MECHANISM and MODULATION

OF EARLY GLUCOCORTICOID INHIBITION IN

ANTERIOR PITUITARY CORTICOTROPHS

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To my parents

DECLARATION

This study was carried out under the guidance of Dr Ferenc A. Antoni at the MRC Brain Metabolism Unit, Department of Pharmacology, University of Edinburgh between October 1989 and September 1992.

The experimental work presented in this thesis is my own and this thesis has been composed by myself. Where contributions from others have been presented these are acknowledged in the text.

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ABSTRACT

Corticotrophin releasing factor (CRF-41) and arginine vasopressin (AVP) are the principal hypothalamic mediators of adrenocorticotrophin (ACTH) secretion by the anterior pituitary gland, while adrenal corticosteroids are the major inhibitors of ACTH output. The aim of this thesis is to characterize *in vitro* the mechanism and potential modulation of early glucocorticoid inhibition of stimulated ACTH release in anterior pituitary corticotrophs.

In perifused rat anterior pituitary corticotrophs early (<2h) glucocorticoid inhibition of CRF-41- and AVP-stimulated ACTH release was mediated through type II glucocorticoid receptors and the induction of new mRNA and protein. Glucocorticoids inhibited the amount of ACTH released by either secretagogue, but had no effect on the time-course of the ACTH secretory response. Significantly, the characteristics of early glucocorticoid inhibition were dependent on the nature of the secretagogue as well as the relative timing of glucocorticoid application. ACTH secretion stimulated by AVP, that acts through the inositol phosphate/protein kinase C pathway, was invariably suppressed by glucocorticoids. In contrast, CRF-41, that activates the cAMP/protein kinase A pathway, inactivated early glucocorticoid inhibition of CRF-41-, but not AVP- stimulated ACTH release when applied at the start of glucocorticoid exposure. When CRF-41 and AVP were applied in combination the characteristics of early glucocorticoid inhibition resembled those observed using CRF-41 alone. Qualitatively similar results were obtained in the mouse corticotroph cell line, AtT20 D16:16, in static incubation: CRF-41, but not phorbol dibutyrate ester (that activates protein kinase C), blocked early inhibition of CRF-41-stimulated ACTH release. The precise mechanism of CRF-41-inactivation of early glucocorticoid inhibition is unknown, however, the time-course of the effect suggests that CRF-41 blocks glucocorticoid-induced gene transcription. To test this hypothesis and further explore the mechanism(s) of early glucocorticoid inhibition in corticotrophs, the mouse corticotroph cell line, AtT20 D16:16, was used to characterize early glucocorticoid-induced protein(s). Functional evidence in AtT20 D16:16 cells, as well as other systems, suggests that early glucocorticoid inhibition involves the suppression of intracellular free calcium levels. Hence, these studies were confined to known calcium-binding proteins. The levels of the calcium-binding proteins lipocortin (annexin) I and chromogranin A were not altered during the period when early glucocorticoid inhibition was maximal in AtT20 cells. Importantly, the mRNA and protein encoding the calcium receptor protein, calmodulin, was induced within the timescale of early inhibition. Induction of calmodulin mRNA was dependent on ongoing protein synthesis suggesting that additional glucocorticoid activated transcription factors may be required for the enhancement of calmodulin gene transcription. Pretreatment with CRF-41, but not phorbol dibutyrate ester, blocked glucocorticoid-induction of calmodulin mRNA accumulation supporting the hypothesis that inactivation of early glucocorticoid inhibition by CRF-41 involves blockade of glucocorticoid-induced gene transcription. Several other glucocorticoid-induced mRNAs were isolated using subtraction hybridization screening of an AtT20 D16:16 cDNA library and await further characterization.

Taken together, these data implicate calmodulin as a glucocorticoid-induced mediator of early inhibition in anterior pituitary corticotrophs. Furthermore, CRF-41, through a cAMP-dependent mechanism, can prevent early glucocorticoid action by the blockade of glucocorticoid-induced gene transcription, as exemplified by the suppression of glucocorticoid-induced calmodulin mRNA accumulation.

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3 CHARACTERISTICS OF EARLY GLUCOCORTICOID INHIBITION IN PERIFUSED RAT ANTERIOR PITUITARY GLAND SEGMENTS

ABBREVIATIONS

A_{260}	Absorbance at 260nm
ACTH	Adrenocorticotrophic hormone
ANF	Atrial natriuretic factor
5'-AMP	Adenosine 5'monophosphate
AtT20	Mouse clonal corticotroph cell line (subclones: D1, D16v, D16:16)
ATP	Adenosine triphosphate
AVh	Amp-Volt hours
AVP	Arginine vasopressin
B	Corticosterone
bp	Base pairs
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CRF-41	41-residue corticotrophin releasing hormone
CaM	Calmodulin
cAMP	Cyclic adenosine 3',5'-monophosphate
cAMP-TME	2'-O-monosuccinyl cAMP tyrosyl methyl ester
cDNA	Complimentary deoxyribonucleic acid
cGMP	Cyclic guanosine 3',5'-monophosphate
cpm	Counts per minute
cps	Counts per second
8-CPT-cAMP	8-(4-chlorophenylthio) 3',5'-cyclic adenosine monophosphate
CREB	cAMP response element binding protein
DAG	Diacylglycerol
DMEM	Dulbecco's modification of Eagle's medium
DMSO	Dimethylsulphoxide
dNTP	deoxy(nucleotide)triphosphate
DNA	Deoxyribonucleic acid
DRB	5,6-Dichloro-1-ß-D-ribofluranosylbenzimidazole (transcription inhibitor)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethylene glycol-bis-(ß-amino ethyl ether)N,N'-tetra-acetic acid
FCS	Foetal calf serum
h	Hour
HPA	Hypothalamic-pituitary-adrenal
IBMX	Isobutylmethylxanthine
i.d.	Internal diameter
IEF	Isoelectric focussing
IP_3	Inositol 1,4,5 trisphosphate
Kd	Kilodaltons
LC1	Lipocortin (annexin) 1
min	Minute
mRNA	Messenger ribonucleic acid
OD_{260}	Optical density at 260nm
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PdBu	Phorbol 12,13 dibutyrate ester
pfu	Plaque forming unit
PKA	Protein kinase A
PKC	Protein kinase C
PMSF	Phenylmethylsulphonylfluoride
POMC	Proopiomelanocorticotrophin

PVN	Paraventricular nucleus
RNA	Ribonucleic acid
RT	Room temperature
RU28362	11ß, 17ß-dihydroxy-6-methyl-17α–(1-propynyl)androsta-1,4,6-trien-3-one: Type II glucocorticoid receptor agonist
RU38486	17ß-hydroxy-11ß(4-dimethylaminophenyl-1)-17α(propy-nyl)-estra-4, 9-dien-3-one: Type II glucocorticoid receptor antagonist
SDS	Sodium dodecylsulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SON	Supraoptic nucleus
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
U	Units

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PUBLICATIONS

Full papers:

Shipston, M. J. & Antoni, F. A. (1991) Early glucocorticoid feedback in anterior pituitary corticotrophs: differential inhibition of hormone release induced by vasopressin and corticotrophin-releasing factor *in vitro*. *Journal of Endocrinology* **129**, 261-268.

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Woods, M. D., **Shipston, M. J.**, Mullens, E. L. & Antoni, F. A. (1992) Pituitary corticotroph tumour (AtT-20) cells as a model system for the study of early inhibition by glucocorticoids. *Endocrinology* **131**, 2873-2880

Shipston, M. J. & Antoni, F. A. (1992) Rapid glucocorticoid induction of calmodulin and its suppression by corticotropin-releasing factor in mouse corticotrope tumor (AtT20) cells. *Biochemical and Biophysical Research Communications* **189**,1382-1388

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Shipston, M. J., McArthur, M. & Antoni, F. A. (1991) Glucocorticoid suppression of stimulated corticotrophin release by rat adenohypophysis *in vitro*: The nature of the secretory stimulus determines the pattern of inhibition. *Journal of Physiology* **434**, 89P.

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Shipston, M. J., Mullens, E. L., Lightman, S. L., Woods, M. D. & Antoni, F. A. (1992) Early glucocorticoid inhibition in mouse corticotrope tumor (AtT20) cells: induction of calmodulin but no change in lipocortin (annexin) I mRNA or protein. *Society for Neuroscience Abstracts* **18**, No. 284.2.

Antoni, F. A., **Shipston, M. J.**, Woods, M. D., Hoyland, J., Mason, W. T. & Mullens, E. L. (1992) Secretagogue glucocorticoid interactions in the control of anterior pituitary adrenocorticotrophin (ACTH) release. *Progress in Endocrinology* (in press).

1

Introduction

1

INTRODUCTION

1.1 General introduction

Stress activates pleiotropic homeostatic control mechanisms including the immune, central nervous, and metabolic control systems. Traditionally the glucocorticoid hormones, released from the adrenal gland in response to stress, were thought to enhance these normal defence mechanisms. However, since the 1940's the glucocorticoid hormones, especially from evidence of their anti-inflammatory actions, were increasingly perceived as feedback inhibitors of these adaptive responses to stress. Indeed if left unchecked these defence mechanisms can promote collapse of body homeostasis. In the seminal review of 1984, Allan Munck and collaborators proposed a new conceptual framework for the physiological role of glucocorticoid hormones in stress. As stated by these workers:

> "....the physiological function of stress-induced increases in glucocorticoid levels is to protect not against the source of the stress itself, but against the normal defense reactions that are activated by stress,.....glucocorticoids accomplish this function by turning off those defense reactions, thus preventing them from overshooting and themselves threatening homeostasis." (Munck, Guyre & Holbrook,1984)

To this end glucocorticoids affect a bewilderingly wide range of physiological control systems including modulation of carbohydrate metabolism, body fluid balance, neural activity as well as the extensively characterised effects on the immune system including anti-inflammatory and immunosuppressive actions (see Table 1.1) (Munck *et al.*,1984; Munck, Mendel, Smith & Orti,1990).

Glucocorticoids exert their effects in at least two phases which will be termed *early* (occurring within minutes to ~3h after glucocorticoid administration) and *late* (requiring

>6h to days) in this thesis. *Early* glucocorticoid inhibition can be demonstrated in a wide range of cell types and may involve a generic mechanism of glucocorticoid action requiring induction of new mRNA and protein. Examples of early inhibition include suppression of glucose transport in lymphocytes, inhibition of adrenocorticotrophin hormone (ACTH) release from anterior pituitary corticotrophs and inhibition of interleukin 1 (IL-1) and IL-2 secretion by cells of the immune system (macrophages and T cells respectively). *Late* inhibition generally involves suppression of differentiated cell function such as suppression of proopiomelanocortin (POMC) synthesis in anterior pituitary corticotrophs and induction of apoptosis in lymphocytes *cf* (Jones & Gillham,1988; Keller-Wood & Dallman,1984; Munck *et al*,1984).

Release of glucocorticoids into the peripheral circulation in response to stress is mediated through activation of the hypothalamic-pituitary-adrenal (HPA) axis with the resultant inhibition of stress-activated defence mechanisms. Increasing evidence suggests that these homeostatic mechanisms, such as the immune response, in turn directly modulate the activity of the HPA axis (Bateman, Singh, Kral & Solomon,1989; Munck *et al.*,1984). The HPA axis is a complex multi-component physiological reflex arc composed of afferent inputs, central processing and integration, efferent output and several levels of negative feedback regulation as depicted schematically in Figure 1.1. The physiological role of the HPA axis is to tightly regulate plasma glucocorticoid levels in order to maintain cellular homeostasis in response to changes in the environment (for reviews see Dallman, Akana, Cascio *et al.*,1987; Jones & Gillham,1988; Keller-Wood & Dallman,1984).

Activation of the HPA axis results in release of stimulus specific 'cocktails' of the major hypothalamic secretagogues, corticotrophin releasing factor (CRF-41) and arginine vasopressin (AVP), into the hypothalamo-hypohysial portal circulation, that stimulate the release of ACTH from anterior pituitary corticotrophs. In turn, ACTH

drives the release of glucocorticoids (principally cortisol in man, and corticosterone in rodents) from the adrenal glands. As well as counteracting stress-induced homeostatic defence mechanisms (Munck *et al.*,1984) glucocorticoids act to regulate their own production by feedback inhibition of HPA axis activity at several feedback sites, including the anterior pituitary corticotroph. Further inhibitory control of HPA axis activity is provided by higher brain centres, controlling CRF-41 and AVP release, as well as purported hypothalamic inhibitors of ACTH release such as atriopeptin (ANF) (Antoni, Hunter, Lowry *et al.*,1992b; Dallman *et al.*,1987; Fink, Dow, Casley *et al.*,1992; Jones & Gillham,1988; Keller-Wood & Dallman,1984).

Table 1.1

Physiological role of glucocorticoids in stress

	Glucocorticoid action	Examples of mediators inhibited
Anti-inflammatory & Immunosuppression	Suppression of cytokine and inflammatory mediator release. Inhibition of macrophage & T-lymphocyte activity	γ-interferon Interleukin-1 Interleukin-2 Tumor necrosis factor Bradykinin Histamine
Carbohydrate metabolism	Stimulates gluconeogenesis and glucagon secretion. Inhibits glucose uptake and insulin release.	Insulin
Fluid Balance	Promotes fluid secretion	Arginine vasopressin
Neural- and pituitary system	Inhibition of hypothalamic-pituitary-adenal axis	Corticotrophin releasing factor Arginine vasopressin Adrenocorticotrophin ß-endorphin

Table 1.1: See section 1.1 and references therein for discussion.

Figure 1.1

The hypothalamic-pituitary-adrenal axis

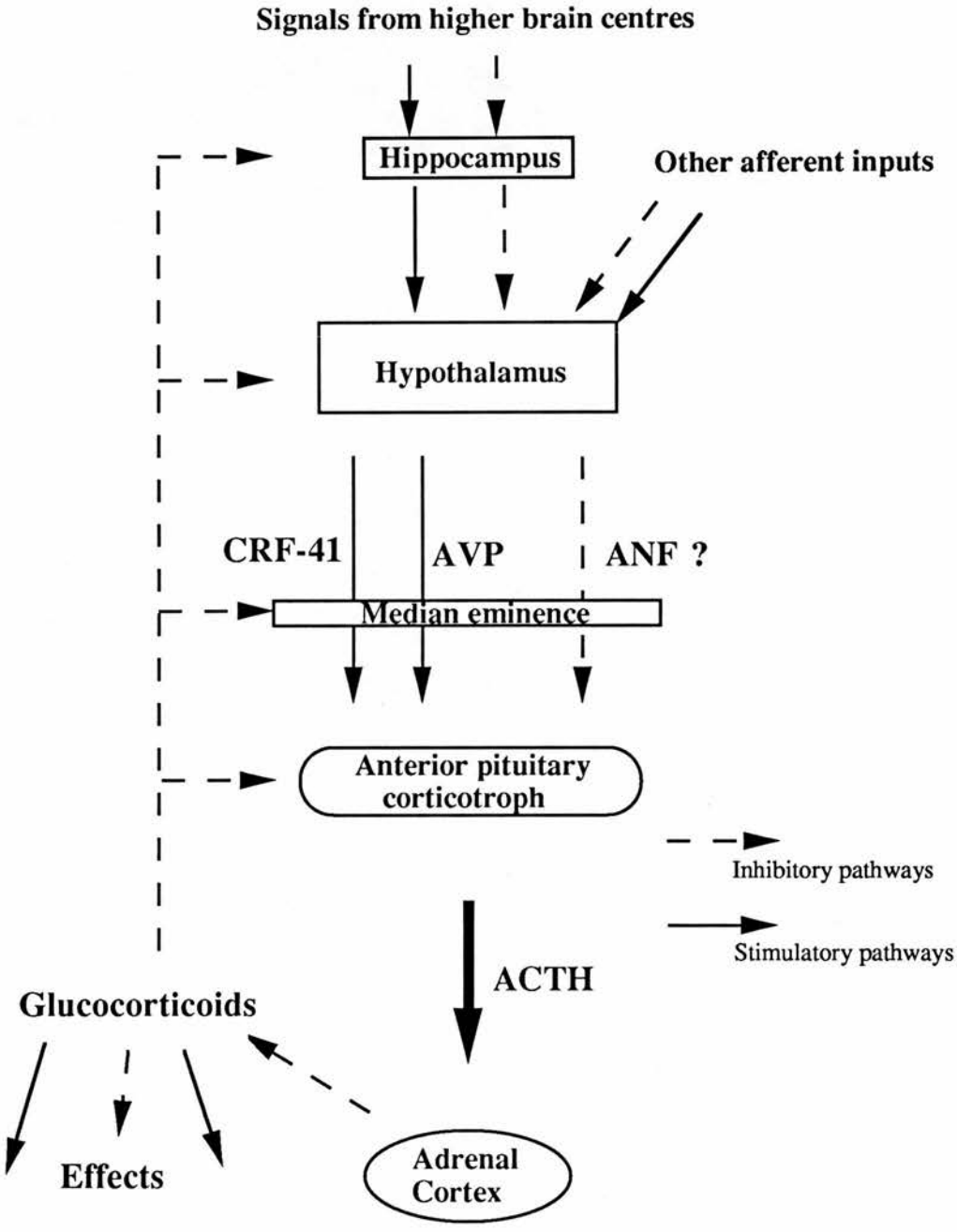


Figure 1.1: CRF-41, 41-residue corticotrophin releasing factor; AVP, arginine vasopressin; ANF, atrial natriuretic factor; ACTH, adrenocorticotrophin.

1.2 The anterior pituitary corticotroph as a model of early glucocorticoid inhibition

As the anterior pituitary corticotroph is central to the integration of HPA axis function, and is a target for HPA axis feedback regulation by glucocorticoids (Jones & Gillham,1988; Keller-Wood & Dallman,1984; Keller-Wood, Leeman, Shinsako & Dallman, 1988) it is a physiologically relevant model in which to explore early glucocorticoid inhibition of cellular function. Furthermore, the corticotroph is a relatively simple system that is accessible to experimental manipulation *in vitro*: the output signal (ACTH) is physiologically relevant and measurable; the corticotroph cell, along with clonal cell lines, are well defined; and the action of ACTH secretagogues is well explored. Thus, the anterior pituitary corticotroph provides a unique system in which to study the interaction between glucocorticoid hormones and neuropeptides (in this case CRF-41 and AVP) in the regulation of cellular function.

As the *in vivo* action of secretagogues and glucocorticoids is complex (see later sections) several *in vitro* corticotroph model systems have been employed to elucidate the intracellular mechanisms of ACTH secretagogue and glucocorticoid action. Such models include perifusion and static incubation of acutely prepared pituitary fragments or dispersed pituitary cells as well as primary cultures of dissociated cells. The advantages and disadvantages of the various systems employed have been extensively reviewed previously (Antoni,1986; Antoni,1992; Watanabe & Orth,1988). Briefly, perifused corticotrophs are more responsive to ACTH secretagogue stimulation than cells in static incubation. Furthermore, dispersion of cells by enzymatic digestion and culturing of corticotrophs for several days probably alters secretagogue and glucocorticoid efficacy through modulation of cell surface receptor/ion channel populations and intracellular pathways. A further model, that responds to CRF-41

and has been extensively used in the characterization of *late* glucocorticoid inhibition, is the mouse clonal corticotroph cell line, AtT20, with various subclones (Sabol,1980; Tashjian Jr.,1979; Yasumura,1968). Unfortunately, although some investigators have described biological responses to AVP in this cell line (Amechi, Norman & Gillham,1991; Johnson, Longenecker, Baxter *et al.*,1982), the majority of workers fail to demonstrate any action of AVP precluding the analysis of the intracellular pathways activated by AVP. However, activation of the protein kinase C pathway, that mediates the action of AVP in normal corticotrophs (see section 1.4.3), stimulates ACTH secretion in AtT20 corticotrophs. The models employed in this thesis are discussed in the relevant results section.

1.3 Aims and Objectives of the Thesis

The aim of this thesis is to further the understanding of the mechanism of early glucocorticoid action using the inhibition of stimulated ACTH secretion from the anterior pituitary corticotroph *in vitro* as an experimental model. Using this system it is hoped that the fundamental mechanisms of early glucocorticoid action in corticotrophs can be exposed and correlated with other model systems (such as lymphocytes) to formulate a potentially generic model of early glucocorticoid action. Furthermore, characterization of glucocorticoid action at the anterior pituitary corticotroph should provide insights into glucocorticoid action at other relatively inaccessible regulatory loci such as the central nervous system.

This thesis has two fundamental objectives that were designed to compliment additional ongoing functional studies of early glucocorticoid action in this laboratory:

1) To determine the characteristics of early glucocorticoid inhibition of CRF-41 and AVP-stimulated ACTH release - in particular to determine the nature and function of putative glucocorticoid-induced proteins in the corticotroph. As corticotrophs represent only ~5% of the total cell population of the anterior pituitary and glucocorticoids affect virtually all cells in the gland, characterization of early glucocorticoid induced proteins is more likely to succeed if a nominally homogenous corticotroph cell line such as the AtT20 cell line is used.

2) To examine whether the hypothalamic ACTH secretagogues, CRF-41 and AVP, interact with glucocorticoids at the level of the anterior pituitary corticotroph to modulate early glucocorticoid inhibition. Furthermore, because the mechanism of early glucocorticoid inhibition is largely unknown investigation of glucocorticoid/secretagogue interactions in the corticotroph could provide useful

information for the identification of glucocorticoid-induced proteins involved in early inhibition.

The rest of this section provides a brief overview of the actions of the ACTH secretagogues, CRF-41 and AVP, followed by a review of the currently proposed mechanisms of early glucocorticoid inhibition in anterior pituitary corticotrophs. A summary of this introduction is presented in section 1.7 and the aims and objectives of the thesis reiterated.

1.4 Mechanism of CRF-41- and AVP- stimulated ACTH secretion

1.4.1 *General considerations*

The major physiological hypothalamic ACTH secretagogues are 41-residue corticotrophin releasing factor (CRF-41) and arginine vasopressin (AVP) (Antoni,1986; Antoni,1992; Plotsky,1991). The functional neuroanatomy of the hypothalamic pathways controlling pituitary ACTH secretion has been extensively reviewed (Antoni,1986; Antoni,1989; Antoni,1992; Plotsky,1991). Over the last decade the 'final common pathway' involving a single hypothalamic projection to the anterior pituitary has given way to a more complex, multiple pathway, of hypothalamic regulation. Anatomical studies suggest that three pathways expressing CRF-41 and/or AVP exist in the hypothalamus (see Figure 1.2): 1) the parvocellular CRF-41 pathway (CRF+/AVP-); 2) the parvocellular CRF-41 and AVP pathway (CRF+/AVP+) that contains neurones expressing AVP and CRF-41 co-packaged in the same secretory granules (Bertini & Kiss,1991; Whitnall, Mezey & Gaiser,1985) and 3) the magnocellular AVP pathway.

Increasing evidence suggests that the various distinct hypothalamic secretagogue pathways may be activated in a stressor specific manner (for reviews see Antoni,1986; Antoni,1992; Plotsky,1991). For example, cytokines such as interleukin I may preferentially activate the parvocellular CRF+/AVP- system *in vivo* and *in vitro* (for example see Bateman *et al.*,1989; Berkenbosch, Van Oers, Del Rey *et al.*,1988; Gaillard, Spinedi, Goya *et al.*,1990; Sapolsky, Rivier, Yamamoto *et al.*,1988 although see Whitnall, Perlstein, Mougey & Neta,1992), whereas ketamine anaesthesia in sheep may preferentially activate the magnocellular AVP system *in vivo* (Canny, Funder & Clarke,1989).

Figure 1.2

Hypothalamic pathways regulating adrenocorticotrophin (ACTH) secretion from the anterior pituitary gland

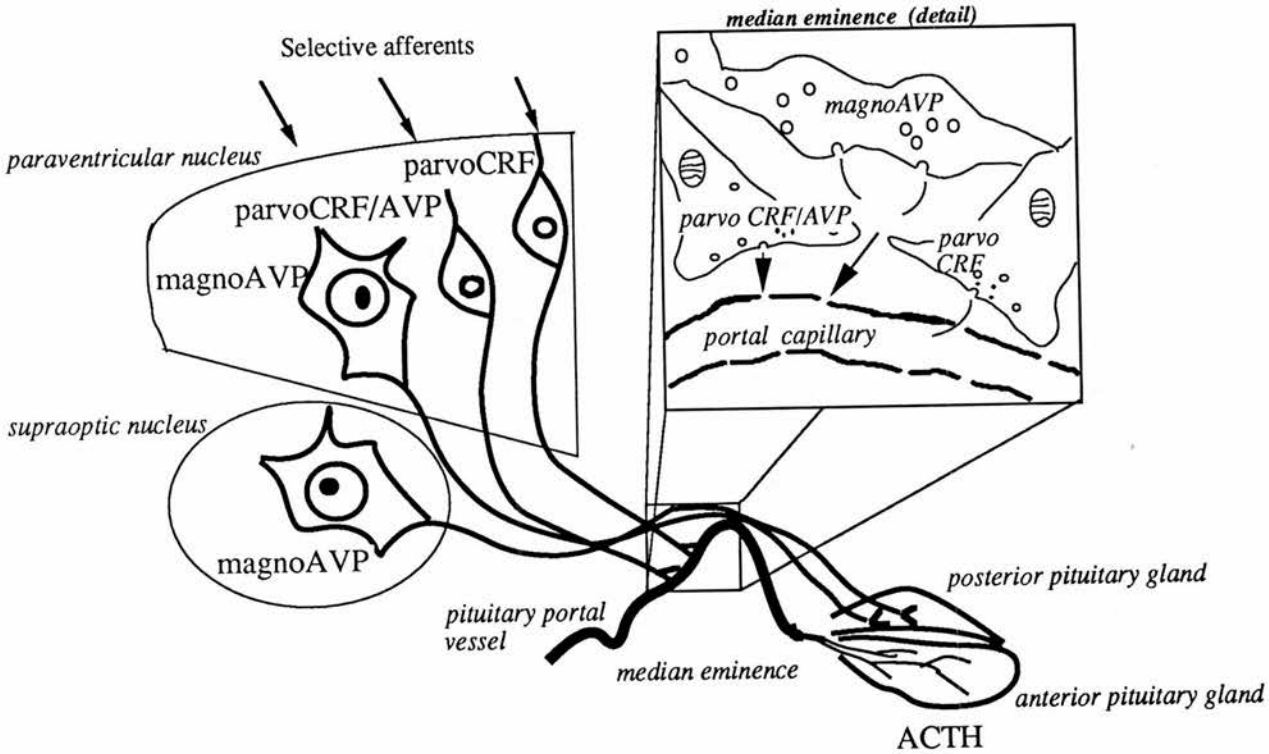


Figure 1.2: See section 1.4.1 and references therein for description of pathways. Parvocellular CRF and CRF/AVP neurones project to the median eminence and release CRF and AVP into the portal vessels that perfuse the anterior pituitary. Magnocellular AVP neurones project to the posterior pituitary and may also release AVP into the portal vessels in the median eminence *en passant*. Key: magno, magnocellular neurones; parvo, parvocellular neurones; AVP, arginine vasopressin; CRF, corticotrophin releasing factor; ACTH, adrenocorticotrophin.

1.4.2 *The anterior pituitary corticotroph as the target cell type- evidence for multiple corticotroph subpopulations*

Anterior pituitary corticotrophs represent approximately 5% of the total anterior pituitary cell population and are distributed throughout the gland (Westlund, Aguilera & Childs,1985). A brief overview of evidence for multiple corticotroph cell populations is presented here (for further reviews see Antoni,1992; Childs,1992; Schwartz,1990). It has been suggested on the basis of morphological, biochemical and functional evidence that the corticotroph cell population may consist of heterogeneous cell types (for example see Childs,1987; Fremeau Jr. & Lundblad,1986; Hatfield, Daikh, Adelman *et al.*,1989; Schwartz,1990; Tanaka & Kurosumi,1986).

The strongest support for such a subpopulation hypothesis has come from functional studies exploring the secretory response of corticotrophs to different secretagogues. Results obtained using the reverse haemolytic plaque assay (RHPA), which allows secretion from individual corticotrophs to be examined, has led several workers to postulate the existence of cell populations differentially responsive to CRF-41 and/or AVP (Childs & Burke,1987; Childs & Unabia,1989; Jia, Canny, Orth & Leong,1991; Leong,1988). Jia & co-workers (Jia *et al.*,1991), using a double RHPA demonstrated three distinct corticotroph cell populations in acutely dispersed rat anterior pituitary cells: 1) cells responsive to CRF-41 alone (approximately 22% of the total ACTH secreting cell population); 2) cells responsive to both CRF-41 and AVP (58%) and, 3) cells responsive to only a combination of CRF-41 and AVP but not either peptide alone (12%), the remaining 8% of cells released ACTH spontaneously. No evidence for cells selectively responsive to AVP alone were observed. Whether the lack of solely AVP responsive corticotrophs is a result of using enzymatically dispersed

pituitary cells, high levels of AVP exposure in these studies (100nM for 2h) that probably desensitizes the AVP response *cf* (Antoni,1986; Antoni,1992), or is dependent on the species used (for example see Schwartz & Vale,1988) remains to be clarified.

Increasing evidence suggests that the purported corticotroph subpopulations are dynamic. For example, Childs & co-workers have reported that CRF-41 pretreatment increases the percentage of cells that are subsequently responsive to AVP and *vice versa* (for review see Childs,1992). Although this view remains controversial (for example see Canny, Jia & Leong, 1992) such recruitment of corticotrophs may underlie the reported subpopulation of corticotrophs that responds only to CRF and AVP in combination (Jia *et al.*,1991).

A recent RHPA study by Canny and co-workers in rat corticotrophs (Canny, Jia & Leong,1992) suggests that increasing the concentration of CRF-41 or AVP generates distinct ACTH secretory responses from single corticotrophs. CRF-41 stimulated ACTH release in a concentration-dependent, graded, fashion from all CRF-41 responsive corticotrophs, no recruitment of corticotrophs at increasing CRF-41 concentrations was observed. In contrast, the amount of ACTH released by individual corticotrophs in response to AVP appeared constant, elevating the concentration of AVP appeared to increase the number of cells responding. Whether these characteristics reflect different pools of ACTH mobilized by AVP and CRF-41 (Schwartz, Pham & Funder,1990) that may, or may not, reside in discrete corticotroph subpopulations remains to be explored.

1.4.3 Intracellular mechanisms of CRF-41- and AVP- stimulated ACTH secretion

Although many investigators report that AVP is a weaker ACTH secretagogue than CRF-41 this probably reflects the different model systems and species used in the

study of ACTH secretagogue action. For example AVP is more potent in ovine than rat corticotrophs and is more effective in acutely dispersed perifused cells than static primary culture incubations (for reviews see Antoni,1986; Antoni,1992; Watanabe & Orth,1988).

Several recent reviews of the intracellular pathways involved in CRF-41 and AVP stimulation of ACTH secretion have been presented (Antoni,1992; King & Baertschi,1990). The intracellular second messenger pathways involved in secretagogue-stimulated ACTH secretion are summarised in Figure 1.3.

41-residue corticotrophin releasing factor (CRF-41)

Since the elucidation of the structure of CRF-41 by Vale & co-workers in 1981 (Vale, Spiess, Rivier & Rivier,1981) a wide body of pharmacological and biochemical evidence has suggested that the CRF-41 receptor is a member of the guanine nucleotide binding protein (G-protein) coupled receptor family linked to the adenylate cyclase system through a G_s type G-protein (King & Baertschi,1990; Taylor,1990).

CRF-41 stimulates accumulation of intracellular cAMP with subsequent activation of cAMP-dependent protein kinase (PKA) (King & Baertschi,1990; Reisine, Rougon & Barbet,1986; Reisine, Rougon, Barbet & Affolter,1985; Schecterson & McKnight,1991; Taylor & Buecler,1990). The mechanisms regulating secretion downstream of PKA activation are unclear, however, several substrates for PKA have been identified in AtT20 D16v cells *cf* (King & Baertschi,1990) including a 19kD family of proteins (now termed Stathmin) that may mediate secretagogue action in a variety of secretory cells (Doye, Soubrier, Bauw *et al.*,1989; Pasmantier, Danoff, Fleischer & Schubart,1986; Sobel,1991; Sobel, Boutterin, Beretta *et al.*,1989).

Figure 1.3

Intracellular second messenger pathways mediating CRF-41- and AVP-stimulated ACTH release

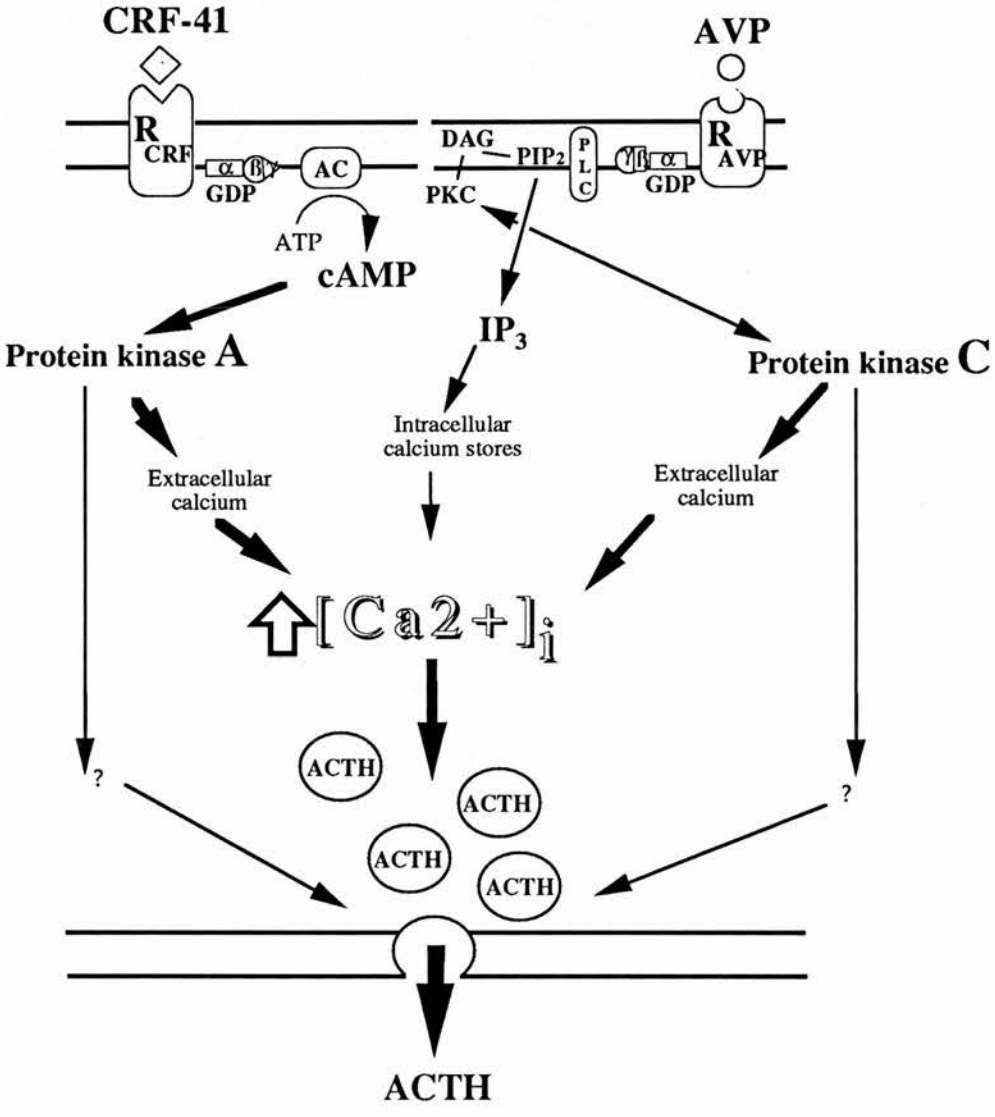


Figure 1.3: See section 1.4.3 for description of intracellular pathways.
Key: AVP, arginine vasopressin; CRF-41, corticotrophin releasing factor; R, receptor; PLC, phospholipase C; AC, adenylate cyclase; PIP_2, phosphatidylinositol bisphosphate; DAG, diacylglycerol; cAMP, cyclic adenosine monophosphate; IP_3, inositol 1,4,5 triphosphate; Ca^{2+}, calcium; ACTH adrenocorticotrophin.

Although some workers have reported that CRF-41 can elicit ACTH release in the absence of Ca^{2+} (Oki, Peatman, Qu & Orth,1991) the majority of evidence to date demonstrates that Ca^{2+} influx through voltage sensitive L and T type Ca^{2+}-channels is an essential component of CRF-41-stimulated ACTH release (Abou-Samra, Catt & Aguilera,1987a; Childs, Marchetti & Brown,1987; Guild & Reisine,1987; Luini, Lewis, Guild *et al.*,1985) in accordance with the Ca^{2+} hypothesis of exocytosis (Almers,1990; Knight, von Grafenstein & Athayde,1989). Furthermore, cobalt, that blocks Ca^{2+} channels, completely prevents CRF-41-stimulated ACTH release (Vale *et al.*,1981) and cAMP cannot stimulate ACTH release in electrically permeabilized AtT20 D16:16 cells maintained in submicromolar levels of Ca^{2+} (Guild,1991).

Corticotrophs from human adenomas and AtT20 D16:16 cells display spontaneous Ca^{2+} transients in the basal state, the frequency and/or amplitude of these transients increase in response to CRF-41 (Antoni, Hoyland, Woods & Mason,1992a; Guérineau, Corcuff, Tabarin & Mollard,1991), however, the relationship between modulation of these Ca^{2+} transients and ACTH secretion remains to be fully elucidated *cf* (Korn, Bolden & Horn,1990). To date evidence for spontaneous Ca^{2+} transients in normal corticotrophs is conflicting (Guérineau *et al*, (1991); Link, Dayanithi, Föhr & Gratzl, 1992) although differences in experimental protocol, such as incubation temperature, may account for these contradictory data. Current evidence suggests that basal (unstimulated) ACTH secretion involves both constitutive and regulated release of ACTH (Burgess & Kelly,1987; Matsuuchi & Kelly,1991). In AtT20 D16v cells the majority (approximately 60%) of basal (unstimulated) secretion is through the regulated pathway (Matsuuchi & Kelly,1991). Thus spontaneous Ca^{2+} transients, and by inference CRF-41-stimulated changes in transient frequency, may play a role in ACTH secretion. However, a word of caution is advisable at this point as the AtT20 D16v cell (as with other clonal pituitary cell lines) displays a higher basal level of

ACTH secretion than normal corticotrophs (Sabol,1980).

The exocytotic events leading to secretion are not understood in the corticotroph. In AtT20 cells GTP-binding proteins have been suggested to have a stimulatory (Guild,1991) as well as inhibitory (Luini & De Matteis,1988; Luini & De Matteis,1990) regulatory role. The nature and the role of such proteins and other components of the exocytotic machinery in mediating CRF-41-stimulated ACTH secretion from the corticotroph are poorly understood (for reviews of the exocytotic machinery see Ahnert-Hilger, Bhakdi & Gratzl,1991; Almers,1990; Aunis & Bader,1988; Schweizer, Schäfer & Burger,1991; Thomas, Suprenant & Almers,1990).

Arginine vasopressin (AVP)

The lack of an homogenous corticotroph system has hampered study of the intracellular mechanisms of AVP action. Thus, to date, all studies of the second messenger pathways activated by AVP have been performed on mixed pituitary cell populations. Important in this regard is the observation that approximately 20% of anterior pituitary AVP binding sites are associated with thyrotropes (Childs, Westlund & Unabia,1989; Lumpkin, Samson & McCann,1987); thus all data for second messengers involved in AVP action must be viewed with a degree of caution. An outline of the intracellular second messenger pathways thought to be involved in AVP-stimulated ACTH secretion is shown in Figure 1.3.

In the anterior pituitary gland AVP increases the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) (Raymond, Leung, Veilleux & Labrie,1985; Todd & Lightman,1987) presumably to increase levels of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). Pituitary AVP receptors are pharmacologically distinct from the classical V_1 receptors and termed $V_{1\beta}$ (Antoni,1984; Antoni,1987; Du Pasquier,

Dreifuss, Dubois-Dauphin & Tribollet,1991). Although the $V_{1\beta}$ receptor has not been isolated or cloned it is likely to be a member of the G-protein coupled receptor family as recently verified for the rat $V_{1\alpha}$ (Morel, O'Carroll, Brownstein & Lolait,1992) as well as the rat and human V_2 AVP receptors (Birnbaumer, Seibold, Gilbert *et al.*,1992; Lolait, O'Carroll, McBride *et al.*,1992).

Using dispersed anterior pituitary cells in a microperifusion system Orth and co-workers (Watanabe, Oki & Orth,1989; Won & Orth,1990) have observed biphasic ACTH release in response to AVP. The initial phase is associated with an elevation of intracellular Ca^{2+} from IP_3-sensitive stores and the more sustained phase is dependent on the influx of extracellular Ca^{2+} through L type Ca^{2+} channels. The initial phase of ACTH release is only evident at high (>100nM) AVP concentrations, thus whether mobilization of intracellular Ca^{2+} stores is a pre-requisite for establishment of the sustained phase of ACTH secretion at physiological AVP concentrations is unknown (Leong,1988; Watanabe *et al.*,1989; Won & Orth,1990). The activation of PKC (through the action of DAG and increase in Ca^{2+}) is essential for the secretagogue activity of AVP (Carvallo & Aguilera,1989; Oki, Nicholson & Orth,1990), furthermore downregulation of PKC by phorbol ester pretreatment (that activates PKC in a similar manner to DAG) blocks the ACTH response to a subsequent AVP stimulus (Bilezikjian, Woodgett, Hunter & Vale,1987).

Although several PKC substrates have been identified, primarily in AtT20 cells *cf* (King & Baertschi,1990) events downstream of PKC activation and Ca^{2+} influx that regulate ACTH secretion are unknown.

Synergism of AVP- and CRF-41- stimulated ACTH release

Release of ACTH in response to co-administration of AVP and CRF-41 in excess of that released by either secretagogue alone has been unequivocally demonstrated *in*

vivo, although such synergism is not universally reported *in vitro* probably as a consequence of the different models employed *cf* (Antoni,1986). Although AVP and phorbol esters potentiate CRF-41-stimulated accumulation of cAMP (Abou-Samra, Harwood, Manganiello *et al.*,1987b; Carvallo & Aguilera,1989; Cronin, Zysk & Baertschi,1986; Giguere & Labrie,1982; Lutz-Bucher, Félix & Koch,1990) the mechanism of synergy is poorly understood. Increasing evidence suggests that modulation of signal transduction pathways occurs by 'crosstalk' at different levels between components of separate second messenger systems *cf* (Burgess, Bird, Obie & Putney Jr,1991; Houslay,1991) thus several sites downstream of cAMP accumulation may also be involved. For example, synergy between AVP and 8-bromo-cAMP has been reported in primary cultures of rat anterior pituitary cells (Bilezikjian & Vale,1987).

1.4.4 *Conclusion*

CRF-41 and AVP are the major hypothalamic secretagogues that act at the anterior pituitary corticotroph to stimulate ACTH release. Activation of the HPA axis releases 'stimulus-specific cocktails' of secretagogue into the hypophyseal-portal circulation. Indirect evidence suggests that corticotrophs differentially responsive to CRF-41 and AVP exist. CRF-41 and AVP activate the cAMP/PKA and inositol phosphate/PKC intracellular pathways respectively and elevate intracellular free calcium levels to stimulate the exocytosis of ACTH. Mechanisms downstream of intracellular calcium regulating ACTH secretion from the corticotroph are poorly understood.

1.5 Early glucocorticoid inhibition of pituitary ACTH secretion

1.5.1 *General considerations*

Glucocorticoids inhibit HPA activity *in vivo* in several time domains and at several loci including the hippocampus, hypothalamus and anterior pituitary corticotroph (see Figure 1.1, section 1.1, p5) (Dallman *et al.*,1987; Jacobson & Sapolsky,1991; Jones & Gillham,1988; Keller-Wood & Dallman,1984).

The complexity of feedback regulation *in vivo* has precluded the direct investigation of the mechanisms of early inhibition at the pituitary level using whole animal paradigms. For example, some stressors appear to be relatively resistant to inhibition (eg laparotomy) as their afferent inputs bypass several feedback sites whereas other stressors act through multiple feedback sites (eg exposure to ether) (Keller-Wood & Dallman,1984). Consequently, in order to determine the cellular mechanisms of early glucocorticoid inhibition at the pituitary gland several *in vitro* models of anterior pituitary corticotroph function have been developed as described in section 1.2. Important in this regard is the fact that glucocorticoids *in vivo* are reported to act in at least three distinct time domains namely: fast (rate sensitive),occurring within seconds to minutes of glucocorticoid exposure; intermediate (rapid & delayed) evident between 0.25h-3h of application; and late (slow) evident after several hours or days. The characteristics of these time domains are summarised in Table 1.2. Fast and intermediate inhibition suppress stimulated ACTH secretion from the corticotroph with no effect on cellular stores of hormone. In contrast, late inhibition generally results through reduction in intracellular ACTH stores, through suppression of POMC gene transcription, and downregulation of receptor/effector systems (Dallman *et al.*,1987; Jacobson & Sapolsky,1991; Jones & Gillham,1988; Keller-Wood & Dallman,1984).

Table 1.2

***In vivo* characteristics of glucocorticoid feedback inhibition of the hypothalamic-pituitary-adrenal (HPA) axis**

	Fast	Intermediate (rapid and delayed)	Slow
Time Domain	secs-mins	0.25h-3h	>6h
Rate Sensitive	Yes	No	No
mRNA and Protein Synthesis	⇔	⇑	⇓
Secretagogue and ACTH release	⇓	⇓	⇓
Intracellular ACTH and secretagogue stores	⇔	⇔	⇓
Site of action	Hippocampus Hypothalamus	Hippocampus Hypothalamus Pituitary	Hippocampus Hypothalamus Pituitary

Table 1.2: For further information and review of the literature see references cited in section 1.5

Table 1.3

Characteristics of glucocorticoid inhibition at the anterior pituitary corticotroph

	Early (rapid & delayed)	**Late** (slow)
Time Domain	0.25h-3h	>6h
mRNA and Protein Synthesis	Induction (proteins unknown)	Generally suppression eg proopiomelanocortin (POMC)
ACTH release:		
Basal	⇔	⇓
Stimulated	⇓	⇓
ACTH stores	⇔	⇓
Mechanism(s)	Suppression of intracellular free Ca^{2+} ?	Suppression of gene transcription eg POMC
	Enhancement of K^+ currents ?	Downregulation of receptor/effector systems

Table 1.3 For further information and review of the literature see section 1.5 and references cited therein

As discussed in the following sections, and summarised in Table 1.3, glucocorticoid feedback at the *anterior pituitary corticotroph* <u>*in vitro*</u> is best described by two time domains namely:

1) ***Early*** (rapid & delayed) apparent within 10min-3h after glucocorticoid exposure resulting through inhibition of stimulated ACTH secretion but not corticotroph ACTH content and;

2) ***Late*** (slow) apparent after several hours to days resulting in depletion of intracellular ACTH stores and downregulation of signalling pathways.

The above nomenclature is used subsequently in this thesis to describe glucocorticoid regulation at the corticotroph. The well-characterised mechanisms of *late* glucocorticoid inhibition at the corticotroph have been discussed in several recent reviews (see above) and are discussed only to highlight the fundamentally different mechanism(s) involved in *early* glucocorticoid inhibition.

The following sections discuss the characteristics and mechanism(s) of early glucocorticoid inhibition of stimulated ACTH release from the anterior pituitary corticotroph.

1.5.2 <u>*Glucocorticoid receptors: structure and function*</u>

Although evidence for non-genomic (membrane) actions of the physiological glucocorticoids have been documented (for reviews see Duval, Durant & Homo-delarche,1983; Johnson *et al.*,1982; McEwen,1991; Schumacher,1990) their action is predominantly mediated through activation of intracellular receptors belonging to the steroid superfamily of ligand activated transcription factors (for extensive reviews see Carson-Jurica, Schrader & O'Malley,1990; Evans,1989; Evans & Arriza,1989;

Miesfeld,1989; Miesfeld,1990; Munck *et al.*,1990; O'Malley,1990; O'Malley, Tsai, Bagchi *et al.*,1991). The salient points of glucocorticoid receptor action with respect to early glucocorticoid inhibition and the outline of the thesis are summarised here.

In the HPA two glucocorticoid receptor systems termed mineralocorticoid (MR or type I) and glucocorticoid (GR or type II) respectively have been unequivocally identified that exert a coordinate regulation of HPA axis function (Dallman *et al.*,1987; De Kloet,1991; Jones & Gillham,1988; McEwen, De Kloet & Rostene,1986; Reul & De Kloet,1985; Reul & De Kloet,1986). In most species analysed to date type I glucocorticoid receptors are predominantly localised in the hippocampus (Jacobson & Sapolsky,1991; Reul & De Kloet,1985; Reul & De Kloet,1986) whereas type II receptors are found throughout the HPA axis including the hypothalamic parvocellular CRF-41- and magnocellular AVP- containing neurones (Fuxe, Harfstrand, Agnati *et al.*,1985; Kiss, van Ekelen, Reul *et al.*,1988) as well as the anterior pituitary corticotroph (Antakly & Eisen,1984). Functional studies suggest that basal HPA axis activity is regulated through hippocampal type I receptors (Dallman, Levin, Cascio *et al.*,1989; Jacobson & Sapolsky,1991) whereas inhibition of 'stress' responses is mediated through type II receptors predominantly in the hypothalamus and pituitary (Dallman *et al.*,1987; Jones & Gillham,1988; Reul & De Kloet,1985).

The type I and type II receptors have been cloned from a variety of species and are structurally similar to other members of the steroid receptor family *cf* (O'Malley,1990). *In vivo* the unliganded glucocorticoid receptor is thought to associate with several proteins including a 90Kd heat shock protein (hsp90) that acts as a negative transcriptional regulator preventing DNA binding of the receptor (Cadepond, Schweizer-Groyer, Segard-Maurel *et al.*,1991; Picard, Khursheed, Garabedian *et al.*,1990; Scherrer, Dalman, Massa *et al.*,1990). On binding of glucocorticoid hsp90 dissociates, the receptor dimerises and interacts with

glucocorticoid response elements (GRE) in target genes (see Figure 1.4). Considerable evidence suggests that glucocorticoid receptors undergo a phosphorylation/dephosphorylation cycle the functional role of which remains unclear although receptor reutilization and DNA transcriptional activation models (amongst others) have been proposed (Mendel, Bodwell & Munck,1986; Munck *et al.*,1990; Orti, Bodwell & Munck,1992).

To date the literature remains controversial regarding the intracellular distribution of unliganded glucocorticoid receptors (receptors bound with ligand are found exclusively in the nucleus). The classical two step model of steroid hormone action (Gorski & Gannon,1976) involving translocation of steroid receptors to the nucleus from the cytoplasm on steroid binding has lost favour for all but the glucocorticoid receptor. However, several recent studies suggest that unliganded glucocorticoid receptors have a lower affinity for nuclear structures than other unliganded steroid receptors and are thus more readily lost from the nucleus during cell disruption or fixation procedures used in localization studies (Brink, Humbel, De Kloet & Vandriel,1992; Carson-Jurica *et al.*,1990; Gasc, Delahaye & Baulieu,1989). Although the translocation model remains controversial the action of intracellular glucocorticoid receptors is unequivocally mediated through binding to GRE's present in the 5'-promoter regions of glucocorticoid responsive genes such as POMC and α-globulin (see review references above and Lundblad & Roberts,1988).

Figure 1.4

Genomic action of glucocorticoid hormones

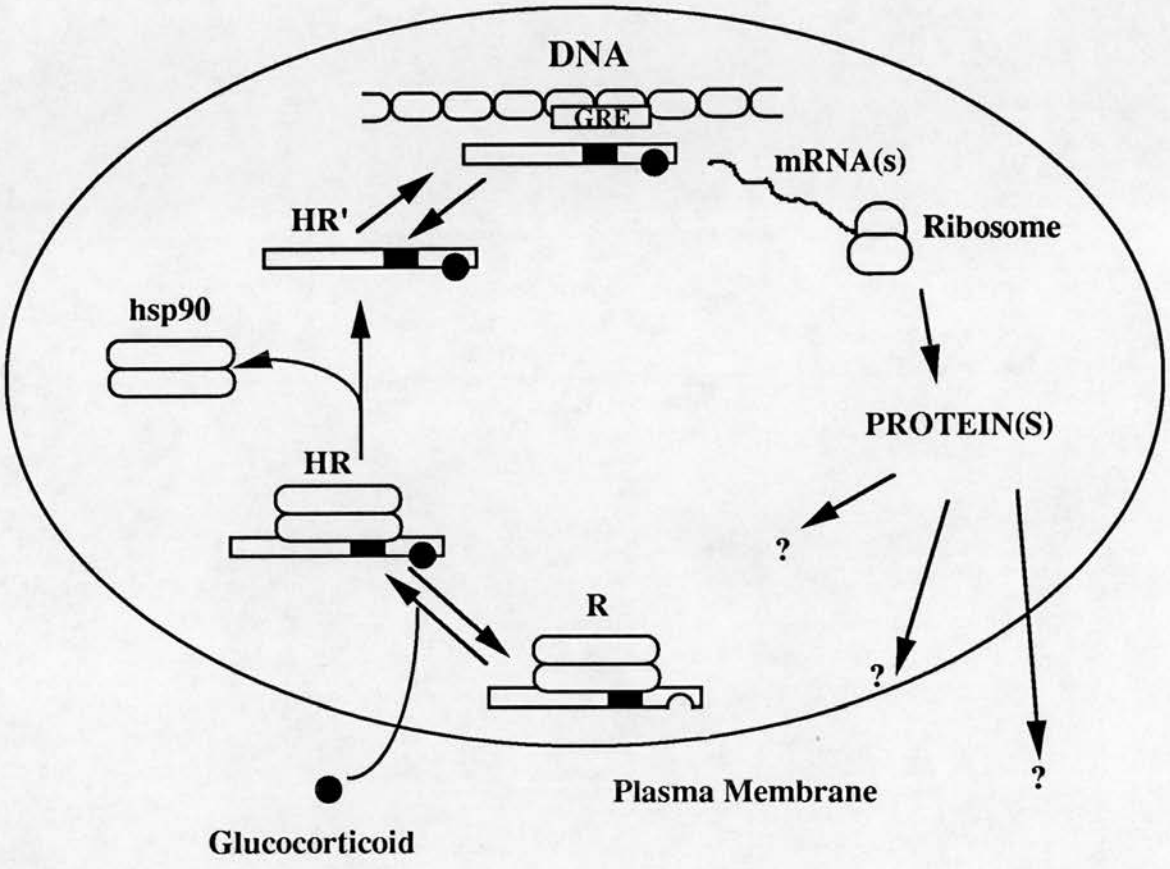


Figure 1.4: Glucocorticoid hormone enters the cell and binds to the unliganded non-activated glucocorticoid receptor (R) to form the non-activated liganded receptor complex (HR). Subsequent release of heat shock protein 90 (hsp90) activates the liganded receptor (HR') which dimerises (not shown) and can associate with nuclear glucocorticoid response elements (GREs) within target genes to activate RNA transcription. Subsequent translation of messenger RNA (mRNA) on ribosomes generates proteins that mediate the action of glucocorticoids (?). Intracellular localization of the unliganded (R) has not been defined as discussed in section 1.5.2. Diagram after Munck, Mendel, Smith & Orti (1990).

