Hormone Binding and Catecholamine secretion studies on PC12 Cells

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To:

My Father, Mother

Ahmed and Youcef

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List of abbreviations:

A	Adrenaline
A I	Angiotensin I
A II	Angiotensin II
ANP	Atrial natriuretic peptide
AP I	Atriopeptin I
AP II	Atriopeptin II
AP III	Atriopeptin III
ATP	Adenosine tri-phosphate
AVP	Arginine-8-vasopressin
BSA	Bovine serum albumin
B_{max}	Receptor density
COMT	Catechol-O-methyl trasferase
cAMP	Cyclic 3',5' adenosine-mono-phosphate
cGMP	Cyclic 3',5' guanosine-mono-phosphate
DHP	dihydropyridine
DOPA	l-dihydroxyphenylalanine
Dopamine	l-dihydroxyphenylethanolamine
DBH	Dopamine-β-hydroxylase
DHMA	3,4 dihydroxymandelic acid
DHPG	Dihydroxyphenylglycol
DMEM	Dulbeco modified Eagle's medium
GPSA	Glutamine, Penicillin, Streptomycin, Amphotericin B.
GFR	Glomerular filtration rate

h-ANP	Human atrial natriuretic peptide
5-HT	5-hydroxytryptamine (serotonin)
HS	Hepes saline (Ca^{2+} and Mg^{2+} free salts solution)
H2	Hank's Hepes (buffer balanced solution)
HBSS	Hank's balanced salt solution
HSA	Human serum albumin
HPLC	High-perfomance liquid chromatography
K_d	Dissociation constant
MAO	Monoamine oxidase
NA	Noradrenaline
NGF	Nerve growth factor
PNMT	Phenylethanolamine-N-methyl transferase
PBS	Phosphate buffered saline
PMSF	Phenyl methane sulphonyl fluoride
RPMI	Medium developed at Roswell Park Memorial Institute
r-ANP	Rat atrial natriuretic peptide
T/V	Trypsin/Versene
TLC	Thin layer chromatography

Summary

Summary

Specific binding sites for atrial natriuretic peptide (ANP) secreted from the heart of several species have been identified in cultured PC12 phaeochromocytoma cells, a cell line derived from a tumour of the rat adrenal medulla.

The ^{125}I-labelled ANP bound specifically to PC12 cells with a dissociation constant (K_d) of 794 pM and a receptor density (B_{max}) of 256 femtomole/mg protein. Fiscus *et al.*, (1987) demonstrated that ANP stimulated the accumulation and efflux of cGMP in cultured PC12 cells. This observation lends strong support to the present study and suggests ANP acts via specific cell surface receptors to exert its second messenger action.

Binding of ^{125}I-ANP (99-126) to a plasma membrane preparation from PC12 cells increased with increasing protein concentration and remained linear up to 100 µg protein /100 µl. The kinetics of ligand binding to the membrane suggested that ANP was degraded in studies performed at 37°C and 22°C but remained almost intact when experiments were carried out at 0°C (on ice). These results were confirmed by HPLC which revealed that 70%, 14% and 3.6 % of ANP remained intact after 1h incubation at 0°C, 22°C and 37°C respectively. 90% of ANP incubated without the membrane remained intact after 1h at 0°C . Hormones unrelated to ANP such as A II, bradykinin and arginine-8-vasopressin did not compete for the binding sites; however, hormones related to ANP competed for the receptors with the following order of affinities: rat atrial peptide fragment 13-28 aa > r-ANP (28 aa) > h-ANP (28 aa) > r-ANP (8-33 aa) rat atrial peptide fragment

(1-11aa)>atriopeptin I.

A II binding sites were not expressed either in normal PC12 cells or NGF-treated cells (neuron-like). A catecholamine radio-enzymatic assay was used to show that dopamine but not adrenaline or noradrenaline was secreted from PC12 cells. This result is in agreement with those of Nagatsu *et al.*, (1987) and Ahnert-Hilger and Gratzel (1987). One of the techniques used for looking at dopamine release from PC12 cells was to incubate them with ^{3}H-dopamine and then expose them to different concentrations of potassium. The release was higher at 37mM K^{+} and even higher at 70mM K^{+} compared with cells incubated with 5mM K^{+}. Since ANP has specific receptors on PC12 cells the effect of ANP on neurotransmitter release were investigsted. PC12 cells were exposed to 5 and 70mM K^{+} in the presence of ANP at final concentration of 10^{-5}M. ANP had no statistically significant effect on the release of dopamine from PC12 cells.

PC12 cells were shown to contain ANP by measuring ANP released either under perfusion conditions or after homogenization. The results from homogenized cells for ANP release were 420 pg/ml for non-trypsinized cells and 284 pg/ml for trypsinized cells.

The supernatant obtained from homogenized PC12 cells was used in another radio-immunoassay to determine renin activity. PC12 cells did contain renin, since angiotensin I was generated after incubating samples with angiotensinogen (renin-substrate) for 5h at 37°C.

The presence of these three major blood pressure controllers (dopamine, ANP and renin) within PC12 cells, shows clearly that these tumour cells provide an excellent model system for investigating biosynthesis, maturation

and secretion of atrial peptides (Nguyen *et al.*, 1988), as well as the physiological role of the co-secretion of neuropeptides and catecholamines in the nervous system (differentiated PC12 cells).

In conclusion, PC12 cells represent an extremely useful model system in investigating many aspects of blood pressure regulation.

Introduction

1. Introduction

The regulation of blood pressure in mammals is under the control of both nervous and endocrine systems. Expansion of extracellular volume, activation of the sympathetic nervous system, or increased activity of the renin-angiotensin system will often result in hypertension (Landsberg and Young,1981). Blood pressure is regulated in two main ways;

1.1 Nervous regulation

The sympathetic nervous system affects the heart, veins, kidneys and arterioles (Landsberg and Young, 1981). The hypothalamus is well established as a major organ of cardiovascular control. When various areas of the hypothalamus are stimulated an increase or decrease in heart rate and modulation of the baroreceptor reflex is observed (Brody *et al.*, 1983).

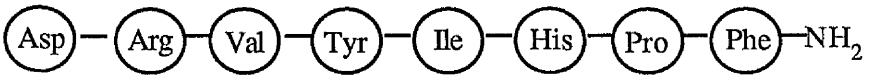
1.2 Hormonal regulation

1.2-1 Angiotensin II

One of the main systems responsible for the maintenance of blood pressure is the renin-angiotensin system. Juxtaglomerular cells in the kidney synthesize and release renin, an enzyme which cleaves renin substrate (angiotensinogen), which is synthesized by the liver, to form angiotensin I. Angiotensin I is cleaved by angiotensin converting enzyme to form an octapeptide angiotensin II (A II) (Fig.1). The conversion of angiotensin I to angiotensin II takes place in the vascular endothelium, particularly in the pulmonary circulation (Vane *et al.*, 1967).

A II increases cardiac output through a positive inotropic effect due to its action on β_2-adrenergic receptors (Freer *et al.*, 1976) and elevates blood pressure by having a direct vasoconstrictor effect on blood vessels (Bohr and Uchida,1967). A II also affects the brain, by stimulating thirst (Epstein *et*

Fig.1 . The sequence of angiotensin II



al., 1970) and the release of vasopressin (Bonjour and Malvin,1970) from the posterior lobe of the hypothalamus gland. The effect of A II on the kidney is to decrease renal blood flow and glomerular filtration rate (Navar and Langford,1974).

Angiotensin II controls the secretion of aldosterone which is released from the zona glomerulosa of the adrenal cortex (Fraser *et al.*, 1981). When high levels of A II are infused into dogs an increase in circulating levels of aldosterone is observed (Fraser *et al.*, 1981). Hyperactivity of the renin-angiotensin system due to high circulating levels of angiotensin II or due to the presence of high levels of renin, from renin-secreting tumours of the kidney, usually results in hyperaldosteronism (that is an excess of aldosterone in the circulation). Hyperaldosteronism leads to an increase in reabsorption of Na^+, Cl^- and water and to decreased reabsorption of K^+. Fluid and sodium retention in turn, cause an expansion of blood volume followed by high blood pressure (Keeton and Gould,1986).

1.2-2 Catecholamines

The adrenal medulla, which is the inner part of the adrenal gland, secretes and releases the catecholamines, adrenaline and noradrenaline into the blood stream. These hormones, which are released under stress conditions, stimulate the liver to supply more glucose to the blood and increase heart rate which in turn elevates blood pressure (Fig. 2).

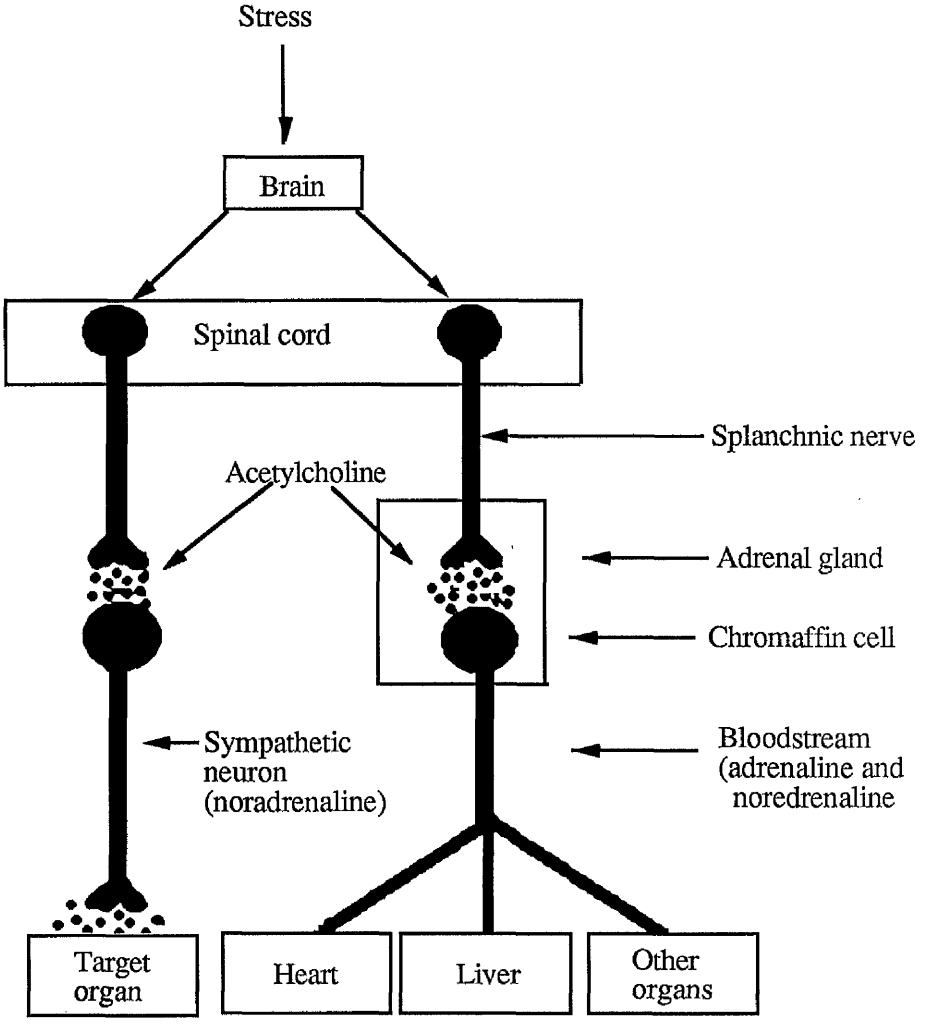
1.2-3 Thyroid hormones

Thyroid hormones, thyroxin-T4- and triiodothyronine-T3-, are secreted into the blood stream from the thyroid gland. Hypothyrodism (low circulating thyroid hormone) is associated with impaired renal water excretion (Derubertis *et al.*, 1971), vasoconstriction and an increased peripheral vascular resistance (Davies *et al.*, 1952); however hyperthyrodism (high

Fig.2. Stress and secretion of catecholamines.

The brain responds to stress by sending impulses to the adrenal glands (located above the kidney) and to sympathetic neurons through nerves in the spinal cord. The adrenal gland secretes adrenaline, noradrenaline and other substances into the bloodstream to act on a number of target organs. Sympathetic nerves however discharge noradrenaline locally.
(After Carmichael and Winkler, 1985).

Fig.2. Stress and secretion of catecholamines



circulating thyroid hormone) is associated with natriuresis and vasodilatation (Bell *et al.*, 1982). Landenson *et al.*, (1988) has demonstrated elevated levels of circulating atrial natriuretic peptide (ANP), a hormone involved in blood pressure regulation, in rats with hyperthyroidism compared with hypothyrodism. This is consistent with the above findings.

1.2-4 Prostaglandins

Under various stimuli, such as nerve stimulation and mechanical disturbances, prostaglandins can be synthesized and released from most tissues. They owe their name to the hydrocarbon skeleton prostane and are derivatives of arachidonic acid. Because of their occurence in the kidney and tendency to lower blood pressure, they might function as antihypertensive substances (Passmore and Robson, 1980).

1.2-5 Pancreatic hormones

Beta and alpha cells in the islets of Langerhans in the pancreas secrete insulin and glucagon into the bloodstream. These hormones may influence sodium balance thus modifying blood pressure indirectly (Kolanowski,1981). Insulin has been shown to stimulate growth in cultured vascular smooth muscle cells and this may be important in the development of hypertension in diabetic patients (Kaiser,1985).

1.2-6 The atrial natriuretic peptides

The atrial natriuretic family of peptides are known to be potent vasorelaxants. They oppose the effect of vasoconstrictors such as adrenaline, noradrenaline and angiotensin II. It is believed that atrial natriuretic peptides have an important role in the regulation of fluid volume and blood pressure and this will be discussed in more depth in section 4.

1.3 Phaeochromocytoma and hypertension

Phaeochromocytomas are tumours of chromaffin cells of the adrenal medulla. Hypertension caused by a phaeochromocytoma is due to an excess of circulating catecholamines (adrenaline and noradrenaline) secreted by the tumour. It is a relatively rare condition as only 0.1% of patients with diastolic hypertension have a phaeochromocytoma (Sleight and Freis,1982). It ends in death if untreated but normally it is cured by surgery. Phaeochromocytoma will be discussed in more detail in section 3.

2. The adrenal medulla

2.1 The adrenal gland

The adrenal gland is a double gland located above the kidney. It is composed of an inner part known as the medulla and an outer part known as the cortex. The adrenal cortex is organized into 3 concentric zones (Fig.3) which secrete a large number of steroid hormones derived from cholesterol (Keeton and Gould, 1986).

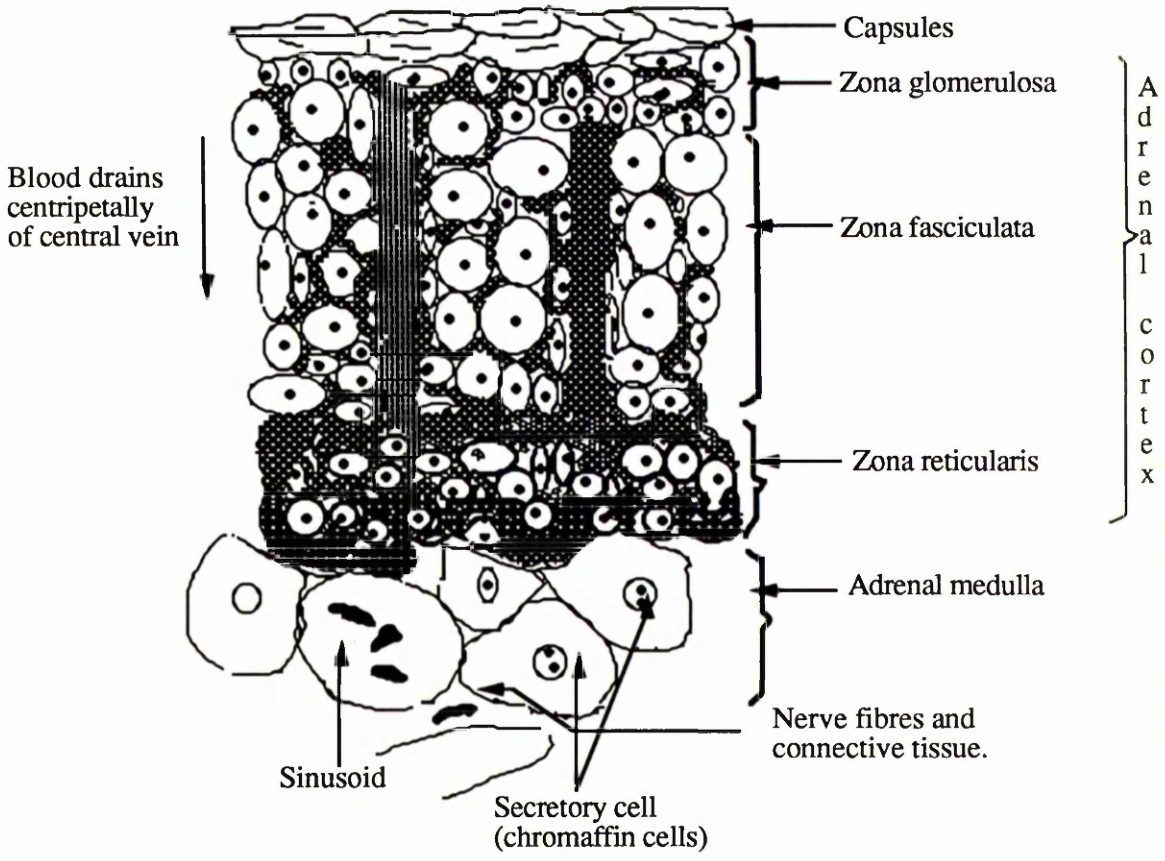
The outer zona glomerulosa secretes mineralocorticoids. These hormones regulate salt and water balance. The zona fasciculata secretes glucocorticoids. These hormones are involved in the regulation of carbohydrate and protein metabolism. Finally the innermost zona reticularis secretes sex hormones which are very similar chemically and functionally to the sex hormones produced by the gonads (Keeton and Gould, 1986).

2.2 Definition and function of the adrenal medulla

The adrenal medulla is viewed as sympathetic ganglia, which are innervated by preganglionic cholinergic fibres (Passmore and Robson, 1980). Chromaffin cells form the adrenal medulla; their name is derived from the observation that adrenaline released from the adrenal medulla reacts with chromium salt to produce a yellowish brown colour (Carmichael and Winkler, 1985). These cells extend axon-like processes in culture suggest the close relationship of these cells with neurons (Carmichael and Winkler, 1985). Chromaffin cells secrete the catecholamines adrenaline, noradrenaline and, to a limited extent, dopamine into the bloodstream. Adrenaline and noradrenaline are found in many animals; dopamine is abundant in insects (Passmore and Robson, 1980). Plants, fruit and

Fig.3. Structure of the adrenal gland (cortex and medulla).

(After Jeffcoate, 1986)



vegetables also contain catecholamines. Because these catecholamines are not absorbed from the gastro-intestinal tract, they do not produce any effect on the organism (Passmore and Robson, 1980).

2.3 Catecholamines and their concentrations in the bloodstream

In normal human peripheral blood the concentrations of catecholamines are extremely low making their accurate measurement difficult. Their ranges are for noradrenaline: 1.2—2.4 nM, dopamine 0.6—1.3 nM and adrenaline about 0.5 nM (Passmore and Robson, 1980).

2.4 Adrenergic receptors and catecholamine effects

Adrenaline, noradrenaline and dopamine function both as hormones and neurotransmitters. Usually adrenaline acts as an endocrine hormone but it is also a neurotransmitter for some neurones. Noradrenaline, however, usually acts as a neurotransmitter (Darnell *et al.*, 1986). Adrenaline and noradrenaline are widely distributed throughout the body and transmit their message through specific receptors on target organs. There are 3 major types of receptors for catecholamines: α-adrenergic, β-adrenergic and dopamine receptors. They can be distinguished by their ligand specificity, their biochemical responses and their responses to specific blockers agonists and antagonists (Levitzki, 1980). Binding of adrenaline or noradrenaline to β-adrenergic receptors on hepatic cells (liver), adipose cells and heart muscle causes glycogenolysis, lipolysis and increases the rate of contraction respectively, thus supplying more oxygen, fatty acids and glucose to fuel the tissues (Darnell *et al.*, 1986). The binding of adrenaline and noradrenaline to β-adrenergic receptors is associated with inceased levels of cAMP as a signal transducer (Levitzki, 1980). Binding of α-agonists to α-adrenergic receptors is associated with constriction of arteries which reduces the blood

supply to peripheral organs. Receptor binding is coupled to an influx of Ca^{++}, which acts as a second messenger, coupling signal and response (Levitzki,1980). A separate class of α–adrenergic receptors is coupled to the enzyme adenylate cyclase in the central nervous system. This discovery was based on studies showing that α–adrenergic blockers inhibited the formation of cAMP (Levitzki,1980). Physiologically α and β–adrenergic receptors oppose each other in target cells (Table 1).

2.5 Biosynthesis of catecholamines in the adrenal medulla

Catecholamines are synthesized in the adrenal medulla , the brain and sympathetic nerve endings (Musacchio *et al.*, 1963) and in cells of neural crest origin throughout the body (Melmon,1974). Tyrosine is the primary substrate for catecholamine synthesis in mammals and it is derived mainly from the diet (Fig.4). The main rate-limiting enzyme involved in the synthesis of catecholamines is tyrosine hydroxylase, which is found only within catecholamine-producing cells. This enzyme convert l-tyrosine to l-dihydroxyphenylalanine (DOPA). DOPA is rapidly converted to l-dihydroxyphenylethanolamine (dopamine) by aromatic l-amino acid decarboxylase, an enzyme which is found in many tissues including the cytoplasm of chromaffin cells (Manger and Gifford, 1977). Dopamine is then taken up into cytoplasmic vesicles by an ATP-dependent mechanism. These vesicles contain dopamine β-hydroxylase (DBH), which hydroxylates dopamine to form noradrenaline when ascorbic acid, oxygen and fumaric acid are present as cofactors. Some of the chromaffin cells can convert l-noradrenaline to l-adrenaline using phenylethanolamine-N-methyl transferase (PNMT), an enzyme found only in the adrenal medulla (except for minute quantities in heart and brain). The activity of PNMT is dependent

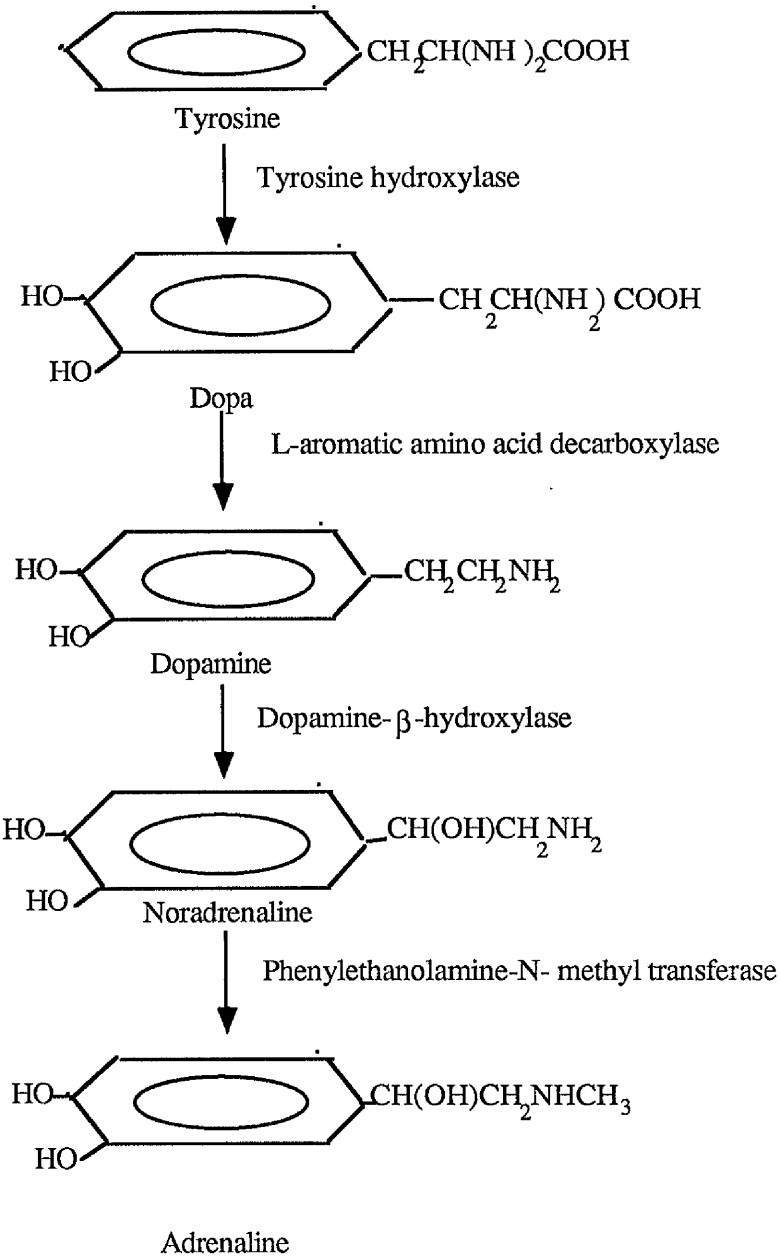
Table 1. Some physiological actions of adrenergic receptors .

