MECHANISMS OF ADRENOCORTICOTROPIN-INDUCED ACTIVATION OF $ERK_{1/2}$ MAP KINASE IN THE HUMAN H295R ADRENAL CELL LINE

A Thesis Presented for the Degree of Doctor of Philosophy (Ph.D.)

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November 2008

ABSTRACT

The role of ACTH in stimulating or inhibiting growth of adrenal cells has been a subject of some controversy. Reports that ACTH may stimulate Erk/MAP kinase in Y1 cells have suggested a role for cAMP in this process. In attempting to extend this work the ACTH responses in the human H295R cell line have been studied. This cell line makes only a very modest cAMP response to ACTH, yet the $Erk_{1/2}$ response is highly reproducible and immediate, but not prolonged. It is minimally reduced by the protein kinase A inhibitor, H89, but unaffected by PKC and calcium inhibitors. Inhibition of EGF receptor or other tyrosine kinase receptor transactivation was without effect, as was inhibition of c-Src activity or c-Src phosphorylation. The most effective inhibitor of this pathway was dansylcadaverine, an inhibitor of receptor internalisation. These findings imply that ACTH-induced $Erk_{1/2}$ activation in H295R cells is dependent on a mechanism distinct from that by which most G protein-coupled receptors activate $Erk_{1/2}$, but which nevertheless seems to depend on receptor internalisation.

ACKNOWLEDGEMENTS

I would firstly like to thank my supervisor Adrian Clark for his patience, support and enthusiasm for the project. I am also very grateful to Julian Challis, my current employer, who has regularly encouraged me to complete the thesis and has given me time off in order to do so. Although my career is no longer in science, I'm so glad to have spent three years in the lab and met the people I have met, who made life in the lab so enjoyable. Finally I would like to thank my mum and dad for their continued love and support.

This thesis is dedicated to the memory of David Newman, who, with his enthusiasm for science and encouragement during my placement year, inspired me to undertake this PhD.

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LIST OF ABBREVIATIONS

AC	Adenylate cyclase
ACTH	Adrenocorticotropic Hormone
AP	Adaptor protein
AT	Angiotensin
ARF	ADP ribosylation factor
BR	Bacteriorhodopsin
cAMP	cyclic adenosine monophosphate
CRE	cAMP response element
CYP	Cytochrome P450
DAG	Diacyl glycerol
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
$ERK_{1/2}$	Extracellular signal related kinase
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
GAP	GTPase activating protein

GDP	Guanosine diphospate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GIT	GTP interacting protein
GPCR	G-protein coupled receptor
GRK	GPCR kinase
GTP	Guanosine triphospate
HRP	Horse radish peroidase
IGF	Insulin-like growth factor
IP_3	Inositol phosphate 3
JNK	c-jun amino terminal kinases
LH	Leutinizing hormone
LPA	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MC2R	Melanocortin 2 receptor
MDC	Monodansylcadaverine
MKP	MAPK phosphatise
MMP	Matrix metalloprotease
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PH	Pleckstrin homology
P13-K	Phosphatidyl-inositol 3 kinase
PIP	Phosphatidyl inositol 3,4,5 triphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
POMC	Proopiomelanocortin
PTX	Pertussis toxin
RNA	Ribonucleic acid
SAPK	Stress activated protein kinase

SF1	Steroidogenic factor 1
SFM	Serum free medium
StAR	Steroidogenic acute regulatory protein
TBS	Tris buffered saline
TNF	Tumour necrosis factor
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
ZF	Zona fasciculata
ZG	Zona glomerulosa
ZR	Zona reticularis

1 INTRODUCTION

1.1 PRO-OPIOMELANOCORTIN (POMC)

The POMC gene encodes a 32 kDa propeptide molecule that is extensively post-translationally modified to produce a number of bioactive hormones (Fig. 1.1). The major site of expression of POMC is in the corticotroph cells of the anterior pituitary however POMC mRNA has been detected in extra-pituitary sites such as neurons of the hypothalamic arcuate nucleus as well as in some peripheral tissues. Many of the roles of POMC peptides in the brain have been elucidated, however their involvement in peripheral tissues is less clear since an alternative, shorter transcript is expressed which does not contain the signal sequence required for targeting of the peptide to the endoplasmic reticulum and its subsequent processing (DeBold et al., 1988). The processing of POMC occurs by proteolytic cleavage in secretory granules by a family of serine proteases, the pro-hormone convertases, which cleave specifically at dibasic amino acid residues (Cool et al., 1997). Processing depends on the tissue specific expression of the prohormone convertase enzymes thus yielding different active peptides in different tissues.

In the anterior pituitary, POMC is cleaved by pro-hormone convertase 1 into pro-adrenocorticotrophic hormone (pro-ACTH) and β-lipotrophin (β-LPH (Fig. 1.1) Pro-ACTH is then further cleaved to yield N-pro-opiocortin (N-POC), joining peptide (JP) and the 4.5 kDa hormone ACTH (White and Gibson, 1998). ACTH is the largest circulating POMC product and its primary role in steroidogenesis is well understood (Haynes, 1958). The role of N-POC is less clear, however it is thought to potentiate ACTH-induced steroidogenesis in human and rat adrenocortical cells and may have a role in adrenal growth which is discussed in section 1.6.5 (Bicknell et al., 2001).

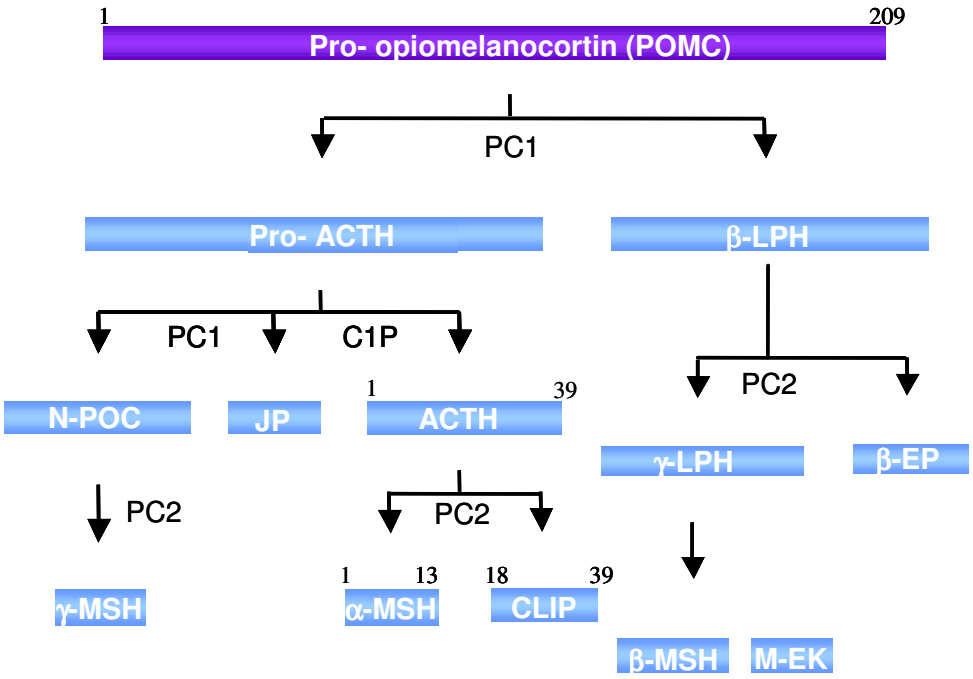


Fig. 1.1 *Post-translational processing of the POMC peptide. ACTH (adrenocorticotrophic hormone), LPH (lipotrophic hormone), N-POC (N-pro-opiocortin), JP (joining peptide),EP (endorphin), CLIP (corticotrophin-like intermediary peptide), MSH (melanocyte stimulating hormone), M-EK (met-enkephalin).*

In extra-pituitary sites however such as the neurons of the hypothalamus, expression of pro-hormone convertase 2 results in further processing of ACTH to α-melanocyte stimulating hormone (α-MSH) which has a key role in appetite control and energy homeostasis (Pritchard et al., 2002). Further processing of N-POC yields γ-MSH, a hormone thought to have effects on centrally mediated cardiovascular control (Li et al., 1996). Other melanocortin peptides in the periphery have diverse biological functions such as the control of skin pigmentation (Abdel-Malek et al., 1999) and the stimulation of adipocyte lipolysis (Boston, 1999).

1.2 THE HYPOTHALMIC-PITUITARY (HPA) AXIS

ACTH, a 39 amino acid peptide was discovered in the 1950's to be a factor secreted from the pituitary gland that was able to regulate steroidogenesis in the adrenal gland (Li et al., 1954). ACTH is secreted from the anterior pituitary in an episodic manner, with a distinct circadian rhythm. In addition to this it is secreted in response to stress. Its plasma levels peak between 6 and 9 am and fall during the day to reach a low point in the evening.

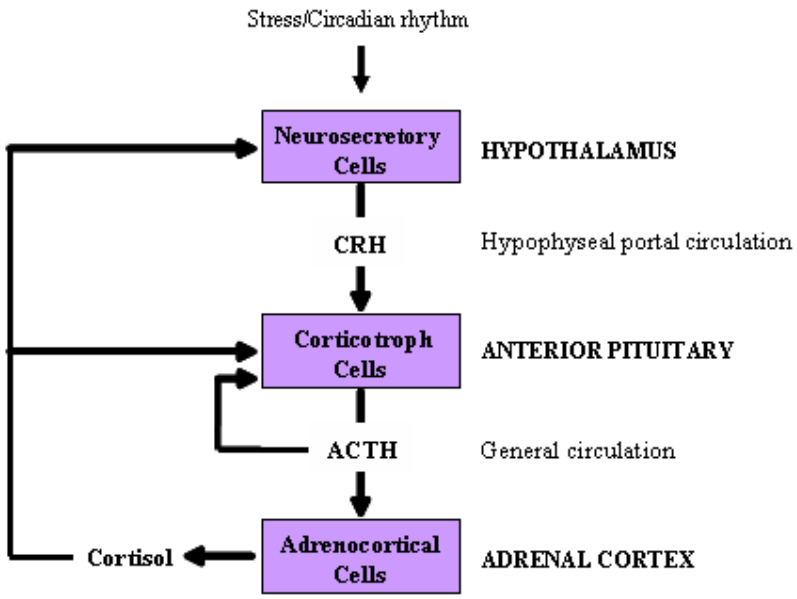


Fig. 1.2 - The hypothalamic-pituitary-adrenal (HPA) axis*. Stress and circadian rhythm induce the secretion of CRH from the hypothalamus. CRH then stimulates the secretion of ACTH from corticotroph cells in the anterior pituitary and this circulates in the blood. ACTH subsequently stimulates cortisol production and release form the adrenal cortex. An inhibitory feedback mechanism exists where cortiosl prevents the release of CRH and ACTH. ACTH also negatively regulates its own synthesis in corticotroph cells.*

ACTH secretion is stimulated by the release of corticotrophic releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus, as well as by arginine vasopressin and certain neurotransmitters (Vinson et al., 1992). CRH also acts to increase the expression of POMC by stimulating cAMP accumulation through its receptor and consequently serine phosphorylation of the cAMP response element

binding protein (CREB) transcription factor. ACTH circulates in the blood and acts on the adrenal gland through the ACTH receptor, to regulate the synthesis and secretion of steroids. This pathway is referred to as the HPA axis and is under tight regulation by a negative feedback loop whereby cortisol inhibits the release of CRH from the hypothalamus and also negatively feeds back to the pituitary to prevent ACTH release.

1.3 THE ADRENAL GLAND AND STEROIDOGENESIS

The adrenal gland consists of two regions formed from distinct embryological origins both of which are involved in coordinating the stress response. The inner medulla region is derived from the neural crest, a sympathetic ganglion modified to secrete its neurotransmitter, primarily adrenaline into the blood. The adrenal cortex develops from the mesoepithelium of the abdominal cavity and during embryogenesis, envelops the medulla to form the adrenal gland. The adrenal cortex responds to a number of circulating peptides to produce glucocorticoids, androgens and mineralocorticoids.

The adrenal cortex itself comprises three histologically distinct functional zones, which alternately express various enzyme systems enabling the production of different steroids. The outer most zone, the zona glomerulosa (ZG), secretes the mineralocorticoid aldosterone which acts to stimulate sodium (and thus water) reabsorption in the renal distal tubule in times of hypovolemia and hyponatremia. Bordering this zone is the zona fasciculata (ZF) which secretes glucocorticoids such as cortisol in response to ACTH. Cortisol functions at times of stress to provide substrates for energy production (Wajchenberg et al., 1984). It achieves this in a number of ways. Firstly, by inhibiting protein synthesis and at high concentrations stimulating protein degradation, thus providing amino acid precursors for glucose production. Secondly, it promotes lipolysis thus providing glycerol for gluconeogenesis (White and Engel, 1958, Ramachandran and Lee, 1976). Thirdly, enzymes critical in reversing glycolysis are upregulated through the action of

cortisol on the glucocorticoid receptor (Lucas and Granner, 1992). Glucocorticoids are also potent anti-inflammatory agents (Getting, 2002). Bordering the ZF is the zona reticularis (ZR) which secretes adrenal androgens. These act as pre-cursors which are later released into the circulation and produce the sex steroids. Fig. 1.3 depicts the steroidogenic pathways present in the different zones of the adrenal cortex where the expression of the enzymes CYP11B1, CYP11B2 and 17, 20 lyase are important in determining the types of steroid produced (Keegan and Hammer, 2002).

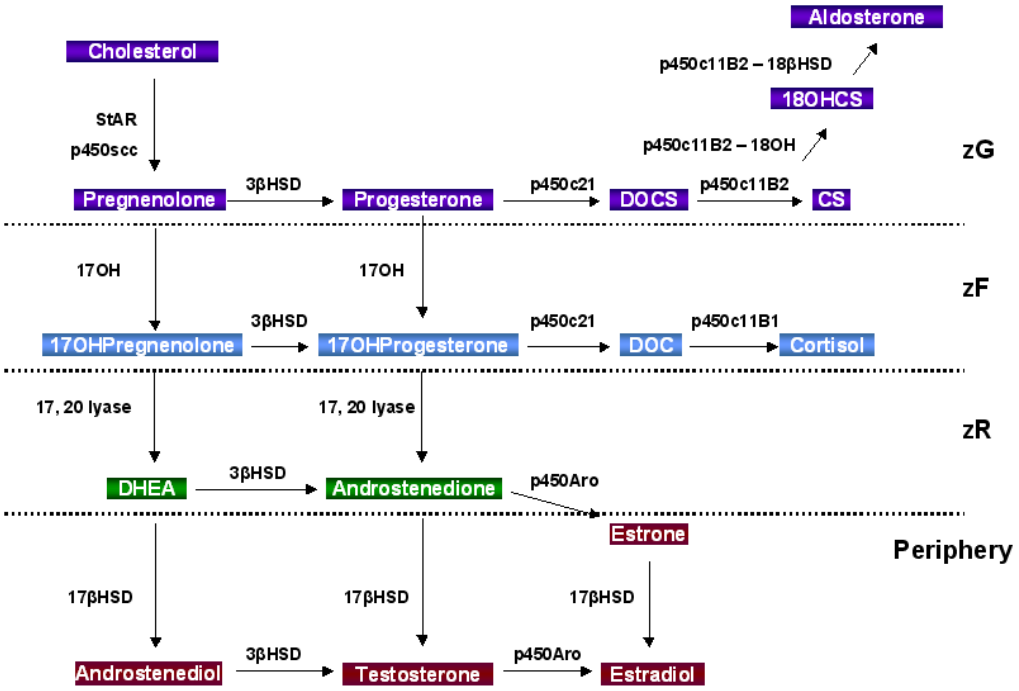


Fig. 1.3 Steroidogenesis in the adrenal cortex *(Adapted from Keegan CE, 2002, Trends In Endo and Metabolism). DHEA (dehhydroepiandrosterone), DOCS (deoxycorticosterone), HCS (hydroxycorticosterone), CS(corticosterone), DOC (deoxycortisol).*

1.4 ACTH AND THE ACTH RECEPTOR

ACTH mediates its steroidogenic response through binding to the ACTH receptor otherwise known as the Melanocortin 2 receptor (MC2R), which is expressed

mainly in the ZF of the adrenal cortex. Early studies looking at the effects of ACTH on the adrenal cortex in terms of increasing phosphorylase activity (now known as protein kinase A or PKA), found this effect to be mediated through cAMP, similar to the stimulation of hepatic phosphorylase by epinephrine (Haynes, 1958). Later experiments demonstrated specific binding of radiolabelled ACTH (I^{125}-ACTH) in adrenal cortex extracts that contained adenylate cyclase (Lefkowitz et al., 1970). However it wasn't until 1992 that the ACTH receptor was isolated. The human ACTH receptor was first cloned in parallel with another melanocortin receptor, the MC1R. cDNA derived from a human melanoma specimen that was known to contain a high number of MSH binding sites, was used in a PCR with degenerate primers for conserved transmembrane domains of G-protein coupled receptors (GPCRs are discussed in further detail in section 1.5) (Mountjoy et al., 1992). The two resultant PCR subclones were found to be expressed in melanocytes and the adrenal cortex, the second of which was probably the result of genomic DNA contamination of the melanoma specimen. These receptors were subsequently named the MC1R and MC2R respectively. In addition to these receptors, three further melanocortin receptors have been cloned and named the MC3, 4 and 5 receptors (Gantz et al., 1993b, Gantz et al., 1993a, Gantz et al., 1994).

The MC2R is the smallest of all the GPCRs and consists of 297 amino acid residues in the mature human protein. In its unmodified state the MC2R is predicted to be 33 kDa in size, however it has two N-linked glycosylation sites in the N-terminal domain and is expressed at the cell surface as a 43 kDa glycoprotein (Hofmann et al., 1988). Functional characterisation of the MC2R protein was initially difficult because unlike the MC1R which could be readily and functionally expressed in 293 cells, the MC2R could only be expressed in cells expressing other melanocortin receptors (Mountjoy et al., 1992). Recent studies in our laboratory have confirmed the requirement of a factor named MRAP that enables functional expression of the MC2R in non adrenal cells (Metherell et al., 2005). However, this problem complicated earlier binding studies because of the presence of other melanocortin receptors capable of binding ACTH. It was later demonstrated that the murine Y1 cell line (Yasumura et al., 1966) expressed a high affinity ACTH-receptor , with an

IC_{50} of 0.13nM (see table 1.1) which did not bind to MSH peptides (Schioth et al., 1996). MSH binding could be restored on expression of the MC1, 3 and 4 receptors in these cells. These studies were important in recognising the MC2R as the ACTH receptor and the high affinity and ACTH exclusive properties of this receptor were consistent with the very specific role of ACTH in regulating steroidogenesis.

1.4.1 The Melanocortin receptors

The melanocortin receptors have affinities for various members of the POMC peptides. MC1, 3, 4 and 5 receptors for example bind with the highest affinity to α-MSH but also with a much lower affinity to ACTH. Conversely, the MC2R binds solely, with very high affinity to ACTH (Schioth et al., 1996). Table 1.1 shows the affinity of naturally occurring POMC peptides to the melanocortin receptors.

LIGAND	**RECEPTOR**				
	MC1R	**MC2R**	**MC3R**	**MC4R**	**MC5R**
ACTH (1-39)	3.95	0.13	135	2,170	4,920
α-MSH	0.12	-	31	660	5,700
β-MSH	1.2	-	13	380	14,000
γ-MSH	2.7	-	7.1	29,000	43,000
TRH	6,400	-	-	-	-

Table 1.1 Melanocortin receptor binding characteristics. *K_i values (nm) of natural POMC peptides for human melanocortin receptors obtained for ACTH, in competition with I^{125}ACTH and for the remainder, in competition with I^{125}NDP-MSH (Schioth et al., 1997, Schioth et al., 1996, Wikberg et al., 2000).*

The melanocortn receptors are distributed across a large number of different tissues. This is in accordance with the wide ranging effects of the melanocortins themselves. The MC1R and MC2R are expressed primarily in melanocytes and the adrenal gland respectively however, the other melanocortin receptors are more

widely distributed where they are expressed in both central and peripheral tissues. Table 1.2 summarises the expression pattern of the melanocortin receptors along with their reported functions.

RECEPTOR	TISSUE EXPRESSION	PRIMARY FUNCTION
MC1R	Melanocytes, melanoma, macrophages, adipose tissue	Pigmentation, inflammation
MC2R	Adrenal, adipose tissue, fetal testis, pituitary	Steroidogenesis
MC3R	Brain, placenta, duodenum, pancreas, stomach	Energy homeostasis
MC4R	Brain, spinal cord, muscle, sympathetic nervous system	Energy homeostasis, erectile function
MC5R	Brain, skin, adrenal, spleen, thymus, testis, ovary, muscle, adipose tissue	Sebaceous gland secretion

Table 1.2 *Properties of the melanocortin receptors (Mountjoy and Wong, 1997, Gantz and Fong, 2003).*

1.4.2 MC2R gene structure and expression

The coding sequence for the human MC2R is contained within a single exon. However, the mature mRNA is composed of multiple exons, which are spliced together to form the 5' untranslated region (5'UTR), upstream of the coding sequence (Fig. 1.4). The majority of human transcripts contain a single upstream exon however in some mRNA species, an alternatively spliced second exon is also present (Kubo et al., 2000). The primary site of expression of the MC2R is in the adrenal cortex as demonstrated by northern blot and in situ hybridisation (Mountjoy et al., 1992). This finding was consistent with the highly conserved role of ACTH in adrenal steroidogenesis. ACTH may also play a role in the maintenance of growth of the adrenal cortex however this will be discussed in greater detail in section 1.6. MC2R mRNA has also been detected in an increasing number of extra-

adrenal sites. These included the detection of mRNA in sympathetic ganglia, where it is upregulated by stress (Nankova et al., 2003) subcutaneous human adipocytes have been shown to express MC2R mRNA (Smith et al., 2003). MC2R mRNA has also been reported in human skin however it was not clear from which cell type the mRNA originated (Slominski et al., 1996). MC2R mRNA was detected in the pituitary gland where it may function as part of a short negative feedback loop, regulating ACTH secretion from corticotroph cells (Morris et al., 2003).

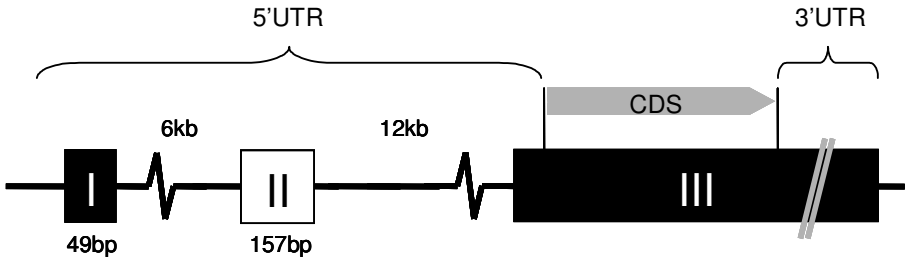


Fig. 1.4 Genomic organisation of the human MC2R*. Exons are drawn as boxes. Exons 1 and 2 are constitutively expressed whereas exon 2 is alternatively spliced The sizes of the exons are indicated in addition to the approximate length of the intronic sequences. CDS indicates the coding sequence.*

Much research has also been undertaken in murine cells where significant amounts of mRNA have been detected in the murine adipocyte where it may play a developmental role (Boston and Cone, 1996). Furthermore, more recently murine leydig cells were found to express the MC2R and respond to ACTH stimulation by secretion of androgens, a response that is absent in adult testes (O'Shaughnessy et al., 2003).

