# STUDIES ON THE CONTROL OF RENIN RELEASE FROM HUMAN CULTURED NEPHROBLASTOMA CELLS

by

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## ABBREVIATIONS

Abbreviations used are as recommended in the Biochemical Journal Instructions to Authors, 1989, with the following additions:

| | |
|---|---|
| $A_I$ | angiotensin I |
| $A_{II}$ | angiotensin II |
| ANP | atrial natriuretic peptide |
| BAEE | benzoyl arginine ethyl ester |
| cAMP | adenosine 3',5'-cyclic monophosphate. |
| cGMP | guanosine 3',5'-cyclic monophosphate. |
| $[cAMP]_i$ | intracellular cAMP concentration |
| $[cGMP]_e$ | extracellular cGMP concentration |
| dbcAMP | dibutyryl cAMP |
| DG | diacylglycerol |
| EM | electron microscopy |
| Enalaprilat | N-{N-[(S)-1-carboxy-3-phenylpropyl]-L-alanyl}-L-proline. An orally-active inhibitor of angiotensin converting-enzyme. |
| G-protein | guanine nucleotide-binding protein |
| $G_s$ | stimulatory G-protein |
| $G_i$ | inhibitory G-protein |
| $G_o$ | 'other' G-protein. |
| H.77 | D-His-Pro-Phe-His-Leu-Leu-Val-Tyr H.77 is an analogue of the $His^{6}Tyr^{13}$ octapeptide sequence of equine angiotensinogen in which the N-terminal |

| | |
|---|---|
| | histidine residue has been replaced with D-histidine and the cleavage site has been modified by the introduction of a reduced peptide bond (R: $-CH_2-NH-$) in place of the peptide bond normally cleaved by renin. |
| IBMX | 3-isobutyl-1-methylxanthine |
| i.v. | intravenous |
| JG | juxtaglomerular |
| NPY | neuropeptide Y |
| PAGE | polyacrylamide gel electrophoresis |
| PDGF | platelet-derived growth factor |
| SBTI | soybean trypsin inhibitor |
| SV40 | simian virus 40 |
| TEMED | N,N,N',N'-tetramethylethylenediamine |
| Tween 20 | polyoxyethylenesorbitan monolaurate |

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## SUMMARY

A novel *in vitro* model system has been used to study the control of renin release. Human cultured nephroblastoma cells, which secrete active renin and prorenin at high levels, were used to investigate the intracellular messengers that are involved in the renin secretory response to hormones (e.g. $A_{II}$, ANP) and drugs (e.g. isoprenaline, Enalaprilat).

1. The present study showed that treatment with isoprenaline, dbcAMP or forskolin stimulated active renin release at 5h.
2. Long-term (up to 12 days) treatment with isoprenaline, dbcAMP or forskolin caused stimulation of renin release and nephroblastoma cell growth.

Isoprenaline and dbcAMP did not increase the amount of renin secreted per cell. The apparent increase in renin secretion was due to an increase in the number of renin-secreting cells.

By contrast, forskolin increased renin secretion per cell.

3. Propanolol blocked the action of isoprenaline on renin secretion and cell growth.
4. ANP inhibited basal renin release and this was blocked by pertussis toxin.
5. ANP reduced $\beta$-agonist-stimulated renin release.
6. ANP reduced isoprenaline- and dbcAMP-stimulated cell growth.

7. Isoprenaline and forskolin stimulated intracellular [cAMP]. Isoprenaline caused an immediate response (within 1 min) which peaked early (at 5 mins) and was followed in time (at 1h) by an increase in renin release.

Forskolin caused a sustained response which was paralleled (in time) by an increase in renin release.

8. ANP caused a significant stimulation of cGMP release from the cells, which paralleled the inhibition of basal active renin release.

9. ANP reduced the isoprenaline-stimulated increase in intracellular [cAMP] and renin release.

10. ANP reduced forskolin-stimulated renin release but potentiated the forskolin-induced rise in $[cAMP]_i$.

11. The ANP-induced rise in cGMP release was reduced by isoprenaline and forskolin, but forskolin was less effective than isoprenaline.

12. $A_{II}$ reduced basal renin release at 5h.

13. Long-term treatment with $A_{II}$ inhibited both active and total renin release. Long-term treatment with $A_{II}$ had no effect on cell growth.

14. $A_{II}$ concentrations were high in the medium to which no $A_{II}$ had been added. These could be reduced by Enalaprilat with a concomitant stimulation of renin release, indicating $A_{II}$ feedback inhibition on the cells.

15. Long-term treatment of the cells with Enalaprilat caused stimulation of active renin but not prorenin release. Long-term treatment with Enalaprilat had no

effect on cell growth.

16. The presence of $G_s$ and $G_i2$ was detected in membrane preparations from cultured nephroblastoma cells, using SDS/PAGE and immunoblotting.

17. Immunocytochemistry, electron microscopy and karyotype analysis showed that the cells exhibited mesenchymal differentiation, had an ultrastructure indicative of some smooth muscle differentiation and had a 46XX karyotype.

In conclusion, results from the present study show that the nephroblastoma cell model system has proved to be a valuable model <u>in vitro</u> for investigating second messenger involvement in the control of human renin release.

# CHAPTER 1

# INTRODUCTION

## 1.1 The aspartyl proteinase, renin

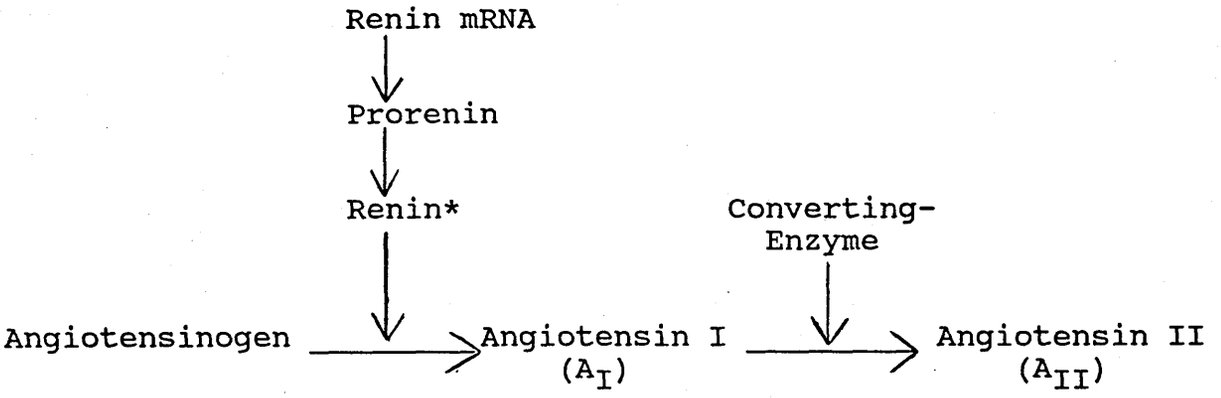
Renin (E.C.3.4.23.15.) is a highly specific aspartyl proteinase. It is the first and rate-limiting enzyme of the renin-angiotensin system, catalysing the formation of angiotensin I ($A_I$) from renin substrate, angiotensinogen (Figure 1.1). Subsequently $A_I$ is cleaved by converting-enzyme to form angiotensin II ($A_{II}$), which is involved in fluid and electrolyte balance and in the control of blood pressure.

Renin is an enzyme which, together with such enzymes as pepsin and cathepsin D, is considered an aspartyl proteinase because of the presence of two aspartic acid residues at the active site which are involved in catalysis. The presence of two catalytically active aspartic $\beta$-carboxyl groups in the active sites of aspartic proteinases was deduced from pH dependence and alkylation experiments (Fruton, 1976). The proposed general acid-general base catalytic mechanism for action of aspartic proteinases in converting substrate to products is depicted in Figure 1.2.

In this mechanism, direct addition of water leads to the formation of a tetrahedral intermediate (Rich *et al.*, 1982) before the peptide bond is broken in the catalytic reaction.

The two aspartyl groups in the enzyme which provide

**Figure 1.1: Renin* is the rate-determining step of the renin-angiotensin system.**



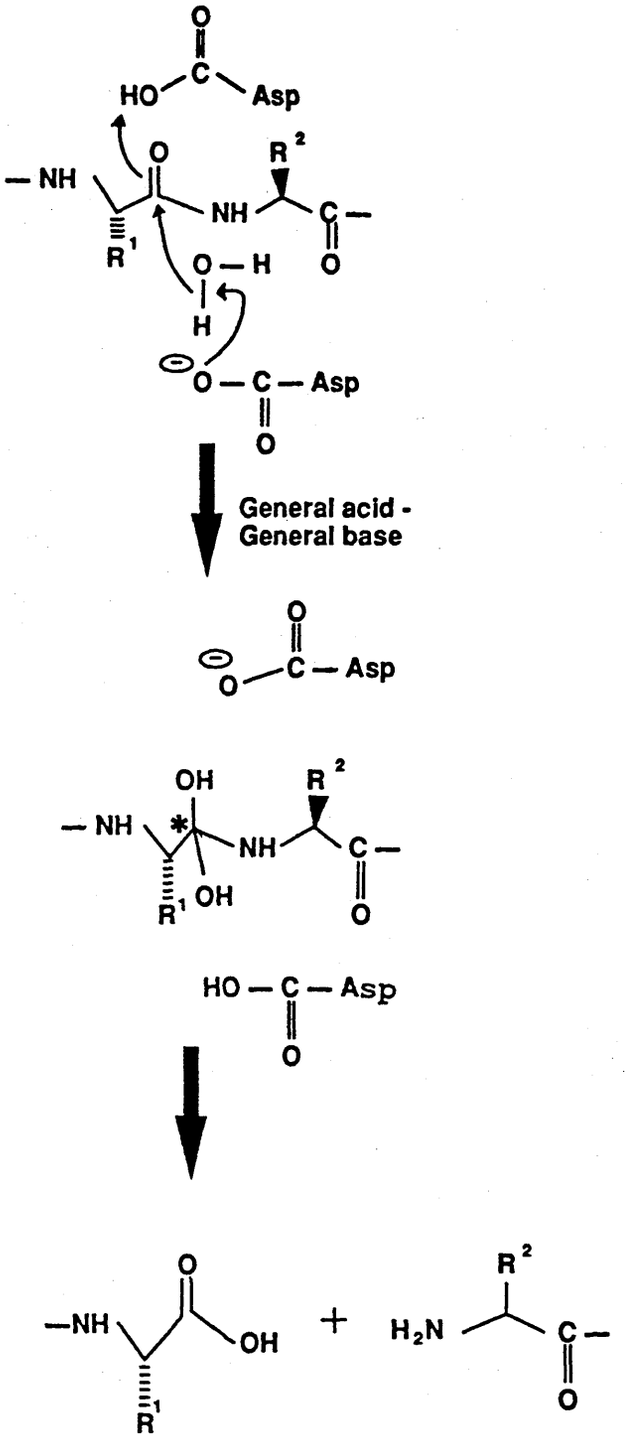



**Figure 1.2: Schematic representation of proposed catalytic mechanism for action of aspartic acid proteinases in converting substrate to products. (Source: Rich, 1985).**
Direct addition of water leads to formation of a tetrahedral intermediate* (Rich et al., 1982).

essential carboxyl groups for the cleavage reaction are a highly conserved feature of the active site of the enzyme, which resembles that of other acid proteinases, in particular that of pepsin.

The human renin gene has been sequenced (Imai et al., 1983; Hardman et al., 1984; Hobart et al., 1984) and the structure of the human renin and human pepsinogen genes are remarkably similar (Sogawa et al., 1983). Both genes are composed of 10 exons (coding sequences) and 9 introns (Hardman et al., 1984). The size of the corresponding exons and the location of introns in the expressed gene sequence for renin and pepsinogen are nearly congruent. For example there is 66% homology between the amino acid sequences of exon 3, which includes the first catalytic aspartyl residues for both human renin and human pepsinogen.

The mouse submaxillary gland renin gene was sequenced by Panthier et al. in 1982, and the mature, human renin peptide is 68% homologous in amino-acid sequence to the mouse enzyme. The two coding nucleotide sequences are 77% homologous (Hobart et al., 1984).

The similarities in the structure of the human renin and human pepsinogen genes support the view that the mammalian aspartyl proteinase family of enzymes (which includes chymosin and cathepsin D) share a common protein structure which is essential for activity (Blundell et al., 1983). This conserved structure is apparently built from peptide domains of similar size that are encoded from

individual exons. Therefore, aspartyl proteinases exhibit conserved homology in their primary, secondary and tertiary structures.

Recently, it has been reported by Lapatto et al. (1989) that the structure of the human immunodeficiency virus-1 (HIV-1) proteinase corresponds closely in certain areas of its primary, much of its secondary and most of its tertiary structure to the established structure of the aspartyl proteinases.

The substrate specificity of the aspartyl proteinases differs widely. For example, pepsin splits its substrate at all the internal aromatic residues. Renin, however, is unique in having a very high degree of specificity and shows no general proteolytic activity. Indeed, renin cleaves at only one site in a single peptide substrate, angiotensinogen and, unlike other aspartyl proteinases, primate renin is species-specific (Braun-Menendèz et al., 1946).

Comparative amino terminal sequences for angiotensinogen from a number of species are listed in Table 1.1. The minimum peptide sequence required for the substrate to be active comprises an octapeptide (Skeggs et al., 1968a). Renin specifically cleaves the peptide bond between the amino acid residues at positions 10 and 11. In the horse, dog and rat, this is a leucyl-leucine bond. In the human, it is a leucyl-valine scissile bond. This reflects the species-specificity of the renin-substrate

**Table 1.1**

**Comparison of the N-terminal sequences of human, equine, canine and rat angiotensinogens.**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 ↓ Renin cleavage site | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Human[a] | asp | arg | val | tyr | ile | his | pro | phe | his | leu | val | ile | his | asn |
| Equine[b] | asp | arg | val | tyr | ile | his | pro | phe | his | leu | leu | val | tyr | ser |
| Canine[a] | asp | arg | val | tyr | ile | his | pro | phe | his | leu | leu | val | tyr | ser |
| Rat[a] | asp | arg | val | tyr | ile | his | pro | phe | his | leu | leu | tyr | tyr | ser |

Key: a. Data from Burton and Quinn, 1988.

b. Data from Skeggs et al., 1968b.

reaction.

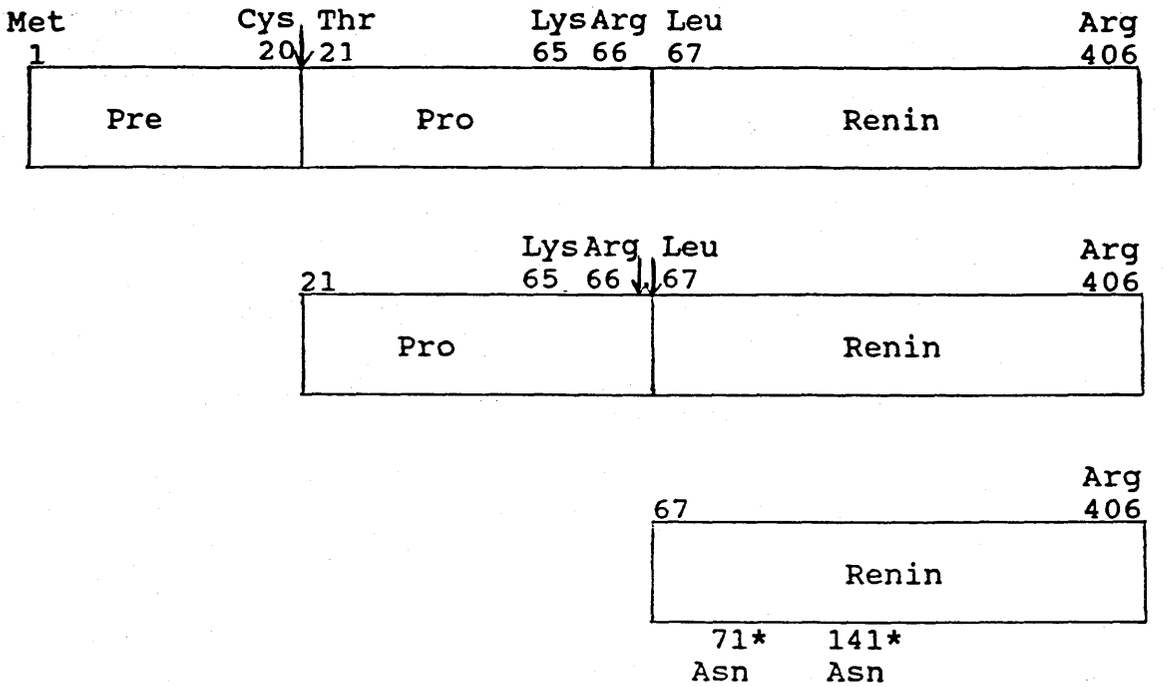
Renin differs from other aspartyl proteinases, such as pepsin, penicillinopepsin and chymosin (Tang, 1973) in that its activity is not optimal at acid pH. The purified animal renins have a broad pH optimum in the range pH 5.5 to pH 7.0 when tested with homologous renin substrates (Inagami et al., 1977). Human kidney renin has a narrower pH optimum when acting on human substrate, at pH 6.0 (Yokosawa et al., 1980).

1.2 Renin biosynthesis

Human renin is synthesized and stored mainly in the juxtaglomerular (JG) cells of the afferent glomerular arteriole of the kidney (Lacasse et al., 1985). Like other proteinases, renin is synthesized as a preproenzyme. Processing of renin involves sequential proteolytic cleavages of the preproform to the active, mature form (Figure 1.3a). Preprorenin is rapidly transferred to the rough endoplasmic reticulum and hydrolysed, probably by a signal peptidase, to produce prorenin. Prorenin is processed at paired basic amino acids in its amino-terminal region and the resultant active, mature renin is stored in granules until its release is stimulated (Figure 1.3b) (Pratt et al., 1983; Hirose et al., 1985). However, the nature of the intracellular sorting signal, the location of renin precursor processing and the enzyme(s) responsible are currently unknown.

Both prorenin and active renin are released by the kidney into the circulation. Recently, the presence of

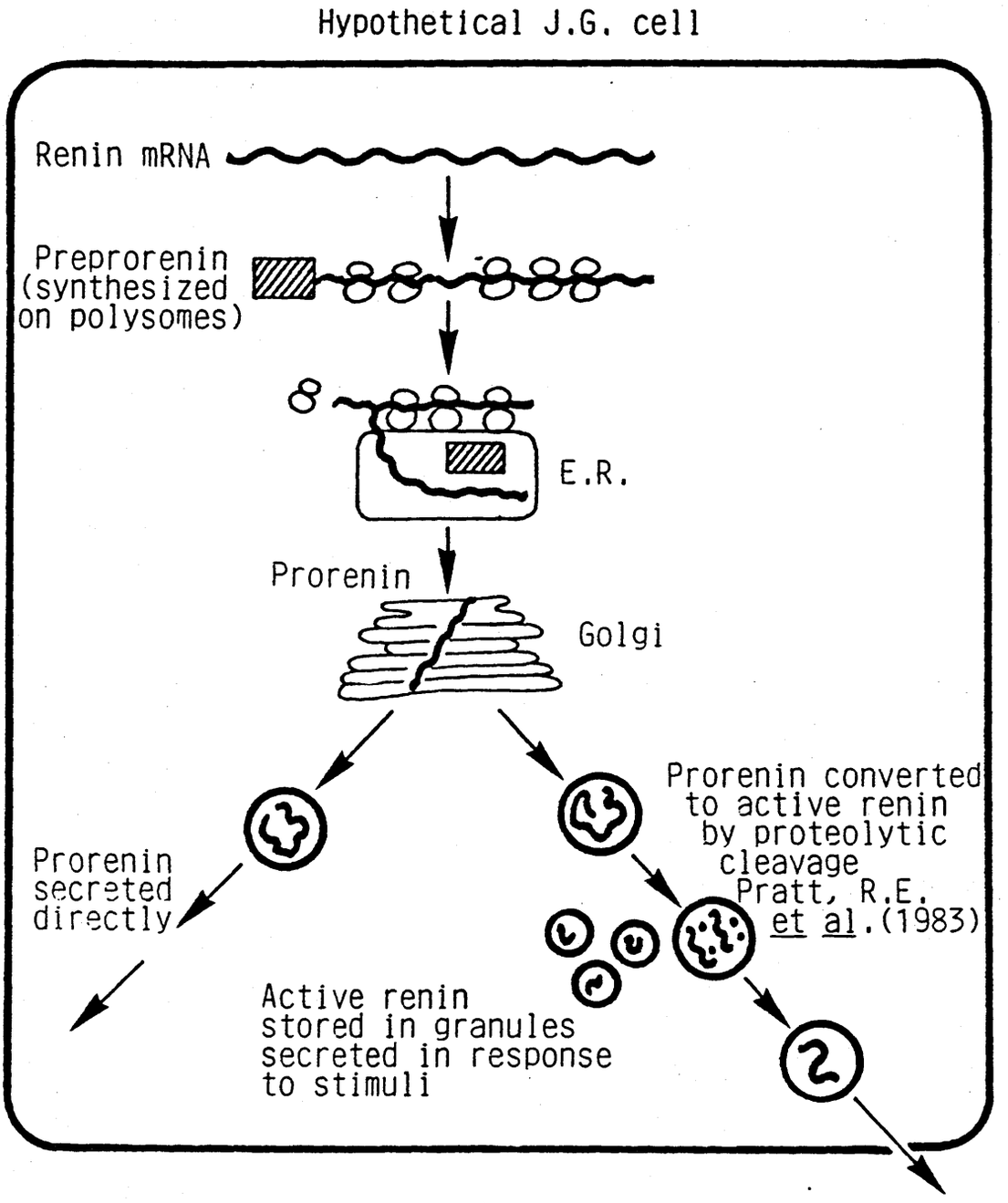
**Figure 1.3a: Schematic representation of the possible maturation process of human kidney renin.**




Adapted from: Imai et al., 1983.

(a) Proposed sites of processing of leader sequence and prosequence are indicated by single and double arrowheads, respectively, as predicted by comparison with the mouse renin precursor (Panthier et al., 1982) and human prepepsinogen (Sogawa et al., 1983). Potential N-glycosylation sites are denoted by asterisks. The mature protein is composed of 340 amino acid residues.

**Figure 1.3.b. Intracellular pathway(s) of renin synthesis and release.**




(b) Proposed intracellular localization of the various forms of renin during biosynthesis and secretion.

more than one pathway of renin secretion has been suggested by several groups of investigators (Fray, 1978; Katz and Malvin, 1982; Pratt et al., 1983). In one pathway (Figure 1.3b), active renin is stored in granules before release, while in another pathway, newly synthesized prorenin is secreted directly.

Although renin is generally considered to be a renal enzyme, it has been found in a number of other tissues such as uterus and placenta (Symonds et al., 1968); blood vessel walls (Re et al., 1982) and brain (Inagami et al., 1982) particularly the anterior pituitary (Saint André, 1986) (for review, see Deboben et al., 1983). Recently, considerable attention has been given to extrarenal sites of renin expression, and there is evidence for renin synthesis in many organs, including the adrenal gland, testis, ovary and brain (Hirose et al., 1980; Naruse et al., 1984; Kim et al., 1987).

Although the significance of such expression is not yet understood, recent studies suggest that local synthesis of $A_{II}$ promotes angiogenesis (Fernandez et al., 1985) and may be important for ovulation (Pellicer et al., 1988). This suggests that the renin-angiotensin system has physiological functions in addition to its classic role in the maintenance of blood pressure.

## 1.3 The renin-angiotensin system

Renin is secreted by the kidney into the bloodstream, where it cleaves the $\alpha_2$-globulin, angiotensinogen, to produce the inactive decapeptide, angiotensin I ($A_I$)

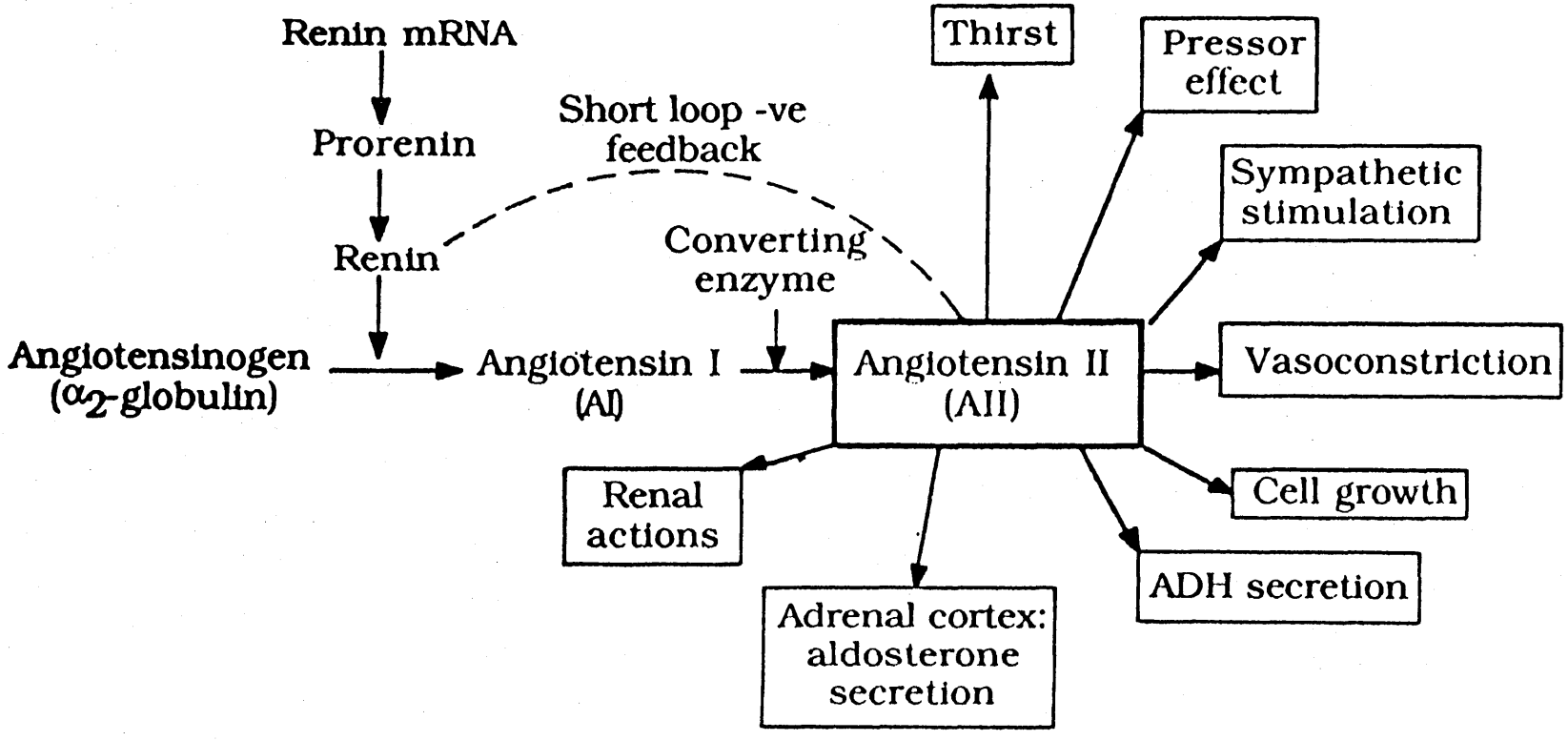
(Figure 1.4). Subsequently, the C-terminal dipeptide of $A_I$ is cleaved by angiotensin converting-enzyme to produce the potent vasoactive octapeptide angiotensin II ($A_{II}$). $A_{II}$ has a very short half-life (approx. 20s.) in the blood (Ferreira and Vane, 1967) and the concentration of circulating renin is the major, rate-limiting step in the production of $A_{II}$ *in vivo*. $A_{II}$ is a potent vasoconstrictor and has other actions (Figure 1.4) such as stimulation of aldosterone secretion from the adrenal cortex and stimulation of smooth muscle cell growth (Lyall *et al.*, 1988a). Therefore, $A_{II}$ is important in cardiovascular homeostasis (Brown *et al.*, 1983).

Up to 90% of human plasma renin exists in an inactive form, which is believed to be the biosynthetic precursor of the active enzyme (Leckie *et al.*, 1977; Sealey *et al.*, 1980; Hsueh, 1984). Conversion of prorenin to active renin probably does not occur in the plasma. However, this inactive renin may be used for local production of active renin by specific tissues. (Section 1.2).

Prorenin, the inactive precursor of active renin can be found in the circulation under certain circumstances when the kidneys are absent (Sealey *et al.*, 1977). However, the kidney is the only organ where secretion of active renin has been conclusively demonstrated (Deboben *et al.*, 1983; Pandey *et al.*, 1985; Ohashi *et al.*, 1985).

Plasma renin levels which are markedly elevated or suppressed in comparison with the normal range are often

**Figure 1.4: The renin angiotensin system and some of the main actions of angiotensin II ($A_{II}$).**



indicative of certain disease states. For example, renal hypertension is often associated with increased levels of plasma active renin (Millar et al., 1978), whereas nephroblastoma patients have elevated levels of circulating prorenin (Carachi et al., 1987a).

1.4 Physiological control of renin release

Renin secretion is under tight control physiologically and it is generally recognized that three groups of mechanisms are involved in the control of renin release: 1) two intrarenal receptors; the renal vascular receptor and the macula densa 2) the renal sympathetic nerves and an adrenergic receptor and 3) several humoral agents including circulating catecholamines, sodium ($Na^+$) and potassium ($K^+$) ions, $A_{II}$, anti-diuretic hormone (ADH) and atrial natriuretic peptide (ANP). The interrelationships of the vascular receptor, the macula densa, the JG cells, the afferent and efferent arterioles and the renal sympathetic nerves are depicted diagrammatically in Figure 1.5.

The three main groups of control mechanisms were identified through the use of animal models, such as conscious dogs (Goldblatt et al., 1934); perfused dog kidneys; sodium depleted dogs with a denervated kidney; isolated rat kidneys; mouse kidney experiments and also studies involving human patients, including tilting in man (Hollenberg et al., 1969). The various physiological mechanisms involved in renin release have been reviewed thoroughly by Davis and Freeman (1976) and also more recently, by Keeton and Campbell (1981).

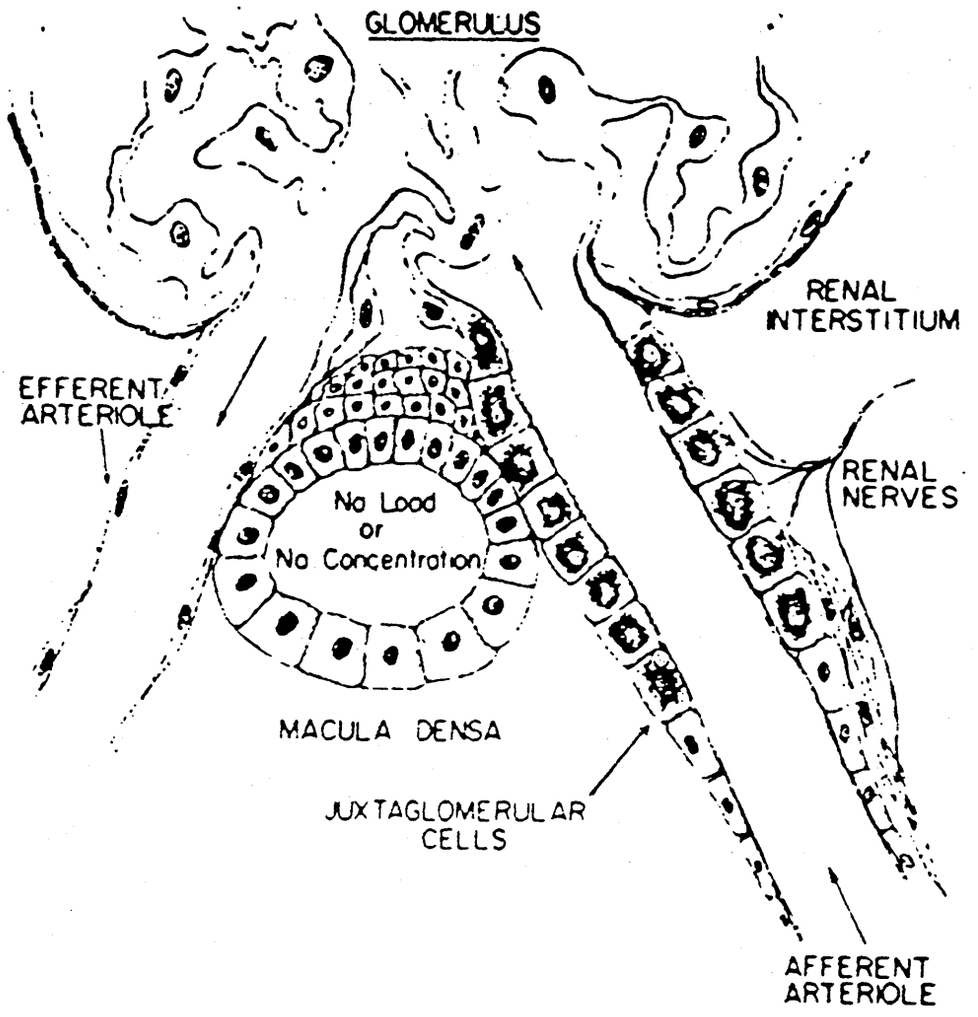



**Figure 1.5: Diagram of renal juxtaglomerular apparatus, which includes the JG cells and macula densa in their close physical relationship.** Dark granules in JG cells are renin-containing granules. Renal vascular receptor includes JG cells and adjacent renal afferent arteriole. Renal nerves are shown ending in both JG cells and smooth muscle cells of renal afferent arteriole. (From, Davis (1971)).

