DESENSITIZATION OF THE ADRENOCORTICOTROPIN RESPONSE TO ARGININE VASOPRESSIN

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CONTENTS

ABSTRACT

The hypothalamic peptide arginine vasopressin (AVP) is an important regulator of adrenocorticotropin (ACTH) release from the anterior pituitary. AVP stimulates ACTH secretion from corticotroph cells by activating the V1b AVP receptor, a member of the G protein-coupled receptor (GPCR) family which activates the phosphoinositide signalling pathway. *In vivo*, persistent or repeated stress can result in reduced ACTH responsiveness and it appears that regulation of the V1b receptor plays an important role in this process. Similarly, repeated or prolonged stimulation of anterior pituitary cells with AVP *in vitro* results in a reduction in ACTH responsiveness, or desensitization. The aims of this study were to characterize this desensitization process and to investigate the mechanisms underlying it.

Desensitization of the ACTH response to AVP was investigated in perifused dispersed ovine anterior pituitary cells. Desensitization was found to be rapid, readily reversible and occurred at relatively low AVP concentrations. Treatment with 5 nM AVP for 10 min reduced the response to a subsequent stimulation with 100 nM AVP by 39.4 ± 8.6%, but treatment for longer durations at this concentration did not increase the magnitude of desensitization. Recovery of responsiveness to a subsequent stimulation with AVP was complete within 40 min following pre-treatment with 10 nM AVP for 15 min. Desensitization was found to occur at concentrations and

durations of AVP pre-treatment which are within the range of endogenous AVP pulses, suggesting that it plays an important physiological role in the regulation of ACTH secretion. The very rapid kinetics of desensitization and resensitization of the ACTH response to AVP suggest that these processes are more important in regulating the acute response to AVP than the alterations of corticotroph responsiveness which have been observed during chronic stress.

The characteristics of the desensitization process suggested that it might be caused by uncoupling of the pituitary AVP receptor from its signalling pathway, a common mechanism of desensitization amongst GPCRs which is often mediated by receptor phosphorylation. Investigations showed that the protein kinases protein kinase C (PKC) and casein kinase 1α, were not involved in desensitization. Activation of PKC by treatment with the diacylglycerol analogue 1,2-dioctanoyl-*sn*-glycerol did not result in desensitization and inhibition of PKC activity by treatment with Ro 31-8220 did not prevent AVP-induced desensitization. Similarly, treatment with the casein kinase 1 inhibitor CK1-7 did not prevent AVP-induced desensitization.

Receptor internalization and dephosphorylation by protein phosphatases are important in the resensitization of many GPCRs. Interestingly, pharmacological inhibition of receptor internalization reduced the extent of *desensitization* which could be induced by AVP pre-treatment, suggesting that sequestration of receptors to an intracellular compartment is important in the desensitization process. Inhibition of protein phosphatase 2B by treatment with FK506 slowed the rate of recovery following desensitization, suggesting that this enzyme plays an important role in the resensitization process. No evidence could be found for the involvement of protein phosphatase 2A in resensitization of the ACTH response to AVP.

ABBREVIATIONS

^{125}I-AVP	(3-[^{125}I]iodotyrosyl^{2})Vasopressin[Arg^{8}]
^{125}I-oACTH	^{125}I-labelled oACTH
ACTH	adrenocorticotropin
ATC	alkali-treated casein
AVP	arginine vasopressin
CalyA	calyculin A
cAMP	cyclic AMP
CHO	Chinese hamster ovary
CK1α	casein kinase 1α
ConA	concanavalin A
CRH	corticotropin-releasing hormone
DAG	diacylglycerol
DB	dispersing buffer
ddH_2O	distilled, deionized H_2O
DiC_8	1,2-dioctanoyl-*sn*-glycerol
DME	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DSS	disuccinimidyl suberate
GFP	green fluorescent protein
GnRH	gonadotropin-releasing hormone
GPCR	G protein-coupled receptor

GRK	G protein-coupled receptor kinase
HPA axis	hypothalamo-pituitary-adrenal
HPLC	high performance liquid chromatography
IB	inhibitor buffer
IP_3	inositol 1,4,5-trisphosphate
KR	Krebs ringer
KR/ATC	Krebs Ringer + 0.05% ATC + 0.005% L-ascorbate
L-VSCC	L-type voltage-stimulated Ca^{2+} channel
MARCKS	myristoylated alanine-rich cytosolic kinase substrate
NCS	new-born calf serum
NS	not significant
NSB	non-specific binding
OA	okadaic acid
oACTH	ovine ACTH
OAG	1-oleoyl-2-acetyl-*sn*-glycerol
P/ATC	0.05 M phosphate buffer + 0.1% ATC
PAB	protein assay buffer
PAO	phenylarsine oxide
PDBu	phorbol-12,13-dibutyrate
PEG	polyethylene glycol solution
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol-12-myristate-13-acetate
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PP2B	protein phosphatase 2B
RIA	radioimmunoassay

SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCA	trichloroacetic acid

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INTRODUCTION

1.1 REGULATION OF PITUITARY ADRENOCORTICOTROPIN SECRETION DURING STRESS

1.1.1 THE HYPOTHALAMO-PITUITARY-ADRENAL AXIS

The survival and independent existence of higher animals is dependent upon their ability to maintain a constant internal milieu despite changes in their external environment. Adaptation to such external disturbances, or stress, requires the concerted action of a variety of behavioural, visceral and endocrine systems (Aguilera 1994).

The key endocrine system involved in the vertebrate response to stress is the hypothalamo-pituitary-adrenal (HPA) axis (Fig. 1.1). Exposure to a stressful stimulus is followed by rapid increases in secretion of adrenocorticotropin (ACTH) by corticotroph cells of the anterior pituitary (Harbuz & Lightman 1992). Once released, ACTH is carried in the circulation to the adrenal cortex where it stimulates the release of glucocorticoid steroid hormones. These mediate the peripheral responses to stress (Johnson *et al.* 1992). The regulation of ACTH secretion from the anterior pituitary is a multifactorial process which is dependent largely upon hypothalamic neuropeptides that are carried to the pituitary in the hypophyseal portal circulation (Guillaume *et al.* 1992b).

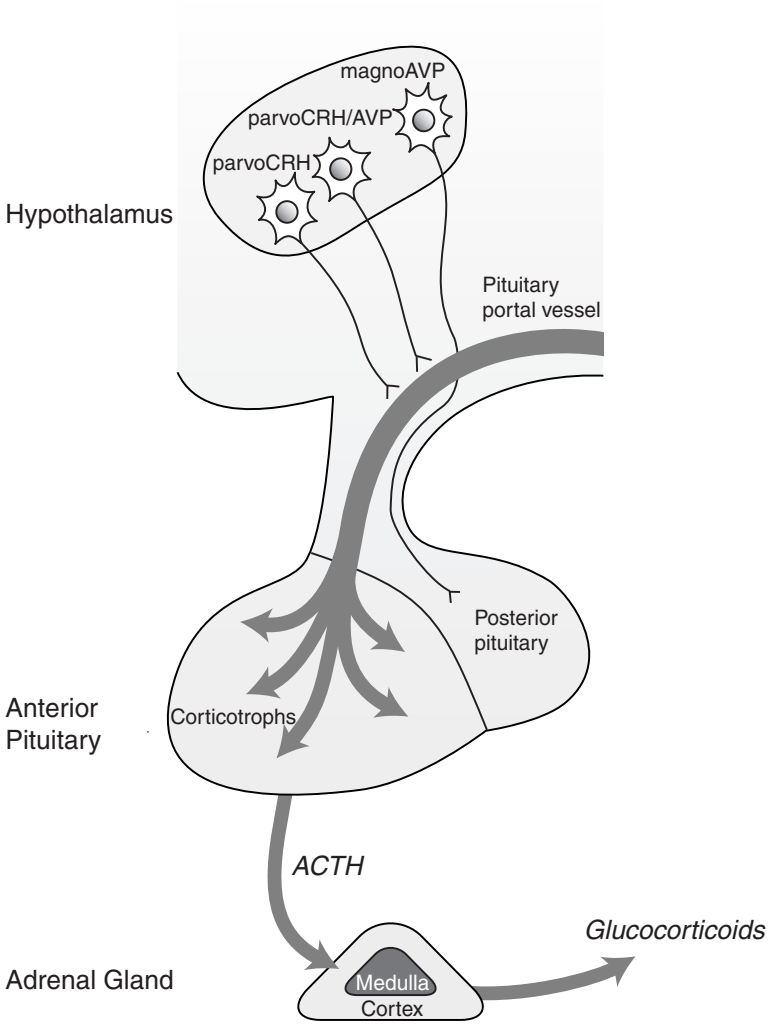

Fig. 1.1. The HPA axis. CRH and AVP are synthesized in parvocellular neurons of the paraventricular nucleus and released from nerve terminals in the median eminence into the hypophyseal portal blood. AVP is also produced by magnocellular neurons of the paraventricular and supraoptic nuclei which project to the posterior pituitary and may release AVP as they pass through the median eminence. AVP and CRH stimulate ACTH secretion from corticotrophs of the anterior pituitary. ACTH induces the release of glucocorticoids from the adrenal cortex.

While a number of hypothalamic factors are capable of acutely stimulating ACTH secretion, the two most potent secretagogues are corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). These interact with specific receptors located in the plasma membrane of the pituitary corticotroph, thereby stimulating ACTH secretion (Aguilera 1994).

During adaptation to chronic stress pituitary ACTH responses undergo regulatory changes (Aguilera 1994). While a variety of mechanisms may be responsible for these changes it has been suggested that because there is a good correlation between ACTH responsiveness and pituitary AVP receptor content, regulation of this receptor may be particularly important in controlling the ACTH responsiveness of corticotrophs (Aguilera *et al.* 1994). Commonly, the response of hormone receptors is regulated by negative feedback loops within the cell which result in a desensitization of the response to either a repeated or continuous stimulus (Lohse 1993). *In vitro* studies have shown that the ACTH response of pituitary cells to AVP is desensitized following prolonged exposure to AVP, however this process is poorly characterized and the molecular mechanisms involved are unknown (Holmes *et al.* 1984; Evans *et al.* 1988). The aims of this study were to use an *in vitro* approach to characterize the desensitization of the ACTH response to AVP in ovine anterior pituitary cells and to investigate the mechanisms involved in this process.

1.1.2 VASOPRESSINERGIC CONTROL OF ACTH SECRETION

Although it has been known since the 1950s that AVP is capable of stimulating ACTH release from the anterior pituitary (*e.g.* see Martini & Morpurgo 1955) it is only relatively recently that the important physiological role played by this neuropeptide in the

HPA axis has become clear. Previously attention had been focused on CRH as the physiological regulator of ACTH secretion (Antoni 1993).

Two types of hypothalamic neuron are capable of producing and secreting AVP: magnocellular neurons of the supraoptic and paraventricular nuclei and parvocellular CRH neurons of the paraventricular nuclei (Antoni 1993). There are two types of parvocellular CRH neuron, one containing CRH only, and a second in which CRH and AVP are co-localized (Whitnall *et al.* 1985; Sawchenko *et al.* 1984), apparently within the same secretory vesicles (Whitnall *et al.* 1987). While magnocellular AVP may be responsible for increased ACTH secretion during some acute stress conditions (*e.g.* saline infusion [Irvine *et al.* 1989]) most evidence suggests that AVP from parvocellular neurons, in combination with CRH, is responsible for the regulation of ACTH secretion during chronic stress (Aguilera 1994).

Exposure to a variety of different types of stress has been shown to increase the secretion of AVP into the hypophyseal portal circulation. In experiments conducted with conscious, unrestrained sheep insulin-induced hypoglycaemia (Engler *et al.* 1989; Caraty *et al.* 1990), audiovisual stress (Engler *et al.* 1989), acute haemorrhagic stress (Caraty *et al.* 1988), anaesthesia (Engler *et al.* 1989) and endotoxin treatment (Battaglia *et al.* 1998) all caused pulsatile secretion of AVP into the portal circulation and these increases were associated with elevated ACTH concentrations in systemic blood. These observations are consistent with the hypothesis that AVP is involved in the regulation of ACTH secretion from the anterior pituitary. Similar patterns of hormone secretion have been shown to occur during stress in the rat (Plotsky *et al.* 1985a; Plotsky *et al.* 1985b), monkey (Zimmerman *et al.* 1973) and horse (Alexander *et al.* 1991; Alexander *et al.* 1994).

Blockade of the action of AVP, either through neutralization using specific antibodies or treatment with selective AVP antagonists, has firmly established that endogenous AVP plays a physiological role in the regulation of ACTH secretion. Using an anti-AVP antibody Guillaume *et al.* (1992b) selectively immunoneutralized AVP in intact rams. This treatment significantly reduced the ACTH and cortisol responses to two types of stress (insulin-induced hypoglycaemia and isolation-restraint). Similar reductions in the ACTH response to both restraint and formalin stress were observed in anti-AVP immunoneutralized rats (Linton *et al.* 1985). In the rat, treatment with the vasopressin antagonist 1-deaminopenicillamine-2-(*O-methyl*)tyrosine vasopressin reduces the ACTH response to insulin-induced hypoglycaemia (Plotsky *et al.* 1985b) and haemorrhagic stress (Plotsky *et al.* 1985a). Additionally, the ACTH and cortisol responses to stress in Brattleboro rats, which are genetically lacking in AVP, are significantly reduced compared to the response observed in normal rats. (Conte-DeVolx *et al.* 1982).

In many instances the secretion of CRH and AVP into the hypophyseal portal circulation occurs simultaneously (Engler *et al.* 1989). Although the data described above indicate a role for AVP in the regulation of ACTH secretion, its relative importance and in particular its interaction with CRH remain a matter of debate. The ability of the two hormones to stimulate ACTH secretion *in vitro* appears to vary depending on the species. In both the rat (Vale *et al.* 1983; Watanabe & Orth 1988) and horse (Evans *et al.* 1993) CRH is a much more potent ACTH secretagogue than AVP. There is a degree of controversy regarding the relative importance of the two hormones in the regulation of ACTH secretion in the sheep (McFarlane *et al.* 1995). Familari *et al.* (1989) found that AVP was considerably more potent than CRH whereas Evans *et al.* (1988) reported that AVP was less potent than CRH. In all cases a marked synergism has been

observed to occur both *in vivo* and *in vitro* (Evans *et al.* 1996). That is, when CRH and AVP are given together ACTH output is greater than the added effect of each hormone alone (see Antoni]1986] for review).

It has been proposed that this synergism may allow CRH to function as a permissive signal, setting the overall responsiveness of the corticotroph, while AVP is the main dynamic hypothalamic ACTH-releasing factor (Antoni 1993). ACTH is released into the portal circulation in a pulsatile manner with two main independent rhythms: one with short duration and a frequency of approximately 3/h and another with ultradian rhythm of approximately 2 h (Carnes *et al.* 1989). Studies in the sheep in which the two hormones are immunoneutralized have shown that CRH and AVP have different effects on these rhythms. Basal ACTH secretion and the number and mean amplitude of ACTH pulses were reduced following immunoneutralization of CRH but were unaffected by immunoneutralization of AVP. In both instances the responses to acute stress were reduced (Guillaume *et al.* 1992a; Guillaume *et al.* 1992b). In the rat administration of a CRH antagonist reduced mean plasma ACTH levels without affecting pulse frequency and duration of the pulses, whereas an AVP antagonist reduced frequency and duration of the pulses but not mean ACTH values (Negro-Vilar *et al.* 1987). Also, short term changes in ACTH secretion have been found to correlate more closely with AVP release than with CRH release in the horse. Using a novel technique, Alexander *et al.* (1991) sampled pituitary venous blood from conscious, unanaesthetized horses at intervals of approximately 30 s during acute exercise. A brief high-speed gallop was found to cause a transient increase in ACTH secretion which occurred at the same time as AVP secretion, while CRH levels were unchanged. Similarly, in the resting horse there is a very close and consistent relationship between the secretion of micropulses of AVP from the hypothalamus and the secretion of ACTH from the pitu-

itary, suggesting that AVP is the primary signal for pulsatile secretion of ACTH (Alexander *et al.* 1994).

In addition to the stimulatory effects of hypothalamic factors such as CRH and AVP, the HPA axis is under negative feedback regulation by glucocorticoids (Keller-Wood & Dallman 1984). At the hypothalamus both expression and release of CRH and AVP are inhibited (Spinedi *et al.* 1991; Ma & Aguilera 1999), and at the pituitary POMC transcription and ACTH secretion are inhibited (Levin & Roberts 1991). As such, removal of glucocorticoid negative feedback by adrenalectomy results in a marked activation of the HPA axis with increases in secretion of CRH, AVP and ACTH (Aguilera 1994).

1.1.3 MECHANISMS OF AVP-STIMULATED ACTH SECRETION

As described above, the main targets of AVP and CRH in the anterior pituitary are the ACTH-producing corticotrophs. Most investigations into the mechanisms of hormone-stimulated ACTH secretion have been carried out *in vitro* using one of four experimental systems: primary cultures of dispersed anterior pituitary cells, acutely prepared isolated cells, anterior pituitary segments or corticotroph adenoma cells (most commonly the ACTH-secreting AtT-20 mouse tumour cell line)(Watanabe & Orth 1988; Antoni 1993). These studies have shown that both AVP and CRH bind to specific, distinct cell surface receptors, thereby triggering intracellular signalling cascades which ultimately result in increased secretion of ACTH (Antoni 1993). Binding of AVP to its receptor results in activation of the phosphoinositide signalling pathway (see Fig. 1.2), whereas the CRH receptor is coupled to adenylate cyclase (see King & Baertschi 1990 for review).

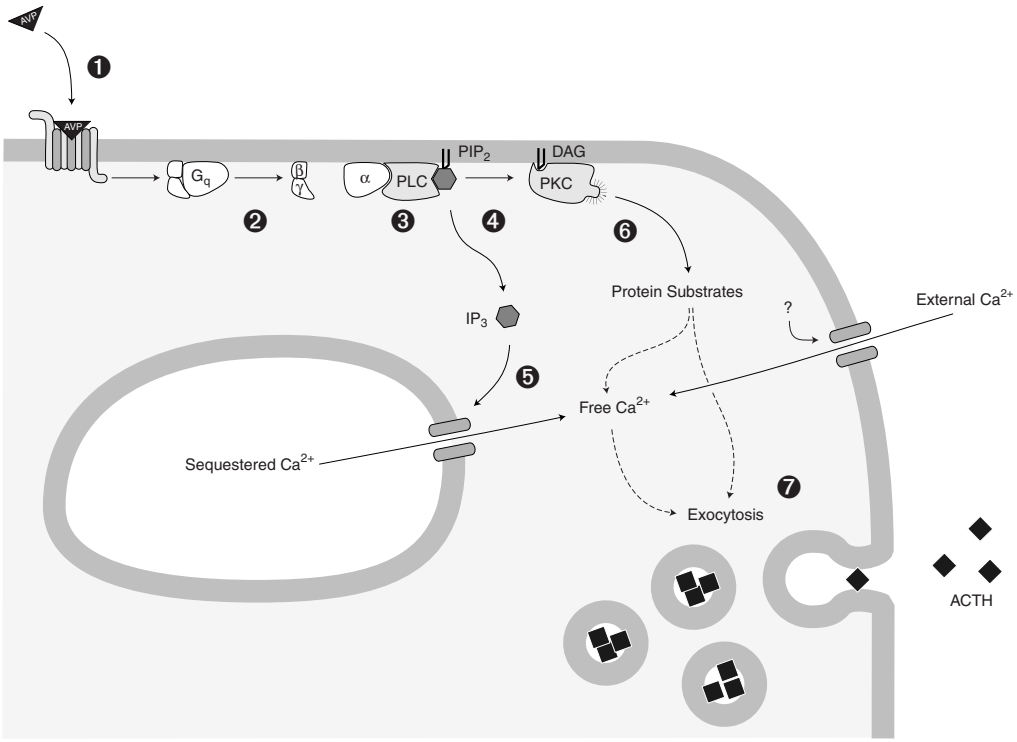

Fig. 1.2. Diagrammatic representation of the intracellular signalling pathways involved in the regulation of AVP-stimulated ACTH secretion from the pituitary corticotroph. Binding of AVP to its specific cell surface receptor (1) causes the α and βγ subunits of G_q to dissociate (2), with $G_{q\alpha}$ going on to activate phospholipase C (PLC)(3). PLC hydrolyzes the membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) to inositol trisphosphate (IP_3) and diacylglycerol (4). Both IP_3 and DAG promote an increase in intracellular free Ca^{2+}—IP_3 causes Ca^{2+} to be released from intracellular stores (5) while DAG increases intracellular Ca^{2+} via the activation of protein kinase C (PKC)(6). This increase in intracellular Ca^{2+} results in the exocytosis of ACTH (7).

1.1.3.1 PITUITARY AVP RECEPTORS

In addition to stimulating ACTH secretion from the anterior pituitary, AVP has a number of other effects on a variety of tissues. In the kidney it has an antidiuretic effect, promoting water reabsorption, in the vasculature it has a pressor effect, while in the liver it promotes glycogenolysis (László *et al.* 1991). The different effects of AVP are mediated by three structurally and pharmacologically distinct receptors. All three are G protein-coupled receptors (GPCRs) and share the characteristic structure of this receptor family, *i.e.* a single polypeptide chain with seven transmembrane domains (De Keyzer *et al.* 1994).

Two groups of AVP receptors have been distinguished on both functional and pharmacological bases. V2 receptors are found in the kidney and mediate their effects via activation of adenylate cyclase and subsequent production of the intracellular second messenger cyclic AMP (cAMP)(Birnbaumer *et al.* 1992). In contrast the V1 receptors mediate signal transduction via activation of phospholipase C (PLC) and the phosphoinositide signalling pathway (Watson & Arkinstall 1994). There are two subtypes of V1 receptors: the V1a receptor which is found at high levels in vascular smooth muscle cells and liver (Morel *et al.* 1992) and the V1b (also known as V3) receptor found in the pituitary (De Keyzer *et al.* 1994). While the two receptors activate the same signalling pathway they are pharmacologically and structurally different (De Keyzer *et al.* 1994). Baertschi & Friedli (1985) first postulated the presence of a pituitary V1 receptor subtype based upon the lack of effect of the antipressor agonists dPTyr(Me)AVP and d$(CH_2)_5$Tyr(Me)AVP on AVP potentiation of the ACTH response to CRH. This hypothesis was supported by investigations in which the efficaciousness of a greater variety of antagonists on pituitary, hepatic and kidney AVP receptors were compared (Jard *et al.* 1986). More recently the V1b receptors from the human (De Keyzer *et al.*

1994; Sugimoto *et al.* 1994), rat (Lolait *et al.* 1995) and mouse (Ventura *et al.* 1999) have been cloned. The putative amino acid sequence of the human V1b receptor has a relatively high degree of sequence identity with the human V1a, oxytocin and V2 receptors (45.5, 44.8 and 37.3% respectively), and also with members of the AVP receptor family in other species (De Keyzer *et al.* 1994). For example, the human and mouse V1b receptors share 81% amino acid identity (Ventura *et al.* 1999).

1.1.3.2 ACTIVATION OF THE PHOSPHOINOSITIDE SIGNALLING PATHWAY BY AVP

The intracellular effects of many extracellular signals are mediated by the phosphoinositide signalling system (Berridge & Irvine 1984). In this pathway the twin intracellular second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) are produced by hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), a minor lipid constituent of the plasma membrane, by the effector enzyme PLC (Michell 1992). Involvement of this signalling pathway in AVP-stimulated ACTH secretion is well established: treatment of rat pituitary cells with AVP results in increased phosphatidylinositol turnover (Raymond *et al.* 1985) and accumulation of inositol phosphates (Todd & Lightman 1987) both of which are markers of increased PIP_2 hydrolysis.

GPCRs regulate the activity of the β isoform of PLC via G proteins of the $G_{q/11}$ class (Katan 1996). Binding of a ligand to the extracellular face of its receptor induces a conformational change which allows the intracellular loops and tail of the receptor to interact with the trimeric $G_{q/11}$ protein. The α and βγ subunits of the G protein dissociate, with the $G_{q\alpha}$ subunit going on to activate PLC-β (Strader 1994). Although it is clear that the V1b receptor couples to $G_{q/11}$ (De Keyzer *et al.* 1994) the molecular mechanisms involved in this interaction are unknown. It seems likely, however, that the

mechanisms are similar to those involved in the coupling of the V1a receptor to $G_{q/11}$, which is the result of an interaction between the G protein and the second intracellular loop of the receptor (Liu & Wess 1996).

As described above, hydrolysis of PIP_2 produces the twin intracellular second messengers IP_3 and DAG. Ultimately, both of these molecules stimulate ACTH secretion by increasing the concentration of intracellular free Ca^{2+} (King & Baertschi 1990). IP_3 is a highly charged molecule and, once produced, diffuses away from the membrane to the cytoplasm where it mediates the release of Ca^{2+} from internal stores (Berridge & Irvine 1984). In contrast to IP_3, DAG remains in the plasma membrane and activates the enzyme protein kinase C (PKC)(Nishizuka 1984). The involvement of PKC in the AVP-induced ACTH response is well documented. Treatment of cells with phorbol esters, which directly activate PKC (Castagna *et al.* 1982), and DAG analogues which have a similar effect (Ebeling *et al.* 1985), stimulate the secretion of ACTH from anterior pituitary cells (Won *et al.* 1990; Abou-Samra *et al.* 1986; Liu *et al.* 1990). Furthermore, both down-regulation (Carvallo & Aguilera 1989; Koch & Lutz-Bucher 1991) and inhibition (Koch & Lutz-Bucher 1991) of PKC reduce AVP-stimulated ACTH secretion. Although the intracellular targets of PKC phosphorylation are still to be identified (Liu 1994; Liu *et al.* 1994), the effects of PKC activation on ACTH secretion appear to be dependent upon an influx of extracellular Ca^{2+} through L-type voltage-stimulated Ca^{2+} channels (L-VSCC)(Abou-Samra *et al.* 1987; Won *et al.* 1990; Corcuff *et al.* 1993; Le Beau & Mason 1994). It has been suggested that PKC might enhance Ca^{2+} entry through L-VSCC by directly phosphorylating the channels (Le Beau & Mason 1998).

1.2 ATTENUATION OF SIGNALLING: DESENSITIZATION

Commonly, stimulation of a target cell by a hormone not only elicits the characteristic cellular response but also regulates the components of the intracellular signalling pathway involved, thereby modifying the response to a subsequent exposure to the hormone (Catt *et al.* 1979). In many systems, responsiveness is reduced following exposure to either a long-lasting or repeated stimulus (Lohse 1993). This reduction in responsiveness, or desensitization, is a commonly observed feature of signalling mediated by GPCRs (Grady *et al.* 1997). Although desensitization of signalling through GPCRs can be achieved by regulatory mechanisms operating at many levels in the signalling pathway, those which are involved in the regulation of the receptor appear to account for most desensitization (Krupnick & Benovic 1998).

Mechanistically, desensitization processes can be divided into two groups: those which affect the ability of a receptor to activate its signalling pathway (often known as uncoupling) and those which affect receptor number (often known as down-regulation). Mechanisms in the first category are generally rapid, occurring within minutes of the onset of the stimulus, and reduce responsiveness by preventing the receptor from transducing the signal, *i.e.* uncoupling it from its intracellular signalling pathway. In contrast, those in the second are comparatively slow, taking place over periods of hours to days, with the reduced response being the result of a reduction in the total number of receptors (Lohse 1993).

1.2.1 RECEPTOR PHOSPHORYLATION

Uncoupling of GPCRs from their signalling pathways is commonly achieved by phosphorylation of receptors on one or more intracellular domains (Ferguson *et al.* 1996b). Phosphorylation of these domains either directly or indirectly inhibits the interaction

between the receptor and its G protein, thereby diminishing the agonist-induced response (Pitcher *et al.* 1998).

A number of different protein kinases are able to phosphorylate GPCRs. Amongst the most important and best studied of these are the G protein-coupled receptor kinases (GRKs). The hallmark characteristic of this family of serine/threonine kinases is that they preferentially phosphorylate activated (*i.e.* agonist-bound) receptors (Premont *et al.* 1995). Interaction of a GRK with a receptor results in a potent activation of the kinase (Pitcher *et al.* 1998), allowing it to phosphorylate specific residues in either the C-terminal tail (*e.g.* the β_2-adrenergic receptor [Fredericks *et al.* 1996]) or the third intracellular loop (*e.g.* the m2 muscarinic receptor [Nakata *et al.* 1994] and the α_2-adrenergic receptor [Eason *et al.* 1995]) of the receptor. Phosphorylation at these sites by a GRK is not in itself sufficient to fully inactivate the receptor (Benovic *et al.* 1987a). Instead, GRK-mediated phosphorylation enhances 10 to 30-fold the binding to the receptor of a class of cytoplasmic inhibitory proteins known as arrestins (Lohse *et al.* 1992). Binding of an arrestin molecule to the receptor prevents receptor-G protein interactions from taking place, resulting in termination of signalling (Lohse *et al.* 1990b).

Seven mammalian GRKs have been identified: GRK1 (also known as rhodopsin kinase), GRK2 (β-adrenergic receptor kinase-1), GRK3 (β-adrenergic receptor kinase-2), GRK4 (IT-11), GRK5, GRK6 and GRK7 (Ferguson 2001). With the exception of GRK1 and GRK7, which are found almost exclusively in the retina (Lorenz *et al.* 1991; Weiss *et al.* 1998), and GRK4, which is found in testis (Premont *et al.* 1996), the GRKs are ubiquitously expressed (Freedman & Lefkowitz 1996). Each of the GRKs is able to phosphorylate many distinct GPCRs although there appears to be no obvious correlation of GRK specificity with any particular receptor or class of receptors (Premont *et al.*

1995). For example, GRK2 phosphorylates not only the β_1-, β_2-, α_{2A}- and α_{2B}-adrenergic receptors but also the distantly related m1, m2 and m3 muscarinic receptors, the D_1 dopaminergic receptor and the NK_1 neurokinin receptor (Böhm *et al.* 1997). Similarly, there appears to be no clear consensus sequence within the receptor substrates for GRK-mediated phosphorylation, although in some instances the putative phosphorylation sites have pairs of acidic residues located at the amino-terminal side of the phosphorylated serine or threonine residue (Ohguro *et al.* 1993; Prossnitz *et al.* 1995; Eason *et al.* 1995; Fredericks *et al.* 1996).

Because the GRKs only phosphorylate agonist-occupied receptors, desensitization through this mechanism is receptor-specific, or homologous, with the cellular responses to other stimuli being unaffected (Premont *et al.* 1995). Many GPCRs are phosphorylated by their effector kinases, namely protein kinase A (PKA) and PKC. This type of desensitizing phosphorylation results in a direct negative feedback in which the effector enzyme turns off its own stimulation. However, because the effector kinases are able to phosphorylate receptors which are not agonist-occupied this mechanism of desensitization allows for a generalized, or heterologous, form of desensitization in which exposure to one agonist can result in the desensitization of responses to another (Lohse 1993). However, it should be noted that although it is not obligatory for its phosphorylation and desensitization by the effector kinases, agonist-occupancy of the β_2-adrenergic receptor increases the rate of phosphorylation of the receptor by PKA *in vitro* (Benovic *et al.* 1985).