1.5 G-PROTEIN COUPLED RECEPTORS (GPCRS)

1.5.1 Structure

Bacteriorhodopsin (BR) was the first GPCR to be structurally characterised using X-ray crystallography (Muller, 2000) however the elucidation of the structure of other GPCRs by this technique has been problematic since GPCRs are difficult to purify and dissolve. For this reason, structural modelling of most other GPCRs has been based on homology with this receptor. GPCRs are a large and diverse family of receptors which have a unique structural conformation within the phospholipid bilayer. The peptide chain contains seven regions of alpha-helical transmembrane domains rich in hydrophobic residues which allows the receptor to span the membrane seven times. All GPCRs have an extracellular amino (N)-terminal domain and an intracellular carboxy (C)-terminal domain, in addition to three hydrophilic extracellular and intracellular loops. The MC2R belongs to this diverse family of receptors and is depicted in fig. 1.5.

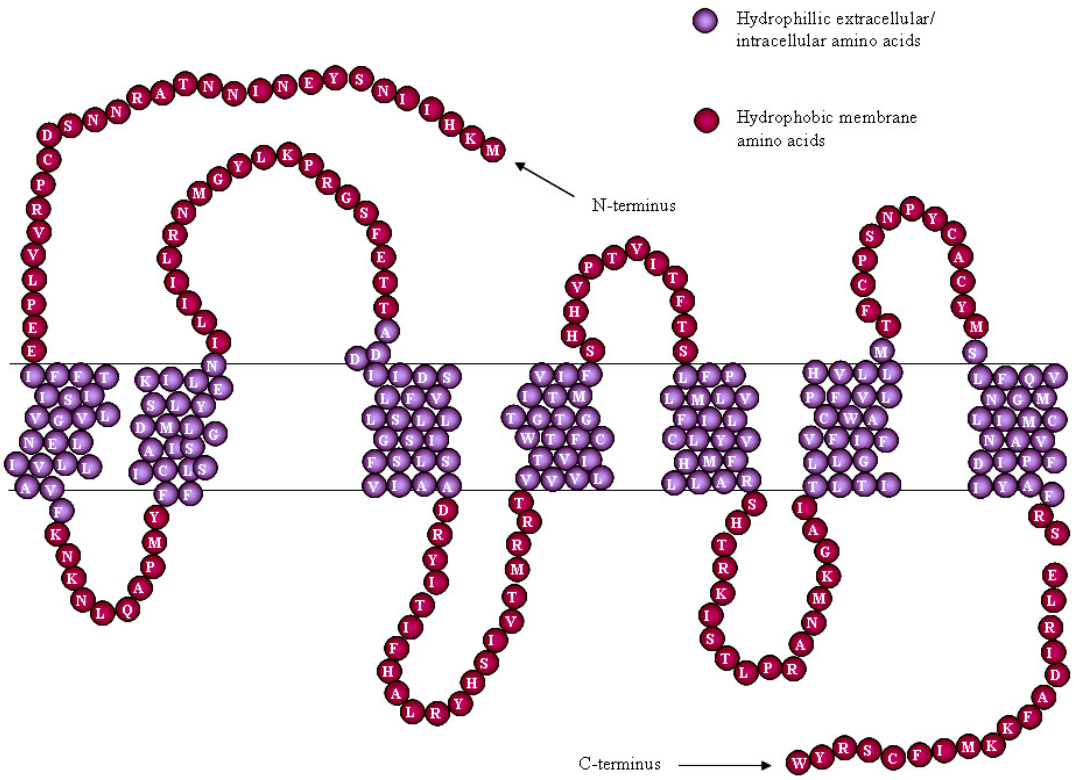


Fig. 1.5 The human ACTH receptor. *The human MC2R is thought to structurally resemble that of the GPCR rhodopsin. It comprises an extracellular N-terminal domain, an intracellular C-terminal tail, three intracellular and three extracellular loops and seven hydrophic transmembrane spanning regions.*

1.5.2 Signalling

GPCRs are so called because they interact with GTP-binding proteins (G-proteins) following agonist stimulation, and this results in the activation or inactivation of an enzyme to mediate a signalling response. GPCRs have very conserved signalling mechanisms. The signalling pathway that follows the binding of an agonist to a GPCR depends on the G-protein with which the receptor interacts. In their inactive state G-proteins consist of a heterotrimeric complex made up of α, β and γ subunits where the α subunit interacts at the cell membrane with the GPCR and is

bound to a GDP molecule. Following agonist binding to the GPCR, a conformational change occurs which allows a GDP/GTP exchange on the α sunbunit which consequently causes the α subunit to dissociate from the βγ subunits. The α subunit, along with the still tightly bound βγ subunits, are then free to bind to an effector molecule and induce a cellular response (Fig. 1.6).

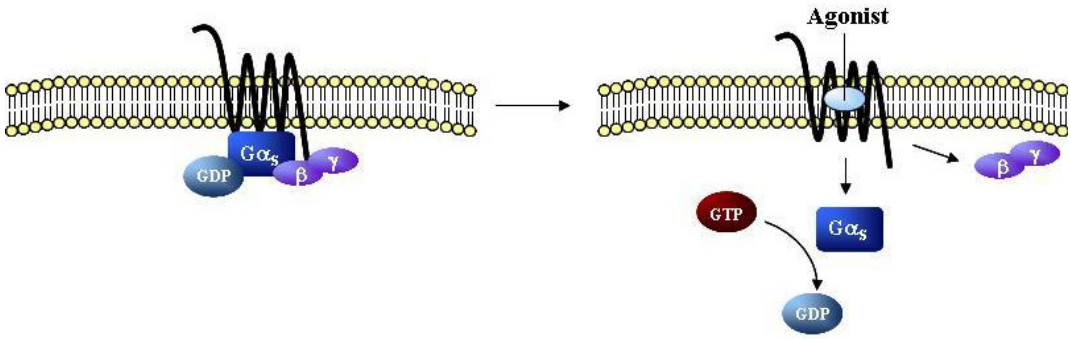


Fig 1.6 Agonist stimulation of GPCRs. *GPCRs in their inactive state remain bound to the heterotrimeric G protein complex consisting of α, β and γ subunits. Binding of the agonist to the GPCR causes a conformational change that leads to GDP/GTP exchange on the α subunit. This causes dissociation of α and βγ subunits which instigate cell signalling cascades.*

There are three major G-protein families which have different second messenger actions, these are G_s, $G_{i/o}$ and $G_{q/12}$. Additional specificity is conferred by the expression of multiple genes encoding both Gα and βγ subunits (Watson and Arkinstall, 1994). α subunits from G_s proteins bind to and activate the enzyme adenylate cyclase which catalyzes the synthesis of cyclic adenosine monophosphate (cAMP) from ATP. cAMP accumulates in the cell and activates various intracellular targets including PKA with a subsequent cascade of events involving cellular targets of PKA. It is through the activation of this pathway that ACTH regulates the synthesis and release of steroids. This classical signalling pathway is depicted in Fig.1.7.

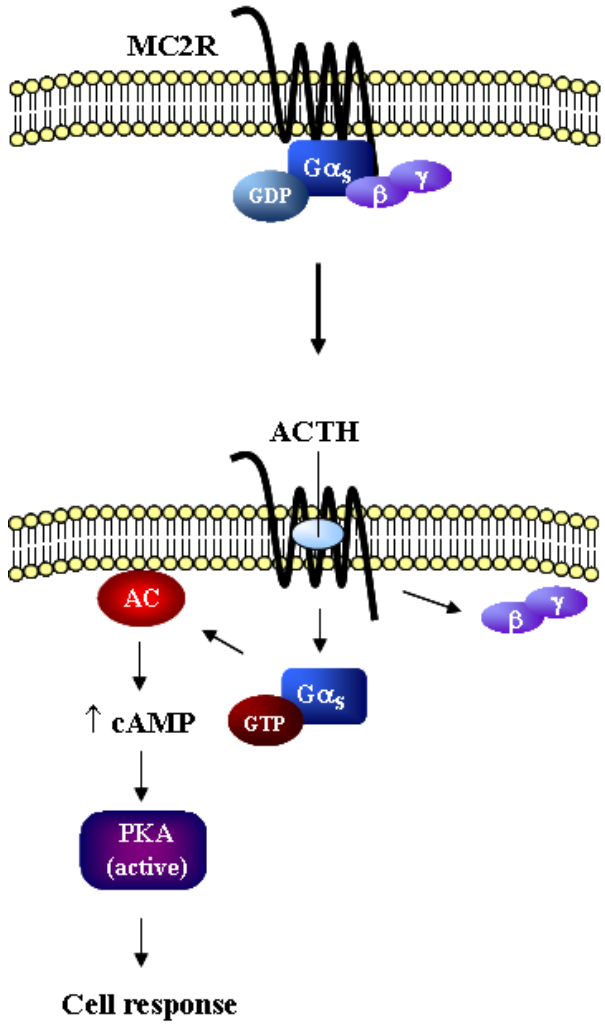


Fig. 1.7 Classical G_s coupled signalling pathway. *ACTH binding to the MC2R results in $G\alpha_s$ mediated activation of the adenylate cyclase (AC) enzyme which catalyses the production of cAMP from ATP which accumulates and activates PKA. PKA, in its active state, phosphoylates its cellular targets which ultimately result in steroidogenesis.*

Conversely, coupling of a receptor to a G_i protein results in the inactivation of adenylate cyclase and therefore has opposing effects within the cell to that of G_s signalling. G_q proteins couple to a different enzyme, phospholipase C (PLC). As a consequence of PLC activation there is increased production of inositol phosphate 3 (IP_3) which acts on IP_3 receptors on the endoplasmic reticulum (ER). Following this, Ca^{2+} is released from the ER leading to an increase in cytosolic Ca^{2+} concentration and the subsequent activation of protein kinase C (PKC) leading to a further cascade of events and a cellular effect. The PKC pathway is utilised in many

biological systems including endocrine systems such as the synthesis and release of gonadotrophins in the reproductive system (Ando et al., 2001).

Although GPCR signalling mechanisms are highly conserved, the specificity of the response is conferred by alternative gene expression in different cell types which then act as targets for the effector molecules (PKA and PKC) in these signalling pathways.

1.5.3 Targets of cAMP and PKA in adrenocortical cells

The cellular responses to ACTH in an adrenocortical cell are numerous and act at both the transcriptional and post-translational level to increase the steroidogenic capacity of the cell. One effect of PKA is the activation of CREB. CREB is a transcription factor which, when activated, can positively regulate the expression of genes containing a cAMP response element (CRE) in the promoter of the gene. In addition to later effects brought on by transcriptional activation, PKA also exerts more immediate effects through the phosphorylation of other cellular targets. One such target is cholesterol ester hydroxylase. This enzyme converts cholesterol esters to free cholesterol and therefore increases the availability of the steroid hormone substrate (Liscum and Dahl, 1992). The rate-limiting step in the production of all steroid hormones is the conversion of cholesterol to pregnenolone, which is catalysed by CYP11A1 (cytochrome P450 11A1) in the inner mitochondrial membrane. For this reaction to take place, cholesterol must be mobilised from intracellular stores and translocated across the mitochondrial membrane by steroidogenic acute regulatory protein (StAR). StAR is rapidly upregulated following ACTH induced cAMP/PKA signalling thus allowing this process to take place (Clark and Combs, 1999). The ACTH induced activation of this second messenger signalling pathway also contributes to the upregulation of all steroidogenic CYP enzymes which are involved in converting pregnenolone to steroid hormones (John et al., 1986). A further level of regulation is in the upregulation of MC2R expression

levels which increases the sensitivity of cells to ACTH and thus steroidogenic capacity (Penhoat et al., 1989b).

Many of the effects seen in reponse to ACTH are the result of the an increase in expression of a transcription factor known as steroidogenic factor 1 (SF1) which binds to the promoter regions of many of the steroidogenic CYP genes (Morohashi et al., 1992, Clark and Combs, 1999, Naville et al., 1999). However, it is still unclear as to what mechanism SF1 is activated although it is thought to be PKA dependent and may also involve activation by the mitogen activated protein kinase (MAPK) family (Gyles et al., 2001).

1.5.4 Modifying agonist/receptor responses

In every signalling cascade, feedback mechanisms exist whereby in some way a response is dampened following continued stimulation of a pathway and conversely, a response is amplified in situations where the pathway is under active. These mechanisms allows for tight regulation of signalling pathways so that appropriate responses are mounted. For GPCRs, these processes include those that allow a rapid modification in the magnitude of a response by altering the ability of the receptor to signal during continued agonist stimulation, termed "desensitisation"; modifying the number of receptors at the cell surface by vesicular uptake mechanisms, termed "internalisation"; as well as longer-term mechanisms of receptor regulation which may involve receptor degradation or the down-regulation of receptor gene expression. Long term regulation of receptor number is illustrated in the ACTH-MC2R system, ACTH was shown to positively regulate MC2R cell surface expression following ACTH treatment for up to 48 hours of bovine fasciculata cells (Penhoat et al., 1989a, Lebrethon et al., 1994, Mountjoy et al., 1994).

1.5.5 *Desensitisation*

The most common mechanisms of desensitisation of GPCR signalling pathways are post-translational modifications to the receptor. Phosphorylation by intracellular kinases such as PKA or GRK results in the attenuation of receptor G protein coupling which dampens the signalling response. However, additional mechanisms exist whereby regulation of the coupling to G proteins is affected which has the same result in that the receptor is densenstised. An example of this is in the interaction of the regulators of G protein signalling (RGS) family members which alter the rate of GTP hydrolysis on G proteins and therefore modulates the signalling to effector enzymes (De Vries et al., 2000, Ross and Wilkie, 2000).

1.5.5.1 Second-messenger protein kinase regulation

Second-messenger protein kinases such as PKA and PKC phosphorylate receptors on serine and threonine residues within specific amino acid consensus sites. This leads to the desensitisation of the response in a classical negative feedback mechanism due to the altering of the receptors affinity for its ligand. This method of desensitisation is termed heterologous because the activation of one receptor can result in the non-specific phosphorylation of agoinst occupied and unoccupied receptors of different types (Hausdorff et al., 1989, Lohse et al., 1990).

In addition to altering the receptors affinity for its ligand, PKA mediated phosphorylation has also been shown to mediate desenstisation of the cAMP response by altering the β_2 AR receptors affinity for G_s and cause a switch in coupling to G_i. This causes inactivation of adenylate cyclase allowing a more rapid dampening of the response (Daaka et al., 1997).

1.5.5.2 GRK-mediated regulation

The GRKs are a family of receptor kinases composed of seven members which share significant sequence homology and functional organisation. Each have a central catalytic domain, an amino terminal domain (which also contains an RGS-like domain) and a c-terminal domain that allows plasma membrane targeting. GRK1 and 7 are specifically expressed in the retina and are farnesylated on their c-terminal domains; GRK2 and 3 share added similarity in their c-terminus each containing a βγ-subunit binding domain with homology to PH domains (Pitcher et al., 1992); GRK5 has a c-terminal region thought to mediate plasma membrane interactions (Kunapuli et al., 1994, Kunapuli and Benovic, 1993); GRK4 and 6 are palmitoylated at cysteine residues which is also thought to allow their localization at the plasma membrane (Stoffel et al., 1994, Stoffel et al., 1998).

The GRKs mediate homologous desensitisation whereby agonist-occupied receptors are phosphorylated leading to desensitisation of only activated receptors (Krupnick and Benovic, 1998). GRK mediated desensitisation also commonly involves receptor interaction with arrestins. Phosphorylation of the receptor by a GRK increases the affinity of β-arrestin for the receptor by 10-30 fold (Pippig et al., 1993). This results in the steric hinderance of GPCR/G-protein coupling and the subsequent uncoupling of the receptor from the G-protein (Benovic et al., 1987, Ferguson et al., 1996b).

1.5.5.3 Arrestin mediated regulation

The Arrestins comprise a family of four proteins, two of which are expressed exclusively in the retina (arrestin 1 and 4) and two that are ubiquitously expressed and are termed β-arrestin 1 and 2 (or arrestin 2 and 3) (Krupnick and Benovic, 1998). ß-arrestins are thought to regulate most of the GPCRs (Attramadal et al., 1992). Their localisation, established using green flourescent protein (GFP) tagged ß-arrestins, is cytosolic in the resting state with translocation to the cell membrane on agonist stimulation (Zhang et al., 1999). The binding of ß-arrestins to

phosphorylated receptors sterically hinders receptor, G-protein coupling and prevents further GTP/GDP exchange in the presence of continued agonist exposure. ß-arrestin further acts to modulate a cAMP response by recruiting phosphodieterases to the membrane which accelerate the degradation of cAMP (Perry et al., 2002).

Although desensitisation by second-messenger protein kinases and GRKs were originally thought to be by distinct mechanisms, recent evidence has suggested some overlap between these two pathways. GPCRs have been shown to spontaneously move to an activated conformation in the absence of agonist suggesting that GRK can act in agonist unoccupied or constitutive states (Pei et al., 1994, Rim and Oprian, 1995). In addition, inhibition of either PKA or GRK results in the complete abolition of olfactory receptor desensitisation suggesting interplay between these two kinases (Schleicher et al., 1993). This is supported by a report demonstrating that PKA and PKC can phosphorylate and activate GRK2 by promoting its $\beta\gamma$ mediated membrane association (Winstel et al., 1996, Cong et al., 2001).

1.5.6 Internalisation of GPCRs

Internalisation or endocytosis of GPCRs is a further mechanism of regulation of receptor signalling. The first evidence of this process was the observation that β-AR was lost from the surface of frog erythrocytes with a corresponding increase in cytosolic binding sites following continued agonist stimulation (Chuang and Costa, 1979). This was supported by a study demonstrating β_2-AR sites in different fractions of a sucrose gradient (Harden et al., 1980). Since then numerous studies have shown the internalisation of a variety of GPCRs visualized using green fluorescent protein (GFP) tagged receptors transfected into cells (Barak et al., 1997, Hudson et al., 2006).

Several mechanisms of internalisation have been elucidated which include, internalisation by clathrin-coated pits (Trowbridge et al., 1993), caveolae

(Anderson, 1993), ubiquitin dependent processes (Katzmann et al., 2002) and non-coated vesicles (Montesano et al., 1982). By far the most common and best characterised mechanism of internalisation is clathrin-mediated endocytosis.

1.5.6.1 Clathrin mediated endocytosis

Clathrin is a fibrous protein which forms triskelions that polymerize to form a polygonal lattice structure with an intrinsic curvature. These so-called clathrin coated pits are assembled by adapter proteins (AP) which have also been shown to bind to membrane proteins to determine which proteins are specifically included within the vesicle (Lodish, 1999). Continued polymerization causes an invagination within the membrane which is then pinched off by the GTPase dynamin thus forming a vesicle and internalising all the components within the region (Damke et al., 1994). This mechanism was first identified for the LDL Receptor (Anderson et al., 1977) however many other GPCRs have also been shown to internalise via clathrin-coated vesicles.

GRKs and ß-arrestins, in addition to desensitising receptors, also regulate the endocytic process by allowing the recruitment of the GPCR to clathrin coated vesicles through direct binding to proteins involved in the process (Shiina et al., 2001). GRK was also shown to bind phosphatidyl-inositol 3 kinase (PI3-K) and recruit it to the cell membrane in an agonist dependent manner where it contributed to receptor internalisation (Naga Prasad et al., 2001). A small GTP binding protein family, ADP ribosylation factor (ARF) is a well-known mediator of vesicular trafficking and receptor internalisation. This proteins GTPase activity is accelerated by the GTPase activating protein (GAP) activity of GRK interactor (GIT) protein which, as its name suggest has been demonstrated to interact with GRK (Premont et al., 2000, Premont et al., 1998). The link between receptor internalisation and ARF were demonstrated by the overexpression of GIT 1 and 2 which reduced receptor endocytosis (Claing et al., 2000).

β-arrestins also serve as adapters to link the receptor to the endocytic machinery (Goodman et al., 1996). Interaction with phosphorylated receptors causes a conformational change in ß-arrestin which exposes a clathrin binding region (Krupnick et al., 1997) and an AP2 binding region (Laporte et al., 2000) to facilitate targeting to the clathrin coated pit.

1.5.6.2 Alternative mechanisms of internalisation

Lipid raft mediated internalisation has attracted much interest in recent years. Lipid rafts are regions of the plasma membrane characterized by an abundance of cholesterol and sphingolipids (Lajoie and Nabi, 2007). These microdomains are more rigid and are detergent insoluble. It is thought that lipid rafts act to compartmentalise complexes of interacting molecules and thereby serve as signalling platforms. One specific type of lipid raft termed caveolae, are flask shaped invaginations characterized by the presence of caveolin-1, a cholesterol binding membrane protein (Rothberg et al., 1992). Dynamin has been shown to mediate the budding of caveolae vesicles in addition to clathrin coated vesicles (Oh et al., 1998).

1.5.7 MC2R desensitisation and internalisation

Desensitisation of the MC2R in the Y1 adrenocortical cell line has been previously investigated in our laboratory (Baig et al., 2001). Experiments suggested a heterologous mechanism of densensitisation whereby phosphorylation of the receptor by PKA uncoupled the receptor from its G-protein. The PKA antagonist H89 was shown to almost completely eradicate the early phase of densensitisation and in addition, when a ser-208 mutant was transfected into Y6 cells (a mutant variant of the Y1 cell line which does not express the MC2R), a similar effect on desensitisation was shown to that of H89 treated Y1 cells. However, treatment of Y1 cells with the β_2 agonist isoproteronol, which caused an increase in cAMP similar to that of ACTH treatment, did not densensitise the ACTH response,

suggestive of a homologous mechanism with the possible involvement of a GRK. These observations are in support of overlapping mechanisms of heterologous and homologous desensitisation as previously discussed (section 1.5.5.3).

Additional experiments in our lab also demonstrated the internalisation of the MC2R by a mechanism most likely to be dependent on clathrin-mediated endocytosis since internalisation could be significantly modulated after pre-treatment with hypertonic sucrose, an inhibitor of internalisation (Baig et al., 2002). In contrast to many other GPCRs however, internalisation of the MC2R is thought to be limited to a small population of receptors since only around 20 % of cell surface receptors internalised following ACTH treatment. In addition, internalisation of the MC2R is slow with maximum internalisation after 1 hour. By contrast, the β-adrenergic receptor and AT1A receptor internalise completely 5 mins after the addition of their respective agonists (Hunyady, 1999).

1.5.8 Non-classical roles for GPCRs

The activation of second messenger signalling cascades by agonist bound GPCRs through interactions with G-proteins, are generally very well characterised, in addition to the cellular effects of these pathways. However, in the last 20 years, alternative roles for GPCRs have emerged where in addition to classical second messenger signalling pathways, other signalling cascades are activated leading to effects such as proliferation, differentiation and apoptosis (Gu et al., 2000, Rozengurt, 1986, van Biesen et al., 1996). Gene knock-out studies have demonstrated that many GPCRs are necessary for cell growth under physiological conditions (Nagata et al., 1996). Further support for the proliferative roles of GPCRs is the observation that activating mutations in GPCRs and G-proteins have been linked to several disease states including cancer. Constitutively activated $G_{\alpha s}$ leads to hyperplasia of endocrine cells found in pituitary and thyroid tumours (Dhanasekaran et al., 1995). Furthermore, a number of GPCRs have been shown to

transform contact inhibited cultures of rodent fibrobalsts when persistantly activated (Allen et al., 1991, Gutkind et al., 1991, Julius et al., 1989).

A further non classical cellular effect of GPCR agonists has been demonstrated for β-adrenergic receptors in S49 cells and for the follicle-stimulating hormone (FSH) receptor, where binding of their respective ligands results in the induction of apoptosis (Gu et al., 2000, Amsterdam et al., 1998).

It has long been debated that in addition to the major role of ACTH in steroidogenesis, ACTH may also be involved in the regulation of growth of the adrenal cortex. Although the mechanisms by which ACTH mediates these effects are not fully elucidated, there may be a direct effect of ACTH on the activation of mitogenic signalling pathways which lead to proliferation of adrenal cells as has been demonstrated for other GPCRs.

