AN INVESTIGATION INTO THE MECANISMS MEDIATING CALCIUM ION-STIMULATED ACTH SECRETION FROM ATT-20 ANTERIOR PITUITARY TUMOUR CELLS

Brian William McFerran

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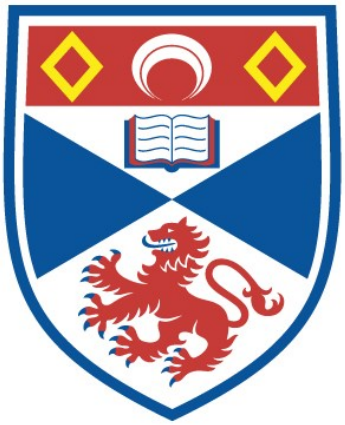
at the
University of St Andrews



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AN INVESTIGATION INTO THE MECHANISMS MEDIATING CALCIUM ION-STIMULATED ACTH SECRETION FROM AtT-20 ANTERIOR PITUITARY TUMOUR CELLS

A thesis submitted to the University Of St. Andrews in candidature for the degree of Doctor of Philosophy in the Faculty of Science.

by

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DECLARATIONS

I, Brian William McFerran, hereby certify that this thesis, which is approximately 40 000 words in length, has been written by me, that it is a record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

March 1, 1996 .....

I was admitted as a research student in October, 1992 and as a candidate for the degree of Doctor of Philosophy in October, 1992; the higher study for which this is a record was carried out in the University of St. Andrews between 1992 and 1995.

March 1, 1996 .....

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Doctor of Philosophy in the University of St. Andrews and that the candidate is qualified to submit this thesis in application for that degree.

March 1, 1996 .....

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ABBREVIATIONS

ACTH	(Adrenocorticotropin)
$AlF_{(3-5)}$	(Aluminium fluoride)
AMP	(Adenosine 5'-monophosphate)
aPKCs	(Atypical PKC isozymes)
ATP	(Adenosine 5'-triphosphate)
AVP	(Arginine-vasopressin)
BSA	(Bovine serum albumin)
CGN	(*Cis*-Golgi network)
cPKCs	(Conventional PKC isozymes)
CRF-41	(Corticotrophin releasing factor, human / rat)
CTX	(Cholera toxin)
Cyclic AMP	(Adenosine 3' : 5' cyclic monophosphate)
DAG	(Diacylglycerol)
DMEM	(Dulbecco's modified Eagle's medium)
DOG	(1,2-Dioctanoyl-sn-glycerol)
dPPA	(12-Deoxyphorbol 13-phenylacetate 20-acetate)
EDTA	(ethylenediamine-tetraacetic acid)
EGTA	(ethylene glycol-bis(β-aminoethyl ether) N, N, N', N'-tetra acetic acid)
GDP- β -S	Guanosine 5'-O-(2-thiodiphosphate))
GTP- γ -S	(Guanosine 5'-O-(3-thiotriphosphate))
HEEDTA	(N-Hydroxyethylethylenediaminetriacetic acid)
HEPES	(N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid])
IBMX	(1-Isobutyl 3-methylxanthine)
IP3	(Phosphatidyl inositol (3, 4, 5) triphosphate)

MOPS	(3-[N-morpholino]propanesulfonic acid)
NAD	(β-Nicotinamide adenine dinucleotide)
NEM	(*N*-ethylmaleimide)
nPKCs	(Novel PKC isozymes)
NSF	(NEM-sensitive fusion protein)
NTA	(Nitrilotriacetic acid)
OAG	(1-Oleoyl-2-acetyl-sn-glycerol)
PDBu	(Phorbol 12, 13-dibutyrate)
PDD	(Phorbol 12, 13-didecanoate)
PIP_2	(Phosphatidyl inositol (4, 5)-bisphosphate)
PIP_3	(Phosphatidyl inositol (3, 4, 5)-triphosphate)
PIPES	(Piperazine-N-N'-bis-[2-ethanesulfonic acid])
PKC	(Calcium ion & phosholipid activated protein kinase)
PLA_2	(Phospholipase A_2)
PLC	(Phospholipase C)
PMA	(Phorbol 12-myristate 13-acetate)
POMC	(Pro-opiomelanocortin)
PS	(Phosphatidylserine)
PTX	(Pertussis toxin)
PVN	(Paraventricular nucleus)
RER	(Rough endoplasmic reticulum)
[^{125}I]-ScAMP-TME	([^{125}I]-Succinyl cyclic AMP-tyrosyl methyl ester)
TAPS	(N-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid)
SNAP	(Soluble NSF-attachment protein)
t-SNARE	(Target membrane associated SNAP receptor)
SRIF	([D-trp^8]-Somatostatin)
TGN	(*Trans*-Golgi network)

TMX	(Thymeleatoxin)
Triton X-100	(t-octylphenoxypolyethoxyethanol)
VIP	(Vasoactive intestinal polypeptide)
v-SNARE	(Vesicle membrane associated SNAP receptor)

PUBLICATIONS

Some of the work detailed herein has been published and can be found in the following communications and papers.

1 PRE-CIRCULATED COMMUNICATIONS

McFerran, B.W., Semple, S.E. & Guild, S.B. (1994). PMA potentiates calcium- but not guanine nucleotide-evoked ACTH secretion from electrically permeabilized AtT-20 cells. *Br. J. Pharmacol.*, **111**, 112P.

McFerran, B.W. & Guild, S.B. (1994). Effects of protein kinase C activators upon the late stages of the ACTH secretory pathway of AtT-20 pituitary tumour cells. *Can. J. Physiol. Pharmacol.*, **72**, (Suppl. 1), P18.10.4.

Guild, S.B. & McFerran, B.W. (1995). The effects of calyculin A upon calcium-, guanine nucleotide- and phorbol 12-myristate 13-acetate-stimulated ACTH secretion from AtT-20 cells. *Br. J. Pharmacol.*, **114**, 66P.

McFerran, B.W. & Guild, S.B. (1995). Effects of mastoparan upon the late stages of the adrenocorticotropin (ACTH) secretory pathway of AtT-20 cells. *Br. J. Pharmacol.*, **114**, 443P.

2 FULL PAPERS

McFerran, B.W. & Guild, S.B. (1994). Effects of protein kinase C activators upon the late stages of the ACTH secretory pathway of AtT-20 cells. *Br. J. Pharmacol.*, **113**, 171-178.

McFerran, B.W. & Guild, S.B. (1995). The effects of calyculin A upon calcium-, guanine nucleotide- and phorbol 12-myristate 13-acetate-stimulated ACTH secretion from AtT-20 cells. *Br. J. Pharmacol.*, **114**, 1571-1576.

McFerran, B.W., MacEwan, D.J. & Guild, S.B. (1995). Involvement of multiple protein kinase C isozymes in the ACTH secretory pathway of AtT-20 pituitary cells. *Br. J. Pharmacol.*, **115**, 307-315.

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ABSTRACT

The mouse AtT-20/D16-16 anterior pituitary tumour cell line was employed as a model system for the study of the mechanisms mediating calcium ion-stimulated adrenocorticotropin (ACTH) secretion. The present study indicates that calcium ion-stimulated ACTH secretion from AtT-20 cells is mediated by a GTP-binding protein which is present in a variety of other cell types and has been dubbed G_e (for reviews see Gomperts, 1990; Lindau & Gomperts, 1991). In AtT-20 cells the nature of G_e remains elusive with the selective heterotrimeric GTP-binding protein activator $AlF_{(3-5)}$ proving not to be a useful pharmacological tool under the conditions employed in the present study. G_e present in this cell line does however display characteristics consistent with it being a heterotrimeric GTP-binding protein. The results of this study would also suggest that in AtT-20 cells G_e is insensitive to both pertussis toxin and cholera toxin.

Both cyclic AMP-dependent protein kinase (PKA) (Guild, 1991) and protein kinase C (PKC) (Guild & Reisine, 1987; Reisine, 1989) have been implicated in the regulation of calcium ion-stimulated ACTH secretion from AtT-20 cells. Results from the present study suggest that calcium ion/G_e-stimulated ACTH secretion from AtT-20 cells is not mediated by PKA, PKC or any other kinase but is in fact mediated by a phosphatase. PKC appears to provide a direct stimulus to secretion, which is independent of calcium ion/G_e-stimulated secretion, in contrast to PKA which is unable to stimulate secretion by itself but seems to play a modulatory role with regard to both calcium ion/G_e- and PKC-stimulated secretion.

INTRODUCTION

1 STRESS RESPONSE

The 19th century physiologist Claude Bernard first suggested that the constancy of the internal milieu is critical for a free and independent existence (Bernard, 1878). The preservation of this internal environment, which was later termed and is now commonly referred to as homeostasis (Cannon, 1929), requires continuous adaptation to external and internal stimuli, or stressors, by means of a wide range of physiological changes throughout the body which are generally referred to as the stress response. The stress response is mediated by the hypothalamus, a small region of the brain which initiates two main stress response pathways (for reviews see Fisher, 1989; Johnson *et al.,* 1992). The first pathway produces an immediate set of physiological changes termed the alarm reaction or fight or flight response. The alarm reaction is a complex of responses initiated by hypothalamic stimulation of the sympathetic division of the autonomic nervous system. These responses are fast, mobilising the resources of the body for immediate physical activity, however they are also short lived. The second pathway is termed the resistance reaction which, unlike the alarm reaction, is mediated by a number of hypothalamic hormones and is a more long term response. The actions of these hypothalamic hormones include the release of adrenocorticotropic hormone (ACTH) from corticotrophs of the anterior pituitary (for reviews see Aguilera, 1994; Antoni, 1986; Jones & Gillham, 1988). ACTH in turn is the dominant, if not sole, physiological regulator of the secretion of glucocorticoids, in particular corticosterone, from the zona fasciculata of the adrenal cortex (for review see Dallman, 1985) which subsequently act upon every cell in the body to cause alterations in differentiated function (for review see Munck *et al.*, 1984). A knowledge of how ACTH release from the anterior pituitary is regulated is therefore of importance in the understanding of how the body

mounts a co-ordinated response to stressors and is the focus of the present study.

2 THE PITUITARY

The fully developed pituitary or hypophysis is an amalgam of hormone-producing glandular cells (the adenohypophysis) and neural cells with secretory function (the neurohypophysis). Endocrine cells of the adenohypophysis, or anterior pituitary gland, synthesise, store and secrete a group of tropic hormones from a number of specialised secretory cells; ACTH from corticotrophs, thyroid stimulating hormone (TSH) from thyrotrophs, gonadotropins (luteinizing hormone (LH) & follicle-stimulating hormone (FSH)) from gonadotrophs, somatotropin (growth hormone, GH) from somatotrophs and prolactin from lactotrophs. Two hormones, antidiuretic hormone (arginine-vasopressin, AVP) and oxytocin, are synthesised by neurones in the hypothalamus but are stored and secreted by the neurohypophysis or posterior pituitary gland.

In order to understand the regulation of hormone release from the anterior pituitary a brief knowledge of the anatomy of the pituitary gland as a whole is useful (for review see Porter *et al.*, 1974). The anterior endocrine portion of the pituitary develops from an upward outgrowth of ectodermal cells from the roof of the oral cavity (Rathke's pouch). This pouch eventually pinches off and becomes separated from the oral cavity by the sphenoid bone of the skull which forms a hollow around the pituitary which is termed the *sella turcica*, the lumen being reduced to a small cleft. The posterior neural portion of the pituitary develops from a downward outgrowth of ectoderm from the brain in the floor of the third ventricle, with

the lumen of this pouch being obliterated inferiorly as the sides fuse. Superiorly the lumen remains contiguous with the third ventricle. The upper portion of this neural stalk expands to invest the lower portion of the hypothalamus and is termed the median eminence. In the anterior pituitary of some species, a third, well defined intermediate lobe is also included, but in humans is rudimentary. In lower vertebrates this lobe secretes melanocyte stimulating hormones (MSHs). A schematic diagram of these anatomical arrangements is displayed in figure 1.

The blood supply to this consortium of neural and endocrine tissue is complex and is also represented in figure 1. In the posterior pituitary the neural tissue of the infundibular process derives its blood supply from the capillary plexus of the inferior hypophyseal artery into which posterior pituitary hormones are released. These hormones subsequently drain into the posterior hypophyseal veins for distribution to the systemic circulation. The neural tissue of the median eminence is supplied largely by the superior hypophyseal artery. The capillary plexus emanating from this artery forms a set of long portal veins that carry the blood downward into the anterior pituitary. There these portal veins give rise to a second capillary plexus that supplies the endocrine cells with the majority of their blood supply, into which anterior pituitary hormones are secreted, which is then drained off into the anterior hypophyseal veins for distribution to the systemic circulation. The anterior pituitary receives its remaining blood supply via a set of short portal veins originating in the capillary plexus of the inferior hypophyseal artery, which carry posterior pituitary hormones. There is therefore little or no direct blood supply to cells of the adenohypophysis.

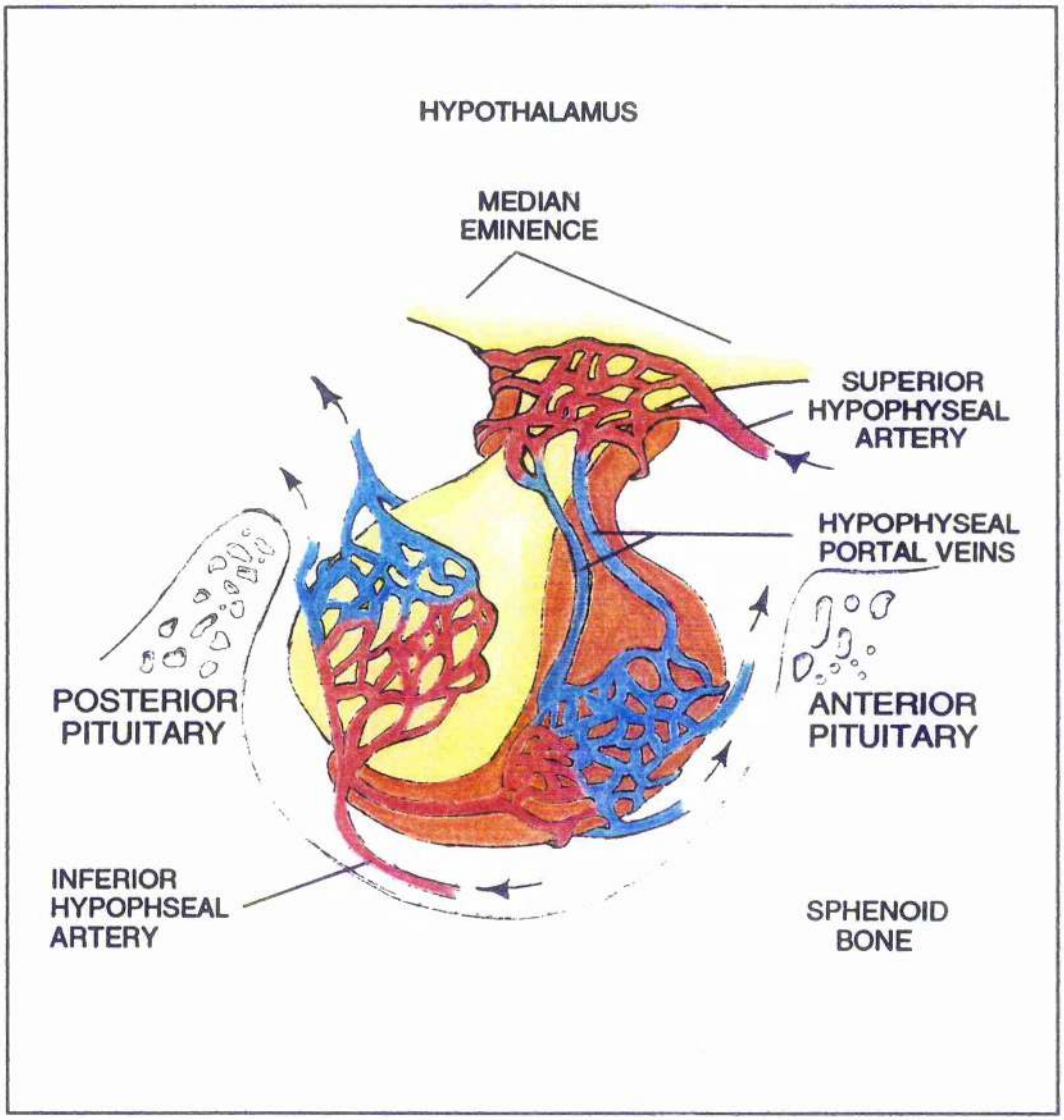

Figure 1 Diagram of the pituitary gland showing its division into the adenohypophysis (anterior pituitary) and the neurohypophysis (posterior pituitary). (Adapted from Tortora & Grabowski, 1992).

Hormone secretion from both the anterior and posterior pituitary is primarily under the control of the hypothalamus. This small region of the brain is the major integrating link between the nervous system and endocrine system receiving and integrating numerous inputs, both neural and hormonal, excitatory and inhibitory. The relationships between the posterior pituitary and the hypothalamus and the anterior pituitary and the hypothalamus, however, are entirely different. The posterior pituitary represents a collection of axons whose cell bodies lie in two well defined clusters of the hypothalamus, the supraoptic and paraventricular nuclei. Peptide hormones are synthesised in the cell bodies of the hypothalamic neurones and travel down their axons in neurosecretory granules to be stored in the nerve terminals lying in the posterior pituitary. Thus secretion from the posterior pathway is under the control of a neural pathway. Upon stimulation the granules release their contents into the capillary plexus of the inferior hypophyseal artery and ultimately the peripheral circulation (for review see Douglas, 1974 a). In contrast to the posterior pituitary, the anterior pituitary is a collection of endocrine cells regulated by blood borne stimuli originating from neural cells of the hypothalamus. Cell bodies of particular hypothalamic neurones, termed hypophysiotropic neurones, synthesise releasing and inhibiting hormones (including corticotrophin releasing factor-41 (CRF-41) and AVP, discussed in detail below) which travel down their axons only as far as the median eminence where they are stored as neurosecretory granules in the nerve terminals. Upon stimulation the releasing or inhibiting hormones are released into the median eminence and enter the primary capillary plexus of the superior hypophyseal artery. They are transported along the long portal veins and exit from the second capillary plexus to reach their specific endocrine target. These cells respond by modulating their release of their tropic hormones into the same second capillary plexus through which they

ultimately reach the peripheral circulation. This unusual capillary to capillary connection allows the releasing and inhibiting hormones to be delivered immediately and directly to the anterior pituitary at relatively high concentrations, completely bypassing the systemic circulation (for review see Riskind & Martin, 1989).

3 REGULATION OF ACTH RELEASE

ACTH secretion from anterior pituitary corticotrophs is now known to be largely under the control of stimulatory corticotropin-releasing factors, which are secreted from the hypothalamus in response to a central circadian rhythm, coupled with a tonic inhibition conferred by glucocorticoids by means of a negative feedback mechanism (for reviews see Aguilera, 1994; Antoni, 1986; Jones & Gillham, 1988).

The discovery of the portal blood supply between the hypothalamus and the anterior pituitary (Popa & Fielding, 1930) together with the observation that the direction of flow was from the hypothalamus to the anterior pituitary (Green & Harris, 1949) gave the first indications of the functional importance of the hypothalamus over the release of tropic hormones from the anterior pituitary. In a series of classic experiments Harris subsequently demonstrated that placing an impenetrable barrier across the cut ends of the portal vessels led to a permanent disruption of anterior pituitary function (Harris, 1955). Such observations led Harris to propose the neurohumoral hypothesis which predicted that the hypothalamus releases specific neurohormones, including a corticotropin-releasing factor (or factors), into the portal blood supply of the median eminence which, upon reaching the anterior pituitary, stimulates the

release of a number of tropic hormones including ACTH (Harris, 1955). This hypothesis was quickly followed by the demonstration that a corticotropin-releasing factor (or factors) existed (Guillemin, 1955; Saffran & Schally, 1955), however it was more than 25 years later before Vale and co-workers purified the first corticotropin-releasing factor and characterised it as a 41 amino acid peptide which was subsequently named corticotropin releasing factor-41 (CRF-41) (Vale *et al.*, 1981). CRF-41 is now known to be contained in hypophysiotropic neurones which send axonal projections from the hypothalamus to the median eminence to control the release of pituitary hormones. 90 % of these CRF-41 containing hypophysiotropic neurones are located in the parvicellular subdivisions of the hypothalamic paraventricular nucleus (PVN) (Bruhn *et al.*, 1984). CRF-41 containing neurones are also present in a second neuronal pathway which projects from the magnocellular neurones of the PVN, the supraoptic and the accessory magnocellular nuclei of the hypothalamus (Antoni, 1986).

ACTH secretion from anterior pituitary corticotrophs is now known to be under the control of a number of other corticotropin-releasing factors, the most important of which is the hypothalamic nonapeptide arginine-vasopressin (AVP). Although this peptide was first proposed as a candidate for a corticotropin-releasing factor as early as the 1950s (Martini & Morpurgo, 1955; McCann, 1957) it was not until the discovery of CRF-41 that this theory was proved conclusively. Since the discovery of CRF-41 it has been shown in preparations derived from rats, primates and humans that although AVP is a minor stimulant to ACTH secretion it does potentiate the stimulatory effect of CRF-41 upon ACTH secretion in a synergistic manner (Abou-Samra *et al.*, 1987; Gillies *et al.*, 1982; Millan *et al.*, 1987; Rivier *et al.*, 1984; Rivier & Vale, 1983). In ovine preparations the opposite is true, AVP is the dominant hormone and CRF-41 is facilitative with regard

to evoked ACTH release (Familary *et al.*, 1989; Liu *et al.*, 1990), however this appears to be the exception. The importance of AVP as a corticotropin releasing factor is emphasised by the finding that this peptide is also present in the parvicellular CRF-41 containing pathway to the median eminence. There is evidence to suggest that AVP may co-localise with up to 50 % of the CRF-41 containing neurones of the parvicellular pathway not only in the same axons but also in the same neurosecretory granules (Whitnall *et al.*, 1985). A number of other corticotropin-releasing factors released into the median eminence which are of particular relevance to the present discussion include angiotensin II (Capponi *et al.*, 1982; Sobel & Vagnucci, 1982), catecholamines (Giguere *et al.*, 1981; Vale & Rivier, 1977) and vasoactive intestinal polypeptide (VIP) (Reisine *et al.*, 1982). It has been proposed that different stressors may evoke the release of specific mixes of ACTH secretagogues in the median eminence (Plotsky *et al.*, 1985) which may go some way to explaining the need for this variety of ACTH secretagogues.

ACTH secretion from anterior pituitary corticotrophs is also regulated by a number of inhibitory factors, the dominant one being glucocorticoids by means of a negative feedback mechanism. Glucocorticoids inhibit ACTH secretion directly at the level of the pituitary corticotroph and also at different sites in the brain including the PVN and the hippocampus (Dallman *et al.*, 1987; DeKloet, 1991). The primary site of feedback inhibition under basal conditions is the hippocampus which contains the high affinity type I steroid receptors which are sensitive to non-stressed levels of circulating glucocorticoids (DeKloet, 1991, Sapolsky & Jacobson, 1991). Lower affinity type II receptors constitute the major proportion of receptors found in the pituitary and PVN which become occupied with stress induced levels of glucocorticoids (Reul & DeKloet,

1985). A number of other agents have been implicated in the inhibition of ACTH secretion from anterior pituitary corticotrophs including, of relevance to the present discussion, the hypothalamic neuropeptide somatostatin (somatotropin release inhibitory factor, SRIF) (Luini *et al.*, 1986; Reisine & Guild, 1985; Reisine *et al.*, 1988).

4 SECRETORY PATHWAY

(i) Protein sorting & processing

Proteins can be released from specialised secretory cells by means of two major routes, that of the regulated secretory pathway and that of the constitutive secretory pathway (for review see Burgess & Kelly, 1987). The majority of secretory proteins secreted from eukaryotic cells originate in the rough endoplasmic reticulum (RER) in the form of a peptide precursor. Secretory proteins are transferred from the RER to the *cis* -Golgi network (CGN) and from one Golgi stack to the next, in shuttle vesicles, finally reaching the *trans* -Golgi network (TGN) (figure 2) (Klausner, 1989; Pfeffer & Rothman, 1987; Rothman & Orci, 1992). At this point proteins to be secreted by means of the regulated secretory pathway are actively sorted into dense core secretory granules in contrast to delivery into constitutive secretory vesicles which proceeds by default (Griffiths & Simons, 1986). Release from the constitutive pathway is a continuous process, limited only by the availability of the product, therefore release from this pathway is regulated at the most proximal of levels, that of biosynthesis (Mains & Eipper, 1981; Tartakoff & Vassalli, 1978). In contrast secretion via the regulated pathway is stimulated by secretagogues which occurs at the level of exocytosis itself, the most distal step in the pathway, and allows for

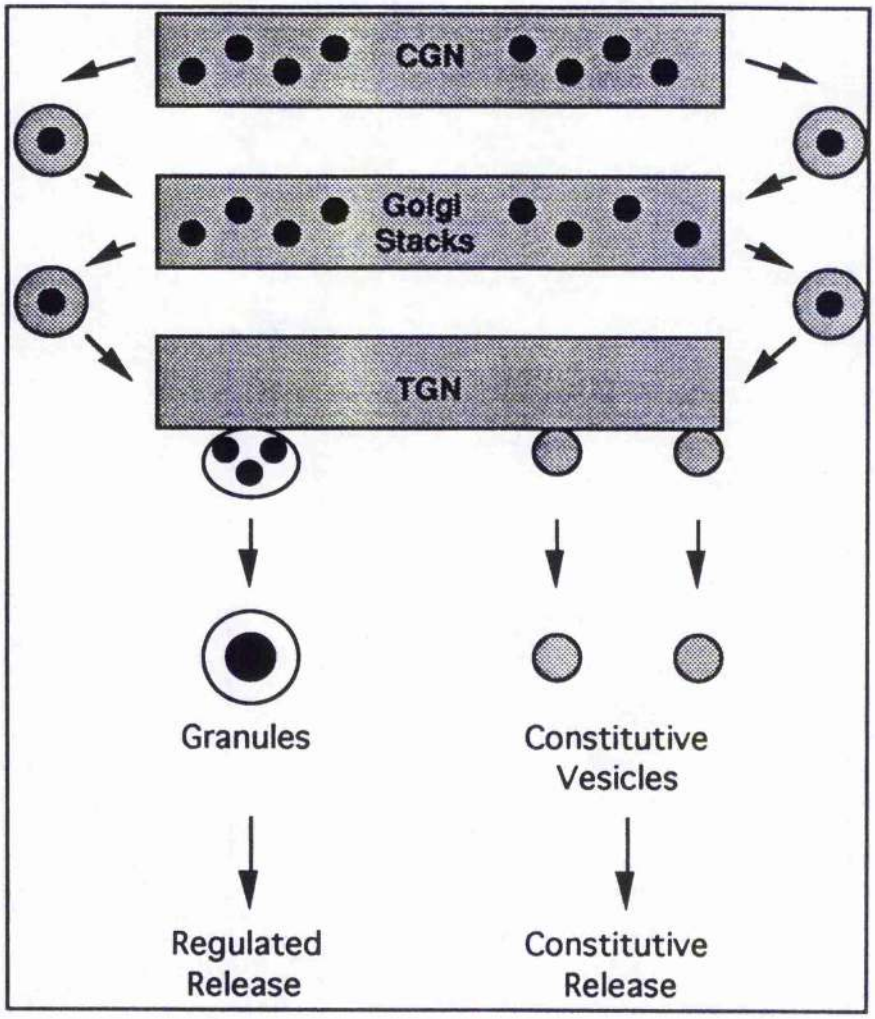

Figure 2 The regulated and constitutive secretory pathways. CGN, *cis*-Golgi network; TGN, *trans*-Golgi network. (Adapted from Halban & Irminger, 1994).

the rapid and massive discharge of proteins stored in dense core secretory granules at the physiologically appropriate moment (Palade, 1975).

The peptide precursor found in corticotrophs is pro-opiomelanocortin (POMC) which is processed to form a number of peptides including ACTH. POMC is processed by a membrane bound conversion endoproteases to form ACTH, β-lipotropic hormone (lipotropin) and POMC (1-74) (figure 3). β-lipotropic hormone in turn can be partially cleaved by a second related endoprotease, which in comparison is poorly expressed, to produce β-endorphin and γ-lipotropic hormone (for reviews see Halban & Irminger, 1994; Loh *et al.*, 1991).

(ii) Fusion machinery

Over recent years the general mechanisms of eukaryotic intracellular transport and in particular the proteins controlling vesicle docking and fusion have been greatly elucidated (for review see Rothman, 1994). Initially Rothman and co-workers identified a number of soluble mammalian proteins involved in intra-Golgi transport using a run down-reconstitution approach (for review see Rothman & Orci, 1992). One of the first proteins to be identified by this method was *N*-ethylmaleimide-sensitive fusion protein (NSF) which was purified by its ability to reconstitute transport through the Golgi after blockade by the alkylating agent *N*-ethylmaleimide (NEM) (Block *et al.*, 1988). The ability of NSF to reconstitute vesicular transport was however shown to be dependent upon additional cytosolic factors, in particular a group of proteins which later became known as the soluble NSF attachment proteins (SNAPs). SNAPs are now known to be a family of three proteins named α-, β- and γ-SNAP which were purified by their ability to reconstitute NSF-dependent transport

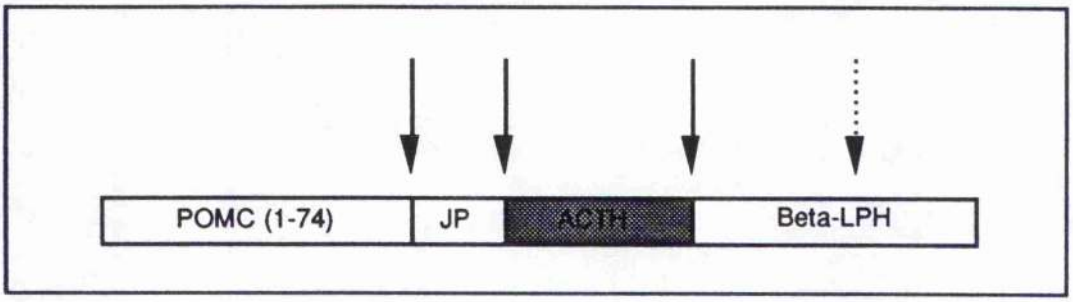

Figure 3 POMC processing in the anterior pituitary. Solid arrows indicate efficient cleavage whereas broken arrows indicate partial cleavage. ACTH, adrenocorticotropic hormone; Beta- LPH, β-lipotropic hormone (lipotropin); JP, joining peptide; POMC, pro-opiomelanocortin. (Adapted from Halban & Irminger, 1994).

through the Golgi (Clary & Rothman, 1990) and were subsequently shown to mediate NSF binding to the membrane (Clary *et al.*, 1990). Employing an immunoprecipitation approach using brain membranes a number of membrane associated proteins involved in vesicle docking and membrane fusion have been identified including three SNAP receptors (SNAREs) which have subsequently been named syntaxin, synaptobrevin and SNAP-25 (Sollner *et al.*, 1993). These three proteins are now known to be targets for the botulinum neurotoxins (Montecucco & Shiavo, 1994), potent inhibitors of neurotransmission, thus implicating NSF and SNAPs in regulated exocytosis at the synapse. Functional evidence also exists for the involvement of NSF and SNAPs in regulated exocytosis at the synapse (Debello *et al.*, 1995) and also in chromaffin cells (Morgan & Burgoyne, 1995 a). It therefore appears that a "fusion complex", comprising membrane bound SNAREs bound to cytosolic SNAPs which in turn bind cytosolic NSF, has some role in vesicle docking and membrane fusion. Additional information comes from the observation that this fusion complex has an intrinsic ATPase activity conferred by NSF (Tagaya *et al.*, 1993) which contains two ATP binding sites (Wilson *et al.*, 1989). This ATPase activity appears crucial to some aspect of vesicle docking and membrane fusion since after ATP hydrolysis this complex of proteins dissociates (Wilson *et al.*, 1992).

These and other observations led Rothman and co-workers to formulate the SNARE hypothesis (Sollner *et al.*, 1993), a schematic diagram of which is displayed in figure 4. This model predicts that SNAREs present on the vesicle membrane (v-SNAREs), of which synaptobrevin is an example, interact with their equivalent on the target membrane (t-SNAREs), of which syntaxin and SNAP-25 are examples, forming a complex to which one or more SNAPs can bind. Inactive cytosolic NSF can

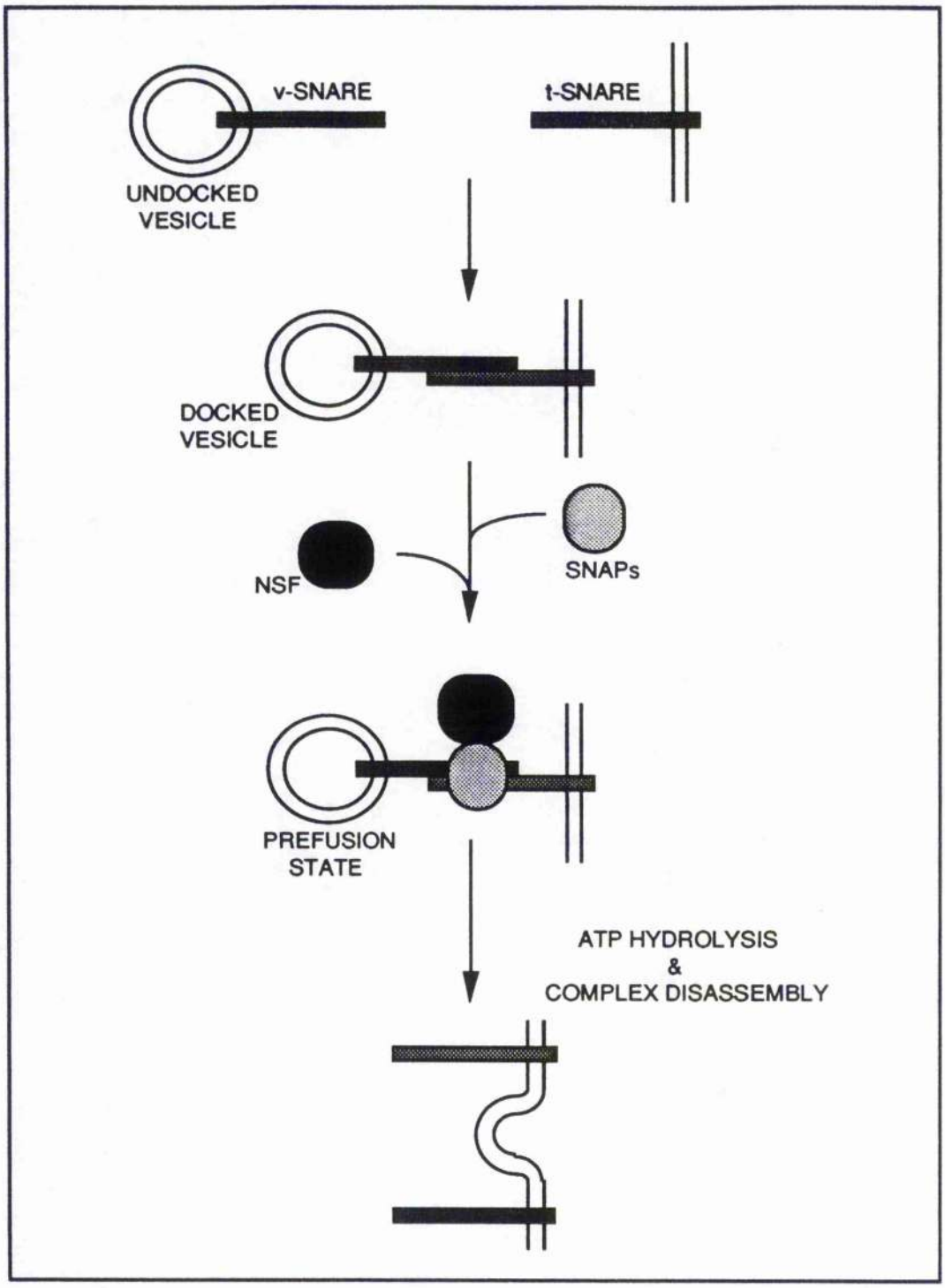

Figure 4 SNARE hypothesis. ATP, adenosine 5'-triphosphate; NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAPs, soluble NSF attachment proteins; t-SNARE, target membrane associated SNAP receptor; v-SNARE, vesicle membrane associated SNAP receptor. (Adapted from Sollner *et al.*, 1993).

then also bind to this complex, via interaction with one or more SNAPs, resulting in an activation of this protein and therefore ATP hydrolysis. This ATPase activity according to Rothman "creates a fusion active conformation which facilitates bilayer fusion" (Rothman, 1994). This model is an attempt to explain how some of the key proteins known to participate in membrane docking and fusion may interact to regulate this process at a number of stages throughout both the regulated and constitutive secretory pathways. In the case of the regulated pathway a "fusion clamp" is included in the hypothesis, a protein which under resting conditions would prevent the full formation of the fusion complex. Upon activation by second messengers this clamp would however be removed and membrane fusion allowed to proceed in a manner similar to that of constitutive secretion.

Although the SNARE hypothesis may prove with time to be an oversimplification (Morgan & Burgoyne, 1995 b) it does highlight the advances made over recent years in the understanding of the general machinery controlling vesicle docking and membrane fusion. In contrast the mechanisms which mediate the effects of extracellular secretagogues upon the final stages of the regulated secretory pathway remain, particularly in non-neuronal secretory cells, only partially understood. This is certainly true of the regulated ACTH secretion from the anterior pituitary corticotroph despite many studies being carried out to this end (Antoni, 1986; Axelrod & Reisine, 1984). The broad aim of this study was therefore to further elucidate the intracellular mechanisms which mediate the effects of corticotropin-releasing factors upon the regulated ACTH secretory pathway.

5 AtT-20 CELLS

(i) Corticotroph model system

Corticotrophs have been estimated to represent only 3-10 % of the cell population of the anterior pituitary (Westlund *et al.*, 1985). The major drawback, therefore, of any preparation derived from normal anterior pituitary tissue is the heterogeneity of this cell population, which makes experiments conducted at an intracellular level difficult. As a result many studies investigating the intracellular mechanisms mediating regulated ACTH secretion in response to corticotropin-releasing factors have been carried out in using the AtT-20 anterior pituitary tumour cell line which provides a homogeneous population of ACTH secreting corticotrophs (Axelrod & Reisine, 1984). The AtT-20 cell line was therefore adopted as a corticotroph model system in the present study. As can be seen in figure 5, the POMC cleavage patterns in anterior pituitary corticotrophs and in AtT-20 cells closely resemble each other and as a result both secrete a similar array of proteins (for reviews see Halban & Irminger, 1994; Loh *et al.*, 1991).

(ii) Derivation

Transplantable mouse pituitary ACTH-secreting tumours, which retain differential function after serial transfer, were first developed by Furth upon exposure of mice to ionising radiation from an atomic detonation (Furth, 1955). One such tumour contained about twice the normal adrenotropic hormone (ACTH) content and was designated AtT-20 (Adrenotropic Tumour # 20) (Cohen & Furth, 1958). AtT-20 cells were subsequently adapted to tissue culture (Yasumura, 1968) by the method of

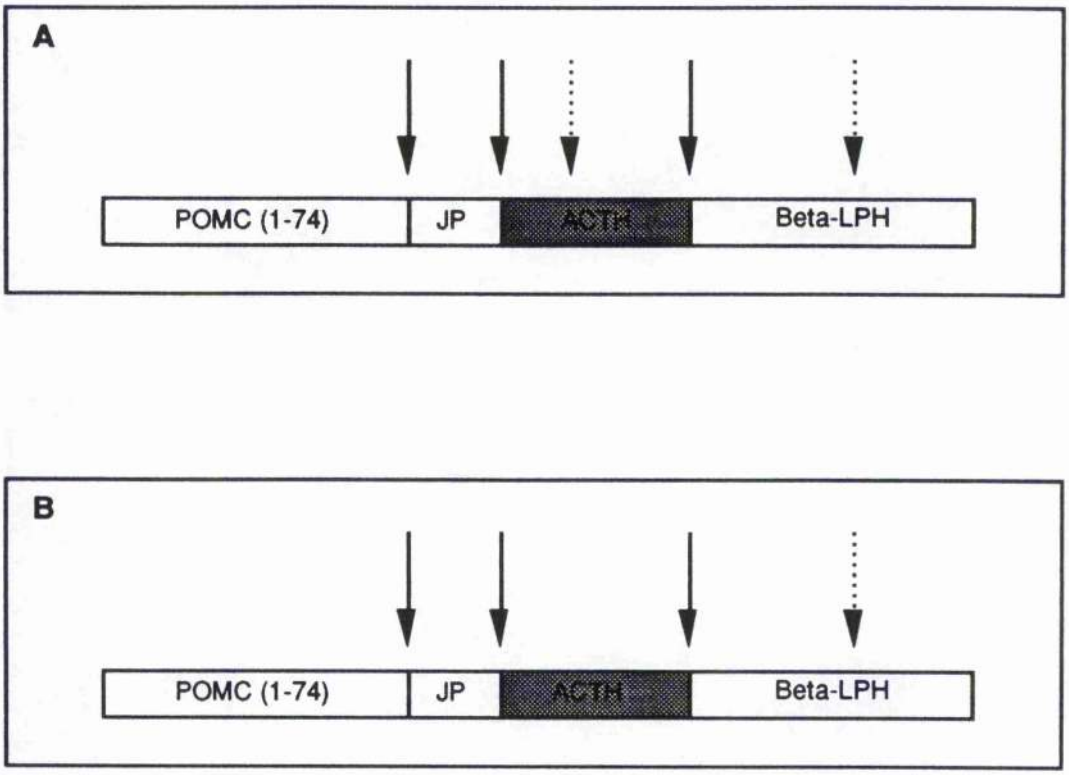

Figure 5 POMC processing in AtT-20 cells in comparison to the anterior pituitary. Panel A represents protein processing in AtT-20 cells whereas panel B represents protein processing in the anterior pituitary. Solid arrows indicate efficient cleavage whereas broken arrows indicate partial cleavage. ACTH, adrenocorticotropic hormone; Beta- LPH, β-lipotropic hormone (lipotropin); JP, joining peptide; POMC, pro-opiomelanocortin. (Adapted from Halban & Irminger, 1994).

alternate animal and cell culture passage (Buonassisi *et al.*, 1962). Briefly hormone producing tumours were cultured and after various times injected into mice and tumours arising from these injected cultures checked for hormonal activity, put back into culture and the whole process repeated. This process allows only those tumour cells which survive culture conditions to contribute to subsequent tumours and appears to give rise to cells with increased growth and enhanced hormonal activity in culture. After completion of this process a cell line of ACTH-secreting cells, designated D-1 (Dai-iti-1), which grows as floating aggregates was finally isolated and cloned (Yasumura, 1968; Yasumura & Sato, 1967). Three variant cell lines, including the AtT-20/D16v (variant) cell line, were subsequently isolated from the D-1 cell line (Yasumura, 1968). Each of these variant cell lines grew as a monolayer and were re-cloned by the single-cell plating technique (Puck, 1959). This AtT-20/D16v mouse anterior pituitary cell line has since provided a simplified model system in which to study POMC processing and subsequent storage and secretion of ACTH (for review see Halban & Irminger, 1994). Because of possible heterogeneity within this AtT-20/D16v cell line a number subclones were derived from single cells and isolated, one of which was chosen, by virtue of its high β-endorphin immunoreactivity, for further experimentation and designated AtT-20/D16-16 (Sabol, 1980). This AtT-20/D16-16 subclone shows identical morphology to that of the AtT-20/D16v cell line (Sabol, 1980).