(After Levitzki, 1980).

System or tissue	Action	Receptor
Heart	Increased force of contraction increased rate	β_1
Blood vessels	Constriction Dilation	α β_2
Respiratory system, tracheal and bronchial smooth muscle	Relaxation	β_2
Iris (radial muscle), smooth muscle, uterus.	Pupil dilated contraction	α α
Spleen	Relaxation Contraction	β_2 α
Bladder	Contraction Relaxation	α β_2
Skeletal muscle	Changes in twitch tension Increased release of acetylcholine Increased glycogenolysis	β_2 α β_2
Adipose tissue	Increased lipolysis	β_1
Parotid gland	Enzyme secretion Water and K^+ secretion	β α

Fig.4. Biosynthesis of catecholamines

(Passmore and Robson 1980)



on glucocorticoids synthesized from the adjacent adrenal cortex (Manger and Gifford, 1977). Noradrenaline has to move to the cytoplasm, where PNMT is located, for the methylation reaction. Adrenaline is then transported back into vesicle for storage (Manger and Gifford, 1977).

2.6 Storage and release of catecholamines

Chromaffin vesicles store not only catecholamines but also ATP and a diverse mixture of proteins. The most abundant components are acidic proteins called chromogranins. Neuropeptides of various sizes are also stored in the adrenal medulla (Carmichael and Winkler, 1985). After their synthesis and storage, catecholamines are released by stimulation with acetylcholine. The cholinergic fibres of the splanchnic nerves make cholinergic synapses on the chromaffin cell membrane, opening gates , which, by plasma membrane calcium channels, trigger the release of hormones by a mechanism known as stimulus-secretion-coupling. Chromogranins, dopamine β–hydroxylase and some other constituents of the vesicle are released along with the catecholamines (Passmore and Robson, 1980). Release of catecholamines are also stimulated by histamine, 5-HT, bradykinin and high concentrations of potassium. Calcium is always required for catecholamine release (Passmore and Robson, 1980). Dihydropyridines such as nitrendipine (a calcium channel antagonist) block the release of catecholamines from adrenal chromaffin cell; release is enhanced by Bay K8644 (calcium channel agonist) (Kongsamut and Miller, 1986). Adrenaline and noradrenaline are released from the adrenal medulla as a result of insulin hypoglycemia. Hillarp and Hokfelt, (1953) suggested that there are two chromaffin cell populations. Hillarp *et al.,* (1954) have shown that adrenaline and noradrenaline are formed in two different cell types and released independently. Substances

such as glucagon (Scian *et al.*, 1960), nicotine, polocarpine (Dale *et al.*, 1912) and angiotensin (Feldberg *et al.*, 1964) stimulate catecholamine secretion from chromaffin cells by depolarizing cells to allow the entry of Ca^{++}. K^+, Na^+, Cl^- and Mg^{++} are not necessary for catecholamine release (Manger and Gifford, 1977).

2.7 Inactivation of catecholamines

After their release into the bloodstream catecholamines are inactivated very rapidly. The enzymes involved in the degradation of catecholamines and the pathway followed in the inactivation are the same as in phaeochromocytoma PC12 cells and this will be discussed in section 3.

2.8 Catecholamines as neurotransmitters

Adrenaline, noradrenaline and dopamine are the three main members of the catecholamine family. Each these has different and characteristic physiological functions, pharmacological actions, sites of production and pathways of metabolism (Passmore and Robson, 1980). They function as both hormones and neurotransmitters. Noradrenaline plays a major role at synapses with smooth muscle cells, and is responsible for the classic "fight or flight" reaction. It is also found at synapses within the central nervous system. When β-receptors are activated at β-adrenergic synapses, adenylate cyclase is activated whereas α-receptor activity involves changes in ion permeability where the first event is the influx of Ca^{++} (Levitzki, 1980). Another class of α-receptor was found to be coupled to the enzyme adenylate cyclase (Levitzki,1980).

3. Phaeochromocytoma

Phaeochromocytoma are tumours of chromaffin cells. About 90% occur in the adrenal medulla, 2% in the chest and 0.1% in the neck (Sleight and Freis, 1982). They are composed of phaeochromocytes which are arranged in cords or in small or large alveolar patterns (Page *at al.,* 1986). Phaeochromocytoma are sometimes located in the abdomen and the organ of Zuckerkandl. Histologically phaeochromocytoma are usually benign but physiologically they are malignant because they secrete catecholamines. In 90% of cases a phaeochromocytoma is curable, but it is fatal if left untreated (Manger and Gifford, 1977). Phaeochromocytoma releasing dopa or dopamine are more likely to be malignant (MacMillan, 1956).

3.1 Isolation of the cell line PC12 from a rat phaeochromocytoma and effects of nerve growth factor

The clonal cell line PC12, isolated by Greene and Tischler, (1976) has been established from a transplantable rat adrenal phaeochromocytoma. Adrenal medullary cells *in vivo* (Aloe and Levi-Montalcini, 1979) and PC12 phaeochromocytoma cells *in vivo* differentiate after exposure to nerve growth factor. They stop dividing, produce elongated neurite like processes, develop presynaptic terminals containing neurotransmitters and develop first calcium and then sodium action potentials (Dichter *et al.,* 1977). After removal of NGF, the processes degenerate within 24h and cells revert to their original phenotype (Greene and Tischler, 1976). The level of catecholamine release is not altered in PC12 cells grown in the presence of NGF when expressed on a per cell basis but does result in a 4 to 6 fold decrease in levels when expressed on a per mg of protein basis (Greene and Tischler, 1976). Another clonal cell line from rat phaeochromocytoma was

established by Goodman *et al.*, (1979) in which NGF could induce tyrosine hydroxylase, the enzyme responsible for converting tyrosine to dopa. This was done by culturing uncloned cells from a rat phaeochromocytoma in tyrosine free-medium containing NGF. PC-G2 was the clone isolated in this fashion and responded to NGF by becoming neural-like (1-10μg NGF/ml) with an induction of tyrosine hydroxylase. PC12 cells have been fused using polyethylene glycol to produce cells up to 300 μm in diameter in order to perform electrophysiological studies. Without this treatment the cells would be damaged by penetration of microelectrodes due to their size (10-20μm in diameter) (O'Lague and Huttner, 1980). Nerve growth factor is a protein known to stimulate neurite outgrowth from sympathetic neurones. It is required for the development and maintenance of these cells (Dichter *et al.*, 1977). PC12 cells respond to NGF by stopping cell division, extending processes and developing neuronal properties and causing changes in the apparent rate of synthesis of cytoplasmic and nuclear proteins (Tiercy and Shooter, 1986). In addition acidic fibroblast growth factor and basic fibroblast growth factor can be used to induce neurite outgrowth from phaeochromocytoma. The binding of basic fibroblast growth factor to the receptors is completely blocked by heparin (Neufeld *et al.*, 1987).

PC12 cells are very useful for physiological studies as well as as a model for cell-cell interactions. In the presence of NGF, PC12 cells resemble neurons and synthesize, store and release acetylcholine (Greene and Rein, 1977). PC12 cells become sensitive to acetylcholine when differentiated with NGF. It would be of interest to investigate if PC12 cells formed synapses with several target cells or with each other (Dichter *et al.*, 1977). PC12 cells express action potential mechanisms for Na^+, Ca^{++}, K^+ conductance and other K^+ conductance mechanisms (O'Lague and Huttner, 1980). PC12

cells, whether they are treated with NGF or not, are like normal chromaffin cells and sympathetic neurones in that they can generate action potentials (Biales *et al.*, 1976). Since NGF plays the major role in promoting the maturation prossess of neuroblasts into neurones, and promoting the differentiation processes of phaeochromocytoma into neuron-like cells, PC12 cells could be a unique model system for studying the development of neuroblasts into mature neurons. Furthermore they could be useful for studies of the development of electrical excitability. Because NGF-treated PC12 cells are sensitive to acetylcholine, they may respond to cholinergic fibers (Dichter *et al.*, 1977).

3.2 Similarities between normal chromaffin cells and PC12 cells

PC12 cells share several characteristics with adrenal medullary chromaffin cells.

a) The adrenal chromaffin cells and PC12 cells are derived from the sympathetic nervous system originating from the neural crest (Carmichael and Winkler, 1985).

b) The normal chromaffin cells and PC12 cells secrete catecholamine (Kongsamut and Miller, 1986).

c) Exocytosis is a common way of releasing catecholamines in both cells (Manger and Gifford, 1977).

d) The various enzymes necessary for catecholamine synthesis and inactivation are present in both cells (Manger and Gifford, 1977).

3.3 Differences between normal chromaffin cells and phaeochromocytoma

Although these two different type of cells have many similarities, they still different in many other characteristics.

a) The adrenal medulla is innervated by the splanchnic nerve so that

acetylcholine release, which is the result of activation of this nerve, induces the release of catecholamines from chromaffin cells. In contrast, there is no evidence showing that phaeochromocytoma is innervated (Cousins and Rubin, 1974). The mechanism of cell stimulation is still not clear.

b) The granules in the adrenal medulla contain a high concentration of ATP while some phaeochromocytoma contain low concentrations of this compound (Winkler and Smith, 1968)

c) Activation of the sympathetic nervous system during hypoglycemia results in the release of catecholamines from the adrenal medulla into the circulation but not from phaeochromocytoma (Cantu *et al.*, 1968).

d) The turnover of catecholamines in phaeochromocytoma is much faster than in normal chromaffin cell. This might explain the continuous release of these substances from phaeochromocytoma (Winkler and Smith, 1972).

e) The activity of nerves stimulating adrenal medulla cells controls the secretion rate of catecholamines but with phaeochromocytoma, the rate of release depends on the rate of synthesis (Winkler and Smith, 1968).

f) The mean size of granules in normal chromaffin cells is 170nm, whereas the mean size of granules in phaeochromocytoma cells is greater than 270nm (Victor *et al.*, 1982).

g) Electron microscopy studies have shown the presence of light and dark cells in phaeochromocytoma, while in normal chromaffin cells only the dark cells were present (Tannen-Baum, 1975).

h) Winkler and Smith, (1968) suggested that the release of catecholamines from phaeochromocytoma was due to bypass of storage sites and diffusion of catecholamines out of the cell (Winkler and Smith, 1972). The granule's membrane could be destroyed by lysosomes that allow the release of catecholamines from phaeochromocytoma (Winkler and Smith, 1968).

i) Phaeochromocytoma preloaded with ^{3}H-dopamine and then permeabilized with Staphylococcal alpha-toxin release ^{3}H-dopamine in the presence of Ca^{++}. In contrast, normal chromaffin cells require an Mg-ATP for this release (Ahnert-Hilger and Gratzl, 1987).

3.4 Biosynthesis and release of catecholamines from PC12 cells

PC12 cells have the enzymes necessary for catecholamine biosynthesis. The amount of catecholamines synthesized by PC12 cells is greater than in the adrenal medulla. PC12 cells secrete a combination of catecholamines and their metabolites and occasionally their precursors (Lightman, 1979). A lack of ordinary control mechanisms regulating synthesis or release of adrenaline or its precursors could help explain the unusual secretion rates and it has been demonstrated that tyrosine hydroxylase does not seem to be inhibited by the normal concentrations of noradrenaline in the cell. The enzyme could be unusual in having a different K_m for its substrate or the amines could be held in compartments after their formation thus not allowing feedback inhibition of the normal enzyme. In addition, the tumour's metabolites have been shown to prevent the ordinary feedback inhibition of tyrosine hydroxylase by noradrenaline (Melmon, 1974). The release of transmitter substances from PC12 cells increases under depolarizing conditions (Shalaby *et al.*, 1984).

PC12 cells are extremely sensitive to dihydropyridines as are normal chromaffin cells, but once they are differentiated (neuron-like) the release becomes predominantly insensitive to dihydropyridines. The release of ^{3}H-noradrenaline from preloaded, differentiated PC12 cells was slightly enhanced by BAY K8644, and very poorly inhibited by nitrendipine even at high concentrations (Ogura and Takahachi, 1984). The release of adrenaline from differentiated PC12 cells is less than in undifferentiated ones upon depolarization by 70mM K^{+} (12.2±0.22% undifferentiated cf 8.88±0.20%

differentiated). Cells can be permeabilized by high-voltage discharge (Baker and Knight, 1978), by saponins (Dunn and Holz, 1983), or by alpha-toxin from *Staphylococcus aureus*.

3.5 Inactivation of catecholamines in PC12 cells

Catecholamines, taken up by either neuronal cells or extraneuronal cells like smooth muscle cells, are degraded very quickly inside the cell. The two main enzymes involved in the degradation of catecholamines are: MAO (monoamine oxidase) and COMT (catechol-O-methyl transferase). The major role of MAO is in the metabolism of tissue stores of catecholamines and in the further metabolism of O-methylated amines. COMT is widely distributed and present in almost all tissues in association with the methyl donor-S-adenosyl methionine. It is the major route of degradation of intravenously administred catecholamines (Manger and Gifford, 1977). Adrenaline can be deaminated by monoamine oxidase (MAO) plus aldehyde dehydrogenase in mitochondria to form unstable aldehydes which are then oxidized to an acid 3,4 dihydroxy mandelic acid (DHMA). Secondly, noradrenaline can be reduced to an alcohol, dihydroxyphenylglycol (DHPG). The second way occurs mainly in the rat whereas oxidation takes place in man. Catechol-O-methyl transferase (COMT) converts noradrenaline released into the circulation to normetanorphine (Manger and Gifford, 1977)

3.6 PC12 responses

Many studies have been carried out on the response of phaeochromocytoma PC12 cells to both inhibitors and stimulators. Dopamine release by high K^+ concentrations (50mM) from PC12 cells was inhibited by a specific inhibitor of myosin light chain kinase (ML-9) (Nagatsu *et al.*, 1987). In response to stimulation by bradykinin, angiotensin II and carbachol, PC12 cells accumulate inositol phosphates (mono, bis and triphosphate) (Van Calker *et al.*, 1987).

Exposing PC12 cells to nicotine (10^{-5}M) result in the release of adrenorphin along with catecholamines (Yanase *et al.*, 1987). It is believed that PC12 cells contain high amounts of guanylate cyclase, which is a proposed second messenger of ANP. Atriopeptin I, atriopeptinII, atriopeptin III and human ANP stimulate cyclic 3', 5' guanosine mono-phosphate (cGMP) accumulation in PC12 cells (Fiscus *et al.*, 1987).

4. Atrial Natriuretic Peptide

Mammalian atrial cardiocytes contain secretory granules which release atrial natriuretic peptide (ANP) into the bloodstream (De Bold *et al.*, 1981). However, in non-mammalian vertebrates, atrial and ventricular cardiocytes are involved in this secretion (Yunge *et al.*, 1980). ANP is localised in the granules of cardiocytes and is released into the blood stream as a result of intravascular or extracellular (Lang *et al.*, 1985) fluid volume expansion whereupon it exerts diverse cardiovascular, renal and endocrine effects. It may therefore be considered as a true circulatory hormone (Hirata *et al.*, 1985).

ANP is secreted at high levels in different conditions such as congestive heart failure, chronic renal failure and atrial distention through a mechanism called stretch-secretion coupling (Trippodo, 1987). The evidence that ANP is secreted from the heart is the high concentrations of this peptide found in coronary sinus blood and low concentrations in peripheral organs (Sugawara *et al.*, 1985). ANP is also secreted from atrial myocytes in culture (Gibson *et al.*, 1987).

4.1 Isolation and sequence

ANP is synthesized as a 151 (152 in the rat) amino acid residue precursor, prepro ANP. The first 25 amino acid (26 amino acid for rat) from the N-terminal end constitute a "signal peptide" which is presumably used in ribosomal processing of the peptide, this is removed before storage as a 126 amino acid chain in granules in atrial cells (Genest, 1986). At the time of release the active 28 amino acid moiety is removed. The nomenclature of atrial peptide is not uniform. In 1987 a joint committee of the international society of hypertension, the world Organisation and the American Heart

Association (Dzau *et al.*, 1987) recommended that peptides should be named from the N-terminal end of the precursor proANP eg: rat ANP becomes ANP (99-126). There is great homology between species such that rat ANP differs only by the substitution of isoleucine for methionine at position 110. Smaller forms of rat ANP have been identified but all those with physiological activity contain a cysteine-cysteine disulphide bridge (cys 105-cys121). The location of the ANP gene has been identified on the distal short arm of chromosome 1 in man, and on chromosome 4 in mice. ANP cDNA was cloned by Yangfeng *et al.*, (1985). Recent studies have reported the presence of a specific gene in the brain (rat) encoding ANP-like related peptides. The fact that highly specific receptors for ANP have been identified in the brain (Table 4) and the recent studies about ANP gene transcript in the brain are strong evidence for the existence of an ANP system within the brain (Quirion, 1988). ANP (99 - 126) has recently been detected together with its precursor (Asn 1-Tyr 126) within bovine chromaffin granules (Ong *et al.*, 1987; Flemminger *et al.*, 1983). They confirmed the maturation process of atrial peptides in the adrenal medulla. Exposure of normal chromaffin cells to nicotine (10μM) or KCl (56mM) induced an increase over the basal secretory level of immunoreactive ANP (from 27 ±12 up to 186±52 femtomole /$2x10^7$ cells) and (from 27± 12 up to 180±53 femtomole/$2x10^7$ cells) respectively (Nguyen *et al.*, 1988). ANP was isolated, synthesized and sequenced by Seidah *et al.*, in 1984 (Fig.5).

4.2 Circulating levels of ANP

Much variation still exists among laboratories but the majority of studies show mean values for basal plasma ANP of healthy volunteers in the range of (10-60pg/ml) (Trippodo, 1987). ANP levels vary together with molecular forms of ANP according to age and cardiac condition (Miyata *et al.*,

1985). In rats the plasma concentration of ANP is between 20-40 pM (60-120pg/ml). High concentrations of ANP in plasma, ranging from 100pg/ml to 450pg/ml according to age and blood pressure, were observed in hypertensive rats. ANP levels increased from 117pg/ml to 238pg/ml during the development of salt induced hypertension in Dahl salt-sensitive rats (Tanaka and Inagami, 1986). Similarly high levels of plasma ANP were found in patients with severe congestive heart failure by Schiffrin *et al.* , (1986) and renal failure (Rascher *et al.*, 1985). Plasma ANP levels are also elevated by infusing intravenously with isotonic fluid (Yamaji *et al.*, 1985).

4.3 The effects of ANP

4.3-1 On the kidney

It is well established that the kidney is the major site of ANP action (Espiner *et al.* 1986) (Table 2). Bolus injections of larger doses cause a rapid, massive and short diuresis and natriuresis within the kidney of dogs (Maack *et al.*, 1984). Glomerular filtration rate (GFR) is increased together with filtration fraction in rats and man after intravenous injections of ANP. In addition, urine volume and sodium excretion is elevated (Genest, 1986). More recently low dose infusions of ANP have shown similar effects on sodium, calcium and magnesium excretion without increases in GFR or a diuretic response (Richards *et al.*, 1988).

Maack *et al.*, (1988) have shown that in rat kidney more than 95% of ANP receptors are biologically silent, in that ANP binding occurs without a functional response or cyclic GMP generation and these receptors were termed as "clearance receptors". Thus the kidney itself may profoundly unfluence the clearance, therefore the circulating plasma concentrations of ANP. Atrial but not ventricular extracts from mammalian species showed a natriuretic and diuretic activity, whereas in frog both atrial and ventricular

Table 2: Summary of ANP effects and its main sites of action in the human body.

Effect	Site of action	Source
Direct blood pressure reducer	vessels and arteries	Sagnella *et al.*, 1985
Stimulates the cotransport of Na^+, Cl^- and K^+	vascular smooth muscle cells	Owen *et al.*, 1987
Rapid, massive & short diuresis & natriuresis	Kidney	De Bold *et al.*,1981
Inhibition of renin secretion	Kidney	Burnett *et al.*, 1984
Increase circulating levels and urinary excretion of cGMP	Kidney	Gerzer *et al.*, 1985
Increase cGMP	Aortic tissues	Hirata *et al.*, 1985
	Adrenal gland	Hinko *et al.*, 1987
	Kidney	Takeda *et al.*, 1986
	Avian epiphyseal growth plate chond--rogenitor cells	Pines &Hurwitz, 1988
	PC12 cells	Fiscus *et al.*, 1987
	Astrocytoma cells	Lyall *et al.*, 1988

Plasma aldosterone and A&NA concen--tration depressed	Blood	Richards *et al.*, 1985
Induces release of arginine-8-vasopressin	Posterior lobe of the hypophysis	Januszewicz *et al.*, 1986
Inhibition of cortisol	Bovine adrenal cortex secretion	Chartier & Schiffrin, 1987.

extracts showed both these actions. Ventricular but not atrial extracts showed diuresis only and not natriuresis in hen . Natriuretic specific activity values were high in rat and mouse atria (De Bold and Salerno, 1983).

Table 3. Summary of the more important effects of ANP (Natriuresis-Diuresis) in different species.

	Site of secretion		Effect	
Species	Atria	Ventricle	Natriuresis	Diuresis
Human	+	—	+	+
Frog	+	+	+	+
Hen	—	+	—	+
Rat & Mouse	+	—	+	—

4.3-2 On the heart and blood circulation

Injections of synthetic atriopeptin III (Ser103-Tyr126) or atriopeptin II (Ser 103 - Arg 125) reduced cardiac output in anaesthetised dogs and rats (Natsume *et al.*, 1985; Smiths and Lappe, 1985) however the output was not significally altered after injection of ANP (Arg101-Tyr126) (Siegel *et al.*, 1984). ANP is a vasorelaxant and blood pressure reducer (Sagnella *et al.*, 1985; Anderson *et al.*, 1986; Richards, 1986). Low dose infusion of ANP (0.75 pM/kg.min. ie: 0.0023 µg/kg.min) in six normal men resulted in a small increase in plasma ANP within the range established for normal men and women (5-24 pM) and a significant reduction in systolic blood pressure (Richards *et al.*, 1988).

