Somatostatin
	Muscarinic M_2, M_3	Vasopressin V_{1A}
Phospholipase C stimulation	α_1 adrenergic*	Muscarinic M_1, M_4, M_5
	Angiotensin II	Neurokinin B
	Bombesin	Oxytocin
	Bradykinin	PAF, TXA_2
	CCK	Purinergic P_{2Y}, P_{2X}
	fMLP*, NAF	5-HT_{1C}, 5-HT_2
	Glucagon G_1	Substance K*
	GnRH	Substance P*
	Histamine H_1	Thrombin
	IgE*	Vasopressin V_{1A}, V_{1B}
	TRH*	VIP*
Phospholipase A_2 stimulation	α_1 adrenergic*	Histamine H_1
	Bradykinin*	Leukotriene C_4, D_4
	GnRH	
Calcium channel	**Opening**	**Closing**
	Angiotensin II	α_2 adrenergic*
	β_1 adrenergic	$GABA_B$
	GnRH	Opioid*
		Somatostatin
Potassium channel	**Opening**	**Closing**
	Adenosine A_1	Muscarinic M_1
	Bradykinin*	
	Dopamine D_2	
	$GABA_B$	
	Muscarinic M_2	
	5-HT_{1A}, 5-HT_{1C}	
	Somatostatin	

nucleotides. Later, the binding of β-adrenergic agonists but not antagonists were shown to be reduced by the presence of the GTP analogue, Gpp[NH]p [Maguire *et al.*, 1976]. Further studies on β-adrenergic receptors revealed that competition curves for antagonist versus radiolabelled antagonist had pseudo Hill coefficients close to 1, competition curves for agonist versus radiolabelled antagonist were shallower with pseudo Hill coefficients less than 1 [De Lean *et al.*, 1980]. These data suggested the existence of two affinity states for agonist. A low affinity state of receptor for agonist was seen in the presence of guanine nucleotides, whereas the receptor adopted a higher affinity for agonist when guanine nucleotides were absent.

In an attempt to account for the effects of guanine nucleotides on the ligand binding a ternary complex model was proposed. In the unstimulated state the inactive α subunit, coupled to βγ subunit, may interact with the receptor (R), leading to the agonist (H) promoted formation of a high affinity ternary complex (H>R>G protein). In the presence of guanine nucleotides which activate the α subunit, the ternary complex is destabilized and both agonist and α subunit can dissociate from the receptor.

A large number of individual G protein-coupled receptors (GPCRs) have now been cloned and sequenced, and the structure and function of these receptors has been the subject of much research in recent years. GPCRs have been subdivided into several classes including those for dopaminergic [Vallar and Meldolesi, 1989], muscarinic cholinergic [Birdsall & Hulme, 1983], serotonergic [Schmidt and Peroutka, 1989], adrenergic [Gilman, 1987] and olfactory [Jones & Reed, 1987] receptors according to their ligands which are capable of activating several effector systems as shown in **Table 1.1.**

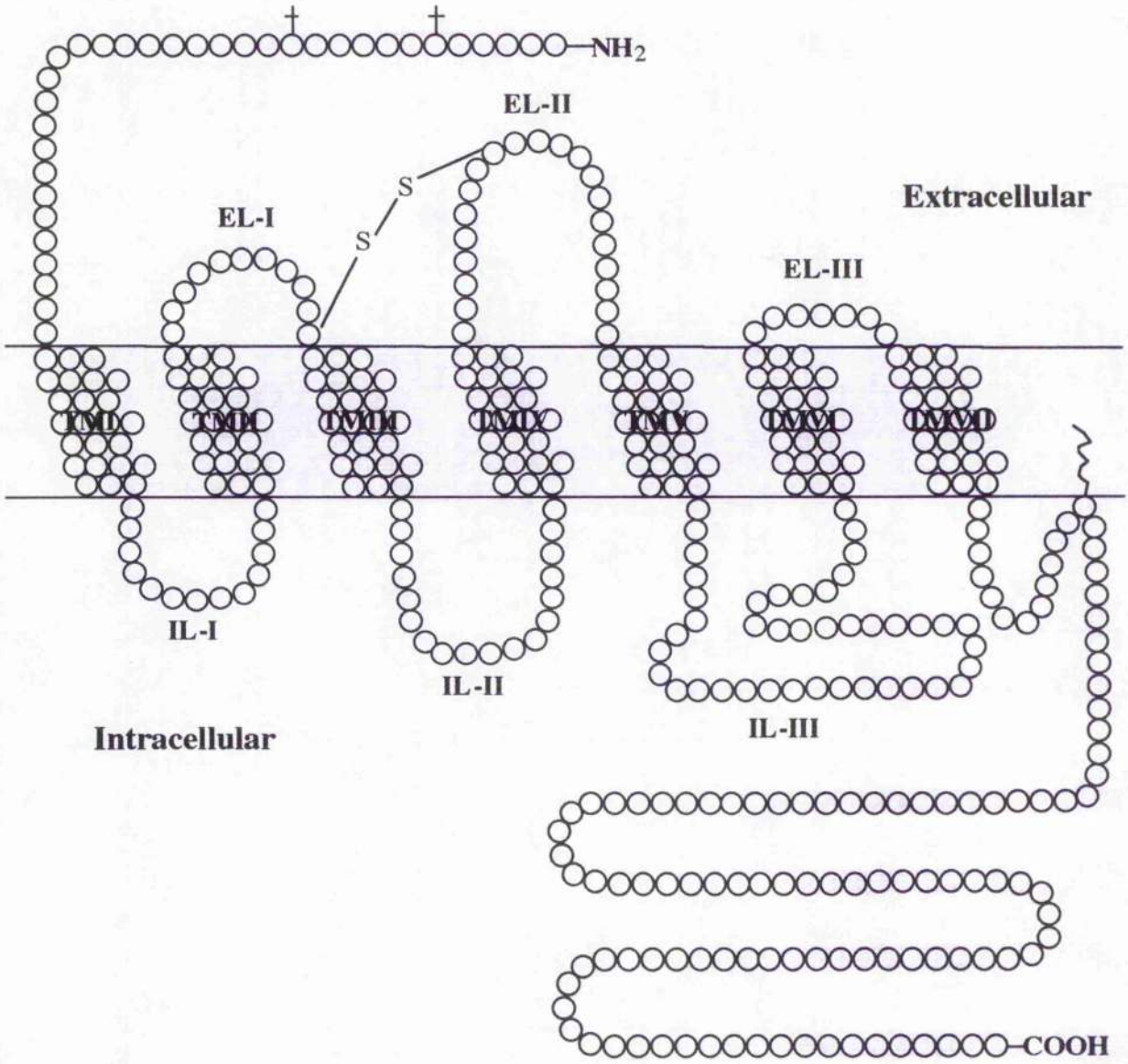
These GPCRs have in common a putative seven transmembrane spanning architecture as shown in **Figure 1.2.** When the amino acid sequences of the various cloned GPCRs were compared, the greatest homology was found in seven

Figure 1.2. Topographical representation of a typical G protein coupled receptor

G protein coupled receptors (GPCRs) have an amino terminus and three loops (**EL-I**, **EL-II** and **EL-III**) that have an extracellular orientation, seven hydrophobic transmembrane α helices (**TM I** through **TM VII**), and a carboxy terminus and three loops with an intracellular orientation (**IL-I**, **IL-II** and **IL-III**). The α helical structure may extend into the extracellular or intracellular spaces. Each circle represents a single amino acid.

Proposed glycosylation sites are represented by a cross based upon the consensus amino acid sequence, N-X-S/T, with N being Asn and X being any amino acid residue. The proposed disulphide bond is shown between cysteine residues in **EL-I** and **EL-II**. A hook (Cys) in the proximal portion of the cytoplasmic tail is the proposed site for palmitoylation in some systems, forming a fourth intracellular loop [O'Dowd *et al.*, 1989; Morrison *et al.*, 1991; Kennedy & Limbird, 1993; Kawate & Menon, 1994].

Figure 1.2


NH2
EL-II
S
S
EL-I
Extracellular
EL-III
TMD
TMD
TMD
TMD
TMD
TMD
TMD
IL-I
IL-II
IL-III
Intracellular
COOH

hydrophobic regions each consisting of between 20-25 amino acids. Each of these hydrophobic regions is thought to comprise a seven transmembrane helix. The regions of greatest diversity are at the extracellular N-terminus and at the intracellular C-terminus and cytoplasmic domains. Both the ligand bound and the G protein activated by the receptor determines the specific cellular response mediated by it. These two structural features of the GPCRs have been studied in great depth and will be discussed below.

1.2.1. Structural and functional features of GPCRs

It is now known that GPCRs have many structural adaptations to their extracellular domains to allow them to respond to diverse signals. The present understanding of the structure of GPCRs is based on the structure of the photoactivated proton pump of *Halobacterium halobium*. This protein, termed bacteriorhodopsin, is folded in such a way that the N-terminus is located on the extracellular surface of the cell and the C-terminus is intracellular. Between these two areas are seven hydrophobic regions which electron microscopy and high resolution electron diffraction indicate as being transmembrane spanning domains arranged in a bundle perpendicular to the plane of the lipid bilayer [Henderson & Unwin, 1975; Engleman *et al.*, 1980; Henderson *et al.*, 1990]. Protease digestion studies and detailed immunological mapping have also provided supporting evidence for the existence of seven transmembrane spanning domains linking an extracellular N-terminus and an intracellular C-terminus [Dohlman *et al.*, 1987].

1.2.1.1. Functions of the N-terminus

It has been shown that the N-terminus of GPCRs is extracellular and the length of amino acid sequence is variable from receptor to receptor. N-terminal Asn residues of this region are considered to be sites for N-linked glycosylation. Endoglycosidase treatment of many GPCRs decreases their molecular mass. For example, such treatment of the purified β_2 adrenergic receptor resulted in a reduction of molecular mass from 65 kDa to 49 kDa [Rands *et al.*, 1990]. Glycosylation is not thought to play a role in defining agonist binding activity since mutations of the two Asn residues in the β_2 adrenergic receptor had little effect on agonist/antagonist binding or on the coupling of this receptor to adenylyl cyclase [O'Dowd *et al.*, 1989]. However, this post-translational modification may function in determining receptor location within the cell [O'Dowd *et al.*, 1989].

1.2.1.2. Functions of the C-terminus

The presence of one or two cysteine residues in a similar position in most G protein coupled receptors suggests that this may be a common structural feature. Among the most well-studied and well-known GPCRs are the β-adrenergic receptor and rhodopsin. They have one and two cysteine residues, respectively, in their C-terminal sequence which have been shown to bind palmitate (C14:0) via a thioester linkage [O'Dowd *et al.*, 1989]. It is believed that the covalently bound palmitate becomes intercalated into the membrane bilayer, thereby creating a fourth cytoplasmic loop. Removal of the cysteine residue in the β_2 adrenergic receptor uncouples the receptor from its associated G protein, $G_s\alpha$ [O'Dowd *et al.*, 1989; Moffett *et al.*, 1993], but equivalent mutations caused no functional effect in rhodopsin [Karnik *et al.*, 1988] and in the α_{2A} adrenergic receptor [Kennedy *et al.*, 1993].

1.2.1.3. Functions of the extracellular disulphide bonds

Members of the GPCR family contain a cysteine residue in the putative first extracellular loop (EC-1) near the top of the third transmembrane domain (TM3) and another cysteine in EC-2. It has been assumed that this pair of Cys residues forms a disulphide bond in most GPCRs [Strader *et al.*, 1994]. This linkage has been proposed to be important to allow the receptor to attain a normal conformation during synthesis, for normal expression on the cell surface or to maintain normal function, in particular, with respect to binding and activation. Data consistent with the presence of a disulphide bond between these two conserved Cys residues have been reported for rhodopsin [Davidson *et al.*, 1994], β_2 adrenergic receptors [Dixon *et al.*, 1987; Dohlman *et al.*, 1990], muscarinic acetylcholine receptors [Savarese *et al.*, 1992], thyroid stimulating hormone receptors [Kosugi *et al.*, 1992; Gustavsson *et al.*, 1994] and thyrotropin releasing hormone receptors [Perlman *et al.*, 1995; Cook *et al.*, 1996].

Evidence in support of this disulphide linkage has been primarily of two types. Firstly, it has been shown that the binding affinity of a number of GPCRs is decreased under reducing conditions. This effect, however, may involve reductions of disulphides other than the proposed bond. Secondly, substitution of one or the other of these Cys residues has led to decreased binding affinity, expression, or activation.

1.2.1.4. Importance of transmembrane domains for ligand binding

Although GPCRs share the same basic structure, differences exist in the method of agonist binding. The seven transmembrane (7TM) domains are known to participate in the formation of the ligand binding pocket. The receptors for small molecules such as light using 11-cis-retinal as a chromophore ligand [Khorana, 1992], small hormone and neurotransmitter molecules such as catecholamines, acetylcholine, histamine and serotonin [Strader *et al.*, 1989; Dohlman *et al.*, 1992; Savarese & Fraser,

1992] bind these agonists in a cleft formed by the folding of the transmembrane helices.

The β_2 adrenergic receptor has been used for many years as a model system for studying the function and regulation of these receptors in transmembrane signalling. Large parts of the intracellular and extracellular domains can be deleted without appreciably altering the ligand binding properties of the receptor. Studies using alkylating agents [Dohlman *et al.*, 1988], photoaffinity labels [Wong *et al.*, 1988] and a fluorescent antagonist [Tota & Strader, 1990] have shown that the ligand binding pocket, formed from the seven transmembrane helices, is located approximately one third of the way down into the core of the protein.

The carboxyl group of the aspartate residue in TM-III (Asp 113) was postulated to act as a counter-ion for the catecholamine nitrogen in the presence of an agonist or an antagonist containing a protonated amine group, like isoprenaline and propranolol. Two conserved serine residues (204 and 207) in TM-V were found to form a hydrogen bond with the para- and meta-hydroxyl group of isoprenaline, respectively [Strader *et al.*, 1989]. This gives a model of isoprenaline binding to the β_2 adrenergic receptor through a carboxylamine salt bridge at aspartate 113 and hydrogen bonds at serines 204 and 207.

Receptors for peptide agonists appear to recognize these agonists on their exofacial surface [Fong *et al.*, 1992a & Fong *et al.*, 1992b] whilst glycoprotein hormones such as thyrotropin bind to the N-terminal tail [Moyle *et al.*, 1991; Nagayama *et al.*, 1991] of the receptor causing a conformational change which leads to the tail binding to the extracellular loops of the receptor. A unique ligand-receptor interaction is described by the thrombin receptor, in which the thrombin molecule acts as a protease which binds to the N-terminal tail of the thrombin receptor and cleaves the first 25 amino acids of the receptor to create a shortened amino terminus which then acts as a "tethered ligand" causing activation of the receptor [Vu *et al.*, 1991a; Vu *et al.*,

1991b]. The metabotropic glutamate receptors and parathyroid Ca^{2+} sensing receptor utilize yet another mechanism to detect their ligands [Tanabe *et al.*, 1992; Brown *et al.*, 1993]. These receptors contain extremely large N-terminal regions which have stretches resembling bacterial transporters for small molecules such as amino acids [O'Hara *et al.*, 1993]. Studies on these bacterial periplasmic binding proteins suggest that glutamate and Ca^{2+} binding to these domains induce a conformational change in the metabotropic glutamate receptors and Ca^{2+} sensing receptors [Oh *et al.*, 1993]. This conformational change may then form another tethered ligand which binds to the exofacial surface of the receptor.

1.2.1.5. Importance of cytoplasmic domains for receptor-G protein interactions

The first and second intracellular loops are relatively well conserved among G protein coupled receptors, but the third intracellular loop and the C-terminal tail are quite divergent, possibly reflecting a molecular basis for variable ligand and G protein binding specificity. Most work in this area has focused on the third intracellular loop and the C-terminal tail of the receptor.

Mutagenesis studies and the use of chimeras have been used to define the regions of GPCRs thought to comprise the site of G protein coupling. Mutagenesis studies of the β_2 adrenergic receptor show that a large portion of the third intracellular loop is important in activation of $G_s\alpha$ and then adenylyl cyclase. Deletion of residues 239-272 of the β_2 adrenergic receptor produced a receptor which could not activate adenylyl cyclase [Dixon *et al.*, 1987]. Deletion of residues 222-229 and 258-270 both greatly reduced the activation of adenylyl cyclase by the receptor [Strader *et al.*, 1987] and deletion of the residues at the C-terminal end of the third intracellular loop of the

human β_2 adrenergic receptor also resulted in stimulation of adenylyl cyclase to only a fraction of the level produced by the wild type receptor [Hausdorff *et al.*, 1990].

By constructing chimeric receptors where by a portion of TM V, all of TM VI and the intervening third intracellular loop of the human β_2 adrenergic receptor was introduced into the corresponding regions of the human α_2 adrenergic receptor, it was found that the chimera could stimulate adenylyl cyclase to approximately one third the level of that produced by the wild type β_2 adrenergic receptor [Kobilka *et al.*, 1988]. If only a portion of the third intracellular loop of the receptor was introduced, however, then the ability to activate adenylyl cyclase was lost. These results indicate that the third intracellular loop of the β_2 adrenergic receptor plays an important role in governing the interaction of the receptor and $G_s\alpha$ and thus stimulation of adenylyl cyclase, but also that this loop is insufficient on its own to fully activate the G protein.

A highly conserved Asp residue at the junction of TM III and the second intracellular loop may be one of these additional determinants for G protein binding. It is thought that the conformational changes which occur upon receptor activation may mask or unmask this residue, as it is located in or near the cytosol. Mutagenesis of this residue and corresponding residues in bovine rhodopsin, the rat m1 muscarinic receptor and the human α_2 adrenergic receptor either prevent receptor-G protein interaction or reduce affinity of receptor for G protein [Fraser *et al.*, 1988; Fraser *et al.*, 1989; Franke *et al.*, 1990; Wong *et al.*, 1991].

Chimeric experiments with α_1 and β_2 adrenergic receptors were performed to delineate the regions of G protein interaction. Such experiments showed that the cytoplasmic tail as well as the carboxyl-terminus of the third intracellular loop are important in G protein binding and in the agonist induced conformational change of the receptor which activates G protein [Cotecchia *et al.*, 1990].

In addition to the importance of the third intracellular loop, the C-terminal tail may be involved in governing the fidelity of interaction of receptors and G proteins.

The splice variants of the EP3 prostanoid receptor which have different C-terminal tails display a marked infidelity in G protein coupling and show remarkable promiscuity in coupling to G proteins [Namba *et al.*, 1993]. Of the 4 splice variants of the EP3 receptor isolated, $EP3_A$ coupled to $G_i\alpha$, $EP3_B$ coupled to $G_s\alpha$ and $EP3_C$ coupled $G_s\alpha$ and $G_o\alpha$, whilst $EP3_D$ coupled to $G_i\alpha$, $G_s\alpha$ and $G_q\alpha$ [Namba *et al.*, 1993; Negishi *et al.*, 1993].

1.2.2. Functional regulation of GPCRs

One of the salient features of GPCRs is that their functions are subject to dynamic regulation by a number of mechanisms such as phosphorylation and phosphorylation associated desensitization which will be discussed in the following sections.

1.2.2.1. Phosphorylation of GPCRs

One of the most intensively studied modification mechanisms involves phosphorylation by a family of G protein-coupled receptor kinases (GRKs). GRKs are a family of serine/threonine protein kinases that specifically recognize agonist-occupied, activated G protein-coupled receptor proteins as substrates. Phosphorylation of an activated GPCR leads to attenuation of receptor-G protein coupling since receptors phosphorylated by a GRK become targets for the binding of an arrestin protein. An arrestin bound to a GRK-phosphorylated receptor prevents coupling of that receptor to its cognate G protein, effectively reducing the level of functional receptor in the cell membrane. Since activation or agonist-occupancy of a receptor is a prerequisite for phosphorylation of a receptor by a GRK, this GRK-arrestin pathway results in the specific or homologous desensitization of receptor responses following agonist

exposure. A role for GRKs and arrestins has been implicated in only a few model GPCR systems. Nevertheless, these model systems provide a framework for understanding the role of receptor regulation in diverse signal transduction pathways [Lefkowitz, 1993; Inglese *et al.*, 1993].

Six distinct mammalian GRKs are known, which differ in tissue distribution and in regulatory properties [Premont *et al.*, 1995]. The intracellular localization of GRKs to membrane-bound receptor substrates is the most important known regulatory feature of these enzymes. Among them rhodopsin kinase (GRK1) requires a post-translationally added farnesyl isoprenoid to bind to light-activated rhodopsin (metarhodopsin II) [Hargrave *et al.*, 1993]. The β adrenergic receptor kinases (GRK2 and GRK3) were named as an activity that phosphorylated the agonist-occupied β_2 adrenergic receptor. They are targetted to the membrane by associating with heterotrimeric G protein βγ subunits released upon receptor activation of G proteins, and their substrate specificity is not limited to adrenergic receptors [Benovic *et al.*, 1986; Benovic *et al.*, 1989].

Within the past few years, a novel subfamily of the GRKs has emerged from molecular cloning studies. This subfamily includes the mammalian GRK4 [Ambrose *et al.*, 1993], GRK5 [Kunapuli & Benovic, 1993], and GRK6 [Benovic & Gomez, 1993; Haribabu & Snyderman, 1993], as well as the Drosophila GPRK2 sequence [Cassill *et al.*, 1991]. These kinases utilize distinct mechanisms for membrane localization, which are just beginning to be defined. GRK5 is the most extensively charaterized of these new kinases and has been shown to phosphorylate rhodopsin, α_2- and β_2 adrenergic receptors, and m2-muscarinic receptors [Premont *et al.*, 1994; Pei *et al.*, 1994; Kunapuli *et al.*, 1994]. All GRKs appear to play the same general cellular role of desensitizing activated GPCRs, but utilize distinctly individual means to the same end. More recently, Fredericks *et al.* [1996] have identified the sites of GRK2- and GRK5-mediated β_2 adrenergic receptor phosphorylation which reside exclusively

in residues of Ser and Thr in a 40-amino acid peptide located at the extreme caboxyl terminus of the receptor.

1.2.2.2. Mechanisms of desensitisation of GPCRs

Many GPCR systems are known to undergo some form of desensitization or adaptation to repeated or prolonged stimulation. The activity of an effector enzyme produced by a particular dose of a hormone is dependent on the previous activity of the system. Desensitization refers to the rapid attenuation of responsiveness to a drug or hormone in the continued presence of the agent. Many distinct mechanisms play a role in the overall desensitization of G protein coupled systems, including protein trafficking of receptors and G proteins among cellular membranes, second messenger-regulated protein phosphorylation, and enhanced degradation or removal of intracellular messengers.

Several distinct phosphorylation patterns have been correlated with different aspects of receptor desensitization. Among GPCRs, desensitization has been studied in the β_2-adrenergic receptor [Hausdorff *et al.*, 1990], cholinergic receptors [Menniti, *et al.*, 1991; Nakahata *et al.*, 1987; Eva *et al.*, 1990], the α_1 adrenergic receptor [Leeb-Lundberg *et al.*, 1987; Lattion *et al.*, 1994], the serotonin $5HT_2$ and $5HT_{1A}$ receptors [Pauwels et al., 1990; Raymond, 1991], the receptors for thrombin [Paris *et al.*, 1988], platelet activating factor [Morrison & Shikla, 1987], histamine [Dickenson *et al.*, 1993], substance P [Menniti *et al.*, 1991; Sugiya *et al.*, 1987], bradykinin [Wolsing & Rosenbaum, 1993], and bombesin [Walshi *et al.*, 1993]. However, despite the large amount of information about the adenylyl cyclase-linked receptors, much less is known about the molecular mechanisms involved in desensitization of the receptors coupled to the phospholipase C signalling pathway.

Several mechanisms have been delineated which are associated with the diminished response to agonist. Upon agonist occupation, the first event appears to be phosphorylation of the receptor at specific sites by the βARK and the cAMP-dependent protein kinase (PKA), which leads to rapid attenuation of receptor-G protein interaction. The sites required for phosphorylation by βARK have been localized to serine and threonine residues in the carboxyl-terminal tail of the β_2 adrenergic receptor [Dohlman *et al.*, 1987; Bouvier *et al.*, 1988]. More recently, Fredericks *et al.* [1996] have identified the sites of GRK2- and GRK5-mediated β_2 adrenergic receptor phosphorylation which reside exclusively in a 40-amino acid peptide located at the extreme caboxyl terminus of the receptor.

Phosphorylation of β_2 adrenergic receptor by PKA occurs at one or both of the consensus sequences located in the third intracellular loop or the cytoplasmic tail [Benovic *et al.*, 1985; Hausdorff *et al.*, 1989]. β adrenergic receptors phosphorylated by either process couple poorly to G_s. Removal of the above-mentioned site of the β_2 adrenergic receptor by mutagenesis significantly alters the patterns of agonist-induced desensitization [Bouvier *et al.*, 1988; Hausdorff *et al.*,1989]. Similarly, β_2 adrenergic receptor desensitization can be selectively altered by the use of specific inhibitors of βARK (heparin) or PKA (PKI) [Lohse *et al.*, 1990]].

A second component of agonist-induced desensitisation of the receptor is sequestration (also referred to as an internalisation or compartmentalisation) of the receptor away from the cell surface to an intracellular location where it is presumably unavailable for interaction with G protein. β_2 adrenergic receptors can eventually be recycled to the cell surface without the need for new protein synthesis. Finally, after a more prolonged (h) exposure to agonist, the total receptor numbers may become dramatically decreased, a process termed receptor down-regulation. This refers to the mechanisms that remove receptors from the cell membrane, leading to their internalisation and ultimately their degradation. Replacement of such down-regulated

receptors generally requires new protein synthesis. The exact molecular determinants of this process are not well defined, it does appear that G_s coupling is an important component in the down-regulation process of β_2 adrenergic receptor [Campbell *et al.*, 1991].

1.2.3. Mechanisms of multifunctional signalling by GPCRs

A number of GPCRs have recently been shown to regulate multiple effector pathways both when expressed endogenously and in heterologous systems. This signal bifurcation can be explained by three different models [Milligan, 1993]. Firstly, each of multiple receptor subtypes is able to interact with a separate G protein and each G protein is able to regulate a separate effector species. An example of this mechanism is the splice variants of the EP3 prostanoid receptor as described in **section 1.2.1.5.** Isoforms of this receptor are predicted to differ in the C-terminus and while they show identical ligand binding properties, they appear to interact with distinct members of the G_i family [Sugimoto *et al.*, 1993].

Secondly, a single receptor activates multiple G proteins, each of which is able to regulate a separate effector. For example, in NG108-15 cells, both a δ-opioid receptor and an α_{2B} adrenergic receptor are able to inhibit adenylyl cyclase and voltage-dependent Ca^{2+} channels in a pertussis toxin-sensitive manner. Opioid-mediated inhibition of adenylyl cyclase is abolished by antibodies that selectively interact with $G_i2\alpha$ and α_2 adrenergic receptor inhibition of Ca^{2+} currents is attenuated by antibodies which identify splice variants of $G_o\alpha$ [McKenzie & Milligan, 1990; McFadzean *et al.*, 1989]. Thirdly, a single receptor interacts with a single G protein and bifurcation of signal is subsequently produced by the regulation of different effectors by the G protein α and $\beta\gamma$ subunits. As certain phospholipases of the C and A_2 classes are activated by $\beta\gamma$ subunits, it is possible that a receptor expressed at sufficiently high levels could

activate sufficient G protein to allow the α subunit to regulate one effector while the associated βγ subunits could regulate another [Camps *et al.*, 1992; Katz *et al.*, 1992].

1.2.4. Constitutively active mutant (CAM) receptors

The unusual property of activating cellular signalling pathways in the absence of agonist have been reported in several GPCRs including the thyrotropin receptor [Parma *et al.*, 1993], the lutcinizing hormone receptor [Shenker *et al.*, 1993], the dopamine D_1 & D_5 receptor [Tiberi & Caron, 1994], $5HT_{1D\alpha}$,$5HT_{1D\beta}$ and $5HT_{2C}$ [Barker *et al.*, 1994; Thomas *et al.*, 1994] and *in vitro* mutants of various receptors. They are termed, consititutively active mutant (CAM) receptors. Recent studies of the properties of several mutationally activated receptors has led to a more detailed understanding of mechanisms by which GPCRs function.

1.2.4.1. The revised ternary complex model

Classic theories of receptor activation [Del Castillo & Katz, 1957] hold that agonist binding by receptors leads to a conformation that is productive for effector activation. Antagonists, on the other hand, are considered to be able to bind effectively with the receptor without requiring an induced conformational change and, thus, to inhibit rather than stimulate receptor signalling.

The ternary complex model [De Lean *et al.*, 1980], which is the most widely accepted model describing the activation of a G protein coupled receptor, relates the active form of the receptor to a ternary complex that involves the interaction of the hormone (agonist), the receptor and the G protein. However, based on recent analyses of ligand binding and activation of many GPCRs, this ternary complex model of receptor activation has been revised [Samama *et al.*, 1993]. Central to this revised

model is the finding that mutated receptors can exist in a constitutively active state, that is, a state that can initiate productive signalling in the absence of agonist.

Analysis of in vitro model CAM and wild-type receptor systems indicates that receptors exist in an equilibrium of inactive and active states or conformations. Agonists [H] or CAM mutations shift the receptor toward the activated conformation [R*]; neutral antagonists bind without shifting the equilibrium between inactive [R] and active [R*] conformations, and inverse agonists or negative antagonists shift the equilibrium toward the inactive conformation [R] as shown in **Figure 1.3.** Accordingly, it has been proposed that similar to channels that spontaneously isomerize between a closed (or resting) and open (or active) conformation, receptors resonate between a basal state [R] and an active state [R*], and that only the active [R*] state can productively interact with G protein to allow effector activation by the [R*G] complex [Bond *et al.*, 1995]. Furthermore, agonists bind with higher affinity to the active state and trap the receptor in the [R*] conformation. Antagonists, on the other hand, can bind with similar affinity to both [R] and [R*].

1.2.4.2. Constitutive activation of receptors

It was discovered that interchange of a short homologous stretch of amino acids between the C-terminal regions of the third cytoplasmic loops of the G_q-PLC-coupled α_{1B} adrenoceptor and the G_s-adenylyl cyclase-coupled β_2 adrenergic receptors resulted, in both cases, in a pattern of constitutive activity [Cotecchia *et al.*, 1990]. The basal agonist-independent signalling activity of the mutant receptors was greatly enhanced to levels comparable to that of a fully agonist-stimulated wild type receptor in the case of the β_2 CAM [Samama *et al.*, 1993]. Comparable mutations in the α_{2A} adrenoceptor also led to constitutive activation [Ren *et al.*, 1993]. More recently the constitutive activity of GPCRs by mutation has been shown not limited to the third

Figure 1.3. The ternary complex model.

This model was originally developed to explain several features of ligand binding to the β_2 adrenergic receptor. First, agonists but not antagonists were able to distinguish between a high- and a low- affinity state of the receptor. Second,the proportion and relative state of affinity of ligands for these two states of the receptor was observed to vary with the intrinsic activity of the agonist. Third, guanine nucleotides appeared to convert receptor from a high to low affinity state. [H] represents hormone; R for receptor; G for G protein.

a) shows the classical form of the model : M, the affinity of R for G; α, the molecular efficacy of the ligand; K, the receptor affinity of the ligand.

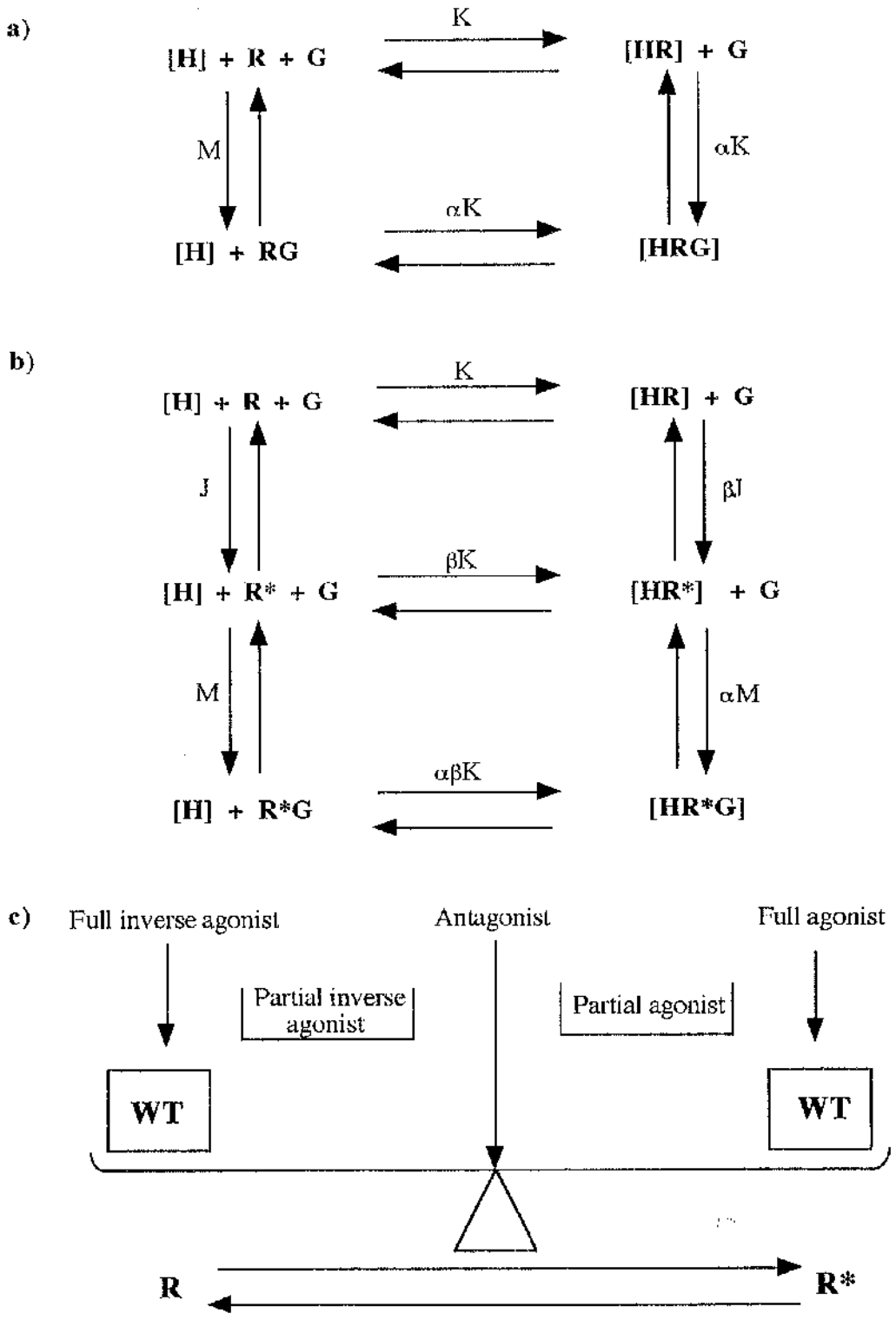
$$K = \frac{[HR]}{[H]\,[R]} \qquad M = \frac{[RG]}{[R][G]} \qquad \alpha = \frac{[HRG]\,[R]}{[HR]\,[RG]}$$

b) shows the extended version of the model or allosteric ternary complex model. This model introduces an explicit isomerization step regulating the formation of the state of the receptor from R to R*, which is capable of binding to the G protein. J represents an equilibrium constant in receptor isomerization.

$$J = \frac{[R^*]}{[R]} \quad K = \frac{[HR]}{[H]\,[R]} \quad M = \frac{[R^*G]}{[R^*][G]} \quad \alpha = \frac{[HR^*G][R^*]}{[HR^*][R^*G]} \quad \beta = \frac{[HR^*][R]}{[HR]\,[R^*]}$$

c) shows how the position of the equilibrium between an inactive state R and an active state R* varies with individual receptors and is altered by the presence of receptor ligands. Agonists function by stabilising R* while inverse agonists preferentially stabilise R. A continuum of ligands between full agonists and full inverse agonists is expected to exist, with antagonists having no effect on the position of the equilibrium at all.

Figure 1.3


a)
[H] + R + G
K
[HR] + G
M
αK
αK
[H] + RG
[HRG]
b)
[H] + R + G
K
[HR] + G
J
βJ
βK
[H] + R* + G
[HR*] + G
M
αM
αβK
[H] + R*G
[HR*G]
c)
Full inverse agonist
Antagonist
Full agonist
Partial inverse agonist
Partial agonist
WT
WT
R
R*

cytoplasmic loop but can be observed in mutations in the C-terminus as well as in the ligand binding domain (TM-III) [Matus-Leibovitch *et al.* 1995; Perez *et al.*, 1996].

Analysis of CAM β_2- and α_2 adrenergic receptors indicates that the CAM receptors are also constitutively desensitised and phosphorylated since the CAM receptors are constitutive (agonist independent) substrates for GRK2 in vitro [Ren *et al.*, 1993; Pei *et al.*, 1994]. Furthermore constitutive GRK2 phosphorylation of CAM β_2 adrenergic receptor can be partially inhibited by addition of an inverse agonist, which reduces the amount of receptor in the activated, GRK substrate state [Samama *et al.*, 1994].

1.2.4.3. Inverse agonism

In constitutively active systems containing mutant receptors [Samama *et al.*, 1994], heterologous expression systems with receptor over-expression [Chidiac *et al.*, 1994; Tian *et al.*, 1994], over-expressed receptor systems from transgenic animals [Milano *et al.*, 1994], and in disease states associated with receptor mutations [Coughlin, 1994; Raymond, 1994] antagonists that were previously thought to lack intrinsic efficacy have been shown to actually depress constitutive receptor activity *in vitro*.

Drugs acting at GPCRs display a spectrum of efficacies from what has routinely been defined as zero for full antagonists to one for full agonists [Stephenson, 1956]. Antagonists inhibit the binding of agonists to receptors, and the physiological effects of the former usually are attributed to their ability to prevent activation of receptors by endogenous hormones and neurotransmitters. In general, it is thought that antagonists do not modulate the activity of unliganded receptors, but a growing body of evidence contradicts this notion. Certain antagonists have been reported to produce

effects opposite to those of the corresponding agonists at GPCRs [Schutz & Freissmuth, 1992].

The direct, agonist-independent modulation of receptor activity by these antagonists has been referred to variously as inverse agonism, negative or reverse intrinsic activity, and negative antagonism [Schutz & Freissmuth, 1992; Ehlert, 1986; Costa & Herz, 1989; Costa *et al.*, 1990]. This property gives 'negative efficacy' due to its ability to actively destabilise spontaneous complexes of receptor and G proteins

Drugs displaying inverse agonism were postulated to have a higher affinity for [R] compared with [R*], resulting in a decrease in the proportion of [R*] and a reduction in the basal regulation of the effector system as shown in **Figure 1.3c** [Milligan *et al.*, 1995]. Therefore, the characteristics of inverse agonists tends to be more apparent in systems that express relatively high receptor levels with associated higher basal effector activity.

Much of the detailed early analysis of inverse agonism at GPCRs was derived from the ability of the δ-opioid receptor ligand ICI174864 to inhibit basal high affinity GTPase acivity in membranes of NG108-15 cells [Costa & Herz, 1989; Costa *et al.*, 1990]. Recent studies have examined this phenomenon directly at a range of receptors, including β_2 adrenoceptor [Adie & Milligan, 1994; Chidiac *et al.*, 1994; Samama *et al.*, 1994], α_2 adrenoceptor [Tian *et al.*, 1994], $5HT_{2C}$ [Barker *et al.*, 1994], $5HT_{1D\alpha}$ and $5HT_{1D\beta}$ [Thomas *et al.*, 1994], D_{1B} dopamine receptor [Tiberi *et al.*, 1994], and the δ-opioid receptor [Mullaney *et al.*, 1996].

1.3. Guanine nucleotide binding proteins (G proteins)

1.3.1. Structure, function and mechanism of action

In all eukaryotic organisms, a family of heterotrimeric GTP-binding and hydrolysing proteins (G proteins) plays an essential signal transducing role in linking many cell surface receptors to effector proteins at the plasma membrane. Structurally, the heterotrimeric G proteins are composed of distinct α, β and γ subunits of molecular masses 39-52, 35-36 and 7-8 kDa, respectively. G protein coupled signalling cascades include the regulation of various intracellular second messenger generating effector systems such as adenylyl cyclase, phosphoinositidase C, phospholipase A_2, phospholipase D and ion channels.

The α subunit has a single high affinity binding site for GDP or GTP and possesses intrinsic GTPase activity which results in hydrolysis of the terminal phosphate of bound GTP to yield bound GDP and free inorganic phosphate. The GDP-bound form of the α subunit binds tightly to $\beta\gamma$ forming an inactive complex, whereas the GTP-bound form dissociates from $\beta\gamma$ and can then interact with an effector protein causing an intracellular response. The β- and γ subunits exist as a tightly associated complex which can itself activate effector molecules.

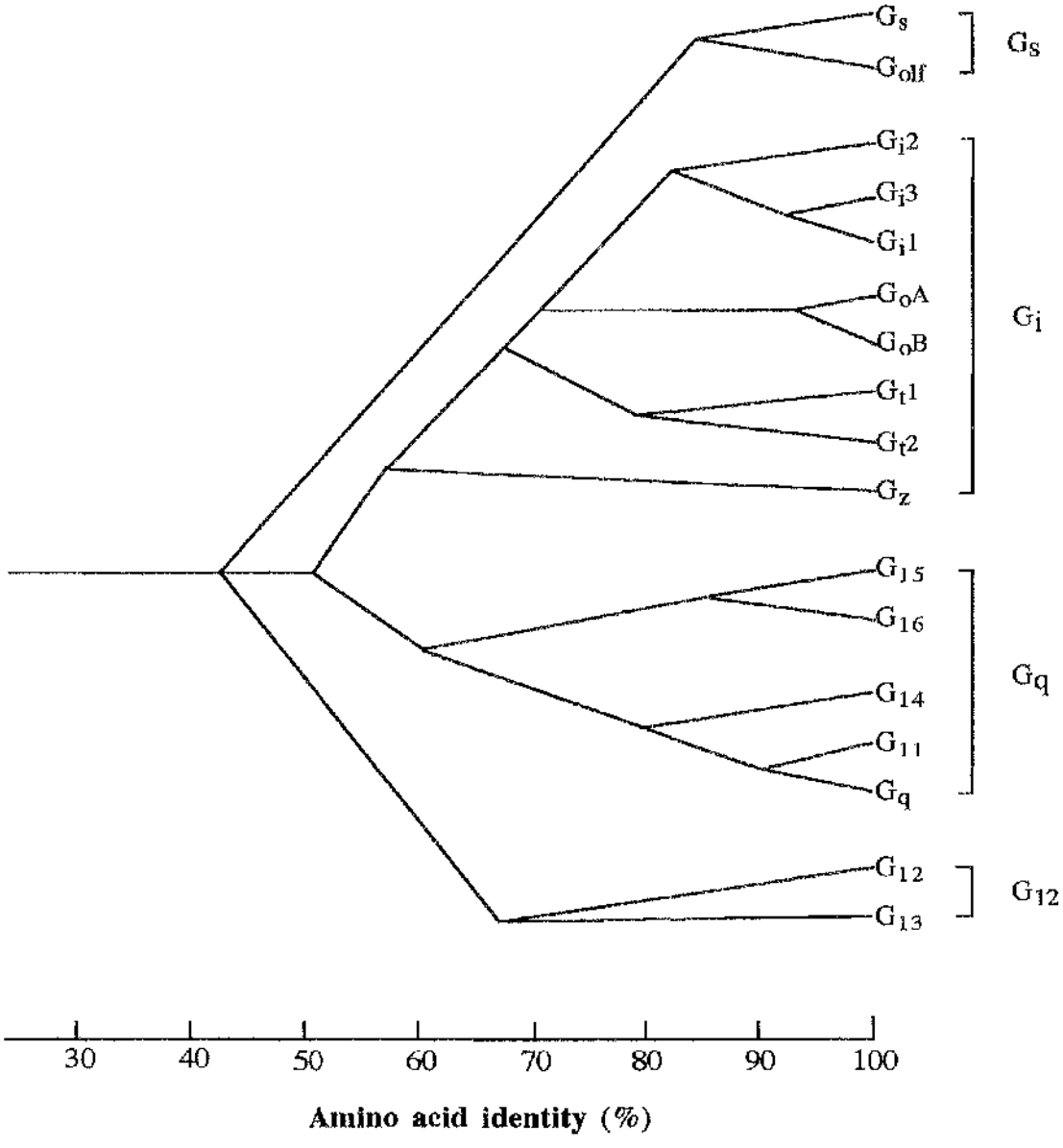
1.3.2. Gα subunits

Molecular cloning techniques have allowed identification of 21 distinct mammalian G protein α subunits which are derived from 16 genes. These can be classified into 4 major subclasses based on structural homology at the amino acid sequence level. These are termed the G_s, G_i, G_q and G_{12} families as shown in **Figure 1.4** [Simon *et al.*, 1991]. All α subunits share sequence homology to a greater or lesser degree which reflects the similar function of these proteins.

Although the length of the polypeptide sequence does not show the homology between the α subunits, regions of high and low homology exist between all α subunits. The regions of highest homology occur in four non-continuous regions that

Figure 1.4. G protein α subunit amino acid identity

The relationship among mammalian Gα subunits is displayed. The α subunits are grouped by amino acid sequence identity and define four distinct classes of Gα subunits. The splice variants of $G_s\alpha$ are not shown [Simon *et al.*, 1991].



form the GTP-binding and hydrolysis domains. Regions of greatest diversity represent areas of functional differences in coupling to βγ subunits, receptors or effectors thereby conferring specificity upon the individual α subunits [Simon *et al.*, 1991].

1.3.2.1. G_s family

$G_s\alpha$ subunit, named for its stimulatory effect on adenylyl cyclase, was first identified by evidence suggesting that a hormone sensitive GTPase was required for the stimulation of adenylyl cyclase [Cassel & Selinger, 1977 & 1978; Ross & Gilman, 1977]. Further studies demonstrated that disruption of hormone stimulated adenylyl cyclase in the cyc^- mutant of S49 lymphoma cells was due to the absence of a GTP binding protein. Ross *et al.* [1978] found that reconstitution *in vitro* of cyc^- cells with adenylyl cyclase-free plasma membrane detergent extracts resulted in restoration of the adenylyl cyclase activity. The latter findings permitted the purification of G_s by Northup *et al.* [1980]. This protein was shown to be a heterotrimer of α, β and γ subunits which could be a mixture of two oligomers made up of two distinct α subunits and indistinguishable βγ dimers. The relative concentrations of these α subunits of molecular masses, 45k and 52kDa, varies between cell types and any functional differences between the two have yet to be identified.

Alternative splicing of a single $G_s\alpha$ gene results in the production of four polypeptides comprising 380, 381, 394 and 395 amino acids [Bray *et al.*, 1986]. The gene consists of 13 exons and 12 introns covering about 20kb [Kozasa *et al.*, 1988]. $G_s\alpha$-1 and $G_s\alpha$-2 are identical except for a single 45 nucleotide stretch absent in $G_s\alpha$-3 and $G_s\alpha$-4. $G_s\alpha$-2 and $G_s\alpha$-4 have an additional 3 nucleotides located 5'end of exon 4.

This is due to the alternative splicing of exon 3 of the gene such that $G_s\alpha$-1 and $G_s\alpha$-2 have exon 3 and $G_s\alpha$-3 and $G_s\alpha$-4 do not [Bray *et al.*, 1986; Kozasa *et al.*, 1988; Kaziro *et al.*, 1990]. The production of an additional serine

residue in $G_s\alpha$-2 and -4 by this differential splicing may represent a potential phosphorylation site on $G_s\alpha$ for protein kinase C and indeed this $G_s\alpha$ has been shown to be the target of phosphorylation by PKC in vitro [Pyne *et al.*, 1992].

$G_s\alpha$ is ubiquitously expressed in all cells although the relative levels of expression of the splice variants vary from each cell and tissue type. The exact purpose of this differential expression is not yet clear at present as there has been no evidence of any functional difference between the splice variants.

1.3.2.2. G_i family

The pertussis toxin-sensitive 40kDa $G\alpha$ subunit was first purified from bovine brain, and has been shown to couple to the inhibition of adenylyl cyclase. $G_i\alpha$ has subsequently proved to be a mixture of three α subunits termed $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ [Jones & Reed, 1987].

$G_i1\alpha$: $G_i1\alpha$ is one of the largest members of the G_i family with a molecular mass of 41 kDa and is a substrate for ADP-ribosylation by a bacterial secretory endotoxin produced by Bordetella pertussis (PTX). This covalent modification occurs via the transfer of an ADP-ribose group, donated by NAD^+, to a cysteine residue 4 amino acids from the C-terminus of the α subunit. Such transfer occurs most efficiently when the G protein is in its holomeric form complexed to its receptor [Gilman, 1987; Milligan, 1988]. PTX-mediated ADP ribosylation results in a stabilization of the trimeric form of the protein and causes the release of the inactive G protein from the receptor thus preventing transduction of the hormone signal to the appropriate effector system as described in **section 1.3.5.1.** The tissue distribution is limited to in neuronal tissues and certain other tissues, which indicate a likely role in regulating ion channels as opposed to adenylyl cyclase.