1.5.3 *Characterization of the time domain of early glucocorticoid inhibition in vitro*

Glucocorticoid inhibition of anterior pituitary ACTH release *in vitro* has been classically subdivided into three distinct time domains: rapid, developing within 30min of steroid application; delayed, evident between 30min to 3h and late, manifest after ~6h to days of glucocorticoid exposure *cf* (Dallman *et al.*,1987; Jones & Gillham,1988; Keller-Wood & Dallman,1984). The rationale for these divisions is the assumption that distinct intracellular mechanisms are responsible for inhibition in each time domain. For example, the classical view of rapid glucocorticoid action proposes non-genomic, plasma membrane associated processes, whereas the well characterised late inhibition is mediated through suppression of POMC gene transcription and modulation of signal transduction systems (for reviews see Dallman *et al.*,1987; Johnson *et al.*,1982; Jones & Gillham,1988; Keller-Wood & Dallman,1984; Lundblad & Roberts,1988; McEwen,1991). The majority of discrepancies in the literature regarding the purported differences in the mechanisms of rapid and delayed glucocorticoid inhibition are because many studies do not clearly define the time-scale of glucocorticoid exposure employed, resulting in some workers discussing "rapid" effects of steroids that were determined using overnight (>18h) steroid preincubation protocols.

Recently Dayanithi & Antoni (1989) demonstrated that both rapid and delayed glucocorticoid inhibition at the pituitary corticotroph requires activation of type II glucocorticoid receptors and induction of new mRNA and protein *ie.* a genomic action (see Figure 1.4 for model of genomic action of glucocorticoids). Thus a unifying concept of *'early"* glucocorticoid inhibition seems more useful when describing the mechanisms of glucocorticoid inhibition within the 10min-3h time-scale at the anterior pituitary corticotroph.

In the following sections evidence in support of this definition of early glucocorticoid inhibition is discussed, furthermore the putative mechanism(s) of early glucocorticoid inhibition of ACTH secretion from the corticotroph are presented. It is important to note that early glucocorticoid inhibition at the corticotroph does not display rate sensitivity (Mahmoud, Scaccione, Scraggs *et al.*,1984) and is thus distinct from the fast, rate sensitive, component observed *in vivo*.

Rapid and delayed glucocorticoid inhibition are characterised by the suppression of stimulated *cf* (Dayanithi & Antoni,1989; Phillips & Tashjian,1982; Widmaier & Dallman,1984) but not basal ACTH secretion, although some reports in both normal and clonal corticotrophs do suggest slight inhibition of basal release (for example see Buckingham & Hodges,1977; Woods, Shipston, Mullens & Antoni,1992), independently of changes in intracellular ACTH content *cf* (Abou-Samra, Catt & Aguilera,1986a; Buckingham & Hodges,1977; Phillips & Tashjian,1982; Sabol,1980). Although glucocorticoids inhibit POMC gene transcription within 15min of steroid application no reduction of intracellular ACTH levels is seen within 6h (Fremeau Jr. & Lundblad,1986; Gagner & Drouin,1985; Gagner & Drouin,1987; Lundblad & Roberts,1988; Sabol,1980).

The majority of studies performed to date report a lag time of 10-30min after glucocorticoid application for rapid inhibition to be manifest in a variety of *in vitro* corticotroph models (Abou-Samra *et al.*,1986a; Brattin & Portanova,1977; Dayanithi & Antoni,1989; Gillies & Lowry,1978; Mahmoud *et al.*,1984; Mulder & Smelik,1977; Portanova & Sayers,1974; Widmaier & Dallman,1984). However, Johnson *et al.* (1982) have reported a virtually immediate action of glucocorticoids in AtT20 cells while Familari & Funder (1989) failed to report rapid or delayed inhibition in perifused primary cultures of rat corticotrophs attached to cytodex beads. Whether

these differences are due to the characteristics of the experimental models used, or the actual experimental paradigms employed, in each study that may modulate the magnitude as well as direction of the glucocorticoid response (Johnson, Lan & Baxter,1979) remain to be established. Although these discrepancies exist it is important to note that studies investigating inhibition at the pituitary level *in vivo*, glucocorticoids inhibit CRF-41- and insulin- stimulated ACTH release within 10min in normal dogs (Keller-Wood,1990; Keller-Wood & Bell,1988).

Although some workers have reported a 'silent', steroid non-responsive, period approximately 30-50min after glucocorticoid application at the pituitary level *in vitro* and *in vivo* when the inhibitory effect of steroids is diminished (Abou-Samra *et al.*,1986a; Mahmoud *et al.*,1984; Mulder & Smelik,1977) this has not been confirmed subsequently *cf* (Dayanithi & Antoni,1989). Furthermore, as discussed by several workers the 'silent' period is not observed when the duration of glucocorticoid exposure is increased while maintaining the same time interval between the start of glucocorticoid application and the secretagogue stimulus (Mahmoud *et al.*,1984; Mulder & Smelik,1977).

Most workers characterise delayed inhibition at the pituitary level developing within 3h of glucocorticoid application *in vitro* (Abou-Samra *et al.*,1986a; Arimura, Bowers, Schally *et al.*,1969; Brattin & Portanova,1977; Buckingham & Hodges,1977; Dayanithi & Antoni,1989; Gillies & Lowry,1978; Mahmoud *et al.*,1984; Mulder & Smelik,1977; Oki *et al.*,1991; Phillips & Tashjian,1982; Woods *et al.*,1992) and *in vivo* (Keller-Wood, Leeman, Shinsako & Dallman,1988). A notable exception is the complete lack of inhibition observed *in vitro* by Familari & Funder (1989), although the lack of inhibition may be a result of the glucocorticoid treatment paradigm employed in this particular study (see section 1.5.5).

1.5.4 *Involvement of type II glucocorticoid receptors and induction of mRNA and protein synthesis in early glucocorticoid inhibition*

Abou-Samra & co-workers concluded on the basis of differences in EC_{50} values for corticosterone inhibition of CRF-41-stimulated ACTH secretion in primary cultures of rat anterior pituitary cells in static incubation that different receptor subtypes mediate rapid and delayed inhibition (Abou-Samra *et al.*,1986a). However, using specific type II glucocorticoid receptor agonists and antagonists in perifused isolated rat anterior pituitary cells Dayanithi & Antoni (1989) demonstrated that both rapid and delayed inhibition of CRF-41-stimulated ACTH release require activation of type II glucocorticoid receptors. This is in agreement with an earlier report suggesting that aldosterone inhibition of ACTH secretion is a result of this mineralocorticoid activating the type II glucocorticoid receptor (Sakly, Philibert, Lutz-Bucher & Koch,1984). The involvement of type II receptors in early inhibition has been confirmed in AtT20 D16:16 corticotrophs (Woods *et al.*,1992), a cell line which only contains type II glucocorticoid receptors (Gannon, Spencer, Lundblad *et al.*,1990).

Although the general consensus suggests that delayed glucocorticoid inhibition in a variety of cellular systems is manifest through induction of new mRNA and *de novo* protein synthesis *cf* (Abou-Samra *et al.*,1986a; Arimura *et al.*,1969; Colbert & Young,1986b; Dayanithi & Antoni,1989; Woods *et al.*,1992), the importance of such a role in rapid inhibition at the corticotroph has been controversial. Portanova & Sayers (1974) using acutely dispersed rat anterior pituitary cells in a static incubation assay reported that rapid glucocorticoid inhibition in normal rat corticotrophs is not blocked by the transcription inhibitor, actinomycin D, at concentrations that blocked >80% of RNA synthesis. Blockade was observed in pituitaries obtained from adrenalectomised rats, suggesting that a tonic induction of mRNA by glucocorticoids

in normal rats overides the action of actinomycin D. However, Dayanithi & Antoni (1989) using 20 fold higher actinomycin D concentrations than employed by Portanova & Sayers (1974) demonstrated a clear blockade of rapid glucocorticoid inhibition in normal perifused rat corticotrophs.

The requirement for *de novo* protein synthesis in rapid inhibition has also been in debate, this discrepancy is probably a result of the protein synthesis inhibitor, cycloheximide, being used in negative reports (Abou-Samra *et al.*,1986a; Brattin & Portanova,1977; Widmaier & Dallman,1983). Indeed as discussed by Brattin & Portanova (1977) and Dayanithi & Antoni, (1989) cycloheximide used at concentrations that block >85% of total pituitary protein synthesis is relatively ineffective in blocking rapid as well as delayed inhibition. Furthermore, higher cycloheximide concentrations may have a deleterious effect on stimulated ACTH secretion (Dayanithi & Antoni,1989). In contrast the protein synthesis inhibitor, puromycin (Gale, Cundliffe, Reynolds *et al.*,1981), blocks rapid (Brattin & Portanova,1977; Dayanithi & Antoni,1989) as well as delayed glucocorticoid inhibition *cf* (Dayanithi & Antoni,1989; Woods *et al.*,1992).

To date reports utilizing transcription or translation inhibitors to block early glucocorticoid action have explored the inhibition of CRF-41- or mixed secretagogue- (such as hypothalamic/median eminence extracts that contain CRF-41 as well as AVP) stimulated ACTH release. Thus it is assumed that the mechanism of inhibition of AVP-stimulated ACTH release also involves induction of new mRNA and protein. Although Phillips & Tashjian (1982) reported a requirement for protein synthesis in delayed inhibition of phorbol ester stimulated ACTH release in perifused AtT20 D16v cells no direct evidence for protein induction has been reported for glucocorticoid inhibition of AVP-stimulated ACTH release in normal corticotrophs. This question requires further clarification.

Although several studies in other systems have reported non-genomic actions of glucocorticoids, that occur within seconds to minutes (for reviews see Duval *et al.*,1983; Johnson *et al.*,1982; McEwen,1991; Schumacher,1990) the time course and requirement for mRNA and protein synthesis described above precludes such non-genomic action for both rapid and delayed inhibition at the pituitary corticotroph. Thus it seems more appropriate to describe glucocorticoid inhibition developing within 10min-3h at the anterior pituitary corticotroph under a single title of '*early*' glucocorticoid inhibition. The characteristics of early glucocorticoid inhibition at the corticotroph are similar to those seen for early glucocorticoid action in other systems such as lymphocytes and mast cells *cf* (Colbert & Young,1986a; Colbert & Young,1986b; Grosman & Jensen,1984; Harrigan, Baughman, Campbell & Bourgeois,1989; Munck *et al.*,1990). To date proteins induced by glucocorticoids in the early time domain at the anterior pituitary corticotroph have not been characterized.

1.5.5 *Modulation of early glucocorticoid inhibition*

Several workers have emphasized that stimulus context (nature and timing) may fundamentally determine the magnitude as well as direction (*ie* inhibition or stimulation) of steroid action in any particular system (De Kloet,1991; Johnson *et al.*,1979). However, few reports in the literature have explored whether the nature of the secretagogue stimulus (*ie* whether CRF-41, AVP or in combination) or the temporal relationship between secretagogue stimulation and glucocorticoid exposure are important in determining the characteristics of early glucocorticoid inhibition at the anterior pituitary corticotroph.

Using primary cultures of rat anterior pituitary corticotrophs Abou-Samra *et al.* (1986a) reported that the early inhibitory action of corticosterone on CRF-41-stimulated ACTH release was impaired if AVP was applied simultaneously with the

CRF-41 stimulus. However, this apparent "blockade" of corticosterone action by AVP is probably a consequence of the combined CRF-41/AVP stimulus releasing more ACTH than CRF-41 alone. Furthermore, the percentage inhibition of ACTH release was identical (~60% of control stimulus) for the respective stimuli (CRF-41 or CRF-41/AVP) suggesting that the efficacy of corticosterone inhibition is not modulated by AVP. Indeed, early glucocorticoid inhibition of CRF-41 and CRF-41/AVP stimulated ACTH release are identical in perifused rat corticotrophs (Antoni & Dayanithi,1990b). In a further study Childs & Unabia (1990) reported that phorbol ester pretreatment blocked early glucocorticoid inhibition of CRF-41-stimulated ACTH release and CRF-41 receptor downregulation, however, this was not mimicked by AVP. An *in vivo* study in humans also reported blockade of dexamethasone suppression of CRF-41-stimulated ACTH release by simultaneous infusion with AVP, however this study did not report the effect of combined CRF-41/AVP stimulus on ACTH release (von Bardeleben, Holsboer, Stalla & Müller,1985). Consequently the apparent 'escape' of CRF-41-stimulated ACTH release reported is probably due to the synergistic enhancement of ACTH release induced by AVP. The above indirect data, in conjunction with the well characterised multifactorial hypothalamic secretagogue stimulation of ACTH secretion (see section 1.4), point to a potential modulatory role of secretagogues on early glucocorticoid inhibition of ACTH release that requires further evaluation.

The importance of the temporal relationship between secretagogue stimulation and glucocorticoid application has been reported by Mulder & Smelik (1977), who found that corticosterone pretreatment (for 15min) was necessary to suppress ACTH release stimulated by stalk-median eminence extract in perifused rat pituitary cells. Corticosterone started simultaneously with, and maintained during, secretagogue stimulation did not result in inhibition of ACTH release. Other early *in vitro* studies

also indirectly suggest the requirement of prior steroid exposure for inhibition to develop *cf* (Arimura *et al.*,1969; Kraicer, Milligan, Gosbee *et al.*,1969) however, other studies provide conflicting evidence *cf* (Widmaier & Dallman,1984). Such a temporal requirement may explain the lack of inhibition observed by Familari & Funder (1989); this phenomenon requires detailed examination and may also provide insights into the nature and function of early glucocorticoid induced proteins.

1.5.6 *Conclusion*

The receptor pharmacology and requirement for *de novo* mRNA and protein synthesis in rapid as well as delayed glucocorticoid inhibition would suggest that similar mechanisms underlie these time domains, the purported difference between the two time domains simply reflects the extent of inhibition (Dayanithi & Antoni,1989). Consequently it is more appropriate to regard rapid and delayed inhibition as *'early'* inhibition when discussing the mechanism of glucocorticoid action that occurs within 3h *at the level of the pituitary*. This would prevent confusion with *in vivo* data reporting fast, rate sensitive, effects of steroids (seconds to minutes) on HPA function that result from inhibition of other components of the HPA axis such as the hypothalamus and hippocampus. Indirect evidence suggests that the characteristics of early glucocorticoid inhibition at the corticotroph is dependent on the nature (whether CRF-41 or AVP) and timing of secretagogue application. No direct evidence for the involvement of mRNA or protein induction has been presented for early inhibition of AVP-stimulated ACTH release. To date early glucocorticoid-induced proteins have not been characterized in the corticotroph. Analysis of the characteristics of early glucocorticoid inhibition of CRF-41 and AVP stimulated ACTH release may provide valuable evidence as to the nature of the glucocorticoid induced protein(s).

1.6 Mechanism(s) of early glucocorticoid inhibition

1.6.1 *General considerations*

How do the putative glucocorticoid-induced proteins inhibit stimulated ACTH secretion in the early time domain?

As discussed in section 1.4 secretagogue stimulation of ACTH exocytosis is not well defined. However, the multiple pathways and levels involved provide numerous sites at which glucocorticoids may exert their inhibitory effect. Important in this respect is the fact that most workers, using a wide variety of experimental models, do not find consistent inhibition of basal ACTH secretion by glucocorticoids. Thus some aspect of stimulus-secretion coupling must be impaired. As discussed earlier, most of the discrepancies in the literature relating to early glucocorticoid action have arisen due to the glucocorticoid exposure protocols employed.

The demonstration of early glucocorticoid inhibition in the corticotroph cell line AtT20 (Antoni *et al.*,1992a; Phillips & Tashjian,1982; Woods *et al.*,1992) suggests that paracrine interactions between different anterior pituitary cell types do not play an important role in early inhibition. Thus, glucocorticoids must interact with target genes within corticotrophs to generate the putative proteins that characterise early inhibition. The rest of this section discusses the currently proposed mechanism(s) of early glucocorticoid inhibition at the corticotroph.

1.6.2 *Modulation of receptor / signal transduction pathways*

Childs & co-workers reported a reduction of CRF-41 plasma membrane binding sites within 10min of glucocorticoid application in enriched primary cultures of rat corticotrophs (Childs & Unabia,1990). Although such downregulation is observed in

late inhibition *cf* (Bilezikjian, Blount & Vale,1987; Rivier & Vale,1987) it is unlikely to be involved in early inhibition. Studies of late glucocorticoid inhibition have reported inhibition of CRF-41-stimulated cAMP accumulation (Bilezikjian *et al.*,1987; Bilezikjian & Vale,1983) *in vitro* , however, other reports on CRF-41-stimulated cAMP accumulation *in vitro* and *in vivo* do not support this hypothesis (Giguere, Labrie, Côté *et al.*,1982; Kant, Mougey, Brown & Meyerhoff,1989). To date no direct evidence for glucocorticoid modulation of secretagogue-stimulated signal transduction pathways in early inhibition has been documented. Furthermore, it is important to note that ACTH secretion is not necessarily impaired when CRF-41 receptors and cAMP responses are downregulated *cf* (Aguilera, Wynn, Harwood *et al.*,1986) suggesting that possible modulation of receptor/signal transduction systems by glucocorticoids are not necessarily involved in ACTH secretion inhibition. Numerous studies report that ACTH secretion stimulated by protein kinase activators are inhibited by glucocorticoids *cf* (Abou-Samra *et al.*,1986a; Abou-Samra, Catt & Aguilera,1986b; Antoni *et al.*,1992a; Miyazaki, Reisine & Kebabian,1984; Phillips & Tashjian,1982; Woods *et al.*,1992) suggesting that the site of glucocorticoid inhibition is downstream of, or parallel to, protein kinase A or C activation. Moreover, Miyazaki *et al.* (1984) demonstrated that glucocorticoid inhibition of forskolin- (that activates adenylate cyclase to elevate cAMP levels directly) stimulated ACTH secretion was not a failure of cAMP to activate protein kinase A in AtT20 D16:16 cells.

A recent study in AtT20 D16:16 cells suggested that the synthetic glucocorticoid, dexamethasone, stabilizes the sub-plasmalemmal actin network with a concommitent inhibition of CRF-41-stimulated ACTH release in the early time domain of inhibition (Castellino, Heuser, Marchetti *et al.*,1992). In this study the inhibitory action of dexamethasone was reversed by cytochalasin B and D, a fungal toxin that severs actin filaments. Furthermore, dexamethasone elevated caldesmon protein levels (although

determination was only performed after 24h dexamethasone treatment) suggesting a modulatory role for this actin-stabilizing protein. However, as elevation of intracellular Ca^{2+} is required to disassemble the actin network (Aunis & Bader,1988) on secretagogue exposure it is likely that an actin-stabilization mechanism is secondary to inhibition of intracellular Ca^{2+} levels by dexamethasone in this cell line (see section 1.6.3 and Antoni *et al.*,1992a; Castellino *et al.*,1992). Furthermore, reports that depolarization-induced ACTH secretion is largely resistant to early glucocorticoid inhibition in AtT20 cells (Antoni & Woods,1992; Phillips & Tashjian,1982) appear to contradict this hypothesis, because it would be assumed that the actin-caldesmon mechanism would inhibit all types of Ca^{2+}-mediated secretion.

As mentioned previously (section 1.5.3) glucocorticoids do not reduce the of ACTH in anterior pituitary corticotrophs within the time-scale of early inhibition. The role of postranslational modification of ACTH in the early suppressive action of glucocorticoids has not been fully explored. A recent study in primary cultures of rat anterior pituitary cells reports that after glucocorticoid exposure CRF-41 releases a modified form of ACTH that has reduced biological activity (Norman, Gurney & Gillham,1992). These observations may also explain some of the variations in the reported degree of early glucocorticoid inhibition depending whether bio- or immuno-assays are used to determine ACTH release. As secretion is an energy dependent process it is also possible that modulation of cellular metabolism, such as inhibition of glucose uptake, is responsible for early glucocorticoid inhibition (Horner, Packan & Sapolsky,1990; Munck,1971).

1.6.3 *Suppression of intracellular free Ca^{2+} responses*

Early *in vitro* studies *cf* (Kraicer *et al.*,1969) suggested a modulatory role for glucocorticoids in the Ca^{2+} handling of corticotrophs, indeed Ca^{2+} is essential for both CRF-41- and AVP-stimulated ACTH secretion (see section 1.4). Recently Orth and co-workers (Oki *et al.*,1991), using an *in vitro* perifusion model, suggested that glucocorticoids inhibit the plateau phase of CRF-41- and AVP-stimulated ACTH release (that is dependent on Ca^{2+} influx through L-type Ca^{2+} channels, see section 1.4) implicating early glucocorticoid inhibition of Ca^{2+} influx through L-type Ca^{2+} channels. In these experiments glucocorticoids did not inhibit the initial phase of AVP-stimulated ACTH release, that is reported to be dependent on IP_3-stimulated intracellular Ca^{2+} release (see section 1.4.3 and Oki *et al.*,1991). This would be in accordance with the site of glucocorticoid inhibition being downstream of protein kinase activation, perhaps preventing opening of plasma membrane Ca^{2+} channels (Antoni & Dayanithi,1990b).

Antoni & co-workers (Antoni *et al.*,1992a) have recently reported early glucocorticoid suppression of CRF-41-induced Ca^{2+} transients as well as reduction of absolute intracellular Ca^{2+} levels using Ca^{2+}-imaging of fura-2 loaded AtT20 D16:16 cells. A similar hypothesis of glucocorticoid suppression of intracellular Ca^{2+} signals has also been proposed in other models of early glucocorticoid inhibition such as B-lymphocytes (Dennis, June, Mizuguchi *et al.*,1987), basophilic leukaemia cells (Her, Weissman & Zor,1990), and pancreatic islet ß-cells (Billaudel, Mathias, Sutter & Malaisse,1984). Whether the glucocorticoid-induced protein(s) modulate Ca^{2+} channels directly (Callewaert, Hanbauer & Morad,1989; Ebersole, Gajary & Molinoff,1988; Janis, Shrikhande, Johnson *et al.*,1988) or restrict agonist induced redistribution of intracellular free Ca^{2+} remains to be explored (see Figure 1.5).

1.6.4 *Hyperpolarization of the membrane potential*

Several workers have demonstrated that glucocorticoids hyperpolarize the plasma membrane potential within 30min in hippocampal CA1 neurones (Joëls & De Kloet,1989; Kerr, Campbell, Hao & Landfield,1989). Such modulation of membrane potential would indirectly inhibit Ca^{2+} influx and thus modulate Ca^{2+} homeostasis. A recent preliminary report using whole cell patch (nystatin) clamp recordings from AtT20 D16:16 cells suggests that dexamethasone increases the proportion of cells expressing an A-type K^+ current, moreover, the amplitude of the A-current was enhanced in dexamethasone treated cells (Pennington, Woods, Kelly & Antoni,1992). Furthermore, ACTH secretion stimulated by K^+ channel blockers (tetraethylammonium and 4-aminopyridine) as well as 50mM extracellular K^+ in AtT20 cells is largely resistant to dexamethasone inhibition (Pennington *et al.*,1992; Phillips & Tashjian,1982; Sabol,1980) suggesting an involvement of membrane hyperpolarization in early inhibition (see Figure 1.5). However, it should be noted that an early study in normal corticotrophs reported that corticosterone blocks ACTH release stimulated by high extracellular K^+ medium (Kraicer *et al.*,1969). Interestingly a similar involvement of K^+ channels for the inhibitory effect of atrial natriuretic factor, ANF, acting through cGMP (Antoni & Dayanithi,1990a; Antoni & Dayanithi,1990b; Dayanithi & Antoni,1990), on pituitary ACTH release has been reported. Although glucocorticoids have been reported to stimulate cGMP production in other systems (Vesely,1980) glucocorticoid and ANF inhibition involve distinct intracellular mechanisms in anterior pituitary corticotrophs (Antoni & Dayanithi, 1990b). In particular, the site of glucocorticoid and ANF inhibition of intracellular free calcium appears to be different as ionomycin, that releases Ca^{2+} from intracellular stores, reversed the inhibitory action of ANF but not glucocorticoid. Whether glucocorticoid-induced proteins modulate K^+ channels directly, for example through

dephosphorylation as reported for the mechanism of somatostatin inhibition of prolactin secretion from the clonal pituitary cell line GH_4C_1 (White, Schonbrunn & Armstrong,1991) is unknown. Recently Levitan & co-workers reported glucocorticoid induction of the Kv1 mRNA, encoding a voltage dependent K^+ channel, within 40min in the anterior pituitary gland as well as clonal GH_3 cells, however, no induction was observed in AtT20 (strain not reported) cells (Levitan, Hemmick, Birnberg & Kaczmarek,1991). The role of early glucocorticoid-induction of other K^+ channel proteins has not been explored (Cook,1988; Salkoff, Baker, Butler *et al.*,1992).

1.6.5 *Glucocorticoid-induced proteins involved in early inhibition?*

As discussed above increasing evidence suggests that early inhibition is manifest through modulation of the regulated secretory process, perhaps by suppression of intracellular free Ca^{2+} levels. Consequently the glucocorticoid-induced proteins are likely to be Ca^{2+}-binding proteins and associated proteins involved in secretion, although other modes of action such as induction of K^+ channel proteins also require examination (see above).

Proteins induced within the time-scale of early glucocorticoid inhibition at the corticotroph have not been identified to date although in other models of early glucocorticoid action several proteins, including the Ca^{2+}-binding proteins calmodulin, lipocortin (annexin) 1 and glucocortin have been isolated (for example see Baughman, Harrigan, Campbell *et al.*,1991; Colbert & Young,1986b; Dowd, MacDonald, Komm *et al.*,1991; Flower,1988; Harrigan *et al.*,1989; Peers & Flower,1990). The functional role of these proteins in early glucocorticoid action remains to be determined.

1.6.6 *Conclusion*

The intracellular mechanisms of early glucocorticoid inhibition of stimulated ACTH release from the anterior pituitary corticotroph are not well defined. Recent evidence suggests that glucocorticoids suppress intracellular free Ca^{2+} levels, whether this action is directly on Ca^{2+} channels or involves hyperpolarization of the plasma membrane potential is unclear (see Figure 1.5). To date proteins involved in early glucocorticoid action at the corticotroph have not been characterised, however, they are likely to be members of the Ca^{2+}-binding family of proteins.

Figure 1.5

Current model of early glucocorticoid inhibition in anterior pituitary corticotrophs

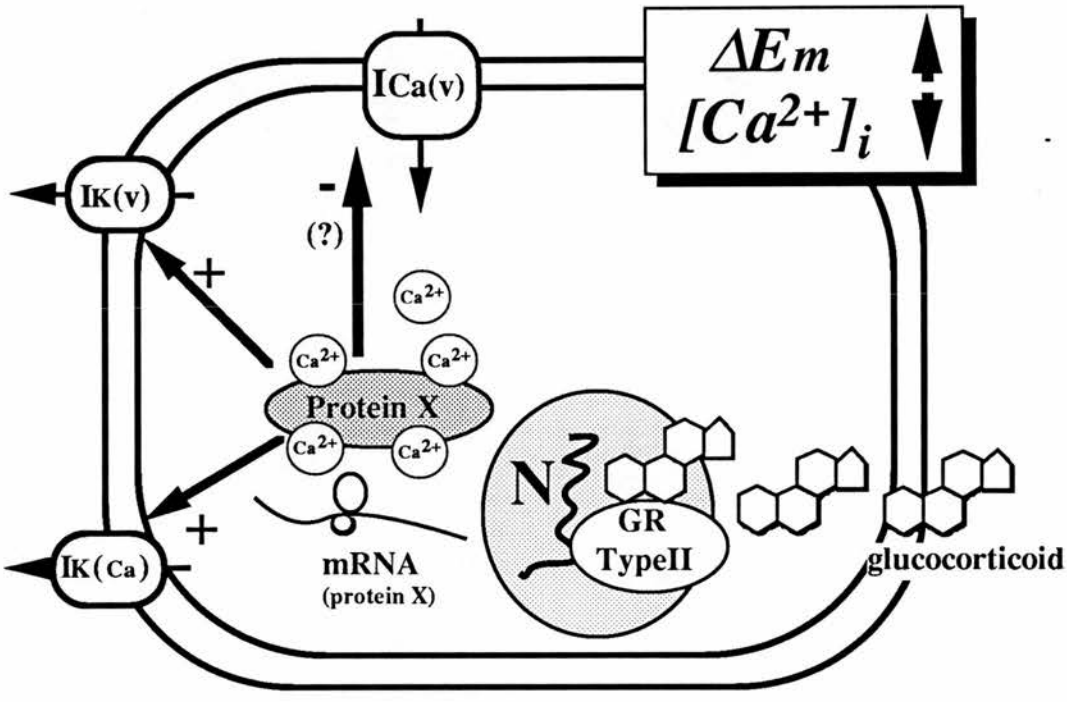


Figure 1.5: Activation of type II glucocorticoid receptors (GR TypeII) stimulates expression of the glucocorticoid-induced protein(s) (protein X) that regulates intracellular free calcium levels [Ca^{2+}] directly, perhaps by sequestration of intracellular Ca^{2+} or inhibition of Ca^{2+} channels, or indirectly by activating voltage (IK(v)) or Ca^{2+}-dependent (IK(Ca2+)) potassium

1.7 Summary

In summary, *early* glucocorticoid inhibition of stimulated ACTH secretion in anterior pituitary corticotrophs is characterised by *induction* of new mRNA and protein that occurs within 3h of glucocorticoid application. This is in contrast to the general *suppression* of POMC gene expression and differentiated cellular function associated with *late* inhibition. Indirect evidence from early *in vitro* studies suggests that the characteristics of early glucocorticoid inhibition is dependent on the nature of the secretagogue employed as well as the temporal relationship between secretagogue and glucocorticoid exposure. Investigation of the characteristics of early glucocorticoid action should provide insights into the nature and function of the glucocorticoid-induced proteins. The precise mechanism of early glucocorticoid inhibition is unknown, however, it is likely to be mediated through alterations in intracellular Ca^{2+} homeostasis or plasma membrane potential as summarised in Figure 1.5. Although proteins mediating early actions of glucocorticoids in corticotrophs have not been identified to date they are likely to belong to the Ca^{2+}-binding protein family.

As discussed in section 1.3 the aim of this thesis is to:

1) Determine the nature and function of the putative glucocorticoid-induced proteins that appear to mediate the early inhibitory action of glucocorticoids in anterior pituitary corticotrophs.

2) To examine whether the hypothalamic ACTH secretagogues, CRF-41 and AVP, interact with glucocorticoids at the level of the anterior pituitary corticotroph to modulate early glucocorticoid inhibition. It is hoped that analysis of such interaction will also shed light on the identity of the induced proteins.

In section 3 I have explored and discussed the characteristics of early glucocorticoid

inhibition of AVP- and CRF-41- stimulated ACTH secretion using a perifusion model of normal rat corticotrophs. The identity of the putative glucocorticoid induced proteins is addressed and discussed in section 4 using the clonal mouse corticotroph, AtT20 D16:16, cell line as a model. The work presented in this thesis is summarised in section 5 and the conclusions presented in sections 3 and 4 reiterated. Finally, proposals for future studies required to consolidation and extend the work presented in this thesis are discussed.

2

Materials and Methods

2

MATERIALS AND METHODS

2.1 Materials

2.1.1 *Animals*

Female ex-breeder rats of mean body weight 250-300g were obtained from Harlan-Olac (Bicester, Oxon, UK) or Charles River Ltd (Margate, Kent, UK) and maintained four to a cage for at least 2 weeks prior to use under controlled temperature (22°C) and lighting (lights on 05:00h, off 19:00h) with free access to standard lab chow and water. Twenty four hours before an experiment rats were placed in pairs and maintained under controlled conditions in a separate quiet room. On the day of experiment rats were decapitated, routinely between 08:30 and 09:30, within 10s of removal from the home cages to avoid undue activation of the hypothalamic-pituitary-adrenal axis.

2.1.2 *Clonal mouse corticotroph cell line, AtT20 D16:16*

The mouse anterior pituitary tumour corticotroph cell line (AtT20 D16:16 passage 13) was obtained from Dr S L Sabol, NIH, Bethesda, Maryland, USA, (Sabol,1980). Cells were maintained as monolayers in Dulbecco's modified Eagle's medium (Gibco-BRL, Paisley, Strathclyde, UK) supplemented with 10% foetal calf serum (FCS, Sera-Lab, Crawley Down, Sussex, UK) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The batch of foetal calf serum used in all subsequent studies contained 30nM cortisol as measured by radioimmunoassay. Cells were passaged every 7 days after reaching 70-80% confluency in 75cm^2 flasks (CelCult, Sterilin Ltd, Hounslow, UK) using phosphate buffered saline (PBS), pH7.4 containing 10mM

glucose and 0.025% w/v EDTA. After pelleting at 100g for 5min, cells were split 1:4, reseeded and fed every 3-4 days. All experiments were performed on cells between passage 16 and 35.

2.1.3 *Biochemicals*

Steroids: Corticosterone and dexamethasone were from Sigma (Poole, Dorset, UK). RU28362 (11ß, 17ß-dihydroxy-6-methyl-17α-(1-propynyl)androsta-1,4,6-trien-3-one) and RU38486 (17ß-hydroxy-11ß(4-dimethylaminophenyl-1)-17α(propy-nyl)-estra-4, 9-dien-3-one) were a generous gift from Roussel-Uclaf (Romainville, France). 18ß-glycyrrhetinic acid was from Aldrich (Poole, Dorset, UK). Working steroid stocks were dissolved in dimethylsulphoxide (DMSO) or ethanol at a concentration of 10mM and stored at -20°C before dilution in the appropriate cell medium.

Peptides: Rat 41-residue corticotrophin releasing factor (CRF-41), rat arginine vasopressin (AVP), and human adrenocorticotrophin hormone ($ACTH_{1-39}$) were obtained from Bachem UK Ltd (Saffron Walden, Essex, UK) or Peninsula Laboratories Ltd (St Helens, Merseyside, UK). Working stock peptides were stored as 0.1mM stocks in 1mM HCl, pH5 (including 0.1mM ascorbate where appropriate to prevent methionine oxidation) at -70°C. Cloned mouse lipocortin I was a generous gift from Dr R B Pepinsky, Biogen Inc., Boston, MA., USA, (Pepinsky, Sinclair, Douglas *et al.*,1990). Bovine brain calmodulin was from Biogenesis Ltd (Bournemouth, Sussex, UK).

Primary antisera: Mouse monoclonal antibodies (MAb 105) against lipocortin I were a generous gift of Dr R B Pepinsky, Biogen Inc, Boston, MA., USA (Pepinsky *et al.*,1990). Rabbit anti-ACTH antiserum (AS6) was a generous gift from Dr G B Makara, MTA KOKI, Budapest, Hungary (Makara, Stark, Rappay *et al.*,1979).

Rabbit anti-cAMP antiserum (cAB4) was supplied by Dr K Catt, NICHD, NIH, Bethesda, MD, USA (Dufau, Watanabe & Catt,1973). Rabbit polyclonal antibodies to bovine chromogranin A were a generous gift of Dr D K Apps, Dept. Biochemistry, University of Edinburgh, Edinburgh, UK. Mouse monoclonal antibodies (IgG_1) to bovine calmodulin were obtained from Upstate Biotechnology Inc., Lake Placid, NY, USA (Sacks, Porter, Ladenson & McDonald,1991).

cDNA clones: cDNA clones for mouse calmodulin (containing a 0.6kbp insert encoding pCAMII in EcoRI site of pBluescript, clone 21) and a putative G-protein linked receptor (1.3kbp insert, clone 4.2) were a generous gift of Dr S Bourgeois, Salk Institute, San Diego, CA, USA (Baughman *et al.*,1991; Harrigan *et al.*,1989). A cDNA clone for chicken calmodulin (0.28kbp insert in EcoRI/PstI site of pGEM-1) was a generous gift of Dr D R Dowd, Arizona Cancer Center, Tucson, Arizona, USA (Dowd *et al.*,1991). A cDNA clone for human lipocortin I (1.38kbp insert in EcoRI site of pUC13) and a cDNA clone for human α-tubulin (1.4kbp insert in Pst1 site of pSP64) were provided by Dr E L Mullens (Lab. of Neuroendocrinology, Charing Cross and Westminster Hospital, London, UK). A cDNA encoding the abundant cytoplasmic 7S RNA (0.28kbp in BamH1 site of pAT153 (Balmain, Krumlauf, Vass & Birnie,1982)) was obtained through Dr J Seckl, Western General hospital, Edinburgh, U K.

Molecular biology reagents: Guanidinium thiocyanate was from Fluka Chemicals Ltd, Glossop, Derbyshire, UK. Bacto-tryptone, bacto-yeast extract and agar were from Oxoid, Unipath Ltd, Basingstoke, Hampshire, UK. Materials used in cDNA library construction were from Stratagene, Cambridge, Cambs, UK. Additional restriction enzymes and nucleotides were from Boehringer Mannheim or Gibco-BRL. All other material was from Sigma (Molecular biology grade) unless otherwise stated.

Miscellaneous: Cyclic 8-(4-Chlorophenylthio) adenosine monophosphate (8-CPT-cAMP) was from Boehringer Mannheim UK (Lewes, Sussex, UK). Isobutyl-methylxanthine (IBMX), ketoconazole, 2'-O-monosuccinyl cAMP tyrosyl methyl ester (cAMP-TME), cyclic adenosine monophosphate (cAMP) and 5,6-Dichloro-1-ß-D-ribofluranosylbenzimidazole (DRB) were from Sigma. Actinomycin D and puromycin were from Aldrich.

All other materials were obtained as described in the relevant methods or results section. General chemicals were obtained from BDH (Poole, Dorset, UK) and were of the highest analytical grade.

2.2 Perifusion of anterior pituitary gland segments

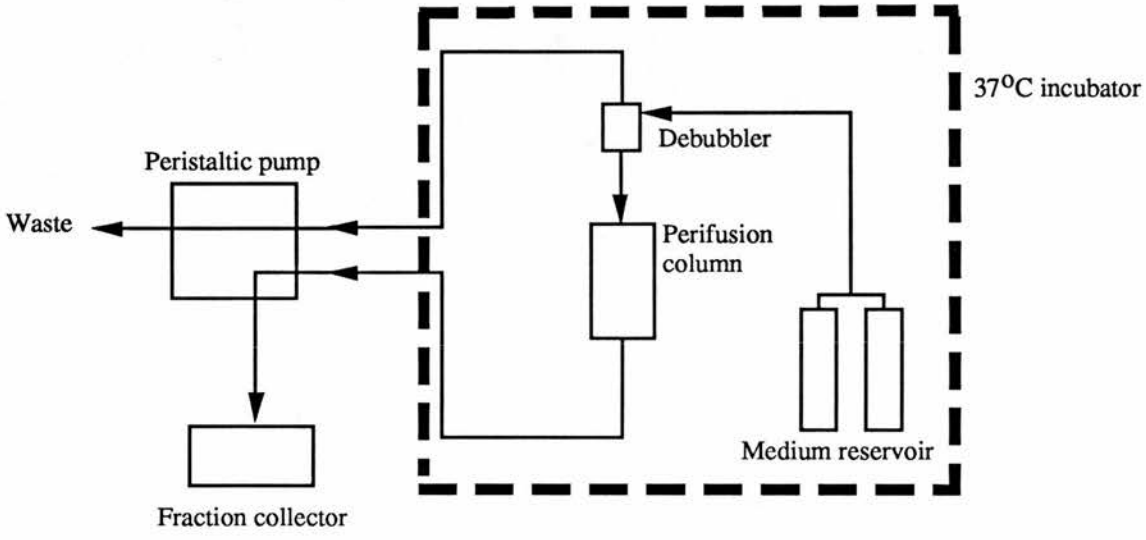
2.2.1 *Apparatus construction*

Perifusion of rat anterior pituitary gland segments was performed using a modification of previous methodologies (Dayanithi & Antoni,1989; Gillies & Lowry,1978; Mulder & Smelik,1977). A flow diagram of the perifusion column apparatus is shown in Figure 2.1a: typically 4 perifusion columns were run in parallel. The perifusion medium was pumped through the system using an eight channel peristaltic pump (IPS, Ismatec, Zürich, Switzerland) using 0.16cc/m peristaltic pump tubing (Altec, Alton, Hampshire, UK). All other connecting tubing was 0.5mm i.d. polypropylene tubing (Altec). Perifusion medium, columns and inlet tubing were maintained at 37°C in a thermostatically controlled incubator (Stuart Scientific, Scotlab, Paisley, UK). Column perifusate was collected on ice using a Gilson microfraction collector modified to collect 4 samples simultaneously.

Figure 2.1

Anterior pituitary gland column perifusion apparatus

a) Flow diagram of perifusion apparatus



b) Perifusion column construction

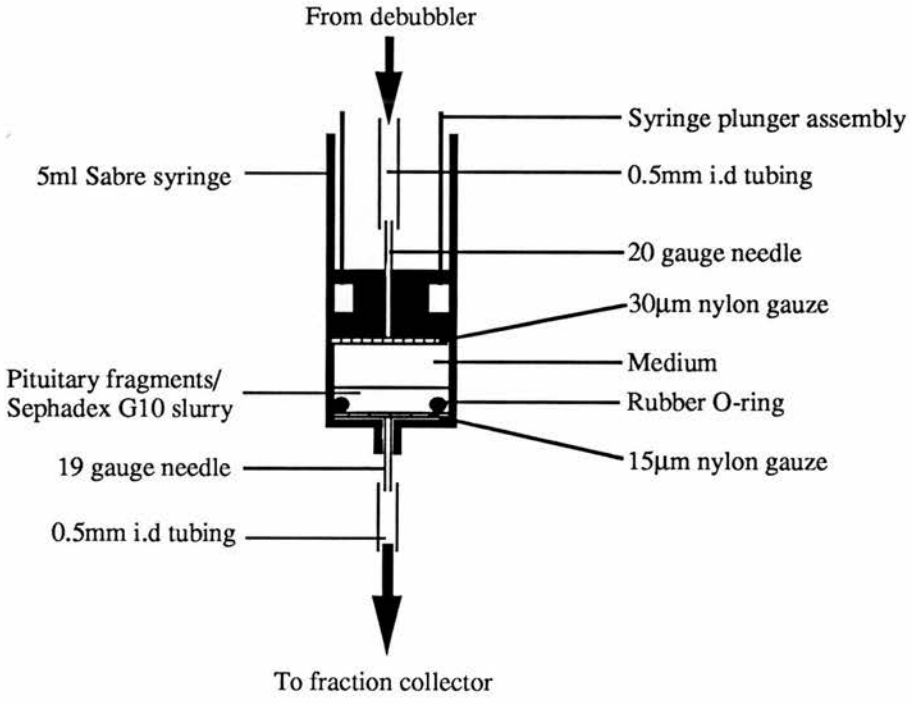


Figure 2.1: See section 2.2.1 for description of perifusion apparatus

Perifusion columns were constructed from 2ml polystyrene syringes (Sabre, Reading, Berkshire, UK) as shown in Figure 2.1b. The syringe plunger was pierced with a 20-gauge needle (Becton Dickson, Wembley, Middlesex, UK) and attached to 0.5mm i.d. polypropylene tubing (Altec). The nylon gauze (R Cadisch & Sons, Finchley, London, UK) prevented Sephadex or cells entering the tubing and causing blockage. A 100µl cushion of Sephadex G10 (Pharmacia. Uppsala, Sweden) was used as a matrix for the pituitary segments and aided complete mixing of the perifusion medium in the column chamber. Sephadex G10 was preswollen overnight in dH_2O and equilibrated with perifusion medium on the day of experiment.

Debubblers (0.2ml volume) were constructed from 1ml polystyrene syringes (Sabre) to prevent entry of air bubbles into the perifusion chamber during transfer of inlet tubing to experimental treatments.

2.2.2 *Perifusion protocol*

Anterior pituitary glands were isolated and cut into approximately equal size (1.5mm x 1.5mm) segments using a scalpel blade and randomly distributed between four perifusion columns. Each column received 1-1.5 pituitary equivalents. The column volume was adjusted to 0.5ml and the segments perifused with Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Paisley, Strathclyde, UK) containing 25mM Hepes, pH7.4, 0.25% bovine serum albumin (BSA), 100U/l penicillin and 100U/l streptomycin for 2h at minimum flow rate (approximately 150µl/5min) to allow the basal ACTH release to stabilize. Segments were perifused for a further hour at the experimental flow rate of 200µl/min during which time the initial basal fractions were collected. At this flow rate the transit time from the medium reservoir to the fraction collector was 100s. Secretagogue stimuli were then applied every 1h for 5min with various treatments applied as shown in the respective figure legends. Column effluent

was collected using a Gilson microfraction collector, modified to collect four fractions simultaneously, and stored at -40°C until assayed for immunoreactive ACTH.

2.2.3 *Calculation of ACTH released by a secretagogue stimulus*

Net ACTH release per 5min exposure to secretagogue was determined for each stimulus as previously described (Dayanithi & Antoni,1989). Net ACTH release is defined as:

$$\text{Net ACTH release} = \frac{V_1+V_2+.....+V_n}{n} - B$$

Where V_1 is the first 5min fraction collected during the stimulus (100sec transit time discarded) and V_2 to V_n are the next n consecutive fractions that are elevated with respect to basal ACTH release (B). Basal ACTH secretion was determined immediately prior to the application of each stimulus. For 10nM CRF-41 or AVP n=5: for 0.1nM CRF-41 n=3 (see Figure 2.2).

Because of the variability in absolute ACTH release between experiments (for example see Figure 3.2, section 3.2) data were standardised by expressing the net ACTH release elicited by a treatment as a percentage of that elicited by secretagogue alone (at the 4h treatment point). The net ACTH release at 4h was defined as the 100% release value in all columns.

Figure 2.2

Time course of ACTH release in response to a stimulus of CRF-41 and AVP in perifused rat anterior pituitary segments

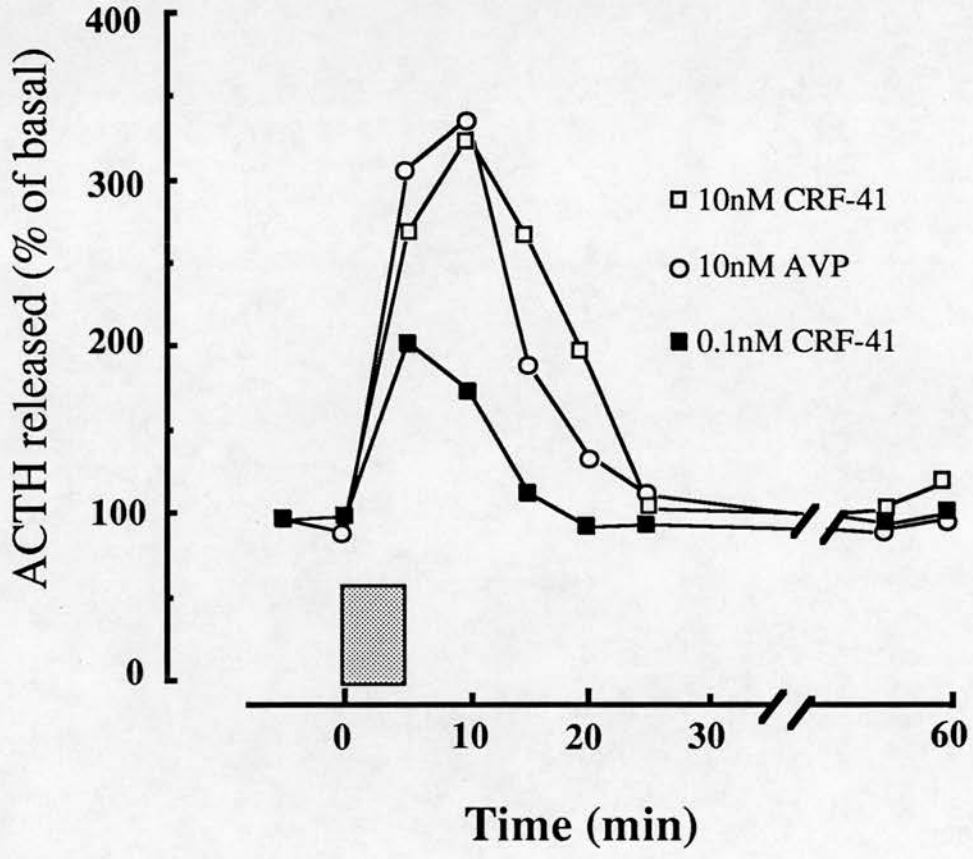


Figure 2.2: The various stimuli were applied for 5min (stippled horizontal box) as described in section 2.2.2 and 1ml fractions of column perifusate collected every 5mins. Data are from typical experiments and expressed as a percentage of the basal ACTH released immediately prior to application of the stimulus to compensate for interexperimental differences in absolute ACTH release. For 10nM CRF-41 and 10nM AVP, 5 fractions after the start of the stimulus are elevated with respect to basal thus subsequent data are expressed as the net ACTH released in 25min after a 5min stimulus as described in section 2.2.3. For 0.1nM CRF-41, 3 fractions after the start of the stimulus are elevated with respect to basal thus subsequent data are expressed as the net ACTH released in 15min after a 5min stimulus.

2.3 Static ACTH release experiments

2.3.1 *Pituitary segments*

Anterior pituitaries were isolated and sectioned into eight approximately equal size (1.5mm x 1.5mm) segments under a dissecting microscope. One pituitary eighth was placed in each well of a 24 well cluster plate (CelCult) and maintained in DMEM containing 25mM Hepes, pH7.4, 0.25% BSA, 100U/l penicillin and 100U/l streptomycin for 3h before experimental manipulation. ACTH release was determined for a 30min secretagogue stimulus in a volume of 0.5ml.

2.3.2 *Primary cultures of anterior pituitary cells*

Primary cultures of rat anterior pituitary cells were established using a modification of the trypsin/mechanical dissociation technique (Dobson & Brown,1985; Gillies & Lowry,1978; Portanova, Smith & Sayers,1970)

Anterior pituitaries from eight rats were aseptically isolated and placed in DMEM, pH 7.4, containing 25mM Hepes, 0.25% BSA (hereafter referred to as DMEM-Hepes). Pituitaries were chopped into *ca* 0.5mm blocks using a razor blade and placed in 5ml DMEM-Hepes containing 0.25% trypsin (TRL, Worthington Biochemical Corporation, NJ, USA) and 5µg/ml DNAse 1 (Sigma, Poole, Dorset). The tissue was incubated at 37°C in a shaking water bath. Every 5min the pituitary pieces were triturated using a 5ml pipetman tip to aid cell dispersion. After 20min the tissue was triturated approximately 40 times using a 10ml Sterilin pipette fitted with a 1ml pipetman tip. The suspension was filtered through 100µm nylon gauze (R Cadisch & Sons, Finchley, London, UK) to remove any remaining tissue debri and diluted with an equal volume of DMEM-Hepes containing 10% FCS to inactivate the trypsin. The supernatant was centrifuged at 200g for 10min and the cell pellet resuspended in

DMEM containing 10% FCS. Cell viability was determined by Trypan blue exclusion using an haematocytometer and cells plated at a density of 3-4x10^4 cells/well in 24 well cluster plates and maintained in a humidified atmosphere of 95% air and 5% CO_2. Typically 1-1.5x10^6 cells per pituitary were obtained with greater than 95% viability.

Secretion studies were performed on the fourth day after plating with a medium change on day three. Cells were washed twice and maintained in DMEM containing 25mM Hepes, pH7.4, 0.25% BSA, 100U/l penicillin and 100U/l streptomycin for two hours to remove FCS before experimental manipulation. Preliminary studies demonstrated that the secretory response was identical for cells used on days 4 to 7 after plating. ACTH release was determined in a volume of 0.3ml after which samples were spun briefly at 200g to pellet floating cells and the supernatant stored at -40°C.

2.3.3 *AtT20 D16:16 corticotroph cell line*

For release studies AtT20 D16:16 cells were plated in 24 well cluster dishes (CelCult) at an initial density of 4x10^4 cells/well, fed 3-4 days after plating and used at 70-80% confluency. Prior to experimental manipulation cells were washed twice and maintained in DMEM containing 25mM Hepes, pH7.4, 0.25% BSA, 100U/l penicillin and 100U/l streptomycin for two hours to remove FCS. ACTH release was determined in a volume of 0.5ml after which samples were spun briefly at 200g to pellet floating cells and the supernatant stored at -40°C before radioimmunoassay.

Determination of intracellular cAMP content was performed as above except that after treatment the incubating medium was removed and the cells lysed in 0.5ml of ice cold 0.1N HCl with ultrasound disruption for 20s, spun at 200g to pellet cellular debri and the acidic cell extract stored at -40°C before radioimmunoassay.

2.4 Radioimmunoassay for ACTH and cAMP

2.4.1 *Iodination of ACTH and cAMP using the Iodogen™ method*

Labelling of ACTH and cAMP with ^{125}Iodine for use in their respective radioimmunoassay was performed using the Iodogen™ method essentially as described by Salacinski, McLean, Sykes *et al.* (1981).

Iodogen™ coated polypropylene 1.5ml conical Eppendorf centrifuge tubes (Sarstedt) were prepared by adding 50µl of 0.04mg/ml Iodogen™ (Sigma) in dichloromethane which was evaporated to dryness in a 37°C water bath in a fan extraction fume hood. Human $ACTH_{1-39}$ (Bachem or Peninsula) was stored in aliquots at 25µM in 0.01N HCl and 2'-O-monosuccinyl cAMP tyrosyl methyl ester (Sigma, cAMP-TME) stored at 25µM in dH_2O at -70°C before use.

Five microlitres of Na^{125}I (ICN Radiochemicals, Irvine, CA, USA) containing 0.5mCi (≈ 0.25nmol Na^{125}I) were incubated with 0.25nmol of ACTH or cAMP-TME in a total volume of 50µl in 0.5M sodium phosphate buffer, pH 7 in an Iodogen™ coated tube. After 12min at room temperature the reaction was terminated by the addition of 1ml 0.1% trifluoroacetic acid (TFA) and applied to a Sep-Pak C18 octadenysilyl (ODS) cartridge.

For ACTH the cartridge was washed with three 2ml aliquots of 0.1% TFA followed by 2ml each of a stepwise gradient of acetonitrile (10-80%) containing 0.1% TFA. Figure 2.3 shows a typical elution profile for labelled ACTH. Free iodine elutes in the initial wash (Peak i, Figure 2.3), the peak typically eluting at 30 and 40% acetonitrile (Peak ii, Figure 2.3) was suitable for use in the ACTH radioimmunoassay.