The mechanisms of heterologous desensitization are best understood for the β_2-adrenergic receptor. This receptor contains two consensus sites for phosphorylation by PKA (Blake *et al.* 1987; Bouvier *et al.* 1989), one in the third intracellular loop, a region that is essential for coupling to G_s, and the other at the amino-terminal part of the car-

boxyl-terminal tail of the receptor, which also appears to be important in receptor-G_s coupling (Strader *et al.* 1987; Okamoto *et al.* 1991). PKC is also able to phosphorylate the β_2-adrenergic receptor, probably at the same sites that are utilized by PKA (Pitcher *et al.* 1992; Bouvier *et al.* 1987; Johnson *et al.* 1990). Phosphorylation at these sites does not enhance binding of arrestin to the receptor and this protein does not appear to be involved in this desensitization process (Pitcher *et al.* 1992; Lohse *et al.* 1992). Indeed it appears that, unlike GRK-mediated phosphorylation, phosphorylation of the β_2-adrenergic receptor by PKA and PKC is, in itself, sufficient to induce receptor desensitization, presumably because phosphorylation in these coupling regions impairs the ability of the receptor to activate G_s (Okamoto *et al.* 1991; Pitcher *et al.* 1992).

In addition to the β_2-adrenergic receptor a variety of other receptors have been shown to be desensitized by their effector kinases. For example, phosphorylation by PKA results in desensitization of the D_1 dopamine receptor (Bates *et al.* 1991) and the PGE_1 prostaglandin receptor (Clark *et al.* 1988) while PKC-mediated phosphorylation has been shown to result in desensitization of the $5HT_2$ serotonin receptor (Smit *et al.* 1992) and the AT_{1A} angiotensin II receptor (Oppermann *et al.* 1996; Zhang *et al.* 1996). However, many receptors do not undergo desensitization by their effector kinases, *e.g.* in contrast to the β_2-adrenergic receptor, the β_3-adrenergic receptor does not undergo PKA-mediated desensitization (Nantel *et al.* 1993).

Although the maximal extent of desensitization which can be induced by the GRKs and effector kinases is similar (50–70% and 20–50% respectively [Lohse 1993]) there are important differences in the characteristics of the two mechanisms of desensitization. Firstly, although both mechanisms of desensitization are considered to be rapid, GRK-mediated desensitization is much faster than effector kinase-mediated desensitization. For example, desensitization of the β_2-adrenergic receptor by GRK2

occurs with a $t_{½}$<15 s compared with desensitization by PKA which occurs with a $t_{½}$ of 3.5 min (Roth *et al.* 1991). Secondly, desensitization by the effector kinases is much more sensitive to agonist concentrations. For example, PKA-mediated desensitization of the β_2-adrenergic receptor occurs at concentrations 100-fold lower than GRK-mediated desensitization (Clark *et al.* 1988; Hausdorff *et al.* 1989; Lohse *et al.* 1990a). These characteristics have suggested that GRK-mediated desensitization may be most important in the regulation of receptor function in rapidly changing environments where agonist concentrations are high, such as the synaptic cleft (Roth *et al.* 1991).

The PLC-coupled m3 muscarinic receptor has been shown to undergo desensitization within seconds of agonist exposure (Tobin & Nahorski 1993). This desensitization was associated with phosphorylation of the receptor but did not appear to be mediated by either a GRK or an effector kinase (Tobin *et al.* 1993; Tobin *et al.* 1996). It was subsequently found that this phosphorylation was mediated by casein kinase 1α (CK1α)(Tobin *et al.* 1997). The casein kinase 1 family is a group of ubiquitous serine/threonine protein kinases that recognize acidic residues in their substrates (Rowles *et al.* 1991). Although much is known about the biochemical properties of the four casein kinase 1 isoforms (α, β, γ and δ) their physiological functions are largely undetermined (McInnes & Leader 1997). Indeed, 'casein kinase' is something of a misnomer. While the enzyme is able to phosphorylate casein *in vitro* it is not the biological substrate for any of the enzymes (Tobin *et al.* 1997). In addition to the m3 muscarinic receptor CK1α has been shown to phosphorylate the m1 muscarinic receptor which, like the m3 receptor, undergoes a rapid desensitization following agonist exposure (Waugh *et al.* 1999). Initially it was thought that phosphorylation by CK1α might provide an alternative mechanism for desensitization of GPCRs (Tobin *et al.* 1997), but

subsequent research has suggested that it might be more important in regulation of the initial IP_3 response following activation of these receptors (Budd *et al.* 2000).

1.2.2 RECEPTOR SEQUESTRATION

Following agonist-exposure many GPCRs are moved from the cell surface to an intracellular compartment, most likely endosomes (von Zastrow & Kobilka 1992; Ferguson *et al.* 1996b). Because they are inaccessible to both their hydrophilic ligands and G proteins once internalized this process of receptor sequestration could contribute to desensitization (Sibley & Lefkowitz 1985). However, further investigation of the β_2-adrenergic receptor system suggested that receptor internalization did not contribute to desensitization in normal circumstances. Firstly, blockade of receptor internalization using pharmacological agents such as hypertonic sucrose and concanavalin A (ConA) did not affect the ability of receptors to desensitize (Yu *et al.* 1993; Pippig *et al.* 1995). Secondly, when compared with desensitization by receptor phosphorylation, receptor internalization was found to be relatively slow ($t_{½}$=10 min for the β_2-adrenergic receptor [Roth *et al.* 1991]) and as such sequestration would mostly affect already desensitized receptors (Ferguson *et al.* 1996b). Finally, receptor mutations which prevented receptor endocytosis had no effect on desensitization (Barak *et al.* 1994). Further investigation showed that rather than being a desensitizing process receptor internalization was important in resensitization, or recovery of responsiveness following desensitization. Thus, pharmacological blockade of receptor internalization prevented resensitization (Yu *et al.* 1993; Pippig *et al.* 1995). Similarly, mutant β_2-adrenergic receptors which did not undergo endocytosis were not resensitized (Barak *et al.* 1994).

These findings suggested that resensitization was dependent upon processing of the receptors which took place following internalization (Böhm *et al.* 1997). This pro-

cessing may include dissociation of the ligand in acidified endosomes, dissociation of arrestins and dephosphorylation of the receptor by protein phosphatases which are associated with the endosomal compartment to which they are transferred (Böhm *et al.* 1997). Dephosphorylation is dependent upon endosomal acidification, presumably because the low pH makes the phosphorylated receptor a substrate for protein phosphatases (Krueger *et al.* 1997). It is somewhat unclear which of the protein phosphatases is involved in this dephosphorylation, although both protein phosphatase 2A (PP2A)(Pitcher *et al.* 1995a) and protein phosphatase 2B (PP2B)(Shih & Malbon 1996) have been implicated in dephosphorylation of the β_2-adrenergic receptor. Following dephosphorylation the receptors are recycled back to the plasma membrane in a fully functional state (Pippig *et al.* 1995).

Similar models for the role of internalization in resensitization have been proposed for a number of GPCRs including the NK_1 neurokinin receptor (Garland *et al.* 1996), the δ-opioid receptor (Hasbi *et al.* 2000) and the CB1 cannabinoid receptor (Hsieh *et al.* 1999). However, in some instances the relationship between receptor internalization and desensitization and resensitization may not be so straightforward. Desensitization of the sst_{2B} somatostatin receptor (Beaumont *et al.* 1998), the secretin receptor (Mundell & Kelly 1998) and the V2 vasopressin receptor (Pfeiffer *et al.* 1998) can be inhibited by pharmacological blockade of receptor internalization. Similarly, desensitization of the m2 muscarinic receptor can be prevented by pharmacological blockade of receptor internalization when the receptor is expressed in Chinese hamster ovary (CHO) cells (Tsuga *et al.* 1998a), but when the receptor is expressed in HEK293 cells desensitization is independent of internalization (Pals-Rylaarsdam *et al.* 1995; Pals-Rylaarsdam & Hosey 1997). The reason for this difference in the pattern of desensitization in

these two cell types in unclear although Tsuga *et al.* (1998a) has suggested that it might be due to a lower level of receptor expression in the CHO cells.

1.2.3 RECEPTOR DOWN-REGULATION

Desensitization can also be achieved through a decrease in the total number of receptors, or down-regulation (Hausdorff *et al.* 1990). Compared with receptor desensitization through uncoupling down-regulation is a slow process, requiring hours or days rather than seconds or minutes to take effect (Lohse 1993). Because resensitization requires the synthesis of new receptors it is similarly slow (Böhm *et al.* 1997). Down-regulation can be achieved by either degradation of existing receptors (Bouvier *et al.* 1989), or, alternatively, through a reduction in the rate of synthesis of new receptors (Hadcock & Malbon 1993).

1.3 DESENSITIZATION OF THE ACTH RESPONSE TO AVP

1.3.1 PREVIOUS STUDIES

A variety of *in vivo* studies have shown that the HPA axis undergoes regulatory changes during chronic stress. A major component of this adaptation to stress is the regulation of CRH and AVP receptors in the pituitary (Aguilera 1994). Depending on the nature of the stress paradigm being employed, responsiveness to a novel stress can be either increased or decreased. Physical-psychological stress paradigms (such as repeated immobilization or ip hypertonic saline injection) are associated with hyper-responsiveness to a novel stress whereas osmotic stress (such as 2% saline in drinking water or water deprivation) results in reduced pituitary ACTH responsiveness (Aguilera *et al.* 1994). In contrast to the CRH receptor, levels of which are either down-regu-

lated or unchanged during adaptation of the HPA axis in response to chronic stress, there is a good correlation between changes in pituitary AVP receptor levels and pituitary ACTH responsiveness (Aguilera 1994). During osmotic stress AVP binding in the rat pituitary is reduced whereas during physical-psychological stress AVP binding is increased. These changes in AVP receptor number are associated with changes in the biological effect of AVP at the pituitary: changes in AVP number correlate well with the ability of AVP to stimulate ACTH secretion from anterior pituitary cells isolated from control and experimental animals (Aguilera *et al.* 1994). Together these results suggest that regulation of AVP receptors in the pituitary is a major determinant of corticotroph responsiveness during adaptation of the HPA axis (Aguilera 1994). In apparent conflict with this hypothesis is the markedly increased ACTH secretion observed following adrenalectomy, despite decreases in pituitary AVP binding (Koch & Lutz-Bucher 1985; Antoni *et al.* 1985) and a reduced ability of AVP to stimulate IP_3 formation in pituitaries from these rats (Todd & Lightman 1987). However, the high concentrations of ACTH observed are due to elevated basal secretion, and responses to stimulation are in fact decreased (Aguilera & Rabadan-Diehl 2000).

Prolonged exposure to AVP results in homologous down-regulation of pituitary AVP receptors *in vivo*. Chronic injection of AVP into normal rats reduces pituitary AVP receptor levels by 80% (Antoni *et al.* 1985; Koch & Lutz-Bucher 1985). Similarly, in Brattleboro rats, which are genetically deficient in AVP, chronic injection of AVP to a level which matches that of normal rats results in a reduction in receptor binding, indicating that physiological levels of AVP can also cause receptor down-regulation (Koch & Lutz-Bucher 1985). The decrease in pituitary AVP binding following adrenalectomy appears to be due, at least in part, to increased secretion of AVP from the hypothalamus following the removal of glucocorticoid negative feedback: surgical

lesioning of the paraventricular nucleus (a treatment which drastically reduces the number of AVP-secreting neurons in the hypothalamus [Kárteszi *et al.* 1982]) reduces the extent of AVP receptor down-regulation (Antoni *et al.* 1985; Lutz-Bucher *et al.* 1986). However, in rats with hypothalamic lesioning cortisol replacement can completely prevent the loss of pituitary AVP receptors, indicating that glucocorticoids can directly regulate the pituitary AVP receptor (Lutz-Bucher *et al.* 1986).

In vitro studies in the sheep, mouse and rat have shown that exposure of both pituitary cells and segments to AVP results in desensitization of their ACTH response to a subsequent stimulation with AVP. When AVP was administered in 10 min pulses at 60 min intervals to dispersed ovine anterior pituitary cells in a multi-column perifusion system there was a loss of responsiveness to the hormone with time (Evans *et al.* 1988). This loss of responsiveness was not appreciable at lower concentrations but at higher concentrations (between 100 and 2000 nM) the effect was marked. This reduction in response was not due to depletion of intracellular ACTH stores as desensitization to AVP did not reduce the response to CRH. Furthermore, cells stimulated continuously with submaximal levels of AVP showed desensitization while ACTH was still available for release to higher levels of the hormone. Taken together these results indicated that a true desensitization process was involved. Desensitization of ACTH responsiveness has also been observed when either pituitary cells or segments are treated with AVP in static culture. Holmes *et al.* (1984) induced desensitization of the ACTH response to AVP in rat anterior pituitary segments by treating them with 100 nM AVP for 4 h. This treatment reduced the response to a subsequent 1 h stimulation with either 10 or 1000 nM AVP by 27% and 60% respectively, while the response to CRH was unaffected. In parallel experiments binding of [^{3}H]-AVP to pituitary membranes isolated from rat anterior pituitary segments which had received a similar AVP treatment

was reduced by 71% (Antoni *et al.* 1985). Murakami *et al.* (1984) found that treatment of dispersed rat pituitary cells with 0.92 nM AVP for 6 h markedly reduced the ACTH response of cells to subsequent stimulation with AVP. When the AVP concentration of the pre-treatment was increased to 92 nM the response to subsequent AVP stimulation was abolished. Desensitization to AVP has also been observed in mouse anterior pituitary cells (Castro 1993). Treatment with AVP at concentrations between 1 and 100 nM for 3 h reduced the response to a subsequent 3 h stimulation with 100 nM AVP by up to approximately 50% (my calculation from Fig. 3B) without affecting the response to CRH.

Although desensitization of the ACTH response to AVP can occur *in vitro* the mechanisms underlying this process are unknown. Phosphorylation of the pituitary AVP receptor has not been reported to date although the third intracellular loop and C terminal tail of the receptor contain phosphorylation consensus sequences for both PKC and the GRKs (Ventura *et al.* 1999). Also, a green fluorescent protein(GFP)-tagged V1b receptor expressed in CHO cells has been shown to associate with PKCα and with GRK5 (but not GRK2, GRK3 or GRK6) during agonist exposure (Berrada *et al.* 2000). Receptor internalization may also play an important role in the regulation of the ACTH response to AVP since the pituitary AVP receptor has been shown to be internalized during AVP treatment (Mogensen *et al.* 1988; Childs *et al.* 1991; Berrada *et al.* 2000). However, in these studies the relationship between internalization and ACTH responsiveness was not investigated.

1.3.2 AIMS OF THIS STUDY

The data described above show clearly that the pituitary AVP receptor is regulated during adaptation to stress. Furthermore, this regulation plays a critical role in the control

of corticotroph responsiveness during the adaptation to chronic stress. Depending on the nature of the stressor, persistent or repeated stress can also result in reduction in pituitary AVP binding and ACTH responsiveness. While *in vitro* studies have shown that the ACTH response to AVP undergoes homologous desensitization following repeated or prolonged exposure to AVP this process is poorly characterized. In particular the relationship, if any, between the desensitization process seen *in vitro* and desensitization, particularly adaptation to chronic stress, that occurs *in vivo* is unclear. Although the desensitization observed *in vitro* appears to be concentration-dependent it is unclear how quickly it occurs or if it can be reversed. Furthermore the AVP treatment regimes used to induce desensitization in these studies do not match the conditions to which corticotrophs are exposed *in vivo*. In the sheep, AVP secretion into the portal circulation is highly pulsatile, with secretory events rarely lasting more than 30 min, and the AVP concentration rarely rising above 5 nM (Caraty *et al.* 1988; Engler *et al.* 1989; Caraty *et al.* 1990; Battaglia *et al.* 1998; Dadoun *et al.* 1998) whereas the studies described above used AVP treatments of supra-physiological concentration (>100 nM), very long duration (up to 4 h), or a combination of both.

The first aim of this research was to characterize the desensitization of the ACTH response to AVP. Chapter 3 describes experiments in which the concentration- and dose-dependency of this process in ovine anterior pituitary cells were investigated using multi-column perifusion. Particular effort was made to ensure that the AVP treatments used to induce desensitization fell within the range of concentrations and durations of endogenous AVP pulses. As such, this study was aimed at investigation of the regulation of ACTH responsiveness during acute rather than chronic stimulation with AVP. Additionally, the ability of cells to become resensitized following desensitization was investigated.

Both *in vivo* and *in vitro* studies have shown that regulation of the pituitary AVP receptor plays an important role in the regulation of corticotroph responsiveness. However the molecular mechanisms which are involved in the regulation of signalling via this receptor during acute stimulation are unknown. As described in Section 1.2, amongst the GPCR family desensitization can be mediated by either a reduction in total receptor number (*i.e.* down-regulation) or by preventing receptors from activating their signalling pathway. The latter mechanism is more rapid and is commonly achieved via receptor phosphorylation. Chapter 4 describes experiments in which the involvement of receptor phosphorylation in the desensitization of the ACTH response to AVP was investigated. Two approaches were taken. Firstly, the involvement of the intracellular protein kinases PKC and CK1α in desensitization of the ACTH response to AVP of ovine anterior pituitary cells was investigated through the use of specific pharmacological inhibitors and activators of these enzymes. Secondly, the involvement of phosphorylation in regulation of the pituitary AVP receptor was investigated directly in experiments aimed at determining whether phosphate groups are incorporated into the receptor during AVP exposure.

Receptor internalization also plays an important role in the regulation of GPCRs, either by allowing resensitization of receptors or, in a few cases, causing desensitization because receptors are moved to a location where they are inaccessible to both their hydrophilic ligands and their associated G proteins. Although the V1b receptor has been shown to internalize during AVP exposure the consequences of this are unknown. Chapter 5 describes experiments in which the role of receptor internalization in regulation of the ACTH response to AVP was investigated by pharmacologically inhibiting receptor internalization. In many instances the role of internalization of GPCRs is to allow dephosphorylation of desensitized receptors by phosphoprotein phosphatases.

The involvement of PP2A and PP2B, two phosphatases which have been implicated in the resensitization of GPCRs (Pitcher *et al.* 1995a; Shih & Malbon 1996), in the regulation of the ACTH response to AVP was investigated using selective inhibitors of these enzymes.

2

METHODS

2.1 MATERIALS

For the sources of all materials used in this study, refer to Appendix A. All reagents used were of analytical grade.

2.2 SOLUTIONS AND MEDIA

For the details of all solutions and media used in this study, refer to Appendix B.

2.3 CELL PREPARATION AND CULTURE

2.3.1 PITUITARIES

Pituitaries were collected from freshly slaughtered, sexually mature (*i.e.* >1 year old) female sheep (*Ovis aires*). Most often, whole heads were collected from either Alliance Group Ltd. (Sockburn, Christchurch) or the Malvern Abattoir Ltd. (Malvern, Canterbury) and transported on ice to the Department of Zoology, University of Canterbury. There the heads were cut posterio-anteriorally with a band saw, allowing the cut to pass just to the left of the pituitary gland, which was subsequently removed. On occasion, pituitary glands were collected from Canterbury Frozen Meats Ltd. (Belfast,

Christchurch). Because heads are routinely split at this plant it was possible to remove pituitary glands on site. Following their removal from the heads the pituitary glands (usually ten were collected) were placed in chilled, sterile dispersing buffer (DB; see Appendix B). Typically, no more than 45 min elapsed between slaughter of the animal and removal of the pituitary gland.

2.3.2 PREPARATION OF DISPERSED OVINE ANTERIOR PITUITARY CELLS

Chilled, sterile DB was used as the medium for preparation of dispersed ovine anterior pituitary cells. The cell preparation was performed in a laminar flow hood (CF43S, Gelman Sciences, Australia), and aseptic conditions were maintained throughout. All instruments and glassware used for cell preparation had previously been either autoclaved (15 min, 121°C, 15 psi), or heat sterilized (170°C, 2 h). Similarly, all plasticware and solutions used were sterile.

The pituitary glands were washed by three brief submersions in fresh DB and then collected in DB on ice. Depending on their size, either seven or eight glands were selected to prepare the dispersed cells. Adhering connective tissue, the median eminence, pituitary stalk and the posterior pituitary were removed. The anterior pituitaries were then rinsed once with DB and minced, with scissors, into pieces less than 2 mm^3 in size. Following mincing, the pituitary pieces were transferred to a trypsinizing flask containing 50 ml of collagenase solution (Type II collagenase [clostridiopeptidase A], 480 U/ml DB). The flask was placed in a 37°C incubator and the tissue suspension was gently stirred with a magnetic stirrer. Cells were collected after an initial incubation period of 20–30 min and again after two subsequent incubations of either 45 or 60 min duration. 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 the pellets obtained from the second and third incubations, a fourth incubation, of 30 min duration, was occasionally required. Dispersed cells were collected by decanting the supernatant from the trypsinizing flask and centrifuging this at 200 × g (CR-412, Jouan, France) for 5 min at 4°C. The supernatant was returned to the trypsinizing 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.

The cells from the 1 h incubations were pooled and washed (as above) four times. After the third wash the cells were, if necessary, filtered through sterile U.S.P. Type VII gauze to remove any connective tissue and/or gelatinous material. Following the final wash the cells were resuspended in 25 to 40 ml of Dulbecco's modified Eagle's medium (DME; see Appendix B), 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 (Dealtry 1992), and was typically greater than 85%, and never less than 80%.

2.3.3 OVERNIGHT CULTURE OF CELLS

For perifusion experiments, a cell suspension containing 4.5×10^6 viable cells/ml DME/NCS was prepared. Aliquots of this suspension (1 ml) were then transferred to plastic petrie dishes containing 19 ml of DME/NCS. One plate was prepared for each of the perifusion columns, plus one for cell viability determination on the day of the perifusion. For the preparation of pituitary cell plasma membranes cells were cultured at a density of 20×10^6 viable cells per plate in 20 ml of DME/NCS. All cells were incubated overnight (approximately 18 h) at 37°C in a 95% air:5% CO_2 environment.

2.4 MULTI-COLUMN PERIFUSION EXPERIMENTS

2.4.1 PERIFUSION SYSTEM

The multi-column perifusion system, as described previously and with modifications (McIntosh & McIntosh 1983, Evans *et al.* 1985, Evans *et al.* 1988, Evans *et al.* 1996), allows for the simultaneous perifusion of up to fifteen cell chambers, or columns. Through the use of a solenoid switching system various trains of AVP and pharmacological agents can be applied to the cells. The perifusion system is shown diagrammatically in Fig. 2.1 and photographically in Fig. 2.2.

Following overnight incubation, the cells from each cell culture plate were transferred in DME/NCS to separate 50 ml centrifuge tubes and pelleted by centrifugation at 275 × g for 5 min at 4°C (CR-412, Jouan, France). The supernatant was then aspirated and replaced with 170 µl of a slurry of Sephadex G-25 (fine) suspended at a ratio of 1:1.5 (vol:vol) in Krebs ringer (KR; see Appendix B), in preparation for transfer to the cell columns. Each of the cell columns has an internal volume of approximately 500 µl and is maintained at a temperature of 37°C by a tubular water jacket which encloses all fifteen columns. Before transfer of the cells to the columns, slurries of Bio-Gel P-2 (80 µl suspended 1:1.5 [vol/vol] in KR) and Sephadex G-25 (80 µl suspended 1:1.5 [vol/vol] in KR) beads were added to each of the columns to form a bead bed on which the cells would be supported. This bed was prevented from draining from the columns by a 10 µm mesh Nybolt nylon gauze. The cell/Sephadex slurry was then placed onto this bead bed using a pasteur pipette, with care being taken to minimize disturbance of the cells. Each of the columns was then filled with KR/ATC (a KR solution containing 0.05% alkali-treated casein [ATC; prepared as described by Livesey & Donald {1982}] and 0.005% L-ascorbate) and sealed with a rubber bung into which the flow inlet tube was inserted.

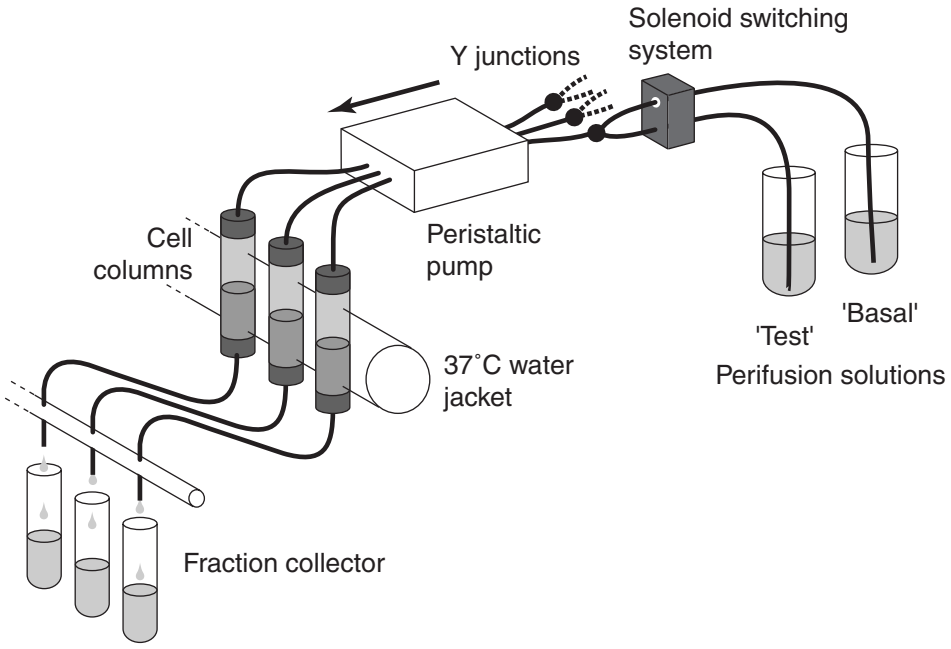

Fig. 2.1. Diagram of the multi-column perifusion system. Arrow indicates direction of flow through the system. Note that while for clarity only three cell columns are shown here, in the actual system there are fifteen.

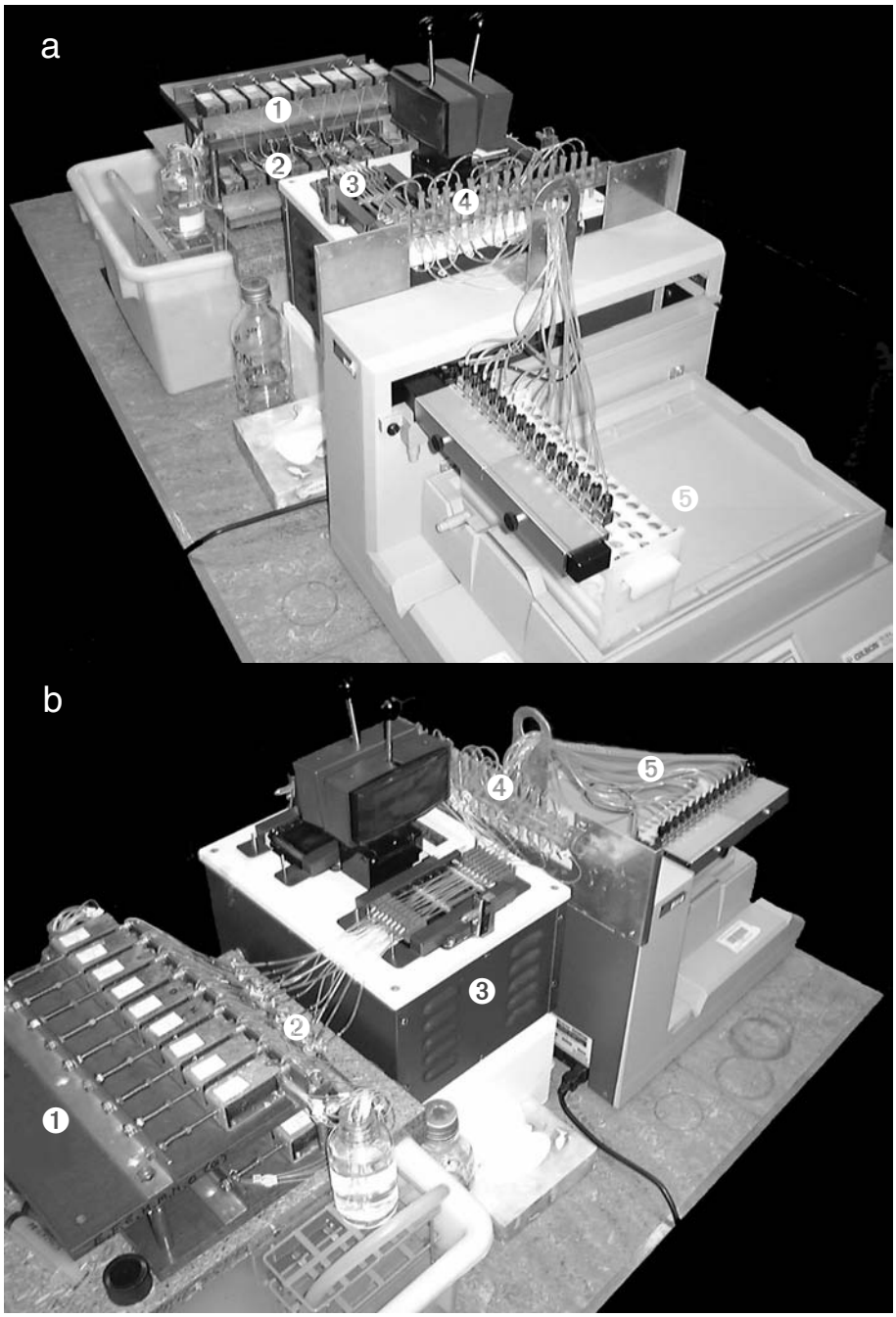

Fig. 2.2. Two views of the multi-column perifusion system showing the solenoid switching system (1), Y junctions (2), peristaltic pump (3), cell columns enclosed in a tubular water jacket (4) and fraction collector (5).

Once cells had been transferred to each of the columns a multi-channel pump (Super Standby CPP15-30, ChemLab, England) was used to pump perifusion medium through fine tubing into the cell columns at a flow rate of 0.157 ml/min. The effluent from the columns was collected in 5 or 10 min fractions using a Gilson FC 204 fraction collector (Gilson, France). These samples were stored at –20°C and assayed for ACTH content by radioimmunoassay (RIA)(see Section 2.4.3). The different types and lengths of tubing used in the perifusion system are summarized in Fig. 2.3.