Sabol and co-workers (Hook *et al.*, 1982), upon isolating the D16-16 subclone, demonstrated that CRF-41 stimulates ACTH release from these cells in a similar manner to CRF-41-stimulated ACTH release from primary cultures of anterior pituitary cells (Turkelson *et al.*, 1981; Vale *et al.*, 1981). It was therefore concluded that AtT-20/D16-16 cells may serve a useful

model system for the study of the cellular and molecular actions of CRF-41 in the regulation of secretion and synthesis of ACTH from the normal corticotroph. Due to its greater degree of homogeneity the AtT-20/D16-16 cell line has since been used extensively as a model system in the investigation of ACTH secretion from the normal corticotroph in response to a number of different secretagogues (Axelrod & Reisine, 1984) and as a result was the particular subclone employed as a model system for the normal corticotroph in the present study.

(iii) Morphology

Many of the studies carried out in the comparison of the regulated and constitutive secretory pathways employed the AtT-20 model system which also promoted an understanding of the morphology of these cells, a photo-micrograph of which is displayed in figure 6. AtT-20 cells are approximately 15 μm long (Richardson, 1978) and as can be seen these cells exhibit a polarised morphology with dense-core secretory granules accumulating in the tips of processes extending from the cell. When AtT-20 cells are stimulated with secretagogues they increase in size become flatter and the tips of these processes enlarge and accumulate ACTH (Buckley & Kelly, 1985; Burgess *et al.*, 1985; Kelly *et al.*, 1983; Matsuuchi *et al.*, 1988; Tooze & Burke, 1987).

6 CALCIUM IONS AS CENTRAL REGULATORS OF ACTH SECRETION

First messengers, including hormones, interact with specific receptors at the cell surface initiating a cascade of molecular events that underlies trans-membrane signalling or signal transduction. In many cases

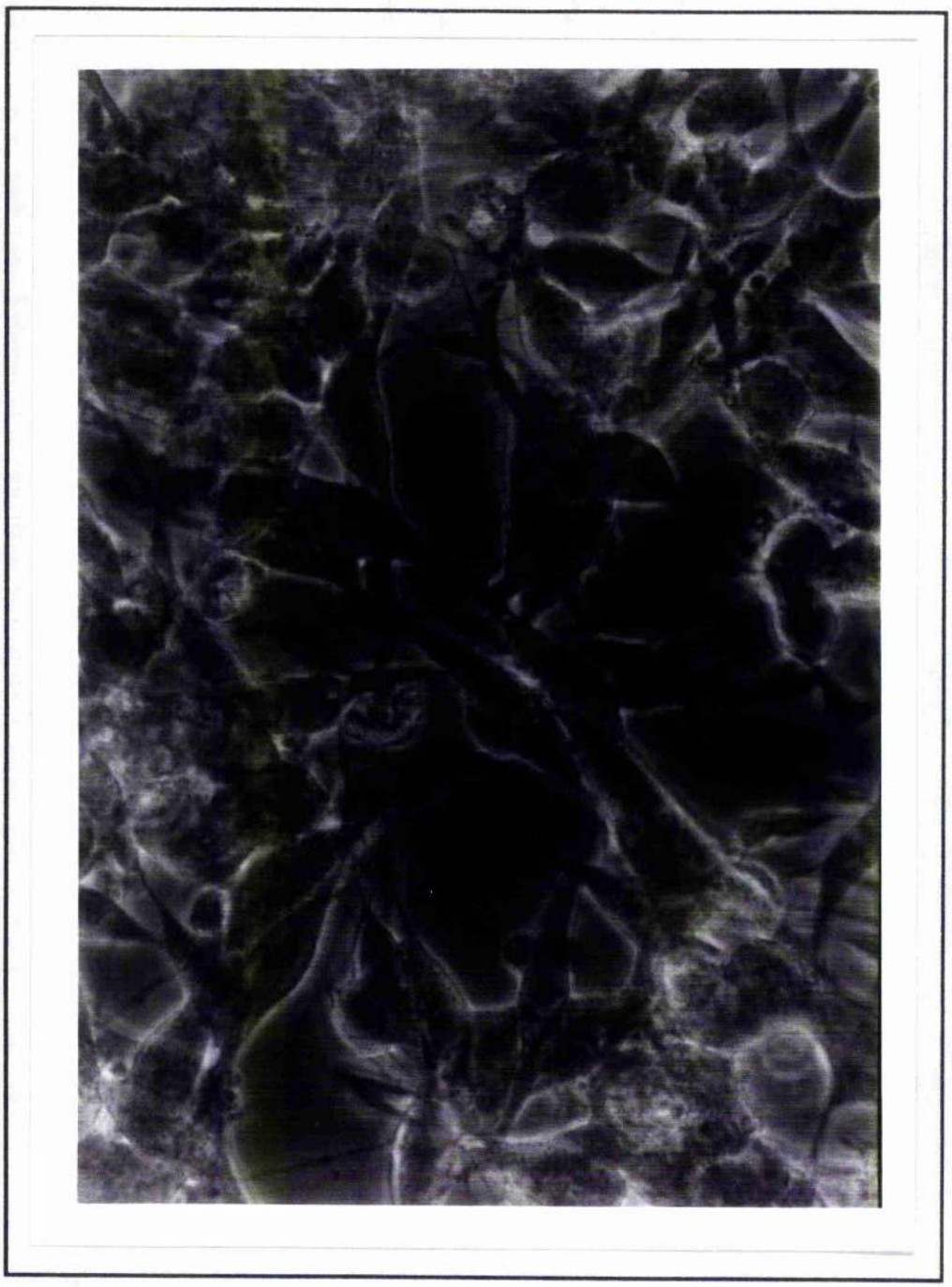

Figure 6 **A photo-micrograph of AtT-20/D16-16 cells.**

stimulation of these receptors results in activation of effector proteins such as enzymes or ion channels which mobilise chemical second messengers that in turn initiate characteristic actions within the cell, a concept first proposed by Sutherland and co-workers (Sutherland & Rall, 1958). Evidence from a variety of studies established calcium ions as one such second messenger, including the work of Douglas who first suggested that changes in cytosolic calcium ions may be a necessary and sufficient trigger to hormone secretion (Douglas, 1968 & 1974 b). Calcium ions are now established as one of the most important second messengers in a variety of systems (for review see Clapham, 1995) and have been shown to directly stimulate a secretory response from a variety of specialised secretory cells (Gomperts, 1990; Knight & Scrutton, 1986; Lindau & Gomperts, 1991) including AtT-20 cells (Guild, 1991; Luini & DeMatteis, 1988).

Calcium ions are central to the actions of both CRF-41 and AVP. The actions of CRF-41 and AVP are both thought to be mediated by second messenger generation, activation of distinct kinases which reversibly add a phosphate onto specific target proteins and in turn stimulate calcium ion entry into the cell (a detailed account of the evidence for which is given below). The reversible addition of a phosphate by a kinase was first elucidated as a regulatory mechanism 40 years ago (Fischer & Krebs, 1955) and is now recognised as one of the most common means of regulating the function of signal transducing proteins in eukaryotic cells (for reviews see Goldsmith & Cobb, 1994; Pawson, 1994; Taylor *et al.*, 1993). There are two major classes of protein kinases; those which transfer a phosphate onto a tyrosine residue and those which do so onto a serine or threonine residue (Krebs & Beavo, 1979). Two prominent serine/threonine-specific kinases, which are activated by second

messenger action and play a central role in signal transduction, are the adenosine 3' : 5' cyclic monophosphate (cyclic AMP)-dependent protein kinase (PKA) (for reviews see Francis & Corbin, 1994; Taylor *et al.*, 1990; Walsh & Van Patten, 1994) and the calcium ion and phospholipid activated protein kinase C (PKC) (for reviews see Dekker & Parker, 1994; Hug & Sarre, 1993; Nishizuka, 1995). PKA and PKC have been implicated in mediating the abilities of CRF-41 and AVP respectively to raise the cytosolic free calcium ion concentration (a detailed account of the evidence for which is given below).

(i) Corticotropin releasing factor-41 & cyclic AMP-dependent protein kinase activation

PKA was first described by Larner and co-workers as a cyclic AMP-dependent glycogen synthase kinase that, in the presence of MgATP, transferred the γ-phosphate of ATP to serine and threonine residues on many cellular enzymes (Friedman & Larner, 1963; Rosell-Perez & Larner, 1964). Not long after, this enzyme was purified and permanently named as PKA (Walsh *et al.*, 1968). PKA is now known to be present in all mammalian tissues and is responsible for the phosphorylation and regulation of a wide variety of proteins which are involved in a wide variety of cellular functions (Francis & Corbin, 1994; Taylor *et al.*, 1990; Walsh & Van Patten, 1994). Cyclic AMP is generated from ATP by agents which activate adenylate cyclases, a family of eight isozymes, which are now known to be multiply regulated by variety of factors including guanosine 5'-triphosphate (GTP)-binding proteins (discussed in detail below), PKC and calcium ions (Cooper *et al.*, 1995; Iyengar, 1993; Tang & Gilman, 1992). Cyclic AMP is degraded by hydrolysis to adenosine 5'-monophosphate (AMP) by a large family of different cyclic nucleotide phosphodiesterases

which appear to be differentially expressed and regulated in different cell types (Beavo & Reifsnyder, 1990).

The effect of CRF-41 upon anterior pituitary corticotrophs has been the focus of many studies and as a result it is now well established that the effects of this peptide are mediated largely through the activation of PKA and subsequent elevation of cytosolic free calcium ion levels (Axelrod & Reisine, 1984). CRF-41-stimulates ACTH secretion from anterior pituitary corticotrophs via activation of specific GTP-binding protein linked receptors (Aguilera *et al.*, 1987) which have seven transmembrane spanning domains (Chen *et al.*, 1993, Perrin *et al.*, 1993). The ability of CRF-41 to stimulate ACTH secretion has been shown to be mediated by cyclic AMP generation, via activation of adenylate cyclase, both in AtT-20 cells (Litvin *et al.*, 1984; Miyazaki *et al.*, 1984) and in rat anterior pituitary corticotrophs (Aguilera *et al.*, 1983). Other agents which increase the cellular content of cyclic AMP such as forskolin, a plant diterpene capable of activating adenylate cyclase (Seamon & Daly, 1981; Seamon *et al.*, 1981), and 8-bromo cyclic AMP, a membrane permeant cyclic AMP analogue (Hei *et al.*, 1991; Meyer & Miller, 1974; Sandberg *et al.*, 1991), have also been shown to stimulate ACTH secretion from both AtT-20 cells (Axelrod & Reisine, 1984; Heisler & Reisine, 1984; Litvin *et al.*, 1984; Miyazaki *et al.*, 1984) and rat anterior pituitary corticotrophs (Aguilera *et al.*, 1983). These findings suggest that elevation of cellular cyclic AMP content and subsequent activation of PKA may be an important step in the stimulation of ACTH secretion by CRF-41. In addition catecholamines (Reisine *et al.*, 1983) and VIP (Reisine *et al.*, 1982) both stimulate ACTH secretion from AtT-20 cells, the effects of which are thought to be mediated by cyclic AMP accumulation and PKA activation (Miyazaki *et al.*, 1984) further

emphasising the importance of PKA in the stimulus-secretion coupling pathway of this cell line.

Extracellular calcium ions have been shown to be necessary for the stimulation of ACTH secretion from AtT-20 cells by secretagogues which generate cyclic AMP, however extracellular calcium is not required for cyclic AMP accumulation (Axelrod & Reisine, 1984, Miyazaki *et al.*, 1984). Furthermore CRF-41, forskolin and 8-bromo-cyclic AMP also elevate cytosolic calcium ion concentrations in AtT-20 cells (Guild *et al.*, 1986, Guild & Reisine, 1987; Luini *et al.*, 1985; Reisine & Guild, 1985). Since agents which raise the cytosolic calcium ion concentration, such as calcium ionophores, had been shown to stimulate ACTH secretion from AtT-20 cells (Richardson, 1983), it was postulated that cyclic AMP and subsequent PKA activation influence ACTH secretion via an interaction with the calcium messenger system. This was later shown to be the case with the finding that cyclic AMP, via activation of PKA, is able to increase the cytosolic free calcium ion concentration of AtT-20 cells by stimulating the entry of extracellular calcium ions by the activation of voltage-dependent calcium channels (Reisine & Guild, 1987, Reisine, 1989).

(ii) Arginine-vasopressin & protein kinase C activation

PKC was originally discovered by Nishizuka and co-workers on the basis of its activation by calcium ions, phospholipids and diacylglycerol (DAG) (Castagna *et al.*, 1982; Inoue *et al.*, 1977; Takai *et al.*, 1979). Because of these requirements PKC is generally thought to be activated in the cell by signal transduction cascades that produce DAG. PKC is also a major receptor for the tumour promoting phorbol esters, which activate PKC in a similar fashion to DAG (Gschwendt *et al.*, 1991), therefore

phorbol esters have been used extensively as a pharmacological tool to investigate the functions of PKC. Mainly through the use of phorbol esters it has been demonstrated that PKC is involved in a variety of diverse processes and, in common with PKA, phosphorylates a wide range of substrates (for reviews see Dekker & Parker, 1994; Hug & Sarre, 1993; Nishizuka, 1995). It soon became clear that PKC is not a single entity however and is now known to consist of a number of PKC isozymes (Huang *et al.*, 1986). At present the ever increasing mammalian PKC family consists of 12 closely related polypeptides designated α, β_1, β_2, γ, δ, ε, ζ, η, θ, ι, λ and μ (Dekker & Parker, 1994). Some isozymes are widespread in their distribution whereas others are restricted to a small number of tissues. In general only a certain subset of PKC isozymes are present in a particular cell (Dekker & Parker, 1994; Hug & Sarre, 1993). The PKC family can be subdivided by means of their dependence upon calcium ions into the calcium-dependent or conventional PKCs (cPKCs) and the calcium-independent or novel PKCs (nPKCs) (Ohno *et al.*, 1991). The α, β and γ isozymes belong to the cPKC subfamily and the δ, ε, η, θ and μ belong to the nPKC subfamily. A third emerging subfamily, designated atypical PKCs (aPKCs) comprising the ζ isozyme and the related ι and λ isozymes appear not to be regulated by phorbol esters or DAG (Akimoto *et al.*, 1994; Nishizuka, 1988; Selbie *et al.*, 1993). There is evidence however to suggest that members of this third subfamily are regulated by phosphatidyl inositol (3,4,5)-triphosphate (PIP_3) (Nakanishi *et al.*, 1993; Singh *et al.*, 1993). All isoforms also appear to require the membrane phospholipid phosphatidyl serine (PS) as a cofactor for their activation (Dekker & Parker, 1994; Hug & Sarre, 1993; Nishizuka, 1995). DAG is generated by a number of signal transduction cascades including that initiated by activation of phospholipase C (PLC). This enzyme upon stimulation cleaves phosphatidyl inositol (4,5) bisphosphate (PIP_2), a minor

membrane phospholipid, to produce two second messengers, DAG and phosphatidyl inositol-(3,4,5)-triphosphate (IP_3) (Divecha & Irvine, 1995). PLC can be activated by a variety of stimuli including GTP-binding proteins, a mechanism through which some hormones are thought to act (Exton, 1994).

There is now evidence to suggest that the hypothalamic neuropeptide AVP stimulates ACTH secretion from anterior pituitary corticotrophs at least partly by the activation of PKC and the elevation of the cytosolic free calcium ion concentration. The investigation of the intracellular mechanisms mediating the actions of AVP were initially hampered by the fact that although AtT-20 cells do posses a AVP receptor it is not a functional one (Lutz-Burcher *et al.*, 1987) therefore the effects of AVP upon ACTH secretion have only been investigated in heterogeneous anterior pituitary preparations. As a result these mechanisms are poorly understood in comparison to those of CRF-41. This peptide however is known to act upon membrane bound receptors belonging to the V_1 subtype similar to the AVP receptors found on smooth muscle and the liver, although pharmacological and structural differences are now known to be present between this pituitary V_1 receptor (designated V_{1b}) and the liver and vascular V_1 receptor (designated V_{1a}) (Antoni, 1987; Jard *et al.*, 1986; Lolait & Brownstein, 1994; Lolait *et al.*, 1992; Morel *et al.*, 1992). The actions of the V_1 receptor subtype are generally known to be mediated by PLC activation (Jard *et al.*, 1987) and indeed AVP has since been shown to activate PLC in anterior pituitary corticotrophs which therefore results in the generation of DAG and subsequent PKC activation as well as the generation of IP_3 (Bilezikjian *et al.*, 1987 a & b; Guillon *et al.*, 1987; Oki *et al.*, 1990; Raymond *et al.*, 1985). The importance of PKC in the ACTH stimulus-secretion coupling pathway in anterior pituitary corticotrophs is

emphasised by the finding that the effects of angiotensin II are also mediated by activation of PLC (Guillon *et al.*, 1987) and subsequent activation of PKC (Abou-Samra *et al.*, 1986). Although AtT-20 cells do not respond to AVP, lithium, an inhibitor of a particular phosphatase involved in the recycling of DAG and IP_3 to PIP_2 (Hallcher & Sherman, 1980), has been shown to be an effective secretagogue in AtT-20 cells indicating that phosphatidyl inositol metabolism is also an important second messenger pathway in the regulation of ACTH secretion from this cell line as well as the normal corticotroph (Reisine & Zatz, 1987; Zatz & Reisine, 1985). In addition activators of PKC have been shown to stimulate ACTH release from these cells indicating that this enzyme is also an important regulator of ACTH secretion from AtT-20 cells as well as the normal corticotroph (Reisine & Guild, 1987; Reisine & Zatz, 1987; Zatz & Reisine, 1985).

In common with CRF-41 the ability of AVP to stimulate a secretory response is thought to be mediated, at least partly, by the elevation of the cytosolic free calcium ion concentration. AVP has been shown to stimulate a biphasic release of ACTH from normal anterior pituitary corticotrophs, an initial spike, which may be mediated by IP_3 stimulated calcium ion mobilisation from intracellular stores, followed by what is in comparison a sustained response, mediated by the generation of DAG and subsequent PKC activation (Oki *et al.*, 1990). Studies employing the AtT-20 cell line have also demonstrated that the ability of PKC to stimulate ACTH secretion is due to an elevation of the cytosolic free calcium ion concentration. Activators of PKC appear to inhibit potassium channels resulting in a depolarisation of the cell which in turn activates voltage-dependent calcium channels allowing calcium ions to enter the cell (Reisine & Guild, 1987; Reisine, 1989).

7 MECHANISMS MEDIATING CALCIUM ION-STIMULATED ACTH SECRETION

Although it is clear that elevation of the cytosolic free calcium ion level is an important trigger to ACTH secretion and is central to the actions of both CRF-41 and AVP the mechanisms by which calcium ions in turn stimulate secretion are poorly understood. The aim of this study was to further the understanding of the mechanisms which mediate the effects of calcium ions upon ACTH secretion from anterior pituitary corticotrophs using the AtT-20 cell model system. There is evidence to suggest that a well established family of transducing proteins, termed GTP-binding proteins, may mediate the ability of calcium ions to stimulate secretion from a number of specialised secretory cells (Gomperts, 1990; Lindau & Gomperts, 1991) including ACTH secretion from AtT-20 cells (Guild, 1991; Luini & DeMatteis, 1988 & 1990). There is also some evidence to suggest that PKA and PKC, which mediate, at least partly, CRF-41- and AVP-stimulated ACTH secretion respectively, in addition to raising the cytosolic free calcium ion concentration may also regulate secretion by actions in the stimulus-secretion coupling pathway distal to changes in cytosolic free calcium ion concentrations (Guild, 1991; Guild & Reisine, 1987; Reisine, 1989). This study therefore focused upon the involvement of these two kinases and GTP-binding proteins and their involvement in calcium ion-stimulated ACTH secretion from AtT-20 cells.

(i) GTP-binding proteins

The observation by Rodbell, Birnbaumer and co-workers that a guanine nucleotide, GTP, was required for hormonal activation of adenylate cyclase (Rodbell *et al.*, 1971) ultimately resulted in the discovery

of a large, diverse and ubiquitous superfamily of GTP-binding and hydrolysing proteins which have been implicated in the regulation of a wide variety of cellular functions (for reviews see Bourne *et al.*, 1990 & 1991; Kaziro *et al.*, 1991). All GTP-binding proteins, however, have certain features in common including the ability to bind and hydrolyse GTP and the ability to undergo two alternate conformations (figure 7). The GTP-bound form is an active conformation in which the protein can recognise and interact with target molecules. Upon hydrolysis of the bound GTP to GDP and inorganic phosphate, by an intrinsic GTPase activity, the conformation is shifted to the inactive GDP-bound form. The conversion of the GDP-bound form to the GTP-bound form is achieved by the exchange of the bound GDP with external GTP. The GTP-binding protein also passes through a transient "empty" state upon releasing GDP during which no guanine nucleotide is bound. Since GTP-hydrolysis is irreversible the cycle is unidirectional. GTP binding proteins are now known to constitute a large superfamily of proteins which can be largely categorised by means of their subunit composition into a number of families including heterotrimeric and monomeric GTP-binding proteins both of which have been implicated in various aspects of the secretory process (Ferro-Novick & Novick, 1993; Nuoffer & Balch, 1994; Pfeffer, 1992).

Heterotrimeric GTP-binding proteins, or classical GTP-binding proteins, consist of an α subunit (36-52 kDa) which binds and hydrolyses GTP, as well as a β (35-37 kDa) and a γ subunit (6-10 kDa) (for reviews see Gilman, 1987; Hepler & Gilman, 1992; Neer, 1995; Taylor, 1990). Mammals have over 20 different GTP-binding protein α subunits, 16 gene products, some with alternatively spliced isoforms. Five β subunits are known which display between 53 and 90 % sequence homology in contrast to the six known γ subunits which display little sequence homology (Neer,

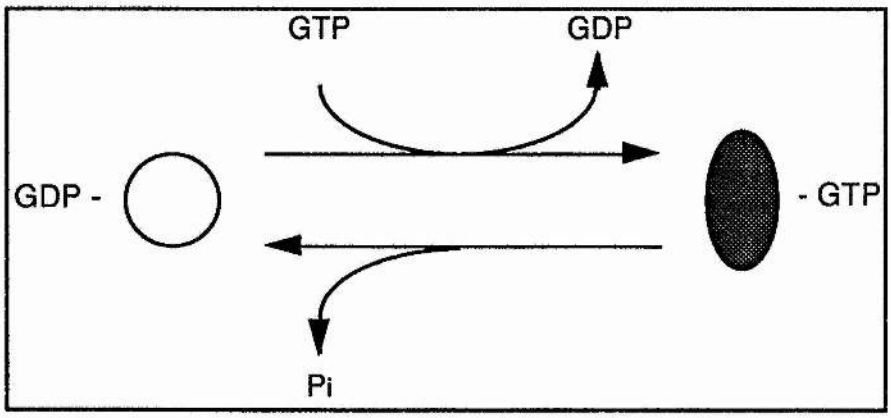

Figure 7 GTP-binding protein cycle. GDP, guanosine 5'-diphosphate; GTP, guanosine 5'-triphosphate; P_i, inorganic phosphate.

1995). Heterotrimeric GTP-binding proteins are generally subdivided by means of the amino acid sequence of their α subunit into four major subfamilies designated G_s, G_i, G_q and G_{12} which display between 56 and 95 % sequence homology (Hepler & Gilman, 1992; Neer, 1995).

In contrast monomeric GTP-binding proteins, or *ras* -related GTP-binding proteins, consist of a single 20-25 kd subunit (for reviews see Hall, 1990; Takai *et al.*, 1992). The *ras* oncogene is known to encode a 21 kDa protein that specifically binds and hydrolyses GTP (Barbacid, 1987). The designation of the *ras* gene product as a GTP-binding protein, and subsequently as the prototypical small GTP-binding protein, was made with the discovery that it displayed regions of homology with the α and γ subunits of heterotrimeric GTP-binding proteins (Hurley *et al.*, 1984). The ever increasing *ras* -superfamily of GTP-binding proteins currently contains over 60 proteins which can be divided by means of their amino acid sequence homology into the ras, rho, rab, ran and arf subfamilies (Hall, 1990; Takai *et al.*, 1992).

Heterotrimeric GTP-binding proteins are well characterised with regard to activation by membrane bound receptors (for reviews see Gilman, 1987; Hepler & Gilman, 1992; Neer, 1995; Taylor, 1990). The GTPase cycle of heterotrimeric GTP-binding proteins is depicted in figure 8. In the GDP-bound conformation, the α-subunit (Gα) binds the β and γ subunits (Gβγ), which function as a dimer, to form a heterotrimer that binds to the receptor. Both Gα and Gβγ subunits are capable of binding to the receptor. Activation of receptors on the plasma membrane by extracellular ligands induces a conformation change in the receptor which in turn induces a conformational change in Gα which results in a decreased affinity of the GTP-binding protein for GDP. As a result Gα releases GDP,

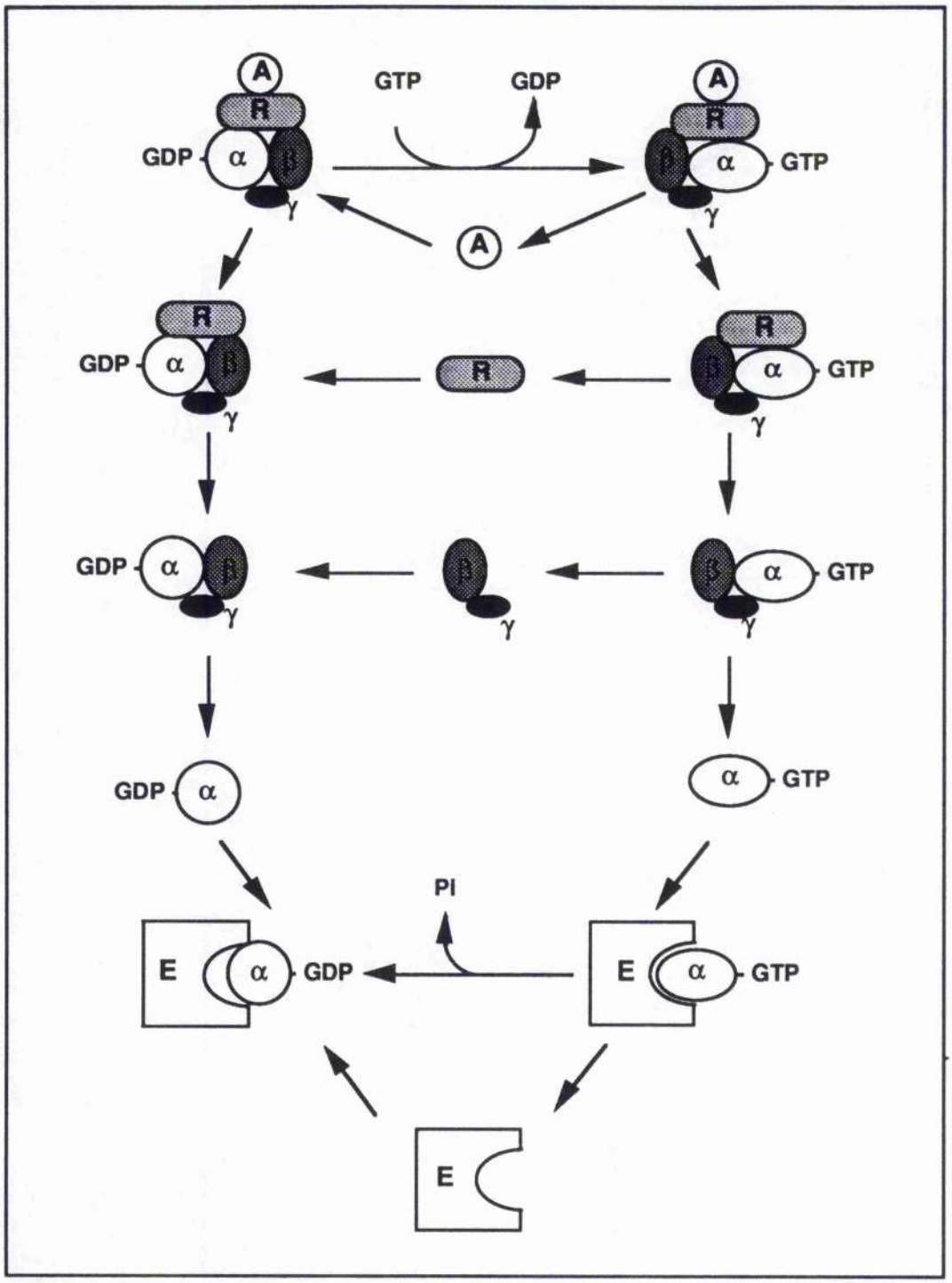

Figure 8 Heterotrimeric GTP-binding protein cycle. A, agonist (extracellular ligand) ; E, effector; GDP, guanosine 5'-diphosphate; GTP, guanosine 5'-triphosphate, P_i, inorganic phosphate; R, receptor. (Adapted from Kaziro *et al.*, 1991).

tightly associated with Gα, and because the concentration of GTP in cells is much higher, the leaving GDP is replaced with GTP. This leads to the dissociation of the receptor from the GTP-binding protein which in turn dissociates into the activated state of Gα-GTP and Gβγ. The dissociated Gα the goes on to activate target molecules. There is evidence to suggest that many Gβγ subunits are also capable of activating target molecules (for reviews see Birnbaumer, 1992; Sternweis, 1994). This activated state lasts until the bound GTP is hydrolysed to form GDP by the intrinsic GTPase action of Gα. Now Gβγ, which has a high affinity to Gα-GDP, can associate to form the Gαβγ-GDP complex, terminating the actions of both Gα and Gβγ and priming the system to respond again.

Monomeric GTP-binding proteins also cycle between an inactive GDP-bound conformation and an active GTP-bound conformation. This cycle has been postulated to be modulated by a several families of regulatory proteins (for reviews see Boguski & McCormick, 1993; Bokoch & Der, 1993; Feig, 1994). The GDP/GTP exchange reaction is extremely slow and is facilitated by a family of regulatory proteins referred to as guanine-nucleotide exchange factors (GEFs). This point in the cycle is also negatively regulated by GDP dissociation inhibitors (GDIs). One major difference between monomeric and heterotrimeric GTP-binding proteins lies in the intrinsic GTPase activity of monomeric GTP-binding proteins. The intrinsic GTPase activity of monomeric GTP-binding proteins is < 0.01 min^{-1} compared to that of heterotrimeric GTP-binding proteins which is 3-5 min^{-1}. This GTPase activity of monomeric GTP-binding proteins is therefore catalysed by another family of regulatory proteins termed GTPase activating proteins (GAPs) and may also be inhibited by GDIs.

The technique of cell permeabilisation has been employed extensively over recent years to elucidate the late stages of the stimulus-secretion coupling pathway of many specialised cell types. This technique is one which permits the composition of the cytosol to be manipulated allowing the complicating influences of membrane bound events to be bypassed. The ability of calcium ions to stimulate secretion from a variety of specialised cell types has been demonstrated using permeabilised cell preparations (Knight & Scrutton, 1986) including ACTH secretion from permeabilised AtT-20 cells (Guild, 1991; Luini & DeMatteis, 1988). GTP-binding proteins are well established as transducing proteins linking cell surface receptors to effector proteins (Bourne *et al.*, 1990 & 1991; Kaziro *et al.*, 1991) however more recently evidence has been obtained, again using this technique, to suggest that GTP-binding proteins may play more than one role in the stimulus-secretion coupling pathway. The finding by Gomperts and co-workers that GTP analogues were capable of stimulating a secretory response from permeabilised neutrophils by an action which was independent of receptor-linked events (Barrowman *et al.*, 1986) prompted the suggestion that a GTP-binding protein, subsequently designated G_e (Gomperts *et al.*, 1986), is also involved at a late stage of the stimulus-secretion coupling pathway. It is now thought that calcium ion-stimulated secretion from a number of specialised secretory cell types is mediated by this GTP-binding protein (Gomperts, 1990; Lindau & Gomperts, 1991). Initial findings from this (Guild, 1991) and other laboratories (Luini & DeMatteis, 1988 & 1990) using the non-hydrolysable GTP analogue guanosine 5'-0-(3-thiotriphosphate) (GTP- γ -S) have suggested that such a GTP-binding protein may mediate the effects of changes in the cytosolic free calcium ion concentration upon ACTH secretion from AtT-20 cells. In addition ACTH secretion is inhibited by a heterotrimeric GTP-binding protein which mediates the effects of SRIF and

also acts at a late stage in the stimulus secretion coupling pathway. This GTP-binding protein can be regarded as the inhibitory equivalent to G_e and has therefore been dubbed G_{ei} (Luini & DeMatteis, 1988 & 1990).

The first aim of this study was to confirm that previously observed effects of GTP-γ-S upon ACTH secretion from permeabilised AtT-20 cells (Guild, 1991; Luini & DeMatteis, 1988 & 1990) were due to activation of G_e and to further characterise G_e-stimulated ACTH secretion. In addition it was hoped to determine, using a number of pharmacological tools, whether this stimulatory GTP-binding protein, which mediates the effects of calcium ions upon ACTH secretion, displays characteristics consistent with it, in common with G_{ei}, being a heterotrimeric GTP-binding protein or whether it displays characteristics which suggest it may be a monomer or perhaps an as yet uncharacterised GTP-binding protein.

(ii) Cyclic AMP-dependent protein kinase & protein kinase C

The technique of cell permeabilisation has also been used to further investigate the mechanisms mediating cyclic AMP-regulated ACTH secretion. By this approach it has been demonstrated that cyclic AMP has a second action in the regulation of ACTH secretion from AtT-20 cells. Cyclic AMP has no effect upon ACTH secretion from permeabilised AtT-20 cells in the absence of cytosolic free calcium ions however it potentiates the ability of cytosolic free calcium ions to stimulate ACTH secretion (Guild, 1991). This synergistic interaction seems to suggest that cyclic AMP may also interact with the calcium ion messenger system at a stage distal to changes in cytosolic calcium ion concentrations. Thus it appears that cyclic AMP, and therefore PKA, has at least two sites of action in the ACTH stimulus-secretion coupling pathway of anterior pituitary corticotrophs both

of which involve an interaction with the calcium ion messenger system. PKA appears capable of increasing the cytosolic free calcium ion concentration and also of modulating the ability of a particular cytosolic free calcium ion concentration to stimulate ACTH secretion.

There is also evidence to suggest that PKC may also have a point of regulation in the ACTH stimulus-secretion coupling pathway which is unrelated to the elevation of cytosolic free calcium ion levels. As already discussed PKC is capable of stimulating ACTH secretion by an elevation of the cytosolic free calcium ion concentration due to an inhibition of potassium channels which in turn results in a depolarisation and an activation of voltage-dependent calcium channels (Guild & Reisine, 1987; Reisine, 1989). This action however is quickly followed by the inhibition of the voltage-dependent calcium channel and as a result the rise in cytosolic free calcium ion concentration observed in response to activation of PKC is a transient one (Guild & Reisine, 1987; Reisine, 1989). In contrast the evoked secretory response is persistent and long outlasts the changes in cytosolic free calcium ion concentrations (Guild & Reisine, 1987; Reisine, 1989) a situation quite unlike that observed with PKA-stimulated ACTH secretion. This may suggest that PKC, in common with PKA, may also have additional actions in the ACTH stimulus-secretion coupling pathway distal to changes in the concentration of cytosolic free calcium ions. The second main aim of this study was to determine using the permeabilised cell preparation whether PKC does indeed have a point of regulation in the ACTH stimulus-secretion coupling pathway which is unrelated to the elevation of cytosolic calcium ion concentration. The role of PKC at this late stage of the stimulus-secretion coupling pathway would then be compared to that of PKA.

8 OBJECTIVES

It is clear that the second messenger pathways mediating the effects of CRF-41 and AVP, as well as a variety of other secretagogues, upon ACTH secretion from anterior pituitary corticotrophs are complex. As detailed above the effects of CRF-41 and AVP are largely mediated by PKA and PKC respectively which in turn evoke ACTH secretion by raising the cytosolic free calcium ion concentration, regarded as the central trigger to hormone secretion. These mechanisms are relatively well understood, however this is not the case with regard to the mechanisms which mediate the effects of calcium ions upon the secretory apparatus. The aim of this study was therefore to investigate the mechanisms mediating calcium ion-stimulated ACTH secretion from anterior pituitary corticotrophs using the AtT-20 anterior pituitary cell line as a model system.

Initial results from the present study confirmed previous findings which suggested that G_e mediates calcium ion-stimulated ACTH secretion from AtT-20 cells (Guild, 1991). As previously mentioned both heterotrimeric and monomeric GTP-binding proteins have been implicated in various aspects of the secretory process (Ferro-Novick & Novick, 1993; Nuoffer & Balch, 1994; Pfeffer, 1992) and as a result both represent candidates for the GTP-binding protein G_e. Having confirmed the existence of this protein in AtT-20 cells it was also an aim of this study to gain evidence to suggest whether or not this GTP-binding protein belongs to the heterotrimeric or monomeric family of GTP-binding proteins. A number of pharmacological agents affecting GTP-binding protein function have been identified which display a degree of selectivity towards heterotrimeric, as opposed to monomeric, GTP-binding proteins. These include aluminium fluoride ($AlF_{(3-5)}$) which is capable of activating heterotrimeric GTP-binding

proteins by binding to GDP and mimicking the γ-phosphoryl group of GTP and thus converting the protein to the active conformation (Higashijima *et al.*, 1991). Monomeric GTP-binding proteins appear in contrast to be insensitive to $AlF_{(3-5)}$ (Kahn, 1991). Another is the amphiphilic tetradecapeptide mastoparan which has a wide variety of actions which are largely attributed to the activation of heterotrimeric GTP-binding proteins (Higashijima *et al.*, 1988 & 1990; Weingarten, 1990). In addition the bacterial exotoxins pertussis toxin (PTX) from *Bordetella pertussis* and cholera toxin (CTX) from *Vibrio cholerae* both posses mono ADP-ribosyl transferase activity. PTX has the capacity to transfer the ADP-ribose moiety of the cofactor β-nicotinamide adenine dinucleotide (NAD) onto a specific cystine residue of the α subunit of certain members of the G_i subfamily of heterotrimeric GTP-binding proteins which prevents activation of these proteins by their endogenous activators. CTX is also capable of transferring the ADP-ribose moiety from NAD in this case to a particular arginine residue on the α subunit of members of the G_s subfamily of heterotrimeric GTP-binding proteins resulting in a persistent activation of these proteins due to an inhibition of their intrinsic GTPase activity (Yamane & Fung, 1993). It was therefore decided to investigate the possibility that G_e may be a heterotrimeric GTP-binding protein by means of a pharmacological approach employing these and other related agents.

Having established that a GTP-binding protein mediates the effects of calcium ions another aim of this study was to determine what mediates the effects of G_e upon ACTH secretion. As outlined above there is evidence to suggest that PKC (Guild & Reisine, 1987; Reisine 1989) has at least one additional action in the regulation of ACTH secretion from AtT-20 cells which is unrelated to the elevation of the cytosolic free calcium ion concentration. These studies were carried out in intact AtT-20 cells and as

a result the evidence for such an action is therefore indirect. In the present study it was however possible to investigate this suggestion further using permeabilised cells which allow a more direct approach. The second main aim of this study was therefore to confirm this suggestion, again using a number of pharmacological agents, that PKC has a point of regulation in the stimulus-secretion pathways of AtT-20 cells which is distal to changes in the cytosolic free calcium ion concentration. Such an approach proved successful in the study of PKA-mediated regulation of calcium ion-stimulated ACTH secretion from AtT-20 cells (Guild, 1991). This study suggested that PKA may have a point of regulation in the ACTH stimulus-secretion coupling pathway of AtT-20 cells which is distal to changes in the cytosolic free calcium ion concentration. It was therefore also an aim of the present study to gain further evidence to implicate PKA at a stage in the ACTH stimulus-secretion coupling pathway distal to changes in the cytosolic free calcium ion concentration.

Results from the present study did indeed confirm that PKC, as well as PKA, does have a post calcium ion-point of regulation in the ACTH stimulus-secretion coupling pathway. One possibility which could not be ruled out is that one or other of these kinases may mediate the effects of this GTP-binding protein. Other explanations include the possibility that PKC or PKA may modulate calcium ion-stimulated ACTH secretion or alternatively these kinases may stimulate a secretory response which is completely independent of calcium ion/G_e-stimulated secretion. The nature of these post-calcium ion points of regulation was therefore also investigated. It was also hoped to gain evidence to suggest whether PKC and PKA act at this late stage in the stimulus-secretion coupling pathway by similar or distinct mechanisms.

As previously mentioned PKC is now known to describe a family of closely related polypeptides (Dekker & Parker, 1994). Having established that PKC has more than one point of regulation in the ACTH stimulus-secretion coupling pathway the possibility that different isozymes contribute to ACTH secretion at different points of the stimulus-secretion coupling pathway was also investigated. A number of agents have been reported to selectively activate particular PKC isozymes (Ryves *et al.*, 1991), therefore a pharmacological approach was again adopted. In particular whether or not different isozymes act at pre- or post-calcium site was determined. This was made possible once again by the use of permeabilised AtT-20 cells in which the cytosolic free calcium ion concentration can be controlled.

Taken together it was hoped that the various aspects of this study could suggest some possible mechanisms by which calcium ions, well established as the central trigger to exocytosis in AtT-20 cells, stimulate ACTH secretion. By gaining a greater knowledge of these late stages of the secretory pathway it may be possible to understand to a greater extent why corticotropin-releasing factors, in particular CRF-41 and AVP, interact in the manner that they do to regulate ACTH secretion.

METHODS

1 CULTURE OF AtT-20 CELLS

Cells of the mouse AtT-20/D16-16 anterior pituitary cell line were grown and subcultured in a growth medium of Dulbecco's modified Eagle's medium (DMEM) containing glucose (4.5 gl^{-1}) supplemented with foetal calf serum (10 % v:v), penicillin (100 units ml^{-1}) and streptomycin (100 µg ml^{-1}). The cells were cultured as a monolayer in 80 cm^2 / 260 ml flasks (Nunc, Gibco, Paisley, U.K.). Passaging required a flask of 80-90% confluent cells from which the growth medium was decanted and the cells liberated from the surface of the flask by incubation with trypsin-EDTA (0.5 g trypsin and 0.2 g EDTA per litre of modified Pucks saline A) at 37 °C. The action of this trypsin solution was terminated by the addition of 5 ml of fresh growth medium. Cells to be used in ACTH release experiments from intact cells were plated in 24-well (16 mm diameter) multiwell plates (Costar, U.S.A.) at a initial density of 10^5 cell per well in a final volume of 2 ml per well and used 5-7 days after subculturing upon reaching 80-90 % confluency. The cells were grown at 37 °C with 10 % carbon dioxide in air and 96 % humidity. Cells to be used in used in ACTH release and cyclic AMP accumulation experiments from electrically-permeabilised cells were plated in 80 cm^2 / 260 ml flasks at an initial density of approximately 2 million cells per flask in a final volume of 20 ml. The cells were grown at 37 °C with 10 % carbon dioxide in air and 96 % humidity and were used for experimental procedures 7-9 days after subculturing upon reaching 80-90 % confluency. Routinely 10-20 million cells were harvested from each flask. These procedures were carried out in aseptic conditions in a Gelaire laminar air flow hood. The growth medium and constituents as well as the trypsin-EDTA solution were kept sterile and manipulated using sterile 10 ml serological pipettes (Falcon, A. & J. Beveridge, Edinburgh, U.K.).