$G_i2\alpha$: $G_i2\alpha$ is the smallest of the three G_i proteins, is a substrate for pertussis toxin catalysed ADP-ribosylation and shares around 88% amino acid identity with $G_i1\alpha$. Although other members of the $G_i\alpha$ family have been shown to cause inhibition of adenylyl cyclase [Taussig & Gilman, 1995], there is greater evidence suggesting that $G_i2\alpha$ is the main physiological regulator of this process. For example, it was demonstrated that $G_i2\alpha$ was responsible for the inhibition of forskolin-stimulated adenylyl cyclase activity in NG108-15 neuroblastoma x glioma hybrid cells by using $G_i2\alpha$ specific antisera to selectively uncouple $G_i2\alpha$ from receptor regulation of adenylyl cyclase [McKenzie & Milligan, 1990].

Another system which $G_i2\alpha$ may interact with is the MAP kinase cascade [van Corven *et al.*, 1993; Alblas *et al.*, 1993]. Hormonal stimulation of $G_i\alpha$-coupled receptors causes activation of a series of protein kinases which ultimately leads to the phosphorylation and activation of MAP kinase which in turn catalyses the phosphorylation of a variety of cellular proteins to cause entry into the cell cycle and stimulation of transcription. It is probable, however, that this stimulation of MAP kinase is also mediated by $\beta\gamma$ [Crespo *et al.*, 1994].

$G_i3\alpha$: $G_i3\alpha$ is the third of the G_i family and is thought to stimulate various ion channels in the plasma membrane. It has been shown that a G_i protein purified from human erythrocyte membranes was capable of stimulating receptor activated K^+ channels in atrial and pituitary membrane patches [Yatani *et al.*, 1987]. This protein was subsequently found to be $G_i3\alpha$. Furthermore, an amiloride sensitive Na^+ channel was identified in renal epithelial cell line A6 which could be activated via $G_i3\alpha$ [Cantiello *et al.*, 1989], apparently via stimulation of phospholipase A_2 [Cantiello *et al.*, 1990]. Ausiello *et al.* [1992] also suggested that the stimulation of this channel proceeds via phospholipase A_2 and generation of second messengers. It was also

reported that $G_i3\alpha$ stimulated a large conductance renal apical Cl^- channel [Schwiebert *et al.*, 1990].

The involvement of $G_i3\alpha$ in trafficking of proteins from the Golgi to the plasma membrane has also been implicated since secretion of the heparin sulphate proteoglycan from LLC-PK1 epithelial cells was inhibited upon overexpression of $G_i3\alpha$ and location of this G protein to the Golgi complex [Stow *et al.*, 1991]. Pertussis toxin treatment reversed this inhibition of secretion of heparin sulphate proteoglycan indicating a role for $G_i3\alpha$ in Golgi trafficking of a contitutively secreted protein.

$G_o\alpha$: Named for G 'other', was first identified as a 39kDa pertussis toxin substrate purified from bovine brain [Neer *et al.*, 1984; Sternweis & Robishaw, 1984; Milligan & Klee, 1985] and is distributed mainly in neuronal and electrically excitable cells. It was demonstrated that this α subunit could inhibit the opening of a voltage sensitive N-type Ca^{2+} channel as well as a K^+ channel [Heschler *et al.*, 1990; van Dongen *et al.*, 1988]. Three $G_o\alpha$ proteins has been identified [Hsu *et al.*, 1990; Strathmann *et al.*, 1990; Nurnberg *et al.*, 1994]. These $G_o\alpha$ splice variants display differential tissue distribution; $G_o1\alpha$ being primarily restricted to neuronal cells whilst $G_o2\alpha$ is found in peripheral tissues such as pituitary, lung and testis. Unlike the splice variants of $G_s\alpha$, those of $G_o\alpha$ have been shown to possess distinct functions [Kleuss *et al.*, 1991; Man-Song-Hing *et al.*, 1992; Nurnberg *et al.*, 1994].

$G_t\alpha$: Vertebrate visual transduction is known to be mediated by $G_t\alpha$. Transducin was first identified as the transducing entity between activated rhodopsin and the phosphodiesterase responsible for lowering levels of cGMP causing influx of Na^+ from the outside of the cell, depolarization and thus initiation of nerve impulses to the brain. Two $G_t\alpha$ subunits, named $G_t1\alpha$ and $G_t2\alpha$, were cloned [Lochrie *et al.*, 1985; Tanabe *et al.*, 1985; Yatsunami & Khorana, 1985; Medinski *et al.*, 1985],

former being distributed in retinal rod membranes as well as in non-retinal taste tissue, whilst the latter was found only in retinal cone membranes [Grunwold *et al.*, 1986; Ruiz-Avila *et al.*, 1995; Lerea *et al.*, 1986]. The two types share 80% amino acid sequence identity.

Gustducin α: Gustducin α , found on the inside face of the membrane of taste receptor cells, is the most recent α subunit to be identified. This subunit was obtained from a taste tissue cDNA library and belongs to the G_i family since it contains a consensus amino acid sequence for pertussis toxin catalysed ADP-ribosylation [McLaughlin *et al.*, 1992]. Rat gustducin shows 79 % , 81 % and 90 % identity with rat $G_t1\alpha$, bovine $G_t1\alpha$ and $G_t2\alpha$, respectively [McLaughlin *et al.*, 1992; Ruiz-Avila *et al.*, 1995]. Hoon *et al.* [1995] have suggested that baculovirus expressed gustducin α could interact with bovine rhodopsin and both bovine brain and retinal βγ and stimulate bovine retinal cGMP phosphodiesterase activity.

Taste transduction is initiated when taste stimuli interact with receptor sites or channels, causing membrane depolarisation and release of transmitter from receptor cells onto gustatory afferent neurons. Ionic stimuli such as salts and acids interact directly with apically located ion channels to depolarise taste-receptor cells, but sugars, amino acids and most bitter-tasting compounds bind to specific receptors on the outside of the cell membrane, most of which are coupled to gustducin and relay the signal to the second-messenger systems via phosphoinositidase C, phosphodiesterase or adenylyl cyclase [Wong *et al.*, 1996].

$G_z\alpha$: $G_z\alpha$, cloned from human brain and retina, is unique as it is the only G_i family member that lacks the cysteine residue which serves as the acceptor for PTX-mediated ADP ribosylation. $G_z\alpha$ shares only 62 % amino acid identity with other members of the family [Matsuoka *et al.*, 1988; Fong *et al.*, 1988; Casey *et al.*, 1990].

It has a limited tissue distribution, being mainly restricted to neurones, and has a very slow guanine nucleotide release rate and a slow intrinsic GTPase activity [Hinton *et al.*, 1990]. As yet, however, $G_z\alpha$ has no clear function in signal transdution, although it may inhibit type I and type V adenylyl cyclases and allow pertussis toxin-insensitive inhibition of adenylyl cyclase [Taussig & Gilman, 1995].

1.3.2.3. G_q family

cDNA cloning techniques have allowed identification of a family of G proteins involved in coupling receptors to the phospholipid hydrolyzing enzyme, phosphoinositidase C. This family comprises 5 isotypes namely, G_q, G_{11}, G_{14}, G_{15}, and G_{16}. One member of this subclass, termed $G_q\alpha$, was predicted to encode a protein of 359 amino acids, equivalent to approximately 42kDa under SDS-PAGE [Strathmann & Simon, 1990; Exton *et al.*, 1990; Pang & Sternweis, 1990]. This α subunit is not a substrate for pertussis toxin catalyzed ADP-ribosylation since it lacks the necessary C-terminal cysteine residue and is ubiquitously distributed.

Another isotype of the G_q subfamily is $G_{11}\alpha$, contained 359 amino acids, which shares 88% amino acid sequence identity with $G_q\alpha$ [Strathmann & Simon, 1990; Simon *et al.*, 1991]. $G_{11}\alpha$ is also pertussis toxin-insensitive and shows no functional difference to $G_q\alpha$. Purified $G_q\alpha/G_{11}\alpha$ from brain was shown to activate phosphoinositidase Cβ1 in a reconstitution assay suggesting that the two α subunits may have identical abilities to activate phosphoinositidase Cβ1 [Blank *et al.*, 1991]. Although they have similar function in the activation of phosphoinositidase C, the tissue distribution of the two proteins shows some variation, i.e., $G_{11}\alpha$ is not present in human haematopoietic cells and the relative amounts of the two subunits are different in both brain regions and peripheral tissues [Milligan *et al.*, 1993].

The differences in primary structure between $G_q\alpha$ and $G_{11}\alpha$ are mostly clustered near the N-terminus of the proteins in regions concerned with $\beta\gamma$ binding and the relative rate of GDP-GTP exchange and hydrolysis, perhaps indicating that $G_q\alpha$ and $G_{11}\alpha$ couple differently to different combinations of β and γ [Simon *et al.*, 1991].

$G_{14}\alpha$ shares approximately 80% identity with $G_q\alpha$ and $G_{11}\alpha$ at the amino acid level. According to analysis of mRNA distribution, this protein was found primarily in stromal and epithelial cells [Wilkie *et al.*, 1991] as well as in liver, lung and kidney [Nakamura *et al.*, 1991]. $G_{15}\alpha$ shares 57% amino acid identity with $G_q\alpha$, $G_{11}\alpha$ and $G_{14}\alpha$ and is found in murine B cells and myeloid cells [Simon *et al.*, 1991; Amatruda *et al.*, 1991]. $G_{16}\alpha$ shares approximately 83% amino acid identity to $G_{15}\alpha$ and is distributed mainly in haematopoietic cells such as T cells and myeloid cells [Amatruda *et al.*, 1991]. It has been shown that $G_{15}\alpha$ and/or $G_{16}\alpha$ was involved in the signalling processes of chemokines and chemoattractants such as interleukin-8 and the complement-derived C5a anaphylatoxin [Wu *et al.*, 1993; Amatruda *et al.*, 1993; Buhl *et al.*, 1993]. More recently, $G_{15}\alpha$ and $G_{16}\alpha$ have been suggested to be a universal G protein adapter since they interact with a variety of GPCRs which are known to couple distinct $G\alpha$ subunits [Milligan *et al.*, 1996].

The structure of these proteins shows differences from other α subunits with two inserts near the C-terminus and a divergent N-terminus. The first insert (a.a.317-323) is similar to the corresponding region in $G_s\alpha$ while the second insert (a.a.331-337) contains several shared residues which are not found in other α subunits [Amatruda *et al.*, 1991]. An additional aspect is a proline residue resides at position 50, a region involved in GTP binding and hydrolysis, where an alanine resides in $G_s\alpha$ and $G_i\alpha$ or a threonine resides in $G_q\alpha$. This might indicate an impaired GTP binding and hydrolysing capacity [Pang & Sternweis, 1990; Casey *et al.*, 1990].

1.3.2.4. G_{12} family

Two isotypes, $G_{12}\alpha$ and $G_{13}\alpha$, have been cloned and are expressed ubiquitously. They share relatively little primary sequence homology with other α subunits, approximately 45%, and have less than 70% identity with each other [Strathmann & Simon, 1991].

Possible roles for these α subunits have recently been reported in the activation of arachidonic acid metabolism [Xu *et al.*, 1993] as well as in the regulation of Na^+/H^+ exchange [Voyno-Yasenetskaya *et al.*, 1994]. $G_{12}\alpha$ and $G_{13}\alpha$ were also shown to be activated by the thrombin and thromboxane A_2 (TXA_2) receptors in platelet membranes using photoreactive azidoanilide GTP analogues [Offermanns *et al.*, 1994]. These polypeptides display a slower basal GDP-GTP exchange rate than $G_s\alpha$ and most $G_i\alpha$ subunits [Pang & Sternweis, 1990; Casey *et al.*, 1990; Wange *et al.*, 1991; Offermanns *et al.*, 1994].

1.3.3. $G_{\beta\gamma}$ subunit

The β and γ subunits of heterotrimeric G proteins are a tightly associated dimer complex in the plasma membrane. Four distinct β subunits isotypes, ranging in molecular mass from 35 to 36 kDa, have been identified sharing around 80% amino acid homology with the differences spread throughout the sequence [Gao *et al.*, 1987; Simon *et al.*, 1991; von Weizsacker *et al.*, 1992], at least 10 γ subunits ranging in size from 8 to 10kDa have been reported, sharing relatively little sequence identity, for example, $\gamma1$ being only 38% identical to $\gamma2$ [Clapham & Neer, 1993].

The β and γ subunit combination shows certain allowed association in cells. The $\gamma1$ subunit associates with only the $\beta1$ subunit. Conversely, complexes can be made of the $\beta1$ subunit with $\gamma1$, $\gamma2$, $\gamma3$, $\gamma5$ and $\gamma7$; $\beta2$ with any of $\gamma2$, $\gamma3$, $\gamma5$ or $\gamma7$,

however β3 does not associate with either γ1 or γ2 [Pronin & Gautam, 1992; Schmidt *et al.*, 1992; Iniguez-Lluhi *et al.*, 1992; Ueda *et al.*, 1994].

The roles of the βγ subunits in membrane signalling are now considered to be many and include such membrane anchoring of α subunits, activation of phospholipase A_2 [Jelsema & Axelrod, 1987] and phosphoinositidase C_β isoforms [Camps *et al.*, 1992; Smrcka & Sternweis, 1993; Lee *et al.*, 1993], modulation of the activities of K^+ channels [Logothetis *et al.*, 1987; Wickman *et al.*, 1994; Reuveny *et al.*, 1994], N- and Q-type Ca^{2+} channels [Ikeda, 1996; Herlitze *et al.*, 1996], stimulation or inhibition of the activity of adenylyl cycase isoforms [Tang & Gilman, 1991; Gao & Giman, 1991; Cooper *et al.*, 1995] and modulation of the activities of kinases including GRKs [Pitcher *et al.*, 1992; Kameyama *et al.*, 1993], MAP kinase [Crespo *et al.*, 1994], and phosphatidylinositol-3-kinase [Stephens *et al.*, 1994].

1.3.4. Mechanism and structural determinants in G protein action

The heterotrimeric G proteins undergo a series of steps leading to interaction with effector systems upon activation of GPCRs as shown in **Figure 1.5**. Activated receptor causes a conformational change in the G protein α subunit such that the bound GDP is released and exchanged for GTP in the nucleotide which triggers two events; the G protein has a lower affinity for receptor in its GTP bound state and thus is released from it, and the trimer dissociates into free α subunit and βγ dimer. The GTP-bound α subunit and/or the βγ complex liberated can then interact with an effector protein causing an intracellular response. The GDP-bound α subunit has a lower affinity for effector and is released to reassociate once more with βγ forming a quiescent GαβγGDP complex. The system returns to a basal state when the holoenzyme associates with a neighbouring unoccupied receptor [Gilman, 1987].

Figure 1.5. G protein-mediated transmembrane signalling cycle

Step A) Basal state : In the basal state of receptor (R), G protein (α, β, γ) and effector (E), G proteins exist as heterotrimers with GDP bound tightly to the α subunit. The receptor is unoccupied and the effector is inactive.

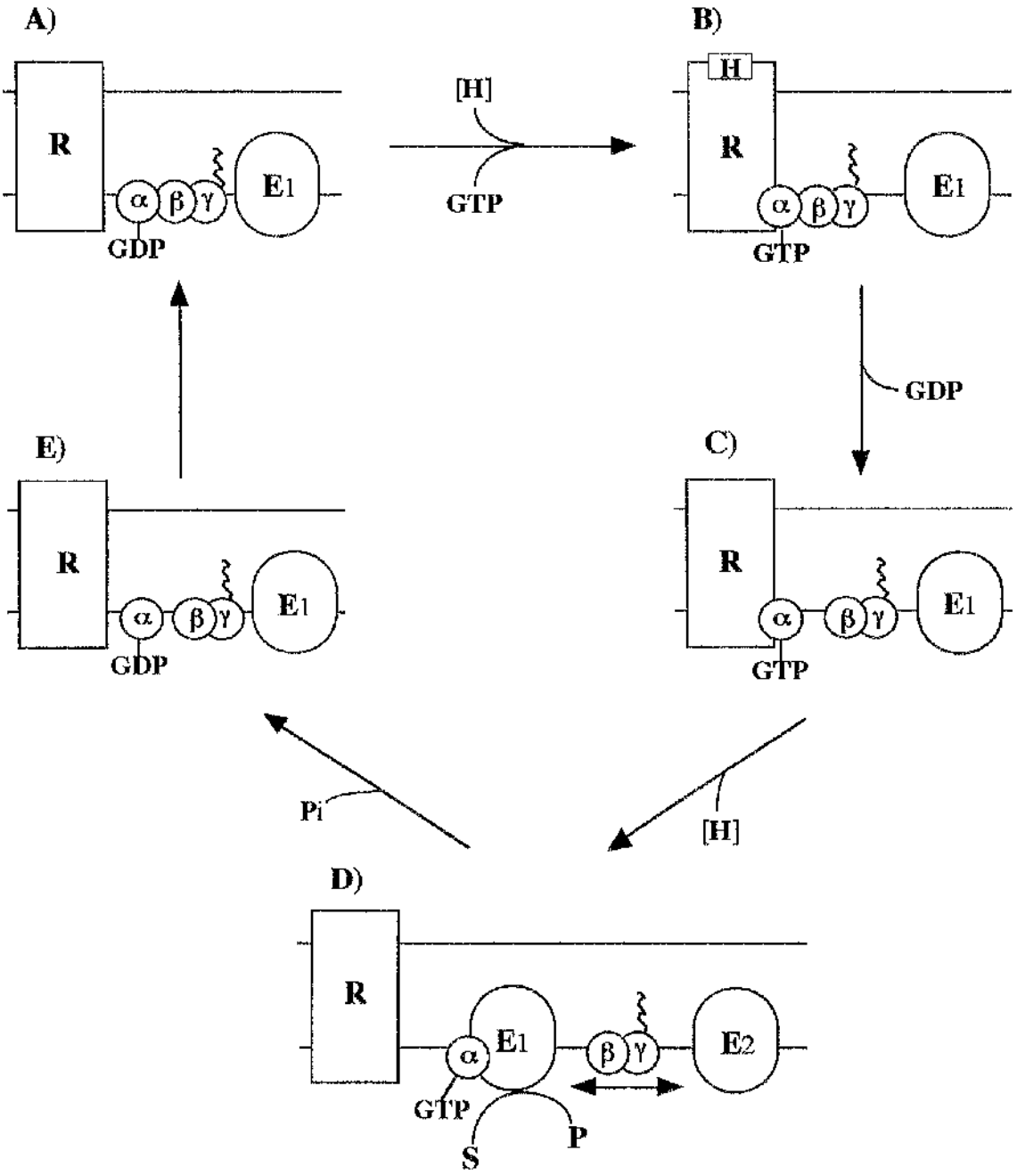
Step B) Receptor activation : When hormone (H) binds to thereceptor (R), [HR] complex interacts with the heterotrimeric G protein (inactive form) to promote a conformational change and dissociation of GDPfrom the guanine nucleotide-binding site. GTP replaces GDP, and induces a conformational change in the α subunit.

Step C) Subunit dissociation : G protein dissociates from the [HR] complex, reducing the affinity of hormone for receptor and, in turn, freeing the receptor for binding with a neighboring quiescent G protein. GTP binding also reduces the affinity of α for $\beta\gamma$, and subunit dissociation occurs.

Step D) Effector activation : Dissociated [α-GTP] fulfills its primary role as a regulator of effectors. The freed $\beta\gamma$ subunit complex may also interact directly with an effector (E_1) thereby modulating the activity of the complex, or it may act independently at another effector (E_2) in some systems.

Step E) GTPase activity : The α subunits possess an intrinsic GTPase activity. The rate of this GTPase determines the lifetime of the active species and the associated physiological response. The α-catalysed hydrolysis of GTP leaves GDP in the binding site and causes dissociation and deactivation of the active complex. The GTPase activity of α is an internal regulator that controls an on/off switch. The GDP bound form of α has high affinity for $\beta\gamma$, and subsequent reassociation of αGDP with $\beta\gamma$ dimer returns the system to the basal state.

Figure 1.5


A)
R
α
β
γ
E1
GDP
[H]
GTP
B)
H
R
α
β
γ
E1
GTP
GDP
C)
R
α
β
γ
E1
GTP
[H]
D)
R
α
E1
β
γ
E2
GTP
S
P
Pi
E)
R
α
β
γ
E1
GDP

The guanine nucleotide binding site on the α subunit is composed of 5 separate streches of amino acid sequence spread throughout the protein. These 5 repeated regions share significant homology to those of p21ras and other members of the GTPase superfamily [Conklin & Bourne, 1993]. The C-terminal region of the α subunit which is thought to be the contact site for the receptor forms a mobile region that prevents release of bound GDP. Upon agonist activation of a receptor, this C-terminal domain moves aside to allow GDP to leave the binding site and allow GTP to enter [Denker *et al.*, 1992a; Noel *et al.*, 1993]. The N-terminal region of the α subunit is important for interaction with βγ such that the N-terminus of $G_{t1}\alpha$ can compete for βγ binding with monoclonal antibodies directed against the N-terminus [Mazzoni & Hamm, 1989; Mazzoni *et al.*, 1991; Kokame *et al.*, 1992; Denker *et al.*, 1992b].

It has been suggested that several regions of the α subunit contact G protein coupled receptors. In particular, several lines of evidence suggest that the α helix at the C-terminus of α subunits is a contact site for GPCRs. PTX-catalysed ADP-ribosylation of $G_i\alpha$ in a cysteine four residues from the C-terminus prevents interaction with the receptor as discussed in **section 1.3.5.1** [West *et al.*, 1985]. Mutations in the C-terminal tail and antibodies directed specifically against it can also cause uncoupling of the receptor-G protein complex [Sullivan *et al.*, 1987; Simonds *et al.*, 1989; Gutowski *et al.*, 1991; Hirsch *et al.*, 1991; Shenker *et al.*, 1991].

Substitution of three amino acids at the C-terminal end of $G_q\alpha$ to those of $G_i2\alpha$ caused a change in the receptor specificity of $G_q\alpha$ to that of $G_i2\alpha$. From this work it was postulated that a C-terminal β-turn, centred on glycine residue played a critical role in specifying receptor interaction of G protein in the G_i subfamily [Conklin *et al.*, 1993]. Moreover, following the crystallisation of $G_t\alpha$, Noel *et al.*[1993] proposed that the C-terminal 5-10 amino acids of $G_t\alpha$ undergo a conformational change upon binding GTP, which may lead to functional dissociation of $G_t\alpha$ from photoactivated rhodopsin.

The N-terminal region of Gα subunit has also been postulated as a possible receptor contact site because the N- and C-termini lie in close proximity in three dimensional space. It has been demonstrated that a peptide corresponding to the N-terminus of $G_t\alpha$ inhibited interactions of $G_t\alpha$ with rhodopsin without affecting interaction with βγ [Hamm *et al.*, 1988; Conklin & Bourne, 1993]. Furthermore, mastoparan, an amphiphilic peptide from wasp venom which causes activation of the G_i family of G proteins, was found to cross-link to a cysteine residue near the N-terminus of $G_o\alpha$ [Higashijima & Ross, 1991]. A third site for receptor interaction has been identified proximal to the C-terminus and is represented by amino acids 311-328 in $G_t1\alpha$, since activation of G_t by rhodopsin was blocked by a peptide corresponding to these residues [Hamm, 1991].

Some of the regions responsible for effector interaction have also been identified. A synthetic peptide corresponding to residues 293-314 of $G_t1\alpha$ was shown in vitro to activate cGMP-PDE [Rarick *et al.*, 1992]. The construction of chimaeric $G_s\alpha$ subunits has provided a means of examining G protein-effector interaction. Systematic mutation of a 121 residue domain within the C-terminal half of chimaeric $G_i\alpha/G_s\alpha$ permitted identification of four effector-activating regions of $G_s\alpha$, corresponding to amino acids 235-240, 261-262, 277-285 and 349-356. Mutations within these four regions of the chimaera prevented activation of adenylyl cyclase [Berlot & Bourne, 1992]. More recently, Skiba *et al.*[1996] have shown that the N-terminal 1-45 region of phosphodiesterase γ (PDEγ) containing the central polycationic region binds to $G_t\alpha$'s α3 helix and α3/α5 loop in both active and inactive states, while the C-terminal region of PDEγ binds to the conserved switch region of activated conformations by using $G_t\alpha/G_i1\alpha$ chimaeras.

1.3.5. Covalent modifications of G proteins

Signalling components are known to undergo several co-translational and post-translational covalent modifications, including ADP ribosylation, phosphorylation and lipid modifications such as myristoylation, palmitoylation, polyisoprenylation and addition of glycosylphosphatidylinositol (GPI) [Turner, 1992]. Lipid modification of proteins plays a major role in regulating their subcellular location and function [Deschenes *et al.*, 1990; Turner, 1992]. Many proteins involved in cellular signalling, i.e.receptors and G protein α subunits, have been shown to be post-translationally acylated by one or more lipid molecules, and these modifications have been shown to promote membrane targeting and attachment [Resh, 1994; Casey, 1994; Wedegaertner *et al.*, 1995].

1.3.5.1. ADP-ribosylation

Some of the α subunits of G proteins are substrates for bacterial toxin-mediated modification. ADP ribosylation is a well known posttranslational modification whereby pertussis toxin, produced by Bordetella pertussis, catalyses mono-ADP ribosylation of $G_i\alpha$ family subunits resulting in attenuation of inhibition of adenylyl cyclase activity. Arginine and cysteine specific mono ADP ribosyltransferases as well as enzymes that catalyse the removal of ADP ribose from ADP ribosylated amino acid residues have been identified in several cell types, suggesting that ADP ribosylation might be a reversible modification [Moss and Vaughan, 1988; Tanuma *et al.*, 1988; Inageda and Tanuma, 1991]. Although the identification of an endogenously ADP ribosylated G protein α subunit has not yet been clearly reported *in vivo*, there has been much evidence to support ADP ribosylation of G protein α subunits by both arginine and cysteine specific mono ADP ribosyltransferases [Tanuma & Endo, 1989; Donnelly *et al.*, 1992].

Another example is that cholera toxin, produced by Vibrio cholera, causes the persistant activation of the adenylyl cyclase system by this enzymatic transfer of ADP ribose from NAD^+ to an arginine residue in $G_s\alpha$ close to the site which houses the GTPase activity of the protein. The $G\alpha$ subunit is locked in its GTP-bound form and causes constitutive activation of adenylyl cyclase [Gilman, 1987].

1.3.5.2. Prenylation

It has been shown that many proteins involved in cellular signalling are modified by the addition of isoprene units, either farnesyl or geranylgeranyl groups, to their C-termini [Deschenes *et al.*, 1990; Casey, 1994]. The presence of a motif consisting of a cysteine-aliphatic-aliphatic-any amino acid (CAAX) at the C-terminus is required for the process. However, this is not the only determinant for isoprenylation since the α subunits of G_i1, G_i2, G_i3 and G_o have such a motif at their C-termini but are not substrates for isoprenylation [Cox *et al.*, 1993; Casey, 1994].

The addition of either farnesyl or geranygeranyl groups to CAAX containing polypeptides is not a random process, but is instead governed by the exact composition of the CAAX motif. $G\gamma$ subunits, γ_1 and γ_2, have the C-terminal sequences CVIS and CAIL, respectively. γ_1 is modified by the addition of a farnesyl residue whilst γ_2, like all other γ subunits, is modified by a geranylgeranyl group, suggesting that the extreme C-terminal residue is responsible for the direction of isoprenylation [Casey *et al.*, 1994]. Isoprenylation of γ subunits plays an important role in membrane binding of the $\beta\gamma$ complex [Simonds, 1994].

1.3.5.3. Myristoylation

It has been shown that the addition of myristic acid, a 14-carbon saturated fatty acid, to a protein may play a role in determining its membrane association properties [Deschenes *et al* , 1990]. The consensus sequence for this modification in Gα subunits is an N-terminal glycine residue at position 2 with a hydroxylamine residue four amino acids downstream. The addition of myristate to glycine-2 via an amide bond has been demonstrated in $G_i1\alpha$, $G_i2\alpha$, $G_i3\alpha$, $G_t\alpha$, $G_o\alpha$ and $G_z\alpha$ but not in $G_s\alpha$. Although $G_s\alpha$ is not myristoylated, the glycine residue at position 2 is nevertheless important in the signalling properties of the molecule. Mutation of the glycine to alanine, or deletion of the N-terminal region of $G_s\alpha$ caused a reduction in the ability of $G_s\alpha$ to activate adenylyl cyclase and to interact with the βγ complex [van der Neut *et al.*, 1993]. This may be due to an inhibition of palmitoylation as gly 2 is required for palmitoylation of $G_i1\alpha$ and $G_z\alpha$ [Galbiati *et al.*, 1994; Hallak *et al.*, 1994].

1.3.5.4. Palmitoylation

Recently, a number of α subunits have been shown to undergo the post-translational process of palmitoylation. These include those that are myristoylated ie, $G_o\alpha$, $G_i1\alpha$, $G_i2\alpha$, $G_i3\alpha$ and $G_z\alpha$, and also $G_s\alpha$ and $G_q\alpha$ which do not undergo myristoylation. Palmitate is generally esterified to cysteine residues via thio-ester bonds [Casey, 1994; Casey, 1995]. The amide bond attaching the myristate is very stable and once attached, it is essentially permanently bound, although there has been a report of a demyristoylation of mature MARCKS protein [Manenti *et al.*, 1994]. The thio-ester bond linking the palmitate is, however, much more labile and can be enzymatically cleaved.

The palmitoylation of some forms of p21ras is part of the overall process of isoprenylation. Following the addition of farnesyl or geranylgeranyl groups, proteolysis and carboxymethylation, a palmitoyltransferase adds palmitate to specific

cysteine residues near the site of isoprenylation [Hancock *et al.*, 1989]. This enzyme is poorly characterised and has yet to be purified. However, the enzyme requires the prior isoprenylation of p21ras. Given this specificity, this enzyme is unlikely to be responsible for addition of palmitate to G protein α subunits and Src family members.

As mentioned above it has been shown that palmitoylation occurs in $G_s\alpha$ [Parenti *et al.*, 1993; Linder *et al.*, 1993], in $G_i2\alpha$, $G_o\alpha$ and $G_q\alpha/G_{11}\alpha$ [Parenti *et al.*, 1993], in $G_{12}\alpha$ and $G_{13}\alpha$ [Viet *et al.*, 1994], and in gustducin [Hoon *et al.*, 1995]. The residues responsible for undergoing this process have been identified as cysteine-3 in $G_o\alpha$ and $G_s\alpha$ [Parenti *et al.*, 1993; Degtyarev *et al.*, 1993a], and cysteine 9 and cysteine 10 which are both palmitoylated in $G_q\alpha$ [Wedegaertner *et al.*, 1993].

The role of palmitoylation of G protein α subunits is not yet clear, although it has been suggested that it may play a role in membrane association since palmitate negative mutants of $G_o\alpha$ have been shown to have a decreased avidity of interaction with the plasma membrane [Grassie *et al.*, 1994]. The ability of this protein to still interact with the plasma membrane reflects the presence of other determinants for membrane attachment, e.g.the presence of myristate at the N-terminus. The ability of palmitate negative mutants of $G_s\alpha$ to interact with the plasma membrane is less clear. Degtyarev *et al.*[1993b] found no effect on membrane attachment of C3S $G_s\alpha$ when expressed in COS-7 cells whilst Wedegaertner *et al.*[1993] found that palmitate was absolutely required for membrane association of $G_s\alpha$ when expressed in HEK-293 cells.

McCallum *et al.* [1995] have shown that the palmitoylation status of the cysteine residues at positions 9 and 10 in murine $G_{11}\alpha$ played a central role in defining membrane assosciation of this G protein and indicated that much of the particulate fraction of the expressed palmitoylation-resistant mutants was to represent non-functional rather than correctly folded protein by mutations of C9S, C10S or a combination of both alterations. Treatment with an inhibitor of myristoyl-CoA:protein

N-myristoyltransferase, 2-hydroxymyristate, prevented myristoylation of $G_i1\alpha$, but not its palmitoylation or membrane association, and prevention of myristoylation by the introduction of the S6D mutation into this G protein also prevented palmitoylation and comprised membrane interaction, suggesting that palmitoylation, but not myristoylation, plays a key role in membrane association of $G_i1\alpha$ [Galbiati *et al.*, 1996].

Palmitoylation of G proteins is a dynamic process. The half life of palmitate on $G_s\alpha$ is approximately 50-90 min [Degtyarev *et al.*, 1993b; Wedegaertner & Bourne, 1994] while the half life of the protein is approximately 11-22h [Levis & Bourne, 1992; Degtyarev *et al.*, 1993b]. Upon agonist activation of a receptor, the half life of the α subunit decreases to around 6h while the half life of the palmitate on the $G_s\alpha$ subunit decreases to around 2 min. It is clear that the regulation of the protein and its attached palmitate on it are under very different control processes. It may be that reversible palmitoylation serves as a dampener of receptor mediated second messenger generation. In removing the palmitate, the G protein α subunit may no longer be able to interact with the target effector system and thus second messenger generation would be curtailed [Wedegaertner *et al.*, 1995].

1.3.5.5. Phosphorylation

Phosphorylation of G proteins is the least well charaterized of the covalent modifications. The first evidence that G proteins may be regulated by phosphorylation came with the observation that treatment of platelet membranes with partially purified PKC resulted in phosphorylation of a 41kDa protein, later demonstrated to be $G_i\alpha$, with a stoichiometry of 1 mole Pi per mole of substrate [Katada *et al.*, 1985]. More studies with recombinant G proteins have shown that $G_z\alpha$ subunit was phosphorylated by partially purified PKC on a serine residue near the N-terminus, whereas

recombinant $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ were not phosphorylated under the same conditions [Lounsbury *et al.*, 1991]. In a reconstituted system containing purified insulin protein kinase and PKC, phosphorylation of α subunits occurred on both serine and tyrosine residues [Krupinski *et al.*, 1989; Pyne *et al.*, 1992].

Recently, Kozasa *et al.* [1996] have shown that $G_{12}\alpha$ serves as a substrate for phosphorylation by various isoforms of PKC *in vitro* suggesting that PKC regulates α_{12}-mediated signalling pathway by preventing their association with $\beta\gamma$. The γ_{12} was shown to be phosphorylated by PKC and the phosphorylated $\beta\gamma_{12}$ associated with $G_o\alpha$ more tightly than the unphosphorylated form [Morishita *et al.*, 1996]. In v-*src* oncogene transformed Rat 1 fibroblasts, $G_q\alpha/G_{11}\alpha$ were phosphorylated on tyrosine residues and markedly increased inositol phosphate generation by phosphoinositidase C_β [Liu *et al.*, 1996].

However, the physiological consequences of this process have yet to be demonstrated although phosphorylation of G proteins may function significantly in cross-talk between signalling systems.

1.4. Effectors interacting with G proteins

A relatively small number of effector systems convert extracellular signals transduced across the plasma membrane by GPCRs and G proteins into intracellular responses. These include various ion channels, cGMP phosphodiesterase, adenylyl cyclase, phospholipase A_2, phosphoinositidase C and phospholipase D.

1.4.1. Adenylyl cyclase family

It is generally known that adenylyl cyclase catalyses the conversion of intracellular ATP to cAMP. This receptor mediated signal is transduced by $G_s\alpha$

subunits for a stimulatory effect on the enzyme or by $G_i\alpha$ subunits ($G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$) for an inhibitory effect, as mentioned earlier. Cloning studies have identified multiple types of adenylyl cyclases which are all stimulated by $G_s\alpha$. All adenylyl cyclases are associated with the plasma membrane and hydropathy analyses suggest that they span the membrane 12 times. The N- and C-termini are both intracellular and the various isotypes all possess two large intracellular domains, one (C1) between transmembrane helices 6 and 7 and another (C2) located at the C-terminus [Taussig & Gilman, 1995].These regions are thought to form catalytic sites, termed C, which are subdivided into 2 domains a and b in this protein. This molecular architecture resembles that of ATP driven pumps such as the P glycoprotein [Cooper *et al.*, 1995] and a K^+pump in *Paramecium* [Schultz *et al.*, 1992].

Portions of the C1 and C2 (C1a and C2a) domains are well conserved displaying ~92% homology, and are very similar to the catalytic domains of a series of membrane bound guanylyl cyclases [Chinkers and Garbers, 1991]. Catalytic activity of adenylyl cyclase requires the N- and C-terminus [Tang *et al*, 1991] and also both C1a and C2a, suggesting that these two domains may interact to produce an active protein [Tang *et al*, 1992]. The exact site on the adenylyl cyclase polypeptide required for catalytic activity and G protein interaction is not yet clear. The amino acid sequence homology is limited beyond the C1a and C2a domains, but the overall structure is probably well conserved, suggesting that the two sets of 6 transmembrane helices may play a certain undiscovered role in the function of the enzyme. Potential for cross-talk between signalling pathways exists at the level of adenylyl cyclase. Nine different adenylyl cyclase isoforms have been identified, each of them being the product of distinct genes. The isoforms of mammalian adenylyl cyclase display differential regulation by $\beta\gamma$ dimers, calcium ions and protein kinase C [Choi *et al.*, 1993; Jacobiwitz *et al.*, 1993; Yoshimura and Cooper, 1993]. The diterpene forskolin stabilizes the interaction with $G_s\alpha$ and adenylyl cyclase C domain causing the

constitutive activation of adenylyl cyclase which gives rise to chronically high levels of cAMP inside the cell which causes a variety of intracellular effects.

1.4.2. Phosphoinositidase C family

The phosphoinositidase C (PIC) superfamily comprises at least 16 isoenzymes which have been classified into 3 subfamilies, β, γ and δ. All the isozymes function in the same manner, hydrolysing phosphatidylinositol-4,5-bisphosphate (PIP_2) to yield the second messenger molecules sn-1,2-diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). This bifurcation of the signal leads to the activation of two distinct pathways. The lipid molecule DAG causes the activation of PKC which is a family of serine/threonine protein kinases and affect many cellular responses including the activation of the Raf kinase, which is then able to activate another series of protein kinases ending in the phosphorylation and activation of MAP kinase. IP_3, being a polar molecule, is released from the membrane to bind to specific receptors located on the endoplasmic reticulum. This causes the release of Ca^{2+} from stores in the ER, again leading to effects on cellular systems.

The phosphoinositidase C isozymes differ in their regulation and only those belonging to the β class are under direct G protein control [Rhee & Choi, 1992] although PLC δ_1 may be stimulated by βγ [Park *et al.*, 1993]. Those of the γ class are activated through their Src Homology 2 (SH2) domains by binding phosphotyrosine residues of growth factor receptors. The β isozymes are activated both by the α subunits of G_q family members and by the βγ dimer, which can be generated from any class of α subunit. Both $G_{14}\alpha$ and $G_{16}\alpha$ activate the β1 isoform, whereas only $G_{16}\alpha$ activates the β_2 isoform to a significant degree [Wu *et al.*, 1992; Lee, C. *et al.*, 1992]. This dual regulation gave rise to pertussis toxin-sensitive activation, caused by the release of βγ from activated α subunits of G_i family members. The sites of action on

Table 1.2. Regulation of phosphoinositidase C_β isoforms by G protein

The β isoforms of phosphoinositidase C are regulated by G protein α and βγ subunits. To date, $\beta 1-\beta 3$ are known to be activated by α and βγ subunits but to different degrees. The $\beta 4$ isoform is not well-characterised. The magnitude of stimulation is described in order ++++ > +++ > ++ > + > +/- [Smrcka & Sternweis, 1993; Lee *et al.*, 1993].

PIC_β isoform	Activation by $G_{q/11}\alpha$	Activation by $G_{\beta\gamma}$
β1	+++	+
β2	+/-	++
β3	++++	+++
β4	?	?

PIC_β for G protein α subunits and $\beta\gamma$ dimers are apparently distinct, however, and the efficacies of their activation differ between the different members of the PIC_β family ($PIC_{\beta 1\text{-}4}$) as displayed in **Table 1.2** [Smrcka & Sternweis, 1993; Lee *et al.*, 1993].

Recently, Liu *et al.* [1996] have shown that a newly discovered $PIC_{\delta 4}$ was dominantly present in nucleus by immunocytochemical staining and nuclear $PIC_{\delta 4}$ was dramatically increased at the transition from G_1- to S-phase, but not in $PIC_{\beta 1}$, $PIC_{\gamma 1}$ and $PIC_{\delta 1}$, suggesting that this might play a role in cell growth as one of the early genes expressed in the cell cycle.

1.4.3. Phospholipase D

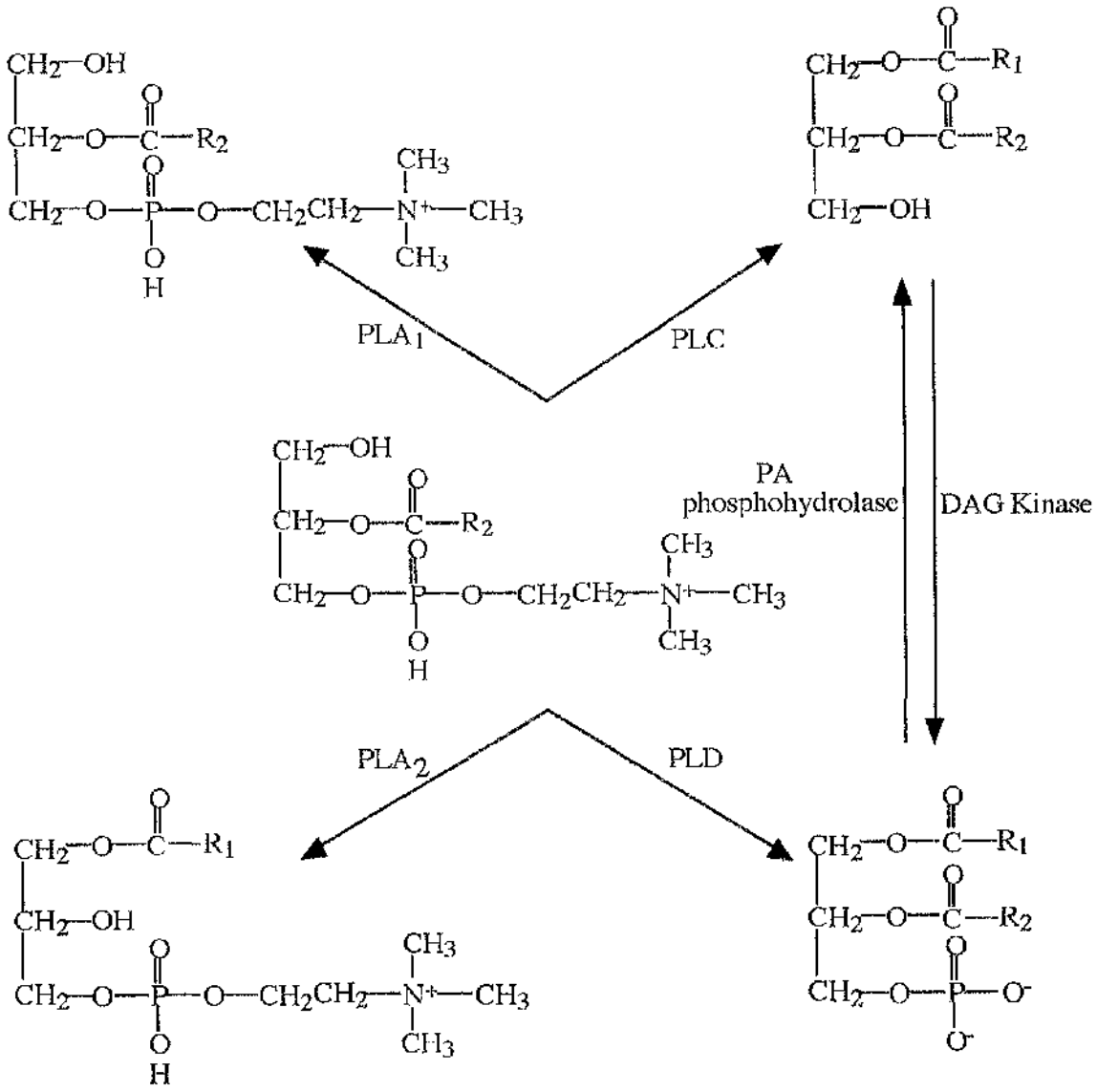
Phospholipase D (PLD) catalyses the hydrolysis of phosphatidylcholine (PtdCho), producing phosphatidic acid(PtdOH) and choline as shown in **Figure 1.6** [Exton, 1990]. PtdOH is a central metabolite in both phospholipid and triglyceride metabolism and an effector in several physiological processes including secretion, DNA synthesis, and cell proliferation [Boarder, 1994; Rose *et al.*, 1995]. PtdOH is metabolized by PtdOH phosphohydrolase to diacylglycerol (DAG), which can activate protein kinase C (PKC) as a second messenger. PLD activity was identified in plants, bacteria, fungi and mammalian tissues and cells including brain, lung, liver, adipose tissue, endothelial cells, HL-60 cells and spermatozoa; lung and brain are the richest sources [Massenberg *et al.*, 1994].

At least two classes of membrane-associated PtdCho-specific PLD enzymes have been described in mammalian tissues. One is stimulated by phosphatidylinositol-4,5-bisphosphate (PIP_2), while the other is stimulated by oleate [Brown *et al.*, 1993; Messenberg *et al.*, 1994]. The PIP_2-dependent PLD activity in monocytic cells and brain can be activated by a number of monomeric GTP-binding proteins such as the ADP-ribosylation factor (ARF). This ARF-regulated PLD is cytosolic and hydrolyses

Figure 1.6. The hydrolysis of phosphatidylcholine (PtdCho) by phospholipases

The activation of phospholipase A_1 and A_2 (PLA_1 and PLA_2) results in the production of one fatty acyl chain and acylphosphatidylcholine, known as lysolecithin, from phosphatidylcholine. The activation of phospholipase C (PLC) results in the production of phosphocholine (not shown) anddiacylglycerol (DAG). The activation of phospholipase D (PLD) results in the production of choline (not shown) and phosphatidic acid (PA). PLA produced arachidonic acid, DAG and PA are possible second messengers in cellular signalling. Diacylglycerol and phosphatidic acid are interconvertible by the actions of diacylglycerol kinase and phosphatidic acid phosphohydrolase. R_1 and R_1 are hydrocarbon chains of long chain fatty acids.

Figure 1.6



phosphatidylethanolamine (PtdEtn) and phosphatidylinositol (PtdIns) as well as PtdCho. This finding has led to the speculation that PLD functions in protein trafficking and vesicular movement by altering the local structural charateristics of membranes [Liscovitch & Cantly, 1995].

However, the mechanism of PLD regulation by G protein coupled receptors is still a matter of debate. A number of reports have described receptors that are coupled to PLD via a G protein including the P_2 purinergic receptor [Martin & Michaelis, 1989], α_2-adrenergic receptor [MacNulty *et al.*, 1992], and m1, m2 and m3 muscarinic acetylcholine receptors [Offermanns *et al.*, 1994]. However, there has been no adequate distinction between receptors that have a direct G protein-linkage and those that are dependent on an intermediate step involving the activation of PIC, since this PLD response is dependent upon Ca^{2+} entry [Boarder, 1994]. Besides the receptor themselves, Ca^{2+}, PKC, tyrosine kinases, and GTP-binding proteins have been shown to be involved in the regulation of cellular PLD activity [Billah, 1993; Exton, 1994].

Singer *et al.* [1995] have shown that multiple cytosolic components including ARF, RhoA and an unidentified factor which activated PLD in a nucleotide-independent manner in a partially purified PLD from membrane of porcine brain were involved in PLD stimulation. Stimulation of PLD by purified PKCα or recombinant PKCα occurred in the absence of any nucleotide and required activators such as Ca^{2+} or phorbol ester and this stimulation was synergistic with either ARF or RhoA, but independent of PKC activity since dephosphorylation of the recombinant PKCα with phosphatase 1 or 2A resulted in a loss of its kinase activity but had little effect on its ability to stimulate PLD either alone or in conjunction with ARF [Singer *et al.*, 1996].

More recently, it has been reported that rho protein plays an essential role in PLD pathway by demonstrating that cytotoxin B of *Clostridium difficile* which glycosylates this protein inhibits PLD activity. This toxin B treatment also inhibited PLD activation by the direct G protein activators, AlF_4^- and GTPγS [Schmidt *et al.*,

1996]. Waksman *et al.* [1996] have identified a phosphtydylcholine-hydrolising PLD gene from chromosome XI of the yeast, *Saccharomyces cerevisiae*.

1.4.4. Phospholipase A_2

The phospholipase A_2 (PLA_2) is one of four enzymes that hydrolyze specific bonds in phospholipids as shown in **Figure 1.6**, e.g., phosphatidylcholine and phosphatidylethanolamine and is an abundant component of some snake venoms, which have the ability to cause hemolysis or rupture of red blood cells. It is also known that PLA_2 has two forms; extracellular and intracellular. The intracellular PLA_2 is found in plasma and other intracellular membranes and also the cytosol [Exton, 1994]. The cytosolic form which is regulated by protein kinases and Ca^{2+} is a substrate for mitogen-activated protein kinase (MAPK) and PKC, phosphorylation of the enzyme leading to its activation [Hazen *et al.*, 1993; Lin *et al.*, 1993].

