The role of Ca^{2+} and other ion channels in AVP-stimulated ACTH release from ovine anterior pituitary cells

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Glossary

ACTH	adrenocorticotropin
ATC	alkali-treated casein
AVP	arginine vasopressin
BSA	bovine serum albumin
Ca_i^{2+}	intracellular calcium
cAMP	cyclic adenosine-monophosphate
cAMP-PK	cyclic adenosine-monophosphate-dependent protein kinase (= PKA)
Cd^{2+}	cadmium
CFM	Canterbury Frozen Meat Company
CI	confidence interval
Co^{2+}	cobalt
CRH	corticotropin-releasing hormone
DAG	diacylglycerol
DB	dispersing buffer
ddH_2O	de-ionised, distilled water
DHP	dihydropyridine
DME	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DOG	dioctanoylglycerol
D600	methoxyverapamil
EGTA	ethyleneglycoltetraacetic acid
E_M	plasma-membrane electric potential
ER	endoplasmic reticulum
FSH	follicle-stimulating hormone
GH	growth hormone
G-protein	guanine nucleotide-binding protein

hACTH	human ACTH-secreting pituitary adenoma cells
HPA	hypothalamic-pituitary-adrenal (axis)
IC_{50}	concentration of inhibitory agent at half-maximal efficacy
IP_2	inositol bisphosphate
IP_3	inositol 1,4,5-trisphosphate
IP_4	inositol 1,3,4,5-tetrakisphosphate
K^+_e	extracellular potassium
KR	Kreb's ringer
LH	luteinizing hormone
L-VSCC	L-type voltage-sensitive calcium channel
NCS	newborn calf serum
NSB	non-specific binding
OAG	1-oleoyl-2-acetylglycerol
P/ATC	0.05 M phosphate buffer containing alkali-treated casein
PEG	polyethylene glycol
PI	phosphoinositide-derived (second messenger system)
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A (= cAMP-PK)
PKC	protein kinase C
PMA	phorbol 12-myristate-13-acetate (= TPA)
PRL	prolactin
RHPA	reverse haemolytic plaque assay
RIA	radioimmunoassay
SEM	standard error of the mean
TEA	tetraethylammonium
TPA	12-*O*-tetradecanoylphorbol 13-acetate (= PMA)
TSH	thyrotropin-stimulating hormone
TTX	tetrodotoxin
T-VSCC	T-type voltage-sensitive calcium channel
VSCC	voltage-sensitive calcium channel
4-AP	4-aminopyridine

Abstract

The role and regulation of Ca^{2+} and other ion channels in the *in vitro* adrenocorticotropin (ACTH) response to arginine vasopressin (AVP), were investigated in static cultures of ovine anterior pituitary cells. Previous evidence suggests that the action of AVP in ACTH secreting (corticotroph) cells involves the activation of the polyphosphoinositide-derived (PI) second-messenger system, and has also been shown to be dependent on Ca^{2+} influx. In this report, a variety of chemically distinct blockers of Ca^{2+} influx, including the organic agents (methoxyverapamil (D600), nifedipine and diltiazem) and the inorganic ions (Cd^{2+} and Co^{2+}) were all found to cause large reductions in the AVP-stimulated ACTH response, providing further evidence that the AVP-induced response is dependent on Ca^{2+} influx to a large degree. However, the entire AVP-induced response was not inhibited by the blockers, suggesting that other factors, such as release of intracellularly stored Ca^{2+} also participates in this response. The blocking agents used in this study are all classified as blockers of L-type (L-) voltage-sensitive Ca^{2+} channels (VSCC), and thus the results suggest that L-VSCC are responsible for the bulk of the Ca^{2+} influx that underlies the AVP-induced response. The inorganic blocking ions also inhibit T-type (T-) VSCC, and thus it is possible that these channels also contribute to the response.

In cells that possess voltage-activated Ca^{2+} channels, raising the extracellular K^+ concentration ($[K^+]_e$) typically evokes hormone secretion, due to depolarisation-induced Ca^{2+} influx via the voltage-sensitive channels. Raising $[K^+]_e$ caused ACTH secretion from ovine corticotrophs, and this response was also sensitive to VSCC blockers. These results provide further support for the presence of VSCC in ovine corticotrophs.

Simultaneous stimulation with AVP and raised $[K^+]_e$ caused a level of ACTH secretion that was less than the sum of the individual responses, when the concentrations of the secretagogues were moderate to high. This result is consistent with the hypothesis that both secretagogues activate, to some extent, the same population of Ca^{2+} channels during

their respective responses. At low concentrations of the secretagogues, a synergistic response was observed. Further experimentation and analysis suggested that this response may be generated at the level of Ca^{2+} influx, and thus raised the possibility that the VSCC may be subject to dual voltage and voltage-independent regulation.

The possibility that voltage-independent regulation of VSCC activity by protein kinase C (PKC), part of the PI second-messenger system, occurred during the response to AVP was explored by down-regulating PKC activity. This was achieved by chronic exposure of pituitary cells to the PKC-activating phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate (TPA). This treatment totally inactivated PKC, reduced the responses to AVP, but not K^+_e, and abolished the synergistic interaction between AVP and K^+_e. Thus these results are consistent with a role for PKC in the AVP-induced ACTH response, and with the hypothesis that the synergistic response occurs due to voltage-independent (chemical) regulation of VSCC. This hypothesis was further supported by the finding that simultaneous stimulation with TPA plus raised $[K^+]_e$ caused synergistic ACTH responses.

The possibility that PKC activates VSCC was investigated by examining the effects of VSCC blockers on TPA-stimulated ACTH release. The organic blocker, D600, and the inorganic ion, Co^{2+}, both reduced the TPA-induced response. However, the patterns of inhibition were not entirely consistent with those previously observed for inhibition of AVP-induced secretion. Thus an additional protocol, reducing or removing Ca^{2+} from the extracellular medium, was employed to investigate the involvement of Ca^{2+} influx during the response to TPA. This protocol reduced both AVP- and TPA-stimulated ACTH release, and thus provided additional evidence that the AVP-induced response is dependent on Ca^{2+} influx, and further suggested that PKC can activate Ca^{2+} influx in ovine corticotrophs. Thus the possibility that AVP-activated PKC can affect VSCC activity in corticotroph cells is a viable hypothesis.

Voltage regulation of VSCC by AVP, and the effects on ACTH secretion, were also investigated. The possibility that AVP may create a depolarisation stimulus via PKC-mediated inhibition of a K^+ current that is active at rest, was examined. Exposure of cells to the K^+ channel blocker tetraethylammonium (TEA), stimulated a small increase in ACTH release that was sensitive to Ca^{2+} channel blockers. These findings are consistent with the hypothesis under investigation.

Exposure of cells to TEA in the presence of AVP or TPA enhanced the responses to these secretagogues, suggesting that a (possibly Ca^{2+}-activated) K^+ current (distinct from the one discussed in the previous paragraph) is present in ovine corticotrophs, and may act to regulate the cellular response to these agents.

Removal of external Na^+ caused a small reduction in AVP-stimulated ACTH release, suggesting that Na^+ channels may play a minor role in the response to AVP.

These investigations extend the current knowledge regarding the regulation of the AVP-induced ACTH response, particularly with respect to ovine cells.

Chapter 1

General Introduction

1.1 Regulated Hormone Secretion

In higher organisms, hormone secretion from the endocrine glands is a fundamental mechanism by which distinct organ systems communicate. Endocrine glands are defined in part by the lack of unique ducts for delivery of their secretory products. Instead, glands of the endocrine system secrete their products directly into the general circulatory system. Such a mechanism allows for a wide distribution of the secreted hormones, to various parts of the body, and thus can generate an integrated response. Targeting of the message to specific organs or cell-types occurs due to the target tissue possessing appropriate, specific receptor molecules. Steroid hormones are able to pass directly through the plasma-membrane, and bind to receptors within the cytoplasm. However peptide hormones, because of their chemical nature, are unable to pass through the cell membrane and instead the receptors for these signals are cell-surface molecules. The function of these receptor molecules is to bind the signalling hormone, and transduce the message across the cellular membrane. Often, one peptide hormone represents a signal that, upon interaction with its specific receptor on the target tissue, leads to a second hormone being secreted from the target tissue. The second hormone thus becomes the signal, which in turn may lead to a third hormone being released, etc. Many other types of secretory systems also occur, but the system in which one peptide hormone induces the release of a second peptide hormone, described here, represents the type under investigation in this study, and so discussion will

be restricted to this form of stimulus-secretion coupling.

Peptide hormone secretion, of the type to be considered here, is an example of the process known as regulated protein secretion. This process is distinguished from constitutive hormone secretion by three major characteristics (Burgess & Kelly 1987). These are: 1) secretion is coupled to an extracellular stimulus; 2) the secretory product undergoes concentration and condensation into specialized membrane-bound organelles, the secretory granules. Hormones secreted via the constitutive pathway do not undergo these processes; and 3) hormones to be secreted via the regulated pathway are stored, in secretory granules, for long periods of time prior to release. Thus a large intracellular pool of the product exists.

The process of regulated secretion of proteins involves the transduction of the primary message, represented by the extracellular signalling molecule, into an intracellular message that reproduces, within the regulatory mechanisms of the cells, the magnitude and temporal aspects of the initial signal. This intracellular message then feeds into the process of exocytosis which is responsible for the actual release of the stored hormone. Thus, the intracellular processes can be loosely divided into 1) the mechanisms responsible for processing the initial signal and generating the integrated, intracellular signal, and 2) the mechanisms involved in converting this signal into the actual cellular response, which in this case is the release of stored hormone. This project is concerned only with the signal processing aspects of hormone secretion and the latter mechanisms, controlling exocytosis, will not be considered further in any detail.

The release of a particular hormone may be under the control of multiple first messengers, often at the same time. These first messengers all have unique cell-surface receptors and may utilise the same, or, to some extent, independent intracellular signal-processing mechanisms. The multiple first messengers can produce synergistic, additive, inhibitory or other effects on hormone release, and the final cellular response will represent the integration of all the individual initial inputs.

Often the transduction of a single initial signal across the membrane, leading to the generation of the intracellular signal, involves the activation of a complex network of interconnecting pathways. The activation of multiple pathways allows for a rich variety of information and control mechanisms, which may be particularly important during the

response to multiple first messengers. In many, perhaps all, cases, the interconnecting pathways activated within the cell, by either a single first messenger or by multiple first messengers, lead to exocytosis through the modulation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of the cell (see Knight *et al.* 1989). It is the Ca^{2+} signal that is thought to regulate exocytosis and in at least some cases, the temporal and spatial profile of $[Ca^{2+}]_i$ may be closely correlated with that of the actual secretory response (Leong 1988).

1.2 The Pituitary Gland

In mammals, the pituitary gland is located at the base of the brain, immediately inferior to the hypothalamus and is subdivided into the anterior and posterior sections. The cells of the posterior pituitary are not of interest to this study, and will not be described further. The cells of the anterior pituitary synthesize, store and secrete a variety of peptide hormones including adrenocorticotropin (ACTH, from the corticotroph cells), thyroid-stimulating hormone (TSH, from thyrotrophs), luteinizing hormone (LH) and follicle-stimulating hormone (FSH, both from gonadotrophs), growth hormone (GH, from somatotrophs) and prolactin (PRL, from lactotrophs). The pituitary gland is perfused by the hypothalamo-pituitary portal blood system, which delivers hypothalamic factors that modulate pituitary cell functions. In turn, pituitary hormones are released from the various cell-types, into the circulatory system, and are thus delivered to target tissues throughout the body. Such target tissues include the adrenal glands (ACTH), the thyroid gland (TSH), and the reproductive organs (LH and FSH). Feedback systems often operate, from the target tissues back to either the hypothalamus (or higher regions) or to the pituitary, or both.

This study is concerned with the functioning of the ACTH storing and secreting sub-type of anterior pituitary cells, the corticotroph cells. However, many of the intracellular factors present in ACTH-secreting cells are also present in other hormone secreting cells of the anterior pituitary, and therefore some information from these other cell types may be relevant to the understanding of corticotroph function. This information will

be given as necessary, but a comprehensive description of functioning of these other cell types is beyond the scope of this thesis.

1.3 Corticotroph Cells and the Regulation of ACTH Release

The release of ACTH is regulated by several agents. The hypothalamic agents corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) both stimulate the release of ACTH and are considered to be the primary secretagogues of ACTH. The major target organ for ACTH is the adrenal cortex, where it stimulates the release of glucocorticoids, mainly cortisol, in response to stress. Glucocorticoids also feed back to negatively modulate the release of ACTH at both the level of the hypothalamus (and higher brain areas) and at the pituitary (see Jones & Gillham 1988, King & Baertschi 1990). Thus this system is often referred to as the hypothalamic-pituitary-adrenal (HPA) or stress axis. Other secretagogues, including oxytocin, angiotensin II, and catecholamines also influence ACTH secretion in vitro (Antoni 1986, Jones & Gillham 1988, King & Baertschi 1990). However, the physiological significance of these agents in vivo, is yet to be established, and they will not be discussed further.

The following discussion deals with the intracellular processes involved in ACTH secretion. These processes are common to many other cell types, displaying a wide variety of functions. Despite many overall similarities in the ways that these processes act in different cells, there are also considerable specific differences in the functioning of the processes. It is not possible to consider all of the variations in the intracellular processes involved in hormone secretion and therefore some generalisations must be made for the sake of readability. When made, these generalisations will tend to favour the situation pertinent to pituitary cells, and in particular, corticotroph cells.

1.3.1 CRH Regulation of ACTH Release

CRH was first isolated and characterised by Vale *et al.* (1981) and since then it has been firmly established that CRH acts intracellularly through the activation of the cAMP-protein kinase A pathway to induce ACTH release (see Antoni 1986, Jones & Gillham 1986, King & Baertschi 1990). Receptors for CRH appear to be expressed in all corticotrophs (Childs *et al.* 1987b, Childs & Unabia 1990) and the binding of these by CRH causes the activation of a specific guanine-nucleotide-binding (G-) protein, probably G_s (Aguilera *et al.* 1983, Perrin *et al.* 1986). The activated G-protein leads, in turn, to the activation of the membrane-bound enzyme adenylate cyclase. Adenylate cyclase catalyses the synthesis of cAMP which then activates protein kinase A (PKA) (see King & Baertschi (1990). PKA regulates the phosphorylation, and therefore the activity, of intracellular proteins. It has been clearly demonstrated that the ACTH response to CRH requires the influx of Ca^{2+} (Abou-Samra *et al.* 1987a, Reisine & Guild 1987, Leong 1988, Reisine 1989, Won & Orth 1990, Guerineau *et al.* 1991) via voltage-dependent Ca^{2+} channels (VSCC) (Childs *et al.* 1987a, Mollard *et al.* 1987, Won & Orth 1990, Guerineau *et al.* 1991), and there is evidence that PKA activation is linked to Ca^{2+} influx (Guild & Reisine 1987).

In most in vitro preparations, CRH and AVP act synergistically in promoting ACTH release (see Antoni 1986, Jones & Gillham 1986, King & Baertschi 1990), with multiple potential sites of interaction having been identified (see King & Baertschi 1990).

1.3.2 AVP Regulation of ACTH Release

AVP was first considered to have ACTH-regulatory properties in the mid 1950s (Martini & Morpurgo 1955, McCann 1957), but this remained controversial until 1979 when Gillies & Lowry (1979) established AVP as an important regulator of ACTH release (see Jones & Gillham 1988). When the CRH molecule was characterised by Vale *et al.* (1981), the role of AVP as a physiological regulator actually became *more* evident, due to the synergistic response that these two agents engendered (see Antoni 1986, Jones & Gillham 1988, King & Baertschi 1990). CRH is often regarded as the most potent ACTH secretagogue, and in

rat this appears to be true (Vale *et al.* 1981, 1983, Gillies *et al.* 1982). However, in the sheep this may not be the case. Liu *et al.* (1990) found AVP to be the more potent secretagogue in the ovine corticotroph, which may be due to an increased ratio of AVP to CRH receptors in ovine compared to rat corticotrophs (Shen *et al.* 1990). In our laboratory a slightly different pattern has been observed. The threshold for stimulation by CRH has been found to be lower than that of AVP (in molar terms), but AVP causes a greater maximal level of ACTH secretion (Johnson 1992).

AVP binding to corticotrophs is via a single class of receptor molecules, which are distinct from AVP receptors identified from other tissues (Antoni *et al.* 1984, Baertschi & Friedli 1985). In ACTH reverse haemolytic plaque assay (RHPA) experiments, 80 - 90% of plaque-forming rat anterior pituitary cells were found to bind AVP (Childs *et al.* 1987b), suggesting most ACTH-producing cells express functional AVP receptors.

Activation of polyphosphoinositide-derived second-messenger system

The actions of AVP on corticotrophs are mediated intracellularly through the activation of the polyphosphoinositide-derived second messenger system (PI pathway) (King & Baertschi 1990). Interaction of AVP and its specific, cell-surface receptor causes the activation of the hormone-receptor complex, and this complex is coupled to the membrane-bound enzyme, phospholipase C, through a specific G-protein (although the specific identity of the G-protein is yet to be determined; see Johnson 1992). Phospholipase C catalyses the hydrolysis of membrane phospholipid, phosphatidylinositol-4,5-bisphosphate (PIP_2), into the two intracellular messenger molecules, inositol 1,4,5 trisphosphate (IP_3) (Berridge & Irvine 1984, 1989, Berridge 1987) and diacylglycerol (DAG) (Nishizuka 1984, Berridge 1987).

Cellular responses, to external factors, that are mediated via the PI pathway have been found in many tissues to produce a characteristic intracellular Ca^{2+} profile (Rasmussen & Barrett 1984, and see Meldolesi *et al.* 1991). This profile consists of an initial spike phase, supported primarily by IP_3-induced release of Ca^{2+} from intracellular stores, followed by a plateau phase, which is sustained by Ca^{2+} influx from the external medium

(Meldolesi *et al.* 1991). Often, for secretory cells at least, the profile of the secretory response is closely correlated, both spatially and temporally, with that of the Ca_i^{2+} profile, leading to the suggestion that the Ca_i^{2+} response is the key integrator of second-messenger systems. In other words, the various intracellular signalling pathways, activated by one or more extracellular messengers, all, ultimately, affect the Ca^{2+} homeostasis of the cytosol, and thus lead to a change in the net $[Ca^{2+}]_i$. It is this factor that then feeds directly into the exocytotic pathways. Although this scheme is almost certainly an oversimplification, and other factors probably also influence exocytotic events together with Ca_i^{2+}, this scheme does reflect the considerable importance of Ca_i^{2+} in intracellular signalling (see Knight *et al.* 1989, Stojilkovic & Catt 1992), and provides a useful framework around which interpretation of experimental data can be made, at least in the first instance. This scheme will be considered further, later in this thesis.

Role of IP_3-induced Ca^{2+} mobilisation in the AVP-induced response

The spike phase of the Ca_i^{2+} response is characterised by a rapid rise-time, to a peak, followed by a similarly rapid declining phase. The rising phase occurs when IP_3, generated by the hydrolysis of PIP_2, leaves the plasma-membrane and moves into the cytosol where it facilitates the release of stored Ca^{2+} from non-mitochondrial membrane-bound organelles (Berridge & Irvine 1984). The identity of the Ca^{2+} store has not been firmly characterised; often the store is regarded to be the endoplasmic reticulum (ER), however other researchers believe the store is not the ER and use other terms, such as the "calciosome" (Meldolesi 1988; also see Tsien & Tsien 1990 and Rossier & Putney 1991, for reviews of this debate). Here the generic term "intracellular store" will be used. IP_3 is thought to act by binding to receptors on the membrane of the Ca^{2+}-storing organelle, and this causes the opening of Ca^{2+}-channels in the organelle membrane, admitting Ca^{2+} into the cytosol. The release of Ca^{2+} from the internal store is often regarded as occurring as a bolus release, depleting the store (see Tsien & Tsien 1990, Berridge 1990a and Negulescu & Machen 1988, Hoth & Penner 1992), although this may not be true in all cases (Tse *et al.* 1993). The store may refill either by sequestration of Ca^{2+} from the cytosol, or by the "capacitative Ca^{2+} entry"

proposed by Putney (1986, 1990). In both excitable and non-excitable cells, oscillations of $[Ca^{2+}]_i$ may occur. There are at least two distinct mechanisms for generating the oscillations - in non-excitable cells the oscillations are generally derived from fluctuations in the release of Ca^{2+} from internal stores, whereas in excitable cells the oscillations are derived from fluctuations in the entry of Ca^{2+}, usually via VSCC. These two mechanisms have been termed the cytoplasmic oscillator and the plasma-membrane oscillator, respectively (Berridge 1988). For a full review of the kinetics of $[Ca^{2+}]_i$ see Putney (1986, 1990), Rink & Hallam (1989), Berridge (1988, 1990a, 1990b 1993), Jacob (1990), Stojilkovic & Catt (1992), Putney & Bird (1993) and Tse *et al.* (1993).

The declining phase of the IP_3-induced Ca^{2+} spike profile also displays rapid kinetics, and results from the dual effects of depletion of the intracellular store and the activation of cytosolic Ca^{2+} buffering systems, such as sequestration to other storage organelles or efflux of Ca^{2+} from the cell (see Carafoli 1987). In general, during the falling phase of the spike response, Ca_i^{2+} does not return to the original, pre-stimulatory level, instead it is usually maintained at a level above basal for the duration of the stimulatory period. This latter phase represents the plateau phase, and, in excitable cells at least, this plateau phase is maintained by Ca^{2+} entry via specific plasma-membrane Ca^{2+} channels, directly into the cytosol (Stojilkovic & Catt 1992, and see below). IP_3 activity is terminated by a phosphatase which dephosphorylates it to the inactive IP_2 form, and further metabolism occurs before the inositol molecule becomes reassociated with the plasma-membrane (Downes *et al.* 1982, Berridge 1987). Alternatively IP_3 may be further phosphorylated to IP_4 (Irvine *et al.* 1986), which may fulfill other roles in the signalling pathway, including regulation of the entry of extracellular Ca^{2+} (Putney 1986, 1990).

For AVP-induced ACTH release from a population of pituitary cells, the schema described above appears to hold, both in terms of the generation of a Ca_i^{2+} response (Leong 1988, Corcuff *et al.* 1993) and the pattern of ACTH release (Won *et al.* 1990, Mason 1988, and see below for details). However, evidence for the generation of the typical Ca_i^{2+} response, in a population of corticotrophs, is only indirect, due to the difficulties associated with Ca^{2+} imaging using mixed cell populations (*ie.* ensuring that the observed effects arise from corticotroph cells, and not other pituitary cell types which may also respond to AVP - see below).

The generation of IP_3 and its subsequent Ca^{2+}-mobilising actions have not been conclusively demonstrated to occur in corticotroph cells, however there is much indirect evidence supporting such events occurring. In mixed cell populations, an increased turnover of polyphosphoinositides (Raymond *et al.* 1985) and, more specifically, an increased production of total inositol polyphosphates (Todd & Lightman 1987) has been found to occur in response to AVP. However, as Leong (1988) states these results do not demonstrate that the observed effects are specific to corticotroph cells. TMB-8, an agent purported to block the release of intracellular Ca^{2+}, has been shown to modestly reduce AVP-induced ACTH release (Le Beau 1989), suggesting that IP_3-induced Ca^{2+} release contributes to the AVP-induced ACTH response. However, the involvement of IP_3 can only be inferred from these observations.

Additional indirect support for the involvement of IP_3 in the ACTH response to AVP comes from observations of a requirement for Ca^{2+} mobilisation during the early phase of the response. Abou-Samra *et al.* (1987a) found, using static cultures of rat pituitary cells, that removal of extracellular Ca^{2+} significantly reduced the response to AVP only during the latter part (between the 2nd and 3rd hours) of the incubation period, suggesting that the late, but not early, phase of secretion is dependent on extracellular Ca^{2+} influx. Furthermore, pretreatment of the cells with EGTA in Ca^{2+}-free medium, in order to deplete the cells of stored Ca^{2+}, abolished both early- and late-phase secretion in response to AVP. However, these conditions are rather severe and thus the results of this latter protocol must be viewed with some caution.

Won *et al.* (1990) present more direct observations concerning the relative Ca^{2+} requirements during the ACTH response to AVP. Using a microperifusion system, these authors demonstrated that the response to AVP caused the characteristic spike/plateau pattern of hormone secretion from rat pituitary cells. When Ca^{2+} was removed from the perifusion medium, a small (32%) decrease in the spike phase secretion was observed, but this was not significant. However, the plateau phase of secretion was significantly reduced, by 67%. The cells were then exposed to the Ca^{2+} ionophore A_{23187}, to deplete the cells of Ca_i^{2+}, and the perifusion was continued with Ca^{2+}-free medium. This treatment was found to dramatically reduce stimulated ACTH release (87% and 83% for spike and plateau phase secretion, respectively). However, once again, this treatment is extreme, and raises

questions over the specificity of the observed effects. Nevertheless, these results support the hypothesis that spike-phase secretion is not greatly, if at all, dependent on extracellular Ca^{2+} influx, and instead is presumably dependent on IP_3-induced release of intracellular Ca^{2+}. On the other hand, plateau-phase secretion was found to display considerable dependence on Ca^{2+} influx. These observations were extended to an analysis of the effects of a Ca^{2+} channel blocker, nimodipine (a member of the dihydropyridine class of Ca^{2+} channel blockers) on the response to AVP. The results were found to be quantitatively identical to those where Ca^{2+} was removed from the perfusate, further supporting the hypothesis.

This group also demonstrated that depletion of intracellular, IP_3-sensitive Ca^{2+} stores by a low (1 μM) concentration of the Ca^{2+} ionophore, ionomycin, decreased the spike phase of the AVP-stimulated ACTH response (Oki *et al.* 1991). The plateau phase of the response to AVP, and the response to CRH were unaffected by this treatment. This is perhaps a less extreme treatment for depleting intracellular Ca^{2+} stores, and therefore the interpretation of a role for IP_3-induced Ca^{2+} release during the response to AVP may be made with greater confidence.

Evidence that a similar situation occurs in ovine corticotrophs arises from work conducted in this laboratory (Mason *et al.* 1989), using a column perifusion system. Ovine pituitary cells also respond to AVP with the spike/plateau pattern of ACTH release. This response is dependent on extracellular Ca^{2+} in a manner that is quantitatively similar to the results of Won *et al.* (1990). The plateau phase of secretion displayed a greater level of dependence on extracellular Ca^{2+} than did the first phase of secretion. Therefore these results are also consistent with the hypothesis that IP_3-induced release of stored Ca^{2+} is primarily responsible for the first phase of ACTH secretion, whereas Ca^{2+} influx supports the plateau phase of secretion.

A problem associated with studies of corticotroph functioning is the difficulty of obtaining pure populations of ACTH secreting cells. Because of this, the Ca_i^{2+} response from a population of corticotrophs has not been directly measured. However, the Ca_i^{2+} responses from single, identified corticotrophs have been demonstrated. Leong (1988) showed that a single ACTH-secreting cell responded to AVP by generating a spike/plateau pattern of Ca_i^{2+}. The plateau, but not the spike phase of the Ca_i^{2+} response to AVP was

abolished following the depletion of extracellular Ca^{2+} by EGTA. This finding therefore further supports the hypothesis that Ca^{2+} mobilisation occurs in response to AVP, and this generates the spike phase of the Ca_i^{2+} response. However, these results come from a review article, where details of the methodology and repeated measures of these results are not given. Therefore, it is difficult to determine the general applicability of these results to a complete population of corticotroph cells. More recently, Corcuff *et al.* (1993), measuring the intracellular Ca^{2+} concentration in normal rat corticotrophs, have found that AVP induces a heterogenous set of Ca_i^{2+} responses. In about half the cells examined, the application of a maximally stimulating concentration of AVP caused cytosolic Ca^{2+} transients - a series of short-lived Ca_i^{2+} transients (a pattern distinct from the spike/plateau profile), whereas the other half of the cells generated the typical spike response, followed by either a steady or oscillating plateau phase. Cells displaying each pattern almost always continued to display the same pattern when repeatedly stimulated with AVP. Thus these results suggest that corticotrophs are "programmed" to generate distinct Ca_i^{2+} responses, and this has important implications for the involvement of intracellular messenger systems. Corcuff *et al.* (1993) found that in transient-producing cells, the Ca_i^{2+} response was completely dependent on Ca^{2+} influx, apparently obviating a role for IP_3 in these cells. Alternatively, in cells responding with a spike/plateau profile, the plateau *but not the spike phase* was sensitive to removal of extracellular Ca^{2+}. The authors conclude that the spike phase must be dependent on the mobilisation of Ca^{2+} from intracellular stores (Corcuff *et al.* 1993).

Taken together, the results of the reports presented above provide a large body of evidence supporting the hypothesis that the mobilisation of stored Ca^{2+} contributes, in part, to the generation of the AVP-induced Ca^{2+} response in corticotrophs, and thus presumably contributes to ACTH secretion from these cells. Although it has not been demonstrated conclusively, it is generally accepted that the mobilisation of stored Ca^{2+} is due to IP_3-induced release mechanisms.

Role of Ca^{2+} influx and PKC activation in AVP-induced ACTH release

The plateau phases of a typical PI pathway-induced Ca^{2+} response, and the correlated secretory response, occur due to the steady influx of Ca^{2+} via specific plasma-membrane channels (Rasmussen & Barrett 1984, Berridge 1987). In excitable endocrine cells, such as pituitary cells, this influx typically (though perhaps not always) occurs via VSCC, *ie.* channels that are sensitive to the plasma-membrane electric potential (E_M) (Stojilkovic & Catt 1992). However, the modulation of these VSCC is almost certainly not entirely voltage-dependent, instead chemical factors such as cyclic AMP (cAMP), protein kinase C (PKC), or direct modulation by G-proteins or Ca^{2+} itself have been implicated in the control of Ca^{2+} influx via VSCC (Kaczmarek 1988, Levitan 1988, Ono & Fozzard 1992, Stojilkovic & Catt 1992), although the precise details of how this occurs are yet to be resolved (see Hartzell 1993).

In a PI-pathway-mediated cellular response, PKC is activated by DAG (which is generated following PIP_2 hydrolysis), in concert with the membrane phospholipid phosphatidylserine and an increase in $[Ca^{2+}]_i$ (from IP_3-induced release of stored Ca^{2+}). These agents promote the translocation and conversion of PKC from its inactive, cytosolic form, to its active, membrane-bound form (Nishizuka 1984). AVP and the PKC-activating phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate (TPA, also known as phorbol 12-myristate-13-acetate - PMA), have been found to induce the translocation of PKC activity from the cytosolic to the membrane fraction in an enriched population of corticotrophs (Carvallo & Aguilera 1989), as did TPA (Liu *et al.* 1990) and AVP (Liu *et al.* 1992) in ovine pituitary cells. Similar results were observed in AtT-20 cells (Zatz *et al.* 1987). TPA was found to induce phosphorylation of multiple proteins in AtT-20 cells, including species from the cytosolic, membrane and nuclear fractions (Rougan *et al.* 1989). Therefore these results suggest the presence of PKC activity in ACTH secreting cells that is sensitive to AVP.

Using either down-regulation of PKC activity, or inhibitors of PKC, several studies have reported that the full expression of the AVP-induced ACTH response requires the activation of PKC activity (Bilezikjian *et al.* 1987, Carvallo & Aguilera 1989, Liu *et al.*. 1990, 1992, Oki *et al.* 1990, Koch & Lutz-Bucher 1991). Furthermore, PKC-activating

phorbol esters or synthetic analogues of DAG have been shown to induce ACTH secretion (Reisine & Guild 1987, Abou-Samra *et al.* 1987b, Bilezikjian *et al.* 1987, Mason 1988, Sobel 1988, Carvallo & Aguilera 1989, Liu *et al.* 1990, 1992, Won *et al.* 1990, Oki *et al.* 1990). However, despite these results, the precise intracellular targets for PKC are yet to identified.

The ACTH response to AVP has been shown to display a considerable level of dependence on Ca^{2+} influx (Abou-Samra *et al.* 1987a, Mollard *et al.* 1987, 1988, Reisine & Guild 1987, Won *et al.* 1990a, Corcuff *et al.* 1993). As is described above, the Ca^{2+} current is thought to be either primarily or totally transmitted by VSCC, and the particular classes identified in corticotrophs correspond to the L (for *L*ong-lasting)-type and the T (for *T*ransient)-type described originally for neuronal tissue (Nowycky *et al.* 1985). However the precise mechanisms leading to VSCC activation are yet to be resolved.

AVP has been found to increase the L-type current, but not the T-type current, in hACTH cells (Mollard *et al.* 1987). Ca^{2+} transients, linked to action potentials in hACTH cells, were also sensitive to the VSCC blockers PN 200-110 and Cd^{2+}, as were Ca^{2+} transients from normal rat corticotrophs (Guerineau *et al.* 1991). These results provide direct evidence that L-type VSCC are activated during the ACTH secretory response to AVP.

Corcuff *et al.* (1993) found that individual rat corticotrophs produce cell-specific Ca^{2+}_i signals and membrane electrical events in response to AVP. Of the two groups of corticotrophs identified, one responded to a high (100 nM) AVP concentration with a series of short-lived Ca^{2+}_i rises (transient pattern), whereas the other responded with a Ca^{2+}_i spike followed by a sustained plateau of raised Ca^{2+}_i (spike/plateau pattern). Both the Ca^{2+} transients and the plateau phase of raised Ca^{2+}_i were reduced by PN 200-110 and Cd^{2+} as well as Ca^{2+}-free extracellular medium. In cells that produced Ca^{2+} transients, these appeared to be linked to the generation of action potentials, suggesting that the electrical state of the plasma-membrane contributes to the regulation of Ca^{2+} influx.

An effect of PKC to increase VSCC activity in corticotrophs, causing influx of Ca^{2+}, has been suggested by the demonstration that blockers of VSCC or removal of extracellular Ca^{2+} reduces the ACTH response induced by phorbol esters (Reisine & Guild 1987, Sobel 1988, Won *et al.* 1990). The activity of the L-VSCC has been found to be sensitive to the

actions of PKC (as well as other kinase enzymes), in several different tissue types (DeReimer *et al.* 1985, Rane & Dunlop 1986, Kaczmarek 1988, Lacerda *et al.* 1988, Levitan 1988, O'Callahan *et al.* 1988), including pituitary cells (Lewis & Weight 1988, Marchetti & Brown 1988, Stojilkovic *et al.* 1991). Both positive and negative effects of PKC-induced phosphorylation of L-VSCC have been observed. In some tissue preparations, including in pituitary cells (Stojilkovic *et al.* 1991), both effects have been observed in the same channel species (Lacerda *et al.* 1988). The differential effects of PKC are thought to yield fine regulatory control over Ca^{2+}-dependent cellular processes (Stojilkovic *et al.* 1991).

In corticotroph cells, the effects of phorbol esters on Ca^{2+} influx might also display this complex pattern of both activation and inhibition. The studies described above (Reisine & Guild 1987, Sobel 1988, Won *et al.* 1990), in which blockers of VSCC were found to attenuate phorbol ester-induced ACTH secretion, are complemented by the demonstration that TPA can induce a rise in $[Ca^{2+}]_i$ in AtT-20 cells (Reisine & Guild 1987, Reisine 1989). However, Lewis & Weight (1988) found that the PKC activator 1-oleoyl-2-acetylglycerol (OAG) reduced the voltage-dependent Ca^{2+} current in AtT-20 cells, and Luini *et al.* (1985) found that TPA reduced the $[Ca^{2+}]_i$ in AtT-20 cells. Therefore PKC may exert differential effects in corticotroph cells (see Chapters 4 and 6 for further discussion).

PKC may also have other effects in the regulation of ACTH release. Activators of PKC have been found to cause the biosynthesis of ACTH in ovine corticotrophs (Liu *et al.* 1990). PKC has also been reported to feed back and regulate ligand-induced IP_3 generation and also to enhance IP_3 metabolism, thus attenuating IP_3-induced release of stored Ca^{2+} (Judd *et al.* 1987).

1.4 Objectives

The objectives of this study are to investigate the role and regulation of Ca^{2+} and other ion channels in the in vitro regulation of ACTH secretion induced by AVP in dispersed ovine anterior pituitary cells. Chapter 3 describes the results of an investigation of the effects of VSCC blockers, of various chemical classes, on the AVP-stimulated ACTH response. This

investigation provides additional evidence for the dependence of the AVP-induced response on Ca^{2+} influx and has appeared in published form (Le Beau & Mason 1994).

Chapter 4 describes the results of an investigation into the potential role of PKC activation on the functioning of VSCC during the response to AVP. The results provide additional support for the role of PKC activation during the AVP-stimulated response, and also for the notion that VSCC activity is regulated, in part, by non-electrical factors. PKC is implicated as a potential regulator of VSCC activity.

Chapter 5 describes the results of investigations into the electrical regulation of VSCC during the response to AVP, and also the role of plasma-membrane Na^+ and K^+ channels in the AVP-induced response. The results provide evidence that K^+ conductance may regulate membrane excitability, and thus VSCC activity, and Na^+ channels may also be involved, to a limited extent in AVP-stimulated ACTH release.

Much of the previous research relating to the regulation of ACTH secretion has utilised either rat pituitary cells or tumour corticotroph cells. AVP appears to be a more potent, and therefore (perhaps) a more important, regulator of ACTH release in ovine corticotrophs, than it is in rat corticotrophs. This study extends the current knowledge regarding ACTH regulation in ovine pituitary cells.

Chapter 2

Materials and Methods

2.1 Materials

For the sources of materials used in this study, refer to Appendix I.

2.2 Methods

For details of all solutions and mediums used refer to Appendix II.

2.2.1 Pituitaries

Pituitaries from sexually mature (> 1 year old) ewes were collected from either of two sources. Preferentially, pituitaries were obtained from the Canterbury Frozen Meat Company (CFM) freezing works (Belfast, Christchurch), where the heads were split ventro-dorsally, allowing the whole, intact pituitary to be dissected out. The glands (usually about 10) were placed in chilled, sterile dispersing buffer (DB) for transportation back to the laboratory.

When pituitaries were not available from Belfast CFM, whole heads were collected from Malvern Abattoir Ltd (Canterbury) and transported, on ice, back to the Department of Zoology, University of Canterbury. Here the heads were cut posterio-anteriorally with

a band saw, allowing the cut to curve around the area of the pituitary gland. The pituitary glands were then dissected out from the heads and placed into cold, sterile DB.

When either source of pituitaries was used, only intact pituitary glands were used.

2.2.2 Cell Preparation and Preliminary Culture

Chilled, sterile DB was used as the medium for preparation of dispersed pituitary cells. All solutions, instruments, plasticware and glassware used for cell preparation had previously been either autoclaved (15 min, 121°C, 15 psi), or heat sterilised (170°C, 4 h). The cell preparation was performed in a laminar flow hood and aseptic conditions were maintained throughout.

The pituitary glands were washed by three brief submersions in fresh DB and then collected in DB on ice. Depending on their size, either six, seven or eight glands were selected to prepare the dispersed cells. Adhering connective tissue, the median eminence, pituitary stalk and posterior pituitary were removed. The anterior pituitaries were then rinsed once with DB, minced, with scissors, into small (less than 2 mm^3) pieces and transferred to a trypsinising flask containing 50 ml of collagenase solution (concentration 480 U/ml DB). The flask was placed in a 37°C incubator and the tissue suspension was stirred with a magnetic stirrer. Cells were collected after an initial incubation period of 20 - 30 min and after two subsequent 1 hour incubations. Generally, the initial incubation produced few cells, but considerable connective tissue, and the tissue was discarded. Depending on the number of cells required and the size of cell pellets obtained from the second and third incubations, a fourth incubation, of up to 1 hour duration, was occasionally required. Dispersed cells were collected by decanting the supernatant from the trypsinising flask and centrifuging this at 300 x g (IEC Centra-8R) or 200 x g (Jouan CR-412) for five minutes at 4°C. The supernatant was returned to the trypsinising flask for subsequent incubations while the cell pellet was washed by resuspending the cells in DB using a 10 ml pipette and centrifuging as before. Following resuspension of the cell pellet any strands of connective tissue or gelatinous material were removed with a Pasteur pipette as necessary.

The cells collected from the 1 h incubations were pooled and washed (as above) four times. After the cells had been resuspended, following each spin, connective tissue was removed, if necessary, with a Pasteur pipette. Following the final wash the cells were resuspended in 30 - 40 ml of Dulbecco's modified Eagle's medium (DME), supplemented with 10% new-born calf serum (NCS). A sample of this suspension was used to perform a cell count using a haemocytometer. Viability was determined by trypan blue exclusion (Hoskins *et al.* 1956) and was typically greater than 90%, and never below 85%.

A cell suspension containing 0.5 x 10^6 viable cells/ml DME + NCS was prepared. One ml aliquots of the suspension was then distributed randomly into 24 well tissue culture plates (Nunc Inter Med., Denmark), to give a final density of 0.5 x 10^6 viable cells/well. The cells were cultured at 37°C under 5% CO_2:95% air in a humidified incubator. This preliminary culture, of 20 ± 2 h duration, allowed the cells to recover from the digestion process, and to adhere to the bottom of the wells.

2.2.3 Hormone Secretion Experiments

The general procedure for the cell experiments was as given below. Specific alterations in this protocol are given in the text where appropriate. Typically 4 - 6 culture plates were used per experiment and for each manipulation (*eg.* removal of overnight culture medium and the addition of the 1 h pre-incubation medium) the entire manipulation was performed on each plate individually. This was done until all the plates had received the particular manipulation. The commencement of the experimental protocol for each plate was staggered so that each plate could receive the correct incubation periods.

Medium was changed by vacuum aspiration of the wells, followed by addition of new medium using a 1 ml automatic pipette (Gilson P1000). Care was taken at all times to minimize disturbance of the cells.

Following the preliminary culture, the medium bathing the cells was removed and replaced with 1 ml of warmed Kreb's Ringer (KR, the experimental medium), containing 5% NCS and 50 μg/ml ascorbic acid (KR + NCS). The cells were incubated in KR + NCS for 60 - 75 min (37°C) to allow them to equilibrate to the new medium. At the completion

of this pre-incubation period the cells were washed once with 1 ml of fresh, warmed KR + NCS (*ie.* the pre-incubation medium was removed, fresh KR + NCS was added and then removed from all wells prior to the addition of the test medium).

One ml of the appropriate, pre-warmed (34 - 37°C) test solution (*ie.* medium containing secretagogues and/or inhibitory agents etc.) was then added to each well. KR + NCS was used to prepare all test solutions used, details of which are given in Appendix II. Each treatment was carried out in quadruplicate. After addition of the test medium, the plate was returned to the incubator. Unless otherwise stated the test incubation lasted 90 minutes, and at the completion of this period 700 μl of the medium was removed (1 ml automatic pipette) and frozen for ACTH analysis. Care was taken to remove the sample from immediately below the meniscus, to avoid contamination of the sample with cells.

2.2.4 Analysis of remaining ACTH content of cells

In the experiments in which cellular PKC activity was down-regulated by chronic exposure to the phorbol ester, TPA (see section 4.2.2), the remaining ACTH content of the cells, at the completion of the hormone secretion experiment, was assessed. This was necessary because exposure to TPA initially stimulates PKC activity, which then induces considerable ACTH release (see Fig. 4.7). The continued presence of TPA results in the down-regulation of PKC activity. However, it has been found that this general protocol often has the drawback that the hormone content of the cells does not recover, compared to control (non TPA-pre-exposed) cells by the time the test incubation is commenced (Stojilkovic *et al.* 1988b). Thus, comparing the hormone responses from the control and TPA pre-exposed cells is invalid unless the actual amount of hormone available to be released is taken into account. To do this, it is necessary to measure the total pool of hormone present in the cells in each well. Therefore, at the completion of the hormone secretion experiment, the remaining ACTH content of the cells was determined, and the sum of this value and the hormone released during the test incubation gave a value for the total releasable hormone

content for the cells in each well. The hormone response for each treatment was then expressed as a percentage of the total hormone pool at the start of the test incubation.

The procedure for determining the ACTH content of the cells was as follows. After removal of the test sample, the remainder of the medium in the wells was removed by aspiration and 1 ml of KR + NCS + 0.1% Triton X-100 was added to solubilise the cells. The plates were left for ≥ 30 min at 4°C. The medium and ruptured cells in each well were re-pipetted with a 1 ml automatic pipette, and the resulting suspension was collected into a sample (3DT) tube. The cell lysates were then frozen for ACTH analysis. To prepare the samples for radioimmunoassay (RIA, see below), they were thawed, mixed (vortex mixer), and then centrifuged at 2500 x g (IEC Centra-8R) for 20 min at 4°C. When the sample was added to the RIA tubes, care was taken to remove the supernatent, without disturbing the pellet of undissolved cellular material.