Comparatively little work has been done on the intracellular control of renin release i.e. from the receptor to the effector. Indeed, the sequence of intracellular events within the JG cell, leading to the release of renin, in response to extrinsic stimuli, remains poorly defined. Extrinsic stimuli, such as β-adrenergic agonists, $A_{II}$ or ANP are thought to affect the activity of the renin-secreting cell by altering its intracellular concentrations of certain second messengers.

## 1.5 Second messengers involved in the control of renin release

The main second messengers reported to be involved in the control of renin release are;

1) Adenosine 3',5'-cyclic monophosphate (cAMP).

2) Calcium and 3) Guanosine 3',5'-cyclic monophosphate (cGMP).

### 1.5.1. cAMP:

cAMP is a stimulatory second messenger in many secretory cells (Rubin, 1982) and is almost certainly, a stimulatory second messenger in renin secretion (Peart et al., 1975; Hofbauer et al., 1978; Churchill and Churchill, 1982a). This is because, renin secretion is stimulated by many substances that are known to activate adenylate cyclase (β-adrenergic agonists (Pinet et al., 1987); $A_2$ adenosine receptor agonists; dopamine; glucagon and forskolin (Seamon and Daly, 1981)) and inhibited by other substances known to inhibit adenylate cyclase (α-adrenergic

agonists and $A_1$ adenosine receptor agonists). Moreover, renin secretion is stimulated by exogenous cAMP (Michelakis et al., 1971) and/or dibutyrylcAMP (dbcAMP) and by phosphodiesterase inhibitors (Peart et al., 1975).

$\beta$-Adrenergic agonists stimulate adenylate cyclase (E.C.4.6.1.1.). The adenylate cyclase enzyme system is composed of at least 3 distinct protein components: a hormone receptor; a guanine nucleotide-binding protein (G-protein) which mediates hormonal activation and a catalytic subunit which converts the substrate ATP to cAMP (Rodbell, 1980). Adenylate cyclase can be regulated in both positive and negative fashion by hormones and neurotransmitters. In each case, receptor control of cAMP production is mediated via a G-protein, $G_S$ for the stimulation of adenylate cyclase activity resulting in an increase in intracellular cAMP levels and $G_i$ for inhibition of enzyme activity, which is associated with reduced signal transduction by adenylate cyclase (Gilman, 1987; Spiegel, 1987).

1.5.2. Calcium:

Calcium is a stimulatory second messenger in most secretory cells (Rubin, 1970). However, the available evidence indicates that calcium is an inhibitory second messenger in renin secretion (Peart et al., 1975; Fray, 1980; Churchill and Churchill, 1982a; Churchill, 1985). But renin is not unique in being a secretory protein triggered by a lowering of cytosolic calcium. Notable other examples are parathyroid-hormone (Wallace and Scarpa, 1982; Habener et al., 1984); glucagon (Leclercg-Meyer and Malaisse, 1983)

and human placental lactogen (Handwerger et al., 1981).

Since JG cells are thought to be derived from smooth muscle cells (Barajas, 1979), a logical conclusion was that smooth muscle contraction and renin inhibition induced by $A_{II}$, depend on the concentration of intracellular calcium ($[Ca^{2+}]_i$).

In normal conditions, JG cells are estimated to contain approximately 0.1μM free $Ca^{2+}$ in the cytoplasmic space. (Park et al., 1986) JG cells in vivo, are bathed by extracellular fluid which usually contains between 1 and 3mM $Ca^{2+}$ (Figure 1.6a). JG cell membranes are also electrically polarized (Fishman, 1976; Buhrle et al., 1985) with the intracellular fluid being approximately 70mV negative, with respect to the extracellular fluid (Figure 1.6a).

Consequently, there is a large electrochemical gradient which favours the influx of $Ca^{2+}$ i.e. the electrical potential and the 1,000 to 10,000-fold difference in concentration (Figure 1.6a). In order to maintain this high concentration gradient for $Ca^{2+}$, the cells utilize at least 3 mechanisms: (1) a low basal permeability of the plasma membrane for $Ca^{2+}$, to prevent excessive influx; (2) a highly efficient $Ca^{2+}$ efflux mechanism(s), to extrude residual $Ca^{2+}$ and (3) intracellular systems to sequester excess $Ca^{2+}$ (e.g. mitochondrial uptake, uptake into calciosomes (Burgoyne, 1989).

Inhibitory first messengers could increase JG cell $[Ca^{2+}]_i$ in several ways; decreased $Ca^{2+}$ efflux across the

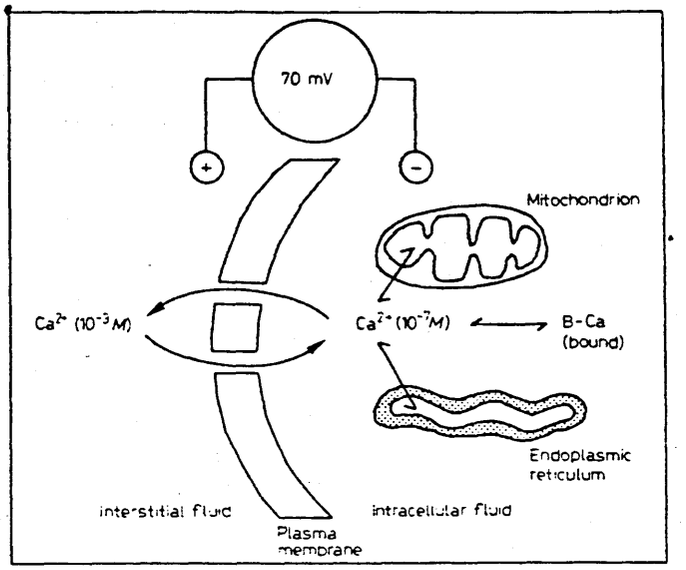



**Figure 1.6.a: Hypothetical JG cell.** Large electrochemical gradient favouring $Ca^{2+}$ influx suggests that efflux pathways must exist even though intracellular $Ca^{2+}$ can be buffered by mitochondrial uptake, by sequestration in endoplasmic reticulum and by $Ca^{2+}$-binding molecules such as proteins and nucleotides (from Churchill, 1985).

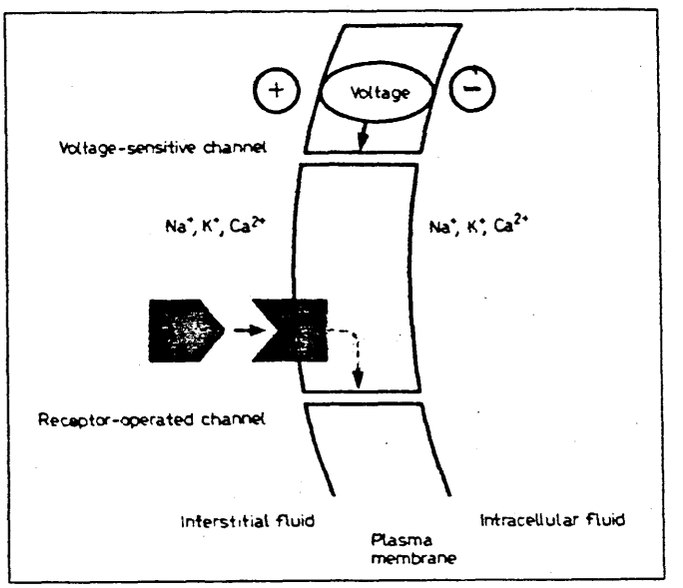



**Figure 1.6.b: Hypothetical JG cell membrane with voltage-sensitive and** proposed receptor-operated **channels.** Voltage sensitive channels for $K^+$, $Na^+$ and $Ca^{2+}$ have been described in many cells. "Receptor-operated" channels are activated when an agonist occupies its receptor on the cell membrane. Angiotensin, vasopressin and adenosine probably activate JG cell "receptor-operated" channels (from Churchill, 1985).

membrane; increased $Ca^{2+}$ influx, and/or mobilization of $Ca^{2+}$ from intracellular sites of sequestration/binding.

The hypothesis that JG cells have two main mechanisms for regulating/changing $[Ca^{2+}]_i$ has been widely reported in the literature. The two mechanisms are: (1) voltage-operated $Ca^{2+}$ channels and (2) proposed receptor-operated $Ca^{2+}$ channels (Figure 1.6b).

Some first messengers increase $[Ca^{2+}]_i$ by depolarizing the cell membrane e.g. membrane-depolarizing concentrations of extracellular $K^+$ (Park and Malvin, 1978; Churchill and Churchill, 1982b) or calcium ionophores. However, the inhibitory effects are blocked by calcium channel antagonists, such as verapamil (Park et al., 1981), which suggests that $Ca^{2+}$ influx mediated (at least partly) the inhibitory effect of; depolarization, or $Ca^{2+}$ ionophores.

Other first messengers have been proposed to activate "receptor-operated" $Ca^{2+}$ channels resulting in a $Ca^{2+}$ influx and/or mobilization, which seems to be independent of membrane potential (Figure 1.6b). It has also been proposed that $A_{II}$ and vasopressin activate "receptor-operated" $Ca^{2+}$ channels. In addition, it has already been shown that $A_{II}$ causes PtdIns4,5$P_2$/PtdIns4P hydrolysis in cultured vascular smooth muscle cells with rapid formation of Ins1,4$P_2$/Ins1,4,5$P_3$ and a diacylglycerol (DG) peak (Griendling et al., 1986). Ins1,4,5$P_3$ has been shown to induce calcium release from nonmitochondrial storage sites (Berridge and Irvine, 1984). Evidence is accumulating which suggests that activation of protein kinase C by DG and increased $[Ca^{2+}]_i$ is the next step in the sequence of

events leading to alterations in the activity of some cells (Churchill and Churchill, 1984; Berridge, 1984).

Whether this is also true for $A_{II}$ inhibition of renin release from JG cells is, as yet, not firmly established.

Also, tumour-promoting phorbol esters, such as 12-0-tetradecanoyl phorbol 13-acetate (TPA) activate protein kinase C by substituting for DG and by increasing the affinity of protein kinase for ambient $Ca^{2+}_i$. TPA was found to inhibit renin secretion, in a concentration-dependent manner, in rat renal cortical slices (Churchill and Churchill, 1984).

Adenosine inhibits renin secretion *in vivo* (Spielman and Thompson, 1982) and adenosine analogues selective for activation of $A_1$ adenosine receptors inhibit renin secretion in isolated, perfused rat kidneys (Murray and Churchill, 1984) and in rat renal cortical slices (Churchill and Churchill, 1985), probably by receptor-dependent hydrolysis of phosphoinositides, in an analogous fashion to that reported for $A_{II}$ and vasopressin.

1.5.3. cGMP:

There is evidence to indicate that renal renin secretion is inhibited by ANP (Garcia *et al.*, 1985); a natriuretic and diuretic peptide produced by the cardiac atria of several mammalian species, including man (De Bold *et al.*, 1981; Kangawa and Matsuo, 1984). In the kidney, ANP interacts with guanylate cyclase-linked receptors (Ohlstein and Berkowitz, 1985) resulting in increased cGMP

concentration in glomeruli and distal tubules. Increased release of cGMP can alter $Ca^{2+}$ fluxes (Cornwell and Lincoln, 1989) and cAMP levels in some cells (Ishikawa et al., 1985; Anand-Srivastava, 1985) and, therefore, cGMP is emerging as a second messenger of increasing importance in the control of renin release.

Kurtz et al. (1986) have reported that inhibition of renin release by ANP in cultured rat JG cells was paralleled by an increase in cellular cGMP levels. The mechanism of elevation of cGMP by ANP is due to activation of the particulate isoenzyme form of guanylate cyclase (Winquist et al., 1984; Tremblay et al., 1985; Murad, 1986) and stimulation of particulate guanylate cyclase by ANF has been demonstrated for a number of different tissues (Winquist et al., 1984; Hamet et al., 1984; Matsuoka et al., 1985).

There are increasing numbers of reports concerning ANP receptor subtypes in various tissues, and cDNAs encoding two separate receptor proteins have recently been cloned (Fuller et al., 1988; Chinkers et al., 1989). Maack et al. (1987) proposed the model of two types of human ANP (hANP) receptors and their functions. One is the 'B' receptor which is coupled with the membrane form of guanylate cyclase (particulate) and is responsible for the biological activity of ANP.

Another is the 'C' receptor which is not coupled with guanylate cyclase and is probably responsible for the clearance of ANP. Presumably a guanylate cyclase-linked

receptor is involved in the ANP-induced suppression of renin release.

However, the effect of ANP on renin secretion is still controversial, as some authors (Burnett et al., 1984) have reported an inhibition of renin release in models such as the perfused dog kidney and others (Hackenthal et al., 1985) have reported a stimulation of renin secretion in the isolated, perfused rat kidney.

Also, pharmacological discrepancies of ANP action, such as those reported by Resink et al. (1988), raise the possibility that changes in cGMP are an associated phenomenon rather than a primary mechanism of ANP action.

Therefore, at present, details of the molecular mechanism by which ANP alters renin secretion are still unclear.

1.6. $Ca^{2+}$-cAMP(-cGMP) interactions

Theoretically, if both $Ca^{2+}$ and cAMP are second messengers, they could act independently or they might act sequentially in either of two ways: increased $[cAMP]_i$ leads to decreased $[Ca^{2+}]_i$ leads to increased renin secretion or alternatively: decreased $[Ca^{2+}]_i$ leads to increased $[cAMP]_i$ leads to increased renin secretion. These theoretical possibilities are illustrated in Figure 1.7 and are supported by the observations that increased $[Ca^{2+}]_i$ can lead to decreased $[cAMP]_i$ (by inhibiting adenylate cyclase and/or by stimulating phosphodiesterase(s)) and that increased $[cAMP]_i$ can lead to decreased $[Ca^{2+}]_i$ by

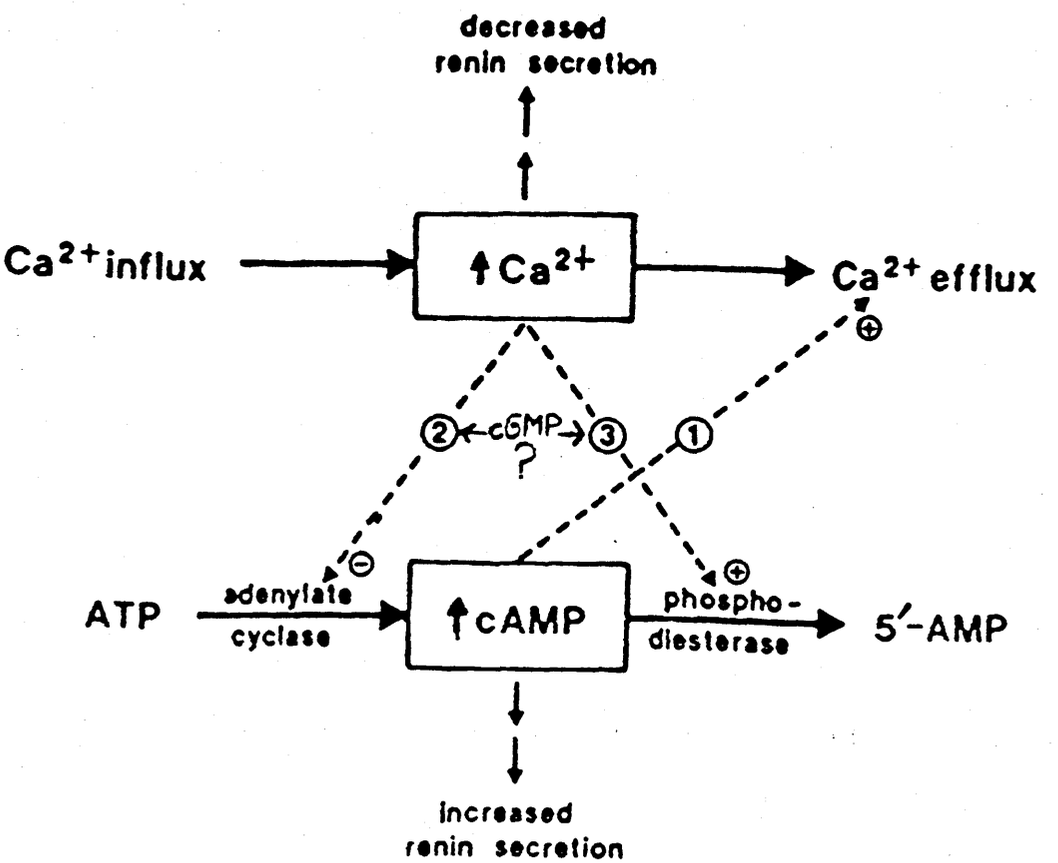



**Figure 1.7: A schematic of possible interactions between $Ca^{2+}$ and cAMP second messenger systems in renin secretion**
Experimental observations indicate that $Ca^{2+}$ is inhibitory (top) and that cAMP is stimulatory (bottom). Increased $cAMP_i$ could decrease $Ca^{2+}{}_i$ by stimulating $Ca^{2+}$ efflux and/or sequestration (pathway 1). Increased $Ca^{2+}{}_i$ could decrease $cAMP_i$ by inhibiting its synthesis (adenylate cyclase; pathway 2) and/or by stimulating its destruction (phosphodiesterase; pathway 3) (as could cGMP*) (from Churchill, 1985).

stimulating $Na^+$-$K^+$-ATP'ase and $Na^+$-$Ca^{2+}$ exchange and/or by stimulating $Ca^{2+}$-ATP'ase) (reviewed by Rasmussen and Barrett, 1984). Thus, if acting sequentially, either $Ca^{2+}$ or cAMP could theoretically be the predominant, or the final, second messenger.

Pinet et al. (1987) showed that $A_{II}$ did not alter basal cAMP release, and was also unable to reduce forskolin-stimulated cAMP release although $A_{II}$ did reduce forskolin-stimulated prorenin secretion. These results support the hypothesis that cAMP and $Ca^{2+}$ act independently on prorenin secretion, but that the inhibitory effect of the rise in $[Ca^{2+}]_i$ predominates over the stimulatory effect of the rise in $[cAMP]_i$.

Pinet et al. (1987) also investigated the role of ANP on forskolin-stimulated prorenin secretion, in a dose-dependent manner, and no change was observed in the forskolin-stimulated cAMP release although ANP did reduce forskolin-stimulated prorenin secretion. However, other authors have reported that ANP inhibits the forskolin and ADH-induced rise in cAMP (Ishikawa et al., 1985). Therefore, cGMP most probably has an interactive role with cAMP (Figure 1.7).

Neuropeptide Y (NPY), a 36 amino-acid first isolated from porcine brain (Tatemoto and Mutt, 1980), possesses direct vasoconstrictor properties that are not dependent on simultaneous adrenergic activation. NPY has been shown to be a potent inhibitor of cAMP accumulation in feline

cerebral blood vessels (Fredholm et al., 1985) and also to inhibit cardiac adenylate cyclase through a pertussis toxin-sensitive G-protein (Kassis et al., 1987). Whether it has the same effects on human JG cells and whether its actions are dependent on $Ca^{2+}$ is, as yet, not firmly established.

The results obtained from cell culture systems, to date, support the view that $\beta$-agonists, $A_{II}$ and ANP affect renin secretion by a direct action on human and rat JG cells. However, the intracellular signals involved and their exact interactions are, as yet, not well defined.

1.7. In vitro model systems used to date

The intracellular control of renin release is not well established. This reflects the limitations of the preparations used to study renin release in vitro. A major obstacle in preparing an in vitro model system is the fact that JG cells represent only 0.03% of the total cell population of the renal cortex (Fray and Park, 1987).

Most studies of renin secretion have been performed in complex systems, such as whole animals; the isolated, perfused kidney; kidney cortex slices and isolated glomeruli with attached afferent arterioles (Hackenthal et al., 1983). These systems included potential influences of endothelial, mesangial and vascular smooth muscle cells that are located close to the JG cells and, in these preparations, it is impossible to measure cellular responses and intracellular events in renin-secreting cells.

The most suitable *in vitro* model appears to be the culture of highly purified JG cells. However, establishing JG cell cultures has, in the past, proved difficult, mainly because of the scarcity of these cells in the kidney. Primary cultures of renin-secreting cells from the rat were recently reported by Kurtz *et al.* (1984). Also, primary cultures of renin-secreting cells have been described in the human in two cases of JG cell tumours (Conn *et al.*, 1972; Galen *et al.*, 1984). Galen *et al.* (1984), cultured renin-secreting tumour cells for longer than one month in both primary and secondary cultures. In this study, renin was synthesized as an inactive precursor that had all of the biochemical and immunological characteristics of pure standard renin.

The sole attempt to maintain a rat JG cell culture has been made by Rightsel *et al.* (1982) (Table 1.2) by subculturing and cloning dissociated cortical cells from neonatal rat kidney.

Pinet *et al.* (1985) obtained continuous JG cell lines producing renin which they maintained by transfection with three different SV40 mutants. These cell lines provided a source of renin-producing cells available for the study of human prorenin regulation *in vitro*. However, the rate of renin secretion was low and more than 95% of the renin secreted into the medium was in an inactive form.

Also, Pratt *et al.* (1988) have transfected the human renin gene into established, cultured mammalian cell lines.

The resultant cell lines contain the human gene stably integrated into the host cell genome, providing a consistent cell line for studies of renin biosynthesis and secretion. These authors compared renin biosynthesis and secretion in 3 transfected cell lines: mouse fibroblast (L929), Chinese hamster ovary (CHO) and mouse pituitary tumour (AtT-20) cells, but they did not carry out extensive studies, designed to look at the control of active and inactive renin release.

For a summary of the main *in vitro* model systems of renin release used to date, see Table 1.2.

## 1.8. Human nephroblastoma cells as an *in vitro* model

In the present study, cultured human nephroblastoma cells have been used as a model *in vitro*, to study the control of renin release at the second messenger level.

Nephroblastoma, or Wilms' tumour, is a paediatric neoplasm, derived from pluripotent mesodermal cells which can differentiate into all known structures of the normal kidney (Lindop *et al.*, 1984). It has previously been shown by Carachi *et al.* (1987a), that patients with nephroblastoma have high, circulating concentrations of prorenin.

Cells were derived from an excised tumour and a primary culture was established by standard methods (Carachi *et al.*, 1987b). Inglis and Leckie (1990) have shown that nephroblastoma cells in culture, secrete both active renin and the enzymatically inactive precursor; prorenin, at high levels.

**Table 1.2:**

**In vitro model systems of renin release used to date.**

| In vitro model. | Authors. |
|---|---|
| Cultured rat JG cells. (primary cultures) | Rightsel et al., 1982<br>Kurtz et al., 1986 |
| Human tumoral JG cells. (primary cultures, secreted mainly inactive renin) | Conn et al., 1972<br>Galen et al., 1984 |
| SV40 transformed tumoral JG cells. (permanent line) | Pinet et al., 1985<br>Pinet et al., 1987 |
| Cultured mouse JG cells. (primary cultures) | Lacasse et al., 1987 |
| Cultured human mesangial cells. (long-term culture) | Chansel et al., 1987 |
| Cultured human chorionic cells. (possible permanent line). | Pinet et al., 1988 |
| Mouse pituitary AtT-20 cells transfected with human renin gene. (permanent) | Fritz et al., 1987<br>Pratt et al., 1988<br>Nakayama et al., 1989 |

Renin from the cultured cells was found to be identical to pure human renin from normal kidney in its binding to renin antibodies and in its behaviour on H.77 affinity columns (Leckie, 1987, personal communication).

## 1.9. Aim of the present study

The control of renin release from human JG cells is, as yet, not clearly defined. As renin is the rate-limiting step in the renin-angiotensin system, it is of particular importance in the control of normal blood pressure and in some forms of hypertension. However, as was discussed in the preceding sections, the precise control mechanisms of renin release, at the second messenger level, are not firmly established.

The aim of the present study was, therefore, to investigate the control of human renin release, using a novel *in vitro* model system. Initially pharmacological agents, known to stimulate or inhibit renin release *in vivo* were tested *in vitro* and subsequently, a study of the possible second messengers involved in the responses, was carried out.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1. Materials.

### 2.1.1. Biochemicals

All reagents used were 'AnalaR' grade. With the exceptions of the compounds listed below the reagents were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Tris(hydroxymethyl)aminomethane(crystallised) ethylene diamine tetra acetic acid (EDTA), bovine serum albumin (Fraction V), human serum albumin (Fraction V), soybean trypsin inhibitor (SBTI), sodium acetate, disodium phosphate, monopotassium phosphate, neomycin sulphate, guanosine 3',5'-cyclic monophosphate($Na^+$ salt), 3-isobutyl-1-methylxanthine (IBMX), polyoxyethylenesorbitan monolaurate (Tween 20), sodium deoxycholate, dithiothreitol, thimerosol, orthodianasidine hydrochloride, (-)isoproterenol HCl(isoprenaline), angiotensin II (human sequence), forskolin, pertussis toxin (islet activating protein), propanolol and dibutyryl cyclic AMP (dbcAMP) were purchased from Sigma Chemical Company Ltd., Poole, Dorset, U.K.

Dextran T70 was purchased from Pharmacia (Great Britain) Ltd., Hounslow, Middlesex.

Human ANP(1-28) was purchased from Bachem Ltd., Saffron Walden, Essex.

Glycine and trichloroacetic acid (TCA) were purchased from Fisons, Loughborough, Leicestershire.

($Ile^5$)-angiotensin I was obtained from Schwartz Bioresearch, Orangeburg, New York.

$Asp^1$-$NH_2$-$Val^5$-angiotensin II ('Hypertensin') was obtained from Ciba-Geigy, Horsham, Sussex.

Enalaprilat was obtained from Merck Sharp and Dohme (U.K.) Ltd.

2.1.2. Reagents for polyacrylamide gel electrophoresis.

Acrylamide and N,N,N',N'-tetramethylethylenediamine (TEMED) were purchased from Sigma Chemical Company Ltd., Poole, Dorset, U.K. N,N'-methylene-bis-acrylamide was obtained from Fisons (Electrophoresis reagent), Loughborough, Leicestershire. Pre-stained molecular mass markers were obtained from Bethesda Research Labs. Ammonium persulphate (AnalaR grade) was purchased from BDH Chemicals Ltd., Poole, Dorset, U.K.

2.1.3. Enzymes

Trypsin (E.C. 3.4.21.4.) from bovine pancreas (type III) was purchased from Sigma Chemical Company Ltd., Poole, Dorset, U.K. The specific activity of trypsin was 10,500 units/mg protein where one B.A.E.E. unit will produce a $\Delta A_{253}$ of 0.001 per min in 3.2 ml at pH 7.6 at 25°C (1 cm light path).

International Reference Standard Human Renin (MRC reagent 68/356) was obtained from the National Institute for Biological Standards and Control, Hampstead, London, U.K.

2.1.4. Radiochemicals

[$^{125}$I] Tyr$^{4}$-Angiotensin I, Angiotensin II (5-L-isoleucine), [tyrosyl-$^{125}$I]- and Guanosine 3',5'-cyclic phosphoric acid, [$^{125}$I] 2'-0-succinyl (iodosine methyl ester), all 2,200 Ci/mmole, were purchased from DuPont (U.K.) Ltd., Biotechnology Systems Division, NEN Research Products, Hertfordshire.

Cyclic AMP assay kits containing [8-$^{3}$H]Adenosine 3',5'-phosphate; 180 pmol/5$\mu$Ci were purchased from Amersham International PLC, Aylesbury, Buckinghamshire.

2.1.5. Cell culture reagents/disposables

Tissue culture flasks (25 cm$^2$, 75 cm$^2$) (Nunc), Medium 199, foetal calf serum, colcemid, glutamine and fungizone were purchased from Gibco BRL, Life Technologies Ltd., Paisley, Renfrewshire, Scotland.

Sterile flaskettes (Lab-Tek) were supplied by Flow Laboratories, Irvine, Scotland.

Penicillin/streptomycin solution, Earle's balanced salt solution (without $Ca^{2+}$ and $Mg^{2+}$), trypsin (0.05% (v/v)/E.D.T.A. (0.02%) (v/v)) and Hank's balanced salt solution (HBSS) were obtained from Flow Laboratories Ltd., Irvine, Scotland.

Tissue culture flasks (125 cm$^2$) were purchased from G. Bibby and Company, Stone, Staffordshire.

Cell scraper was purchased from Costar (Costar Corporation, Cambridge MA 02139) (suppliers: Northumbria Biologicals Ltd., Northumberland, U.K.).

24 well tissue culture plates were obtained from Corning (Corning Glass Works, Corning, New York 14831).

Sterile, 0.22μm (low protein binding) filter units were obtained from Millipore S.A., 67, Molsheim, France.

Microcentrifuge tubes were obtained from Scotlab, Bellshill, U.K. and were autoclaved for cell culture work using a pressure cooker (Prestige).

Sterile, disposable pipettes (10 ml) and sterile bijoux (7 ml) were purchased from Sterilin Ltd., Hounslow, U.K.

Sterile, disposable pastettes and sterile universals (30 ml) were purchased from Alpha Labs, Eastleigh, Hampshire.

Dimethylsulphoxide (DMSO) 'AnalaR' grade was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Sterile cryotubes for cell storage in liquid nitrogen were purchased from L.I.P., Shipley, West Yorkshire.

2.1.6. Miscellaneous materials

Glass microscope slides (frosted) (Chance) were supplied by Gallenkamp, Loughborough.

Filter papers were purchased from Whatman Ltd.

Liquid scintillator NE260 was obtained from N.E. Technology Ltd., Sighthill, Edinburgh, U.K.

Scintillation vials were obtained from L.I.P., Shipley, West Yorkshire.

2.2. General methods

2.2.1. Glassware

(i) All items of glassware were washed in solutions of the detergent Decon 75 (Decon Laboratories Ltd., Hove, U.K.), rinsed thoroughly with tap water, then distilled

water, and dried in an oven at 60°C.

(ii) Glass plates used for SDS/PAGE were thoroughly cleaned with alcohol (70% EtOH) before procedure (i).

2.2.2. Micropipetting

Solution volumes in the range 10µl to 1ml were transferred reproducibly using Gilson pipettes (Gilson Medical Electronics (France) S.A., Villiers-Le-Bel, France) or using BCL 8000 automatic dispensers and BCL 8000 syringes (Boehringer Mannheim, Lewes, East Sussex).

Graduated pipette tips were obtained from Alpha Labs, Eastleigh, Hampshire.

Volumes in the range 0.5µl to 50µl were transferred accurately, using Hamilton microsyringes (BDH apparatus, BDH Ltd., Thornliebank, Glasgow, U.K.).

2.2.3. pH measurement

Measurements of pH were carried out using an Elcomatic Instruments Ltd. (Kent), model 7065 digital pH meter fitted with a combination electrode (Gallenkamp and Company Ltd., East Kilbride, Glasgow, U.K.). This apparatus was standardised regularly, using solutions of pH 7.0, pH 4.0 and pH 9.2 prepared using buffer tablets (BDH Chemicals Ltd., Poole, Dorset, U.K.).

2.2.4. Centrifugation

Accelerations of up to 1000g were obtained using a 'Centra-7R' benchtop centrifuge (Damon/I.E.C. (U.K.) Ltd., Dunstable, Beds., U.K.). Accelerations of up to 100,000g were obtained using an LKB 2332 ULTROSPIN 85

ultracentrifuge (LKB, Pharmacia House, Milton Keynes, Bucks.) fitted with a 45 Ti rotor (Beckman RIIC Ltd., Glenrothes, Fife, U.K.).

For small samples, accelerations of 10,000g were obtained using an MSE microcentrifuge 'Microcentaur', MSE Scientific Instruments, Crawley, West Sussex, U.K.

2.2.5. Measurement of protein concentration.

Protein concentration was measured by a modification of the method described by Lowry et al. (1951).

Stock solutions were prepared as follows:

Buffer A: 2% (w/v) $Na_2CO_3$ (anhydrous) in 0.1M NaOH.

Buffer $B_1$: 4% (w/v) sodium potassium tartrate tetrahydrate in distilled water.

Buffer $B_2$: 2% (w/v) $CuSO_4.5H_2O$ in distilled water.

Solution 1 was prepared by mixing Buffers A, $B_1$ and $B_2$ in the ratio 40:1:1, respectively, shortly before use. 1ml of solution 1 was added to 100$\mu$l of each of the samples (in 10mM Tris pH 7.4) to be assayed. These were then vortexed and left to stand for 10 minutes at room temperature. Next, 100$\mu$l of solution 2 was added, the mixture vortexed immediately and allowed to stand for at least 30 minutes prior to determination of the absorbance at 700nm. Protein standards (0-25$\mu$gs) were prepared using bovine serum albumin (Fraction V) dissolved in distilled water. Normally, 5 protein dilutions and 5 blanks of the appropriate buffer were taken for each sample.

2.2.6. Distilled water

Glass distilled water stored in glass containers was

used in all experiments.

## 2.3. Cell culture.

### 2.3.1. General equipment.

The general items of equipment used in cell culture with their suppliers are;

| | | |
|---|---|---|
| Class II Microbiological Safety Cabinet | : | Medical Air Technology Ltd., Denton, Manchester. |
| Heraeus $CO_2$ incubator | : | McQuilken Ltd., Polmadie, Glasgow. |
| Zeiss Invertoscope IDO2 | : | Carl Zeiss (Oberkochen Ltd.) Welwyn Garden City, Herts., U.K. |
| Electronic Coulter Counter (ZM) | : | Coulter Electronics Ltd., Luton, Beds., U.K. |

### 2.3.2. Routine culture

Nephroblastoma cells were cultured (Nunc flasks) in Medium 199, supplemented with 15% (v/v) foetal calf serum (FCS), 2mM glutamine, 2.5$\mu$g of fungizone/ml and 50 I.U. penicillin/50$\mu$g of streptomycin/ml and used before the 10th subculture.

The cells were incubated in an atmosphere containing 5% $CO_2$ at 37$^{o}$C.

2.3.3. Agonist experiments

2.3.3.(i) Short-term experiments

In short-term release experiments, cells were seeded and grown to confluence in 24-well tissue culture plates. At the end of a basal incubation period (18h), the medium was removed and replaced with medium containing the appropriate agent, and the cells were incubated for 5 hours.