There is some evidence that G proeins may control PLA_2 activity in permeabilized neutrophils, HL60 promyelocytes, platelets, RBL basophilic cells, Swiss 3T3 fibroblasts, thyroid cells and MC3T3-E1 osteoblasts [Exton, 1994]. In addition, there have been reports of guanine nucleotide and AlF^-_4 effects on PLA_2 activity in isolated membranes from platelets and thyroid cells [Silk *et al.*, 1989]. In rod outer segment, light and GTPγS activate $cPLA_2$ activity and both cholera and pertussis toxins inhibit it [Jelsema, 1987]. The addition of transducin βγ subunits markedly activated PLA_2, however, transducin α subunits which by themselves were slightly stimulatory inhibited the stimulatory effect of βγ subunit [Jeselma & Axelrod, 1987]. Antibodies to βγ subunits inhibited PLA_2 response to histamine, but not thrombin [Murayama *et al.*, 1990]. Conversely, antibodies to $G_i\alpha$ and $G_o\alpha$ inhibited both responses [Murayama *et al.*, 1990].

Recently, Xu *et al.* [1993] transfected NIH 3T3 fibroblasts with wild-type $G_{12}\alpha$ and also a mutant form of $G_{12}\alpha$ in which glutamine 228 was changed to leucine. The mutation decreased the GTPase activity and enhanced transformation activity. There was no change in PI metabolism, cytosolic Ca^{2+} or PLD activity, but production of arachidonic acid was enhanced in the presence of serum. However, the nature of the G proteins that regulate PLA_2 activity remain uncertain and direct coupling between a pure G protein subunit and a pure enzyme has not been demonstrated.

1.4.5. Ion channels

It has been shown that $G_s\alpha$ and $G_i\alpha$ can regulate more than a single effector system. In addition to stimulating adenylyl cyclase, $G_s\alpha$ can stimulate Ca^{2+} channels of the L-type and a subclass of smooth muscle Ca^{2+}-activated voltage-dependent K^+ channels (K_{Ca} channels). These are functions that are expressed only in specific tissues and cell types so that responses to receptors that activate $G_s\alpha$ may vary from tissue to tissue [Birnbaumer *et al.*, 1994]. The inhibition of voltage-dependent Ca^{2+} channels through the muscarinic and somatostatin receptors was shown to be mediated by the pertussis toxin-sensitive G proteins, $G_o1\alpha$ and $G_o2\alpha$, in secretory cells [Kleuss *et al.*, 1991]. Recently, it has been demonstrated that $G_{\beta\gamma}$ subunits can modulate N-type Ca^{2+} channels in synaptic neurons [Ikeda, 1996]. The $G\gamma$ subunit is ineffective by itself, however, overexpression of exogenous $G\beta$ subunits is sufficient to cause this channel modulation [Herlitze *et al.*, 1996].

At least two classes of K^+ channels, the muscarinic-type inwardly rectifying K^+ channel which is found primarily in cardiac artrial cells and in neuroendocrine cells, and the ATP-sensitive K^+ channel found in cardiac ventricle cells and pancreatic islet cells, can be activated by $G_i\alpha$ subunits ($G_i1\alpha, G_i2\alpha$ and $G_i3\alpha$). These channels are also referred to as G protein-gated K^+ channels [Birnbaumer *et al.*, 1994].

Na^+ channels are another example of ion channels regulated by heterotrimeric G proteins. One such type regulated by aldosterone has been shown in patch clamp studies to be gated by G proteins, in a similar manner to other ion channels, and the $G_i3\alpha$ subunit is known to be topographically localized with the channel [Ausiello *et al.*, 1992]. Aldosterone stimulates the expression of $G_i3\alpha$ subunit protein which is palmitoylated and co-localized with the Na^+ channel, and sodium transport is significantly inhibited by inhibition of $G_i3\alpha$ palmitoylation in A-6 cells [Rokaw *et al.*, 1996].

1.4.6. Regulation of MAP kinase activity

Mitogen activated protein kinases (MAP kinases) are activated by many extracellular stimuli that influence cell proliferation and differentiation, including growth factors, cytokines and hormones. Signalling via growth factors such as epidermal growth factor (EGF) involves phosphorylation of tyrosine residues and a series of protein-protein interactions, mediated via Src homology 2 and 3 (SH2/SH3) domains, leading to serial activation of $p21^{ras}$ (ras), Raf-1 kinase and MAP kinases [Blenis, 1993; Crews & Erikson, 1993]. Agonist binding to the EGF receptor leads to receptor dimerization and autophosphorylation, resulting in a phosphotyrosine-dependent association with Shc. The subsequent interaction between Tyr(P)-phosphorylated Shc and the Grb2 adaptor protein causes a translocation of the Grb2-Sos complex to the membrane, where Sos mediates guanine nucleotide exchange on Ras [Li *et al.*, 1993].

Two members of the MAP kinase family are the p44 MAP kinase (extracellular signal-regulated kinase 1, ERK1) and p42 MAP kinase (ERK2). When these two isoforms are activated by phosphorylation catalyzed by Raf-1 kinase or other MAP kinase kinases (MEKs) they in turn catalyse the phosphorylation of a large group of

substrates located at the cell membrane, cytoplasm and nucleus [Davis, 1993]. Inactivation of MAP kinase is by dephosphorylation of the same threonine and tyrosine residues catalysed by dual-specificity protein phsophatases [Nebreda, A.R., 1994].

Several G_i-coupled receptors, including the α_2 adrenergic receptor, lysophosphatidic acid (LPA) receptor, m2 muscarinic acetylcholine (m2ACh) receptor, and platelet-activating factor (PAF) receptor, have been shown to stimulate MAP kinase activity in various cell types as shown in **Figure 1.7** [Howe & Marshall, 1993; van Corven *et al.*, 1993; Cook *et al.*, 1993; Hordijk *et al.*, 1994; Alblas *et al.*, 1993; Winitz *et al.*, 1993; Honda *et al.*, 1994]. The signalling pathways by which G_i-coupled receptors activate MAP kinase, however, are not yet clear, but there is evidence for both Ras-dependent and Ras-independent activation of MAP kinases [Koch *et al.*, 1994]. The primary G protein subunit(s) responsible for G_i-mediated Ras and/or MAP kinase activation has not been identified, but reports have implicated $G_{\beta\gamma}$ as an activator of overexpressed p44 MAP kinase (ERK1) in COS-7 cells [Crespo *et al.*, 1994].

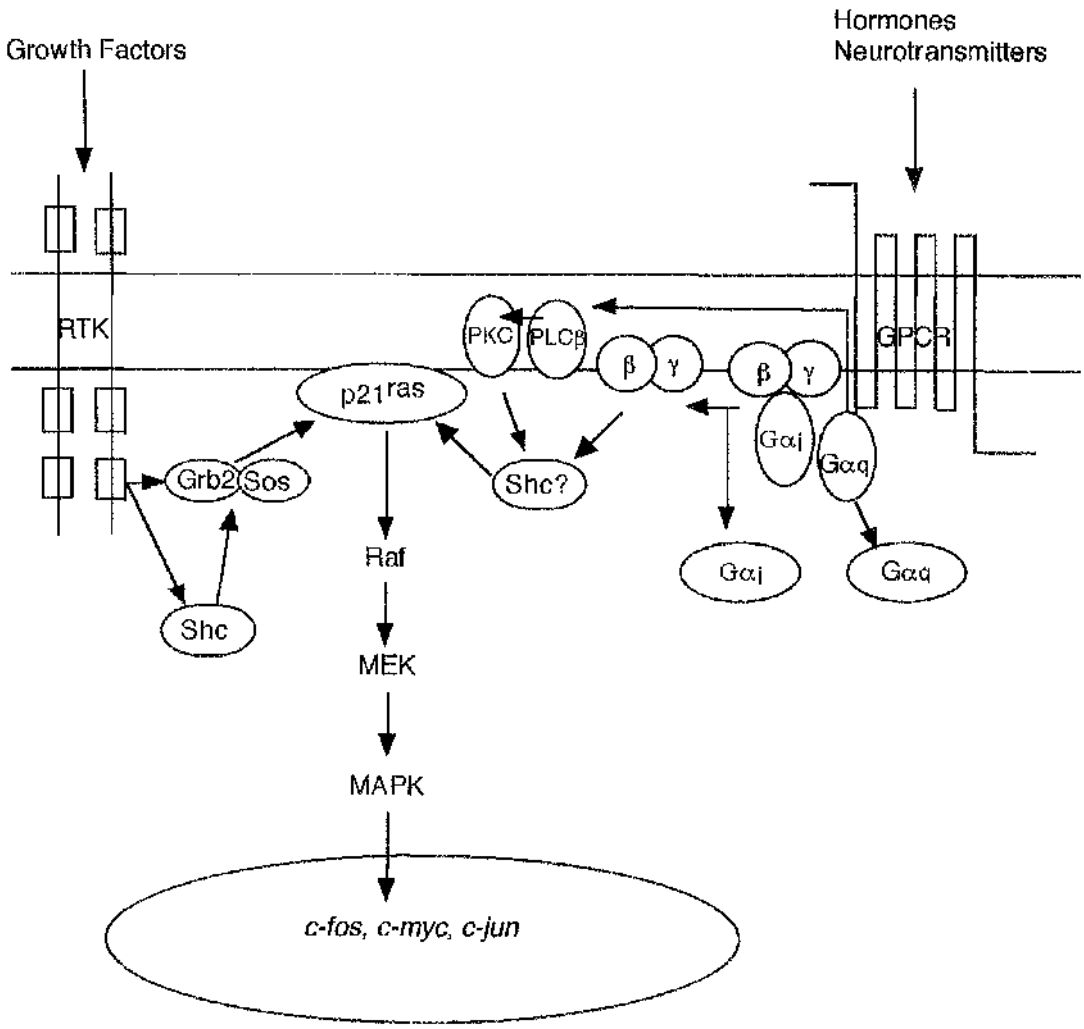
van Biesen *et al.* [1995] have shown that $G_{\beta\gamma}$ subunits derived from PTX-sensitive G proteins were mediated by Ras-dependent MAPK activation. Release of $G_{\beta\gamma}$ promotes the tyrosine phosphorylation of Shc and its subsequent association with Grb2-Sos. Both RTK- and $G_{\beta\gamma}$-mediated MAPK activation were completly blocked by the expression of dominant negative mutants of mSos1 and Ras, demonstrating that RTKs and $G_{\beta\gamma}$ activate MAPK via a common signalling pathway involving Shc, Grb2, Sos and Ras [van Biesen *et al.*, 1995].

MAPK activation via G_i-coupled receptors was sensitive to inhibition by the C-terminal fragment of βARK1 (βARK1ct), a competitive inhibitor of $G_{\beta\gamma}$-mediated signals [Koch et al., 1994]. However, not all GPCRs mediate MAPK activation exclusively via receptor-catalysed release of $G_{\beta\gamma}$ subunits. For example, MAPK activation via receptors coupled to members of the PTX-insensitive G_q family. such as

Figure 1.7. Scheme of the activation of the Ras-MAP kinase signal transduction pathway through growth factor and G_i-coupled receptors.

G_i-coupled receptor activation (e.g., LPA) of Ras is mediated by the $G_{\beta\gamma}$ subunit complex resulting from receptor-mediated dissociation of the heterotrimeric G_i protein. $G_{\beta\gamma}$ alone or through the recruitment of one or more cytoplasmic or membrane associated factors, possibly by pleckstrin homology (PH) domain-directed binding, leads to Ras activation. Growth factor receptor activation (e.g. EGF) activates Ras through the recruitment, directed by SH2/SH3 domains, of the cytoplasmic proteins Grb2 and Sos1. The convergence of these two different surface receptor signalling pathways on Ras activation in turn leads to the sequential activation of the Raf-1 and MAP kinase cascade, producing activation of nuclear genes and cellular responses including growth and differentiation.

Figure 1.7


Growth Factors
Hormones
Neurotransmitters
RTK
GPCR
PKC
PLCβ
β
γ
β
γ
p21ras
Gαi
Gαq
Grb2
Sos
Shc?
Raf
Gαi
Gαq
Shc
MEK
MAPK
c-fos, c-myc, c-jun

m1 muscarinic acetylcholine receptor and the α_1 adrenergic receptor, was insensitive to the $G_{\beta\gamma}$ sequestrant βARK1ct peptide [Hawes *et al.*, 1995], instead, MAPK activation occured predominantly via a PKC-dependent pathway. The GTP-bound α subunit of the G_q/G_{11} protein activated phosphoinositide hydrolysis and PKC and subsequently the activated PKC stimulated MAPK activity via a poorly understood mechanism involving the activation of Raf kinase [Kolch *et al.*, 1993; Troppmair *et al.*, 1994].

Recently, van Biesen *et al.* [1996] have shown that Ras-independent MAPK activation was mediated by the α subunit of the PTX-sensitive G_o protein at the m1 muscarinic acetylcholine receptor and platelet-activating factor receptor, demonstrating that G_o-mediated MAPK activation was sensitive to treatment with pertussis toxin but insensitive to inhibition by a $G_{\beta\gamma}$ sequestrating peptide (βARK1ct).

However, a direct role for $G_{\beta\gamma}$ in Ras activation or in the regulation of endogenous MAP kinase activity has not been established.

1.5. Aims of research

It has been known that the basic unit of information processing via G protein-coupled mechanisms consists of a receptor, a heterotrimeric G protein, and an effector enzyme. Despite intense investigation of the individual components of such signal transduction cascades and the basic features of cellular response to the presence of receptor ligands, little is currently known about regulation of each component.

Chapter 3 aims to examine signalling properties of G protein-coupled receptor isoforms which are co-expressed in the cell, and whether they display differences in agonist potency, G protein coupling, and activation of effector systems.

Chapter 4 aims to investigate the mechanism of how receptor agonists can control the cellular content of G proteins which interact with that receptor.

Chapter 5 aims to investigate signalling charateristics of a constitutivcly active mutant (CAM) receptor. The regulation of signalling cascade elements and the action of inverse agonist at this receptor will be discussed.

Chapter 2. Materials and Methods

Chapter 2. Materials and Methods

2.1. Materials

2.1.1. Chemicals

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Amersham International Plc., Buckinghamshire

Amplify, Hyperfilm

Applied Biosystems, Warrington, Cheshire

Amplitaq Dyedcoxy terminator cycle sequencing kit (part no.401150), phenol:water:chloroform reagent (68:18:14) (part no. 400765)

Appligene, Birtley, Co. Durham

Aquaphenol

Boehringer Mannheim UK, Lewes, East Sussex

BSA, aprotinin, DNase free RNase

Calbiochem-Novabiochem (UK) Ltd., Beeston, Nottinghamshire

Pansorbin cells, DTT

Difco, Detroit, Michigan, USA

Yeast extract, Bacto agar, Bacto tryptone

Gibco Life Technologies, Paisley, Lanarkshire

DMEM, inositol free DMEM, glutamine, sodium bicarbonate, newborn calf serum, penicillin/streptomycin solution, Lipofectin, Lipofectamine, ultrapure urea, agarose, 1kb DNA ladder

National Diagnostics, Aylesbury, Buckinghamshire

Sequagel-6

Porton Products, Porton Down, Wiltshire

Pertussis toxin

Promega Ltd., Southampton, Hampshire

DNA minipreps, DNA maxipreps, PCR preps, DNA clean-up system, calf intestinal alkaline phosphatase (CIAP), T7 promoter primer, Taq DNA polymerase, 4 dNTPs, T4 DNA ligase, all restriction endonucleases

Qiagen Ltd., Surrey

DNA extraction kit

Scottish Antibody Production Unit, Lanarkshire

Horseradish peroxidase conjugated donkey anti-rabbit IgG

Sigma Chemical Co. Ltd., Poole, Dorset

Trypsin, NP-40, gelatin, o-dianisidine hydrochloride, 7-deoxycholic acid, bromophenol blue, TEMED, forskolin, Coomassie Blue R-250, sodium azide, geneticin sulphate, soybean trypsin inhibitor, PMSF, Triton X-100, thimerosal, ampicillin, low melting point agarose, mineral oil, Ponceau S,

TRH, PMA, endothelin-1, phentolamine, phenylephrine, noradrenaline, prazosin

Stratagene

pfu DNA polymerase

All other reagents and chemicals were of analytical grade and obtained from Fisons Scientific Equipment, Loughborough, Leicestershire.

2.1.2. Radiochemicals

Amersham International plc., Buckinghamshire

[α-^{32}P]ATP, cyclic [^{3}H]AMP, myo-[2-^{3}H]inositol (17.1-17.6 Ci/mmol), [^{3}H]prazosin (24 Ci/mmol), [9,10-^{3}H(N)]palmitic acid (40-60 Ci/mmol)

Du Pont NEN (UK) Ltd., Stevenage, Hertfordshire

[^{3}H]TRH (45-70 Ci/mmol)

ICN Flow, Irvine, Lanarkshire

Trans ^{35}S label (1180 Ci/mmol)

2.1.3. Antisera

All antisera used in this study are detailed in **Table 2.1**. The methods of production of the antisera have been described previously [Goldsmith *et al.*,1988].

2.1.4. Plasmids

Table 2.1. Specificity of antisera

The antisera used in this study are shown along with the peptide sequences to which they were raised, the corresponding areas of the G protein α subunits which correspond to these amino acids, the α subunits which the antisera identify and the references dealing with their production. All of the Gα antisera were made in house. ERK-1 antipeptide antisera were provided by Dr.N.G.Anderson, Hannah Research Institute, Ayr.

Table 2.1.

Antiserum	Peptide	a.a. Sequence	Idenditification	Reference
CS3	RMHLRQYELL	$G_s\alpha$ 385 - 394	$G_s\alpha$	Milligan & Unson [1989]
SG1	KENLKDCGLF	$G_{i1}\alpha$ 341- 350	$G_{i1}\alpha$, $G_{i2}\alpha$, $G_{i1}\alpha$, $G_{i2}\alpha$	McKenzie & Milligan [1990]
CQ4	QLNLKEYNLV	$G_q\alpha$ 351 - 360 $G_{11}\alpha$ 350 - 359	$G_q\alpha$, $G_{11}\alpha$	Mitchell & Milligan [1991]
ERK-1		ERK-1 325 - 345	p42/p44 MAP kinase	Anderson & Milligan [1994]

All plasmids used in this study are house kept except plasmid pBluescript SK$^-$ with a full length version of the rat thyrotropin-releasing hormone receptor short isoform was provided by Dr. Pilar de la Pena, University of Oviedo, Spain.

2.2. Cell culture

2.2.1. Cell Growth

Cells were grown in continuous monolayer culture in 75 cm^2 sterile tissue culture flasks (Nunc, Roskilde, Denmark) in sodium pyruvate-free Dulbecco's Modified Eagle's medium supplemented with 5 - 10 % (v/v) serum (as indicated for each cell line), 2 mM L-glutamine, 100 I.U./ml penicillin, 100 µg/ml streptomycin. Buffering of the medium was achieved by the addition of 0.375 % (W/V) sodium bicarbonate and growing the cells in an atmosphere of air : CO_2 in the ratio of 95 % : 5 %. Cells were incubated in 10 ml of the above medium in a VSL incubator (Scotlab, Strathclyde) at 37°C and allowed to reach confluency. The medium was changed regularly,generally every 4 days.

2.2.2. Maintenance of cells

Parental Rat 1 fibroblasts and clones derived from these cells expressing the long and the short TRH receptor cDNA isoforms were grown in the above medium containing 5 % newborn calf serum. The receptor expressing clones were further maintained in the presence of 750 µg/ml geneticin sulphate. Rat 1 fibroblasts transfected to stably express the rat $\alpha_{1A/D}$, the hamster α_{1B}, and the bovine α_{1C} adrenoceptor cDNA species were obtained from Dr.D.E.Clark (Syntex, Palo Alto, CA) under license to Syntex from Dr. L.F. Allen (Duke University, NC) and were grown

in the same medium. Rat 1 fibroblasts transfected to stably express the hamster wild type and constitutively active mutant α_{1B} adrenergic receptor cDNA species were obtained from Dr.Susanna Cotecchia (University De Lausanne, Switzerland) and were maintained in the presence of 300μg/ml geneticin sulphate. COS-7 cells and HEK 293 cells were grown in DMEM containing 10 % (v/v) fetal bovine serum.

2.2.3. Passaging of confluent cell cultures

Upon reaching confluency, each flask of cells was usually split 1: 8 into new flasks. Medium was removed and the monolayer trypsinized by addition of a solution containing 0.1% (w/v) trypsin, 10mM glucose, 0.67mM EDTA, pH7.4. When cells had detached from the flask, trypsinization was terminated by the addition of 10 ml DMEM containing 5 - 10 % newborn calf serum. The cell suspension was then decanted into a sterile 50 ml polypropylene centrifuge tube (Nunc,Roskilde, Denmark) and centrifuged in a bench top centrifuge (MSE) at 1000 rpm for 5 mins. The supernatant was discarded and the pellet of cells resuspended in 8 ml of serum containing DMEM and aliquots of 1 ml were each added to 8 new flasks containing 9 ml of medium and placed in an incubator.

2.2.4. Storage and recovery of cells

To provide a stock of cells for further use, cells were cryogenically stored in liquid nitrogen. After trypsinization and centrifugation as above, cells were resuspended in 1 ml per confluent flask of freezing medium. This was DMEM supplemented with 20 % (v/v) of the appropriate serum and 10 % (v/v) dimethyl sulphoxide. 1 ml of the cell suspension was added to sterile freezing vials (Nunc, Denmark). To prevent formation of ice crystals in the cells, the freezing vials were

placed in a polystyrene box packed with cotton wool and frozen slowly overnight at -80°C. The cells were then transferred to liquid nitrogen.

To bring the frozen cells up, vials were removed from liquid nitrogen and thawed at 37°C . The contents were placed in a 50 ml centrifuge tube and diluted with 13 ml of DMEM containing 10 % serum and centrifuged at 1000 rpm for 5 mins as above. The supernatant was then discarded and the pellet resuspended in 10 ml DMEM containing 5-10 % serum and the whole added to a 75 cm^2 flask. Cells were maintained as described above.

2.2.5. Treatment of cells with drugs and toxins

Cells which were to be pretreated with agonist, antagonist and pertussis toxin were grown to approximately 70 % confluency. Medium was then removed by aspiration and 9 ml of fresh DMEM was then added to each flask. Agonist, antagonist and toxin were prepared fresh as a 10x stock in DMEM and filter sterilized as described above. 1 ml was added to each flask to a desired final concentration. Cells were then grown for various times prior to harvesting as described in **section 2.2.8**.

2.2.6. Labelling of cells with [^{3}H]palmitic acid

150 μCi/ml of [9,10-^{3}H]palmitate was added to cells stably expressing the α_{1B} adrenergic receptor in DMEM containing 5 % (v/v) dialyzed newborn calf serum, 5mM sodium pyruvate and incubated for 4 hrs. Cells were then harvested and total lysates immunoprecipitated as described in **section 2.5.2**.

2.2.7. Pulse-chase of cells with Trans ^{35}S label

Cells were trypsinized and seeded in 6-well culture plates. At about 70 % confluency, 2/3 of the growth medium was replaced with DMEM lacking methionine and cysteine, supplemented with glutamine, antibiotics,and 50 μCi/ml Tran^{35}S-label (final concentration in well). After the labeling period (16hrs), the radioactive medium was removed, and the now close to confluent cell layer was washed once with 2ml of normal DMEM culture medium. They were subsequently incubated in 1.5 ml/well normal culture medium in the presence or absence of 100μM phenylephrine. At appropriate times, the medium was removed and cells were dissolved in 200 μl/well of 1 % (w/v) SDS and scraped. The cell suspension was transferred to a screw-cap test tube and heated to 100°C for 20 min to denature proteins and nucleic acids, and then the samples were either stored at - 20°C or processed directly for immunoprecipitation as described in **section 2.5.2.**

2.2.8. Harvesting of cells

After cells reached confluency or the particular treatment time had elapsed they were harvested by scraping the monolayer into the medium and the cells collected in a 50 ml centrifuge tube on ice. The tubes were centrifuged at 2000 rpm for 5 min at 4°C in a Beckman TJ-6 benchtop centrifuge. The supernatant was discarded and the cell pellet was resuspended in 50 ml of ice-cold PBS (137 mM NaCl, 4 mM Na_2HPO_4, 0.27 mM KCl, 0.15 mM KH_2PO_4, pH 7.4) and centrifuged as before. Again the supernatant was discarded and the pellet was resuspended in 1x PBS and re-centrifuged. The supernatant was again discarded and the pellet was stored at -80°C until required.

2.2.9. Transient transfection of cells

For the transient expression of receptor cDNA, COS-7 cells or HEK 293 cells were used. These cells were grown to approximately 50 % confluency in 100 mm tissue culture dishes as described above. DNA (5 - 10 μg) was added to a 13 ml sterile polypropylene centrifuge tube containing 20-30 μl of Lipofectin and sterile Milli Q H_2O, to a final volume of 300 μl. DNA/liposome complexes were allowed to form for 15 min during which time the cells were washed twice with serum free DMEM. 10 ml of serum free DMEM was then added to the DNA/liposome complexes and the whole added to one 100 mm dish of cells. Cells were then incubated for 8-12 hrs to allow the DNA to enter the cell. Medium was then replaced with DMEM containing 10 % newborn bovine serum and cells were incubated for approximately 72 h before being harvested as described in **section 2.2.8**.

2.2.10. Stable transfection of cells

Rat 1 fibroblasts were grown to approximately 60 % confluency in 100 mm tissue culture dishes. cDNA (5-10 μg) encoding either the long or short isoform of the rat TRH receptor together with 0.5-1 μg of the plasmid pSV2-neo (Invitrogen) was added to a sterile 13 ml polypropylene tube containing 40 μl of Lipofectin and serum free medium to a final volume of 200 μl. DNA/liposome complexes were allowed to form for 15 min during which time the cells were washed twice with serum free DMEM. 10 ml of serum free DMEM was then added to the DNA/liposome complexes and the whole added to one 100 mm dish of cells.

After 16 h medium was replaced with normal DMEM containing 5 % newborn bovine serum, and 48 h later cells were trypsinized and split 1:3 into new 100mm tissue culture dishes and were maintained in the above medium supplemented with 750 μg/ml geneticin sulphate to initiate selection. Resulting geneticin-resistant clones were picked and seeded into 24-well plates with normal medium containing geneticin

sulphate (750 μg/ml) and 1 week later cells were transferred into 25 cm^2 flasks and again transferred into 75 cm^2 flasks when they were confluent and expanded for further use.

2.3. Preparation of plasma membrane fractions

Membranes were prepared according to the method of Koski and Klee (1981). Frozen cell pastes were thawed on ice and resuspended in 2 ml of ice-cold TE buffer (10 mM Tris HCl, pH7.5, 0.1 mM EDTA) and transferred to a pre-chilled glass homogenizer tube. The cells were then homogenized, on ice, with 25 strokes of a hand-held teflon-on-glass homogenizer. The resulting homogenates were transferred to polypropylene centrifuge tubes and the homogenizer tube washed with a further 1 ml of ice-cold TE buffer and the contents added to the centrifuge tube.

Centrifuge tubes were placed in a type 50Ti rotor (Beckman) and centrifuged at $500g_{av}$ for 10 min at 4°C in a Beckman L5-50B ultracentrifuge. The pellet, which contained nuclei and unbroken cells, was discarded and the supernatant transferred to fresh pre-chilled centrifuge tubes and centrifuged at $48{,}000g_{av}$ for 10 min at 4°C. The supernatant was discarded and pellet resuspended in 5 ml of TE buffer and recentrifuged at $48{,}000g_{av}$ for 10 mins at 4°C. Again the supernatant was discarded and pellet resuspended in TE buffer to give an approximate protein concentration of 1-3 mg/ml. The samples were then frozen at -80°C in 100μl fractions until required.

2.4. Determination of protein concentration

Protein concentrations were determined by the method of Lowry *et al.*[1951], using BSA as standard. The samples were read at 750 nm on a Shimadzu UV-1201 UV visible spectrophotometer.

2.5. Preparation of samples for SDS-PAGE

2.5.1. TCA/deoxycholate precipitation of proteins

The required amount of crude plasma membranes as indicated for each experiment, was placed in a 1.5 ml microcentrifuge tube on ice and 6.3 µl of 2 % (w/v) 7-deoxycholic acid, sodium salt added. 700 µl of dH_2O and 250 µl of 24 % (w/v) trichloroacetic acid were then added sequentially and the tubes were vortexed briefly and incubated on ice for 15 mins before being centrifuged on an MSE microcentrifuge at 13,000 rpm for 5 min. The supernatant was discarded and the pellet dissolved by the addition of 20 µl of 1 M Tris base. Samples were then prepared for SDS-PAGE by the addition of 20 µl of SDS-PAGE sample buffer containing 5 mM urea, 0.4 mM DTT, 0.4 mM Tris HCl, pH8.0, with a few crystals of bromophenol blue.

2.5.2. Immunoprecipation of G-proteins

To 200 µl of SDS-denatured cell suspension from **section 2.2.7** was added 800 µl of solubilization buffer (1 % (w/v) Triton X-100, 10mM EDTA, 100mM NaH_2PO_4, 10 mM NaF, 100 mM Na_3VO_4, 50 mM HEPES, pH 7.2) and 100 µl of Pansorbin (Calbiochem). Samples were incubated at 4°C with continuous rotation for 1-2 h for nonspecific preclearing. Following centrifugation of the samples at 13,000x*g* for 1 min at 4°C, the supernatant was collected and subjected to immunoprecipitation by addition of 100 µl protein A-agarose along with 10 µl of the specific G-protein antiserum and rotated at 4°C for 3 h.

Immune complexes were then recovered by centrifugation at 13,000x*g* for 1min and washed by resuspension-centrifugation three times each with 1 ml of wash

buffer (1% (w/v) Triton X-100, 100 mM NaCl, 100 mM NaF, 50 mM NaH_2PO_4, 50 mM HEPES, pH 7.2, 0.5 % (w/v/) SDS). The final protein A-agarose pellet was resuspended in 50 µl of SDS-PAGE sample buffer containing only 5mM DTT and incubated at 100°C for 5 min. Samples were cooled down at room temperature for 5 min, spun briefly, and the supernatant was loaded onto the gel.

2.6. SDS-Polyacrylamide gel electrophoresis

2.6.1. 10% SDS-PAGE ; Lower resolving gel

SDS-PAGE was carried out on vertical slab gels of typical dimensions 11 cm x 14 cm x 1.5 mm containing 10 % (w/v) acrylamide using the discontinuous buffer system of Laemmli [1970]. Resolving gels were prepared by mixing stock acrylamide solution [30% (w/v) acrylamide, 0.8% (w/v) N,N'-methylene bisacrylamide], buffer 1 [1.5 M Tris HCl, pH 8.8, 0.4 % (w/v) SDS], 50 % (v/v) glycerol and dH_2O to give final concentrations of 10 % (w/v) acrylamide, 0.24 % (w/v) N,N'-methylene bisacylamide, 0.375 M Tris HCl, pH 8.8, 0.1 % (w/v) SDS, and 3 % (v/v) glycerol. Gel polymerization was achieved by the addition of TEMED and freshly prepared ammonium persulphate to final concentration of 0.1 % (v/v) and 0.05 % (w/v) respectively. Upper stacking gels were as described in **section 2.6.3** and electrophoresis was carried out as described in **section 2.6.4**.

2.6.2. 6M urea SDS-PAGE ; Lower resolving gel

In order to separate $G_q\alpha$ and $G_{11}\alpha$, SDS-PAGE was carried out on vertical slab gels of dimension 16 cm x 20 cm x 1.5 mm with the addition of 6 M urea to the

acrylamide mix described in **section 2.6.1** [Kim & Milligan, 1994]. Gels were allowed to polymerise for 4-5 h prior to electrophoresis.

2.6.3. Upper stacking gels

The upper stacking gel was prepared by mixing stock acrylamide solution (30 % (w/v), 0.8 % (w/v) N,N'-methyllene bisacrylamide), buffer 2 (0.5 M Tris HCl, pH 6.8, 0.4 % (w/v) SDS and dH_2O to give final concentrations of 3 % (w/v) acrylamide, 0.08 % (w/v) N,N'-methylene bisacrylamide, 0.125 M Tris HCl, pH 6.8, and 0.1 % (w/v) SDS. Gel polymerization was performed as described in **section 2.6.1**.

2.6.4. Electrophoresis running conditions

The running buffer contained 25 mM Tris HCl, pH 8.5, 0.192 M glycine and 0.1 % (w/v) SDS. Electrophoresis was toward the anode at 60 V, 30 mA per slab until the bromophenol blue dye front was 0.5 cm from the bottom (standard 10 % gels) or 100 V, 50 mA per slab until the prestained lactate dehydrogenase molecular weight marker was around 5 cm from the bottom (urea containing gels).

2.7. Staining of SDS-PAGE gels

2.7.1. Staining of gels with Coomassie Blue

Following electrophoresis, gels were soaked, with gentle shaking on a rotary shaker, for 1 hrs in 45 % (v/v) methanol, 10 % (v/v) acetic acid containing 0.25 % (w/v) Coomassie Blue R-250. Destaining was achieved by washing gels in several changes of 45 % (v/v) methanol, 10 % (v/v) acetic acid.

2.7.2. Staining of [^{3}H]containing gels

Following Coomassie Blue staining and destaining of the gels containing [^{3}H]palmitate labelled samples, gels were subjected to Amplify (Amersham) with gentle shaking for 1 hour to intensify the signal before being dried and subjected to autoradiography as described in **section 2.11**.

2.8. Western blotting

2.8.1. Transfer of proteins to nitrocellulose

Electrophoresed proteins were electroblotted onto nitrocellulose membranes essentially as described by Towbin *et al.*[1979]. Transfer was performed toward the anode at 2A for 2hrs in 25 mM Tris HCl, pH 8.3, 192 mM glycine and 20 % (v/v) methanol. Following transfer, blotted proteins were visualised by staining the nitrocellulose membrane in 0.1 % (w/v) Ponceau S, 3 % (w/v) trichloroacetic acid for approximately 2 min. After the positions of molecular mass standards were marked, the membrane was destained fully by washing in 1x PBS.

2.8.2. Incubation of nitrocellulose membranes with antisera

Non-specific binding sites on the membrane were then blocked by incubation in 5 % (w/v) gelatin in 1x PBS for 2 h at 37°C. The membrane was then incubated sequentially over night at 37°C in an appropriate dilution of primary antibody followed by a 1:250 dilution of donkey anti-rabbit IgG horseradish peroxidase conjugate. Membranes were washed sequentially for 3 x 10 min at room temperature between

each incubation with PBS containing 0.2 % (v/v) NP-40 followed by 1x PBS. All antibody dilutions were carried out in 1 % (w/v) gelatin in PBS containing 0.2 % (v/v) NP-40.

2.8.3. Development of immunoblots

1 ml of freshly prepared 1 % (w/v) o-dianisidine hydrochloride was added to 40 ml of PBS, pH7.4, and the whole added to the newly washed nitrocellulose membranes. Visualization of cross-reacting polypeptides was initiated by addition of 5 µl of stock H_2O_2 (30 % v/v). Immediate termination of the peroxidase reaction was achieved by immersing the membranes in 1 % (w/v) sodium azide. The developed immunoblot was rinsed in H_2O and allowed to dry.

2.9. Densitometric analysis of immunoblots

Immunoblots were densitometrically scanned on a Bio-Rad GS-360 imaging densitometer and analyzed on an Apple Macintosh Quadra 800 microcomputer.

2.10. Autoradiography

Destained gels from experiments containing ^{35}S-labelled protein or Amplify (Amersham) treated gels were dried onto Whatman No.3 chromatography paper under a vacuum line attached to a Bio-Rad 583 gel drier at 80°C for 2 h. Autoradiogrphy was performed at -80°C for an appropriate time on Fuji X-ray film (^{35}S-labelled protein) or Hyperfilm ([^3H]-labelled proteins) in a Kodak X-o-matic cassette with intensifying screens. Films were developed in a Kodak X-o-mat developing machine.

2.11. Phosphorimaging

Gels containing ^{35}S-labelled proteins were dried as described in the preceding section and subjected to phosphorimaging for the indicated time before being analysed on a Fujix BAS 1000 phosphorimager linked to an Apple Macintosh Quadra 650 microcomputer.

2.12. Radioligand binding assays

2.12.1. Binding experiments with [^{3}H]TRH

These were performed routinely with approximately 10 nM [^{3}H]TRH at 30°C for 1 h in 20 mM Tris/HCl (pH 7.4)/ 50 mM sucrose/ 20 mM $MgCl_2$/ 100 μM guanosine 5'-[$\beta\gamma$– imido]triphosphate (Gpp[NH]p) (buffer B) in the absence and presence of 100 μM TRH to define maximal and non-specific binding, respectively. Specific binding, defined as above, represented ~80 % of the total binding of [^{3}H]TRH. In experiments designed to assess the maximal binding capacity of membranes of transfected cells for this ligand, the specific radioactivity of a single concentration (~10 nM) of [^{3}H]TRH was varied by addition of TRH in a final volume of 50 μl, and measured specific binding was subsequently corrected on this basis.

All binding experiments were terminated by rapid filtration through Whatman GF/C filters which had been presoaked in assay buffer, followed by three washes of the filter with 5 ml ice-cold buffer B using a Brandell Cell Harvester. Filters were soaked overnight in 10 ml of Ultima Flo scintillation fluid cocktail prior to counting in a Rackbeta scintillation counter. In some instances, the binding data were manipulated according to the equations derived by Scatchard [1949] or DeBlasi *et al.* [1989].

2.12.2. Binding experiments with [^{3}H]prazosin

Binding assays were performed by the methods of Morrow & Crese [1986] and initiated by the addition of 10-20 µg of protein to an assay buffer (50 mM Tris-HCl, 0.5 mM EDTA, pH 7.4) containing [^{3}H]prazosin (0.005 - 1 nM in saturation assays and 1 nM for competition assays) in the absence or presence of increasing concentrations of the test drugs in a final volume of 250 µl. Nonspecific binding was determined in the presence of 10 µM phentolamine. Reactions were incubated for 30 min at 25°C, and bound ligand was separated from free by vacuum filtration through Whatman GF/C filters as indicated in **section 2.12.1.**

2.13. Adenylyl cyclase assay

This was essentially the method of Salomon and co-workers [1979], except the amount of [α-^{32}P]-ATP was reduced to 1µCi per sample. This assay measures the production of [^{32}P]-cAMP from the substrate [α-^{32}P]-ATP. The cAMP thus produced is separated from the unreacted [α-^{32}P]-ATP by a two-step column method.

2.13.1. Sample preparation and reaction

Assays were performed in a reaction volume of 100 µl using 50 µl of an assay mix comprising 4 mM creatine phosphate, 100 mM NaCl, 100 U/ml creatine phosphokinase, 200 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.4 mM ATP (pH 7.5), 2 mM cAMP, 2 µM GTP (pH 7.5), [α-^{32}P]-ATP ($1x10^6$ cpm), 10-20 µg of membrane protein and an appropriate ligand. Reaction tubes were kept on ice at all times and the reaction started by removal to a 30°C water bath. After 15 min the reactions were terminated by placing onto an ice-slurry and adding 100 µl stopper

solution which comprised 2 % (w/v) SDS, 45 mM ATP, 1.3 mM cAMP. 50 µl of [8-^{3}H]-3'5'cAMP (approximately 10000 cpm) was added to measure recovery from the coulumn. Reactions were then boiled for 10 min, 750 µl of dH_2O was added, and the [^{32}P]- and [^{3}H]-cAMP content determined.

2.13.2. Preparation of Dowex and alumina columns

The methods used to quantitate the amount of cAMP produced by each sample was identical to that of Salomon [1979] and involves the separation of cyclic AMP from other nucleotides by Dowex and then Alumina chromatography. Dowex H^+ 50x4 (200-400) was washed sequentially in twice its volume with 1 M HCl, 1 M NaOH and finally 1 M HCl. The Dowex was mixed with dH_2O to a slurry (1:1,v/v) and then 2 ml added to glass wool stoppered columns. The water was allowed to drain out and the columns washed with 2 ml 1 M HCl and stored at room temperature. Prior to use, the columns were washed with 4 ml 1 M NaOH and with 4 ml of 1 M HCl followed by 20 ml of dH_2O.

Alumina columns were prepared by the addition of 1 g dry neutral alumina to glass wool stoppered columns and the columns washed with 12 ml 1 M imidazole buffer (pH 7.3) followed by 15 ml of 0.1 M imidazole (pH 7.3) and then stored at room temperature. On the day of use, each column was washed with 8 ml of 0.1 M imidazole (pH 7.3).

2.13.3. Separation of cAMP on Dowex columns

Prior to sample chromatography, the nucleotide elution profiles for each column were determined. This was performed by applying [^{3}H]-cAMP to the columns and determining the elution volume. Stock [^{3}H]-cAMP was diluted in dH_2O to give

approximately 10000 cpm in 50 µl. 50 µl cAMP solution was added to 950 µl of dH_2O and applied to a Dowex column. The cAMP was eluted from the column by successive washes of the column with 0.5 ml H_2O. Fractions were collected and mixed with 5ml of Ultima Flo scintillation cocktail and radioactivity determined by scintillation counting using a dual label programme. The elution volumes required to elute the cAMP from the Dowex columns were then determined graphically. Recovery from the Dowex columns was always greater than 70 %. The elution volume required to elute the cAMP from the alumina columns was determined as for the Dowex columns except the eluting buffer was 0.1 M imidazole (pH 7.3). Recoveries were similar to that obtained for the Dowex columns.

2.13.4. Determination of cAMP produced by membrane fractions

Samples (total volume of 950 µl) were added to prepared Dowex columns and the ATP eluted with 0.5 ml water. Dowex columns were then washed with 6 ml dH_2O and the combined eluate was applied directly onto the alumina columns. The cAMP fraction was eluted into vials containing 14 ml scintillation fluid with 6 ml imidazole (pH 7.3). The recovery of cAMP from the columns was routinely greater than 75 %. When recovery fell below 60 % the columns were discarded and fresh columns prepared.

The cAMP fractions obtained were counted on a dual label scintillation counting programme which automatically corrected for spillover from each channel. The amount of cAMP produced by each sample was calculated by taking into account the recovery from each column, based on the recovery of the [^{3}H]cAMP internal standard. Data were then calculated as pmoles of cAMP produced per min per mg membrane protein, and the assay was sensitive to approximately 5 pmoles/min/mg.

2.14. Total inositol phosphate assay

2.14.1. Preparation of Dowex formate

The conversion of Dowex chloride (1x8-200, Sigma) to Dowex formate was achieved by the addition of formic acid in two steps. 100 g of Dowex chloride beads were swirled gently with 1 L of dH_2O, allowed to settle, and the water containing fines decanted. This process was repeated for a total of 3 washes. The beads were then transferred to a sintered funnel and washed with 2 L of 2 M NaOH to exchange X-Cl with X-OH. Subsequently, they were washed with 5 - 6 L of dH_2O to remove NaCl and excess NaOH, and then washed with 1 L of 1 M formic acid. Finally 20-30 L of dH_2O were applied to wash away excess formic acid until the pH was constant at 5-5.5 as measured with narrow range pH paper (pH4-6). The prepared Dowex was stored at room temperature in an approximately 1:1 ratio of Dowex : dH_2O.

Characterizationof the Dowex-formate was achieved by applying a standard amount of [^{3}H]IP_3 in 1 ml of 5 mM NaTB/0.5 mM EDTA to 1 ml of the resin. The eluate was collected in 1 ml fractions which were then mixed with 4 ml scintillation fluid. [^{3}H]IP_3 was then quantified by liquid scintillation counting and % recovery of the standard calculated.

2.14.2. Preparation of samples

Cells were seeded in 24-well plates and labelled to isotopic equilibrium by incubation with 1 μCi/ml myo-[2-^{3}H]inositol in 0.5 ml inositol-free DMEM containing 2 % (v/v) dialyzed newborn calf serum for 24 hrs. On the day of experiments, the labeling medium was removed, and the cells were washed twice with 0.5 ml of Hank's modified balanced solution (1.25 mM $CaCl_2$, 5.4 mM KCl, 0.5 mM $MgCl_2$, 0.85 mM

$MgSO_4$, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.33 mM NaH_2PO_4, pH7.4), containing 2 % (w/v) bovine serum albumin and 10 mM glucose (HBG). Cells were then washed twice for 10 min with HBG supplemented with 10 mM LiCl (HBG/LiCl) and subsequently stimulated with agonist in HBG/LiCl for 20 min at 37°C. Reactions were terminated by the addition of 0.5 ml of ice-cold methanol, and then cells were scraped and transferred to insert vials. Chloroform was added to a ratio of 1:2 ($CHCl_3$: MeOH) and samples extracted for 1 h prior to the addition of chloroform and water to a final ratio of 1 : 1 : 0.9 ($CHCl_3$: MeOH : dH_2O). To separate the phases of water and lipid completely, the vials were centrifuged at 3000 rpm for 5 min in a tabletop centrifuge, and 800 µl of the upper phase was taken and transferred into new vials. Lower phase (300 µl) was allowed to evaporate and then samples were subjected to liquid scintillation counting.

Dowex-formate (0.5 ml) was added to each 800 µl upper phase fraction containing inositol phosphates together with 3 ml of dH_2O. The Dowex-formate beads were allowed to settle out and the supernatant was removed by aspiration. Unbound label was then removed by succesive washing of the beads with 3 ml volumes of dH_2O, solution I (5 mM NaTB/60 mM $HCOONH_4$), and with dH_2O again. Finally, after aspiration, 1 ml of solution II (1 M $HCOONH_4$/0.1 M HCOOH) was added, beads were allowed to settle, and 800 µl of the supernatant was mixed with 4 ml of scintillation fluid and counted as above.

2.15. Phospholipase D assay

2.15.1. Preparation of solvents and TLC plates

To prepare the TLC running solvent, 2,2,4-Trimethylpentane (isooctane) : ethylacetate : glacial acetic acid : dH_2O (solvent I) of a final ratio 5:11:2:10 were mixed

and shaken in a 500 ml separating funnel. After settling down, the lower phase was drained and discarded, and the organic upper phase was placed in a glass TLC tank. TLC plates (Whatman LK5 DF) were pre-run in solvent I for 2 hrs to equilibrate each lane, dried for 10 min in a fume hood, and baked at 120°C for 20 min prior to loading samples.

2.15.2. Preparation of samples

Cells were seeded in 24-well plates in DMEM containing 5 % newborn calf serum. When the cell confluency reached about 70 %, the medium was replaced with 500 µl of the above medium containing 4 µCi/ml of [9,10 (n)-^{3}H]palmitic acid (51 mCi/ml) . 24 - 36 hrs later, the medium was replaced with 500 µl of HBG as described in **section 2.14.2** to wash away un-incorporated [^{3}H] palmitic acid and incubated at 37°C for 20 min. Cells were then incubated with 500 µl of HBG containing 0.3 % (v/v) butan-1-ol (HBG/butanol) at 37°C for 10min. After aspirating the HBG/butanol, cells were treated with 200 µl HBG/butanol containing test drugs for 20 min. Reactions were terminated by removal of the drug containing solution and immediately adding 500 µl of ice-cold Analar grade methanol. Cells were scraped into screw-capped glass vials, and remaining cells were removed with a further 200 µl methanol.

Samples were extracted with 700 µl of analar grade chloroform at room temperature for 15 min and 585 µl dH_2O then added to give a final ratio of chloroform : methanol : water of 1 : 1 : 0.8 prior to centrifugation at 1200 rpm in a table top centrifuge for 5 min. 450 µl of the lower chloroform phase was taken into new glass vials and dried down by vacuum centrifugation in a Jouan RC centifugal evaporator for 1 hr. Dried samples were resupended in 2 x 25 µl of solvent II [chloroform : methanol, (19 : 1, v/v)] and loaded onto TLC plates by using a positive displacement pipette. Samples were applied to the adsorbant strip at the bottom of each lane between 1 cm

from the bottom and 0.5 cm from the top of the adsorbant strip. 7 µl of standard phosphatidylbutanol was applied to alternate lanes. The plates were run to 1 - 2 cm from the top in solvent I as indicated in **section 2.15.1**, and then removed to dry in the fume hood for 10 min. The position of phosphatidylbutanol was located by staining with iodine for 2-3 min. This area on each lane was then scraped and transferred to insert vials contained 4 ml scintillation fluid and counted.

2.16. Regulation of activity/mobility of ERK-1

The activation of extracellularly regulated kinase-1(ERK-1) was determined by an electrophoretic-mobility shift assay [Marshall *et al.*,1992]. Cells were grown in DMEM containing 5 % newborn calf serum in 100mm tissue culture dishes. When cells were nearly confluent, they were maintained in serum-free DMEM for 48 h. The cells were then stimulated by an appropriate ligand in serum-free DMEM at 37°C for 5 min. Plates were transferred to 4°C and medium was removed. Cells were washed with 10 ml ice-cold 1xPBS and subsequently lysed in 450µl of lysis buffer containing 25 mM Tris/HCl, pH7.5, 40 mM *p* -nitrophenol, 25 mM NaCl, 10 % (v/v) ethylene glycol, 10 µM dithiothreitol, 0.2 % (w/v) NP-40, 1 µg/ml aprotinin, 1 mM sodium vanadate, 3.5 µg/ml pepstatin A and 200 µM phenylmethanesulphonyl fluoride (PMSF) at pH 7.5.

