EFFECT OF HORMONAL INTERACTION ON DESENSITIZATION OF THE ADRENOCORTICOTROPIN RESPONSE TO ARGININE VASOPRESSIN IN OVINE ANTERIOR PITUITARY CELLS

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ABSTRACT

Corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) are major physiological stimulators of adrenocorticotropin (ACTH) secretion from the pituitary gland, while glucocorticoids act as inhibitors. In addition to acting alone, CRH, AVP and glucocorticoids interact with each other to regulate ACTH release in response to stress. Prolonged or repeated stimulus results in attenuated ACTH responsiveness, a process termed as desensitization. The aim of this study was to investigate the effects of interactions between CRH, AVP and steroids on desensitization of the ACTH response to AVP.

Perifused ovine anterior pituitary cells were stimulated with three 5-min pulses of 100 nM AVP at 120, 200 and 280 min, and were continuously exposed to CRH (0.2 nM) from 80 min and/or cortisol (10-500 nM) from 0 min onwards. Desensitization was induced by a 15 min pre-treatment with 5 nM (0.5 nM for CRH alone) AVP immediately preceding the second AVP pulse. When CRH was absent, pre-treatment with 0.5 nM AVP did not influence the ACTH response to the second AVP pulse. In the presence of CRH, the response to the second AVP pulse was reduced to 66.7±2.2% of control (n=6, $P<0.0001$, *t*-test).

On the other hand, following 5 nM AVP pre-treatment, continuous perifusion with cortisol (100 nM) results in a significantly smaller reduction in the response to the second AVP pulse compared with that seen in its absence (78.4±1.7% c.f. 66.7±1.9% of control; n=10, $P<0.001$, *t*-test). In contrast to this, following 5 nM AVP pre-treatment, continuous CRH and cortisol in combination results in a greater reduction in the response to the second AVP pulse compared with that obtained in the absence of these two hormones (46.5±1.7% c.f. 66.2±1.7 of control; n=8, $P<0.0001$, *t*-test).

Taken together, these data suggest that desensitization of the ACTH response to AVP can be modulated by CRH and/or cortisol: CRH or CRH and cortisol in combination amplify this desensitization, whereas cortisol reduces it.

ABBREVIATIONS

ACTH	adrenocorticotropin
^{125}I-$_{O}$ACTH	^{125}I-labelled ovine ACTH
ATC	alkali-treated casein
AVP	arginine vasopressin
BK channels	calcium-activated potassium channels
cAMP	cyclic AMP
CaM	calmodulin
CRH	corticotropin-releasing hormone
CRH-R1	type 1 CRH receptor
CSPs	calcium-sensor proteins
DAG	diacylglycerol
DB	dispersing buffer
ddH2O	distilled, deionized H2O
DEX	dexamethasone
DME	Dulbecco's modified Eagle's medium
DME/NCS	DME + 10 % newborn calf serum
GPCR	G protein-coupled receptor
GR	glucocorticoid receptor
GRK	G-protein coupled receptor kinase
HPA	hypothalamo-pituitary-adrenal
IP_3	inositol 1.4.5-trisphosphate
KR	Krebs ringer
KR/ATC	Krebs Ringer + 0.05% ATC + 0.005% L-ascorbate
MR	mineralocorticoid receptor
NCS	newborn calf serum
NS	not significant

NSB	non-specific binding
P/ATC	0.05 M phosphate buffer + 0.1% ATC
PEG	polyethylene glycol solution
PIP_2	phosphatidylinositol 4.5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholiphase C
PVN	paraventricular nucleus
RIA	radioimmunoassay

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INTRODUCTION

1.1 The Hypathalamo-Pituitary-Adrenal Axis: Regulation of Adrenocorticotropin Release

To function properly, the various organs of an animal must communicate well with each other to maintain a constant internal environment (i.e., homeostasis) and respond appropriately to any changes (e.g. stressful stimuli) in the internal and external environments (Hiller-Sturmhofel & Bartke 1998). A key component involved in the vertebrate response to stress is activation of the hypothalamo-pituitary-adrenal (HPA) axis. As shown in Figure 1.1.1, corticotrophin (ACTH) secretion from pituitary gland is the central part of the HPA axis. Upon input of stressful stimuli, increased corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) are released from the hypothalamus into the pituitary portal circulation, where they stimulate ACTH secretion. This, in turn, triggers an increase in glucocorticoid release from the adrenal gland and these steroid hormones act on multiple targets to restore homeostasis (Buckingham *et al.* 1997).

Regulation of ACTH secretion within the HPA axis is a multifactorial process, with the hypothalamic peptides CRH and AVP being important physiological stimulators, and adrenal glucocorticoids as a major inhibitory factor by feedback loops (Aguilera 1994). In addition to steroid inhibition, ACTH has been shown to elicit an inhibitory effect on hypothalamic release of CRH/AVP, but the physiological significance of this is unclear (Buckingham *et al.* 1997).

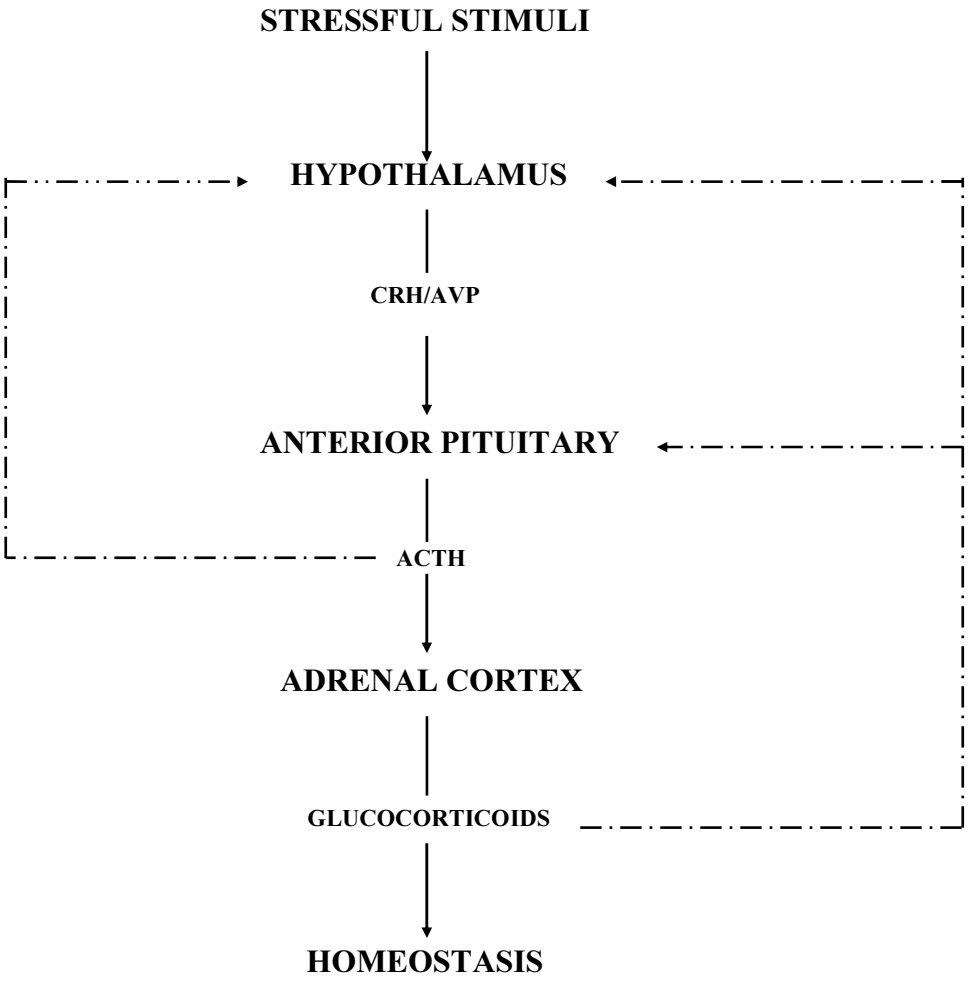

Figure 1.1.1 Activation of the hypothalamo-pituitary-adrenal axis. Solid lines represent positive stimulation, dashed lines represent negative feedback.

1.1.1 Control of ACTH release by stimulators

CRH and AVP are the most important physiological stimulators of ACTH release from the pituitary gland (Antoni 1993). CRH is mainly released from the parvocellular division of the paraventricular nucleus (PVN). About half of the CRH-containing neurons in the PVN also contains AVP and thus release both CRH and AVP (Antoni 1993). AVP is also released from magnocellular neurons of the supraoptic and paraventricular nuclei (Antoni 1993; Scott & Dinan 1998). It has been suggested that AVP from both origins is involved in stimulating ACTH secretion, although these two vasopressinergic systems appear to respond to different stress paradigms (Antoni 1993; Aguilera 1994).

AVP had already been structurally characterized and implicated in the regulation of ACTH secretion from the anterior pituitary by the mid-1950s (Martini & Morpurgo 1955), but this remained controversial for nearly 30 years. Meanwhile, most studies focused on CRH as a potent physiological stimulator for ACTH release (Antoni 1993). When the CRH molecule was characterized and synthetic 41-CRH became available in the early 1980s (Vale *et al.* 1981), studies using rat anterior pituitary cells confirmed that AVP significantly potentiates the effect of CRH on ACTH secretion, although vasopressin alone is a weak ACTH secretagogue in this species (Gillies *et al.* 1982; Bilezikjian & Vale 1987). Studies in mouse, horse and fallow deer pituitary cells show that, similar to the situations in the rat, AVP is less potent in stimulating ACTH release than CRH in these species (Hoffman *et al.* 1985; Evans *et al.* 1993a; Willard *et al.* 1995). In contrast to this, experiments conducted in ovine anterior pituitary cells provide conflicting results. Whereas some studies suggest that CRH is more effective in stimulating ACTH release from perifused ovine anterior pituitary cells than AVP (Evans *et al.* 1988; Hassan *et al.* 2003), other researchers have found that AVP is a much more potent stimulator than CRH in ovine pituitary static cultures (Familari *et al.* 1989; Liu *et al.* 1990). Overall, all studies in sheep pituitary cells have

confirmed that AVP alone is a strong stimulus for ACTH secretion (Evans *et al.* 1988; Familari *et al.* 1989; Liu *et al.* 1990; Hassan *et al.* 2003).

Exposure of normal sheep to various stimuli, such as insulin-induced hypoglycemia (Engler *et al.* 1989; Caraty *et al.* 1990), audiovisual stress (Engler *et al.* 1989), transport (Smith & Dobson 2002), and endotoxin challenge (Battaglia *et al.* 1998), result in pulsatile secretion of AVP and CRH into pituitary portal circulation, and this is associated with increased plasma ACTH and/or cortisol concentration. Similar results have been observed in horse with insulin-induced hypoglycemia (Alexander *et al.* 1996). In addition, exogenous administration of CRH and/or AVP into normal rats (Tizabi & Aguilera 1992; Hauger & Aguilera 1993) and sheep (Keller-Wood 1998) also results in increased plasma ACTH, while immunoneutralization of AVP or CRH in intact sheep significantly reduced the ACTH and cortisol responses to isolation-restraint and insulin-induced hypoglycemia (Guillaume *et al.* 1992a; 1992b). These studies, together with the *in vitro* results, clearly demonstrate the important physiological roles of AVP and CRH in stimulating ACTH release from the pituitary gland.

In addition to individual effects on ACTH secretion, CRH and AVP exert a synergistic effect on ACTH response when these two secretagogues are given together. Extensive studies using isolated pituitary cells or segments have shown that the ACTH response to CRH and AVP in combination is higher than the summed responses to CRH or AVP alone in sheep (Evans *et al.* 1988; Familari *et al.* 1989; Liu *et al.* 1990; Kemppainen & Clark 1993), rat (Gillies *et al.* 1982; Bilezikjian & Vale 1987; Watanabe & Orth 1987), horse (Evans *et al.* 1993a) and fallow deer (Willard *et al.* 1995). Similarly, simultaneous administration of CRH and AVP in human (Debold *et al.* 1984), rat (Rivier & Vale 1983) and sheep (McFarlane *et al.* 1995) significantly increased the amount of ACTH release to a level that is much higher than that in response to either CRH or AVP alone infusion. Furthermore, blockade of either endogenous AVP or CRH by chronic immunoneutralization markedly reduced the

ACTH response to infusion of the other one (Guillaume *et al.* 1992a; 1992b; Rivier & Vale 1983). Together these studies confirm a synergistic effect of CRH and AVP on ACTH release.

1.1.2 Control of ACTH release by glucocorticoid inhibition

In contrast to CRH and AVP, excessive levels of glucocorticoids inhibit the activation of the HPA axis by feedback loops (Keller-wood & Dallman 1984).The inhibitory effect of glucocorticoids on ACTH secretion at the pituitary level has been well established both *in vivo* and *in vitro*. Reduction in circulating steroids, such as following adrenalectomy, are usually associated with an increased ACTH secretion, which can be reversed by glucocorticoid replacement (Buckingham *et al.* 1997). For example, lowering plasma cortisol levels by metyrapone treatment increased the pituitary venous concentrations of CRH, AVP and ACTH in the horse (Alexander *et al.* 1993), whereas the ACTH response to exogenous CRH and/or AVP is diminished by cortisol replacement in adrenalectomized sheep (McFarlane & Coghlan 1995). Increasing steroid levels by infusion of cortisol and corticosterone significantly suppressed the ACTH response to insulin injection in the dog (Keller-Wood & Bell 1988). Consistent with this, addition of cortisol or corticosterone to the medium markedly reduced the ACTH response of perifused horse and sheep anterior pituitary cells (Evans *et al.* 1993b; Kemppainen & Clark 1995), and rat pituitary segments (Shipston & Antoni 1991), to AVP or CRH pulses. The effects of glucocorticoids are dose-dependent (Evans *et al.* 1993b; Clark & Kemppainen 1994). Similar results have been observed using static culture of isolated pituitary cells (Abou-Samra *et al.* 1986; Bilezikjian & Vale 1987). Taken together, it is evident that glucocorticoids play an important physiological role in regulating ACTH release from the pituitary gland.

1.1.3 Control of ACTH release by interaction between CRH, AVP and glucocorticoids

ACTH secretion is regulated not only by stimulatory factors such as CRH and AVP, and inhibitory factors such as glucocorticoid feedback, but also by a complex

interaction between these factors (Evans *et al.* 1996). Several lines of *in vitro* evidence indicate that glucocorticoid inhibition of CRH- or AVP-stimulated ACTH response can be overcome by the combined action of CRH and AVP (Abou-Samra *et al.* 1986; Shipston & Antoni 1992a; Evans *et al.* 1996; Livesey *et al.* 2000; Lim *et al.* 2002). For example, exposure of horse anterior pituitary cells to continuous cortisol (100 nM) significantly suppressed the ACTH response to successive AVP pulses. This inhibitory effect of cortisol was prevented by the presence of CRH perifusion (0.02 nM), suggesting a role of CRH in protecting AVP-stimulated ACTH secretion against steroid feedback (Evans *et al.* 1996). In adrenalectomized sheep, suppression of the ACTH response to CRH infusion by cortisol replacement is blocked by combined CRH and AVP administration (McFarlane & Coghlan 1995). The above data indicate the complex regulation of ACTH secretion under physiological conditions, where all three hormones are likely to be present.

1.2 Molecular Mechanisms of Regulation of ACTH Secretion

1.2.1 Actions of AVP and CRH in stimulating ACTH release

The primary targets for AVP and CRH in the anterior pituitary are the corticotrope cells, where they initiate activation of different intracellular signalling pathways through binding to distinct receptors. The action of CRH in the pituitary corticotrope is mediated through binding to the type 1 CRH receptor (CRH-R1), which is a member of the G-protein coupled receptor (GPCR) family (Aguilera *et al.* 2004). Upon ligand binding, CRH-R1s are coupled to the Gs heterotrimeric G-protein, which in turn, activates adenylyl cyclase. Subsequently, cyclic AMP (cAMP) is produced as a second messenger, thereby stimulating protein kinase A (PKA), resulting in influx of extracellular Ca^{2+} via voltage-sensitive L- (L-VSCC) and T-type channels (reviewed in Antoni 1993; Mason *et al.* 2002).

AVP binds to the V1b receptor, another member of the GPCR family. V1b receptors associate with the $Gq/_{11}$ protein, which subsequently, stimulates the activity of

phospholiphase C (PLC) (Birnbaumer 2000). Activated PLC promotes hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), generating two intracellular second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Nishizuka 1984; Berridge & Irvine 1984; Bilezikjian & Vale 1987). Although both of these intracellular messengers stimulate ACTH secretion by increasing the concentration of intracellular free Ca^{2+}, the underlying mechanisms are different. IP_3 mediates the release of Ca^{2+} from intracellular stores (Berridge & Irvine 1984), whereas DAG increases cytosolic Ca^{2+} through activation of protein kinase C (PKC) leading to influx of extracellular Ca^{2+} (Nishizuka 1984; Liu *et al.* 1990). This dual mechanism results in the two phases of ACTH release upon exposure to AVP: a rapid 'spike' phase dependent on release of Ca^{2+} from intracellular stores, followed by a sustained 'plateau' phase, which requires Ca^{2+} influx mediated by activation of PKC (Oki *et al.* 1991).

It has been suggested that AVP- and CRH-triggered signalling pathways interact at multiple sites to potentiate the effect on ACTH secretion (Bilezikjian & Vale 1987). Incubation of rat anterior pituitary cells with either 10 nM AVP or 100 nM phorbol 12-myristate 13-acetate in combination with 10 nM CRH significantly increased both the ACTH release and cAMP formation in response to CRH (Abou-Samra *et al.* 1987). A synergistic effect on ACTH release has also been observed with application of 8-Br-cAMP and AVP together in rat pituitary cells (Bilezikjian & Vale 1987). These effects appear to be mediated by PKC-dependent inhibition of cAMP degradation and enhancement of adenylyl cyclase activity (Abou-Samra *et al.* 1987). Regulation of Ca^{2+} is implicated as another interacting site. Depletion of IP_3-sensitive Ca^{2+} stores of rat anterior pituitary cells by thapsigargin pretreatment inhibits the ACTH response to CRH in a dose- and time-dependent manner, indicating that this intracellular Ca^{2+} store is crucial for the sustained activity of adenylyl cyclase (Won & Orth 1995).

