

**PURINERGIC REGULATION OF STEROID SECRETION IN THE BOVINE
ADRENAL CORTEX (INNER ZONE).**

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**A thesis submitted for the degree of Doctor of
Philosophy, University of Edinburgh, 1995.**



DECLARATION OF ORIGINALITY

I declare that the composition of this thesis and the work
presented herein is my own.

Dorothy, Elizabeth, Elaine Hoey.

I would like to dedicate this thesis to my parents, for their encouragement, faith and support throughout this project and my life.

"The fear of the Lord is the beginning of wisdom; all who follow his precepts have good understanding. To him belongs eternal praise".

Psalm 111: v10.

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ABSTRACT

Although ACTH is the main stimulus to cortisol secretion from zona fasciculata/reticularis (zfr) cells of the adrenal cortex, recent work has established the importance of other hormones and neurotransmitters in the regulation of steroidogenesis. In view of the close association of the adrenal cortex and medulla and the morphological evidence for cell-cell contacts, the possibility that chromaffin cell secretory products might influence adrenocortical cell function merits closer investigation. One such secretory product is the purine, ATP which is known to bind to cell surface receptors and influence cell function in a variety of cell types. Preliminary work in this department has indicated that ATP is able to stimulate cortisol secretion from bovine zfr adrenocortical cells.

The aims of this thesis were: a) to investigate the mechanisms by which ATP, adenosine and other purines stimulate cortisol secretion from bovine adrenal zfr cells maintained in primary culture, b) to study the interactions of ATP with the other adrenocortical hormones and finally c) to investigate the acute and chronic effects of ACTH, All and ATP on the regulation of the steroidogenic pathways.

The purinergic agonists ATP and ADP stimulated cortisol secretion in a dose-dependent manner in both freshly-isolated and cultured cells (ATP: E_{\max} (the maximum stimulatory response) = 423 ± 220 nmol/l/h; ACTH: $E_{\max} = 1205 \pm 481$ nmol/l/h). Peak cortisol secretion to these agonists was observed on day 3 (48 h) of cell culture. Cells were also responsive to the pyrimidine nucleotide, UTP. EC_{50} values for ATP, ADP and UTP were $5.83 \pm 3.98 \times 10^{-6}$ M, $13.7 \pm 5.67 \times 10^{-6}$ M and $7.33 \pm 4.52 \times 10^{-7}$ M respectively. In the absence of specific P2 purinergic receptor antagonists, the receptor subtype(s) mediating the steroidogenic responses to the purines were characterised using selective agonists and second messenger studies. The potency order for a range of purine analogues was as follows: ATP = UTP > ADP > 2-methylthio ATP > α,β -methylene ATP = β,γ -methylene ATP > AMP. ATP, ADP and UTP all dose-dependently stimulated

the accumulation of total [³H]-labelled phosphoinositols from cells whose phosphoinositides had been pre-labelled with ³H-inositol. Examination of the purinergic response in bovine zfr cells demonstrated that it fulfills the criteria presently used to define the P2u (nucleotide) receptor.

The possibility that cortisol secretion to ATP might be accounted for by the degradation of ATP to adenosine over the course of the incubation was excluded. Although there was degradation, largely to ADP (30%) and AMP (20%), there was no significant formation of adenosine after 1 h.

Bovine zfr cells also demonstrated a dose-dependent increase in intracellular cAMP in response to ATP. It was not possible to account for the cAMP on the basis of conversion of ATP to adenosine which then stimulated a P1 purinoceptor.

Adenosine, like ATP, stimulated cortisol in a dose-dependent manner, with a threshold at 10⁻⁶ M. A parallel dose-dependent increase in cAMP was also measured, but there was no effect on phosphoinositide turnover. Highly selective A₁ and A₂ sub-type antagonists showed an order of potency for inhibition of the adenosine response as follows: CGS 15943A > DPCPX > 8-CPT; indicative of an A₂ purinoceptor sub-type. Evidence is also presented that the cAMP component of the dual second messenger response to ATP may also occur via the A₂ receptor sub-type. Thus CGS 15943A also inhibited the cortisol response to ATP by 54.15 ± 4.00%. The % inhibition of cAMP formation (P1) and phosphoinositide turnover (P2) was also measured with ATP in the presence of CGS 15943A. The cAMP response to ATP was inhibited by 50.44 ± 10.9% while phosphoinositide turnover was inhibited by only 7.66 ± 4.41%.

A novel superfusion system was used to characterise the interaction of ATP with ACTH, the main regulator of steroidogenesis. Co-infusion of the two agonists produced a secretion of cortisol that was greater than the sum of the individual agonist responses, consistent with a synergistic interaction. In the adrenal cortex we are faced with a myriad of cell surface receptors, connected to particular signal transduction systems. ATP is

unique in the sense that it activates both the cAMP and phosphoinositide pathways. Further experiments were therefore performed to determine which of these two component second messenger systems was responsible for the synergism. However, co-infusion of ACTH with acetylcholine (activator of the phosphoinositide pathway alone) and with adenosine (activator of the cAMP pathway alone) evoked synergistic secretory responses in bovine zfr cells in both cases. These findings would suggest that synergism in the adrenal cortex involves in some cases the interaction or "cross-talk" between the same signalling systems and in other cases between different signalling systems.

Finally, a method, using gas-chromatography mass spectroscopy (GC-MS), was established for the identification and quantification of the endogenous steroids secreted by bovine zfr cells in culture. Once this was set-up, the acute and chronic effects of ACTH, All and ATP on the steroidogenic pathways were examined. Chronic stimulation with ACTH, favoured the androgen pathway, while the production of corticosterone was nearly completely inhibited. In contrast, chronic stimulation with All favoured both the mineralocorticoid and the glucocorticoid pathways, with very little change in the androgen pathway products. ATP with its dual second messenger effect, reflected the actions of both ACTH and All implicating differential effects of the two systems on the longer-term regulation of the steroidogenic pathways.

These results suggest that purinergic mechanisms could play a role in the regulation of adrenocortical function *in vivo*.

ABBREVIATIONS

ACAT	Cholesterol acyl transferase
ACE	Angiotensin converting enzyme
Ach	Acetylcholine
AChE	acetylcholinesterase
ACTH	Adrenocorticotrophic hormone
ADP	Adenosine diphosphate
AI	Angiotensin I
All	Angiotensin II
Alll	Angiotensin III
AIP	Aldosterone-induced protein
AMP	Adenosine monophosphate
5'AMP	Adenosine kinase
AR	Adenosine receptors
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
BSA	Bovine serum albumin
cAMP	adenosine 3',5' cyclic monophosphate
CEH	Cholesterol ester hydroxylase
CGRP	Calcitonin gene-related peptide
CRH	Corticotropin-releasing hormone
%CV	% Coefficient of Variation
DAG	Diacylglycerol
DHEA	Dehydroepiandrosterone
DHP	1,4 dihydropyridine
DMSO	Dimethyl Sulphoxide
DPM	Disintegrations per minute
EBS	Earle's balanced salt solution
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethyleneglycol tetraacetic acid
ER	Endoplasmic reticulum
FPLC	Fast Protein Liquid Chromatography
GC-MS	Gas chromatography mass spectroscopy
G proteins	Guanine nucleotide regulatory proteins
HDL	High density lipoprotein
HMDS	Cyclohexane/pyridine/Hexamethyldisilazane
HPA	Hypothalamic-pituitary-adrenal
3 β -HSD	3 β -Hydroxysteroid dehydrogenase
IL-1	Interleukin I
IS	Internal standard
IU/ml	International units per millilitre
LDL	Low density lipoprotein
MKB	Modified Krebs Ringer solution
MO-TMS	Methyloxime-trimethylsilyl
MR	Mineralocorticoid receptor
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NPY	Neuropeptide Y
OT	Oxytocin
PCR	Polymerase chain reaction
PFTBA	Perfluorotributylamine
PGI ₂	Prostacyclin

PGP 9.5	Protein gene product 9.5.
PIP ₂	Phosphatidyl inositol 4,5 bisphosphate
PKC	protein kinase C
PL-C	Phosphoinositidase C
PNMT	phenylethanolamine N-methyltransferase
POMC	Pro-opiomelanocortin
RAS	Renin-angiotensin system
SAH	S-adenosyl homocysteine
SCP ₂	Sterol carrier protein 2
SIM	Selected ion monitoring
TSIM	Trimethylsilylimidazole
UTP	Uridine triphosphate
VIP	Vasoactive intestinal peptide
v/v	volume per volume
w/v	weight per volume
zf	zona fasciculata
zfr	zona fasciculata/reticularis
zg	zona glomerulosa
zi	zona intermediate
zr	zona reticularis

CHAPTER 1

INTRODUCTION

This chapter presents a general introduction to the adrenal cortex and to the purines adenosine triphosphate (ATP), uridine triphosphate (UTP) and adenosine. The chapter starts with a general description of the anatomy and physiology of the adrenal cortex followed by a detailed review of adrenocortical steroids and the enzymes involved in their production as described in sections 1.1 and 1.2. Section 1.3 considers the mechanisms involved in the control of steroid biosynthesis and in particular the role played by the second messengers, cyclic AMP, Ca^{2+} and phosphoinositols. Section 1.4 introduces the purines ATP and adenosine with a general historical background for each agonist. Classification of the purinoceptors according to agonist potency orders and signal transduction systems is described in detail in sections 1.5 and 1.6. The source and fate of purines and current knowledge of their role in the adrenal is briefly described in sections 1.7 and 1.8. The final section of this introductory chapter summarises the aims of the thesis.

1.1. THE ADRENAL GLAND

1.1.1. Embryology and Development.

The adrenal glands in the human lie between the vertebral column central line and the medial aspect of each kidney and are localised near the cranial end of the eleventh thoracic vertebra. The gland consists of two morphologically and functionally distinct regions, the outer steroid-synthesising cortex and the inner catecholamine-producing medulla. The two regions of the adrenal have different embryological origins, the medulla developing from the anlagen of the sympathetic ganglia and the cortex from the Wolffian body in the mesoblast. Development of the adrenocortical tissue is intimately bound with that of the kidney, gonads and posterior venous system (Chester-Jones et al 1957).

The primordium of the cortex appears at about 25 days of gestation in humans in the ventral portion of the celomic epithelium just medial to the urogenital ridge. Following this, sympathochromaffin cells migrate to the region of the cortical anlagen and form a single compact mass. By 6-8 weeks gestation, it is possible to differentiate between the fetal zone first described by Elliot in 1913 and the permanent definitive cortex. For three months there is only irregular separation of the fetal and permanent cortex and medulla. The fetal zone rapidly degenerates after birth and is completely lost by 1 year, being replaced by typical normal cortex. The last phase in the development of the cortex occurs by the 28th - 30th week where the zona glomerulosa, zona fasciculata and medulla begin to delineate. All adult zones of the adrenal cortex are present by 6 months after birth. During the first year of life, the weight of both the male and female adrenal gland falls by 50% as a result of degeneration of the fetal cortex. After this period of time however, the gland will continue to enlarge until the age of twenty.

1.1.2. Anatomy and Blood supply.

The adrenal glands vary in shape from almost spherical in fetal life and in the adult of some species (eg the rat and mouse) though somewhat flattened to more or less triangular (eg humans and cattle). The combined weight of a pair of adrenals in the human species is about 8g in the adult and about 10-12g in the bovine species. The gland is usually greater in weight in the female (5.5g) than in the male (4g) after puberty (for example, in humans). The human adult adrenal measures about 50mm in length, 25mm in width and about 5mm in thickness. The right adrenal is triangular and touches the inferior vena cava whereas the left adrenal is crescent shape in outline.

The blood supply to the adrenal gland arises from three main groups of arteries: 1) the inferior phrenic artery, 2) the aorta and 3) the renal artery (reviewed in Dobbie et al 1966). These three main vessels break up as arteriae comitantes and penetrate the capsule of the gland to form an extensive subcapsular arterial plexus. In the rat, the subcapsular arterioles have muscular walls suggesting that they may be a site for the control of bloodflow through the gland. Arterial blood flows through capillary loops which surround the cortical cells of the zona glomerulosa (zg). The capillaries widen as they course through the fasciculata layer before opening out to form the plexus reticularis. In the zona glomerulosa/zona fasciculata (zg/zf) the capillary networks are located in the membranous septa, while in the zona reticularis (zr) they are embedded in the fibrillar stroma.

There are three sources of blood supply to the medulla: 1) Small arteries from the subcapsular plexus pass directly through the cortex to supply the medulla (Flint 1900), 2) arteriae comitantes accompanying the central vein and 3) effluent cortical blood drains directly across the cortico-medullary boundary into the medullary sinusoids (reviewed in Dobbie et al 1966). The vasculature of the human adrenal is rather more complex than

that of the rat adrenal. Effluent blood from the medulla and the cortex is collected into a single central vein engulfed by a cuff of cortical tissue, which exists at the hilus of the suprarenal vein. Only in the head region of the gland do small muscular venous radicles lie free in the medullary tissue, unencompassed by this cortical sleeve. The three zones of the cortex are recognisable in the cuff region which is supplied with blood derived from the arteriae comitantes. In humans, this vein discharges either into the renal vein as in the left gland or directly into the vena cava as in the right gland. In rat, the central vein is very thin as it traverses the gland, although it becomes thicker as it is exteriorised. The vasculature of the adrenal plays a unique and important role in cortico-medullary interactions and in the functional zonation of the cortex.

1.1.3. Adrenocortical Zonation.

The human adrenal was described as early as 1611 (Bartholinus)(Schumacker et al 1936) though very little was known about its function at that time. In 1856 Brown-Sequard showed the adrenal gland to be essential for life but it was not until 1917 that Wheeler et al. proved that the outer cortex and not the inner medulla was responsible for the sustenance of life. In 1858 Harley (Schumacker et al 1936) recognised that the cortex was arranged into three concentric shells which were later named by Arnold in 1866. The different zones of the cortex were originally distinguished by the shape and size of the cells and their arrangement and position within the gland rather than by any functional differences. The three zones making up the cortex are, the zona glomerulosa (zg), the zona fasciculata (zf) and the zona reticularis (zr). Figure 1.1a shows a diagrammatic representation of the adrenal cortex and figure 1.1b shows a light micrograph of a section through the bovine adrenal cortex.

The zg lies just below the connective tissue capsule and has been described as

FIGURE 1.1.

A.

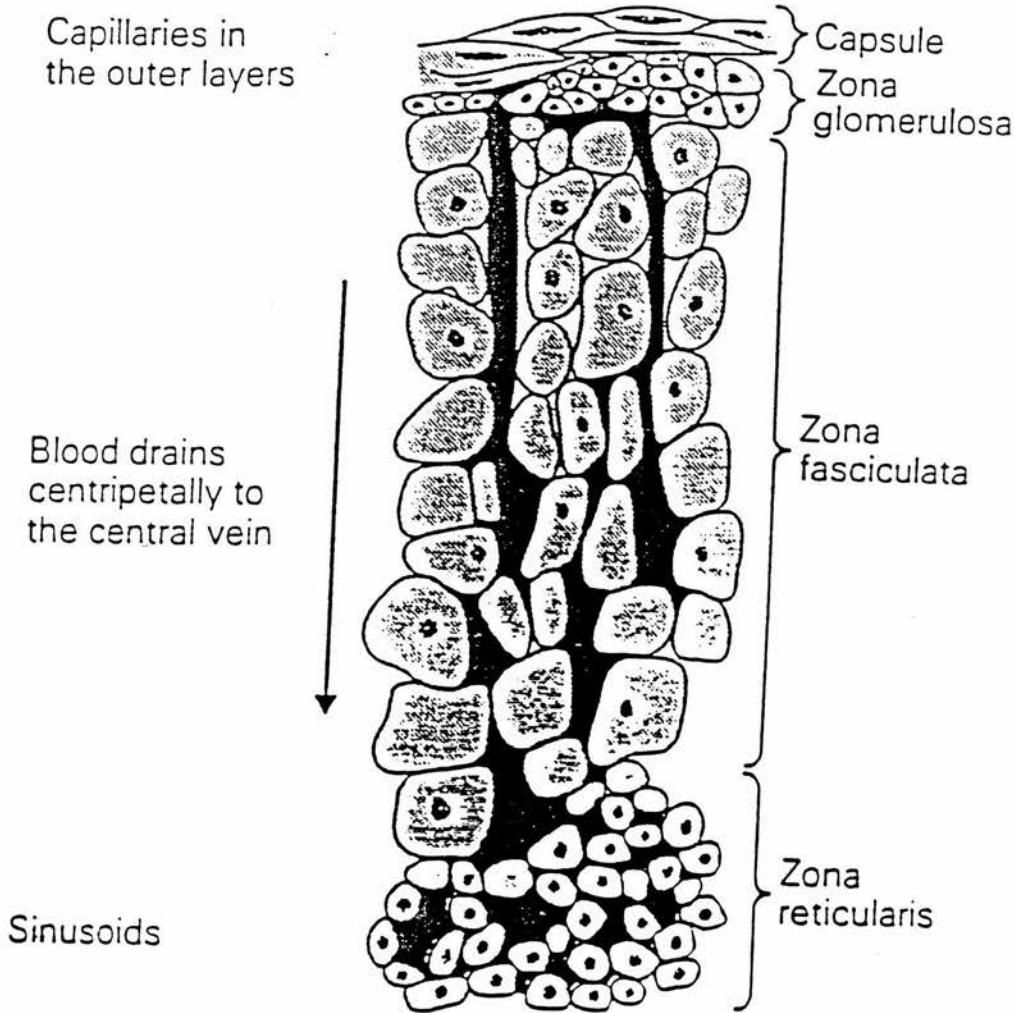


FIGURE 1.1.

Zonation of the mammalian adrenal cortex. Diagrammatic representation of the arrangement of cells in a cross-section of the adrenal gland.

B.



FIGURE 1.1b.

Light micrograph of a paraffin-embedded section of the bovine adrenal gland stained with Haematoxylin α Eosin (\times 200 magnification).

a large connective tissue housing large masses of cells arranged as whorls, loops or baskets. The cells are small and round with a high nuclear/cytoplasmic ratio. The mitochondria are larger than those found in the other zones with cristae that are shelf-like in appearance and the cytoplasm contains relatively little lipid. According to Neville et al (1982) the zg seldom takes up more than 5% of the total cortical volume of a normal gland. In the zf the cells are arranged as a series of centripetally orientated cords and are larger than those present in the zg. These cells are characterised by their low nuclear/cytoplasmic ratio, lipid-rich cytoplasm, small ovoid mitochondria with tubulovesicular cristae and are described as "clear cells". They make up approximately 70% of the cortex. The rest of the cortex (about 20%) comprises the innermost zr; these cells are intermediate in size exhibiting a network arrangement and are often referred to as "compact" cells. They have an eosinophilic cytoplasm with few mitochondria and lipid droplets. The cells are easily characterised by their pigmentation due to the presence of lipofuscin granules.

The exact cellular arrangement of the cortex varies from species to species. In the bovine adrenal cortex, the zg is sharply defined from the inner zones while the zf/zr merge together to form a single layer. In contrast, the zf cells in the human species are observed directly under the capsule due to the integration of these cells with the zg.

The traditional three zones of the adrenal cortex may not always be present due to differences between species. In 1938, Howard and co-workers discovered a zone in the adrenals of young mice and called it the X-zone (Chester-Jones et al 1957). The X-zone lies between the zr and the medulla. It involutes at puberty in the male and first pregnancy in the female leaving behind a band of connective tissue in the mouse. In the male rat this does not occur, instead the X-zone forms the zr in the adult (Shire 1979). The X-zone seems to play a role in fetal or post-natal life. Rat, cattle and sheep exhibit a fourth zone in the adrenal cortex situated between the zg and zf consisting of 2-3 layers

of cells termed the zona intermediate (zi) (Deane 1962). The fetal adrenal in humans and primates but not in other mammals exhibits two zones in the adrenal cortex: the fetal zone and the definitive cortex. The cells of the fetal zone are 20-50 μ m in diameter and are responsible for the large size of the fetal adrenal during gestation. An additional inner adrenocortical zone has been found in the marsupial. It has been termed the "special zone" and only occurs in the adult female.

Originally in 1866 the division of the adrenal cortex into the zg, zf and the zr was devised by Arnold on the basis of histological and anatomical differences between these zones. It is now clear that there is functional zonation of the cortex as well. Differences in the enzyme complements of each zone result in differences in the steroids produced by a particular zone. The zg is the only site of production of aldosterone in the cortex whereas the inner zfr produces mainly cortisol (in primates) and corticosterone (in rodents). Androgens are secreted principally by the zr and the inner zones of the fetal adrenal.

The mechanism(s) by which each adrenocortical zone is formed and replenished with the cells having the function characteristic of that particular zone are still uncertain. The "zonal theory", suggests that each zone replenishes its own cells independently and that the cells die locally (Chester-Jones 1948), a concept that became popular when distinct functional differences between the zones became clear. It was first proposed by Greep et al (1949) that functional division of the cortex into different steroid-secreting zones was provided by the unusual centripetal system of the adrenal gland. The role of the vascular system was to create a steroid gradient across the cortex from the subcapsular capillary bed to the medulla (reviewed in Hornsby et al 1987). It was proposed that the steroidogenic enzymes can be regulated by steroids, allowing the activation or inhibition of a particular cellular enzyme depending upon its position in the steroid gradient. Another hypothesis, the "cell migration theory" states that the cells in the

three zones arise from a common stem cell in and beneath the capsule or in the subcapsular region and then migrate centripetally to the inner zones, with concomitant changes in their specific functions, finally dying in the zr (Ford et al 1963, Zajicek et al 1986). However, neither theory fits the recent findings by Mitani and co-workers (1994) who revealed the presence of a cell-layer devoid of mineralocorticoid- and glucocorticoid-synthesising enzymes (ie. aldosterone synthase and cytochrome P450 11 β respectively) between the zg and zf in the rat adrenal. In addition, since the rat adrenal cortex lacks the steroid 17 α -hydroxylase, these cells were also incapable of producing adrenal androgens. Further studies examining the distribution of 5'-bromo-2'-deoxyuridine incorporation in the rat adrenal cortex revealed that the stained nuclei were concentrated in this same cell layer and pulse-chase experiments showed that the labelled cells migrated out of this layer and into the zfr. On the basis of these findings and in accordance with the suggestion by Deane and Greep (1946) that the intermediate zone is the stem cell zone of the cortex, Mitani and co-workers (1994) suggested that this new cell layer is the progenitor cell zone of the rat adrenal cortex.

1.1.4. Cortico-medullary Interactions.

Although anatomically separated, interactions between cortex and medulla have been hypothesised for years. Steroid hormones (glucocorticoids) secreted from the adrenal cortex have been shown to exert selective trophic actions on the medulla promoting the growth, maturation and maintenance of chromaffin cells, as well as modulating the activity of the constitutive enzyme, phenylethanolamine N-methyltransferase (PNMT), most characteristic of the medulla (Carballeira et al 1980). Recently, it has been suggested that catecholamines regulate the synthesis of aldosterone in the zg (De Lean et al 1984). Other medullary products were also found to regulate the

production of aldosterone; for example vasopressin (Gallo-Payet et al 1986), adrenaline and noradrenaline (De Lean et al 1984) stimulate aldosterone production whilst dopamine (Racz et al 1984), natriuretic factor-like peptides (De Lean et al 1985) and enkephalins (Racz et al 1980) inhibit aldosterone secretion. In addition, medullary products were also found to regulate steroidogenesis in the inner zone cells of the adrenal cortex, for example, catecholamines and ATP both stimulate the production of cortisol (Lightly, PhD Thesis 1991 and Hoey et al 1994). The centripetal blood flow from cortex to medulla would however seem to preclude any humoral effect of medullary products on the cortex.

The morphological evidence for a close proximity of medullary and adrenocortical cells is strong. In the rat adrenal, rays of medullary tissue (15 - 20 μm in diameter) have been reported to extend across the outer zones of the cortex, up to the capsular zone. In some cases, these rays followed the connective fibres of the large adrenal vein. The inter-relationship between chromaffin and cortical cells persists after cell isolation and therefore provides an explanation for the actions of exogenous medullary products on aldosterone secretion *in vitro* (Gallo-Payet et al 1987). Bornstein et al (1991), using the adrenals of perfused fixed rats, detected chromaffin cells that contained organelles identical to adrenocortical mitochondria and smooth endoplasmic reticulum. The possibility therefore exists that these cortico-chromaffin hybrid cells have the capacity to carry out steroid biosynthetic reactions.

Similar projections of chromaffin cells appear to exist in porcine adrenal, extending across all three zones, as demonstrated with the immunostaining for chromogranin A (Bornstein et al 1991). In addition, small islets and single neuroendocrine cells could be detected in the zf and zr of the cortex. Immunostaining for 17α -hydroxylase (characteristic of adrenocortical cells) showed immunoreactive cells in the medulla. In the bovine adrenal, cortical cells could also be detected in the medulla especially around the blood vessels. This would suggest a regulatory function of secretagogues from medullary cells to cortical

cells and vice-versa by paracrine mechanisms.

To date, evidence supporting cortico-medullary interactions has been based exclusively on non-human experimental systems. Bornstein et al (1994), with the aid of specific immunohistochemical markers, identified the occurrence of chromaffin cells in all three zones of the human adrenal cortex. In an ultrastructural analysis, cortical and chromaffin cells were shown to be in close apposition, not separated by fibrous tissue, thus providing sufficient contact zones for widespread paracrine interactions.

Usadel et al (1993) demonstrated the existence of cell-to-cell communications in bovine adrenocortical cells in culture. With the help of an interactive laser cytometer, these cell communications were shown to be functionally active gap junctions. In the light of the vast amount of evidence supporting the direct apposition of chromaffin and cortical cells, it may be postulated that gap junctions could play an important role in the rapid mediation of paracrine signals, for example, in the case of ATP which is present in high concentrations in chromaffin cells.

1.1.5. Adrenocortical Innervation.

It is well established that the adrenal medulla is richly innervated by preganglionic fibres of the sympathetic nervous system. Although, reports of intrinsic innervation of the adrenal cortex date back to 1894 (Dogiel), only in recent years has conclusive evidence emerged to show both efferent post-ganglionic adrenergic and sensory (afferent) nerve endings distributed throughout all three zones of the cortex of different species. Direct innervation of endocrine cells, in the mammalian adrenal, was illustrated by both Unsicker et al (1969) and Garcia-Alvarez et al (1970) by application of a catecholamine fluorescence technique. This demonstrated innervation of the basal lamina of cortical cells by autonomic axons, terminating close to the plasma membrane. In the rat adrenal,

it has been postulated that the innervation of the gland may arise from two different sources; 1) one set of nerves has cell bodies outside the gland and enters the adrenal capsule parallel to blood vessels and 2) the other set of nerves has cell bodies in the adrenal medulla and is regulated by the splanchnic nerve (Holzwarth et al 1987). A range of peptides and amines have been identified in nerves supplying the adrenal cortex in several mammalian species, of which the following are briefly discussed.

(i) Cholinergic.

Schulkes et al (1975) observed a fine plexus of acetylcholinesterase (AChE)-positive nerve fibres in sheep with ACTH-induced hypertension. Robinson et al (1977) described a complex innervation of the ovine adrenal cortex. Thick bundles of acetylcholinesterase (AChE)-positive nerve fibres were described which were dispersed throughout the cortex forming a distinct plexus in the zr. These fibres were believed to be derived from the splanchnic nerve. More recently, Charlton et al (1991) has used AChE-histochemistry to demonstrate cholinergic innervation in normal human adrenocortical tissue. A subcapsular nerve plexus was evident which sometimes extended through the cortex forming a second plexus in the zr. The pattern of AChE positive cortical innervation is similar to the distribution of capillaries and therefore consistent with a nerve supply to the cortical cells and/or blood vessels.

(ii) Adrenergic and Noradrenergic.

Histofluorescent methods for catecholamines, immunostaining for tyrosine hydroxylase (the rate-limiting enzyme for catecholamine biosynthesis) and dopamine- β -hydroxylase (which converts dopamine to noradrenaline) have shown that fibres are

located in the capsular and zg regions of the gland (Kleitman et al 1985 and Vizi et al 1993). Noradrenergic (dopamine β -hydroxylase-positive) nerve fibres were also reported in the human adrenal cortex with a similar pattern of distribution to the cholinergic innervation except that there was no penetration to the innermost zr (Charlton et al 1992). The noradrenergic nerves appear to be independent of the splanchnic nerve supply to the medulla (Kleitman et al 1985) but are associated with the vascular supply to the gland, in particular the blood vessels in the superficial adrenal cortex (Carlsson et al 1993).

Recent studies, using immunohistochemical detection of the neurone-specific ubiquitin hydrolase protein gene product 9.5 (PGP 9.5) applied to the normal adult human adrenal cortex, revealed three distinct types of nerve morphology: 1) large nerve trunks traversing the cortex to the medulla, 2) a complex branching network of slender nerve fibres and 3) individual nerve fibres. Many of these nerve fibres demonstrated varicosities, indicative of neurotransmitter release. In addition, nerves were identified in the muscular wall of the central vein which were not identified using acetylcholinesterase histochemistry or dopamine β -hydroxylase immunohistochemistry implying that there are non-noradrenergic, non-cholinergic neurotransmitter types involved in the vascular responses of the human adrenal gland (McNicol et al 1994).

(iii) VIPergic.

Stimulation of the splanchnic nerve in conscious calves causes the release of vasoactive intestinal peptide (VIP) from the adrenal gland (Bloom et al 1987b). Holzwarth et al (1987) also described a plexus of nerves containing VIP (intrinsic to the adrenal cortex) which showed enhanced immunostaining following ligation of the splanchnic nerves. VIP has been identified in nerve fibres supplying the adrenal gland of both the rat (Hokfelt et al 1981) and the pig (Kong et al 1989). These fibres are distributed mainly

within the capsule, the zg and the medulla and have been found to be important in relation to the regulation of aldosterone, corticosterone and cortisol (Bloom et al 1987b and Hinson et al 1994). In addition, Bornstein et al (1992) reported that VIP appears to be a modulator of adrenal androgen release and given that adrenal androgens are produced in the zr, which is in direct apposition with the adrenal medulla, VIP may be involved in a local neuroendocrine control mechanism for adrenal androgen secretion.

(iv) Other peptides.

These include calcitonin gene-related peptide (CGRP), methionine-enkephalin, leucine enkephalin and neuropeptide Y (NPY).

CGRP has been identified in nerve fibres in the adrenal cortex of the pig (Kong et al 1989) and the rat (Kuramoto et al 1987). Bundles of fibres containing CGRP are present in the subcapsular regions and the zg where they lie in close contact with cortical cells. CGRP receptors have been described in the rat adrenal (Goltzman et al 1985) and administration of CGRP has been reported to increase corticosterone and aldosterone secretion in the intact perfused rat adrenal (Hinson et al 1990).

Extensive evidence exists for the presence of met- and leu-enkephalin in the adrenal cortex. Hinson et al (1994) reported that both of these opioid peptides stimulated corticosterone secretion in the intact perfused rat adrenal gland *in situ*. Most of the adrenal content of these peptides is located in the chromaffin tissue of the medulla (Kong et al 1989), although in both pig and man, both met- and leu-enkephalin have been identified in islets of chromaffin cells adjacent to the capsule.

NPY has been identified in intra-adrenal nerves in several mammalian species including rat (Kuramoto et al 1986) and bovine (Majane et al 1985). Receptors for NPY have been identified in the bovine zg (Torda et al 1988), but not in other areas of the

adrenal gland. However, Hinson et al (1994) reported that NPY had only a minor effect on corticosteroid secretion in the intact perfused rat adrenal gland *in situ*. NPY immunoreactive fibres are usually associated with the blood vessels in both the cortex and the medulla (Kong et al 1989).

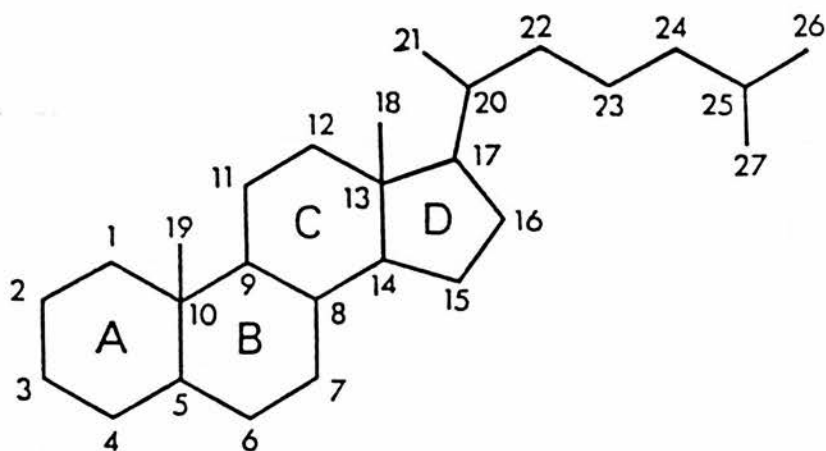
The distribution of the various types of nerve fibres occurs in the outer part of the cortex, mainly within the capsule and the zg. Therefore two distinct possibilities exist, 1) since blood flow itself regulates steroidogenesis, the major regulatory role of the neurotransmitters on steroidogenesis could be indirect, mediated by action on the vasculature system and/or 2) the neurotransmitters released could also have a direct effect on adrenocortical cells and steroidogenesis.

1.2. THE ADRENOCORTICAL STEROIDS

The majority of hormones secreted by the adrenal cortex are steroids, a class of compounds widely found in nature and of which over forty have so far been isolated. The main classes of steroids found in the adrenal cortex are mineralocorticoids, glucocorticoids, and the "sex steroids" androgens, estrogens and progestogens. All steroids have the same basic stem structure consisting of four carbon atom rings, three of six carbon atoms ("cyclohexane")(rings A,B and C) and one of five ("cyclopentane")(ring D) linked together as illustrated in figure 1.2.

The biological properties of a steroid are determined by the number and structure of the functional groups attached to this basic steroid nucleus. There are four parent hydrocarbon molecules from which all the major steroids are derived. 1) Cholestane (C_{27}) is not found in nature but may be considered chemically to be the parent compound for cholesterol and its related metabolites which include the bile acids (C_{24}). 2) Pregnane (C_{21}) is the parent hydrocarbon for a group of steroids which are characteristic of the

FIGURE 1.2.

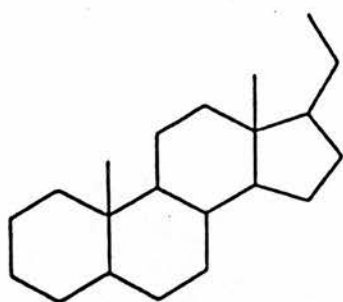


Cholestane

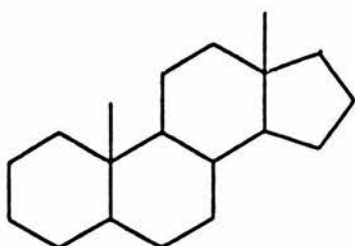
FIGURE 1.2.

The basic steroid structure. Cholestane, which has 27 carbon atoms in the positions shown, is not found in nature, but may be considered chemically as the parent hydrocarbon for cholesterol and related molecules.

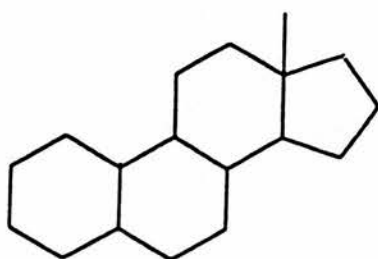
FIGURE 1.3.



Pregnane



Androstane



Estrane

FIGURE 1.3.

The basic structures of the three parent hydrocarbon molecules for the C_{21} steroids (pregnane), the C_{19} steroids (androstane) and the C_{18} steroids (estrane).

adrenal cortex and have 21 carbon atoms (figure 1.3). This group includes the glucocorticoids, corticosterone and cortisol, and the mineralocorticoid, aldosterone. 3) Androstane (C₁₉) has no side chain on ring D (figure 1.3). Major compounds in this group include androstenedione, dehydroepiandrosterone and testosterone. 4) Estrane (C₁₈) (figure 1.3) which includes the phenolic C₁₈ steroids such as estradiol 17 β and estriol.

1.2.1. Mineralocorticoids.

Aldosterone is the most potent mineralocorticoid produced by the adrenal cortex and is secreted exclusively by the zg though the zf can catalyse all the steps leading to aldosterone synthesis except for the final one. The mineralocorticoids which also include corticosterone and 11-deoxycorticosterone are characterised by the presence of a hydroxyl group at C-11.

Aldosterone and the other steroids with mineralocorticoid activity act primarily on the distal convoluted tubule of the kidney, promoting reabsorption of Na⁺ ions in exchange for K⁺ or H⁺ ions. Other sites of action include the secretory epithelia, most notably the intestinal mucosa, cardiac fibroblasts and the salivary and sweat glands. It is also possible that aldosterone affects the Na⁺/K⁺ exchange into non-epithelial cells in the body such as the arterial smooth muscle and brain.

The molecular mechanism of action of aldosterone on modulating the excretion of Na⁺ and K⁺ ions has been most extensively studied in the kidney. Originally amphibian tight epithelia, in particular the urinary bladder of the toad, proved a useful *in vitro* model system for the study of transepithelial Na⁺ and K⁺ transport. In the last 10-15 years, cell cultures originating from these tissues have been available for the study of the acute actions of mineralocorticoids (Handler et al 1983).

The mode of action of aldosterone on ion transport is characterised by three

distinct phases; 1) a latent period of 1 hour, 2) a large increase of Na^+ transport with a paralleled decrease of the transepithelial resistance and finally 3) an increase in Na^+ transport for 6-12 hours accompanied by a constant stable transepithelial resistance (Garty et al 1986). Aldosterone enters a cell by simple diffusion and binds to a specific cytosolic receptor which then undergoes structural changes associated with activation. This facilitates the transfer of the hormone receptor complex to the nucleus where it interacts with the hormone responsive elements of DNA to activate or repress specific genes. Actinomycin D, a RNA synthesis inhibitor, inhibits the mineralocorticoid effects of aldosterone on the urinary excretion of Na^+ and K^+ . In the amphibian tight epithelia, aldosterone stimulates an increase in the transcription of the α and β subunits of the Na^+, K^+ -ATPase although no other effector proteins have been characterised as an aldosterone-induced protein (AIP) (Verrey et al 1990). One possible candidate for AIP is citrate synthase which may be the rate-limiting enzyme involved in ATP synthesis in the kidney.

1.2.2. Glucocorticoids.

Glucocorticoids are produced primarily by the cells of the zfr, however zg cells are capable of the production of corticosterone (Tait et al 1980). Cortisol is the major glucocorticoid in human, bovine, sheep and guinea-pig while corticosterone is the major glucocorticoid produced by rats, mice and rabbits.

Glucocorticoids are characterised by the absence or presence of hydroxyl groups at C-11 and C17. The effects of glucocorticoids on a number of tissues is much more diverse than those of mineralocorticoids or androgens (reviewed in David et al 1970). The characteristic metabolic effects of glucocorticoids in the whole animal include protein catabolism, hepatic glycogenesis and gluconeogenesis, with decreased glucose uptake

and utilisation in the peripheral tissues. Glucocorticoids effect carbohydrate (increase the deposition of glycogen and the rate of hepatic gluconeogenesis during fasting), lipid (increase in lipolysis) and protein metabolism (increase in protein catabolism resulting in muscle wasting) in a way that is generally antagonistic to that of insulin and also can be seen as providing a protection against long-term glucose deprivation. In one respect, the glucocorticoids act in the same way as insulin in that both cause an increased deposition of glycogen in the liver.

Glucocorticoids have a number of effects on calcium and bone which may be involved in the development of osteoporosis. Increased bone loss and decreased bone accretion are caused in part to the direct actions of glucocorticoids as well as their actions in concert with parathyroid hormone and 1,25-dihydroxycholecalciferol (Baxter et al 1987).

Glucocorticoids play an important anti-inflammatory role in response to infection or injury, an observation that was first made by Hench et al (1949). They decrease the release of mediators of the vascular response and reduce the accumulation of tissue fluid. These actions are thought to be mediated by inhibition of the production of the intercellular mediators of inflammation, for example the cytokines.

It has been proposed that the glucocorticoid-induced inhibition of arachidonic release is due to the ability of these steroids to block the activity of phospholipase A₂ through the induction of endogenous inhibitors referred to as lipocortins (Flower et al 1988). Thus their effect is to reduce the activity of the enzymes which degrade membrane phospholipids and release arachidonic acid. This, in turn, reduces the production of both prostaglandins and leukotrienes. Glucocorticoids also act as immunosuppressants by reducing the number of lymphocytes and inhibitory cells that secrete interleukin I and II, thus lowering the proliferative effects of antigens on lymphocytes (O'Malley et al 1991).

1.2.3. Androgens.

Androgens are one of the major groups of steroids secreted by the human adrenal and are preferentially produced by cells of the zr in human and guinea-pig but by the zf and zr in rat. All androgens are C₁₉ steroids with an oxo group at C₁₇.

The most abundant androgens are androstenedione and dehydroepiandrosterone (DHEA). The sulphated form of the latter is produced in large amounts by the zr of the human adrenal and is thought to be largely inactive. The adrenal androgens are metabolised further by other body tissues to oestrogen and testosterone depending on the sex of the mammal. Androgens are believed to play an important part in zonation of the fetal adrenal gland and in its growth during childhood. Early in gestation, cortisol is not produced by the human fetal adrenal *in vivo* (because it does not express 3 β -hydroxysteroid dehydrogenase (3 β -HSD)), whereas androgen production occurs in the transitional and fetal zones (which express P450_{scc} (cholesterol side-chain cleavage enzyme) and P450_{c17} (steroid 17 α hydroxylase enzyme)). Later in gestation, the definitive zone may produce mineralocorticoids and the transitional zone may produce glucocorticoids, whereas the fetal zone continues to produce androgens (Mesiano et al 1993). Thus, late in gestation the functional zonation of the human fetal adrenal cortex may be similar to the adult. Plasma levels of the adrenal androgens increase gradually when adrenarche occurs (latter half of first decade) reaching a plateau in the second decade of life, before declining at some point in the fifth decade (Vermeulin et al 1980).

While ACTH is necessary for androgen biosynthesis, alone it is not sufficient. Other factors responsible for the control of androgen production are not yet clear, though fragments from the precursor molecule, proopiomelanocortin (POMC) (McKenna et al 1991) and a postulated androgen stimulatory hormone isolated from bovine pituitaries have been suggested as possible candidates. Another possibility arises from the

increasing evidence in support of a local neuroendocrine regulation of the adrenal cortex (Bornstein et al 1990). VIP-ergic nerve fibres have been shown to occur in the entire adrenal cortex including the zr and also to be important stimulators of aldosterone and cortisol respectively (Ehrhart-Bornstein et al 1991). Adrenal androgens are produced in the zr in direct apposition with the adrenal medulla and therefore may be under a local neuroendocrine influence. Recent studies supporting this, showed that adrenaline caused an immediate release of the C₁₉ steroid androstenedione from isolated perfused preparations of porcine adrenals when added to the arterial line of the perfusion system. Immunohistochemical staining of paraffin sections of bovine and porcine adrenals for P450_{scc} revealed that the zr and the medulla are closely interwoven. In addition electrical stimulation of the splanchnic nerves caused a significant release of androstenedione; an effect that could be mediated in a paracrine manner through the release of adrenaline from chromaffin cells located in direct apposition with cortical cells (Ehrhart-Bornstein et al 1994).

1.2.4. Adrenocortical Steroid Biosynthesis.

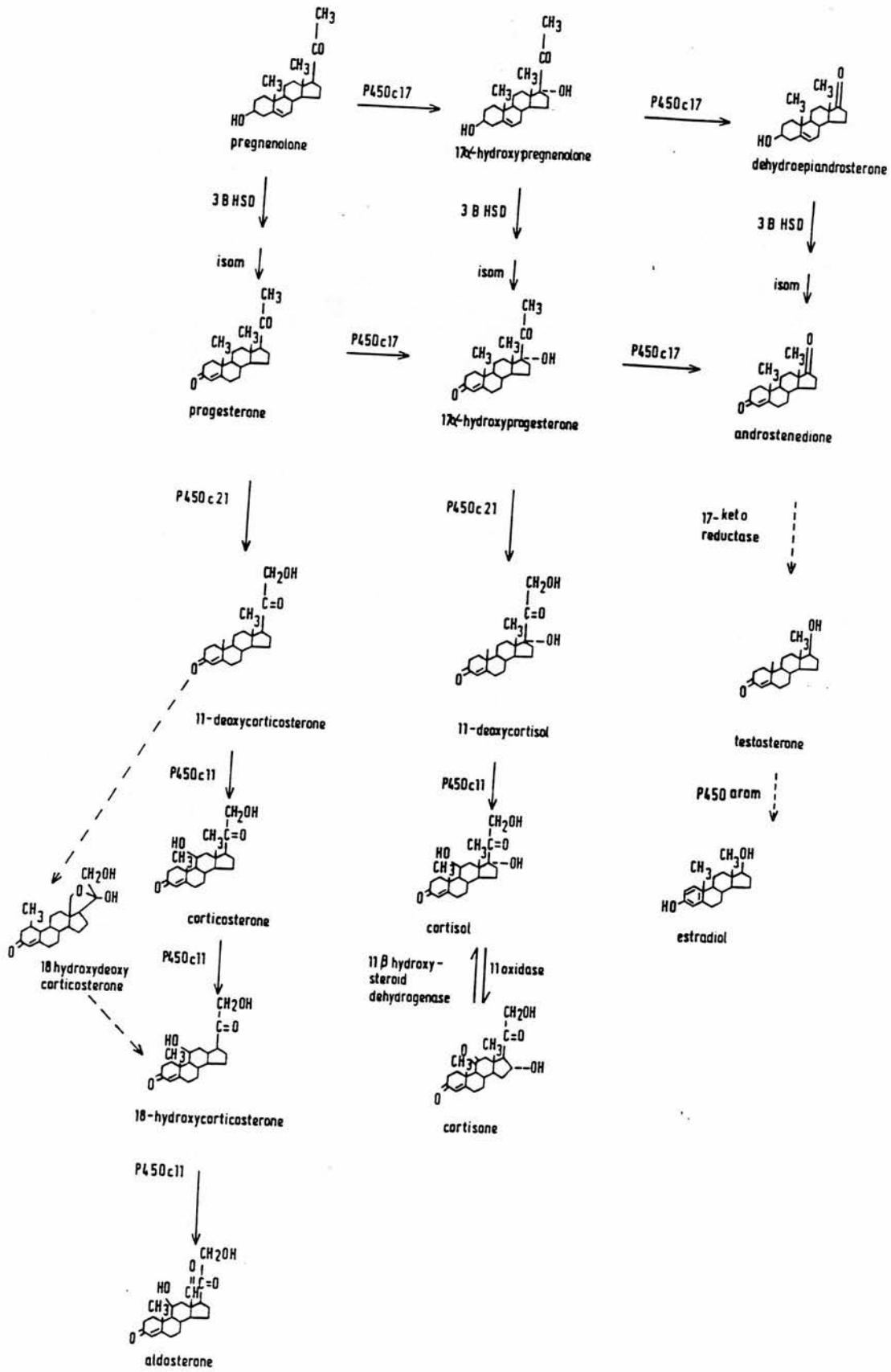
Two major types of enzymes are involved in the steroid biosynthetic pathway: cytochrome P-450s and other steroid oxidoreductases (figure 1.4). Molecular studies have shown that almost every one of the enzymes involved in this pathway is encoded by a single gene which carries all the information required for its regulation in a diverse number of cell types.

Cytochrome P450s are found in most tissues and are mainly located in the mitochondria and endoplasmic reticulum. They are b-type cytochromes with a heme group at the centre which acts as the active site of the enzyme, binding the substrate. Steroidogenic P450s catalyse either a single hydroxylation at a specific position of the

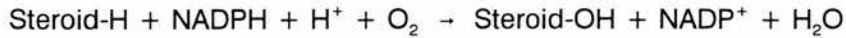
FIGURE 1.4.

Major pathways of adrenocortical steroidogenesis in mammals. Abbreviations are
3 β HSD: 3 β Hydroxy-steroid dehydrogenase; P450_{c17}: 17 α Hydroxylase; P450_{c21}:
21 β Hydroxylase; P450_{c11}: 11 β Hydroxylase; P450_{arom}: P450 aromatase.

FIGURE 1.4



steroid or a series of consecutive hydroxylations which result in C-C bond cleavage or aromatisation of the steroid ring (Hanukoglu et al 1992). The hydroxylation involves the transfer of electrons from NADPH to the cytochrome P450 via specific electron carrier proteins.



In the mitochondria, P450s receive electrons from the iron-sulphur protein adrenodoxin via NADPH-adrenodoxin oxidoreductase whereas the microsomal P450s depend on the membrane bound flavoprotein P450 reductase and cytochrome b_5 (figure 1.5).

Cholesterol is the precursor of all adrenocortical steroids and is stored as cholesterol esters in lipid droplets. Its storage in the form of esters is regulated by two opposing enzymes, cholesterol acyl transferase (ACAT) and cholesterol ester hydroxylase (CEH). Two sources of cholesterol exist in the adrenal cortex. It is either synthesised de novo from acetate (Hechter et al 1953) or it is transported from the plasma into the cell with the plasma-derived low density lipoproteins (LDL), as is the case with the human and bovine species or with the high density lipoproteins (HDL), as in the rat. Transport of free cholesterol from the lipid droplets to the mitochondria may involve elements of the cytoskeleton (Crivello et al 1980) in particular the microfilaments and intermediate filaments in conjunction with a sterol carrier protein (SCP₂) (Chanderbhan et al 1982).

The first metabolic step (side-chain cleavage) of cholesterol to pregnenolone comprises of three distinct processes; cholesterol supply, intracellular transfer and metabolism, each of which is potentially rate-limiting (Jefcoate et al 1992) and therefore will determine the total steroid output of each adrenal cell. This first metabolic step involves three separate reactions, 20 α hydroxylation, 22-hydroxylation and scission of the

FIGURE 1.5.

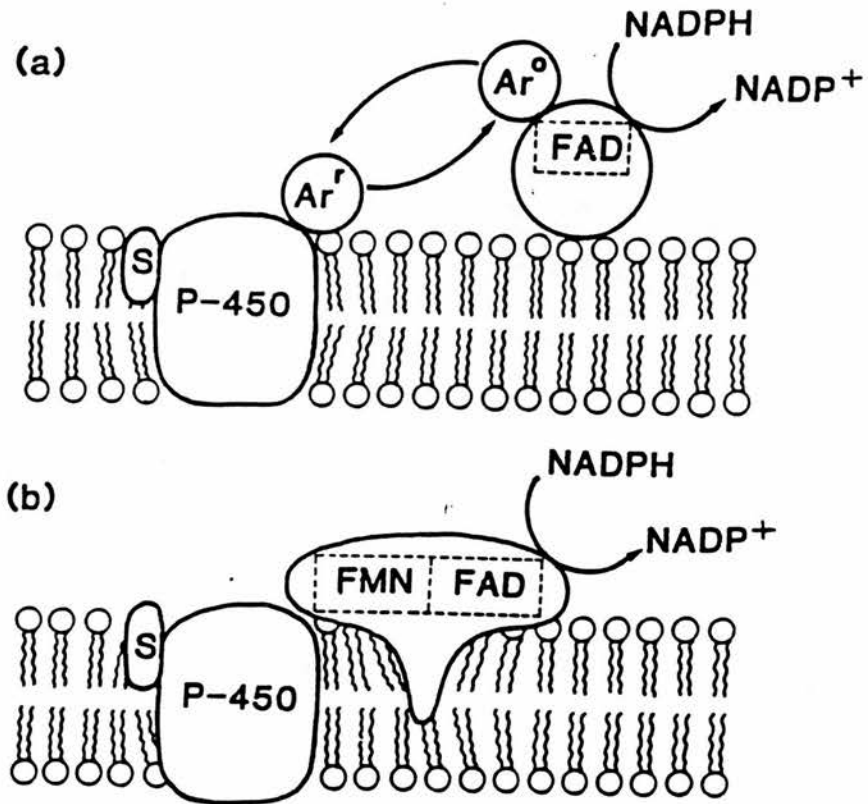


FIGURE 1.5.

Schematic representation of the steroidogenic electron transfer system of mitochondria (a) and microsomes (b). In the mitochondria, the FAD-containing adrenodoxin reductase receives electrons from NADPH which transfers them to the adrenodoxin. The reductase/adrenodoxin complex then dissociates, and the reduced adrenodoxin (Ad^r) forms a new complex with P-450 to which the steroid substrate is bound (S). In the microsomes, a single FMN and FAD containing reductase transfers electrons between NADPH and cytochrome P-450.

cholesterol side chain to yield pregnenolone and isocaproic acid (figure 1.6). There are four mammalian P450 gene families that produce the enzymes almost exclusively involved in the steroidogenic pathway and the mitochondrial P450_{scc} belongs to the P450 X1B1 gene family. P450_{scc} is expressed in all three zones of the adrenal cortex (Hanukoglu et al 1992) and has been cloned in the case of the human, bovine and rat species. The human and bovine P450_{scc} share 82% amino-acid homology and 72% nucleotide homology (Miller et al 1988). The full-length cDNA for bovine P450_{scc} encodes a protein of 520 amino-acids synthesised by membrane-free-polyribosomes. The protein interacts with the inner mitochondrial membrane, a process mediated by the phospholipid cardiolipin and undergoes post-translational modification, where the NH₂ terminal peptide is cleaved off by a specific protease to form the mature protein (Nebert et al 1987).

Pregnenolone produced by this reaction leaves the mitochondria and travels to the endoplasmic reticulum. The microsomal P450_{c₁₇α}, a member of the P450 XV11 gene family, catalyses two key reactions: a) 17α hydroxylation of C₂₁ steroids and b) cleavage of the C₁₇ - C₂₀ bond of C₂₁ steroids and thus is the key branch point directing steroid biosynthesis towards the production of glucocorticoids and androgens depending on its activity and on steroid concentrations.

The availability of electrons to P450 reductase appears to control the activity of P450_{c₁₇α}; increasing the ratio of P450 reductase to P450_{c₁₇α} increases the ratio of 17,20 lyase activity to 17α hydroxylase activity (Onoda et al 1982). If hydroxylase activity exists, the P450_{c₁₇α} converts progesterone and pregnenolone to 17α hydroxyprogesterone and 17α hydroxypregnenolone respectively which can then be converted to cortisol (figure 1.4). The 17α hydroxylated steroids can be further metabolised by 17,20 lyase activity to dehydroepiandrosterone (DHEA) and (after 3β-HSD activity) to androstenedione. However in both the human and bovine species, P450_{c₁₇} can cleave the C₁₇ - C₂₀ bond of 17α hydroxypregnenolone but not of 17α hydroxyprogesterone, so that androgen biosynthesis

FIGURE 1.6.

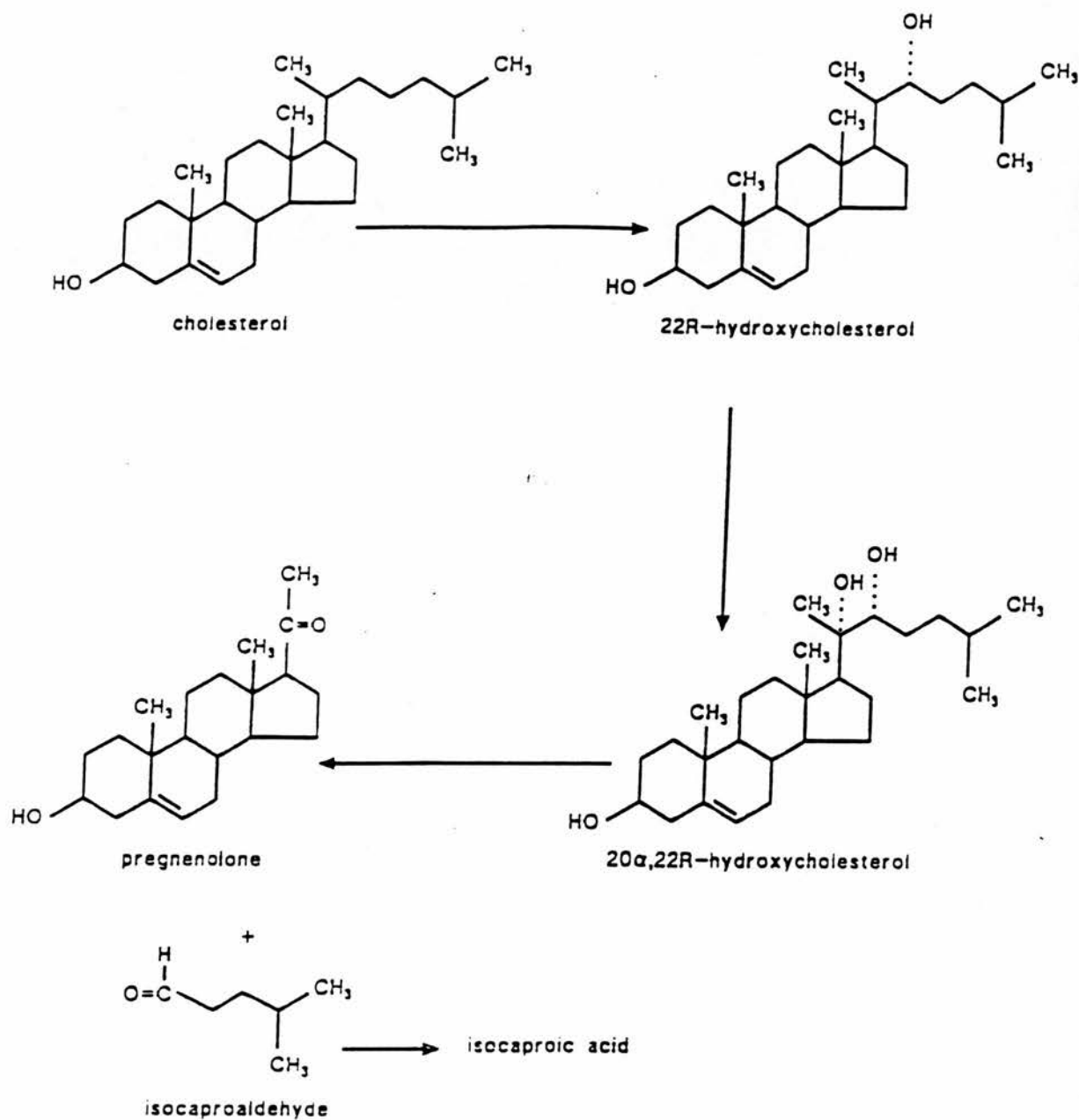


FIGURE 1.6.

Reaction sequence for the conversion of cholesterol to pregnenolone and isocaproic acid by P-450scc.

proceeds mainly from pregnenolone rather than progesterone. If there is no hydroxylase or 17,20 lyase activity, pregnenolone is converted to progesterone by the 3 β -hydroxysteroid dehydrogenase/ Δ^{5-4} isomerase (3 β -HSD). The adrenals of rats, rabbits, and hamsters lack the P450_{c17 α} enzyme, so corticosterone and not cortisol is the major glucocorticoid in these species.

The membrane bound enzyme 3 β -HSD catalyses an essential step in the formation of 3-keto-4-ene steroids from all 5 pregnene-3 β -ol and 5-androsten-3 β -ol steroids and is an obligatory step in the biosynthesis of glucocorticoids, mineralocorticoids, androgens and oestrogens (Mason et al 1993, Labrie et al 1992). The enzyme system consists of two closely linked components, the 5-ene-3 β HSD:NAD⁺ oxidoreductase and the 5-ene-3-oxo steroid-4,5 isomerase. It is expressed in a wide variety of steroidogenic tissues and to date two types of human 3 β -HSD cDNA clones have been characterised. Type I is expressed in non-endocrine tissues such as the skin whereas the type II isoform is found in steroidogenic tissues such as the adrenal cortex and the gonads (Lachance et al 1991). The genes encoding the two types of isoenzymes in human share 93.5% homology. Labrie et al (1992) have described a predicted type III 3 β -HSD in rat liver that shares 80% homology with the other two isoenzymes. Immunohistochemical studies have revealed the presence of 3 β -HSD in the same steroidogenic cells in all three zones of the cortex as P450_{scc}.

The most actively studied P450 enzyme in the steroidogenic pathway is the 21-hydroxylase which catalyses the formation of the precursors of cortisol and corticosterone. P450_{c21} is expressed in all three zones of the cortex and two copies of the gene arranged in tandem have been reported in human, mice and cattle. Both P450_{c21} genes are active in cattle whereas in human and mice one of the two genes is non-functional (Miller et al 1988).

The final enzyme involved in the synthesis of cortisol and aldosterone is P450_{c11}.

Analogous with all other steroidogenic P450 enzymes, this is a multi-functional enzyme displaying 11 β -hydroxylase, 18-hydroxylase and aldehyde synthetase activities. It is present throughout the adrenal cortex, although aldosterone biosynthesis is confined solely to the zg. The P450_{c11} enzymes purified from bovine and porcine zg and zfr are biochemically identical yet the mitochondria from the zg are much more active in aldehyde synthesis than those of the zfr and hence are able to synthesise aldosterone. It appears that the aldehyde synthetase activity is affected by the mitochondrial environment and that the mitochondria from the zg may in some way be able to stabilise this labile enzyme and promote aldosterone synthesis (Yanagibashi et al 1986). Phospholipids or calmodulin are possible candidates as mitochondrial regulators of this particular enzyme.

In contrast, humans and rats have distinct 11 β hydroxylase isoenzymes that are responsible for cortisol and aldosterone production. Humans carry two genes on chromosome 8q (Mornet et al 1989) that encode the 11 β hydroxylase isoenzymes with 93% amino-acid sequence homology (White et al 1992). One gene CYP11B1 is regulated by ACTH and is required for cortisol biosynthesis while the other gene CYP11B2 is regulated by All and has 11 β -hydroxylase, 18-hydroxylase and 18-oxidase activities resulting in the production of aldosterone. In the rat, Lauber and coworkers (1989) reported that the adrenal mitochondria may be capable of producing two forms of P450_{c11 β} which differ in molecular weight (49kDa and 51kDa). The 51kDa protein catalysed 18 and 11 β hydroxylation of deoxycorticosterone but was unable to produce aldosterone even in stimulated zg cells. The 49kDa protein, exclusively found in the zg, could convert deoxycorticosterone not only to 18-OH deoxycorticosterone and corticosterone but also to 18-OH corticosterone and aldosterone. This zone-specific effect of 11 β hydroxylase was further supported by the findings of Kirita et al (1988) who reported the existence of two P450_{11 β} genes with expression products that possessed different catalytic activities. Data suggested that in both human and rat, the CYP11B1 gene synthesises cortisol in the

zfr whereas the CYP11B2 synthesises aldosterone in the zg and that the production of both hormones is controlled at the transcriptional level.

The interconversion of cortisol and cortisone is mediated by two distinct non-P450 microsomal enzymes (Lakshmi et al 1985). 11 β -hydroxysteroid dehydrogenase (11 β -HSD) (NADP⁺-dependent) activity converts cortisol to cortisone, while 11-oxidase (NADPH-dependent) converts cortisone to cortisol.

11 β -HSD is a "short-chain" dehydrogenase which is encoded by a single gene on human chromosome 1 (White et al 1992). The mammalian liver and kidney enzymes possess both dehydrogenase and reductase activities (Lakshmi et al 1988) and were initially thought to comprise of two distinct enzymes. However, following cloning of the cDNA encoding this enzyme and its subsequent transfection into the vaccinia virus, a single protein species with both dehydrogenase and reductase activities was expressed (Agarwal et al 1990). Congenital deficiency of 11 β -HSD (the syndrome of apparent mineralocorticoid excess) results in cortisol acting as a potent mineralocorticoid (Stewart et al 1993). In contrast, cortisol lacks mineralocorticoid effects in normal patients suggesting a crucial role for 11 β -HSD in the tissue control of mineralocorticoid activity. Recent studies have indicated that 11 β -HSD is responsible for conveying specificity for the mineralocorticoid receptor (MR) (Edwards et al 1988) and therefore protects the MR from higher circulating concentrations of the glucocorticoids. In the case of congenital deficiency of 11 β -HSD, the *in vivo* specificity for the renal MR is lost.

However, several lines of evidence suggest the existence of at least two isoforms of 11 β -HSD, one found predominantly in glucocorticoid receptor rich tissues and the other restricted to aldosterone-selective mineralocorticoid target tissues and placenta. The human placental 11 β -HSD has a much higher affinity for cortisol than that reported for the enzyme in rat liver and also appears to prefer NAD⁺ as a co-factor rather than NADP⁺. This protein may have a "protective" function in the fetus, inactivating the maternal

glucocorticoids, so that the low fetal glucocorticoid levels derive almost exclusively from the fetal adrenal (Lopez-Bernal et al 1980). This NAD⁺-dependent 11 β -HSD isoform in the placenta may be similar or identical to the 11 β -HSD isoform in the renal distal tubule that completely protects the MR from glucocorticoids (Seckl et al 1993).

1.3 STIMULATION OF STEROIDOGENESIS

1.3.1. Mechanism of action of ACTH on adrenocortical cells.

ACTH stimulates an increase in adrenocortical steroidogenesis mainly via the action of the second messenger adenosine 3' 5' cyclic monophosphate (cAMP) (Haynes et al 1957). This finding was later confirmed by Grahame-Smith and co-workers (1967), who reported that the production of cAMP in response to ACTH was dose-dependent and preceded the increase in steroidogenesis. Schimmer et al (1968), using ACTH linked to a large molecule, established that ACTH exerts its steroidogenic effect by acting through a receptor located in the plasma membrane. Studies on both rat zf cells and human adrenocortical cells maintained in primary culture, using a labelled analogue of ACTH, Phe², Nle⁴-ACTH, showed in each case a single class of ACTH receptor (Buckley et al 1981). However, Gallo-Payet et al (1985), using freshly-isolated rat zf cells, demonstrated two classes of ACTH receptor, a high affinity receptor (Kd 1.1×10^{-11} M) and a low affinity site (Kd 2.9×10^{-9} M). Following purification of the ACTH receptor, from the murine adrenocortical tumour cell line, ACTH binding was shown to occur to the largest of its four subunits (Bost et al 1986). The purified receptor was also shown to possess two binding affinities, confirming the work of Gallo-Payet.

Two temporally distinct actions of ACTH on steroidogenesis exist. In the case of acute stimulation, ACTH has an early pathway effect regulating the supply of substrate to

the P450 enzyme whereas chronic ACTH stimulation causes increased biosynthesis of the steroidogenic enzymes, by increasing the expression of the genes for all the cytochrome-P450 species involved in steroid biosynthesis as well as the iron-sulphur protein, adrenodoxin (Simpson et al 1987).

A major site of action of this trophic hormone is the rate-limiting step involving the conversion of cholesterol to pregnenolone (Stone et al 1954). This action requires a supply of cholesterol, transport of the steroid to the inner mitochondrial membrane, binding to the side-chain cleavage P-450 enzyme and finally release of the product pregnenolone into the cytosol.

The most important source of cholesterol for the side-chain cleavage reaction is the intracellular store of cholesterol ester present in lipid droplets. ACTH induces phosphorylation by cAMP-dependent protein kinases of cholesteryl ester hydrolase and cholesterol acyl transferase (Bristow et al 1980) leading to the activation of these enzymes and an increase in the availability of the unconjugated form of cholesterol in the lipid droplets. In human fetal adrenal cells, ACTH also increases the activity of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA reductase) involved in the conversion of acetate to cholesterol, thus replenishing the lipid droplet stores of the free steroids which have been depleted as a result of the transfer of cholesterol to the mitochondria.