2 MEASUREMENT OF ACTH SECRETION FROM INTACT AtT-20 CELLS

(i) Cell preparation

The growth medium was removed, cells adhering to the substrate in each well were washed 3 times with 1 ml of Dulbecco's modified Eagle's medium supplemented with 0.1 % (w:v) bovine serum albumin (fraction V) (DMEM/BSA) then incubated for 1 h in 1 ml of fresh DMEM/BSA at 37 °C in 10 % carbon dioxide in air and 96 % humidity.

(ii) Experimental procedure

The DMEM was decanted and replaced with 1 ml of fresh DMEM / BSA supplemented with the appropriate drugs. Zero time samples were removed at this point, centrifuged (10,000 *g*, 20 s) and the supernatants removed. The remaining samples were incubated for the appropriate period at 37 °C in 10 % carbon dioxide in air and 96 % humidity. Incubations were terminated by removing the DMEM/BSA bathing the cells, centrifugation of these samples (10,000 *g*, 20 s) and removal of the supernatant. The ACTH content of the supernatants were measured by radioimmunoassay (RIA). In each experiment sextuplicate samples were run for each condition.

3 MEASUREMENT OF ACTH SECRETION FROM PERMEABILISED AtT-20 CELLS

(i) Cell preparation

The growth medium was decanted and the cells washed in 10 ml of a balanced salt solution of the following composition (mM): NaCl 145, KCl 5.6, $CaCl_2$ 2, $MgCl_2$ 0.5, glucose 5.6, HEPES 5, sodium ascorbate 0.5, pH 7.4, containing 0.1 % (w:v) bovine serum albumin (BSA). Cells were liberated from the surface of the flask by incubation with trypsin-EDTA (0.5 g trypsin and 0.2 g EDTA per litre of modified Pucks saline A) at 37 °C. The actions of this trypsin solution were terminated by adding the original growth medium and the balanced salt solution back into each flask. The cells were washed twice by centrifugation (200g, 5 min, room temperature) and resuspension in the balanced salt solution, then counted by means of a Coulter Counter and resuspended at a density of 1 million cells ml^{-1} and incubated for 30 min at 37°C. After this time the cell suspension was centrifuged (200g, 5 min, room temperature) and the cell pellet washed twice by centrifugation (200g, 5 min, room temperature) and resuspension in a standard permeabilisation medium of the following composition (mM): potassium glutamate 129, PIPES (potassium salt) 20, glucose 5, adenosine 5'-triphosphate (ATP) 5, EGTA 5, pH 6.6 containing 0.1 % (w:v) BSA. Finally after resuspension at a density of 40 million cells ml^{-1} in the standard permeabilisation medium the cells were electrically-permeabilised by subjection to intense electric fields of brief duration (Knight & Baker, 1982).

(ii) Electrical permeabilisation

AtT-20 cells were permeabilised using apparatus similar to that first described by Knight & Baker (1982), a schematic diagram of which is shown in figure 9. Cells suspended at a density of 40 million cells ml^{-1} in the basic permeabilisation medium were exposed to electric fields of brief duration by discharging a capacitor through the cell suspension. The 2 μF capacitor was charged to a known voltage by a 0-3 kV high voltage power supply, isolated from the source, and discharged through a cell suspension of 1 ml between two platinum electrodes 1 cm apart at room temperature. Routinely cell suspensions were exposed to 10 discharges each of 3 kV cm^{-1}. The effectiveness of these parameters were confirmed using the nuclear stain ethidium bromide which is normally impermeable to cells and fluorescent only when in contact with nucleic acid. This stain can therefore be used as an indicator of cell permeability. Cells were subjected to the standard permeabilisation parameters and subsequently stained with ethidium bromide (10 μg/ml) as well as the cellular stain acridine orange (3 μg/ml). Control AtT-20 cells are displayed in figure 10 while AtT-20 cells exposed to the electric fields are displayed in figure 11 both in the presence of an ethidium bromide/acridine orange mixture and viewed by means of a Leitz Dialux 20 fluorescence microscope. Of the cells exposed to the above permeabilisation parameters more than 98 % appear to be permeabilised compared to less than 5 % of the control cells. Cells subjected to the standard permeabilisation parameters remained permeable to the same extent for up to two hours.

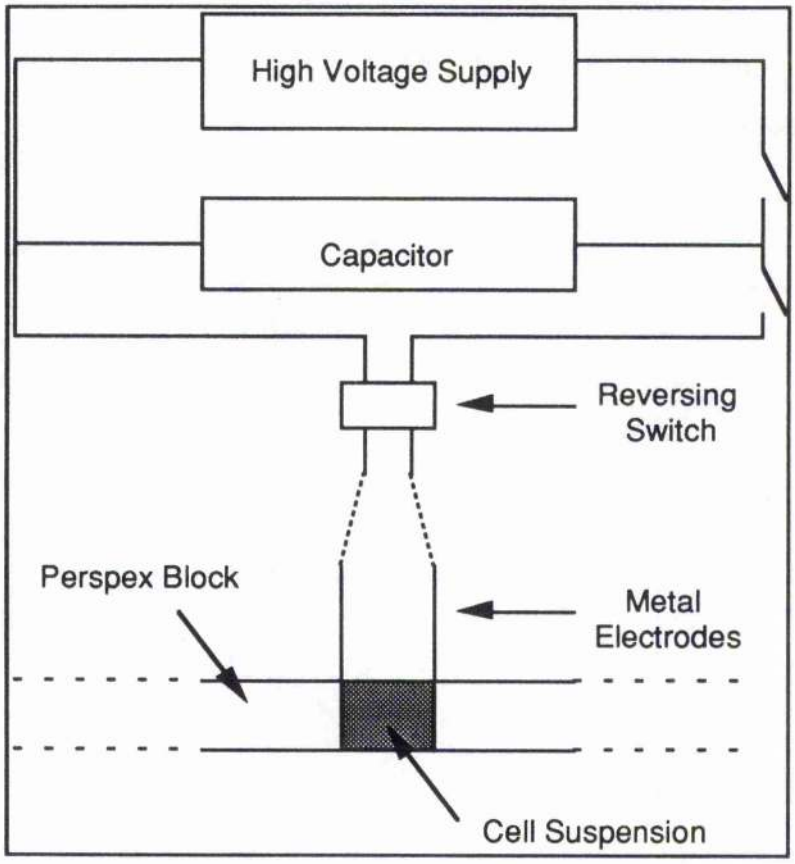

Figure 9 Schematic representation of the apparatus used to expose cell suspensions to brief electric fields. (Adapted from Knight & Baker, 1982).

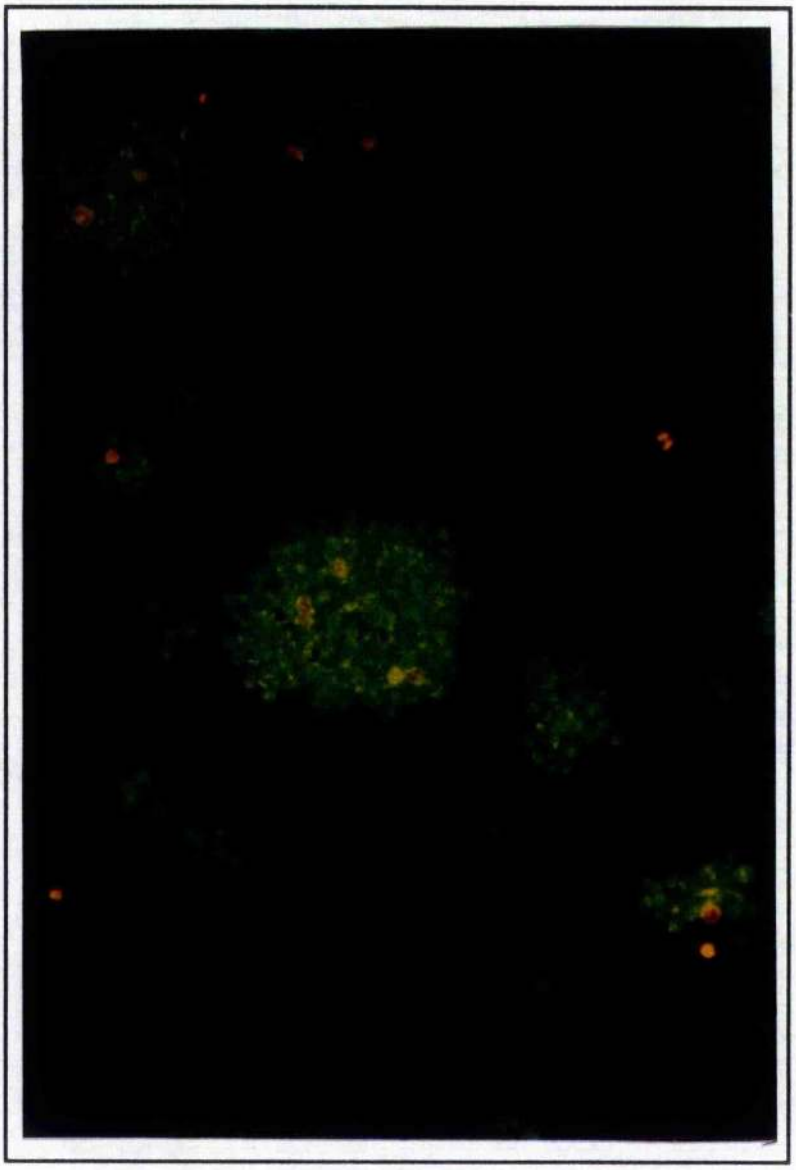

Figure 10 Control AtT-20 cells in the presence of ethidium bromide / acridine orange. Cells are viewed by means of a fluorescence microscope in the presence of ethidium bromide / acridine orange as described. When in the presence of nucleic acid the nuclear stain ethidium bromide will fluoresce red however under normal circumstances stained by acridine orange the cells will fluoresce green.

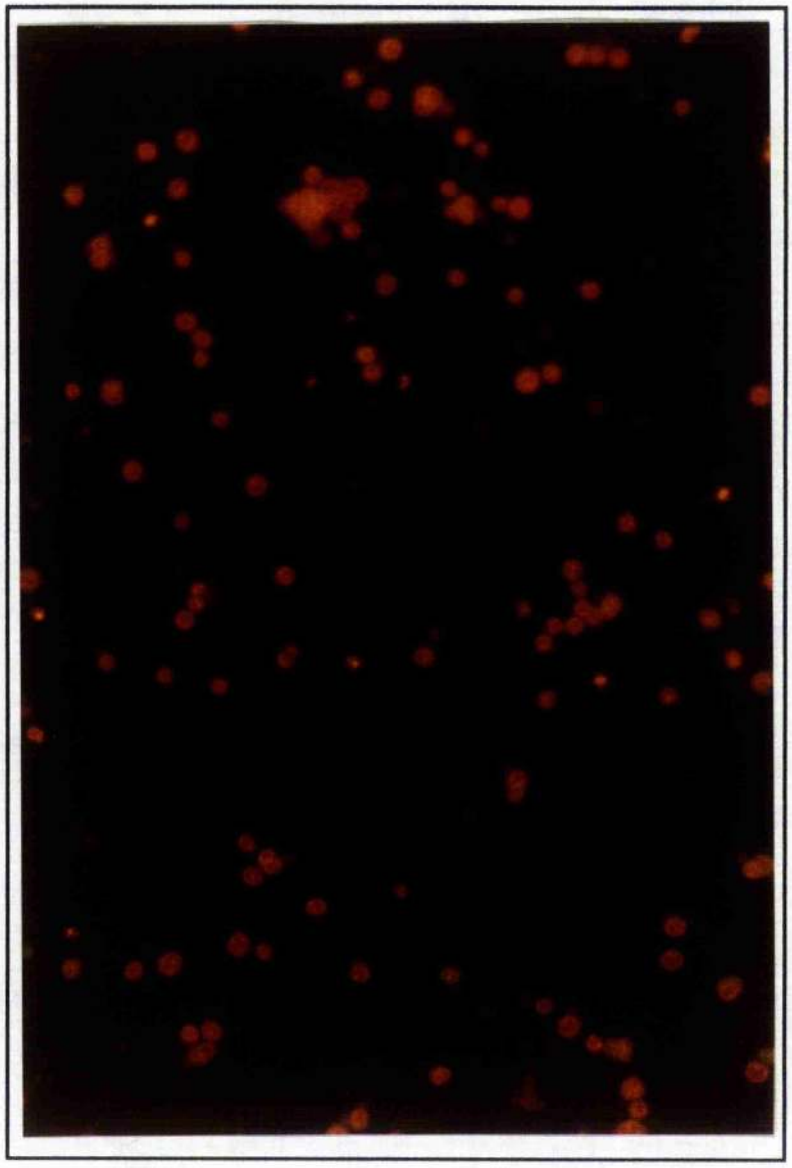

Figure 11 Permeabilised AtT-20 cells in the presence of ethidium bromide / acridine orange. Cells were exposed to 10 discharges each of 3 kV cm^{-1} and viewed by means of a fluorescence microscope in the presence of ethidium bromide / acridine orange as described. When in the presence of nucleic acid the nuclear stain ethidium bromide will fluoresce red however under normal circumstances stained by acridine orange the cells will fluoresce green.

(iii) Experimental procedure

Calcium-stimulated ACTH secretion. Permeabilised cells were suspended at a density of 10^5 cells ml^{-1} in a series of permeabilisation buffers designed to establish and maintain free calcium ion concentrations over the range of 10^{-9}M to 10^{-4}M. At this point zerotime samples were centrifuged (200 *g*, 5 min, 4 °C) and samples of the supernatant stored for subsequent measurement of ACTH content. The remaining cell suspensions were incubated at 37 °C in the presence and absence of the appropriate drugs for the appropriate incubation period at which point the incubations were terminated by centrifugation (200 *g*, 5 min, 4 °C) and samples of the supernatant stored for subsequent measurement of ACTH content by radioimmunoassay. In each experiment sextuplicate samples were run for each condition.

Calcium-independent ACTH secretion. Permeabilised cells were suspended at a density of 10^5 cells ml^{-1} in a permeabilisation buffer designed to establish and maintain a free calcium ion concentration of 10^{-9}M (effectively zero calcium). At this point zerotime samples were centrifuged (200 *g*, 5 min, 4 °C) and samples of the supernatant stored for subsequent measurement of ACTH content. The remaining cell suspensions were incubated at 37 °C in the presence and absence of the appropriate drugs for the appropriate incubation period at which point the incubations were terminated by centrifugation (200 *g*, 5 min, 4 °C) and samples of the supernatant stored for subsequent measurement of ACTH content by radioimmunoassay. In each experiment sextuplicate samples were run for each condition.

(iv) Permeabilisation buffers

A series of calcium-EGTA permeabilisation buffers were employed to establish and maintain a range of free calcium ion concentrations within the cytosol of permeabilised cells (Portzehl *et al.*, 1964). Various quantities of 1 M calcium chloride and 1 M magnesium chloride solutions were added to a basic permeabilisation medium of the following composition (mM): glutamate 129, PIPES 20, glucose 5, ATP 5, EGTA 5, pH 6.6, containing 0.1 % (w:v) BSA to give the required calcium concentration over the range of 1 nM to 1 mM and a magnesium concentration of 1 mM when in equilibrium with 5 mM EGTA and 5 mM MgATP (both present in the basic permeabilisation medium) at pH 6.6. The exact quantities of calcium chloride and magnesium chloride (standard 1M stock solutions) added to 100 ml of basic medium were calculated by a computer programme written on the basis of programmes for hand held calculators (Fabiato & Fabiato, 1979; Perrin & Sayce, 1967) and are displayed in table 1. A cytosolic free calcium ion concentration of 10^{-9}M was considered to be essentially calcium ion free and was adopted for experiments carried out in the effective absence of cytosolic free calcium ions. The calcium concentration of these buffers was checked using a calcium sensitive electrode (Russel pH Limited) which was calibrated by means of a series of calcium standards with known free calcium ion concentrations over the range of 10^{-9}M - 10^{-3}M, the composition of which are shown in table 2, as previously described (Tsien & Rink, 1980). A calibration curve was constructed in order to convert electrode potential readings obtained by the electrode into a measure of the free calcium ion concentration of the permeabilisation buffers. A typical calcium electrode calibration curve is shown in figure 12.

[free Ca^{2+}] (log M)	-9	-8	-7	-6	-5	-4	-3
$CaCl_2$ (µl / 100 ml)	0	2.0	19.7	146.0	405.0	516.0	734.0
$MgCl_2$ (µl / 100 ml)	531.0	530.0	530.0	529.0	525.0	510.0	411.0

Table 1 Calcium chloride and magnesium chloride composition of permeabilisation buffers. The indicated quantities of calcium chloride ($CaCl_2$) and magnesium chloride ($MgCl_2$) (standard 1M solutions) were added to 100 ml of basic medium as described to produce a series of permeabilisation buffers with the indicated free calcium ion concentration.

[free Ca^{2+}] (Log M)	[$CaCl_2$] (mM)	Ligand (10 mM)	[KCl] (mM)	pH buffer (10 mM)	pH
-3	1	none	98	MOPS	7.30
-4	5	NTA	90	HEPES	7.39
-5	5	NTA	90	TAPS	8.42
-6	5	HEEDTA	90	HEPES	7.70
-7	5	EGTA	90	MOPS	7.29
-8	5	EGTA	90	HEPES	7.80
-9	0	EGTA	100	HEPES	7.80

Table 2 Composition of calcium electrode calibrating solutions. Each different calibrating solution contained 10 mM of the specified pH buffer and 10 mM of the specified calcium ligand (except 1 mM calcium which contained no ligand). The pH of each solution was brought to the desired level by titration with potassium hydroxide. The final Cl^- concentration was always 100 mM. NTA, nitrilotriacetic acid.

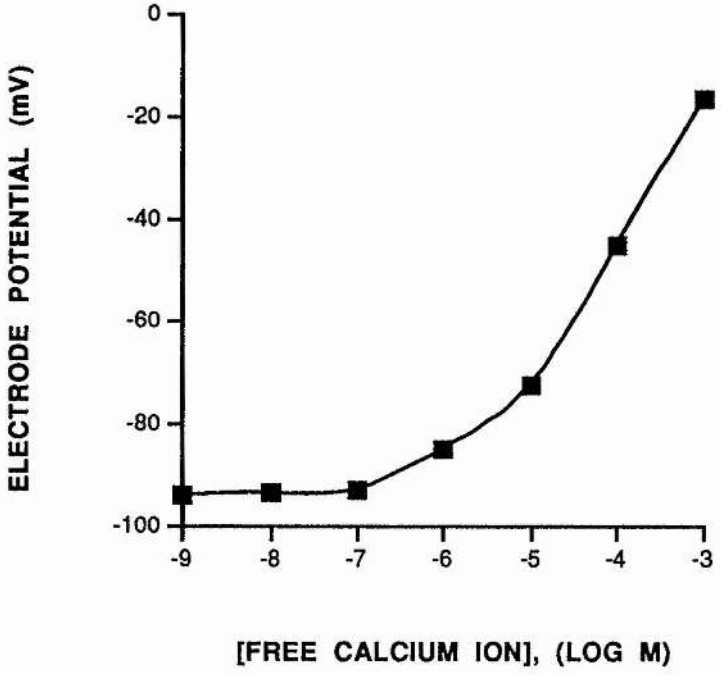

Figure 12 A representative calcium electrode standard curve. Curve shows electrode potential readings from calcium electrode calibrating solutions containing the indicated concentrations of free calcium ions. The results are expressed as the means from duplicate determinations; s.e. mean shown by the vertical bars. Absence of error bars indicate that they lie within the symbol.

4 ACTH RADIOIMMUNOASSAY

The ACTH content of experimental samples was initially determined by a radioimmunoassay based upon a method previously described by Hook *et al.* (1982). This assay relies upon the competition between ACTH, present in experimental samples, and radioactively labelled ACTH to a fixed quantity of ACTH antibody. The greater the quantity of unlabelled ACTH present the smaller the amount of labelled ACTH will be bound to the antibody, that is to say there is an inverse relationship. To each 100 µl of experimental sample containing unknown amounts of ACTH, 100 µl of rabbit anti-human ACTH serum was added at a final dilution of 1:40 000 in an ACTH-RIA buffer of the following composition; 0.02 M barbital buffer, pH 8.6, containing 0.3% (w:v) BSA and 0.2 % (v:v) 2-mercaptoethanol. This ACTH antiserum dilution also contained 0.02 % normal rabbit serum. 200 µl of [^{125}I] ACTH was also added to each sample, diluted with ACTH-RIA buffer to give approximately 10 000 counts min^{-1} in each sample. The reagents were allowed to equilibrate by overnight incubation (16-24 h) at 4°C after which time the antibody-ACTH complex was precipitated by addition of a second antibody, donkey anti-rabbit immunoglobulin G. 100 µl of this second antibody, at a dilution of 1:20 in ACTH-RIA buffer was added to each sample and again incubated overnight (16-24 h). The precipitate was pelleted by centrifugation (3200 *g* , 30 min, 4°C). The supernatant which contains unbound ACTH was removed by aspiration and the radioactivity associated with the pellet was determined by a Packard Cobra II auto gamma counter. Each sample was assayed in duplicate.

The amount of ACTH in each sample was quantified by constructing a standard curve using known concentrations of human ACTH 1-39. This hormone was diluted in ACTH-RIA buffer to give 100 µl aliquots containing

varying amounts of human ACTH 1-39 ranging from 0-5000 picogrammes, the expected range of hormone secretion. These standard ACTH dilutions, again in duplicate, were subjected to the same protocol as the experimental samples. By comparing each count in the standard curve to a sample containing only [^{125}I]-ACTH the percentage of the [^{125}I]-ACTH bound to the ACTH antiserum was calculated. Non specific [^{125}I]-ACTH binding was calculated by means of a sample containing [^{125}I]-ACTH and 200 µl ACTH-RIA buffer and was taken into consideration in calculating the percentage [^{125}I]-ACTH bound. By plotting the percentage of [^{125}I]-ACTH bound against the ACTH concentration the standard curve was obtained. A typical standard curve for this ACTH-RIA is shown in figure 13. A new standard curve was constructed with each set of experimental samples. Therefore by knowing the amount of radioactivity associated with each experimental sample the percentage of the [^{125}I]-ACTH bound can be calculated and by means of the standard curve converted to a measure of ACTH present in each experimental sample.

For unknown reasons the ACTH radioimmunoassay initially employed became highly variable. The ACTH content of experimental samples was therefore measured by an alternative radioimmunoassay based upon the previously described method of Antoni *et al.* (1983) which proved to be more reliable. To each 100 µl of experimental sample, containing unknown amounts of ACTH, 100 µl of rabbit anti human ACTH serum was added to give a final dilution of 1: 32000 in a modified ACTH-RIA buffer of the following composition; 0.05 M sodium phosphate, 6 % (w:v) polyethylene glycol 8000, 0.1% (w:v) BSA, 0.1% (v:v) triton-X 100, 2.5 mM EDTA, pH 7.4. This ACTH-antiserum dilution also contained 0.02 % (v:v) normal rabbit serum. 100 µl of [^{125}I]-ACTH was also added to each sample, diluted with modified ACTH-RIA buffer to give approximately 10

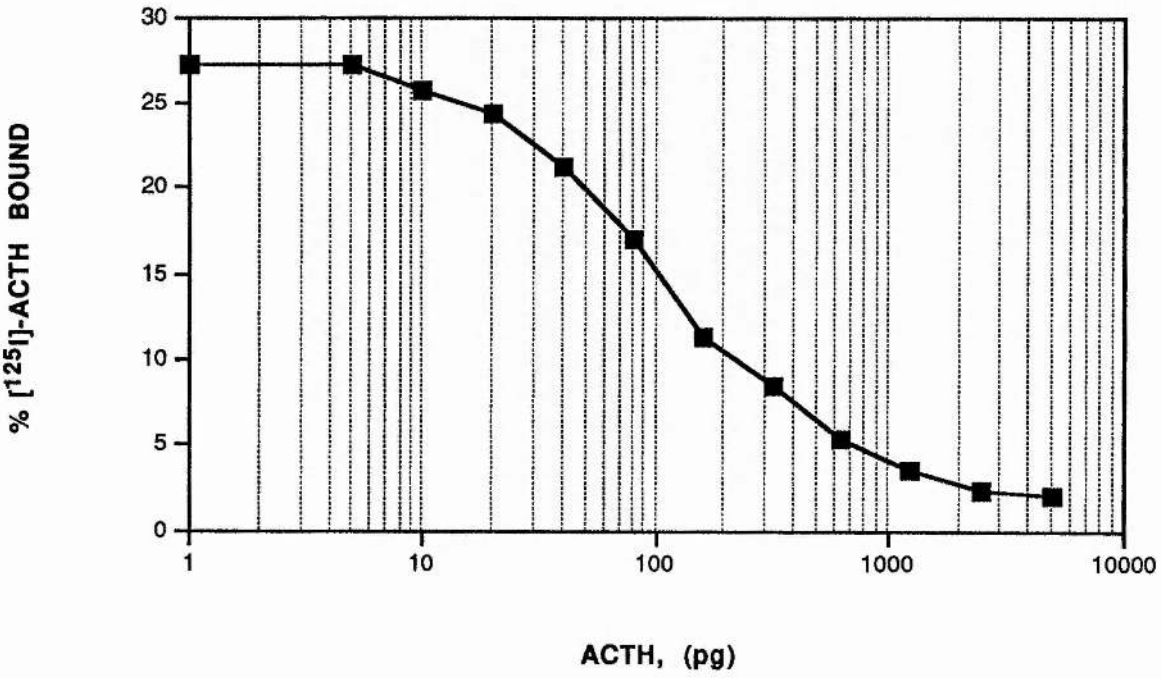

Figure 13 A representative ACTH radioimmunoassay standard curve using the original protocol. Standard concentrations of ACTH were incubated in the presence of [^{125}I]-ACTH as described. The results are expressed as the means from duplicate determinations; s.e. mean shown by the vertical bars. Absence of error bars indicate that they lie within the symbol.

000 counts min^{-1} in each tube. The reagents were allowed to equilibrate by overnight incubation (16-24 h) at 4°C. The antibody-ACTH complex was precipitated by addition of a second antibody, donkey anti-rabbit immunoglobulin G. 100 µl of this second antibody, diluted in ACTH-RIA buffer at a dilution of 1: 10 containing 0.5% (v:v) normal rabbit serum, was added to each sample and further incubated at room temperature for 3 h after which time 1 ml of 3% (w:v) polyethylene glycol 8000 was added to each tube. The precipitate was pelleted by centrifugation (3200 *g* , 30 min, 4°C). The supernatant which contains unbound ACTH was removed by aspiration and the radioactivity associated with the pellet was determined by a Packard Cobra II auto gamma counter. Each sample was assayed in duplicate. The amount of ACTH in each experimental sample was quantified by constructing a standard curve by the same method as previously described using however the modified ACTH-RIA buffer. A typical standard curve for this modified ACTH-RIA is shown in figure 14.

5 MEASUREMENT OF CYCLIC AMP ACCUMULATION FROM PERMEABILISED AtT-20 CELLS

AtT-20 cells were prepared and electrically-permeabilised as previously described. Permeabilised cells were then suspended at a density of 10^6 cells ml^{-1} in a standard permeabilisation medium designed to establish and maintain a free calcium ion concentration of 10^{-9}M (effectively zero calcium) supplemented with the appropriate drugs. The medium was also supplemented with the phosphodiesterase inhibitor 1-isobutyl 3-methylxanthine (IBMX, 10^{-3}M). At this point zero time samples were centrifuged (10,000 *g* , 20 s) and samples of the supernatant stored for subsequent measurement of cyclic AMP content. The cell suspensions

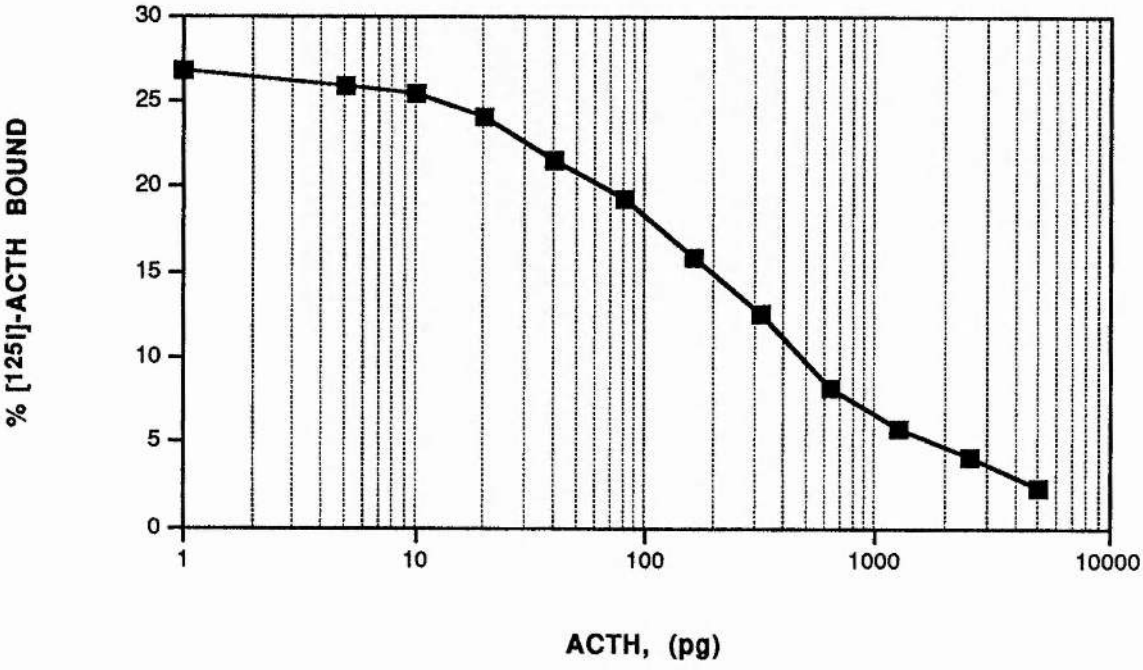

Figure 14 A representative ACTH radioimmunoassay standard curve using the modified protocol. Standard concentrations of ACTH were incubated in the presence of [^{125}I]-ACTH as described. The results are expressed as the means from duplicate determinations; s.e. mean shown by the vertical bars. Absence of error bars indicate that they lie within the symbol.

were incubated at 37 °C for the appropriate incubation period at which point the incubations were terminated by centrifugation (10,000 *g* , 20 s) and samples of the supernatant removed and stored for subsequent measurement of cyclic AMP content. In each experiment sextuplicate samples were run for each condition.

6 CYCLIC AMP RADIOIMMUNOASSAY

The cyclic AMP content of experimental samples was determined by a radioimmunoassay based upon a method previously employed to measure cyclic GMP accumulation (McCartney & Cramb, 1993). Again this assay relies upon a competition between cyclic AMP, present in experimental samples, and a radioactively labelled cyclic AMP derivative to a fixed quantity of cyclic AMP antibody. To each 100 µl of experimental sample, containing an unknown amount of cyclic AMP, 250 µl of rabbit cyclic AMP anti-serum was added at a final dilution of 1: 5 000 in a cyclic AMP-RIA buffer of the following composition; 50 mM sodium acetate, pH 4.75, containing 0.5 % (w:v) BSA. Cyclic AMP antiserum was prepared by the method of Richman *et al.* (1980) and was the gift of Dr Gordon Cramb, University of St Andrews. 100 µl [^{125}I]-succinyl cyclic AMP tyrosyl methyl ester ([^{125}I]-ScAMP-TME) was also added to each sample diluted with cyclic AMP-RIA buffer to give approximately 10 000 counts min^{-1}. The reagents were allowed to equilibrate by overnight incubation (16-24 h) at 4 °C. The antibody-cyclic AMP complex was precipitated by the addition of ice cold 96 % ethanol, vortexing and incubation at room temperature for 30 min The resulting precipitate pelleted by centrifugation (2000 *g* , 30 min, 4 °C). The supernatant was removed by aspiration and the radioactivity

associated with the pellet determined by a Packard Cobra II auto gamma counter. Each sample was assayed in duplicate.

The amount of cyclic AMP in each sample was quantified by constructing a standard curve using cyclic AMP diluted in cyclic AMP-RIA buffer to give 100 µl aliquots containing 0.0625 - 32 pmol cyclic AMP. These standard cyclic AMP dilution's, again in duplicate, were subjected to the same protocol as the experimental samples. By comparing each count in the standard curve to a sample containing only [^{125}I]-ScAMP-TME the percentage of the [^{125}I]-ScAMP-TME bound to the antibody was calculated. Non-specific [^{125}I]-ScAMP-TME binding was determined by means of a sample containing [^{125}I]-ScAMP-TME and 350 µl cyclic AMP-RIA buffer and was taken into consideration when calculating the percentage [^{125}I]-ScAMP-TME bound. By plotting the percentage of the [^{125}I]-ScAMP-TME bound to the antibody against the cyclic AMP concentration the standard curve was obtained. A typical standard curve for this cyclic AMP-RIA is shown in figure 15. A new standard curve was constructed with each set of experimental samples. Therefore by knowing the amount of radioactivity associated with the pellet of each experimental sample the percentage of the [^{125}I]-ScAMP-TME bound to the antibody can be calculated and by means of the standard curve can be converted to an accurate measure of cyclic AMP present in each experimental sample.

7 IODINATIONS

[^{125}I] labelled ACTH for radioimmunoassay use was produced using the Iodo-gen reagent (1,3,4,6-tetrachloro-3α,6α-diphenyl glycouril) which was first described as a reagent for iodination by Fraker and Speck (1978).

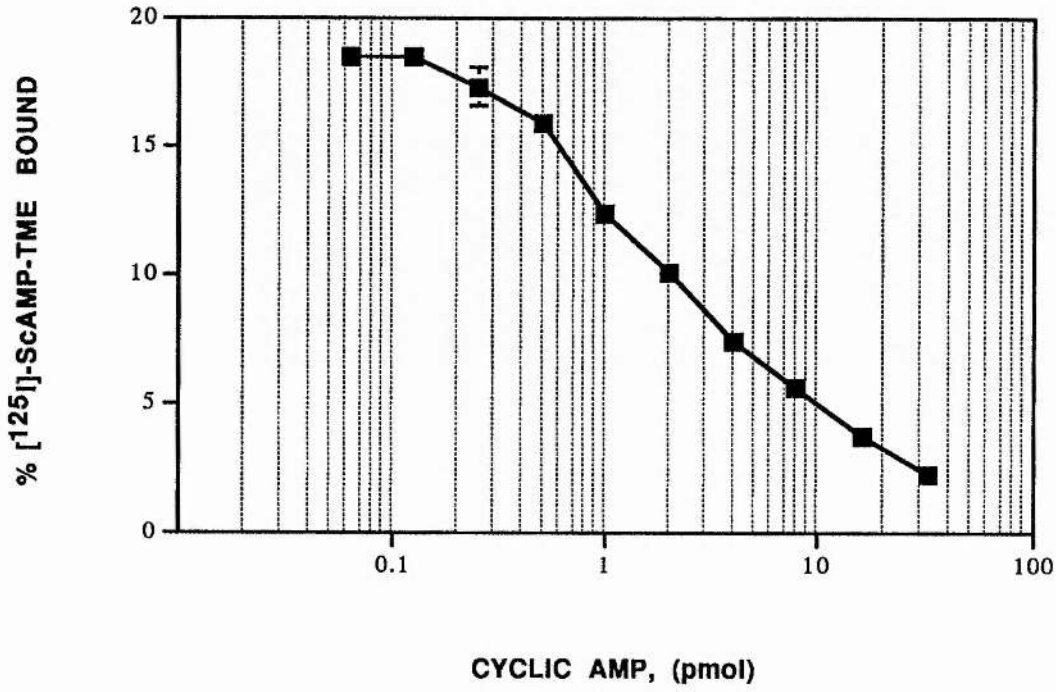

Figure 15 A representative cyclic AMP radioimmunoassay standard curve. Standard concentrations of cyclic AMP were incubated in the presence of [^{125}I]-succinyl cyclic AMP tyrosyl methyl ester as described. The results are expressed as the means from duplicate determinations; s.e. mean shown by the vertical bars. Absence of error bars indicate that they lie within the symbol.

5 mg of Iodo-gen was diluted in 25 ml of chloroform and divided into 50 µl aliquots (i.e. 10 µg Iodo-gen/tube). The tubes were placed in a water bath at 37°C and evaporated to dryness allowing the tubes to become coated with Iodo-gen. Standard human ACTH 1-39 was diluted in a 0.5 M sodium phosphate buffer pH 6.0-7.4 to give a final ACTH concentration of 6×10^{-6}M. 40 µl of this ACTH was added to an iodogen coated tube along with approximately 1 mCi of Na [^{125}I] and allowed to react for 30 min at room temperature. During this time the reagent iodinates exposed tyrosine residues by an oxidative reaction. The reaction was halted by adding 1 ml of 0.1 % (v:v) trifluoroacetic acid. An octyl C8 amprep column (Amersham, Buckinghamshire, U.K.) was prepared by washing with 4 ml methanol followed by 4 ml distilled water, the iodinated ACTH sample added and washed again with 6 ml 0.1 % (v:v) trifluoroacetic acid. 2 ml of 10, 20, 30, 40, 50, and 60 % (v:v) acetonitrile each containing 0.1 % (v:v) trifluoroacetic acid were passed through the cartridge in turn and the six eluted samples containing varying amounts of iodinated ACTH collected. 1 ml of methanol was added to each of these samples. The amount of radioactivity incorporated into each eluted sample was determined by counting a fraction of each sample in a Packard Cobra II auto gamma counter. The 30-40 % acetonenitrile solutions produced the eluted samples which gave the highest degree of radioactivity incorporation and therefore optimum results in the radioimmunoassay. A typical elution profile is displayed in figure 16. [^{125}I]-succinyl cyclic AMP tyrosyl methyl ester was produced by a similar method and was the gift of Dr Gordon Cramb, University of St Andrews.

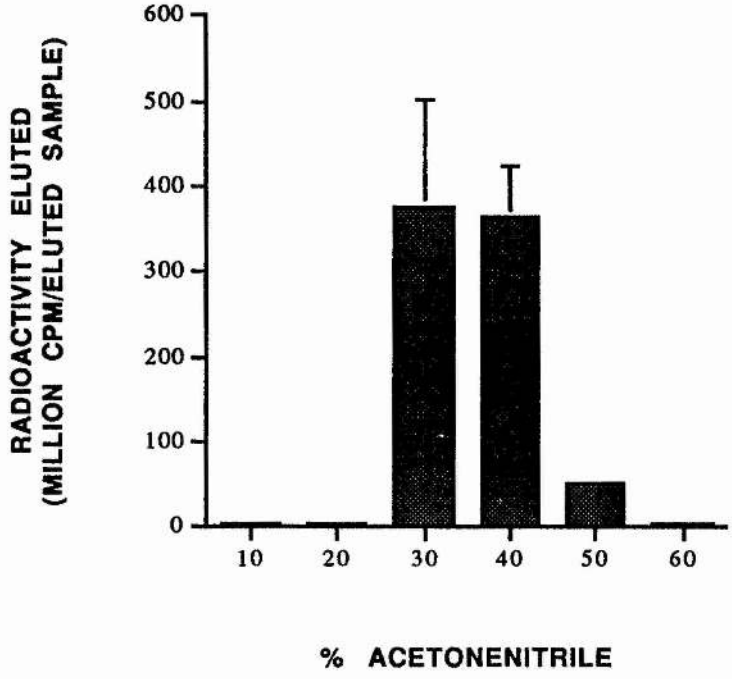

Figure 16 [^{125}I] ACTH elution profile. ACTH was iodinated and eluted from an octyl C8 cartridge (Amersham) by the indicated concentration of acetonitrile as described. The results are expressed as the mean ± s.e. mean from three separate iodinations. Absence of error bars indicate that they lie within the columns.

8 STATISTICAL ANALYSIS

In each experiment sextuplicate determinations at each experimental condition were made and each experiment was repeated three times on different days. ACTH secretion is expressed as the mean ± s.e. mean. Statistical significance was determined by means of either a paired t-test or in cases of multiple comparisons an ANOVA test with Scheffe's F-test post hoc analysis.

9 MATERIALS & ABBREVIATIONS

The following substances (with their sources) were used: Dulbecco's modified Eagle's medium containing glucose 4.5 gl^{-1} (DMEM), foetal calf serum, normal rabbit serum, penicillin (5000 units/ml)/ streptomycin (5000 µg/ml) and trypsin-EDTA (0.5 g trypsin and 0.2 g EDTA per litre of modified Pucks saline A) from Gibco, Paisley, U.K.; adenosine 3' : 5' cyclic monophosphate (cyclic AMP), adenosine 5'-triphosphate (ATP), aluminium chloride, barbital (sodium salt), bovine serum albumin (BSA) (fraction V) , cholera toxin (CTX), EDTA (ethylenediamine-tetraacetic acid), forskolin, glutamate (monopotassium salt), EGTA (ethylene glycol-bis(β-aminoethyl ether) N, N, N', N'-tetra acetic acid), HEEDTA (N-hydroxyethylethylenediaminetriacetic acid), HEPES (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]), 1-isobutyl 3-methylxanthine (IBMX), mastoparan (Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2), 2-mercaptoethanol, MOPS (3-[N-morpholino]propanesulfonic acid), β-nicotinamide adenine dinucleotide (NAD), nitrilotriacetic acid (NTA), pertussis toxin (PTX), 4α phorbol, phorbol 12, 13-dibutyrate (PDBu),

phorbol 12, 13-didecanoate (PDD), phorbol 12-myristate 13-acetate (PMA), polyethylene glycol 8000, PIPES (piperazine-N-N'-bis-[2-ethanesulfonic acid]), potassium fluoride, sodium fluoride, sodium phosphate (monosodium phosphate), TAPS (N-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid), and Triton X-100 (t-octylphenoxypolyethoxyethanol) from Sigma, Poole, U.K.; calyculin A, chelerythrine chloride, 12-deoxyphorbol 13-phenylacetate 20-acetate (dPPA), 1,2-dioctanoyl-sn-glycerol (DOG), 1-oleoyl-2-acetyl-sn-glycerol (OAG) and thymeleatoxin (TMX) from L.C. Laboratories distributed by Calbiochem-Novabiochem, Nottingham, U.K.; guanosine 5'-O-(2-thiodiphosphate) (GDP- β -S) and guanosine 5'-O-(3-thiotriphosphate) (GTP- γ-S) from Boehringer Mannheim, Lewes, U.K.; mas-7 (Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Ala-Leu-Leu-NH_2), Corticotrophin releasing factor (CRF-41, human / rat), mas-17 (Ile-Asn-Leu-Lys-Ala-Lys-Ala-Ala-Leu-Ala-Lys-Lys-Leu-Leu-NH_2) and [D-trp^8]-somatostatin (SRIF) from Peninsula Laboratories, Belmont, U.S.A.; anti-rabbit immunoglobulin G was the gift of the Scottish Antibody Production Unit, Carluke, U.K.; human ACTH antiserum and standards were the gift of the National Hormone and Pituitary programme, N.I.A.D.D.K., N.I.H., U.S.A.; cyclic AMP antiserum and standards and [^{125}I]-succinyl cyclic AMP-tyrosyl methyl ester ([^{125}I]-ScAMP-TME) were the gift of Dr Gordon Cramb, University of St Andrews, U.K.; Iodo-gen reagent from Pierce & Warriner, Chester, U.K.; radioisotopes from Dupont, Stevenage, U.K. All other chemicals were of analar grade and readily commercially available.