1.2.2 Actions of glucocorticoids in regulating ACTH release

The effects of glucocorticoids are mediated through two types of intracellular cytoplasmic receptors, namely mineralocorticoid receptor (MR) and glucocorticoid receptor (GR; Deak *et al.* 1999). Although the affinity of GRs for glucocorticoids is lower than that of MRs, GRs are abundant in the anterior pituitary gland and are predominantly involved in the control of ACTH secretion (Willard *et al.* 1995). The activated receptors are translocated to nucleus, where they initiate specific changes in DNA transcription and therefore in protein synthesis (Buckingham *et al.* 1997).

Inhibition of the ACTH response to CRH and AVP by glucocorticoids falls into three patterns depending on the length of exposure to steroid hormones: fast (within seconds to minutes), intermediate (2 to 10 h) and slow (hours to days) feedback (Keller-Wood & Dallman 1984). These are mediated by different mechanisms. Fast feedback inhibits ACTH secretion very rapidly without requiring new protein synthesis, whereas intermediate feedback requires mRNA and protein synthesis (reviewed in Keller-Wood & Dallman 1984). Both fast and intermediate feedback inhibit ACTH release rather than synthesis, while the slow feedback appears to affect both ACTH release and synthesis.

Most *in vitro* experiments looking at glucocorticoid inhibition are conducted in isolated pituitary tissues, and usually within the timeframe of intermediate steroid feedback. Multiple sites for the action of glucocorticoids have been indicated from those studies. One is modulation of extracellular Ca^{2+} influx via L-VSCC by glucocorticoids. Dexamethasone (DEX) pretreatment of perifused rat anterior pituitary cells for 2 h significantly reduced the ACTH response to CRH and the sustained plateau phase of the response to AVP, but had no effect on the spike phase of the response to AVP (Oki *et al.* 1991). It has been suggested that calcium-activated potassium (BK) channels are a primary target for glucocorticoid action in AtT20 cells (Shipston *et al.* 1996). Pretreatment with 1 μM DEX for 2 h prevented the inhibition

of the outward steady-state potassium current by cAMP analogue or CRH. This was accompanied by a significant reduction in the ACTH response to CRH. In the absence of PKA, no effect of DEX on mean current density was observed, indicating that DEX pretreatment blocked PKA-mediated inhibition of BK channels (Shipston *et al.* 1996). Additional mechanisms of glucocorticoid action include stabilization of actin filament, which may interfere with ACTH exocytosis (Castellino *et al.* 1992), and an action on paracrine factors (Jia *et al.* 1992).

The inhibitory effect of glucocorticoids can be overcome by the combined action of CRH and AVP, as described in Section 1.1.3. Mechanisms underlying this are unclear. It has been suggested that the ACTH response to AVP is less sensitive to glucocorticoid feedback than the response to CRH (Abou-Samra *et al.* 1986; Bilezikjian & Vale 1987; Evans *et al.* 1993b). This is probably due to lack of glucocorticoid inhibition on the ACTH response to AVP-stimulated IP_3 signalling pathway (Oki *et al.* 1991). DEX treatment of normal rats for 7 days even resulted in significant increase in AVP-stimulated IP_3 formation (Rabadan-Diehl & Aguilera 1998).

CRH and AVP have also been shown to activate the *c-fos* gene in mouse AtT20 cells and in rat aortic smooth muscle cells, respectively, which interferes with glucocorticoid-induced gene transcription (Nambi *et al.* 1989; Autelitano & Sheppard 1993). Furthermore, both CRH and AVP are capable of regulating GRs in rat hippocampus and anterior pituitary (Hügin-Flores *et al.* 2003). All of these mechanisms may contribute to the regulation of ACTH secretion when CRH, AVP, and glucocorticoids are present.

1.3 Desensitization of the ACTH Response to AVP

The ACTH response to stimuli is influenced by other factors in addition to those already mentioned. For example, prolonged or repeated stimulus leads to attenuated

responsiveness, i.e. adaptation of the system. This can also be seen *in vitro*. Exposure of isolated anterior pituitary cells to repeated or prolonged CRH or AVP stimulation results in gradual loss of ACTH responsiveness, a process termed as desensitization. Under physiological conditions, this mechanism acts as a negative feedback signal to prevent the HPA axis from overstimulation.

1.3.1 Desensitization of G-protein coupled receptors

The principal function of GPCRs is to transmit extracellular information to the interior of cells by interacting with heterotrimeric G-proteins (Böhm *et al.* 1997; Ferguson & Caron 1998). In intact cells or tissues, exposure of GPCRs to their agonists usually results in a rapid loss of responsiveness, or desensitization (Lohse *et al.* 1990). GPCR desensitization plays an important physiological role by acting as a feedback signal limiting both acute and chronic overstimulation of GPCR signal transduction pathways, but it also limits the therapeutic usefulness of many pharmacological treatments (Ferguson & Caron 1998).

Many studies of GPCR desensitization have been conducted with the β_2-adrenergic receptor (β_2AR). Although most mechanisms elucidated from studies with this receptor are representative, there are many subtle differences in the regulation of different GPCRs (Mason *et al.* 2002). The major mechanisms of GPCR desensitization include receptor uncoupling from its associated G-protein due to receptor phosphorylation, receptor internalization, and loss of cellular receptors due to degradation or reduced receptor synthesis (Ferguson 2001). Desensitization through the first mechanism is usually rapid, occurring within seconds to minutes of the onset of the stimulus. Two classes of protein kinases have been shown to be involved in phosphorylation of GPCRs, namely second messenger-dependent protein kinases (e.g. PKA and PKC) and G-protein-coupled receptor kinases (GRKs) (Ferguson 2001). Whereas the former class of kinases phosphorylates both agonis*t*-occupied receptors and free receptors (receptors that are not bound with agonist), GRKs selectively

phosphorylate agonis*t*-activated receptors (Böhm *et al.* 1997; Ferguson 2001). Consequently, two types of desensitization can be distinguished based on the above mechanisms, homologous and heterologous desensitization. Homologous desensitization, which is mediated by phosphorylation of agonis*t*-bound receptors, does not affect the cellular responses to other stimuli. On the other hand, heterologous desensitization results from phosphorylation of both free and agonis*t*-occupied receptors. It can therefore cause desensitization of more than one type of receptor (Lohse *et al.* 1990; Mason *et al.* 2002). In addition, GRK-mediated desensitization occurs much faster (e.g. $t_{½}$<15 s) than that induced by second messenger-dependent kinases such as PKA ($t_{½}$=3.5 min), suggesting that GRK-mediated desensitization may be most important in the regulation of receptors in rapidly changing situations such as the synaptic cleft **(**Roth *et al.* 1991).

GPCR internalization from the cell surface to an intracellular compartment was initially considered as a primary factor contributing to receptor desensitization because it sequestrated GPCR from its effectors (Sibley & Lefkowitz 1985). This was subsequently reconsidered as the desensitization process is usually more rapid than receptor endocytosis, and pharmacological blockade of β_2AR internalization did not affect the receptor desensitization profile (Ferguson 2001). It is now recognized that GPCR internalization is often more important for receptor resensitization (cell recovery from desensitization) than for desensitization. This was demonstrated by the studies in which inhibition of endocytosis blocked resensitization of cellular responsiveness to agonists of the β_2AR with no effect on its desensitization (reviewed in Böhm *et al.* 1997). Nevertheless, although internalization is not important for β_2AR, receptor internalization may contribute to desensitization of some receptors by depleting the cell surface receptors (Böhm *et al.* 1997).

Compared with desensitization through receptor uncoupling, receptor down-regulation, a decrease in the total number of receptors due to long-term exposure to agonist (hours to days), results in a slow desensitizing process (Böhm *et al.* 1997). As

described above, down-regulation can be achieved either by a reduction of receptor synthesis, or degradation of existing receptors (Mason *et al.* 2002).

1.3.2 Desensitization of the V1b receptor

Regulation of the V1b receptor appears to play a critical role in most chronic stress paradigms as shown by the good correlation between changes in pituitary V1b receptor levels and pituitary corticotrope responsiveness (Aguilera & Rabadan-Diehl 2000). For example, pituitary V1b receptor levels decreased during chronic osmotic stimulation, which is associated with reduced ACTH responsiveness; in contrast to this, stressors that results in hyper-responsiveness to a novel stress have shown an increased V1b receptor level (Volpi *et al.* 2004). Down-regulation of V1b receptors can also be caused by continuous AVP administration or by adrenalectomy. Chronic injection of AVP into normal rats reduces pituitary AVP receptor levels by ~80%, which is followed by decreased ACTH release (Koch & Lutz-Bucher 1985). The pituitary AVP binding was also decreased in adrenalectomized rats (Antoni *et al.* 1985), which may be due to increased exposure of the pituitary to AVP following adrenalectomy (Aguilera *et al.* 2004).

The reduction in ACTH response to repeated or prolonged stimuli, i.e. desensitization, can also be seen *in vitro* using either anterior pituitary cells or segments from different species. Exposure of rat anterior pituitary segments to 100 nM AVP for 4 h reduced the ACTH response to subsequent stimulation with either 10 or 1000 nM AVP by ~50% and ~80% respectively, whereas the response to CRH was not affected (Holmes *et al.* 1984). Similar results have been reported by Murakami *et al.* (1984) and by Castro (1993) using rat and mouse anterior pituitary cells, respectively. Both groups found that pre-treatment of cells with AVP at concentrations as low as 1 nM was capable of eliciting a significant reduction in response to subsequent AVP application. AVP-induced desensitization also occurs in perifused ovine anterior pituitary cells. Repetitive 10-min AVP (100-2000 nM) pulses at 60 min intervals resulted in a

gradual loss of cellular ACTH responsiveness with time, which was defined as homologous desensitization to AVP as it did not reduce the response to CRH (Evans *et al.* 1988). In a more recent study (Hassan *et al.* 2003), a rapid AVP desensitization process was observed in perifused ovine anterior pituitary cells. In this study, both the concentration of AVP (0-20 nM) and the durations (0-25 min) used for pre-treatment were closely matched with endogenous AVP pulses in the sheep. It was found that pre-treatment with AVP for 25 min reduced the response to the subsequent 5-min AVP (100 nM) pulse in a dose-dependent manner, with a threshold concentration at 2 nM (Hassan *et al.* 2003). The authors suggest that the rapid desensitization and recovery of the ACTH response to AVP is more likely to be involved in regulating the ACTH response to AVP during acute stress than chronic stress. A later study indicated that the desensitization process involved V1b receptor internalization and phosphorylation, as blockade of V1b internalization with concanavalin A significantly reduced the extent of desensitization, whereas inhibition of receptor dephosphorylation extended the time for cell recovery (Hassan & Mason 2005). Taken together, these studies suggest that V1b receptor desensitization plays an important role in regulating ACTH release during stress.

The protein kinase(s) responsible for V1b receptor phosphorylation is unknown, but it has been shown that neither PKC nor casein kinase 1α is involved in this process (Hassan & Mason 2005). Since V1b receptor contains one proximal GRK consensus motif at its C terminus (Berrada *et al.* 2000), there is a great opportunity for GRKs being involved in V1b receptor phosphorylation. However, this has not so far been demonstrated.

1.3.3 Influence of CRH and glucocorticoids on desensitization of the ACTH response to AVP

Since interaction between CRH, AVP and glucocorticoids is known to play an important role in regulating ACTH secretion, one might predict that CRH and/or cortisol may have some effect on desensitization of the ACTH response to AVP.

Previously, the effect on AVP-induced ACTH desensitization of continuous perifusion with a low level of CRH (0.01 nM) has been tested in our laboratory (Hassan *et al.* 2003). This experiment was aimed at testing the idea that CRH acts to set the gain of corticotrope responsiveness to fluctuations in AVP. Although, at this concentration, CRH alone did not stimulate ACTH secretion, it enhanced the ACTH response to pulses of AVP, but it had no effect on desensitization. However, CRH at concentrations higher than 0.01 nM was not tested in this previously study.

Other researchers have also observed possible effects of hormonal interaction on the AVP desensitization process. Using perifused sheep anterior pituitary cells, Evans and coworkers (1988) have shown that the decrease in response to repetitive AVP or CRH pulses (defined as desensitization of the ACTH response to AVP or CRH) was enhanced by the presence of AVP and CRH in combination. In perifused horse anterior pituitary cells, the decrease in response to successive AVP pulses (determined by the ratio between the responses to the sixth pulse and to the first pulse) is not affected by the presence of cortisol perifusion (100 nM), whereas it is greater when both CRH (0.02 nM) and cortisol are present, and greatest with CRH alone (Evans *et al.* 1996). In addition, the effect of CRH (1-25 pM) alone seems concentration-dependent (Evans *et al.* 1993a). However, in the studies using horse anterior pituitary cells, the authors did not address whether the decrease in response to repeated AVP pulses was due to desensitization or other factors.

Since V1b receptor phosphorylation and internalization have been found to be important in the AVP desensitization process (Hassan & Mason, 2005), the actions of CRH and/or glucocorticoids in regulating these two procedures may influence the extent of AVP-induced ACTH desensitization. No evidence to date has been found for the involvement of PKA in V1b receptor phosphorylation. Instead of phosphorylating V1b receptors, PKA has been shown to phosphorylate GRK2 (Kohout & Lefkowitz 2003). This phosphorylation promotes the activity of GRK2, thereby enhancing GRK2-mediated desensitization of β2AR (Kohout & Lefkowitz 2003). It is possible

that CRH influences GRK activity through activation of PKA, which in turn, influences V1b receptor phosphorylation and thus desensitization.

Calcium sensor proteins (CSPs), such as recoverin and calmodulin (CaM), are able to alter the activities of GRKs in different GPCRs (Pronin *et al.* 1997; Sallese *et al.* 2000). DEX treatment of mouse AtT20 cells for 90 min significantly enhanced both CaM mRNA levels (10-fold) and protein expression (2-fold), while simultaneous application of CRH with DEX blocked the induction of CaM mRNA by DEX (Shipston & Antoni 1992b; Shipston 1995). This suggests the possibility that both CRH and cortisol affect the AVP desensitization process via CSPs, particularly CaM, acting on GRK activity.

It has been shown that CRH (21 nM) significantly increases the rate of V1b receptor internalization within 30 min in rat anterior pituitary cells (Mogenson *et al.* 1988). However, in the same study, AVP binding to V1b receptors is not affected. It is not sure whether this effect of CRH will contribute to desensitization of the ACTH response to AVP.

Recently, actin polymerization has been reported to play a role in clathrin-mediated endocytosis (Kaksonen *et al.* 2006), which is a major pathway for GRK-induced GPCR internalization (Ferguson 2001). However, there are conflicting results about the effect of actin filaments on receptor-mediated endocytosis. Whereas some studies have shown that stabilization of actin filament is crucial for receptor endocytosis (Lamaze *et al.* 1996), other observations suggest the opposite effect in different cell types (Lamaze *et al.* 1997). It has been reported that glucocorticoids can stabilize actin filaments by preventing drug-induced disruption or by thickening actin bundles in different cells including AtT20 cells (Castellino *et al.* 1992). Taken together, these suggest that glucocorticoids may influence the V1b receptor endocytosis by acting on actin filaments.

1.3.4 Aims of this study

Since desensitization of the ACTH response to AVP may have an important physiological role in ACTH regulation (Hassan *et al.* 2003), it is important to investigate whether factors that influence ACTH secretion directly may also have some impact on this process, as this could contribute significantly to the overall regulation of ACTH release.

The overall aim of this project was to investigate the effects of hormonal interaction on desensitization of the ACTH response to AVP in ovine anterior pituitary cells. Specifically, the first objective of this study was to examine whether CRH at a higher concentration than previously tested (but still in the physiological range) affects AVP-induced ACTH desensitization. The second objective was to investigate the influence of cortisol, a major inhibitory factor for ACTH release, on desensitization of the ACTH response to AVP. The third part of this study was aimed at investigating the effect of combined CRH and cortisol on AVP-induced ACTH desensitization using ovine anterior pituitary cells.

2

MATERIALS AND METHODS

2.1 Materials, Solutions and Media

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

2.2 Preparation of Anterior Pituitary Cells

2.2.1 Collection of sheep pituitaries

Pituitaries were collected from sexually mature ewes shortly after they were slaughtered. Whole heads were collected from either PPCS Ltd. (Belfast, Christchurch) or the Malvern Abattoir Ltd. (Malvern, Canterbury) and put on ice for transporting back to the School of Biological Sciences, University of Canterbury. The heads were then cut posterior-anteriorally with a band saw, and the pituitary gland was exposed and dissected out using sterile forceps and scalpel. After removal from the head the pituitary glands were placed into chilled, sterile dispersing buffer (DB) and transferred to the laboratory.

2.2.2 Cell dispersion

Cell preparation was carried out under aseptic condition. Pituitary cells were prepared in a laminar flow hood (CF43S, Gelman Sciences, Australia). All instruments and glassware used for cell preparation had previously been either autoclaved (15 min, 121ºC, 15 psi), or heat sterilized in an oven (≥170ºC, 2 h). Similarly, all glassware and solutions used were sterile.

The pituitary glands were put into a petri dish, and usually 7 intact one were selected. The pituitaries were then washed briefly by submerging three times in fresh DB. After washing, the pituitaries were put into DB on ice. The connective tissue, the median eminence, pituitary stalk and posterior pituitary were removed using scissors and forceps. The anterior pituitaries were then placed into a 50 ml centrifuge tube containing 20 ml DB that had been put on ice. Following a rinse with 45 ml DB, the anterior pituitaries were minced into small pieces (less than 2 mm^3) with scissors. These pieces were transferred to a trypsinizing flask along with 50 ml of collagenase solution (Type Π collagenase; 450 U/ml). The flask was then placed in a 37ºC incubator and the tissue suspension was gently stirred on a magnetic stirrer. Usually three digestions were done (the first was 20 min long, and the subsequent two were 1 h each). Cells were collected as described below following each digestion. Firstly, the digestion flask was taken out of the incubator, and the supernatant containing the dispersed cells was decanted into a 50 ml plastic centrifuge tube. Then the supernatant was centrifuged at 200 × g (CR-412, Jouan, France) for 5 min at 4ºC. After centrifugation, the cell-free supernatant was returned to the digestion flask for the subsequent digestion. The cell pellet was washed once by resuspending the cells in 45 ml DB using a 10 ml pipette, and then centrifuged as before. The supernatant was aspirated, and the cell pellet was resuspended in 10 ml DB, and placed on ice. Depending on the size of the pellets obtained from the three digestions, a fourth digestion (~30 min), was occasionally done to increase the number of cells.