The accumulation of cholesterol at the inner mitochondrial membrane is the driving force behind steroidogenesis. The intramitochondrial movement of cholesterol is blocked by the protein synthesis inhibitor, cycloheximide, indicating that a newly synthesised protein factor (labile protein) is involved in the ACTH-stimulating process (Ferguson 1963). This cycloheximide-sensitive labile factor is envisaged to be activated by a cAMP-dependent protein kinase. Over the years several phosphoproteins have been proposed as possible candidates. For example, Krueger and co-workers (1983) identified a 28k phosphoprotein, protein i, in adrenocortical cells stimulated with ACTH, with a time course

and dose-dependency paralleling the steroidogenic response. In contrast to this, a peptide factor with a molecular weight of 2,200 composed predominantly of basic amino-acids has been identified in rat adrenals (Pederson et al 1983) which seems to fulfill many of the criteria required for the labile protein. However, the most convincing argument for the discovery of the candidate protein involved in the transfer of cholesterol across the mitochondrial membranes was proposed by Clark et al (1994), who characterised a novel 30kDa mitochondrial protein in MA-10 mouse Leydig tumour cells that is synthesised in response to luteinising hormone. This protein is newly-synthesised in a time- and dose-responsive manner to hormone stimulation and its synthesis is sensitive to cycloheximide, but most importantly, the expression of this protein (Steroidogenic Acute Regulatory protein (StAR)) in MA-10 cells in the absence of hormone stimulation is sufficient to induce steroid production.

Cholesterol traverses the inner mitochondrial membrane without the aid of translocating proteins. Farese et al (1979) reported that ACTH rapidly increases the concentration of rat adrenal phospholipids, in particular those with a high degree of unsaturation. Bilayer phases are resistant to solute permeability whereas hexagonal phases are more favourable structures, therefore the possibility exists for hexagonal phase-mediated transport of cholesterol without the aid of translocating proteins (Kimura et al 1986).

These acute actions of ACTH are solely directed at the stimulation of the formation of pregnenolone. The chronic effects of this peptide hormone are exerted at every step in the steroidogenic pathway. Long-term treatment with ACTH causes the increased transcription and accumulation of mRNAs specific for P-450 steroid hydroxylases involved in steroid biosynthesis (John et al 1986), as well as the iron-sulphur protein adrenodoxin (Simpson et al 1987). ACTH increases the transcriptional activation of five adrenocortical steroid hydroxylases that catalyse the conversion of cholesterol to cortisol; P-450scc (John

et al 1984), P-450_{c17 α} (Zuber et al 1986), P-450_{11 β} (John et al 1985), P-450_{c21} (John et al 1986) and 3 β -HSD (Naville et al 1991). Furthermore, as is the case with acute stimulation, these responses are cAMP-dependent and the effect is blocked by cycloheximide (Simpson et al 1988), suggesting a role for a regulatory protein in controlling mRNA levels of the steroid pathway enzymes.

The adrenal steroidogenic response to ACTH is dependent on the presence of extracellular calcium (Birmingham et al 1953). Yanagibashi (1979), proposed that Ca²⁺ may be a primary "second messenger" of ACTH with or without cAMP to regulate steroidogenesis in rat and bovine adrenocortical cells. Its primary site of action includes the activation of adenylate cyclase and the stimulation of the hexagonal phase of the inner mitochondrial membrane to facilitate the attachment of cholesterol to P-450_{sc} (Kimura et al 1986). Ca²⁺ influx and steroid secretion experiments with 1,4 dihydropyridine (DHP) Ca²⁺ antagonists suggested that bovine adrenal zona fasciculata cells express L-type Ca²⁺ channels which function in regulating ACTH-stimulated cortisol production (Yanagibashi et al 1990). However, Mlinar et al (1993), using the whole cell version of the patch clamp technique, showed that bovine adrenal zf cells express only low voltage-activated, rapidly inactivating Ca²⁺ current with properties of T-type Ca²⁺ current. Studies by Enyeart and co-workers (1993), also demonstrated that T-type Ca²⁺ channels are required for ACTH-stimulated cortisol synthesis in bovine adrenal zf cells. This serves as the main portal for voltage-gated Ca²⁺ entry into the cell, coupling membrane depolarisation to the synthesis of cortisol. The sensitivity of T-type Ca²⁺ channels in bovine adrenal zf cells to DHP Ca²⁺ channel antagonists provides an explanation for these apparent contradictory results (Enyeart et al 1992). ACTH is known to depolarise adrenal cortical cells, trigger Ca²⁺ uptake and stimulate cortisol secretion at concentrations much lower than those which produce measurable increases in cAMP (Mlinar et al 1993), indicating that Ca²⁺ may be a primary intracellular messenger regulating corticosteroid production.

1.3.2. The Hypothalamic-pituitary-adrenal axis.

The control of glucocorticoid secretion by the zf is, in part, affected by the activity of the hormone ACTH. ACTH is a single chain peptide of 39 amino-acids and is derived from a large precursor, pro-opiomelanocortin (POMC). In addition to increasing the rate of secretion of adrenal steroids, acute stimulation by ACTH induces vasodilatation of the adrenocortical blood vessels, a process thought to be mediated by mast cells present in the connective tissue capsule (Hinson et al 1988). Long term effects of ACTH include cell proliferation and adrenocortical hypertrophy.

ACTH is synthesised and released from the corticotroph cells of the anterior pituitary in an episodic manner with a distinct circadian rhythm. In humans, ACTH levels are highest in the morning, thereafter falling to a low point in the late evening. The major physiological stimulus to ACTH release is stress which includes fear, emotion, pain, hypoglycaemia, hypoxia or haemorrhage (reviewed in Lilly et al 1992).

Several factors act on the anterior pituitary to initiate the release of ACTH. The most important of these is corticotropin-releasing hormone (CRH) which is a 41 amino-acid peptide synthesised in the parvocellular neurones of the paraventricular nucleus located in the hypothalamus. This peptide is transported through axons to nerve endings in the median eminence which are in contact with the hypothalamo-hypophysial portal vessels leading to the anterior pituitary (Lilly et al 1992). CRH binds to specific membrane receptors on the pituitary corticotrophs activating cAMP-dependent protein kinases, resulting in the release of ACTH. In addition to stimulating the secretion of ACTH, CRH also increases the expression of POMC mRNA in corticotrophs.

Other factors such as Arginine vasopressin (AVP), oxytocin (OT) and the catecholamines act at the level of the pituitary and hypothalamus indirectly stimulating adrenal steroid biosynthesis. In fact, each of these peptides interacts with CRH in the

anterior pituitary and potentiates the effects of this hormone by increasing the levels of intracellular calcium (Antoni et al 1986). The most important of these is AVP which is co-secreted with CRH by the parvocellular neurones within the paraventricular nucleus.

The release of both ACTH and CRH is inhibited by the corticosteroids. The feedback inhibition mechanism exerted by the corticosteroids on CRH/ACTH release comprises of three different time domains; immediate (seconds to minutes), fast (several minutes) and slow (hours to days) (Keller-Wood et al 1984). The immediate and fast negative feedback effects involve inhibition of CRH release from the hypothalamus (Buckingham and Hodges 1978) while the delayed response involves the inhibition of CRH and ACTH synthesis (Arimura et al 1969). In cases of haemorrhage and hypoxia, corticosteroids potentiate the responses of ACTH and thus the hypothalamic-pituitary-adrenal (HPA) system has a positive facilitatory mechanism which on activation can override this negative feedback.

Stimulation of secretion of the growth hormone somatostatin is attenuated on activation of the pituitary-adrenal axis (Martin et al 1976). Richardson (1983) observed that unlike the other secretagogues such as the neurohypophysial peptides and catecholamines, somatostatin inhibited CRH-induced ACTH release from mouse pituitary tumour cells.

In recent years, it has been suggested that cytokines, involved in the inflammatory response, activate elements of the HPA system. Most work has concentrated on the cytokine interleukin 1 (IL-1) which occurs in two forms (α and β) and is secreted by leukocytes at the site of infection. IL-1 is reported to act at the hypothalamus through a prostaglandin-dependent mechanism. Evidence indicates that this action is quite specific as IL-1 activates the parvocellular neurones that express CRH alone (Berkenbosch et al 1989). The resulting increase in CRH in the portal vessels leads to release of ACTH from the anterior pituitary and subsequently an increase in corticosteroid secretion by the

adrenocortical cells. The increase in ACTH release in response to IL-1- β may be mediated by factors other than CRH such as noradrenaline and dopamine (Palazzolo et al 1990).

These findings point to the possibility of two-way humoral communication between HPA and immune systems comprising the immunosuppressive activity of the corticosteroids and the stimulatory activity of the cytokines on the immune system (Lilly et al 1992). The increased production of corticosteroids in response to IL-1 will negatively feedback onto the HPA system thus restraining the inflammatory response.

1.3.3. The intra-adrenal CRH-ACTH axis.

Since the first isolation of CRH (Vale et al 1983), CRHs have been identified in a variety of different species including rat (Rivier et al 1983), human (Shibahara et al 1983), bovine (Esch et al 1984) and porcine (Patty et al 1985). CRH-like substances have been found not only in neuronal tissues but also in the pancreas, gut and adrenal gland (Hashimoto et al 1984). Pro-opiomelanocortin-derived peptides such as ACTH were identified in the adrenal medulla (Evans et al 1983), a finding later confirmed by Suda (1986) who described CRH and ACTH-like immunoreactivity localised mainly in the human adrenal medulla although small amounts were also present in the cortex.

In cattle, CRH-like immunoreactivity (Minamio et al 1988) and POMC gene expression has also been reported in the adrenal medulla (Thorne et al 1991). This raises the possibility that the adrenal gland may possess an active CRH-ACTH system which may locally regulate steroidogenesis. This possibility has been further strengthened by the recent finding of CRH receptors coupled to adenylate cyclase in primate adrenal medulla, which when activated, stimulated the production of catecholamines (Udelsman et al 1986). There are also reports of stimulatory effects of exogenous CRH on

cells in culture (Udelsman et al 1986). Thus CRH could be locally released in the adrenal to regulate both cortical and medullary stress hormones in a paracrine manner during short and long-term exposures to stress.

1.3.4. Mechanism of action of All in adrenocortical cells.

All is the main stimulant of aldosterone production in rat and bovine glomerulosa cells (Kaplan et al 1962). In rat zg cells, All promotes a rise in intracellular Ca^{2+} implicating the involvement of phosphoinositidase C (PL-C) in the activation of steroidogenesis (Williams et al 1981). Prelabelling of adrenocortical cells with [^3H]-inositol was used to study the effects of All on PL-C activity. In both rat (Enyedi et al 1985) and bovine (Kojima et al 1984) zg cells, All increased phosphoinositol formation with an early rise in InsP_3 and InsP_2 followed by an increase in $\text{Ins}(1)\text{P}$. In superfused rat zg cells All stimulated a rapid and sustained rise in $[\text{Ca}^{2+}]_i$ with a dose-dependency correlating with that of aldosterone secretion (Braley et al 1986). The initial sharp rise in $[\text{Ca}^{2+}]_i$ in response to All occurs through an $\text{Ins}(1,4,5)\text{P}_3$ -mediated release of Ca^{2+} from intracellular stores (Balla et al 1989 and Kojima et al 1984) while the sustained rise in Ca^{2+} is maintained by an influx of Ca^{2+} across the plasma membrane (Aquilera et al 1986).

Electrophysiological evidence points to the activation of voltage-dependent Ca^{2+} channels as a common pathway for Ca^{2+} entry into zg cells during stimulation. Membrane voltage and current measurements demonstrate the existence of a Ca^{2+} conductance in both rat and bovine zg cells (Matsunaga et al 1987, Matsunaga et al 1987). In bovine and human zg cells, both L-type and T-type voltage-activated Ca^{2+} channels have been characterised (Cohen et al 1988, Payet et al 1994) and All-induced depolarisation has been shown to result from an inhibition of potassium conductances by protein kinase-C (Kanazirska et al 1992). Recently another pathway for Ca^{2+} entry, resulting from

intracellular Ca^{2+} pool depletion, has been described in bovine (Rossier et al 1993) and rat (Hajinoczky et al 1991) glomerulosa cells. This pathway is activated by thapsigargin, an agent leading to Ca^{2+} release from intracellular stores and subsequent activation of aldosterone secretion. Burnay and co-workers (1994) showed that only a minor part of the Ca^{2+} influx response to All involves voltage-operated Ca^{2+} channels whereas the rest of the response is thapsigargin-sensitive. Interestingly, in rat cells, the steroidogenic response to thapsigargin was highly potentiated by K^+ (Hajinoczky et al 1991), suggesting that various Ca^{2+} entry pathways into glomerulosa cells act synergistically to regulate the production of aldosterone.

In both bovine and rat zfr cells All stimulated PL-C activity though only in bovine cells is there a subsequent increase in steroid output (Bird et al 1989). The effects of All on $[\text{Ca}^{2+}]_i$ in zfr cells has received comparatively less attention. The failure of All in rat zfr cells to have an effect on $^{45}\text{Ca}^{2+}$ efflux or $[\text{Ca}^{2+}]_i$ (Williams et al 1981) is in agreement with the lack of effect of the peptide on steroid secretion. In contrast, All stimulates a rapid increase in $[\text{Ca}^{2+}]_i$ in bovine zfr cells (Walker et al 1990) which is reported to be mobilised from a common intracellular pool (Walker et al 1991).

Calcium is involved in the regulation of a number of enzymes, including protein kinase C (PKC). PKC is present in the adrenal cortex (Kojima et al 1984) and the factors necessary for its activation, diacylglycerol (DAG) and Ca^{2+} mobilisation, are stimulated by All (Catt et al 1987). Tumour promoters, such as the phorbol ester tetradecanoylphorbol β -acetate (TPA) can substitute for DAG and activate PKC (Pelosin et al 1991). Nakano et al (1990) reported that All caused translocation of PKC activity from the cytosol to the membrane in rat adrenal zg cells. However, further experiments demonstrated that PKC activity is not directly linked to All-stimulated production of aldosterone, although in bovine zg cells, where the effect of All on PKC translocation is more prominent (Lang et al 1987), experiments suggested that PKC activation by All may modulate the steroidogenic

response. Rat glomerulosa cells expressed only the α and ϵ isoforms of PKC and All increased membrane-bound levels of both of these isoforms. In addition, DAG arising from All action can also be metabolised to arachidonic acid, which is then further metabolised by the lipoygenase (LO) pathway to products such as hydroxyeicosatetraenoic acids (HETEs). 12-HETE was shown to stimulate PKC enzyme activity in rat zg cells with the same potency as All and could specifically activate PKC- ϵ without altering the subcellular distribution of PKC- α (Natarajan et al 1994). PKC has emerged as a potential intermediate in a number of cellular transregulation processes, for example, in bovine adrenocortical cells, TPA treatment induces a sensitisation of the ACTH-dependent adenylate cyclase activity (Pelosin et al 1991).

1.3.5. The Renin-Angiotensin system.

The renin-angiotensin system (RAS) is activated under conditions of sodium depletion, haemorrhage and inferior vena constriction. A decrease in sodium filtration results in the release of renin and the subsequent formation of All. Other factors that stimulate the release of renin are a decreased renal perfusion pressure, decreased sodium delivery to the distal convoluted tubule and sympathetic stimulation of the juxtaglomerular cells.

Renin is a 42k proteolytic enzyme secreted mainly by the juxtaglomerular cells located in the afferent arteriole of the kidney. After release into the circulation, renin acts on an α_2 globulin plasma protein, angiotensinogen, cleaving it at the leu₁₀-leu₁₁ bond to form a decapeptide, angiotensin I (AI) which has relatively little biological activity. Angiotensin converting enzyme (ACE), found primarily in the lungs, cleaves AI at a leucine and a histidine residue to form an octapeptide, angiotensin II (AII). Angiotensinogen may also be directly converted to AII by the action of the serine protease, tonin, which is found

in the venous effluent of the submaxillary gland. All has a very short half-life and is rapidly broken down by aminopeptidases present in various tissues to produce inactive peptide fragments. Aminopeptidase A removes the N-terminal aspartate residue from All to form the heptapeptide angiotensin III (Alll).

All initiates a complex series of physiological events which work in concert to restore plasma volume, blood pressure and sodium concentration. It is a powerful vasoconstrictor in most species being around 40 times more potent than noradrenaline. A major site of action of All is the kidney where it regulates renal blood flow and can also directly inhibit renin secretion. Circulating All stimulates thirst via receptors located in the subfornical organ and the paraventricular nuclei. An important action of All is its effect on the adrenal gland which is discussed in detail in section 1.3.4. In the case of the medulla, it stimulates the release of catecholamines enhancing its hypertensive actions whereas in the cortex it is a potent stimulator of aldosterone secretion which, in turn, regulates electrolyte and fluid balance.

1.3.6. The intra-adrenal renin-angiotensin system.

A renin-like enzyme was first identified in the rabbit adrenal (Ryan et al 1967) which reacted with a renin substrate to form AI. Since this observation, renin-like activity has been detected in a wide number of tissues including the uterus, placenta, brain, testis, heart and vascular smooth muscle (Gross et al 1964, Hirose et al 1978, Naruse et al 1984). These findings may suggest that the renin-angiotensin system acts as a local hormone system in each of these tissues. In the adrenal cortex, renin has been found in various species including rabbits, dogs, cattle, rats and humans (Wang et al 1992) and its mRNA has been detected in rats (Dzau et al 1987) and mice (Field et al 1984). Each component participating in the formation of All has been identified in the rat adrenal

cortex, supporting the presence of an intra-adrenal RAS system (Mulrow et al 1988).

Localised mainly in the zg cells, the site of aldosterone formation, biosynthesis and secretion of adrenal renin was increased in sodium-depleted or potassium-loaded rats (Doi et al 1985). Under these physiological conditions, the increase in renin activity was demonstrated to be linked to elevated levels of renin mRNA indicating that adrenal renin is synthesised locally rather than taken up from the extensive vascular system supplying the gland (Brecher et al 1989 and Wang et al 1992).

The physiological role of intra-adrenal renin has not been established, but many observations have shown that the concentration of this enzyme in the zg parallels that of the mineralocorticoid aldosterone. In cultured rat zg cells, ACTH, All and K^+ stimulated both aldosterone secretion and renin activity. The increase in renin activity correlated with increased levels of renin mRNA (Wang et al 1992) indicating an increase in the transcriptional rate of the renin gene or an increase in the stability of the mRNA.

Further evidence supporting a function for the RAS system in the control of aldosterone secretion by zg cells has been described by Le Houx et al (1992). Results suggested that the RAS system was involved in the control of $P450_{\text{sc}}c$ and $P450_{\text{c}11}$ gene expression in response to sodium restriction or potassium supplementation. $P450_{\text{c}11}$ which catalyses the formation of aldosterone from corticosterone is dependent solely on the presence of All, whereas $P450_{\text{sc}}c$ is modulated by several factors including All, emphasising the key part played by this peptide in the control of steroid biosynthesis.

Less information is available regarding the existence of a functional RAS system in zfr cells. Renin is present in both the zg and the zfr of the mouse adrenal cortex (Naruse et al 1984) whereas in the rat it is confined to the zg (Brecher et al 1989). Transgenic rats (mRen-2)27 transfected with the Ren-2 mouse renin gene developed severe hypertension (Mullins et al 1990) and differed from normal rats in that there was an increase in the expression of renin activity and aldosterone biosynthesis in both the

zg and zfr (Yamaguchi et al 1992).

However, it was proposed that there was no functional RAS system present in the inner zone cells as there were no changes in the levels of renin and mRNA (Doi et al 1984) in response to variation in the levels of Na^+ and K^+ . Unlike the zg, this region of the cortex appeared to be insensitive to these physiological stimuli. Further work is required to clarify whether the inner zone cells do possess a separate functional RAS or whether the activities of an intra-adrenal RAS system present in the adrenal cortex are largely confined to the zg.

1.3.7. Adenylate Cyclase.

Cyclic AMP was the first agent to be positively identified as an intracellular second messenger. It has been implicated as the second messenger involved in the action of a number of hormones and neurotransmitters at their respective target tissues, for example, ACTH (adrenal), adrenaline (heart), thyroid stimulating hormone (thyroid) and glucagon (liver) (Rodbell et al 1980). The concentration of cAMP is regulated by two separate enzymes; 1) adenylate cyclase which catalyses the conversion of ATP to cAMP and 2) phosphodiesterase which degrades cAMP into 5'AMP.

The adenylate cyclase system comprises of at least three distinct components (figure 1.7): the hormone receptor, a catalytic subunit and a guanine nucleotide-binding regulatory protein (Gp). Two forms of Gp exist, termed Gs and Gi. Gs mediates the stimulation of adenylate cyclase whereas Gi mediates the inhibition of this enzyme (Hildebrandt et al 1983). Both G proteins are composed of three subunits α , β and γ . The β and γ subunits of both G proteins are identical (Hildebrandt et al 1984), while the α subunits, though highly homologous, possess many variable regions in particular the amino terminus which may be necessary for $\beta\gamma$ binding (Lochrie et al 1988). The $G\alpha$

FIGURE 1.7

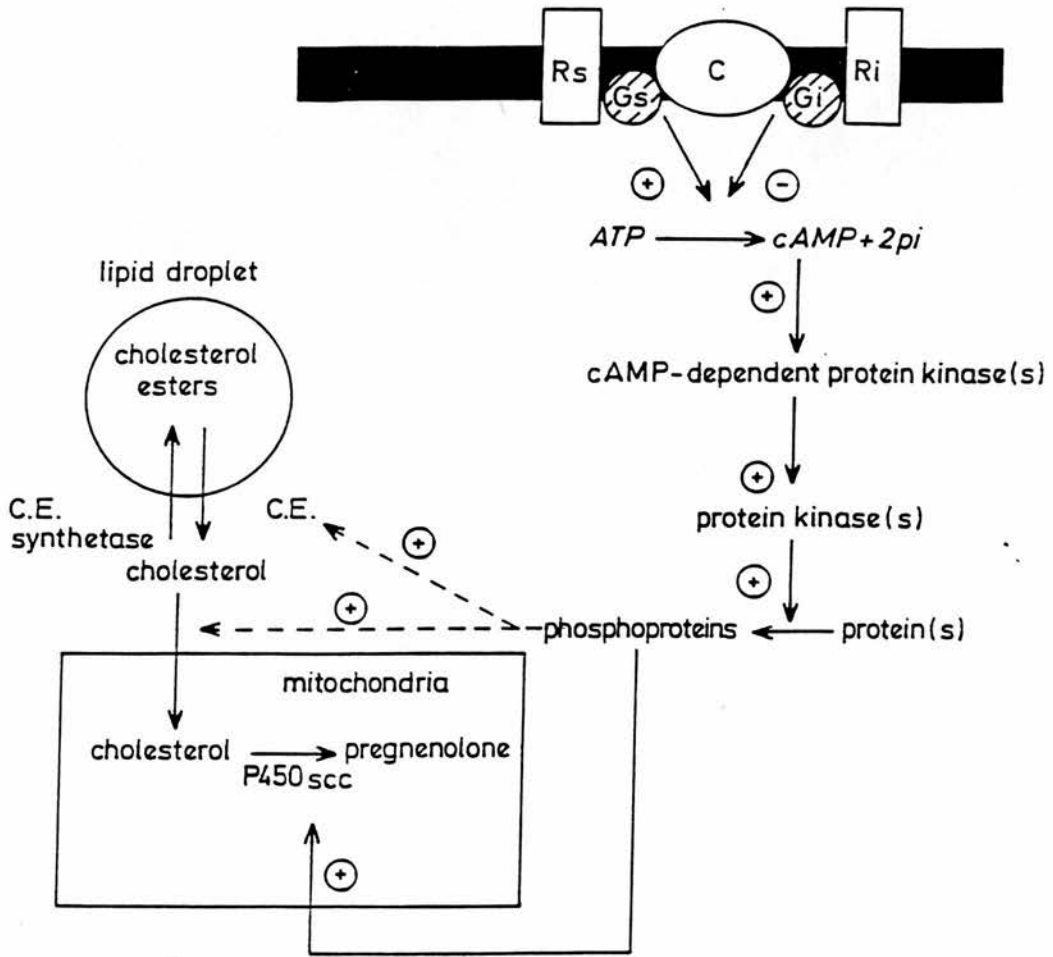


FIGURE 1.7

Structure of the hormonally regulated adenylate cyclase. Agonists interact with stimulatory receptors (R_s) which activate the enzyme catalytic unit (C) via a pertussis toxin-insensitive G protein (G_s). The enzyme can also be inhibited by agonists which interact with receptors linked to the inhibitory G proteins (G_i). The cAMP thus formed on the activation of R_s , stimulates cAMP-dependent protein kinases, eventually leading to the activation of steroidogenesis, through the phosphorylation of specific enzymes (P450_{scc}).

subunits also differ in their response to bacterial toxins. The α subunit of Gs is permanently activated by cholera toxin whereas the α subunit of Gi is ADP-ribosylated by pertussis-toxin leading to inactivation of this G protein.

The cAMP generated interacts with a specific binding protein present in the cytosol of the adrenocortical cells (Gill et al 1969). This binding protein is closely associated with a catalytic protein and together these two subunits comprise a cAMP-dependent protein kinase. Dissociation of these two components results in activation of the protein kinase (Garren et al 1971) and phosphorylation of cytosolic and microsomal proteins. Important enzymes that are activated by this cascade of reactions include cholesterol ester hydrolase (Boyd et al 1983) which causes an increase in the pool of free metabolically active cholesterol in the adrenal cell and P450_{scc} which converts the unconjugated cholesterol to pregnenolone, thus promoting steroidogenesis.

Many agents are now known to stimulate steroidogenesis in adrenocortical cells via cAMP. These include ACTH (Haynes et al 1957), serotonin (Fujita et al 1979), K⁺ (Albano et al 1974), catecholamines (Walker et al 1988) and ATP (Hoey et al 1994).

1.3.8. Phosphatidylinositol metabolism.

The involvement of inositol phospholipids present in the plasma membrane in translating hormonal and neurotransmitter signals was first described by Hokin and Hokin (1953), who reported that acetylcholine increased the incorporation of ³²Pi into phospholipids in the pancreas, brain and various other tissues. Michell (1975) proposed a link between agonist stimulation of phospholipid metabolism and elevation of [Ca²⁺]_i, a concept proved to be correct in later years by Streb et al (1983) and Irvine et al (1984), the latter establishing that inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) was responsible for the release of [Ca²⁺]_i from non-mitochondrial stores.

The major pathways of phosphoinositide and phosphoinositol metabolism are shown in figure 1.8. A dynamic equilibrium exists between the three phosphoinositides, phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdIns4P) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) involving specific kinases and phosphomonoesterases. Agonist stimulation initiates the hydrolysis of PtdIns(4,5)P₂ by activating a hormone-sensitive phospholipase, phospholipase C (phosphoinositidase C), to produce the second messengers Ins(1,4,5)P₃ and diacylglycerol (DAG). Ins(1,4,5)P₃ can be metabolised by two separate pathways. The first involves sequential dephosphorylation by phosphatases to free inositol via Ins(1,4)P₂ and Ins4P and the second requires phosphorylation via a cytosolic 3-kinase to Ins(1,3,4,5)P₄ (inositol tetrakisphosphate) followed by dephosphorylation to free inositol via Ins(1,3,4)P₃, a reaction catalysed by the same 5-phosphatase that acts on Ins(1,4,5)P₃. The biosynthetic pathways for the synthesis of IP₅ and IP₆ have not been adequately characterised. In homogenates of both brain (Stephens et al 1988) and chromaffin cells (Sasakawa et al 1990), added [³H](1,4,5)IP₃ can be converted to [³H]IP₅. According to a recent report, Ins(1,3,4,5,6)P₅ is synthesised, by the phosphorylation of Ins(1,4,5,6)P₄, by a kinase that shows specificity for the 3-hydroxy group (Stephens et al 1988). Presumably, InsP₅ is further phosphorylated to InsP₆. The inositol monophosphate isomers, Ins(1)P₁ and Ins(3)P₁, are dephosphorylated to free inositol by the same lithium-sensitive enzyme. The DAG kinase phosphorylates DAG to form phosphatidic acid (PtdOH) which is converted to PtdIns via cytidine diphosphodiacylglycerol (CDP-DG). DAG also acts as an intracellular second messenger by stimulating protein kinase-C (PKC) which exerts its effects on receptor-linked cellular responses through the phosphorylation of specific intracellular proteins, for example, fibrinogen (Humble et al 1984), insulin receptor (Jacobs et al 1983) and Ca²⁺ transport ATPase (Limaç et al 1980). PKC requires Ca²⁺ and phospholipid, particularly phosphatidylserine, for its activation. In some cells, such as the adrenal medulla, PKC may

FIGURE 1.8.

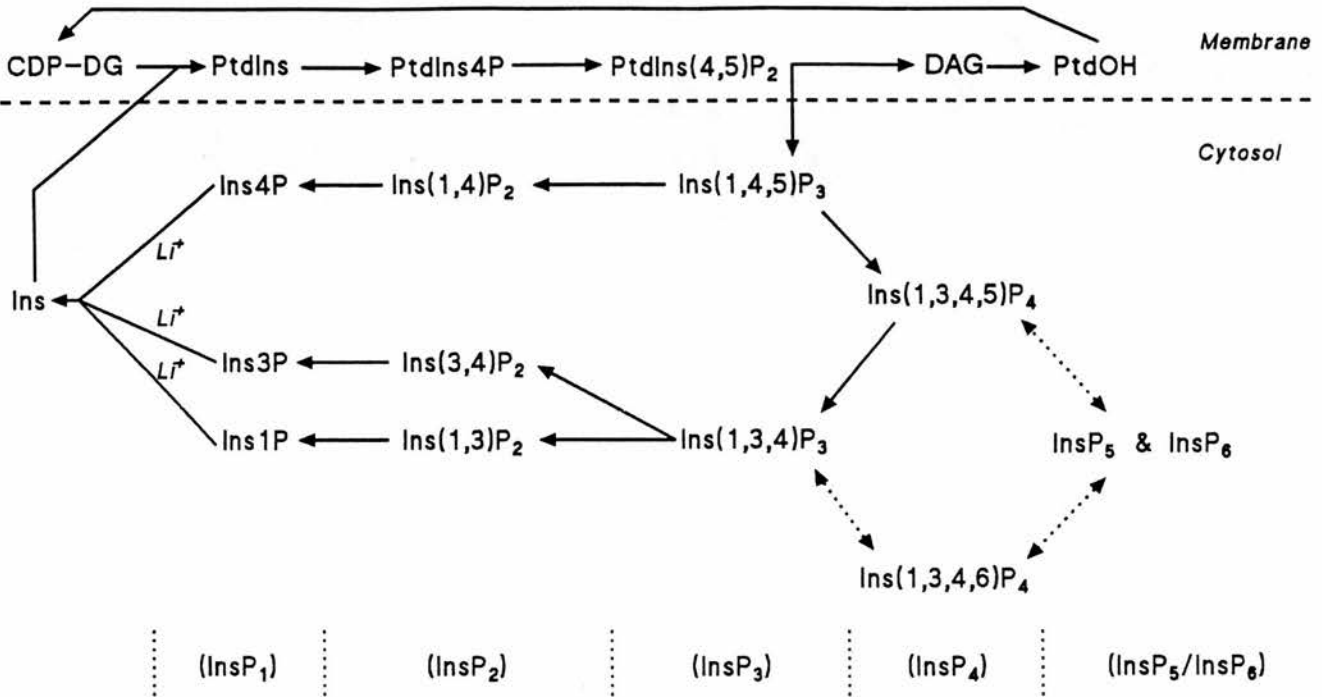


FIGURE 1.8.

An outline of the major metabolic pathways which are activated as a consequence of phosphoinositidase C action on phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P₂) to produce the second messengers inositol (1,4,5) trisphosphate (Ins(1,4,5)P₃) and diacylglycerol (DAG). Ins(1,4,5)P₃ is recycled back to free inositol (Ins) by sequential phosphorylation/dephosphorylation, the final steps being inhibited by lithium (Li⁺). DAG is recycled by phosphatidic acid (PtdOH) and cytidine diphosphodiacylglycerol (CDP-DG), the latter combining with free inositol to reform phosphatidylinositol (PtdIns).

be activated solely through calcium entering via voltage-dependent calcium channels (Berridge et al 1987). However, in most cells, the bifurcation of the pathway provides the versatility necessary to control the final response of the cell by acting either cooperatively or synergistically.

1.3.9. Ins(1,4,5)P₃ and calcium mobilisation.

A biphasic mobilisation of calcium involving an initial transient release of sequestered Ca²⁺ from the endoplasmic reticulum (ER) followed by an influx of extracellular Ca²⁺ through voltage-sensitive or receptor-operated Ca²⁺ channels in the plasma membrane, is believed to involve inositol phosphates in both processes.

Evidence that Ins(1,4,5)P₃ was responsible for the release of Ca²⁺ from non-mitochondrial intracellular stores was first reported by Streb et al (1983) for permeabilised rat pancreatic acinar cells. Subsequently, a growing number of studies have demonstrated this property of Ins(1,4,5)P₃ in a variety of different permeabilised cell types and from isolated ER preparations of skeletal muscle (Volpe et al 1985), platelets (O'Rourke et al 1985) and pancreas (Streb et al 1984). Cell fractionation experiments have confirmed that Ins(1,4,5)P₃ releases Ca²⁺ from a membrane fraction that is a component of the ER. Recently it has been proposed that the ER is not the only source of Ca²⁺ and that discrete specialised Ins(1,4,5)P₃-responsive organelles named calciosomes exist (Volpe et al 1988).

Ins(1,4,5)P₃ exerts its effects on the ER through specific receptors resulting in the opening of Ca²⁺ channels. Binding sites for Ins(1,4,5)P₃ have been identified in microsomal fractions of bovine adrenal cortex (Baukal et al 1985) which is consistent with the observations that adrenocortical cells exhibit calcium-mediated steroidogenic responses to All. The Ins(1,4,5)P₃ receptor undergoes a large conformational change on

binding of $\text{Ins}(1,4,5)\text{P}_3$ which is thought to be due to the coupling of the receptor to the Ca^{2+} channel (Berridge 1993), both of which (ie receptor and channel) may even reside in the same protein (Berridge et al 1989).

$\text{Ins}(1,4,5)\text{P}_3$ is not directly involved in the influx of extracellular Ca^{2+} as it does not itself promote the release of Ca^{2+} from plasma membrane vesicles derived from rat brain synaptosomes (Ueda et al 1986) and exocrine pancreas (Streb et al 1984). Electrophysiological studies using sea urchin eggs concluded that $\text{Ins}(1,4,5)\text{P}_3$ does mediate Ca^{2+} entry into cells (Slack et al 1986) but only if the metabolite $\text{Ins}(1,3,4,5)\text{P}_4$ is also present (reviewed in Berridge et al 1993). In addition to this synergism between the two inositol phosphates, $\text{Ins}(1,3,4,5)\text{P}_4$ may also control the movement of Ca^{2+} between $\text{Ins}(1,4,5)\text{P}_3$ -sensitive and insensitive intracellular compartments. It has been argued that the influx of extracellular Ca^{2+} is regulated by the levels of Ca^{2+} present in the ER and extracellular Ca^{2+} has been demonstrated to flow into the ER when its stores are depleted by the opening of a channel in its membrane under the control of $\text{Ins}(1,4,5)\text{P}_3$ (Putney 1986). Thus $\text{Ins}(1,4,5)\text{P}_3$ may itself promote the influx of extracellular Ca^{2+} or because $\text{Ins}(1,3,4,5)\text{P}_4$ controls the transfer of calcium between pools, $\text{Ins}(1,4,5)\text{P}_3$ may act in conjunction with the tetrakisphosphate to increase sequestration of Ca^{2+} from intracellular pools therefore aiding the influx of Ca^{2+} across the plasma membrane.

1.4. PURINES

The physiological consequences of adenosine and adenine nucleotides have been recognised for more than 60 years. These substances are unusual in that they produce their effects both by intracellular and extracellular mechanisms. Both adenosine and ATP are found in all living cells and their concentrations are dynamically regulated in a variety of pathophysiological conditions (Berne et al 1963, Olsson et al 1990). In addition, under appropriate conditions, these compounds can be released from cells where they can interact with specific cell surface receptors to modulate cellular function in an autocrine or paracrine manner (Olsson et al 1990, Berne et al 1980).

1.4.1. Historical Background.

1.4.1.1. Adenosine.

The first reported physiological action of adenosine was its ability to cause bradycardia, coronary vasodilation and blood pressure decreases; these findings were initially reported by Drury and Szent-Gyorgi in 1929. This stimulated a flurry of interest in the use of adenosine as an antihypertensive agent, though clinical use of adenosine in the 1930's as an anti-hypertensive agent was short-lived due to its poor efficiency, which was a result of its short half-life. This led to a degree of negativity about its therapeutic potential (Honey et al 1930).

For the next three decades, basic research concentrated on the physiological rather than the pharmacological aspects of adenosine action. Adenosine has general



vasodilatory actions (Berne et al (1983), Fredholm et al (1986)) and is a vasodilator in all vascular beds thus far studied with the exception of the kidney (Osswald et al (1983)), where it is a potent vasoconstrictor.

The effect of adenosine on the release of neurotransmitters was first shown using electrophysiological techniques by Ginsborg and Hirst in 1972. Soon after, it was directly demonstrated that release of noradrenaline and acetylcholine could be reduced by this compound (Fredholm et al (1974) and Hedquist et al (1976)). Since that time it has become clear that adenosine is able to inhibit the release of a variety of neurotransmitters both centrally and peripherally (Fredholm et al (1983) and (1987)). It controls neurotransmitter release via presynaptic receptors under conditions of hypoxia, hypoglycaemia or ischaemia.

Although the physiological effects of adenosine have been studied for more than 60 years, the cellular mechanisms of action have only been investigated for the past 10 years. It is not surprising that interest in adenosine and its receptors has seen a resurgence in recent years, considering that adenosine is a ubiquitous biological compound being present in every cell of the body and the fact that all cells under stress can release adenosine, which can "feedback" in an autocrine manner to modulate the function of the cell. The short half-life of adenosine in the circulation makes it highly unlikely that adenosine acts as a circulatory hormone (Olsson et al (1990)) but rather as a local regulator or as Newby (1984) has termed it a "retaliatory metabolite".

Adenosine once released can activate its receptors which in turn regulate a diverse set of physiological functions. These include actions on the cardiovascular system. Adenosine can regulate coronary blood flow (Drury et al 1929) and has negative chronotropic and inotropic effects on heart contractility (Fredholm et al 1986). Recently, Van Belle (1993) focused on the growing evidence for a major role of adenosine as a "natural defence" for cardioprotection. Adenosine is produced and released in response

to ischaemia and may be important in protecting the myocardium. Infusion of adenosine into the coronary artery of closed-chest dogs, following periods of regional ischaemia lasting up to 2 hours, resulted in a much better functional recovery compared to the control animals (Babbitt et al 1989). Other physiological functions include modulating gastric acid secretion (Gerber et al 1985), inducing bronchoconstriction when administered to asthmatic patients (Cushley et al 1984), decreasing spontaneous motor activity (Snyder et al 1985) and inhibiting mitogenic stimulation of lymphocytes (Marone et al 1984). Thus almost every organ system of an animal is regulated by the local release of adenosine (Ramkumer et al (1988)).

1.4.1.2. Adenosine Triphosphate (ATP).

The biological activity of extracellular ATP was reported for the first time in 1929 by Drury and Szent-Gyorgyi, who showed that its i.v injection in guinea pigs induced a transient slowing of the heart rate. Following this report there was considerable activity in the field, with particular emphasis initially being placed on the action of ATP on the cardiovascular system and its resulting shock-inducing properties (Green et al 1950).

A causal link between ATP and traumatic shock was first established by Kalckar (1947) (reviewed in Gordon et al (1986)). One of the two components of the role of ATP in circulatory shock could be explained by the findings of Holton (1959); that the potent vasodilator released on stimulation of sensory nerves in rabbit ear vessels was ATP. This property of ATP in shock results in a significant decrease in systolic blood pressure and progressive hypoxia. The second component of shock was the release of ATP into the plasma where it reaches levels as high as 200 μ M (Green et al 1950). At these levels erythrocytes become semi-permeable, leading to the release of more ATP into the plasma to levels at which the ectoenzymes, which normally metabolise nucleotides, can no longer

cope (Trams et al 1980).

In the 1960s two further important discoveries were made: 1) ADP induced platelet aggregation resulting in the release of both ATP and ADP from the granules (Born 1962). The nucleotides released influence both vascular tone and haemostasis, and 2) The discovery of a component in the autonomic nervous system that was neither adrenergic nor cholinergic (Burnstock et al 1964, 1969). Burnstock proposed that the active substance released from some of these nerves was ATP as assessed by Eccles (1964) for neurotransmitters. Early evidence supporting this hypothesis was that ATP was synthesised, stored and released from nonadrenergic, noncholinergic nerves supplying the smooth muscle of the intestine and that it mimicked the effects of nerve stimulation on these muscles (Burnstock et al 1970). As a consequence, these nerves were termed "purinergic" (Burnstock et al 1971).

ATP acts at neuroeffector junctions by modulating the release of other neurotransmitters. It reduces the release of noradrenaline from sympathetic nerves in a wide variety of tissues and the release of acetylcholine at both the neuromuscular junction (Ginsburg et al 1972) and the cholinergic-smooth muscle junction (Sawynok et al 1976).

The first indication that ATP might be released as a co-transmitter with noradrenaline was the demonstration that release of both [³H] ATP and noradrenaline was blocked by guanethidine during stimulation of sympathetic nerves supplying the guinea pig taenia coli (Su et al 1971). Acetylcholine is also released with ATP at both somatic and autonomic nerve endings (Burnstock et al 1986).

Since then a number of findings have confirmed that when present in extracellular domains, ATP can elicit functional responses in a large number of tissues and cells (Gordon et al 1986). These functions include stimulation of exocrine (for example, acinar cells from the parotid gland (Gallacher 1982)) and endocrine secretion (for example, catecholamine release from chromaffin cells isolated from the adrenal medulla (Kim et al

1990), relaxation and contraction of smooth muscle, modulation of neuronal excitability, vascular tone and cardiac function (Boeynaems et al 1990). While exogenous ATP can be rapidly catabolised to adenosine by a number of ectophosphohydrolases, many of the observed actions of ATP can be distinguished from those triggered by occupation of extracellular adenosine receptors (Dubyak et al 1990). Current opinion suggests that extracellular ATP may be a signalling agent in a number of biological systems. Many of these functional effects are observed at low micromolar concentrations well below the Michaelis constant (K_m) values for most adenosine triphosphatases (ATPases) and other ATP-utilising enzymes (Dubyak et al 1990). This would support the existence of specific cell surface receptors for ATP.

1.5. SUBCLASSIFICATION OF PURINOCEPTORS.

In 1978, Burnstock proposed a system for classification of the receptors at which adenosine and adenine nucleotides act to produce their diverse and numerous effects. This classification system proposed that there were two major subtypes, the P1 and P2 purinoceptors. This original classification was based on four criteria: 1) the relative potencies of ATP, ADP, AMP and Adenosine. 2) the selective actions of antagonists, particularly methylxanthines. 3) the activation of adenylate cyclase by adenosine but not ATP, and 4) the induction of prostaglandin synthesis by ATP but not by adenosine (Burnstock et al 1978).

In the initial nomenclature for purinoceptors, those receptors sensitive to adenosine were termed P1 and those sensitive to ATP were termed P2.

1.5.1. The P1 receptor and receptor subtypes.

Classification systems for adenosine receptors were independently proposed by Van Calker et al (1979) and Londos et al (1977). In their original work on adipocytes, Londos and Wolff (1977) considered that adenosine could activate two distinct receptor forms, one responsible for the inhibition of adenylate cyclase and termed R_i and the other responsible for the stimulation of adenylate cyclase and termed R_a . At the same time, Van Calker et al (1979) also reported a dual modulation of adenylate cyclase activity by adenosine analogues. The inhibitory receptor was referred to as A_1 and the stimulatory site as A_2 . The A_1/A_2 classification does not inherently imply any activation or inhibition of adenylate cyclase and many examples are now known where adenosine analogues have biological effects with potency orders suggesting an A_1 or A_2 site but without an apparent involvement of adenylate cyclase. Accordingly, the A_1/A_2 nomenclature has become more widely adopted and is recommended for general use (Stone et al 1985).

Adenosine analogues have been used extensively to determine which P1 purinoceptors belong to the A_1 subtype and which belong to the A_2 subtype. Receptors of the A_1 subtype are preferentially activated by adenosine agonists with substitutions in the N^6 position of the purine moiety such as N^6 -cyclopentyladenosine (CPA), N^6 -cyclohexyladenosine (CHA) and R-phenylisopropyladenosine (R-PIA). Analogues substituted in the 5' position of the ribose moiety most notably 5' N-ethylcarboxamidoadenosine (NECA) and 2,5-disubstituted CGS 21680 (2-[4-(2-carboxyethyl)phenethyl]adenosine-5-N-ethylcarboxamide) show activity at the A_2 receptor (Williams et al 1987). Prototypical A_1 receptors in adipose tissue have the potency order R-PIA > 2-chloroadenosine (CADO) \geq NECA. A_2 receptors in platelets and vascular smooth muscle have the potency order NECA > CADO > R-PIA (Linden et al 1991, Stone et al 1990).

Table 1. Pharmacological classification of P1 purinergic receptors.

<u>Subtype</u>	<u>Agonist Selectivity</u>	<u>Antagonist(s)</u>	<u>Effectors</u>
A ₁	CPA > R-PIA > CADO > NECA > CV- 1808	DPCPX > 8- CPT	cAMP ↓, K ⁺ channel, Ca ²⁺ channel ↓
A ₂	NECA > CADO > CV- 1808 = R-PIA > CPA	CGS 15943A	cAMP ↑

CPA, Cyclopentyladenosine; R-PIA, R-N⁶phenylisopropyladenosine; CADO, 2-chloroadenosine; NECA, 5' N-ethylcarboxamido adenosine; CV-1808, 2-phenylaminoadenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; 8-CPT, 8-cyclopentyltheophylline; CGS 15943A.

It has been proposed that the A_1 sites exist in two distinct pharmacological forms: the A_{1a} and the A_{1b} . This distinction has been made on the basis of a different rank order of potencies of agonists including CV-1674 [2-(4-methoxyphenyl)-adenosine] (Gustafsson et al 1989). This compound is effective at displacing the binding of adenosine at the A_1 receptor found in the central nervous system but has little binding displacement activity on presynaptic terminals in the peripheral nervous system.

A_2 receptors were first subdivided into A_{2a} and A_{2b} by Bruns et al (1986) on the basis of a difference in affinity for adenosine analogues. Central A_{2a} receptors are localised primarily in the striatum, nucleus accumbens and olfactory tubercle and bind adenosine and NECA with higher affinity than A_{2b} receptors; the latter are found to be more widely distributed throughout the brain and are coupled to an elevation of cyclic AMP levels. However agonists that are selective for the A_{2b} subtype of the receptor are not yet available.

In 1986, Ribeiro and Sebastiao postulated the existence of a third category of adenosine receptor based on a thorough review of the existing literature. This stemmed from observations of adenosine receptor agonists that were not linked to adenylate cyclase but led to an inhibition of Ca^{2+} influx and/or mobilisation. The agonist potency order was determined to be NECA = CHA = R-PIA > CADO, quite distinct from the original A_1 receptor classification derived from work on the adipocytes. It was suggested that the A_3 receptor was present on excitable tissues and mediated inhibition of transmitter release from central and peripheral neurones. In 1992, Zhou and colleagues reported the cloning, expression and functional characterisation of a novel adenosine receptor, that exhibited 58% sequence identity with the rat A_1 and A_{2a} adenosine receptors. The cloned A_3 receptor now stands on solid ground because it is defined by sequence, by its link through pertussis toxin sensitive G protein to adenylate cyclase and by a distinct pharmacology. Moreover, evidence in support of a functional correlate of the site in the

cardiovascular system of the rat has recently appeared (Fozard et al 1993).

A novel A_4 subtype has also been proposed based on pharmacological and electrophysiological criteria. It may play an important role in responses to hypoxia by activating K^+ channels (reviewed in Dalziel et al 1994).

The use of selective and specific antagonists is necessary in order to fully classify a receptor. The first compounds identified as adenosine receptor antagonists were the naturally occurring xanthines, caffeine and theophylline. The utility of these is limited because they are weak and non-selective for A_1 or A_2 adenosine receptors and also inhibit cyclic nucleotide phosphodiesterase and calcium mobilisation (Van Galen et al 1992). Since then a multitude of xanthines have been synthesised and studied as antagonists at A_1 and A_2 receptors. One of the most potent antagonists for the A_1 receptor subtype is 1,3 dipropyl-8-cyclopentylxanthine (DPCPX), also known as CPX, and PD116,948 (Bruns et al 1987). The latter compound has greater than 20-fold increase in A_1 affinity and 6-fold increase in selectivity compared to the parent compound 8-cyclopentyltheophylline (CPT). Numerous structurally diverse non-xanthine antagonists have also been identified during the last decade. CGS 15943A, a member of the triazoloquinazoline family, originally synthesised as a benzodiazepene antagonist was unexpectedly found to have potent antagonist properties (Williams et al 1987). It was shown to have 7-fold selectivity for the A_2 receptor compared to the A_1 receptor and an IC_{50} of 3nM at the A_2 receptor (Francis et al 1988). Antagonist potency orders for the A_1 and A_2 subtypes are shown in table 1. In general adenosine antagonists are planar, aromatic (or having a high π electron density), nitrogen-containing heterocycles (Van Galen et al 1990).

1.5.2. The P2 receptor and receptor subtypes.

The design, synthesis and pharmacology of analogues of ATP, particularly by

Burnstock and Kennedy (1985) has provided clear evidence of at least four P2 receptor subtypes (Cusack et al 1985) which have been termed P2x, P2y, P2z and P2t. The excitatory P2x purinoceptor was found on visceral and vascular smooth muscle and sensory neurones: the inhibitory P2y purinoceptor on visceral and vascular smooth muscle, endothelial cells, hepatocytes, parotid acinar type II alveolar cells and pancreatic B cells; the P2z purinoceptor on mast cells, macrophages, lymphocytes and epithelial cells and P2t purinoceptor uniquely on blood platelets (Cusack et al 1990).

The purinoceptor subclassification has been postulated on the basis of the relative potencies of ATP analogues (and on the basis of selective antagonism). Burnstock and Kennedy proposed that P2 purinoceptors could be divided into 2 subtypes, P2x and P2y respectively, based on the relative order of potencies of ATP, $\alpha\beta$ -methylene ATP, $\beta\gamma$ -methylene ATP and 2-methylthio ATP as displayed in table 2. Cusack had also synthesised and examined the actions of a number of adenine nucleotides that have provided further evidence for P2x and P2y subclassification.

The rank order of potency of 2-Me-S-ATP \gg ATP $>$ $\alpha\beta$ -MeATP = $\beta\gamma$ -MeATP was designated to typify the P2y subtype. 2Me-S-ATP is 700-fold more potent than 2Me-L-ATP, at this purinoceptor, whereas the P2x subtype shows no selectivity towards the D- and L- enantiomers of ATP, ADP and 2-chloro-ATP. Based on the knowledge that $\beta\gamma$ methylene ATP is a potent agonist at the P2x purinoceptors, Cusack synthesised $\beta\gamma$ -Me-L-ATP which was later found to be the most potent agonist known at the P2x purinoceptor while having no activity at the P2y receptors. Adenosine 5,2 flurodiphosphate (ADP- β -F) may be a selective agonist at the P2y receptors (Hourani et al 1988).

A number of studies using phosphorothioate analogues of ATP (where an ionised oxygen on the phosphate chain of ATP is replaced by sulphur) also produced results consistent with the P2x/P2y subdivision. In the guinea-pig vas deferens and urinary bladder, contractions were evoked with a potency order ATP- γ -S = ATP- β -S $>$ ATP =

Table 2. Pharmacological classification of P2 purinergic receptors,

<u>Subtype</u>	<u>Agonist Selectivity</u>	<u>Antagonist(s)</u>	<u>Effectors</u>
P _{2t}	2-MeSADP > ATP	ATP	Intrinsic ion channel
P _{2x}	L-AMPP-C-P ≥ AMP-C-PP = ATP = ADP > AMP	Suramin	Intrinsic ion channel
P _{2z}	BzATP > ATP = ATP _γ S >>> ADP,AMP	2Me-S-L-ATP	Intrinsic channel/pore
P _{2y}	2-MeSATP = ADPβS > ATP = ADP >> AMP-C-PP = AMPP-C-P = UTP	Suramin	G protein activation/IP ₃ / DAG
P _{2u}	UTP ≥ ATP = ATP _γ S > ADP > 2-MeSATP > AMPP-C-P = AMP-C-PP	?	G protein activation/IP ₃ / DAG

2-MeSADP, 2-methylthio-ADP; 2-MeSATP, 2-methylthio-ATP; AMPP-C-P, βγ-methylene-ATP; AMP-C-PP, αβ-methylene-ATP; BzATP, benzoylbenzoic-ATP; ATP_γS, adenosine 5'-O-(3-thiotriphosphate); IP₃, inositol trisphosphate; DAG, diacylglycerol; ?, not characterised.

ATP- α -S (P2x receptor) but in the guinea-pig taenia coli relaxations were evoked with a potency order ATP- α -S > ATP- β -S = ATP- γ -S = ATP (P2y receptor) (Burnstock et al 1984a,1985).

Shortly after Burnstock and Kennedy proposed the P2x/P2y subdivision in 1985, Gordon (1986) also came to the conclusion that the P2 purinoceptor does not form a homogenous group. He proposed two further P2 purinoceptor subtypes. The P2t purinoceptor was proposed to mediate the aggregation of platelets in response to ADP whilst the P2z receptor was proposed to exist on mast cells where it mediates the degranulating effects of ATP (usually acting in the form of the ATP⁴⁻ anion).

A more definite characterisation of the P2 receptor continues to be hindered (except for the P2t receptor) by the lack of selective, competitive receptor antagonists. In 1985, only one specific antagonist for the P2 purinoceptor was available, arylazido-amino-propionyl ATP (ANAPP₃). ANAPP₃ is a structural analogue of ATP which is thought to form a specific covalent attachment to the P2 purinoceptor following irradiation with visible light. However, the irreversible nature of its action prevents the calculation of pA₂ values and so the comparison of the antagonist potency of ANAPP₃ in different tissues. ANAPP₃ was therefore of limited use in studying subtypes of the P2 purinoceptor. Older agents such as reactive blue 2 and the oligopeptide apamin are also unsuitable for this purpose.

Recently considerable interest has been generated by studies on the trypanocide suramin which shows competitive antagonist properties at the P2x purinoceptor in the guinea-pig vas deferens (Dunn et al 1988) and urinary bladder and also at the P2y purinoceptor in the guinea-pig taenia coli (Den Hartog et al 1989). However suramin is unlikely to assist receptor classification significantly since it demonstrates similar affinity for P2x and P2y subtypes.

1.5.3. Nucleotide Receptors.

There are also a variety of tissues where ATP activates phospholipase C but in which the potency order for the P2y purinoceptor does not apply. In these tissues 2-Me-S-ATP has also little or no activity. Examples include Ca^{2+} release from the human neutrophil (Walker et al 1991), inositol phosphate production in sheep pituitary cells (Davidson et al 1990) and prostacyclin (PGI_2) production from bovine aortic smooth muscle cells (Seifert et al 1989). This evidence suggests that there may be a subpopulation of phospholipase-C linked ATP receptors which cannot be correctly classified as P2y purinoceptors (see section 1.6.2). This argument is strengthened by the observation that UTP has similar agonist potency to ATP in many of the tissues that are also unresponsive to 2-Me-S-ATP. The equipotency of UTP and ATP means that "purinoceptor" is an inappropriate description for this putative subpopulation (which also responds to pyrimidine nucleotides). Indeed, this led Davidson and his colleagues to introduce the term "nucleotide" receptor for the ADP/UTP-sensitive site on sheep pituitary cells. The nucleotide receptor or P2u receptor may be characterised by the following potency order $\text{UTP} = \text{ATP} > \text{ADP} > \alpha\beta\text{MeATP}, 2\text{Me-ATP}$ (table 2). In addition, $\text{ATP}\gamma\text{S}$ appears to be a potent agonist at the nucleotide receptor. Observations that support this include the displacement of [^{35}S] $\text{ATP}\gamma\text{S}$ binding in heart sarcolemma by both ATP and UTP (Zhou et al 1990) and the ability of $\text{ATP}\gamma\text{S}$ to desensitise responses to both ATP and UTP in human neutrophils (Walker et al 1991).

Tissues like rat aorta may contain a heterogeneous population of receptor types, possibly P2y and nucleotide receptors (reviewed in O'Connor et al 1993). The agonist potency order for tissues with heterogeneous "mixed" receptor population proposed by O'Connor et al (1991) is $2\text{Me-S-ATP} > \text{ATP} = \text{UTP} = \text{ADP}$.

There is growing evidence to suggest that there are UTP-sensitive receptors

(pyrimidinoceptors) (Seifert et al 1989) which can be distinguished from ATP-recognising receptors on the basis of cross-desensitisation experiments in human neutrophils and HL-60 cells. Further studies are necessary to clarify the similarities and dissimilarities between pyrimidinoceptors and nucleotide receptors.

1.5.4. Molecular cloning of the Purinoceptors.

Adenosine receptors were one of the first G protein coupled orphan receptors, identified by a homology screening protocol whereby oligonucleotides were designed on the basis of similarities among genes that encode receptors with seven transmembrane helices (Linden et al 1991). Eight adenosine receptors have been cloned so far; the A_1 receptor from three different species, the A_{2a} from two species, the rat A_{2b} and the A_3 receptor from both rat and sheep.

The polymerase chain reaction (PCR) was used by Libert et al (1989) to clone the receptor RDC8, from a canine thyroid cDNA library, which was subsequently identified as that coding for the A_2 receptor. This cDNA fragment was further characterised as an A_{2a} receptor based on ligand binding and signal transduction criteria when expressed in Y1 mouse adrenal tumour cells, dog thyrocytes and in *Xenopus* oocytes (Maenhaut et al 1990, Schiffmann et al 1990). In situ hybridisation was also used to show that RCD8 had a tissue distribution similar to A_{2a} binding sites in rat brain.

A second cDNA fragment RDC7, with a high degree of homology to RDC8 was identified as the A_1 receptor in the canine species based on the binding of [3 H] cyclohexyladenosine and inhibition of adenylylase in transfected cells (Libert et al 1991). Clones for the A_1 receptor showing a high degree of homology with the corresponding canine receptor have also been detected in the rat (Reppert et al, Mahan et al 1991) and bovine species (Tucker et al, Olah et al 1992).

An adenosine receptor cloned using PCR in the rat testis by Meyerhof et al (1991) and referred to as TGPCR is clearly related to but different from the A₁ and A₂ receptors; Zhou et al (1992) re-cloned and further characterised the same receptor and designated it A₃. A homolog of the rat A₃ adenosine receptor (72% identical) has recently been cloned in sheep but unlike the rat, the sheep transcript is most abundant in the lung, spleen and the pineal gland (Linden et al 1993). It is important to note that these A₃ receptors are probably unrelated to the A₃ receptors proposed by Ribeiro et al (1986) on the basis of pharmacological criteria.

At least five subtypes of the P₂ purinoceptors have been proposed pharmacologically, with P_{2x} and P_{2y} purinoceptors the best described. However none have been characterised at the molecular level until recently. A novel subtype of the P_{2y} purinoceptor subtype designated P_{2y₁} (Webb et al 1993) from the brain of a chick and the P_{2u} (or nucleotide) receptor from mouse neuroblastoma cells (Lustig et al 1993) have recently been cloned. In each case, sequence analysis of the isolated clones revealed a novel member of the G-protein-coupled receptor (GCR) superfamily, that had only a low sequence identity with the adenosine receptor. Further characterisation was achieved by agonist potency orders and signal transduction studies similar to those performed for the adenosine receptors. The findings demonstrated that the pharmacological profile of the P₂ sites could be attributed to a single gene product. Cloning of the purinoceptors should provide a useful tool for examining the structure, function and expression of members of this receptor family.

1.6. SIGNAL TRANSDUCTION.

1.6.1. P1 Purinoceptors.

The most extensively studied effector system coupled to adenosine receptors (AR) is the adenylate cyclase system. In all tissues studied so far, A_1 receptors inhibit adenylate cyclase activity whereas A_2 receptors stimulate the activity of this enzyme. The A_2 AR's are still thought to couple only to adenylate cyclase. In contrast, A_1 AR's are promiscuous in that they couple to a variety of effector systems including adenylate cyclase, guanylate cyclase, potassium channels, calcium channels, chloride channels, phospholipase A_2 and C and the sodium-calcium exchange system.

The adenosine receptors regulate the adenylate cyclase indirectly by activating guanine nucleotide regulatory proteins (G proteins). A_2 AR's appear to act uniquely through G_s whereas the myriad of effector systems mediated by A_1 receptors would suggest variable coupling to different G proteins. Recent reconstitution studies (Freissmuth et al 1991) using purified A_1 AR's and recombinant G protein α subunits have demonstrated that A_1 receptors couple with highest affinity to α_{i3} and α_o which are pertussis-toxin sensitive (Munshi et al 1990).

A_1 AR acting through pertussis toxin-sensitive G_i proteins opens K^+ channels in cardiac tissue (Bohm et al 1986). Adenosine has subsequently been found to activate similar K^+ channels in other tissues including the brain (Schubert et al 1985). A_1 receptors are also coupled to another type of potassium channel which is activated by a fall in intracellular ATP and is referred to as the ATP-sensitive K^+ channel (Kirsch et al 1990).

A wealth of information exists on the ability of A_1 AR's to inhibit Ca^{2+} channel opening in neurones (Olsson et al 1990). Two distinct mechanisms are involved in this action; 1) an increase in K^+ conductance leading to hyperpolarisation of the membrane

and inhibition of calcium influx through voltage sensitive N-type channels and 2) the activation of the G protein directly inhibiting the Ca^{2+} channels (Olsson et al 1990). Subsequent activation of A_1 receptors (for example, in brain) produces presynaptic effects which inhibit the release of excitatory transmitters by these mechanisms.

Chloride channels have been found to be activated by adenosine in the brain and kidney. This activation may require diacylglycerol released from membrane phosphoinositides through activation of PL-C. More recently, the connection between adenosine receptors and accumulation of inositol phosphates has come to light in various tissues, where both stimulatory and inhibitory effects are described. In general, the effect of adenosine appears to be indirect in that it potentiates the activity of other neurotransmitters to activate PL-C. For instance, in guinea-pig brain slices, adenosine augments histamine-stimulated inositol phosphate accumulation. Other examples include the potentiation of the actions of noradrenaline in the spinal cord (Sawynok et al 1990) and A_{11} in the kidney (Deray et al 1990). In mouse brain, however, adenosine inhibits histamine-induced phosphoinositide turnover. Similar inhibitory actions have been reported in brown adipose tissue, frog ganglia (Rubio et al 1989) and pituitary cells (Enjalbert et al 1990). Evidence suggests that adenosine inhibits neurotransmitter release at the frog neuromuscular junction in part by inhibiting PL-C (Sebastiao et al 1990). These responses may act in part via G proteins coupled to inhibition of PL-C.

Additional inhibitory mechanisms of adenosine appear to operate at motor neurones by reducing intraneuronal Ca^{2+} mobilisation and by decreasing the activity of protein kinase C. However much more work needs to be performed before any true understanding of how adenosine influences inositol phosphate metabolism can be established.

1.6.2. P2 Purinoceptors.

Many of the diverse biological actions of extracellular ATP acting at the different P2 receptor subtypes, can be ascribed to or correlated with alterations in cellular Ca^{2+} homeostasis. The different mechanisms that mediate ATP/UTP/ADP induced changes in cytosolic $[\text{Ca}^{2+}]$ are summarised in figure 1.9 (Dubyak et al 1993). Two of the ATP selective receptors, P2y and P2u, function as G protein-coupled receptors, P2x and P2t receptors act as ligand gated ion channels and P2z receptors are associated with ATP-induced pore formation.

Most of the actions of ATP that result in a rise in cytoplasmic Ca^{2+} are a consequence of the hydrolysis of phosphatidyl inositol 4,5 bisphosphate (PIP_2) by PL-C, resulting in the generation of inositol (1,4,5) trisphosphate. It is now well established that the P2y and P2u purinoceptors mediate this effect via G proteins.

The coupling between typical P2y purinoceptors and PL-C has been demonstrated in hepatocytes, turkey erythrocytes and vascular endothelial cells. In turkey erythrocytes there is evidence that the P2y receptor is coupled to an $\alpha\beta\gamma$ heterotrimeric G protein (Boyer et al 1989). Regulation by G proteins has been characterised in numerous cell-free or permeabilised cell systems including membrane ghosts prepared by hypo-osmotic lysis of [^3H]-inositol labelled turkey erythrocytes (Harden et al 1988) and permeabilised endothelial cells (Brock et al 1988). G proteins associated with the P2y receptor tend to be pertussis toxin insensitive as described in aortic endothelial cells, human neutrophils (Cockcroft et al 1989) and FRTL-5 thyroid cells (Okajima et al 1988), where pretreatment of these cells with pertussis toxin partially inhibited accumulation of inositol phosphates in response to ATP and UTP but the action of 2-methylthio ATP was unaffected (Piroton et al 1987). A new class of heterotrimer G proteins, Gq/G_{11} , has been purified from turkey erythrocytes which mediates the activation of a form of PI-PLC (phospholipid-specific PLC)

FIGURE 1.9.