ANP relaxes rabbit aorta precontracted with noradrenaline or histamine

(Currie *et al.*, 1984), but gives poor relaxation with those contracted with K^+ (Garcia *et al.*, 1984). Atriopeptin II (10^{-7}M) inhibits intracellular Ca^{++} release in vascular smooth muscle caused by noradrenaline, histamine (10^{-5}M) and caffeine (25 mM) (Meicheri *et al.*, 1986). The decrease of cytosolic Ca^{++} in vascular smooth muscle cells (rat) by the activation of cGMP dependent protein kinase, results in relaxation of these cells (Lincoln and Johnson, 1984). Owen *et al.*, (1987) concluded that ANP stimulates Na^+ -K^+ -Cl^- cotransport in vascular smooth muscle cells through the elevation of intracellular cGMP.

4.3-3 On the adrenal gland

An infusion of 15pmol/min of α-hANP inhibited angiotensin II-stimulated aldosterone release in normal subjects (Anderson *et al.*, 1986). Natriuresis was enhanced, plasma aldosterone and adrenaline concentrations were depressed and blood pressure lowered due to an intravenous injection of ANP (Richards *et al.*, 1985). ANP induces the secretion of arginine-vasopressin from the isolated posterior lobe of the hypophysis (Januszewicz *et al.*, 1986) but inhibits aldosterone and cortisol secretion from adrenocortical tissue stimulated with angiotensin II, ACTH and K^+ (Chartier and Schiffrin, 1987).

4.4 ANP receptors

ANP receptors with different K_ds are distributed in several tissues (table 4) according to the degree of involvment of these areas in the regulation of the blood pressure and the other fluids in the body. The K_d is in the picomolar range in most studies. Although most of the binding studies revealed a single class of high affinity binding sites whether radio-iodinated synthetic ANP or a quantitative autoradiographic method was used, few of these studies showed more than one class of binding sites. The number of sites per cell or

per mg of protein varies according to the tissue and their involvment in translating ANP signals. Receptors for ANP have been detected in the zona glomerulosa of the adrenal cortex, renal glomeruli, collecting ducts and all areas in the brain involved in regulation of blood pressure, sodium and water (Genest, 1986). Binding sites were found in vascular and epithelial tissue in the lung, the bases of intestinal villi, in ciliary bodies of the eye (Bianchi *et al.,* 1985) and in the anterior pituitary (Quirion *et al.,* 1984) although ANP had no effect on the anterior pituitary hormone secretion (Abousamra *et al.*, 1987). Receptors for ANP were found in cultured neuroblastoma cells (N4TG1) of peripheral origin (Pandy *et al.,* 1987). Human platelets contain specific receptors for ANP; but red and white blood cells do not (Schiffrin *et al.*, 1986). Specific binding sites for ANP were detected in cultured vascular smooth muscle cells (VSMC) of the rat aorta (Hirata *et al.,* 1984). Blood vessels (mesenteric & renal arteries) and adrenal capsules bear specific receptors for r-ANP (Schiffrin *et al.,* 1985). ANP specific receptors were detected in the subfornical organ and choroid plexus in water-deprived rats (Saavandra *et al.,* 1987). Gibson *et al.,* (1986) have studied ANP receptors distribution in rat brain using a quantitative autoradiographic method. They found the highest levels of ANP receptor in the circumventricular organs and the olfactory apparatus and moderate levels in the mid brain and brain stem but the lowest levels were found in the forbrain dienciphalon, basal ganglia, cortex and cerebellum. All the areas in the brain, which are known to be involved in the regulation of blood pressure, renin-dependent hypertension and in the regulation of water and sodium homeostasis, contain ANP receptors (Genest, 1986). Specific receptors for ANP have been identified in cultured mesenchymal nonmyocardial cells in rat heart (Hirata *et al.,* 1985). Vollmar *et al.,* (1988) have demonstrated specific receptors on the corpus

Table 4: ANP receptors .

K_ds are in the pM range in most studies however the number of sites per cell or per mg of protein varies greatly.

Binding sites for ANP	Kd (x 10^{-10} M)	Binding sites	Source
Cultured neuroblastoma (mouse)	10	110,000-150,000 /cell	Pandy *et al.* (1987)
Cultured vascular smooth muscle cells (human)	20	200,000-300,000 /cell	Hirata *et al.* (1984)
Human platelet	0.3	9.8±1.4/cell	Schiffrin *et al.* (1986)
Rat blood vessels			
mesenteric arteries	0.7	68±22 femtomole/mg protein	Schiffrin *et al.* (1985)
renal arteries	1.8	23±5 femtomole/mg protein	
adrenal capsules	0.6	90±30 femtomole/mg protein	

[Cont]			
mesenteric arteries	0.2 100	17 femtomole/mg protein $5x10^6$ femtomole/mg protein	Lyall and Morton 1987
Subfornical organ *(rat)*	83	138.7±23 femtomole/mg protein	Saavandra *et al.* 1987
Choroid plexus	66	126.2±9femtomole/mg protein	
Cultured mesenchymal non-myocardial cells	2 (atria) 2.8 (ventricle)	189,000 /cell 300,000 /cell	Hirata *et al.* 1985)
Rat central nervous system	6	0.12±0.02—3.13±0.1 femtomole/mg protein	Gibson *et al.* (1986)
Corpus luteum	1.2	18 femtomole/mg protein	Vollmar *et al.* (1988)
Astrocytoma (human)	1.0	105 femtomole/mg protein	Lyall *et al.* (1988)

luteum. Specific high-affinity binding sites for ANP in the clone D384 derived from the human astrocytoma cell line have been recently demonstrated and furthermore, these binding sites were coupled to guanylate cyclase (Lyall *et al.*, 1988).

4.5 Signal transduction

Receptors for ANP are localized on the external surface of the plasma membrane and the cyclic nucleotide guanosine 3', 5' monophosphate phosphate (cGMP) mediates the action of this peptide on target cells. ANP was found to inhibit cyclic adenosine 3', 5' monophosphate activity (cAMP) (Anand-Srivastava *et al.*, 1984). It is worth pointing out that ANP is unique in selectively activating the particulate form (membrane-associated) of guanylate cyclase in PC12 cells (Fiscus *et al.*, 1987).

The increase of cGMP in LLC-PK1 kidney epithelial cells, was time and concentration dependent after addition of ANP, AVP and oxytocin. However, unlike ANP that AVP and oxytocin activated the soluble guanylate cyclase instead of the particulate form. In the presence of methylene blue, which is an inhibitor of the soluble form of the enzyme, the ANP-induced increase in cGMP was not altered nor was the accumulation of cAMP by AVP and oxytocin. This has confirmed the activation of the soluble form of the enzyme by AVP and oxytocin (Leitman *et al.*, 1988).

The increase of cGMP accumulation in aortic smooth muscle cells induced by ANP is inhibited by angiotensin II and other calcium mobilizing stimuli. (Smith and Lincoln, 1987). ANP increases the production of cGMP in aortic tissue (Hirata *et al.*, 1985), adrenal gland (Hinko *et al.*, 1987), kidney (Takeda *et al.*, 1986) and PC12 rat pheochromocytoma cells in culture (Fiscus *et al.*, 1987). Human and rat ANPs stimulated cGMP in avian epiphyseal growth plate chondroprogenitor cells and avian skin fibroblasts but

they did not alter the basal cAMP concentrations (Pines and Hurwitz, 1988). The accumulation of cGMP in many areas of the brain by ANP was pointed out by Fiscus *et al.*, (1987). In summary cGMP appears to play a major role in the translation of ANP signals in the cell.

5. The aim of the project

The PC12 cell line used in these studies is derived from a phaeochromocytoma of rat adrenal chromaffin cells. They have been investigated as firstly a site of action and as a source for vasoactive hormones and secondly as a possible model of normal chromaffin cell secretory function.

Receptors for the pressor hormone angiotensin II and for the vasorelaxing hormone, atrial natriuretic peptide were sought and characterised since brain and normal medullary tissue are known to secrete vasoactive hormones, PC12 cells were investigated for the presence of ANP and for elements of the renin angiotensin system. Preleminary attempts were made to study the content of ANP secretion.

The type(s) of catecholamine(s) released by PC12 cells were also identified. The influences of K^+ depolarisation, ANP on the release of the major catecholamine secretory product, dopamine, were also considered using both static incubations and perfusion system .

For future studies the NGF-treated PC12 cells in culture (which resemble sympathetic neurons) may well be a useful system for studying ANP presence within the brain, since it is difficult to handle neurons for long periods in culture.

Materials and Methods

6. Materials and methods

6.1 Materials

All and ANP fragments were obtained from Peninsula Laboratories (UK). Angiotensin II, bradykinin, arginine-8-vasopressin and Tween 20 were obtained from Sigma (UK).^{125}I-rANP (99-126) and ^{125}I-A II (human) and ^{3}H-dopamine were obtained from Amersham International. Foetal calf serum and other media were obtained from GIBCO BRL. The remaining chemicals were also obtained from Sigma (UK).

6.2 Cell culture

PC12 cells were obtained from Dr. A. McCruden (University of Strathclyde, Scotland) and Dr. Peter Clark (University of Glasgow). Cells ($5x10^6$) were frozen at -70 °C in 1 ml of freezing mix composition (9 ml of foetal calf serum + 1 ml of dimethyl sulfoxide (DMSO). To start a culture 1 ml of frozen cells ($5x10^6$) was thawed out and added to 75cm^2 tissue culture flasks containing 20ml of growth medium (section 6.8-1) and left for 2 days to allow the cells to recover. The medium was changed daily thereafter. When the cells became confluent they were passaged as follows. The medium was removed and the monolayer rinsed twice with 10ml of hepes saline (HS) and then 5mls of trypsin/versene [5 ml of trypsin (0.25%) in HS (section 6.8-2)+20 ml of versene] (T/V) was added. After approximately 5 min the cells started detaching from the flasks. To stop further effects of T/V, 10ml of growth medium was added to the flasks. The cell suspension was collected in a sterile universal container and then trituated using a pasteur pipette until a single cell suspension was obtained. Cells were counted with a haemocytometer and added to 75cm^2 tissue culture flasks at a concentration of 4 million cells per flask. Each flask contained 20ml of growth medium

(6.8-1) and was maintained at 37°C in a 5 % $C0_2$, 95 % air, humidified atmosphere. Cells were used at confluence approximately one week later.

6.3 Binding studies

6.3-1 Plasma membrane preparation

3 flasks ($75cm^2$) of confluent cells were used for each experiment (Fig.7). Cells were scraped from the flasks using a cell scraper (Costar , Cambridge, MA.), pelleted by centrifugation at 2000 rpm for 5min at 10°C and then resuspended in ice-cold 250 mM sucrose (5ml). They were then homogenized (2x12sec) with a Polytron PTA 20S homogenizer (Kinetic, Denmark). The homogenate was then centrifuged (40 000 rpm for 1h at 4°C) to obtain the membrane fraction. The membrane was then resuspended in buffer Tris/NaCl containing(mM) : Tris (50), NaCl (150), KCl (3.6), $MgCl_2$ (1.8), $CaCl_2$ (5), pH 7.35, at 0°C. Membranes were stored on ice and used fresh on the day of preparation.

6.3-2 Protein estimation

Protein concentration was estimated using the method of Lowry *et al.*, (1951).

The following solutions were prepared:

A) 2% W/V Na_2CO_3 (anhydrous) in 0.1 NaOH

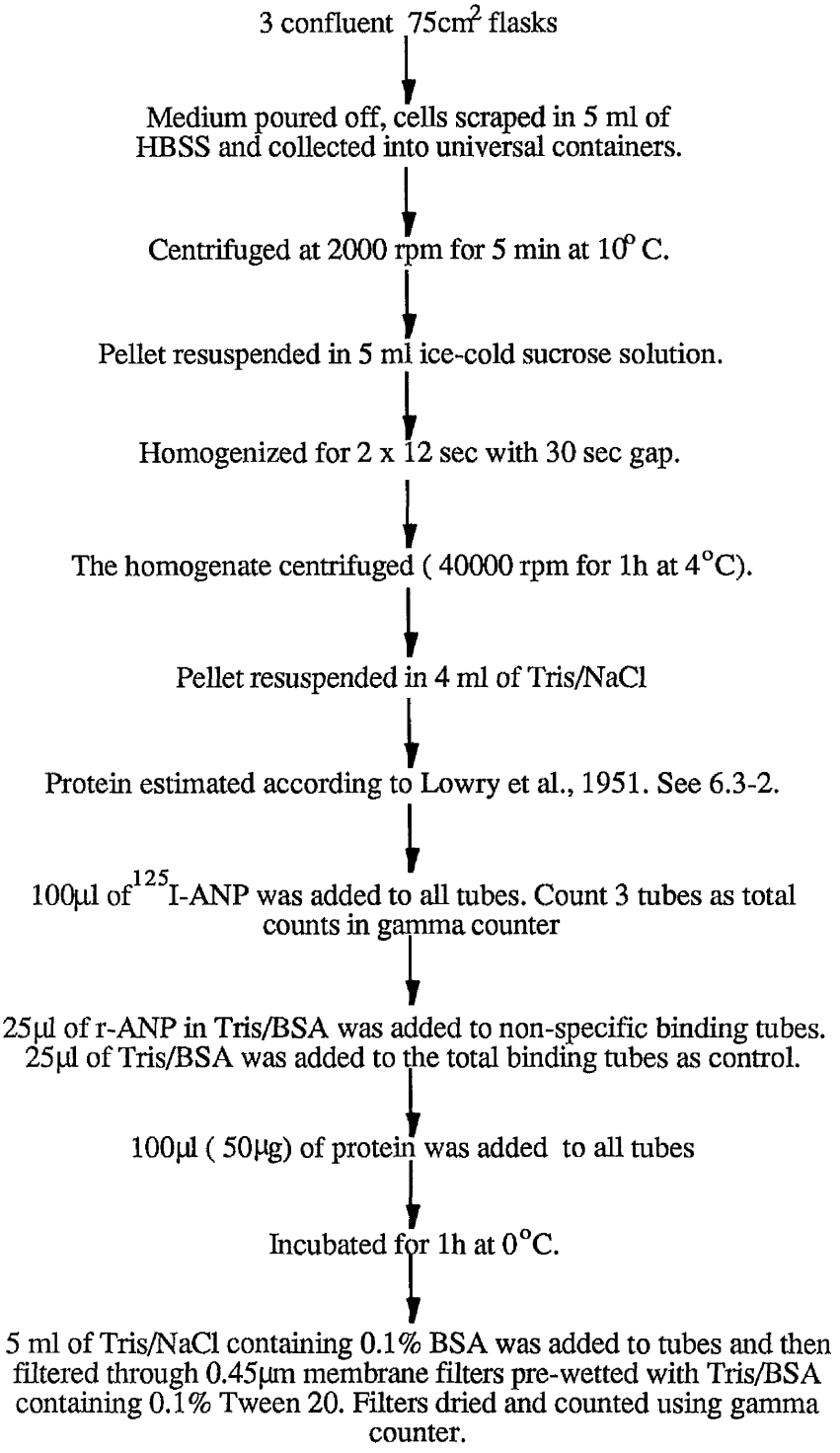
B1) 2% W/V Cu SO_4

B2) 4% NaK tartarate tetrahydrate in H_2O

C) 100ml of (A) was added to [2.5ml (B1)+2.5ml (B2)]

D) Folin (yellow) Ciocaltean's Phenol Reagent was prepared by adding 5ml of the stock solution to 3ml of distilled water. Bovine serum albumin (BSA) (0.1mg/ml-1mg/ml) dissolved in distilled water was used as a protein

Fig.7. The binding experiments



standard.

Procedure

200µl of each standard, membrane preparation or Tris/ NaCl (used as a blank) was added to plastic disposable tubes. 2.5ml of solution C was added to each tube, mixed, left for 10 min and then 250 µl of solution D was added, left 60 min at room temperature, and then the absorbance was read at 700nm using a Unicam SP 1800 Ultraviolet Spectrophotometer.

6.3-3 Binding conditions

Tubes contained membrane (100 µl, 50 µg of protein) in Tris/NaCl and 100 µl of ^{125}I-rANP (99-126) (7.8 pM-1 nM) in Tris/NaCl containing 1% bovine serum albumin (Tris/BSA) as a carrier. Non-specific binding was defined as radioactivity not displaceable by the addition (25 µl) of rANP (99-126) at a final concentration of 1 µM. Initial kinetic experiments were performed at 0°C, 22°C or 37°C but all subsequent experiments were performed at 0°C. Bound tracer was separated from free tracer by the addition of 5ml of ice-cold Tris/NaCl containing 0.1% BSA followed by immediate filtration through 0.45 µm membrane filters (Millipore HAWP) pre-wetted with Tris/BSA containing 0.1% Tween 20. The filters were washed with a further 5ml of filter buffer, dried and radioactivity counted in a Nuclear Enterprises 1612 gamma counter. Non-specific binding to the filters, estimated throughout the range of tracer used, amounted to less than 0.5% of total counts.

6.3-4 Tracer degradation

Membrane breakdown of ANP tracer during incubation was determined by high-performance liquid chromatography. After 1h the incubation mixture was centrifuged to pellet the membrane and the supernatant removed for analysis using a Gilson model 303 HPLC with a C18 ultrasphere ODS column (5µm, 4.6 cm x 25 cm), a gradient of 0-60% acetonitrile in 0.1%

trifluoroacetic acid and a flow of 0.5ml/min. The fractions collected were measured for radioactivity.

6.4 Catecholamine assay

Most phaeochromocytoma cells synthesise noradrenaline and rarely adrenaline as final catecholamine products (Kuchel, 1983). The release of noradrenaline from cultured PC12 cells under depolarizing conditions has been documented (Shalaby *et al.*, 1984) (Kongsamut and Miller, 1986), while Melega and Howard, (1984) Nagatsu *et al.*, (1987) and Ahnert-Hilger and Gratzl, (1987) have reported the release of dopamine from cultured PC12 cells. This present study was to find out whether this clone of cultured PC12 cells secreted adrenaline, noradrenaline or dopamine.

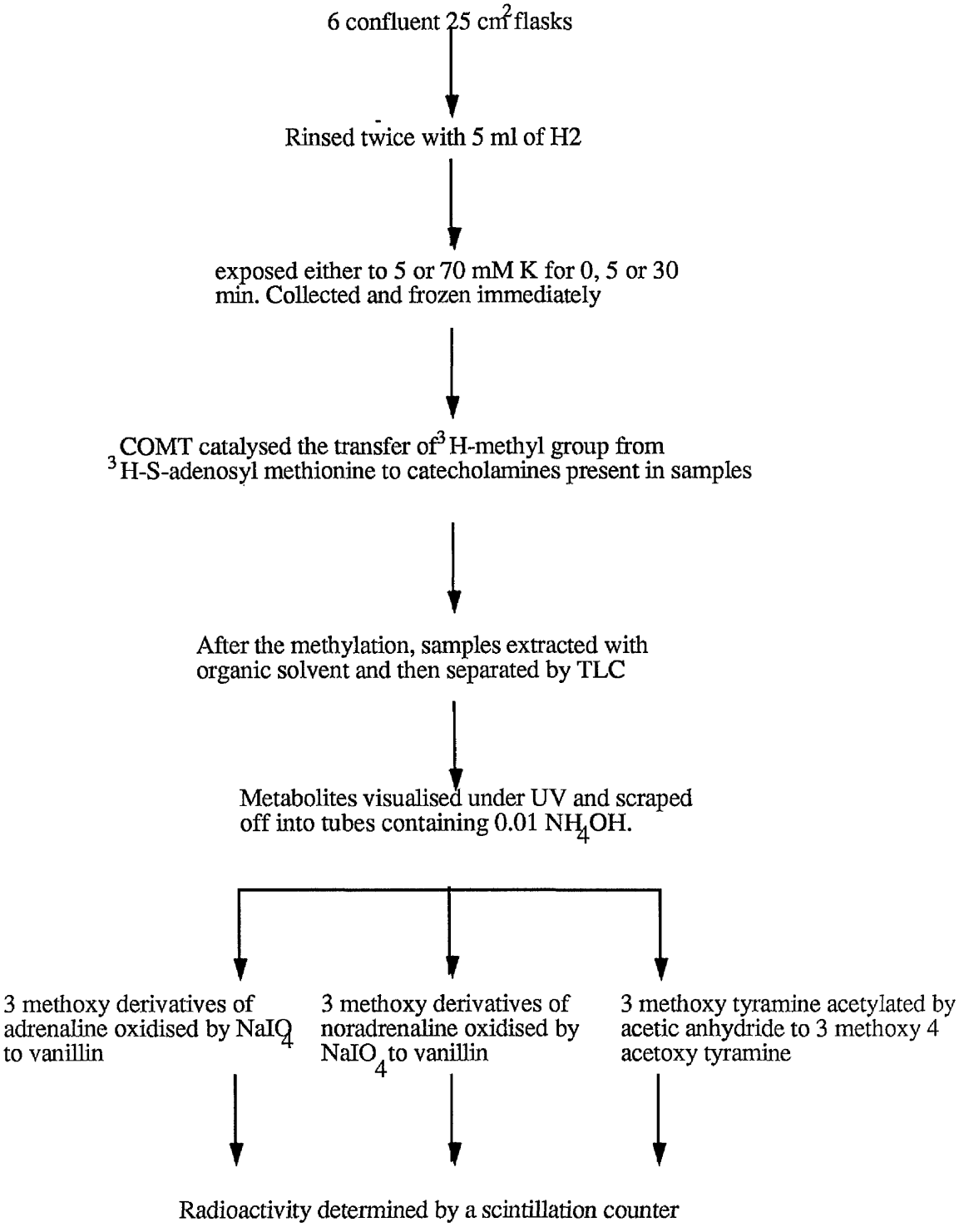
6.4-1 Method

6 flasks ($25cm^2$) of confluent PC12 cells were used for the experiment (Fig.8). Flasks were rinsed twice with H2 medium and then exposed to either 5 or 70 mM K^+ for either 0, 5 or 30 min. The solutions were decanted and frozen immediately at -70 °C.

A radio-enzymatic assay (Ball *et al.*, 1981) was used to identify the catecholamines released. With this method the enzyme Catechol-O-Methyl Transferase (COMT) catalyses the transfer of the ^3H-methyl group from ^3H-S-adenosyl-methionine (^3H-SAM) to catecholamines (adrenaline, noradrenaline and dopamine) present in the sample. The 3-methoxy derivatives formed were thus labelled with a methyl group.

After methylation the samples were extracted with an organic solvent and then separated by thin layer chromatography (TLC). The metabolites were visualised under UV light and scraped off into a tube containing 0.01 M NH_4OH to extract the samples from the silica. The 3 methoxy derivatives of

Fig.8. Catecholamine assay



noradrenaline and adrenaline were then oxidised to vanillin and the 3 methoxy tyramine acetylated to facilitate their uptake into scintillation fluid. The radioactivity in the samples was then determined in a scintillation counter.