RESULTS

1 AN INVESTIGATION INTO THE INVOLVEMENT OF GTP-BINDING PROTEINS MEDIATING CALCIUM ION-STIMULATED ACTH SECRETION FROM AtT-20 CELLS

(i) Effects of calcium ions upon the late stages of the ACTH secretory pathway of AtT-20 cells

Calcium ions stimulated ACTH secretion from permeabilised AtT-20 cells, over a period of 30 min, in a concentration-dependent manner becoming significantly greater than control secretion (defined here and subsequently as ACTH secretion in standard permeabilisation medium in the absence of added secretagogues) at concentrations above 10^{-6}M with an EC_{50} of $1.6 \pm 0.3 \times 10^{-6}$M (figure 17). A 30 min incubation period has previously been shown to be suitable for the investigation of calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells (Guild, 1991) and therefore was adopted for all experiments investigating calcium ion-stimulated ACTH secretion. A free calcium ion concentration of 10^{-5}M was found to be maximal with regard to ACTH secretion. ACTH secretion in response to a free calcium ion concentration of 10^{-5}M was 218 ± 17 pg/10^5 cells/30 min compared to control secretion which was 50 ± 3 pg/10^5 cells/30 min.

(ii) Effects of GTP-γ-S upon the late stages of the ACTH secretory pathway of AtT-20 cells

The involvement of GTP-binding proteins in the late stages of the ACTH secretory pathway in AtT-20 cells was investigated using the non-hydrolysable GTP analogue GTP-γ-S. A suitable incubation period for the investigation of GTP-γ-S upon ACTH secretion from permeabilised AtT-20

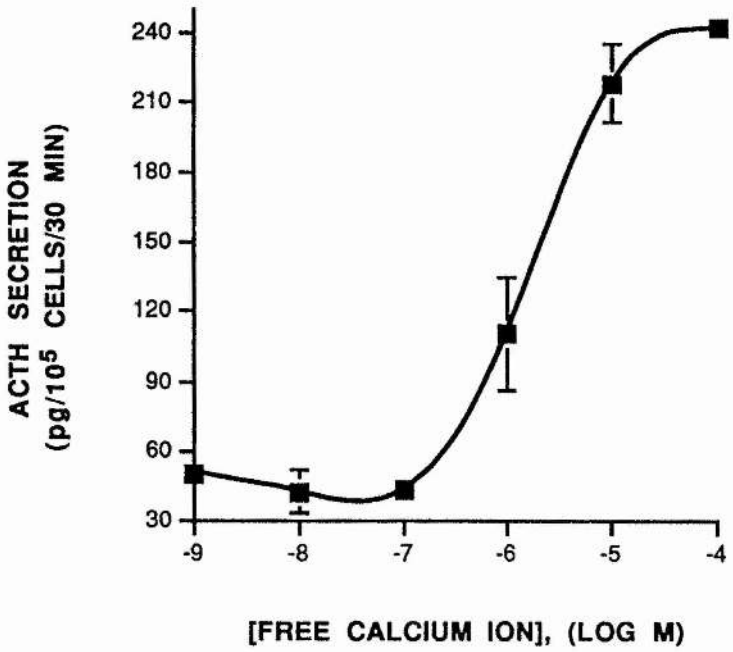

Figure 17 Effect of calcium ions upon ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated for 30 min as described in the methods in a series of calcium/EGTA buffers such that the indicated cytosolic free calcium ion concentration resulted and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

cells was initially determined. ACTH was secreted in a time-dependent manner from permeabilised AtT-20 cells in standard permeabilisation medium in the absence of added secretagogues becoming significant after 60 min (figure 18). GTP- γ -S (10^{-4}M) enhanced this control secretion in a time-dependent manner becoming significantly greater than control secretion after 20 min (figure 18). Secretion in response to GTP- γ -S after 30 min was 60 $\pm$ 2 pg/10^5 cells compared to a control secretion which was 40 $\pm$ 2 pg/10^5 cells after the same time period. A standard incubation period of 30 min was chosen for all subsequent experiments in which GTP- γ -S-stimulated ACTH secretion from permeabilised AtT-20 cells was being investigated. GTP- γ -S stimulated ACTH secretion from permeabilised AtT-20 cells in standard permeabilisation medium in a concentration-dependent manner becoming significantly greater than control secretion at concentrations above 10^{-6}M (figure 19). ACTH secretion in response to GTP- γ -S (10^{-4}M) was 98 $\pm$ 8 pg/10^5 cells/30 min compared to control secretion which was 33 $\pm$ 3 pg/10^5 cells/30 min. The ability of GTP- γ -S to stimulate ACTH secretion in standard permeabilisation medium, which contains effectively no calcium ions, suggests that a GTP-binding protein (or proteins) contributes to ACTH secretion from AtT-20 cells at a stage in the stimulus-secretion coupling pathway at a stage distal to changes in cytosolic calcium ion levels. In the present study higher concentrations of GTP- γ -S proved too expensive therefore complete concentration response curves were unfortunately not obtained.

Having established that calcium ions and GTP- γ -S both stimulate ACTH secretion from permeabilised AtT-20 cells the possibility that they do so via a common mechanism, i.e. by the activation of a GTP-binding protein, was investigated by studying the effects of these two secretagogues in combination. Calcium ions stimulated ACTH secretion

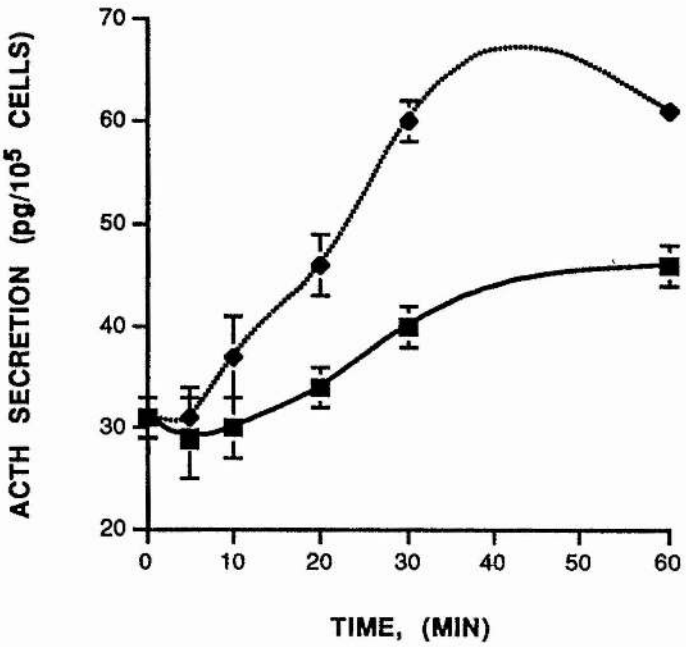

Figure 18 Time course of GTP- γ -S-stimulated ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated for the indicated time periods as described in the methods in standard permeabilisation medium in the presence (◆) or absence (■) of GTP- γ -S (10^{-4}M) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

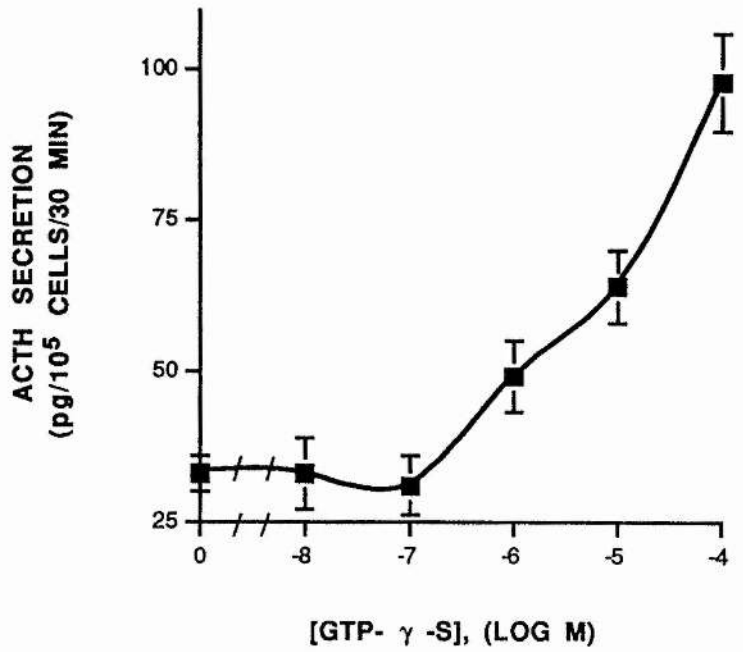

Figure 19 Effect of GTP- γ-S upon ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated as described in the methods in standard permeabilisation medium for 30 min supplemented with the indicated concentration of GTP- γ-S and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments.

from permeabilised AtT-20 cells in a concentration-dependent manner becoming significantly greater than control secretion at concentrations above 10^{-7}M (figure 20). GTP- γ - S (10^{-5}M) significantly stimulated ACTH secretion in standard permeabilisation medium (free calcium ion concentration of 10^{-9}M), however in contrast was unable to enhance ACTH secretion observed in response to a free calcium ion concentration of 10^{-5}M (figure 20). ACTH secretion in standard permeabilisation medium was 57 $\pm$ 6 pg/10^5 cells/30 min in the absence of GTP- γ -S compared to 159 $\pm$ 5 pg/10^5 cells/30 min in the presence of GTP- γ -S. ACTH secretion in a free calcium ion concentration of 10^{-5}M was 216 $\pm$ 29 pg/10^5 cells/30 min in the absence of GTP- γ -S compared to 208 $\pm$ 18 pg/10^5 cells/30 min in the presence of GTP- γ -S. These results demonstrate that GTP- γ -S is able to stimulate ACTH secretion from permeabilised AtT-20 cells in the effective absence of calcium ions however is unable to enhance calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells. This convergence may suggest that the effects of calcium ions and GTP- γ -S are mediated by a common mechanism i.e. the GTP-binding protein G_e.

(iii) Mechanisms mediating calcium ion- and G_e-stimulated ACTH secretion from AtT-20 cells

Chelerythrine chloride is a potent and selective inhibitor of PKC (Herbert *et al.*, 1990) which was employed in the present study to investigate the possibility that activation of PKC mediates calcium ion- and GTP- γ -S-stimulated ACTH secretion from permeabilised AtT-20 cells. Calcium ions stimulated ACTH secretion from permeabilised AtT-20 cells in a concentration-dependent manner becoming significantly greater than control secretion at concentrations above 10^{-6}M (figure 21). This calcium ion-stimulated secretion was not significantly different in the presence and

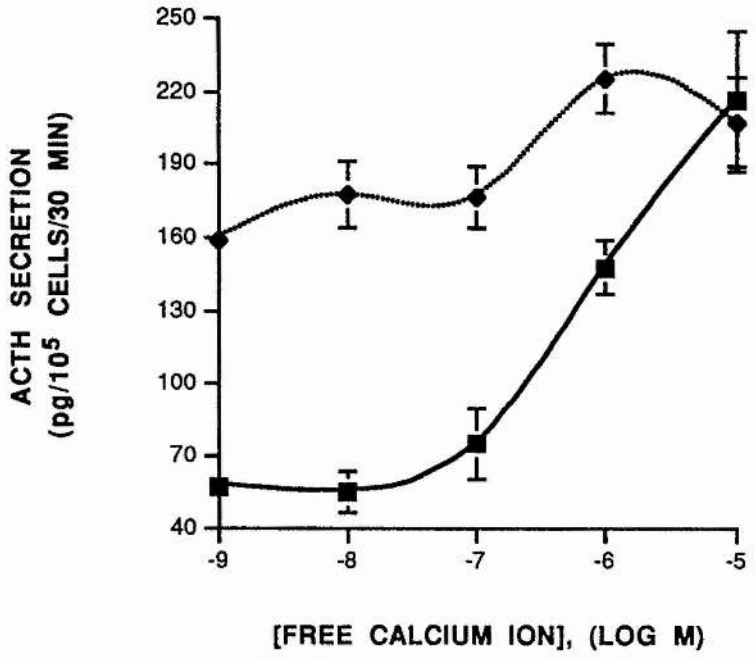

Figure 20 Effect of GTP- γ-S upon calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated for 30 min as described in the methods in a series of calcium/EGTA buffers such that the indicated cytosolic free calcium ion concentration resulted in the presence (◆) and absence (■) of GTP- γ-S (10^{-5}M) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

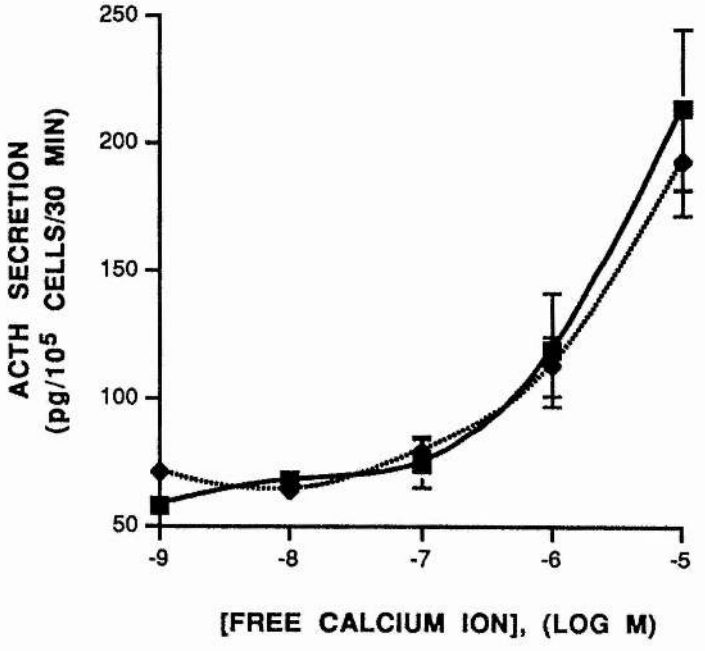

Figure 21 Effect of chelerythrine chloride upon calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated for 30 min as described in the methods in a series of calcium/EGTA buffers such that the indicated cytosolic free calcium ion concentration resulted in the presence (◆) and absence (■) of chelerythrine chloride (10^{-5}M) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

absence of chelerythrine chloride (10^{-5}M) (figure 21). ACTH secretion in response to a free calcium ion concentration of 10^{-5}M was 213 ± 32 pg/10^5 cells/30 min in the absence of chelerythrine chloride compared to 193 ± 21 pg/10^5 cells/30 min in the presence of chelerythrine chloride. GTP- γ -S stimulated ACTH secretion from permeabilised AtT-20 cells in standard permeabilisation medium in a concentration-dependent manner becoming significantly greater than control secretion at concentrations above 10^{-6}M (figure 22). This GTP- γ -S-stimulated ACTH secretion cells was also not significantly different in the presence and absence of chelerythrine chloride (10^{-5}M) (figure 22). ACTH secretion in response to GTP- γ -S (10^{-4}M) was 248 ± 37 pg/10^5 cells/30 min in the absence of chelerythrine chloride compared to 246 ± 72 pg/10^5 cells/30 min in the presence of chelerythrine chloride. Results from the present study indicate that chelerythrine chloride (10^{-5}M) is capable of completely inhibiting the effects of PKC activators in AtT-20 cells under similar conditions (a detailed account of this positive control can be found on p73) therefore the inability of chelerythrine chloride to inhibit calcium ion- and GTP- γ -S-stimulated secretion suggests that neither of these secretagogues stimulate ACTH secretion from permeabilised AtT-20 cells via activation of PKC.

As discussed above the enzyme responsible for the generation of cyclic AMP, adenylate cyclase, can be regulated by calcium ions and by GTP-binding proteins (Cooper *et al.*, 1995; Iyenger, 1993; Tang & Gilman, 1992). In addition the present study demonstrated that GTP- γ -S (10^{-4}M) was capable of stimulating cyclic AMP accumulation (table 3). The possibility that the effects of calcium ions and GTP- γ -S upon ACTH secretion from permeabilised AtT-20 cells were mediated by activation of adenylate cyclase and subsequent generation of cyclic AMP was investigated. Cyclic AMP (10^{-4}M) had no significant effect upon ACTH

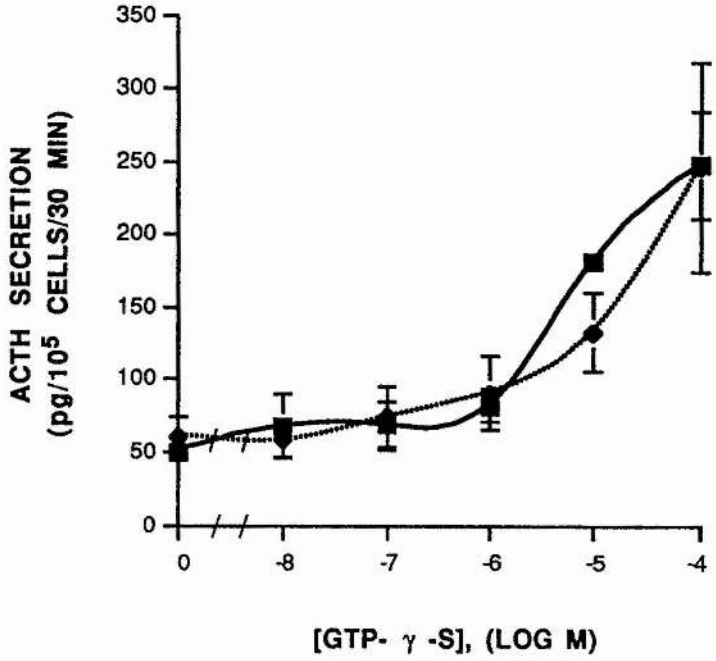

Figure 22 Effect of chelerythrine chloride upon GTP- γ -S-stimulated ACTH secretion from permeabilised AtT-20 cells.
Permeabilised cells were incubated for 30 min as described in the methods in standard permeabilisation medium in the presence of the indicated concentration of GTP- γ -S in the presence (◆) and absence (■) of chelerythrine chloride (10^{-5}M) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

	CYCLIC AMP ACCUMULATION (pmol/10^5 CELLS/30 MIN)
CONTROL	1.3 ± 1.0
GTP-γ-S (10^{-4}M)	15.0 ± 3.5

Table 3 Effect of GTP-γ-S upon cyclic AMP accumulation from permeabilised AtT-20 cells. Permeabilised cells were incubated as described in the methods in standard permeabilisation medium for 30 min in the presence and absence of GTP-γ-S (10^{-4}M) as indicated and cyclic AMP accumulation measured by radioimmunoassay. The results are expressed as the mean $\pm$ s.e. mean from three separate experiments.

secretion from permeabilised AtT-20 cells in standard permeabilisation medium over a period of 30 min (figure 23). ACTH secretion was 90 ± 16 pg/10^5 cells/30 min in the presence of cyclic AMP compared to control ACTH secretion which was 68 ± 11 pg/10^5 cells/30 min. In addition the effects of agents, the effects of which are already known to be mediated by cyclic AMP, upon ACTH secretion were investigated. The diterpene forskolin, from the roots of *Coleus forskohlii* is a potent activator of adenylate cyclase and is thus capable of stimulating the generation of cyclic AMP (Seamon *et al.*, 1981; Seamon & Daly, 1981). As discussed above the effects of CRF-41 upon ACTH secretion are also mediated by cyclic AMP generation. Forskolin (10^{-5}M) (figure 24) and CRF-41 (10^{-7}M) (figure 25) both significantly stimulated cyclic AMP accumulation from permeabilised AtT-20 cells in standard permeabilisation medium in the presence of IBMX (10^{-3}M) over a period of 30 min. In contrast forskolin (10^{-5}M) (figure 26) and CRF-41 (10^{-7}M) (figure 27) both had no significant effect upon ACTH secretion from permeabilised AtT-20 cells in standard permeabilisation medium over a period of 30 min. Thus it appears that cyclic AMP and agents which stimulate ACTH secretion by raising cyclic AMP levels are unable to stimulate ACTH secretion from permeabilised AtT-20 cells in standard permeabilisation medium. It can therefore be assumed that calcium ion- and GTP- γ -S-stimulated ACTH secretion from permeabilised AtT-20 cells are not mediated by the generation of cyclic AMP and subsequent activation of PKA and that these agents must stimulate ACTH secretion from AtT-20 cells by a different mechanism of action.

GTP- γ -S-stimulated ACTH secretion from permeabilised AtT-20 cells was found to be independent of ATP, normally present in the permeabilisation medium at a concentration of 5 x 10^{-3}M. GTP- γ -S

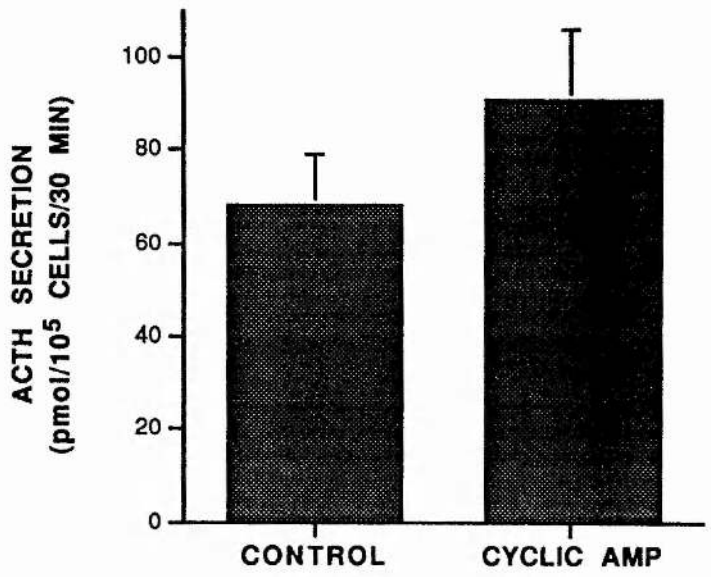

Figure 23 Effect of cyclic AMP upon ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated as described in the methods in standard permeabilisation medium for 30 min in the presence and absence of cyclic AMP (10^{-4}M) as indicated and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments.

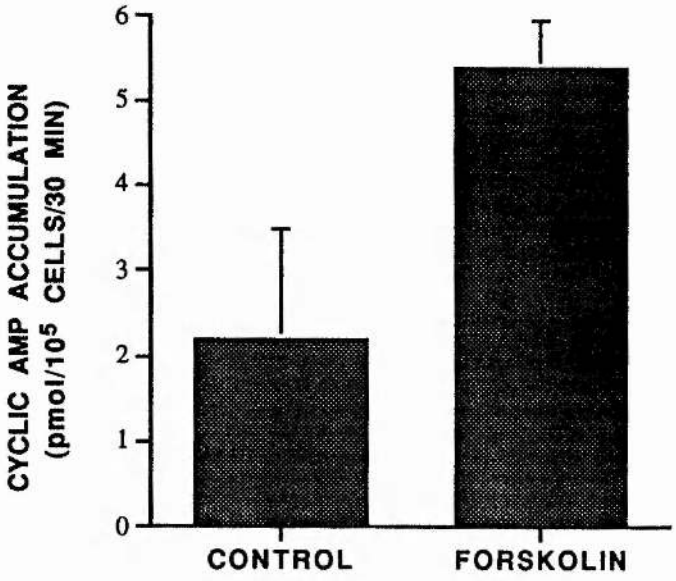

Figure 24 Effect of forskolin upon cyclic AMP accumulation from permeabilised AtT-20 cells. Permeabilised cells were incubated as described in the methods in standard permeabilisation medium for 30 min in the presence and absence of forskolin (10^{-5}M) as indicated and cyclic AMP accumulation measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments.

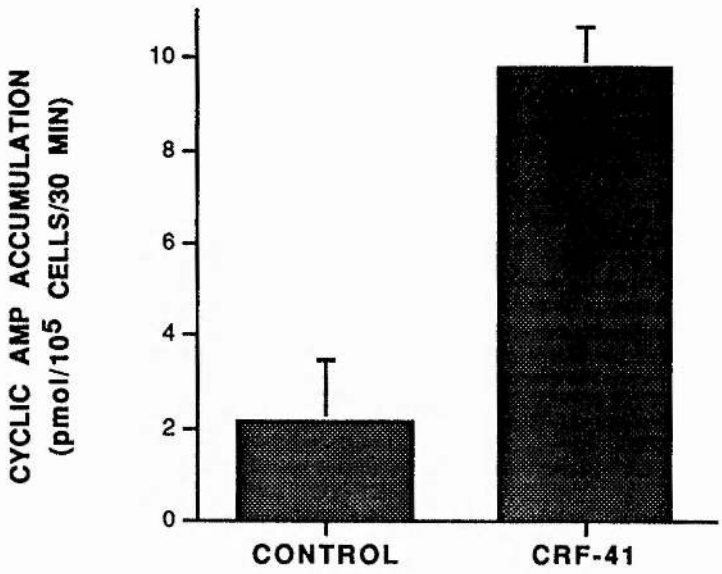

Figure 25 Effect of CRF-41 upon cyclic AMP accumulation from permeabilised AtT-20 cells. Permeabilised cells were incubated as described in the methods in standard permeabilisation medium for 30 min in the presence and absence of CRF-41 (10^{-7}M) as indicated and cyclic AMP accumulation measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments.

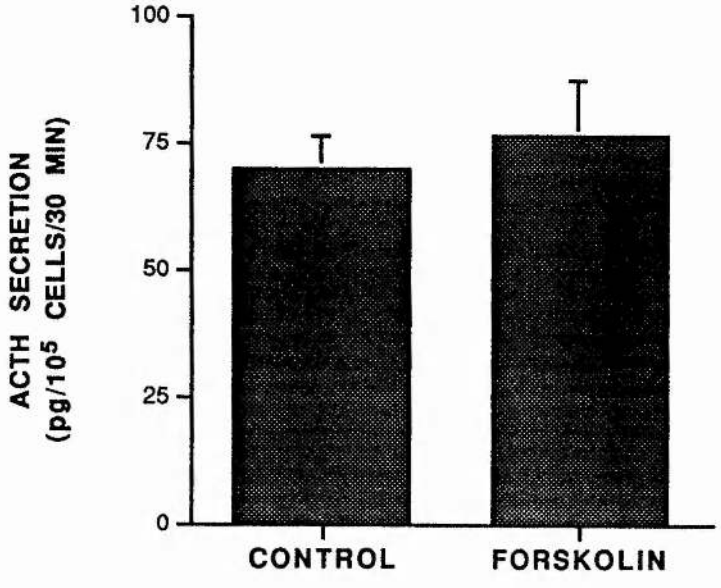

Figure 26 Effect of forskolin upon ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated as described in the methods in standard permeabilisation medium for 30 min in the presence and absence of forskolin (10^{-5}M) as indicated and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments.

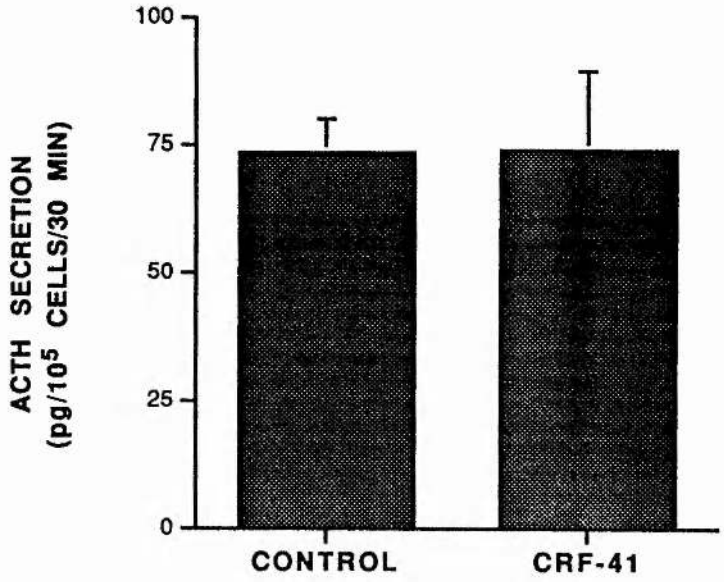

Figure 27 Effect of CRF-41 upon ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated as described in the methods in standard permeabilisation medium for 30 min in the presence and absence of CRF-41 (10^{-7}M) as indicated and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments.

stimulated ACTH secretion from permeabilised AtT-20 cells in standard permeabilisation medium in a concentration-dependent manner (figure 28) becoming significantly greater than control secretion at concentrations above 10^{-6}M. Omitting ATP from the permeabilsation medium had no significant effect upon this GTP- γ -S-stimulated ACTH secretion at every concentration of the guanine nucleotide investigated (figure 28). ACTH secretion in response to GTP- γ -S (10^{-4}M) was 96 $\pm$ 12 pg/10^5 cells/30 min in the presence of ATP compared to 91 $\pm$ 15 pg/10^5 cells/30 min in the absence of ATP. Since ATP is a requirement for kinase activation (for reviews see Goldsmith & Cobb, 1994; Pawson, 1994; Taylor *et al.*, 1993) this again suggests that GTP- γ -S-stimulated ACTH secretion is not mediated by a kinase.

The protein phosphatase inhibitor calyculin A (Ishihara *et al.*, 1989) was employed in the present study to test the possibility that the effects of cytosolic free calcium ions and GTP- γ -S upon ACTH secretion from AtT-20 cells are mediated by a phosphatase. Initially increasing concentrations of the phosphatase inhibitor upon calcium ion- and GTP- γ -S-stimulated ACTH secretion from permeabilised AtT-20 cells were investigated. Calcium ions (10^{-5}M) and GTP- γ -S (10^{-4}M) both stimulated ACTH secretion over 30 min from permeabilised AtT-20 cells (figure 29) which in both cases was significantly greater than control ACTH secretion. Calyculin A had no significant effect upon control ACTH secretion from permeabilised AtT-20 cells however did inhibit calcium ion (10^{-5}M)- and GTP- γ -S (10^{-4}M)-stimulated ACTH secretion in a concentration-dependent manner (figure 29) becoming significant at 10^{-8}M in the case of GTP- γ -S and 10^{-7}M in the case of calcium ions. Secretion in response to GTP- γ -S (10^{-4}M) was 102 $\pm$ 7 pg/10^5 cells/30 min in the absence of calyculin A compared to 61$\pm$ 2 pg/10^5 cells/30 min in the presence of

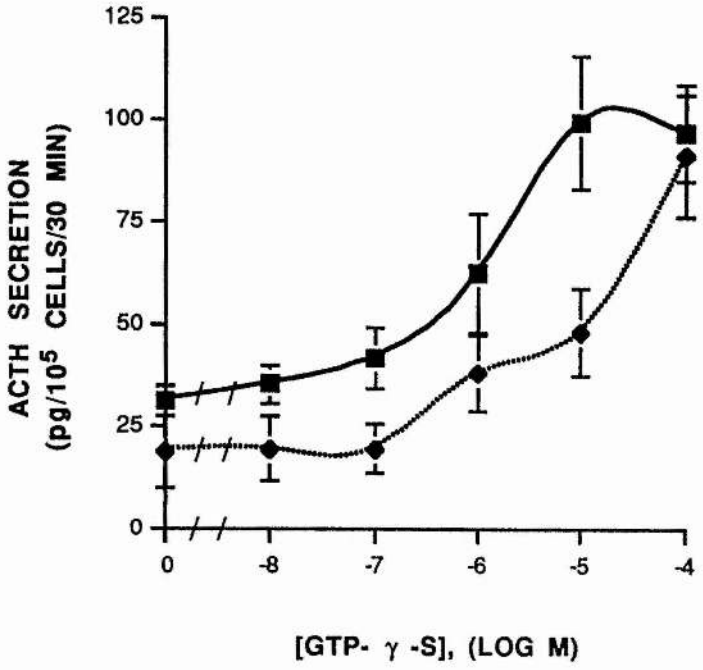

Figure 28 Effect of GTP- γ -S upon ACTH secretion from permeabilised AtT-20 cells in the presence and absence of ATP. Permeabilised cells were incubated for 30 min as described in the methods in the presence of the indicated concentration of GTP- γ -S either in standard permeabilisation medium which contains ATP (5 x 10^{-3}M) (■) or permeabilisation medium containing no ATP (◆) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments.

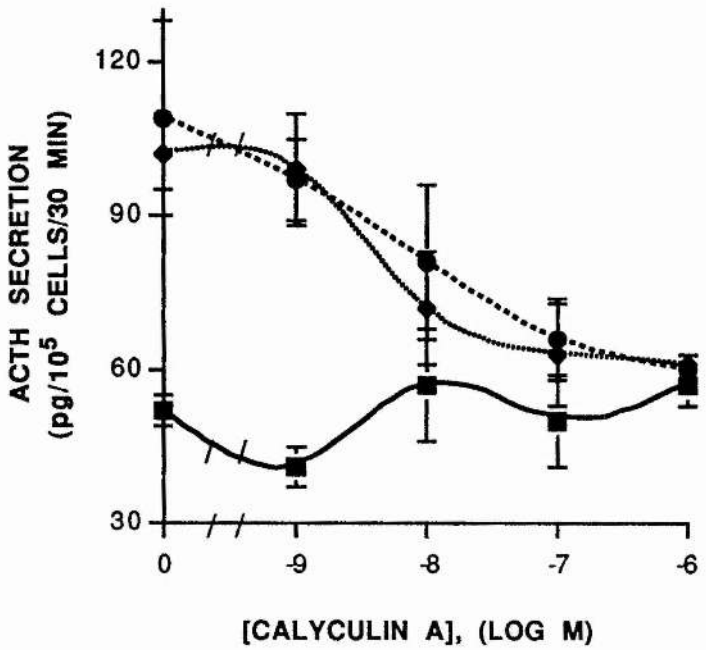

Figure 29 Effect of calyculin A upon calcium ion- and GTP-γ-S-stimulated ACTH secretion from permeabilised AtT-20 cells.
Permeabilised cells were incubated for 30 min as described in the methods in the indicated concentrations of calyculin A in the absence of added secretagogues (■) and in the presence of GTP-γ-S (10^{-4}M) (◆) both in standard permeabilisation medium and also in the presence of calcium ions (10^{-5}M) (●) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments.

calyculin A (10^{-7}M). Secretion in response to calcium ions (10^{-5}M) was 109 ± 19 pg/10^5 cells/30 min in the absence of calyculin A compared to 60 ± 3 pg/10^5 cells/30 min in the presence of calyculin A (10^{-7}M). A calyculin A concentration of 10^{-7}M was chosen as standard for all subsequent experiments investigating the effects of the phosphatase inhibitor upon stimulated ACTH secretion.

Calcium ions stimulated ACTH secretion from permeabilised AtT-20 cells in a concentration-dependent manner becoming significantly greater than control secretion at concentrations above 10^{-6}M (figure 30). Calyculin A (10^{-7}M) significantly inhibited calcium ion-stimulated ACTH secretion to the extent that there was no significant difference between control secretion and secretion in response to any free calcium ion concentration investigated (figure 30). Secretion in response to calcium ions (10^{-5}M) was 123 ± 6 pg/10^5 cells/30 min in the absence of calyculin A compared to 42 ± 4 pg/10^5 cells/30 min in the presence of calyculin A. GTP-γ-S stimulated ACTH secretion from permeabilised AtT-20 cells in a concentration-dependent manner becoming significantly greater than control secretion at concentrations above 10^{-6}M (figure 31). Calyculin A (10^{-7}M) significantly inhibited GTP-γ-S-stimulated ACTH secretion to the extent that there was no significant difference between control secretion and secretion in response to any concentration of GTP-γ-S investigated (figure 31). Secretion in response to GTP-γ-S (10^{-4}M) was 135 ± 15 pg/10^5 cells/30 min in the absence of calyculin A compared to 84 ± 10 pg/10^5 cells/30 min in the presence of calyculin A. The ability of calyculin A to inhibit calcium ion- and GTP-γ-S-stimulated ACTH secretion from permeabilised AtT-20 cells appears to suggest that the effects of these two secretagogues are mediated by a phosphatase.

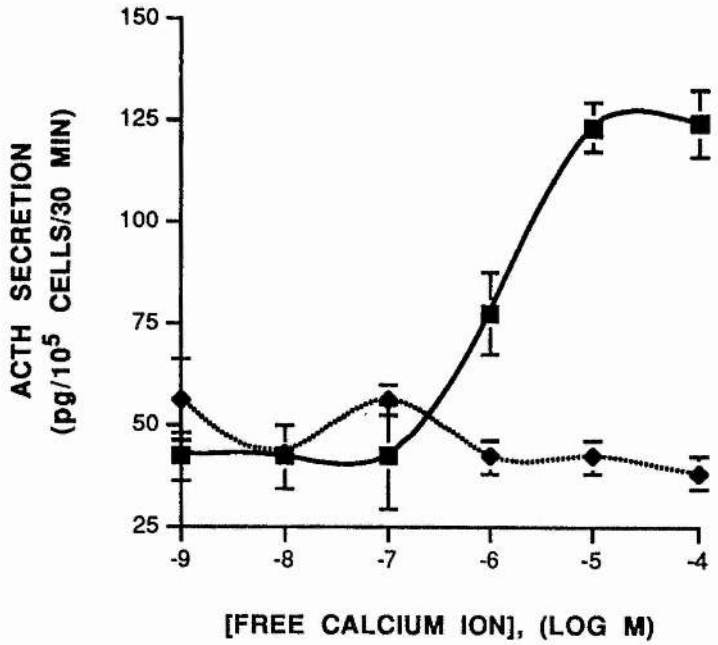

Figure 30 Effect of calyculin A upon calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated for 30 min as described in the methods in a range of calcium/EGTA buffers such that the indicated concentrations of calcium resulted in the absence (■) and presence (◆) of calyculin A (10^{-7}M) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments.

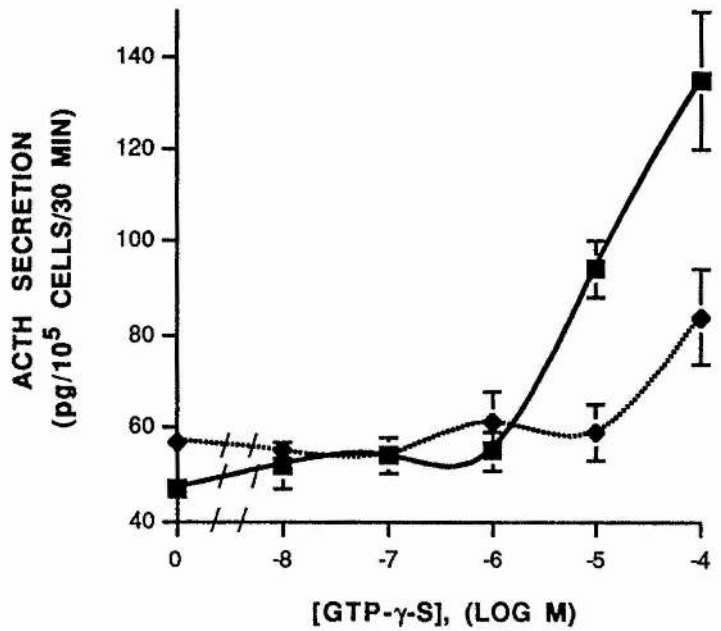

Figure 31 Effect of calyculin A upon GTP- γ -S-stimulated ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated for 30 min as described in the methods in standard permeabilisation medium supplemented with the indicated concentration of GTP- γ -S in the absence (■) and presence (◆) of calyculin A (10^{-7}M) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

2 AN INVESTIGATION INTO THE NATURE OF THE GTP-BINDING PROTEIN MEDIATING CALCIUM ION-STIMULATED ACTH SECRETION FROM AtT-20 CELLS

(i) Effects of aluminium fluoride upon the late stages of the ACTH secretory pathway of AtT-20 cells

Aluminium fluoride ($AlF_{(3-5)}$) is capable of activating heterotrimeric GTP-binding proteins by binding to GDP and mimicking the γ-phosphoryl group of GTP and thus converting the protein to the active conformation (Higashijima *et al.*, 1991). Monomeric GTP-binding proteins appear in contrast to be insensitive to $AlF_{(3-5)}$ (Kahn, 1991). The effects of $AlF_{(3-5)}$, provided in the present study by the combination of potassium or sodium fluoride with aluminium chloride, were initially investigated upon cyclic AMP accumulation in order to obtain effective parameters for heterotrimeric GTP-binding protein activation. $AlF_{(3-5)}$ should be able to generate cyclic AMP by an action on the heterotrimeric GTP-binding protein G_s which is known to regulate adenylate cyclase (Iyenger, 1993). The effects of aluminium chloride (5×10^{-5}M), potassium fluoride (1, 5, 10 & 20 $\times 10^{-3}$M) and sodium fluoride (1, 5, 10 & 20 $\times 10^{-3}$M) upon cyclic AMP accumulation in standard permeabilisation medium in the presence of IBMX (10^{-3}M) over a 90 min incubation period were investigated. Sodium fluoride (1, 5, 10 & 20 $\times 10^{-3}$M) and potassium fluoride (1, 5, 10 & 20 $\times 10^{-3}$M) both had no significant effect upon cyclic AMP accumulation from permeabilised AtT-20 cells (figure 32). However aluminium chloride (5×10^{-5}M) in combination with potassium fluoride was able to stimulate cyclic AMP accumulation from permeabilised AtT-20 cells becoming significant at potassium fluoride concentrations of 5×10^{-3}M and above (figure 32). The combination of aluminium chloride (5×10^{-5}M) and sodium fluoride also produced an

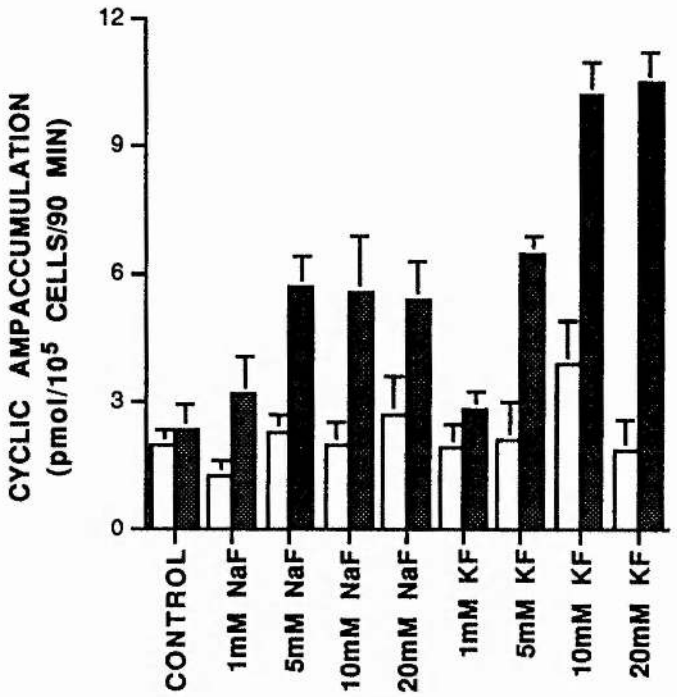

Figure 32 Effect of $AlF_{(3-5)}$ upon cyclic AMP accumulation from permeabilised AtT-20 cells. Permeabilised cells were incubated as described in the methods in standard permeabilisation medium for 90 min supplemented with IBMX (10^{-3}M) and the indicated concentration of sodium fluoride or potassium fluoride in the presence (shaded columns) or absence (open columns) of aluminium chloride (5×10^{-5}M) and cyclic AMP accumulation measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments.

enhancement of cyclic AMP production, however this was not statistically significant. Cyclic AMP accumulation in response to aluminium chloride (5 x 10^{-5}M) in combination with potassium fluoride (2 x 10^{-2}M) was 10.5 ± 0.7 pmol/10^5 cells/90 min compared to control cyclic AMP accumulation (defined here and subsequently as cyclic AMP accumulation in standard permeabilisation medium, in the presence of IBMX (10^{-3}M), in the absence of added secretagogues) of 2.0 ± 0.4 pmol/10^5 cells/90 min. The combination of aluminium chloride (5 x 10^{-5}M) and potassium fluoride (2 x 10^{-2}M) was subsequently adopted as standard for further experiments in which the effects of $AlF_{(3-5)}$ were being investigated.