The cells were then scraped and transferred into microfuge tubes, and solubilized by passing approximately 10 times through a 1 ml syringe with a 26G needle. Following centrifugation of the lysed samples in a microfuge at 13,000 rpm for 5 min at 4°C, the supernatant was transferred into a new microfuge tube and 100 µl of Laemmli loading buffer was added as described in **section 2.5.1** prior to boiling for 5 min.

Samples were applied to 10 % SDS-PAGE containing 6 M urea as described in **section 2.6.2** and samples were immunoblotted, as described in **section 2.9**, using an anti-peptide antiserum raised against amino acids 325 - 345 of ERK-1, also referred to as p44 MAP kinase.

2.17. Growth of *Escherichia coli* strains

E.coli strains were routinely grown in Luria-Bertani medium (LB-medium) which consisted of 10 g/L bacto-tryptone, 5 g/L of bacto-yeast extract, 10 g of NaCl, pH 7.0. *E.coli* cells harbouring plasmids with antibiotic resistance genes were selected on LB agar plates supplemented with the appropriate antibiotics. Liquid cultures of transformed strains were also supplemented with the relevant antibiotics and were routinely propagated at 37°C with continuous shaking.

All media and solutions, unless otherwise stated, were sterilized by autoclaving for 15 min at 121°C. Ampicillin (50 mg/ml dH_2O), kanamycin (10 mg/ml dH_2O) and tetracyclin (5 mg/ml ethanol) were prepared as stock solutions and sterilized by filtration through 0.22 μm syringe filters.

Stock *E.coli* cells were streaked onto LB agar plates (15 g/L Bacto-agar in LB medium) in the absence of any antibiotic. The agar plates were incubated overnight at 37°C. A single colony was picked for expansion and grown up overnight at 37°C in 5 ml of L-broth. The liquid culture was then used to prepare competent cells. MC 1061/P3 cells (Invitrogen) transformed with plasmid pcDNA I, and JM 109 cells transformed with plasmid pCMV and DH5α cells transformed with plasmid pcDNA3 or recombinant plasmids derived from them were grown in LB medium or on LB agar plates supplemented with 50 μg/ml of ampicillin. A full length version of the rat thyrotropin-releasing hormone (TRH) receptor long isoform cDNA (2.2 kb) was sucloned into the EcoR I site of pcDNA I. A full length version of the rat TRH receptor

short isoform was obtained in pBluescript SK⁻ from Dr.Pilar de la Pena, University of Oviedo, Spain. This was subcloned into pCMV5 between the Xba I and BamH I sites of the multiple cloning region.

For long term storage, bacteria were conserved in 15 % (v/v) glycerol and 85 % (v/v) LB medium at -80°C. Basically, sterile 80 % (v/v) glycerol was added to the overnight culture to a final concentration of glycerol of 15 % (v/v). 1 ml aliquots were snap-frozen in a slurry of dry ice and ethanol and stored at -80°C until required.

2.18. Preparation of chemically competent *E.coli*

Competent cells for heat-shock transformation were prepared according to Hanahan [1985]. A liquid culture (5 ml) of *E.coli* cells which had been grown overnight at 37°C, was inoculated into 500 ml of LB medium and cultured at 37°C in a shaking incubator until an OD_{600} of approximately 0.25 absorbance units (equivalent to approx. 1 x 10^8 cells/ml) was reached. The cell suspension was then cooled on ice for approximately 30 min and cells recovered by centrifugation at 10,000 rpm for 10 min at 4°C in a Beckman J-2-21 centrifuge containing a JA 14 rotor. *E.coli* cell pellets were resuspended in a total volume of 40 ml of ice-cold sterile buffer I [100 mM $RbCl_2$, 50 mM $MnCl_2$, 30 mM CH_3COOK, 10 mM $CaCl_2 \cdot 2H_2O$, 15 % (w/v) glycerol, pH 5.8]. The suspension was left on ice for 15 min and recentrifuged as before. Cell pellets were resuspended in a total volume of 3.5 ml of ice-cold buffer II [10 mM $RbCl_2$, 10 mM MOPS, 75 mM $CaCl_2 \cdot 2H_2O$, 15 % (w/v) glycerol, pH 6.8] and pooled. The suspension was left on ice for 15 min and then 250 µl aliquots were snap frozen in a slurry of dry-ice and ethanol, and stored at - 80 °C.

2.19. Transformation of chemically competent *E.coli* with plasmid DNA

Competent cells were thawed on ice and 250 µl added to plasmid DNA (50-100 ng of supercoiled plasmid DNA or 5 µl of a ligation reaction) followed by incubation on ice for 15 min. The cells were then heated at 42°C for 90 seconds and returned to ice for 2 min prior to the addition of 800 µl of LB medium. Cells were allowed to recover by incubation at 37°C for 1 h in a shaking incubator.

Aliquots of 100 µl and 200 µl were spread onto LB agar plates containing 50 µg/ml of ampicillin. Plates were incubated overnight at 37°C and transformed colonies selected and grown up overnight in 10 ml LB medium containing 50 mg/ml ampicillin.

2.20. Preparation of plasmid DNA

2.20.1. Preparation of double stranded plasmid DNA by alkaline lysis

This method of plasmid DNA isolation was used to prepare plasmid DNA for diagnostic restriction digestion only [Birnboim and Doly, 1979]. A total of 3 ml liquid overnight culture was sedimented by centrifugation in a micricentrifuge at 13,000 rpm for 3 min. Cell pellets were resuspended in 200 µl of solution I (50 mM glucose, 25 mM Tris/HCl, pH 8.0, 10 mM EDTA). Cells were then lysed by the addition of 200 µl of freshly prepared solution II [0.2 M NaOH, 1 % (w/v) SDS]. After several inversions, samples were neutralized with 200 µl of solution III (60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of dH_2O, giving final concentrations of 3 M potassium and 5 M acetate). After mixing by gentle inversion, plasmid DNA was recovered in the supernatant following centrifugation in a microfuge at 13,000 rpm for 5 min. Plasmid DNA was then isolated by precipitation with ethanol

as described in **section 2.22.** DNA precipitates were resuspended in 50 µl of 20 µg/ml DNase-free RNase in sterile dH_2O.

2.20.2. Small scale preparation of double stranded plasmid DNA

This was carried out as described by the manufacturer (Promega). A total of 3 ml of overnight culture was centrifuged as described in **section 2.20.1** and the resulting pellet resuspended in 200 µl buffer 1 (50 mM Tris/HCl, pH 7.5, 10 mM EDTA, 100 µg/ml RNase A). Buffer 2 (200 µl) [0.2 M NaOH, 1 % (w/v) SDS] was added and the cells lysed by inversion. Buffer 3 (200 µl) (1.32 M potassium acetate) was added to neutralize the solution, again by inversion, and centrifuged at 13,000 rpm in a microcentrifuge for 5 min. Purification resin (1 ml) was added to the supernatant and plasmid DNA was isolated by purification on a minicolumn according to the manufacturers instructions.

2.20.3. Preparation of double stranded plasmid DNA for automated DNA sequencing

This was achieved by a modification of the method in the preceding section. An entire 10 ml overnight culture was centrifuged in a Beckman TJ6 benchtop centrifuge for 15 min at 3000 rpm. The cell pellet was resuspended in 300 µl of buffer 1 (**section 2.20.2**), lysed using 300 µl of buffer 2 and then neutralized with 300 µl of buffer 3 as decribed in **section 2.20.2.** Following centrifugation of the lysate, purification resin (1 ml) was added and the whole applied to a minicolumn. Resin was washed with 3 ml of wash buffer (0.09 M NaCl, 10 mM Tris/HCl, pH 7.5, 2.2 mM EDTA, 55 % (v/v) analar ethanol) and purified DNA eluted using 100 µl H_2O. The resulting DNA solution was stored at -20°C until required.

2.21. Ethanol precipitation of DNA

DNA was precipitated from solution with 0.1 vol of 3 M sodium acetate, pH 7.0 and 2.5 vol of absolute ethanol at - 20°C for 20 min. DNA was recoved by centrifugation at 13,000 rpm for 10 min. The pellet was subsequently washed with 70 % (v/v) ethanol at - 20°C, dried at 65°C on a heating block, and then resuspended in deionized H_2O.

DNA from the preparation of plasmid DNA described in **section 2.20.1** was precipitated at room temperature without the addition of 3 M sodium acetate.

DNA obtained from PCR amplication which was to be used in automated sequencing was precipitated on ice in the presence of 0.1 volumes of 3 M sodium aceteate, pH 5.2.

2.22. Quantitation of DNA and RNA

The concentraion of DNA in a given sample was determined by measuring the absorbance at 260 nm (A_{260}) of a 1:200 dilution of the sample in sterile Milli Q H_2O, assuming 1 absorbance unit was equivalent to 50 µg/ml of double stranded DNA, 40 µg/ml for single-stranded DNA and RNA, and 20 µg/ml of single stranded oligonucleotide. The purity of the DNA was assessed by measuring the A_{280} in parallel and calculating the A_{260} : A_{280} ratio. A ratio of approximately 1.8 for DNA and 2.0 for RNA was considered to be sufficiently pure for use otherwise a phenol : chloroform extraction was carried out as described in **section 2.23.**

2.23. Phenol/chloroform extraction of DNA

DNA was initially extracted with phenol:chloroform (50:50, v/v) and then with chloroform:isoamyl alcohol (24:1, v/v). Extracted DNA was then precipitated with ethanol as described in section 2.21.

2.24. Digestion of plasmid DNA with restriction endonucleases

Plasmid DNA, generally 1 µg, was digested in a volume of 10 µl using buffer condition recommended by the manufacturers and with 4-10 units of appropriate restriction enzymes. The composition of the buffers used are indicated in **Table 2.2.a** and the enzymes used in these buffers are indicated in **Table 2.2.b**.

2.25. Separation of digested plasmid DNA by electrophoresis

Digested DNA was routinely analysed by agarose gel electrophoresis as described by Sambrook *et al.*[1989]. Samples were prepared by the addition of 6x loading buffer [30 % (v/v) glycerol, 0.25 % (w/v) bromophenol blue] to a 1x final concentration. Samples between 0.5 and 5 kb were electrophoresed through 0.8 % (w/v) agarose gels containing 1x TAE buffer (40mM Tris acetate, 1 mM EDTA, pH 8.0) and 2.5 mg/ml ethdium bromide. For DNA fragments smaller then 0.5 kb, electrophoresis was performed using 1.5-2 % (w/v) agarose.

Electrophoresis was carried out, towards the anode, at 75 - 100 mA at room temperature in a horizontal electrophosis tank containing 1x TAE buffer. Ethidium bromide stained DNA fragments were visualized under UV light and photographed. Size was assessed by comparison with a 1 kb ladder.

2.26. Purification of DNA from agarose gels

Table 2.2. Restriction enzyme buffer compositions and use by various restriction enzymes

Digestion of DNA by restriction enzymes was carried out using the buffer conditions recommended by the manufacturer. The conmposition of the buffers used are listed in **panel A**. Enzymes used together with their respective buffers and reaction conditions are shown in **panel B**.

Table 2.2.

A.Buffers

Buffer	pH (at 37°C)	Tris/HCl (mM)	$MgCl_2$ (mM)	NaCl (mM)	$C_4H_6O_4Mg$ (mM)	DTT (mM)
A	7.5	6	6	6	-	1
B	7.5	6	6	50	-	1
C	7.9	10	10	50	-	1
D	7.9	6	6	150	-	1
E	7.5	6	6	100	-	1
H	7.5	90	10	50	-	-
J	7.5	6	7	50	-	1
M=	7.8 ^	25+	-	100 *	10	1

B.Enzymes

Buffer	Enzymes	Buffer	Enzymes
A	*Apa* I	**B**	*Stu* I
C	*Cla* I, *Hae* III	**D**	*Bgl* I, *Bgl* II, *Nco* I
			Not I, *Sal* I, *Xba* I, *Xho* I
E	*BamH* I, *Hind* III	**H**	*EcoR* I, *Pst* I

+ Tris Acetate * Potassium Acetate ^ at 25°C
= Buffer M was used only in double digestions

In order to purify a desired DNA fragment, DNA was electrophoresed through low-melting point agarose at appropriate concentrations at 50 mA at 4°C. The fragment of interest was excised from the gel and was then melted in a heating block (Techne) at 70°C for 5 min. Immediately, 1 ml of Promega PCR purification resin was added and the contents mixed by vortexing for 30 sec. The resin was applied to a Promega mini column and was then washed with 80 % (v/v) isopropanol. Purified DNA was eluted with preheated sterile Milli Q H_2O as described in **section 2.20.2.**

2.27. Phosphatase treatment of DNA fragments

To prevent recircularization of vector DNA during ligation, digested vector DNA was treated with CIAP to remove the 5'phosphate group. CIAP treatment was performed using 0.5 units of enzyme in a buffer (50 mM Tris/HCl, pH 9.0, 1mM $MgCl_2$, 100 mM $ZnCl_2$, 1 mM spermidine) at 37°C for 2 h. The reaction was stoped by the addition of SDS and EDTA to final concentrations of 0.5 % (w/v) and 5 mM, respectively, followed by heat treatment at 75°C for 10 min. Vector DNA was purified from the reaction mixture by extraction with phenol:chloroform (50:50, v/v) followed by extraction with chloroform:isoamyl alcohol (24:1, v/v) and precipitation with ethanol as described in **section 2.21.**

2.28. Ligation of DNA fragments

Ligation of vector and insert DNA was routinely carried out overnight at 10-16°C in a reaction volume of 10 µl containing 1x ligation buffer (30 mM Tris/HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP), 1.5 units of T4 DNA ligase plus vector and insert DNA fragments. Reactions were performed using ratios of

vector:insert of 1:1, 1:5, and 1:10. Ligated DNA was transformed as described in **section 2.19.**

2.29. Reverse transcriptase-PCR

2.29.1. RNA extractions

Total RNA was extracted by the acid phenol/guanidinium thiocyanate method of Chomczynski and Sacchi [1987]. Growth medium was withdrawn from a 75cm^2 flask and 750 µl RNAzol (Biogenesis) was added. Cells were scraped into a sterile microfuge tube and solubilized by passing the lysate a few times through a sterile pipette and then freezing at -80°C for 30 min. RNA was extracted from the lysate by adding 100 µl of chloroform, mixing vigorously and placing on ice for 5 min. After centrifugation at 13,000 rpm for 15 min at 4°C, an equal volume of isopropanol was added and total RNA was allowed to precipitate on ice for 30 min. Following recovery by centrifugation as above, RNA pellets were washed with 75 % (v/v) ethanol (-20°C), dried and resuspended in 30 µl of 1 mM EDTA, pH 7.0.

2.29.2. Reverse transcription

Prior to reverse transcription, residual genomic DNA was removed by digestion with RNase-free DNase. RNA (30 µg) was ethanol precipitated and dissolved in 40 mM Tris/HCl, pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$. RNAguard(17 units) and RNase-free DNase (5 units) were added and digestion was carried out at 37°C for 15 min. Reactions were terminated by purification of the RNA through a clean-up column (Promega). RNA was then precipitated with NaCl/ethanol as above and redissolved in 20 µl of Milli Q H_2O.

Table 2.3. Oligonucleotide primers and PCR conditions

Oligonucleotide primers for the RT-PCR amplification of TRH receptor isoforms, α_1 adrenergic receptor subtypes and CAM α_{1B} adrenergic receptor cDNA used in this study were synthesized by Dr. V. Math on an Applied Biosystems DNA synthesiser at the Biochemistry department, University of Glasgow.

Table 2.3

A.Oligonucleotide primers

TRH receptor isoforms	Sense	5'-CAACCTCATGTCTCAGAAGTTTCG-3'
	Antisense	5'-AATGAAGACCTTCGATCAGTTGG-3'
$\alpha_{1A/D}$ adrenocptor	Sense	5'-TTGGAAGGAGCCAGTGC-3'
α_{1B} adrenoceptor	Sense	5'-ACAAGGAATGCGGAGTC -3'
$\alpha_{1A/D}$ & α_{1B} AR	Antisense	5'-GAAGGCGCGCTTGAACT-3'
α_{1C} adrenoceptor	Sense	5'-TTCTCCGTGAGACTGCT-3'
	Antisense	5'-CCAGGTCCTTGTGCTGT-3'
CAM α_{1B} AR	Sense	5'-GACGACAAGGAATGCGGAGTC-3'
	Antisense	5'-GTCCACGGCCGATAGGTGTAA-3'

B.PCR conditions

Receptor	**Denaturation**	**Annealing***	**Extension**	**Cycles**
TRHr isoforms	95°C/5min	60°C/1min	72°C/2min	1
	95°C/1min	60°C/1min	72°C/3min	30
	95°C/1min	60°C/1min	72°C/5min	1
α_1AR subtypes	95°C/5min	49°C/30sec	72°C/1min	1
	95°C/30sec	49°C/30sec	72°C/1min	30
	95°C/30sec	49°C/30sec	72°C/5min	1
α_{1B} CAM	95°C/5min	60°C/2min	72°C/2min	1
	94°C/1min	60°C/2min	72°C/3min	33
	94°C/1min	60°C/2min	72°C/5min	1

* The annealing temperature was calculated to be 5-10°C lower than the melting temperature of the oligonucleotides.

Reverse transcription was carried out with 0.5-10 µg of DNase-treated RNA using a First-strand cDNA synthesis kit (Pharmacia). Basically RNA was denatured by heating at 65°C for 10 min. Bulk cDNA synthesis reaction mix (5-10 µl) with 0.2 µg of oligo $d(T)_{18}$ primer, 1.8 mM each dNTP and buffer (45 mM Tris, pH 8.3, 68 mM KCl, 15 mM DTT, 9 mM $MgCl_2$, 0.08 mg/ml BSA) was added and reverse transcription was allowed to proceed at 37°C for 1 h. Reactions were stopped by heating at 90°C for 5 min. Reverse transcribed RNA was then stored at -20°C or used directly for PCR.

2.29.3. PCR

Amplifications were routinely performed in a reaction volume of 50 µl containing 50-200 ng of DNA template, dNTPs (0.2 mM each dATP, dCTP, dGTP, dTTP), 25 pmoles each of sense and antisense oligonucleotide primers, as indicated in **Table 2.3a**, 1.5 mM $MgCl_2$, 1x thermophilic buffer (50 mM KCl, 10 mM Tris/HCl, pH 9.0, 0.1 % Triton X-100) and 2.5 units of *Taq* DNA polymerase (Promega). Samples were overlayed with light mineral oil to prevent evaporation, and the reaction was carried out on a Hybaid Omnigene thermal cycler as indicated in **Table 2.3b** for each PCR condition.

For site-directed mutagenesis or for the creation of new restriction enzyme cleavage sites, PCR was carried out with *Pfu* DNA polymerase (Stratagene). Reactions were performed in 20 mM Tris/HCl, pH 8.2, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.1 % Triton X-100, 10 µg/ml BSA using 25 pmol each primer and dNTPs as above. *Pfu* DNA polymerase (5 units) was added after the reaction mixture had been heated to 95°C for 10 min. The temperature cycling condition (30 cycles) were 94°C for 42 sec (denaturation), annealing (temperature as noted in **Table 2.3b**) for 1 min,

and 72°C for 3-10 min (extension), depending on the DNA fragment size to be amplified.

2.30. DNA sequencing

In order to yield accurate sequence data for ss and ds DNA templates, symmetrical PCR fragments, and site-directed mutants, cycle DNA squencing was performed.

2.30.1. Sample preparation for cycle sequencing

Sequencing of double stranded DNA was performed using automated cycle squencing with the *Taq* DyeDeoxy DNA sequencing kit (Applied Biosystems). Reactions were performed in a volume of 20 µl using 1 µg of plasmid DNA in 5 µl of dH_2O together with 4 µl of 5x TACS buffer, 1 µl dNTP mix, 1 µl each DyeDeoxy A, T, G, C Terminator, 2 µl ampliTaq DNA polymerase and 3.2 pmol of primer for double stranded DNA sequencing (0.8 pmol for ssDNA). dH_2O was added to give a final reaction volume of 20 µl.

Reactions were overlayed with mineral oil and cycle sequencing (30 cycles) was performed on a Hybaid OmniGene thermal cycler preheated to 96°C with cycling conditions of 96°C, 30 sec (denaturation), 50°C, 50 sec (annealing), and 60°C, 4 min (extension). Excess terminator dyes were then removed by two extractions with phenol:water:chloroform (68:18:14) and DNA was recovered by precipitation with ethanol as descibed in **section 2.21**. DNA pellets were dried at 85°C for 1 min in a thermal cycler and then either stored at -20°C or resuspended in 4 µl of formamide reagent (a mixture of 5 µl deionized formamide and 1 µl 50 mM EDTA, pH8.0) and electrophoresed immediately.

2.30.2. Preparation of PAGE gels for DNA sequencing

Samples were analysed by polyacrylamide electrophoresis using gels comprising 6 % acrylamide Sequagel-6 solution (National Diagnostics) and ran in 1x TBE buffer (0.9 M Tris, 0.9 M boric acid, 0.02 M EDTA, pH 8.0). Polymerization was initiated by addition of 600 µl of 10% APS per 75 ml of gel solution. Gels were cast using an ABI 373 DNA sequencing apparatus (Applied Biosystems) and were pre-run at 1200 V/ 30 mA for 30 min. Prior to loading, samples were heated to 95°C for 2 min to ensure complete denaturation. Electrophoresis was performed overnight as pre-run conditions. Automated sequencing data were then analysed using Gene Jockey software program.

Chapter 3

Comparison of the signalling properties of the long and short isoforms of the rat thyrotropin-releasing hormone (TRH) receptor

Chapter 3. Comparison of the signalling properties of the long and short isoforms of the rat thyrotropin-releasing hormone (TRH) receptor

3.1. Introduction

The hypothalamic neuropeptide thyrotropin-releasing hormone (TRH) plays pivotal roles in the pituitary-thyroid axis. This tripeptide (pyroglutamyl-histidyl-prolineamide) is known to rapidly stimulate the synthesis and release of pituitary thyroid stimulating hormone (TSH) as well as prolactin (PRL) [Murdoch *et al.*, 1985; Laverriere *et al.*, 1983; Kourides *et al.*, 1984; Shupnik *et al.*, 1986]. Although TRH was first discovered in the hypothalamus and characterised by its ability to stimulate TSH production from pituitary cells, it has since been identified in several extrahypothalamic brain structures and in the gastrointestinal tract [Hokfelt *et al.*, 1989]. Putative TRH receptors have been characterised in detail in terms of affinity for TRH and related peptides [Hinkle *et al.*, 1974; Vale *et al.*, 1977; Sharif & Burt, 1983; Szirtes *et al.*, 1986; Phillips & Hinkle, 1989; Ladram *et al.*, 1992], and TRH interaction with those plasma membrane receptors has been shown to influence several potential second messenger systems.

The isolation of cDNA clones encoding mouse [Straub, *et al.*, 1990] and rat [de la Pena, *et al.*, 1992, Sellar *et al.*, 1993] versions of the TRH receptor confirmed these to be single-polypeptide putative seven-transmembrane-element receptors, as anticipated for G-protein coupled receptors. Although a series of reports have indicated that these receptors couple in a pertussis-toxin-insensitive fashion to the stimulation of inositol phosphate and diacylglycerol production [Hsieh and Martin, 1992, Kim *et al.*,

1994], and indeed have been shown directly to do so by interaction with $G_q\alpha$ and $G_{11}\alpha$, many groups have shown coupling to other G proteins, $G_s\alpha$ and $G_i\alpha$.

A number of reports in which TRH responses have been analysed in GH3 and other related cell lines of pituitary origin have indicated both a direct interactionof the TRH receptor with G_s to cause activation of adenylyl cyclase [Paulssen *et al.*, 1992] and a TRH-mediated activation of G_i2 and thence an increase in Ca^{2+} flux through L-type Ca^{2+} channels [Gollasch *et al.*, 1993], although it is uncertain whether the TRH receptor directly interacts with G_i2 or whether this occurs subsequent to stimulation of protein kinase C.

The longest open reading frame in the largest TRH receptor cDNA clone encodes a protein of 412 amino acids known as long isoform. A second isoform of the rat TRH receptor was subsequently cloned from a GH3-cell library [de la Pena *et al.*, 1992]. This short isoform of the receptor, which appeared to be derived from the same gene as the long isoform by differential splicing mechanisms, was also shown to be expressed in the pituitary. Sequence analysis indicates identical sequence with the notable exception of a 52-base pair deletion located between nucleotides 1392 and 1443 at the 3' end of the coding region and thus results in a clear variation in the carboxyl terminus of the receptor protein. Thus, the open reading frame in this short splice variant encodes a 387 amino acids protein as displayed in **Figure 3.1**.

Hence, the existence of two splice variants of the rat TRH receptor raises the possibility that the range of effects reported in response to TRH treatment of pituitary-derived GH-cell lines may reflect the activation of two pharmacologically similar, but genetically distinct, receptor species. To examine this question, cDNA species encoding each of the long and the short isoforms of the rat TRH receptor have been expressed in Rat 1 fibroblast cells which do not endogenously express the receptor and the signalling properties of the two isoforms then examined.

3.2. Results

3.2.1. Correlation between TRH-receptor expression levels and TRH-stimulated inositol phosphate generation for Rat 1 fibroblasts expressing long and short receptor isoforms.

Rat-1 fibroblasts were stably transfected with either 10 μg of plasmid pcDNA I, containing the long isoform of the rat TRH receptor [Kim *et al.*, 1994], or 10 μg of plasmid pCMV5, into which the short isoform of the rat TRH receptor [de la Pena *et al*, 1992] had been subcloned. In both cases 1 μg of plasmid pSV2neo was co-transfected along with the receptor-containing plasmids to allow for selection of clones expressing resistance to geneticin sulphate. Resistant colonies were selected and expanded and expression of the TRH-receptor isoforms initially screened for by the ability of 10 μM TRH to promote generation of [^{3}H]inositol phosphates after labelling of cells with 1 μCi/ml of myo-[^{3}H]inositol as indicated in **section 2.14**.

A number of clones producing elevated levels of [^{3}H]inositol phosphates in response to TRH were further defined (**Figure 3.2**) by analysis of the specific binding to membranes of a single concentration of [^{3}H]TRH (10 nM) in the presence of the poorly hydrolysed analogue of GTP, Gpp[NH]p (100 μM) as described in **section 2.12.1**. Such clones exhibited a range of levels of TRH-receptor binding as shown in **Figure 3.2** and the correlation between receptor levels and fold stimulation of inositol phosphate levels above basal was high in cells transfected with plasmids incorporating cDNA for either the long or short splice variants of the TRH receptor ($r = 0.94$).

3.2.2. Specific detection of mRNAs encoding either the long or short isoforms of the rat TRH receptor

Clone 32, transfected to express the long isoform of the receptor, and clone 1, transfected with the short-isoform cDNA, were selected for detailed study. To confirm solely the expression of the expected TRH-receptor isoforms in these cells, RNA was isolated and subjected to reverse transcription followed by PCR (RT-PCR) by using a primer pair which straddles the splice variation site in the TRH-receptor gene as described in **section 2.29 and Table 2.3**. A single PCR product of 376 bp was obtained from RNA isolated from clone 32, and a single PCR product of 324 bases from RNA isolated from clone 1 as shown in **Figure 3.3**. These were the expected products from the long and short isoforms, respectively. Confirmation of this was obtained from PCR reactions performed with the two cDNA species ligated into the plasmids used for their expression. No product was obtained either when template was excluded from the PCR reaction or when RNA isolated from parental Rat 1 fibroblasts was subjected to RT-PCR with these primers.

3.2.3. [^{3}H]TRH-binding characteristics of long and short splice variants of the rat TRH receptor

Maximal levels and the affinity of TRH binding to the long and short isoforms of the receptor expressed in membranes of clones 32 and 1 were examined by analysis of competition curves between [^{3}H]TRH (approximately 10nM) and various concentrations of TRH in the presence of 100 μM Gpp[NH]p as decribed in **section 2.12.1**. Analysis of such data indicated that the long isoform of the receptor was expressed at 750±30 fmol/mg of membrane protein in clone 32, and the short isoform of the receptor was expressed at 860±170 fmol/mg of membrane protein in clone 1 as shown in **Figures 3.4a** and **3.4b**.

The estimated K_d for the binding of TRH to these receptors in these assay conditions was 85±3 nM for the long isoform and 71±34 nM for the short isoform

(mean±S.E.M.;n=3 in each case). These values were not significantly different (p =0.76). These values must, however, be interpreted with caution, as binding parameters based on analysis of binding of [^{3}H]-labelled agonist ligands can only be expected to provide approximate values. Similar binding experiments were also performed in the absence of Gpp[NH]p as shown in **Figures 3.4c and 3.4d**. In such experiments, the Hill coefficient (slope) of the displacement curve of specific [^{3}H]TRH binding by TRH was substantially less than 1. Analyses of such data by a two-site fit model indicated that 57±8% (mean±S.D., n =3) of the long isoform of the receptor was in the high-affinity state in these experiments.

This was not significantly different (p =0.31) from the short isoform, in which some 48±11% (mean±S.D., n =3) of the receptors were estimated to be in the same high-affinity state. The estimated K_d for the binding of TRH to the high affinity state of the long isoform was 8.2±4.0 nM (mean±S.D., n = 3), and for the short isoform it was 12.1±3.8 nM (mean±S.D., n =3). These values were not significantly different from one another (p =0.29). The characteristics of the low-affinity state of the receptor for TRH for each isoform were not amenable to analysis by this means, as the estimated errors were at least as great as the values obtained.

3.2.4. Comparison of the ability of TRH to stimulate inositol phosphate generation in Rat 1 fibroblast clones expressing either the long or the short isoforms of the rat TRH receptor

Examination of the ability of TRH to stimulate inositol phosphate generation in *myo* -[^{3}H]inositol-labelled clone 32 and clone 1 cells in the presence of 10 mM LiCl, as described in **section 2.14**, demonstrated that the EC_{50} for TRH was highly similar for the two receptors (7.5±1.7 nM for long isoform, 6.0±0.9 nM for short isoform) as shown in **Figure 3.5**. This was unaffected by pretreatment of the cells with 25 ng/ml

of pertussis toxin for 16 hrs, conditions able to cause ADP-ribosylation of essentially the entire population of pertussis toxin-sensitive G proteins in these cells (data not shown).

3.2.5. Inositol phosphate generation by either the long or the short TRH receptor isoforms : time-course studies

Examination of time courses of inositol phosphate generation by the two TRH-receptor splice variants was assessed over a period of 25 min as described in **section 2.14.2.** In both clone 32 (long isoform) and clone 1 (short isoform) the rate of inositol phosphate generation was linear over this time period. As clone 1 produced a larger fold stimulation of inositol phosphate generation than clone 32, a second clone (clone 7) expressing the short form of the receptor was also tested in parallel as displayed in **Figure 3.6.** This clone also demonstrated linear kinetics of inositol phosphate production in response to TRH.

3.2.6. Inability of TRH to alter the adenylyl cyclase activity in membranes of cells expressing either the long or short isoforms of the rat TRH receptor

The potential for either the long or short isoforms of the TRH receptor to regulate adenylyl cyclase activity was assessed in membranes of clones 1 and 32 as described in **section 2.13.** TRH (10μM) was unable, however, either to stimulate basal or to inhibit forskolin (10μM)-amplified adenylyl cyclase activity as displayed in **Table 3.1.**

3.2.7. Similar expression levels of both phosphoinositidase C and adenylyl cyclase regulatory G proteins

In order to examine if this lack of regulation of adenylyl cyclase might reflect the G-protein profile of Rat 1 fibroblasts expressing either the long or the short isoforms of the rat TRH receptor, levels of expression of the α subunits of the G-proteins associated with stimulation of phosphoinositidase C (G_q G_{11}), and stimulation (G_s) and inhibition (G_i2, G_i3) of adenylyl cyclase were assessed by immunoblotting as described in **section 2.8**. Each of these polypeptides was demonstrated to be expressed in membranes of these cells as shown in **Figure 3.7.**

3.2.8. Inability to regulate ERK-1 activity substantially in Rat 1 fibroblasts expressing either the long or short isoforms of the rat TRH receptor

TRH stimulation of the ERK-1 (MAP kinase) cascade has recently been reported in pituitary GH3 cells [Ohmichi *et al.*, 1994]. This observation was confirmed as shown in **Figure 3.8a** by demonstrating that addition of 10 μM TRH for 5 min to GH3 cells resulted in the characteristic decrease in mobility of the ERK-1 (44 kDa MAP kinase) in SDS/PAGE, as described in **section 2.16**, which is routinely correlated with phosphorylation and activation of this protein [Leevers and Marshall, 1992; de Vries-Smits *et al.*, 1992; Hordijk *et al.*, 1994]. In clones of Rat 1 fibroblast cells expressing either the long or the short isoform of the TRH receptor which had been maintained in the absence of serum for 48 hrs, treatment of 10 μM TRH for 5 min had only a small modulatory effect on the fraction of the cellular population of this polypeptide in the activated form as shown in **Figure 3.8b**.

Treatment of the cells with the phorbol ester, phorbol 12-myristate 13-acetate (PMA), equally resulted in only minimal modulation. In parallel experiments both epidermal growth factor (10 nM) and lysophosphatidic acid (10 μM), which have both previously been noted to cause stimulation of ERK activity in Rat 1 cells and clones derived from them [Hordijk *et al.*, 1994; Alblas *et al.*, 1994; Anderson & Milligan, 1994; Winitz *et al.*, 1993] produced this characteristic mobility shift in essentially the total cellular population of ERK-1 (**Figure 3.8b**). Pertussis-toxin (25 ng/ml, 16 hrs) treatment of the cells prevented lysophosphatidic acid regulation of ERK-1 mobility, but not that produced by epidermal growth factor (**Figure 3.8c**).

3.3. Discussion

It has generally been observed that TRH acts as a phosphoinositidase-C-linked hormone, the receptor for which interacts with pertussis toxin-insensitive G proteins of the G_q class [Hsieh & Martin, 1992; Kim *et al.*, 1994; Aragay *et al.*, 1992]. In a number of reports, however, particularly in GH3 cells and related rat anterior-pituitary cell lines, which are perhaps the most widely used model cell systems for analysis of TRH receptor function, interactions of TRH receptors with other G proteins [Paulssen *et al.*, 1992; Gollasch *et al.*, 1993], most prominently G_s and G_{i2} which are viewed traditionally as being responsible for stimulatory and inhibitory regulation of adenylyl cyclase have been demonstrated.

The identification of expression of splice variants of the TRH receptor in GH3 cells [de la Pena *et al.*, 1992] offered a potential explanation of these findings and begs the question of whether these splice variants display different functional properties. A number of molecularly distinct receptors are now appreciated to result from differential splicing of pre-mRNA derived from a single gene and, particularly in the cases of the splice variants of the EP3 prostanoid receptor [Namba *et al.*, 1993] and the pituitary

adenylate cyclase-activating-polypeptide (PACAP) receptor [Spengler *et al.*, 1993], very different second-messenger generation functions have been recorded for the individual forms.

Splice variation in the rat TRH receptors results from the presence or absence of a 52-bp segment in the C-terminal tail to yield either a 412 amino acid polypeptide (long isoform) or a 387 amino acid version (short isoform) [de la Pena *et al.*, 1992]. This is a reflection that the absence of a 52-bp fragment not only removes genetic information, but also results in a frame shift such that the amino acid sequence downstream of the deletion is altered and a different stop codon is encountered, which is not in frame in the long isoform.

In this study, I have examined whether the signalling characteristics of the two isoforms differ following stable expression of each isoform in Rat 1 fibroblasts, to assess whether reported regulation of effector systems other than phosphoinositidase C might reflect activation of one isoform rather than another. The initial preconceptions were that this might be a property of the short isoform, as assessed by RT-PCR, this is the minor form of the receptor in both normal pituitary and GH3 cells [de la Pena *et al.*, 1992].

After isolation of clones expressing specific [^{3}H]TRH-binding sites, it was determined that the clones expressed only the expected splice variant form of the receptor by reverse transcription of RNA isolated from these clones and subsequent PCR using a primer pair designed to generate fragments which would demonstrate the presence or absence of the 52-bp section which differs between the two isoforms (**Figure 3.3**).

Both receptor isoforms displayed similar binding affinity for [^{3}H]TRH (**Figure 3.4**). Most of the binding experiments were performed in the presence of 100 μM Gpp[NH]p. The rationale for this approach was that no radiolabelled antagonists are available for the TRH receptor, and when binding experiments with [^{3}H]TRH were

performed in the absence of Gpp[NH]p the data indicated a mixture of apparent low- and high-affinity sites with *h* values substantially below 1 (**Figures 3.4c** and **3.4d**).

However, Gpp[NH]p converts all G protein linked receptors into a G protein free state, which displays lower but uniform affinity for agonists and thus allows easier estimation of the binding parameters of B_{max} and K_d for a ligand. Under these conditions the *h* values for the self-competition experiments were close to 1. These were assessed by using the formalisms of DeBlasi *et al.* [1989] as these relate specifically to self-competition experiments in which the *h* value from the data is close to 1.0 as described in the legends of **Figures 3.4a** and **3.4b** for confirmation of the suitability of this approach in this case. However, even under such conditions the estimated values for the binding parameters should be treated with a degree of caution, as agonist-binding characteristics are difficult to analyse unambiguously. Under these conditions both the long and short isoforms of the rat TRH receptor bound TRH with an estimated K_d of some 80 nM (**Figure 3.4**).

A series of [^{3}H]TRH-binding experiments in the absence of Gpp[NH]p were performed and analysed using two-site displacement models. The estimated K_d for TRH at the high-affinity state for each isoform was close to 10 nM. These values are similar to the measured EC_{50} of 5-10 nM for TRH stimulation of inositol phosphate production observed for clones expressing both the long and short TRH-receptor splice variants (**Figure 3.5**), and, hence, indicate that it is the high-agonist-affinity state of the receptor which is able to interact with the cellular G protein population to produce effector regulation.

This similarity of EC_{50} values for TRH-mediated stimulation of inositol phosphate production is in agreement with analysis of TRH regulation of Cl^- flux in *Xenopus* oocytes after injection of cRNA species corresponding to the two isoforms [de la Pena *et al.*, 1992]. Given the location of the splice variation site in the C-terminal tail and the demonstration that Tyr-106 in the third transmembrane helix of the receptor

plays a key role in the binding of TRH [Perlman *et al.*, 1994], it should not be considered surprising that the two splice variants display very similar binding and activation affinities for TRH.

In contrast with the reported activation of adenylyl cyclase by TRH in GH cells, which has been demonstrated to occur via a direct activation of $G_S\alpha$ [Paulssen *et al.*, 1992], I was not able to record any ability of either TRH receptor splice variant to alter adenylyl cyclase activity either positively or negatively in membranes of the TRH receptor expressing clones of Rat 1 cells (**Table 3.1**). This is not a reflection of a lack of expression of the relevant G-proteins, which have been demonstrated to be expressed by these cells (**Figure 3.7**).

It may of course be a reflection of the adenylyl cyclase isoform distribution in the individual cell lines, an area which remains essentially uncharted, but, as all forms of hormonally regulated adenylyl cyclase are activated by G_s [Tang & Gilman, 1992], it seems unlikely. It is, therefore, concluded that neither isoform of the rat TRH receptor displays appreciable ability to interact with either G_s or indeed with G_i2, at least when expressed in this genetic background. As such the results can provide no evidence in this study for either of the splice variants of the TRH receptor providing a locus for bifurcation of signalling information, as has recently been described for a variety of receptors, including the TSH receptor [Allgeier *et al.*, 1994, and for review Milligan, 1993]. Recent data have also noted the ability of TRH to stimulate MAP kinase (ERK) activity in GH3 cells [Ohmichi *et al.*, 1994], but it was unable to produce a significant effect in clones of Rat 1 cells expressing either the long or short isoforms of the rat TRH receptor (**Figure 3.8**).

In such cells it has previously been reported that both receptors with intrinsic tyrosine kinase activity such as the epidermal growth factor receptor and G protein-coupled receptors, which are able to cause activation of $G_i2\alpha$ and hence inhibition of adenylyl cyclase activity, cause activation of this cascade, e.g. the endogenously

expressed receptor for lysophosphatidic acid or the transfected α_{2C10} adrenoceptor [Alblas *et al.*, 1993; Anderson & Milligan, 1994; Winitz *et al.*, 1993]. It has also previously been noted that the endogenously expressed receptor for the peptide endothelin, and indeed stimulation of the cells with phorbol esters, has little ability to cause activation of ERK in these cells [Hordijk *et al.*, 1994]. Hence, as TRH also functions to cause activation of a phosphoinositidase C, and thus stimulation of protein kinase C, it is not surprising that in this genetic background neither of the TRH receptor isoforms regulated a signalling cascade necessary to produce this effect.

The data produced herein demonstrate that no obvious functional differences are observed for the long and the short isoforms of the rat TRH receptor, at least when expressed in Rat 1 fibroblasts, and further demonstrate a high selectivity of coupling of both of these isoforms to G_q/G_{11} stimulation of phosphoinositidase C over regulation of adenylyl cyclase.

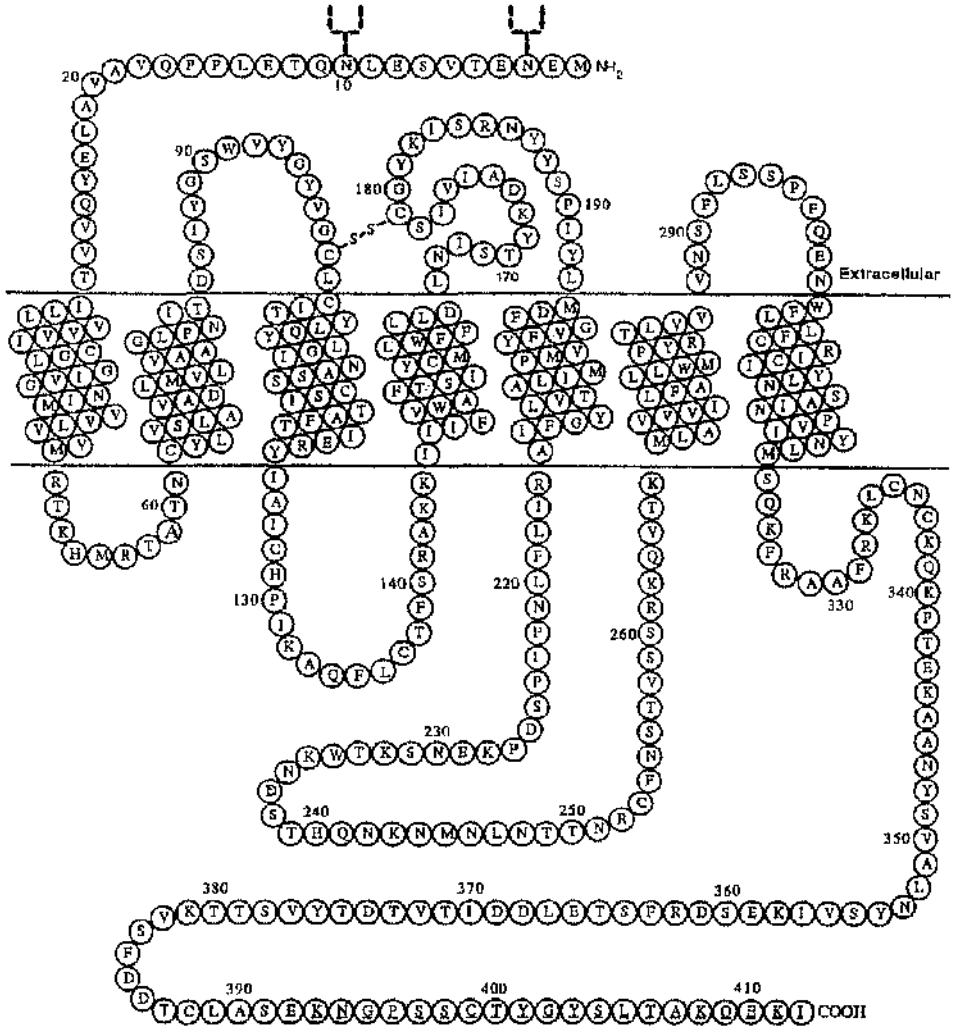
Figure 3.1. Seven transmembrane spanning model of the TRH receptor

Panel A shows the proposed topology of the TRH receptor cloned from rat pituitary cells with respect to the plasma membrane [de la Pena *et al.*, 1992a; Zhao *et al.*, 1992]. The receptor is proposed to form seven transmembrane helices (TM1-TM7) based on hydropathy analysis of the receptor sequence. Asn3 and Asn10 are proposed to be glycosylated based upon the consensus glycosylation sequence, N-X-S/T, with X being any amino acid residue. Cys 98 and Cys 179 are proposed to form a disulfide bond [Cook *et al.*, 1996; Han & Tashjian, 1995].

Panel B shows the divergent carboxyl-terminal amino acid sequences of the long (412 a.a.) and short (387 a.a.) isoforms of the rat TRH receptor [de la Pena *et al.*, 1992b]. Amino acid sequences of the deleted sequence and its boundaries in the short isoform are shown with the numbers indicating the amino acid position in the open reading frame. Putative sites for phosphorylation by protein kinase C (©) and casein kinase II (*) are indicated in the carboxyl terminal domain.

Figure 3.1.

A. Amino acid sequences of the TRH receptor



B. Divergent C-terminal sequence of TRH receptor isoforms

```
                375                                 393                                 412
Long isoform : TDTYVSTTKVSFDDTCLASEKNGPSSCTYGYSLTAKQEKI
                  ©     *           ©                 ©

                375                 376             387
Short isoform: TDT· · · · · · · · · · · · · · · · · RMDPVLVHMDIL
```

Figure 3.2. Correlation between TRH-receptor expression levels and TRH-stimulated inositol phosphate generation for Rat 1 fibroblasts expressing long and short receptor isoforms.

The specific binding of a single concentration of [^{3}H]TRH (10nM) defined by the absence and presence of 10μM TRH in the presence of 100μM Gpp[NH]p was measured as was the ability of 10μM TRH to stimulate production of inositol phosphates as described in **section 2.14** in a number of clones of Rat 1 fibroblasts transfected to express either the short (closed circle) or long (open square) isoforms of the rat TRH receptor. The receptor-binding data have not been corrected for receptor occupancy. Clones which were transfected with receptor-containing plasmids and which became resistant to geneticin sulphate, but failed to display specific binding of [^{3}H]TRH, also failed to accumulate inositol phosphates. Data are taken from the following clones: long isoform, clones 25, 32, 34 and 42; short isoform, clones 1, 4, 5, and 7. The maximal levels of inositol phosphate stimulation were obtained from clone 1 (4110 dpm) and clone 32 (2040 dpm). The results represent the means of 3 determinations. Similar results were obtained in 2 further experiments.

Figure 3.2

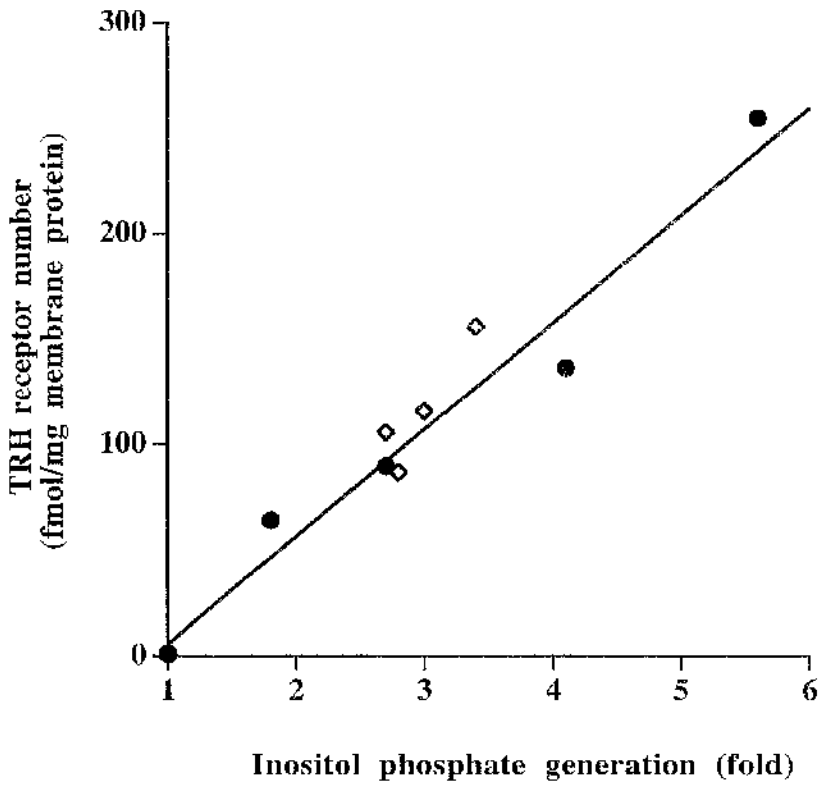

300
200
100
0
1
2
3
4
5
6
TRH receptor number
(fmol/mg membrane protein)
Inositol phosphate generation (fold)

Figure 3.3. Specific detection of mRNAs encoding either the long or short isoforms of the rat TRH receptor

RNA isolated from either clone 32 (transfected with long-isoform cDNA) (lane 2) or clone 1 (short isoform) (lane 3) was reverse-transcribed and PCR was performed with the primers as described in **section 2.9** and **Table 2.3**. Positive controls were provided by the long (lane 5) and short (lane 6) cDNA species, and a negative control (lane 4) was obtained by omission of template. A 1 kb ladder provided size markers (lane 1).