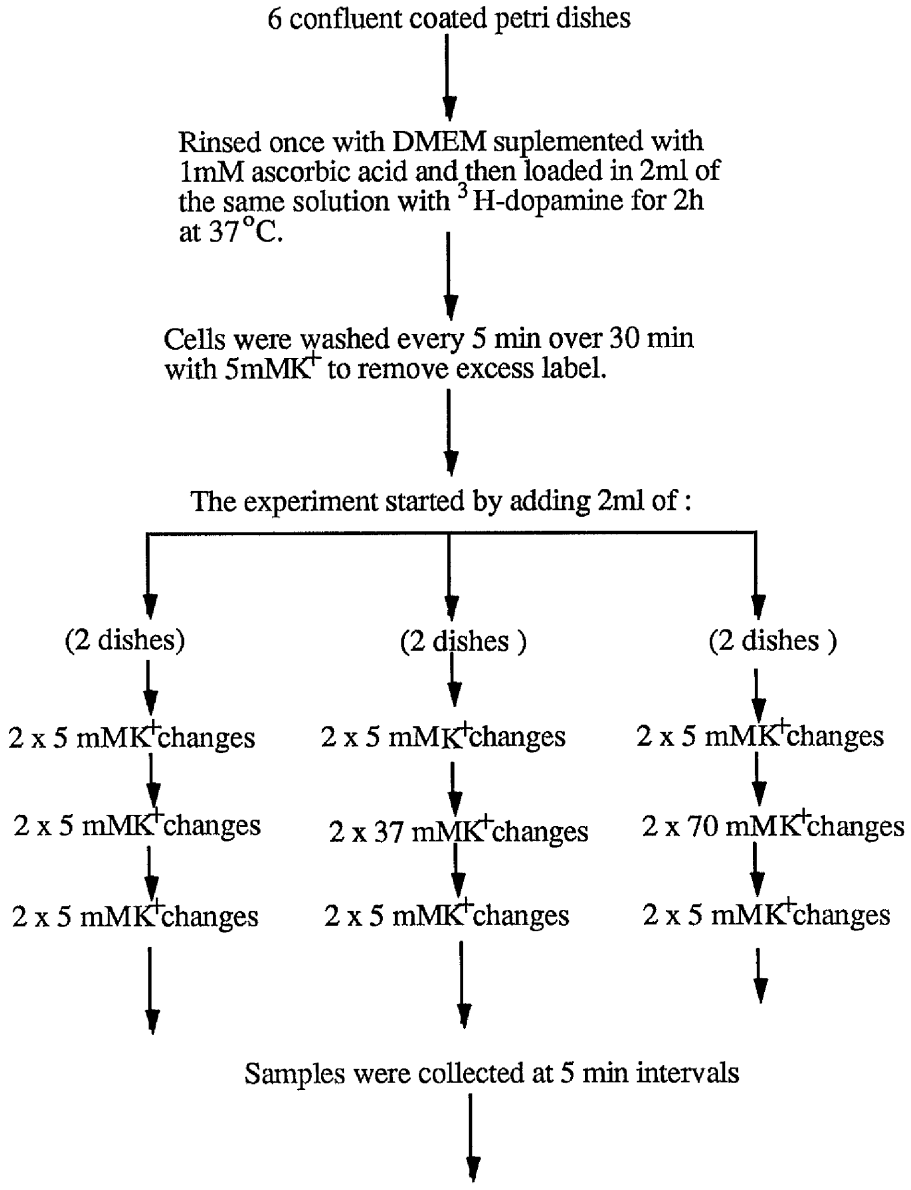
6.5 Effect of ANP on ^{3}H-Dopamine release from PC12 cells

Having identified ANP receptors on PC12 cells, and that the cells secreted dopamine, it was of interest to test the hypothesis that ANP might act directly to inhibit catecholamine release. Two methods were used. The dish method had the advantage of simplicity, whereas the perfusion method reduced the artefacts associated with solution changes, and has the potential to achieve better time resolution.

6.5-1 Dish method

These experiments were performed using a method modified from Shalaby *et al.*, (1984), Kongsamut and Miller, (1986) and Ahnert-Hilger and Gratzl, (1987) (Fig.9). Cells were plated in petri-dishes (60 x 15 mm) treated with Gelatin/Poly-L-Lysine [20mls of 2% gelatin+2mls of poly-L-lysine (10 µg/ml working solution)] at a concentration of 1 million per dish and grown in the standard PC12 cell culture medium (section 6.8-1). Cells were used at confluence approximately 3-4 days later. Cells were washed once with DMEM, supplemented with 1mM ascorbic acid as an anti-oxidant, and then loaded with (2,5,6 ^{3}H-dopamine (0.1µ Ci/ µl) at a concentation of 3 µM for 2h in 2 mls of DMEM supplemented with 1mM ascorbic acid. This was followed by a wash period whereby for a total of 30 minutes a 2ml solution containing 5mM K^+ (composition 6.8-5) was added to the cell monolayer, left 5min, removed and replaced with fresh solution. For the experimental period, six further solution changes were made at 5 min intervals. To study the effect of increasing the concentration of K^+ on ^{3}H-Dopamine release, control dishes received 5mM K for all 6 samples and experimental dishes

Fig.9. Dish method.



At the end of each 30 min 2 ml of lauryl sulphate was added to each dish, collected into vials and all vials counted using in a scintillation counter

received either 37mM K^+ or 70mM K^+ (composition section 6.8-6—6.8-7) in the third and fourth sample followed by 5mM K^+ for the remaining samples, as for the control. The 2ml samples, after being removed from the cells were saved, and radioactivity in them counted in a scintillation counter. At the end of each experiment cells were still attached to the dishes and 2ml of lauryl sulphate (0.2%) was added to each dish to determine remaining intracellular ^{3}H-dopamine. For each experiment the release of ^{3}H-dopamine from the cells is expressed as a percentage of radioactivity present in the cells prior to the paticular release period. These experiments were carried out at 37°C in duplicate. The three different concentrations of potassium were used because of the difference in their ability to depolarize these cells (Hodgkin and Huxley, 1952).

Even though much care was taken not to disturb the attached cells when exchanging the solutions (5, 37 or 70 mM K^+) a few cells still came off especially from the edges. This might affect the radioactivity measured in the supernatant.

6.5-2 Perfusion system (Fig.10).

Each experiment used 2 million PC12 cells in suspension (Fig.11). To avoid risk of proteolytic damage to ANP receptors through trypsinisation, cells were left in 10 ml of normal medium at room temperature for 5-10 min until they detached from their culture dishes and triturated to produce a single cell suspension. Cells were then loaded with ^{3}H-dopamine for 2h as described above. They were then loaded onto Whatman GF/B Glass microfibre filter paper. A wash period followed whereby for a total of 30 min cells were washed with 5 mM K^+ solution and then perfused with 5 mM K^+ or 70 mM K^+ solutions using the following modified method of Whitton *et al.*,

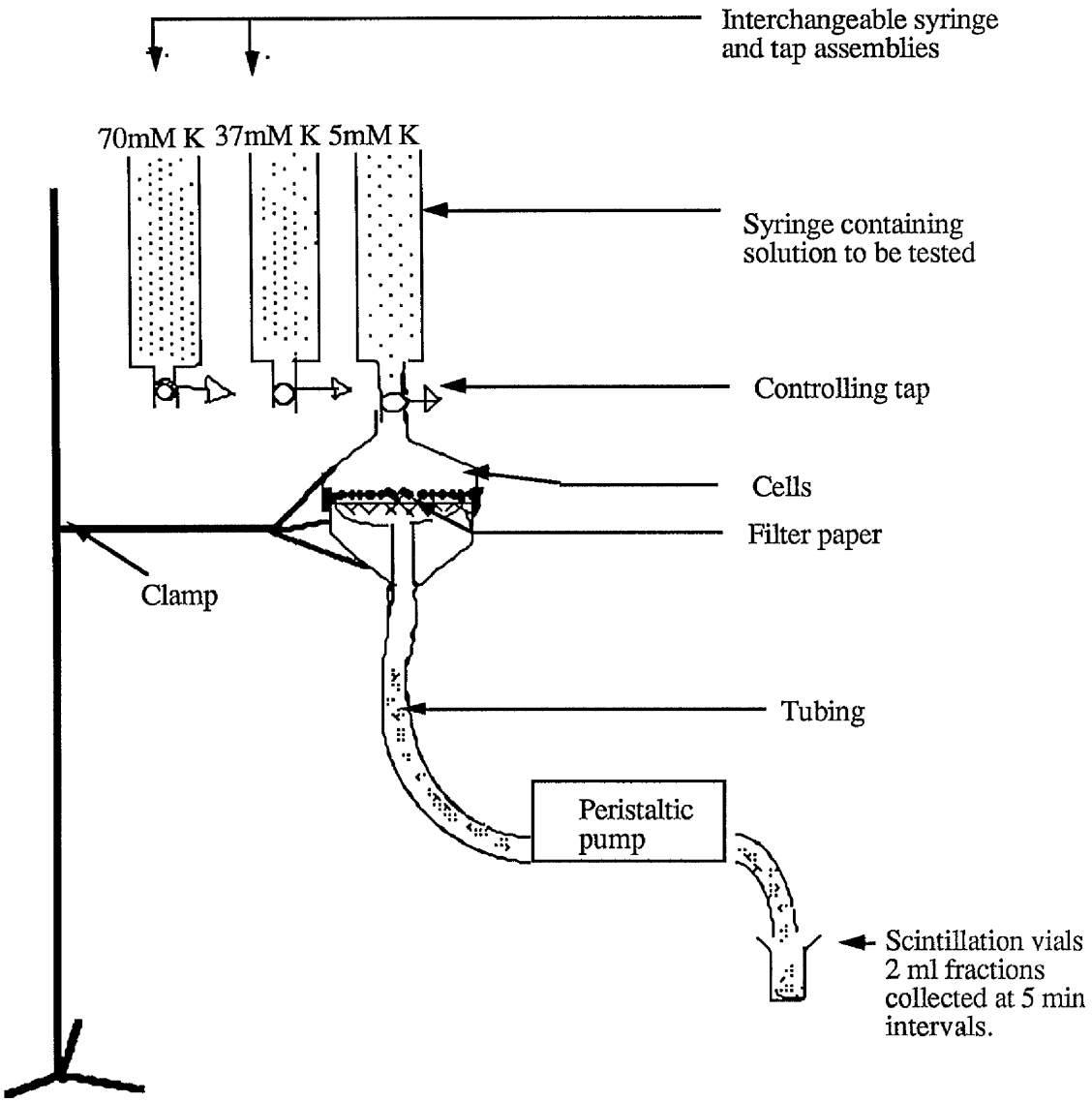
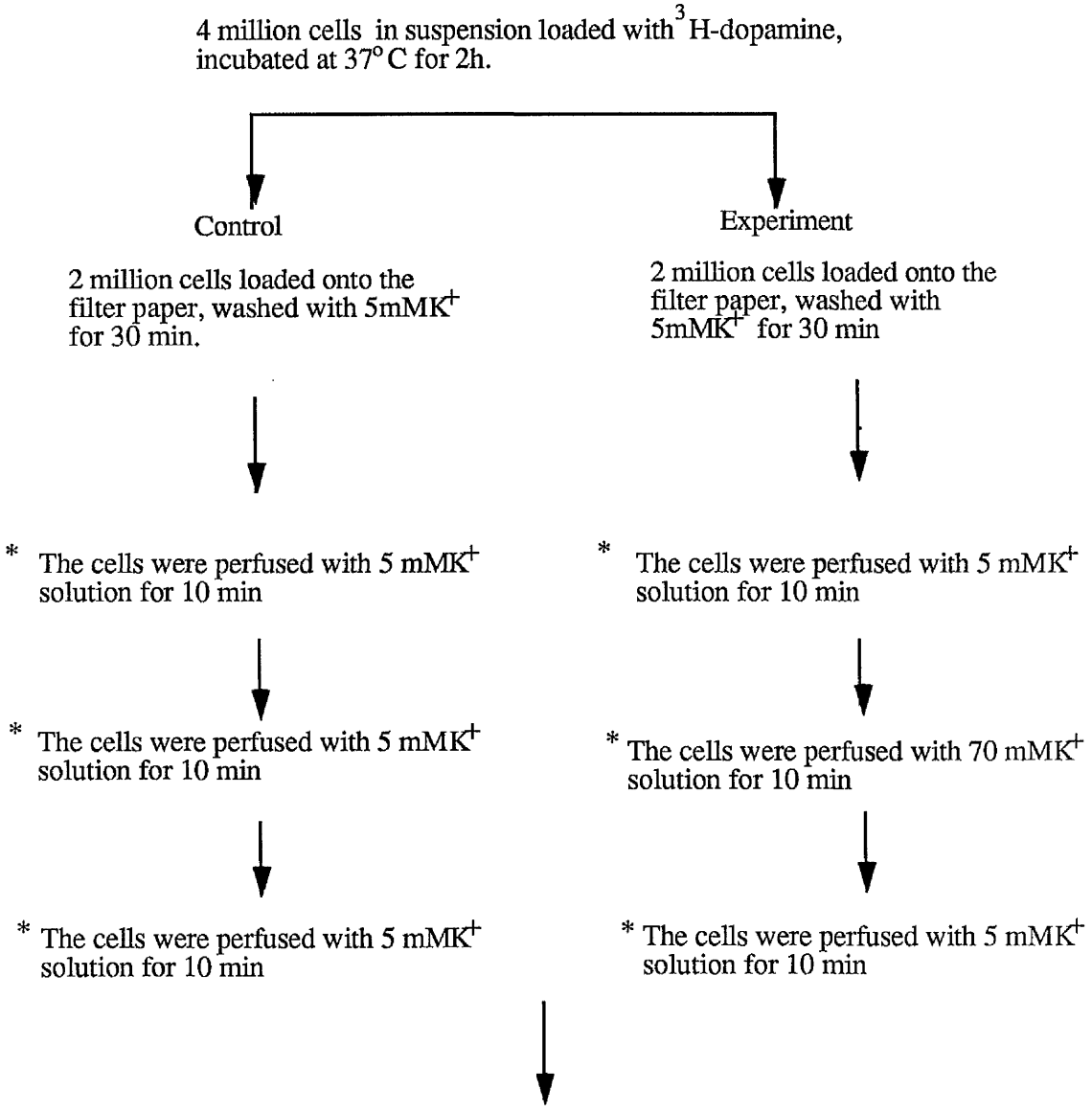


Fig.10. The perfusion system

Fig.11. The perfusion method



* For ANP modulation experiments, ANP was added at final concentration of 10^{-5} M to all perfusion solutions.

(1988). The filter paper was held in a clamped 10 mm diameter filter holder (Swinnex filters) and a 5 ml syringe with tap was attached to the filter to allow washing with defined solutions (Fig.10). After the wash period, the pump was stopped, the syringe removed and a prepared 5 ml syringe filled with 5 mM K^+ was replaced, the pump was restarted and the collection of the 2 ml of perfused solution started after the last drop of previous solution was pumped out. The same procedure was employed for solution changes with other syringes filled with 5 mM K^+ +ANP, 70 mM K^+ or 70 mM K^+ +ANP. 2ml samples were collected into scintillation vials (6 samples over 30 min) through a Microperpex (LKB) peristaltic pump. The syringe changing procedure took less than 1 min. To test the effect on ^{3}H-dopamine release from PC12 cells perfused with either 5 mM K^+ or 70 mM K^+ the following procedure was carried out. In the first experiment two parallel systems were set up whereby one set of cells was perfused with 5 mM K^+ for 30 min and the other with 5 mM K^+ containing a final concentration of 10^{-5}M ANP. ANP was used at 10^{-5}M because this was well above the K_d determined previously in the binding studies (nM range), receptors would be saturated and so any effects would be maximal. In a second set of two parallel experiments, cells were perfused for 10 min with 5 mM K^+, then for a further 10 min with 70 mM K^+ and finally for the last 10 min with 5 mM K^+. Again, one of the two experiments contained 10^{-5}M ANP throughout. 20ml of scintillation fluid was added to each vial and then radioactivity in the samples was determined in a scintillation counter.

The delay calculated between the changes of solutions in this perfusion system was less than 1 min in total, in other words the tubing system was short to reduce the lengh of time used by the solutions reaching the cells on

the filter and moving along the tubing to the vials. The volume of the filter and tubing downstream of the cells was <1 ml .

6.6 ANP secretion

Recently Ong *et al.*, (1987) have demonstrated the co-existence of ANP (99-126) and its precursors within the bovine chromaffin granules. These investigators have comfirmed the maturation process of atrial peptides in the adrenal medulla.

The presence of ANP in the adrenal medulla suggested the question as to whether these PC12 cells secrete ANP or not.

For this assay, several methods were used ;

1) PC12 cells were left in 10 ml of normal medium at room temperature until they detached from their culture flasks (Fig.12). 2 million cells were then loaded onto Whatman GF/BG Glass micro filter paper and perfused with 2 different concentrations of potassium (5 or 70mM K^+) using the same modified method of Whitton *et al.*, (1988) described in section 6.5-2. 2 ml samples were collected into scintillation vials dipped in liquid nitrogen to freeze them immediately six samples were taken at 5 min intervals and tested for ANP secretion.

2) Six (25 cm^2) coated confluent flasks were used (Fig.13). The culture medium was removed and cells were rinced twice with H2 at 37 °C. 2ml of 5mM K^+ were added to 2 flasks as a control, 2ml of 70mM K^+ were added to 2 flasks and 2 ml of the same medium supplemented with protease inhibitors were added to the other 2 flasks. The six flasks were incubated for 5 min at 37 °C; the medium was then collected and frozen immediately in liquid nitrogen. The samples were then tested for ANP secretion.

3) The supernatant prepared for renin assay (section 6.7-1) was also assayed for ANP presence in PC12 cells.

Fig.12. Perfused PC12 cells

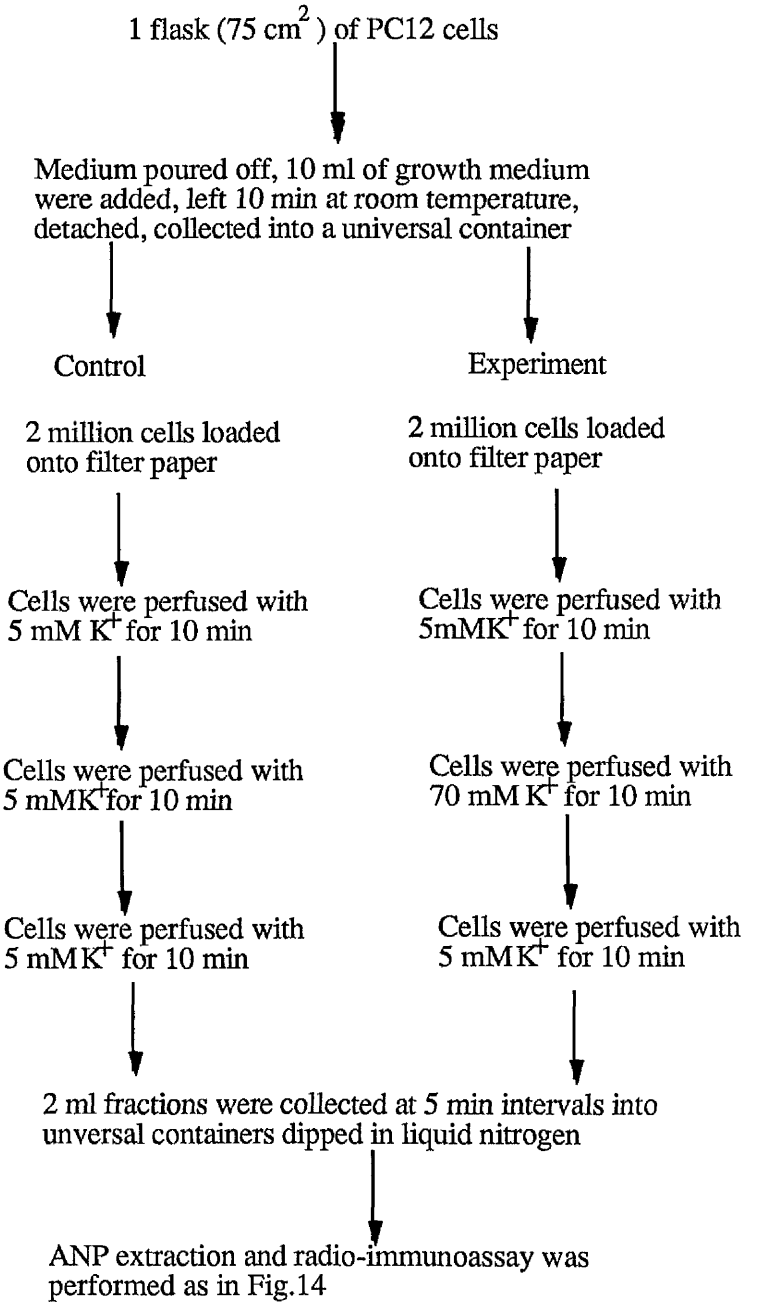
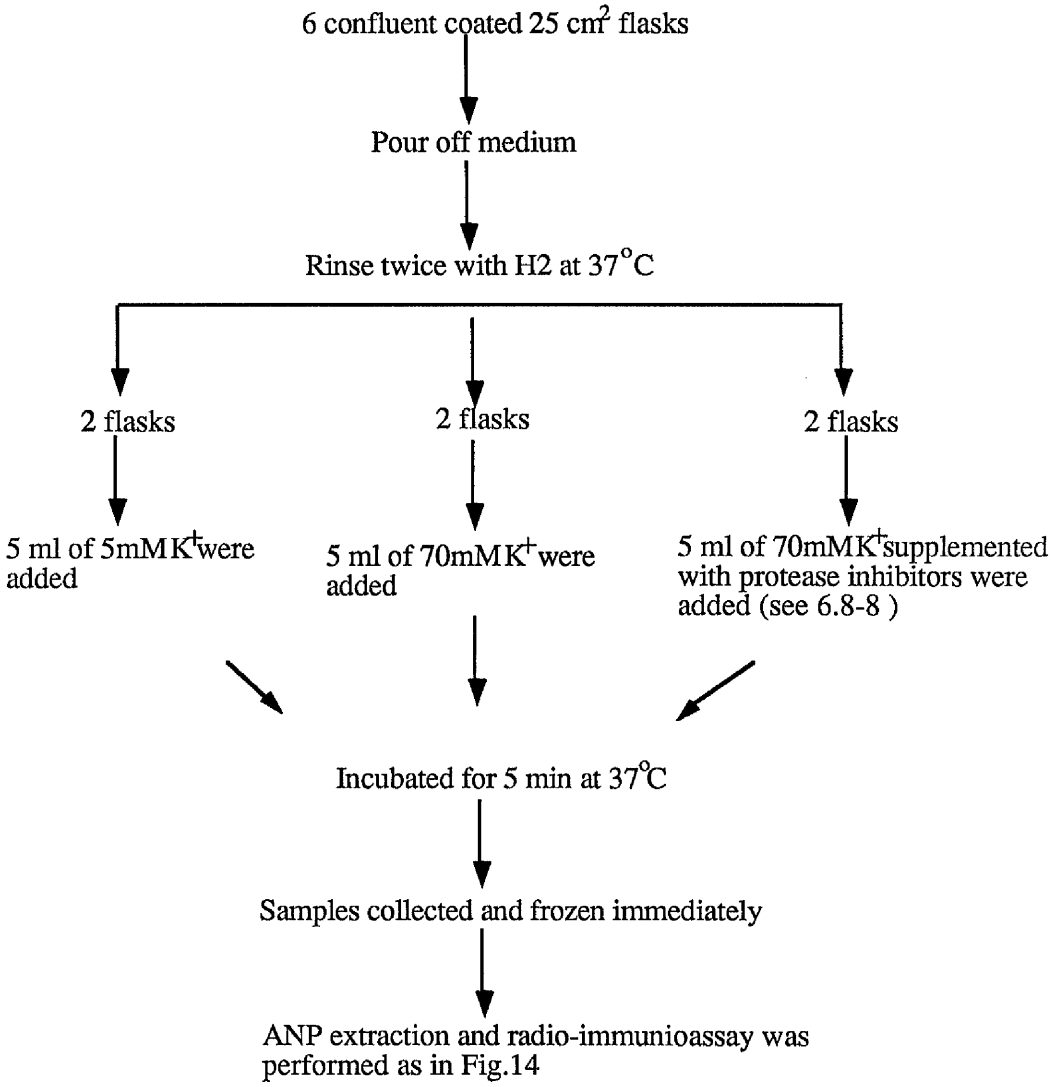


Fig.13. Flask method



6.6-1 ANP extraction

All these three preparations were assayed according to Richards *et al.*, (1987). Samples were acidified by adding each 1 ml of sample to 0.25 ml of 2N HCl. The acidified samples were centrifuged (2000 rpm, at 4°C for 10 min).