After all digestions were completed, all of the cells obtained were pooled and washed (as above) four times. After the third wash the cells were resuspended and filtered through sterile gauze to remove tiny pieces of connective tissue. Following the final centrifugation the cells were resuspended in 25 ml of Dulbecco's modified Eagle's medium (DME; see Appendix B) containing 10% newborn calf serum (DME/NCS), and placed on ice.

2.2.3 Cell viability determination

Cell viability was determined by the trypan blue exclusion method. A small volume (100-200 µl) of cell suspension was used to do a cell count using a haemocytometer. Typically, the viability varied between 75-90 %, and the average was 82.2%.

2.2.4 Overnight culture

For perifusion experiments, 20 ml of 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 petri dishes containing 19 ml of DME/NCS. Fifteen of these dishes were used for the perifusion experiment (one per perifusion column), and one was used for cell viability determination on the day of the perifusion. All dishes were then incubated overnight (~18 h) at 37 ºC in a 95% air:5% CO_2 incubator.

2.3 Perifusion Experiments

2.3.1 The multi-column perifusion system

Experiments were performed using a multi-column perifusion system, as shown diagrammatically in Figure 2.3.1. Cells were put into cell chambers, or columns, and solutions were pumped over the cells using a peristaltic pump. Up to 15 columns can be perifused simultaneously. Each of the columns has an internal volume of approximately 600 µl and can be maintained at a temperature of 37ºC with a water jacket during experiments. Using a solenoid switching system the solutions pumped over the cells can be rapidly and precisely changed from 'basal' solutions (containing no hormone) to 'test' solutions (containing hormone). This enables the solutions in all columns to be changed simultaneously. The effluent from columns was collected via a Gilson FC 204 fraction collector (Gilson, France).

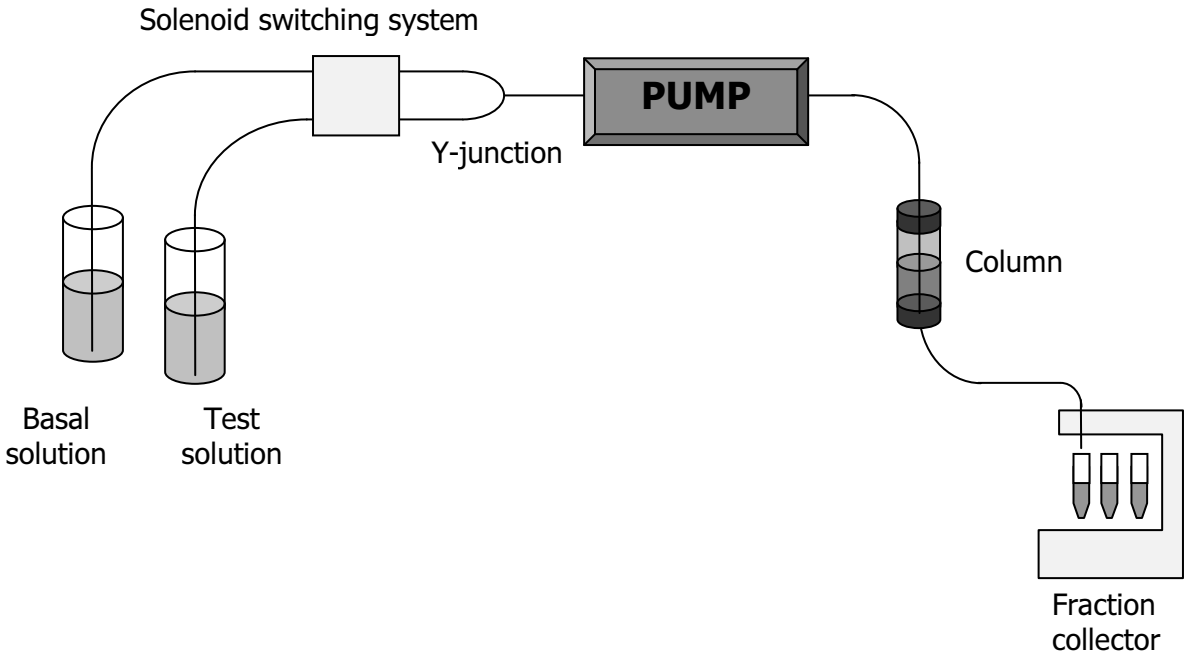

Figure 2.3.1 Diagram showing one column from the multi-column perifusion system. Note that there are 15 columns arranged in parallel in the actual system

2.3.2 Experimental procedure

On the day of a perifusion experiment, the cells from each plate, that had been cultured overnight, were transferred along with the DME/NCS to separate 50 ml centrifuge tubes (one plate to one tube) and centrifuged at 275 x g (CR-412, Jouan, France) for 5 min at 4ºC. After the supernatant was aspirated, the cells were resuspended in 170 µl of a slurry of Sephadex G-25 (fine). The tubes were placed on ice prior to loading the cells into the perifusion columns. Before loading the cells, all lines were flushed with Krebs Ringer (KR) containing 0.05% alkali-treated casein (ATC) and 0.005% L-ascorbate (KR/ATC). The test lines leading to the Y-junctions were primed with test solutions. Slurries of Biogel P-2 and Sephadex G-25 (80 µl of each) were added sequentially to each of the columns to form a bead bed on which the cells would be supported. The bead bed was prevented from draining out by a 10 µm mesh Nybolt nylon gauze. The cells, suspended in the slurry of Sephadex, were transferred onto this bead bed using a Pasteur pipette. All columns were then filled with KR/ATC and sealed with a rubber bung attached to the inflow tube.

Once cell loading had been finished, the pump was switched on to start the experiment (0 min). During an experiment, cells were perifused with either a 'basal' solution (e.g. KR/ATC) or a 'test' solution of AVP dissolved in KR/ATC. Changes between 'basal' and 'test' were done using the solenoid switch system. When cells were perifused with KR/ATC plus two or more other treatment solutions, a manual change was required for transfer between KR/ATC and the second solution. The solutions were put into separate tubes (normally 50 ml centrifuge tubes) and placed in a 37ºC water bath. These solutions were pumped through the perifusion system tubing at a flow rate of ~0.156 ml/min. The effluent from the columns was collected as 5 or 10 min fractions. These samples were stored at -20ºC, and assayed for ACTH content by radioimmunoassay (RIA; usually within one week of sample collection).

2.3.3 Design of protocols

The experimental protocols used in this study were based on a protocol used by Hassan *et al.* (2003) to characterize desensitization of the ACTH response to AVP. During the experimental procedure cells were repeatedly stimulated three times at 80 min intervals with 5-min AVP pulses (100 nM). Desensitization of the ACTH response to AVP was induced by pretreating the cells for 15 min with 5 nM AVP (excepted where indicated otherwise) immediately preceding the second AVP pulse. Cells were perifused with either KR/ATC or KR/ATC containing cortisol for 80 min at the beginning of the experiment, which allowed them to recover from loading. During this time ACTH secretion fell to a constant, low (basal) level. CRH (0.2 nM) was added to the perifusion solution from 80 min onwards, when necessary. AVP pulses were given at 120, 200, and 280min. The duration of each experiment was 320 min.

There were 4-5 different treatments within one experiment. Generally, each treatment was assigned to 2-3 columns per experiment and repeated in at least three experiments. The details of different concentrations and specific treatment regimes used in individual experiments can be found in the following 'Results' chapter.

2.4 ACTH Radioimmunoassay

2.4.1 Assay reagents

Solutions

For the details of all solutions used in the RIAs, see Appendix B

Tracer

^{125}I-labelled ovine ACTH (^{125}I-oACTH) was used as the tracer for the RIAs in this project. Tracer was prepared by Dr. Drusilla Mason using a chloramine T radioiodination method, and was carried out in the dedicated facilities of the Endolab,

Department of Endocrinology, Christchurch Hospital. In brief, the iodination involved incubation of oACTH (a kind gift from Dr C. H. Li; see Appendix A) with ^{125}I for 1 min in the presence of chloramine T, stopping the iodination reaction with sodium metabisulphite, and separating the labelled ACTH from the reaction mixture by high performance liquid chromatography.

The tracer was aliquoted into appropriate volumes and stored at -20ºC for up to thirteen weeks before use. On the day of use, the tracer was diluted in a 0.05 M phosphate buffer containing 0.1% ATC (P/ATC) to give 10,000 cpm/100µl.

ACTH standards

The standards used in this study were prepared by Dr Drusilla Mason. One ml of an ovine ACTH stock solution (500 µg/L) was diluted in P/ATC to give a concentration of 10 µg/L. This solution was further diluted in different volumes of P/ATC to give a series of ACTH solutions of 10, 5, 3, 2, 1.5, 1, 0.5 and 0.25 µg/L. These standards were then aliquoted into 300 or 600 µl aliquots and stored at -20ºC (or -80 ºC for long-term storage). P/ATC was used as the zero standard.

ACTH antiserum

Antiserum (AS) was raised in rabbits by injection with two porcine ACTH preparations (ACTH with carboxymethylcellulose and ACTH zinc hydroxide). Two batches of AS were used in this study. The initial batch used, Athol Pool 1 (containing Athol Bleeds 4, 5, 9, 11, 12, 16; 1975), was provided by Professor Richard Donald (Department of Endocrinology, Christchurch Hospital). This was used for assays 1 - 43 and was diluted 1:1400 in P/ATC. The second batch was Athol Bleed 7 (1975), which was provided by Dr Margaret Evans (Department of Endocrinology, Christchurch Hospital). This was used in assays 44 - 55 at 1:1000 dilution. All diluted AS were stored at -20ºC.

ACTH replicates

Replicate samples used in this study were prepared by Ali Hassan (12/1997). Replicates at three concentrations (high, ~3.0 µg/L; medium, ~1.3 µg/L; low, ~0.4 µg/L) were included at both the beginning and the end of each assay, and were used to indicate the quality of assay. The major steps involved in the preparation of replicates were as follows. Surplus cells from a cell preparation were incubated overnight. On the following day the cells were exposed to high concentrations of AVP and CRH for 2 h. After centrifugation, the supernatant was collected and the cell pellet was resuspended in KR/ATC, vortexed, and frozen, and thawed to rupture the cells, releasing ACTH. The resulting suspension was centrifuged and the supernatant was mixed with the supernatant from the first centrifugation. These replicates were stored as 1 ml aliquots at -20ºC.

A total of 55 assays were done in this study. The intra-assay coefficients of variation were 8.99, 6.12 and 6.17% for the low, medium and high replicates, respectively. The inter-assay coefficients of variation were 10.30, 9.68 and 7.85% for low, medium and high replicates, respectively.

2.4.2 Assay set up

All assays were set up in duplicate in an ice/water bath. From assays 1 to 43, P/ATC buffer, ACTH sample (including standards, replicates and experimental samples), ^{125}I-oACTH tracer and ACTH antiserum (Athol Pool 1) were added sequentially on the same day to the appropriate assay tubes (Table 2.1). Non-specific binding tubes were included for each set of standards, replicates and samples. The assay tubes were then incubated at 4ºC for ~ 24 h prior to separation.

Tube Type	Buffer	Standard Sample Replicate	Tracer	Antiserum
NSB (zero std)	150	100	100	0
Zero std	50	100	100	100
Other stds	50	100	100	100
NSB (assay zero)	250	0	100	0
Zero (assay)	150	0	100	100
NSB	150	100	100	0
Replicates	50	100	100	100
NSB	150	100	100	0
Set of samples	50	100	100	100
NSB	150	100	100	0
Replicates	50	100	100	100
NSB (assay zero)	250	0	100	0
Zero (assay)	150	0	100	100

Table 2.1 Protocol for the RIA set up. All volumes are in microlitres. After addition of all reagents the tubes were mixed briefly using a rack vortex mixer, and then incubated at 4ºC for 20 - 24 h.

From assay 44 to 55, Athol Bleed 7 AS was used to replace Athol Pool 1 AS. The assay procedure was similar to the above protocol except that the tracer was added after the other three reagents had been incubated together for ~ 24 h at 4ºC (referred to as a "pre-incubation"). Following addition of the tracer, the tubes were mixed again and a "post-incubation" was carried out for ~ 48 h at 4ºC.

2.4.3 Assay separation

The antibody-bound ^{125}I-oACTH was separated from the free labelled hormone by fractional precipitation at room temperature. The basic procedure was as follows. At the end of the incubation with tracer, 1.2% bovine γ-globulin (50 µl) was added immediately to each tube. After the racks were shaken briefly by hand, 18% polyethylene glycol solution (1.5 ml/tube) was added. Following vigorous vortex mixing the tubes were incubated at room temperature for 15 minutes. The tubes were then centrifuged at 4,010 x g, for 15 min at 4ºC (Heraeus Varifuge 3.0R) to precipitate the antiserum-bound ACTH. The supernatant was aspirated, and the radioactivity (counts/2 min) in each tube was measured using a gamma scintillation counter (1275 Minigamma counter, LKB Wallac). The data obtained were saved as a computer file for later analysis.

2.5 Data Analysis

2.5.1 RIA output

The count file obtained for each RIA was analyzed using the MRIAC RIA program (Livesey 1974). This program fits the raw data of the standards into a standard curve. This standard curve is then used to calculate the ACTH concentration ([ACTH]) in each sample assayed. The value shown in the output is given as the mean of the duplicates. A confidence limit of 95% is given for each duplicate.

2.5.2 Calculation of ACTH secretion

ACTH content in each fraction was calculated using the equation:

ACTH content (ng) = [ACTH] in fraction x fraction duration x flow rate
(ng/ml) (min) (ml/min)

The values for the ACTH concentrations of the fractions were obtained from the RIA output. Thus, total ACTH secretion during a certain period could be calculated as a sum of the ACTH contents in all fractions collected during that period.

In terms of the above, ACTH secretion stimulated by each pre-treatment or test pulse was calculated as below:

Stimulated ACTH secretion = Total ACTH secretion – Basal ACTH secretion

For 100 nM AVP or 100 mM KCl test pulse, total ACTH secretion was calculated for a 20 min period from the onset of the pulse; while for the AVP pre-treatment, total ACTH secretion was calculated for a 15 min duration (covering the whole pre-treatment period).

Basal ACTH secretion was calculated using the mean [ACTH] from the three fractions immediately before a test pulse or a pre-treatment (when there was a pre-treatment preceding the second test pulse). It is assumed that these values remain constant and equal to the basal secretion during the response.

2.5.3 Assessment of desensitization

In this project, the method for assessing the extent of desensitization was adapted from Hassan *et al.* (2003). They showed that, when the ACTH response to the second 100 nM AVP pulse was compared with the mean of the responses to the first and third pulses (Figure 2.5.1a), there was no significant difference between them. Thus, the mean of the responses to the first and third pulses was assigned as an intra-column control (100%) for the response to the second AVP pulse. The magnitude of desensitization was assessed by expressing the response to the second AVP pulse following AVP pre-treatment as a percentage of this control. There are two

advantages of using this intra-column control. First, it avoids analytical difficulties that can arise due to variations in the absolute values of ACTH release between individual columns. Second, the control protocol can be omitted once the relationship mentioned above has been established, so that there are more columns available to be used for other treatments.

In view of this, a series of protocols similar to those used by Hassan *et al.* (2003) were used in present study to test whether the above relationship still held, and to assess the impact of additional treatments on this relationship (Figure 2.5.1). In the first three experiments in which cells were perifused with KR/ATC before and after three 5 min pulses of 100 nM AVP (Figure 2.5.1a), the response to the second AVP pulse was significantly less than the mean response to the first and third pulses (88.8±2.1%, n=7, $P<0.01$, *t*-test). However, this percentage value increased to 100.7±3.2% (n=9, NS, *t*-test) in the subsequent experiments. As a result, there were two different control values for this treatment. For the first three experiments, 88.8% of the mean response to the first and third pulses was taken as a control with the same column. For each column that received AVP pre-treatment, the response to the second AVP pulse was expressed as a percentage of the mean response to the first and third pulses. This percentage value was then normalized by dividing by 88.8% to show the actual magnitude of desensitization. In the later experiments, the magnitude of desensitization with the same treatment was directly assessed by expressing the response to the pre-treated AVP pulse as a percentage of the mean response to the first and third pulses.

In the presence of continuous perifusion with CRH or cortisol alone (see Figure 2.5.1 b & c), the response to the second AVP pulse was not significantly different from the mean of the responses to the first and third pulses (99.0±4.1% [n=9, NS, *t*-test] and 105.0±2.1% [n=7, NS, *t*-test], respectively). Thus, with these two treatments, the mean of the responses to the first and third AVP pulses was taken as a control to assess the extent of desensitization.