Figure 2.3

Purification of ^{125}Iodine labelled ACTH on a Sep-Pak ODS cartridge

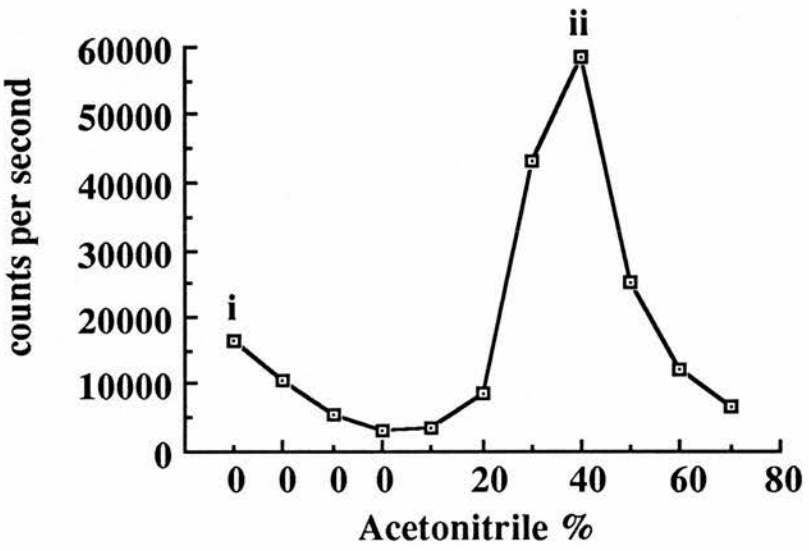


Figure 2.3: ^{125}Iodine labelled ACTH was eluted from a Sep-Pak ODS cartridge with 0.1% trifluoroacetate and a stepwise acetonitrile gradient as described in section 2.4.1. The peaks at i) and ii) represent free iodine and iodinated ACTH respectively. The 30-40% acetonitrile fractions were used in the ACTH radioimmunoassay as described in section 2.4.2.

For cAMP-TME the cartridge was washed as for ACTH except that a 10-80% methanol gradient containing 0.1% TFA was used, the 20% fraction was used in the cAMP radioimmunoassay.

2.4.2 *Measurement of ACTH by radioimmunoassay*

Duplicate 50µl aliquots of experimental medium were assayed for immunoreactive ACTH using a double antibody precipitation radioimmunoassay (Antoni, Holmes & Jones,1983) after appropriate dilution in RIA buffer (0.05M sodium phosphate buffer, pH7.4, 0.1% BSA, 0.1% Triton X-100, 2.5mM EDTA and 100 kallikrein inhibitor units (KIU) of aprotinin per ml).

Fifty microlitres of ACTH antiserum (AS6, courtesy of GB Makara, MTA KOKI Budapest, Hungary) typically at a final titre of 1:70,000 (see Figure 2.4a) in RIA buffer containing 6% polyethylene glycol 6000 (PEG-6000), were mixed with 50µl of sample in a polypropylene microtiter RIA vial (Sarstedt). The assay was incubated with 10µl of ^{125}I-ACTH (approximately 10k cpm in 10µl sodium phosphate buffer without PEG-6000) for 24h at 4°C. Subsequently donkey anti-rabbit IgG (SAPU, Carluke, Lanarkshire, UK) and non-immune rabbit serum (SAPU) were added to a final titre of 1:30 and 1:200, respectively, and incubated at 4°C for 3hrs. Three hundred microlitres of ice-cold 6% PEG-6000 were added and the bound label separated from free by centrifugation at 1,500g for 20min at 4°C. The resultant supernatant was decanted, tubes washed and blot dried, and the radioactivity remaining in the pellet counted on a Packard autogamma counter.

Standard curves were constructed in the range 0.125 to 64.0 fmol ACTH/50µl using human $ACTH_{1-39}$ diluted in DMEM or RIA buffer as appropriate. Non-specific binding was determined by either incubating in the presence of excess unlabelled

ACTH or by omitting the ACTH antiserum and was typically <10% of the total bound counts. Inter- and intra- assay coefficients of variance were typically <10% and <5% respectively. α-Melanotrophin stimulating hormone significantly (<10%) cross-reacts with this antiserum (Makara, Stark, Rappay *et al.*,1979).

Assayed ACTH content was determined by interpolation from the % Bound counts *vs* log ACTH concentration curve (see Figure 2.4b), or its logit transformation, generated using the respective algorithm of the Packard Cobra autogamma analysis package. Specific bound counts were typically 35% of total counts applied. Percent bound counts (%B) is defined as:

$$\frac{\text{X-NSB}}{\text{Bo-NSB}} \times 100\%$$

Where X = cpm for standard/unknown sample, B_0 = cpm in absence of ACTH, and NSB is the non-specific cpm.

Logit %B is defined as:

$$\log \frac{\%B}{100-\%B}$$

2.4.3 *Measurement of cAMP by radioimmunoassay*

Duplicate 25µl samples of sonicated cell extract in 0.1N HCl were assayed for immunoreactive cAMP using a double antibody precipitation reaction (Dufau *et al.*,1973) using antiserum cAB4 at a final dilution of 1:50,000 supplied by Dr K Catt, NIH, Bethesda, USA. All assays were performed in a final volume of 275µl in polypropylene tubes (Alpha-Labs) in 50mM sodium acetate buffer, pH 6.0 containing 0.25% BSA. After overnight incubation with 10K cpm of ^{125}I-cAMP-TME, donkey anti-rabbit IgG (SAPU) and non-immune rabbit serum (SAPU) were added to a final titre of 1:40 and 1:400 respectively and incubated at 4°C. After 3hrs one millilitre of

ice-cold 6% PEG-6000 was applied and the bound label separated from free by centrifugation at 1,500g for 20min at 4°C. Non-specific binding was typically <10% of total bound counts, approximately 40% of total counts bound to the antiserum. Inter- and intra- assay coefficients of variance were typically <10% and <5% respectively. Standard curves were generated in the range 0.025 to 12.8 pmole/tube using cAMP as standard (Sigma). Assayed cAMP content was determined as described for ACTH radioimmunoassay.

Figure 2.4

Characterization of primary ACTH antiserum (AS6)

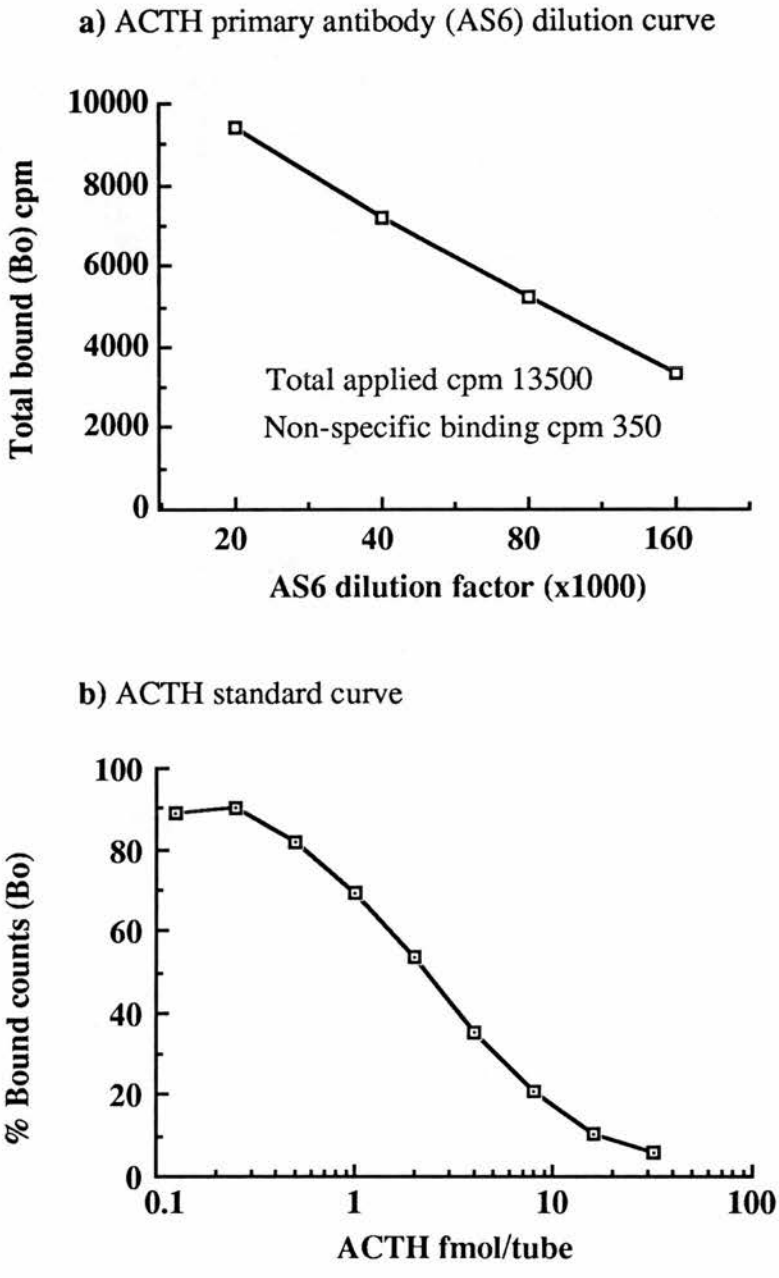


Figure 2.4: Characterization of primary ACTH antibody (AS6) used in ACTH radioimmunoassay. **a)** Linear relationship of total bound counts with increasing AS6 dilution and **b)** ACTH concentration standard curve using AS6 at a final dilution of 1:70,000. Both curves were generated using a 100µl assay as described in section 2.4.2.

2.5 Protein labelling and isolation

2.5.1 *Treatment and harvesting of AtT20 D16:16 cells for protein isolation*

In the following protein isolation procedures (unless otherwise stated) cells were seeded at an initial density of $1x10^6$ cells per $75cm^2$ flask and grown to 70-80% confluency in DMEM and 10% FCS. Before experimental manipulation the medium was replaced with DMEM containing 25mM Hepes, pH 7.4, 0.25% BSA and 100U/l penicillin and 100U/l streptomycin for 2h. Cells were removed from the flask by washing with PBS, pH7.4 containing 10mM glucose and 0.025% EDTA, and pelleted at 200g for 5min.

2.5.2 *Preparation of crude pellet and supernatant protein homogenates*

Cells (approximately $1x10^7$) were homogenised by sonication (3x20s) on ice in 250µl of 10mM Tris/HCl, pH8.0, 1mM EDTA and 0.25mM phenylmethylsulphonylfluoride (PMSF) and spun at 100g to remove large cellular debris. For isolation of calmodulin 2mM EGTA was also included in the homogenisation buffer. The supernatant was centrifuged at 12,000g for 30min at 4°C in an Eppendorf microfuge. The resultant pellet was resuspended in 50µl of homogenisation buffer; both supernatant and pellet fractions were stored at -40°C before use.

2.5.3 *Isolation of Ca^{2+}-binding proteins from AtT20 D16:16 cells*

Calcium binding proteins were isolated using a modification of the calcium precipitation-elution method described by Woolgar, Boustead & Walker (1990) for the isolation of annexins from mammalian brain. All subsequent steps were performed at 4°C. Cells (approximately $5x10^7$) were homogenised by sonication (3x 20s) in 0.5ml of 10mM Hepes, pH7.4 containing 150mM NaCl, 5mM EGTA and 0.25mM PMSF

and centrifuged for 30min at 40,000g in a Beckmann TL-100 fixed angle ultracentrifuge. The supernatant was removed and $CaCl_2$ added to 1mM excess (final concentration 6mM); after 15min, the sample was centrifuged at 40,000g for 30min. The pellet was washed sequentially with 2vols of 10mM Hepes, pH7.4, 150mM NaCl and 1mM $CaCl_2$ followed by 10mM Hepes, pH7.4 and 1mM $CaCl_2$. The pellet was resuspended in 0.1vol of 10mM Hepes, pH7.4, 10mM EGTA and centrifuged for 30min at 100,000g. The resultant supernatant contained typically ~18% of total cell protein and was stored at -40°C before use. After 2D-gel electrophoresis (see section 2.6.1) proteins were detected using the Pierce silver staining kit (Pierce Inc, Rockford, Illinois, USA).

2.5.4 *^{32}Phosphate labelling of cellular proteins in AtT20 D16:16 cells.*

^{32}Phosphate (^{32}P) labelling of cellular proteins in AtT20 D16:16 cells was performed using a method modified from Rougon, Barbet & Reisine (1989). Under these conditions the cells equilibrate ^{32}P with the intracellular phosphate pool within 3h with approximately 5-6% of the ^{32}P taken up into the cells in this period.

Cells were seeded at an initial density of 150,000/well in 6 well culture plates (CelCult) and grown to 70-80% confluency in DMEM and 10% FCS. Cells were washed and preincubated for 1h in 3ml of phosphate free MEM (ICN Biomedicals, High Wycombe, Bucks, UK), pH7.4 containing 0.25% BSA and 100U/l penicillin and 100U/l streptomycin in a humidified atmosphere of 95% air and 5% CO_2. After 3h in fresh medium containing 150μCi/ml of ^{32}P-orthophosphoric acid (DuPont, Stevenage, Herts, UK) 10nM dexamethasone was applied for 90min. 10nM CRF-41 was applied in the last 5min of dexamethasone treatment. Phosphorylation was terminated by harvesting cells as described in section 2.5.1 except that 10mM NaF was included as an inhibitor of phosphatase activity. Cells were homogenised in 50μl

of IEF lysis buffer (9M urea, 2%w/v Nonidet P40, 10mM NaF, 5%w/v pH3-10 Pharmalyte) by trituration and centrifuged at 8000g for 2min at RT to remove undissolved material. Protein samples containing equivalent TCA precipitable ^{32}P counts were used immediately in 2D-gel electrophoresis. TCA precipitable counts were approximately 14% of the total cellular ^{32}P counts. After overnight fixation of the second dimension slab gels in 40% MeOH and 10% acetic acid gels were autoradiographed for 4 weeks at -70°C using ß-max Hyperfilm (Amersham, Aylesbury, Bucks) with intensifying screens (Agfa).

2.5.5 *^{35}S-methionine labelling of newly synthesized proteins in AtT20 D16:16 cells.*

Cells were seeded at an initial density of 50,000/well in 24 well cell culture plates (CelCult) and grown to 70-80% confluency in DMEM and 10% FCS. Cells were washed and preincubated for 2h in 0.5ml of methionine free MEM (Gibco), pH7.4 containing 0.25% BSA and 100U/l penicillin and 100U/l streptomycin in a humidified atmosphere of 95% O_2 and 5% CO_2. Fresh medium containing 440µCi/ml of ^{35}S-methionine (DuPont, Stevenage, Herts, UK (1000Ci/mmol) with or without 10nM dexamethasone was applied for 90min. Incorporation was terminated by triturating cells in the well with 50µl of IEF lysis buffer (9M urea, 2%w/v Nonidet P40, 5%w/v pH3-10 Pharmalyte) and samples frozen on solid CO_2. Samples were homogenised by trituration and centrifuged at 8000g for 2min at RT to remove insoluble material. Protein samples containing equivalent TCA precipitable ^{35}S-methionine counts were used immediately in 2D-gel electrophoresis. Uptake of label was approximately 44% of total applied ^{35}S-methionine of which 20% was incorporated into protein as determined by TCA precipitation. After overnight fixation of the second dimension slab gels in 40% MeOH and 10% acetic acid gels were autoradiographed for 4 days at -70°C using ß-max Hyperfilm (Amersham, Aylesbury, Bucks, UK) with intensifying screens (Agfa).

2.5.6 *Protein determination*

The method of (Bradford,1976) was used with bovine serum albumin (ICN Biomedicals, High Wycombe, Bucks, UK) as standard in the range 10-100µg protein.

2.6 Protein gel electrophoresis

2.6.1 *Two-dimensional gel electrophoresis*

All two dimensional gel electrophoresis was kindly performed by Mr Jim Simpson (MRC BMU, University of Edinburgh, Edinburgh, UK) using a modification of the method of O'Farrell (1975) as described below.

For first dimension focusing, samples containing approximately 15µg protein were homogenised in IEF lysis buffer (9M urea, 2%w/v Nonidet P40, 5%w/v pH3-10 Pharmalyte) and applied to pre-focused (250V for 1h at 18°C) 125mm x 1.5mm cylindrical focusing gels. Gels consisted of 8.11g urea (BDH, Poole, Dorset) 6ml dH_2O, 300mg Nonidet P40, 1.5ml 30%w/v acrylamide/bisacrylamide 19:1 (Bio-Rad, Hemel Hempstead, Herts, UK), 300µl pH4-6.5 Pharmalyte (Pharmacia, Uppsala, Sweden), 300µl pH6.7-7.7 Pharmalyte, 150µl pH3-10 Pharmalyte, 15µl 10%w/v ammonium persulphate and 10.5µl TEMED (Sigma). Samples were focused for 17h at 400V and 4h at 1000V using 20mM NaOH cathodic and 10mM orthophosphoric acid anodic buffer at 18°C. Calibration of IEF gels was performed using separate pH and reference surface microelectrodes (Microelectrodes Inc, Londonderry, NH, USA). For second dimension SDS-PAGE the first dimension cylindrical gels were equilibrated for 4min in 1ml of 2.3% SDS, 62.5mM Tris-HCl, pH6.8 and applied to

the top of SDS slab gels (139mm long x 163mm wide x 1.5mm thick). Second dimension gels (consisting of 40ml 1.5M Tris-HCl, pH8.8, 66.7ml 30% acrylamide/bisacrylamide 37.5:1 (Bio-Rad Laboratories Ltd, Watford, Herts, UK), 53.3ml dH_2O, 533µl 10% ammonium persulphate and 80µl TEMED) were electrophoresed at 30mA/gel at 15°C in 250mM Tris/HCl, pH 7.5, 1.92M glycine and 1%w/v SDS using the Protean II 2-D Multi-Cell electrophoresis system (Bio-Rad). Molecular mass calibrations were performed using ^{14}C-methylated protein standards (Amersham).

2.6.2 *One dimensional SDS-PAGE and Western blotting*

Samples containing 1-2µg/µl of protein were boiled for 5min in the presence of 2.5% sodium dodecylsulphate and 5% ß-mercaptoethanol and applied in 1µl to 0.45mm thick 10-15% continuous gradient Phastgel SDS-PAGE gels (Pharmacia, Uppsala, Sweden). Gels were run at 15°C at 10mA for 70AVh in buffer containing 0.2M Tris-HCl pH7.5, 0.2M tricine and 0.55% SDS using the Phastsystem gel electrophoresis apparatus (Pharmacia). After electrophoresis, proteins were transferred to Immobilon PVDF membranes (Millipore, Watford, Herts, UK) by electroblotting after equilibrating gels in 25mM Tris-HCl pH8.3, 192mM glycine and 20% methanol for 5min followed by 45min transfer at 25mA using carbon electrodes (Pharmacia). For improved electrotransfer of calmodulin 2mM $CaCl_2$ was included in the transfer buffer and the blot immediately fixed in fresh 0.25% glutaraldehyde/PBS for 15min as described by McKeon & Lyman (1991).

Following transfer, blots were wetted in methanol followed by dH_2O and background blocked in 50mM Tris/HCl, pH7.6 containing 3% low fat milk powder (Marvel) for 1h. All following steps were performed with gentle agitation. After 5x 5min washes in Tris/HCl containing 0.1% Tween-20 (Tris/Tween), blots were incubated overnight

at 4°C with the respective concentration of primary antibody. Blots were washed for 5x 5min at RT in Tris/Tween, incubated with a 1:200 dilution of the appropriate biotinylated antisera to the primary antibody (Dakopatts, Denmark) in Tris/HCl for 1h, washed 5x 5min Tris/Tween and incubated with a 1:300 dilution of streptavidin biotinylated-conjugated peroxidase (Amersham, Aylesbury, Bucks) for 45min. Blots were developed after a 3x 5min wash in Tris/Tween using diaminobenzidine tetrahydrochloride hydrate (Aldrich, Poole, Dorset) at a concentration of 0.75mg/ml in Tris/HCl containing 0.02% H_2O_2. After 5-10min at RT the reaction was terminated by washing extensively in dH_2O. Alternatively, for calmodulin immunoblotting ^{125}I-labelled anti-mouse IgG (DuPont) was used as second antibody at a final activity of 50Bq/µl and blots autoradiographed for 3 days at -70°C using Hyperfilm-MP (Amersham). Blot lanes containing molecular weight markers (Pharmacia LMW) were excised and stained using 0.1% Coomassie blue R250 in 50% methanol.

2.7 General molecular biology methods

All routine molecular biology techniques such as restriction digestion, TCA precipitation etc were performed as described in Sambrook, Frisch & Maniatis (1989).

Table 2.1

Standard buffers referred to in molecular biology methods

SSC 150mM NaCl, 15mM trisodium citrate, pH 7.0

TBE 90mM Tris-borate, 90mM boric acid, 2mM EDTA, pH8.3

TE 10mM Tris-HCl, 1mM EDTA, pH8.0

SSPE 150mM NaCl, 2.5mM NaH_2PO_4, 0.25mM EDTA, pH7.4

SM 50mM Tris-HCl, pH 7.5, 100mM NaCl, 8mM $MgSO_4$, 0.01% gelatin

LB 10g Bacto-tryptone, 5g Bacto-yeast extract, 10g NaCl per litre, pH 7.5

NZY 5g NaCl, 2g $MgSO_4$, 5g Bacto-yeast extract, 10g bacto-tryptone per litre, pH7.5

2.7.1 *Total RNA and Poly A$^+$mRNA extraction*

Total RNA was extracted using the single step acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski & Sacchi,1987). Approximately 10^7 AtT20 D16:16 cells (70-80% confluency) were lysed directly as a monolayer from a 75cm^2 flask using 1ml of GTC solution (4M guanidinium thiocyanate, 25mM sodium citrate, pH 7, 0.5% N-laurylsarcosine and 0.1M ß-mercaptoethanol) and homogenised by trituration. For pituitary fragments tissue was homogenised in a glass hand homogeniser using approximately 200µl GTC solution per pituitary. Sequentially, 0.1vol 2M sodium acetate pH4, 1vol water saturated phenol and 0.2vol chloroform:isoamyl alcohol (49:1) were applied to the homogenate with vortexing. The homogenate was cooled on ice for 15min, centrifuged 10,000g for 20min at 4°C and the aqueous phase mixed with 1vol isopropanol at -20°C for 1h to precipitate RNA. After centrifugation at 12,000g for 20min at 4°C the RNA pellet was resuspended in 0.15vol GTC solution and precipitated with 1vol isopropanol at -20°C for 1h followed by centrifugation at 12,000g, 10min at 4°C. After washing the pellet with 75% ethanol RNA was vacuum dried and resuspended in TE buffer. RNA quality and quantity was determined using the absorbance ratio at 260 and 280nm and by ethidium bromide staining of agarose gels.

Poly A$^+$ messenger RNA (mRNA) was selected using two rounds of poly dT cellulose spun column chromatography (Pharmacia, Uppsala, Sweden). Typically 2-5% of the starting RNA was isolated as mRNA.

2.7.2 *Generation of radiolabelled subtraction probe*

Single strand ^{32}Phosphate labelled cDNA (ss cDNA*) was generated using mRNA isolated from AtT20 D16:16 cells treated for 90min with 10nM dexamethasone as shown schematically in Figure 2.5. A large scale synthesis (4µg mRNA in a 100µl reaction) was performed as for cDNA library construction (section 2.8.2) except that poly $dT_{(12-18)}$ (Pharmacia) was used as primer (1µg/µg mRNA), and the reaction contained 0.5mM each of dATP, dGTP, dTTP and 150µM dCTP (Boehringer Mannheim, Mannheim, Germany) and 400µCi α-^{32}P-dCTP (3000Ci/mmol, DuPont). First strand synthesis was terminated by adjusting the reaction to 20mM EDTA, 0.4% SDS, and template mRNA hydrolysed with 0.2N NaOH at 68°C for 30min. After cooling to RT the reaction was neutralized with HCl, the ss cDNA* was phenol-chloroform extracted and unincorporated label removed by centrifugation through a Nu-Clean R50 spin column (IBI Ltd., Cambridge, UK). The specific activity of the generated ss cDNA* was routinely >2.2x10^8cpm/µg. The ss cDNA* was hybridized in a heat sealed silanized capillary tube to a 30-fold mass excess of uninduced mRNA from AtT20 D16:16 cells for 48h to a R_0t of 1,000 (mol/litre x s) in 0.5M sodium phosphate (pH6.8), 1mM EDTA and 0.1% SDS at 68°C. Unhybridized single stranded ss cDNA* was collected by hydroxylapatite chromatography (Sambrook *et al.*,1989). Optimal separation was achieved using 0.14M sodium phosphate pH6.8, 0.1%SDS to elute ss cDNA* using a 1ml packed volume of hydroxylapatite (DNA-grade HAP, Bio-Rad, Watford, Herts) in a 5ml polypropylene syringe maintained at 68°C. After a single round of hybridization 7.4% of the input ss cDNA* remained unhybridized, this ss cDNA* was used to screen the unamplified dexamethasone induced library (see section 2.8.8 and Figure 2.5).

Figure 2.5

Generation of ss cDNA* probe used in cDNA library screening strategy

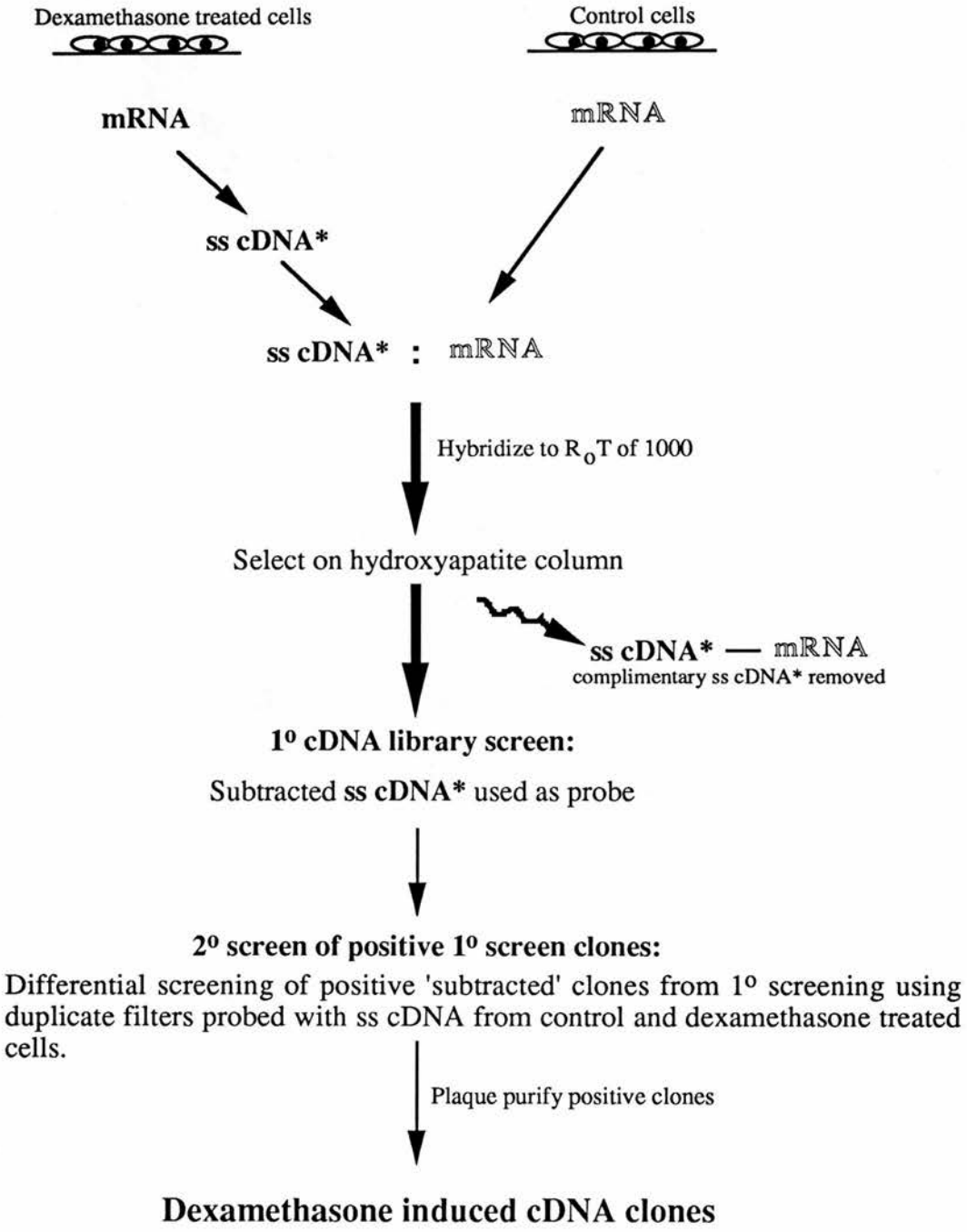


Figure 2.5: See sections 2.7.2 and 2.8.8 for generation of ss cDNA* probe and cDNA library screening strategy methodology

2.7.3 *Random prime labelling of cDNA inserts*

cDNA inserts were cut overnight from vector DNA using the appropriate restriction enzymes (Sambrook *et al.*,1989) and gel purified on a 1.2% low-melting point agarose (Gibco-BRL) gel run in 1xTBE buffer. The excised insert was heated to 65°C and extracted with phenol. The DNA in the aqueous phase was phenol/chloroform (24:1) extracted and precipitated at -20°C (Sambrook *et al.*,1989). Twenty-five nanograms of heat denatured DNA insert was used as template in a reaction volume of 50µl containing 50mM Tris-HCl, pH8.0, 5mM $MgCl_2$, 2mM dithiothreitol, 0.2M Hepes, pH6.6, 20µM each of dATP, dGTP, dTTP, 20µg BSA, $0.05A_{260}$ U random hexanucleotide primers, 50µCi α^{32}P-dCTP (DuPont, 3000Ci/mmol) and 5 U Klenow DNA polymerase using the Prime-a-gene kit (Promega Ltd, Southampton, UK). The reaction was continued at RT for 1.5-3h and terminated by heat inactivation (95°C for 2min) and adjusting the reaction mix to 20mM EDTA. Labelled DNA was purified by Sephadex G-25 column chromotography (Nu-Clean, IBI Ltd, Cambridge, UK) and used directly in hybridization. DNA was typically labelled to a specific activity of $>1x10^9$ cpm/µg.

2.7.4 *Northern blotting and hybridization*

Total RNA or polyA$^+$ mRNA was denatured for 15min at 65°C in MOPS/EDTA buffer (20mM MOPS, pH7.0 (3-(N-morpholino)propanesulfonic acid) containing 0.66M formaldehyde, 5mM sodium acetate and 1mM EDTA). Samples were electrophoresed for 2h at 100V on a 1% agarose gel (Gibco-BRL) containing 0.66M formaldehyde using MOPS/EDTA as running buffer (Fourney, Miyakoshi, Day III & Paterson,1987). After equilibrating the gel in 10xSSC (2x 20min washes) RNA was transferred overnight at 4°C by capillary blotting to nitrocellulose (Schleicher &

Schull, Dassel, West Germany) using 10xSSC (Sambrook *et al.*,1989). RNA was fixed to nitrocellulose by baking for 2h at 80°C before use in hybridization.

Northern blots were typically prehybridized for 2h at 55°C in 50% v/v deionized formamide (Sigma), 5x SSPE, 0.5x Denhardts (Sigma), 0.1% SDS and 0.2mg/ml salmon sperm carrier DNA (Sigma). Labelled DNA was applied directly to the prehybridization mixture (typically $2x10^6$ cpm/ml) and incubated for 16-18h. Filters were washed for 15min with 2xSSC, 0.1%SDS at 65°C and then twice with 0.1xSSC, 0.1%SDS for 15min. Any modification of the hybridization and washing protocols for individual probes is indicated in the respective figure legend. Filters were exposed at -70°C to Fuji-RX X-ray film (Fuji) using intensifying screens. Blots were reprobed with the abundant cytoplasmic 7S RNA (Balmain *et al.*,1982) to verify equal loading and transfer of RNA. Densitometric analysis of RNA blots was performed as described in section 2.9.2.

2.7.5 *Maintenance and transformation of competent E. Coli cells*

Long term glycerol stocks of the *E. Coli* strains PLK-F', XL-1 Blue, JM109 and HB101 (Stratagene) were prepared using 25% glycerol in LB medium (10g NaCl, 10g tryptone, 5g yeast extract per litre, pH 7.5) containing 12.5µg/ml tetracycline and stored at -80°C (Sambrook *et al.*,1989). For use bacteria were streaked onto a LB-agar plate containing 12.5µg/ml tetracycline and incubated overnight at 37°C.

Bacteria used in library titration, screening, amplification and *in vivo* excision were grown in overnight 50ml liquid cultures of LB medium containing 0.2% maltose (to induce the lambda bacteriophage receptor) and 10mM $MgSO_4$ at 37°C with vigorous shaking from a single colony selected from an LB-agar tetracycline plate. Cells were pelleted at 1000g for 10min and resuspended to the appropriate optical density at 600nm in 10mM $MgSO_4$.

Transformation was performed using the $CaCl_2$ method (Sambrook *et al.*,1989). Fifty millilitres of preequilibrated LB-broth were inoculated with 0.5ml of an overnight 50ml culture of bacteria (PLK-F', XL-1 Blue, JM-109 or HB101) and incubated at 37°C with shaking to an OD_{600} of 0.2. Flasks were cooled on ice and bacteria pelleted at 2,500g for 5min at 4°C. The bacterial pellet was resuspended in 25ml ice cold 50mM $CaCl_2$ and incubate at 4°C for 1h before pelleting bacteria. Cells were resuspended in 5ml ice cold 50mM $CaCl_2$ and incubated at 4°C for >2h. Plasmid DNA (25ng) was incubated for 30min at 4°C with 200µl of the above freshly prepared competent bacteria. Bacteria were heat shocked for 5min at 37°C, cooled on ice and plated overnight at 37°C on LB-agar plates containing 50µg/ml ampicillin.

2.7.6 *Plasmid DNA minipreps*

Minipreps were prepared from 3ml overnight cultures using the rapid boiling-lysozyme method (Holmes & Quigley,1981). Cultures (1.5ml) were pelleted, resuspended in 110µl STETL (50mM Tris, pH 8.0, 50mM EDTA, 8% sucrose, 0.5% Triton X-100, 0.5mg/ml lysozyme (Sigma)) and boiled for 30s in a water bath. Cell debri was pelleted (12,000g) at 4°C for 15min and DNA in the supernatant immediately precipitated with 1 vol isopropanol followed by centrifugation at 12,000g for 15min at RT. The DNA pellet was washed with 75% ethanol, dried and resuspended in TE buffer, pH 8.0. DNA was used for restriction digest as described in Sambrook *et al.* (1989).

2.8 cDNA Library construction

2.8.1 *General description of vector and cDNA library construction*

Representative cDNA libraries were constructed using the lambda ZAP vector (Short, Fernandez, Sorge & Huse,1988) after synthesis of cDNA using the ZAP-cDNA kit (Stratagene, Cambridge, Cambs, UK). A flow chart of cDNA library formation is shown in Figure 2.6.

The Uni-ZAP-XR™ vector is a lambda vector that allows the uni-directional insertion of cDNA (0-10kbp) in a sense orientation (EcoR I - Xho I) with respect to the vector lacZ promoter by using a 50 base oligo poly dT primer containing a Xho I recognition site in the synthesis of first strand cDNA. The sequence of the poly dT primer is shown below:

5' GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAGTTTTTTTTTTTTTTTTTT 3'
Xho I site

Methylated dCTP (5'-Me-dCTP) is included in the first strand reaction to prevent the digestion of cDNA containing internal Xho I sites in the subsequent generation of uni-directional cDNA. The second strand synthesis reaction contains an excess of dCTP to prevent 5'-Me-dCTP incorporation into the second strand allowing the Xho I site in the linker primer to be digested with Xho I. Following second strand synthesis EcoR I adaptors are ligated to the cDNA and the cDNA restricted with Xho I to generate cDNA with a 5' EcoR I site and a 3' Xho I site. After size fractionation the cDNA is ligated to the Uni-Zap-XR™ lambda vector arms containing complimentary EcoR I and Xho I termini and packaged to generate an infective lambda bacteriophage particle. As hemi-methylated DNA is digested by the mcrA and mcrB restriction system the bacteriophage are initially infected into mcrA- and mcrB- PLK-F'.

Figure 2.6

Schema for cDNA library construction in Uni-ZAP-XR™ vector

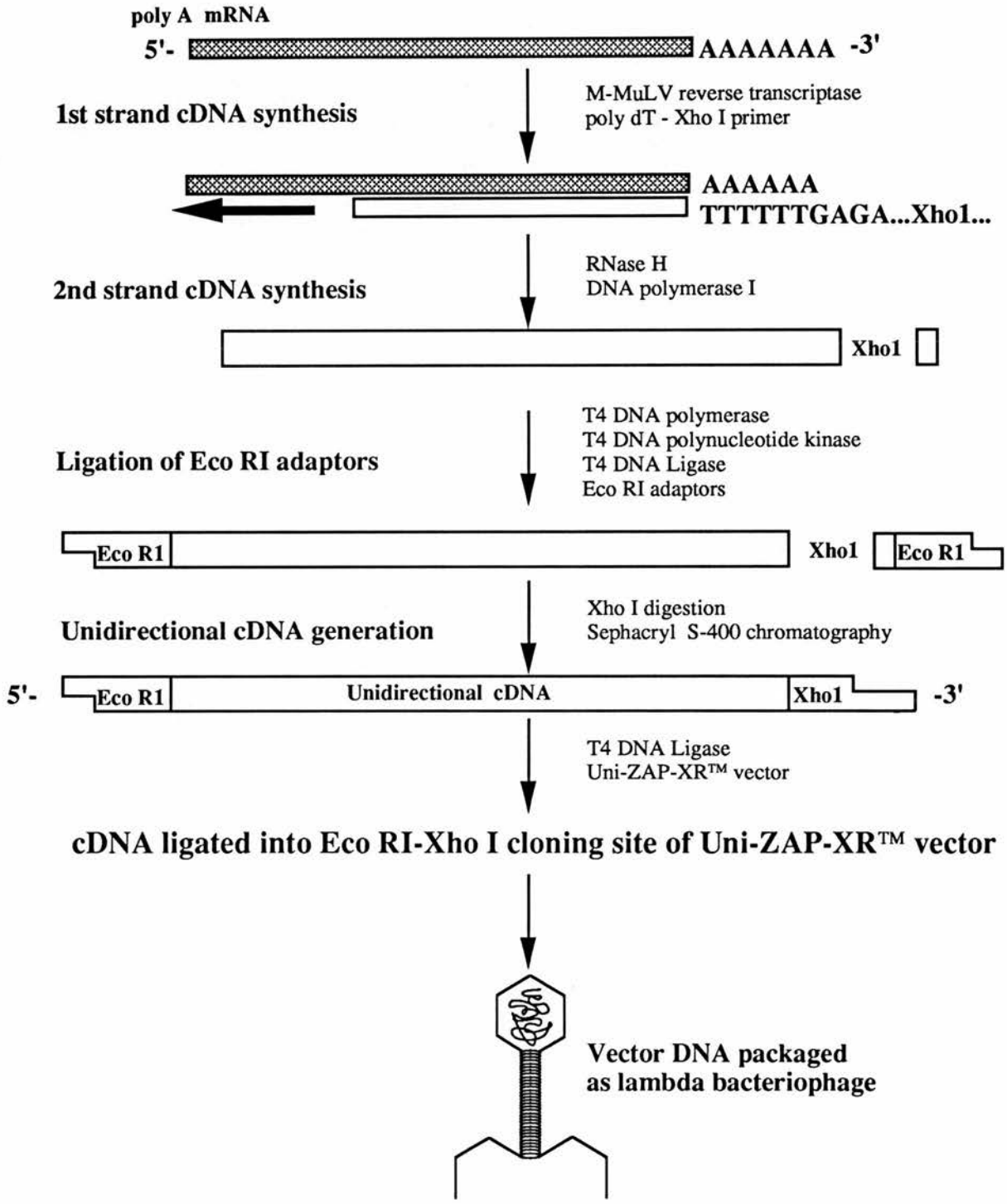


Figure 2.6: See section 2.8 for cDNA library construction methodology

After passage of the library through PLK-F' the DNA is no longer methylated and is grown on the mcrA+ and mcrB+ bacterial strain XL-1 Blue. Following identification of clones of interest the pBluescript plasmid is excised from the lambda bacteriophage in an *in vivo* reaction circumventing the lengthy subcloning procedures required for subsequent analysis of the cDNA insert.

2.8.2 *First strand cDNA synthesis*

First strand cDNA was synthesized from 4.5µg of poly (A^+) mRNA in a total reaction volume of 45µl containing 50mM Tris-HCl, pH 8.3, 75mM KCl, 3mM $MgCl_2$, 10mM dithiothreitol (DTT), 0.6mM each of dATP, dGTP, dTTP, 0.3mM 5'-Me-dCTP, 2.8µg poly dT-Xho1 primer linker, 1U RNase block II. The reaction was started by addition of 45U Moloney-Murine Leukaemia Virus reverse transcriptase and incubated at 37°C for 1h. A small scale reaction (0.5µg mRNA in 5µl) was performed in parallel for subsequent gel analysis and calculation of 1st strand cDNA yield using 5µCi α-^{32}P-dATP (Dupont, 800Ci/mmol). First strand cDNA yield was typically 8-15% of input mRNA as determined by incorporation of α-^{32}P-dATP into TCA precipitated material.

2.8.3 *Second strand cDNA synthesis*

The non-radioactive 45µl first strand synthesis was carried through to a 400µl volume second strand synthesis reaction containing: 25mM Tris-HCl, pH 8.3; 100mM KCl; 2mM $MgCl_2$; 2mM DTT; 0.2mM each of dATP, dGTP, dTTP; 0.5mM dCTP; 20µCi α-^{32}P-dATP (Dupont, 800Ci/mmol). The reaction was started by addition of 3.6U RNase H and 75U DNA polymerase I and incubated for 2.5h at 16°C. After synthesis the cDNA was extracted sequentially with an equal volume of phenol:chloroform (24:1) and chloroform. The subsequent aqueous cDNA phase was

precipitated overnight at -20°C with 0.1vol 3M sodium acetate and 2.5vol 100% ethanol and pelleted by centrifugation at 4°C for 1h at 12,000g. After washing the pellet with 75% ethanol the cDNA was vacuum dried and resuspended in sterile dH_2O. An aliquot was removed for subsequent second strand yield determination and gel electrophoresis. Typically second strand synthesis yield was greater than 80% of first strand cDNA as determined by TCA precipitation (Sambrook *et al.*,1989).

2.8.4 *Generation of uni-directional cDNA inserts*

The uneven termini of double stranded cDNA were blunted using 10U T4 DNA polymerase in a 50µl reaction containing 30mM Tris acetate, pH 7.9; 70mM potassium acetate; 10mM magnesium acetate; 0.5mM DTT; 125µM each of dATP, dCTP, dGTP, dTTP at 37°C. After 30min 50µl of dH_2O was added to the reaction and the cDNA extracted with an equal volume of phenol:chloroform (24:1) followed by chloroform. The supernatant was precipitated at -20°C for 1h using 0.1vol 3M sodium acetate and 2.5vol 100% ethanol. After centrifugation at 12,000g for 1h at RT the cDNA pellet was washed with 75% ethanol and vacuum dried.

EcoR I adaptors were ligated at 4°C to the blunted cDNA in a 10µl reaction containing: 50mM Tris-HCl, pH 7.5; 7mM $MgCl_2$; 1mM DTT; 1mM ATP and 4 Weiss U of T4 DNA ligase. After 36h the ligase was heat inactivated at 70°C for 10min. EcoR I ends were kinased in a 20µl reaction for 30min containing: 50mM Tris-HCl, pH 7.5; 7mM $MgCl_2$; 1mM ATP and 10 U T4 Polynucleotide kinase, the reaction was terminated by heat inactivation at 70°C.

The heat inactivated kinase reaction was carried through to a 50µl Xho I digest reaction containing: 50mM Tris-HCl, pH7.5; 10mM $MgCl_2$; 100mM NaCl; 1mM DTT and 135 U Xho I. This generates cDNA that possess a 5' EcoR I site and a 3' Xho I

recognition site that allows uni-directional insertion of the cDNA into the Uni-Zap-XR™ vector.

2.8.5 *cDNA size fractionation on Sephacryl S-400 spin column*

The 50µl Xho I reaction was cooled to RT and adjusted to 100mM NaCl and 1mM EDTA, pH8.0. The cDNA was applied to a 1ml column of Sephacryl S-400 preequilibrated with STE buffer (10mM Tris-HCl, pH8.0, 100mM NaCl and 10mM EDTA) in a sterile 1ml polypropylene syringe barrel (Sabre). The cDNA was eluted from the column by spinning at 600g. Two sequential 60µl aliquots of STE were applied and the the three cDNA fractions pooled. These fractions contained cDNA of >500bp whereas cDNA fragments <500bp and unincorporated nucleotides remained on the column. Typically 30-100cps were detected per fraction on a Geiger-Muller tube. The pooled fractions were extracted sequentially with phenol:chloroform (24:1) and chloroform and precipitated overnight with 2vols 100% ethanol at -20°C. The cDNA was pelleted by centrifugation for 1h at 12,000g, washed with 75% ethanol, vacuum dried and resuspended in 10µl sterile dH_2O. The cDNA was quantitated by spotting 0.5µl of the sample on an ethidium bromide agarose plate (Sambrook *et al.*,1989), using salmon sperm testes DNA as standard (Sigma).

2.8.6 *Ligation and packaging of cDNA into Uni-Zap -XR ™ vector*

Optimum ligation was achieved using 100ng of cDNA per 1µg of calf intestinal alkaline phosphatase (CIAP) treated Uni-Zap-XR™/Eco RI and Xho I prepared arms in a 5µl reaction containing: 50mM Tris-HCl, pH 7.5; 7mM $MgCl_2$; 1mM DTT and 1mM ATP. The reaction was started by addition of 2 Weiss U of T4 DNA Ligase, continued for 48h at 4°C, followed by 2h at RT before packaging.

Packaging was performed using the Gigapack II Gold packaging extract (Stratagene (Kretz & Short,1989)). Test packaging reactions of the ligated cDNA/vector were performed by mixing 1-2.5µl aliquots on ice with the thawing freeze/thaw extract. Fifteen microlitres of sonic extract were applied to the cDNA, gently mixed and incubated for 2h at RT. Five hundred microlitres of SM buffer (50mM, pH 7.5; 100mM NaCl, 8mM $MgSO_4$ and 0.01% gelatin) and 20µl chloroform were added, spun for 2s at 12,000g to sediment debri. The supernatant was titered and stored at 4°C. If greater than 200,000 pfu/µl ligation was obtained the remaining ligated cDNA/vector was packaged in the same ratio as for the test package. The background titre of vector arms alone ligated to themselves was $<4x10^4$ pfu/µg vector arms. Vector ligated with cDNA resulted in a 25-70 fold increase in efficiency compared to arms alone. Primary libraries resulted in $>1.3x10^6$ independent clones from 100ng cDNA ligated per 1µg vector arms.

2.8.7 *Amplification of primary cDNA library*

Primary libraries of lambda bacteriophage are relatively unstable, thus the majority of each library was amplified immediately into a high titre stock library ($>1x10^{10}$ pfu/ml). Aliquots of the packaged lambda clones containing 50,000pfu were incubated with 600µl of (OD_{600} = 0.5) PLK-F' host E. coli bacteria for 15min at 37°C and plated on prewarmed (37°C) NZY agar plates (NZY broth + 15g/l agar) using 6.5ml NZY top agar (NZY broth + 0.7% agarose) per 150mm plate (CelCult). Plates were incubated for 5-8h at 37°C until pinpoint plaques were generated. Bacteriophage were eluted overnight at 4°C using 10ml SM buffer per plate with gentle rocking. After rinsing the plates with a further 2mls of SM buffer the bacteriophage suspension was pooled, chloroform added to 5% and incubated for 15min at RT. Cell debris was removed by centrifugation at 5min at 4000g, the

supernatant recovered and chloroform added to 0.3%. Aliquots (1ml) containing $>1x10^{10}$ pfu/ml were stored at 4°C. For longer term storage aliquots were stored at -70°C with 7% dimethylsulphoxide.

2.8.8 *Screening of dexamethasone-induced cDNA library*

A summary of the screening strategy employed to isolate dexamethasone-induced cDNA clones from a cDNA library of AtT20 D16:16 cells treated with dexamethasone is shown in Figure 2.5, section 2.7.2.

1° screen: Fifty thousand unamplified plaques were plated at a density of 12,500pfu per 150mm plate on NZY-agar plates using PLK-F' as host as described for amplification of libraries. Phage DNA was transferred to nitrocellulose filters (Millipore, Watford, Hertfordshire, UK), denatured with 0.5M NaOH, 1.5M NaCl, neutralized with 0.5M Tris-HCl, pH 8, 1.5M NaCl and washed with 0.2M Tris-HCl pH7.5, 2xSSC and baked for 2h at 80°C (Sambrook *et al.*,1989). Preybridization was performed at 42°C in a solution of 20mM Pipes, pH6.5, 0.8M NaCl, 50% deionized formamide, 0.5% SDS and 0.1mg/ml salmon sperm DNA for 2h. Radiolabelled single stranded cDNA (ss cDNA*), enriched for dexamethasone induced sequences as described in section 2.7.2, was added directly to the prehybridisation mixture and incubated for 16-18h. Filters were sequentially washed for 15min with 2x SSC, 0.1% SDS at 42°C, 0.1x SSC, 0.1%SDS at 42°C and 0.1xSSC, 0.1% SDS at 55°C and exposed to Fuji-RX X-ray film for 2 weeks at -70°C.

2° screen: Positive plaques were transferred to a cell lawn of XL-1 Blue cells on 150mm NZY-agar plates and rescreened as a grid using duplicate lifts hybridized to single stranded ^{32}P-cDNA probes generated from dexamethasone induced or control AtT20 D16:16 cell mRNA.

2.8.9 *In vivo excision of pBluescript plasmid from Uni-ZAP-XR ™ bacteriophage vector*

Two hundred microlitres of recombinant bacteriophage stock (containing>$1x10^5$pfu) were incubated with 200µl of (OD_{600} = 1.0) XL-1 Blue bacteria and 1µl of R408 helper phage (Stratagene) at 37°C. After 5min the mixture was incubated with 5ml of 2x YT buffer (10g NaCl, 10g yeast extract and 16g tryptone per litre, pH7.5) with shaking at 37°C for 3h in a 50ml conical tube. Bacteria were heat inactivated for 20min at 70°C and debri pelleted at 1000g for 5min. The supernatant contained the pBluescript phagemid packaged as a filamentous phage particle and was used to reinfect XL-1 Blue cells and maintained on LB-agar plates containing 50µg/ml ampicillin for recombinant selection.

2.9 Miscellaneous

2.9.1 *Statistics*

Statistical evaluation between different treatments in the perifusion studies were performed using the non-parametric Kruskal-Wallis test with multiple comparisons for several independent samples or the Mann-Whitney U-test for two independent samples (Conover,1980). Alternatively Students t-test (unpaired, 2-tail) was used where appropriate.

2.9.2 *Densitometric analysis*

2D-gels: Analysis was performed manually by two separate observers (MJS and JS) using grid overlays after protein detection using autoradiography or silver staining. Only protein spots that differed in intensity/migration in all gels of a treatment group compared to control (typically 6 gels per group run simultaneously) were analysed

further for optical density measurements using peak grey scale levels (0-256) determined by the Optomax V Image analyser (Analytical Measuring Systems (Synoptics), Cambridge, Cambs, UK).

Northern- and Immuno-blots: The areas and optical densities of the respective bands on blots and autoradiographs were determined using the area densitometry algorithm on the Optomax V Image analyser (Analytical Measuring Systems (Synoptics), Cambridge, UK).

For northern blot analysis of calmodulin mRNA, optical density measurements were divided by the respective values for 7S RNA yielding the normalized hybridization intensity. Finally, because of slight variability in the control levels of calmodulin mRNA between experiments (see section 4.3.2), the values for each experimental treatment were divided by the normalized hybridization intensity of the control group for each experiment to obtain the relative hybridization intensity.

For immunoblots, standard curves were constructed with the respective antigen and protein levels in unknown samples determined by interpolation of the respective optical density measurements.

3

Characteristics of early glucocorticoid inhibition in perifused rat anterior pituitary gland segments

3

CHARACTERISTICS OF EARLY GLUCOCORTICOID INHIBITION IN PERIFUSED RAT ANTERIOR PITUITARY GLAND SEGMENTS

3.1 Introduction

In order to investigate the characteristics of early glucocorticoid inhibition a reproducible ACTH secretory response is required. To date the most reliable *in vitro* model is the column perifusion system (Antoni,1986; Watanabe & Orth,1988), furthermore, the composition and timing of experimental treatments is easily manipulated in this system allowing glucocorticoid-secretagogue interactions to be studied. Perifused anterior pituitary gland segments were used as a model because they provide the closest approximation to *in vivo* corticotroph function as cell morphology, intercellular communication and plasma membrane receptors/channels are relatively conserved compared to mechanically and enzymatically dispersed cells.

In the following section the characteristics of early inhibition of CRF-41-, and AVP-, stimulated ACTH release are investigated. The interactions observed between CRF-41 and glucocorticoid during these studies were then explored further to investigate whether the physiological ACTH secretagogues, CRF-41 and AVP, interact at the adenohypophysis to modulate glucocorticoid action. The results presented in this section have been published as full papers (Shipston & Antoni,1991; Shipston & Antoni,1992).

Results

3.2 Early glucocorticoid inhibition of CRF-41-stimulated ACTH release

3.2.1 *Response to CRF-41*

To evaluate the characteristics of early glucocorticoid inhibition it is essential to use a model that consistently responds to ACTH secretagogues. Figure 3.1 shows the dose-response relationship of net ACTH release and 5min pulses of CRF-41 in perifused female ex-breeder Wistar (Harlan Olac) rat anterior pituitary segments *in vitro*. CRF-41 was used at the submaximal concentration of 10nM in subsequent experiments using Harlan Olac rats. After a 3h preperifusion period, to allow basal ACTH release to stabilize, the release of ACTH at 4h in response to a 5min pulse of 10nM CRF-41 was enhanced (net ACTH release was 121.0±5.2% of the release at 3h, mean±SEM n=46, $p<0.05$) with respect to the response at 3h in the columns. Subsequent stimuli released similar amounts of ACTH to that released at 4h, up to 7h after the start of perifusion (see Figures 3.2a and 3.3a). Typical ACTH release profiles for a control CRF-41 and a synthetic glucocorticoid (RU28362)-treated column are shown in Figure 3.2.

These data demonstrate that perifused rat anterior pituitary gland segments consistently respond in a dose-dependent fashion to CRF-41 and are thus a viable model in which to explore early glucocorticoid inhibition of CRF-41-stimulated ACTH secretion.