During an experiment cells were perifused with a 'basal' solution (*i.e.* KR/ATC) and a 'test' solution containing AVP and/or various pharmacological agents. See Appendix B for details of the preparation of 'test' solutions. The tubing carrying these solutions was connected to the pump tubing by a Y-junction. At any given time one of these lines was clamped closed. Through the use of a solenoid-actuated switching system the perifusion solution could be rapidly and precisely changed between the 'test' and 'basal' solutions. When the cells were perifused with 'basal' KR/ATC plus two separate 'test' solutions, the basal line was manually transferred between KR/ATC and the second 'test' solution.

2.4.2 DESIGN AND ANALYSIS OF MULTI-COLUMN PERIFUSION EXPERIMENTS

2.4.2.1 EXPERIMENTAL DESIGN

A variety of different experimental protocols were used in multi-column perifusion experiments. While the protocol used depended on the aim of the particular experiment, the design and analysis of all experiments shared some common features. These are described below while the details of the specific treatment regimes used in individual experiments can be found in subsequent chapters.

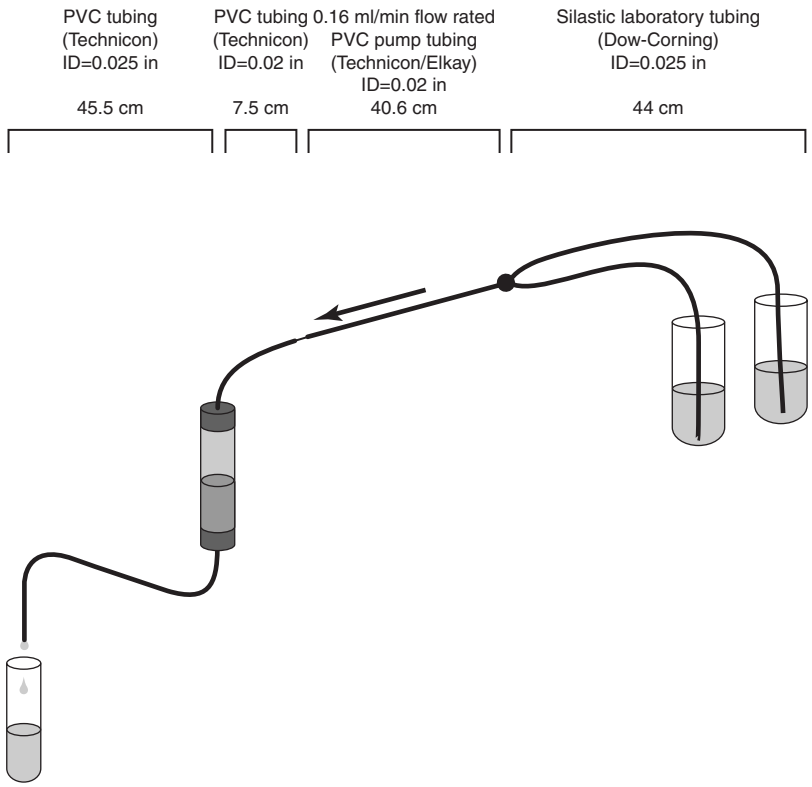

Fig. 2.3. Types and lengths of tubing used in the multi-column perifusion system. The internal diameter (ID) of all tubing is indicated in inches.

Each of the fifteen columns in the perifusion system was randomly assigned an experimental treatment. Typically, each of the different treatments was repeated in at least two independent columns. In all experiments cells were perifused with 'basal' KR/ATC for at least 90 min at the beginning of the experiment, allowing them to recover from transfer into the columns. During this period ACTH secretion dropped to a consistently low level. The ability of a particular treatment to induce desensitization was assessed by measuring its effect on a subsequent stimulation with 100 nM AVP for 5 min. The effect of this 'pre-treatment' on the subsequent 100 nM AVP 'pulse' was assessed quantitatively by expressing the response to the AVP test pulse as a percentage of the response to similar pulses that had not been pre-treated (*i.e.* controls).

2.4.2.2 DATA ANALYSIS

ACTH responses to the pre-treatments and test pulses were calculated as follows from the raw data obtained from RIA. Firstly, the 'total ACTH secretion' during a period of stimulation was calculated from the ACTH concentration measured in the appropriate fractions using the equation:

$$\text{Total ACTH secretion (ng)} = \sum \left\{ \begin{array}{c} \text{[ACTH] in fraction} \\ \text{(ng/ml)} \end{array} \times \begin{array}{c} \text{Fraction Duration} \\ \text{(min)} \end{array} \times \begin{array}{c} \text{Flow rate} \\ \text{(ml/min)} \end{array} \right\}$$

For the pre-treatment the 'total ACTH secretion' during the entire pre-treatment was calculated whereas for the test pulse 'total ACTH secretion' was calculated for a 20 min period from the onset of the pulse.

The term 'response' refers to AVP-stimulated ACTH secretion. This was determined by subtracting basal ACTH secretion from the values calculated for 'total ACTH secretion', *i.e.*:

AVP-stimulated ACTH secretion = Total ACTH secretion – Basal ACTH secretion

Basal ACTH secretion was calculated using the mean ACTH secretion in the three fractions immediately preceding a pre-treatment or pulse. Where the fractions immediately preceding a pulse were collected during the pre-treatment, basal ACTH secretion was calculated using the fractions immediately prior to the pre-treatment.

2.4.2.3 STATISTICAL ANALYSIS

Data from perifusion experiments were statistically analyzed using a variety of software packages, namely Prism 3.0, Instat 1.0 (both from GraphPad Software, San Diego, CA) and Excel 8 (Microsoft, Redmond WA). Details of the different types of analyses used are indicated in the text. $P<0.05$ was considered significant. Actual levels of significance are as indicated in the text. All data are reported as mean ± SEM.

2.4.3 ACTH RADIOIMMUNOASSAY

The ACTH concentration in perifusion samples was measured by direct RIA. The procedures for the RIA of ovine ACTH (oACTH) have been described previously by Evans *et al.* (1985).

2.4.3.1 TRACER

^{125}I-labelled oACTH (^{125}I-oACTH) was prepared using a chloramine T radioiodination procedure (Hunter & Greenwood 1962). Briefly, 2.5 µg of highly purified oACTH (a kind gift from the late Dr C.H. Li; see Appendix A) was incubated with ^{125}I in the presence of chloramine T for 1 min. At the end of this incubation the reaction was stopped by the addition of sodium metabisulphite and the reaction mixture was separated from the labelled hormone either by elution from a cellulose column with horse plasma or by high performance liquid chromatography (HPLC). HPLC was carried out on a reverse phase C8 column (300 Å pore size; Brownlee RP300) with a 0 to 60% acetonitrile gradient. The tracer was aliquoted at volumes appropriate for use in the RIA and was stored frozen at –20°C for up to six weeks before use.

Tracer purified on a cellulose column was repurified on the day of assay using the following procedure. 15 mg of silicic acid (100 mesh) was added to an aliquot of tracer and mixed by vortexing for 20 s. This was then centrifuged for 90 s at 1200 × g (benchtop centrifuge, Griffen and George Ltd., England). The supernatant was discarded and the pellet was washed twice by resuspending it in 1 ml of distilled, deionized H_2O (ddH_2O) with vortexing followed by centrifugation as before. To elute the hormone from the silicic acid mesh 1 ml of an acetone/acetic acid solution (see Appendix B) was added, followed by vortexing and centrifugation. The supernatant containing the repurified tracer was removed using a pasteur pipette and diluted in assay buffer (0.05 M phosphate buffer containing 0.1% ATC [P/ATC; see Appendix B]) to 10,000 cpm/100 µl. Repurification of HPLC-purified tracer was not necessary and this was diluted directly into P/ATC.

2.4.3.2 ANTISERUM

Antiserum, kindly provided by Professor Richard Donald (formerly of the Department of Endocrinology, Christchurch Hospital) was raised in rabbits by injection with two porcine ACTH preparations (ACTH with carboxymethylcellulose and ACTH zinc hydroxide). The antiserum was diluted 1:1400 in P/ATC for assay.

2.4.3.3 ACTH STANDARDS

A 100 g/l oACTH (a gift from Dr C.H. Li; see Appendix A) solution was prepared by dissolving the dry powder in 0.003 M HCl. This was diluted in P/ATC to give a 500 µg/l stock solution. This stock solution was stored at –20°C in 1 ml aliquots. To prepare standards for use in RIA one of these aliquots was thawed and diluted with P/ATC to produce a stock solution of 10 µg/l oACTH. This was then diluted with appropriate volumes of P/ATC to produce standard solutions with the following concentrations: 10, 5, 3, 2, 1.5, 1, 0.5, and 0.25 µg/l. Each standard was aliquoted into a mixture of 0.6 ml and 0.3 ml aliquots and stored at –20°C. P/ATC was used as the zero standard.

2.4.3.4 REPLICATION STANDARDS

Replicate samples of ACTH at three concentrations (high, ~3.0 µg/l; medium ~1.3 µg/l; low ~0.4 µg/l) were included in each assay for quality control purposes. The inclusion of these samples at the beginning and end of each assay allowed assay precision to be monitored. Comparison of the ACTH concentration obtained for the replicates was used to give an indication of the variation both within individual assays and between separate assays. The intra-assay co-efficients of variation for the high, medium and low ACTH replicates were 6.77%, 9.25%, and 14.86% respectively. The inter-assay

co-efficients of variation for the high, medium and low ACTH replicates were 8.25%, 19.29% and 15.42% respectively.

Replicate standards were prepared as follows. Surplus 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 and CRH for 2 h. The medium was collected after centrifugation and the pellet was resuspended in KR/ATC, vortexed vigorously and frozen and thawed to rupture the cells, releasing ACTH. The resulting suspension was centrifuged and the ACTH-containing supernatant was recovered and mixed with the supernatant from the first centrifugation. 1 ml aliquots of these replicates were stored at –20°C.

2.4.3.5 ASSAY PROCEDURE

Assays were set up in duplicate in an ice/water bath using the protocol described in Table 2.1. P/ATC buffer, ACTH sample (either standard, experimental sample or replicate), ^{125}I-oACTH tracer and ACTH antiserum were added to the appropriate assay tubes in sequential fashion. 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. Once the addition of all reagents was complete the tubes were vortexed briefly (Multi-tube Vortex, SMI, Miami FL) and incubated at 4°C for 18 to 20 h.

2.4.3.1 ASSAY SEPARATION

The antibody-bound ^{125}I-oACTH was separated from the free labelled hormone by fractional precipitation, as follows. At the end of the incubation period, 50 µl of either horse plasma or a 1.2% solution of bovine γ-globulins in P/ATC was added to each

Table 2.1. Assay procedure for the RIA of oACTH. All volumes are in microlitres unless otherwise indicated.

Tube Number	Standard or sample	Buffer	Std/ sample	Tracer	Anti-serum		γ-globulin	PEG
1	Blank (0 μg/l std)	150	100	100	—		50	1.5 ml
2	0 μg/l std	50	100	100	100		⇓	⇓
3	0 μg/l std	⇓	⇓	⇓	⇓			
4	0.25 μg/l std							
5	0.5 μg/l std							
6	1 μg/l std							
7	1.5 μg/l std							
8	2 μg/l std							
9	3 μg/l std							
10	5 μg/l std							
11	10 μg/l std							
12	Blank (assay zero)	250	—	100	—			
13	Assay zero	150	—	100	100			
14	Blank (High replicate)	150	100	100	—			
15	High replicate	50	100	100	100			
16	Blank (medium replicate)	150	100	100	—			
17	Medium replicate	50	100	100	100			
18	Blank (low replicate)	150	100	100	—			
19	Low replicate	50	100	100	100			
20	Blank (samples)	150	100	100	—			
	Samples (undiluted)	50	100	100	100			
	⇓	⇓	⇓	⇓	⇓			
	Blank (½ dilution)	200	50	100	—			
	Samples (½ dilution)	100	50	100	100			
	⇓	⇓	⇓	⇓	⇓			
	Blank (High replicate)	150	100	100	—			
	High replicate	50	100	100	100			
	Blank (medium replicate)	150	100	100	—			
	Medium replicate	50	100	100	100			
	Blank (low replicate)	150	100	100	—			
	Low replicate	50	100	100	100			
	Blank (assay zero)	250	—	100	—			
	Assay zero	150	—	100	100			

Overnight incubation at 4°C

tube, followed by 1.5 ml of an 18% polyethylene glycol solution (PEG; see Appendix B). After vigorous vortex mixing the tubes were incubated at room temperature for a total of 15 min. The antibody-bound hormone was then pelleted by centrifugation for 15 min at 4,010 × g (Heraeus Varifuge 3.0R) at 4°C, and the supernatant was removed by vacuum aspiration. The radioactivity present was measured using a γ scintillation counter (1275 Minigamma counter, LKB Wallac) and the data obtained were stored as a computer file.

2.4.3.2 ANALYSIS OF RIA OUTPUT

The raw data (counts/2 min) were analyzed 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 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.

2.5 PHOSPHORYLATION OF THE PITUITARY AVP RECEPTOR

To investigate phosphorylation of the pituitary AVP receptor plasma membranes were isolated from both AVP-treated and control cells and retardation of the electrophoretic mobility of AVP receptors from these preparations was used as an indicator of phosphorylation.

2.5.1 PREPARATION OF PLASMA MEMBRANES

Prior to preparation of plasma membranes, cells were treated with AVP to induce desensitization. Following overnight culture, 20 μM AVP stock solution was added to plates to give a final concentration of 50 nM. The medium was mixed by gently swirling the plates which were then incubated for a further 30 min at 37°C. A similar number of control plates, which were not treated with AVP, were also prepared. To ascertain whether this treatment was capable of inducing desensitization 1 ml aliquots from treated and untreated plates were transferred to sterile culture tubes, centrifuged at 625 × g for 10 min at 4°C (CR-412, Jouan), and the supernatant was replaced with either DME/NCS or DME/NCS containing 100 nM AVP. After a 90 min incubation the tubes were centrifuged again. Following centrifugation an aliquot of the supernatant was removed and its ACTH concentration was determined by RIA. It was found that the ACTH response in cells pre-treated with 50 nM AVP was reduced by 41.0 ± 5.5%.

Following treatment with 50 nM AVP for 30 min the cells were transferred in DME/NCS from the culture plates to 50 ml centrifuge tubes and recovered by centrifugation at 625 × g for 10 min at 4°C (CR-412, Jouan). After aspiration of the supernatant the cells were resuspended in 4 ml of ice-cold inhibitor buffer (IB; see Appendix B). This cell suspension was then transferred to a 15 ml Dounce homogenizer (Wheaton, Millville NJ) and homogenized with fifteen passes of a tight fitting pestle. One homogenizer was used for all cells from control plates and a second was used for all cells from AVP-treated plates. The homogenates were then centrifuged at low speed (1,049 × g; Varifuge 3.0R, Heraeus) to remove undamaged cells, partially ruptured cells, and nuclei. The resulting supernatant was centrifuged at 50,000 × g (RC-M150GX, Sorvall), and the sealed tubes were then sliced open using a hot scalpel

blade. The supernatant was aspirated and the plasma membrane-containing pellets were resuspended in 400 µl of protein assay buffer (PAB; see Appendix B). A small aliquot (~25 µl) was removed for protein assay and the remainder was frozen in liquid nitrogen and stored at -20°C. The protein concentration of the membrane fraction was determined using a bincinchonic acid protein assay (BCA Protein Assay Kit, Pierce, Rockford IL). Each culture plate of 20×10^6 cells yielded approximately 200 µg of membrane protein.

2.5.2 CROSS-LINKING OF ^{125}I-AVP TO THE PITUITARY AVP RECEPTOR

Sufficient membrane preparation to give 100 µg of protein was transferred to microtubes and centrifuged at 20,800 × g for 30 min (5417, Eppendorf Hamburg, Germany). The supernatant was aspirated and the pellet resuspended in PAB. This membrane preparation was incubated with ~0.25 nM (3-[^{125}I]iodotyrosyl^2)Vasopressin[Arg^8] (^{125}I-AVP) for 2 h either alone or in the presence of 2.5 µM unlabelled AVP according to the protocol shown in Table 2.2.

Table 2.2. Incubation of pituitary cell membrane preparations with ^{125}I-AVP.

	NSB	Experimental
Membrane protein	100 µg	100 µg
PAB	200 µl	225 µl
~2.5 nM ^{125}I-AVP (final concentration ~0.25 nM)	25 µl	25 µl
25 µM AVP (final concentration 2.5 µM)	25 µl	—
Total	250 µl	250 µl

After incubation for two hours at room temperature 10.4 µl of a 25 mM solution of the cross linker disuccinimidyl suberate (DSS) in dimethylsulfoxide (DMSO) was added to give a final concentration of 1 mM. The incubation was then continued for a further 20 min before 250 µl 1 M Tris·HCl (pH 7.5) was added to stop the reaction.

2.5.3 ELECTROPHORESIS OF MEMBRANE PREPARATIONS

Following cross-linking, the membrane proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the labelled bands were visualized by autoradiography. The cross-linked membrane preparations were centrifuged at 20,800 × g for 30 min (5417, Eppendorf), the supernatant aspirated, and the pellets resuspended in 20-30 µl of SDS-PAGE sample buffer (commercially prepared 2× concentrate [4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris·HCl, pH ~6.8; Sigma Chemical Co., St Louis MO] diluted in PAB). The samples were then placed in a boiling water bath for 3 min, before separation on unidirectional polyacrylamide slab gels prepared according to the method of Laemmli (1970). Details of the preparation of gels can be found in Appendix B. Best separation was achieved using 24 cm long, 1.5 mm thick, 12% polyacrylamide gels (with a 4% stacking gel) run at 20 A constant voltage. Molecular weight markers (SigmaMarkers) were electrophoresed alongside the samples. Gels were stained overnight in a Brilliant Blue G-TCA solution (prepared as described by Blakesley & Boezi [1977]) and destained in ddH_2O for a further 24 h. Gels were dried at 60°C for 2 h (Model 543, Biorad Laboratories, Hercules CA). Dried gels were apposed to XAR-5 film (Eastman Kodak, Rochester NY) with X-Omatic intensifying screens (Eastman Kodak, Rochester NY) for 2 weeks at −70°C.

3

CHARACTERISTICS OF THE DESENSITIZATION OF THE ACTH RESPONSE TO AVP

3.1 INTRODUCTION

In vivo studies have shown that the pituitary AVP receptor undergoes regulatory changes during adaptation to chronic stress (Aguilera 1994; Aguilera & Rabadan-Diehl 2000). Similarly, a number of *in vitro* studies have shown that anterior pituitary cells become desensitized following prolonged or repeated exposure to AVP (Holmes *et al.* 1984; Evans *et al.* 1988; Castro 1993). The AVP treatments used to induce desensitization in these *in vitro* experiments were either of long duration, high concentration or a combination of both. For example, Holmes *et al.* (1984) observed desensitization of the ACTH response to AVP in dispersed rat anterior pituitary cells following treatment with 100 nM AVP for 4 h (see Section 1.3.1 for a more extensive discussion).

In contrast to these AVP treatment regimes the secretion of AVP into the hypophyseal portal circulation is highly pulsatile with peak concentrations of relatively low magnitude. In the sheep, sampling of the hypophyseal portal blood has been possible in unrestrained and conscious animals. Using this technique it has been shown that pulsatile AVP secretion into the portal blood is stimulated by a variety of stressors including

insulin-induced hypoglycaemia (Engler *et al.* 1989; Caraty *et al.* 1990), audiovisual stress (Engler *et al.* 1989); haemorrhagic stress (Caraty *et al.* 1988) and endotoxin infusion (Battaglia *et al.* 1998; Dadoun *et al.* 1998). In all of these studies basal AVP secretion rates were very low, with exposure to a stressor resulting in peaks of AVP secretion. There was a high degree of inter-animal variation, but the maximal AVP concentration in the portal plasma following exposure to a stressor ranged from approximately 1 nM for acute haemorrhage (Caraty *et al.* 1988), to ~6 nM for insulin-induced hypoglycemia (Caraty *et al.* 1990) and ~4 nM for endotoxin exposure (Battaglia *et al.* 1998). Assessment of the duration of endogenous AVP pulses and the inter-pulse period is somewhat more problematic. In the studies described above a 10 min sampling period was used. At this sampling frequency it is difficult to estimate the duration of endogenous pulses with great precision (Alexander *et al.* 1994; Gudmundsson & Carnes 1997), although it appears that in most cases secretory events rarely last more than about 30 min and occur approximately once an hour. In the horse a unique venous pathway exists which allows pituitary venous blood to be collected as soon as it leaves the pituitary (Alexander *et al.* 1994). Because of the large size of horses, samples can be taken more frequently than is possible in the sheep. Using this technique, with a 5 min sample period, Redekopp *et al.* (1986) found that pulses of AVP were surprisingly brief, usually less than 10 min in duration, and frequent, occurring at 15 to 25 min intervals.

It is unclear whether the desensitization of the ACTH response to AVP which has been shown to occur *in vitro* takes place under conditions that are characteristic of AVP pulses *in vivo*. The aim of the following experiments was to determine whether desensitization occurs *in vitro* in response to AVP pulses that are similar in magnitude and duration to those measured in the hypophyseal portal blood. Also, the time required for resensitization to occur was investigated.

The rapidity with which desensitization and resensitization occur may give some clues as to the mechanisms involved in the desensitization process. Amongst the GPCR family desensitization can be achieved by either a reduction in total receptor number (down-regulation) or by preventing receptors from activating their signalling pathway (uncoupling), which is commonly mediated by phosphorylation of the receptor by intracellular protein kinases. Alternatively, uncoupling can be achieved by moving receptors to an intracellular location where they are inaccessible to both their hydrophilic ligands and G proteins. Compared with down-regulation, which takes place over a period of hours or days, uncoupling is rapid, occurring within seconds or minutes of the onset of the stimulus. Furthermore, because it does not require the synthesis of new receptors, the effects of uncoupling are much more readily reversed than those of down-regulation (Lohse 1993).

3.2 RESULTS

3.2.1 DESENSITIZATION OF THE ACTH RESPONSE TO AVP

The first series of experiments was designed to determine the concentrations and durations of AVP treatment which cause desensitization. The experimental protocol for these experiments was based on that used by Weiss *et al.* (1995) to investigate desensitization of gonadotropin-releasing hormone (GnRH)-stimulated luteinizing hormone secretion. In this procedure cells are repeatedly stimulated with short pulses of a secretagogue at a high concentration. In order to induce desensitization one of these pulses is preceded by a treatment with a lower concentration of the secretagogue. The extent of desensitization is assessed quantitatively by expressing the response to this

pre-treated pulse as a percentage of the response to the other pulses, which act as controls.

Using the multi-column perifusion system (see Section 2.4), dispersed ovine anterior pituitary cells were stimulated with 5 min pulses of 100 nM AVP after 100, 180 and 260 min of perifusion. This resulted in three similar peaks of ACTH secretion (Fig. 3.1a). When the response to the second pulse was expressed as a percentage of the mean of the responses to the first and third pulses no significant difference could be found (n=6, not significant [NS], Student's *t*-test). Therefore, when the second pulse was preceded by a desensitizing AVP pre-treatment the first and third pulses were used as controls. Pre-treatment of the second 100 nM AVP pulse for 25 min with 5 nM AVP reduced the response by 37.9 ± 3.7% (n=7, $P<0.0001$, *t*-test) compared to the controls (Fig. 3.1b). This pre-treatment had no effect on the response to the third 100 nM AVP pulse: the response to this pulse was unchanged following pre-treatment of the second pulse compared to the response observed in the absence of pre-treatment.

It can be seen from Fig. 3.1a that there was a small reduction in response from one 100 nM AVP pulse to the next. The responses to the second and third pulses were found to be reduced by 10.7 ± 5.2% (n=6, NS, *t*-test) and 22.2 ± 2.1% (n=6, $P<0.0001$, *t*-test) compared to the first pulse. This reduction in response did not appear to be due to specific (*i.e.* receptor-mediated) desensitization processes since a similar reduction in response from one pulse to the next was also observed when cells were stimulated with 5 min pulses of 50 mM KCl in place of 100 nM AVP, although these reductions in response were not found to be statistically significant (Fig. 3.2a). Response to the second and third 50 mM KCl pulses dropped by 6.7 ± 3.5% (n=3, NS, *t*-test) and 16.1 ± 4.5% (n=3, NS, *t*-test). KCl does not activate a cell surface receptor and stimulates ACTH secretion by directly depolarizing cells (Oki *et al.* 1990). Also, when cells

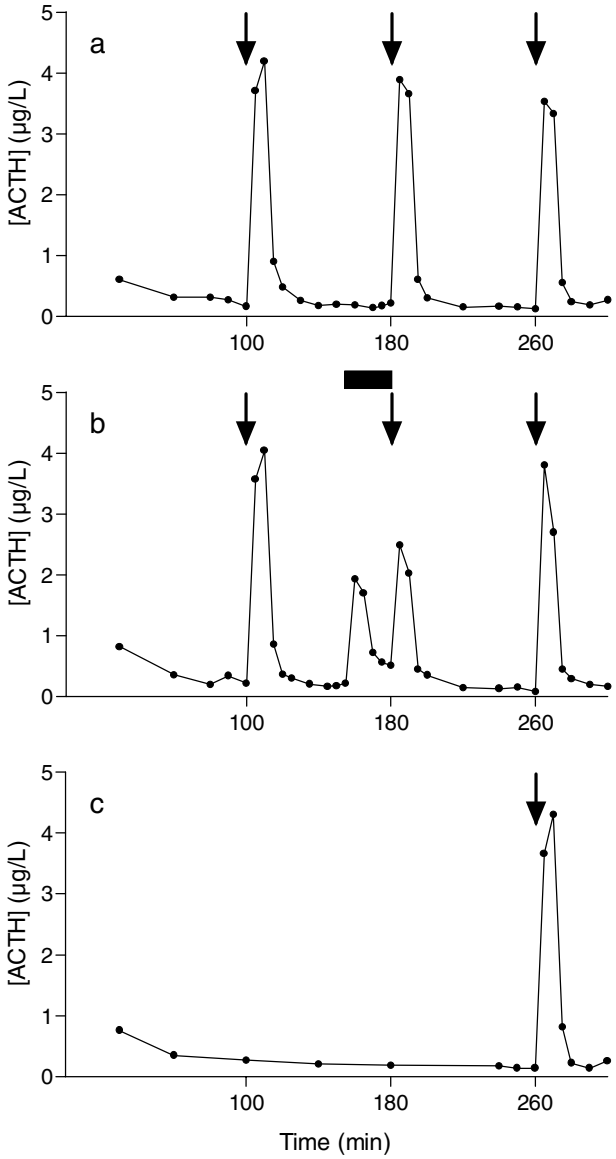

Fig. 3.1. Desensitization of the ACTH response to AVP. Representative data from three independent columns of the perifusion system illustrating the experimental protocol used in these experiments are shown. Cells were treated with 5 min pulses of 100 nM AVP after 100, 180 and 260 min of perifusion (indicated by arrows). To induce desensitization the second pulse was preceded by a pre-treatment with 10 nM AVP for 25 min (indicated by the black bar). In the column shown in (c) the cells were treated with a single 100 nM pulse after 260 min of perifusion.

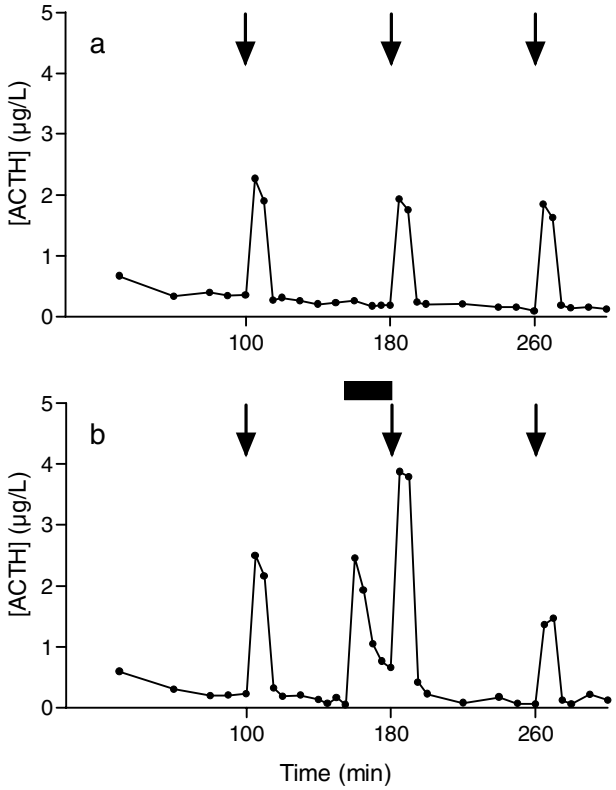

Fig. 3.2. Effect of treatment with KCl on ACTH secretion. Cells were treated with 5 min pulses of 50 mM KCl after 100, 180 and 260 min of perifusion (indicated by arrows). Pre-treatment of the second KCl pulse with 5 nM AVP for 25 min (indicated by black bar) resulted in an increase in ACTH secretion compared to the control pulses. Representative data from two indepedent perifusion columns are shown.

were treated with a single 100 nM AVP pulse after 260 min of perifusion, the response to this pulse was not significantly different to the response to a 100 nM AVP pulse at 100 min (Fig. 3.1c). Together these data suggest that the reduction in response from one pulse to the next was not the result of a true desensitization but was due to non-specific processes, probably depletion of intracellular ACTH stores.

In contrast, the reduction in response to AVP following pre-treatment with AVP seen in Fig. 3.1b reflected a specific desensitization of the cells to AVP. This was demonstrated by replacing the three AVP pulses with 5 min pulses with 50 mM KCl. Following pre-treatment with 5 nM AVP for 25 min prior to the second pulse it was found that there was no reduction in the response to the second KCl pulse compared to the responses to the control pulses (Fig. 3.2b). In fact, there was a significant increase in ACTH secretion in response to the KCl pulse following AVP pre-treatment (ACTH secretion increased to 198.8 ± 18.4% of control, n=3, $P<0.05$, *t*-test). This is consistent with a report by Le Beau & Mason (1998) who found that AVP and KCl were synergistic at low concentrations. Also, pre-treatment with 300 μM 1,2-dioctanoyl-*sn*-glycerol (DiC_8), a specific PKC activator and ACTH secretagogue (Watanabe & Orth 1988; Won *et al.* 1995), did not reduce the response to a subsequent stimulation with 100 nM AVP, indicating that stimulation of ACTH secretion was not, in itself, sufficient to induce a reduction in response to a subsequent stimulation with 100 nM AVP (see Section 4.2.1 for details). Overall, these results clearly show that the reduction in response to an AVP pulse following pre-treatment is the result of an AVP-specific desensitization process.