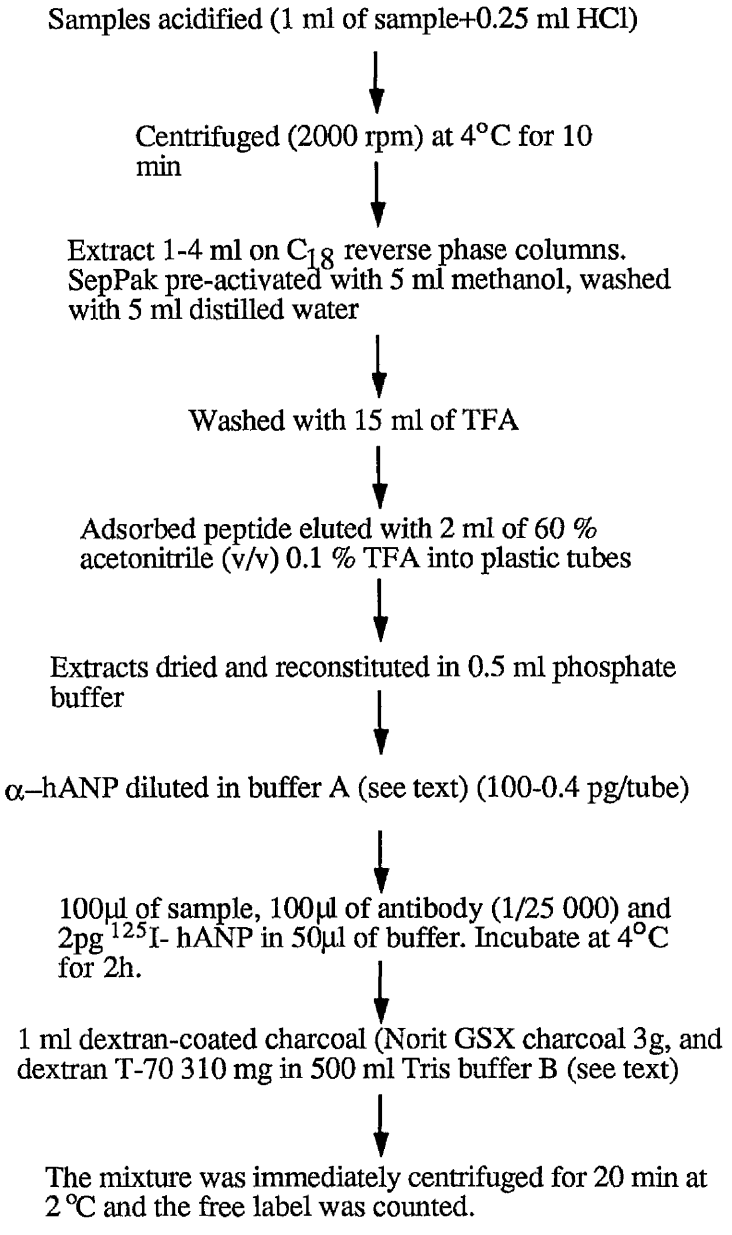
The extraction of ANP from 1-4 ml acidified samples took place on C_{18} reverse phase columns (Sep-Pak Waters Associates). Sep-Paks were pre-activated with 5 ml methanol and washed with 5 ml distilled water prior to application of the acidified sample.

A wash period followed where 15 ml of trifluoroacetic acid (TFA, 3 x 5 ml) were run through and the adsorbed peptide was eluted with 2 ml of 60% acetonitrile (v/v) 0.1% TFA into plastic tubes. The extracts were dried under compressed air and reconstituted in 0.5 ml buffer (Fig.14).

6.6-2 ANP radio-immunoassay

Synthetic α-hANP was diluted (100—0.4 pg/tube) in buffer A (100 mM sodium phosphate pH 7.4 containing 50 mM NaCl, 0.1 % w/v bovine serum albumin, 0.1 % w/v Triton x-100 and 50 kallikrein inhibitor units/ml trasylol). For extracted methods, 100 µl of the sample (standard solution, or reconstituted plasma extract) 100 µl of the ready prepared antibody at a final dilution of 1/25 000 and 2 pg ^{125}I- α-hANP in 50 µl of the buffer, were incubated at 4 °C for 24h. All assays were set up in duplicate. Separation of free and bound ligand was achieved by mixing with 1 ml dextran-coated charcoal (Norit GSX charcoal 3g, and dextran T-70 310mg in 500 ml Tris/buffer B: 50mM Tris/HCl pH 7.4. 5mM EDTA, 0.1% BSA). The mixture was immediately centrifuged for 20 min at 2°C and the free label was counted (Fig.14).

Fig.14. ANP extraction



6.7 Measurement of intracellular renin in PC12 cells in culture

Patients with hypertension caused by an excess of circulating catecholamines released by phaeochromocytoma have been reported to show increased plasma renin activities and aldosterone levels (Vetter, 1975). For such reasons these rat PC12 cells were tested for their ability to secrete renin.

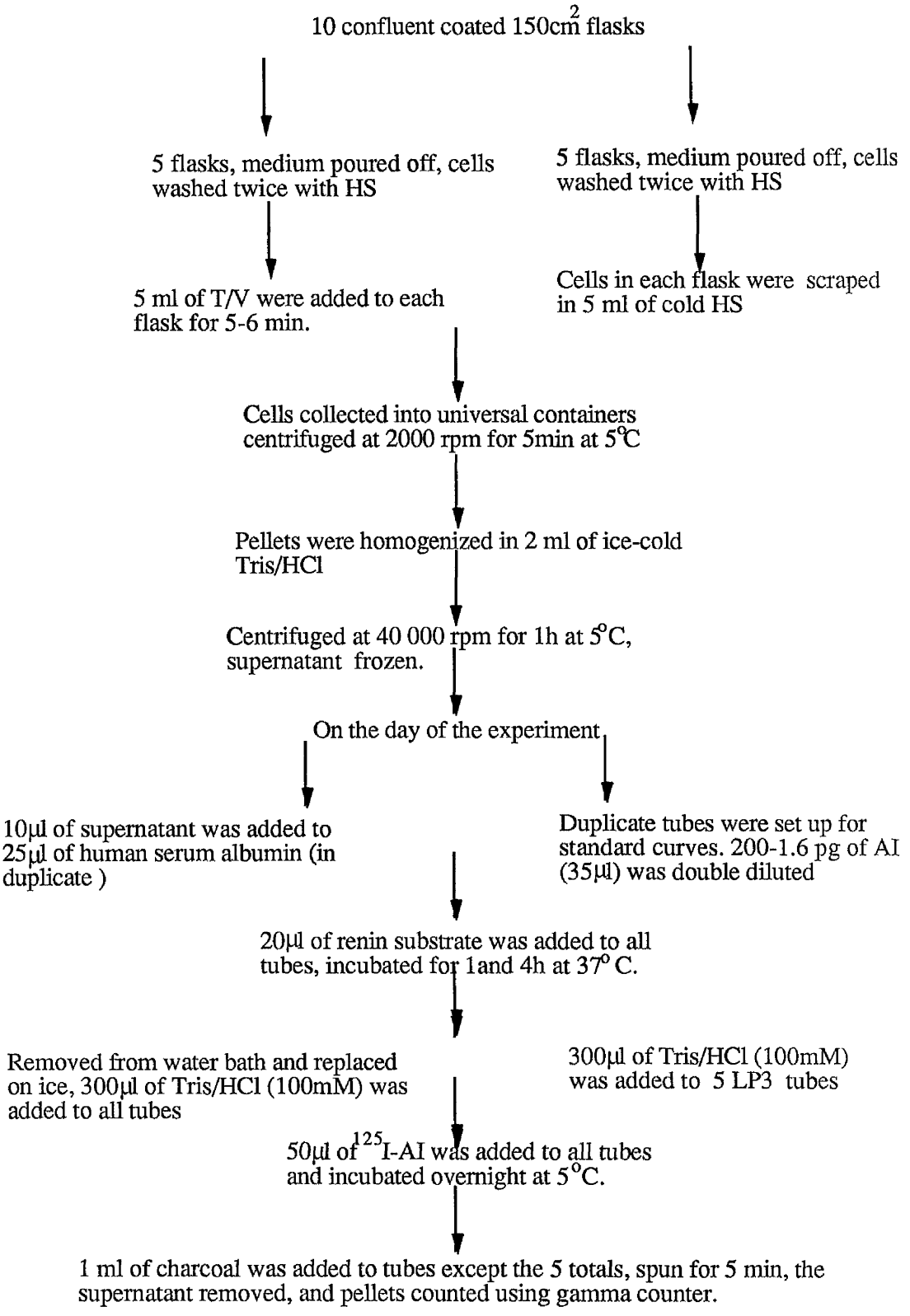
6.7-1 Cell preparation

PC12 cells were cultured as described earlier. Ten (150 cm^2) confluent collagen-coated flasks were used for the experiment (Fig.15). Half of the cells were trypsinized since trypsin is known to activate renin. The other half of the cells were scraped from the flasks. The medium was poured off and the monolayers then washed twice with HS (2x10ml). Any remaining traces of HS were removed using a pasteur pipette. A further 5ml of either cold HS or trypsin were then added to each flask. Half of the cells were scraped from the flasks using a cell scraper (Costar, Cambridge, MA). Trypsinized and non-trypsinised cells were collected into universal containers and centrifuged at 200g for 5min at 5°C. Two ml ice cold Tris/HCl (100 mM) pH 7.4 was then added to each pellet. The pellets were then homogenized (2x10 sec) with a polytron PTA20S homogenizer (Kinematic, Denmark), care being taken to keep the preparation cold and pelleted by centrifugation at 40 000 rpm for 1h at 5°C. The two supernatants were removed and frozen immediately at -70 °C until the renin radioimmunoassay was carried out .

6.7-2 Renin assay

Renin was assayed by an indirect radioimmunoassay (Morton and Wallace, 1983) as follows. Renin samples were incubated with renin substrate (angiotensinogen) for predetermined times. At the end of the incubation the reaction was stopped by placing tubes on ice and the AI generated was

Fig.15. Renin assay



detected by standard radioimmunoassay.

On the day of the experiment, duplicate samples of the stored supernatant (10 µl) were added to 25 µl of human serum albumin (HSA) and 20 µl of ready prepared angiotensin I antiserum (981/7 diluted 1:80 in 3M Tris/HCl pH 7) and rat renin substrate in a ratio of 1: 6. The mixture was then centrifuged for 5 min at 5°C and incubated at 37°C for various times (0, 1 and 5h). Duplicate control samples were kept on ice and these were called time 0 samples. Standard curves (Fig. 16) were set up as follows. Duplicate tubes contained 200-1.6 pg of A I (35 µl). To these tubes 20 µl of A I antiserum/ renin substrate was added and then the tubes were incubated for the same length of time as the renin samples. At the end of the required incubation period samples were removed from the water-bath, and 300 µl of ice cold Tris (100 mM) pH 7.4 was added to stop further renin activity.

50 µl (5pg) of ^{125}I-A I was added to all tubes. Samples were mixed and then incubated at 5°C overnight. 1ml of charcoal was added to each sample to bind free ^{125}I-A I, and then the samples were spun for 5 min at 5°C to pellet the charcoal. The supernatant was then removed carefully and the charcoal pellets were counted using a gamma counter (Nuclear Enterprises 1612).

6.8 Media recepes

6.8-1 Growth medium

	ml
Eagle's water	134
x 10 concentrated GMEM	16
7.5% bicarbonate sodium	5
*Antibiotics (GPSA)	5
Horse serum	20
Foetal calf serum	20

	Final concentration in the growth medium.
*Glutamine	2.85 mM
*Penicillin	47.6 units/ml
*Streptomycin	27.3 µg/ml
*Amphotericin B	0.29 µg/ml

6.8-2 Calcium and magesium free Hepes Saline (HS)

	g/l	mM
NaCl	8	140
KCl	0.4	5
Glucose	1	5
Hepes	2.388	10
Phenol red (0.5%) add 2ml/l		0.003

pH to 7.4 with 5M NaOH

Sterilised by autoclaving at 121 °C for 20 min and stored at 4 °C

6.8-3 Balanced salts solution (Hank's Hepes,H2)

	g/l	mM
NaCl	8	140
KCl	0.4	5
$CaCl_2$	0.14	1
$MgCl_2$	0.2	1
D-Glucose	1	5
Hepes	2.388	10
Phenol red (0.5%) add 2ml/l		0.003

pH to 7.4 with 5M NaOH.

Sterilised by autoclaving at 121 °C for 20 min and stored at 4 °C

6.8-4 Versene

	g/l	mM
NaCl	8	140
KCl	0.2	2.5
Na_2HPO_4	1.15	8
KH_2PO_4	0.2	1.5
EDTA (di-sodium salt)	0.2	0.5
0.5 phenol red: add 2 ml /l		0.003

Sterilised by autoclaving at 121 °C for 20 min and stored at 4 °C

6.8-5 5mM K^+ solution

Reagent	g/l	mM
Choline chloride	11.97	86.1
NaCl	2.92	50
KCl	0.37	5
$CaCl_2$	0.14	1.3
KH_2PO_4	0.05	0.44
Na_2HPO_4	0.04	0.34
$NaHCO_3$	0.22	2.62
$MgSO_4$	0.09	0.81
Ascorbate	0.17	1
Pargyline	0.015	0.1
Desmethylimipramine	0.019	0.1
Glucose	1.008	5.6
Hepes	4.7	20

0.5 % Phenol red add 2ml/l		0.003

Adjust the pH to 7.4 with HCl

6.8-6 70mM K^+ solution

As above except for :	g/l	mM
Choline chloride	21.1	2.93
KCl	70	5.21

6.8-7 37mM K^+ solution Made up as required as 1:1 dilution of 5mM K^+ and 70mM K^+ solutions.

6.8-8 The secretion medium:

For ANP secretion assay these proteases were added at the final concentration shown below to the 70mM K solution.

	g/l	mM
Aprotonin	0.0065	0.001
Leupeptin	0.00047	0.001
Pepstatin A	0.00068	0.001
1,10 Phenanthroline	0.018	0.01

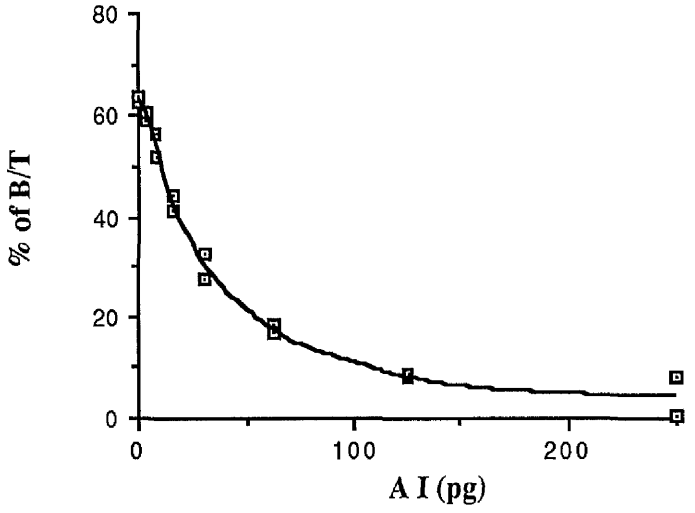


Fig.16. Renin standard curve for 1h.

Results

7. Characterization of the PC12 cell ANP receptor

In this section a characterization of the rat PC12 cell ANP receptor is described. A comparison of the results of this study with those already published for other ANP adrenal receptors is included.

7.1 Specific binding as function of membrane protein concentration

The binding of ^{125}I-labeled ANP to the PC12 membrane preparation increased with increasing concentrations of protein, and remained linear up to 100 µg/100 µl (Fig.17). These experiments were carried out on ice for 60 min.

7.2 Kinetics of specific binding

Specific binding of ^{125}I-ANP to the membrane fraction was time and temperature dependent. 100 µl (50 µg protein of the PC12 cell membrane preparation was incubated with 100 µl of ^{125}I-labeled ANP (0.2nM) and 25 µl of either ANP (final concentration 1 µM) or Tris/BSA for increasing times up to 2h. At 0°C there was a progressive increase in binding with time, plateauing after 45 min (Fig.18 a). In contrast at 22°C, binding was maximal by 30 min and declined rapidly thereafter (Fig.18 b). At 37°C binding was very rapid, but unstable (Fig.18 c).

7.3 Degradation of ^{125}I-ANP

Membrane degradation of r-ANP tracer during the incubation was determined by high performance liquid chromatography (HPLC). HPLC showed marked degradation of the tracer in the presence of membrane at 22°C and 37°C (14% and 3.6% remaining intact after 1 hour respectively); whereas at 0°C over

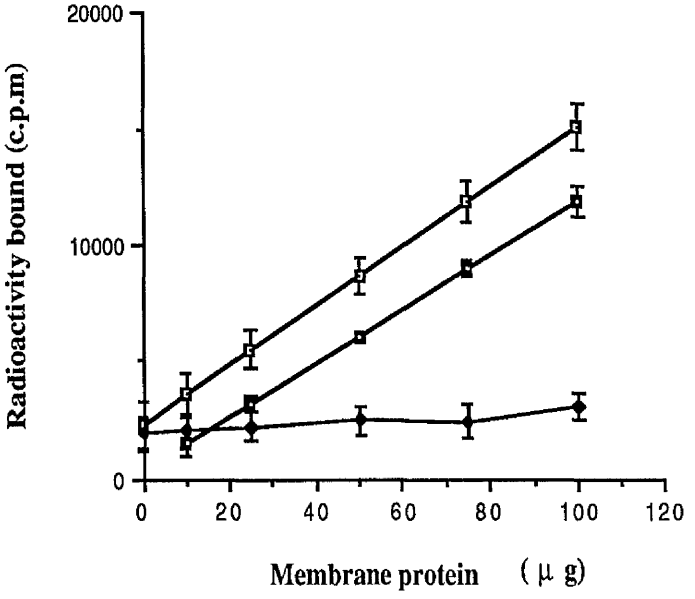
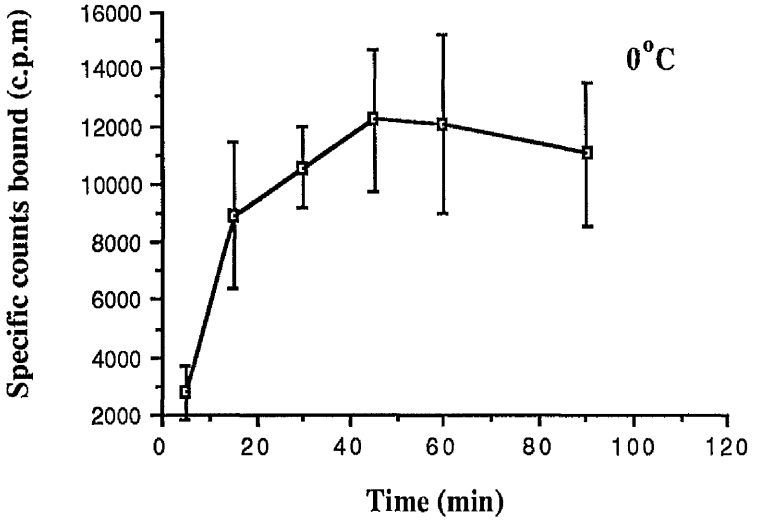
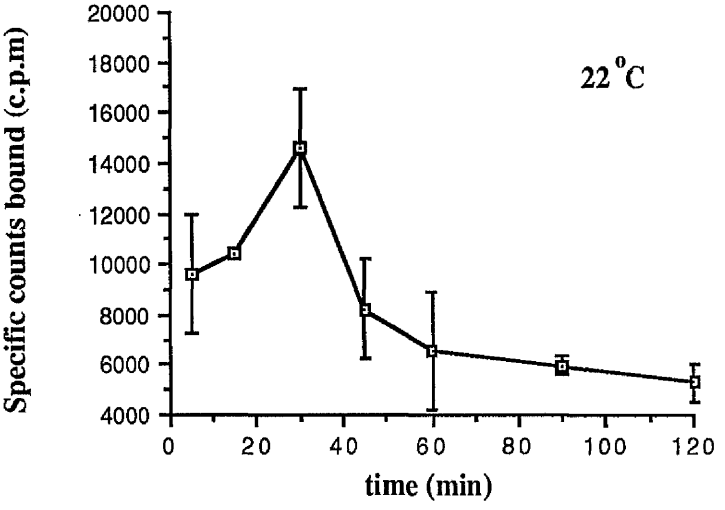


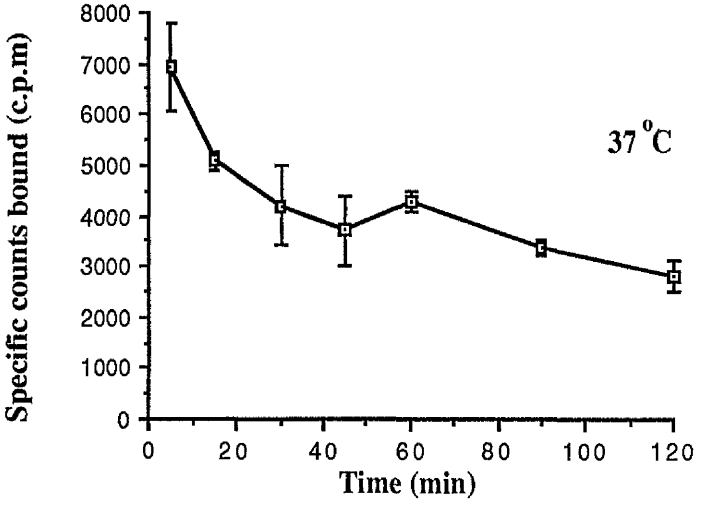
Fig.17: Binding of ^{125}I-rANP (99-126) to a plasma membrane preparation as a function of membrane protein concentration. Incubation was for 60 min at 0 °C. Points are the mean ± SE of 4 separate experiments each performed in triplicate. Empty squares denote total binding; filled squares specific binding; dots, nonspecific binding.

Fig.18: Specific binding of ^{125}I-rANP (99-126) to a plasma membrane preparation as a function of time at 0°C, 22°C and 37°C.

Points are the mean ± SE of 4 separate experiments each performed in triplicate.


Specific counts bound (c.p.m)
16000
14000
12000
10000
8000
6000
4000
2000
0
20
40
60
80
100
120
Time (min)
0°C


Specific counts bound (c.p.m)
20000
18000
16000
14000
12000
10000
8000
6000
4000
0
20
40
60
80
100
120
time (min)
22°C


Specific counts bound (c.p.m)
8000
7000
6000
5000
4000
3000
2000
1000
0
0
20
40
60
80
100
120
Time (min)
37°C

70% remained intact compared with ANP which had not been incubated with membrane (90% intact) (Fig.19). Subsequent experiments were therefore carried out at 0°C.