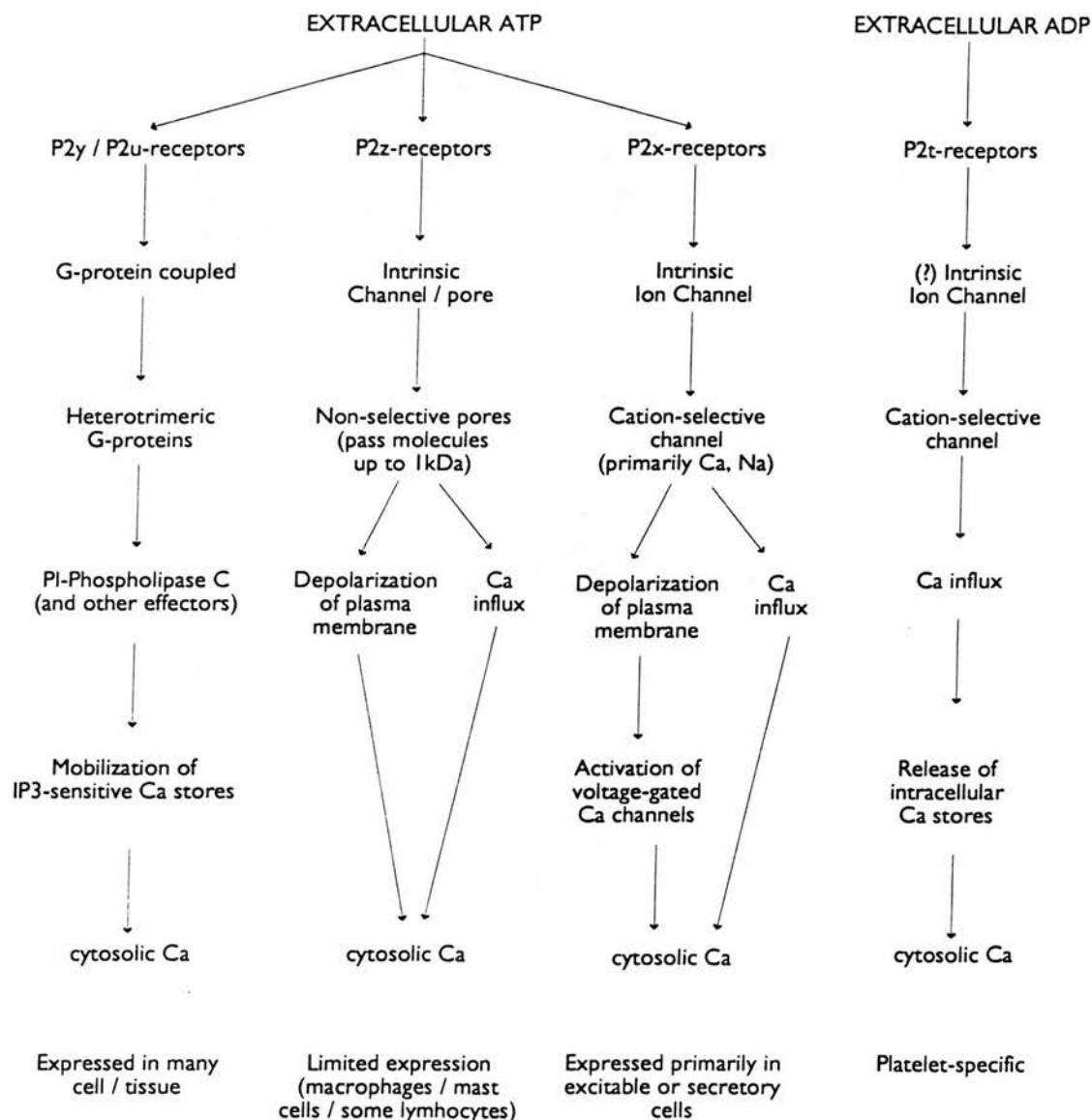


FIGURE 1.9.

Diverse mechanisms by which extracellular ATP and ADP can increase cytosolic Ca²⁺ concentration (Dubyak et al 1993).

(related to the mammalian PI-PLC β) by P2y purinoceptors. It appears likely that the P2y receptors present in mammalian cells trigger a similar Gq/PI-PLC β 1 signalling cascade (Dubyak et al 1993).

The P2u receptor, as described in pituitary cells (Davidson et al 1990), human fibroblasts (Fine et al 1989) and a wide variety of leukocytes (Dubyak et al 1990) is functionally similar to the P2y receptor class, in so far as its effects are mediated via PL-C dependent mobilisation of cytoplasmic free Ca^{2+} . G protein coupled P2u receptors have been expressed in *Xenopus* oocytes injected with mRNA from differentiated HL-60 granulocytes (Murphy et al 1990). This was followed by a report that both ATP and UTP activate Ca^{2+} mobilisation in the same expression system injected with mRNA from guinea-pig brain (Honore et al 1991). Unlike the P2y receptor, the G protein coupled to the P2u receptor appears to be capable of activating either pertussis toxin-sensitive (HL-60 and human fibroblasts) or insensitive pathways (NCB-20 cells) in a cell-type specific manner. This diversity indicates that in different cell types, different G proteins may mediate the signal transduction pathway of the P2u receptor.

In rat cardiac myocytes, ATP activates a Gs-type G protein coupled to a L-type Ca^{2+} channel, whilst in neurones and endothelial cells, K^+ channels are controlled by ATP (reviewed in Dubyak et al 1993). In contrast, in rat parotid acinar cells (McMillan et al 1988) and thymocytes (Lin et al 1985), ATP acts exclusively on Ca^{2+} influx by a mechanism which does not involve the hydrolysis of phosphoinositides.

Other phospholipase-based signal transduction systems are activated by P2y and P2u purinoceptor occupancy. These include phospholipases A_2 and D (Boeyneams et al 1990); the latter is involved in the selective hydrolysis of phosphatidylcholine (Piroton et al 1990), a mechanism which is dependent on protein kinase C and phosphatidylethanolamine in fibroblasts (Kiss et al 1990).

The other major effector system that has been characterised in some detail is the

ATP-activated cation conductance pathway in the plasma membranes of visceral and vascular smooth muscle cells, sensory neurones and platelets. P2x and P2t are the two purinoceptor classes linked to this signal transduction mechanism. Patch clamp electrophysiological recordings of both whole cell currents and single channel currents have facilitated the identification of P2x receptors that act on ligand-gated ion channels in excitatory cells such as neurones and muscle (Dubyak et al 1993). Activation of the P2x receptor in most cell types is associated with a direct influx of extracellular Ca^{2+} and the depolarisation of the plasma membrane as a result of sodium acting as the predominant charge carrier. This is followed by a secondary activation of voltage-dependent Ca^{2+} channels leading to an increase in cytosolic Ca^{2+} . These ligand-gated ion channels are very characteristic of excitatory neurotransmitter-gated channels such as the nicotinic acetylcholine receptor and thus may be important in mediating fast excitatory neurotransmission at various synapses.

The P2t purinoceptor, present exclusively on platelets, is also believed to be a ligand-gated ion channel receptor. Activation results in an initial wave of Ca^{2+} influx followed by the release of intracellular Ca^{2+} stores. This triggers a second wave of Ca^{2+} influx which is responsible for the rapid change in shape of the platelets before aggregation. This ADP-stimulated ion channel is also permeable to Na^+ , K^+ and Ba^{2+} .

Finally, Rozengurt et al (1977) reported that extracellular ATP was responsible for a change in the membrane permeability of certain transformed murine fibroblasts. ATP pore-forming receptors, later classified as P2z purinoceptors, were reported to be responsible for these phenomena and found to be expressed in a limited number of cell types, namely macrophages, mast cells and fibroblasts. These receptors exhibited little selectivity for cations over anions or for monovalent cations over divalent cations (Dubyak et al 1993).

The wide-ranging signal transduction mechanisms associated with the P2

purinoceptors may explain how a small molecule such as ATP can produce a wide variety of biological responses in many tissues and cells.

1.7. SOURCES AND FATE OF PURINES.

The first source of ATP was probably cell lysis. External receptors for ATP may have originally evolved in single-celled organisms as a means of chemotaxis towards potential food sources. Crustaceans, for example, can detect adenine nucleotides in the surrounding sea-water via receptors located on their antennae (Carr et al 1986).

ATP is a ubiquitous intracellular constituent being present in most cells at a cytosolic concentration of 3-5mM. Therefore any cell on sudden breakage could potentially serve as a source of extracellular ATP, as may occur during tissue trauma. Extracellular levels of the nucleotide are normally maintained at extremely low levels due to the actions of ectophosphatases.

Two additional sources of extracellular ATP exist, 1) the exocytotic release of ATP from storage granules or vesicles and 2) the release of cytosolic ATP via intrinsic plasma membrane channels or pores (Dubyak et al 1993).

Exocytotic release of ATP occurs from storage granules in non-neuronal cells and neuronal cells. The former has been studied in the most detail and includes cells such as platelets, adrenal chromaffin cells, mast cells and basophilic leukocytes. The "dense granules" in blood platelets are packed with amines and nucleotides. Both ATP and ADP are contained in platelet storage granules at approximately 40nmol per mg of protein (Da Prada et al 1978) and can be released on platelet aggregation. The serum concentration of ATP and/or ADP can be transiently raised as high as 50 μ M on degranulation. ATP is also packaged with catecholamines and enkephalins in adrenal chromaffin granules representing 15% of the dry weight of adrenal granules (Hillarp et al 1959) and thus may

contribute to the local release of ATP with potential effects on both the cortex and medulla.

ATP is present in all neural tissue and is released from storage vesicles during nerve stimulation of many tissues including: rabbit ear artery (Holton et al 1959) and nerve fibres in the gastrointestinal tract (Burnstock et al 1979). Non-adrenergic, non-cholinergic nerves associated with the smooth muscle in the gastrointestinal tract and elsewhere release ATP as a neurotransmitter. Burnstock (1971) thus proposed that these nerve terminals were "purinergic". ATP is packaged and released as a co-transmitter with noradrenaline from sympathetic nerves supplying the vas deferens, aorta, smooth muscle and some blood vessels. There is similar evidence that ATP is released with acetylcholine in certain types of smooth muscle and at both somatic and autonomic motor nerve endings.

The second source of cytoplasmic ATP release is mediated by specific transporter proteins and is not due to cell lysis. ATP is released during the activation of cells by physiological or pathological stimuli, as described by Forrester (1990) in isolated cardiac myocytes that have been metabolically compromised by hypoxia. The multi-drug resistant (mdr) gene product, P-glycoprotein has been proposed by Abraham et al (1993) as an ATP channel in Chinese hamster ovary cells and various other cell lines.

These various sources of released ATP, suggest that significant concentrations of this purine accumulates locally at important physiological extracellular sites producing its diverse effects via specific cell surface receptors.

Extracellular ATP is rapidly catabolised by a variety of extracellular ATPases and nucleotidases. These enzymes have a widespread distribution amongst tissues and isolated cells. There are apparently three separate enzymes that sequentially catabolise ATP → ADP → AMP → Adenosine and these were classified as ecto-triphosphatase, ecto-diphosphatase and 5'nucleotidase. The last enzyme catalyses the hydrolysis of AMP to

adenosine and is inhibited by both ATP and ADP.

These enzymes play at least two major roles in cells. Firstly they may be important in termination of ATP/ADP induced signal transduction. Different tissues will express on their cell surfaces ectonucleotidases with different activities depending on the sensitivities of these cells to sudden changes in purine concentration. For example, endothelial cells in the vasculature express very active ectoenzymes resulting in ATP having a very short half-life. This may be important in limiting platelet activation and thrombus formation at the site of local tissue damage (Slakely et al 1990). Sudden changes in the extracellular concentration of ATP may result in secondary tissue damage. Secondly these ectoenzymes catalyse the generation of adenosine which on re-uptake into the cells will replete intracellular purine nucleotide and nucleoside resources. The ubiquity and efficiency of these ectonucleotidases is central to the regulatory role played by extracellular ATP in all tissues and cells.

Adenosine is present in every cell, therefore like ATP, any cell could serve as a source of extracellular adenosine. It is produced intracellularly via two distinct metabolic pathways. The first requires the hydrolysis of AMP by an intracellular 5'nucleotidase. The second depends upon catabolism of S-adenosyl homocysteine (SAH). Once produced in the cell, its concentration is maintained below $1\mu\text{M}$ by several enzymes. These can bring about the deamination of adenosine to inosine (adenosine deaminase), the phosphorylation of adenosine to 5'AMP (adenosine kinase) and finally the coupling to SAH (SAH hydroxylase) (Spielman et al 1991).

Adenosine may also arise from the metabolism of released ATP, which is rapidly broken down by ectonucleotidases, for example, at nerve endings. This source of adenosine is probably only important in specialised circumstances, for example, at synapses or sites of injury, whereas the adenosine generated intracellularly predominates at a more macroscopic level.

Once produced adenosine can pass through the cell membrane by facilitated diffusion, a concentration-dependent process. Physiological stimuli that lower the energy charge (eg hypoxia, ischaemia and exercise), greatly increase the production and release of adenosine from cells. Extracellular adenosine, like ATP, has a very short half-life (sub-minutes) *in vivo* due to efficient re-uptake by the nucleoside transporter and extracellular deamination. All these various processes regulate the local production of adenosine and its extracellular concentration for signalling roles in tissues and cells.

1.8. ROLE OF PURINES IN THE ADRENAL.

Biological responses to ATP and adenosine have been documented in virtually every major organ and/or tissue system that has been studied. Subsequently, the receptors for purine nucleotides and nucleosides have been extensively studied and classified into their different subtypes.

The adrenal itself has received very little attention. Chromaffin granules release high concentrations of ATP (amounting to 10-20% of total cellular ATP) (Rojas et al 1985) and adenosine along with the catecholamines which may inhibit (Chern et al 1987) or excite (Chern et al 1988) the further release of catecholamines in a feedback manner. Chern et al (1988) reported the presence of an A₂ receptor on chromaffin cells that on activation by adenosine, enhances catecholamine secretion through a rise in adenylate cyclase activity. An "atypical" P_{2y} purinoceptor has also been reported to be present in the adrenal medulla (Allsup et al 1990). The agonist potency order for this receptor was inconsistent with that reported for a typical P_{2y} or P_{2x} purinoceptor, even though it was linked to phosphoinositide hydrolysis (Sasakawa et al 1989, Allsup et al 1990).

Due to the close proximity of chromaffin cells to the vascular endothelium, it would seem likely that ATP and adenosine released from the medullary cells, bathe the adjacent

endothelial cells at high concentrations. Forsberg et al (1987), reported that ATP acts on adrenal medullary endothelial cells via a P₂ purinergic receptor to activate both inositol phosphate production and prostacyclin release. Prostacyclin released by the action of ATP promotes vasodilation in the medulla while adenosine is known to cause the relaxation of blood vessels, primarily through A₂ receptors (Luty et al 1989), thus controlling the delivery of hormones to areas such as the adrenal cortex. Both ATP and adenosine may thus regulate local blood flow.

In the case of the adrenal cortex, most of the work has concentrated on the effect of purines on steroidogenesis and to date the receptors for ATP or adenosine have not been classified. Kowal et al (1969) was the first to report that ATP, ADP, AMP and adenosine stimulated steroidogenesis in monolayer cultures of adrenal cells derived from transplantable murine adrenal tumours. The pyrimidine UTP gave a partial response. This work was verified by Wolff et al (1977) who described similar responses to purines in Y1 adrenal tumour cells from mice. In the adrenal tumour cells, adenosine appears to stimulate adenylate cyclase at an external site in a manner that is antagonised by theophylline. Contrary to these results, Cooper et al (1978) showed that in normal rat adrenocortical cells both adenosine and theophylline had no effect on basal steroidogenesis and therefore argued against a direct action of the nucleoside on adenylate cyclase activity. However both compounds enhanced ACTH-stimulation of steroid production, possibly by their inhibition of cAMP phosphodiesterase (Schoenbaum et al 1959). In capsulated and decapsulated rat adrenocortical membranes, experiments suggested the presence of A₁ receptor sites (Cooper et al 1978) whereas in the adrenal tumours, inhibition of adenylate cyclase activity by theophylline would support the presence of the external stimulatory A₂ receptor for the action of adenosine. Further investigation may identify the differences between normal and tumour cells which results in such divergent actions of adenosine in these two cell types.

1.9. AIMS OF THESIS.

- 1) To examine the effects of purines on the secretion of cortisol in bovine zfr cells in primary culture and to characterise this response.
- 2) To classify the purinoceptor subtype(s) in these cells using sub-type-selective agonists and antagonists and second messenger studies.
- 3) To investigate the acute interaction of purines with other regulatory hormones in the adrenal cortex, namely ACTH, All and Acetylcholine.
- 4) To establish a method for the identification and quantification of endogenous steroids secreted by bovine zfr cells in culture and subsequently to investigate the acute and chronic effects of ATP on the regulation of the steroidogenic pathway and to compare the findings with both ACTH and All.

CHAPTER 2

MATERIALS AND METHODS

2.1. SOURCE OF MATERIALS

Aldrich Chemical Co Ltd, Gillingham, Dorset.

1,1,2-trichlorotrifluoroethane; Tri-n-octylamine.

Amersham International, Aylesbury, Bucks.

Cortisol tracer (cortisol-3-(O-carboxymethyl) oximino-(2-[¹²⁵I] iodohistamine); ³[H]-myo-inositol; ³[H] inositol polyphosphate marker set.

BDH, Thornlibank, Glasgow, Strathclyde.

Acetic anhydride; Charcoal; Cyclohexane; Dimethylchlorosilane; Ethyl acetate; Pyridine; Dimethyl Sulphoxide (DMSO); Gelatin; Triethylamine; Triton X-100.

Bio Rad Laboratories, Watford, Herts.

AG1-X8 anion exchange resin; Biogel P2.

Boehringer Mannheim, Lewes, East Sussex.

Adenosine; Adenosine 5' monophosphate; Adenosine 5' diphosphate; Adenosine 5' triphosphate; Ethylenediamine tetraacetic acid (EDTA); Uridine 5' triphosphate; Fura-2 AM.

Canberra Packard, Pangbourne, Berks.

Lipidex 5000; Scintillation Fluid; Scintillation Vials.

CIBA Laboratories, Horsham.

ACTH (Synacthen).

Flow Laboratories, Rickmansworth.

Amphotericin B; Glutamine; Penicillin; Streptomycin.

Henry Simon, Stockport, Cheshire.

Nylon Gauze.

ICN Biomedial, High Wycombe, Bucks.

Bovine Serum Albumin (Fraction V); 8-Cyclopentyl dimethylxanthine;

8-Cyclopentyl dipropylxanthine; 5'-N-(ethylcarboxamido)adenosine.

Lorne Diagnostics, Bury St. Edmonds.

Collagenase (Type 1).

Millipore (UK) Ltd, Watford, Herts.

Sep-pak C₁₈ cartridges.

Northumbria Biologicals Ltd, Cramlington, Northumberland.

Earle's Balanced Salts; Hams' F-10.

National Institute for Biological Standards & Control, Potters Bar, Herts.

Angiotensin II (Asp¹ - Val⁵) (WHO Standard 64/15).

Pierce & Warriner, 44 Upper Northgate St, Chester.

Hexamethyldisilazane; Methoxyamine Hydrochloride; Trimethylsilylimidazole.

Pharmacia, Milton Keynes.

Sephadex G-10 (40 - 120 μm); Sephadex G-50 (100 - 300 μm).

Scottish Antibody Production Unit, Carluke, Strathclyde.

Antiserum to cortisol (Product code: s004-201).

Semat Technical (UK) Ltd, St. Albans, Herts.

2-Methylthioadenosine triphosphate.

Sigma Chemical Co, Poole, Dorset.

αβ-Methylene ATP; βγ-Methylene ATP; Citric acid; Cyclopentyladenosine;

CPSR-5; Digitonin; Ethyleneglycol tetraacetic acid (EGTA); Formic Acid (Ammonium salt);

Glucose; Hepes; Hydrocortisone; Inositol; Lithium chloride; Sodium azide; Steroids.

The following were obtained from non-commercial sources:

Adenosine 3',5'-cyclic phosphoric acid 2'-O-succinyl-3 [¹²⁵I] iodotyrosine:

Dr. B.C. Williams, Department of Medicine, Western General Hospital,
Edinburgh, Scotland.

CGS-15943A:

Dr. R.A. Lovell, Ciba-Geigy Corp, 556 Morris Ave, Summit, NJ 07901, U.S.A.

Suramin:

Bayer U.K. Ltd, Bayer House, Strawberry Hill, Newbury, Berkshire, England.

All other chemicals were obtained from BDH.

2.2. METHODS

2.2.1. Cell Isolation and Culture.

Bovine adrenocortical zfr cells were isolated and purified from adrenal glands obtained from freshly slaughtered steers (age range 18 months to 2 years, Gorgie Abattoir), according to the method of Williams et al (1989). The glands were collected into Earle's balanced salt solution (EBS) and transported within 30 minutes to the laboratory on ice. All further procedures were carried out aseptically (in a class II laminar flow cabinet, MDH Ltd).

After removal of the surrounding fat with scissors, separation of the zones of the cortex was performed using a Stadie-Riggs microtome which sectioned the tissue into 100 μ m slices. The first slice consisting of capsule, zg and the outer part of the zf was discarded. The subsequent slice comprising of zf/zr tissue with no visible contamination by the adrenal medulla was collected into EBS containing 0.2% (w/v) bovine serum albumin (BSA). 3.0 - 4.0g of zfr tissue were routinely collected for each primary cell isolation. The slices were finely chopped into 1-2 mm fragments, washed thoroughly with EBS/0.2% BSA and digested for 2 hours, at 37°C, in a solution consisting of EBS/2% (w/v) BSA with 2mg/ml of collagenase type I. At 30 minute intervals, the tissue was dispersed mechanically by vigorous shaking. After incubation, the dispersed cells were separated from any undigested material by filtration through a 250 μ m mesh nylon gauze. The cells were then harvested at 450g for 30 minutes, resuspended in EBS/BSA and filtered through a 100 μ m mesh nylon gauze followed by a 30 μ m mesh nylon gauze to remove large aggregates of cells.

Further purification of the zfr cells was achieved using the column filtration method of M^CDougall et al (1979). The cell suspension was applied to a scintered glass Allihn funnel (100mm \times 20mm disc / 16-40 μ m pore size) containing 15ml Sephadex G10 (40-

120 μ m bead size) and 5ml Sephadex G50 (100-300 μ m bead size) layered on top. Following equilibration of the column with EBS/BSA, gentle suction with a syringe was used to pull the cell suspension through the column which was then washed with approximately 20ml of EBS/BSA to remove red blood cells, cell debris and small damaged cells.

The zfr cells were harvested from the column by resuspending the Sephadex and trapped cells in EBS/BSA and filtering through a 30 μ m mesh nylon gauze. This allowed the zfr cells but not the Sephadex beads to pass through. The cell suspension was centrifuged at 450g for 30 minutes and resuspended in 50ml of growth medium (Ham's F-10 medium supplemented with 10% CPSR-5, penicillin (50IU/ml), streptomycin (50 μ g/ml) and amphotericin B (2.5 μ g/ml)). Cells were counted using an improved Neubauer haemocytometer.

Freshly-isolated cells were either used immediately or plated out at a density of 330,000 cells/ml in 12 well plates. In some experiments, the cells were seeded in 75 cm³ flasks at 10⁷ cells / flask (15ml). In all cases, the cells were cultured at 37°C in an atmosphere of 5% CO₂ and 100% humidity. After 24h (day 2), the medium was removed and replaced by 0.5ml (15ml for 75 cm³ flasks) of fresh medium and the cells used between the day of plating (day 1) and day 5.

2.2.2 Stimulation of Cortisol Secretion

(i) Freshly-isolated cells.

Freshly-isolated cells (in growth medium) were centrifuged at 450g for 20 minutes and the pellet resuspended in EBS with added BSA (0.2% w/v) and glucose (0.1% w/v) (EBSBG) at a density of 330,000 cells/ml. The centrifugation step was repeated and the cells resuspended in EBSBG. Stimulation was carried out in 3ml plastic tubes (LP4)

containing 330,000 cells in 0.45ml EBSBG. Following preincubation at 37°C for 5-10 minutes; agonists made up in the same EBSBG solution were added to give a final volume of 0.5ml and stimulation was allowed to proceed for 60 minutes. Incubations were terminated by placing the tubes on ice, before the cells were pelleted by centrifugation at 500g for 3 minutes in a Heraeus Christ Labofuge B. The supernatants were decanted and stored at -20°C prior to cortisol radioimmunoassay.

(ii) Cultured Cells.

To study the effects of agonists and antagonists on cortisol secretion, growth medium was removed from each well and the cells were washed twice with 1ml EBS. Cells were then incubated (5 min) in 0.45ml EBSBG/well. Agonists and antagonists were added (50µl/well) to the appropriate final concentration and the cells incubated at 37°C in 5% CO₂ for 60 minutes (or other times as stated in the Results). At the end of the incubation, the EBSBG was aspirated from each well and stored at -20°C prior to assay for cortisol.

2.2.3. Stimulation of cAMP.

Cells were washed in EBS and agonist incubations set up in EBSBG, exactly as described above (Section 2.2.2 (ii)) for cortisol measurements. After 1 min in the absence (basal) or presence of agonist, the overlying EBSBG was rapidly removed and replaced by 500µl of ice-cold 75% ethanol. Plates were left for at least 5 mins before the base of each well was scraped using the rubber tip from the plunger of a 1ml syringe and the contents transferred to plastic tubes. A further 500µl of 75% ethanol was added to each well and the washings combined. The extracts were centrifuged for 15 mins at 450g in a Heraeus Christ Labofuge B. The supernatant was decanted into a glass tube and the

ethanol evaporated under a stream of air while being warmed simultaneously to 37°C on a heating block (Techne Dri-block : DB-3 fitted with a sample concentrator : SC-3 Jencons). This takes about 1-2 hours. The dried pellets were reconstituted in 500µl of 50mM acetate buffer pH 5.0 and acetylated immediately. This was carried out as follows: a 2:1 (v/v) mix of triethylamine and acetic-anhydride was prepared in a glass tube and 15µl added to each sample tube. The contents of the tube were mixed immediately and stored at -20°C prior to radioimmunoassay of cyclic AMP.

2.2.4. Stimulation of Phospholipase C.

Activation of a hormone-sensitive phospholipase C results in the rapid catabolism of the polyphosphoinositides to form the two second messengers inositol 1,4,5 trisphosphate (Ins (1,4,5) P₃) and diacylglycerol (DAG). The inositol (1,4,5) trisphosphate in turn can be further metabolised via a complex pathway to other inositol phosphate isomers of the general formula IP_n (n = 1,2,3,4,5 or 6); all of which are water soluble.

Cells in culture are pre-labelled with [³H]-inositol which then becomes incorporated into the membrane inositol lipids through basal turnover. Phospholipase-C activation was determined by measuring the agonist stimulated production of the water soluble [³H] phosphoinositols resulting from the turnover of the phosphatidylinositol lipids.

(i) Cell labelling with [³H]-Inositol.

When studies of phosphoinositide metabolism were performed, bovine zfr cells were prepared and plated out as described before in Section 2.2.1. The cells were incubated for 24h and the overlying culture medium replaced with fresh medium supplemented with myo [³H] inositol (10µCi/ml). Cells were incubated at 37°C for a minimum of 42h to allow membrane phosphoinositides to be labelled to isotopic steady

state. Measurement of the ^3H -total phosphoinositol head groups was carried out on day 4 cells.

(ii) Agonist stimulation of Phospholipase-C.

On day 4 of cell culture, the labelled medium was removed, replaced with EBSBG (0.5ml/well) and the cells were incubated for 15 mins at 37°C. Medium was again removed, replaced with 0.45ml of EBSBG/LiCl (10mM)/inositol (10mM) and the incubation continued for a further 15 mins.

Agonists and/or antagonists were added (50 μl /well) and stimulation allowed to proceed for 15 mins (unless indicated otherwise), after which stimulation was terminated by the rapid addition of 250 μl /well of ice-cold 15% perchloric acid. The cells were scraped from each well with the rubber end on a plunger from a 1ml syringe and the total acid lysate was transferred (with 0.5ml of water wash from each well) to a 1.5ml Eppendorf tube. Following centrifugation at 3000g (3min), the aqueous radiolabelled-containing supernatant was transferred to glass tubes for deacidification by freon octylamine and neutralisation of perchloric acid. This was achieved by the addition of 1.5ml of freshly prepared 1:1 (v/v) 1,1,2-trichloro-trifluoroethane ("freon") and tri-n-octylamine to each sample tube. After brief centrifugation to separate the three phases, 0.9ml of the top phase (containing the neutral, aqueous radiolabelled products) was recovered and stored at -20°C prior to [^3H] phosphoinositol assay.

2.2.5. Measurement of intracellular calcium.

The cells were prepared as described above in Section 2.2.1 and cultured in 75 cm^3 flasks (10^7 cells / flask), changing the medium at 24h. Measurements were carried out between 48-72h after initial isolation.

The cells were detached from the flasks by removing the overlying medium, washing twice with Ca^{2+} -free EBS containing 1mM EDTA and then incubating the cells at 37°C in 5ml of the same buffer until the cells could be detached mechanically by shaking (generally after 10-15 min). Detachment of cells using trypsin was avoided to limit any damage to the purinoceptor. Detached cells were collected into Ca^{2+} -free EBS, centrifuged and washed in 50ml of modified Krebs-Ringer buffer (MKB)(pH 7.4) containing (mM): NaCl 136; KCl 1.8; KH_2PO_4 1.2; MgSO_4 1.2; NaHCO_3 5.0; CaCl_2 1.2; EGTA 0.21; glucose 5.5; HEPES 20.0 containing 0.5% BSA and 0.1% added glucose. After a further centrifugation step, the cells were finally resuspended in the same buffer at a density of 5×10^6 cells/ml and left at 37°C for 60 min to allow intracellular stores of Ca^{2+} to be replenished. Fura-2 AM ($15\mu\text{l}$ per tube; final concentration of $15\mu\text{M}$) or vehicle (DMSO) was added to the overlying cell suspension and cells left for 45 min (optimal) at 37°C . Following loading, 1ml portions of the fura-2 loaded cells were centrifuged at 450g for 2 min in Eppendorf tubes and the pellet resuspended in 2ml of MKB (without BSA) to give a final cell density of 2.5×10^6 cells/ml. The cell suspension was then transferred to a quartz cuvette (1cm path length) and fluorescence measurements were carried out at a single excitation wavelength of 362nm, recording the fluorescence signal at the fura-2 emission wavelength of 505nm on a Perkin-Elmer model LS-5 luminescence spectrophotometer with continuous stirring at 37°C , using a magnetic microstirrer. The fluorescence values were corrected for autofluorescence obtained by cells loaded with DMSO only. The intracellular concentration of calcium was determined using the following equation: $[\text{Ca}^{2+}]_i = [(F - F_{\min}) / (F_{\max} - F)] \times K_d$ where the dissociation constant (K_d) for fura-2 is 224nM. F_{\min} is the fluorescence of fura-2 in the absence of Ca^{2+} (by the addition of 0.4M EGTA to remove extracellular Ca^{2+}), F_{\max} the fluorescence of fura-2 when saturated with Ca^{2+} (by the addition of 100mM CaCl_2 until no further increase in fluorescence was observed) and F is the resultant fluorescence (Grynkiewicz et al 1985).

2.2.6. Assay Procedures.

(i) Cortisol RIA

Cortisol production by adrenocortical cells in culture was measured using an "in-house" radioimmunoassay described in Gray et al (1983) with modifications as described by Walker et al (1988). The assay employed a double antibody preprecipitate to separate bound and free tracer.

Cortisol standards (1 to 2000 nM) were prepared by dilution of stock cortisol (10mM) in EBSBG and stored at -20°C. Three quality control samples (10, 80 and 800 nM respectively) were prepared to check for intra-assay drift and inter-assay precision. Cortisol tracer (cortisol-3-(O-carboxymethyl) oximino-(2-[¹²⁵I] iodohistamine) in methanol:water (9:1) was diluted in 0.1M citrate buffer (0.2% (w/v) gelatine, pH 4.0) to 10⁴ cpm/700µl. Antibody preprecipitate solution contained: 650µl reconstituted sheep anti-cortisol, 500µl normal sheep serum, 15 ml donkey anti-sheep (goat), 10 ml 0.1M citrate buffer and was left overnight at 4°C to equilibrate. The suspension was centrifuged, at 230g for 15 min and the pellet reconstituted in a total volume of 500ml 0.1M citrate buffer.

The assay was set up with a Clinicon Dilutrend automatic diluter. Each assay tube contained 700µl tracer, 100µl standard/QC/sample and 250µl antibody preprecipitate. The contents were mixed on a multivortex and then incubated at 37°C for 2h. Following centrifugation at 1800g for 30 min, the supernatant was decanted and the pellets containing the bound fraction counted for 120 seconds in a gamma counter. Standard curves were fitted to a 4 parameter logistic model using the software package RIACALC (LKB/Pharmacia) on an IBM PC. The intra-assay drift was < 10% over the range 20-2000nM. The inter-assay precision was calculated to be < 10% for each of the QC's (10nM, 80nM and 800nM) used. Figure 2.1 shows an example of a standard curve

FIGURE 2.1.

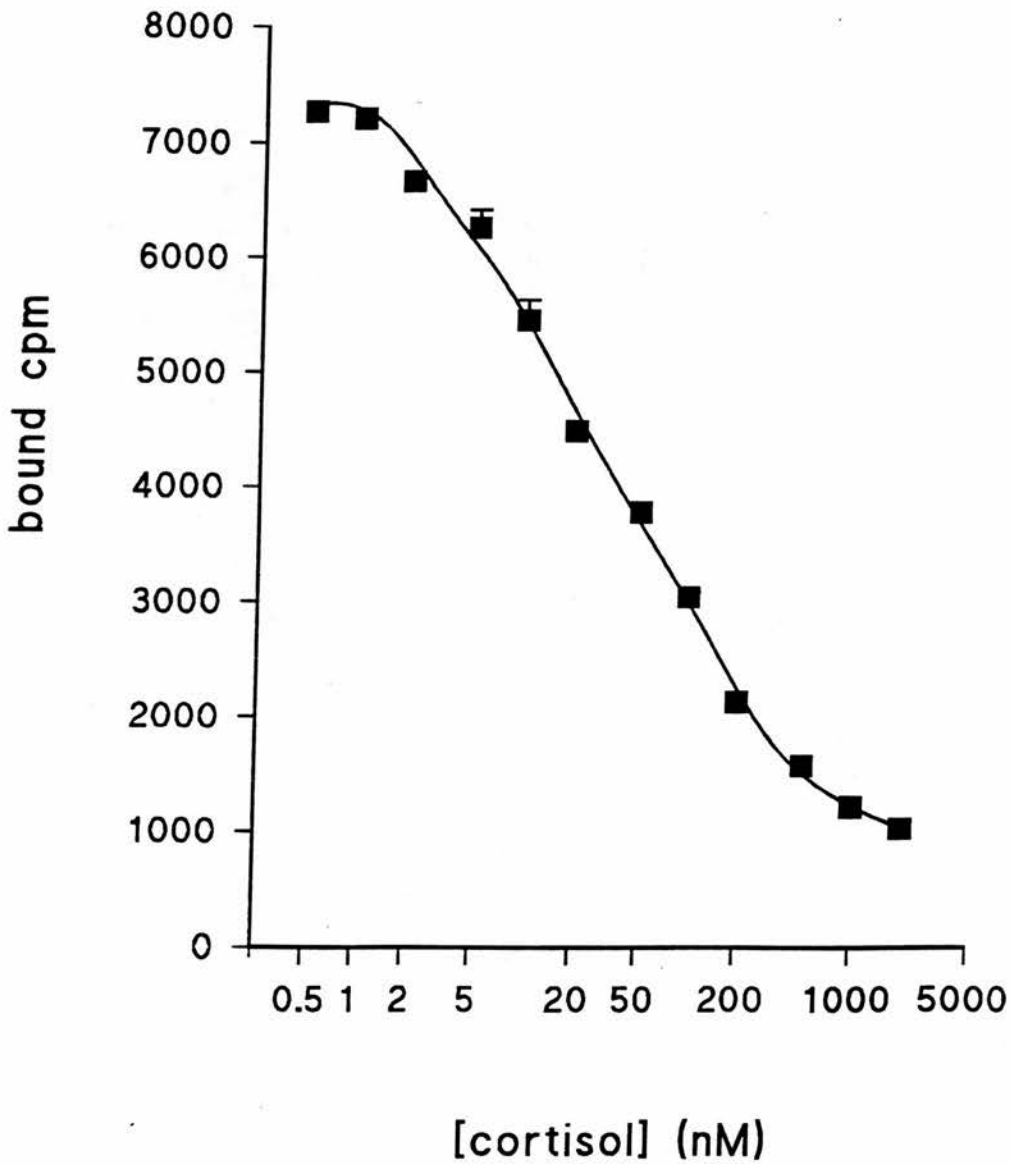


FIGURE 2.1.

Representative standard curve for the ^{125}I radioimmunoassay of cortisol. Standard solutions of cortisol (in EBS/BSA/glucose, 1-2000nM) were assayed in duplicate and the inhibition curve fitted to a four parameter logistic equation. Label binding in the absence of cortisol was typically 60-65% of total.

obtained by this assay.

(ii) cAMP RIA.

The cAMP assay was carried out essentially as described by Harper and Brooker (1975) involving an overnight incubation of the standard/sample with the cAMP antibody and label followed by separation of bound and free cAMP using activated charcoal on the following day.

cAMP standards (0.0625-16nM) were prepared by dilution of a stock solution of 32 μ M of the sodium salt of cAMP in 50mM acetate buffer pH 5.0 and stored at -20°C until required. Pre-acetylated standards, QC's and sample (50 μ l) were added to 250 μ l of antibody/label mix and incubated overnight at 37°C (Antibody/label mix consisted of 50mM acetate buffer, 0.1% BSA, pH 5.0 containing anti-cAMP antibody (1/1000) and tracer (adenosine 3', 5'-cyclic phosphoric acid 2'-O-succinyl-3 [¹²⁵I] iodotyrosine, 10⁴ cpm/tube).

The charcoal mixture used to separate bound from free cAMP consisted of 0.6% charcoal, 0.06% dextran T-70 and 0.03% gelatin in 50mM phosphate buffer pH 7.4. 0.7ml of activated charcoal/buffer was added to each assay tube at 4°C and the tubes centrifuged for 30 minutes at 1720g at 4°C. The supernatant was decanted and the pellets containing the free fraction counted for 200s in an LKB/Pharmacia 1261 multigamma counter. The standard curve was constructed using a smooth spline fit (LKB/Pharmacia RIACALC package). Intra-assay % CV values were < 15% over the working range 0.0625-10nM and values were judged to be acceptable within these limits. Figure 2.2 shows a representative cAMP RIA standard curve.

(iii) Total [³H] phosphoinositol assay.

Total aqueous radiolabelled phosphoinositol (total head groups) products were

FIGURE 2.2.

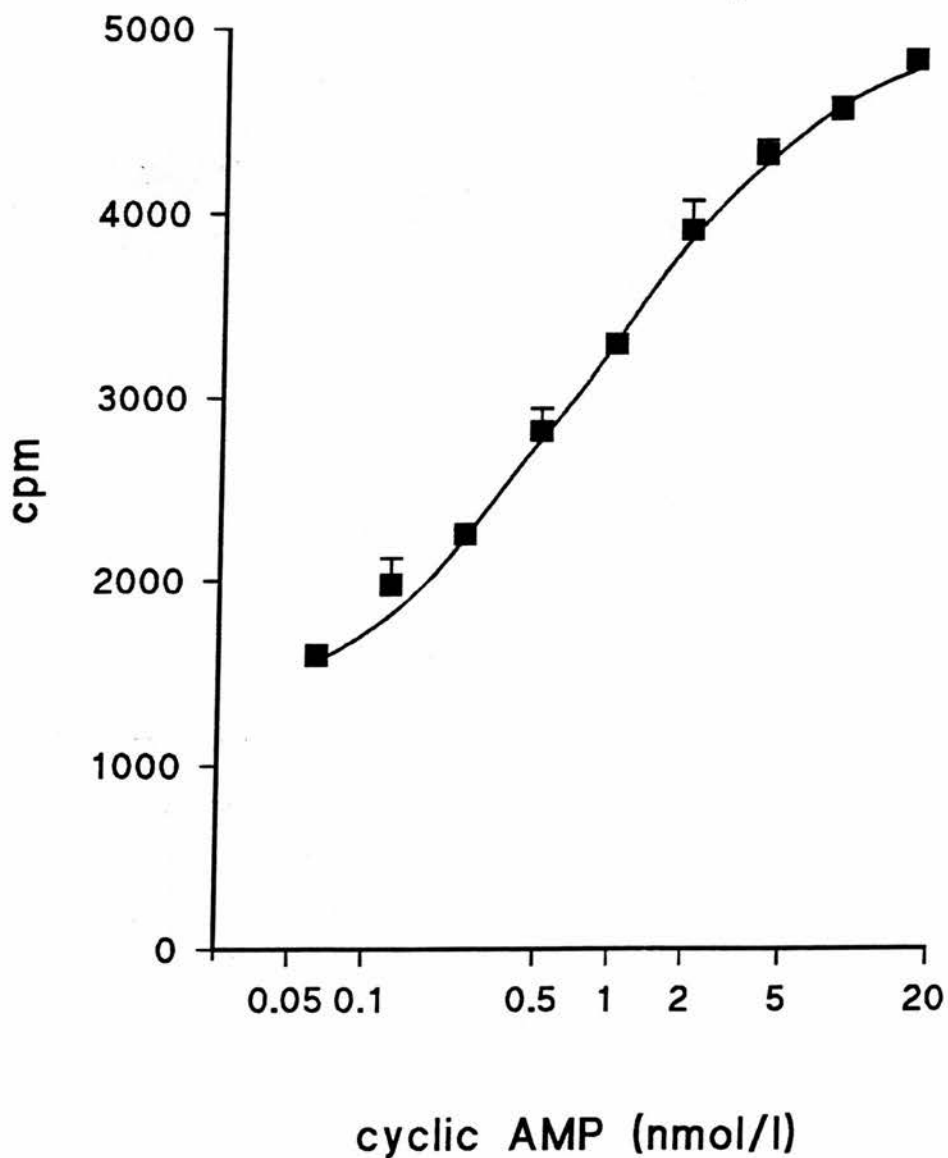


FIGURE 2.2.

Representative standard curve for cAMP radioimmunoassay. Standard solutions of the sodium salt of cAMP (in 50mM acetate buffer, pH 5.0, 0.0625-32nM) were assayed in duplicate and a smooth spline curve fitted. Label binding in the absence of cAMP was typically 55-65% of total.

analysed by making the neutral sample obtained from each culture well 1mM with respect to EDTA. Samples were loaded onto individual columns of AGI-X8 anion exchange resin (0.25ml) and the columns washed twice with 4ml of distilled water which was allowed to run to waste (free [³H] inositol fails to bind under these conditions and is therefore also lost to waste). Bound [³H] phosphoinositols were eluted from the resin into scintillation vials with 1M ammonium formate/0.1M formic acid (2 × 2ml). 3ml of Hydroluma scintillation fluid was added to each vial and the radioactivity was measured on a Canberra Packard 1900CA liquid scintillation counter.

Samples were counted for 10 min and the ³H-counts in the total phosphoinositol products measured as disintegrations per minute (DPM).

(iv) Protein Assay.

Cellular protein content was measured on a minimum of three wells for each experiment. Wells were first washed twice with 1ml of 0.9% saline and cells then taken up in 0.5ml 1% (v/v) Triton X-100. After diluting the sample 1:10 with water, protein content was determined by the method of Bradford (1976), automated for use on the Cocas Fara (Roche) centrifugal analyser. The assay standards used bovine serum albumin made up in 0.1% Triton X-100 (range of standards; 0 - 100µg/ml).

2.2.7. Separation of Inositol Phosphates.

More detailed analysis of the aqueous radiolabelled head groups was achieved by differential elution using a discontinuous salt gradient from the anion exchange column. The individual classes of phosphoinositols (IP₁, IP₂ and IP₃) but not their isomeric forms, can be separated and quantitated using a method originally described by Ellis et al (1963), subsequently modified by Berridge et al (1983) and Batty et al (1985).

In order to separate the phosphoinositols completely the system had to be fully optimised. Unlabelled cell extracts were processed as described in sections 2.2.4 (i) and (ii). Each blank extract was spiked with 10 μ l of [³H] Ins 4 P or [³H] Ins (1,4) P₂ or [³H] Ins (1,4,5) P₃ all at (10 μ Ci/ml) to which EDTA was added to a final concentration of 1mM. Samples were loaded onto individual columns of AGI-X8 anion exchange resin and the columns washed with distilled water (5 \times 2ml) to allow elution of the [³H] inositol which does not bind to the resin; this was collected into a scintillation vial. Following this, each column was sequentially eluted with a series of ammonium formate buffers (5 \times 2ml) ranging in concentration from 60mM - 1.2M. Scintillation fluid (3ml) was added to each of the collected fractions which were subsequently counted (as described in section 2.2.6 (iii)) to determine the minimum buffer strength which led to the elution of the inositol phosphate standards.

Tissue extracts of cells that had been pre-labelled with [³H] inositol on day 2 of cell culture (section 2.2.4 (i)), followed by agonist stimulation on day 4 were then processed as described above, to allow identification of InsP₁, InsP₂ and InsP₃ species.

2.2.8. Metabolism of ATP by cells in culture.

The metabolic fate of ATP in EBSBG added to cells in culture (at 10⁻⁴ M) was examined by following its conversion to the products ADP, AMP and adenosine. Separation of these products was performed using High Performance Liquid Chromatography (HPLC) on a Mono Q HR5/5 column (Pharmacia) with a Fast Protein Liquid Chromatography (FPLC) solvent delivery system to deliver a defined salt gradient.

Individual wells containing bovine zfr cells were stimulated with ATP (10⁻⁴ M) in EBSBG for 1 min, 30 min, 60 min and 120 min respectively. The contents of three wells were pooled before application to the Mono Q column. In parallel, EBSBG containing ATP (10⁻⁴ M) was incubated in multi-well plates at 37°C (no cells present), to determine whether

any significant chemical breakdown of ATP occurred under these conditions.

Each of the pooled samples were made up to 4.0ml with distilled water and injected onto a Mono Q column pre-equilibrated with deionised distilled water (buffer A). 1M ammonium formate (pH 4.5 with formic acid) (buffer B) was then used to generate the gradient.

The flow rate was maintained at 2.0ml/min throughout the gradient run. At 25 min after sample injection, gradient elution with 1M ammonium formate/formic acid (buffer B) was started, rising to 100% buffer B at 85 min and remaining at 100% for the next 5 min. Elution was then returned to water only and the column washed for a further 10 min before application of the next sample.

Elution of the purines from each sample was monitored at 280nm and characterised by their retention times which had earlier been determined by the injection in turn of adenosine and each of the adenine nucleotides (ATP, ADP and AMP) (0.01mg/ml in 4.0ml of distilled water) onto the Mono Q column.

After analysis of nine samples, the column was cleaned successively with 4ml each of 50% acetic acid, 100% methanol, 1M NaCl and 2M NaOH while the column was eluted with water. After cleaning of the column, a blank gradient run was carried out before the further application of samples for analysis.

2.2.9. Data handling and Statistics.

Tests of statistical significance employed Students t-test and the Mann Whitney U-test where stated.

Unless otherwise stated in the figure legends, data from 3 separate experiments using 3 separate cell isolates were combined. Within each experiment each dose or time point was determined using the data from 3 combined wells. In order to take account of variation in cell number and basal output, data from some experiments were normalised

with respect to basal secretion. Statistical differences between mean values were assessed using unpaired Student's t test and the Mann Whitney U-test (Chapter 5) with a value of $p < 0.05$ considered significant.

To determine the EC_{50} and E_{max} values for cortisol secretion elicited by ATP, ADP and UTP each individual dose-response curve was fitted using a sigmoid power model on Biosoft Fig P (Biosoft, PO Box 10938, Ferguson, MO 63135, U.S.A.) on a Nimbus (Research Machines) PC-486/25^SX personal computer. The mean EC_{50} and E_{max} values were then determined from three combined experiments for each agonist.

CHAPTER 3

CORTISOL SECRETION IN CULTURED BOVINE ZONA FASCICULATA/RETICULARIS CELLS IN RESPONSE TO ATP, UTP, AND CHARACTERISATION OF THE PURINERGIC RECEPTOR SUBTYPE WHICH MEDIATES THIS RESPONSE.

3.1. INTRODUCTION.

Purine nucleosides and nucleotides have a major role in cell function. In addition to a central function in cellular energy metabolism and as a co-factor for many enzyme reactions, extracellular ATP (and ADP, adenosine and possibly other purines) is (are) now known to influence many biological processes via specific purine receptors. These include platelet aggregation, neurotransmission (peripheral and central), cardiac function, muscle contraction, histamine release by mast cells and release of prostacyclin and relaxing factor by endothelial cells (Gordon et al 1986, Williams et al 1987 and Dubyak et al 1990).

While exogenous ATP can be rapidly catabolised to adenosine by a number of ectophosphohydrolases, many of the observed actions of extracellular ATP can be distinguished from those mediated by extracellular adenosine receptors. Many of these functional effects are observed at low micromolar concentration, well below the Michaelis constant (K_m) for most adenosine triphosphatases (ATPases) and other ATP utilising enzymes (Dubyak et al 1990).

Receptors sensitive to adenosine were originally termed P1 and those sensitive to ATP termed P2. The adenosine P1 receptor is now known to be heterogeneous and to consist of A1

and A2 subtypes (Londos et al 1980). The extracellular ATP receptor is also heterogeneous, based on agonist sensitivity and differences in signal transduction mechanisms. The major subdivision was initially thought to be between the P2x and P2y subtypes (Burnstock et al 1985), however this has been broadened to include P2t (platelets) and P2z (mast cells) subtypes (Williams et al 1987).

The P2x subtype displays a rank order of agonist potency α,β -methyleneATP ($\alpha\beta$ MeATP) = β,γ -methyleneATP ($\beta\gamma$ MeATP) > ATP = 2-methylthioATP and is selectively de-sensitised by $\alpha\beta$ MeATP. Using whole cell and patch clamp electrophysiological recording techniques, this receptor appears to activate a conductance which is non-selective for mono- or divalent cations. In contrast, the P2y subtype displays a different rank order of potency of 2-methylthioATP (2-Me-S-ATP) >> ATP > $\alpha\beta$ MeATP = $\beta\gamma$ MeATP and is only weakly or not de-sensitised by $\alpha\beta$ MeATP (Kennedy et al 1990). In a variety of tissues examined, including hepatocytes, erythrocytes, leucocytes (Boeynaems et al 1990) and vascular smooth muscle (Tada et al 1992) the responses associated with P2y receptor stimulation appears to be linked to the activation of phosphoinositidase C.

With the exception of the P2t receptor, a more definite characterisation of P2 receptor subtypes is hampered by the lack of potent and specific competitive antagonists. Suramin is a competitive antagonist of the P2 purinoceptor, but is unable to distinguish between P2x and P2y subtypes.

Although an "atypical" P2y purinoceptor has been identified in the adrenal medulla (Allsup et al 1990), the possibility that the adrenal cortex is responsive to extracellular purines through a specific receptor has received little attention. Kawamura et al (1991) presented preliminary evidence that ATP led to a dose-dependent stimulation of cortisol secretion from bovine inner zone cells. These workers argued, on the basis of agonist potency, that the response was mediated by a P2y receptor, though the most potent agonist for the P2y receptor, 2-methylthioATP, was not tested. Moreover, the ability of ATP to stimulate membrane phosphoinositide turnover was not studied in this or subsequent studies of the effects of ATP on

adrenocortical steroidogenesis. Niitsu (1992) reported a steroidogenic effect of ATP on bovine zona fasciculata cells and observed an increase in cyclic AMP in response to the highest dose of ATP used; this was attributed to breakdown of ATP to adenosine, which was then postulated to stimulate cyclic AMP through a P1 purinoceptor. A requirement for Ca^{2+} in the steroidogenic action of ATP has also been reported (Matsui et al 1991 and Niitsu et al 1992).

There is an increasing awareness that cortico-medullary interactions may be important in the function of the adrenal gland as a whole. Adrenoceptors (β_1) have been identified on inner zone adrenocortical cells and both outer and inner zone cells secrete steroids in response to adrenaline (Walker et al 1991). Purines, such as ATP and UTP are also stored in and secreted from chromaffin granules in medullary cells (Douglas et al 1966 and Winkler et al 1977). In view of the close contact between these cell types (Bornstein et al 1991 and Gallo-Payet et al 1987), the possibility exists for a paracrine effect of released purines, such as ATP.

The aims of this chapter were; 1). to determine if bovine zfr cells in culture responded to ATP and UTP with respect to steroidogenesis and to characterise this response and 2). to characterise the purinoceptor subtype present in these cells using agonist potency orders and second messenger studies.

RESULTS.

3.2. Characterisation of the steroidogenic response of bovine zfr cells to purinergic agonists.

3.2.1. The effect of ATP, ADP, AMP and UTP on cortisol secretion from ZFR cells.

The effects of a range of doses of ATP, ADP, AMP and UTP on the secretion of cortisol over a 60 min period from bovine zfr cells at 48h in primary culture are shown in fig. 3.1 and 3.2. Both ATP and ADP (fig 3.1) produced a dose-dependent increase in cortisol secretion when compared to the basal secretion (no agonist) over the same period. The lowest concentrations of ATP and ADP which elicited a significant increase in steroid secretion was 10^{-6} M for both agonists ($p < 0.05$ in each of the three separate experiments); the maximum response (the maximally observed value) occurred by 10^{-4} M (10^{-3} M in some experiments). Similar results were obtained for the pyrimidine UTP. The EC_{50} values were $5.83 \pm 3.98 \times 10^{-6}$ M for ATP, $13.7 \pm 5.67 \times 10^{-6}$ M for ADP and $7.33 \pm 4.52 \times 10^{-7}$ M for UTP (mean \pm S.D., $n=3$ experiments for each agonist (table 3.1). The EC_{50} for UTP was significantly lower than for ADP ($p < 0.05$), but not ATP.

AMP was a poor agonist and significant stimulation of cortisol secretion (relative to basal) was not observed until 10^{-4} M ($p < 0.05$ in each of the three experiments) (fig. 3.2). The potency order was thus ATP = UTP > ADP > > AMP.

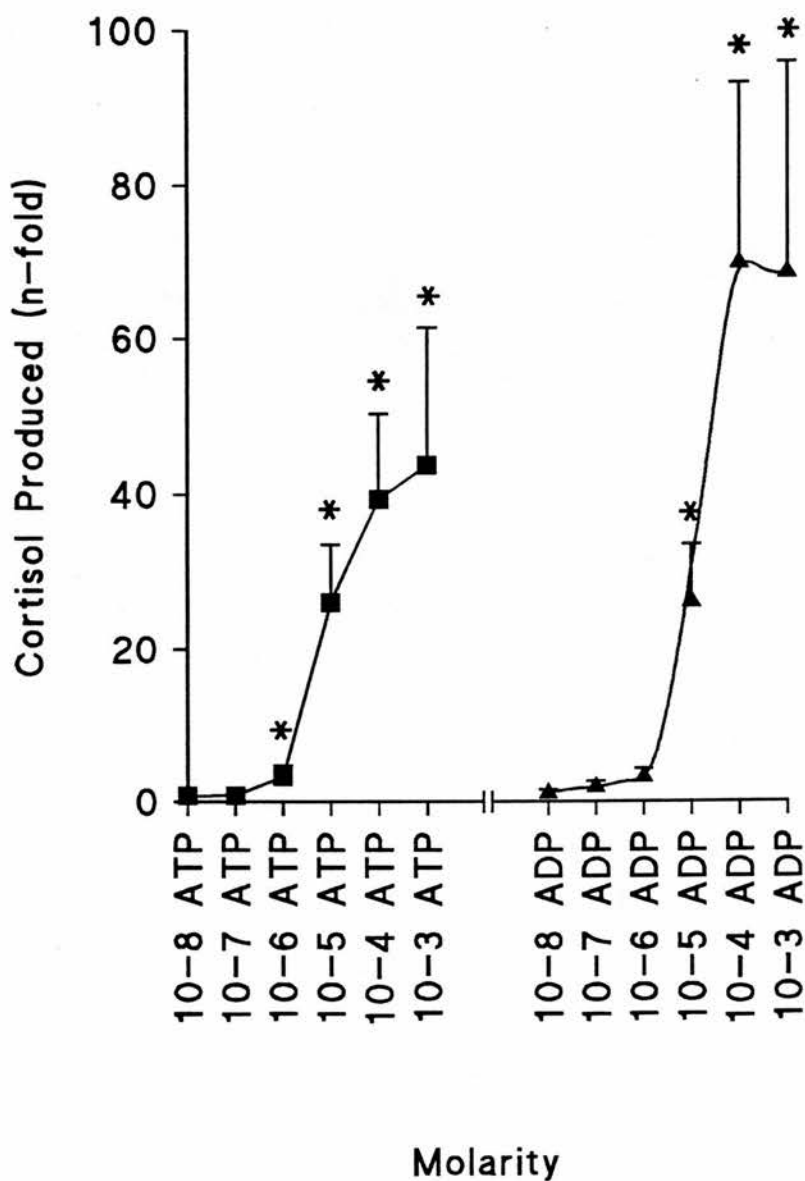


FIGURE 3.1.

Cortisol secretion over a period of 60 minutes was measured in the overlying medium from cells stimulated with ATP and ADP (dose range: 10^{-8} - 10^{-3} M) after 48 hours in primary cell culture. The threshold concentration eliciting a significant increase above basal secretion was 10^{-6} M for both ATP and ADP in each of 3 different experiments (* $p < 0.05$). Results are expressed as n-fold stimulation ratios, and show the mean \pm S.D. of triplicate determinations from three separate experiments.

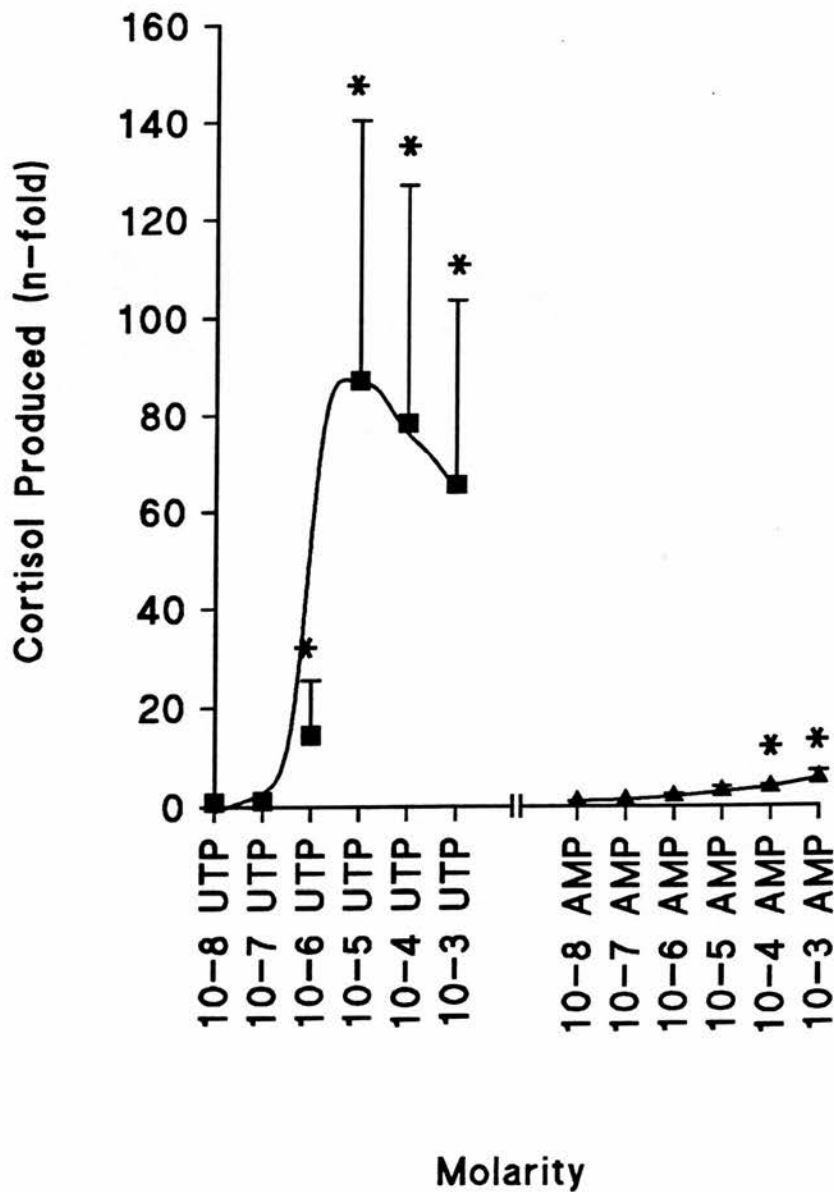


FIGURE 3.2.

Cortisol secretion over a period of 60 minutes was measured in the overlying medium from cells stimulated with UTP and AMP (dose range: 10^{-8} M - 10^{-3} M) at 48 hours in primary culture. The threshold concentrations of UTP and AMP eliciting a significant increase in cortisol secretion above basal secretion were 10^{-6} M and 10^{-4} M, respectively, in each of 3 different experiments (* $p < 0.05$). Results are expressed as n-fold stimulation ratios, and show the mean \pm S.D. of triplicate determinations from three separate experiments.

3.2.2. Changes in the cortisol secretory response to ATP and ADP with cell culture.

Fig. 3.3 shows the changes in the purine-stimulated cortisol secretion of the zfr cells using fixed doses of ATP or ADP on a day-by-day basis. Cortisol secretion was measured over 60 min in freshly isolated cells (0h) and at 24, 48, and 72h in primary culture using 10^{-6} M and 10^{-4} M doses of ATP and ADP. Freshly dispersed cells, used immediately after collagenase digestion of adrenocortical tissue, showed no significant secretion to any dose of ATP or ADP. By 24h (day 2) in culture, the response had appeared reaching a maximum by 48h (day 3) in culture. Cells were still responsive at 72h (day 4), although cortisol production had decreased, as compared to cells at 48h (day 3).

3.2.3. Time course of cortisol secretion to ATP and ADP.

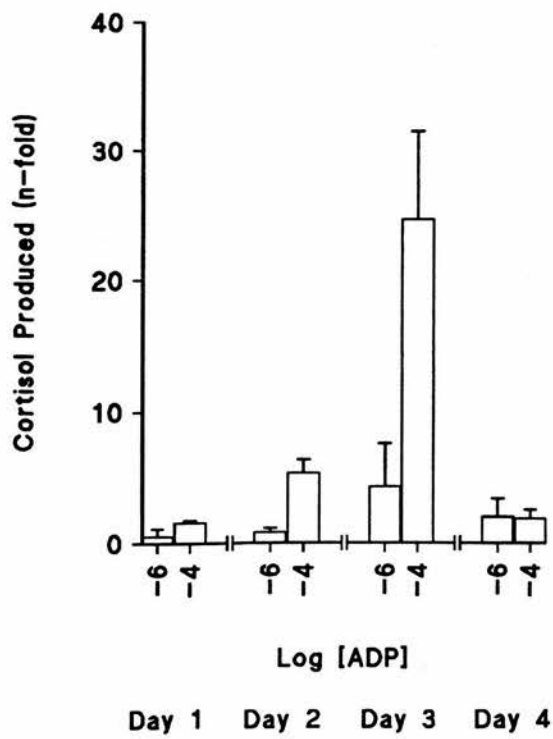
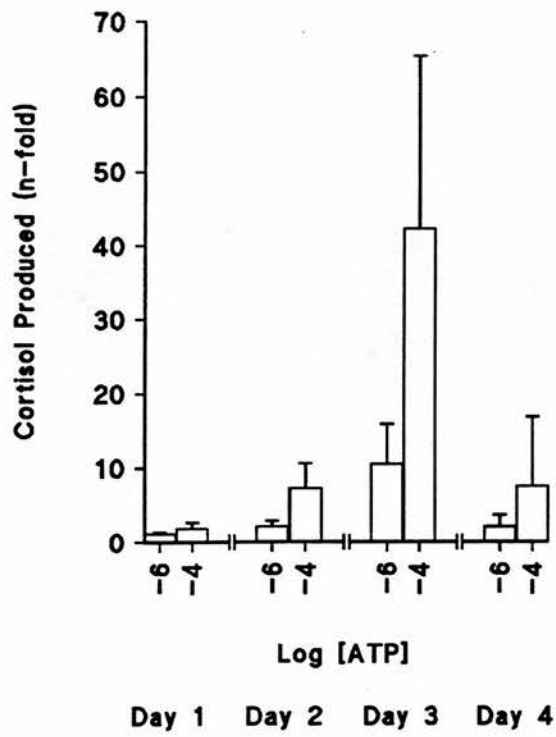
Since maximum secretion of cortisol was achieved by 10^{-4} M ATP or ADP, this dose was used to follow the time-course of cortisol secretion over a 4 h period at 48 h in primary culture. Incubation of zfr cells with ATP or ADP resulted in a time-dependent increase in cortisol secretion. As shown in fig. 3.4, this response was continuous for 60 min, thereafter declining.

3.2.4. Additivity of the responses to ATP and UTP.

To investigate possible additivity, UTP and ATP were added simultaneously to inner zone adrenocortical cells at 48 h in primary culture and the responses compared with those induced by the same concentration of each agonist alone. Fig. 3.5 shows that the response of a maximally stimulatory dose of ATP (10^{-4} M) was not significantly affected ($p > 0.05$) by the addition of a maximally stimulatory dose of UTP (10^{-4} M). On the contrary, the combination of the maximal dose of ATP (10^{-4} M) and All (10^{-7} M or 10^{-8} M) gave an additive response ($p < 0.05$). The lack of additivity of ATP and UTP would suggest that the effects of these agonists were mediated through

FIGURE 3.3.

Cortisol secretion over a 60 min period was measured in response to a threshold dose of (10^{-6} M) and a maximum dose (10^{-4} M) of ATP and ADP in freshly-isolated cells in suspension at 0h (day 1) and thereafter in primary cell culture at 24 h (day 2), 48 h (day 3) and 72 h (day 4). The mean values of protein content on days 1-4 were 81.9 ± 20.2 , 84.8 ± 17.6 , 91.0 ± 12.4 and 98.6 ± 10.2 μg /well. Results are the mean \pm SD from three measured experiments; see Methods for details.



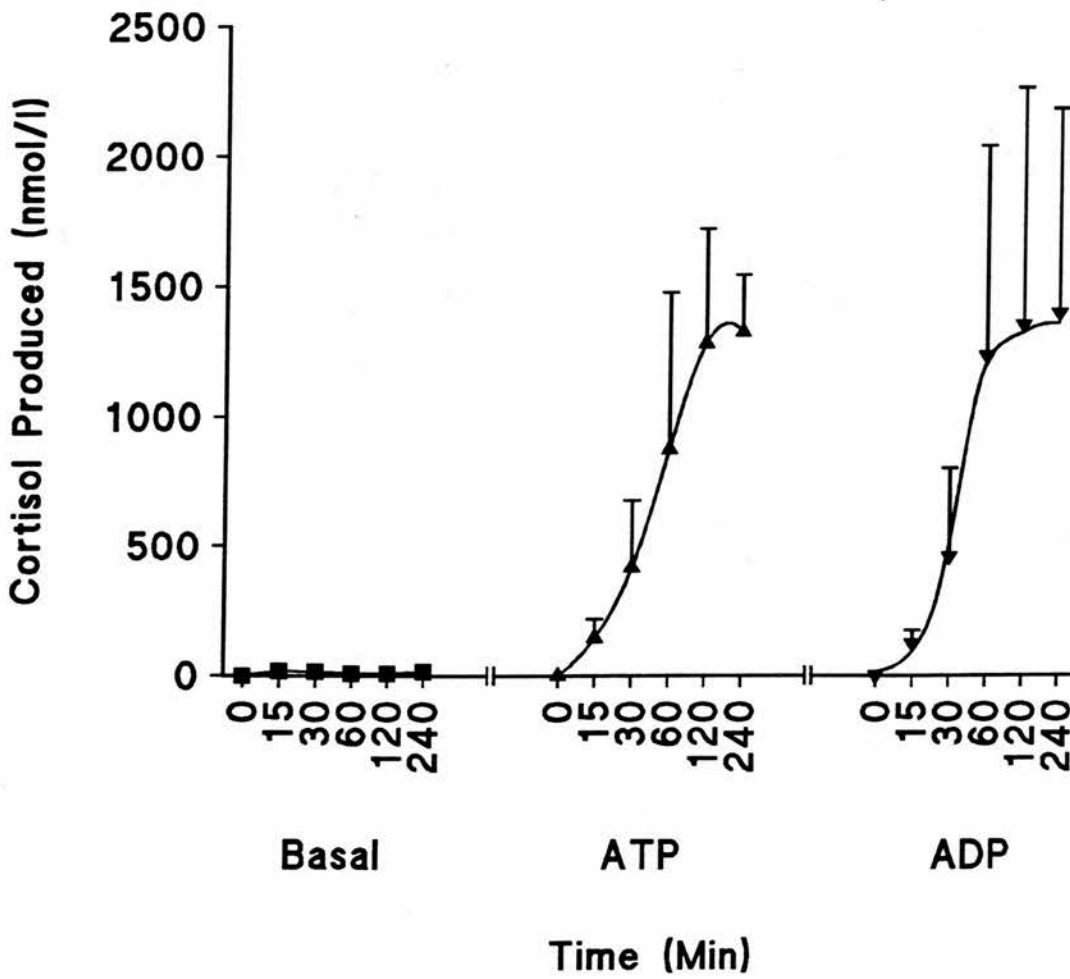


FIGURE 3.4.