3.2.2 EFFECT OF CONCENTRATION OF AVP PRE-TREATMENT ON DESENSITIZATION

To investigate the concentration-dependency of desensitization, the duration of the pre-treatment was held constant at 25 min while the AVP pre-treatment concentration applied to different columns was varied from 0.1 to 50 nM. Representative results from five perifusion columns are shown in Fig. 3.3 and the combined data are summarized in Fig. 3.4. AVP pre-treatment desensitized the response to a subsequent AVP pulse in a concentration-dependent manner. A sigmoidal concentration-response curve was fitted to the data yielding an r^2 of 0.93 and a predicted value for 50% desensitization (IC_{50}) of the ACTH response of 6.54 nM AVP. The lowest concentration of AVP tested which was capable of causing a significant reduction in response was 2.0 nM (n=3, $P<0.02$, *t*-test). The ACTH release following pre-treatment with 50 nM AVP was maintained at a plateau by the second AVP pulse, rather than the pulse causing a response peak as seen at lower pre-treatment concentrations.

3.2.3 EFFECT OF DURATION OF AVP PRE-TREATMENT ON DESENSITIZATION

The degree of desensitization of the response to an AVP pulse was also found to be dependent on the duration of the pre-treatment. A similar protocol to that described above was used except that the concentration of the pre-treatment was held constant at 5 nM while the duration varied from 0 to 25 min. Data from five independent perifusion columns are shown in Fig. 3.5, while the combined data are summarized in Fig. 3.6. A pre-treatment for as little as 5 min resulted in a significant reduction (21.5 ± 1.5% of control, n=3, $P<0.005$, *t*-test) in the response to the second AVP pulse compared to the mean of the responses to the first and third pulses. A greater reduction in the response to the second AVP pulse (39.4 ± 8.6%, n=3, $P<0.05$, *t*-test) was observed when the duration of the 5 nM AVP pre-treatment was increased to 10 min.

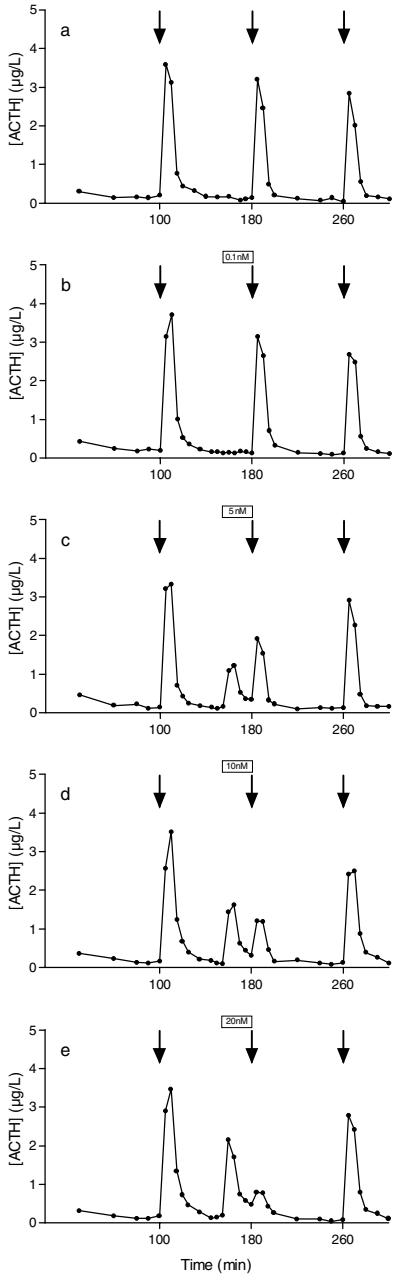

Fig. 3.3. Effect of pre-treatment concentration on desensitization of the ACTH respone to AVP. Representative data from five independent perifusion columns are shown. The second AVP pulse was pre-treated with AVP at the concentration indicated for 25 min.

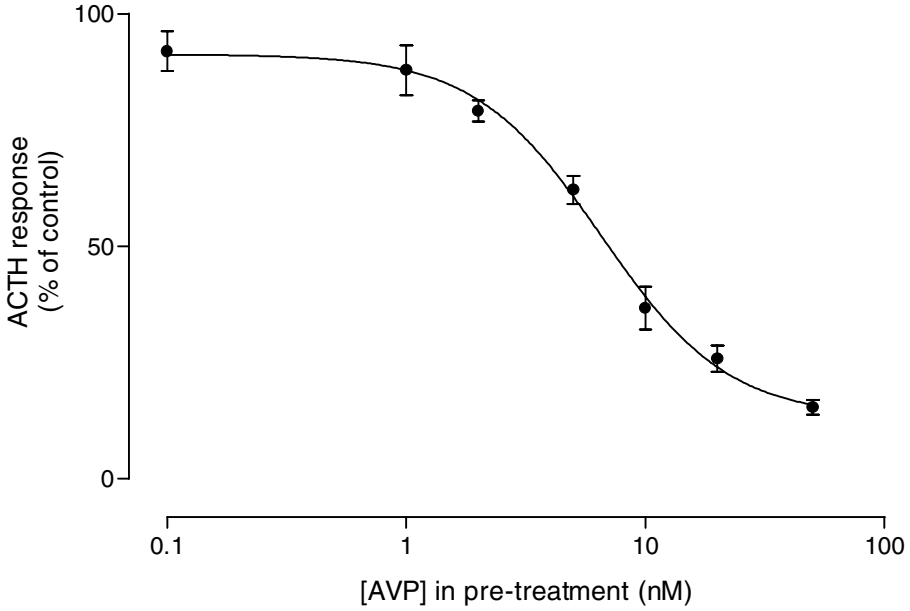

Fig. 3.4. Concentration-dependence of the desensitization of the ACTH response to AVP, determined using the experimental protocol shown in Fig. 3.3. The ACTH response to the second AVP pulse was expressed as a percentage of the control pulses. The concentrations used for the 25 min pre-treatment are plotted on a log scale. Data are mean ± SEM (n=2–7 for each treatment).

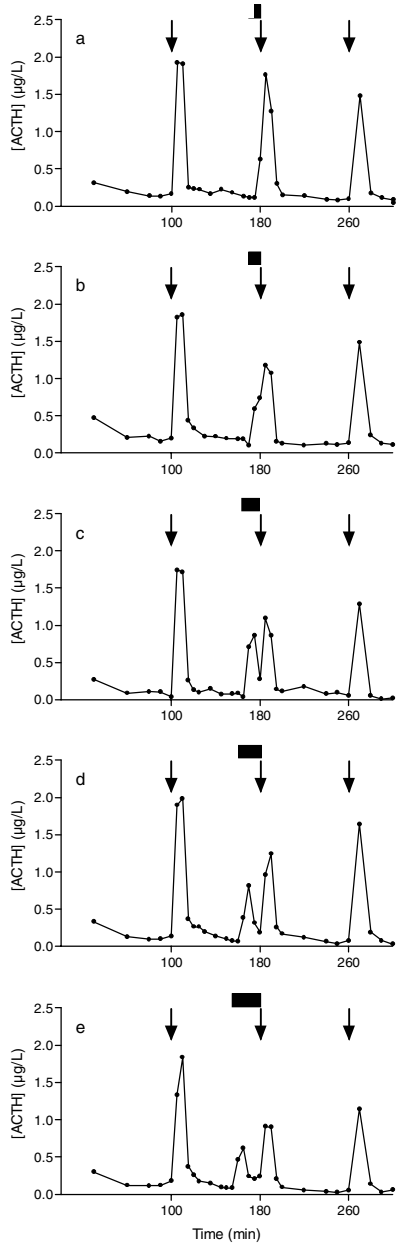

Fig. 3.5. Effect of pre-treatment duration on desensitization of the ACTH response to AVP. The concentration of AVP in the pre-treatment was held constant while the duration was varied. Pre-treatment durations were a) 5 min; b) 10 min; c) 15 min; d) 20 min and e) 25 min. Representative data from five independent perifusion columns are shown.

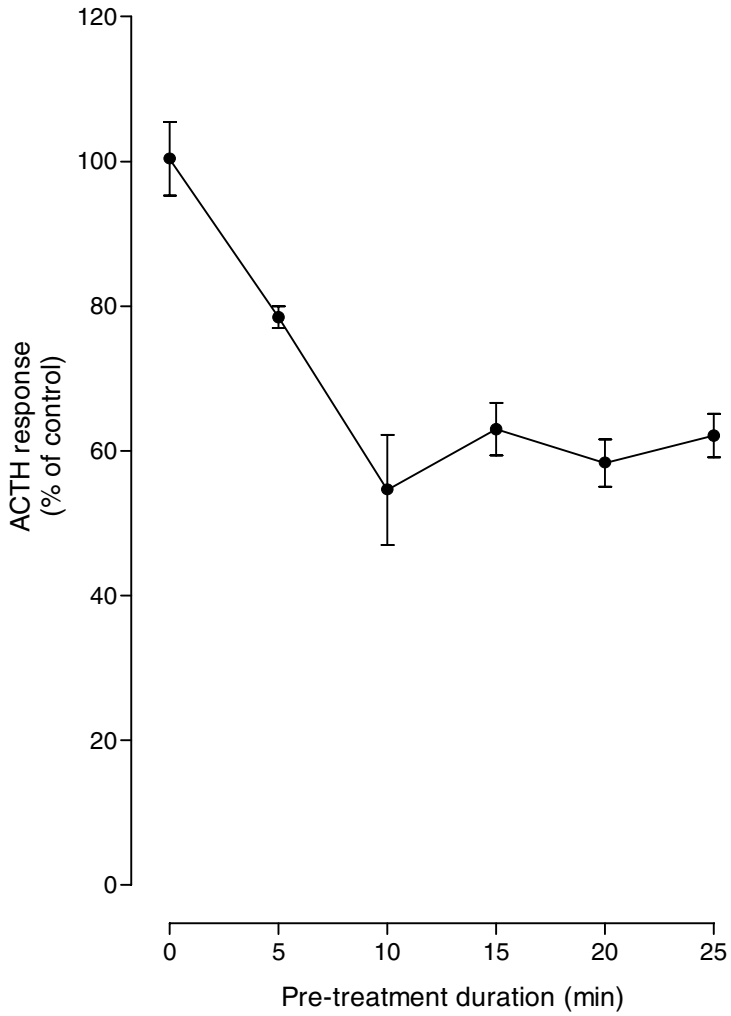

Fig. 3.6. Time-dependence of the desensitization of the ACTH reponse to AVP. The ACTH response to the second AVP pulse, as a percentage of the control pulses is plotted against the duration of the AVP pre-treatment. The concentration of AVP used for the pre-treatment was 5 nM. Data are mean ± SEM (n=3–7 for each treatment).

However, extending the pre-treatment period beyond 10 min did not result in any further increase in the magnitude of desensitization.

3.2.4 RESENSITIZATION OF THE ACTH RESPONSE TO AVP

Perifused anterior pituitary cells were clearly quite sensitive to desensitization of the ACTH response to AVP. To characterize this process further, the ability of desensitized cells to recover their responsiveness to AVP was investigated. Since it was anticipated that the effects of desensitization would take somewhat longer to be reversed than they did to occur, an experimental protocol different to that used to investigate the time- and concentration-dependency of desensitization was employed. Cells were stimulated with a single 5 min pulse of 100 nM AVP after 200 min of perifusion. This pulse was preceded by a 15 min pre-treatment with 10 nM AVP. Unlike the experimental protocol used in the first series of experiments the cells were allowed a recovery period of up to 80 min between the pre-treatment and the AVP pulse. During this recovery period they were perifused with medium alone. In order to quantitatively assess the extent of resensitization, the response of the pre-treated cells to 100 nM AVP was expressed as a percentage of the mean response of the control cells (*i.e.* cells that had not been pre-treated) to a 100 nM AVP pulse.

Data from five independent perifusion columns are shown in Fig. 3.7. Combined data are summarized in Fig. 3.8. When the AVP pulse was applied immediately upon termination of the pre-treatment (*i.e.* 0 min recovery time) the magnitude of the response to the test pulse was reduced to 36.7 ± 5.4% (n=7, $P<0.01$, One way ANOVA with Dunnett's test) of the response observed in controls that were not pre-treated. When the cells were allowed a 10 min recovery period between the pre-treatment and

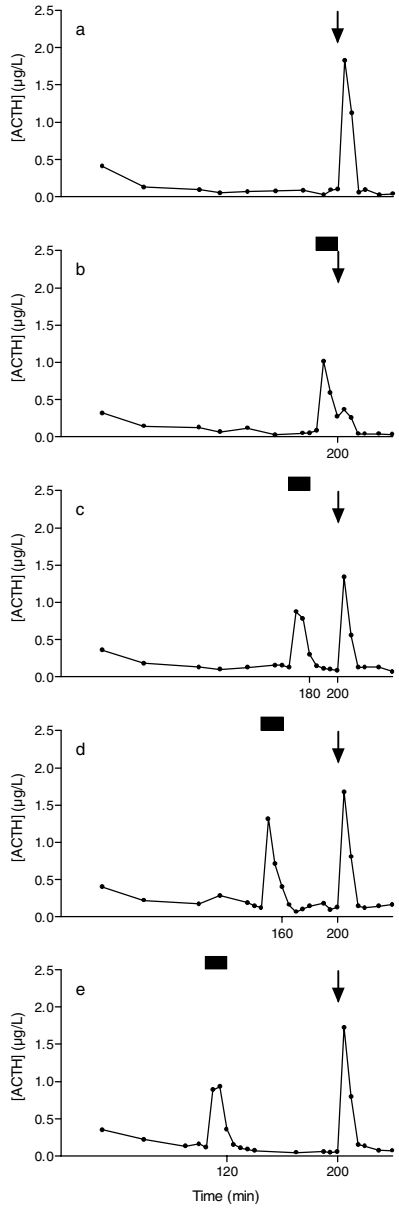

Fig. 3.7. Recovery of the ACTH response to AVP following desensitization. Cells were treated with a single 100 nM AVPpulse after 200 min of perifusion (indicated by arrows). Pre-treatment with 10 nM AVP for 15 min decreased the response to the AVP pulse (b) compared to the control (a). When a recovery period was allowed between the pre-treatment and the pulse the response to the pulse increased. Durations of the recovery periods shown above were c)2 0min; d) 40 min; e) 80 min. Representative data from five independent perifusion columns are shown.

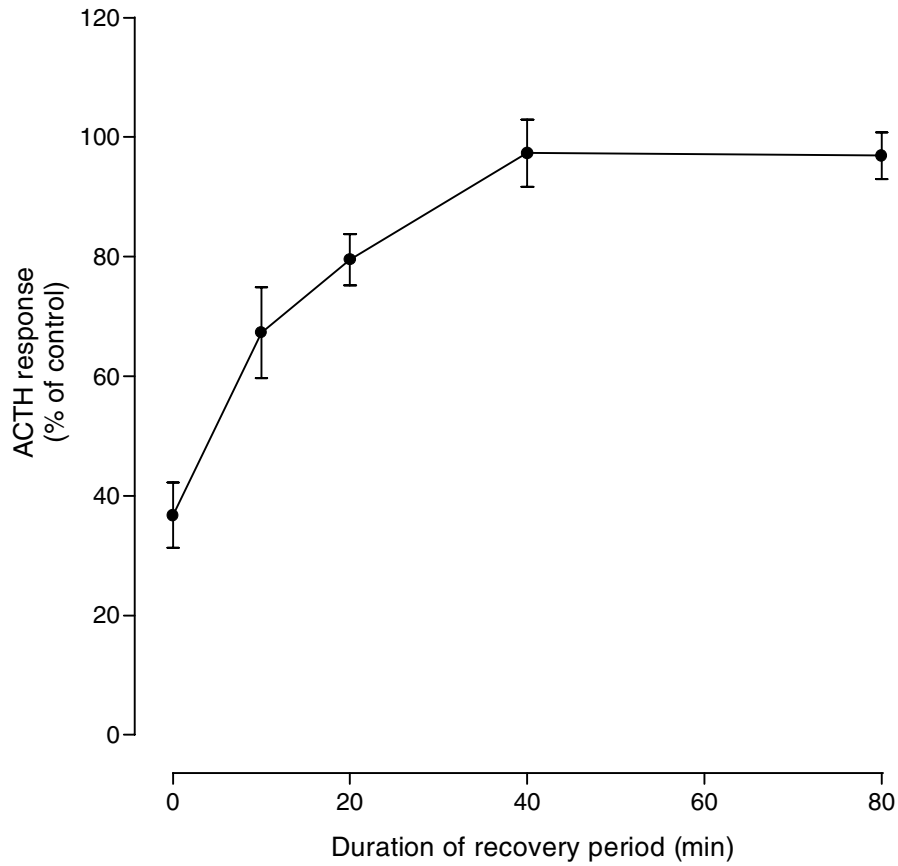

Fig. 3.8. Time-course of resensitization of the ACTH response to AVP. The ACTH response after pre-treatment with 10 nM AVP for 15 min is plotted against the duration of the recovery period (*i.e.* the period of time betweeen the pre-treatment and the AVP pulse during which the cells were perifused with medium alone). Data are mean ± SEM (n=7–15 for each treatment).

the test pulse there was a partial recovery in response to 67.3 ± 7.6% (n=8, $P<0.01$, Dunnett's test) of control. Recovery was complete after 40 min.

3.3 DISCUSSION

This study shows that desensitization of the ACTH response of cultured ovine anterior pituitary cells to AVP occurs more rapidly and at lower AVP concentrations than has previously been reported. Previous *in vitro* studies have shown that desensitization to AVP following stimulation with AVP at either high concentrations or for long periods of time (Holmes *et al.* 1984; Antoni *et al.* 1985; Evans *et al.* 1988; Castro 1993). These experimental treatments contrast with the endogenous pattern of AVP secretion into the hypophyseal portal circulation which is highly pulsatile, with the AVP concentration of pulses normally being less than 5 nM (Engler *et al.* 1989; Caraty *et al.* 1990; Engler *et al.* 1989; Caraty *et al.* 1988; Battaglia *et al.* 1998; Dadoun *et al.* 1998).

Using an experimental protocol based on that described by Weiss *et al.* (1995) I have investigated desensitization of the ACTH response to AVP pulses at concentrations and durations which more closely match those of endogenous pulses. Using this experimental paradigm it was found that pre-treatment with AVP at concentrations as low as 2 nM was capable of eliciting a significant reduction in response to a subsequent 100 nM AVP pulse. The IC_{50} for this desensitization process was calculated to be 6.54 nM. Furthermore it was found that the desensitization was rapid, with the maximal desensitization to 5 nM AVP being reached after 10 min of pre-treatment. The resensitization of the ACTH response was also investigated and it was found that the response returned to a level not significantly different to that of the controls after a recovery period of 40 min.

The comparison of these results with the results of studies which examined the endogenous AVP pulse characteristics is interesting. As indicated above, in the sheep, peak concentrations of AVP in the hypophyseal portal blood during stress are less than 6 nM and secretory events appear to last no more than about 30 min. Taken together, these data suggest that while most endogenous AVP pulses would be long enough to cause desensitization their low concentrations would elicit only partial desensitization. This raises the question of whether this type of rapid desensitization plays a role in normal physiology. Weiss *et al.* (1995) investigated the desensitization of perifused pituitary cells to GnRH and found a qualitatively similar relationship between *in vitro* desensitization and endogenous pulse characteristics: endogenous GnRH pulses would have been long enough, although not of great enough concentration to evoke desensitization. It was suggested that the role of desensitization was not to prevent gonadotropin secretion, but rather to limit the maximum secretory response to GnRH. It is possible that desensitization to AVP plays a similar role in the regulation of ACTH secretion. If this were the case, it would suggest that corticotrophs have an intrinsic set-point, beyond which they become refractory to further stimulation with AVP. Furthermore, the rapidity of the desensitization of the ACTH response to AVP might act to limit the duration of secretory episodes. Together these two properties of the desensitization process could result in a stereotyping of ACTH pulse amplitude and duration (Leng & Brown 1997).

The characteristics of the desensitization which has been observed give some clues to the mechanisms which may underlie it. As described above, amongst the G protein-coupled receptors there are two types of mechanism which result in desensitization: those which affect the ability of receptors to activate their signalling pathways (uncoupling) and those that result from a reduction in total receptor number (down-regula-

tion)(Lohse 1993). Uncoupling of receptors from their signalling pathways is rapid, occurring within seconds or minutes of the stimulus onset (Roth *et al.* 1991), and is readily reversible (Lohse 1993). In contrast, down-regulation is a relatively slow process, requiring hours to take effect (Hausdorff *et al.* 1990). Furthermore, because it involves degradation of existing receptors (Hausdorff *et al.* 1990) or the inhibition of the production of new receptors (Hadcock & Malbon 1993) it is relatively slow to reverse. The speed with which desensitization of the ACTH response to AVP occurs and its rapid resensitization strongly suggest that the mechanism(s) underlying it involve uncoupling of the receptor from its signaling pathway. Commonly uncoupling is mediated by receptor phosphorylation which, either directly or indirectly, prevents receptors from interacting with their associated G proteins (Grady *et al.* 1997). Desensitization of both the V1a (Innamorati *et al.* 1998a) and V2 (Innamorati *et al.* 1997) AVP receptors is mediated by receptor phosphorylation, and it is possible that desensitization of the V1b receptor is mediated in a similar manner, although the involvement of other mechanisms (such as receptor sequestration) cannot be excluded.

The rapid resensitization of the ACTH response to AVP suggests that this rapid desensitization is more likely to be involved in regulating the acute response to AVP rather than altering the responsiveness of the corticotroph to chronic stress. The changes in AVP receptor number and associated changes in corticotroph responsiveness observed by Aguilera *et al.* (1994) following exposure to various chronic stress paradigms are most likely the result of a down-regulation process rather than the rapid desensitization observed here.

The regulation of ACTH secretion is a complex process which is influenced not only by the independent action of stimulatory factors (such as CRH and AVP) and inhibitory factors (such as glucocorticoids) but also by complex interactions of these

factors with one another (Evans *et al.* 1996). It is possible that some of these factors are capable of modulating the degree of desensitization of pituitary cells to AVP. In the sheep, since ACTH secretion is dependent upon the co-ordinated action of AVP and CRH, it is important to consider the effect of CRH on the desensitization of the ACTH response to AVP. Using an experimental protocol similar to that described above, it has been shown that this desensitization is unaffected by treatment with CRH (Chacko 2000; Chacko *et al.* 2000). Presence of a low concentration of CRH (0.01 nM) during perifusion neither prevented nor enhanced the desensitization of the ACTH response to AVP. It remains possible that other factors, such as glucocorticoids, may modulate the desensitization process, thereby providing an additional level of control of ACTH secretion.

In summary, it has been shown that the ACTH response to AVP undergoes rapid desensitization. The concentrations and durations of AVP exposure which are capable of eliciting desensitization suggest that this desensitization process may play an important physiological role in the regulation of ACTH secretion from the anterior pituitary.

4

ROLE OF PROTEIN KINASES IN THE DESENSITIZATION OF THE ACTH RESPONSE TO AVP

4.1 INTRODUCTION

The results of the experiments described in the previous chapter show that the ACTH response to AVP of perifused dispersed ovine anterior pituitary cells is rapidly and reversibly desensitized. These characteristics of the desensitization suggest that the molecular mechanisms underlying it involve uncoupling of the pituitary AVP receptor from its signalling pathway. This is a common mechanism of rapid desensitization amongst the GPCR family and is most commonly mediated by phosphorylation of the receptor on serine and/or threonine residues by one or more protein kinases (Lohse 1993). A variety of different protein kinases are capable of phosphorylating GPCRs. These include the GRK family (Premont *et al.* 1995), the effector kinases PKA and PKC (Pitcher *et al.* 1992) and CK1α (Tobin *et al.* 1997). The aim of the following research was to investigate the involvement of receptor phosphorylation in desensitization of the ACTH response to AVP. In particular, the involvement of PKC and CK1α in the desensitization process was investigated.

The AVP receptor expressed in the anterior pituitary is of the V1b subtype (Jard *et al.* 1986; Sugimoto *et al.* 1994). It shares a relatively high degree of homology (45.5%) with the V1a AVP receptor and both are coupled to the phosphoinositide signalling pathway (De Keyzer *et al.* 1994). Like the V1b receptor, signalling through the V1a receptor undergoes desensitization following exposure to AVP (Cantau *et al.* 1988; Grier *et al.* 1989; Caramelo *et al.* 1991; Gallo-Payet *et al.* 1991; Nathanson *et al.* 1994; Ancellin *et al.* 1997). This desensitization process is both concentration-dependent and rapid, with significant desensitization occurring within 30 s of AVP exposure in vascular smooth muscle cells (Caramelo *et al.* 1991). Evidence suggests that desensitization of the V1a receptor is mediated, at least in part, by PKC-mediated phosphorylation. Firstly, desensitization of signalling through the V1a receptor can be induced by activating PKC. Both pre-treatment with specific PKC activators such as 1,2-dicotanoyl-*sn*-glycerol (DiC_8), 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), phorbol-12-myristate-13-acetate (PMA) and phorbol-12,13-dibutyrate (PDBu)(Gallo-Payet *et al.* 1991; Ancellin *et al.* 1997; Ancellin & Morel 1998), and direct injection of the catalytic subunit of PKC into cells (Ancellin *et al.* 1997) cause a reduction in response to a subsequent stimulation with AVP. (Note that Nathanson *et al.* [1994] did not observe significant desensitization of the V1a receptor following treatment with PDBu. This was probably because a PDBu treatment of insufficient duration was used to induce desensitization [Ancellin & Morel 1998]). Secondly, desensitization can be significantly reversed by down-regulation of PKC by 24 h treatment with PMA (Caramelo *et al.* 1991). Finally, an increase in phosphorylation of the V1a receptor has been observed following treatment with PMA (Innamorati *et al.* 1998a) and activation of the PLC-coupled m3 muscarinic receptor (Ancellin *et al.* 1999), indicating that PKC is able to phosphorylate the receptor.

Given that there are structural similarities between the V1a and V1b receptors and that the two receptors have similar signalling properties it is possible that the molecular mechanisms involved in their desensitization are also similar. Consistent with this hypothesis, Berrada *et al.* (2000) have shown that during agonist exposure PKCα interacts with a GFP-tagged V1b receptor expressed in CHO cells. Alternatively, the mechanism involved may be different. It has been proposed that such differential desensitization may explain the existence of receptor subtypes with otherwise similar pharmacological and signalling properties (Kurose & Lefkowitz 1994; Nantel *et al.* 1993). I have used two approaches to investigate the involvement of PKC in desensitization of the ACTH response to AVP. Firstly, the ability of the specific PKC activator DiC_8 to induce desensitization of the ACTH response to AVP was assessed. Secondly, the effect of inhibition of PKC on AVP-induced desensitization was investigated using the highly selective PKC inhibitor Ro 31-8220.

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GRKs have been found to be involved in the regulation of some PLC-coupled GPCRs, including the α_{1B}-adrenergic receptor (Diviani *et al.* 1996), the AT_{1A} angiotensin II receptor (Oppermann *et al.* 1996) and the substance P receptor (Kwatra *et al.* 1993). However, changes in the intracellular environment following activation of PLC appear somewhat unfavourable to activation of GRKs. Following activation of PLC levels of PIP_2 in the plasma membrane fall by up to 80% (Willars *et al.* 1996; Wilson *et al.* 1985; Koreh & Monaco 1986). PIP_2 plays an important role in the regulation of GRK activity: a synergistic interaction between PIP_2, $G_{\beta\gamma}$ subunits and the pleckstrin homology domain in the C-terminus of GRK2 promotes translocation of the enzyme

to the plasma membrane, thereby enhancing its ability to phosphorylate agonist-occupied receptors (Pitcher *et al.* 1995b; DebBurman *et al.* 1996). Similarly, PIP_2 regulates membrane association of GRK4, GRK5 and GRK6, although in this instance this is the result of interactions of the phospholipid with the NH_2-terminus of the enzymes (Pitcher *et al.* 1996). Furthermore, the rise in intracellular Ca^{2+} which follows activation of PLC-coupled receptors may also discourage the activity of GRKs: translocation of GRK2 and GRK3 to the plasma membrane is inhibited by the ability of Ca^{2+}/calmodulin to compete for the binding of $G_{\beta\gamma}$ subunits (Chuang *et al.* 1996; Haga *et al.* 1997) and GRK5 translocation is inhibited by a direct interaction of Ca^{2+}/calmodulin with the kinase (Chuang *et al.* 1996). The inhibitory effect of Ca^{2+}/calmodulin on GRK2 activity only occurs at relatively high calmodulin concentrations (IC_{50} ~2 μM) suggesting that it might only be physiologically important in tissues such as brain where calmodulin levels are high (Pitcher *et al.* 1998). Finally, PKC-mediated phosphorylation has been shown to inhibit GRK5 (Pronin & Benovic 1997), although, on the other hand, it has also been shown to activate GRK2 (Chuang *et al.* 1995; Winstel *et al.* 1996).

Since these conditions may be unfavourable for activity of the GRKs, particularly GRK5, it has been suggested that in some cases alternative mechanisms might be involved in the regulation of PLC-coupled GPCRs. Recently it has been shown that CK1α phosphorylates the PLC-coupled m3 muscarinic receptor (Tobin *et al.* 1997). This phosphorylation was agonist-dependent and was associated with a rapid desensitization of the cellular responses to stimulation. Subsequently it has been shown that CK1α can also phosphorylate rhodopsin (Tobin *et al.* 1997) and the m1 muscarinic receptor (Waugh *et al.* 1999), suggesting that it may have a broad substrate specificity amongst the GPCRs. Interestingly, two yeast casein kinase 1 homologues, Yck1p and

Yck2p, have been shown to phosphorylate a GPCR, namely the α pheromone receptor ste2p (Hicke *et al.* 1998). Although it was originally suggested that CK1α-mediated phosphorylation might provide an alternative mechanism for desensitization of PLC-coupled receptors, replacing GRKs in some instances (Tobin *et al.* 1997), more recent evidence suggests that CK1α-mediated phosphorylation may not mediate desensitization of the m3 muscarinic receptor but is instead involved in the regulation of the initial IP_3 response to stimulation. Expression of a catalytically inactive mutant CK1α was found to have no effect on desensitization but resulted in an increase in the initial IP_3 response. Excision of the third intracellular loop, which contains the residues phosphorylated by CK1α, had a similar effect on the IP_3 response. These changes in response may have reflected enhanced coupling of the receptor to $G_{q/11}$ (Budd *et al.* 2000).

As the V1b receptor is coupled to PLC the involvement of CK1α in the regulation of signalling through this receptor was investigated. This was achieved using the specific casein kinase 1 inhibitor CK1-7. The effect of treatment with this agent on both the ability of AVP pre-treatment to induce desensitization and its effect on the initial ACTH response to AVP were investigated.

While the experiments described above were designed to investigate the involvement of specific protein kinases in the desensitization of the ACTH response to AVP they do not show directly whether the effects of these protein kinases are mediated by phosphorylation of the receptor. Like the V1a and V2 AVP receptors (both of which are phosphorylated following agonist exposure [Innamorati *et al.* 1997; Innamorati *et al.* 1998a; Ancellin *et al.* 1999]) the V1b receptor contains a number of serine residues in its intracellular loops and C terminal tail that are potential sites for phosphorylation (De Keyzer *et al.* 1994; Ventura *et al.* 1999). In light of this, phosphorylation of the

pituitary AVP receptor during agonist-exposure was investigated directly. Stadel *et al.* (1983) showed that desensitized β-adrenergic receptors migrated more slowly on polyacrylamide gels than receptors isolated from control cells. This retarded mobility was due to the incorporation of phosphate groups into the receptor. A similar approach was used to investigate the phosphorylation of the V1b receptor, with the receptor being visualized on polyacrylamide gels by the cross-linking of a radiolabelled ligand.