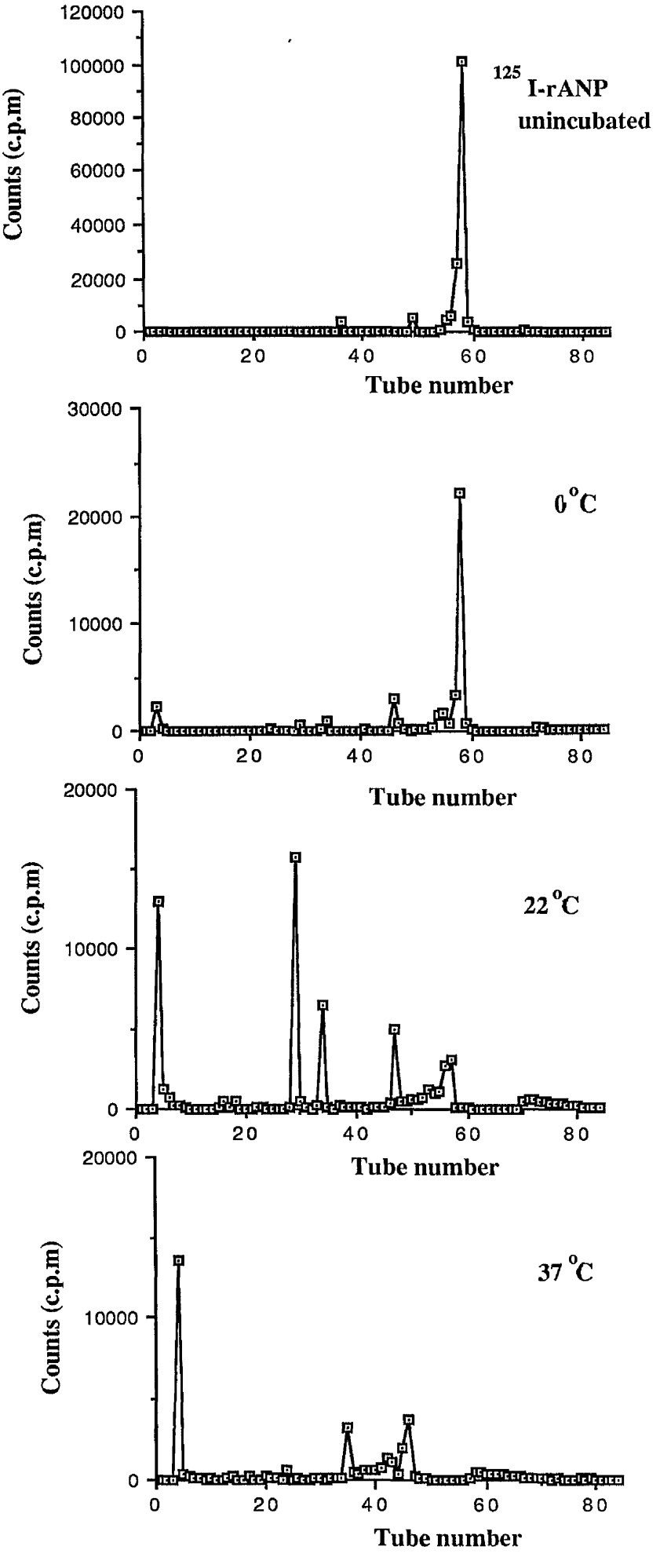
7.4 Saturability of specific binding

Binding of ^{125}I-labeled ANP to the PC12 membrane preparation with increasing ligand concentration was then studied. 100 µl of membrane preparation (50 µg of protein) was incubated at 0°C for 60 min in tubes containing either 25 µl of ANP (1 µM) or Tris/BSA and 100 µl of ^{125}I-rANP (7.8 pM-1 nM). Binding increased with increasing ligand concentration and the Scatchard transformation of the data is shown in Fig.20. From the plot a dissociation equilibrium constant (K_d) of 794 pM and receptor number (B_{max}) of 254 fmol/mg protein was calculated.

7.5 Specificity of binding

The specificity of the ANP binding sites for ANP-related and unrelated peptides was examined in competitive binding experiments. ANP nomenclature is based on the sequence of the prepropeptide starting at the end of the signal peptide (Oikawa *et al.*, 1984); thus α h-ANP (99-126)= human ANP (28 amino acid), rANP fragment (111-126)= rat atrial peptide fragment (13-28), ANP fragment (99-109)= rat atrial peptide fragment (1-11). As shown (Fig.21), unrelated peptides such as angiotensin II, arginine-8-vasopressin and bradykinin did not compete for the ANP-binding sites. Of the ANP-related peptides which competed for the receptor, the following order of affinity was established; rat atrial peptide fragment 13-28 > rANP (99-126) > hANP > rat atrial peptide fragment 8-33 > rat atrial peptide fragment 1-11 >atriopeptin I.

Fig.19: HPLC profile of pure ^{125}I-ANP (99-126), and supernatant after incubation of ^{125}I-ANP (99-126) with membrane for 1h at 0°C, 22°C and 37°C.


120000
100000
80000
60000
40000
20000
0
Counts (c.p.m)
125 I-rANP
unincubated
0
20
40
60
80
Tube number
30000
20000
10000
0
Counts (c.p.m)
0 °C
0
20
40
60
80
Tube number
20000
10000
0
Counts (c.p.m)
22 °C
0
20
40
60
80
Tube number
20000
10000
0
Counts (c.p.m)
37 °C
0
20
40
60
80
Tube number

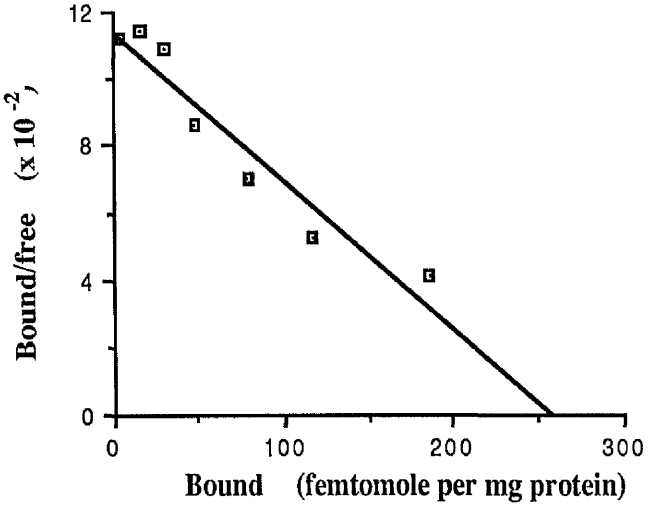


Fig.20. Scatchard plot.

Scatchard analysis of specific binding of ^{125}I-ANP (99-126) to membrane preparation as function of tracer concentration up to 2 nM. Points are mean of 3 separate experiments each performed in triplicate. Non-specific filter binding estimated throughout the range of tracer concentrations used amounted to less than 1 % of total counts.

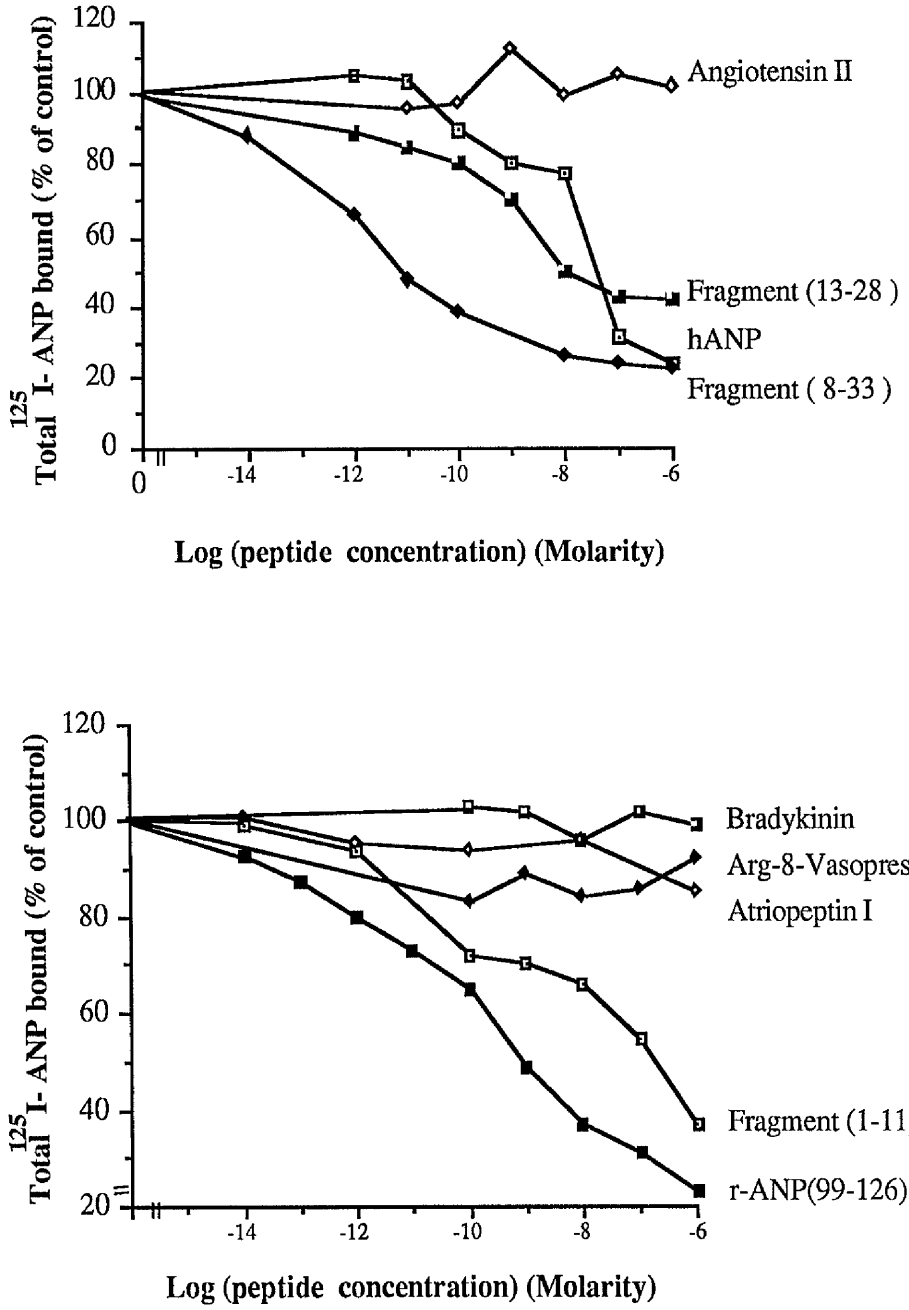


Fig.21: Competition for ANP (99-126) binding sites by ANP related and unrelated peptides.

^{125}I-ANP (99-126) (200pM) was incubated with membrane for 1h at 0°C in the presence of increasing concentrations of the appropriate peptide. Apart from r-ANP (99-126) which shows the mean of 9 seperate experiments, each plot represent the mean of two experiments carried out in triplicate.

Hill coefficients for ANP and its competitors were calculated as follows; rANP (99-126) 0.21; fragment (1-11) 0.21; fragment (13-28) 0.18; fragment (8-33) 0.36; hANP 0.13. Shallow competitive binding curves have previously been observed in ANP receptor binding studies (Lyall *et al.*, 1987) the significance of this is unclear.

6.6 Comparison with other studies

Many reports have been published which show that binding of ANP to its receptors on a number of target cells results in increasing levels of the second messenger cGMP (section 4.5).

Previous studies have shown that ANP specific binding sites are present in rat adrenal capsules revealing a K_d of 0.1 nM (Schiffrin *et al.*, 1985). Since this work was completed, Rathinavelu and Isom, (1988) have characterized specific receptors for ANP in rat PC12 cells with a K_d of 670 pM and receptor density of 29 femtomole/10^5 cells.

The K_d (0.794 nM) obtained from the present studies with rat PC12 cells confirms that the receptor expressed in this cell line resembles closely that found in PC12 cells by Rathinavelu and Isom, (1988), and in the intact adrenal gland.

8. Binding studies with angiotensin II: Effect of nerve growth factor

Angiotensin II is an vasoactive hormone known to elevate blood pressure. Specific receptors for A II have been identified in the adrenal gland. These receptors were reported to be concentrated in the zona glomerulosa (Douglas *et al.*, 1978). Specific binding sites for A II also have been identified in a number of other target tissues (McQueen, 1986).

Van Calker *et al.*, (1987) have reported that rat PC12 cells preincubated with ^{3}H-inositol responded to stimulation by bradykinin, A II and carbachol with an increased formation of labeled inositol phosphates. It was thus of interest to find out whether or not this response was mediated by A II receptors and indeed if this clone of PC 12 cells expressed A II receptors. To investigate this possibility PC12 membranes (50 μg/100 μl) were prepared as described previously for ANP binding experiments. Competitive binding studies were performed again as described in section 7.5 in the presence of A II ranging from (10^{-6}M—10^{-12}M). Competitive binding experiments were chosen for preliminary experiments because they used less ^{125}I-A II (125 pM), which is expensive. When these experiments were carried out no specific binding of A II to the membrane preparation could be detected. Interestingly, the amount of non-specific binding of ^{125}I-A II to the membrane preparation was much less than that observed using ^{125}I-ANP. This is in agreement with results found by Morton, (1988) using membrane preparations derived from rat vascular smooth muscle (personal communication).

It was possible that these PC12 cells might have lost their receptors over an unknown number of passages. To test this possibilty an experiment was designed to try to make the cells re-express the receptors. Cells were treated

daily with NGF at a final concentration of 50ng/ml and 1mM dibutyryl cAMP for 6 days. Since NGF is known to differentiate PC12 cells to become neuronal-like (section 3.1) it was possible that this might restore the A II receptors. When NGF and cAMP were added to PC12 cells, they stopped dividing, extended neurites and generally became neuronal-like (Plate 1). A plasma membrane preparation was prepared from nerve growth factor-treated PC12 cells as described in section 6.3-1 Plasma membrane preparations from non-treated cells were prepared as a control.

Although PC12 cells regained their original phenotype, they did not re-express angiotensin II receptors. It can be concluded therefore that NGF differentiates PC12 cells but it does not cause re-expression of A II receptors.

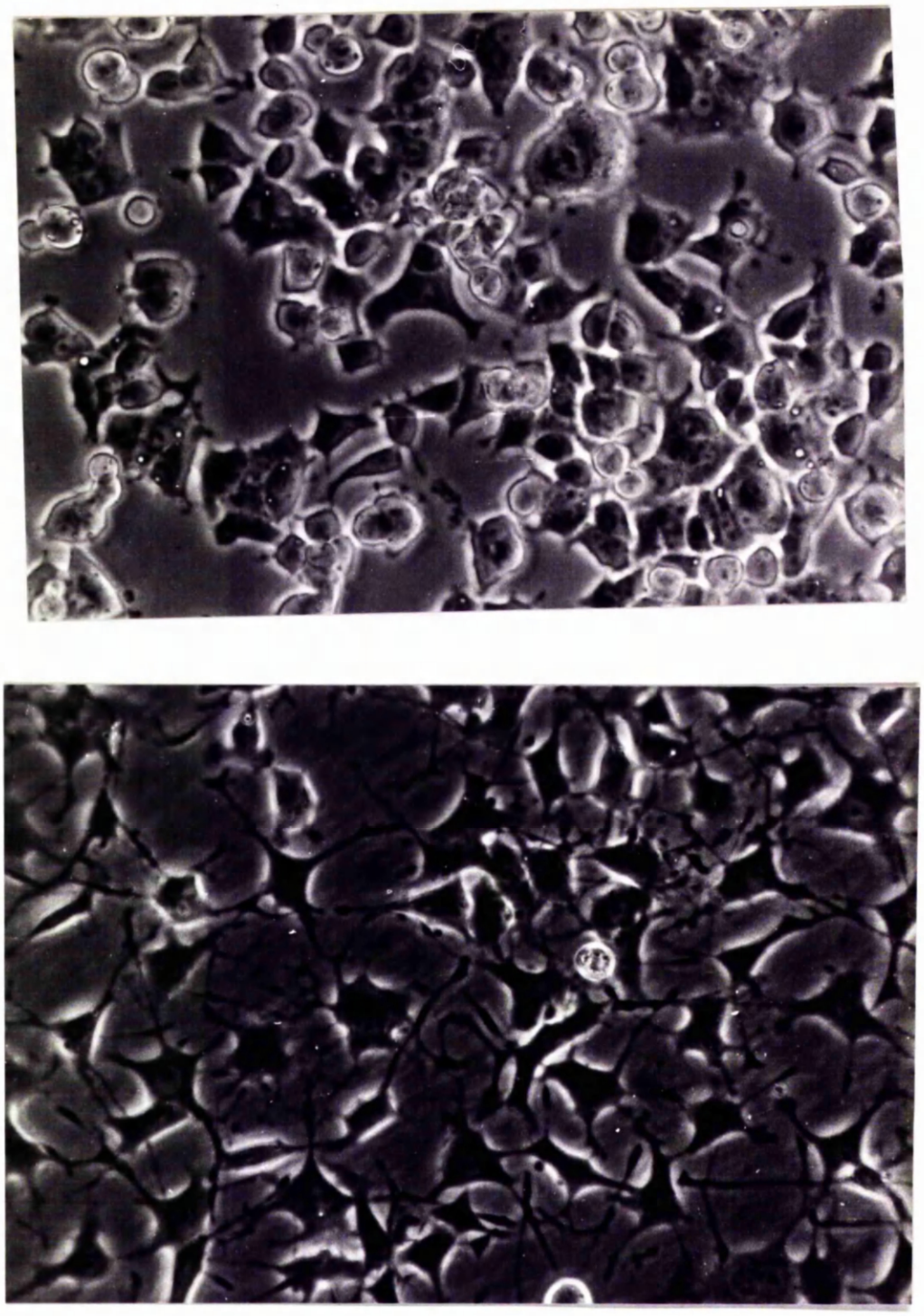


Plate 1. Normal PC12 cells grown on collagen coated flasks (top photograph) and PC12 cells on collagen coated flasks then treated with NGF (final concentration 50 ng/ml) and cAMP (final concentration 1mM) daily for 6 days (bottom photograph).

9. Characterization of catecholamines released by PC12 cells

Greene and Tischler, (1976) have reported that PC12 cells synthesize and store noradrenaline and dopamine. Rebois *et al.*, (1980) and Schubert and Klier, (1977) have also reported that PC12 cells store and secrete dopamine and acetylcholine. It was of interest therefore to identify which catecholamines were released by this PC12 cell line. Six flasks (25 cm^2) of confluent PC12 cells were exposed to 2 ml of 5 or 70mM K^+ solution for 0, 5 or 30 min. In the presence of 5mM K^+ dopamine concentrations released into the medium after 0, 5 and 30 min were 44, 101.5 and 120 nM respectively, however in the presence of 70mM K^+ dopamine released after 0, 5 and 30 min was 90, 425 and 460 nM respectively (Fig. 22). Adrenaline and noradrenaline levels were too low (> 0.1nM) to be detected using this radio-enzymatic assay.

9.1 ^{3}H-dopamine release from PC12 cells

The final event in stimulus-exocytosis coupling is the fusion of secretory vesicles with the plasma membrane. To investigate this event cells were permeabilized using different procedures for different cells. Kongsamut and Miller, (1986) studied the release of noradrenaline from normal PC12 cells and PC12 cells treated with NGF in order to see if NGF treatment of PC12 cells altered the drug sensitivity of neurotransmitter release. PC12 cells became insensitive to dihydropyridines after their differentiation. One of the techniques used for looking at dopamine release from PC12 cells is to incubate them with ^{3}H-dopamine. The ^{3}H-dopamine is taken up across the plasma membrane into vesicles until released by various agonists. Melega and Howard, (1984) have showed that 2-(4-phenyl piperidino) cyclohexanol (AH 5183) at 40 μM had slight inhibitory effect on the loading of

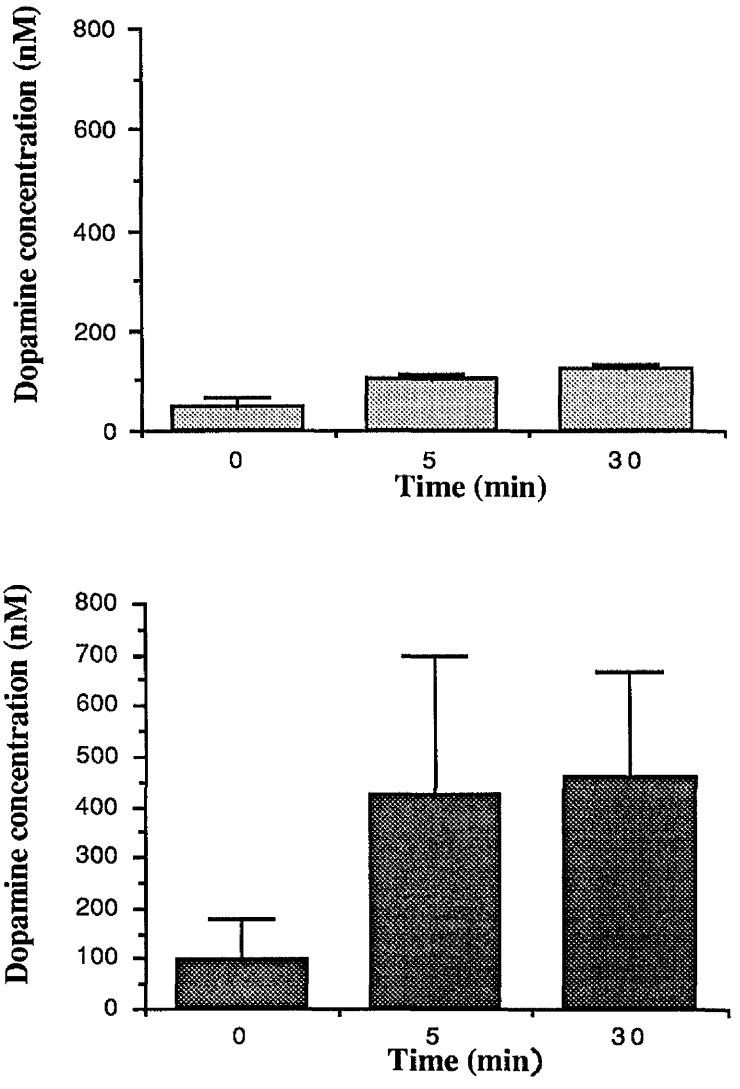


Fig.22: Release of dopamine from PC12 cells.

PC12 cells were exposed to 5mM K^+ (top graph) and 70mM K^+ (bottom graph) for 0, 5 and 30min respectively. Only dopamine was detectable in this radio-enzymatic assay. The points represent the mean of 2 experiments± SE.

^{3}H-dopamine into vesicles. Ahnert-Hilger and Gratzl, (1987) have used α-toxin from *Staphylococus aureus* to permeabilize PC12 cells after loading them with ^{3}H-dopamine.

Since it had been established in the previous experiments that PC12 cells secrete dopamine, cells were loaded with ^{3}H-dopamine and the effects of high potassium or ANP on ^{3}H-dopamine release from the cells was investigated. Fig.23 illustrates the release of ^{3}H-dopamine from PC12 cells after various stimuli. Cells were loaded with ^{3}H-dopamine and then exposed to various potassium concentrations as described in section 6.5.

Three separate experiments were set up and cells were exposed to 3 different concentrations of potassium (5, 37 or 70mM K^+). When the cells were exposed to 5mM K^+ the release of ^{3}H-dopamine in the first 5 min was 6.4 % of total ^{3}H-dopamine taken up by the cells and then remained at ~3.06 % /5min for the remaining 25 min. The higher level of ^{3}H-dopamine released in the first 5min was probably due to free ^{3}H-dopamine which had not been washed from the flasks after the loading and washing steps. The exposure to 5mM K^+ was considered as basal release (control). In the second set of experiments cells were exposed to 5mM K^+ for the first 10 min , 37mM K^+ for the second 10 min (Fig.23) and 5mM K^+ again for the last 10 min. The release of dopamine when cells were exposed to 37mM K^+ (15.8 % over 10 min) was higher than that released by 5mM K^+ but once the 37mM K^+ solution was removed and replaced by 5mM K the release returned to normal (basal release). In the third set of experiments when the 37mM K^+ solution was replaced by 70mM K^+ solution, which depolarizes PC12 cells, even

Fig.23. ^{3}H-dopamine release from PC12 cells.