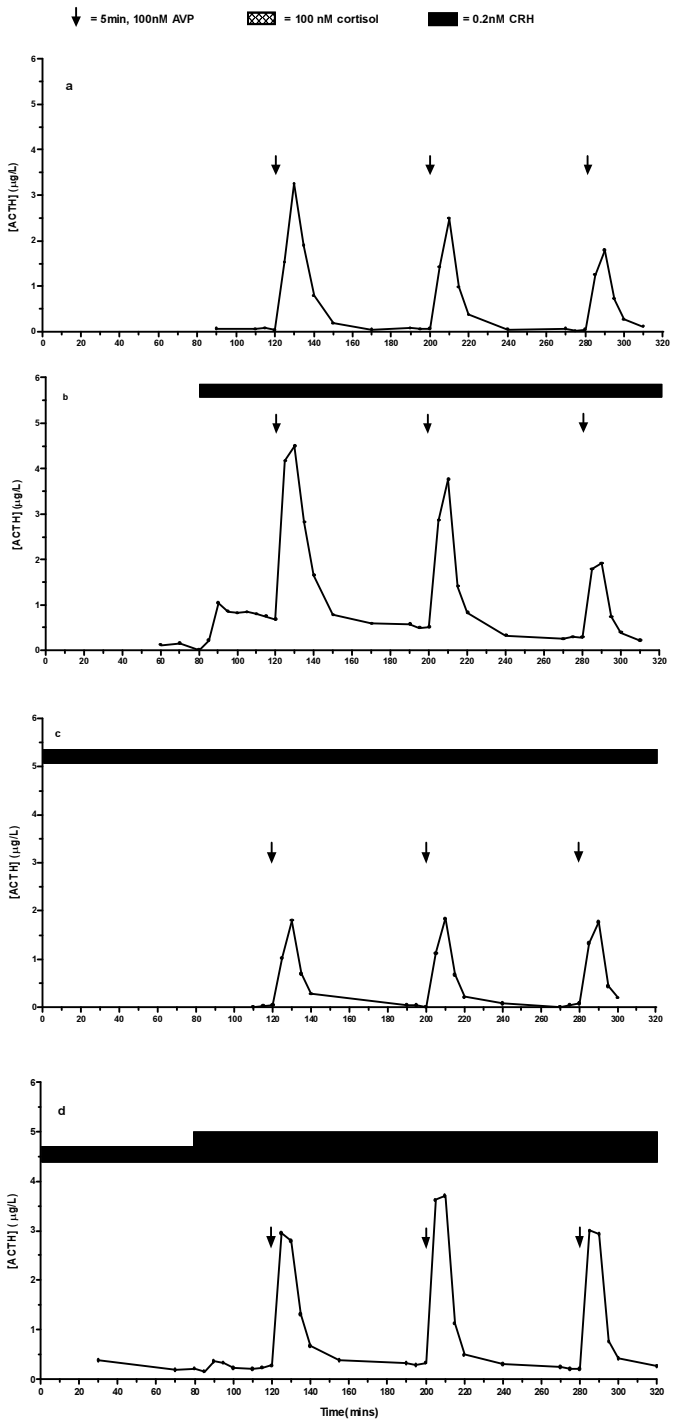

Figure 2.5.1 ACTH response to AVP with different treatments. Representative data are shown from four perifusion columns illustrating the control protocols used in this study. Typically, cells were treated with 100 nM AVP for 5 min at 120, 200, and 280 min, with the perifusion medium containing (a) no hormone, (b) 0.2 nM CRH (from 80 min), (c) 100 nM cortisol (from 0 min), and (d) CRH and cortisol in combination.

When both CRH and cortisol were present (see Figure 2.5.1d), the response to the second AVP pulse was significantly greater than the mean response to the first and third pulses (110.0±2.0%, n=8, $P<0.01$, *t*-test). With this treatment, for each column that received AVP pre-treatment, the response to the second AVP pulse was expressed as a percentage of the mean response to the first and third pulses. This percentage value was then normalized by dividing by 110% to show the extent of desensitization in the presence of CRH and cortisol in combination.

2.5.4 Statistics

Microsoft Excel and GraphPad Prism 4 were used for data analysis. Data were statistically analyzed using Student *t*-test and one-way or two-way ANOVA. Details of the analyses are indicated in the text. $P<0.05$ was considered as significant. All data were reported as mean ± SEM.

3

RESULTS

3.1 Effect of CRH on Desensitization of the ACTH Response to AVP

Both CRH and AVP are important stimulators for ACTH release. In many species such as rats, sheep and horses, they can act alone, or act together to give synergistic effects (Gillies *et al.*1982; Evans *et al.* 1988; Liu *et al.* 1990; Evans *et al.* 1993). In addition to stimulate ACTH secretion, prolonged or repeated exposure of the pituitary gland to CRH or AVP results in attenuated ACTH responsiveness, or desensitization. Recently Hassan *et al.* (2003) have characterised desensitization of the ACTH response to AVP and CRH in perifused ovine anterior pituitary cells, and suggested that desensitization may play an important role in regulating ACTH secretion *in vivo*. In view of the suggestion that CRH may act in a permissive role, setting the overall responsiveness of corticotrope cells, while AVP is the main dynamic ACTH-releasing factor (Antoni 1993; Evans *et al.* 1996), Hassan and coworkers (2003) also investigated whether a constant perifuson with CRH could overcome or enhance AVP-induced ACTH desensitization. Data demonstrated from their studies show that a low level, constant CRH 'background' (0.01 nM) did not have any significant effect on AVP desensitization. However, pituitary portal CRH concentrations can increase dramatically to levels more than an order of magnitude greater than this under certain conditions in conscious sheep. For example, maximal CRH concentrations of ~0.2 nM have been reported for acute haemorrhage (reviewed in Mason *et al.* 2002) and ~0.4 nM for insulin-induced hypoglycaemia (Caraty *et al.* 1990). It was therefore important to investigate whether a CRH concentration in this range (0.2 nM) modifies desensitization of the ACTH response to AVP.

3.1.1 Development and validation of CRH protocols

In earlier experiments investigating the effect of 0.01 nM CRH on AVP desensitization, CRH was included in the perifusion medium at a constant concentration from 80 min onwards (Hassan *et al.* 2003). This treatment was used in conjunction with the protocol described in Section 2.3.3, in which AVP (100 nM) was given as three 5 min pulses at 120, 200 and 280 min, and desensitization was induced by pretreating cells for 15 min with 5 nM AVP immediately before the second AVP pulse. At a concentration of 0.01 nM, CRH on its own did not stimulate ACTH secretion. Therefore basal ACTH secretion in CRH-treated cells was comparable to control cells perifused with KR/ATC alone.

In the present study, a CRH concentration of 0.2 nM was used. From earlier work in the laboratory (Hassan *et al.* 2003) it was clear that this concentration would stimulate ACTH secretion with a peak at ~10-15 min after the onset of CRH stimulation, and that secretion was then likely to drop over the next 5-10 min to a slowly declining "plateau", or fall gradually from the peak. It is necessary to have a relatively constant ACTH secretion immediately prior to each AVP pulse in order to calculate AVP-stimulated ACTH secretion (see Section 2.5.2). Therefore, the timing of the onset of CRH perifusion relative to the first AVP pulse needed to be assessed in initial experiments looking at the effect of CRH on AVP desensitization.

In the first experiment, cells were exposed to CRH from 90 min (i.e. 30 min before the first 100 nM AVP pulse). It was observed that for some columns perifused with CRH, the initial ACTH response to CRH had not reached a "plateau" before the first AVP pulse was given (Figure 3.1.1a). Thus, in subsequent experiments, cells were perifused with CRH from 80 min, as shown in Figure 3.1.1b. As a result, the baseline immediately before the first AVP pulse was relatively steady.

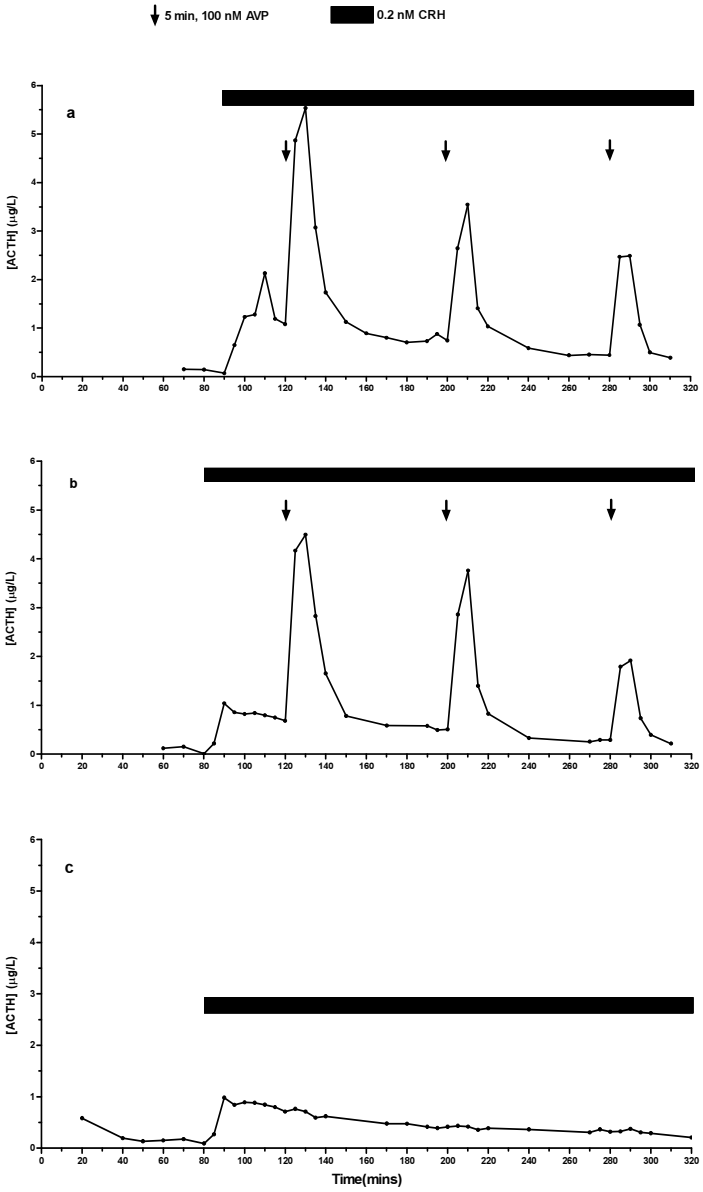

Figure 3.1.1 ACTH secretion in response to continuous CRH exposure. Data from three individual columns illustrating the initial experimental protocols are shown. Cells were treated with 5-min pulses of 100 nM AVP after 120, 200, and 280 min of perifusion, with a 0.2 nM CRH 'background' from (a) 90 min and (b) 80 min. In (c), cells were treated with 0.2 nM CRH alone from 80 min, resulting in variations in basal ACTH secretion.

To assess the impact of prolonged CRH exposure on basal ACTH secretion throughout the experiment, CRH alone was perifused from 80 min onwards (Figure 3.1.1c). It can be seen that after the high secretion during the initial 30-40 min, the ACTH release in response to CRH fell very slowly during the remainder of the perifusion. Despite this, the basal secretion immediately before 120, 200 and 280 min was not dramatically different from the basal secretion immediately after the same time point. Since these three time points are corresponding to the onset of each AVP pulse in Figure 3.1.1b, this indicates that variations in basal secretion in the presence of CRH should have minimal effect on the calculation of ACTH release from each AVP pulse.

3.1.2 Desensitization of the ACTH response to AVP in the presence of CRH

In the absence of CRH, 15 min AVP (5 nM) pre-treatment significantly reduced the response to the second AVP pulse (to 77.6±3.3% of control, n=10, $P<0.001$, *t*-test) as shown in Figure 3.1.2. A significantly greater reduction ($P<0.001$, *t*-test) was seen in the presence of CRH (0.2 nM): to 48.1±4.3% (n=12, $P<0.0001$, *t*-test) of control, i.e. the mean of the responses to the first and third pulses. Data are summarized in Figure 3.1.3.

More ACTH was released in response to 100 nM AVP when cells were continuously exposed to CRH, than in its absence (see Figure 3.1.2). This raises the question as to whether the greater reduction in response to the second AVP pulse seen in the presence of CRH is due to a true desensitization or to depletion of ACTH. This was assessed by replacing the second AVP pulse with a 5-minute KCl pulse (100 mM), with or without AVP pre-treatment in the presence of continuous CRH perifusion (Figure 3.1.4). The mean of the responses to the first and third pulses was taken as a control. Both the responses to a KCl pulse and the second AVP pulse were expressed as a percentage of this control for the same column.

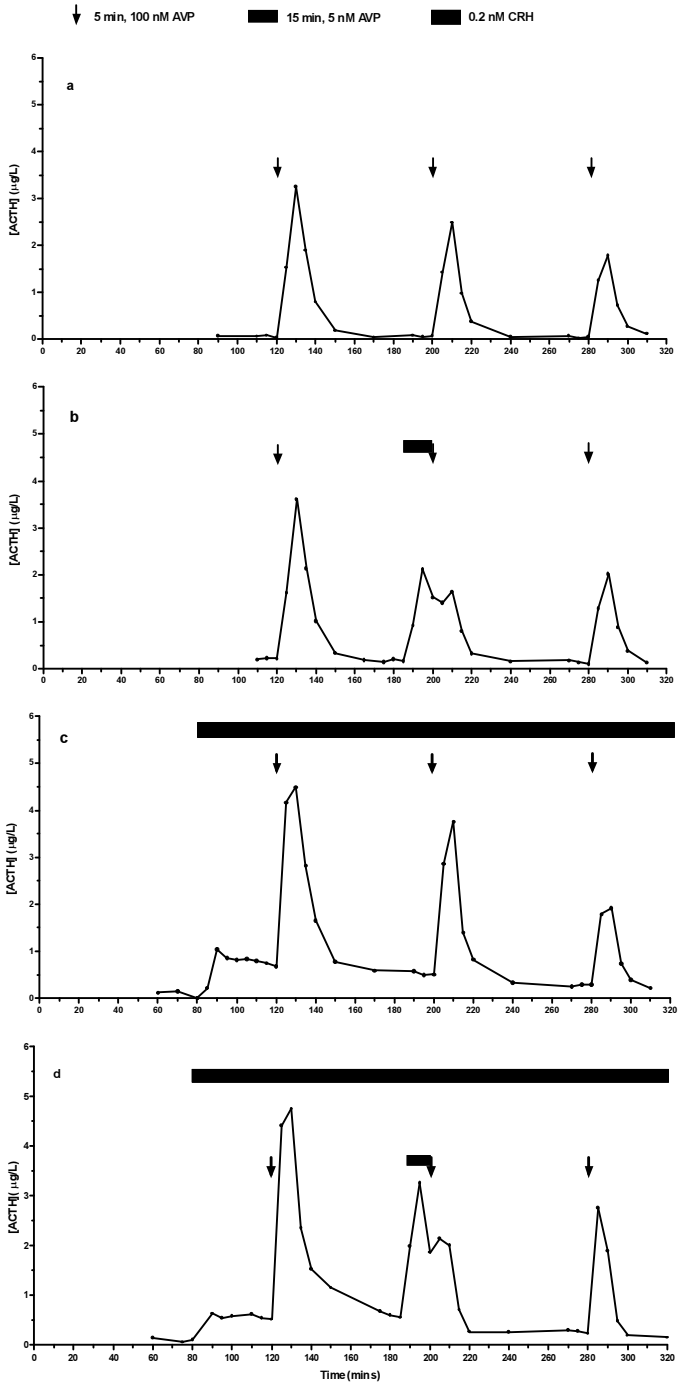

Figure 3.1.2 Desensitization of the ACTH response to AVP in the presence of CRH. Data from four individual columns illustrating the experimental protocols are shown. Cells were treated with 5-min pulses of 100 nM AVP after 120, 200, and 280 min of perifusion in all columns. To induce desensitization the second AVP pulse was preceded by a pre-treatment with 5 nM AVP for 15 min in either (b) the absence or (d) presence of 0.2 nM CRH (from 80 min).

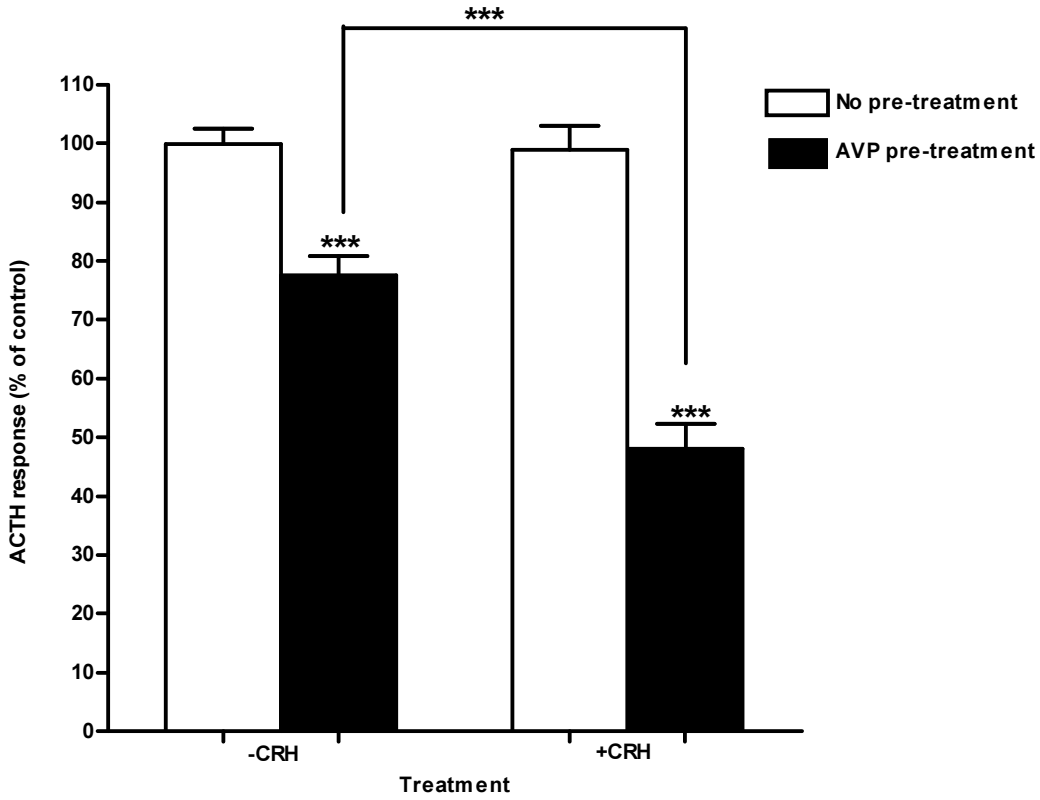

Figure 3.1.3 Effect of CRH on desensitization of the ACTH response to AVP. Data show the ACTH response to the second AVP pulse in either the absence or presence of a pre-treatment with 5 nM AVP for 15 min, with (+CRH) or without (-CRH) a 0.2 nM CRH 'background'. Asterisks indicate a statistically significant difference between the 'No pre-treatment' and 'AVP pre-treatment' groups, and between the two 'AVP pre-treatment' groups. Data are mean ± SEM (*** $P<0.001$, *t*-test, n=7-12 for each group).