Figure 3.1

Release of ACTH by perifused rat anterior pituitary segments in response to CRF-41 and AVP

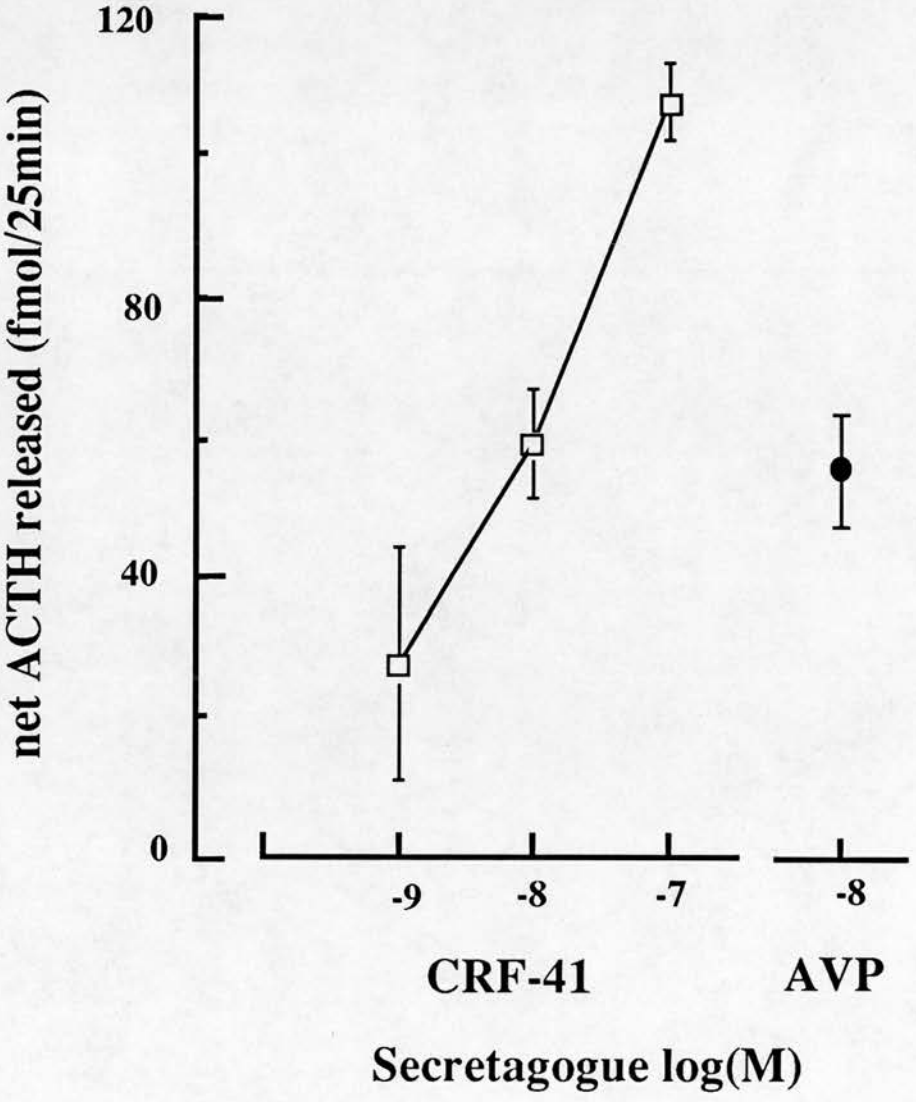


Figure 3.1: Four columns of segments were exposed to various concentrations of secretagogue for 5min at 1h intervals in a randomized complete block design after initial exposure to two doses of 10nM CRF-41. Data are the means of the net release of hormone ± SEM (n=4/group). The basal secretory rate in this experiment was 62.1± 4.3 fmol ACTH/25min (n=24).

Figure 3.2

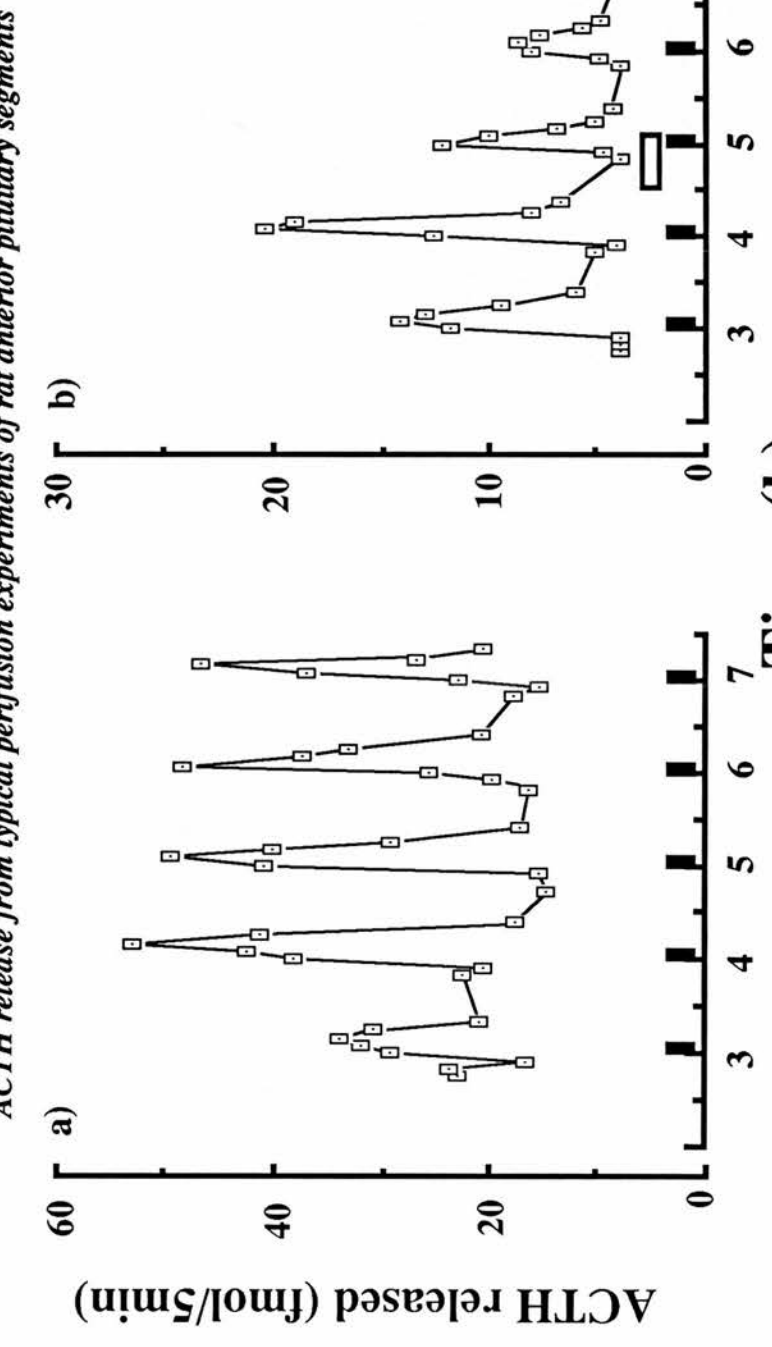


Figure 3.2: a) The release of ACTH in response to repeated 5min pulses of CRF-41 (10nM, solid vertical bars) and; **b)** the effect of a 35min exposure (open horizontal bar) to the synthetic glucocorticoid type II receptor agonist, RU28362 (1 μM), on CRF-41-stimulated ACTH secretion. Data are expressed as the amount of immunoreactive ACTH present in each 5min collection fraction. Interexperimental variations in absolute ACTH release (compare abscissa of a with b) required subsequent data to be standardized as described in section 2.2.3.

3.2.2 *Effect of glucocorticoids on CRF-41- stimulated ACTH release*

To verify that glucocorticoids suppress CRF-41-stimulated ACTH release in the early time domain (within 2h) in perifused rat anterior pituitary segments the time course, receptor pharmacology and transcription/translation inhibitor sensitivity of glucocorticoid action were characterized.

Exposure of Harlan Olac rat pituitary segments to the physiological rat glucocorticoid, corticosterone, at 0.1 μM applied 30min before and during the 10nM CRF-41 pulse at 5h resulted in a significant ($p<0.05$) attenuation of stimulated ACTH release after 30min; the inhibitory effect was maintained at 6h. At 7h (2.5h after the application of corticosterone) the release of ACTH returned towards the control value (Figure 3.3b). The degree of inhibition of ACTH release was not altered by increasing the corticosterone concentration to 1μM. The inhibition of CRF-41-stimulated ACTH release by corticosterone exhibited a lag-time of at least 15min (Figure 3.3c). Surprisingly, when corticosterone application was started simultaneously with the CRF-41 pulse at 5h (and continued for a total of 35min) no inhibition of ACTH secretion occurred at any subsequent time point (Figure 3.3d).

The potent glucocorticoid type II receptor agonist, RU28362 (Philibert & Moguilewsky,1983), at a concentration of 1μM had no significant inhibitory effect after 35min of exposure when applied 30min before a 10nM CRF-41 stimulus (Figure 3.4). However, at this time-point the release of ACTH was highly variable: there was a marked inhibition in two out of six experiments. After 90min ACTH release was significantly ($p<0.05$) reduced to 50% of control and, unlike corticosterone, the inhibitory effect was maintained 2.5h after application using 1μM and 0.1μM RU28362 (compare application of corticosterone and RU28362 before a CRF-41 stimulus at 5h in Figures 3.3b and 3.4 respectively). Figure 3.5 demonstrates that the

synthetic type II glucocorticoid receptor agonist, RU28362, suppresses the amount of ACTH released by a CRF-41 pulse, but has no apparent effect on the time course of the ACTH secretory response.

To investigate whether the inhibitory action of corticosterone observed after 30min exposure is manifest through the type II glucocorticoid receptor the potent type II glucocorticoid receptor antagonist RU38486 (Philibert,1984) at 1μM was applied 15min before and during the 35min exposure to corticosterone. RU38486 completely blocked the action of corticosterone at this time point. In two experiments net ACTH release was 109% and 86% of control at 5h in columns receiving corticosterone and RU38486, compared to 61.3±3.4 (n=6) in columns treated with corticosterone alone as in Figure 3.3b. RU38486 alone had no effect on stimulated or basal ACTH secretion.

Actinomycin D (0.1mM), an irreversible inhibitor of DNA-dependent RNA synthesis (Gale *et al.*,1981), completely blocked the inhibitory action of RU28362 on CRF-41-induced ACTH secretion at all time points when given 5min before and during the application of the steroid (Figure 3.4a) and had no effect on CRF-41-stimulated ACTH release (in 2 columns net ACTH release was 104 and 120 % of the respective controls at 5h; 115 and 90% at 6h; 107 and 101% at 7h). Initial experiments using the reversible transcription inhibitor α-amanitin at concentrations up to 1μM and applied as for puromycin (see Figure 3.4b) failed to block the action of RU28362. However, at higher concentrations α-amanitin was insoluble. Furthermore, although α-amanitin has been used extensively in cell-free analysis of RNA-polymerase II action, its cyclic structure and molecular weight prevent its transfer across the plasma membrane in many cell types (Kuwano & Ikehara,1973; Wieland & Faulstich,1978).

Figure 3.3

Corticosterone inhibition of CRF-41-stimulated ACTH release in perifused rat anterior pituitary segments

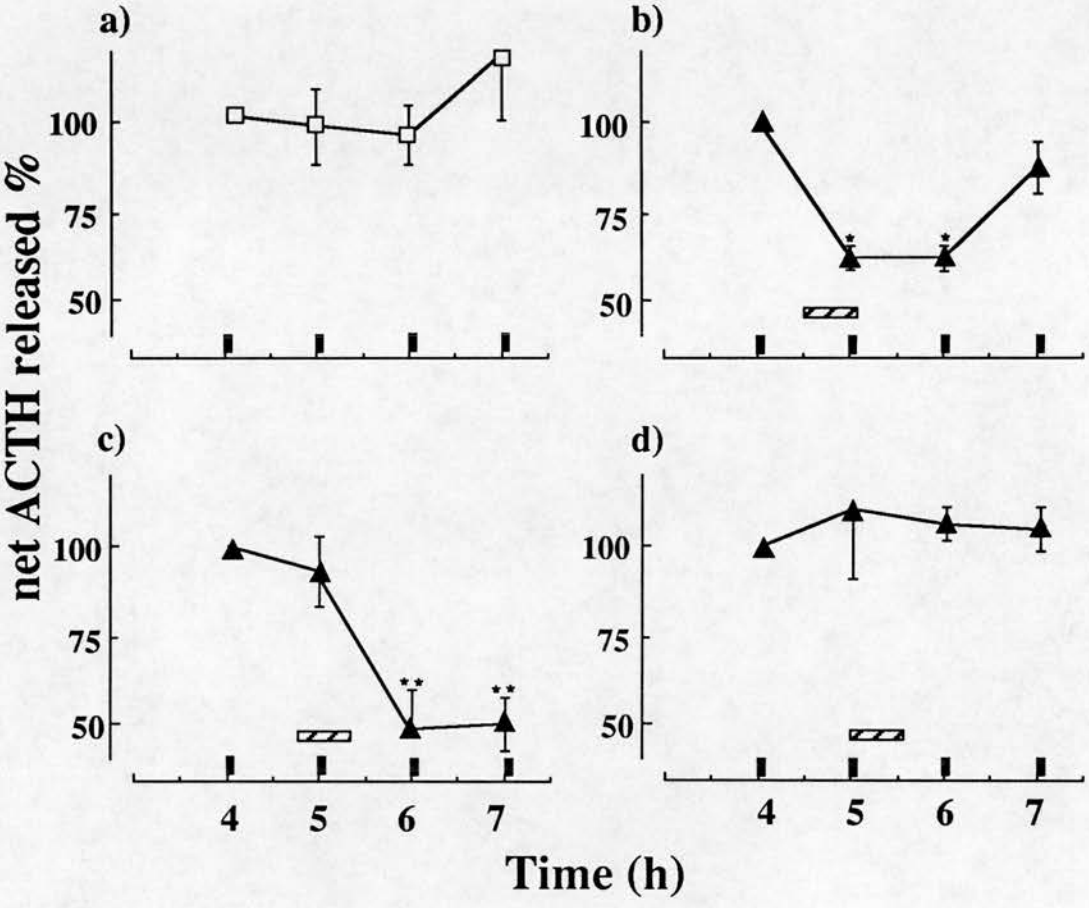


Figure 3.3: The control group **a)** received repeated 5min pulses of 10nM CRF-41, (solid vertical bars). Other groups received corticosterone (0.1 μM, 35min total exposure; horizontal hatched bars): 30min before, **b)**; 15min before **c)** or simultaneously with **d)** the start of the CRF-41 pulse at 5h. Data are expressed as a percentage of the net ACTH released by a 5min pulse of 10nM CRF-41 received at time 4h by all groups. Data are means ± SEM (n=4-6/group). The range of absolute ACTH release at 4h (100%) was 75.6-494.8 fmol ACTH/25min. * $p<0.05$, ** $p<0.01$ (non-parametric Kruskal-Wallis test) compared with the control group.

Figure 3.4

Antagonism of RU28362 inhibition of CRF-41-stimulated ACTH secretion from perifused rat anterior pituitary segments by inhibitors of: a) transcription (actinomycin D) and b) translation (puromycin)

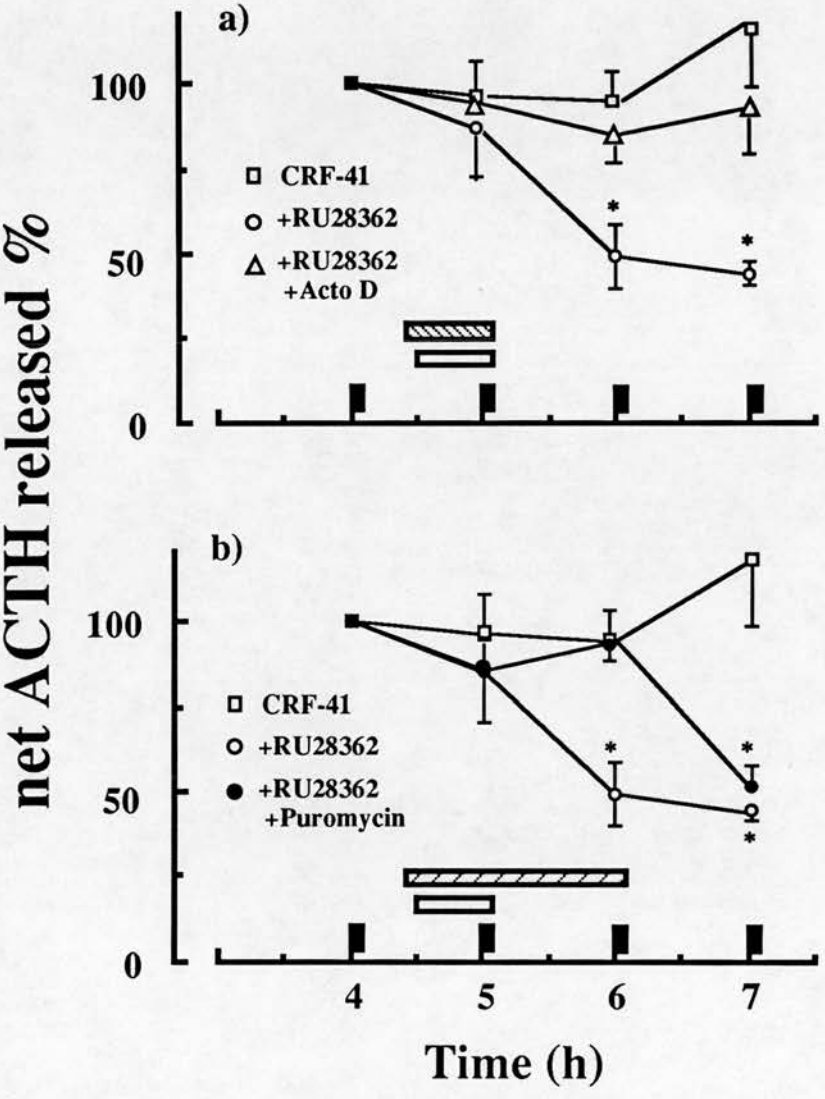


Figure 3.4: Data are expressed as a percentage of the net amount of ACTH released after the 5min pulse of CRF-41 (10nM; solid vertical bars) at 4h. The synthetic glucocorticoid, RU28362, (1 μM) was applied for 35min, (open horizontal bar); actinomycin D (0.1mM) for 40min,(short hatched horizontal bar in a; puromycin (0.1mM) for 100min, (long hatched horizontal bar in b. Data are means ± SEM (n=4-6/group). The range of absolute ACTH releaseat 4h was 75.6-494.8 fmol ACTH/25min. * $p<0.05$ (non-parametric Kruskal-Wallis test) compared with CRF-41 control.

Figure 3.5

Effect of the synthetic type II glucocorticoid agonist, RU28362, on the profile of CRF-41-stimulated ACTH release in perifused rat anterior pituitary segments

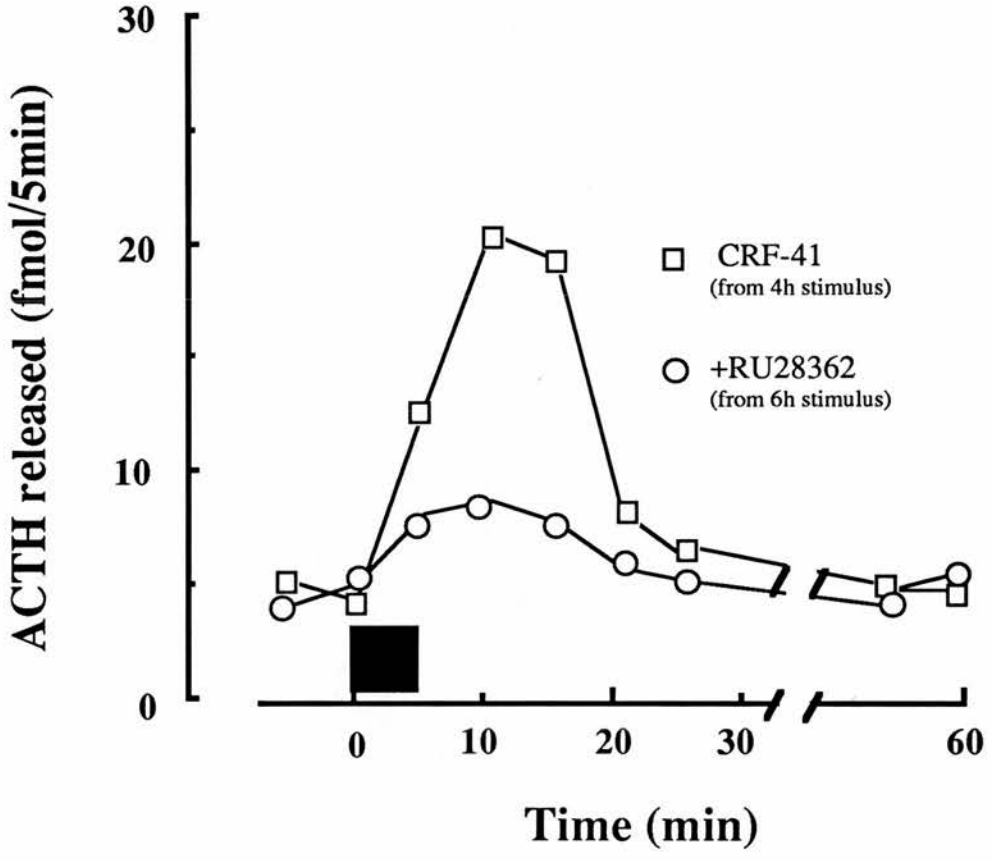


Figure 3.5: Typical CRF-41-stimulated ACTH release profiles from a representative experiment performed as in Figure 3.2 a & b. The ACTH release profile for CRF-41 (10nM for 5min, solid horizontal box) alone are from the 4h stimulus. The CRF-41-stimulated ACTH release profile after RU28362 treatment is from the 6h stimulus in the same column. RU28362 (0.1μM 35min total duration) was started 30min before the 5h stimulus as in Figure 3.2b. Data are expressed as the amount of immunoreactive ACTH in each 5min collection fraction.

The inhibition of CRF-41-stimulated ACTH release by RU28362 was prevented during exposure of the segments to the reversible protein synthesis inhibitor, puromycin (Gale *et al.*,1981), at a concentration of 0.1mM (Figure 3.4b). The full inhibitory action of RU28362 was observed 1h after withdrawal of puromycin, indicating that translatable mRNA was still present in the cells. Puromycin applied at the same concentration and duration as for the RU28362 experiments completely blocked the inhibitory action of corticosterone on CRF-41-stimulated ACTH release at all time points: In two experiments the net ACTH release expressed as a percent of control was 117% and 135% at 5h, 103% and 105% at 6h, and 101% and 119% at 7h. Puromycin alone did not modify CRF-41-stimulated ACTH release (in 2 experiments net ACTH release was 117% and 135 % of the respective controls at 5h, 103% and 105% at 6h , 101% and 109 % at 7h).

Increasing the concentration of glucose in the perifusion medium to 20mM (compared to the normal 5.6mM) had no effect on RU28362 inhibition of CRF-41-stimulated ACTH release. The mean net ACTH release expressed as a percentage of the 4h stimulus using medium supplemented with 20mM glucose was 96% and 101% at 5h, 87% and 40% at 6h, and 100% and 35% at 7h for control and RU28362 treated experiments respectively.

In summary, these data support the hypothesis of Dayanithi & Antoni (1989) implicating activation of type II glucocorticoid receptors and induction of new mRNA and protein in the early (within 2h) inhibitory action of glucocorticoids.

3.2.3 *The relative timing of CRF-41 and glucocorticoid application is essential for development of early glucocorticoid inhibition of CRF-41-stimulated ACTH release*

During analysis of the time course of early glucocorticoid inhibition of CRF-41-stimulated ACTH release, presented in section 3.2.2, it was observed that when corticosterone was started simultaneously with the CRF-41 pulse at 5h (and continued for a total of 35min) no inhibition of ACTH secretion occurred at any subsequent time point (Figure 3.3d). This apparent modulation of glucocorticoid action was further explored with the aim of providing information that would be useful in identification of proteins mediating early inhibition.

Because the time between the start of corticosterone administration and the stimuli at 6 and 7h is longer (by 30min) for columns receiving corticosterone 30min before, rather than simultaneously with, the 5h CRF-41 pulse, experiments were performed to verify that the absence of inhibition upon simultaneous application of corticosterone and CRF-41 was not a result of the 'silent' (steroid non-responsive) period reported by some workers (Abou-Samra *et al.*,1986a; Mahmoud *et al.*,1984). Four perifusion columns were treated with 0.1μM corticosterone applied simultaneously with the CRF-41 stimulus at 5h, stimuli were then applied at 6.5h and 7.5h. No significant inhibition was observed at either time point (net ACTH released, at 6.5h, 101.0±7.9%; at 7.5h, 102.0±7.1%).

The duration of exposure to corticosterone is also important in determining the characteristics of early inhibition in this system. When 10nM CRF-41 was applied at the start of a continuous corticosterone infusion (0.1μM started at 5h) no inhibition of CRF-41 stimulated ACTH release developed 1h after the start of corticosterone treatment (Figure 3.6). However, significant inhibition of CRF-41-stimulated ACTH release developed after 2h of steroid exposure.

Figure 3.6

Inhibition of stimulated ACTH secretion by a continuous exposure to corticosterone started simultaneously with a CRF-41 stimulus in perifused rat anterior pituitary segments.

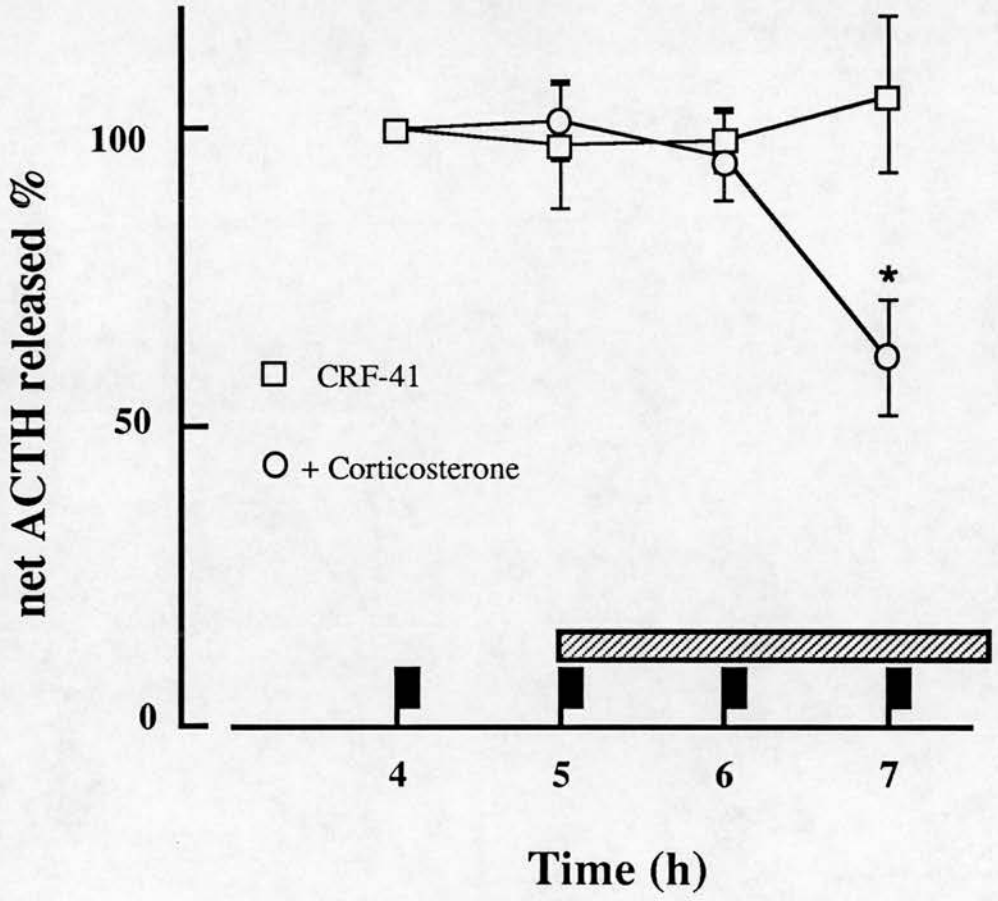


Figure 3.6: The control group received repeated 5min pulses of 10nM CRF-41 (solid vertical bars) every 1h. The other group received 0.1μM corticosterone (horizontal hatched bar) started simultaneously with the 5h stimulus and maintained to the end of the experiment. Data are expressed as a percentage of the net ACTH released by a 5min CRF-41 stimulus applied at 4h. Data are means ± SEM (n=4-7/group). The range of absolute ACTH released at 4h (100%) by CRF-41 was: 75.6-494.8 fmol ACTH/25min. *P<0.05, **p<0.01 (non-parametric Mann-Whitney U test) compared with the CRF-41 control group.

The above observations do not resolve whether CRF-41 applied after the start of glucocorticoid exposure (as in Figure 3.3b) is actually required to activate early inhibition or, alternatively, that CRF-41 applied at the start of glucocorticoid exposure (as in Figure 3.3d) inactivates the inhibitory action of glucocorticoids.

To determine whether CRF-41 is required to activate early inhibition corticosterone was applied 30min before the 5h time point and the 10nM CRF-41 stimulus at 5h omitted. Using this paradigm no subsequent inhibition developed (Table 3.1). Application of 0.1nM or 10nM CRF-41 at 5h during the exposure to corticosterone resulted in significant inhibition of stimulated ACTH release within 30min before returning towards control at 7h (Table 3.1). Application of the 0.1nM CRF-41 stimulus or omission of the stimulus at 5h had no effect on subsequent ACTH release thus their respective controls were pooled with those receiving 10nM ACTH at 5h. Furthermore, if corticosterone (0.1μM, for total duration of 35min) was applied for 20min before and 15min after, but not during, the 5h stimulus no subsequent inhibition developed (Table 3.1). These data suggested that CRF-41 is required to activate some aspect of early glucocorticoid inhibition

However, the Harlan Olac rats used in the work suggesting an activation mechanism were subsequently reported to be infected with Sendai virus. Consequently, the timing characteristics of glucocorticoid action had to be re-investigated in rats obtained from an alternative supplier, Charles River.

Repeated exposure of female ex-breeder Wistar (Charles River) rat anterior pituitary segments with 10nM CRF-41 resulted in downregulation of subsequent ACTH responses. However, anterior pituitary glands from Charles River rats consistently responded to 0.1nM CRF-41 (Figure 3.7), a concentration approximating that recorded in rat hypophysial portal blood (Plotsky,1991).

Table 3.1

CRF-41 activates early glucocorticoid inhibition of CRF-41-stimulated ACTH release in perifused anterior pituitary segments from virally infected Harlan-Olac rats

net ACTH released %			
4h CRF-41	**5h** Treatment	**6h** CRF-41	**7h** CRF-41
100	CRF-41 (10)	97.3±5.4	105.4±13.2
100	Corticosterone (4)	94.5±4.9	74.3±10.3
100	Corticosterone + 10nM CRF-41 (6)	61.3±3.7**	87.0±7.4
100	Corticosterone + 0.1nM CRF-41 (4)	69.4±9.4*	81.0±8.3
100	Corticosterone + 10nM CRF-41 (4)	97.3±6.96	71.8±5.4

Table 3.1: All groups received 5min pulses of 10nM CRF-41 at 4, 6 and 7h. The various treatments applied, along with their temporal relationship of application, are shown in the 5h treatment column. CRF-41 was applied for 5min at either 0.1nM or 10nM. Because omission of the 5h stimulus or application of 0.1nM CRF-41 at 5h did not affect the subsequent stimulated ACTH release (see section 3.2.3) the corresponding controls for these experiments were pooled with the 10nM CRF-41 controls. Corticosterone (0.1μM, 35min total exposure) was started 30min before the 5h stimulus. In the last row corticosterone was terminated 10min before the 5h stimulus and reapplied for a further 15min starting 15min after the 5h stimulus. Data are expressed as a percentage of the net ACTH released by a 5min pulse of CRF-41 at 4h (100%) in all groups. Values are means ± SEM; the number of experiments in each treatment group are shown in parentheses. The range of absolute net ACTH release between experiments at 4h (100%) was 108.4-494.8 fmolACTH/25min. **$p<0.01$, *$p<0.05$ compared to control using the nonparametric Kruskal-Wallis test.

Figure 3.7

Early corticosterone inhibition of CRF-41-stimulated ACTH secretion in perifused rat (Charles River) anterior pituitary segments

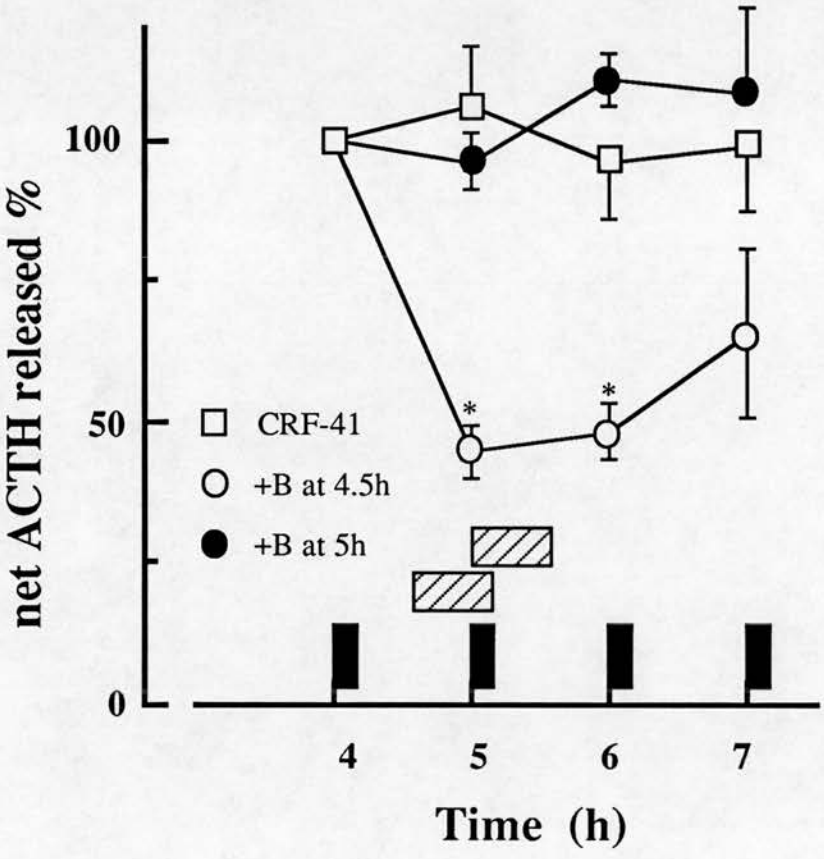


Figure 3.7: The control group (CRF-41) received 5min pulses of 0.1nM CRF-41 every 1h. Other groups also received 0.1μM corticosterone (B) (total exposure 35min; horizontal hatched bar) applied 30min before (+B at 4.5h) or simultaneously with (+B at 5h) the CRF-41 pulse at 5h. Data are expressed as a percentage of the net ACTH released by a 5min pulse of 0.1nM CRF-41 received at time 4h (100%) by all groups. Data are means ± SEM (n=4-7/group). The range of absolute ACTH release between experiments for the 4h (100%) stimulus was 113.4-466.8 fmol/15min. *$p<0.05$ compared to control using the nonparametric Kruskal-Wallis test.

Table 3.2

Inactivation of corticosterone inhibition of CRF-41-stimulated ACTH secretion by CRF-41 and the cyclic adenosine 3',5'-monophosphate analogue, 8-CPT-cAMP, in perifused (Charles River) rat anterior pituitary segments

net ACTH released %			
4h CRF-41	**5h** Treatment	**6h** CRF-41	**7h** CRF-41
100	CRF-41 (7)	98.0±8.4	99.0±9.9
100	Corticosterone (4)	61.5±9.9[a]	54.3±9.3[a]
100	Corticosterone +CRF-41 (5)	108.4±4.3[b]	111.2±12.4[b]
100	8-CPT-cAMP (8)	103.5±15.1	76.3±12.2
100	Corticosterone + 8-CPT-cAMP (6)	101.2±13.2[b]	106.0±13.6[b]

Table 3.2: All groups received 5min pulses of 0.1nM CRF-41 at 4, 6 and 7h. The various treatments applied, along with their temporal relationship of application, are shown in the the 5h treatment column. 8-CPT-cAMP was applied for 5min at 0.1mM. Corticosterone (0.1μM, 35min total exposure) was started simultaneously with the 5h stimulus. Data are expressed as a percentage of the net ACTH released by a 5min pulse of 0.1nM CRF-41 applied at 4h (100%) in all groups. Because omission of the 5h CRF-41 stimulus did not affect subsequent stimulated ACTH release data from control columns receiving no CRF-41 at 5h were pooled with the CRF-41 control group. Values are means ± SEM, the number of experiments in each treatment group are shown in parentheses. The range of absolute ACTH release between experiments at 4h (100%) was 113.4-466.8 fmol/15min. [a]$P<0.05$ compared to CRF-41 control, [b]$P<0.05$ compared to corticosterone alone using non-parametric Kruskal-Wallis test.

The timing of glucocorticoid application was essential for early inhibition of CRF-41-stimulated ACTH release in Charles River rats (Figure 3.7). Furthermore, no inhibition was observed when the potent type II glucocorticoid receptor agonist RU28362 (0.1μM, 35min) was applied simultaneously with the 0.1nM CRF-41 stimulus at 5h as in Figure 3.7: in two experiments the mean net ACTH release expressed as a percentage of that released by CRF-41 alone at 4h was 109% at 5h, 90% at 6h and 107% at 7h. Thus the timing phenomenon is intact in rats obtained from Charles River.

In contrast to the results obtained with virally infected Harlan Olac rats, CRF-41-stimulated ACTH release in Charles River rats was significantly inhibited when no CRF-41 stimulus was applied during the exposure to corticosterone (Table 3.2). This suggests that application of CRF-41 simultaneously with the onset of corticosterone exposure inactivates early inhibition. Omission of the CRF-41 stimulus at 5h had no effect on the responses to subsequent pulses of CRF-41, thus these controls were pooled with controls receiving CRF-41 at 5h. The cell membrane permeant cyclic adenosine 3',5'-monophosphate analogue, 8-(4-chlorophenylthio)- cyclic adenosine 3',5'-monophosphate (8-CPT-cAMP; 0.1mM for 5min (Miller, Beck, Simon & Meyer,1975)) also prevented the subsequent inhibition of CRF-41-induced ACTH release when applied at the start of corticosterone exposure (0.1μM, 35min duration) at 5h (Table 3.2). At this concentration a 5min pulse of 8-CPT-cAMP elicited 26.0±5.1% of the ACTH released by 0.1nM CRF-41 and did not modify the ACTH release to subsequent CRF-41 pulses (Table 3.2). Preliminary experiments were performed to determine whether forskolin at 1-10μM (an activator of adenylate cyclase (Barber & Goka,1985)) and Rolipram at 1-100μM (a specific blocker of cAMP-dependent phosphodiesterase (Beavo & Reifsnyder,1990; Weishaar,1987)) could mimic the action of CRF-41 by raising intracellular cAMP levels. Although both

compounds alone elicited ACTH secretion (10μM forskolin and 100μM rolipram applied for 5min released 20% and 36% of the ACTH released by 0.1nM CRF-41 respectively) subsequent responses to CRF-41 stimulation were impaired. This action may be a result of incomplete washout of these compounds from the perifusion system as basal levels remained elevated for up to 2h after their withdrawal. In summary, CRF-41 inactivates early glucocorticoid inhibition of CRF-41-stimulated ACTH release, and this can be reproduced with an analogue of cAMP.

Several workers have demonstrated that intracellular cAMP levels and adenylate cyclase activity are modulated in cells infected with Sendai virus (Cohen & Cuatrecasas,1976; Vallier, Farjanel, Bata & Deviller,1981). Thus, the apparent divergent mechanism(s) of the CRF-41/glucocorticoid timing phenomenon reported in Charles River rats and virally infected Harlan Olac rats could be a result of differential sensitivity of corticotrophs to cAMP stimulation in the two strains. In order to test this hypothesis the ACTH secretory response of anterior pituitary gland segments to CRF-41 and 8-CPT-cAMP in static incubation were examined. The dose response characteristics for CRF-41 in ex-breeder (Figure 3.8a) as well as age matched (6month) virgin female rats (Figure 3.8b) were identical for Charles River rats and virally-infected Harlan Olac rats. However, in response to the cell permeant cAMP analogue, 8-CPT-cAMP, virally-infected Harlan-Olac rats released significantly lower amounts of ACTH compared to Charles River rats in both ex-breeder and 6month virgin females (Figure 3.8c & d) suggesting that Sendai virus infection modulates the cAMP-activated pathways. As the inactivation mechanism reported in Charles River rats is cAMP-dependent the impaired cAMP response in virally infected Harlan-Olac rats may explain the discrepant findings observed. Furthermore, as cAMP action is modulated in virgin as well as ex-breeder rats obtained from Harlan-Olac any differences of breeding protocol between the two suppliers cannot explain the

discrepant results. Significantly, in two subsequent perifusion experiments with virus free rats obtained from Harlan Olac CRF-41-inactivated early glucocorticoid inhibition.

In conclusion, the relative timing of application of glucocorticoid and CRF-41 is important for development of early inhibition: importantly, CRF-41 appears to inactivate early glucocorticoid inhibition of CRF-41-stimulated ACTH release.

Figure 3.8

Dose response characteristics of CRF-41- and 8-CPT-cAMP-stimulated ACTH secretion in anterior pituitary segments in static incubation from Charles River and virally infected Harlan Olac rats

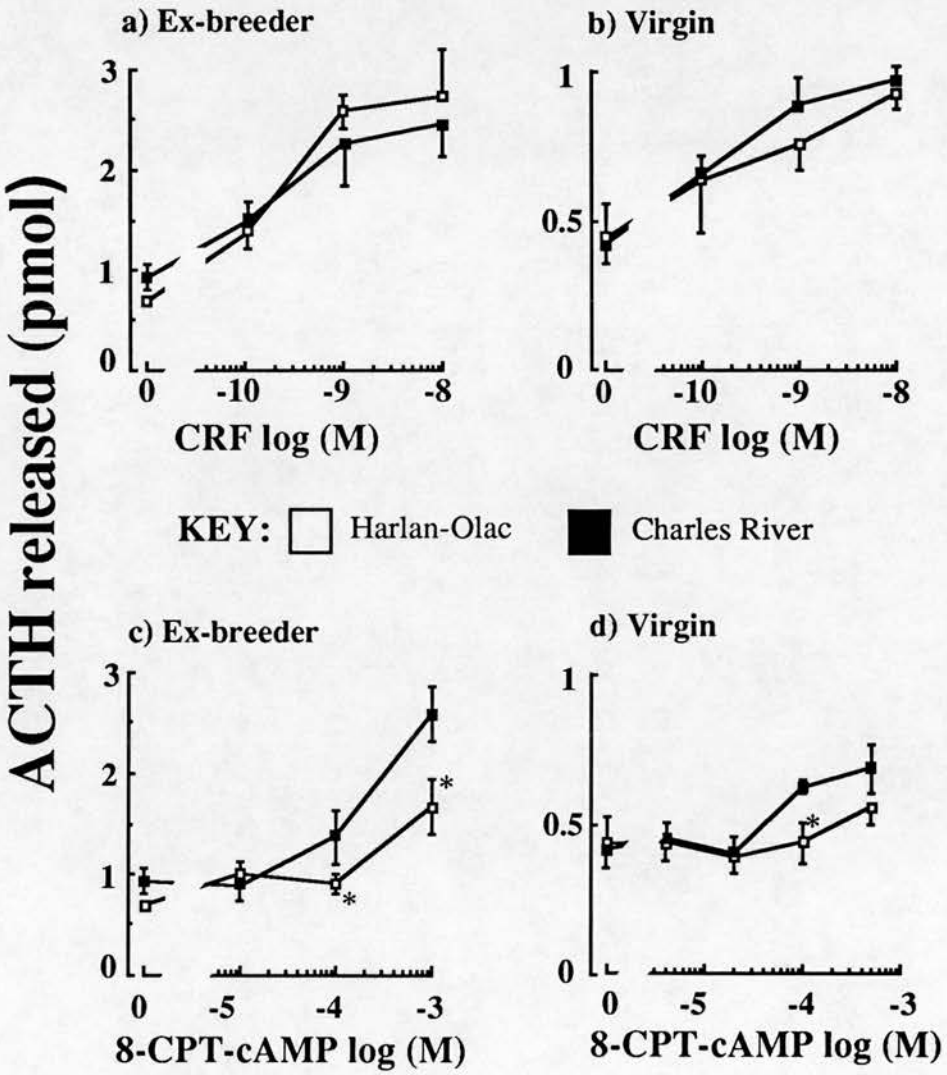


Figure 3.8: Anterior pituitary segments were incubated with different concentrations of the respective secretagogue for 20min as described in section 2.3.1. Data are expressed as pmoles of ACTH released into the medium, note the difference in scale for ex-breeder and virgin rats. Data are means ± SEM (n=6/group). *p<0.05 compared to Charles River rats (Student two-tailed, unpaired t-test)

3.3 Early glucocorticoid inhibition of AVP-stimulated ACTH release

AVP is a major hypothalamic ACTH secretagogue, however, relatively few studies have investigated early glucocorticoid inhibition of AVP-stimulated ACTH release. In the following sections the time course, receptor pharmacology and transcription/translation inhibitor sensitivity of early inhibition were examined to determine whether glucocorticoids suppress AVP-stimulated ACTH secretion with a similar mechanism to that observed using CRF-41 as secretagogue. Furthermore, as CRF-41 appears to inactivate early inhibition of CRF-41-stimulated ACTH release the importance of the temporal relationship of AVP and glucocorticoid application was studied to examine whether AVP could prevent early inhibition of AVP-stimulated ACTH release.

3.3.1 *Response to AVP*

In perifused anterior pituitary gland segments from Harlan Olac rats 10nM AVP appeared equipotent with 10nM CRF-41 (see Figure 3.1, section 3.2.1). The release of ACTH in response to 10nM AVP was 186±24.0% (n=14, $p<0.05$) of a 0.1nM CRF-41 stimulus in Charles River rats. Results obtained with rats from either supplier were identical in all respects of AVP action and interaction with glucocorticoids and are thus pooled in the following sections. After a 3h preperifusion period, to allow basal ACTH release to stabilize, the release of ACTH at 4h in response to a 5min pulse of 10nM AVP was enhanced (net ACTH release at 4h was 164.2±12.3% of that at 3h, mean±SEM n=28, $p<0.05$) with respect to the response at 3h in the columns. Subsequent stimuli released similar amounts of ACTH to that released at 4h, up to 7h after the start of perifusion (see Table 3.3). In all subsequent experiments AVP was used at the submaximally effective concentration of 10nM. The

reproducibility of AVP-stimulated ACTH release in perifused rat anterior pituitary gland segments demonstrate that the perifusion system is a viable model in which to investigate early glucocorticoid inhibition of AVP-stimulated ACTH release.

3.3.2 *Effect of glucocorticoids on AVP- stimulated ACTH release*

Few studies to date have directly investigated early inhibition using AVP solely as secretagogue thus the characteristics of early glucocorticoid suppression of AVP-stimulated ACTH release were examined.

The synthetic type II glucocorticoid receptor agonist RU28362 (1μM, total exposure 35min) significantly ($p<0.05$) inhibited AVP-stimulated ACTH secretion to 64% of control within 35min of application (Table 3.3) implicating type II glucocorticoid receptors in the mechanism of early glucocorticoid inhibition of AVP-stimulated ACTH release. The inhibition was maintained 2.5h after initial exposure to the steroid. Basal ACTH release was unaffected by RU28362. Figure 3.9 demonstrates that RU28362 reduces the amount of ACTH released by an AVP pulse, but has no apparent effect on the time course of the ACTH secretory response as reported for CRF-41-stimulated ACTH release in section 3.2.2.

To date no direct evidence for involvement of glucocorticoid-induced proteins in early inhibition of AVP-stimulated ACTH release have been presented. Surprisingly, AVP-stimulated ACTH release was inhibited by puromycin alone (Table 3.3). After withdrawal of puromycin from the perifusion medium AVP-stimulated ACTH release returned towards control. No further inhibition of ACTH release by RU28362 occurred in puromycin treated tissue (Table 3.3). At 7h in columns treated with both RU28362 and puromycin significantly lower ACTH release was observed compared to columns receiving puromycin alone (Table 3.3) suggesting that removal of puromycin at 6h allows the inhibition of RU28362 to be manifest at 7h similar to that

seen for CRF-41-stimulated ACTH release (see Figure 3.4b, section 3.2.1). Actinomycin D also reduced AVP-stimulated ACTH release to approximately 70% of control at 6 and 7h.

To further examine whether RU28362 inhibition of AVP-stimulated ACTH secretion is dependent on induction of new mRNA and protein experiments were performed using primary cultures of rat anterior pituitary cells in static incubation. Initial experiments failed to elicit ACTH release in response to AVP at concentrations up to 25nM even after preincubation of cells for 6h with CRF-41 (2nM) or 8-bromo-cyclic AMP (0.1mM) (Miyazaki *et al.*,1984). Furthermore, no consistent stimulation of ACTH release by AVP (10-50nM for 30min) was observed using anterior pituitary segments in static incubation. Changes in responsiveness to AVP and other ACTH secretagogues in primary culture, compared to freshly isolated tissue, have been widely noted (for review see Antoni, 1986). Furthermore, the ACTH secretory response to AVP is generally more robust in the perifusion model (Antoni,1986; Watanabe & Orth,1988). To mimic the action of AVP in the corticotroph (King & Baertschi,1990) the protein kinase C activator phorbol-12,13-dibutyrate (PdBu) was used as secretagogue (Figure 3.10a & b). RU28362 (10nM for 90min) significantly inhibited a 20min stimulus of 50nM PdBu in this system (Figure 3.10c). The reversible transcription inhibitor 5,6-Dichloro-1-ß-D-ribofluranosylbenzimidazole (DRB, (Tamm & Sehgal,1978)) blocked RU28362 inhibition of PdBu-stimulated ACTH release (Figure 3.10c). DRB suppressed basal ACTH release by ~20% in this system, thus data are presented after subtraction of the respective basal values. Taken together, these data are consonant with the hypothesis that early inhibition of AVP-stimulated ACTH release requires the induction of new mRNA and protein.

In conclusion, the time course and pharmacology of early glucocorticoid suppression of AVP-stimulated ACTH release appeared similar to that observed when CRF-41 is

used as secretagogue. However, AVP-stimulated ACTH release was blocked by inhibitors of transcription and translation alone. Whether protein induction is required for suppression of AVP-stimulated ACTH release could not be fully resolved in this study, however, the findings are consonant with the protein induction hypothesis.

3.3.3 *Early glucocorticoid inhibition of AVP-stimulated ACTH release is independent of the relative timing of application of AVP and glucocorticoid*

In perifused rat anterior pituitary gland segments significant corticosterone (0.1μM, 35min duration) inhibition of AVP-stimulated ACTH release was observed only 90min after the start of corticosterone exposure (Figure 3.11). However, this is probably a result of the wide scatter in AVP-stimulated ACTH release at 5h as 2 out of 4 experiments showed significant corticosterone-inhibition at this time point, furthermore RU28362 inhibition was manifest within 30min (Table 3.3).

In contrast to the results observed with CRF-41, early inhibition of AVP-stimulated ACTH release was independent of the timing of application of corticosterone (Figure 3.11). Furthermore, when AVP was applied at the start of a continuous corticosterone infusion (0.1μM started at 5h) inhibition of AVP-stimulated ACTH release developed within 1h (Figure 3.12).

In conclusion, early glucocorticoid inhibition of AVP-stimulated ACTH release was independent of the relative timing of glucocorticoid and AVP application.

Table 3.3

Inhibition of AVP-stimulated ACTH release from perifused rat anterior pituitary gland segments by the synthetic glucocorticoid, RU28362, and the protein synthesis inhibitor, puromycin

Treatment	net ACTH released %			
	4h	5h	6h	7h
AVP 10nM (5)	100	103.6±12.6	103.8±17.8	80.8±7.2
+RU28362 1μM (6)	100	64.3±4.9*	59.7±5.4*	48.0±14.0
+Puromycin 0.1mM (4)	100	49.0±7.6**	32.3±3.4**	60.3±11.0
+Puromycin 0.1mM +RU28362 1μM (4)	100	58.8±6.1	25.8±8.0**	39.0±4.9*[a]

Table 3.3: Data are expressed as a percentage of the net ACTH released in response to a 5min pulse of 10nM AVP received by all groups at 4h. 5min pulses of AVP were applied every hour as described in Figure 3.11. RU28362 was applied 30min before and during the AVP stimulus at 5h. Puromycin was applied 35min before the stimulus at 5h and continued until the end of the stimulus at 6h as for Figure 3.4b. The number of experiments per group are shown in parentheses. Data are means ± SEM. The range of absolute ACTH release at 4h (100%) was 194-813 fmol ACTH/25min. ** $p<0.01$, *$p<0.05$ compared with the control (AVP) group, [a] $p<0.05$ versus the puromycin alone group, using the non-parametric Kruskal-Wallis test.

Figure 3.9

Effect of the synthetic type II glucocorticoid agonist, RU28362, on the profile of AVP-stimulated ACTH release in perifused rat anterior pituitary segments

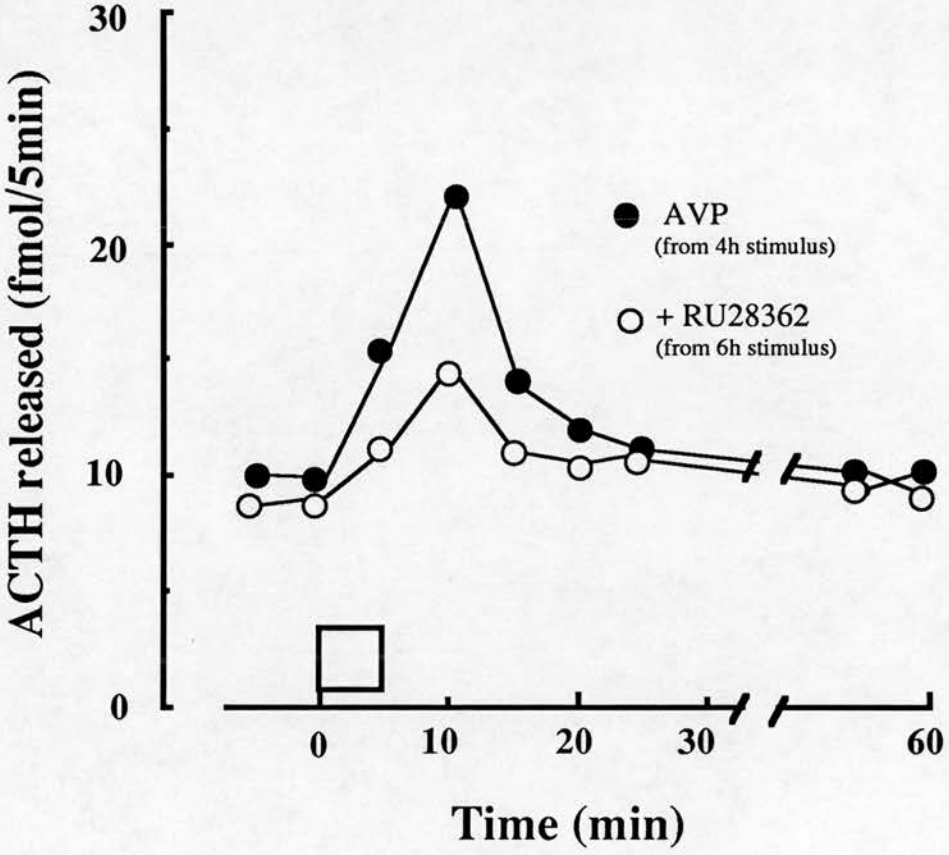


Figure 3.9: Typical AVP-stimulated ACTH release profiles from a representative experiment performed as in Figure 3.2 a & b. The ACTH release profile for AVP (10nM for 5min, open horizontal box) alone are from the 4h stimulus. The AVP-stimulated ACTH release profile after RU28362 treatment is from the 6h stimulus in the same column. RU28362 (0.1μM 35min total duration) was started 30min before the 5h stimulus as in Figure 3.2b. Data are expressed as the amount of immunoreactive ACTH in each 5min collection fraction.