The time-course for the accumulation of cortisol in the overlying medium was measured under basal conditions (no agonist) and in response to a maximally stimulatory dose of either ATP (10^{-4} M) or ADP (10^{-4} M) after 48 hours in primary cell culture. Results are mean \pm SD ($n = 3$ wells) for 3 combined experiments.

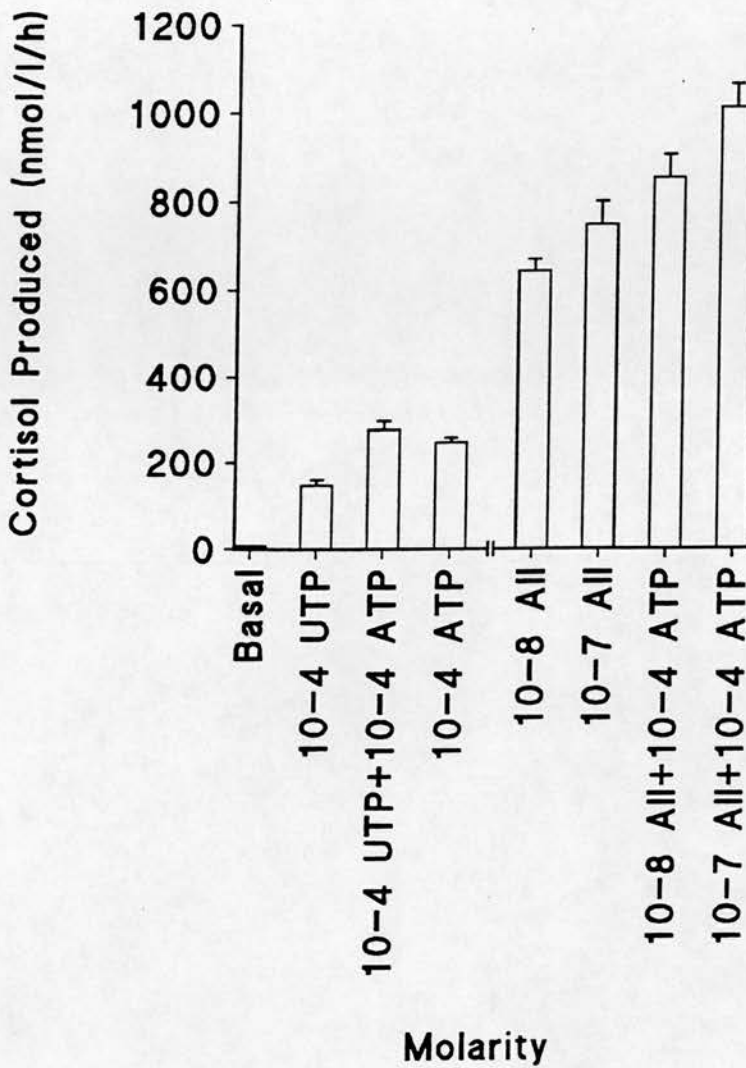


FIGURE 3.5.

Representative experiment showing additivity of the effects of ATP and UTP on cortisol secretion. Day 3 zfr cells in culture were incubated for a 60 min period with the maximal doses of both ATP and UTP (10^{-4} M). The response to ATP (10^{-4} M) was not significantly affected by the addition of UTP (10^{-4} M) ($p > 0.05$). A control was set up involving the combination of All (10^{-7} M or 10^{-8} M) and the maximal dose of ATP which gave an additive response ($p < 0.05$). The data are expressed as mean \pm S.D. of triplicate determinations from one representative experiment of three.

the same receptor.

3.2.5. Characterisation of the purinergic receptor subtype.

The non-specific P2 receptor antagonist, suramin, had no significant effect on the cortisol secretion over 60 min (10^{-4} M ATP or 10^{-4} M UTP), except at the highest suramin dose used (10^{-3} M), which led to an approximate halving of the response (two separate experiments; data not illustrated).

In the absence of any available specific receptor subtype antagonists, the potency order of a range of ATP analogues was used in an attempt to characterise the purinergic receptor subtype present in bovine zfr cells. Cortisol secretion was measured over a concentration range of 10^{-9} M to 10^{-3} M for the following agonists: ATP, UTP, ADP, AMP, $\alpha\beta$ MeATP, $\beta\gamma$ MeATP and 2-Me-S-ATP. The potency order established was as follows: ATP = UTP > ADP > 2Me-S-ATP > $\alpha\beta$ MeATP = $\beta\gamma$ MeATP = AMP (table 3.1).

3.2.6. Time course of ATP degradation in bovine zfr cells in culture.

The possibility that ATP was degraded by ectophosphatase activity was studied by monitoring the possible conversion of the ATP to ADP, AMP or adenosine. Each individual adenine nucleotide was first characterised by determining its retention time (min) using standard solutions of the appropriate nucleotide. Sodium salt preparations of adenosine, AMP, ADP and ATP (0.1mg/ml) were made up in distilled H₂O and injected separately onto a MonoQ column and eluted with 1M ammonium formate/0.1M formic acid (pH 4.5) (Buffer B) as shown in fig. 3.6. Retention times of 1 min (Adenosine), 17 min (AMP), 38 min (ADP) and 54 min (ATP) were obtained.

When a 10^{-4} M solution of ATP was added to the cells and the nucleotide composition of the overlying medium determined at 1 min, 30 min, 60 min and 120 min, a progressive conversion

TABLE 3.1.

Agonist	Active concentration range (threshold - maximum)	EC ₅₀ ±S.D. (n=3)	E _{max} ±S.D. (n=3) nmol/l/h
ATP	10 ⁻⁶ M - 10 ⁻⁴ M	5.83±3.93 x 10 ⁻⁶ M	423±220
ADP	10 ⁻⁶ M - 10 ⁻⁴ M	13.7±5.67 x 10 ⁻⁶ M	545±288
UTP	10 ⁻⁶ M - 10 ⁻⁴ M	7.33±4.52 x 10 ⁻⁷ M	317±233
2Me-S-ATP	10 ⁻⁵ M - 10 ⁻³ M	*	*
αβMeATP	10 ⁻⁴ M - 10 ⁻³ M	*	*
βαMeATP	10 ⁻⁴ M - 10 ⁻³ M	*	*
AMP	10 ⁻⁴ M - 10 ⁻³ M	*	*

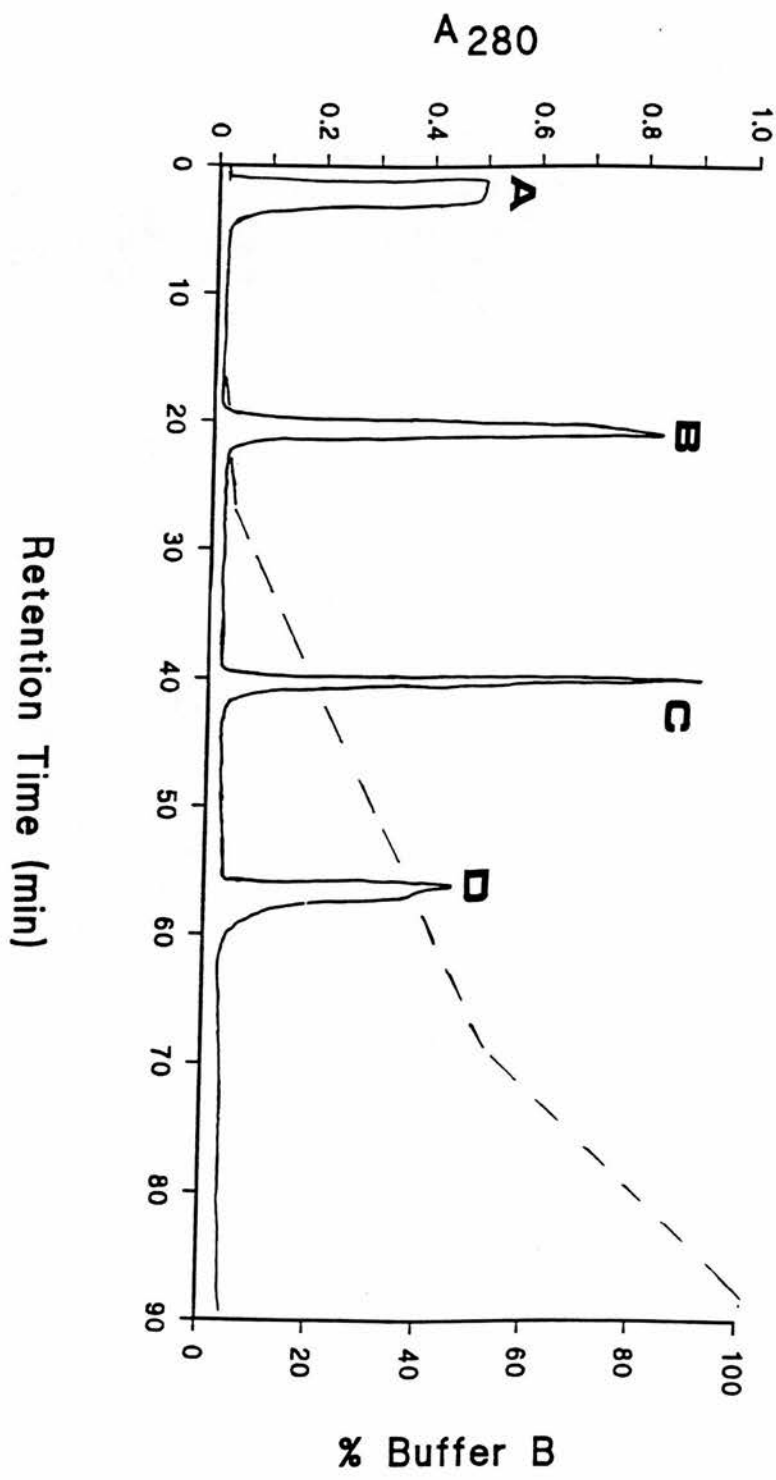
TABLE 3.1.

Relative agonist potencies for cortisol secretion from adrenocortical inner zone cells after 48h in primary culture for a range of purine nucleotides. Agonists were tested on three separate cell preparations.

*EC₅₀ values and E_{max} values are shown for the most potent agonists ATP, ADP and UTP; these values were not calculated over the narrow dose-range and high agonist concentrations required to elicit a response for the other agonists shown in the table. A maximal stimulatory dose of ACTH (10⁻¹⁰M) (Williams et al 1989) was included as a positive control in all experiments. The E_{max} for ACTH was 1205±481 (n=3) nmol/l/h cortisol.

FIGURE 3.6.

The separation of the adenine nucleosides and nucleotides (detected by absorption at 280nm) by FPLC on a Mono Q HR5/5 eluted with a 90 min linear gradient of 1M ammonium formate/0.1M formic acid (pH 4.5) (Buffer B) are shown. The gradient program used is indicated by the broken line. Sodium salt preparations of adenosine, AMP, ADP and ATP (0.1mg/ml) were prepared and injected separately (0.25ml made up to 4.0ml with dH₂O) onto the column. Retention times on this system are 1 min for adenosine (A), 17 min for AMP (B), 38 min for ADP (C) and 54 min for ATP (D).



of ATP to ADP and AMP (but without significant formation of adenosine) was observed over 2h. After 1 min incubation time with the cells no degradation of ATP was observed (fig. 3.8a). However after 30, 60 and 120 min incubation time with the cells, ATP was degraded to ADP (23%, 30% and 25%) and AMP (17%, 20% and 40%) respectively (fig. 3.8b, c and d). Control experiments showed that, in the absence of cells, there was insignificant chemical degradation of ATP over the same period (fig. 3.7a and b).

3.3. Characterisation of the phosphoinositol response to the purinergic agonists.

3.3.1. Stimulation of membrane phosphoinositide turnover in response to ATP, ADP and UTP.

The accumulation of water-soluble, [³H]-inositol labelled head groups was measured in the presence of increasing doses of ATP, ADP or UTP. Cells were pre-labelled to isotopic steady-state with [³H]-inositol for 48h and experiments carried out in the presence of 10mM Li⁺ to block the recycling of [³H]-inositol monophosphates. Fig. 3.9 shows the dose-dependent increase in labelled head groups in response to ATP, ADP and UTP. The dose of ATP, ADP or UTP required to elicit threshold and maximal responses were similar to those for cortisol.

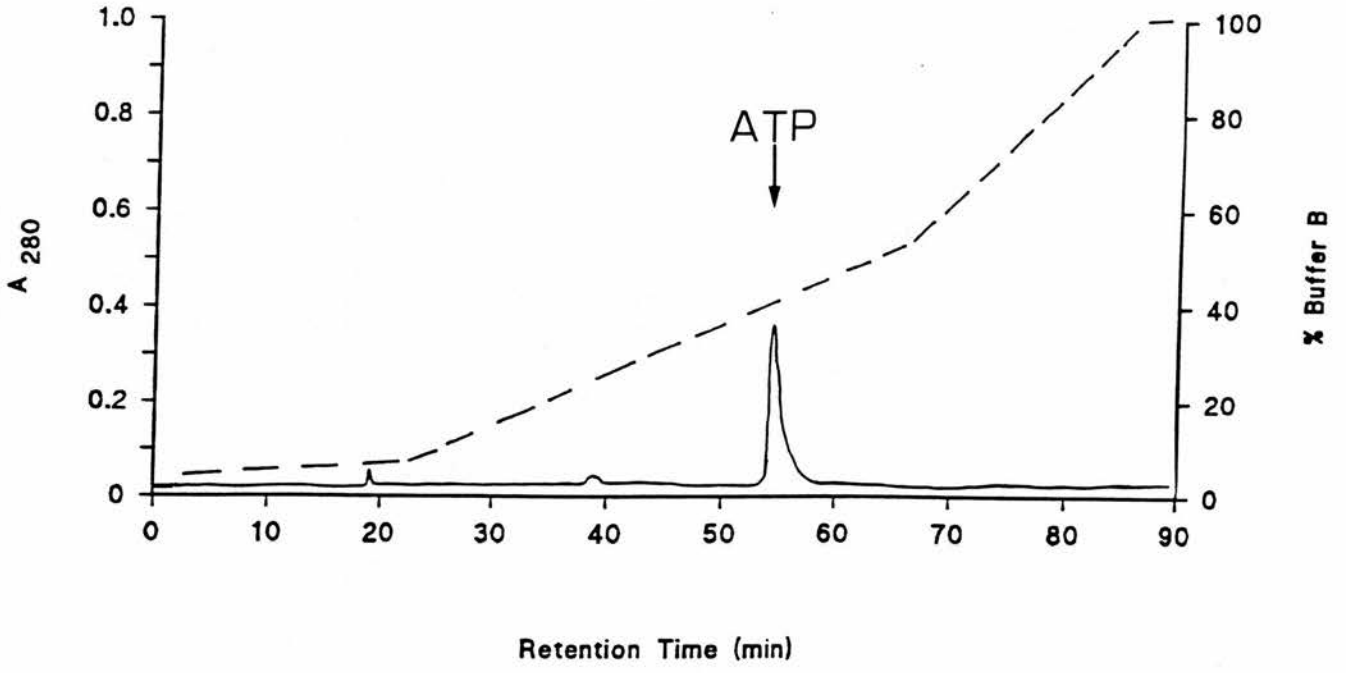
3.3.2. Time course of [³H]-inositol head group response to ATP.

The time-dependent changes in [³H]-inositol labelled head groups in response to a maximally stimulatory dose of ATP (10⁻⁴M) on day 4 cells in culture are illustrated in fig. 3.10. The total head group response remained linear for about 20min but decreased after

FIGURE 3.7.

Control experiments were first set up to determine if there was any significant chemical degradation of ATP in the absence of cells under the experimental conditions used. Figure 3.7a shows the profile of the sodium salt preparation of ATP (0.1 mg/ml) that was injected immediately onto the MonoQ column. A similar profile was obtained (fig. 3.7b) for the same ATP solution that had been incubated for a 60 min period at 37°C in the absence of cells, establishing that there was no significant chemical degradation of ATP under these conditions.

a.



b.

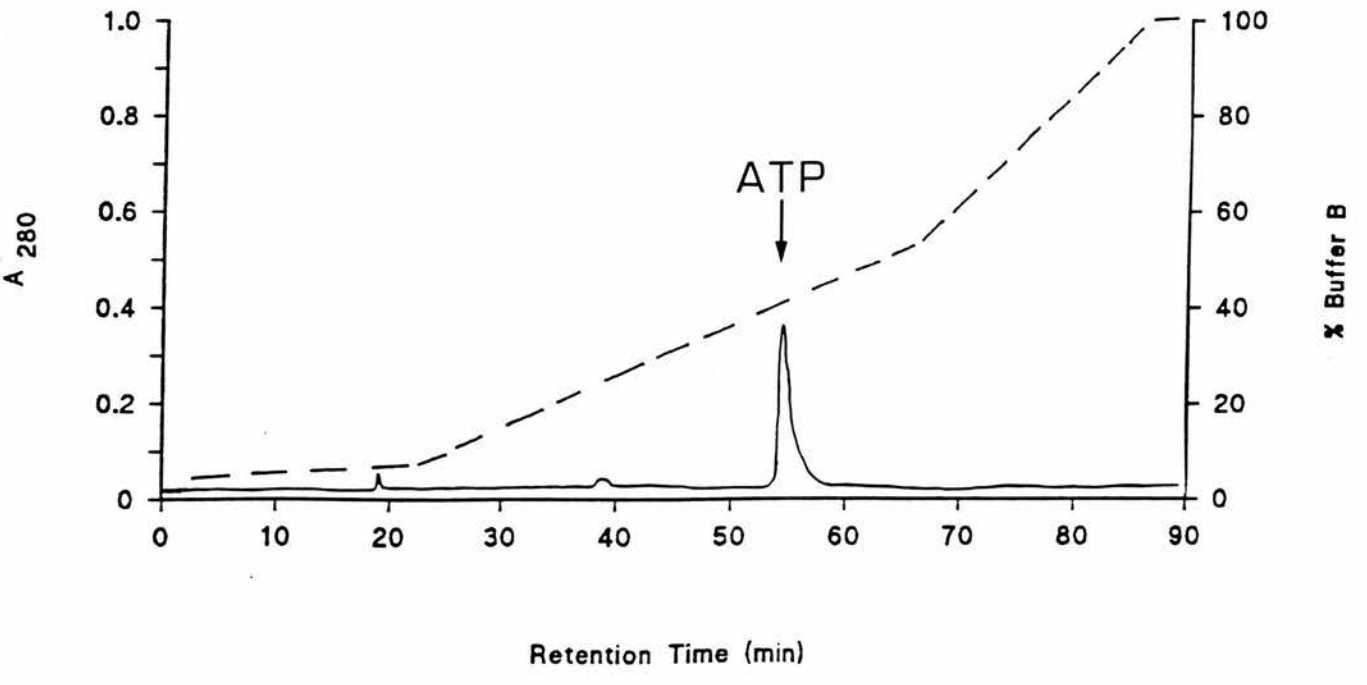
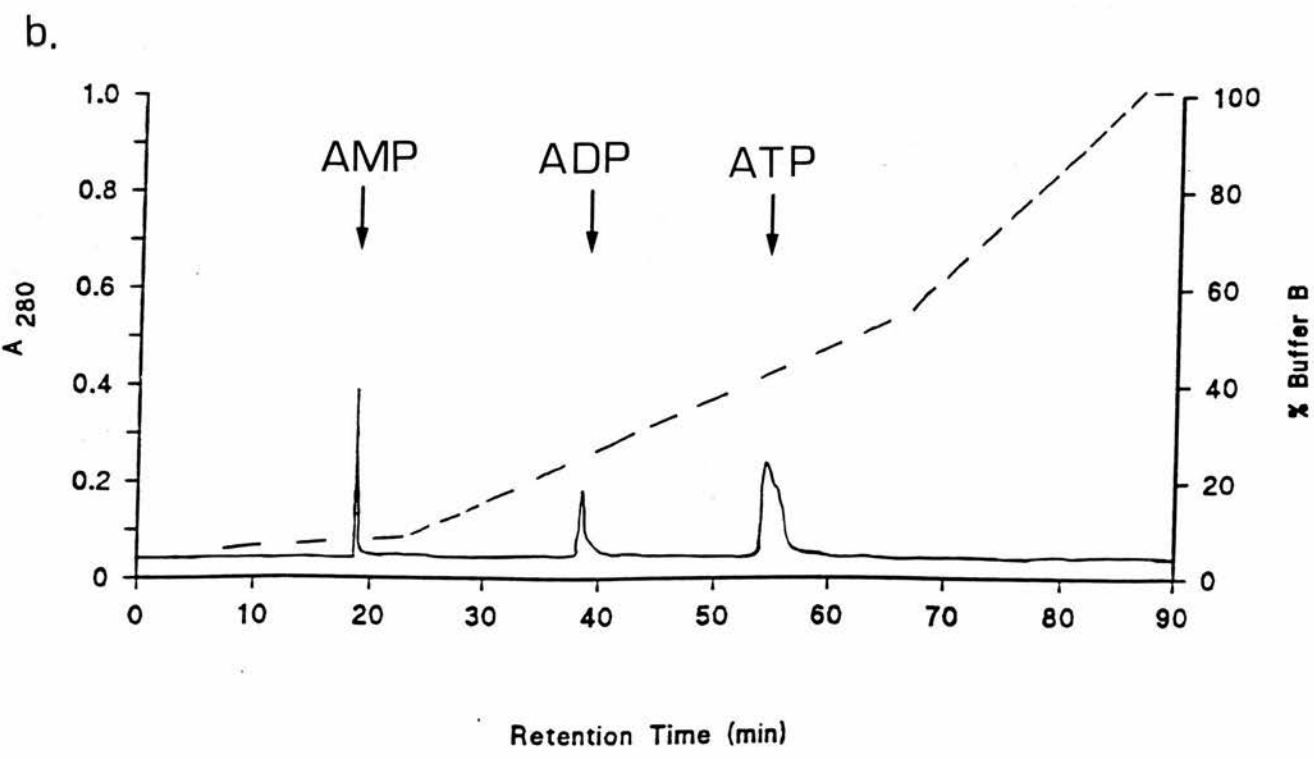
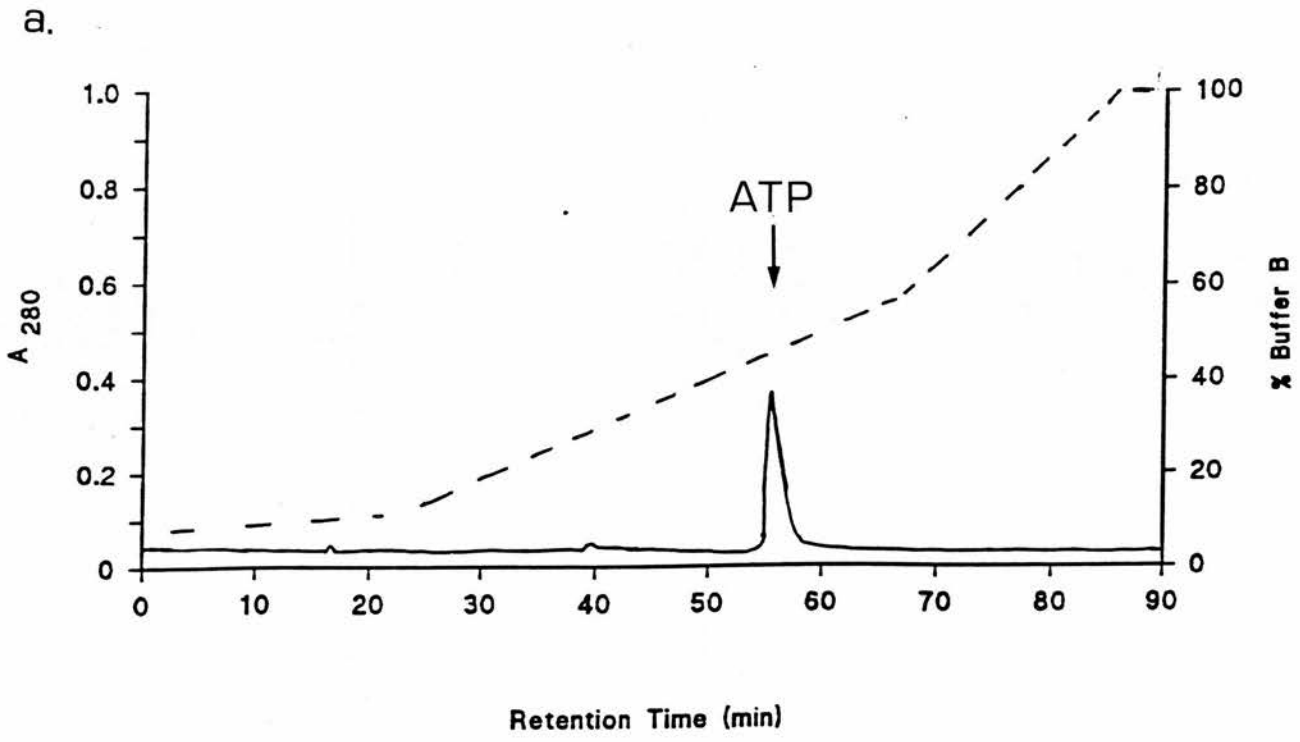
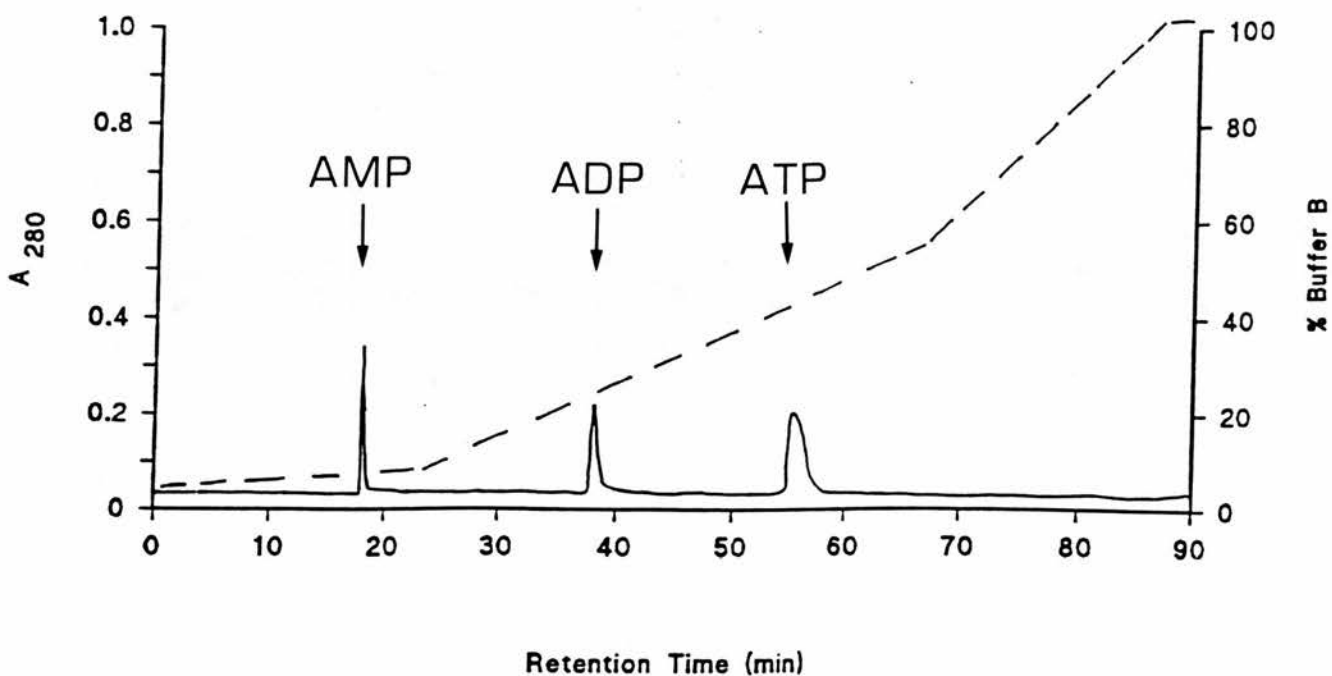


FIGURE 3.8.

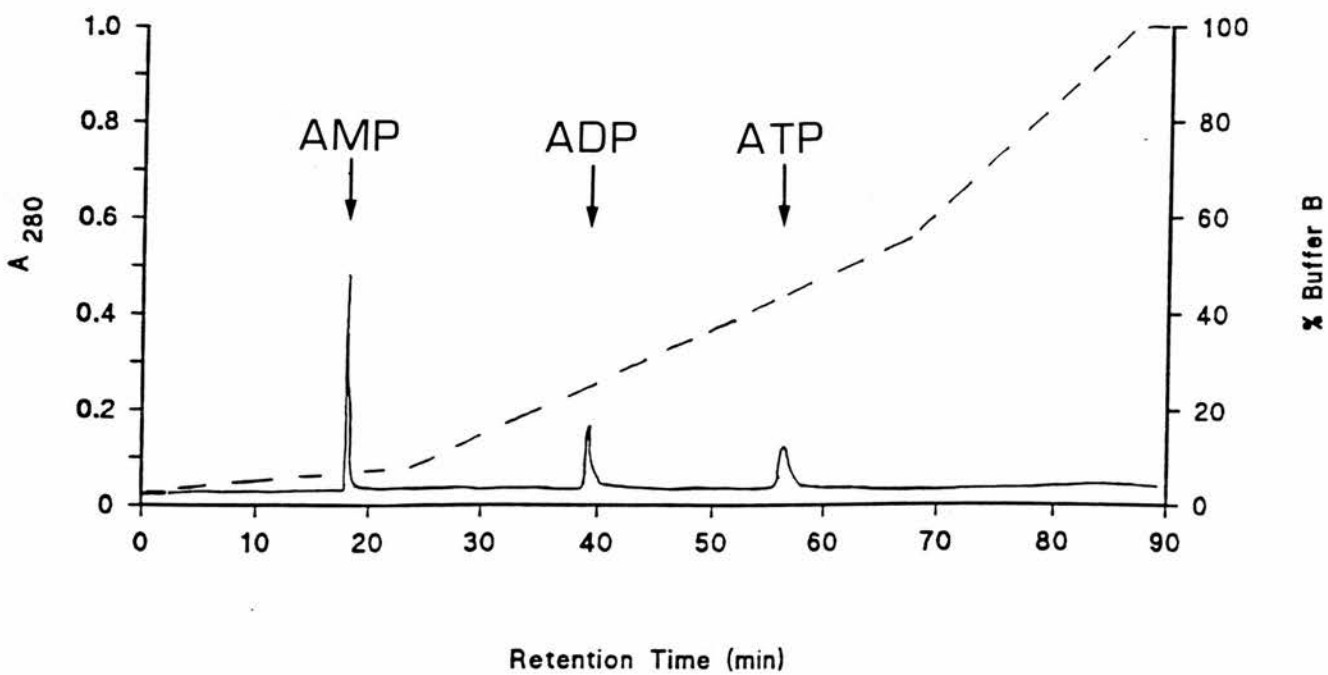
Time course for the degradation of ATP in the medium overlying the cells was monitored over a 2h period using the FPLC. Individual wells containing bovine zfr cells were stimulated with ATP (10^{-4}M) in EBSBG for 1 min, 30 min, 60 min and 120 min. The contents of 3 wells were pooled before application to the MonoQ column. After 1 min incubation time, there was no degradation of ATP (fig. 3.8a). However after 30 (fig.3.8b), 60 (fig.3.8c) and 120 (fig.3.8d) min incubation time progressive conversion of ATP to ADP and AMP was observed but without significant formation of adenosine (% conversion was calculated by measuring the area under each peak). The individual adenine nucleotides were identified by their respective retention times.



c.



d.



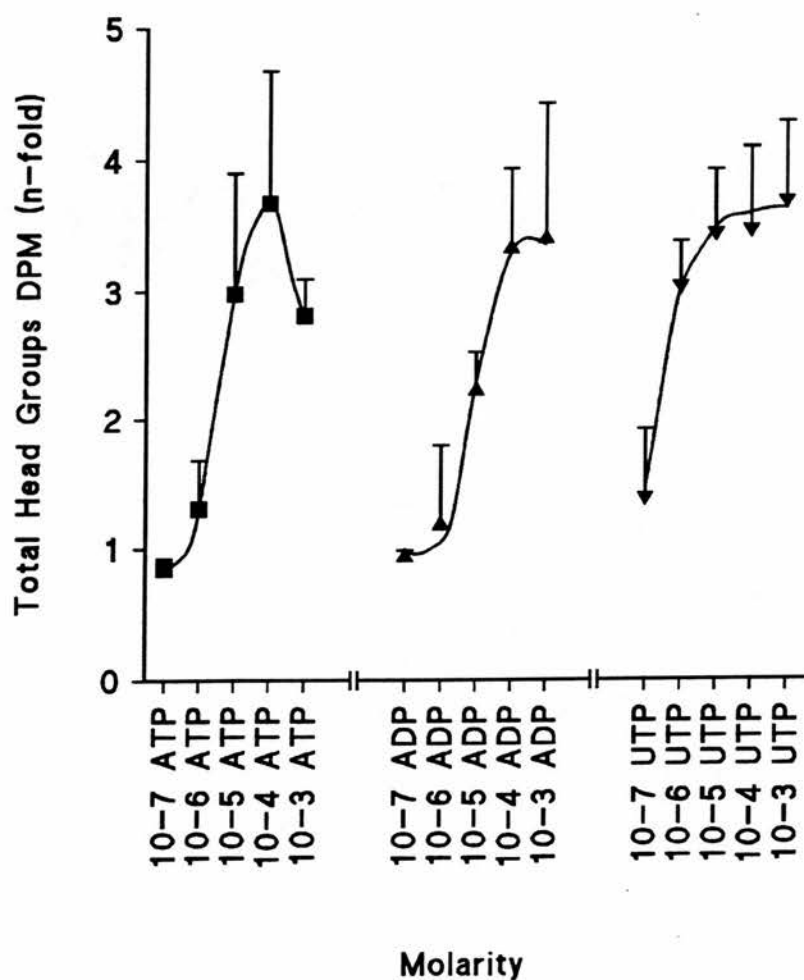


FIGURE 3.9.

Cells were pre-labelled to isotopic steady state for 48h with ³H-inositol and the accumulation of ³H-label in the total aqueous phosphoinositol/ glycerophosphoinositol pool (total head groups) measured after 15 min exposure of the cells to ATP, ADP or UTP over the concentration range shown. Experiments were carried out in the presence of 10 mM/L LiCl. The threshold response eliciting a significant increase above basal was 10⁻⁶M for ATP, ADP and UTP in each of the three different experiments (p < 0.05). Results are normalized with respect to basal, in order to allow different experiments to be combined, (mean ± SD for 3 combined experiments).

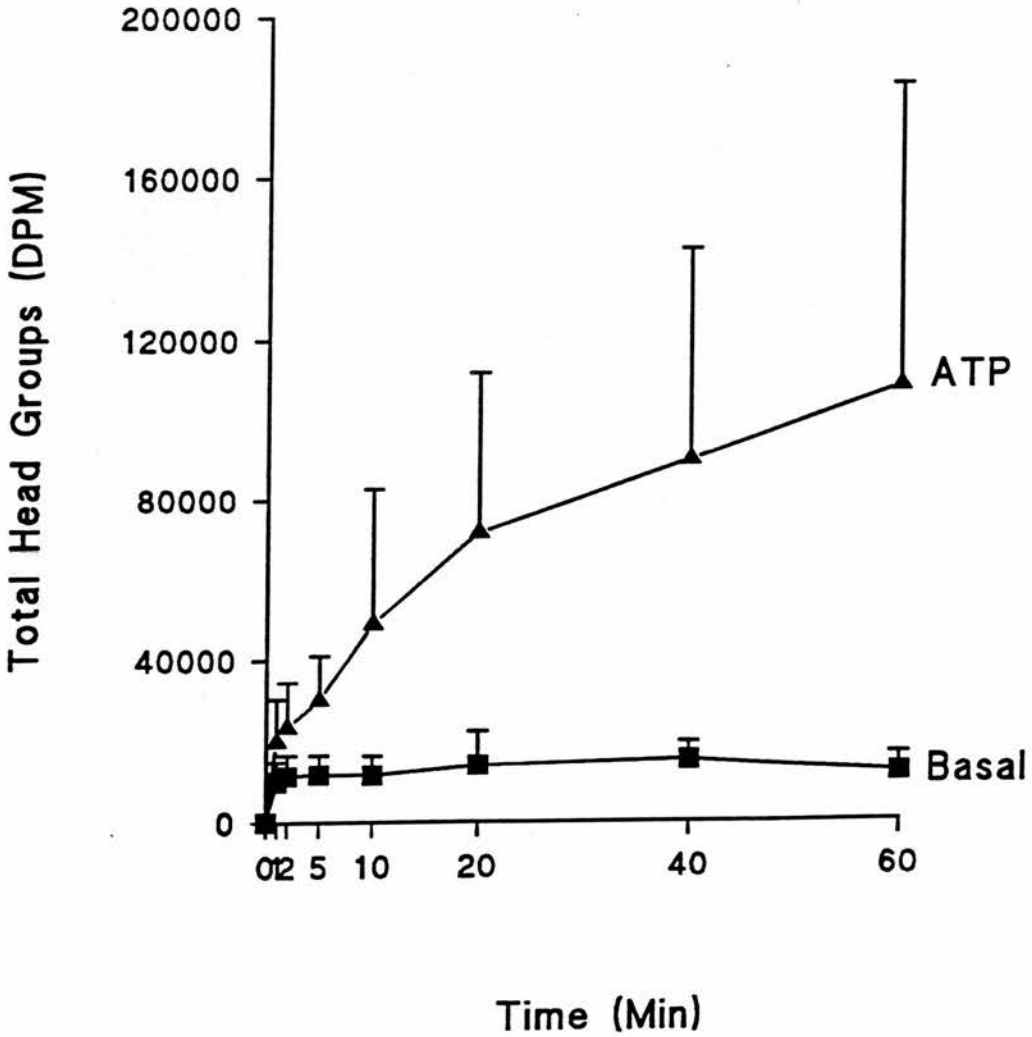


FIGURE 3.10.

Cells were pre-labelled to isotopic steady state for 48h with ^3H -inositol and the accumulation of ^3H -label in the total aqueous phosphoinositol/glycerophosphoinositol pool (total head groups) measured over a 60 min period under basal conditions (no agonist) and in response to ATP (10^{-4}M). Experiments were carried out in the presence of LiCl (10mM/L). Each time-point is the mean \pm SD from 3 combined experiments; see Methods for details.

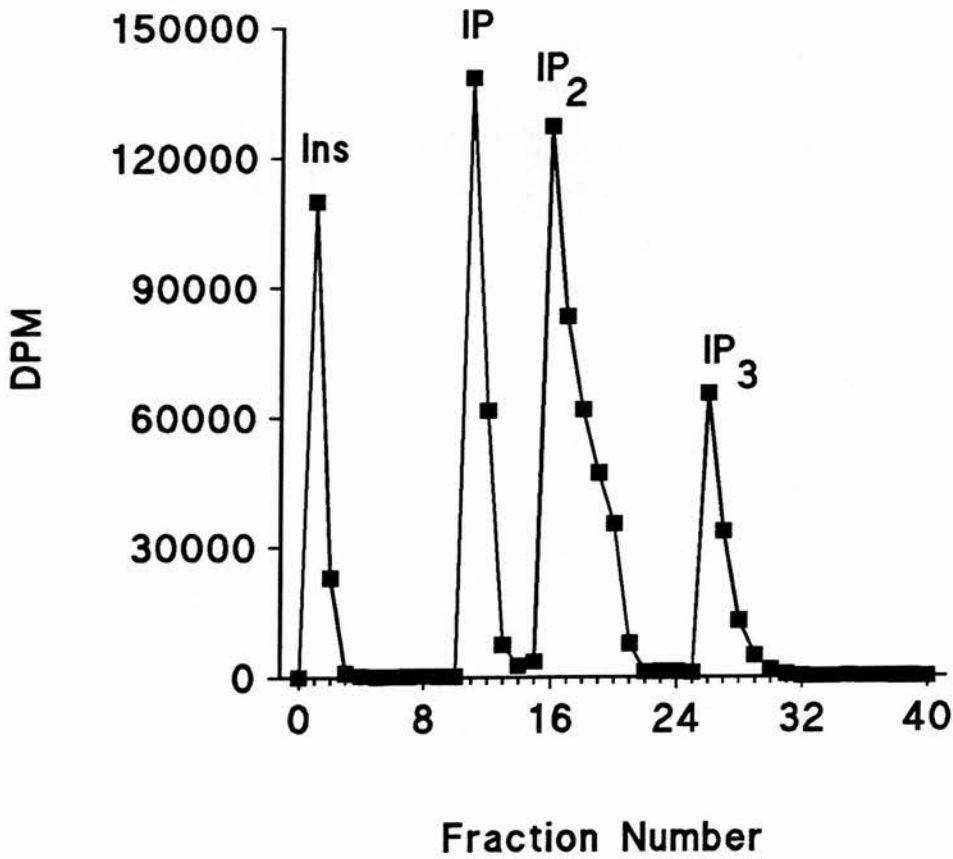


FIGURE 3.11.

A standard mixture of ^3H -labelled Ins, InsP, InsP² and InsP³ is separated using anion exchange chromatography with 100 -200 mesh AG1X8 resin as described in Materials and Methods Section 3.3.3. The inositol phosphate standards were eluted sequentially with 5 × 2ml formic acid containing 60mM (Fractions 6-10), 120mM (Fractions 11-15), 200mM (Fractions 16-20), 400mM (Fractions 21-25), 800mM (Fractions 26-30), 1M (Fractions 31-35) and 1.2M (Fractions 35-40) ammonium formate respectively. The radioactivity in each fraction was determined by liquid scintillation counting.

this time point. The response was statistically significant ($p < 0.05$) when compared to the basal response at 1min and all later times investigated.

3.3.3. Separation of phosphoinositols by anion exchange chromatography.

The separation of both [^3H] phosphoinositol standards and [^3H] phosphoinositol products from tissue extracts using 100-200 mesh AGIX8 resin (see methods) are shown in fig. 3.11 and 3.12. Standards chosen were unbound free inositol (eluted with distilled H_2O), InsP (eluted with 120mM ammonium formate buffer), InsP_2 (eluted with 200-400mM ammonium formate buffer) and InsP_3 (eluted with 800mM ammonium formate buffer (fig. 3.11).

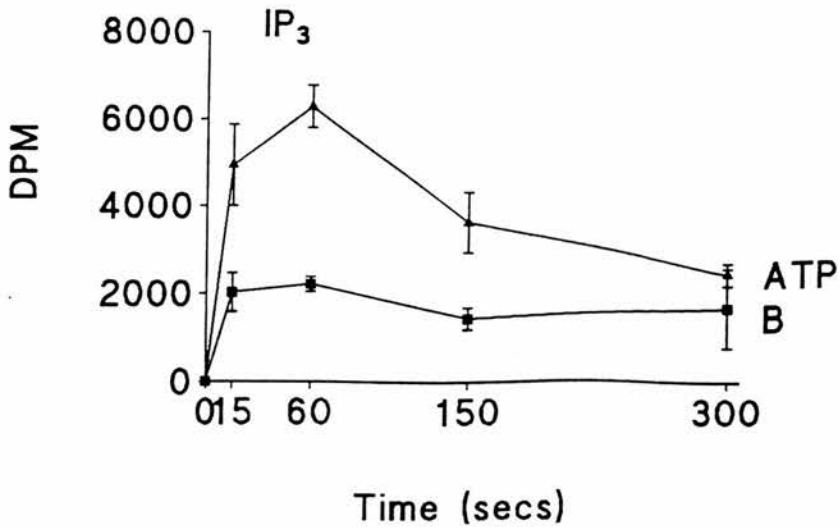
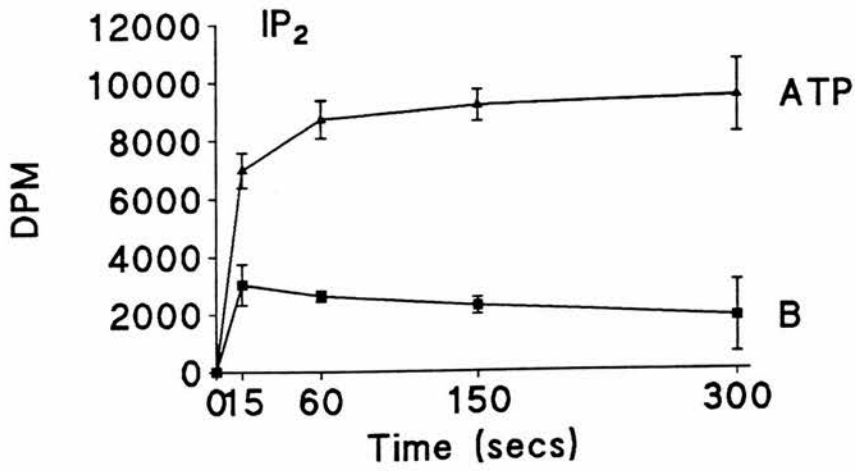
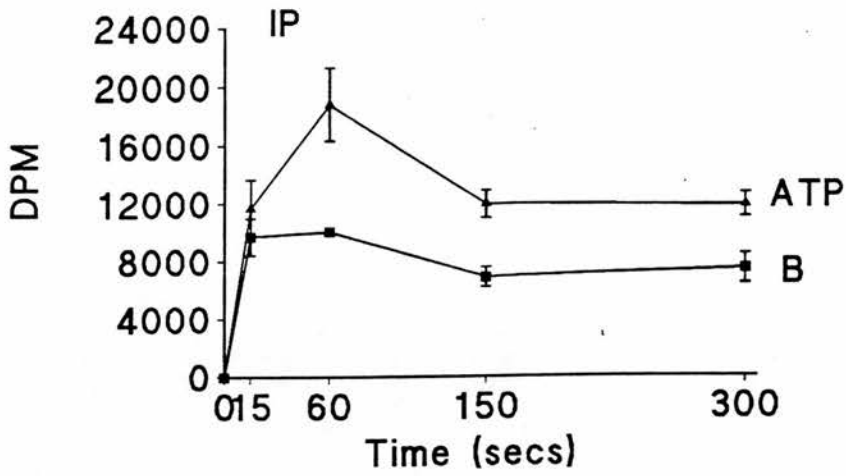
Aqueous radiolabelled products recovered from cells incubated with medium containing 10^{-4}M ATP or medium alone (basal) in the presence of Li^{2+} (10mM) and inositol (10mM) for the times indicated were subjected to chromatography as described in Materials and Methods and fig. 3.12. Cellular levels of InsP_2 and InsP_3 were increased significantly ($p < 0.05$) within 15 sec of exposure to extracellular ATP. InsP increased at a slower rate, reaching a significant increase ($p < 0.05$) over basal levels after 60 sec exposure to ATP. The levels of all the inositol phosphate species remained elevated as compared to basal for at least 5mins.

3.3.4. Measurement of the intracellular [Ca^{2+}] response.

The effect of a maximal steroidogenic dose of ATP (10^{-4}M) on the fluorescence signal from fura-2-loaded cells in suspension was examined. Basal intracellular [Ca^{2+}] measured between 48-72h in culture was found to be 57.3 ± 39.3 nmol/l (mean \pm S.D., $n=12$ cell suspensions derived from 4 different cell preparations) rising to 171 ± 84.2 nmol/l (mean \pm S.D., $n=12$ cell suspensions derived from 4 different cell preparations) in

FIGURE 3.12.

Time course of appearance of ^3H -labelled phosphoinositol species in response to ATP. Cells were incubated in the presence of 10^{-4}M ATP or medium alone (control) in the presence of Li^+ (10mm) and the aqueous radiolabelled products from the cells were subjected to anion exchange chromatography at the times indicated. The radiolabel accumulated in the peaks corresponding to InsP , InsP_2 and InsP_3 is shown. After 15 s, both InsP_2 and InsP_3 labelling increased significantly ($p < 0.05$) with respect to basal stimulation with 10^{-4}M ATP. By 60 s, the labelling of InsP was also significantly increased by this dose of ATP as compared to control values. These results are the mean \pm S.D. of values of triplicate incubations in a single experiment.



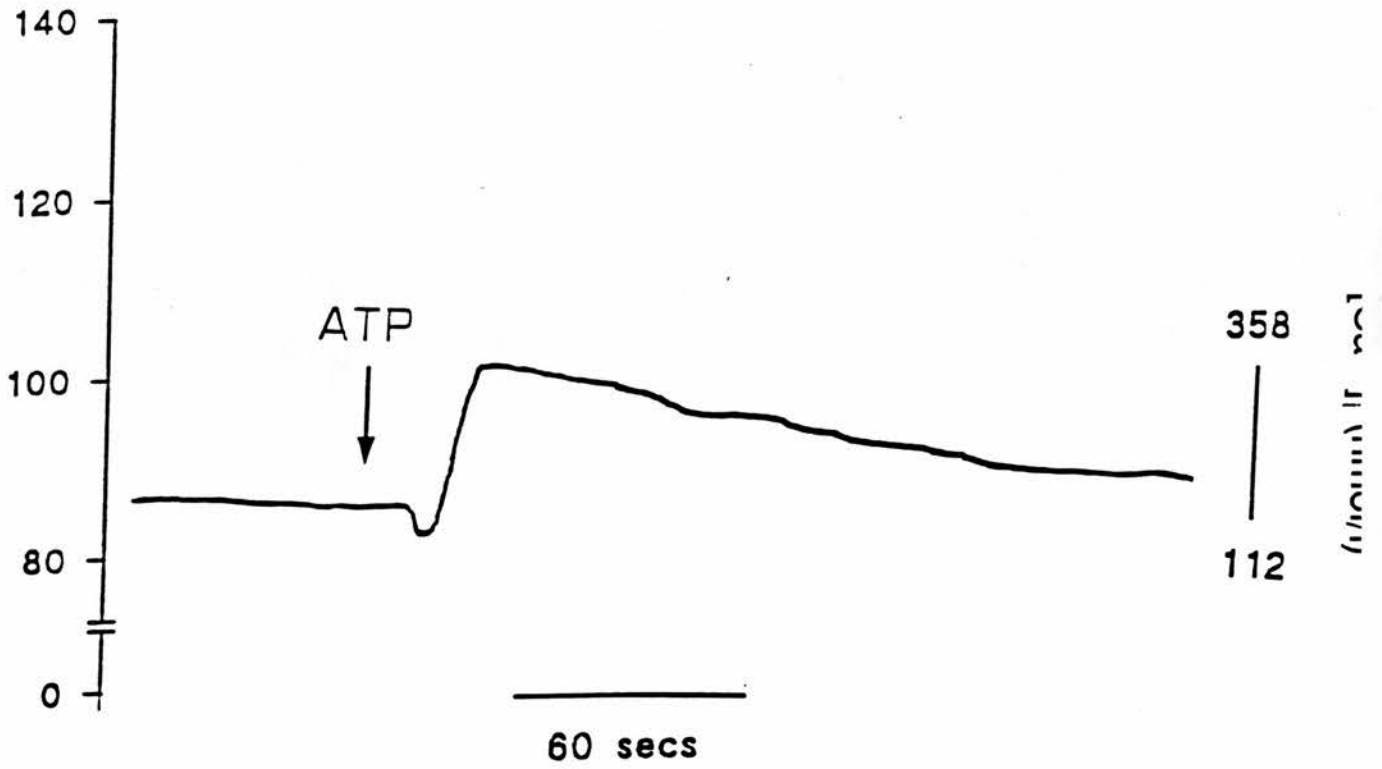


FIGURE 3.13.

Day 3 cells in culture were harvested and resuspended before being loaded with fura-2 AM as described in Materials and Methods. The figure illustrates a representative trace of the fluorescence signal from a cell suspension loaded with fura-2 AM (excitation at 340nm and emission at 505nm) and the increase in fluorescence when the cells are exposed to ATP (10^{-4} M). Intracellular $[Ca^{2+}]$ was calculated using a calibration equation as described in Section 2.2.5. These values are given in the text.

response to ATP. Fig. 3.13 shows a representative trace in which the fura-2 fluorescence was measured after addition of ATP. Details of the calibration equation for fura-2 which was used to calculate $[Ca^{2+}]_i$ are discussed in Materials and Methods.

3.4. DISCUSSION.

The experimental data establish that the purines ATP and ADP dose-dependently stimulate cortisol secretion from primary cultures of zfr cells isolated from bovine adrenal cortex (fig. 3.1). Both agonists exhibited a similar threshold at 10^{-6} M reaching a maximum response by 10^{-4} M purine. The EC_{50} for ATP and ADP were similar in magnitude and not significantly different (table 3.1). In addition, it was observed that the pyrimidine nucleotide, UTP, also elicited cortisol secretion from these cells, though AMP was relatively ineffective (fig. 3.2). The EC_{50} for UTP was significantly lower than for ADP but not ATP; E_{max} values for ATP, ADP and UTP were not significantly different and were approximately one third the E_{max} for ACTH (table 3.1).

Cortisol secretion was linear over the 60 min time period used for these studies. Over a longer time-course some flattening of the response was evident (fig. 3.4); this may reflect degradation of added ATP (see below), though a component of desensitization of the response cannot be ruled out. It is unlikely that the response is limited by reduced (cholesterol ester) substrate, as the cells secrete cortisol linearly for several hours with ACTH. It has been reported in many different cell types, including aortic endothelial cells (Kitazona et al 1992), HL60 cells (Xing et al 1991) and fibroblasts (Gonzalez et al 1989), that incubation with ATP (minutes to hours) results in desensitisation of P2 purinergic receptors.

The possibility that cortisol secretion in response to ATP might be accounted for by metabolism of ATP to adenosine (via ADP and AMP), which then acts at an A_1 or A_2 receptor, was excluded. Analysis of the nucleotides in the medium overlying the cells established that ATP was broken down by the cells but not to adenosine. After 30, 60 and 120 min about 40%, 50% and 60% of the ATP had degraded, largely to ADP and AMP, but with no significant formation of adenosine (fig. 3.8 a,b,c and d). There was no significant chemical breakdown of ATP under the same conditions, but in the absence of

cells (fig. 3.7). The increasing accumulation of the weakly potent agonist AMP, at the expense of ATP and ADP, might contribute to the decline in cortisol secretion at later points on the time-course (fig. 3.4). In guinea-pig urinary bladder, ATP, ADP and AMP were dephosphorylated by ectonucleotidases with a half-life of 15min (Welford et al 1987) while the half-life of ATP in blood perfused through the lung vasculature is 0.2s (Ryan et al 1971). The activities of these ectoenzymes are variably expressed on the cell surface of different cells and tissues, probably depending on the extent of effect of the purines on their function.

Both ATP and ADP were ineffective cortisol secretagogues in static incubations of freshly isolated cells, although a steroid response was evident by 24 h in culture, reaching a maximum at 48 h and declining thereafter (fig. 3.3). The rise and subsequent fall in cortisol response to ATP in culture is also observed for ACTH, adrenaline and angiotensin II; the change in responsiveness from freshly-isolated cells to cells in culture may be due to an increase in the number of viable receptors for the purines in the cultured cells or a greater availability of steroidogenic substrates. It is also possible that appearance of the response is an artefact of cell culture (this possibility is further addressed in Chapter 5). The subsequent decline in response after day 3 may reflect an uncoupling of the second messenger from steroidogenic capacity accompanied by a probable decline in steroidogenic enzyme activity (Walker et al 1991). Certainly, there is little or no reduction in cell number as determined by well protein content.

The ability of ATP and ADP to elicit a steroid response, the relative lack of potency of AMP, and the failure to explain the response on the basis of nucleotide degradation to adenosine initially suggested that the response was mediated by a purinergic P2 receptor. In the absence of specific purine receptor antagonists, purine receptor classification has largely depended on relative agonist potencies combined with second messenger studies. The agonists $\alpha\beta$ MeATP and $\beta\gamma$ MeATP are both potent at P2x receptors but were found to have low potency in bovine adrenal inner zone cells, arguing that the response is not

mediated by this receptor subtype (table 3.1). Furthermore, the P2x response is believed to operate ligand-gated ion channels, without the apparent involvement of G proteins or stimulation of membrane phosphoinositide turnover. The receptor is also clearly different from that found in platelets (P2t receptor), which is sensitive only to ADP and not to ATP, and the pore-forming ATP receptors (P2z) present in mast cells and fibroblasts.

Kawamura et al (1991) suggested, on the basis of agonist potency, that the steroid response occurred through a P2y receptor. However, the possible occurrence of ATP-stimulated membrane phosphoinositide turnover, as predicted on the basis of this classification, was not investigated. ATP, ADP and UTP all dose-dependently stimulated the accumulation of total [³H]-labelled phosphoinositols from cells whose phosphoinositides had been pre-labelled with ³H-inositol (figs. 3.9). The phosphoinositol head group response was linear for approximately 20 min (fig. 3.10) but declined thereafter indicating that the purinoceptor present in the inner zone cells of the adrenal cortex does not become rapidly desensitised with the continuous presence of the agonist. This contrasts with the rapid desensitisation (half-time 0.5 - 2 min) of the phospholipase C activity coupled to the P2y receptor described in turkey erythrocytes (Martin et al 1989) and the 5'-nucleotide receptor in human airway epithelial cells (Brown et al 1991). Thus, the experimental findings make it probable that the nucleotides activate a phosphoinositidase C.

This was confirmed by investigating the time-course of accumulation of the radiolabelled products InsP, InsP₂ and InsP₃ after stimulation by ATP (10⁻⁴ M)(fig. 3.12). Within 15s, the levels of [³H]IP₂ and [³H]IP₃ had increased significantly (p < 0.05) as compared to control (no ATP) reaching a maximum after 60s. Radiolabelling in InsP₁ lagged behind InsP₂ and InsP₃; a significant increase being apparent only after 60s (p < 0.05). The radiolabelling in each of the inositol phosphate species remained elevated above basal throughout the time-course (300s), consistent with the linear increase in total [³H]-inositol phosphates observed in fig. 3.10. The rapid and early increase in both InsP₂

and InsP_3 and before the increased labelling in InsP_1 is consistent with the earliest post-receptor event being a phospholipase C-dependent hydrolysis of either PtdInsP or $\text{PtdIns}(4,5)\text{P}_2$. The increased labelling of InsP_2 within 15s may also reflect a rapid formation and equally rapid dephosphorylation of $\text{Ins}(1,4,5)\text{P}_3$.

On this basis it is concluded that at early time points the phospholipase C is polyphosphoinositide-specific. This pattern of time-dependent phosphoinositol isomer accumulation as a result of ATP stimulation has been reported in a wide range of different tissues and cells including endothelial cells (Brock et al 1988), turkey erythrocytes (Berrie et al 1988), neuronal cells (Murrin et al 1991) and sheep pituitary cells (Davidson et al 1990). Recently, P2 purinergic receptors were reported in cells derived from the microvasculature of the adrenal medulla (Allsup et al 1990, Forsberg et al 1987) and in cultured bovine adrenal chromaffin cells (Sasakawa et al 1989). In both the adrenal medullary cells and the adjacent endothelial cells, ATP stimulated a rapid increase in InsP_2 and InsP_3 consistent with the activation of a polyphosphoinositide-specific phospholipase C.

The formation of inositol 1,4,5-trisphosphate released by the hydrolysis of PIP_2 by phosphoinositidase C would be expected to release $[\text{Ca}^{2+}]$ from an intracellular site (Berridge et al 1989). Concomitant rises in $[\text{Ca}^{2+}]_i$ and $\text{Ins}(1,4,5)\text{P}_3$ levels have been demonstrated in rat adrenal glomerulosa cells stimulated by All (Balla et al 1989) and in bovine adrenal chromaffin cells in culture stimulated by ATP (Sasakawa et al 1989). Experiments on fura-2 loaded inner zone cells demonstrated a clear effect of ATP (10^{-4}M) in elevating the intracellular $[\text{Ca}^{2+}]$ from a mean basal (resting) value of 57.3 nmol/L to a mean stimulated value of 171 nmol/l. Matsui et al (1992) also reported that ATP led to an increase in intracellular $[\text{Ca}^{2+}]$ in bovine adrenocortical inner zone cells and that extracellular Ca^{2+} was required for a sustained elevation of the intracellular $[\text{Ca}^{2+}]$. Similar findings were obtained in bovine zona fasciculata cells (Niitsu et al 1992), adrenal chromaffin cells (Sasakawa et al 1989), pulmonary artery endothelial cells (Lustig et al

1992), Ehrlich Ascites tumor cells (Dubyak et al 1985), rat hepatocytes (Okajima et al 1987) and human glomerular epithelial cells (Pavenstadt et al 1992). Niitsu (1992) also found that the steroid response to ATP was inhibited by calmodulin antagonists, but apparently unaffected by dihydropyridine Ca^{2+} channel blockers.

Recently, a further type of purine receptor has been provisionally identified to which both ATP and the pyrimidine nucleotide, UTP, bind. The term nucleotide receptor was originally introduced by Davidson and colleagues (1990) to describe the ATP/UTP sensitive site on sheep pituitary cells, though it was pointed out that a heterogeneous receptor population might explain the findings, with classical P_{2y} purinoceptors and nucleotide receptors present, each of which resulted in the same functional response. Two separate, co-existing receptor populations ; P_{2y} purinoceptors and nucleotide receptors (P_{2u}) are present in aortic endothelial cells (Wilkinson et al 1993) which both activate phospholipase C. In mammalian tissues, nucleotide receptors may be coupled to a variety of signal transduction mechanisms involving phospholipase C, phospholipase A₂, phospholipase D and the activation of membrane Ca^{2+} channels (Lushy et al 1992).

Closer examination of the purinergic response in bovine adrenocortical inner zone cells demonstrates that it fulfils a number of the criteria presently used to define this nucleotide receptor; in particular, an ability to respond to UTP, in addition to ATP, and the relative lack of potency of 2-methylthioATP which is a superactive agonist at the classical P_{2y} receptor described in endothelial cells (Martin et al 1985), taeni coli (Burnstock et al 1983) and pancreatic β cells (Bertrand et al 1987).

A more definite classification of ATP and UTP receptors is not yet possible due to the lack of selective, competitive receptor antagonists. Although a number of compounds have been tested for this purpose, only the anti-trypanosomal drug, suramin, has proven to be an efficacious and reversible antagonist. Dunn et al (1988) provided the first evidence that suramin was a competitive antagonist at P_{2x} purinoceptors. They showed that in mouse vas deferens 100 μM suramin inhibited the contractile response to the P_{2x}

purinoceptor agonist $\alpha\beta$ methylene ATP. Subsequently, suramin has been shown to inhibit responses at both P2x and P2y purinoceptors in guinea-pig urinary bladder, taeni coli (Hoyle et al 1990), vas deferens DDT, MF-2 smooth muscle cells (Hoiting et al 1990), mast cells and basophilic leukocytes (Osipchuk et al 1992). Suramin was relatively ineffective at inhibiting ATP or UTP-stimulated cortisol secretion in the bovine inner zone cells. Partial inhibition was only observed (in 2 experiments) at the highest dose of suramin (10^{-3}M). Similar findings were reported in aortic endothelial cells (Wilkinson et al 1993) and mouse myotubules (Henning et al 1992) where suramin was less effective at the nucleotide receptors in these cells indicating that it may have a differential effect on the response to P2 and nucleotide receptor stimulation. This finding further supports the view that the P2y purinoceptor is not responsible for the actions of ATP and UTP in bovine zfr cells.

It is important to determine whether ATP and UTP are acting at the same receptor or two separate recognition sites (nucleotide receptor and pyrimidine receptor) linked to the same functional response through a common transduction pathway. Evidence for the existence of a common extracellular receptor, for both ATP and UTP, has been investigated in many tissues by performing simple additivity experiments. In bovine zfr cells, the ATP and UTP responses at maximal concentrations (10^{-4}M) were not additive ($p > 0.05$) after simultaneous application compared to each agonist alone (fig. 3.5). In marked contrast, the responses of maximally effective concentrations of All and ATP were additive ($p < 0.05$) when compared with the response observed with All and ATP alone. Similar findings were reported in neuronal cells (Murrin et al 1991), pituitary cells (Davidson et al 1990), human airway epithelia cells (Brown et al 1991), aortic endothelial cells (Wilkinson et al 1993) and mouse myotubules (Henning et al 1992) as evidence for the presence of a nucleotide receptor.

In conclusion, a more definite pharmacological characterisation will probably await the application of a suitable radioligand binding assay along with the development of more selective analogues. However the following points arise from the data presented

here as evidence of a nucleotide receptor; 1). the relative agonist potency order obtained was identical to that previously reported for a P_{2u} purinoceptor (O'Connor 1992), 2). the responses measured (cortisol and phosphoinositide turnover) showed no dissociation between ATP and UTP, 3). there was no apparent additivity of the responses elicited by maximally stimulatory doses of ATP and UTP and 4). the P₂ purinergic antagonist, suramin, was relatively ineffective consistent with observations made in other tissues that express the nucleotide receptor.

Finally, the importance of these observations with regard to normal regulation of adrenocortical function is unknown and must remain speculative at present; as yet there is no physiological evidence for ATP and UTP effects on adrenocortical function. The experimental findings reported here, with other circumstantial evidence, suggest that ATP may regulate adrenocortical function *in vivo*. This is discussed in more detail in Chapter 7.

CHAPTER 4

CHARACTERISATION OF THE ADENOSINE A₂ RECEPTORS IN PRIMARY CULTURES OF BOVINE INNER ZONE ADRENOCORTICAL CELLS AND EVIDENCE THAT ATP IS A LIGAND AT THIS RECEPTOR SUBTYPE.

4.1. INTRODUCTION.

In 1970, Sattin et al showed that adenosine stimulated the formation of cyclic AMP in guinea-pig cerebral cortex and suggested the existence of specific extracellular receptors coupled to adenylate cyclase. Subsequently, extracellular adenine nucleotides have been shown to interact with a variety of receptor subtypes to influence many biological processes, including platelet aggregation, neurotransmission, cardiac function and muscle contraction (Gordon et al 1986).

Initially, Burnstock (1978) introduced two major purine receptor subclasses termed, P1 (Adenosine) and P2 (ATP). The adenosine receptors have since been classified into two major subtypes according to their ability to increase (A₂) or decrease (A₁) adenylate cyclase activity. Further sub-classification of the P1-purinergic receptors was made on the

basis of agonist structure-activity relationships and the use of the antagonists 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (Daly et al 1986) and CGS 15943A (Williams et al 1987). For the A_1 receptor the rank order of agonist potencies is such that N^6 -derivatives of adenosine such as N^6 -cyclopentyladenosine (CPA) are more potent than 5'-analogues such as 5'-N-ethylcarboxamidoadenosine (NECA), while the reverse is true for the A_2 receptor (Daly et al 1986). Although the existence of A_1 receptor subclasses is not clear cut, these agents also provided the tools by which adenosine receptors were classified into A_1 , A_{2a} and A_{2b} subtypes. Bruns et al (1986) further proposed that A_2 receptors with a high affinity for adenosine be designated A_{2a} and those with a low affinity A_{2b} .