Figure 3.3

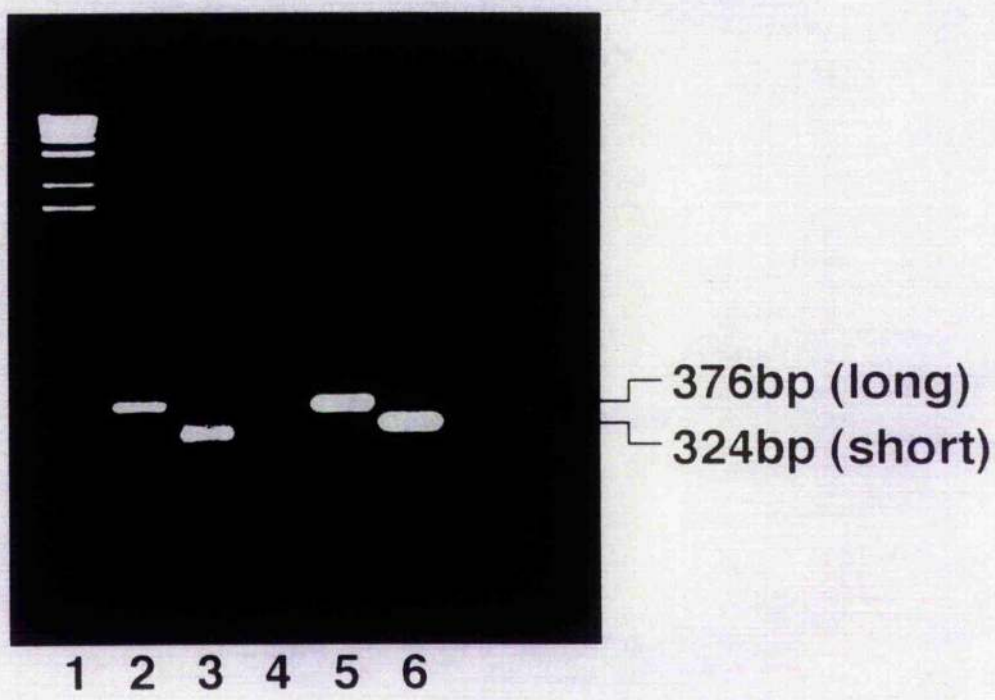


Figure 3.4. [^{3}H]TRH-binding characteristics of long and short splice variants of the rat TRH receptor

The ability of various concentrations of TRH to compete with [^{3}H]TRH for binding to the TRH-receptor isoforms expressed in membranes (50μg) of clones 1 (short isoform) and 32 (long isoform) was assessed in the presence (**a,b**) (closed symbols) or absence (**c,d**) (open symbols) of 100μM Gpp[NH]p as described in **section 2.12.1.**

(**a**) Clone 1: In the example displayed, [^{3}H]TRH was 9.9 nM and the estimated IC_{50} for TRH was 150 nM; *h* was 1.11. Application of the formalisms of DeBlasi *et al.*[1989] therefore allows estimation of a K_d for TRH of 140 nM.

(**b**) Clone 32: In the example displayed, [^{3}H]TRH was 10.7 nM and estimated IC_{50} for TRH was 93 nM; h was 1.09. These data provide an estimate of K_d for TRH of 82 nM.

(**c**) Clone 1: In the example displayed, [^{3}H]TRH was 11.0 nM and the estimated IC_{50} for TRH was 27 nM; *h* was 0.78. A *h* value of less than 1 implies the likely existence of more than one detected affinity state for TRH and precludes use of the formalisms of DeBlasi *et al.*[1989]. Application of two-component fit model allowed estimation of the K_d of the high-affinity site for TRH of 8.6 nM.

(**d**) Clone 32: In the example displayed, [^{3}H]TRH was 11.0 nM and the estimated IC_{50} for TRH was 38 nM; *h* was 0.6. Application of a two-component fit model allowed estimation of the K_d of the high-affinity site for TRH of 10.5 nM.

Figure 3.4 (a)

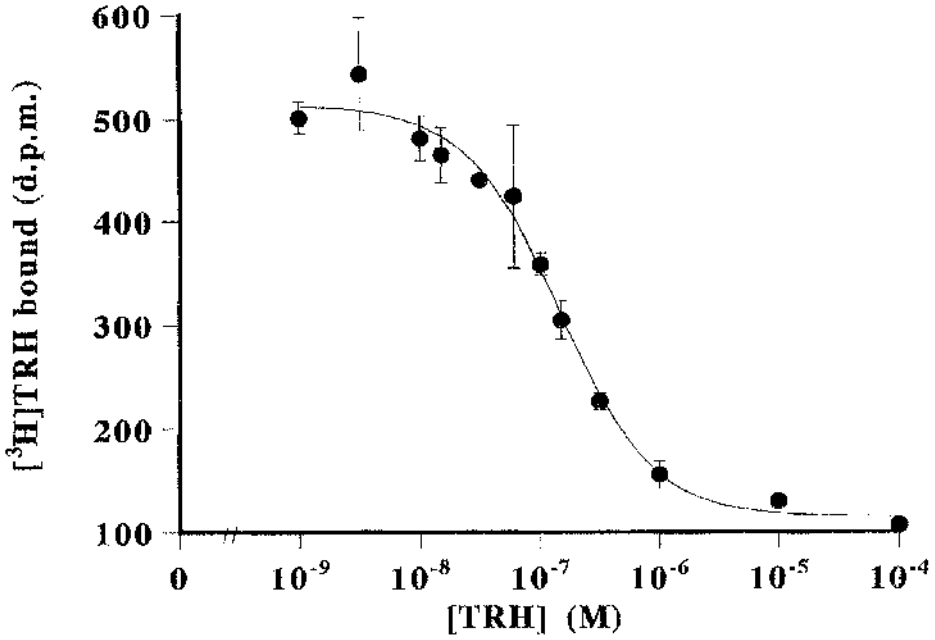


Figure 3.4 (b)

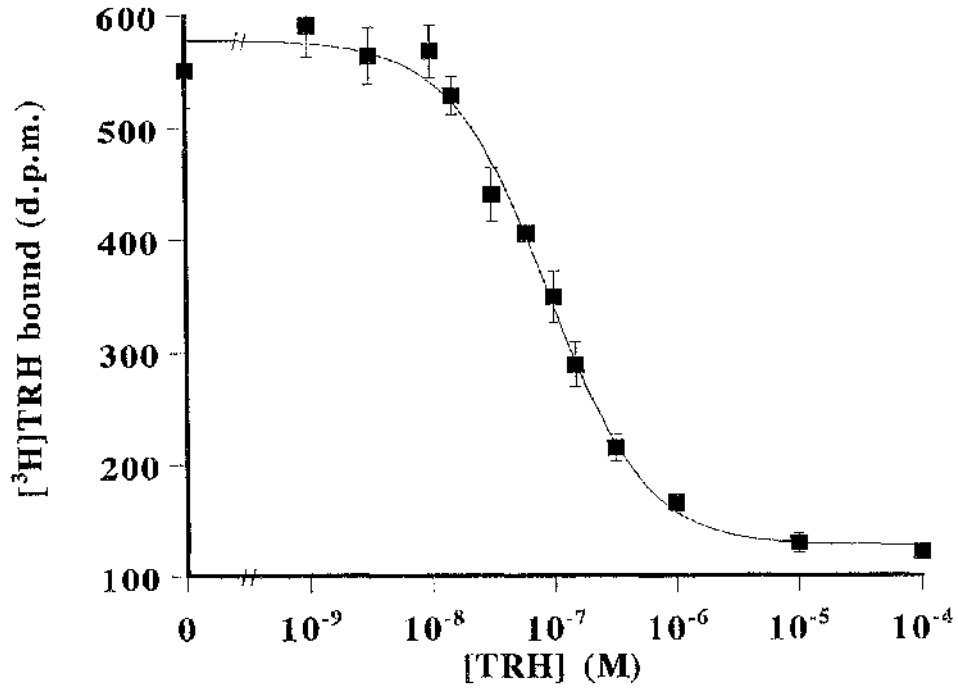


Figure 3.4 (c)

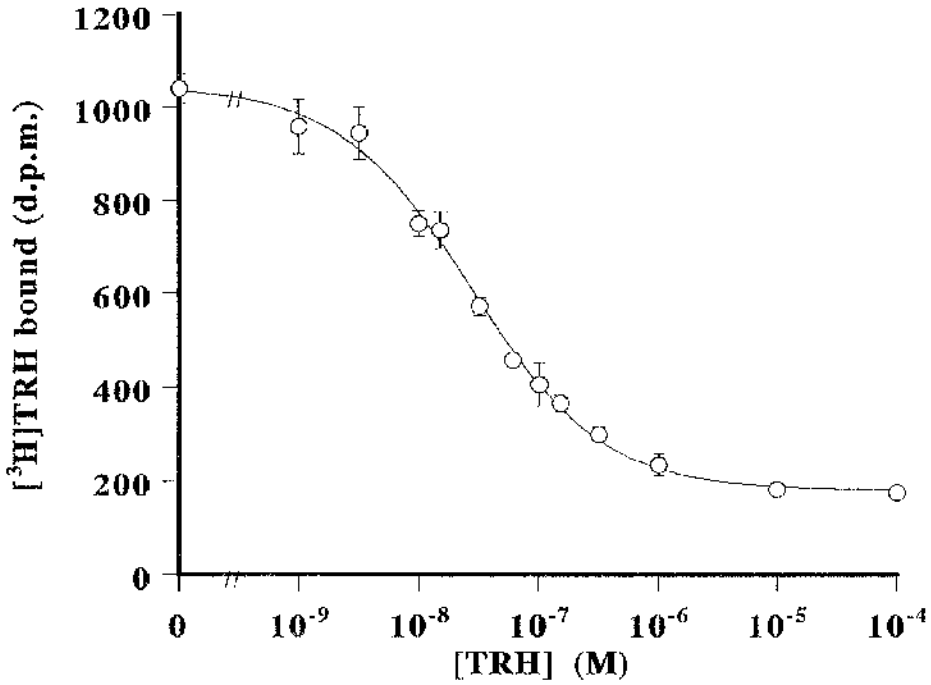


Figure 3.4 (d)

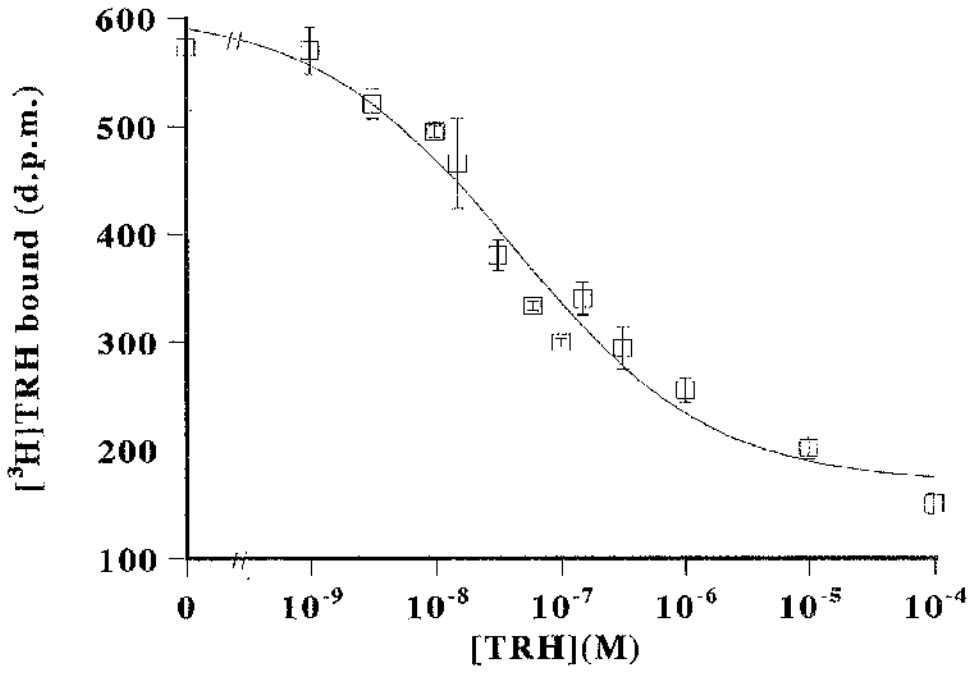


Figure 3.5. Comparison of the ability of TRH to stimulate production of inositol phosphate generation in clones expressing either the long or the short isoforms of the rat TRH receptor

The ability of various concentrations of TRH to stimulate production of inositol phosphates was assessed in cells of clones 1 (short isoform) (open circles) and 32 (long isoform) (closed squares) as described in **section 2.14**. Results are presented as percentage of maximal effect (defined as the stimulation obtained with 10μM TRH).

The basal levels of inositol phosphate in this experiment were 1110 dpm for clone 1 and 595 dpm for clone 32. The maximal levels in clones 1 and 32 were 8730 dpm and 1980 dpm, respectively. The data displayed are the mean of triplicate assays from representitive experiments (n=3).

Figure 3.5

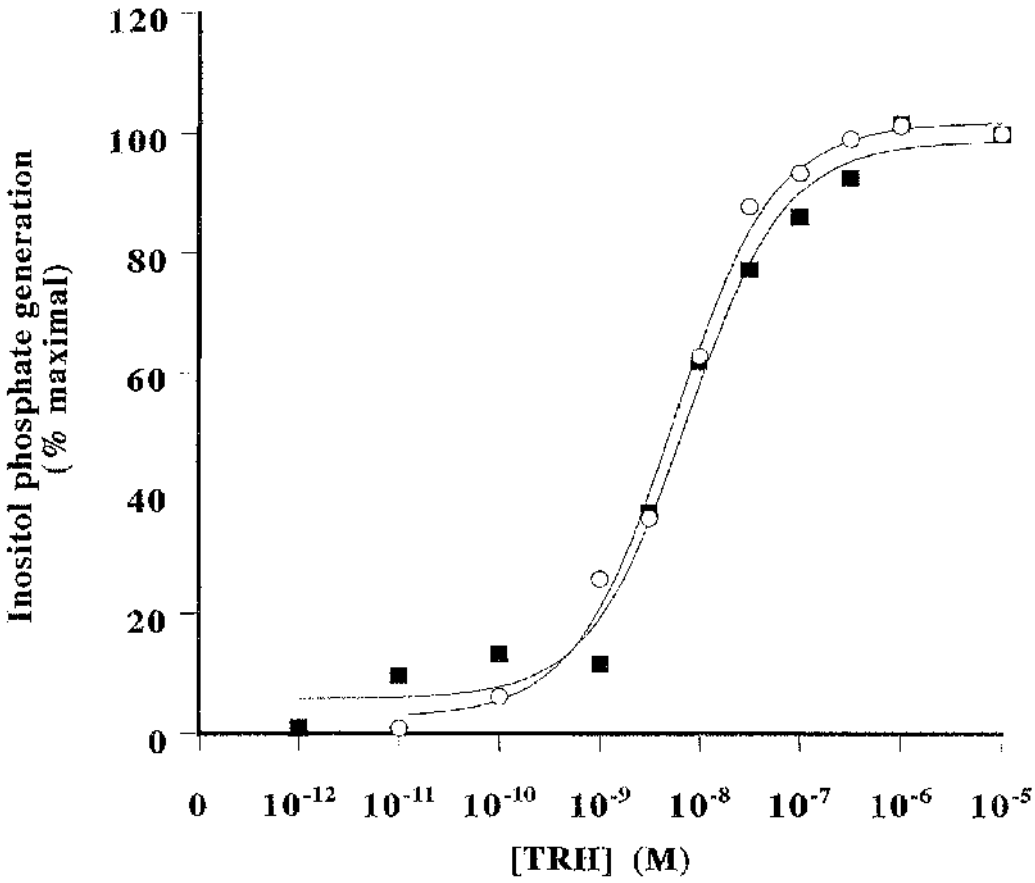

Inositol phosphate generation
(% maximal)
120
100
80
60
40
20
0
0
10-12
10-11
10-10
10-9
10-8
10-7
10-6
10-5
[TRH] (M)

Figure 3.6. Inositol phosphate production by either the long or the short TRH-receptor isoforms : time-course studies

The ability of TRH (10μM) to stimulate inositol phosphate production was assessed as described in **section 2.14** at various time points over a period of 25 min in clones 1 (closed circles) and 7 (closed triangles) (both short isoform) and clones 32 (open squares) (long isoform). The higher fold stimulation of inositol phosphate generation in clone 1 in response to TRH in these studies was largely a reflection of lower levels of inositol phosphates being present in incubations performed in the absence of TRH.

The basal levels of inositol phosphate in clones 1, 7, and 32 were 460 dpm, 830 dpm, and 750 dpm respectively. The results represent the mean of triplicate determinations. The experiment shown is typical of three independent experiments which gave similar results.

Figure 3.6

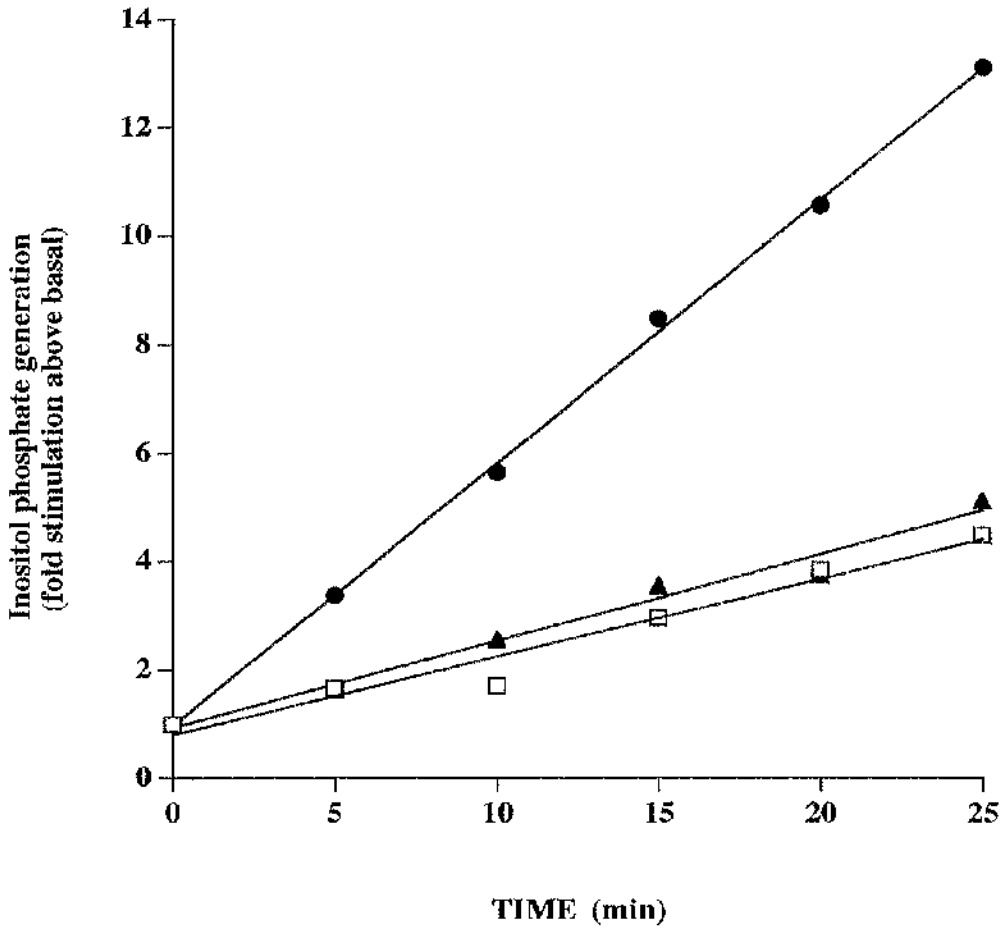

Inositol phosphate generation
(fold stimulation above basal)
14
12
10
8
6
4
2
0
0
5
10
15
20
25
TIME (min)

Figure 3.7. Rat 1 fibroblasts expressing either the long or the short isoforms of the rat TRH receptor express similar levels of both phosphoinositidase C and adenylyl cyclase regulatory G proteins.

Membranes (15 μg, panel **A**; 30 μg, panel **B**; or 20 μg, panel **C**) from Rat 1 fibroblast clone 32 (long isoform) (lanes 1 and 2) and clone 1 (short isoform) (lanes 3 and 4) were resolved by SDS/PAGE (10% acrylamide), transferred to nitrocellulose and subsequently immunoblotted with specific antisera as described in **section 2.8 and Table 2.1** to detect the presence of the α subunits of the phosphoinositidase C-linked G proteins G_q/G_{11} (**A**), and the adenylyl cyclase-stimulatory, G_s (**B**), and inhibitory, G_i2 (**C**), G proteins.

Figure 3.7

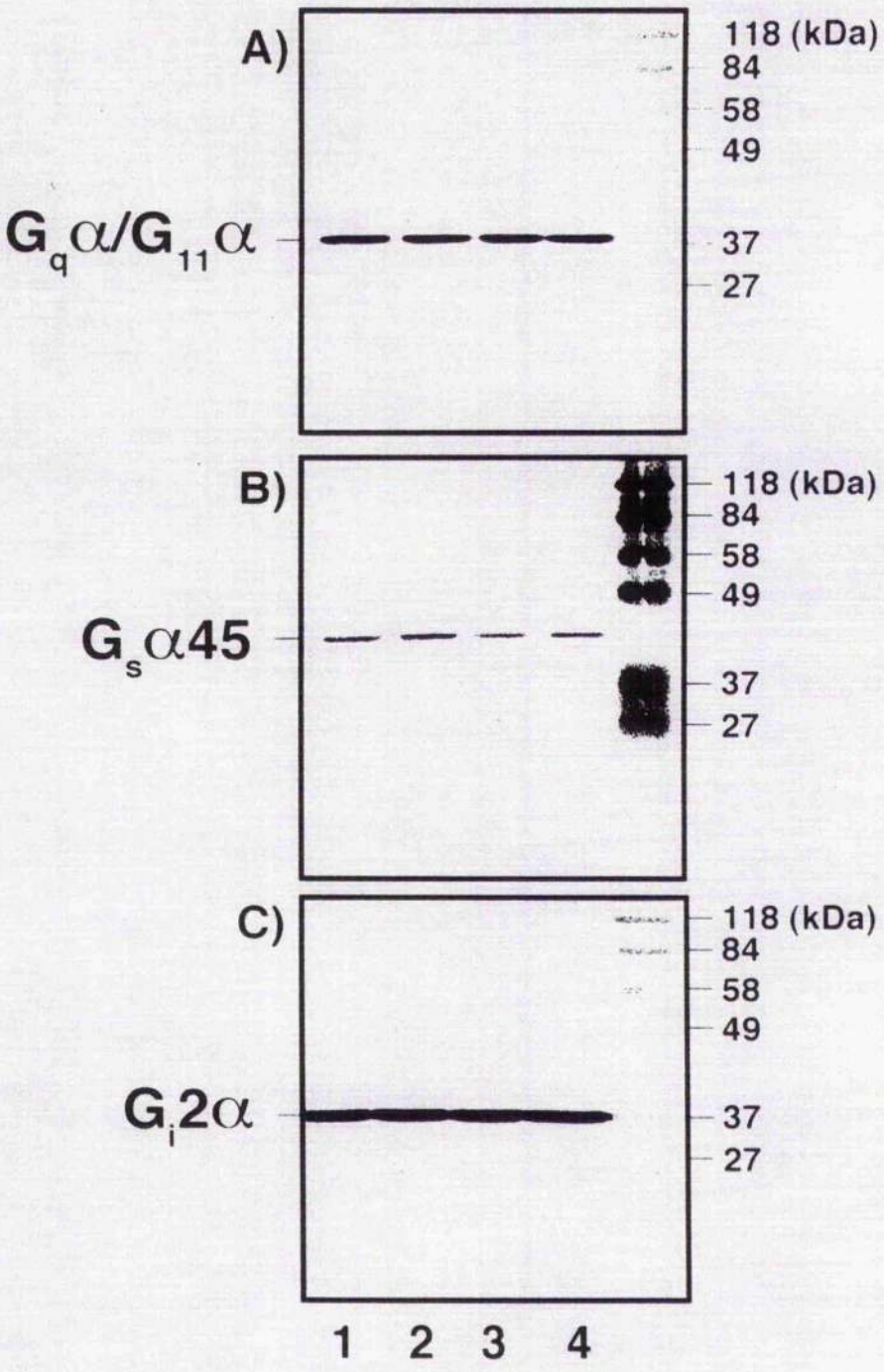

A)
$G_q\alpha/G_{11}\alpha$
B)
$G_s\alpha45$
C)
$G_i2\alpha$
118 (kDa)
84
58
49
37
27
1 2 3 4

Figure 3.8. Neither the long or the short isoforms of the rat TRH receptor is able to regulate ERK-1 substantially in Rat 1 fibroblasts

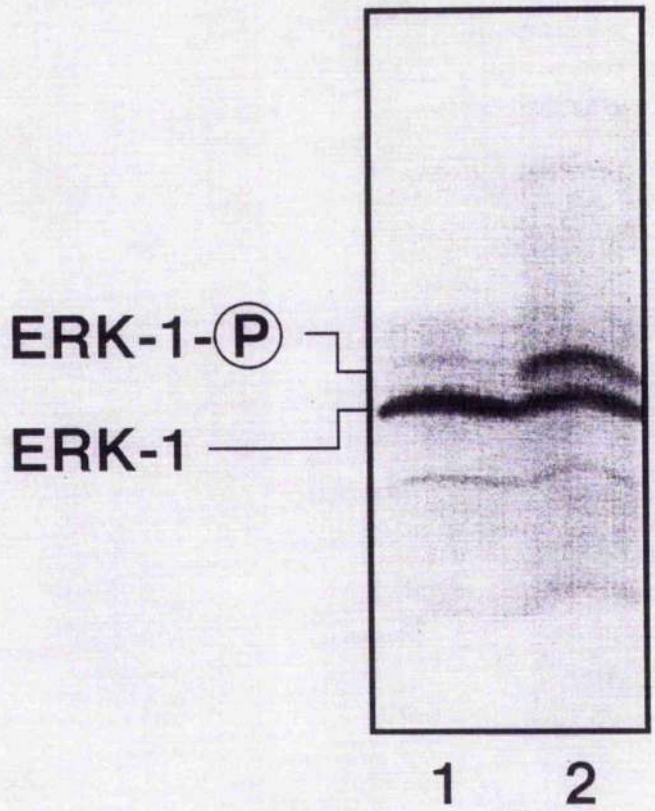
Figure 3.8a shows that TRH alters the gel mobility of ERK-1 in GH3 cells. GH3 cells were deprived of serum for 48 h and then challenged with (2) or without (1) TRH (10μM) for 5 min. Cell lysates were resolved by SDS/PAGE (10% acrylamide containing 6M urea) and immunoblotted as described in **section 2.16** for the presence and mobility of ERK-1. After challenged with TRH a substantial fraction of this polypeptide migrated through the gel with decreased mobility, a characteristic associated with phosphorylation and activation of this polypeptide.

Figure 3.8b shows that epidermal growth factor (EGF) and lysophosphatidic acid, but not TRH or phorbol myristate acetate, regulate ERK-1 in Rat 1 fibroblasts expressing short or long forms of the TRH receptor. Cells of clone 32 (TRH-receptor long isoform, lanes 1-6) and clone 1 (TRH-receptor short isoform, lanes 7-12) were deprived of serum for 48h and then subsequently challenged with vehicle (lanes 1, 7), 10μM lysophosphatidic acid (lanes 2,8), 10 nM EGF (lanes 3,9), 10 nM EGF plus 10 μM TRH (lanes 4,10), 10μM TRH (lanes 5,11) or 1 μM phorbol myristate acetate (lanes 6,12) for 5 min. Cell lysates were resolved by SDS-PAGE (10% acrylamide containing 6M urea) and immunoblotted as above for the presence and mobility of ERK-1.

Pertussis toxin (25ng/ml, 16h) treatment of the cells expressing the long isoform of the Rat TRH receptor prevented lysophosphatidic acid regulation of ERK-1 mobility, but not that produced by epidermal growth factor as shown in **Figure 3.8c** with lanes of vehicle (lane 1), 10μM lysophosphatidic acid (lane 2), 10 nM EGF (lane 3), 10μM TRH (lane 4), and 1 μM phorbol myristate acetate (lane 5).

Figure 3.8

a)



b)

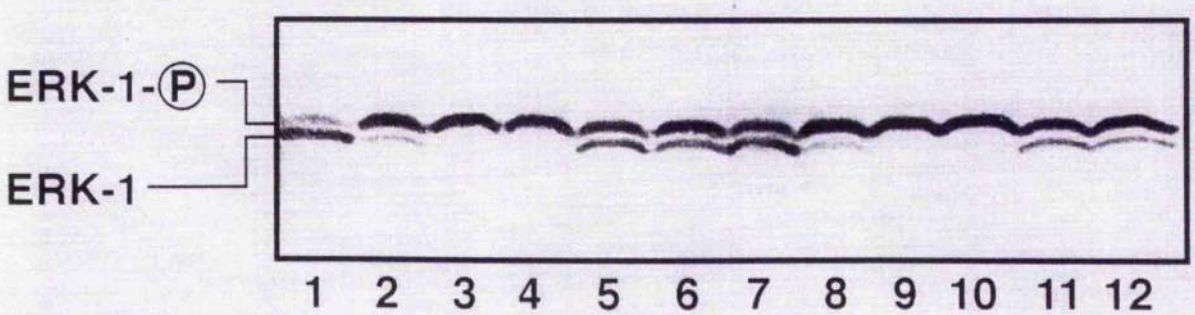


Figure 3.8

c)

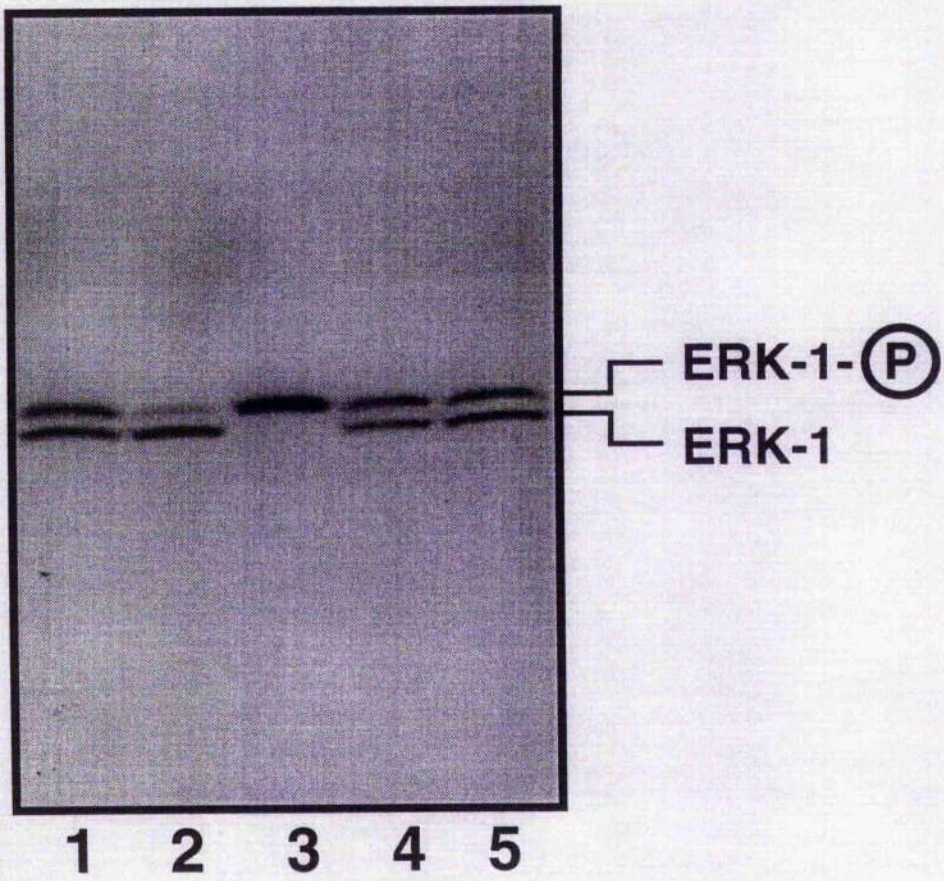


Table 3.1. Both the long and short isoforms of the rat TRH receptor are unable to regulate adenylyl cyclase activity in membranes of receptor-expressing Rat 1 fibroblast clones

Adenylyl cyclase activity was measured as described in **section 2.13** in membranes of clones 1 and 32. Results are presented as means ± S.D. (n = 4). A similar lack of ability to regulate adenylyl cyclase activity was observed in membranes from two further clones [(clone 34, long isoform; clone 7, short isoform (results not shown)].

Adenylyl cyclase activity (pmol/min/mg membrane protein)

	Receptor Isoform	
Condition	**Long (clone 32)**	**Short (clone 1)**
Basal	76 ± 0.1	100 ± 3.0
TRH (10μM)	74 ± 0.8	99 ± 1.0
Forskolin (10μM)	160 ± 3.0	220 ± 11.0
Forskolin (10μM) + TRH (10μM)	160 ± 5.0	230 ± 6.0

Chapter 4

Accelerated degradation of $G_{11}\alpha/G_q\alpha$ by agonist occupancy of α_1 adrenergic receptor subtypes

Chapter 4. Accelerated degradation of $G_{11}\alpha/G_q\alpha$ by agonist occupancy of α_1 adrenergic receptor subtypes

4.1. Introduction

The natural catecholamines norepinephrine (NE) and epinephrine activate adrenergic receptors in three families, α_1, α_2, and β. Multiple subtypes have been cloned within each family: three α_1 adrenergic receptors (A/D, B, and C), three α_2 adrenergic receptors (A, B, and C; the human forms are also called C10, C2, and C4 adrenergic receptors, respectively, based on their chromosomal location), and three β adrenergic receptors (1, 2, and 3) [Lomasney *et al.*, 1995]. The reasons for conservation of multiple subtypes remain uncertain, since all subtypes in each family couple preferentially to the same effector when overexpressed, α_1 adrenergic receptors to activation of phosphoinositidase C, α_2 adrenergic receptors to inhibition of adenylyl cyclase, and β adrenergic receptors to activation of adenylyl cyclase.

There are many closely related α_1 adrenergic receptor subtypes that have been indicated by comparisons of the pharmacological profiles of ligands in different tissues [Ford *et al.*,1994]. Three distinct α_1 adrenergic receptor cDNA species have currently been isolated [Cotecchia *et al.*, 1988, Schwinn *et al.*, 1990, Lomasney *et al.*, 1991], $\alpha_{1A/D}$, α_{1B}, α_{1C} as shown in **Table 4.1**, but there has been considerable debate as to how closely these reflect the pharmacologically defined subtypes [for review, Ford *et al.*, 1994], even if all of these cDNA species show the same signal transduction mechanisms following their heterologous expression in cell lines.

This has arisen partially because of the relative pharmacological similarity of the subtypes and partially because the first isolated cDNA species were derived from a number of different species [Ford *et al.*, 1994, Milligan *et al.*, 1994]. Current opinion

favours the view that the cloned α_{1A} adrenergic receptor correponds to the pharmacologically defined α_{1D} adrenergic receptor, that the cloned and pharmacologically defined α_{1B} adrenergic receptor are identical, and that the cloned α_{1C} adrenergic receptor may be the equivalent of the pharmacologically defined α_{1A} adrenergic receptor [Ford *et al.*, 1994].*

Despite these ongoing concerns, it is generally accepted that they share similar structural features with other G protein coupled receptors as shown in **Figure 4.1** and mediate neurotransmission, vasoconstriction, cardiac inotropy, chronotropy, and glycogenolysis. The primary signalling function of α_1 adrenergic receptor subtypes is to stimulate the hydrolysis of inositol-containing phospholipids via interaction with pertussis toxin-insensitive G proteins of the G_q/G_{11} family [Wu *et al.*, 1992] with subsequent activation of phosphoinositidase Cβ activity [Wu *et al.*, 1992].

Phosphorylation and desensitization to the effects of agonist, epinephrine, in α1 adrenergic receptors are mediated by β adrenergic receptor kinase (βARK) 1 and 2 as well as arrestin proteins [Diviani *et al.*, 1996]. On the other hand, truncation of the serine- and threonine rich carboxyl portion of the α_{1B} adrenergic receptor abolished agonist induced phosphorylation and greatly impaired homologous desensitization of the receptor [Lattion *et al.*, 1994].

Although it has been well established that sustained exposure of many G protein-coupled receptors to agonist can result in a reduction in cellular levels of the receptor (a process known as down-regulation), it has only been in the recent past that agonist-mediated reduction in cellular levels of G proteins has also been observed [Milligan G., 1993]. Even in such cases, information on the mechanism(s) responsible for these effects is fragmentary. To attempt to address this point directly in this study, clonal cell lines derived from rat 1 fibroblasts following stable transfection with the cloned rat $\alpha_{1A/D}$, the hamster α_{1B}, and the bovine α_{1C} adrenergic receptor cDNA species were used.

It is noted that in cells expressing each of the three receptor species, sustained exposure to phenylephrine results in a large, selective downregulation of $G_{11}\alpha$ and $G_q\alpha$ as well as in downregulation of the receptors. These are the G proteins that have been demonstrated to couple α_1 adrenergic receptors to phosphoinositidase C activity and the hydrolysis of inositol-containing phospholipids [Wu *et al.*, 1992]. In each case, it is demonstrated that the basal rate of turnover of these G proteins is described adequately by a monoexponential with a $t_{0.5}$ between 33 and 40 h, while upon exposure to agonist, this rate of degradation is markedly increased, such that a substantial fraction of the cellular content of these G proteins now has a $t_{0.5}$ of between 7 and 10 h.

This study shows that G proteins activated by a receptor are degraded considerably more rapidly than those in the inactive state and provide a mechanistic explanation for how receptor agonists can control the cellular content of G proteins which interact with that receptor.

---

* The nomenclature for the α_1 adrenergic receptor subtypes was that originally assigned to the cDNA species which were used in this study, except that the cDNA originally named the α_{1A} adrenergic receptor is referred to as the $\alpha_{1A/D}$ adrenergic receptor, as this is now widely accepted to be equivalent to the pharmacologically defined α_{1D} adrenergic receptor.

* It has been suggested that the α_{1C} should be renamed the α_{1A}, and $\alpha_{1A/D}$ should be renamed α_{1D} according to the recent International Union of Pharmacology (IUPHAR) meeting report [Hieble *et al.*, 1995].

4.2. Results

4.2.1. Specific detection of mRNAs encoding α_1 adrenergic receptor subtypes

Rat 1 fibroblast cells transfected to stably express each of the three cloned α1 adrenergic receptor subtypes were obtained from Dr.D.E.Clarke (Syntex, Palo Alto, CA) under the license to Syntex from Dr.L.F.Allen (Duke University, NC) and used for this study. The presence of $\alpha_{1A/D}$, α_{1B} and α_{1C} adrenergic receptor mRNA in appropriately transfected cells only was confirmed by reverse transcriptase-PCR analysis of RNA isolated from untransfected parental rat 1 fibroblast and each of the clonal cell lines examined in this study as described in **section 2.29** using oligonucleotide primers specific for each of the three molecularly defined α1 adrenoreceptor subtypes as described in **Table 2.3**. and is shown in **Figure 4.2.**

4.2.2. The ability of phenylephrine to displace [^{3}H]prazosin binding in membranes of α_1 adrenergic receptor expressing cells

Membranes derived from all three clonal cell lines were examined for their levels of expression of the α1 adrenergic receptor subtypes by measuring the specific binding of the α1 adrenergic receptor antagonist [^{3}H]prazosin as described in **section 2.12.2.** $\alpha_{1A/D}$, α_{1B} and α_{1C} adrenergic receptor subtypes were found to be expressed at levels of 2.1 ± 0.1, 2.8 ± 0.5, and 7.0 ± 0.9 pmol/mg membrane protein, with K_d values for the binding of [^{3}H]prazosin of 110 ± 20, 76 ± 13, and 150 ± 26 pM, respectively (data provided by Dr.Christine Brown). Displacement of specific [^{3}H]prazosin binding by varying concentrations of the α_1 adrenergic receptor agonist

phenylephrine was achieved with pIC_{50} values (and Hill coefficients) of 5.1 ± 0.1 (n_H= 0.76 ± 0.09), 4.7 ± 0.2 (n_H = 1.06 ± 0.07), 5.2 ± 0.1 (n_H = 0.90 ± 0.06), respectively, for the $\alpha_{1A/D}$, α_{1B}, and α_{1C} adrenergic receptor subtypes as displayed in **Figure 4.3.**

Addition of the poorly hydrolysed analogue of GTP, Gpp[NH]p (100 μM), to such assays produced no significant alterations in the positions of such displacement curves [5.1 ± 0.1 (n_H = 0.99 ± 0.03), 4.6 ± 0.1 (n_H = 0.89 ± 0.05), 4.9 ± 0.2 (n_H = 1.12 ± 0.16) for the $\alpha_{1A/D}$, α_{1B}, and α_{1C} adrenergic receptor subtypes] as shown in **Figure 4.3.**

4.2.3. Stimulation of inositol phosphate production by all the α_1 adrenergic receptor subtypes

Accumulation of inositol phosphates in LiCl (10mM)-treated α1 adrenergic receptor subtype expressing cells, which had been labeled for 24 h with *myo*-[2-^{3}H]inositol as described in **section 2.14**, was found to be markedly stimulated by phenylephrine as shown in **Figure 4.4**. However, it was in a manner that was insensitive to pretreatment of the cells with pertussis toxin (25ng/ml) for 16 h (data not shown), confirming a functional coupling of these receptors to the cellular G protein machinery. Half-maximal stimulation of inositol phosphate generation in response to phenylephrine was produced with between 0.3 and 1 μM agonist in a range of experiments with each of the three α1 adrenergic receptor subtype expressing cell lines as shown in **Figure 4.4.**

4.2.4. Phenylephrine-mediated downregulation of $G_q\alpha/G_{11}\alpha$ levels in membranes of α_1 adrenergic receptor subtype-expressing Rat 1 fibroblasts

Sustained exposure of cells expressing each of the three α_1 adrenergic receptor subtypes to varying concentrations of phenylephrine resulted in a reduction of membrane-associated levels of a combination of the phosphoinositidase C-linked G proteins $G_q\alpha$ and $G_{11}\alpha$ by, maximally, approximately 50-70% as determined by immunoblotting of membranes of agonist-treated and untreated cells with antiserum CQ as indicated in **Table 2.1**, which identifies the C-terminal decapeptide, which is entirely conserved between these two closely related G proteins. No significant alterations in immunologically detected levels of other G protein α subunits expressed by these cells ($G_s\alpha$, $G_i2\alpha$) were noted to be associated with phenylephrine treatment (data not shown). Downregulation of $G_q\alpha/G_{11}\alpha$ was found to be dose dependent with EC_{50} for phenylephrine close to 600 nM for all three α_1 adrenergic receptor subtype-expressing cells as shown in **Figure 4.5**.

4.2.5. Time course of agonist-mediated downregulation of $G_q\alpha/G_{11}\alpha$

In cells expressing the $\alpha_{1A/D}$ and α_{1B} adrenergic receptor subtypes, half-maximal effects in response to a maximally effective concentration of phenylephrine (100μM) were produced after some 8h, whereas in cells expressing the α_{1C} subtype, this figure was determined to be 4h as shown in **Figure 4.6**, a difference that might reflect the higher levels of expression of the receptor in these cells. In all cells treated with agonist over a sustained period (16h), a similar membrane-bound plateau level of $G_q\alpha/G_{11}\alpha$ was established at some 30-50% of that present in untreated cells.

4.2.6. Phenylephrine treatment of α_1 adrenergic receptor subtype expressing cells results in downregulation of both $G_q\alpha$ and $G_{11}\alpha$.

$G_q\alpha$ and $G_{11}\alpha$ comigrate in 10% (w/v) acrylamide SDS-PAGE. Therefore, to determine both the relative levels of expression of these two phosphoinositidase C-linked G proteins in the rat 1-derived clonal cell lines and whether phenylephrine-mediated downregulation of $G_q\alpha/G_{11}\alpha$ in any of these cells was selective for either of these highly homologous G protein α subunits, membranes from untreated and phenylephrine (100μM) treated cells were resolved by SDS-PAGE using 12.5% (w/v) acrylamide gels containing 6M urea as indicated in **section 2.6. and section 2.8**, conditions shown to be effective in resolving these G proteins by Mullaney *et al.*[1993], Shah & Milligan [1994], and Svoboda & Milligan [1994].

Figure 4.7 demonstrates that steady-state levels of $G_{11}\alpha$ were substantially (over 2-fold) greater than $G_q\alpha$ in these cells and that phenylephrine-driven down-regulation of membrane-associated $G_q\alpha$ and $G_{11}\alpha$ from the clonal cell lines expressing each of the three α1 adrenergic receptor subtypes was non-selective between these G protein α subunits.

4.2.7. Time course of α_1 adrenergic receptor subtype-stimulated enhancement of $G_q\alpha/G_{11}\alpha$ protein degradation

In order to address the mechanism(s) of phenylephrine-mediated reduction in membrane-bound levels of $G_q\alpha/G_{11}\alpha$, cells of the α1 adrenergic receptor subtype-expressing clones were incubated with Trans ^{35}S-label for 16h, and subsequently the decay of ^{35}S with time in immunoprecipitated $G_q\alpha/G_{11}\alpha$ was monitored in cells that were either untreated or treated with phenylephrine (100μM) as described in **section 2.2.7** and **section 2.5.2.**

Analysis of the rate of decay of ^{35}S-labeled $G_q\alpha/G_{11}\alpha$ indicated that in the untreated $\alpha_{1A/D}$ (**Figure 4.8a**), α_{1B} (**Figure 4.8b**), and α_{1C} (**Figure 4.8c**) adrenergic receptor subtype-expressing cells, this process was described adequately by

monoexponentials with estimated half-time ($t_{0.5}$) ranging from 33 to 40h. However, the decay of ^{35}S-labeled $G_q\alpha/G_{11}\alpha$ in each of the cell lines in the presence of phenylephrine was more rapid, and the data were more effectively modeled by a two-component fit than by a single exponential as shown in **Figure 4.8a, b, and c**. Addition of phenylephrine was associated with a substantial component of the decay in which $t_{0.5}$ for $G_q\alpha/G_{11}\alpha$ was markedly accelerated to 10.2, 10.9, and 7.7h in rat 1 fibroblasts expressing $\alpha_{1A/D}$, α_{1B}, and α_{1C} adrenergic receptor subtypes, respectively.

There was, however, a second component of the decay rate for $G_q\alpha/G_{11}\alpha$ that was not enhanced compared to the single phase decay observed in the untreated cells. Because of the lower steady-state level of expression of $G_q\alpha$ relative to $G_{11}\alpha$, it was not attempted to examine whether the agonist-induced acceleration of $G_q\alpha/G_{11}\alpha$ degradation could be observed independently for both of these G-proteins. In contrast to the effect of phenylephrine on the rate of removal of ^{35}S-labeled $G_q\alpha/G_{11}\alpha$, this agonist had no effect in any of the α_1 adrenergic receptor subtype-expressing cells on the rate of decay of ^{35}S-labeled $G_i2\alpha$, which had been immunoprecipitated with antiserum SG as indicated in **Table 2.1** (data not shown). This G protein has been shown to be involved in receptor-mediated inhibition of adenylyl cyclase [McKenzie & Milligan, 1990; Simonds *et al.*,1989].

4.3.Discussion

Although the basic observation that maintained agonist activation of a G protein-linked receptor can result in a marked and selective reduction in cellular levels of the G protein(s) activated by that receptor is now well established [Milligan, 1993], far less is known about the mechanisms responsible for such phenomena. Studies from Hadcock *et al.*[1990, 1991] have noted complex regulation of G proteins following

receptor stimulation, including alterations in both protein and mRNA stability of a variety of G proteins, which is then integrated to result in upregulation of some G proteins and downregulation of others. By contrast, Mitchell *et al.*[1993] noted that muscarinic m1 acetylcholine receptor-mediated downregulation of the α subunits of the phosphoinositidase C-linked G proteins G_q/G_{11} was accompanied by a selective accelerated rate of degradation of these G proteins. In this sudy, it has been sought to further analyse such effects by examining the process of downregulation of the α subunits of G_q and/or G_{11} in rat 1 cells transfected to express individual molecularly defined α_1 adrenergic receptor subtypes.

In such cells, expressing one of the rat $\alpha_{1A/D}$, the hamster α_{1B}, and the bovine α_{1C} adrenergic receptors and labeled with myo-[^{3}H]inositol, exposure to the α_1 adrenergic receptor agonist phenylephrine resulted in stimulation of inositol phosphate production in a fashion that was resistant to pretreatment of the cells with pertussis toxin. Such a feature was hardly unexpected, but is the pattern anticipated for receptors that couple to phosphoinositidase C-linked G proteins of the G_q family. Maintained exposure of these cells to phenylephrine resulted in downregulation of each of the receptor subtypes as shown in **Table 4.2** and selective downregulation of some combination of the α subunits of G_q/G_{11}.