Having established effective parameters for the activation of GTP-binding proteins by $AlF_{(3-5)}$ the effects of $AlF_{(3-5)}$ upon ACTH secretion from permeabilised AtT-20 cells were subsequently investigated over various time periods. Control ACTH from AtT-20 cells became significant after 30 min (figure 33). The addition of potassium fluoride (2 x 10^{-2}M) inhibited control ACTH secretion becoming significantly lower than control secretion after 20 min (figure 33). The addition of aluminium chloride (5 x 10^{-5}M) had no significant effect upon control secretion and was also unable to enhance secretion observed in response to potassium fluoride (figure 33). Control secretion after 30 min was 144 ± 6 pg/10^5 cells which was reduced to 106 ± 2 pg/10^5 cells in the presence of potassium fluoride. Secretion in response to $AlF_{(3-5)}$ was 93 ± 6 pg/10^5 cells. It therefore appears that potassium fluoride inhibits ACTH secretion from permeabilised AtT-20 cells, an inhibition which $AlF_{(3-5)}$ is unable to overcome.

The ability of GTP-γ-S to stimulate ACTH secretion in the presence of $AlF_{(3-5)}$ was investigated in order to determine whether or not G_e-

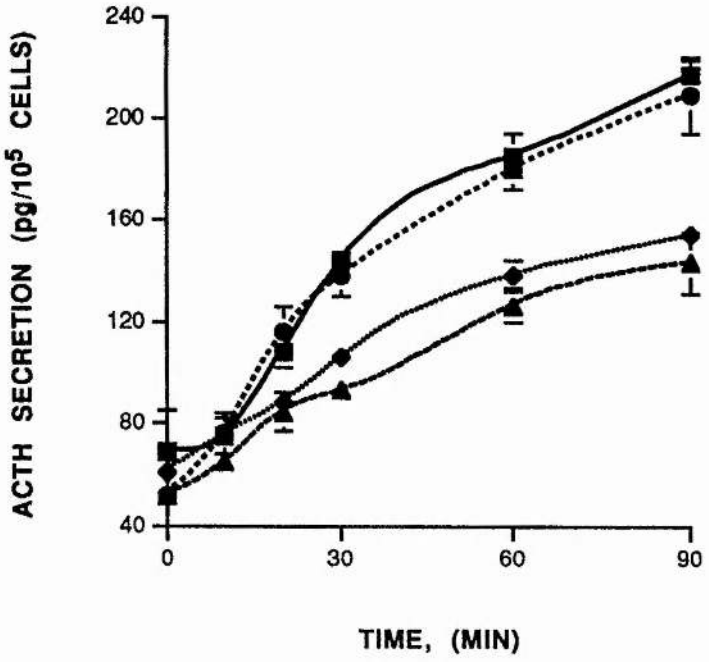

Figure 33 Time course of the effects of $AlF_{(3-5)}$ upon ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated for the indicated time periods as described in the methods in standard permeabilisation medium in the absence of added substances (■), in the presence of potassium fluoride (2×10^{-2}M) (◆), aluminium chloride (5×10^{-5}M) (●) or $AlF_{(3-5)}$, provided by the combination of potassium fluoride (2×10^{-2}M) and aluminium chloride (5×10^{-5}M) (▲), and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

stimulated secretion was possible under these conditions. GTP- γ -S (10^{-4}M) significantly stimulated ACTH secretion from permeabilised AtT-20 cells which was significantly greater than control ACTH secretion (figure 34). Aluminium chloride (5 x 10^{-5}M) alone had no significant effect upon control ACTH secretion or GTP- γ -S-stimulated ACTH secretion (figure 34). Potassium fluoride (2 x 10^{-2}M) alone significantly inhibited GTP- γ -S-stimulated ACTH secretion (figure 29). Control ACTH secretion was significantly inhibited in the presence of $AlF_{(3-5)}$, provided by the combination of potassium fluoride (2 x 10^{-2}M) and aluminium chloride (5 x 10^{-5}M) (figure 34). GTP- γ -S in the presence of $AlF_{(3-5)}$ was unable to stimulate ACTH secretion above that observed in response to $AlF_{(3-5)}$ alone (figure 34). Control ACTH secretion was 51 ± 3 pg/10^5 cells/30 min in the absence of GTP- γ -S compared to 150 ± 14 pg/10^5 cells/30 min in the presence of GTP- γ -S. In the presence of $AlF_{(3-5)}$ ACTH secretion was 11 ± 5 pg/10^5 cells/30 min in the absence of GTP- γ -S (10^{-4}M) compared to 18 ± 7 pg/10^5 cells/30 min in the presence of GTP- γ -S (10^{-4}M). It is clear that activation of G_e in the presence of $AlF_{(3-5)}$ does not result in ACTH secretion and therefore $AlF_{(3-5)}$ seems not to be a useful tool with regard to the investigation of G_e in AtT-20 cells.

(ii) Effects of mastoparan upon the late stages of the ACTH secretory pathway of AtT-20 cells

The amphiphilic tetradecapeptide mastoparan has a wide variety of actions which are largely attributed to the activation of heterotrimeric GTP-binding proteins (Higashijima *et al.*, 1988 & 1990; Weingarten, 1990). This peptide was also employed in the present study, again in order to investigate the possibility that the GTP-binding protein which mediates calcium ion-stimulated ACTH secretion from AtT-20 cells may be a

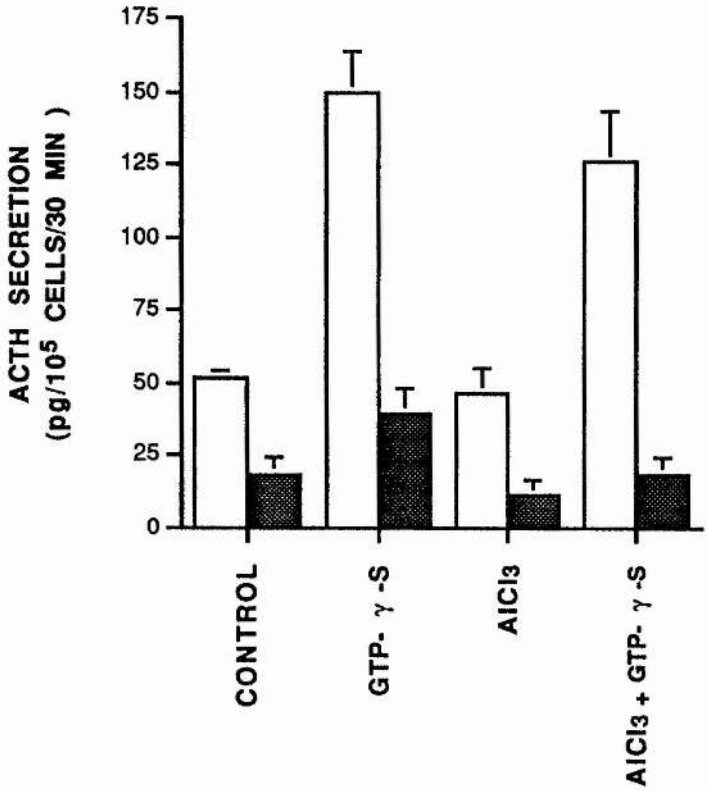

Figure 34 Effect of $AlF_{(3-5)}$ upon GTP- γ -S-stimulated ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated for 30 min as described in the methods in standard permeabilisation medium supplemented as indicated with GTP- γ - S (10^{-4}M), aluminium chloride (5×10^{-5}M), and both GTP- γ - S and aluminium chloride as indicated in the presence (shaded columns) and absence (open columns) of potassium fluoride (2×10^{-2}M) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments.

heterotrimeric GTP-binding protein. Mastoparan stimulated ACTH secretion from intact AtT-20 cells over 2 h becoming significantly greater than control secretion (defined here and subsequently as secretion in DMEM/BSA in the absence of added secretagogues) at concentrations above 10^{-6}M (figure 35). ACTH secretion in response to mastoparan (10^{-5}M) was 550 ± 65 pg/well/2 h compared to control secretion which was 303 ± 42 pg/well/2h. In order to determine if mastoparan directly activates the GTP-binding protein G_e the effects of this peptide were investigated in permeabilised AtT-20 cells.

A suitable incubation period for the investigation of mastoparan upon ACTH secretion from permeabilised AtT-20 cells was initially determined. Control ACTH secretion became significant after 60 min (figure 36). Mastoparan (10^{-5}M) significantly stimulated ACTH secretion from permeabilised AtT-20 cells in a time-dependent manner becoming significantly greater than that of control secretion after 10 min (Figure 36). Secretion in response to mastoparan after 30 min was 104 ± 6 pg/10^5 cells compared to a control secretion of 40 ± 3 pg/10^5 cells. Thirty minutes was chosen as the standard incubation period for all subsequent experiments in which mastoparan-stimulated secretion from permeabilised AtT-20 cells was being investigated. Mastoparan stimulated ACTH secretion in standard permeabilisation medium in a concentration-dependent manner becoming significantly greater than control secretion at concentrations above 10^{-6}M (figure 37). ACTH secretion in response to mastoparan (10^{-5}M) was 359 ± 25 pg/10^5 cells/30 min compared to control ACTH secretion which was 42 ± 10 pg/10^5 cells/30 min. Again in the present study higher concentrations of mastoparan proved too expensive therefore complete concentration response curves were unfortunately not obtained.

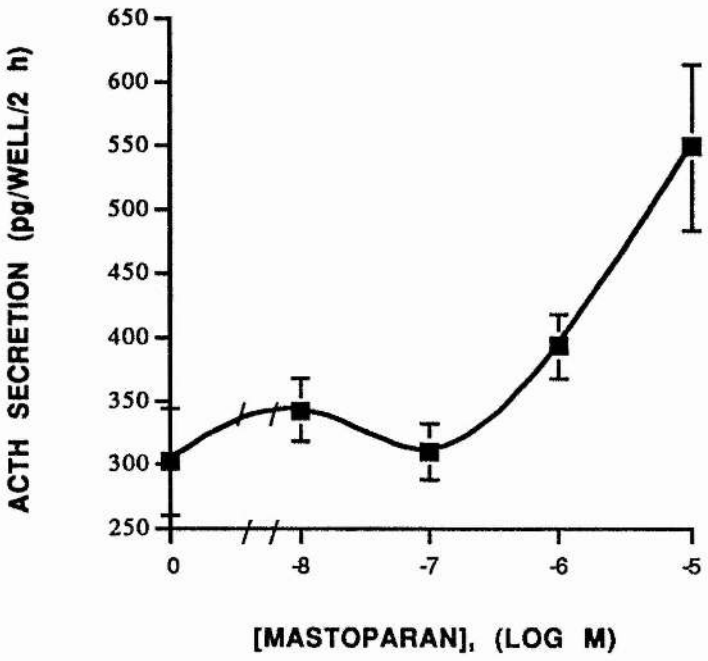

Figure 35 Effect of mastoparan upon ACTH secretion from intact AtT-20 cells. Intact cells were incubated as described in the methods in (DMEM/BSA) for 2 h supplemented with the indicated concentration of mastoparan and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments.

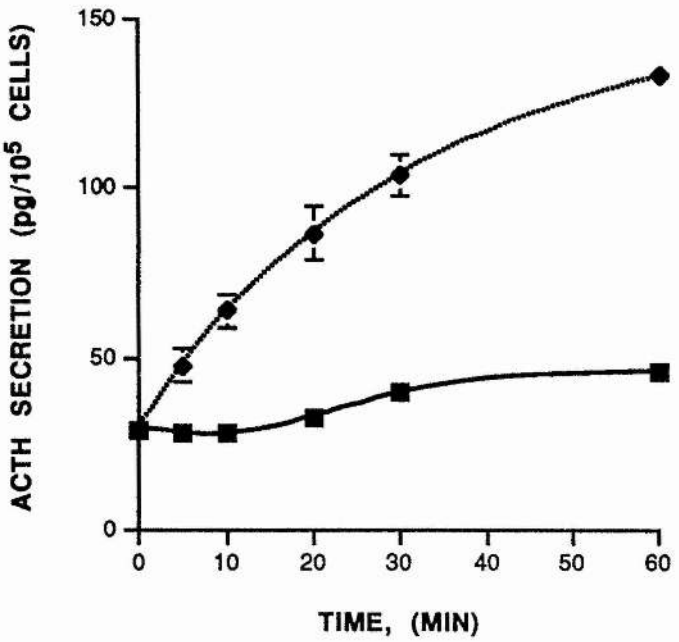

Figure 36 Time course of mastoparan-stimulated ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated for the indicated time periods as described in the methods in standard permeabilisation medium in the presence (◆) or absence (■) of mastoparan (10^{-5}M) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

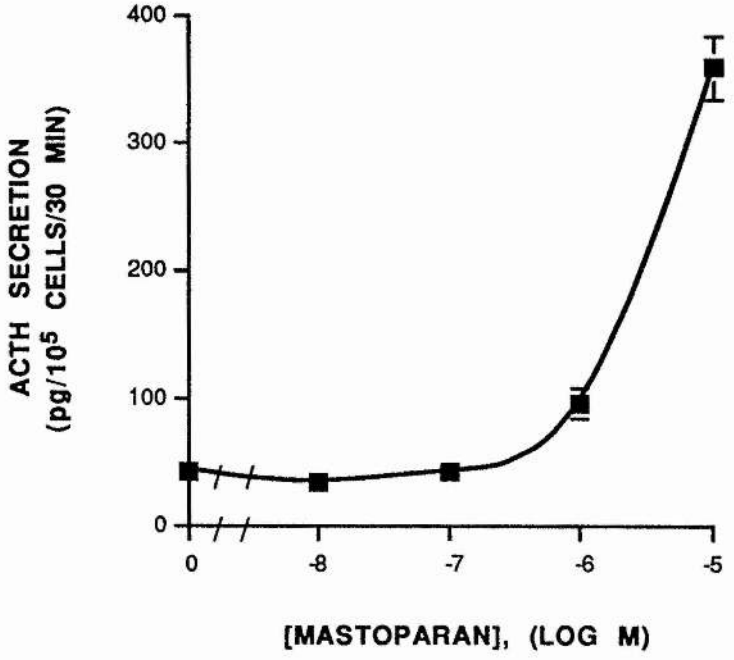

Figure 37 Effects of mastoparan and upon ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated as described in the methods in standard permeabilisation medium for 30 min supplemented with the indicated concentration of mastoparan and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

Mastoparan-stimulated ACTH secretion from permeabilised AtT-20 cells was reversed by the stable GDP analogue GDP- β -S. This guanine nucleotide is used as a means of inhibiting GTP-binding protein activation by virtue of its ability to compete with GTP for the guanine nucleotide binding site, and once bound to maintain the GTP-binding protein in the inactive conformation. Mastoparan stimulated ACTH secretion from permeabilised AtT-20 cells in standard permeabilisation medium in a concentration-dependent manner becoming significantly greater than control secretion at concentrations above 10^{-6}M (figure 38). GDP- β -S (10^{-4}M) significantly reduced mastoparan-stimulated ACTH secretion from permeabilised AtT-20 cells (figure 38). Secretion evoked by mastoparan (10^{-5}M) was reduced from 340 ± 9 pg/10^5 cells/30 min in the absence of GDP- β -S to 211 ± 23 pg/10^5 cells/30 min in the presence of GDP- β -S.

The effects of two mastoparan related peptides upon ACTH secretion from permeabilised AtT-20 cells were also investigated. The mastoparan analogue mas-7 has been reported to be a highly effective GTP-binding protein activator whereas the mastoparan analogue mas-17 is unable to activate GTP-binding proteins (Higashijima *et al.*, 1990). Mastoparan and mas-7 both stimulated ACTH secretion, in standard permeabilisation medium, in a concentration-dependent manner becoming significantly greater than control secretion at concentrations above 10^{-6}M (Figure 39). ACTH secretion in response to mas-7 (10^{-5}M) was 430 ± 13 pg/10^5 cells/30 min compared to 291 ± 11 pg/10^5 cells /30 min obtained in response to mastoparan (10^{-5}M). In contrast, mas-17 (10^{-8}M-10^{-5}M) was unable to stimulate ACTH secretion from permeabilised AtT-20 cells. No significant difference was observed between control secretion and secretion in response to every concentration of mas-17 investigated (Figure 40).

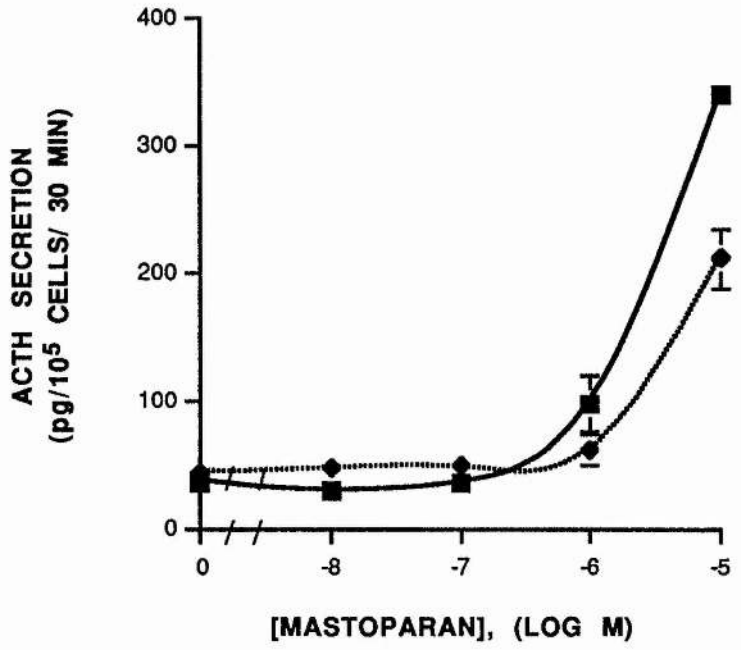

Figure 38 Effect of GDP- β -S upon mastoparan-stimulated ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated as described in the methods in standard permeabilisation medium for 30 min supplemented with the indicated concentration of mastoparan in the absence (■) or presence (◆) of GDP- β -S (10^{-4}M) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

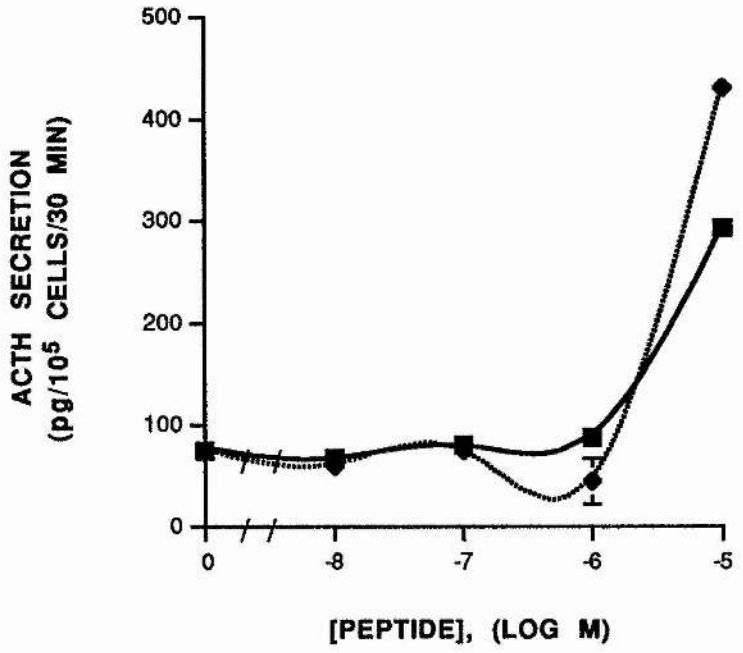

Figure 39 Effects of mastoparan and mas-7 upon ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated as described in the methods in standard permeabilisation medium for 30 min supplemented with the indicated concentration of mastoparan (■) or mas-7 (◆) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

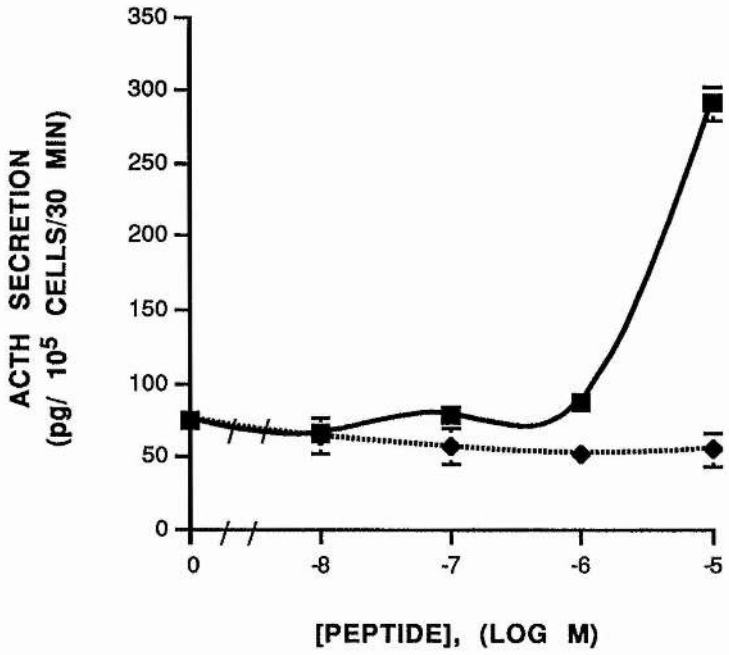

Figure 40 Effects of mastoparan and mas-17 upon ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated as described in the methods in standard permeabilisation medium for 30 min supplemented with the indicated concentration of mastoparan (■) or mas-17 (◆) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

The ability of GDP- β -S to reverse mastoparan-stimulated ACTH secretion from permeabilised AtT-20 cells would suggest that mastoparan stimulates secretion, at least partly, via activation of a GTP-binding protein. The GDP- β -S concentration of 10^{-4}M was chosen as this concentration of the guanine nucleotide completely reverses calcium- and GTP- γ -S-stimulated ACTH secretion from permeabilised AtT-20 cells (Guild, 1991). Higher concentrations of GDP- β -S were unable to reverse mastoparan-stimulated ACTH secretion any further (data not shown). The ability of mas-7, the highly effective GTP-binding protein activator, to stimulate ACTH secretion to a greater extent than mastoparan coupled with the inability of mas-17, unable to activate GTP-binding proteins, to stimulate ACTH secretion is also consistent with mastoparan-stimulated ACTH secretion from permeabilised AtT-20 cells being mediated, at least partly, by activation of a GTP-binding protein.

The PKC inhibitor chelerythrine chloride was employed to determine whether mastoparan-stimulated ACTH secretion from permeabilised AtT-20 cells was mediated by this kinase. Mastoparan stimulated ACTH secretion in standard permeabilisation medium in a concentration-dependent manner becoming significantly greater than control secretion at concentrations of above 10^{-6}M (figure 41). Chelerythrine chloride (10^{-5}M) had no significant effect upon control secretion or secretion in response to any concentration of mastoparan investigated (figure 36). ACTH secretion in response to 10^{-5}M mastoparan was 321 ± 11 pg/10^5 cells/30 min in the absence of chelerythrine chloride (10^{-5}M) and 307 ± 22 pg/10^5 cells/30 min in the presence of chelerythrine chloride (10^{-5}M). Results from the present study indicate that chelerythrine chloride (10^{-5}M) is capable of completely inhibiting the effects of PKC activators in AtT-20 cells under similar

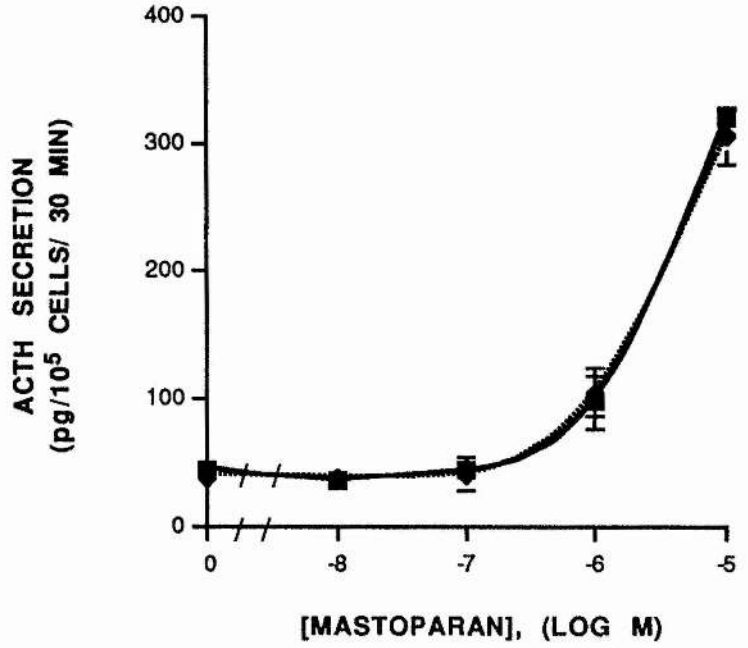

Figure 41 Effect of chelerythrine chloride upon mastoparan-stimulated ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated as described in the methods in standard permeabilisation medium for 30 min supplemented with the indicated concentration of mastoparan in the absence (■) or presence (◆) of chelerythrine chloride (10^{-5}M) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

conditions (a detailed account of this positive control can be found on p73). The inability of chelerythrine chloride to reverse the effects of mastoparan therefore suggest that mastoparan-stimulated ACTH secretion from permeabilised AtT-20 cells is not mediated by activation of PKC.

As demonstrated above cyclic AMP is unable to stimulate ACTH secretion from permeabilised AtT-20 cells. It can therefore be assumed that the effects of mastoparan upon ACTH secretion from permeabilised AtT-20 cells are not mediated by the generation of cyclic AMP. This was confirmed by investigating the effect of mastoparan upon cyclic AMP accumulation from permeabilised AtT-20 cells. Mastoparan had no significant effect upon cyclic AMP accumulation from permeabilised AtT-20 cells over a 30 min incubation period. No significant difference was observed between control cyclic AMP accumulation and cyclic AMP accumulation from permeabilised cells in response to mastoparan (10^{-5}M) (figure 42). Cyclic AMP accumulation in the presence of mastoparan was 1.5 $\pm$ 10.1 pmol/10^5 cells/30 min compared to control cyclic AMP accumulation which was 1.4 $\pm$ 0.3 pmol/10^5 cells/30 min. Mastoparan was however able to stimulate ACTH secretion from the same cells under the same conditions (data not shown). This confirms that mastoparan-stimulated ACTH secretion from permeabilised AtT-20 cells is not mediated by generation of cyclic AMP and subsequent activation of PKA.

It therefore appears that mastoparan stimulates ACTH secretion from permeabilised AtT-20 cells by activation of a GTP-binding protein and that the effects of this peptide are not mediated by activation of either PKC or PKA. Mastoparan-stimulated ACTH secretion from permeabilised AtT-20 cells therefore displays characteristics highly consistent with those of calcium ion- and GTP- γ -S-stimulated secretion. Since mastoparan is an

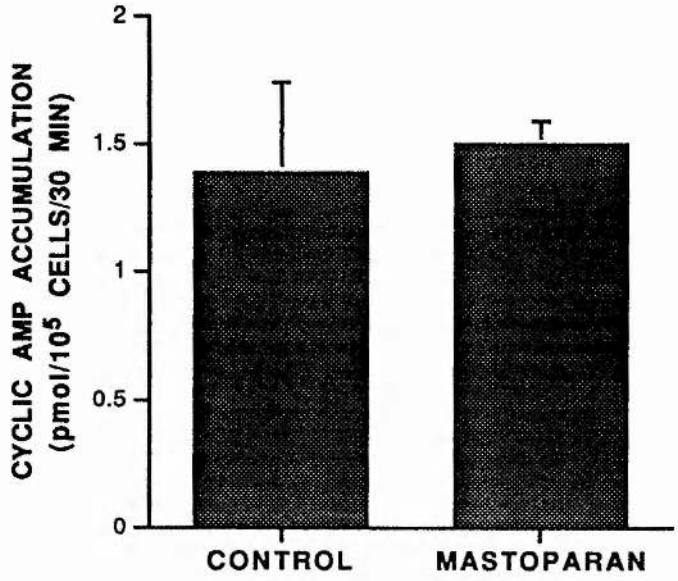

Figure 42 Effect of mastoparan upon cyclic AMP accumulation from permeabilised AtT-20 cells. Permeabilised cells were incubated as described in the methods in standard permeabilisation medium for 30 min supplemented with IBMX (10^{-3}M) in the presence or absence of mastoparan (10^{-5}M) as indicated and cyclic AMP accumulation measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments.

activator of heterotrimeric GTP-binding proteins this may suggest that the GTP-binding protein which mediates calcium ion-stimulated ACTH secretion from AtT-20 cells is a heterotrimeric GTP-binding protein.

Omitting ATP from the permeabilisation medium, normally present in the standard permeabilisation medium at a concentration of 5 x 10^{-3}M, significantly reduced mastoparan-stimulated ACTH secretion from permeabilised AtT-20 cells. Mastoparan significantly stimulated ACTH secretion from permeabilised AtT-20 cells, in standard permeabilisation medium, in a concentration-dependent manner becoming significantly greater than control secretion at concentrations above 10^{-7}M (figure 43). Omitting ATP from the standard permeabilisation medium however reduced control secretion, although not statistically significant, and significantly reduced mastoparan (10^{-6}-10^{-5}M)-stimulated ACTH secretion (figure 43). Secretion obtained in the absence of mastoparan was reduced from 31 $\pm$ 3 pg/10^5 cells/30 min to 13 $\pm$ 1 pg/10^5 cells/30 min. ACTH secretion in response to 10^{-5}M mastoparan was reduced from 274 $\pm$ 13 pg/10^5 cells/30 min in the presence of ATP to 128 $\pm$ 11 pg/10^5 cells/30 min in the absence of ATP. It should be noted however that mastoparan (10^{-5}M)-stimulated ACTH secretion in the absence of ATP was still significantly greater than control. This is consistent with previous results which demonstrated that calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells is similarly ATP-dependent (Guild, 1991).

(iii) Effects of pertussis toxin upon the late stages of the ACTH secretory pathway of AtT-20 cells

The bacterial exotoxin pertussis toxin (PTX) from *Bordetella pertussis* possesses mono ADP-ribosyl transferase activity and has the

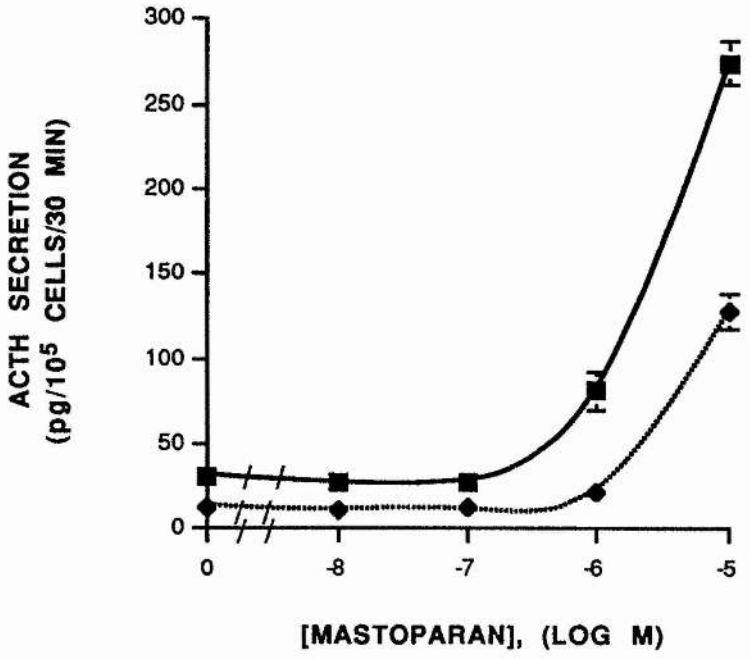

Figure 43 Effect of mastoparan upon ACTH secretion from permeabilised AtT-20 cells in the presence and absence of ATP. Permeabilised cells were incubated for 30 min as described in the methods supplemented with the indicated concentration of mastoparan either in standard permeabilisation medium containing ATP (5 x 10^{-3}M) (■) or permeabilisation medium containing no ATP (◆) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

capacity to transfer the ADP-ribose moiety of the cofactor β-nicotinamide adenine dinucleotide (NAD) onto a specific cysteine residue of the α subunit of certain members of the G_i subfamily of heterotrimeric GTP-binding proteins which prevents activation of these proteins by their endogenous activators (Yamane & Fung, 1993). PTX was therefore employed in the present study in an attempt to determine whether or not the GTP-binding protein which mediates calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells belongs to the G_i subfamily of heterotrimeric GTP-binding proteins. The abilities of calcium ions, GTP- γ -S and mastoparan to stimulate ACTH secretion were investigated using permeabilised AtT-20 cells which prior to permeabilisation were incubated for 16 h either in control growth medium or growth medium supplemented with 1μg ml^{-1} PTX.

Calcium ions stimulated ACTH secretion from permeabilised AtT-20 cells in a concentration-dependent manner becoming significantly greater than control secretion at concentrations above 10^{-6}M (figure 44). No significant difference was observed between calcium ion-stimulated ACTH secretion from control cells and from PTX (1μg ml^{-1}, 16 h) pretreated cells (figure 44). Secretion in response to calcium ions (10^{-5}M) from control cells was 132 ± 10 pg/10^5 cells/30 min compared to 134 ± 11 pg/10^5 cells/30 min from PTX pretreated cells. GTP- γ -S stimulated ACTH secretion from permeabilised AtT-20 cells in a concentration-dependent manner, in standard permeabilisation medium, which became significantly greater than control secretion at a concentrations above of 10^{-6}M (figure 45). No significant difference was observed between GTP- γ -S-stimulated ACTH secretion from control cells and from PTX (1μg ml^{-1}, 16 h) pretreated cells (figure 45). Secretion in response to GTP- γ -S (10^{-4}M) from control cells was 145 ± 15 pg/10^5 cells/30 min compared to 151 ± 15 pg/10^5 cells/30

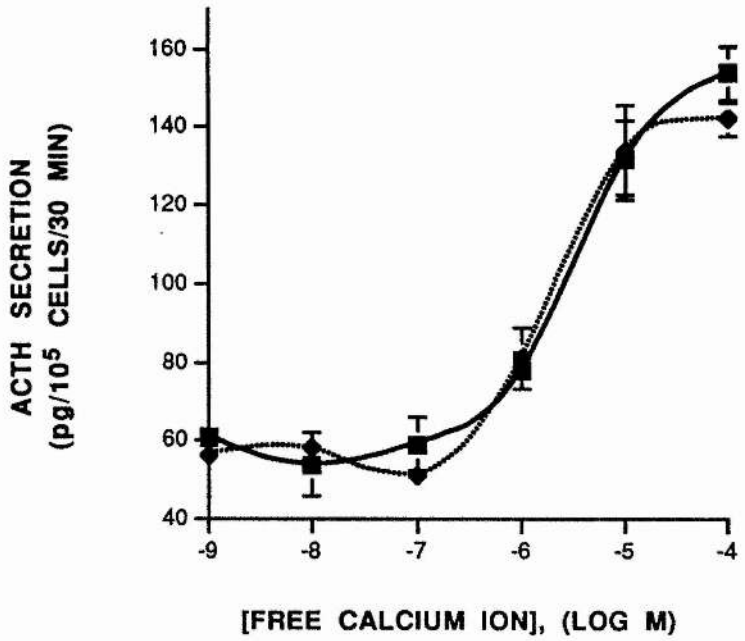

Figure 44 Effect of PTX pretreatment upon calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells. Control cells (■) and PTX pretreated cells (1 μg ml^{-1}, 16 h, ◆) were permeabilised and incubated as described in the methods for 30 min in a series of calcium-EGTA buffers such that the indicated concentrations of free calcium resulted and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

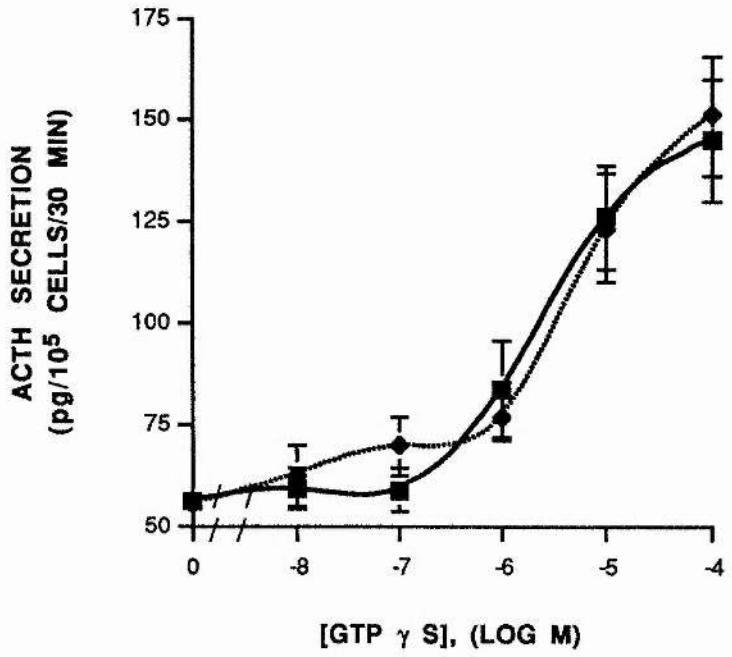

Figure 45 Effect of PTX pretreatment upon GTP-γ-S-stimulated ACTH secretion from permeabilised AtT-20 cells. Control cells (■) and PTX pretreated cells (1 μg ml^{-1}, 16 h, ◆) were permeabilised and incubated as described in the methods for 30 min in standard permeabilisation medium containing the indicated concentrations of GTP-γ-S and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

min from PTX pretreated cells. Mastoparan stimulated ACTH secretion from permeabilised AtT-20 cells in a concentration-dependent manner in standard permeabilisation medium becoming significantly greater than control secretion at concentrations above 10^{-6}M (figure 46). No significant difference was observed between mastoparan-stimulated ACTH secretion from control cells and from PTX (1μg ml^{-1}, 16 h) pretreated cells (figure 46). Secretion in response to mastoparan (10^{-5}M) from control cells was 281 ± 35 pg/10^5 cells/30 min compared to 283 ± 16 pg/10^5 cells/30 min from PTX pretreated cells.

Raising the extracellular calcium chloride concentration has been shown to result in an increase in the cytosolic calcium ion concentration of AtT-20 cells (Luini *et al.*, 1985). In the present study raising the extracellular concentration of calcium chloride from 1.8 mM (normally present in DMEM / BSA) to 4 mM and to 6 mM in both cases resulted in an increase in ACTH secretion over 2 h which was significantly greater than control secretion from intact AtT-20 cells (figure 47). No significant difference was observed between calcium chloride-stimulated ACTH secretion from control cells and from PTX pretreated cells (figure 47). Secretion in response to calcium chloride (6 mM) from control cells was 411 ± 12 pg/well/2 h compared to 450 ± 33 pg/well/2 h from PTX pretreated cells (1μg ml^{-1}, 16 h). Forskolin (10^{-4}M) stimulated ACTH secretion from intact AtT-20 cells over 2 h and was significantly greater than control secretion (figure 48). No significant difference was observed between forskolin-stimulated ACTH secretion from control cells and from PTX pretreated cells (figure 48). Secretion in response to forskolin from control cells was 817 ± 40 pg/well/2 h compared to 746 ± 43 pg/well/2 h from PTX pretreated cells.

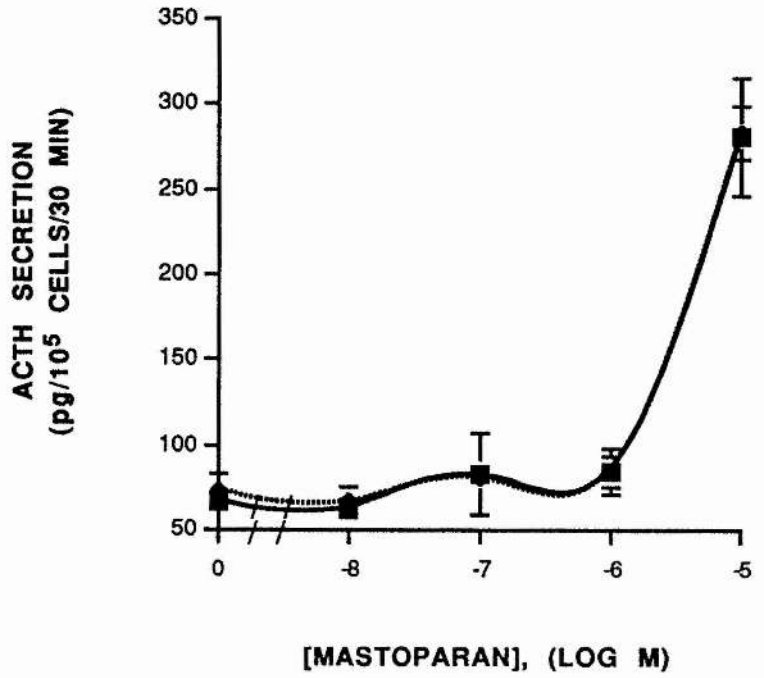

Figure 46 Effect of PTX pretreatment upon mastoparan-stimulated ACTH secretion from permeabilised AtT-20 cells. Control cells (■) and PTX pretreated cells (1 μg ml^{-1},16 h, ◆) were permeabilised and incubated as described in the methods for 30 min in standard permeabilisation medium containing the indicated concentrations of mastoparan and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

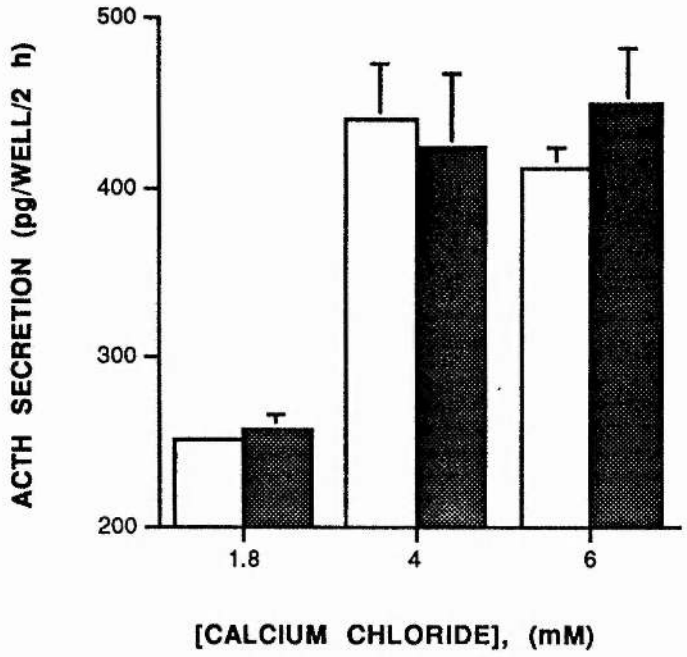

Figure 47 Effect of PTX pretreatment upon calcium chloride-stimulated ACTH secretion from intact AtT-20 cells. Control cells (open columns) and PTX pretreated cells (1 μg ml^{-1}, 16 h, shaded columns) were incubated as described in the methods for 2 h in DMEM/BSA supplemented with the indicated concentration of calcium chloride and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the column.