Kowal et al (1969) first reported that ATP and adenosine stimulated steroidogenesis in monolayer cultures of adrenal cells derived from transplantable murine adrenal tumours. In contrast to this, Cooper et al (1978) reported that adenosine had no effect on basal corticosteroid production by isolated rat adrenocortical cells. However, recently it was reported that ATP stimulates cortisol secretion from bovine zona fasciculata/reticularis cells in culture, via a $P2_u$ ("nucleotide") purinoceptor, linked to an activation of phospholipase C and of intracellular Ca^{2+} (Hoey et al 1994).

The aim of this chapter is to characterise the adenosine response with respect to steroidogenesis and second messenger generation and the $P1$ purinoceptor subtype, present in the inner zone cells of the adrenal cortex, through which purines may mediate these responses.

4.2. RESULTS.

4.2.1. The effect of adenosine and its analogues on cortisol secretion from zona fasciculata/reticularis (zfr) cells.

The effects of adenosine, N⁶-cyclopentyladenosine (CPA) and 5'-N-(ethylcarboxamido)adenosine (NECA) on the secretion of cortisol over a 60 min period from bovine zfr cells at 48h in primary culture are shown in figs. 4.1 and 4.2. Adenosine produced a dose-dependent increase in cortisol secretion when compared to basal (no agonist) over the same time period. The threshold concentration of adenosine eliciting a significant increase in steroid secretion was 10⁻⁶ M or lower (p < 0.05) in each of the three experiments carried out (n = 3 experiments). In the case of the A₁ receptor agonist, CPA, and the A₂ receptor agonist, NECA, the lowest concentrations that elicited a significant increase in steroid secretion were 10⁻⁵ M and 10⁻⁷ M respectively (p < 0.05 in each of the 3 experiments). The potency order was thus NECA > Adenosine > CPA.

4.2.2 Second messenger responses to adenosine.

The accumulation of water-soluble, [³H]-inositol labelled head groups was measured in the presence of increasing doses of adenosine (10⁻⁸ M - 10⁻³ M), over a 15 min incubation. In two separate experiments, adenosine failed to increase total labelled head groups above basal (data not shown).

Fig. 4.3a. illustrates the effect of increasing doses of adenosine on the intracellular accumulation of cyclic AMP (measured at 1 min) in the inner zone cells after 48h in primary culture; a dose-dependent increase in cyclic AMP formation was observed in each of the three separate experiments, with a threshold increase (relative to basal) achieved at 10⁻⁶ M adenosine or lower.

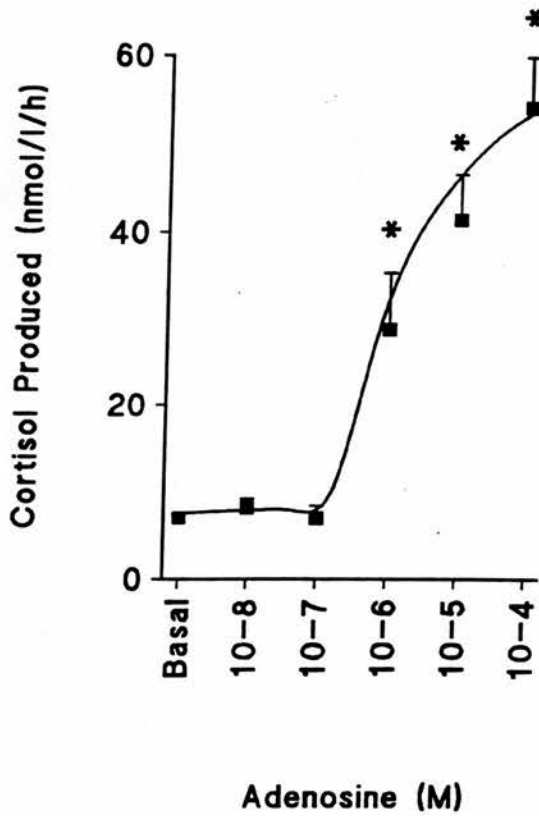


FIGURE 4.1.

Cortisol secretion over a period of 60 minutes was measured in the overlying medium from cells stimulated with adenosine (dose-range: 10^{-8} - 10^{-4} M) at 48 hours in primary culture. The threshold concentration of adenosine eliciting a significant increase in cortisol secretion above basal secretion was 10^{-6} M in each of three different experiments (* $p < 0.05$). Each point is the mean \pm S.D. of triplicate determinations from a representative experiment carried out on 3 separate occasions.

FIGURE 4.2.

Cortisol secretion over a period of 60 minutes was measured in the overlying medium from cells stimulated with the A₁ agonist cyclopentyladenosine (CPA) and the A₂ agonist 5'-N-ethylcarboxamide adenosine (NECA) (dose range: 10⁻⁸ -10⁻⁴ M) at 48 hours in primary culture. The threshold concentrations of CPA and NECA eliciting a significant increase in cortisol secretion were 10⁻⁵ M and 10⁻⁷ M, respectively, in each of three different experiments (* p < 0.05). Each point is the mean ± S.D. of triplicate determinations from a representative experiment carried out on 3 separate occasions.

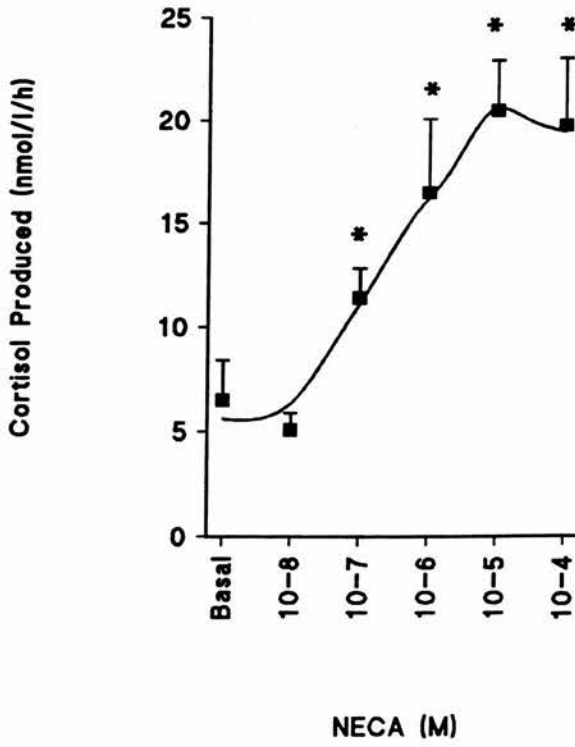
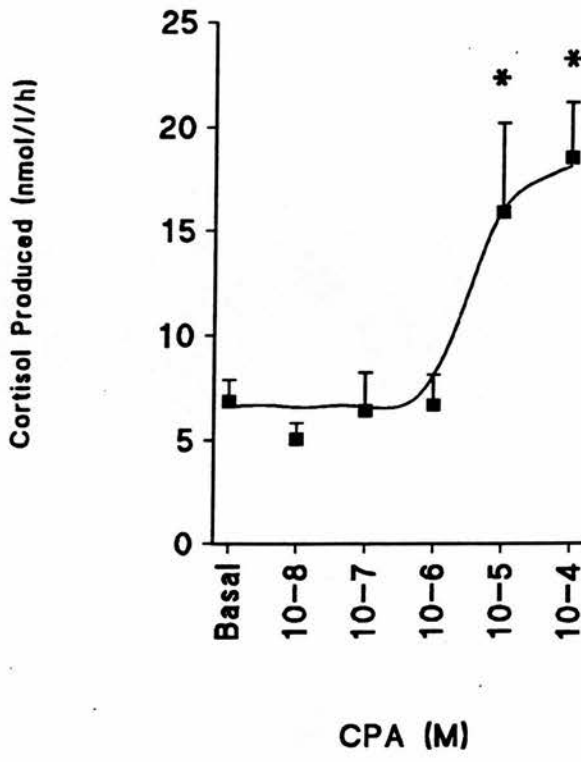
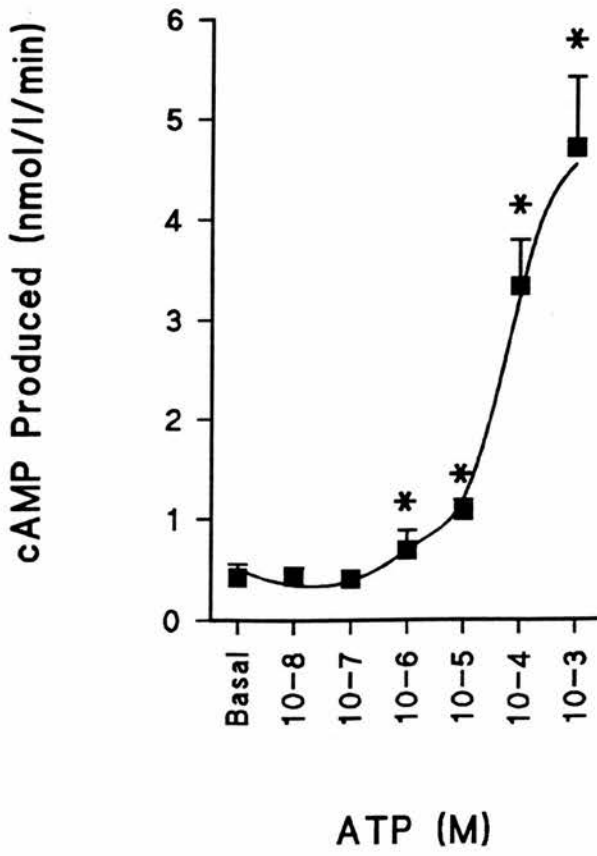
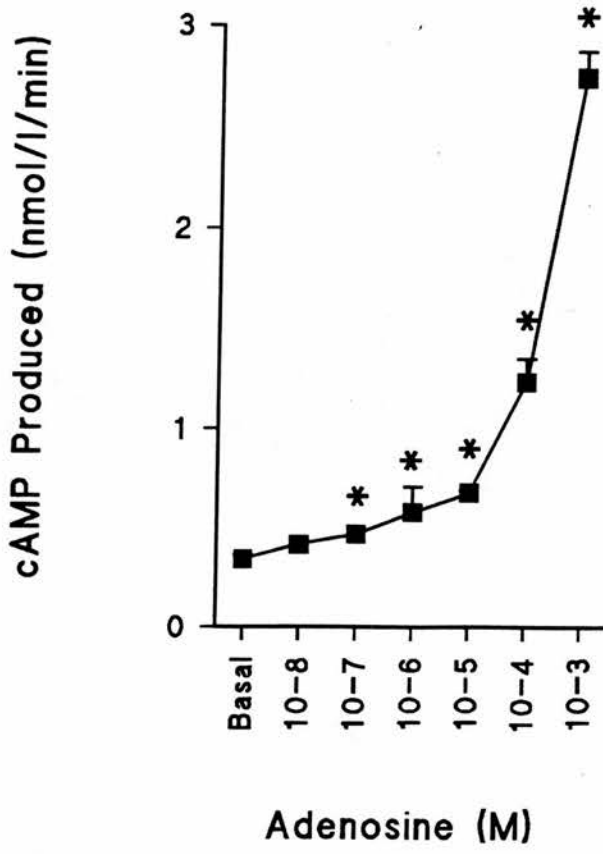


FIGURE 4.3.

The cellular cyclic AMP production was measured over a one minute period from cells stimulated with the concentrations of adenosine and ATP shown after 48 hours in primary culture. Basal cyclic AMP formation is also shown. Each point is the mean \pm S.D. for 3 wells from a representative experiment. Significant stimulation of cyclic AMP relative to basal was observed at 10^{-6} M adenosine and ATP or lower (* $p < 0.05$) in all 3 experiments.



4.2.3. The effects of ATP, ADP and UTP on cAMP Formation.

It is known that, unlike adenosine, ATP, ADP and UTP stimulate the production of phosphoinositols (Chapter 3). In bovine zfr cells, each of these agonists elicit a dual second messenger response; also producing a dose-dependent increase in the intracellular cyclic AMP measured at 1 min (fig. 4.3a, 4.4a and 4.4b). The threshold concentration producing a significant increase in cyclic AMP production occurred at 10^{-6} M or lower for ATP and UTP and at 10^{-5} M for ADP in each of the 3 separate experiments.

4.2.4. The effect of A₁ and A₂ receptor antagonists on adenosine-stimulated cortisol production.

The class of adenosine receptor mediating the cortisol response was further investigated by using the selective A₁ receptor antagonists DPCPX and 8-CPT and the A₂-selective receptor antagonist CGS 15943A. Inhibition concentration-response curves were obtained under antagonist equilibrium conditions (30 min preincubation) in the presence of a constant adenosine concentration (10^{-4} M). The data are expressed as a % of the response to 10^{-4} M adenosine. In order to take account of variation in cell number, data were normalised as follows: the % response was calculated as $R = (S_a - B_a)/(S - B) \times 100\%$, where S and S_a are the agonist responses in the absence and presence, respectively of the antagonist and B and B_a are the basal steroid responses (no agonist) in the absence and presence, respectively, of antagonist. The IC₅₀ for CGS 15943A was $6.8 \pm 1.5 \times 10^{-6}$ M (n=3) compared to the weaker antagonist DPCPX which had an IC₅₀ value of $5.5 \pm 2.1 \times 10^{-5}$ M (n=3) (fig 4.5). The A₁ antagonist 8-CPT failed to inhibit the adenosine response over the dose-range used in two separate experiments (10^{-8} M - 10^{-3} M) (data not shown). The order of potencies for inhibition of the adenosine response, based on the IC₅₀ values is thus; CGS 15943A > DPCPX > CPT.

FIGURE 4.4.

The cellular cyclic AMP production was measured over a one minute period from cells stimulated with the concentrations of ADP and UTP shown after 48 hours in primary culture. Basal cyclic AMP formation is also shown. Each point is the mean \pm S.D. for 3 wells from a representative experiment. Significant stimulation of cyclic AMP relative to basal were observed at 10^{-6} M UTP and 10^{-5} M ADP, respectively, (* $p < 0.05$) in all 3 experiments.

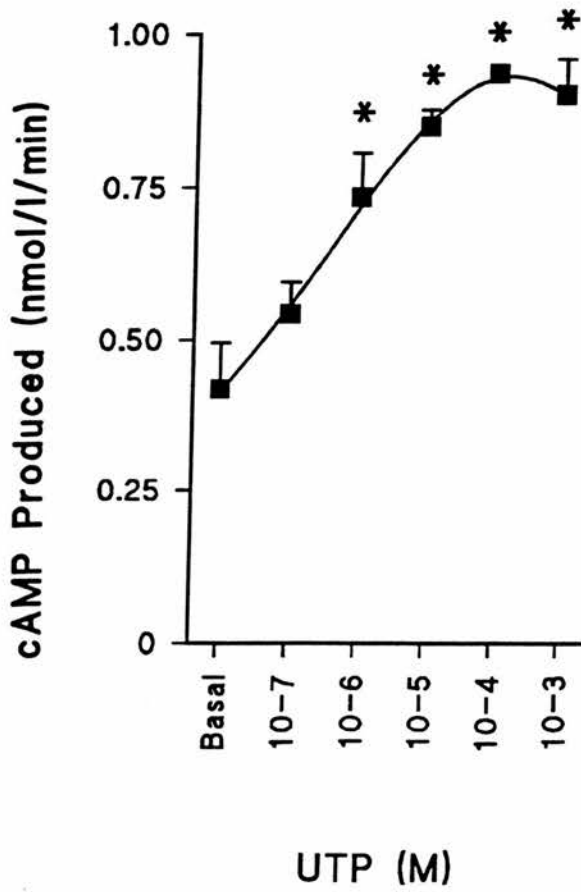
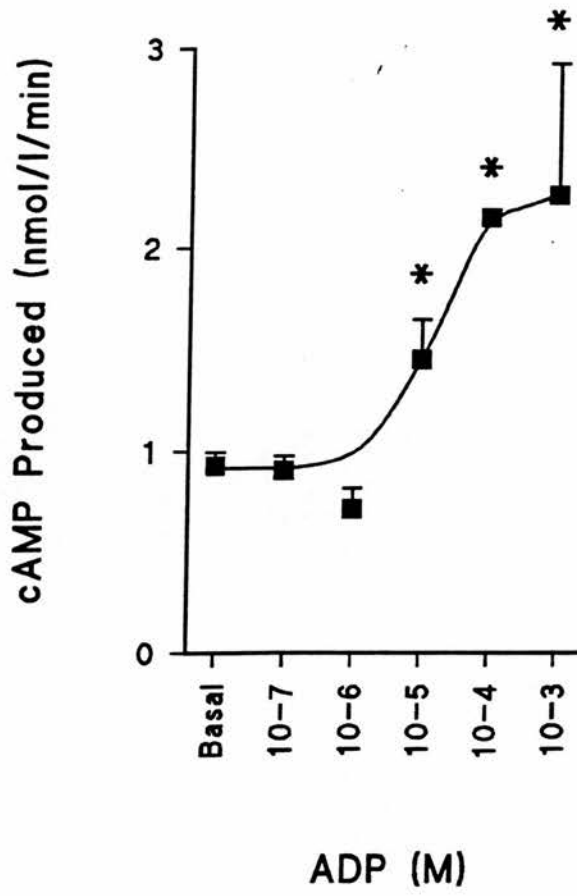
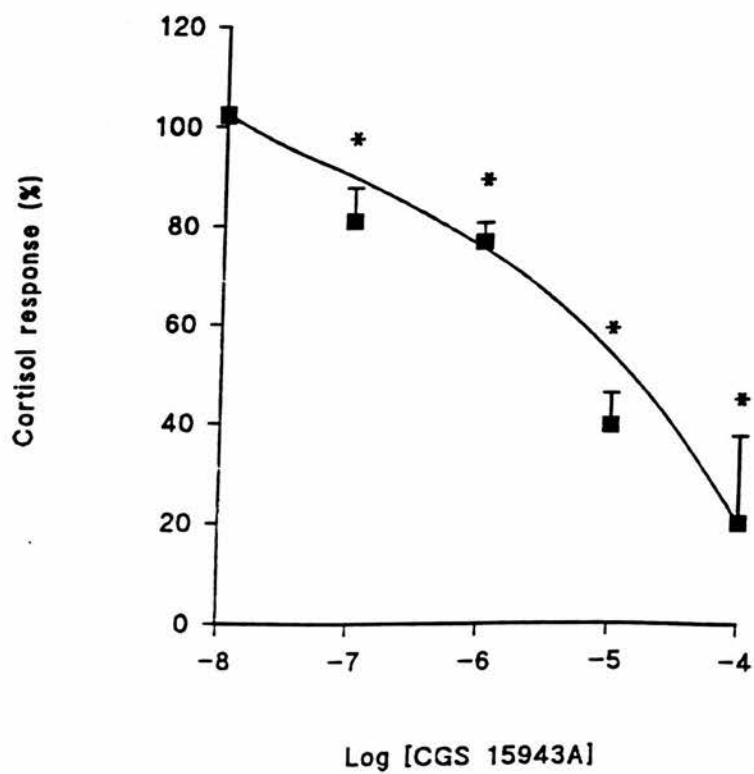
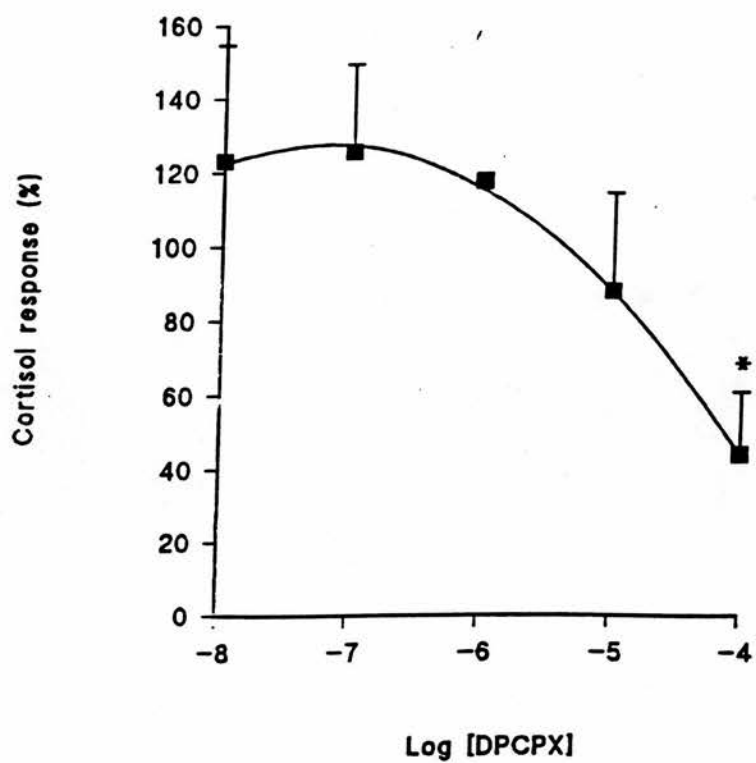


FIGURE 4.5.

Inhibition of adenosine-stimulated cortisol secretion by the A₁ antagonist, DPCPX, and the A₂ antagonist, CGS 15943A is shown. The incremental (i.e. above basal) response to adenosine (10⁻⁴ M) alone is set at 100%. Cells at 48 hours in primary culture were incubated for 30 minutes with the indicated drug concentrations before the addition of adenosine (10⁻⁴ M) for a 60 minute period. Significant inhibition of the incremental increase in cortisol secretion in response to adenosine was observed with 10⁻⁴ M DPCPX and 10⁻⁷ M CGS 15943A, respectively, (* p < 0.05 or less). Each point represents the mean ± S.D. from 3 combined experiments.



4.2.5. The effect of A₁ and A₂ receptor antagonists on ATP-stimulated cortisol production.

In the case of the A₁ antagonist DPCPX, significant inhibition of ATP-stimulated cortisol secretion was observed at 10⁻⁴ M DPCPX and at 10⁻⁷ M for the A₂ antagonist CGS 15943A (*p* < 0.05 in all 3 experiments) (fig. 4.6). The A₁ antagonist 8-CPT failed to inhibit the ATP response over the dose-range used in two separate experiments (10⁻⁸ M - 10⁻³ M)(data not shown). The IC₅₀ values for the antagonists, CGS 15943A and DPCPX, are 1.6 ± 1.0 × 10⁻⁵ M and 1.1 ± 0.4 × 10⁻⁴ M (n=3) respectively. The order of potencies for inhibition of the ATP response is therefore the same as that for adenosine; CGS 15943A > DPCPX > CPT.

4.2.6. The effect of the A₂ antagonist, CGS 15943A, on second messenger responses to ATP.

Fig. 4.6b. shows that CGS 15943A inhibited the steroidogenic response of the inner zone cells to ATP. Approximately 50-60 % of the cortisol response to ATP was inhibited using 10⁻⁴ M of the antagonist (table 4.1). To eliminate the possibility that CGS 15943A may be non-selective and antagonise the steroidogenic response to ATP at both the P1 and P2 purinoceptors, the % inhibition (100 - R) of the second messenger responses (cAMP (P1) and phosphoinositide turnover (P2)) were also measured in the presence of 10⁻⁴ M CGS 15943A. The cAMP response was inhibited by approximately 50% though the phosphoinositide turnover was not significantly altered (table 4.1).

FIGURE 4.6.

Inhibition of ATP-stimulated cortisol secretion by the A₁ antagonist, DPCPX, and the A₂ antagonist, CGS 15943A is shown. The incremental (i.e. above basal) response to ATP (10⁻⁴ M) alone is set at 100%. Cells at 48 hours in primary culture were incubated for 30 minutes with the indicated drug concentrations before the addition of ATP (10⁻⁴ M) for a 60 minute period. Significant inhibition of the incremental increase in cortisol secretion in response to ATP was observed with 10⁻⁴ M DPCPX and 10⁻⁷ M CGS 15943A, respectively, (* p < 0.05 or less). Each point represents the mean ± S.D. from 3 combined experiments.

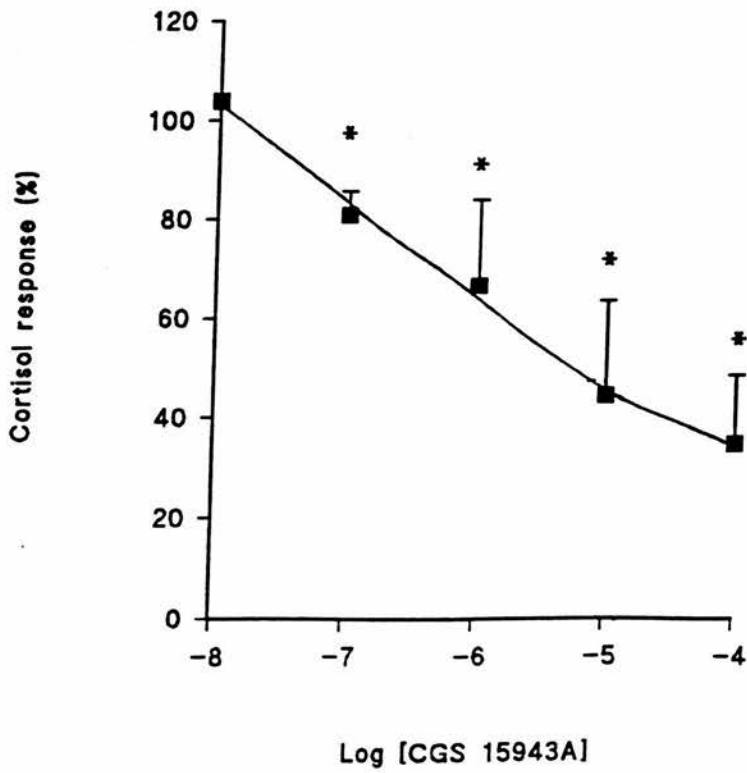
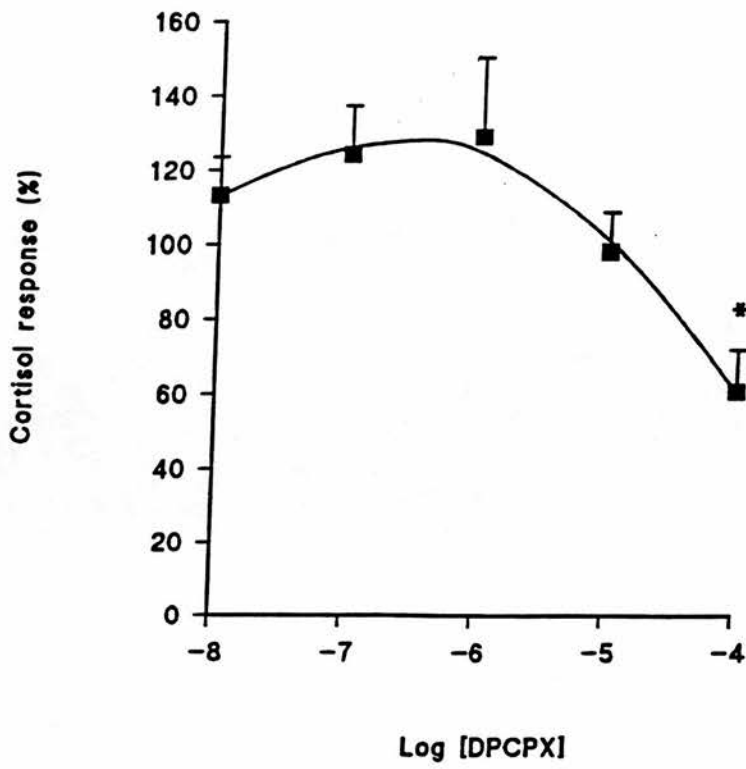


Table 4.1.

Response measured	% Inhibition (by CGS 15943A) of the cortisol or 2nd messenger response to ATP (10⁻⁴M).
Cortisol	54.15 ± 4.00 (n=9 wells, 3 expts)
³ H-labelled total head groups	7.66 ± 4.41 (n=6 wells, 2 expts)
cAMP	50.44 ± 10.9 (n=6 wells, 2 expts)

Table 4.1. Inhibition of the ATP-stimulated cortisol and second messenger responses in bovine zfr cells in culture by the A₂ antagonist CGS 15943A. The % inhibition of the increment in cortisol or second messenger (phosphoinositide turnover and cAMP) responses to ATP (10⁻⁴ M) above basal were measured in the presence of a drug concentration of 10⁻⁴ M.

4.3. DISCUSSION.

Although, it has been known for some time that acute administration of adenosine to rats elevated plasma steroid levels (Formento et al 1975), early studies on the mechanism of action of adenosine on adrenocortical cells were contradictory. In the mouse adrenal tumour cell line (Y-1) adenosine was found to stimulate both steroidogenesis (Kowal et al 1969) and adenylate cyclase activity (Wolff et al 1977, Londos et al 1980) while in normal cells (zfr) from decapsulated rat adrenal glands, adenosine was shown to inhibit both steroid secretion and adenylate cyclase activity (Cooper et al 1978, Shima et al 1986). It is unclear if this discrepancy simply reflects a striking contrast between normal adrenal tissue and adrenal tumour or whether other factors are involved.

Whilst several conflicting studies have been carried out on the effects of adenosine on rat adrenal tissue, little information exists in relation to its effects upon normal bovine adrenocortical cells. The data in this study show that adenosine dose-dependently stimulates the production of cortisol, with a threshold response at 10^{-6} M, from primary cultures of zfr cells isolated from the bovine adrenal cortex.

Several thousand adenosine agonists have been synthesised over the past 40 years. Two agonists were selected to characterise the P1 subtype in zfr cells; N^6 -cyclopentyladenosine (CPA), which is 400-800 fold selective for the A_1 subtype (Trivedi et al 1989) and NECA which has been extensively used to define tissue responses mediated by the A_2 receptor. Each of these agonists elicited cortisol secretion from these cells, although NECA was much more potent than CPA with a threshold response at 10^{-7} M compared to 10^{-5} M for the A_1 agonist (fig. 4.2). The order of potency in bovine adrenocortical cells in culture, NECA > adenosine > CPA, is consistent with that observed in other tissues which possess stimulatory A_2 receptors (Lazarowski et al 1992 and Hourani et al 1993).

The presence of A₂ receptors in the inner zone of the cortex would imply that adenosine was capable of stimulating adenylate cyclase activity. Data from this study showed that adenosine dose-dependently increased intracellular cyclic AMP measured at 1 min (fig. 4.3); previous studies from this laboratory have shown that the intracellular cyclic AMP is already maximal at 1 min, thereafter falling and accumulating over a longer period in the overlying medium (Lightly, PhD thesis 1991).

More recently, additional transduction mechanisms, such as the stimulation of phospholipase C activity and increases in intracellular calcium, have also been implicated in the effects of adenosine on target cells, particularly those mediated by the A₁ receptor subtype (Cooper et al 1989 and White et al 1992). A recent report showed that adenosine stimulates phospholipase C and Ca²⁺ mobilisation in the rat tumour-derived mast cell line RBL-2H3 with a rank order of potency of agonists characteristic of an A₂ adenosine receptor (Ali et al 1990). However, in bovine zfr cells, there was no increase in total [³H]-labelled phosphoinositols in response to adenosine (in 2 separate experiments; data not shown).

A more convincing argument for the characterisation of the P1 receptor subtype lies in the use of highly selective antagonists that are active *in vivo*. 8-CPT and DPCPX are both highly selective antagonists at the A₁ subtype: DPCPX, in particular, has selectivity for this particular subtype (Lohse et al 1987). The stimulatory effect of adenosine on cortisol secretion was antagonised by DPCPX (fig. 4.5.). At low concentrations, DPCPX elevates the cortisol response to adenosine thus appearing to act as a partial agonist (fig. 4.5. and 4.6.). However at the highest concentration examined (10⁻⁴ M) this antagonist reduced the stimulatory effect of adenosine by about 45%. It is now clear that DPCPX has nanomolar affinity at A₁ receptors whereas it has micromolar affinity at A₂ receptors (Jacobson 1990, Collis 1990). Thus the calculated IC₅₀ for DPCPX of 5.5 ± 2.1 × 10⁻⁵ M, along with the observation that 8-CPT failed to inhibit the adenosine response even at the highest concentration used (10⁻⁴ M) argues strongly that the adenosine

receptor is of the A₂ class.

The triazoloquinazoline CGS 15943A, is a novel non-xanthine adenosine antagonist with a 7-fold selectivity for the A₂ subtype (Francis et al 1988). This compound is a potent inhibitor of the adenosine response in bovine zfr cells showing significant inhibition at 10⁻⁷ M (15%) (fig. 4.5), rising to about 60% at the highest concentration used (10⁻⁴ M). Similar observations were reported in guinea-pig synaptoneurosome, where approximately 50% of the adenosine-stimulated adenylate cyclase activity was inhibited by 10⁻⁴ M CGS 15943A (Williams et al 1987). The antagonist potency order is thus, CGS 15943A > DPCPX > 8-CPT, which is again strongly indicative of an A₂ receptor subtype for the adenosine-stimulated cortisol response in bovine zfr cells.

It has been claimed recently that in several cell types, ATP can induce a stimulation of cAMP production. Acetylcholine and AII are known to secrete cortisol from inner zone cells through a stimulation of phosphoinositidase C but in both cases no effect on cellular cyclic AMP formation was observed (Bird et al 1990). In contrast, both ATP and ADP produced a dose-dependent increase in intracellular cyclic AMP, at 1 min, with a threshold at 10⁻⁶ M and 10⁻⁵ M respectively, with both achieving a maximum at 10⁻⁴ M (Hoey et al 1994). An area of continuing controversy is whether adenine nucleotides are capable of activating the adenosine receptor directly or whether they must first be converted to adenosine by ectonucleotidases. At 1 min (the time at which our cyclic AMP measurements were made) the FPLC confirmed that there was no detectable degradation of ATP (Chapter 3, fig. 3.8a), establishing that the cyclic AMP response must result from the action of ATP itself. Niitsu (1992) also found that ATP increased cyclic AMP from bovine inner zone cells, but only at the highest dose used (10⁻³ M). Moreover, the measurements were made after 1h, so that degradation of ATP to AMP (which might increase cyclic AMP by acting as a weak agonist at the A₂ receptor) would confound the interpretation of these data (Chapter 3, fig. 3.8c). The FPLC results make it clear that a significant accumulation of AMP has occurred by 1h, presumably as a result of

ectophosphatase activity, since chemical degradation is insignificant at 1h.

It should be noted that the zfr cultures were obtained using a column purification system which leads to very low contamination with zona glomerulosa (zg) cells (Williams et al 1989). It is therefore unlikely that the dual second messenger response can be explained by the presence of two distinct cell types or, indeed, that the increase in cortisol occurs through indirect release of zg products acted upon by zfr cells.

A further possibility is that the stimulation of cyclic AMP by ATP may be solely due to leaky membranes in the bovine zfr cells, possibly as a result of the collagenase treatment they are subjected to during their preparation. If this was the case, ATP could diffuse into the cell and be converted, by adenylate cyclase, to cyclic AMP. However, this explanation seems unlikely since, like both ATP and ADP, UTP stimulated the production of cyclic AMP in a dose-dependent manner with a threshold at 10^{-6} M and a maximum at 10^{-4} M (Hoey et al 1994). Also, experiments were carried out at 48h after primary isolation, by which time any membrane damage is likely to be fully repaired.

The most likely explanation is that, ATP activates two different effector pathways and that this could be due either to the action at P2 receptors coupled to both adenylate cyclase and phospholipase C, as reported in hepatocytes (Okajima et al 1987), or alternatively to the separate actions of ATP at the P1 receptor (coupled to adenylate cyclase) and the P2 receptor (coupled to phospholipase C). By examining the effects of P1 receptor antagonists on ATP-induced cortisol secretion and second messenger function it was possible to distinguish between these possibilities.

In the case of adenosine, the A_2 antagonist, CGS 15943A was the most potent inhibitor of ATP-induced steroidogenesis (IC_{50} of $1.6 \pm 1.0 \times 10^{-5}$ M compared to $1.1 \pm 0.4 \times 10^{-4}$ M for DPCPX), with about 65% inhibition at the highest dose used (10^{-4} M) (fig. 4.6). The pyrimidine, UTP, gave similar results to ATP (data not shown). The following antagonist potency order for ATP (and UTP) was established; CGS 15943A > DPCPX > 8-CPT, suggesting that both ATP and UTP act via the same receptor as adenosine, the

A₂ subtype, to mediate cAMP formation. This conclusion is further strengthened by the effects of the A₂ antagonist, CGS 15943A, on the two second messengers responses activated by ATP (cAMP and inositol phosphates). Table 4.1 shows that CGS 15943A (10⁻⁴ M) inhibited approximately 50% of the ATP-stimulated cAMP production but inositol phosphate production was not significantly altered, implying that the two different effector pathways activated by ATP are linked to two different receptors.

In conclusion, with the aid of agonist potency orders, second messenger studies and selective antagonists, the effect of adenosine in bovine zfr cells, has been shown to be mediated by the A₂ subtype of the P1 receptor. The purines ATP and UTP activate two different effector systems; inositol phosphate formation which is linked to the P2u purinoceptor (Hoey et al 1994) and cAMP formation which is linked to the same P1 receptor as adenosine (A₂ subtype). The physiological significance of adenosine in the regulation of adrenocortical function is as yet unknown. Chromaffin granules release high concentrations of ATP and adenosine along with catecholamines which may inhibit or excite (Chern et al 1988) the further release of purines in a feedback manner. In view of the morphological evidence for a close proximity of medullary and adrenocortical cells (Bornstein et al 1991, Gallo-Payet 1987) the possibility clearly exists for a paracrine effect of released purines, on adenocortical function.

CHAPTER 5

STUDIES ON THE PURINERGIC REGULATION OF CORTISOL SECRETION FROM BOVINE ZONA FASCICULATA/RETICULARIS CELLS USING A SUPERFUSION SYSTEM.

5.1. INTRODUCTION

Cell cultures have been used extensively to address specific physiological and biological functions in endocrine tissues. Cortisol secretion from bovine zfr cells has also been extensively studied in static incubation experiments (Walker et al 1988), although this *in vitro* method still inadequately mimics an ideal adrenal microenvironment despite its many attractive features.

The major disadvantages of cell culture are; 1) the medium remains unchanged and unstirred over an extended period of time resulting in an accumulation of products which may lead to uncontrolled conditions within culture, 2) the bioavailability of hormones added to the culture medium is not adjustable and 3) the measured morphological attributes and physiological functions of cells in culture may differ from *in vivo* as a result of dedifferentiation. To study the dynamics of adrenocortical steroid secretion *in vitro*, it is desirable to have a cell population that is not only stable for long durations but is also easily manipulated and can be continuously monitored without compromising cellular integrity.

Over the years, superfusion has shown great promise in satisfying the requirements of a more physiological environment. The word superfusion was originally used in a pharmacological context to describe "experiments in which suitable liquids run over tissues suspended in air " and where the drug under investigation could be tested by injecting it into the stream of fluid (Gaddum et al 1953).

Superfusion methods are not uncommon in the study of steroid production, allowing systematic investigation without the extra-adrenal metabolism of steroids or the dilution of secretory products *in situ*. Tait et al (1967) studied the time-course of changes of production of the four major adrenal steroids of the rat using a superfusion system. This was followed by reports that physiological doses of ACTH caused an increase in 11-OH corticosteroids (Lowry et al 1974) and corticosterone (Al-Dujaili et al 1981, Persengiev et al 1989) following *in vitro* superfusion of rat adrenals. Isolated perfused human adrenal cells, containing a mixture of zg and zfr cells, behaved similarly to the rat adrenal cells by producing an increase in corticosterone and aldosterone in response to ACTH (Al-Dujaili et al 1981).

In this chapter, in addition to investigating the interactions of ATP with the two main regulators of adrenocortical steroidogenesis *in vivo*, ACTH and All, the novel superfusion system was used to determine if the ATP response was present in freshly-isolated cells. Freshly dispersed cells used immediately after collagenase digestion of adrenocortical tissue, showed no significant secretion to ATP, when incubated in a static system (see Chapter 3). One possibility is that it may be necessary to continuously replenish ATP, if ATPase activity is high after initial cell isolation, thereby rapidly destroying added ATP. This would be overcome by using the superfusion system. To maximise sensitivity and to allow the measurement of steroid output at short time intervals, cortisol was chosen for measurement as an indication of steroidogenesis and quantified using a sensitive radioimmunoassay technique.

5.2. METHODS.

5.2.1. Superfusion

The isolation and purification of bovine zona fasciculata/reticularis cells is described in detail in section 2.2.1. The freshly-isolated cells were either used immediately or stored overnight, in Hams F10 medium, at 4°C. In both situations, the cells were centrifuged at 450g for 15 minutes, the supernatant discarded and the cells resuspended in EBS/0.2% BSA/0.1% (w/v) glucose at 10⁶ cells/ml. Preliminary experiments (see Results p160-164) found that cells kept overnight at 4°C were at least as responsive as freshly isolated cells used immediately. Accordingly, most experiments used the cells stored overnight at 4°C.

The superfusion technique followed in broad outline the protocol described by Fenske et al (1983). Cells (10⁶) were mixed with 0.2g of moist Bio-Gel P2 (200-400 mesh) which had previously been swollen in 0.2ml of 0.9% NaCl and washed in the superfusion medium (EBS/0.2% BSA/0.1% (w/v) glucose). The cells were placed in each superfusion chamber/column and the columns perfused simultaneously at 37°C in each experiment, using a multichannel peristaltic pump, with oxygenated medium (95% oxygen/5% carbon dioxide, pH 7.4) and a flow rate of 0.25ml/min. The perfusion medium was equilibrated for 60 min without sample collection prior to each experiment, to stabilise basal secretion of cortisol. During the perfusion experiment, fractions were collected every 5 min. Basal conditions, using only medium in the superfusion lines, were interrupted every 30 (6 fractions) or 50 (10 fractions) minutes, depending on the type of experiment, by a 5 min stimulation with agonist solution.

Fractions were removed and assayed for cortisol content using a sensitive radioimmunoassay technique. Figure 5.1 shows a schematic representation of the superfusion set-up used.

FIGURE 5.1.

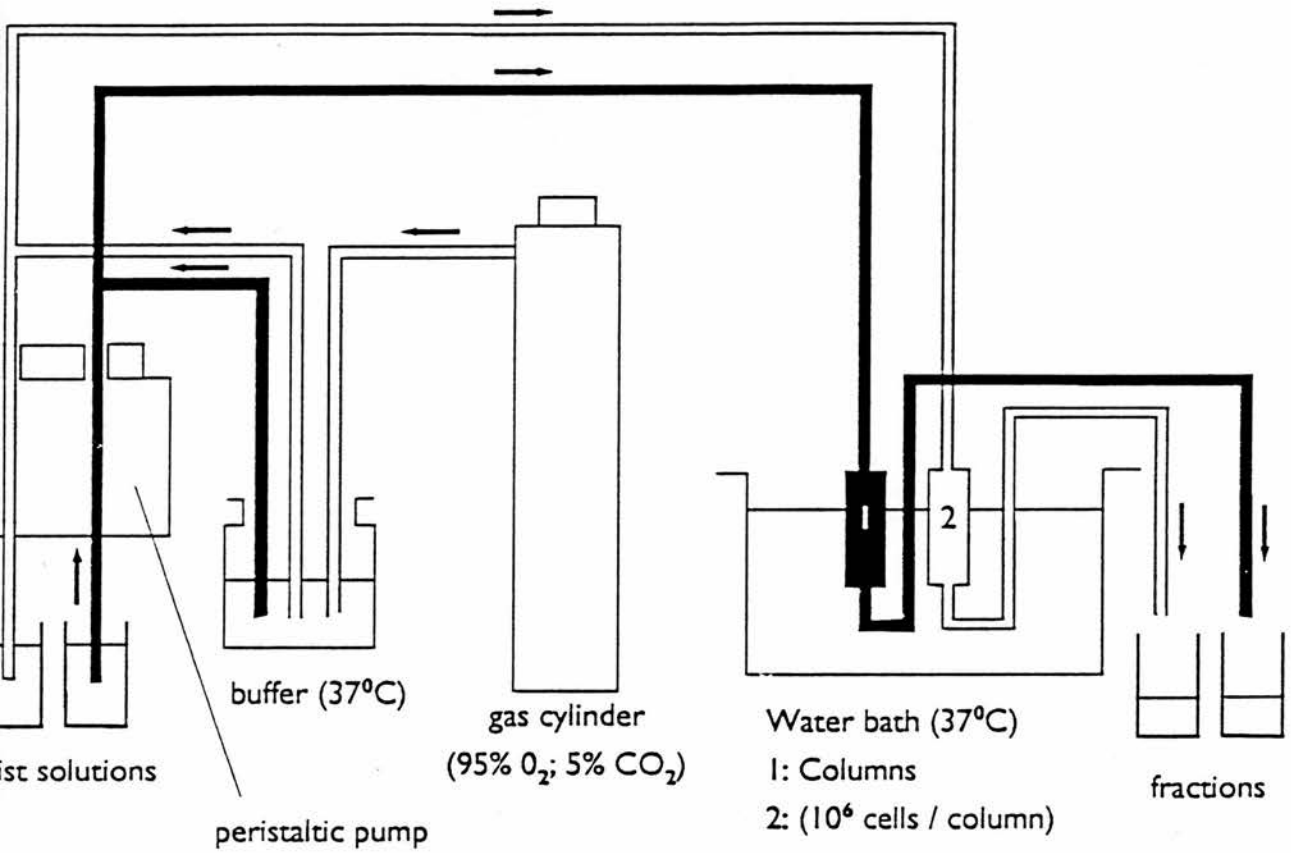


FIGURE 5.1.

A schematic representation of the superfusion set-up used to study the secretion of cortisol from bovine adrenocortical cells. The flow rate used was 0.25ml/min for the main line (buffer) and 0.025ml/min for the stimulus line (agonist solutions).

5.2.2. Sensitive Cortisol Radioimmunoassay.

Additional sensitivity was gained by using separate 1st and 2nd antibody steps, rather than the precipitated 1st antibody previously described. Whilst the antibody pre-precipitate was entirely satisfactory for the high levels of cortisol measured in the cell culture wells, it lacked sufficient sensitivity for the dilute fractions pertaining to the superfusion set-up.

Cortisol secretion by perfused adrenocortical cells was measured using an in-house radioimmunoassay similar to that described in section 2.2.6 (i) except that the assay employed two antibodies to separate bound from free tracer.

Cortisol standards (0 - 341.4 nmol/l) were prepared by dilution of stock cortisol (10mM) in EBSBG and stored at -20°C. To determine the concentration of the first antibody used, an antibody dilution curve for the lowest standard (zero) and the middle standard (5.33nM) was set up. This involves the incubation of a fixed amount of tracer with different concentrations of the binder, for example, serial doubling dilutions of the first antibody. Plotted as percentage of tracer bound against serial dilution of the antibody, the closest approximation (dilution of antibody which produces the greatest difference in the binding of tracer between the two standards used, ie the greatest sensitivity) was a 1:8 initial dilution of the antibody. The specificity of the anti-cortisol serum was calculated according to the method used by Abraham et al (1969). Cross reaction with the steroids corticosterone, cortisone and 11-deoxycortisol was calculated to be 0.18%, 0.07% and 0.58% respectively. Three quality control values were also prepared to check for inter-assay precision and intra-assay drift.

The assay was set up with a Clinicon Dilutrend automatic diluter. Each assay tube contained 290µl of assay diluent (100mM citrate-phosphate buffer pH 4.0), 50µl tracer (10000 counts per tube), 10µl standard/QC/sample and 50µl first antibody (sheep anti-cortisol)(final dilution of 1:6400) and was mixed on a multi-vortex and then incubated at

FIGURE 5.2.

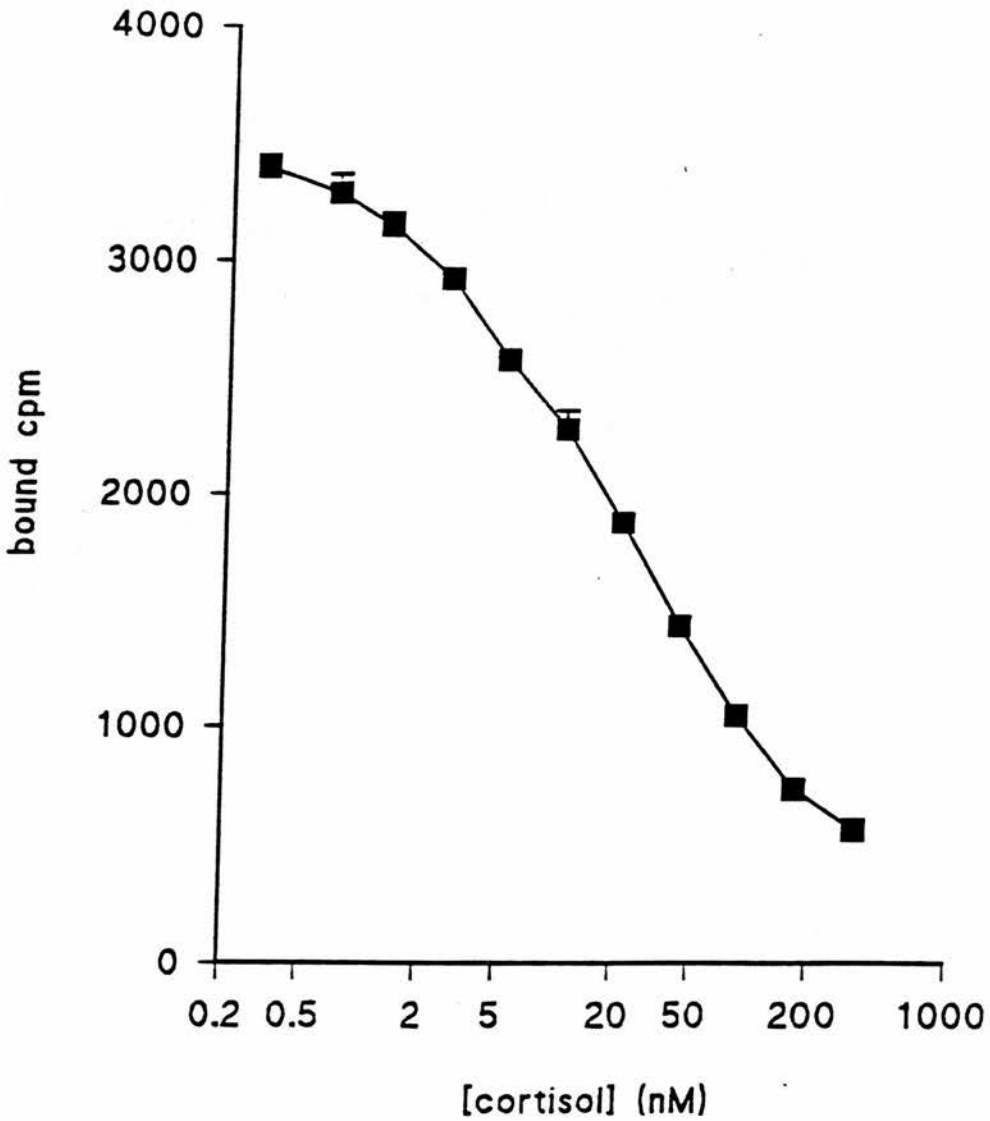


FIGURE 5.2.

Representative standard curve for the ^{125}I radioimmunoassay of cortisol. Standard solutions of cortisol (in EBS/BSA/glucose, 0.03 - 341.4 nM) were assayed in duplicate and the inhibition curve fitted to a four parameter logistic equation. Label binding in the absence of cortisol was typically 60-65% of total.

room temperature for 4 hours. This was followed by the addition of the second antibody (50 μ l of donkey anti-sheep in normal sheep serum) and the samples left overnight at 4°C. 2ml of cold (4°C) assay diluent was added to each tube and the samples centrifuged for 45 min at 3000rpm (1720g) at 4°C. Following centrifugation, the supernatant was decanted and the pellets counted for 120 seconds in a gamma counter. Standard curves were fitted to a 4 parameter logistic model using the software package RIACALC (LKB/Pharmacia) on an IBM PC. The minimal detection limit for this assay was calculated to be 0.44 nmol/l. This was determined from a mean precision profile of 10 sequential assays and defined as the dose of cortisol which had a mean CV of 22% as suggested by M^cConway et al (1989). The inter-assay precision was < 15% for the each of the quality controls (0.66 nM, 5nM and 170nM) used. Figure 5.2 shows an example of a standard curve obtained by this assay.

RESULTS.

5.2.1. The effect of ACTH on cortisol secretion from superfused zfr cells.

The effects of 10^{-12} M and 10^{-11} M ACTH on the secretion of cortisol from freshly-isolated bovine zfr cells are shown in fig. 5.3 (top panel). The cells were freshly-isolated and placed immediately onto the Biogel column and equilibrated in the perfusion medium for 60 min prior to the addition of ACTH. Agonist stimulation lasted for a period of 5 min, as did all subsequent agonist stimulations, irrespective of the agonist used.

ACTH produced a dose-dependent increase in cortisol secretion when compared with basal secretion. ACTH (10^{-11} M) elicited a maximum response of 49.79 nmol/l/5 min cortisol, compared with basal cortisol levels of between 0.33 - 0.66 nmol/l/5 min.

The effects of 10^{-12} M ACTH and 10^{-11} M ACTH on the secretion of cortisol from freshly-isolated bovine zfr cells left overnight at 4°C, before being placed on the Biogel column, are also shown in fig. 5.3 (bottom panel). ACTH again produced a dose-dependent increase in cortisol secretion with a similar magnitude of response to that measured in the freshly-isolated cells. If anything, responsiveness was higher after overnight storage. Thus, 10^{-11} M ACTH produced a maximum cortisol response of 129.20nmol/5 min, compared with basal cortisol levels of between 0 - 0.33 nmol/l/5 min.

5.2.2. The effect of ATP on cortisol secretion from superfused zfr cells.

Fig. 5.4 (top panel) illustrates that freshly-isolated zfr cells placed immediately onto the Biogel secrete cortisol in a dose-dependent manner when challenged with ATP. ATP (10^{-5} M) produced a maximum cortisol response of 2.99 nmol/l/5 min, whilst 10^{-4} M ATP caused a maximum cortisol output of 10.52 nmol/l/5 min. Freshly-isolated zfr cells kept overnight at 4°C produced a similar magnitude of response, with a dose-dependent

FIGURE 5.3.

An experiment showing the effect of ACTH on the secretion of cortisol from freshly-isolated zfr cells (top panel) and freshly-isolated cells kept overnight at 4°C (bottom panel). Cells were superfused with EBS/BSA/glucose buffer (0.25ml/min) containing stated concentrations of the peptide hormone (10^{-11} and 10^{-12} M respectively). Each bar represents the cortisol secretory response (nmol/ 10^6 / 5 min) as measured the in-house radioimmunoassay. Each fraction was collected for 5 min. This experiment was performed only once.

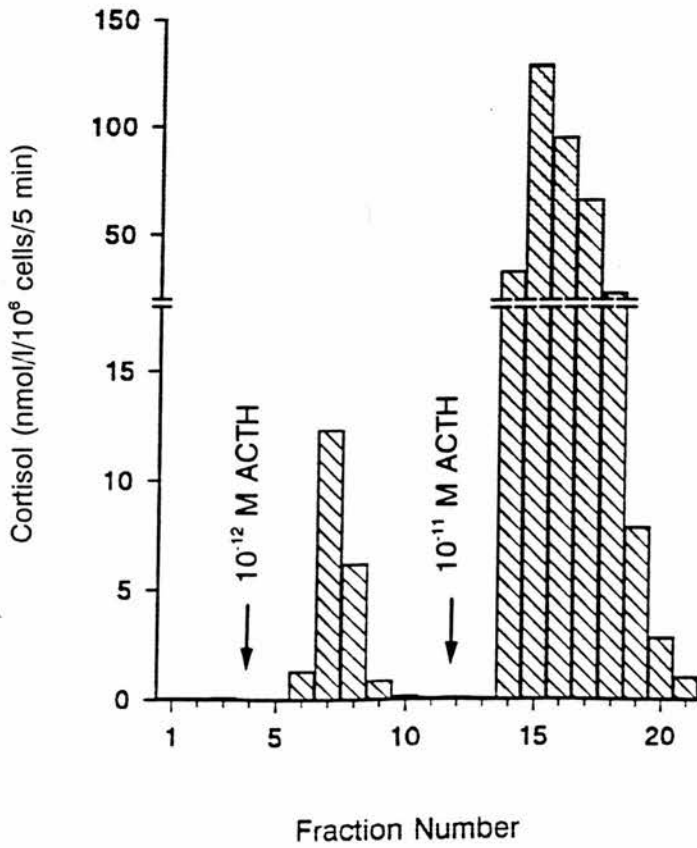
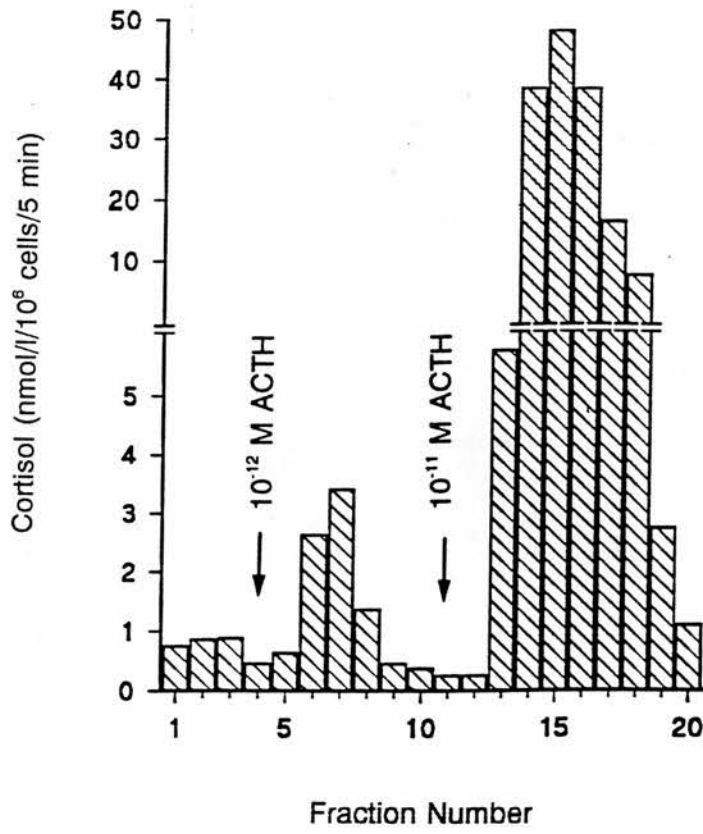
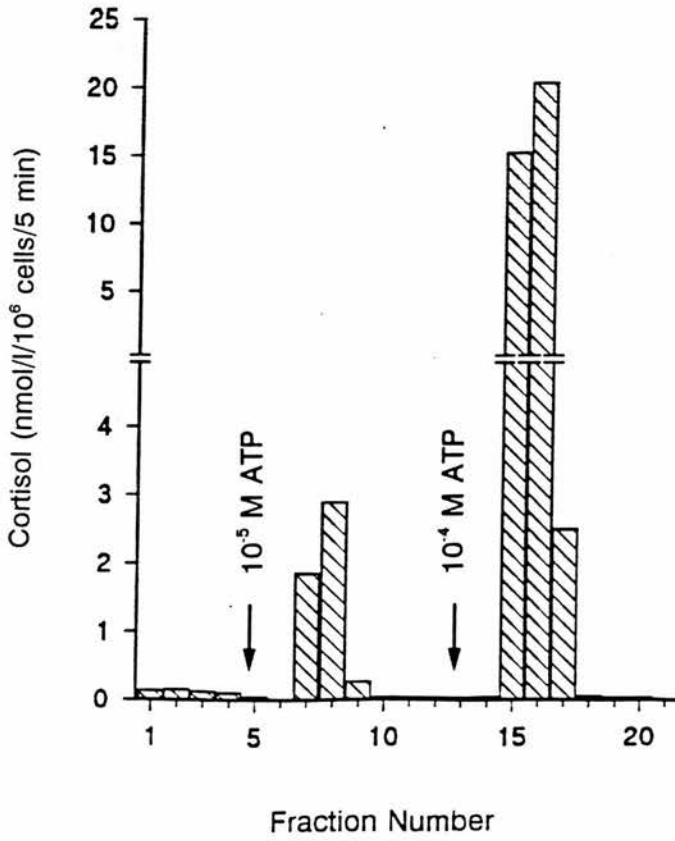
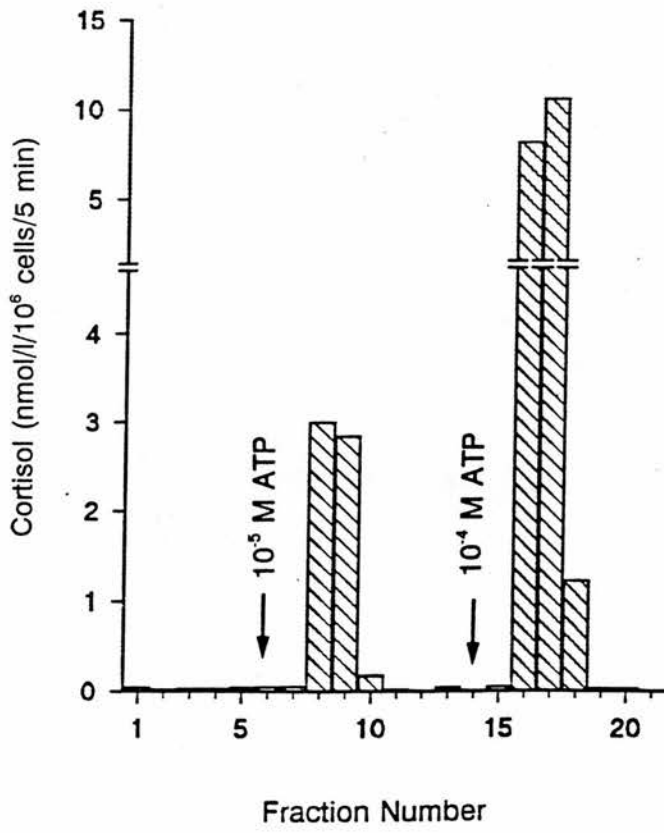


FIGURE 5.4.

An experiment showing the effect of ATP on the secretion of cortisol from freshly-isolated zfr cells (top panel) and freshly-isolated cells kept overnight at 4°C (bottom panel). Cells were superfused with EBS/BSA/glucose buffer (0.25ml/min) containing stated concentrations of the peptide hormone (10^{-11} and 10^{-12} M respectively). Each bar represents the cortisol secretory response (nmol/ 10^6 / 5 min) as measured the in-house radioimmunoassay. Each fraction was collected for 5 min. This experiment was performed only once.



increase in cortisol secretion in response to ATP (bottom panel). ATP (10^{-5} M) produced a maximum response of 2.90 nmol/l/5 min, whilst 10^{-4} M ATP caused a maximum cortisol response of 20.35 nmol/l/5 min. Basal cortisol levels ranged from 0 - 0.33 nmol/l/5 min in both experiments. As with ACTH, cells kept overnight at 4°C appeared more responsive to ATP than freshly-isolated cells.

5.2.3. The effect of ADP and UTP on cortisol secretion from freshly-isolated cells left overnight at 4°C .

Fig. 5.5 shows that both ADP and UTP produced a dose-dependent increase in cortisol secretion in freshly-isolated zfr cells subsequently left overnight at 4°C . In this superfusion system, the lowest concentration of UTP that produced a significant increase in steroid secretion above basal level was 10^{-6} M UTP. A maximum dose of 10^{-4} M UTP produced a maximal cortisol response of 5.78 nmol/l/5 min. The threshold concentration of ADP eliciting a significant increase in cortisol secretion above basal secretion was 10^{-5} M. The maximal cortisol output of 4.73 nmol/l/5 min occurred at 10^{-4} M ADP.

5.2.4. The effect of ATP and ACTH on the cortisol secretory response when combined as a double agonist solution.

The previous Figs. 5.3 and 5.4 establish the fact that both ATP and ACTH stimulate the production of cortisol in freshly-isolated zfr cells left overnight at 4°C . Taking this into account, an experimental protocol was set up to try and establish whether submaximally stimulatory doses of ACTH and ATP, if added simultaneously, would produce a simple additive effect on cortisol secretion or whether the cortisol secretory response would be potentiated or reduced by their combined addition. The results are illustrated in fig. 5.6 and in table 5.1.

FIGURE 5.5.

An experiment showing the effect of ADP and UTP on the secretion of cortisol from freshly-isolated zfr cells kept overnight at 4°C. Cells were superfused with EBS/BSA/glucose buffer (0.25ml/min) containing stated concentrations of the purine (10^{-5} and 10^{-4} M respectively) and the pyrimidine (10^{-6} , 10^{-5} and 10^{-4} M respectively). Each bar represents the cortisol secretory rate over 5 min for each fraction. This experiment was performed only once.

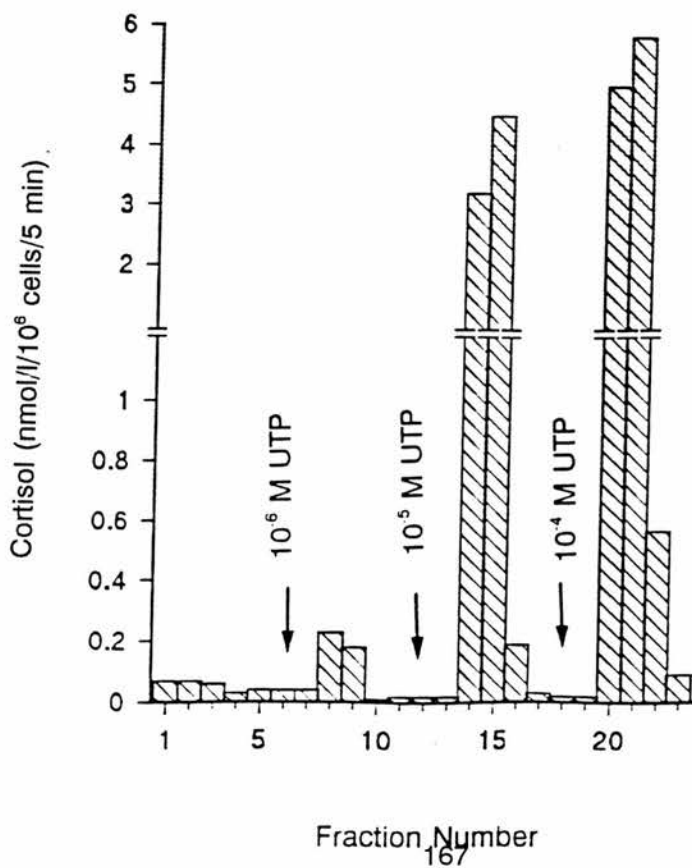
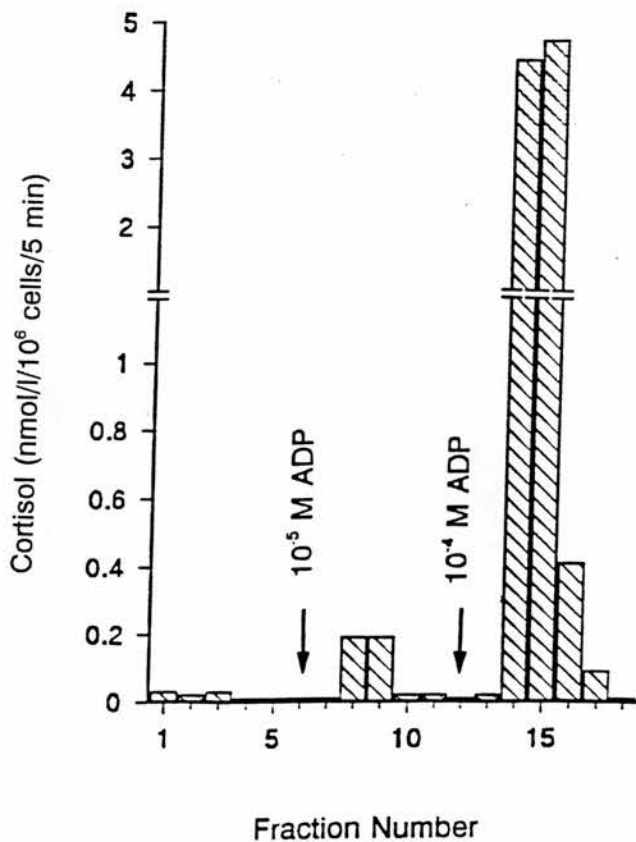


FIGURE 5.6.