4.2 RESULTS

4.2.1 EFFECT OF PRE-TREATMENT WITH 1,2-DIOCTANOYL-SN-GLYCEROL ON THE ACTH RESPONSE TO AVP

In order to investigate the involvement of PKC in desensitization of the ACTH response to AVP the effect of pre-treatment with DiC_8 on a subsequent stimulation with AVP was assessed. DiC_8 is a synthetic diacylglycerol that strongly activates PKC (Go *et al.* 1989), and as such treatment with this agent results in a marked stimulation of ACTH secretion from anterior pituitary cells (Watanabe & Orth 1988; Won *et al.* 1995). If PKC were involved in the desensitization process one would expect that pre-treatment with DiC_8 would reduce the response to a subsequent stimulation with AVP.

Experiments were carried out using the multi-column perifusion system (see Section 2.4). The experimental design and analysis used were essentially similar to those used to investigate the desensitization of the ACTH response to AVP and are described in detail in Section 3.2.1. Briefly, cells were stimulated with 5 min pulses of 100 nM AVP after 100, 180 and 260 min of perifusion resulting in three similar peaks of ACTH secretion (Fig. 4.1a). The second pulse was preceded by either a 15 min pre-treatment with 10 nM AVP (Fig. 4.1b) or a 15 min pre-treatment with DiC_8 at con-

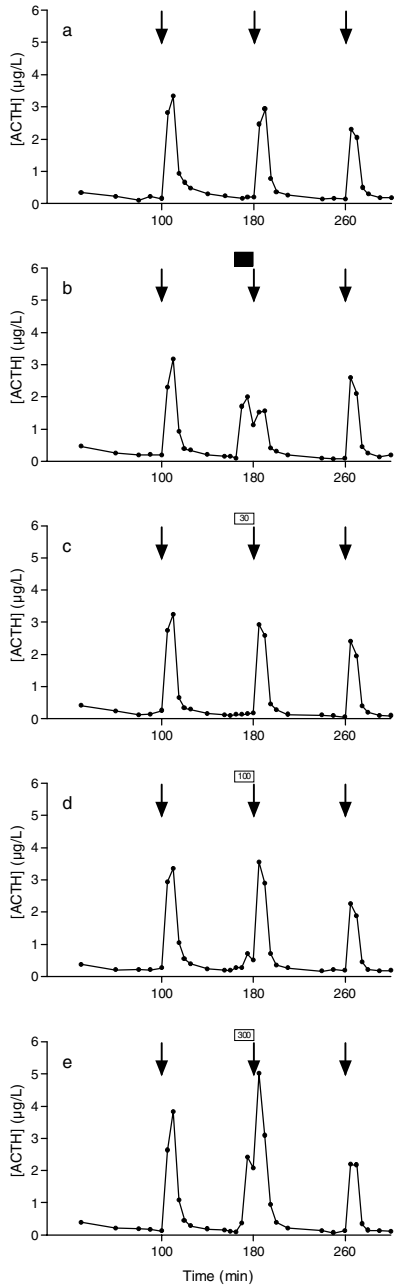

Fig. 4.1. Effect of pre-treatment with DiC_8 on the ACTH response to AVP. Data in each panel are from single, representative columns in which prior to the second 100 nM AVP pulse the cells received either no pre-treatment (a); or a 15 min pre-treatment with 10 nM AVP (black bar; b); 30 µM DiC_8 (open bar; c); 100 µM DiC_8 (d); 300 µM DiC_8 (e). AVP pulses are indicated by arrows.

centrations of either 30, 100 or 300 μM (Fig. 4.1c, d, e). The effect of this pre-treatment on the ACTH response to AVP was assessed quantitatively by expressing the response to the second pulse as a percentage of the response to the first and third pulses.

Secretion of ACTH during pre-treatment with DiC_8 was concentration-dependent (Fig. 4.2). Treatment with 30 μM DiC_8 caused a small but significant increase in ACTH secretion above basal (n=6, $P<0.01$, Student's *t*-test). When the concentration was increased to 300 μM, stimulated ACTH secretion was not significantly different from that observed during pre-treatment with 10 nM AVP (One way ANOVA with Dunnett's test).

Despite stimulating ACTH secretion at a level equivalent to AVP, pre-treatment with DiC_8 did not induce desensitization. In contrast to pre-treatment with 10 nM AVP, which caused a reduction in response of 48.6 ± 1.6% (n=6, $P<0.0001$, Student's *t*-test) to a subsequent stimulation with AVP, the ACTH response to AVP was increased in a concentration-dependent manner following pre-treatment with DiC_8 (Fig. 4.3). While pre-treatment with 30 μM DiC_8 had no significant effect on the response to the subsequent 100 nM AVP pulse, pre-treatment with 100 μM DiC_8 caused a 9.9 ± 3.7% (n=6, $P<0.05$, *t*-test) increase compared with controls, and after a 300 μM DiC_8 pre-treatment the response was increased by 51.3 ± 4.4% (n=6, $P<0.0001$, *t*-test).

4.2.2 EFFECT OF PRE-TREATMENT WITH RO 31-8220 ON DESENSITIZATION OF THE ACTH RESPONSE TO AVP

The involvement of PKC in the desensitization of the ACTH response to AVP was investigated further using Ro 31-8220. Ro 31-8220 (or, more fully, 2-{1-[3-(amidinothio)propyl]-1*H*-indol-3-yl}-3-(1-methylindol-3-yl)-maleimide methanesulfonate), is a potent, selective inhibitor of PKC derived from the bacterial metabolite staurosporine

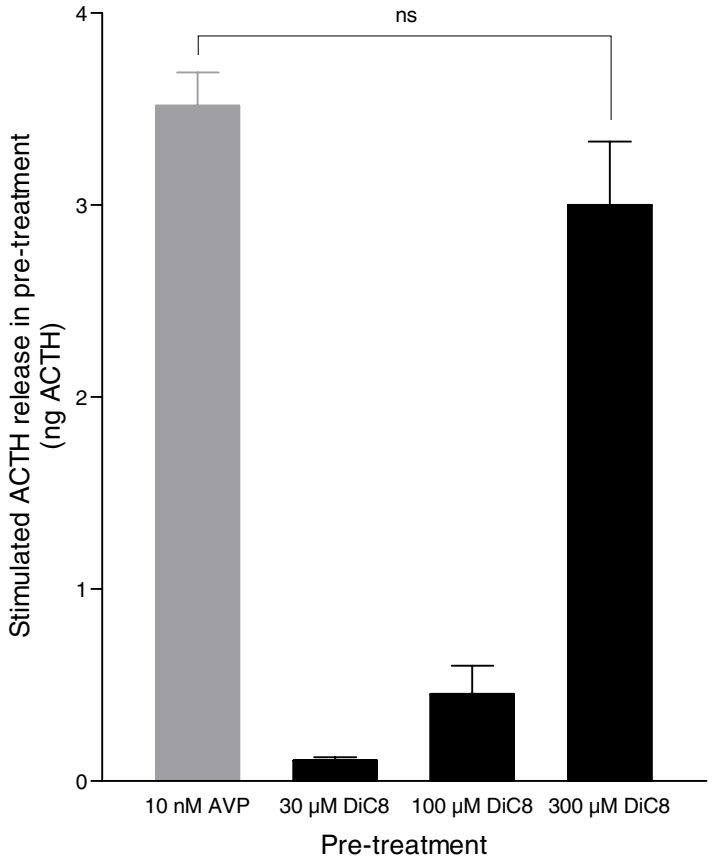

Fig. 4.2. Effect of treatment with DiC_8 for 15 min on ACTH secretion. Treatment with DiC_8 caused significant increases in ACTH secretion above basal at all concentrations tested. DiC_8-stimulated ACTH secretion was concentration-dependent, with treatment with 300 µM DiC_8 causing ACTH secretion equivalent to that stimulated by 10 nM AVP (Dunnett's test). Data are mean ± SEM (n=6 for each treatment).

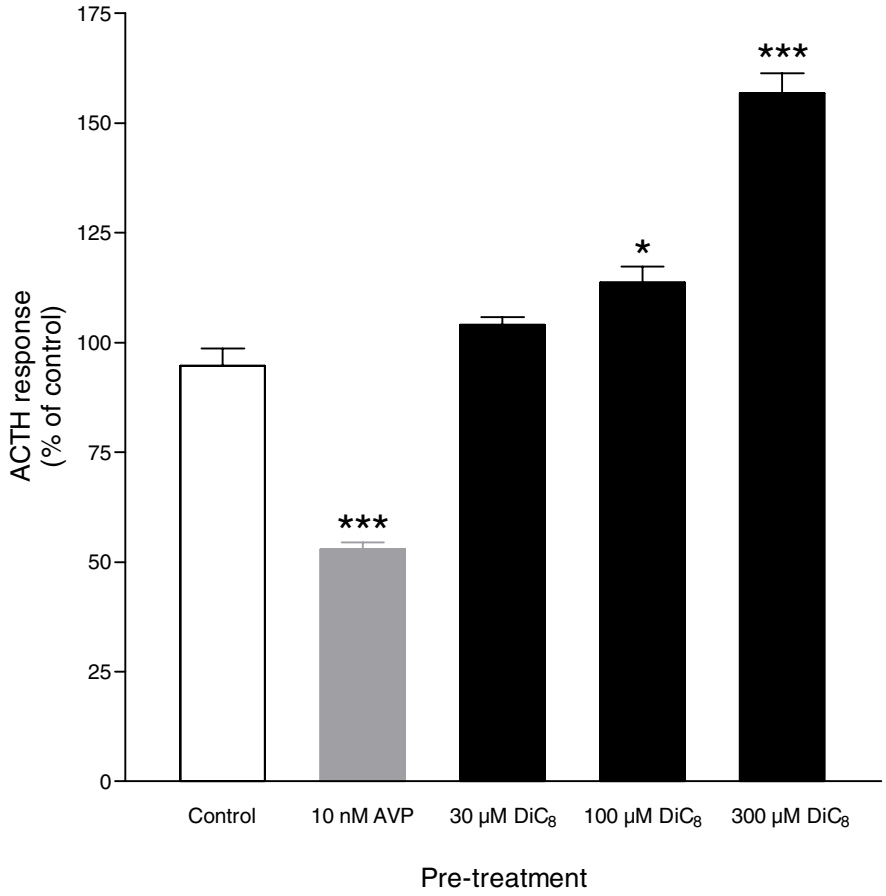

Fig. 4.3. Effect of pre-treatment with DiC_8 on the ACTH response to a subsequent stimulation with AVP. The data shown are the responses, expressed as a percentage of control, to a 5 min, 100 nM AVP pulse following pre-treatment for 15 min with the indicated solutions. Data are mean ± SEM (n=5–6 for each treatment). Asterisks indicate a statistically significant difference between the results observed and 100% (*t*-test, * $P<0.05$, *** $P<0.001$).

(Davis *et al.* 1992). It was provided as a kind gift by Dr G. Lawton of the Roche Research Centre, Welwyn Garden City, UK. Ro 31-8220 was used to inhibit PKC activity during treatment with AVP. If PKC were involved in the desensitization process then treatment with Ro 31-8220 would be expected to impair the ability of an AVP pre-treatment to induce desensitization of the ACTH response to a subsequent stimulation with AVP.

Again, experiments were carried out using the multi-column perifusion system with an experimental design similar to that described in Section 3.2.1. To induce desensitization the second 100 nM AVP pulse was preceded by a 15 min pre-treatment with 10 nM AVP. In initial experiments the effect of PKC inhibition of the development of this desensitization was assessed by treating the cells with 2 μM Ro 31-8220 for a short period (20 min) prior to the second AVP pulse. This experimental design was similar to that used to investigate the involvement of CK1α in desensitization (see Section 4.2.3). Unfortunately it was found that the effects of treatment with Ro 31-8220 were not confined to the second AVP pulse: the response to the third 100 nM AVP pulse was significantly reduced by a prior treatment with Ro 31-8220 alone. Because of these long-lasting effects of treatment with Ro 31-8220 it was not possible to use the first and third pulses as controls. In an attempt to avoid this problem it was decided to treat cells with Ro 31-8220 at a constant 'background' concentration for the duration of the experiment. Treatment with 2 μM Ro 31-8220 began at 90 min and continued throughout the experiment until its completion (*i.e.* 300 min). This treatment with Ro 31-8220 reduced the ACTH response to each of the 100 nM AVP pulses. This effect was not uniform however, with the reduction in response being more marked for the second and third pulses. As a result the response to the second AVP pulse was 28.8 ± 8.4% less than the mean of the response to the first and third pulses in

the absence of AVP pre-treatment. Although this reduction was not significant (P=0.0749, n=3, t-test) it was decided that it was not appropriate to use this method of analysis to assess the extent of desensitization induced by AVP pre-treatment. Instead, the response to the second AVP pulse following pre-treatment was compared to the response to the second AVP pulse in control columns which received no pre-treatment. This analysis was performed both when 2 μM Ro 31-8220 was present in the perifusion medium and when it was absent. During treatment with 2 μM Ro 31-8220 the ACTH response to the 100 nM AVP was reduced by 45.1 ± 3.5% (n=7, P<0.0001, t-test) compared to controls which were not treated with Ro 31-8220. Despite being able to inhibit ACTH secretion, treatment with Ro 31-8220 did not reduce the extent of desensitization observed. Following pre-treatment with AVP in combination with 2 μM Ro 31-8220 the response to a subsequent stimulation with 100 nM AVP was reduced by 88.8 ± 4.5% (n=4, P<0.01, t-test) compared to the corresponding control (Fig. 4.4a, b). In contrast, following AVP pre-treatment in the absence of Ro 31-8220 the response to a 100 nM AVP pulse was reduced by 69.8 ± 2.4% (n=3, P<0.05, t-test)(Fig. 4.4c, d). This difference in the magnitude of reduction in response caused by AVP pre-treatment was significant. (n=7, P<0.05, t-test). Results are summarized in Fig. 4.5.

4.2.3 EFFECT OF PRE-TREATMENT WITH CKI-7 ON DESENSITIZATION OF THE ACTH RESPONSE TO AVP

The role of CK1α in the regulation of AVP-stimulated ACTH secretion was investigated using the potent casein kinase 1 inhibitor CK1-7 (*N*-(2-aminoethyl)-5-chloro-isoquinoline-8-sulfonamide). This cell-permeable isoquinoline sulfonamide compound is a highly selective inhibitor of casein kinase I activity (IC_{50}=9.5 μM), with a much

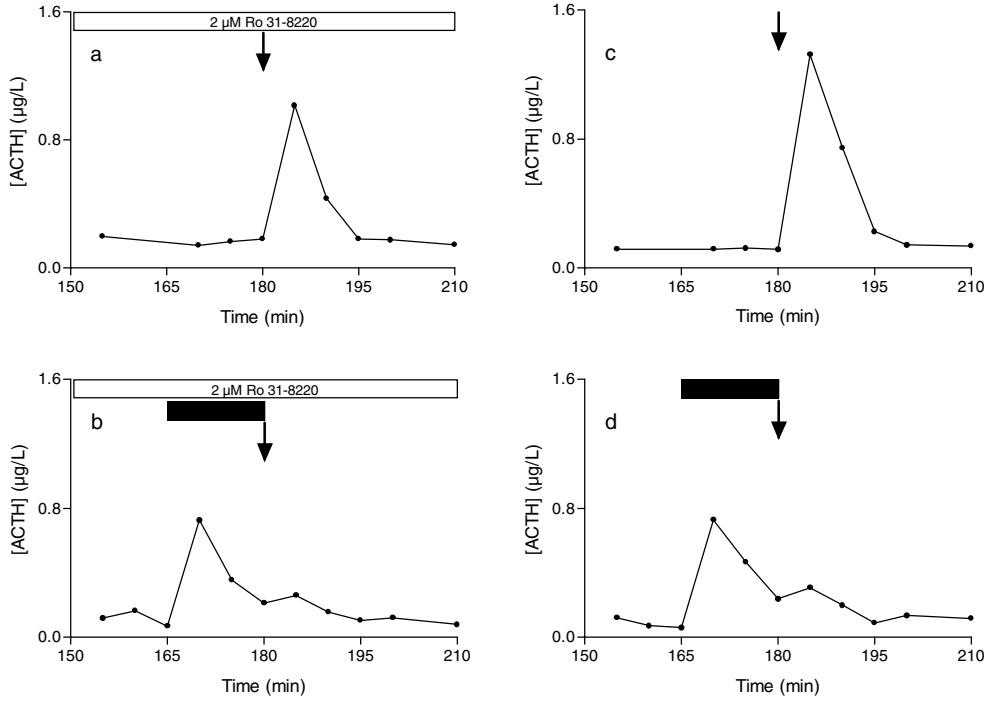

Fig. 4.4. Effect of treatment with Ro 31-8220 on desensitization of the ACTH response to AVP. Treatment with a 100 nM AVP pulse (indicated by arrows) resulted in a peak of ACTH secretion both in the presence (a) and absence (c) of 2 μM Ro 31-8220. Pre-treatment with 10 nM AVP for 15 min (indicated by black bars) resulted in a reduction in response to a subsequent stimulation with 100 nM AVP, both in the presence (b) and absence (d) of 2 μM Ro 31-8220. Representative data from four independent perifusion columns are shown.

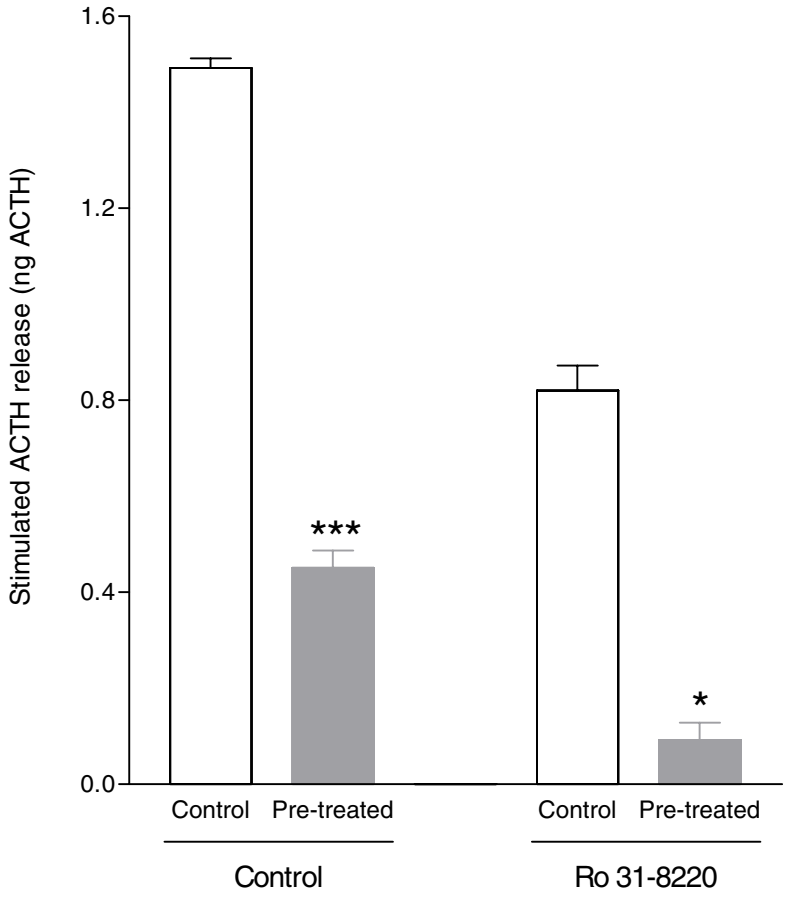

Fig. 4.5. Effect of treatment with Ro 31-8220 on desensitization of the ACTH response to AVP. Data are the ACTH secreted in response to a 100 nM AVP pulse both with and without pre-treatment with 10 nM AVP for 15 min and in the presence and absence of 2 μM Ro 31-8220. Data are mean ± SEM (n=3–4 for each treatment). Asterisks indicate statistically significant difference between the pre-treated and control value for each pair of responses (*t*-test, * $P<0.05$, *** $P<0.001$).

weaker effect on casein kinase II and other protein kinases (Chijiwa *et al.* 1989). The involvement of CK1α in desensitization of the ACTH response to AVP was investigated by testing the effect of treatment with CK1-7 on the ability of AVP pre-treatment to induce desensitization. If CK1α were involved in desensitization then the presence of CK1-7 would be expected to reduce the extent of desensitization induced by AVP pre-treatment.

Once again, experiments were carried out using the multi-column perifusion system with an experimental protocol similar to that described in Section 3.2.1. Pre-treatment of the cells for 25 min with 10 nM AVP prior to the second 100 nM AVP pulse caused a reduction in response of 49.0 ± 3.1% (n=9, $P<0.001$, *t*-test)(Fig. 4.6a) compared with controls. To investigate the involvement of CK1α in desensitization the second 100 nM AVP pulse was immediately preceded by a 30 min treatment with CK1-7 at concentrations of 30, 100 or 300 μM (*i.e.* for 5 min before the pre-treatment and then concurrently with it for 25 min)(Fig. 4.6b, c, d). Treatment with CK1-7 at all three concentrations had no effect on AVP-induced desensitization, with the reduction in response induced by AVP pre-treatment not being affected by any of these concentrations of CK1-7 (Dunnett's test). In order to assess the effect of treatment with CK1-7 alone on the response to a subsequent AVP stimulation, the second pulse was preceded by a pre-treatment with 30 μM CK1-7 for 30 min. Following this pre-treatment the response to the second AVP pulse was not significantly altered compared to the controls (n=6, NS, *t*-test)(Fig. 4.6e). Results are summarized in Fig. 4.7.

Treatment with CK1-7 both alone and in combination with AVP had no effect on ACTH secretion. During pre-treatment with 30 μM CK1-7 alone ACTH secretion was not significantly increased above basal (n=6, NS, *t*-test). Similarly, treatment with 10 nM AVP in combination with CK1-7 had no effect on stimulated ACTH secretion

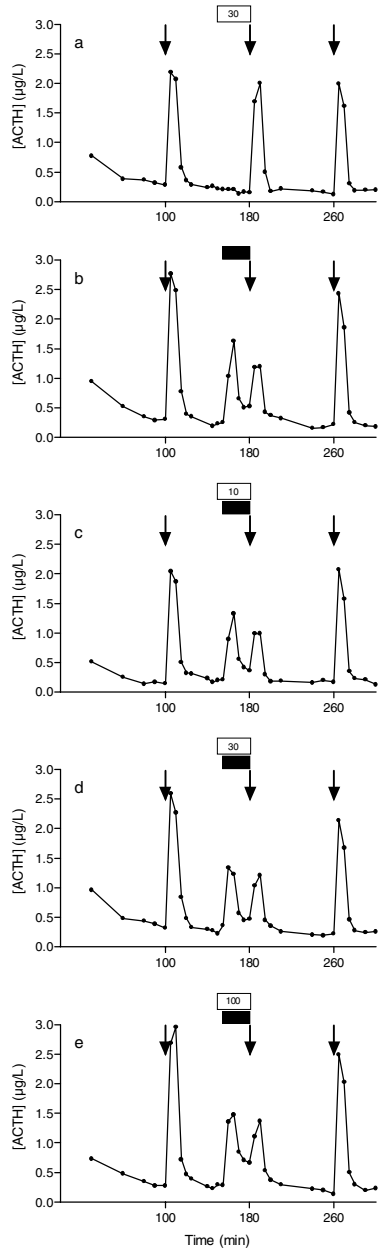

Fig. 4.6. Effect of pre-treatment with CK1α on desensitization of the ACTH response to AVP. Data in each panel are from single, representative perifusion columns in which the second 100 nM AVP pulse has been preceded by a treatment with either 30 µM CK1-7 for 30 min (a); 10 nM AVP for 25 min (b); 10 nM AVP in combination with 10 µM CK1-7 (c); 10 nM AVP in combination with 30 µM CK1-7 (d); or 10 nM AVP in combination with 100 µM CK1-7 (e). Black bars indicate the 10 nM AVP pre-treatment, open bars indicate CK1-7 treatment and arrows indicate 100 nM AVP pulses.

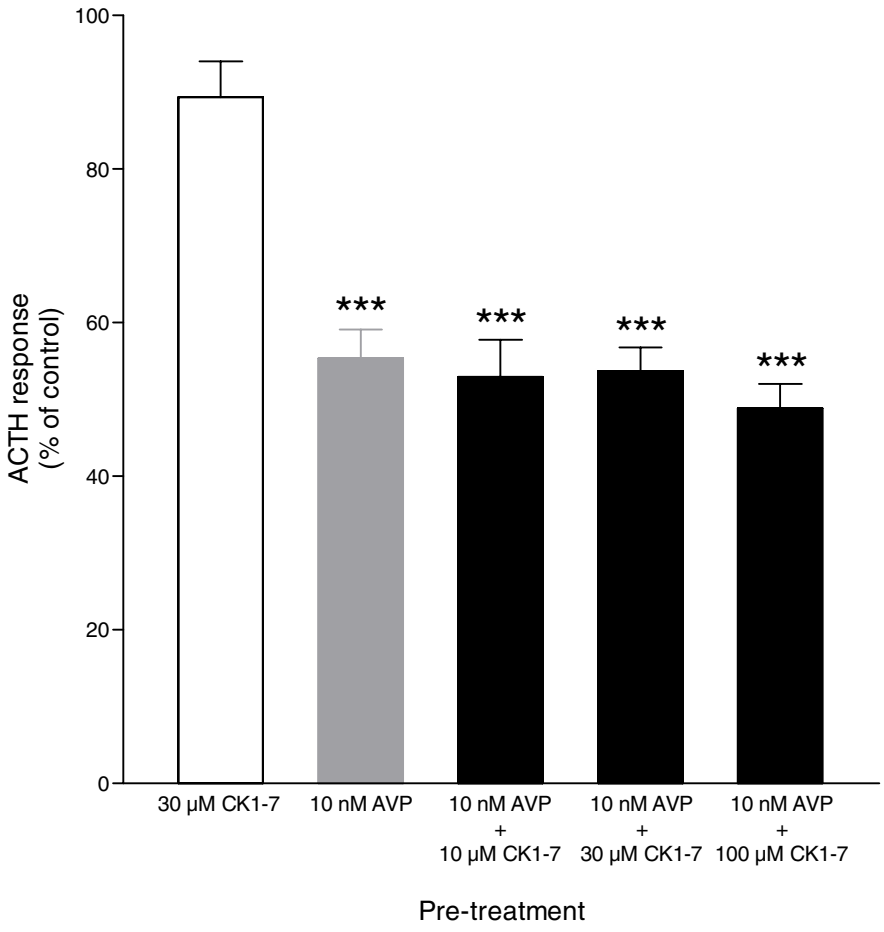

Fig. 4.7. Effect of pre-treatment with CK1-7 on the desensitization of the ACTH response to AVP. The data shown are the responses, expressed as a percentage of control, to a 5 min, 100 nM AVP pulse following pre-treatment with the indicated solutions. Data are mean ± SEM (n=6–9 for each treatment). Asterisks indicate a statistically significant difference between the results observed and 100% (*t*-test, *** $P<0.001$).

during the 25 min 10 nM AVP pre-treatment. (NS, Dunnett's test). Results are summarized in Fig. 4.8.

4.2.4 PHOSPHORYLATION OF THE PITUITARY AVP RECEPTOR

Cell culture of dispersed ovine anterior pituitary cells was performed as described in Section 2.3.3. Treatment of these cells with AVP, preparation of plasma membranes, cross-linking of ^{125}I-AVP to these membrane preparations and SDS-PAGE were carried out as described in Section 2.5. When samples of plasma membrane preparation containing 100 μg of protein were electrophoresed without incubation with, or cross-linking to, ^{125}I-AVP a satisfactory separation of proteins on the gel was achieved. Staining with Brilliant Blue G-TCA solution showed that many distinct protein bands were present within the 46 to 76 kD range expected to be occupied by the pituitary AVP receptor (The human V1b receptor is a 424 amino acid protein with a predicted molecular weight of 47 kD [De Keyzer *et al.* 1994]. The rat V1a receptor is a 424 amino acid protein [Innamorati *et al.* 1996] with a predicted molecular weight of 46 kD, but migrates between 62 and 76 kD on SDS-PAGE, with the difference probably attributable to receptor glycosylation [Innamorati *et al.* 1998a]). When these membrane preparations were incubated with ^{125}I-AVP and cross-linked to the radiolabelled ligand with DSS there was a marked change in the banding pattern observed. Although discrete bands had previously been clearly visible they were now no longer distinct. Autoradiography showed that ^{125}I-AVP had bound and been cross-linked to proteins in the membrane preparation. However, binding of ^{125}I-AVP to these proteins appeared to be non-specific as it was not affected by the presence of an excess of 'cold' AVP. No difference in the pattern of ^{125}I-AVP binding to membranes from control and AVP-treated cells could be observed.

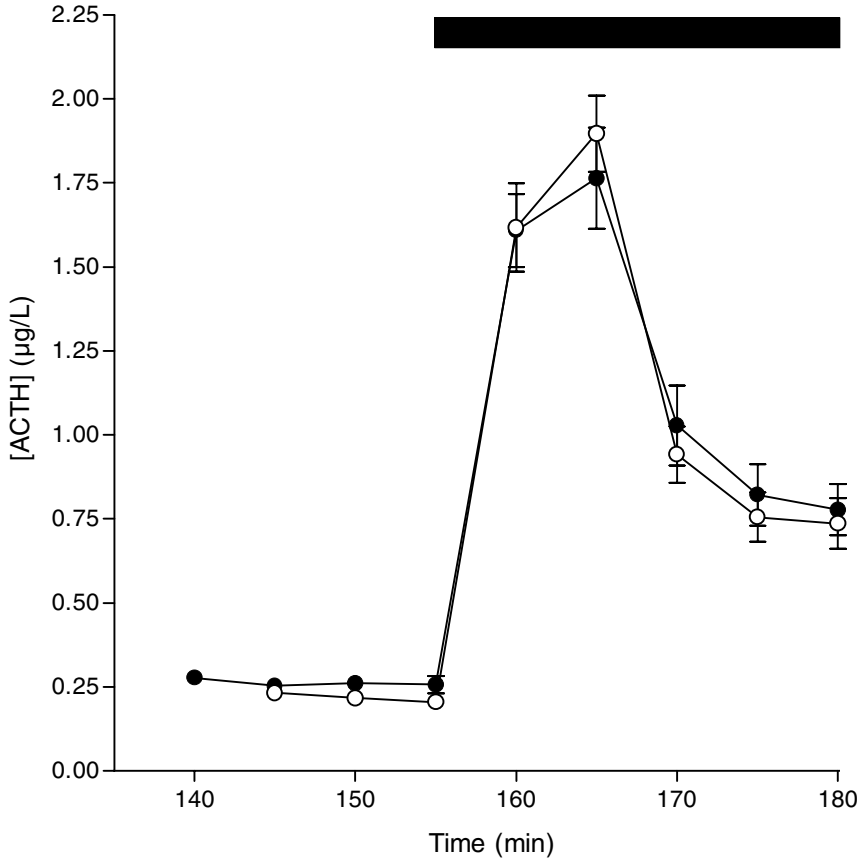

Fig. 4.8. Effect of treatment with CK1-7 on AVP-stimulated ACTH secretion. Black bar indicates a 25 min treatment with 10 nM AVP, beginning at 155 min of perifusion. The response of cells to 10 nM AVP both alone (●), and in combination with 100 µM CK1-7 (○). Treatment with CK1-7 began after 150 min of perifusion and continued until the end of the AVP treatment. Data are mean ± SEM (n=9 for each treatment).