2.2.5 ACTH Radioimmunoassay

Antiserum

ACTH was measured by direct radioimmunoassay (RIA) of the test incubation medium, using rabbit anti-porcine ACTH antiserum, which was a gift from Professor Richard Donald (Department of Endocrinology, Christchurch Hospital). The antiserum was diluted 1:1400 with assay buffer - 0.05 M phosphate buffer containing 0.1% alkali treated casein (P/ATC, see Appendix II).

Tracer

^{125}I-labelled ovine ACTH (^{125}I-oACTH, the oACTH was a gift from Dr CH Li, see Appendix II) was prepared by chloramine-T radioiodination of oACTH, and purified on a

cellulose column (Greenwood *et al.* 1963), by either myself or Dr DR Mason. The tracer was aliquoted (0.5 ml/aliquot) and frozen at -20°C for up to six weeks prior to use in the RIA.

Before use the tracer was repurified by adding 15 mg of silicic acid powder to a 0.5 ml aliquot of tracer. Damaged ACTH and free ^{125}I were removed by 2 washes (centrifugation at approximately 1200 x g (Griffen and George Ltd, bench top centrifuge) and re-suspension in de-ionised, distilled H_2O (ddH_2O)). After the final spin, the hormone was eluted from the silicic acid pellet by the addition of 1 ml of acetone/acetic acid solution (2.5:0.1:10 acetone:glacial acetic acid:ddH_2O). The suspension was centrifuged, as before, and the supernatant, containing the labelled hormone, was collected. The repurified tracer was then diluted with assay buffer (P/ATC) to give approximately 10 000 cpm/100 μl.

ACTH Standards

A 1 ml frozen aliquot of 500 μg/l oACTH was thawed and diluted with P/ATC to produce a stock solution of 10 μg/l oACTH. This was then used to produce standard solutions of the following concentrations: 10, 5, 3, 2, 1.5, 1.0, 0.5 and 0.25 μg/l by a single dilution of the stock solution with an appropriate volume of P/ATC. Each standard was aliquoted into 1 ml aliquots and stored frozen. P/ATC was used as the zero standard.

Replication standards

Replicate standards were prepared as follows. Unused cells from a cell preparation were incubated overnight in a 50 ml centrifuge tube. The next day the medium was changed and the cells were exposed to high concentrations of AVP for 90 min. The medium was collected after centrifugation, to prevent collection of any cells, and the ACTH content of the supernatent was assayed and then diluted to give ACTH concentrations of approximately:

High replicate	6.0 μg/l
Medium replicate	2.0 μg/l
Low replicate	0.6 μg/l

These were prepared as 1.0 ml aliquots and stored at -20°C.

Intra-assay coefficients of variation were 7.0%, 3.5% and 4.4% for the low, medium and high replicates, respectively. Interassay coefficients of variation were 5.8%, 4.6% and 5.5% for low, medium and high replicates, respectively.

Assay Procedure

Assays were set up in duplicate with the assay tubes placed in an ice/water bath. P/ATC buffer, ACTH sample (either standard (Std), experimental sample or replicate), ^{125}I-oACTH tracer and ACTH antiserum were added to the appropriate assay tubes in sequential fashion (Table 2.1). Non-specific binding (NSB) tubes were included for each set of standards, replicate, and set of samples. The concentration of ACTH in the experimental samples was such that dilution in the assay was generally not necessary.

Assay Separation

The antibody-bound ^{125}I-oACTH was separated from the free labelled hormone by fractional precipitation, as follows: at the completion of the incubation period, horse plasma (50 μl) and polyethylene glycol solution (PEG, 1.5 ml, Appendix II) were added to each tube. After vigorous vortex mixing the tubes were incubated at room temperature for a total of 15 minutes. The antiserum-bound hormone was then precipitated by centrifugation at 2450 x g (IEC Centra-8R centrifuge) for 25 min at 4°C, or 3900 x g (Jouan GR4-22 centrifuge) for 15 min at 4°C. The supernatant was removed by vacuum aspiration and the radioactivity (counts/2 min) of each pellet was measured by a gamma scintillation counter

Tube type	Reagents (μl/tube)			
	P/ATC	Std/Sample/Replicate	Tracer	Antiserum
NSB (zero std)	150	100	100	-
zero std	50	100	100	100
standards	50	100	100	100
NSB (assay zero)	250	-	100	-
assay zero	150	-	100	100
replicates	50	100	100	100
samples	50	100	100	100

Table 2.1. Protocol for addition of reagents to RIA tubes. After addition of all reagents the tubes were mixed briefly (vortex mixer) and then incubated at 5°C for 20-24 h.

(1275 minigamma counter, LKB Wallac). The data obtained were stored as a computer file on an IBM-compatible microcomputer.

Analysis of RIA output

The raw data (counts/2 min) were analysed using the MRIAC RIA program (Livesey 1974). The basis of this program is to fit a transformed standard curve by overlapping two or more straight line segments in order to achieve approximate linearity across the whole range of the standards used. Each segment is as long as is consistent with linearity. The transformation used on the standards is related to the logit. The standard curve is then used to calculate the hormone concentration for each of the samples assayed. The value given by the program output is the mean for the duplicates, and a 95% confidence limit is set for each of the duplicates.

2.2.6 Analysis of results

The following describes the general procedures used to analyse the raw data and derive values that would allow comparisons between individual experiments. Specific departures from these procedures are described when the data concerned are presented.

Data obtained from MRIAC analysis were defined as either: 1) "basal" or "unstimulated" release, *ie.* the amount of ACTH released in the absence of (putative) stimulating agents, or 2) "total ACTH released", *ie.* the amount of ACTH released in the presence of stimulating agents (with or without other agents that may modulate the cellular response).

For each treatment, the mean of the quadruplicate values from the MRIAC was calculated and expressed ± the standard error of the mean (SEM), in units of ng ACTH/0.5 x 10^6 cells/90 min (or the time period used if different). In most cases the data was converted to values of "stimulated ACTH release" (with the same units) by subtracting the mean basal release from the mean total ACTH released. When agents that may affect the cellular response (such as Ca^{2+} channel antagonists) were used, the values for stimulated release were normalised by subtracting the value for basal release in the presence of the agent from the total release in the presence of the agent. This was done because the agents used occasionally had effects on unstimulated ACTH release (see Chapter 3).

Secretagogue-induced ACTH release in the presence of agents that modify cellular function (*eg.* Ca^{2+} channel blockers) was calculated as follows (with AVP as the secretagogue in this case). For each antagonist concentration, M ($[Ant]_M$):

$$\text{AVP-stimulated ACTH release at } [Ant]_M = \text{Total ACTH release in presence of AVP} + [Ant]_M - \text{Unstimulated ACTH release at } [Ant]_M$$

In most cases the percentage inhibition caused by this concentration of the antagonist was calculated as follows:

$$\%\ \text{inhibition at } [\text{Ant}]_M = \frac{\text{AVP-stimulated release at } [\text{Ant}]_0 - \text{AVP-stimulated release at } [\text{Ant}]_M}{\text{AVP-stimulated release at } [\text{Ant}]_0} \times 100$$

where $[\text{Ant}]_0$ represents the absence of antagonist. Analogous calculations were performed for K_e^+-, TPA- or TEA-stimulated ACTH release. In some situations, the ACTH response (total or stimulated) of some treatment, X, was expressed relative to some control value (either unstimulated ACTH release, or another value, as appropriate), *ie.*:

$$\text{response relative to control} = \frac{\text{ACTH response of X}}{\text{control ACTH response}} \times 100$$

This calculation gives a value termed the "response relative to control" (which was set to 100%). Further variations of this procedure were occasionally used, depending on what question was being addressed in the experiments and the subsequent analyses. Specific details of the way the data were analysed are given, where appropriate, when the data are presented.

The absolute values for unstimulated, total, and stimulated ACTH release varied somewhat between individual experiments. However, the relative levels of secretion for different treatments within replicates of a particular experimental protocol were generally consistent between experiments, and therefore the calculations described above allowed pooling of data from replicate experiments. However, where possible, the raw values for total and/or stimulated ACTH release are presented.

Experimental protocols

Following the initial trial of a particular experiment, the protocol (with modification if necessary), was repeated, using further cell preparations, at least twice more. The "n" value quoted when results are described therefore refers to the number of individual experiments conducted using the particular protocol (unless specifically stated otherwise).

Determination of IC_{50} values

IC_{50} values for inhibition of stimulated ACTH release by Ca^{2+} channel antagonists were calculated using the *FLEXIFIT* curve analysis software (Guardabasso *et al.* 1987).

2.2.7 Statistics

Statistical analysis of the data was calculated using the *Statistics* (version 4.0) computer package. Analysis of the data from dose-response experiments and intra-treatment comparisons of means were performed using one way ANOVA and subsequent comparisons of means tests (Bonferroni method), to determine which means differed by a significant margin (*P* level = 0.05). For all other experiments, inter-comparisons of means, and subsequent determinations of significance, were done using the two sample Student's T-test. Levels of significance are as indicated. Values of percentage inhibition or relative responses were defined as statistically different from 0% or 100% inhibition by constructing confidence intervals for the experimental data. The data was considered significantly different from the parameter being compared with, at the significance level indicated, if the parameter was outside the confidence interval.

Chapter 3

The effects of Ca^{2+} channel antagonists on AVP- and K_e^+-stimulated ACTH release

3.1 Introduction

Several studies have used organic blockers of L-type VSCC, agents which bind to the channel complex and prevent the movement of Ca^{2+} through the channel (Janis & Triggle 1991), to investigate the role of Ca^{2+} influx during CRH- and AVP-stimulated ACTH release. However, these have produced conflicting results. Giguere *et al.* (1982) found no effect of verapamil (a phenylalkylamine) on CRH-stimulated ACTH release, and Murakami *et al.* (1985) found no significant effect of nifedipine on AVP-stimulated ACTH release. In contrast, more recent studies have demonstrated inhibition by nitrendipine and nimodipine of the AVP- (Abou-Samra *et al.* 1987a, Won *et al.* 1990) and the CRH-stimulated (Abou-Samra *et al.* 1987a, Won & Orth 1990, Childs *et al.* 1987a) responses. Nifedipine, nitrendipine and nimodipine are all members of the 1,4-dihydropyridine (DHP) class of L-type VSCC blockers.

Electrophysiological and combined electrophysiological/Ca^{2+}-imaging observations have shown that human ACTH-secreting pituitary adenoma (hACTH) cells, and normal

(small ovoid) rat corticotroph cells, exhibit putative L-type currents that could be blocked by the DHPs nifedipine (Mollard *et al.* 1988) and PN 200-110 (Guerineau *et al.* 1991). Furthermore, the inorganic divalent ions Co^{2+} and Cd^{2+}, which block both L-type- and T-type VSCC (Nowycky *et al.* 1985), were found to reduce L- and T-type currents in hACTH cells and in normal rat corticotrophs (Mollard *et al.* 1987, 1988, Guerineau *et al.* 1991). Of particular note was the finding that AVP was able to increase the L-type current, but not the T-type current, in hACTH cells (Mollard *et al.* 1988). Ca^{2+} transients, linked to action potentials in hACTH cells, were also sensitive to both PN 200-110 and Cd^{2+}, as were Ca^{2+} transients from normal rat corticotrophs (Guerineau *et al.* 1991). These results provide direct evidence that L-type VSCC, thought to be activated during the ACTH secretory response to both CRH and AVP, are sensitive to both organic and inorganic blocking agents.

It has recently been reported (Corcuff *et al.* 1993) that individual rat corticotrophs produce cell-specific intracellular Ca^{2+} (Ca_i^{2+}) signals and membrane electrical events in response to AVP. Of the two groups of corticotrophs identified, one responded to a high (100 nM) AVP concentration with a series of short-lived Ca_i^{2+} rises (transient pattern), whereas the other responded with a Ca_i^{2+} spike followed by a sustained plateau of raised Ca_i^{2+} (spike/plateau pattern). Both the Ca^{2+} transients and the plateau phase of raised Ca_i^{2+} were reduced by PN 200-110 and Cd^{2+} as well as Ca^{2+}-free extracellular medium. Furthermore, injection of hyperpolarising current reversibly reduced the plateau phase of raised Ca_i^{2+}. This report also provides evidence that Ca^{2+} released from intracellular stores contributes to the AVP-stimulated response in corticotrophs exhibiting the spike/plateau pattern.

Discrepancies between various reports as to the effectiveness of Ca^{2+} channel antagonists, may be due, at least in part, to methodological differences. Thus, it is appropriate to evaluate the efficacy of the various types of Ca^{2+} channel blockers in a particular experimental system, before using any of these agents to investigate the mechanism of the response under investigation, in this case the AVP-stimulated ACTH response. Information regarding the potencies of these agents also allows comparison of the characteristics of corticotroph Ca^{2+} channels with those of other tissues that have been examined.

In this study, organic agents representing three chemically distinct families of L-type VSCC antagonists, as well as two inorganic blocking ions, were used to investigate the role of extracellular Ca^{2+} influx during AVP-stimulated ACTH release. The antagonists used were: 1) methoxyverapamil (D600), a phenylalkylamine, 2) nifedipine, a DHP, 3) diltiazem, a benzothiazepine, 4) Co^{2+} and 5) Cd^{2+}. Results of comparative studies, similar to the present one, have been reported for rat pituitary gonadotrophs (Conn *et al.* 1983) and for the prolactin (PRL)- and growth hormone (GH)-secreting, GH_4C_1 pituitary tumour cell line (Enyeart *et al.* 1985), but not as yet for pituitary corticotrophs.

3.2 Results

AVP stimulated the release of ACTH from ovine corticotrophs in a concentration-dependent manner. Maximum stimulation by AVP occurred at concentrations of approximately 100 nM and above, and the threshold AVP concentration for stimulation of ACTH release was found to be approximately 1.0 nM AVP (Fig. 3.1).

3.2.1 Effects of VSCC antagonists on unstimulated ACTH release

Table 3.1 shows the effects of the three organic Ca^{2+} channel antagonists on unstimulated ACTH release during the 90 min test incubation. For most of the D600 and nifedipine concentrations used, there was no effect of the antagonist on unstimulated ACTH release. However, occasionally, a small but significant increase was observed (Table 3.1). For this reason, AVP-stimulation in the presence of each concentration of antagonist was corrected for by subtracting the unstimulated value obtained at the same antagonist concentration (see Chapter 2, Materials and Methods).

Diltiazem had a greater effect on unstimulated release (Table 3.1). Three of the four diltiazem concentrations used caused significant changes in unstimulated ACTH release, although the changes seen with 10 and 100 μM diltiazem were small.

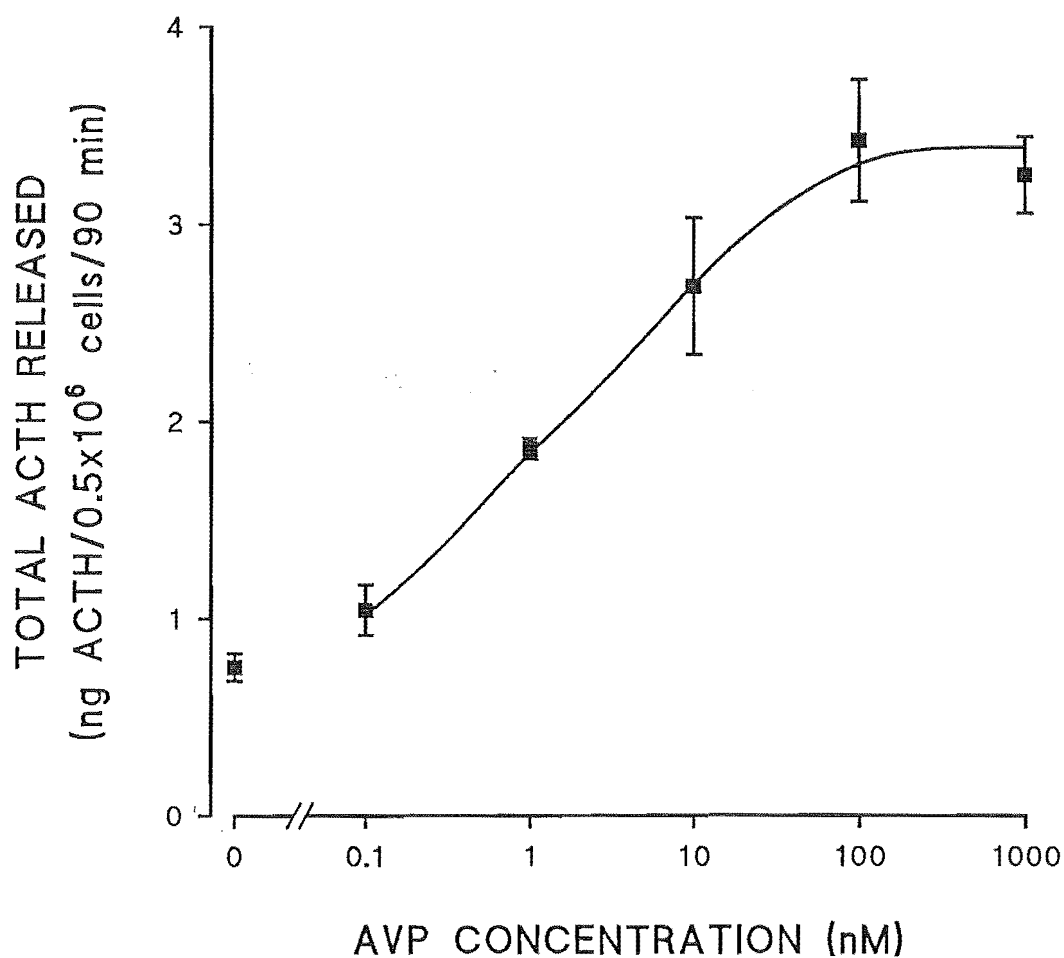

Fig. 3.1. Dose-response curve for AVP-stimulated ACTH release. Data are mean ± SEM, n = 4, from a single representative experiment. Quantitatively similar levels of AVP-induced secretion (in terms of the relative response *cf.* basal) were obtained throughout the work described in this thesis.

	ACTH release relative to control						
	[Antagonist]						
	10^{-8} M	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M	$3x10^{-4}$ M	10^{-3} M
D600	107.2 (7.2)	106.2 (8.7)	105.3 (4.9)	106.8 (4.2)	105.3 (7.8)	112.3** (4.6)	-
Nifedipine	96.1 (6.1)	110.7** (3.7)	108.4 (5.6)	107.0 (7.4)	-	-	-
Diltiazem	-	-	91.3 (4.5)	94.0** (1.4)	112.0* (4.7)	-	140.9** (6.5)

Table 3.1. Effects of organic Ca^{2+} channel antagonists on unstimulated ACTH release. Data are expressed as a percentage of control (*ie.* absence of antagonists), and are "means (± SEM)", n ≥ 3; *, $P < 0.05$; **, $P < 0.01$.

The inorganic ions Co^{2+} and Cd^{2+}, both caused alterations of unstimulated ACTH release (Table 3.2). Co^{2+} caused significant, but minor changes in unstimulated release at 0.2 and 0.5 mM ($P < 0.01$), but at 5 and 10 mM, larger effects (elevations) were observed ($P < 0.01$).

Cd^{2+} caused a significant ($P < 0.01$) increase in unstimulated ACTH release when used at concentrations of 50 μM or greater (Table 3.2); this effect was apparently concentration-dependent.

3.2.2 D600 inhibition of AVP-stimulated ACTH release

D600 inhibited AVP (1 μM)-stimulated ACTH release in a concentration-dependent manner (Fig. 3.2). Significant inhibition ($P < 0.05$, by confidence interval (CI)) was observed at D600 concentrations of 1 μM and above. The greatest inhibition of AVP-stim-

	ACTH release relative to control									
	[Antagonist] (M)									
	$2x10^{-6}$	$2x10^{-5}$	$5x10^{-5}$	10^{-4}	$2x10^{-4}$	$5x10^{-4}$	10^{-3}	$2x10^{-3}$	$5x10^{-3}$	10^{-2}
Cd^{2+}	100.0	110.0	112.3*	123.7*	145.4*	148.9*	155.6*	-	-	-
	(1.2)	(10.4)	(1.1)	(6.5)	(10.8)	(15.9)	(13.2)	-	-	-
Co^{2+}	-	101.7	-	-	87.8*	92.0*	-	109.2	137.7*	154.0*
	-	(9.8)	-	-	(4.2)	(1.2)	-	(5.7)	(12.4)	(16.1)

Table 3.2. Effects of inorganic blocking ions on unstimulated ACTH release. Data are expressed as percentage of control (*ie.* absence of antagonists) and are "means (± SEM)", n ≥ 3; *, $P < 0.01$.

ulated ACTH release was seen with 300 μM D600 (71.6 ± 2.3%), and although this value was obtained at the highest concentration of D600 used, the data suggest that the magnitude of inhibition has reached a plateau in this region (Fig. 3.2). The IC_{50} for D600 inhibition of AVP-stimulated release was calculated to be 1.62 ± 0.32 μM.

Percentage inhibition by D600 of 1 nM AVP-stimulated ACTH release was not significantly different from inhibition of 1 μM AVP-stimulated release ($P > 0.05$), despite a 4- to 5-fold difference in the absolute amounts of ACTH released in response to these two concentrations of AVP (data not shown). This suggests that as the level of stimulation by AVP is increased, there is no change in the relative dependence of the response on extracellular Ca^{2+} influx.

3.2.3 Nifedipine inhibition of AVP-stimulated ACTH release

Nifedipine, a member of the DHP class of L-type VSCC antagonists, was also found to inhibit AVP-stimulated ACTH release (Fig. 3.2). The low solubility of this agent precluded

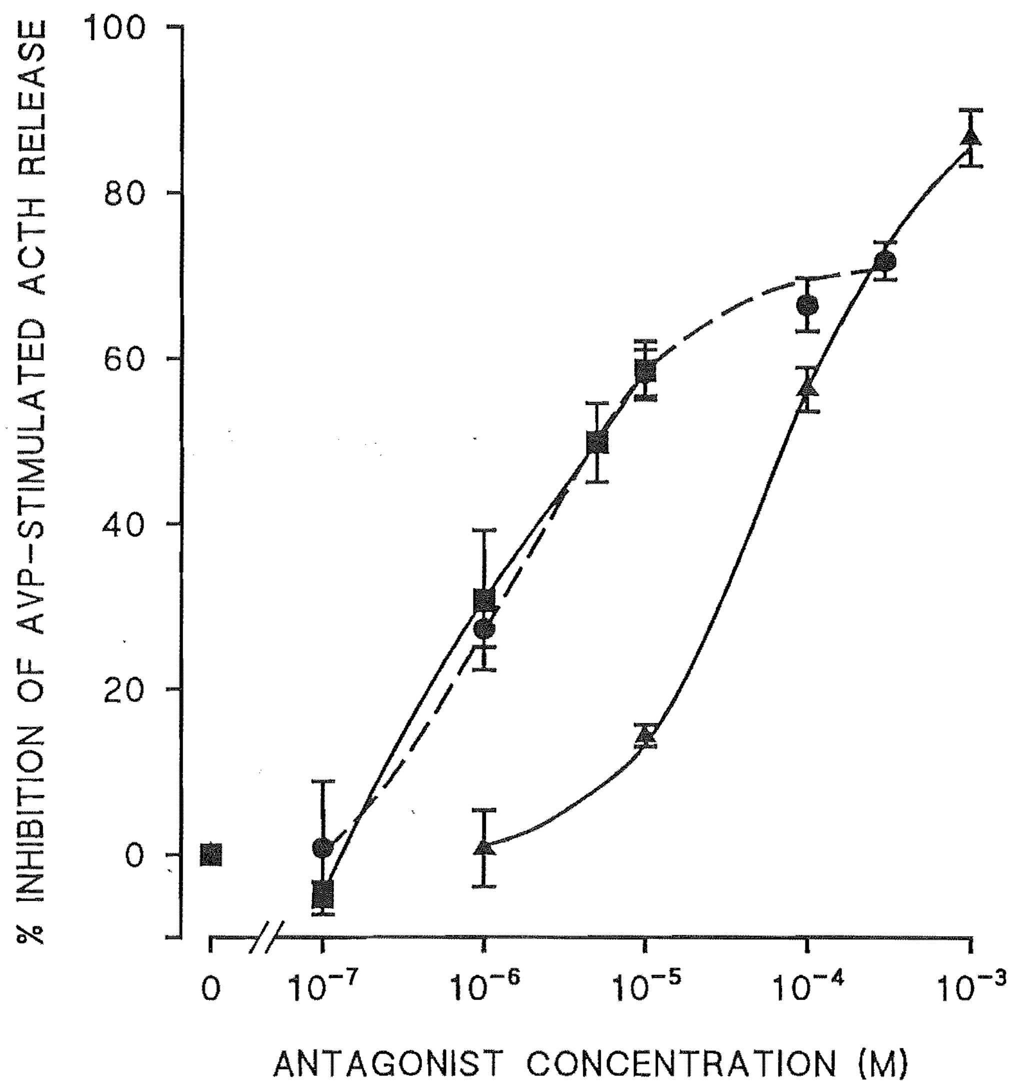

Fig. 3.2. Effects of organic blockers on maximal AVP-stimulated ACTH release (100 nM AVP for nifedipine (squares) and diltiazem (triangles) curves, 1000 nM AVP for D600 (circles) curve). Values are means ± SEM, $n \geq 3$.

its use at high concentrations, comparable to those used for D600. However, for equimolar concentrations of the two antagonists, the magnitude of inhibition caused by nifedipine was equivalent to that caused by D600. Significant inhibition ($P < 0.05$, by CI) of stimulated ACTH release was observed at nifedipine concentrations of 1 μM and above, and the greatest level of inhibition of AVP (100 nM)-stimulated release, was 58.4 ± 3.6% at 10 μM nifedipine. As with D600, inhibition by nifedipine was unaffected by the level of stimulation by AVP ($P > 0.05$ for 1 nM *vs.* 100 nM AVP-stimulated release; 1 nM AVP data not shown).

3.2.4 Diltiazem inhibition of AVP-stimulated ACTH release

Diltiazem, a member of the benzothiazepine class of L-type VSCC antagonists, was also found to cause inhibition of AVP-stimulated ACTH release (Fig. 3.2). At molar concentrations corresponding to those used for D600 and nifedipine, this agent was a less potent inhibitor of the AVP-stimulated secretory response. However, because of its greater solubility, diltiazem could be used at higher concentrations than D600 or nifedipine. Thus, at 1 mM, diltiazem caused greater inhibition (86.5 ± 3.4%) of the response to a maximally stimulating AVP concentration (100 nM) than was observed for either D600 or nifedipine ($P < 0.05$). Also, for the concentrations used, the curve for diltiazem shows no sign of reaching a plateau below 100% inhibition, in contrast to the inhibition curve obtained for D600 (Fig. 3.2).

3.2.5 Inorganic ion inhibition of AVP-stimulated ACTH release

As with the three organic blockers used, the inorganic Ca^{2+}-entry blocking ions, Co^{2+} and Cd^{2+}, were found to be effective blockers of AVP-stimulated ACTH release. Cd^{2+} was the more potent ion, causing significant inhibition of AVP (100 nM)-stimulated ACTH release at 50 μM ($P < 0.05$, CI), and maximal inhibition (87.6 ± 2.6%) at 1000 μM (Fig. 3.3). The IC_{50} for inhibition by Cd^{2+} was calculated as 74.4 ± 6.3 μM.

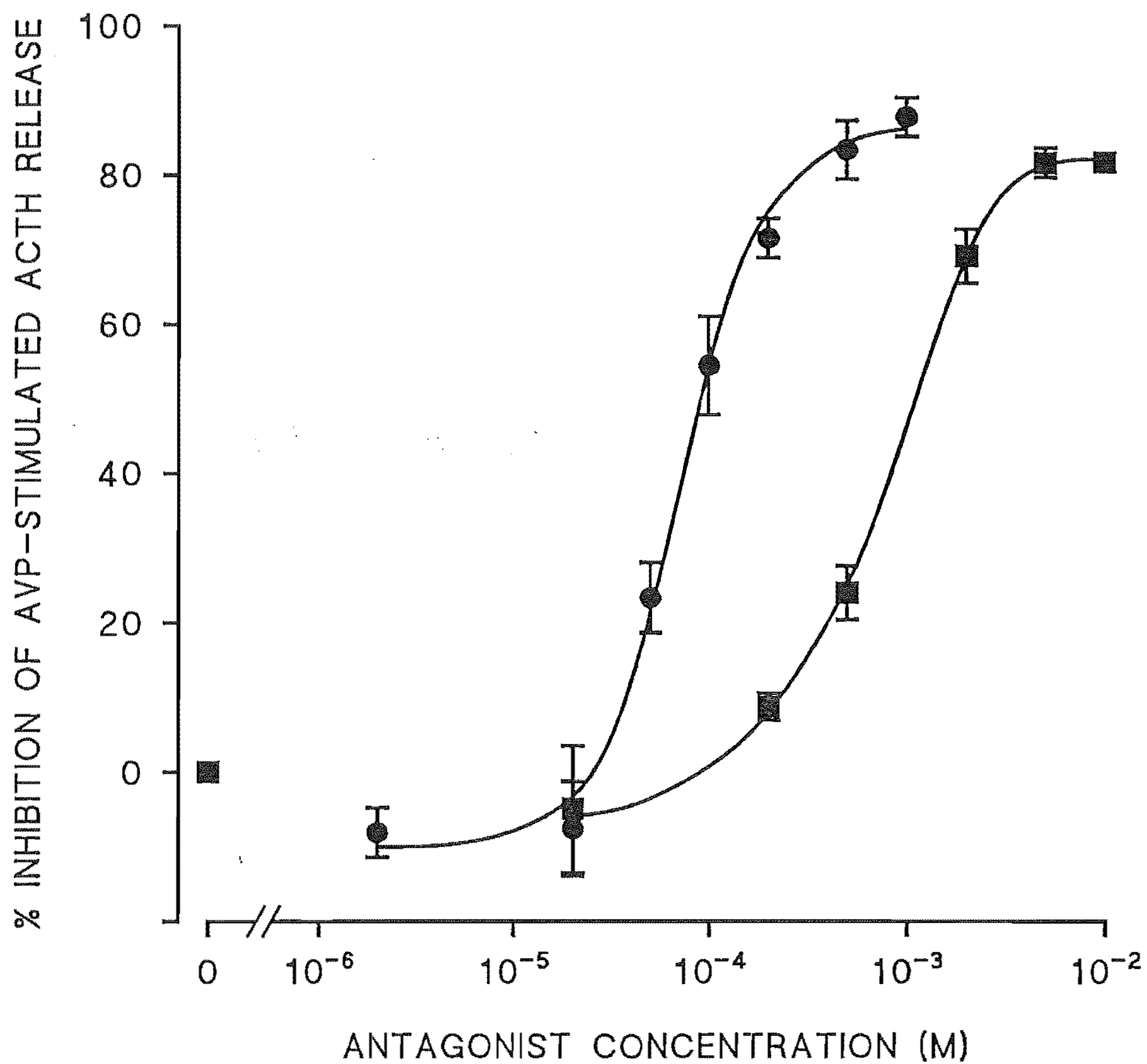

Fig. 3.3. Effects of inorganic blocking ions on AVP (100 nM)-stimulated ACTH release. Symbols denote inhibition by Cd^{2+} (circles) and Co^{2+} (squares). Values are means ± SEM, $n \geq 3$.

Significant inhibition of AVP (100 nM)-stimulated release by Co^{2+} occurred at 0.5 mM, and maximal inhibition (81.6 ± 1.2%) was observed at 10 mM Co^{2+} (Fig. 3.3). Inhibition by Co^{2+} appeared to have a reached a plateau at this point. The IC_{50} was calculated as 0.80 ± 0.12 mM.

3.2.6 D600 and nifedipine inhibition of K^+-stimulated ACTH release

Raising the extracellular K^+ ion concentration ($[K^+]_e$) causes release of ACTH from cultured pituitary cells (Fig. 3.4), presumably via a depolarization-activated opening of VSCC, and subsequent influx of Ca^{2+} ions (Mollard *et al.* 1987). The rationale for using this form of stimulation of ACTH release was to provide a response whereby the role of VSCC activation in ACTH release could be assessed without the involvement of other mechanisms that are activated during AVP stimulation. Maximum release induced by raised K^+_e occurred at 60 to 80 mM $[K^+]_e$ (Fig. 3.4). The secretory responses to 60 mM (Fig. 3.5) and 80 mM $[K^+]_e$ (not shown) were sensitive to the actions of both D600 and nifedipine. As with inhibition of AVP-stimulated secretion, the actions of D600 and nifedipine on K^+_e-stimulated release were both concentration-dependent and quantitatively equivalent. However, K^+_e-stimulated ACTH release was more sensitive to antagonism by D600 and nifedipine than was the AVP-induced response: the threshold concentration for significant inhibition was lower (100 nM for both antagonists) and the greatest level of inhibition observed was higher. In fact, D600, at 100 μM, totally blocked the K^+_e (60 mM)-stimulated release (inhibition 99.4 ± 1.5%, $P > 0.05$ *cf.* 100%, by CI). The IC_{50} for inhibition was found to be 0.29 ± 0.06 μM. D600 inhibition of ACTH release induced by 80 mM $[K^+]_e$ was similarly complete (not shown). The greatest level of inhibition of K^+_e-stimulated release observed for nifedipine (10 μM), was 88.0 ± 2.2%.

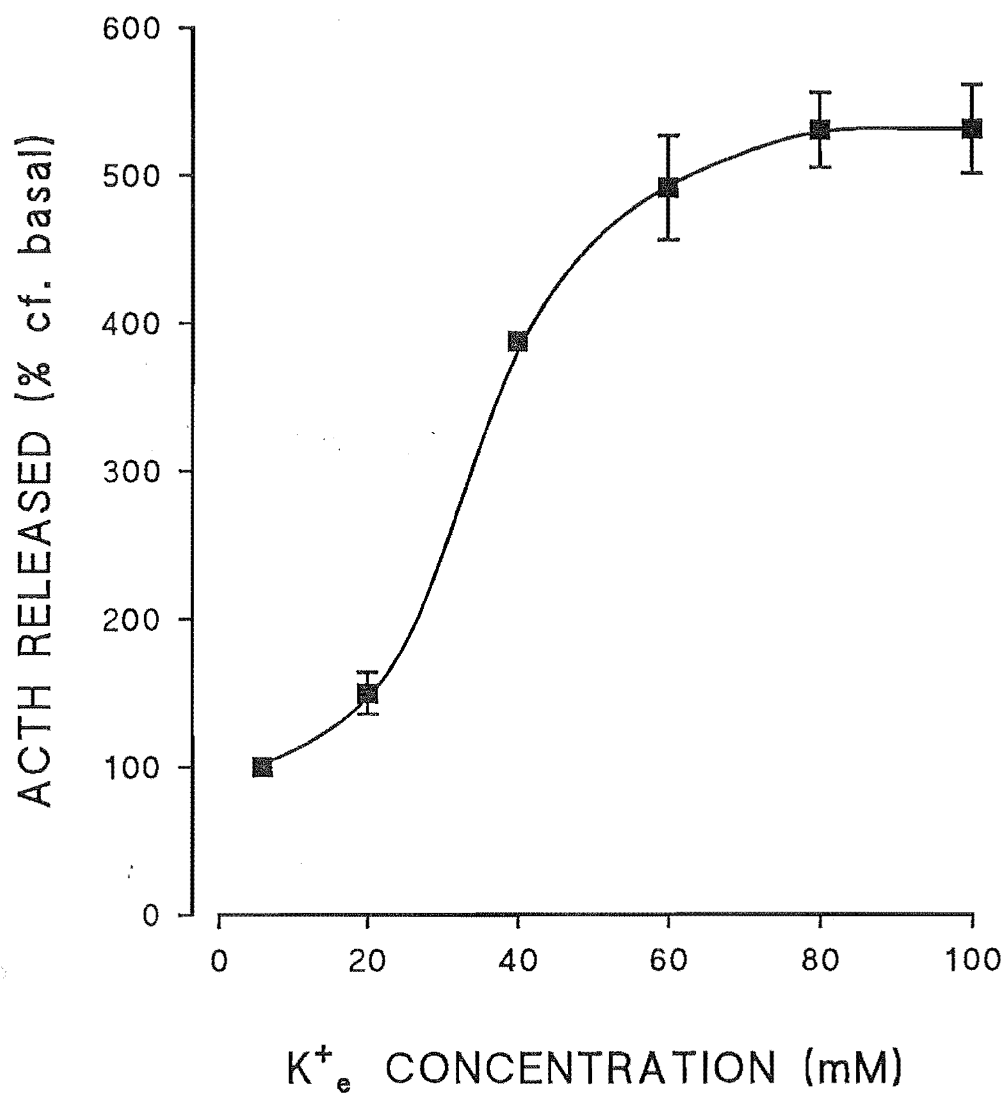

Fig. 3.4. Dose-response curve for K_e^+-stimulated ACTH release. Data are mean ± SEM, n = 3.

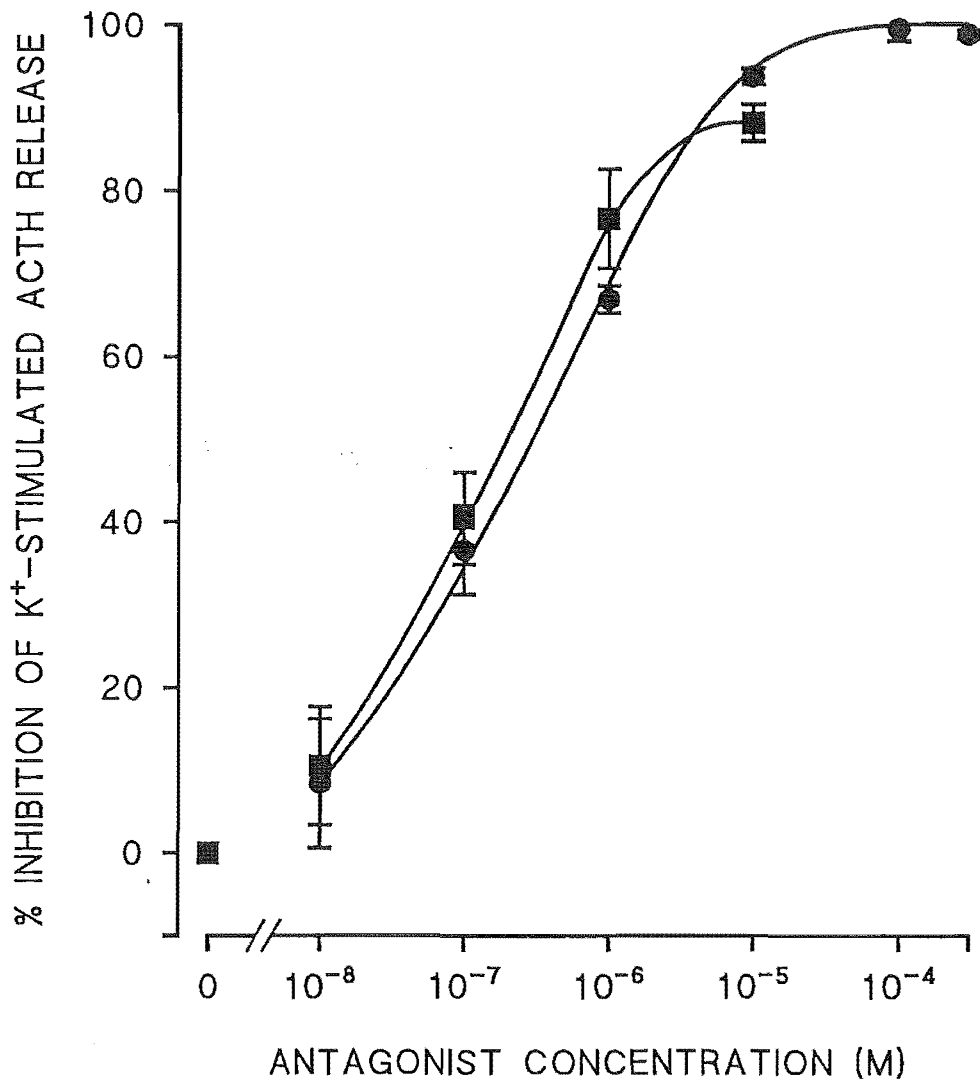

Fig. 3.5. Effects of organic blockers on ACTH release induced by 60 mM $[K^+]_e$. Symbols denote inhibition by D600 (circles) and nifedipine (squares). Values are means ± SEM, $n \geq 3$.

3.2.7 Inorganic ion inhibition of K^+-stimulated ACTH release

As with the organic blockers, the inorganic blocking ions, Co^{2+} and Cd^{2+}, were found to be effective inhibitors of K^+_e-stimulated ACTH release (Fig. 3.6). Cd^{2+} was again the more potent ion, and at a concentration of 1000 μM totally blocked the response (inhibition 99.9 ± 3.3%, $P > 0.05$ *cf.* 100%, by CI). Maximum inhibition by Co^{2+} (96.1 ± 0.5% at 5 μM Co^{2+}) was slightly less than 100% ($P < 0.05$, by CI). IC_{50} values for Cd^{2+} and Co^{2+} inhibition of K^+_e-stimulated ACTH release were 38.1 ± 2.8 μM and 0.63 ± 0.08 mM, respectively.

3.3 Discussion

In this study, all three of the organic L-type VSCC antagonists and both inorganic blocking ions were effective at partially inhibiting the AVP-stimulated ACTH response in ovine corticotrophs. At equimolar concentrations, D600 and nifedipine caused similar levels of inhibition of both AVP- and K^+-stimulated ACTH release. On a molar basis, diltiazem was less potent than the other two antagonists, but at the highest concentration used, diltiazem produced the greatest level of inhibition observed for the organic blockers, and showed no signs of having reached a plateau at this point (but see below).

The inorganic Ca^{2+}-entry blocking ions, Co^{2+} and Cd^{2+}, were also effective inhibitors of the AVP-stimulated ACTH response. Cd^{2+} was the more potent ion, although both ions caused similar maximum levels of inhibition.

On the basis of the results presented here, it appears that D600 is the most suitable organic blocker to use for future investigation of the mechanism of AVP-stimulated ACTH release in the ovine corticotroph. D600 was found to have little or no effect on unstimulated ACTH release, up to relatively high concentrations, suggesting that D600 has very minor, if any, non-specific effects on non-stimulated ovine corticotrophs. Nifedipine also had no apparent effects on unstimulated ACTH release, and on a molar basis, showed

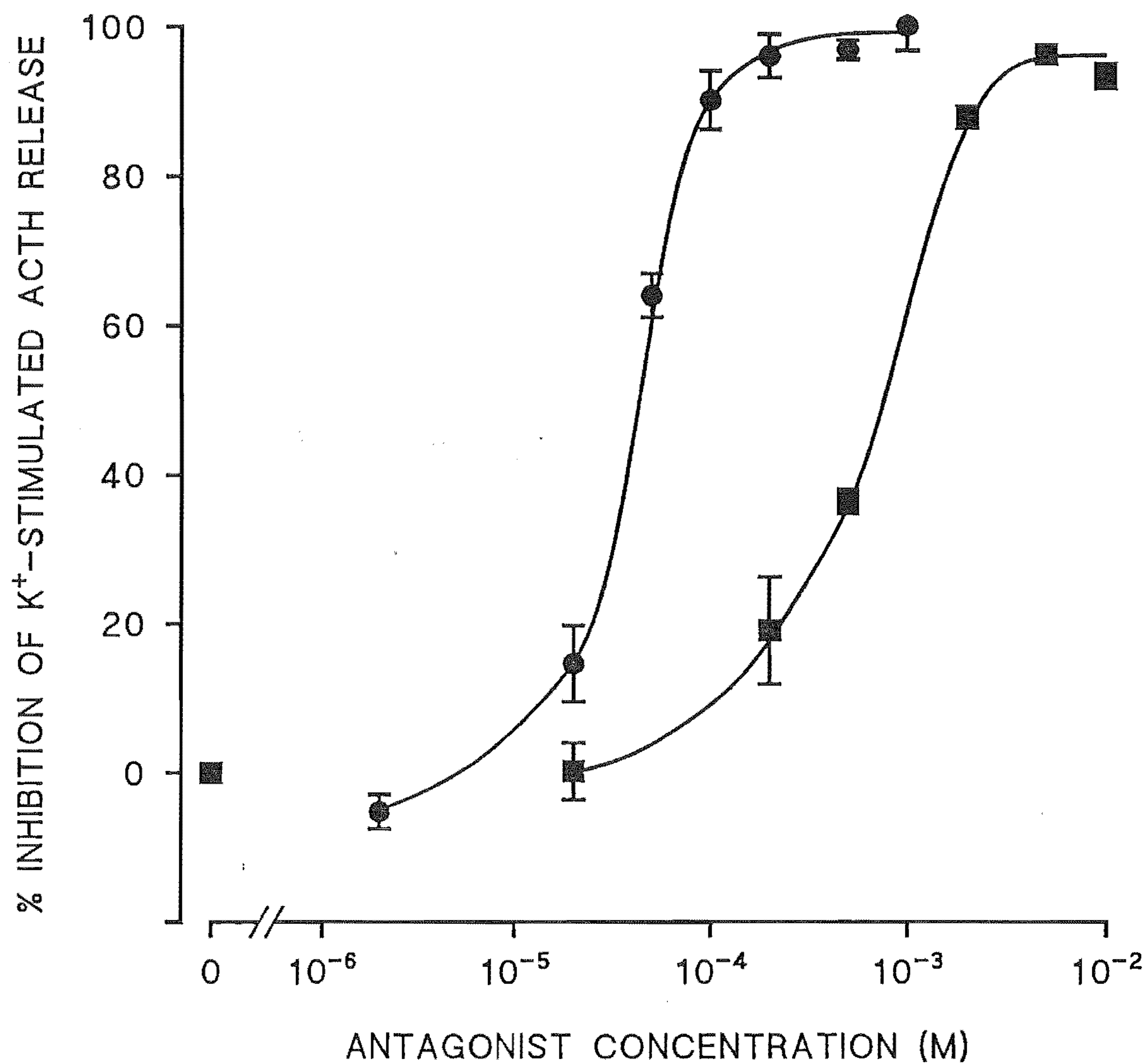

Fig. 3.6. Effects of inorganic blocking ions on ACTH release induced by 60 mM $[K^+]_e$. Symbols denote inhibition by Cd^{2+} (circles) and Co^{2+} (squares). Values are means ± SEM, $n \geq 3$.

very similar effects on stimulated ACTH release to those of D600. However, practical considerations of a lower solubility and sensitivity to light, make nifedipine a less useful tool than D600.