Synergistic effects of ACTH and ATP on cortisol secretion from freshly-isolated zfr cells left overnight at 4°C. A 5 min infusion of each agonist was applied to superfused adrenal cells followed by a wash out period and fractions collected at 5 min intervals. Each bar represents the cortisol secretory response over 5 min for each fraction. A representative experiment is shown which was repeated on three separate occasions.

Cortisol (nmol/l/10⁶ cells/5 min)

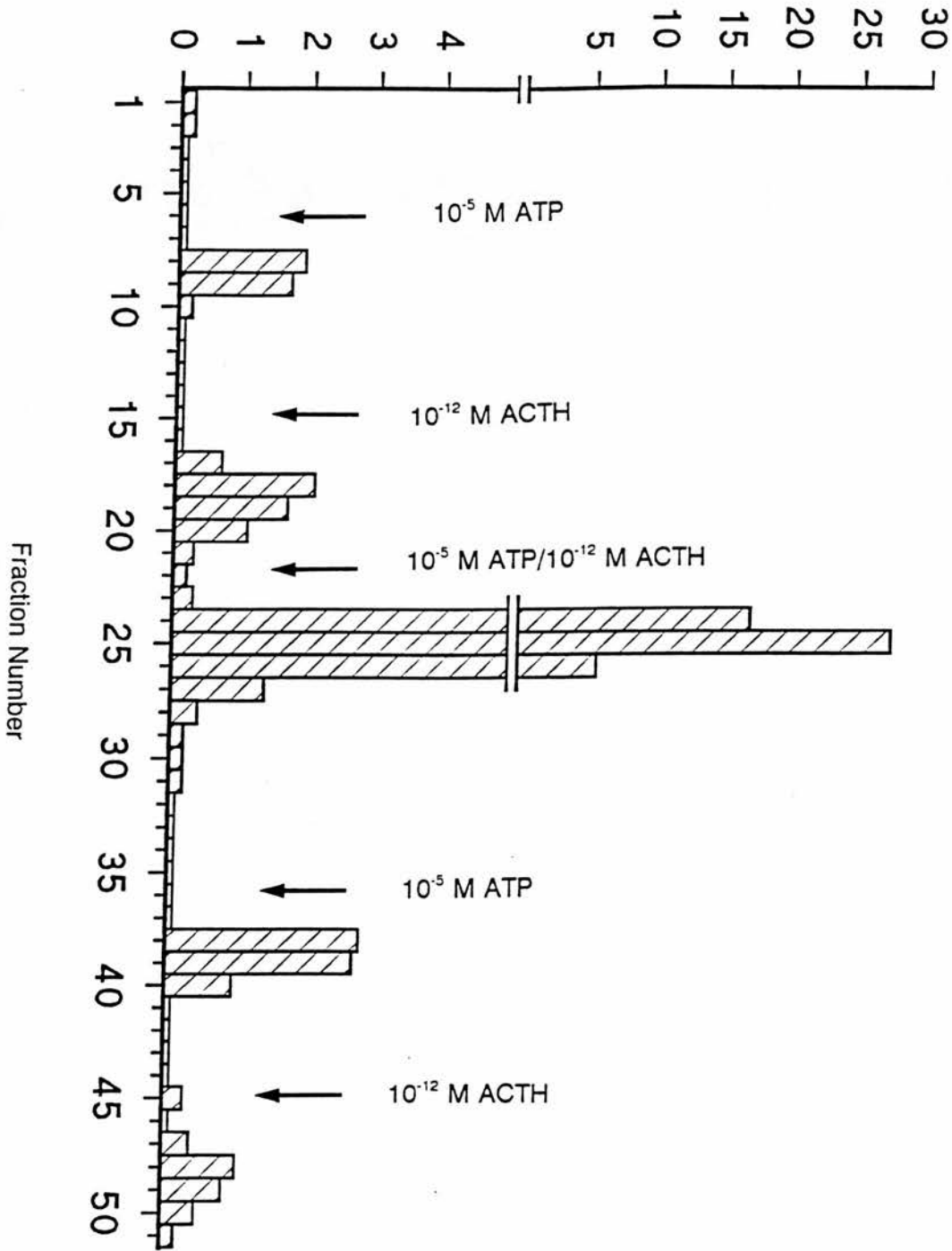


Table 5.1.

	Agonist(s)	Cortisol (n-fold)
1)	ATP (10^{-5} M)	58.00 ± 38.82
	ACTH (10^{-12} M)	10.50 ± 5.67
	<i>ACTH (10^{-12} M)/ATP (10^{-5} M)</i>	<i>68.50 ± 39.62</i>
	ACTH (10^{-12} M)/ATP (10^{-5} M)	170.13 ± 53.20
2)	Adenosine (10^{-4} M)	2.12 ± 0.29
	ACTH (10^{-12} M)	10.56 ± 3.56
	<i>Adenosine (10^{-4} M)/ACTH (10^{-12} M)</i>	<i>12.54 ± 4.04</i>
	Adenosine (10^{-4} M)/ACTH (10^{-12} M)	25.54 ± 5.28
3)	ATP (10^{-5} M)	3.72 ± 1.94
	All (10^{-10} M)	1.79 ± 0.66
	<i>ATP (10^{-5} M)/All (10^{-10} M)</i>	<i>5.51 ± 2.48</i>
	ATP (10^{-5} M)/All (10^{-10} M)	12.96 ± 3.13
4)	Ach (10^{-6} M)	3.08 ± 1.24
	ACTH (10^{-12} M)	1.21 ± 0.52
	<i>Ach (10^{-6} M)/ACTH (10^{-12} M)</i>	<i>4.29 ± 1.57</i>
	Ach (10^{-6} M)/ACTH (10^{-12} M)	8.27 ± 1.90

Table 5.1.

Figures for the cortisol production from isolated superfused adrenocortical cells expressed as an n-fold with respect to the basal response (no agonist present). To determine the n-fold, the basal value was calculated as the mean ± S.D. (n = 5 fractions) of the cortisol secretory response, before the addition of the agonist. Data are the mean ± S.D. of results from three separate experiments (n = 3), except in the case of ATP/ACTH where n = 4. The values in italics are the predicted n-folds, for the combined agonist additions, obtained by adding together the individual n-fold stimulations for each individual agonist. There was significant potentiation (p < 0.05) for each of the agonist combinations, as confirmed by the Mann-Whitney U-test.

An initial stimulus with 10^{-5} M ATP evoked a cortisol response of 58.00 ± 38.82 (n-fold, mean \pm S.D., n=4 cell preparations). Once basal cortisol secretion had been re-established, stimulation with 10^{-12} M ACTH produced a cortisol response of 10.50 ± 5.67 (n-fold, mean \pm S.D., n=4 cell preparations). After another wash-out period to re-establish basal cortisol levels, the Biogel cell columns were perfused with a combined agonist solution of 10^{-12} M ACTH/ 10^{-5} M ATP. This stimulus produced a cortisol response of 170.13 ± 53.20 (n-fold, mean \pm S.D., n=4 cell preparations). Once cortisol levels had returned to basal (after the addition of the combined agonist), ATP (10^{-5} M) and ACTH (10^{-12} M) were added singly to determine whether or not their individual responses were affected by the addition of the combined agonist during the experiment.

The additive effects of separate single doses of the two agonists was calculated to be 68.50 ± 39.62 (n-fold, mean \pm S.D., n=4 cell preparations). However, the combined effect of the two agonist added together produced a cortisol response of 170.13 ± 53.20 (n-fold, mean \pm S.D., n=4 cell preparations). This value is significantly higher when compared to the predicted (calculated) value ($p < 0.05$) determined by adding together the separate agonist responses. It appears that the effects of ATP and ACTH when added together are synergistic and not simply additive.

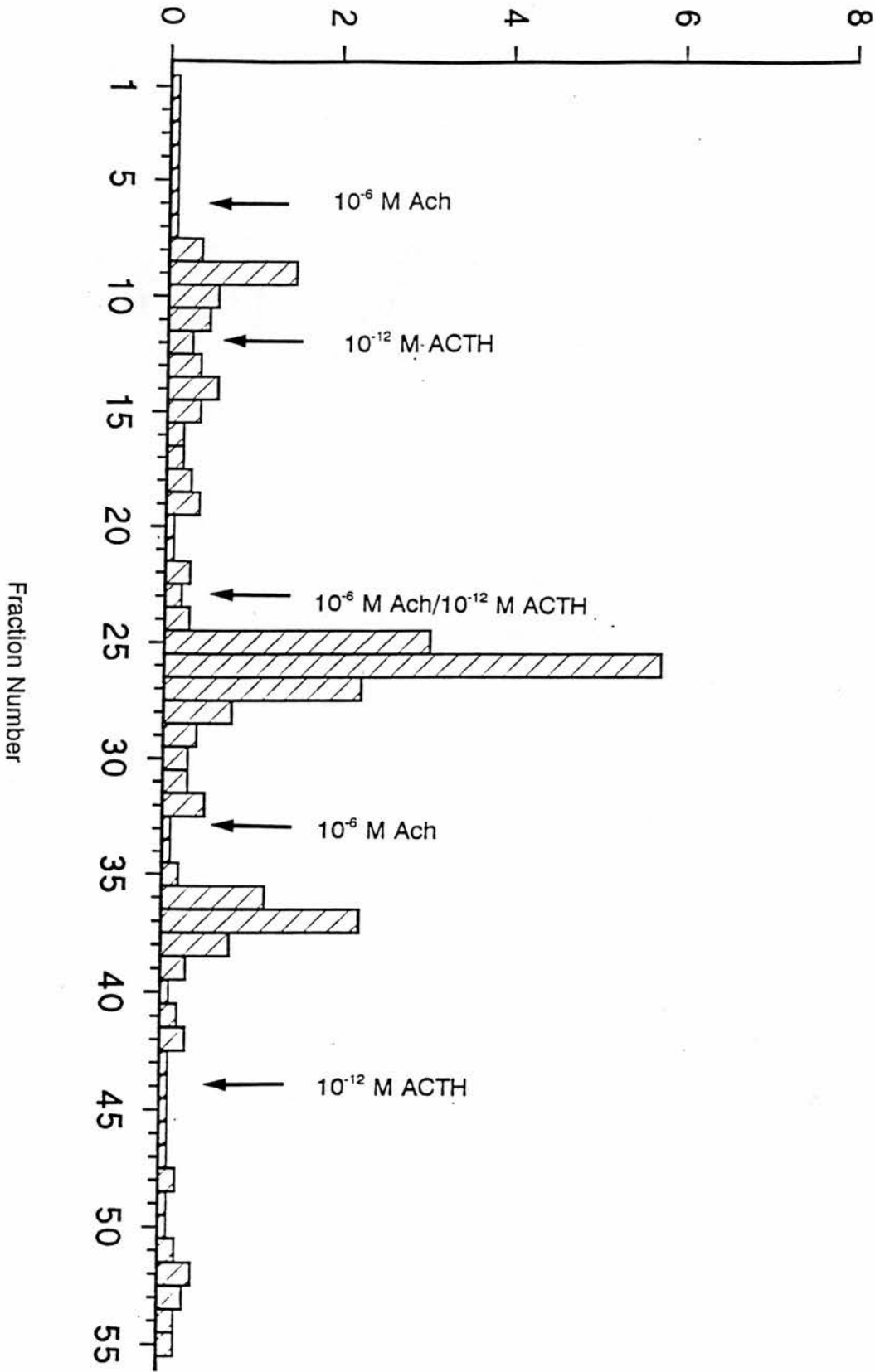
5.2.5. The effect of Acetylcholine and ACTH on the cortisol secretory response as a combined agonist solution.

Initial stimulation with submaximally stimulatory doses of acetylcholine (10^{-6} M) and ACTH (10^{-12} M), added separately, evoked cortisol responses of 3.08 ± 1.24 and 1.21 ± 0.52 respectively (n-fold, mean \pm S.D., n=3 cell preparations) (table 5.1). The additive effect on cortisol secretion of separate doses of the two agonists was calculated to be 4.29 ± 1.57 (n-fold, mean \pm S.D., n=3 cell preparations). However, the combined effect elicited by a combined agonist solution achieved a cortisol response of 8.27 ± 1.90 (n-

FIGURE 5.7.

Synergistic effects of ACTH and acetylcholine on cortisol secretion from freshly-isolated zfr cells left overnight at 4°C. A 5 min infusion of each agonist was applied to superfused adrenal cells followed by a wash out period and fractions collected at 5 min intervals. Each bar represents the cortisol secretory response over 5 min for each fraction. A representative experiment is shown which was repeated on two further occasions.

Cortisol (nmol/l/10⁶ cells/5 min)



fold, mean \pm S.D., n=3 cell preparations). This is significantly higher when compared to the predicted value ($p < 0.05$) obtained by adding together the separate agonist responses. It is apparent that the effects of acetylcholine and ACTH when added together are synergistic and not simply additive.

5.2.6. The effect of Adenosine and ACTH on the cortisol secretory response when combined as a combined agonist solution.

A similar experimental protocol was set up as described in section 5.2.4 except that adenosine and ACTH were added to the column-perfused cells as a combined agonist solution. Initial stimulation with adenosine (10^{-4} M) and ACTH (10^{-12} M), added separately, evoked cortisol responses of 2.12 ± 0.29 and 10.56 ± 3.56 respectively (n-fold, mean \pm S.D., n=3 cell preparations) (table 5.1). The additive effect on cortisol secretion separate single doses of the two agonists was calculated to be 12.54 ± 4.04 (n-fold, mean \pm S.D., n=3 cell preparations). However, the combined effect evoked by a combined agonist solution produced a cortisol response of 25.54 ± 5.28 (n-fold, mean \pm S.D., n=3 cell preparations). This is significantly higher when compared to the predicted value ($p < 0.05$). It is evident that the effects of adenosine and ACTH when added together are synergistic and not simply additive.

5.2.7. The effect of All and ATP on the cortisol secretory response as a combined agonist solution.

Initial stimulation with All (10^{-10} M) and ATP (10^{-5} M), added separately, evoked cortisol responses of 1.79 ± 0.66 and 3.72 ± 1.94 respectively (n-fold, mean \pm S.D., n=4 cell preparations)(table 5.1). The additive effect on cortisol secretion of separate doses of the two agonists was calculated to be 5.51 ± 2.48 (n-fold, mean \pm S.D., n=3 cell

FIGURE 5.8.

Synergistic effects of ACTH and adenosine on cortisol secretion from freshly-isolated zfr cells left overnight at 4°C. A 5 min infusion of each agonist was applied to superfused adrenal cells followed by a wash out period and fractions collected at 5 min intervals. Each bar represents the cortisol secretory response over 5 min for each fraction. A representative experiment is shown which was repeated on two further occasions.

Cortisol (nmol/l/10⁶ cells/5 min)

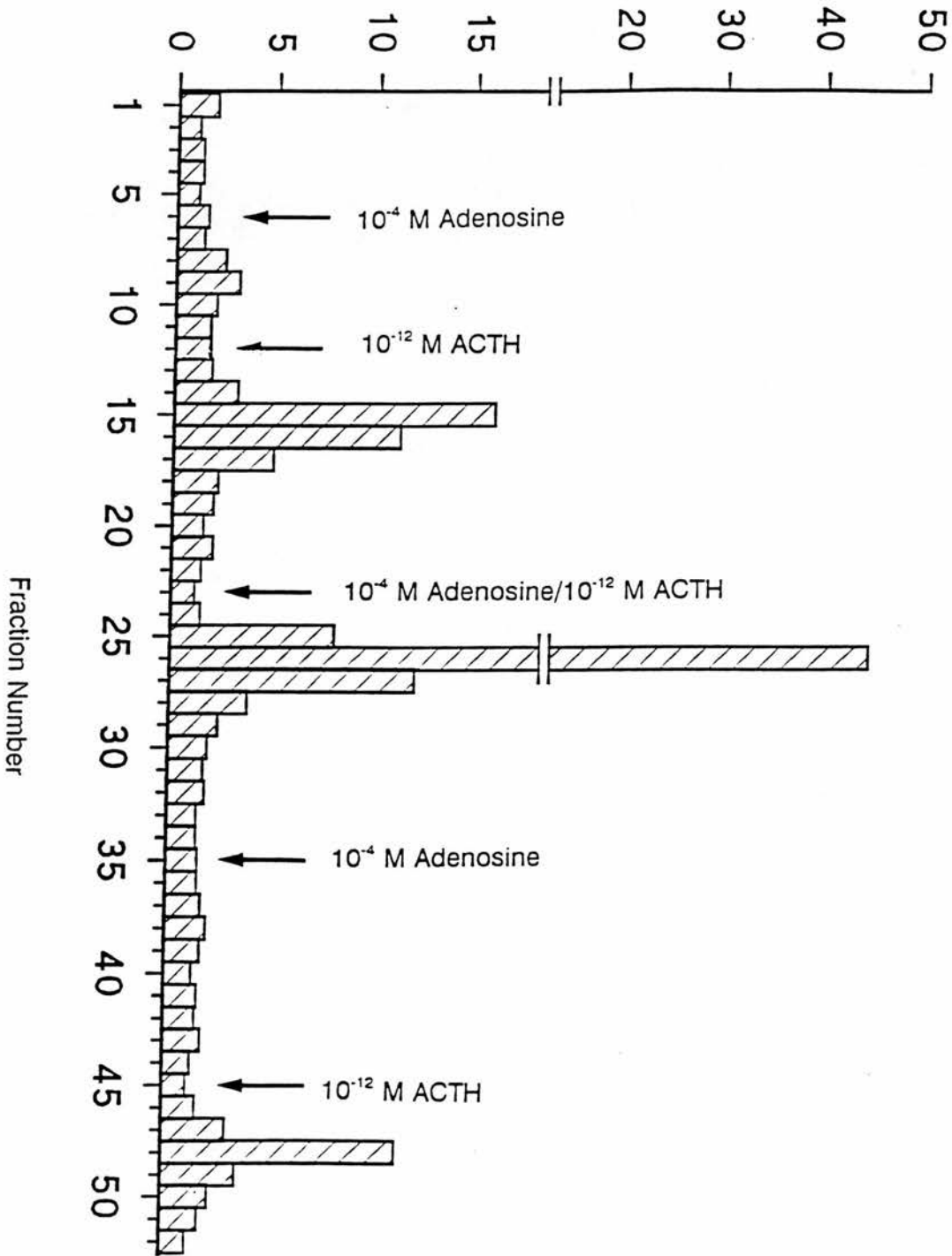
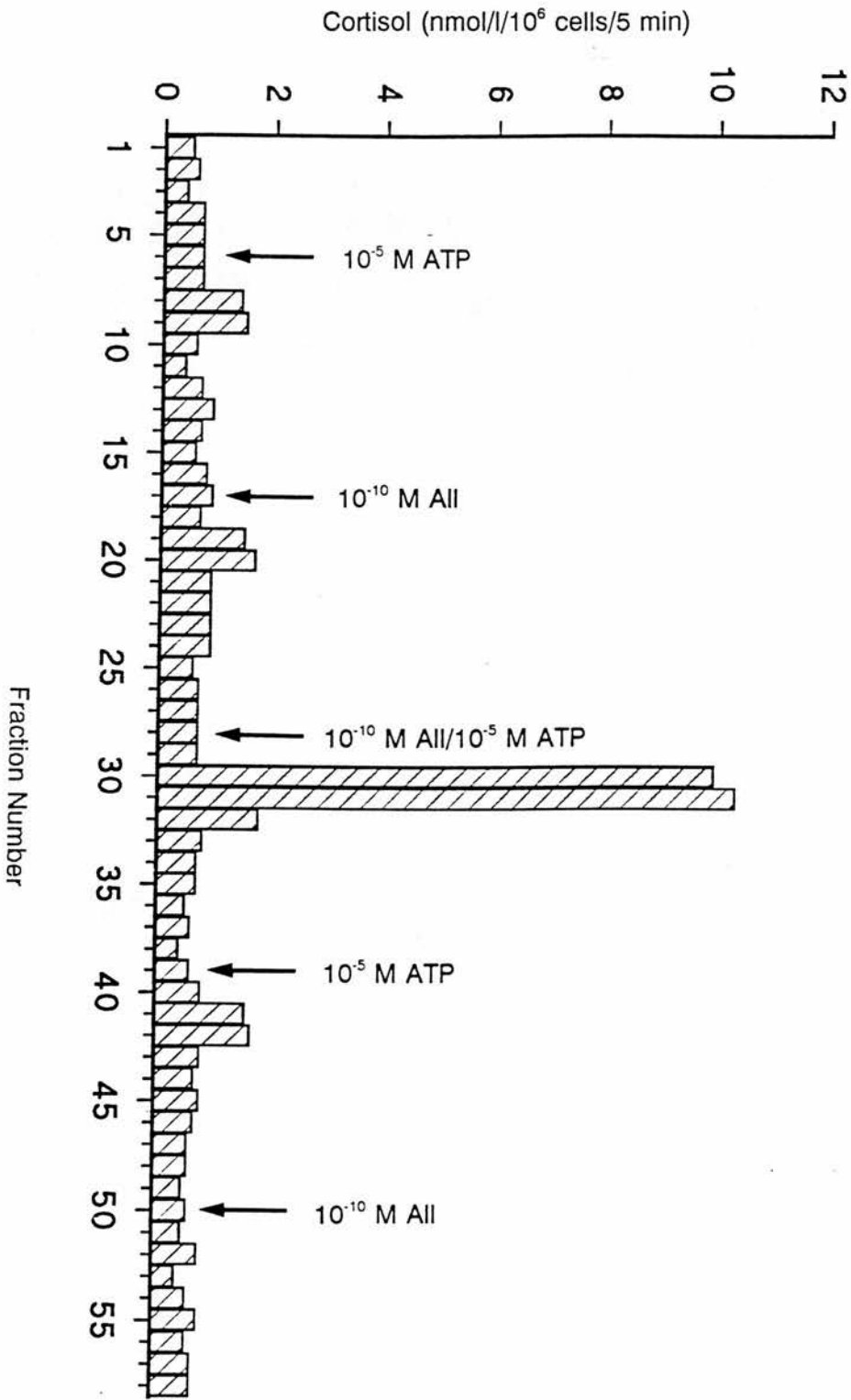


FIGURE 5.9.

Synergistic effects of All and ATP on cortisol secretion from freshly-isolated zfr cells left overnight at 4°C. A 5 min infusion of each agonist was applied to superfused adrenal cells followed by a wash out period and fractions collected at 5 min intervals. Each bar represents the cortisol secretory response over 5 min for each fraction. A representative experiment is shown which was repeated on two further occasions.



preparations). However, the combined effect evoked by a combined agonist solution achieved a cortisol response of 12.96 ± 3.13 (n-fold, mean \pm S.D., n=3 cell preparations). This is significantly higher when compared to the predicted value ($p < 0.05$). It is evident that the effects of All and ATP when added together are also synergistic.

5.3. DISCUSSION

A number of informative studies investigating steroidogenesis in the adrenal cortex have been carried out using intact adrenals *in vivo* (Beaven et al 1964), adrenal quarters *in vitro* (Grahame-Smith et al 1967) and tissue culture.

However, Matthews et al (1967) developed a flow system where the adrenal tissue was constantly bathed in the incubation medium which was then collected over fixed periods of time. Such a superfusion technique provides many practical and theoretical advantages in the study of the dynamics of steroid secretion in the adrenal. Cell column superfusion techniques, in which cells are suspended in a gel matrix of Bio-Gel offer the advantage of relatively low dead volumes in the system and therefore provide rapid cell responses to changes in the extracellular environment.

Firstly, the medium bathing the cells is continuously replenished during the steroid secretory process. This allows the population of cells to be subjected to a continual flow of agonists *in vitro*, thereby both supplying fresh modifier and also removing the products of the response, a situation more akin to that *in vivo*. As the steroid product is not removed from the bathing medium in static incubations, the possibility exists that the released steroid could exercise a positive or negative autocrine effect on the cell response. Similarly, released intermediates, on the steroid pathway, could exert positive or negative feedback effects on the measured steroid response, thus altering the dynamics of steroidal interconversion. Secondly, response/time relationships are easily studied in a superfusion set-up without the need to stop the "reaction" artificially. This could be especially useful in performing more sensitive studies on zfr cell regulation such as receptor desensitisation, agonist interactions and signal transduction mechanisms on a response/time scale.

The purpose of the superfusion system used here was 1) to investigate a steroidogenic response to ATP in freshly-isolated cells and 2) to study the interaction of

ATP with established steroid agonists such as ACTH and All, as determined by the steroid (cortisol) response.

ACTH produced dose-dependent increases in cortisol secretion in both freshly-isolated cells applied immediately to the Bio-Gel column and in freshly-isolated cells stored overnight at 4°C before being placed on the column (fig. 5.3). In both cases, the cells were sensitive to physiological doses of ACTH with a threshold response at 10^{-12} M, supporting the findings previously reported by Williams et al (1989) for primary cultures of bovine zfr cells. The onset and delay (≈ 5 min) in the response to a 5 min infusion of ACTH compares favourably to that reported by Schulster et al (1970) using superfused rat adrenals and may partially be accounted for by the time needed for the medium containing ACTH to reach the cells in the Bio-Gel column and displace the control medium inside it.

ATP is an ineffective cortisol secretagogue in static incubations of freshly-isolated cells, although a steroid response was evident by 24 h in culture (Chapter 3). However, in a superfusion system, it was observed that ATP produced dose-dependent increases in cortisol secretion in both freshly-isolated zfr cells and in the same cells kept overnight at 4°C, with a maximum response at 10^{-4} M (fig. 5.4). This observation provides strong supportive evidence that the purinergic receptor is present *in vivo* and not an artefact of the culture system. The reason for the absent response in static incubations is unknown, but, speculatively, it could be due to a number of factors; 1) the local release of ATP from the adrenal chromaffin granules during the "mechanical" stage of the cell preparation such that high local levels of ATP may desensitise the purinergic response, taking up to 24 h for the cells to recover and 2) the extensive ATPase activity from dead or dying cells during the cell isolation procedure and maintained by continued cell death over the initial few hours of cell isolation. In the superfusion set-up, the medium bathing the cells is continuously cleared of agonists, thereby removing any high local concentrations of ATP or ATPase activity that may be present.

Since there is little or no difference in the responsiveness of fresh cells compared to cells that were kept overnight at 4°C, all subsequent experiments were performed using zfr cells left overnight at 4°C. This also had the advantage that the difficulties in carrying out both the cell preparation and the required experiments on the same day were overcome.

The presence of a nucleotide receptor in freshly-isolated bovine zfr cells was further confirmed in that both UTP and ADP produced dose-dependent increases in cortisol secretion, with threshold responses at 10^{-6} M and 10^{-5} M respectively (fig. 5.5.). Thus the experimental findings reported here, along with other circumstantial evidence, suggest that the purines could regulate adrenocortical function *in vivo*.

How do the purines interact with the main regulatory hormone in the adrenal cortex, namely ACTH? The approach used was to co-administer submaximally stimulatory doses of the agonists under investigation (ACTH and ATP), to determine whether the presence of both agonists resulted in an alteration of cortisol secretion that was greater or smaller than the additive effect of individual agonists, ie whether the interaction is synergistic, antagonistic or neutral. The additive effects of separate single doses of ACTH (10^{-12} M) and ATP (10^{-5} M) was calculated to be 68.50 ± 39.62 (n-fold above basal; mean \pm S.D. (n = 4)). The combined effect elicited by a combined agonist solution of these agonists produced a cortisol response of 170.13 ± 53.20 (n-fold above basal; mean \pm S.D. (n = 4)) (table 5.1). The combined agonist effect was significantly higher than the sum of the individual agonist responses ($p < 0.05$), consistent with a synergistic interaction.

ATP is unique in the sense that it activates both the cAMP and phosphoinositide pathways. Thus, the possibility arises that the synergistic interaction of ATP with ACTH may depend exclusively on the stimulation of one second messenger pathway (ie cAMP dependent protein kinase A or Ca^{2+} /phospholipid dependent protein kinase C) or possibly both pathways may interact synergistically with ACTH. In order to dissect out these

possibilities, the interaction of ACTH with acetylcholine (an agonist for the phosphoinositidase C pathway via the M₃ muscarinic receptor (Walker et al 1990) and adenosine (an agonist for the cAMP-dependent pathway via the A₂ purine receptor, Chapter 4 of this thesis) were studied. Note that, unlike ATP, neither acetylcholine nor adenosine stimulates the alternative signalling pathway, in bovine adrenocortical cells. The possible mechanisms underlying this potentiation were therefore examined in a further series of experiments.

The additive effects of separate single doses of ACTH (10⁻¹² M) and acetylcholine (10⁻⁶ M) was calculated to be 4.29 ± 1.57 (n-fold above basal), whilst the steroid response elicited by a combined agonist solution produced a cortisol response of 8.27 ± 1.90 (n-fold above basal) (table 5.1). A number of other investigations have clearly established a synergistic interaction between these two signalling systems, ie cAMP-dependent mechanism and phosphoinositidase-dependent mechanism. Synergistic interactions include the effect of opioid and purinergic receptors in NG 108-15 cells (Okajima et al 1993), the interaction of ACTH and All in bovine adrenal fasciculata cells (Langlois et al 1992) and the synergistic effects of All and adenosine in the renal microvasculature (Weihprecht et al 1994). Yoshimasa et al (1991) produced the first direct demonstration of a covalent modification of the catalytic subunit of adenylate cyclase (protein kinase C-mediated phosphorylation of adenylate cyclase) in frog erythrocytes which thus provided a potential biochemical mechanism for a regulatory link between the two transmembrane signalling systems.

Co-infusion of ACTH and adenosine (both activators of the cAMP pathway) also evoked a synergistic secretory response in bovine zfr cells (table 5.1). Synergism was also reported by Chern et al (1988) in the stimulation of secretion and in the elevation of cAMP levels in bovine adrenal chromaffin cells in response to adenosine and forskolin (activates adenylate cyclase). In addition, Penhoat et al (1989) reported synergism between ACTH and insulin-like growth factor 1 (IGF-1) (activates cAMP pathway) for the

ACTH-induced cortisol response in cultured bovine adrenocortical cells. However, the effects of both peptides on ACTH-induced cAMP production was only additive, which could be related to an increase in phosphodiesterase activity.

The other main hormone involved in the regulation of adrenal steroidogenesis *in vivo* is All, which exerts its effects mainly through the phosphoinositide pathway. All, like ACTH, also potentiates ATP-induced cortisol secretion in bovine zfr cells (figure 5.9). These findings would suggest that the clear synergism seen with the co-infusion of ACTH/ATP and All/ATP, involves the interaction or "cross-talk" between different signalling systems. Taking into account the complexity of both the cAMP and phosphoinositide pathways, interaction between these pathways may occur at various points. The following examples outline some of the potential sites where the occurrence of "cross-talk" might be expected to elicit a synergistic response.

One possible explanation would be to surmise that "cross-talk" involves a common component of the two receptor coupling systems. One obvious candidate for such a role is calcium. There are two mechanisms which increase intracellular calcium; mobilisation of intracellular stores following an increase in cellular Ins (1,4,5) P₃ or increased influx of extracellular calcium. Electrophysiological evidence now exists for the activation of voltage-dependent Ca²⁺ channels as a common pathway for Ca²⁺ entry into adrenocortical cells during stimulation by ACTH (T-type Ca²⁺ channel; Enyeart et al 1993) and All (both T- and L-type Ca²⁺ channels; Payet et al 1994, Cohen et al 1988). At present there is no evidence to suggest that purines such as ATP increase intracellular calcium through the activation of voltage-dependent channels in bovine adrenocortical cells. ATP-induced Ca²⁺ influx has however been described in several endocrine/ exocrine secretory cells (Dubyak et al 1993).

Isales et al (1991) observed synergism between All and parathyroid hormone (PTH) on aldosterone secretion from bovine adrenal zg cells. PTH elicited an increase in intracellular Ca²⁺ and cyclic AMP. Evidence to suggest that the elevation of cAMP levels

by PTH may influence calcium influx was obtained. All is known to elicit increases in Ca^{2+} influx through L-channels which are present in bovine adrenal zg cells (Payet et al 1994), and these channels are modulated by a cAMP-dependent protein kinase. Thus the synergism between these two agonists may be attributable to a synergistic effect in elevating intracellular calcium.

Another possible site of interaction was suggested by Langlois et al (1992), who reported that All potentiated ACTH-induced cyclic AMP production in bovine adrenal cells in culture. Experiments showed that nifedipine, a specific calcium channel antagonist, did not modify the amplifying effects of All. However, trifluoperazine (a calmodulin inhibitor) in association with staurosporine (a PKC inhibitor), although not individually, blocked the All potentiation of the ACTH-induced response. This would imply that All mediates its effect through the two branches of the phosphoinositide pathway. The existence of at least two distinct species of adenylate cyclase, calmodulin-dependent and calmodulin-independent, has been proposed (Smigel, 1986) which may explain the potentiating effects of All on ACTH-induced cAMP production.

Further experiments are required to dissect out the possible points of interaction between the cAMP and phosphoinositide pathways, which results in a synergistic cortisol response to the co-infusion of ACTH/ATP and All/ATP of superfused bovine adrenocortical cells. This would involve the use of specific calcium channel antagonists, protein kinase C and calmodulin inhibitors to elicit the possible interaction(s) involved in "cross-talk" between receptors in the adrenal cortex.

What is the physiological basis for the synergistic effects reported in these experiments?

Control of glucocorticoid secretion from the adrenal cortex is widely believed to be almost exclusively under the control of ACTH. Initially, it was proposed that the corticosteroid rhythm was the result of passive adrenal response to a rhythm in plasma ACTH (Krieger et al 1977). However, there are a number of examples in the literature

where there is a dissociation between ACTH and cortisol, with no visible correlation between the concentrations of the two hormones (Dallman et al 1978, Ottenweller et al 1982), thus arguing for the existence of extrapituitary mechanisms involved in the regulation of adrenocortical function. Following experiments that demonstrated plasma corticosteroid rhythms in hypophysectomised gulf killifish (Srivastava et al 1972) and in hypophysectomised rats implanted with beewax pellets containing ACTH (Meier et al 1976), Ottenweller and co-workers (1982) suggested that an extrapituitary mechanism involving adrenal innervation may be able to generate a rhythm of adrenal responsiveness which is responsible for plasma corticosteroid rhythm in hypophysectomised rats treated with ACTH. It must be concluded that ACTH is required for glucocorticoid secretion but is not sufficient to explain all physiological situations and other factors, for example humoral and neural, should be considered.

In particular two different adrenocortical mechanisms have been postulated involving; 1) an intra-adrenal paracrine mechanism within the adrenal gland and 2) direct innervation of the cortex itself.

1). Corticomedullary Interactions.

ATP is present in high concentrations (150mM) in chromaffin cells, representing 15% of the dry weight of these granules. In addition, ATP has a direct stimulatory effect on adrenocortical cells (Kawamura et al 1991, Hoey et al 1994). A prerequisite for an intra-adrenal paracrine mechanism would involve close cellular contacts between chromaffin cells and adrenocortical cells. Extensive morphological evidence now exists, in a range of species, showing interactions between the cortex and the medulla and this is discussed in Section 1.1.4. CRH-like immunoreactivity (Minamio et al 1988) and POMC gene expression has also been reported in the adrenal medulla of cattle (Thorne et al 1991), raising the possibility that the adrenal gland may possess an active CRH-ACTH system. Hadjian et al (1982) in cattle and Benyamina et al (1987) in frog reported clear synergism on co-administration of acetylcholine and ACTH by adrenal tissue. The

possibility therefore exists that the steroidogenic action of ACTH is potentiated by an intra-adrenal paracrine mechanism involving acetylcholine and possibly ATP.

2) Innervation.

It is now recognised that the adrenal cortex receives direct innervation. A range of peptides and amines have been identified in nerves supplying the cortex (see Section 1.1.5.); these include vasoactive intestinal polypeptide (VIP) which appears to be under the control of the splanchnic nerve. Electrical stimulation of the splanchnic nerve increases adrenocortical sensitivity to ACTH in the hypophysectomised animal (Edwards et al 1987) while sectioning of the splanchnic nerves reduces the cortisol response to ACTH by about 50% (Edwards et al 1986b). This implies a potentiation of the ACTH-stimulated response by VIP, a concept that may be applicable to the other neuropeptides and amines released into the adrenal cortex.

Histofluorescent methods for catecholamines and acetylcholinesterase-histochemistry have shown noradrenergic and cholinergic innervation of the entire adrenal cortex (Kleitman et al 1985, Charlton et al 1991). ATP is packaged and released as a co-transmitter with noradrenaline in certain sympathetically innervated smooth muscles, mainly the vas deferens and blood vessels. There is similar evidence showing that ATP is co-released with acetylcholine in certain smooth muscles for example the urinary bladder, with cholinergic innervation (Dubyak et al 1993). The possibility exists that ATP is co-released with noradrenaline or acetylcholine in the adrenal cortex, giving rise to a synergistic response.

CHAPTER 6

GAS CHROMATOGRAPHY-MASS SPECTROSCOPY (GC-MS) OF ADRENOCORTICAL STEROIDS IN CELL CULTURE.

6.1. INTRODUCTION.

ATP is unique in the adrenal cortex in that it has a dual second messenger effect, activating both adenylate cyclase and PL-C (Chapter 3 and 4). It was therefore of particular interest to compare the acute and chronic effects of ATP with those of ACTH (cAMP pathway only) and All (phosphoinositol pathway only) and to determine if different second messengers regulate distinct parts of the steroidogenic pathway or whether, irrespective of the second messenger system, does ATP produce its own unique "stimulated" fingerprint? Instead of focusing on one particular steroid in the steroidogenic pathway, for example cortisol (as in previous chapters), the aim was to establish a method for the identification and quantification of all the major steroids in the pathway.

The identification of steroids in biological extracts is made difficult by the large number, structural variety and low concentrations of closely related compounds which may be present. The methods used in adrenal tissue culture are essentially non-separative methods such as

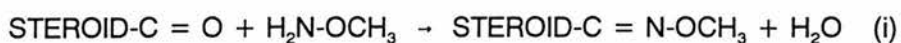
fluorimetry (Hornsby et al 1978), radioimmunoassay (Dazord et al 1977) and competitive protein binding (Goodyer et al 1976). This prompted the need for accurate and sensitive methods of assay which are also separative in order to distinguish between similar metabolites, for instance in the same metabolic pathway.

The word "profile" was first used to represent multicomponent chromatographic analysis and according to purists it implies "the collection of all relevant data in a single chromatogram" (Shackleton et al 1986). Horning (1968) was the first to use the term in relation to the analysis of steroids. Various techniques over the years have been used in steroid profiling, including paper chromatography, liquid chromatography and thin-layer chromatography. Vandenneuval et al (1960) was the first to describe the separation of steroids using gas chromatography (GC). A more positive identification of the steroids emerging from the GC column has been made possible by passing the effluent directly into a mass spectrometer (MS). This technique, gas chromatography-mass spectroscopy (GC-MS) was first introduced in the mid 1960's and was only made possible by the research of Ryhage (1964), who constructed an interface (molecular separator) connecting the two parts which had the role of preferentially removing the carrier gas thereby concentrating the sample. This reduced the vast difference in operating pressures, between the two instruments, prior to transmission to the ion source.

Problems in the differentiation of closely related steroids by GC-MS, have been greatly improved by the introduction of efficient capillary columns and more recently fused silica columns that permit clear separation of steroids with similar retention times. These columns can operate at very low flow rates, so it is possible to introduce the whole of the gaseous sample into the MS. Another advantage is that the column can be taken right into the ion source, so there is no requirement for an interface between the GC and MS.

Over the last few years, the coupling of computers into GC-MS systems, has greatly improved the collection and analysis of mass spectra. One of the major roles of the computer is to assign mass values to each of the ion peaks and to subtract background peaks where necessary, to produce a normalised spectrum for each eluted peak of a GC-MS run.

The principal disadvantage of GC-MS in steroid profiling, relates to the necessity to protect thermally labile compounds, from the high temperatures required for volatisation and separation. The most favoured derivatives for steroid analysis are the methyloxime-trimethylsilyl (MO-TMS) ethers, which were introduced in 1966 by Gardiner and Horning to allow them to perform steroid profiling with flame ionisation detection. The ketone groups are oximated first with methylhydroxylamine hydrochloride (i) and then the hydroxyls are silylated, using trimethylsilylimidazole (TSIM) (ii).



If the ketones are not derivatised, exchange reactions between these and adjacent hydroxyls may occur during silylation (keto-enol transformation). Due to the involatility of TSIM, which would damage the capillary column if injected directly, Axelson et al (1974) developed a method of reagent removal using a lipophilic Sephadex, Lipidex 5000, which is now widely accepted.

6.2. METHODS.

6.2.1. Stock Solutions.

(i) Internal Standards.

These are prepared by making 0.3mg/ml solutions in ethanol of 5 α -androstane-3 α ,17 β -diol, Stigmasterol and Cholesterol butyrate and stored in silanized sealed glass tubes at -20°C.

(ii) Steroids.

These are prepared by making 0.5mg/ml solutions in ethanol of each of the authentic steroids and are stored at -20°C in silanized sealed glass tubes.

6.2.2. Incubations.

Bovine adrenocortical zfr cells were isolated and purified according to the method described in Section 2.1, except that the cells were plated out in 75cm³ flasks at 10⁷ cells/flask. Agonists were made up in growth medium and on day 3 of cell culture, the cells were initially stimulated acutely for 2h in the presence of the agonist. After further exposure for 24h to the same concentration of the same agonist, the cells were restimulated for a further 2h with the same concentration of agonist. The % change was calculated as the amount of steroid produced in the final 2h stimulus divided by the amount of steroid produced in the initial 2h stimulus, multiplied by 100. For each agonist experiment, 40 × 10⁶ cells were stimulated (4 flasks) and the cell medium, containing the secreted steroids, collected at the end of each incubation (40 ml; 2h or 24 h). The culture media were stored at -20°C until steroid extraction was performed.

6.2.3. Solid-phase extraction.

The steroid extraction was performed on Sep-Pak C₁₈ solid phase extraction columns. These are cartridges, about 1 cm in diameter and 1.5 cm high, with octadecylsilane-bonded packing between two filters. Based on the procedure suggested by the manufacturer, Sep-Pak

C₁₈ cartridges were primed with 2ml of methanol followed by 5ml of distilled water. The cell culture medium (40ml) was then passed through the column using gentle vacuum. The cartridges were washed with distilled water (10ml) and subsequently eluted with methanol (3ml) which elutes the steroids. The eluate was collected in a silanized glass tube and evaporated to dryness under a stream of nitrogen and dissolved in ethyl acetate (1ml). Before derivatisation, the samples were washed initially with 0.2ml of 8% sodium bicarbonate, to remove any acidic impurities, followed by two washes with distilled water (0.2ml). Finally, the organic layer was evaporated under a stream of nitrogen at 60°C.

6.2.4. Derivative Formation.

There are two steps in the preparation of methoxime-trimethylsilyl (MO-TMS) derivatives,

1) Oxime Formation and 2) Trimethylsilylether Formation.

To the dry residue, 250 μ l of 2% methoxyamine hydrochloride in distilled pyridine was added and allowed to react at 60°C for 2h. To the methyl-oxime derivative, 100 μ l of trimethylsilylimidazole (TSIM) was added and incubated overnight at 100°C. Excess pyridine was removed under nitrogen at 60°C.

6.2.5. Lipidex purification of derivatised samples.

The purification on the Sep-Pak cartridge does not remove a number of low molecular weight impurities, which do not interfere with sample analysis but tend to cause deterioration of the capillary columns. Excess reagents and these polar residues were separated from the steroid MO-TMS derivatives by chromatography on Lipidex 5000 column containing dry gel prepared in the Lipidex solvent-system, cyclohexane/pyridine/hexamethyldisilazane (HMDS)(98:1:1). Before application to the column, 1ml of Lipidex solvent was added to the sample residue. The reaction mixture is passed through the column using Lipidex solvent (1.5 ml), concentrated under nitrogen at 60°C and taken up in 20 μ l of the same solvent mixture.

6.2.6. Gas chromatography Mass spectroscopy (GC-MS).

GC-MS was carried out on a Hewlett-Packard 5890 gas chromatograph connected to a Hewlett Packard 5971a mass selective detector equipped with a 25m × 0.2mm × 0.33 μ m DB-5 fused silica capillary column. Helium was used as the carrier gas, with a column head pressure of 55 kilopascals. The oven temperature was programmed from 150 - 255°C at a rate of 35°C per min and then at 3°C per min to 300°C and held there for the rest of the run time. 1 μ l samples were injected onto the column using a splitless mode of injection. Injector and detector temperatures were both 300°C.

6.3. RESULTS.

6.3.1. Evaluation of the Sep-Pak method.

10 ml sample of growth medium containing the steroid cortisol (0.1mg) were extracted by the Sep-Pak procedure. The internal standards stigmasterol (IS_2) and cholesterol butyrate (IS_3 ; 0.05mg of each) were subsequently added (as they are not quantitatively recovered after Sep-pak extraction) and the sample derivatised and washed, before being processed by GC-MS. Fig.6.1 (top panel) shows a GC chromatograph of time of elution versus abundance of steroid for this sample. A comparable sample was processed in the same manner except that extraction using the Sep-Pak procedure was omitted. Fig. 6.1 (bottom panel) demonstrates the GC chromatograph for the directly derivatised steroids. The efficiency of extraction was determined by comparing the peak heights of cortisol in the extracted sample with that in the directly derivatised sample. Approximately 85% of the cortisol was recovered, suggesting that there was very little loss of cortisol during the Sep-Pak extraction procedure.

6.3.2. Tuning of the Spectrometer.

The tuning process can be automated (autotune) or operator controlled (manual tune). For both, this involves the introduction of a chemical compound into the mass selective detector. PFTBA (perfluorotributylamine, $(C_4F_9)_3 N$) is used as the tuning compound since it produces fragments throughout the entire mass range. The computer selects, from the various ion peaks produced from this compound, three ions; 69, 219 and 514 and scans over a small range of ± 5 mass units of each of these values. Fig 6.2 (top panel) illustrates a gas chromatogram for IS_2 and cortisol after autotuning of the spectrometer. Only the molecular ion for IS_2 is prominent (m/z 394); the molecular ion for cortisol (m/z 605) is masked by the baseline peaks.

The MO-TMS derivatives of the adrenocortical steroids produce molecular ions of relatively high molecular mass. To increase sensitivity at the high masses, the MS was manually tuned, and the three ion peaks selected were 69, 263 and 624. Fig. 6.2 (bottom panel) shows how the

FIGURE 6.1.

Evaluation of the Sep-Pak procedure. Analysis by GC of cortisol (0.1 mg in 10ml of growth medium) extracted by Sep-Pak cartridges before derivatisation (top-panel) and the directly derivatised steroid (bottom panel). The internal standards, IS₂ and IS₃, are stigmasterol and cholesteryl butyrate respectively. Both internal standards (0.05mg (top panel) and 0.1 mg (bottom panel) were added after extraction and before MO-TMS derivatisation.

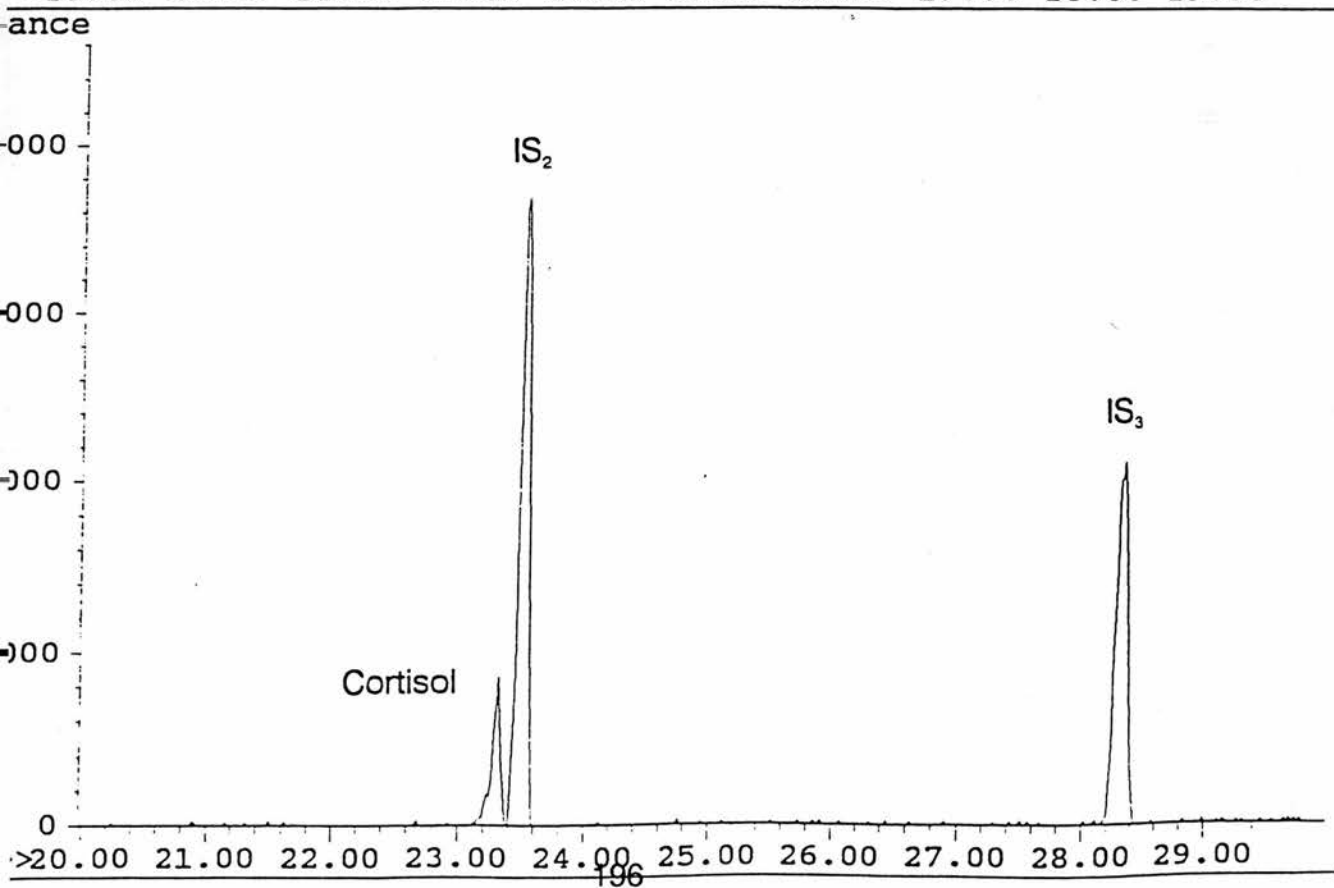
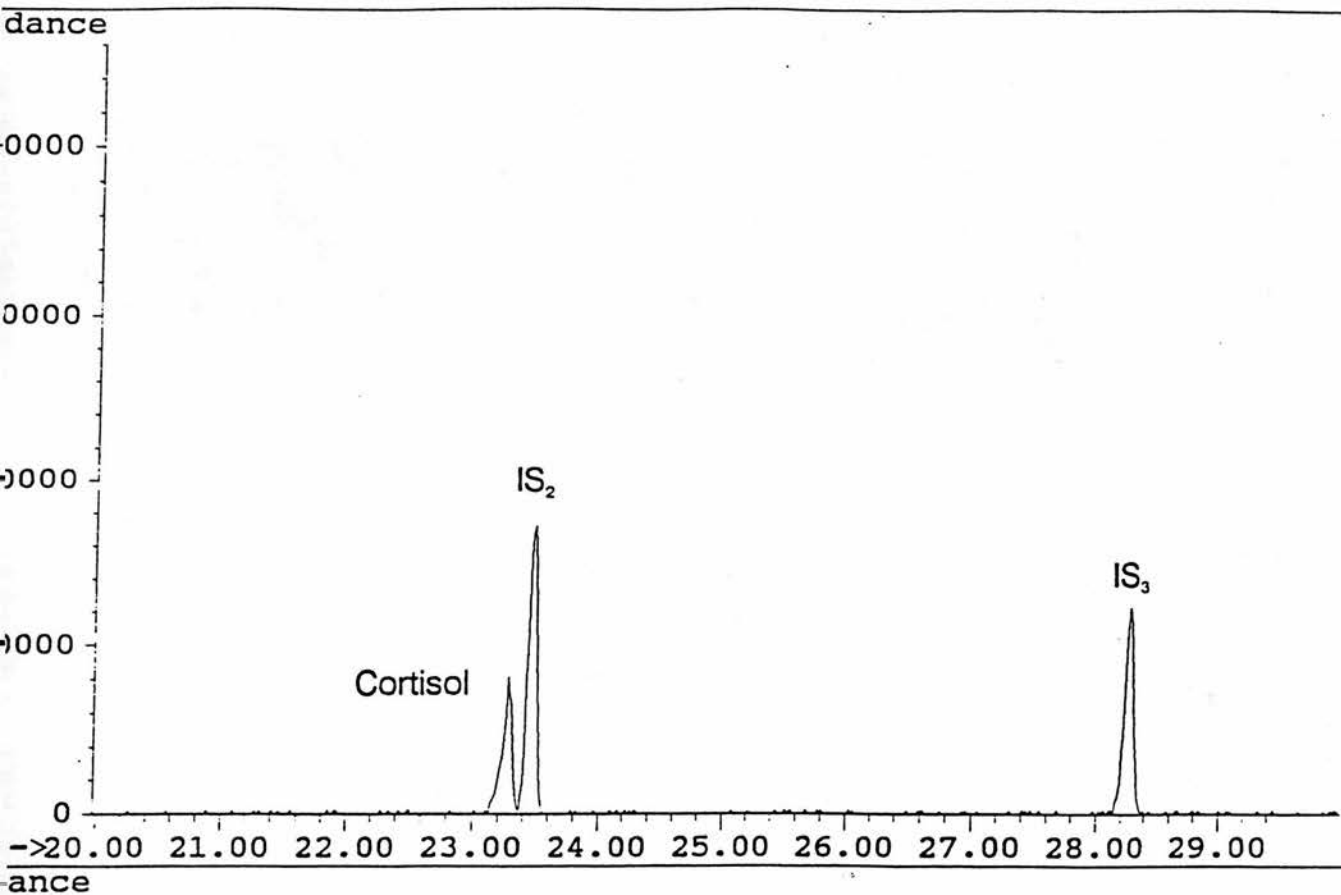
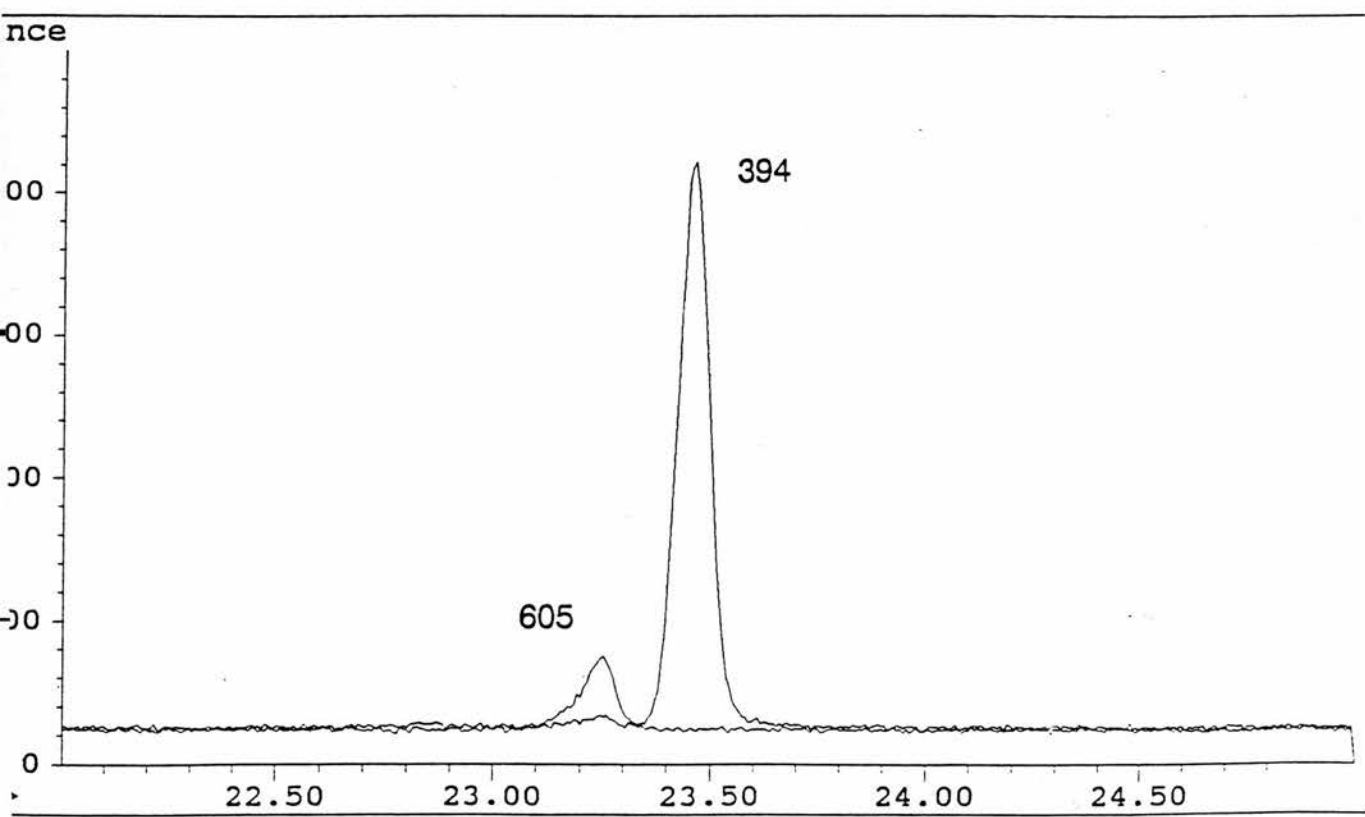
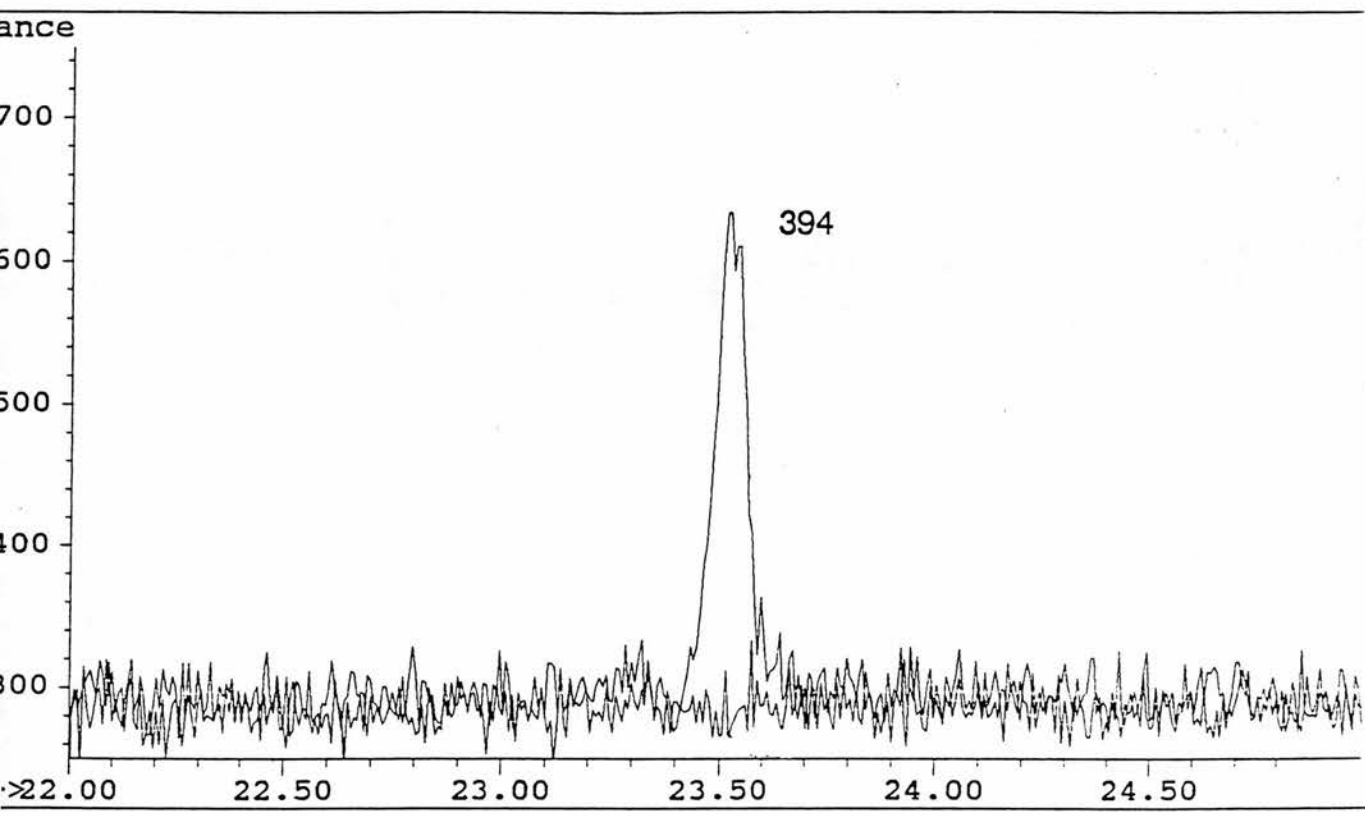


FIGURE 6.2.

GC analysis of the MO-TMS derivative of cortisol (0.1mg) using; a) auto-tuning, where the computer selects three ion peaks (m/z 69, 219 and 514) and b) high mass tuning, where the ion peaks are manually selected (m/z 69, 263 and 624). The internal standard is stigmasterol (I_{S_2})(1mg). The large peak present in the top panel is the molecular ion for stigmasterol (m/z 394). The molecular ion for cortisol (m/z 605), is masked by the baseline peaks. Tuning of the MS at high masses increases the sensitivity and the signal/noise ratio which is evident by the steady baseline in the bottom panel. The molecular ions for both cortisol and stigmasterol are now clearly apparent.



molecular ion for cortisol is no longer masked by the baseline peaks due to an improvement in the signal/noise ratio (lower background signal) and can easily be distinguished from the molecular ion for IS₂.