Results of all short-term (5h) experiments are expressed as 'active renin release, % change'. Active renin concentrations were determined for basal and experimental samples using the radioimmunoassay method of Millar et al., 1980 (Section 2.4.1.). These were then corrected to rate of renin release per hour of cell culture and the percentage change was calculated.

2.3.3.(ii) 24 hour experiments

In 24 hour experiments, the protocol was identical to 2.3.3.(i) above except that the experiments were continued for up to 24 hours. The active renin concentrations were measured (Millar et al., 1980) in the cell culture supernatants, at 24 hours.

Cells were harvested at 24 hours, using Earle's balanced salt solution (without $Ca^{2+}$ and $Mg^{2+}$), followed by a 0.05% (v/v) trypsin/0.02% (v/v) EDTA solution.

The cell suspensions were used to determine cell number using a ZM electronic coulter counter.

2.3.3.(iii). Long-term experiments

In long-term release and growth-rate experiments, cells were seeded and grown for 3-4 days. Medium was removed and replaced with medium containing the appropriate agent, and growth was continued for up to 12 days.

On day 3 of the experiments, the culture medium was changed, and at 3 day intervals (except in the isoprenaline/propanolol experiments (Section 3.2.3.)) samples were taken for active and total renin estimations (Section 2.4.1, 2.4.2).

Cells were harvested at intervals (Section 3.2.3) and counted, as previously described in Section 2.3.3.(ii).

In short-term release experiments, a minimum of four wells were used per agent and, in all other experiments, a minimum of 6 wells per agent were used. All experiments were carried out at least twice.

2.4. Assays

2.4.1. Renin assay

Renin concentration was measured using the method of Millar et al., 1980. This method is based on the radioimmunoassay of the angiotensin I generated during the incubation of renin with excess ox renin substrate, using an antibody trapping technique.

The following stock reagents were prepared:

Buffer A: 3M-Tris/HCl(pH 6.9)/0.2M EDTA.

Buffer B: 50mM-Tris/HCl (pH 7.4)/5mM EDTA
0.5% (w/v) bovine serum albumin
(Fraction V)/0.2% (w/v) neomycin sulphate.

Buffer C: 0.05M-Tris/HCl (pH 7.5)/0.18% (w/v) human serum albumin (fraction V)/0.35% (w/v) neomycin sulphate.

Dextran coated charcoal: 6.3g of charcoal and 0.125g of Dextran T-70 were suspended in 100 ml of Buffer B.

Assays were carried out by incubating 35µl of cell culture medium at 37°C with 35µl of a pre-mixed solution consisting of ox renin substrate (Millar et al., 1980), Buffer A and antiserum to angiotensin I, diluted in Buffer B (1:80 (v/v)). The ratio of the pre-mixed components was 2:2:1 by volume. The enzyme reaction was terminated by adding 0.5mls of ice-cold Buffer B.

10pg (5,000 - 10,000 cpm) of ($^{125}$I)angiotensin I in 50µl of Buffer C was added to the mixture and the radioimmunoassay was completed by further incubation at 4°C for 48 hours.

The separation of free and bound ligand was achieved by adding 200µls of Dextran coated charcoal and immediately centrifuging the mixture for 10 minutes at 1,000g and 4°C. The supernatant solution was removed by suction and the ($^{125}$I)angiotensin I adsorbed to the charcoal pellet was then measured using a model NE 1612 gamma counter (Nuclear Enterprises Ltd., Sighthill, Edinburgh, U.K.).

Standard curves for angiotensin I were prepared by serial dilution of (Ile$^{5}$)angiotensin I in 35µls of 7% human serum albumin (Fraction V) from 1,000pg to 8pg angiotensin I per tube in duplicate. Tubes containing no angiotensin I

*Data for intra- and inter-assay variation, recoveries of active renin across activation and assay steps, recovery of angiotensin I, detection limit of renin and radioimmunoassay sensitivity for angiotensin I have been reported previously (Miller et al., 1980).

were also included. 35$\mu$l of the pre-mixed substrate solution was added to each tube and the standards were then treated in an identical manner to the unknown assay samples.

Originally, for each cell culture sample, the velocity of angiotensin I generation was determined using one timed incubation in duplicate and a zero time measurement. In practice, angiotensin I concentrations at zero time were undetectable and therefore only a 3 hour incubation was used.

The assay results were expressed in International Units of renin by comparison with the International Reference Standard Preparation of Human Renin (M.R.C. reagent 68/356).

One hundred $\mu$Units/ml of renin generated angiotensin I at a rate of 13.14ng/ml/hr in the above assay system*.

2.4.2. Inactive renin

Inactive renin was assayed by measuring the increase in renin activity produced by the treatment of samples with trypsin.

The activation of inactive renin was carried out using a modification of the method, as described by Millar et al., 1980.

The following stock solutions were prepared:

Trypsin solution: Crystalline trypsin 10,500 B.A.E.E. Units/mg protein (Section 2.1.3.), was dissolved in 1mM-HCl to a final concentration of 10mgs/ml. Fresh

solutions of the enzyme were prepared each day. The crystalline enzyme was stored at -20°C with dessicant.

SBTI solution : Soybean trypsin inhibitor was dissolved in 1mM HCl to a final concentration of 20mgs/ml.

Usually 500µl of cell culture supernatant was activated by treatment with 50µls of trypsin solution and the solution was incubated for 5 minutes at 4°C. The reaction was stopped by adding 50µl of SBTI solution and the renin concentration was then measured, as described in Section 2.4.1. Often, trypsin activated samples required dilution (in Buffer B - see Section 2.4.1.), prior to renin concentration measurement. Initially, zero time samples were included in the renin assay to allow for angiotensin I immunoreactive material generated during the incubation of unknown samples with trypsin. As angiotensin I concentrations were undetectable, this step was omitted from subsequent assays.

The renin concentration measured after trypsin-activation was defined as the total renin concentration. The active renin concentration was measured after an identical incubation at 4°C, except that the samples had not been trypsin-activated. The inactive renin concentration was calculated as the difference between the

total renin concentration and the active renin concentration*.

2.4.3. Angiotensin II ($A_{II}$) assay

The preparation of antisera and the radioimmunoassay for angiotensin II ($A_{II}$) were performed, essentially, using the methods as described by Morton et al. 1976; Morton and Webb (1985) (except that Sep-pak extraction was not carried out).

For the radioimmunoassay of $A_{II}$, the following stock reagent was prepared:

Buffer A: 0.05M-Tris/HCl (pH 7.5)/0.18% (w/v) human serum albumin (fraction V)/0.35% (w/v) neomycin sulphate.

Briefly, $A_{II}$ antiserum (100$\mu$l at 1:30,000) was mxied with standard (100$\mu$l of 0-100pg) or unknown (100$\mu$l) and $^{125}$I-labelled $A_{II}$ (50$\mu$l, 2.5pg) in Buffer A for 18h at 4$^{o}$C.

Bound label was separated from free using dextran-coated charcoal.

2.4.4. cGMP assay

At the end of agonist experiments, typically 0.25mls of cell culture supernatant was extracted into 1ml of chilled methanol (100%) (Williams, B.C., 1988, personal communication). The purpose of the chilled methanol is to precipitate out protein and to effectively extract cGMP and also, to concentrate the sample. The extraction mixture was centrifuged, at 1000g for 10 mins at 4$^{o}$C, to pellet out the protein. The supernatant was carefully removed and the pellet was washed with a further 0.5mls of chilled methanol

(100%). The mixture was once again, centrifuged and the supernatants were pooled for each sample.

For radioimmunoassay of cGMP, the following stock reagents were prepared:

Buffer A: 0.05M-Tris pH 7.5 containing 0.5mmol/l 3-isobutyl-1-methyl xanthine (IBMX).

Buffer B: 50mM-Tris pH 7.5.

Buffer C: 50mmol/l sodium acetate, pH 6.2.

The extracts were dried down under compressed air and reconstituted in Buffer A. The radioimmunoassay for cGMP was performed using the method as described by Lyall et al., 1988b.

Briefly, cGMP antiserum (100µl at 1:20,000, in Buffer B) was mixed with standard (100µl of 0-5pmol/ml or 0-2pmol/ml in Buffer B) or unknown (100µl) and $^{125}$I-labelled cGMP (50µl; 2pg) in Buffer C for 18 hours at 4°C.

Bound label was separated from free using dextran-coated charcoal.

2.4.5. cAMP assay

At the end of agonist experiments, incubations were terminated, after removal of the medium, by the addition of 1ml chilled ethanol (100%) (Friedl et al., 1985; Williams, B.C., 1988, personal communication).

This was immediately spun, at 1000g for 10 mins at 4°C. The supernatant was carefully removed and the pellet was re-extracted/washed with a further 0.5mls of chilled ethanol (100%). This supernatant was pooled with the first

and the extract was dried under compressed air. The dried extracts were reconstituted in 0.05M Tris pH 7.5/4mM EDTA containing 100$\mu$M IBMX.

Intracellular cAMP concentrations were then measured using a cAMP radioimmunoassay (Amersham International PLC).

This method is based on the competition between unlabelled cAMP and a fixed quantity of the $^{3}$H-labelled cAMP for binding to a cAMP-binding protein.

2.5. Membrane preparations/immunological analysis.

2.5.1. Nephroblastoma cell membrane preparations

Nephroblastoma cells, which had been maintained in culture, up to the 7th subculture (P7), were grown to confluence in 125cm$^{2}$ tissue culture flasks. Confluent flasks were harvested by scraping the cells from the flask surface, using a cell scraper (Costar) into, approximately 40mls of HBSS (Flow Laboratories). Cell membranes were prepared, using a modification of the procedure as described by Jakobs et al., 1982.

The following stock buffers were prepared:

Buffer A: 10mM-Tris/1mM EDTA pH 7.5.

Buffer B: 10mM-Tris pH 7.4.

The protocol used was as follows:

1. Cells were centrifuged at 1,000g for 5 mins, at 4$^{o}$C, to pellet the cells.
2. The pellet was resuspended in approximately 5mls of Buffer A.
3. The re-suspended pellet was homogenized using a small polytron (Polytron PT205) (Kinematica, GmbH,

Switzerland), setting '6' for 2 x 12 seconds.

(The preparation was kept on ice throughout this procedure).

4. A clearing spin, in which the suspension was centrifuged at 1000g for 5 mins at 4°C, was carried out to remove any unbroken cells.
5. The suspension was ultracentrifuged at 35,000g in an LKB 2332 ultrospin 85 ultracentrifuge, fitted with a 45 Ti rotor (Beckman RIIC Ltd.) for 1 hour at 4°C, in order to collect a crude membrane pellet.
6. The crude membrane pellet was then resuspended in approximately, 3mls of Buffer B and then hand homogenized, using a small Teflon/glass homogenizer (about 10 strokes) to form a homogeneous membrane suspension.

The protein concentration of the cell membrane preparation was determined, using the method as previously detailed in Section 2.2.5. The membranes were then aliquoted into suitable fractions, in practice about 260µls (depending on protein concentration), and stored at -70°C, until required for use. Membranes were found to remain stable, in these conditions, for at least 3 months.

2.5.2. SDS/PAGE and immunoblotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE) was performed according to Laemmli, 1970.

Standard amounts of membranes from cultured

nephroblastoma cells; human platelets; cultured vascular smooth muscle cells (Jakobs et al., 1982); rat (kidney heart and adipocytes) (Sharma et al., 1982); and from cerebral cortices of rat brain (McKenzie et al., 1988) were precipitated using trichloroacetic acid (TCA)/deoxycholate (Bensadoun and Weinstein, 1976). These, along with pre-stained molecular mass markers (Bethesda Research Labs) were resolved by SDS/PAGE [10% (w/v) acrylamide], in vertical slab gels overnight at 40-50V.

Proteins were transferred, using an Electroblotting Unit (LKB BROMMA 2005 Transphor Electroblotting Unit) with constant current (100V) for 1½ hours, to nitrocellulose (Schleicher and Schuell, Dassel, W. Germany). The nitrocellulose was then blocked for 2 hours at 37°C with 3% (w/v) skimmed milk (Marvel) in Tris-buffered saline (TBS) (20mM Tris/HCl, pH 7.5/500mM-NaCl). This was removed and the blots were washed extensively with distilled water.

Primary antiserum (1:200 dilution) (which was kindly provided by Dr. G. Milligan (Section 7.2.)) in 1% gelatin/TBS was then added, and left overnight. The primary antiserum was then removed and the blot was washed extensively with distilled water followed by washes with TBS containing 0.05% (v/v) Tween-20 (TTBS) and then TBS.

Secondary antiserum (donkey anti-rabbit IgG coupled to horseradish peroxidase) (Scottish Antibody Production Unit, Wishaw, Scotland) (1:100 dilution) in 1% gelatin/TBS was added and left for 3 hours. Removal of the secondary antiserum was followed by the same series of extensive

washes of the nitrocellulose as detailed following removal of the primary antiserum. Orthodianasidine hydrochloride was employed as the substrate for detection of the antibody complex.

2.6. Immunocytochemical studies

Nephroblastoma cells (no older than the 10th subculture) were grown in flaskettes on glass coverslips. When the slides were confluent, as judged by light microscopy, the slides were fixed and immunostained with antisera to vimentin and a range of epithelial cell markers such as cytokeratins, epithelial membrane antigen (EMA) (Polak and Van Noorden, 1986). Also the cells were immunostained using two renin antisera, as described previously by Lindop et al. (1984).

2.7. Electron Microscopy

This was carried out by the Electron Microscopy Unit, Department of Pathology, Western Infirmary, Glasgow, using routine methods.

Briefly, cells were fixed in 2% glutaraldehyde buffered with Sörenson's phosphate buffer, pH 7.38 and post-fixed with osmium tetroxide. Following embedding in araldite, sections were cut employing an LKB Ultratome III, double stained with uranyl acetate and lead citrate then viewed in a Philips CM10 microscope.

2.8. Karyotyping

Young nephroblastoma cells which were actively dividing in culture (typically at subculture 3 or 4) were

used to determine the karyotype.

2.8.1. Preparation of chromosome spreads

At least 24 hours after subculturing, cells were treated with colcemid (as an arresting agent), for 2-3 hours. The cells were then harvested using trypsin/E.D.T.A. and treated with 37.5mM KCl (hypotonic solution) at 37°C. The cells were then centrifuged and fixed in three changes of 1:3 (v/v) acetic acid:methanol.

2.8.2. Staining techniques

2.8.2.(i) Solid Staining

The following stock solutions were required:

Solution A: (Leishman's stain) 3g of Leishman's powder in 2 litres of methanol. Filter using Whatman filter paper.

Solution B: (Gurr's buffer) 1 tablet of Gurr pH 6.8 buffer in 1 litre of distilled water.

The method used for solid staining was:

(i) Mix 1:3 (v/v) Solution A : Solution B.

(ii) Apply this mixture to the slide for a few minutes.

(iii) Wash off solution with tap water and dry slide on hotplate (or carefully blot dry with paper towel).

2.8.2.(ii) G-banding

The following stock solutions were required:

Solution A: (Sörenson's Buffer) 0.067M $Na_2HPO_4$; 0.067M $KH_2PO_4$ in 1 litre of distilled water.

Solution B: (Trypsin Solution) 1.2g of Difco powdered trypsin in 1 litre of Solution A.

For G-banding, slides should be 'aged' by hardening at 80°C for 2 hours and then banded, using a modification of

the method as described by Gallimore and Richardson (1973). Briefly;

(i) The slide is flooded with solution B for 5-25 seconds (timing varies).

(ii) Solution B is then washed off using Solution A.

(iii) Slides are then stained as for solid staining.

2.9. Statistical analysis

All results were compared by analysis of variance using Newman-Keuls multiple range test where appropriate.

## CHAPTER 3

## The effects of β-adrenergic agonists on renin release and cell growth.

### 3.1. Introduction

Innervation of the renal arterioles and the JG apparatus was first observed in 1952 when De Muylder identified nerves travelling to the afferent renal arterioles in the region of the granular JG cells. A decade later, Barajas (1964) examined the innervation of the JG apparatus by electron microscopy and found that non-myelinated nerve fibres, possibly noradrenergic, were associated with the afferent and efferent arterioles in monkeys and rats. In the ensuing years, other researchers (Biava and West, 1966) have confirmed that the JG cells of rats, dogs and humans are innervated by sympathetic fibres.

Vander (1965) was the first to show that direct stimulation of the renal nerves or intravenous (i.v.) infusion of catecholamines in anaesthetized dogs caused stimulation of renin release. In man, the renal nerves are involved in the renin release which occurs after upright tilting, exercise, cold stress or i.v. frusemide (Sonkodi et al., 1982). β-Adrenergic receptor blockade with propanolol diminishes, but does not abolish the rise in plasma renin concentration and plasma $A_{II}$ concentration, on upright tilting or frusemide injection. These results suggest that the renal nerves probably control renin secretion.

Catecholamines exert their effects through stimulation

of α- and β-adrenergic receptors. Renal nerve stimulation increased the release of renin via the activation of β-adrenergic receptors (Osborn et al., 1981). Direct stimulation of the renal β-adrenergic receptors with isoprenaline stimulates renin release from kidney slices (Capponi and Vallotton, 1976). Also the rate of renin release from isolated rat glomeruli, superfused in vitro, is stimulated when the preparation is exposed to noradrenaline, adrenaline or isoprenaline (Morris et al., 1976).

The intracellular signal of the β-adrenergic pathway is often cAMP, but it is uncertain whether cAMP is the final signal which couples β-stimulation with renin secretion.

In the present study, therefore, experiments were carried out to investigate the mechanisms involved in β-adrenergic-controlled renin release.

It was first necessary to establish if β-agonists would stimulate renin release in this model.

It seemed possible that the long-term control of renin release may occur through additional or different mechanisms to the short-term. Thus, experiments were carried out using different times of exposure to β-agonists.

### 3.2. β-Adrenergic agonist experiments

#### 3.2.1. Short-term release experiments

Cells were seeded at 2 x $10^4$ cells/ml and grown to

confluence (Section 2.3.3.(i)). At the end of the basal incubation period, the medium was removed and replaced with medium containing isoprenaline, forskolin or dbcAMP at concentrations ranging from 0.1mM to 1μM, and the cells were incubated for 5 hours. The concentration of active renin released at 5 hours was measured as detailed in Section 2.3.3.(i).

3.2.2. 24 hour release/growth rate experiments

24 hour experiments were carried out as detailed in Section 2.3.3.(ii). At the end of the basal incubation period, medium was removed and replaced with medium containing isoprenaline (1μM), forskolin (0.1mM) or dbcAMP (0.1mM). Active renin levels were measured, at 24 hours, and cells harvested and counted as detailed in Section 2.3.3.(ii).

3.2.3. Long-term release and growth rate experiments

In long-term release and growth-rate experiments, cells were seeded at a density of $2 \times 10^5$ cells/ml (Figures 3.2, 3.3) or $3 \times 10^4$ cells/ml (Figure 3.4) (1 ml/well in each experiment), and grown for 3-4 days (Section 2.3.3.(iii)). Medium was removed and replaced with medium containing isoprenaline (1μM) alone, isoprenaline (1μM) and propanolol (1μM), propanolol (1μM) alone, forskolin (0.1mM) or dbcAMP (0.1mM) and growth was continued for up to 12 days.

On day 3 of the experiments, the culture medium was changed and at 3 day intervals (except in the isoprenaline and propanolol experiments, where intervals were 3 and 4

days) samples were taken for active and (in the isoprenaline experiments (Figure 3.2)) total renin estimations (Sections 2.4.1., 2.4.2.). At 6 day intervals (Figure 3.2), on day 6 and day 10 (Figure 3.3) and on day 6 and day 9 (Figure 3.4) cells were harvested and counted (Section 2.3.3.(ii)).

## 3.3 Results and discussion

### 3.3.1. Short-term experiments

In short-term release experiments (5h), active renin release was stimulated by 0.1mM- but not by 1$\mu$M - isoprenaline (Figure 3.1.a). Stimulation of renin secretion also occurred with 0.01mM forskolin or 0.1mM dbcAMP ($P < 0.01$) (Figure 3.1.b).

These results confirm that renin secretion is stimulated by $\beta$-adrenergic receptor agonists and by agents (forskolin and dbcAMP) which raise intracellular cAMP concentration.

### 3.3.2. 24 hour experiments

In 24 hour experiments, the levels of active renin release (Table 3.1) were significantly stimulated by 1$\mu$M isoprenaline ($P < 0.01$), 0.1mM forskolin ($P < 0.01$) and 0.1mM dbcAMP ($P < 0.01$).

Cell counts done in one set of the experiments showed that cell growth was stimulated by 1$\mu$M isoprenaline ($P < 0.01$) and 0.1mM dbcAMP ($P < 0.01$) at 24 hours.

The increase in cell growth, noted in this experiment was an unexpected finding, and suggests that renin production may be stimulated by different mechanisms during

Figure 3.1: Short-term (5h) exposure to (a) Isoprenaline (ISO) (0.1mM , 1$\mu$M) and (b) dbcAMP (0.1mM, 1$\mu$M), forskolin (0.01mM).

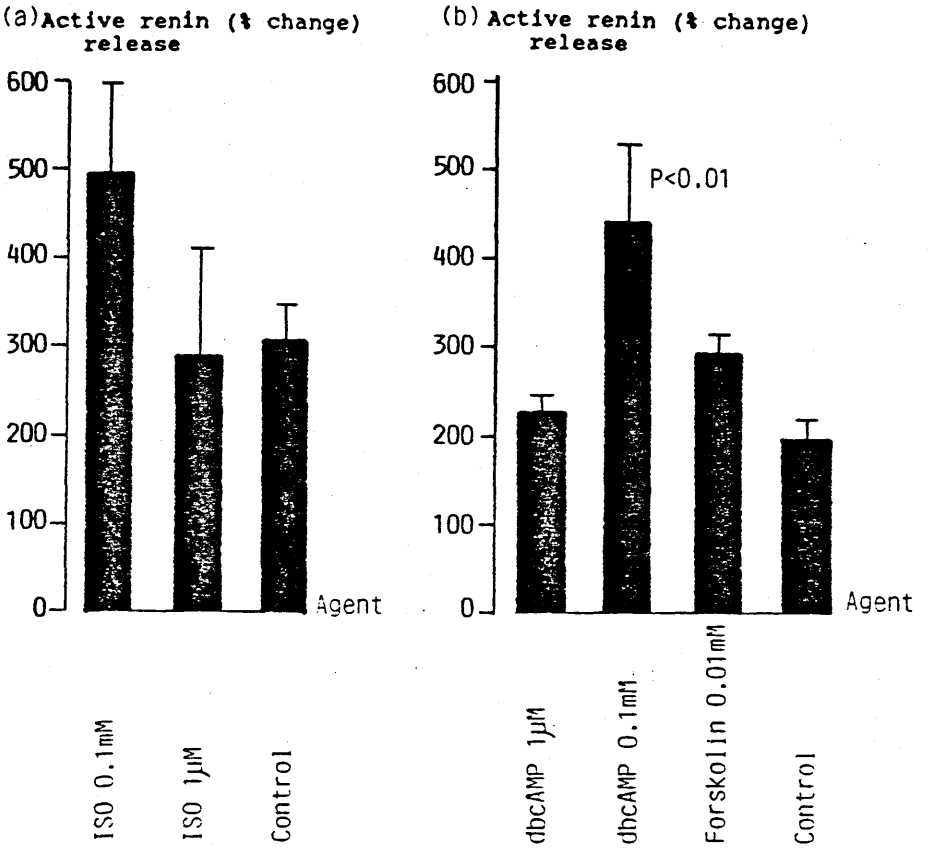


**Table 3.1**

**Active renin concentrations in cell culture supernatants at 24 h.**

Results are expressed as mean ± S.E.M. (n = 6).

| | Renin activity (μUnits/ml) | | |
|---|---|---|---|
| | Control | Agent | P value |
| isoprenaline (1μM) | 6.8±0.4 | 14.3±0.6 | <0.01 |
| dbcAMP (0.1mM) | 9.4±0.4 | 11.5±0.3 | <0.01 |
| forskolin (0.1mM) | 6.5±0.4 | 14.0±0.5 | <0.01 |

short-term and long-term exposure to β-agonists . This was further investigated in long-term experiments in which the cells were grown in the presence of isoprenaline and related agents for up to 12 days.

The specificity of the isoprenaline response was tested by using a β-adrenergic receptor antagonist; propanolol.

3.3.3. Long-term experiments

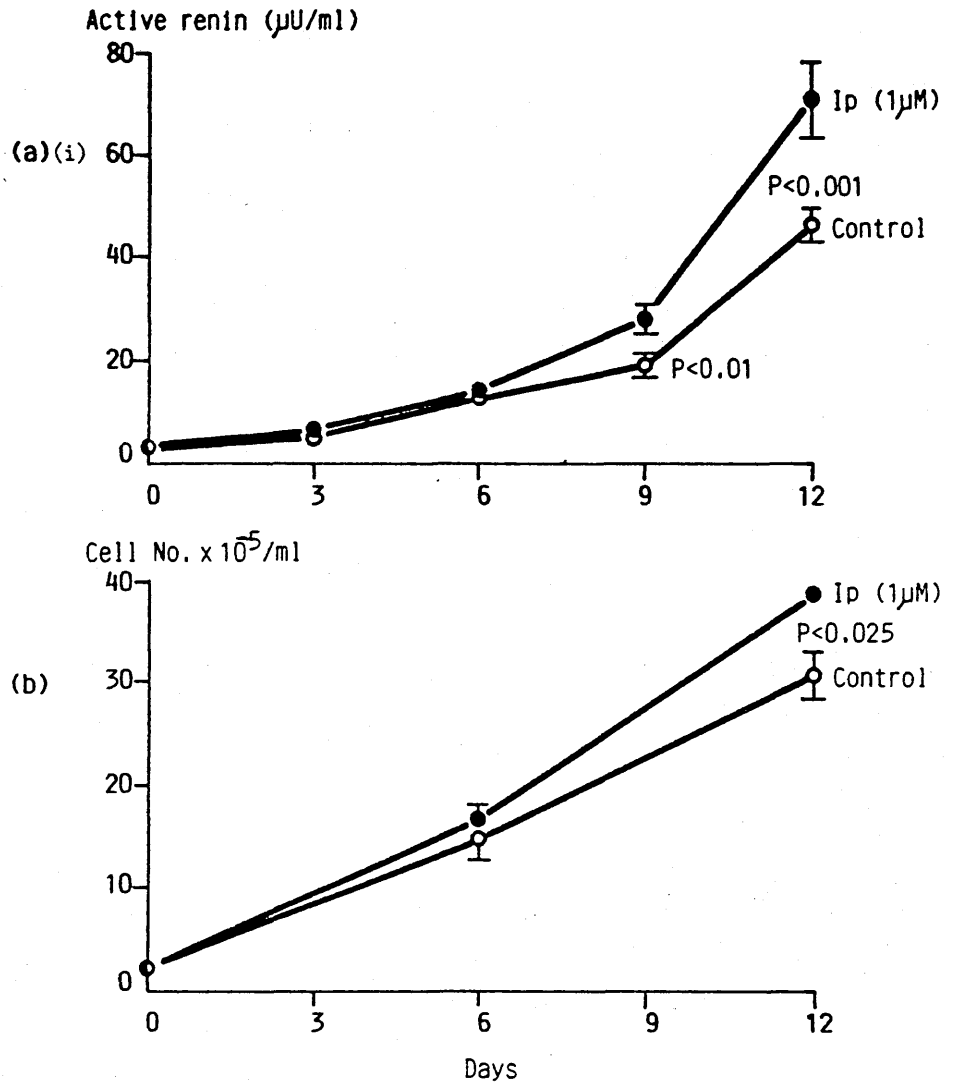
3.3.3.(i). Isoprenaline

After 9 days exposure to 1μM isoprenaline (Figure 3.2.a(i)), a significant stimulation of both active ($P < 0.01$) and total ($P < 0.01$) (Figure 3.2.a(ii)) renin release was observed. This occurred in three separate experiments. Cell number was significantly increased after 12 days exposure to 1μM isoprenaline in all three experiments ($P < 0.025$) (Figure 3.2.b). In one experiment, the increased cell growth was significant by day 6 ($P < 0.01$) (results not shown).

When the increased renin secretion was related to cell number (Figure 3.2.c), it was found that the increase in renin release per cell was not significantly different between the isoprenaline-treated cells and controls, in all three experiments. Therefore, the increase in renin secretion after long-term treatment with 1μM isoprenaline, can be accounted for by stimulated cell growth.

The long-term action of isoprenaline on renin secretion and nephroblastoma cell growth was blocked by the

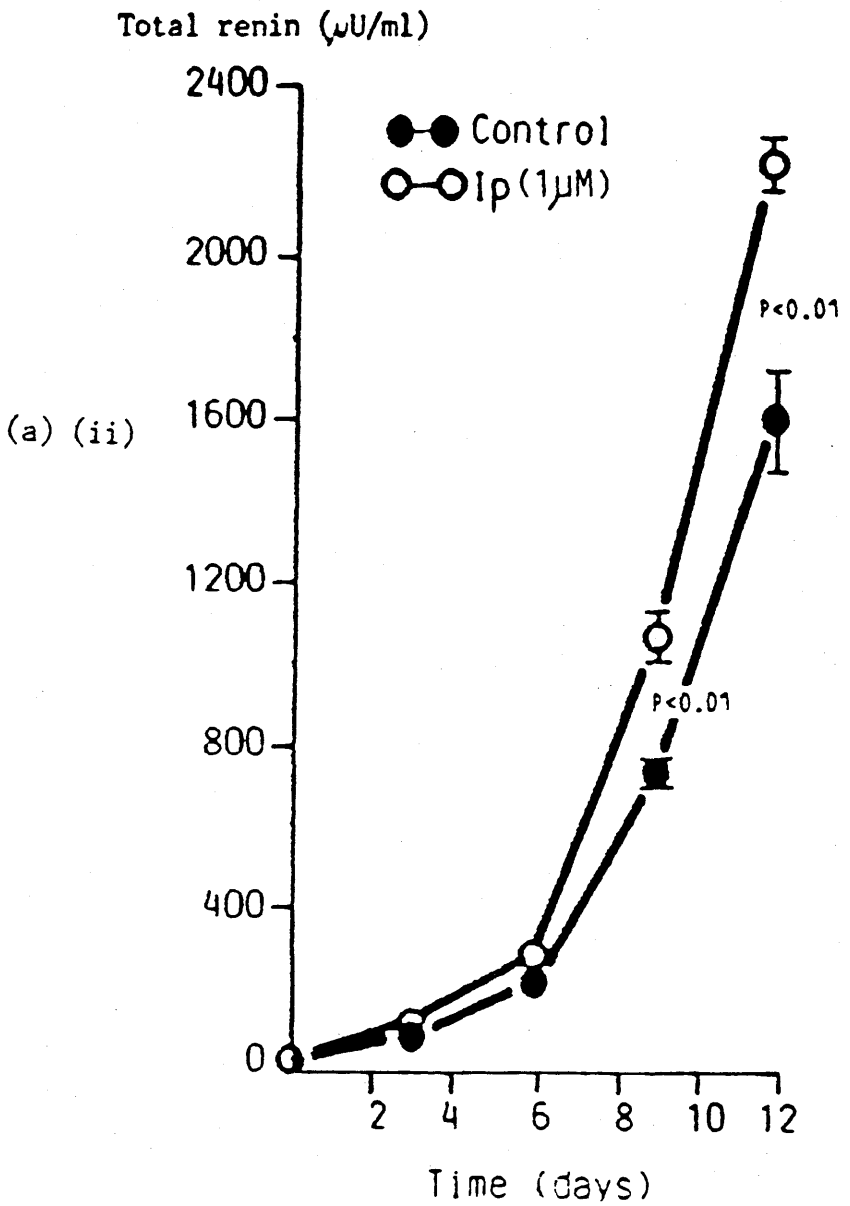
**Figure 3.2: Long-term action of isoprenaline (1μM) on (a)(i) active renin release and (b) cell growth (where IP = isoprenaline).**




Points are mean values ± standard errors.

| Figure 3.2. (c). | Renin release per cell number μU/10⁶ cells | | |
|---|---|---|---|
| **Time** | **Control** | **IP (1μM)** | **P value** |
| Day 12 | 14.16 ± 1.40 | 17.34 ± 0.90 | P>0.05 N.S.D. |

**Figure 3.2.(a)(ii): Long-term action of isoprenaline (1μM) on total renin release.**




(a) (ii)

addition of the β-blocking agent, propanolol (1μM) (Figures 3.3.a,b). This indicates that isoprenaline causes its effects by a direct, specific action on β-adrenergic receptors. There was also observed to be a slight stimulation of renin release, in response to long-term treatment with 1μM propanolol alone. This is probably because, at high doses, the β-adrenoceptor antagonist may have a partial agonist effect.

3.3.3.(ii). Forskolin, dbcAMP

Active renin release was stimulated by long-term exposure to 0.1mM forskolin at day 6 ($P < 0.05$) and 0.1mM dbcAMP also caused stimulation of active renin release by day 9 ($P < 0.05$) (Figure 3.4.a). Cell number was significantly increased by 0.1mM forskolin at day 9 ($P < 0.05$) and 0.1mM dbcAMP at day 6 ($P < 0.01$) (Figure 3.4.b.).

When renin secretion was related to cell number (Figure 3.4.c.), it was found that the increase in active renin secretion caused by 0.1mM dbcAMP could also be accounted for by an increase in cell growth. However, the increase in active renin secretion caused by 0.1mM forskolin was caused by an increase in renin secretion per cell. This was observed in three separate forskolin experiments.