4.3 DISCUSSION

The results described above show that PKC does not mediate the desensitization of the ACTH response to AVP. Indeed, the data suggest that rather than acting as a feedback mechanism, activation of PKC primes the phosphoinositide signalling pathway, thereby increasing the response to subsequent stimulation.

Despite being able to potently activate PKC, treatment with DiC_8 was not capable of inducing desensitization. During the 15 min pre-treatment 300 μM DiC_8 stimulated ACTH secretion equivalent to that stimulated by pre-treatment with 10 nM AVP. However, in contrast to the marked desensitization observed following 10 nM AVP pre-treatment, pre-treatment with DiC_8 did not reduce the response to a subsequent stimulation with 100 nM AVP. In fact, the opposite was true, with DiC_8 pre-treatment markedly increasing the response to a subsequent stimulation with 100 nM AVP. While the data presented here give no clue as to which PKC substrates are responsible for this increase in AVP-stimulated ACTH secretion, a possible explanation is provided by previous studies of the role of PKC in the regulation of Ca^{2+} influx into corticotrophs. The ACTH response to AVP is dependent to a large extent upon the entry of extracellular Ca^{2+} through L-VSCC (Abou-Samra *et al.* 1987; Won *et al.* 1990; Corcuff *et al.* 1993; Le Beau & Mason 1994). In many tissues the activity of L-VSCC is modulated by phosphorylation of the channels by a variety of protein kinases (see McDonald 1994 for review). Depending on the tissue being studied the effects of PKC on Ca^{2+} entry can be either positive (*e.g.* smooth and skeletal muscle [Navarro 1987; Fish *et al.* 1988]) or negative (*e.g.* sensory neurons [Rane & Dunlap 1986]). In human ACTH-secreting pituitary adenoma cells AVP enhances Ca^{2+} entry through L-VSCC (Mollard *et al.* 1988). This suggests that some factor activated by AVP, possibly PKC, modulates the activity of L-VSCC in corticotrophs. In dispersed

ovine anterior pituitary cells there is a synergistic interaction between low concentrations of AVP and raised extracellular K^+ on ACTH secretion (Le Beau & Mason 1998). This synergistic interaction was dependent upon PKC activity and it has been suggested that it might have been the result of modulation of L-VSCC activity by PKC resulting in increased Ca^{2+} entry, and thus increased ACTH secretion. Such a modulation of L-VSCC activity by PKC could explain the increase in AVP-stimulated ACTH secretion following treatment with DiC_8. Activation of PKC by DiC_8 could increase the activity of L-VSCC, resulting in an increase in Ca^{2+} entry during the subsequent 100 nM AVP pulse. This would, in turn, result in increased ACTH secretion. While such a mechanism could explain the results which have been observed in this study other mechanisms cannot be excluded. PKC phosphorylates a number of cytosolic (Liu *et al.* 1994) and membrane (Liu 1994) proteins in ovine anterior pituitary cells. Any of these PKC substrates might be responsible, either directly or indirectly, for the increase in AVP-stimulated ACTH secretion following DiC_8 treatment which has been observed in this study.

Since activation of PKC plays a critical role in mediating AVP-stimulated ACTH secretion (Abou-Samra *et al.* 1986; Carvallo & Aguilera 1989; Liu *et al.* 1990), it was not surprising that treatment with the specific PKC inhibitor Ro 31-8220 markedly inhibited the ACTH response to AVP. However, effects of Ro 31-8220 treatment on ACTH secretion made it somewhat difficult to devise an experimental protocol which allowed a meaningful assessment of the effect of PKC inhibition on the ability of AVP pre-treatment to induce desensitization. Specifically, treatment with 2 μM Ro 31-8220, either for a short period prior to the second AVP pulse or as a background throughout the experiment, affected the relationship between the AVP pulses. Because of this it was not possible to assess the effect of pre-treatment on the second pulse by

using the first and third pulses as controls for the second. A partial solution to this problem was to express the response to a 100 nM AVP pulse following pre-treatment as a percentage of the mean response to similar, but unpre-treated control pulses. When analyzed in this way the results were not consistent with the hypothesis that PKC mediates the desensitization of the ACTH response to AVP. As indicated above, treatment with 2 μM Ro 31-8220 reduced AVP-stimulated ACTH secretion: the response to the second 100 nM AVP pulse was reduced by 45.1% by Ro 31-8220 treatment. This reduction in response indicates that treatment with Ro 31-8220 at this concentration was capable of inhibiting PKC activity. Despite its ability to inhibit PKC the presence of Ro 31-8220 did not reduce the extent of desensitization induced by 15 min pre-treatment with 10 nM AVP. Indeed, when expressed as a percentage of the appropriate control, the reduction in response induced by AVP pre-treatment was actually greater when Ro 31-8220 was present in the perifusion medium than when it was absent. It seems highly unlikely this could be the result of reduced phosphorylation of the V1b receptor by PKC. There is no evidence in the literature to suggest that a reduction in PKC-mediated phosphorylation of a GPCR can enhance its desensitization. Recent evidence has shown that when stably transfected in CHO cells a GFP-tagged human V1b receptor associates with GRK5 (but not with GRK2, GRK3 or GRK6) during agonist exposure, although it was not demonstrated whether the receptor becomes phosphorylated during this interaction (Berrada *et al.* 2000). One possible explanation for the increased reduction in response observed during treatment with Ro 31-8220, is that inhibition of GRK5 by PKC-mediated phosphorylation was reduced, thereby resulting in enhanced phosphorylation and desensitization of the receptor. However, given that the intracellular environment following activation of PLC is very unfavourable to activation of GRK5 (see above) it would be surprising if

this enzyme were involved in the desensitization of the ACTH response to AVP. The interaction between the GFP-tagged V1b receptor and GRK5 may have been an artefact of expression of the receptor in the CHO cells. A more plausible explanation is that this effect is the reverse side of the increase in responsiveness to AVP observed following treatment with DiC_8. In this instance treatment with 2 μM Ro 31-8220 reduces the extent of PKC activation during the 10 nM AVP pre-treatment. As a result PKC-mediated 'priming' of the signalling pathway during the AVP pre-treatment is reduced and subsequent stimulation with 100 nM AVP is less effective.

Receptor phosphorylation by the effector kinases is an heterologous mechanism of desensitization and agonist-occupancy of receptors is not obligatory for their phosphorylation and desensitization (Lohse 1993). However there is a small increase in the rate of phosphorylation of the β-adrenergic receptor by PKA *in vitro* when the receptor is isoproterenol bound (Benovic *et al.* 1985). Use of Ro 31-8220 to inhibit PKC allowed the role of this enzyme in desensitization to be investigated while the receptor was AVP-bound, which was not possible in experiments in which DiC_8 was used to activate PKC. The results obtained do not support the involvement of PKC in desensitization of the pituitary AVP receptor, even when agonist-bound.

No evidence could be found for the involvement of CK1α in the regulation of AVP-stimulated ACTH secretion. Casein kinase 1 activity was inhibited using the specific inhibitor CK1-7 at three concentrations, all of which were greater than the IC_{50} for this inhibitor measured in a cell-free enzyme system (Chijiwa *et al.* 1989). Treatment with CK1-7 had no effect on the magnitude of desensitization induced by a 25 min pre-treatment with 10 nM AVP, nor did it have any effect on basal and AVP-stimulated ACTH secretion.

Attempts to biochemically determine whether the pituitary AVP receptor is phosphorylated during desensitization were unsuccessful. Non-specific binding and cross-linking of the radiolabelled ligand to proteins present in the membrane preparation made it impossible to determine whether the electrophoretic mobility of the receptor had been altered.

The lack of involvement of PKC in desensitization of the ACTH response to AVP suggests AVP receptor subtype-specific differences in mechanisms of desensitization: the V1a vasopressin receptor, which is expressed largely in the liver and vasculature (Watson & Arkinstall 1994), has been shown to be desensitized via PKC-mediated receptor phosphorylation (Gallo-Payet *et al.* 1991; Ancellin *et al.* 1997; Ancellin & Morel 1998; Caramelo *et al.* 1991). Differential desensitization of the V1a and V1b receptors might explain, at least in part, the existence of two receptors with otherwise similar signalling properties. These differences in desensitization might play an important role in the regulation of responsiveness to AVP in different tissues. It is interesting to note that the patterns and maximal concentrations of AVP secretion into the pituitary portal circulation are quite different from those in the peripheral circulation. AVP is released into the portal blood in a highly pulsatile manner and reaches relatively high concentrations, *e.g.* up to ~6 nM during insulin-induced hypoglycaemia in sheep (Caraty *et al.* 1990; see Section 3.1 for a more extensive discussion). In contrast, changes in AVP concentration in the peripheral circulation are slow and of low magnitude, *e.g.* following hypertonic saline infusion into ewes there is a steady increase in plasma AVP concentration from ~2 pM to ~7 pM over a period of 90 min (Keller-Wood 1994). Since GRK-mediated desensitization of the β-adrenergic receptor is very rapid ($t_{½}$<15 s) and requires relatively high agonist concentrations it has been suggested that this process might be particularly important in environments such as the synaptic cleft

where agonist concentrations increase rapidly to high concentrations (1–100 μM)(Lohse *et al.* 1990a; Roth *et al.* 1991). Correspondingly, the desensitization of the β-adrenergic receptor mediated by the PKA is slower ($t_{½}$=3.5 min) and occurs at lower agonist concentrations, suggesting that it is better suited to the regulation of responsiveness at non-synaptic receptors (Roth *et al.* 1991). Differences in the mechanisms underlying the desensitization of the V1a and V1b receptors could allow appropriate regulation of the responsiveness to AVP to occur in the different tissues in which the two receptors are expressed.

While neither PKC nor CK1α appear to be involved in the desensitization of the ACTH response to AVP it remains likely that an intracellular protein kinase is involved in this process. Perhaps the best remaining candidates for phosphorylation of the V1b receptor are members of the GRK family. Using immunocytochemistry for GRK2, GRK3, GRK6 and β-arrestins Neill *et al.* (1998) showed that these proteins are expressed in rat anterior pituitary cells, indicating that the intracellular machinery necessary for the regulation of the V1b receptor by GRKs is present in pituitary cells. Furthermore, as described above, recent evidence has shown that the V1b receptor can interact with GRKs (Berrada *et al.* 2000). For a number of reasons the investigation of the involvement of GRKs in the desensitization of the ACTH response to AVP is somewhat difficult. Although a number of substances, including heparin (Lohse *et al.* 1989), Zn^{2+} (Benovic *et al.* 1987b) and the wasp venom peptide mastoparan (Tang *et al.* 1998), have been reported to modulate GRK activity, each has non-specific effects. Heparin also inhibits casein kinase 2 (Hathaway *et al.* 1980) and is an IP_3 receptor antagonist (Kobayashi *et al.* 1989), Zn^{2+} reduces cell viability (Hasbi *et al.* 1998), while mastoparan activates G_o and G_i (Higashijima *et al.* 1988; Tanaka *et al.* 1998). Furthermore, neither heparin nor mastoparan are cell permeable and cells must be permea-

bilized in order for these compounds to gain access to GRKs (Verspohl & Wienecke 1998; Tang *et al.* 1998). Another technique used to investigate the involvement of GRKs in desensitization is to suppress the activity of the enzymes, either through the expression of dominant-negative mutant GRKs (Pitcher *et al.* 1998), or by reducing expression of the enzymes by treatment with antisense oligonucleotide sequences (Shih & Malbon 1994) or by stable expression of antisense mRNA (Shih *et al.* 1999). Experiments using dominant-negative GRKs could be carried out most easily in a cell line endogenously expressing or transfected with the V1b receptor. Unfortunately the AtT-20 mouse corticotroph tumour cell line is not suitable since it does not respond to AVP (Lutz-Bucher *et al.* 1987). Antisense oligonucleotides reduce expression of proteins by binding to mRNA, thereby preventing it from being translated (Phillips & Gyurko *et al.* 1997). Because cells can be treated with antisense sequences by adding the oligonucleotides directly to the culture medium (Shih & Malbon 1994; Aiyar *et al.* 2000), this is probably the best technique for the investigation of the involvement of GRKs in desensitization of the pituitary AVP receptor in dispersed ovine anterior pituitary cells. Although the ovine GRKs have not yet been cloned the GRK family is highly conserved, both amongst the different members of the family and across species (Pitcher *et al.* 1998), and as such it should be possible to design an oligonucleotide sequence which could inhibit expression of the ovine GRKs.

5

INVOLVEMENT OF RECEPTOR INTERNALIZATION AND PROTEIN PHOSPHATASES IN THE REGULATION OF THE ACTH RESPONSE TO AVP

5.1 INTRODUCTION

The results described in Chapter 3 show that the rapid desensitization of the ACTH response to AVP is readily reversible. Resensitization is itself rapid, with complete recovery of the response occurring within 40 min. The aim of the research described in this chapter was to investigate the molecular mechanisms underlying this process. In particular, the involvement of receptor internalization and protein phosphatase activity in the regulation of the ACTH response to AVP was investigated.

Receptor endocytosis plays an important role in the regulation of many GPCRs (Ferguson 2001). However, evidence suggests the role of this process in both desensitization and resensitization varies from one receptor to the next. Based largely upon investigation of the β_2-adrenergic receptor, a model has been proposed in which receptor internalization does not mediate desensitization but instead is necessary for receptor resensitization (Ferguson *et al.* 1996b). In this model internalization allows dephosphorylation of phosphorylated, desensitized receptors to take place. The receptors are sub-

sequently recycled back to the plasma membrane in a fully functional state. Studies of a variety of GPCRs, including the NK_1 neurokinin receptor (Garland *et al.* 1996), the CB1 cannabinoid receptor (Hsieh *et al.* 1999) and the δ-opioid receptor (Hasbi *et al.* 2000), have produced results consistent with this model. However more recently receptor internalization has been shown to be necessary for the desensitization of a small number of receptors. These include the sst_{2B} somatostatin receptor (Beaumont *et al.* 1998), the m2 muscarinic receptor when expressed in CHO cells (Tsuga *et al.* 1998a), and the secretin receptor (Holtmann *et al.* 1996; Mundell & Kelly 1998)(see Section 1.2.2 for a more extensive discussion).

Both the V1a and V2 AVP receptors are internalized following agonist exposure and this process plays an important role in their regulation (Lutz *et al.* 1991). However, the role of internalization in the regulation of these two receptors appears to differ. Whereas the V1a receptor is rapidly dephosphorylated and recycled back to the plasma membrane, the V2 receptor is retained in an intracellular compartment (Innamorati *et al.* 1998a; Innamorati *et al.* 1998b). This is apparently due to a cluster of serine residues in the C terminal tail of the receptor which prevents dephosphorylation and recycling of the receptor back to the cell surface. Pfeiffer *et al.* (1998) found that treatment with hypertonic sucrose, which disrupts receptor-mediated endocytosis (Daukas & Zigmond 1985), significantly reduced the extent of V2 receptor desensitization, suggesting that desensitization was dependent upon receptor sequestration.

The pituitary AVP receptor is internalized during AVP treatment. Following exposure of rat anterior pituitary cells to [^{3}H]-AVP the amount of bound radiolabelled AVP that was acid-resistant (*i.e.* internalized) increased to a maximum over approximately 20 min (Mogensen *et al.* 1988). Internalization of bound biotinylated AVP and GFP-tagged V1b receptors has been visualized following agonist exposure in rat ante-

rior pituitary cells (Childs *et al.* 1991) and stably transfected CHO cells (Berrada *et al.* 2000) respectively. The role of receptor internalization in the regulation of responsiveness is unknown. The first aim of the following experiments was to investigate the role of receptor endocytosis in both desensitization and resensitization of the ACTH response to AVP, by observing the effect on these two processes of pharmacologically blocking internalization.

As described above, a key role of receptor internalization is to allow dephosphorylation by protein phosphatases before they are recycled back to the plasma membrane (Sibley *et al.* 1986; Pippig *et al.* 1995). Two different types of protein phosphatase, PP2A and PP2B have been implicated in the dephosphorylation of GPCRs. Pitcher *et al.* (1992) were the first to demonstrate the involvement of PP2A in the dephosphorylation of a GPCR, showing that the enzyme dephosphorylated both β_2-adrenergic and α_2-adrenergic receptors. Consistent with its involvement in resensitization, pharmacological inhibition of PP2A has been shown to attenuate the recovery of responsiveness of a number of GPCRs including the β_2-adrenergic receptor (Pippig *et al.* 1995) and the NK_1 neurokinin receptor (Grady *et al.* 1995; Garland *et al.* 1996). Similarly, pharmacological inhibition of the Ca^{2+}/calmodulin-dependent protein phosphatase PP2B (also known as calcineurin) or suppression of its expression can slow the rate of resensitization of the β_2-adrenregic receptor (Shih & Malbon 1996; Shih *et al.* 1999). This phosphatase has been implicated in the resensitization of the 5-HT_{1C} serotonin receptor (Boddeke *et al.* 1991), the δ-opioid receptor (Ueda *et al.* 1995) and muscarinic receptors in *Xenopus* oocytes (Sakuta *et al.* 1991).

Both PP2A and PP2B have been shown to be involved in the regulation of responsiveness of AtT-20 mouse corticotroph tumour cells. Inhibition of PP2A reduces CRH-stimulated cAMP accumulation (Koch & Lutz-Bucher 1994; Antaraki *et al.*

1997). In contrast, inhibition of PP2B increases basal ACTH secretion and CRH-stimulated cAMP accumulation and ACTH secretion (Antoni *et al.* 1993). Although this suggests that these two enzymes are expressed in corticotrophs their roles in the regulation of the ACTH response are unclear. The second aim of the following experiments was to investigate the involvement of PP2A and PP2B in the regulation of resensitization of the ACTH response to AVP. Selective inhibitors of these enzymes were used to reduce their activity, and the effect of this treatment on the time course of resensitization was assessed.

5.2 RESULTS

5.2.1 EXPERIMENTAL PROTOCOL

Experiments were carried out using the multi-column perifusion system (see Section 2.4.1). The protocol was similar to that used to investigate the resensitization of the ACTH response to AVP, which is described in detail in Section 3.2.4. Briefly, cells were stimulated with a single 5 min pulse with 100 nM AVP after 160 min of perifusion, resulting in a characteristic peak of ACTH secretion. To induce desensitization this pulse was immediately preceded by a 15 min pre-treatment with 10 nM AVP. A recovery period of either 20 or 40 min duration was allowed between the end of the pre-treatment and the start of the pulse. The extent of desensitization present was assessed quantitatively by expressing the response to the 100 nM AVP pulse following pre-treatment as a percentage of the response of control cells (which were not pre-treated) to similar 100 nM AVP pulses.

The involvement of both receptor internalization and protein phosphatase activity in the regulation of the ACTH response to AVP was investigated by using pharmaco-

logical agents to inhibit these two processes. Cells were treated with the appropriate pharmacological agent for 70 min prior to the 100 nM AVP pulse (*i.e.* from 90 min to 160 min). The agent was present at a constant concentration throughout this period, *i.e.* before, during and after the pre-treatment. The effect of this treatment on the extent of AVP-induced desensitization was assessed quantitatively by expressing the response to the 100 nM AVP pulse following pre-treatment as a percentage of the response to AVP pulses of control cells which were treated with the pharmacological agent but which were not pre-treated with 10 nM AVP.

5.2.2 EFFECT OF BLOCKADE OF RECEPTOR INTERNALIZATION ON THE REGULATION OF THE ACTH RESPONSE TO AVP

5.2.2.1 CONCANAVALIN A

Concanavalin A (ConA) is a lectin derived from the Jack Bean (*Canavalia ensiformis*) and has an affinity for α-D-mannosyl and α-D-glucosyl residues (Reeke *et al.* 1974; Lis & Sharon 1986). Treatment with this agent irreversibly cross-links glycosylated membrane proteins such as GPCRs to one another (Lis & Sharon 1986), thereby preventing them from being internalized (Waldo *et al.* 1983; Pippig *et al.* 1995). Treatment with ConA has been widely used to inhibit the internalization of a variety of GPCRs, *e.g.* see Lohse *et al.* (1990a), Blaukat & Müller-Esterl (1997), Mundell & Kelly (1998) and Gardner *et al.* (2001).

In the control experiment, in which cells were not treated with ConA, pre-treatment with 10 nM AVP for 15 min immediately prior to the 100 nM AVP pulse caused a reduction in response of 58.4 ± 4.4% ($P<0.001$, One way ANOVA with Bonferroni's test) compared with control (Fig. 5.1a, b). When a recovery period was allowed between the pulse and the pre-treatment, resensitization occurred (Fig. 5.1c, d). After a

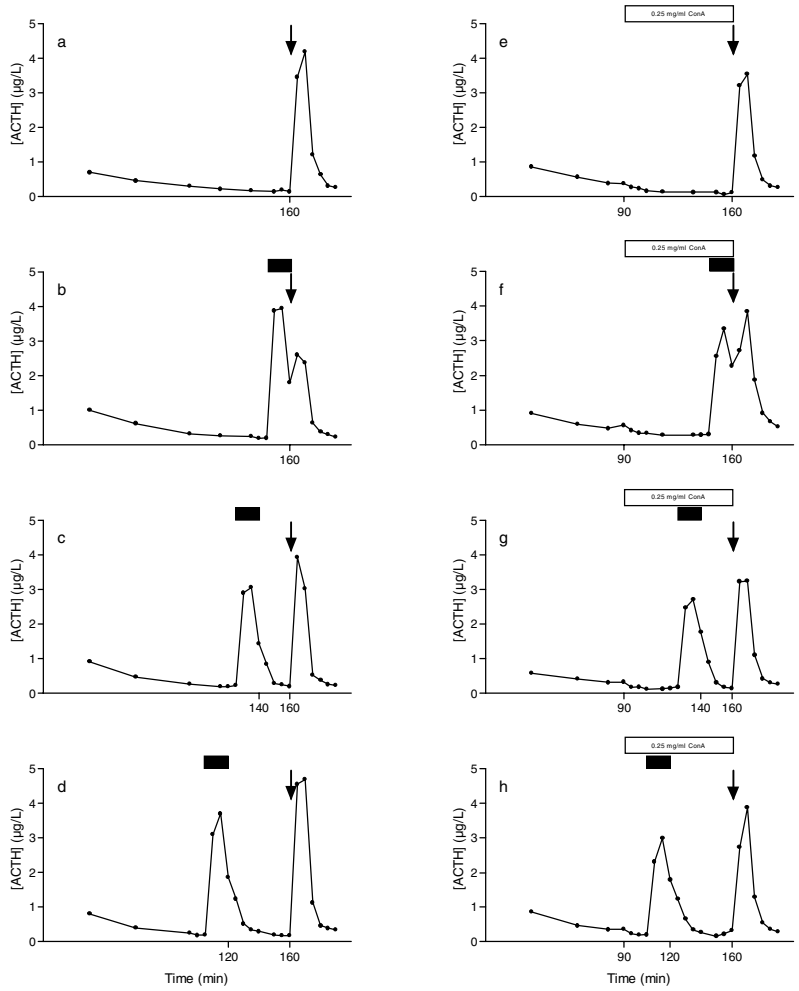

Fig. 5.1. Effect of treatment with 0.25 mg/ml ConA on the ACTH response to AVP. Representative data from 8 independent perifusion columns are shown. Cells were treated with a 5 min pulse of 100 nM AVP after 160 min of perifusion (indicated by arrows). To induce desensitization this AVP pulse was immediately preceded by a 15 min pre-treatment with 10 nM AVP (indicated by black bars) (b, f). When recovery periods of 20 or 40 min were allowed between the pre-treatment and the pulse resensitization occurred (c, d, g, h). The effect of pharmacological blockade on regulation of the ACTH response to AVP was investigated by treating cells with 0.25 mg/ml for 70 min prior to the 100 nM AVP pulse (e to h).

20 min recovery period no significant desensitization remained (NS, Bonferroni's test); *i.e.* resensitization was complete. When cells were treated with 0.25 mg/ml ConA for 70 min prior to the 100 nM AVP pulse AVP pre-treatment was still able to induce a desensitization but it was significantly reduced compared to that observed in the control experiment. When the AVP pre-treatment immediately preceded the 100 nM AVP pulse the response was reduced by 22.7 ± 3.7% ($P<0.05$, Bonferroni's test) compared to the response of cells which were not pre-treated but were treated with ConA (Fig. 5.1e, f). This effect of treatment with ConA on the ability of pre-treatment with 10 nM AVP for 15 min to induce desensitization was statistically significant ($P<0.01$, Bonferroni's test). Following recovery periods of 20 and 40 min there was no significant difference between the extent of desensitization observed in control and ConA-treated cells. Results are summarized in Fig. 5.2.

Treatment with ConA had no significant direct effect on ACTH secretion. Although the ACTH response to the 10 nM AVP pre-treatment was slightly less (approximately 15% at each of the time points) during treatment with 0.25 mg/ml ConA this was not statistically significant (Fig. 5.3). Similarly, in columns which received no AVP pre-treatment the ACTH response to the 100 nM AVP pulse was unaltered by prior exposure to 0.25 mg/ml ConA. Basal ACTH secretion was unaffected by treatment with ConA.

5.2.2.2 HYPERTONIC SUCROSE

Treatment with hypertonic sucrose inhibits receptor-mediated endocytosis without affecting fluid phase endocytosis (Daukas & Zigmond 1985). This selective inhibition of receptor-mediated endocytosis occurs because the treatment prevents the normal formation of clathrin-coated pits (Heuser & Anderson 1989). Treatment with hyper-

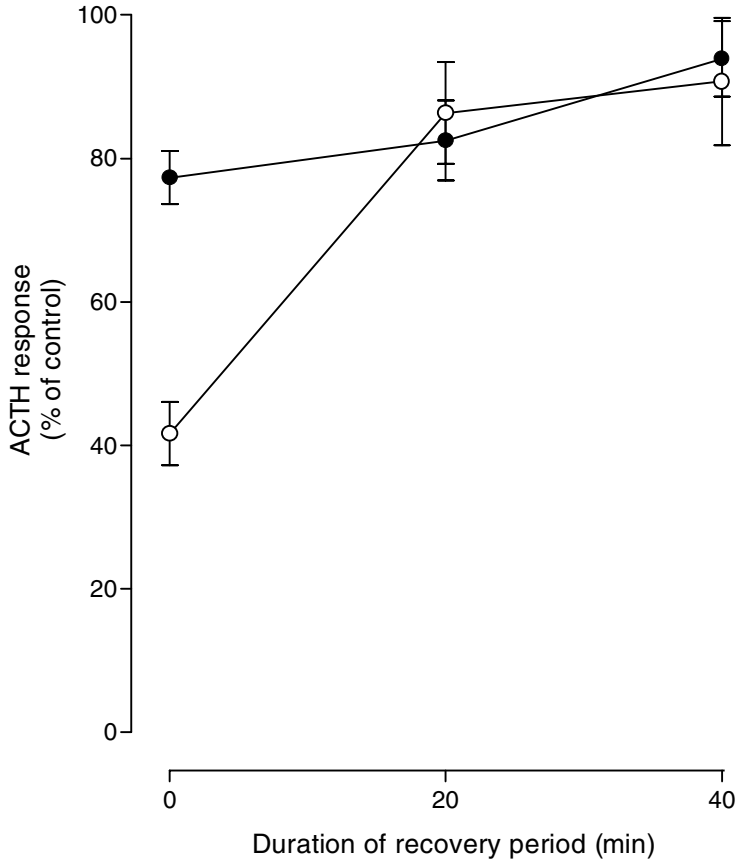

Fig. 5.2. Effect of treatment with 0.25 mg/ml ConA on desensitization and resensitization of the ACTH response to AVP. The ACTH response after pre-treatment with 10 nM AVP for 15 min is plotted against the duration of the recovery period. Combined results from columns in which ConA was present (●) and absent (*i.e.* controls; ○) are shown. Data are mean ± SEM (n=5–8).

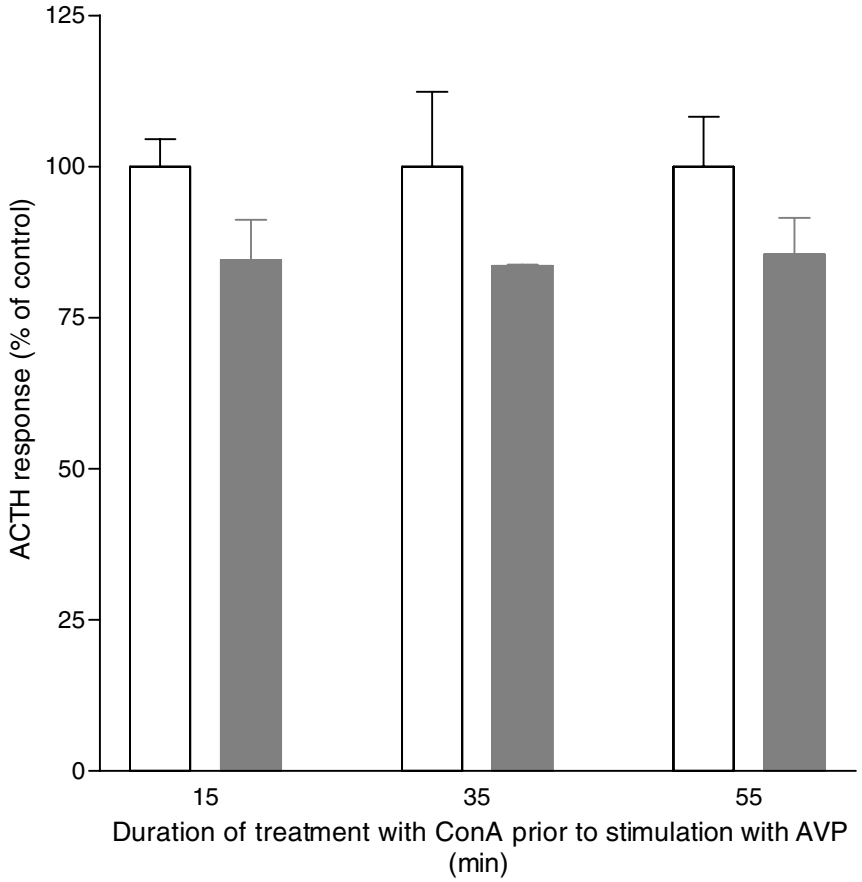

Fig. 5.3. Effect of treatment with 0.25 mg/ml ConA on the ACTH response to the 15 min pre-treatment with 10 nM AVP. The ACTH response to the AVP pre-treatment during ConA treatment (closed bars) is expressed as a percentage of the response to similar pre-treatments in control columns (open bars). Because the AVP pre-treatments started at different times depending upon the duration of the recovery period cells were treated with ConA for different durations prior to the beginning of the AVP pre-treatment. Data are mea n ±SEM (n=2–6).

tonic sucrose is commonly used to inhibit the internalization of GPCRs, *e.g.* see Pippig *et al.* (1995), Ng *et al.* (1995), Mundell & Kelly (1998) and Pfeiffer *et al.* (1998).