Agonist-induced downregulation of G protein-linked receptors is a common regulatory feature. However, in the subfamily of α_2 adrenergic receptors, while both the α_2C_{10} and α_2C_2 receptor are readily down-regulated by agonist treatment, the α_2C_4 receptor has been reported to be largely resistant to downregulation [Eason & Liggett, 1992]. Mutation of the site for palmitoylation in the C-terminal tail of the α_2C_{10} adrenergic receptor has been reported to render it resistant to agonist-mediated downregulation without altering its ability to couple to the G_i-like G proteins [Eason *et al.*, 1991; Kennedy & Limbird, 1993]. Although direct information is not currently available, all three α_1 adrenergic receptor cDNA species used in this study have

cysteine residues in their predicted C-terminal tail in a context that makes them likely sites of palmitoylation. It would be interesting to examine if mutation of these residues results in an agonist-mediated downregulation resistant form of these receptors and whether this interferes with agonist-mediated downregulation of G_q/G_{11}.

Agonist-mediated downregulation of the α subunits of G_q/G_{11} has now been reported for a variety of receptors, including the muscarinic m1 acetylcholine receptor [Mullaney *et al.*, 1993; Mitchell *et al.*, 1993], the long isoform of the thyrotropin-releasing hormone receptor [Kim *et al.*, 1994], and the gonadotropin-releasing hormone receptor [Shah & Milligan, 1994]. However, only for the first of these has any mechanistic analysis been provided. In Chinese hamster ovary cells transfected to express the rat muscarinic m1 receptor, accelerated degradation of a combination of G_q/G_{11} was recorded without detectable alteration in levels of mRNA of either of these polypeptides [Mitchell *et al.*, 1993].

In this study, those observations were expanded to show that in the genetic background of rat 1 fibroblasts, the basal half-life of the α subunits of G_q/G_{11} can be adequately modelled as a single monoexponential consistent with $t_{0.5}$ in the region of 33-40h and that agonist occupancy of any of the α_1 adrenergic receptor subtypes leads to a fraction of the cellular G_q/G_{11} population being degraded much more rapidly. Data from each of the systems, however, are not consistent with all of the cellular content of these G proteins being degraded more rapidly in the presence of agonist. A maximally effective concentration of phenylephrine was able to cause downregulation of between 50 and 70% of the total $G_q\alpha/G_{11}\alpha$ population in these cells in a range of experiments.

As the immunoprecipitation experiments that were performed in the G protein turnover studies made use of an antiserum that identifies $G_q\alpha/G_{11}\alpha$ equally [Mitchell *et al.*, 1991], as it is directed at an epitope that is identical in these two G proteins as idicated in **Table 2.1**, and these two G proteins are widely co-expressed [Strathmann & Simon, 1990], I then wished to examine the possibility that each of the α_1

adrenergic receptor subtypes was able to cause downregulation of only one of these two G proteins. To do so, observations that separation of these two polypeptides can be achieved in SDS-PAGE systems that incorporate high concentrations of urea were used [Mullaney *et al.*, 1993; Svoboda & milligan, 1994; Shah & Milligan, 1994].

Immunoblotting of membranes of the clones used in this study with antiserum CQ following their resolution in such gels demonstrated that the steady-state levels of $G_{11}\alpha$ expression was over 2-fold higher than that of $G_q\alpha$. Furthermore, they indicated that sustained phenylephrine occupancy of each of the α_1 adrenergic receptor subtypes resulted in a downregulation of both $G_{11}\alpha$ and $G_q\alpha$. Although it was not analysed in these clonal cell lines, it was previously shown in cellular fractionation studies of Chinese hamster ovary cells on sucrose density gradients that the subcellular distribution of these two G proteins is identical [Svoboda & Milligan, 1994].

An epitope-tagged constitutively activated mutant of $G_s\alpha$ has been demonstrated to have a substantially reduced half-life compared with the epitope-tagged wild type protein when expressed in S49 lymphoma cyc$^-$ cells [Levis & Bourne, 1992], and agonist activation of an IP prostanoid receptor can result in downregulation of this epitope-tagged variant of $G_s\alpha$ when this G protein is stably expressed in neuroblastoma NG108-15 cells [Mullaney & Milligan, 1994]. Thus, although it has not been formally demonstrated for any G protein other than $G_s\alpha$, it is reasonable to surmise that activation of the G protein might be the key feature that controls its rate of degradation.

The palmitoylation status of both the activated mutant of $G_s\alpha$ and the wild type protein following activation of a $G_s\alpha$-linked receptor is altered compared with the basal state of the wild type protein [Mumby *et al.*, 1994; Degtyarev *et al.*, 1993; Wedegaertner & Bourne, 1994]. Kinetic evidence indicates that this is likely to reflect accelerated depalmitoylation [Wedegaertner & Bourne, 1994]. How relevant this is to agonist-mediated downregulation of $G_s\alpha$ remains to be explored, but it is certainly true

that in a number of systems, agonist occupation of $G_s\alpha$-linked receptors has been noted to result in large selective downregulation of this G protein [McKenzie & Milligan, 1990; Adie *et al.*, 1992; Adie & Milligan, 1994].

It will thus now be of considerable interest to examine whether agonist activation of the α_1 adrenergic receptor subtypes in these cells results in an alteration in the palmitoylation status of the α subunits of G_q/G_{11}. As with the previous sudies on muscarinic m1 receptor regulation of $G_q\alpha/G_{11}\alpha$ levels [Mitchell *et al.*, 1993], Northern blot analysis of mRNAs corresponding to these G proteins in untreated and phenylephrine treated cells did not provide evidence for regulation at the mRNA level [Wise *et al.*, 1995].

The data provided herein demonstrate that agonist occupation of α_1 adrenergic receptor subtypes can selectively regulate the cellular levels of both $G_q\alpha$ and $G_{11}\alpha$. The mechanism of this effect is a selective acceleration of the rate of degradation of these G proteins.

Table 4.1. Classification of α_1 adrenergic receptor subtypes

Native	Cloned	Peptide Length	Chromosomal Location (Human)	Tissue Distribution	Potency	
					Agonist	Antagonist
α_{1A}	α_{1a} (α_{1c})*	466 a.a.	C8	vas deferens brain prostate	oxymetazoline(+++) norepinephrine(++) phenylephrine(+)	prazosin(+++) (+)niguldipine(++) 5-methylurapidil(+)
α_{1B}	α_{1b} (α_{1b})*	515 a.a.	C5	liver spleen blood vessels	norepinephrine(++) phenylephrine(+)	prazosin(++) chloethyl- clonidine(+)
α_{1D}	α_{1d} ($\alpha_{1a/d}$)*	560 a.a.	C20	aorta	norepinephrine(++) phenylephrine(+)	prazosin(++) BMY 7378(+)

* The subtypes in the parentheses are historical nomenclature, meanwhile the subtypes without parentheses are the newly suggested cloned nomenclature by IUPHAR [1995].

(+) The potency of drugs is represented in order of +++>++>+.

Figure 4.1. Seven transmembrane spanning model of the hamster α_{1B} adrenergic receptor

The deduced amino acid sequence and putative topography of the hamster α_{1B} adrenergic receptor is depicted. Seven transmembrane domains (7TMD) are defined by hydropathy analysis of the receptor sequence. The amino acid identities with residues in 7TMD of the rat α_{1A} and the bovine α_{1C} adrenergic receptor are 73% and 65%, respectively. Solid circles indicate amino acids identical in the corresponding position in the hamster β_2 adrenergic receptor which shows 45% identity in 7TMD. Potential sites of N-linked glycosylation are indicated by crosses at Asn 10, 24, 29 and 34 [Lomasney *et al.*, 1991].

Figure 4.1.

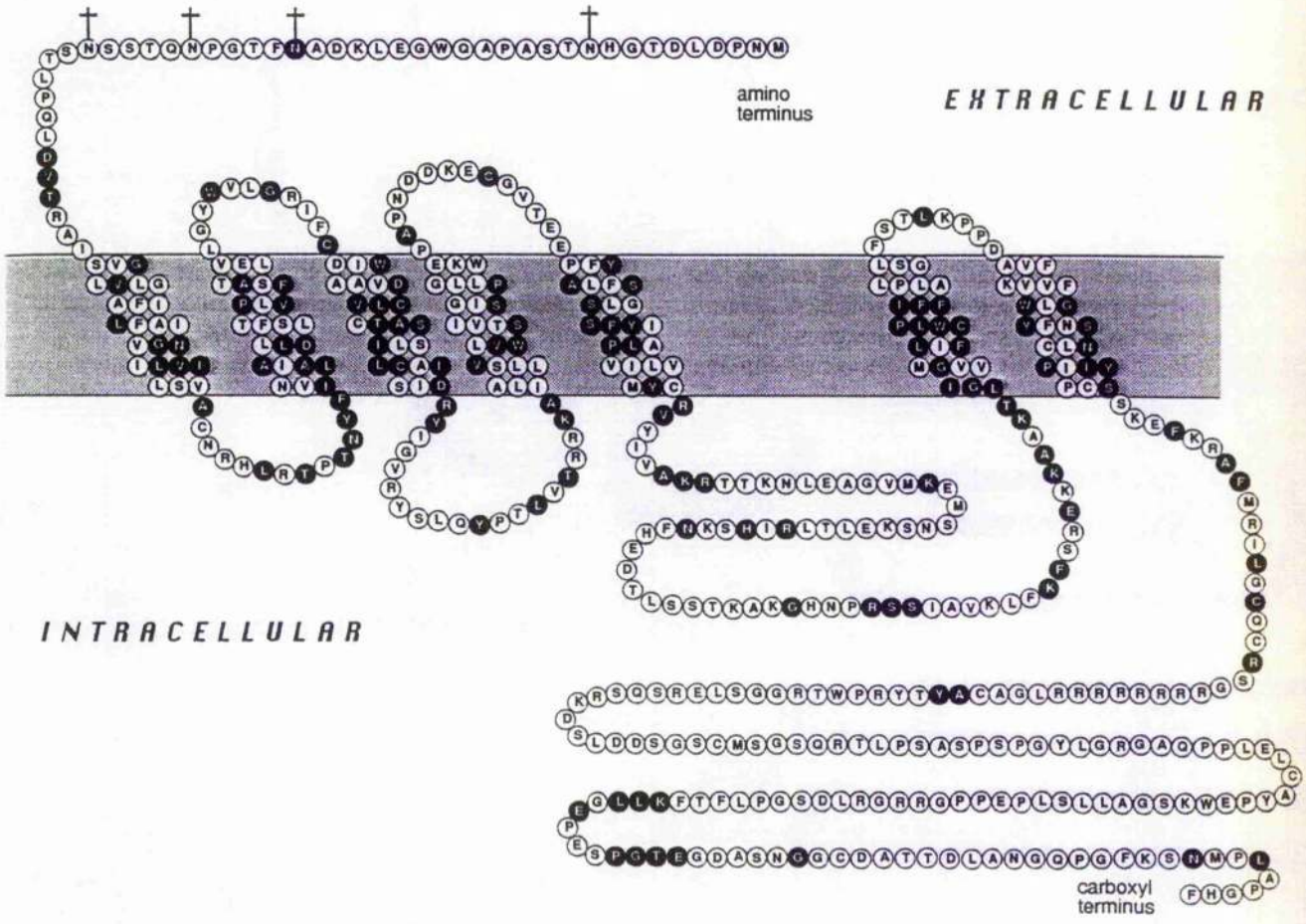

amino
terminus
EXTRACELLULAR
INTRACELLULAR
carboxyl
terminus

Figure 4.2. Specific detection of mRNAs encoding three subtypes of cloned α_1 adrenergic receptor

The presence of the three cloned α_1 adrenergic receptor subtypes in appropriately transfected cells was confirmed by reverse-transcriptase PCR analysis as described in **section 2.29.1.** RNA was isolated from untransfected parental and transfected rat 1 fibroblast cells which stably express the $\alpha_{1A/D}$, α_{1B}, and α_{1C} adrenergic receptor mRNA, reverse transcribed and subjected to PCR with the oligonucleotide primers specific for each of the three molecularly defined α_1 adrenergic receptor subtypes as described in **Table 2.3.**

Parental cells (lanes 1-3) and cells transfected $\alpha_{1A/D}$ (lanes 4-6), α_{1B} (lanes 7-9), and α_{1C} (lanes 10-12) adrenergic receptor cDNA are shown with specific primers of $\alpha_{1A/D}$ (lanes 1, 4, 7, and 10), α_{1B} (lanes 2, 5, 8, and 11), and α_{1C} (3, 6, 9, and 12). No message was detected in untranfected parental cells. On the other hand, each mRNA encoding α_1 adrenergic receptor subtypes was detected specifically in cells expressing $\alpha_{1A/D}$ (lane 4), α_{1B} (lane 8), and α_{1C} (lane 12) with their appropriate primers.

Figure 4.2

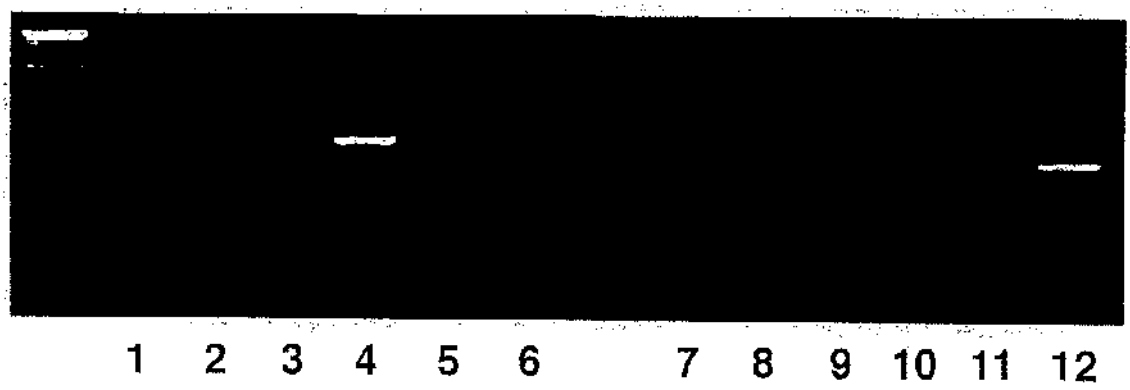


Figure 4.3. The ability of phenylephrine to displace [^{3}H] prazosin binding in membranes of α_1 adrenergic receptor expressing cells.

The ability of phenylephrine in the absence (filled symbols) or presence (open symbols) of Gpp[NH]p (100μM) to displace the specific binding of [^{3}H] prazosin to membranes of $\alpha_{1A/D}$-**panel (I)**, α_{1B}-**panel (II)** and α_{1C}-**panel (III)**-adrenergic receptor expressing Rat 1 fibroblasts was assessed as described in **section 2.12.2**. Typical examples, representative of 4 independent experiments, are displayed (see Results for details).

Figure 4.3

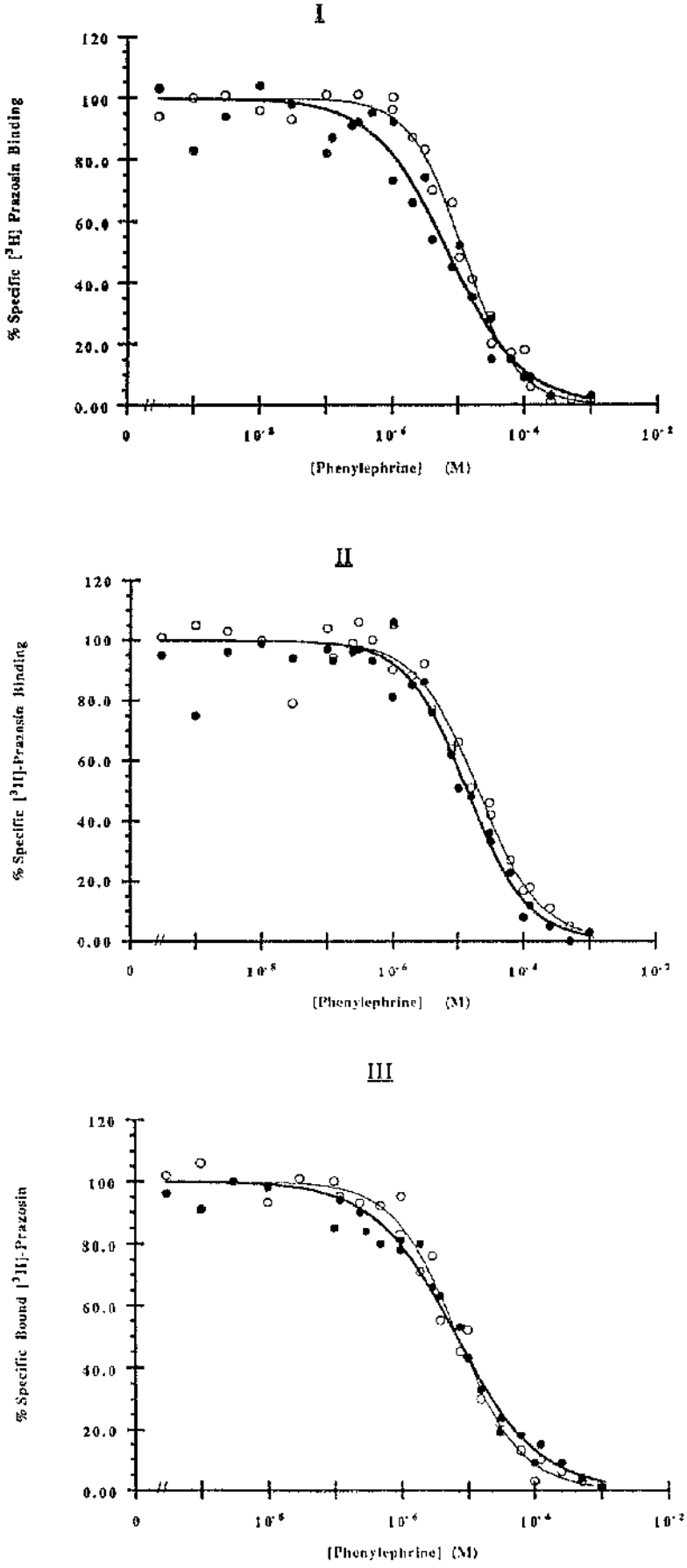
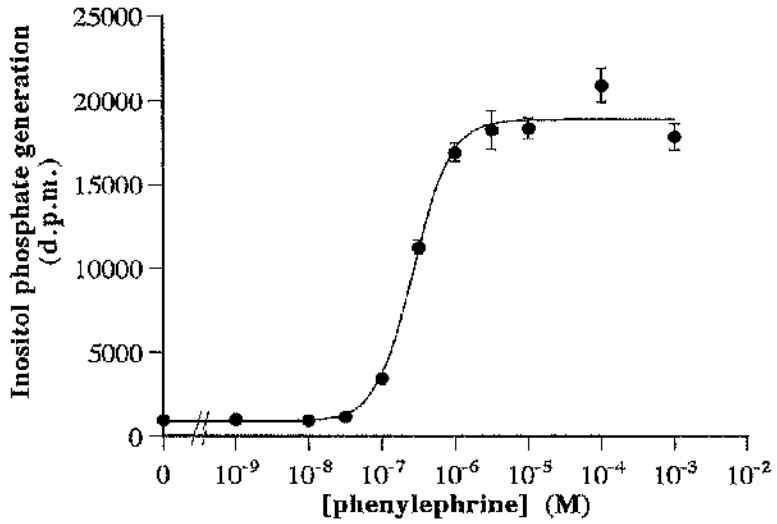

I
% Specific [3H] Prazosin Binding
120
100
80.0
60.0
40.0
20.0
0.00
0
10-8
10-6
10-4
10-2
[Phenylephrine] (M)
II
% Specific [3H]-Prazosin Binding
120
100
80.0
60.0
40.0
20.0
0.00
0
10-8
10-6
10-4
10-2
[Phenylephrine] (M)
III
% Specific Bound [3H]-Prazosin
120
100
80.0
60.0
40.0
20.0
0.00
0
10-8
10-6
10-4
10-2
[Phenylephrine] (M)

Figure 4.4. The α_1 adrenergic receptor subtypes all cause stimulation of inositol phosphate production.

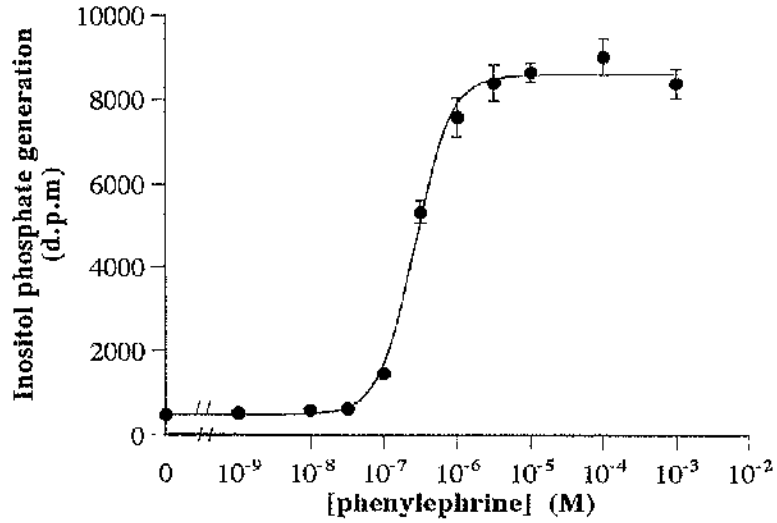
Rat 1 fibroblasts transfected to stably express the $\alpha_{1A/D}$ (**panel a**), α_{1B} (**panel b**) and α_{1C}-adrenergic receptor subtypes (**panel c**) were labelled with *myo*-[2-^{3}H]inositol (1 μCi/ml) for 36 h prior to treatment with varying concentrations of phenylephrine for 20 min. Total inositol phosphates were measured as described in **section 2.14**. Stimulation of inositol phosphate generation was found to be dose-dependent with an EC_{50} for phenylephrine between 0.3 and 1.0 μM for all three cell lines in a range of experiments. The data displayed are the mean of triplicate assays from representative experiments; bars represent S.E.M.

Figure 4.4

a)



b)



c)

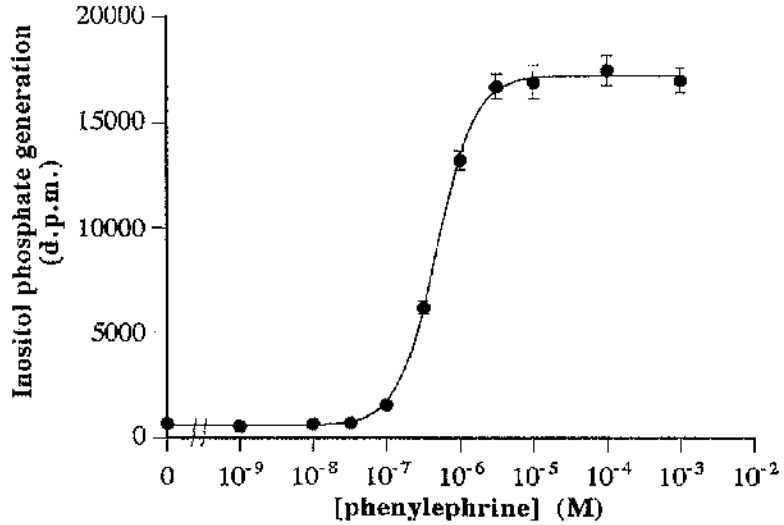
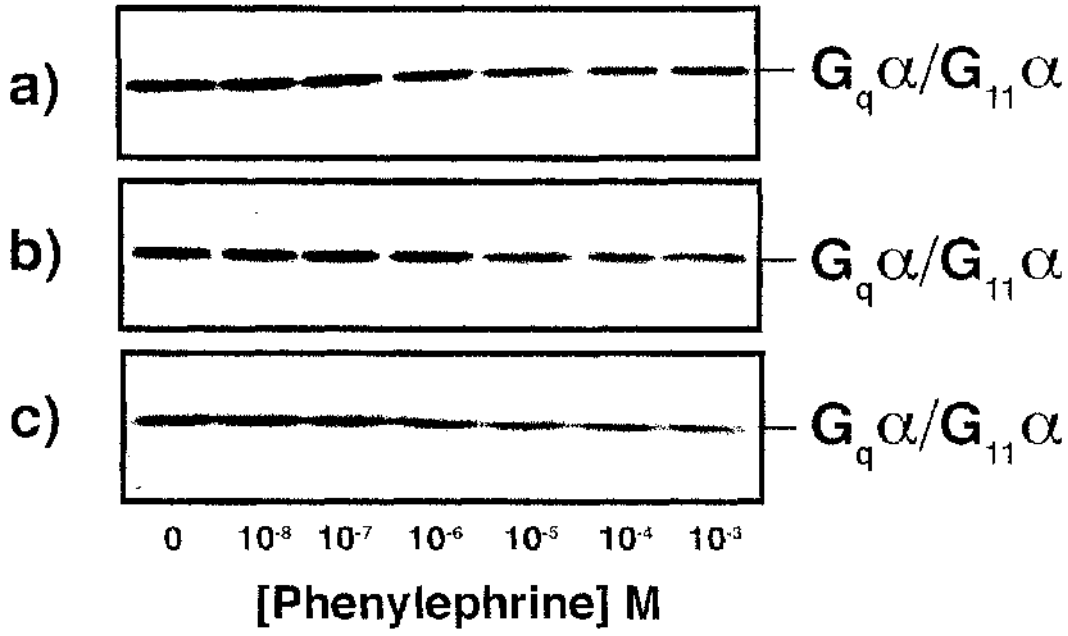


Figure 4.5. Phenylephrine-mediated downregulation of $G_q\alpha/G_{11}\alpha$ levels in membranes of α_1 adrenergic receptor subtype-expressing Rat 1 fibroblasts.

Membranes (15 μg) derived from $\alpha_{1A/D}$- **panel (a)**, α_{1B}- **panel (b)** and α_{1C}- **panel (c)** adrenergic receptor-expressing rat 1 fibroblasts which were either untreated or had been treated with varying concentrations of phenylephrine for 16h were resolved by SDS-PAGE [10 % (w/v) acrylamide] as described in **section 2.6** and then immunoblotted using the anti-$G_q\alpha/G_{11}\alpha$ antiserum CQ as primary reagent as described in **section 2.8**. Quantitative analysis, as described in **section 2.9** from these studies on the α_{1C} adrenergic receptor expressing cell line is displayed in **panel (d)**.

Figure 4.5


a)
b)
c)
$G_q\alpha/G_{11}\alpha$
$G_q\alpha/G_{11}\alpha$
$G_q\alpha/G_{11}\alpha$
0 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3}
[Phenylephrine] M

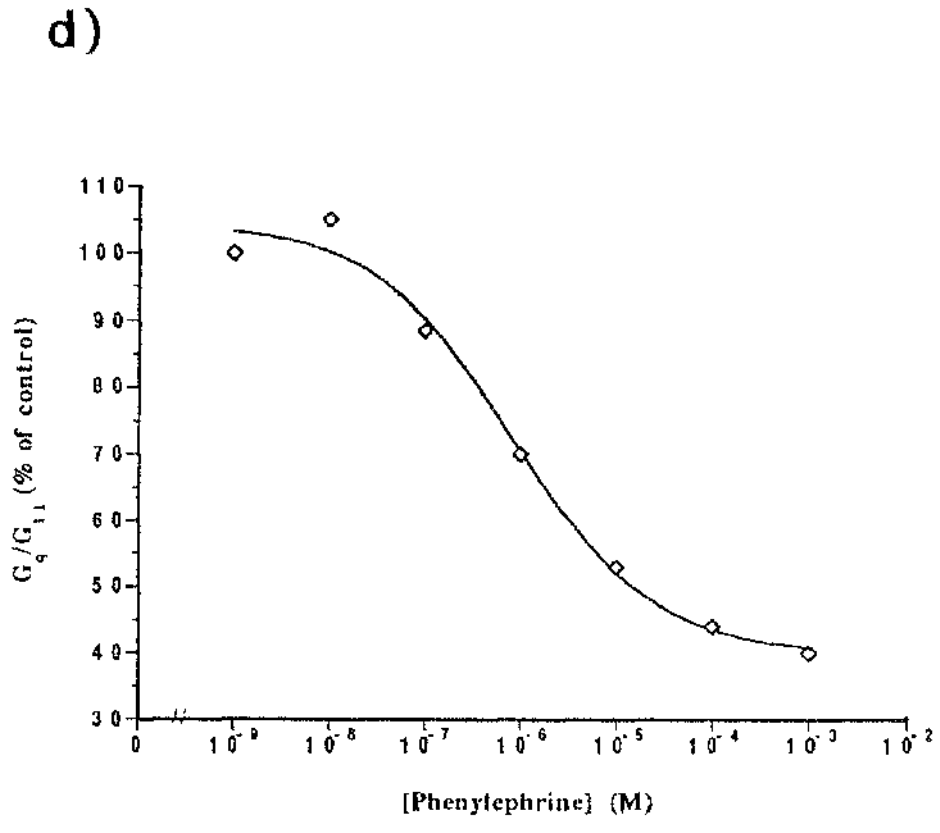
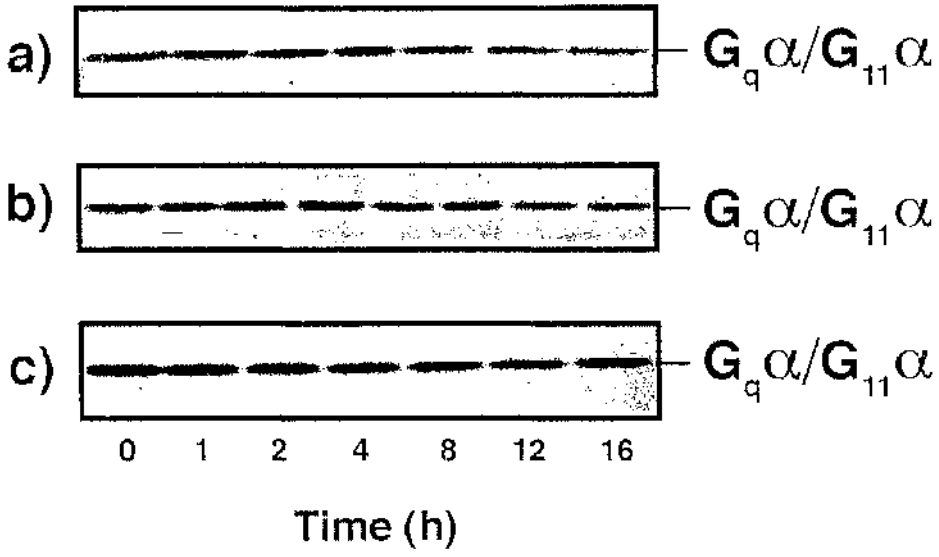

d)
G_q/G_{11} (% of control)
110
100
90
80
70
60
50
40
30
0 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3} 10^{-2}
[Phenylephrine] (M)

Figure 4.6. Time course of agonist-mediated downregulation of $G_q\alpha/G_{11}\alpha$.

Membranes (15 μg) derived from $\alpha_{1A/D}$-**panel (a)**, α_{1B}-**panel (b)** and α_{1C}-**panel (c)** adrenergic receptor-expressing rat 1 fibroblasts either untreated or treated with 100 μM phenylephrine for the times indicated were resolved by SDS-PAGE [10 % (w/v) acrylamide] as described in **section 2.6** and subsequently immunoblotted using antiserum CQ as described in **section 2.8**. Quantitative analysis, as described in **section 2.9**, from these studies on the α_{1C}-adrenoceptor expressing cell line is displayed in **panel (d)**.

Figure 4.6


a)
b)
c)
$G_q\alpha/G_{11}\alpha$
$G_q\alpha/G_{11}\alpha$
$G_q\alpha/G_{11}\alpha$
0 1 2 4 8 12 16
Time (h)

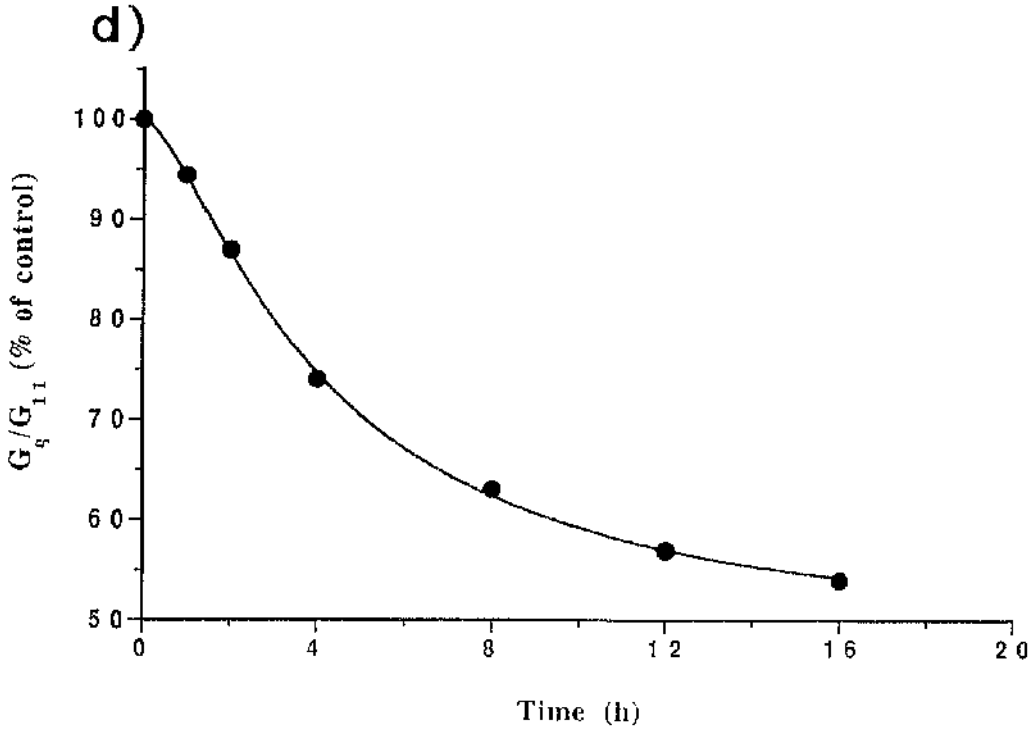

d)
G_q/G_{11} (% of control)
100
90
80
70
60
50
0 4 8 12 16 20
Time (h)

Figure 4.7. Phenylephrine treatment of α_1 adrenergic receptor subtype expressing cells results in downregulation of both $G_q\alpha$ and $G_{11}\alpha$.

Membranes (60 μg) from $\alpha_{1A/D}$- (lanes 1 and 2), α_{1B}- (lanes 3 and 4) and α_{1C}- (lanes 5 and 6) adrenergic receptor-expressing rat 1 fibroblasts, untreated (lanes 2, 4 and 6), or treated with phenylephrine (100 μM, 16 h) (lanes 1, 3 and 5) were resolved by SDS-PAGE in a 12.5 % (w/v) acrylamide, 0.0625 % (w/v) bis-acrylamide gel system containing 6 M urea as described in **section 2.6.2** and subsequently immunoblotted as described in **section 2.8**.

Figure 4.7

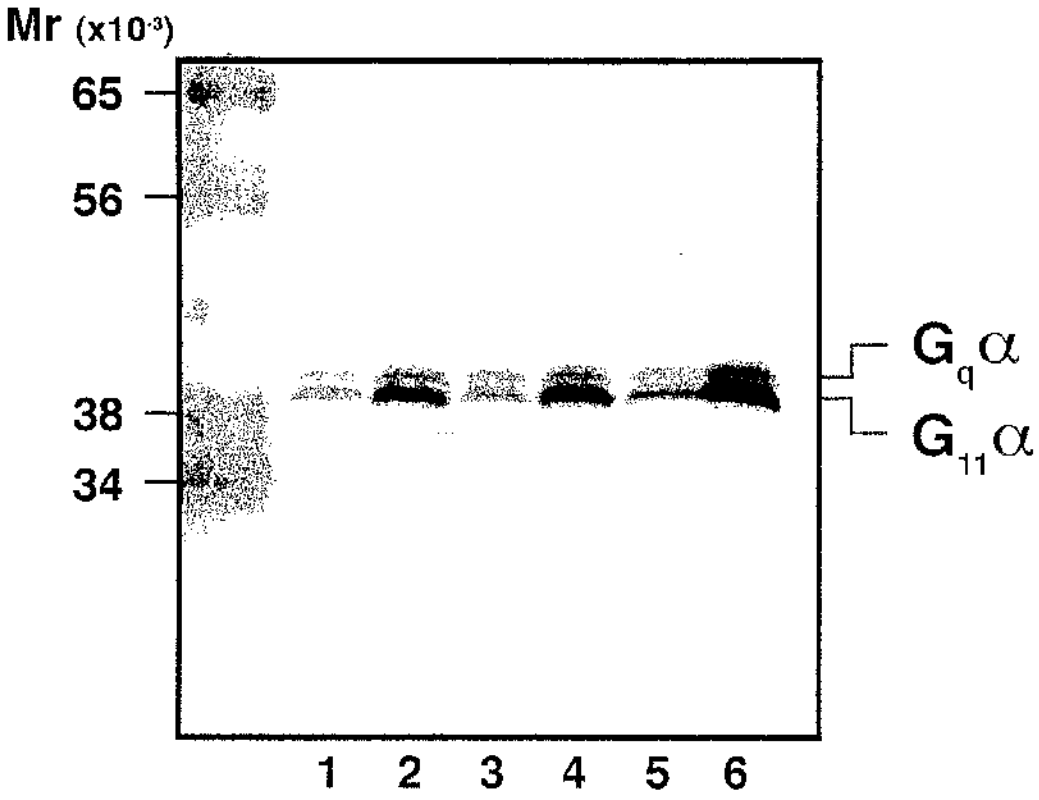


Figure 4.8. Time course of α_1 adrenergic receptor subtype-stimulated enhancement of $G_q\alpha/G_{11}\alpha$ protein degradation

Whole cell [^{35}S]-amino acid pulse-chase analysis of $G_q\alpha/G_{11}\alpha$ from each α_1 adrenergic receptor-expressing rat 1 cells isotopically labelled and experimentally processed as described in **section 2.2.7**. In the chase phase cells expressing $\alpha_{1A/D}$-**panel (a)**, α_{1B}-**panel (b)**, and α_{1C} adrenergic receptor-**panel (c)** were incubated with medium containing non-radiolabelled amino acids in the absence (filled symbols) or presence (open symbols) of 100 μM phenylephrine. Cell activity was stopped at the indicated time and the cell extract processed for immunoprecipitation as described in **section 2.5.2**. Following resolution of the proteins, the dried gels were exposed to phosphor storage screen autoradiography and the radiolabelled $G_q\alpha/G_{11}\alpha$ analysed as described in **section 2.11**. Results represent the means from four independent experiments.

Figure 4.8

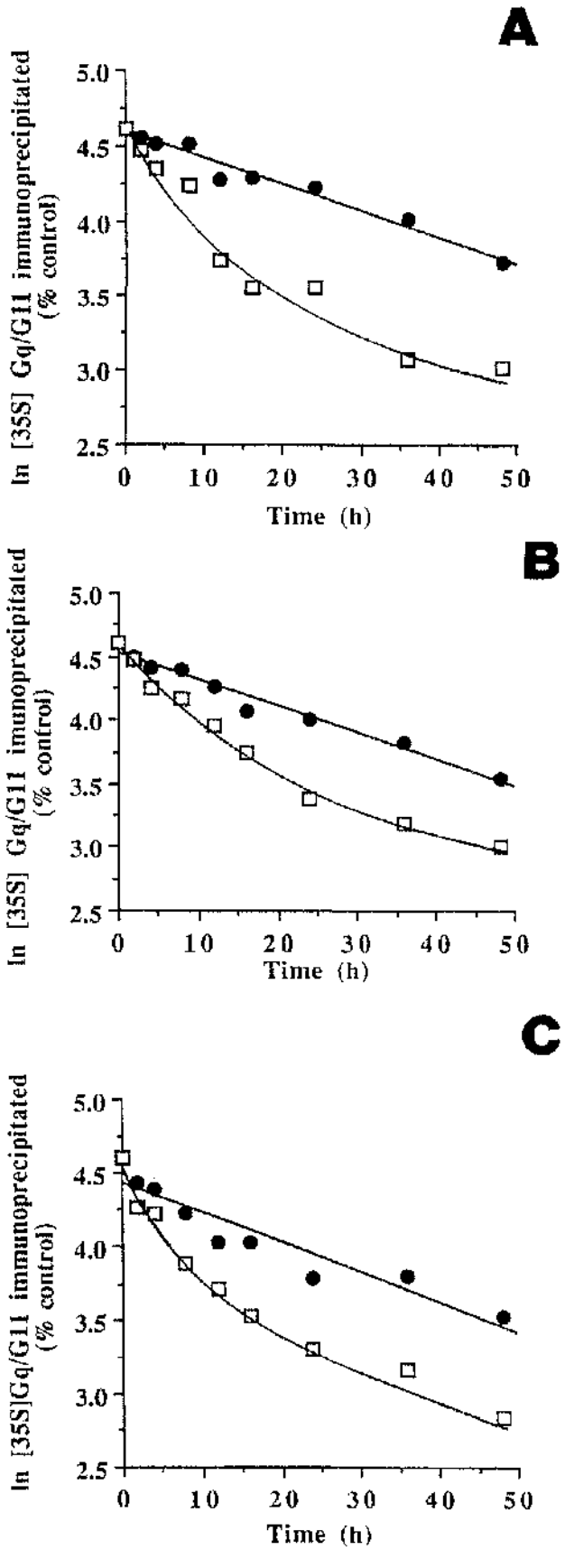

A
B
C
ln [35S] Gq/G11 imunoprecipitated (% control)
ln [35S]Gq/G11 immunoprecipitated (% control)
5.0
4.5
4.0
3.5
3.0
2.5
0
10
20
30
40
50
Time (h)

Table 4.2. Exposure of rat 1 cells expressing α_1 adrenergic receptor subtypes results in downregulation of these receptors.

Rat 1 cells expressing the three α_1 adrenoceptor subtype cDNAs were exposed to amaximally effective concentration of phenylephrine (100μM) or vehicle for 6hrs. Membranes were prepared from these cells as described in **section 2.3** and [^{3}H] prazosin binding experiments performed as described in **section 2.12.2**. Results are presented as % of the [^{3}H] prazosin binding sites present after treatment with phenylephrine compared to treatment with vehicle.

Condition	Receptor subtype		
[^{3}H]prazosin binding	$\alpha_{1A/D}$	α_{1B}	α_{1C}
% control	26 ± 9	53 ± 4	38 ± 7

Chapter 5

Signalling characteristics of a constitutively active mutant (CAM) of the α_{1B}-adrenergic receptor

Chapter 5. Signalling characteristics of a constitutively active mutant (CAM) of the α_{1B}-adrenergic receptor

5.1. Introduction

Different receptors for adrenaline and noradrenaline couple preferentially to different subfamilies of G proteins to allow the same hormones to regulate distinct effector pathways [Milligan *et al.*, 1994]. The α_1 adrenergic receptor subtypes represent the prototypic examples of G protein-coupled receptors (GPCRs) which interact with pertussis toxin-insensitive G proteins to cause stimulation of phosphoinositidase C activity as described in **section 4.1** [Bylund, 1992].

In experiments designed to analyse which segments of the intracellular loops of receptors define the identity of the G proteins regulated by different adrenergic receptors, substitution of a small region of the human β_2-adrenergic receptor in the extreme distal end of the third intracellular loop with the equivalent region from the hamster α_{1B}-adrenergic receptor resulted in the expressed construct displaying elevated basal activation of adenylyl cyclase activity compared to the wild type receptor in the absence of an agonist ligand [Samama *et al.*, 1993, Pei *et al.*, 1994].

This construct was designated as the CAM (constitutively active mutant) β_2-adrenergic receptor. Generation of a reciprocal construct in which a small section of the α_{1B}-adrenergic receptor was replaced with the equivalent region of the β_2-adrenergic receptor resulted in this construct (CAMα_{1B}-adrenergic receptor) as shown in **Figure 5.1** [Cotecchia *et al.*, 1990, Kjelsberg *et al.*, 1993, Allen *et al.*, 1991] displaying elevated basal activation of phosphoinositidase C.

Although it has been well established that sustained exposure of many GPCRs to agonist can result in a reduction in cellular levels of the receptor (a process known as downregulation) [Dohlman *et al.*, 1991] it is only in the recent past that it has also become clear that agonist-occupation of GPCRs can result in a reduction in cellular levels of the G protein(s) activated by the receptor [Milligan, G., 1993]. The mechanism responsible for agonist-mediated G protein regulation seems primarily to be enhanced proteolysis of the activated G protein α subunit as it has been demonstrated in clones of Rat 1 fibroblasts transfected to express individual α_1 adrenergic receptor subtypes [see chapter 4]. Thus, in the first half of this study the ability of the α_1 adrenergic receptor agonist phenylephrine to regulate cellular levels of $G_q\alpha$ and $G_{11}\alpha$ in Rat 1 fibroblasts transfected to express stably either the wild type or the CAMα_{1B}-adrenergic receptor were compared.

A recent reappraisal of receptor theory has concluded that even wild type receptors are not entirely quiescent in the absence of agonist ligands and that the position of the equilibrium between an inactive conformation of the receptor [R] and a conformation [R*] capable of activating a cognate G protein may vary between different receptors and can be modulated by mutation [Samama *et al.*, 1993; Lefkowitz *et al.*, 1993; Tiberi & Caron, 1994; Bond *et al.*, 1995; Milligan *et al.*, 1995; Mullaney *et al.*, 1996].

The current view is that agonist ligands function because they display preferential affinity for [R*], act to selectively stabilize this form of the receptor and thus move the equilibrium between [R]<----->[R*] to the right [Samama *et al.*, 1993; Lefkowitz *et al.*, 1993; Tiberi & Caron, 1994; Bond *et al.*, 1995; Milligan *et al.*, 1995]. Ligands which display preferential affinity for [R] will move the equilibrium in the other direction, will reduce agonist-independent activity and, on this basis, have been termed "inverse agonists" as described in **section 1.2.4.3** [Costa & Herz, 1989; Samana *et al.*, 1993; Lefkowitz *et al.*, 1993; Tiberi & Caron,

1994; Leeb-Lundberg *et al.*, 1994; Adie & Milligan, 1994; Chidiac *et al.*, 1994; Samama *et al.*, 1994; Bond *et al.*, 1995; Milligan *et al.*, 1995; Mullaney *et al.*, 1996].

If CAM variants provide useful models for an [R*] state of a receptor it must be predicted that as long as such mutations have the inherent capacity to relax to the [R] conformation then they will potentially provide useful models for detection of inverse agonists and a means to explore their mechanisms of action. In the second half of this study the regulation of the CAMα_{1B}-adrenergic receptor by the α-adrenergic receptor inverse agonist phentolamine is assessed whether this CAM receptor provides a useful screening system for inverse agonists.

5.2. Results.

5.2.1. Cells transfected to express the wild type or CAMα_{1B}-adrenergic receptor express only the anticipated form of the receptor.

RNA was isolated from clones of Rat-1 fibroblasts stably transfected with cDNA species encoding either the wild type hamster α_{1B}-adrenergic receptor or an altered form of this receptor (CAMα_{1B}-adrenergic receptor [Cotecchia *et al.*, 1990, Kjelsberg *et al.*, 1993, Allen *et al.*, 1991]) in which a fragment of the distal end of the third intracellular loop was replaced with the equivalent segment of the human β_2-adrenergic receptor. The RNA samples were subjected to reverse transcription (RT) followed by polymerase chain reaction (PCR) using a primer pair designed to straddle the region altered to generate the CAMα_{1B}-adrenergic receptor as described in **section 2.29** and **Table 2.3.** Such RT-PCR resulted in the anticipated generation of a fragment of 597bp from both cell lines as shown in **Figure 5.2.**

The alteration in sequence to produce the CAMα_{1B}-adrenergic receptor introduces a specific site for the restriction enzyme *Stu*-I which should cleave the PCR product virtually in the centre to generate fragments of 292 and 305 bp. Treatment of the PCR product from the clone transfected with the CAMα_{1B}-adrenergic receptor cDNA resulted in all of the fragment being digested whereas equivalent treatment with the PCR fragment derived from the cells transfected with the wild type α_{1B}-adrenergic receptor cDNA had no effect as shown in **Figure 5.2.** These results confirmed the identity of the expressed receptors and indicated that the entire cellular population of the CAMα_{1B}-adrenergic receptor cDNA transfected cells reflects the modified receptor.