3.4. Discussion

The results of the present study show that 0.1mM isoprenaline, 0.01mM forskolin and 0.1mM dbcAMP ($P < 0.01$) stimulate renin secretion from cultured nephroblastoma cells at 5 hours, a time within the cell cycle. The

**Figure 3.3: The effect of 1μM propanolol on (a) isoprenaline-stimulated renin release and (b) cell growth.**

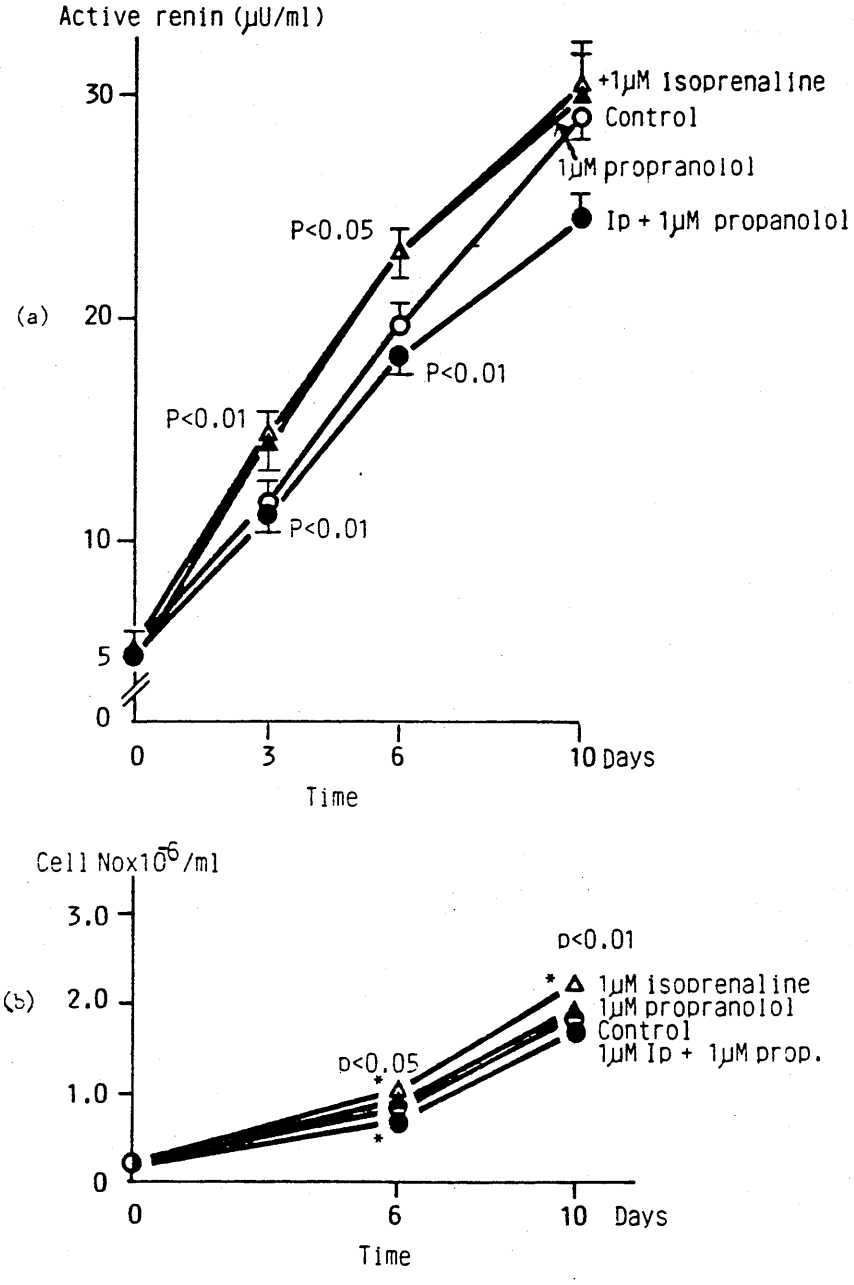
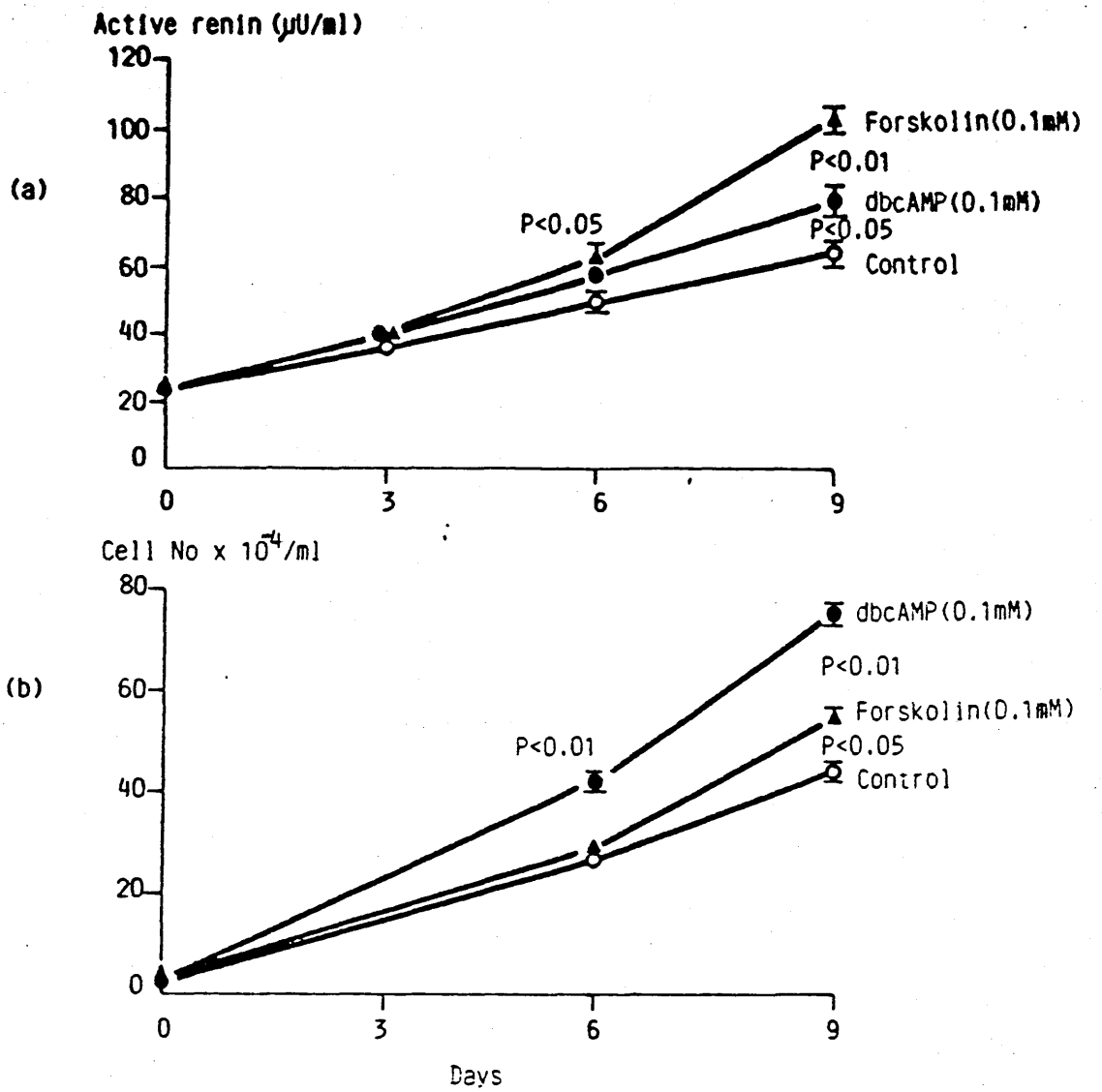


**Figure 3.4: Long-term action of 0.1mM forskolin and 0.1mM dbcAMP on (a) active renin release and (b) cell growth.**




Points are mean values ± standard errors.

| Figure 3.4. (c). | Renin release per cell number μU/10⁶cells | | |
|---|---|---|---|
| Time | Control | Forskolin(0.1mM) | dbcAMP(0.1mM) |
| Day 9 | 92.96 ± 3.15 | 144.69 ± 7.5<br>P<0.01 | 73.9 ± 6.5<br>P>0.05<br>N.S.D. |

increased secretion may be due to either an increase in exocytosis alone, or to increased renin synthesis, gene expression and exocytosis. In the normal JG cell, renin is stored in granules and the immediate response to a stimulus is likely to be an increase in the rate of exocytosis. However, for secretion to be maintained, an increase in the rate of renin synthesis must eventually occur.

Isoprenaline-stimulated renin release from nephroblastoma cells has been shown to be a $\beta$-receptor-mediated event (Section 3.3.3.(i)). In contrast, the diterpene compound, forskolin, is a direct (receptor-independent) stimulant of the catalytic subunit of adenylate cyclase (Seamon and Daly, 1981) and, therefore, by-passes the $\beta$-receptor.

The increased renin secretion after long-term exposure to isoprenaline mainly occurs through increased cell growth. In contrast, forskolin causes an increase in renin release per cell. The increased cell growth appears to be mediated via adenylate cyclase, since dbcAMP also stimulates cell growth. However, forskolin was not as effective (at increasing cell number) as isoprenaline or dbcAMP in this system in vitro.

At this stage, it can be concluded that stimulated nephroblastoma cell growth, is a $\beta$-receptor-mediated event, involving adenylate cyclase stimulation and increased concentration of intracellular cAMP. Also, it can be tentatively suggested that forskolin may have an

additional role in these cells, different to those of isoprenaline and dbcAMP, which may render it ineffective in stimulating cell growth.

It is known that forskolin acts by directly activating the catalytic subunit of adenylate cyclase. However, there have been recent reports (Laurenza et al., 1989) that forskolin has other actions (such as inhibition of membrane transport proteins) which do not involve production of cAMP.

The intracellular mechanisms whereby these agents (i.e. isoprenaline, dbcAMP and forskolin) mediate their effects was investigated, in subsequent experiments.

## CHAPTER 4

## The effect of ANP on basal and β-adrenergic-stimulated renin release and cell growth.

### 4.1. Introduction

Atrial natriuretic peptides (ANP) from mammalian atria have been purified, sequenced and characterized (Currie et al., 1984; Kangawa and Matsuo, 1984). Specific receptors for ANP occur in diverse tissues such as lung (Kuno et al., 1986), adrenal (Meloche et al., 1986), kidney (Yip et al., 1985) and smooth muscle (Schenk et al., 1985; Hirata et al., 1984).

Two major effects of ANP on renal function have been described, namely the enhancement of sodium and water excretion (c.f. De Bold et al., 1981 and Maack et al., 1985) and the suppression of renin release (Maack et al., 1984; Burnett et al., 1984; Anderson et al., 1987).

The diversity of receptor distribution and biological effects suggests that the cellular mechanism of ANP action may involve different receptors and different intracellular second messenger cascade systems.

In addition to cGMP (Section 1.5.3.), ANP has also been recognized to operate through cAMP, because ANP has been demonstrated to be an inhibitor of adenylate cyclase in certain tissues (Anand-Srivastava et al., 1984; Anand-Srivastava et al., 1985). Also, Ishikawa et al. (1985), showed that ANP diminished the stimulatory effect of arginine vasopressin (AVP) on cellular cAMP production from rat cultured collecting tubule cells.

Receptors for many hormones and growth factors which are able to modulate the rate of production of second messengers, such as cAMP are dependent upon the intermediate activation of one or more, G-proteins. Each G-protein is a heterotrimer, in which distinct $\alpha$ subunits share a common population of $\beta/\delta$ subunits.

Initial identification of G-protein $\alpha$-subunits were based on the known ability of toxins elaborated by Vibrio cholerae and Bordatella pertussis to modulate the adenylate cyclase cascade. The observation that pertussis toxin was able to catalyse ADP-ribosylation of the inhibitory G-protein of the adenylate cyclase cascade and consequently attenuate receptor-mediated inhibition of adenylate cyclase (Katada and Ui, 1981), provided a tool with which to identify $G_i$. ADP-ribosylation occurs at a cysteine residue located four amino-acids from the C-terminus and the modification appears to be sufficient to prevent productive interactions between receptors and $G_i$.

Therefore, pertussis toxin is a useful agent, which can be used to investigate whether or not a hormone causes $G_i$-mediated inhibition of adenylate cyclase.

The mechanism by which ANP inhibits renal renin release is, as yet, a matter of debate (Maack et al., 1985) and the exact mechanisms underlying the cellular and molecular action of ANP are still unknown.

Therefore, in the present study, using the nephroblastoma cells as a model in vitro, it was decided to

examine whether or not ANP inhibits renal renin release *in vivo* by a direct action on the JG cell and, if so, which second messengers were involved.

4.2. The effect of ANP on basal renin release

4.2.1. Short-term ANP experiments

Cells were seeded and grown to confluence (Section 2.3.1.(i)). At the end of the basal incubation period, the medium was removed and replaced with medium containing 1μM ANP and the cells were incubated for 5 hours.

Also, in order to test for the presence of a pertussis toxin-sensitive G-protein, short-term experiments were carried out in which the cells were treated with medium containing 1μM ANP alone; 1μM ANP and 100ngs/ml pertussis toxin, and 100ngs/ml pertussis toxin for 5 hours.

The concentration of active renin released at 5 hours was measured (Section 2.3.3.(i), 2.4.1).

4.2.2. 24 hour release/growth rate experiments

24 hour experiments were carried out as detailed in Section 2.3.3.(ii). At the end of the basal incubation period, the medium was removed and replaced with medium containing a range of doses (1pM to 1μM) of ANP.

In order to determine whether ANP had any effect on isoprenaline, forskolin or dbcAMP-stimulated renin release, cells were grown in medium containing 1μM isoprenaline, 0.1mM forskolin or 0.1mM dbcAMP, in the presence or absence of 1μM ANP, for 24 hours.

Active renin levels were measured at 24 hours and cells harvested and counted (Section 2.3.3.(ii)).

## 4.3. Results and discussion

### 4.3.1. Short-term experiments

ANP (1$\mu$M) was found to inhibit active renin release in short-term (5h) experiments ($P < 0.01$) (Figure 4.1). This inhibition was released by the presence of 100ngs/ml pertussis toxin (Figure 4.2). This means that short-term ANP-mediated inhibition of active renin release is possibly mediated by a pertussis toxin-sensitive G-protein.

### 4.3.2. 24 hour experiments

#### 4.3.2.(i). Dose-response to ANP

The dose-response experiment which was carried out for 24 hours showed that ANP inhibited active renin release to 60% of control values at the optimal dose of 1$\mu$M (results not shown).

#### 4.3.2.(ii). The effect of ANP on isoprenaline-induced renin release and cell growth

Active renin release was significantly ($P < 0.01$) stimulated by 1$\mu$M isoprenaline at 24 hours (Figure 4.3.a). This confirms our previous results (Section 3.3.2.). Isoprenaline-induced renin release was significantly attenuated by 1$\mu$M ANP ($P < 0.01$) (Figure 4.3.a.). Cell number was significantly increased by 1$\mu$M isoprenaline treatment for 24 hours ($P < 0.01$) (Figure 4.3.b.), and this was significantly reduced ($P < 0.01$) by 1$\mu$M ANP.

Table 4.3.c shows renin release related to cell number. As described in Section 3.3.2., treatment with 1$\mu$M isoprenaline apparently increased renin secretion at 24 hours, but this was due to an increase in cell number.

**Figure 4.1:Short-term (5h) exposure to 1μM ANP**

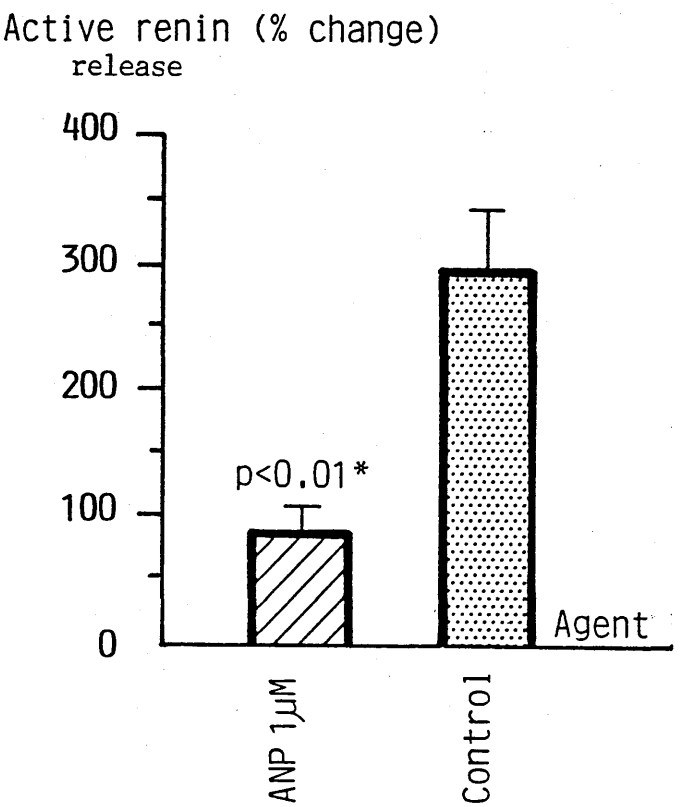


**Figure 4.2: The effect of pertussis-toxin (100ngs/ml) on ANP-inhibited renin release.**

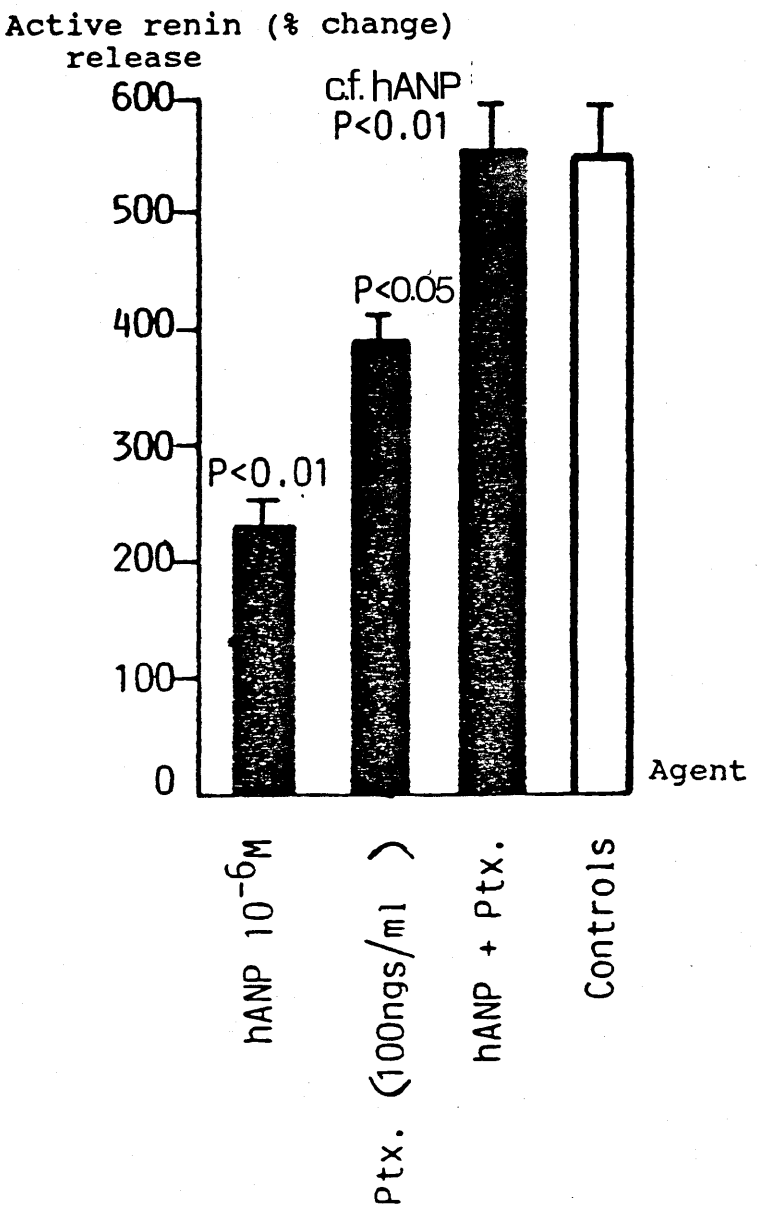
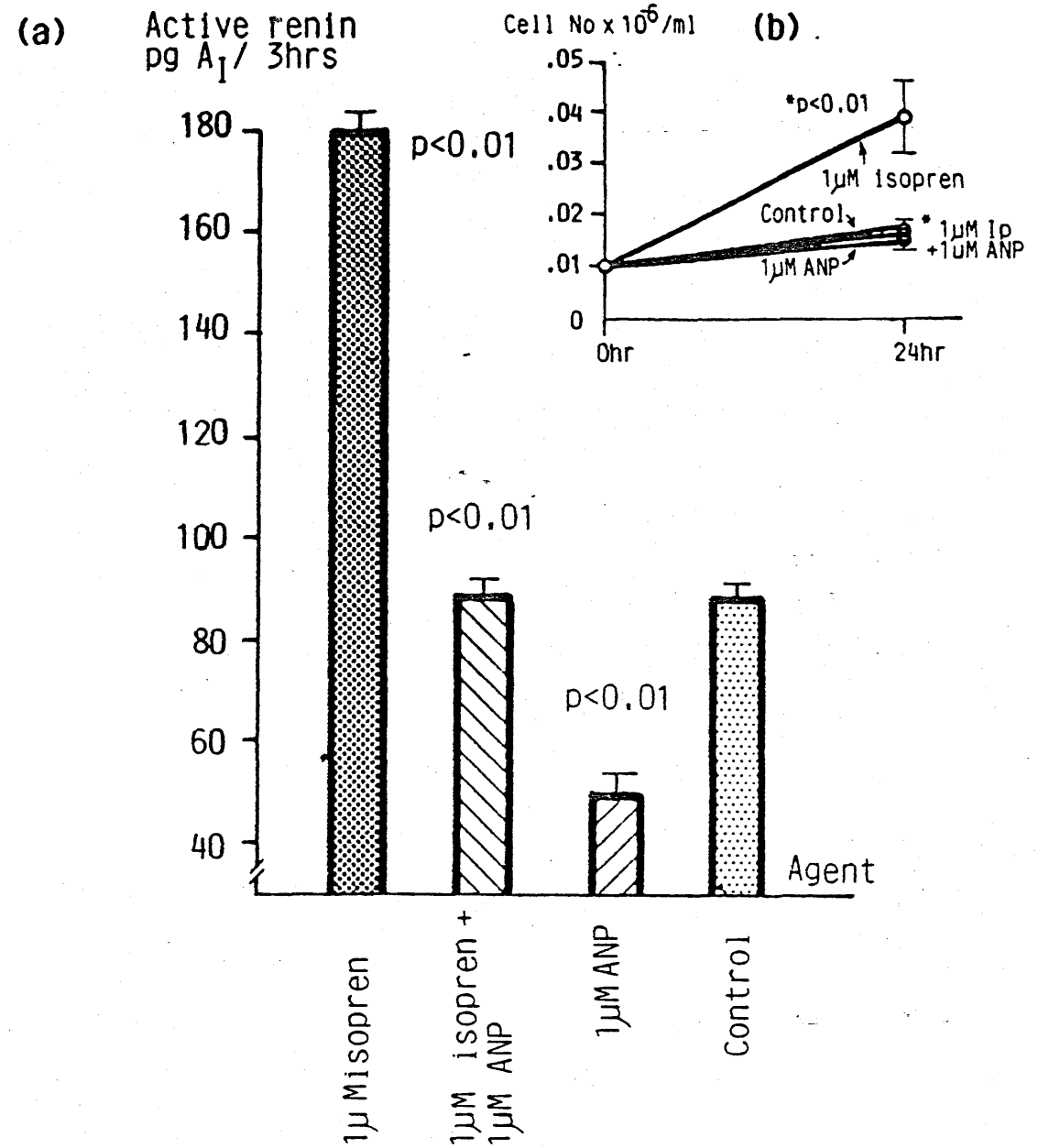


Figure 4.3:

# The effect of ANP on (a) isoprenaline- stimulated renin release and (b) cell growth at 24h




| Renin release per Cell number at 24h | |
|---|---|
| Agent | μU/$10^6$ cells |
| Control | 361.9±18 |
| 1μM isoprenaline | 389.0± 76 NSD |
| 1μM Isoprenaline + 1μM ANP | 402.9 ± 25 NSD |
| 1μM ANP | 217.0 ± 57 NSD |

Table 4.3.(c)

Results are expressed as Mean ± S.E.M.

Thus renin release per cell remained the same.

Treatment with 1$\mu$M ANP for 24 hours lowered renin release by decreasing cell number. Therefore, the response at 24 hours was due mainly to an effect on cell growth.

4.3.2.(iii). The effect of ANP on dbcAMP-induced renin release and cell growth

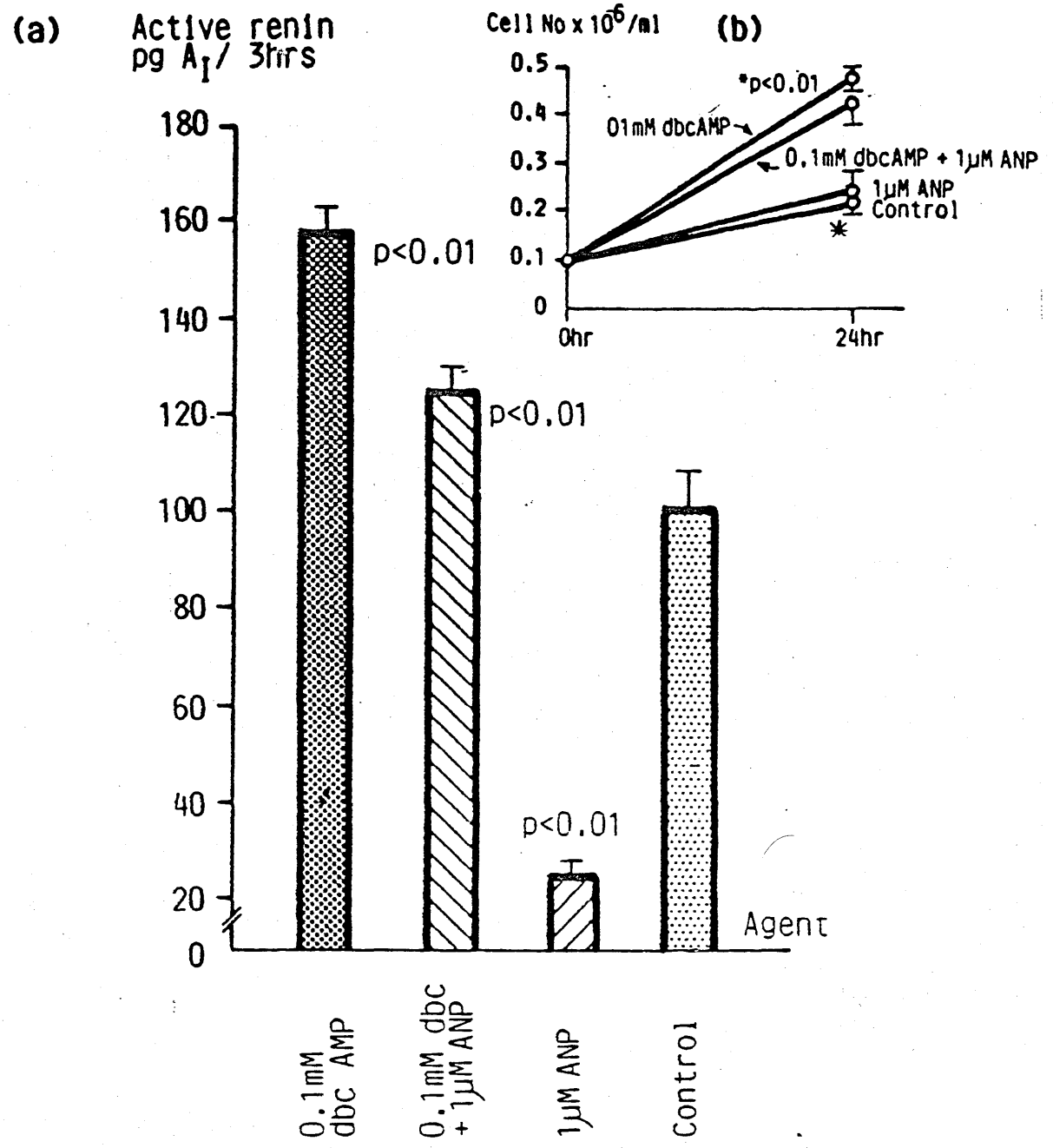
Active renin release (Figure 4.4.a) was significantly stimulated ($P < 0.01$) by 24 hour treatment with dbcAMP (0.1mM). ANP (1$\mu$M) significantly attenuated dbcAMP-stimulated renin release ($P < 0.01$). Cell growth was significantly increased after 24 hour treatment with 0.1mM dbcAMP ($P < 0.01$) (Figure 4.4.b). This was reduced slightly by the presence of 1$\mu$M ANP but the difference was non-significant. 1$\mu$M ANP alone, did not inhibit cell growth.

Table 4.4.c. shows renin release related to cell number. As described in Chapter 3, treatment with 0.1mM dbcAMP caused an increase in renin secretion at 24 hours. This increase in renin release was due to an increase in cell number and the rate of renin release per cell remained the same.

Treatment with 1$\mu$M ANP for 24 hours, lowered renin release through a suppression of dbcAMP-stimulated cell growth, although the effect was not as marked as that of ANP on isoprenaline-stimulated cell growth. Renin release per cell number at 24 hours was not significantly different and so ANP must reduce dbcAMP stimulated renin release by an effect on cell growth.

Figure 4.4:

# The effect of ANP on (a) dbcAMP-stimulated renin release and (b) cell growth at 24h




| Renin release per Cell number at 24h | |
|---|---|
| Agent | μU/$10^6$ cells |
| Control | 310.0±63 |
| 0.1mM dbcAMP | 280.0±9 NSD |
| 0.1mM dbcAMP + 1μM ANP | 392.0±108 NSD |
| 1μM ANP | 95.0±27 p<0.01 |

Table 4.4.(c)

Results are expressed as Mean ± S.E.M.

4.3.2.(iv). The effect of ANP on forskolin-induced renin release.

Active renin release was significantly stimulated by 0.1mM forskolin ($P < 0.01$) at 24 hours (Figure 4.5.a). 1$\mu$M ANP treatment for 24 hours, significantly reduced forskolin-stimulated renin release ($P < 0.01$). However, in contrast to that found with isoprenaline and dbcAMP, 0.1mM forskolin had no effect on cell growth at 24 hours (Figure 4.5.b.).

Table 4.5.c. shows renin release related to cell number. As previously mentioned (Section 3.3.2.), treatment with 0.1mM forskolin caused an increase in renin release, but did not apparently cause an increase in cell growth, at 24 hours.

Treatment with 1$\mu$M ANP for 24 hours lowered renin release by decreasing the rate of renin release per cell. Thus, in contrast to that found with 1$\mu$M isoprenaline and 0.1mM dbcAMP, the response to 0.1mM forskolin at 24 hours, was due to an effect on renin release per cell.

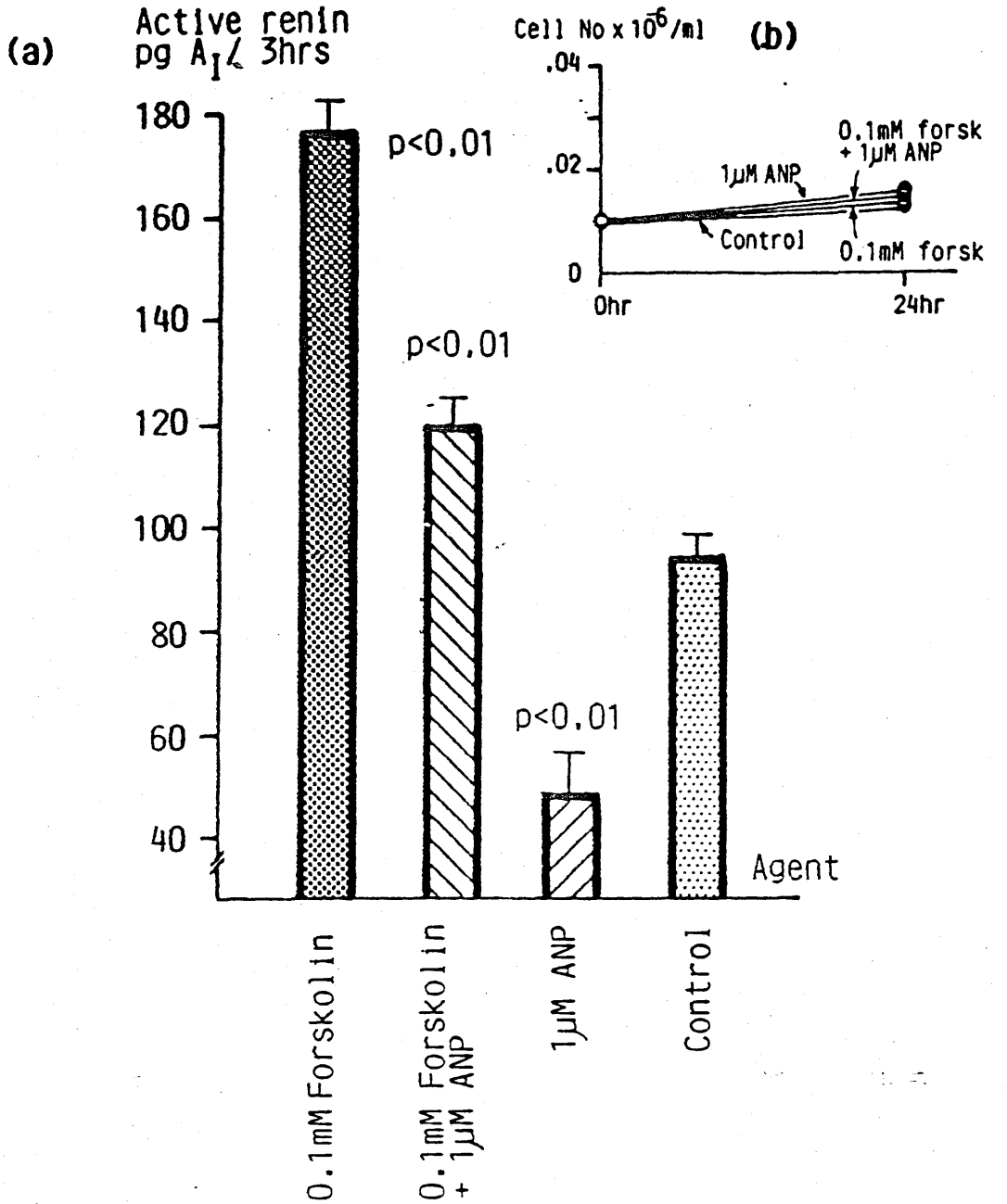
4.4. Conclusions

From the present study, it can be concluded that ANP inhibits basal renin release from human cultured nephroblastoma cells, possibly via a pertussis toxin-sensitive G-protein and inhibition of adenylate cyclase activity.