Figure 3.10

Antagonism of RU28362 inhibition of phorbol ester stimulated ACTH release in rat anterior pituitary primary culture by the transcription inhibitor 5,6-Dichloro-1-ß-D-ribofluranosylbenzimidazole (DRB)

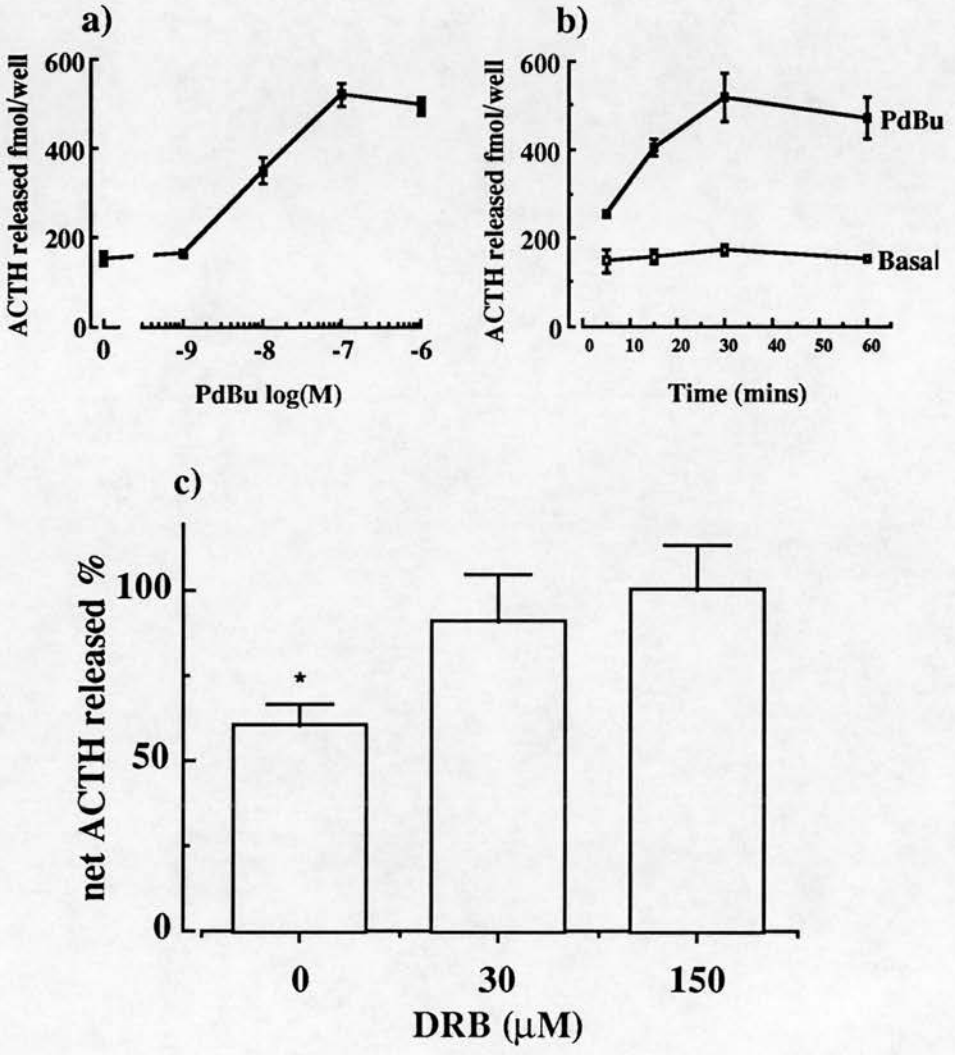


Figure 3.10: **a)** Dose response curve and **b)** time course of 50nM phorbol 12,13 dibutyrate (PdBu) stimulated ACTH release from primary culture of rat anterior pituitary in static incubation. Data are expressed as fmol/well of ACTH released, PdBu was applied for 20min in a). **c)** Dose dependent reversal of RU28362 inhibition of 50nM PdBu stimulated ACTH release by DRB. Data are means ± SEM, expressed as the percent of net ACTH released by PdBu alone. RU28362 (10nM) was applied for 90min before the stimulus, DRB was started 20min before and continued during the exposure to RU28362. *$p<0.05$ compared to control (Students two-tailed, unpaired t-test)

Figure 3.11

Corticosterone inhibition of AVP-stimulated ACTH release from perifused rat anterior pituitary segments

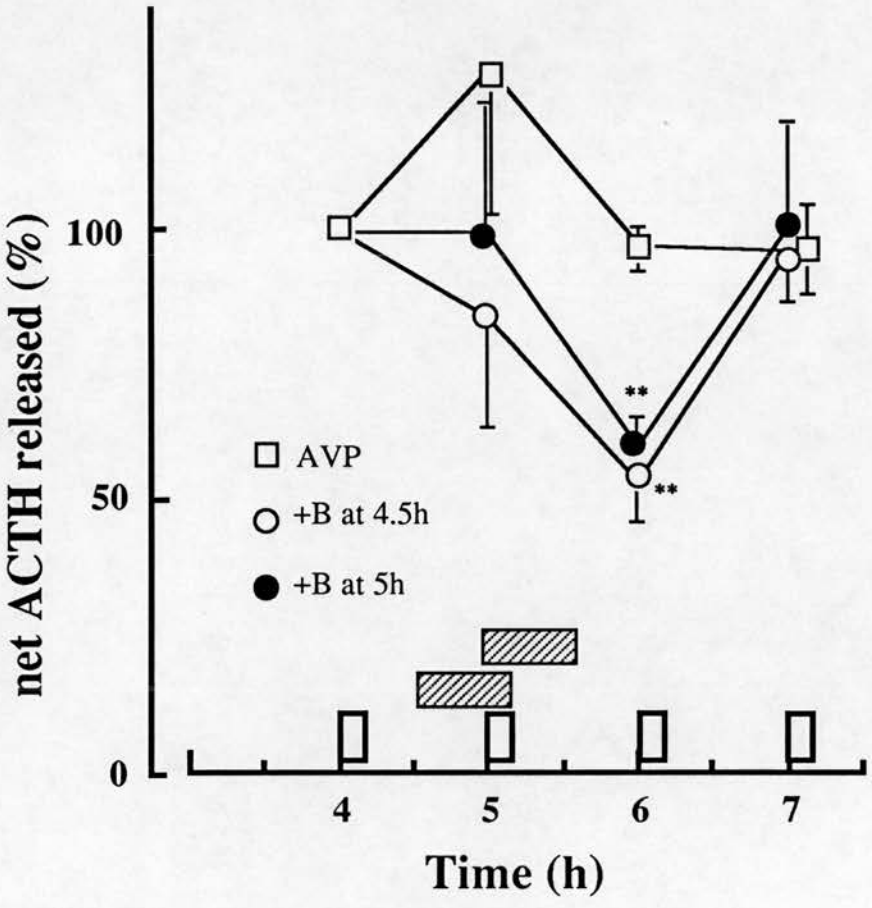


Figure 3.11: The control group (AVP) received repeated 5min pulses of 10nM AVP (open vertical bars). Other groups received 0.1 μM corticosterone (B, total exposure 35min; horizontal hatched bar) applied 30min before (+B at 4.5h) or simultaneously with (+B at 5h) the AVP pulse at 5h. Data are expressed as a percentage of the net ACTH released by a 5min pulse of 10nM AVP received at time 4h by all groups. Data are means ± SEM (n=4/group). The absolute range of ACTH release at 4h (100%) was 82.6-577.0 fmol ACTH/25min. ** $p<0.01$ (non-parametric Kruskal-Wallis test) compared with the AVP group.

Figure 3.12

Inhibition of AVP-stimulated ACTH secretion by a continuous exposure to corticosterone started simultaneously with an AVP stimulus in perifused rat anterior pituitary segments.

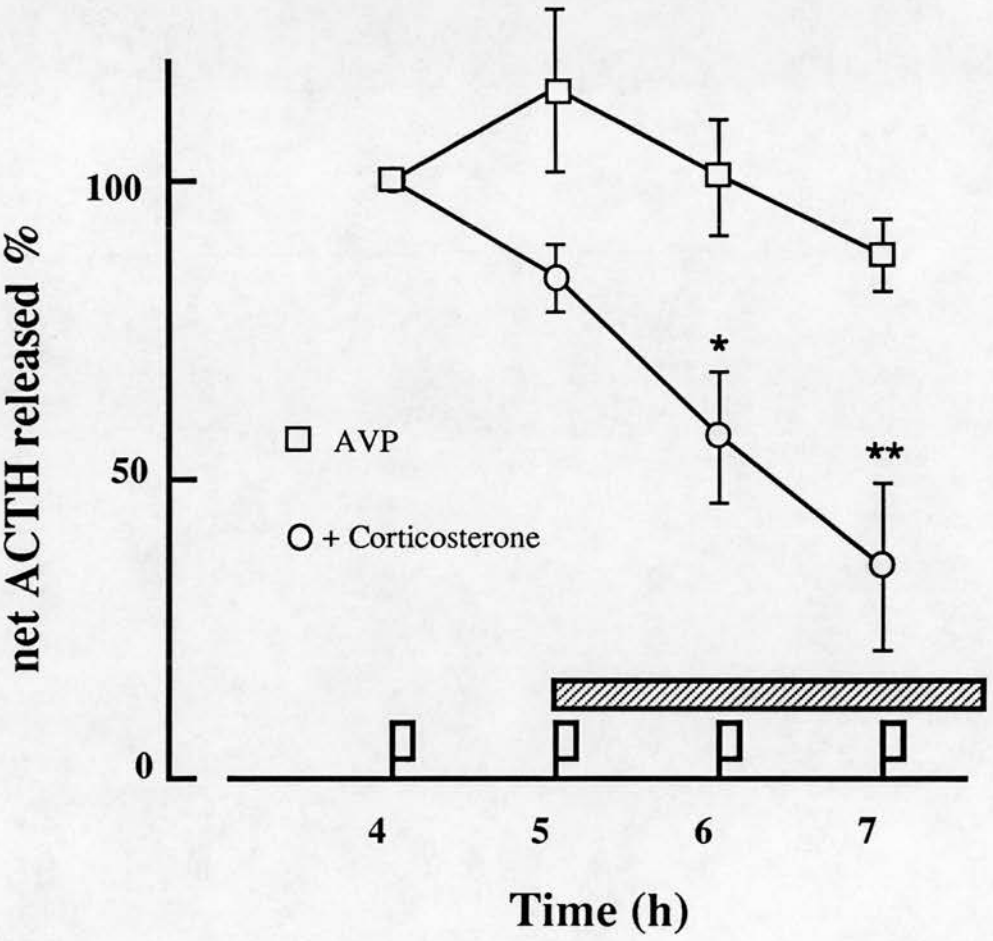


Figure 3.12: The control group received repeated 5min pulses of 10nM AVP (open vertical bars) every 1h. The other group received 0.1μM corticosterone (horizontal hatched bar) started simultaneously with the 5h stimulus and maintained to the end of the experiment. Data are expressed as a percentage of the net ACTH released by a 5min AVP stimulus applied at 4h. Data are means ± SEM (n=4-9/group). The range of absolute ACTH released at 4h (100%) by AVP was: 82.6-481.8 fmol ACTH/25min. *P<0.05, **p<0.01 (non-parametric Mann-Whitney U test) compared with the AVP control group.

3.4 Interactions between CRF-41, AVP and glucocorticoids

In vivo, ACTH secretion is stimulated by AVP and CRF-41 acting in concert. Thus it was important to examine the nature of interactions between CRF-41, AVP and glucocorticoids in combination in order to determine whether new properties of early glucocorticoid inhibition would be found.

3.4.1 *Early glucocorticoid inhibition of AVP-stimulated ACTH release is not modulated by CRF-41*

As CRF-41 inactivates early glucocorticoid inhibition of CRF-41-stimulated ACTH secretion it was of interest to examine whether CRF-41 could block inhibition of AVP-stimulated ACTH release. For example, if glucocorticoids inhibit CRF-41-, and AVP-stimulated ACTH secretion with a similar mechanism it would be expected that CRF-41 would inactivate early inhibition of AVP-, as well as CRF-41-, stimulated ACTH release.

CRF-41 (0.1nM) applied simultaneously with the start of corticosterone exposure (0.1μM, 35min duration) at 5h could not prevent the corticosterone inhibition of AVP-stimulated ACTH release at 6h (Table 3.4) in perifused rat anterior pituitary segments. Although CRF-41 appears to decrease the subsequent response to AVP at 7h (Table 3.4) this effect is not statistically significant and is a result of one experiment out of the five in the group releasing a very low amount of ACTH at 7h. Increasing the concentration of CRF-41 to 10nM also failed to modify the inhibition by corticosterone: in two experiments the release of ACTH at 6h was 21% & 48%, at 7h 93% & 128% of the ACTH released by AVP alone at 4h.

Table 3.4

CRF-41 does not inactivate early glucocorticoid inhibition of AVP-stimulated ACTH secretion in perifused rat anterior pituitary segments

net ACTH released %			
4h AVP	**5h Treatment**	**6h AVP**	**7h AVP**
100	AVP (9)	100.2±9.7	87.1±5.7
100	Corticosterone +AVP (5)	60.4±4.5[a]	101.0±18.6
100	CRF-41 (5)	94.8±9.5	65.6±16.1
100	Corticosterone + CRF-41 (5)	41.4±12.0[b]	63.8±17.2

Table 3.4: All groups received 5min pulses of 10nM AVP at 4, 6 and 7h. The various treatments applied, along with their temporal relationship of application, are shown in the 5h treatment column. Corticosterone (0.1μM, 35min total exposure) was started simultaneously with the 5h stimulus. CRF-41 was applied for 5min at 0.1nM. Data are expressed as a percentage of the net ACTH released by a 5min pulse of 10nM AVP applied at 4h in all groups. Values are means ± SEM, the number of experiments is shown in parentheses. The range of absolute ACTH released at 4h (100%) between experiments was 194.0-826.7 fmol ACTH/25min. [a]$P<0.05$ compared to AVP control, [b]$P<0.01$ compared to CRF-41 treated control using non-parametric Kruskal-Wallis test.

Because these columns received AVP before the application of CRF-41 and corticosterone at 5h, it was essential to examine whether this AVP pretreatment could prevent CRF-41-inactivation of glucocorticoid inhibition. In order to test this, CRF-41 (0.1nM) stimuli were applied at 3h, 4h and 5h and 10nM AVP applied at 6 and 7h. Corticosterone (0.1μM, 35min duration) started simultaneously with CRF-41 at 5h resulted in significant ($p<0.05$, using non-parametric Kruskal Wallis test) inhibition of AVP-stimulated ACTH release compared to control even under these conditions. The net ACTH release expressed as a percentage of the 4h CRF-41 stimulus for control and corticosterone treated groups respectively was: at 6h 186.7±45.4% *vs* 67.8±16.6% and at 7h 214.7±48.0% *vs* 97.8±31.2% (n=3-4/group. Note: the ACTH response to a 10nM AVP stimulus is 186.2±24.0% of that elicited by 0.1nM CRF-41 as described in section 3.3.1). Furthermore, CRF-41 prevented corticosterone inhibition of CRF-41-stimulated ACTH release after pretreatment of the segments with 10nM AVP applied at 3h and 4h; 0.1nM CRF-41 stimuli were applied at 5, 6 and 7h respectively with corticosterone (0.1μM, 35min duration) applied at 5h. The ACTH release for corticosterone and control treated columns was: at 6h 119.0±20.9 *vs* 82.8±8.2% , at 7h 76.3±15.7% *vs* 101.3±25.3% of that released by the CRF-41 stimulus at 5h.

In summary, CRF-41 does not influence early glucocorticoid inhibition of AVP-stimulated ACTH release.

3.4.2 *AVP cannot inactivate early glucocorticoid inhibition of CRF-41-stimulated ACTH release*

To investigate whether inactivation of early glucocorticoid inhibition of CRF-41 stimulated ACTH release was specific to the CRF-41/cAMP pathway, 10nM AVP (that acts via the inositol phosphate/protein kinase C pathway (King &

Baertschi,1990)) was applied for 5min at 5h. The CRF-41 stimulus at 6h released similar amounts of ACTH compared to CRF-41 controls however, downregulation of the CRF-41 response at 7h occurred in columns treated with AVP at 5h (Table 3.5). Importantly, when AVP was applied at the start of corticosterone exposure significant ($p<0.01$) inhibition of CRF-41-stimulated ACTH release occurred at 6h (Table 3.5). The desensitization of the CRF-41 response after application of AVP is not understood in this system, however, it is likely to involve modification of receptor/postreceptor systems rather than a result of the depletion of releasable ACTH stores. Indeed, in primary culture of rat anterior pituitary cells AVP pretreatment reduces the subsequent ACTH response to CRF-41, but not to other secretagogues, independently of changes in intracellular ACTH content (Hoffman, Ceda & Reisine,1985). Moreover, AVP pretreatment has been shown *in vivo* and *in vitro* to reduce the number of pituitary CRF-41 binding sites (Holmes, Catt & Aguilera,1987). It should also be noted that to date corticotrophs responding solely to AVP have not been documented, for example recent RHPA studies suggest that cells that respond to AVP are also responsive to CRF-41 (Jia *et al.*,1991). Thus heterologous desensitization is possible without involving depletion of (putatively) differentially regulated ACTH stores.

In conclusion, inactivation of early glucocorticoid inhibition would appear to be specific to the CRF-41/cAMP pathway.

Table 3.5

AVP cannot prevent early glucocorticoid inhibition of CRF-41-stimulated ACTH release in perifused rat anterior pituitary segments

net ACTH released %			
4h CRF-41	**5h** Treatment	**6h** CRF-41	**7h** CRF-41
100	CRF-41 (7)	98.0±8.4	99.0±9.9
100	Corticosterone (4)	61.5±9.9[a]	54.3±9.3[a]
100	Corticosterone +CRF-41 (5)	108.4±4.3[b]	111.2±12.4[b]
100	AVP (4)	82.5±4.3	48.0±15.6
100	Corticosterone + AVP (4)	32.3±18.7[c]	50.0±12.4

Table 3.5: All groups received 5min pulses of 0.1nM CRF-41 at 4, 6 and 7h. The various treatments applied, along with their temporal relationship of application, are shown in the the 5h treatment column. AVP was applied for 5min at 10nM. Corticosterone (0.1μM, 35min total exposure) was started simultaneously with the 5h stimulus. Data are expressed as a percentage of the net ACTH released by a 5min pulse of 0.1nM CRF-41 applied at 4h (100%) in all groups. Because omission of the 5h CRF-41 stimulus did not affect subsequent stimulated ACTH release data from control columns receiving no CRF-41 at 5h were pooled with the CRF-41 control group. Values are means ± SEM, the number of experiments in each treatment group are shown in parentheses. The range of absolute ACTH release between experiments at 4h (100%) was 113.4-466.8 fmol/15min. [a]$P<0.05$ compared to CRF-41 control, [b]$P<0.05$ compared to corticosterone alone, [c]$P<0.05$ compared to AVP control using non-parametric Kruskal-Wallis test.

3.4.3 *Early glucocorticoid inhibition of CRF-41/AVP-stimulated ACTH release resembles that when CRF-41 is used alone*

The physiological ACTH secretagogue stimulus *in vivo* is a cocktail of CRF-41 and AVP, thus it was important to analyse the interaction between glucocorticoids and a combined CRF-41/AVP stimulus in the time-scale of early inhibition. When CRF-41 and AVP were used in combination as secretagogue at concentrations approximating levels recorded in rat hypophysial portal blood (0.1nM and 0.2nM respectively *cf* Plotsky,1991) and corticosterone applied 30min before the 5h stimulus significant inhibition developed within 90min (Figure 3.13). When the combined stimulus was applied simultaneously with the start of corticosterone exposure no subsequent inhibition developed (Figure 3.13). Thus early glucocorticoid inhibition of the 'physiological' CRF-41/AVP stimulus resembled that when CRF-41 was used alone (*ie* no inhibition was observed when the secretagogue pulse was applied simultaneously with the start of corticosterone) suggesting that CRF-41-inactivation is physiologically relevant.

In summary, CRF-41 only inactivates early glucocorticoid inhibition of CRF-41-stimulated ACTH release furthermore, inactivation is specific to the CRF-41/cAMP pathway. Importantly, early glucocorticoid inhibition of the 'physiological' stimulus, CRF-41/AVP in combination, resembles that when CRF-41 is used alone.

Figure 3.13

Early corticosterone inhibition of CRF-41/AVP-stimulated ACTH secretion in perifused rat anterior pituitary segments

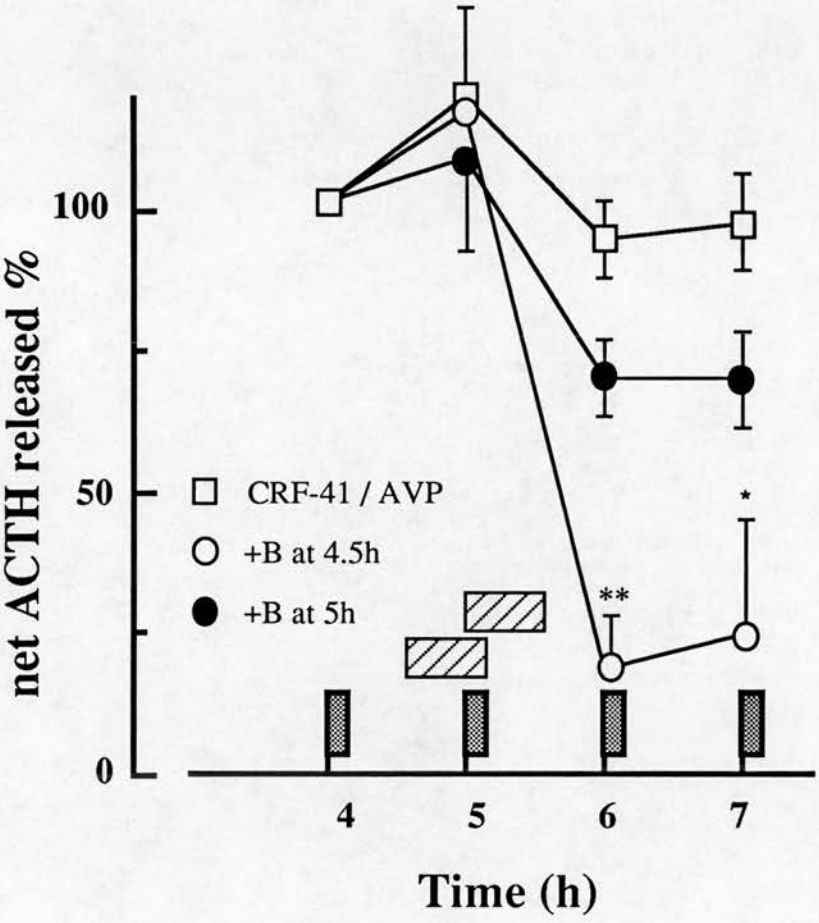


Figure 3.13: The control group (CRF-41 / AVP) received 5min pulses of the combined stimulus of 0.1nM CRF-41 and 0.2nM AVP every 1h. Other groups also received 0.1μM corticosterone (B) (total exposure 35min; horizontal hatched bar) applied 30min before (+B at 4.5h) or simultaneously with (+B at 5h) the CRF-41 / AVP pulse at 5h. Data are expressed as a percentage of the net ACTH released by the CRF-41 / AVP pulse received at time 4h (100%) by all groups. Data are means ± SEM (n=4/group). The range of absolute ACTH release at 4h (100%) between experiments was 156.8-420.8 fmol/25min. **p<0.01, *p<0.05 compared to control using the nonparametric Kruskal-Wallis test.

3.5 Discussion

These data suggest that perifused anterior pituitary segments are a valid model in which to study the characteristics of early glucocorticoid inhibition. The hallmarks of early glucocorticoid inhibition: onset within 2h, requirement for type II glucocorticoid receptor activation and mRNA and protein induction were demonstrated for early suppression of CRF-41 and AVP-stimulated ACTH secretion. Significantly, the timing and nature of the applied secretagogue was important in determining early inhibition. CRF-41, applied at the start of glucocorticoid exposure, inactivated early glucocorticoid inhibition of CRF-41-, but not AVP-, stimulated ACTH release and this action was mimicked by cAMP. Early inhibition of CRF-41/AVP-stimulated ACTH release resembled that when CRF-41 was used alone. Finally, CRF-41- and AVP-stimulated ACTH release were differentially sensitive to transcription and translation inhibitors suggesting that distinct pools of ACTH are mobilized by these neurohormones.

3.5.1 *Response to secretagogue stimulation: do CRF-41 and AVP mobilize distinct pools of ACTH?*

The suppression of AVP-, but not CRF-41-induced ACTH release by inhibitors of transcription and translation suggest that the secretagogues may activate different intracellular pools of ACTH, for example, AVP may release newly synthesized ACTH as opposed to CRF-41, which may preferentially mobilize stored hormone (Figure 3.14). Schwartz and co-workers using the lysosmotropic drug, chloroquine (that purportedly diverts ACTH from the stored regulatory pathway to the constitutive secretory pathway (Moore, Gumbiner & Kelly,1983)) suggested that CRF-41 mobilized ACTH from the stored regulated pathway whereas AVP mobilized ACTH from a pool associated with unregulated (constitutive) ACTH release (Schwartz *et*

al.,1990). However, as AVP-stimulated ACTH is Ca^{2+}-dependent (see section 1.4.3 and King & Baertschi,1990; Oki *et al.*,1991), and constitutive secretion is Ca^{2+}-independent (Brion, Miller & Moore,1992; Matsuuchi & Kelly,1991; Miller & Moore,1991) it seems unlikely that such a constitutive pool is in fact mobilized by AVP. Thus AVP probably releases a regulated pool of newly synthesized hormone. Protein translation inhibitors have also been reported to block components of the regulated secretory pathway independently of hormone synthesis inhibition (Brion *et al.*,1992), thus it is possible that the AVP-responsive ACTH pool has distinct regulatory properties (in terms of translation inhibitor action) compared to the CRF-41 activated ACTH pool.

Several workers have reported subpopulations of corticotrophs that are differentially responsive to hypothalamic secretagogues (see section 1.4.2 and Childs,1992; Jia *et al.*,1991; Schwartz, Canny, Vale & Funder,1989) however, to date, no evidence for corticotrophs responding solely to AVP have been reported. Thus on the basis of current evidence it is likely that these potentially distinct ACTH pools reside in a single cell population (Figure 3.14). Alternatively, it cannot be excluded at present that actinomycin D and puromycin interfere with the signal transduction pathways activated by AVP. However, if such interference does occur it must be distal to AVP-receptor activation as puromycin also blocks a combined phorbol ester/ionomycin stimulus in acutely isolated perifused rat anterior pituitary cells (Antoni,1992).

Interestingly, CRF-41 and AVP also differentially regulate corticotroph ACTH content in the long-term. CRF-41 stimulates POMC gene expression thereby increasing intracellular ACTH stores, in contrast, AVP does not stimulate POMC gene expression, but may enhance the rate of processing of POMC pre-mRNA transcripts (Levin & Roberts,1991).

Figure 3.14

CRF-41 and AVP mobilize distinct pools of ACTH from anterior pituitary corticotrophs

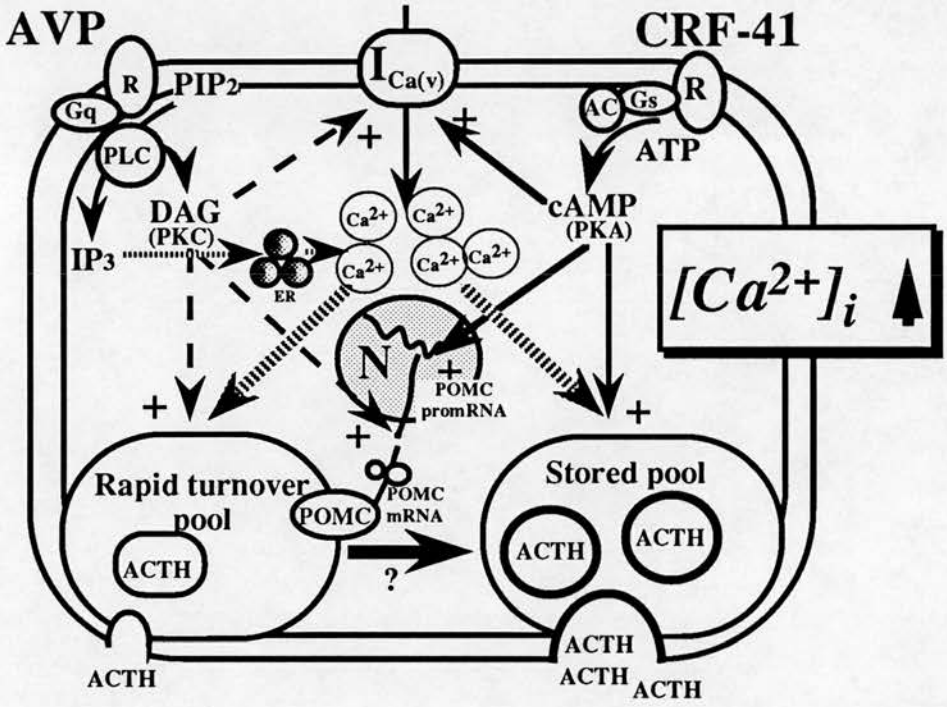


Figure 3.14: CRF-41 mobilizes a stored pool of ACTH whilst AVP activates a rapid turnover, newly synthesized, pool in anterior pituitary corticotroph cells. Abbreviations are as follows: R, receptor; G, G-protein; PLC, phospholipase C; DAG, diacylglycerol; IP3, inositol 1,4,5 triphosphate; ER, endoplasmic reticulum; ICa(v), voltage operated calcium channel; AC, adenylyl cyclase; $[Ca^{2+}]_i$, intracellular free calcium concentration; POMC, proopiomelanocortin, the precursor of ACTH. See section 3.5.1 for discussion.

3.5.2 *Involvement of type II glucocorticoid receptors and requirement for mRNA and protein synthesis in the early inhibitory action of glucocorticoids*

As discussed in section 1.5.4 it has been suggested that early glucocorticoid inhibition at the corticotroph involves rapid and delayed effects that are mediated through different receptor subtypes (Abou-Samra *et al.*,1986a; Keller-Wood & Dallman,1984). However, current evidence suggests that both rapid and delayed inhibition are manifest through the type II glucocorticoid receptor; the purported time-domains differing in the extent rather than the cellular mechanism of inhibition (see Dayanithi & Antoni (1989) and section 1.5.4).

The data presented here demonstrate that both CRF-41- and AVP-stimulated ACTH secretion are inhibited in the early time-domain by glucocorticoids acting through the type II glucocorticoid receptor. The lack of a statistically significant inhibition of CRF-41-stimulated ACTH release within 30min of application of RU28362 in the present study may suggest mediation of rapid feedback by a non-type II glucocorticoid receptor. However, because the rapid effect of corticosterone was blocked by the potent type II receptor antagonist, RU38486, it seems reasonable to assume that type II receptors are involved in this time domain. Thus these data support the hypothesis that similar mechanisms underlie rapid and delayed inhibition and that these time domains differ only in the extent of inhibition as suggested by Dayanithi & Antoni (1989). Hence it would seem appropriate to designate rapid and delayed action under a common title of '*early* ' glucocorticoid inhibition. The more prolonged inhibitory action of RU28362, with respect to corticosterone, on both CRF-41- and AVP-stimulated ACTH release may be a consequence of differences in tissue traffic (metabolism and diffusion) between RU28362 and corticosterone rather than mediation of the response by different types of glucocorticoid receptor. For example,

corticosterone binds to transcortin-like binding proteins and is metabolised by 11ß-hydroxysteroid dehydrogenase whereas RU28362, similar to other synthetic glucocorticoids, does not bind to transcortin and is largely resistant to degradation by 11ß-hydroxysteroid dehydrogenase (De Kloet, Burbach & Mulder,1977; Funder, Pearce, Smith & Smith,1987; Stewart, Wallace, Valentino *et al.*,1987).

Since inhibitors of transcription (actinomycin D) and translation (puromycin) blocked the suppressive action of glucocorticoids on CRF-41-stimulated ACTH release, the present data are consonant with the hypothesis that early glucocorticoid action involves the synthesis of new protein(s) (Arimura *et al.*,1969; Dayanithi & Antoni,1989; Munck,1971). Increasing the concentration of glucose in the medium had no effect on the inhibitory effect of type II receptor stimulation suggesting that a reduction of glucose uptake (Munck,1971) by the glucocorticoid induced protein is unlikely to be the primary cause of inhibition of secretagogue-stimulated ACTH release. Whether glucocorticoid-inhibition of AVP-stimulated ACTH secretion requires a similar protein could not be resolved fully in this study because of the inhibition of AVP-induced ACTH secretion by actinomycin D or puromycin given alone. However, RU28362 produced no additional inhibition of ACTH release in the presence of puromycin and RU28362 inhibition appeared to be present on withdrawal of the translation inhibitor. Furthermore, the transcription inhibitor, DRB, blocked RU28362 inhibition of phorbol ester-stimulated ACTH release in static incubation of rat anterior pituitary primary cultures. These results are compatible with the protein induction hypothesis for glucocorticoid inhibition of AVP-stimulated ACTH release.

3.5.3 *Secretagogue context (nature and timing) determines the characteristics of early glucocorticoid inhibition*

Early glucocorticoid suppression of CRF-41-stimulated ACTH secretion is dependent upon the relative timing of secretagogue and glucocorticoid application, whereas this is not the case for AVP-induced ACTH secretion.

The importance of the temporal relationship between secretagogue stimulation and glucocorticoid exposure has been reported by Mulder & Smelik (1977) (see also section 1.5.5), who found that corticosterone pretreatment was necessary to achieve an early inhibitory effect in isolated perifused rat anterior pituitary cells stimulated by short pulses of stalk-median eminence extract (that contains CRF-41 and AVP). Mahmoud *et al.*(1984) have reported a biphasic time-course of the early effects of corticosterone on CRF-41-stimulated ACTH secretion, with a "silent" period at 30-50 min after exposure to the steroid. This latter phenomenon, however, appears to be different from the results presented here, since a long time-interval after the application of corticosterone simultaneously with CRF-41 has been covered and no subsequent inhibition of ACTH release was observed. It is interesting to note that the timing phenomenon reported here may underlie the complete lack of early glucocorticoid inhibition reported by Familari & Funder (1989) who applied steroid simultaneously with a CRF-41 stimulus in perifused rat anterior pituitary cells, although other factors, such as use of primary cultures of rat anterior pituitary cells attached to cytodex beads as opposed to pituitary segments in this thesis, may also underlie this discrepant finding.

When CRF-41 and AVP were used in combination as secretagogue at concentrations approximating levels recorded in rat hypophysial portal blood (0.1nM and 0.2nM respectively *cf* Plotsky,1991) early glucocorticoid inhibition was blocked by a

secretagogue pulse started at the onset of the exposure to glucocorticoids *i.e.* in this respect the 'physiological' CRF-41/AVP stimulus resembles CRF-41 alone. The timing dependence of CRF-41/AVP-stimulated ACTH release may be a result of the reported synergistic enhancement of CRF-41-stimulated cAMP accumulation by AVP (Abou-Samra *et al.*,1987b; Carvallo & Aguilera,1989; Cronin *et al.*,1986; Giguere *et al.*,1982; Lutz-Bucher *et al.*,1990). Interestingly, the release of ACTH elicited by CRF-41/AVP in perifused isolated rat anterior pituitary cells is suppressed by puromycin (FA Antoni, personal communication). Thus it will be of interest to examine whether varying the relative proportions of CRF-41 and AVP in the combined secretagogue paradigm will modulate the subsequent characteristics (timing dependence and translation inhibitor sensitivity) of early glucocorticoid inhibition of CRF-41/AVP-stimulated ACTH release.

3.5.4 *CRF-41 inactivates early glucocorticoid inhibition of CRF-41-, but not AVP-, stimulated ACTH release*

The dependence of early glucocorticoid inhibition of CRF-41-stimulated ACTH secretion on the relative timing of glucocorticoid and CRF-41 application could potentially be a result of two separate mechanisms as summarised in Figure 3.15:

1) CRF-41 is required to activate some step of early glucocorticoid inhibition (for example post-translational modification of the induced protein) when CRF-41 is applied following the start of corticosterone exposure (Figure 3.15a)

2) CRF-41 inactivates early glucocorticoid inhibition, perhaps by preventing activation of the gene(s) encoding the early glucocorticoid induced protein(s), when applied simultaneously with the start of corticosterone exposure (Figure 3.15b).

Figure 3.15

Alternative models of CRF-41/glucocorticoid timing interaction

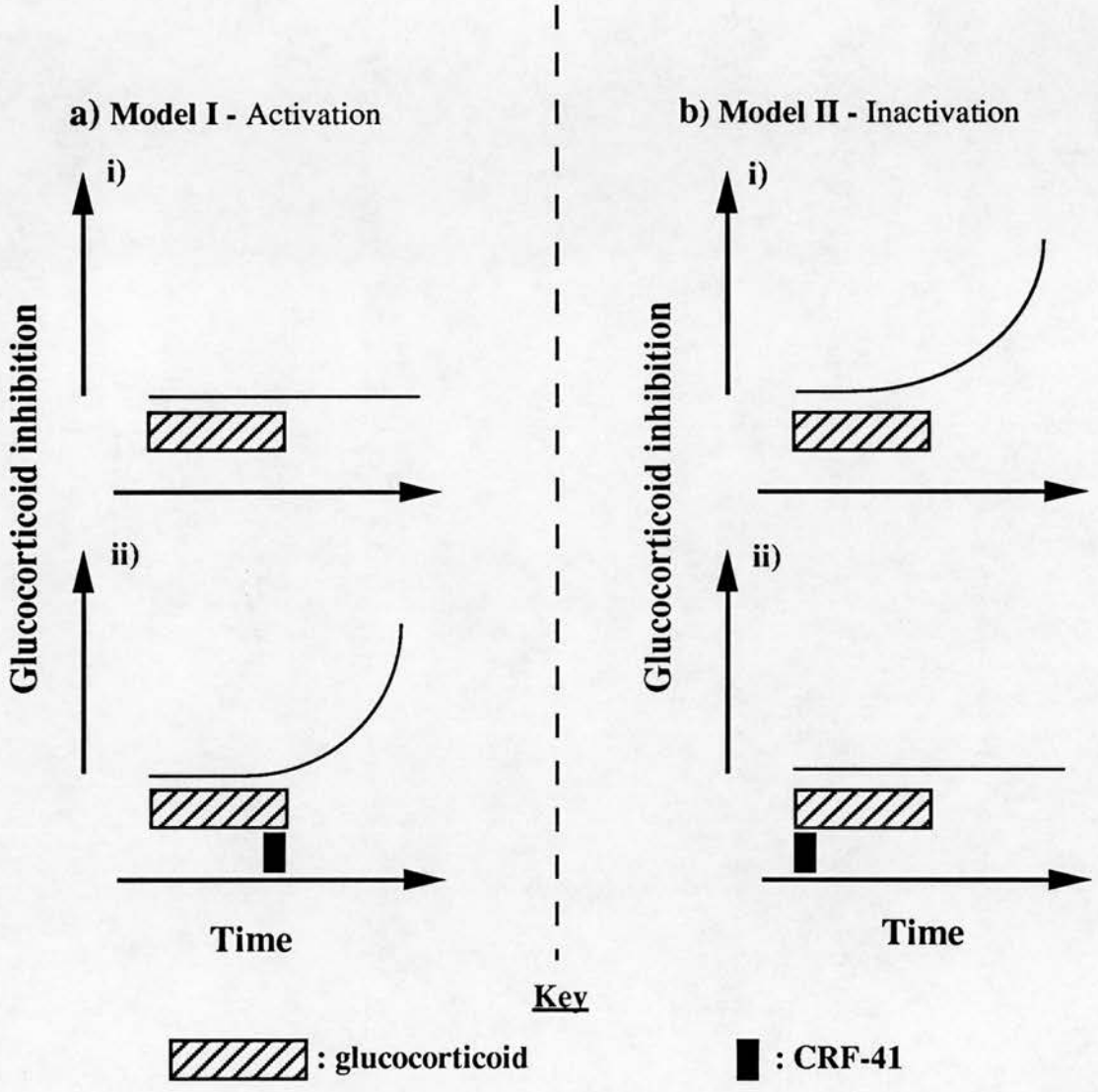


Figure 3.15: **a) Model I - activation.** In this model glucocorticoid inhibition does not fully develop (a_i) unless CRF-41 is applied after the start of glucocorticoid exposure (a_{ii}) *ie* CRF-41 is required to activate some aspect of early glucocorticoid inhibition (for further discussion see section 3.5.4).

b) Model II - inactivation. In this model glucocorticoid inhibition develops independently of CRF-41 exposure (b_i) however, CRF-41 applied simultaneously with the onset of glucocorticoid application inactivates early inhibition (b_{ii}) perhaps by preventing glucocorticoid-induced gene transcription (for further discussion see sections 3.5.4 & 3.5.5).

Initial experiments using Wistar rats obtained from Harlan-Olac suggested that CRF-41 was required to activate the early inhibitory action of corticosterone. However, these experiments were performed using rats infected with Sendai virus and could not be reproduced in rats obtained from Harlan-Olac that had been cleared of infection by the supplier. Furthermore, subsequent experiments revealed a mechanism of inactivation in virus-free rats as discussed below for Charles River rats.

In anterior pituitary glands of Charles River rats CRF-41-stimulated ACTH release was significantly inhibited by corticosterone when no CRF-41 stimulus was applied during the exposure to corticosterone or the type II glucocorticoid receptor agonist RU28362. Furthermore, application of CRF-41 or its intracellular second messenger analogue, 8-CPT-cAMP, simultaneously with the onset of corticosterone exposure prevented the subsequent inhibition of CRF-41-stimulated ACTH release. Thus, it appears that CRF-41 prevents the action/induction of the glucocorticoid-induced protein(s) when applied at the start of glucocorticoid exposure.

Because the parameter of glucocorticoid inhibition being measured in this system, stimulated ACTH secretion, requires CRF-41 stimuli to be used as an assay system after the exposure to glucocorticoid (as in Table 3.1) it is possible that these subsequent stimuli are required to activate some component of early inhibition. Thus inactivation and activation may occur in concert in the same cell. To establish whether activation is also important characterization of the intracellular molecular pathways of glucocorticoid inhibition are required so that other models of early inhibition using markers other than ACTH secretion, for example gene transcription, can be employed.

Intriguingly, CRF-41 failed to prevent glucocorticoid inhibition of AVP-stimulated ACTH secretion. From amongst the several possible explanations to this phenomenon, it is reasonable to exclude on the basis of the data presented in this thesis that priming of the tissue segments with AVP inhibits the action of a subsequent CRF-41 pulse to prevent glucocorticoid inhibition, or that priming with CRF-41 is required for this effect of CRF-41. The differential glucocorticoid inhibition of CRF-41- and AVP- stimulated ACTH release reported in this thesis suggests that glucocorticoids may suppress CRF-41- and AVP- stimulated ACTH secretion through different mechanisms.

Current evidence suggests two alternatives for the apparent differences in the mechanism for early glucocorticoid inhibition of CRF-41- and AVP-stimulated ACTH release. Evidence from other workers (for review see Schwartz *et al.*,1990) combined with the inhibition of AVP-stimulated ACTH release by the protein synthesis inhibitor, puromycin, presented in section 3.3.2 indicates that distinct pools of ACTH are mobilized by these neurohormones: CRF-41 releasing a stored, slow-turnover pool of ACTH, whereas AVP-induced secretion is derived from a rapid-turnover pool of hormone. A further possibility, (this in fact does not exclude the previous one) is that distinct populations of corticotrophs are involved *cf* (Childs,1992; Jia *et al.*,1991; Schwartz *et al.*,1990) in which cAMP phosphorylation targets are expressed in a cell-specific manner. For example, the mechanism of glucocorticoid inhibition of CRF-41, and AVP-stimulated ACTH release may be identical, however, CRF-41-mediated inactivation of early glucocorticoid inhibition may only occur in corticotrophs that respond solely to CRF-41 but not in corticotrophs that respond to both CRF-41 and AVP.

3.5.5 *Putative mechanism(s) of CRF-41-inactivation of early glucocorticoid inhibition*

The precise mechanism by which CRF-41 prevents early glucocorticoid inhibition of ACTH release is unclear, however, because it can be reproduced with a cAMP analogue, it seems reasonable to suggest that it is in fact mediated by cAMP and/or cAMP-dependent phosphorylation. The time window during which CRF-41 must be applied for inactivation to occur (*ie* within 15min after the start of glucocorticoid exposure) would imply that CRF-41 must activate the cAMP/protein kinase pathway before the intracellular pathways mediating glucocorticoid action are fully activated. As the functional activity of the glucocorticoid receptor is dependent upon its state of phosphorylation (Mendel *et al.*,1986; Munck *et al.*,1990; Orti *et al.*,1992) CRF-41, through cAMP, may interfere with glucocorticoid action through modulation of the state of glucocorticoid receptor phosphorylation (Singh & Moudgil,1985).

Although Sheppard and co-workers recently reported a reduction in glucocorticoid receptor mRNA after exposure of AtT20 D16:16 cells to activators of cAMP synthesis no reduction in glucocorticoid receptor mRNA was seen until 6h after treatment suggesting that inactivation does not involve blockade of glucocorticoid receptor synthesis (Sheppard, Roberts & Blum,1991).

A further possibility, and probably the most plausible explanation of the timing characteristics of inactivation, is the observation that transcription factors such as c-fos, c-jun, the cAMP response element binding protein (CREB) and octamer factors which are induced or activated by cAMP, interfere with glucocorticoid-induced gene transcription (Beato,1991; Brindle & Montminy,1992; Diamond, Miner, Yoshinaga & Yamamoto,1990; Lucibello, Slater, Jooss *et al.*,1990; Schüle, Rangarajan, Kliewer *et al.*,1990; Wieland, Döbbeling & Rusconi,1991; Yang-Yen, Chambard, Sun *et al.*,1990).

Figure 3.16

Proposed model of CRF-41 inactivation of early glucocorticoid inhibition

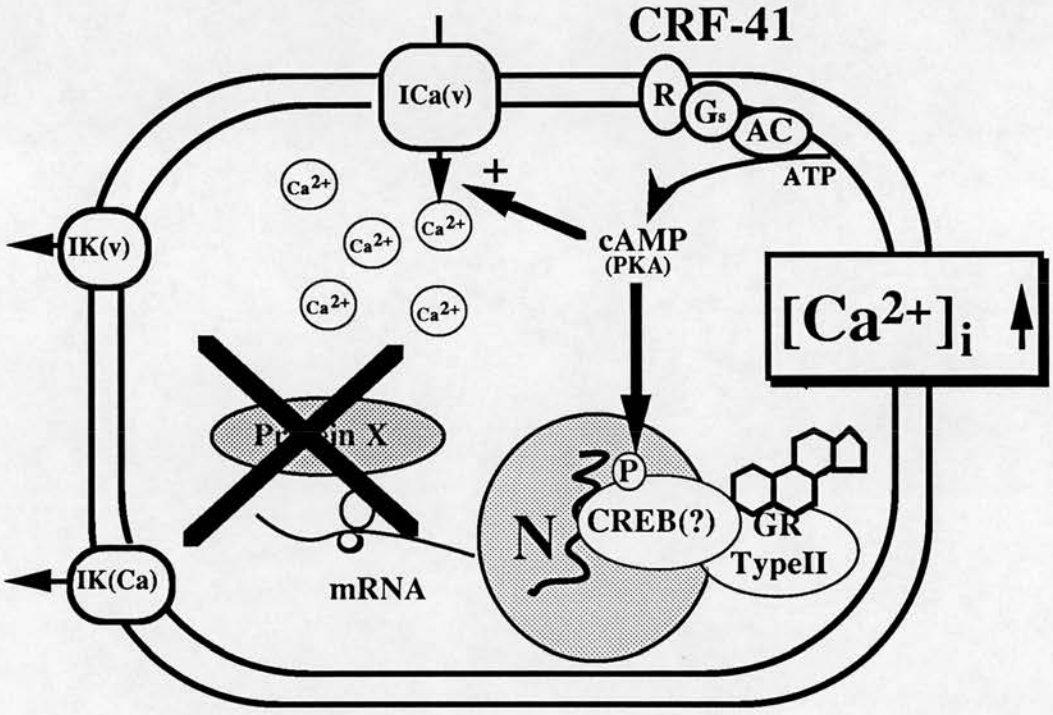


Figure 3.16: CRF-41 blocks early glucocorticoid inhibition in a cAMP-dependent mechanism perhaps by activating or inducing additional transcription factors (such as the cAMP response element binding protein, CREB) that interfere with glucocorticoid-induced gene transcription to prevent expression of the glucocorticoid-induced protein (protein X). Consequently, intracellular free Ca^{2+} levels are not suppressed allowing subsequent CRF-41 stimuli to release ACTH. For further discussion see section 3.5.5. Abbreviations are as follows: IK(Ca), calcium activated potassium channel; IK(v), voltage regulated potassium channel; ICa(v) ,voltage dependent calcium channel; GR, glucocorticoid receptor.

CRF-41 stimulates accumulation of c-fos mRNA in AtT20 D16v cells (Boutillier, Sassone-Corsi & Loeffler,1991) suggesting that such a regulatory mechanism may be involved in inactivation. Interestingly, transcriptional interference between c-fos and the glucocorticoid receptor has been reported for the CRF-41-induced blockade of glucocorticoid suppression of POMC gene transcription (Autelitano & Sheppard,1992; Levin & Roberts,1991; Lundblad & Roberts,1988).

However, it is important to note that c-fos mRNA in AtT20 D1 cells is also induced by glucocorticoids within the timescale of early inhibition (Lin, MacLeod & Hardin,1992). It remains to be explored whether activation of CREB, or other transcription factors, are involved in the mechanism of CRF-41-inactivation of early glucocorticoid inhibition. If such an hypothesis is correct it would be predicted that CRF-41 prevents glucocorticoid induction of genes encoding protein(s) mediating early inhibition (Figure 3.16). CRF-41-inactivation could be a useful marker that may aid the identification of protein(s) involved in early glucocorticoid inhibition.

3.5.6 *Physiological relevance of differential early glucocorticoid inhibition of ACTH secretion*

The implications of differential early glucocorticoid inhibition of stimulated ACTH secretion for the control of ACTH release *in vivo* are that glucocorticoid feedback at the pituitary level is modulated in a differential manner by secretagogue neurohormones. In general, stimuli involving CRF-41 may be relatively resistant to early glucocorticoid inhibition, and those involving primarily AVP are invariably suppressed. For example, some of the major cytokines released during inflammation, interleukin-1 and tumour necrosis factor, are thought to stimulate the release of CRF-41 by hypothalamic neurones but not that of vasopressin (for example see Berkenbosch *et al.*,1988; Sapolsky *et al.*,1988 although see Whitnall *et al.*,1992).

Moreover, in certain autoimmune inflammatory diseases, adrenocortical steroid secretion is enhanced for several days (MacPhee, Antoni & Mason,1989; Mason, MacPhee & Antoni,1990; Sternberg, Hill, Chrousos *et al.*,1989), suggesting that the feedback mechanism may have been modified. Furthermore, several reviews of glucocorticoid feedback inhibition *in vivo* have emphasized that certain 'stressors' are relatively resistant to glucocorticoid inhibition (Dallman *et al.*,1987; Jones & Gillham,1988; Keller-Wood & Dallman,1984), it remains to be examined whether these 'stressors' differentially mobilize CRF-41 or AVP as secretagogue. These observations illustrate the possible functional importance of the escape of CRF-41-induced ACTH release from glucocorticoid feedback inhibition at the pituitary level.

Interestingly, a recent study employing the CRF-41 gene stably transfected in AtT20 cells suggests that CRF-41-gene transcription activated by protein kinase A activators is more resistant to glucocorticoid inhibition than that evoked by the protein kinase C pathway (Rosen, Majzoub & Adler,1992). However, as discussed by these authors this evidence is derived from two different cell lines: the AtT20 cell stably transfected with the human CRF-41 gene and the human primary liver carcinoma cell line NPLC that endogenously expresses the CRF-41 gene. CRF-41 gene expression in AtT20 and NPLC cells is induced by activators of the protein kinase A and protein kinase C pathways respectively but not *vice versa,* thus these data may be a result of different tissue specific factors expressed in the two cell lines. In contrast, Bilezikjian and co-workers (Bilezikjian *et al.*,1987) have suggested ACTH release evoked by protein kinase C activation is more resistant to late glucocorticoid inhibition than ACTH release stimulated by protein kinase A activators. It is of interest that a reduction in glucocorticoid receptor number and glucocorticoid receptor mRNA levels develop 6h after exposure to activators of cAMP synthesis in AtT20 D16:16 corticotroph tumour cells (Sheppard *et al.*,1991). Furthermore, glucocorticoid/CRF-41 interaction also

occurs at the proopiomelanocortin (POMC) gene; glucocorticoids cannot inhibit POMC gene transcription when the gene is fully activated by CRF-41 (see section 3.5.5). Thus, CRF-41 acting via cAMP exerts both short- (present findings) and long-term control over glucocorticoid action at the pituitary level. In turn, reciprocally opposing actions are provided by early and late glucocorticoid inhibition of CRF-41 action on ACTH release and biosynthesis *cf* (Dallman *et al.*,1987; Jones & Gillham,1988).

3.6 Conclusion

In summary the data presented in this section suggest that:

1) CRF-41 and AVP mobilize distinct pools of ACTH from the anterior pituitary gland.

2) Type II glucocorticoid receptors and synthesis of new protein(s) are involved in early glucocorticoid inhibition of AVP- and CRF-41-stimulated ACTH release.

3) The timing of glucocorticoid application is important in determining the early glucocorticoid inhibition of CRF-41-, but not AVP-stimulated ACTH release.

4) CRF-41 inactivates early inhibition of CRF-41-stimulated ACTH release, and this is mimicked by a cell permeant analogue of cAMP suggesting an acute interaction between the cAMP/protein kinase A and glucocorticoid responsive signalling pathways.

Such differential modulation of early glucocorticoid inhibition may be of functional significance *in vivo*. CRF-41-inactivation may be a useful tool in characterization of glucocorticoid-induced proteins explicitly involved in early inhibition.