Unlike ConA treatment, which did not affect ACTH secretion, treatment with KR/ATC made hypertonic with 0.4 M sucrose had significant effects on both basal and AVP-stimulated ACTH secretion. Following the onset of hypertonic sucrose treatment there was a small but significant increase in basal ACTH secretion (*e.g.* see Fig. 5.4e, to h). Furthermore, following treatment with hypertonic sucrose there was a 4.0-fold increase in the ACTH response to the 100 nM AVP pulse (n=4, $P<0.01$, *t*-test)(*e.g.* compare Fig. 5.4a and Fig. 5.4e).

Despite these effects on ACTH secretion, treatment with hypertonic sucrose appeared to prevent AVP-induced desensitization from occurring. When 0.4 M sucrose was present in the perifusion medium pre-treatment with 10 nM AVP was unable to induce any reduction in the response to a subsequent stimulation with 100 nM AVP (NS, Bonferroni's test). There was a significant difference between the extent of desensitization induced by AVP pre-treatment in control and hypertonic sucrose-treated cells ($P<0.001$, Bonferroni's test). There was no significant difference between the ACTH response of control and hypertonic sucrose-treated cells following recovery periods of either 20 or 40 min (NS, Bonferroni's test). Results of experiments with hypertonic sucrose are summarized in Fig. 5.5.

5.2.2.3 PHENYLARSINE OXIDE

Treatment with the trivalent oxidizing agent phenylarsine oxide (PAO) inhibits receptor internalization (Wiley & Cunningham 1982; Knutson *et al.* 1983; Griendling *et al.* 1987; Garland *et al.* 1996; Sanders & LeVine 1996). PAO prevents endocytosis by irreversibly cross-linking membrane proteins containing sulfhydryl groups to one another

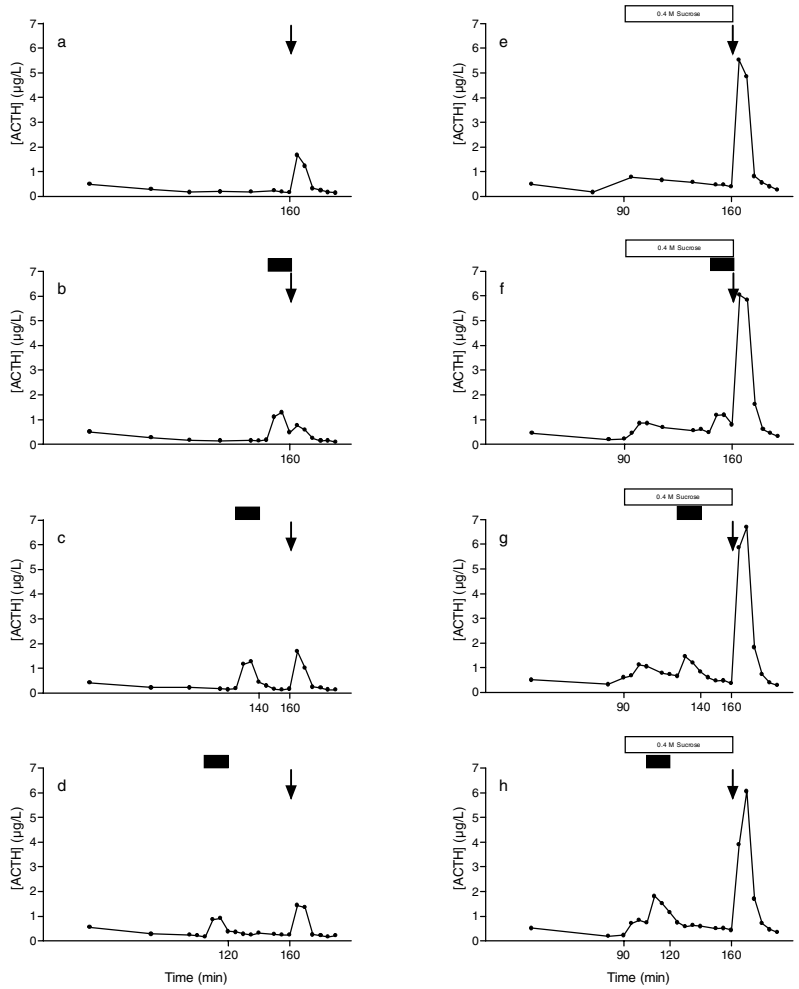

Fig. 5.4. Effect of treatment with hypertonic sucrose on the ACTH response to AVP. Representative data from 8 independent perifusion columns are shown. The experimental protocol used was identical to that described in Fig. 5.1, except that instead of ConA cells were treated with KR/ATC made hypertonic with 0.4 M sucrose for 70 min immediately prior to the 100 nM AVP pulse (e to h).

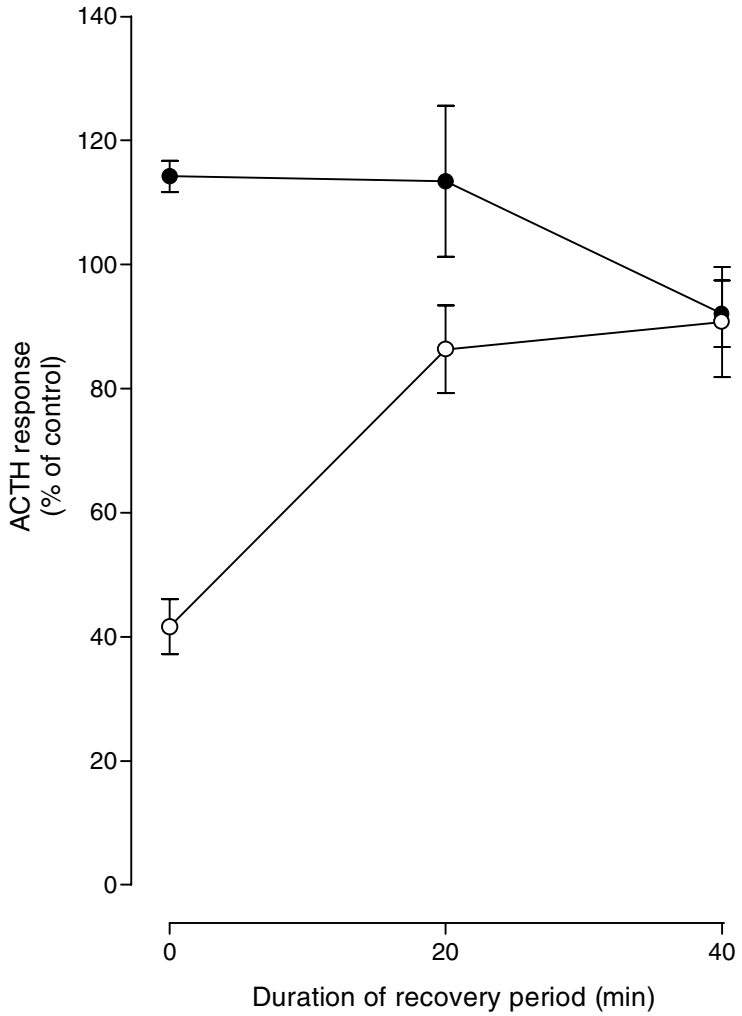

Fig. 5.5. Effect of treatment with 0.4 M hypertonic sucrose on desensitization and resensitization of the ACTH response to AVP. The ACTH response after pre-treatment with 10 nM AVP for 15 min is plotted against the duration of the recovery period. Combined results from columns in which hypertonic sucrose was present (●) and absent (*i.e.* controls; ○) are shown. Data are mean ± SEM (n=2–8).

(Schelling & Linas 1994). In addition to preventing receptor internalization treatment with PAO has been reported to have effects on a variety of other cellular processes. These effects include the inhibition of tyrosine phosphatase activity (Massol *et al.* 1998), the inhibition of NADPH oxidase (Le Cabec & Maridonneau-Parini 1995) and the inhibition of caspases (Takahashi *et al.* 1997). Treatment with 5 μM PAO for 70 min completely abolished the ACTH response to a subsequent stimulation with 100 nM AVP. Therefore this was not a useful agent for assessing the role of internalization in either desensitization or resensitization.

5.2.3 EFFECT OF INHIBITION OF PROTEIN PHOSPHATASES ON RESENSITIZATION OF THE ACTH RESPONSE TO AVP

5.2.3.1 FK506

FK506 is a macrocyclic lactone derived from *Streptomyces* sp. which specifically inhibits PP2B (Schreiber 1991). This inhibition is indirect: FK506 first binds to an FK506-binding protein and the resulting complex inhibits PP2B (Liu *et al.* 1991; O'Keefe *et al.* 1992). The FK506 used in the following experiments was provided as a kind gift by Dr. M. Tomoi of the Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan.

Cells were treated with FK506 at two concentrations: 50 nM and 1 μM. At these concentrations FK506 did not change the magnitude of desensitization induced by pretreatment with 10 nM AVP for 15 min (54.6 ± 6.4% in the presence of 1 μM FK506 compared with 53.7 ± 3.5% in controls not treated with the inhibitor [$P<0.01$, Bonferroni's test]). However, treatment with FK506 decreased the rate of resensitization. In the absence of FK506 recovery from desensitization was complete after 20 min (9.2 ± 5.1% less than control; NS, Bonferroni's test). In contrast, when cells were treated with 1 μM FK506 a significant desensitization still remained at 20 min

(38.1 ± 3.3% less than control; $P<0.001$, Bonferroni's test). Resensitization was not complete until 40 min. Representative data from individual columns in which cells were treated with 1 μM FK506 are shown in Fig. 5.6 and summarized results are shown in Fig. 5.7.

In addition to this effect on the rate of resensitization, treatment with FK506 also caused a small but reproducible increase in AVP-stimulated ACTH secretion. Treatment with both 50 nM and 1 μM FK506 increased the ACTH response to the 15 min 10 nM AVP pre-treatment (see Fig. 5.8). Treatment with FK506 had no significant effect on either the ACTH response to the 100 nM AVP pulse or basal ACTH secretion.

5.2.3.2 OKADAIC ACID

Okadaic acid (OA) is a polyether fatty acid which inhibits protein phosphatase activity (Cohen 1990). It specifically inhibits the activity of both protein phosphatase 1 (PP1) and PP2A potently (Herzig & Neumann 2000). PP2A is completely inhibited by 1 nM OA in a cell-free enzyme system, whereas PP1 is unaffected by this concentration, its IC_{50} being 10-15 nM (Cohen *et al.* 1989). Similar experiments have shown that PP2B is relatively unaffected by OA, with an IC_{50} of 3600 nM (Bialojan *et al.* 1988). Treatment with 10 nM OA for 70 min prior to the 100 nM AVP pulse had no effect on the pattern of desensitization and resensitization observed following pre-treatment with 10 nM AVP for 15 min. Results are summarized in Fig. 5.9. No evidence could be found for an effect of treatment with 10 nM OA on either basal or AVP-stimulated ACTH secretion.

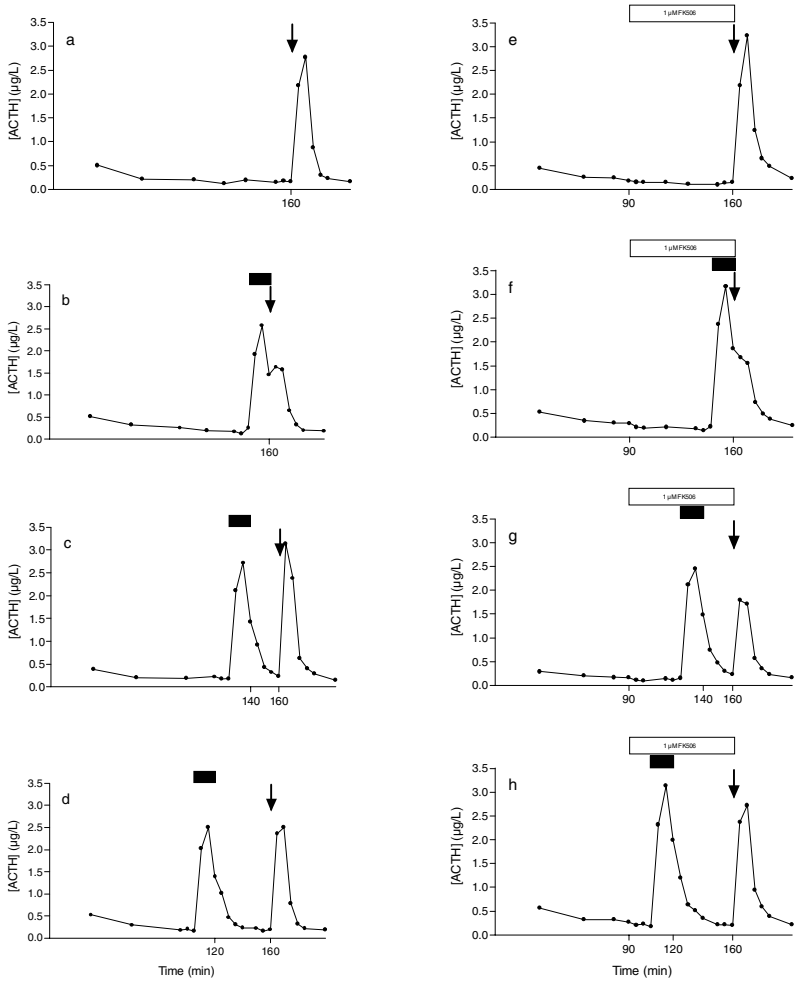

Fig. 5.6. Effect of treatment with 1 µM FK506 on the ACTH response to AVP. Representative data from 8 independent perifusion columns are shown. The experimental protocol used was identical to that described in Fig. 5.1, except that instead of ConA cells were treated with 1µM FK506 for 70 min immediately prior to the 100 nM AVP pulse (e to h).

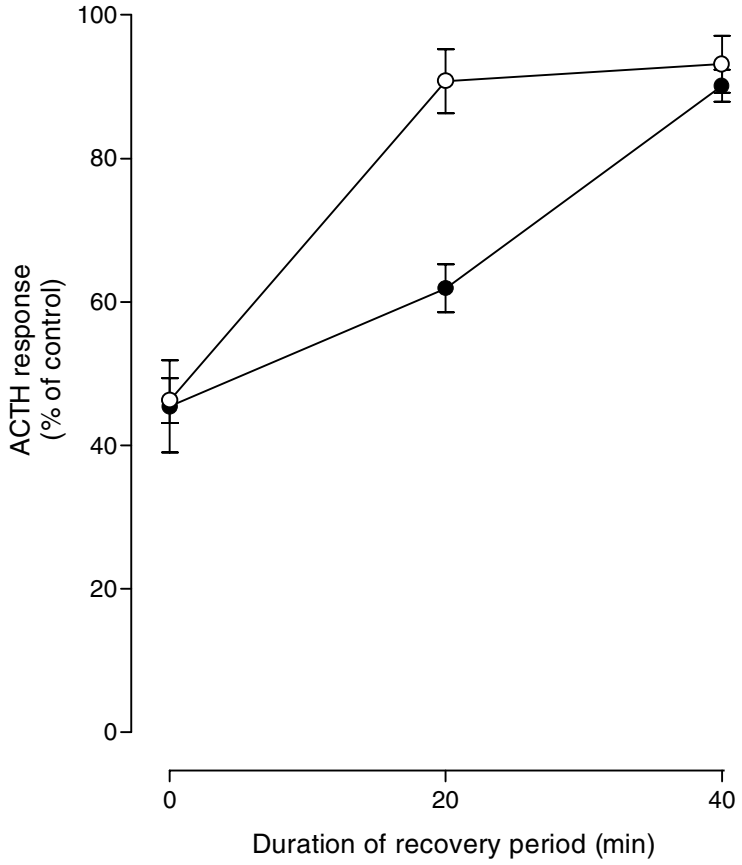

Fig. 5.7. Effect of treatment with 1 μM FK506 on desensitization and resensitization of the ACTH response to AVP. The ACTH response after pre-treatment with 10 nM AVP for 15 min is plotted against the duration of the recovery period. Combined results from columns in which FK506 was present (●) and absent (*i.e.* controls; ○) are shown. Data are mea n ±SEM (n=4–10).

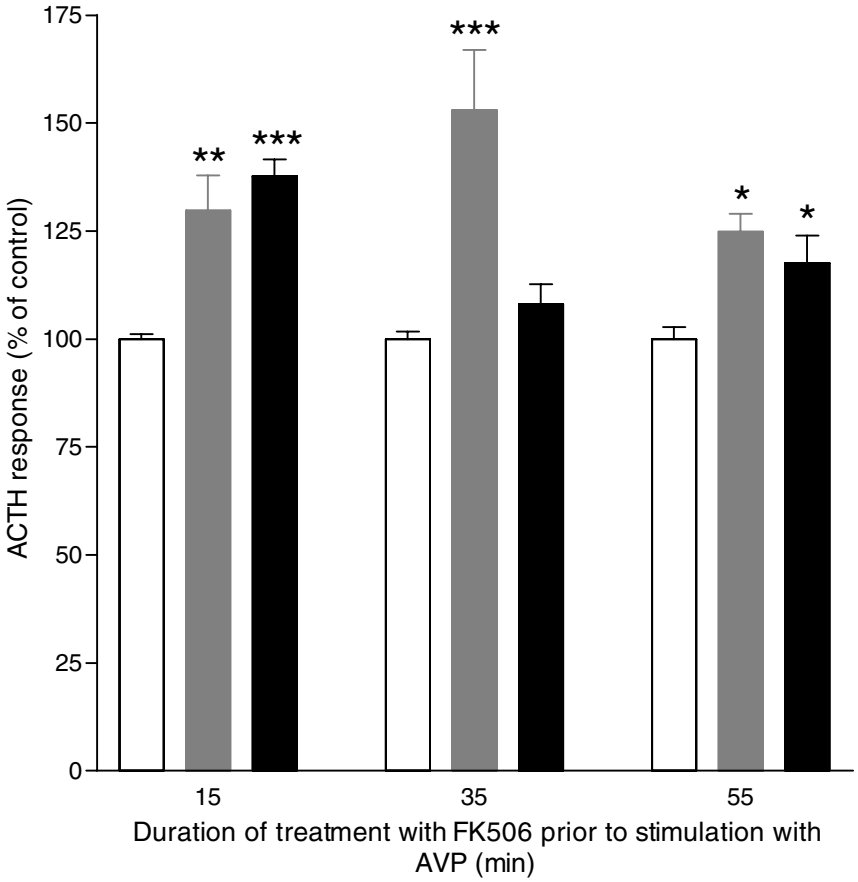

Fig. 5.8. Effect of treatment with FK506 on the ACTH response to the 15 min pre-treatment with 10 nM AVP. The ACTH response to the AVP pre-treatment during treatment with 50 nM (grey bars) and 1 μM FK506 (black bars) is expressed as a percentage of the response to similar pre-treatments in control columns (open bars). Because the AVP pre-treatments started at different times depending upon the duration of the recovery period cells were treated with FK506 for different durations prior to the beginning of the AVP pre-treatment. Asterisks indicate a statistically significant difference between the results observed FK506 treatment and the controls (Bonferroni's test, * $P<0.05$, ** $P<0.01$, *** $P<0.001$). Data are mea n ±SEM (n=2–6).

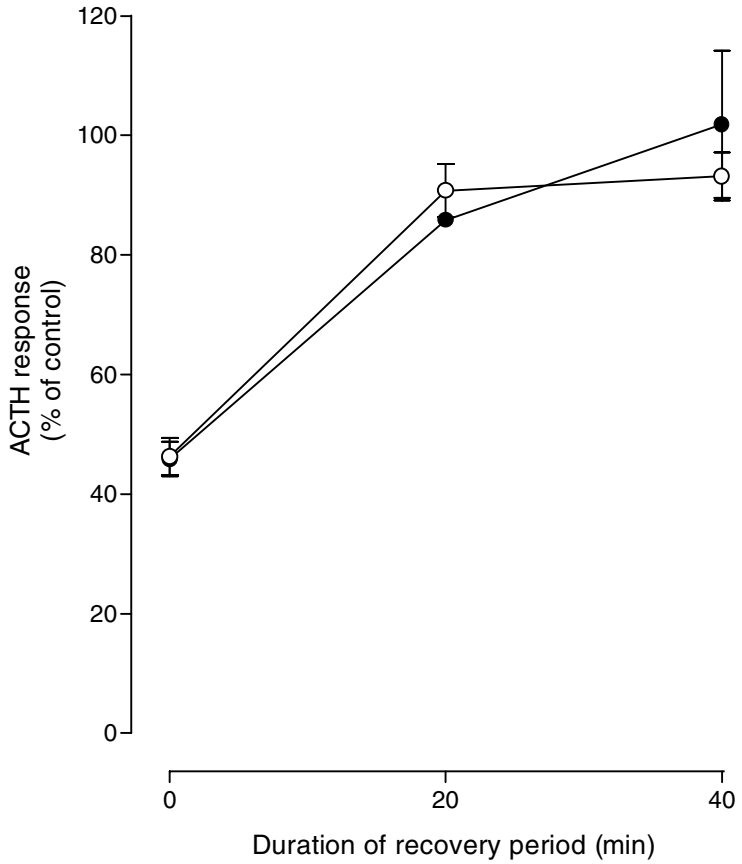

Fig. 5.9. Effect of treatment with 10 nM OA on desensitization and resensitization of the ACTH response to AVP. The ACTH response after pre-treatment with 10 nM AVP for 15 min is plotted against the duration of the recovery period. Combined results from columns in which OA was present (●) and absent (*i.e.* controls; ○) are shown. Data are mean ± SEM (n=2–10).

5.2.3.3 CALYCULIN A

Like OA, Calyculin A (CalyA) is a phosphatase inhibitor which inhibits both PP1 and PP2A (Herzig & Neumann 2000). It is equally potent against PP1 and PP2A (Ishihara *et al.* 1989). After the commencement of treatment with 50 nM CalyA there was a steady increase in the rate of basal ACTH release. ACTH secretion increased from 35.5 ± 4.4 pg/min at 90 min to 129.1 ± 7.2 pg/min at 160 min ($P<0.01$, n=4, *t*-test). This effect on basal ACTH secretion confounded interpretation of this experiment and it was not possible to assess the effect of treatment with CalyA on either resensitization of the ACTH response to AVP or on AVP-stimulated ACTH secretion.

5.3 DISCUSSION

Taken together, the results of experiments in which receptor internalization was blocked pharmacologically indicate that internalization plays an important role in the regulation of the ACTH response to AVP. Treatment with 0.25 mg/ml ConA reduced the extent of desensitization induced by pre-treatment with 10 nM AVP for 15 min by approximately two-thirds. This effect on desensitization occurred without any significant effect on either basal or AVP-stimulated ACTH secretion. The small and statistically insignificant decrease in AVP-stimulated ACTH secretion during the pre-treatment might have been due to ConA hindering the interaction of AVP with its receptor. In any event such a small reduction in stimulation could not account for the large reduction in the magnitude of desensitization that was caused by ConA treatment.

Additionally, treatment with hypertonic sucrose (which blocks receptor-mediated endocytosis (Daukas & Zigmond 1985; Heuser & Anderson 1989) reduced the ability of AVP pre-treatment to induce a reduction in response to a subsequent AVP stimula-

tion. Hypertonic sucrose completely abolished the AVP-induced reduction in response. However, this treatment caused significant non-specific effects: basal ACTH secretion was increased and the response to the 100 nM AVP pulse was greatly increased. These results suggest that the effects of hypertonic sucrose on the cells were not confined to the inhibition of receptor-mediated endocytosis. Because of these apparently non-specific effects of hypertonic sucrose on ACTH secretion it is difficult to unambiguously interpret these results with regard to the effects of this treatment on the processes of desensitization and resensitization.

Treatment with 5 μM PAO for 70 min prevented 100 nM AVP from stimulating any ACTH secretion. Thus this agent could not be used to assess the role of internalization in either desensitization or resensitization. Garland *et al.* (1996) have reported that they were unable to maintain the viability of CHO cells treated with 80 μM PAO for 60 min. A similar problem may have been encountered in these experiments or, alternatively, a non-specific effect of PAO may have prevented ACTH secretion.

In summary, the data obtained with ConA showed that desensitization of the ACTH response to AVP is dependent to a large extent upon receptor internalization. Given the effects of hypertonic sucrose on both basal and AVP-stimulated ACTH secretion there is doubt whether the effect of this treatment on the ability of AVP to induce desensitization was in fact the result of blockade of receptor internalization. However, while hypertonic sucrose treatment is unlikely to be entirely specific (Beaumont *et al.* 1998), it is important to note that it affected AVP-induced desensitization in the same way as ConA, *i.e.* it inhibited it.

The most convincing results in this series of experiments were those obtained by using ConA to inhibit internalization. It is interesting that treatment with 0.25 mg/ml ConA only partially inhibited the ability of AVP pre-treatment to induce desensitiza-

tion. Treatment with the agent at this concentration has been shown, in some instances to completely inhibit receptor internalization. Lohse *et al.* (1990a) showed that during treatment with isoproterenol for 10 min, 30% of β_2-adrenergic receptors became inaccessible to the hydrophilic ligand CGP 12177, as measured by its ability to displace ^{125}I-cyanopindolol binding. This sequestration of the receptor could be prevented by 20 min pre-treatment with 0.25 mg/ml ConA. Pippig *et al.* (1995) obtained identical results using the same experimental system. Similarly, Ng *et al.* (1995) found that treatment with 0.25 mg/ml ConA for 45 min completely inhibited internalization of the D_1 dopamine receptor, as measured by subcellular fractionation and radioligand binding. In contrast, Trincavelli *et al.* (2000) found that treatment with the same concentration of ConA for 15 min inhibited internalization of the A_3 adenosine receptor (as measured by radioligand binding) by only 51%. If receptor internalization was completely inhibited in the perifused anterior pituitary cell system used in these experiments then the remaining desensitization must have been the result of another desensitization process or processes (see below). Alternatively, ConA treatment may have only partially inhibited receptor internalization and as a result only partially inhibited the development of desensitization.

The results of experiments in which selective inhibitors were used to inhibit phosphatase activity show that dephosphorylation by PP2B plays an important role in the resensitization of the ACTH response to AVP. Treatment with the highly specific PP2B inhibitor FK506 at a concentration of 1 μM significantly attenuated the rate of resensitization. No evidence could be found for the involvement of PP2A in resensitization of the ACTH response to AVP. Treatment with 10 nM OA did not affect the rate of resensitization. Because of the non-specific effects of 50 nM CalyA treatment

on ACTH secretion this agent could not be used to assess the role of dephosphorylation in resensitization.

The involvement of PP2B in resensitization the ACTH response to AVP strongly suggests that phosphorylation of one or more components of the signalling pathway plays a role in the desensitization of the ACTH response to AVP. As described in Section 1.2.1 receptor phosphorylation is a very common mechanism of rapid desensitization amongst the GPCRs. The role of PP2B in resensitization has been most extensively investigated for the β_2-adrenergic receptor. Inhibition of PP2B activity, either by treatment with 122 nM FK506 (Shih & Malbon 1996) or expression of a PP2B antisense oligonucleotide sequence (Shih *et al.* 1999), prolongs recovery from desensitization of the cAMP responses to isoproterenol stimulation of this receptor. In the same system desensitization was found to be dependent upon GRK2 activity, indicating that the receptor was phosphorylated during desensitization (Shih & Malbon 1994). Although it has not been shown directly that PP2B dephosphorylates the β_2-adrenergic receptor, co-immunoprecipitation experiments have shown that PP2B associates with the receptor soon after agonist stimulation (Shih *et al.* 1999; Fraser *et al.* 2000) providing strong circumstantial evidence that the role of PP2B in resensitization is to dephosphorylate the receptor. It is possible that PP2B plays a similar role in the regulation of the ACTH response to AVP, mediating the dephosphorylation of V1b receptors phosphorylated during desensitization. However, because a wide variety of membrane and cytosolic proteins are phosphorylated during AVP stimulation (Liu 1994; Liu *et al.* 1994) it is not possible, based on the data presented here, to exclude the possibility that dephosphorylation of proteins other than the AVP receptor is involved in resensitization. However, much of the phosphorylation which occurs during AVP stimulation is mediated by PKC and is involved in ACTH secretion (Abou-Samra *et al.*

1986; Carvallo & Aguilera 1989; Liu *et al.* 1990; Liu 1994; Liu *et al.* 1994). Results described in Chapter 4 show that PKC is not involved in the desensitization of the ACTH response to AVP: activation of PKC did not cause desensitization and inhibition of PKC activity did not prevent AVP pre-treatment from inducing desensitization. While reversal of PKC-mediated phosphorylation by PP2B might be involved in the regulation of ACTH secretion (see below) this is unlikely to play a role in resensitization, given that PKC does not mediate desensitization of the ACTH response to AVP. Furthermore this observation suggests that desensitizing phosphorylation is not mediated by PKC or any protein kinase downstream from it in the signalling pathway. This is consistent with the involvement of GRK-mediated phosphorylation in desensitization of the V1b receptor.