1.6 ACTH IN THE REGULATION OF ADRENAL GROWTH

There are a number of lines of evidence that support a growth promoting role of ACTH on the adrenal. These include clinical observations of syndromes of ACTH excess and ACTH deficiency, *in vivo* studies in rats looking at the effects of ACTH on adrenal weight and cell number, and the reversal of adrenal atrophy by ACTH administration to hypophysectomised rats (Farese and Reddy, 1963a, White et al., 1987a, White et al., 1987b, Bransome and Reddy, 1964, Farese and Reddy, 1963b, Nussdorfer et al., 1973). Equally, in many studies ACTH has been demonstrated to have an anti-mitogenic effect on the adrenal cortex. The majority of this evidence arises from *in vitro* studies where ACTH inhibits the growth of primary cultures and cell lines (Gospodarowicz et al., 1977) however, there is also further *in vivo* evidence from a model of adrenal compensatory growth which suggests that a factor other than ACTH is responsible for the rapid growth of the remaining adrenal gland that arises in this model (Engeland et al., 1975).

1.6.1 Clinical observations

Clinicians have long held the view that ACTH plays a role in the regulation of adrenal cortex growth, mainly due to the correlation between levels of circulating ACTH and adrenal cortex size. This is clearly demonstrated in a well-characterised in-born error of steroidogenesis in humans, congenital adrenal hyperplasia, where there is a deficiency of cortisol production, leading to chronic elevations in circulating ACTH and resulting in hyperplasia of the adrenal cortex (New, 1998). Studies in our laboratory have shown constitutive activation of the MC2R in a point mutation present in a patient with ACTH-independent Cushings syndrome. The mutated receptor, when transfected into Y6 cells had impaired desensitisation and internalisation compared to wild type receptor leading to constitutive activation of the receptor, a possible mechanism for the adrenal hyperplasia seen in the patient (Swords et al., 2002). In addition, inactivating mutations in the ACTH receptor are associated with adrenocortical hypoplasia (Clark and Weber, 1998).

1.6.2 In vivo studies

1.6.2.1 Intact animals

Over the last 30 years, many studies have attempted to elucidate the growth or growth inhibitory effects of ACTH on the adrenal gland using several *in vivo* and *in vitro* models. Early studies in the 1960's supported the still widely held view that ACTH is involved in adrenal growth since administration of ACTH was found to increase adrenal weight, protein synthesis and nucleic acid content in rats (Bransome and Reddy, 1964, Farese and Reddy, 1963a). However, an increase in adrenal weight does not necessarily indicate a true proliferative effect on the adrenal gland. ACTH has been shown to cause a morphological change where cell size is greatly increased (Nussdorfer et al., 1978). This effect is hypertrophic, possibly caused by a rapid vasodilatory effect of ACTH on the adrenal gland where there is increased blood flow to the gland and thus increased volume (Vinson et al., 1992). One study measured changes in RNA and DNA content in adrenals following

ACTH administration (Dallman et al., 1980). An initial increase in RNA after 12 hours was seen but an increase in DNA and therefore cell number was only seen after 72 hours of treatment implying that this proliferative effect only occurs as a result of ACTH induced cellular changes. A review in 1984 by Dallman suggests that ACTH primarily causes adrenal hypertrophy followed by adrenal hyperplasia (Dallman, 1984).

Other studies looked at changes in cell number as a direct measure of proliferation. A series of studies by Nussdorfer and co-workers in the 1970's demonstrated that chronic treatment of rats with ACTH increased the number and volume of ZF cells where the effects on the other zones of the cortex were limited to increased cell volume (Belloni et al., 1978, Nussdorfer et al., 1977, Nussdorfer and Mazzocchi, 1971, Mazzocchi et al., 1976). Even after 36 days treatment, they found no proliferative effect on ZR cells (Mazzocchi et al., 1976). In a study carried out in hamsters, ACTH treatment was shown to increase cell number from day 3 by 50% in the ZF and by 58% in the ZR following 9 days of treatment however there was no change in ZG cell number (Malendowicz and Dembinska, 1990). This is supported by another *in vivo* study whereby the numbers of mitotic cells in the rat adrenal cortex in response to a synthetically made ACTH peptide (1-24) were measured (Payet and Lehoux, 1980). ACTH treatment caused a dose-dependent increase in mitoses in the ZG but not in the ZF however the increase in the size of the gland was due to the ZF and not the ZG. This suggests that newly proliferated cells in the ZG quickly differentiate into fasciculata cells.

1.6.2.2 Hypophysectomy

In the experimental hypophysectomy model, the pituitary gland is surgically removed. causing marked adrenal atrophy thus suggesting the requirement of one or more pituitary peptides in the maintenance of adrenal growth. In addition it was also demonstrated that administration of ACTH can reverse the adrenal atrophy caused by hypophysectomy (Nussdorfer et al., 1973). It stimulated growth and

steroidogenic capacity of the ZG and this effect could be mimicked by cAMP. Another study showed that ACTH induced adrenal gland regeneration following hypophysectomy, involves cellular proliferation (Tchen et al., 1977).

1.6.2.3 Adrenal compensatory model

The experimental model of compensatory adrenal growth following unilateral adrenolectomy in rats, whereby the remaining adrenal undergoes hypertrophy and hyperplasia, has been extensively used as an experimental model for adrenal growth in the adult. This model has provided most of the evidence against the role of ACTH as an adrenal mitogen. One group measured plasma ACTH concentrations and found that levels did not increase following adrenalectomy and thus could not account for the rapid growth of the remaining adrenal (Engeland et al., 1975). In the same study, it was demonstrated that compensatory adrenal hypertrophy occurred in adrenalectomised and hypophysectomised rats therefore suggesting that a pituitary peptide is not involved in this response. This was supported by two other reports, one which demonstrated that ACTH actually inhibited the growth response seen in adrenalectomised rats and the other which showed that administration of ACTH antiserum was able to reduce steroidogenesis but had no effect on adrenal weight (Dallman et al., 1980, Rao et al., 1978). This was confirmed more recently (Nakayama et al., 1993).

1.6.3 In vitro studies

In vitro cell culture studies often represent a cleaner system in which to study cell signalling mechanisms. However, earlier experiments that investigated the effects of ACTH *in vitro* were often on mixed populations of cells. One such study found that treatment of primary bovine adrenocortical cells with ACTH inhibited DNA synthesis and stimulated steroidogenesis and furthermore, that ACTH inhibited the growth stimulatory effect of FGF (Gospodarowicz et al., 1977). These findings

were confirmed in subsequent studies and a further study reported the same effect in adult and neonatal rat adrenal cells where ACTH inhibited thymidine incorporation (Hornsby and Gill, 1978, Ramachandran and Suyama, 1975, Simonian et al., 1982). In contrast to these findings one investigation found a significant increase in thymidine incorporation and in actual cell number in response to ACTH in adult rat inner zone cells (Armato and Nussdorfer, 1972). Arola et al however found a biphasic effect of ACTH on foetal rat adrenal cells where it was initially inhibitory of growth, a cAMP independent response, and then strongly stimulatory after 48 h, which was mimicked by cAMP analogues (Arola et al., 1993). Since the early work in the 1970's, groups have switched to using cell lines as supposedly more reproducible systems to try to determine the signalling mechanisms involved in the responses to ACTH.

1.6.3.1 The Y1 adrenocortical cell line

The Y1 cell line is an ACTH responsive, steroid secreting, clonal mouse cell line. First isolated in 1966 (Yasamura et al., 1966), it is a widely used cell line in studying the ACTH receptor and has been used extensively in recent years to look at mitogenic mechanisms in response to ACTH.

ACTH has been shown to inhibit cell proliferation in the Y1 adrenocortical cell line by interfering with progression through the G_1 phase of the cell cycle (Masui and Garren, 1971). This growth inhibitory effect has been further demonstrated to be mediated through a cAMP-dependent mechansim, since cell lines expressing dominant inhibitory mutations in PKA, which disrupt cAMP-dependent signalling pathways, are resistant to the growth inhibitory actions of ACTH and cAMP analogues (Armelin et al., 1996, Schimmer and Schulz, 1985). Contrastingly, in the same cell line, ACTH has been demonstrated to induce the expression of genes associated with cell cycle progression and proliferation. For example, ACTH induced the expression of fos and jun protooncogenes (Kimura et al., 1993, Kimura and Armelin, 1990). The fos and jun family of transcription factors are the

immediate early genes conventionally upregulated following the binding of growth factors to tryosine kinase receptors (Angel and Karin, 1991). They form homo or heterodimers, widely termed AP1, which play a vital role in inducing genes involved in cell cycle progression. This data suggests a growth-promoting action of ACTH. One study examined the regulation of the p42/p44 MAPK pathway otherwise called the extracellular signal related kinase ($Erk_{1/2}$) pathway, one of the major pathways regulating mitogenesis, in response to ACTH, to reconcile this conflicting data (Lotfi et al., 1997). They demonstrated that ACTH induced $Erk_{1/2}$ phosphorylation of serum-starved cells by 5 mins and over a range of concentrations. Using the kin-8 (PKA defective) mutant they demonstrated that ACTH could still activate this pathway over the same concentration range, suggesting that this was a cAMP-independent mechanism. They also showed that ACTH, given as a short pulse of up to 2 hours, stimulated an increase in BrdU labelling by 2 fold over unstimulated cells which approached the effects of serum and FGF. ACTH was also found to stimulate cell cycle progression as demonstrated by an increase in thymidine incorporation compared to unstimulated cells. To ascertain whether this signal could progress into an increase in mitosis, cells were stimulated for 5 mins and then left in SFM for 32 hours. A 1.5 fold increase in cell number was found compared to unstimulated cells. This study went on to look further at the signalling mechanism initiated by ACTH. They found that the ACTH effect could be mimicked by the phorbol ester, phorbol 12-myristate 13-acetate (PMA) but not by the inactive 4α-hydroxyl isomer of PMA suggesting a Protein kinase C (PKC) dependent mechanism.

The above data do suggest a mitogenic component to ACTH signalling however studies comparing the strength of its mitogenic activity with that of FGF suggest that it is only a weak mitogen (Armelin et al., 1996). A 2 h pulse of 1nM ACTH lead to a 2.6 fold increase in BrdU-labelling index whereas under the same conditions, 0.2nM FGF2 caused a 7-fold increase. Furthermore, anti-sense oligonucleotides to either c-fos and c-jun were able to abolish DNA synthesis stimulated by ACTH, however to abolish the FGF2 mitogenic response, antisense to both c-fos and c-jun were required (Lotfi and Armelin, 2001). In another study,

it was demonstrated that higher concentrations of the MEK 1 inhibitor PD98059 (MEK being an upstream component in the $Erk_{1/2}$ pathway) were required to inhibit an FGF induced $Erk_{1/2}$ response than an ACTH $Erk_{1/2}$ response (Le and Schimmer, 2001).

Armelin and co-workers have focused on characterising the anti-mitogenic mechanisms of ACTH in the Y1 cell line by looking at the antagonising effects of ACTH on FGF stimulated mitogenesis. They demonstrated that FGF2 elicits a strong mitogenic response in G_0/G_1 arrested Y1 cells that includes a rapid and transient activation of $Erk_{1/2}$ (2 to 10 mins); transcriptional activation of c-fos, c-jun and c-myc (another protooncogene) genes (10 to 30 mins); induction of c-fos and c-myc proteins by 1 h and cyclin D1 protein by 5 h; and the stimulation of DNA synthesis within 8 h (Lotfi et al., 2000). They found that a combination of FGF2 and ACTH for a 30 min pulse, reduced the mitogenic activity of FGF2 by 50%, however, an initial 30 mins pulse of FGF2 followed by a 2 h pulse of ACTH 4-6 hours later, completely abolished the mitogenic response (Lotfi et al., 1997). They suggest that this inhibitory effect is due to ACTH being more effective in the middle of G_1 phase (Armelin and Lotfi, 1999). They have further dissected this mechanism by looking at another mitogenic pathway, the PI3-K/Akt pathway. Akt is a mitogenic and anti-apoptotic serine/threonine protein kinase that is activated by phosphorylation of Thr308 and Ser473, through a process dependent on PI3-K and stimulated by growth factors (Chan et al., 1999). They demonstrated that Y1 cells have a constitutively high level of activated (phosphorylated) Akt, which may be significantly decreased with ACTH treatment within 2-5 min, and remaining up to 4 h (Forti and Armelin, 2000). This response was abolished after pre-incubation with 7-38 ACTH, implying that the effect is specifically through the MC2R. In PKA defective cells, ACTH did not dephosphorylate Akt suggesting a cAMP/PKA dependent mechanism. In another study, this response was correlated with an induction of the cell cycle inhibitor $p27^{kip1}$ after a 2 h treatment of ACTH (Forti et al., 2002). Further, this group has demonstrated that ACTH post-transciptionally down-regulates c-myc and imply that this results in the growth inhibition seen in these cells (Lepique et al., 2000).

1.6.4 Conclusions

With respect to *in vivo* ACTH effects, in the intact animal, it appears that ACTH is growth promoting, however the evidence for this is only suggestive. The initial methods used such as adrenal wet weight may not be indicative of a proliferative effect, and the later studies showed conflicting evidence with regard to cell numbers in the different zones after ACTH treatment. The hypophysectomy model is further supportive of an ACTH growth-promoting effect where a lack of ACTH coincides with adrenal atrophy, an effect that is reversed on administration of ACTH. The adrenal compensatory model is unsupportive of an ACTH mitogenic role, however results have also suggested that a pituitary peptide is not involved in this response, which does not concur with the hypophysectomy model of adrenal atrophy. Furthermore, from POMC knockout studies, it is clear that a pituitary peptide is involved in adrenal growth since the knockout has demonstrated the presence of atrophy (Coll et al., 2004). Thus, the adrenal compensatory model may be the result of an altogether different mechanism, which may not be physiologically relevant.

Early cell studies into the growth effects of ACTH on adrenocortical cells have yielded mixed and confusing results. ACTH was clearly inhibitory of growth in primary bovine adrenocortical cells whereas in rat inner zone cells, ACTH caused a significant proliferation (Gill, 1976, Nussdorfer et al., 1977). These studies however have further been complicated because of the likelihood that these experiments were carried out on mixed populations of cells. In addition there is some doubt over the purity of ACTH preparations used in these early experiments, which also applies to early *in vivo* experiments (Lotfi et al., 2000). Some would argue that many of the proliferative effects seen were from a contaminant in these preparations.

In recent years, synthetic ACTH has been commonly used but this still hasn't solved the contradictory results. Two groups have carried out the majority of more recent work on ACTH signalling mechanisms in Y1 cells. It is clear that in these

cells ACTH does initiate a mitogenic pathway and in short pulses can bring about a proliferative response, an effect that is probably mediated by the PKC pathway. However, ACTH also initiates an anti-mitogenic mechanism with activation of the cAMP/PKA pathway leading to blockade of the PI3-K/Akt pathway (Lepique et al., 2000). Physiologically, ACTH plasma concentrations are variable throughout the day dependent on circadian rhythms (Vinson et al., 1992). This may be important when assessing the effects of long periods of treatment of cells with ACTH. It may be that the concentrations of ACTH required to initiate a response in the adrenal cortex (mitogenic or otherwise) may physiologically be experienced for shorter periods of time than those used in some cell studies.

The local release of certain factors following treatment with ACTH has also been thought to play a role in the regulation of growth of the adrenal. One group demonstrated by slot blot and ribonuclease protection analysis that bFGF mRNA was present in very low amounts in total RNA from primary cultures of unstimulated human fetal adrenal cells but was increased 2-3 fold in cells exposed to 10nM ACTH 1-24 for 24 h (Mesiano et al., 1991). Another study has suggested that hyperplasia of adrenal cells may be the result of the effects of ACTH on the development of adrenal vasculature where ACTH stimulates Vascular endothelial growth factor (VEGF) production (Thomas et al., 2003).

The most likely scenario is that mitogenic signalling through ACTH is a complex process involving the interplay between ACTH and many other factors including those produced locally. Further investigation into pathways initiated by ACTH is required to elucidate these mechanisms further.

1.6.5 Other adrenal mitogens

Whilst some investigators have concentrated on the role of ACTH in adrenal growth regulation, other groups have concentrated on alternative pituitary peptides

that may be involved. This is based on the premise that since hypophysectomy leads to adrenal atrophy, a pituitary derived peptide must be crucial for adrenal growth. In the 1980's the group of Lowry and co-workers demonstrated adrenal mitogenic activity in hypophysectomised rats treated with peptides derived from the N-terminus of the POMC peptide (Estivariz et al., 1982, Lowry et al., 1983, Lowry et al., 1984). However, 1-76 POMC, which accounts for the pro-γ-MSH peptide secreted by the pituitary, had no mitogenic activity on the adrenals (Estivariz et al., 1980). Shorter fragments, that did not contain γ-MSH (1-28 POMC), did have a growth effect on hypophysectomised rats (Estivariz et al., 1988, Lowry et al., 1984). Furthermore, anti-sera to 1-48 POMC administered to rats after unilateral adrenalectomy, inhibited adrenal compensatory growth (Lowry et al., 1983). Most of the evidence for an N-terminal POMC adrenal mitogen, was based on the use of pituitary extracts, which may have been contaminated with other pituitary peptides. More recently however, the case for an N-terminal POMC mitogen has been strengthened after the discovery of an adrenal serine protease that specifically cleaves rat POMC to derive a short fragment of N-terminal POMC that has already been shown to stimulate growth of the adrenal *in vivo* (Bicknell et al., 2001). One study has looked at the effect of a sythetic 1-28 POMC on human H295R and Y1 cells (Fassnacht et al., 2003). They demonstrated that 1-28 POMC stimulated cell proliferation and rapidly activated $Erk_{1/2}$ in both cell types and in primary bovine adrenal cells in a concentration dependent manner. Although this data is convincing, there has as yet been no receptor found to 1-28 POMC.

1.7 GROWTH PROMOTING SIGNALLING PATHWAYS

There are a number of well-characterised signalling pathways that are thought to initiate the progression of the cell cycle and ultimately lead to proliferation of cells. The MAPK signalling pathway comprises a highly conserved cascade of at least three protein kinases, sequentially activated by phosphorylation, which culminates in the activation of a multi-functional MAPK. Fig. 1.8 shows the basic components

found in MAPK pathways and highlights some of the proteins involved in these pathways.

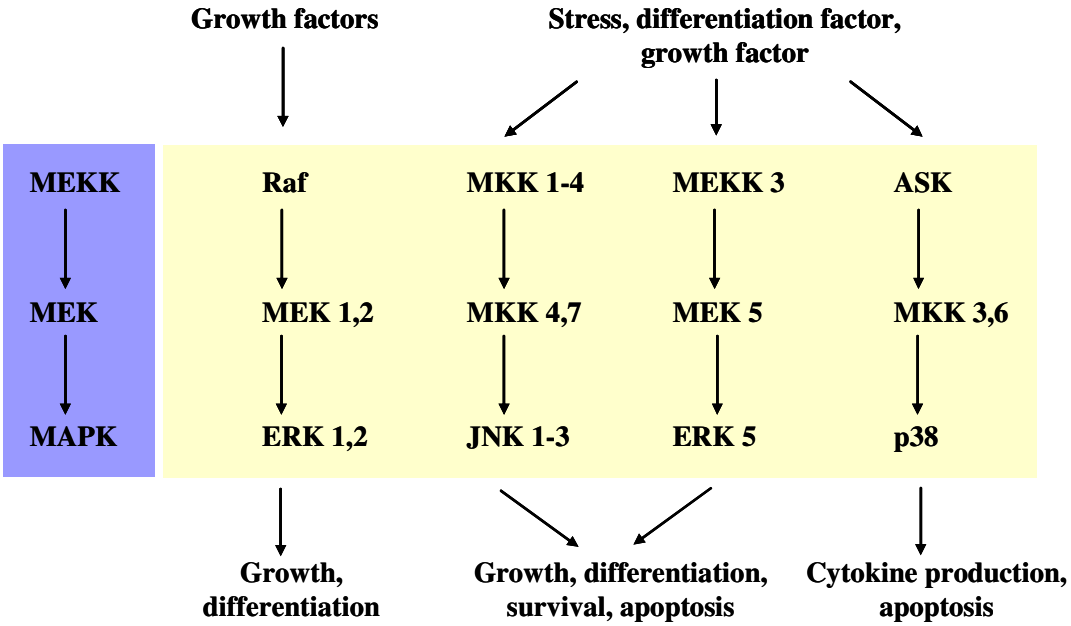


Fig. 1.8 MAPK pathways. *The MAPK pathways consist of MEKKs that respond to a variety of extracellular signals including growth factors and stress. The MEKKs can then activate one of several MEKs (or MKKs). MEKs are relatively specific for their target MAPKs. In turn MAPKs activate various targets including transcription factors which regulate their target gene expression bringing about effects such as growth, differentiation and apoptosis.*

MAPK pathways are activated by a diverse array of stimuli and act to transduce signals from the plasma membrane to exert a physiological effect. Transcription factors are the major targets of MAPKs. On phosphorylation, transcription factors are targeted to the nucleus whereon they regulate expression of their target genes. For example $Erk_{1/2}$ activates AP1 (activating protein 1), an immediate early gene involved in cell cycle progression (Chen et al., 1993). A further target of MAPK is RSK2. Activated RSK2 can phosphorylate histone H3, an important protein in the restructuring of chromatin (Sassone-Corsi et al., 1999).

In addition to being involved in cell proliferation, MAPK pathways have also been implicated in differentiation, development, apoptosis and inflammatory responses (Raman et al., 2007). Although MAPK pathways are ubiquitous, specificity is conferred by the availability of cellular targets and by their association with scaffolding and anchoring proteins (Garrington and Johnson, 1999).

Within the MAPK family several distinct pathways exist, the most widely studied of which is the $Erk_{1/2}$ pathway. There are also MAPK pathways activated by cellular stress known as the stress-activated protein kinase (SAPK) pathway. This includes both the c-jun amino terminal kinases (JNKs) and p38 MAPK. In recent years the emerging role of Erk 5 has been investigated where is has been implicated in mediating the effects of numerous oncogenes (Wang and Tournier, 2006).

The duration of MAPK signals is thought to be an important factor in determining the strength and type of a response. MAPK signalling is negatively regulated by MAPK phosphatases (MKPs) of which isoforms can be found in both the nucleus and the cytosol (Kondoh and Nishida, 2007) and it is these enzymes that are thought to determine the length of duration of the $Erk_{1/2}$ signal. It has been suggested that the immediate early gene product c-Fos, acts as a sensor for $Erk_{1/2}$ signal duration where prolonged $Erk_{1/2}$ activation allows the stabilization of c-Fos and thus c-Fos signalling in contrast to shorter periods of $Erk_{1/2}$ activation (Murphy et al., 2002). This may explain why shorter periods of $Erk_{1/2}$ activation do not necessarily result in cell cycle progression.

1.7.1 The $Erk_{1/2}$ Pathway

Erk 1 and 2 are proteins of 44 and 42kDa respectively. They share 85% sequence homology with a greater homology in the regions involved in substrate binding. Both contain a Threonine-glutamine-tyrosine (TEY) motif within an activation loop and it is here that their tyrosine and threonine residues are phosphorylated enabling

their activation (Payne et al., 1991). Both Erk 1 and 2 are ubiquitously expressed however their relative abundance is variable between cell types. Due to their homology and the fact that both are activated by a pair of closely related MEKs, MEK 1 and MEK 2, Erk 1 and 2 are most commonly treated as one entity. MEK1 and 2 are activated by Raf isoforms. This family of kinases is made up of Raf-1, A-Raf and B-Raf. Each Raf contains three conserved regions, two of which are found in the N-terminus and are thought to regulate their catalytic domain (Whitehurst et al., 1995). The third is contained in the kinase domain. Raf-1 is found ubiquitously whereas B-raf is most highly expressed in neuronal tissues and testes and A-ras mainly in urogenital tissue.