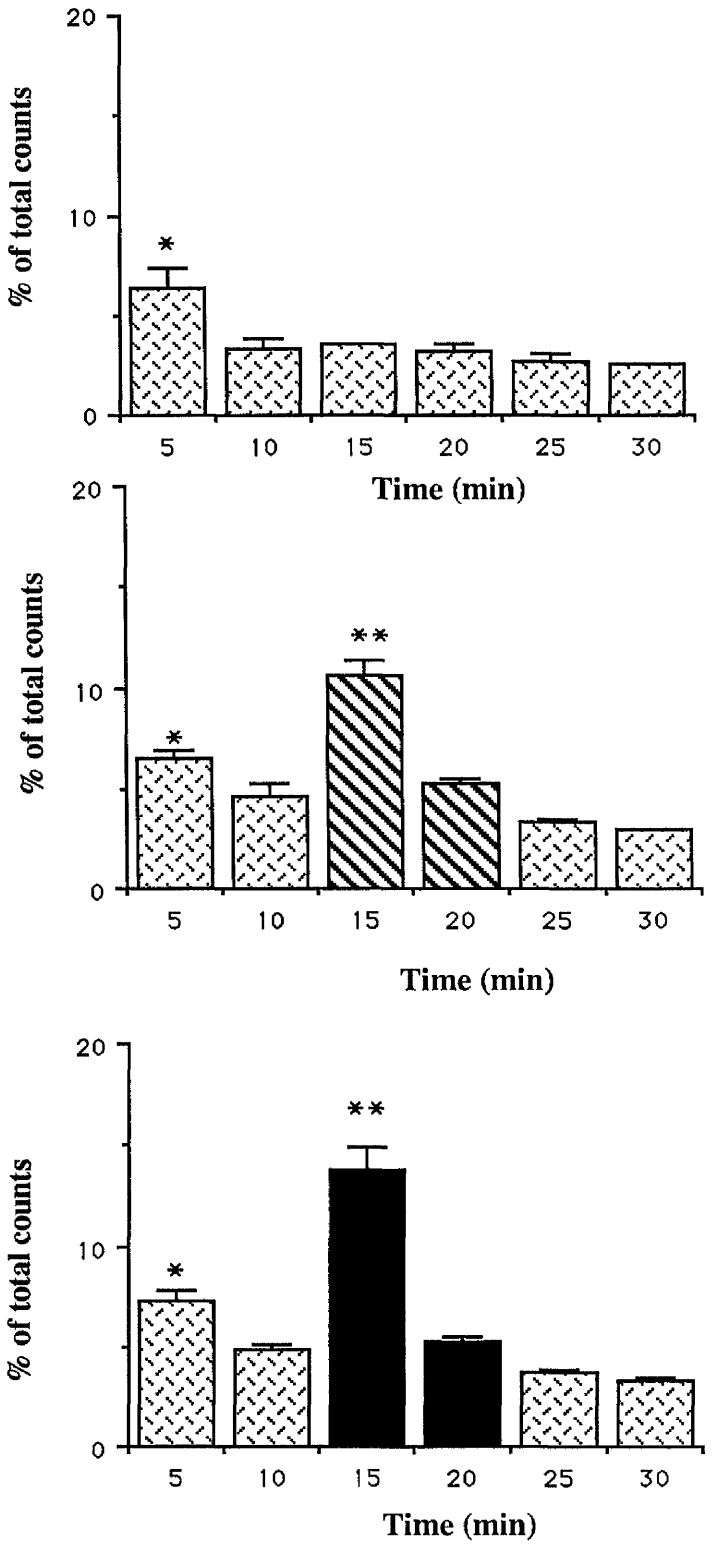
Cells were incubated with ^{3}H-dopamine for 2h at 37°C, washed to remove the excess label and then exposed to different concentrations of potassium (5, 37 or 70mM K^{+}). Points represent the mean of 2 experiments each performed in triplicate ± SE.

** differs from * $p < 0.001$.

5mM K^{+}

37mM K^{+}

70mM K^{+}


20
10
0
% of total counts
*
5
10
15
20
25
30
Time (min)
20
10
0
% of total counts
*
**
5
10
15
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25
30
Time (min)
20
10
0
% of total counts
*
**
5
10
15
20
25
30
Time (min)

higher levels (19 %) of ^{3}H-dopamine was released over 10 minutes. It can be concluded that this PC12 cell line secretes only dopamine and secretion of dopamine is elevated when the cells are depolarized using 70mM K^{+}.

9.2 The effect of ANP on ^{3}H-dopamine release from PC12 cells.

Since this study has shown that ANP receptors are present on PC12 cells and that these same cells secrete dopamine, the question remained, did ANP have any direct effect on dopamine release ?

To test this possibility ANP (final concentration 10^{-5}M) was added to either the 5mM K^{+} or 70mM K^{+} solutions. Cells were then perfused with these two solutions (composition as in section 6.8-5—6.8-6). ANP had no statistically significant effect on the release of dopamine from cells (Fig.24 and 25).

9.3 ANP secretion

It is now well established that ANP is located within and secreted from the heart of many species. ANP is stored as 126 amino acid precursor (proANP) and cleaved upon its release to the circulating form (3000) (Thibault *et al.*, 1987). Studies on isolated perfused heart *in vivo* and *in vitro* has suggested that the atrial distension is a common physiological stimulus for ANP secretion (Thibault *et al.*, 1987).

Recently Ong *et al.*, (1987) demonstrated the co-existence of ANP (99-126) and its precursor ANP (1-126) within bovine chromaffin granules, comfirming the maturation process of atrial peptides in the adrenal medulla.

Nguyen *et al.*, (1988) have demonstrated that nicotinic activation (10μM) and depolarization by KCl (56mM) increased secretion of ANP and its precursors from cultured chromaffin cells. In this study PC12 cells, derived from a

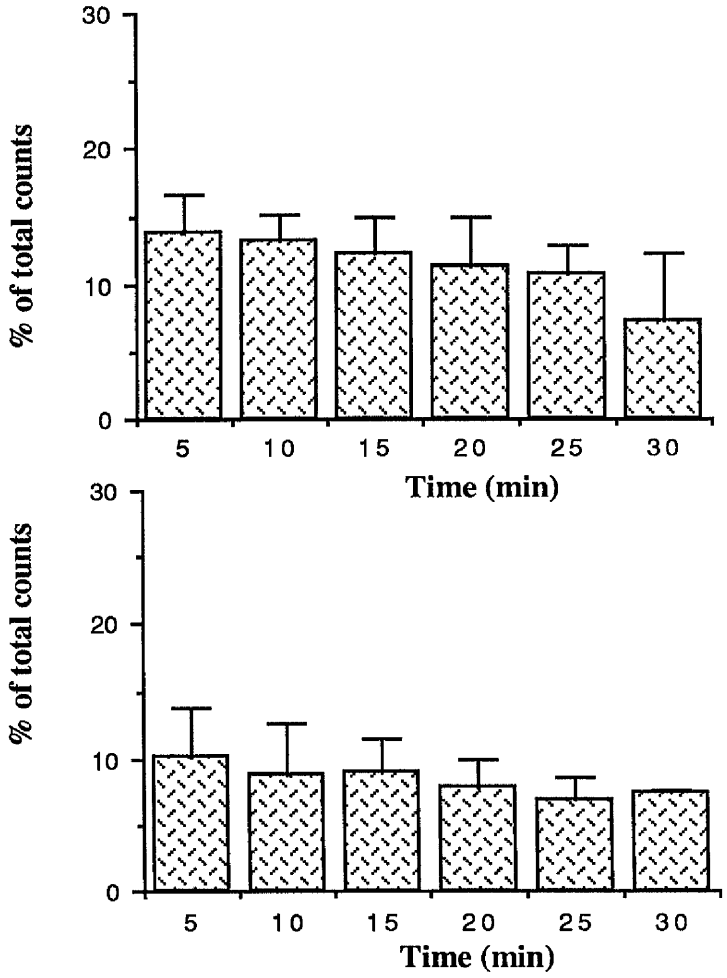


Fig.24.The effect of ANP on dopamine release from PC12 cells.
Release of ^{3}H-dopamine from PC12 cells exposed to 5mM K (top graph) or 5mM K solution containing 10^{-5}M ANP (bottom graph). Points are the mean of 3 experiments each performed in triplicate ±SE. None of the lower bars differed significantly ($p< 0.05$) from the corresponding control value. (Student t test).

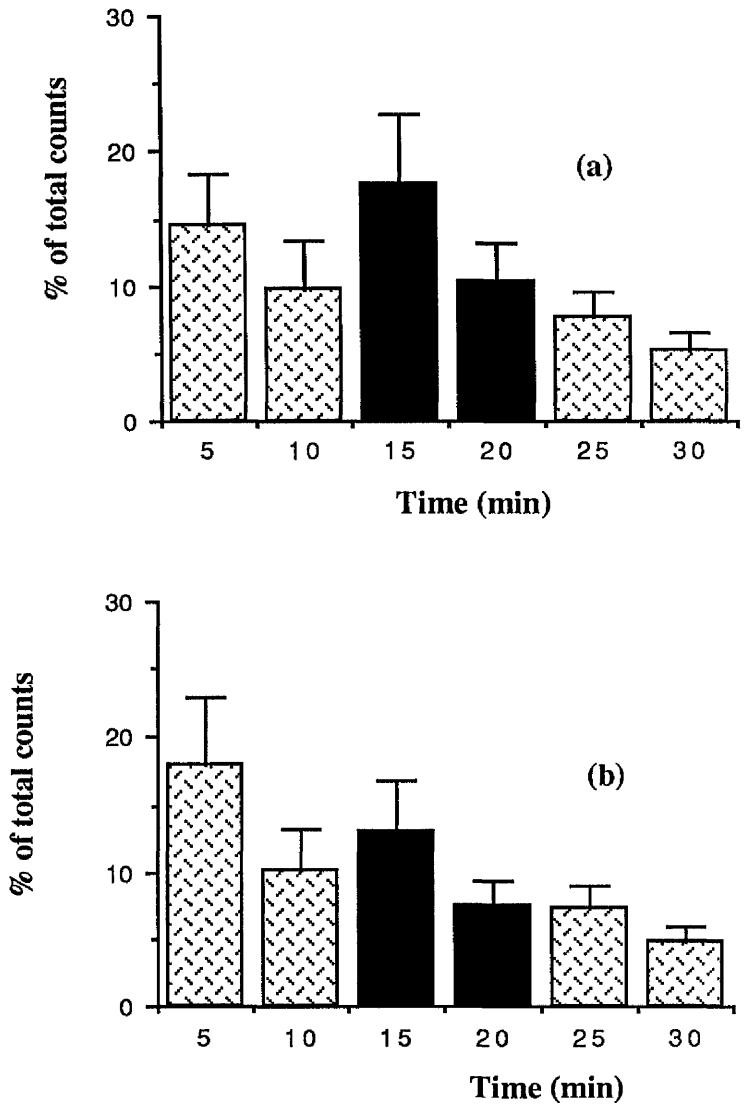


Fig.25. The effect of ANP on ^{3}H-dopamine release from PC12 cells depolarized by 70mM K^{+}.

Release of dopamine from PC12 cells exposed to 70mM K^{+} solution (a) or 70mM K^{+} containing ANP at final concentration of 10^{-5}M (b). ANP was also added to the 5mM K^{+} solution. Points are the mean of 11 separate experiments ±SE. None of the lower bars differed significantlty ($p<0.05$) from the corresponding control value. (Students t test).

5mM K^{+}

70mM K^{+}

tumour of these chromaffin cells, were used to look for ANP secretion. The results obtained from the perfusion system when cells were exposed to 5mM K^+ for 30 min are shown in Fig.26. These experiments show that ANP is present in the cells, but when cells were perfused with 70mM K^+ (Fig.27) the response of cells in releasing ANP was clear after the removal of the 70mM K^+ and replacement by 5mM K^+. Both set of experiments have indicate that ANP is present within PC12 cells. In a separate experiment, where six ($25cm^2$) confluent flasks of PC12 cells were exposed to 5mM K^+, 70mM K^+ and 70mM K^+ containing protease inhibitors at 37°C for 5 min (Fig.28). ANP released to the media was below the resolution of the assay (> 5 pg/ml) . The results, as can be seen, are not reproducible. To clarify this uncertainity requires further investigation. In a third set of experiments, where the supernatant of cells (homogenized) was assayed for ANP secretion, 420 pg/ml was detected in non-trypsinized cells and 248 pg/ml in trypsinized cells. Although PC12 cells were not exposed to trypsin for longer than 5-6 min, trypsin does seem to affect the content of ANP in PC12 cells.

Fig.26. The release of ANP from PC12 cells exposed to 5mM K^+. In a perfusion system PC12 cells were exposed to 5mM K^+ for 30 min. 2 ml samples were collected at 5 min intervals and assayed for ANP release (Fig.14). 3 individual experiments are shown in this Fig. and the bottom graph is the mean of all 3 ± SE.

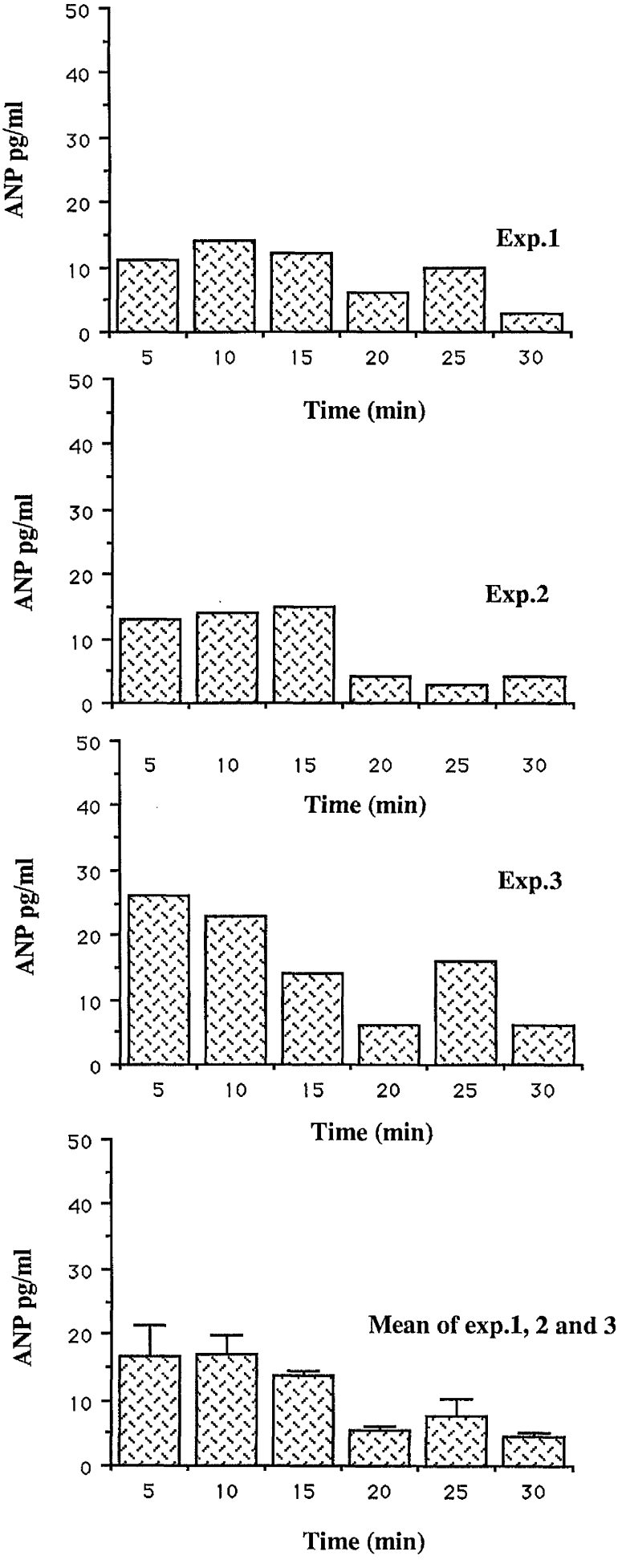
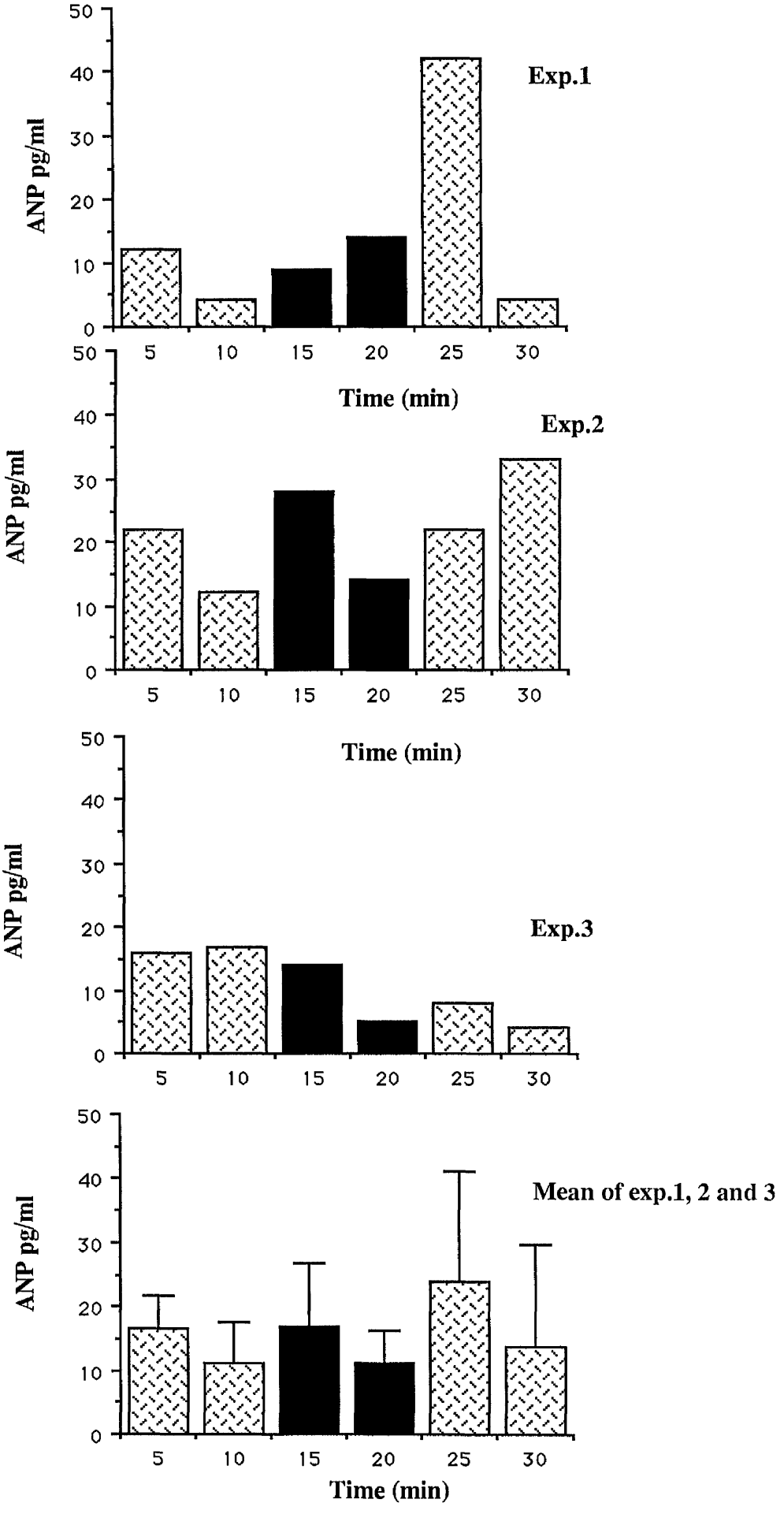

ANP pg/ml
50
40
30
20
10
0
Exp.1
5
10
15
20
25
30
Time (min)
ANP pg/ml
Exp.2
Time (min)
ANP pg/ml
Exp.3
Time (min)
ANP pg/ml
Mean of exp.1, 2 and 3
Time (min)

Fig.27. The release of ANP from depolarised PC12 cells .

In a perfusion system PC12 cells were exposed to 5mM K^+ for the first 10 min and then depolarised with 70mM K^+ for 10 min. 5mM K^+ replaced the 70mM K^+ for the last 10 min. 2 ml samples were collected at 5 min intervals and assayed for ANP release (Fig.14). 3 individual experiments are shown in this Fig. and the fourth is the mean of the 3 ± SE.

5mM K^+

70mM K^+


ANP pg/ml
50
40
30
20
10
0
5
10
15
20
25
30
Time (min)
Exp.1
Exp.2
Exp.3
Mean of exp.1, 2 and 3

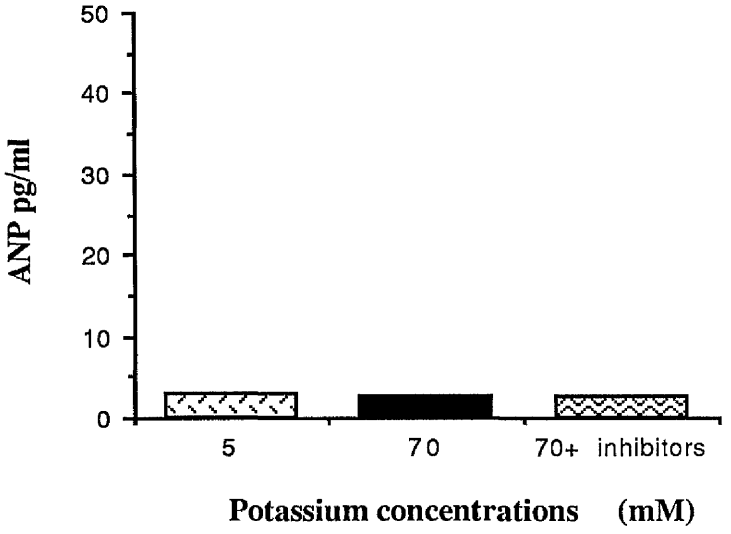


Fig.28. The release of ANP from PC12 cells.

6 confluent flasks ($25cm^2$) received either 5mM K^+. 70mM K^+ or 70mM K^+ containing protease inhibitors. The ANP released was below the normal range of plasma ANP concentration (5-50pg/ml). Points are one experiment each carried out in duplicate.

10. The measurement of renin in PC12 cells

As described in section 6.7 patients with phaeochromocytoma are reported to have increased plasma renin activity. To investigate the possibility that PC12 cells secrete renin the following experiments were carried out. 10 ($150cm^2$) collagen coated confluent flasks were prepared. Cells from five flasks were isolated by trypsinization. However trypsin is known to activate renin. Cells from five flasks were scraped from the flasks. Cells from each preparation were homogenized in 2 ml (Tris/HCl 100mM) in a polytron homogenizer and spun for 1h at 40000 rpm at 5°C. The supernatant was removed and frozen until the assays were carried out.

The results from the radio-immunoassay were obtained after the incubation of samples (supernatant of lysed PC12 cells) with angiotensin I antiserum and rat renin substrate (see methods) for 0 and 5h at 0°C and at 37°C. At 0°C , where renin would not be active, angiotensin I was undetectable in both the trypsinized and non-trypsinized PC12 cells. After incubation for 5h at 37°C, angiotensin I generated was 37 pg/h for non-trypsinized cells and 42 pg/h for the trypsinized cells (roughly $150x10^6$ cells).

Discussion

11. Discussion

General

The atrial natriuretic family of peptides isolated from the heart of mammalian and non-mammalian species, has been shown to possess powerful vasorelaxant and natriuretic activity (Garcia *et al.*, 1984). Since the discovery of these bioactive peptides (De Bold *et al.*, 1981), a great deal of interest has been generated in them. In that time the atrial natriuretic peptide has been purified, (Thibault *et al.*, 1983) sequenced (Seidah *et al.*, 1984) and the cDNA sequence of its precursor determined (Maki *et al.*, 1984).

The heart is an endocrine gland (Cantin and Genest, 1987) and releases the atrial natriuretic family of peptides into the bloodstream. This method of release allows these peptides to act at a wide range of target organs. ANP receptors have been identified in many organs involved in regulation of blood pressure and fluid and electrolyte homeostasis.