Diltiazem was a less potent inhibitor of AVP-stimulated ACTH release than either D600 or nifedipine. This, combined with a pronounced effect on unstimulated ACTH release, makes diltiazem an undesirable choice for use in future experimentation. Diltiazem, at the highest concentration used (10^{-3} M), did cause greater inhibition of AVP-stimulated release than was observed for D600, but at this concentration diltiazem had a disturbingly large effect on unstimulated ACTH release (see Table 3.1). At high concentrations (> 10^{-6} M) diltiazem has previously been found to have additional, intracellular effects on Ca^{2+} kinetics (Saida & van Breeman 1983).

The blockers D600, Co^{2+} and Cd^{2+} all produced clear plateaus for inhibition of AVP-stimulated release. In each case the maximum value for inhibition was below 100% (*ie.* inhibition was not complete) whereas inhibition of the K_e^+-stimulated response was complete (for D600 and Cd^{2+}) or virtually complete (96.1% for Co^{2+}). This greater inhibition of K_e^+-stimulated release by the blockers was a very consistent finding not only at the plateau of inhibition, but also over the ranges of concentrations used for the various blockers.

The finding that D600 was able to completely block the response to 60 mM K_e^+-stimulated ACTH release, when both agents were given simultaneously, indicates: 1) that the K_e^+-evoked response is totally dependent on Ca^{2+} influx via L-type VSCC, and 2) that D600 can act virtually instantaneously, and inhibit completely, K_e^+-stimulated Ca^{2+} channels in ovine corticotrophs. A similar interpretation can also be made for Cd^{2+}.

In contrast to the actions of D600 and Cd^{2+} on K_e^+-stimulated ACTH release, neither blocker completely inhibited the response to AVP. This suggests that the response to AVP contains a component that is independent of Ca^{2+} influx via VSCC; an interpretation that is in agreement with other recent reports (Abou-Samra *et al.* 1987a, Mollard *et al.* 1988, Won *et al.* 1990, Corcuff *et al.* 1993).

Alternatively, the difference in the degree of inhibition seen with AVP- *vs.* K_e^+-stimulated release may be due to a reduced efficacy of action of the blockers on AVP-stimulated channels. Examination of the IC_{50} values, however, indicates that for Cd^{2+} and

Co^{2+}, at least, this latter explanation is unlikely to be true. Comparison of the IC_{50} values obtained for each ion for blocking AVP- *vs.* K^+-stimulated release shows that they are very similar (< 2-fold difference). On the other hand, the IC_{50} values for D600 inhibition indicate that D600 blocked the K_e^+-evoked response almost 6-times more effectively than it did AVP-stimulated release. Although not great, this difference may explain why D600 inhibited AVP-stimulated release to a lesser extent (71.6%) than either Co^{2+} (81.6%) or Cd^{2+} (87.6%).

The observation that D600 was less effective at inhibiting AVP-stimulated release, compared to its effects on K_e^+-induced release, whereas the inorganic ions displayed almost equal inhibitory effects on AVP- *vs.* K_e^+-stimulated release, might result from the different mechanisms by which both the stimulating and the blocking agents act. The response of VSCC to K_e^+-induced depolarisation of the plasma-membrane is probably a purely electrical response, however there is mounting evidence that activation of VSCC by their physiological secretagogues (*ie.* AVP in this case) possesses a chemical as well as an electrical component (Mollard *et al.* 1988, Ma *et al.* 1992). With regard to the blocking agents, the inorganic ions act by competing with Ca^{2+} ions for the Ca^{2+}-binding site/s in the VSCC (Lakshminarayanaiah 1991), whereas the organic antagonists block by binding to the channel complex, and preventing the channel from re-assuming the open (conducting) state (Janis & Triggle 1991). This is thought to occur by the organic blocker preventing the re-phosphorylation of the inactivated channel, and thus preventing the conversion of the channel from the inactivated state to the closed state, where the channel would be able to open in response to the appropriate stimulus. Considering the combinations of effects, the inorganic ions may be able to block VSCC activated by either AVP or raised K_e^+ equally well, because it is the presence of an open channel (and thus the Ca^{2+}-binding site) which is required, and differences in the mechanisms of activation may not affect the ability of the ions to block the channels. In contrast, a chemical modification of the VSCC by AVP-activated processes could affect the ability of D600 to bind and/or exert its inhibitory actions. Armstrong and Kalman (1990) have found that agents that promote cAMP-dependent phosphorylation of L-type VSCC substantially reduce the inhibitory efficacy of nimodipine (a DHP) in intact cells and cell-free patches of the GH_3 rat pituitary tumour cell line. Thus, following AVP stimulation (and, possibly, PKC-catalysed phosphorylation),

the activated L-type VSCC may be less sensitive to the actions of organic blockers than channels activated by K_e^+, since K_e^+ would not be expected to cause these modifications. This type of chemical modification of VSCC, in the presence of AVP, could account for the observed differences in IC_{50} values for D600.

In addition to the possibility that the binding or action of organic blockers is affected by chemical modification of VSCC, it is possible that these processes may be influenced by membrane potential. The ability of these blockers to bind and inhibit VSCC has been found to be voltage-dependent in other tissue types (Janis & Triggle 1991). Raised $[K^+]_e$ would cause a profound and essentially constant depolarisation of corticotrophs which may enhance the sensitivity of the VSCC to D600. In contrast, AVP has been shown to cause less dramatic electrical responses in rat corticotrophs, including bursts of action potentials and low amplitude plateau depolarisation (Corcuff *et al.* 1993). Under these voltage conditions, the blocking efficacy of D600 may be reduced relative to that seen following K_e^+-induced depolarisation.

As noted above, although Cd^{2+}, Co^{2+} and D600 all failed to completely block the AVP-stimulated ACTH response, inhibition by the inorganic ions was higher than that observed for D600. Also discussed above is the possibility that some, or all, of this difference may be due to D600 not being able to fully exert its inhibitory actions on AVP-induced release. Another possible explanation for the greater inhibition by the inorganic ions is that these agents are known to inhibit Ca^{2+} influx via T-type VSCC as well as L-type channels. Thus inhibition of AVP-stimulated ACTH release by inorganic ions might reflect the combined inhibition of both L- and T-type VSCC. Similarly, Corcuff et al (1993) found that Ca^{2+} transients from single rat corticotrophs were reduced and abolished by PN200-110 (a DHP) and Cd^{2+} respectively, suggesting that both DHP-sensitive (L-type?) and DHP-insensitive (T-type?) Ca^{2+} currents contribute to Ca^{2+} influx in corticotrophs.

The role of T-type channel activation in the response to AVP has yet to be fully determined; Childs & Marchetti (1987), working on rat corticotrophs, have postulated that T-VSCC may act in a "trigger" role, to accentuate the initial depolarisation to the point where L-VSCC are activated. Since T-type channels have properties of low conductance and fast inactivation, Ca^{2+} influx via T-VSCC might not be expected to contribute greatly to the actual amount of ACTH released, although their participation may be important, if

not obligatory, in the response to AVP.

T-type VSCC may not contribute significantly to the K_e^+-stimulated response because the depolarisation induced by 60 mM K_e^+ is likely to raise the membrane potential of the cells past the inactivation potential of these channels (Stojilkovic *et al.* 1990a). Thus both the inorganic ion, Cd^{2+}, and the organic blocker, D600, were able to totally inhibit the K_e^+-stimulated response.

Previous investigations of the comparative effects of organic VSCC antagonists on pituitary cell function have demonstrated considerable variability in the responses observed. Enyeart *et al.* (1985) found that in normal rat lactotrophs and in PRL- and GH-secreting, GH_4C_1 pituitary tumour cells, K_e^+-stimulated prolactin release was very sensitive to DHPs (including nifedipine), whereas verapamil and diltiazem were effective only at much higher concentrations ($\geq$ 2 orders of magnitude - our interpretation of their data). In contrast to this, Conn *et al.* (1983) found that for the rat gonadotroph, D600 and verapamil (both phenylalkylamines), were effective inhibitors of GnRH-stimulated LH release, whereas diltiazem was effective only when cells were pre-exposed to this antagonist for at least 15 min, and DHPs (including nifedipine) were entirely without effect. The lack of an effect of DHPs in this latter report is, however, at variance with more recent accounts which demonstrate sensitivity of rat gonadotrophs to the actions of DHPs (Shangold *et al.* 1988, Stojilkovic *et al.* 1990b).

Taken together, the results of the present study, and those of the studies described above, indicate that there may be differences in the comparative effects of organic VSCC antagonists in different pituitary cell types. Some of this dissimilarity, especially directly contradictory findings obtained by different workers examining the same pituitary cell type, may be the result of methodological factors, such as tissue preparation, culture conditions etc. However, it is doubtful whether methodological factors could be totally responsible for such dramatic differences. That real differences do exist between pituitary cell types is suggested by other reports (Drouva *et al.* 1988, Stojilkovic *et al.* 1988c) which have compared the effects of a single class of VSCC antagonist (DHPs for both reports) on the responses of various subtypes of pituitary cells. In these reports, any methodological influences, if present, would presumably be constant across the various cell sub-types, and thus the differences in the effects of VSCC antagonists observed in these reports should

reflect actual differences in the number and/or properties of VSCC in the pituitary subtypes. Different classes of cloned L-type VSCC α_1-subunits have been characterized from the pituitary gland (Snutch & Reiner 1992).

Whatever the cause of the contrasting results, it is clear that for studies of pituitary-cell VSCC it is advisable to investigate the effects of a range of VSCC antagonists, of differing chemical classification, on the cell type of interest before such agents can be used to investigate the mechanisms of the response under observation. Another advantage of using a range of antagonists, including using both organic and inorganic agents, is that it allows for better assessment of non-specific actions of these agents. On the basis of the similarities in the effects of the five antagonists used in this study, it can be concluded that extracellular Ca^{2+} entry is an important contributor to the AVP-induced response in ovine corticotrophs. In addition, in view of these similarities it is unlikely that the results obtained with any one antagonist were due (primarily) to non-specific effects on the AVP-stimulated response.

Previous studies using rat anterior pituitary cells have found that unstimulated release (Abou-Samra *et al.* 1987a, Childs *et al.* 1987a) and AVP-stimulated ACTH release (Abou-Samra *et al.* 1987a) were inhibited by antagonists of L-type VSCC, suggesting a role for L-type VSCC in both unstimulated and AVP-stimulated ACTH release. In this study, none of the blocking agents used caused a reduction in unstimulated ACTH release. Apart from a few minor and inconsistent increases in unstimulated release the organic blockers D600 and nifedipine had no effects on ACTH release in the absence of AVP or raised $[K^+]_e$. Diltiazem had more pronounced effects on unstimulated release, and, largely because of this, this agent was not used for further experimentation. Both of the inorganic ions caused significant increases in unstimulated release; an effect that was apparently concentration-dependent. From the general lack of effects of D600 and nifedipine, it is unlikely that L-type VSCC contribute to unstimulated ACTH release under the experimental conditions used in this study. Stojilkovic *et al.* (1988c) drew the same conclusions concerning basal ACTH secretion from rat pituitary cells. The effects observed with inorganic ions might reflect an effect of the ions on T-type channels, or alternatively, the effects of the inorganic ions could be due to non-specific actions of these agents.

In summary, the results presented in this chapter demonstrate that Ca^{2+} influx via

L-type VSCC plays a pivotal role in ACTH release induced by AVP in ovine corticotrophs. However, although necessary for a large component of the AVP-stimulated hormone release, such Ca^{2+} influx is insufficient to entirely account for this ACTH release since the response could not be completely abolished by antagonists of Ca^{2+} entry. The same Ca^{2+}-entry antagonists, despite their chemical diversity, were consistently more effective at inhibiting ACTH release induced by raised $[K^+]_e$. In fact, D600 and Cd^{2+} both completely abolished the response to high K_e^+.

Chapter 4

Modulation of L-Voltage Sensitive Calcium Channels: Chemical Regulation

4.1 Introduction

Ca^{2+} influx via VSCC is of fundamental importance in the AVP-stimulated ACTH response. This has been demonstrated previously for rat corticotrophs, AtT-20 cells and hACTH cells, and now for ovine corticotrophs (see Chapter 3 and Abou-Samra *et al.* 1987, Mollard *et al.* 1987, 1988, Reisine *et al.* 1987, Guerineau *et al.* 1991, Corcuff *et al.* 1993). However, modulation of the activity of the VSCC following exposure to AVP is yet to be fully elucidated. That the channels involved display voltage-sensitivity does not conclusively prove that voltage changes of the membrane are the sole driving force for increasing channel activity. Current evidence suggests that a combination of both electrical and chemical factors might control VSCC activity during the response to AVP. An investigation of the potential roles of these factors, in the AVP-induced ACTH response, is the focus of the remainder of this study. In this chapter, the role of chemical modulation of VSCC activity is considered, and electrical modulation is investigated in Chapter 5.

Chemical modulation, in the form of cycles of phosphorylation/dephosphorylation, has been found to occur in L-VSCC from many different tissues (DeReimer *et al.* 1985,

Rane & Dunlop 1986, Kaczmarek 1988, Lacerda *et al.* 1988, Levitan 1988, O'Callahan *et al.* 1988), including pituitary cells (Lewis & Weight 1988, Marchetti & Brown 1988, Stojilkovic *et al.* 1991). In fact, it appears that some degree of phosphorylation is obligatory for the channels to become activated (Armstrong & Eckert 1987, Ono & Fozzard 1992). Recent evidence suggests that two (or possibly more) phosphorylation sites, which control the kinetic functions of channel activity, exist on L-VSCC (Ono & Fozzard 1993, Hartzell 1993). Channel activity is reduced by phosphatase activity (Ono & Fozzard 1993). It is not clear whether phosphorylation of a closed channel will itself cause the channel to be converted to the open state, or whether a combination of voltage changes and phosphorylation together enhance the open probability of the channels.

In corticotroph cells, PKC has been implicated as having modulatory actions on VSCC activity during the response to AVP (Reisine & Guild 1987, Lewis & Weight 1988, Sobel 1988, Won *et al.* 1990), however the details are yet to be firmly established. Specific actions of PKC on Ca^{2+} channels have been described: PKC was found to increase the open probability of skeletal muscle Ca^{2+} channels (Ma *et al.* 1992). In ACTH secreting hACTH cells, AVP was shown to enhance the kinetic features of the L-type current (Mollard *et al.* 1987). AVP did not alter the activation threshold for action potential firing, suggesting that the facilitation of the L-type current represents a direct effect on L-VSCC. Thus these results suggest that some factor activated by AVP, possibly PKC, enhances the activity of L-VSCC in corticotrophs. The facilitation of the Ca^{2+} current resulted in an enhanced influx of Ca^{2+} during exposure to AVP, and therefore would presumably be translated into enhanced ACTH release, although this was not measured in this study (Mollard *et al.* 1987).

Chemical modulation of T-VSCC has only recently been observed (Lu *et al.* 1994), and in many situations it appears there may be no chemical modulation of this channel type. T-type channels more typically display only voltage-dependent kinetics; activating at low thresholds and inactivating rapidly with a rising E_M (Miller 1987, Tsien *et al.* 1988).

The aim of the work presented in this chapter is to investigate mechanisms whereby AVP causes the influx of Ca^{2+} leading to the release of ACTH. The work consists of secretion studies, which attempt to complement and corroborate the electrophysiological studies which have demonstrated the presence of Ca^{2+} currents in corticotrophs, and the

possible involvement of these currents in the AVP-induced ACTH response. In the first set of experiments described here, dispersed pituitary cells were exposed simultaneously to AVP and raised $[K^+]_e$. The rationale for these experiments was to further characterise (following the findings of Chapter 3) the Ca^{2+} channels involved in the AVP-induced response. Specifically, this research was aimed at investigating whether the populations of channels underlying the individual responses to both secretagogues (see Chapter 3) are separate, or whether they are shared, at least to some degree (see section *4.3.1 Theoretical analysis of simultaneous stimulation*). If it was found that the channels are shared, to some extent, this would strengthen the notion that the AVP response in ovine corticotrophs involves the activation of *voltage-sensitive* channels, as was suggested by the results in Chapter 3. In other words, a demonstration of a shared set of channels would reduce the likelihood that the channel blockers used in the experiments of Chapter 3, could be acting on non-voltage-sensitive channels when they reduced the magnitude of the AVP-induced response.

The experiments presented in this chapter provided both expected and unexpected outcomes; the expected outcome agreed with the hypothesis of a shared channel population, while the unexpected outcome was that under certain conditions, a moderate level of synergism was found to occur in response to the simultaneous exposure to AVP and raised $[K^+]_e$. This result suggested that a positive interaction between the two responses occurred during the simultaneous response, and further analysis suggested that the most plausible locus for the point of interaction is the shared Ca^{2+} channel population. This raised the possibility that chemical modulation of the channels may occur during the response to AVP, and led to further experimentation to investigate whether PKC-induced effects were responsible for the synergistic response. The results of these experiments supported the role of PKC in the synergism, which led to a comprehensive investigation into the role of PKC in the activation of VSCC in ovine corticotrophs.

4.2 Results

4.2.1 Simultaneous stimulation with AVP plus raised $[K^+]_e$

The standard procedures, as described in Chapter 2, were used for the simultaneous exposure experiments. The cells were exposed to both AVP and raised $[K^+]_e$ at the same time (at the start of the test incubation period). For these experiments, a range of raised $[K^+]_e$ media from 20 mM (approximately threshold for stimulation of ACTH release) to 100 mM (supramaximal, see Fig. 3.3) was used. The range of AVP concentrations was 1 to 1000 nM. Fig. 4.1 shows the results of a typical experiment in which the AVP concentrations used were 10 nM and 1000 nM, and raised $[K^+]_e$ ranged from 20 to 100 mM (the effects at the control value of 5.9 mM $[K^+]_e$ are also shown). As can be seen, the responses to simultaneous exposure were always greater than either of the individual AVP- or K_e^+-induced responses. To further analyse the effects of simultaneous exposure, relative to the individual responses, the simultaneous responses were divided by the sum of the individual responses (eg the simultaneous response to the combination of 10 nM AVP + 40 mM K_e^+, was divided by the sum of the individual responses to 10 nM AVP and to 40 mM K_e^+). Thus if the resulting value, termed the "response ratio" was equivalent to "1" (within statistical significance), then the level of the simultaneous response is additive with respect to the sum of the individual responses. If the response ratio value is less than, or greater than 1, then the simultaneous response is less than additive or synergistic, respectively, with respect to the sum of the individual responses.

The pooled results of a number of experiments are presented in Fig. 4.2, and these demonstrate that all three patterns of response to simultaneous stimulation with AVP and raised K_e^+ were obtained. Synergism was found at lower levels of stimulation, and, as the level of stimulation was increased (*ie.* as the concentration of the secretagogues was increased), the response to simultaneous stimulation became additive and ultimately it was less than additive. This trend is obvious when both the AVP and $[K^+]_e$ concentrations are considered (Fig. 4.2).

It is important to note that although the level of the synergism decreased with an

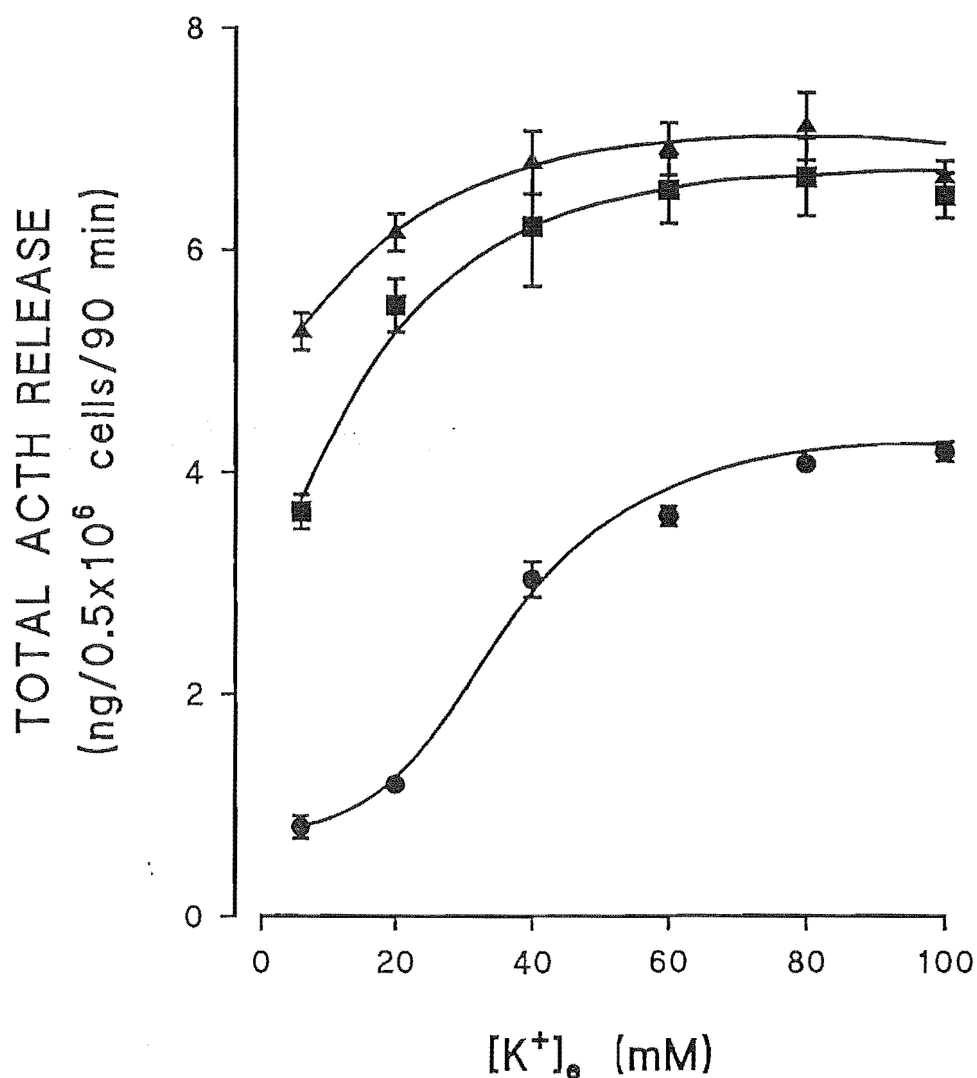

Fig. 4.1. Effect on ACTH secretion of raising $[K^+]_e$, either alone (circles) or in combination with AVP at 10 nM (squares) or 1000 nM (triangles). Data are mean ± SEM from a single representative experiment with n = 4 within the experiment. This experiment was repeated partly or wholly at least two more times, with quantitatively similar results, in terms of the relative magnitude of hormone release. The normal $[K^+]$ of the bathing medium is 5.9 mM, and thus hormone release at this $[K^+]$ (in the absence of AVP) represents basal (unstimulated) hormone release.

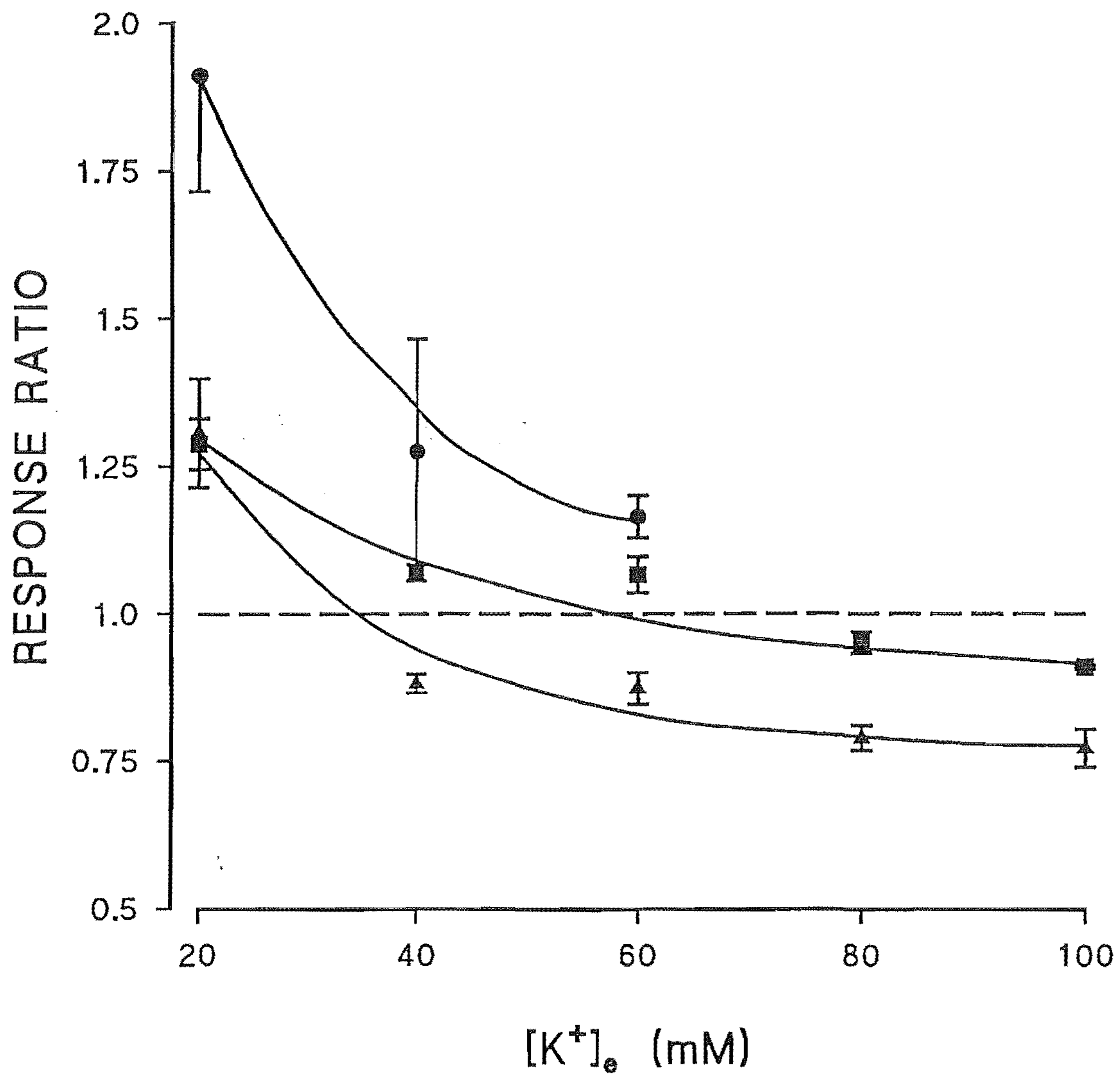

Fig. 4.2. Response ratios for simultaneous stimulation by AVP plus raised $[K^+]_e$. AVP concentrations are 1 nM (circles), 10 nM (squares) and 1000 nM (triangles). Data are mean ± SEM with each point representing the mean of at least 3 separate determinations.

increasing level of stimulation, the actual *amount* of ACTH released either continued to increase with the level of stimulation, or remained constant once the plateau of the response had been reached (Fig. 4.1).

Loss of the synergistic response as the level of stimulation was increased, resulting eventually in the response becoming less than additive, suggests that one (or more) factor(s) underlying the simultaneous response became maximally activated, limiting the rate of increase of this response as the concentration of the secretagogues was increased. In contrast, for the individual responses, this factor(s) became limiting at higher concentrations of the secretagogues (see the discussion in section 4.3.1 for a more detailed analysis). In Chapter 1, it was noted that a close correlation between the Ca_i^{2+} and secretory profiles in response to AVP has been reported, and that this finding suggests that the Ca_i^{2+} signal feeds directly into the exocytotic pathway. Therefore, once the Ca_i^{2+} profile is determined, the secretory profile may also be determined. This putative feature of the ACTH response in corticotrophs allows the response to be divided into those factors which give rise to the Ca_i^{2+} response, and those factors which become activated following the generation of the Ca_i^{2+} profile. This division of the cellular response can be used to attempt to elucidate how the interaction between the AVP- and K_e^+-induced responses might lead to the generation of the synergistic response. Given the following assumptions: 1) if the interaction between the secretagogue-induced responses that changes the response ratio (*ie.* from > 1 to < 1) occurs *distal* to the generation of the Ca_i^{2+} response, and 2) if the less than additive response at high levels of stimulation is a result of some factor being maximally activated, and therefore limiting, then the greatest ACTH response observed in response to simultaneous stimulation (where a less than additive response is clearly observed) should represent the largest possible ACTH response from corticotrophs under the conditions used for these experiments. Alternatively, if a significantly larger ACTH response could be elicited, then this would suggest that at least one limiting factor for the simultaneous response occurs prior to the generation of the Ca_i^{2+} profile (or, at the very least, that it is not the exocytotic capability of the cells that is causing the less-than-additive responses at high levels of stimulation).

To investigate this question, data is presented (Table 4.1) in which the ACTH responses to 1000 nM AVP plus 60 mM $[K^+]_e$ (a combination that causes a less than

additive response) from a series of experiments are compared with TPA-stimulated ACTH responses from the same experiments (see later in this chapter for a description of TPA-stimulated release). [In this analysis, the TPA-induced responses are used because this agent was found to elicit a high level of ACTH secretion, and therefore represents a minimum estimate for the maximum secretory capacity of the cells. Thus the TPA-induced response is used here purely as an indicator of the exocytotic capability of the cells, and the details of the factors involved in TPA-stimulated secretion are not important for this analysis. They are considered in a later section of this chapter.]

In three of four identical experiments, the magnitude of the response to 1000 nM AVP + 60 mM $[K^+]_e$ was significantly less than that elicited by 150 nM TPA (Table 4.1). In the other experiment, these responses were not significantly different, however, in this particular experiment, a different treatment, 150 nM TPA + 60 mM $[K^+]_e$ *did* cause a level of secretion significantly greater than that of the AVP plus K_e^+ response (Table 4.1). Thus, in all four experiments, there was one treatment that generated an ACTH response that was significantly larger than the response to 1000 nM AVP plus 60 mM $[K^+]_e$.

To further characterise the nature of the synergistic response, and to investigate the possible involvement of L-VSCC in the simultaneous response, experiments were performed in which cells were exposed to various treatments, including the combination of 10 nM AVP + 60 mM $[K^+]_e$, for the time periods 15, 30 60 and 90 min. The results of this investigation are expressed in two separate ways: 1) for each experiment, stimulated release induced by each treatment, for each time period, was expressed as a percentage of the stimulated release in response to 10 nM AVP + 60 mM $[K^+]_e$ at 90 min (which was therefore set to 100%). The data from four experiments were then pooled (Fig. 4.3). Thus the magnitude of each treatment, for each time period, can be compared. 2) The second method used to analyse the data was to take the data from each experiment, and then, for each individual treatment, express the stimulated release at each time period as a percentage of the stimulated release at 90 min. The data from the four experiments were then pooled (Fig. 4.4). This allows a comparison, within and between treatments, of the relative contribution of each time period to the total secretory response.

Fig. 4.4 shows that the AVP, K_e^+, and AVP + K_e^+ responses all follow a very similar time course. The general pattern of the profiles shows that the greatest rate (*ie.* steepest

	1000 nM AVP + 60 mM $[K^+]_e$	150 nM TPA	*P*	Response ratio of AVP + K_e^+ response	Simultaneous response/ response ratio
1	3.393 ± 0.089	3.943 ± 0.023	< 0.05	0.931	3.599
2	3.580 ± 0.147	4.528 ± 0.233	< 0.05	0.923	3.816
3	2.416 ± 0.035	3.282 ± 0.057	< 0.001	0.965	2.491
4	1.830 ± 0.037	2.886 ± 0.064*	< 0.001	0.945	1.917
				0.941 ± 0.009 (P < 0.01, *vs.* 1.0)	

Table 4.1. Comparison of simultaneous response with an estimate of maximum secretory capacity. Values for 1000 nM AVP plus 60 mM $[K^+]_e$, and 150 nM TPA are total ACTH released ± SEM (n = 4), from four individual experiments. * indicates that this value was obtained in response to stimulation with 150 nM TPA *plus 60 mM* $[K^+]_e$. Response ratio values from the individual experiments, and the mean ± SEM of these, are also given to demonstrate that the AVP + K_e^+ responses caused less than additive responses in these experiments. The values in the last column were obtained by dividing the values for the simultaneous responses (column 2) by their respective response ratios (column 5) (adjusted for the fact that the data presented are total ACTH release, and response ratios are calculated on stimulated release data) and therefore represent the ACTH response that would be necessary to obtain a response ratio of 1.0 (*ie.* the simultaneous response would be equal to the sum of the individual responses). The calculated values therefore indicate the magnitude of the secretory responses that would be necessary for there to be no "limitation" of the simultaneous response (with respect to the individual responses). Note that in all cases the calculated values are less than the responses to 150 nM TPA in the same experiment, and therefore limitation of the simultaneous responses does not appear to result from limitation of the secretory capacity of the cells.

slope) of ACTH secretion occurred in the first 15 min, after which the rate of secretion declined during the 15 to 30 min period, and further during the 30 to 60 min period. This time course is consistent with the spike/plateau profile observed from perifusion data (see Chapter 1). However, from 60 to 90 min, the rate of secretion appeared to increase for all treatments, relative to the preceding rate. The cause of this effect is unclear.

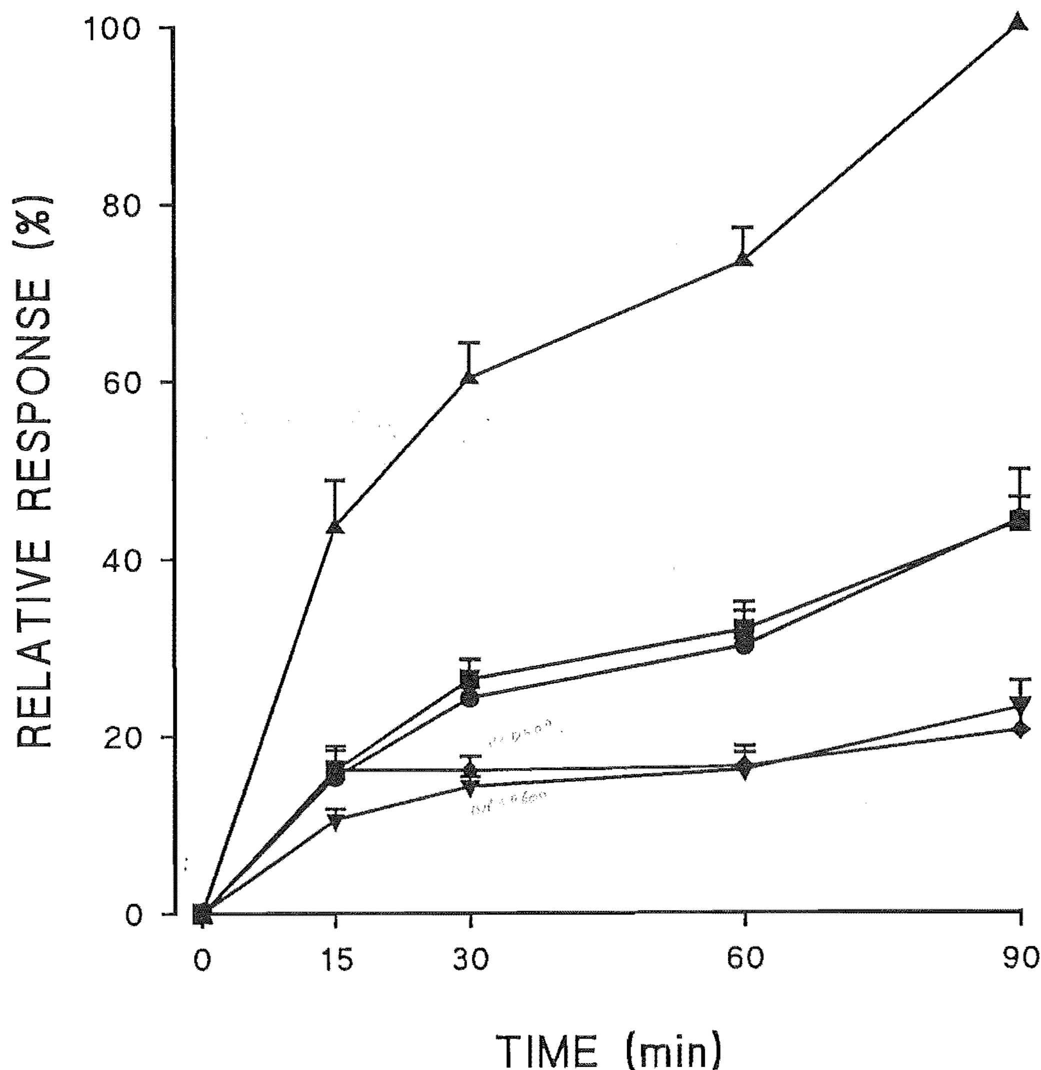

Fig. 4.3. Effect of test incubation duration on the development of secretory responses to 10 nM AVP (circles), 60 mM $[K^+]_e$ (squares), 10 nM AVP plus 60 mM $[K^+]_e$ (triangles), 10 nM AVP plus 10 μM D600 (inverted triangles) and 10 nM AVP plus 60 mM $[K^+]_e$ plus 10 μM D600 (diamonds). Data are mean ± SEM from 4 identical experiments. The values were obtained as follows: for each experiment, the stimulated (*ie.* total minus basal) release induced by 10 nM AVP plus 60 mM $[K^+]_e$, at 90 min, was set to 100%, and the values for stimulated release for all the other treatments, for each time period, were normalised to this (100%) value. The data from the 4 experiments were then pooled. The (unsmoothed) lines connecting the data points are added principally for the sake of clarity, and should not be taken as indicating hormone release rates *between the points*. No such interpolation is used in the text. This feature is pertinent to Figs. 4.4 and 4.5 as well.

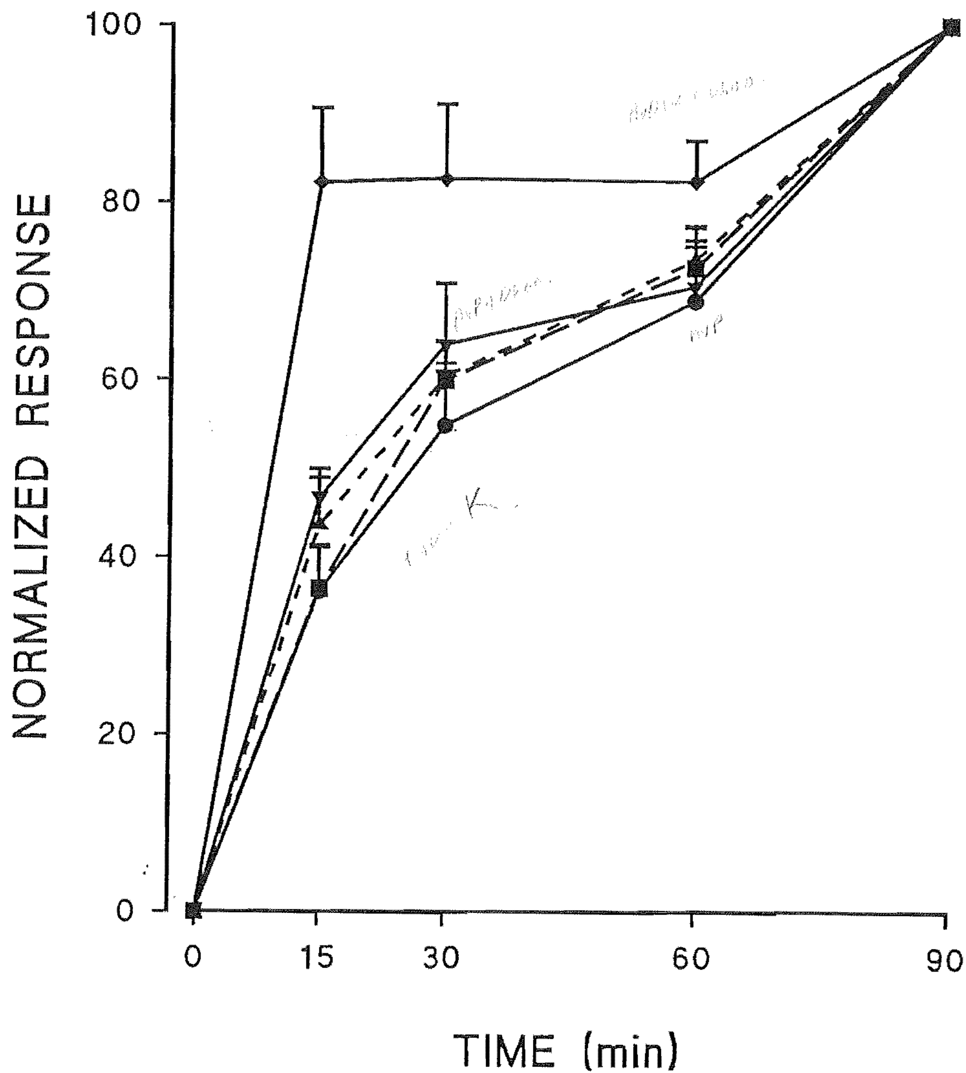

Fig. 4.4. Contribution of hormone release at each time period to the overall 90 min responses. The original data (stimulated release), which are presented in an alternative form in Fig. 4.3, were expressed as follows: *for each treatment* of each experiment, the response at 90 min was set to 100%, and the responses at 15, 30 and 60 min were expressed relative to this. The data from the 4 experiments were then pooled. Thus the relative contribution of secretion during each time period could be compared within and between treatments. The values are mean ± SEM, n = 4, and the symbols represent the same treatments as for Fig. 4.3.

Addition of 10 μM D600 had no significant effect on the time course of the AVP-induced secretory response (Fig. 4.4), although the magnitude of the secretion was affected (Fig. 4.3). This concentration of D600 causes 58.1 ± 2.81% inhibition of AVP-induced ACTH secretion at 90 min (see Fig. 3.1) - a level which is less than the maximum level of inhibition attainable with D600 (71.6 ± 2.26). This concentration of D600 also causes 93.6 ± 1.0% inhibition of the K^+-induced response (see Fig. 3.3).

Both the magnitude and the time course of the simultaneous response to 10 nM AVP + 60 mM $[K^+]_e$ were greatly affected by the addition of 10 μM D600 (Figs. 4.3 & 4.4). Fig. 4.3 shows that the magnitude of the simultaneous response was considerably reduced in the presence of D600 at all four time points, and Fig. 4.4 shows that in the presence of D600, virtually all of the secretion that does occur (> 80%), occurs in the first 15 min of the response, and from 15 to 60 min there is virtually no further secretion.

The response ratios for 10 nM AVP + 60 mM $[K^+]_e$, in the presence and absence of 10 μM D600 were calculated and are shown in Fig. 4.5. For both datasets, the response ratios were highest at 15 min, and declined with time - regression analysis gave significant, negative slopes for both datasets ($P < 0.005$ and 0.02 for absence and presence of D600, respectively).

4.2.2 The effects of PKC down-regulation on ACTH secretion

Since L-VSCC appear to be involved in both the responses to AVP and raised $[K^+]_e$, they are one potential point where the two responses might interact, to cause the synergistic response observed at low levels of simultaneous stimulation (see section 4.3.1 for more comprehensive analysis and rationale). Modulation of L-VSCC activity by PKC occurs in many different cell types, and this interaction has also been suggested to occur in corticotrophs (see Chapter 1 for details). Therefore, experiments were designed to investigate whether PKC activity, as a putative modulator of L-VSCC activity, was involved in the generation of the synergistic response.