1.7.2 Stress activated protein kinase pathways

The JNKs were first isolated and characterized as stress activated kinases since they were activated in response to the inhibition of protein synthesis (Kyriakis et al., 1994). The SAPK/JNK pathway is induced by cellular stress and extracellular signals including UV radiation, hyperosmolarity, toxins and inflammatory cytokines. Activation requires the sequential dual phosphorylation of tyrosine and throenine residues located in a TPY motif in its activation loop within its kinase domain. This pathway is extremely complex and may be phosphoylated by many MKKKs. SAPK has been reported to phosphorylate transcription factors such as c-Jun, ATF 2, Elk 1 and c-Myc among others thus giving rise to its important roles in regulating cell proliferation, differentiation, transformation and apoptosis (Chang and Karin, 2001, Davis, 2000, Nishina et al., 2004).

The p38 MAPK cascade is another stress activated MAPK pathway. The most well studied of these is the p38α pathway which is expressed in most cell types. These kinases were first discovered in a screen for drugs inhibiting TNFα induced inflammatory responses (Lee et al., 1994). P38 MAPK is activated by many stimuli including osmotic shock, heat shock, ligands for GPCRs and hormones, and its effects are most commonly in regulating cytokine production in the inflammatory response.

1.7.3 Other MAPK pathways

MEK 5 was identified by two groups using DNA cloning strategies (Zhou et al., 1995, English et al., 1995). Following this its downstream substrate, Erk 5, was identified independently by two groups using a yeast two-hybrid approach and a degenerate PCR strategy (Zhou et al., 1995, Lee et al., 1995). The catalytic domain of Erk 5 appears most similar to Erk 2 however Erk 5 contains a stretch of 400 amino acids, c-terminal to the kinase domain, that displays no similarity to any known proteins. Erk 5 contains 10 concensus sites for MAPK phosphorylation all of which may be autophosphorylated (English et al., 1998).

1.7.4 Non MAPK growth promoting pathways

The PI3-K/Akt pathway is a widely studied pathway that promotes cell survival and proliferation. The Akt protein consists of an amino terminal PH domain, a central kinase domain and a carboxy-terminal regulatory domain. The PH domain of Akt interacts with membrane lipid products such as phosphatidylinositol (3,4,5) triphosphate (PIP_3) produced by PI3-K. Akt may also be activated independently of PI3-K. For example, it was shown that Akt could be activated by Ca^{2+}/calmodulin dependent kinase (Perez-Garcia et al., 2004). Akt activation provides the cell with a survival signal allowing them to withstand apoptotic stimuli and is thus thought to be one of the most important pathways in regulating cell survival (Yao and Cooper, 1995). Many of its targets are transcription factors involved in the regulation of apoptosis. These include the Forkhead family of transcription factors whose upregulation by Akt prevents the transcription of pro-apoptotic genes (Burgering and Medema, 2003). Akt is also involved in the regulation of CREB activity and as a consequence, the transcriptional regulation of CREB regulated survival genes.

In addition to its role in cell survival, Akt has also been shown to have important roles in cross-talk with other signalling pathways. For example, Akt was

demonstrated to form complexes with $Erk_{1/2}$, to regulate apoptosis in kidney proximal tubular cells (Sinha et al., 2004).

1.8 ACTIVATION OF MAPK PATHWAYS BY GPCRS

Since the discovery of the involvement of GPCRs in non-classical roles such as growth and apoptosis, an extensive amount of research into the steps that lead to these responses has been undertaken. Numerous GPCRs, including the MC2R, have been demonstrated to activate pathways such as the $Erk_{1/2}$ signalling pathway however, of greater interest is the way in which such pathways are activated by GPCRs because this is important in determining the outcome of the response.

1.8.1 G-protein involvement in the mediation of $Erk_{1/2}$ signalling

All three of the major G proteins (G_s, $G_{i/o}$ and $G_{q/12}$) have been demonstrated to be involved in the transduction of $Erk_{1/2}$ signals through agonist binding to GPCRs (Faure et al., 1994, Crespo et al., 1994).

1.8.1.1 G_s coupled receptors

In the case of G_s coupled receptors the effector molecule is most commonly the α subunit of the G-protein. $Erk_{1/2}$ activation may be mediated by direct binding of the activated subunit to an effector molecule such as the tyrosine kinase *src* (Ma et al., 2000) or through mechanisms downstream of the activation of the cAMP/PKA pathway (Norum et al., 2003, Klinger et al., 2002) Since the MC2R is classically coupled to a G_s protein, this is a likely mechanism of activation of $Erk_{1/2}$ by this receptor. This mechanism will be discussed in further detail later.

1.8.1.2 $G_{i/o}$ coupled receptors

Pertussis toxin (PTX) has been widely used to assess the involvement of $G_{i/o}$ proteins in the transduction of the $Erk_{1/2}$ cascade from $G_{i/o}$ coupled GPCRs. PTX ribosylates G-protein α chains of $G_{i/o}$ proteins in the $\alpha\beta\gamma$ state therefore preventing the dissociation of these subunits. Many groups have shown the activation of $Erk_{1/2}$ to be PTX sensitive suggesting that the signal is dependent on coupling to either G_i or G_o (Cook and McCormick, 1996). Further elucidation of these mechanisms has shown that the main effector molecules transducing signals to $Erk_{1/2}$ from PTX sensitive GPCRs are the free $\beta\gamma$ subunits. One study looked at Ras and $Erk_{1/2}$ activation through endogenous G_i-coupled receptors, in response to lysophosphatidic acid (LPA), in a fibroblast cell line transfected with a polypeptide derived from the c-terminus of the GPCR kinase GRK2 (this acts as a $G\beta\gamma$ antagonist). They found that $Erk_{1/2}$ activation was attenuated in transfected cells compared to wild-type cells, implying that $G\beta\gamma$ subunits dissociated from G_i mediate the $Erk_{1/2}$ response (Koch et al., 1994). This data was supported by similar studies where overexpression of $G\beta\gamma$ subunits activated the $Erk_{1/2}$ cascade (Crespo et al., 1994, Faure et al., 1994).

$G\beta\gamma$ proteins are thought to transduce signals through binding to proteins that contain a pleckstrin homology (PH) domain, a well-known lipid-binding domain (Lemmon et al., 1996). In addition a number of proteins that contain a PH domain have also been shown to regulate ras activity suggesting that $G\beta\gamma$ effects on ras regulatory proteins may mediate the activation of the $Erk_{1/2}$ pathway (Langhans-Rajasekaran et al., 1995). Fig. 1.9 depicts the activation of $Erk_{1/2}$ through G_i coupled receptors through $\beta\gamma$ subunits.

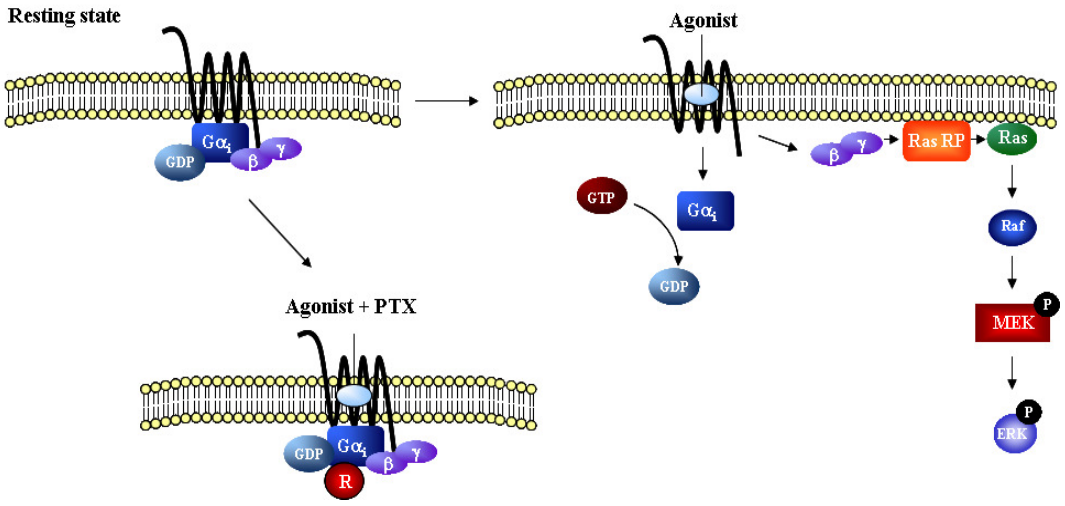


Fig. 1.9 ßγ activation of Erk$_{1/2}$ pathway. *On binding of an agonist to a G_i coupled GPCR, βγ subunits transduce the signal through binding to a ras regulatory protein (Ras RP) that contains a PH domain. The signal is then transduced through Ras to activate the Erk$_{1/2}$ pathway. With the addition of agonist and pertussis toxin, Erk$_{1/2}$ activation is prevented because pertussis toxin ribosylates (R) the G_i protein and prevents dissociation of the αβγ subunits.*

PI3-K has also been shown to be involved in Gβγ responses. A PI3-K responsive to Gβγ proteins has been cloned (Stoyanov et al., 1995) which contains a potential PH domain near its amino terminus. In addition, inhibitors of the PI3-K/Akt pathway, wortmannin and LY294002 block the Erk$_{1/2}$ activation induced by LPA, α_2-adrenergic receptor or Gβγ proteins (Hawes et al., 1996). Events downstream of Gβγ binding to proteins containing PH regions tend to be the stimulation of Shc and further the activation of growth factor tyrosine kinase receptor pathways suggesting that Gβγ mediated activation of Erk$_{1/2}$ converges with classical tyrosine kinase receptor pathways (Touhara et al., 1995, Hordijk et al., 1994). This process of activation will be discussed in more detail later.

1.8.1.3 $G_{q/11}$ coupled receptors

Coupling of GPCRs to G_q proteins results in a stimulation of PKC activity through the production of diacylglycerol (DAG) and IP_3. This process also involves Ca^{2+} influx through channels in the plasma membrane as well as intracellular Ca^{2+} release from the endoplasmic reticulum. Receptors coupled to G_q proteins often activate the $Erk_{1/2}$ cascade through the activation of this classical second messenger pathway where $Erk_{1/2}$ activation is down stream of Ca^{2+} mobilisation or PKC activation. PKCα was demonstrated to activate Raf-1 directly by phosphorylation (Kolch et al., 1993). In addition, activation of $Erk_{1/2}$ following stimulation of the α1B adrenergic and M1 muscarinic receptors in fibroblasts occurs through a raf dependent but ras independent mechanism which can be blocked by PKC downregulation and mimicked by overexpression of PLC-β2 (Faure et al., 1994). The influx of Ca^{2+} ions can also cause the activation of $Erk_{1/2}$ independently of PKC activation (Schliess et al., 1996). Fig 1.10 shows some of the mechanisms by which G_q coupled receptors are able to initiate $Erk_{1/2}$ activation. Although the MC2R is not thought to couple to G_q directly, Ca^{2+} has long been thought to play a role in the binding of ACTH to its receptor (Cheitlin et al., 1985). Furthermore, intracellular Ca^{2+} has also been demonstrated to enter adrenal cells after stimulation with ACTH through a PKA mediated depolarisation of cells (Enyeart and Enyeart, 1998, Catalano et al., 1986). This may suggest a mechanism through which ACTH mediates $Erk_{1/2}$ activation and indeed previous experiments in Y1 cells demonstrated that PMA, an activator of PKC, could mimic ACTH induced $Erk_{1/2}$ activation (Lotfi et al., 1997).

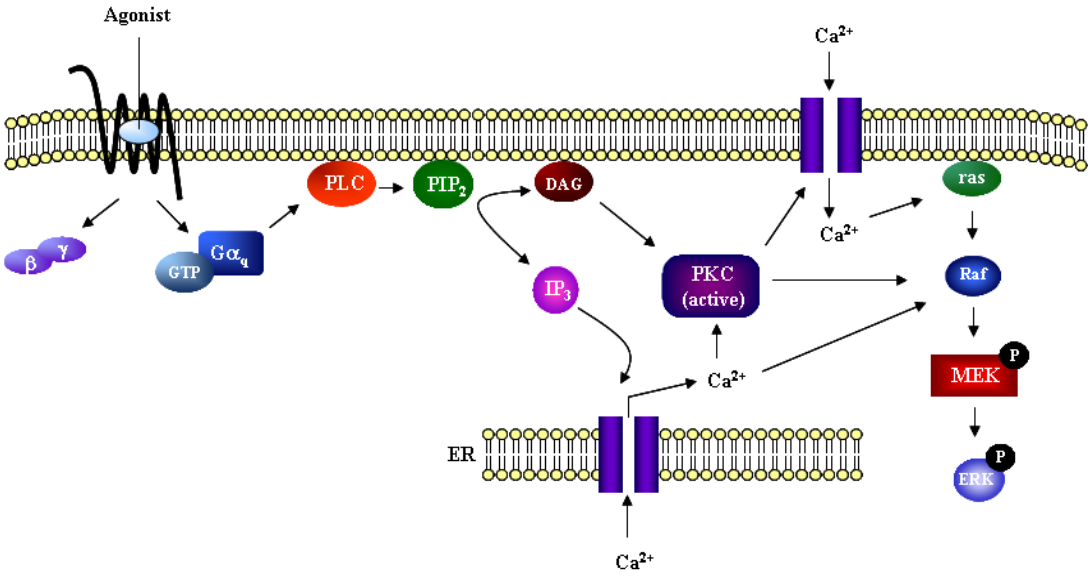


Fig. 1.10 G_q coupled receptor mediation of $Erk_{1/2}$ activation. *Following agonist binding, PKC is activated in the usual manner along with an increase in Ca^{2+} concentration following release from the ER and influx through calcium channels. Active PKC may then bind and activate Raf and initiate the $Erk_{1/2}$ pathway. In addition Ca^{2+} ions may activate the $Erk_{1/2}$ pathway via Raf or Ras.*

1.8.1.4 Switching of coupling of GPCRs

An interesting additional mechanism of $Erk_{1/2}$ activation by GPCRs has been described in which PKA phosphorylation of the β_2 adrenergic receptor (Daaka et al., 1997) and the murine prostacyclin receptor (Lawler et al., 2001) leads to a switch in coupling from $G\alpha_s$ to $G\alpha_i$ with subsequent $Erk_{1/2}$ activation through $\beta\gamma$ subunits (Lefkowitz et al., 2002). Numerous other receptors have also been shown to couple to a variety of G proteins in heterologous cells (reviewed in (Rashid et al., 2004). However, highlighted in this review is the fact that many of these studies involved the expression of recombinant receptors in heterologous cell types making the observations slightly artificial. For ACTH responses, a switching of coupling of its receptor from a G_s to a G_i protein is unlikely since our laboratory has previously shown that PKA phopshorylation of the MC2R does not lead to a switch of G protein coupling in Y1 cells (Baig et al., 2001).

1.8.2 cAMP/PKA mediated activation of $Erk_{1/2}$

Many G_s coupled GPCRs have been previously shown to activate the $Erk_{1/2}$ cascade, many of which by mechanisms at least partially dependent on the activation of the cAMP/PKA pathway. Earlier studies indicated that a constitutively active $G\alpha_s$ mutant, forskolin and a cAMP analogue could activate the $Erk_{1/2}$ pathway in COS-7 cells (Faure et al., 1994). This effect in some cases was dependent on PKA and Ras activation (Norum et al., 2003, Klinger et al., 2002). However, in many cases the mechanism of $Erk_{1/2}$ phosphorylation was found to be dependent on the activation of a small Ras superfamily guanine nucleotide binding protein Rap1A, independently of PKA activation (Kawasaki et al., 1998). In 1998, a guanine nucleotide exchange factor (GEF) that is activated by binding to cAMP was cloned (de Rooij et al., 1998). It was found that once activated this factor is able to activate Rap1 by promoting its release of the guanine nucleotide GDP and its binding to GTP. This factor was named 'exchange protein directly activated by cAMP or EPAC. Following Rap1 activation, B-Raf is preferentially activated giving rise to $Erk_{1/2}$ activation (Ohtsuka et al., 1996, Vossler et al., 1997). This mechanism has since been shown to be a common way in which G_s coupled receptors mediate $Erk_{1/2}$ signals (Schinelli et al., 2001, Fujita et al., 2002). It is also worth noting that in some cell types, Rap 1 may negatively interfere in Ras signalling to $Erk_{1/2}$ (Carey et al., 2000, Cook et al., 1993, Kitayama et al., 1989). Figure 1.11 depicts the pathway by which $Erk_{1/2}$ may be activated by G_s coupled GPCRs.

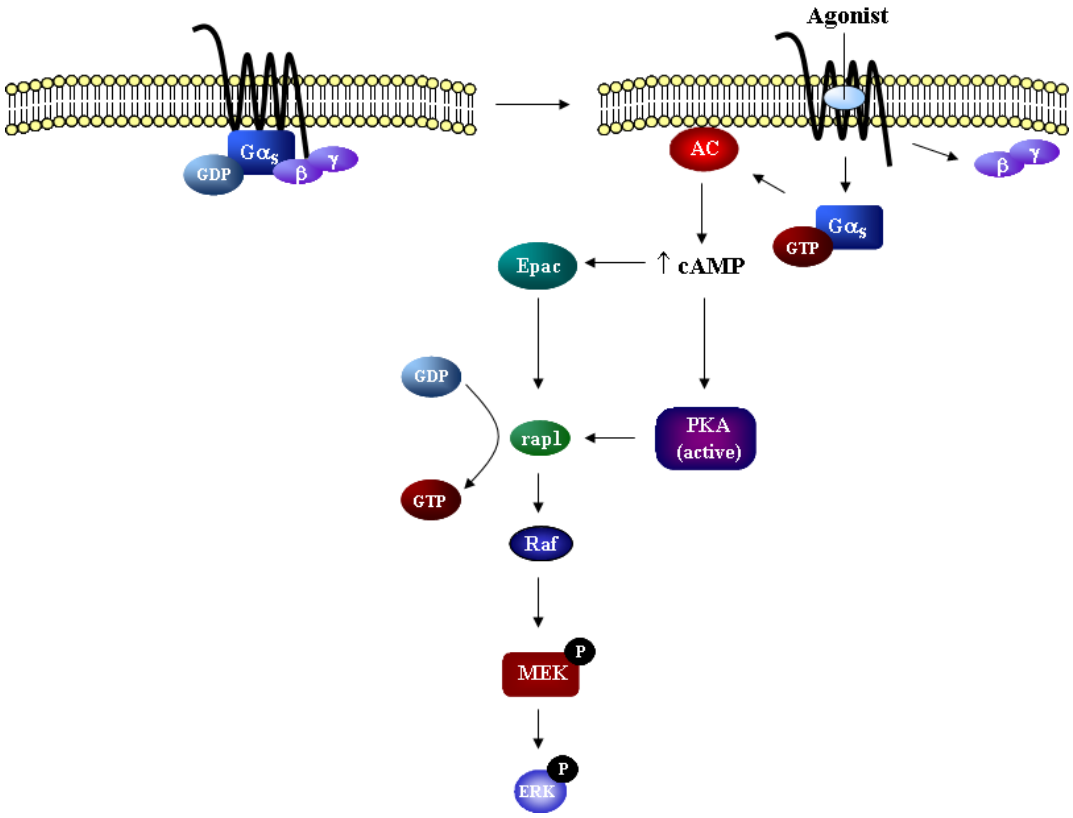


Fig. 1.11 Activation of Erk$_{1/2}$ by G$_s$ coupled GPCRs dependent on the cAMP/PKA pathway. *Agonist binding to the GPCR promotes an increase in intracellular cAMP and the subsequent activation of PKA. PKA may act to enhance Rap 1 activity. In addition cAMP may directly activate Epac which in turn enables activation of Rap 1 by promoting GDP/GTP exchange. Rap 1 may then promote the Erk$_{1/2}$ pathway through Raf activation.*

1.8.3. Transactivation of tyrosine kinases in GPCR mediated activation of Erk$_{1/2}$

Research into the mechanisms that activate MAPK cascades over the past decade has identified converging pathways linking GPCR stimulation and classical growth factor receptor stimulation in the activation of these cascades. The first evidence linking a GPCR to tyrosine kinase activation was the use of genistein, a non-specific tyrosine kinase inhibitor that prevented LPA induced p21ras activation in Rat-1

fibroblasts (van Corven et al., 1993). This group also demonstrated that protein tyrosine kinase phosphorylation could be induced by LPA (Hordijk et al., 1994). Since then numerous groups have identified GPCRs that transduce signals to the $Erk_{1/2}$ cascade through tyrosine kinase dependent processes.

1.8.3.1 Transactivation of receptor tyrosine kinases

The most notable mechanism involving tyrosine kinases is through the transactivation of the epidermal growth factor (EGF) receptor following stimulation with GPCR agonists (Daub et al., 1996, Mifune et al., 2005, van Biesen et al., 1995). In addition, the PDGF receptor has been implicated in the transduction of an $Erk_{1/2}$ signal through the dopamine D2 receptor and the P2Y receptor (Wang et al., 2005, Milenkovic et al., 2003); and the IGF-1 receptor is transactivated in response to angiotensin II in smooth muscle cells (Zahradka et al., 2004).

The mechanisms that result in the transactivation of tyrosine kinase receptors are not fully understood however they may involve the direct interaction of a GPCR with the receptor as in the case of the angiotensin II receptor type I which interacts with the EGF receptor in rat hepatic C9 cells (Olivares-Reyes et al., 2005). A perhaps more common mechanism however is the cleaving of membrane anchored isoforms of EGF receptor ligands by the activation of matrix metalloproteases (MMPs) (reviewed in Higashiyama and Nanba, 2005). These enzymes constitute a large family of endopeptidases that have important roles in development, wound healing and a variety of pathological processes. An initial study demonstrated the activation of a MMP by $G\beta\gamma$ subunits that released the growth factor heparin-binding (HP) EGF, which subsequently activated the $Erk_{1/2}$ cascade (Prenzel et al., 1999). Since then numerous MMPs have been shown to be involved in GPCR mediated growth factor receptor transactivation (Thomas et al., 2005, Yano et al., 2004). Fig. 1.12 shows the steps involved in the transactivation of growth factor receptors by GPCRs leading to $Erk_{1/2}$ activation.