4

Characterization of early glucocorticoid-induced proteins in the mouse corticotroph cell line, AtT20 D16:16

4

CHARACTERIZATION OF EARLY GLUCOCORTICOID-INDUCED PROTEINS IN THE MOUSE CORTICOTROPH CELL LINE, AtT20 D16:16

4.1 Introduction

The low proportion of corticotrophs in the adenohypophysis (Westlund *et al.*,1985) and the fact that glucocorticoids affect virtually all cells in the gland make the characterisation of glucocorticoid induced proteins and messenger ribonucleic acids (mRNA) in normal corticotrophs extremely difficult. A more attractive alternative is to employ a nominally homogenous corticotroph cell population such as the mouse clonal anterior pituitary corticotroph cell line, AtT20, derived from a radiation induced tumour (Furth, Gadsden & Upton,1953; Sabol,1980). Recent data from this laboratory (see following results and Woods *et al.*,1992) has confirmed that the D16:16 strain of the AtT20 cell line, that is maintained as a monolayer and derived from the D1 and D16v strains (Sabol,1980; Tashjian Jr.,1979; Yasumura,1968), is a valid model in which to examine the mechanism of early glucocorticoid inhibition. As CRF-41 inactivates early glucocorticoid inhibition in normal corticotrophs (as discussed in section 3) CRF-41/glucocorticoid interaction in AtT20 D16:16 cells was also analysed in an attempt to shed light on the nature of the glucocorticoid induced protein(s).

With respect to characterization of proteins involved in early glucocorticoid inhibition in AtT20 D16:16 cells two major methodological approaches were used:

1) Northern and Western blotting for glucocorticoid-induced mRNAs and proteins in AtT20 D16:16 cells using probes complimentary to mRNAs/proteins that have been identified in other models of early inhibition. As functional evidence in AtT20 D16:16 cells, as well as other systems (see section 1.6.3), suggest glucocorticoid suppression of intracellular free Ca^{2+} levels these studies were predominantly confined to known Ca^{2+}-binding proteins which may act as sensors of intracellular free Ca^{2+}.

2) Complimentary DNA library technology has proved invaluable for the identification of differentially regulated mRNAs (for example see Davis, Cohen, Nielsen *et al.*,1984; Duguid, Rohwer & Seed,1988; Harrigan *et al.*,1989; Sargent & Dawid,1983). Thus a cDNA library constructed from glucocorticoid-induced AtT20 D16:16 cells was screened using subtraction and differential hybridization methodologies. Attempts were also made to characterize novel proteins using 2D-gel electrophoresis. Messenger RNAs (mRNAs) or proteins identified in this way could be further screened with known probes to establish potentially novel as well as previously characterised proteins.

Glucocorticoid-induced proteins identified in AtT20 D16:16 corticotrophs will allow the mechanism and modulation of early inhibition to be examined in detail. Furthermore, proteins characterized in this system can be used as probes to characterize early inhibition in normal corticotrophs as well as other models of early inhibition in an attempt to dissect a generic mechanism of early glucocorticoid inhibition.

Results

4.2 Early glucocorticoid inhibition of CRF-41-stimulated ACTH release in AtT20 D16:16 corticotrophs

4.2.1 *Effect of glucocorticoids on CRF-41-stimulated ACTH secretion*

As the ACTH secretory response of normal corticotrophs is most reliable using a perifusion system initial studies were designed to examine the secretory response of AtT20 D16:16 corticotrophs using cells (approximately $2x10^6$ cells/column) perifused in a matrix of Sephadex G-10 (as described by Dayanithi & Antoni,1989) or perifused on glass coverslips coated with $2.5\mu g/cm^2$ poly-L-lysine (Sigma). The poly-L-lysine was required to prevent cells detaching from the coverslip during the time course of an experiment. The ACTH secretory response declined progressively on repeated application of a submaximal (10nM) dose of CRF-41 irrespective of the duration of stimulation (2.5-5min) or interstimulus period (30-60min). Typically, stimulated ACTH release declined to 50% of the initial evoked release after 3 consecutive pulses of CRF-41. Lower CRF-41 concentrations did not result in reproducible ACTH responses, thus AtT20 D16:16 cells grown as a monolayer in static incubation were examined as a model.

AtT20 D16:16 cells consistently responded in a time and dose dependent manner to CRF-41 (Figure 4.1a & b) in static monolayer incubations. Stimulus to basal ratios were optimal at 20min of incubation with 10nM CRF-41, typically giving a 1.5-2fold increase over basal ACTH release. Such short incubation periods were used in order to stay within the time domain of early inhibition, other workers typically incubate with secretagogue for 3h to improve stimulus to basal ratios *cf* (Miyazaki *et al.*,1984). Pretreatment with the synthetic glucocorticoid, dexamethasone, (10nM for 90min)

inhibited CRF-41-stimulated ACTH release to approximately 50% of control (Figure 4.1c). Dexamethasone had no significant inhibitory effect on basal ACTH release. The transcription inhibitor, 5,6-Dichloro-1-D-ribofluranosylbenzimidazole (DRB) prevented the inhibitory action of dexamethasone: DRB had no effect on basal or CRF-41-stimulated ACTH release (Figure 4.1c). Dexamethasone had no effect on 10nM CRF-41-stimulated accumulation of cAMP in the presence of 0.5mM IBMX, a phosphodiesterase inhibitor (Table 4.1). The peak of CRF-41-stimulated cAMP accumulation (~7 fold above basal) was evident at 2min after 10nM CRF-41 application and remained elevated (~2 fold above basal) after 30min exposure. No accumulation of cAMP was observed in the absence of IBMX, furthermore no enhancement of basal cAMP accumulation was observed on addition of IBMX.

Further data from this laboratory have confirmed that early glucocorticoid inhibition in this cell line requires activation of type II receptors and induction of mRNA and protein. Significant inhibition is demonstrable after 45min of glucocorticoid application, maximal inhibition (to 50-90% of control CRF-41-stimulated ACTH release) is evident using 10nM dexamethasone for 1.5-2h (Woods *et al.*,1992). Thus the AtT20 D16:16 cell line is a valid model in which to characterize proteins involved in early inhibition.

Figure 4.1

Reversal of dexamethasone inhibition of CRF-41-stimulated ACTH release in AtT20 D16:16 cells by the transcription inhibitor 5,6-Dichloro-1-ß-D-ribofluranosylbenzimidazole (DRB)

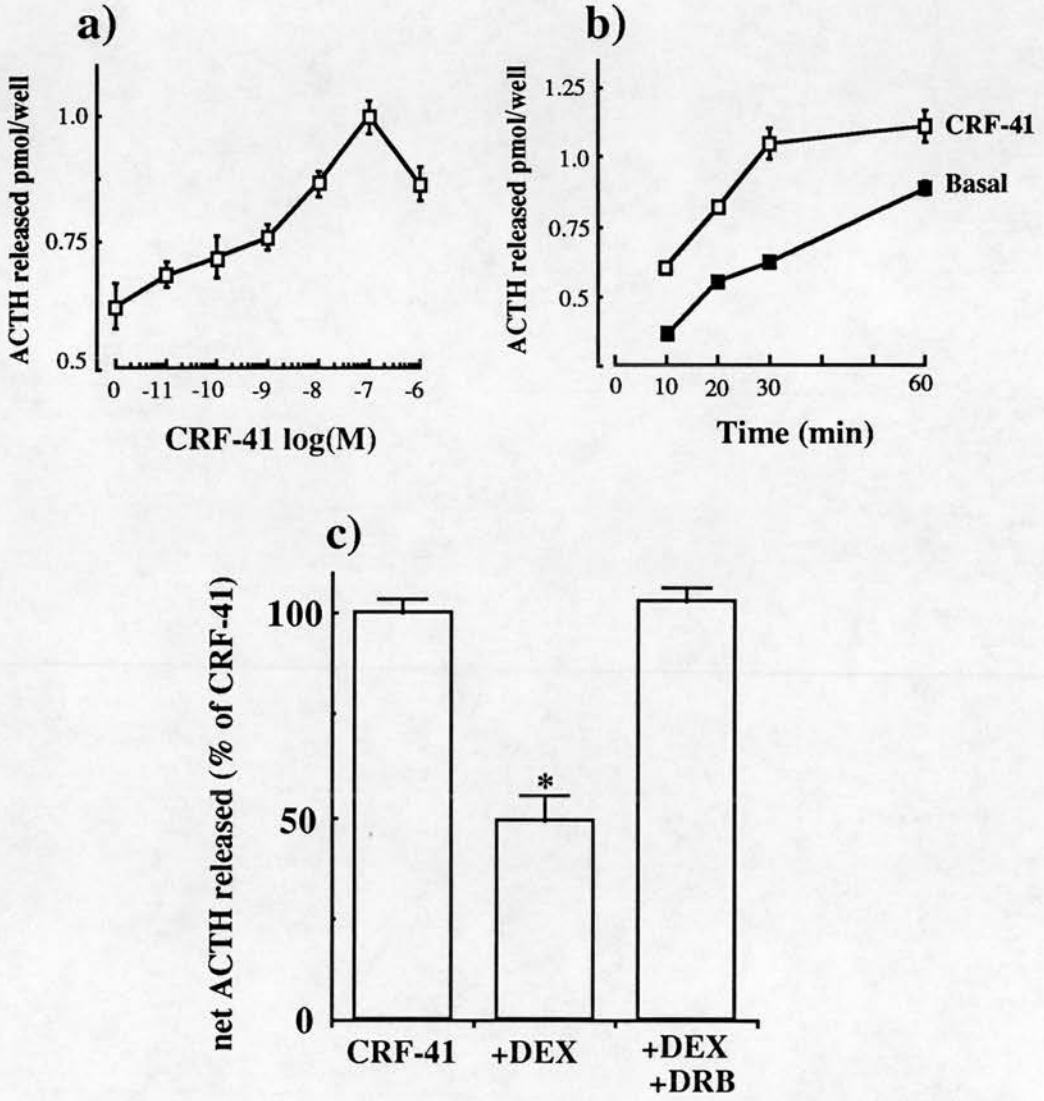


Figure 4.1: **a**) Dose response curve and **b**) time course of CRF-41-stimulated ACTH release from AtT20 D16:16 cells in static monolayer incubation. Data are expressed as pmol/well of ACTH released, CRF-41 was applied for 20min in a). **c**) Reversal of dexamethasone inhibition of 10nM CRF-41-stimulated ACTH release by 150µM DRB. Data are means ± SEM (n=3), expressed as a percent of the net ACTH released by CRF-41 alone. Dexamethasone (10nM) was applied for 90min before the stimulus, DRB was started 20min before and continued during the exposure to dexamethasone. *p<0.05 compared to CRF-41 control and +DEX+DRB (Students unpaired 2-tail t-test)

Table 4.1

Effect of dexamethasone and ketoconazole on CRF-41-stimulated cAMP accumulation in AtT20 D16:16 cells

	pmol cAMP/ well					
	Time (min)					
Treatment	0'	2'	5'	10'	20'	30'
Basal (4)	3.16±0.35	3.43±0.08	3.43±0.39	3.55±0.47	3.78±0.27	3.40±0.39
10nM CRF-41 (3)	3.47±0.23	21.33±1.05	16.34±1.99	9.98±0.74	8.46±0.23	6.47±0.23
10nM CRF-41 + Dexamethasone (3)	3.16±0.35	19.50±1.56	15.10±0.78	9.67±0.59	7.45±0.66	7.41±1.05
1nM CRF-41 (3)	2.93±0.27	9.59±0.08	8.27±0.46	5.66±0.35	4.91±0.23	4.88±0.31
1nM CRF-41 + Ketoconazole (3)	2.69±0.23	8.66±0.78	6.86±0.12*	4.64±0.19*	5.07±0.27	5.11±0.19

Table 4.1: Data are expressed as pmol of cAMP generated per well for the stated time period. IBMX (0.5mM) was applied for 15min before time 0' and fresh IBMX was applied with the stimulus at time 0'. No accumulation of cAMP in response to CRF-41 was observed in the absence of IBMX. Dexamethasone and ketoconazole alone had no effect on cAMP accumulation at any time point. Total intracellular cAMP was determined as described in section 2.3.3. Dexamethasone (10nM) was applied for 90min before the CRF-41 stimulus; ketoconazole (200μM) was started 15min before and continued during the stimulus. Data are means ± SEM, the number of experiments is shown in parentheses. *$p<0.05$ compared with the respective 1nM CRF-41 control group using Students unpaired 2-tail t-test.

4.2.2 *CRF-41 inactivates early glucocorticoid inhibition of CRF-41-stimulated ACTH release*

As CRF-41 inactivates early inhibition in normal corticotrophs CRF-41/glucocorticoid interactions in the AtT20 D16:16 cell line were examined in an attempt to provide markers that may prove useful for the identification of proteins involved in early glucocorticoid inhibition. Treatment of AtT20 D16:16 cells with 1nM CRF-41 during the first 30min of exposure to 10nM dexamethasone (total duration 90min) blocked early inhibition of subsequent CRF-41-stimulated ACTH release (Figure 4.2). Inactivation was not observed if 1nM CRF-41 pretreatment was started 15min (total duration 30min) after the onset of dexamethasone exposure (Figure 4.2). Pretreatment with 10nM CRF-41 or 1-100μM 8-CPT-cAMP alone resulted in a reduction of the subsequent ACTH response to CRF-41 to 26% and 63% respectively of control CRF-41-stimulated ACTH release. Pretreatment with 0.1nM CRF-41 alone resulted in subsequent 10nM CRF-41 stimulated ACTH release of 90.0±12.0% (n=6) of control. However, 0.1nM CRF-41 pretreatment failed to reverse the action of dexamethasone (ACTH release was 4.2±6.5% (n=6) of control). Phorbol dibutyrate, PdBu, pretreatment alone (10nM for 30min as for CRF-41 in Figure 4.2) reduced the subsequent CRF-41-stimulated ACTH release to 66.0±10.3% (n=4) of control, however, PdBu pretreatment did not block early glucocorticoid inhibition of CRF-41-stimulated ACTH release (this paradigm resulted in net ACTH release of 12.0±7.0% (n=3) compared to control).

Experiments were performed to examine whether blockade of CRF-41-induced cAMP accumulation could prevent CRF-41-inactivation of early inhibition. Ketoconazole, that maximally inhibits CRF-41-stimulated cAMP accumulation in normal rat corticotrophs when used at 200μM (Stalla, Stalla, Huber *et al.*,1988) had no

significant effect on the time course of cAMP accumulation induced by 1nM CRF-41 (Table 4.1) although a small but significant inhibition was observed at the 5 and 10min time point. A similar lack of action was observed using 20μM ketoconazole. Concentrations greater than 200μM could not be achieved as ketoconazole required greater than 1% DMSO to remain soluble, a DMSO concentration that was deleterious to the secretory function of AtT20 D16:16 cells.

In conclusion, early glucocorticoid inhibition in AtT20 D16:16 cells develops within 2h, requires the activation of type II glucocorticoid receptors and the synthesis of new mRNA and protein. Furthermore, CRF-41, but not phorbol dibutyrate, inactivates early inhibition of CRF-41-stimulated ACTH secretion.

Figure 4.2

CRF-41 inactivates early glucocorticoid inhibition in AtT20 D16:16 cells in static monolayer culture

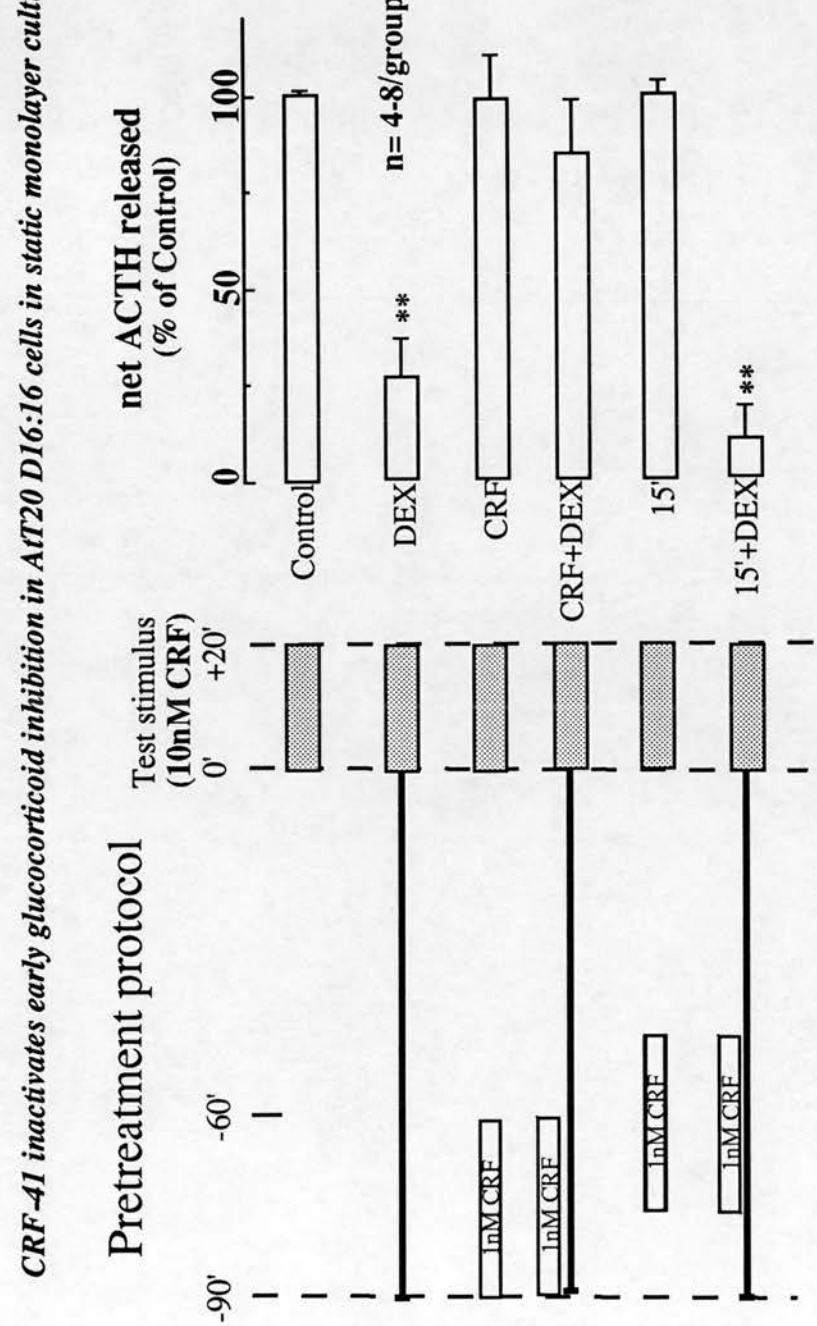


Figure 4.2: Dexamethasone was applied for 90min at 10nM (DEX, solid horizontal line), CRF-41 pretreatment was for 30min at 1nM (horizontal open box) applied simultaneously with (CRF) or started 15min (15') after the start of dexamethasone exposure before washout. After 90min the pretreatment media was removed and the10nM CRF-41 test stimulus applied for 20min (horizontal stippled box). Data are expressed as a percentage of the net ACTH released by 10nM CRF-41 alone (Control), after subtraction of the respective basal ACTH release. Data are means ± SEM. **p<0.01 compared to all groups (Students unpaired 2-tail t-test).

4.3 Characterization of known glucocorticoid-induced Ca^{2+}-binding, and other, proteins in AtT20 D16:16 corticotrophs

4.3.1 *Lipocortin (annexin) I and chromogranin A*

Lipocortin I has been proposed as a mediator of early glucocorticoid action in macrophages and other systems (for reviews see Flower,1988; Peers & Flower,1990). Lipocortin I mRNA or protein was undetectable in AtT20 D16:16 cells even after 90min of 10nM dexamethasone treatment (Figure 4.3 and 4.4a respectively). Lipocortin I mRNA was constitutively expressed in AtT20 D1 cells, however, no induction of lipocortin I mRNA was seen after 24h of 1μM dexamethasone treatment (Figure 4.3). Northern hybridization for lipocortin I mRNA was kindly performed by Dr E L Mullens, Department of Medicine, Charing Cross and Westminster Hospital Medical School, London, UK.

Recently, chromogranin A has been reported as a paracrine inhibitor mediating late (slow >24h) glucocorticoid inhibition in AtT20 D16v cells thus it was of interest to examine whether this protein is involved in early inhibition. Immunoblotting for chromogranin A using a polyclonal antiserum (courtesy Dr D K Apps, Dept. Biochemistry, University of Edinburgh, UK) in Ca^{2+}-precipitated protein fractions from AtT20 D16:16 cells revealed two major immunoreactive bands (that appeared as doublets, a common feature of chromogranin A immunoblots (Simon & Aunis,1989)) at ~74kDa and ~33kDa respectively (Figure 4.4b). However, no induction of either chromogranin A-like immunoreactive band was observed after 90min of 10nM dexamethasone treatment (n=3). Although chromogranin A has a calculated molecular weight of ~50kDa the protein is heavily glycosylated, binds SDS weakly and displays an apparent molecular weight of approximately 75kDa in SDS-PAGE electrophoresis.

Figure 4.3

Northern blotting for lipocortin I mRNA in AtT20 D1 and D16:16 corticotrophs

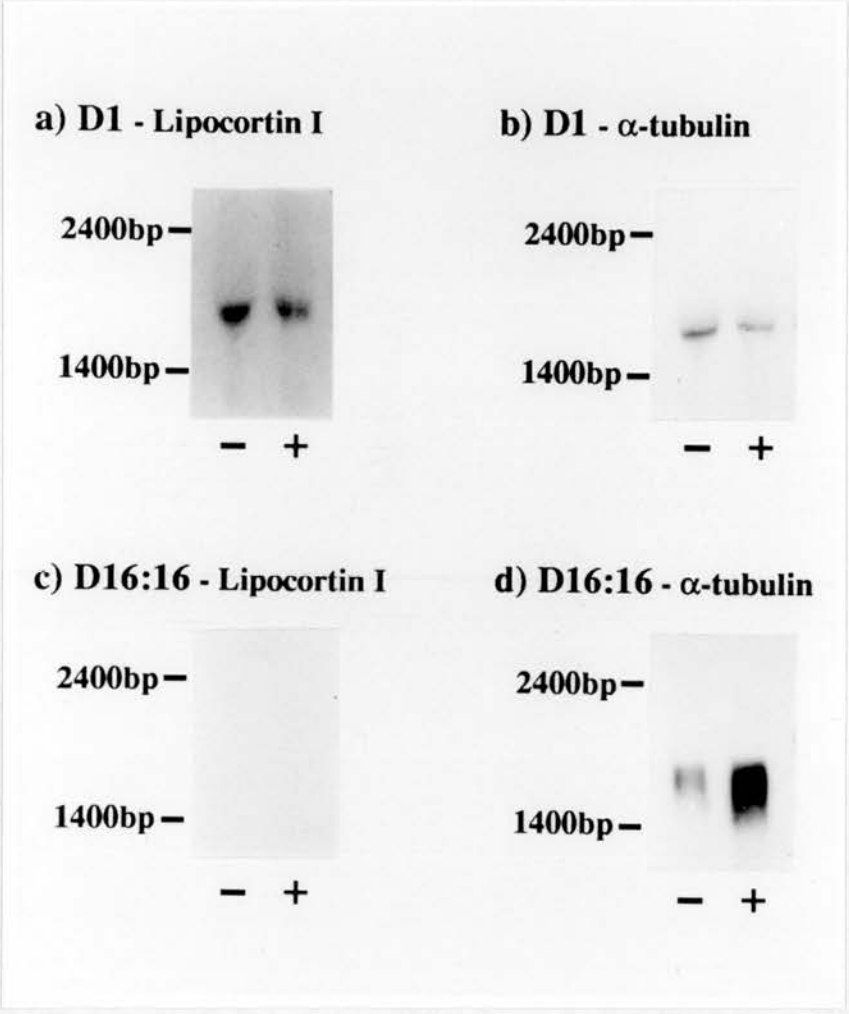
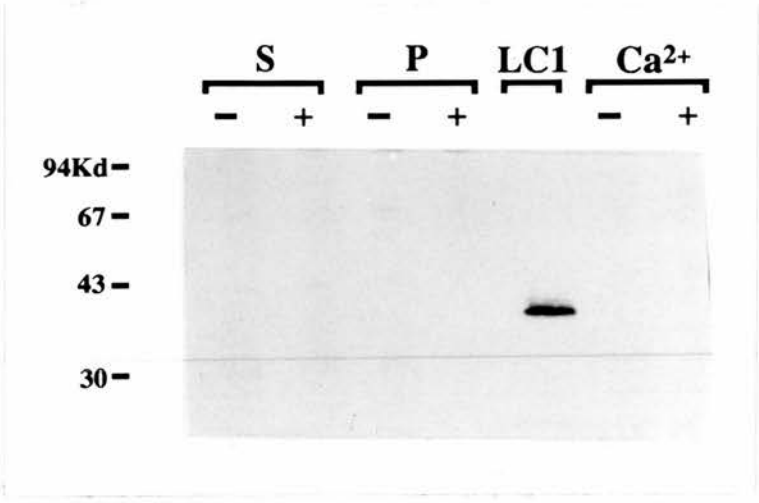


Figure 4.3: **a)** Autoradiograph of 20µg of total RNA from AtT20 DI cells hybridized with (α^{32}P) dCTP labelled lipocortin I cDNA and exposed for 5 days. Cells were incubated for 24h in the presence (+) or absence (-) of 1µM dexamethasone. **b)** Blot a) rehybridized with (α^{32}P) dCTP labelled α-tubulin cDNA and exposed for 5h. **c)** Autoradiograph of 2µg of poly A^+ mRNA from AtT20 D16:16 cells hybridized with (α^{32}P) dCTP labelled lipocortin I cDNA and exposed for 5 days. Cells were incubated for 90min in the presence (+) or absence (-) of 10nM dexamethasone. **d)** Blot c) rehybridized with (α^{32}P) dCTP labelled α-tubulin cDNA and exposed for 5h. All blots were hybridized with the respective probe ($1x10^6$ cpm/ml) overnight at 65°C in 10% dextran sulphate, 1M NaCl, 1% SDS and 100µg/ml salmon sperm DNA. Washing was performed at 65°C for 30min in 1xSSC, 0.1%SDS.

Figure 4.4

Immunoblotting for lipocortin I and chromogranin A protein in AtT20 D16:16 corticotrophs

a)



b)

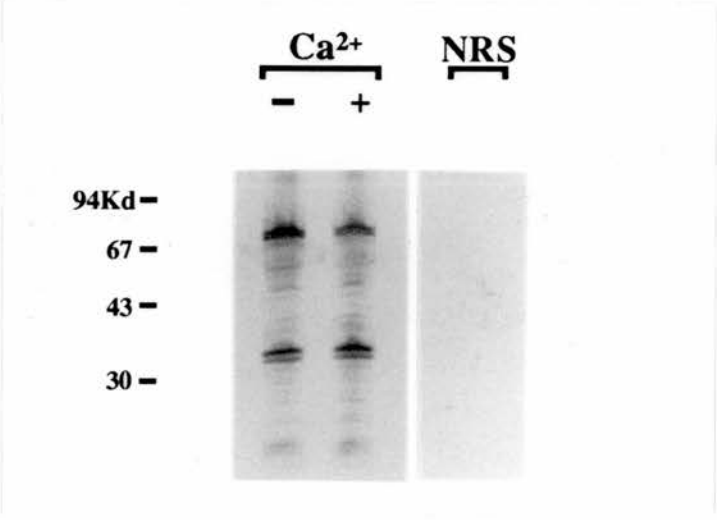


Figure 4.4: **a)** Lipocortin I immunoblot. **b)** Chromogranin A immunoblot. Cells were incubated in the presence (+) or absence (-) of 10nM dexamethasone for 90min. Crude supernatant (S) and calcium precipitated (Ca^{2+}) samples contained 2µg/lane, crude pellet samples (P) contained 1µg/lane and cloned lipocortin I standard (LC 1) contained 25ng/lane. Non-immune rabbit serum (NRS) was used to assess non-specific binding. Samples were electrophoresed on a 10-15% SDS-PAGE Phastgel, electroblotted to Immobilon PVDF and reacted for lipocortin I, using monoclonal MAb105 at 1:1000 dilution, or chromogranin A, using a polyclonal rabbit antiserum at 1:500 dilution.

The identity of the 33kDa and other weaker bands is unknown, however, to date all polyclonal antisera directed against chromogranin A cross react with several other Ca^{2+}-binding proteins (Simon & Aunis,1989).

The lack of induction of lipocortin I or chromogranin A in the time-scale of early inhibition suggest that these proteins are not essential components of early glucocorticoid action.

4.3.2 *Calmodulin*

Recently, glucocorticoid-induction of the intracellular calcium receptor protein, calmodulin, has been reported in several lymphocyte-derived models of early inhibition (Dowd *et al.*,1991; Harrigan *et al.*,1989). AtT20 D16:16 cells constitutively expressed a single major ~1.6kb mRNA species that hybridized to a calmodulin cDNA probe encoding the mouse pCAMII calmodulin mRNA (clone 21 cited in Baughman *et al.*,1991 and Harrigan *et al.*,1989) or a chicken calmodulin cDNA probe (Dowd *et al.*,1991). Constitutive expression was slightly variable between different passage of cells (see Figures 4.5, 4.6a and 4.7). In three separate experiments the normalized hybridization intensity (see section 2.9.2) of control (untreated) samples was 0.062, 0.096 & 0.105 respectively. Furthermore, in 3 out of 9 experiments weak hybridization signals were also seen at ~2.4 and ~4.2kb using the above probe (for example see Figure 4.5). Dexamethasone treatment for 90min increased the level of the ~1.6kb calmodulin mRNA. Time-course studies showed that calmodulin mRNA increased at 45min but not at 15min after treatment with 10nM dexamethasone (Figure 4.5), in contrast the cytoplasmic 7S RNA transcript was unaffected by dexamethasone pretreatment and was used for normalization of all subsequent blots. After normalization calmodulin mRNA in control cells was 1.0±0.2 (relative hybridization intensity units (see section 2.9.2 for calculation), n=3 mean ± SEM) and increased to

7.4±1.7 (n=5) and 10.8±1.1 (n=3) after a 90min treatment with 10nM and 100nM dexamethasone, respectively. Identical results were obtained using a cDNA probe encoding chicken calmodulin (Dowd *et al.*,1991) hybridized at lower stringency (for example see Figure 4.6a). Pretreatment of cells with the protein synthesis inhibitor, puromycin (0.1μM, 15min before and during the exposure to 100nM dexamethasone) blocked the induction of calmodulin mRNA (Figure 4.7). This concentration of puromycin fully blocks early glucocorticoid inhibition of CRF-41-stimulated ACTH release in AtT20 D16:16 cells (Woods *et al.*,1992).

Application of 10nM CRF-41 during the first 30min of dexamethasone pretreatment (as described in Figure 4.2, section 4.2.1) blocked the dexamethasone-induced (100nM, 90min) accumulation of calmodulin mRNA. CRF-41 pretreatment alone had no significant effect on calmodulin mRNA accumulation (Figure 4.6 and 4.7). The protein kinase C activator, phorbol dibutyrate (10nM) alone, using the same pretreatment protocol as for CRF-41, appeared to induce calmodulin mRNA accumulation to ~6fold above control. Importantly, phorbol dibutyrate did not block dexamethasone induced calmodulin mRNA accumulation (Figure 4.7).

Acutely isolated rat anterior pituitary glands incubated *in vitro* as described in section 2.3.1 constitutively expressed 2 major RNA species of equal intensity at ~1.6kb and ~4.2kb, constitutive expression in rat anterior pituitary was consistently greater than that observed in AtT20 D16:16 cells (Figure 4.5). No induction of either calmodulin RNA species was observed in rat anterior pituitary after 90min dexamethasone (10-100nM) pretreatment (Figure 4.5).

Dexamethasone pretreatment (90min at 100nM) significantly elevated immunoreactive calmodulin protein levels (band ~18Kd) to 2.1±0.3 fold above basal (n=3) in Ca^{2+}-precipitated protein extracts of AtT20 D16:16 cells (Figure 4.8a).

Figure 4.5

Effect of dexamethasone on calmodulin mRNA expression in AtT20 D16:16 corticotrophs and normal rat anterior pituitary gland

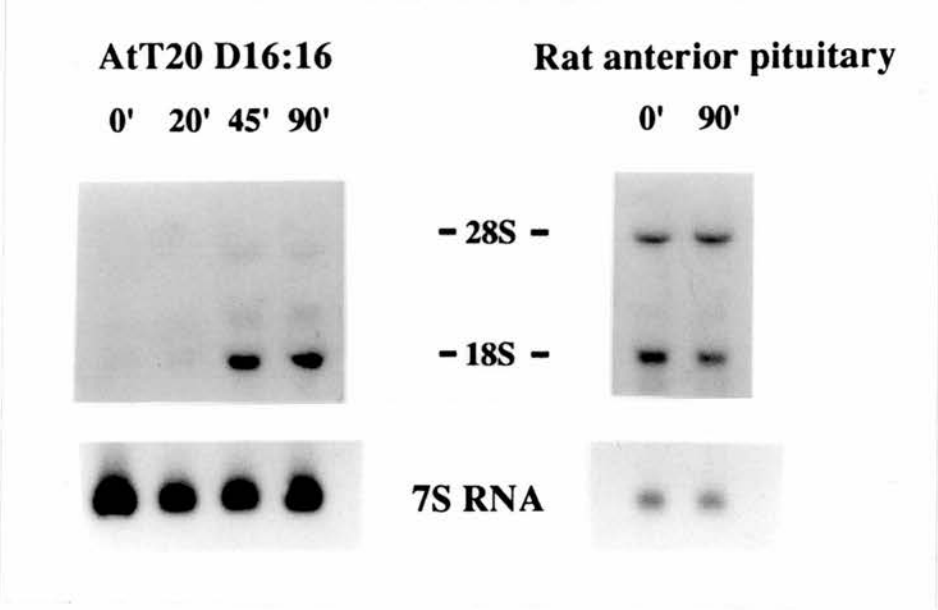


Figure 4.5: AtT20 D16:16 corticotrophs and rat anterior pituitary gland segments were treated with 10nM and 100nM dexamethasone respectively for the indicated time (min) *in vitro*. Total RNA from AtT20 D16:16 corticotrophs (20µg/lane) or normal rat anterior pituitary segments (10µg/lane) were hybridized with a (α^{32}P) dCTP labelled 0.4kbp EcoRI fragment of the mouse calmodulin (pCAMII) cDNA derived from the mouse WEHI-7TG lymphocyte cell line (clone 21). Hybridization was performed as described in section 2.7.4, autoradiographs were exposed for 3days. Blots were reprobed with a 0.2kbp BamHI mouse cDNA fragment encoding the abundant cytoplasmic 7S RNA and exposed for 2h.

Figure 4.6

CRF-41 blocks dexamethasone induction of calmodulin mRNA in AtT20 D16:16 cells

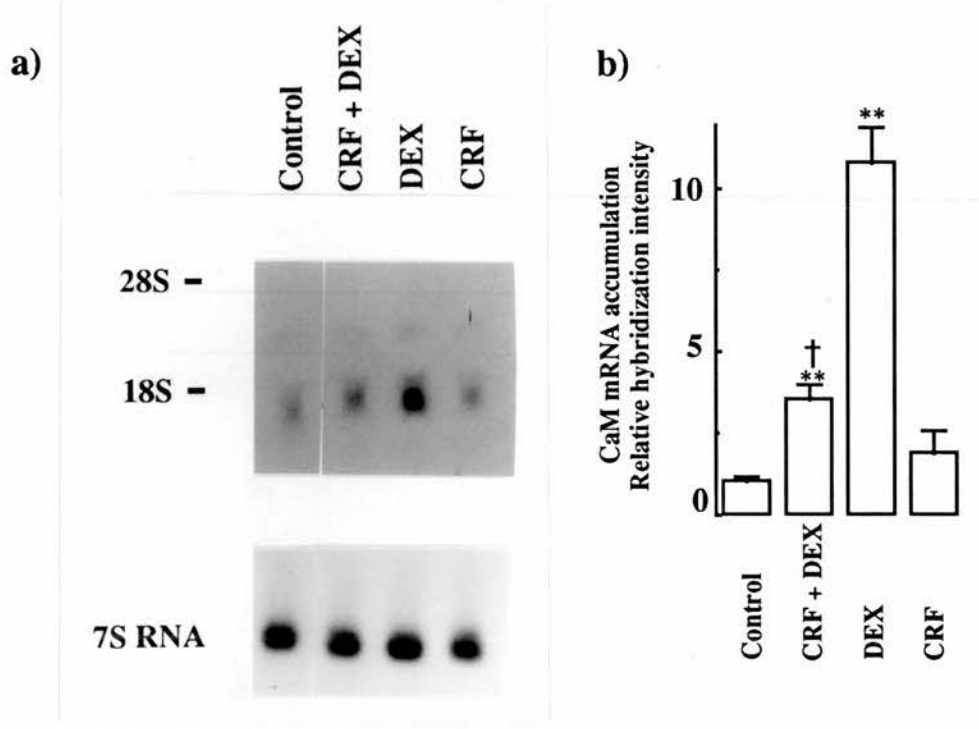


Figure 4.6: **a)** Autoradiograph from a typical experiment. CRF-41 (10nM) was applied during the first 30min of dexamethasone exposure (100nM, 90min total duration) as in Figure 4.2. Total RNA (20μg/lane) was hybridized with a (α^{32}P) dCTP labelled 0.28kbp EcoRI/PstI cDNA fragment of chicken calmodulin. Blots were hybridized as described in section 2.7.4, except that the final wash was at 1xSSC, 0.1%SDS at 65°C and exposed for 3days. **b)** Summary of results from 3 independent experiments performed as in a). Data are expressed as the relative hybridization intensity of calmodulin mRNA as described in section 2.9.2. **p<0.01 with respect to control, †p<0.01 with respect to dexamethasone treated cells (Students unpaired 2-tailed t-test).

Figure 4.7

Effect of phorbol dibutyrate, and the translation inhibitor, puromycin, on dexamethasone-induced calmodulin mRNA expression in AtT20 D16:16 cells

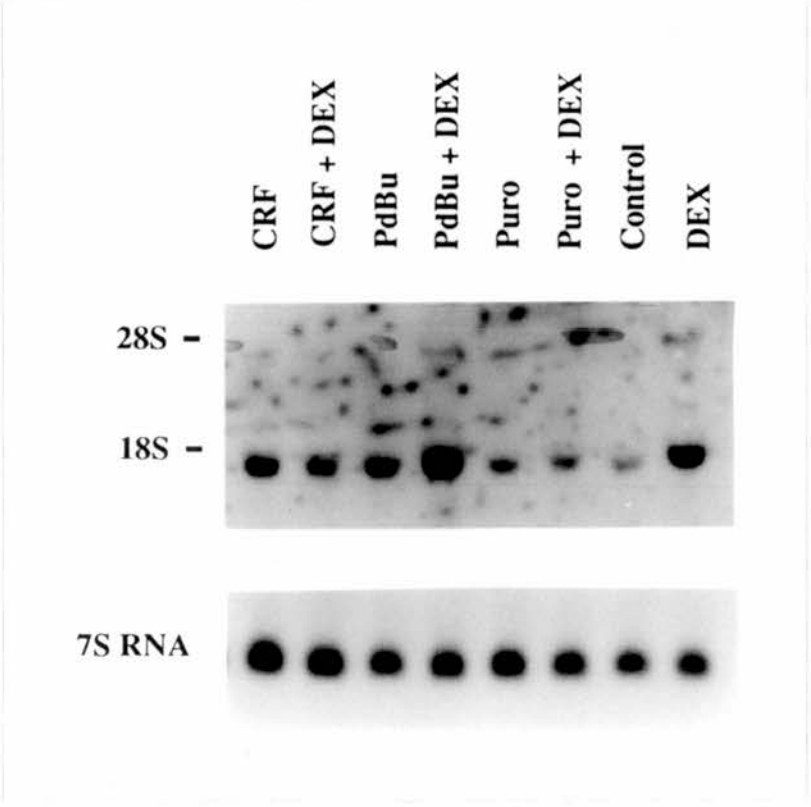


Figure 4.7: Puromycin (0.1mM) was applied 15min before and during the exposure to dexamethasone (100nM for 90min). Phorbol dibutyrate (a protein kinase C activator, PdBu, 10nM), and CRF-41 (10nM) were applied during the first 30min of dexamethasone exposure as in Figure 4.2. Total RNA (20μg/lane) was hybridized with a (α^{32}P) dCTP labelled 0.4kbp EcoRI fragment of the mouse calmodulin (pCAMII) cDNA as described in section 2.7.4, autoradiographs were exposed for 3days. Equal loading of RNA was determined by reprobing blots for 7S RNA. In this experiment relative hybridization intensities (Control = 1.0) were as follows: CRF, 3.2; CRF+DEX, 2.9; PdBu, 6.2; PdBu+DEX, 11.9, Puro, 1.9; Puro+DEX, 1.8, Control, 1.0; DEX, 9.4.

Figure 4.8

Induction of immunoreactive calmodulin protein by dexamethasone in AtT20 D16:16 corticotrophs

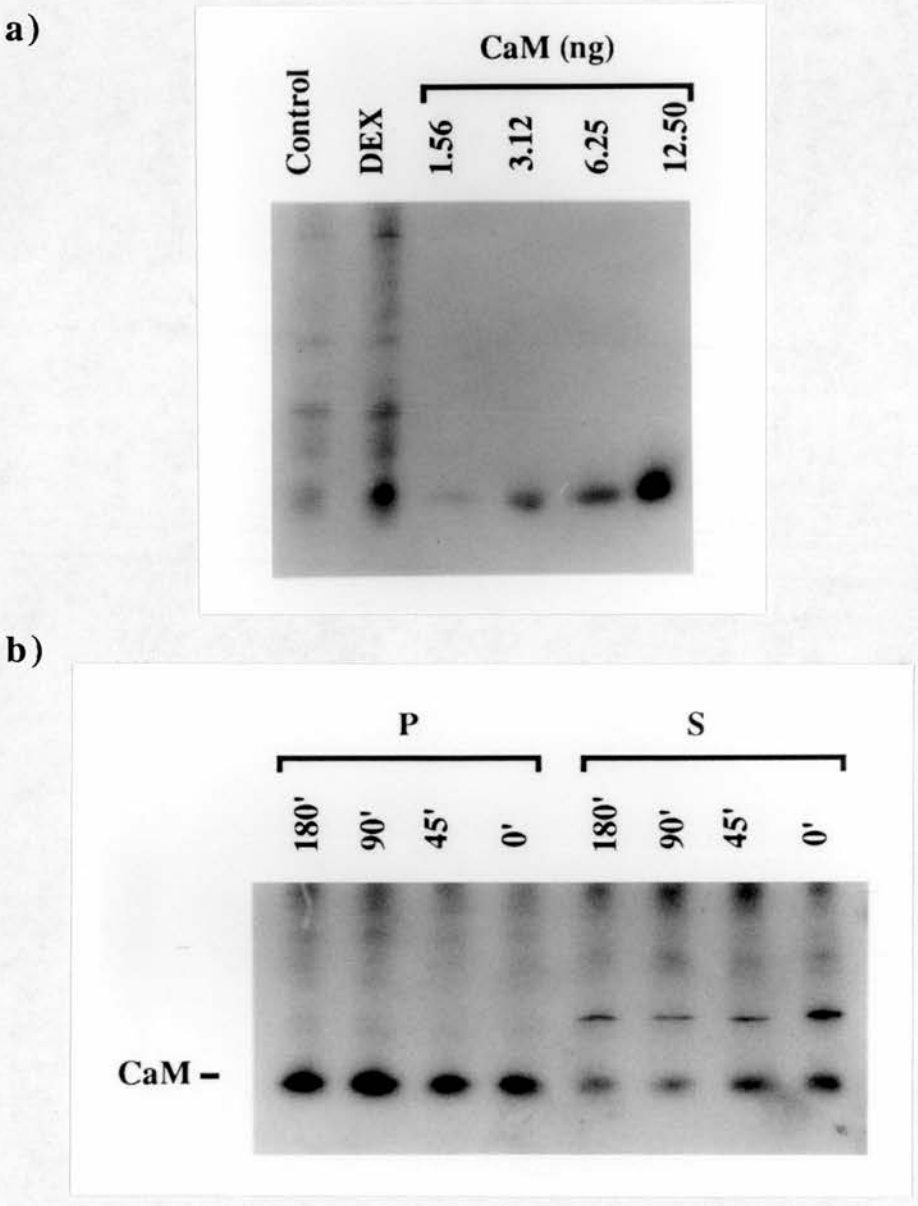


Figure 4.8: **a)** AtT20 D16:16 cells were incubated in the presence (DEX) or absence (Control) of 10nM dexamethasone for 90min. Calcium precipitated extracts (1µg/lane) and bovine calmodulin standards (CaM, 1.56-12.50ng/lane) were run in parallel. **b)** AtT20 D16:16 cells were incubated with 100nM dexamethasone for the stated time periods (min). Crude supernatant (S) and pellet (P) samples contained 2µg/lane. All samples were electrophoresed on a 10-15% SDS-PAGE Phastgel, electroblotted to Immobilon PVDF and probed for calmodulin, using a mouse monoclonal antibody at 1µg/ml. ^{125}I-labelled anti-mouse IgG was used as second antibody, autoradiographs were exposed for 3days.

Calmodulin was detectable in both crude supernatant and pellet fractions from AtT20 D16:16 cells isolated as described in section 2.5.2 except that 2mM EGTA was included in the homogenization buffer. In the crude supernatant fraction a slight decrease (maximally 30-40% at 90min) was observed on increasing exposure to 100nM dexamethasone (Figure 4.8b). Cross reaction with a protein band at ~36Kd was also observed in crude supernatant fractions. Calmodulin protein levels in the pellet fraction were maximally elevated (mean 2.8 fold above control in two experiments) after 90min of 100nM dexamethasone exposure (4.8b)

In summary, glucocorticoids induce the mRNA and protein encoding the calcium receptor protein, calmodulin, in the time-scale of early inhibition in AtT20 D16:16 corticotrophs. Furthermore, dexamethasone may also promote redistribution of calmodulin from the cytosol to membrane structures.

4.3.3 *Other mRNAs induced by glucocorticoids in the mouse T-lymphocyte cell line, WEHI-7TG*

No expression of an mRNA hybridizing to clone 4.2, a putative G-protein linked receptor clone related to the tachykinin receptor family in the WEHI-7TG cell line (Harrigan, Campbell & Bourgeois,1991), was observed in total RNA extracts from AtT20 D16:16 cells even after 90min of 10nM dexamethasone treatment. Two cDNA clones encoding early glucocorticoid-induced proteins of unknown function from the mouse T-lymphocyte cell line, WEHI-7TG (clones 58 and 213 respectively cited in Baughman *et al.*,1991 and Harrigan *et al.*,1989) were used to probe Northern blots of total RNA from AtT20 D16:16 cells. Filter hybridizations were kindly performed by Drs G Baughman and S Bourgeois, Salk Institute, San Diego, California, USA. Both clones were constitutively expressed and slightly induced (maximally 2 fold after 45min) in AtT20 D16:16 cells treated with 10nM dexamethasone (Figure 4.9). These

data demonstrate that common, as well as cell-specific, mRNAs are induced by glucocorticoids in the early time domain.

In summary, glucocorticoids induce the accumulation of the Ca^{2+}-binding protein, calmodulin, but not lipocortin I or chromogranin A in the time-scale of early inhibition in AtT20 D16:16 cells. Furthermore, several other unidentified mRNAs were induced by glucocorticoids and await further characterization.

Figure 4.9

Northern blotting for the glucocorticoid-induced clones 58 and 213, derived from the WEHI-7TG lymphocyte cell line, in AtT20 D16:16 cells

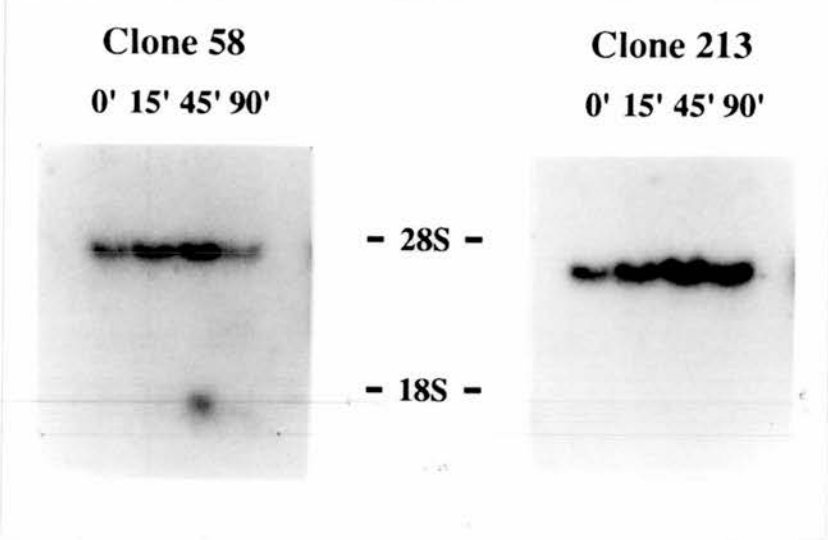


Figure 4.9: AtT20 D16:16 cells were treated with 10nM dexamethasone for various times as indicated (min). Total RNA (20μg/lane) was hybridized with (α^{32}P) dCTP labelled EcoRI fragments of cDNAs encoding clones 58 and 213 respectively derived from the lymphocyte cell line, WEHI-7TG. Blots were hybridized and washed as described in section 2.7.4 except that RNA was fixed to nylon (Hybond-N, Amersham) membranes. Equivalent RNA loading was assessed by ethidium bromide staining (not shown).

4.4 Attempts to identify novel glucocorticoid-induced mRNAs and proteins in AtT20 D16:16 corticotrophs

4.4.1 *Subtraction hybridization screening of a cDNA library constructed from dexamethasone-induced AtT20 D16:16 cells*

Subtraction hybridization screening of cDNA libraries has proved a powerful tool in the characterization of differentially expressed mRNAs and was used to identify glucocorticoid-induced mRNAs in AtT20 D16:16 cells. Two representative cDNA libraries (control and 10nM dexamethasone treated for 90min) containing $>1x10^6$ independent clones with average insert size >1kbp were constructed using the Uni–ZAP-XR™ cloning vector. Screening of 50,000 independent clones from the unamplified dexamethasone-induced library revealed 394 positive clones hybridizing to a subtracted cDNA probe enriched for dexamethasone-induced cDNA (see section 2.7.2 and 2.8.8 for methodology). Rescreening of these primary screen positive plaques using differential hybridization (see section 2.8.8) revealed 30 plaques that strongly hybridized only to cDNA derived from dexamethasone treated cells. The signal from the remaining plaques was not detectable above background using this differential screening method. For representative autoradiographs of duplicate plaque filter lifts used in secondary screening see Figure 4.10.

Preliminary characterization of these secondary screen positive plaques revealed multiple cDNA clones within a single plaque. Further plaque purification and screening is required before sequencing and complete characterization of these dexamethasone-induced cDNAs. However, at least two plaques from the 30 secondary positive plaques hybridised with a cDNA probe encoding mouse calmodulin (clone 21 cited in Baughman *et al.*,1991 and Harrigan *et al.*,1989),

suggesting induction of calmodulin mRNA in the time-course of early glucocorticoid inhibition in AtT20 D16:16 cells.

4.4.2 *2-D gel electrophoresis of Ca^{2+}-precipitated, ^{35}S-methionine- and ^{32}Phosphate - labelled, proteins*

Experiments were designed to analyse glucocorticoid-induced proteins using two dimensional gel electrophoresis of proteins metabolically labelled with ^{35}S-methionine that incorporates into newly synthesized protein. After first dimension isoelectric focusing, over 1,000 proteins could be resolved using 10cm x 16cm second dimension SDS-PAGE gels, however, no reproducible difference between control (Figure 4.11a) and dexamethasone treated (Figure 4.11b) protein extracts could be determined during the time-scale in which the inhibitory action of dexamethasone is maximal (see section 4.2.1 and Woods *et al.*,1992). Furthermore, no consistent induction of protein(s) by dexamethasone was observed using Ca^{2+}-precipitated protein extracts (Figures 4.12a and 4.12b for control and dexamethasone treated cells respectively) or phosphoprotein (^{32}Phosphate labelled) extracts (Figures 4.13a and 4.13b for control and dexamethasone treated cells respectively). Further analysis using this methodology would require 'giant' 2D-gel electrophoresis (typically 100cm x 100cm second dimension gels) (Colbert & Young,1986a; Colbert & Young,1986b) for resolution of glucocorticoid-induced proteins and was not pursued further in this study.

Induction of calmodulin protein was undetectable using 2D-gel electrophoresis however, this is probably a consequence of the highly acidic nature of the protein. Several workers have reported loss of the protein from gels during the routine overnight fixation and washing procedures used in this study (McKeon & Lyman,1991) (see section 2.6.1), furthermore re-examination of all 2D gels revealed a

wide variation in signal intensity between control samples for proteins of similar electrophoretic mobility as reported for calmodulin.

In summary, cDNA technology revealed several putative glucocorticoid-induced mRNAs in AtT20 D16:16 corticotrophs. Further work is required to isolate and characterize these mRNAs however dexamethasone-induced clones corresponding to calmodulin were identified.

Figure 4.10

Representative autoradiographs of differential hybridization screening of 'dexamethasone-induced' cDNA clones from an AtT20 D16:16 cDNA library

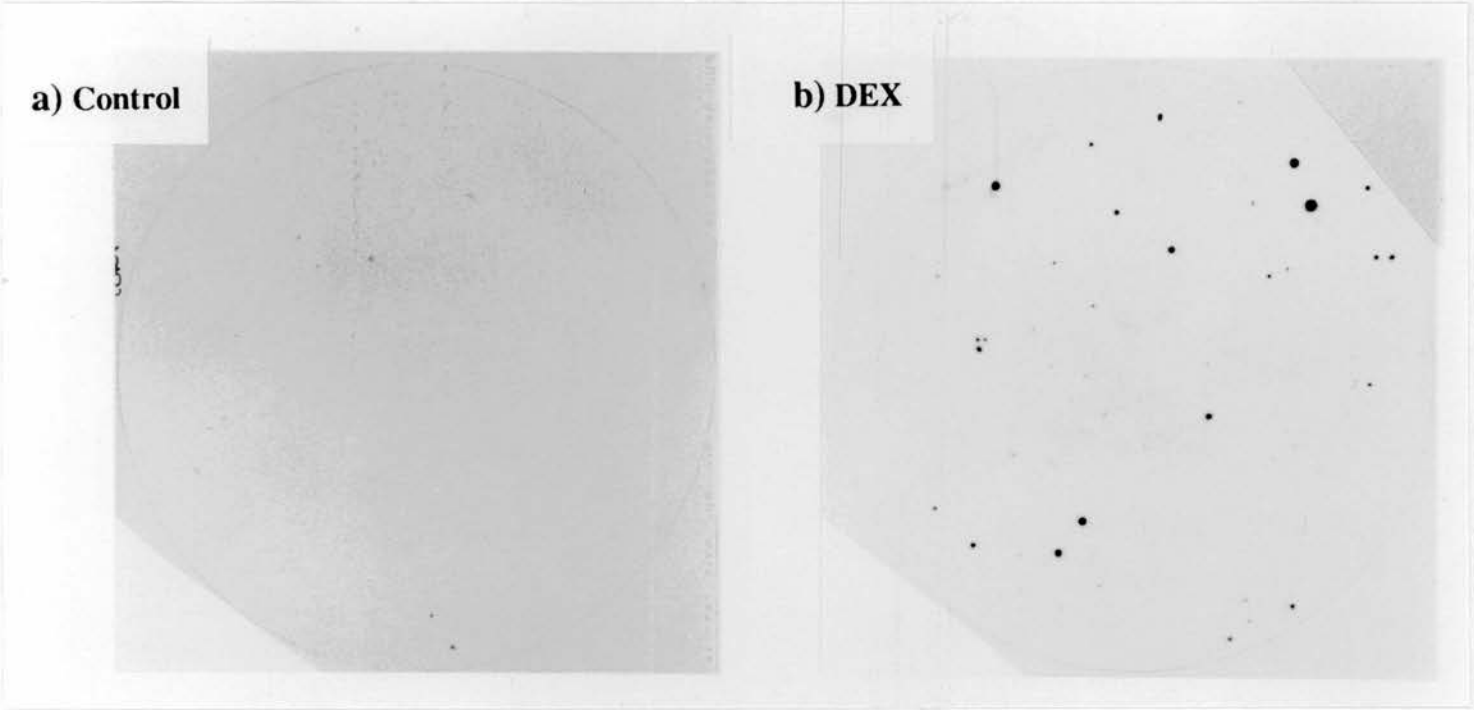


Figure 4.10: The 394 first round screen positive clones from the primary cDNA library of dexamethasone-induced AtT20 D16:16 cells hybridizing to a subtracted cDNA probe enriched for dexamethasone-induced sequences (see section 4.2.3) were replated and duplicate plaque filter lifts taken. Filters were hybridized with total (α^{32}P) dCTP labelled cDNA probes generated from either control **a)** or dexamethasone treated (90min, 10nM) **b)** AtT20 D16:16 mRNA as described in section 2.8.8. Filters were hybridized as described in section 2.8.8, autoradiographs were exposed for 3days.