As described in section 4.1, chronic exposure to phorbol esters results in the down-

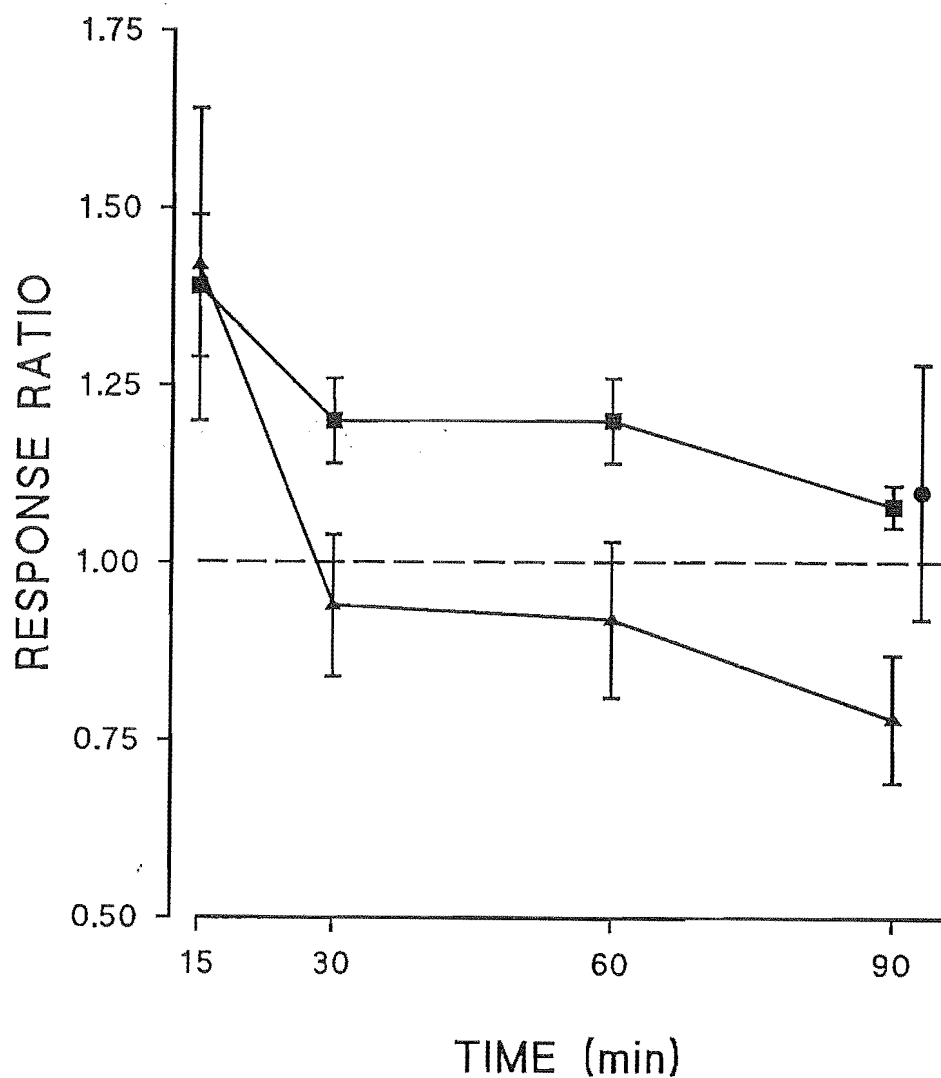

Fig. 4.5. Effect of test incubation duration on response ratios for simultaneous stimulation by AVP plus raised $[K^+]_e$. Data derived from same original data as presented in Fig. 4.3. Treatments are 10 nM AVP plus 60 mM $[K^+]_e$ (squares) and 10 nM AVP plus 60 mM $[K^+]_e$ plus 10 μM D600 (triangles). The datapoint denoted by the circle represents the re-calculated response ratio for 10 nM AVP plus 60 mM $[K^+]_e$ plus 10 μM D600, at 90 min (offset for clarity), allowing for the possibility that D600 was more effective at blocking AVP-activated VSCC during the simultaneous response, compared to when AVP was the only secretagogue present.

regulation of PKC activity. Experiments were conducted in which the PKC activity was abolished using this procedure (see legend for Fig. 4.6), and the effects of this on the ACTH secretory response to a number of secretagogues, including simultaneous exposure to AVP plus raised $[K^+]_e$, were determined.

The effects of PKC down-regulation on ACTH secretion can be seen in Fig. 4.6. In control (non-pre-treated) cells, 150 nM TPA induced the release of 57.1 ± 1.4% of the total cellular ACTH stores. Following pre-exposure to TPA, this response was completely abolished ($P < 0.001$ control vs pre-treated responses). The response to 1 nM AVP following the down-regulation procedure was reduced by 35.7%, but this was not significant ($P > 0.05$). However, the response to 1000 nM AVP was significantly reduced, by 51.8% ($P < 0.05$).

CRH, which acts independently of PKC activation (see Chapter 1), was included as a control to test for the specificity of the PKC down-regulation. The secretory response to 1 nM CRH was unaffected by the down-regulation procedure ($P > 0.05$, Fig. 4.6).

The ACTH responses to 20 and 60 mM $[K^+]_e$ were also unaffected by the pre-exposure to TPA ($P > 0.05$ for both, Fig. 4.6). The responses to simultaneous exposure to AVP (1 and 1000 nM) plus raised $[K^+]_e$ (20 and 60 mM) were tested, and of the four possible combinations, the responses of three were significantly reduced following pre-exposure to TPA. The responses to 1000 nM AVP plus 20 mM $[K^+]_e$, 1 nM AVP plus 60 mM $[K^+]_e$ and 1000 nM AVP plus 60 mM $[K^+]_e$ were reduced by 43.6% ($P < 0.05$), 22.6% ($P < 0.05$) and 39.4% ($P < 0.005$), respectively. Although the response to 1 nM AVP plus 20 mM $[K^+]_e$ was also reduced (by 43.1%) it was not significantly different from control ($P > 0.05$).

When the data for the simultaneous responses were expressed as response ratios, it became evident that the synergistic responses observed in control cells for both 1- and 1000 nM AVP plus 20 mM $[K^+]_e$ were lost following down-regulation of PKC activity (Table 4.2). Following pre-exposure to TPA, none of the combinations of secretagogues resulted in simultaneous responses that were significantly greater than the sum of the individual secretory responses.

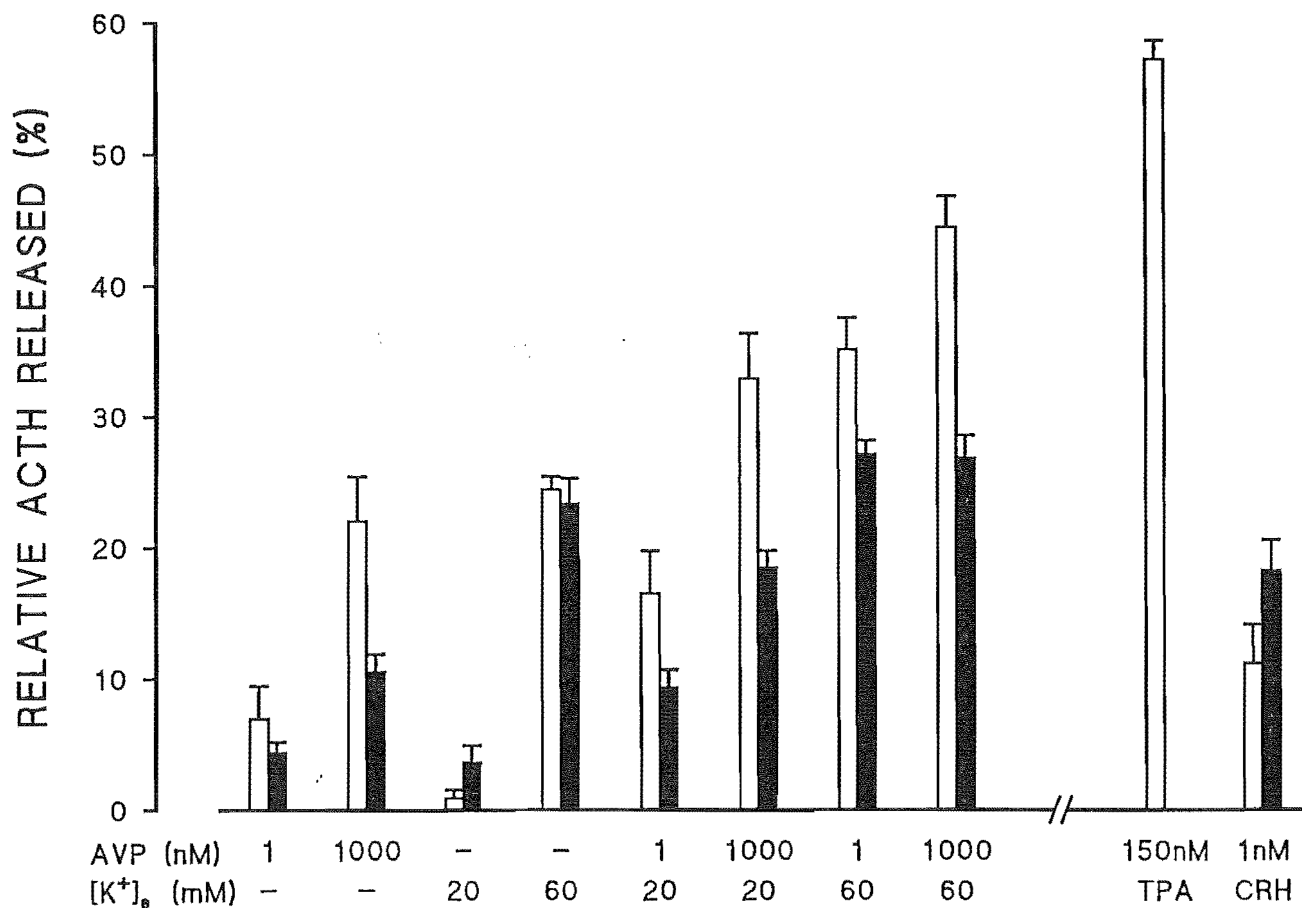

Fig. 4.6. Effect of PKC down-regulation on ACTH secretion. For TPA pre-exposure, cells were exposed to 150 nM TPA during the overnight (20 ± 2 h) incubation. TPA was not included in the medium for either the 1 h pre-incubation or wash procedure, which preceded the test incubation (see Chapter 2). Open and filled bars represent control (non-pre-treated) and TPA pre-treated conditions, respectively. Since TPA pre-exposure caused a reduction in the ACTH content in the pre-treated cells, relative to the control cells, the amount of hormone left in the cells at the end of the test incubation was determined for the cells in each well. The stimulated hormone release was then expressed as the percentage release relative to the total cellular ACTH content (*ie.* the sum of the release during the test incubation and the remaining ACTH content at the completion of the test incubation). Data are mean ± SEM from three separate experiments.

Treatment	Control/Test	Response ratio	P (*cf.* 1.0)
20 mM $[K^+]_e$ + 1 nM AVP	c	2.155 ± 0.133	< 0.001
	t	1.135 ± 0.080	> 0.05
20 mM $[K^+]_e$ + 1000 nM AVP	c	1.447 ± 0.080	< 0.05
	t	1.304 ± 0.115	> 0.05
60 mM $[K^+]_e$ + 1 nM AVP	c	1.114 ± 0.036	> 0.05
	t	0.976 ± 0.039	> 0.05
60 mM $[K^+]_e$ + 1000 nM AVP	c	0.956 ± 0.008	< 0.05
	t	0.793 ± 0.023	< 0.05

Table 4.2. Effect of PKC down-regulation on response ratio values. Values for response ratios are mean ± SEM, n = 3. Control (c) and test (t) conditions represent non-pre-treated and TPA-pre-treated conditions, respectively.

4.2.3 Acute exposure of corticotrophs to TPA, and TPA plus raised $[K^+]_e$

The results of the preceding section suggest that PKC activation is necessary for the generation of the synergistic response arising from the simultaneous exposure to AVP and raised $[K^+]_e$. To further investigate this possibility, experiments were performed in which TPA was used as an acute stimulator of PKC activity (*ie.* TPA was present only during the test incubation). Cells were exposed to the combination of TPA plus raised $[K^+]_e$; thus TPA acted as a replacement for AVP, as the PKC-activating agent, in an attempt to reproduce the synergistic response.

Prior to being used in combination with raised $[K^+]_e$, the potency of TPA under acute secretory conditions was determined. The threshold concentration of TPA required for a significant stimulation of ACTH release was found to be approximately 0.1 nM (Fig. 4.7), although this concentration of TPA did not always produce a significant ACTH response. Increasing the TPA concentration resulted in a typical sigmoidal dose-response

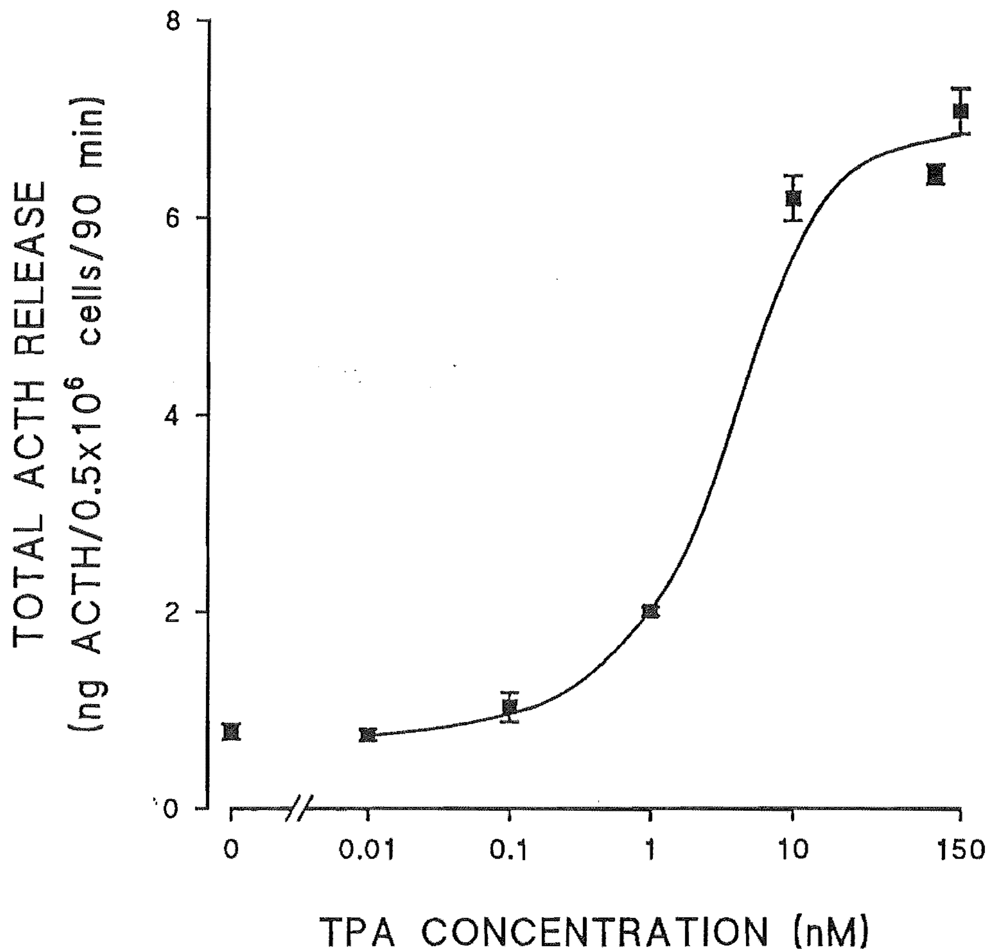

Fig. 4.7. Dose-response curve for TPA-stimulated ACTH release. Data are mean ± SEM, n = 4, from a single experiment. Although the full dose-response experiment was not repeated, when used in other experiments the relative responses of the TPA concentrations were consistent with those obtained in this experiment.

curve with maximal stimulation occurring at approximately 100 nM, and the steepest section of the curve occurred between 1 and 10 nM TPA.

The effects of simultaneous stimulation with TPA plus raised $[K^+]_e$ can be seen in Fig. 4.8. For most of the combinations of concentrations used, ACTH release was greater than that observed from either of the individual responses, similar to the effects of AVP plus raised $[K^+]_e$. When the data were expressed as response ratios (Fig. 4.9), a significant level of synergism was found to occur in some of the simultaneous responses. The magnitude of the synergism at its maximum level was more pronounced than that observed for AVP plus raised $[K^+]_e$ (compare Figs. 4.2 and 4.9). There were also differences regarding the pattern of the synergism with respect to the concentration of the stimulating agents. For TPA plus raised $[K^+]_e$, there was no effect of $[K^+]_e$ on the level of the synergistic response ($P > 0.05$); for each concentration of TPA, none of the three $[K^+]_e$ used caused significantly different response ratios from the other two. However, the influence of the TPA concentration was considerable (Fig. 4.9). At 0.1 nM TPA, the synergistic response was slightly developed (highest value 1.24 ± 0.05 at 40 mM K^+_e), but rose markedly when the TPA concentration was increased to 1 nM (maximum value 3.11 ± 0.74 at 20 mM $[K^+]_e$, $P < 0.05$ vs 0.1 nM TPA plus 40 mM K^+_e). When the TPA concentration was raised further to 10 nM, the synergistic response declined sharply (maximum value 1.49 ± 0.12, $P < 0.05$ vs 1 nM TPA plus 20 mM K^+_e), to values similar to those obtained from 0.1 nM TPA. The TPA concentration at which this reduction in the magnitude of the synergistic response occurs, coincides with the steepest part of the TPA dose-response curve (compare Figs. 4.4 and 4.6).

4.2.4 The effects of VSCC blockers on secretion evoked by TPA

The results presents so far in this chapter provide support not only for the involvement of AVP-activated PKC in ACTH release, but the results also suggest that there may be a chemical modulation of these channels, by PKC, during the ACTH response. Previous reports have demonstrated that ACTH release evoked by PKC-activating agents (phorbol esters or synthetic diacylglycerols) could be reduced when Ca^{2+} influx was compromised

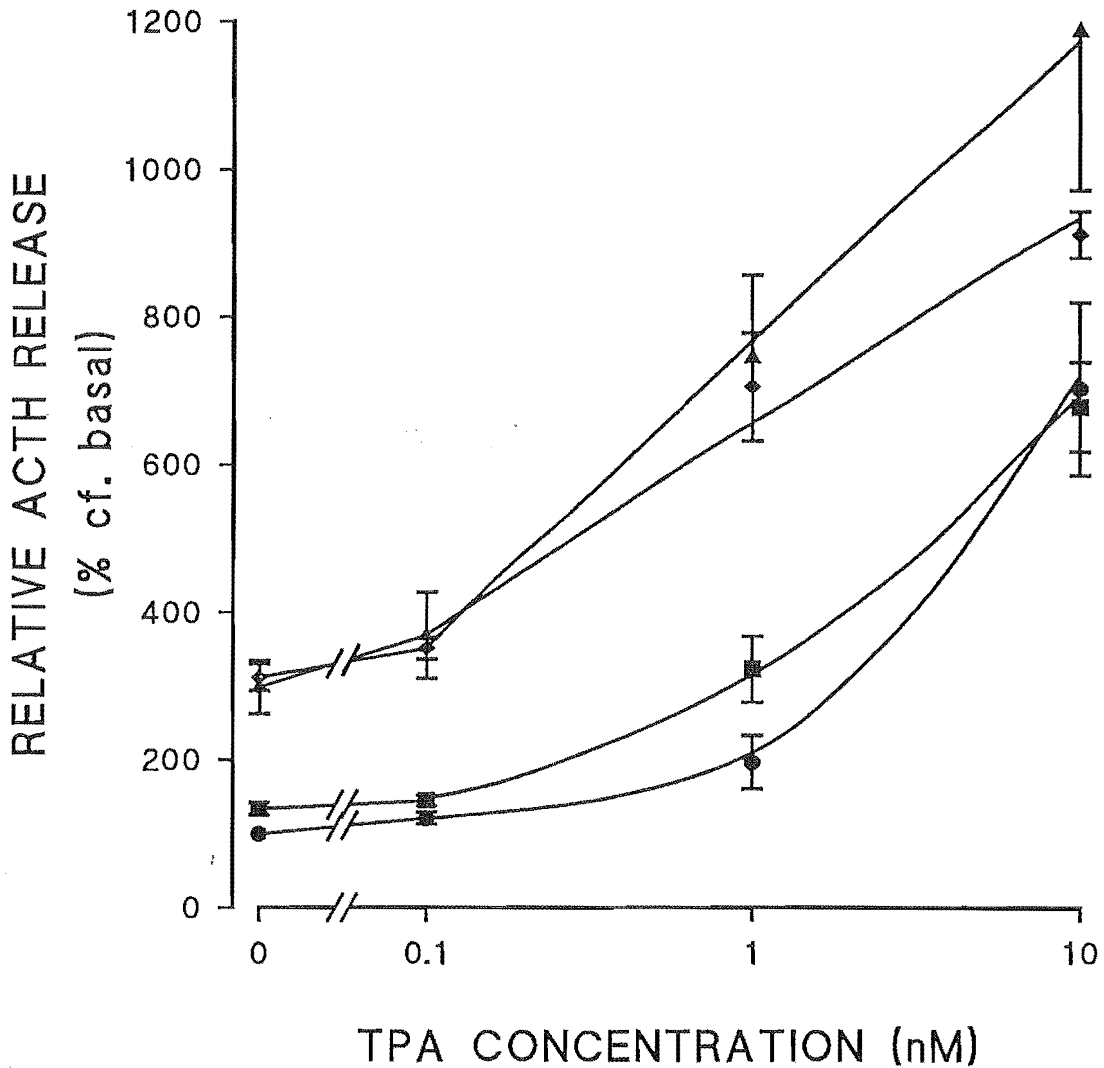

Fig. 4.8. Effect of simultaneous stimulation with TPA plus raised $[K^+]_e$. $[K^+]_e$ are 5.9 mM (normal, circles), 20 mM (squares), 40 mM (triangles) and 60 mM (diamonds). Data are mean ± SEM, n ≥ 3, and are expressed relative to basal (unstimulated release), which is set at 100%, to allow pooling between experiments with variable absolute levels of hormone secretion.

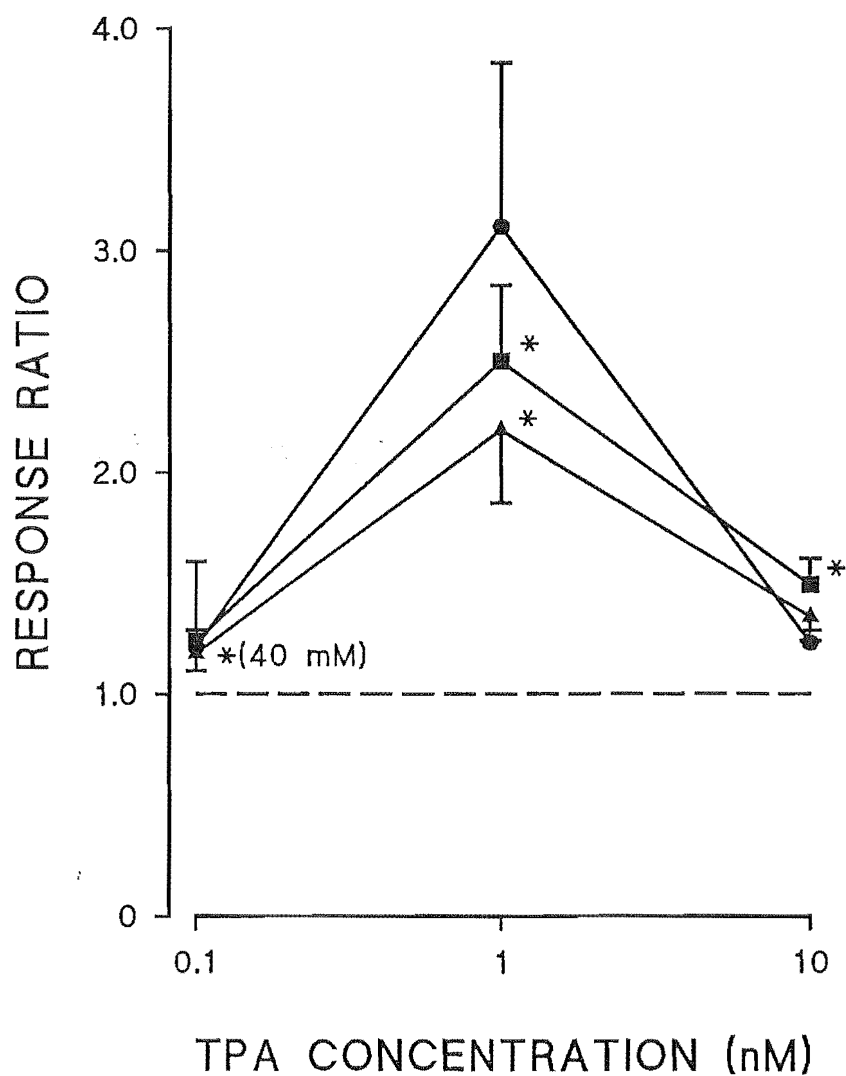

Fig. 4.9. Response ratios for simultaneous stimulation by TPA plus raised $[K^+]_e$. Data are mean ± SEM, n ≥ 3. $[K^+]_e$ are 20 mM (circles), 40 mM (squares) and 60 mM (triangles). Values that are significantly different from 1.0 are indicated with an asterisk.

by either the addition of VSCC blockers or by exposing the cells to Ca^{2+}-free medium (Reisine & Guild 1987, Sobel 1988, Won *et al.* 1990). To further characterise the dependence of the TPA/synthetic diacylglycerol-induced ACTH response on Ca^{2+} influx (and thus the potential influence of PKC on VSCC), and to extend these findings to ovine corticotroph cells, experiments were performed in which static cultures of ovine pituitary cells were exposed to TPA in the presence and absence of the L-VSCC blockers D600 and Co^{2+}. Both of these agents have previously been shown to inhibit AVP-induced ACTH secretion in ovine corticotrophs (see Chapter 3).

Two concentrations of TPA were used in conjunction with D600: 1 nM, which evokes a moderately sized secretory response, and 150 nM TPA, which induces maximal, or near maximal, secretion (Fig. 4.7). The effects of D600 on the responses to these TPA concentrations can be seen in Fig. 4.10. On secretion evoked by 1 nM TPA, D600 was ineffectual up to 10 μM, but at higher concentrations D600 readily blocked TPA-stimulated ACTH release, to a maximum value of 71.4 ± 12.8% at 300 μM D600. The response to 150 nM TPA was almost completely insensitive to D600, with a significant level of inhibition occurring only at the highest concentration of D600 used (300 μM), and the level of inhibition was only 9.1 ± 1.3%. The actions of D600 on TPA-stimulated ACTH release were different to the effects observed on AVP-stimulated release (see Fig. 3.1) in two respects: 1) D600 was an effective inhibitor of the AVP-stimulated response at much lower concentrations, and 2) there was no difference in the potency of D600 (*ie.* % inhibition) on different levels of stimulation by AVP (see Chapter 3).

Further experiments were conducted in which the inorganic ion Co^{2+} was used as the blocking agent. For this investigation three concentrations of TPA were used: 1, 10 and 150 nM. Co^{2+} completely blocked the response induced by 1 nM TPA ($P > 0.05$ compared to 100% inhibition), and the threshold for significant inhibition was similar to that required for significant inhibition of AVP-induced ACTH release (2 vs 0.5 mM, for inhibition of TPA- (Fig. 4.11) and AVP-induced (Fig. 3.2) secretion, respectively). At Co^{2+} concentrations lower than 2 mM, the TPA-stimulated response was occasionally enhanced (Fig. 4.11).

Co^{2+}, at 2 mM, also significantly reduced the ACTH response to 10 nM TPA (Fig. 4.11). However, the maximum level of inhibition of the 10 nM TPA-induced response by

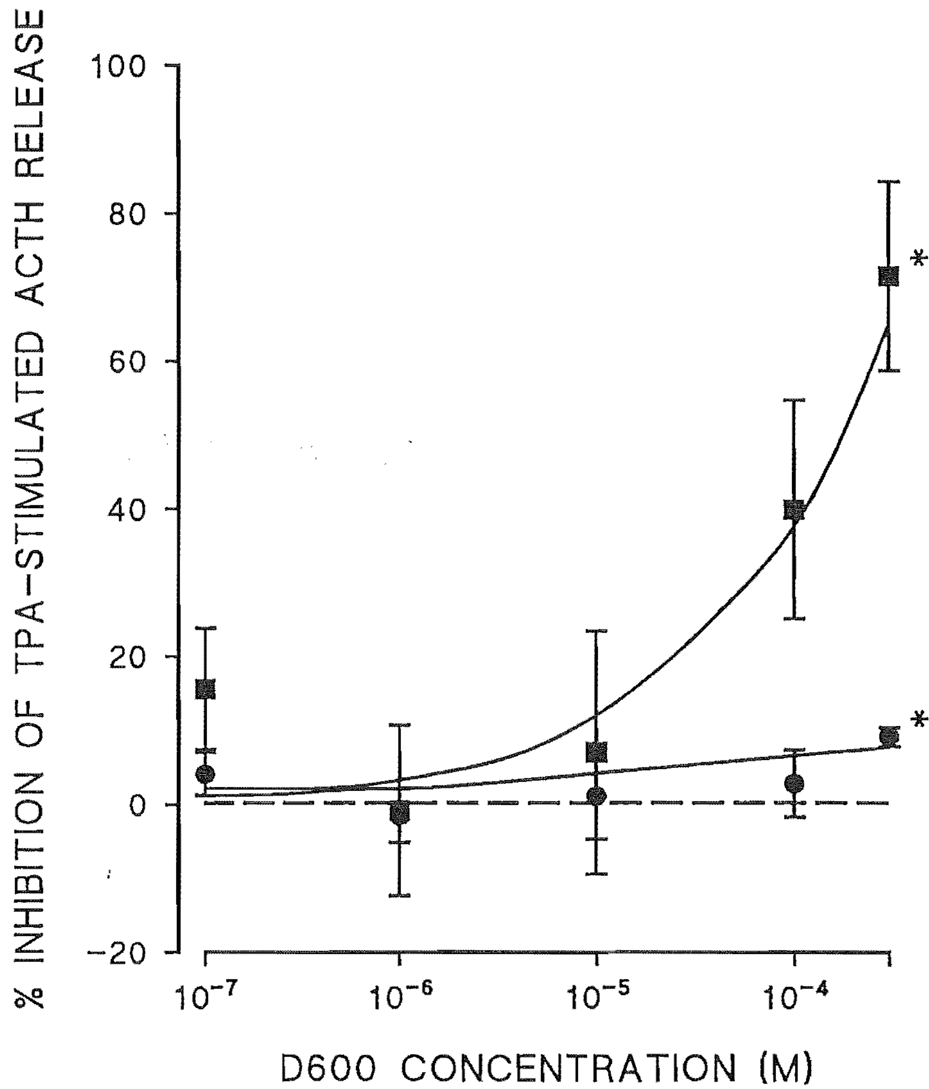

Fig. 4.10. Effect of D600 on secretion evoked by TPA at 1 nM (squares) and 150 nM (circles). Values are mean ± SEM, n ≥ 3, and values significantly different from 0% inhibition are indicated with an asterisk.

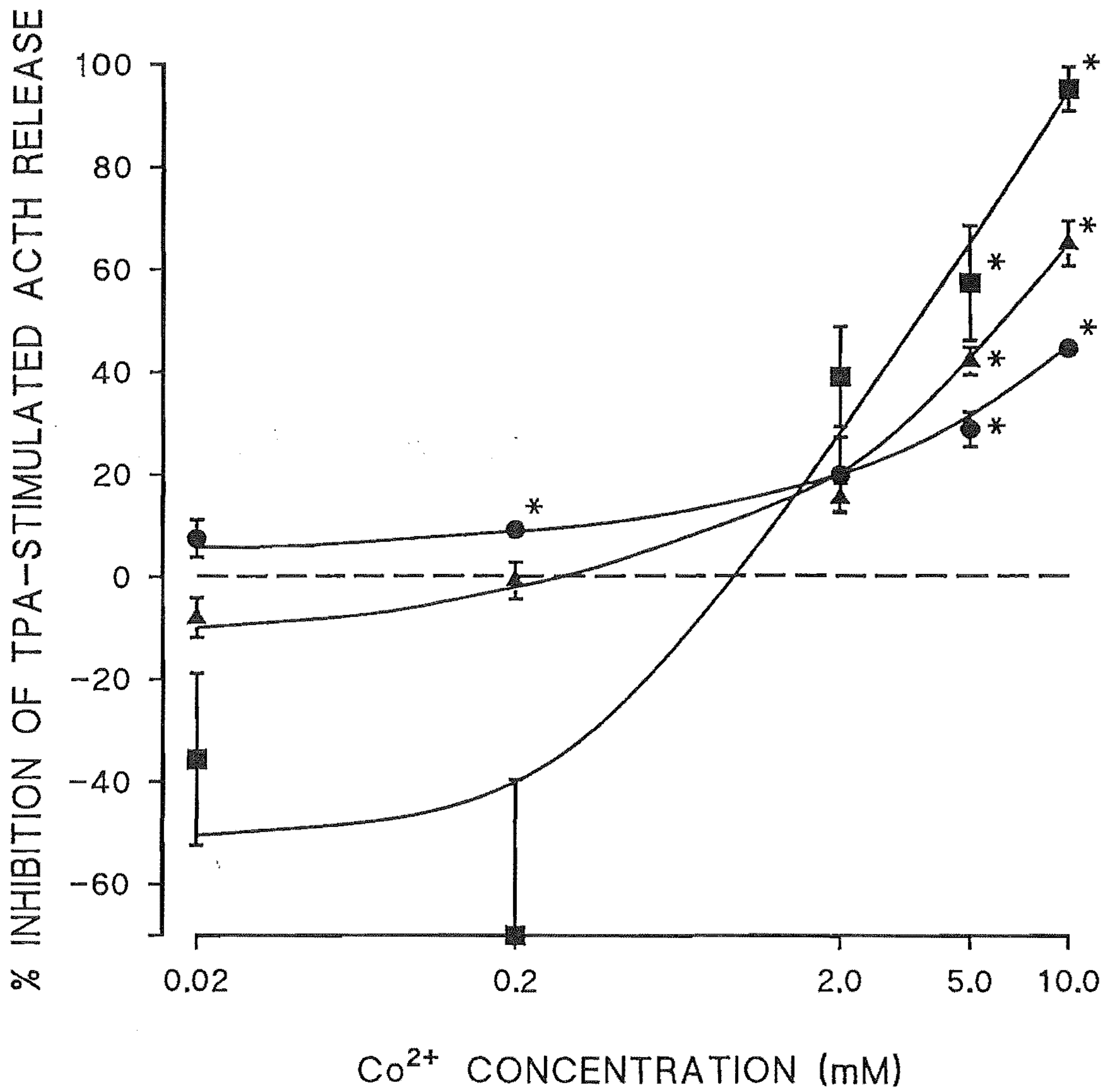

Fig. 4.11. Effect of Co^{2+} on secretion evoked by TPA at 1 nM (squares), 10 nM (triangles) and 150 nM (circles). Values are mean ± SEM, n ≥ 3, and those significantly different from 0% inhibition are indicated with an asterisk.

Co^{2+} was reduced compared to that observed for inhibition of the 1 nM TPA response (65.0 ± 4.4% compared with 95.2 ± 4.3, respectively, $P < 0.05$), and inhibition of the 10 nM TPA response was not complete ($P < 0.05$ compared to 100% inhibition). The ACTH response to 150 nM TPA was significantly inhibited at 0.02 mM Co^{2+} ($P < 0.01$), although the level of inhibition was small (9.3 ± 0.5%), and inhibition at 2 mM Co^{2+}, despite being higher, was not significant ($P > 0.05$). Maximal inhibition of the response to 150 nM TPA by Co^{2+} was 44.6 ± 0.5% and this was significantly different from both 0 and 100% inhibition ($P < 0.001$ for both comparisons), and further reduced compared to maximal inhibition for the 10 nM TPA-induced response ($P < 0.05$).

For both D600 and Co^{2+} the concentration of TPA used was found to affect the level of inhibition of the observed response. This is a very different effect from that observed for the inhibition of AVP-stimulated release (see Chapter 3). To further analyse the effects of channel blockers on TPA-stimulated release, the data were expressed in an alternative form. Previously, the difference between stimulated ACTH release in the presence and absence of a particular VSCC blocker was related back to stimulated release in the absence of the blocker, to give the percentage inhibition (see Chapter 2, section 2.2.5). For this alternative analysis, the second step of this process was omitted, thus the data were expressed as the reduction in ACTH release in the presence of the blocker, relative to stimulated release in the absence of the blocker (see Chapter 2, section 2.2.5 for full details of the procedures).

When the effects of D600 on TPA-stimulated ACTH release are expressed in this alternative form, it can be seen that D600 inhibits 1- and 150 nM TPA-stimulated release by approximately the same absolute amount over the range of D600 concentrations used (Fig. 4.12). At 300 μM D600 (the only concentration that caused significant percentage inhibition of both 1- and 150 nM TPA-stimulated release, Fig. 4.10), the absolute reduction was not different for the two concentrations of TPA ($P > 0.05$). However, whereas this level of reduction in the absolute response corresponds to approximately 70% inhibition of the 1 nM TPA-stimulated response, it corresponds to only about 10% inhibition of the response to 150 nM TPA, because this latter response is so much larger than the 1 nM TPA-induced response (see Fig. 4.7).

When the results from the TPA plus Co^{2+} experiments are analysed in the same

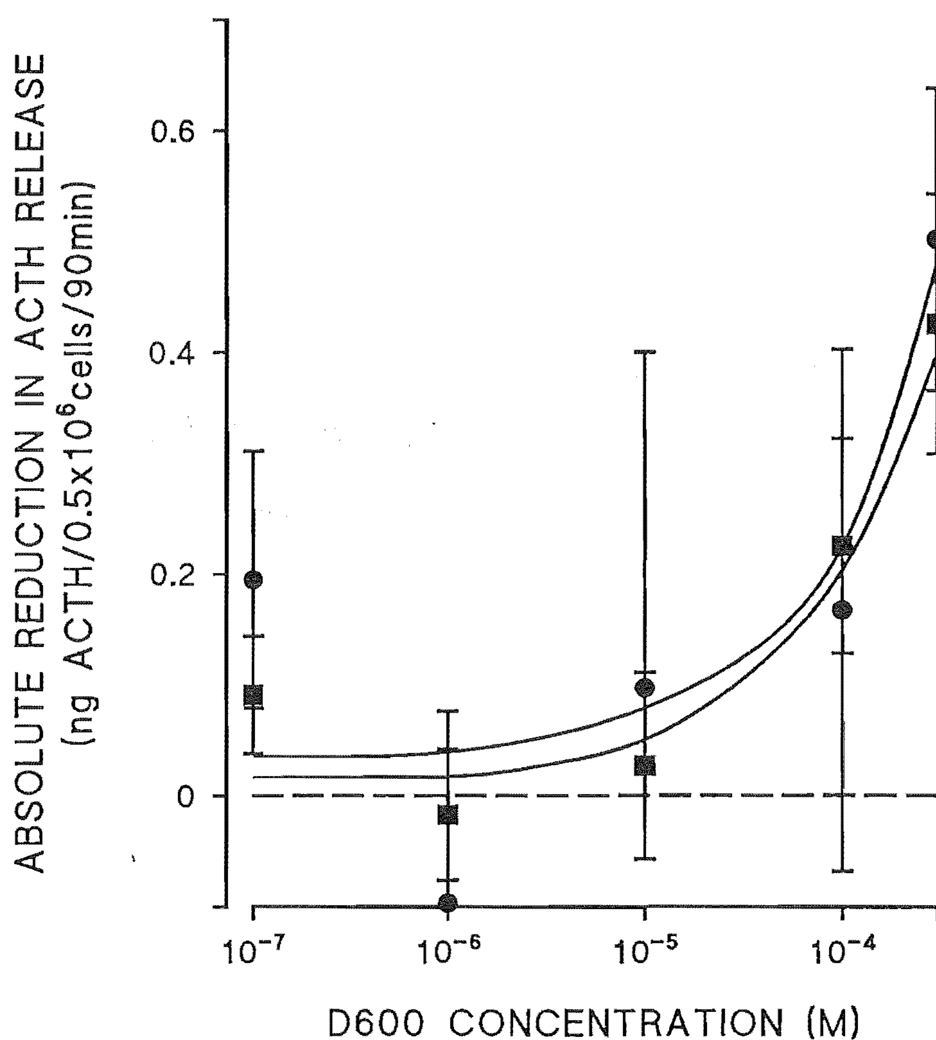

Fig. 4.12. Data from Fig. 4.10, expressed as the absolute reduction in ACTH release induced by D600. TPA concentrations are 1 nM (squares) and 150 nM (circles). Data are mean ± SEM, n ≥ 3. For these data, the absolute reduction is used because the levels of hormone release were similar between the separate experiments (*cf.* Fig. 4.13).

manner, a similar, although not identical pattern, was observed (Fig. 4.13). When the effects of 10 mM Co^{2+} are considered, it can be seen that the magnitude of the reduction of the 10 nM TPA-stimulated response was larger than that of the 1 nM TPA response, although percentage inhibition was actually lower for the response to 10 nM (Fig. 4.11). From the 10 nM- to the 150 nM TPA-stimulated responses, 10 mM Co^{2+} caused no further increase in the magnitude of the absolute reduction in ACTH release (Fig. 4.13), but percentage inhibition continued to fall with an increase in TPA concentration (Fig. 4.11).

4.2.5 The effects of Ca^{2+}-free medium on stimulation by AVP and TPA

VSCC blockers were able to inhibit TPA-stimulated ACTH release, suggesting that Ca^{2+} influx is involved in this response, and therefore, that PKC may directly, or indirectly, modulate VSCC activity. However, the pattern of inhibition by the blockers was more complex than that observed for inhibition of AVP-stimulated release and therefore experiments were conducted to further analyse the role of Ca^{2+} influx during the TPA-stimulated ACTH response. This involved investigating the effects of the removal of Ca^{2+} from the extracellular medium on TPA-stimulated ACTH release. The effects of Ca^{2+} removal on AVP-stimulated release were also determined, to allow correlation of the effects of this procedure with those of the VSCC blockers.

"Ca^{2+}-deficient" Kreb's Ringer was prepared by omitting $CaCl_2$ and increasing the [NaCl] to maintain osmolarity. For "Ca^{2+}-free" medium, 1 mM EGTA was added to Ca^{2+}-deficient medium. Since NCS (5%) was added to both of these media, the Ca^{2+} -deficient medium would be expected to have a [Ca^{2+}] of approximately 125 μM, while the EGTA should reduce the [Ca^{2+}] of the Ca^{2+}-free medium to nM levels (Stojilkovic *et al.* 1988).