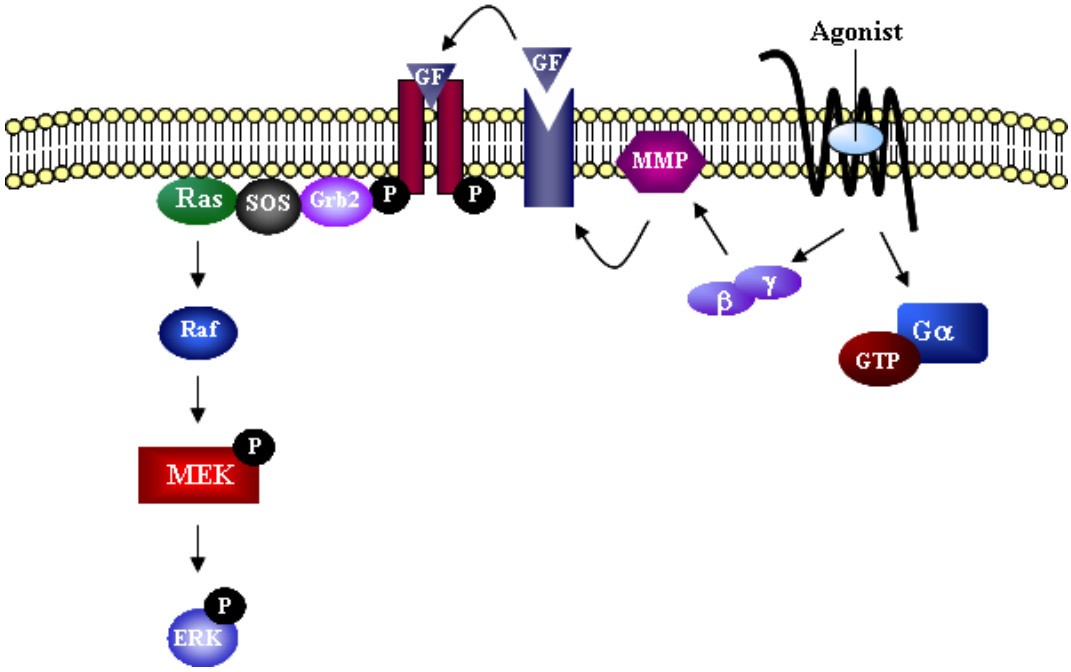


Fig. 1.12 Binding of an agonist to a GPCR causes the activation of a MMP possibly via βγ subunits. *This causes the cleavage of a membrane bound heparin-binding growth factor enabling binding and activation of a growth factor receptor with the subsequent activation of the Erk$_{1/2}$ cascade.*

1.8.3.2 Src tyrosine kinase involvement in GPCR mediated Erk$_{1/2}$ activation

An additional family of non-receptor tyrosine kinases shown to be involved in GPCR mediated signalling to the Erk$_{1/2}$ cascade are the *src* family tyrosine kinases (Dikic et al., 1996, Ptasznik et al., 1995). The viral-*src* (*v-src*) oncogene was found to be a transmissible agent responsible for tumorigenesis in chickens and since then the cellular homologue *c-src* has been found to be over expressed in a number of human cancers (Irby and Yeatman, 2000). *Src* recruitment and activation is a pre-requisite of Erk$_{1/2}$ activation by several GPCRs. Overexpression of *c-src* kinase, (CSK) an inhibitor of *src* activity, impairs Gβγ mediated LPA and α_2 adrenergic receptor stimulation of EGF receptor phosphorylation (Luttrell et al., 1997). In addition, activation of *c-src* was evident following stimulation of angiotensin II type 1 receptors via a G_q and Ca^{2+} dependent process in vascular smooth muscle cells (Eguchi et al., 1998). In the case of G_s coupled receptors it has been shown that the

activated α subunit can interact directly with *src* leading to its activation (Ma et al., 2000, Ma and Huang, 2002). *Src* has also been shown to bind directly to agonist occupied GPCRs as in the case of the β_2 and β_3 adrenergic receptors (Fan et al., 2001, Cao et al., 2000). *Src* phosphorylates a number of cytoskeletal-associated proteins and probably contributes to the activation of raf-1 and PI3-K (Erpel and Courtneidge, 1995). Conversely to its role in activating the $Erk_{1/2}$ pathway, *src* has also been demonstrated to be involved in mediating the inhibition of cell growth via cAMP and Rap1 (Schmitt and Stork, 2002).

1.8.4 Internalisation and scaffolding of signalling complexes

In addition to regulating cell surface receptor number as previously discussed, internalisation of a number of GPCRs has been demonstrated to be a pre-requisite for other signalling pathways including $Erk_{1/2}$ signalling. For example, LPA, thrombin and bombesin activation of $Erk_{1/2}$ was sensitive to 4 different chemical inhibitors of internalisation (Luttrell et al., 1997); β_2AR mediated $Erk_{1/2}$ signalling through G_i coupled receptors is sensitive to dominant negative dynamin transfection (Daaka et al., 1998); and $Erk_{1/2}$ activation by the serotonin $5HT_{1A}$ receptor requires Ca^{2+}/calmodulin dependent internalisation of the receptor (Della Rocca et al., 1999).

In recent years, much research has been conducted into the role of β-arrestins in transducing signalling pathways. For example it was shown in HEK 293 cells that stimulation of the β_2 adrenergic receptor triggers colocalisation of the receptor with β-arrestins and *src* kinases in clathrin coated pits (Luttrell et al., 1999). This has lead to the speculation that it is β-arrestin that acts as a scaffold for recruitment of components of signalling pathways since it was found that *src* can directly bind to β-arrestin 1 (Miller et al., 2000). This interaction has also been linked to activation of the $Erk_{1/2}$ pathway (Luttrell et al., 1999, DeFea et al., 2000b). Assembly of a β-arrestin-$Erk_{1/2}$ complex was shown to be required for the activation of $Erk_{1/2}$ by

the PAR2 receptor since expression of a truncated form of β-arrestin that inhibits endocytosis, prevented $Erk_{1/2}$ activation (DeFea et al., 2000b). A further example is the NK1 receptors where activation of these receptors by substance P causes formation of complexes containing *src*, β-arrestin and $Erk_{1/2}$ (DeFea et al., 2000a). β-arrestin has additionally been found to form complexes with components of other MAPK pathways for example β-arrestin 2 was shown to interact with JNK 3 in a yeast two-hybrid system (McDonald et al., 2000).

The functional role of β-arrestin-$Erk_{1/2}$ complexes is not entirely understood however it has been shown that such complexes may be retained within the cytoplasm of the cell thus preventing nuclear translocation and subsequent transcription factor activation. For example, a mutant PAR2 receptor that is unable to bind to β-arrestin, induces nuclear translocation of $Erk_{1/2}$ and cell proliferation whereas the wild type receptor does not (DeFea et al., 2000b). A further example is in a paper that utilised chimeric V2 vassopressin (V2R) and β_2 adrenergic receptors (β_2R) (Tohgo et al., 2003). The wild-type V2R formed a stable complex with β-arrestin whereas the wild-type β_2R formed a transient complex. However, when the c-terminal tails were exchanged, the complexes formed corresponded to the level of β-arrestin binding. It was shown that the wild-type V2R generated a larger cytosolic pool of phospho-$Erk_{1/2}$ and a smaller amount of nuclear $Erk_{1/2}$ than that of the chimeric receptor. In addition, the chimeric V2R was able to elicit a mitogenic response unlike the wild-type receptor.

It may be therefore that a cytosolic pool of $Erk_{1/2}$ has non-nuclear substrates and thus differing roles to that of an $Erk_{1/2}$ pathway generated where β-arrestin is not involved. Fig. 1.13 shows the possible mechanisms involved in the activation of $Erk_{1/2}$ via a mechanism involving β-arrestin.

These mechanisms of $Erk_{1/2}$ activation are of relevance to the MC2R since our lab has previously demonstrated that the MC2R is internalised via a clathrin coated pit

mechanism in Y1 cells (Baig et al., 2001). This raises the possibility that internalisation may be a pre-requisite for $Erk_{1/2}$ signalling by ACTH.

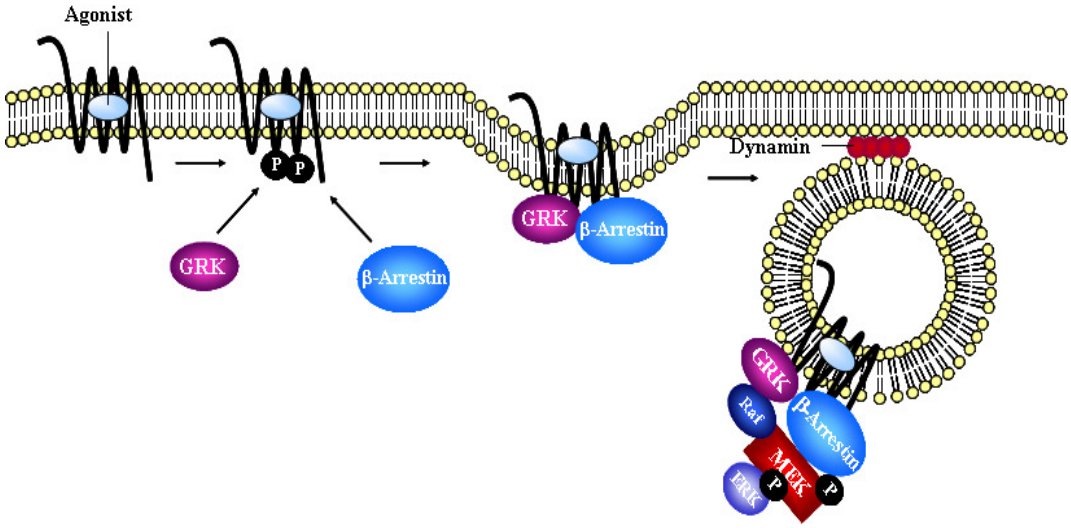


Fig. 1.13 Binding of an agonist to its receptor leads to activation of its classical second messenger pathway. *GRK phosphorylates agonist occupied receptors on their third intracellular loop and c-terminal domains and this increases receptor affinity to β-arrestins. Following this β-arrestin recruits components of the clathrin-dependent endocytic machinery and allow internalisation to occur. β-arrestins also recruit components of the $Erk_{1/2}$ pathway resulting in its activation.*

1.9 THESIS AIMS

Although much work has been undertaken in the past to establish the proliferative and non-proliferative roles of ACTH on the adrenal gland, there is only limited data available on the mechanisms behind these responses. Studies in the Y1 adrenocortical cell line have established that the $Erk_{1/2}$ pathway is activated in response to ACTH and that this response may lead to a proliferative effect over short exposure times (Lotfi et al., 1997) however the mechanisms that lead to initiation of this pathway are as yet uncharaterised. Studies with other GPCRs have revealed interesting and complex mechanisms of activation of MAPK pathways including transactivation of growth factor receptors, cross-talk with other signalling pathways and the requirement of β-arrestin for $Erk_{1/2}$ signalling. It is the aim of this thesis therefore to investigate the mitogenic signalling mechanisms initiated in adrenal cells in response to ACTH in order to give a clearer understanding of the regulation of adrenal growth, and to contribute to the expanding field of GPCR induced mitogenic signalling.

2 MATERIALS AND METHODS

Laboratory reagents were supplied by Sigma-Aldrich (Poole, UK) unless otherwise stated. Chemicals were all certified as analytical grade. Plasticware was purchased from Greiner Bio-one Ltd (Gloucestershire, UK) unless otherwise stated. Ultra pure water (dH_2O) was obtained using the Purite Select Analyst HP water purifier, and used when making all solutions. Where sterility was required, solutions were autoclaved using (Priorclave tactrol 2) or filtered through a 0.2 μm membrane (Nalgene).

2.1 GENERAL BUFFERS AND SOLUTIONS

PBS - 0.2g/L KCl, 0.2g/L KH_2PO_4 (anhydrous), 8.0g/L NaCl, 1.15g/L Na_2HPO_4 (anhydrous), pH 7.4 (Sigma)

Phenol - Add 10ml of equilibration buffer (10mM Tris pH 10 - Sigma) to 100ml of equilibrated phenol (pH6.7) (Sigma) to adjust to pH 7.9. Add 8-Hydroxyquinoline to final concentration of 0.1%. Store in the dark at 4ºC.

3M Sodium Acetate - Dissolve 40.8g of sodium acetate.$3H_2O$ in 80ml of H_2O. Adjust to pH5.2 with glacial acetic acid, make up to a final volume of 100 ml and autoclave.

TE - 10mM Tris pH 8.0, 1mM EDTA

TAE (50x) - 2M Tris-acetate and 100mM Na_2EDTA (pH 8.3 at 1x conc.) (National Diagnostics, UK)

6X loading dye - 0.5mM EDTA, 40% sucrose, 0.25% Orange G.

RNAase free dH_2O - Diethyl pyrocarbonate (DEPC) was added to dH_2O to a final concentration of 0.1 % and incubated overnight in a fume cupboard prior to autoclaving.

Ethidium bromide - Dissolve 0.5g ethidium bromide in 50ml of 0.1M EDTA solution and store at RT in a light sealed container.

dNTPs - 100 mM dNTPs (New England Biolabs, MA, US) were combined in a 1:1:1:1 ratio of dATP:dCTP:dGTP:dTTP and diluted in dH_2O to make a 10 mM stock solution which was stored at –20 °C.

Tris buffers – Solutions of 1 M Tris-OH and 1 M Tris-HCl were prepared. To prepare a solution of Tris buffer at a particular pH, the two solutions were mixed at the appropriate ratio according to the table 2.1 and pH confirmed using a calibrated pH meter.

pH	1 M Tris-HCl	1 M Tris-OH
6.8	969	31
7.2	889	111
7.3	867	133
7.4	837	163
7.5	804	196
7.6	767	233
7.7	724	276
7.8	673	327
7.9	618	382
8.0	562	438
8.1	509	491
8.2	448	552
8.3	389	611
8.4	334	666
8.5	208	792
8.6	232	768
8.7	190	810
8.8	156	844
8.9	122	878
9.0	96	904

Table 2. 1 *Ratios Tris-HCl to Tris-OH for required pH.*

2.2 CELL CULTURE

All work involving the culture of cells was carried out in a class II sterile cell culture hood. Hoods were regularly cleaned with virkon (Antec International, Sudbury, UK) and sprayed down with 70 % ethanol before and after use. All sterile culture-ware was purchased from Greiner unless otherwise stated. Other laboratory plastic-ware and media was autoclaved prior to use and opened only in the cell culture hood. Gloves were worn at all times during cell culture procedures. All cells were incubated in a humidified Sanyo MCO-17A/C incubator (Jencons-PLS, East Grinstead, UK) at 37 °C and 5 % CO_2.

2.2.1 Maintenance and sub-culture of H295R cells

Solutions:

- **Dulbecco's Modified Eagle's Medium (DMEM)** – 4500 mg/L glucose, 0.584 g/L L-Glutamine, 3.7 g/L $NaHCO_3$ (Sigma #D5796)
- **F12 nutrient media (Ham)** – 1802 mg/L glucose, 0.146 g/L l-glutamine, 1.18 g/L $NaHCO_3$ (Gibco, Paisley, UK)
- **Penicillin/Streptomycin** – formulated to contain 10,000 units/ml penicillin and 10 mg/ml streptomycin in 0.9% sodium chloride (Sigma)
- **ITS (100 x)** – 1 mg/ml insulin, 0.55 mg/ml human transferrin, 0.5 μg/ml sodium selenite, 50 mg/ml bovine serum albumin and 0.47 mg/ml linoleic acid (Sigma #I3146)
- **Trypsin-EDTA 1x Solution** (T/E) – 0.5 g/L of trypsin and 0.2 g/L EDTA.4Na in Hanks' balanced salt solution (Gibco).

Where serum free medium was required, a 1:1 mix of DMEM and F12 was made with the addition of 10 ml/L of penicillin/streptomycin solution. Fully supplemented media contained a 1:1 mix of DMEM:F12, 10 ml/L penicillin/streptomycin, 10 % foetal bovine serum (FBS – Gibco) and 1 X ITS

solution. H295R cells were grown in fully supplemented media, which was replaced every three days. Cells were not allowed to reach confluence but were passaged when around 80 % confluent. Passaging of cells involved aspirating the media and then washing the cells in pre-warmed PBS. Cells were then incubated in 3 ml trypsin solution for 3 min at 37 °C. The flask was then gently tapped to release the cells from the plastic surface and allow them into solution. An equal volume of fully supplemented medium was added to inactivate the trypsin and the solution pipetted up and down in order to fully disperse the cells. This solution was then diluted 1 in 5 into new cell culture flasks.

2.2.2 Maintenance and sub-culture of Y1 adrenocortical cells

Y1 cells (ATCC, US) were maintained in a 1:1 mix of Ham's F10 (Gibco) and DMEM (Sigma) with the addition of 2 mM Glutamine, 10 ml/L penicillin/streptomycin solution and 15 % heat-inactivated horse serum and 2.5 % heat inactivated FBS (Gibco). Media was changed every 3 days and cells were passaged 1 in 10 when 80 % confluent.

2.2.3 Maintenance and sub-culture of primary bovine adrenal cells (SBACs)

SBACs (ECACC) were maintained in Ham's F12 media containing 500 pg/ml recombinant FGF (Sigma), 2 mM Glutamine, 10 ml/L penicillin/streptomycin solution and 10 % heat inactivated FBS (Gibco). Media was changed every 3 days and sub-confluent cells were passaged 1:3 to 1:6.

2.2.4 Maintenance and sub-culture of immortilised bovine adrenal cells

Immotalised bovine adrenal cells (a kind gift from P. J. Hornsby) were maintained in a 1:1 mix of Ham's F12 and DMEM media containing 100 pg/ml recombinant FGF, 2 mM Glutamine, 10 ml/L penicillin/streptomycin solution, 10 % heat-

inactivated horse serum and 10 % heat inactivated FBS. Cells were seeded onto collagen coated plates (see section 2.2.7) and sub-confluent cultures were passaged 1:3 to 1:6.

2.2.5 Storage of cells

Solutions:

- Freezing medium - 90 % FBS and 10 % DMSO

In order to keep stocks of cells for long-term storage, cells were frozen in liquid nitrogen. Cells were trypsinised as previously described and pelleted in 15 ml polypropylene tubes by centrifugation at 100 g for 5 min. The supernatant was aspirated and the pellet resuspended in the required volume of freezing medium. Cells were pipetted into 2 ml cryogenic vials (Nalgene), wrapped in bubble wrap (to ensure a slow freezing process) and transferred to the –80 °C freezer overnight. The next day tubes were transferred to liquid nitrogen for long-term storage.

2.2.6 Thawing cells

Vials were removed from liquid nitrogen storage and quickly thawed in a 37 °C waterbath. Cells were plated in warm, fully supplemented medium. The next day, after the cells had adhered, the medium was changed for fresh fully supplemented medium in order to remove DMSO.

2.2.7 Collagen coating of plates

Solutions:

- **Collagen solution – 1 mg/ml collagen in 0.1 N acetic acid (Sigma #C8919)**

In some circumstances it was necessary to facilitate attachment of H295R cells to plasticware by coating plates with collagen. Collagen solution was diluted 1:100 in sterile dH_2O and pipetted onto cell culture plates so that the entire area was just covered. The solution was left on overnight at room temperature. The next day, the solution was aspirated and the plates dried at 37 °C for 2 hours or overnight at room temperature. Plates were washed twice with PBS prior to use.

2.3 TRANSFECTION OF CELLS

2.3.1 Transient transfection of H295R cells

H295R cells were transiently transfected using the FuGENE 6 Transfection Reagent (Roche, UK). This lipid based reagent forms complexes with DNA and then transports it into cells. To ensure the highest possible protein expression, the optimal ratio of FuGENE 6 reagent to DNA was determined, utilising the renilla-luciferase assay system (Promega, UK). In this system, a Renilla expression plasmid is transfected into cells and transfection efficiency is quantified in an assay where the substrate coelenterazine is converted to coelenteramide + light catalysed by renilla luciferase.

H295R cells were transfected in 6 well plates when they were 50-70 % confluent. Various ratios of FuGENE to Renilla DNA were tested according to the manufacturers guidelines. Complexes were pre-formed in a tube by adding serum free medium (SFM) followed by FuGENE followed by DNA, where the final volume was 100 µl. The cells were washed twice in SFM and the pre-formed complexes added. After 4 h, FBS was added to the wells to give a final concentration of 10 %. The transfection reagent remained in the culture dish until the cells were harvested 48 h later.

Cells were harvested and assayed according to the instructions given by Promega. A BioOrbit 1253 luminometer was used to measure luciferase activity. The optimal ratio of FuGENE to DNA was found to be 6 μl FuGENE to 1 μg DNA when transfecting a well of a 6 well plate. These amounts were scaled up or down depending on the surface area of the culture dish. Where more than one expression plasmid was transfected, the total amount of DNA remained the same. Generally, where Renilla luciferase was used to control for transfection efficiency, only one tenth of this plasmid was transfected compared to co-transfected plasmids to ensure that all cells expressing Renilla would also be likely to be expressing the co-transfected plasmid(s).

2.3.2 Stable transfection

Solutions:

- **G418 (100 mg/ml)** – 1 g of geneticin (Sigma #A1720) was dissolved in 10 ml dH_2O and sterile filtered through a 2 μm filter.

Stable H295R cell lines were produced by transfecting constructs containing a neomycin resistance gene, and cells expressing the construct were preferentially selected based on their ability to grow in media containing G418. Colonies of cells expressing the constructs were isolated individually and expanded. To determine the concentration of G418 in which to select, a kill curve was performed in which untransfected cells were grown in media containing a range of concentrations of G418 from 0.1-1 mg/ml. The minimum concentration that caused complete cell death in 7 days was selected and for H295R cells this was found to be 500 μg/ml.

H295R cells that were 50 % confluent were transfected in 9 cm dishes, overnight, using FuGENE as previously described. For each stable transfection, a mock transfection (one which contained no DNA) was carried out. The next day, the

media was aspirated and replaced with G418 containing media. This was changed every three days resulting in the eventual death, over 7 days, of most of the cells. Cells that were expressing the construct were resistant to G418 and started to form in isolated colonies by around two weeks. These colonies were likely to be specific if the mock-transfected plate of cells were completely dead.

2.3.2.1 Monoclonal cell lines

When the colonies were approximately 2 mm in diameter, 3 ml of trypsin was added to the dish and incubated for 3 min at 37 °C. The trypsin was then aspirated without disturbing the cells and colonies harvested by pipetting around 10 µl of medium onto the colony and then aspirating the colony. For each construct 10-20 colonies were isolated and plated into individual wells of a 96 well plate. Cells were pipetted up and down in order to disperse them. Cells were progressively moved into larger culture dishes by trypsinisation when they had reached near confluence. To confirm that the cells were expressing the desired construct, RNA was extracted from the cells and an RT-PCR carried out with a forward primer designed to the plasmid polylinker and the reverse primer to the gene encoded within the plasmid.

2.3.2.2 Polyclonal cells

Once the mock transfected cell had died, a polyclonal stable cell line could be obtained by passaging the plasmid transfected cells. Cells were maintained as usual in G418 containing medium.

2.4 TREATMENT OF CELLS WITH INHIBITORS

H295R cells were plated into 6 well plates and when around 70 % confluent were washed once in SFM and left subsequently in SFM overnight. The next day, inhibitors were added in SFM to the indicated concentrations, one hour (unless otherwise stated) prior to adding a 10x stock of the appropriate stimulant for the

times indicated. Cells were harvested as described for Western blotting or cAMP assays.

2.5 MANIPULATION OF DNA AND RNA

2.5.1 Agarose gel electrophoresis

Nucleic acids were separated according to their size using the Sub-Cell GT® Agarose Gel Electrophoresis System (BioRad). An appropriate amount of agarose (Sigma #A9539) was dissolved in 1 X TAE to make a final concentration of 1% - 2% (w/v) depending on the size of the fragment to be visualised. Agarose solution was heated until it had completely dissolved before adding ethidium bromide solution to a final concentration of 0.1 μg/ml. The agarose was poured into the moulding apparatus and left to set. Samples were loaded with Orange G loading dye (6x) and electrophoresed in 1 x TAE buffer at 120 V for around 30 min alongside 0.5 μg GeneRuler™ 1kb DNA ladder (MBI Fermentas). The gel was visualised on a UV light box and the Kodak EDAS 120 digital camera system (Gibco). For agarose separation of RNA see section 2.4.8.

2.5.2 Nucleic acid quanitification

The concentration and purity of a nucleic acid preparation was determined using the GeneQuant RNA/DNA calculator (Pharmacia Biotech). A sample was appropriately diluted and its OD_{260} measured in a glass cuvette. An OD_{260} of 1 corresponds to approximately 50 μg/ml for double stranded DNA and 40 μg/ml for single stranded DNA and RNA. Thus the following formula was used:

OD_{260} x f x d = concentration of nucleic acid (μg/ml)

Where: OD_{260} = optical density of the sample at 1 cm path length

f = 40 for RNA or 50 for DNA

d = dilution factor of sample

The purity of a nucleic acid sample could be ascertained by determining the ratio of OD_{260}/OD_{280}. Pure preparations of DNA and RNA have a ratio of 1.8 and 2.0 respectively. These values were much reduced when there was contamination with either phenol or protein.

2.5.3 Phenol Extraction and Precipitation

In order to purify a DNA or RNA sample from contaminating proteins, for example from an enzymatic reaction, samples were first phenol extracted followed by ethanol precipitaion. An equal volume of phenol to sample was added and vortexed vigorously for 2 min. The tube was centrifuged at 12,000 g for 2 min to separate the aqueous from the organic phase. RNA was partitioned in the aqueous phase whereas contaminating proteins associate with the organic phase. The aqueous phase was carefully transferred to a fresh microcentrifuge tube for ethanol precipitation.