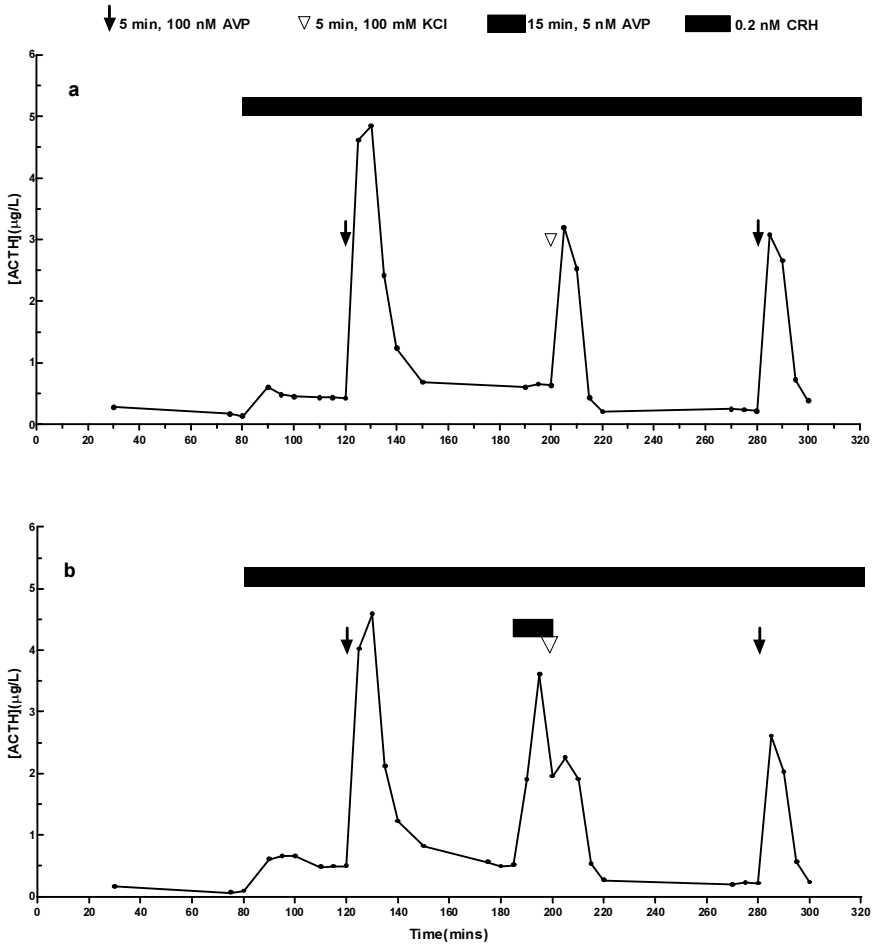

Figure 3.1.4 ACTH response to a KCl pulse in the presence of CRH. Representative data are shown from two perifusion columns illustrating the protocol used in these experiments. In the presence of 0.2 nM CRH, cells were treated with a 100 mM KCl pulse (starting at 200 min) for 5 min in (a) the absence or (b) presence of 5 nM AVP pre-treatment.

Data from two experiments showed that, the response to a KCl pulse following AVP pre-treatment (39.1±2.0% of control, n=10) was significantly less than the response to the KCl pulse that were not pre-treated (63.3±6.1% of control, n=10, $P<0.01$, *t*-test). Since KCl stimulates ACTH release by directly depolarizing the cell membrane rather than by activating a cell surface receptor, desensitization of the ACTH response to AVP should not reduce the ACTH response to a KCl pulse. In view of this, the reduction in response to a pre-treated KCl pulse could be due to depletion of cellular ACTH.

The response to the second AVP pulse in the presence of CRH was reduced from 100% of control, in the absence of AVP pre-treatment , to 37.1±1.7% following pre-treatment (n=9, $P<0.0001$, *t*-test). This reduction (62.9%) is greater than that seen with AVP pre-treatment of the KCl pulse (from 63.3±6.1% to 39.1±2.0%, i.e. 24.2%). This suggests that the reduction in response to AVP following AVP-pre-treatment in the presence of CRH involves both a specific desensitization process and ACTH depletion. Taken together, these results indicate that depletion of cellular ACTH contributes, at least partially, to the greater reduction in response to the second AVP pulse following pre-treatment in the presence of CRH. Results are summarized in Figure 3.1.5.

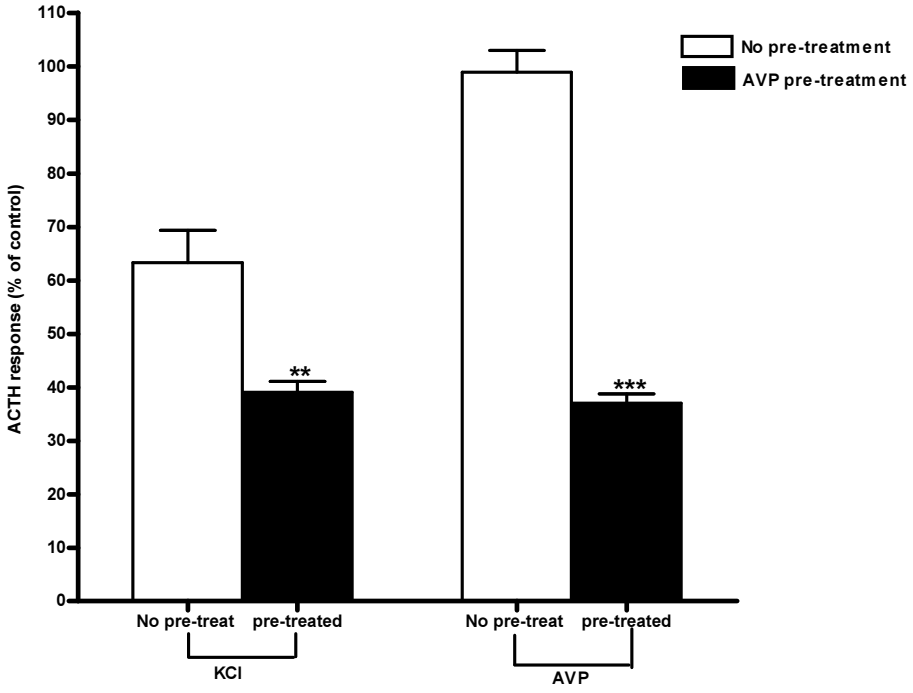

Figure 3.1.5 Effect of AVP pre-treatment on the ACTH responses to KCl and AVP pulses in the presence of CRH. Data show the response to a KCl pulse or the second AVP pulse in either the absence (open bar) or the presence (black bar) of a pre-treatment with 5 nM AVP for 15 min, with a 0.2 nM CRH 'background'. Asterisks indicate a statistically significant difference between the 'No pre-treatment' and 'AVP pre-treatment' groups. Data are mean ± SEM (** $P<0.01$, *** $P<0.001$, *t*-test, n=9-10 for each group).

In view of the ACTH depletion seen with the protocol discussed above, the effect of CRH on AVP desensitization could not be accurately determined from this data. Thus, a slightly modified experimental protocol was tested. The concentration of AVP used for the pre-treatment was reduced from 5 to 0.5 nM, while the rest of the protocol remained the same (Figure 3.1.6). In a previous study, 0.5 nM AVP pre-treatment did not have a significant effect on the response to a subsequent AVP pulse (Hassan *et al.* 2003). Results obtained in the current study were consistent with this: In the absence of CRH, following 0.5 nM AVP pre-treatment, the response to the second pulse was 99.1±3.7% of control (Figure 3.1.6a, n=4, NS, *t*-test). In contrast to this, in the presence of CRH, pre-treatment with 0.5 nM AVP substantially reduced the response to the second pulse to 66.7±2.2 % of control (Figure 3.1.7, n=6, $P<0.0001$, *t*-test).

Again, a KCl pulse protocol was used to determine whether the reduction in response seen following 0.5 nM AVP pre-treatment in the presence of CRH was due to AVP-specific desensitization or to depletion of ACTH (Figure 3.1.8). In the presence of CRH, AVP pre-treatment did not reduce the response to a KCl pulse compared with the response of the cells that were not pre-treated (73.0±4.5% c.f. 65.6±3.0%, respectively, of control, i.e. mean of the responses to the first and third AVP pulses; n=3, NS, *t*-test). In contrast, 0.5 nM AVP pre-treatment in the presence of CRH reduced the response to the second AVP pulse from 100% to 63.6±1.1% of control (n=3, $P<0.001$, *t*-test). Since 0.5 nM AVP pre-treatment had no effect on the ACTH response to the subsequent KCl pulse, it was obvious that the reduction in response to the second AVP pulse induced by the same pre-treatment was due to an AVP-specific desensitization process rather than depletion of cellular ACTH.

In summary, these data indicate that CRH (0.2 nM) can interact with AVP (0.5 nM) to augment AVP-induced desensitization of the ACTH response.

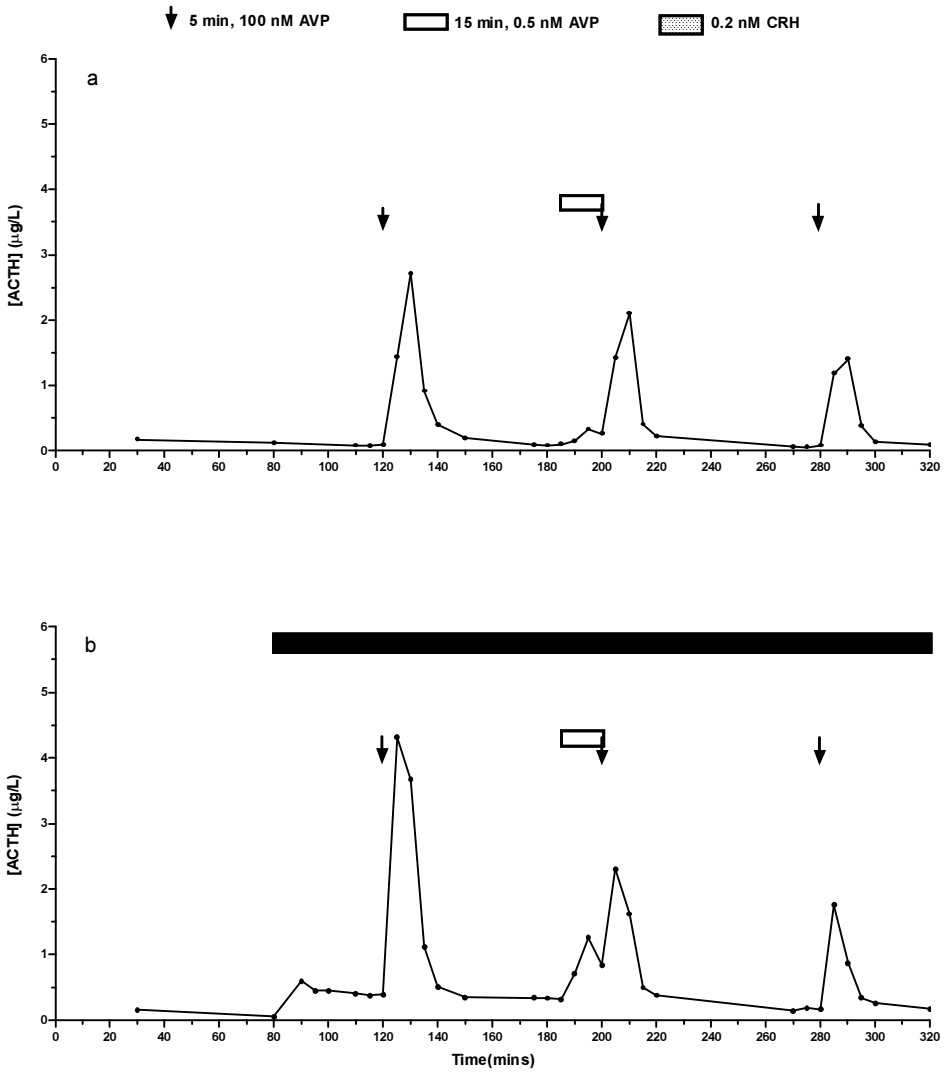

Figure 3.1.6 Desensitization of the ACTH response to AVP in the presence of CRH. Representative data are shown from two perifusion columns illustrating the protocols used in these experiments. Desensitization was induced using a pre-treatment with 0.5 nM AVP prior to the second AVP pulse in either (a) the absence or (b) presence of 0.2 nM CRH 'background'.

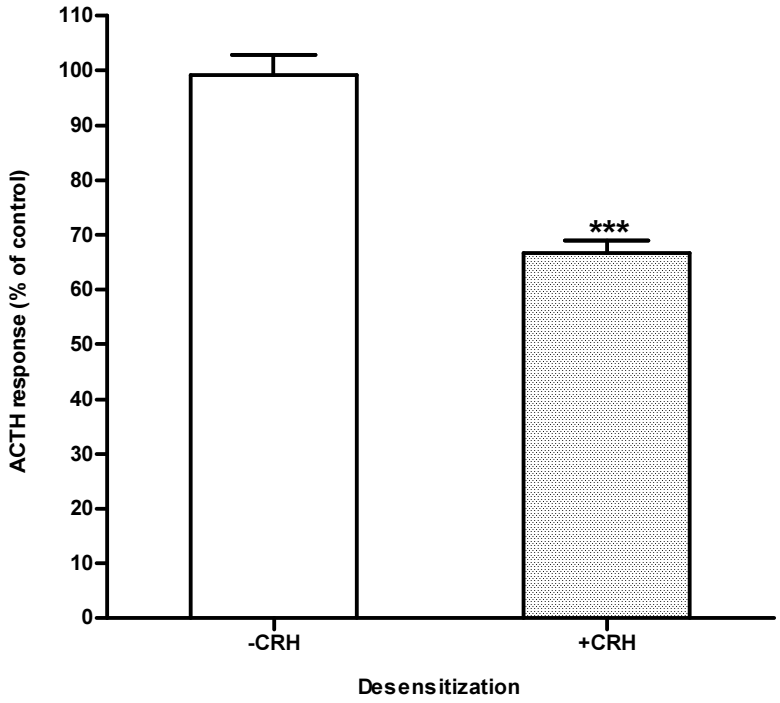

Figure 3.1.7 Effect of CRH on desensitization of the ACTH response to AVP. Data show the ACTH responses to the second AVP pulse following a 15 min pre-treatment with 0.5 nM AVP, in either the absence (-CRH) or the presence (+CRH) of a 0.2 nM CRH 'background'. Data are reported as mean ± SEM (*** $P<0.001$, *t*-test).

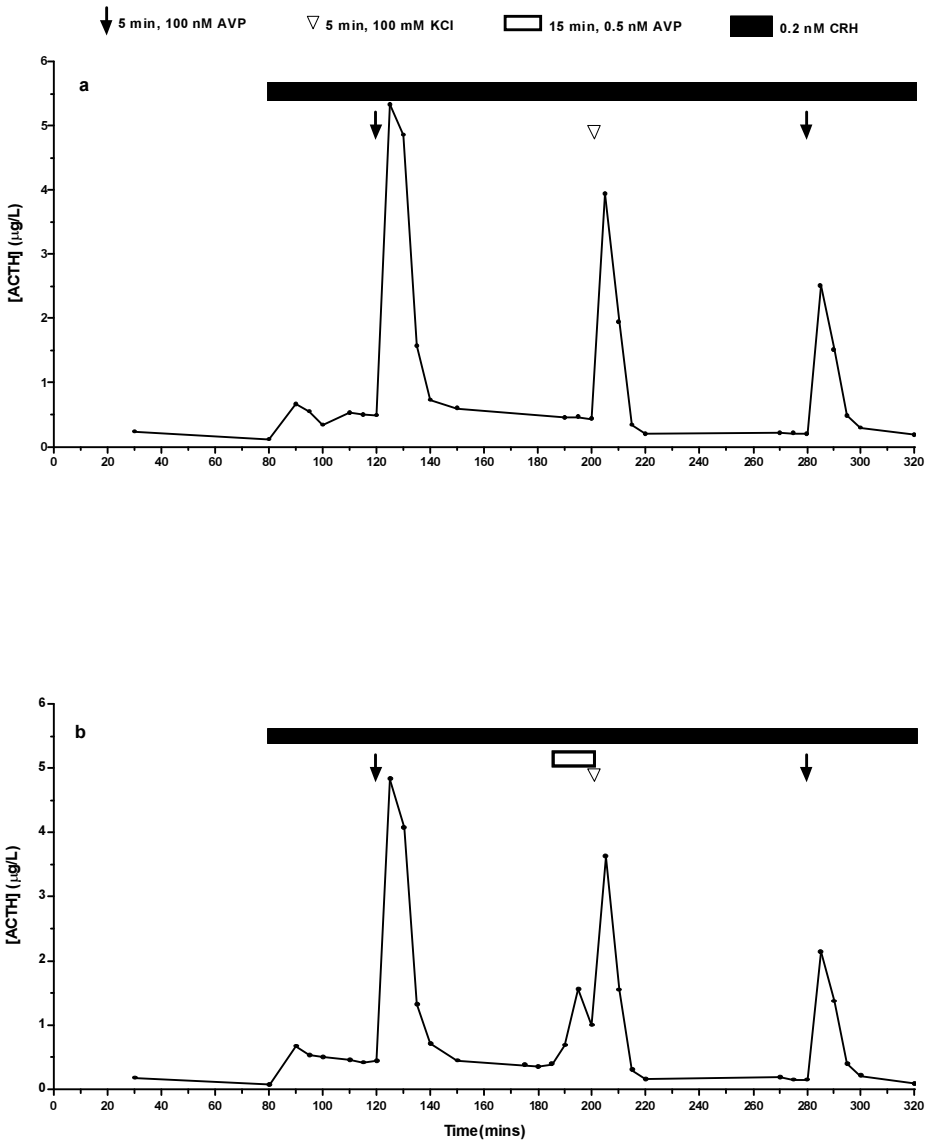

Figure 3.1.8 ACTH responses to KCl in the presence of CRH. Representative data are shown for two perifusion columns illustrating the protocols used in these experiments. In the presence of 0.2 nM CRH, cells were treated with a 5 min KCl pulse in (a) the absence or (b) presence of 0.5 nM AVP pre-treatment.