ANP (1$\mu$M) also antagonises $\beta$-adrenergic stimulated renin release and it does so by either attentuation of $\beta$-stimulated cell growth (in the case of isoprenaline,

Figure 4.5:

# The effect of ANP on (a) forskolin-stimulated renin release and (b) cell growth at 24h




| Renin release per Cell number at 24h | | |
|---|---|---|
| Agent | μU/$10^6$ cells | |
| Control | 508.9±71 | |
| 0·1mM forskolin | 993.5±80 p<0.025 | p<0·01 |
| 0·1mM forskolin + 1μM ANP | 643.9±29 NSD | p<0·01 |
| 1μM ANP | 188.0±94 p<0.05 | |

Table 4.5.(c)

Results are expressed as Mean ± S.E.M.

dbcAMP) or by reduction of $\beta$-stimulated renin release per cell (as in the case of forskolin).

It was decided to investigate in detail the intracellular second messenger responses to these agents, in order to try and decipher their interactions, which could account for the responses observed so far.

## CHAPTER 5

## The role of second messengers in the control of renin release.

### 5.1 Introduction

The role of second messengers involved in the control of renin release, is not well established (Section 1.6). Using the nephroblastoma cells as a model *in vitro*, it has been determined that active renin release is stimulated by isoprenaline, dbcAMP and forskolin on a short-term basis, which is probably mediated by adenylate cyclase.

However, stimulation at 24 hours, revealed a difference in the mechanisms of isoprenaline (and dbcAMP) and forskolin. Isoprenaline (and dbcAMP) caused a stimulation of cell growth at 24 hours, in contrast to forskolin which acted mainly by increasing renin release per cell. Furthermore, this effect was maintained in the long-term experiments (up to 12 days) and was specific (in the case of isoprenaline).

Therefore, it can be concluded that isoprenaline and forskolin stimulate renin release by different mechanisms and this is first evident at 24 hours.

ANP is a peptide hormone which is becoming increasingly important in the control of renin release. The present study has shown that ANP inhibits basal renin release from cultured nephroblastoma cells directly, over a period of 5 hours.

The results of 24 hour experiments showed that ANP

(1μM) significantly attenuated β-adrenergic-stimulated renin release. This was specifically (in the case of isoprenaline and dbcAMP) by blocking the increase in cell growth.

As the interactions of ANP with β-stimulated renin release and cell growth were very interesting, it was decided to investigate the second messenger responses in detail.

5.2. Second messenger time course experiments

Cells were seeded and grown to confluence (Section 2.3.3.(i)). At the end of the basal incubation period, the medium was removed and replaced with medium containing; isoprenaline (1μM) alone, ANP (1μM) alone, isoprenaline (1μM) and ANP (1μM). All medium contained the phosphodiesterase inhibitor; 3-isobutyl-1-methylxanthine (IBMX), at 0.5mM.

Identical experiments were set up in which the cells were treated with forskolin (0.1mM) alone, ANP (1μM) alone, or forskolin (0.1mM) and ANP(1μM). Again, all medium contained IBMX at 0.5mM.

It was decided to look at cGMP and cAMP concentrations in each well, in response to agents.

The extracellular appearance of cGMP is used as a marker of the biological activity of ANP (Hamet et al., 1986; Lewis et al., 1988).

Therefore, it was decided that extracellular cGMP concentrations (Section 2.4.5.), intracellular cAMP concentrations (Section 2.4.6) as well as active renin

concentrations (Section 2.3.3.(i)) should be measured at the following times: basal, 1 minute, 5 minutes, 1 hour and 6 hours after the addition of the agents.

## 5.3. Results and discussion

### 5.3.1. Comparison of second messenger responses in medium with and without phosphodiesterase inhibitor

Initial experiments were carried out, in order to look at second messenger levels in response to treatment of the cells (for up to 24 hours) with individual agonists.

Intracellular cAMP concentrations were significantly raised in response to administration of 0.1mM forskolin, at 5 mins ($P < 0.05$) and in response to 0.1mM dbcAMP at 6 hours ($P < 0.05$). 1$\mu$M ANP had no significant effect on intracellular cAMP concentrations (results not shown).

However, 1$\mu$M ANP caused stimulation of extracellular cGMP concentrations, over a 24 hour period (Figure 5.1). These initial experiments were carried out in medium without IBMX.

IBMX is needed to effectively inhibit phosphodiesterase activities (Wells and Kramer, 1981).

The inclusion of IBMX was found to cause an increase in the concentrations of intracellular cAMP and extracellular cGMP, in response to agonist (Table 5.1). Therefore, all subsequent second messenger experiments were carried out in medium containing IBMX at 0.5mM (Section 5.2).

**Figure 5.1: cGMP release in response to 1μM ANP**

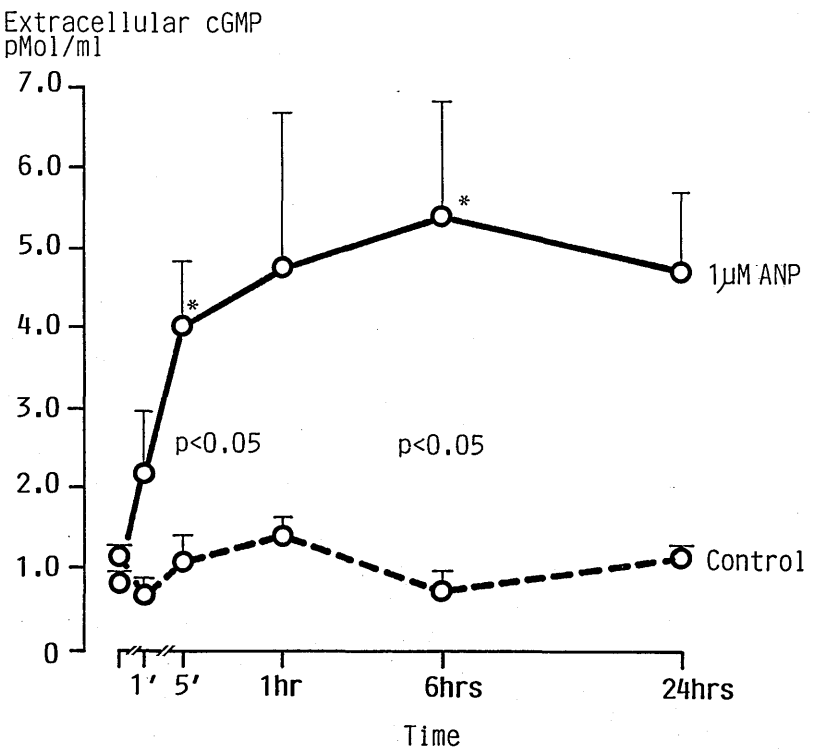


**Table 5.1.**

**Comparison of second messenger concentrations in the absence and presence of IBMX.**

| | Medium (-IBMX) | Medium (+ IBMX) 0.5mM |
|---|---|---|
| [a] 0.1mM forskolin | $[cAMP]_i$ (pMol/ml) | |
| 5 mins | 37.0±9.0 | 38.9±0.9 |
| 1 hour | 25.2±2.0 | 42.7±3.0 |
| 6 hours | 70.0±5.0 | 85.8±7.5 |
| [b] 1μM ANP | $[cGMP]_e$ (pMol/ml) | |
| 5 mins | 4.0±0.8 | 5.2±0.7 |
| 1 hour | 4.8±2.0 | 12.6±0.3 |
| 6 hours | 5.4±1.5 | 11.6±1.2 |

Results are expressed as Mean ± S.E.M.

[a] is representative experiment (n=3)

[b] is representative experiment (n=4).

5.3.2. Second-messenger responses during ANP-isoprenaline interaction

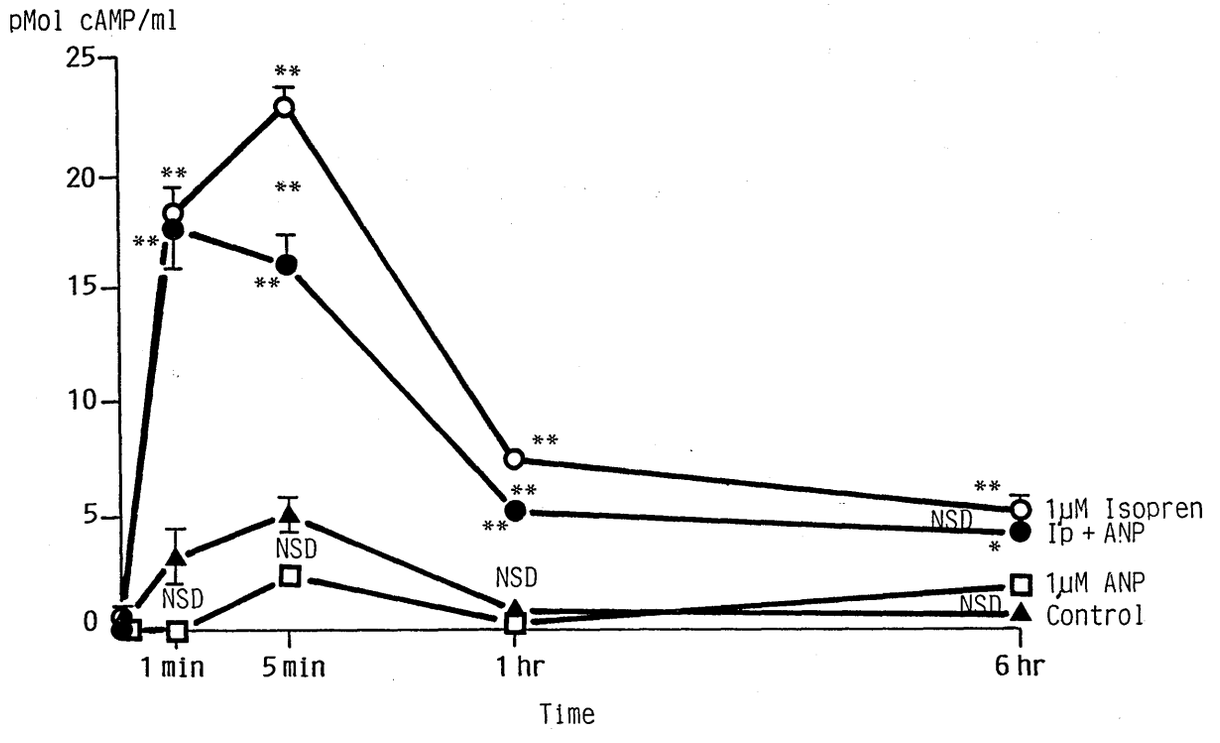
Treatment of cells with 1$\mu$M isoprenaline caused a rapid (within 1 min) increase in intracellular cAMP levels ($P < 0.01$), which peaked at 5 mins ($P < 0.01$) (Figure 5.2). Thereafter, the levels declined, but were still significantly higher than control levels, at 6 hours ($P < 0.01$).

ANP (1$\mu$M) did not significantly alter basal intracellular cAMP concentration, at any time. However, 1$\mu$M ANP did attenuate the isoprenaline-induced cAMP response (Figure 5.2.). This was found to be significant at 5 mins ($P < 0.01$) and 1 hour ($P < 0.01$).

Extracellular cGMP concentration was significantly stimulated by 1$\mu$M ANP, at 1 hour and 6 hours ($P < 0.01$) (Figure 5.3.). This result was very similar to that obtained in the initial experiments (Figure 5.1.). Isoprenaline (1$\mu$M) significantly reduced the ANP-stimulated cGMP response at 1 hour ($P < 0.01$) and 6 hours ($P < 0.01$). Throughout the course of the experiment, isoprenaline (1$\mu$M) alone caused no change in extracellular cGMP concentration.

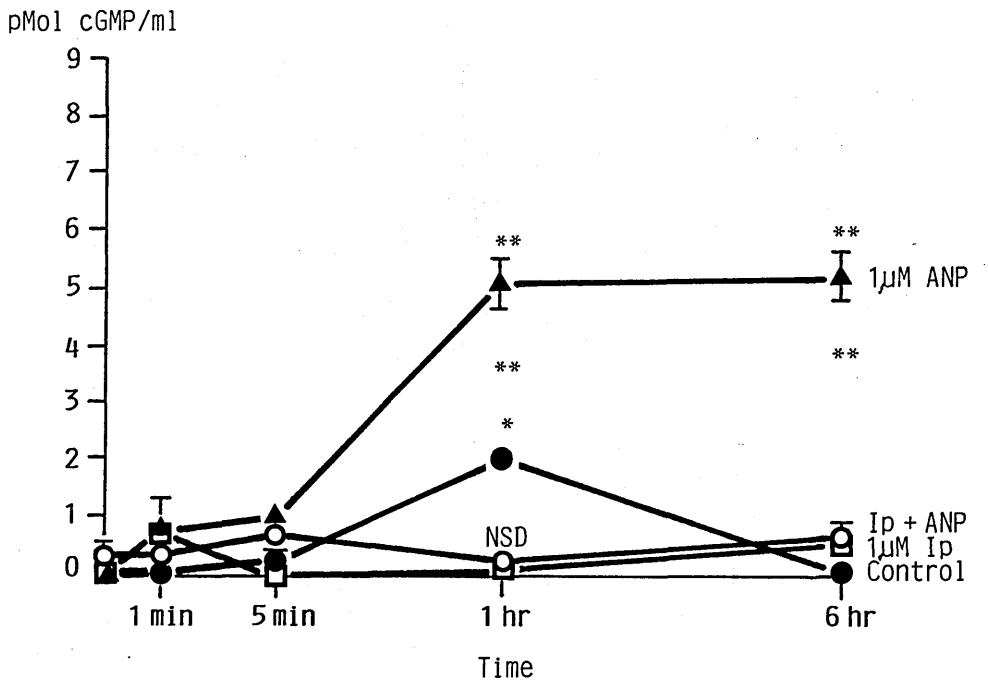
In these experiments, it was observed that renin release was significantly stimulated as early as 1 hour after treatment with isoprenaline ($P < 0.05$) (Figure 5.4.). Furthermore, ANP (1$\mu$M) significantly reduces $\beta$-stimulated renin release at 1 hour ($P < 0.05$). At 6 hours, active renin release was stimulated by 1$\mu$M isoprenaline ($P <$

## Figure 5.2: Intracellular cAMP response during ANP-isopenaline interaction



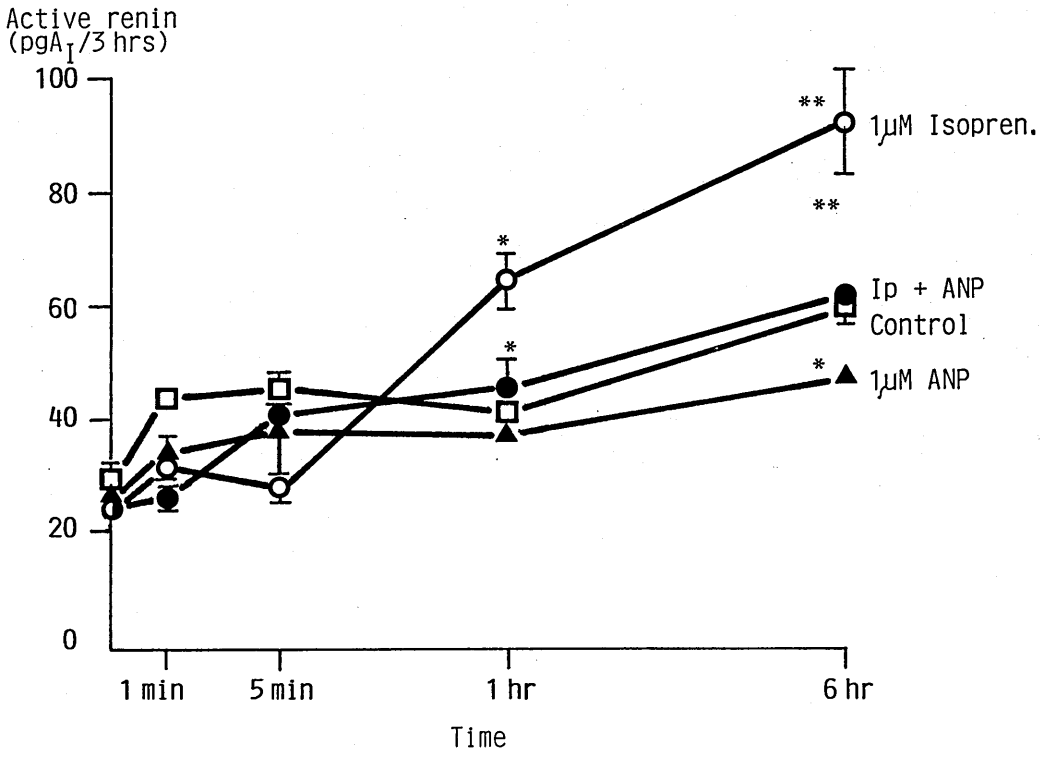

Each point is Mean ± S.E.M.
** p<0.01 * p<0.05
NSD - no significant difference

## Figure 5.3: Extracellular cGMP response during ANP-isoprenaline interaction




Each point is Mean ± S.E.M.
** p<0.01 * p<0.05
NSD - no significant difference

## Figure 5.4: Active renin concentrations during ANP-isoprenaline interaction




Each point is Mean ± S.E.M.
** p<0.01 * p<0.05
NSD - no significant difference

0.01). This was antagonised by 1μM ANP ($P < 0.01$). Also, 1μM ANP caused inhibition of active renin release, at 6 hours ($P < 0.05$) (Figure 5.4.).

These results confirm and extend those previously presented in Sections 3.3.1, 4.3.1, 4.3.2(ii-iv). Furthermore, the present study has shown that ANP interacts with β-stimulated renin release as early as 1 hour after treatment.

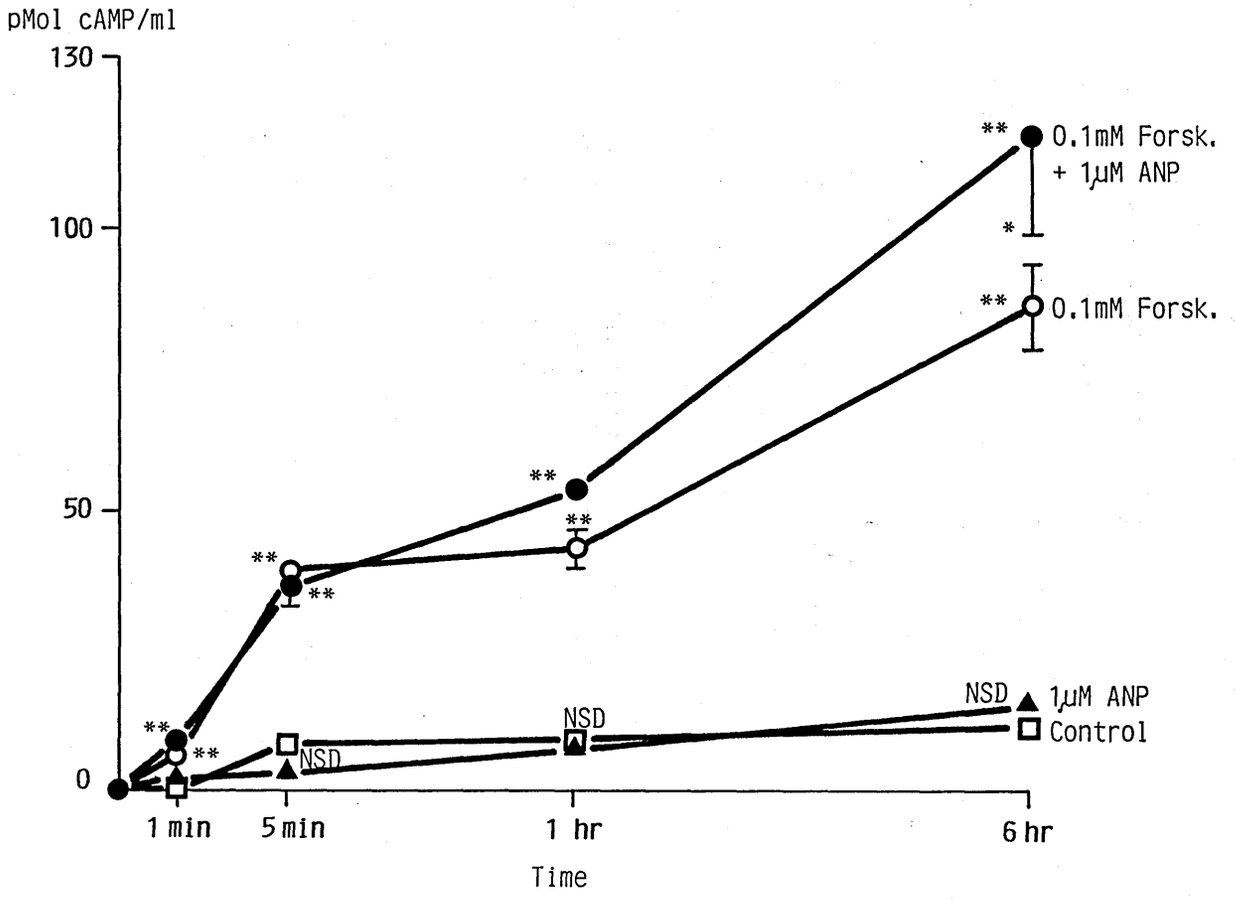
5.3.3. Second-messenger responses during ANP-forskolin interaction

Treatment of the cells with 0.1mM forskolin (Figure 5.5.) also caused an increase in intracellular cAMP concentration, but in quite a different manner to that observed in response to isoprenaline. The increase in intracellular cAMP is gradual and continuous up until 6 hours. The increase, in response to 0.1mM forskolin is significant at 1 min, 5 mins, 1 hour and 6 hours after treatment.

Treatment of the cells with 1μM ANP and 0.1mM forskolin caused a very similar, gradual increase in intracellular cAMP concentration (Figure 5.5.). However the inclusion of 1μM ANP with forskolin (0.1mM) significantly potentiated the forskolin-evoked increase in intracellular cAMP concentration at 1 hour ($P < 0.01$) and 6 hours ($P < 0.05$) (Figure 5.5.).

After treatment with 0.1mM forskolin, at 1 min, 1 hour and 6 hours, active renin release is significantly

**Figure 5.5: Intracellular cAMP response during ANP-forskolin interaction**




Each point is Mean ± S.E.M.
** p<0.01 * p<0.05
NSD - no significant difference

stimulated ($P < 0.05$) (Figure 5.6.). Forskolin-stimulated renin release is significantly reduced by the inclusion of 1$\mu$M ANP at 1 hour ($P < 0.05$) and also at 6 hours ($P < 0.01$) (Figure 5.6.).

Extracellular cGMP levels (Figure 5.7.) are stimulated by 1$\mu$M ANP, but not by 0.1mM forskolin. Both forskolin and isoprenaline cause attenuation of the cGMP response to ANP (Figures 5.3, 5.7.). Isoprenaline is very efficient whereas forskolin is less efficient and is ineffective at 6 hours (c.f. isoprenaline).

## 5.4. Conclusions

### 5.4.1. ANP-isoprenaline

Isoprenaline (1$\mu$M) causes a rapid increase in intracellular [cAMP], which peaks early (at 5 mins) and is followed at 1 hour, by stimulation of active renin release. At 24 hours, the effect of isoprenaline (1$\mu$M) on cell growth is first evident.

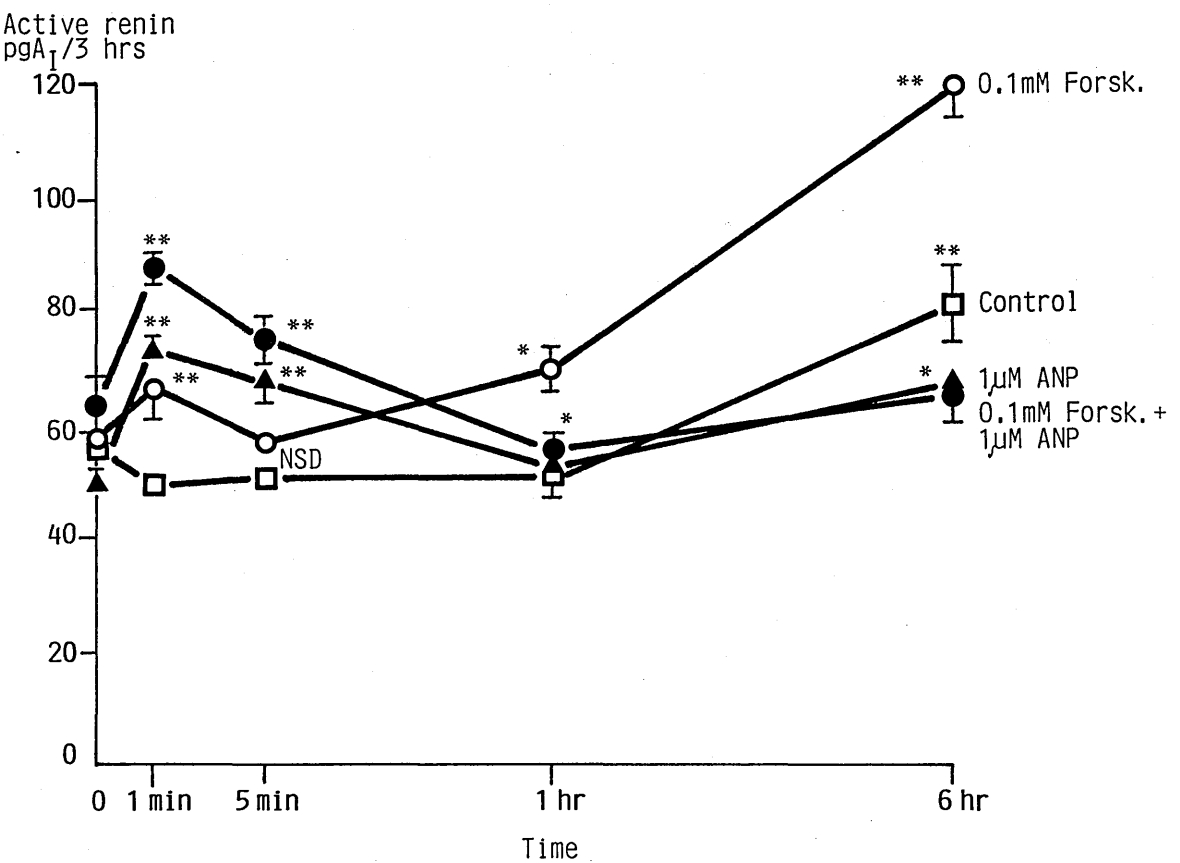
The cAMP response to isoprenaline is reduced by the inclusion of 1$\mu$M ANP. Therefore ANP probably attenuates isoprenaline-stimulated renin release (and possibly cell growth) by reducing intracellular cAMP concentration.

ANP (1$\mu$M) does not alter basal cAMP concentration. The observed stimulation of extracellular cGMP in response to 1$\mu$M ANP coincided with inhibition of active renin release at 6 hours and seems likely to be the mechanism whereby ANP inhibits basal renin release.

### 5.4.2. ANP-forskolin

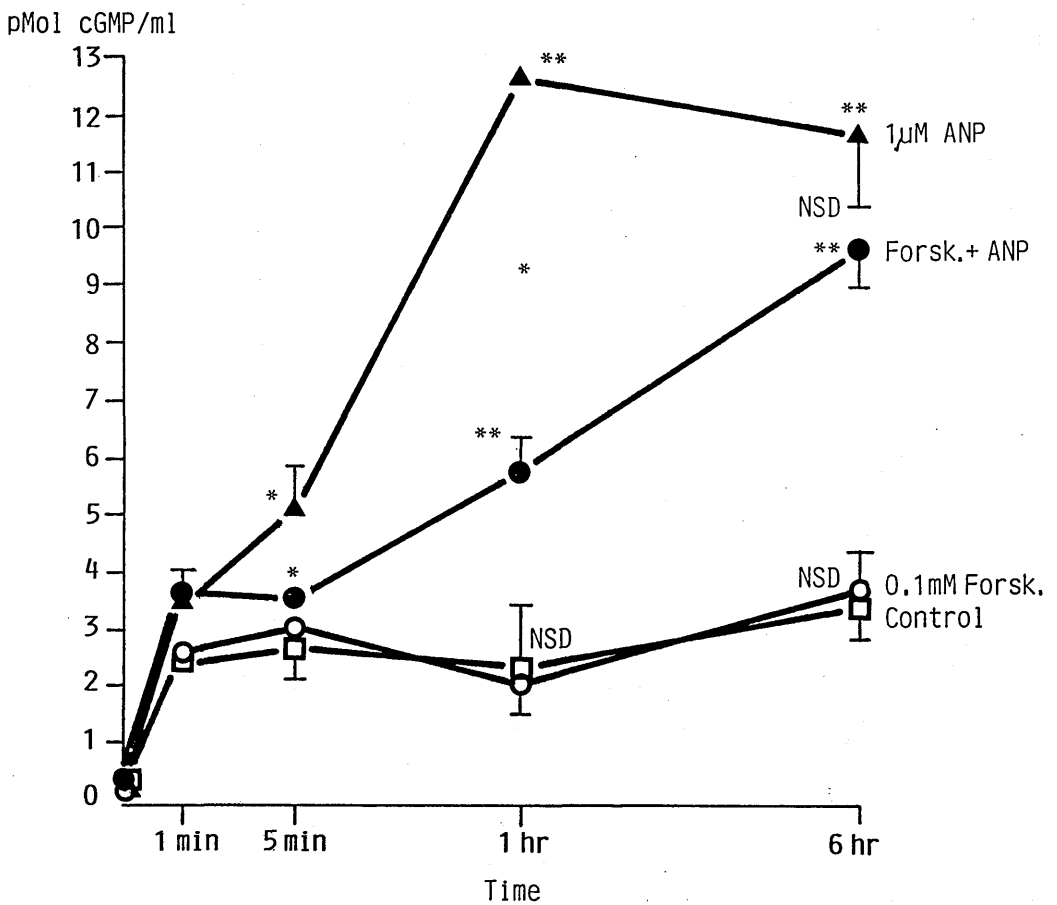
Forskolin (0.1mM) also causes a rapid increase in

## Figure 5.6: Active renin concentrations during ANP-forskolin interaction




Each point is Mean ± S.E.M.
** p<0.01 * p<0.05
NSD - no significant difference

**Figure 5.7: Extracellular cGMP response during ANP-forskolin interaction**




Each point is Mean ± S.E.M.
** p<0.01 * p<0.05
NSD - no significant difference

intracellular [cAMP] but this response is sustained and is paralleled by stimulation of active renin release.

ANP (1$\mu$M) does not reduce forskolin-stimulated $[cAMP]_i$ (rather ANP potentiates it). It was, however, noticeable that forskolin was not as effective at lowering ANP-stimulated cGMP release, as isoprenaline was. Therefore, ANP may, through guanylate cyclase linked receptors, cause inhibition of forskolin-stimulated renin release in this model.

5.5. Summary

(i) Isoprenaline and forskolin stimulate adenylate cyclase.
(ii) ANP stimulates guanylate cyclase (probably particulate).
(iii) ANP inhibits isoprenaline-stimulated renin release (and cell growth) and causes a reduction in $[cAMP]_i$.
(iv) ANP inhibits forskolin-stimulated renin release and forskolin is not as efficient as isoprenaline is at lowering $[cGMP]_e$.

From this summary we can therefore deduce;
(v) ANP has receptors which could be linked, in a negative fashion with adenylate cyclase (Section 4.3.1) and in a positive fashion with guanylate cyclase.
(vi) Increased $[cAMP]_i$ and reduced $[cGMP]_e$ could be important in stimulation of cell growth in this model system, in vitro.

## CHAPTER 6

**The effect of $A_{II}$ and converting-enzyme inhibition on renin release and cell growth.**

### 6.1. Introduction

The vasoactive effector peptide, angiotensin II ($A_{II}$) is known to inhibit renin release from the kidney (Keeton and Campbell, 1981). This effect on renin release can be considered as a physiological negative feedback loop (Figure 1.4.) in the regulation of blood pressure (Davis and Freeman, 1976).

It has also been shown that $A_{II}$ is active in inhibiting renin release from kidney slices and nonfiltering kidneys (Shade et al., 1973). The inhibitory effect of $A_{II}$ on renin release has been shown to be dependent on extracellular calcium (Vandongen, 1975; Park et al., 1981) and functioning calcium channels (Park et al., 1981).

It has been postulated that $Ca^{2+}$ influx is involved in the inhibitory response to $A_{II}$ (Kurtz et al., 1984). In a later study Kurtz et al. (1986) used the fluorescent $Ca^{2+}$ dye, quin-2, to measure changes in $[Ca^{2+}]_i$ and showed a prompt rise in cytosolic $Ca^{2+}$ in response to $A_{II}$, in cultured rat JG cells.