Exposing cells to Ca^{2+}-deficient or Ca^{2+}-free media did not affect unstimulated hormone release ($P > 0.05$ for both, not shown). However, secretion induced by 1 and 100 nM AVP was reduced by 50.7 ± 5.3% and 35.1 ± 5.2%, respectively, in Ca^{2+}-deficient medium (Fig. 4.14). These levels of inhibition were not significantly different from each other ($P > 0.05$). The Ca^{2+}-free medium reduced AVP-stimulated release to a greater extent; by 91.4 ± 11.1% and 90.5 ± 6.8% for 1- and 100 nM AVP, respectively, and again,

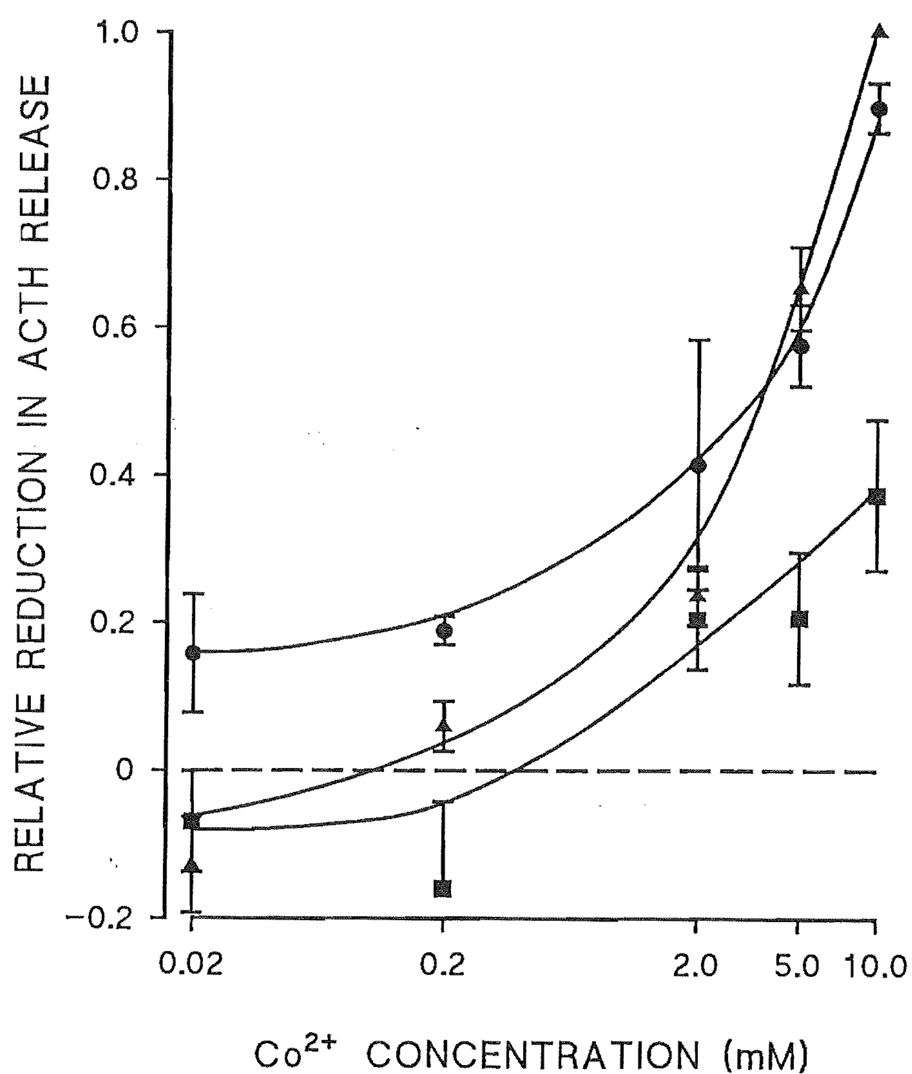

Fig. 4.13. Data from Fig. 4.11, expressed as the relative reduction in ACTH release induced by Co^{2+}. TPA concentrations are 1 nM (squares), 10 nM (triangles) and 150 nM (circles). Data are mean ± SEM, n ≥ 3. For the data described in this figure, the absolute levels of hormone release were variable between experiments. Therefore, for each experiment, the value for the absolute reduction induced by 10 mM Co^{2+} of the 10 nM TPA-stimulated response was set to 1.0, and all of the other values for inhibition by Co^{2+} were expressed relative to this value. The data for the separate experiments were then pooled.

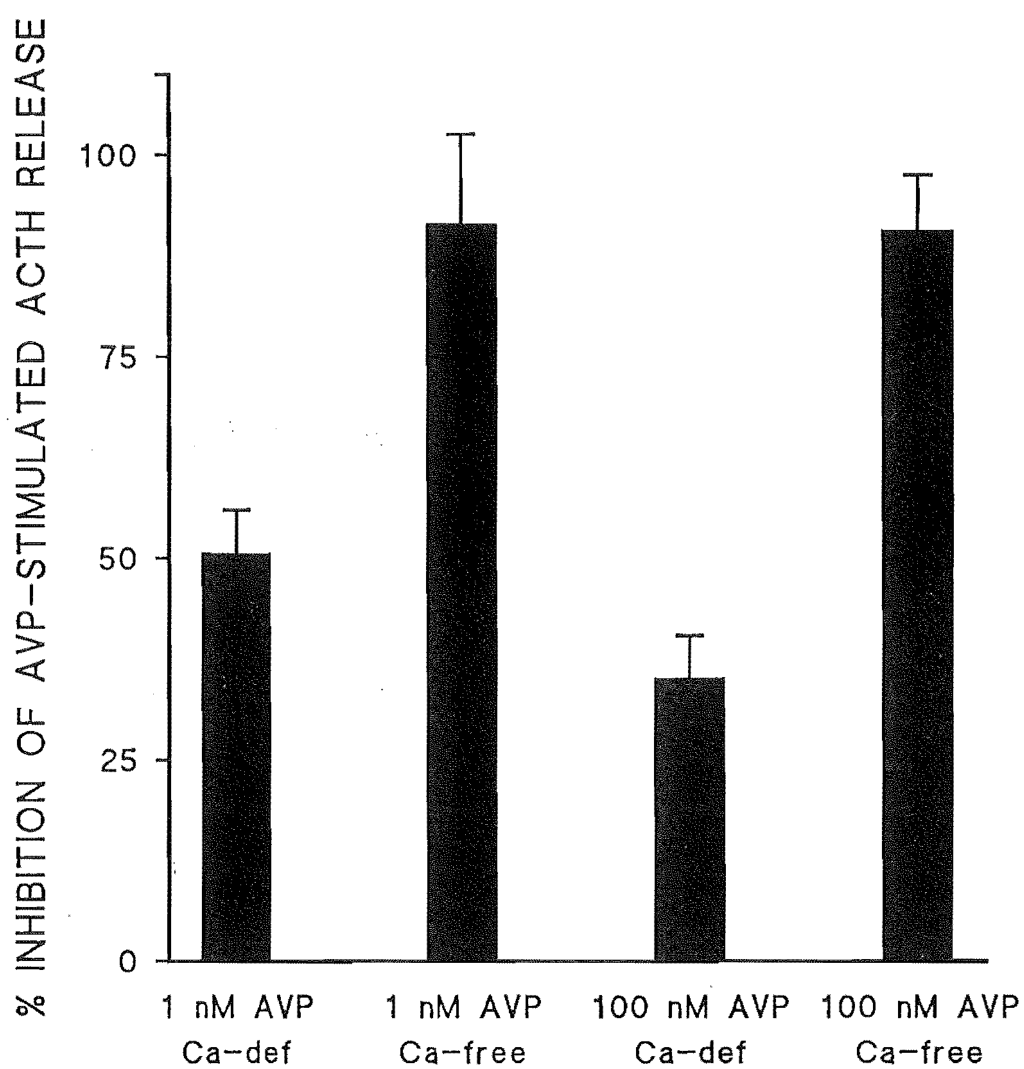

Fig. 4.14. Effect of Ca^{2+}-deficient/free media on ACTH release induced by AVP. Values are mean ± SEM, n = 2.

these values are not significantly different from each other ($P > 0.05$). The levels of inhibition induced by the Ca^{2+}-free medium were also not significantly different from 100% inhibition ($P > 0.05$, for both).

TPA-induced release was also found to be sensitive to both Ca^{2+}-reduced media. Ca^{2+}-deficient medium reduced the responses to 1- and 10 nM TPA by 95.4 ± 14.7% and 62.0 ± 7.4%, respectively (Fig. 4.15). Although there is a substantial numerical difference between these values, they are not significantly different ($P > 0.05$). Ca^{2+}-free medium reduced 1- and 10 nM TPA-stimulated release by 114.2 ± 8.1% and 91.2 ± 6.2%, respectively. These values are not significantly different from each other, nor from 100% inhibition ($P > 0.05$).

4.3 Discussion

4.3.1 Simultaneous stimulation with AVP plus raised $[K^+]_e$

Theoretical analysis of simultaneous stimulation

The simultaneous stimulation protocols were used to attempt to characterise the mechanisms by which the Ca^{2+} channels were modulated during the AVP-stimulated ACTH response. The rationale, in simple terms, was that if both AVP and raised $[K^+]_e$ caused Ca^{2+} influx via the same channel type, as was suggested by earlier observations (see Chapter 3), then the magnitudes of the individual responses should not be able to be maintained when these two secretagogues were given simultaneously; *ie.* the VSCC should become limiting, at least at high concentrations of the secretagogues. This rationale, and the theoretical outcomes of these experiments are described more formally and comprehensively in the following discussion.

In the first instance, it is necessary to consider the nature of the populations of channels that might be involved in the individual responses to AVP and raised $[K^+]_e$.

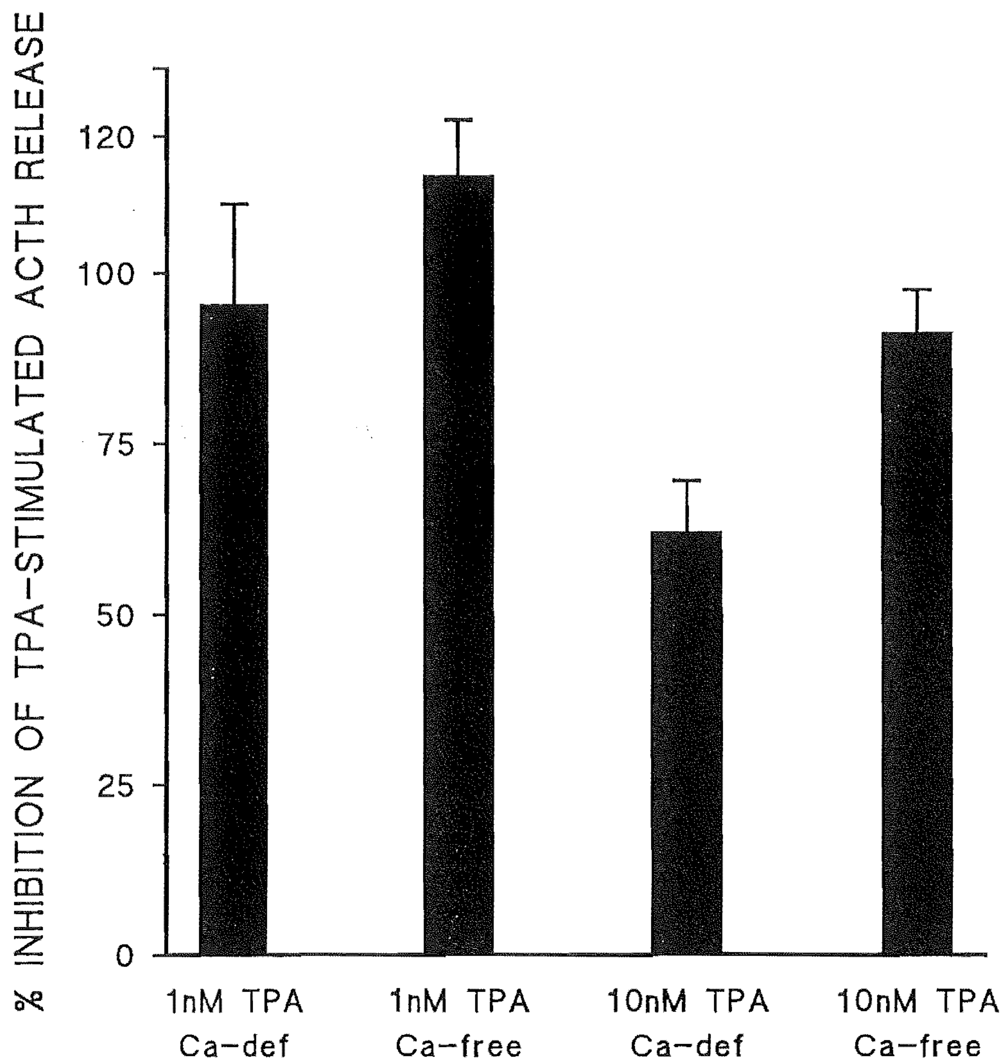

Fig. 4.15. Effect of Ca^{2+}-deficient/free media on ACTH release induced by TPA. Values are mean ± SEM, n = 2.

The results presented in Chapter 3 suggest that somewhere between 71.6 to 87.6% of the AVP-stimulated ACTH response is sensitive to blockers of VSCC (these values represent the maximum levels of inhibition caused by D600 and Cd^{2+}, respectively, see Figs. 3.1 and 3.2). Thus it would appear that under the conditions used in this study, the activation of VSCC by AVP is obligatory for *up to* 87.6% of the response. However, no VSCC-blocker was able to totally block the AVP-induced response. Thus there is a component of the AVP-induced response that is quite resistant to the effects of VSCC blockers, and the most conservative estimate of the magnitude of this component is 12.4% of the response over a 90 min period.

In contrast, the response to raised $[K^+]_e$ was totally dependent on VSCC activation, as determined by the ability of D600 and Cd^{2+} to abolish this response (see Figs. 3.3 and 3.4). This indicates that raised $[K^+]_e$ is unable to activate the part (estimated at 12.4%) of the AVP-induced response that is not sensitive to the actions of VSCC blockers. In the entire population of pituitary cells composing one experimental unit (*ie.* one well of a tissue culture plate), the channels that become activated in response to K_e^+-induced membrane depolarisation would presumably be all those channels that have the appropriate activation/inactivation kinetics. That is, the K_e^+-induced response is relatively non-specific; channels from all different cell types will be able to respond if they display the appropriate attributes for activation. [This does not present a problem for this study, because it is the ACTH response that is measured, and therefore only the effects of channels that are functionally linked to ACTH release are observed.] Therefore, K_e^+-induced ACTH release should arise from any cell that: 1) has channels that respond to membrane depolarisation, and 2) has channels whose activation leads ultimately to ACTH release. In a more complex scenario, the activation of channels in one cell could lead to the generation of a paracrine factor resulting in ACTH release from another cell. As yet, there is no evidence for such a mechanism.

There is now considerable evidence that the total population of ACTH-secreting cells is not a homogenous group (see Chapter 1 and Jia *et al.* 1991, Canny *et al.* 1992, Corcuff *et al.* 1993). Rat corticotroph cells that respond to CRH but not AVP have been identified (Jia *et al.* 1991), and it is believed that the response to CRH also involves the activation of VSCC (see Chapter 1). Therefore the VSCC in the membranes of cells that

do not respond to AVP would presumably not become activated during exposure to AVP, but are likely to be activated by exposure to raised $[K^+]_e$.

Therefore, it is suggested that the AVP- and K^+-induced ACTH responses both contain a common component (the subset of VSCC that are activated by both secretagogues) and also a component that is unique to each secretagogue. Using this hypothesis, a theoretical analysis of the effects of simultaneous exposure to AVP and raised $[K^+]_e$ can be derived:

The individual responses are arbitrarily assigned an ACTH response value, *ie.*

AVP-stimulated ACTH release = (xACTH + y_iACTH) (4.1), and

K_e^+-stimulated ACTH release = (y_{ii}ACTH + zACTH) (4.2).

The values xACTH and zACTH correspond to the unique components of the AVP and K^+ responses, respectively. For AVP-stimulated release this represents secretion that is not sensitive to VSCC blockers, and probably reflects secretion arising from the release of intracellular Ca^{2+}. For K_e^+-stimulated release this unique component represents secretion dependent on the activation of VSCC that are not activated during exposure to AVP. The values y_iACTH and y_{ii}ACTH correspond to the components of the individual responses that are common to both secretagogues. The "y" part of the notation refers to the nature of the pathway used to produce this component of the response (as does "x" and "z"), and the "$_i$" and "$_{ii}$" notation reflects the fact that the size of the components is not necessarily the same. For simplicity sake, however, y_iACTH and y_{ii}ACTH will be considered to be equal (unless specifically stated to be otherwise) and so are redefined as y_{ind}ACTH, for "individual yACTH" (*ie.* to denote y-type secretion induced by the secretagogues when given individually). Eqs. 4.1 and 4.2 can thus be re-written as:

AVP-stimulated ACTH release = (xACTH + y_{ind}ACTH) (4.3), and

K_e^+-stimulated ACTH release = (y_{ind}ACTH + zACTH) (4.4).

Therefore, the sum of the individual responses is given by:

sum of individual responses = (xACTH + $2y_{ind}$ACTH + zACTH) (4.5).

Considering the outcome of simultaneous stimulation with AVP and raised $[K^+]_e$, the two secretagogues should still contribute the unique components (xACTH and zACTH, respectively), but the size of yACTH (denoted as y_{sim}ACTH, for "simultaneous yACTH") will depend on the level of stimulation by the two agents. Firstly, let it be assumed that, when given individually, AVP and raised $[K^+]_e$, at maximal stimulatory concentrations, cause the yACTH pathway to be maximally activated (= y_{max}ACTH). This assumption is made because both of the individual responses would appear to utilise the common component (*ie.* VSCC sensitive to *both AVP and raised* $[K^+]_e$) to a large degree, suggesting that at high levels of stimulation this component is activated to a large extent. Thus, when the agents are given simultaneously, it seems reasonable to assume that the level of activation of the common component that would be needed to faithfully maintain the responses at the levels that occurs when they are given individually, would be greater than the maximal possible activity of this component. For the sake of simplicity in this theoretical analysis, it has been assumed that at maximally stimulating concentrations, both agents, when given individually, stimulate the common component to its maximally active state. This may not actually be the case, but the important aspect of this assumption is that the maximal activity of the common component is exceeded by the combined level of activity of this factor that occurs in response to the stimulating agents when give individually.

Given this assumption, for simultaneous stimulation, when the two agents are given at maximally stimulating concentrations, y_{sim}ACTH can only be as large as y_{ind}ACTH and both of these quantities are equal to y_{max}ACTH. This gives, at maximal stimulation:

simultaneous ACTH release = (xACTH + y_{sim}ACTH + zACTH) (4.6), or

simultaneous ACTH release = (xACTH + y_{ind}ACTH + zACTH) (4.7), or

simultaneous ACTH release = (xACTH + y_{max}ACTH + zACTH) (4.8).

Comparing Eq. 4.7 to Eq. 4.5, when the stimulating agents are given at maximally stimulating concentrations, the difference between the sum of the individual responses and the simultaneous response is y_{ind}ACTH (= y_{max}ACTH). The value y_{ind}ACTH is qualitative only (*ie.* the actual value of ACTH cannot be determined), but the significant aspect is that, under conditions where the stimulating agents are given at maximally-stimulating concentrations, the simultaneous response should be lower than the sum of the individual responses. However, from this theoretical analysis, it can also be determined that the simultaneous response should always be greater than *either* of the individual responses (compare Eq. 4.7 with Eqs. 4.3 and 4.4).

At sub-maximal levels of stimulation, y_{ind}ACTH will not equal y_{max}ACTH (or y_{sim}ACTH). If the level of stimulation is low enough that the yACTH component of both of the individual responses, when combined, does not exceed y_{max}ACTH (*ie.* $2y_{ind}$ACTH $\leq y_{max}$ACTH), then the yACTH pathway will not be limiting and the simultaneous response should be able to match the sum of the individual responses:

sum of individual ACTH responses = (xACTH + $2y_{ind}$ACTH + zACTH) (4.5), and

simultaneous ACTH release = (xACTH + y_{sim}ACTH + zACTH) (4.9).

Since $2y_{ind}$ACTH $\leq y_{max}$ACTH,

then $2y_{ind}$ACTH $\ngtr y_{sim}$ACTH

and so in the simplest case it would be expected that:

sum of individual ACTH responses = simultaneous ACTH release (4.10).

Once again, the simultaneous response will always be greater than either of the individual responses.

To summarize, when comparing the simultaneous response to the sum of the individual responses there should be a range of outcomes, depending on the level of stimulation. At low levels of stimulation (using concentrations of the secretagogues that elicit modest levels of ACTH release), the simultaneous response should match the sum of the individual responses (*ie.* the comparison will be additive). As the level of stimulation is increased, the cellular mechanisms that are common to both AVP- and K^+_e -stimulated responses will become activated to a high (perhaps maximal) level, and therefore limiting, and the simultaneous response will not be able to match the sum of the individual responses (*ie.* a less-than-additive response will occur). This theoretical profile is represented schematically in Fig. 4.16. At all times, the simultaneous response should be greater than either of the individual responses.

Comparison of theoretical outcomes and experimental findings

The theoretical analysis of simultaneous stimulation suggests three key aspects to be identified when analysing the experimental data. These are:

1) At high levels of stimulation, the simultaneous response should be less than the sum of the individual responses.

2) At modest levels of stimulation, the simultaneous response is likely to be equivalent to the sum of the individual responses.

3) At all concentrations, the simultaneous response should be greater than either of the individual responses.

The experimental data was found to be completely consistent with aspects "1)" and "3)". With respect to aspect "1)", at high levels of stimulation the simultaneous response *was* smaller in magnitude than the sum of the individual responses (see Fig. 4.2), and with respect to aspect "3)", the simultaneous response was always larger than either of the individual responses (see Fig. 4.1). With respect to aspect "2)", additive responses were observed for several combinations of [AVP] and raised $[K^+]_e$, however the unexpected finding of synergism was also observed (see Fig. 4.2). This unexpected finding does not

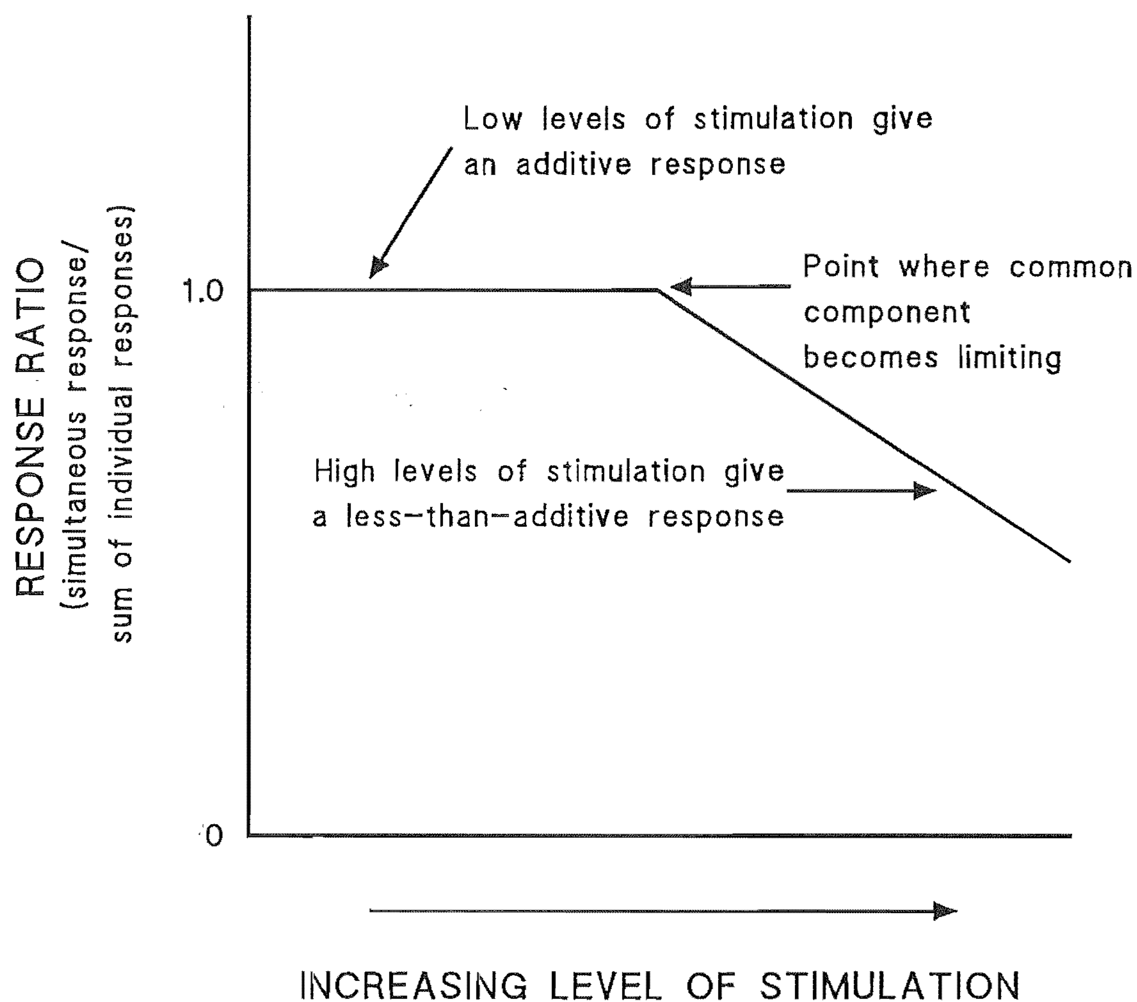

Fig. 4.16. Schematic figure representing the theoretical relationship between response ratios and an increasing level of simultaneous stimulation by AVP plus raised $[K^+]_e$.

necessarily weaken the theoretical explanation of the data, but it does suggest an extra level of complexity in the simultaneous response. This added complexity will be considered in later discussion.

Overall the data is consistent with the hypothesis that AVP- and K_e^+-induced ACTH responses utilise, to some (probably large) extent, a common set of VSCC. In the theoretical analysis, given above, the common set of channels represents the limiting factor for the simultaneous response, such that when the AVP and K_e^+ concentrations are high, the simultaneous response cannot be maintained at a level comparable to the sum of the individual responses. However, the data do not *prove* this, and may be consistent with other explanations of the processes underlying the simultaneous response. For example, considering the simplest alternative to the "common set of channels" hypothesis, it is possible that AVP and K^+ *do not* act through the same channels (*ie.* they utilise distinct channel populations). In this case, the limiting factor could be some aspect distal to Ca^{2+} influx. This is a reasonable possibility, given that both responses generate Ca_i^{2+} responses to initiate exocytosis. The limiting factor could be the actual secretory capability of the cells. Either the actual amount of hormone may be limiting (*ie.* high levels of simultaneous stimulation might deplete the cells of hormone), or some factor, distal to the generation of the Ca_i^{2+} response, may be present that controls the magnitude of the secretory response, so as to not deplete the cells of hormone. It is therefore necessary to attempt to determine if the limiting factor occurs at the level of Ca^{2+} influx, at the level of exocytosis, or some other possibility.

From Fig. 4.1 it is evident that the absolute ACTH response to 1000 nM AVP plus 60 mM $[K^+]_e$ lies on the plateau for simultaneous stimulation, which suggests that the simultaneous response has reached a maximum at these concentrations of the secretagogues. The data from Table 4.1 shows that the response ratios for 1000 nM AVP plus 60 mM $[K^+]_e$ were significantly below 1.0 (1.0 represents additivity). Therefore, at these concentrations, the simultaneous response was significantly less than the sum of the individual responses, and thus it appears that some part of the simultaneous response was being limited. Table 4.1 also shows that in the same experiments, there was a TPA-induced secretory response that was significantly larger than the response to 1000 nM AVP plus 60 mM $[K^+]_e$. The TPA-induced responses, which were the largest that occurred in these

experiments, can be taken as best estimates for the maximum possible secretory responses, under the conditions used, and in these particular experiments. The simultaneous responses can be divided by their respective response ratios to give the amount of secretion that would be necessary for the responses to be additive with respect to the sum of the individual responses, and thus not be limiting (Table 4.1). None of the calculated responses were found to be larger than the TPA-induced responses. Therefore, these results suggest, purely on the size of the secretory responses, that the limitation of the simultaneous response is not due to a restriction (by whichever means) on the absolute magnitude of the secretory response. Therefore the results support the possibility that the limiting factor is at the level of Ca^{2+} influx (or some other locus), above the possibility that it is at the level of exocytosis.

The above analysis has the potential drawback that the comparisons between the simultaneous responses and the estimates for the maximum possible secretory responses (the TPA-induced responses) were done purely on the absolute magnitude of the responses. TPA might induce ACTH release via processes that are unique to TPA-induced stimulation (or at least are not activated during the simultaneous response to AVP plus raised $[K^+]_e$). Thus it is possible that the maximum simultaneous response observed for AVP plus raised $[K^+]_e$ *does* reflect the maximum possible secretory response *for these particular agents.* If this was the case, then the limiting factor could be at the level of exocytosis. The validity of the above analysis depends on whether TPA induces cellular effects that AVP plus raised $[K^+]_e$ can also activate. On the currently available evidence, it is not possible to determine whether this is the case, and this must be taken into account when considering the validity of the above analysis.

Analysis of the synergistic response

The unexpected finding from the simultaneous stimulation experiments was the observation of synergism of the simultaneous response with respect to the individual responses. The

enhancement of ACTH release was relatively small, but the expectation was for values equivalent to, or less than, the sum of the individual responses. This was based on the fact that the individual responses are thought to utilise common intracellular components. It must be stressed that the synergism is non-physiological, since the raised $[K^+]_e$ used here would never occur *in vivo*, and it should not be confused with the synergism observed in response to the combined presence of AVP and CRH (see Chapter 1). However, this does not make the observation of the synergism inconsequential, as analysis of the possible causes of the synergism may provide further insights into how the AVP-stimulated response is generated.

The question is raised as to what factor(s) leads to the generation of the synergistic response. Without any evidence to the contrary, the simplest case must be considered first, and this is that the facilitatory effect which causes the synergism, occurs at a single locus. It is possible that this point of interaction is the same as that which becomes the limiting factor (as described above), when the concentrations of AVP plus K_e^+ are high. Alternatively, the point where facilitation occurs may be distinct from the limiting factor described above. In this instance the synergism would occur at one point, and then at some point further along the pathway the full message could not be maintained due to one or more components of the response becoming maximally activated and therefore the level of the synergism would be reduced.

The previous discussion highlighted the likelihood that the AVP- and raised $[K^+]_e$-induced ACTH responses utilise a common set of VSCC. The activation of VSCC are a relatively early event in both of the individual secretory responses, and it was suggested previously that the loss of the synergistic response occurs due to the maximum activation (and thus limitation) of a factor proximal to the regulation of exocytosis. Thus the temporal location of VSCC activation fits with the scheme of when the synergism might occur.

Therefore the role of VSCC in the generation of the synergistic response was investigated, and the results of these experiments were presented in Figs. 4.3 - 4.5. In these experiments, D600 (at 10 μM, a level that caused significant, but not maximal levels of inhibition of the AVP- and K_e^+-stimulated responses, see Figs. 3.2 and 3.4) was used to inhibit L-VSCC activity, and a variable test incubation duration was used to investigate the temporal development of the synergism. The effects of D600 on the temporal aspects of

the AVP-induced response were investigated, to provide a background comparison for the effects of D600 on the simultaneous response. This particular investigation extends the work presented in Chapter 3, and will be dealt with here briefly before further analysis of the simultaneous response.

Although the amount of secretion was reduced (Fig. 4.3), addition of D600 did not significantly affect the profile of AVP-stimulated release (Fig. 4.4). This might initially seem surprising, since the first phase of AVP-induced secretion is thought to involve, principally, the release of Ca^{2+} from intracellular stores. D600 would be expected to have little effect during this time, but have a greater effect during the later stages (after the first 15 min) of the response, when Ca^{2+} influx predominates over release of intracellular Ca^{2+}. Thus, the addition of D600 would be expected to result in the proportion of the entire secretory response to AVP plus D600 being higher during the first 15 min compared to that from the AVP-only response. However, the lack of a significant difference between the temporal profiles of the two responses may simply reflect the relative contributions of intracellular Ca^{2+} release and Ca^{2+} influx over a 90 min period. The results from Chapter 3 demonstrate that for a 90 min time period, up to 87.6% (*ie.* the level of inhibition observed in the presence of 1000 μM Cd^{2+}, Fig. 3.3) of the AVP-induced response is dependent on Ca^{2+} influx. Also, the concentration of D600 used (10 μM) caused a level of inhibition (58.1%) that was lower than the maximum level of inhibition observed for D600 (71.6%, see Fig. 3.2), which itself was less than the 87.6% inhibition observed for 1000 μM Cd^{2+}. Thus some Ca^{2+} influx would presumably still occur during exposure to AVP plus 10 μM D600. A theoretical temporal response profile was calculated assuming that 12.4% (*ie.* the residual component that is insensitive to Cd^{2+}) of the response to a 90 min exposure to AVP is dependent on Ca^{2+} released from intracellular stores, and that this release of Ca^{2+} occurs only in the first 15 min of the response (which corresponds with perifusion data from this laboratory, DR Mason, in prep.). The value for the level of inhibition caused by 10 μM D600 on AVP-stimulated release (Fig. 3.2, corrected for the contribution to the response of Ca^{2+} mobilisation) is applied to the level of secretion induced by AVP (only) during 15 to 90 min time period (the data in Fig. 4.3). The temporal response profile that results from this calculation was no different from that obtained for AVP plus D600, or for the AVP only response (not shown). Thus this result is consistent with the interpretation

that the lack of a significant difference between the observed temporal profiles for the AVP only, and AVP plus D600 responses is expected given the relative contributions of intra- and extracellular Ca^{2+} during the AVP-induced response under the prevailing conditions, and the level of inhibition induced by 10 μM D600. Furthermore, these results are consistent with the notion that release of intracellular Ca^{2+} occurs during the early phase of AVP-induced ACTH secretion, and that Ca^{2+} influx occurs during the later stages. If a higher concentration of D600 (or a high concentration of Cd^{2+}) was used, it would be expected that a greater proportion of the AVP-activated L-VSCC would be blocked, and therefore a significantly different temporal response profile would be expected to occur.

When the profile for AVP + K_e^+ + D600 is compared to that for AVP + K_e^+ (Fig. 4.4), it can be seen that in the former profile, the vast majority of the secretion occurs in the first 15 min of the response. This is quite different from the AVP plus K_e^+ response. In the AVP + K_e^+ + D600 response, there is almost no further secretion after the first 15 min. In contrast, for AVP + D600, although the level of secretion for the entire response is modest, the level of secretion after 90 min is more then double that which had occurred at 15 min. The secretion that occurs during the 15 to 90 min period is probably due to Ca^{2+} influx, and occurs because 10 μM D600 causes a level of inhibition less than the maximal level that can be obtained by D600 (see above and Fig. 3.2). This raises the question that even if 10 μM D600 can inhibit virtually the entire 60 mM K^+-induced response (approximately 93%, Fig. 3.5), why, in the AVP + K_e^+ + D600 response, was there not the level of secretion during the 15 to 60 min time periods attributable to incomplete inhibition of AVP-induced Ca^{2+} influx (*ie.* corresponding to that observed in the AVP + D600 response)? A possible interpretation is that the effect of adding the raised $[K^+]_e$ was to increase the effectiveness of D600 on AVP-activated channels. In Chapter 3 the possibility was raised that the enhanced effectiveness of D600 on K_e^+-activated VSCC, compared to inhibition of AVP-activated VSCC, might be due the level of activation of the channels. That is, the greater level of inhibition of K_e^+-activated channels may be due to the more intense, permanent depolarisation of the plasma-membrane. The findings presented in this chapter are consistent with this hypothesis.

When the data from the variable test-incubation-duration experiments were converted to response ratios (Fig. 4.5), it was found (through regression analysis) that the

response ratios decreased with an increasing test incubation duration. Previously, it was found that the response ratios dropped as the level of stimulation was increased. Another way to state this is that the response ratios dropped as the magnitude of the simultaneous response increased. For the variable test-incubation-duration experiments, a similar pattern occurred, at least with respect to a reduction in the response ratios with the largest responses (*ie.* those that occurred at the longer test incubations). The analysis showed that the decrease in the response ratios with increasing test incubation duration was observed both in the presence and absence of D600. This finding provides further evidence that the loss of the synergistic simultaneous response, resulting eventually in less-than-additive response ratios, is not due to the simultaneous response reaching the secretory limit of the cells. If this was the case, then in the presence of D600, the response ratios should be maintained irrespective of the duration of the test incubation, since the absolute levels of secretion would never approach the maximum secretory capability of the cells. Therefore, there must be some other factor, proximal to the level of actual exocytosis, that becomes limiting, and causes the reduction in the response ratios as the level of stimulation is increased.

Analysis of the data from Fig. 4.5 can be extended further in an attempt to investigate whether the synergism observed in response to low levels of simultaneous stimulation occurs at the level of the VSCC. The following rationale will be used. For a 90 min test incubation, stimulation by 10 nM AVP plus 60 mM $[K^+]_e$ resulted in a response ratio of 1.08 ± 0.03 (*ie.* additivity). If the enhancement of the simultaneous response that occurs when lower concentrations of the secretagogues are used does not directly involve VSCC (*ie.* Ca^{2+} influx might be necessary, but the actual facilitation does not occur at the level of the VSCC), then the effect of adding a VSCC blocker should be to lower the effective concentration of the secretagogues, by inhibiting part of the response and therefore reducing the magnitude of the response. For example, 10 μM D600 causes 93% inhibition of the 60 mM K_e^+-stimulated ACTH response (see Fig. 3.3). If it is assumed that the VSCC that are activated by raised $[K^+]_e$ have an equal chance of being blocked by D600, then the addition of 10 μM D600 should result in the 60 mM $[K^+]_e$-induced response being reduced to approximately 7% of its original magnitude. The typical response to 20 mM $[K^+]_e$ is 12.7 ± 2.6% of the 60 mM response. Therefore, 10 μM D600 inhibition of 60

mM $[K^+]_e$-induced secretion represents an ACTH response that is quantitatively and (probably) qualitatively similar to the 20 mM $[K^+]_e$-induced response.

In contrast, 10 μM D600 inhibits the AVP-induced response by 58.1 ± 2.8% (see Fig. 3.2), meaning that approximately 42% of the response is left intact. Comparing 1 and 10 nM AVP-induced ACTH release, 1 nM AVP induces 50.4% of the ACTH secreted in response to 10 nM AVP. Therefore, the ACTH response in the presence of 10 μM D600 plus 10 nM AVP and the 1 nM AVP-induced response are also quantitatively similar. Qualitatively, they will be somewhat different due to the fact that ACTH released due to mobilisation of intracellular-stored Ca^{2+} is presumably unaffected by D600 and would therefore make up a greater proportion of the response to 10 nM AVP plus D600, compared with the 1 nM AVP-induced response. However, this effect should be small since the contribution of mobilised Ca^{2+} for a 90 min test incubation has been estimated at only 12.4% (see above).

Therefore, the overall effect of adding 10 μM D600 to the response induced by 10 nM AVP plus 60 mM $[K^+]_e$ should be to roughly simulate the response to 1 nM AVP plus 20 mM $[K^+]_e$. If the interaction that produces the synergism does *not* occur at the level of the VSCC, then the effect of exposure to AVP + K_e^+ + D600 should be to raise the response ratio relative to that found for AVP + K_e^+ (at the same concentrations). The rationale for this is that in this situation, D600 can be viewed as a regulating the magnitude of the response distal to the activation of VSCC. Therefore the limiting factor that causes the reduction in the synergistic response as the level of stimulation is increased (and which previously has been suggested to occur distal to VSCC activation, see above), would be stimulated to a lesser extent, compared to when D600 was not present. The result should be a higher response ratio compared to when D600 is absent. Alternatively, if the interaction causing the synergism *does* occur at the level of the VSCC, then such an effect would not be expected. This is because although the effective "level of stimulation" is reduced by the addition of D600, so too is the component (VSCC) that produces the enhanced response, and therefore an increase in the response ratios would not be expected.

The results show that the addition of D600 to the 10 nM AVP plus 60 mM $[K^+]_e$-induced response did not enhance the response ratio (Fig. 4.5). In fact, in the presence of D600, the response ratio was significantly reduced ($P < 0.05$) at 90 min compared to when

D600 was not present (see below for explanation). Therefore the data supports the hypothesis that the enhancement of the response occurs at the level of the VSCC. The only other possibility that would be consistent with this data is that the interaction occurred prior to the activation of VSCC. However, the activation of VSCC is probably the *first* point of interaction between the responses to AVP and K_e^+, and thus an effect at the level of VSCC activation is more plausible than an effect prior to VSCC activation.

As noted above, the response ratio for the AVP plus K_e^+-induced response, at 90 min, was significantly reduced by the addition of D600. Considering the previous findings, a plausible explanation for this difference is that the addition of raised $[K^+]_e$ increased the effectiveness of D600 at blocking AVP-activated VSCC. If true, this means that the calculations used to generate the response ratios in the presence of D600 (the data in Fig. 4.5) are not accurate because VSCC activated by AVP during the simultaneous response are inhibited to a greater extent than are the AVP-activated VSCC in the individual response. Thus, when the simultaneous (+ D600) response is divided by the sum of the individual responses (in the presence of D600), the value used for the AVP (+ D600) individual response is an overestimate of the magnitude of the contribution by AVP-activated processes during the simultaneous response. This inaccuracy has the potential to invalidate the previous interpretation (that enhancement of the simultaneous response occurs at the level of the VSCC), since the calculation that the interpretation is based on is not accurate. However, the interpretation is not necessarily invalidated, because it is not immediately obvious how much of an effect the putative increased efficacy of D600 has on AVP-activated processes during the simultaneous response. It is possible that the enhanced effect is reflected by the difference (at 90 min) between the simultaneous response and the simultaneous plus D600 response (Fig. 4.5), in which case the conclusion would still hold. However, this cannot be determined from the data as it is presented. It is therefore necessary to attempt to re-calculate the response ratios taking into account the possibility that the level of inhibition by D600 may be greater during the simultaneous response compared to the individual response (for AVP activated channels). From Fig. 3.2, the maximum level of inhibition by D600 on AVP-stimulated ACTH release was 71.6%. If it occurs, the increased effectiveness of D600 should not exceed the maximum level of inhibition (*ie.* 71.6%), instead it should reduce the concentration necessary to obtain this

level of inhibition. Therefore this value will be used in the following calculation as an estimate of the maximal effectiveness of D600 on AVP-activated VSCC during the simultaneous response.

The calculation can only be performed for the 90 min time point, since values for inhibition by D600 are only available for this period. Also, this was the only time point where a significant difference between the response ratios for the simultaneous responses, with or without D600, was observed. Using the adjusted value for the effect of D600 on AVP-activated VSCC during the simultaneous response, the response ratio in the presence of D600 was calculated to be 1.10 ± 0.18 (Fig. 4.5). This value is very similar to the value for the simultaneous response in the absence of D600 and therefore these results are consistent with the hypothesis that the difference between the response ratios originally calculated for AVP + K_e^+ and AVP + K_e^+ + D600 was due to the increased effectiveness of D600 on AVP-activated VSCC when raised $[K^+]_e$ was also present. Thus the results also support the hypothesis that the synergistic response of simultaneous stimulation occurs at the level of the VSCC.

If the interaction that produces the synergistic response does occur at the level of the VSCC then a plausible explanation is that the facilitation is caused by the combination of the extreme depolarisation induced by K_e^+ and chemical modification arising from the AVP signal. An alternative explanation is that the facilitation is caused by the dual electrical effects of AVP and K_e^+. However, this explanation seems unlikely, since any electrical effects of AVP are almost certainly negligible with respect to the effects of raised $[K^+]_e$. Thus it is quite likely that any electrical effects normally induced by AVP (*ie.* when given alone) are "swamped" by the K_e^+-induced depolarisation (when the agents are given together), and it is therefore unlikely that electrical effects of AVP could act with the depolarisation induced by K_e^+ to produce the synergistic response. Therefore, the results suggest that AVP does cause modulation of VSCC activity that is distinct from electrical modulation during the ACTH secretory response.

4.3.2 Down-regulation of PKC activity

Following the results of the simultaneous stimulation experiments, further investigation was undertaken into the hypothesis that the synergistic response observed arose from the combination of chemical modification of the channels by AVP plus the extreme plasma-membrane depolarisation caused by the high $[K^+]_e$. PKC, which is activated by AVP, has been found to modulate L-VSCC activity (see section 4.1), and therefore is a candidate for an agent that might modulate L-VSCC activity during the response to AVP (including during the simultaneous response). If true, this hypothesis would suggest that an intact PKC activity should be necessary for the generation of the synergistic response. The PKC down-regulation experiments were designed to test this hypothesis.

The down-regulation protocol was effective at abolishing PKC activity, as demonstrated by the complete removal of the TPA-induced ACTH response in cells that were pre-exposed to TPA (Fig. 4.6). The responses to 1 and 1000 nM AVP were inhibited by 35.7 and 51.8%, respectively, which, in general, supports previous findings that PKC activity is necessary for the full expression of the AVP-stimulated response (Bilezikjian *et al.* 1987, Carvallo & Aguilera 1989, Oki *et al.* 1990, Liu *et al.* 1990). However, in the first two of these reports, in which rat cells were used, the AVP-induced response was virtually abolished by pre-exposure to phorbol ester. Oki *et al.* (1990), also using rat cells, observed only 33% inhibition of the AVP-induced response. However, the pre-exposure protocol used by Oki *et al.* did not abolish the TPA-induced response, instead this response was inhibited by 79%. Liu *et al.* (1990), using ovine cells and a down-regulation protocol similar to the one used in this report, found that TPA pre-exposure caused about 50% inhibition of the response to 1000 nM AVP. [Note that this is my extrapolation of their data (Fig. 8F) corrected for an increased basal secretion from the pre-exposed cells.] These authors present a figure of the data. However, they do not give values in the text for the level of inhibition of the AVP-induced response following pre-exposure, but state that "the ability of AVP to increase the release of ACTH...was markedly attenuated" (following pre-exposure to TPA). Thus, the observed differences in the level of inhibition of the AVP-induced ACTH response following PKC down-regulation between the reports described

above, and the data presented here, might reflect a species difference where in rat cells the AVP-induced ACTH response is more susceptible to PKC down-regulation. Alternatively, the differences might have been caused by the slightly varying protocols used in the different studies, or by the way the data is handled, given that the pre-exposure protocol often reduces the cellular ACTH content that is available for secretion. However, despite the reported differences, all the reports are in agreement that PKC activation is an important component of the AVP-induced ACTH response.