Phaeochromocytoma are tumours of chromaffin cells of the adrenal gland. PC12 cells share many characteristics with normal chromaffin cells (section 3.2) but once PC12 cells are treated with NGF, they cease dividing and extend neurites to become neuronal-like cells (Greene and Tischler, 1976). The ability of these PC12 cells to take on neuronal properties has made these cells a useful model system for physiological studies; in particular, for studying the differentiation of neuroblasts into neurons and also to investigate the role and mechanisms of action of NGF on its target cells (Dichter *et al.*, 1977). The cGMP response in PC12 cells to ANP may provide important clues concerning the molecular mechanisms of action of ANP in the central and peripheral nervous systems (Fiscus *et al.*, 1987).

11.1 Binding studies with ANP

The present study demonstrates the presence of specific receptors for ANP in PC12 cells, a cell line derived from a tumour of chromaffin cells in the rat adrenal medulla. Binding studies revealed a K_d of 794pM and a receptor density (B_{max}) of 256 fmol/mg of protein. The peptides related to ANP compete for the binding sites in the following order of affinities: rat atrial peptide fragment 13-28 aa > r-ANP (28 aa) > h-ANP (28 aa) > r-ANP (8-33 aa) rat atrial peptide fragment (1-11aa) > atriopeptin I.

The data obtained from these binding studies was published in October 1988. In the same month a paper was published by Rathinavelu and Isom, (1988) which contained data that was in close agreement with the present study. They characterized specific receptors for ANP in intact PC12 cells. Their binding studies, using rat ANP (8-33), revealed a K_d of 670pM and receptor density of 29 femtomole/10^5 cells. In their study, intact PC12 cells (5x 10^5 cells/tube) were incubated in suspension with ^{125}I-ANP (0.2-2nM) in the binding medium (RPMI medium containing 0.2 % BSA) in a total volume of 200 µl at 0°C for 2h. All assays were performed with cells in suspension in polystyrene tubes. Separation of bound from free radioligand was obtained by filtration through GF/C glass fibre filters treated with 0.02% Tween 20 in PBS for 2h. Filters were washed with 10 ml of ice cold 0.15 M NaCl and then counted in a gamma counter. Although this method used intact cells to characterize ANP receptors in PC12 cells, the K_d calculated from this method was still very similar to the K_d found in the present study using membrane preparations. Rathinavelu and Isom, (1988) showed that ANP receptors were internalized by PC12 cells when cells were incubated

with ^{125}I-ANP at 37°C. They demonstrated internalization of receptors as follows: at the end of the incubation period, tubes were placed on ice and 0.5 M NaCl, 0.2M acetic acid was used to extract ANP bound to the cell surface. The internalized ligand, represented by the radioactivity remained in the cells, was determined by solubilizing the cells in 1N NaOH (Rathinavelu and Isom, 1988). This work on intact cells which was published at the same time and is in close agreement with the present study, further suggests a regulatory role for ANP in the adrenal gland.

Both of these studies confirm the prediction of Fiscus *et al.*, (1987), that these cells would express high levels of ANP receptors since they have shown that ANP is coupled to cGMP accumulation in PC12 cells. This project demonstrates the presence of specific ANP binding sites, on PC12 cells. The accumulation of cGMP in C6-2B rat glioma cells and in phaeochromocytoma PC12 cells was stimulated by ANP related peptides in the following order of affinity (Fiscus *et al.*, 1987) AP III = AP II > h-ANP > AP I and this order of affinity of h-ANP and AP I is in agreement with the order of receptor affinity found in the present study. This data is also consistent with the idea that the biological response of ANP is via cell surface receptors since Fiscus *et al.*, (1987) have shown that atriopeptin II (100nM) activates particulate forms (membrane-associated) of the enzyme guanylate cyclase responsible for cGMP synthesis, rather than the soluble form. This isoenzyme form of guanylate cyclase binds ANP not only in PC12 membrane preparations but also in several rat tissues. Furthermore, this group have shown that the ANP receptor coupled to cGMP synthesis in rat lung is a glycoprotein that copurifies with particulate guanylate cyclase (Kuno *et al.*, 1986). Chinkers *et al.*, (1989) have reported the isolation, sequence and expression of a complementary DNA clone encoding the

membrane form of guanylate cyclase from rat brain. This cDNA was transfected into cultured mammalian cells and resulted in expression of guanylate cyclase activity and ANP-binding activity. This ANP receptor/guanylate cyclase represents a new class of mammalian cell-surface receptors which contain an extracellular ligand-binding domain and an intracellular guanylate cyclase catalytic domain. Cyclic GMP accumulation by atriopeptin II (23 aa) was enhanced in the presence of the phosphodiesterase inhibitor 3-isobutyl-l-methylxanthine (IBMX) (250μM) with both C6-2B and PC12 cells. This observation that addition of the phosphodiesterase inhibitor IBMX enhanced intracellular cGMP levels further suggests that ANP directly activates guanylate cyclase (Fiscus *et al.*, 1987).

Specific receptors for ANP have been identified in cultured N4TG1 neuroblastoma cells . These cells have a single class of receptors for ANP with a K_d of $1x10^{-10}$ M (Pandy *et al.*, 1987). ANP also stimulated cGMP accumulation (K_d : 100nM) in these neuronal cells (Pandy *et al.*, 1987). The K_d calculated from Pandy's binding study agrees closely with the K_d value (794 pM) obtained from the present study on PC12 cells. The discrepancy of the K_ds of the ANP receptor and the stimulation of intracellular cGMP by ANP in a number of cell types (Hirata *et al.*, 1984) has not yet been explained. The presence of ANP receptors in three neuronally derived cell lines, the N4TG1 neuroblastoma (mouse), C6-2B rat glioma cells and phaeochromocytoma PC12 cells further supports the idea that ANP has an important function in the central and peripheral nervous system acting through its second messenger cGMP. Furthermore Schiffrin *et al.*, (1985) have identified specific binding sites for ANP (K_d of 0.1nM) in a membrane

fraction isolated from rat adrenal capsules. The K_d value obtained from PC12 cells confirms that the receptor expressed in this cell line closely resembles that found in the intact adrenal gland.

Initially, binding experiments were performed on cells attached to plastic tissue culture. However, during the incubation both at 0°C and 22°C the cells detached from the dishes. Because of the rapid breakdown of the ^{125}I-ANP at 37°C experiments on intact cells were not carried out at this temperature. At 37°C there was also the possibility that the ligand-receptor complex would become internalized by the cells. Since performing these experiments it has become clear that using collagen coated dishes might have overcome this problem by stopping the cells detaching at lower temperatures for the duration of the experiment and it would be of interest to go back and do further binding experiments on intact cells plated on collagen coated dishes. In a second attempt to characterize the ANP receptor on intact cells, PC12 cells were used in suspension. For these experiments cells were grown on flasks and when confluent the flasks were left at 22°C for 5-10 min. The cells could then be gently shaken from the flasks without enzyme treatment which could possibly have destroyed surface receptors. This method allowed binding experiments to be performed on cells in suspension at 0°C where breakdown of the ligand would be minimized. However, when the samples were filtered, they rapidly (0.45 μm Whatman filters) blocked the filters, thus preventing separation of bound and free ligand. At a later date, while performing the perfusion experiments, it became clear that glass fibre (GF/C) filters would have been much more suitable in the binding studies with cells in suspension. Because of the reasons outlined above binding studies were performed on membrane preparations at 0°C.

11.2 Degradation of ^{125}I-ANP

The temperature used in ANP binding experiments by other investigators usually varied between 0-37 °C. Most investigators do not appear to have performed HPLC on the ANP to check the breakdown of the label after incubation with the membrane preparation or cells .

Lyall *et al.*, (1988), have shown that, after the incubation of ^{125}I-ANP for 1h at 0°C with a membrane preparation from the human astrocytoma cell line clone D 384. 63 % of the ligand remained intact. By adding the protease inhibitors 0.5mmol phenylmethane sulphonyl fluoride/l, 0.1% (w/v) bacitracin and 1umol aprotonin/l (PMSF) , they found that up to 85% of ^{125}I-labelled ANP remained intact. These inhibitors, however, adversely affected binding of the ligand to the membrane. Because the complete elimination of tracer degradation was not obtained in the presence of these inhibitors and because of adverse effects on ligand binding they chose to perform their experiments at 0°C for 1h without them. Schiffrin *et al.*, (1985) have examined the degradation of the ^{125}I-ANP in mesenteric and adrenal membranes. The incubation of ^{125}I-ANP with mesenteric or adrenal membranes in the presence of proteinase inhibitors (bacitracin, PMSF and aprotinin) resulted in more than 90 % of the labelled ANP remaining intact as shown by HPLC. Adrenal membranes were incubated with ^{125}I-ANP and then centrifuged, the label in the supernatant was removed and used in another binding experiment with a fresh membrane preparation, 92 % of this label was found to bind to these membranes on re-incubation . This result was in agreement with the results obtained from their HPLC experiments (Schiffrin *et al.*, 1985).

In the present study about 70 % of the labelled ANP remained intact in the

presence of the membrane after incubation for 1h at 0°C. The percentage of intact label fell to 14 % and 3.6 % when the incubation was at 22°C and 37°C respectively. Breakdown of the ligand could affect both the accuracy of the estimated k_d value of the binding sites and also the number of binding sites if the receptors themselves were damaged at 37°C.

11.3 Specificity of binding

The specificity of the binding sites for ANP related and unrelated peptides was examined in competitive binding experiments as shown in Fig.20. In this study, hormones unrelated to ANP, such as A II, bradykinin and arginine-8-vasopressin, did not compete for the binding sites. ANP-related peptides which compete for the binding sites were in the following order of affinity : Rat atrial peptide fragment (13-28) > r-ANP (28 aa) > h-ANP (28 aa) >r-ANP (8-33 aa) rat atrial peptide fragment (1-11aa) > atriopeptin I. This clearly shows the specificity of these binding sites for ANP. It is worth pointining out that peptides without disulphide bridges, like ANP fragment (1-11), did not compete in ANP binding studies using rat mesenteric artery membrane preparations (Lyall and Morton, 1987). Schiffrin *et al.*, (1985) have examined the displacement of labelled ANP (0.05 nM) in mensenteric and adrenal membranes (0.5 mg/ml) in a competitive binding study with unrelated peptides such as A II and vasopressin (AVP) (10^{-7} to 10^{-6}M). The related peptides displaced ^{125}I-ANP in the following order of affinity. ANP (101-126) > ANP (101-125) > ANP (101-123) > ANP (102-126). Hormones unrelated to ANP, such as A II and AVP, did not compete for the binding sites.

Affinity cross-linking studies were performed by Rathinavelu and Isom, (1988) using ^{125}I-ANP and PC12 cells. Two labelled protein components of

the PC12 cell plasma membrane which bound the ^{125}I-ANP migrated at molecular weight of 70000 and 130000 on SDS polyacrylamide gels. The same authors have shown that ^{125}I-ANP was bound to a single class of high affinity binding sites in PC12 cells thus suggesting that these PC12 cells might possess two classes of receptors, one of which is linked to the particulate form of guanylate cyclase.

12. Angiotensin II binding studies

ANP relaxes vascular smooth muscle cells by stimulating the synthesis of cGMP (Atlas *et al.*, 1986; Winquist, 1986) and antagonizing the vasoconstrictor effects of other hormones such as A II. ANP also inhibits A II actions on other cell types such as the adrenal cells, for example ANP decreases A II evoked secretion and steroidogenesis in adrenal glomerulosa cells (Elliot *et al.*, 1986). Feldberg and Lewis, (1964) have reported that angiotensin is a potent releaser of the medullary hormones, mainly adrenaline, from the adrenal gland.

Smith and Lincoln, (1987) have shown that the angiotensin II and other calcium-mobilizing stimuli markedly inhibited the accumulation of cGMP that is evoked by ANP in cultured smooth muscle cells. ANP inhibits the contractile effects of a variety of other hormones beside angiotensin II (Kleinert *et al.*, 1984). The antagonistic effect of these two hormones (ANP and A II) has been known for a long time. Since this study has shown that functional receptors for ANP are located on PC12 cells it was of interest to investigate whether or not these PC12 cells have receptors for A II. Competitive binding studies were performed with ^{125}I-A II as described previously for ANP binding experiments. When these experiments were performed no specific binding of ^{125}I-A II to the membrane preparation could be detected. In addition the amount of non-specific binding of ^{125}I-A II to the membrane preparation was much less than that observed using ^{125}I-ANP and this finding is in agreement with the results found by Morton using a membrane preparation derived from rat vascular smooth muscle (personal communication).

Many investigators have added nerve growth factor to PC12 cells to cause them to differentiate into neuronal-like cells, for a number of different

studies. An experiment was designed in which PC12 cells were treated daily with NGF at a final concentration of 50ng/ml and 1mM dibutyryl cAMP for 6 days in order to try to restore the A II receptors. Although the differentiation of PC12 cells was achieved when binding studies with ^{125}I-A II were carried out, these cells still did not express A II receptors.

It would have been interesting to look at ANP binding sites with differentiated PC12 cells to see whether or not the same receptors for ANP would be expressed when cells are differentiated. Since the radiolabelled ANP is expensive this experiment was not performed. Recently Drewett *et al.*, (1988) have reported that ANP (101-126) inhibited the release of carbachol-induced noradrenaline release from differentiated PC12 cells. This further proves that undifferentiated PC12 cells express ANP binding sites and that differentiated PC12 cells probably express them too.

Van Calker *et al.*, (1987) have found that bradykinin, A II and carbachol accumulated inositol phosphates in PC12 cells and this was increased by adding 10mM lithium. To produce such an effect, it would have been expected that PC12 cells expressed A II receptors. However, the PC12 cells used in this project did not appear to express any receptors for A II. Thus PC12 cells either do not have surface receptors for A II or only certain clones of PC12 cells express receptors for A II. Interestingly, it has been shown by a number of investigators that A II may be internalized and have intracellular effects. For example it has been reported that rat liver and spleen nuclei have specific nuclear binding sites for angiotensin II (Re *et al.*, 1981). Autoradiographic studies have shown that ^{3}H-A II was localized in the nucleus of smooth muscle cells (Robertson and Khairallah, 1971). This finding has suggested that A II or its metabolic fragments have specific effects on nuclear function.

The discrepancy between the results of A II binding studies in this project and the results showing A II mediated IP_3 production suggest the possibility that A II may be internalized with its receptors and may have some intracellular effect in PC12 cells. It would be of interest to incubate PC12 cells with A II and use the techniques described by Rathinavelu and Isom, (1988) to show whether A II is internalized. If so, autoradiography could be performed to check where the A II was located.

If autoradiographic studies showed that A II was located on the nucleus then it would be of interest to perform ^{125}I-A II binding studies on nuclear membrane preparations from these cells. Furthermore since ANP has been shown to be internalized by PC12 cells (Rathinavelu and Isom, 1988) this suggests that ANP may have some intracellular effect on these cells, and it would be interesting to perform ANP-binding studies on nuclear membrane preparations from these cells.

13. ^{3}H-dopamine release from PC12 cells

PC12 cells synthesize, store and release noradrenaline and dopamine at higher rates than normal chromaffin cells (Greene and Tischler, 1976), however, the normal adrenal medulla synthesizes, stores and releases adrenaline, noradrenaline and dopamine. Recently Drewett *et al.*, (1988) have shown that carbachol-induced PC12 cells derived from rat adrenal phaeochromocytoma released noradrenaline. Noradrenaline was assayed by HPLC with electrochemical detection. Many other investigators have shown that PC12 cells secrete noradrenaline, while some others have shown that PC12 cells secrete dopamine (section 6.4). It was thus of interest to find out which catecholamines were released by the PC12 cells used in this study.

Radio-immunoassay revealed that only dopamine was detectable in medium derived from these PC12 cells. The dopamine released was 44, 101.5 and 120 nM after 0, 5 or 30 min in the presence of 5mM K^{+}. In the presence of 70mM K^{+} dopamine detected was 90, 425 and 460 nM after 0, 5 or 30 min. Adrenaline and noradrenaline were undetectable (< 0.1 nM).

The next part of this work was to study the conditions under which dopamine was released. The catecholamine assay is a sensitive but time-consuming technique. Another approach to this work is to pre-load cells with ^{3}H-dopamine and then quantify release of the radiolabel, following exposure to various potassium concentrations, using a scintillation counter. In the first set of experiments when PC12 cells were exposed to 5mM K^{+} solution the ^{3}H-dopamine released in the first 5 min was 6.4 % and then remained at ~3.06 % for the remaining 25 min. This release was considered as basal (control). In the second set of experiments PC12 cells were exposed to 5mM K^{+} for the first 10min, where the release was at almost the same

level as the previous set of experiments, and then exposed to 37mM K^+ for the second 10 min, where the release was 15.8 % over 10min. Once the 37mM K^+ was removed and replaced by 5mM K^+ for the last 10 min the release returned to normal (basal release). The third set of experiments were performed in the same way as the previous experiments except 70mM K^+ replaced the 37mM K^+ solution. The release in the first 10min was much the same as the basal release, however, the release after the exposure to 70mM K^+ was 19 % over 10 min. This response of the PC12 cell line to depolarization conditions further shows that these cells are a useful model for looking at signal-response coupling in neuronal cells.

13.1 The effect of ANP on the release of ^{3}H-dopamine release from PC12 cells

Since PC12 cells express binding sites for ANP and secrete dopamine, it was of interest to see whether ANP had any effect on dopamine release from these cells. In this study ANP had no statistically significant effect on inhibition of potassium-stimulated release of dopamine from PC12 cells. Takagi *et al.*, (1988) have shown that ANP (10^{-8} M) did not inhibit the K^+ induced increase in Ca^{++} influx rate from rat adrenal glomerulosa cells.

The normal adrenal medulla modulates blood pressure by releasing catecholamine hormones (adrenaline, noradrenaline and dopamine) into the bloodstream. These hormones help maintain normal blood volume and pressure. ANP and other vasorelaxant peptides may modulate blood pressure by acting through their specific receptors found on adrenal cells to inhibit the release of catecholamines which in turn relaxes smooth muscle.

ANP has been reported to inhibit adrenergic neurotransmission from presynaptic terminals in rat mesenteric arteries (Nakamura and Inagami,

1986). Very recently Drewett *et al.*, (1988) have reported a pre-synaptic neuromodulatory effect of ANP on PC12 cells. It has already been shown that differentiated PC12 cells synthesize and accumulate noradrenaline and also release it when treated with the nicotinic agonist carbachol (Greene and Rein, 1977; Koike and Takashima, 1986). Drewett *et al.*, (1988) further showed that ANP (101-126) significantly inhibited carbachol-induced noradrenaline release in a concentration-dependent manner. Half maximal inhibition was produced at an ANP concentration of 0.1nM. This study suggests that, at least with differentiated PC12 cells, ANP is an inhibitory neuromodulator.

In a different study, Nakamura and Inagami, (1986) showed that ANP (100nM) only reduced electrically-induced ^{3}H-noradrenaline release by 12% from PC12 cells, whereas in the above paper 100nM ANP resulted in total inhibition of carbachol-induced noradrenaline release.

In this project, ANP effects on neurotransmitter release were only performed on undifferentiated PC12 cells. It would be of interest to differentiate these cells and see whether: a) The catecholamines produced by the cells are the same and b) Whether ANP had any effect on dopamine release. It is worth noting that the experiments in this study involved electrical stimulation with KCl rather than nicotinic agonists.

13.2 ANP secretion

Recently Ong *et al.*, (1987) demonstrated the co-existence of ANP (99-126) and its precursor ANP (1-126) within bovine chromaffin granules, comfirming the maturation process of atrial peptides in the adrenal medulla. Nguyen *et al.*, (1988) have demonstrated that nicotinic activation (10μM) and depolarization by KCl (56mM) increased secretion of ANP and its precursors from cultured chromaffin cells. Because of these observations, it was of

interest to see whether ANP could be detected in the PC12 cells.

As discussed in section 6.6, three different methods were used to investigate this. In the first set of experiments cells were perfused with either 5mM K^+ or 70mM K^+. The results (9.3) showed that PC12 cells do appear to release ANP into the medium but that depolarizing conditions did not have a reproducible effect on this release. In the second experiment, PC12 cells were exposed to either 5 or 70mM K^+ in flasks (25 cm^2). Although there were almost double the number of cells on the flasks, ANP was not detectable using this method. However, in the third experiment, the supernatant from homogenized cells (supernatant prepared for renin experiments) was assayed for ANP. The results were for trypsinized cells 248 pg/ml and for non-trypsinized cells (scraped from the flasks) 420 pg/ml . 1ml represented the ANP obtained from $75x10^6$ cells. Although the trypsin was external and the cells were not exposed to it for long time, it still affected internal ANP content. This experiment showed that ANP is present within the cells. The presence and release of ANP from PC12 cells suggest a very important role played by this hormone, in normal adrenal cells, possibly through its specific receptors. (autocrine signaling) which in turn regulates blood pressure.

The conclusion that could be drawn from these experiments is that PC12 cells contain intracellular ANP but it was not released under the conditions studied in these experiments. Further experiments are required to confirm whether or not it is indeed ANP (that is being released), or whether the antibody is cross-reacting with similar peptide. This could be investigated by performing HPLC on the samples (Richards *et al.*, 1987).

14. Measurement of intracellular renin in PC12 cells

Ganten *et al.,* (1974) reported the presence of renin-like activity in rat adrenal glands. In latter studies, investigators localized adrenal renin to the zona glomerulosa (Naruse *et al.,* 1984). Nakamaru *et al.,* (1985) have suggested that adrenal renin is involved in the regulation of aldosterone production. The interrelationship of catecholamines and renin angiotensin-aldosterone system (Peach, 1971) has been established by many investigators. Phaeochromocytoma are catecholmine-secreting tumours. They were found to be associated, in patients, with increased plasma renin activities (PRA) and aldosterone levels (Vetter *et al.,* 1975). The present study has shown the presence of renin within rat phaeochromocytoma PC12 cells . This adds further support to the findings reported by Vetter *et al.,* (1975) that phaeochromocytoma cells secrete renin and therefore raises the possibility that these cells may be a useful model system for looking at renin release. At 0°C renin in the supernatant obtained from homogenized PC12 cells [(roughly 150 x 10^6) homogenized in 2 ml of Tris 100mM pH 7.4] was not detectable; however after incubation of the samples of this supernatant for 5h at 37°C, the renin detected was for trypsinized cells, 42 pg/h of A I, and for non-trypsinized cells, 37 pg/h of A I. There was not much difference between the results obtained from trypsinized and non-trypsinized cells, but still it can be said that trypsin contributed to the activation of renin even though PC12 cells were exposed to trypsin only for 5-6 min until they started detaching.

Human nephroblastoma cells were reported by Inglis and Leckie, (in press 1989) to secrete renin and prorenin over 18 days in culture. Renin secreting cell cultures (human transfected juxtaglomerular cells) have been described by Pinet *et al.,* (1987). Therefore, PC12 cells being a cultured tumour cell

line and secreting renin, share these characteristics with human transfected juxtaglomerular cells (culture) and nephroblastoma (tumour).

PC12 cells are known to release catecholamines into the bloodstream elevating blood pressure. The elevation of blood pressure stimulates the release of ANP from the heart, which in turn reduces blood pressure by relaxing smooth muscle cells. Also the renin-angiotensin system plays a major role in regulating blood pressure.

The presence of these three main blood pressure control elements (catecholamines, ANP and the renin -angiotensin system) within PC12 cells, tumours derived from normal adrenal chromaffin cells, shows clearly that these PC12 cells could be an excellent model system for investigating biosynthesis, maturation and secretion of atrial peptides (Nguyen *et al.*, 1988), as well as for the physiological role of the co-secretion of neuropeptides and catecholamines in the nervous system (differentiated PC12 cells).

In conclusion, PC12 cells represent an extremely useful model system in investigating many aspects of blood pressure regulation.

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