3.2 Effect of Cortisol on Desensitization of the ACTH Response to AVP

The pituitary gland has been considered as an important site of steroid feedback. In many species such as rat (Abou-Samra *et al.* 1986; Bilezikjian & Vale 1987), horse (Evans *et al.* 1993b) and sheep (Clark *et al.* 1994), continuous exposure to glucocorticoids (2-4 h) significantly inhibited CRH- or AVP-stimulated ACTH secretion from cultured anterior pituitary cells. However, this inhibitory effect appears more efficient on the ACTH response to CRH than that to AVP (Bilezikjian & Vale 1987; Evans *et al.* 1993b), indicating that AVP-stimulated ACTH release is somewhat resistant to steroid feedback. As a support of this, DEX pretreatment of perifused rat anterior pituitary cells for 2 h significantly reduced the ACTH response to CRH and the sustained plateau phase of the response to AVP, but had no effect on the spike phase of the response to AVP, which is mediated by AVP-stimulated IP_3 formation (Oki *et al.* 1991). Long-term DEX treatment even resulted in significant increase in AVP-induced IP_3 production, but is accompanied with a reduction in V1b receptor (Rabadan-Diehl & Aguilera 1998). These indicate that a complex interaction between AVP and glucocorticoids is involved in regulating ACTH secretion in response to AVP, and this may also affect AVP-induced ACTH desensitization. Therefore, the following experiments were designed to investigate the effect of cortisol, a major glucocorticoid in sheep, on desensitization of the ACTH response to AVP stimulation in sheep anterior pituitary cells.

The inhibitory effect of glucocorticoids on ACTH secretion falls into three timeframes, namely fast (within minutes), intermediate (2-10 h) and slow (hours to days) steroid feedback, which are mediated by different mechanisms (Keller-Wood & Dallman 1984). In view of this, in the present study, continuous cortisol perifusion starts at 0 min, which is 2 h before the first AVP pulse is given, so that the action of cortisol in the responses to the three AVP pulses involves similar mechanisms to the intermediate feedback.

The experimental protocols used were similar to those in Section 3.1, except that cells were perifused with cortisol rather than CRH. Cells were stimulated with three 5-min AVP (100 nM) pulses and desensitization was induced by a 15 min pre-treatment with 5 nM AVP. In sheep, the total plasma cortisol concentration under basal conditions is ~50 nM, with ~65% bound to cortisol-binding globulin, ~20% bound to albumin, and ~15% (i.e. ~8 nM) free (derived from Figures 1 & 4, Gayrard *et al.* 1996). Some stressful stimuli, such as transport and scrapie, can increase plasma cortisol levels up to 60 ng/ml (~180 nM; Schelcher *et al.* 1999; Smith & Dobson 2002). Therefore, concentrations of cortisol used in this study ranged from 10-500 nM.

3.2.1 Effect of continuous perifusion with cortisol on AVP-stimulated ACTH release

Consistent with previous reports (Abou-Samra *et al.* 1986; Evans *et al.* 1993b; Clark *et al.* 1994), cortisol exhibited an overall inhibitory effect on AVP-stimulated ACTH release (Figure 3.2.1). For example, in the presence of 100 nM cortisol, the ACTH response to the first AVP pulse was reduced to ~40% of controls that were not exposed to cortisol (Figure 3.2.1a). It was also noted that in the presence of cortisol the responses to the three AVP pulses were very similar (see Figure 3.2.1b). This contrasts with the progressive decrease in magnitude of the three ACTH peaks seen in the absence of cortisol in this study (see Figure 3.2.1a).

Because cortisol was prepared as a 1 mM stock in 95% ethanol (see Appendix B), and subsequently diluted into KR/ATC to give the final cortisol concentrations used for perifusion, possible effects of the vehicle on the ACTH response to AVP were tested. Cells were stimulated with three AVP pulses in both the absence and presence of continuous ethanol perifusion from 0 min. AVP-stimulated ACTH release was not affected by ethanol at the maximum concentration used in the cortisol experiments (0.0475%, NS, Two-way ANOVA with Bonferroni's test, n=3).

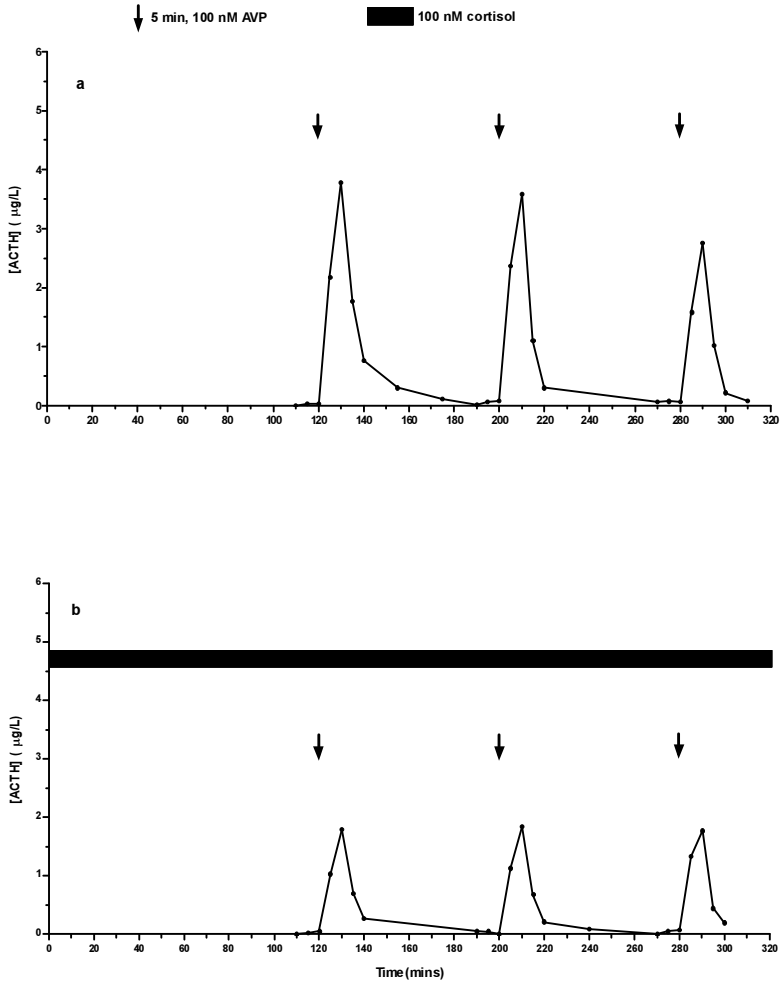

Figure 3.2.1 Continuous cortisol on the ACTH response to AVP. Data from two individual columns illustrating the experimental protocols are shown. Cells were treated with 5-min pulses of 100 nM AVP after 120, 200, and 280 min of perifusion in either (a) the absence or (b) presence of 100 nM cortisol from 0 min.

3.2.2 AVP desensitization in the presence of cortisol

Following a 15 min AVP (5 nM) pre-treatment in the absence of cortisol, the response to the second pulse was reduced to 66.7±1.9% of control (Figure 3.2.2a, n=10). In the presence of 100 nM cortisol, a significantly smaller reduction in the response to the second pulse was seen following the same pre-treatment (to 78.4±1.7% of control, n=10, $P<0.001$, *t*-test, Figure 3.2.2c).

3.2.3 Effect of cortisol concentration on AVP desensitization

The inhibitory effect of cortisol on AVP-stimulated ACTH secretion is dose-dependent (Evans *et al.*, 1993). To investigate whether the inhibitory effect of cortisol on desensitization of the ACTH response to AVP is also dose dependent, three concentrations of the steroid were tested. In the presence of 10, 100 and 500 nM cortisol, the response to the second AVP pulse following pre-treatment was 79.5±4.4% (n=6), 78.4±1.7% (n=10), and 84.0±4.8% (n=5) of control, respectively. The magnitude of AVP desensitization was significantly decreased with all three cortisol concentrations compared with that seen in the absence of cortisol (66.7±1.9% of control, $P<0.01$, One way ANOVA with Tukey's test, n=5-10). However, the magnitude of the effect of cortisol on AVP desensitization did not vary with the cortisol concentration used (NS, One-way ANOVA with Tukey's test, n=5-10). Representative results from four columns are shown in Figure 3.2.2. Combined data are summarized in Figure 3.2.3.

Overall, these data suggest that cortisol reduces the magnitude of desensitization of the ACTH response to AVP. However, over the range of cortisol concentrations investigated in this project, there is no evidence that this effect is dose-dependent.

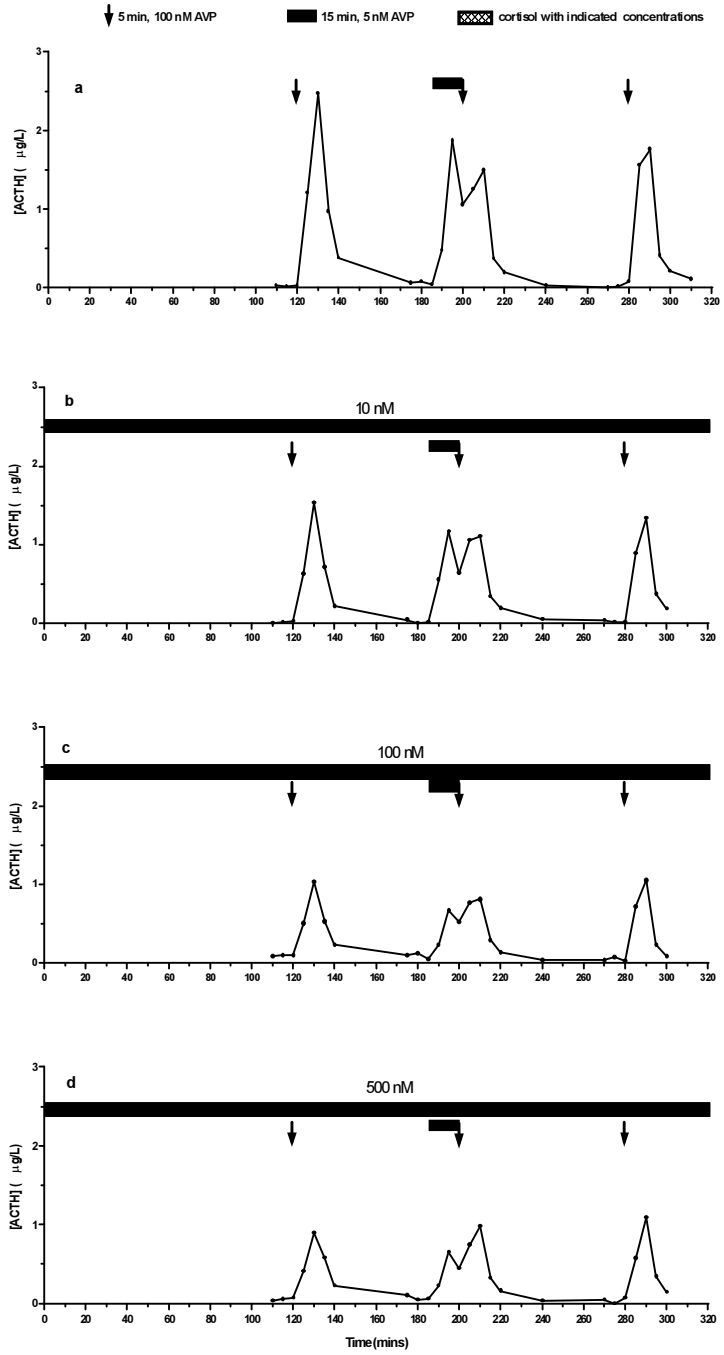

Figure 3.2.2 Different concentrations of cortisol on desensitization of the ACTH response to AVP. Representative data from four independent perifusion columns are shown. The second AVP pulse was pre-treated with 5 nM AVP for 15 min in (a) the absence, or the presence of (b) 10 nM, (c) 100 nM, and (d) 500 nM cortisol from 0 min.

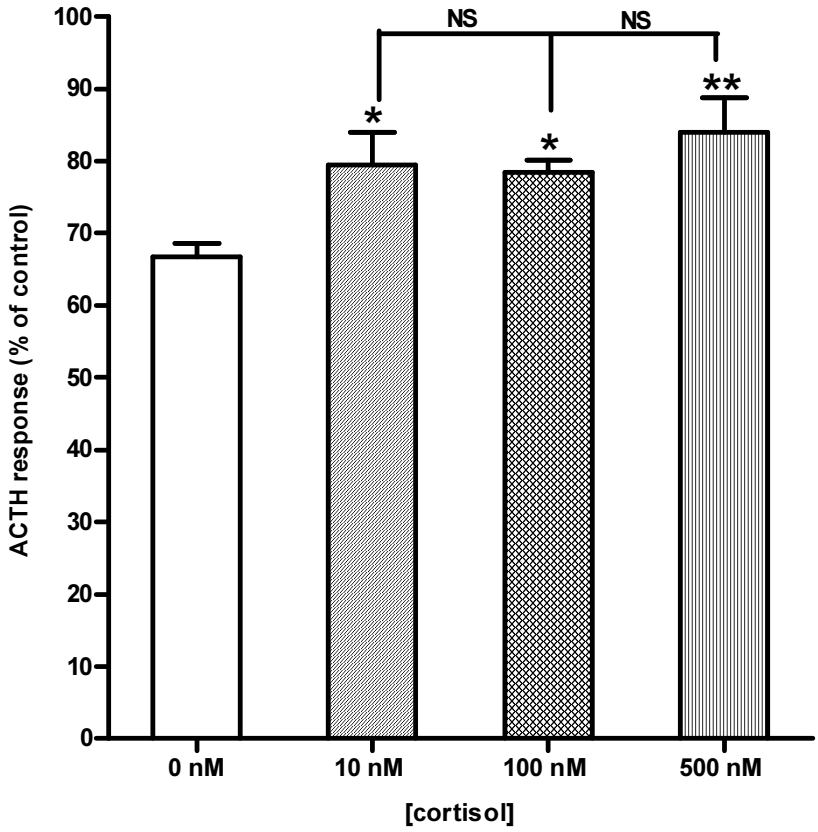

Figure 3.2.3 Effect of concentration of cortisol on desensitization of the ACTH response to AVP. Data show the ACTH response to the second AVP pulse in either the absence (0 nM) or the presence of a 10, 100 or 500 nM cortisol 'background' when the pulse was immediately preceded by a 15 min pre-treatment with 5 nM AVP. Asterisks indicate a statistically significant difference between that treatment and the control (open bar). Effect of cortisol between 10, 100 and 500 nM were not significantly different from each other (NS). Data are mean ± SEM (* $P<0.05$, ** $P<0.01$, One-way ANOVA with Tukey's test, n=5-10).

3.3 Effect of CRH and Cortisol in Combination on AVP Desensitization

ACTH secretion is influenced not only by individual actions of AVP, CRH or glucocorticoid, but also by a complex interaction between these hormones (Evans *et al.* 1996). Several studies have suggested that the inhibitory effect of glucocorticoids on ACTH secretion at the pituitary gland in response to CRH or AVP can be overcome by CRH application together, or by the combined action of CRH and AVP (Abou-Samra *et al.* 1986; Shipston & Antoni 1991; 1992a; McFarlane *et al.* 1995; Evans *et al.* 1996). For example, in perifused horse anterior pituitary cells, continuous presence of CRH (0.02 nM from 0 min) protected the ACTH response to 5-min pulses at 30 min intervals with 10 nM AVP (from 30 min) from cortisol (starts at -100 min) inhibition (Evans *et al.*, 1996). In rat anterior pituitary cells, the magnitude of corticosterone inhibition of CRH-induced ACTH response could be reduced by simultaneous CRH application (Shipston & Antoni, 1992a) or the presence of CRH and AVP together (Lim *et al.* 2002). Results obtained in adrenalectomized sheep show that an intravenous cortisol replacement equivalent to the upper limit of the normal unstressed range was sufficient to abolish the ACTH response to exogenous CRH and AVP alone, but not to CRH and AVP in combination (McFarlane *et al.* 1995). Taken together, these results clearly suggest that a complex interaction between CRH, AVP and glucocorticoids plays an important role in regulating ACTH secretion from the anterior pituitary. Based on this, and the results presented in Section 3.1 & 3.2, experiments were aimed at investigating the effects of CRH and cortisol in combination on AVP desensitization.

3.3.1 Effect of CRH plus cortisol on AVP-induced ACTH secretion

CRH (0.2 nM) and cortisol (100 nM) were perifused continuously in combination from 80 min and 0 min, respectively. Cells were treated with three 5-min AVP pulses (100 nM) at 120, 200 and 280 min in both the absence and presence of CRH and cortisol in combination. A 15 min pre-treatment with 5 nM AVP was given prior to

the second pulse. The response to the first AVP pulse was not significantly different in either the absence or the presence of CRH and cortisol in combination (Figure 3.3.1, NS, *t*-test, n=3). Furthermore, in the presence of both hormones, the response to the second pulse was the largest of the responses to the three AVP pulses (see Figure 3.3.1c). This differs from the profile seen in the absence of CRH and cortisol, where the responses to the three AVP pulses decreased gradually and progressively (see Figure 3.3.1a).

3.3.2 AVP desensitization in the presence of CRH and cortisol in combination

Results combined from three experiments showed that, following 15 min AVP (5 nM) pre-treatment, in the absence of CRH and cortisol, the response to the second pulse was 66.2±1.7% of control (Figure 3.3.1b, n=7). When both CRH and cortisol were present (see Figure 3.3.1d), AVP pre-treatment caused a significantly greater reduction in the response to the second AVP pulse (to 46.5±1.7% of control, n=8, $P<0.0001$, *t*-test). These results are summarized in Figure 3.3.2.