5.2.2. The wild type receptor is expressed at higher levels than the CAMα_{1B} adrenergic receptor.

Inositol phosphate generation in the absence of added agonist, measured using a Li^+ blockade accumulation assay, was some 2 fold higher in cells of the clone expressing the CAMα_{1B}-adrenergic receptor compared to the wild type receptor when the results were modified to account for the level of incorporation of [^{3}H]inositol into cellular phospholipids and around 3-4 fold higher when the generation of [^{3}H]inositol phosphates was not corrected in this manner (data not shown but see **Figure 5.5**) as has previously been recorded [Cotecchia *et al.*, 1995]. This was despite ligand binding studies, using the specific binding of the α_1-adrenoceptor antagonist [^{3}H]prazosin as described in **Section 2.12**, which demonstrated that the CAMα_{1B}-adrenergic receptor was expressed at lower levels than the wild type receptor as shown in **Figure 5.3**.

5.2.3. Antagonists bind the wild type and CAMα_{1B} adrenergic receptor with similar affinity but agonists show substantially higher affinity for the CAM receptor.

The estimated affinity for [^{3}H]prazosin binding as described in **Section 2.12.2** to the wild type and the CAMα_{1B}-adrenergic receptor were very similar (K_d = 8. 6 ± 1.5 x 10^{-11} M at wild type and 9.6 ± 1.9 x 10^{-11} M at the CAM receptor, mean ± S.E.M. n = 3 in each case). Competition for the binding of [^{3}H]prazosin by the α_1 antagonist phentolamine to the wild type (K_i = 2.1± 0.1 x 10^{-8} M) and the CAM (K_i = 1.1 ± 0.1 x 10^{-8}M) α_{1B}-adrenergic receptor was achieved with similar affinities as shown in **Figure 5.4a,** but the potencies of the agonists phenylephrine and noradrenaline to compete for the binding of [^{3}H]prazosin were approximately 160 fold higher at the CAMα_{1B}-adrenergic receptor (IC_{50} (corrected for radioligand occupancy) for noradrenaline = 5.3 ± 0.2 x 10^{-9} M, for phenylephrine = 2.4 ± 0.2 x 10^{-8} M) than at the wild type receptor (IC_{50} (corrected for radioligand occupancy) for noradrenaline = 9.3 ± 0.5 x 10^{-7} M, for phenylephrine = 4.6 ± 0.3 x 10^{-6} M) (means ± S.E.M., n= 3 in all cases) as shown in **Figure 5.4b**.

5.2.4. Phenylephrine, but not endothelin 1, stimulates inositol phosphate generation to both greater levels and more potently in cells expressing the CAMα_{1B}-adrenergic receptor compared to the wild type receptor.

Stimulation of inositol phosphate generation from [^{3}H]inositol-labelled cells as described in **Section 2.14** by the agonists noradrenaline (EC_{50}s = 5.3 ± 0.6 x 10^{-9} M compared to 7.7 ± 0.6 x 10^{-7} M) and phenylephrine (EC_{50}s = 5.1 ± 0.4 x

10^{-9} M compared to 5.9 ± 0.1 x 10^{-7} M) (means ± S.E.M. n = 3) was also greater than 100 fold more potent in cells of the clone expressing the CAMα_{1B}-adrenergic receptor than in those expressing the wild type receptor as shown in **Figure 5.5a** and **5.5b.** Furthermore, the maximal ability of phenylephrine to stimulate inositol phosphate production was substantially greater (170±20 %, mean±S.E.M., n=3) at the CAMα_{1B}-adrenergic receptor as shown in **Figure 5.5a.**

These effects could be clearly attributed to the specific forms of the α_{1B}-adrenergic receptor expressed and not simply to some trivial example of clonal variation as inositol phosphate generation in response to endothelin 1, acting at the endogenously expressed ET_A endothelin receptor, was similar both in maximal effect (in the CAMα_{1B}-adrenergic receptor expressing cells endothelin 1 produced 108.6±5.8% of the inositol phosphate response observed in the cells expressing the wild type receptor) and in the potency of the ligand (EC_{50} = 4.8±2.1 x 10^{-9}M at the CAMα_{1B}-adrenergic receptor and 3.0±1.2 x 10^{-9}M at the wild type receptor, mean±S.E.M., n=3 in each case) as shown in **Figure 5.5c.**

5.2.5. Phenylephrine stimulates phosphatidylbutanol generation to both greater levels and more potently in cells expressing the CAMα_{1B}-adrenergic receptor compared to the wild type receptor.

Stimulation of phosphatidylbutanol generation from [^{3}H]palmitate-labelled cells as described in **Section 2.15** by the agonist phenylephrine was approximately 30 fold more potent in cells of the clone expressing the CAMα_{1B}-adrenergic receptor than in those expressing the wild type receptor as shown in **Figure 5.6.** EC_{50}s were 3.2 x 10^{-7}M for wild type and 9.7 x 10^{-9}M for the CAM receptor, respectively (means ± S.E.M., n=3). Furthermore, the maximal ability of

phenylephrine to stimulate phosphatidylbutanol production was about 2 fold greater at the CAMα_{1B}-adrenergic receptor as shown in **Figure 5.6.**

5.2.6. Membrane levels of $G_q\alpha$ and $G_{11}\alpha$ are reduced by phenylephrine treatment of both wild type and CAMα_{1B}-adrenergic receptor expressing cells but the effect is greater in the CAM receptor expressing cells.

Sustained exposure of the two clones to a concentration of phenylephrine (100μM) which resulted in maximal generation of [^{3}H]inositol phosphates in both cell lines resulted in a reduction in cellular levels of some combination of the α subunits of the phosphoinositidase C-linked, pertussis toxin-insensitive, G proteins G_q and G_{11} when immunoblotting experiments were performed using SDS-PAGE (10% (w/v) acrylamide) conditions which are unable to resolve these two polypeptides and with an antiserum (CQ) which identifies a common epitope shared at the extreme C-terminus of these two polypeptides (36±5% for the wild type and 72±8% reduction for the CAM receptor, repectively).

Resolution of these two G protein α subunits by SDS-PAGE (10% (w/v) acrylamide) which incorporated 6M urea into the resolving gel as described in **Section 2.6** indicated that cellular steady-state levels of $G_{11}\alpha$ were higher than $G_q\alpha$ but that very similar levels of each of these G proteins were present in membranes of untreated wild type receptor and CAMα_{1B}-adrenoceptor expressing cells as shown in **Figure 5.7.** Phenylephrine treatment resulted in a downregulation of both of these polypeptides in cells expressing both wild type and the CAMα_{1B}-adrenoceptor as shown in **Figure 5.7.** Importantly, however, the maximal degree of $G_q\alpha$ and $G_{11}\alpha$ downregulation which could be achieved in

response to phenylephrine treatment was substantially greater in the clone expressing the CAMα_{1B}-adrenoceptor.

5.2.7. More pronounced down-regulation of $G_q\alpha/G_{11}\alpha$ is produced by the CAM than the wild type α_{1B}-adrenergic receptor by all concentrations of phenylephrine.

Sustained exposure of the wild type and the CAMα_{1B}-adrenoceptor expressing cells to varying concentrations of phenylephrine indicated that the enhanced capacity to cause downregulation of these two G proteins was observed at all concentrations of the agonist by immunoblotting as described in **Section 2.8** and as shown in **Figure 5.8a.** However, in contrast to the differences in affinity of phenylephrine at the wild type and CAMα_{1B}-adrenoceptor measured in binding assays and for inositol phosphate generation, the potency of phenylephrine to cause downregulation of G_q/G_{11} was only slightly greater in cells expressing the CAMα_{1B}-adrenoceptor ($1.5\pm1.1 \times 10^{-8}$M) than in those expressing the wild type α_{1B}-adrenoceptor ($6.5\pm1.8 \times 10^{-8}$M)(mean±S.E.M, n=3 in each case) as shown in **Figure 5.8b**.

5.2.8. Phenylephrine treatment of both wild type and CAMα_{1B}-adrenergic receptor expressing cells accelerates the rate of degradation of $G_q\alpha/G_{11}\alpha$.

Wild type and CAMα_{1B}-adrenergic receptor expressing cells were labelled with Trans[^{35}S]label and immunoprecipitated as described in **Section 2.2.7.** Little difference in the turnover of $G_q\alpha/G_{11}\alpha$ could be observed between the wild type and CAMα_{1B}-adrenergic receptor expressing cells in the absence of agonist

ligand as shown in **Figure 5.10.** Half-lives for these G proteins, as assessed in [^{35}S]amino acid pulse-chase experiments were adequately described by monoexponential function, being between 33-36 hours in individual experiments.

As anticipated from previous studies [**Chapter 4**], the rate of degradation of $G_q/G_{11}\alpha$ in the cells expressing the wild type α_{1B}-adrenergic receptor was accelerated by the presence of 100μM phenylephrine (estimated $t_{0.5}$ = 21.1 ± 2.7 h) as shown in **Figure 5.9a**. A similar effect of phenylephrine was observed in the cells expressing the CAMα_{1B}-adrenoceptor, except that the rate of degradation ($t_{0.5}$ = 14.1 ± 1.6 h)(means±S.E.M., n=3 in each case) as shown in **Figure 5.9b** was somewhat more pronounced than in the wild type expressing cells.

5.2.9. Upregulation of the CAMα_{1B}-adrenergic receptor following exposure to α_1 antagonists

Sustained (24h) treatment of CAMα_{1B}-adrenergic receptor expressing cells with α_1 antagonists resulted in upregulation of CAMα_{1B}-adrenergic receptor levels, but not in wild-type α_{1B}-adrenergic receptor expressing cells as shown in **Figure 5.10a**. Phentolamine, HV-723, and WB 4101 were able to increase the receptor levels approximately 3 fold. Furthermore, this upregulation of CAMα_{1B}-adrenergic receptor levels was also observed in response to YM12617, corynanthine, and 5-methyl urapidil with approximately 2 fold over vehicle (control) as shown in **Figure 5.10b**.

5.2.10. Time-dependent upregulation of the CAMα_{1B}-adrenergic receptor following exposure to phentolamine

As phentolamine produced a large upregulation of the CAMα_{1B}-adrenergic receptor levels, this effect was examined over a range of time courses. The bulk of upregulation of the CAMα_{1B}-adrenergic receptor was produced in response to the presence of phentolamine in around 36h as shown in **Figure 5.11**.

5.2.11. Phentolamine produces an upregulation of the CAMα_{1B}-adrenergic receptor as measured by Scatchard analysis of [^{3}H]prazosin binding.

In order to confirm that the enhanced binding of a single concentration of [^{3}H]prazosin in membranes of phentolamine-treated cells expressing CAMα_{1B}-adrenergic receptor represented a true upregulation Scatchard analyses were conducted. As shown in **Figure 5.12** saturation analysis of [^{3}H]prazosin binding demonstrated the total number of receptor sites to be increased greatly following phentolamine treatment (about 3 fold) whereas the affinity for [^{3}H]prazosin for the CAMα_{1B}-adrenergic receptor was similar following treatment with or without phentolamine.

5.2.12. Phentolamine treatment of the CAMα_{1B}-adrenergic receptor expressing cells increases agonist-stimulated phosphoinositidase C and phospholipase D activity

I investigated whether the large increase in CAMα_{1B}-adrenergic receptor levels in response to phentolamine (24h) could cause increase in the basal and maximal level of phosphoinositidase C (PIC) as well as phospholipase D (PLD) activity, but not in cells expressing wild-type α_{1B}-adrenergic receptor. As shown in **Figure 5.13a** the maximal level of phosphoinositidase C activity which could be

achieved by these cells with a maximally effective concentration of phenylephrine was increased 2-3 fold in CAMα_{1B}-adrenergic receptor expressing cells treated with phentolamine for 24h.

An approximately 2 fold increase in basal phosphoinisitidase C activity was observed in these cells with phentolamine treatment. Despite the increase in CAMα_{1B}-adrenergic receptor levels in these cells in response to phentolamine the EC_{50} for phenylephrine was not changed. The same was also true when the maximal and basal levels of phospholipase D activity were measured in phentolamine pretreated cells expressing the CAMα_{1B}-adrenergic receptor. The EC_{50} for phenylephrine in the treated cells was again similar in the phentolamine untreated cells as shown in **Figure 5.13b**.

5.2.13. α_1 antagonists lower phentolamine-upregulated basal phospholipase D acivity in cells expressing CAMα_{1B}-adrenergic receptor

As phentolamine produced an upregulation of the CAMα_{1B}-adrenergic receptor and increased basal phospholipase D activity this system could provide a model to detect α_1 adrenergic receptor inverse agonists. Phentolamine, HV-723, YM-12617, corynanthine, 5-methyl urapidil, and WB-4101 were clearly capable of acting as inverse agonists at this CAMα_{1B}-adrenergic receptor as measured by their ability to inhibit basal phospholipase D activity. As shown in **Figure 5.14** phentolamine, HV-723, corynanthine, and WB 4101 greatly decreased (approximately 80%) the phentolamine-upregulated PLD basal activity. Meanwhile YM12617 and 5-methyl urapidil reduced this to a lesser degree (51% and 38% over control, respectively).

5.3. Discussion

Modifications of GPCRs which result in the receptors displaying agonist-independent second messenger regulatory capacity have been observed both by designed mutation in the laboratory [Samama *et al.*, 1993, Pei *et al.*, 1994, Cotecchia *et al.*, 1990, Kjelsberg *et al.*, 1993, Allen *et al.*, 1991] and in spontaneous mutations [Shenker *et al.*, 1993, Parma *et al.*, 1993, Raymond, J.R., 1994, Coughlin, S.R., 1994] within the human population. GPCRs of this form have been termed constitutively active mutant (CAM) receptors and much recent interest has centred on to what degree these provide useful models, at least in certain aspects, of the conformational changes which must occur upon agonist binding to wild type receptors to allow efficient transmission of a signal via activation of heterotrimeric G proteins [Lefkowitz *et al.*, 1993].

Evidence, derived initially from observations that varying degrees of overexpression of wild type GPCRs resulted in an increase in basal activity (i.e. in the absence of agonist) of the effector system coupled to that receptor and that there was a strong correlation between the level of expression of the GPCR and the amount of effector regulation [Milano *et al.*, 1994, Adie & Milligan, 1994, Chidiac *et al.*, 1994], has indicated that adrenergic GPCRs must have the capacity to exist and spontaneously interconvert between a minimum of two (and possibly a continuum of) states, an inactive ground-state conformation R and an active conformation R* [Milano *et al.*, 1994, Samama *et al.*, 1994, Milligan *et al.*, 1995, Gether *et al.*, 1995].

In efforts to define elements of α_1 adrenergic receptors responsible for activation of pertussis toxin-insensitive G proteins, Cotecchia and co-workers generated a series of receptor chimaeras in which different elements of the third intracellular loop of the hamster α_{1B}-adrenergic receptor were replaced by

equivalent sequences from the human β_2-adrenergic receptor [Cotecchia *et al.*, 1990, Kjelsberg *et al.*, 1993, Allen *et al.*, 1991]. One of these chimaeras which was derived by replacement of the region 288-294 of the α_{1B}-adrenergic receptor with the corresponding sequence of the β_2-adrenoceptor causes a triple mutation (Ala293Leu, Lys290His, Arg288Lys) as shown in **Figure 5.1** and results in the expressed receptor displaying constitutive activation of inositol phosphate generation and a capacity to act as a protooncogene as measured by an ability to induce focus formation in fibroblasts transfected with this construct [Allen *et al.*, 1991].

This is the CAMα_{1B}-adrenergic receptor used in the present study. Although the CAMα_{1B}-adrenergic receptor was inherently able to produce this effect, addition of agonist potentiated this effect greatly, indicating that this form of the receptor did not function in a fully agonist-independent manner [Allen *et al.*, 1991]. Similarly, although the degree of agonist-independent inositol phosphate generation produced by the CAMα_{1B}-adrenergic receptor is markedly greater than by the wild type receptor, the CAM receptor still produces a further robust stimulation upon addition of agonist [Cotecchia *et al.*, 1995].

In **Chapter 4** I showed that sustained agonist treatment of Rat 1 fibroblasts transfected to express high levels of each of the wild type $\alpha_{1A/D}$, α_{1B} and α_{1C}-adrenergic receptors results in a substantial downregulation of the phosphoinositidase C-linked $G_q\alpha$ and $G_{11}\alpha$ and in this chapter I explored whether the CAM-mutant of the α_{1B}-adrenergic receptor has the capacity to act more effectively in this regard than the wild type receptor. This is clearly the case as shown in **Figures 5.7** and **5.8**. Addition of 100μM phenylephrine produced a markedly greater degree of G protein downregulation in the cells expressing the CAMα_{1B}-adrenergic receptor than in those expressing the wild type receptor (**Figure 5.7**).

This was despite the fact that the CAMα_{1B}-adrenergic receptor was expressed at somewhat lower steady-state levels than the wild type receptor (**Figure 5.3**). Particular attention was paid to this point by routinely performing [^{3}H]prazosin binding studies on membranes isolated from the control cells in such experiments (i.e. minus agonist or zero time points) to confirm the levels of expression in specific experiments. As shown for the wild type α_{1B}-adrenergic receptor in **Chapter 4**, downregulation of $G_q\alpha$ and $G_{11}\alpha$ by phenylephrine occupancy of the CAMα_{1B}-adrenergic receptor appeared to be relatively non-selective for these two G proteins, indicating equivalent interactions of these with the activated receptor [Milligan, 1993].

Although the CAMα_{1B}-adrenergic receptor did result in higher level generation of inositol phosphates in the absence of agonist than the wild type receptor, this was also true in response to maximally effective concentrations of agonist (**Figure 5.5**), confirming the data of Cotecchia *et al.* [1995]. Indeed, the basal inositol phosphate generation from both receptor variants was only a fraction of that produced in response to agonist. Inositol phosphate generation in response to maximally effective concentrations of phenylephrine or noradrenaline was approximately two-fold higher in the CAMα_{1B}-adrenergic receptor expressing cells than in those expressing the wild type receptor. The same was also true when phosphatidylbutanol generation by phospholipase D was measured (**Figure 5.6**). By contrast, there was little difference in the maximal ability of endothelin-1 to stimulate inositol phosphate generation in the two cell lines. These data indicate that it is an inherent property of the CAMα_{1B}-adrenergic receptor, rather than some undefined element of clonal cell line variation, which was responsible for the greater response to α_1 agonists.

There was certainly no higher steady-state levels of $G_q\alpha$ or $G_{11}\alpha$ in the CAMα_{1B}-adrenergic receptor expressing cells (**Figure 5.7**) and thus it seems

likely that the CAMα_{1B}-adrenergic receptor is able to activate these G proteins with higher stoichiometry than the wild type receptor. As it has been previously recorded that the sustained presence of agonist is required to cause downregulation of G_q and G_{11} via the wild type α_{1B}-adrenergic receptor (and indeed for other phosphoinositidase C-coupled receptors [Mitchell *et al.*, 1993, Shah *et al.*, 1995, van de Westerlo *et al.*, 1995]) and because activating mutants of G proteins have shorter half-lives than their native counterparts [Levis & Bourne, 1992] for example), then an enhanced G protein activation stoichiometry would be fully consistent with the observed greater degree of downregulation of these G proteins by agonist occupation of the CAMα_{1B}-adrenergic receptor.

Although the EC_{50} for agonist stimulation of inositol phosphate generation was greater than 100 fold lower at the CAMα_{1B}-adrenoceptor compared to the wild type receptor, this was not true when agonist-mediated $G_q\alpha/G_{11}\alpha$ downregulation was measured. In this assay the difference in measured EC_{50} was only approximately 4-5 fold. I do not have a ready explanation for these differences but it should be remembered that agonist-occupation of the wild type receptor resulted in a relatively small % downregulation of the G proteins making quantitation of the EC_{50} value substantially more difficult to quantitate reliably than in the other end points measured.

I also examined whether the expression of the CAMα_{1B}-adrenergic receptor would result in an agonist-independent downregulation of these G proteins. Although this is impossible to assess fairly in different clones of cells, I did not detect substantially lower steady-state levels of $G_q\alpha$ and $G_{11}\alpha$ in the CAMα_{1B}-adrenergic receptor expressing cells. However, given the difficulties of accurately measuring small alterations by the use of relative intensity immunoblotting and, as noted above, the still relatively small degree of phosphoinositidase C (and thus G protein) activation produced by the CAMα_{1B}-adrenergic receptor in the absence of

agonist compared to that achieved in the presence of agonist, it is perhaps to be anticipated that very small (and perhaps technically undetectable) alterations are the most which could be expected in the absence of agonist.

Further support for the contention that the CAM form of the α_{1B}-adrenergic receptor provides only limited signalling capacity in the absence of agonist was derived from examining the rate of turnover of $G_q\alpha/G_{11}\alpha$ in the cells. Analysis of immunoprecipitated $G_q\alpha/G_{11}\alpha$ following pulse labelling with Trans [^{35}S] label and a chase performed in the absence of phenylephrine indicated the half-life of these proteins, which was between 33 and 37h, to be very similar in the two cell lines. No differences could be detected between cells harbouring the wild type or the CAMα_{1B}-adrenergic receptor. This value is also very similar to data for the half life of $G_q\alpha/G_{11}\alpha$ on a completely independent series of clones derived from Rat 1 fibroblasts in **Chapter 4.**

Addition of a maximally effective concentration of phenylephrine during the chase phase of the experiments resulted in an acceleration in the rate of degradation of $G_q\alpha/G_{11}\alpha$ in cells expressing both the wild type and the CAMα_{1B}-adrenergic receptor. The acceleration in the rate of degradation of these G proteins due to agonist occupancy of the wild type α_{1B}-adrenergic receptor in this study (measured mean half-life in the presence of phenylephrine was approximately 21h) was not as great as noted previously and this is entirely consistent with the degree of downregulation of the G proteins produced by the receptor being less than that observed in chapter 4.

The acceleration in the rate of degradation of $G_q\alpha/G_{11}\alpha$ was more pronounced with agonist occupancy of the CAMα_{1B}-adrenergic receptor (measured half-life in the presence of 100μM phenylephrine was approximately 14h) and this may be primarily responsible for the greater degree of $G_q\alpha/G_{11}\alpha$ downregulation produced by agonist treatment of the CAMα_{1B}-adrenergic receptor expressing

cells. However, it must be stated that this difference seems too limited to account, in isolation, for the very marked differences in G protein regulation observed.

The data provided herein confirms previous reports that this CAMα_{1B}-adrenergic receptor displays a degree of agonist-independent signalling capacity which is greater than the wild type receptor. However, the CAM receptor is far from being fully agonist-independent and indeed is able to cause greater maximal output from the $G_q\alpha/G_{11}\alpha$ to phosphoinositidase C pathway as well as phospholipase D pathway than equivalent levels of the wild type α_{1B}-adrenergic receptor upon addition of agonist.

As steady-state basal levels of $G_q\alpha$ and $G_{11}\alpha$ are not appreciably different in the cell lines expressing the two forms of the receptor and the phosphoinositidase C response of the endogenously expressed endothelin ET_A receptor is also not different in the wild type vs CAMα_{1B}-adrenergic receptor expressing cells then the differences in signalling effectiveness of the two receptor variants are not due to trivial differences in the individual cell lines. It has been previously established that, for a range of wild type receptors expressed in various cellular backgrounds, agonist-dependent G protein downregulation is limited to the G proteins activated by a receptor [Mitchell *et al.*, 1993, Shah *et al.*, 1995, Mullaney *et al.*, 1995, Adie & Milligan, 1994].

Furthermore, it reflects a measure of sustained activation of the G protein pool. The extent of the effect must be dependent upon the fraction of the appropriate G protein pool which is activated by the receptor because high level receptor expression is substantially more effective at producing G protein downregulation than low level expression [Mullaney *et al.*, 1995, Adie & Milligan, 1994], even in situations in which the lower level of receptor is capable of causing maximal activation of second messenger generation due to the effector enzyme being the limiting component for the output of the signalling cascade [Adie &

Milligan, 1994, MacEwan *et al.*, 1995]. The key difference in the present study is thus likely to reflect a greater stoichiometry of activation of the G proteins by the CAM version of the α_{1B}-adrenergic receptor compared to the wild type receptor.

Meanwhile, there has been considerable recent attention given to the concept that inverse agonist ligands may be useful therapeutic agents in a range of clinical conditions [Lefkowitz *et al.*, 1993; Hieble & Bond, 1994; Milano *et al.*, 1994 & 1995; Bond *et al.*, 1995; Milligan *et al.*, 1995; Bond & Lefkowitz, 1995]. As such, simple means to detect compounds with this characteristic would be extremely useful. The concept that CAM variants of G protein coupled receptors represent at least a partial model of the activated [R*] state of a receptor suggests that they might provide the basis of such a system as they are able to produce greater activation of G proteins in a partly agonist-independent manner.

In this study this question has been assessed for the α_1 adrenergic inverse agonist phentolamine using clones of Rat 1 fibroblast cells transfected to express either the hamster wild-type or CAMα_{1B}-adrenergic receptor. The sustained presence of phentolamine in the medium surrounding the CAMα_{1B}-adrenergic receptor expressing cells was able to cause a dramatic upregulation of levels of this receptor as assessed in [^{3}H]prazosin saturation binding analyses (**Figure 5.12**). An increase in levels of the CAMα_{1B}-adrenergic receptor was also observed following sustained treatment of cells with HV-723, WB 4101, YM12617, corynanthine, and 5-methyl urapidil (approximately two fold over control) (**Figure 5.10**).

Interestingly, phentolamine-mediated upregulation of the CAMα_{1B}-adrenergic receptor levels was accompanied by a substantial increase in the maximal phosphoinositidase C activity in response to phenylephrine with a similar potency (**Figure 5.13**). This phenomenon was also observed in phospholipase D activity measurement in response to phenylephrine. Unlike other effector enzymes, e.g.

adenylyl cyclase, this may reflect phosphoinositidase C and phospholipase D are not the quantitatively limiting protein component of these signalling cascades. This provides a useful system to overcome low basal enzyme activity for the selection of inverse agonists.

As it was expected that the increase in the CAMα_{1B}-adrenergic receptor levels in response to phentolamine could cause an increase in the basal level of phospholipase D activity, this system was used for screening inverse agonists of the receptor. All of the tested α_1 antagonists appeared to be clearly capable of acting as inverse agonists at this CAMα_{1B}-adrenergic receptor as measured by their ability to inhibit phentolamine-stimulated basal phospholipase D activity (**Figure 5.14**).

This study provides a basis to suggest that upregulation of CAM G-protein coupled receptors may provide a useful and technically simple approach for the screening and detection of novel inverse agonist ligands.

Figure 5.1. Structural model of the constitutively active mutant (CAM) α_{1B} adrenergic receptor

Substitution of residues at the carboxyl terminus of the third intracellular loop of the α_{1B} adrenergic receptor appears to render the receptor constitutively active, resulting in the stimulation of phosphatidylinositol hydrolysis in the absence of agonist-induced receptor activation. The enlarged region illustrates the carboxyl terminus of the third intracellular loop and the sixth transmembrane domain. Solid circles indicate the amino acids of the wild type α_{1B} adrenergic receptor that were mutated; the amino acid residues replaced in the α_{1B} adrenergic receptor mutant are shown to the right (Arg288Lys, Lys290His, and Ala293Leu).

Figure 5.1

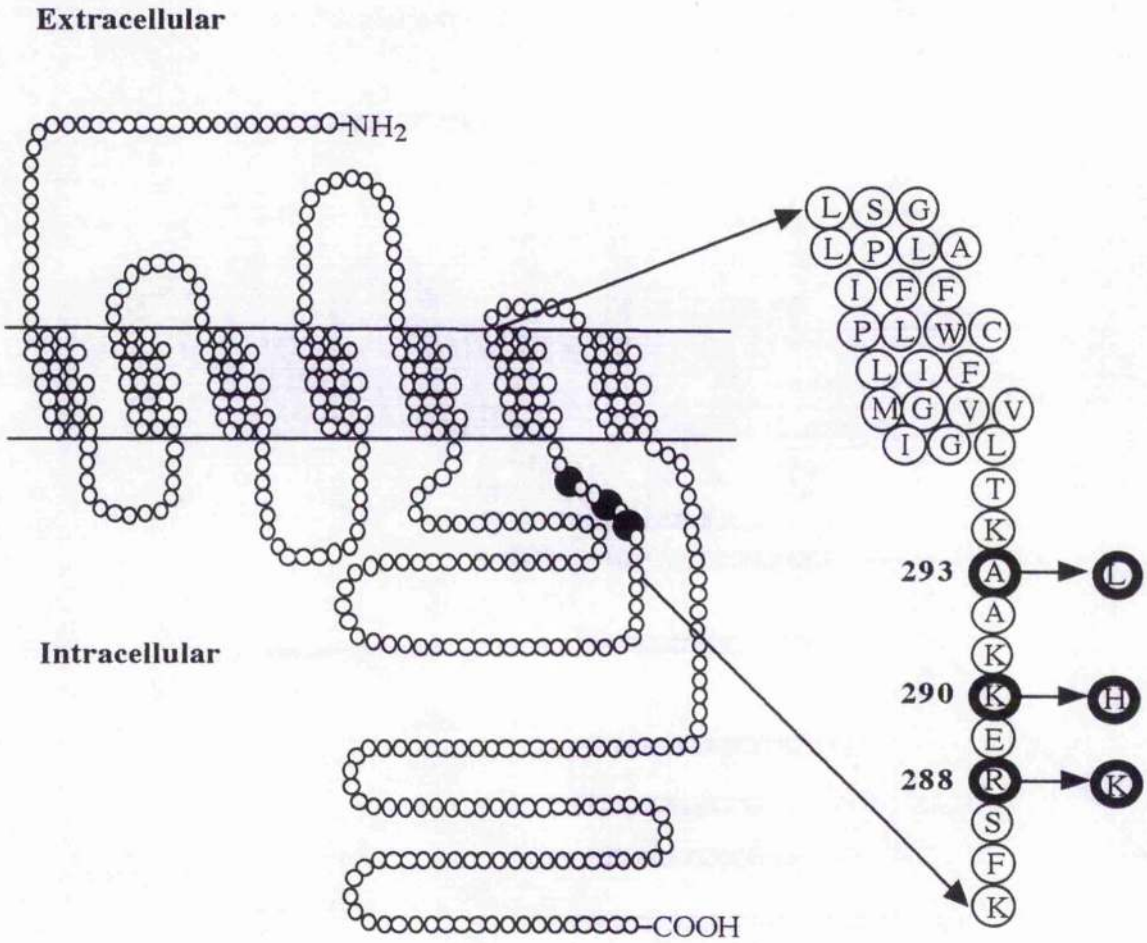

Extracellular
NH2
L S G
L P L A
I F F
P L W C
L I F
M G V V
I G L
T
K
293 A L
A
K
290 K H
E
288 R K
S
F
K
Intracellular
COOH

Figure 5.2. Cells transfected to express the wild type or CAM α_{1B}-adrenergic receptor express only the anticipated form of the receptor.

RNA isolated from wild type (lanes 2-3) and CAM (lanes 4-6) α_{1B}-adrenergic receptor transfected cells was reverse transcribed and polymerase chain reaction then performed with primers (which straddle the region of difference between the two forms of the receptor) designed to amplify a 597 bp fragment as described in **section 2.29**. Samples were then treated (lanes 3, 5-6) or not (lanes 2,4) with the restriction enzyme *Stu*-I. Product derived from the CAM form of the receptor is cleaved to yield fragments of 305 and 292bp (lanes 5-6) whereas that derived from the wild type receptor is resistant to the enzyme (lane 3). Lanes 1 and 7 contain molecular size markers.

Figure 5.2

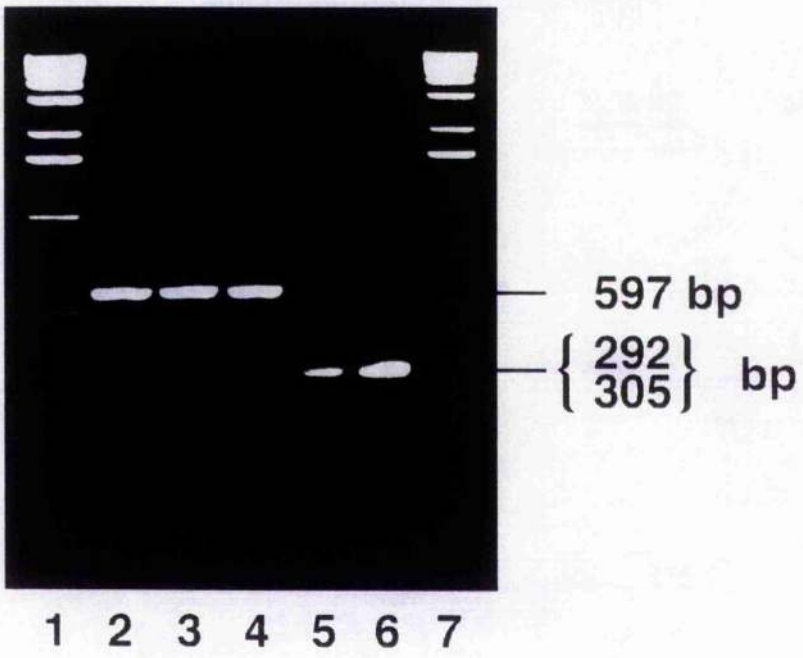


Figure 5.3. The wild type receptor is expressed at higher levels than the CAMα_{1B}-adrenergic receptor.

Membranes prepared from cells expressing either the wild type α_{1B} adrenergic receptor (squares) or the CAMα_{1B}-adrenergic receptor (circles) were analysed for their ability to bind a range of concentrations of [^{3}H]prazosin in a specific manner as descibed in **section 2.3 and 2.12.2.** In the experiment displayed, the two forms of the receptor bound the [^{3}H]ligand with very similar affinity (K_d = 100pM) but the levels of expression of the wild type receptor (2.8 pmol/mg membrane protein) was greater than that of the CAM receptor (1.7 pmol/mg membrane protein). Similar results were obtained in two further experiments.

Figure 5.3

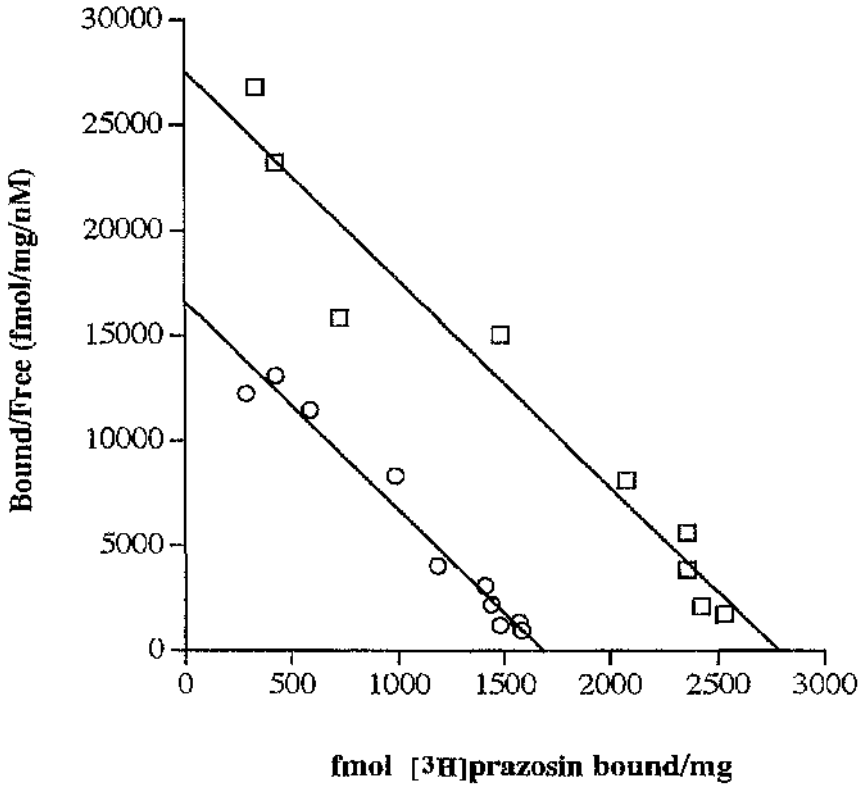
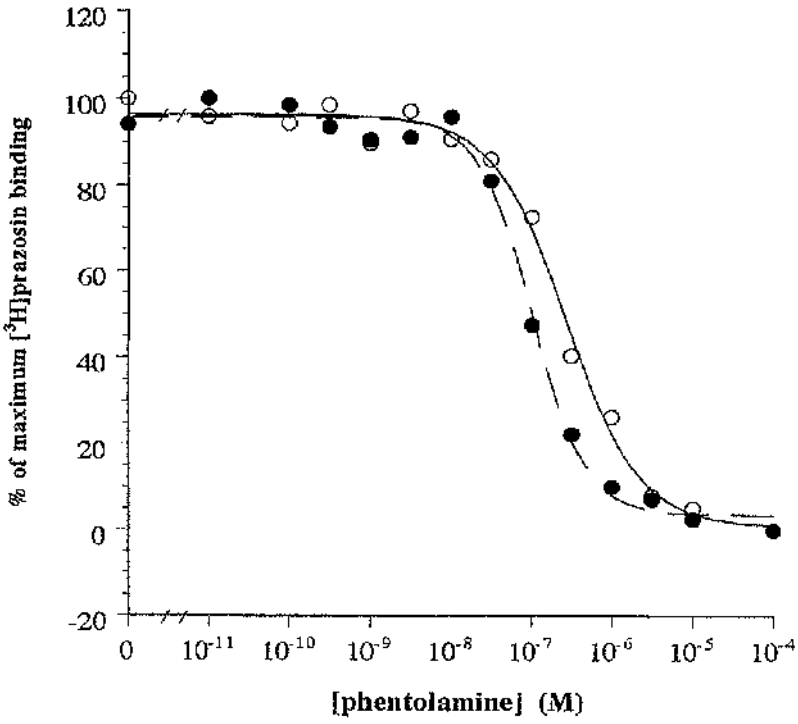

30000
25000
20000
15000
10000
5000
0
Bound/Free (fmol/mg/nM)
0
500
1000
1500
2000
2500
3000
fmol [3H]prazosin bound/mg

Figure 5.4. Antagonists bind the wild type and CAMα_{1B}-adrenergic receptor with similar affinity but agonists show substantially higher affinity for the CAM receptor.

The specific binding of [^{3}H]prazosin (0.88nM) to membranes of cells expressing the wild type (open symbols) or the CAM (closed symbols) α_{1B} adrenergic receptor was competed for by the antagonist phentolamine (**Figure 5.4a**) or (**Figure 5.4b**) by the agonists phenylephrine (circles) and norepinephrine (squares) as described in **section 2.12.2**.

Figure 5.4

a)


120
100
80
60
40
20
0
-20
% of maximum [3H]prazosin binding
0
10-11
10-10
10-9
10-8
10-7
10-6
10-5
10-4
[phentolamine] (M)

b)

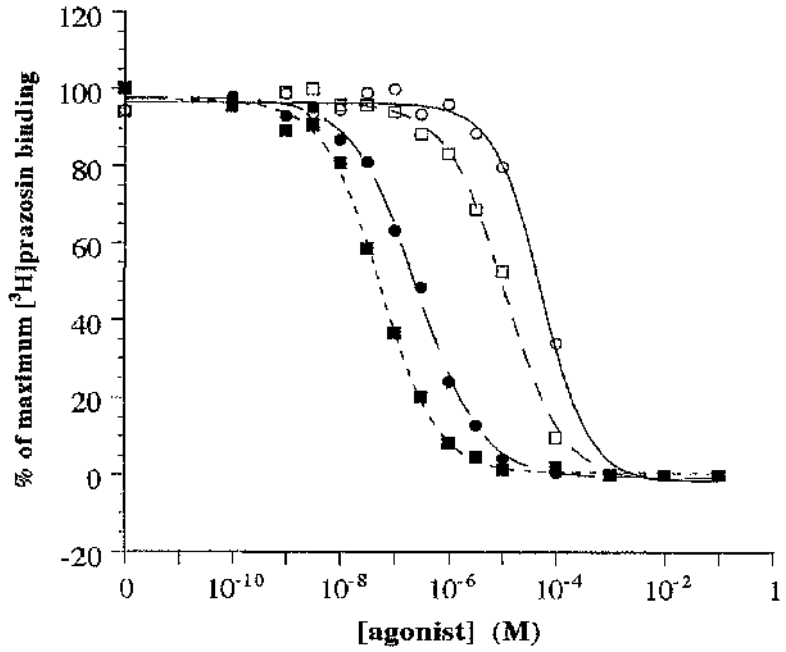

120
100
80
60
40
20
0
-20
% of maximum [3H]prazosin binding
0
10-10
10-8
10-6
10-4
10-2
1
[agonist] (M)

Figure 5.5. Phenylephrine, but not endothelin 1, stimulates inositol phosphate generation to both greater levels and more potently in cells expressing the CAMα_{1B}-adrenergic receptor compared to the wild type receptor.

Cells expressing the wild type (open symbols) and the CAM (closed symbols) α_{1B}-adrenergic receptor were labelled with [^{3}H]inositol and stimulated with either varying concentrations of phenylephrine (**Figure 5.5a** and **5.5b**) or endothelin 1 (**Figure 5.5c**). The generation of [^{3}H]inositol phosphates was then measured as described in **section 2.14**.

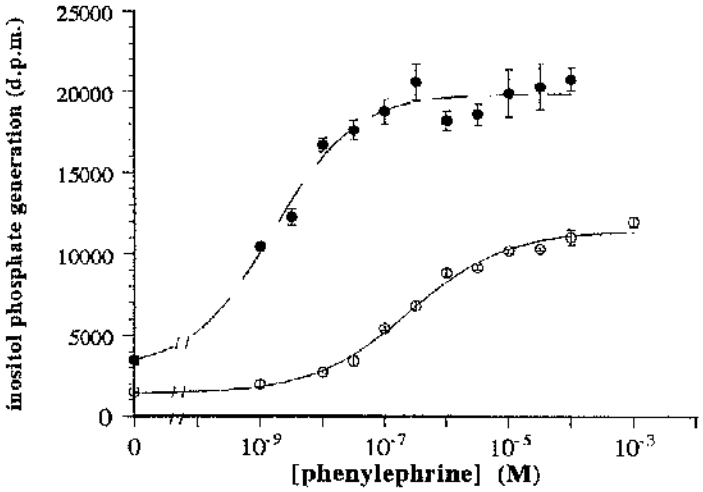
In the experiment shown in **Figures 5.5a** and **5.5b** the EC_{50} and Hill coefficient for phenylephrine stimulation of inositol phosphate production in the CAMα_{1B} adrenergic receptor expressing cells were 1.6×10^{-9}M and 0.55 and in the wild type receptor expressing cells they were 2.4×10^{-7}M and 0.65.

In **Figure 5.5b** the data for phenylephrine in **Figure 5.5a** is presented as % of maximal effect achieved. Similar results were obtained in two further experiments.

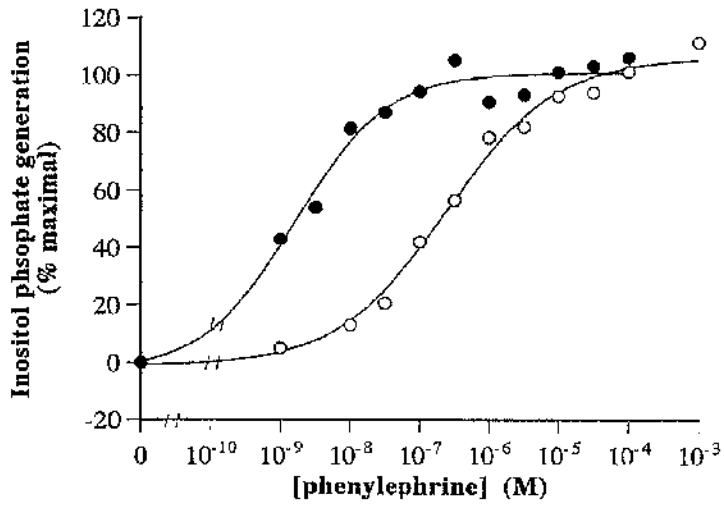
In **Figure 5.5c**, the EC_{50} and Hill coefficient for endothelin 1 stimulation of inositol phosphate generation were 3.4×10^{-9}M and 0.8 for the wild type α_{1B} adrenergic receptor expressing cells and 2.4×10^{-9}M and 1.1 in the CAM α_{1B} adrenergic receptor expressing cells. Similar results were obtained in two further experiments using each agonist.

Figure 5.5

a)



b)



c)

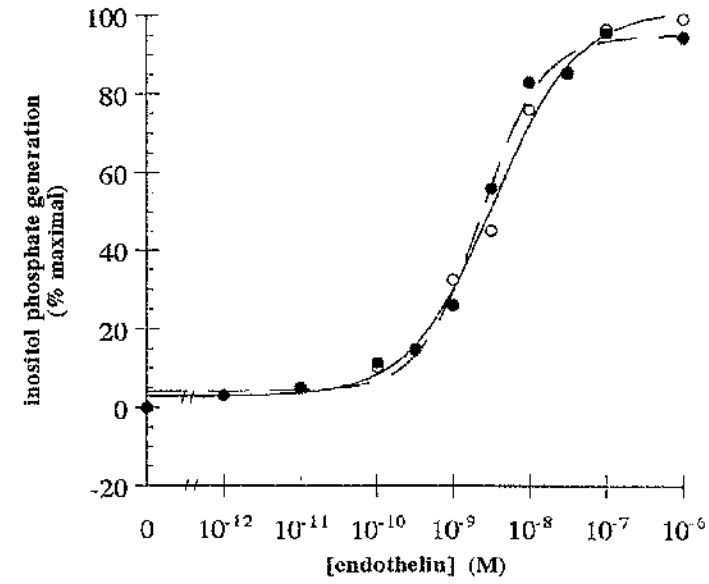


Figure 5.6. Phenylephrine stimulates phosphatidylbutanol generation to both greater levels and more potently in cells expressing the CAMα_{1B}-adrenergic receptor compared to the wild type receptor.

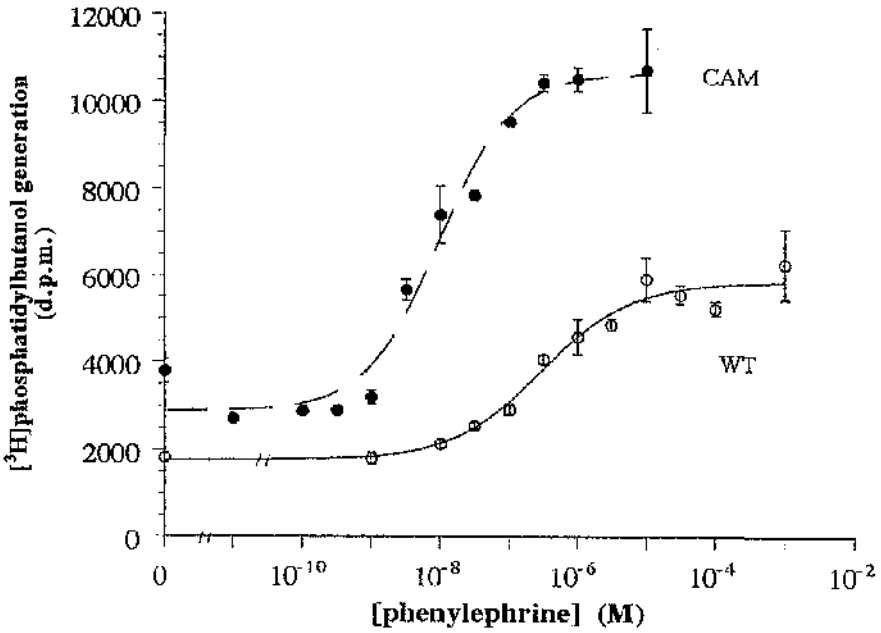
Cells expressing the wild type (open symbols) and the CAM (closed symbols) α_{1B}-adrenergic receptor were labelled with [^{3}H]palmitate and stimulated with varying concentrations of phenylephrine. The generation of [^{3}H]phosphatidylbutanol was then measured as described in **section 2.15.**

In the experiment shown in **Figures 5.6a** and **5.6b** the EC_{50} and Hill coefficient for phenylephrine stimulation of phosphatidylbutanol production in the CAMα_{1B} adrenergic receptor expressing cells were 9.7 x 10^{-9}M and 0.84 and in the wild type receptor expressing cells they were 3.2 x 10^{-7}M and 0.67.

In **Figure 5.6b** the data for phenylephrine in **Figure 5.6a** is presented as % of maximal effect achieved.