However, a characteristic feature of calcium-mobilizing receptors is that they initiate a signal cascade leading to the formation of a number of putative second messengers (Berridge, 1981, 1982; Irvine et al., 1982; Takai et al., 1982). The initial event in this cascade is

specific hydrolysis of membrane phosphoinositides. $A_{II}$ has been shown to stimulate phosphoinositide metabolism in cultured vascular smooth muscle cells (Alexander et al., 1985; Brock et al., 1985; Griendling et al., 1986).

Agonists (such as $A_{II}$) act by stimulating the hydrolysis of PtdIns4,5$P_2$ (Figure 6.1) to form diacylglycerol and Ins1,4,5$P_3$. These two products then function as second messengers to activate the two independent, but parallel signal pathways.

Diacylglycerol (Figure 6.1) functions within the plane of the membrane to increase protein phosphorylation by activating C-kinase (Kaibuchi et al., 1982; Takai et al., 1982; Nishizuka, 1983). Inositol trisphosphate is released to the cytosol and is thought to function as a second messenger to mobilize calcium from intracellular stores (Streb et al., 1983; Joseph et al., 1984). These two signal pathways appear to function in a synergistic manner to stimulate a wide variety of cellular processes.

It is as yet not known, however, whether $A_{II}$ exerts similar effects on phosphoinositide metabolism in human JG cells.

Relatively few studies of the direct effects of $A_{II}$ on isolated and/or cultured human JG cells have been carried out. Pinet et al. (1987) have studied the effects of $A_{II}$ on prorenin secretion from cultured transfected JG cells, but they did not investigate the regulation of active renin release.

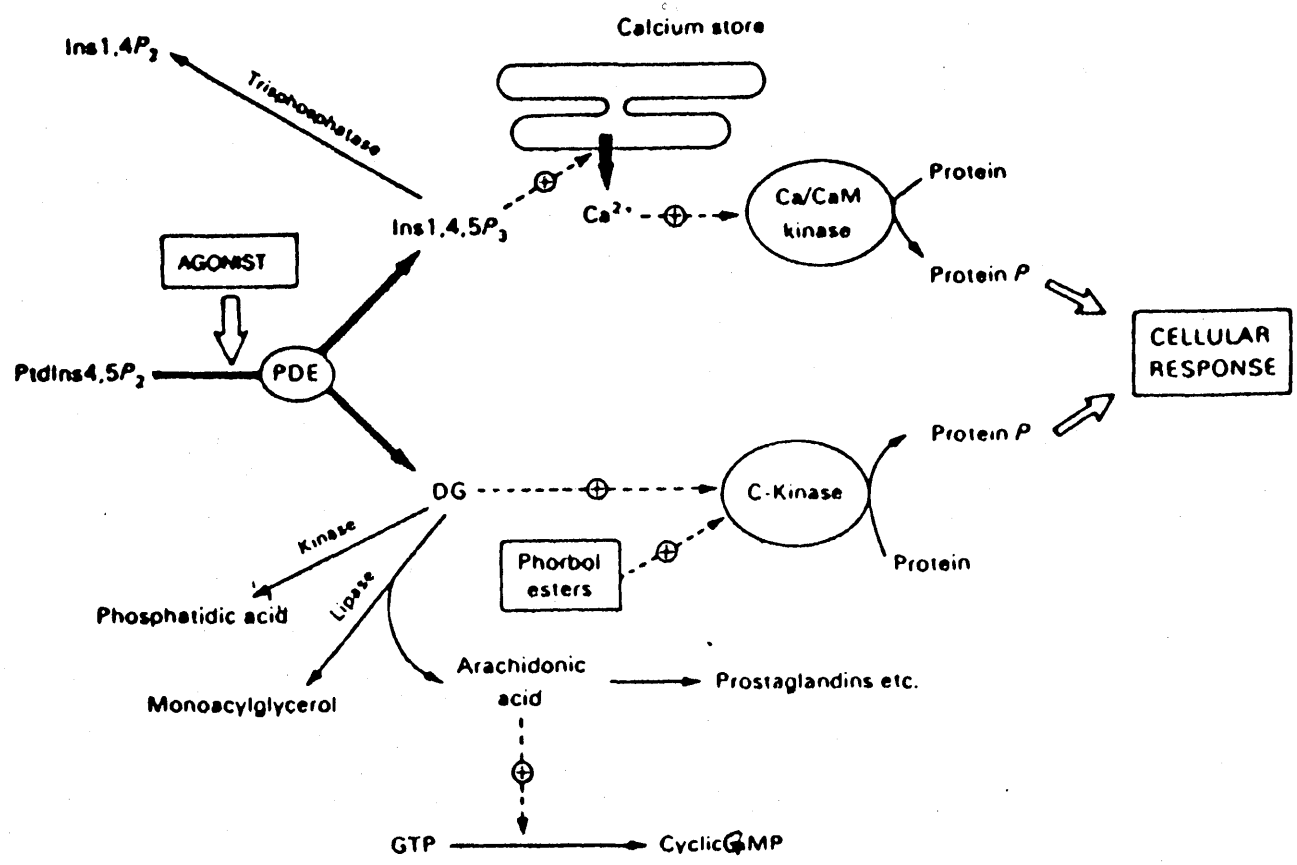



**Figure 6.1.:** *Second messenger role of diacylglycerol (DG) and inositol trisphosphate (Ins1,4,5$P_3$)*
A large number of cellular processes may be switched on through the combined action of these two parallel signal pathways that begin with the agonist-dependent hydrolysis of PtdIns4,5$P_2$ to give Ins1,4,5$P_3$ and diacylglycerol. The former is removed by a trisphosphatase, whereas diacylglycerol is converted to either phosphatidic acid or monoacylglycerol. Ins1,4,5$P_3$ acts to mobilize intracellular calcium, whereas diacylglycerol stimulates the same C-kinase that can be activated by phorbol esters. Abbreviations used: PDE, phosphodiesterase; CaM, calmodulin.

(Source: Berridge, 1984)

It was decided to investigate whether addition of exogenous $A_{II}$ had any effect on active and prorenin release from cultured nephroblastoma cells, and whether $A_{II}$ had any effect on cell growth. We were also interested in determining whether the cells synthesized and released $A_{II}$ or whether $A_{II}$ was formed extracellularly.

Subsequently, it was decided to determine if the increase in renin induced by the converting-enzyme inhibitor Enalapril(at) *in vivo*, is mediated through interruption of $A_{II}$ feedback, using the nephroblastoma cells as an *in vitro* system.

### 6.2. The effect of $A_{II}$ on renin release and cell growth

#### 6.2.1. Short-term experiments

Cells were seeded and grown to confluence (Section 2.3.3.(i)). At the end of the basal incubation period, the medium was removed and replaced with medium containing 1$\mu$M $A_{II}$ and the cells were incubated for 5 hours. The concentration of active renin released at 5 hours was measured as detailed in Section 2.3.3.(i).

#### 6.2.2. Long-term release and growth rate experiments

In long-term release and growth rate experiments, cells were seeded at 2 x $10^5$ cells/ml and grown for 3-4 days (Section 2.3.3.(iii)). Medium was removed and replaced with medium containing $A_{II}$ (1$\mu$M) or Enalaprilat (1$\mu$M) and growth was continued for up to 12 days. On day 3 of the experiments, the culture medium was changed and at 3 day intervals (except in the $A_{II}$ effect on total renin release in which the first sample was at 2 days) samples

were taken for active and total renin estimations (Sections 2.4.1., 2.4.2.).

Also, in order to determine whether these agents had any effect on cell growth, cells were harvested and counted at 6 day intervals, as described previously (Section 2.3.3.).

6.2.3. Long-term dose-response experiment to Enalaprilat.

In this experiment, the cells were seeded at 2 x $10^5$ cells/ml and grown for 3-4 days. The medium was removed and replaced with medium containing Enalaprilat in the range 1$\mu$M to 0.1mM and growth was continued until day 9. On day 3 of the experiment, the culture medium was changed and on day 3 and day 9 samples were taken for active renin estimations and $A_{II}$ concentration measurement (Sections 2.4.1. and 2.4.3. respectively).

6.3. Results and discussion

6.3.1. Short-term experiments

Addition of exogenous $A_{II}$ (1$\mu$M) was found to inhibit active renin release from cultured human nephroblastoma cells, over a period of 5 hours ($P < 0.01$) (Figure 6.2).

When measurements of $A_{II}$ concentration were made (Section 2.4.3.), it was found that $A_{II}$ levels were high in the cell culture medium, to which no $A_{II}$ had been added (Table 6.1.). This $A_{II}$ was found to be formed by renin (secreted by the cells) acting on substrate present in the foetal calf serum (Table 6.1.).

**Figure 6.2.: Short-term (5h) exposure to 1μM $A_{II}$.**

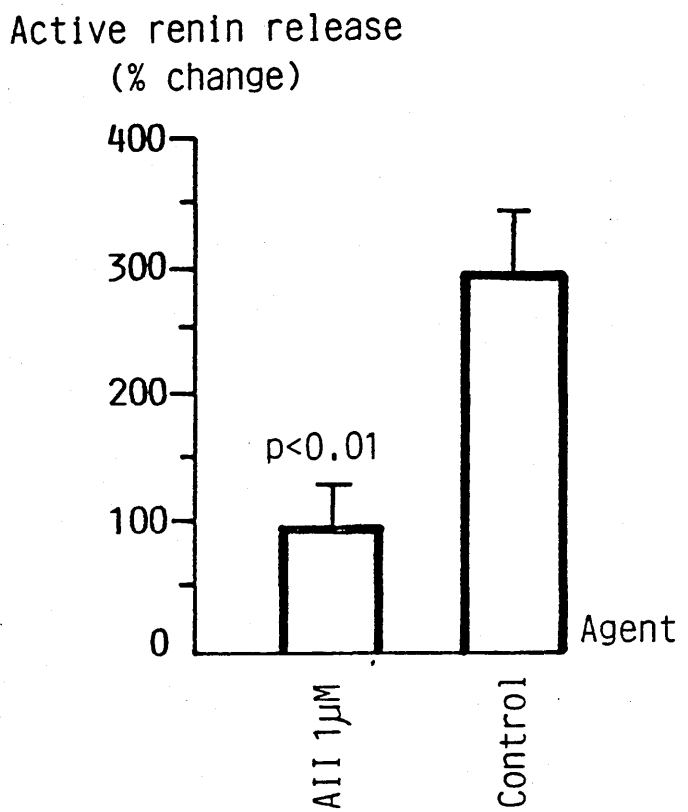


**Table 6.1.: $A_{II}$ concentrations in the cell culture medium.**

| | $[A_{II}]$pg/ml. | | |
|---|---|---|---|
| | Basal | 4h | 8h |
| Medium + F.C.S. | 125.0±6.1 | 117.4±2.5 | 113.8±14.0 |
| Medium - F.C.S. | Undetectable | Undetectable | Undetectable |

Results are presented as Mean ± S.E.M. (results of a representative experiment).

6.3.2. Long-term release and growth rate experiments.

Long-term treatment of cells with 1$\mu$M $A_{II}$ inhibited active ($P < 0.01$ at day 3) (Figure 6.3.a.(i)) and total ($P < 0.01$ at day 6) (Figure 6.3.a.(ii)) renin release but had no effect on cell growth over the 12 day study period.

Long-term treatment with Enalaprilat (1$\mu$M) for 12 days caused significant stimulation of active renin release ($P < 0.025$) at day 9 (Figure 6.4.a.(i)), but does not cause a sustained increase of prorenin release ($P < 0.025$ at day 6 only) (Figure 6.4.a.(ii)). 1$\mu$M Enalaprilat was observed to have no effect on cell growth, over a 12 day period, in this in vitro system.

6.3.3. Long-term dose response experiment to Enalaprilat.

The results from the long-term dose response experiments to Enalaprilat are presented in Figures 6.5.a,b. Enalaprilat was found to suppress extracellular $A_{II}$ concentrations, in a dose-dependent manner, with a concomitant stimulation of active renin release (Figures 6.4.a,b).

6.4. General discussion

In this study it was found that $A_{II}$ acts directly on renin-secreting cells to reduce secretion of both active renin and prorenin. Furthermore, $A_{II}$ measurements showed that extracellular endogenous $A_{II}$ levels in contact with the cells were high. Therefore, it is likely that these cells are basally suppressed by high endogenous $A_{II}$ levels.

In order to test this, cells were incubated in the presence of converting-enzyme inhibitor. Long-term

**Figure 6.3.: Long-term action of $A_{II}$ (1μM) on (a)(i) active renin release and (b) cell growth.**

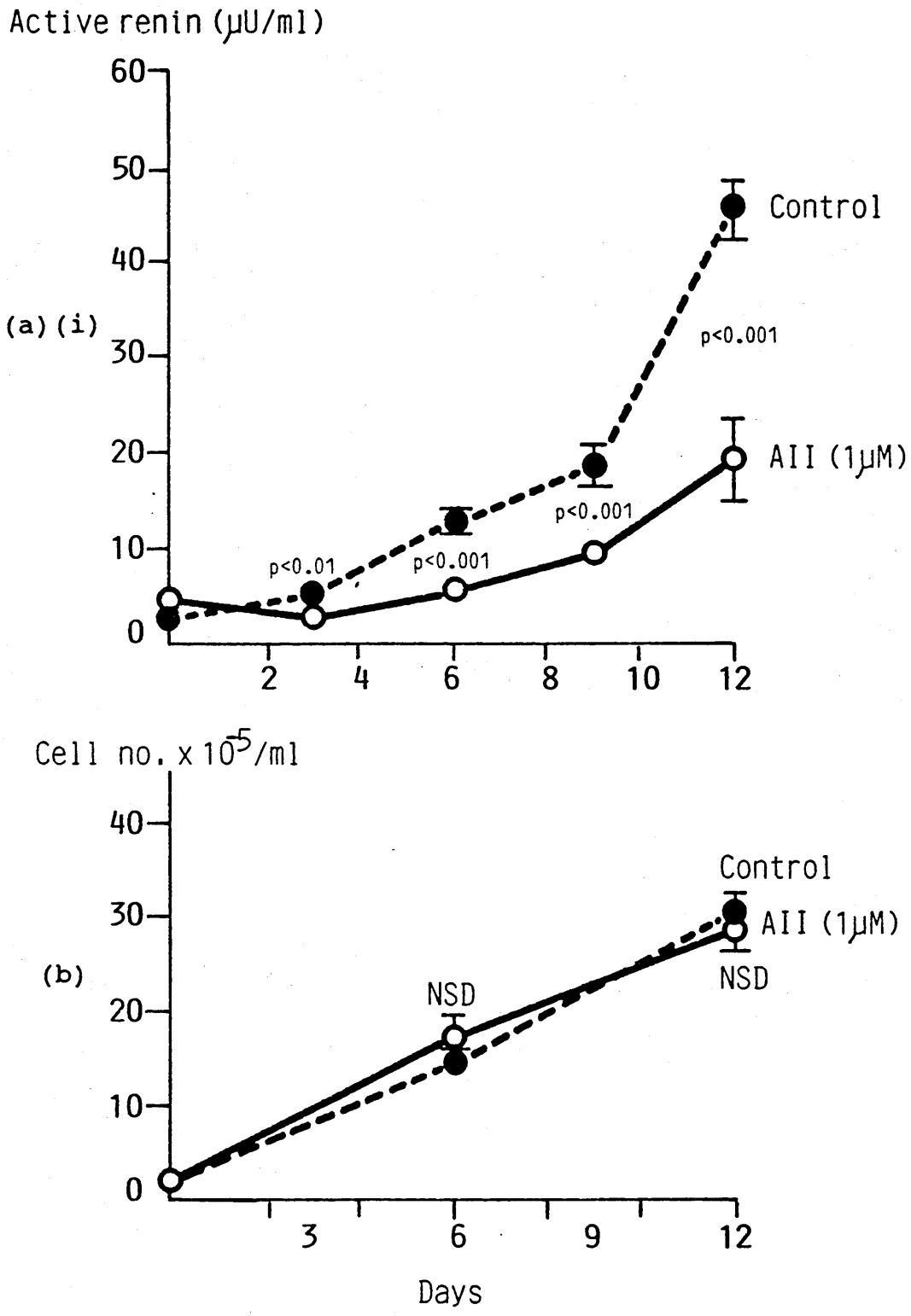


**Figure 6.3.(a)(ii): Long-term action of $A_{II}$ (1μM) on total renin release.**

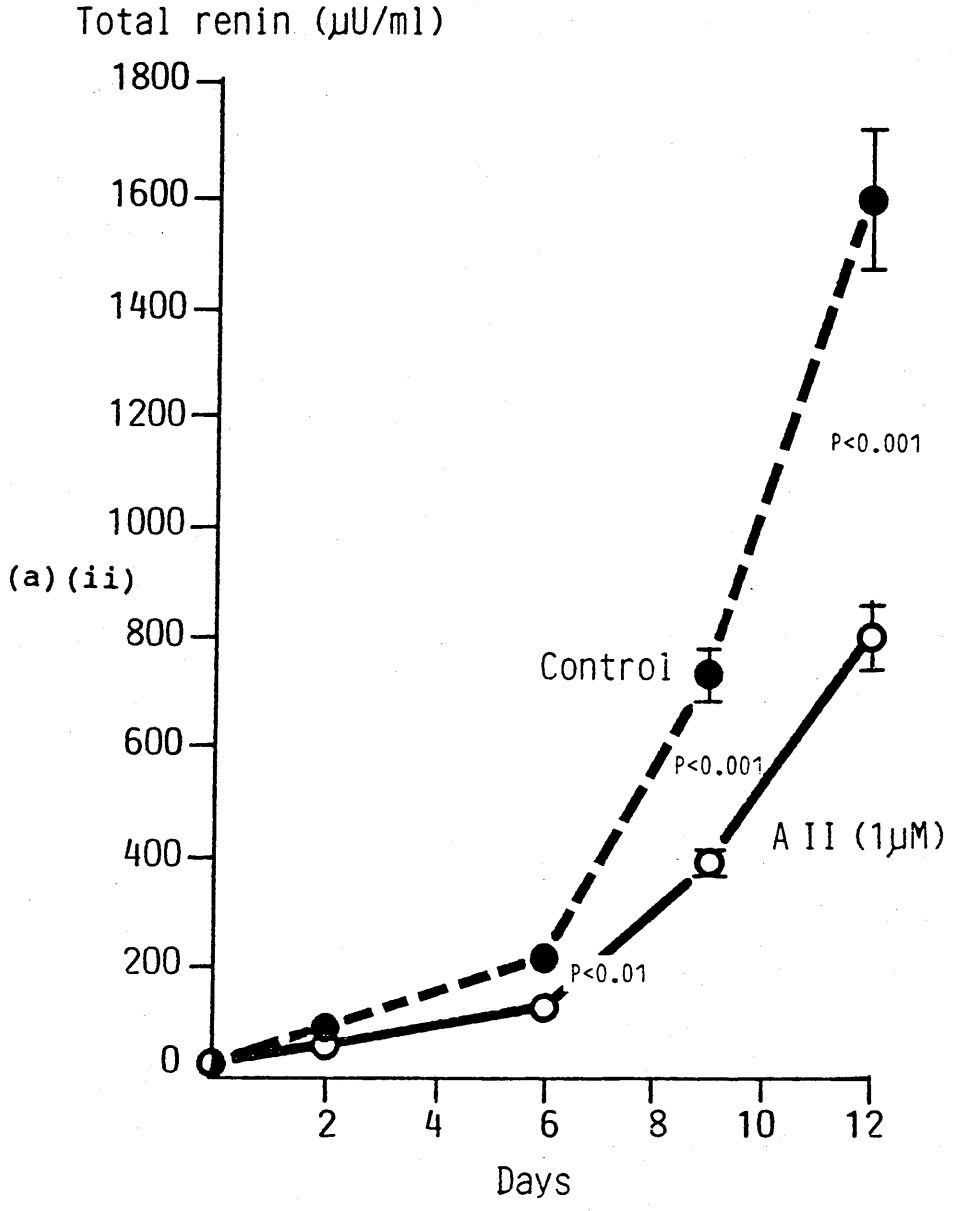


**Figure 6.4.: Long-term action of Enalaprilat (1μM) on (a)(i) active renin release and (b) cell growth.**

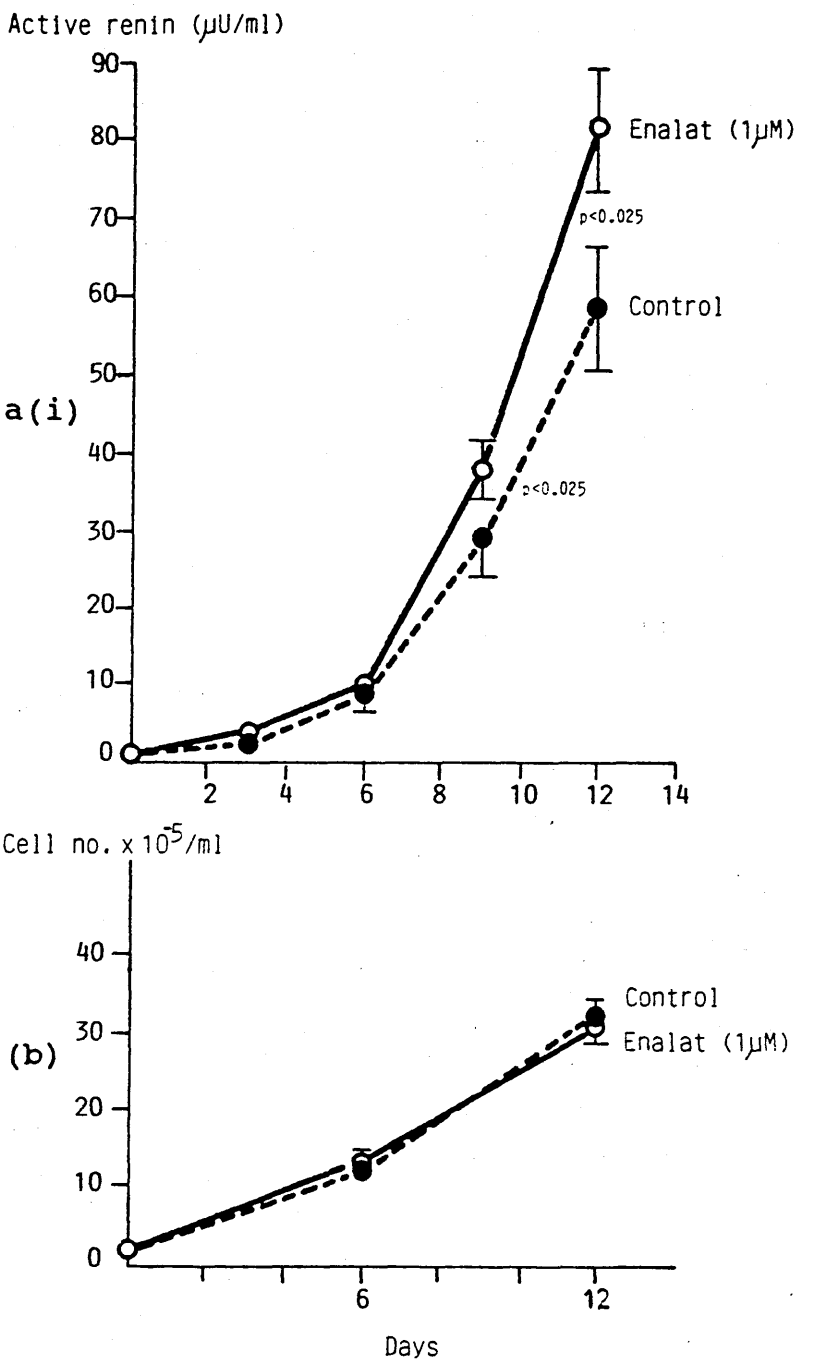


**Figure 6.4.(a)(ii): Long-term action of Enalaprilat (1μM) on total renin release.**

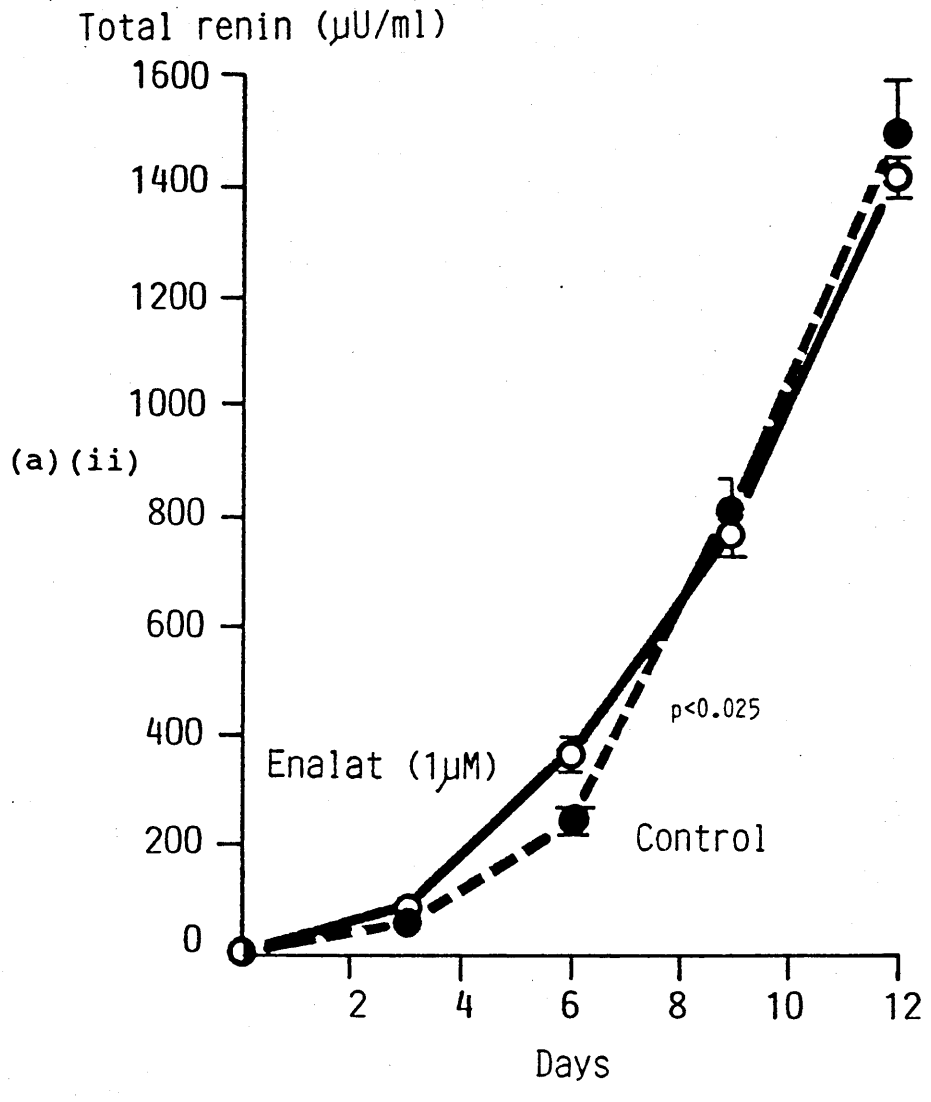
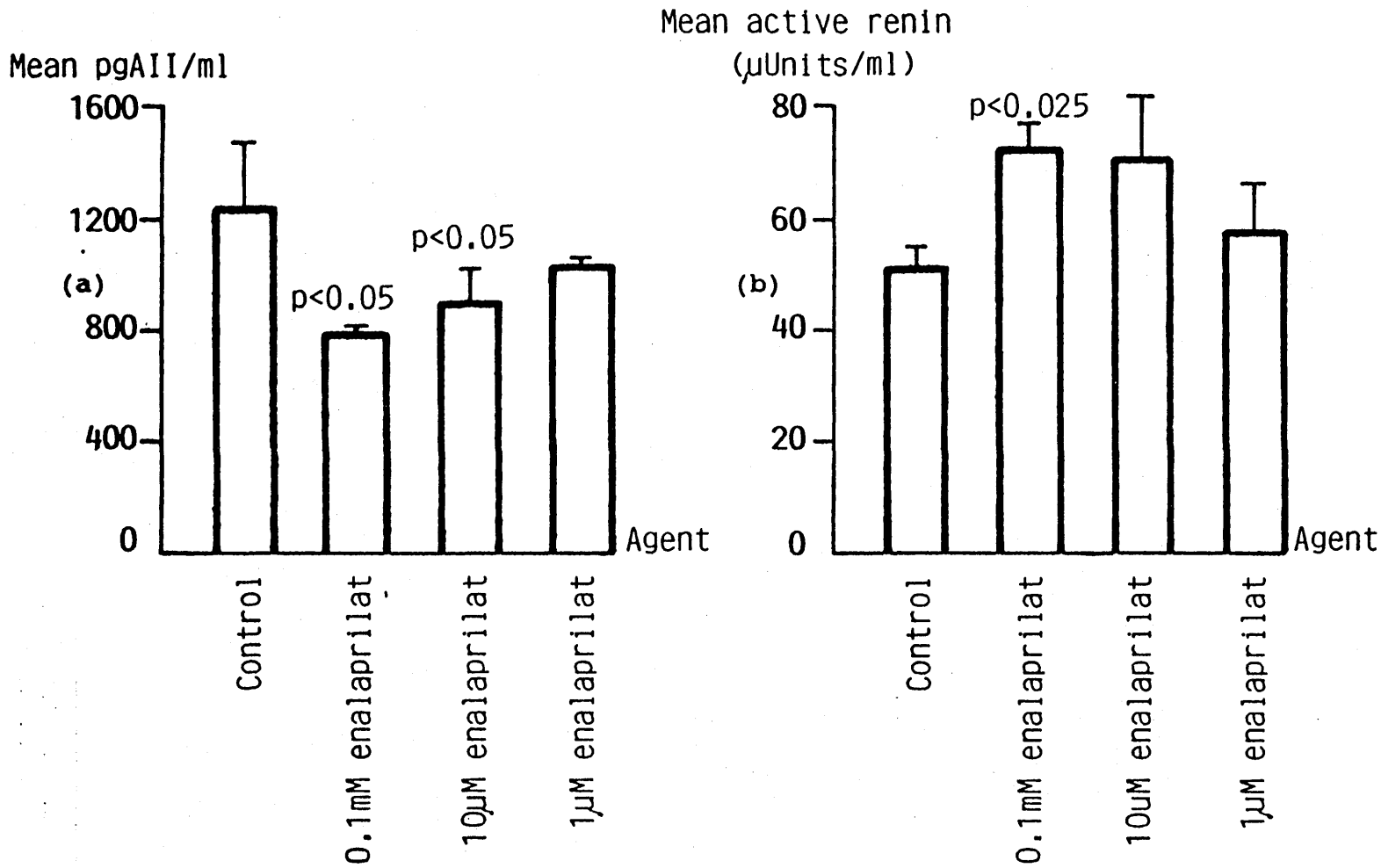


**Figure 6.5.**

**Long-term dose-response experiment to Enalaprilat (a) $A_{II}$ concentrations in medium (b) active renin concentrations in medium at day 9.**



treatment of the cells with 1$\mu$M Enalaprilat caused stimulation of active, but not prorenin release. Enalaprilat was found to be effective at lowering endogenous $A_{II}$ levels and this is probably the mechanism whereby it increases active renin release (i.e. intervention of $A_{II}$ negative feedback).

However, Enalaprilat (1$\mu$M) is less effective at stimulating prorenin release and this may be because $A_{II}$ is less effective at reducing prorenin compared with active renin in this system.

$A_{II}$, which has been reported to promote the growth of vascular smooth muscle cells in culture (Lyall et al., 1988a) had no effect on the growth of cultured nephroblastoma cells throughout the 12-day study period.

Geary et al. (1990) have shown (using the reverse hemolytic plaque assay) an increase in renin secretion by recruitment of renin-secreting cells in rats treated with Enalapril. In contrast, this study showed that Enalaprilat had no significant effect on cell growth.

Also, Geary et al. (1990) found that Enalapril did not affect the amount of renin secreted by each cell. In contrast, this study showed that Enalaprilat causes increased release of active renin per cell.

The feedback suppression by $A_{II}$ acts by inhibiting active renin and prorenin release per cell. Johns et al. (1987) have reported that $A_{II}$ suppresses renin gene expression in rat kidney.

Whether or not the observed inhibition of renin

release (in response to $A_{II}$) from cultured nephroblastoma cells reflects suppressed renin synthesis and gene expression is, as yet, unknown. Further studies would be necessary to resolve this point.

## CHAPTER 7

## G-protein expression in membranes prepared from human cultured nephroblastoma cells.

### 7.1. Introduction

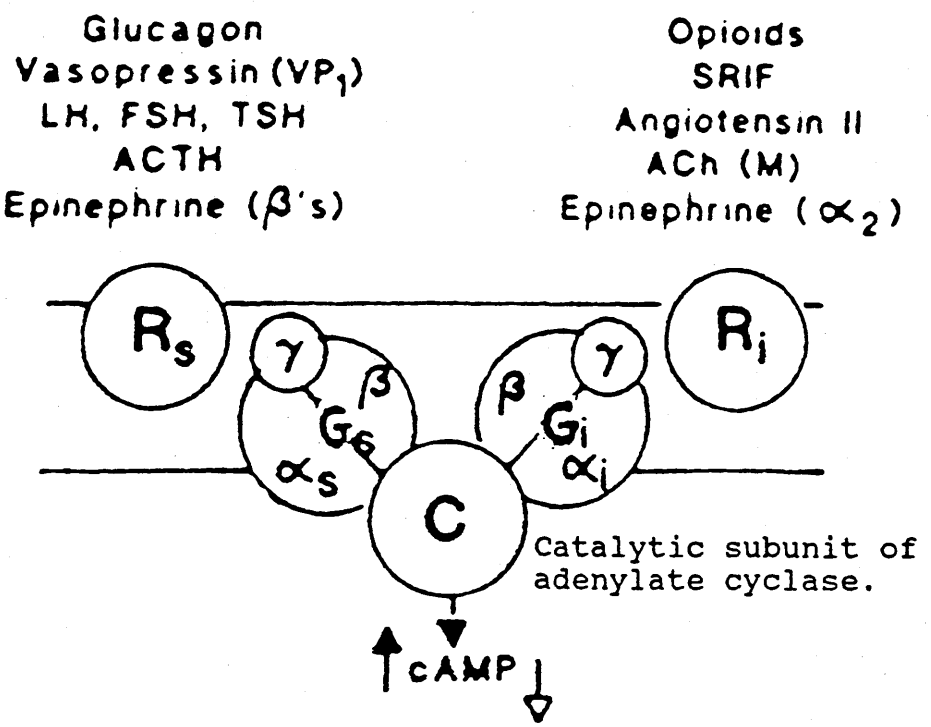
Guanine nucleotide-binding proteins (G-proteins) are a family of closely related membrane proteins that are involved in the transduction of signals from cell surface receptors to their effectors. Receptors for many hormones, growth factors and neurotransmitters, which control the rate of production of second messengers, such as Ins $1,4,5P_3$ and cAMP, are dependent upon the intermediate activation of one, or more G-protein(s).