Figure 4.11a

Two-dimensional gel analysis of ^{35}S-methionine incorporation into newly synthesized cellular proteins in control AtT20 D16:16 cells

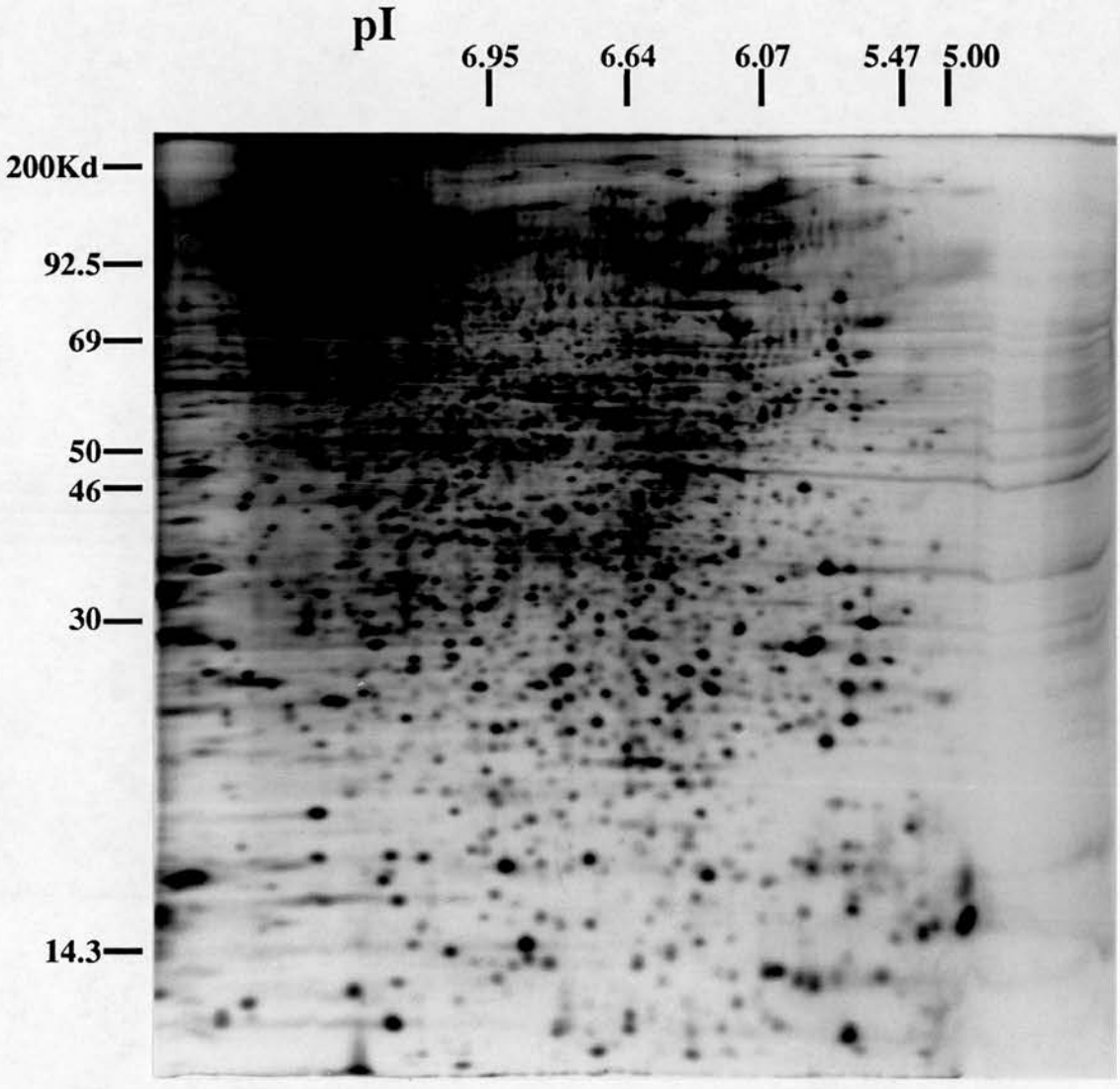


Figure 4.11a: Incorporation of ^{35}S-methionine into newly synthesized proteins in control AtT20 D16:16 cells was performed as described in section 2.5.5. Protein samples containing equal TCA precipitable cpm were loaded and electrophoresed by IEF in the first dimension followed by SDS-PAGE electrophoresis in the second dimension. The pH and molecular weight calibrations are shown. The gel is representative of six control samples run in parallel with six dexamethasone treated samples (see Figure 4.11b for comparison with dexamethasone treated samples). Gels were dried and exposed to ß-max hyperfilm at -70°C with intensifying screens for 4 days.

Figure 4.11b

Two-dimensional gel analysis of ^{35}S-methionine incorporation into newly synthesized cellular proteins in dexamethasone treated AtT20 D16:16 cells

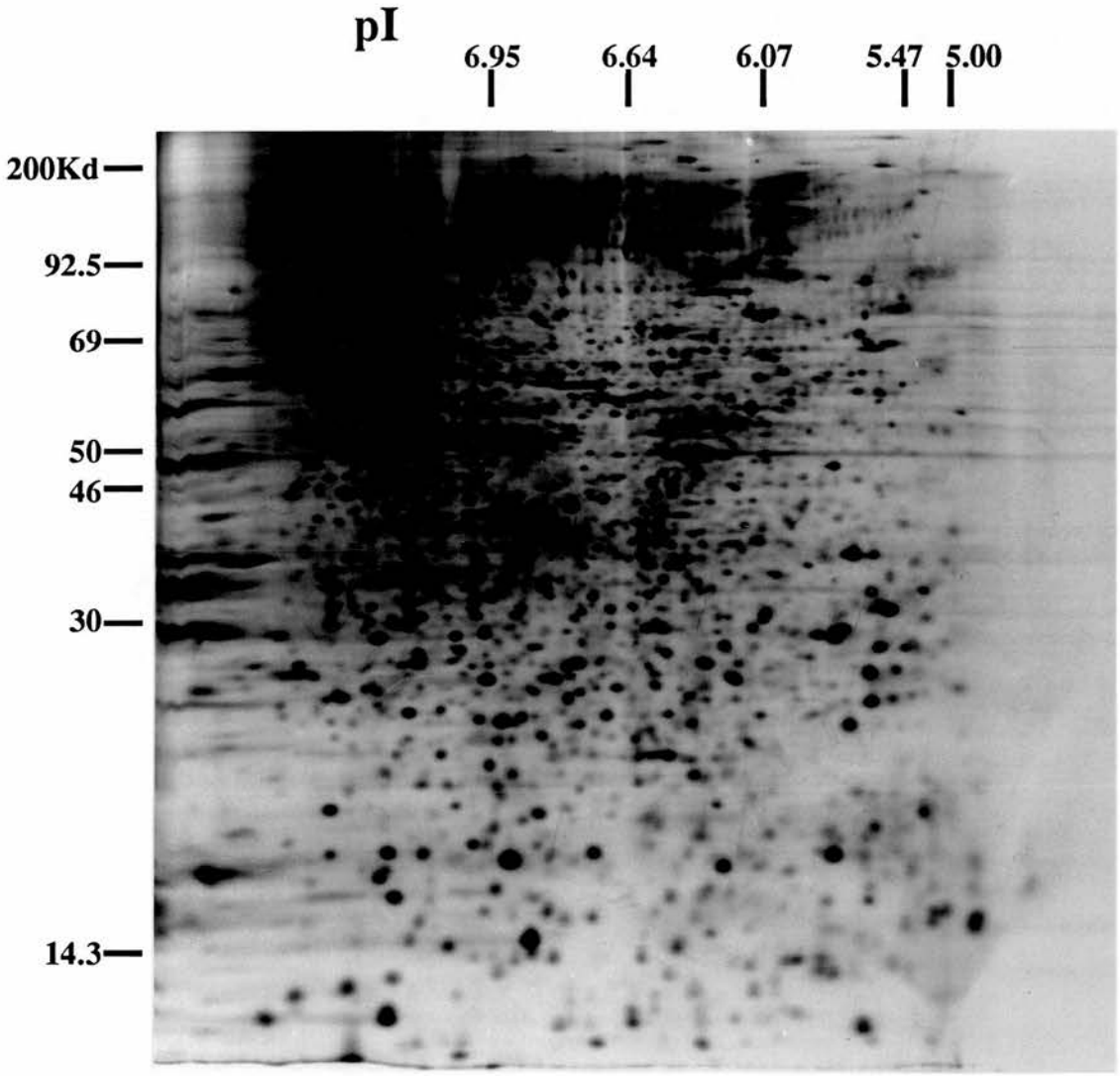


Figure 4.11b: Incorporation of ^{35}S-methionine into newly synthesized proteins in AtT20 D16:16 cells treated with 10nM dexamethasone for 90min was performed as described in section 2.5.5. Protein samples containing equal TCA precipitable cpm were loaded and electrophoresed by IEF in the first dimension followed by SDS-PAGE electrophoresis in the second dimension. The pH and molecular weight calibrations are shown. The gel is representative of six dexamethasone treated samples run in parallel with six control samples (see Figure 4.11a for comparison with control samples). Gels were dried and exposed to ß-max hyperfilm at -70°C with intensifying screens for 4 days.

Figure 4.12a

Two-dimensional gel analysis of Ca^{2+}-precipitated proteins from control AtT20 D16:16 cells

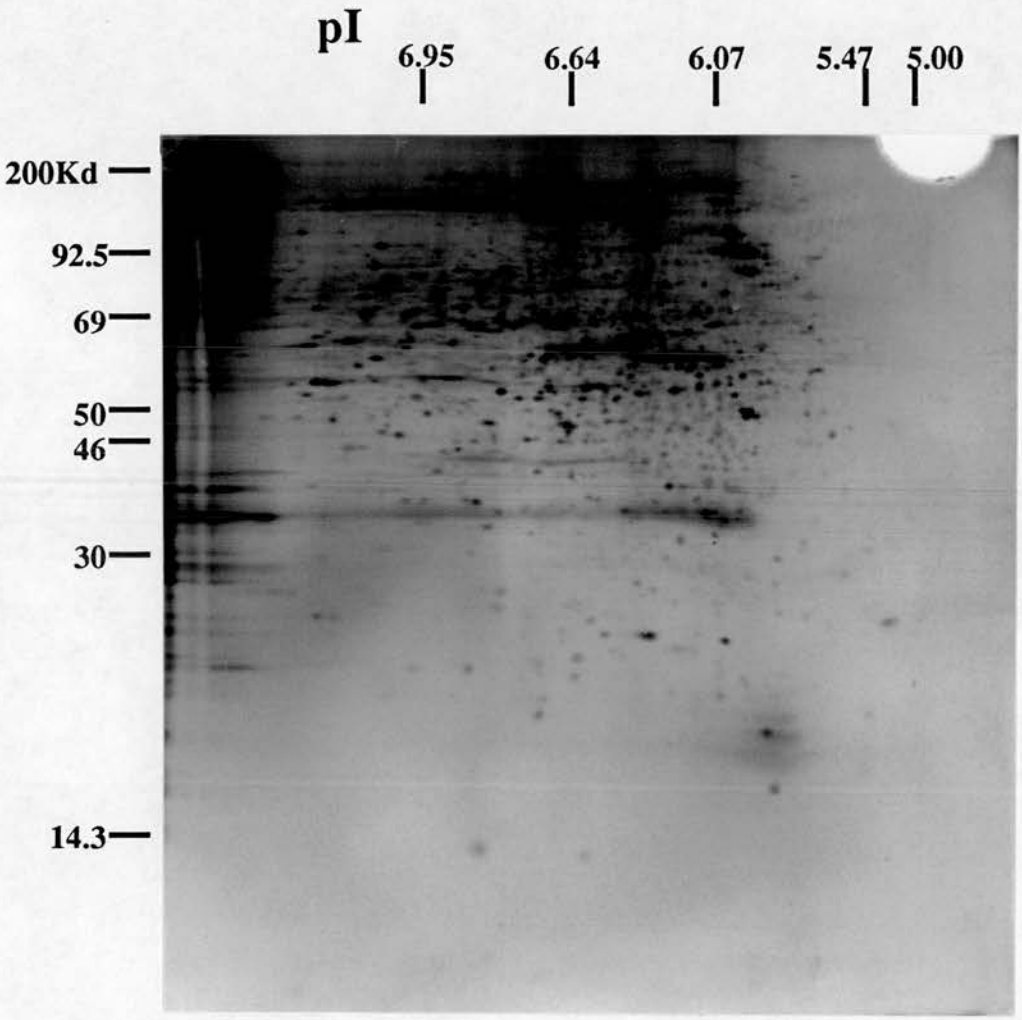


Figure 4.12a: Isolation of Ca^{2+}-precipitated proteins from control AtT20 D16:16 cells was performed as described in section 2.5.3. Equivalent amounts of protein were loaded and electrophoresed by IEF in the first dimension followed by SDS-PAGE electrophoresis in the second dimension. The pH and molecular weight calibrations are shown. The gel is representative of six control samples run in parallel with six dexamethasone treated samples (see Figure 4.12b for comparison with dexamethasone treated samples). Proteins were detected using the Pierce silver staining procedure as described in section 2.5.3.

Figure 4.12b

Two-dimensional gel analysis of Ca^{2+}-precipitated proteins from dexamethasone treated AtT20 D16:16 cells

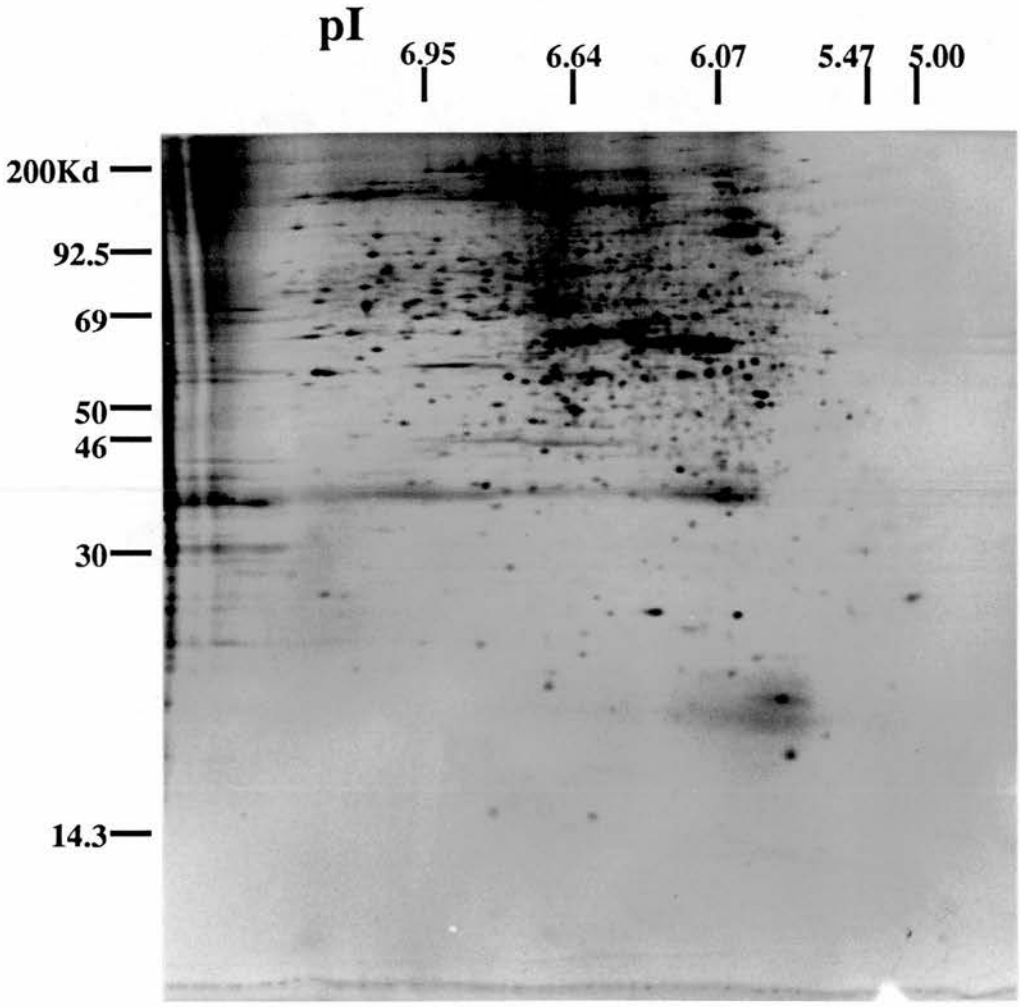


Figure 4.12b: Isolation of Ca^{2+}-precipitated proteins from dexamethasone treated (10nM, 90min) AtT20 D16:16 cells was performed as described in section 2.5.3. Equivalent amounts of protein were loaded and electrophoresed by IEF in the first dimension followed by SDS-PAGE electrophoresis in the second dimension. The pH and molecular weight calibrations are shown. The gel is representative of six dexamethasone treated samples run in parallel with six control samples (see Figure 4.12a for comparison with control samples). Proteins were detected using the Pierce silver staining procedure as described in section 2.5.3.

Figure 4.13a

Two-dimensional gel analysis of ^{32}Phosphate incorporation into total cellular protein of control AtT20 D16:16 cells

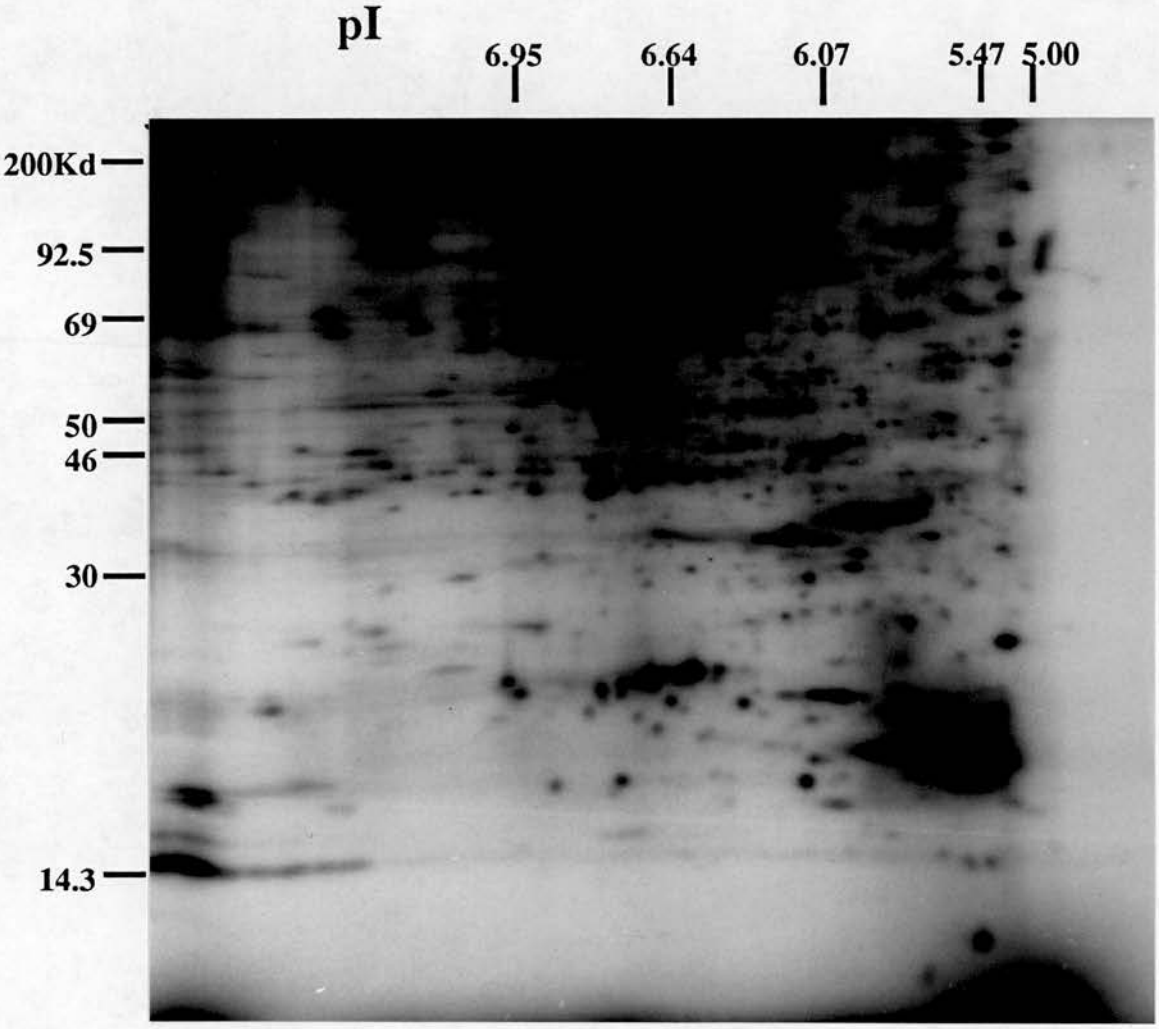


Figure 4.13a: Incorporation of ^{32}Phosphate into total protein from AtT20 D16:16 cells was performed as described in section 2.5.4. Protein samples containing equal TCA precipitable cpm were loaded and electrophoresed by IEF in the first dimension followed by SDS-PAGE electrophoresis in the second dimension. The pH and molecular weight calibrations are shown. The gel is representative of six control samples run in parallel with six dexamethasone treated samples (see Figure 4.13b for comparison with dexamethasone treated samples). Gels were dried and exposed to ß-max hyperfilm at -70°C with intensifying screens for 4 weeks.

Figure 4.13b

Two-dimensional gel analysis of ^{32}Phosphate incorporation into total cellular protein of dexamethasone treated AtT20 D16:16 cells

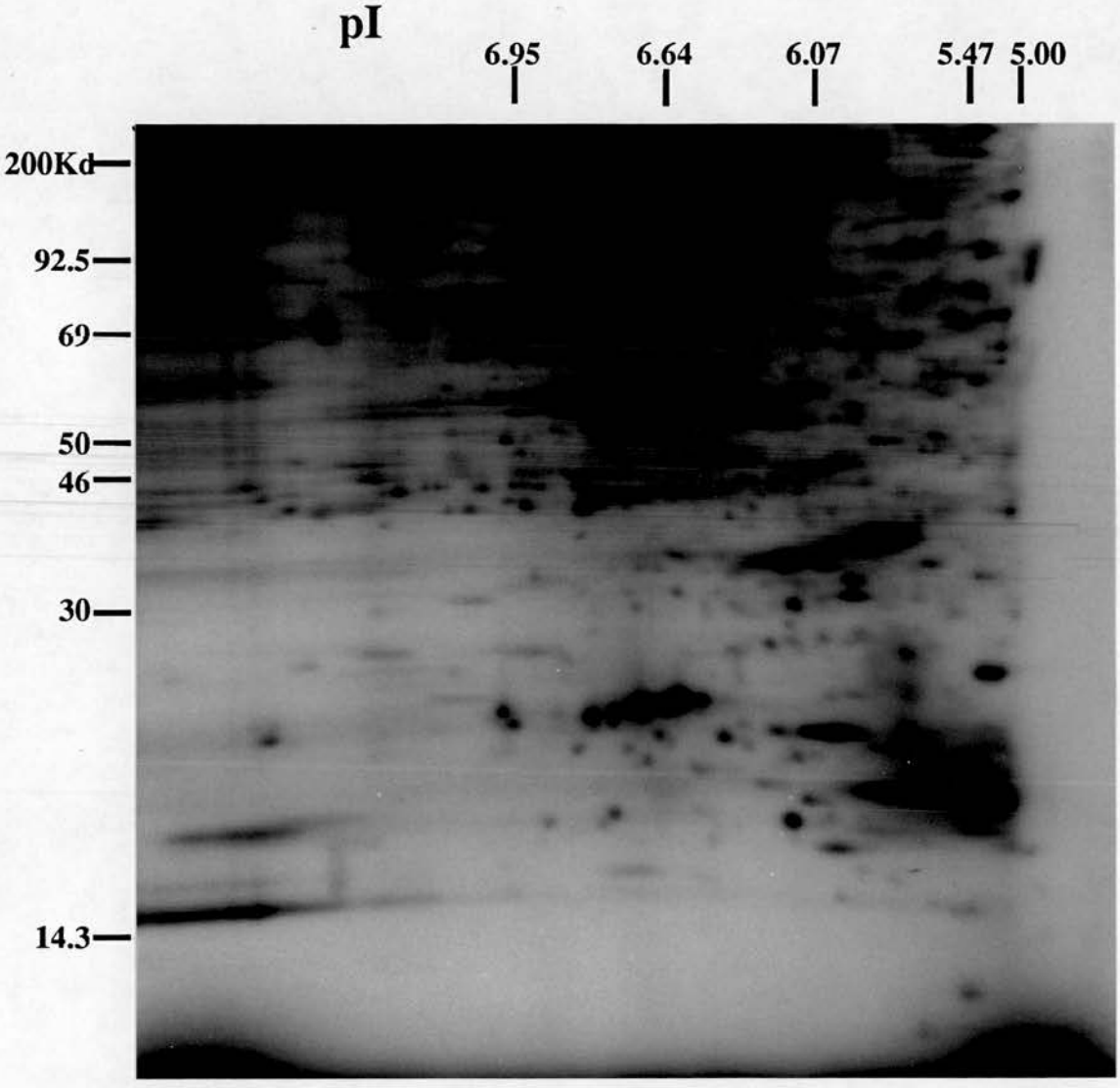


Figure 4.13b: Incorporation of ^{32}Phosphate into total protein from AtT20 D16:16 cells treated for 90min with 10nM dexamethasone was performed as described in section 2.5.4. Protein samples containing equal TCA precipitable cpm were loaded and electrophoresed by IEF in the first dimension followed by SDS-PAGE electrophoresis in the second dimension. The pH and molecular weight calibrations are shown. The gel is representative of six dexamethasone treated samples run in parallel with six control samples (see Figure 4.13a for comparison with control samples). Gels were dried and exposed to ß-max hyperfilm at -70°C with intensifying screens for 4 weeks.

4.5 Discussion

The results presented in this section, combined with further data from this laboratory (Woods *et al.*,1992), suggest that the mouse corticotroph cell line AtT20 D16:16 retains the fundamental hallmarks of early inhibition: onset within 2h, activation of type II glucocorticoid receptors and mRNA and protein induction, and is thus a valid model in which to characterize early glucocorticoid-induced proteins. Furthermore, CRF-41-inactivation of early inhibition demonstrated in AtT20 D16:16 cells as for normal corticotrophs, may be a useful tool with which to identify proteins involved in early glucocorticoid inhibition.

With regards to the nature of the glucocorticoid-induced protein(s), lipocortin (annexin) I mRNA or protein was not detectable or induced in AtT20 D16:16 cells at a time when the early inhibitory effect of glucocorticoids is maximal. Importantly, accumulation of mRNA and protein encoding the calcium receptor protein, calmodulin, was observed within the time-scale of early inhibition. Furthermore, CRF-41 blocked glucocorticoid-induced accumulation of calmodulin mRNA. Complimentary DNA technology techniques revealed several other glucocorticoid-induced mRNAs in AtT20 D16:16 cells. These mRNAs require further characterization.

4.5.1 *Early glucocorticoid inhibition in AtT20 D16:16 cells*

Because corticotrophs represent only 5% of the anterior pituitary gland cell population and the fact that virtually all cells in the anterior pituitary gland are responsive to glucocorticoids a homogenous corticotroph cell model is required to characterize the molecular mechanisms of early glucocorticoid inhibition.

The results presented in section 4, combined with further work in this laboratory (Woods *et al.*,1992), indicates that the AtT20 D16:16 cell line retains the fundamental hallmarks of early glucocorticoid inhibition observed in normal corticotrophs. The mechanism of early glucocorticoid inhibition in AtT20 D16:16 cells does not involve suppression of CRF-41-stimulated cAMP production. Whether glucocorticoids suppress absolute cAMP levels by phosphodiesterase activation could not be determined in these studies as CRF-41-stimulated cAMP accumulation was only observed when the phosphodiesterase inhibitor, IBMX, was present in the incubation medium. However, the majority of evidence to date in normal as well as AtT20 corticotrophs suggests that the site of early inhibition is distal to second messenger production (cAMP or IP_3/DAG for CRF-41 and AVP respectively) (Abou-Samra *et al.*,1986a; Abou-Samra *et al.*,1986b; Antoni *et al.*,1992a; Miyazaki *et al.*,1984; Phillips & Tashjian,1982; Woods *et al.*, 1992).

CRF-41 pretreatment blocked early glucocorticoid inhibition in AtT20 D16:16 cells. In contrast PdBu, that activates the protein kinase C pathway, could not block early glucocorticoid inhibition of CRF-41-stimulated ACTH release. Taken together these results parallel the data obtained in section 3 using normal rat corticotrophs in a perifusion model. The observed desensitization of CRF-41-stimulated ACTH release after pretreatment with PdBu, and higher doses of CRF-41 is probably through heterologous and homologous desensitization of CRF-41 receptor/postreceptor systems respectively rather than depletion of ACTH stores (Hoffman *et al.*,1985). CRF-41-inactivation should prove useful in identification of proteins involved in early inhibition of stimulated ACTH release. Moreover, the AtT20 D16:16 cell line should serve as a model in which to examine the molecular interactions between the glucocorticoid and cAMP/protein kinase A activated intracellular pathways.

4.5.2 *No evidence for involvement of lipocortin (annexin) I or chromogranin A in early glucocorticoid inhibition in AtT20 D16:16 cells*

It was of interest to examine whether lipocortin (annexin) I, the purported mediator of early glucocorticoid action in macrophages and other systems (for reviews see Flower,1988; Peers & Flower,1990), is involved in early glucocorticoid inhibition of stimulated ACTH release from the the model corticotroph cell line AtT20 D16:16. Lipocortin I protein or mRNA could not be detected in AtT20 D16:16 cells even after dexamethasone pretreatment. Although a recent preliminary report has suggested that a N-terminal fragment of lipocortin 1 mimics dexamethasone inhibition of hypothalamic extract stimulated ACTH release in rat pituitary segments in a static incubation assay, the lipocortin 1 fragment had no inhibitory action in AtT20 D16:16 cells (Taylor, Antoni, Croxtall *et al.*, 1992). Furthermore, although lipocortin I is present in the anterior pituitary gland, as well as the D16v and D1 strains of the AtT20 cell line, no evidence for its induction by glucocorticoids has been demonstrated in these systems (Woods, Kiss, Smith *et al.*,1990). Work on the the role of lipocortin I in other systems has also cast doubt on its suggested primary and generic function as a mediator of glucocorticoid action (for example see Brönnegard, Andersson, Edwall *et al.*,1988; Wong, Frost & Nick,1991).

Chromogranin A, a secretory vesicle associated protein (Deftos,1991; Huttner, Gerdes & Rosa,1991; Simon & Aunis,1989), has been implicated as a glucocorticoid responsive paracrine inhibitor in slow (>24h) inhibition of ACTH release in AtT20 D16v cells (Wand, Takiyyuddin, O'Connor & Levine,1991). Proteolytic fragments of chromogranin A also inhibit secretion in other secretory cells such as adrenal chromaffin cells (Galindo, Rill, Bader & Aunis,1991; Simon, Bader & Aunis,1988). No induction of two major chromogranin A immunoreactive protein bands in AtT20

D16:16 cells was observed in the time-interval of early inhibition in this system. Furthermore, chromogranin A mRNA and protein is only expressed in gonadotrophs of normal rat anterior pituitary even after glucocorticoid treatment (Fischer-Colbrie, Wohlfarter, Schmid *et al.*,1988; Grino, Wohlfarter, Fischer-Colbrie & Eiden,1989).

The nature and function of other unidentified glucocorticoid-induced mRNAs reported in this study using: 1) subtraction hybridization screening of AtT20 D16:16 cDNA libraries and; 2) Northern blotting of AtT20 D16:16 mRNA with probes from the glucocorticoid-treated mouse lymphocyte cell line, WEHI-7TG (Baughman *et al.*,1991; Harrigan *et al.*,1989), require further evaluation.

4.5.3 *Early glucocorticoid induction of calmodulin and its suppression by CRF-41 in AtT20 D16:16 cells*

Dexamethasone stimulated accumulation of a single calmodulin mRNA species (~1.6kb) maximally 10 fold with a concomitant elevation (~2 fold) in calmodulin protein level within 90min of glucocorticoid application in AtT20 D16:16 cells. Subtraction hybridization screening of a cDNA library generated from dexamethasone treated AtT20 D16:16 cells identified several calmodulin cDNA clones confirming glucocorticoid induction of calmodulin mRNA. Accumulation of calmodulin mRNA was dependent on ongoing protein synthesis. Whether the induction requires constitutive expression of rapid turnover proteins, as reported for the rat α_1-acid glycoprotein gene (Klein, Reinke, Feigelson & Ringold,1987), or is dependent on glucocorticoid-induction of additional transcription factors could not be determined in these studies. The rapid and transient induction of mRNAs (maximal within 45min, reduced by 90min) in AtT20 D16:16 cells complimentary to clones 58 and 213 from WEHI-7TG cells (Baughman *et al.*,1991; Harrigan *et al.*,1989) would suggest that glucocorticoids in fact activate a 'cascade' of potential transcription factor genes. In

support of this hypothesis Lin & co-workers recently reported that glucocorticoids rapidly induce the transcription factor c-fos in AtT20 D1 cells (Lin *et al.*,1992). Potential glucocorticoid as well as cAMP and AP-2 response elements have been identified in the 5' promoter region of the rat calmodulin II gene indicating, in accordance with the findings described above, that interactions between multiple transcription factors are involved in the regulation of calmodulin gene expression. The promoter regions of other calmodulin genes, including those encoding the mouse calmodulin genes have not been fully characterized (Nojima,1989).

As CRF-41 pretreatment prevents early glucocorticoid inhibition as well as glucocorticoid-induced calmodulin mRNA accumulation in AtT20 D16:16 cells the data strongly suggest that calmodulin is an essential component of early glucocorticoid inhibition. The pleiotropic action of calmodulin (see section 4.5.4) along with functional evidence from this laboratory, implicating glucocorticoid-suppression of intracellular free Ca^{2+} levels and enhancement of K^+ currents in this cell line (Antoni *et al.*,1992a; Pennington *et al.*,1992), are consistent with this hypothesis.

Studies using calmodulin antagonists have implicated calmodulin in the mechanism of CRF-41- as well as AVP-stimulated ACTH secretion (Murakami, Hashimoto & Ota,1985). However, these calmodulin antagonists are non-specific and also block a wide range of cellular enzymes *cf* (Klee & Vanaman,1982) precluding the direct evaluation of these results.

In normal rat anterior pituitary, no induction of calmodulin mRNA (major mRNAs at ~1.6kb and ~4.2kb) was observed after dexamethasone pretreatment. However, glucocorticoids have divergent effects on different cells of the anterior pituitary gland (for example see Borski, Helms, Richman III & Grau,1991; Briski & Sylvester,1991; Suter & Schwartz,1985). Corticotrophs represent only 5-10% of the total anterior

pituitary cell population and thus induction of calmodulin mRNA confined to corticotrophs may be masked by the constitutive expression of calmodulin in other cell types. Furthermore, although only one isoform of calmodulin protein exists in all mammalian cells the protein is encoded by several independent genes (at least 3 in rat and 2 in mouse) with multiple mRNA transcripts (Bender, Dedman & Emerson Jr,1988; Fischer, Koller, Flura *et al.*,1988; Nojima,1989; Nojima & Sokabe,1987). It is likely that each calmodulin gene has a separate function, for example a housekeeping gene to maintain constitutive calmodulin expression and regulated genes to respond to changes in homeostasis. Development of transcript-specific probes for *in situ* hybridization studies are required to examine glucocorticoid regulation of calmodulin mRNAs expressed exclusively in normal rat corticotrophs.

4.5.4 *Is calmodulin a generic mediator of early glucocorticoid action?*

Calmodulin is an ubiquitous, highly conserved, EF-hand, 17Kd acidic calcium binding protein that constitutes approximately 0.2% of total cellular protein in anterior pituitary cells. Calmodulin acts as a multi-functional Ca^{2+} receptor/effector system that has been implicated in a wide range of physiological processes including secretion, cell growth and cell motility through modulation of a wide range of intracellular proteins including cyclic nucleotide phosphodiesterases, adenylate cyclase, calcium-transport enzymes, cytoskeletal proteins, metabolic enzymes and ion channels (for reviews see Harper,1988; Klee & Vanaman,1982; Manalan & Klee,1984; Means,1988; Wang, Pallen, Sharma *et al.*,1985).

The demonstration that CRF-41 blocks both early glucocorticoid inhibition of CRF-41-stimulated ACTH secretion as well as glucocorticoid-induction of calmodulin mRNA in AtT20 D16:16 corticotrophs strongly supports the hypothesis that calmodulin is involved in early glucocorticoid inhibition at the corticotroph. In several

lymphocyte-derived models of early inhibition glucocorticoids stimulate accumulation of calmodulin (for example see Dowd *et al.*,1991; Harrigan *et al.*,1989). Interestingly, calmodulin displays the same electrophoretic mobility in 2D-gel electrophoresis (IEF and SDS-PAGE) as the glucocorticoid-induced protein, glucocortin, proposed as a generic mediator of glucocorticoid action (Colbert & Young,1986a; Colbert & Young,1986b). Sequencing of glucocortin is required to verify that this protein is indeed calmodulin.

Functional evidence from this laboratory, implicating early glucocorticoid suppression of intracellular free Ca^{2+} responses and enhancement of K^+ currents (Figure 4.14) are consistent with the known properties of calmodulin (see above). Glucocorticoid suppression of intracellular Ca^{2+} signals has been proposed in other models of early glucocorticoid inhibition such as B-lymphocytes (Dennis *et al.*,1987), basophilic leukaemia cells (Her *et al.*,1990) and pancreatic islet ß-cells (Billaudel *et al.*,1984). Interestingly, Epstein & co-workers recently reported that early glucocorticoid suppression of pancreatic insulin release could be mimicked by over-expression of a calmodulin transgene (intracellular calmodulin protein levels were elevated 5fold) in mouse pancreatic ß-cells. This inhibition was also present in transgenes expressing a mutated calmodulin gene that sequestered Ca^{2+} but did not allow activation of calmodulin dependent proteins suggesting that calmodulin acts as a Ca^{2+} sink in this system (Epstein, Ribar, Decker *et al.*,1992). Furthermore, in other models of early inhibition, including CA1 hippocampal neurones and AtT20 D16:16 cells (Joëls & De Kloet,1989; Kerr *et al.*,1989; Pennington *et al.*,1992) glucocorticoids activate K^+ currents that may have an indirect effect on intracellular free Ca^{2+} levels.

Figure 4.14

Current model of early glucocorticoid inhibition in anterior pituitary corticotrophs

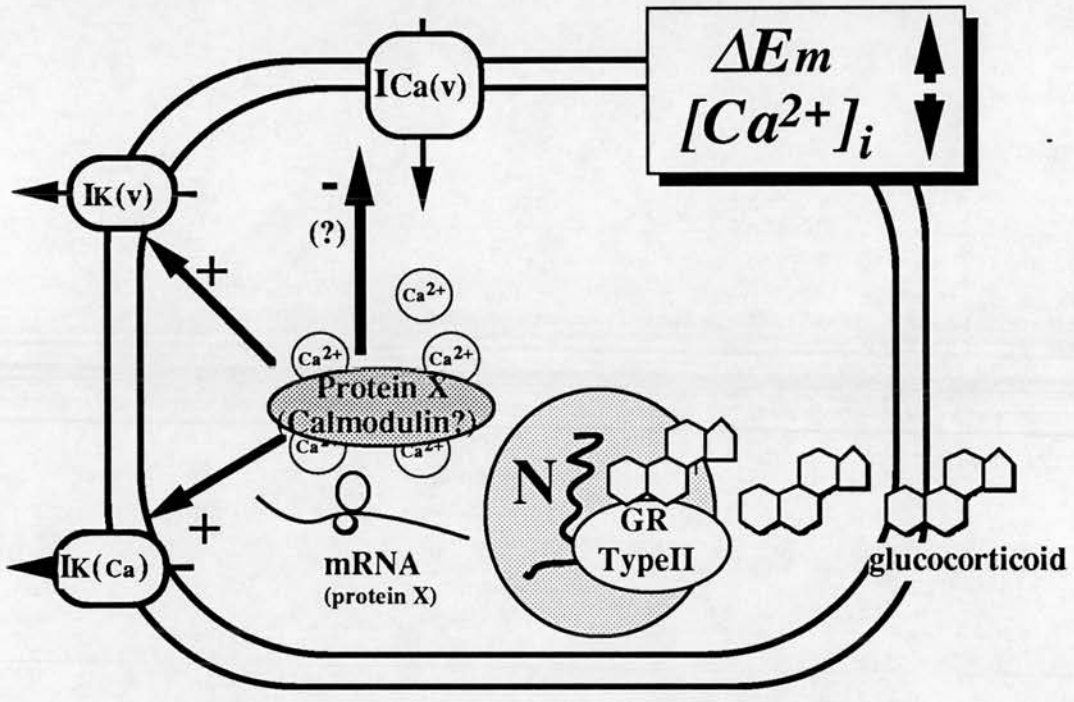


Figure 4.14: Activation of type II glucocorticoid receptors (GR TypeII) induces expression of glucocorticoid induced protein(s), protein X, that include calmodulin. The induced protein acts to inhibit stimulated hormone secretion by suppressing intacellular free calcium levels $[Ca^{2+}]i$ directly, perhaps by sequestration of intracellular Ca^{2+} or inhibition of Ca^{2+} influx, or indirectly by activating voltage (IK (v)) or Ca^{2+} dependent (IK(Ca)) potassium channels causing membrane hyperpolarisation (ΔEm).

Thus as Ca^{2+} is essential for cellular function, and calmodulin is a highly conserved protein, glucocorticoid-induction of calmodulin may act as a mediator of early inhibition in several systems. Whether calmodulin modulates K^+ currents, or acts as an intracellular Ca^{2+} sink remains to be explored. However, elevation of intracellular calmodulin levels probably restricts agonist-induced redistribution of intracellular free Ca^{2+} (see Figure 4.14) thus blocking activation of Ca^{2+}-dependent processes such as secretion.

4.6 Conclusion

The clonal AtT20 D16:16 mouse corticotroph cell line displays the major hallmarks of early glucocorticoid inhibition described for normal corticotrophs. Early inhibition requires activation of type II glucocorticoid receptors and induction of new mRNA and protein. Furthermore, CRF-41 inactivates early glucocorticoid inhibition of CRF-41-stimulated ACTH release. With respect to the nature of the induced protein(s) glucocorticoids elevated the mRNA and protein coding for the ubiquitous intracellular calcium binding protein, calmodulin, within the time-scale of early inhibition in AtT20 D16:16 cells. Moreover, CRF-41 blocked glucocorticoid-induced accumulation of calmodulin mRNA implicating this intracellular calcium receptor protein in the mechanism of early glucocorticoid inhibition. Importantly, glucocorticoid-induction of the calcium binding proteins lipocortin (annexin) I and chromogranin A could not be detected in the time-scale of early glucocorticoid inhibition. Finally, cDNA technology revealed several unidentified mRNAs induced by glucocorticoids, further work is required to characterise these mRNAs.

5

Summary, conclusions and perspectives

5

SUMMARY, CONCLUSIONS AND PERSPECTIVES

5.1 Introduction

Two major lines of investigation were pursued in this thesis to characterize the mechanism and modulation of early (within 2h) glucocorticoid inhibition in the anterior pituitary corticotroph:

1) Perifused normal rat anterior pituitary gland segments were used as a model to characterize early glucocorticoid inhibition of ACTH released stimulated by the two major hypothalamic ACTH secretagogues, CRF-41 and AVP (section 3). Because the mechanism of early glucocorticoid inhibition is largely unknown, especially with regard to the nature of the induced proteins, it was hoped that analysis of the characteristics of early inhibition of CRF-41- and AVP-stimulated ACTH secretion would prove useful in subsequent characterization of glucocorticoid-induced proteins specifically involved in early inhibition.

2) The clonal mouse corticotroph cell line, AtT20 D16:16, was analyzed as a nominally homogenous corticotroph cell population model of early glucocorticoid inhibition that could be used for the characterization of glucocorticoid-induced proteins (section 4).

The experimental findings are discussed in detail in the relevant sections and are summarised below. The proposed direction of future research based on the results presented in this thesis are then presented.

5.2 Early glucocorticoid inhibition in anterior pituitary corticotrophs: differential inhibition of ACTH release induced by CRF-41 and AVP

In perifused rat anterior pituitary corticotrophs early glucocorticoid inhibition of CRF-41-, as well as AVP-, stimulated ACTH release requires activation of type II glucocorticoid receptors and induction of new mRNA and protein. Whether similar proteins mediate inhibition of hormone release stimulated by the two secretagogues remains to be examined. Glucocorticoids inhibited the amount of ACTH released by either secretagogue, but had no effect on the time-course of the ACTH secretory response. The characteristics of early glucocorticoid inhibition were dependent on the nature of the secretagogue as well as the relative timing of glucocorticoid application. AVP-stimulated ACTH release was invariably suppressed by glucocorticoids. In contrast, CRF-41 inactivated early glucocorticoid inhibition of CRF-41-stimulated ACTH release when applied at the start of glucocorticoid exposure. CRF-41 inactivation of early glucocorticoid inhibition was mediated by cAMP. Qualitatively similar results were obtained in the mouse corticotroph cell line, AtT20 D16:16, in that CRF-41, but not phorbol ester, blocked early inhibition of CRF-41-stimulated ACTH release.

The precise mechanism of CRF-41 inactivation of early glucocorticoid inhibition is unknown. CRF-41 only blocks early glucocorticoid action when applied simultaneously with the start of steroid exposure, these timing characteristics suggest that CRF-41 blocks glucocorticoid gene transcription as depicted in Figure 5.1b. In support of this hypothesis, CRF-41 blocked glucocorticoid-induced calmodulin mRNA accumulation in AtT20 D16:16 cells. Whether CRF-41 blocks glucocorticoid receptor activation/translocation or activates additional transcription factors that

interfere with glucocorticoid-induced gene activation remains to be explored. Interestingly, the latter mechanism has been implicated in CRF-41 blockade of glucocorticoid suppression of POMC gene transcription in normal and AtT20 D16:16 corticotrophs (Autelitano & Sheppard,1992; Levin & Roberts,1991; Lundblad & Roberts,1988). The AtT20 D16:16 cell line appears to be a useful model in determining the mechanisms of glucocorticoid/secretagogue interaction in various time domains and levels of cellular control processes.

During the analysis of early glucocorticoid inhibition in perifused rat anterior pituitary corticotrophs it was noted that AVP and CRF-41 appear to mobilize distinct pools of ACTH. AVP may mobilize a rapid turnover (newly synthesized) pool of ACTH whereas CRF-41 activates a stored, slow turnover pool. Current evidence would suggest that these potentially distinct ACTH pools reside within a single cell.

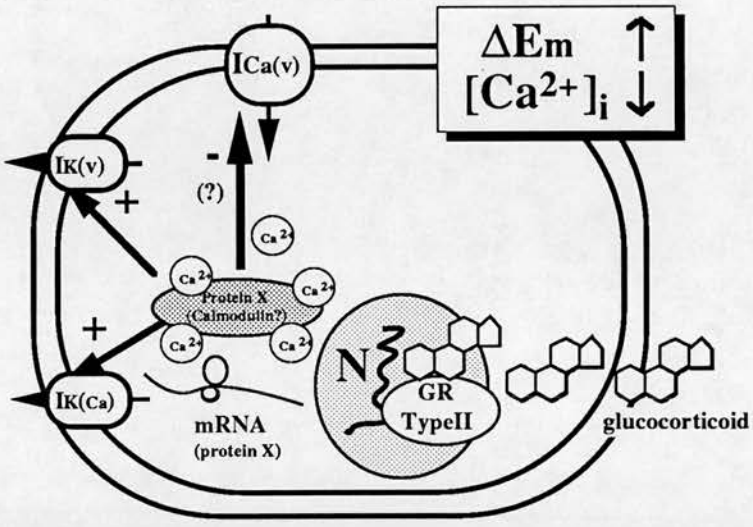
Secretagogue-dependent modulation of glucocorticoid action raises an important concept for the study of glucocorticoid action at other potential feedback sites in the HPA axis such as the hypothalamic AVP and CRF-41 containing neurones, as well as hippocampal neurones. Target cells that express glucocorticoid receptors are not necessarily prone to glucocorticoid inhibition, the presence or absence of other regulatory inputs may dictate the functional role of glucocorticoids at that site. Several workers have emphasized the importance of such stimulus context in relation to to glucocorticoid action (for example see De Kloet,1991 and Johnson *et al.*,1979).

The implications of differential modulation of early glucocorticoid inhibition *in vivo* is that stimuli that are primarily dependent upon CRF-41 as secretagogue may be relatively resistant to early inhibition whereas stimuli evoking AVP release will be invariably suppressed. Such regulation would allow fine tuning of the stress response to enable the organism to respond appropriately to diverse stressors.

Figure 5.1

Glucocorticoid / secretagogue interactions in anterior pituitary corticotrophs

a) Current model of early glucocorticoid inhibition



b) Proposed model of CRF-41 inactivation of early glucocorticoid inhibition

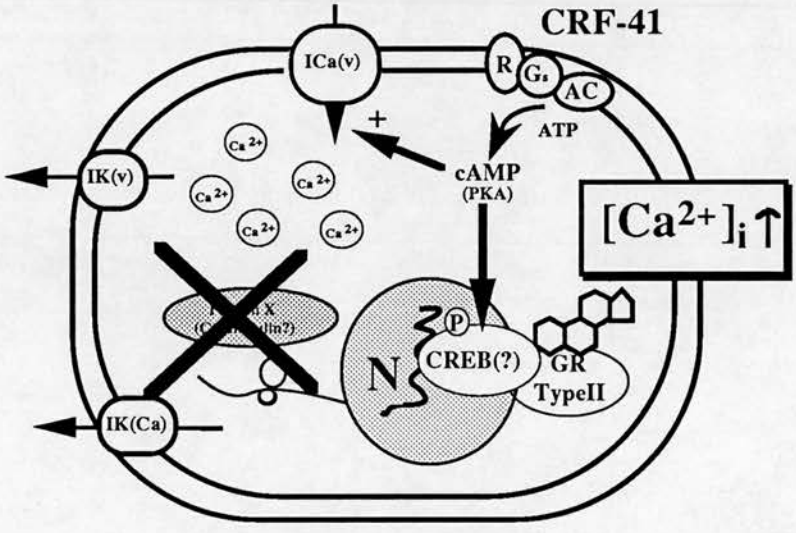


Figure 5.1: **a)** Activation of type II glucocorticoid receptors (GR TypeII) stimulates expression of glucocorticoid induced protein(s), protein X, that include calmodulin. The induced protein acts to inhibit stimulated hormone secretion by suppressing intacellular free calcium levels $[Ca^{2+}]i$ directly, perhaps by sequestration of intracellular Ca^{2+} or inhibition of Ca^{2+} influx, or indirectly by activating voltage (IK(v)) or Ca^{2+} dependent (IK(Ca)) potassium channels causing membrane hyperpolarisation (ΔEm). **b)** CRF-41 inactivates early glucocorticoid inhibition probably by activating or inducing additional transcription factors such as CREB that prevent glucocorticoid induction of protein X as demonstrated for calmodulin. See section 3.5.5 for further discussion.

5.3 Calmodulin: a role in early glucocorticoid inhibition in anterior pituitary corticotrophs ?

With regards to the identity of the glucocorticoid-induced protein(s) lipocortin (annexin) I, the purported mediator of glucocorticoid inhibition in macrophages (Flower,1988; Peers & Flower,1990), and chromogranin A, that has been implicated in late inhibition (Wand *et al.*,1991), are not central components of early glucocorticoid inhibition in AtT20 cells. Furthermore, there is little, if any, support for a role of either protein in early glucocorticoid inhibition in normal corticotrophs.

The demonstration that CRF-41 inactivates early glucocorticoid inhibition of CRF-41-stimulated ACTH release and also prevents glucocorticoid-induced accumulation of calmodulin mRNA in AtT20 D16:16 corticotrophs strongly suggests that calmodulin is involved in early glucocorticoid inhibition. Furthermore, functional evidence from this laboratory, implicating early glucocorticoid-suppression of intracellular free Ca^{2+} levels and enhancement of K^+ currents (see Figure 5.1a) (Antoni *et al.*,1992a; Pennington *et al.*,1992) are consistent with known actions of calmodulin. As discussed in section 4.5.4 calmodulin has been implicated in the mechanism of early glucocorticoid inhibition in other systems. Taken together, these data would suggest that a potentially generic model of early glucocorticoid inhibition would involve induction of calmodulin protein that plays a role in the restriction of agonist-induced redistribution of intracellular free Ca^{2+}. Future studies should be directed at analysis of the mechanism of action of calmodulin in these models of early inhibition as outlined in section 5.4 below.

Studies using dual *in situ* hybridization and histochemical probes are required to examine whether glucocorticoids regulate calmodulin mRNA and protein expression in normal corticotrophs. Furthermore, analysis of calmodulin regulation in individual

normal corticotrophs in conjunction with functional studies of secretion are required to determine whether early glucocorticoid inhibition of CRF-41- and AVP-stimulated ACTH release is mediated through the same protein(s).

5.4 Future perspectives

Characterization of the mechanism and modulation of early glucocorticoid inhibition requires identification of proteins involved in early inhibition. The work presented in this thesis, in conjunction with functional studies in this laboratory (Antoni *et al.*,1992a; Pennington *et al.*,1992) strongly suggest that calmodulin is involved in the mechanism of early glucocorticoid inhibition. The direction of future studies required to consolidate and extend these proposals are outlined below:

1) Do glucocorticoids induce calmodulin expression in normal corticotrophs? Studies employing dual *in situ* hybridization and histochemical probes are required to analyze regulation of calmodulin mRNAs expressed exclusively in normal corticotrophs.