Oki *et al.* (1990), using their micro-perifusion system, found that the spike phase of secretion was unaffected by the TPA pre-exposure, whereas the plateau phase was inhibited by 57%. This finding is consistent with the hypothesis that PKC and VSCC activity are linked, by demonstrating that both factors act at the same stage of the AVP-induced response.

The data reported here show that complete inhibition of PKC activity (as determined by the abolition of the TPA-induced response following the pre-exposure protocol) did not reduce the AVP-induced response as much as was observed following exposure to VSCC blockers (up to 90% inhibition, see Chapter 3). Whether this difference is real is arguable, considering the findings of the reports detailed above, in which PKC down-regulation inhibited the response to AVP to a greater extent. However, the difference was observed, and bears further consideration. It has been suggested in this study that AVP-induced VSCC activity involves both electrical and chemical components, with PKC (or factors activated proximal to it) providing the chemical signal. The difference in the levels of inhibition induced by VSCC blockers and PKC down-regulation suggests that in the absence of PKC activity, VSCC can still become activated. Two possibilities are raised: 1) that there is a subset of VSCC that respond only to electrical signals whether PKC is activated or not, or 2) that all channels are normally influenced by PKC, but in the absence of PKC activity, they can still respond, although the activity of the channels is reduced. In the case of the latter explanation, PKC activity would act as a facilitator of VSCC activity, enhancing the response to membrane depolarisation, but the presence of activated PKC would not be obligatory for the response to occur. From the data currently available, it is not possible to distinguish between these possibilities.

PKC down-regulation had no effect on CRH-stimulated ACTH release. Similarly,

Bilezikjian *et al.* (1987), Liu *et al.* (1990) and Oki *et al.* (1990) all found that CRH-induced ACTH release was unaffected by PKC down-regulation. However, Carvallo & Aguilera (1989) reported a 30% decrease in the CRH-stimulated response following their down-regulation procedure. Again, the reason for this discrepancy is unclear, however, overall, these reports and the data presented here are in agreement with the hypothesis that CRH acts through the cAMP-dependent protein kinase pathway and does not influence PKC activity.

The responses to 20 and 60 mM $[K^+]_e$ were also unaffected by the TPA pre-exposure, in agreement with Oki *et al.* (1990). This suggests that the K^+_e-induced response does not require PKC activation. Armstrong & Eckert (1987) found that the DHP-sensitive VSCC (assumed to be the L-type channel) must be phosphorylated in order to respond to depolarisation. This interpretation was reached after channels were found to lose their ability to respond to depolarisation when excised in membrane patches. The loss of responsiveness could be prevented or reversed by the addition of ATP to the cytoplasmic face of the patch. This finding raises the question as to how the L-VSCC can respond, and continue to respond over a 90 min test incubation, to K^+_e-induced depolarisation, since this treatment does not activate PKC or, presumably, PKA - the two kinase molecules known to interact with L-type VSCC. That the channels "lost" their activity when excised in the patch suggests that the channels are already phosphorylated, to some extent, in resting cells. This explanation deals with how VSCC are able to respond initially to the K^+_e-induced depolarisation. However, L-VSCC inactivation is thought to be at least partly Ca^{2+}-dependent (Carbone & Swandulla 1991) and involves dephosphorylation of the channel complex (Armstrong & Eckert 1987). Presumably, K^+_e-activated L-VSCC undergo the same process of Ca^{2+}-induced inactivation. Therefore, during a 90 min exposure to raised K^+_e, the channels would need to recover from inactivation (*ie.* be transformed from the inactive state to the resting state) in order to re-respond to the continued K^+_e-induced depolarisation stimulus. It is not clear how this might occur. Armstrong & Eckert (1987) found that L-VSCC, from excised membrane patches, could be prevented from losing their activity, or could recover from "run-down", if the channels were exposed to ATP and the catalytic subunit of cAMP-PK. This treatment was completely successful at maintaining/recovering channel activity. When the channels were exposed to ATP only,

the results were more variable, but maintenance of activity was observed in most of the channels. Since these experiments were conducted on excised patches, the observations suggest the presence of a basal level of kinase activity, associated with, or at least close to, the plasma membrane. This activity could be a low level of either (or both) PKC or PKA, or it could be a different kinase activity. The fact that most, but not all of the channels were prevented from losing their activity, suggests that this kinase activity might not be closely associated with every channel. It is possible that the kinase activity is free to move about the membrane, and thus prevent run-down by phosphorylating only those channels that it was able to temporarily associate with. This basal kinase activity could re-phosphorylate inactivated channels during exposure to high K^+_e, allowing them to re-respond to the depolarisation. In cells that display spontaneous membrane electrical activity, the membrane-associated kinase activity might function to transform channels that open, and then inactivate, in response to the electrical activity back to the resting state.

Following pre-exposure to TPA, the responses to simultaneous stimulation were reduced by between 22.6 and 43.6%. When this data was expressed as response ratios, it was evident that the synergistic effect seen at 20 mM $[K^+]_e$ plus either concentration of AVP, in the control cells, was abolished in the TPA pretreated cells. Following the pre-exposure protocol, only additive or less-than-additive responses were observed. Therefore these results support the hypothesis that PKC-activated processes are necessary for the generation of the synergistic response.

From the data presented in Fig. 4.6, it can be seen that in PKC down-regulated cells, the responses to simultaneous exposure to both concentrations of AVP plus 20 mM $[K^+]_e$, were greater than the individual response to 20 mM $[K^+]_e$. For 1 nM AVP plus 20 mM K^+_e the difference was not significant (9.40% ± 1.28% *vs.* 3.73% ± 1.25%, $P > 0.05$), however the 1000 nM AVP plus 20 mM K^+_e response was almost 5 times greater than the individual response to 20 mM K^+_e (18.47% ± 1.24% *vs.* 3.73% ± 1.25%) and this was a significant difference ($P < 0.05$). Part of the increased level of secretion observed in the simultaneous response can be attributed to AVP-induced release of intracellular Ca^{2+}. However, the results from Chapter 3 show that over a 90 min test incubation the contribution of mobilised Ca^{2+} to the AVP-induced ACTH response is small, and would be less than the difference between the simultaneous and individual K^+_e response discussed here. These

results suggest that part of the increased level of secretion observed in the simultaneous response is due to AVP-activated VSCC. PKC activity is not present in these cells, and therefore these results suggest that the channels can still be activated in the absence of PKC. This interpretation is the same as that raised in previous discussion, when it was noted that VSCC blockers reduced the AVP-induced response to a greater extent than was observed when PKC was down-regulated. At this stage, it is not clear whether, in the absence of PKC activity, AVP activates the channels by purely electrical means or by some PKC-independent, non-electrical factor(s).

In the simultaneous responses to 60 mM $[K^+]_e$ plus AVP in PKC down-regulated cells, the ability of AVP to activate VSCC by PKC-independent means appeared to be lost. Fig. 4.6 show that in PKC down-regulated cells, the three responses (to 60 mM $[K^+]_e$, 1 nM AVP plus 60 mM $[K^+]_e$, and 1000 nM AVP plus 60 mM $[K^+]_e$) were not significantly different from each other ($P > 0.05$). The two simultaneous responses were numerically slightly greater, but this effect can be attributed to AVP-mobilised intracellular Ca^{2+} release. In non-PKC down-regulated cells, the simultaneous responses are both significantly greater than the K_e^+-only response ($P < 0.05$), which suggests that the VSCC population is not maximally activated during the individual response to 60 mM K_e^+. Thus the loss of the AVP-activated VSCC activity, in PKC down-regulated cells, cannot be attributed to the VSCC becoming maximally activated.

In previous discussion, it was suggested that the ability of AVP to activate VSCC during the AVP plus 20 mM $[K^+]_e$ response, in PKC down-regulated cells, could occur by either electrical or PKC-independent, non-electrical means. The results of AVP plus 60 mM $[K^+]_e$ would tend to support the former possibility, since it is reasonable to consider that the more profound depolarisation induced by 60 mM $[K^+]_e$ might "swamp" any membrane electrical events induced by AVP. On the other hand, it seems less likely that the increased $[K^+]_e$ would prevent the effect of AVP to activate VSCC through a PKC-independent, non-electrical factor. This interpretation provides support for the ability of AVP to activate VSCC by purely electrical means, and suggests that PKC, while apparently able to modulate VSCC activity during the response to AVP, is not obligatory for VSCC to be opened.

4.3.3 Acute exposure of corticotrophs to TPA, and TPA plus raised $[K^+]_e$

To further investigate the involvement of PKC activation in the generation of the synergistic response, TPA was used as an acute stimulator of PKC activity. Exposure of cells to TPA during the test incubation caused considerable ACTH release, a result that was consistent with the numerous previous reports demonstrating that PKC activity is linked to ACTH secretion (see Chapter 1). Phorbol esters and synthetic diacylglycerols have been used very widely to directly activate PKC and thus examine the involvement of PKC in various cellular processes. However, the effects of these agents cannot be expected to be totally specific, and therefore, results of studies using these agents, particularly at high concentrations, must be viewed with some caution (Kikkawa & Nishizuka 1986). Furthermore, with regard to phorbol esters, these agents differ from DAG in that they are not readily metabolized, and therefore their effects may be longer lasting than the physiological agent, and this could potentially lead to production of non-physiological phenomena. Thus although phorbol esters represent useful tools for the examination of the actions of PKC, the results of such investigations must be interpreted with care.

Simultaneous stimulation by TPA plus raised $[K^+]_e$ caused significant synergistic responses (Fig. 4.9). Therefore, these results are consistent with the previous finding that PKC activation, possibly affecting VSCC activity, is involved in the generation of the synergistic response.

The synergistic response to TPA plus raised $[K^+]_e$ displayed considerable sensitivity to TPA concentration and relative insensitivity to the $[K^+]_e$. At 0.1 nM TPA plus raised $[K^+]_e$, the response ratios were not much greater than 1.0 (*ie.* additivity, although the value at 40 mM $[K^+]_e$ was significantly above 1.0), but the ratios increased considerably when the TPA concentration was raised to 1 nM. This suggests that although a small synergistic effect may occur at concentrations of TPA (only) that are at the threshold for significant stimulation of ACTH release (see Fig. 4.7), a supra-threshold TPA concentration is required to consistently induce synergistic responses. The possibility has been raised above that the synergism occurs due to PKC-induced modulation of Ca^{2+} channels. If this was the case,

then an explanation of the effects of 0.1 *vs.* 1 nM TPA, during the simultaneous response, could be that the level of PKC activity induced by 0.1 nM TPA is not sufficient to cause the modulation of enough Ca^{2+} channels to produce large synergistic responses.

When the TPA concentration was increased from 1 to 10 nM, the response ratios were reduced to levels similar to those observed at 0.1 nM TPA (plus raised $[K^+]_e$). A reduction in the response ratios with an increasing level of stimulation was also observed for AVP plus raised $[K^+]_e$, and analysis of these results suggested that the reduction was not a result of the secretory capacity of the cells having been reached (see section 4.3.1). Instead, a limiting factor, proximal to the level of actual exocytosis, was suggested to operate to restrict the magnitude of the simultaneous response to AVP plus raised $[K^+]_e$, at high levels of stimulation, and thus cause the reduction in the response ratios. For TPA plus K_e^+, it is possible that a similar (or the same) factor is present, and acts in the same manner as for AVP plus K_e^+, but the data do not specifically support this hypothesis. This can be seen if the response ratios for the responses to 1 nM TPA plus K_e^+ are applied to the sum of the individual responses to 10 nM TPA and K_e^+, when these latter data are expressed as the percentage ACTH released relative to the total hormone content of the cells (*ie.* in the units of the data presented in Fig. 4.6). Therefore this procedure tests the hypothesis of whether, *purely on the basis of the secretory capacity of the cells*, it is possible to maintain the response ratios observed for 1 nM TPA (plus K_e^+) as the TPA concentration is increased to 10 nM.

The responses to 10 nM TPA, 20 mM K_e^+ and 40 mM K_e^+ were not actually measured in the experiments depicted in Fig. 4.6 (the only experiments in which the cellular hormone content was assayed), but the values of these, in terms of the percentage released relative to total cellular hormone content, can be estimated by comparing the magnitudes of these responses to the 60 mM K_e^+-induced response (which was measured in both the experiments described in Fig. 4.6 and in Fig. 4.8). From Fig. 4.6, 60 mM K_e^+ induced the release of 24.4% ± 0.99% of the total hormone content. For example, to estimate the value for 10 nM TPA, the ratio of the 10 nM TPA to the 60 mM K_e^+ responses (from the data presented in Fig. 4.8) are multiplied by the percentage released by 60 mM K_e^+ (*ie.* 24.4%). Thus the estimated percentage released by 10 nM TPA is 601.9/211.1 x 24.4 = 69.5%. Applying the response ratios from the simultaneous response to 1 nM TPA

plus K_e^+ to the sum of the individual responses to 10 nM TPA plus K_e^+, gave estimated values of 228.1%, 230.8% and 205.4% for 10 nM TPA plus 20 mM, 40 mM and 60 mM K_e^+, respectively. The theoretical maximum hormone content (and thus maximum secretory response) is 100%. Therefore, given the results of the calculations, it would not be possible for the response ratios observed at 1 nM TPA plus K_e^+ to be maintained at the same level for 10 nM TPA plus K_e^+, purely on the basis of the secretory capacity of the cells. Thus it is not possible to determine, on the currently available data, whether the limiting factor that is proposed to limit the simultaneous responses to AVP plus K_e^+, also acts on the TPA plus K_e^+ response.

Mollard *et al.* (1988) and Corcuff *et al.* (1993) provide some direct evidence that chemical modulation of VSCC occurs during the response to AVP. These effects were positive, *ie.* Ca^{2+} influx was enhanced. Support for the involvement of PKC in the regulation of Ca^{2+} influx comes from Reisine & Guild (1987) and Reisine (1989), who showed that TPA can induce a rise in $[Ca^{2+}]_i$ in AtT-20 cells. However, Lewis & Weight (1988) found that the PKC activator 1-oleoyl-2-acetylglycerol (OAG) reduced the voltage-dependent Ca^{2+} current in AtT-20 cells, and Luini *et al.* (1985) found that TPA reduced the $[Ca^{2+}]_i$ in AtT-20 cells. These results suggest that PKC-activating agents can decrease Ca^{2+} influx, *ie.* the opposite effect to that which has previously been suggested. However, a possible explanation for these disparate findings comes from Stojilkovic *et al.* (1991), who found that in quiescent gonadotrophs, TPA *stimulates* Ca^{2+} influx, whereas when Ca^{2+} influx was already occurring (in cells that were spontaneously active or that had been stimulated 2 min prior by raised $[K^+]_e$), TPA exerted an immediate and predominantly *inhibitory* action. This suggests that the current state of the Ca^{2+} channels (*ie.* closed or open) influences the effects that are exerted by PKC. Thus, both positive and negative effects of PKC on Ca^{2+} channels might occur during the same response. Initially, PKC may act to increase the activity of Ca^{2+} channels, and thus promote Ca^{2+} influx, but as the response develops the actions of PKC may be altered to become inhibitory on channel activity. This scheme would provide the system with feedback regulation.

A similar mechanism may occur in corticotroph cells. In the report of Lewis & Weight (1988), OAG was introduced while the cells were undergoing a stimulation paradigm invoked by a series of depolarising steps from the holding potential. The

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maximum observed current was reversibly reduced in the presence of OAG. However, the recording solution used contained a high concentration of Ca^{2+} (10 mM), and therefore, during a paradigm of repeated voltage steps, it would expected that considerable Ca^{2+} influx through the VSCC would occur, presumably resulting in raised cytosolic [Ca^{2+}]. These conditions might promote OAG-activated PKC to act only in its secondary, inhibitory manner, thus reducing Ca^{2+} influx. Support for this hypothesis comes from other work reported in Lewis & Weight (1988). The authors found that phorbol esters also reduced the Ca^{2+} current, however, when the cytosolic [Ca^{2+}] was buffered by the addition of EGTA, this effect did not occur. This suggests that the intracellular [Ca^{2+}] may influence the effects exerted by PKC.

The apparent dual activity of PKC, to both promote and inhibit Ca^{2+} influx, in corticotrophs (AtT-20 cells) was demonstrated by Reisine & Guild (1987). Both TPA (referred to as PMA in this report) and raised $[K^+]_e$ induced a rise in cytosolic [Ca^{2+}] that was sensitive to Ca^{2+} channel blockers. However, prior exposure to TPA (which raised cytosolic Ca^{2+}) reduced the Ca^{2+} response of a subsequent exposure to raised $[K^+]_e$. The magnitude of the reduction of the response to K_e^+ was dependent on both the concentration of TPA used and the interval separating the exposure to TPA and raised $[K^+]_e$. Inhibition occurred after 1 min of exposure to TPA, but not when the agents were given simultaneously. Thus this finding is consistent with the results of this study, in which simultaneous stimulation with TPA and raised $[K^+]_e$ caused ACTH responses greater than either of the individual responses (see Fig. 4.8). Reisine & Guild (1987) found that when the order of the treatments was reversed, TPA reduced cytosolic Ca^{2+} to basal levels, whereas exposure to raised $[K^+]_e$ alone maintained the Ca^{2+} response above basal for several minutes. These results provide support for the hypothesis that PKC can both activate and inhibit Ca^{2+} influx in corticotroph cells. Furthermore, pre-exposure to TPA (5 minutes prior) reduced the rise in cytosolic Ca^{2+} induced by both CRH and forskolin (a direct activator of adenylate cyclase). This suggests that CRH-mediated, and PKC-mediated Ca^{2+} influx occurs, at least in part, via the same channels. It is not clear whether the secondary, negative effects of PKC on Ca^{2+} influx are translated through to negative effects on ACTH secretion, although this seems likely.

4.3.4 TPA plus VSCC blockers

The experiments discussed so far in this chapter have suggested, albeit indirectly, that the PKC activity, induced by AVP can affect VSCC activity. To investigate directly whether PKC can activate L-VSCC, experiments were performed in which the sensitivity of the TPA-induced response to blockers of VSCC was examined. This was done using the organic blocker D600 and the inorganic blocker Co^{2+}.

D600 was found to inhibit secretion evoked by 1 nM TPA to quite a high maximum value of 71.4% ± 12.8%, at 300 μM. However, this was the only concentration of D600 that caused a significant inhibition of TPA-induced release (see Fig. 4.10). In contrast, the AVP-induced response was significantly reduced at 1 μM D600 (see Fig. 3.1). D600 was even less effective at inhibiting secretion evoked by 150 nM TPA. Again, only at 300 μM D600 was there a significant level of inhibition, and this was only a 9.1% ± 1.3% reduction. This difference in the effectiveness of D600 for different concentrations of the secretagogue also contrasts with the situation found for AVP-induced release, where a particular concentration of the blocker caused the same level of inhibition, irrespective of the concentration of AVP.

These data suggest that Ca^{2+} influx does occur during the response to TPA, in agreement with previous reports (Reisine & Guild 1987, Sobel 1988, Won *et al.* 1990). However, this is a qualified interpretation, since the evidence in this report is not, as yet, convincing. The fact that only high concentrations of D600 were able to significantly reduce the TPA-stimulated response raises the possibility that these effects of D600 are non-specific, and do not reflect inhibition of L-VSCC. Also, the difference in the level of inhibition of the two TPA concentrations is not consistent with the effect of D600 on AVP-stimulated release.

To further characterise the role of Ca^{2+} influx in the TPA-induced response, the inorganic ion Co^{2+} was used, and this was also found to inhibit TPA-induced secretion. The pattern of inhibition by Co^{2+} shared some similarities with that of D600: for a particular concentration of Co^{2+} the level of inhibition was different for 1, 10 and 150 nM TPA-induced release (Fig. 4.11). However, for 1 and 150 nM TPA-stimulated release, Co^{2+} was

a more effective blocker than D600, completely inhibiting the 1 nM response (95.2% ± 4.3%, $P > 0.05$ *vs.* 100% inhibition), and causing 44.6% ± 0.5% inhibition of the 150 nM response. Also, Co^{2+}, unlike D600, was not much less effective, if at all, on TPA-stimulated release compared to its effects on AVP-stimulated release. Co^{2+} caused significant inhibition of TPA-stimulated release at 2 mM (0.02 mM for inhibition of the 150 nM response), and of AVP-stimulated release at 0.5 mM. Since Co^{2+} was not used at 0.5 mM for inhibition of TPA-induced release, it is possible that this concentration could also cause significant inhibition of the TPA-induced response. Thus the potency of Co^{2+} appears to be similar for AVP- and TPA-stimulated ACTH release.

The effects of Co^{2+} provide further support for the hypothesis that the TPA-induced response does involve Ca^{2+} influx. However, the finding that the concentration of TPA influences the level of inhibition is consistent between both blockers. One possible explanation for this effect, is that the cellular mechanisms that are activated in response to a high concentration of TPA are not the same as those activated at low TPA concentrations. In other words, the ACTH response to an increasing concentration of TPA does not represent a simple "scaling up" of the activity of the initial response elements, but instead (or as well as), distinct intracellular mechanisms, that also cause ACTH secretion, may be invoked at higher TPA concentrations.

To investigate this possibility, the data for TPA plus the VSCC blockers were expressed as the reduction in ACTH secretion caused by the blockers. For D600-induced inhibition (Fig. 4.12), the absolute magnitude of the reduction in ACTH release was virtually identical for both concentrations of TPA (although there was a vast difference in the level of hormone secretion induced by these two concentrations, see Fig. 4.7). This suggests that there is a component of the TPA-induced response that involves the activation of L-VSCC, and therefore is sensitive to D600. This component is activated at the threshold of the TPA-induced response and is responsible for more than 70%, at least, of the response to 1 nM TPA. However, the data suggest that the response to 150 nM TPA has no greater dependence on Ca^{2+} influx via D600-sensitive channels, and therefore the magnitude of the reduction in ACTH release was the same (Fig. 4.12) but percentage inhibition drops, to less than 10% (Fig. 4.10).

When the TPA plus Co^{2+} data was expressed as the reduction in ACTH release (Fig.

4.13) a similar, but not identical pattern is evident. In this case, the magnitude of the reduction in ACTH release was the same for inhibition of the 10 nM and 150 nM TPA-induced responses, but these were both higher than the reduction of the 1 nM response. Therefore, in this case, the dependence of the TPA-induced response on Ca^{2+} influx continues above the response induced by 1 nM TPA, but has reached a maximum level at 10 nM TPA.

Therefore, overall, the data from both blockers showed some similarities and some differences, in their effects on TPA-stimulated ACTH release. The results from both blockers suggest that at low concentrations of TPA, there is a considerable, possibly complete, dependence of the response on Ca^{2+} influx. As the TPA concentration was increased, the relative dependence on Ca^{2+} influx decreased, possibly due to the recruitment of intracellular mechanisms that do not require Ca^{2+} influx. Since the intracellular concentration of PKC is closely regulated by rapid metabolism of DAG and autoregulation of PKC itself (Kikkawa & Nishizuka 1986), it is likely that the effects observed at low, rather than high, concentrations of TPA reflect the more physiologically relevant actions of PKC in corticotrophs. Cellular effects observed at high TPA concentrations are more likely to represent non-specific, or at least, non-physiological actions of TPA, particularly if the threshold(s) for activation of these effects are higher than for other TPA-induced processes.

Although the effects of D600 and Co^{2+} on TPA-stimulated ACTH release showed some similarities, there were differences in the relative effectiveness of the two agents. Firstly, Co^{2+} inhibited TPA-stimulated ACTH release to a higher level than did D600. Secondly, D600 was a very much less effective inhibitor of the TPA- versus the AVP-stimulated response, whereas there was no evidence to suggest that Co^{2+} was any less effective at inhibiting the TPA-induced response. Taken together, these data suggest that the inhibitory efficacy of D600 may have been reduced by the actions of TPA. This suggestion is similar to that raised in Chapter 3, and again in this chapter (see above), to explain why D600 was, in molar terms, a less potent inhibitor of the AVP- versus the K_e^+-induced response. The suggestion made in Chapter 3 was that chemical modification of the VSCC (possibly by PKC-activated processes) during the AVP-induced response affected the ability of D600 to block the channels. The absence of PKC activation during the K_e^+-

induced response meant that these modifications do not occur, and so the efficacy of D600 is not compromised. Since the inorganic blockers (*eg.* Co^{2+}) act more as a "plug" to block the VSCC, they may not be susceptible to PKC-induced modification of the VSCC (see Chapter 3 for full discussion). For the TPA-induced response, a similar effect may occur. In fact, the effect may occur to an even greater extent, since the PKC-activating agent (TPA) is not under the control of the normal PKC-regulatory processes.

4.3.5 The effects of Ca^{2+}-free medium on stimulation by AVP and TPA

The investigation of the effects of VSCC blockers on TPA-stimulated ACTH release gave results that suggested, overall, that Ca^{2+} influx does occur during this response, but the results differed in some respects to those obtained for the effects of the blockers on the AVP-stimulated response. To further characterise the role of Ca^{2+} influx during the TPA-stimulated response, and thus further investigate whether PKC can act to promote Ca^{2+} influx, the effects of reducing and removing Ca_e^{2+} on secretion evoked by TPA, were examined. However, it was first necessary to characterise the effects of reduction/removal of Ca_e^{2+} on the response to AVP, to act as a comparison and control for potentially differential effects of VSCC blockers and Ca_e^{2+} reduction/removal on corticotroph function.

Both Ca^{2+}-deficient and Ca^{2+}-free media inhibited 1 and 100 nM AVP-stimulated release. Ca^{2+}-free medium caused levels of inhibition (90.5% ± 6.8% inhibition of 100 nM AVP) that were very similar to the maximum levels of inhibition induced by Cd^{2+} (87.6%). Therefore these findings further strengthen the hypothesis that the AVP-stimulated response is dependent on Ca^{2+} influx for almost the entire response over a 90 min incubation period. However, although the levels of inhibition induced by Cd^{2+} and Ca-free medium were not statistically different from each other ($P > 0.05$), the values for the latter treatment were not significantly different from 100% inhibition, unlike the value for Cd^{2+}-induced inhibition (see Chapter 3). Thus complete dependence of the AVP-induced response on Ca^{2+} influx cannot be ruled out on the basis of the Ca^{2+}-free data. Therefore

it is possible that the component of the AVP-induced response that cannot be blocked by the VSCC blockers might reflect Ca^{2+} influx via channels that are not sensitive to any of the blockers used in this study. However, such a mechanism is not consistent with the considerable amount of (indirect) evidence for the involvement of IP_3-induced release of stored Ca^{2+} in the AVP-induced response (see Chapter 1). The VSCC-insensitive component of a 90 min exposure to AVP is certainly small (possibly as little as 12.4%), and if this component of the AVP-stimulated response *is* due to Ca^{2+} mobilisation, it is not unreasonable that the Ca^{2+}-free data would be unable to resolve this small effect.

Ca^{2+}-deficient medium caused lower levels of inhibition of AVP-stimulated ACTH release (50% - 65%, Fig. 4.14) than was found for the Ca^{2+}-free medium. This medium was prepared free of Ca^{2+}, but the addition of the NCS would be expected to add Ca^{2+} to give a concentration in the medium of approximately 100 μM. The results of Chapter 3 suggest that under the conditions used in this study, the level of dependence of the AVP-induced response on influx of Ca^{2+} via VSCC is in the range 71.6% to 87.6% (the maximum levels of inhibition by D600 and Cd^{2+}, respectively). Since Ca^{2+}-deficient medium inhibited AVP-induced release to a lesser extent than both of these estimates, it appears that the low level of Ca^{2+} in the Ca^{2+}-deficient medium (which was removed by the addition of EGTA in the Ca^{2+}-free medium) was sufficient to allow some Ca^{2+} influx. Stojilkovic *et al.* (1988a) found that the ED_{50} for Ca^{2+}-dependence of GnRH- or TPA-stimulated LH release was approximately 140 μM, and therefore the AVP-stimulated response appears to have a sensitivity to the removal of Ca_e^{2+} that is similar within an order of magnitude.

TPA-induced ACTH release was also sensitive to Ca^{2+}-deficient and Ca^{2+}-free media. The responses to both 1 nM and 10 nM TPA were completely abolished by the Ca^{2+}-free medium. The 1 nM TPA-stimulated release was also abolished by the Ca^{2+}-deficient medium, and therefore these results are consistent with the effects of Co^{2+}, and further supports the complete dependence of the 1 nM TPA response on Ca^{2+} influx. However, the finding that Ca^{2+}-free medium caused a level of inhibition of 10 nM TPA-stimulated release that was not different from 100% inhibition (Fig. 4.15), is not consistent with the effects of Co^{2+} on 10 nM TPA-stimulated release. The differential effects of Ca^{2+}-free medium and Co^{2+}, may either be due to a dependence of the 10 nM TPA-stimulated response on Ca^{2+} influx via channels that are not sensitive to Co^{2+}, or alternatively, the Ca^{2+}-

free medium may have exerted some intracellular effects that reduced stimulated ACTH release.

Overall, the data from the channel blockers, and Ca^{2+}-deficient/free media, support a role for Ca^{2+} influx during the TPA-induced ACTH response. The data are consistent with VSCC being at least partly involved in carrying this inward ion flux (see Chapter 6 for a fuller discussion).

Previous reports examining the sensitivity of TPA- or synthetic DAG-stimulated ACTH release have all demonstrated sensitivity of these responses to either VSCC blockers, or Ca^{2+}-free medium (Reisine & Guild 1987, Sobel 1988, Won *et al.* 1990). Reisine & Guild (1987) found that in AtT-20 cells, the VSCC blockers nifedipine (100 nM) and verapamil (1 μM) both completely abolished the response to 1 μM TPA. This result differs from the results presented here with respect to the level of inhibition that can be induced by VSCC blockers. This difference may reflect a species difference (murine *vs.* ovine) or it could be due to the fact that tumour cells were used by Reisine & Guild (1987). Sobel (1988), using rat cells, states in the text of this report that nifedipine (10 μM) caused 35% inhibition of 3 nM TPA-induced ACTH release. However, examination of the figure presenting the data (Fig. 3) suggests that when basal secretion is subtracted from the responses (*ie.* to make the data analogous to the results presented here) the value for inhibition is approximately 60%. Won *et al.* (1990) examined the effects of Ca_e^{2+} removal on secretion evoked by the synthetic DAG, dioctanoylglycerol (DOG), in rat cells, and found that the response to 1 mM DOG was inhibited 73% by Ca^{2+}-free medium. Therefore, the data presented here are generally consistent with previously reported data. Certainly, all the reports demonstrated sensitivity of the ACTH responses induced by PKC-activating agents to the prevention of Ca^{2+} influx.

The results of this study suggest that the TPA-induced response requires Ca^{2+} influx, possibly, in part, through VSCC. This interpretation has come after the use of both VSCC blockers and Ca^{2+}-deficient/free media. Furthermore, the effect was found to be particularly evident at low concentrations of TPA, where the most physiologically relevant effects would be expected to occur. Thus the data provides evidence supporting the hypothesis that PKC can affect VSCC activity in ovine corticotrophs, and thus it is possible that a similar effect occurs during the AVP-activated ACTH response.

Chapter 5

Modulation of L-Voltage Sensitive Calcium Channels: Voltage Regulation

5.1 Introduction

It has been quite clearly demonstrated, in this report and in previously published data (Abou-Samra *et al.* 1987a, Mollard *et al.* 1987, 1988, Reisine & Guild 1987, Won *et al.* 1990a, Corcuff *et al.* 1993), that the full expression of the ACTH response to AVP requires the influx of Ca^{2+}, via plasma-membrane ion channels. However, the processes that lead to Ca^{2+} channel activation have not been fully determined. Evidence from previously reported investigations (Corcuff *et al.* 1993), plus data presented here, suggests the possibility that dual modulation of VSCC by electrical (via changes in the membrane potential, E_M) and chemical (via PKC) factors might occur. Chemical modulation of VSCC was investigated in Chapter 4, and this chapter presents the results of experiments aimed at investigating the potential role of electrical modulation of VSCC in AVP-stimulated ACTH release.

Pituitary cells have been known to be electrically active since 1975 (Kidokoro 1975). Several cell types, including the GH3/4 tumour cell lines and normal lactotrophs

have been found to exhibit spontaneous action potential generation (see Ozawa & Sand 1986, for review). However, it has become clear that not all normal pituitary cell-types exhibit spontaneous action potentials, at least not all of the time (Mason & Waring 1985, 1986), and Mason *et al.* (1988) suggest that in some pituitary cells "hormone secretion (can) occur independently of significant membrane depolarization." Therefore, just because a cell-type has been shown to possess VSCC does not necessarily mean that the channels are (always, if at all) activated by voltage changes of the plasma-membrane, during the *normal* signal transduction processes of the cell.

Stojilkovic *et al.* (1988c) examined the effects of various treatments, designed to manipulate VSCC activity, on hormone secretion from various rat pituitary cell types. This report is particularly useful because it examines the responses of various pituitary cell types that have been prepared and studied under the same conditions, and therefore any effects of the preparation procedure for the cells are likely to be constant for the various cell types. Thus differences in the responses of the cell types are more likely to reflect real differences in cellular function, compared to the differences observed when cell types are prepared and studied under differing conditions. Furthermore, since it is the cellular hormone response that is the measured parameter, the effects of manipulating cellular activity are described in terms of their actual effects on hormone release. The results of the report demonstrate that lactotrophs and somatotrophs (which secrete PRL and GH, respectively) displayed the highest levels of spontaneous membrane electrical activity. Raising the $[Ca^{2+}]_e$ caused an increase in basal secretion of both PRL and GH, whereas basal FSH, LH, ACTH and TSH secretion was unaffected. Furthermore, VSCC blockers decreased basal secretion of PRL and GH but not the other hormones. However secretion from all of the cell types was evoked by exposure to raised $[K^+]_e$ and the DHP Ca^{2+} channel agonist, BK 8644. This latter result suggests that although lactotrophs and somatotrophs displayed the greatest level of spontaneous activity, spontaneously active Ca^{2+} channels are present in gonadotrophs, corticotrophs, and thyrotrophs, since BK 8644 increases the open time of *already activated* channels (Kokubin & Reuter 1984). Stojilkovic *et al.* (1988c) suggest that differences exist in the number of spontaneously active Ca^{2+} channels in the various cell types. In lactotrophs and somatotrophs, the level of spontaneous activity was apparently high enough to cause the generation of action potentials, leading to the activation of VSCC, and thus

Ca^{2+} influx, to a level that caused hormone secretion *dependent on this Ca^{2+} influx.* Blockers of VSCC activity reduced basal secretion in these cells. In the other three cell types, the level of spontaneous membrane activity caused Ca^{2+} influx that was not sufficient to elicit hormone secretion, in the absence of secretagogues. Therefore, in these cells, basal release was not sensitive to blockers of VSCC. A lack of sensitivity of basal ACTH release to blockers of VSCC is consistent with results presented in Chapter 3 (VSCC blockers) and Chapter 4 (Ca^{2+}-deficient medium) of this study. Thus these results suggest that spontaneous electrical activity in corticotrophs is low, at least with respect to the extent to which spontaneous activity causes basal ACTH release.

Electrophysiological studies, however, have demonstrated that ACTH-secreting cells do display spontaneous electrical activity, including the generation of action potentials. Suprenant (1982) found that most AtT-20 cells (82%) generated spontaneous action potentials, and action potentials could be elicited from all cells following injection of depolarising current. Ca^{2+} was found to be the major inward-carrying current source. In hACTH cells, spontaneous Ca^{2+}-action potentials were observed, and the characteristics of these were modulated by factors that caused ACTH release - AVP, CRH and raised $[K^+]_e$ (Mollard *et al.* 1987, 1988). Guerineau *et al.* (1991) showed that both spontaneously generated, and CRH-evoked action potentials were closely correlated with Ca_i^{2+} transients in hACTH cells. Spontaneously generated, and CRH-evoked Ca_i^{2+} transients, which were sensitive to VSCC blockers, were also observed in *normal* rat corticotrophs, suggesting that these cells also display spontaneous electrical activity of the plasma-membrane (Guerineau *et al.* 1991). This group have more recently demonstrated that AVP induces an increase in action potential generation and Ca_i^{2+} transients (which were correlated) in normal rat corticotrophs (Corcuff *et al.* 1993). Thus these results suggest that the frequency of action potential generation is modulated by both AVP and CRH, and that this electrical activity leads to the influx of Ca^{2+}, and therefore, most probably also leads to ACTH release.

If an electrical stimulus to VSCC, via a change in the plasma-membrane electric potential, is involved in the AVP-induced ACTH response, this raises the question of how the chemical signal of AVP is transformed into this electrical signal. In neuronal cells, the initial signal is electrical in nature (the action potential), but this is transformed into a chemical signal at the pre-synaptic terminal (release of neurotransmitter). At the post-

synaptic terminal, the chemical signal is transformed back into an electrical signal due to the neurotransmitter receptors being receptor-operated Na^+ channels. Interaction of the neurotransmitter and its receptor causes the activation of the Na^+ channels, and concomitant influx of Na^+, which depolarises the neuronal membrane sufficiently to activate voltage-sensitive Na^+ channels and thus re-generate the action potential.

In corticotrophs, there is as yet no evidence for AVP receptors being directly linked to Na^+- or any other ion channels. This may be a result of a lack of probes (*ie.* pharmacological agents) that target these channel types, and therefore it is possible that a mechanism analogous to the one described above for nerve cells may occur in corticotrophs. However, in the absence of any evidence for this type of mechanism, an alternative scheme for the generation of the electrical signal (change in the E_M), based on what evidence is available, must be considered. The state of the E_M at any time is determined by the distribution of ions on either side of the membrane, and the relative conductances of the membrane to each ion. To change the E_M, it is necessary to change the conductance of the membrane for one or more ions, and thus alter the distribution of the ions across the membrane.

Activation of ion channels by direct coupling of activated G-proteins to the channels (*ie.* in the absence of the generation of a second messenger molecule), is a mechanism that has been described in other cell-types, but again, there is no reported evidence of this phenomenon occurring in corticotrophs. One possible mechanism for generating a depolarisation of the plasma-membrane from a chemical stimulus (*ie.* an extracellular first messenger) is to close K^+ channels that are active at rest (and which therefore contribute to the resting E_M). Such a scheme for obtaining a depolarisation has been hypothesised to occur in other cell types. González *et al.* (1992) presented a model of chemoreception in carotid bodies. The carotid bodies sense blood Po_2, Pco_2 and pH, and transduce this information into a neurosecretory response that is dependent upon external Ca^{2+} influx via L-VSCC. The chemoreceptor cells have an O_2-sensitive K^+ current that is reversibly inhibited at low Po_2. The depolarisation induced by K^+ channel closure results in the activation of L-VSCC. Voltage-sensitive Na^+ channels are also activated, and the resulting depolarisation is thought to enhance the activation of the Ca^{2+} channels. According to this model, low Po_2 also activates adenylate cyclase and thus the cAMP second messenger

cascade, which also modulates K^+ channel activity. The model does not define the precise mechanism that is responsible for K^+ channel closure.

Associative learning of the B cell photoreceptors of the gastropod *Hermissenda* has been found to be associated with changes in membrane currents of the B cells (Farley & Auerbach 1986). Training was found to induce decreases in two (outward) K^+ currents and an increase in an (inward) Ca^{2+} current. The net effect of these conductance changes was to enhance the B cell's depolarizing response to light, and cause a decrease in the resting membrane conductance. These changes could be reproduced by both the addition of serotonin and the application of phorbol ester or direct injection of PKC. These results suggested that PKC caused an increase in the excitability of these cells through changes in K^+ and Ca^{2+} conductance. Such a mechanism is analogous to that reported for the carotid body chemoreceptor cells reported by González *et al.* (1992).

Some evidence exists to suggest that secretagogue-induced inhibition of a K^+ current, which is active at rest, may lead to depolarisation of the plasma-membrane, and activation of VSCC in corticotroph cells. Firstly, in pituitary tissue, as well as many other cell types, K^+ channel activity has been shown to be sensitive to the effects of cAMP-dependent protein kinase and PKC (Reeves *et al.* 1986, Sikdar *et al.* 1989, Chung *et al.* 1991). Both positive and negative actions of the kinases have been observed.

Secondly, direct evidence that PKC may cause Ca^{2+} influx via the closing of K^+ channels in corticotrophs, has come from Reisine & Guild (1987) and Reisine (1989). In these reports, the effect of various treatments on cytosolic Ca^{2+} levels in the AtT-20 tumour cell-line were assessed. TPA, CRH and raised $[K^+]_e$ were all effective at raising $[Ca^{2+}]_i$. Reisine & Guild proposed that TPA might act by depolarising the plasma-membrane, leading to an increased activity of voltage-gated Ca^{2+} channels, and that this depolarisation could be achieved by blocking potassium channels. They showed that addition of the K^+ channel blocker tetraethylammonium (TEA, 10 mM) caused a small, rapid, transient increase in $[Ca^{2+}]_i$. This suggests that blocking K^+ channels that are active at rest in these cells causes a depolarisation of the plasma-membrane that leads to the activation of VSCC, and the influx of Ca^{2+} into the cells. Following exposure to TEA, TPA was no longer able to raise $[Ca^{2+}]_i$, whereas CRH and raised $[K^+]_e$ still produced the same maximal rise in $[Ca^{2+}]_i$. These results suggest that TPA causes Ca^{2+} influx via a membrane depolarisation

triggered by inhibition of K^+ channels that are active at rest, since prior inhibition of these channels prevented the response to TPA. These findings were confirmed and extended in a later report (Reisine 1989), which demonstrated that other K^+ channel blockers, Cs^+ and 4-aminopyridine (4-AP), caused a rise in $[Ca^{2+}]_i$ and both also blocked TPA-induced rises in $[Ca^{2+}]_i$.

Mollard *et al.* (1987) investigated the effects of TEA on the resting membrane potential of ACTH secreting human adenoma cells (hACTH), grown in culture. These cells fired spontaneous action potentials, a characteristic that has not been demonstrated in all normal corticotroph cells. Mollard *et al.* found that TEA (10 mM) did not alter the resting E_M. However, TEA did produce an increase in action potential amplitude and duration, and decreased the level of the afterhyperpolarisation. Since the current underlying the action potentials in hACTH is generated by Ca^{2+} entry (Mollard *et al.* 1987) these TEA-induced changes would be expected to cause an increase in Ca^{2+} influx. These results suggest that the effects of TEA arise due to TEA-induced inhibition of the large conductance Ca^{2+}-activated K^+ channels, which are normally activated following action potential firing to repolarise the membrane and control Ca^{2+} influx. This might explain the lack of an effect of TEA on the resting E_M, since any effects of TEA on other K^+ channels may be negligible if large conductance K^+ channels were being activated regularly (*ie.* due to regular firing of spontaneous action potentials). However, Mollard *et al.* (1987) did report that CRH caused a slight depolarisation of the resting E_M in hACTH cells, and they attributed this to a reduction of a K^+ conductance that is normally active at resting potentials. Furthermore, Corcuff *et al.* (1993) found that exposure of normal rat corticotrophs to AVP caused a small (~ 5 mV) depolarisation of the membrane potential and low level fluctuations of the membrane potential, as well as an increase in action potential activity. This increased membrane activity was correlated with Ca^{2+} influx in these cells. Thus, electrophysiological data, combined in some cases with Ca^{2+} imaging data, is consistent with the possibility that inhibition of a K^+ current may depolarise the plasma-membrane and lead to activation of voltage-sensitive channels. However, the electrophysiological data demonstrates that in ACTH-secreting tumour cells, and in some normal corticotrophs, spontaneous action potentials are observed, and therefore, in these cells, the membrane potential often exceeds the activation potential for VSCC anyway. How these spontaneous

action potentials are generated is not understood.

The spontaneous action potentials that occur in some ACTH-secreting cells, mean that voltage-sensitive channels are occasionally active in the absence of secretagogues. Therefore, the addition of, AVP or CRH would only need to increase the frequency of action potentials (as was shown to occur by Corcuff *et al.* 1993), to increase the rate of Ca^{2+} influx. However, not all corticotrophs have been shown to generate spontaneous action potentials, and therefore, in these cells at least, a mechanism is required that can generate an electrical stimulus following exposure to a secretagogue. Therefore it is necessary to investigate mechanisms whereby chemical messages might influence the electrical excitability of the plasma-membrane. Thus, the involvement of K^+ and Na^+ channels in the AVP-induced ACTH response in ovine corticotrophs was investigated using agents and protocols which would be expected to alter the activity of either channel type.

5.2 Results

5.2.1 Effect of TEA on ACTH release

The effect of TEA (10 and 50 mM) on ACTH release was assessed and was found to cause a small, but consistent increase in ACTH release (Fig. 5.1). At 10 and 50 mM TEA, ACTH release was $114.4 \pm 2.8\%$ and $180.4 \pm 9.3\%$ ($P < 0.01$ and 0.001, respectively) compared with control (100%).