1/10 of the volume of 3 M sodium acetate, pH 5.2, 2.5 times the volume of absolute ethanol and approx. 20 μg glycogen (Ambion, UK) was added to the nucleic acid sample. This solution was vortexed briefly and left on dry-ice for 15 min. The solution was centrifuged at 12,000 g for 10 min to pellet the precipitated nucleic acid. The supernatant was removed and discarded and the pellet washed with 1 x volume of 70 % ethanol. The sample was centrifuged again for 2 min, the ethanol aspirated and the pellet allowed to air dry for 10 min. The pellet was then resuspended in an appropriate volume of dH_2O.

2.5.4 Amplification of plasmid DNA

To amplify plasmid DNA, bacterial cells were made to take up plasmid DNA and those that contained the plasmid were selected based on their ability to grow on agar plates containing antibiotics. These bacteria could then be expanded in culture, and plasmid DNA extracted from these bacterial cells.

Solutions:

- **LB** - 10 g of LB (Sigma #L-3022) was dissolved in 500 ml dH_2O and autoclaved
- **LB Agar** - 10g of LB and 7.5 g of Agar (Sigma #A-5054) was made up to 500 ml in dH_2O and autoclaved. This could be allowed to cool to 50°C and then used to pour plates or could be allowed to cool completely and melted in the microwave when required
- **Ampicillin stock solution** (Sigma #A-9518) - 1 g of ampicillin was dissolved in 10 ml of dH_2O to give a 100 mg/ml stock solution and stored at 4 °C until required

2.5.4.1 Pouring Agar plates

Ampicillin stock solution (or another appropriate antibiotic) was added to melted LB agar (at no higher than 50 °C) to a final concentration of 100 μg/ml. Around 20 ml of this solution was poured into each 90 mm sterile petri dish in a sterile environment, and the agar left to solidify. Dishes could be wrapped in parafilm and stored at 4 °C for up to one month.

2.5.4.2 Plasmid transformation

JM109 Competent Cells (>10^8cfu/μg, Promega #L2001) were thawed on ice for 5 min and then 50 μl of cells were aliquoted into sterile microcentrifuge tubes (one per transformation reaction). 1-50 ng of plasmid DNA was added, mixed and incubated on ice for 10 min. Cells were then heat-shocked at 42 °C for 50 s and returned immediately to ice for a further 2 min. 950 μl of SOC medium (Invitrogen #15544-034) was added and the cells incubated for 1 h at 37 °C with constant shaking. Cells were then pelleted by centrifugation at 6000 g and resuspended in 100 μl of SOC medium. This was spread onto an agar/ampicillin plate using a sterile glass spreader and incubated overnight at 37 °C.

2.5.4.3 Amplification and preparation of plasmid DNA

A sterile wire loop was used to pick an individual colony from the agar plate and innoculate a solution of LB containing 100 μg/ml ampicillin. This was grown overnight at 37 °C in a shaking incubator. Depending on the volume of culture, plasmid DNA was extracted using a Qiagen mini or midi kit according to the manufacturers instructions. Briefly, cells were pelleted by centrifugation at 3000 g for 10 min. Supernatant was removed and the pellet resuspended in the supplied resuspension solution. Lysis buffer was added and the cells were left on ice for 5 min prior to the addition of neutralisation buffer. Lysates were passed through spin columns containing Qiagen Anion-Exchange Resin in a low salt buffer. A series of wash buffers were passed through the column before eluting the plasmid DNA.

2.5.5 Extraction of RNA from cultured cells

Solutions:

- RLN buffer: 5mM Tris-HCL, pH8, 140mM NaCl, 1.5mM $MgCl_2$, 0.5% (v/v) Nonidet P-40, 1000 U/ml RNase inhibitor, 1mM DTT (added fresh). This was made with RNAse free dH_2O.
- RLT buffer: As supplied but with the addition of 10µl/ml of β-mercaptoethanol prior to use.

For the isolation of RNA from cultured cells, RNAase free tips were used with all reagents. To avoid contamination of RNA with genomic DNA, the method of cytoplasmic RNA extraction was used thus yielding a sample that contained only mRNA that had been exported from the nucleus. The Qiagen RNeasy® Mini kit was used for this procedure according to the manufacturers protocol. To extract RNA from approximately 1 x 10^7 cells, the cells were first scraped in 1 ml of ice-cold PBS, transferred to a microfuge tube and pelleted at 13,000 g for 15 s in a microcentrifuge. The PBS was aspirated and the pellet resuspended in 175 µl of cold RLN buffer and incubated on ice for 5 min to lyse the cells. The lysate was centrifuged at 300 g at 4 °C for 2 min to pellet the nuclei. The supernatant was transferred to a new tube and 600 µl of RLT buffer added. Since RNA is rapidly degraded by RNAases that are present within the cell, a combination of both β-mercaptoethanol, a reducing agent, and guanidine salt, a potent denaturing agent are essential to allow isolation of intact RNA. These agents are both present in RLT buffer. 430 µl of absolute ethanol were added and the lysate applied to an RNeasy column where RNA selectively binds to the silica-gel membrane. A series of wash steps using the supplied buffers removed any contaminants prior to eluting with 50 µl dH_2O.

2.5.6 DNase I digestion

Although isolation of cytoplasmic RNA should reduce genomic DNA contamination, DNase digestion was also carried out as an extra measure. A reaction containing 2 µg RNA, 1 x transcription optimised buffer (Promega), 30 Units RNasin (Promega #N211A) and 208 U DNase I (0.75 µl)(Sigma #D-7291)

made up to a final volume of 15 µl, was incubated at 37 °C for 15 min. The reaction was terminated by the addition of 100 µl of TES (10 mM Tris-HCl, pH 8, 1 mM EDTA and 0.1 % w/v SDS). RNA was then phenol extracted and precipitated as described previously.

2.5.7 Agarose gel electrophoresis of RNA

Since RNA degrades very rapidly, the gel tank, combs and moulding apparatus were soaked for 1 h in a solution of 0.1M NaOH and 1mM EDTA and rinsed in RNAase free dH_2O prior to running the gel.

To assess the quality of an RNA preparation, a small amount of sample was separated on a 1 % non-denaturing agarose gel and visualised. Ribosomal RNA (rRNA), the most abundant type of RNA in the cell, could be seen to run as two sharp bands of 28S and 18S rRNA. The 28S rRNA was approximately twice as bright as the 18S rRNA for good quality RNA.

2.5.8 Reverse Transcription

First strand synthesis of cDNA from mRNA is catalysed by the enzyme RNA-dependent DNA polymerase or reverse transcriptase (RT). In order to initiate DNA synthesis, this enzyme requires an RNA or DNA primer.

First, a reaction containing 2 µg of RNA, 0.5 µg/µl of random primers (Promega #C118A) and RNAase free dH_2O up to a final volume of 12 µl was incubated at 70 °C for 4 min to allow binding of the random hexamers throughout the mRNA. The tubes were immediately returned to ice for 3 min before adding 8 µl of the RT reagents as follows:

- 1 µl of 10 mM dNTP mix (final concentration of 0.5 mM)

- 1 µl M-MLV (Moloney murine leukaemia virus RT, Promega) (200 U)
- 1 µl RNasin ribonuclease inhibitor (20 U)
- µl 5 X M-MLV RT buffer (Promega)
- 1 µl dH_2O (RNase free)

This was added to the RNA/random primer mix and incubated at 37 °C for 1 h and the resulting cDNA stored at –20 °C until required. RT enzyme was omitted from one reaction to serve as a negative control for genomic DNA contamination in the subsequent PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a general house-keeping gene to compare the amount of cDNA in each sample by PCR (see list of primers, table 2.2)

2.5.9 Polymerase chain reaction (PCR)

The PCR reaction is used to amplify a segment of DNA that lies between two areas of known sequence catalysed by the enzyme DNA polymerase. Two oligonucleotides that flank the region of interest and that are complimentary to sequences that lie on opposite strands of the DNA are synthesized. The template is first denatured by heating to 95 °C and then cooled to a temperature that is optimal for the annealing of the oligonucleotide primers (which are in excess). After this, DNA polymerase extends the annealed primers in the presence of excess of the four dNTPs. This cycle is repeated and, since each new product can serve as a template for the next cycle, there is a doubling in quantity of the desired DNA fragment with each cycle. The DNA polymerase used in the reaction is derived from the thermophilic bacterium, *Thermus Aquaticus* and this is used because it is not denatured at high temperatures.

Target	Forward (5' to 3')	Reverse (5' to 3')	Ta (°C)	Amplicon size
GAPDH	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC	55	198
hGRK2	GTGATCAGGAGCTCTACCGC	GCTATCGCACTGCAAAATGA	55	407
hGRK3	CTTCTGAGAGGTCACAGCCC	CAGCGTTCAGAGATGACCAA	55	401
hGRK4	GAAAGGCAACCCGTAACAAA	TGCCCAGGTTGTAAATGTGA	55	310
hGRK5	CCTGGGCTGGAGTGTTACAT	AGAGGATCTCTGCCGCATAA	55	667
hGRK6	CGAGAACATCGTAGCGAACA	GGTGTTTTTGGTCACTGGCT	55	547
hDyn 1	CGAGCTGGCTTACATGAACA	CACGGTCTTGTTGACAATGG	55	589
hDyn2	GGACTTACGACGGGAGATCA	TCTGCTCCGTGTTGAAGATG	55	635
hßarr1	GCCTCTAGCACCCTGTTGAG	CCTTGTCATCCTTCATGCCT	55	286
hßarr2	TGGAGCTGCCTTTTGTTCTT	TCATCCTTCATCCCCTTCAG	55	193
GRK stable	TAATACGACTCACTATAGGG	CCTCTGTCTCCAGCTTCTCG	55	343
Dyn stable	TAATACGACTCACTATAGGG	AGTGACTCGGTCGGTCTCAG	55	400
ßarr stable	CCTCATGTCGGACAAGCC	TCCACAGCCACGTCACCG	60-50 TD	482

Table 2.2 Primers. Primer sequences, annealing temperatures used and size of resulting amplicon. TD (touch down).

2.5.9.1 Designing primers

Where possible primers were designed as intron spanning. The full intron/exon sequence of the gene of interest was obtained from www.ensembl.org, and forward and reverse primers designed so that the product would span at least two exons. Generally, a product size of between 200 and 400 base pairs (bp) was favoured to increase the chances of the PCR working on the first occasion. To design the primers, a few guidelines were followed:

- One or more G or C residues were included at the 3' end of the primer to increase binding efficiency
- The primers were made at least 18 bp long to minimize secondary hybridisation
- Long stretches of single bases were avoided, especially G or C
- Primers with secondary structure were avoided
- Primers that could hybridize to form dimers were avoided
- Ideally, primers with melting temperatures (Tm) of between 55 °C - 65 °C were designed

Alternatively, PCR primer pairs were designed using Biology Workbench software. Primers were synthesized by Sigma-Genosys.

2.5.9.2 PCR Reaction

- **PCR buffer (10x) (Sigma #D6677):** 100mM Tris-HCl pH 8.3, 500mM KCl, 15mM $MgCl_2$

The following reagents were combined in PCR tubes (Abgene):

µl	Reagent	Final concentration
2.5	10x PCR buffer	1x
0.5	dNTPs (10 mM)	0.2 mM
0.25	*Taq* DNA polymerase (5u/µl, Sigma #D56677)	1.25 U
0.5	forward primer (10 mM)	0.2 mM
0.5	reverse primer (10 mM)	0.2 mM
X	Template cDNA	50-200 ng

dH_2O made up to a final volume of 25 µl

Thermal cycling was performed in a GeneAmp® PCR System 9700 (Applied Biosystems). Cycle number and annealing temperature (T_a) were optimised for each

primer pair. However, the T_a could be estimated by multiplying the number of G /C residues by 4 and the number of A/T residues by 2 and adding the two numbers together. Extension times were 1 min/kb of product.

2.6 HARVESTING CELLS FRACTIONS

2.6.1 Harvesting cell lysates

Solutions:

- **10x Cell lysis buffer -** 200 mM Tris pH 7.5, 1.5 M NaCl, 10mM EDTA, 10 % Triton X-100
- **Protease inhibitor cocktail (Sigma #P-8340) -** 104 mM AEBSF, 0.08 mM Aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, 1.4 mM E-64 in DMSO. Added to lysis buffer at a dilution of 1:1000.
- **Phosphatase inhibtor cocktail I (Sigma, #P-2850)** - phosphatase inhibitors in DMSO. Added to lysis buffer at 1:100.
- **Phosphatase inhibtor cocktail II (Sigma, #P-5726) -** phosphatase inhibitors in DMSO. Added to lysis buffer at 1:100.
- **1x Cell lysis buffer** - 1 x cell lysis buffer with protease and phosphatase inhibitor cocktails added just prior to use.

Cell plates were placed on ice and the culture medium aspirated. Cells were washed once in ice-cold PBS before adding an appropriate volume (100 µl for a well of a 6 well plate) of 1 x cell lysis buffer with additives, and incubated on ice for 5 min. Lysates were transferred to microcentrifuge tubes and left on ice for a further 15 min with intermittent vortexing. Cell debris was removed by centrifugation at 13,000 g for 3 min at 4 °C, and the supernatant frozen at –80 °C until required.

2.6.2 Preparation of nuclear extracts

Nuclear proteins were extracted using Nonidet P-40 mediated cytoplasmic lysis (Whiteside et al., 1992) adapted from original protocol (Dignam et al., 1983).

Solutions:

- **Buffer A** - 10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, protease inhibitor cocktail (DTT and protease inhibitors were added just prior to use).
- **Buffer C** - 20 mM Hepes pH 7.9, 25 % (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, protease inhibitor cocktail (DTT and protease inhibitors were added just prior to use).

The following volumes were suitable for cells in a well of a 6 well plate. Cells were washed in ice-cold PBS and then scraped in 1 ml of PBS and transferred to a microcentrifuge tube. Cells were pelleted by centrifugation for 15 s at 13,000 g. All remaining PBS was aspirated and the cell pellet resuspended in 400 µl buffer A. This solution was incubated on ice for 5 min to allow the cells to swell. 5 µl of 10 % Nonidet P-40 was added to lyse the plasma membrane, and the tube immediately mixed. The solution was centrifuged for 30 s at 13,000 g to pellet the nuclei. The supernatant (cytoplasmic fraction) was stored at –70 °C if required. The nuclear pellet was resuspended in 25 - 50 µl of buffer C and nuclear proteins extracted by vigorous shaking at 4 °C for 1 h. The solution was centrifuged for 10 min at 13,000 g and at 4 °C and the supernatant retained as pure nuclear protein.

2.7 IMMUNOPRECIPITATION

Lysates were precleared using around 0.5µg of a nonspecific antibody and 25µl (bed volume) protein A/G agarose beads (Roche) for 30 mins at 4°C on a rotating

wheel. The mixture was then centrifuged for a few seconds at 10,000 g and the beads discarded. 0.5μg of the primary antibody of interest was then added to the solution and incubated again at 4°C on a rotating wheel over night. Protein A/G beads (50μl bed volume) were then added for a further 1 hour incubation under the same conditions. The sample was centrifuged and the beads retained and washed in ice cold lysate buffer a further 3 times (with centrifugation and retaining of the beads on each occasion). Finally, bound proteins were dissociated from the beads by the addition of SDS sample buffer (see below for recipe) and heating of the sample for 5 mins at 95°C. Samples were subsequently anaylsed by SDS-polyacrylamide gel electrophoresis (see 2.6).

2.8 SDS - POLYACRYLAMIDE GEL ELECTROPHORESIS

Solutions:

- **10 x Tris-glycine** - 0.25 M Tris base, 2 M glycine
- **3X SDS Sample Buffer -** 187.5mM Tris-HCl pH6.8 at 25°C, 6% SDS (w/v), 30% glycerol (v/v), 150mM dithiothreitol (DTT), 0.03% bromophenol blue (w/v).

Denaturing polyacrylamide gel electrophoresis was carried out using the mini-protean II system (Biorad) and Tris-HCl ready gels (Bio-rad). Samples were prepared by adding an appropriate volume of 3 x SDS sample buffer to make a 1 x solution and subsequently denaturing the proteins by heating at 95 °C for 5 min. Samples were then loaded on to the gel and run alongside 10μl of 10-250 kDa Full Rainbow Molecular Weight Markers (Amersham). Gels were electrophoresed in 1 x Tris-glycine buffer with 0.1 % SDS for 1 h at 120 V. Gels were then subjected to Western Blotting.

2.9 WESTERN BLOTTING

Solutions:

- **Blotting buffer** - 1 x Tris-glycine (pre-chilled)
- **10 x Tris-buffered saline (TBS)** - 0.2 M Tris pH 7.6, 1.46 M NaCl
- **Wash buffer** - 1 x TBS, 0.1 % (v/v) tween 20
- **Blocking buffer** - 5 % Marvel in wash buffer

The mini-protean II system was used for the transfer of fractionated proteins in the gel to a PVDF membrane to allow for probing with antibodies. Gels were removed from the apparatus and soaked in blotting buffer. Sponge pads, provided with the system were also pre-soaked in blotting buffer along with 2 pieces per gel of 3mm Whatman filter paper (pre-cut to the same size as blotting pads). Hybond PVDF membrane (Amersham) was soaked for 1 min in methanol prior to soaking in blotting buffer. A sandwich containing all these components was created as in Fig. 2.1.

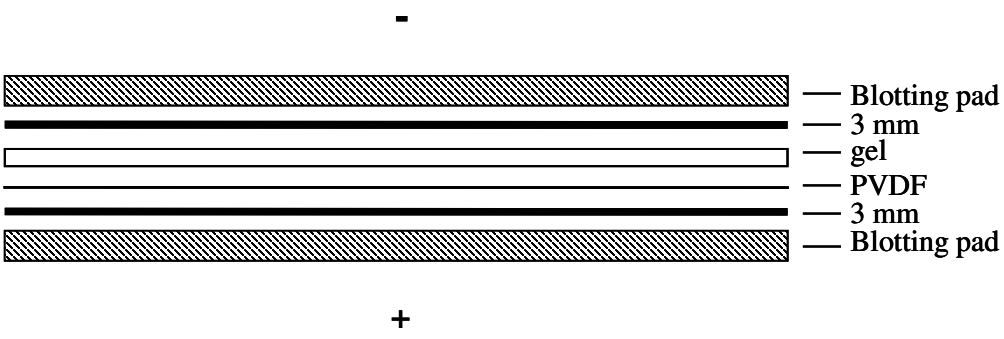


Fig. 2.1 ***Assembly of Western blotting components***

With each added layer, blotting buffer was poured on to ensure proper transfer. Electrophoresis was carried out at 80 V for 1 h in blotting buffer. Following this, the PVDF membrane was removed, inserted into a 50 ml tube and incubated in 5 ml blocking buffer, either overnight at 4 °C or for 1 h at room temperature, on a

rolling platform. Primary antibody was diluted in blocking buffer (the dilution was either recommended by the antibody manufacturer or determined by a dose titration) and 5 ml incubated with the membrane, again either over night at 4 °C or for 1 – 2 h at room temperature on a rolling platform. Following this, the blot was washed for 3 x 10 min in washing buffer, prior to incubation with 5 ml of horseradish peroxidase (HRP) labelled secondary antibody for 1 h at room temperature. The secondary antibody recognised the species in which the primary antibody was raised. Peroxidase labelled rabbit anti-mouse antibodies were from Cell Signalling Technology and peroxidase labelled donkey anti-rabbit from Amersham. The membrane was washed with 3 x 10 min of washing buffer and incubated for 5 min with the ECL Plus Western Blotting Detection Reagent (Amersham). This was then drained from the membrane, the membrane wrapped in Saran wrap (VWR, UK) and exposed to film (Hyperfilm ECL, Amersham, UK).

2.9.1 Stripping of membranes

Solutions:

- **Stripping buffer** - 62.5 mM Tris pH 6.8, 100 mM β-mercaptoethanol, 2 % SDS

Membranes were regularly stripped so that they could be reprobed with a different antibody. For this, membranes were incubated at 50 °C in stripping buffer for 30 min with occasional agitation. Membranes were then washed for 3 x 20 min with 30 ml wash buffer. Membranes could then be blocked and reprobed as described previously.

2.9.2 Densitometry

Bands on x-ray film were scanned on an HP ScanJet 6300C scanner. Densitometry was then performed using Image J Software with adjacent background sections of

the same area subtracted from each band. Each experiment was repeated at least 3 times.

2.10 ERK1/2 IN VITRO KINASE ASSAY

The $Erk_{1/2}$ *in vitro* kinase assay (Cell Signaling Technology) was used to give a measure of the activity of phosphorylated $Erk_{1/2}$ in the sample. The assay was carried out according to the manufacturer's instructions. The following solutions were supplied by the manufacturer.

Solutions:

- **10X Kinase Buffer** - 1X concentration: 25mM Tris (pH 7.5), 5mM b-Glycerolphosphate, 2mM DTT, 0.1mM Na3VO4, 10mM MgCl2
- **10X Cell Lysis Buffer** - 1X concentration: 20mM Tris (pH 7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton, 2.5mM sodium pyrophosphate, 1mM b Glycerolphosphate, 1mM Na3VO4, 1μg/ml Leupeptin
- **ATP** - 10mM (50 μl)
- **Immunoprecipitation antibody** - Immobilized Phospho- p44/42 MAP Kinase (Thr202/Tyr204) Monoclonal Antibody:
- **Primary antibody** - phospho-Elk-1 (Ser383) antibody (rabbit polyclonal IgG, affinity purified)
- **Substrate** - Elk-1 Fusion Protein: GST fused to Elk-1 codons 307–428.

Briefly, Cells were appropriately treated and then to harvest, the media was aspirated and washed once with ice-cold PBS. 200 μl of ice-cold 1X cell lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and plates incubated on ice for 5 minutes. Cells were transferred to cold microcentrifuge tubes and sonicated

on ice. Lysates were subsequently centrifuged at 10,000 g for 5 minutes at 4°C and the supernatant transferred
to a new tube. Lysates were stored at –80°C until required.

15 µl of immobilized antibody bead slurry were added to 200 µL of cell lysate and incubated with gentle rocking overnight at 4°C. Samples were centrifuged at 10,000 g for 30 seconds at 4°C. Pellets were washed twice 500 µL of 1X cell lysis buffer (samples were kept on ice during washes).

Pellets were then washed twice with 500 µL of 1X Kinase Buffer. Pellets were then suspended in 50 µl of 1X Kinase Buffer supplemented with 200µM ATP and an appropriate quantity of Elk-1 fusion protein. Reactions were incubated for 30 minutes at 30°C. The reaction was subsequently terminated with 25 µL 3X SDS sample Buffer (see 2.8). Samples were vortexed then centrifuged at 10,000g for 30 seconds.

Samples were analysed according to the western blotting protocol (2.6). Blots were probed with the phospho-Elk-1 (Ser383) antibody (rabbit polyclonal) and secondary antibody was that described in section 2.6.

2.11 GAL 4-ELK LUCIFERASE ASSAY

In order to quantify the transcriptional activity of MAPK activated in response to ACTH, a Gal4-Elk-1 luciferase reporter assay system was used (Roberson et al., 1995). This system consisted of a Gal4 Elk-1 expression vector encoding the DNA binding domain of Gal4 (amino acid residues 1-147) linked to the carboxy-terminal transcription activation domain of Elk-1 (residues 307-428) and a 5 x Gal4-E1b-luciferase (referred to as Gal4-luc) expression vector containing Gal4 binding sites upstream of the luciferase gene. On MAPK activation, Gal4-Elk-1 is phosphorylated leading to transcription of the luciferase reporter.