6.3.3. Identification of Steroids.

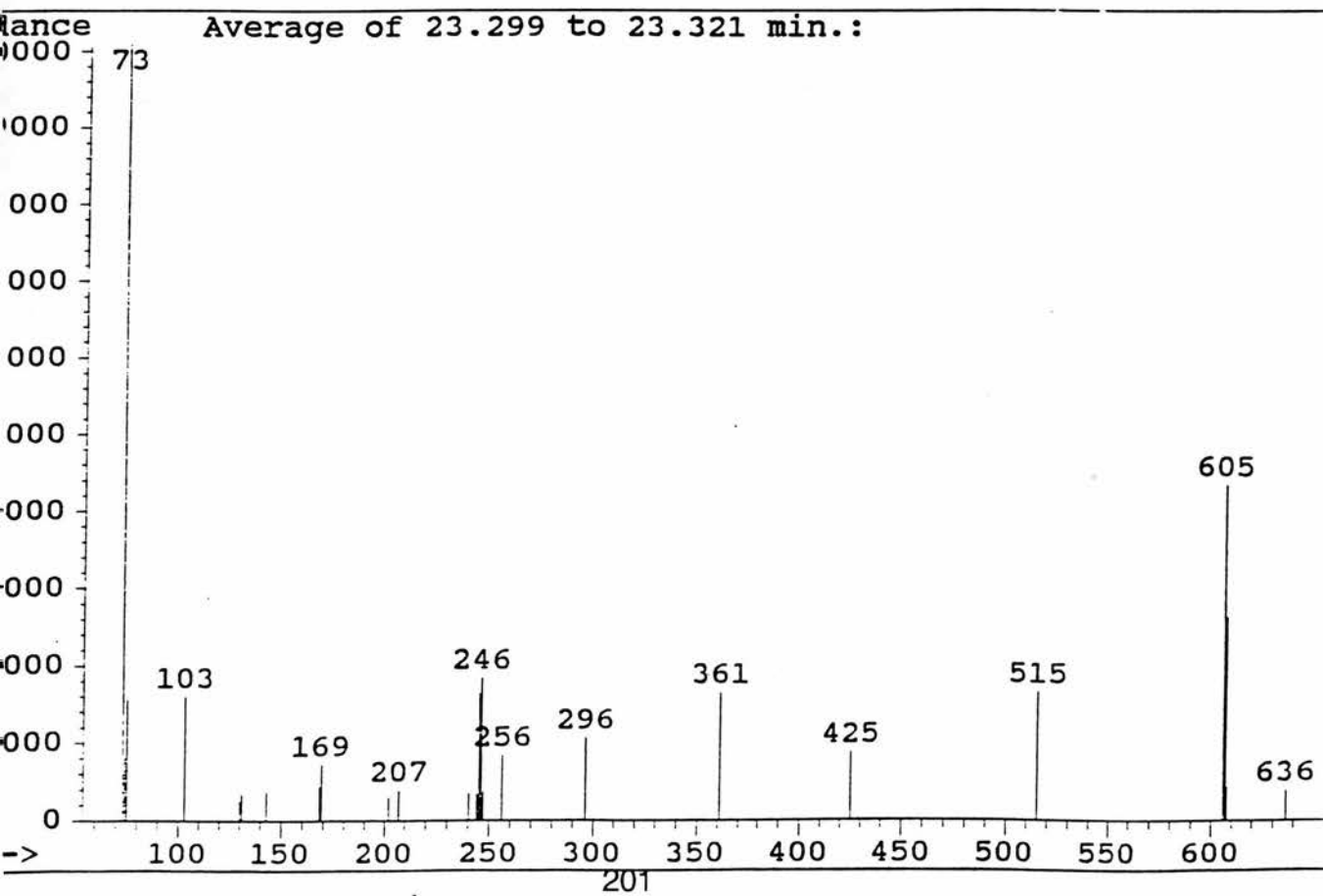
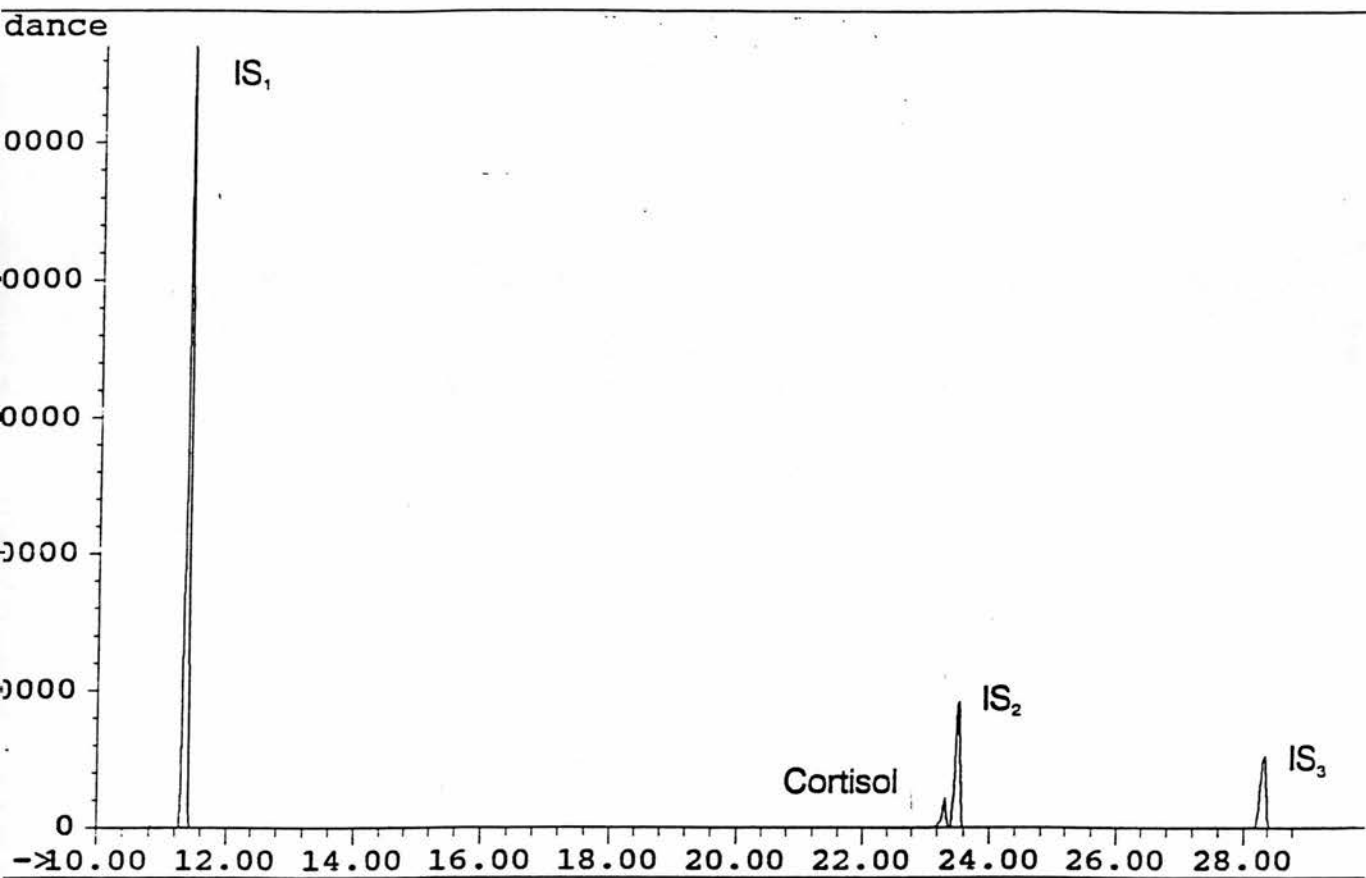
In order to identify specific steroids in the culture medium, it was first necessary to characterise the GC-MS spectrum of each individual steroid sought in the medium, using pure samples of each steroid. The following steroids were studied in this way; pregnenolone, progesterone, 17 α -hydroxypregnenolone, 17 α -hydroxyprogesterone, deoxycorticosterone, 18-hydroxydeoxycorticosterone, corticosterone, 11-deoxycortisol, cortisol, cortisone, dehydroandrosterone (DHAS), 4 androstene-3,17-dione and 4 androstene-11- β -ol-3,17-dione.

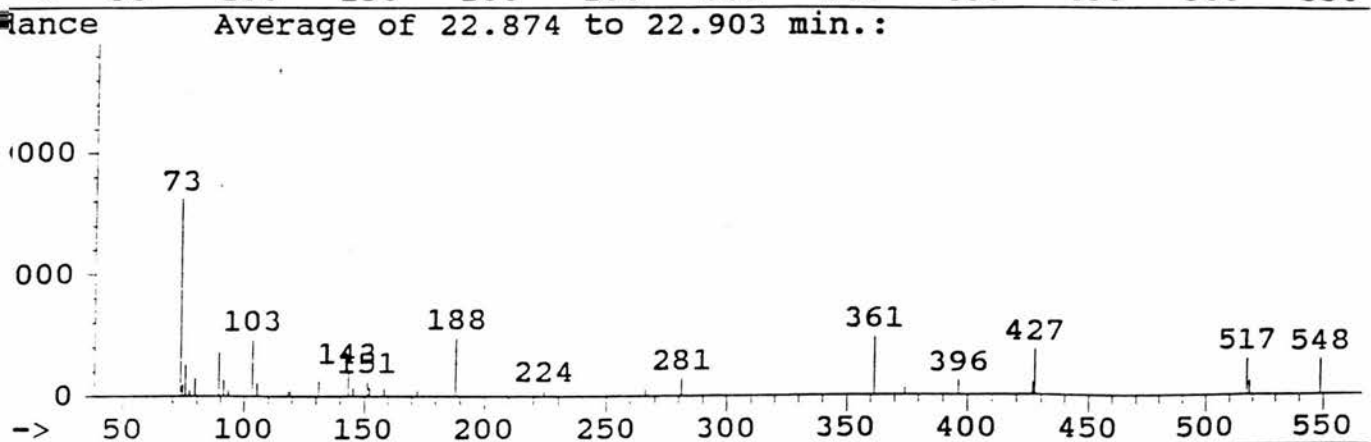
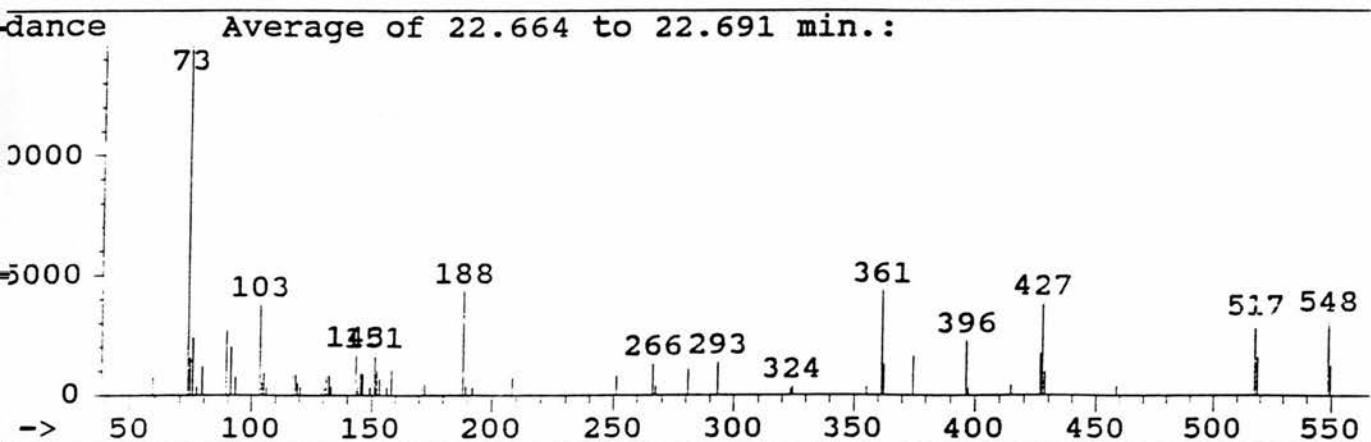
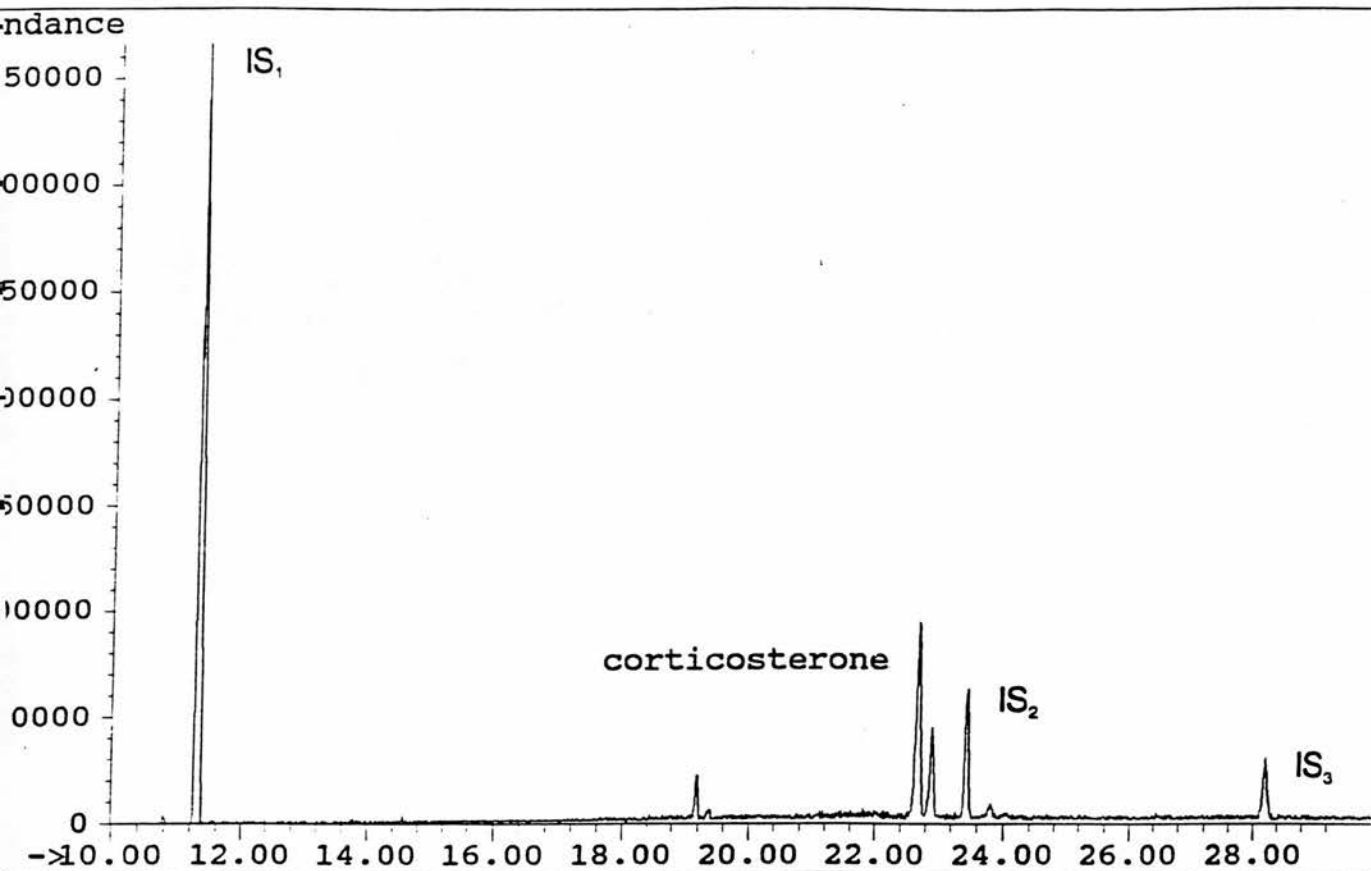
The characterisation of each of the above steroids required the following steps. Stock solutions of each individual steroid (3mg/10ml) and internal standards (0.3mg/ml) were prepared. Portions of 1ml of each steroid were made up in 40ml of growth medium (to which was added 0.01mg of IS₁) and extracted on a Sep-Pak column. 0.01mg of IS₂ and IS₃ were added and the steroids evaporated to dryness singly. MO-TMS derivatives were formed as described and analysed by GC-MS.

Two sets of information were obtained for each steroid analysed. 1) The GC-MS produced a chromatogram of time of elution versus abundance for each steroid. Cortisol, corticosterone and 4 androstene-11 β -ol-3,17-dione are shown as examples (fig. 6.3 a,b,c). The internal standards are required for GC analysis to identify steroids of interest by the position of the latter relative to the standards. From this it was possible to establish the retention time (RT) that is characteristic for a particular steroid (table 6.1). The mass spectrometer produces an electron-impact mass spectrum for each steroid. The ion of highest mass in the spectrum is the molecular ion (M⁺). In the case of cortisol this occurs at m/z 605. Table 6.1 summarises the M⁺ ions for each of the steroids. All the other ions are formed from the fragmentation of this molecular ion. This provides a "fingerprint" which can be used to identify a particular steroid.

FIGURE 6.3.

A gas chromatogram of time of elution versus concentration of steroid and a mass spectra for each of the MO-TMS derivatised steroids, cortisol, corticosterone and 4 androstene 11 β -ol,3,17-dione (a,b and c). The two major peaks in the gas chromatograms for corticosterone and 4 androstene 11 β -ol,3,17-dione correspond to the syn and anti isomers of the steroid. The mass spectra for each of these isomers is the same (b and c). The major ions and retention times for each of these steroids are summarised in table 6.1.





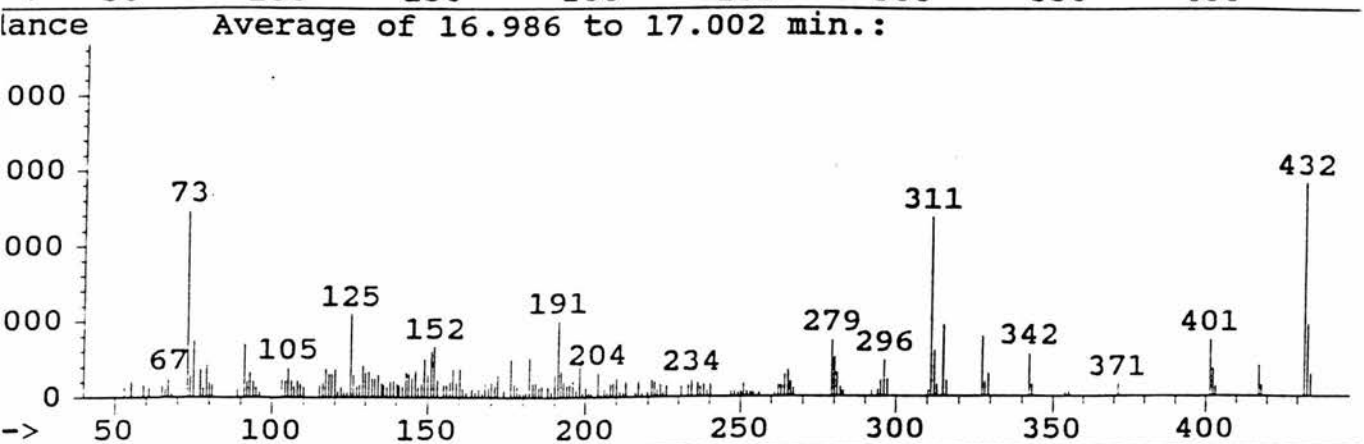
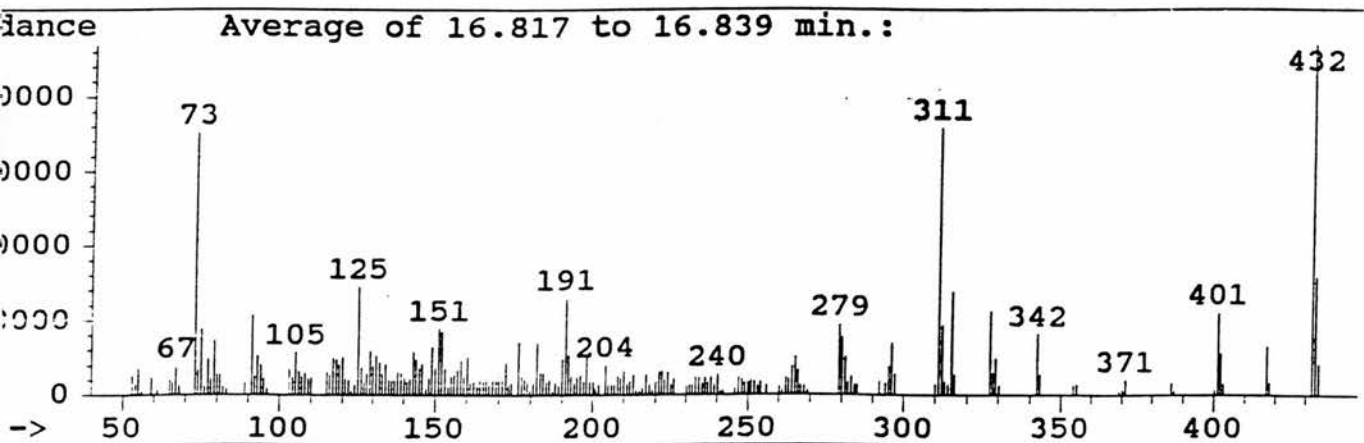
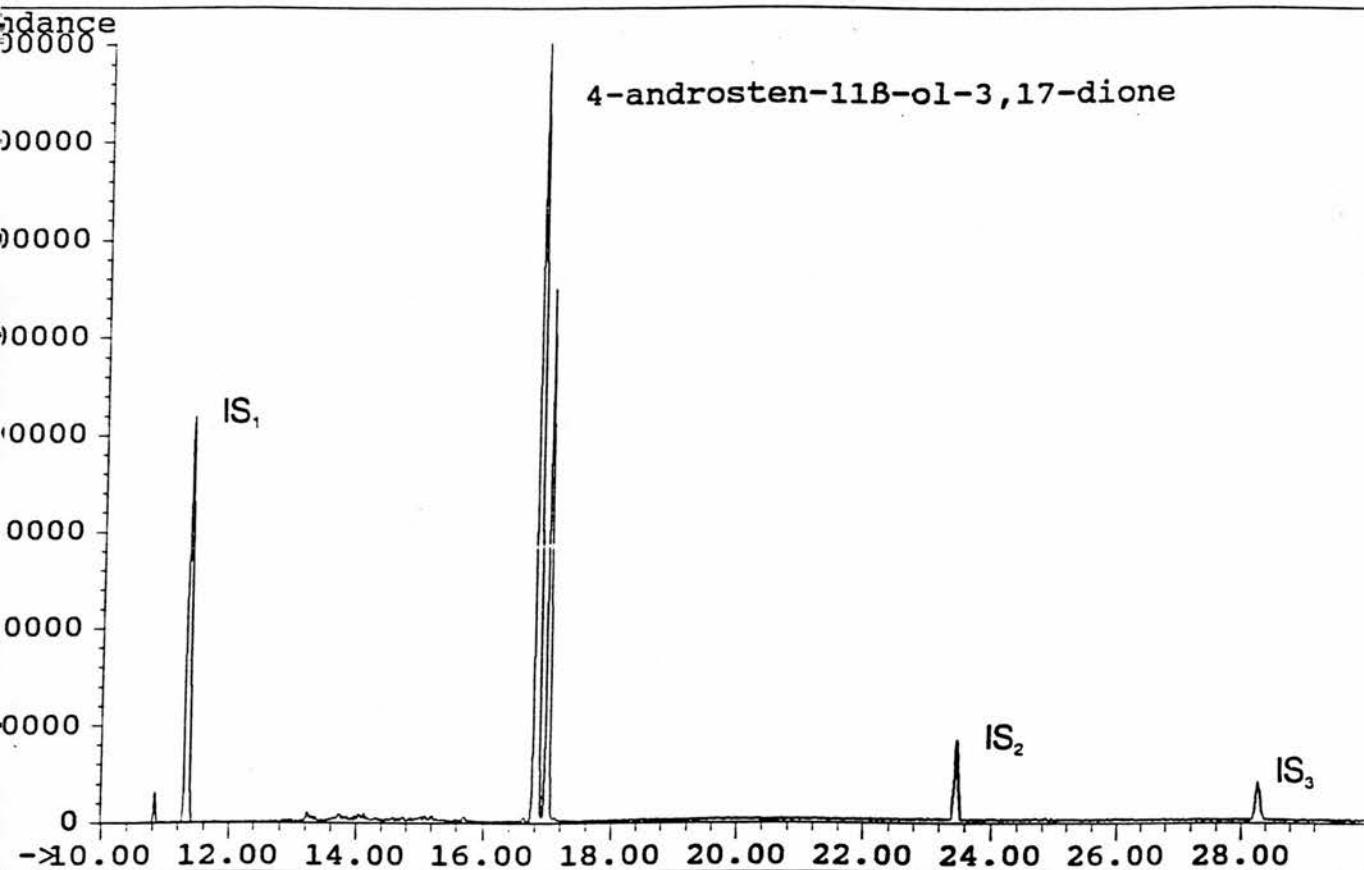


Table 6.1.

STEROID	T.I.	Q.I. 1	Q.I. 2	Q.I. 3	Major Peak	Minor Peak
IS ₁	436.3	421.3	346.2	331.2	10.640	
IS ₂	484.4	394.3	355.3	255.2	21.363	
IS ₃	368.3	369.3	353.3	247.2	25.620	
DHAS	358.2	389.2	374.2	284.2	11.725	
Pregnenolone	386.3	417.3	402.3	312.2	14.242	
Progesterone	372.3	341.2	286.2	273.2	15.601	15.675
17 α OH-pregnenolone	474.3	505.4	384.2	362.2	15.088	
17 α OH-progesterone	429.3	460.3	339.2	273.2	16.287	16.196
4-androstene3,17-dione	344.2	329.2	313.2	267.2	12.824	12.898
4-androsten-11 β -ol-3,17	432.3	401.3	342.2	311.2	15.514	15.662
Deoxycorticosterone	429.3	460.3	357.3	286.2	18.230	
18OH-deoxycorticosterone	396.3	548.4	517.4	427.3	19.518	
18OH-deoxycortisol	484.4	606.5	515.4	425.3	22.491	22.677
11-deoxycortisol	517.4	548.4	445.3	427.3	18.526	18.372
Cortisol	605.5	515.4	425.3	361.2	21.266	
Cortisone	531.4	441.3	562.4	459.3	20.459	20.281
Corticosterone	361.2	548.4	517.4	427.3	20.704	20.892

Table 6.1.

Summarises the molecular ions (M^+), which have been designated as the target ions (T.I.), and the qualifier ions (Q.I.) for each of the major adrenocortical steroids and which were employed, using SIM, in their identification. The retention times (min) of the major peaks (and for some steroids, the minor peaks) are also recorded.

The principle, known as selected ion monitoring (SIM), for identifying compounds from a few of their fragment ions is now widely used in steroid analysis. Designated ions were chosen for each of the steroids, based on their relative abundance and specificity for the steroid of interest. In the case of cortisol the ions chosen were m/z 605, 515, 425 and 361 respectively. The molecular ion 605, is the most abundant ion and is referred to as the target ion. The other ions then serve as qualifier ions. Table 6.1 shows the target and qualifier ions that were designated to each of the authentic steroids. The peak areas of the qualifier ions relative to the target ion are calculated for each of the authentic steroids and expressed as a percentage. These values are then compared to those calculated for the endogenous steroids secreted by the adrenocortical cells in culture. The observed and expected values should be similar, if the correct steroid has been identified. Fig. 6.4. illustrates examples of this for cortisol and IS_1 .

6.3.4. Quantitation of steroids.

The position of the peaks on the chromatogram identifies the steroids qualitatively, the size of the peaks gives an indication of the relative amounts of steroid present. Quantitation of any steroid is feasible as long as a suitable derivative and internal standard are available. 5α androstane $3\alpha,17\beta$ diol (IS_1) was chosen to quantitate the steroids as both IS_2 and IS_3 did not extract quantitatively using the Sep-pak procedure.

For each of the steroids the linearity of the response factors was tested. Various concentrations of steroids ranging from 0-100 μ g in the case of cortisol and corticosterone and 0-10 μ g for the other steroids were made up in 40ml of growth medium. To each solution a known concentration of IS_1 was added before extraction and the sample processed as described above. A standard curve was plotted for each steroid, of response ratio (peak area of the target ion of the steroid as measured against the peak height or area of IS_1) against the concentration of the steroid in the buffer (fig. 6.5). Quantification was performed by the computer.

FIGURE 6.4.

Analysis by GC using SIM of the endogenous steroid cortisol, from a cell culture incubation medium, and IS₁. The major ions for cortisol are m/z 605 (molecular ion), 515, 425 and 361 (qualifier ions) (top panel). The peak areas of the qualifier ions relative to the molecular ion for the authentic steroid, in this case cortisol, are calculated and expressed as a %. The expected values for each of the qualifier ions were 26.8% (Q1; 515), 8.3% (Q2; 425) and 13.9% (Q3; 361). The observed values for cortisol were calculated to be 26.8% (Q1), 9.2% (Q2) and 14.8% (Q3) respectively, indicating that these peaks correspond to the steroid cortisol. The major ions for IS₁ are, m/z 436 (molecular ion), 421, 346 and 331 (qualifier ions). The expected values for each of the qualifier ions were 58.6% (Q1), 47.6% (Q2) and 60.6% (Q3). The observed values for IS₁ were calculated to be 58.1% (Q1), 48.1% (Q2) and 65.2% (Q3).

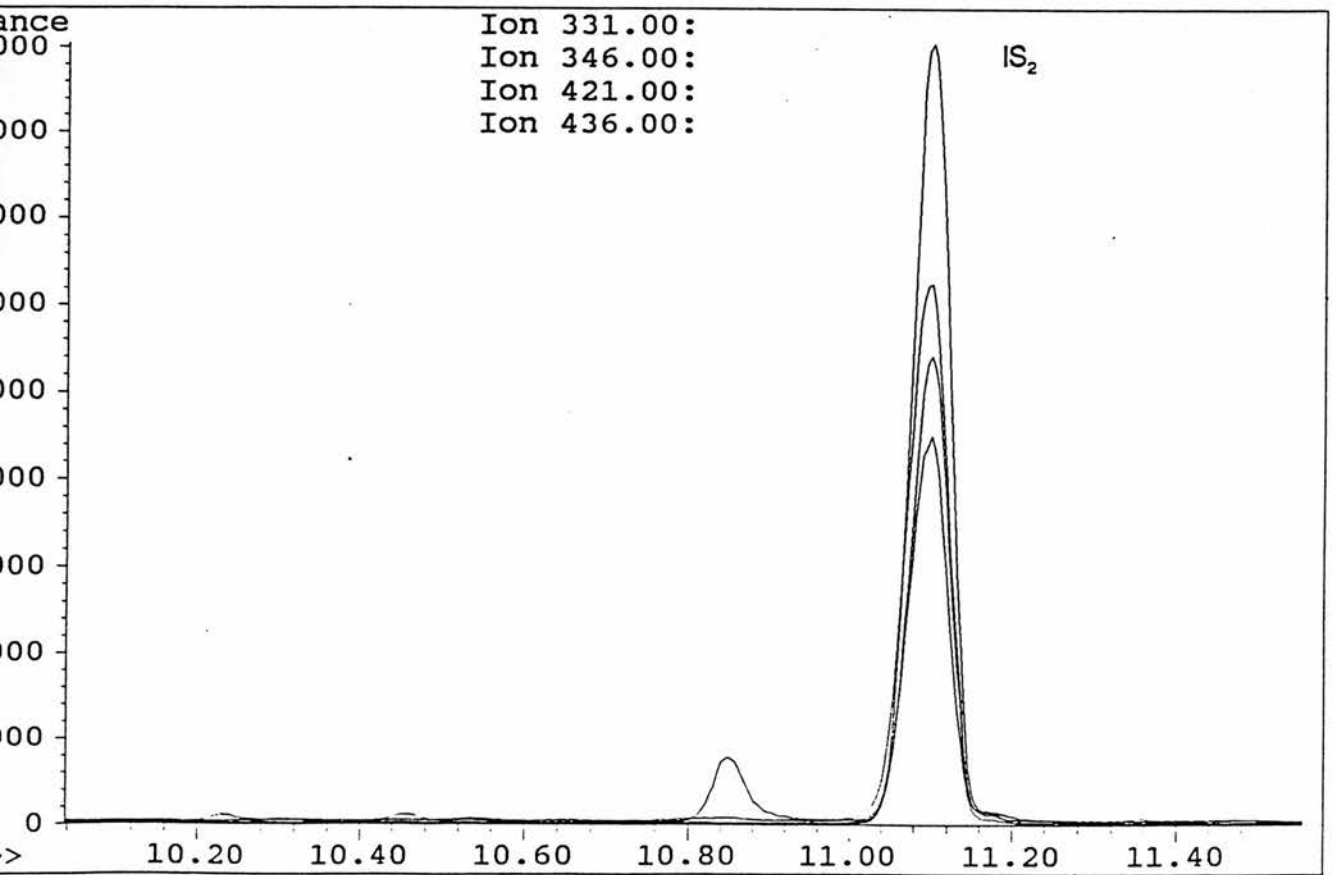
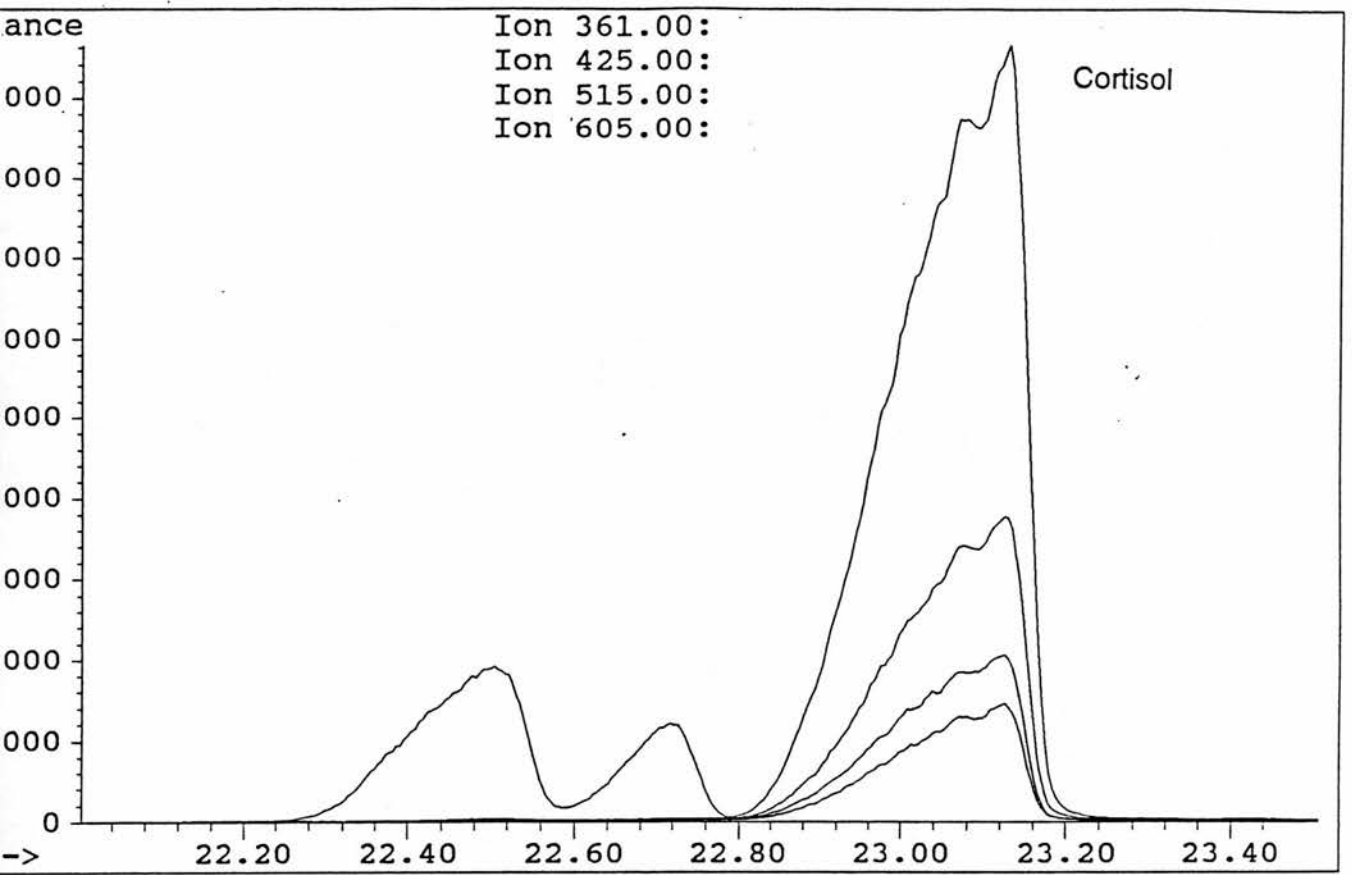
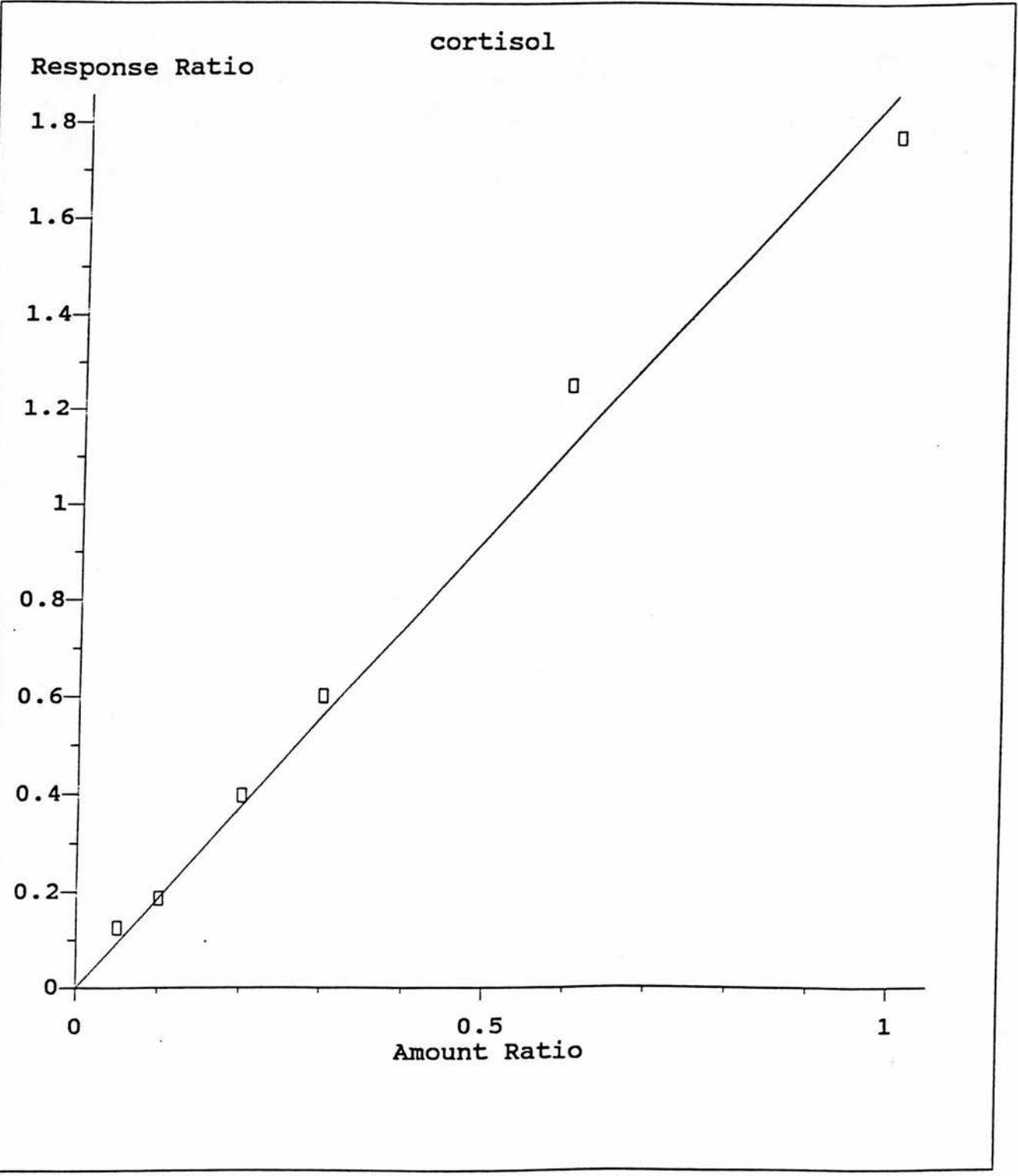


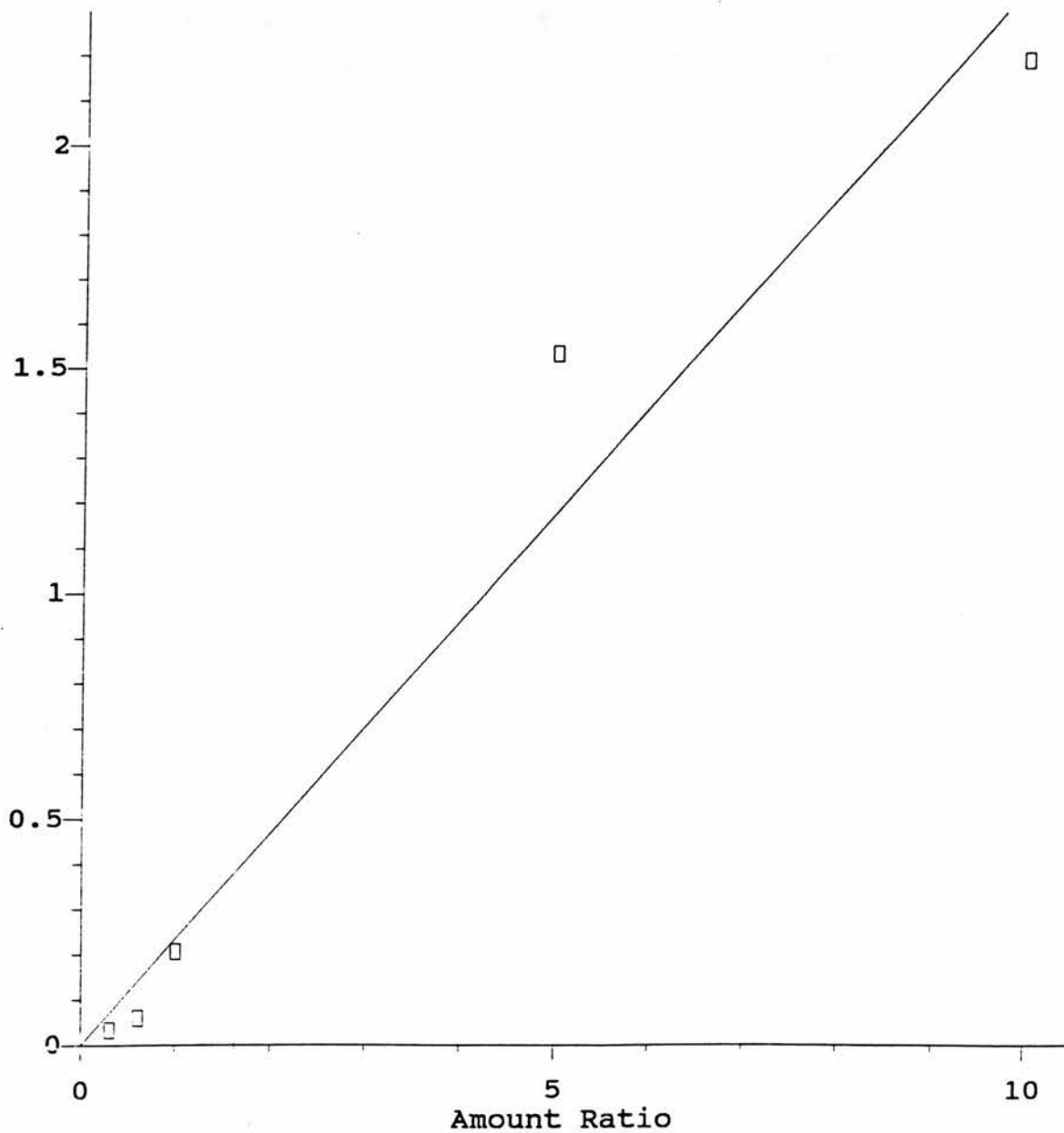
FIGURE 6.5.

Standard curves for cortisol (a), corticosterone (b) and 4 androstene 11 β -ol,3,17-dione (c). Various concentrations of the steroid (0-100 μ g) were made up in ethanol and taken through the complete analytical procedure. The ratio of the areas of the steroid and IS₁ were plotted against the concentration of steroid in the buffer relative to IS₁ (amount ratio).



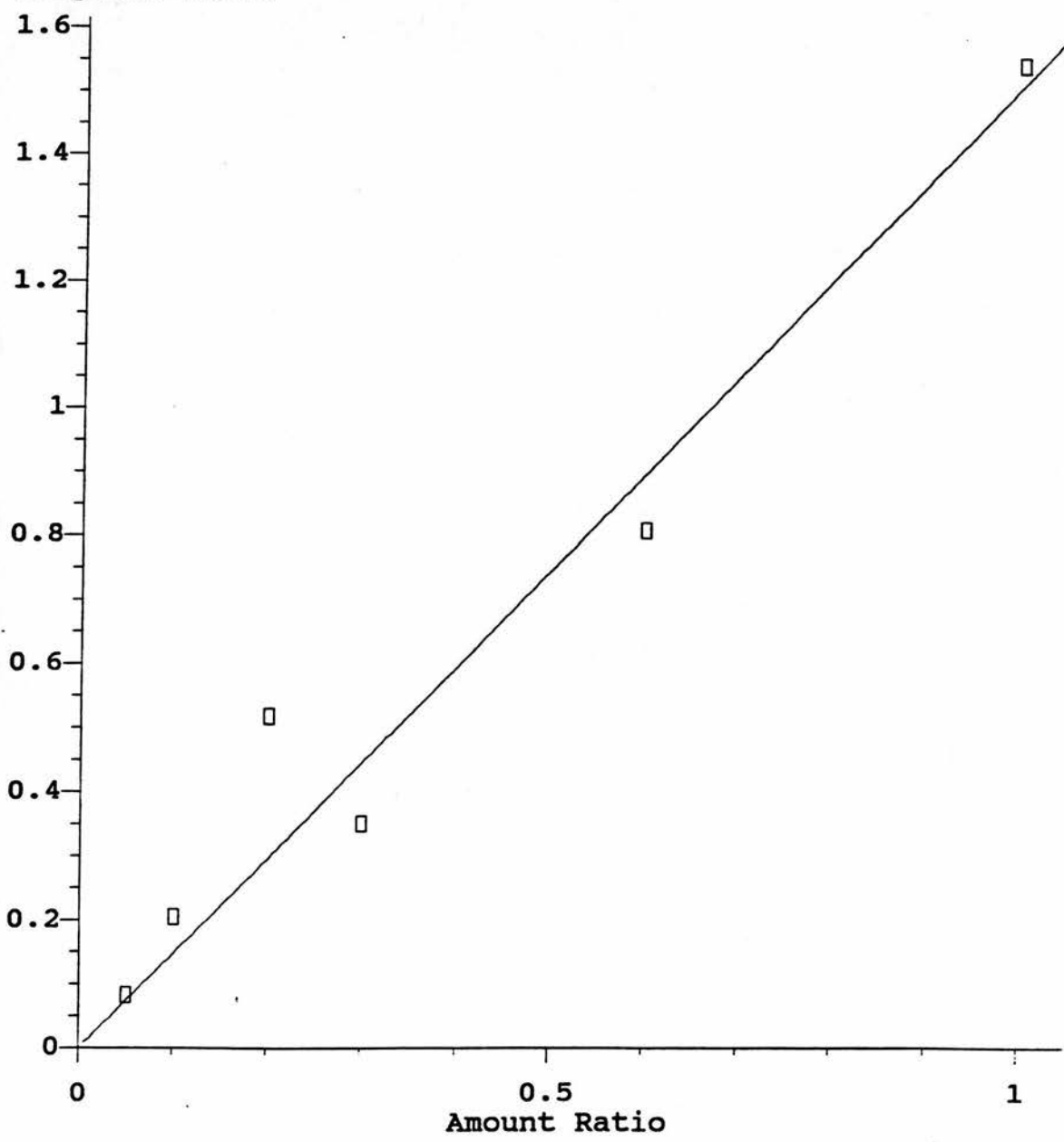
corticosterone

Response Ratio



4-androsten-11beta-ol-3,17-dione

Response Ratio



6.3.5. Full scan and SIM analysis of endogenous steroids.

40×10^6 bovine zfr cells in culture were acutely (2h) and chronically (24h) stimulated with 10^{-9} M ACTH. The medium containing the secreted steroids was collected and processed for GC-MS analysis as described earlier. Fig. 6.6 shows the chromatograms obtained for the endogenous steroids, recorded using full scan monitoring. According to this approach, the whole of the mass range of interest (50-650) was scanned automatically and repetitively. Only 3-4 of the endogenous steroids were identified using this method. The major differences between the two profiles is an increase in the production of cortisol and androstene-11 β -ol,3,17-dione on chronic stimulation with ACTH. The major advantage of this method is that all unknown material as well as the steroids under investigation are recorded. For example, a major unidentified peak (U) (fig.6.6 bottom panel) appears after chronic stimulation.

If a mass range of 50-650 is being scanned rapidly and repetitively, the abundance of each ion is only being examined for a relatively short period of time. Higher sensitivity is achieved if the instrument is calibrated such that only selected ions of a particular steroid are monitored, as previously explained. Fig. 6.7 shows a chromatogram recorded using SIM. Most of the key adrenocortical steroids present in the steroidogenic pathway, secreted after acute stimulation with ACTH (10^{-9} M), can be identified using this technique.

6.3.6. GC-MS analysis of the adrenocortical steroids secreted in response to ACTH, All and ATP.

Bovine zfr cells were plated out in 75cm³ flasks at 10^7 cells/flask. On day 3 of cell culture the cells were stimulated with ACTH (10^{-9} M), All (10^{-7} M) or ATP (10^{-4} M). For each agonist, 40×10^6 cells (4 flasks) were used, so that there was enough steroid present for quantitation. The cells were initially stimulated for a period of 2h with each of the agonists and the medium, containing the endogenous steroids, collected and stored, ready to be processed for GC-MS analysis. The cells were then washed twice (2×10 ml) using growth medium, before being chronically stimulated (24h) with the same agonists. The medium was removed and the cells washed, before

FIGURE 6.6.

Total adrenocortical steroid profile of MO-TMS derivatives. Bovine zfr cells in culture (40×10^6) were stimulated acutely (2h) (bottom panel) and chronically (24h) (top panel) with ACTH (10^{-9} M). Analysis of the separated steroids is by GC with full scan monitoring. The steroids identified were; a (4-androstene 11β -ol,3,17-dione), b (cholesterol), c (corticosterone), d (cortisol) and u (unidentified compound). Peaks a and u could not be identified after acute stimulation of the inner zone cells.

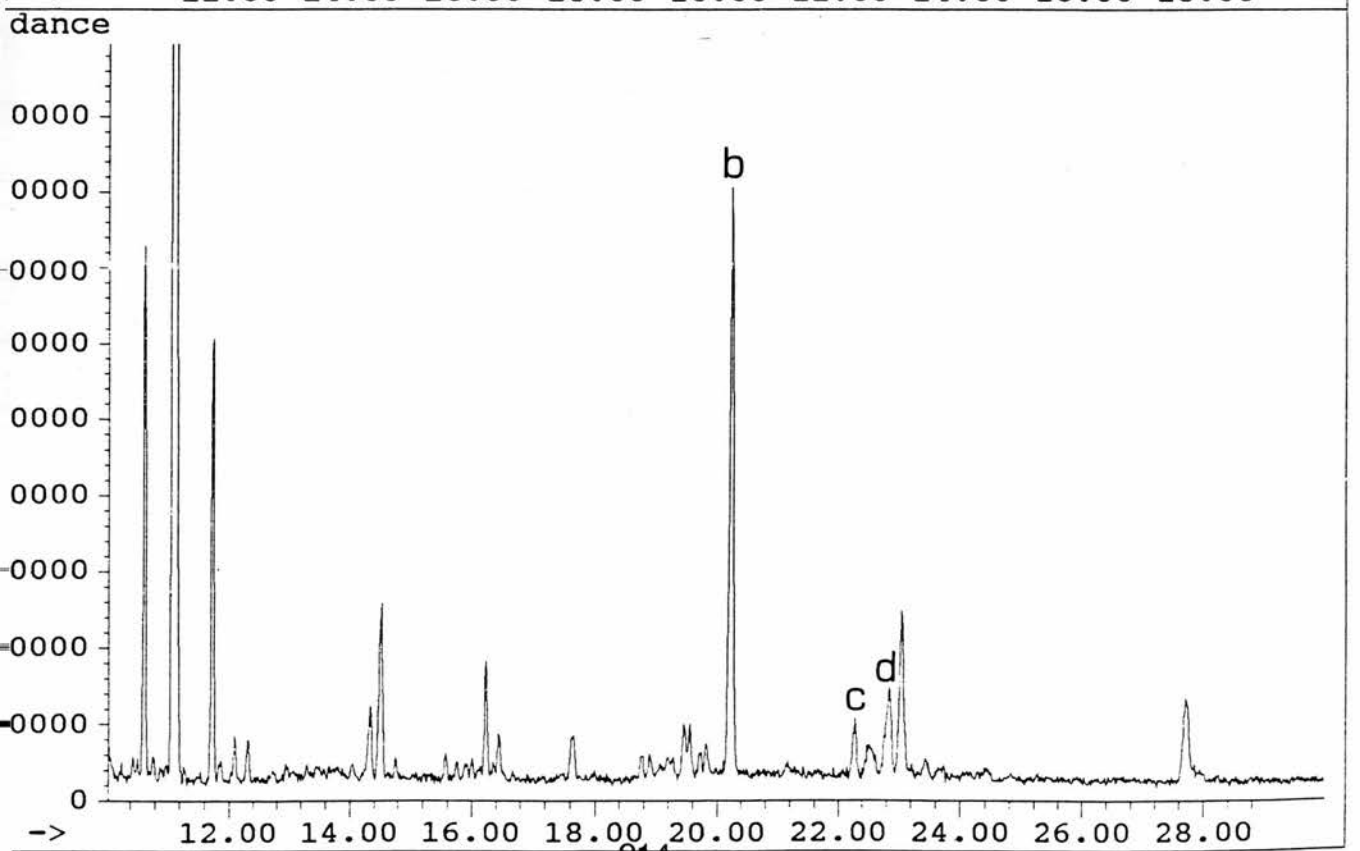
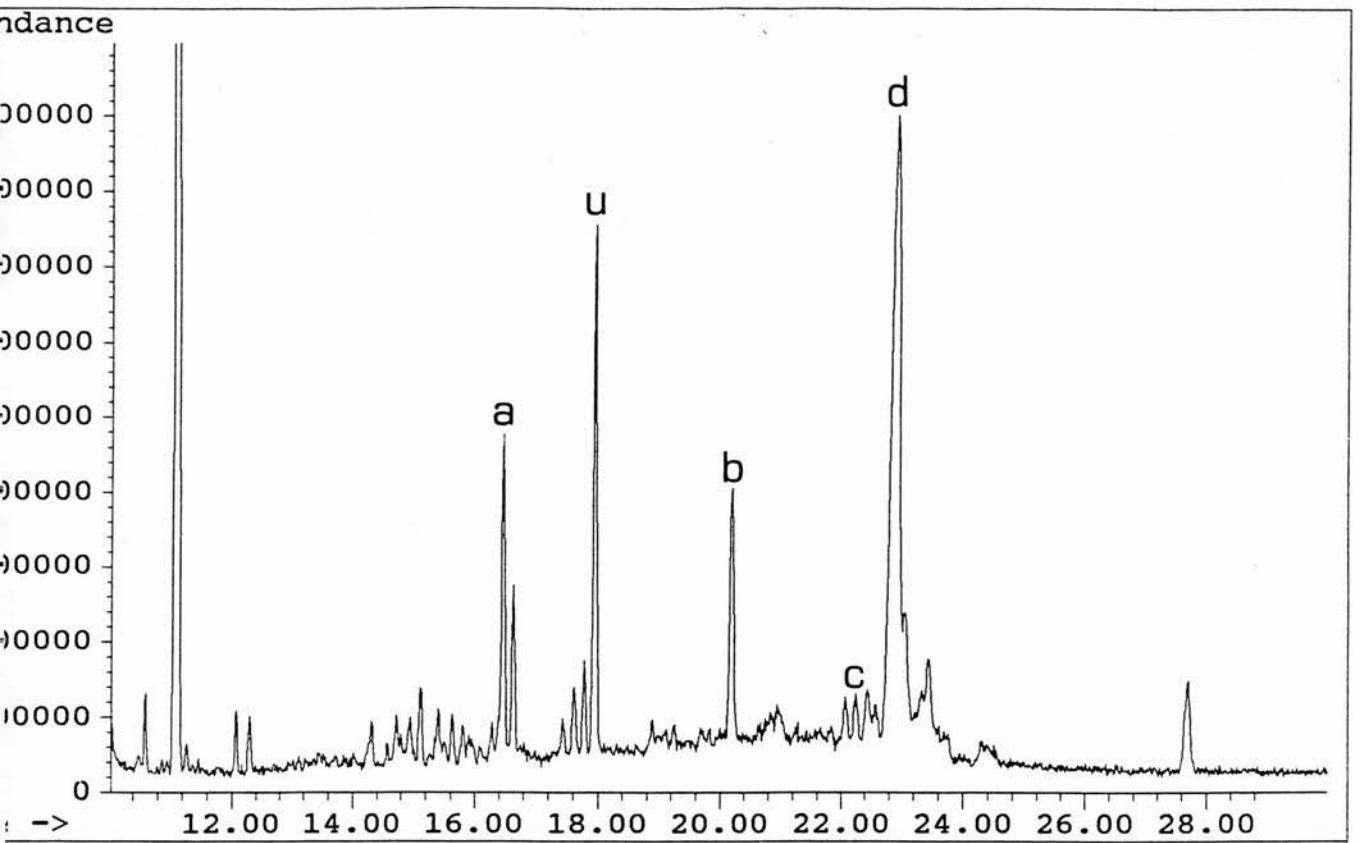
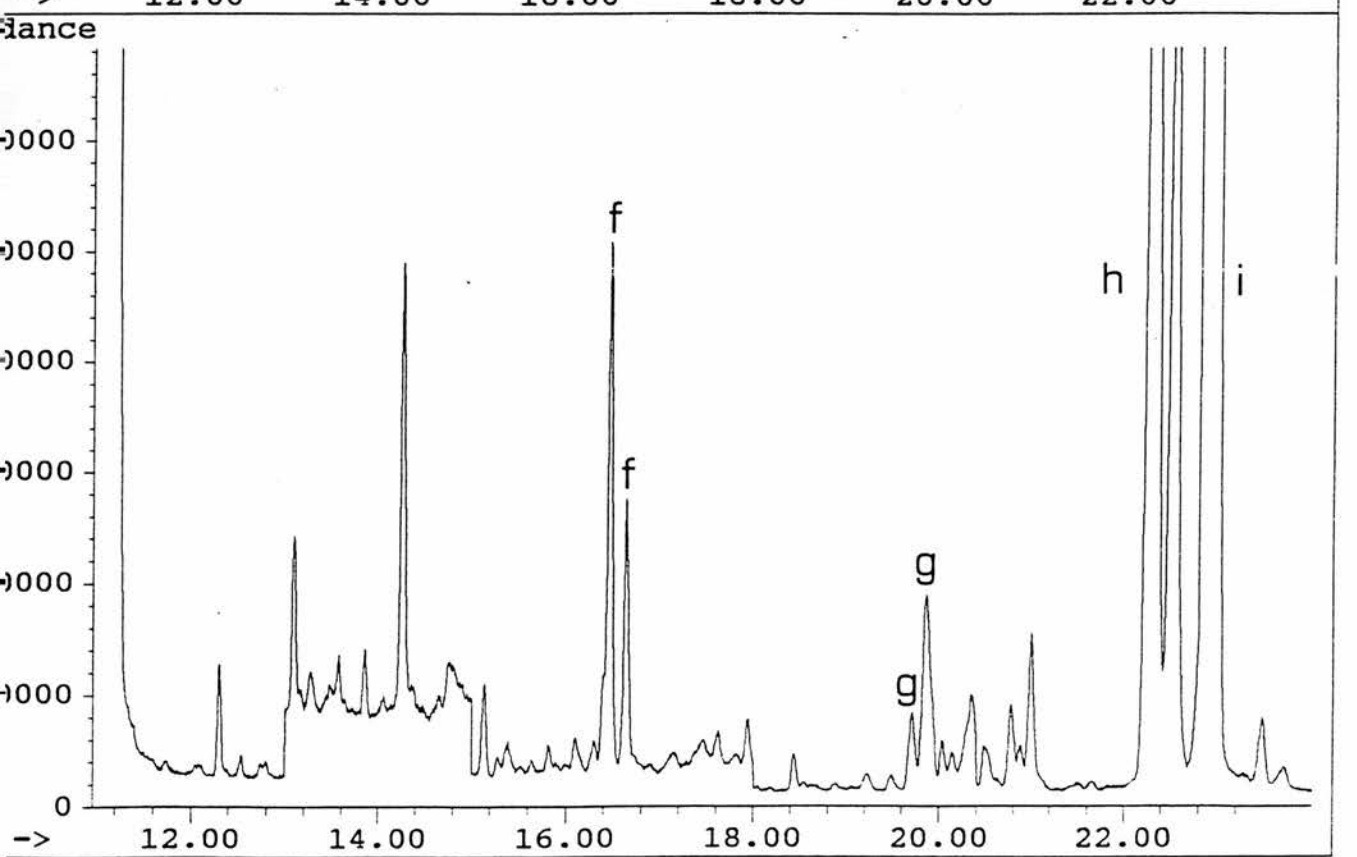
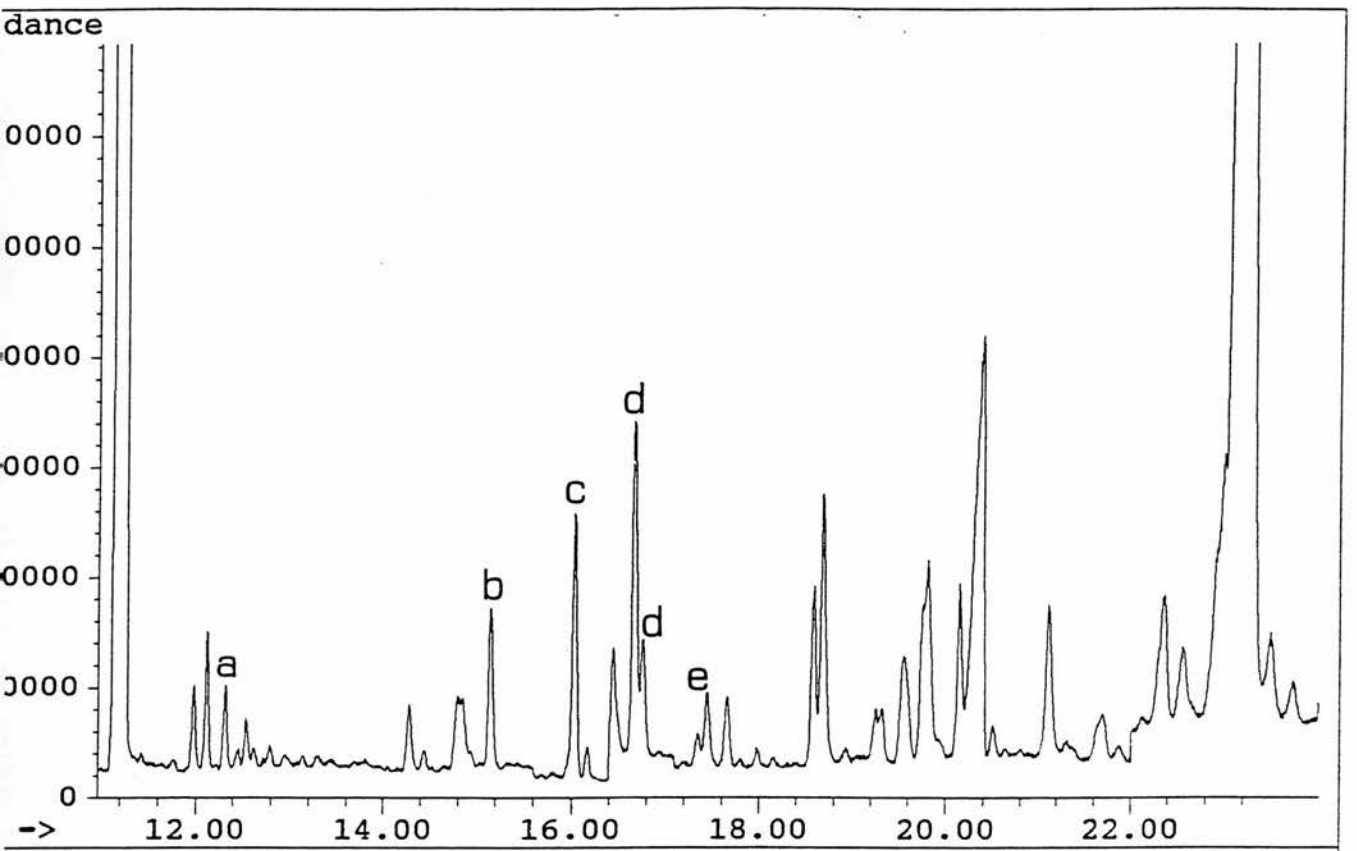


FIGURE 6.7.

Total adrenocortical steroid profile of MO-TMS derivatives, recorded using SIM. Bovine zfr cells in culture (40×10^6) were stimulated acutely (2h) with ACTH (10^{-9} M) and the incubation medium taken through the complete analytical procedure. The following steroids were identified; a (DHA), b (pregnenolone), c (17α -hydroxypregnenolone), d (progesterone), e (17α -hydroxyprogesterone), f (4 androstene 11β -ol,3,17-dione), g (11-deoxycortisol), h (corticosterone) and i (cortisol). Some steroids have similar retention times, therefore their peaks overlap. To overcome this, two separate injections (1μ l) of the sample were necessary; hence two traces are shown to represent this.



a final acute stimulation (2h). Again, the medium was collected and stored for quantitation.

The steroids were quantified as described in section 6.3.4 and the % change between initial 2h stimulus and final 2h stimulus (after 24h chronic exposure to agonist) for each agonist calculated (table 6.2). The major changes were the following; for ACTH, there was a large increase in the androgen, 4 androstene 11 β -ol,3,17-dione, and nearly complete inhibition in the production of corticosterone after chronic stimulation. All gave major changes in the production of cortisol (574.1 %) and corticosterone (360.4 %) with relatively little change in the androgens. Finally, for ATP, the major changes found were an increase in both androgen and cortisol secretion. All these experiments were conducted only once, except in the case of ATP where qualitatively similar results were obtained in a further experiment.

Table 6.2.

STEROID	Final steroid/Initial steroid (% change)		
	ACTH (10^{-9} M)	All (10^{-7} M)	ATP (10^{-4} M)
17 α -hydroxyprogesterone	231.9	Increase	133.3
17 α -hydroxypregnenolone	221.4	146.9	ND
18-hydroxydeoxycorticosterone	Large decrease	202.8	187.5
Cortisol	67.4	574.1	198.3
4 androstene-11 β -ol,3,17-dione	741.5	137.8	184.5
Deoxycorticosterone	11.6	ND	ND
Cortisone	120.9	27.5	16.7
Pregnenolone	Decrease	145.6	Decrease
Progesterone	5.9	304.6	ND
11-deoxycortisol	1055.1	542.2	136.7
Corticosterone	0.64	360.4	111.6
18-hydroxycorticosterone	4.78	134.9	74.1

Table 6.2.

40×10^6 bovine inner zone cells were initially stimulated acutely for 2h with the above agonists. After further exposure for 24h to the same concentration of the same agonist, the cells were restimulated for a further 2h with the same concentration of agonist. The % change between the initial and final 2h stimulus, for a range of adrenocortical steroids, was calculated. A few of the steroids are present in such small quantities, that they are not detectable (ND). In some instances, either after initial or final stimulation, the steroid is not detectable and the % change is just recorded as an increase or decrease in steroid concentration.

6.4 DISCUSSION.

Gas chromatography-mass spectroscopy (GC-MS) combines one of the most adaptable methods for the separation of complex organic mixtures of structurally related compounds with one of the most sensitive, powerful and reliable techniques for their detection. The utility of GC-MS is now widespread in several fields of biochemistry, including the analysis of steroids. This stems from the stability of the uniform nuclear carbon skeleton and the characteristic ions in the mass spectra, produced from the fragmentation-directing effects of the double bonds and the oxygenated substituents.

GC-MS has been used in the identification of previously unknown steroids in human urine, blood, faeces, amniotic fluid, fetal tissue and bile, providing a more complete understanding of the development of the human adult. The steroid "profile" is a valuable screening test in the studies of inborn errors of metabolism, for instance, steroid 21-hydroxylase deficiency and 3 β hydroxysteroid dehydrogenase deficiency (noted for reduced excretion of cortisol metabolites) and also in the mapping of the metabolic profile in other diseased states like, Cushing's syndrome, adrenal tumours, hepatic disorders (Shackleton et al 1986), hypertension (Stockl et al 1991) and cancer.

Originally, the method used in the extraction and separation of adrenocortical steroids secreted by cells in primary culture, involved the classical solvent extraction method using adsorbents such as Amberlite XAD-2 or Sephadex derivatives (Bradlow 1968, Sjoval et al 1979) followed by thin layer chromatography (TLC) or some other form of column chromatography to yield pure samples. Major disadvantages of this technique included differential steroid extraction according to their polarity, which potentially leads to errors in quantification and also the inconvenience in the length of time taken for this extraction-purification method.

Ramirez et al (1982) developed a one step extraction procedure using a C₁₈ Sep-pak cartridge system and reported approximately 90% yields of the extracted steroids, pregnenolone, progesterone, corticosterone and cortisol from cell culture incubation medium. Extraction of free

cortisol using Sep-pak C₁₈ cartridges and subsequent elution with methanol gave a recovery of approximately 85 % (fig. 6.1), similar to the findings of Shackleton et al (1980) for the recoveries of cortisol and aldosterone in urine.

The individual steps of the sample preparation and the derivative formation procedure were not tested individually, since such information is available in the literature. The derivatisation of the steroids employed is a standard procedure (Theriot et al 1972) with small modifications. All functional groups, except at position C₁₁, are derivatised by this method. Excess silylation reagent and non-polar lipids are removed using hydroxyalkyl-Sephadex LH-20, known as Lipidex 5000. The major advantages of lipophilic gels are inertness and low bleed, giving high yields and low contamination, ensuring clean chromatograms. They are also capable of dealing with high sample capacities.