If the increase in ACTH release following exposure to TEA is the result of increased Ca^{2+} influx, then this effect should be blocked in the presence of VSCC blockers. Therefore the sensitivity of the TEA-induced response to the VSCC blockers, D600 and Co^{2+}, was assessed. Both agents were found to inhibit the TEA-induced response (Fig. 5.1). At 300 μM and 2 mM, respectively, D600 and Co^{2+} completely inhibited the response to 50 mM TEA ($P > 0.05$, for both, vs control). Exposing cells to Ca^{2+}-deficient medium also abolished the normal response to 50 mM TEA (Fig. 5.1, $P > 0.05$ vs control). Thus TEA-

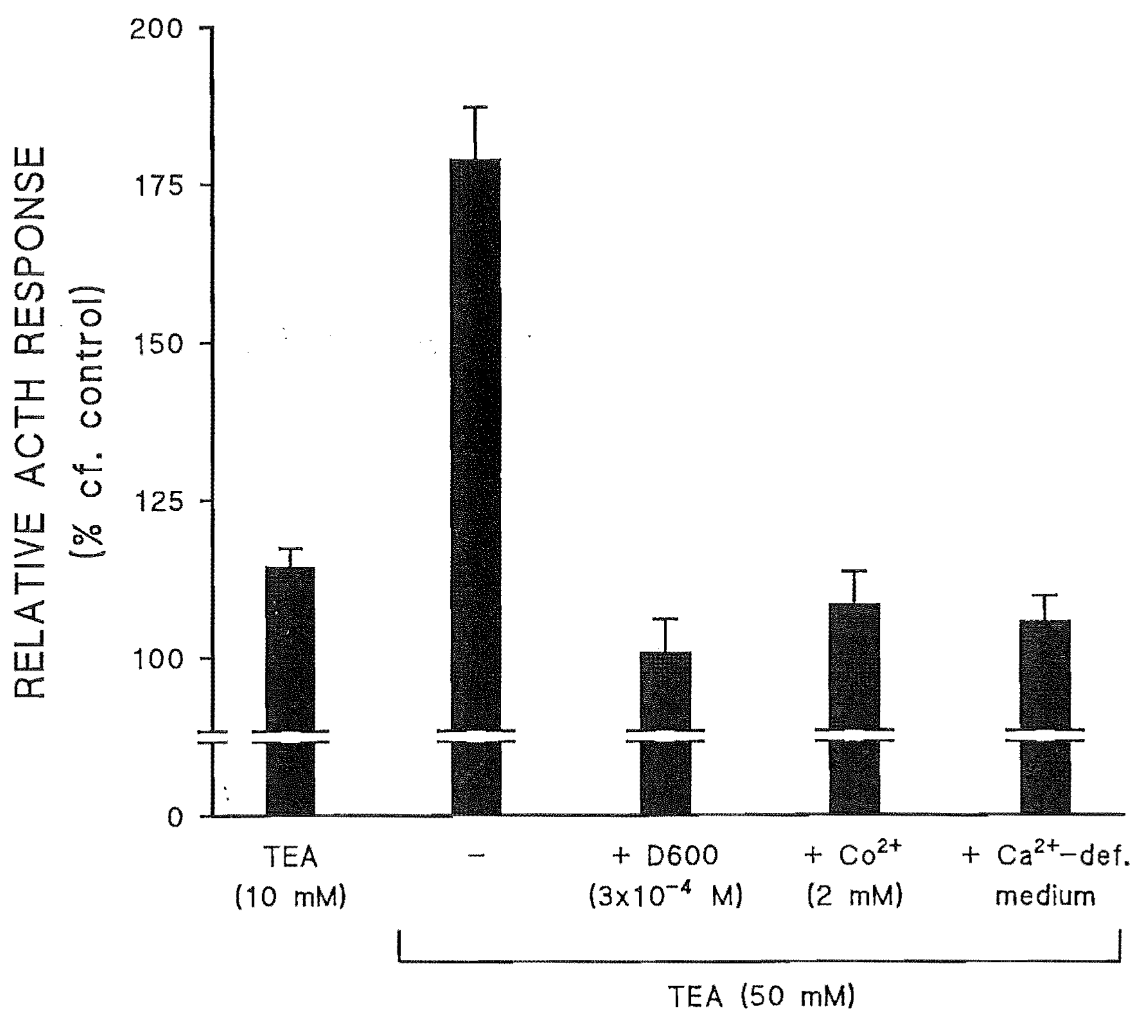

Fig. 5.1. ACTH responses to TEA, and the effect on this of Ca^{2+} influx blockers, D600 and Co^{2+}, and Ca^{2+}-deficient medium. Data are mean ± SEM, n ≥ 3, relative to control (basal release = 100%).

induced ACTH response appears to be absolutely dependent on the influx of Ca^{2+} ions.

To investigate the involvement of K^+ channel activity during the ACTH responses to AVP and TPA, cells were simultaneously exposed to TEA (10 and 50 mM) plus either AVP or TPA. For both AVP (100 nM) and TPA (10 nM), simultaneous exposure with TEA caused an enhanced ACTH response (Fig. 5.2). Response ratios were calculated (as before, by dividing the simultaneous response by the sum of the individual responses), and it was found that for TPA plus both concentrations of TEA, and for AVP plus 50 mM TEA, the simultaneous response was significantly greater than the sum of the individual responses (Table 5.1). There was no significant difference in the level of these synergistic responses between the four combinations of AVP/TPA plus TEA ($P > 0.05$).

Further experiments investigating the involvement of K^+ channel activity in the responses to AVP and TPA were performed. The aim of these experiments was to investigate the putative role of K^+ channel activity in the generation of the electrical signal leading to VSCC activation. The basis for these experiments was the work of Reisine & Guild (1987) and Reisine (1989), in which it was demonstrated that prior exposure to TEA (resulting in a small, short-lived rise in $[Ca^{2+}]_i$) prevented TPA from generating a rise in $[Ca^{2+}]_i$, as it had in control experiments. The explanation given for this finding was that TPA caused a rise in $[Ca^{2+}]_i$ by blocking K^+ channels, which caused the plasma-membrane to become depolarised, thus activating VSCC. The prior exposure to TEA prevented this response because "since TEA probably depolarises AtT-20 cells, its addition to these cells may have removed the means by which phorbol esters could depolarise the plasma-membrane to open voltage-gated Ca^{2+} channels and elevate intracellular Ca^{2+} concentrations" (Reisine & Guild 1987).

In an attempt to produce effects analogous to those of Reisine & Guild (1987) and Reisine (1989) on ACTH secretion (rather than $[Ca^{2+}]_i$), experiments were performed in which cells were pre-exposed to TEA (50 mM) during the 1 h pre-incubation period and wash procedure. The cells were then exposed to various treatments during the test incubation, and the effects of the pre-exposure to TEA was assessed by comparison with control (*ie.* non-TEA pre-exposed) cells.

Pre-exposure to TEA had no apparent effect on unstimulated ACTH release (Table

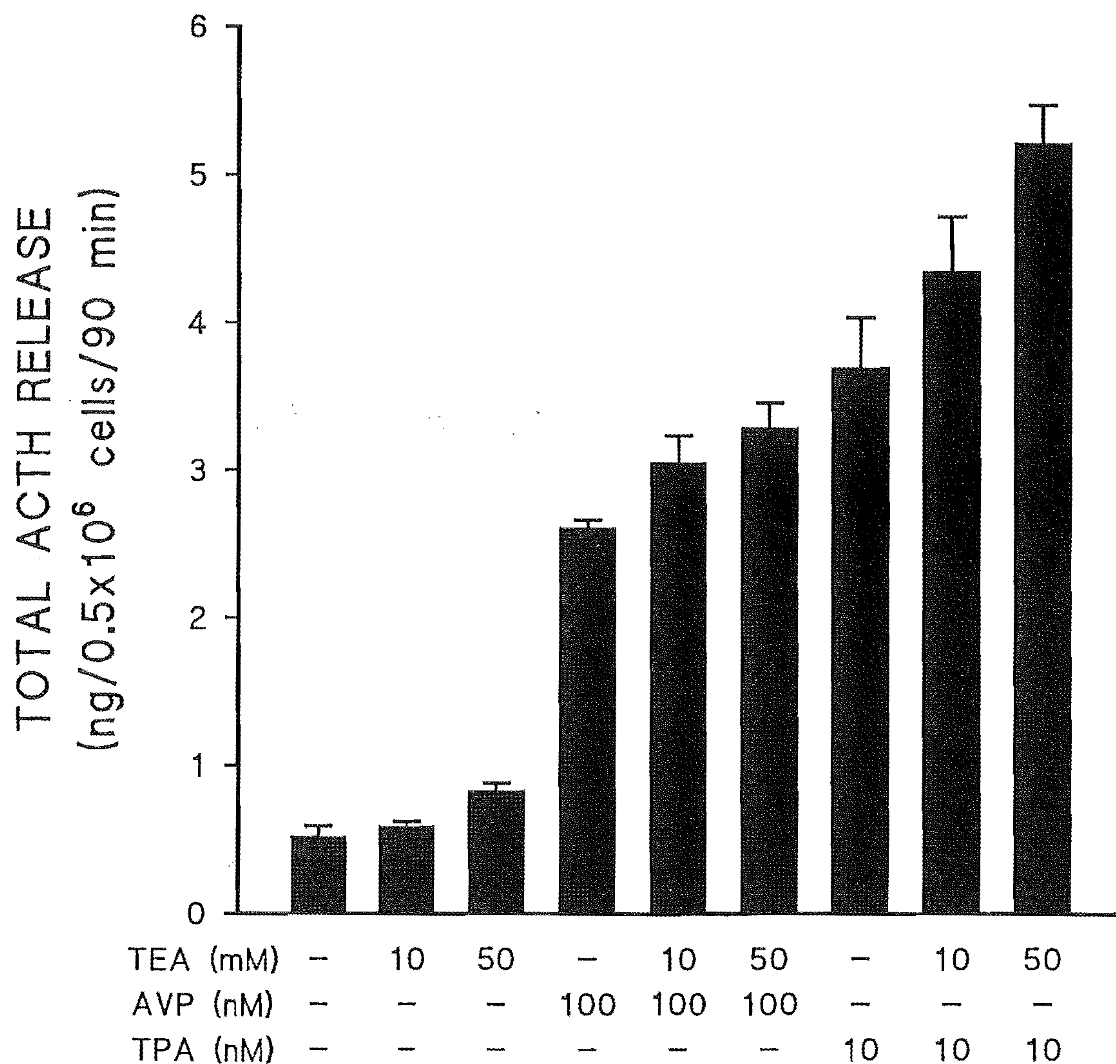

Fig 5.2. ACTH responses to simultaneous exposure to TEA plus either AVP or TPA. Data are mean ± SEM, n = 4, from a single representative experiment. Quantitatively similar responses, relative to control (basal release), were obtained in at least two other experiments using the same protocols. A representative figure is used here because this was the only individual experiment in which the effects of TEA plus AVP and TEA plus TPA were both assessed. Pooled data, expressed as response ratios, are given in Table 5.1.

Treatment	Response ratio	Significance
100 nM AVP + 10 mM TEA	1.258 ± 0.085	ns
100 nM AVP + 50 mM TEA	1.247 ± 0.040	$P < 0.01$
10 nM TPA + 10 mM TEA	1.174 ± 0.016	$P < 0.01$
10 nM TPA + 50 mM TEA	1.376 ± 0.047	$P < 0.05$

Table 5.1. Comparison of response to simultaneous exposure to TEA plus either AVP or TPA, relative to exposure to the agents given individually. Response ratios were obtained by dividing the response to simultaneous exposure by the sum of the individual responses. Data represent mean ± SEM, n ≥ 3, ns - not significant.

5.2). The effect of TEA pre-exposure on a subsequent challenge with 50 mM TEA was not consistent. In 1 of 4 identical experiments, the pre-exposure abolished the response to a subsequent exposure to TEA (107.8% inhibition). Within this particular experiment, the raw values for total ACTH release (quadruplicate determinations) in response to TEA, with or without TEA pre-exposure, were significantly different ($P < 0.05$). The data were then converted to stimulated release by subtracting the appropriate value (*ie.* with or without TEA pre-exposure) for the mean unstimulated release, in order to control for any effects of the pre-exposure on unstimulated release. When this was done, the responses to TEA with or without TEA pre-exposure remained significantly different - in fact the significance was more pronounced ($P < 0.005$).

In the remaining experiments, however, there was no effect of the TEA pre-exposure on a subsequent challenge with TEA. The values for percentage inhibition were -11.3%, 3.3% and -2.4%. Thus there appeared to be no effect whatsoever of the TEA pre-exposure, in contrast to the results of the experiment described above. The protocols used for these experiments were all identical, however, one possible explanation for the different effects was that the pituitaries used for the experiments where there was no effect of TEA pre-exposure were obtained from Malvern Abattoir, whereas the pituitaries from the other experiment were obtained from CFM Belfast freezing works. It is not obvious why the

Treatment	Response (% relative to control)	Significance
Unstimulated (basal)	94.4 ± 7.8	ns
TEA	75.7 ± 28.0	ns
AVP	100.0 ± 6.8	ns
AVP plus TEA	116.4 ± 6.1	ns

Table 5.2. Effect of 1 h pre-exposure to TEA (50 mM) on ACTH responses to TEA, AVP and AVP plus TEA during the 90 min test incubation. Values are mean ± SEM, n ≥ 3, and are expressed as the percentage relative to the response from cells that received the same test incubation treatments, but were not pre-exposed to TEA.

different source of the tissue might affect the results of this particular investigation. Certainly the results of other treatments in these experiments were apparently consistent between all of the experiments. Thus, the cause of the disparate results cannot be determined, although the source of the tissue remains a possible explanation.

When the data from all four experiments were pooled there was no significant effect of TEA pre-exposure on a subsequent challenge with TEA (Table 5.2).

The effect of 50 mM TEA pre-exposure on 100 nM AVP-stimulated release was found to follow a similar pattern to the effect of pre-exposure on TEA-stimulated release. In the experiment where pre-exposure abolished the subsequent response to TEA, the AVP-stimulated response was inhibited by 19.4%. Although this level of inhibition was small, the values for total release in the presence of AVP, with or without TEA pre-exposure, were significantly different ($P < 0.05$), as were the calculated values for AVP-stimulated release (*ie.* calculated as described above) ($P < 0.05$). However, in the three other, identical experiments, there was no significant effect of TEA pre-exposure on ACTH release in the presence of AVP (inhibition was -9.8%, -0.4% and -9.1%), and when the values were pooled there was no apparent effect of TEA pre-exposure (Table 5.2).

The effect of TEA pre-exposure on the combined response to AVP plus TEA was also tested, and the response was found to be unaffected by the pre-exposure to TEA (Table

5.2). However, this particular effect was tested in only three of the four experiments described above, and these experiments do not include the experiment in which a significant effect of TEA pre-exposure on the responses to TEA and AVP was observed.

5.2.2 Involvement of Na^+ channels - Effect of Na^+-free treatment

The involvement of Na^+ channels in the response to AVP was investigated by exposing cells to AVP while they were bathed with medium in which the Na^+ ions had been replaced with choline. In these experiments, alkali-treated casein (ATC) replaced NCS as the protein component of the KR to maintain the Na^+-free nature of the medium (Livesey & Donald 1982). This protein substitution caused a small, but not significant, increase in unstimulated ACTH release (Table 5.3). The ATC substitution also caused a small increase in AVP (1 and 100 nM) stimulated release. The magnitude of the increase was similar for both concentrations of AVP, but the effect on the response to 100 nM AVP was more consistent and reached significance at the 5% level (Table 5.3). Subsequent data analysis of ACTH responses in ATC-containing medium were expressed relative to these control responses in ATC-containing KR.

The effects of removing extracellular Na^+ can be seen in Table 5.4. There was no effect on unstimulated release, but 100 nM AVP-stimulated release was significantly inhibited, albeit to a small extent ($15.8 \pm 2.9\%$, $P < 0.05$). The pooled data show that 1 nM AVP-stimulated response was, numerically, inhibited to a greater extent than the 100 nM response, but the former response was more variable, and the reduction was not significant for this response.

Treatment	Response (% relative to control)	Significance
Unstimulated (basal)	133.9 ± 17.3%	ns
1 nM AVP	110.8 ± 10.8%	ns
100 nM AVP	108.5 ± 1.6%	$P < 0.05$

Table 5.3. Effect of substituting ATC for NCS in KR on ACTH responses. Data are mean ± SEM, n = 3, and represent the percentage response relative to the same treatment with NCS as the protein supplement of the bathing medium.

Treatment	Response (% relative to control)	Significance
Unstimulated (basal)	96.7 ± 1.2%	ns
1 nM AVP	75.4 ± 13.7%	ns
100 nM AVP	84.2 ± 2.9%	$P < 0.05$

Table 5.4. Effect of Na^+-free medium on ACTH responses. Data are mean ± SEM, n = 3, and represent the percentage response relative to the same treatment in normal KR.

5.3 Discussion

5.3.1 Experiments with TEA

Exposure to TEA was found to raise ACTH release above control levels demonstrating that inhibition of K^+ channels can increase the release of ACTH. Thus these results are consistent with the observations of Reisine & Guild (1987) and Reisine (1989) who found that application of K^+ channel antagonists to AtT-20 pituitary tumour cells caused a small, transient increase in $[Ca^{2+}]_i$. It is reasonable to expect that these Ca^{2+} transients would result in ACTH release, since their magnitude is similar to that obtained for TPA and CRH, which are known to stimulate ACTH release.

The magnitude of the TEA-induced response reported here was relatively small, although it was consistent between individual cell preparations. If, as is suggested by this and other data, a similar response occurs following exposure to AVP, then once the initial depolarisation is accomplished (in the response to AVP, at least), there are both electrical (Na^+ channels, T- and L-VSCC) and chemical (modulation of L-VSCC) mechanisms present, that would be able to amplify the response.

As it is suggested to occur here, the ACTH response to TEA requires the influx of Ca^{2+}. Therefore the sensitivity of the TEA-induced response to the inhibition of Ca^{2+} entry was tested. The response was found to be sensitive, in fact complete abolishment occurred following exposure to D600, Co^{2+} and Ca^{2+}-deficient medium. This finding supports the hypothesis that TEA induces ACTH release via Ca^{2+} influx, and provides a link between the results presented here (where ACTH release was measured), and the work of Reisine & Guild (1987) and Reisine (1989) (where changes in Ca_i^{2+} were measured). The sensitivity of the TEA-induced response to the inhibition of Ca^{2+} entry does not prove that K^+ channel closure generates a depolarisation during the response to AVP, but it is consistent with the proposed model.

In addition to their possible involvement in the generation of electrical activity

during the AVP-induced response, K^+ channels may also have a regulatory role in membrane electrical events. In hACTH cells, application of TEA (10 mM) produced an increase in both action potential amplitude and duration, suggesting that a K^+ conductance participates in the repolarisation phase of action potentials in these cells (Mollard *et al.* 1987). A K^+ conductance was also found in normal rat corticotrophs and was suggested to be Ca^{2+}-activated, following a secretagogue-induced rise in $[Ca^{2+}]_i$ (Corcuff *et al.* 1993). However, the hyperpolarisation associated with this particular K^+ conductance was observed in cells that did not produce action potentials. Instead, the hyperpolarisation appeared to be caused by Ca^{2+}-activated K^+ channels responding to the increase in Ca_i^{2+} that resulted from AVP induced release (of Ca^{2+}) from intracellular stores. The hyperpolarisation was followed by a mild depolarisation of the plasma-membrane during the phase of steady Ca^{2+} influx (Corcuff *et al.* 1993, see Chapter 1, section 1.3.2 for a more complete description of the results of Corcuff *et al.*). These results suggest that in cells that generate action potentials, and thus Ca^{2+} transients, the activation of K^+ channels may be required for repolarisation of the membrane, and thus regulate cellular activity. In cells that generate a spike/plateau profile of $[Ca^{2+}]_i$, the hyperpolarisation generated by Ca^{2+}-activated K^+ channels may also serve a regulatory function (although the exact nature of this is unclear), but the transformation from hyperpolarisation to depolarisation during the plateau phase of Ca^{2+} entry suggests that either the K^+ conductance is inactivated or the depolarising effects of the Ca^{2+} influx is sufficient to overcome the hyperpolarising effects of the K^+ conductance.

If K^+ channels do act to regulate AVP-induced membrane electrical effects, then simultaneous exposure to TEA plus AVP (*ie.* exposure to both agents commencing at the same time) would be expected to increase ACTH release relative to that induced by AVP given alone. The increased level of ACTH release would occur due to an enhancement of the depolarisation phase of the plasma-membrane electrical response, which would be expected to enhance Ca^{2+} entry, and thus the cellular response.

Simultaneous exposure to either TEA plus AVP or TEA plus TPA caused ACTH release that was greater than the sum of the individual responses to TEA and either AVP or TPA. These findings are consistent with a scheme whereby the actions of TEA in blocking K^+ channels enhanced membrane electrical events induced by either AVP or TPA,

and therefore also enhanced Ca^{2+} influx induced by these agents. [Thus this refers to modulation of K^+ channels which normally act to repolarise the membrane following electrical activity, and do not refer to K^+ channels that are normally active at rest, and which, when closed, might lead to the generation of a depolarisation stimulus.] Therefore these findings are consistent with the results of Mollard *et al.* (1987) and Corcuff *et al.* (1993) in that: 1) they support the presence of a TEA-sensitive (probably Ca^{2+}-activated) K^+ conductance in corticotrophs which may act to repolarise the cells following electrical activity, and therefore regulate Ca^{2+} influx, and 2) they support the hypothesis that the activation (opening) of these K^+ channels occurs during stimulation with AVP. Furthermore, the finding that the response to TPA was enhanced by co-incubation with TEA suggests that the PKC arm of the AVP-activated intracellular signalling pathway is at least partly responsible for the modulation of membrane electrical events which lead to Ca^{2+} influx. As a whole, these results support the hypothesis that modulation (probably both depolarisation and hyperpolarisation) of the plasma-membrane is an important factor in the regulation of cellular activity during the response to AVP.

Reisine & Guild (1987) and Reisine (1989) demonstrated that prior exposure to TEA and other K^+ channel blockers prevented the normal Ca_i^{2+} response to a subsequent challenge with TPA. These results were taken as suggesting that TPA-activated PKC caused an increase in $[Ca^{2+}]_i$ via closure of K^+ channels (leading to depolarisation of the plasma-membrane and activation of VSCC). This putative effect would involve a distinct set of K^+ channels from the Ca^{2+}-activated K^+ channels discussed above (which are thought to repolarise the membrane and thus regulate the cellular response). Instead the PKC-inhibited K^+ channels would need to be active at rest, and therefore contribute to the resting E_M. Closure of the channels would generate a small depolarisation of the plasma membrane, and thus create an electrical stimulus from an originally chemical messenger. In this study, experiments analogous to those of Reisine & Guild (1987) and Reisine (1989) were performed in which the effect of pre-exposure to TEA on secretion evoked by TEA, AVP and AVP plus TEA was assessed. To correspond to the results of Reisine & Guild (1987) and Reisine (1989), the TEA pre-exposure would be expected to reduce the secretory responses to TEA, AVP and AVP plus TEA, since these agents would be unable to elicit the small membrane depolarisation by the closure of the K^+ channels.

Pre-exposure to TEA was found to have no effect on unstimulated ACTH release. This suggests that the typical ACTH response to TEA, which presumably occurred during the pre-exposure period, was completed by the end of this period (1 h). If this was not the case (*ie.* ACTH release above unstimulated levels was still occurring at the completion of the pre-exposure period), then it would be expected that some residual, TEA-induced ACTH release would still occur immediately following the start of the test incubation period (which is the point where wash-out of TEA first occurred since TEA was present in the wash procedure as well as the pre-incubation period). Thus it appears that by the end of the pre-exposure period, the cells have re-equilibrated their E_M and ACTH release has returned to unstimulated levels.

Overall, pre-exposure to TEA had no significant effects on the responses to a subsequent challenge with TEA, AVP or AVP plus TEA. In one of four experiments, the response to a second exposure to TEA was abolished, but in the remaining experiments the TEA-induced response was completely unaffected. In the one experiment where the TEA-induced response was abolished, and in this experiment only, a reduction in the AVP-stimulated response was also observed. The source of the pituitaries for this experiment (CFM Belfast) was different from the source of the pituitaries for the three experiments that gave similar results (Malvern Abattoir), and therefore it is possible that this was the cause for the disparate results. However, there is no obvious reason why the different effects should occur, and the results from other treatments in these four experiments did not display a similar disparity. Furthermore, there were no problems encountered during the experiment that might explain why the results of this experiment differ from those of the other three identical experiments, so the data cannot simply be discarded. Taken together the disparate results of these experiments may suggest that an effect of TEA pre-exposure, to reduce subsequent secretagogue-induced ACTH release, may occur, but that the protocol used here is not optimal for observing this effect. The use of perifusion experiments, where the time course of the responses can be followed, would allow this possibility to be assessed.

On the other hand, it is possible that the observations of the one variant experiment were caused by some unknown factor, and that pre-exposure to TEA does not affect TEA- or AVP-induced secretion during a subsequent exposure. This interpretation would argue

against the hypothesized effect of PKC to close K^+ channels and thus generate a depolarisation of the plasma-membrane. However, the pre-exposure protocol used here for secretion studies and those used by Reisine & Guild (1987) and Reisine (1989) in which the effect on $[Ca^{2+}]_i$ was described were somewhat different, particularly in the duration of the pre-exposure period (1 h here, and seconds to a few minutes in the previous reports). Furthermore, the previous findings presented here describing the effects of TEA on ACTH release, and the results of Reisine & Guild (1987), Reisine (1989) and Corcuff *et al.* (1993) all provide support for the possibility that PKC-induced inhibition of a K^+ conductance may occur during the early stages of the cellular response to AVP. CRH-induced ACTH may also utilise this mechanism (Mollard *et al.* 1987), using a different factor to close the K^+ channels (ie. not PKC). Clearly, further investigation is necessary to fully determine the putative role of K^+ channels in the generation of electrical stimuli in corticotroph cells.

It still remains to be determined whether the actual *generation* of an electrical stimulus is required during the response to AVP. Given the results of Corcuff *et al.* (1993), there must be some doubt to this. The group of cells that generated Ca^{2+} transients in response to AVP, also generated spontaneous action potentials. Therefore, in these cells, an intermittent electrical stimulus is already present and it would seem necessary to *increase* the rate of action potential firing, rather than actually *generate* a novel (electrical) stimulus. Mollard *et al.* (1987) found that application of TEA to hACTH cells produced an increase in the amplitude and duration, and a decrease in the frequency of spontaneously generated action potentials. These effects could easily be attributed to the effects of TEA on Ca^{2+}-activated K^+ channels. If normal *ovine* corticotrophs generate spontaneous action potentials, then the effects observed in this study of exposure to TEA (a Ca^{2+}-influx-dependent rise in ACTH secretion) are entirely consistent with an effect solely on Ca^{2+}-activated K^+ channels. The rationale for this statement is as follows: Stojilkovic *et al.* (1988c) and Corcuff *et al.* (1993) have suggested that a low level of Ca^{2+} influx does occur in resting corticotrophs (due to a low level of membrane electrical activity), but this level of Ca^{2+} influx may be below the threshold for inducing ACTH release, as is suggested by the inability of VSCC blockers to reduce basal ACTH release (Stojilkovic *et al.* 1988c, Chapter 3 of this report). However, the low level of Ca^{2+} influx may be sufficient to activate Ca^{2+}-activated K^+ channels, perhaps to regulate the level of the membrane electrical

activity. If these K^+ channels are blocked by TEA, this regulation would be removed and Ca^{2+} influx would presumably increase, perhaps to a level that would induce ACTH release. Thus, solely on the results presented here, a distinction between this scheme and the previous hypothesis of a PKC-induced inhibition of K^+ channels cannot be made. The results are consistent with both schemes.

In cells that generated a spike/plateau pattern of Ca_i^{2+}, Corcuff *et al.* (1993) found that exposure to AVP caused a release of intracellular, stored Ca^{2+}, which was associated with a large membrane hyperpolarisation. This hyperpolarisation was suggested to be generated by the activation of a Ca^{2+}-dependent K^+ conductance. The release of stored Ca^{2+} was followed by a period of sustained Ca^{2+} influx. Evidence suggested that this influx was due to entry via VSCC. This raises an interesting point: it is unlikely that these channels could be activated by a depolarisation stimulus, since the channels appear to be activated while the hyperpolarisation that occurs during the spike Ca_i^{2+} response, is still present (see Fig. 13 of Corcuff *et al.* 1993). Thus other factors may serve as the initial stimulus to activate VSCC in these particular cells. The situation is complicated further, however, by the demonstration by Corcuff *et al.* (1993) that during this period of sustained Ca^{2+} influx (when the membrane potential is moderately depolarised, but no action potentials are generated), the injection of hyperpolarising current caused the Ca^{2+} influx to be reduced towards the basal level. This suggests that during this phase of the response, the channels are sensitive to E_M, in fact depolarisation seems essential for Ca^{2+} influx. When the hyperpolarising current was removed, the E_M returned to its previous depolarised level and the Ca^{2+} plateau was restored to its original level. This last finding is particularly important, because it indicates that a stimulus other than membrane depolarisation must be present, otherwise the membrane would not be expected to return to its depolarised level. Overall, this work of Corcuff *et al.* (1993) suggests strongly that both electrical and non-electrical factors control VSCC activity in the cells that generate spike/plateau profiles of $[Ca^{2+}]_i$.

5.3.2 Experiments with Na^+-free medium

The results of experiments using Na^+-free medium demonstrated that this medium caused a modest reduction in AVP-stimulated ACTH release, suggesting that in corticotrophs, there is a moderate dependence on Na^+ channel activity for the full expression of the AVP-induced ACTH response. Previous literature has not been conclusive in determining the extent of Na^+ channel involvement in the functioning of corticotroph cells. Suprenant (1982) found no evidence supporting the involvement of Na^+-conducting currents occurring in AtT-20 pituitary tumour cells, whereas, in the same cell type, Adler *et al.* (1983) found considerable evidence for Na^+ currents. Both reports used similar methodologies to investigate the presence of Na^+ currents (electrophysiological measurements and exposure to tetrodotoxin (TTX) and Na^+-free medium) and both reports also provide similar information regarding the characteristics of Ca^{2+} currents observed. Therefore it is unclear why the findings of these reports are so different with respect to the presence of Na^+ currents. Adler *et al.* (1983) found that exposure to TTX or Na^+-free medium caused the firing pattern of spontaneous action potentials to become irregular, and concluded that Na^+ spikes provide a rhythmic firing pattern and cause the activation of Ca^{2+} channels in these cells.

The presence of membrane currents in rat corticotrophs (Marchetti *et al.* 1987) and the effect on hormone secretion of these (Childs *et al.* 1987), were reported in complementary papers from the same group. This group reported the presence of a TTX-sensitive current that activated at approximately -50 mV and two Ca^{2+} currents, one that activated at approximately -50 mV and was maximal at -10 mV and a second that was activated at -10 mV and was maximal at 10 mV (Marchetti *et al.* 1987). These Ca^{2+} currents display kinetics similar to those ascribed to T- and L-type currents, respectively. The involvement of these currents in unstimulated and CRH-invoked ACTH release was reported in Childs *et al.* (1987). Both unstimulated and CRH-stimulated release were found to be sensitive to the actions of TTX and Ca^{2+} channel inhibitors. Thus, these results suggest that in rat corticotrophs, at least, Na^+, as well as Ca^{2+} channels, are involved in the normal functioning of these cells. These authors presented a tentative model whereby Na^+ channels are involved in the generation of action potentials, which lead to activation of high

threshold (L-type) Ca^{2+} channels.

In their study of the electrical properties of hACTH cells, Mollard *et al.* (1987) found that although these cells fire spontaneous action potentials, there is apparently no involvement of Na^+ channel activation in this response. This conclusion was reached due to the ineffectiveness of TTX or Na^+-free medium to influence action potential activity. In a paper investigating the Ca_i^{2+} responses from both hACTH and normal (small ovoid - a morphologically distinct population of) rat corticotrophs, the same group (Guerineau *et al.* 1991) found that TTX did not alter inward currents in hACTH cells and did not affect spontaneous or CRH-induced Ca_i^{2+} responses in normal rat corticotrophs. These findings contrast with a more recent report from this group, in which they found clear evidence that Na^+ currents were involved in the functioning of some normal rat corticotrophs (Corcuff *et al.* 1993). In this report, about half of the cells tested were found to generate Ca^{2+} transients in response to AVP that were dependent on influx of extracellular Ca^{2+}. In one third of these cells, the AVP-induced Ca^{2+} transients were found to be reversibly blocked by TTX. In the other two thirds of the Ca^{2+}-transient-generating cells, TTX was completely without effect. The authors suggest that in the TTX-sensitive cells, Na^+ channel activation is responsible for the establishment of action potentials, whereas T-VSCC fulfill this function in the TTX-insensitive cells (Corcuff *et al.* 1993). There is no information regarding the TTX sensitivity of the half of the cells that did not generate Ca^{2+} transients (*ie.* cells that generated a spike/plateau pattern of Ca_i^{2+}). Thus the cells that were found to be sensitive to TTX would compose about one sixth of the normal rat corticotrophs studied by Corcuff *et al.* (1993), and this provides a minimum estimate of the number of TTX-sensitive cells in a normal population of (rat) corticotrophs.

The reports described above do not provide a completely consistent picture regarding the involvement of Na^+ channels in corticotroph function. Overall it appears that Na^+ channels may play some part in the regulation of ACTH secretion, but the precise details are yet to be resolved. Given the results from Corcuff *et al.* (1993), where Na^+ currents were found in only some of the cells, the lack of effects of TTX observed from hACTH cells (Mollard *et al.* 1987) could reflect a situation in which the adenoma tissue used may have arisen from proliferation of cells that did not contain functioning Na^+ channels. It is also possible, as suggested by Guerineau *et al.* (1991), that discrepancies

(between the results of their report and those of Childs *et al.* 1987) could be due to either culture conditions, or the fact that this group (Guerineau *et al.*) identified ACTH-containing cells by morphological features. Thus there may be other, morphologically distinct, ACTH-containing cells, that do possess Na^+ channels which are involved in the response to CRH. However, the small ovoid corticotrophs made up about 10% of the rat pituitary cell population (Guerineau *et al.* 1991) which is close to the upper estimate given for ACTH-containing cells in a pituitary cell population (Westland *et al.* 1985). Therefore the small ovoid population of corticotrophs would appear to comprise most of the ACTH-secreting cells in a mixed population of normal rat anterior pituitary cells. Guerineau *et al.* (1991) do not report the effect of TTX on unstimulated or CRH-induced ACTH secretion from normal rat corticotrophs, but it is unlikely that TTX could affect CRH-induced ACTH secretion while displaying no effect on CRH-induced Ca_i^{2+} transients.

Therefore, while it is difficult to assess the overall importance of Na^+ channel activity in corticotroph function, the report of Corcuff *et al.* (1993) suggests that Na^+ channels do play a part in *AVP*-induced corticotroph activity (this is the only previously published report to investigate the role of Na^+ channels in AVP-induced ACTH secretion). Sensitivity to TTX occurred in at least one sixth of the cells investigated and it is reasonable to assume that the effects of TTX to reduce Ca_i^{2+} transients would be translated into inhibitory effects on the processes controlling the secretory activity of these cells. If these findings are extrapolated to the effects on AVP-stimulated ACTH secretion from a population of pituitary cells, it is likely that secretion would be reduced, but only moderately, following any treatment that reduces Na^+ influx in corticotrophs. Therefore this interpretation is consistent with the results found in this study, and, taken together, these findings provide further support for the involvement of an electrical stimulus (membrane depolarisation) during the ACTH response to AVP.

Chapter 6

General Discussion

6.1 General discussion

The objectives of this study were to investigate the role and regulation of Ca^{2+} and other ion channels in the in vitro regulation of ACTH secretion induced by AVP, in dispersed ovine anterior pituitary cells. The investigations described in the preceding three chapters involved examining the effects of manipulating cellular function on the ACTH response from a population of anterior pituitary cells. This type of investigation imposes certain features on the nature of the data that is obtained, and thus on the analysis and interpretation of the data. A particularly important aspect is that the factor, whose involvement in the cellular ACTH response is under investigation, is not measured directly. Instead, it is the *effects of manipulating the factor on the cell population ACTH response* that is the measured parameter. Thus the possibility that any observed effects are due to either non-specific actions, or factors that are not appreciated (although these may become apparent with further investigation), is always present. Furthermore, because the actions of the factor under investigation are only inferred, it is more likely that the observed effects (on the ACTH response), may be consistent with more than one interpretation. These two features represent potential problems for effective analysis of the data.

On the other hand, investigating the ACTH responses from a population of cells

is important for confirming that intracellular factors, that have been shown (by direct investigation) to be influenced by ACTH secretagogues, do actually affect *the cellular response*. Thus direct and indirect investigations of intracellular factors are complementary.

Investigations of the population response are important given the heterogeneity of ACTH-secreting cells (Jia *et al.* 1991, Corcuff *et al.* 1993), since the response of a single cell cannot be simply scaled up to represent a population response. Furthermore, intercellular effects may be present, which would not be apparent in single cell studies (although intercellular effects that may occur *in vivo* may not occur in any *in vitro* situation).

The influence of both of the potential problems raised above (non-specific actions and multiple viable interpretations of results) can be minimized by the use of several different approaches to investigate the same question. Although, potentially, each approach may have its own non-specific effects, these will (hopefully) not be the same for each approach. Thus any non-specific effects should be identifiable and can be taken into account when analysing the data and forming conclusions. This approach was used to investigate the role of Ca^{2+} influx during AVP-stimulated ACTH release. In total, three chemically distinct organic Ca^{2+} channel blockers, two inorganic Ca^{2+} channel-blocking ions, and removal of Ca^{2+} from the extracellular medium were used to investigate this question. Considering the diversity of these agents and approaches, the results that were obtained displayed considerable consistency, and thus some confidence can be placed in the interpretation that Ca^{2+} influx plays a major role in the AVP-induced ACTH release, over 90 min, in ovine anterior pituitary cells.

The Ca^{2+} channel blockers used (both organic and inorganic) are all defined as blockers of VSCC. Since all were effective blockers of the AVP-induced response, it seems reasonable to conclude that VSCC are present in corticotrophs, and that they are activated during the AVP-induced response. To date, non-voltage-activated channels (*eg.* second-messenger operated channels) have not been observed in corticotroph cells. However, there are fewer agents available that can be used as probes for non-voltage-sensitive Ca^{2+} channels, and it is not clear at present whether the lack of evidence for these Ca^{2+} channels is because they are not *actually* present, or because they cannot, at present,

be easily identified. Furthermore, since non-voltage-activated channels are not well characterised, it is also not clear what effect the VSCC blockers might have on these. Thus, while the results of the investigations with the VSCC blockers support the presence and involvement of *voltage-sensitive* Ca^{2+} channels in the AVP-induced ACTH response in ovine corticotrophs, the involvement of non-voltage-activated channels cannot be ruled out.

It is possible that the component of the AVP-induced response that was resistant to the actions of the VSCC blockers (see Chapter 3) could represent Ca^{2+} influx via non-voltage-activated channels. However, Cd^{2+} was found to block 87.6% of the AVP-induced response, and thus if the remaining portion was ascribed to Ca^{2+} influx via non-voltage-activated channels, this would mean that no factors independent of Ca^{2+} influx were involved in the AVP-induced ACTH response. However, there is considerable evidence for the involvement of IP_3-induced release of intracellular stored Ca^{2+} in the response to AVP (see Chapter 1, section 1.3.2). Also, removal of Ca^{2+} from the extracellular medium (Ca^{2+}-free medium), which should prevent influx of Ca^{2+} from all channels that participate in the response to AVP, caused a level of secretion that was very similar to that observed for Cd^{2+}. This suggests that Cd^{2+} is able to block all AVP-induced Ca^{2+} influx, and thus: 1) the remaining component of the AVP-induced response is not due to Ca^{2+} influx, and therefore may be due to release of stored Ca^{2+}, and 2) that if non-voltage-activated channels *are* involved in the response to AVP, they are blocked by Cd^{2+}.

The level of inhibition of AVP-induced secretion by Cd^{2+} and Ca^{2+}-free medium (approximately 87% - 90%) was higher than that observed for the organic blocker D600 (71.6%). D600 acts primarily on L-type VSCC, whereas the inorganic ion and Ca^{2+}-free medium should block both L- and T-VSCC (although at high concentrations D600 may also act on T-VSCC, Janis & Triggle 1991). Thus the difference in the level of inhibition between D600 and the other two agents may reflect Ca^{2+} influx via T-VSCC, or possibly, non-voltage-activated channels.

The ability of raised $[K^+]_e$ to induce ACTH release supports the presence of VSCC in corticotrophs. That this release was inhibited by the blockers that were used demonstrates that the blockers are able to block VSCC in corticotrophs. Thus these results support the hypothesis that AVP induces Ca^{2+} influx via VSCC. However, they do not prove the hypothesis, since it still not determined that the same channels participate in the

AVP-induced response, or, if they do, that the channels are activated by electrical effects during the response to AVP (*ie.* they may be sensitive to voltage changes, but may not actually be voltage-activated during the response to AVP). However, Corcuff *et al.* (1993) do provide direct evidence supporting voltage-activated Ca^{2+} influx in ACTH-secreting cells.

Recent literature has demonstrated that L-VSCC are typically sensitive to chemical modulation in the form of phosphorylation and dephosphorylation, and that the activity of the channels is influenced by these effects (see Chapter 4, section 4.1). These effects have been observed in pituitary cells. Thus, if dual modulation of Ca^{2+} channels by electrical and non-electrical (*ie.* chemical, via, for example second-messengers), does occur in corticotrophs, then it would appear that the VSCC blockers *are* able to act on Ca^{2+} channels that are influenced by non-electrical factors, since the channels are also activated by electrical effects.

The role of non-electrical (*ie.* chemical) modulation of AVP-induced Ca^{2+} influx was investigated in Chapter 4. Initially, the effects of simultaneous stimulation by AVP plus raised $[K^+]_e$ were investigated, in an attempt to determine whether the Ca^{2+} channels activated by both agents represent the same population of channels (to some extent, at least). The results suggested that this is in fact the case, and thus further support the suggestion that the Ca^{2+} channels activated by AVP can be voltage-activated.

The simultaneous stimulation experiments also produced the unexpected finding that AVP and K^+_e caused a synergistic ACTH response, when the concentrations of the agents were low. Although this effect is non-physiological, as the raised $[K^+]_e$ would never occur *in vivo*, analysis of the results may provide insight into the intracellular regulation of the AVP-stimulated ACTH response. Therefore, an investigation was undertaken into what cellular factors were responsible for the generation of the synergistic response. As discussed in Chapter 4, section 4.3.1 (sub-section *Analysis of the synergistic response*), the VSCC are a candidate for the factor that is responsible for the generation of the synergistic response. Part of this investigation involved using a sub-maximally inhibiting concentration of D600. The importance of using a sub-maximally inhibiting concentration of the blocker was that part of the Ca^{2+}-influx-dependent component of the secretagogue-induced responses remained intact. The analysis of the data is described in depth in

Chapter 4 (section as above) and see below. The analysis relies unavoidably on some assumptions regarding corticotroph cell functioning, but these have been described clearly, and readers must judge for themselves the validity of the assumptions.

When analysed, the data were found to support the possibility that the synergistic response is generated at the level of the VSCC, or at least, at the level of Ca^{2+} influx. The interpretation, therefore, is that when AVP and raised $[K^+]_e$ are present simultaneously, some feature of the Ca^{2+} influx mechanism is enhanced. It is not clear what form this enhancement might take: the channels may be activated for longer, more often, or sooner than occurs when either secretagogue is present individually. Mollard *et al.* (1987) found that the L-type current from hACTH cells was enhanced by AVP in a way that suggested a chemical modification of the channels. This is consistent with many other reports that have demonstrated that chemical modulation, in the form of phosphorylation and dephosphorylation, of the L-VSCC occurs (see above and Chapter 4, section 4.1). Thus it is possible that the putative enhancement of Ca^{2+} influx during the simultaneous response is due, at least in part, to chemical effects arising originally from AVP (and PKC is a likely candidate). This effect may act in concert with the more pronounced and long-lasting depolarisation that is likely to occur with raised $[K^+]_e$ (compared to the lower-level, transient electrical activity induced by AVP, *eg.* Corcuff et al 1993) to enhance Ca^{2+} entry during the simultaneous response. Thus this scheme suggests a role for chemical modulation of (perhaps, voltage-sensitive) Ca^{2+} channels during the response to AVP. Evidence suggests that PKC is activated by AVP in pituitary cells (Zatz *et al.* 1987, Carvallo & Aguilera 1989, Rougan *et al.* 1989, Liu *et al.* 1990, 1992), and that PKC can elicit ACTH release (Bilezikjian *et al.* 1987, Carvallo & Aguilera 1989, Liu *et al.*. 1990, 1992, Oki *et al.* 1990, Koch & Lutz-Bucher 1991), via a mechanism which has been shown to be sensitive to the inhibition of Ca^{2+} influx (Reisine & Guild 1987, Sobel 1988, Won *et al.* 1990). Therefore, PKC represents a likely candidate for an AVP-activated factor that could cause chemical modulation of VSCC.