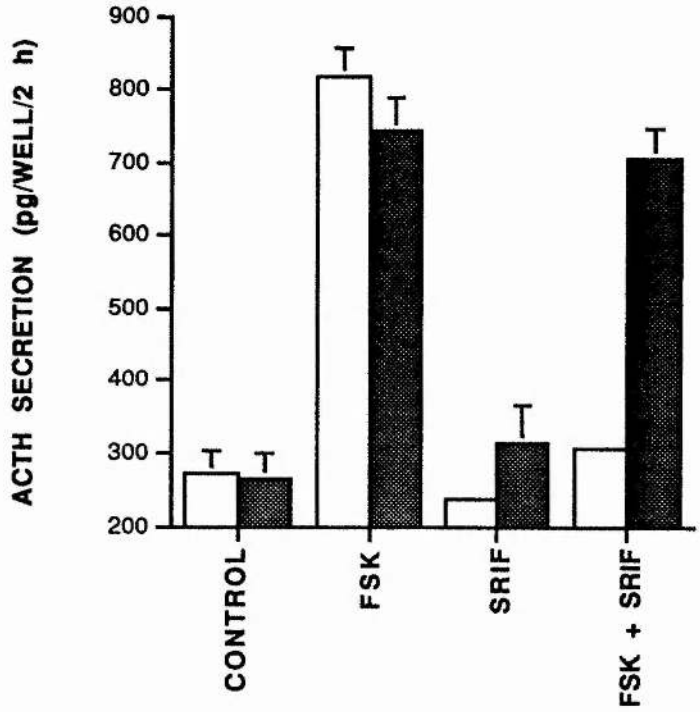

Figure 48 Effect of PTX pretreatment upon forskolin-stimulated ACTH secretion and [D-trp^8]-SRIF mediated inhibition of ACTH secretion from intact AtT-20 cells. Control cells (open columns) and PTX pretreated cells (1 μg ml^{-1},16 h, shaded columns) were incubated as indicated as described in the methods for 2 h in DMEM/BSA supplemented with forskolin (10^{-4}M, FSK), [D-trp^8]-SRIF (SRIF, 10^{-7}M) and both forskolin and [D-trp^8]-SRIF and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the column.

Thus it appears that calcium ion-, mastoparan- and GTP- γ -S-stimulated ACTH secretion from permeabilised AtT-20 cells and calcium chloride- and forskolin-stimulated ACTH secretion from intact AtT-20 cells are all insensitive to PTX pretreatment suggesting that the GTP-binding protein which mediates calcium ion-stimulated ACTH secretion from AtT-20 cells is not sensitive to PTX.

[D-trp^8]-SRIF has previously been shown to inhibit forskolin-stimulated ACTH secretion from intact AtT-20 cells by a PTX-sensitive mechanism of action (Reisine & Guild, 1985). [D-trp^8]-SRIF is employed in preference to SRIF itself as it is six to eight times more potent than the native peptide (Rivier *et al.*, 1975). In order to confirm that in the present study the parameters adopted, a PTX pretreatment of 1μg ml^{-1} for 16 h, were effective for inhibition of certain heterotrimeric GTP-binding proteins the effect of PTX upon [D-trp^8]-SRIF-mediated inhibition of forskolin-stimulated ACTH secretion was investigated. [D-trp^8]-SRIF (10^{-7}M) alone over 2 h had no significant effect upon ACTH secretion from control secretion from control cells or from PTX (1μg ml^{-1}, 16 h) pretreated cells (figure 48). [D-trp^8]-SRIF however significantly inhibited forskolin (10^{-4}M)-stimulated ACTH secretion from control cells (figure 48). This ACTH secretion in response to the combination of forskolin and [D-trp^8]-SRIF was not significantly different from control secretion. In contrast [D-trp^8]-SRIF had no significant effect upon forskolin-stimulated ACTH secretion from cells pretreated with PTX (1μg ml^{-1}, 16 h) (figure 48). ACTH secretion from control cells was 817 $\pm$ 40 pg/well/2 h in the presence of forskolin (10^{-4}M) and 271 $\pm$ 32 pg/well/2h in the absence. ACTH secretion from PTX pretreated cells was 746 $\pm$ 43 pg/well/2 h in the presence of forskolin (10^{-4}M) and 266 $\pm$ 34 pg/well/2 h in the absence. Therefore it appears that PTX pretreatment completely inhibited [D-trp^8]-SRIF-mediated inhibition of

forskolin-stimulated ACTH secretion from intact AtT-20 cells. Thus it appears that the parameters adopted in the present study were highly effective with regard to inhibiting PTX-sensitive GTP-binding proteins.

(iv) Effects of cholera toxin upon the late stages of the ACTH secretory pathway of AtT-20 cells

The bacterial exotoxin CTX from *Vibrio cholerae* possesses ADP-ribosyl transferase activity and is capable of transferring the ADP-ribose moiety from NAD to a particular arginine residue on the α subunit of members of the G_s subfamily of heterotrimeric GTP-binding proteins resulting in a persistent activation of these proteins due to an inhibition of their intrinsic GTPase activity (Yamane & Fung, 1993). CTX was therefore employed in the present study to determine whether or not the GTP-binding protein which mediates calcium ion-stimulated ACTH secretion from AtT-20 cells belongs to the G_s subfamily of heterotrimeric GTP-binding proteins.

The effects of CTX were initially investigated upon ACTH secretion using intact AtT-20 cells in order to obtain a maximal CTX concentration. CTX stimulated ACTH secretion from intact AtT-20 cells in a concentration-dependent manner over 2 h becoming significantly greater than control secretion at concentrations above 10^{-10}M (figure 49). A maximal response was observed in response to 10^{-8}M, a concentration which was therefore adopted as standard for further experiments in which the effects of CTX were being investigated. ACTH secretion in response to CTX (10^{-8}M) was 471 $\pm$ 37 pg/well/2 h compared to control secretion of 272 $\pm$ 21 pg/well/2 h.

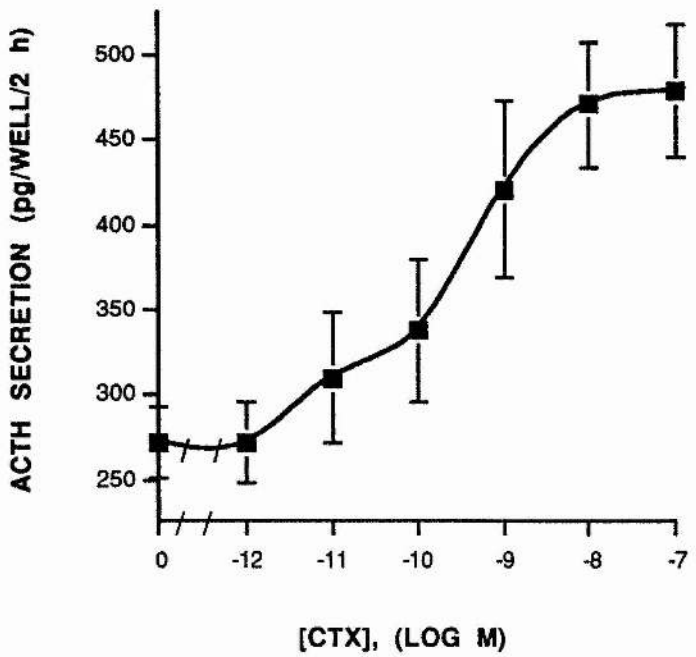

Figure 49 Effect of CTX upon ACTH secretion from intact AtT-20 cells. Intact cells were incubated as described in the methods for 2 h in DMEM/BSA supplemented with the indicated concentrations of CTX and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments.

Having established a maximal CTX concentration using intact AtT-20 cells, the effects of CTX in permeabilised cells were investigated. In order to confirm that CTX was able to activate heterotrimeric GTP-binding proteins under these conditions the effects of CTX upon cyclic AMP accumulation, in the presence of the cofactor NAD, were initially investigated. The combination of CTX (10^{-8}M) and NAD (10^{-5}M), in standard permeabilisation medium in the presence of IBMX (10^{-3}M), stimulated cyclic AMP accumulation in a time-dependent manner becoming significantly greater than control cyclic AMP accumulation after a period of 120 min (figure 50). The combination of CTX (10^{-8}M) and a higher concentration of NAD (10^{-4}M) stimulated cyclic AMP production above control cyclic AMP accumulation from permeabilised AtT-20 cells in a time-dependent manner becoming significantly greater than control cyclic AMP accumulation after a period of 90 min (figure 50). Control cyclic AMP accumulation after 120 min was 1.6 $\pm$ 0.4 pmol/10^5 cells compared to 3.3 $\pm$ 0.5 pmol/10^5 cells in response to CTX (10^{-8}M) and NAD (10^{-5}M) and 3.4 $\pm$ 0.2 pmol/10^5 cells in response to CTX (10^{-8}M) and NAD (10^{-4}M). The ability of CTX, in combination with NAD, to stimulate cyclic AMP production from permeabilised AtT-20 cells suggests that the toxin is capable of ADP-ribosylation under these conditions.

Having established that CTX is capable of ADP-ribosylation in permeabilised AtT-20 cells the effects of CTX, in combination with NAD, upon ACTH secretion from permeabilised AtT-20 cells were investigated. Control ACTH secretion became significant after 60 min (figure 51). No significant difference was observed between control secretion and secretion in response to CTX (10^{-8}M) in combination NAD (10^{-5}M or 10^{-4}M) (figure 51). Control ACTH secretion after 120 min was 189 $\pm$ 15 pg/10^5 cells compared to 188 $\pm$ 23 pg/10^5 cells in response to CTX (10^{-8}M) and

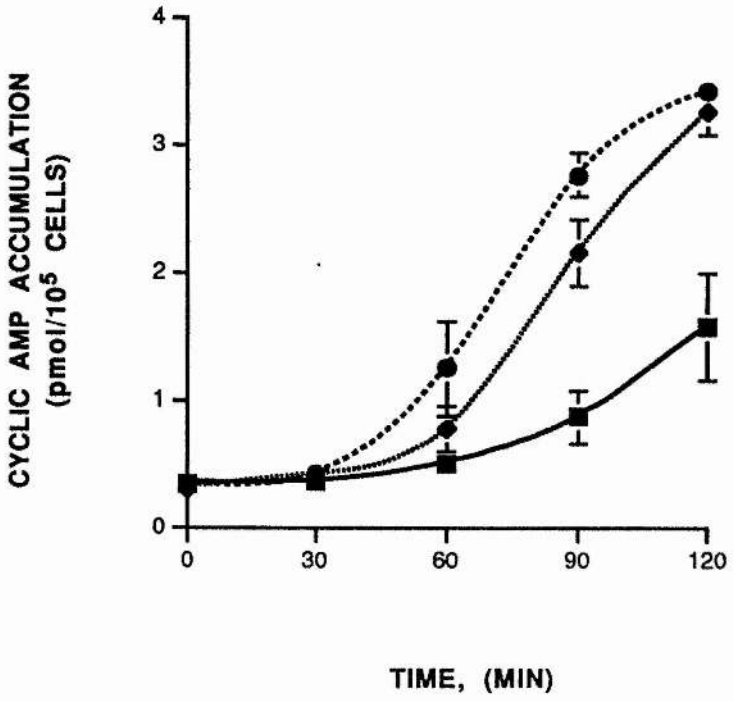

Figure 50 Effect of CTX and NAD upon cyclic AMP accumulation from permeabilised AtT-20 cells. Permeabilised cells were incubated for the indicated time period in standard permeabilisation medium supplemented with IBMX (10^{-3}M) in the absence of CTX and NAD (■), presence of CTX (10^{-8}M) and NAD (10^{-5}M) (◆) or in the presence of CTX (10^{-8}M) and NAD (10^{-4}M) (●) and cyclic AMP accumulation measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

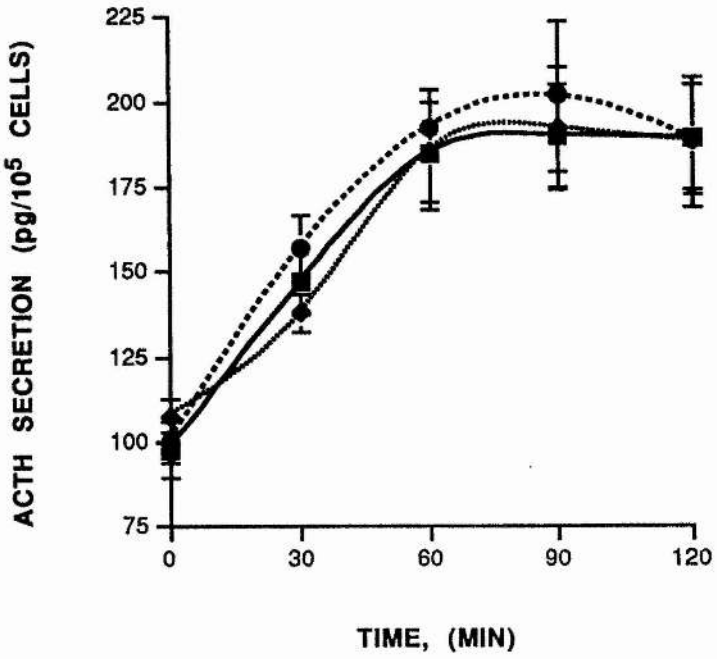

Figure 51 Effect of CTX and NAD upon ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated for the indicated time period in standard permeabilisation medium in the absence of CTX and NAD (■), presence of CTX (10^{-8}M) and NAD (10^{-5}M) (◆) or in the presence of CTX (10^{-8}M) and NAD (10^{-4}M) (●) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments.

NAD (10^{-5}M) and 189 ± 19 pg/10^5 cells in response to CTX (10^{-8}M) and NAD (10^{-4}M). It therefore appears that CTX has no effect upon ACTH secretion from permeabilised AtT-20 cells which therefore suggests that the GTP-binding protein that mediates calcium ion-stimulated ACTH secretion is not CTX sensitive.

3 AN INVESTIGATION INTO THE REGULATION OF ACTH SECRETION FROM AtT-20 CELLS BY PROTEIN KINASE C AND CYCLIC AMP-DEPENDENT PROTEIN KINASE AT A LATE STAGE IN THE ACTH SECRETORY PATHWAY

(i) Effects of protein kinase C activators upon ACTH secretion from intact AtT-20 cells

The involvement of PKC in the ACTH stimulus-secretion coupling pathway of AtT-20 cells was investigated using the PKC activator phorbol 12-myristate 13-acetate (PMA). The effects of PMA were initially investigated upon ACTH secretion using intact AtT-20 cells in order to obtain a suitable PMA concentration for further investigation. PMA stimulated ACTH secretion from intact AtT-20 cells in a concentration-dependent manner over 1 h becoming significantly greater than control secretion at concentrations above 10^{-9}M (figure 52). ACTH secretion in response to PMA (10^{-7}M) was 1607 $\pm$ 102 pg/well/h compared to control secretion of 363 $\pm$ 30 pg/well/h. The DAG analogues 1-oleoyl-2-acetyl-*sn*-glycerol (OAG, 10^{-4}M) and 1,2-dioctanoyl-*sn*-glycerol (DOG, 10^{-4}M) both significantly stimulated ACTH secretion from intact AtT-20 cells (figure 53) as did the phorbol esters phorbol 12, 13-didecanoate (PDD, 10^{-7}M) and phorbol 12, 13-dibutyrate (PDBu, 10^{-7}M) (figure 54). The inactive phorbol ester 4 α phorbol (10^{-7}M) however had no significant effect upon ACTH secretion from intact AtT-20 cells (figure 54). These results suggest that PKC plays an important role in the regulation of ACTH secretion from AtT-20 cells. In order to determine whether PKC has a point of regulation distal to changes in the cytosolic free calcium ion concentration the effects of PMA were investigated upon permeabilised cells.

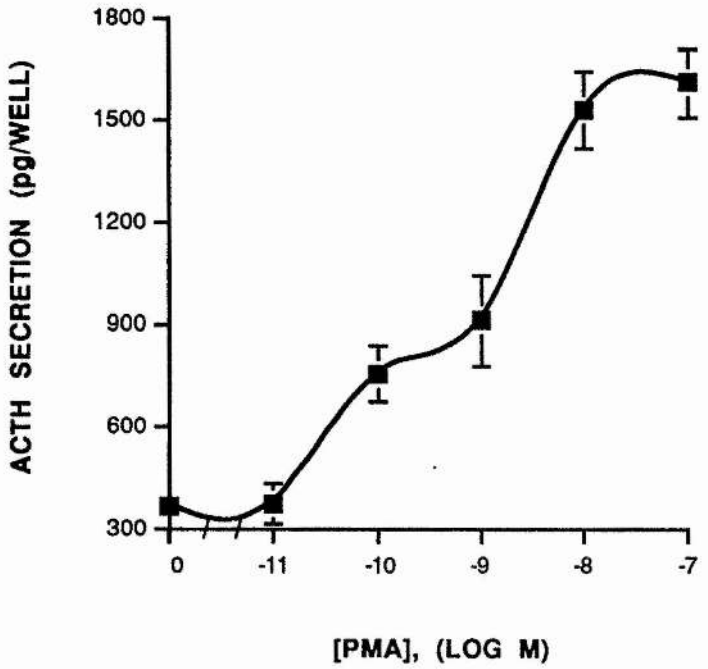

Figure 52 Effect of PMA upon ACTH secretion from intact AtT-20 cells. Intact cells were incubated as described in the methods for 1 h in DMEM/BSA supplemented with the indicated concentrations of PMA and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

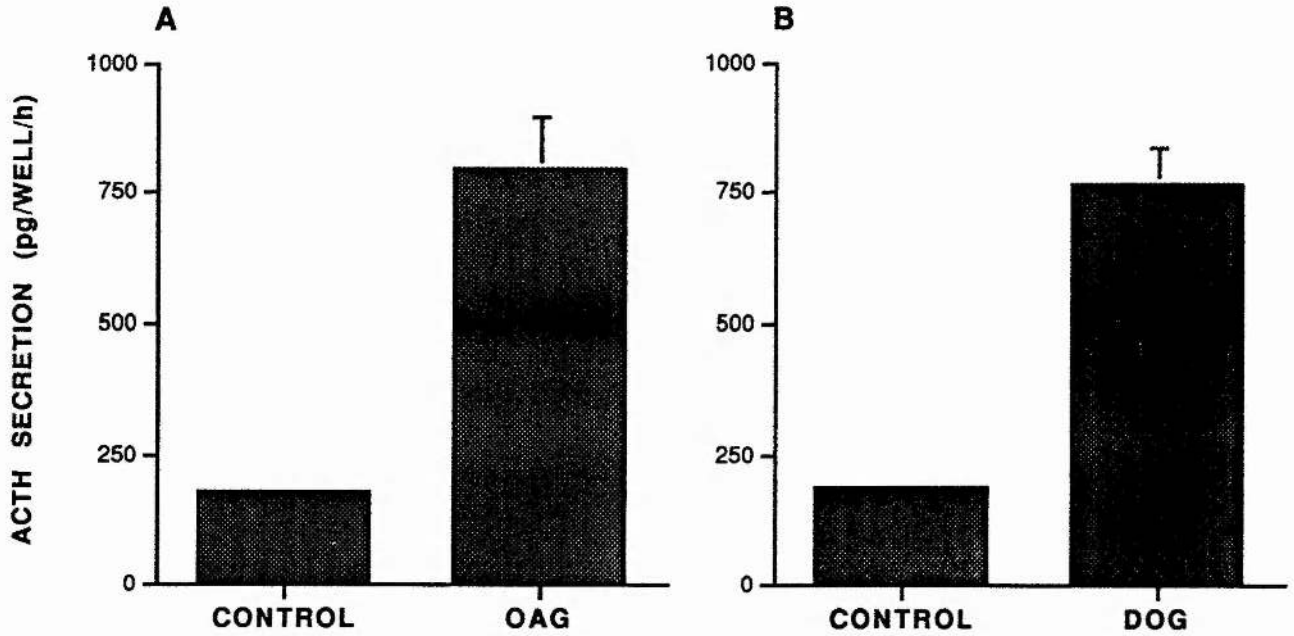

Figure 53 Effect of DAG analogues upon ACTH secretion from intact AtT-20 cells. Intact cells were incubated as described in the methods for 1 h in DMEM/BSA supplemented with OAG (10^{-4}M, panel A) or DOG (10^{-4}M, panel B) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the column.

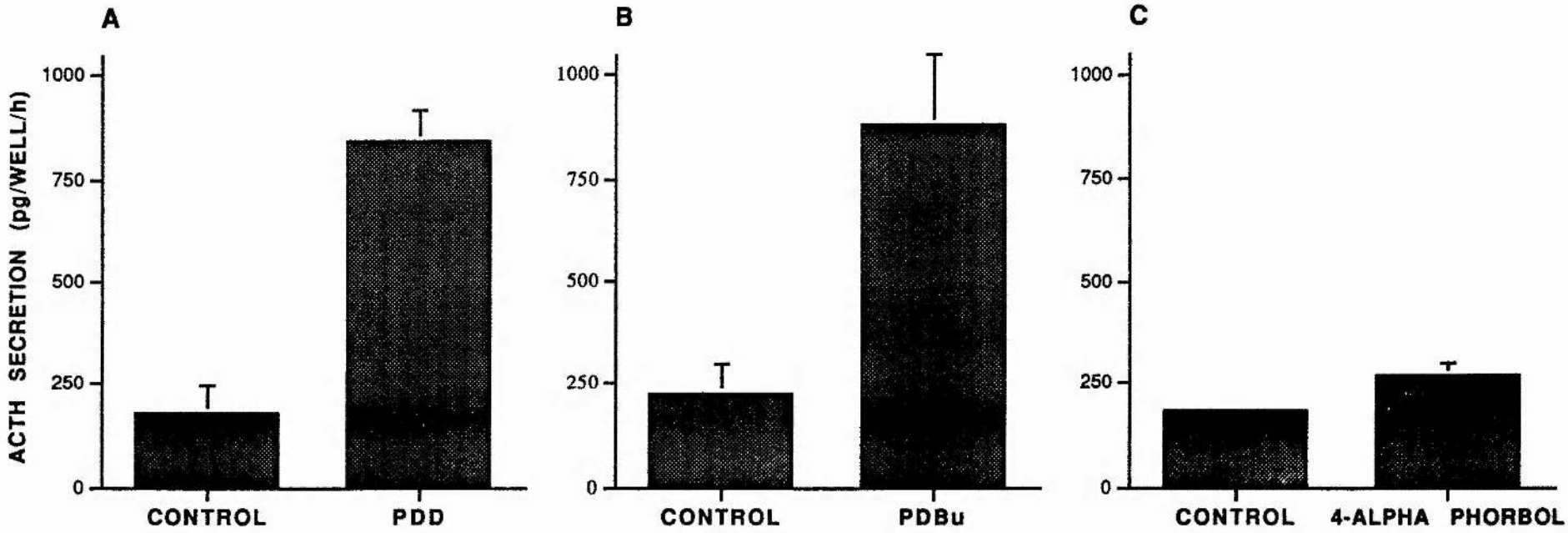

Figure 54 Effect of phorbol esters upon ACTH secretion from intact AtT-20 cells. Intact cells were incubated as described in the methods for 1 h in DMEM/BSA supplemented with PDD (10^{-7}M, panel A), PDBu (10^{-7}M, panel B) or 4 α phorbol (10^{-7}M, panel C) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the column.

(ii) Effects of PMA upon the late stages of the ACTH secretory pathway of AtT-20 cells

Calcium ions stimulated ACTH secretion from permeabilised AtT-20 cells in a concentration-dependent manner (figure 55). This calcium ion-evoked secretion became significantly greater than control secretion at concentrations above 10^{-7}M. This stimulated secretion was enhanced at every free calcium ion concentration investigated by PMA (10^{-7}M) including in the effective absence of free calcium ions (figure 55). ACTH secretion in standard permeabilisation medium was 55 ± 5 pg/10^5 cells/30 min in the absence of PMA compared to 108 ± 2 pg/10^5 cells/30 min in the presence of PMA. ACTH secretion in response to a free calcium ion concentration of 10^{-5}M was 215 ± 13 pg/10^5 cells/30 min in the absence of PMA compared to 491 ± 27 pg/10^5 cells/30 min in the presence of PMA. The ability of PMA to enhance calcium ion-stimulated secretion suggests that PKC can stimulate a secretory response from AtT-20 cells by an action which is unrelated to the elevation of the cytosolic free calcium ion concentration. In addition the ability of PMA to stimulate ACTH secretion in standard permeabilisation medium, which contains effectively no calcium ions, may also suggest that nPKC isozymes stimulate ACTH secretion from AtT-20 cells by an action which is unrelated to calcium ion/G_e-stimulated secretion. Calcium ions and PMA however did interact in a synergistic manner indicating that there may be some kind of interaction between calcium ions and PKC with regard to ACTH secretion. One explanation is that in the presence of calcium ions cPKCs make an increasingly important contribution to the secretory response. As demonstrated in the present study AtT-20 cells contain both calcium ion-dependent cPKCs and calcium ion-independent nPKCs (see p74). At lower calcium ion concentrations PMA will activate only the nPKCs (Ryves *et al.*, 1991) whereas at higher

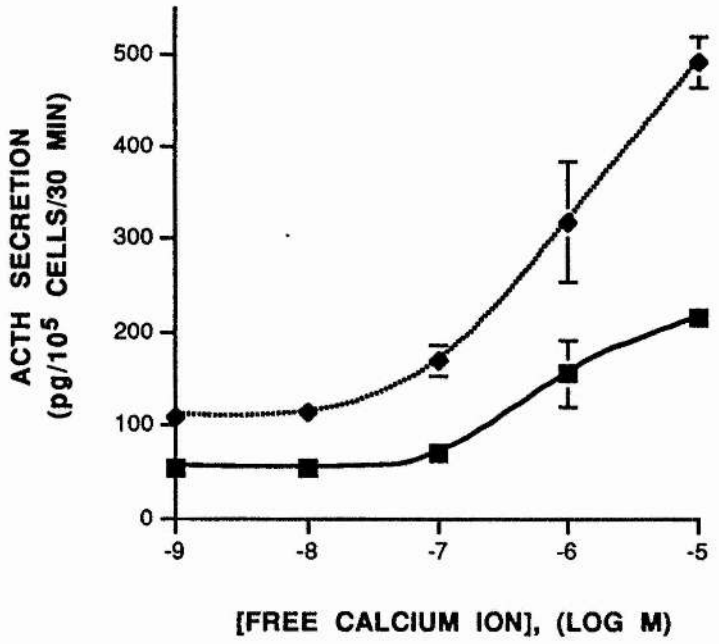

Figure 55 Effect of PMA upon calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated for 30 min as described in the methods in a series of calcium/EGTA buffers such that the indicated cytosolic free calcium ion concentration resulted in the presence (◆) and absence (■) of PMA (10^{-7}M) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

calcium ion concentrations PMA will activate both nPKCs and cPKCs (Ryves *et al.*, 1991). It may be the case that cPKCs as well as nPKCs contribute to the ACTH secretory response by a mechanism which is independent of calcium ions and G_e which therefore could account for this synergistic interaction.

The effect of chelerythrine chloride upon PMA-stimulated secretion was investigated in order to determine the effectiveness of this PKC inhibitor under the conditions employed in the present study. PMA (10^{-7}M) significantly stimulated ACTH secretion from permeabilised AtT-20 cells in standard permeabilisation medium over 30 min (figure 56). This PMA-stimulated ACTH secretion was completely reversed by the PKC inhibitor chelerythrine chloride (10^{-5}M) (figure 56). Control secretion was 56 $\pm$ 1 pg/10^5 cells/30 min compared to PMA-stimulated secretion which was 129 $\pm$ 22 pg/10^5 cells/30 min. This PMA-stimulated secretion was reduced to 74 $\pm$ 12 pg/10^5 cells/30 min in the presence of chelerythrine chloride. PMA (10^{-7}M) also significantly stimulated ACTH secretion from permeabilised AtT-20 cells in a free calcium ion concentration of 10^{-5}M over 30 min (figure 57). This PMA-stimulated ACTH secretion was also completely reversed by the PKC inhibitor chelerythrine chloride (10^{-5}M) (figure 57). Control secretion was 199 $\pm$ 26 pg/10^5 cells/30 min compared to PMA-stimulated secretion which was 341 $\pm$ 30 pg/10^5 cells/30 min. This PMA-stimulated secretion was reduced to 254 $\pm$ 19 pg/10^5 cells/30 min in the presence of chelerythrine chloride. These findings suggest that chelerythrine chloride under the conditions employed in the present study is an effective inhibitor of PKC both in the presence and absence of calcium ions.

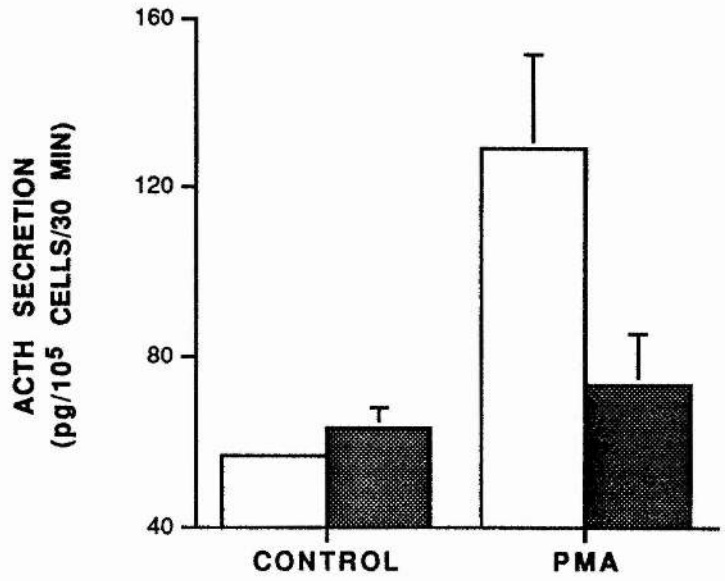

Figure 56 Effect of chelerythrine chloride upon PMA-stimulated ACTH secretion from permeabilised AtT-20 cells in the effective absence of calcium ions. Permeabilised cells were incubated as described in the methods in standard permeabilisation medium for 30 min in the presence of PMA (10^{-7}M) as indicated and also in the presence of (shaded columns) or absence (open columns) of chelerythrine chloride (10^{-5}M) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the column.

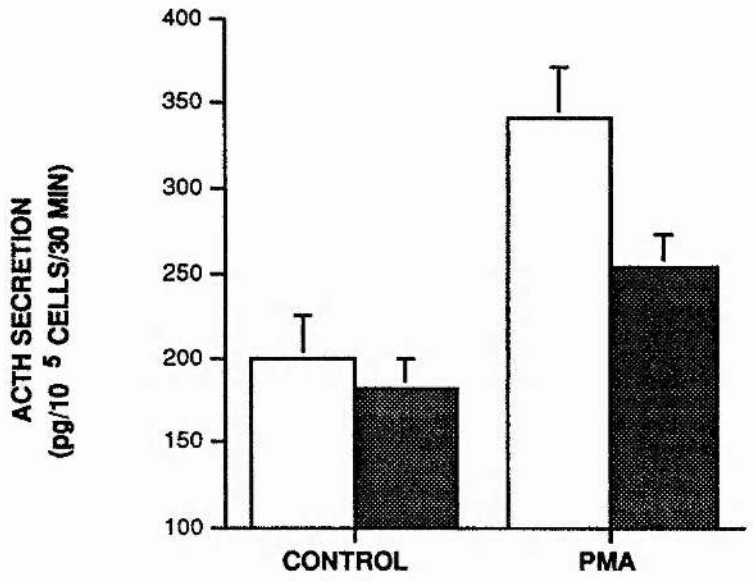

Figure 57 Effect of chelerythrine chloride upon PMA-stimulated ACTH secretion from permeabilised AtT-20 cells in the presence of calcium ions. Permeabilised cells were incubated as described in the methods in calcium/EGTA buffers such that a free calcium ion concentration of 10^{-5}M resulted for 30 min in the presence of PMA (10^{-7}M) as indicated and also in the presence of (shaded columns) or absence (open columns) of chelerythrine chloride (10^{-5}M) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments.

Further studies indicate that PMA is also able to enhance secretion in response to GTP- γ -S in the absence of calcium ions and also in the presence of a variety of free calcium ion concentrations. Calcium ions stimulated ACTH secretion from permeabilised AtT-20 cells in a concentration-dependent manner becoming significantly greater than control secretion at concentrations above 10^{-7}M (figure 58). As previously observed in standard permeabilisation medium, free calcium ion concentration of 10^{-9}M, GTP- γ - S (10^{-5}M) significantly stimulated ACTH secretion however was unable to enhance ACTH secretion observed in response to a free calcium ion concentration of 10^{-5}M (figure 58). PMA (10^{-7}M) enhanced secretion in response to every concentration of calcium ions investigated both in the presence and absence of GTP- γ -S (figure 58). PMA generally appeared to enhance GTP- γ -S-stimulated secretion in an additive manner at every concentration of calcium ions investigated. This finding seems to confirm that PKC is able to stimulate secretion by an action which is quite separate from calcium ion/G_e-stimulated secretion.

(iii) Effects of isozyme-selective protein kinase C activators upon the late stages of the ACTH secretory pathway of AtT-20 cells

Having established that PKC has at least two points of regulation with regard to ACTH secretion from AtT-20 cells the possibility that different PKC isozymes regulate secretion at different points in the stimulus-secretion coupling pathway was investigated. PKC isoform immunoreactivity of whole AtT-20 cells, detected by Western analysis using isoform-specific antisera, revealed detectable amounts of PKCs α, β, ε and ζ, however γ, δ and η could not be detected under the conditions employed although standards were readily detectable (figure 59).

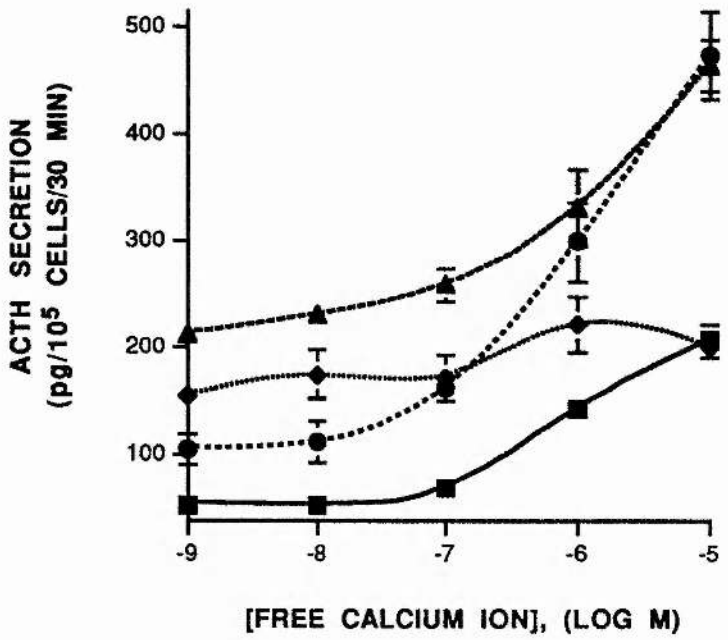

Figure 58 Effects of GTP- γ-S and PMA upon calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells.
Permeabilised cells were incubated for 30 min as described in the methods in a series of calcium/EGTA buffers such that the indicated cytosolic free calcium ion concentration resulted in the absence of added secretagogues (■), presence of GTP- γ-S (10^{-5}M, ◆), presence of PMA (10^{-7}M, ●) and the presence of both GTP- γ-S and PMA (▲) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

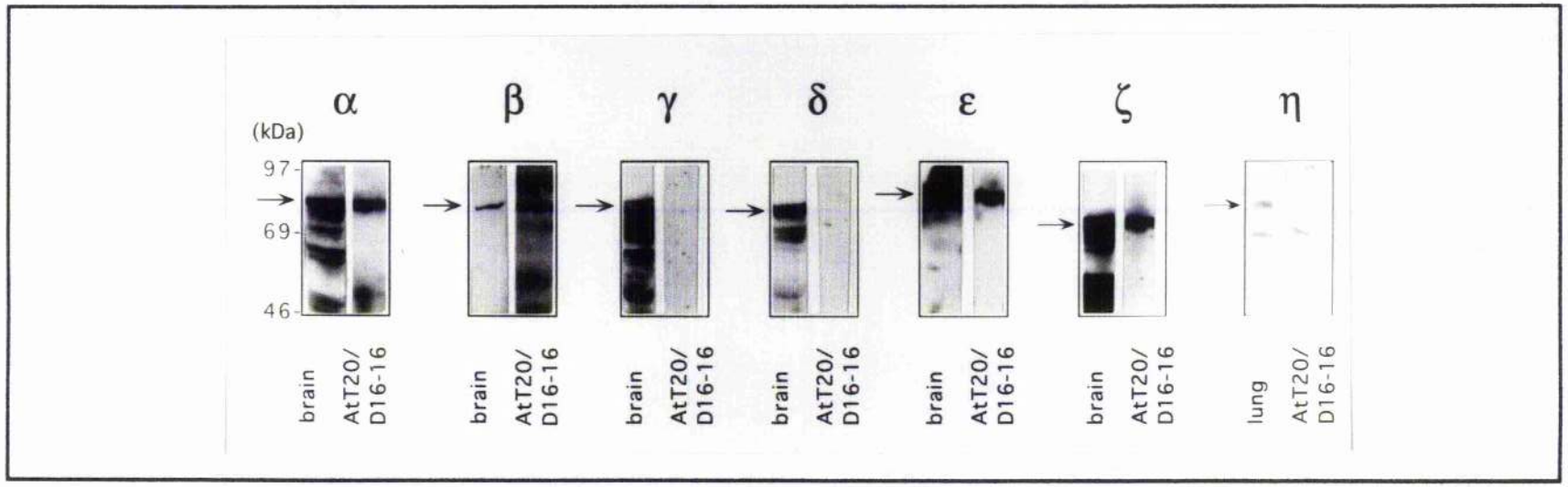

Figure 59 Protein kinase C immunoreactivity of whole AtT-20 cells. One dimensional SDS/PAGE followed by Western analysis with PKC-specific anti-sera was performed upon whole male rat brains (lung for η) as positive controls (left lanes) and whole AtT-20 cells (right lanes). The above study was carried out by the method of Strulovici *et al.* (1989 & 1991) by Dr D.J. MacEwan.

Particular PKC isozymes were activated by thymeleatoxin (TMX), reported to be selective for the cPKCs α, β and γ isozymes, and 12-deoxyphorbol 13-phenyl acetate 20-acetate (dPPA), reported to be selective for the β_1-isozyme (Ryves *et al.*, 1991). TMX and dPPA both stimulated ACTH secretion from intact AtT-20 cells in a concentration-dependent manner over 1 h becoming significantly greater than control secretion in both cases at concentrations above 10^{-8}M in the case of TMX and 10^{-9}M in the case of dPPA (figure 60). In both cases 10^{-7}M was adopted as a standard concentration for further experimentation. ACTH secretion in response to TMX (10^{-7}M) was 917 $\pm$ 143 pg/well/h compared to control secretion of 237 $\pm$ 22 pg/well/h. ACTH secretion in response to dPPA (10^{-7}M) was 631 $\pm$ 73 pg/well/h compared to control secretion of 265 $\pm$ 46 pg/well/h. This finding may suggest that activation of the α and β isozymes or simply the β_1 isozyme can stimulate ACTH secretion from intact AtT-20 cells. Whether the actions of these isozymes were proximal or distal to changes in cytosolic free calcium ion levels was subsequently investigated.

Calcium ions stimulated ACTH secretion from permeabilised AtT-20 cells in a concentration-dependent manner becoming significantly greater than control secretion at concentrations above 10^{-7}M (figure 61). Calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells was not significantly different in the presence and absence of dPPA (10^{-7}M) (figure 61). ACTH secretion in response to a free calcium ion concentration of 10^{-5}M was 1603 $\pm$ 175 pg/10^5 cells/30 min in the absence of dPPA compared to 1340 $\pm$ 147 pg/10^5 cells/30 min in the presence of dPPA. The inability of dPPA to potentiate calcium ion-stimulated secretion indicates that the β_1 isozyme does not contribute to secretion by an action distal to changes in

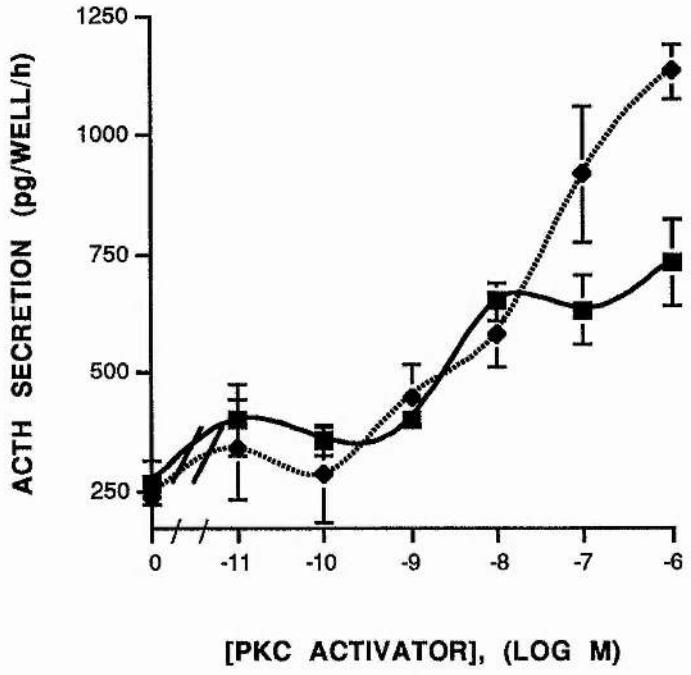

Figure 60 Effect of protein kinase C activators upon ACTH secretion from intact AtT-20 cells. Intact cells were incubated as described in the methods for 1 h in DMEM/BSA supplemented with the indicated concentrations of dPPA (■) or TMX (◆) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

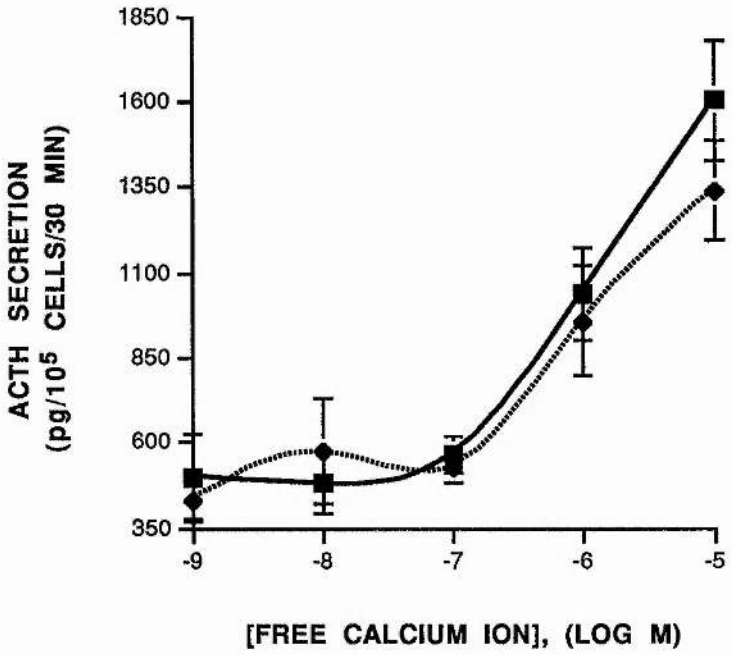

Figure 61 Effect of dPPA upon calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated for 30 min as described in the methods in a series of calcium/EGTA buffers such that the indicated cytosolic free calcium ion concentration resulted in the presence (◆) and absence (■) of dPPA (10^{-7}M) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

cytosolic free calcium ion concentrations and therefore may be at a point proximal to calcium ions in the stimulus-secretion coupling pathway.