To determine whether the greater reduction in response to the second pulse seen after AVP pre-treatment in the presence of cortisol plus CRH was caused by depletion of ACTH stores or a specific desensitization, a KCl experiment similar to those described previously was performed (Figure 3.3.4). After being expressed as a percentage of the mean of the responses to the first and third pulses, the response to a KCl pulse without pre-treatment was not significantly different from the response following pre-treatment (90.9±3.8% e.f. 98.3±4.3%, respectively, n=3, NS, *t*-test). These results differ greatly from those described in section 3.3.2 and clearly show that the greater reduction seen in response to the second AVP pulse in the presence of both CRH and cortisol was not due to ACTH depletion.

In summary, desensitization of the ACTH response to AVP was significantly enhanced in the presence of CRH (0.2 nM) and cortisol (100 nM) in combination.

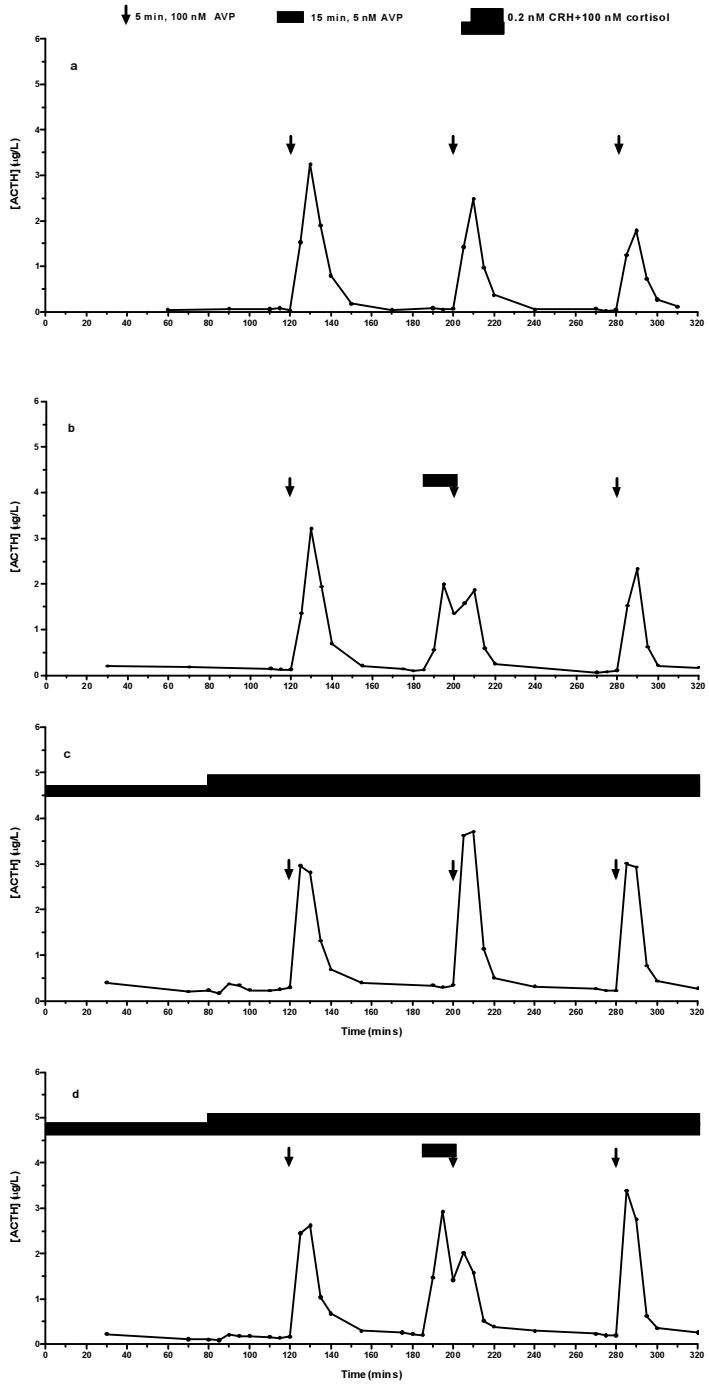

Figure 3.3.1 Desensitization of the ACTH response to AVP in the presence of CRH & cortisol. Data from four individual columns illustrating the experimental protocols are shown. Cells were treated with 5-min pulses of 100 nM AVP after 120, 200 and 280 min of perifusion in all columns. To induce desensitization the second AVP pulse was preceded by a pre-treatment with 5 nM AVP for 15 min in either (b) the absence or (d) presence of 0.2 nM CRH (from 80 min) and 100 nM cortisol (from 0 min) in combination.

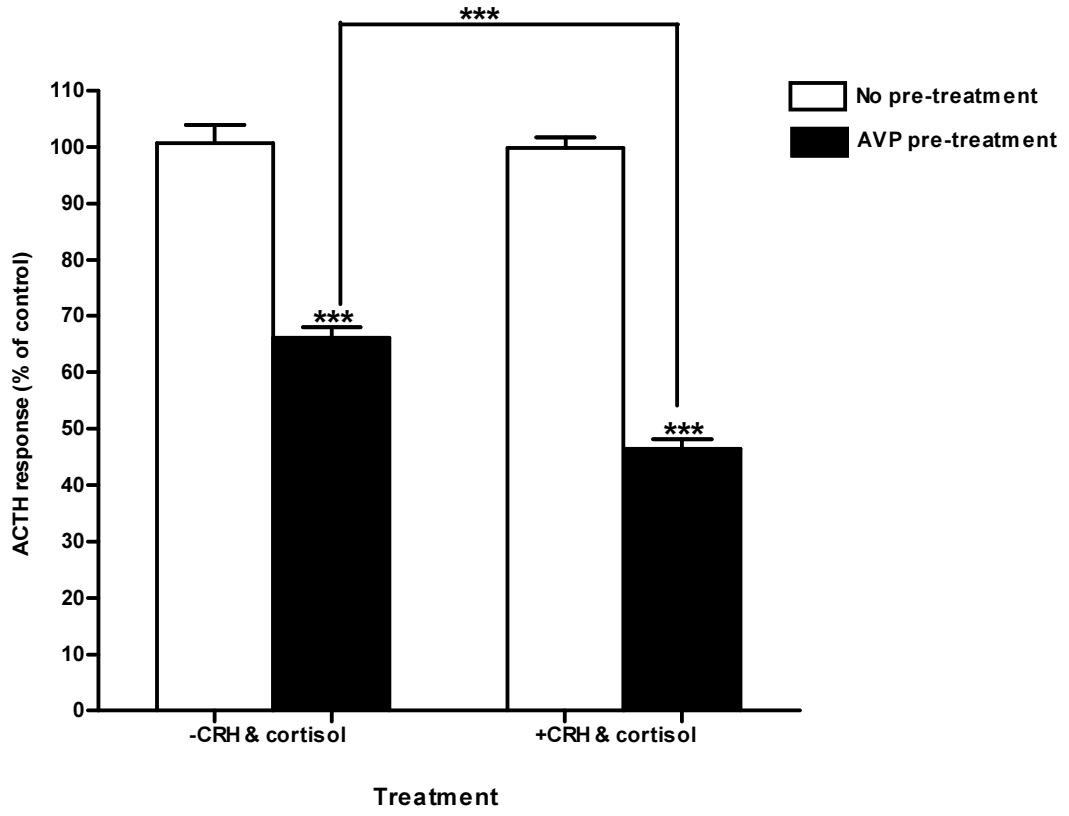

Figure 3.3.2 Effect of CRH and cortisol in combination on desensitization of the ACTH response to AVP. Data show the ACTH response to the second AVP pulse in either the absence (open bar) or presence (black bar) of a pre-treatment with 5 nM AVP for 15 min, with (+CRH & cortisol) or without (-CRH & cortisol) 0.2 nM CRH and 100 nM cortisol in combination. Asterisks indicate a statistically significant difference between the 'No pre-treatment' and the 'AVP pre-treatment' groups, and between the two 'AVP pre-treatment' groups. Data are mean ± SEM (*** $P<0.001$, *t*-test, n=7-9 for each group).

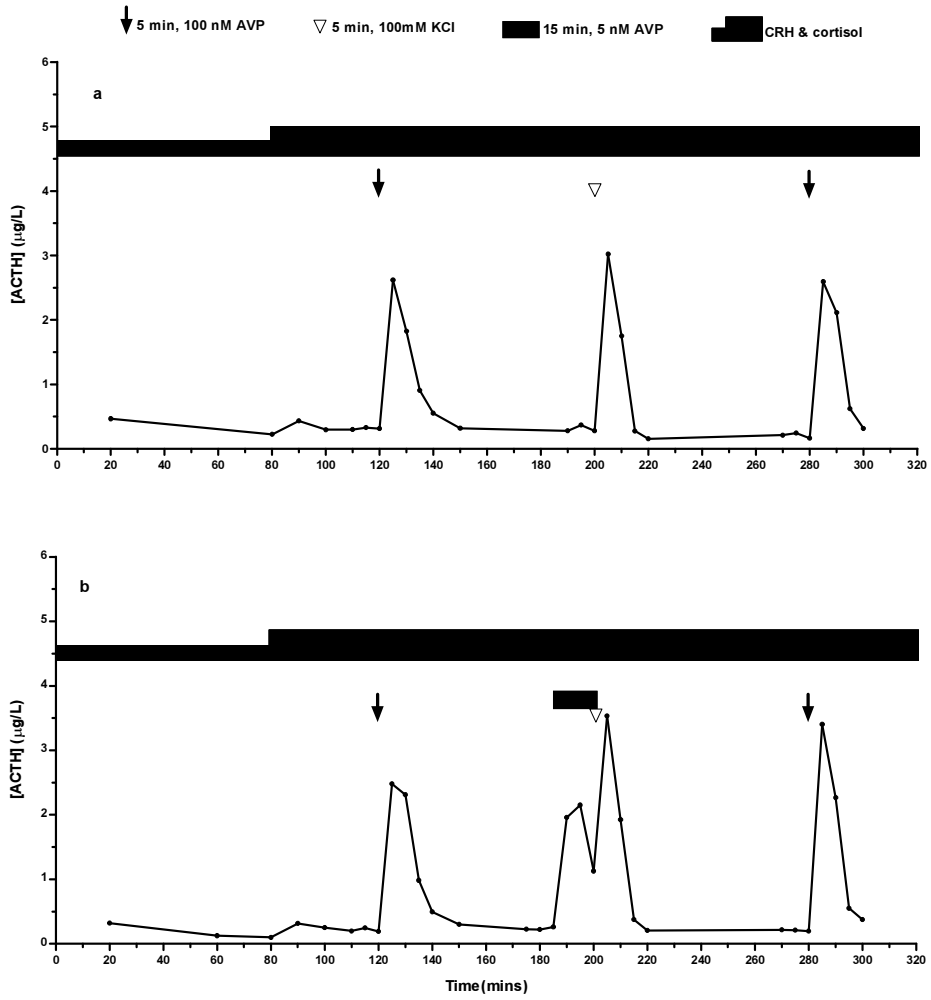

Figure 3.3.3 ACTH responses to KCl in the presence of CRH & cortisol. Representative data are shown for two perifusion columns illustrating the protocol used in these experiments. In the presence of 0.2 nM CRH and 100 nM cortisol, cells were treated with a 5 min KCl pulse (starting at 200 min) in the (a) absence or (b) presence of 5 nM AVP pre-treatment.

4

DISCUSSION

4.1 Influence of CRH on Desensitization of the ACTH response to AVP

This study showed that in the presence of continuous CRH (0.2 nM from 80 min onwards), AVP pretreatment (0.5 nM) induced significant desensitization of the ACTH response to subsequent 100 nM AVP pulse, whereas no desensitization was observed in the absence of CRH. This demonstrated that CRH (0.2 nM) promoted AVP-induced ACTH desensitization in perifused ovine anterior pituitary cells. This is consistent with the findings of Evans *et al.* (1988). These researchers observed that repetitive AVP or CRH pulses resulted in gradual loss of ACTH responsiveness (defined as desensitization of the ACTH response) in perifused sheep anterior pituitary cells. This decrease in responsiveness was greater when CRH and AVP were given together as pulses than when either was given alone (Evans *et al.* 1988), indicating that CRH can augment AVP-induced ACTH desensitization. Later studies in perifused horse anterior pituitary cells showed that the decrease in responsiveness to repeated AVP pulses was influenced in a concentration-dependent manner by continuous perifusion of CRH (1-25 pM): the higher the CRH concentration, the greater the decrease in ACTH responsiveness (Evans *et al.* 1993a). The results presented in this thesis with 0.2 nM CRH and those reported previously from our laboratory (Hassan *et al.* 2003) showing a lack of effect on AVP-induced desensitization of a low level of CRH (0.01 nM) are qualitatively similar, but quantitatively different, to those of Evans *et al.* (1993a). It is likely that in perifused sheep anterior pituitary cells, the effect of CRH on desensitization of the ACTH

response to AVP is concentration-dependent, but that it has a higher threshold which falls between 0.01 – 0.2 nM.

General mechanisms involved in GPCR desensitization include receptor uncoupling due to phosphorylation, receptor internalization and receptor down-regulation, as described in Section 1.3.1. Data indicate that rapid AVP-induced desensitization of the ACTH response in sheep anterior pituitary cells may involve both phosphorylation and internalization of V1b receptor (Hassan & Mason 2005). Therefore, it is possible that CRH influences the magnitude of AVP desensitization by modifying V1b receptor phosphorylation and/or internalization. There is evidence that CRH enhances V1b receptor endocytosis. Incubation of rat anterior pituitary cells with CRH (~21 nM; converted data) for 30 min significantly increased the rate of V1b receptor internalization (Mogenson *et al.* 1988). It is not known whether the CRH concentration used in the current study (0.2 nM) is sufficient to influence V1b receptor internalization.

CRH may affect the AVP desensitization process by directly or indirectly modulating V1b receptor phosphorylation. The protein kinase(s) responsible for anterior pituitary V1b receptor phosphorylation has not been identified to date, but it has been shown that neither PKC nor casein kinase 1α is involved in this process (Hassan & Mason 2005). Since the V1b receptor contains a C-terminus GRK consensus motif (Berrada *et al.* 2000), it is likely that a specific GRK(s) is involved in V1b receptor phosphorylation. If this were the case, the effect of CRH on AVP-induced ACTH desensitization may be achieved by enhancing GRK activity. For example, PKA can increase the activity of GRK2 by phosphorylation, and thus enhancing GRK2-mediated desensitization of β_2AR (Kohout & Lefkowitz 2003). Since CRH leads to activation of PKA in pituitary cells, this could provide a mechanism for enhancement of AVP-induced ACTH desensitization. CSPs, such as recoverin and CaM, are able to alter the activities of GRKs in different GPCRs, however since they exhibit inhibitory effects on GRK activity in all cases investigated (Pronin *et al.* 1997; Sallese *et al.*

2000) it is unlikely that CSP regulation of GRKs contributes to enhancement of AVP-induced ACTH desensitization by CRH.

It was also noted that the ACTH response to AVP pulses was potentiated by the presence of continuous CRH, with the effect on the response to the first pulse being most evident. This is not surprising as the synergistic effect on ACTH secretion between CRH and AVP is well established. The possible mechanisms include AVP stimulating CRH-induced cAMP generation (Abou-Samra *et al.* 1987), CRH promoting AVP-stimulated IP_3 production (Grammatopoulos *et al.* 2001), and interactions between Ca^{2+} and activation of PKA (Won & Orth 1995; Lim *et al.* 1998). As described in Section 1.2.1, depletion of IP_3-sensitive Ca^{2+} stores suppressed the ACTH response to CRH, suggesting a crucial role for this Ca^{2+} store in the ACTH response to PKA-dependent pathway (Won & Orth 1995). In addition, increased intracellular free Ca^{2+} potentiates the ACTH response to cAMP in mouse At*T*-20 cells (Lim *et al.* 1998). It appears that a complex interaction between CRH, AVP, and Ca^{2+} mobilization contributes to the regulation of the ACTH release in response to CRH and AVP together.

The gradual loss of synergism with CRH apparent in the responses to the second and third AVP pulses may be due to CRH-R1 desensitization, resulting in loss of CRH activation with time. It has been reported that preincubation of rat anterior pituitary segments with CRH for 4 h significantly reduced the CRH-R1 activity, abolishing the ACTH response to subsequent CRH challenge (Holmes *et al.* 1984)

In the present study, an initial pretreatment with 5 nM AVP caused a large decrease in response to a subsequent AVP pulse in the presence of CRH (0.2 nM). However, the same pretreatment also reduced the ACTH response to a subsequent KCl (100 mM) pulse. This indicated the involvement of other factors, such as depletion of cellular ACTH. Newly synthesized ACTH might not be enough to compensate for the

substantial ACTH release in response to CRH and AVP in combination, resulting in reduced ACTH stores.

In previous study, the IC_{50} value for desensitization of the ACTH response to AVP (6.5 nM; Hassan *et al.* 2003) is comparable to hypophyseal portal AVP (up to 6 nM in the sheep; reviewed in Mason *et al.* 2002). This has been interpreted as an indication that the physiological role of rapid AVP desensitization seen *in vitro* is to limit the maximum ACTH response to AVP rather than to prevent ACTH secretion (Hassan *et al.* 2003). Since it has been suggested that CRH acts in a permissive role to set the overall responsiveness of the corticotrope to fluctuations in AVP secretion (Antoni 1993; Evans *et al.* 1996), it is possible that, the major role of a lower level of CRH (0.01 nM), which was used previously, is to potentiate the ACTH response to AVP. When CRH increases during stressful conditions, to a level similar to that used in the present study (0.2 nM), CRH may function not only as a synergistic factor in the response to AVP, but may also help to limit the maximum response by enhancing the magnitude of AVP-induced desensitization of the ACTH response. Furthermore, in the presence of CRH, pre-treatment with an AVP concentration as low as 0.5 nM induced significant desensitization to a subsequent AVP pulse. This provides further support for the suggestion that AVP desensitization occurs under physiological conditions.

Overall, the present study demonstrates that CRH significantly enhances desensitization of the ACTH response to AVP in ovine anterior pituitary cells. The concentrations of CRH and AVP used in this study suggest that this influence may be important under physiological conditions, especially in response to stressful stimuli.