To investigate this possibility, two strategies were employed. The first of these was to repeat the simultaneous stimulation experiments in cells that had had their PKC activity removed by chronic exposure to the phorbol ester, TPA. Following this treatment, the synergistic response observed in control cells was abolished, suggesting that PKC, or

another factor dependent on PKC for its activation, was involved in the generation of the synergistic response. The second strategy was to perform simultaneous stimulation experiments in which AVP was replaced with TPA. Synergistic responses were observed from these treatments, and so these results are also consistent with PKC playing a role in the generation of the synergism. Although these data do not themselves provide any direct support for PKC affecting VSCC in the regulation of ACTH secretion, when combined with the previous suggestion, that the synergistic response occurs at the level of Ca^{2+} influx, the results do implicate PKC as a modulator of VSCC activity in AVP-stimulated ACTH secretion, in ovine cells.

In an attempt to more directly demonstrate whether PKC can affect the activity of VSCC in ovine corticotrophs, the effects of inhibiting Ca^{2+} influx on the ACTH response to TPA was investigated. Again, a range of agents/approaches were used to minimize the possibility of non-specific effects suggesting erroneous interpretations. The blockers D600 and Co^{2+} were used, along with Ca^{2+}-deficient and Ca^{2+}-free media. All of these treatments caused a reduction in TPA-stimulated ACTH release to some extent. However, the patterns of inhibition were more complex than observed previously for AVP-stimulated release. Most notably, the concentration of TPA used affected the level of inhibition induced by the blockers, with lower levels of inhibition occurring as the concentration of TPA was increased. This situation is very different from that observed for the inhibition of AVP-induced secretion in which the level of inhibition induced by the blockers was unaffected by the concentration of the secretagogue (see Chapter 3). The effect was more pronounced with the VSCC blockers than with the Ca^{2+}-reduced media, although it was still apparent in the latter case. These results suggest that at low TPA concentrations, Ca^{2+} channels are activated and are sensitive to the blockers, but higher concentrations of TPA either: 1) afford the Ca^{2+} channels some resistance to the effects of the blockers, or 2) promote the activation of previously inactive mechanisms, which cause ACTH release, but which are not sensitive to the actions of the blockers.

These two possibilities are not mutually exclusive, and in fact there is support for both mechanisms occurring. The analyses depicted in Figs. 4.12 and 4.13 provide support for the second option, *ie.* that Ca^{2+}-influx independent, TPA-activated mechanisms are present, that induce ACTH secretion, and which have higher thresholds for activation by

TPA (compared with Ca^{2+}-influx dependent TPA-stimulated mechanisms). At low levels of TPA-induced stimulation, most (D600) or all (Co^{2+}) of the ACTH response was inhibited. At higher concentrations of TPA, more ACTH was released, but the same or similar absolute levels of reduction of the ACTH response occurred when the blockers were present. Thus the percentage inhibition by the blockers decreased.

Although this general pattern was observed for both blockers (and thus provides support for the second option given above), Co^{2+} was a more effective blocker of the TPA-induced ACTH response, in terms of the level of percentage inhibition at each TPA concentration. Furthermore, there was no evidence to suggest that Co^{2+} was a less effective inhibitor of the TPA- *vs.* the AVP-induced responses, whereas D600 was less effective on the TPA-induced response. Taken together, these results suggest that the actions of D600 during the TPA-induced response are compromised relative to its effects on the AVP-induced response. This interpretation is similar to the findings of Chapter 3 in which, in molar terms, D600 was found to be a more potent inhibitor of the K_e^+-induced response than the AVP-induced response. K_e^+-induced VSCC activity probably does not involve chemical modulation of the channels, at least not to the same extent as is proposed to occur during the AVP-induced response (certainly PKC appears not to be activated, see Fig. 4.6). This leads to the suggestion that chemical modulation of the channels may reduce the ability of the organic blockers (which bind to the channel complex) to inhibit the channels whereas the inorganic blockers (which are thought to bind to the Ca^{2+}-binding sites of the channels) may not be affected by any chemical modulation of the channels (see Chapter 3, section 3.3, for a fuller discussion, and Armstrong & Kalman 1990). However, since D600 was only effective at inhibiting the TPA-induced response at high concentrations (300 μM) it is also possible that this effect is due to non-specific actions of D600.

When cells were exposed to Ca^{2+}-deficient or Ca^{2+}-free media, the TPA-induced ACTH response was inhibited, to levels similar to (at 1 nM TPA) or greater than (at 10 nM TPA) those observed for Co^{2+}. Thus these data support the hypothesis that TPA- (and thus PKC) induced ACTH release requires a substantial level of Ca^{2+} influx. The data are also consistent with the possibility that chemical modulation of Ca^{2+} channels (possibly VSCC) can occur in corticotrophs (Mollard *et al.* 1987, Corcuff *et al.* 1993), and thus suggest that AVP-induced modulation of VSCC during ACTH release is a viable hypothesis. However,

in this study, there is no actual evidence of direct interaction between PKC and Ca^{2+} channels.

The results of this, and previously published reports, provide a sound base of evidence supporting some sort of functional link between PKC activation and Ca^{2+} influx during the ACTH response to AVP (although the nature of the link is yet to be fully determined). However there is also considerable evidence suggesting that Ca^{2+} influx is influenced by electrical events of the plasma-membrane. It is yet to be determined exactly what level of electrical activity occurs normally in corticotrophs, although Mason *et al.* (1988) and Stojilkovic *et al.* (1988c) suggest that the level of electrical activity is less than in some other pituitary cell-types (see Chapter 5, section 5.1 for more information). When there is less spontaneous membrane electrical activity, the question is raised as to how the chemical first messenger (*ie.* the extracellular secretagogue) can create an electrical signal. Reisine & Guild (1987) and Reisine (1989) found that in AtT-20 cells, a rise in cytosolic [Ca^{2+}] occurs following exposure to TPA. Evidence suggested that this effect was due to PKC-induced inhibition of a K^+ current that is active at rest in corticotrophs, and this creates a small depolarisation of the membrane, which in turn may activate VSCC and thus Ca^{2+} influx (see Chapter 5, section 5.1).

This putative mechanism was tested in this report, by examining the effects of inhibition of resting K^+ current, by TEA, on ACTH secretion. If this mechanism does occur in ovine corticotrophs, it would be expected that TEA would induce ACTH secretion, and that this response would be sensitive to blockers of Ca^{2+} influx. The results of the investigations were consistent with these expectations, and therefore support the results of Reisine & Guild (1987) and Reisine (1987). Attempts were also made to reproduce effects reported by these authors, in which prior exposure of cells to TEA prevented the rise in cytosolic Ca^{2+} in response to a subsequent exposure to PKC. The effects described by Reisine & Guild (1987) and Reisine (1989) could not be consistently reproduced in this report, in which ACTH rather than cytosolic [Ca^{2+}] was the measured parameter. This result suggests that effects described in the original reports may not occur in ovine corticotrophs, or, if they do, the effects are not linked to ACTH release. Alternatively, the system and protocol used in this report may not be able to resolve the effects of prior exposure to TEA.

Thus, overall, the investigation of the role of inhibition of resting K^+ currents on the generation of an electrical signal, in response to AVP, provided some evidence that supported this hypothesis. However, not all the evidence was consistent with this mechanism. Clearly further investigation of this hypothesis is required for this question to be resolved.

The experiments with TEA did provide evidence that is consistent with the presence of K^+ currents in corticotrophs that may be activated by Ca^{2+}, and which are thought to regulate the cellular response by repolarising the cells after depolarisation events of the plasma-membrane.

Finally, investigations were made into the role of Na^+ channels in the ACTH response to AVP. Exposing cells to Na^+-free medium caused a small level of inhibition of the AVP-induced response. Corcuff *et al.* (1993) studied the membrane currents of individual rat corticotroph cells, and found evidence for Na^+ currents in only a small proportion of the cells. If it is assumed that the AVP-activated Na^+ currents that were observed in some cells participate in the generation of the cellular ACTH response, then it is reasonable to expect that the effects of inhibiting the Na^+ current, on the response of a population of pituitary cells, would be a small reduction in ACTH secretion, which is consistent with the results described in this report.

6.2 Suggestions for further research

Many aspects of the regulation of Ca^{2+} influx during the ACTH response to AVP are yet to be resolved. As typically occurs, the results of this study have not so much answered specific questions, rather they have helped to make the questions to be asked more specific.

There is considerable evidence that AVP-activated Ca^{2+} influx occurs via VSCC. The development of probes for non-voltage-sensitive Ca^{2+} channels will allow the role, if any, of these channel types to be determined.

Further investigation is required to determine what intracellular factors (*ie.* not

voltage) modulate AVP-activated Ca^{2+} channels, and the pathway(s) leading to the generation of these factors. Specifically, what role does PKC play, does it regulate VSCC activity, and, if so, does PKC interact directly with the channels, or are one or more intermediate factors involved? Do other PKC-independent intracellular factors regulate VSCC activity, or Ca^{2+} influx via other channel types?

The *precise* role of voltage effects in the ACTH response to AVP also require further investigation. It is necessary to determine the exact causal relationship between changes in the E_M and the activity of VSCC. It is important to determine the level of spontaneous membrane electrical activity *in vivo*, as this will help to define which of the cellular electrical responses, that are observed *in vitro*, relate to the normal functioning of the cells, and which are products of the experimental conditions. These investigations will help to determine the possible roles of T-VSCC and Na^+ channels in the response to AVP. These channels types may act to deepen a small membrane depolarisation, resulting in a level of depolarisation sufficient to activate L-VSCC. However, if a high level of spontaneous activity normally occurs, T-VSCC and Na^+ channels may not be of great importance in the AVP-induced response.

The role of PKC-dependent inhibition of K^+-channels that are active at rest, also requires further investigation. It is necessary to determine what factor (PKC, or another factor activated by PKC) acts on the K^+ channels. The level of spontaneous membrane activity that normally occurs in corticotrophs is also important for defining a role for this mechanism, as the mechanism would seem only to be necessary if the level of spontaneous membrane activity is low.

Corcuff *et al.* (1993) have demonstrated that ACTH-secreting cells display heterogenous electrical and intracellular Ca^{2+} responses to AVP. This aspect requires further investigation, and ultimately it will be necessary to define a quantitative relationship between the number of cells that generate each Ca^{2+} response type, and the level of ACTH release induced by these responses. This will allow estimations of the population response to be extrapolated from the single-cell experimental data, which can be compared to responses from populations of pituitary cells receiving similar experimental manipulations (*eg.* exposure to Ca^{2+} channels blockers).

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References

Abou-Samra A-B, Catt KJ & Aguilera G (1987a). Calcium-dependent control of corticotropin release in rat anterior pituitary cell cultures. *Endocrinology* 121: 965-971.

Abou-Samra A-B, Harwood JP, Manganiello VC, Catt KJ & Aguilera G (1987b). Phorbol 12-myristate 13-acetate and vasopressin potentiate the effect of corticotropin-releasing factor on cyclic AMP production in rat anterior pituitary cells. *The Journal of Biological Chemistry* 262: 1129-1136.

Adler M, Wong BS, Sabol SL, Busis N, Jackson MB & Weight FF (1983). Action potentials and membrane ion channels in clonal anterior pituitary cells. *Proceedings of the National Academy of Sciences, USA* 80: 2086-2090.

Aguilera G, Harwood JP, Wilson JX, Morell J, Brown JH & Catt KJ (1983). Mechanisms of action of corticotropin-releasing factor and other regulators of corticotropin release in rat pituitary cells. *The Journal of Biological Chemistry* 258: 8039-8045.

Antoni FA (1986). Hypothalamic control of adrenocorticotropin secretion: advances since the discovery of 41-residue corticotropin-releasing factor. *Endocrine Reviews* 7: 351-378.

Antoni FA, Holmes MC, Makara GB, Karteszi M & Laszlo FA (1984). Evidence that the effects of arginine-8-vasopressin (AVP) on pituitary corticotropin (ACTH) release are mediated by a novel type of receptor. *Peptides* 5: 519-522.

Armstrong D & Eckert R (1987). Voltage-activated calcium channels that must be phosphorylated to respond to membrane depolarisation. *Proceedings of the National Academy of Sciences, USA* 84: 2518-2522.

Armstrong D, Kalman D (1990). Dihydropyridines modulate Ca^{++} channels by altering their availability to protein phosphorylation and its removal. *Biophysical Journal* 57: 516a.

Baertschi AJ & Friedli M (1985). A novel type of vasopressin receptor on anterior pituitary corticotrophs? *Endocrinology* 116: 499-502.

Berridge MJ (1987). Inositol phosphate and diacylglycerol: Two interacting second messengers. *Annual Review of Biochemistry* 56: 159-193.

Berridge MJ (1988). Cytosolic calcium oscillators. *FASEB Journal* 2: 3074-3082.

Berridge MJ (1990a). Temporal aspects of calcium signalling. In: *The Biology and Medicine of Signal Transduction* (ed. Y Nishizuka et al) pp. 108-114. Raven Press, New York.

Berridge MJ (1990b). Calcium oscillations. *The Journal of Biological Chemistry* 265: 9583-9586.

Berridge MJ (1993). Inositol trisphosphate and calcium signalling. *Nature* 361: 315-325.

Berridge MJ & Irvine RF (1984). Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* 312: 315-321.

Berridge MJ & Irvine RF (1989). Inositol phosphates and cell signalling. *Nature* 341: 197-205.

Bilezikjian LM, Woodgett JR, Hunter T & Vale WW (1987). Phorbol ester-induced down-regulation of protein kinase C abolishes vasopressin-mediated responses in rat anterior pituitary cells. *Molecular Endocrinology* 1: 555-560.

Burgess TL & Kelly RB (1987). Constitutive and regulated secretion of proteins. *Annual Review of Cell Biology* 3: 243-293.

Canny BJ, Jia L-G & Leong DA (1992). Corticotropin-releasing factor, but not arginine vasopressin, stimulates corticotropin-dependent increases in ACTH secretion from a single corticotrope. *The Journal of Biological Chemistry* 267: 8325-8329.

Carafoli E (1987). Intracellular calcium homeostasis. *Annual Review of Biochemistry* 56: 395-433.

Carbone E & Swandulla D (1991). Calcium channel inactivation. In: *Calcium Channels: Their Properties, Functions, Regulation, and Clinical Relevance* (ed. L Hurwitz, LD Partridge, JK Leach), pp. 35-60. CRC Press, Boca Raton/Ann Arbor/Boston/London.

Carvallo P & Aguilera G (1989). Protein kinase C mediates the effect of vasopressin in pituitary corticotrophs. *Molecular Endocrinology* 3: 1935-1943.

Childs GV, Marchetti C & Brown AM (1987a). Involvement of sodium channels and two types of calcium channels in the regulation of adrenocorticotropin release. *Endocrinology* 120: 2059-2069.

Childs GV, Unabia G, Burke JA & Marchetti C (1987b). Secretion from corticotrphs after aviden-fluoroscein stains for biotinylated ligands (CRF or AVP). *American Journal of Physiology* 252: E347-E356.

Childs GV & Unabia G (1990). Rapid corticotsterone inhibition of corticotropin-releasing

hormone binding and adrenocorticotropin release by enriched populations of corticotropes: counteractions by arginine vasopressin and its second messengers. *Endocrinology* 126: 1967-1975.

Chung S, Reinhart PH, Martin BL, Brautigan D & Levitan IB (1991). Protein kinase activity closely associated with a reconstituted calcium-activated potassium channel. *Science* 253: 560-562.

Conn PM, Rogers DC, Seay SG (1983). Structure-function relationship of calcium ion channel antagonists at the pituitary gonadotrope. *Endocrinology* 113: 1592-1595.

Corcuff J-B, Guerineau NC, Mariot P, Lussier BT, Mollard P (1993). Multiple cytosolic calcium signals and membrane electrical events evoked in single arginine vasopressin-stimulated corticotrophs. *The Journal of Biological Chemistry* 268: 22313-22321.

DeReimer SA, Strong JA, Albert KA, Greengard P & Kaczmarek LK (1985). Enhancement of calcium current in *Aplysia* neurones by phorbol ester and protein kinase C. *Nature* 313: 313-316.

Downes CP, Mussat MC & Michell RN (1982). The inositol trisphosphate phosphomonoesterase of the human erythrocyte membrane. *Biochemical Journal* 203: 169-177.

Drouva SV, Rerat E, Bihoreau C, Laplante E, Rasolonjanahary R, Clauser H & Kordon C (1988). Dihydropyridine-sensitive calcium channel activity related to prolactin, growth hormone, and luteinizing hormone release from anterior pituitary cells in culture: Interactions with somatostatin, dopamine, and estrogens. *Endocrinology* 123: 2762-2773.

Enyeart JJ, Aizawa T, Hinkle PM (1985). Dihydropyridine Ca^{2+} antagonists: potent

inhibitors of secretion from normal and transformed pituitary cells. *American Journal of Physiology* 248: C510-C519.

Farley J & Auerbach S (1986). Protein kinase C activation induces conductance changes in *Hermissenda* photoreceptors like those seen in associative learning. *Nature* 319: 220-223.

Giguere V, Lefevre G, Labrie F (1982). Site of calcium requirement for stimulation of ACTH release in rat anterior pituitary cells in culture by synthetic ovine corticotropin-releasing factor. *Life Sciences* 31: 3057-3062.

Gilles GE, Linton E & Lowry P (1982). Corticotropin releasing activity of the new CRF is potentiated several times by vasopressin. *Nature* 299: 355-357.

Gillies GE & Lowry PJ (1979). The relationship between vasopressin and corticotopin releasing factor. In: *Interactions between the Brain-Pituitary-Adrenocortical System*, ed MT Jones, B Gillham, MF Dallman & S Chattopadhyay. London, Academic, 1979 p. 51-61.

Gonzalez C, Almarez L, Obeso A & Rigual R (1992). Oxygen and acid chemoreception in the carotid body chemoreceptors. *Trends in Neurosciences* 15: 146-153.

Greenwood FC, Hunter WM & Glover JS (1963). The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. *Biochemical Journal* 89: 114-123.

Guardabasso V, Rodbard D, Munson PJ (1987). A model-free approach to estimation of relative potency in dose-response curve analysis. *American Journal of Physiology* 252: E357-E364.

Guerineau N, Corcuff J-B, Tabarin A & Mollard P (1991). Spontaneous and corticotropin-releasing factor-induced cytosolic calcium transients in corticotrophs.

Endocrinology 129: 409-420.

Guild S & Reisine T (1987). Molecular mechanisms of corticotropin-releasing factor stimulation of calcium mobilization and adrenocorticotropin release from anterior pituitary tumor cells. *The Journal of Pharmacology and Experimental Therapeutics* 241: 125-130.

Hartzell HC (1993). Filling the gaps in Ca^{2+} channel regulation. *Biophysical Journal* 65: 1358-1359.

Hoskins JM, Meynell GG & Sanders FK (1956). A comparison of methods for estimating the viable count of a suspension of tumor cells. *Experimental Cell Research* 11: 297-305.

Hoth M & Penner R (1992). Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature* 355: 353-356.

Irvine RF, Letcher AJ, Heslop JP & Berridge MJ (1986). The inositol tris/tetrakisphosphate pathway - demonstration of Ins(1,4,5)P_3 3-kinase activity in animal tissues. *Nature* 320: 631-634.

Jacob R (1990). Calcium oscillations in electrically non-excitable cells. *Biochimica et Biophysica Acta* 1052: 427-438.

Janis RA & Triggle DJ (1991). Drugs acting on calcium channels. In: *Calcium Channels: Their Properties, Functions, Regulation, and Clinical Relevance* (ed. L Hurwitz, LD Partridge, JK Leach), pp. 195-249. CRC Press, Boca Raton/Ann Arbor/Boston/London.

Jia L-G, Canny BJ, Orth DN & Leong DA (1991). Distinct classes of corticotropes mediate corticotropin-releasing hormone- and arginine vasopressin-stimulated

adrenocorticotropin release. *Endocrinology* 128: 197-203.

Johnson AJ (1992). The role of G proteins in ACTH secretion from ovine corticotrophs. Unpublished MSc Thesis, University of Canterbury, Christchurch, New Zealand. 83 pp.

Jones MT & Gillham B (1988). Factors involved in the regulation of adrenocorticotropic hormone/β-lipotropic hormone. *Physiological Reviews* 68: 743-818.

Judd AM, Jarvis WD & MacLeod RM (1987). Attenuation of pituitary polyphosphoinositide metabolism by protein kinase C activation. *Molecular and Cellular Endocrinology* 54: 107-114.

Kaczmarek LK (1988). The regulation of neuronal calcium and potassium channels by protein phosphorylation. In: *Advances in Second Messenger and Phosphoprotein Research, Vol. 22* (ed. P Greengard & GA Robison) pp. 113-138. Raven Press, New York.

Kidokoro Y (1975). Spontaneous Ca action potentials in a clonal pituitary cell line anf their relationship to prolactin secretion. *Nature* 258: 741-742.

Kikkawa U & Nishizuka Y (1986). The role of protein kinase C in transmembrane signalling. *Annual Review of Cell Biology* 2: 149-178.

King MS & Baertschi AJ (1990). The role of intracellular messengers in adrenocorticotropin secretion in vitro. *Experientia* 46: 26-40.

Koch B & Lutz-Butcher B (1991). Inhibition of protein kinase C activity in cultured pituitary cells attenuates both cyclic AMP-independent and dependent secretion of ACTH. *Molecular and Cellular Endocrinology* 77: 57-65.

Kokubin S & Reuter H (1984). Dihydropyridine derivatives prolong the open state of Ca channels in cultured cardiac cells. *Proceedings of the National Academy of Sciences, USA* 81: 4824--4827.

Knight DE, von Grafenstein H & Athayde CM (1989). Calcium-dependent and calcium-independent exocytosis. *Trends in Neurosciences* 12: 451-458.

Labrie F, Veilleux R, Lefevre G, Coy DH, Sueiras-Diaz J & Schally AV (1982). Corticotropin-releasing factor stimulates accumulation of adenosine 3',5'-monophosphate in rat pituitary corticotrophs. *Science* 216: 1007-1008.

Lacerda AE, Rampe D & Brown AM (1988). Effects of protein kinase C activators on cardiac Ca^{2+} channels. *Nature* 335: 249-251.

Lakshminarayanaiah N (1991). Effects of inorganic ions on calcium channels. In: *Calcium Channels: Their Properties, Functions, Regulation, and Clinical Relevance* (ed. L Hurwitz, LD Partridge, JK Leach), pp. 195-249. CRC Press, Boca Raton/Ann Arbor/Boston/London.

Le Beau AP (1989). Relative roles of intra- and extracellular calcium during vasopressin stimulated ACTH release from ovine anterior pituitary cells. Unpublished BSc (Hons) Project, University of Canterbury, Christchurch, New Zealand. 74 pp.

Le Beau AP & Mason DR (1994). The effects of a chemically diverse range of calcium channel antagonists on the AVP-stimulated ACTH response in ovine corticotrophs. *Cell Calcium* 16: 47-58.

Leong DA (1988). A complex mechanisms of facilitation in pituitary ACTH cells: Recent single cell studies. *Journal of Experimental Biology* 139: 151-168.

Levitan IB (1988). Modulation of ion channels in neurons and other cells. *Annual Review*

of Neurosciences 11: 119-136.

Lewis DL & Weight FF (1988). The protein kinase C activator 1-oleoyl-2-acetylglycerol inhibits voltage-dependent Ca^{2+} current in the pituitary cell line AtT-20. *Neuroendocrinology* 47: 169-175.

Liu J-P, Engler D, Funder JW & Robinson PJ (1992). Evidence that the stimulation by arginine vasopressin of the release of adrenocorticotropin from the ovine anterior pituitary involves the activation of protein kinase C. *Molecular and Cellular Endocrinology* 87: 35-47.

Liu J-P, Robinson PJ, Funder JW & Engler D (1990). The biosynthesis and secretion of adrenocorticotropin by the ovine anterior pituitary is predominantly regulated by arginine vasopressin (AVP). *The Journal of Biological Chemistry* 265: 14136-14142.

Livesey JH (1974). Computation of radioimmunoassay data using sequentially linearized curves. *Computers Biomedical Research* 7: 7-20.

Livesey JH & Donald RA (1982). Prevention of absorption losses during radioimmunoassay of polypeptide hormones: effectiveness of albumins, gelatin, casein, Tween 20 & plasma. *Clinica Chimica Acta* 123: 193-198.

Lu H-K, Fern RJ, Nee JJ & Barrett PQ (1994). Ca^{2+}-dependent activation of T-type Ca^{2+} channels by calmodulin-dependent protein kinase II. *American Journal of Physiology* 267: F183-F189.

Luini A, Lewis D, Guild S, Corda D & Axelrod J (1985). Hormone secretagogues increase cytosolic calcium by increasing cAMP in corticotropin-secreting cells. *Proceedings of the National Academy of Sciences, USA* 82: 8034-8038.

Ma J, Gutiérrez LM, Hosey MM & Ríos E (1992). Dihydropyridine-sensitive skeletal muscle Ca channels in polarized planar bilayers. *Biophysical Journal* 63: 639-647.

Marchetti C & Brown AM (1988). Protein kinase activator 1-oleoyl-2-acetyl-*sn*-glycerol inhibits two types of calcium currents in GH3 cells. *American Journal of Physiology* 254: C206-C210.

Marchetti C, Childs GV & Brown AM (1987). Membrane currenst of identified isolated rat corticotropes and gonadotropes. *American Journal of Physiology* 252: E340-E346.

Martini L & Morpurgo C (1955). Neurohumoral control of release of adrenocorticotropic hormone. *Nature* 175: 1127-1128.

Mason DR (1988). The effect of vasopressin, phorbol ester and a calcium ionophore on ACTH release from ovine pituitary cells. *Proceedings of the Endocrine Society of Australia*, Abstract no. 532.

Mason DR, Le Beau AP & Hagan C (1989). Relative roles of intra- and extra-cellular calcium during vasopressin stimulated ACTH release from ovine pituitary cells. *Proceedings of the Endocrine Society of Australia*, Abstract no. 47.

Mason WT, Rawlings SR, Cobbett P, Sikdar SK, Zorec R, Akerman SN, Benham CD, Berridge MJ, Cheek T & Moreton RB (1988). Control of secretion in anterior pituitary cells - linking ion channels, messengers and exocytosis. *Journal of Experimental Biology* 139: 287-316.

Mason WT & Waring DW (1985). Electrophysiological recordings from gonadotrophs. Evidence for Ca^{2+} channels mediated by gonadotrophin-releasing hormone. *Neuroendocrinology* 41: 258-268.

Mason WT & Waring DW (1986). Patch clamp recordings of single ion channel activation by gonadotrophin-releasing hormone in ovine pituitary gonadotrophs. *Neuroendocrinology* 43: 205-219

McCann SM (1957). The ACTH-releasing activity of extracts of the posterior lobe of the pituitary in vivo. *Endocrinology* 60: 664-676.

Meldolesi J, Volpe P & Pozzan T (1988). The intracellular distribution of calcium. *Trends in Neurosciences* 11: 449-452.

Meldolesi J, Clementi E, Fasolato C Zacchetti D & Pozzan T (1991). Ca^{2+} following receptor activation. *Trends in Pharmacological Sciences* 12: 289-291.

Miller RJ (1987). Multiple calcium channels and neuronal function. *Science* 235: 46-52.

Mollard P, Vacher P, Guerin J, Rogawski MA & Dufy B (1987). Electrical properties of cultured human adrenocorticotropin-secreting adenoma cells: Effects of high K^{+}, corticotropin-releasing factor, and angiotensin II. *Endocrinology* 121: 395-405.

Mollard P, Vacher P, Rogawski MA & Dufy B (1988). Vasopressin enhances a calcium channel in human ACTH-secreting pituitary adenoma cells. *FASEB Journal* 2: 2907-2912.

Murakami K, Hashimoto K & Ota Z (1985). The effect of nifedipine on CRF-41 and AVP-induced release in vitro. *Acta Endocrinologica* 109: 32-36.

Negulescu PA & Machen TE (1988). Release and reloading of intracellular Ca stores after cholinergic stimulation of the parietal cell. *American Journal of Physiology* 254: C498-C504.

Nishizuka Y (1984). The role of protein kinase C in cell surface transduction and tumour

promotion. *Nature* 308: 693-698.

Nowycky MC, Fox AP & Tsien RW (1985). Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature* 316: 440-443.

O'Callahan CM, Ptasienski J & Hosey MM (1988). Phosphorylation of the 165-kDa dihydropyridine/phenylalkylamine receptor from skeletal muscle by protein kinase C. *The Journal of Biological Chemistry* 263: 17342-17349.

Oki Y, Nicholson WE & Orth DN (1990). Role of protein kinase C in the adrenocorticotropin secretory response to arginine vasopressin (AVP) and the synergistic response to AVP and corticotropin-releasing factor by perifused rat anterior pituitary cells. *Endocrinology* 127: 350-357.

Oki Y, Peatman TW, Qu Z-C & Orth DN (1991). Effects of intracellular Ca^{2+} depletion and glucocorticoid on stimulated adrenocorticotropin release by rat anterior pituitary cells in a microperifusion system. *Endocrinology* 128: 1589-1596.

Ono K & Fozzard HA (1992). Phosphorylation restores activity of L-type calcium channels after rundown in inside-out patches from rabbit cardiac cells. *Journal of Physiology* 454: 673-688.

Ono K & Fozzard HA (1993). Two phosphatase sites on the Ca^{2+} channel affecting different kinetic functions. *Journal of Physiology* 470: 73-84.

Ozawa S & Sand O (1986). Electrophysiology of excitable endocrine cells. *Physiological Reviews* 66: 887-952.

Penner R, Fasolato C & Hoth M (1993). Calcium influx and its control by calcium release. *Current Opinion in Neurobiology* 3: 368-374.

Perrin MH, Haas Y, Rivier J & Vale WW (1986). Corticotropin-releasing factor binding to the anterior pituitary receptor is modulated by divalent cations and guanyl nucleotides. *Endocrinology* 118: 1171-1179.

Putney JW Jr (1986). A model for receptor-regulated calcium entry. *Cell Calcium* 7: 1-12.

Putney JW Jr (1990). Capacitative calcium entry revisited. *Cell Calcium* 11: 611-624.

Putney JW Jr & Bird G St. J. (1993). The signal for capacitative calcium entry. *Cell* 75: 199-201.

Rane SG & Dunlop K (1986). Kinase C activator 1,2-oleoylacetylglycerol attenuates voltage-dependent calcium currents in sensory neurons. *Proceedings of the National Academy of Sciences, USA* 83: 184-188.

Raymond V, Leung PCK, Veilleux R & Labrie F (1985). Vasopressin rapidly stimulates phosphatidic acid-phosphatidylinositol turnover in rat anterior pituitary cells. *FEBS Letters* 182: 196-200.

Reeves R, Farley J & Rudy B (1986). c-AMP dependent protein kinase opens several K^+ channels from mammalian brain. *Society of Neuroscience Abstracts* 13: 1343.

Reisine T (1989). Phorbol esters and corticotropin releasing factor stimulate calcium influx in the anterior pituitary tumor cell line, AtT-20, through different intracellular sites of action. *The Journal of Pharmacology and Experimental Therapeutics* 248: 984-990.

Reisine T & Guild S (1987). Activators of protein kinase C and cyclic AMP-dependent protein kinase regulate intracellular calcium levels through distinct mechanisms in mouse anterior pituitary tumor cells. *Molecular Pharmacology* 32: 488-496.

Rink TJ & Hallam TJ (1989). Calcium signalling in non-excitable cells: Notes on oscillations and store refilling. *Cell Calcium* 10: 385-395.

Rossier MF & Putney JW Jr (1991). The identity of calcium-storing, inositol 1,4,5-trisphosphate-sensitive organelle in non-muscle cells: calciosome, endoplasmic reticulum...or both? *Trends in Neurosciences* 14: 310-314.

Rougon G, Barbet J & Reisine T (1989). Protein phosphorylation induced by phorbol esters and cyclic AMP in anterior pituitary cells: possible role in adrenocorticotropin release and synthesis. *Journal of Neurochemistry* 52: 1270-1278.

Saida K & van Breemen C (1983). Mechanism of Ca^{++} antagonist-induced vasodilation. *Circulation Research* 52: 137-142.

Shangold GA, Murphy SN & Miller RJ. (1988). Gonadotropin-releasing hormone-induced Ca^{2+} transients in single identified gonadotropes require both intracellular Ca^{2+} mobilization and Ca^{2+} influx. *Proceedings of the National Academy of Sciences, USA* 85: 6566-6570.

Shen PJ, Clarke IJ, Canny BJ, Funder JW & Smith AI (1990). Arginine vasopressin and corticotropin releasing factor: binding to ovine anterior pituitary membranes. *Endocrinology* 127: 2085-2089.

Sikdar SK, McIntosh RP & Mason WT (1989). Differential modulation of Ca^{2+}-activated K^+ channels in ovine pituitary gonadotrophs by GnRH, Ca^{2+} and cyclic AMP. *Brain Research* 496: 113-123.

Snutch TP & Reiner PB (1992). Ca^{2+} channels: diversity of form and function. *Current Opinion in Neurobiology* 2: 247-253.

Sobel D (1988). Characterization of 12-O-tetradecanoyl-phorbol-13-acetate mediated ACTH release. *Endocrine Research* 14: 149-163.

Stojilkovic SS & Catt KJ (1992). Calcium oscillations in anterior piruitary cells. *Endocrine Reviews* 13: 256-280.

Stojilkovic SS, Chang JP, Izumi S-I, Tasaka K & Catt KJ (1988a). Mechanisms of secretory responses to gonadotropin-releasing hormone and phorbol esters in cultured pituitary cells. *Journal of Biological Chemistry* 263: 17301-17306.

Stojilkovic SS, Chang JP, Ngo D & Catt KJ (1988b). Evidence for a role of protein kinase C in luteinizing hormone synthesis and secretion. *Journal of Biological Chemistry* 263: 17307-17311.

Stojilkovic SS, Iida T, Virmani MA, Izumi S-I, Rojas E & Catt KJ (1990a). Dependence of hormone secretion on activation-inactivation kinetics of voltage-sensitive Ca^{2+} channels in pituitary gonadotrophs. *Proceedings of the National Academy of Sciences, USA* 87: 8855-8859.

Stojilkovic SS, Iida T, Merelli F, Torsello A, Krsmanovic LZ & Catt KJ (1991). Interactions between calcium and protein kinase C in the control of signaling and secretion in pituitary gonadotrophs. *The Journal of Biological Chemistry* 266: 10377-10384.

Stojilkovic SS, Izumi S & Catt KJ (1988c). Participation of voltage-sensitive calcium channels in pituitary hormone release. *The Journal of Biological Chemistry* 263: 13054-13061.

Stojilkovic SS, Stutzin A, Izumi S, Dufour S, Torsello A, Virmani MA, Rojas E & Catt KJ (1990b). Generation and amplification of the cytosolic calcium signal during secretory responses to gonadotropin-releasing hormone. *The New Biologist* 2: 272-

283.

Suprenant A (1982). Correlation between electrical activity and ACTH/β-endorphin secretion in mouse pituitary tumor cells. *Journal of Cell Biology* 95: 559-566.

Todd K & Lightman SL (1987). Vasopressin activation of phosphatidylinositol metabolism in rat anterior pituitary in vitro and its modification by changes in the hypothalamo-pituitary-adrenal axis. *Neuroendocrinology* 45: 212-218.

Tse A, Tse FW, Almers W & Hille B (1993). Rhythmic exocytosis stimulated by GnRH-induced calcium oscillations in rat gonadotropes. *Science* 260: 82-84.

Tsien RW, Lipscombe D, Madison DV, Bley KR & Fox AP (1988). Multiple types of neuronal calcium channels and their selective modulation. *Trends in Neurosciences* 11: 431-438.

Tsien RW & Tsien RY (1990). Calcium channels, stores, and oscillations. *Annual Review of Cell Biology* 6: 715-760.

Vale W, Spiess J, Rivier C & Rivier J (1981). Characterisation of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and β-endorphin. *Science* 213: 1394-1397.

Vale W, Vaughn J, Smith M, Yamamoto G, Rivier J & Rivier C (1983). Effects of synthetic ovine corticotropin-releasing factor, glucocorticoids, catecholamines, neurohypophysial peptides, and other substances on cultured corticotropic cells. *Endocrinology* 113: 1121-1131.

Westland KN, Aguilera G & Childs GV (1985). Quantification of morphological changes in pituitary corticotropes produced by in vitro corticotropin-releasing factor stimulation and adrenalectomy. *Endocrinology* 116: 439-445.

Won JGS, Oki Y & Orth DN (1990). Roles of intracellular and extracellular calcium in the kinetic profile of adrenocorticotropin secretion by perifused rat anterior pituitary cells. II. Arginine vasopressin, oxytocin, and angiotensin-II stimulation. *Endocrinology* 126: 858-868.

Won JGS & Orth DN (1990). Roles of intracellular and extracellular calcium on the kinetic profile of adrenocorticotropin secretion by perifused anterior pituitary cells. I. Corticotropin-releasing factor stimulation. *Endocrinology* 126: 849-857.

Zatz M, Mahan LC & Reisine T (1987). Translocation of protein kinase C in anterior pituitary cells. *Journal of Neurochemistry* 48: 106-110.

Appendix I

The sources of materials used in this study are given below. All reagents were analytical reagent grade.

Material	Source
Adrenocorticotropin (ACTH, highly purified)	courtesy of Dr CH Li, Hormone Research Laboratory, University of California, San Francisco, CA, USA.
Alkali-treated casein (ATC)	prepared by Dr DR Mason
Anti-ACTH antiserum	courtesy Professor RA Donald, Department of Endocrinology, Christchurch Hospital, Christchurch, New Zealand
Amphotericin B	Sigma Chemical Co., St. Louis, Missouri, USA
[Arg^8(inine)]- vasopressin (AVP)	Sigma Chemical Co.
Bovine serum albumin (BSA)	Behring, Germany
Cadmium chloride ($CdCl_2$)	Ajax Chemical Ltd., Sydney, Australia
Calcium chloride ($CaCl_2$)	May & Baker Ltd., Australia
Choline chloride	BDH Chemicals Ltd.
Cobalt chloride ($CoCl_2$)	Sigma Chemical Co.
Collagenase (Type II) (*Clostridium histolyticum*)	Sigma Chemical Co.
D(+)-glucose	May & Baker Ltd.
Diltiazem	Sigma Chemical Co.
Dimethylsulfoxide (DMSO)	BDH Chemicals Ltd.
di-Sodium hydrogen orthophosphate (Na_2HPO_4, anhydrous)	BDH Chemicals Ltd.
Dulbecco's modified eagle medium (DME) (commercial powder)	Sigma Chemical Co.
Ethylenediaminetetraacetic acid (Na_2EDTA, disodium salt)	BDH Chemicals Ltd.
Ethyleneglycoltetraacetic acid (EGTA)	Sigma Chemical Co.
HEPES (N-2-hydroxyethylpiperazine -N'-2-ethanesulfonic acid)	Sigma Chemical Co.

Horse plasma	c/o Dr SL Alexander, Lincoln University, Lincoln, New Zealand
L-glutamate	Sigma Chemical Co.
Magnesium sulphate ($MgSO_4$, anhydrous)	BDH Chemicals Ltd.
Methoxyverapamil (D600)	Sigma Chemical Co.
Newborn calf serum (NCS)	Life Technologies
Nifedipine	Sigma Chemical Co.
Non-essential amino acids	Sigma Chemical Co.
Penicillin G	Sigma Chemical Co.
Phenol red	Sigma Chemical Co.
Polyethyleneglycol 6000 (PEG)	BDH Chemical Ltd.
Potassium chloride (KCl)	May & Baker Ltd.
Potassium dihydrogen orthophosphate (KH_2PO_4)	BDH Chemicals Ltd.
Silicic acid powder (100 mesh)	Mallinkrodt
Sodium azide (NaN_3)	BDH Chemicals Ltd.
Sodium chloride (NaCl)	May & Baker Ltd.
Sodium hydrogen carbonate ($NaHCO_3$)	May & Baker Ltd.
Streptomycin sulphate	Sigma Chemical Co.
12-*O*-tetradecanoylphorbol 13-acetate (TPA)	Sigma Chemical Co.
Tetraethylammonium chloride (TEA-Cl)	Sigma Chemical Co.
Tissue culture plates	Nunc Intermed
Triton X-100	Sigma Chemical Co.
Trypan blue	BDH Chemicals Ltd.
Tubes (polystyrene, length 75 mm, diameter 12 mm)	Gallanti, Auckland, New Zealand

Appendix II

Solutions used for cell culture

Dispersing buffer (DB)

The reagents listed below were dissolved in deionised, distilled water (ddH_2O), and the solution made up to 1 l, such that the reagents were at the concentration shown. The pH was adjusted to 7.3 with concentrated HCl or NaOH and the solution filter-sterilised (0.45 μM filter) by negative pressure filtration.

NaCl	137 mM
KCl	5.0 mM
Glucose	10.0 mM
HEPES	25 mM
Phenol red	0.056 mM
Penicillin G	100 U/ml
Streptomycin sulphate	100 μg/ml
Amphotericin B	0.25 μg/ml

Dulbecco's Modified Eagle's Medium (DME)

One bottle of commercially prepared powered medium (Sigma Chemical Co.) was dissolved in ddH_2O and supplemented with the reagents listed below to the concentrations shown. The solution was made up to 1 l (with ddH_2O), adjusted to pH 7.1 and filter sterilized, as above. After 2 - 3 weeks storage fresh L-glutamate was added.

$NaHCO_3$	44.0 mM
HEPES	25 mM
MEM non-essential amino acids	1.0 ml
Penicillin G	100 U/ml
Streptomycin sulphate	100 μg/ml
Amphotericin B	0.25 μg/ml

Solutions used for experimental procedures

Krebs Ringer (KR)

The reagents listed below were dissolved in ddH_2O and the solution was made up to 500 ml, such that the reagents were at the concentrations shown. The pH was adjusted to 7.3 and the solution was filter sterilized, as above. Before use, 5% NCS and ascorbic acid (50 μg/ml) were added.

NaCl	125 mM
KCl	4.7 mM
$CaCl_2.2H_2O$	2.5 mM
KH_2PO_4	1.2 mM
$MgSO_4.7H_2O$	1.2 mM
$NaHCO_3$	3.6 mM
HEPES	25 mM
Glucose	10 mM
Phenol red	0.00065 g
Penicillin G	100 U/ml
Streptomycin sulphate	100 μg/ml
Amphotericin B	0.25 μg/ml

KR containing AVP

Stock solutions of AVP were prepared at concentrations of 200 μM or 2000 μM by dissolving the peptide directly into KR + bovine serum albumin (BSA) + ascorbic acid. The solution was aliquoted and stored at -80°C. Serial dilution of the stock solution was used to prepare the appropriate concentrations of AVP for cell experiments and these were kept chilled until use.

KR containing Ca^{2+} channel antagonists, with or without AVP

Solutions containing the channel blockers were prepared on the day of each experiment, under low light conditions where necessary. Nifedipine, diltiazem, $CoCl_2$ and $CdCl_2$ were dissolved directly in KR+NCS. For $CoCl_2$, the concentration of NaCl was adjusted to maintain osmolarity. D600 was first dissolved in dimethylsulfoxide (DMSO), and then diluted to appropriate concentrations with KR+NCS. The final concentration of DMSO in the experimental buffer did not exceed 0.2%. This concentration of DMSO did not, in itself, affect ACTH release. DMSO was found to be an unsuitable vehicle for nifedipine.

Solutions for RIA

Stock phosphate buffer (0.5 M)

The reagents listed below were dissolved into ddH_2O. The solution was made up to 1 l and stored at -20°C.

KH_2PO_4 (anhydrous)	132 mM
Na_2HPO_4 (anhydrous)	368 mM
Na_2EDTA	0.27 mM
NaN_3	31 μM

P/ATC (0.05 M phosphate buffer + 0.1% alkali treated casein)

A 0.05 M phosphate buffer containing 0.1% alkali treated casein (ATC) was prepared by diluting the stock 0.5 M phosphate buffer 1:10 (v:v) with ddH_2O and adding 0.1% ATC. The pH of the solution was adjusted to 7.4.

Polyethylene glycol 6000 Solution (PEG)

An 18% PEG/0.1 M NaCl solution in ddH_2O was prepared with the reagents listed below and stored at room temperature.

PEG 6000	18%
NaCl	0.1 M
0.5 M stock phosphate buffer	0.05 M
Triton X-100	0.1%