Calcium ions stimulated ACTH secretion from permeabilised AtT-20 cells in a concentration-dependent manner becoming significantly greater than control secretion at concentrations above 10^{-7}M (figure 62). Calcium ion-stimulated ACTH secretion was significantly enhanced by TMX (10^{-7}M) at every concentration of calcium ions investigated (figure 62). ACTH secretion in response to a free calcium ion concentration of 10^{-5}M was 2654 ± 150 pg/10^5 cells/30 min in the absence of TMX compared to 3897 ± 279 pg/10^5 cells/30 min in the presence of TMX. It therefore appears that TMX enhanced calcium ion-stimulated ACTH secretion from permeabilised cells in a manner similar to PMA. In particular the ability of TMX to stimulate ACTH secretion from permeabilised AtT-20 cells in the effective absence of calcium ions may suggest that this drug is not completely selective for cPKCs under these conditions.

There have been suggestions that dPPA is not completely selective for the PKC β_1 isozyme (Kiley *et al.*, 1994), a point which was investigated in the present study. The effects of concentrations of dPPA greater than 10^{-7}M (shown to be supramaximal with regard to ACTH secretion from intact AtT-20 cells) upon control and calcium ion (10^{-5}M)-stimulated ACTH secretion were investigated. As previously demonstrated dPPA at a concentration of 10^{-7}M did not have any significant effect upon control or calcium ion stimulated ACTH secretion from permeabilised AtT-20 cells however dPPA at the higher concentration of 10^{-5}M did significantly enhance calcium ion-stimulated secretion (table 4). Higher concentrations of dPPA also enhanced control ACTH secretion from permeabilised AtT-20 cells although this proved not to be statistically significant. It appears that

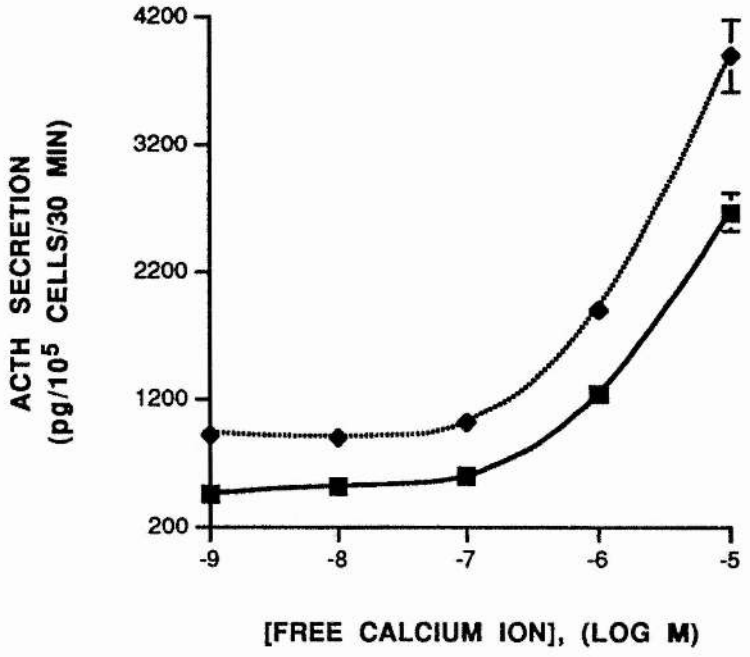

Figure 62 Effect of TMX upon calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated for 30 min as described in the methods in a series of calcium/EGTA buffers such that the indicated cytosolic free calcium ion concentration resulted in the presence (◆) and absence (■) of TMX (10^{-7}M) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

[dPPA]	ACTH SECRETION (pg/10^5 CELLS/30 MIN)	
	CONTROL	CALCIUM (10^{-5}M)
0	373 ± 37	737 ± 68
10^{-7}M	390 ± 40	860 ± 95
10^{-6}M	460 ± 46	1120 ± 113
10^{-5}M	490 ± 16	1240 ± 100

Table 4 Effect of higher concentrations of dPPA upon ACTH secretion from permeabilised AtT-20 cells in the presence and absence of calcium ions. Permeabilised cells were incubated for 30 min as described in the methods in calcium/EGTA buffers such that a cytosolic free calcium ion concentration of 10^{-9}M (control) or 10^{-5}M resulted in the presence of the indicated concentration of dPPA and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments.

higher concentrations of dPPA may activate PKC in a non-selective manner however the concentration of 10^{-7}M adopted in the present study as standard appears to have selective properties.

As can be seen figures 61 & 62 as well as table 4 indicate that in these particular experiments the absolute amount of ACTH released was relatively high in comparison to that from other experiments. This is thought to be due to variability within the assay and is therefore not likely to be physiologically important.

(iv) Effects of cyclic AMP upon the late stages of the ACTH secretory pathway of AtT-20 cells

Although cyclic AMP, in the absence of calcium ions and guanine nucleotides, is unable to stimulate ACTH secretion from permeabilised AtT-20 cells there is evidence to suggest that cyclic AMP can modulate calcium ion- and G_e-stimulated secretion (Guild, 1991). In the present study calcium ions (10^{-5}M) significantly stimulated ACTH secretion from permeabilised AtT-20 cells (figure 63). Cyclic AMP (10^{-4}M) as previously observed had no significant effect upon secretion in the absence of calcium ions (free calcium ion concentration of 10^{-9}M) however did significantly enhance secretion in response to a free calcium ion concentration of 10^{-5}M (figure 63). Control secretion was 68.1 $\pm$ 10.9 pg/10^5 cells/30 min in the absence of cyclic AMP and 90.4 $\pm$ 15.8 pg/10^5 cells/30 min in the presence of cyclic AMP (10^{-4}M). Calcium ion-stimulated secretion was 268.3 $\pm$ 31.7 pg/10^5 cells/30 min in the absence of cyclic AMP and 409.5 $\pm$ 23.1 pg/10^5 cells/30 min in the presence of cyclic AMP (10^{-4}M).

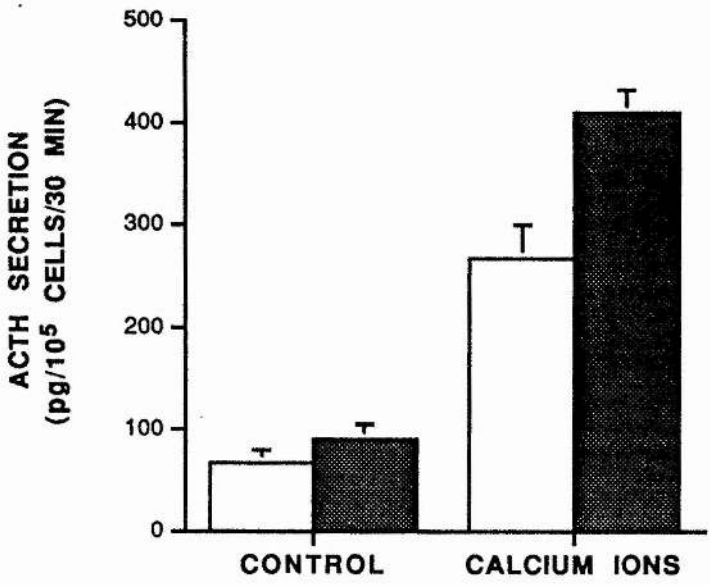

Figure 63 Effect of cyclic AMP upon calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated for 30 min as described in the methods in calcium/EGTA buffers such that a cytosolic free calcium ion concentration of 10^{-9}M (control) or 10^{-5}M (calcium ions) resulted in the presence (shaded columns) and absence (open columns) of cyclic AMP (10^{-4}M) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments.

GTP- γ -S (10^{-4}M) significantly stimulated ACTH secretion from permeabilised AtT-20 cells (figure 64). Cyclic AMP (10^{-4}M) had no significant effect upon secretion in the absence of GTP- γ -S however did significantly enhance secretion in response to GTP- γ -S (figure 64). Control secretion was 31.7 $\pm$ 3.7 pg/10^5 cells/30 min in the absence of cyclic AMP and 22.7 $\pm$ 6.0 pg/10^5 cells/30 min in the presence of cyclic AMP (10^{-4}M). GTP- γ -S-stimulated secretion was 125.2 $\pm$ 15.8 pg/10^5 cells/30 min in the absence of cyclic AMP and 217.6 $\pm$ 21.6 pg/10^5 cells/30 min in the presence of cyclic AMP (10^{-4}M).

PMA (10^{-7}M) significantly stimulated ACTH secretion from permeabilised AtT-20 cells (figure 65). Cyclic AMP (10^{-4}M) had no significant effect upon secretion in the absence of PMA however did significantly enhance secretion in response to PMA (figure 65). Control secretion was 68.1 $\pm$ 10.9 pg/10^5 cells/30 min in the absence of cyclic AMP and 90.4 $\pm$ 15.8 pg/10^5 cells/30 min in the presence of cyclic AMP (10^{-4}M). PMA-stimulated secretion was 137.9 $\pm$ 11.5 pg/10^5 cells/30 min in the absence of cyclic AMP and 252.4 $\pm$ 29.6 pg/10^5 cells/30 min in the presence of cyclic AMP (10^{-4}M).

It therefore appears that cyclic AMP, in the absence of calcium ions and guanine nucleotides, is unable to stimulate ACTH secretion from permeabilised AtT-20 cells however does potentiate calcium ion and GTP- γ -S-stimulated ACTH secretion in a synergistic manner. This confirms previous studies which suggested that PKA is capable of modulating calcium ion/G_e-stimulated ACTH secretion from AtT-20 cells (Guild, 1991). The present study suggests that PKA may also have a similar modulatory role with regard to PKC-stimulated ACTH secretion from AtT-20 cells.

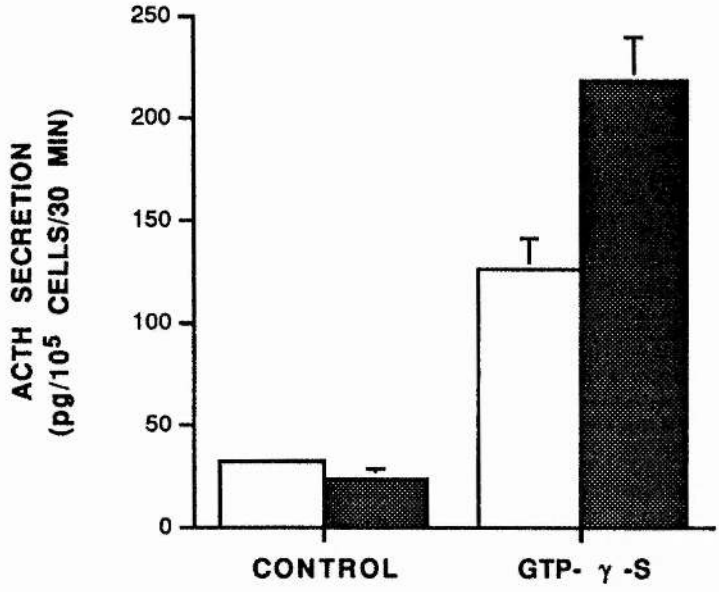

Figure 64 Effect of cyclic AMP upon GTP- γ -S-stimulated ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated for 30 min as described in the methods in standard permeabilisation medium in the presence and absence of GTP- γ -S (10^{-4}M) as indicated in the presence (shaded columns) and absence (open columns) of cyclic AMP (10^{-4}M) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the column.

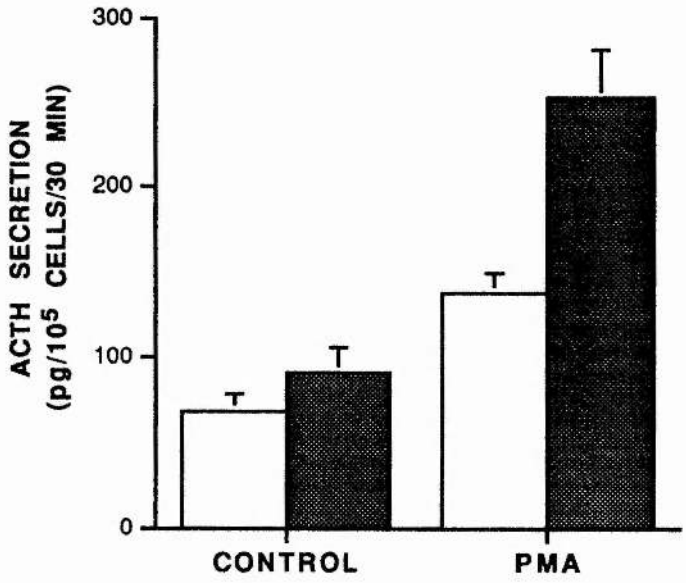

Figure 65 Effect of cyclic AMP upon PMA-stimulated ACTH secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated for 30 min as described in the methods in standard permeabilisation medium in the presence and absence of PMA (10^{-7}M) as indicated in the presence (shaded columns) and absence (open columns) of cyclic AMP (10^{-4}M) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments.

The effects of cyclic AMP upon calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells were compared to those of PMA. Calcium ions stimulated ACTH secretion from permeabilised AtT-20 cells in a concentration-dependent manner becoming significantly greater than control secretion at concentrations above 10^{-6}M which was significantly enhanced by cyclic AMP (10^{-4}M) and PMA (10^{-7}M) with the characteristics previously described (figure 66). This data highlights important differences between PMA and cyclic AMP mediated enhancement of calcium ion-stimulated ACTH secretion. PMA is capable of stimulating ACTH secretion in the effective absence of calcium ions whereas cyclic AMP is not. In addition PKC is capable of enhancing calcium ion-stimulated ACTH secretion to a greater extent than cyclic AMP. Secretion in response to a cytosolic free calcium ion concentration of 10^{-5}M was 268.3 $\pm$ 31.7 pg/10^5 cells/30 min in the absence of PMA and cyclic AMP, 681.7 $\pm$ 44.7 pg/10^5 cells/30 min in the presence of PMA and 409.5 $\pm$ 23.1 pg/10^5 cells/30 min in the presence of cyclic AMP. This observation highlights the fact that calcium ions can not elicit a full secretory response and that co-operation between calcium and PKA and in particular PKC is required to elicit what might be a maximal secretory response.

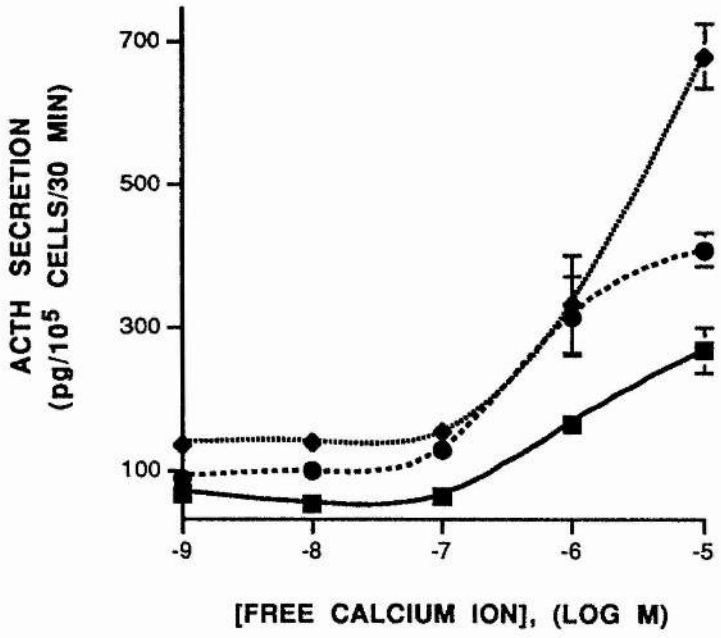

Figure 66 Effects of cyclic AMP and PMA upon calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells.
Permeabilised cells were incubated for 30 min as described in the methods in a series of calcium/EGTA buffers such that the indicated cytosolic free calcium ion concentration resulted in the absence of added secretagogues (■), presence of PMA (10^{-7}M, ◆) and the presence of cyclic AMP (10^{-4}M, ●) and ACTH secretion measured by radioimmunoassay. The results are expressed as the mean ± s.e. mean from three separate experiments. Absence of error bars indicate that they lie within the symbol.

DISCUSSION

1 AN INVESTIGATION INTO THE INVOLVEMENT OF GTP-BINDING PROTEINS MEDIATING CALCIUM ION-STIMULATED ACTH SECRETION FROM AtT-20 CELLS

(i) Effects of calcium ions upon the late stages of the ACTH secretory pathway of AtT-20 cells

Signal transduction mechanisms controlling exocytosis have been studied extensively in recent years using the technique of cell permeabilisation which overcomes the plasma membrane barrier and allows the manipulation of the intracellular environment. The effects of calcium ions upon ACTH secretion and the mechanisms mediating calcium ion-stimulated secretion were largely investigated in the present study using AtT-20 cells permeabilised by the high voltage electric discharge technique. Electrical permeabilisation produces membrane breakdown which is predictable, highly selective for the plasma membrane and localised to certain areas of the plasma membrane (Knight & Baker, 1982). The resulting pores, which are generally 2-4 nm in diameter, are large enough to allow cytosolic dialysis and the introduction of ions and small molecules yet small enough to prevent leakage of cytosolic macromolecules and therefore allow the cell to retain a relatively intact secretory response (Knight & Scruton, 1986). In contrast cells permeabilised using the detergent digitonin (Dunn & Holz, 1983; Wilson & Kirshner, 1983) or the protein streptolysin-O (Bader *et al.*, 1986; Bader *et al.*, 1989), which are other commonly employed methods of cell permeabilisation, display a significant decay in the secretory response with time. In addition since both digitonin (Gogelein & Huby, 1984) and streptolysin-O (Bhakdi & Tranum-Jensen, 1987) facilitate cell permeabilisation by interacting with membrane cholesterol it is likely that

these methods are not completely selective for the plasma membrane. This technique of electrical permeabilisation has previously been successfully employed to study calcium ion-stimulated ACTH secretion from AtT-20 cells (Guild, 1991).

It was Douglas who first suggested that raising the concentration of calcium ions in the cytosol of specialised cells may result in exocytosis (Douglas, 1968 & 1974 b). Direct evidence for this theory was subsequently obtained when it was demonstrated that raising the concentration of free calcium ions in the cytosol of permeabilised adrenal medullary cells to the micromolar range resulted in catecholamine secretion (Baker & Knight, 1978). This has since been shown to be the case in a variety of other permeabilised secretory cell types (Gomperts, 1990; Knight & Scrutton, 1986; Lindau & Gomperts, 1991). In the present study it was demonstrated that raising the concentration of free calcium ions in the permeabilisation medium stimulated ACTH secretion from electrically-permeabilised AtT-20 cells in a concentration-dependent manner with a threshold of 10^{-7}M reaching a maximal stimulation by 10^{-5}M. This data is highly consistent with previous studies investigating calcium ion-stimulated ACTH secretion from electrically-permeabilised (Guild, 1991) and digitonin-permeabilised (Luini & DeMatteis, 1988) AtT-20 cells and also with a variety of other cell types (Knight & Scrutton, 1986). It therefore appears that permeabilising AtT-20 cells by means of the high voltage electric discharge technique does not impair their ability to undergo evoked exocytosis and that electrically-permeabilised AtT-20 cells serve as a useful model system for the investigation of calcium ion-stimulated ACTH secretion.

(ii) Effects of GTP- γ -S upon the late stages of the ACTH secretory pathway of AtT-20 cells

Using permeabilised neutrophils Gomperts and co-workers observed that guanine nucleotides participate in the stimulus-secretion coupling pathway at two distinct points (Barrowman *et al.*, 1986). The first was thought to be the GTP-binding protein G_q, now well established as being responsible for the activation of PLC (Smrcka *et al.*, 1991; Taylor *et al.*, 1991) which results in the generation of DAG and subsequent activation of PKC (Nishizuka, 1995). The other was thought to be a second GTP-binding protein which was involved at a stage in the stimulus-secretion coupling pathway distal to changes in the cytosolic free calcium ion concentration and was also independent of PKC activation. Since this second GTP-binding protein represented a more direct means of regulating exocytosis it was subsequently termed G_e (Gomperts *et al.*, 1986). Using a variety of permeabilisation techniques guanine nucleotides have since been shown to stimulate secretion from a variety of specialised secretory cell types. In addition evidence exists to suggest that a similar G_e-like GTP-binding protein is present in a variety of specialised cell types (Gomperts, 1990; Lindau & Gomperts, 1991).

A number of studies have suggested that a similar mechanism operates in the ACTH stimulus-secretion coupling pathway of AtT-20 cells. The stable GTP analogue GTP- γ -S has been shown to be capable of stimulating ACTH secretion independently of changes in the cytosolic free calcium ion concentration from both electrically-permeabilised (Guild, 1991) and digitonin-permeabilised (Luini & DeMatteis, 1988 & 1990) AtT-20 cells. This was confirmed in the present study with the observation that GTP- γ -S stimulated ACTH secretion from permeabilised AtT-20 cells in a

concentration-dependent manner in the effective absence of calcium ions. Since any changes in the cytosolic free calcium ion concentration induced by GTP- γ -S would have been buffered by the calcium/EGTA buffers, designed to establish and maintain the required cytosolic free calcium ion concentration which in the case of this particular set of experiments was 10^{-9}M (effectively zero), it can be assumed that this guanine nucleotide-evoked secretion is independent of changes in the cytosolic free calcium ion concentration. This observation is therefore consistent with the findings of previous studies (Guild, 1991; Luini & DeMatteis, 1988 & 1990) which first suggested that a GTP-binding protein (or proteins) is involved in the ACTH stimulus-secretion coupling pathway of AtT-20 cells at a stage distal to changes in cytosolic free calcium ion concentrations.

The finding that the stable GDP analogue GDP- β -S inhibits calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells (Guild, 1991) may suggest that this GTP-binding protein involved in the stimulus-secretion coupling pathway at a stage distal to calcium ions in fact mediates the effects of calcium ions upon the secretory response of this cell line. This was confirmed in the present study by investigating the effects of calcium ions and GTP- γ -S in combination upon ACTH secretion from permeabilised AtT-20 cells. Although GTP- γ -S was able to stimulate ACTH secretion from permeabilised AtT-20 cells in the absence of calcium ions this stable GTP analogue was unable to enhance secretion in response to a free calcium ion concentration of 10^{-5}M which is maximal with regard to ACTH secretion. The inability of GTP- γ -S to enhance calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells would suggest that calcium ions and GTP- γ -S are stimulating secretion via a common mechanism. Since the effects of GTP- γ -S are not mediated by raising the cytosolic free calcium ion concentration it is likely that the

effects of calcium ions upon ACTH secretion from AtT-20 cells are mediated by activation of a GTP-binding protein (or proteins). This observation is again consistent with a previous study which observed a similar interaction between GTP- γ -S and calcium ions with regard to evoked ACTH secretion from AtT-20 cells (Guild, 1991) as well as studies carried out in permeabilised chromaffin cells (Bittner & Holz, 1986) and RINm5F cells (Valler *et al.*, 1987).

It can therefore be concluded from the findings of the present and previous (Guild, 1991; Luini & DeMatteis, 1988 & 1990) studies that a GTP-binding protein mediates calcium ion-stimulated ACTH secretion from AtT-20 cells. This finding is consistent with the model for the late stages of the stimulus-secretion coupling pathway proposed by Gomperts and co-workers in which a GTP-binding protein, G_e, plays a similar role (Gomperts, 1990; Gomperts & Lindau, 1991). As a result the GTP-binding protein present in AtT-20 cells which mediates calcium ion-stimulated ACTH secretion will subsequently be referred to as G_e.

(iii) Mechanisms mediating calcium ion- and G_e-stimulated ACTH secretion from AtT-20 cells

There is evidence to suggest that both PKA (Guild, 1991) and PKC (Guild & Reisine, 1987; Reisine 1989), in addition to raising cytosolic free calcium ion levels, may regulate ACTH secretion from AtT-20 cells by an action in the stimulus-secretion coupling pathway distal to changes in the cytosolic free calcium ion concentration. Therefore the possibility that calcium ion- and G_e-stimulated ACTH secretion from AtT-20 cells was mediated by activation of either PKA or PKC was considered.

The possibility that calcium ion- and G_e-stimulated ACTH secretion from AtT-20 cells is mediated via activation of PKC was investigated using the naturally occurring alkaloid chelerythrine chloride which appears to be a potent (IC_{50} of 6.6 x 10^{-7}M) inhibitor of PKC (Herbert *et al.*, 1990). In addition chelerythrine chloride has been shown to be without effect upon a variety of other enzymes (Cohen *et al.*, 1978; Herbert *et al.*, 1990) and from the information available to date it appears that chelerythrine chloride displays a high degree of selectivity towards inhibition of PKC. Chelerythrine chloride, at the concentration used in this study, has been shown in the present study to be an effective PKC inhibitor in AtT-20 cells by completely inhibiting the effects of the PKC activator PMA. This attenuation was demonstrated in both the absence and presence of calcium ions indicating that chelerythrine chloride under the present conditions may inhibit both cPKCs and nPKCs present in this cell line. Calcium ions and GTP- γ -S were both able to stimulate ACTH secretion from permeabilised AtT-20 cells to the same extent in the presence or absence of chelerythrine chloride (10^{-5}M). The inability of chelerythrine chloride to inhibit calcium ion- and GTP- γ -S-stimulated ACTH secretion from permeabilised AtT-20 cells therefore suggests that the effects of calcium ions and G_e upon secretion are not mediated by PKC. This finding rules out the possibility that activation of G_q (or another GTP-binding protein responsible for PLC activation), generation of DAG and subsequent PKC activation contributes to secretion from AtT-20 cells under these conditions.

Previous studies using the Walsh PKA inhibitor (PKI) (Walsh *et al.*, 1971) also argues against the suggestion that PKA may mediate calcium ion-stimulated ACTH secretion from AtT-20 cells. The calcium ionophore ionomycin has been shown to raise the cytosolic free calcium ion

concentration of AtT-20 cells which in turn results in an ACTH secretory response (Guild & Reisine, 1987). Introduction of the PKA inhibitor by means of a targeted liposome technique had no effect upon this ionomycin/calcium ion-induced secretory response (Guild & Reisine, 1987). Since PKI is an effective inhibitor of PKA in AtT-20 cells (Miyazaki *et al.*, 1984) this finding suggests that calcium ion-stimulated ACTH secretion from this cell line is not mediated by PKA.

A number of observations from the present study also suggest that PKA does not contribute to ACTH secretion from permeabilised AtT-20 cells in the absence of calcium ions. The present study demonstrated that cyclic AMP was unable to stimulate ACTH secretion from permeabilised AtT-20 cells in the absence of calcium ions, a result consistent with a previous study (Guild, 1991). In addition agents known to stimulate cyclic AMP, namely CRF-41 and forskolin, were unable to stimulate ACTH secretion from permeabilised AtT-20 cells in the absence of calcium ions. It therefore appears that activation of PKA does not result in ACTH secretion from AtT-20 cells under these conditions. Since GTP- γ -S was able to stimulate ACTH secretion from permeabilised AtT-20 cells in the effective absence of calcium ions it seems that calcium ion/G_e-stimulated ACTH secretion from AtT-20 cells is not mediated by activation of PKA. This observation rules out the possibility that activation of G_s, the GTP-binding protein responsible for adenylate cyclase activation and subsequent cyclic AMP generation (Iyenger, 1993), contributes to ACTH secretion from AtT-20 cells under these conditions.

The possibility that a kinase may mediate calcium ion- and G_e-stimulated ACTH secretion from AtT-20 cells was further investigated by omitting ATP from the permeabilisation medium. ATP is included in the

standard permeabilisation medium as initial studies reported that calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells was almost completely dependent upon the presence of ATP (Guild, 1991; Luini & DeMatteis, 1988). The present study indicates that GTP- γ -S-stimulated ACTH secretion from permeabilised AtT-20 cells can still proceed when ATP is omitted from the permeabilisation medium. Since ATP is a requirement for kinase activation (Goldsmith & Cobb, 1994; Pawson, 1994; Taylor *et al.*, 1993) this would suggest that GTP- γ -S-stimulated ACTH secretion from permeabilised AtT-20 cells and therefore the actions of G_e are not mediated by kinase activation.

It therefore appears that calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells is ATP-dependent (Guild, 1991; Luini & DeMatteis, 1988) whereas GTP- γ -S-stimulated secretion is ATP-independent. This apparent contradiction could indicate that calcium ions and G_e stimulate ACTH secretion from AtT-20 cells via different mechanisms, however this may not be the case. Calcium ions stimulate ACTH secretion from AtT-20 cells via activation of a GTP-binding protein, therefore a requirement for this evoked secretion must be GTP in order for activation of G_e to occur. The standard permeabilisation medium employed in the present study contains no GTP yet calcium ions are capable of stimulating ACTH secretion from permeabilised AtT-20 cells under these conditions. It is possible that ATP, via conversion by the ubiquitous enzyme nucleoside diphosphate kinase, provides a source of GTP which would allow calcium ion-stimulated ACTH secretion to proceed (Gomperts, 1990). Since GTP- γ -S is itself a source of GTP, if this hypothesis was correct, ACTH secretion from permeabilised AtT-20 cells in response to this guanine nucleotide would not be expected to be dependent upon ATP. This was indeed shown to be the case in the present study, therefore it

appears that calcium ion-stimulated ACTH secretion from AtT-20 cells is dependent upon ATP to provide a source of GTP in order to allow activation of the GTP-binding protein G_e.

Protein dephosphorylation is now known to play an equally important role in signal transduction to that of phosphorylation (for review see Hunter, 1995) therefore having established that a kinase does not mediate calcium ion/G_e-stimulated ACTH secretion from AtT-20 cells the possibility that a phosphatase does was considered. It has already been postulated that protein dephosphorylation may well be a step mediating the effects of G_e upon secretion (Gomperts, 1990; Lindau & Gomperts, 1991) following the observation that ATP retards the onset of secretion in permeabilised mast cells (Tatham & Gomperts, 1989). In addition studies carried out in AtT-20 cells have also observed a dephosphorylation of a 14 kDa protein which shows a significant correlation with evoked secretion (Bishop *et al.*, 1987).

Previous studies carried out in AtT-20 cells have indicated that approximately 60 % of the total phosphatase activity of this cell line can be attributed to type 1 or 2A serine/threonine specific phosphatases (Antoni *et al.*, 1993). The physiological relevance of this observation is however unclear therefore the possibility that a type 1 or 2A phosphatase mediates the effects of changes in the cytosolic free calcium ion concentration and also of G_e upon ACTH secretion from AtT-20 cells was therefore investigated. This was achieved using calyculin A, originally isolated from the marine sponge *Discodermia calyx* (Kateo *et al.*, 1986), now established as an inhibitor of type 1 and type 2A serine/threonine specific phosphatases (Ishihara *et al.*, 1989). Calyculin A inhibits type 2A phosphatases with a similar potency (IC_{50} of 10^{-10}-10^{-9}M) to okadaic acid,

however calyculin A is some 20- to 300-fold more potent than okadaic acid with regard to inhibition of type 1 phosphatases (Ishihara *et al.*, 1989). Therefore in certain cases calyculin A represents a more practical alternative to okadaic acid and was adopted as a phosphatase inhibitor in the present study.

Calcium ion- and GTP-γ-S-stimulated ACTH secretion from permeabilised AtT-20 cells were both completely inhibited by calyculin A at every concentration of these secretagogues investigated. This indicates that dephosphorylation, perhaps by a type 1 or 2A phosphatase, plays an important role in the ACTH stimulus-secretion coupling pathway of AtT-20 cells and is involved in regulating the effects of calcium ions and G_e upon ACTH secretion from these cells. This finding is consistent with a study carried out in permeabilised pancreatic acinar cells (Wagner *et al.*, 1992) in which the late stages of the amylase secretory pathway also appear to involve a calcium ion/G_e mechanism which is mediated by a calyculin A-sensitive phosphatase.

(iv) Summary

Initial results from the present study are highly consistent with a number of other studies investigating calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells (Guild, 1991; Luini & Guild, 1988 & 1990). It appears that the effects of calcium ions upon ACTH secretion from AtT-20 cells are mediated by a GTP-binding protein (or proteins) which has been shown to perform a similar function in a variety of specialised cells and has been dubbed G_e (Gomperts, 1990; Lindau & Gomperts, 1991). Previous studies have indicated that calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells is highly

dependent upon the presence of ATP in the permeabilisation medium (Guild, 1991; Luini & DeMatteis, 1988). Results from the present study indicate that ATP may be required as a source of GTP, a requirement for G_e activation, and may account for the ATP dependency of calcium ion-stimulated ACTH secretion from AtT-20 cells.

Both PKA (Guild, 1991) and PKC (Guild & Reisine, 1987; Reisine, 1989) have been implicated in the regulation of ACTH secretion from AtT-20 cells at a stage distal to changes in the cytosolic free calcium ion concentration. Results from the present study suggest that the effects of calcium ions and G_e upon ACTH secretion from AtT-20 cells are independent of activation of PKA, PKC or any other kinase. In contrast studies carried out using the phosphatase inhibitor calyculin A indicate that calcium ion/G_e-stimulated secretion is in fact mediated by a phosphatase. A schematic diagram outlining a proposed model for the mechanisms mediating calcium ion-stimulated ACTH secretion from AtT-20 cells is displayed in figure 67.

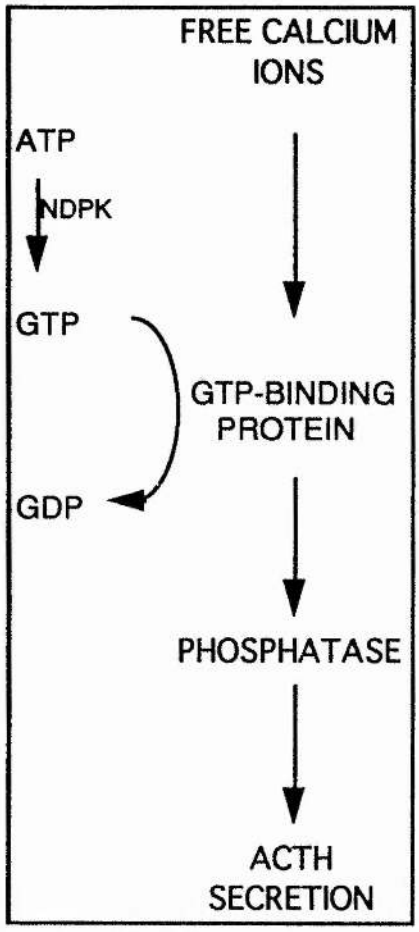

Figure 67 Schematic diagram showing the mechanisms postulated to mediate calcium ion-stimulated ACTH secretion from AtT-20 cells. ATP, adenosine 5'-triphosphate; GDP, guanosine 5'-diphosphate; GTP, guanosine 5'-triphosphate; NDPK, nucleoside diphosphate kinase.

2 AN INVESTIGATION INTO THE NATURE OF THE GTP-BINDING PROTEIN MEDIATING CALCIUM ION-STIMULATED ACTH SECRETION FROM AtT-20 CELLS

GTP-binding proteins represent a large and diverse family of transducing proteins which are involved in a variety of cellular functions (for reviews see Bourne *et al.*, 1990 & 1991; Kaziro *et al.*, 1991). Many of these GTP-binding proteins have been implicated in various aspects of the secretory process, both as part of the secretory apparatus itself and also as part of the signal-transduction pathways regulating secretion (for reviews see Ferro-Novick & Novick, 1993; Nuoffer & Balch, 1994; Pfeffer, 1992). Having established that a GTP-binding protein mediates the effects of calcium ions upon ACTH secretion from AtT-20 cells (Guild, 1991; Luini & DeMatteis, 1988 & 1990 & present study) the aim of this section of the present study was therefore to further characterise this protein G_e.

Results already obtained using AtT-20 cells may give some indication of the nature of G_e in this particular cell type. Many organelles involved in intracellular protein transport have at least one monomeric GTP-binding protein on their cytoplasmic surface and these proteins have been implicated in the regulation of both the constitutive and regulated secretory pathways (Ferro-Novick & Novick, 1993; Nuoffer & Balch, 1994; Pfeffer, 1992). In particular monomeric GTP-binding proteins belonging to the rab subfamily are thought to be associated with the ACTH containing secretory vesicles of AtT-20 cells (Ngsee *et al.*, 1993) and as a result represent a candidate for G_e. These small molecular weight GTP-binding proteins which participate in membrane traffic are postulated to cycle between a GTP and a GDP bound state (Pfeffer, 1992) therefore the non-hydrolysable GTP analogue GTP- γ -S would arrest this cycle in the GTP

bound conformation and result in an inhibition of this process in contrast to heterotrimeric GTP-binding proteins which upon binding GTP- γ -S become persistently activated (Gilman, 1987; Taylor, 1990). The ability of GTP- γ -S to stimulate ACTH secretion from permeabilised AtT-20 cells by an action upon G_e (Guild, 1991; Luini & DeMatteis, 1988 & 1990 & present study) does not rule out the possibility that G_e present in AtT-20 cells is a monomer however it does suggest that this GTP-binding protein does not function in a similar manner to these monomeric GTP-binding proteins which are already known to participate in vesicular traffic.

Heterotrimeric GTP-binding proteins are also thought to regulate intracellular protein transport (Ferro-Novick & Novick, 1993; Nuoffer & Balch, 1994; Pfeffer, 1992) and have been located on intracellular membranes including those of chromaffin (Toutant *et al.*, 1987) and β (Konrad *et al.*, 1995) cell secretory granules. In addition an inhibitory form of G_e, designated G_{ei}, has been identified in AtT-20 cells as a pertussis toxin-sensitive heterotrimeric GTP-binding protein (Luini & DeMatteis, 1988 & 1990). Heterotrimeric GTP-binding proteins therefore also represent a possible candidate for G_e and as a result the possibility that G_e, which mediates calcium ion-stimulated ACTH secretion from AtT-20 cells, displays characteristics consistent with it also belonging to the heterotrimeric rather than the monomeric subfamily of GTP-binding proteins was investigated.

(i) Effects of aluminium fluoride upon the late stages of the ACTH secretory pathway of AtT-20 cells

$AlF_{(3-5)}$ does not activate monomeric GTP-binding proteins (Kahn, 1991) but does however activate heterotrimeric GTP-binding proteins by

binding to GDP and mimicking the γ -phosphoryl group of GTP and hence shifting the GTP-binding protein into the active conformation (Higashijima *et al.*, 1991). $AlF_{(3-5)}$ is therefore a useful pharmacological tool which can be used to discriminate between heterotrimeric and monomeric GTP-binding protein involvement in cellular functions. The effect of $AlF_{(3-5)}$ upon the GTP-binding protein G_e, present in AtT-20 cells, was therefore investigated in the present study in an attempt to determine whether this protein belongs to the heterotrimeric family of GTP-binding proteins.

The effect of $AlF_{(3-5)}$ upon cyclic AMP production from permeabilised AtT-20 cells was initially investigated in order to obtain parameters which were capable of GTP-binding protein activation. $AlF_{(3-5)}$, provided by aluminium chloride in combination with potassium fluoride, evoked cyclic AMP production presumably via activation of G_s, or a similar GTP-binding protein, and subsequent activation of adenylate cyclase and cyclic AMP production (Iyenger, 1993). Of all the conditions investigated the combination of 2×10^{-2}M potassium fluoride and 5×10^{-5}M aluminium chloride stimulated cyclic AMP production to the greatest extent and as a result were adopted as a standard concentrations for further experimentation. The effect of $AlF_{(3-5)}$ upon ACTH secretion from permeabilised AtT-20 cells was subsequently investigated and it was discovered that this heterotrimeric GTP-binding protein activator was unable to stimulate secretion under these conditions. In fact potassium fluoride inhibited control ACTH secretion from permeabilised AtT-20 cells, an inhibition that $AlF_{(3-5)}$ was unable to overcome.

The inability of this heterotrimeric GTP-binding protein activator to stimulate ACTH secretion from permeabilised AtT-20 cells would seem to suggest that in this particular cell type G_e is not a heterotrimeric GTP-

binding protein. However the possibility that $AlF_{(3-5)}$ has additional actions which may inhibit G_e-stimulated exocytosis from AtT-20 cells was investigated. GTP- γ -S-stimulated ACTH secretion from AtT-20 cells, previously shown to be due to a direct activation of G_e (Guild, 1991; Luini & DeMatteis, 1988; 1990 & present study), was partially inhibited by potassium fluoride and completely inhibited in the presence of $AlF_{(3-5)}$. It is therefore clear that under these conditions activation of G_e does not result in ACTH secretion.

It may well be the case that $AlF_{(3-5)}$ is indeed activating G_e however this activation does not result in a secretory response due to additional actions of this heterotrimeric GTP-binding protein activator. It has been demonstrated that potassium fluoride in addition to contributing to GTP-binding protein activation is capable of inhibiting a number of enzymes including phosphatases (Chabre, 1990). Since results from the present study demonstrate that a phosphatase mediates calcium ion/G_e-stimulated ACTH secretion from AtT-20 cells one possibility is that the ability of $AlF_{(3-5)}$ to inhibit G_e-stimulated secretion is due to a phosphatase inhibition. In addition the inhibitory equivalent to G_e designated G_{ei}, which is known to be a heterotrimeric GTP-binding protein (Luini & DeMatteis, 1988 & 1990), will be activated by $AlF_{(3-5)}$ and may also contribute to an inhibition of G_e-stimulated ACTH secretion. It may be the case that additional actions of $AlF_{(3-5)}$ unrelated to activation of GTP-binding proteins, perhaps a phosphatase inhibition, coupled with activation of an inhibitory heterotrimeric GTP-binding protein result in a complete inhibition of G_e-stimulated ACTH secretion from AtT-20 cells. In the present study $AlF_{(3-5)}$ therefore did not prove to be a useful tool in the characterisation of G_e in AtT-20 cells.

$AlF_{(3-5)}$ does however stimulate histamine secretion from digitonin- (Sorimachi *et al.*, 1988) and streptolysin-O- (Aridor *et al.*, 1993) permeabilised rat mast cells, amylase secretion from streptolysin-O-permeabilised rat pancreatic acini (Morrison *et al.*, 1994) and noradrenaline secretion from streptolysin-O-permeabilised bovine chromaffin cells (Vitale *et al.*, 1994) indicating the involvement of heterotrimeric GTP-binding proteins in the late stages of the secretory pathways of these cell types. The ability of $AlF_{(3-5)}$ to stimulate a secretory response from these cell types may indicate that there are important differences in the intracellular mechanisms regulating secretion from AtT-20 cells and other secretory cell types.

(ii) Effects of mastoparan upon the late stages of the ACTH secretory pathway of AtT-20 cells

Mastoparan is an amphiphilic tetradecapeptide (Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2) toxin originally isolated from wasp venom which has been noted for its ability to stimulate histamine secretion from mast cells (Argiolas & Pisano, 1984; Bueb *et al.*, 1990; Mousli *et al.*, 1989) and it is after this action that the peptide is named. Mastoparan is now known to stimulate secretion from a number of other cell types including catecholamines from chromaffin cells (Kuroda *et al.*, 1980), insulin from pancreatic islets (Komatsu *et al.*, 1992; Yokokawa *et al.*, 1989) and the RINm5F β-cell line (Komatsu *et al.*, 1993), prolactin from anterior pituitary lactotrophs (Kurihara, *et al.*, 1986; Mau *et al.*, 1994), serotonin from platelets (Ozaki *et al.*, 1990) and surfactant from pulmonary alveolar cells (Joyce-Brady *et al.*, 1991). Results from the present study indicate that ACTH secretion from intact AtT-20 cells can also be added to this list.