Members of this family include both rod and cone transducins, $G_s$ and $G_i$, which respectively couple receptors (Figure 7.1) to the stimulation or inhibition of adenylate cyclase (Birnbaumer et al., 1985; Gilman, 1987; Spiegel, 1987) and also $G_o$. $G_o$ is highly concentrated in the brain (Rouot et al., 1987), where it accounts for approximately 1% of the total cortical membrane protein, and other neural tissue. $G_o$ is thought to be involved in the functional coupling of opiate receptors to neuronal voltage-dependent calcium channels (Hescheler et al., 1987).

#### 7.1.1. G-protein structure

G-proteins consist of three non-identical subunits termed $\alpha$, $\beta$ and $\gamma$. The unique $\alpha$-subunit appears to function to regulate its specific effector system, with the exceptions of receptor-regulation of phospholipase $A_2$ activity (Jelesma and Axelrod, 1987), and perhaps some

**Figure 7.1: Basic constituents of a hormone-sensitive adenylate cyclase system.**




Receptors which, when occupied, cause increases in cAMP levels are termed $R_s$ and those which bring about decreases in cAMP are termed $R_i$. At the centre of the coupling process between the receptors and the altered adenylate cyclase activity are two signal-transducing proteins which bind $Mg^{2+}$ and guanine nucleotides. One, $G_s$, mediates the effects of $R_s$, the other, $G_i$, mediates the effects of $R_i$ receptor binding (from Birnbaumer *et al.*, 1985).

degree of the control of inhibition of adenylate cyclase. The α-subunits vary in size from 39 to 52 kDa however at the level of primary amino acid sequence they are very highly conserved. They also bind guanine nucleotides with high affinity and contain an intrinsic GTP'ase activity which is essential for their normal function (for reviews see Casey and Gilman, 1988; Milligan, 1988).

Heterogeneity of $G_s$ α subunits was first noted during the purification of $G_s$ (Northup et al., 1983). Most tissues contain a mixture of two major forms of $G_s$ α. Individual forms of $G_s$ α can be generated by differential splicing of the pre mRNA transcribed from the gene for $G_s$ α (Bray et al., 1986). In all of the individual forms, however, the C-terminal decapeptide is conserved.

Two forms of the β-subunit with molecular masses 35kDa and 36kDa have been identified immunologically (Roof et al., 1985) and, more recently, the identification of cDNA clones for two separate forms of the β-subunit has confirmed the presence of at least two genes encoding β-subunits (Gao et al., 1987).

The γ -subunits are polypeptides of between 8 and 10kDa. Immunological evidence suggests the expression of a number of forms, however, relatively little work has been performed on their identification and function. In all physiological situations, the β-subunit remains tightly associated with its relevant γ -subunit.

7.1.2. G-protein function

The function of the G-proteins is to couple agonist-

activated receptors to the effector systems that alter intracellular concentrations of second messengers. As this process must be of limited duration, the G-protein is required to undergo a cyclical pattern of activation followed by a subsequent deactivation (Figure 7.2).

In the resting state the G-protein exists in a complex consisting of $\alpha$, $\beta$ and $\gamma$ subunits, with GDP bound to the nucleotide binding site of the $\alpha$-subunit. Upon receptor activation of the G-protein (Figure 7.2), the rate of release of GDP (which appears to be the rate-limiting step in the G-protein activation/deactivation) is enhanced and the released GDP is replaced by GTP (reviewed by Gilman, 1987).

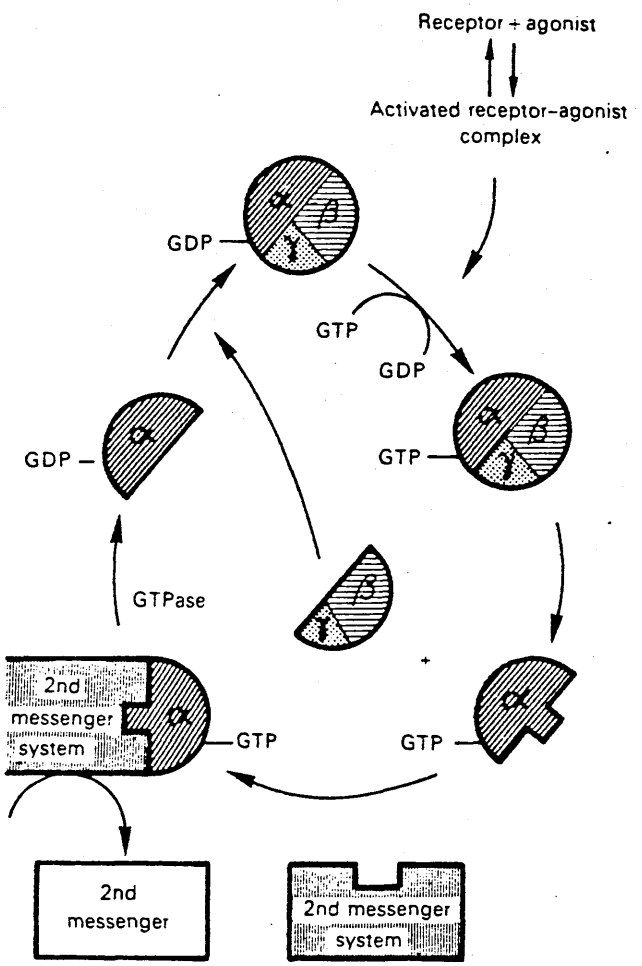
With GTP in the nucleotide binding site and in the presence of $Mg^{2+}$, then the holomeric G-protein can dissociate into an active $\alpha$-subunit with GTP bound and free $\beta/\gamma$ subunits. This active $\alpha$-subunit is then able to interact with the catalytic moiety of a particular second-messenger-generation system to alter the rate of synthesis of the second messenger.

Hydrolysis of the terminal phosphate of the bound GTP by the intrinsic GTP'ase activity deactivates the $\alpha$ subunit and in this GDP-bound form, it is then able to reassociate with $\beta/\gamma$ subunits to restore the G-protein to the deactivated state.

### 7.1.3. G-protein identification

The traditional means of identifying $G_s$ and $G_i$ has

**Figure 7.2: The role of GTP binding and hydrolysis in the activation and deactivation of a typical G-protein.**




Source: Milligan, 1988

relied on the ability of cholera toxin to catalyse the transfer of [$^{32}$P]ADP-ribose from $^{32}$P-NAD$^{+}$ to $G_s$ $\alpha$ (Figure 7.1) and of pertussis toxin to catalyse an analogous ADP-ribosylation of $G_i$ $\alpha$ (Katada and Ui, 1982; Milligan et al., 1985).

ADP-ribosylation of the $\alpha$ subunit of $G_s$ inhibits its GTP'ase activity thereby permanently stimulating adenylate cyclase. Pertussis toxin ADP-ribosylates the $\alpha$ subunit of $G_i$ and this blocks its effect on adenylate cyclase and thereby removes the inhibitory action of $G_i$.

However, limitations of these procedures have included either, for cholera toxin, the requirement for a proteinaceous cofactor (ARF) (Kahn et al., 1988) or, for pertussis toxin, the identification of a considerable number of substrates for the toxin (Milligan and Klee, 1985). These include $G_o$ (Goldsmith et al., 1988) as well as G-proteins named $G_i1$, $G_i2$ and $G_i3$ (Jones and Reed, 1987).

This has led to the generation of both polyclonal and anti-peptide antisera able to identify specifically a number of different G-proteins (Backlund et al., 1988; McKenzie et al., 1988). Information taken from cDNA clones, corresponding to the various G-proteins allowed the generation of anti-peptide antisera against sequences which are unique to the various gene products (Backlund et al., 1988; Mullaney et al., 1988).

## 7.2. Basis for assessment of G-protein expression in nephroblastoma cell membranes

In this study it has previously been shown (in Chapters 3, 4 and 5) that renin release from cultured nephroblastoma cells is stimulated by the $\beta$-adrenergic agonist, isprenaline. This was found to involve an increase in intracellular cAMP concentration and is thought to involve adenylate cyclase activation. In the present study, it has also been shown that ANP inhibited renin release and, furthermore this inhibition was removed by the presence of pertussis toxin. This would therefore imply the involvement of a pertussis toxin-sensitive G-protein in ANP inhibition of renin release in this model, *in vitro*.

In this study, highly selective anti-(G-protein) anti-peptide antisera have been used to assess the expression of G-proteins in membranes prepared from human cultured nephroblastoma cells.

## 7.3. Immunological analysis

Membrane preparations from the cultured nephroblastoma cells were carried out, as described in Section 2.5.1. Membrane protein concentration was determined using the method of Lowry *et al.*(1951) (Section 2.2.5.). Membrane samples were resolved by SDS/PAGE and immunoblotted, as detailed in Section 2.5.2.

The generation and specificities of the various antisera used in this study are defined in Table 7.1.

Antiserum SG2 is an anti-peptide antiserum produced

**Table 7.1: Generation and specificities of antisera used.**

| Anti-serum: | Peptide employed: | Corresponding G-protein sequence: | Antiserum identifies: |
|---|---|---|---|
| SG2 | KENLKDCGLF | TD1(341-350) | TD1, TD2, $G_i1$, $G_i2$ |
| I1C | LDRIAQPNYI | $G_i1$(159-168) | $G_i1$ |
| CS1 | RMHLRQYELL | $G_s$ (385-394) | $G_s$ |
| ON2 | GCTLSAEERAALERSK | $G_o$ (1-16) | $G_o$ |

against the C-terminal decapeptide of rod transducin. This antiserum identifies forms of $G_i$ as well as transducin. However, as transducin is limited in distribution to photoreceptor-containing tissues, this antiserum (SG2) can be used as a probe for forms of $G_i$ in other tissues (Backlund et al., 1988; McKenzie et al., 1988).

Antiserum I1C (Table 7.1) was raised against a peptide corresponding to amino-acids 159-168 of $\alpha_i1$ (Green et al., 1990).

Antiserum CS1 was raised against a peptide (Table 7.1) corresponding to the C-terminal decapeptide of $G_s$ $\alpha$ (Milligan and Unson, 1989).

Antiserum ON2 was raised against a synthetic peptide corresponding to a specific region of the $\alpha$-subunit (1-16) of $G_o$ (Mullaney and Milligan, 1990).

For molecular-mass estimations on immunoblots, prestained molecular-mass markers were subjected to SDS/PAGE in parallel with the test samples.

Immunoblotting with the above antisera was then performed as described in Section 2.5.2.

## 7.4. Results and discussion

The results obtained using the above antisera are summarized in Table 7.2.

### 7.4.1. Assessment of $G_i$ expression

Antiserum SG2 identifies $G_i1$ and $G_i2$ equally, as these two proteins have an identical sequence in the region used to generate the antiserum. This antiserum does not identify $G_i3$.

**Table 7.2: G-protein identification in nephroblastoma cell membranes.**

| Antiserum employed: | Antiserum identifies: | Presence or absence of G-protein: |
|---|---|---|
| SG2 | TD1, TD2, $G_i1$, $G_i2$ | present |
| I1C | $G_i1$ | absent |
| CS1 | $G_s$ | present |
| ON2 | $G_o$ | absent |

Antiserum SG2 identified a single polypeptide in human nephroblastoma cell membranes of 39-40kDa (Figure 7.3.).

In order to determine whether this polypeptide corresponded to $G_i1$ or $G_i2$, an antipeptide antiserum which is able to discriminate between $G_i1$ and $G_i2$ was employed. Antiserum I1C identifies only $G_i1$. No immunoreactivity to this antiserum was observed in human nephroblastoma cell membranes (results not shown), thus demonstrating that the 39-40kDa polypeptide was indeed $G_i2$.

### 7.4.2. Assessment of $G_s$ expression

The presence of $G_s$ $\alpha$ was identified using the antiserum CS1. Two major forms of $G_s$ $\alpha$ (Section 7.1.1.) are present, of molecular mass 40kDa and 42kDa (Figure 7.4), in membranes from cultured nephroblastoma cells.

### 7.4.3. Assessment of $G_o$ expression

Antiserum ON2 recognises $G_o\alpha$. However, no immunoreactivity to antiserum ON2 was observed in membranes from cultured nephroblastoma cells (results not shown).

Interestingly, $G_o$ has previously been reported to be expressed in small amounts, in bovine kidney medulla (Huff *et al.*, 1985) and also in a variety of cultured cell lines (e.g. Mullaney *et al.*, 1988).

## 7.5. Conclusions

In conclusion, the present study has shown that $G_s$ and $G_i2$ are expressed in membranes of human cultured nephroblastoma cells.

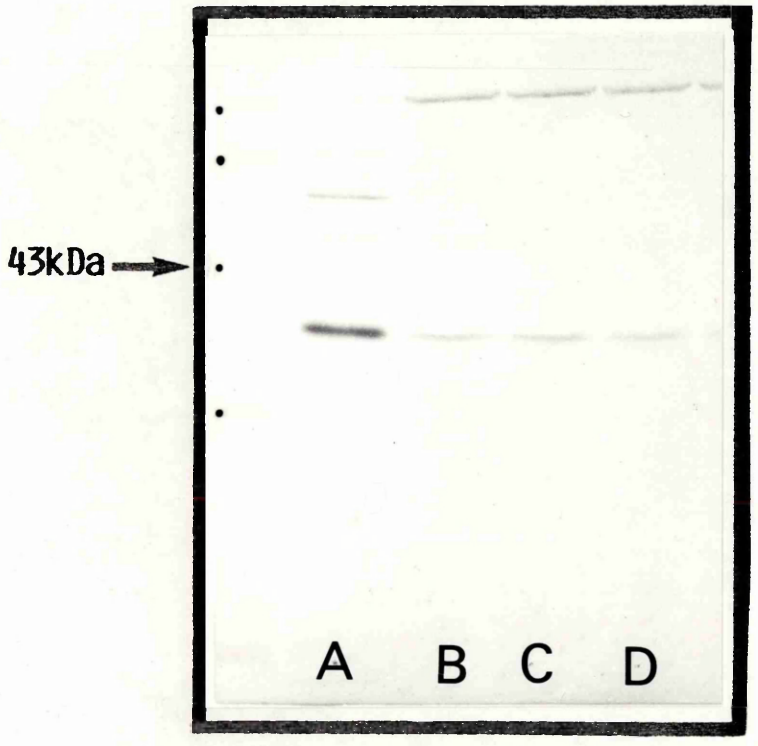



**Figure 7.3: Identification of $G_i2$ in human nephroblastoma cell membranes.**

Membranes from (A) rat cerebral cortex (100μg), (B), (C) vascular smooth muscle cells (330, 280μg), (D) human nephroblastoma cells (120μg) were resolved and immunoblotted, as described in Section 2.5.2. The blot was developed using antiserum SG2 (1:200 dilution) as the primary reagent.

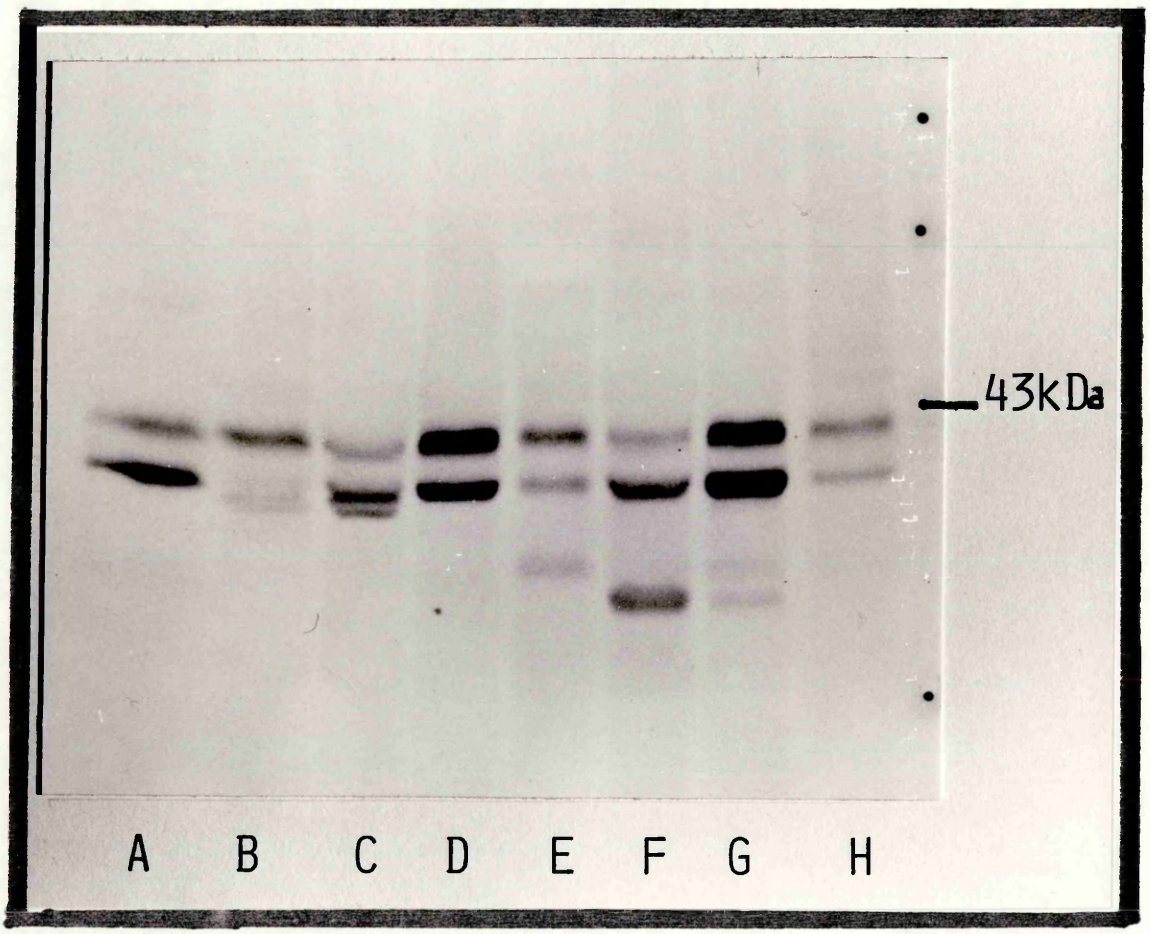



**Figure 7.4: Identification of $G_s$ in human nephroblastoma cell membranes.**

Membranes from (A) human nephroblastoma cells (120$\mu$g), (B)-(H): rat; brain (B), liver (C), heart (D), adipocytes (E), renal cortex (F), renal cortex (partially purified) (G) and vascular smooth muscle cells (H) (all 100$\mu$g), were resolved and immunoblotted, as described in Section 2.5.2. The blot was developed using antiserum CS1 (1:200 dilution) as the primary reagent.

These results are reassuring, because, in this study, it has been demonstrated that $\beta$-stimulated renin release (and cell growth) by isoprenaline involves increased $[cAMP]_i$ probably mediated by adenylate cyclase stimulation. Also, this study has already demonstrated (in Section 4.3.1) that ANP-inhibited renin release was blocked by the presence of pertussis toxin, which suggested the involvement of a pertussis toxin-sensitive G-protein, which probably mediated inhibition of adenylate cyclase.

However, the detection of both $G_s$ and $G_i2$ in the nephroblastoma cell membranes is not surprising since both $G_s$ and $G_i2$ are ubiquitous; being found in essentially all cells of vertebrates (Spiegel, 1987).

Nevertheless, to date, as far as we are aware, no-one has reported the expression of $G_s$ and $G_i2$ in human cultured nephroblastoma cell membranes.

In addition however, further studies will be necessary in order to determine whether $G_s$ and $G_i2$ are functional in the stimulation and inhibition of adenylate cyclase activity, respectively.

## CHAPTER 8

# Immunocytochemistry and electron microscopy studies of human cultured nephroblastoma cells.

## 8.1. Introduction

### 8.1.1. JG granular cells

It is now certain that the juxtaglomerular (JG) granular cell is the site of intrarenal synthesis of renin, as judged by immunohistochemistry (Taugner et al., 1979; Faraggiana et al., 1982; Lindop and Downie, 1984a; Lacasse et al., 1985).

The fine structure of normal, human JG cells has been studied in detail, both at the light and electron microscopic levels by Biava and West (1966), who confirmed the view that the JG cells represent modified arteriolar smooth muscle cells. The persistence of cytoplasmic myofibrils, the general organization of the endoplasmic reticulum and the structural appearances of mitochondria closely relate the JG cells to arteriolar smooth muscle cells.

However, one feature which is specific to the JG cell is the appearance of renin storage granules. Rhomboid protogranules, containing a crystalline (immature) form of renin were observed by Lacasse et al. (1985) in the JG cells of newborn mice.

The coalescence and eventual fusion of these protogranules, results in the formation of mature renin granules (Pratt et al., 1983; Galen et al., 1984; Lacasse et al., 1985).

The concentration of renin has been reported to increase from rough endoplasmic reticulum to Golgi complex to secretory granules (Lacasse et al., 1985), and about 60% of the cellular renin activity is thought to be localized in the mature granules (Morimoto et al., 1979).

The mature granules are located mostly in the periphery of the cell (Taugner et al., 1984). Smaller renin granules, of varying homogeneity, may be found clustering around the Golgi complex (Barajas, 1966).

8.1.2. Nephroblastoma cells

The renin secreting cells in the kidney are thought to be derived from undifferentiated mesodermal cells adjacent to the blood vessels in the metanephric renal cortex (Phat et al., 1981).

Nephroblastoma or Wilms' tumour is a tumour of pluripotent mesodermal cells which can differentiate not only into all structures of the normal kidney, but also other mesodermal tissues, such as bone and skeletal muscle.

It is therefore, not surprising that nephroblastoma cells can differentiate into renin secreting cells and that these renin secreting cells would be associated with the blood vessels (Lindop et al., 1987), mimicking the known developmental pattern of JG cells (Phat et al., 1981).

There have been previous descriptions of renin secreting nephroblastomas (Mitchell et al., 1970; Day and Luetscher, 1974; Carachi et al., 1987a). The evidence is based on high concentrations of prorenin in the pre-

operative plasma and normal plasma prorenin concentrations after removal of the tumour.

Inglis and Leckie (1990) have shown that prorenin (and active renin) were secreted from the tumour cells, by growing the nephroblastoma cells *in vitro* and monitoring the secretion of renin into the growth medium.

### 8.1.3. Characterization of cultured nephroblastoma cells

#### 8.1.3.(i) Immunocytochemistry

In order to further characterize the cells, an immunocytochemical study was carried out, using standard techniques (Section 2.6). This involved staining the cells with antisera to vimentin (which is the main protein subunit of intermediate filaments of cells of mesenchymal origin) and to a battery of epithelial cell markers, such as cytokeratins and epithelial membrane antigen (EMA).

In order to determine if the cells contained intracellular (stored) renin, immunostaining was carried out , using two separate renin antisera; one was raised in rabbits using human renin purified from a JG cell tumour. The purity of the renin and the specificity of this antisera have been documented (Galen *et al.*, 1979).

The other antiserum was raised in rabbits against pure human renin from cadaver kidney cortex (McIntyre *et al.*, 1983). The specificity of this antiserum has also been determined (Lindop *et al.*, 1987; Inglis and Leckie, 1990).

These antisera were used in the peroxidase-anti-peroxidase (PAP) technique, which has been described in detail elsewhere (Lindop *et al.*, 1983,a,b; Lindop and

Downie, 1984a; Lindop et al., 1984b, 1987).

8.1.3.(ii) E.M. studies

It was decided that a study of the ultrastructure of the cultured nephroblastoma cells should be carried out; in particular, to determine whether or not the cells contained storage granules.

Therefore, E.M. studies of human cultured nephroblastoma cells (at the 7th subculture) were carried out using standard techniques (Section 2.7.).

8.2. Results and discussion

8.2.1. Immunocytochemistry

There was negligible intracellular staining of renin, using the two antisera described previously (Section 8.1.3.(i)).

It is likely that renin is secreted from the nephroblastoma cells soon after synthesis.

The nephroblastoma cells were also stained with antisera to vimentin and to a battery of epithelial cell markers.

The cells were only positive for vimentin, which is consistent with mesenchymal differentiation (results not shown).

8.2.2. E.M. studies

E.M. studies of human cultured nephroblastoma cells showed the presence of myofilaments and dense condensations (Figure 8.1). Also, the cells were observed to contain glycogen granules (Figure 8.2) and an abundant and dilated, rough endoplasmic reticulum (Figures 8.1, 8.2) indicative

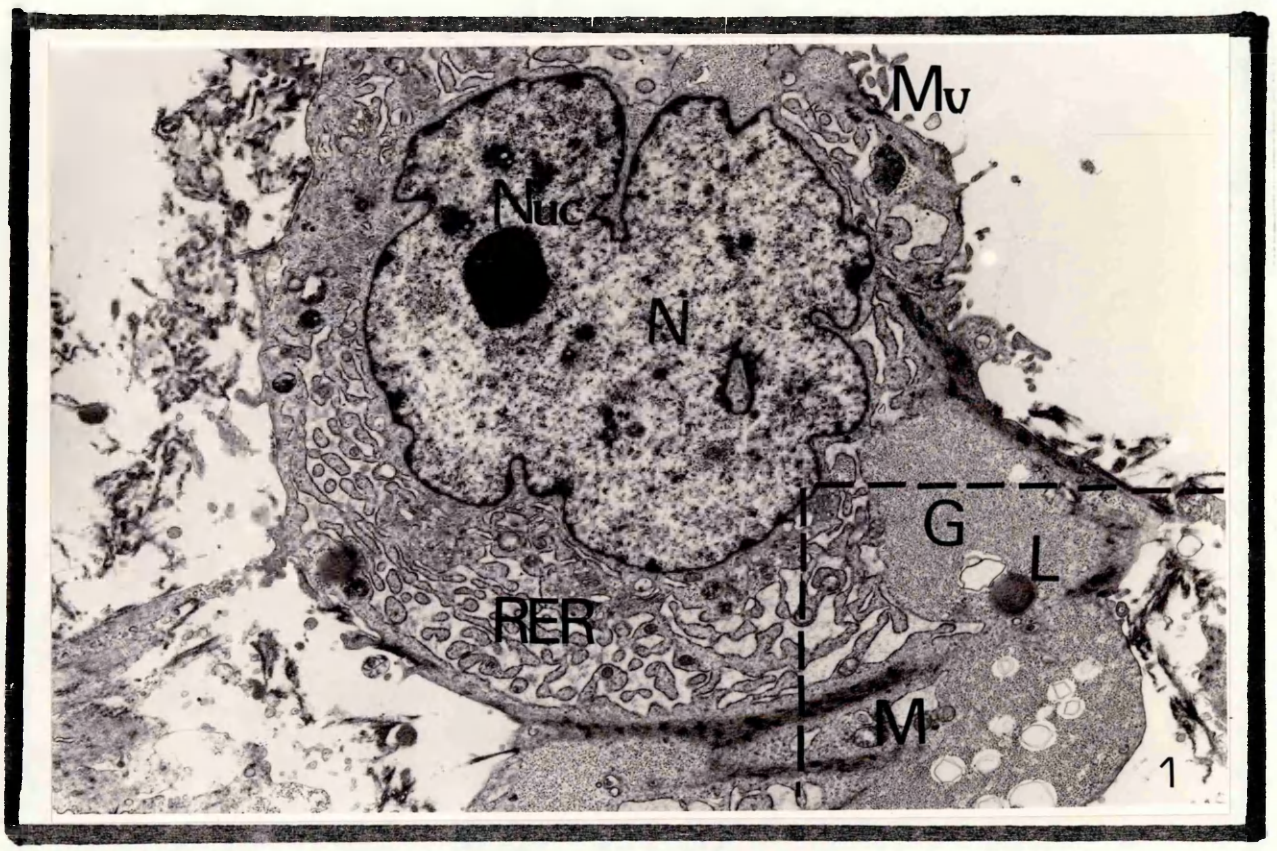



**Figure 8.1.: Low-power E.M. photograph of human nephroblastoma cell in culture.**

A prominent nucleus (N) with nucleolus (Nuc) is present. Note also the presence of myofilaments (M); microvilli (Mv); dilated rough endoplasmic reticulum (RER); liposomes (L) and glycogen (G).
(Final magnification: X8000).

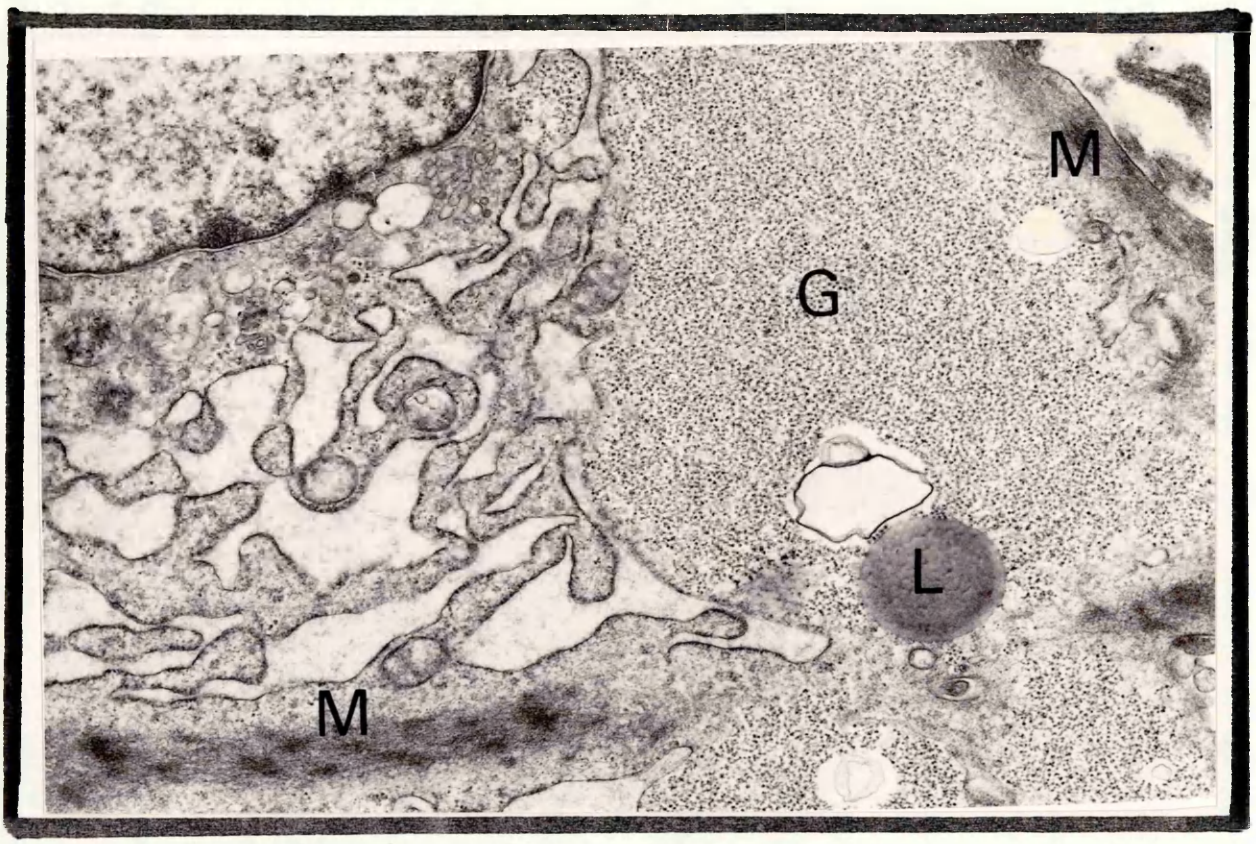



**Figure 8.2.: High-power E.M. photograph of intracellular features of nephroblastoma cell in culture.**

This is an enlargement of the area outlined and marked '1' in Figure 8.1. which, at greater magnification, shows, myofilaments (M); liposome (L) and glycogen (G).
(Final magnification: X 23000).

of a cell which is active in protein synthesis.

All of these features were suggestive of myoid differentiation. These results are consistent with those of Lindop and Gardiner (1986) who described the JG cells as myoepitheliod because their morphology is intermediate between that of endocrine and smooth muscle cells.

At higher magnifications (Figures 8.2, 8.3) the cells were also observed to have poorly differentiated Z-bands (Section 8.1.2), which is consistent with nephroblastoma.