H295R cells were plated into 6 well plates and transfected as previously described (2.3.1) with 0.45 μg Gal4 Elk-1, 0.45 μg Gal4-luc and 0.1 μg Renilla per well. After 24 hours cells were transferred to SFM and incubated over night. The next day cells were treated with 10^{-7} M ACTH in SFM, SFM alone or medium containing 10 % serum for the indicated times. After stimulations cells were transferred back to SFM and were left for a total of 6 hours from the beginning of treatment. Cells were harvested and luciferase activity assayed using the Dual Luciferase reporter assay system (Promega) according to the manufacturer's instructions.

2.12 PROLIFERATION ASSAY

Solutions:

- **MTS/PMS solution** - Just prior to use 50 μl of PMS solution (0.92 mg/ml in PBS) was added to every 1 ml of MTS solution (0.5 mg/ml in PBS)

Proliferation assays were carried out on H295R cells using the CellTiter 96® AQueous proliferation assay (Promega). This assay relies on a novel tetrazolium compound, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, salt) and an electron-coupling reagent, PMS (phanazine methosulphate). MTS is reduced by dehydrogenase enzymes in metabolically active cells, into a soluble formazan product (Fig. 2). The quantity of formazan produced is measured by reading the absorbance at 490 nm, and this measurement is proportional to the viable cell number.

In order to increase the sensitivity of the assay, the assay was performed in 12 well plates in preference to 96 well plates. Plates were also coated with collagen to facilitate adherence of the cells as described previously. Briefly, H295R cells were plated so that the next day they were around 30 % confluent. Cells were washed twice in SFM and left for 48 h in the incubator to induce growth arrest. Following this, cells were treated with appropriate reagents for 72 h. Medium was aspirated

and 500 µl of SFM added to each well. 100 µl of MTS/PMS solution was added directly to each well and incubated for 1 h at 37 °C and 5 % CO_2. 100 µl of each well was pipetted into a 96 well plate and the absorbance read at 490 nm on a Molecular Devices Kinetic Microplate Reader. There were 6 replicates for each treatment and experiments were performed at least three times.

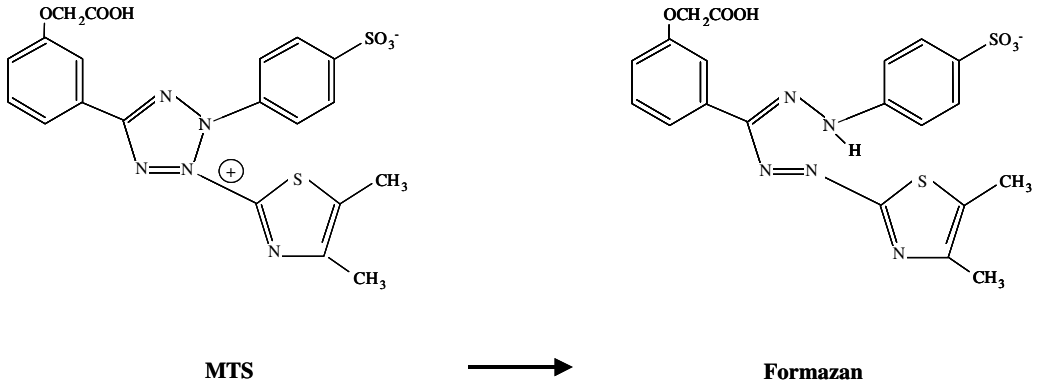


Fig. 2.2 ***Structures of MTS tetrazolium salt and its soluble formazan product***

2.13 MEASUREMENT OF CELLULAR CAMP

This assay is based on a saturation assay method designed by Brown et al (Brown et al., 1971). It is a competitive binding assay that allows the measurement of cAMP accumulation in response to ACTH or other stimulators of the cAMP pathway. The assay is based on the ability of binding protein, prepared from bovine adrenals, to bind cAMP in the sample in competition with a fixed amount of ^{3}H-cAMP. Activated charcoal is added to bind to and remove (by centrifugation) unbound cAMP, and a proportion of the ^{3}H-cAMP in the remaining sample is counted in a scintillation counter. The resultant count is thus inversely proportional to the amount of cAMP in the original sample. The principle of the assay is summarised in Fig. 2.3.

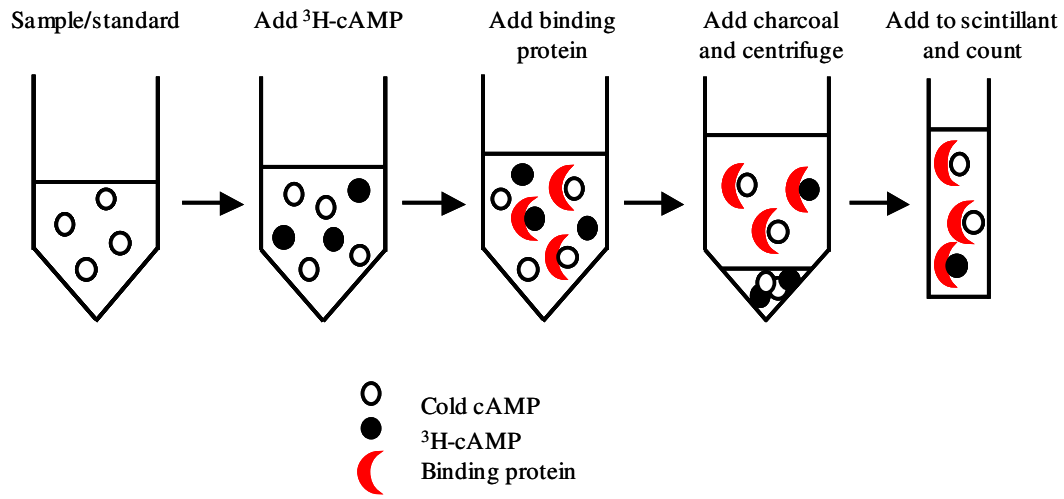


Fig. 2.3 Summary of competitive cAMP assay. *A fixed amount of ^{3}H-cAMP is added to a sample containing an unknown amount of cAMP. Binding protein competitively binds cAMP and sequesters it in solution. Activated charcoal binds to unbound cAMP and is pelleted leaving bound cAMP in solution, a proportion of which is counted in a scintillation counter.*

2.13.1 Preparation of binding protein

Solutions:

- Buffer – Tris- HCl pH 7.4, 250 mM sucrose, 25 mM KCl, 5 mM $MgCl_2$, 50 mM
- 10 bovine adrenal glands

10 bovine adrenal glands were collected as soon as possible after slaughter and transported on ice. The fat and connective tissue were removed and the cortices separated from the medulla and weighed. This was subsequently finely chopped and homogenised in 1.5 volumes (based on the weight of the tissue) of ice-cold buffer. The resultant homogenate was filtered through nylon mesh and then centrifuged at

2000 g at 4 °C for 5 min. The supernatant was centrifuged at 5000 g at 4 °C for 15 min and the supernatant aliquoted into 1 ml aliquots and stored at –70 °C until required. A working solution of binding protein was prepared by diluting 1/10 to 1/20 in Tris buffer. This dilution was determined for each batch of binding protein prepared so that B_o/Total count ratio was 30-50 % (the B_0 control is explained later).

2.13.2 Stimulating and harvesting of samples

Cells were plated into 6 well plates and grown to around 90 % confluence. Prior to stimulation, cells were washed and left in SFM for 1 h. Treatments were carried out in 1 ml of SFM in the presence of 1 mM IBMX (Sigma #I5879). To harvest, plates were transferred to ice and cells were scraped into their stimulation medium. Cells and media were transferred to 1.5 ml microcentrifuge tubes, boiled for 5 min and centrifuged at 10,000 g for 5 min. The supernatant was transferred to fresh tubes and stored at –20 °C until the cAMP assay was performed.

2.13.3 cAMP assay

Solutions:

- **Tris buffer** - 50 mM Tris-HCl pH 7.4, 1 mM IBMX, 6 mM β-Mercaptoethanol.
- **Charcoal solution** - Tris buffer containing 5 % (w/v) activated charcoal (BDH#332034F), 0.15 % (w/v) bovine serum albumin. This was prepared a day before the assay and kept at 4 °C with constant mixing until required.
- **[2,8-3H] cAMP** - 1 μCi [2,8-3H] adenosine 3'5'cyclic phosphate NH_4 salt (35 Ci/mmol, Amersham) was diluted 1:6000 in dH_2O and stored at 4 °C until required.

- **Binding protein** - prepared as described above and diluted to the working concentration
- **cAMP** - (Sigma A-4137) reconstituted in dH_2O to give a stock solution of 100 pmol/µl and aliquoted. Stock was diluted to 1 pmol/µl in Tris buffer prior to use.

Binding reactions were prepared as follows in 0.5 ml microcentrifuge tubes:

Vol	**Reagent**
100 µl	sample/standard/tris buffer
50 µl	[2,8-^{3}H] cAMP
100 µl	Binding protein

Standards were prepared by making serial dilutions of cAMP standard in tris buffer between 0.5 and 32 pmol. Samples with unknown amounts of cAMP were quantified by assaying alongside these standards. Binding reactions were mixed by vortexing and incubated at 4 °C for 1.5 – 2 h. After this time, 200 µl of charcoal solution was added to each sample in batches of 16 tubes, vortexed and centrifuged at 10,000 g for 7 min. 200 µl of supernatant was added to 4 ml of scintillation fluid (Ecoscint A, National Diagnostics) vortexed and counted for 2 min using a Wallac D1409 DSA Liquid Scintillation Counter (Perkin Elmer Lifesciences). A number of controls were included for each assay:

Non-specific binding: A reaction containing no cAMP and no binding protein controlled for the ability of charcoal to remove all of the unbound cAMP in the sample. The resultant count was subsequently subtracted from each count.

Total count: Binding protein and cold cAMP were substituted for tris buffer and charcoal was not added. This gave a measure of the total ^{3}H-cAMP added to the sample.

B_o: Sample/standard was substituted with tris buffer to control for the ability of binding protein to sequester cAMP in the solution. This was affected by the concentration of binding protein, which as mentioned previously, was determined for each batch of binding protein so that B_o/Total count ratio was 30-50%. To control for each centrifugation step and a possible change in Bo with time, a Bo was also included for every 16 tubes.

Samples, standards and controls were assayed in duplicate. After subtraction of non-specific binding, each reaction was averaged and expressed as a percentage of the Bo. The standard curve was a plot of % Bo for each standard against log10 of the cold cAMP concentration. An equation of the line was obtained (via Excel) and the amount of cAMP in a sample could be calculated using this equation.

2.14 ^{125}I-ACTH BINDING ASSAY

Solutions:

- **^{125}I-ACTH** - 10 μCi [^{125}I] (iodotyrosyl^{23}) ACTH (2000 Ci/mmol Phoenix Pharmaceuticals) was reconstituted in 100 μl of dH_2O, aliquoted and frozen at -20°C until required.
- **Acid glycine** - 50mM glycine, 100 mM NaCl, pH 3
- **Wash solution** - 0.9 % (w/v) NaCl
- **Lysis buffer** - 0.5 M NaOH, 0.4 % (w/v) sodium deoxycholate

A competitive binding assay was performed to determine specific binding of ACTH to the surface of H295R cells (Penhoat et al., 1993). Cells were seeded onto 12 well plates and when confluent left in SFM for 1 hour. Cells were then washed twice in wash solution and then incubated for 10 mins in 500 μl of acid glycine solution to remove surface bound ligands. Cells were subsequently washed a further two times in wash solution and then incubated with 0.025 pmol of ^{125}I-ACTH with increasing

concentrations of "cold" ACTH (10^{-5}-10^{-12} M) in SFM containing 0.5 % BSA for 60 mins at 20 °C. After this time, solutions were removed and the cells washed three times in ice-cold wash solution. Cells were then lysed in 500 µl of lysis buffer for 10 mins, and radioactivity assessed by γ counting. A displacement curve was obtained where specific binding, expressed as the percentage of total binding where no "cold" ACTH was present, was plotted against the concentration of "cold" ACTH.

2.15 ^{125}I-ACTH INTERNALISATION ASSAY

Solutions used are the same as those used in the ACTH binding assay (2.14). H295R cells were seeded into 12 well plates and when confluent were transferred to SFM for 1 hour. Cells were then washed twice in wash solution and then incubated in acid glycine for 10 mins. Cells were washed a further two times in wash solution before incubation for the indicated times with 0.025 pmol ^{125}I-ACTH in SFM with 0.5 % BSA (Incubations were started at different times so that all the harvesting could be done at the same time). Cells were then placed on ice and washed three times in ice-cold wash buffer and incubated in 500 µl of acid glycine on ice for 10 mins. This fraction was harvested and its radioactivity assessed as the extracellular bound ACTH. Cells were washed once more in wash solution and then lysed in 500 µl of lysis buffer and the radioactivity assessed as the internalised ACTH fraction. Results were expressed as the proportion of internalised ACTH (i.e. intracellular / (extracellular + intracellular) against time. For the zero time-point, cells were incubated on ice for 5 mins (to eliminate internalisation) before the addition of ^{125}I-ACTH for around 30 seconds. This reading was deducted from all subsequent readings to correct for background. Where inhibitors were used, cells were pre-treated for 1 h in SFM containing 0.5 % BSA, prior to the addition of ^{125}I-ACTH for the appropriate times.

2.16 BIOTIN METHOD OF INTERNALISATION

This method is based on that described in Roberts et al (Roberts et al., 2001). Cells were serum starved over night prior to pretreatment with 0.3mM Monodansyl cadaverine (MDC) in PBS or PBS alone for one hour. Surface labelling was performed at 37°C with 0.2 mg/ml NHS-SS-biotin (Pierce) in PBS for 30 min. Labelled cells were washed in 50mM Tris pH 8 and transferred to SFM with or without 10^{-7}M ACTH with or without MDC, at 37°C.

At the indicated times, the medium was aspirated, and the dishes were rapidly transferred to ice and washed twice with ice-cold PBS. Biotin was removed from proteins remaining at the cell surface by incubation with a non cell permeable reducing agent containing 20 mM 2-Mercaptoethanesulphonic acid sodium salt (MesNa) in 50 mM Tris (pH 8.6) and 100 mM NaCl for 15 min at 4°C. MesNa was quenched by the addition of 20 mM iodoacetamide (IAA) for 10 min, and the cells were lysed in PBS with protease inhibitor cocktail (Sigma #P-8240 at 1:1000).

Samples were subsequently immunoprecipitated (see 2.7) using 0.5µg of an antibody (goat) that recognises the C-terminal portion of the MC2R (Santa Cruz, CA). Samples were run on an SDS-PAGE gel and immunoblotted with 1:100 dilution of anti-biotin antibody (Cell Signaling Technology, MA).

3 CHARACTERISATION OF ADRENAL CELL LINES AS MODELS OF ACTH INDUCED $ERK_{1/2}$ SIGNALLING

3.1 INTRODUCTION

Previous *in vitro* studies concerning the mitogenic role of ACTH, have used a variety of primary adrenal cells from diverse species, in addition to transformed cell lines. As it has already been discussed, these investigations have yielded mixed results and as yet no clear mechanisms have been established at a molecular level to describe the effects on adrenal growth seen *in vivo*. Finding a suitable cell system in order to continue these studies is complicated by several factors. Firstly, the reproducibility of experiments using primary cells is inherently difficult because of differences between animals, in addition to the variation involved in making cell preparations. Furthermore, obtaining sufficient numbers of cells for experiments limits the size of animal that can be used. A further complication though, is the fact that studying mitogenic pathways is intrinsically difficult in transformed cell lines because these cells usually display abnormal growth regulation. An additional consideration when looking at ACTH signalling in adrenal cells is that the majority of primary adrenal cells and adrenal cell lines have lost ACTH responsiveness and are incapable of producing a steroid output, therefore looking at growth mechanisms in response to ACTH may be futile. This introduction aims to give a brief overview of some of the cell systems available for the study of the proliferative mechanisms initiated by ACTH.

3.1.1 The murine Y1 adrenocortical tumour cell line

The Y1 cell line was derived from an adrenal tumour that arose in an adult LAF_1 male mouse following exposure of the mouse to radiation from an atomic blast (Cohen et al., 1957). The tumour was propagated by intramuscular transplantation and was initially found to be highly metastatic. The tumour was able to produce

corticosterone and responded to ACTH by increasing steroid output and was thus thought to be ZF in origin. In subsequent passages, the tumour lost its metastatic properties and the ability to produce corticosterone however it maintained its ACTH responsiveness and steroidogenic capacity (Bloch and Cohen, 1960). The tumour was adapted to grow *in vitro* by alternately dispersing cells and growing the cells as a monolayer, followed by propagating the tumour in the mouse (Buonassisi et al., 1962). The Y1 cell was subsequently cloned from this adapted tumour (Yasamura et al., 1966). The American Type Culture Collection (ATCC) and the European Collection of Cell Cultures (ECACC) currently hold the Y1 cell line. However, the current passage of cells held by ATCC, although able to produce steroids has lost its ACTH responsiveness. When originally isolated, the Y1 cell line produced steroids at a rate of 3.7 μg/mg protein per hour at maximal stimulation (Yasamura et al., 1966) however this output has been shown to decline with continual culturing (Schimmer, 1979) and consequently many strains of Y1 cells exist with varying phenotypes.

The use of the Y1 cell line in characterising the MC2R has already been discussed in section 1.4 however this cell line has also proven to be an invaluable tool in elucidating some of the key steps in steroidogenesis. In contrast to the normal mouse adrenal gland whose major steroid output is corticosterone, Y1 cells produce 20α-dihydroxyprogesterone and 11β-20α-dihydroxyprogesterone (Pierson, 1967). This abnormal profile is due to the lack of the 21-hydroxylase enzyme (CYP 21) and an increase in 20-keto-reductase activity (Parker et al., 1985). Despite this slight abnormality, the regulation of steroid synthesis is similar to that seen in cells cultured from normal adrenal glands with regard to ACTH responses. Thus important discoveries such as the role of Steroid acute regulatory protein (StAR) have utilised the Y1 cell line (Lin et al., 2001, Lin et al., 1995).

The Y1 cell line has also been of major importance in characterising some of the mitogenic and anti-mitogenic properties of ACTH as discussed in section 1.6.3.1 (Lotfi et al., 1997). However, these cells are not ideal in studying growth

mechanisms for a number of reasons. Firstly, Y1 cells have a very fast doubling time of 30-40 h and therefore have markedly different growth rates to that of *in vivo* adrenal growth. Secondly, Y1 cells have been shown to over express c-Ki-ras due to an amplification of the protooncogene, leading to over activation of the $Erk_{1/2}$ pathway (Schwab et al., 1983). Constitutive over activation of this pathway and potentially other growth promoting pathways in this cell line would make the study of the effects of ACTH on these pathways difficult.

3.1.2 NCI-H295 and H295R cell lines

The NCI-H295 cell line is a human adrenocortical carcinoma cell line initially established from an invasive primary adrenocortical carcinoma present in a 48 year old female (Gazdar et al., 1990). The tumour was finely minced and the resulting suspension cultured for a year in a variety of SFM and serum containing media. Many of the steroids secreted by these cells were identified and it was found that the cells seemed to contain all of the major adrenocortical enzyme systems spanning all three zones of the adrenal cortex. From this suspension cell line, sub-strains have been derived using alternative growth conditions and by encouraging growth of clones that attached to a cell culture flask. These cells have been designated H295R cells. In order to maintain steroidogenic capacity and to increase cell growth rate, a number of supplements were added to the cell culture media such as Ultroser and Nu-serum, both low protein serum substitutes which replace the need for BSA (Hornsby and McAllister, 1991).

It has been established through binding studies using ^{125}I Angiotensin II (Ang II) that H295R cells express AT1 receptors (Bird et al., 1994, Bird et al., 1993). Consequently the cell line has been used to look at intracellular signalling pathways in response to Ang II in addition to looking at the regulation of Ang II mediated aldosterone production (Bird et al., 1993, Wu et al., 2001). By contrast, the H295R cell line is thought to be only mildly responsive to ACTH in some strains and completely resistant to ACTH in others. The lack of response to ACTH may reflect

the low level expression of MC2R in this cell line (Mountjoy et al., 1994). Some experiments aimed towards looking at the cAMP pathway, have used forskolin to directly activate adenylate cyclase or cAMP analogues, to mimick the effect of ACTH (Bird, 1993).

3.1.3 Bovine adrenocortical cells

Primary cultures of bovine adrenal cells have been widely used to study adrenal cell function. These are attractive because of the large size of the organ and therefore the relative abundance of primary cells that can be isolated from them. They are also readily available from abattoirs. Previous studies have investigated an ACTH growth promoting effect and found ACTH to be inhibitory to DNA synthesis and to reverse the growth promoting effect of FGF (Gospodarowicz et al., 1977).

Bovine adrenocortical cells also require very specific growth conditions to maintain expression of 11ß hydroxylase and 21-hydroxylase including the addition of IGF-1 and cAMP (Cheng and Hornsby, 1992).

An alternative may be the use of an immortalized bovine cell line derived from the forced expression of telomerase ribonucleoprotein complex (TERT) (Thomas and Hornsby, 1999). In this study it was found that transplantation of immortalized cell clones into SCID mice, formed functional tissue with a similar rate of cell division to transplanted bovine cells with a finite lifespan. This would suggest that these cells behave as functioning adrenal cells and do not have the cell cycle abnormalities associated with other cancer derived cell lines. These cells may therefore be of use in our studies as they are likely to behave consistently with each passage. Furthermore, they have not previously been used in studies of mitogenic pathways and may prove a useful tool.

3.1.4 Other cell lines

Several attempts have been made to establish cell lines from adrenal tumours in mice carrying Simian Virus 40 T-antigen as a transgene (Mellon et al., 1994, Kananen et al., 1996). These cells however were not responsive to ACTH but required the use of 8-bromo-cAMP for progesterone synthesis. In addition a number of attempts have been made to produce ras transformed rat adrenal cells however these cells do not maintain their steroidogenic capacity (Auersperg et al., 1977, Auersperg et al., 1981). Furthermore since they have a ras transformation, they would not be ideal for investigating ras signalling pathways.

3.1.5 Strategies

For these studies, it was decided to attempt to use the Y1 adrenocortical cell line in order to reproduce previous work by Armelin and co-workers on the activation of the $Erk_{1/2}$ signalling pathway in response to ACTH and its downstream proliferative effects, and to further characterise the signalling molecules and protein complexes that transduce a signal from binding of ACTH, through to the activation of the $Erk_{1/2}$ pathway. Should this cell line prove unresponsive to ACTH, alternative cell systems would be sought to investigate the mitogenic effects of ACTH.

The primary method of investigation will be to use immunoblotting to detect levels of phospho $Erk_{1/2}$ vs total $Erk_{1/2}$ in cell lysates derived from ACTH treated and untreated cells. If necessary, to ensure that phospho $Erk_{1/2}$ detected is actually capable of activating a downstream kinase, an $Erk_{1/2}$ activity assay will be performed whereby its ability to phosphorylate Elk-1 in an *in vitro* kinase reaction will be determined (Marshall, 1995).

The ability of ACTH to induce a proliferative effect will be measured using the colorimetric MTS assay (see 2.8). According to the manufacturers, this assay is equally as sensitive as a [^{3}H] thymidine incorporation assay and gives a measure

proportional to viable cell number. It is important that it is a sensitive measure as the effects of ACTH would be expected to be minimal as has been previously shown in Y1 cells (Lotfi et al., 1997). Furthermore, it is also an extremely simple method requiring the addition of the solution to the culture dish and measurement 1-4 hours later and thus should reduce experimental error in comparison to other methods such as cell counting.

3.2 METHODS

3.2.1 Reagents

ACTH (1-39) was obtained from Bachem as a lyophilized solid. It was reconstituted in dH_2O to give a stock solution of 10^{-4} M and aliquoted and stored at –20 °C. Basic fibroblast growth factor (bFGF) was obtained from BD Biosciences (Bedford, UK), reconstituted in dH_2O to 10 μg/ml, aliquoted and stored at –20 °C. Epidermal growth factor was obtained from Sigma and reconstituted in SFM containing 0.1 % BSA to give a stock solution of 10 μg/ml. This was aliquoted and stored at –20 °C.