When mastoparan binds to a phospholipid bilayer it forms an α helix that lies parallel to the plane of the membrane (Higashijima *et al.*, 1983; Wakamatsu *et al.*, 1983) a structure which is mimicked by some GTP-binding protein linked receptors at an area near the inner surface of the membrane which is important for GTP-binding protein activation (Ross, 1989). This prompted speculation that the effects of mastoparan upon secretion may be mediated by GTP-binding protein activation which has indeed recently been shown to be the case with the finding that mastoparan activates heterotrimeric GTP-binding proteins by an action at the carboxyl terminus of G_α an area of the GTP-binding protein which interacts with agonist-bound receptors (Higashijima *et al.*, 1988 & 1990; Higashijima & Ross, 1991; Weingarten *et al.*, 1990). The possibility that mastoparan was capable of stimulating ACTH secretion from AtT-20 cells by an action on the postulated GTP-binding G_e was investigated in the present study. If mastoparan was capable of stimulating secretion by such a mechanism it would be consistent with this GTP-binding protein being a heterotrimeric GTP-binding protein.

In order however to determine whether mastoparan was capable of stimulating ACTH secretion by an action upon G_e the effects of this peptide were investigated using permeabilised AtT-20 cells which allow a direct manipulation of the intracellular environment. Mastoparan stimulated ACTH secretion from permeabilised AtT-20 cells in a concentration-dependent manner in the effective absence of calcium ions. Any changes in the cytosolic free calcium ion concentration induced by mastoparan would have been buffered by the calcium/EGTA buffers designed to establish and maintain the required cytosolic free calcium ion concentration which in the case of this particular set of experiments was 10^{-9}M (effectively zero), therefore mastoparan evoked ACTH secretion from permeabilised AtT-20

cells is independent of changes in the cytosolic free calcium ion concentration which is therefore consistent with this peptide evoking a secretory response by a G_e-like mechanism of action.

Previous results from the present study have indicated that calcium ion/G_e-stimulated ACTH secretion from AtT-20 cells is not mediated by the activation of PKC. Therefore if mastoparan-stimulated secretion is mediated by activation of G_e this secretory response should be similarly PKC-independent. This hypothesis was tested using the PKC inhibitor chelerythrine chloride (Herbert, *et al.*, 1990). Mastoparan was able to stimulate ACTH secretion from permeabilised AtT-20 cells to the same extent in the presence or absence of chelerythrine chloride. Mastoparan is therefore able to stimulate ACTH secretion from AtT-20 cells independently of PKC which is once again consistent with this peptide evoking a secretory response by a G_e-like mechanism of action.

Activation of PKA by cyclic AMP is also known to stimulate ACTH secretion from permeabilised AtT-20 cells however this evoked secretion does not occur in the absence of either calcium ions or guanine nucleotides (Guild, 1991). Since mastoparan stimulated secretion in the absence of guanine nucleotides and the effective absence of calcium ions it is probable that activation of PKA by means of cyclic AMP generation does not contribute to these actions of mastoparan. This point however was confirmed by investigating the effects of mastoparan upon cyclic AMP accumulation from permeabilised AtT-20 cells. Mastoparan under conditions which result in ACTH secretion was unable to stimulate cyclic AMP accumulation. It can therefore be concluded that mastoparan stimulated ACTH secretion from permeabilised AtT-20 cells does not

proceed via cyclic AMP generation again consistent with this peptide evoking a secretory response by a G_s-like mechanism of action.

Mastoparan however has been reported to have a number of actions which may not be mediated by heterotrimeric GTP-binding proteins. These include non-specific cell lysis in chromaffin cells (Wilson, 1989); binding to calmodulin (Malenick & Anderson, 1983); direct activation of phospholipase A_2 (Argiolas & Pisano, 1983) or PLC (Wallace & Carter, 1989); stimulation of nucleoside diphosphate kinase (Kikkawa *et al.*, 1992; Kowluru *et al.*, 1995); inhibition of PKC, calcium/calmodulin kinase II, Na-K ATPase and the Na pump (Raynor *et al.*, 1991). Some of these actions will not be relevant in the permeabilised cell system employed here where changes in the cytosolic free calcium ion concentrations, calmodulin activity, membrane bound ion channels and pumps are circumvented. In addition results from the present study indicate that the actions of mastoparan are not mediated by activation of PLC and subsequent activation of PKC. It was however important to establish whether the effects of mastoparan in AtT-20 cells were due to GTP-binding protein activation or to a non GTP-binding protein-mediated event.

The stable GDP analogue GDP- β -S, which inhibits GTP activation of GTP-binding proteins by means of competitive antagonism, partially inhibited mastoparan-evoked ACTH secretion from permeabilised AtT-20 cells. Mastoparan-stimulated ACTH secretion from AtT-20 cells is therefore mediated, at least partly, by a GTP-binding protein. This is consistent with calcium ion-evoked ACTH secretion from permeabilised AtT-20 cells which is similarly inhibited by GDP- β -S (Guild, 1991). In addition mas-17, an analogue of mastoparan unable to activate GTP-binding proteins (Higashijima *et al.*, 1990), was unable to stimulate ACTH secretion from

permeabilised AtT-20 cells. This is in contrast to the mastoparan analogue mas-7, a highly active GTP-binding protein activator (Higashijima *et al.*, 1990), which stimulated ACTH secretion to an even greater extent than mastoparan. This again suggests that the ability of mastoparan to stimulate ACTH secretion from permeabilised AtT-20 cells independently of changes in calcium and PKC is, at least partly, due to a direct activation of a GTP-binding protein.

The ability of mastoparan to stimulate ACTH secretion from permeabilised AtT-20 cells independently of calcium ions, of PKC and of PKA suggests that this peptide is acting via a G_e-like mechanism of action which may suggest that this GTP-binding protein may belong in this particular cell line to the heterotrimeric family of GTP-binding proteins. These findings are consistent with a number of studies which demonstrate that mastoparan stimulates a secretory response by directly activating G_e in a variety of specialised cells including pancreatic β-cells (Jones *et al.*, 1993), mast cells (Aridor *et al.*, 1990) and platelets (Wheeler-Jones *et al.*, 1992).

The characteristics of mastoparan-stimulated ACTH secretion from permeabilised AtT-20 cells were further investigated in order to compare them to that of calcium ion- and GTP- γ -S-stimulated ACTH secretion. Mastoparan-evoked ACTH secretion from permeabilised AtT-20 cells was significantly, but not completely, reduced when ATP was omitted from the permeabilisation medium. This is again consistent with calcium ion-evoked ACTH secretion from permeabilised AtT-20 cells which also displays a dependency upon ATP (Guild, 1991) however is in contrast to GTP- γ -S-stimulated secretion which displays little or no ATP dependency. This finding may suggest that calcium ions and mastoparan activate G_e by a

similar mechanism which is different to that of GTP- γ -S. Mastoparan may therefore provide a useful alternative to GTP- γ -S as a means of directly activating G_e with the advantage that it activates this GTP-binding protein in a manner more analogous to calcium ions.

Although mastoparan and calcium ions stimulate ACTH secretion from permeabilised AtT-20 cells by similar mechanisms important differences do exist between secretory responses to these two secretagogues which are consistent with mastoparan having an additional action which contributes to ACTH secretion. Calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells is almost completely inhibited in the presence of the GTP-binding protein inhibitor GDP- β -S (Guild, 1991) whereas it should be noted that the present study demonstrates that this guanine nucleotide can only partially inhibit ACTH secretion in response to mastoparan. A similar observation was made using pancreatic β-cells in which mastoparan-stimulated secretion from permeabilised cells was only partially inhibited by GDP- β -S (Jones *et al.*, 1993). Calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells is also almost completely dependent upon the presence of ATP in the permeabilisation medium (Guild, 1991; Luini & DeMatteis, 1988) in contrast to mastoparan-stimulated ACTH secretion which is only partially attenuated by the omission of ATP from the permeabilisation medium. These important differences between calcium ion- and mastoparan-stimulated ACTH secretion from permeabilised AtT-20 cells point towards mastoparan having an additional site of action in the stimulus-secretion coupling pathway regulating ACTH release. This point of regulation would in common with G_e be distal to changes in cytosolic free calcium ion levels, independent of PKC activation and of PKA activation however may not be mediated by a GTP-binding protein.

One possibility which cannot be ruled out is that mastoparan may activate the enzyme phospholipase A_2. As previously mentioned mastoparan, in addition to its ability to stimulate GTP-binding proteins, may also have a direct stimulatory action upon phospholipase A_2 (Argiolas & Pisano, 1983). Evidence exists to suggest that activation of phospholipase A_2 and subsequent release of arachadonic acid and related metabolites may result in ACTH secretion from both the normal corticotroph (Abou-Samra *et al.*, 1986; Won & Orth, 1994) and from AtT-20 cells (Luini & Axelrod, 1985). It is therefore a possibility that activation of phospholipase A_2 is involved at some stage of the stimulus-secretion coupling pathway of AtT-20 cells and is an important trigger to exocytosis therefore this enzyme must be a likely candidate for the second site of action of mastoparan with regard to ACTH secretion from this cell line.

In addition the effects of a number of secretagogues including CRF-41, forskolin, membrane permeant cyclic AMP analogues, potassium and phorbol esters upon ACTH secretion from AtT-20 cells have been shown to be attenuated to some extent by agents which interfere with phospholipase A_2 activity and subsequent arachadonic acid metabolism (Luini & Axelrod, 1985). This diverse array of secretagogues stimulate ACTH secretion from AtT-20 cells by a variety of actions however one common feature is the fact that their effects are mediated to a greater or lesser extent by an elevation in the cytosolic free calcium ion concentration (Guild *et al.*, 1986, Guild & Reisine, 1987; Luini *et al.*, 1985; Reisine & Guild, 1985; Reisine, 1989). Therefore one possibility is that the effects of calcium ion- and G_e-stimulated ACTH secretion from AtT-20 cells may be mediated by activation of phospholipase A_2. Such a mechanism whereby the effects of a diverse variety of secretagogues are all mediated, to some extent, by a common mechanism would provide a convenient point of regulation for

inhibitory influences. Since glucocorticoids, which are the main inhibitory influence upon ACTH secretion from anterior pituitary corticotrophs (Aguilera, 1994; Antoni, 1986; Jones & Gillham, 1988), are known to inhibit phospholipase A_2 (Flowers, 1985) it is tempting to speculate that this is indeed the case.

(iii) Effects of pertussis toxin upon the late stages of the ACTH secretory pathway of AtT-20 cells

PTX-sensitive heterotrimeric GTP-binding proteins have been implicated in the late stages of the ACTH secretory pathway of AtT-20 cells. The inhibitory form of G_e (G_{ei}) which mediates SRIF-regulated inhibition of ACTH secretion from AtT-20 cells is known to be PTX-sensitive (Luini & DeMatteis, 1988 & 1990). Since many of the actions of mastoparan, now known to activate G_e in AtT-20 cells, have been attributed to the activation of PTX-sensitive heterotrimeric GTP-binding proteins (Higashijima *et al.*, 1988) the possibility that the stimulatory form of G_e present in AtT-20 cells is similarly PTX-sensitive was investigated.

Calcium ions, mastoparan and GTP- γ -S all stimulated ACTH secretion from permeabilised AtT-20 cells, at least party, by a direct action upon G_e (Luini & DeMatteis, 1988 & 1990; Guild, 1991 & present study). Pretreatment with PTX did not inhibit calcium ion-, mastoparan- or GTP- γ -S-stimulated ACTH secretion from permeabilised AtT-20 cells. It can therefore be concluded that in permeabilised AtT-20 cells calcium ion-, mastoparan- and GTP- γ -S-stimulated ACTH secretion are not PTX-sensitive. As mastoparan is generally thought to act through activation of PTX-sensitive GTP-binding proteins (Higashijima *et al.*, 1988) this observation would seem to contradict previous findings of the present

study suggesting that mastoparan stimulates ACTH secretion from permeabilised AtT-20 cells, at least partly, by an action upon a GTP-binding protein, namely G_e. Several studies however have shown that mastoparan has been implicated in the activation of PTX-insensitive GTP-binding proteins (Nakahata *et al.*, 1990; Vitale *et al.*, 1994; Wheeler-Jones *et al.*, 1992; Mau *et al.*, 1994).

Calcium chloride- and forskolin-stimulated secretion from intact AtT-20 cells also proved to be PTX-insensitive. The addition of calcium chloride to the extracellular medium of AtT-20 cells has been shown to raise cytosolic calcium ion concentrations (Luini *et al.*, 1985). Raising the extracellular calcium chloride concentration resulted in ACTH secretion from intact AtT-20 cells which was insensitive to PTX pretreatment. Forskolin is known to stimulate ACTH secretion from intact AtT-20 cells by raising the intracellular cyclic AMP content of the cell which in turn results in an elevation of the cytosolic calcium ion concentration (Luini *et al.*, 1985; Reisine & Guild, 1985; Guild *et al.*, 1986; Guild & Reisine, 1987). Forskolin-stimulated ACTH secretion from intact AtT-20 cells was also insensitive to PTX pretreatment.

PTX pretreatment was however able to inhibit the effects of SRIF upon AtT-20 cells. Forskolin stimulated ACTH secretion from intact AtT-20 cells was inhibited by SRIF. This SRIF-mediated inhibition was completely reversed by PTX pretreatment. This confirms previous reports suggesting that the effects of SRIF in AtT-20 cells are mediated by a PTX-sensitive GTP-binding protein (Luini *et al.*, 1986; Luini & DeMatteis, 1988 & 1990; Reisine & Guild, 1985; Reisine *et al.*, 1988) and also suggests that PTX was highly effective under the conditions employed in this study. PTX pretreatment was therefore unable to inhibit evoked ACTH secretion by a

variety of stimuli from both intact or permeabilised AtT-20 cells. It can therefore be concluded that in AtT-20 cells G_e is not a PTX-sensitive GTP-binding protein. PTX has been shown to inhibit the secretion, mediated by activation of G_e, of histamine from streptolysin-O-permeabilised rat mast cells (Aridor *et al.*, 1993) and noradrenaline secretion from streptolysin-O-permeabilised bovine chromaffin cells (Vitale *et al.*, 1994) suggesting that in these cell types G_e is PTX-sensitive and may belong to the G_i subfamily of GTP-binding proteins. Again this seems to indicate that there are important differences between the intracellular mechanisms regulating secretion from these cells and AtT-20 cells.

(iv) Effects of cholera toxin upon the late stages of the ACTH secretory pathway of AtT-20 cells

Mastoparan has been reported to activate purified PTX-sensitive GTP-binding proteins, namely G_i and G_o, however is unable to stimulate G_s to the same extent (Higashijima *et al.*, 1988). It has however been reported that under more physiological conditions mastoparan is able to generate cyclic AMP indicating a possible action upon G_s (Higashijima *et al.*, 1988; Wheeler-Jones *et al.*, 1992). In addition kinetic studies carried out in permeabilised rat mast cells suggested that G_e displayed the characteristics consistent with those of a heterotrimeric GTP-binding protein and of heterotrimers of which the kinetics are understood these characteristics most resemble those of G_s (Lillie & Gomperts, 1993). To determine whether G_e present in AtT-20 cells belongs to the G_s subfamily of heterotrimeric GTP-binding proteins the effects of CTX upon ACTH secretion were investigated.

CTX stimulated ACTH secretion from intact AtT-20 cells in a concentration-dependent manner indicating that a member of the G_s subfamily was involved at some stage of the stimulus-secretion coupling pathway of AtT-20 cells. However activation of G_s is known to generate cyclic AMP (Iyenger, 1993) and agents known to elevate cellular cyclic AMP levels in AtT-20 cells are known to stimulate ACTH secretion from intact AtT-20 cells (Axelrod & Reisine, 1984; Heisler & Reisine, 1984; Litvin *et al.*, 1984; Miyazaki *et al.*, 1984). Therefore to distinguish between the actions of CTX upon the GTP-binding protein associated with cyclic AMP production and G_e a permeabilised cell preparation was employed. The effects of CTX, in the presence of the cofactor NAD, upon both cyclic AMP production and ACTH secretion from permeabilised AtT-20 cells were investigated. CTX in combination with NAD stimulated cyclic AMP production from permeabilised AtT-20 cells but did not stimulate ACTH secretion under similar conditions. It can be concluded that CTX was effective under the conditions employed in this study and that in AtT-20 cells G_e is insensitive to CTX. These results from permeabilised cells suggest that CTX-stimulated secretion from intact AtT-20 cells can be attributed to the generation of cyclic AMP and that G_e appears not belong to the G_s subfamily of heterotrimeric GTP-binding proteins. CTX has been shown to stimulate amylase secretion from streptolysin-O-permeabilised rat pancreatic acini (Morrison *et al.*, 1994) prompting the suggestion that in these cells G_e may belong to the G_s subfamily of GTP-binding proteins and again highlights the fact that different secretory cell types appear to have differences in the intracellular mechanisms regulating secretion from different cell types.

In AtT-20 cells G_e is therefore insensitive to both PTX and CTX. This study appears to be most consistent with studies carried out in the

insulin-secreting RINm5F β-cell line. Calcium ions and GTP- γ-S (Valler *et al.*, 1987) as well as mastoparan (Komatsu *et al.*, 1993) stimulate secretion from RINm5F cells by a G_e-like mechanism of action. It also appears that in the RINm5F cell line G_e is insensitive to PTX (Valler *et al.*, 1987; Komatsu *et al.*, 1993) and to CTX (Valler *et al.*, 1987).

(v) Summary

In conclusion in AtT-20 cells the nature of G_e remains elusive. Despite the inability of $AlF_{(3-5)}$ to stimulate ACTH secretion by an action upon G_e this GTP-binding protein does however still display characteristics consistent with it belonging to the heterotrimeric family of GTP-binding proteins. The results of this study would also suggest that in AtT-20 cells G_e is insensitive to both PTX and CTX. Studies carried out in some specialised secretory cell types, including the present study, have proposed a number of different candidates for G_e. The present study is highly consistent with results obtained using the RINm5F β-cell line in which G_e is also insensitive to both PTX and CTX (Valler *et al.*, 1987; Komatsu *et al.*, 1993). However it contrasts with work carried out in mast cells (Aridor *et al.*, 1993) and chromaffin cells (Vitale *et al.*, 1994) in which G_e appears to be PTX-sensitive and also with studies carried out in the exocrine pancreas in which G_e appears to be CTX-sensitive (Morrison *et al.*, 1994). Studies to date therefore would suggest that G_e present in a peptide secreting endocrine cell is insensitive to both PTX and CTX (Valler *et al.*, 1987; Komatsu *et al.*, 1993). This contrasts with amine secreting endocrine cells in which G_e appears to be PTX sensitive (Aridor *et al.*, 1993; Vitale *et al.*, 1994) and also to exocrine cells in which G_e may be CTX sensitive (Morrison *et al.*, 1994). It may therefore be the case that the identity of G_e in a particular cell type depends upon the nature of secretion

for which that cell is specialised and that G_e may share a common identity between different cell types specialised for similar forms of secretion.

3 AN INVESTIGATION INTO THE REGULATION OF ACTH SECRETION FROM AtT-20 CELLS BY PROTEIN KINASE C AND CYCLIC AMP-DEPENDENT PROTEIN KINASE AT A LATE STAGE IN THE ACTH SECRETORY PATHWAY

(i) Effects of protein kinase C activators upon ACTH secretion from intact AtT-20 cells

As already discussed in detail the effects of AVP are thought to be largely mediated by the activation of PLC (Jard *et al.*, 1987) and subsequent generation of DAG leading to activation of PKC (Bilezikjian *et al.*, 1987 a & b; Guillon *et al.*, 1987; Oki *et al.*, 1990; Raymond *et al.*, 1985). As a result the involvement of PKC in the ACTH stimulus-secretion coupling pathway has been the focus of a number of studies. The phorbol ester PMA stimulates ACTH secretion from both the normal corticotroph (Abou-Samra *et al.*, 1986) and AtT-20 cells (Reisine & Guild, 1987; Reisine, 1989) confirming that PKC plays an important role in the ACTH stimulus-secretion coupling pathway. Initial results from the present study were consistent with this suggestion with the observation that the phorbol esters PMA, PDD and PDBu all stimulated ACTH secretion from intact AtT-20 cells. The ability of diacylglycerol analogues to stimulate ACTH secretion coupled with the inability of 4 α phorbol, an inactive phorbol ester, to stimulate a similar secretory response suggests that the effects of these phorbol esters upon ACTH secretion are mediated by activation of PKC and are not due to a simple biophysical effect.

Previous studies have suggested that the ability of PKC to stimulate ACTH secretion from AtT-20 cells is due to an elevation of the cytosolic free calcium ion concentration (Reisine & Guild, 1987; Reisine, 1989). This

rise in the cytosolic calcium ion concentration is however a transient one in contrast to the resultant hormone release which is sustained, an observation which lead to speculation that PKC may have additional points of regulation in the ACTH stimulus-secretion coupling pathway which are unrelated to the elevation of cytosolic free calcium ion levels (Reisine & Guild, 1987). Using the permeabilised cell preparation, in which the cytosolic free calcium ion concentration can be controlled, it was one aim of this study to determine whether this hypothesis was in fact the case.

(ii) Effects of PMA upon the late stages of the ACTH secretory pathway of AtT-20 cells

The present study demonstrated that PMA enhances calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells. Since any changes in cytosolic free calcium ion concentrations would be counteracted by the calcium/EGTA buffers employed in the present study it appears that PKC is indeed able to stimulate ACTH secretion from permeabilised AtT-20 cells by an action which is unrelated to an elevation of the cytosolic free calcium ion concentration. Therefore it can be concluded that PKC has at least two sites of action with regard to stimulated ACTH secretion from AtT-20 cells. This second site of action uncovered in the present study could account for the sustained nature of PKC-stimulated secretion from these cells (Reisine & Guild, 1987). Having established that PKC has more than one point of regulation in the ACTH stimulus-secretion coupling pathway the nature of this second site of action was considered.

PMA was shown to stimulate ACTH secretion from permeabilised AtT-20 cells in the effective absence of calcium ions (free calcium ion

concentration of 10^{-9}M) and in the absence of guanine nucleotides. This may suggest that this second site of action of PKC is one which is capable of stimulating secretion by a mechanism which is independent of calcium ion/G_e-stimulated secretion. PKC is known to represent a family of isozymes comprising in part of cPKCs and nPKCs (Ohno *et al.*, 1991), both of which are now known to be present in AtT-20 cells. In the absence of calcium ions PMA is therefore only able to activate nPKCs (Ryves *et al.*, 1991) and it can be concluded that secretion from permeabilised AtT-20 cells observed in response to PMA in the effective absence of calcium ions is due to activation of these calcium independent isozymes of PKC.

The present study also indicates that calcium ions and PMA interact in a synergistic manner with regard to ACTH secretion from permeabilised AtT-20 cells. It may be the case that cPKCs, which can be activated by PMA only in the presence of calcium ions (Ryves *et al.*, 1991), also stimulate secretion from AtT-20 cells by a pathway which is independent of calcium ion/G_e-stimulated secretion in a manner similar to nPKCs. Activation of these additional isozymes may result in an enhanced secretory response and could therefore account for the observed synergy between calcium ions and PMA. Consistent with this argument is the finding that there appears to be no interaction between PMA and GTP-γ-S with regard to evoked ACTH secretion from permeabilised AtT-20 cells. In the absence of calcium ions PMA and GTP-γ-S stimulated ACTH secretion from AtT-20 cells in an additive manner indicating no interaction between nPKCs and G_e and therefore confirming that nPKCs stimulate secretion by an action which is independent of calcium ion/G_e-stimulated secretion. In addition PMA and GTP-γ-S also stimulated ACTH secretion in an additive manner at every free calcium ion concentration investigated indicating that there is also no interaction between cPKCs and G_e which is

therefore consistent with cPKCs also stimulating secretion by an action which is independent of calcium ion/G_e-stimulated ACTH secretion. These findings therefore suggest that cPKCs as well as nPKCs may contribute to an ACTH secretory response from AtT-20 cells which is independent of calcium ion/G_e-stimulated secretion.

It therefore appears that at a late stage in the ACTH stimulus-secretion coupling pathway of AtT-20 cells two distinct parallel pathways can be distinguished both of which appear to require calcium ions in some capacity. The first is that of G_e-stimulated secretion which is directly activated by calcium ions and the second is that of PKC-stimulated secretion. This PKC-stimulated secretion can proceed in the absence of calcium ions, however since both cPKCs and nPKCs appear to contribute to this pathway the presence of calcium ions greatly enhances the secretory response mediated by this route. A schematic diagram outlining a proposed model for the involvement of PKC in the ACTH stimulus-secretion coupling pathway of AtT-20 cells is displayed in figure 68. The ability of PKC to stimulate a secretory response in a calcium ion-independent manner from AtT-20 cells is consistent with a number of studies carried out in GH_3 cells (Ronning & Martin, 1986), mammary epithelial cells (Turner *et al.*, 1992), natural killer cells (Ting *et al.*, 1992), neutrophils (Barrowman *et al.*, 1986; DiVirgilio *et al.*, 1984) and RINm5F cells (Valler *et al.*, 1987).

The above model predicts that secretagogues which produce a sustained elevation of the cytosolic free calcium ion concentration, such as CRF-41 (Axelrod & Reisine, 1984; Miyazaki *et al.*, 1984), should be capable of evoking a significant ACTH secretory response from anterior pituitary corticotrophs. In contrast agents the effects of which are mediated

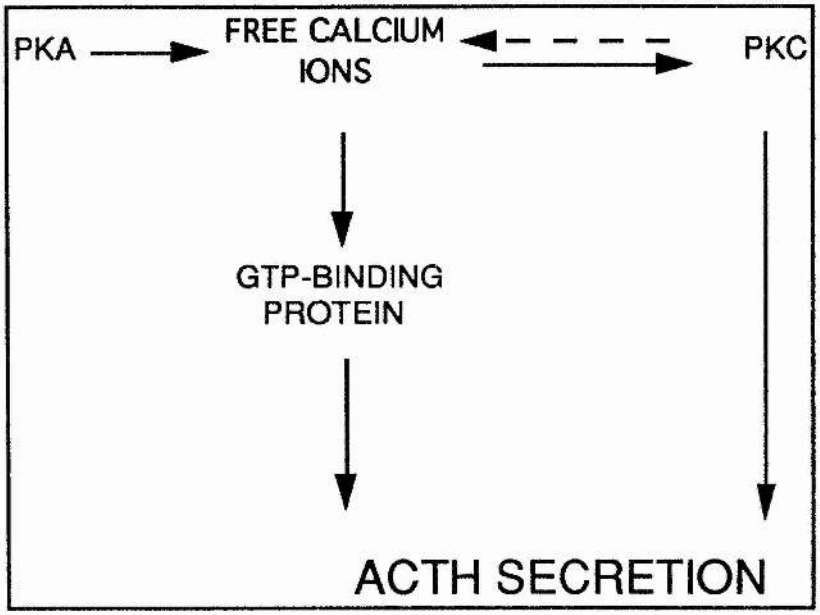

Figure 68 Schematic diagram showing a possible model for the involvement of protein kinase C in the late stages of the ACTH stimulus-secretion coupling pathway of AtT-20 cells.

by activation of PKC, such as AVP (Oki *et al.*, 1990), will produce in a relatively poor secretory response. This model however also predicts that this poor secretory response will be largely potentiated by agents such as CRF-41 which raise the cytosolic free calcium ion concentration. In rats, primates and humans this is indeed the case with CRF-41 being the dominant regulator of ACTH secretion from the anterior pituitary corticotroph. In contrast AVP is a weak stimulator of ACTH release, however this hormone can markedly potentiate CRF-41-stimulated secretion (Aguilera, 1994; Antoni, 1986). Although CRF-41 (Abou-Samra *et al.*, 1987; Antoni, 1986) and AVP (Oki *et al.*, 1990) may have actions which are unrelated to elevation of the cytosolic free calcium ion concentration and to the activation of PKC respectively, the proposed model does seem consistent with the general patterns of hormone release observed in response to these two major regulators of ACTH secretion.

(iii) Effects of isozyme-selective protein kinase C activators upon the late stages of the ACTH secretory pathway of AtT-20 cells

To date the family of PKC isozymes is known to comprise 12 closely related polypeptides (Dekker & Parker, 1994). Western blot analysis revealed that AtT-20/D16-16 cells, employed in the present study, contain a variety of these isozymes including the cPKC α and β isozymes, the nPKC ε isozyme and the atypical PKC ζ isozyme, a profile identical to that of the AtT-20/D16v cell line (Dr D.J. MacEwan, personal communication). Since it has been demonstrated that particular isozymes of PKC have distinct roles in signal-transduction, stimulus-secretion coupling and growth (Akita *et al.*, 1990; Housay *et al.*, 1988; Kiley *et al.*, 1992; Leli *et al.*, 1992; Naor *et al.*, 1989; Pai *et al.*, 1991; Sharma *et al.*, 1991) the present study attempted to establish physiological roles for some of the isozymes present

in AtT-20 cells and in particular establish whether some of these isozymes contribute to PKC-stimulated ACTH secretion by interacting with the calcium ion messenger system (Reisine & Guild, 1987; Reisine, 1989) or by an action which is independent of calcium ion/G_e-stimulated secretion.

This problem was approached in the present study using a number of substances thought to selectively activate particular isozymes of PKC. PMA, employed extensively in the present study, is regarded as a general activator of PKC isozymes although calcium ions are required for maximal activation of cPKCs (Ryves *et al.*, 1991). It should also be noted that certain isozymes, including the ζ isozyme present in AtT-20 cells, do not appear to be regulated by phorbol esters or DAG (Akimoto *et al.*, 1994; Nishizuka, 1988; Selbie *et al.*, 1993) and therefore these isozymes will be unaffected by PMA. TMX is reported to be a selective activator of the α, β and γ isozymes (Ryves *et al.*, 1991) and was employed in the present study in order to activate the α and β isozymes present in AtT-20 cells. Another phorbol ester dPPA was employed in the present study as an activator of the β isozyme for which it is has been reported to be selective (Ryves *et al.*, 1991). The effects of these activators were originally investigated in intact AtT-20 cells from which PMA, dPPA and TMX all stimulated ACTH secretion, a result which appears to suggest that general activation of PKC isozymes, activation of the α and β isozymes or simply activation of the β isozyme may evoke a secretory response.

In order to determine whether these isozymes participate in the stimulus-secretion coupling pathway by raising the cytosolic free calcium ion concentration or by a calcium ion/G_e-independent site of action the effects of these agents upon ACTH secretion were investigated using the permeabilised cell preparation in which the cytosolic free calcium ion

concentration can be controlled. The β isozyme activator dPPA, in contrast to PMA, was unable to enhance calcium ion-stimulated ACTH secretion from permeabilised AtT-20 cells. This finding may therefore suggest that dPPA and therefore that the β isozyme does not contribute to the calcium ion/G_e-independent site of action by PKC. Experiments carried out in intact AtT-20 cells however demonstrated that dPPA is capable of evoking a secretory response indicating that the β isozyme does play a role in the regulation of ACTH secretion. The role of the β isozyme may therefore be at an early stage in the stimulus-secretion coupling pathway where PKC has been shown to stimulate calcium ion entry into the cell across the plasma membrane (Reisine & Guild, 1987; Reisine, 1989). It may therefore be the case that the calcium-dependent α isozyme and the calcium-independent ε isozyme may contribute to the regulated secretory response in a calcium ion/G_e-independent manner.

As previously suggested the relative contributions of these PKC isozymes to the secretory response will be determined to a certain extent by the availability of calcium ions. At lower calcium ion concentrations the ε isoform may be dominant in AtT-20 cells contributing to ACTH secretion by the calcium ion/G_e-independent pathway. At higher calcium ion concentrations however the α and β isoforms will also contribute to the ACTH secretory response, the α isozyme perhaps by a mechanism similar to that of the ε isozyme and the β isozyme perhaps by raising the cytosolic free calcium ion concentration. These findings from the present study are consistent with investigations into the role of PKC in the regulation of prolactin secretion from the GH_3 anterior pituitary cell line which adopted a similar approach and proposed a similar role for the β isozyme in the stimulus-secretion coupling pathway (MacEwan & Mitchell, 1991; MacEwan *et al.*, 1991; Thomson *et al.*, 1993). In addition this involvement

of both cPKCs and nPKCs in the regulation of exocytosis has been shown to be the case in both rat basophilic RBL-2H3 cells (Ozawa *et al.*, 1992) and GH_4C_1 rat anterior pituitary cells (Akita *et al.*, 1990).

The ability of TMX to stimulate ACTH secretion from permeabilised AtT-20 cells in the absence of calcium ions in a similar manner to PMA was surprising since TMX has been reported to be a selective activator of cPKCs (Ryves *et al.*, 1991). This may therefore suggest that TMX is not as selective as previously anticipated, further evidence for which comes from the finding that this PKC activator is capable of binding to the ε isozyme, although TMX was 10-20 fold less potent in this regard in comparison to its effects upon the α or β isozymes (Kazanietz *et al.*, 1993). Although binding of TMX to the ε isozyme does not give any indication of activation it may suggest that caution is required when attributing selective properties to these agents and in particular to TMX.

Similar doubts have been expressed over the selectivity of dPPA with recent findings suggesting that dPPA may activate PKC isozymes in a non-selective manner (Kiley *et al.*, 1994). This non-selective action however required a dPPA concentration in excess of 10^{-7}M which was the concentration employed in the present study. In addition dPPA has been demonstrated *in vitro* to require a concentration in excess of 2×10^{-6}M for non-specific PKC activation to occur whereas activation of the β isozyme occurs at a concentrations of 2×10^{-8}M (Ryves *et al.*, 1991). These findings indicate that dPPA may well facilitate a non-selective activation of PKC isozymes however this action requires a relatively high concentration of this PKC activator. These same studies also suggest that when employed at an appropriate concentration, including the concentration of 10^{-7}M adopted in the present study, dPPA is in fact selective for activation

of the β isozyme of PKC. Consistent with these findings are results from the present study which suggest that dPPA is capable of stimulating ACTH secretion from permeabilised AtT-20 cells both in the presence and absence of calcium ions only at concentrations of 10^{-6}M and above indicating that dPPA may activate other PKC isozymes only when present in relatively large quantities. Results from the present study also indicate clearly that PMA and TMX display different characteristics from dPPA with regard to ACTH secretion which would not be the case if these agents were all non-selective in their activation of PKC isozymes. It therefore does appear that dPPA can be employed as a selective activator of the β isozyme of PKC.

In conclusion it appears that different isozymes of PKC contribute to the ACTH secretory response by actions at distinct points in the stimulus-secretion coupling pathway of AtT-20 cells. In particular the β isozyme may interact with the calcium ion messenger system at an early stage in the stimulus-secretion coupling pathway perhaps by facilitating calcium ion entry into the cell across the plasma membrane. Other PKC isozymes, perhaps the α and ε isozymes, may regulate ACTH secretion from AtT-20 cells by an action which is not due to an interaction with the calcium ion messenger system. The involvement of different isozymes at different points in the ACTH stimulus-secretion coupling pathway of AtT-20 cells may go some way to explaining the need for the variety of PKCs now known to be present in these cells.

(iv) Effects of cyclic AMP upon the late stages of the ACTH secretory pathway of AtT-20 cells

Elevation of the intracellular cyclic AMP content of both AtT-20 cells and the normal corticotroph induced by a number of agents, including CRF-41, has been demonstrated to result in ACTH secretion (Aguilera *et al.*, 1983; Axelrod & Reisine, 1984; Heisler & Reisine, 1984; Litvin *et al.*, 1984; Miyazaki *et al.*, 1984) indicating that PKA is an important part of the stimulus-secretion coupling pathway of these cells. Evidence exists to suggest that PKA stimulates ACTH secretion from AtT-20 cells by interacting with the calcium ion messenger system by producing a sustained elevation of the cytosolic free calcium ion concentration (Luini *et al.*, 1985; Guild & Reisine, 1987). More recently it has also been suggested that PKA has a second point of regulation by which it is capable of modulating the effects of changes in the cytosolic free calcium ion concentration, including those evoked by PKA itself, upon ACTH secretion from AtT-20 cells by an action in the stimulus-secretion coupling pathway distal to calcium ions (Guild, 1991). This modulatory action of PKA was confirmed in the present study with the demonstration that cyclic AMP, although incapable of stimulating ACTH secretion from permeabilised AtT-20 cells itself, can interact in a synergistic manner with both calcium ions and GTP- γ -S to elicit a secretory response. These findings suggest that PKA is capable of modulating calcium ion/G_e-stimulated secretion by an action in the stimulus-secretion coupling pathway which is distal to both calcium ions and to G_e and are consistent with previous results obtained using this cell line (Guild, 1991).

The possibility that PKA may also have a similar modulatory role with regard to calcium ion/G_e-independent PKC-stimulated ACTH secretion

from permeabilised AtT-20 cells was subsequently investigated. PMA-stimulated ACTH secretion from permeabilised AtT-20 cells was enhanced by cyclic AMP in a similar way to that of calcium ion- and GTP- γ -S-stimulated secretion. Cyclic AMP enhanced PMA-stimulated ACTH secretion from permeabilised AtT-20 cells in a synergistic manner indicating that PKA may play a modulatory role with regard to calcium ion/G_e-independent PKC-stimulated secretion. It therefore appears that PKA is capable of enhancing ACTH secretion by two distinct pathways, that of calcium ion/G_e-stimulated secretion and that of PKC-stimulated ACTH secretion. A schematic diagram displaying a possible model for the involvement of PKA in the late stages of the ACTH secretory pathway of AtT-20 cells is displayed in figure 69. Whether this modulatory action of PKA is due to a single point of regulation by PKA which for instance may enlarge a readily releasable pool of stored hormone making more ACTH available for stimulated secretion (Dannies, 1982) or by a separate point of regulation on each pathway is unclear from the present study.

(v) Summary

Results from the present study indicate that both PKC and PKA, in addition to raising cytosolic free calcium ion levels (Luini *et al.*, 1985; Guild & Reisine, 1987; Reisine, 1989), contribute to the ACTH secretory response of AtT-20 cells by an action at a late stage in the stimulus-secretion coupling pathway. It appears however that these kinases contribute at this late stage in quite different ways, PKC providing a direct stimulus to secretion in contrast to PKA which is unable to stimulate secretion by itself but which can modulate both calcium ion/G_e- and PKC-stimulated secretion. Although the present study demonstrates that PKC is capable of stimulating ACTH secretion from AtT-20 cells in the effective

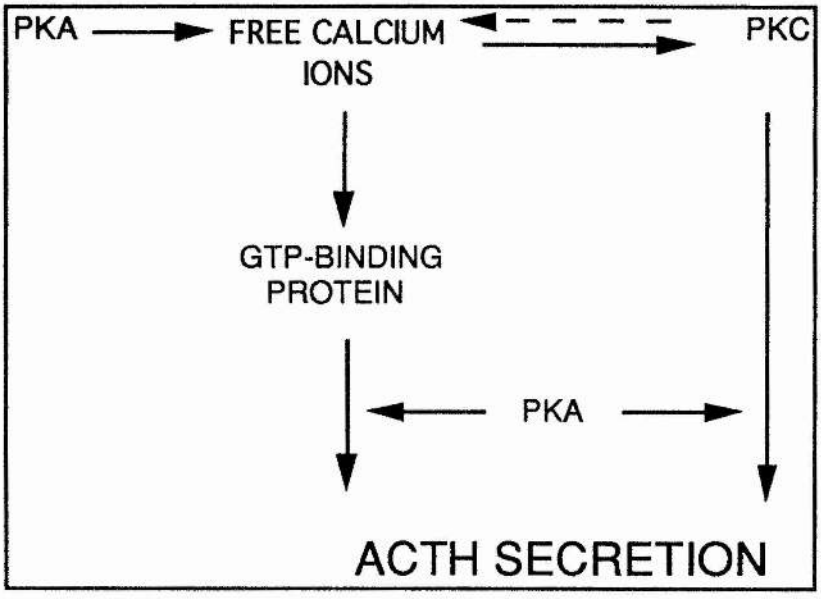

Figure 69 Schematic diagram showing a possible model for the involvement of cyclic AMP-dependent protein kinase in the late stages of the ACTH stimulus-secretion coupling pathway of AtT-20 cells.

absence of calcium ions the secretory response evoked by this route is greatly enhanced by the presence of calcium ions. It therefore appears that calcium ions can still be regarded as the central intracellular regulator of ACTH release from AtT-20 cells however co-operation with PKA and PKC is required to uncover what might be a maximal secretory response.

4 CONCLUDING REMARKS

In conclusion it appears that the mechanisms mediating calcium ion-stimulated ACTH secretion from AtT-20 cells can be separated into two distinct pathways. The first is mediated by the GTP-binding protein G_e the effects of which are mediated in turn by a phosphatase. This pathway is activated by calcium ions and represents the mechanism by which these ions directly stimulate ACTH secretion. In contrast the second pathway which is mediated by PKC is not dependent upon calcium ions but is enhanced to a large extent by their presence. Although it is now clear that calcium ions are not an absolute requirement for ACTH secretion they can still be regarded as the central intracellular regulator of ACTH secretion since this 'calcium-independent' secretory response is relatively small. How two pathways mediated by opposing cellular actions, namely a dephosphorylation and a phosphorylation, result in the same physiological response remains puzzling however one possibility is that two pools of readily releasable ACTH exist, one sensitive to the actions of a phosphatase and the other to a kinase. It is also clear from the present study that PKC contributes to the regulation of ACTH secretion from AtT-20 cells by quite a different mechanism from PKA which appears to be a largely modulatory with regard to both calcium ion/G_e- and PKC-stimulated secretion. It may be the case that PKA may play a regulatory role at this late stage controlling the availability of readily releasable pools of ACTH from a more long term storage pool (Dannies, 1982).

Both PKC and PKA have been shown in AtT-20 cells to have a wide variety of target proteins, some distinct and some shared (Bishop *et al.*, 1987; Rougon *et al.*, 1989). It is therefore likely that both of these kinases have additional points of regulation with regard to the control of ACTH

secretion, that the stimulus-secretion coupling pathways in this cell line are only partially understood and that the models proposed in the present discussion represent an oversimplification. In addition whether these post-calcium ion mechanisms do indeed represent the 'late stages of the ACTH secretory pathway' or simply an intermediate stage remains to be elucidated.

The present study also indicates that G_e, the GTP-binding protein mediating calcium ion-stimulated ACTH secretion, displays characteristics consistent with it being heterotrimeric in AtT-20 cells. Further studies, perhaps adopting a more molecular biological approach, are required to confirm this hypothesis and to establish the identity of this GTP-binding protein which may in turn give indications as to possible effector proteins for G_e. In addition GTP-binding proteins are known to be regulated by phosphorylation (Yamane & Fung, 1993) therefore the possibility that G_e may be a target for a number of kinases could also be investigated. Having established the existence of G_e in AtT-20 cells these follow-up studies may determine the importance of this GTP-binding protein in the stimulus-secretion coupling pathway and suggest why calcium ion-stimulated secretion should be mediated by a GTP-binding protein.

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