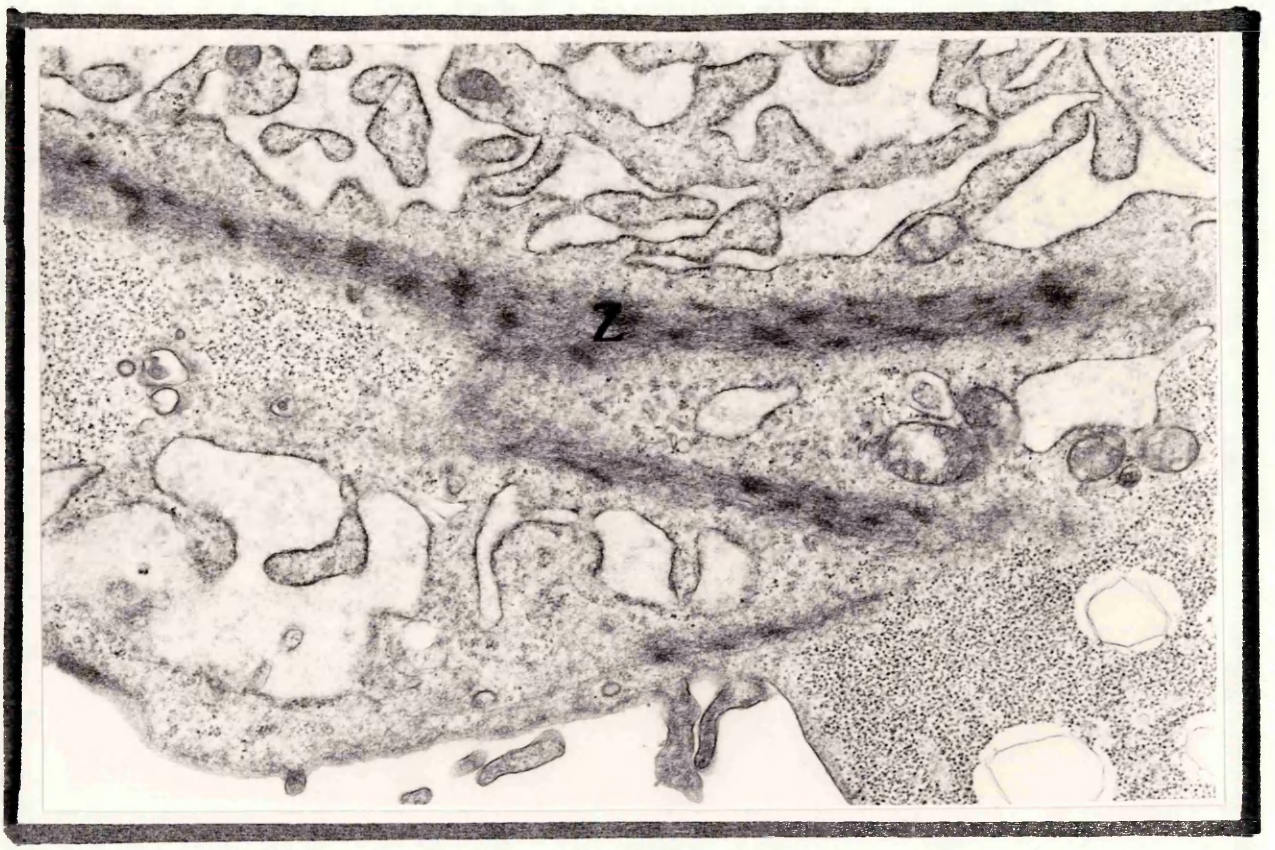



**Figure 8.3: High power E.M. photograph of myofilaments present in nephroblastoma cell in culture.**

Note the presence of poorly-differentiated 'Z' bands (Z). (Final magnification: X 23000).

## CHAPTER 9

# Karyotype analysis of human cultured nephroblastoma cells.

## 9.1. Introduction

### 9.1.1. Nephroblastoma (Wilms' tumour).

Nephroblastoma or Wilms' tumour, is one of the most common paediatric malignancies (Cutler and Young, 1975).

Two distinct forms of Wilms' tumour have been described i.e. hereditary (or familial) and non-hereditary (or sporadic) and the disease can be either unilateral, bilateral or multifocal in terms of the affected kidney(s). The hereditary form is the rarer, comprising only 1% of the diagnosed cases (Matsunaga, 1981) but is inherited as an autosomal dominant trait with variable penetrance (Matsunaga, 1981).

Whereas the hereditary form has a greater frequency of bilateral disease (i.e. both kidneys affected), the great majority of cases are of the sporadic form and affect only one kidney (99% of cases, 97% unilateral) (Maitland et al., 1989).

### 9.1.2. Identification of a possible Wilms' tumour gene

Both sporadic and syndrome-associated Wilms' tumours are accompanied by an increased frequency of abnormalities of the urinary tract and genitalia (Breslow and Beckwith, 1982).

Deletion analysis of individuals with the WAGR syndrome (Davis et al., 1988; Compton et al., 1988) (for, Wilms' tumour, aniridia, genitourinary abnormalities and mental retardation) showed that a Wilms' tumour gene lies

at chromosomal position 11p13 (ISCN, 1985) (Figure 9.1).

This led to the isolation of a candidate Wilms' tumour gene (Gessler et al., 1990), encoding a zinc-finger protein which is likely to be a transcription factor.

Recently, it has been reported by Pritchard-Jones et al. (1990) that the candidate Wilms' tumour gene (WT2-1) (Call et al., 1990) is expressed specifically in the condensed mesenchyme, renal vesicle and glomerular epithelium of the developing kidney, in the related mesonephric glomeruli and in cells approximating these structures in tumours.

The data obtained by this group suggest that the candidate gene, which lies at chromosomal position 11p13 is indeed a Wilms' tumour gene which has a specific role in kidney development and a wider role in mesenchymal-epithelial transitions.

9.1.3. Karyotype analysis of nephroblastoma cells

In order to characterize the nephroblastoma cells more fully, the karyotype of the cells was determined, using the methods as detailed in Section 2.8.

It was decided to determine whether the cultured cells contained any karyotype abnormalities previously reported to be associated with the Wilms' tumour phenotype and also whether the cells were composed of one or more cell population(s).

9.1.4. G-banding of nephroblastoma cell chromosome spreads

Subsequently, banding of the chromosomes was carried out (Section 2.8.2.(ii)). Banding techniques and what they

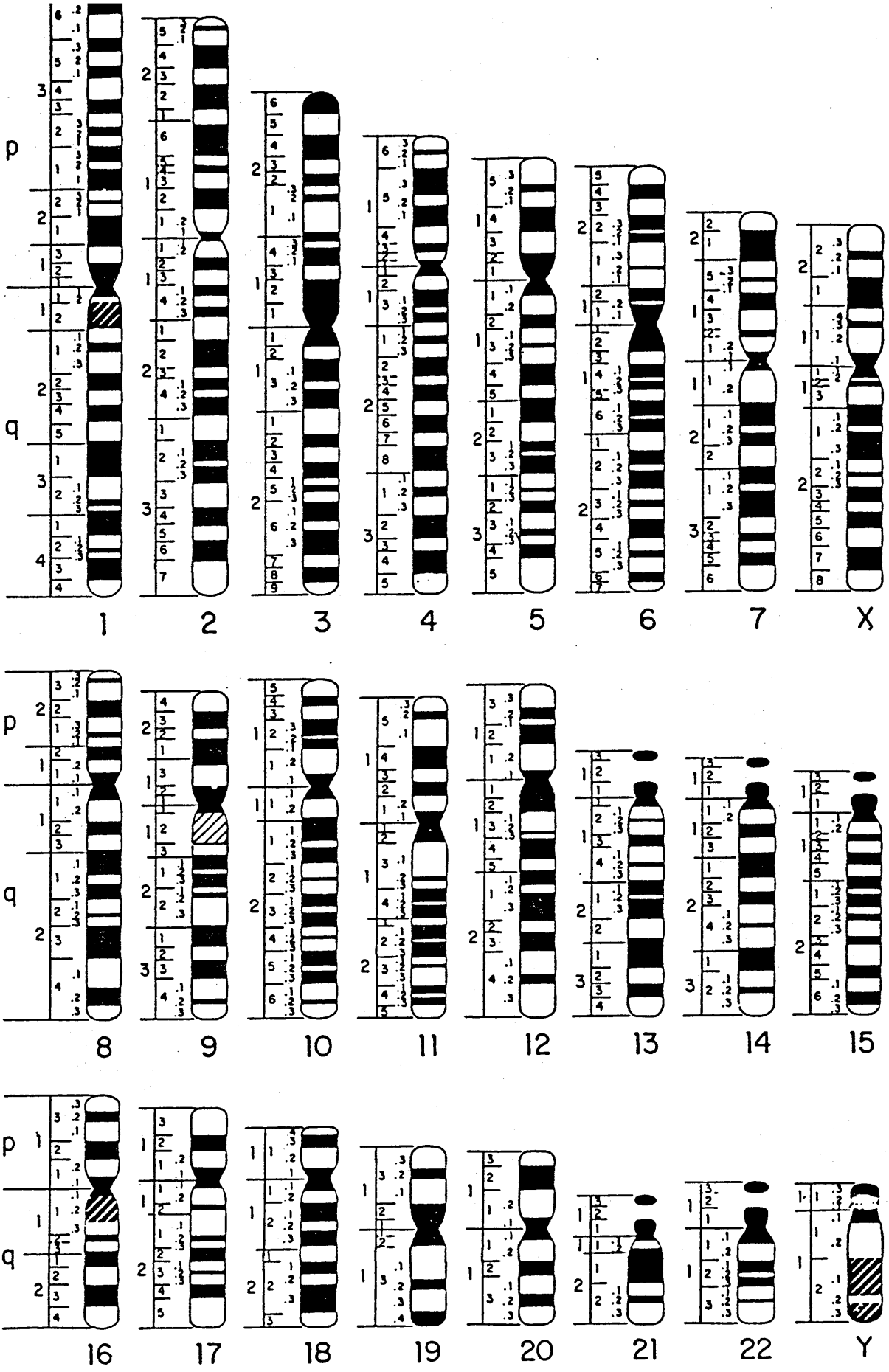



**Figure 9.1: Diagram of a banded human karyotype.**
**(ISCN 1985)**

reveal about chromosome structure have been reviewed in many articles (e.g. Dutrillaux, 1977; Ris and Korenberg, 1979).

This enables abnormalities on individual chromosomes to be detected. Of particular interest were chromosomes 11 and 1 on which the genes for Wilms' tumour (Pritchard-Jones et al., 1990) and control of renin activity (Wilson and Taylor, 1982), respectively, are reported to be located.

## 9.2. Results

### 9.2.1. Karyotype analysis

The karyotype of human cultured nephroblastoma cells (at the 8th subculture) was examined.

Several cells were analysed, the modal number of chromosomes was 46 and the karyotype was determined as 46XX (female) (Figure 9.2).

Therefore, the cells did not have any visible abnormality associated with a malignant phenotype and were clonal.

### 9.2.2. G-banding

Banding of the chromosomes revealed no abnormal chromosomes (Figure 9.3). Both copies of chromosome 1 and chromosome 11 appeared normal.

A typical banded karyotype is shown in Figure 9.3.

## 9.3. Discussion

In the light of recent reports concerning a candidate Wilms' tumour gene, it would of course, be very interesting to determine whether or not, the nephroblastoma cells in culture (which have already been shown to indicate

**Figure 9.2: Typical unbanded karyotype of human cultured nephroblastoma cells.**

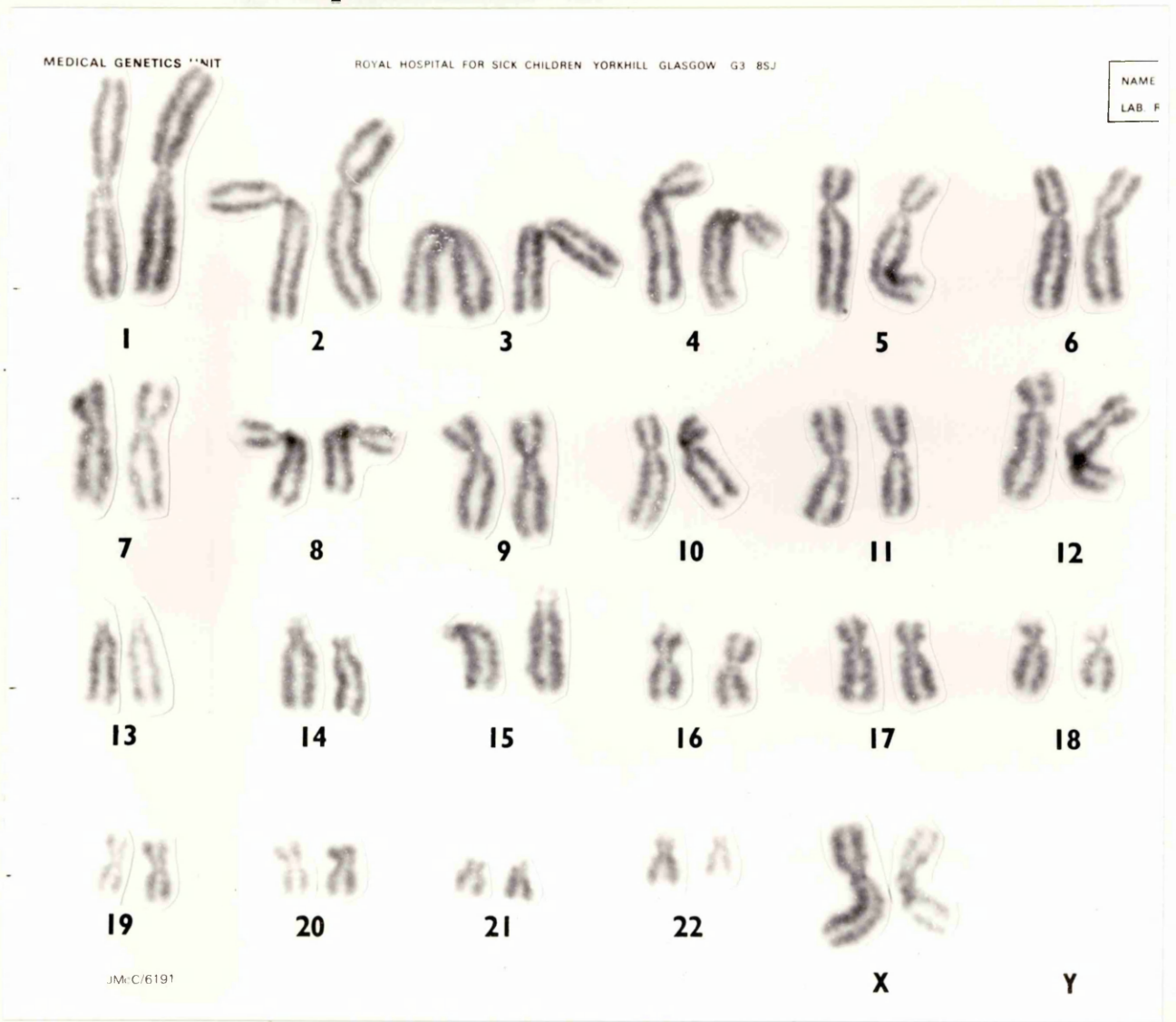
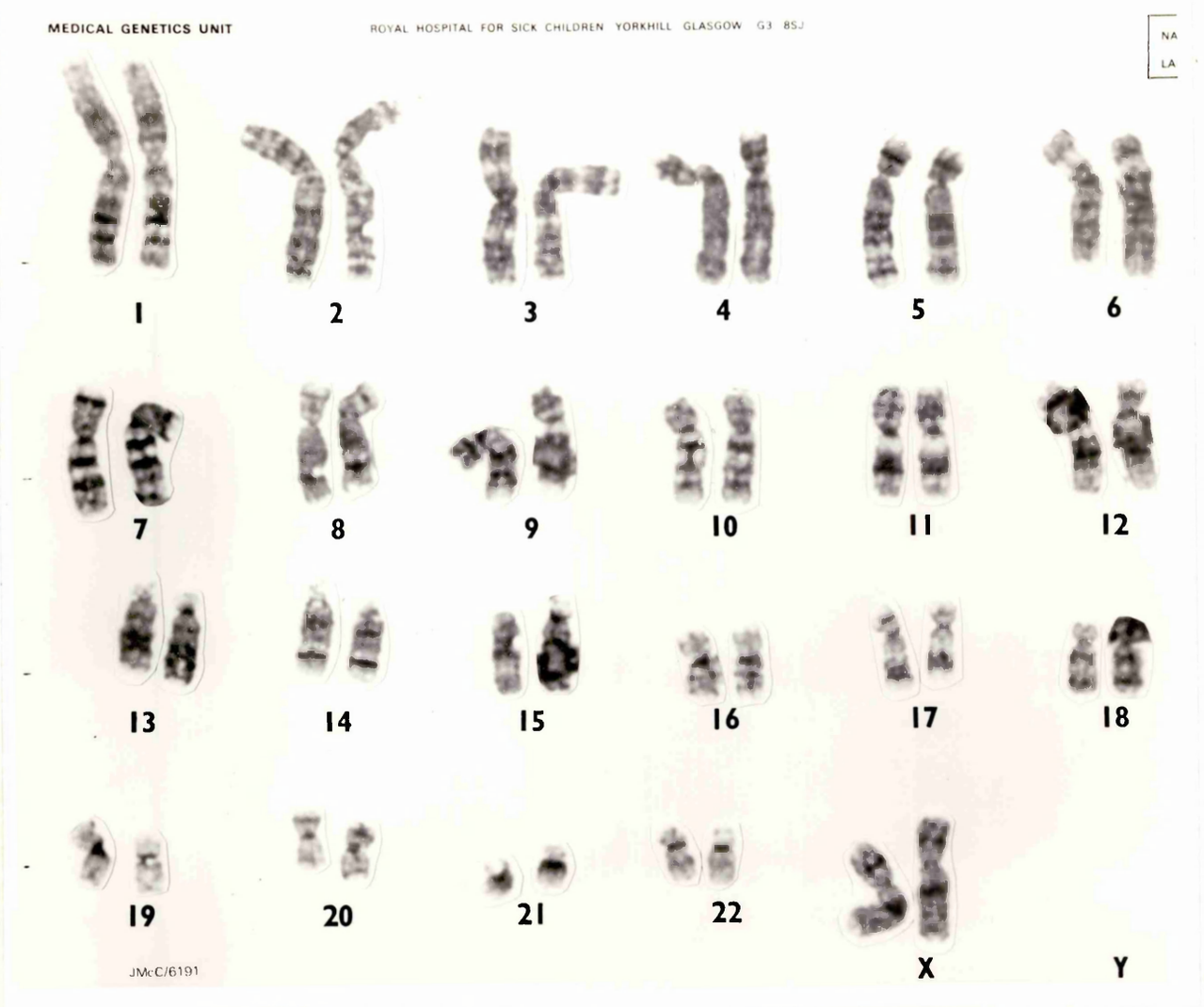


**Figure 9.3:** **Typical banded karyotype of human cultured nephroblastoma cell.**



mesenchymal differentiation (Section 8.2.1)) express the candidate Wilms' tumour gene (WT2-1).

To our knowledge analysis of WT2-1 expression has only been carried out in Wilms' tumour tissue sections and not in nephroblastoma cells which have been maintained in culture.

However, more than one locus has been implicated in the disease (Grundy et al., 1988; Huff et al., 1988) and it is possible that different loci may be implicated in different tumours even though the tumours are histologically indistinguishable (Grundy et al., 1988).

However, to conclude, no obvious abnormalities in the karyotype or banding of chromosomes in the cultured nephroblastoma cells were found.

This does not exclude the possibility that some chromosomal abnormalities at the level of the gene may be present.

# CHAPTER 10

# GENERAL DISCUSSION

## 10.1. Introduction

The aim of the present study was to investigate the control of renin release, using a novel *in vitro* model system: cultured human nephroblastoma cells. Studies of the intracellular control of renin release are extremely difficult to perform in cells derived from normal kidney, since JG cells form only a small proportion of the total cell population (Fray and Park, 1987).

Renin-secreting tumour cells have been described previously. Human tumoral JG cells were maintained in culture by Conn *et al.* (1972), and also by Galen *et al.*, (1984). However, in both instances the cells were primary cultures secreting mostly prorenin. Pinet *et al.* (1985) carried out SV40 transformation of human tumoral JG cells, thereby establishing a continuous culture of human JG cells. However, the level of renin released from the cells was low and more than 95% of the renin secreted into the medium was in an inactive form.

In the nephroblastoma cell model system, active renin represents 10% of the total renin released from the cells (Table 10.1). The proportions of active : total renin released by the cells are very similar to those found in the nephroblastoma patient's plasma (Carachi *et al.*, 1987a).

Although the cells were derived from a tumour, renin

**Table 10.1: Renin concentrations in nephroblastoma cell cultures.**
**(grown in Medium 199 + 15% F.C.S.).**

| | Renin (μUnits/ml) ± S.EM(n=3) | |
|---|---|---|
| | Active | Inactive |
| Cell supernatant : | 11.0 ± 0.1 | 90.0 ± 0.2 |
| Control (Medium 199) : | 0.0 | 0.6 ± 0.0 |

(Values obtained from Day 4 samples, 8th subculture)

release was controlled by some of the agents that are known to regulate secretion from the normal human kidney.

10.2. β-adrenergic agonists stimulate renin release and nephroblastoma cell growth.

The present study has shown that isoprenaline (0.1mM); forskolin (0.01mM) and dbcAMP (0.1mM) stimulate active renin release at 5 hours. Treatment of the cells with 1μM isoprenaline for 12 days stimulated active and total renin release from the cells.

An unexpected and potentially important finding was that when given long-term, isoprenaline (1μM) stimulated nephroblastoma cell growth. The present study has shown that 1μM isoprenaline causes an increase in renin release by stimulating cell growth, which is first evident at 24h. The action of isoprenaline on both renin secretion and cell growth was blocked by propanolol, indicating a specific action on the β-receptor.

The intracellular messenger involved in signal transduction from the β-receptor is cAMP and we found that its analogue, dbcAMP also increased renin secretion by stimulating cell growth (increased cell growth was also first evident at 24h).

Forskolin stimulates the catalytic subunit of adenylate cyclase directly, leading to increased intracellular concentrations of cAMP (Seamon et al., 1981). Forskolin (0.1mM) also caused stimulation of renin release and cell growth. However, unlike isoprenaline and dbcAMP,

forskolin caused an increase in the long-term by significantly increasing renin release per cell.

This result is in contrast to recent work reported by Everett et al. (1990). This group has reported (using the reverse hemolytic plaque assay) that forskolin administration resulted in an increase in the number of renin-secreting microvascular cells without changes in the amount of renin secreted by individual cells.

The differences observed could be due to:

1) Different doses (and number of additions) of forskolin; 0.01mM added at 1h intervals for 3h (Everett et al., 1990) compared with 0.1mM added at the start of a 24h experiment (the present study).

2) Different in vitro model systems used; freshly isolated developing kidney microvessels compared with cultured nephroblastoma cells.

Alternatively, it could be possible that forskolin, acting via an increase in $[cAMP]_i$, can stimulate two pathways to cause either 1) an increase in renin release per cell number or 2) an increase in the number of renin-secreting cells.

### 10.3. Possible mechanism(s) involved in β-adrenergic-stimulated cell growth

The actions of β-receptor agonists in stimulating cell growth were unexpected.

The mechanism of the effect of β-receptor agonist stimulated cell growth is unknown. However, other workers e.g. Rozengurt et al. (1983) have reported that a sustained

increase in cellular cAMP concentrations acts as a mitogenic signal for Swiss 3T3 cells in culture.

The catalytic subunit of cAMP dependent protein kinase may be involved in the regulation of the proto-oncogene, c-fos (Riabowol et al., 1988). c-fos, in association with another proto-oncogene c-jun, binds to the tetradecanoylphorbol acetate responsive element which is found in the promoter region of several genes. c-fos/c-jun are involved in both cell proliferation and cell differentiation (Zelenka, 1990). It is likely that β-receptor agonists are not themselves mitogenic but augment the action of mitogens (e.g. PDGF; Rozengurt et al., 1983), either in the foetal calf serum or even possibly secreted by the cells themselves.

From this work, it seems that β-agonists have two actions: in the short-term they stimulate renin release, probably through increased rate of exocytosis or renin gene expression; longer-term administration of isoprenaline (or dbcAMP) also led to an increased number of renin-secreting cells and consequently a sustained and long-term increase in renin release. This finding could be applicable to other mammalian hormones, namely agonists, effective in the short-term may have a dual role as growth factors on the hormone-secreting target cell, allowing a sustained hormone response to increased demand.

10.4. ANP inhibits basal renin release and β-stimulated renin release and cell growth

The present study has also shown that renin secretion from human cultured nephroblastoma cells is responsive to ANP.

ANP (1μM) caused a marked inhibition of basal renin release at 5h and this was paralleled by an increase in extracellular cGMP concentration.

Furthermore, this study has shown that ANP attenuated isoprenaline (1μM)- and forskolin (0.1mM)-stimulated renin release and (in the case of isoprenaline) β-stimulated cell growth, at 24 hours.

10.5. Second-messenger involvement in the control of renin release

Subsequently, an investigation into the second messengers involved in the observed responses, was carried out. Isoprenaline (1μM) caused an immediate stimulation of $[cAMP]_i$, which peaked early, and this was followed by an increase in active renin release at 1h. Forskolin (0.1mM) caused a sustained increase in $[cAMP]_i$ which was paralleled by an increase in active renin release.

ANP was found to be effective at lowering isoprenaline-stimulated intracellular [cAMP] in cultured nephroblastoma cells. This has also been observed to occur in rat adrenal cortical membrane preparations (Anand-Srivastava et al., 1985); aorta washed particles, mesenteric artery and renal artery homogenates (Anand-

Srivastava et al., 1984).

The involvement of cAMP in the control of renin release has been suspected but the only direct study involving cAMP measurements was by Pinet et al. (1987). This group found that prorenin and cAMP release were stimulated by forskolin. They also found that ANP reduced forskolin-stimulated prorenin release but did not affect cAMP release.

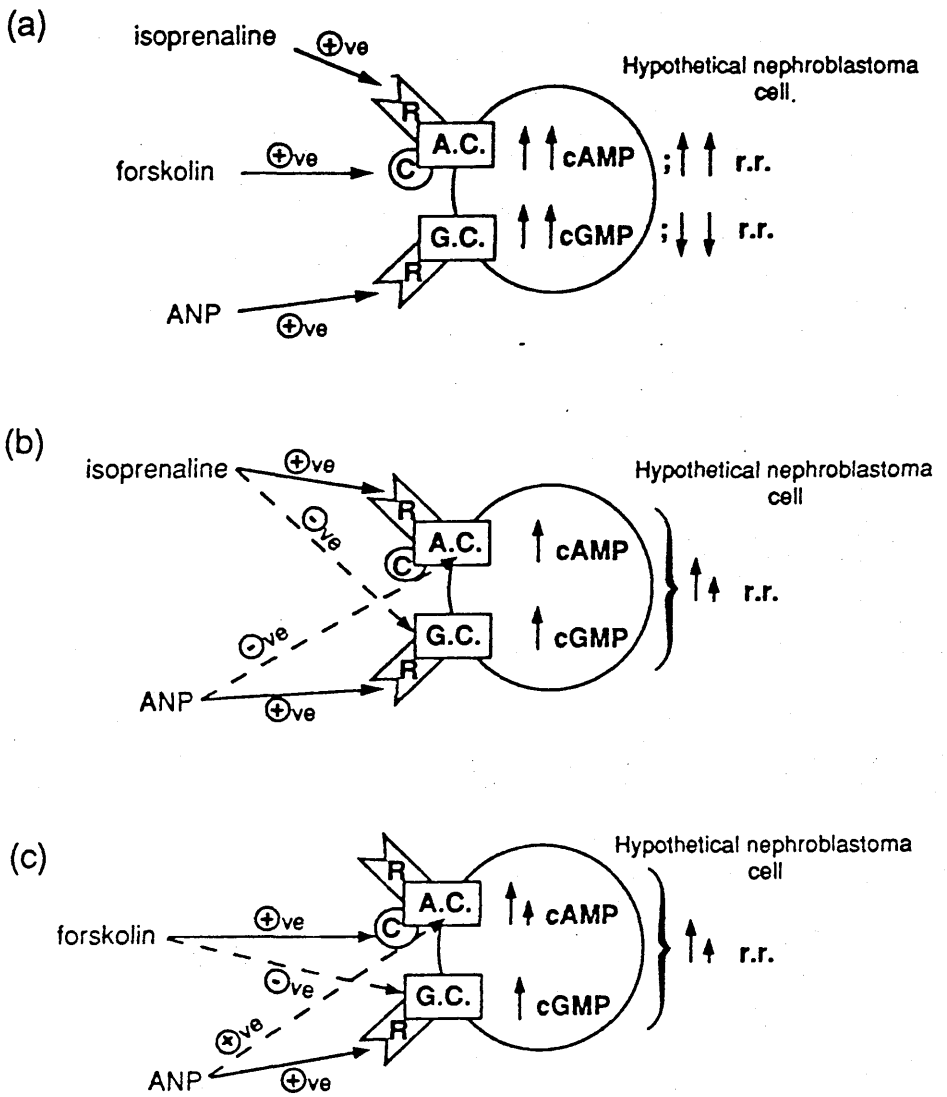
The results from the present study have shown that ANP did not reduce forskolin-stimulated increases in $[cAMP]_i$, but was effective at lowering forskolin-stimulated active renin release. The present study also showed that ANP caused a significant potentiation of forskolin-evoked intracellular [cAMP] (c.f. Pinet et al., 1987).

Results of the present study showed that ANP reduced isoprenaline-stimulated renin release and $[cAMP]_i$ (Figure 10.1). The action of ANP on reducing adenylate cyclase activity has been shown in several systems (e.g. Anand-Srivastava et al., 1984, 1985) but not in conjunction with changes in renin release.

The present study showed that ANP stimulated cGMP release in this cell line. This observation agrees with the literature in that ANP has been shown to act through cGMP in other systems (Winquist et al., 1984; Ishikawa et al., 1985; Kurtz et al., 1986). The present study suggests that cGMP could inhibit basal renin release. It may be the mechanism by which forskolin-stimulated renin release is suppressed (Figure 10.1).

**Figure 10.1**

**A highly schematic representation of second messenger involvement in the control of renin release from cultured nephroblastoma cells.** (a) The effects of; β-agonists and ANP when added separately (b) The effects of isoprenaline and ANP when added together and (c) The effects of forskolin and ANP when added together.
(Solid lines indicate known mechanisms of hormone (or drug) action, dotted lines indicate putative mechanisms of action; + ve and - ve represent stimulatory and inhibitory effects, respectively).




Where A.C. and G.C. represent adenylate cyclase and guanylate cyclase. 'R' represents receptor; catalytic subunit of adenylate cyclase is represented by 'C'. Upward pointing and downward pointing arrows represent increases and decreases in cAMP or cGMP formation and in renin release (r.r.).

## 10.6. Expression of $G_s$ and $G_i2$ in nephroblastoma cell membranes

Results from the present study show that nephroblastoma cells in culture have adenylate cyclase and guanylate cyclase (probably particulate) activity.

They also have ANP receptors, which are linked, in a negative fashion to adenylate cyclase and in a positive fashion to guanylate cyclase (Figure 10.1).

In the present study, the presence of $G_s$ and $G_i2$ (the G-proteins reported to be involved in the stimulation and inhibition of adenylate cyclase, respectively) has also been demonstrated, using pertussis toxin and immunoblotting techniques. However, further studies would be necessary in order to determine whether the G-proteins detected were functional or not.

## 10.7. $A_{II}$ exerts feedback inhibition on renin release from cultured nephroblastoma cells, which can be released by Enalaprilat.

The present study has also shown that $A_{II}$ suppresses basal renin release from nephroblastoma cells on a short-term and long-term basis. $A_{II}$ had no effect on cell growth. $A_{II}$ levels were high in the medium to which no $A_{II}$ had been added and this could be blocked by Enalaprilat treatment which caused stimulation of active renin (but not prorenin) release. Enalaprilat had no effect on cell growth (c.f. Geary et al., 1990). It would be interesting to look at the effect of $A_{II}$ on renin gene expression in nephroblastoma cells.

10.8. Nephroblastoma cells as an immortal line.

At this stage, it would be very useful to establish the nephroblastoma cells as a permanent cell line. This is because, at present, the cells can be maintained in culture for up to 3 months only. Thereafter they start to senesce and reach a crisis from which they do not recover. The cells therefore have a finite lifespan. Thus, it would be useful to 'immortalise' the nephroblastoma cells.

However, while this has been shown to be successful using tumoral JG cells (Pinet et al., 1985), there have been few attempts to immortalise a Wilms' tumour line. Maitland et al. (1989) attempted SV40 transformation by transfection of SV40 tumour antigen genes into the cells soon after establishment (Southern and Berg, 1982). These workers have reported that despite an extended life span of up to 35-40 passages, the SV40-WT cell lines also eventually reached a crisis, from which most of them did not recover. This group has also carried out cell fusions between immortalised kidney cells of normal and tumour origin and an indicator cell line (HeLa). However, they did not carry out these fusions for the purpose of studying renin release and there have therefore been no reports on the suitability of the cells for such studies.

10.9. Conclusions.

In conclusion, therefore, the present study has demonstrated that human cultured nephroblastoma cells are responsive to some of the agents known to regulate renin

release *in vivo*. Also that the cells possess adenylate cyclase which is probably the mediator of β-stimulated cell growth. The cells also possess guanylate cyclase activity which is probably the mediator of ANP-inhibited basal renin release.

In addition, ANP-stimulated cGMP probably interacts with β-stimulated cAMP and in so doing can alter renin release and cell growth. It should be noted, however, that these observations do not rule out a role for $Ca^{2+}$.

Furthermore, the present study has reported differences in the mechanisms by which isoprenaline and forskolin cause a long-term increase in renin release.

In the present study, second-messenger responses to these agents were investigated and it was deduced that an increase in $[cAMP]_i$ (and possibly a decrease in $[cGMP]_e$) is instrumental in stimulating cell growth in this system.

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