2) Is calmodulin overexpression the cause or consequence of inhibition? Use of antisense DNA technology to block glucocorticoid-stimulated calmodulin protein accumulation, and use of calmodulin expression vectors to directly elevate calmodulin levels, in AtT20 D16:16 cells should indicate the causal relationship between calmodulin overexpression and early inhibition. Furthermore, analysis of the other putative glucocorticoid-induced mRNAs isolated in section 4 will establish whether multiple protein(s) are involved in inhibition.

3) What is the mechanism of action of calmodulin in early inhibition? Overexpression studies using mutant forms of calmodulin will provide insights into the mechanism and locus of action, for example, if the primary action of calmodulin is to act as a Ca^{2+}

sink then calmodulin mutants that only display Ca^{2+} binding should mimic early inhibition.

4) What is the mechanism of CRF-41-inactivation? The AtT20 D16:16 corticotroph should provide a useful model in which to examine the molecular interactions between CRF-41/cAMP and glucocorticoid regulated intracellular signalling pathways. Identification of the intracellular pathways involved in early glucocorticoid inhibition will allow functional assays to be developed that will expose the locus of CRF-41/glucocorticoid interaction.

5) Do similar glucocorticoid-induced proteins mediate inhibition of CRF-41 and AVP-stimulated ACTH secretion. Analysis of the response of individual corticotrophs to secretagogues and glucocorticoids should establish whether similar mechanism(s) and protein(s) are involved in early inhibition of AVP- and CRF-41-stimulated ACTH release. This work will require development of corticotroph purification techniques and will also shed light on the potentially distinct intracellular pools of ACTH released by the major hypothalamic ACTH secretagogues.

In conclusion, the work presented in this thesis provides a conceptual framework in which to further examine the mechanism of early glucocorticoid inhibition at the anterior pituitary corticotroph as well as other potential glucocorticoid feedback sites.

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Published papers

PUBLISHED PAPERS

Reprints of the following full papers are enclosed:

Shipston, M. J. & Antoni, F. A. (1991) Early glucocorticoid feedback in anterior pituitary corticotrophs: differential inhibition of hormone release induced by vasopressin and corticotrophin-releasing factor *in vitro*. *Journal of Endocrinology* **129**, 261-268.

Shipston, M. J. & Antoni, F. A. (1992) Inactivation of early glucocorticoid feedback by corticotrophin-releasing factor *in vitro*. *Endocrinology* **130**, 2213-2218.

Early glucocorticoid feedback in anterior pituitary corticotrophs: differential inhibition of hormone release induced by vasopressin and corticotrophin-releasing factor *in vitro*

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ABSTRACT

Vasopressin and 41-residue corticotrophin-releasing factor (CRF-41) are physiological mediators of the hypothalamic control of pituitary ACTH secretion, whilst adrenocortical glucocorticoids are the major inhibitory factors regulating ACTH output. In the present study it was investigated *in vitro* whether the characteristics of early glucocorticoid inhibition of stimulated ACTH secretion would differ depending on the nature of the stimulus and the temporal relationship between secretagogue and steroid.

The experiments were carried out using perifused segments of rat adenohypophysis obtained from randomly cycling female rats. Repeated pulses (5 min) of CRF-41 or vasopressin were given at 1-h intervals for up to 7 h. The net release of ACTH became stable after the second secretagogue pulse. Administration of 0·1 μmol corticosterone/l 30 min before and during a 5-min pulse of 10 nmol CRF-41/l inhibited CRF-41-stimulated ACTH release to 60% of control. Stimulated hormone release remained suppressed at 90 min after the start of the corticosterone infusion and returned to control levels by 150 min. If corticosterone treatment (35 min total exposure) was started simultaneously with the CRF-41 pulse, no inhibitory effect of the steroid was observed at any subsequent time-point examined (60, 90, 120 and 150 min). In contrast, vasopressin-stimulated ACTH release was inhibited by approximately 50% when corticosterone was applied before, or simultaneously with, a 5-min pulse of 10 nmol vasopressin/l. The synthetic glucocorticoid type II receptor agonist RU28362, administered 30 min before and during a 5-min pulse of 10 nmol CRF-41/l, reduced CRF-41-stimulated ACTH release to 50% of control up to 2·5 h after the start of RU28362 application (although inhibition after 35 min exposure was not statistically significant). Inhibition of ACTH release stimulated by 10 nmol vasopressin/l was observed within 35 min of steroid application and was maintained up to 2·5 h after the initial application of RU28362. The action of RU28362 on CRF-41-stimulated ACTH release was blocked by inhibitors of transcription (actinomycin D) and translation (puromycin); notably these drugs did not modify the ACTH response to CRF-41. In contrast, actinomycin D as well as puromycin reduced vasopressin-stimulated ACTH release.

The data suggest that: (1) the timing of steroid application is important in determining the early glucocorticoid inhibition of CRF-41- but not vasopressin-stimulated ACTH secretion; (2) CRF-41 and vasopressin mobilize different pools of ACTH from the anterior pituitary gland; (3) type II glucocorticoid receptors and synthesis of new protein(s) are involved in the early inhibitory action of glucocorticoids; (4) depending on the timing and nature of the incident secretagogue, differential negative feedback inhibition of ACTH secretion may occur at the pituitary level *in vivo*.

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INTRODUCTION

Adrenocortical glucocorticoid hormones inhibit the stimulated release of adrenocorticotrophin (ACTH) by the anterior pituitary gland. Rapid feedback develops within 30 min of glucocorticoid exposure; delayed feedback has the same effect but is maximally apparent at 1–2 h. Slow feedback requires several hours to be manifested and results in a reduction of basal and stimulated ACTH secretion as well as a decrease in the rate of

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ynthesis of the pro-opiomelanocortin precursor (for eviews see Keller-Wood & Dallman, 1984; Dallman, Akana, Cascio *et al.* 1987; Jones & Gillham, 1988; Lundblad & Roberts, 1988). There appears to be no difference between the characteristics of rapid and delayed feedback in the pituitary model used in these tudies (Dayanithi & Antoni, 1989); they will therefore e referred to as early feedback in this paper.

The mechanism of action of early feedback is not stablished; in fact, some investigators have failed to demonstrate it altogether (Familari & Funder, 1989). Earlier in-vitro studies have suggested that prior ddition of steroid was necessary for an early gluco-orticoid inhibition of stimulated ACTH secretion Mulder & Smelik, 1977; Mahmoud, Scaccione, Scraggs *et al.* 1984). Thus it was of interest to deter-nine whether the early inhibitory action of glucocorti-oids at the pituitary level is influenced by the timing f glucocorticoid application in relation to the stimu-ation by secretagogues and whether new mRNA and rotein are required for the inhibitory action of gluco-orticoids. Moreover, the possibility of differential ffects of 41-residue corticotrophin-releasing factor-CRF-41) and vasopressin-stimulated ACTH release as examined.

MATERIALS AND METHODS

Animals

Female ex-breeder Wistar rats of mean body weight 50–300 g were obtained from Harlan-Olac (Bicester, Oxon, U.K.) and maintained with free access to pelleted food and tap water for at least 1 week before use.

Reagents

Rat CRF-41, [Arg^8]-vasopressin and human ACTH(1–9) were supplied by Bachem Inc. (Saffron Walden, Essex, U.K.); puromycin dihydrochloride hydrate and actinomycin D were from Aldrich Chemical Company Ltd (Gillingham, Dorset, U.K.); corticosterone was rom Sigma Chemical Company Ltd (Poole, Dorset, U.K.). The synthetic glucocorticoid type II agon-st, RU28362 (11β,17β-dihydroxy-6-methyl-17α-(1-ropynyl)androsta-1,4,6-trien-3-one), was generously upplied by Roussel Uclaf (Romainville, France). General chemicals were from BDH (Poole, Dorset, U.K.).

Perifusion of rat pituitary segments

n each experiment five or six randomly cyclic rats ere decapitated between 08.45 and 09.30 h. After the posterior lobe had been discarded, the anterior pituitaries were immediately placed in Dulbecco's modified Eagle's medium buffered with 25 mmol Hepes/l (Gibco-BRL, Paisley, Strathclyde, U.K.), at pH 7·4 containing 2·5 g bovine serum albumin/l (ICN Biomedicals, High Wycombe, Bucks, U.K.) and antibiotic/antimycotic solution (Sigma). This medium is subsequently referred to as DMEM–Hepes.

Each pituitary was cut into eight approximately equal sized segments using a scalpel blade. Two segments from each pituitary were transferred on to a 100 µl cushion of preswollen Sephadex G-10 slurry (Pharmacia LKB Biotechnology, Milton Keynes, Bucks, U.K.) in each of four perifusion columns per experiment. Each column thus contained 1·2 to 1·5 pituitary equivalents.

The columns were connected to a multichannel peristaltic pump (Ismatec, Zürich, Switzerland) using 0·5 mm internal diameter plastic tubing (Altec, Alton, Hants, U.K.); column effluent was collected every 5 min on ice using a Gilson fraction collector modified to collect four channels simultaneously. Media, columns and tubing were maintained at 37 °C in a thermostatically controlled incubator.

The column volume was adjusted to 0·5 ml and the segments were perifused with DMEM–Hepes for 2 h at minimum flow rate (approximately 150 µl/5 min) to allow the basal ACTH release to stabilize. The segments were perifused for a further hour at the experimental flow rate of 200 µl/min during which time the initial basal fractions were collected. At this flow rate the transit time from the medium reservoir to the fraction collector was 100 s. Various treatments were then applied as shown in the figure legends. Column effluent was stored at −40 °C until assayed.

ACTH radioimmunoassay

Duplicate 50 µl aliquots of effluent were sampled for immunoreactive ACTH content using antiserum no. 6 (courtesy of G. B. Makara, MTA KOKI, Budapest, Hungary) in a radioimmunoassay described by Antoni, Holmes & Jones (1983). ^{125}I-Labelled ACTH was produced using the iodogen method (Salacinski, McLean, Sykes *et al.* 1981) using Na^{125}I from ICN Biomedicals. Second antibodies were supplied by the Scottish Antibody Production Unit, Carluke, Lanarkshire, U.K.

Analysis of data

ACTH content in the column effluent was elevated above basal over five consecutive fractions after a 5 min exposure to 10 nmol CRF-41/l or 10 nmol vasopressin/l; thus net ACTH release/25 min was determined for each stimulus. Net ACTH release is defined as ((*V*+

$W+X+Y+Z)/5)-B$ where V is the first 5-min fraction collected during the stimulus (100 s transit time discarded) and W, X, Y, Z are the next four consecutive fractions. B is the basal secretion value immediately before application of the stimulus.

Because of the variability in absolute ACTH release between experiments, data were standardized by expressing the net ACTH release elicited by a treatment as a percentage of that elicited by 10 nmol CRF-41/l (or vasopressin) alone, applied to all columns at 3 h and 4 h. The net ACTH release at 4 h was defined as the 100% release value in all columns due to the response enhancement observed between 3 h and 4 h in all treated columns (see the Results section).

Statistical evaluation between different treatments at each time-point was performed using the non-parametric Kruskal–Wallis test with multiple comparisons (Conover, 1980) for several independent samples.

RESULTS

Response to CRF-41 and vasopressin

Figure 1 shows the dose–response relationship of net ACTH release and 5-min pulses of CRF-41. In all subsequent experiments CRF-41 was used at the submaximally effective concentration of 10 nmol/l. In this system 10 nmol vasopressin/l appeared equipotent with 10 nmol CRF-41/l (Fig. 1). The relatively high levels of secretagogue used were necessary to ensure stabilization of the release response to repeated stimuli.

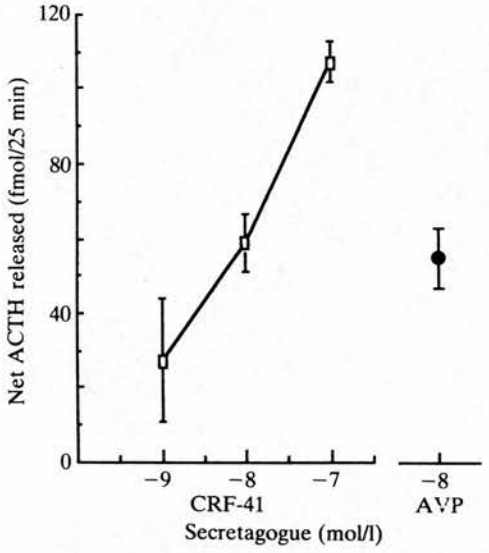

FIGURE 1. Release of ACTH by perifused rat anterior pituitary segments in response to 41-residue corticotrophin-releasing factor (CRF-41) and [Arg]-vasopressin (AVP). Four columns of segments were exposed to various concentrations of secretagogue for 5 min in a randomized complete block design after initial exposure to two doses of 10 nmol CRF-41/l. Data are the means of the net release of hormone ± S.E.M. ($n=4$/group). The basal secretory rate in this experiment was 62·1 ± 4·3 fmol ACTH/25 min ($n=24$).

The release of ACTH at 4 h in response to a 5-min pulse of 10 nmol CRF-41/l was enhanced (net ACTH release was 121·0 ± 5·2% of the release at 3 h, mean ± S.E.M, $n=46$) with respect to the response at 3 h in the columns. Subsequent stimuli released similar amounts of ACTH to that released at 4 h, up to 7 h after the start of perifusion. Typical release profiles for a control and an RU28362-treated column are shown in Fig. 2. Response enhancement was also observed (net ACTH release at 4 h was 164·2 ± 12·3% of that at 3 h, mean ± S.E.M, $n=28$) between the stimulus at 3 and 4 h when vasopressin was used as a secretagogue.

Effect of glucocorticoids on CRF-41- and vasopressin-stimulated ACTH release

Exposure of the pituitary segments to 0·1 μmol corticosterone/l for 30 min before and during the CRF-41 pulse at 5 h resulted in a significant ($P<0·05$) attenuation (60% of control) of stimulated ACTH release after 30 min; the inhibitory effect was maintained at 6 h. At 7 h (2·5 h after the application of corticosterone) the release of ACTH returned towards the control value (Fig. 3).

The degree of inhibition of ACTH release was not altered by increasing the corticosterone concentration to 1 μmol/l, or by increasing the glucose concentration in the DMEM–Hepes from the normal 5·6 mmol/l to 20 mmol/l (data not shown).

When corticosterone application was started simultaneously with the CRF-41 pulse (and continued for a total of 35 min) no inhibition of hormone secretion occurred at any subsequent time-point (Fig. 3). Because the time between the start of corticosterone administration and the stimuli at 6 and 7 h was longer (by 30 min) for columns receiving prior rather than simultaneous corticosterone application, experiments were also performed to verify that the absence of inhibition upon simultaneous application of steroid and CRF-41 was not a result of the 'silent' (steroid non-responsive) period reported by other workers (Mahmoud *et al.* 1984; Abou-Samra, Catt & Aguilera, 1986). Four columns were treated with 0·1 μmol corticosterone/l applied simultaneously with the CRF-41 stimulus at 5 h, stimuli were then applied at 6·5 h and 7·5 h. No significant inhibition was observed at either time-point (net ACTH released, at 6·5 h, 101·0 ± 7·9%; at 7·5 h, 102·0 ± 7·1%). In contrast to the findings with CRF-41, the early inhibition of vasopressin-stimulated ACTH release was independent of the timing of application of corticosterone (Fig. 4). In all experiments, basal ACTH release was unaffected by corticosterone.

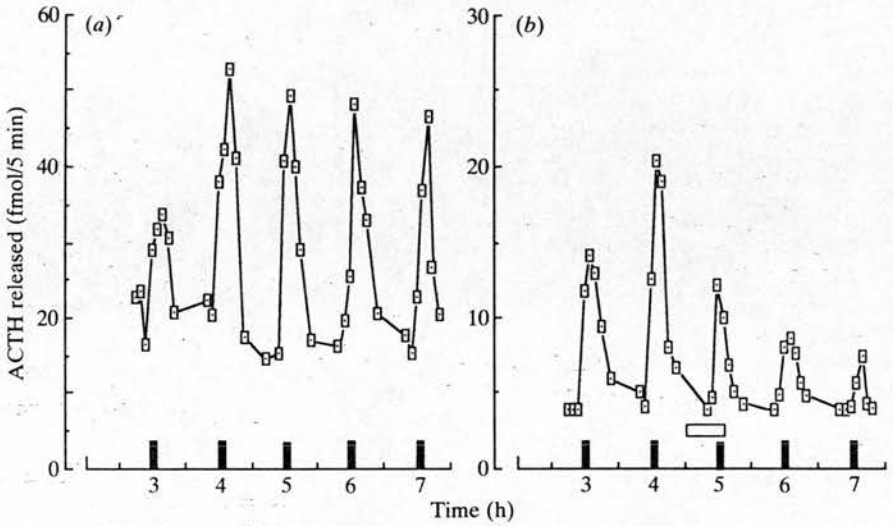

FIGURE 2. Data from typical perifusion experiments of rat anterior pituitary segments showing (*a*) the release of ACTH in response to repeated 5-min pulses of 41-residue corticotrophin-releasing factor (CRF-41, 10 nmol/l, solid vertical bars) and (*b*) the effect of a 35-min exposure (open horizontal bar) to the synthetic glucocorticoid type II receptor agonist, RU28362 (1 μmol/l), on CRF-41-stimulated ACTH secretion. Data are expressed as the amount of immunoreactive ACTH present in each 5-min collection fraction. Interexperimental variations in absolute ACTH release (compare abscissa of *a* with *b*) required subsequent data to be standardized.

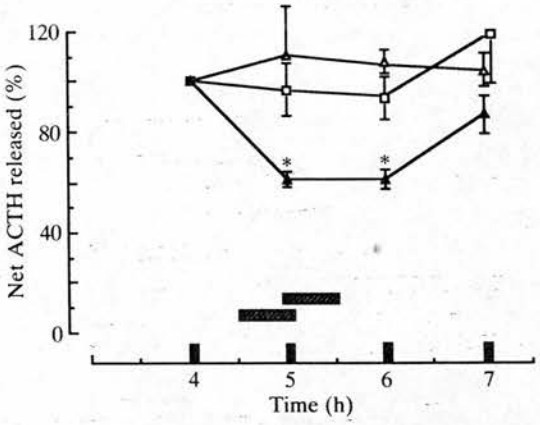

FIGURE 3. Corticosterone feedback inhibition of ACTH release by perifused rat anterior pituitary segments upon simultaneous application of 41-residue corticotrophin-releasing factor (CRF-41) and corticosterone. The control group (□) received repeated 5-min pulses of 10 nmol CRF-41/l (solid vertical bars). Other groups received corticosterone (0·1 μmol/l, 35 min total exposure; horizontal hatched bars) 30 min before (▲) or simultaneously with (△) the start of the CRF-41 pulse at 5 h. Data are expressed as a percentage of the net ACTH released by a 5-min pulse of 10 nmol CRF-41/l received at 4 h by all groups. Data are means ± S.E.M. ($n = 4$–6/group). *$P < 0{\cdot}05$ (non-parametric Kruskal–Wallis test) compared with the control group.

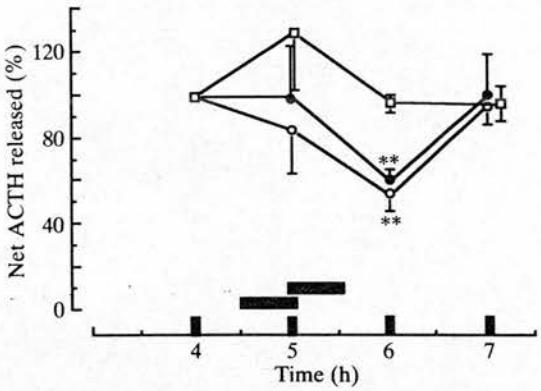

FIGURE 4. Inhibition of vasopressin-stimulated ACTH release from perifused rat anterior pituitary segments by corticosterone. The control group (□) received repeated 5-min pulses of 10 nmol vasopressin/l (solid vertical bars). Other groups received 0·1 μmol corticosterone/l (total exposure 35 min; horizontal hatched bar) applied 30 min before (○) or simultaneously with (●) the vasopressin pulse at 5 h. Data are expressed as a percentage of the net ACTH released by a 5-min pulse of 10 nmol vasopressin/l received at 4 h by all groups. Data are means ± S.E.M. ($n = 4$/group). **$P < 0{\cdot}01$ (non-parametric Kruskal–Wallis test) compared with the control group.

The potent glucocorticoid type II receptor agonist, RU28362 (Philibert & Moguilewsky, 1983), at a concentration of 1 μmol/l had no significant inhibitory

ffect after 35 min of exposure when applied 30 min efore a CRF-41 stimulus (Fig. 5). However, at this me-point the release of ACTH was highly variable: iere was a marked inhibition in two out of six experiments. After 1·5 h ACTH release was significantly $P<0.05$) reduced to 50% of control and, unlike rticosterone, the inhibitory effect was maintained ·5 h after application using both 1 μmol (compare pplication of corticosterone and RU28362 before a RF-41 stimulus at 5 h in Figs 3 and 5 respectively) nd 0·1 μmol RU28362/l (data not shown). RU28362 μmol/l, total exposure 35 min) significantly $P<0.05$) inhibited vasopressin-stimulated ACTH ecretion to 64% of control within 35 min of appliation (Table 1). The inhibition was maintained 2·5 h fter initial exposure to the steroid. Basal ACTH reease was unaffected by RU28362.

ffect of inhibitors of transcription and translation on ie action of glucocorticoids

ctinomycin D (0·1 mmol/l), an irreversible inhiitor of RNA synthesis, completely blocked the inhiitory action of RU28362 on CRF-41-induced ACTH ecretion at all time-points when given 5 min before nd during the application of the steroid (Fig. 5*a*) and ad no effect on CRF-41-stimulated ACTH release (in wo columns net ACTH release was 58 and 64 fmol/25 in at 4 h, 104 and 120% of the respective controls at h, 115 and 90% at 6 h and 107 and 101% at 7 h).

The inhibition of CRF-41-stimulated ACTH release y RU28362 was prevented during exposure of the segents to the reversible protein synthesis inhibitor, uromycin, at a concentration of 0·1 mmol/l (Fig. 5*b*). he full inhibitory action of RU28362 was observed 1 h fter withdrawal of puromycin, indicating that transatable mRNA was still present in the cells. Puromycin pplied at the same concentration and duration as for ie RU28362 experiments completely blocked the inhiitory action of corticosterone on CRF-41-stimulated CTH release at all time-points (data not shown). uromycin alone did not modify CRF-41-stimulated CTH release (in two columns net ACTH release was 2 and 74 fmol/25 min at 4 h, 117 and 135% of the espective controls at 5 h, 103 and 105% at 6 h and 101 nd 109% at 7 h).

In contrast to CRF-41, vasopressin-stimulated CTH release was inhibited by puromycin alone Table 1). After withdrawal of puromycin from the erifusion medium vasopressin-stimulated ACTH elease returned towards control. No inhibition of CTH release by RU28362 occurred in puromycinreated tissue (Table 1). Actinomycin D also reduced asopressin-stimulated ACTH release to approxiately 70% of control at 6 and 7 h (data not hown).

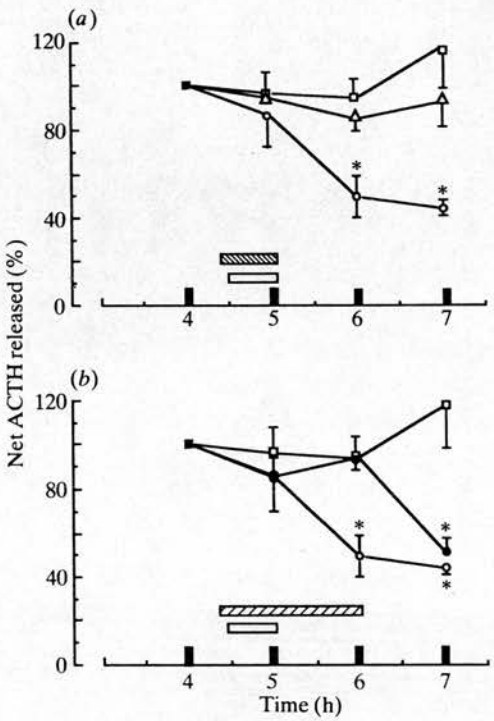

FIGURE 5. Antagonism of the synthetic glucocorticoid receptor agonist (RU28362)-induced inhibition of CRF-41-stimulated ACTH secretion from perifused rat anterior pituitary segments by inhibitors of (*a*) transcription (actinomycin D) and (*b*) translation (puromycin). Data are expressed as the net amount of ACTH released after the 5-min pulse of 41-residue corticotrophin-releasing factor (CRF-41; 10 nmol/l; solid vertical bars) at 4 h. CRF-41 control (□), RU28362 treated (○), RU28362 + actinomycin D treated (△), RU28362 + puromycin treated (●), RU28362 (1 μmol/l) for 35 min (open horizontal bar), actinomycin D (0·1 mmol/l) for 40 min (short hatched horizontal bar) or puromycin (0·1 mmol/l) for 100 min (long hatched horizontal bar). Data are means ± S.E.M. ($n=4$–6/group). $^*P<0.05$ (non-parametric Kruskal–Wallis test) compared with control.

DISCUSSION

The present study demonstrates that the timing of application of glucocorticoids is critical for the early inhibition of CRF-41- but not vasopressin-induced ACTH secretion. Moreover, vasopressin- but not CRF-41-stimulated ACTH release is suppressed by actinomycin D and puromycin.

Response to stimulation by CRF-41 and vasopressin

The mechanism(s) underlying the augmentation of ACTH release induced by CRF-41 or vasopressin in this system are unknown. Similar response enhancement has been observed in acutely dispersed cells

TABLE 1. Inhibition of vasopressin-stimulated ACTH release from perifused anterior pituitary gland segments by the synthetic glucocorticoid receptor agonist, RU28362, and the protein synthesis inhibitor, puromycin. Data are expressed as a percentage of the net ACTH released in response to a 5-min pulse of 10 nmol vasopressin/l received by all groups at 4 h. Values are means ± S.E.M., the number of experiments are shown in parentheses

	ACTH released (%)			
	4 h	5 h	6 h	7 h
Treatment				
Vasopressin, 10 nmol/l (5)	100	103·6 ± 12·6	103·8 ± 17·8	80·8 ± 7·2
+ RU28362, 1 μmol/l (6)	100	64·3 ± 4·9*	59·7 ± 5·4*	48·0 ± 14·0*
+ Puromycin, 0·1 mmol/l (4)	100	49·0 ± 7·6**	32·3 ± 3·4**	60·3 ± 11·0
+ Puromycin, 0·1 mmol/l, + RU28362, 1 μmol/l (4)	100	58·8 ± 6·1*	25·8 ± 8·0**	39·0 ± 4·9*

*$P < 0.05$, **$P < 0.01$ compared with the control group (vasopressin only) (non-parametric Kruskal–Wallis test). The range of absolute ACTH release values at 4 h (100% value) for each group (expressed as fmol ACTH released/25 min) were: vasopressin, 194–577; + RU28362, 269–813; + puromycin, 247–416; + RU28362 + puromycin, 290–480. 5-min pulses of vasopressin were applied every hour as described in the Materials and Methods section. RU28362 was applied 30 min before and during the vasopressin stimulus at 5 h. Puromycin was applied 35 min before the stimulus at 5 h and continued until the end of the stimulus at 6 h.

using CRF-41, vasopressin or a combination of the secretagogues. The rapid stabilization of the hormonal response to subsequent stimuli by 1 h after application of the first secretagogue pulse is probably a result of the relatively high secretagogue concentrations used in the present study (Antoni & Dayanithi, 1990*b*).

Involvement of type II glucocorticoid receptors and requirement for mRNA and protein synthesis in the early inhibitory action of glucocorticoids

It has been suggested that rapid and delayed effects of glucocorticoids are mediated through different receptor subtypes (Keller-Wood & Dallman, 1984; Abou-Samra *et al.* 1986). However, it has been shown recently (Dayanithi & Antoni, 1989) that both rapid and delayed inhibition are manifested through the type II glucocorticoid receptor and that the time-domains differ only in the extent rather than the cellular mechanism of inhibition.

Our data demonstrate that both CRF-41- and vasopressin-stimulated ACTH secretion are inhibited in the early time-domain by glucocorticoids acting through the type II glucocorticoid receptor. The lack of a statistically significant inhibition of CRF-41-stimulated ACTH release within 30 min of application of RU28362 in the present study may suggest mediation of rapid feedback by a non-type II glucocorticoid receptor. However, the rapid effect of corticosterone was blocked by the potent type II receptor antagonist, RU38486 (Philibert, 1984) (M. J. Shipston & F. A. Antoni, unpublished observations). The more prolonged inhibitory action of RU28362, with respect to corticosterone, on both CRF-41- and vasopressin-stimulated ACTH release may be a consequence of differences in tissue traffic (metabolism and diffusion) between RU28362 and corticosterone rather than mediation of the response by different types of glucocorticoid receptor.

Since inhibitors of transcription (actinomycin D) and translation (puromycin) blocked the suppressive action of glucocorticoids on CRF-41-stimulated ACTH release, the present data are consonant with the hypothesis that early glucocorticoid action involves the synthesis of new protein(s) (Arimura, Bowers, Schally *et al.* 1969; Munck, 1971; Dayanithi & Antoni, 1989). Increasing the concentration of glucose in the medium had no effect on the inhibitory effect of type II receptor stimulation suggesting that a reduction of glucose uptake (Munck, 1971) by the glucocorticoid-induced protein is unlikely to be the cause of inhibition of secretagogue-stimulated ACTH release. Whether glucocorticoid inhibition of vasopressin-stimulated ACTH secretion requires a similar protein could not be resolved fully in this study because of the inhibition of vasopressin-induced ACTH secretion by actinomycin D or puromycin given alone. However, RU28362 produced no additional inhibition of ACTH release in the presence of puromycin which is compatible with the protein induction hypothesis.

Do CRF-41 and vasopressin release different pools of ACTH?

The suppression of vasopressin-, but not CRF-41-, induced ACTH release by inhibitors of transcription and translation suggest that the secretagogues may activate different intracellular pools of ACTH, e.g. vasopressin may release newly synthesized ACTH as opposed to CRF-41, which may preferentially mobilize stored hormone. It has been suggested, on the basis of experiments with the lysosomotropic drug chloroquine and a cytotoxic conjugate of CRF-41 (Schwartz,

Familari, Wallace & Funder, 1989; Schwartz, Pham & Funder, 1990), that CRF-41 releases a stored pool of ACTH whereas vasopressin-stimulated secretion is more closely associated with the basal output of hormone. Whether such distinctly regulated pools of hormone reside within a single population of cells or are secreted by different sets of corticotrophs remains to be determined. Alternatively, it cannot be excluded at present that actinomycin D and puromycin interfere with the signal transduction pathways activated by vasopressin.

Importance of timing of glucocorticoid application

The pattern of glucocorticoid inhibition of CRF-41- and vasopressin-induced hormone release appeared different in this study.

First, significant inhibition of vasopressin-induced release was manifest only 90 min after the application of corticosterone, whereas CRF-41-stimulated release was suppressed within 35 min. This, however, is likely to be due to the larger variation in the vasopressin data and probably does not reflect any fundamentally distinct intracellular mechanisms of glucocorticoid action (Antoni & Dayanithi, 1990*a*). In accordance with this suggestion, the synthetic glucocorticoid type II receptor agonist RU28362 significantly inhibited vasopressin-stimulated ACTH release within 35 min of RU28362 exposure.

Secondly, the early suppression of CRF-41-stimulated ACTH secretion is dependent upon the relative timing of secretagogue and corticosterone application, whereas this is not the case with vasopressin-induced ACTH secretion.

The importance of the temporal relationship between secretagogue stimulation and glucocorticoid exposure has been reported by Mulder & Smelik (1977), who found that pretreatment with corticosterone was necessary to achieve an early inhibitory effect in isolated perfused rat pituitary cells stimulated by short pulses of stalk-median eminence extract. Several workers (Mahmoud *et al.* 1984; Abou-Samra *et al.* 1986) have reported a biphasic time-course of the early effects of corticosterone on CRF-induced ACTH secretion, with a 'silent' period at 30–50 min after exposure to the steroid. This latter phenomenon, however, appears to be different from what we have observed in the present study, since we have covered a long time-interval after the application of corticosterone simultaneously with CRF-41 and found no inhibition of ACTH release at any time-point examined.

The mechanism(s) underlying the timing dependence of early glucocorticoid inhibition of CRF-41- but not vasopressin-induced ACTH secretion is unknown. The present observations do not resolve whether CRF-41 inactivates the steroid hormone–receptor complex before it reaches the cell nucleus, or whether it is actually required to activate some process in the line of cellular events which leads to the suppression of ACTH release. The latter possibility is favoured by a report showing that cyclic AMP-dependent protein kinase activation enhances the cytolytic action of glucocorticoids in cell lines derived from lymphoma cells (Gruol, Rajah & Bourgeois, 1989).

The present data point to a regulatory mechanism which may allow differential glucocorticoid control of stimulated ACTH release from the anterior pituitary gland depending on the timing and nature of the incident secretagogue. Intriguingly, some of the major cytokines released during inflammation, interleukin-1 and tumour necrosis factor, stimulate the release of CRF-41 by hypothalamic neurones but not that of vasopressin (Berkenbosch, Van Oers, Del Rey *et al.* 1988; Sapolsky, Rivier, Yamamoto *et al.* 1988). Moreover, in certain autoimmune inflammatory diseases, adrenocortical steroid secretion is enhanced for several days (MacPhee, Antoni & Mason, 1989; Sternberg, Hill, Chrousos *et al.* 1989; Mason, MacPhee & Antoni, 1990), suggesting that the feedback mechanism may have been modified. These observations illustrate the possible functional importance of the escape of CRF-41-induced ACTH release from glucocorticoid feedback inhibition at the pituitary level.

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ctivation of Early Glucocorticoid Feedback by ticotropin-Releasing Factor *in Vitro*

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BSTRACT. We have investigated the interaction between ypothalamic ACTH secretagogues and adrenocortical glucocorcoids in rat anterior pituitary tissue using an *in vitro* perifusion ystem.

Repeated 5 min pulses of 41-residue CRF (CRF-41) or argiine vasopressin (AVP) were applied at 1 h intervals for up to 7 . Administration of 0.1 μM corticosterone 30 min before and uring the 5 min 0.1 nM CRF-41 stimulus at 5 h resulted in a gnificant inhibition of CRF-41 stimulated ACTH release ithin 30 min. Inhibition of ACTH release also developed if no RF-41 stimulus was applied in conjunction with steroid at 5 h. ı contrast, if the exposure to corticosterone (0.1 μM, 35 min otal duration) was started simultaneously with the application f CRF-41 at 5 h, no inhibition of ACTH release ensued. Simiırly, no inhibition of CRF-41-stimulated ACTH release was bserved when corticosterone was started simultaneously with a 5 min pulse of cyclic 8-(4-Chlorophenylthio) AMP (8-CPT-cAMP), a cell membrane permeant analog of cAMP.

In contrast to CRF-41 and 8-CPT-cAMP, AVP failed to modify glucocorticoid-induced inhibition of AVP- or CRF-41-stimulated ACTH release. Moreover, CRF-41 did not prevent the glucocorticoid-induced inhibition of AVP-stimulated ACTH release.

In summary: 1) CRF-41 inactivates early glucocorticoid inhibition of CRF-41-stimulated ACTH secretion, and this is mimicked by a cell membrane permeant analog of cAMP; 2) AVP does not inactivate glucocorticoid-induced inhibition of stimulated ACTH release; 3) the data point to an acute interaction between the cAMP/protein kinase A and glucocorticoid-responsive intracellular pathways. Such differential modulation of feedback inhibition by CRFs may be of functional importance *in vivo*. (*Endocrinology* **130:** 2213–2218, 1992)

RTICOTROPIN releasing factor-41 (CRF-41) and ırginine vasopressin (AVP) are the major physio- hypothalamic regulators of ACTH secretion from ır pituitary corticotrophs while the adrenocortical ırticoids, released in response to elevated ACTH inhibit ACTH release over several time domains views see Refs. 1–4). At the pituitary level early- glucocorticoid inhibition decreases stimulated release within 30 min; the maximal effect is nt within 1–2 h of glucocorticoid application. This is mediated through type II glucocorticoid recepıd requires the synthesis of new messenger RNA as protein (5–8).

g an *in vitro* perifusion model we have reported e characteristics of early-onset glucocorticoid in- n of stimulated ACTH release are dependent on ıe nature of the secretagogue as well as the temelationship between secretagogue and glucocortiplication. Notably, early-onset glucocorticoid inhibition of CRF-41 stimulated ACTH release did not develop if CRF-41 was applied at the start of corticosterone exposure. In contrast, AVP had no such action on early-onset inhibition of AVP-stimulated ACTH release (7).

In this study we have investigated whether CRF-41 blocks early-onset glucocorticoid inhibition and whether the action of CRF-41 is reproduced by its intracellular second messenger cAMP. Furthermore, we have investigated whether there is any interaction between CRF-41 and AVP with respect to the blockage of glucocorticoid-induced inhibition.

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Materials and Methods

Animals

Female exbreeder Wistar rats (250–300 g body wt) were obtained from Charles River Ltd (Margate, Kent, UK) or Harlan-Olac (Bicester, Oxon, UK) and maintained four to a cage under controlled conditions of lighting (lights on 0500 h, off 1900 h) and temperature for at least 2 weeks before use with free access to standard lab chow and water.

Reagents

Rat CRF-41, AVP, and human $ACTH_{(1-39)}$ were supplied by Bachem Inc. (Saffron Walden, Essex, UK); corticosterone was

igma Chemical Company Ltd (Poole, Dorset, UK). 3-(4-Chlorophenylthio) AMP (8-CPT-cAMP) was from ager Mannheim UK (Lewes, Sussex, UK). General als were from BDH (Poole, Dorset, UK).

ion

usion of isolated rat pituitary segments was performed iously described (7). Briefly, anterior pituitary glands t into eight segments and perifused at 200 μl/min with co's modified Eagle's medium buffered with 25 mM S (Gibco-BRL, Paisley, Strathclyde, UK), pH 7.4, con- 0.25% BSA, 100 U/liter penicillin, and 100 U/liter mycin (Gibco-RBL, Paisley, Strathclyde, UK) at 37 C. ly four perifusion columns were run in parallel with one y equivalent of randomly distributed pituitary segments er perifusion column. Column effluent was collected min on ice and stored at −40 C before assaying for immunoreactivity. After an initial preperifusion period o allow basal ACTH release to stabilize, 5 min pulses of gogue were applied every 1 h for up to 7 h after the start xperiment. Various treatments were applied as described igure subscripts.

d data analysis

RIA for immunoreactive ACTH in the column effluent formed as previously described (9) using antiserum no.6 by G. B. Makara, MTA-KOKI, Budapest, Hungary). ACTH release per 5 min exposure to secretagogue was ned for each stimulus as previously described (6). Net release is defined as $\{(V_1 + V_2 + \ldots\ldots + V_n)/n\} - \{B\}$ V_1 is the first 5 min fraction collected during the stimulus transit time discarded) and V_2 to V_n are the next n tive fractions that are elevated with respect to basal release (B) immediately before the application of the s. For 10nM CRF-41 or AVP, n = 5. For 0.1 nM CRF- 3. Because of the variability in absolute ACTH release experiments data were standardized by expressing the TH release elicited by a treatment as a percentage of ited by CRF-41 (or AVP) alone. The net ACTH release as defined as the 100% release value in all columns. tical evaluation between different treatments at each int was performed using the Kruskal-Wallis test with comparisons for several independent samples, or the Whitney U-test for two independent samples (10).

Results

and 8-CPT-cAMP inactivate glucocorticoid on of CRF-41-stimulated ACTH release

costerone pretreatment (0.1 μM for 35 min) sig- ly ($P < 0.05$) inhibited CRF-41-stimulated secretion within 30 min of corticosterone appli- (Fig. 1) before ACTH release returned toward 2.5 h after steroid application. Corticosterone inhibition of CRF- and AVP-stimulated ACTH developed with a similar time profile (data not

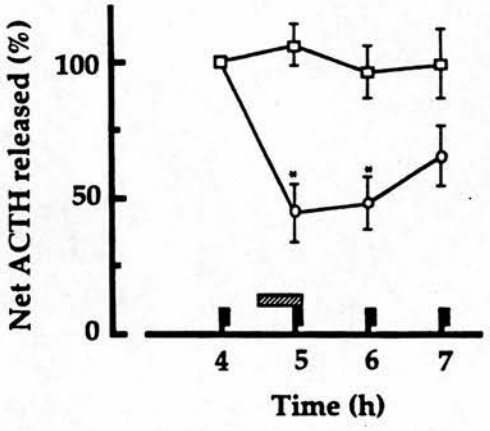

FIG. 1. Early-onset corticosterone-induced inhibition of CRF-41-stimulated ACTH secretion in perifused rat anterior pituitary gland segments. The control group (□) received repeated 5 min pulses of 0.1 nM CRF-41 (*solid vertical bars*); the other group (○) received corticosterone (0.1 μM, 35 min total exposure; *horizontal hatched bar*) 30 min before the start of the CRF-41 pulse at 5 h. Data are expressed as a percentage of the net ACTH released by a 5-min pulse of CRF-41 applied at 4 h. Data are means ± SEM (n = 4–7/group). *, $P < 0.05$ (nonparametric Mann Whitney U-test) compared with the control group.

CRF-41 stimulated ACTH release was also significantly inhibited when no CRF-41 stimulus was applied during the exposure to corticosterone (Table 1). Omission of the CRF-41 stimulus at 5 h had no effect on the responses to subsequent pulses of CRF-41; thus these controls were pooled with controls receiving CRF-41 at 5 h. Early-onset glucocorticoid inhibition did not develop when CRF-41 was applied at the start of corticosterone exposure, suggesting that CRF-41 inactivates glucocorticoid-induced inhibition (Table 1). Furthermore, the cell membrane permeant cAMP analog, 8-CPT-cAMP [0.1 mM for 5 min (11)] also prevented the subsequent inhibition of CRF-41 induced ACTH release when applied at the start of corticosterone exposure (0.1 μM, 35 min duration) at 5 h (Table 1). At this concentration a 5 min pulse of 8-CPT-cAMP elicited 26.0 ± 5.1% of the ACTH released by 0.1 nM CRF-41 and did not modify the ACTH release to subsequent CRF-41 pulses (Table 1).

To investigate whether the inactivation of glucocorticoid-induced inhibition was specific to the cAMP pathway, 10 nM AVP (that acts via the inositol phosphate/protein kinase C pathway) was applied for 5 min at 5 h. The release of ACTH in response to a 5 min pulse of 10 nM AVP at 5 h was 186.2 ± 24.0% (n = 14) of the 0.1 nM CRF-41 stimulus applied at 4 h. The CRF-41 stimulus at 6 h released similar amounts of ACTH compared to CRF-41 controls; however, down-regulation of the CRF-41 response at 7 h occurred in columns treated with AVP at 5 h (Table 1). Importantly, when AVP was applied at the start of corticosterone exposure, significant ($P < 0.01$) inhibition of CRF-41-stimulated ACTH release occurred at 6 h (Table 1). These data suggest that inac-

1. Inactivation of corticosterone-induced inhibition of CRF-ulated ACTH secretion by CRF-41 and the cAMP analog, 8-.MP, in perifused rat anterior pituitary segments

-41	Net ACTH released (%)		
	5 h Treatment	6 h CRF-41 ■	7 h CRF-41 ■
-%	CRF-41 (7) ■	98.0 ± 8.4	99.0 ± 9.9
	Corticosterone (4) ▨	61.5 ± 9.9[a]	54.3 ± 9.3[a]
	Corticosterone + CRF-41 (5) ▨ ■	108.4 ± 4.3[b]	111.2 ± 12.4[b]
	8-CPT-cAMP (8) □	103.5 ± 15.1	76.3 ± 12.2
	Corticosterone + 8-CPT-cAMP (6) ▨ □	101.2 ± 13.2[b]	106.0 ± 13.6[b]
	AVP (4) ▩	82.5 ± 4.3	48.0 ± 15.6
	Corticosterone + AVP (4) ▨ ▩	32.3 ± 18.7[c]	50.0 ± 12.4

oups received 5 min pulses of 0.1 nM CRF-41 at 4, 6, and 7 h. ous treatments applied, along with their temporal relationship :ation, are shown in the 5 h treatment column. 8-CPT-cAMP ied for 5 min at 0.1 mM. AVP for 5 min at 10 nM. Corticosterone 35 min total exposure) was started simultaneously with the 5 lus. Data are expressed as a percentage of the net ACTH by a 5-min pulse of 0.1 nM CRF-41 applied at 4 h in all groups. omission of the 5 h CRF-41 stimulus did not affect subsequent ed ACTH release data from control columns receiving no CRF- were pooled with the CRF-41 control group. Values are means he number of experiments in each treatment group are shown theses.

0.05 compared to CRF-41 control.

0.05 compared to corticosterone alone.

0.05 compared to AVP control using nonparametric Kruskal- st.

1 is specific to the CRF-41/cAMP pathway.

duration of corticosterone exposure is also impor- determining the characteristics of early-onset ion. When CRF-41 was applied at the start of a ious corticosterone infusion inhibition of CRF- ulated ACTH release developed 2 h after the start costerone treatment (Fig. 2).

rticoid inhibition of AVP-stimulated ACTH is not modulated by CRF-41

-41 (0.1 nM) applied simultaneously with the start costerone exposure (35 min duration) at 5 h could vent the corticosterone-induced inhibition of imulated ACTH release at 6 h (Table 2). Al-

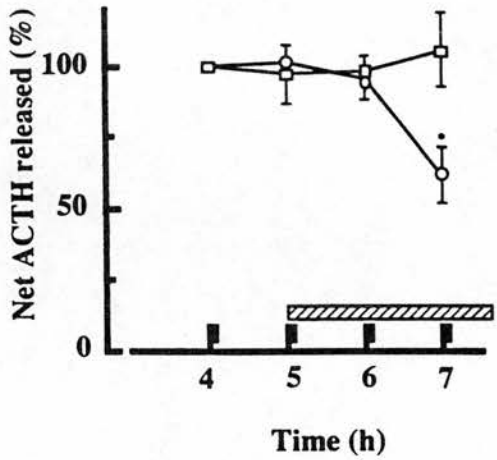

FIG. 2. Inhibition of CRF-41-stimulated ACTH secretion by continuous exposure to corticosterone started simultaneously with a 5 min CRF-41 stimulus in perifused rat anterior pituitary segments. The control groups (□) received repeated 5 min pulses of 10 nM CRF-41 (*solid vertical bars*) every 1 h. The other group (○) received 0.1 μM corticosterone (*horizontal hatched bar*) started simultaneously with the 5 h stimulus and maintained to the end of the experiment. Data are expressed as a percentage of the net ACTH released by a 5-min pulse of CRF-41 applied at 4 h. Data are means ± SEM (n = 4–9/group. *, $P < 0.05$ (nonparametric Mann-Whitney U test) compared with the control group.

TABLE 2. CRF-41 does not inactivate corticosterone-induced inhibition of AVP-stimulated ACTH secretion in perifused rat anterior pituitary segments

	Net ACTH released (%)			
	4 h AVP ▩	5 h Treatment	6 h AVP ▩	7 h AVP ▩
100%		AVP (9) ▩	100.2 ± 9.7	87.1 ± 5.7
		Corticosterone + AVP (5) ▨ ▩	60.4 ± 4.5[a]	101.0 ± 18.6
		CRF-41 (5) ■	94.8 ± 9.5	65.6 ± 16.1
		Corticosterone + CRF-41 (5) ▨ ■	41.4 ± 12.0[b]	63.8 ± 17.2

All groups received 5 min pulses of 10 nM AVP at 4, 6, and 7 h. The various treatments applied, along with their temporal relationship of application, are shown in the 5 h treatment column. Corticosterone (0.1 μM, 35 min total exposure) was started simultaneously with the 5 h stimulus; CRF-41 was applied for 5 min at 0.1 nM. Data are expressed as a percentage of the net ACTH released by a 5-min pulse of 10 nM AVP applied at 4 h in all groups. Values are means ± SEM; the numbers of experiments in each treatment group are shown in parentheses.

[a] $P < 0.05$ compared to AVP control.

[b] $P < 0.01$ compared to CRF-41 treated control using nonparametric Kruskal-Wallis test.

igh CRF-41 appears to decrease the subsequent re-
ıse to AVP at 7 h (Table 2) this effect is not statis-
ly significant and is a consequence of one column
of the five in the group releasing a very low amount
CTH at 7 h. Increasing the concentration of CRF-
o 10 nM also failed to modify the inhibition by
costerone (data not shown). Because these columns
ved AVP before the application of CRF-41 and
costerone at 5 h, we examined whether this AVP
eatment could prevent the CRF-inactivation of glu-
rticoid inhibition. In order to test this, CRF-41 (0.1
stimuli were applied at 3 h, 4 h, and 5 h, and 10 nM
applied at 6 and 7 h. Corticosterone (0.1 μM, 35
duration) started simultaneously with CRF-41 at 5
ulted in significant ($P < 0.05$, using nonparametric
kal Wallis test) inhibition of AVP-stimulated
H release compared to control even under these
itions. The net ACTH release expressed as a per-
ge of the 4 h CRF-41 stimulus for control and
osterone-treated groups, respectively, was: at 6 h
± 45.4% *vs.* 67.8 ± 16.6% and at 7 h 214.7 ± 48.0%
7.8 ± 31.2% (n = 3–4/group). Furthermore, CRF-41
nted the corticosterone-induced inhibition of CRF-
imulated ACTH release after pretreatment of the
ents with 10 nM AVP (data not shown) further
sting that CRF-41 can only inactivate glucocorti-
induced inhibition of CRF-41-stimulated ACTH re-

Discussion

e present study demonstrates that CRF-41 inacti-
early glucocorticoid inhibition of CRF-41-stimu-
ACTH secretion from perifused rat anterior pitui-
corticotrophs *in vitro*, and that this action is me-
l by cAMP. Furthermore, the data suggest that
corticoids act via distinct mechanisms to inhibit
41- and AVP-stimulated ACTH secretion.

41 inactivates early glucocorticoid inhibition of 41-stimulated ACTH release

data confirm our previous observations showing
he development of early glucocorticoid inhibition
RF-41-stimulated ACTH secretion is dependent
the relative timing of application of CRF-41 with
d (7). However, our previous data did not resolve
er the timing phenomenon was a result of CRF-41
ting some step in the glucocorticoid-induced inhi-
(for example posttranslational modification of the
d protein) when CRF-41 was applied after the
of corticosterone exposure, or alternatively, that
41 inactivates glucocorticoid-induced inhibition,
ps by preventing the protein induction, when ap-
simultaneously with the start of corticosterone
exposure.

CRF-41-stimulated ACTH release was significantly inhibited by corticosterone when no CRF-41 stimulus was applied during the exposure to corticosterone; similar results were also observed with the type II glucocorticoid receptor agonist RU28386 (M. J. Shipston and F. A. Antoni, unpublished data). Furthermore, application of CRF-41 or an analog of its intracellular messenger cAMP, simultaneously with the onset of corticosterone exposure, prevented the subsequent inhibition of CRF-41-stimulated ACTH release. Thus, it appears that CRF-41 actually prevents the action/induction of the glucocorticoid-induced protein(s) when applied at the start of glucocorticoid exposure. Interestingly, the duration of corticosterone exposure also plays a role in the characteristics of early glucocorticoid inhibition of CRF-41-stimulated ACTH release because if CRF-41 is applied simultaneously with the start of corticosterone then inhibition of CRF-41-stimulated ACTH release develops after 2 h of continuous steroid exposure.

The precise mechanism by which CRF-41 prevents glucocorticoid inhibition of ACTH release is not clear. However, because it can be reproduced with a cAMP analog but not with AVP (an activator of the inositol phosphate/protein kinase C pathway), it seems reasonable to suggest that the inactivation of glucocorticoid action is, in fact, mediated by cAMP and/or cAMP dependent phosphorylation. In turn, this may interfere with glucocorticoid action through modulation of the state of glucocorticoid receptor phosphorylation (12), and possibly preventing translocation of the hormone-receptor complex into the nucleus (13–15). Furthermore, transcription factors such as c-*fos* or cAMP response element binding protein (CREB) which are induced or activated by cAMP (16), have been shown to interfere with glucocorticoid-induced gene transcription (17–21). Alternatively, CRF-41 may induce some posttranslational modification in, or alter events downstream of, the glucocorticoid-induced protein to prevent the inhibition of CRF-41 stimulated ACTH secretion.

It is of interest that a reduction in glucocorticoid receptor number and glucocorticoid receptor mRNA levels develops 6 h after exposure to activators of cAMP synthesis in AtT-20 corticotroph tumor cells (22). Thus, CRF-41 acting via cAMP exerts both short- (present findings) and long-term (22) control over glucocorticoid action at the pituitary level. In turn, reciprocally opposing actions are provided by early- and late-onset glucocorticoid inhibition of CRF-41 action on ACTH release and biosynthesis (*cf* Refs. 3 and 23 for reviews).

CRF-41 does not inactivate glucocorticoid-induced inhibition of AVP-stimulated ACTH release.

Intriguingly, CRF-41 failed to prevent glucocorticoid induced-inhibition of AVP-stimulated ACTH secretion.

m among the several possible explanations to this menon, on the basis of the present data we can le that the priming of the tissue segments with inhibits the action of a subsequent CRF-41 pulse vent glucocorticoid inhibition, or that priming with 41 is required for this effect of CRF-41. Thus, it rs that glucocorticoids suppress CRF-41- and AVP- d ACTH secretion through different mechanisms. l, previous evidence indicates that distinct pools of I are mobilized by these neurohormones: CRF-41 s a stored, slow-turnover pool of ACTH, whereas nduced secretion is derived from a rapid-turnover f hormone (7, 24). A further possibility, (this in oes not exclude the previous one) is that distinct tions of corticotrophs are involved (*cf* Refs. 24 and which cAMP phosphorylation targets are ex- d in a cell-specific manner.

n CRF-41 and AVP were used in combination as agogue at concentrations approximating levels re- in rat hypophysial portal blood (0.1 nM and 0.2 spectively, *cf* Ref. 1), early-onset glucocorticoid ion can be prevented by a secretagogue pulse l at the onset of the exposure to glucocorticoids e. in this respect the 'physiological' CRF-41/AVP us resembles CRF-41 alone. This is in agreement he AVP-induced enhancement of CRF-41-stimu- ccumulation of cAMP (*cf* Ref. 23).

implications of these findings for the control of release *in vivo* are that glucocorticoid feedback at uitary level is modulated in a differential manner retagogue neurohormones. In general, stimuli in- g CRF-41 may be relatively resistant to inhibition cocorticoids, and those involving primarily AVP ariably suppressed. For instance, it may be pre- that a prolonged increase in ACTH release such t found in autoimmune inflammation (*cf* Ref. 27) ore on CRF-41 while during acute stimulation the component is more dominant and consequently suppressed by glucocorticoids. Finally, it is of ance to determine whether cAMP-mobilizing i have similar actions on glucocorticoid-mediated ion at central nervous sites.

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