4.2 Influence of Cortisol on Desensitization of the ACTH Response to AVP

Consistent with previous findings (Abou-Samra *et al.* 1986; Evans *et al.* 1993b; Clark *et al.* 1994), continuous perifusion with cortisol showed an overall inhibitory effect on the ACTH response to AVP pulses in the present study. In addition to this general effect, this study demonstrated that cortisol application also influences AVP-induced desensitization of the ACTH response. In contrast to CRH, continuous cortisol perifusion significantly reduced the magnitude of AVP desensitization in ovine anterior pituitary cells. The reduction in response to the second AVP pulse following AVP pretreatment was significantly smaller in the presence of cortisol than that in its absence. These results conflict with previous observations, which showed the presence of continuous cortisol (100 nM) did not affect the reduction in response to repeated AVP pulses in perifused horse pituitary cells (Evans *et al.* 1996). Since the physiological levels of cortisol in the horse (111-322 nM; Alexander *et al.* 1993) are much higher than those in the sheep (~50 nM; Gayrard *et al.* 1996), the sensitivity of horse and sheep anterior pituitary cells to cortisol may be different. However, in the present study, cortisol at 10, 100 and 500 nM reduced the magnitude of AVP desensitization to a similar extent, providing no evidence that this effect of cortisol is dose-dependent. Other factors, such as species, steroid application regimes and experimental design (Antoni 1993) may influence the effect of cortisol. It has been shown that proteins in the medium can affect the action of cortisol (Kemppainen *et al.* 1991).

Since cortisol and CRH elicit opposite effect on desensitization of the ACTH response to AVP, it is likely that cortisol influence V1b receptor phosphorylation and internalization in a way that is contrary to that of CRH, such as inhibiting receptor phosphorylation or slowing down the rate of internalization. As described by Keller-Wood & Dallman (1984), intermediate steroid feedback (2-10 h application) induces new protein synthesis. Since AVP pretreatment was given at 185 min from the onset

of cortisol perifusion in this study, it is likely that some cortisol-induced proteins were involved in the regulation of cortisol effect on AVP desensitization. It has been shown that both CaM mRNA (10-fold) and protein level (2-fold) are enhanced after 90 min DEX administration in mouse AtT20 cells (Shipston & Antoni 1992b; Shipston 1995). Since CaM is implicated to inhibit the activities of multiple GRKs including GRK2 to GRK6 in different GPCRs (Pronin *et al.* 1997; Sallese *et al.* 2000), enhancement of CaM levels by glucocorticoids promotes the inhibitory effect on GRKs, thereby preventing GRK-mediated GPCR desensitization.

Actin polymerization is indicated to play a role in clathrin-mediated endocytosis (Kaksonen *et al.* 2006). However, there are conflicting results about the effect of actins on receptor-mediated endocytosis. For example, Lamaze and coworkers (1996) have shown that stimulation of actin filament assembly at the cortex by GTPase activation inhibits clathrin-mediated endocytosis, whereas a later study in A431 cells demonstrates that sequestration of actin monomers by actin-binding proteins or disruption of actin filaments by drugs also inhibits the rate of endocytosis (Lamaze *et al.* 1997). It has been reported that glucocorticoids can stabilize actin filaments (Castellino *et al.* 1992). Pretreatment of AtT20 cells with DEX (100 nM) (at least 2 h) partially prevents drug-induced disruption of actin filaments. Furthermore, exposure of human pneumocytes type II cells to DEX for 12 h significantly thickens actin bundles (Castelllino *et al.* 1992). It is not sure whether glucocorticoids have similar effect in corticotrope cells, and if it were, how this would affect the rate of receptor internalization. However, given that the findings by Castellino *et al.* (1992) and Lamaze *et al.* (1996), in the present study, the possibility that cortisol perifusion affects AVP-induced desensitization by inhibiting V1b receptor internalization could not be excluded.

It has been suggested that intermediate steroid feedback occurs under physiological conditions when circulating glucocorticoids increase in response to moderate stress (Keller-Wood & Dallman 1984). In addition, AVP is implicated to play a major role

in maintaining pituitary responsiveness despite of elevated plasma steroids during stress adaptation (Aguilera & Rabadan-Diehl 2000). The present findings may provide a relevant mechanism for AVP regulating of ACTH secretion during stress: the circulating glucocorticoids may contribute to maintain ACTH responsiveness by AVP through partially preventing desensitization of the ACTH response to AVP.

In summary, data from present study have demonstrated that cortisol significantly reduces the magnitude of desensitization of the ACTH response to AVP in perifused ovine anterior pituitary cells. However, no evidence from this study indicates the effect of cortisol is dose-dependent. In view of the relatively low concentrations of cortisol used in the present study, these *in vitro* findings may be relevant *in vivo* also.

4.3 Influence of CRH and Cortisol in Combination on Desensitization of the ACTH Response to AVP

The current study investigated the effect of combined CRH and cortisol on AVP-induced ACTH desensitization. In the presence of CRH (0.2 nM) and cortisol (100 nM) in combination, the magnitude of desensitization of the ACTH response to AVP was significantly enhanced, compared with the magnitude of this desensitization in the absence of combined CRH and cortisol.

As discussed in Section 4.1, factors that influence the AVP desensitization process are likely to act through modulating V1b receptor phosphorylation and /or internalization. Since GRKs may be involved in V1b receptor phosphorylation, regulation of GRK activity could influence the desensitization process. As mentioned before, cortisol administration may decrease GRK activation through stimulation of CaM mRNA and protein levels (Shipston 1995; Sallese *et al.* 2000). However, the increase of CaM mRNA by DEX treatment in mouse AtT20 cells was blocked by simultaneous addition of CRH (Shipston & Antoni 1992b). This suggests that CRH can counteract the effect of cortisol on GRK activity. In addition to this, CRH alone is able to

enhance AVP-induced ACTH desensitization, which is possibly through PKA-dependent phosphorylation of GRKs (see Section 4.1). It is likely that CRH not only blocks the inhibitory effect of cortisol on GRK activity, but also enhances GRK activation via other mechanisms. It has also been suggested that CRH or cortisol alone may affect receptor endocytosis (see Section 4.1 & 4.2; Mogenson *et al.* 1988; Castellino *et al.* 1992; Kaksonen *et al.* 2006). However, it is difficult to predict how CRH and cortisol in combination influences the internalization of V1b receptor based on these previous findings. Overall, the effect of combined CRH and cortisol on AVP-induced ACTH desensitization appears to be mediated by complex interactions between these hormones.

It has previously been demonstrated that CRH can protect the ACTH secretory response to AVP against cortisol inhibition in perifused horse anterior pituitary cells (Evans *et al.* 1996). This effect of CRH was also observed in the present study: the response to the first AVP pulse was similar in either the absence or the presence of CRH and cortisol in combination. Some transcription factors, such as *c-fos*, have been shown to interfere with glucocorticoid-induced gene transcription (Diamond *et al.* 1990). In rat anterior pituitary cells, CRH application activates *c-fos*, helps to prevent the inhibitory effect of DEX on proopiomelanocortin gene transcription (Autelitano & Sheppard 1993). Furthermore, the blockade of DEX inhibition by CRH was impaired by the presence of puromycin, suggesting that this effect of CRH depends on the rapid synthesis of c-*fos* (Autelitano & Sheppard 1993).

CRH may also prevent the actions of glucocorticoids through modulating GR activity. A recent report showed that injection of a CRH receptor antagonist into normal rats resulted in a significant increase in GR binding to endogenous corticosterone in the anterior pituitary (Hügin-Flores *et al.* 2003), suggesting that CRH can regulate GR activity. Furthermore, purified rat liver GRs can be phosphorylated by PKA, and this phosphorylation may inactivate GRs by keeping them in cytosol (Singh & Moudgil 1985). Since CRH application stimulates cAMP

accumulation, GRs in pituitary gland might undergo a similar phosphorylation to that seen in rat liver cells, thereby resulting in loss of glucocorticoid action.

Alternatively, as pointed out by Antoni (1993), the efficacy of glucocorticoid inhibition of the ACTH response is actually not reduced, since the response to AVP in the presence of CRH is much greater than that to AVP alone. It thus appears that, in the presence of CRH and cortisol in combination, the synergistic effect of CRH and AVP counteracts the inhibition of ACTH secretion by cortisol.

The relatively high concentrations used in the present study (0.2 nM CRH & 100 nM cortisol) indicates that the effect of CRH and cortisol in combination on AVP-induced ACTH desensitization may be important in the regulation of ACTH secretion under stressful conditions, where circulating levels of CRH and /or cortisol are much higher than basal conditions. Although the precise role of this effect in the regulation of ACTH secretion in response to stress is not well known, it is clear that the pituitary responsiveness during stress is regulated not only by the direct actions of CRH and glucocorticoids on the ACTH release, but also by their indirect actions such as modulating the extent of AVP-induced desensitization of the ACTH response.

In summary, data from the present study have demonstrated that in the presence of combined CRH and cortisol, desensitization of the ACTH response to AVP was significantly augmented. This effect of CRH and cortisol in combination resembles the effect of CRH alone, but contrasts with that of cortisol alone.

4.4 Suggestions for Further Research

The present study explored the influence of hormonal interaction on AVP-induced ACTH desensitization in perifused ovine anterior pituitary cells. Either CRH alone or CRH and cortisol in combination enhanced the magnitude of desensitization of the ACTH response to AVP. In contrast, continuous perifusion of cortisol alone reduced

the magnitude of this desensitization. However, mechanisms underlying these are not well understood.

With PKC and casein kinase 1α being not involved in V1b receptor phosphorylation (Hassan & Mason 2005), it is not known which kinase(s) is responsible for this receptor phosphorylation during the desensitization process. This makes it very difficult to further characterize this process and investigate possible factors which contribute to or affect this process. An investigation whether GRK(s) is involved in desensitization of the ACTH response to AVP is currently in progress in our laboratory using antisence oligonucleotides to inhibit expression of GRKs.

While V1b receptor internalization has been shown to be important in AVP desensitization (Hassan & Mason 2005), it is unsure whether the effect of CRH or cortisol on this desensitization process is dependent on changes in the rate of receptor internalization. More information about the effects of CRH and/or cortisol treatment on V1b receptor internalization in the pituitary gland is required to elucidate the mechanisms involved in the regulation of AVP-induced ACTH desensitization.

Mechanisms involved in glucocorticoid feedback vary with the time of steroid application (Keller-Wood & Dallman 1984). In the present study, all AVP pulses were given between 2-5 h from the onset of cortisol perifusion. This falls into intermediate steroid feedback (2-10 h of steroid exposure), suggesting that the effect of glucocorticoids on ACTH secretion requires new protein synthesis (Keller-Wood & Dallman 1984). In our laboratory, a project is currently being conducted to investigate whether protein synthesis induced by cortisol exposure influences the AVP desensitization process. This is done using protocols similar to that used in Section 3.2, but by simultaneous perifusion with a protein synthesis inhibitor, cycloheximide and cortisol together. This enables us to see the effect of inhibition of protein synthesis on AVP-induced desensitization of the ACTH response. In future, it may be

of interest to see whether changing the time of the onset of cortisol perifusion will result in different effect of cortisol on desensitization of the ACTH response to AVP.

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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 filter	Millipore Corporation, Bedford MA
Biogel P-2	Biorad Laboratories, Hercules CA
Bovine γ-globulin	Sigma Chemical Co.
$CaCl_2.2H_2O$	Reidel-De Haen AG, Hannover, Germany
Chloramine T	BDH Chemical Ltd.
Corticotropin-releasing hormone	Sigma Chemical Co.
D-glucose	BDH Laboratory Supplies
DME powder	Sigma Chemical Co.
HEPES	Sigma–aldrich, Inc.
Hydrocortisone	Sigma–aldrich, Inc.
KCl	May and Baker Australia Ltd, West Footscray, Victoria, Australia
KH_2PO_4	BDH Chemical Ltd.
L-ascorbic acid	Sigma Chemical Co.
L-glutamate	Sigma Chemical Co.
Non-essential amino acids	Sigma Chemical Co.
$MgSO_4.7H_2O$	BDH Chemical Ltd.
Na_2HPO_4	BDH Laboratory Supplies
NaCl	BDH Laboratory Supplies
$NaHCO_3$	BDH Laboratory Supplies
NaN_3	BDH Chemical Ltd.

NaOH	BDH Laboratory Supplies
Newborn calf serum (NCS)	Invitrogen/Life Technologies,
Nybolt nylon gauze	Seidengaze, Zurich, Switzerland
Okadaic acid	Sigma Chemical Co.
Ovine ACTH	Dr C.H.Li, Hormone Research Laboratory, University of California, USA
Penicillin G (sodium salt)	Sigma–aldrich co.
Phenol red (sodium salt)	Sigma Chemical Co.
Plastic petri dishes	Labserv, Auckland
Polyethylene glycol 6000	Scharlau Chemie, Spain
Sephadex G-25 fine	Sigma Chemical Co.
Sodium metabisulphite	BDH Chemical Ltd.
Streptomycin sulphate	Sigma Chemical Co.
Triton X-100	Sigma Chemical Co.
Trypan blue	BDH Chemical Ltd.
Type II collagenase	Sigma Chemical Co

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APPENDIX B

SOLUTIONS

1. Cell preparation

1.1 Dispersing Buffer (DB)

This solution was made up by dissolving the following reagents in deionised, distilled water (ddH_2O) to give the final concentrations shown below. Usually 5 L was made up for each time. After all reagents completely dissolved, the pH was adjusted to 7.3 using concentrated NaOH or HCl. In the laminar flow hood, the solution was sterilised by pumping filtration through 0.22 μm polyethersulfone filter, and then was stored at 4ºC.

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)	53 nM
Penicillin G	100 U/ml
Streptomycin sulphate	100 μg/ml
Amphotericin B	0.25 μg/ml

1.2 Dulbecco's modified eagles medium (DME)

Usually 1 L was made up for each time. One bottle of commercially prepared, powdered medium was dissolved in ddH_2O in a 1 L volumetric flask. The quantities and final concentrations of the other reagents were shown below. After all the reagents dissolved, the solution was made up to 1 L with ddH_2O, and the pH was adjusted to 7.1. The medium was then sterilized by filtering through 0.45

μm MCE filter in the flow hood, and stored at 4ºC for up to two months. 10% newborn calf serum was added before use. If the solution was 4 weeks old, fresh L-glutamate was also added (final concentration: 2 mM) before use in the cell culture.

$NaHCO_3$	44 mM
HEPES	25 mM
Non-essential amino acids	1.0 ml
Phenol red (sodium salt)	42 nM
Penicillin G	100 U/ml
Streptomycin sulphate	100 μg/ml
Amphotericin B	0.25 μg/ml

2. Perifusion experiment

2.1 Stock solutions for Krebs ringer (KR)

Five stock solutions were made up and stored for late KR preparation. The reagents given below were dissolved in ddH_2O to give the concentrations shown. Usually 250 ml NaCl stock solution and 100 ml for each of other stock solutions were made up for each time. These stocks were stored at 4ºC.

NaCl	5 M
KCl	0.47 M
$CaCl_2.2H_2O$	0.25 M
KH_2PO_4	0.12 M
$MgSO_4.7H_2O$	0.12 M

2.2 Krebs Ringer (KR)

Usually 5 L or 2 L was made up for each time. The reagents listed below (Stock-containing reagents were dissolved by adding the appropriate volumes of stock solution in ddH_2O) were dissolved in ddH_2O to give the concentrations shown. After the pH was adjusted to 7.3 the solution was filter-sterilized as either for DB

(5 L made up) or for DME (2 L made up), then stored at 4ºC. On the day of perifusion, 0.05% ATC and 0.005% L-ascorbate were added to the solution to make up KR/ATC (Check the pH was between 7.3-7.4 after the addition of ATC and ascorbate). KR/ATC was the basic perifusion medium used in this research.

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
D-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

2.3 KCl (100 mM) Krebs Ringer

This solution was produced similarly with the preparation of KR, except that the final concentrations of NaCl and KCl were changed. Besides, the pure KCl powder rather than KCl stock solution was dissolved in ddH_2O to give the expected concentration. After the pH was adjusted to 7.3, the solution was filter-sterilized through 0.22 μm filter and stored at 4ºC.

NaCl	85 mM
KCl	99 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

D-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

2.4 Sephadex and Biogel preparation

Weigh out the reagents into small sterile glass bottles before hand (Sephadex ≥0.8 g, Biogel≥0.4g). Add some KR in to swell the bead on the day before perifusion. Store at 4ºC. Change KR once on that day. On the day of perifusion, change KR once, allowing the beads to settle down. Then adjust the height of gel and the KR to a ratio of 2:1 for use in the perifusion experiment.

2.5 Preparation of treatment solutions

Treatment solutions (solutions containing hormones) were prepared by diluting a stock solution with KR/ATC (or KR/ATC containing another hormone) to give the desired concentration. Stock solutions of AVP (20 μM) and CRH (1 μM) were prepared by Dr. Drusilla Mason, and stored at -80 ºC. Stock solution of cortisol (1 mM) was prepared by dissolving hydrocortisone in 95% ethanol, and stored at -20ºC. On the day of experiment, appropriate volumes of stock solutions were thawed and diluted in KR/ATC to make up the treatment solutions at required concentrations.

3. Radioimmunoassay Solutions

3.1 Phosphate buffer stock (0.5 M)

This solution was used as a stock solution for preparing other solutions used in RIA. The reagents listed below were dissolved in ddH_2O and made up to 1 L in a volumetric flask. The buffer was stored frozen until use.

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

3.2 Phosphate/ATC buffer (P/ATC)

Usually 1 L was made up for each time. 0.5 M phosphate buffer was diluted (1/10) with ddH_2O, and appropriate amount of ATC was added according to 1 g/L. After the pH was adjusted to 7.4, the solution was stored frozen until use.

3.3 Polyethylene Glycol solution (PEG)

18% PEG solution was prepared according to protocols shown below and stored at room temperature.

PEG6000 (Scharlau)	360 g
NaCl	11.7 g
0.5 M phosphate buffer	210 g
ddH_2O	1500 ml
Triton X-100	2 ml

3.4 γ-globulin

This solution was prepared by dissolving γ-globulin in P/ATC solution to give a final concentration of 12 g/L (1.2%). It was stored at -20ºC and usually used within one month.