If the role of PP2B in resensitization of the ACTH response to AVP is to dephosphorylate desensitized V1b receptors, then the question of what role receptor phosphorylation plays in desensitization is raised. Typically, phosphorylation of GPCRs causes desensitization by inhibiting the interaction of the receptor with its associated G protein, uncoupling it from its signalling pathway (Lohse 1993). Phosphorylation of receptors by the effector kinases (*i.e.* PKA or PKC) is, in itself, sufficient to cause desensitization whereas GRK-mediated phosphorylation is not (Benovic *et al.* 1988). Binding of β-arrestins to the phosphorylated receptor is necessary to terminate signalling (Lohse *et al.* 1990b). Therefore receptor phosphorylation is usually, directly or indirectly, sufficient to induce desensitization, *i.e.* internalization of the receptor is not required for its desensitization. However, it has been shown here that desensitization of the ACTH response to AVP can, to a large extent, be inhibited by pharmacological blockade of receptor internalization, indicating that receptor sequestration is important in the desensitization process. One possibility is that there are dual mecha-

nisms of desensitization, with receptor phosphorylation mediating the proportion of desensitization which could not be inhibited by treatment with ConA. A model of desensitization similar to this has been proposed for the secretin receptor. This receptor is a member of the Class II GPCR family, members of which share little structural homology with the Class I (or β-adrenergic) family of receptors of which the V1b receptor is a member (Gether 2000). During agonist exposure the secretin receptor undergoes GRK-mediated phosphorylation at serine and threonine residues in its C terminal tail (Ozcelebi *et al.* 1995; Shetzline *et al.* 1998). The receptor is also internalized following agonist exposure (Holtmann *et al.* 1996). Truncation of the C terminal tail of the receptor abolished phosphorylation of the receptor and partially inhibited desensitization. Internalization of the receptor was unaffected by this truncation and it was presumed that this process accounted for the remainder of desensitization (Holtmann *et al.* 1996). This conclusion was supported by studies in which pharmacological blockade of internalization with either hypertonic sucrose or ConA was shown to reduce the extent of desensitization induced by secretin treatment (Mundell & Kelly 1998).

Another possible role for receptor phosphorylation in the regulation of the V1b receptor is as a signal which acts to promote receptor sequestration. Phosphorylation, and in particular GRK-mediated phosphorylation, plays an important role in the internalization of a variety of GPCRs including the β_2-adrenergic receptor (Ferguson *et al.* 1995; Ménard *et al.* 1996), the m2 muscarinic receptor (Tsuga *et al.* 1998b), the endothelin A receptor (Bremnes *et al.* 2000) and the follicle stimulating hormone receptor (Lazari *et al.* 1999). In the case of the β_2-adrenergic receptor GRK-mediated phosphorylation is not absolutely required for internalization but instead increases the affinity of the receptor for β-arrestins which, in addition to uncoupling the receptor

from its signalling pathway, bind the β_2-adaptin subunit of the AP-2 adaptor complex which targets the receptor for internalization via clathrin-coated pits (Ferguson *et al.* 1996a; Laporte *et al.* 1999; Laporte *et al.* 2000; Ferguson 2001). It is possible that phosphorylation of the V1b receptor occurs at a site which does not prevent the receptor from interacting with $G_{q/11}$ but targets the receptor for internalization. It should be noted that receptor internalization can occur in the absence of receptor phosphorylation (Hausdorff *et al.* 1989; Ferguson *et al.* 1995). Indeed phosphorylation-independent internalization appears to be the main mechanism of desensitization of the sst_{2B} somatostatin receptor. Desensitization of signalling via this receptor is unaffected by inhibition of the activity of GRK2, GRK3, PKA, PKC and protein kinase G but can be abolished by treatment with PAO, hypertonic sucrose and ConA (Beaumont *et al.* 1998).

These two hypotheses for a role of receptor phosphorylation in the desensitization of the pituitary AVP receptor are not necessarily mutually exclusive. The actual mechanism could consist of a combination of the two. Receptor phosphorylation and subsequent binding of β-arrestins could impair, but not completely prevent, coupling of the V1b receptor to $G_{q/11}$, with subsequent phosphorylation-dependent internalization of the receptor resulting in full desensitization.

The increase in AVP-stimulated ACTH secretion during treatment with FK506 is an interesting phenomenon. As described above, protein phosphorylation, and particularly PKC-mediated phosphorylation, plays an important role in AVP-stimulated ACTH secretion. Clearly, for this protein phosphorylation to act as a reversible signalling system these target proteins must be dephosphorylated by protein phosphatases, resulting in termination of the response. The increase in ACTH secretion which we have observed during FK506 treatment is likely to have been the result of persistent

phosphorylation of these target proteins as a consequence of PP2B inhibition. PP2B has been reported to be involved in the regulation of a variety of cellular processes (Armstrong 1989), but the data presented here do not give any clue as to the sites of action of PP2B in ovine corticotrophs. One possible target of PP2B-mediated dephosphorylation is the myristoylated alanine-rich cytosolic kinase substrate (MARCKS) protein. This widely expressed protein is phosphorylated by PKC during AVP stimulation of ovine corticotrophs and this is associated with ACTH secretion (Liu *et al.* 1994). *In vitro*, PP2B has been shown to dephosphorylate MARCKS (Seki *et al.* 1995). Inhibition of PP2B-mediated dephosphorylation of MARCKS could result in increased ACTH secretion. Another possible substrate for dephosphorylation are L-VSCC. In Section 4.3 the possibility that PKC-mediated phosphorylation may positively modulate L-VSCC in ovine corticotrophs was discussed. PP2B has been shown to play an important role in the regulation of L-VSCC, dephosphorylating the channels and rendering them inactive (Armstrong *et al.* 1989; Lai *et al.* 1993; Schuhmann *et al.* 1997). Removal of a feedback mechanism of this type by inhibition of PP2B could prolong L-VSCC activation in corticotrophs, thereby increasing AVP-stimulated ACTH secretion. Interestingly, PKC and PP2B have been shown to regulate the activity of VSCC in this manner in rat lactotrophs (Fomina & Levitan 1997).

Antoni *et al.* (1993) have reported that treatment with 1 μM FK506 increases basal ACTH secretion of AtT-20 cells. No evidence for such an effect in ovine anterior pituitary cells could be found in these experiments. The magnitude of such an effect may have been too small to detect.

In summary, these results show that desensitization of the ACTH response to AVP depends to a large extent, if not entirely, upon receptor internalization. As such the pituitary AVP receptor joins the small group of GPCRs for which internalization is

important in desensitization. Also, the activity of PP2B plays an important role in resensitization, most likely by dephosphorylating pituitary AVP receptors.

6

DISCUSSION

6.1 SUMMARY

Desensitization of the ACTH response to AVP was investigated in a perifused dispersed ovine anterior pituitary cell system. Characterization of the desensitization process showed that it was rapid, readily reversible and occurred at relatively low AVP concentrations (see Chapter 3). Significant desensitization was found to occur at concentrations and durations of AVP treatment which were within the range of endogenous AVP pulses, suggesting that desensitization might play an important physiological role in the regulation of ACTH secretion. The rapidity of desensitization and resensitization suggested that these processes might be more important in regulating the acute ACTH response to AVP than in the alterations of corticotroph responsiveness which have been observed during chronic stress.

The characteristics of the desensitization process also suggested that it might be mediated by phosphorylation of the pituitary AVP receptor, a common mechanism of desensitization amongst the GPCR family (Lohse 1993). No evidence could be found for the involvement of either PKC or CK1α in desensitization of the ACTH response to AVP (see Chapter 4), but it remains possible that the V1b receptor is phosphory-

lated by another intracellular protein kinase, *e.g.* a member of the GRK family. The lack of involvement of PKC in desensitization suggests that there are AVP receptor subtype-specific differences in the mechanism of desensitization since the hepatic/vascular V1a AVP receptor has been shown to be desensitized by PKC (Caramelo *et al.* 1991; Gallo-Payet *et al.* 1991; Ancellin *et al.* 1997; Ancellin & Morel 1998).

Internalization of GPCRs is commonly associated with their resensitization, allowing dephosphorylation of desensitized receptors before they are recycled back to the plasma membrane in a fully functional state (Ferguson 2001). However, it was found that pharmacological blockade of receptor internalization reduced the extent of desensitization induced by AVP treatment, indicating that receptor sequestration played a critical role in the *desensitization* process. Inhibition of PP2B had no effect on desensitization but slowed the rate of recovery from desensitization, indicating that this enzyme was important in resensitization. The involvement of a protein phosphatase in resensitization is consistent with receptor phosphorylation playing an important role in desensitization (see Chapter 5).

6.2 GENERAL COMMENTS ON METHODS USED IN THIS RESEARCH

In this study the molecular mechanisms involved in the desensitization of the ACTH response to AVP have largely been investigated by examining the effects of various pharmacological agents on this desensitization process. Because the effects of these agents on cellular function have been assessed indirectly, without directly measuring their effects on the intended target, the possibility that observed effects are due to non-specific actions is always present. This problem is particularly acute where the activity of a complex cellular function (such as endocytosis) rather than a specific target (such as an enzyme) is being modulated. Where possible this potential problem has been mini-

mized through the use of multiple agents to investigate the involvement of a particular cellular process or enzyme in desensitization. Although each of the pharmacological agents used may have its own non-specific effects, these are likely to be different for each agent and can be identified and taken into account during analysis of the data. A good example of this approach is provided by the investigation of the involvement of receptor internalization in regulation of the ACTH response to AVP (see Chapter 5). Three agents (ConA, PAO and hypertonic sucrose), each of which is purported to inhibit internalization through a mechanistically distinct mechanism, were used to inhibit internalization. While none of these agents could be considered to be entirely specific (Beaumont *et al.* 1998), the combined results provided strong evidence that desensitization was largely dependent upon internalization of the V1b receptor.

The use of this approach to investigate the mechanisms of desensitization was dictated in part by the use of dispersed ovine anterior pituitary cells as the experimental system. Carrying out experiments in primary cultures made the use of a number of experimental techniques, particularly those involving molecular biology (*e.g.* expression of dominant negative mutant GRKs, truncation of the V1b receptor tail), either impractical or impossible. While in one sense this could be seen as a disadvantage, use of this system did provide a distinct advantage. Recent evidence has shown that the level of expression of GPCRs can affect their regulation. For example, at low density the thromboxane A2 receptor becomes desensitized during agonist-exposure but at high receptor densities desensitization is not observed (Spurney 1998). Furthermore, regulation of GPCRs can vary depending upon the nature of the cellular environment in which they are expressed (Ferguson 2001). For example, the CXCR1 interleukin-8 receptor does not undergo agonist-promoted internalization when expressed in HEK293 cells but does when expressed in the neutrophil-like RBL-2H3 cell line,

which expresses substantially higher levels of GRK2 and β-arrestin (Barlic *et al.* 1999). Similarly, the extent of agonist-promoted internalization of the β_2-adrenergic receptor varies depending on the cell line in which the receptor is expressed, and the extent of receptor internalization was found to correlate well with the level of expression of GRKs and β-arrestins (Ménard *et al.* 1997). These data suggest that the regulation of GPCRs is dependent not only on the presence or absence of specific proteins but also on the level of expression of these proteins. Because the cellular milieu in immortalized cell culture systems may be different to that found in physiological systems it is possible that there are important differences in the regulation of a given receptor in these two environments. (Ferguson 2001). This is especially likely to be the case in heterologous expression systems such as those described above. In contrast, in the anterior pituitary cell system used in this research the expression of all components of the signalling pathway were under physiological regulation. As such the mechanisms which were found to be involved in desensitization of the ACTH response to AVP are likely to be of physiological significance and not artefacts of the system being investigated.

This investigation of the mechanisms of desensitization of the ACTH response to AVP has focused on the regulation of the receptor, as in many instances regulation of GPCRs appears to account for most, if not all, of the observed desensitization (Krupnick & Benovic 1998). However, because ACTH secretion has been used as the indicator of responsiveness to AVP it is possible that post-receptor mechanisms are involved in desensitization. For example, down-regulation of IP_3 receptors plays an important role in desensitization of signalling through the PLC-coupled GnRH receptor (McArdle *et al.* 1999). The involvement of similar mechanisms in desensitization of the ACTH response to AVP cannot be excluded.

6.3 PHYSIOLOGICAL ROLE OF DESENSITIZATION OF THE ACTH RESPONSE TO AVP

Secretion of AVP into the hypophyseal portal circulation is highly pulsatile, with secretory episodes rarely lasting more than 30 min during acute stress in the sheep (Caraty *et al.* 1988; Engler *et al.* 1989; Caraty *et al.* 1990). As described above and in greater depth in Chapter 3, desensitization was found to occur at concentrations and durations of AVP treatment which were within the ranges observed in endogenous AVP pulses. This suggests that this rapid desensitization might play an important physiological role in the regulation of ACTH secretion. Because desensitization was shown to be both rapid and readily reversible it is unlikely that it is responsible for the down-regulation of pituitary AVP receptors which has been observed *in vivo* during treatments such as prolonged AVP infusion (Antoni *et al.* 1985), adrenalectomy (Antoni *et al.* 1985; Lutz-Bucher *et al.* 1986), and chronic osmotic stress such as hypertonic saline injection and 2% saline in the drinking water (Aguilera *et al.* 1994).

Down-regulation of GPCRs typically occurs following long-term agonist exposure (*i.e.* hours or days) and can be achieved by either a reduction in the rate of receptor synthesis, increased degradation of existing receptors or a combination of these two processes (Lohse 1993). Internalization is known to play a key role in the resensitization of GPCRs. For example, following phosphorylation and desensitization β_2-adrenergic receptors are transferred from the cell surface to an intracellular compartment (most likely endosomes [von Zastrow & Kobilka 1992]), where they are dephosphorylated by protein phosphatases and then returned to the cell surface in a fully functional state (Yu *et al.* 1993; Pippig *et al.* 1995; Ferguson 2001). However, internalization also plays an important role in the down-regulation of GPCRs. During long-term treatment with isoproterenol (a treatment which induces down-regulation) GFP-tagged β_2-adrenergic receptors expressed in HEK293 cells accumulate in lysosomes (Kallal *et al.*

1998). Gagnon *et al.* (1998) found that this internalization of the β_2-adrenergic receptor played an important role in down-regulation. Expression of dominant-negative mutants of dynamin and β-arrestin inhibited both internalization and down-regulation of the receptor. It was concluded that during long-term stimulation some of the internalized receptors were sorted from endosomes to lysosomes for degradation.

In this study it has been shown that receptor internalization played a critical role in the desensitization of the ACTH response to AVP (see Chapter 5). Given that following treatment with 10 nM AVP for 15 min complete resensitization occurred within 20 to 40 min, it is likely that receptors were recycled back to the plasma membrane rather than targeted for lysosomal degradation. Because endogenous AVP pulses observed during acute stress are of similarly short duration and low concentration it is unlikely that they are able to cause down-regulation of the pituitary AVP receptor. Indeed, one function of the pulsatile secretion of AVP into the hypophyseal portal circulation may be to avoid prolonged stimulation of the receptor, thereby preventing its down-regulation. However, it is possible that increases in the duration, concentration or frequency of AVP pulses during chronic stress could result in targeting of pituitary AVP receptors for lysosomal degradation rather than recycling back to the cell surface. Interestingly, Rabadan-Diehl *et al.* (1995) found little correlation between V1b receptor mRNA content and AVP binding in anterior pituitary cells isolated from rats exposed to various chronic stress paradigms. Although this lack of correlation could be explained by phenomena such as increased mRNA utilization, especially in situations where the receptor was up-regulated (Aguilera & Rabadan-Diehl 2000), it also suggests that degradation of existing receptors may play an important role in the down-regulation of pituitary AVP receptors.

It is possible that factors such as CRH and glucocorticoids are able to modulate the desensitization of the ACTH response to AVP. As described in Chapter 3, it has been demonstrated in our laboratory that desensitization of the ACTH response to AVP in perifused ovine anterior pituitary cells is unaffected by the presence of a low (0.01 nM) 'background' concentration of CRH in the perifusion medium (Chacko 2000; Chacko *et al.* 2000). However, given that receptor internalization was found to be important in desensitization of the ACTH response to AVP it is interesting to note that treatment with CRH can increase the rate of internalization of the pituitary AVP receptor. Working with rat anterior pituitary cells, Mogensen *et al.* (1988) have shown that treatment with 21 nM CRH causes a marked increase in the rate of receptor-mediated endocytosis of [^{3}H]AVP. This raises the possibility that CRH might be able to modulate the desensitization process in some way. CRH concentrations greater that the 0.01 nM treatment used by Chacko (2000) might be able to enhance desensitization by promoting receptor internalization. Alternatively, a consequence of a CRH-induced increase in the rate of AVP receptor internalization might be enhanced recycling of the receptor, resulting in more rapid resensitization.

6.4 SUGGESTIONS FOR FURTHER RESEARCH

This study has shown that in perifused ovine anterior pituitary cells the ACTH response to AVP undergoes rapid desensitization, and has elucidated some of the mechanisms which underlie this desensitization process. However, a great many questions remain to be answered with regard to both the characterization of this process and the mechanisms which are involved.

While Chacko (2000) has shown that treatment with CRH at physiological concentrations has no effect on desensitization of the ACTH response to AVP, neither

enhancing nor 'protecting' against desensitization, the effect of glucocorticoids on this process is unknown. Furthermore, it is possible that there are complex interactions between AVP, CRH and glucocorticoids (and possibly other factors) which affect the desensitization process in unanticipated ways. Further investigation of the effects of both CRH and glucocorticoids on desensitization could provide important insights into the physiological regulation of ACTH secretion.

Although PKC and CK1α were not involved in desensitization it remains likely that receptor phosphorylation plays an important role in the regulation of the ACTH response to AVP. In particular, the involvement of members of the GRK family in this process is yet to be elucidated. An investigation of the involvement of GRKs in desensitization is currently in progress in our laboratory using treatment with oligonucleotide sequences to inhibit expression of GRKs.

Attempts to show directly that the pituitary AVP receptor is phosphorylated during agonist exposure were unsuccessful. Incorporation of phosphate into the V1b receptor could be shown by incubating cells with ^{32}P and subsequently immunoprecipitating the receptor as has been described for V1a receptor (Ancellin *et al.* 1999) and the V2 receptor (Innamorati *et al.* 1997). Use of this technique would require either anti-V1b receptor antibodies or an epitope-tagged V1b receptor.

While receptor internalization has been shown to be important in desensitization of the ACTH response to AVP it is not clear what signals cause the receptor to become internalized. Both phosphorylation-dependent and independent internalization of GPCRs has been observed (Ferguson 2001). If a protein kinase were shown to be involved in desensitization it would be interesting to assess the effect of inhibition of this enzyme on internalization of the AVP receptor, which could be measured by radioligand binding.

Internalization of many GPCRs (*e.g.* β_2-adrenergic receptor [Pippig *et al.* 1995], NK_1 neurokinin receptor [Garland *et al.* 1996]) plays an important role in resensitization, allowing the dephosphorylation of receptors at an intracellular location (Ferguson *et al.* 1996b). On the other hand, Bogatkewitsch *et al.* (1996) have shown that inhibition of internalization of the m4 muscarinic receptor delays its resensitization. The role of receptor internalization in resensitization of the ACTH response to AVP remains unclear. One approach to this problem would be to inhibit recycling of the pituitary AVP receptor. Both monensin (an ionophore) and bafilomycin A_1 (an H^+/ATPase inhibitor) inhibit receptor recycling by raising endosomal pH (Pippig *et al.* 1995; Garland *et al.* 1996; Beaumont *et al.* 1998). If internalization were necessary for resensitization then treatment with either of these agents would be expected to slow the rate of resensitization.

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APPENDIX A

MATERIALS

[Arg^8]-vasopressin, acetate salt	Sigma Chemical Co.
0.22 μm polyethersulfone filter	Millipore Corporation, Bedford MA
0.45 μm mixed cellulose ester filter	Millipore Corporation, Bedford MA
1,2-dioctanoyl-*sn*-glycerol (DiC_8)	Sigma Chemical Co.
^{125}I	Amersham International, Buckinghamshire, UK
^{125}I-AVP	Amersham International, Buckinghamshire, UK
Acetone	BDH Laboratory Supplies
Acetonitrile	BDH Laboratory Supplies
Acrylamide	Sigma Chemical Co.
ACTH with carboxymethylcellulose	Nordic Biochemicals Ltd., Montreal, Canada
ACTH zinc hydroxide	Organon Laboratories, Surrey, UK
Ammonium persulphate	Sigma Chemical Co.
Amphotericin B	Sigma Chemical Co.
Bio-Gel P-2	Biorad Laboratories, Hercules CA
Bovine γ-globulins	Sigma Chemical Co.
Bovine serum albumin (purified)	Behring, Marburg, Germany

Brilliant Blue G	Sigma Chemical Co.
$CaCl_2.2H_2O$	Reidel-De Haen AG, Hannover, Germany
Calyculin A	Sigma Chemical Co.
Cellulose	Whatman Ltd, Springfield Mill, Kent, UK
Chloramine T	BDH Laboratory Supplies
CK1-7	Seikagaku Corporation, Tokyo, Japan
Concanavalin A	Sigma Chemical Co.
D-glucose	BDH Laboratory Supplies
Dimethylsulphoxide (DMSO)	BDH Laboratory Supplies
Disuccinimidyl suberate (DSS)	Pierce, Rockford IL
DME powder	Sigma Chemical Co.
EGTA	BDH Laboratory Supplies
FK506	Dr. M. Tomoi, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan
Glacial acetic acid	BDH Laboratory Supplies
Glycine	Sigma Chemical Co.
HCl	BDH Laboratory Supplies
HEPES	Sigma Chemical Co.
Horse plasma	Endolab, Christchurch Hospital, Christchurch
KCl	May and Baker Australia Ltd, West Footscray, Victoria, Australia
KH_2PO_4	BDH Laboratory Supplies
L-ascorbic acid	Sigma Chemical Co.
L-glutamate	Sigma Chemical Co.
MEM non-essential amino acids	Sigma Chemical Co.
$MgSO_4.7H_2O$	BDH Laboratory Supplies
N,N'-Methylene bis-Acrylamide (Bis)	Sigma Chemical Co.
Na_2HPO_4	BDH Laboratory Supplies
$Na_2S_3O_5$	BDH Laboratory Supplies

Na_3VO_4	Sigma Chemical Co.
$Na_4P_2O_7.10H_2O$	Sigma Chemical Co.
NaCl	BDH Laboratory Supplies
NaF	BDH Laboratory Supplies
$NaHCO_3$	BDH Laboratory Supplies
NaN_3	BDH Laboratory Supplies
NaOH	BDH Laboratory Supplies
New-born calf serum (NCS)	Life Technologies, Rockville MD
Nybolt nylon gauze	Seidengaze, Zurich, Switzerland
Okadaic acid	Sigma Chemical Co.
Ovine ACTH	Dr C.H. Li, Hormone Research Laboratory, University of California, San Francisco CA
Penicillin G	Sigma Chemical Co.
Phenol red (sodium salt)	Sigma Chemical Co.
Phenylarsine oxide	Sigma Chemical Co.
Plastic petrie dishes	Labserv, Auckland
Polyethylene glycol 6000	BDH and Scharlau Chemie, Barcelona, Spain
Ro 31-8220	Dr G. Lawton, Roche Research Centre, Welwyn Garden City, UK
Sephadex G-25 fine	Sigma Chemical Co.
SigmaMarkers	Sigma Chemical Co.
Silicic acid (100 mesh)	Mallinckrodt, St Louis MO
Sodium dodecyl sulphate (SDS)	Sigma Chemical Co.
Sterile culture tubes	Falcon Labware, Franklin Lakes NJ
Streptomycin sulphate	Sigma Chemical Co.
Sucrose	Sigma Chemical Co.
TEMED	Sigma Chemical Co.
Trichloroacetic acid (TCA)	Sigma Chemical Co.
Tris	Sigma Chemical Co.
Triton X-100	Sigma Chemical Co.

Trypan blue	BDH Laboratory Supplies
Type II collagenase (clostridiopeptidase A)	Sigma Chemical Co.
U.S.P. Type VII gauze	Kendall Healthcare Products, Boston MA

APPENDIX B

SOLUTIONS

B.1 CELL PREPARATION AND CULTURE

B.1.1 DISPERSING BUFFER (DB)

This solution was prepared by dissolving the reagents listed below in ddH_2O to give the concentrations shown. The pH was adjusted to 7.3 using concentrated NaOH or HCl and the solution was sterilised by either negative pressure filtration (0.45 μm mixed cellulose ester [MCE] filter) or positive pressure filtration (0.22 μm polyethersulfone [PES] filter). The solution was stored at 4°C.

NaCl	137 mM
KCl	5 mM
N-[2-hydroxyehtyl]piperazine-N'-2-ethanesulfonic acid (HEPES)	25 mM
D-Glucose	10 mM
Phenol red (sodium salt)	42 nM
Penicillin G	100 U/ml

Streptomycin sulphate	100 μg/ml
Amphotericin B	0.25 μg/ml

B.1.2 DULBECCO'S MODIFIED EAGLES MEDIUM (DMEM)

One bottle of commercially prepared, powdered medium was dissolved in ddH_2O. The reagents listed below were added in quantities which gave the concentrations shown. The solution was made up to 1 L with ddH_2O, adjusted to pH 7.1 and sterilized by negative pressure filtration (0.45 μm MCE filter). If the solution to be used in cell culture was between 2 and 4 weeks old fresh L-glutamate was added to give a final concentration of 2 mM. The solution was stored at 4°C. Before use in cell culture 10% NCS was added.

$NaHCO_3$	44 mM
HEPES	25 mM
MEM non-essential amino acids	1.0 ml
Phenol red (sodium salt)	53 nM
Penicillin G	100 U/ml
Streptomycin sulphate	100 μg/ml
Amphotericin B	0.25 μg/ml

B.2 MULTI-COLUMN PERIFUSION

B.2.1 KREBS RINGER (KR)

The reagents listed below were dissolved in ddH_2O to give the concentrations shown. The pH was adjusted to 7.3 and the solution was filter-sterilised as for dispersing buffer. The solution was store at 4°C. On the day of use in a perifusion experiment

0.05% ATC and 0.005% L-ascorbate were added to give KR/ATC. If necessary the pH was adjusted to 7.3 after the addition of ATC and ascorbate.

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 (sodium salt)	3.5 nM
Penicillin G	100 U/ml
Streptomycin sulphate	100 μg/ml
Amphotericin B	0.25 μg/ml

B.2.2 50 mM KCl Krebs Ringer

This was produced by mixing normal KR (with a K^+ concentration of 5.9 mM) with an appropriate volume of a 100 mM KCl solution. 100 mM KCl was produced by dissolving the reagents listed below in ddH_2O to give the concentrations shown. The pH of the solution was adjusted to 7.3. It was filter-sterilised and stored at 4°C.

NaCl	85 mM
KCl	98.8 mM
KH_2PO_4	1.2 mM
$CaCl_2.2H_20$	2.5 mM

KH_2PO_4	1.2 mM
$MgSO_4.7H_20$	1.2 mM
$NaHCO_3$	3.6 mM
HEPES	25 mM
Glucose	10 mM
Phenol red	3.5 nM
Penicillin G	100 U/ml
Streptomycin sulphate	100 μg/ml
Amphotericin B	0.25 μg/ml

B.2.3 PREPARATION OF 'TEST' SOLUTIONS

'Test' solutions for perifusion were prepared by either dissolving the hormone or pharmacological agent directly in KR/ATC or diluting a stock solution with KR/ATC. Stock solutions of AVP were prepared at concentrations of 20 μM by dissolving the peptide ([Arg^8]-vasopressin, acetate salt) in KR containing 0.3% bovine serum albumin and 0.005% L-ascorbic acid. These stock solutions were stored at –80°C and diluted in KR/ATC to give the desired concentration for perifusion experiments. DiC_8, Ro 31-8220, Calyculin A, okadaic acid and FK506 were prepared as stock solutions in DMSO. ConA and sucrose were dissolved directly in KR/ATC.

B.3 RADIOIMMUNOASSAY SOLUTIONS

B.3.1 STOCK 0.5 M PHOSPHATE BUFFER

The following reagents were made up to 1 L in ddH_2O. This solution was used as a stock solution for other solutions and was stored frozen.

KH_2PO_4 (anhydrous)	18 g
Na_2HPO_4 (anhydrous)	52.3 g
Ethylenediamine tetraacetic acid (disodium salt; Na_2EDTA)	0.1 g
NaN_3	2 g

B.3.2 0.05 M PHOSPHATE/0.1% ATC BUFFER (P/ATC)

0.5 M stock phosphate buffer was diluted 1:10 [v:v] to give a 0.05 M solution. 0.1% ATC was added and the pH was adjusted to 7.4. The solution was stored frozen.

B.3.3 ACETONE/ACETIC ACID

This was used in the repurification of ACTH tracer. It was prepared on the day of the repurification by mixing 2.5 ml of acetone and 100 µl of glacial acetic acid with 10 ml of ddH_2O.

B.3.4 POLYETHYLENE GLYCOL SOLUTION (PEG)

An 18% polyethylene glycol solution was prepared using the reagents listed below and was stored at room temperature.

PEG6000	360 g
NaCl	11.7 g

0.5 M stock phosphate buffer	210 g
ddH_2O	1500 g
Triton X-100	2 ml

B.4 SOLUTIONS FOR PREPARATION OF PLASMA MEMBRANES AND ELECTROPHORESIS

B.4.1 INHIBITOR BUFFER (IB) AND PROTEIN ASSAY BUFFER (PAB)

To prepare IB the reagents listed below were dissolved in ddH_2O. The solution was made up to 250 ml, the pH adjusted to 7.4 and stored at –20°C. PAB was similar to IB but did not contain leupeptin or EGTA. PMSF was prepared in dried ethanol as a 100× stock solution and added immediately before use.

HEPES	25 mM
NaCl	104 mM
NaF	10 mM
Na_2EDTA	2 mM
Ethyleneglycolbis(aminoethylether)-tetraacetic acid (EGTA)	2 mM
$Na_4P_2O_7.10H_2O$	20 mM
Na_3VO_4	0.1 mM
Leupeptin	1 µg/ml
Phenylmethylsulfonylfluoride (PMSF)	1 mM

B.4.2 POLYACRYLAMIDE GEL ELECTROPHORESIS

B.4.2.1 RESERVOIR BUFFER

Reservoir buffer for SDS-PAGE was prepared as a 5× solution and diluted immediately before use. The reagents listed below were dissolved in ddH_2O and the solution was made up to 1 L.

Tris	0.125 M
Glycine	0.96 M
Sodium dodecyl sulphate (SDS)	0.5%

B.4.2.2 GEL CASTING

Resolving gels (12%) were prepared according to the protocol below and dispensed into an electrophoresis unit (SE410, Hoefer, San Francisco CA). 1.5% ammonium persulfate was prepared fresh immediately before use. After layering ddH_2O over the top of the gel it was allowed to polymerise for approximately 1 h.

Acrylamide/Bis Stock (30%T:2.7%C)	20 ml
Resolving gel buffer (3.0 M Tris·HCl; pH 8.8)	6.25 ml
10% SDS	0.75 ml
ddH_2O	21.75 ml
deaerate 1 min	
1.5% ammonium persulphate	1.5 ml
TEMED	0.015 ml

The ddH_2O layered over the gel was then removed and a 4% stacking gel was cast over the resolving gel. This was prepared according to the portocol described below. A comb was inserted into the stacking gel and it was allowed to polymerise for 30 min. The comb was then removed and the wells filled with reservoir buffer.

Acrylamide/Bis Stock (30%T:2.7%C)	2.5 ml
Stacking gel buffer (0.5 M Tris·HCl; pH 6.8)	5.0 ml
10% SDS	0.2 ml
ddH_2O	11.3 ml
deaerate 1 min	
1.5% ammonium persulphate	1.5 ml
TEMED	0.015 ml