Identification of steroids in the incubation medium of adrenocortical cells in culture is difficult, primarily because of the low concentrations in which they are present. All MO-TMS derivatised adrenocortical steroids, on fragmentation, produce molecular ions of high mass. In order to maximise sensitivity of the MS in detecting these steroids, the spectrometer was manually tuned using the tuning compound, PFTBA. This is integral to the identification of the endogenous steroids, as much more interference is seen at lower masses which inevitably decreases the signal/ noise ratio and sensitivity, resulting in the masking of the molecular ion for cortisol (m/z 605) by the baseline ions (fig. 6.2 top panel). By manually tuning the MS for high masses, as described in section 6.3.2, the signal/noise ratio increases, which is evident by the steady baseline. The molecular ion for cortisol is now clearly apparent, due to the increased sensitivity at the high masses.

The endogenous steroids under investigation were characterised by comparing their retention times with those of the steroid standards and verified by the similitude of their mass spectra to those of the authentic samples. Preliminary experiments showed that the levels of aldosterone secreted by stimulated (ACTH) and unstimulated (no agonist) adrenocortical cells in culture were similar, therefore this steroid was excluded from measurement. Internal standards

are required by GC analysis to identify the steroids of interest by their position relative to these standards. The internal standards should be as chemically similar to the unidentified steroids as possible and be capable of separate detection and measurement. Three internal standards were chosen; 5 α -androstene-3 α ,17 β -diol (IS₁) which elutes before the naturally occurring steroids, cholesterol butyrate (IS₃) which elutes after them and stigmasterol (IS₂) which is included because it is relatively difficult to silylate and its peak height, in comparison to the other standards, provides a check on the completeness of derivatisation. The internal standards should ideally be added at the beginning of the extraction procedure, though two of the chosen standards, IS₂ and IS₃, were not quantitatively recovered after Sep-pak extraction and were therefore added after this process.

The greatly improved resolution achieved by the introduction of fused silica capillary columns for the separation of steroids has significantly assisted in their detection. The chromatograms of time of elution versus concentration of steroid in fig 6.3 a, b and c of the steroids; cortisol, corticosterone and 4 androstene 11 β -ol,3,17-dione are compared with that of the internal standards. Table 6.1 gives the retention times of the 14 steroids of interest for the quantitative study of the adrenal biosynthetic pathway. In his first studies of the methoxime derivatives, Horning (1968) noticed the formation of geometric isomers of the syn/anti type. Separation of the isomers depends on the other functional groups attached to the carbon skeleton. Of the 14 steroids under investigation, eight produced two peaks on the gas chromatogram representing syn/anti isomer pairs. These isomers can be divided into three categories; i) type 1, giving two well separated peaks, the first peak eluting being the higher; this group includes corticosterone and 4 androstene 11 β -ol 3,17-dione; ii) type 2, gives two well separated peaks, the first eluted being the lower; this group includes 11-deoxycortisol and the 17 α hydroxysteroids; and iii) type 3, giving a separation of two peaks of the same height; this group includes progesterone.

MS is one of the most powerful methods for the identification of micro-amounts (picogrammes) of a compound. It produces a spectrum of mass/charge ratio versus abundance,

for each steroid, which is unique for that steroid. The ion of highest mass is the molecular ion and all other ions are formed from the fragmentation of this ion. The mass spectrum can also provide extensive detail about the structure of the steroid. If a steroid has a derivatisable hydroxyl group, this would be expected to lead to a loss of 90 mass units, in the case of cortisol (fig. 6.2a), for example there are two losses of 90 mass units (605 to 515 and 515 to 425) consistent with the presence of two hydroxyl groups in cortisol. If a derivatised carbonyl group is attached to the steroid backbone, then the first loss seen from the molecular ion would be 31 mass units, equivalent to O-CH_3 . Cortisol, corticosterone and 4-androstene-11 β -ol-3,17-dione, each have at least one carbonyl group (fig. 6.3 a, b and c). Most of the other fragmentations are related to the construction and position of the functional groups in the steroid. However, characteristic ions are present in the spectra for each of the steroids and these may represent decomposition products of the stationary phase of the column.

A much more reliable measure to undertake in the identification of a particular steroid in a sample is target compound analysis which takes advantage of known information that is characteristic of the steroid. For instance the mass spectrum of the steroid allowed the selection of characteristic ions for the steroid, so that it could then be identified in the presence of other steroids. One of the ions chosen is designated as the target ion (this is usually the molecular ion of the steroid) and the other ions can then be used as qualifier ions (table 6.1). The qualifier ions must be present in the correct amounts relative to the target ion for the peak to qualify as the target compound. For example, from the mass spectrum of cortisol, ions at 605, 515, 425 and 361 were chosen. In the target compound analysis, ion 605 was selected as the target ion, as it is the most abundant and thus increases the sensitivity of the analysis. The other ions were relatively specific for cortisol and were chosen as qualifier ions. Fig. 6.4 illustrates an example of the identification of cortisol in the cell culture medium using the target compound analysis procedure.

Two different modes of operation of the MS can be used in the identification and quantification of steroids; full scan and selected ion monitoring (SIM). In full scan mode, the MS scans linearly across every mass in a specified range (m/z 50 - 650). The mass filter of the MS

is scanned in discrete steps (0.1m/z) from high mass to low mass. In SIM, instead of dividing the mass range into 0.1m/z increments and recording information at each, the mass filter is programmed to select a few specific m/z values and only these few ions are scanned and measured.

Fig. 6.6 shows a total ion chromatogram of total abundance of all the ions in all the scans versus the run time using full scan mode, for bovine adrenocortical cells in culture that have been stimulated both acutely (bottom panel) and chronically (top panel) with ACTH. The major advantage of this technique is that all the data from the analysis is recorded and the mass spectra of any GC peak can be studied, which is important in the identification of unknown compounds (peak U in fig. 6.6 top panel). However the main drawback of full scan is that when spectra are recorded at the typical rapid scan rates, only the order of 1 - 5 msec is spent on each m/z value. Narrow GC peaks may only contain 1 or 2 scans and therefore the peak shape may not be properly recorded with the potential for poor quantitation. Another important consequence of this is that full scan thus lacks the sensitivity to permit detection of the characteristic ions at low concentrations. Only three of the endogenous steroids were identified using this method; cholesterol, corticosterone and cortisol (fig 6.6 bottom panel). Even when the cells in culture were chronically stimulated with ACTH to maximise steroid production, the only additional steroid detected was the androgen, 4-androstene 11 β -ol, 3,17-dione (fig. 6.6 top panel).

SIM provides greater sensitivity, as all of the run time is devoted to looking at the few selected ions rather than scanning large areas where there may be no signal, therefore a peak can be sampled more often (dwell time 100 times greater than that possible in a full scan), allowing correct recording of peak shape and therefore the potential for more accurate quantitation. By monitoring ions characteristic of each peak, overlapping peaks in a complex sample can also be resolved. Fig. 6.7 illustrates the advantage of using SIM, compared to full scan, in improving detection and allowing most of the major steroids in the pathway (produced on acute stimulation with ACTH) to be identified and quantitated.

The quantitative analysis required the setting up of calibration curves for each steroid

under investigation. Mixtures containing known amounts of the authentic steroids were prepared such that the sample injected covered the range 0 - 10 μ g (0 - 100 μ g in the case of the more abundant steroids cortisol, corticosterone and 4-androstene,11 β -ol,3,17-dione) with a fixed amount of IS₁ (0.1mg). Each mixture was then analysed as a MO-TMS derivative under SIM conditions. The response ratio (peak area of the target ion of the steroid/peak area of the target ion of IS₁) was plotted against the amount ratio (amount of steroid/amount of IS₁). Fig. 6.5 shows examples of the calibration curves for the steroids cortisol (a), corticosterone (b) and 4-androstene 11 β -ol,3,17-dione (c). The amount of the endogenous steroids present in the overlying medium is then obtained by reading the calculated response ratio off the appropriate standard curve. The target ion is chosen as this is usually the most abundant ion and therefore meets the requirement for high sensitivity and specificity in the quantitation of steroids using SIM. Quantitative analysis of steroids by the designated ion-internal standard method has been widely used by Axelson et al (1974; bovine corpus luteum) and Adlercreutz et al (1974; pregnancy urine). This technique is capable of measuring steroids in the nanogramme and picogramme level and therefore can compete with radioimmunoassays for sensitivity.

Steroidogenesis in the adrenal cortex is under a complex hormonal control. The mechanisms involved in the regulation of differential expression of the steroidogenic enzymes are important in determining the levels of the various glucocorticoids, mineralocorticoids and androgens.

The two major determinants of glucocorticoid secretion from the adrenal cortex are the peptides, ACTH and All, each mediating its effects through different second messenger pathways. The involvement of cAMP in the mechanism of ACTH-induced adrenal cell steroidogenesis was first demonstrated by Grahame-Smith et al (1967), who showed a good correlation between increases in cAMP and increases in corticosterone secretion in response to ACTH. The steroidogenic effects of All on aldosterone secretion from zg cells are well documented (Tait et al 1980a) and all species studied so far respond to All at the "zg" level. All does not stimulate cAMP formation in zg cells but binds to specific plasma membrane receptors and stimulates PLC

resulting in an early increase in the hydrolysis of phosphatidylinositol phosphate (Marie et al 1983). Over the past decade it has become clear that All also stimulates glucocorticoid secretion from zfr cells, but only in some species, for example bovine (Bird et al 1989) and human (M^cKenna et al 1978).

The principal locus of the acute steroidogenic action of ACTH is confined to the P450_{scc} - mediated synthesis of pregnenolone. ACTH does however exert long-term chronic effects directly at the level of the steroidogenic enzymes. It has been reported to stimulate the synthesis of the following enzymes; P450_{scc}, P450_{c21}, P450_{c11}, P450_{c17} and adrenodoxin in bovine adrenocortical cells (reviewed in Miller, 1988) and to increase mRNA levels for P450_{17 α} , P450_{c21}, P450_{11 β} and 3 β -HSD (Simpson et al 1988 and M^cAllister et al 1988). All has opposing acute and chronic effects on steroid hormone production, including in some species, acute stimulation of basal cortisol production, for example bovine, but chronic inhibition of ACTH-stimulated cortisol production, for example in bovine adrenocortical cells (Rainey et al 1991b) and fetal ovine adrenocortical cells (Rainey et al 1991c). Mason et al (1992) reported that co-treatment of ACTH with All inhibited the ACTH-stimulated expression of P450_{17 α} and to a lesser extent 3 β -HSD (ovine adrenal cortex) and therefore concluded that All potentially has a role in the control of cortisol secretion and in the long term maintenance of these two enzymes.

Bovine adrenocortical zfr cells in primary culture have been useful as an isolated cell system for the study of steroid enzyme gene expression, but in this study, primary cell culture was used to observe the quantitative and qualitative changes in the pattern of steroids secreted, after acute and chronic stimulation, rather than changes in enzyme activities or altered gene expression. Table 6.2 summarises the results for both ACTH and All as a % change, between the acute and the chronic stimulation of the cells in the adrenocortical steroids secreted.

During the first 2 hr period of stimulation with ACTH, both the cortisol and corticosterone pathways are favoured; the two major products being cortisol and corticosterone (data not shown). After chronic stimulation, corticosterone decreases and androgens increase suggesting that corticosterone production is almost entirely switched to the androgen, 4-androsten11 β -ol,3,17-

dione, while the levels of cortisol are decreased by a third.

The basis of the redistribution of these products can be explained by considering the partitioning of pregnenolone metabolism between 17α -hydroxylase and 3β -HSD. The $17\alpha/3\beta$ -HSD partitioning of pregnenolone increases after chronic stimulation by ACTH indicating an elevation of cellular 17α hydroxylase activity. This is shown in table 6.2 where the % changes (final steroid/initial steroid \times 100) in the levels of progesterone and 17α -hydroxypregnenolone were 5.9 and 221.4 respectively, consistent with other data that cAMP may be involved in the induction of enzymes required for the synthesis of 17α -hydroxylated steroids (McAllister et al 1988). Further evidence of this was provided by Lund et al (1990) with the identification of cAMP responsive regions in the 17α -hydroxylase genes. Naville et al (1991) reported that ACTH increased the levels of 3β -HSD mRNA in bovine adrenocortical cells in culture, but this was only detectable after 48 hr chronic stimulation whereas the levels of 17α -hydroxylase mRNA increased much more quickly. The difference in induction of these two enzymes by ACTH has profound effects on the steroid profile. In addition to the induction of the 17α -hydroxylase and the $17,20$ lyase enzyme, a large % change was observed in 11-deoxycortisol (1055.1), suggesting that ACTH increases 21 -hydroxylase activity after chronic stimulation. Increases in 21 -hydroxylase mRNA by ACTH or cAMP have also been described in bovine adrenocortical cells (John et al 1986) and in mitotically quiescent bovine adrenocortical cells in culture (Chang et al 1991). Naseeruddin et al (1990) showed that the enzyme activity of 11β -hydroxylase was biphasic but declined quite rapidly in response to high levels of cAMP. This may explain why, after chronic stimulation with ACTH when levels of cAMP are high, the levels of cortisol decrease even though there was such a large % increase in 11-deoxycortisol.

As previously reported, the mRNA levels of the steroidogenic enzymes can be regulated by All as well as ACTH; All potentiates ACTH induced increases in the mRNA for 3β -HSD and $P450_{C21}$ in human (McAllister et al 1988, Hornsby et al 1990) but inhibits ACTH induced increases in mRNA for $P450_{17\alpha}$ and $P450_{11\beta}$ in bovine and human (McAllister et al 1988, Naseeruddin et al 1990). It is also reported to weakly induce the enzyme 11β hydroxylase (Hornsby et al 1980).

Analogous to ACTH, acute stimulation with All favours the activation of both the cortisol and corticosterone pathways. Chronic stimulation with All, in contrast to ACTH, leads to both pathways being further stimulated with large % changes in the intermediates and in particular the end-products of both pathways; corticosterone (360.4) and cortisol (574.1) (table 6.2). In complete contrast to ACTH, the effect of All on the androgen pathway is trivial.

The conversion of cortisol to cortisone by the 11β -HSD is a major step in the catabolism of cortisol. Preincubation of adrenocortical cells in culture with ACTH (24hr) which were then rechallenged for 2hr with ACTH resulted in a decrease (relative to acute stimulation of 2hr with the same agonist) in cortisol (% change of 67.4) but an increase in cortisone (% change of 120.9). In contrast to ACTH, stimulation with All, under the same conditions, results in an increase in cortisol (% change of 574.1) and a decrease in cortisone (% change of 27.5) respectively. Many of the enzymatic steps in the steroid pathway require the cofactor NADPH whereas the conversion of cortisol to cortisone depends on NAD^+ or $NADP^+$. Stimulation with ACTH may increase the $NADP^+ : NADPH$ ratio, therefore increasing the production of cortisone while All decreases this ratio favouring cortisol formation. The physiological significance of this is unknown though it may be involved in the regulation of local effects of cortisol, as cortisone itself is thought to be physiologically inactive.

ATP is unique in the adrenal cortex in that it has a dual second messenger response (Chapter 3 and 4). It is therefore of particular interest to compare both acute and chronic effects of ATP with those described above for ACTH and All. Acute stimulation with ATP, paralleled the results for both ACTH and All, with corticosterone and cortisol being the major steroids produced (data not shown). Chronic stimulation of the inner zone cells with ATP produced % changes in each of the steroids measured that showed similarities with the actions of both ACTH and All (table 6.2). This can be detected in each of the three branches of the steroid pathway. The glucocorticoid and androgen pathways were both stimulated, after chronic incubation, with large increases in cortisol and 4-androstene 11β -ol³,17-dione, suggesting the induction of the enzyme 17-hydroxylase (including lyase activity) and 3β -HSD, thereby reflecting the actions of both ACTH

and All. This in particular is clearly seen with the corticosterone, whose production is neither stimulated (as is the case with All) or inhibited (as is the case with ACTH), after chronic incubation with ATP.

In conclusion, it must be emphasised that this is only a semi-quantitative method with the ability to detect alterations in the amounts of a particular steroid rather than being able to measure exactly the quantity of steroids. The very large changes in some steroids observed on chronic incubation are of sufficient magnitude to overcome defects in accurate quantitation and give confidence to the general conclusions put forward. The methodology involves many individual steps each of which may be susceptible to variation between samples. Future work may be necessary to improve the quantitative profiling technique using the following measures; 1) quantitative extraction of all reference steroids using the Sep-pak cartridges, 2) quantitative conversion of all adrenocortical steroids into volatile derivatives, 3) baseline GC resolution of all the steroids of interest, 4) linearity of the standard curves over a wider concentration range (0 - 100 μ g) for each steroid, 5) reproducibility of inter and intra-assays of individual steroids and 6) absence of impurities.

For the requirements of this chapter, semi-quantitative GC-MS analysis of adrenocortical steroids secreted in primary cell culture in response to different agonists was sufficient. While several agonists regulate steroid secretion, only a few second messengers seem to be involved. Experimental findings in this chapter have shown that both cAMP and PL-C are involved in the hormonal control of the steroidogenic pathway of bovine adrenocortical cells, but are quite distinct in their actions on the pathway and hence what enzymes they may regulate. ATP, with its dual second messenger response, may play a complementary role by modulating the actions of ACTH and All in the regulation of adrenocortical function *in vivo*.

CHAPTER 7

CONCLUDING REMARKS

In the past few years, the purines, ATP and adenosine in particular, have received considerable attention as their multiple effects are recognised, receptors cloned and their metabolism and mechanisms of action revealed. Due to the close structural and metabolic relationship between ATP and adenosine, their roles both intracellularly and extracellularly are closely intertwined and have been documented in virtually every major organ and/or tissue system that has been studied.

The data in this thesis have extended the knowledge of purinergic effects on the bovine adrenal zfr at the cellular level. The main findings of the thesis are the following; 1) ATP and UTP activate two different effector systems in bovine zfr cells in culture; inositol phosphate formation which is linked to the P2u purinoceptor and cAMP formation which is linked to the P1 purinoceptor, 2) Adenosine stimulates the secretion of cortisol from bovine inner zone cells via the A₂ receptor subtype of the P1 purinoceptor. With the aid of specific antagonists the cAMP component of the dual second messenger response elicited by ATP was shown to be mediated through the same P1 purinoceptor subtype as adenosine, 3) Coadministration of ATP and ACTH to freshly-isolated bovine adrenocortical cells, using a novel superfusion system, resulted in a clear synergistic interaction. Further experiments were performed with different combinations of agonists, each combination giving a synergistic response irrespective of the second messenger systems involved and finally 4) Acute and chronic effects of ATP, ACTH and All on the regulation of the steroidogenic pathway were compared using GC-MS. Chronic stimulation with ACTH, favoured the androgen pathway, while the production of corticosterone was nearly completely inhibited. In contrast chronic stimulation with All, favoured both the mineralocorticoid and the glucocorticoid pathways. ATP with its dual second messenger

response mirrored the actions of both ACTH and All implicating differential effects of the two systems on the longer term regulation of the steroidogenic pathway.

A vital question however concerns the physiological relevance of purinergic regulation of steroidogenesis in the adrenal zfr cells and how the purines interact with the other stimulatory factors of the gland.

First of all it is important to speculate on the possible sources of purines in the adrenal and how they may gain access to the cortex. Chromaffin granules contain high concentrations of the purines ATP (150mM) and ADP (20mM) (Forsberg et al 1987). Acetylcholine releases 30% total cellular content of ATP from the chromaffin cells. Silinsky et al (1975) reported that splanchnic nerve terminals located in the adrenal medulla also contain other biologically active substances such as ATP which may be released on nerve stimulation concomitantly with acetylcholine. Thus the adrenal medulla is potentially a rich source of purines. The released ATP may interact with purinergic receptors ("atypical P2y" (Allsup et al 1990)) and act as a neuromodulator on stimulus-secretion coupling in the chromaffin cells or it may be degraded by the ectophosphohydrolases to adenosine which activate A₂ receptors on the chromaffin cells enhancing secretion through a rise in adenylate cyclase (Chern et al 1988).

By analogy with thrombin-activated platelets (when plasma ATP levels exceed 10⁻⁵ M) (Gordon et al 1986), it is likely that locally released concentrations of ATP would be well within the range to elicit a cortisol response from adjacent adrenocortical cells. Since the main blood flow in the adrenal is believed to be directed centripetally from the cortex to the medulla (Vinson et al 1985b), a regulatory function of the medullary purines on adrenocortical steroidogenesis should therefore be mediated in a paracrine manner. In turn, this would imply a close morphological relationship between cortical and medullary cells.

The morphological evidence for a close proximity of medullary and adrenocortical cells is strong. The existence of medullary rays extending well into the cortex with

cytoplasmic extensions and direct cellular contact between the two cell types has been described by several authors (Bornstein et al (1991), Bornstein et al (1994) and Gallo-Payet et al (1987)).

Purines have a central role in vascular tone (Luty et al 1989) and therefore may regulate local blood flow to the cortex. Mast cells can release ATP/ADP from granule stores. Hinson et al (1988) reported the presence of mast cells close to the adrenal arterioles as they penetrate the connective tissue of the gland. The mast cells are thus appropriately positioned for the purines to reach the subcapsular arterioles which is thought to be the site at which bloodflow through the gland is controlled (Vinson et al 1985b). Stimulation of the splanchnic nerves results in the release of ATP which induces the release of two vasoactive factors, prostacyclin and endothelium-derived releasing factor (EDRF), both of which cause vasodilation in the adrenal medulla (Martin et al 1985). This lowers vascular resistance thereby regulating blood flow and hence modulating the concentrations of stimulatory factors (e.g. All and catecholamines) to the cortex.

Other potential sites of action include the peripheral segments of the central vein that drain the adrenal cortex and supply portal-blood rich in steroids to the medulla (Coupland et al 1975). The vascular endothelium also plays a vital role in the interaction of platelets and the vessel wall. EDRF and prostacyclin act synergistically to inhibit platelet aggregation and the release of purines. Platelets are a rich source of ATP (600mM), ADP (400mM) and UTP (8% of nucleotide content) (Boeynaems et al 1990) which upon activation, in the adrenal vasculature, might provide a local source of purines.

One of the neuronal controls of vascular tone is mediated through activation of α_1 adrenoceptors and β_2 adrenoceptors (Wiener et al 1993). In conditions of shear stress, these receptors are triggered initiating the release of ATP from the vascular endothelial cells in the cortex (Bodin et al 1991). This may explain why blood plasma levels of ATP and adenosine are increased under conditions of stress to levels greater than 10mM. This is well above the amount (5×10^{-5} M) proposed by Trams et al (1980) for the process to

become self-sustaining by the continuous addition of ATP from erythrocytes and other cell types. However little is known about the physiological roles of the purines on cellular function in the adrenal cortex. Under conditions of stress ACTH is released from the hypothalamus and the sympathetic nervous system is activated. A possible physiological role for adenosine in the regulation of this pituitary-adrenocortical axis was suggested by Scaccianoce et al (1989), who reported an increase in adenosine content in the pituitary following restraint stress. Adenosine stimulates adenylate cyclase in cultured anterior pituitary cells which is a prerequisite for the release of pituitary ACTH. In addition *in vitro* studies have shown synergism between CRF and adenosine in ACTH release and therefore it may be an important factor in the regulation of the HPA under stress conditions. Sympathetic splanchnic nerve stimulation results in the release of ACh and ATP concomitantly within the medulla. The released ATP may have a paracrine effect on the cortex inducing steroidogenesis via the nucleotide receptors present in the zfr cells.

In the medulla neurochemical studies have identified ATP in adrenergic and cholinergic synaptic vesicles from where the nucleotide is co-released with either NAd or Ach (Wiener et al 1993). It is not known as yet if the co-release of ATP occurs from adrenergic or cholinergic nerves within the cortex. However this seems possible given the presence of adrenergic and cholinergic nerve terminals derived from the splanchnic nerve within the cortex (Charlton et al 1991).

Although ACTH is central to the mammalian stress response its action on the adrenal cortex would be expected to take several minutes from its release from the hypothalamus to its stimulation of steroidogenesis. Direct innervation of the adrenocortical cells would be one mechanism to effect an instantaneous response to stress, with ATP possibly involved in the initial stimulus. In addition, innervation of the blood vessels plus the vasoactive nature of ATP (released from platelets and mast cells) would imply a primary role in the control of the adrenal blood supply and hence the delivery of ACTH in response to acute and chronic stress. In Chapters 3 and 4, it was shown that ATP elicited

a dual second messenger response in bovine adrenocortical cells in culture. In addition, in Chapter 5, it was found that co-administration of ATP with ACTH or All to isolated bovine adrenocortical cells, using a superfusion system, resulted in a clear synergistic interaction. The possibility therefore exists that the steroidogenic actions of ACTH and All *in vivo* are potentiated by an intraadrenal paracrine mechanism involving ATP.

The inducement of steroidogenesis by purines either individually or in combination with other agonists, may be an appropriate system in adapting the body to stressors, although the protective actions of the corticosteroids remain ill-defined.

In a wider context, commercial interest in the therapeutic potential of purines as drug entities in the treatment of diseases has expanded rapidly. Latest developments include the involvement of a purinergic component in the arthritic process (Green et al 1991), the use of pentoxifylline in the treatment of AIDS-related infections because of its ability to modulate tumour necrosis factor formation (Scrip 1991) and the use of aerosolised ATP and UTP, in conjunction with the sodium channel blocker, amiloride, to stimulate chloride secretion in cystic fibrosis patients, possibly involving interactions with nucleotide receptors (Knowles et al 1991). Rainey et al (1993) reported that the human adrenal *in vivo* can produce almost equal amounts of glucocorticoids and C₁₉ steroids; however the relative ratio between these two steroids changes throughout the course of life, with the levels of the C₁₉ steroids decreasing with aging. In Chapter 6, it was shown that chronic stimulation of the inner zone cells in culture with ATP favoured the androgen pathway and therefore it is possible that purines may produce the appropriate steroid profile to counteract the changes in C₁₉ steroids that occur with aging.

It is important to recognise the problems that may arise in purinergic therapy due to the ubiquitous nature of adenosine and ATP effects, but with a better understanding of the role of purines as the "signal of life" (Engler et al 1991) in tissue function, a new class of therapeutic agents may emerge to effectively treat the disease challenges of the future.

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Primary Cultures of Bovine Inner Zone Adrenocortical Cells Secrete Cortisol in Response to Adenosine 5'-Triphosphate, Adenosine 5'-Diphosphate, and Uridine 5'-Triphosphate via a Nucleotide Receptor That May Be Coupled to Two Signal Generation Systems

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ABSTRACT

Cultured inner zone cells isolated from bovine adrenal cortex secreted cortisol in a dose-dependent fashion in response to ATP and ADP. The threshold response was at 10^{-6} M ATP, reaching a maximum at 10^{-4} M ATP, at which concentration the n-fold increase relative to basal was 43.8 ± 22.3 (mean \pm SD; $n = 3$). Cells were also responsive to the pyrimidine nucleotide UTP. EC_{50} values for ATP, ADP, and UTP were $5.83 \pm 3.98 \times 10^{-6}$, $13.7 \pm 5.67 \times 10^{-6}$, and $7.33 \pm 4.52 \times 10^{-7}$ M, respectively (mean \pm SD; $n = 3$). The response to 10^{-4} M ATP was linear for at least 60 min, and the cells appeared morphologically normal after removal of the stimulus. The purinergic antagonist suramin was relatively ineffective.

The potency order of a range of purines was as follows: ATP = UTP > ADP > 2-methyl-S-ATP > α,β -methylene ATP = β,α -methylene ATP = AMP. Stimulation of cortisol secretion by ATP was evident after 24 h in primary culture and reached a maximum after 48–72 h, thereafter declining. No response was detected in static incubations of freshly isolated cells.

The possibility that added ATP was degraded over the course of the incubation was investigated by separating ATP, ADP, AMP, and adenosine by high resolution anion exchange chromatography after different times of exposure to the cells. Although there was degradation, largely to ADP, about 50% of the ATP remained at 1 h.

Cells grown in the presence of [3 H]inositol (10μ Ci/ml) for 48 h (to

prelabel the membrane phosphoinositide pool to isotopic equilibrium) showed a time- and dose-dependent increase in [3 H]inositol-labeled total phosphoinositols to ATP or ADP; the response was linear for at least 20 min.

Cells labeled with the Ca^{2+} indicator fura-2 showed an increase in intracellular calcium to 10^{-4} M ATP on days 3 and 4 of culture. The basal intracellular Ca^{2+} concentration was 57.3 ± 39.3 nmol/liter (mean \pm SD; $n = 12$ cell suspensions), rising to 171 ± 84 nmol/liter (mean \pm SD; $n = 12$ cell suspensions) after the addition of ATP (10^{-4} M).

Bovine inner zone cells also demonstrated a dose-dependent increase in intracellular cAMP measured after 1 min of stimulation with ATP. It was not possible to account for the cAMP response on the basis of conversion of ATP to adenosine, which then acted at an A2 receptor.

In conclusion, ATP elicits cortisol secretion from bovine inner zone adrenocortical cells via a receptor that exhibits the characteristics of the so-called nucleotide receptor: equipotency of UTP and ATP, a potency order in which 2-methyl-S-ATP was less potent than ATP itself, and a stimulation of membrane phosphoinositide turnover. Whether the dual second messenger response is a characteristic of the nucleotide receptor or reflects receptor heterogeneity remains unknown at present. The findings lend further support to existing evidence for paracrine and neuroendocrine control of adrenocortical cell function. (*Endocrinology* 134: 1553–1560, 1994)

URINE nucleosides and nucleotides have a major role in cell function. In addition to a central function in cellular energy metabolism and as a cofactor for many enzyme reactions, extracellular ATP (and ADP, adenosine, and possibly other purines) is now known to influence many biological processes via specific purine receptors. These include platelet aggregation, neurotransmission (peripheral and central), cardiac function, muscle contraction, histamine release from mast cells, and release of prostacyclin and relaxing factor by endothelial cells (1–4).

Although exogenous ATP can be rapidly catabolized to adenosine by a number of ectophosphohydrolases, many of

the observed actions of extracellular ATP can be distinguished from those mediated by extracellular adenosine receptors. Many of these functional effects are observed at low micromolar concentration, well below the Michaelis constant (K_m) for most adenosine triphosphatases and other ATP-using enzymes (3).

Receptors sensitive to adenosine were originally termed P1, and those sensitive to ATP were termed P2. The adenosine P1 receptor is now known to be heterogeneous and to consist of A1 and A2 subtypes (5). The extracellular ATP receptor is also heterogeneous, based on agonist sensitivity and differences in signal transduction mechanisms. The major subdivision was initially between the P2x and P2y subtypes (6); soon afterward, this was broadened to include P2t (platelets) and P2z (mast cells) subtypes (2, 7).

The P2x subtype displays a rank order of agonist potency α,β -methylene ATP ($\alpha\beta$ MeATP) = β,γ -methylene ATP ($\beta\gamma$ MeATP) > ATP = 2-methylthio-ATP and is selectively

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desensitized by $\alpha\beta$ MeATP. Using whole cell and patch clamp electrophysiological recording techniques, this receptor appears to activate a conductance that is nonselective for mono- or divalent cations. In contrast, the P2y subtype displays a different rank order of potency of 2-methylthio-ATP (2-Me-S-ATP) \gg ATP $>$ $\alpha\beta$ MeATP = $\beta\gamma$ MeATP and is only weakly or not desensitized by $\alpha\beta$ MeATP (8). In a variety of tissues examined, including hepatocytes, erythrocytes, leukocytes (4), and vascular smooth muscle (9), the responses associated with P2y receptor stimulation appear to be linked to the activation of phosphoinositidase-C.

With the exception of the P2t receptor, a more definite characterization of P2 receptor subtypes is hampered by the lack of potent and specific competitive antagonists; suramin is a competitive antagonist of the P2 purinoceptor, but is unable to distinguish between P2x and P2y subtypes.

Although an atypical P2y purinoceptor has been identified in the adrenal medulla (10), the possibility that the adrenal cortex is responsive to extracellular purines through a specific receptor has received little attention. Kawamura *et al.* (11) presented preliminary evidence that ATP led to a dose-dependent stimulation of cortisol secretion from bovine inner zone cells. These workers argued, on the basis of agonist potency, that the response was mediated by a P2y receptor, although the most potent agonist for the P2y receptor, 2-methylthio-ATP, was not tested. Moreover, the ability of ATP to stimulate membrane phosphoinositide turnover was not studied in this or subsequent studies of the effects of ATP on adrenocortical steroidogenesis. Niitsu (12) reported a steroidogenic effect of ATP on bovine zona fasciculata cells and observed an increase in cAMP in response to the highest dose of ATP used; this was attributed to breakdown of ATP to adenosine, which was then postulated to stimulate cAMP through a P1 purinoceptor. A requirement for Ca^{2+} in the steroidogenic action of ATP has also been reported (12, 13).

In this study we report that ATP leads to a dose-dependent secretion of cortisol from bovine inner zone cells. We show that it leads to a stimulation of membrane phosphoinositide turnover and that the receptor involved is not a typical P2y receptor, but has properties of the UTP-sensitive nucleotide receptor. The cAMP response is shown to occur across a wide dose range for ATP and not to result from the breakdown of ATP to adenosine.

There is an increasing awareness that cortico-medullary interactions may be important in the function of the adrenal gland as a whole. Adrenoceptors (β_1) have been identified on inner zone adrenocortical cells, and both outer and inner zone cells secrete steroids in response to adrenaline (14). Purines, such as ATP, are also stored in and secreted from chromaffin granules in medullary cells (15, 16). In view of the close contact between these cell types (17, 18), the possibility exists for a paracrine effect of released purines, such as ATP.

Materials and Methods

Materials

Bovine adrenal glands were collected on ice within 30 min of slaughter from 1- to 2-yr-old steers at the local slaughterhouse. Ham's F-10 growth

medium, Earle's Balanced Salt Solution (EBS), glutamine, BSA (fraction V), penicillin, streptomycin, and amphotericin β were purchased from ICN Flow Laboratories (High Wycombe, United Kingdom). Collagenase was obtained from Lorne Diagnostics (Bury St Edmunds, United Kingdom).

Cortisol, cAMP, ATP, ADP, UTP, $\alpha\beta$ MeATP, $\beta\gamma$ MeATP, control processed serum replacement (no. 5; CPSR5), and Sephadex G-10 and G-50 were obtained from Sigma Chemical Co. (Poole, United Kingdom). The suramin was obtained from Bayer U.K. (Newbury, United Kingdom), and the 2-methylthio-ATP from Semet Technical (RBI, St. Albans, United Kingdom). Cortisol label [cortisol-3-(*O*-carboxymethyl)oximino-([125 I]iodohistamine)] was purchased from Amersham International (Aylesbury, United Kingdom). The fura-2 (free acid and ester) was purchased from Boehringer Mannheim (Lewes, United Kingdom). The cortisol antiserum was kindly provided by the Scottish Antibody Production Unit (Carlisle, United Kingdom).

The materials used for the measurement of 3 H-labeled phosphoinositols are listed in the report by Bird *et al.* (19). All other chemicals were obtained from BDH (Glasgow, United Kingdom).

Methods

Cell preparation, culture, and radiolabeling. The details of the isolation and purification of bovine zona fasciculata/reticularis (ZFR) cells, and the properties of the ZFR cells in culture were described by Williams *et al.* (20). Cells were plated at a density of 330,000 cells/ml in 12-well plates in Ham's F-10 medium supplemented with 10% CPSR5 (bovine serum from which immunoglobulins and endotoxin are removed), penicillin (50 IU/ml), streptomycin (50 μ g/ml), and amphotericin-B (2.5 μ g/ml). After 24 h (day 2), medium was removed and replaced with 0.5 ml fresh medium. When studies of phosphoinositide metabolism were carried out, medium was also supplemented with [3 H]inositol (10 μ Ci/ml), which was added at the routine medium change at 24 h. Initial studies showed that cells were maximally responsive 48–72 h after initial isolation. Experiments in which the cortisol secretory response was measured were mostly carried out 48 h after plating, whereas measurement of the 3 H-labeled total phosphoinositol head groups was performed 72 h after plating (after 48 h of labeling, from 24–72 h, with [3 H]inositol) to allow membrane phosphoinositides to be labeled to isotopic steady state.

Cortisol secretion. To study the effects of agonists and antagonists on cortisol secretion, growth medium was removed from each well, and cells were washed twice with 1 ml EBS. Cells were then incubated (5 min) in EBS with 0.1% added glucose and 0.2% added BSA (EBSBG; 0.45 ml/well). Agonists and antagonist (suramin) were added (50 μ l/well) to the appropriate final concentration, and the cells were incubated at 37 C in 5% CO_2 for 60 min (or other times, as stated in *Results*). At the end of the incubation, EBSBG was aspirated from each well and stored at -20 C before assay for cortisol.

Cortisol was measured using an in-house RIA (21), with the modifications described by Walker *et al.* (22). The between-assay coefficient of variation was less than 10% over the working range of 10–2000 nmol/liter.

Measurement of cAMP. Cells were washed in EBS, and agonist incubations were performed in EBSBG, exactly as described above for cortisol measurements. After 1 min in the absence (basal) or presence of agonist, the overlying EBSBG was rapidly removed and replaced by 75% (vol/vol) ethanol in water. The base of each well was scraped using the rubber tip from the plunger of a 1-ml syringe, and the contents were transferred to glass tubes. The ethanol was evaporated at room temperature under a stream of nitrogen, and the residue was reconstituted in EBS (0.5 ml). Standards were also prepared in EBS. The acetylation of samples and standards and the further details of the cAMP assay were previously described (23). Over the working range of 0.0625–16 nmol/liter, the interassay coefficient of variation was 15% or less.

Metabolism of ATP by cultured cells. The metabolic fate of ATP in the EBSBG added to the cells (at 10^{-4} M) was examined by following its conversion to the products ADP, AMP, and adenosine by separating the products using HPLC on MonoQ HR5/5 (Pharmacia, Piscataway, NJ) and a fast protein liquid chromatography (Pharmacia) solvent delivery

system. Details of the nucleotide separation method were described by Bird (24). Individual wells containing bovine ZFR cells were stimulated with ATP (10^{-4} M) in EBSBG for 1, 30, 60, and 120 min. The contents of three wells were pooled before application to the MonoQ column. In parallel, EBSBG containing ATP (10^{-4} M) was incubated in multiwell plates at 37 C (no cells present) to determine whether any significant chemical breakdown of ATP occurred under these conditions.

Measurement of ^3H -labeled total head groups. Cells were prelabeled to isotopic steady state with [^3H]inositol from 24–72 h in culture. The procedure for the preparation of cells before agonist stimulation in the presence of 10 mM LiCl, perchloric acid termination, deacidification (using 1,1,2-trichlorotrifluoroethane/tri-*n*-octylamine extraction), and assay of [^3H]inositol-labeled water-soluble head groups were described in detail by Bird *et al.* (19).

Measurement of intracellular Ca^{2+} . Cells were prepared as described above and cultured in 50-ml flasks (10^7 cells/flask), with a medium change at 24 h. Measurements were carried out between 48–72 h after initial isolation. The cells were detached from the flasks by removing the overlying medium, washing twice with Ca^{2+} -free EBS containing 1 mM EDTA, and then incubating the cells at 37 C in 5 ml of the same buffer until the cells could be detached mechanically by shaking (generally, after 10–15 min). Trypsin was avoided to limit any damage to the purine receptor. Detached cells were collected into Ca^{2+} -free EBS, centrifuged, and washed in 50 ml Krebs-Ringer buffer. After a further centrifugation step, the cells were finally resuspended in Krebs-Ringer buffer at a density of 5×10^6 cells/ml and left at 37 C for 60 min to allow intracellular Ca^{2+} stores to be replenished. Fura-2AM was then added to a final concentration of 10 μM ; a loading time of 45 min was found to be optimal. The subsequent procedure for measurement of intracellular Ca^{2+} using fura-2AM was described in detail previously (25).

Cellular protein content. This was measured on a minimum of three wells for each experiment. Wells were first washed twice with 1 ml normal saline, and cells were taken up in 0.5 ml 1% (vol/vol) Triton X-100. After diluting the sample 1:10 with water, the protein content was determined by the method of Bradford (26), automated for use on the Cobas Fara (Roche, Welwyn Gardens City, Hertfordshire, UK) centrifugal analyzer. The assay standard was BSA made up in 0.1% Triton X-100.

Data handling and statistics. Statistical significance was determined by Student's *t* test. To determine the EC_{50} and maximum stimulatory response (E_{max}) values for cortisol secretion elicited by ATP, ADP, and UTP, each individual dose-response curve was fitted using a sigmoidal power model on Biosoft FigP (Biosoft, Ferguson, MO) on a Nimbus (Research Machines, Oxford, UK) PC-486/25x personal computer. The mean EC_{50} and E_{max} values were then determined from three combined experiments for each agonist.

For all figures (apart from Fig. 6), data from three separate experiments using three separate cell isolates were combined. Within each experiment, each dose or time point was determined using the data from three combined wells. To take account of variation in cell number and basal output, data were normalized with respect to basal secretion (Figs. 1, A and B; 3; and 4). For the time course studies (Figs. 2 and 5), the basal and stimulated responses are separately shown for the three combined experiments. In the case of Fig. 6, although the qualitative responses were very similar for individual experiments, the quantitative response was less for one experiment than for the other two, obscuring the response pattern when data were combined. Accordingly, the three separate experiments are individually shown in this figure.

Results

Effects of ATP, ADP, AMP, and UTP on cortisol secretion from ZFR cells (Figs. 1 and 2)

The effects of a range of doses of ATP, ADP, AMP, and UTP on the secretion of cortisol over a 60-min period from bovine ZFR cells after 48 h in primary culture are shown in

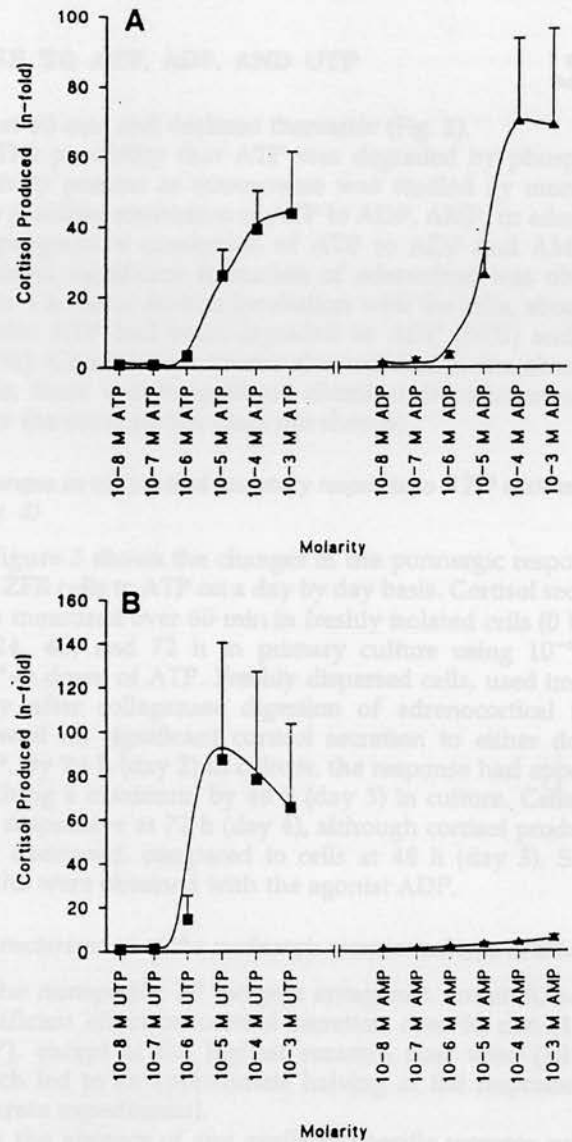


FIG. 1. A, Cortisol secretion over a period of 60 min was measured in the overlying medium from cells stimulated with ATP and ADP (dose range, 10^{-8} – 10^{-3} M) after 48 h in primary cell culture. The threshold concentration eliciting a significant increase above basal secretion was 10^{-6} M for both ATP and ADP in each of three different experiments ($P < 0.05$). Combined data from the three separate experiments are shown (mean \pm SD); see *Materials and Methods* for details. B, Cortisol secretion over a period of 60 min was measured in the overlying medium from cells stimulated with UTP and AMP (dose range, 10^{-8} – 10^{-3} M) at 48 h in primary culture. The threshold concentrations of UTP and AMP eliciting a significant increase in cortisol secretion above basal secretion were 10^{-6} and 10^{-4} M, respectively, in each of three different experiments ($P < 0.05$). Combined data from three separate experiments are shown (mean \pm SD); see *Materials and Methods* for details.

Fig. 1. Both ATP and ADP (Fig. 1a) produced a dose-dependent increase in cortisol secretion compared to the basal secretion (no agonist) over the same period. The lowest concentration of ATP or ADP that elicited a significant increase in steroid secretion was 10^{-6} M ($P < 0.05$ in each of three separate experiments); the maximum response occurred at 10^{-4} M (10^{-3} M in some experiments). The EC_{50} values were

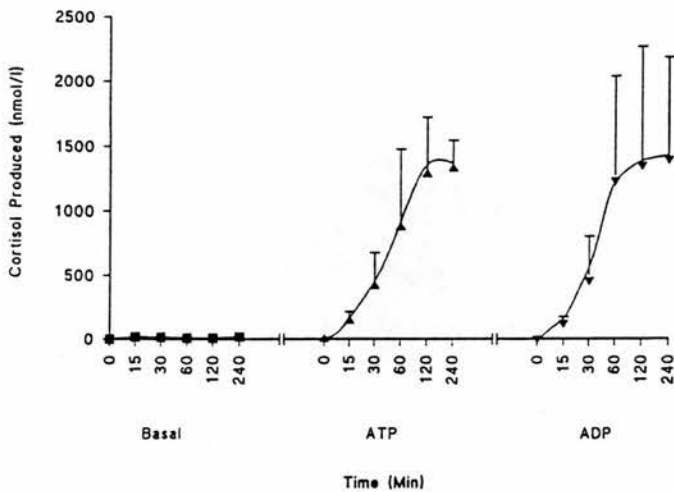


FIG. 2. The time course for the accumulation of cortisol in the overlying medium was measured under basal conditions (no agonist) and in response to maximally stimulatory doses of ATP (10^{-4} M) and ADP (10^{-4} M) after 48 h in primary cell culture. Results are the mean \pm SD ($n = 3$ wells) for three combined experiments; see *Materials and Methods* for details.

TABLE 1. Relative agonist potencies on cortisol secretion from adrenocortical inner zone cells after 48 h in primary culture for a range of purine nucleotides

Agonist	Active concentration range (threshold maximum) (M)	EC ₅₀ \pm SD (n = 3) (M)	E _{max} \pm SD (n = 3) (nmol/liter·h)
ATP	10^{-6} – 10^{-4}	$5.83 \pm 3.98 \times 10^{-6}$	423 ± 220
ADP	10^{-6} – 10^{-4}	$13.7 \pm 5.67 \times 10^{-6}$	545 ± 288
UTP	10^{-6} – 10^{-4}	$7.33 \pm 4.52 \times 10^{-7}$	317 ± 233
2Me-s-ATP	10^{-6} – 10^{-3}		
$\alpha\beta$ MeATP	10^{-4} – 10^{-3}		
$\beta\alpha$ MeATP	10^{-4} – 10^{-3}		
AMP	10^{-4} – 10^{-3}		

Agonists were tested on three separate cell preparations. EC₅₀ values and E_{max} values are shown for the most potent agonists ATP, ADP, and UTP; these values were not calculated over the narrow dose range and high agonist concentrations required to elicit a response for the other agonists shown in the table. A maximal stimulatory dose of ACTH (10^{-10} M) (20) was included as a positive control in all experiments. The E_{max} for ACTH was 1205 ± 481 ($n = 3$) nmol/liter·h cortisol.

$5.83 \pm 3.98 \times 10^{-6}$ M for ATP, $13.7 \pm 5.67 \times 10^{-6}$ M for ADP, and $7.33 \pm 4.52 \times 10^{-7}$ M for UTP (mean \pm SD; $n = 3$ experiments for each agonist; Table 1). The EC₅₀ for UTP was significantly lower than that for ADP ($P < 0.05$), but not that for ATP.

AMP was a poor agonist, and significant stimulation of cortisol secretion (relative to basal) was not observed until 10^{-4} M AMP ($P < 0.05$ in each of three experiments; Fig. 1B). The potency order was, thus, ATP = UTP > ADP \gg AMP.

Time course of cortisol secretion in response to ATP (Fig. 2)

As maximum secretion of cortisol was achieved by 10^{-4} M ATP (or ADP), this dose of ATP (or ADP) was used to follow the time course of cortisol secretion over a 4-h period at 48 h in primary culture. The response was continuous for at

least 60 min and declined thereafter (Fig. 2).

The possibility that ATP was degraded by phosphatase activity present as ectoenzyme was studied by monitoring the possible conversion of ATP to ADP, AMP, or adenosine. A progressive conversion of ATP to ADP and AMP (but without significant formation of adenosine) was observed over 4 h. After 60-min incubation with the cells, about 50% of the ATP had been degraded to ADP (30%) and AMP (20%). Control experiments showed that in the absence of cells, there was insignificant chemical degradation of ATP over the same period (data not shown).

Changes in the cortisol secretory response to ATP with culture (Fig. 3)

Figure 3 shows the changes in the purinergic response of the ZFR cells to ATP on a day by day basis. Cortisol secretion was measured over 60 min in freshly isolated cells (0 h) and at 24, 48, and 72 h in primary culture using 10^{-6} - and 10^{-4} -M doses of ATP. Freshly dispersed cells, used immediately after collagenase digestion of adrenocortical tissue, showed no significant cortisol secretion to either dose of ATP. By 24 h (day 2) in culture, the response had appeared, reaching a maximum by 48 h (day 3) in culture. Cells were still responsive at 72 h (day 4), although cortisol production had decreased, compared to cells at 48 h (day 3). Similar results were obtained with the agonist ADP.

Characterization of the purinergic receptor subtype (Table 1)

The nonspecific P2 receptor antagonist, suramin, had no significant effect on cortisol secretion over 60 min (10^{-4} M ATP), except at the highest suramin dose used (10^{-3} M), which led to an approximate halving of the response (two separate experiments).

In the absence of any available specific receptor subtype antagonists, the potency order of a range of ATP analogs was used in an attempt to characterize the purinergic receptor

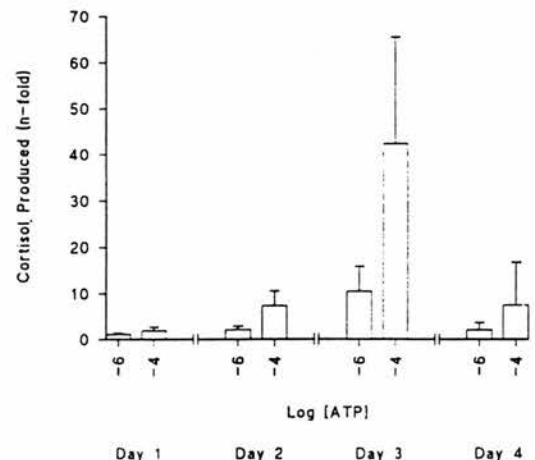


FIG. 3. Cortisol secretion over a 60-min period was measured in response to a threshold dose (10^{-6} M) and a maximum dose (10^{-4} M) of ATP in freshly isolated cells in suspension at 0 h (day 1) and thereafter in primary culture at 24 h (day 2), 48 h (day 3), and 72 h (day 4). Results are the mean \pm SD from three combined experiments; see *Materials and Methods* for details.

subtype present in bovine ZFR cells. Cortisol secretion was measured over a concentration range of 10^{-9} – 10^{-3} M for the following agonists: ATP, UTP, ADP, AMP, $\alpha\beta$ MeATP, γ MeATP, and 2-Me-S-ATP. The potency order established was as follows: ATP = UTP > ADP > 2Me-S-ATP > β MeATP = $\beta\gamma$ MeATP = AMP (Table 1).

Stimulation of membrane phosphoinositide turnover in response to ATP and ADP (Figs. 4 and 5)

The accumulation of water-soluble [3 H]inositol-labeled head groups was measured in the presence of increasing

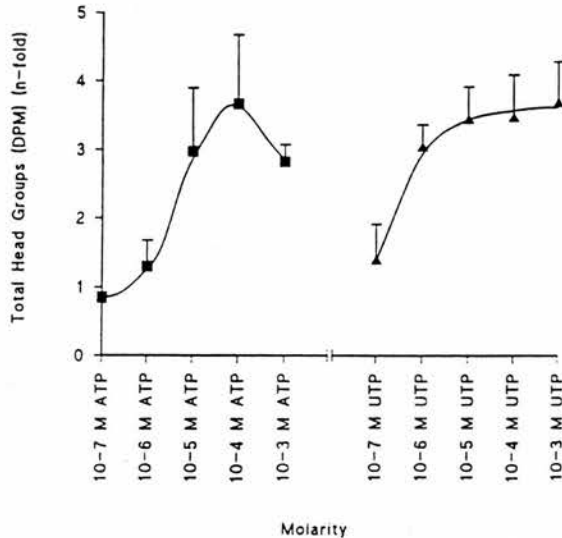


FIG. 4. Cells were prelabeled to isotopic steady state for 48 h with [3 H]inositol, and the accumulation of 3 H label in the total aqueous phosphoinositol/glycerophosphoinositol pool (total head groups) was measured after 15-min exposure of the cells to ATP or UTP over the concentration range shown. Experiments were carried out in the presence of 10 mM/liter LiCl. Results are expressed as the mean \pm SD for three combined experiments; see *Materials and Methods* for details.

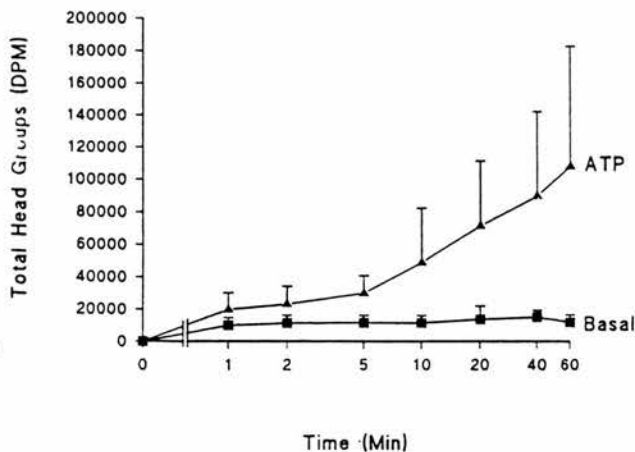


FIG. 5. Cells were prelabeled to isotopic steady state for 48 h with [3 H]inositol, and the accumulation of 3 H label in the total aqueous phosphoinositol/glycerophosphoinositol pool (total head groups) was measured over a 60-min period under basal conditions (no agonist) and in response to ATP (10^{-4} M). Experiments were carried out in the presence of LiCl (10 mM/liter). Each time point is the mean \pm SD from three combined experiments; see *Materials and Methods* for details.

doses of ATP, ADP, or UTP. Cells were prelabeled to isotopic steady state with [3 H]inositol for 48 h, and experiments were carried out in the presence of 10 mM Li $^+$ to block recycling of [3 H]inositol monophosphates. Figure 4 shows the dose-dependent increase in labeled head groups in response to both ATP and UTP. The doses of ATP or UTP required to elicit threshold and maximal responses were similar to those for cortisol secretion. ADP also stimulated membrane phosphoinositide turnover (data not shown). The formation of [3 H]inositol-labeled head groups in response to a maximally stimulatory dose of ATP (10^{-4} M) increased linearly for about 20 min, but decreased after this time point (Fig. 5).

Effects of ATP on cellular cAMP formation (Fig. 6)

Figure 6 illustrates the effects of increasing doses of ATP on the intracellular accumulation of cAMP (measured at 1 min) in the inner zone cells at 48 h in primary culture; a dose-dependent increase in cAMP formation was observed in each of three separate experiments, with a threshold increase (relative to basal) achieved at 10^{-6} M ATP or lower.

Measurement of the intracellular Ca $^{2+}$ response

Basal intracellular Ca $^{2+}$ measured between 48–72 h in culture was 57.3 ± 39.3 nmol/liter (mean \pm SD; n = 12 cell suspensions derived from 4 different cell preparations) and rose to 171 ± 84.2 nmol/liter (mean \pm SD; n = 12 cell suspensions derived from 4 different cell preparations) in response to ATP (10^{-4} M).

Discussion

The stimulation of cortisol from bovine inner zone cells by ATP and other purines has recently been reported by Kawamura *et al.* (11). Our data confirm that the purines ATP and ADP dose-dependently stimulate cortisol secretion from primary cultures of ZFR cells isolated from bovine adrenal cortex. Both agonists exhibited a similar threshold response at 10^{-6} M, reaching a maximum response by 10^{-4} M purine. The EC $_{50}$ values for ATP and ADP were similar in magnitude and not significantly different. In addition, we observed that the pyrimidine nucleotide UTP elicited cortisol secretion from these cells, although AMP was relatively ineffective. The EC $_{50}$ for UTP was significantly lower than that for ADP, but not that for ATP; E $_{max}$ values for ATP, ADP, and UTP were not significantly different and were approximately one third the E $_{max}$ for ACTH.

Cortisol secretion was linear over the 60-min period used for these studies. Over a longer time course, flattening of the response was evident; this may reflect degradation of added ATP (see below), although a component of desensitization of the response cannot be ruled out.

The possibility that cortisol secretion in response to ATP might be accounted for by metabolism of ATP to adenosine (via ADP and AMP), which then acts at an A $_2$ receptor, was excluded. Analysis of the nucleotides in the medium overlying the cells established that ATP was broken down by the cells. After 60 min, about 50% of the ATP had degraded,

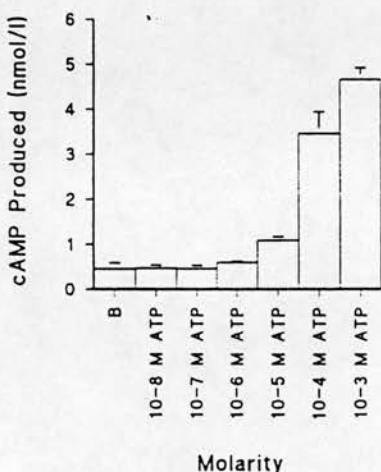
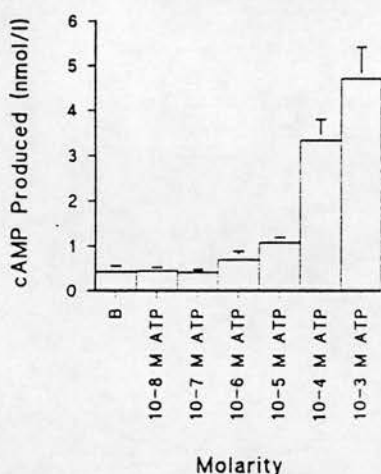
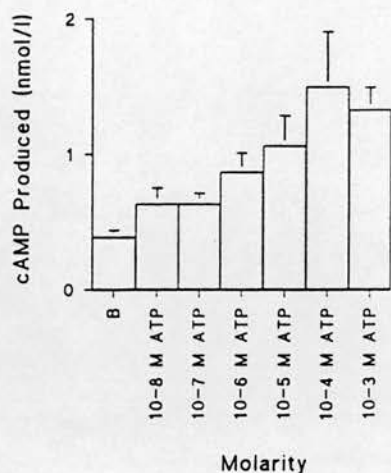


FIG. 6. Cellular cAMP production was measured over a 1-min period from cells stimulated with the concentrations of ATP shown after 48 h in primary cell culture. Basal cAMP formation (B) is also shown. The results from three experiments are separately illustrated; for each experiment, each bar is the mean \pm SD for three wells. Significant stimulation of cAMP relative to the basal level was observed at 10^{-6} M ATP or less ($P < 0.05$) in all experiments.

largely to ADP (30%) and AMP (20%), but with no significant formation of adenosine. There was no significant chemical breakdown of ATP under the same conditions, but in the absence of cells. The increasing accumulation of the weakly potent agonist AMP, at the expense of ATP and ADP, might thus explain the decline in cortisol secretion at later points on the time course. It should also be noted that adenosine itself is a relatively weak steroid agonist in bovine inner zone cells (Hoey, E., unpublished observation).

Both ATP and ADP were ineffective cortisol secretagogues in static incubations of freshly isolated cells, although a steroid response was evident by 24 h in culture, reaching a maximum at 48 h and declining thereafter. We observed that freshly isolated cells clearly respond to ATP and UTP in a superfusion system (unpublished observations), indicating that the purinergic receptor is present *in vivo* and not an artefact of culture. The reason for this difference is not known, but one possibility is that adenosine triphosphatase activity is higher in the freshly isolated cells with a requirement to continuously supply the agonist. The rise and subsequent fall in the cortisol response to ATP in culture is also observed for ACTH, adrenaline, and angiotensin-II; the changing responsiveness reflects an uncoupling of the second messenger from steroidogenic capacity with a probable later decline in steroidogenic capacity (14, 27).

The ability of ATP and ADP to elicit a steroid response, the relative lack of potency of AMP, and the failure to explain the response on the basis of nucleotide degradation to adenosine initially suggested that the response was mediated by a purinergic P2 receptor. In the absence of specific purine receptor antagonists, purine receptor classification has largely depended on relative agonist potencies combined with second messenger studies. The agonists $\alpha\beta$ MeATP and $\beta\gamma$ MeATP are both potent at P2x receptors, but have low potency in bovine adrenal inner zone cells, arguing that the response is not mediated by this receptor subtype. Furthermore, the P2x response is believed to operate via an ion channel mechanism, without stimulation of membrane phosphoinositide turnover. The receptor is also clearly different from that in the platelet (P2t receptor), which is sensitive only to ADP, not to ATP.

Kawamura *et al.* (11) suggested, on the basis of agonist potency, that the steroid response occurred through a P2y receptor. However, the possible occurrence of ATP-stimulated membrane phosphoinositide turnover, as predicted on the basis of this classification, was not investigated. In this study we demonstrated that ATP does stimulate membrane phosphoinositide turnover, a finding that initially led us to believe that a P2y receptor was indeed responsible for the steroidogenic effect of ATP.

ATP, ADP, and UTP all dose-dependently stimulated the accumulation of total 3 H-labeled phosphoinositols from cells whose phosphoinositides had been prelabeled with [3 H]inositol. The phosphoinositol head group response was linear for approximately 20 min, but declined thereafter. This contrasts with the rapid desensitization (half-time, 0.5–2 min) of the phospholipase-C activity coupled to the P2y receptor in turkey erythrocytes (28). Thus, the experimental findings

make it probable that the nucleotides activate a phosphoinositidase-C. The formation of inositol 1,4,5-trisphosphate from the action of phosphoinositidase-C upon phosphoinositide 4,5-bisphosphate would be expected to release Ca^{2+} from an intracellular site (29). Experiments on fura-2-loaded inner zone cells demonstrated a clear effect of ATP (10^{-4} M) in elevating the intracellular Ca^{2+} concentration from a mean basal (resting) value of 57.3 nmol/liter to a mean stimulated value of 171 nmol/liter. Matsui (13) also reported that ATP led to an increase in intracellular Ca^{2+} in bovine adrenocortical inner zone cells and that extracellular Ca^{2+} was required for a sustained elevation of the intracellular Ca^{2+} . Similar findings were obtained by Niitsu (12), who also observed that cortisol secretion in response to ATP was abolished in the absence of extracellular Ca^{2+} . Niitsu (12) found that the steroid response to ATP was inhibited by calmodulin antagonists, but was apparently unaffected by dihydropyridine Ca^{2+} channel blockers.

Recently, another type of purine receptor has been provisionally identified to which both ATP and the pyrimidine nucleotide, UTP, bind. The term nucleotide receptor was originally introduced by Davidson and colleagues to describe the ATP/UTP-sensitive site on sheep pituitary cells (30), although it was pointed out that a heterogeneous receptor population might explain the findings, with classical P2y purinoceptors and nucleotide receptors present, each of which resulted in the same functional response. In mammalian tissues, nucleotide receptors may be coupled to a variety of signal transduction mechanisms involving phospholipase-C, phospholipase-A2, phospholipase-D, and the activation of membrane Ca^{2+} channels (31).

Closer examination of the purinergic response in bovine adrenocortical inner zone cells demonstrates that it fulfills a number of the criteria presently used to define this nucleotide receptor; in particular, an ability to respond to UTP, in addition to ATP, and the relative lack of potency of 2-methylthio-ATP (which should be more potent than ATP at the classical P2y receptor) (32).

Acetylcholine and angiotensin-II are also known to secrete cortisol from bovine inner zone cells through stimulation of phosphoinositidase-C. In both cases, no effect on cellular cAMP formation was observed (33). In contrast, ATP also led to a dose-dependent increase in intracellular cAMP measured at 1 min; previous studies from our laboratory have shown that the intracellular cAMP concentration is already maximal at 1 min, thereafter falling and accumulating over a longer period in the overlying medium. At 1 min, the HPLC confirmed that there was no detectable degradation of ATP, establishing that the cAMP response must result from the action of ATP itself. Niitsu (12) also found that ATP increased cAMP formation from bovine inner zone cells, but only at the highest dose used (10^{-4} M). Moreover, the measurements were made after 1 h, so that degradation of ATP to AMP (which might increase cAMP by acting as a weak agonist at the A2 receptor) (9) would confound the interpretation of this data. We observed that at 1 min, cAMP was significantly increased and at a much lower concentration of ATP, namely 10^{-6} M.

The ZFR cultures were obtained using a column purification system, which leads to very low contamination with zona glomerulosa cells (20). It is unlikely that the dual second messenger response can be explained by the presence of two distinct cell types or, indeed, that the increase in cortisol occurs through indirect release of zona glomerulosa products acted upon by ZFR cells.

The ATP-induced cortisol secretion in these inner zone cells, thus, has a number of unusual features. A classical P2y response does not explain the agonist potencies and response to UTP, which are best explained by the presence of a nucleotide receptor, distinct from P2y. Moreover, ATP clearly stimulates both cAMP production and membrane phosphoinositide turnover, an unusual observation whose significance is uncertain at this stage. In this connection, studies by Tada *et al.* (9) also found that ATP led to stimulation of both phosphoinositide turnover and cAMP formation in cultured bovine vascular smooth muscle cells. Until more specific purinergic or nucleotide receptor antagonists become available or the appropriate receptors are cloned, it is not clear whether this dual second messenger response results from ATP acting at more than one receptor or whether one receptor is linked to both second messenger systems.

Finally, the importance of these observations with regard to the normal regulation of adrenocortical function is unknown and must remain speculative at present; as yet, there is no physiological evidence for the effects of ATP on adrenocortical function. The experimental findings reported here, with other circumstantial evidence, suggest that ATP may regulate adrenocortical function *in vivo*. Chromaffin granules contain high concentrations of ATP (15, 16). Acetylcholine releases 30% of the total cellular ATP from chromaffin cells, whereas the ATP concentration within chromaffin granules has been estimated to be as high as 160 mmol/liter (34). By analogy with thrombin-activated platelets (when plasma ATP levels exceed 10^{-5} M) (1), it is likely that locally released concentrations of ATP would be well within the range required to elicit a cortisol response from adjacent adrenocortical cells. The morphological evidence for a close proximity of medullary and adrenocortical cells is strong; the existence of medullary rays extending well into the cortex, with cytoplasmic extensions and direct cellular contacts between the two cell types has been described (17, 18). A further possibility is that platelet activation within the adrenal vasculature might provide a local source of ATP.

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