Figure 5.6

a)


12000
10000
8000
6000
4000
2000
0
[^{3}H]phosphatidylbutanol generation (d.p.m.)
CAM
WT
0
10^{-10}
10^{-8}
10^{-6}
10^{-4}
10^{-2}
[phenylephrine] (M)

b)

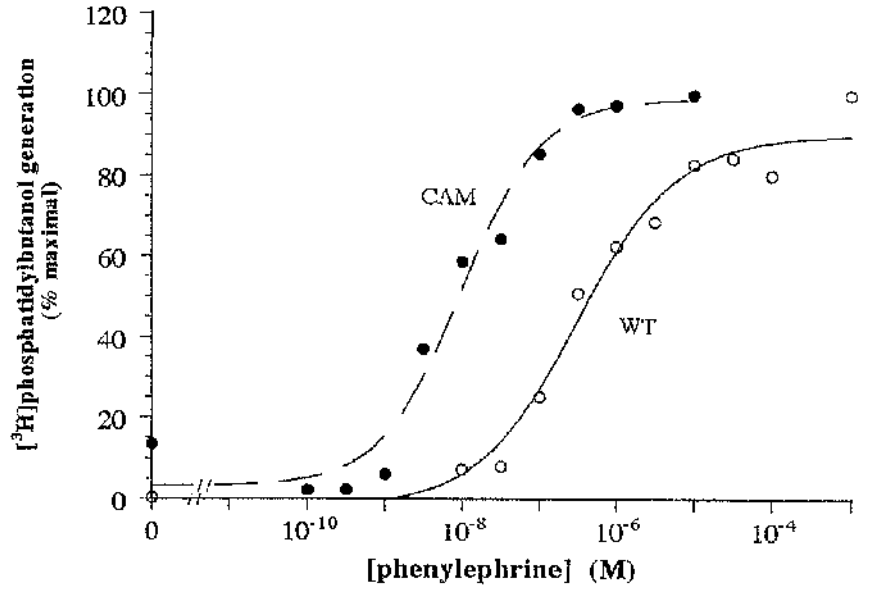

120
100
80
60
40
20
0
[^{3}H]phosphatidylbutanol generation (% maximal)
CAM
WT
0
10^{-10}
10^{-8}
10^{-6}
10^{-4}
[phenylephrine] (M)

Figure 5.7. Membrane levels of $G_q\alpha$ and $G_{11}\alpha$ are reduced by phenylephrine treatment of both wild type and CAMα_{1B}-adrenergic receptor expressing cells but the effect is greater in the CAM receptor expressing cells.

Membranes were isolated from both wild type (lanes 1-2) and CAM (lanes 3-4)-α_{1B}-adrenergic receptor expressing cells following treatment with either vehicle (lanes 1,3) or phenylephrine (100μM) (lanes 2,4) for 16h. The samples were then resolved by SDS-PAGE under conditions which can resolve $G_q\alpha$ from $G_{11}\alpha$ as described in **Section 2.8** and the presence of these polypeptides detected with antiserum CQ as indicated in **Table 2.1** which identifies the C-terminal decapeptide which is completely conserved in these two G proteins. Similar results were obtained on three separate sets of membrane preparations.

Figure 5.7

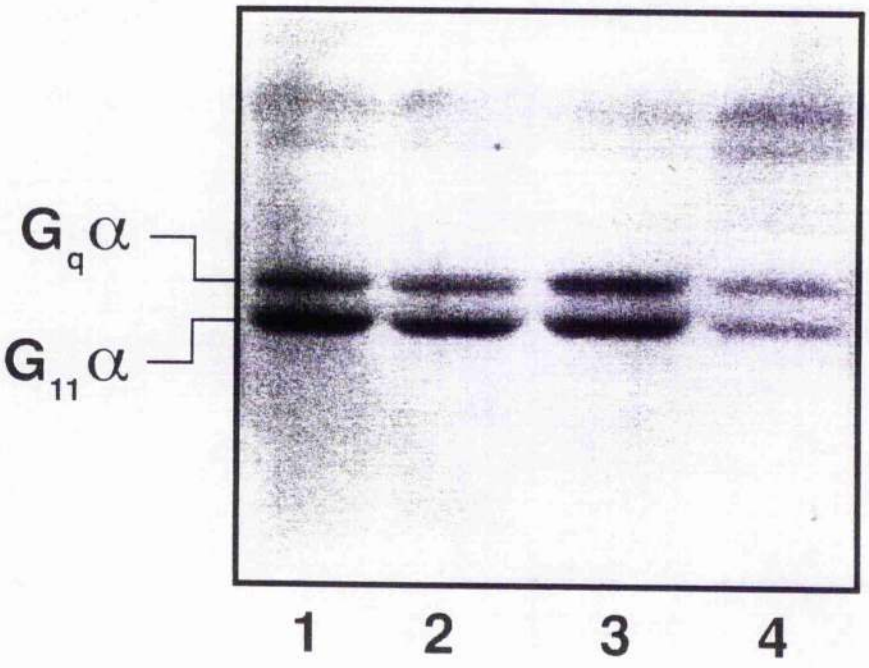
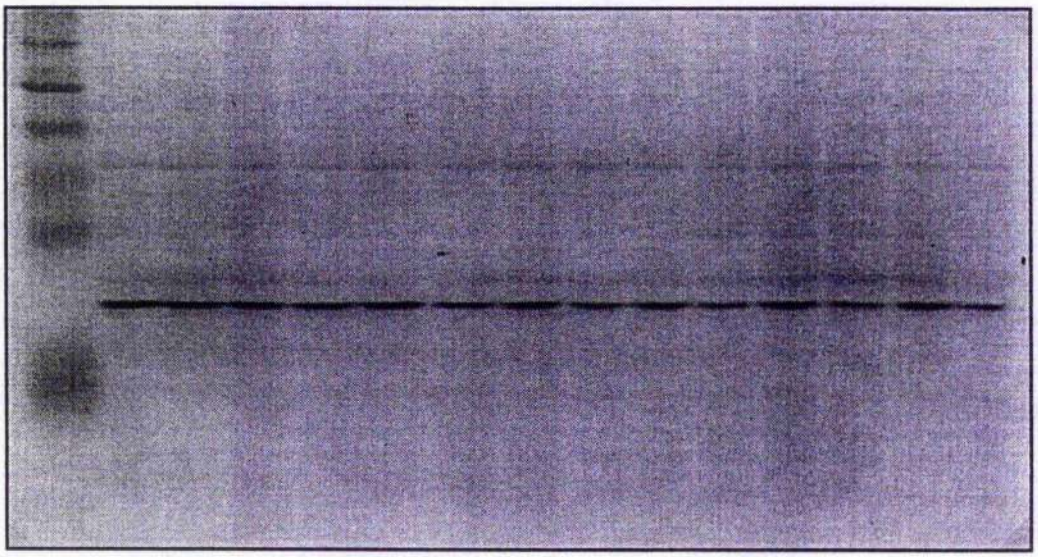


Figure 5.8. More pronounced downregulation of $G_q\alpha/G_{11}\alpha$ is produced by the CAM than the wild type α_{1B}-adrenergic receptor by all concentrations of phenylephrine.

Figure 5.8a shows a $G_q\alpha/G_{11}\alpha$ immunoblot. Cells expressing either the wild type (odd numbered lanes) or the CAM (even numbered lanes) α_{1B}-adrenergic receptor were exposed to varying concentrations (lanes 1&2: untreated, lanes 3&4: 10^{-9}M, lanes 5&6: 10^{-8}M, lanes 7&8: 10^{-7}M, lanes 8&10: 10^{-6}M, lanes 11&12: 10^{-5}M, lanes 13&14: 10^{-4}M) of phenylephrine for 16h. Membranes were prepared as described in **Section 2.3**, resolved by SDS-PAGE under conditions which do not resolve $G_q\alpha$ from $G_{11}\alpha$ and immunoblotted to detect the combined total levels of these two G proteins as described in **Section 2.8**. Such immunoblots were scanned and displayed in **Figure 5.8b**. The data presented as mean ± S.E.M., n=3, derived from three independent experiments on separate membrane preparations. M represents for the standard molecular size markers.

Figure 5.8

a)



M 1 2 3 4 5 6 7 8 9 10 11 12 13 14

b)

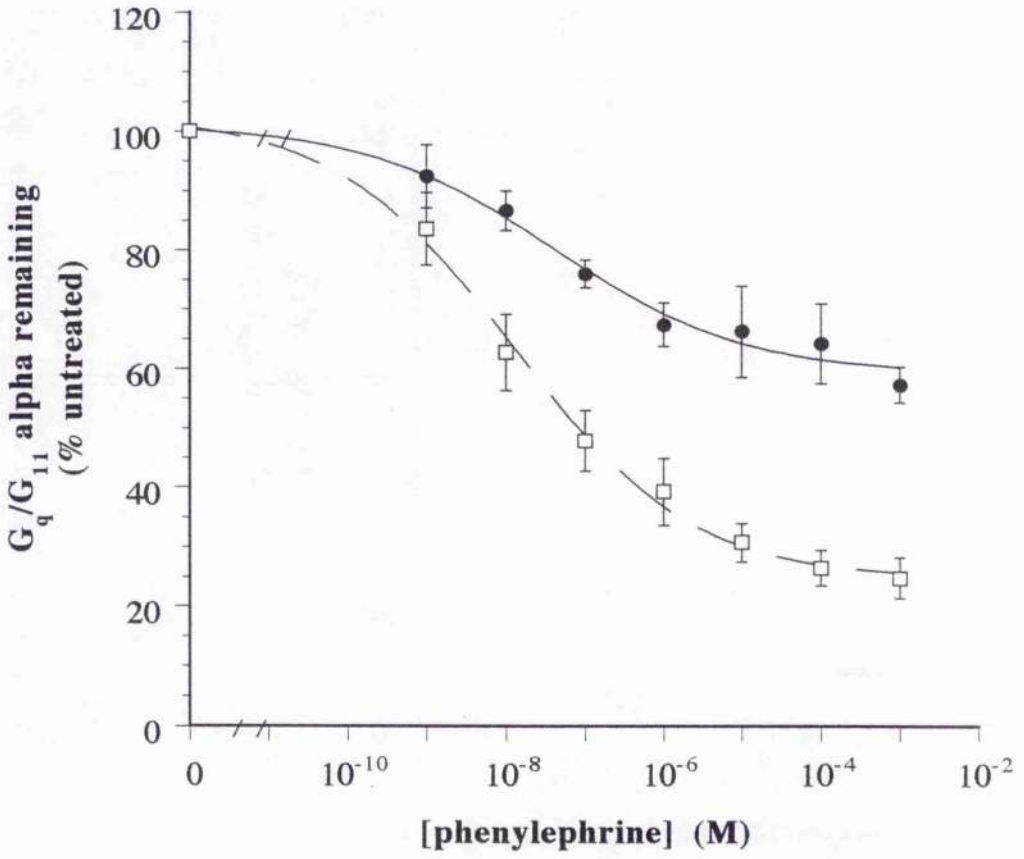
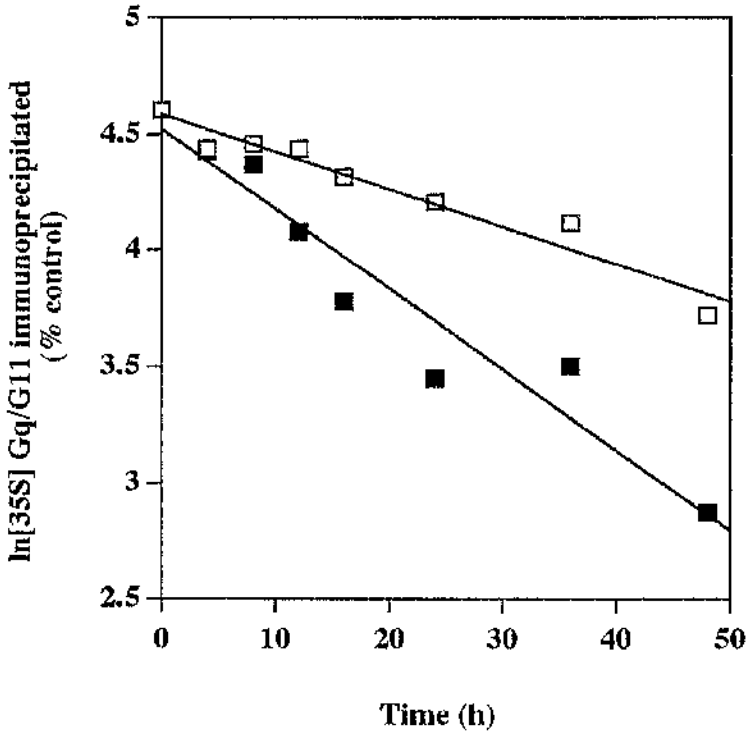


Figure 5.9. Phenylephrine treatment of both wild type and CAMα_{1B}-adrenergic receptor expressing cells accelerates the rate of degradation of $G_q\alpha/G_{11}\alpha$.

Wild type (**a**) (squares) and CAM (**b**) (circles) α_{1B}-adrenergic receptor expressing cells were labelled with Trans[^{35}S]label as described in **Section 2.2.7**. The radiolabel was removed and the cells maintained in the presence (filled symbols) or absence (open symbols) of 100μM phenylephrine for varying periods of time. Samples were taken and $G_q\alpha$ and $G_{11}\alpha$ immunoprecipitated with antiserum CQ. The immunoprecipitates were resolved by SDS-PAGE under conditions which do not resolve the two G proteins and subsequently exposed to a phosphoimager plate for 12h. The radiolabel remaining in $G_q\alpha/G_{11}\alpha$ was then quantitated as described in **section 2.9**. Data are represented as best fit to a monoexponential function from one of the three independent experiments performed.

Figure 5.9

a)


ln[35S] Gq/G11 immunoprecipitated (% control)
5
4.5
4
3.5
3
2.5
0
10
20
30
40
50
Time (h)

b)

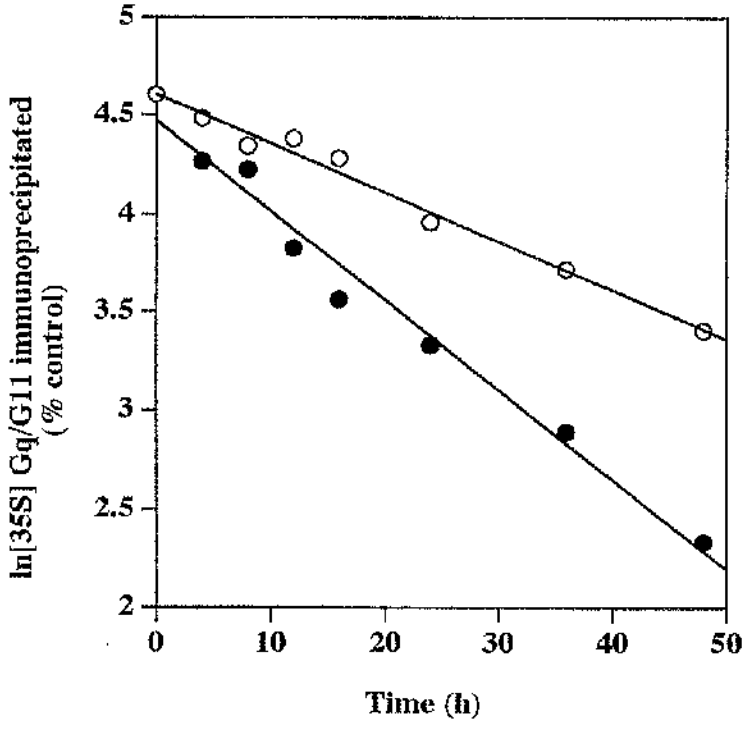
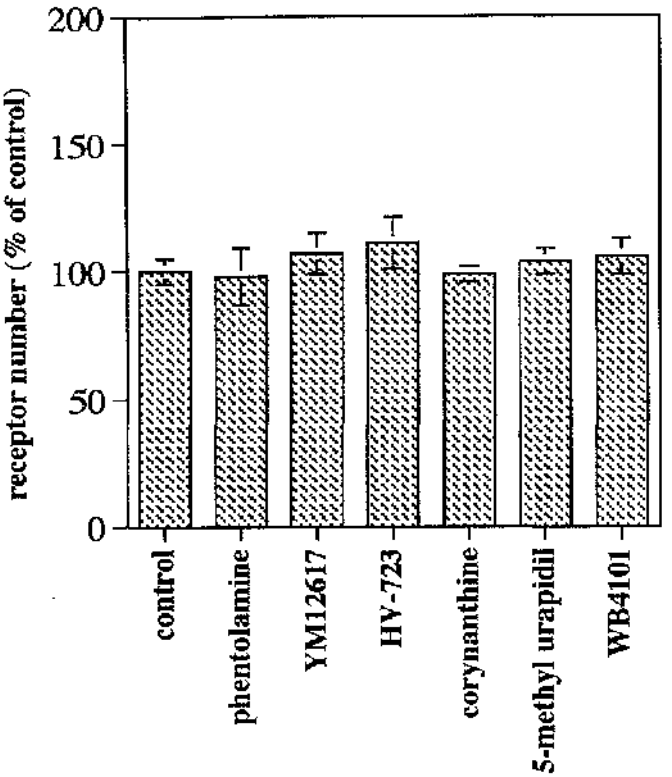

ln[35S] Gq/G11 immunoprecipitated (% control)
5
4.5
4
3.5
3
2.5
2
0
10
20
30
40
50
Time (h)

Figure 5.10

a)



b)

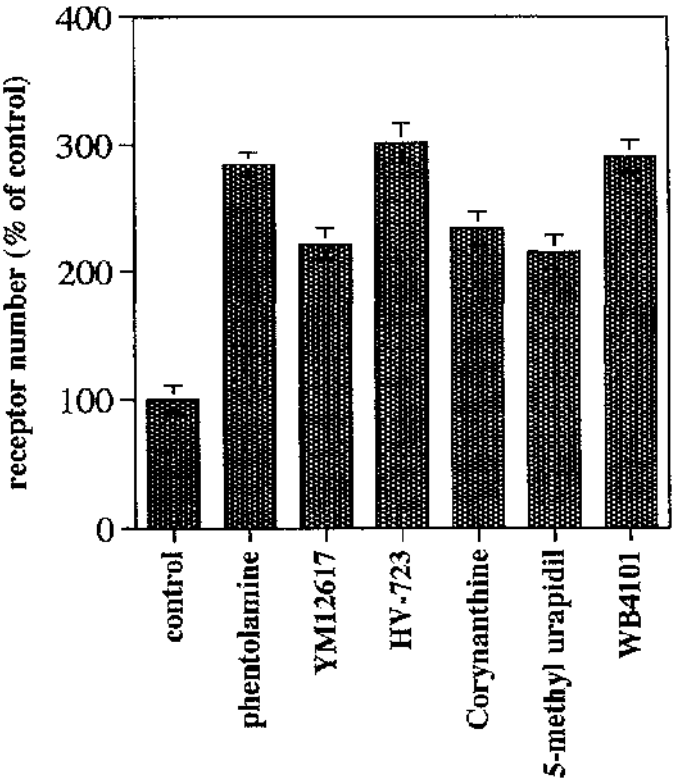


Figure 5.10. Upregulation of the CAMα_{1B}-adrenergic receptor following exposure to α_1 antagonists

Cells expressing either the wild type (**Figure 5.10a**) or CAM (**Figure 5.10b**) α_{1B}-adrenergic receptor were exposed to vehicle (control) or 10μM of phentolamine, YM12617, HV-723, corynanthine, 5-methyl urapidil or WB 4101 for 24h. Membranes were prepared as descibed in **section 2.3** and the specific binding of [^{3}H]prazosin (1nM) measured as described in **section 2.12.2**. Results are presented relative to the levels measured in the vehicle treated cells and represent the mean ± S.E.M. of triplicate determinations. The experiment shown is typical of two other independent experiments.

Figure 5.11. Time-dependent upregulation of the CAMα_{1B}-adrenergic receptor following exposure to phentolamine

Cells expressing CAMα_{1B}-adrenergic receptor were treated for varying times with phentolamine (1 μM). Membranes prepared from these cells were then assessed for levels of the CAMα_{1B}-adrenergic receptor as measured by the specific binding of [^{3}H] prazosin. The results represent the mean ± S.E.M. of triplicate determinations. This is typical of three independent experiments which gave similar results.

Figure 5.11

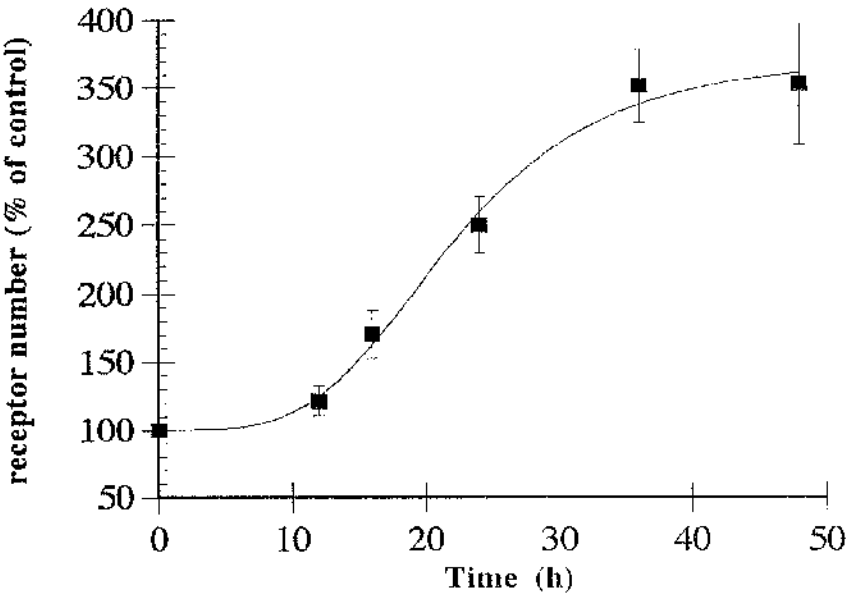

receptor number (% of control)
400
350
300
250
200
150
100
50
0
10
20
30
40
50
Time (h)

Figure 5.12. Scatchard analysis of the CAMα_{1B}-adrenergic receptor treated with or without phentolamine

Cells expressing CAMα_{1B}-adrenergic receptor were incubated for 36h with (closed circles) or without (open squares) phentolamine (1μM) and then membranes prepared as descibed in **section 2.3.** Scatchard analyses were then performed with [^{3}H]prazosin as descibed in **section 2.12.2.** The results represent the mean of triplicate determinations. In this experiment the CAMα_{1B}-adrenergic receptor levels were in control (1.7 pmol/mg membrane protein) and phentolamine-treated (6.6 pmol/mg membrane protein), and the K_d values of the CAMα_{1B} adrenergic receptor for [^{3}H] prazosin in control and phentolamine-treated preparations were estimated to be 118pM and 176pM, respectively. The experiment shown is typical of two other independent experiment.

Figure 5.12

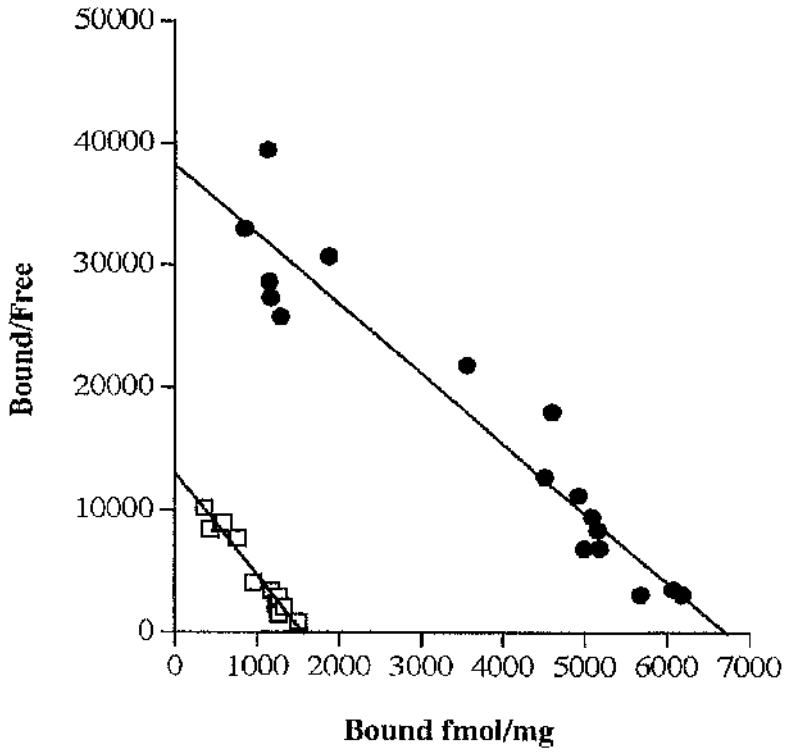

50000
40000
30000
20000
10000
0
Bound/Free
0
1000
2000
3000
4000
5000
6000
7000
Bound fmol/mg

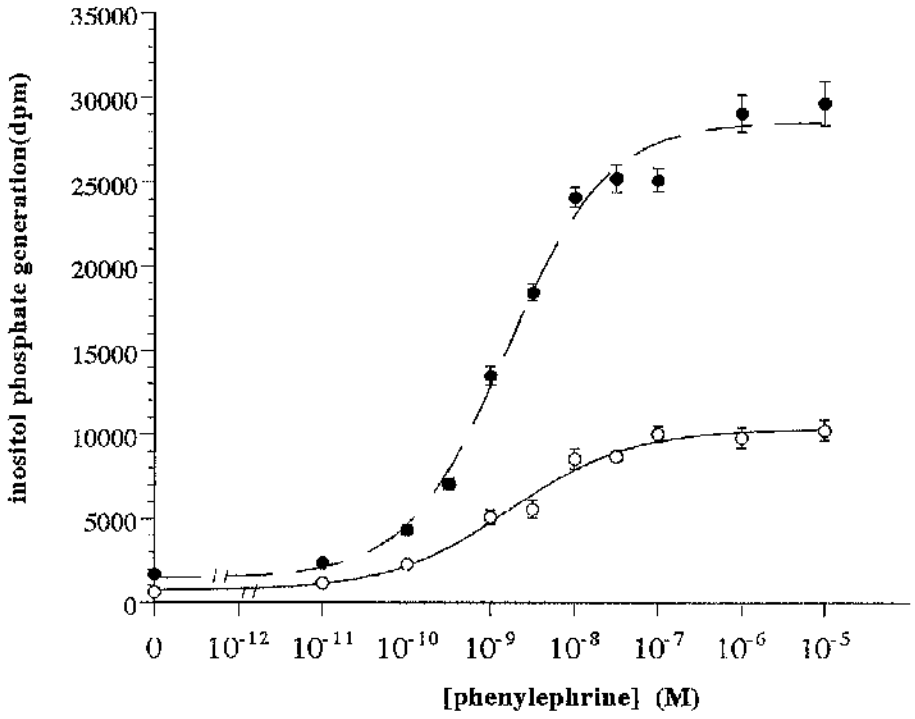
Figure 5.13. Phentolamine treatment of the CAMα_{1B}-adrenergic receptor expressing cells increases agonist-stimulated phosphoinositidase C and phospholipase D activity

Cells expressing CAMα_{1B}-adrenergic receptor were labelled with either [^{3}H]inositol or [^{3}H]palmitate and incubated for 24h with (closed symbols) or without (open symbols) phentolamine (1μM) and then stimulated with varying concentrations of phenylephrine. The generation of [^{3}H]inositol phosphates (**Figure 5.13a**) or [^{3}H]phosphatidylbutanol (**Figure 5.13b**) was then measured as described in **section 2.13** and **2.14**.

In the experiment shown in **Figures 5.13a** the EC_{50} for phenylephrine stimulation of inositol phosphate production in the vehicle were 1.6 x 10^{-9}M and in the phentolamine (1μM) treated cells they were 1.5 x 10^{-9}M. In the experiment shown in **Figures 5.13b** the EC_{50} for phenylephrine stimulation of phosphatidylbutanol production in the vehicle were 1.9 x 10^{-8}M and in the phentolamine (1μM) treated cells they were 1.1 x 10^{-8}M. The results represent the mean ± S.E.M. of triplicate determinations.The experiment shown is typical of two independent experiments which gave similar results.

Figure 5.13

a)



b)

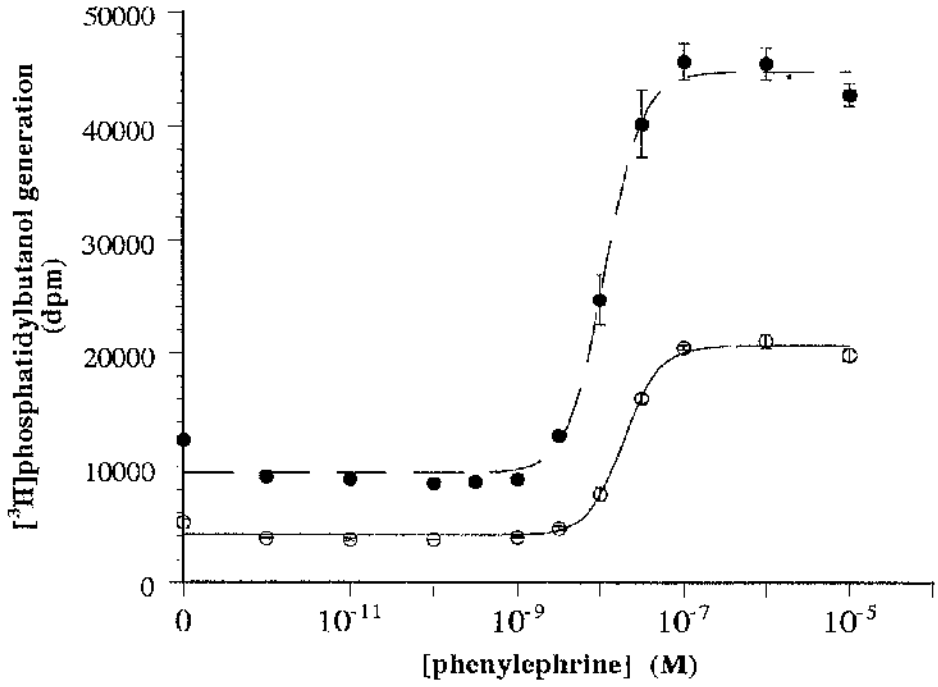
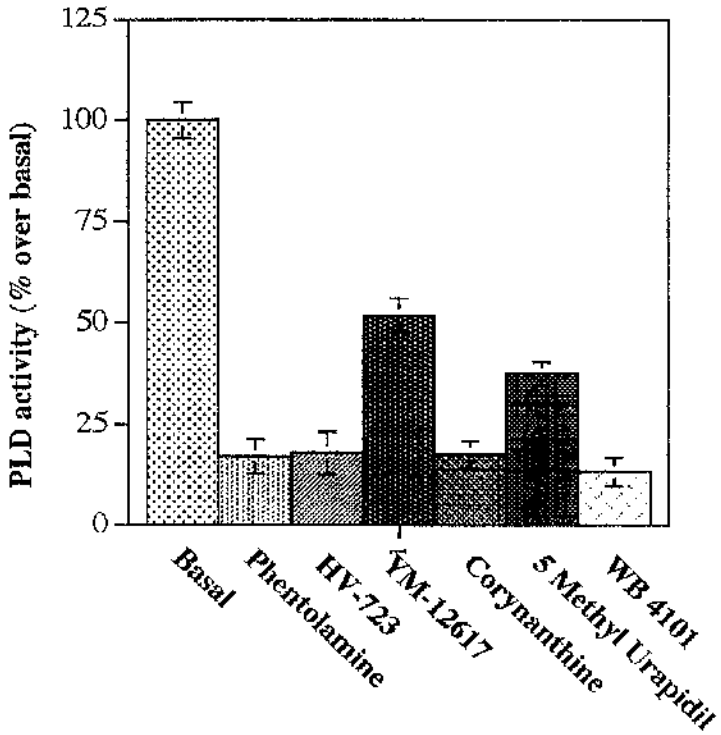


Figure 5.14. α_1 antagonists lower phentolamine-upregulated basal phospholipase D acivity in cells expressing CAM α_{1B}-adrenergic receptor.

Upregulated (phentolamine-mediated) basal phospholipase D activity and its regulation by various α_1 antagonists (phentolamine, HV-723, YM-12617, corynanthine, 5-methyl urapidil, and WB-4101) (1μM each) are shown in cells expressing CAMα_{1B}-adrenergic receptor. Cells were pretreated with 1μM phentolamine for 24h and washed away four times with the assay buffer, and then the ability to inhibit [^{3}H]phosphatidylbutanol generation was measured as described in **section 2.15**.

Figure 5.14


PLD activity (% over basal)
125
100
75
50
25
0
Basal
Phentolamine
HV-723
YM-12617
Corynanthine
5 Methyl Urapidil
WB 4101

Chapter 6. Final discussion

Chapter 6. Final discussion

When G protein-coupled receptors (GPCRs) are encountered by external stimuli including hormones, neurotransmitters, growth factors, light, and agonists they initiate their biological actions to activate or to inhibit the activity of specific effector systems via intermediary signal tranducers, guanine nucleotide binding protiens (G proteins).

Over one hundred receptors have been shown to be coupled to members of the G protein family to facilitate the activation of effectors and their activities are regulated by many well-known mechanisms including downregulation, internalisation, desensitisation, etc. The receptor is coupled to a GDP-liganded G protein in the resting state and the agonist activation of the receptor causes a conformational change in both proteins leading to the exchange of GDP for GTP which promotes dissociation of the G protein from its trimeric form into a free active α and a $\beta\gamma$ complex. The GTP-bound α subunit then interacts with the final executor effector system before its intrinsic GTPase activity hydrolyses the GTP to GDP, promoting reformation of the holomeric protein which reassociates with the receptor [Gilman, 1984; Gilman, 1987].

Many variants of α, β, and γ subunits of G proteins have been identified and the functional roles for individual G proteins have been revealed. GTP-binding α subunits and the $\beta\gamma$ subunit complex can influence the activity of effector systems including adenylyl cyclase, phosphoinositidase C, phospholipase A_2, Phospholipase D, various ion channels and MAP Kinase activity independently or simultaneously, either synergistically or in opposition [Hepler & Gilman, 1992; Clapham & Neer, 1993; Sternweis, 1994; Inglese *et al.*, 1995].

Recently, with the advent of molecular cloning techniques, a number of molecularly distinct receptors were shown to result from differential splicing of pre-mRNA derived from a single gene. For example, in the cases of the splice variants of the EP3

prostanoid receptor [Namba *et al.*, 1993] and the pituitary adenylyl cyclase-activating-polypeptide (PACAP) receptor [Spengler *et al.*, 1993], very different second-messenger generation functions have been reported for the individual forms.

In **chapter 3** I have focused on analysis of potential functional differences of splice variants of the TRH receptor which are co-expressed in GH3 cells [de la Pena *et al.*, 1992]. It has been shown that TRH receptors interact with pertussis toxin-insensitive G proteins of the G_q class to activate a phosphoinositidase C [Hsieh & Martin, 1992; Kim *et al.*, 1994; Aragay *et al.*, 1992]. In a number of reports, interactions of TRH receptors with other G proteins [Paulssen *et al.*, 1992; Gollasch *et al.*, 1993], most prominently G_s and G_i2 which are viewed traditionally as being responsible for stimulatory and inhibitory regulation of adenylyl cyclase, have been demonstrated.

I examined whether the signalling characteristics of the two isoforms differ following stable expression of each isoform in Rat 1 fibroblasts, to assess whether reported regulation of effector systems other than phosphoinositidase C might reflect activation of one isoform rather than another. The initial preconceptions were that this might be a property of the short isoform. After isolation of clones expressing specific [^{3}H]TRH-binding sites, it was determined that the clones expressed only the expected splice variant form of the receptor by RT-PCR based on the presence or absence of the 52-bp section which differs between the two isoforms.

Both receptor isoforms displayed similar binding affinity for [^{3}H]TRH, with an estimated K_d of approximately 80 nM in the presence of Gpp[NH]p. Attempts to measure the affinity of binding in the absence of Gpp[NH]p were restricted but the best estimates for the high affinity site were similar to the measured EC_{50} of 5-10 nM for TRH stimulation of inositol phosphate production observed for clones expressing both the long and short TRH-receptor splice variants, and suggest that it is the high-agonist-affinity state of the receptor which is able to interact with the cellular G protein population to produce effector regulation. This similarity of EC_{50} values for TRH-mediated stimulation of

inositol phosphate production is in agreement with analysis of TRH regulation of Cl^- flux in *Xenopus* oocytes after injection of cRNA species corresponding to the two isoforms [de la Pena *et al.*, 1992]. It should not be considered surprising that the two splice variants display very similar binding and activation affinities for TRH because Tyr-106 in the third transmembrane helix of the receptor has been shown to play a key role in the binding of TRH [Perlman *et al.*, 1994] and the location of the splice variation site is in the C-terminal tail.

In contrast with the reported activation of adenylyl cyclase by TRH in GH cells [Paulssen *et al.*, 1992], no ability of the TRH receptor splice variants to alter adenylyl cyclase activity, either positively or negatively, in membranes of the TRH receptor expressing clones of Rat 1 cells was detected. This was not a reflection of a lack of expression of the relevant G proteins which were demonstrated to be expressed by these cells. It may of course be a reflection of the adenylyl cyclase isoform distribution in the individual cell lines, an area which remains essentially uncharted, but, it seems unlikely as all forms of hormonally regulated adenylyl cyclase are activated by G_s [Tang & Gilman, 1992].

Therefore, it was concluded that neither isoform of the rat TRH receptor displays appreciable ability to interact with either G_s or indeed with G_i2, at least when expressed in this genetic background. As such the results this study can provide no evidence for either of the splice variants of the TRH receptor providing a locus for bifurcation of signalling information, as has recently been described for a variety of receptors, including the TSH receptor [Allgeier *et al.*, 1994, and for review Milligan G., 1993].

The ability of TRH to cause MAP kinase activation was studied in the clones, as recent reports have shown TRH to stimulate MAP kinase (ERK) activity in GH3 cells [Ohmichi *et al.*, 1994]. I was unable to note a significant effect in clones of Rat 1 cells expressing either the long or short isoforms of the rat TRH receptor. The data produced herein demonstrate no obvious functional differences for the long and the short isoforms of

the rat TRH receptor, at least when expressed in Rat 1 fibroblasts, and further demonstrate a high selectivity of coupling of both of these isoforms to G_q/G_{11} stimulation of phosphoinositidase C over regulation of adenylyl cyclase.

Signalling functions of α_1 adrenergic receptor subtypes and the mechanism of G protein regulation were explored in **chapter 4.** α_1 adrenergic receptors have been known to stimulate the hydrolysis of inositol-containing phospholipids via interaction with pertussis toxin-insensitive G proteins of the G_q/G_{11} family with subsequent activation of phospholipase Cβ activity [Wu *et al.*, 1992].

In cells expressing one of the rat $\alpha_{1A/D}$, the hamster α_{1B}, and the bovine α_{1C} adrenergic receptors, exposure to the α_1 adrenergic receptor agonist phenylephrine resulted in stimulation of inositol phosphate production in a fashion that was resistant to pretreatment of the cells with pertussis toxin. Maintained exposure of these cells to phenylephrine resulted in down-regulation of each of the receptor subtypes which is a common regulatory feature and in selective downregulation of some combination of the α subunits of G_q/G_{11}.

Although the basic observation that maintained agonist activation of a GPCRs can cause a marked and selective reduction in cellular levels of the G protein(s) is now well established [Milligan, 1993] far less is known about the mechanisms responsible for such phenomena. Studies from Hadcock *et al.* [1990, 1991] have noted complex regulation of G proteins following receptor stimulation, including alterations in both protein and mRNA stability of a variety of G proteins, which is then integrated to result in upregulation of some G proteins and downregulation of others. By contrast, it was noted that m1 muscarinic acetylcholine receptor-mediated downregulation of the $G_q/G_{11}\alpha$ subunits was accompanied by a selective accelerated rate of degradation of these G proteins [Mitchell *et al.*, 1993].

In this sudy, I sought to further analyse such effects by examining the process of downregulation of the α subunits of G_q and/or G_{11} in Rat 1 cells transfected to express

individual molecularly defined α_1 adrenergic receptor subtypes. Agonist-mediated downregulation of the α subunits of G_q/G_{11} has now been reported for a variety of receptors, including the muscarinic m1 acetylcholine receptor [Mullaney *et al.*, 1993; Mitchell *et al.*, 1993], the long isoform of the TRH receptor [Kim *et al.*, 1994], and the GnRH receptor [Shah & Milligan, 1994]. However, only for the first of these has any mechanistic analysis been provided. In Chinese hamster ovary cells transfected to express the rat muscarinic m1 receptor, accelerated degradation of a combination of G_q/G_{11} was recorded without detectable alteration in levels of mRNA of either of these polypeptides [Mitchell *et al.*, 1993].

This study expanded those observations to show that in the genetic background of Rat 1 fibroblasts, the basal half-life of the α subunits of G_q/G_{11} can be adequately modeled as a single monoexponential consistent with $t_{0.5}$ in the region of 33-40h and that agonist occupancy of any of the α_1 adrenergic receptor subtypes leads to a proportion of the cellular G_q/G_{11} population being degraded much more rapidly. Data from each of the systems, however, were not consistent with all of the cellular content of these G proteins being degraded more rapidly in the presence of agonist. A maximally effective concentration of phenylephrine was able to cause downregulation of between 50 and 70% of the total $G_q\alpha/G_{11}\alpha$ population in these cells in a range of experiments.

Immunoblotting of membranes of the clones demonstrated that the steady-state levels of $G_{11}\alpha$ expression was over 2-fold higher than that of $G_q\alpha$. Furthermore, they indicated that sustained phenylephrine occupany of each of the α_1 adrenergic receptor subtypes resulted in a downregulation of both $G_{11}\alpha$ and $G_q\alpha$. The mechanism of this effect was a selective acceleration of the rate of degradation of these G proteins.

Signalling characteristics of a constitutively active mutant (CAM) α_{1B} adrenergic receptor; its regulation of signalling cascade elements and the action of inverse agonists at this receptor were explored in **chapter 5**. Modifications of GPCRs which result in the receptors displaying agonist-independent second messenger regulatory capacity have been

observed both by designed mutation *in vitro* [Samama *et al.*, 1993, Pei *et al.*, 1994, Cotecchia *et al.*, 1990, Kjelsberg *et al.*, 1993] and in spontaneous mutations [Shenker *et al.*, 1993, Parma *et al.*, 1993, Raymond, 1994, Coughlin, 1994]. The CAM receptors have shown an increase in basal activity of the effector system coupled to that receptor in the absence of agonist.

It has been suggested that the CAM receptors could provide useful models of the conformational changes which must occur upon agonist binding to wild type receptors to allow efficient transmission of a signal via activation of heterotrimeric G proteins [Lefkowitz *et al.*, 1993]. There is strong correlation between the level of expression of the GPCR and the amount of effector regulation [Milano *et al.*, 1994, Adie & Milligan, 1994, Chidiac *et al.*, 1994] and this has indicated that GPCRs must have the capacity to exist and spontaneously interconvert between a minimum of two states, an inactive ground-state conformation R and an active conformation R* [Milano *et al.*, 1994, Samama *et al.*, 1994, Milligan *et al.*, 1995, Gether *et al.*, 1995].

Although the CAMα_{1B} adrenergic receptor was inherently able to produce this effect, addition of agonist potentiated this effect greatly, indicating that this form of the receptor did not function in a fully agonist-independent manner [Allen *et al.*, 1991]. Similarly, although the degree of agonist-independent inositol phosphate generation produced by the CAMα_{1B} adrenergic receptor was markedly greater than by the wild type receptor, the CAM receptor produced a further robust stimulation upon addition of agonist [Cotecchia *et al.*, 1995].

I explored whether the CAMα_{1B} adrenergic receptor had the capacity to act more effectively than the wild type receptor and whether this CAMα_{1B} adrenergic receptor had greater ability to downregulate $G_q\alpha/G_{11}\alpha$ than the wild type receptor upon sustained agonist treatment of Rat 1 fibroblasts expressing each of the receptors. Addition of the α_1 agonist phenylephrine produced a markedly greater degree of G protein downregulation in the cells expressing the CAMα_{1B} adrenergic receptor than in those expressing the wild

type receptor, despite of the fact that the CAMα_{1B} adrenergic receptor was expressed at somewhat lower steady-state levels than the wild type receptor.

The CAMα_{1B} adrenergic receptor showed a higher level generation of inositol phosphates in the absence of agonist than the wild type receptor, this was also true in response to maximally effective concentrations of agonist. The same was also true when phosphatidylbutanol generation by phospholipase D was measured. By contrast, there was little difference in the maximal ability of endothelin 1 to stimulate inositol phosphate generation and phosphatidylbutanol generation in the two cell lines indicating that it is an inherent property of the CAMα_{1B} adrenergic receptor, rather than some undefined element of clonal cell line variation, which was responsible for the greater response to α_1 agonists.

Certainly, no higher steady-state levels of $G_q\alpha$ or $G_{11}\alpha$ were detected in the CAMα_{1B} adrenergic receptor expressing cells and thus it seems likely that the CAM receptor activates these G proteins with higher stoichiometry than the wild type receptor. Although the EC_{50} for agonist stimulation of inositol phosphate generation was greater than 100 fold lower in the CAMα_{1B} adrenergic receptor expressing cells compared to those expressing the wild type receptor this was not true when agonist-mediated $G_q\alpha/G_{11}\alpha$ downregulation was measured. I do not have a ready explanation for these differences.

From the analysis of [^{35}S]pulse-chase experiments on immunoprecipitated $G_q\alpha/G_{11}\alpha$ the α_{1B} adrenergic receptors showed only limited signalling capacity in the absence of agonist and indicated that the half-life of these proteins was very similar in the two cell lines in the absence of agonist. Addition of a maximally effective concentration of phenylephrine during the chase phase of the experiments resulted in an acceleration in the rate of degradation of $G_q\alpha/G_{11}\alpha$ in cells expressing both the wild type and the CAM receptor. The acceleration in the rate of degradation of $G_q\alpha/G_{11}\alpha$ was more pronounced with agonist occupancy of the CAM receptor and this may be primarily responsible for the greater degree of $G_q\alpha/G_{11}\alpha$ downregulation produced by agonist treatment of the CAMα_{1B} adrenergic receptor expressing cells. However, it must be stated that this

difference seems too limited to account for the very marked differences in G protein regulation observed.

There has been considerable recent attention given to the concept of inverse agonism because such ligands may be useful therapeutic agents in a range of clinical conditions [Lefkowitz *et al.*, 1993; Bond *et al.*, 1995; Milligan *et al.*, 1995]. CAM variants of GPCRs have represented at least a partial model of the activated [R*] state of a receptor.

The activity of the α_1 adrenergic inverse agonist phentolamine has assessed in cells expressing either the wild type or CAMα_{1B} adrenergic receptor. The sustained presence of phentolamine in the CAMα_{1B} adrenergic receptor expressing cells caused a dramatic upregulation of levels of this receptor, but not those expressing the wild type receptor. An increase in levels of the CAMα_{1B} adrenergic receptor was also observed in response to HV-723, YM 12617, corynanthine, 5 methyl urapidil and WB 4101 following sustained treatment of cells.

As it was expected, phentolamine-mediated upregulation of the CAMα_{1B} adrenergic receptor levels was accompanied by a substantial increase in the maximal phosphoinositidase C as well as phospholipase D activity in response to agonist in a concentration-dependent manner. Unlike other effector enzymes, e.g. adenylyl cyclase, this may reflect that phosphoinositidase C and phospholipase D are not the quantitatively limiting protein components on these signalling cascades. This provided a useful system to overcome low basal enzyme activity for the detection of inverse agonists since the increase in the CAMα_{1B} adrenergic receptor levels in response to phentolamine caused an increase in the basal level of phospholipase D activity. All of the tested α_1 antagonists appeared to be clearly capable of acting as inverse agonists at this CAMα_{1B} adrenergic receptor as measured by their ability to inhibit phentolamine-stimulated basal phospholipase D activity. This study, therefore, provides a basis to suggest that upregulation of CAM GPCRs may be a simple approach for the screening and detection of novel inverse agonist ligands.

List of Publications

LIST OF PUBLICATIONS

This thesis includes data from the following;

1. Milligan, G., Wise, A., MacEwan, D. J., Grassie, M. A., Kennedy, F. R., **Lee, T.W.**, Adie, E.J., Kim, G.D., MacCallum, J.F., Burt, A., Carr, I.C., Svoboda, P., Shah, B.H., and Mullaney, I. (1995) "Mechanisms of agonist-induced G-protein elimination." *Biochem. Soc. Trans.* 23, 166-170.

2. **Lee, T. W.**, Anderson, L. A., Eidne, K. A. and Milligan, G. (1995) "Comparison of the signalling properties of the long and short isoforms of the rat thyrotropin-releasing hormone receptor following expression in Rat 1 fibroblasts." *Biochem.J.* 310, 291-298.

3. Wise, A., **Lee, T.W.**, MacEwan, D. J. and Milligan, G. (1995) "Degradation of $G_{11}\alpha/G_q\alpha$ is accelerated by agonist occupancy of $\alpha 1_{A/D}$, $\alpha 1_B$, $\alpha 1_C$ adrenergic receptors." *J. Biol. Chem.* 270, 17196-17203.

4. Cook, J.V., McGregor, A., **Lee, T. W.**, Milligan, G. and Eidne, K.A. (1996) "A disulfide bonding interaction role for cysteines in the extracellular domain of the thyrotropin-releasing hormone receptor." *Endocrinology* 137, 2851-2858.

5. Milligan, G., **Lee, T.W.** and Wise, A. (1996) " Regulation of cellular G-protein levels by phospholipase C coupled receptors." In: *The phosphoinositidase C pathway. Its regulation and desensitization.* (Tobin, A.B. ed). p 159-176. R.G. Landes & Co. Austin .

6. **Lee, T. W.**, Wise, A., Cotecchia, S. and Milligan, G. (1996) " A constitutively active mutant of the α_{1B}-adrenergic receptor can cause greater agonist-dependent downregulation of the G proteins $G_q\alpha$ and $G_{11}\alpha$ than the wild type receptor." *Biochem. J.* (in press).

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