3.3 RESULTS

3.3.1 *Activation of Erk1/2 in Y1 adrenocortical cells*

Previous studies in Y1 cells that looked at the activation of the $Erk_{1/2}$ pathway, used long periods of serum starvation in order to growth arrest cells so that differences in phosphorylated $Erk_{1/2}$ could be seen. Armelin and co-workers used a 72 h period of serum starvation in order to achieve growth arrest at the G0/G1 phase of the cell cycle prior to looking at the effects of ACTH (Lotfi et al., 1997). To visually assess the viability of cells after long periods of serum starvation of Y1 cells, cells were grown to around 60% confluence and then maintained in SFM for up to 72 h. After a period of 24 h of serum starvation, cells were generally unchanged in

appearance and had continued to grow. After 48 h, cells had become more confluent and by 72 hours there was no obvious increase in growth. By this time however, cells have become very clustered and were only loosely attached to the cell culture flask. Cells were not suitable for use at this stage because they became detached with a change of medium. Since cells were unusable at 72 h of serum starvation and were still proliferating at 24 h, it was decided to use 48 h of serum starvation in order to growth arrest cells, prior to stimulations.

In an initial experiment, intended to confirm the findings of Armelin and co-workers, Y1 cells were grown in 6 well dishes and serum starved for 48 hours at 60% confluence. The medium was then replaced with SFM containing a range of ACTH concentrations from 10^{-12} M to 10^{-6} M, along with positive and negative controls of SFM supplemented with FBS and SFM alone, respectively. Cells were placed on ice after 5mins and protein extracts made. These were then electrophoresed and western blotted with antibodies against phospho $Erk_{1/2}$ and total $Erk_{1/2}$, which served as a loading control. Fig. 3.1A shows the results of these experiments where densitometry of the resulting films was performed, normalised to total $Erk_{1/2}$ in each case. The positive control (serum) produced an approximately three-fold increase in $Erk_{1/2}$ phosphorylation and there is a clear dose response to ACTH which at 10^{-7} M gives an equivalent increase to that of serum.

However, these findings proved not to be very reproducible due to an often very high basal activation of $Erk_{1/2}$, even after 48 h of serum starvation. A time-course of $Erk_{1/2}$ activation was also very inconsistent with peak phosphorylation ranging between 5 mins and 2h (data not shown). There was a possibility that this was a consequence of the methodology employed to assess $Erk_{1/2}$ activation and so an $Erk_{1/2}$ *in vitro* kinase assay was carried out in order to confirm these findings. This was to give a measure of the activated $Erk_{1/2}$ that was capable of phosphorylating a down-stream transcription factor, Elk-1. Fig 3.1B is a representative blot demonstrating high basal activation of $Erk_{1/2}$ leading to phosphorylation of Elk-1.

There was no significant increase in Elk-1 phosphorylation across the ACTH time course compared to a large increase in the serum control.

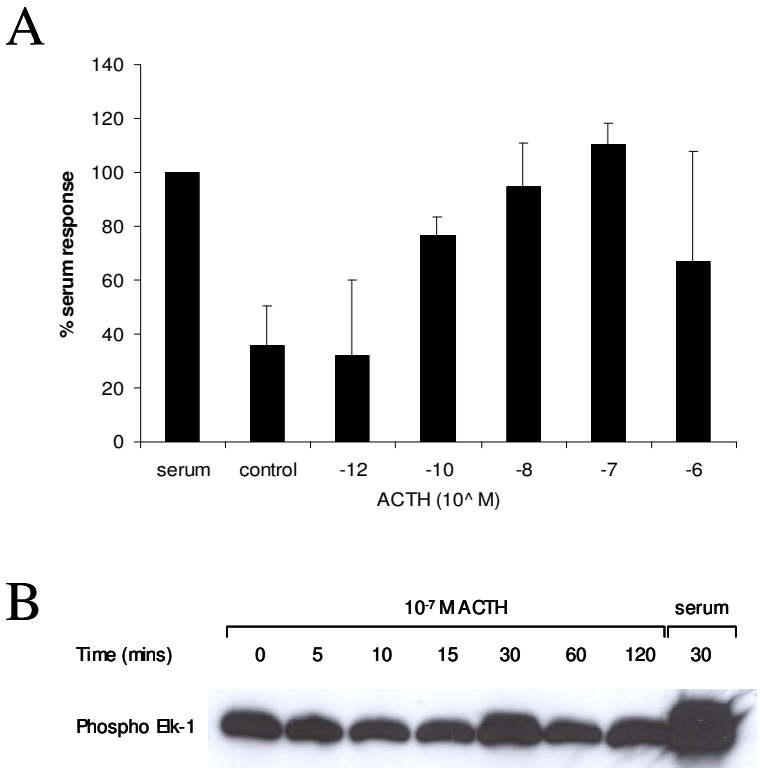


Fig. 3. 1. ACTH induced Erk$_{1/2}$ responses in Y1 cells. *A, 48 h serum starved cells were stimulated for 5 min with increasing concentrations of ACTH or serum supplemented media. Cell lysates Western blotted and probed using phospho-erk$_{1/2}$ antibody. X-ray films were scanned and the densitometry of bands determined as a percentage of the serum response. N=3. Error bars represent SEM. B, Y1 cells were serum-starved and stimulated with 10^{-7} M ACTH or serum supplemented media for the times shown. Lysates were used in an Erk$_{1/2}$ in vitro kinase assay and resultant reactions Western blotted and probed for phospho Elk-1.*

After initial success with this cell line, the high constitutive levels of activated Erk$_{1/2}$ already present in the serum starved cells made any subtle differences between treatments very difficult to visualise. In order to try to reduce constitutive activation

of $Erk_{1/2}$, cells were pre-treated for 24 h with 1 μM dexamethasone after an initial 24 h serum starvation, prior to stimulation with ACTH. Dexamethasone is known to increase MAPK phosphatase (MKP-1) expression, which acts to dephosphorylate $Erk_{1/2}$ (Lasa et al., 2002). Cells were also pre-treated for 1 h with a MEK1 inhibitor U0126 in order to try to reduce constitutive activation of this pathway. Pre-treatment of Y1 cells with dexamethasone did not reduce basal $Erk_{1/2}$ activation (Fig. 3.2A) and thus treatment with FGF, a known activator of this pathway, did not induce $Erk_{1/2}$ activation above basal levels. Pre-treatment of cells with U0126 abolished constitutive levels of $Erk_{1/2}$ activation (Fig.3.2B lane 1) however this effect was removed with the addition of SFM or PBS for 5 min, with phosphorylated $Erk_{1/2}$ levels increasing back to those of untreated cells maintained in serum (lanes 2 and 3 compared to lane 4).

Although other groups have shown a positive effect of ACTH on $Erk_{1/2}$ activation, our data, using our sub-line of Y1 cells, has demonstrated a constitutive activation of this pathway. It was felt that the Y1 cell line would not be a useful system in which to study the potentially weak mitogenic activity of ACTH and it was decided to investigate other adrenal cells to find an appropriate model for the study of ACTH-MC2R mitogenic signalling mechanisms.

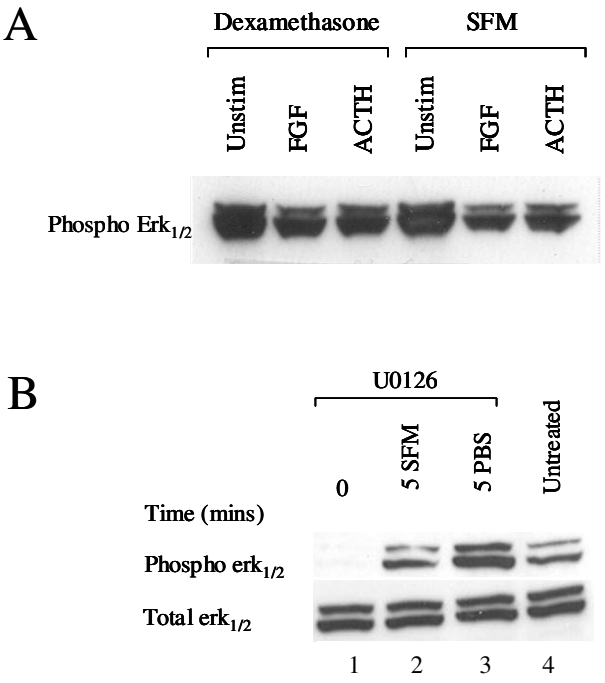


Fig. 3.2 Treatment of Y1 cells with dexamethasone and U0126 to reduce basal Erk$_{1/2}$ stimulation. *A, Y1 cells were left in SFM for 24 h before addition of dexamethasone (1μM) or SFM for a further 24 h. Cells were either untreated (in SFM) or treated with 100 ng/ml FGF or 10^{-7} M ACTH for 5 mins. Cell lysates were immunoblotted for phosho-erk$_{1/2}$. B, Y1 cells were treated with U0126 (1μM) in SFM overnight or left in serum supplemented medium (untreated). Cells were then washed twice in SFM and harvested immediately or washed twice before the addition of fresh medium or PBS for 5 mins. Immunoblotting for phospho Erk$_{1/2}$ was carried out on cell lysates.*

3.3.2 Bovine adrenal cells

Primary bovine adrenal cells (SBAC) obtained from ECACC and a telomerase ribonucleoprotein complex (TERT) immortalized bovine adrenal cell line, a kind gift from P.J. Hornsby (Thomas et al., 2000, Thomas and Hornsby, 1999), were assessed for their suitability for studying ACTH and MC2R interactions. Responses to ACTH were assessed by cAMP assay in comparison to Y1 cells (Fig. 3.3).

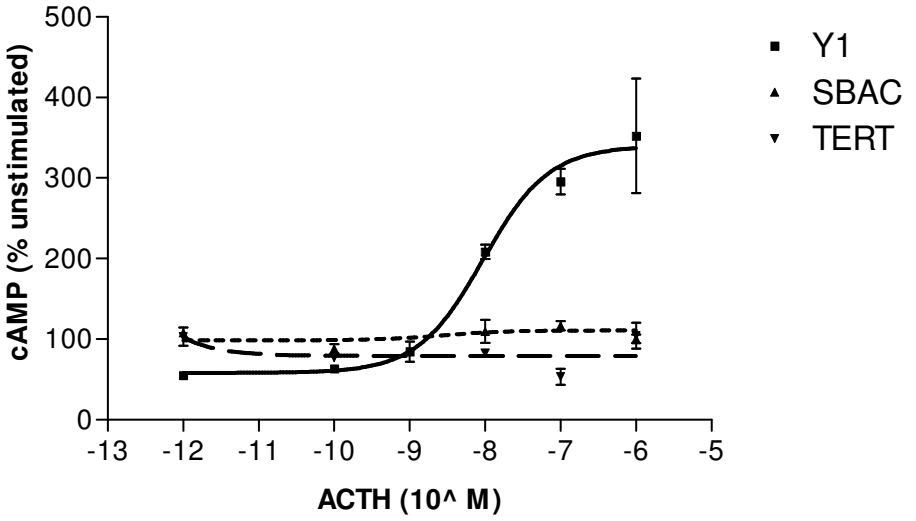


Fig. 3.3 Comparison of adrenal cell responses*. Y1, SBAC and TERT cells were treated for 45 min with increasing concentrations of ACTH and analysed for cAMP accumulation. Results are expressed as a percentage of unstimulated for each cell line and are results from one experiment with three replicates. Error bars represent SEM.*

Y1 cells produced a typical dose response to ACTH with an EC_{50} of 5nM. SBAC and TERT immortalized adrenal cells however did not respond to ACTH. In addition to cAMP assays, cells were also assessed for the activation of $Erk_{1/2}$ following ACTH treatment over a time-course of 1 hour. Neither cell type activated the $Erk_{1/2}$ pathway compared to untreated cells (data not shown). These cells were clearly not useful for these studies.

3.3.3 The H295R cell line as a model for ACTH induced mitogenic signalling pathways

To test the viability of the H295R cell line for our studies we first sought to check the expression of the MC2R and the response of the cells in terms of cAMP production after ACTH stimulation (Fig 3.4).

Fig. 3.4A demonstrates the presence of the MC2R in H295R cDNA, comparable to that of the positive control human adrenal cDNA. MC2R expression was not present in a negative control, HeLa cDNA. To ascertain whether this MC2R was functionally expressed, cells were treated with varying concentrations of ACTH, and assessed for cAMP accumulation in the presence of the phosphodiesterase inhibitor IBMX. Forskolin was used as a positive control. Although the results were not initially significant, the trend suggested that the cells were responding in a dose dependent manner (Fig 3.4B). In order to establish significance, three of the points (unstimulated, 10^{-7} M Forskolin and 10^{-7} M ACTH) were repeated and after performing this experiment seven times indicated a significant increase in cAMP accumulation ($p<0.001$) (Fig 3.4B) although this was only a 0.8-fold increase compared to a 6-fold increase with forskolin treatment.

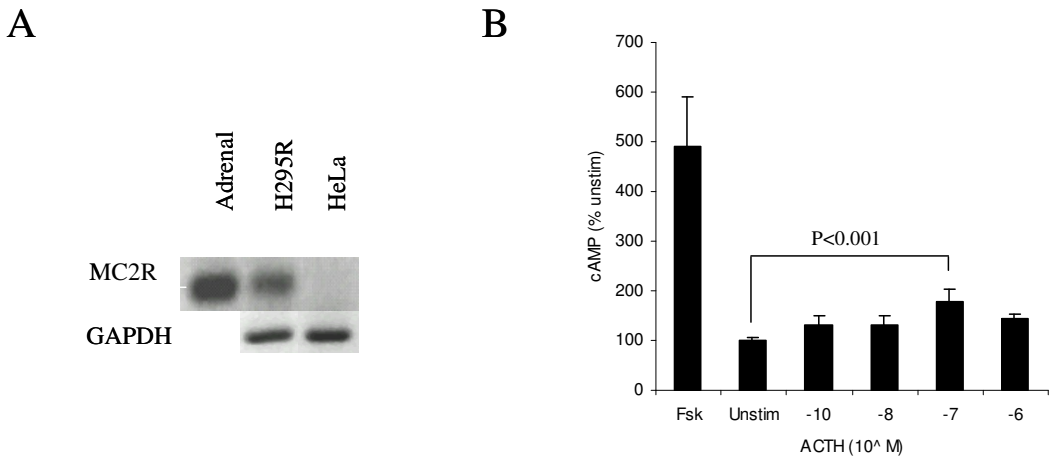


Fig 3.4 Characterisation of a functional MC2R*. A, PCRs using MC2R primers on human adrenal, H295R and HeLa cDNA (35 cycles). B, H295R cells were treated with various concentrations of ACTH or 10 $^{-5}$ M forskolin for 45 min. Cells were subsequently harvested and analysed for cAMP content (n=3 for 10^{-10}, 10^{-8} and 10^{-6}M, for other conditions n=7). Error bars represent SEM*

To further assess whether the MC2R was functionally expressed at the cell surface, I^{125} ACTH binding experiments were undertaken (Fig. 3.5). A displacement-binding

curve was performed where 0.025 pmol of ^{125}I-ACTH was incubated with increasing concentrations of "cold" ACTH (Fig. 3.5). These results showed specific binding with an IC_{50} of 4.68 x 10^{-7} M. Taken together, these results demonstrate that ACTH induces a significant, albeit it weak, cAMP response in H295R cells and furthermore that ACTH is able to specifically bind to the surface of these cells. This suggests that the H295R cell line may be a useful model for the study of the effects of ACTH on mitogenic pathways since they express a functional MC2R.

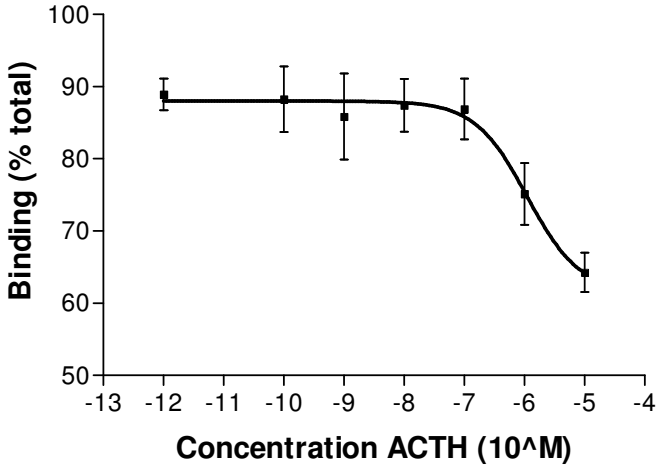


Fig. 3.5 Binding of I^{125} ACTH in H295R cells. *A, a fixed quantity of I^{125} ACTH (0.025 pmol) was added to cells in the presence or absence of various concentrations of "cold" ACTH (1-39) for 1 h. Specific binding was expressed as a percentage of total binding i.e. in the presence of no cold ACTH. The experiment was performed 4 times in triplicate. Error bars represent SEM.*

3.3.4 Characterisation of activated pathways in H295R cells

Having established that H295R cells express functional MC2R, it was possible to determine whether ACTH initiates any mitogenic signalling pathways in this adrenal cell line. It was decided first to look at the activation of the $Erk_{1/2}$ pathway in response to ACTH. An initial study determined that an overnight incubation in SFM was sufficient to give very low basal activation of $Erk_{1/2}$ (Fig 3.6A, lane 1). H295R cells were grown in 6 well dishes, serum starved overnight and then treated with 10^{-7} M ACTH over a time course. This concentration of ACTH was chosen because it appeared to give the maximal response in the cAMP assay (Fig 3.5B). Cells were harvested over a 1h time course and $Erk_{1/2}$ phosphorylation was visualised by western blotting. Fig 3.6A shows that $Erk_{1/2}$ phophorylation was transiently induced by treatment with 10^{-7} M ACTH maximally at 5 min and this activation was maintained to a lesser extent up to 30 min (Fig. 3.6A and B). The extent of activation was comparable to that of a 15 min treatment with medium containing 10 % serum. Furthermore, $Erk_{1/2}$ was activated in a dose dependent manner at 5 mins (Fig. 3.7). Densitometry of this dose response gave an EC_{50} for $Erk_{1/2}$ phosphorylation of 3 nM.

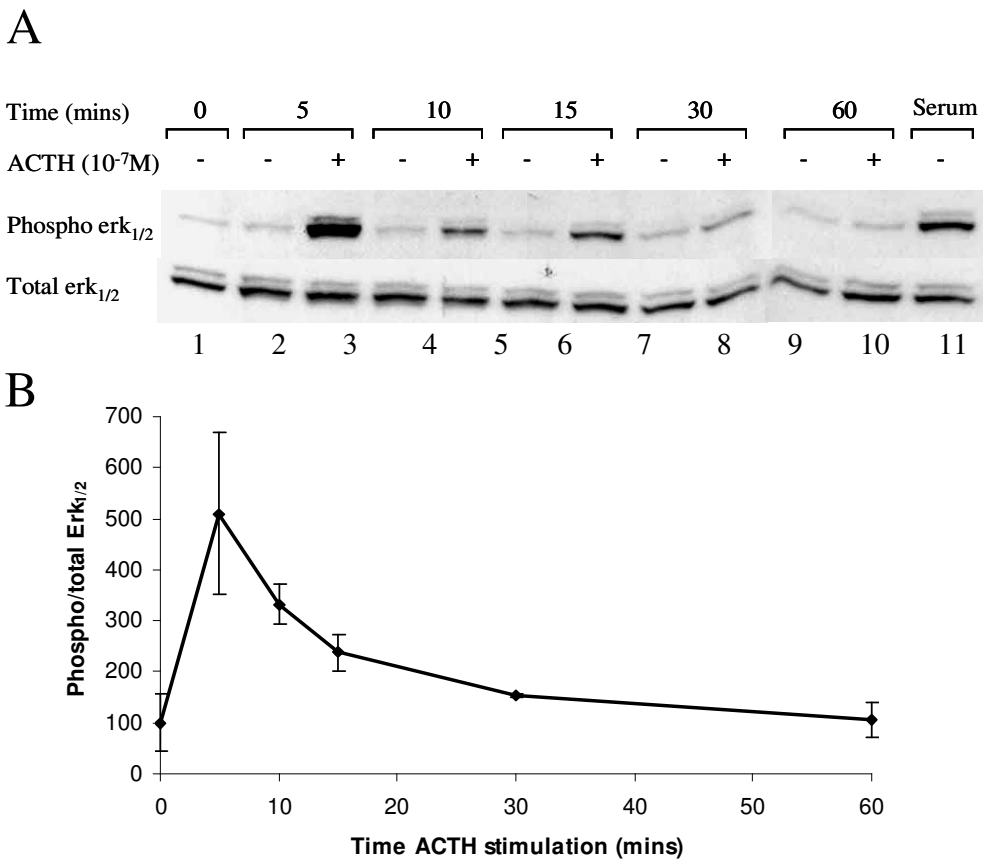


Fig. 3.6 ACTH stimulation of Erk in H295R cells. *H295R cells were serum starved overnight. The next day media was changed to leave 1 ml SFM and 30 mins later were stimulated by adding 10x stock solutions of ACTH directly to the well or the same volume of SFM or 10 % serum (15 min) for the times indicated. Cell lysates were analysed for phopsho* $erk_{1/2}$. *Blots were stripped and reprobed for total* $erk_{1/2}$. *A, time-course of ACTH stimulation. B, densitometry where net intensity of phospho* $erk_{1/2}$ */ total* $erk_{1/2}$ *was determined and expressed as a percentage of unstimulated (n=3). Error bars represent SEM.*

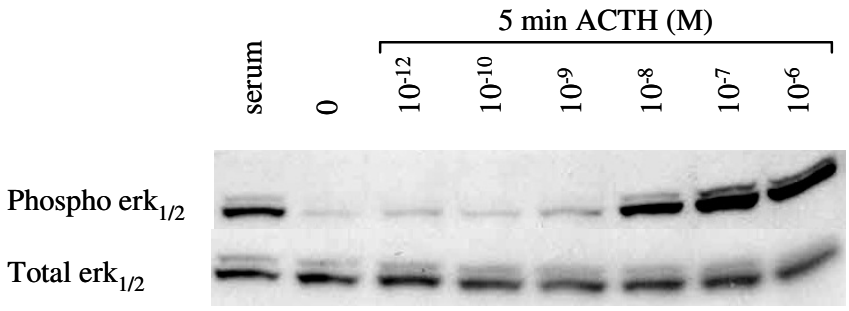


Fig. 3.7 Erk$_{1/2}$ dose response to ACTH*. H295R cells were serum starved overnight. The next day media was changed to leave 1 ml SFM and 30 mins later cells were stimulated by adding 10x stock solutions of ACTH, serum or the same volume of SFM, directly to the well for 5 mins Cell lysates were analysed for phospho-erk$_{1/2}$ (upper panel). Blots were stripped and re-probed for total erk$_{1/2}$ (lower panel).This is representative of three independent experiments.*

These findings suggest that, as has been found previously in Y1 cells, ACTH is able to activate the Erk$_{1/2}$ signalling pathway in H295R cells (Lotfi et al., 1997). However, we wanted to investigate the activation of other classical mitogenic pathways to characterise the mitogenic potential of ACTH in H295R cells. The stress activated MAPK pathways, JNK and p38 MAPK were investigated for this reason in addition to PI3-K/Akt pathway.

H295R cells were stimulated with ACTH over a time course and cell lysates analysed by western blotting for phospho JNK (Fig. 3.8A). As a positive control, cells were treated for 5 and 30 minutes with hypertonic sucrose (0.4 M) in order to induce osmotic shock and thus JNK activation. Treatment for 30 minutes with sucrose caused an increase in levels of phospho p46 and p54 JNK however treatment with ACTH caused no increase above that of untreated. The effect of ACTH on p38 MAPK activity was also analysed in the same way however no induction was seen (data not shown).

Levels of activated Akt were determined following ACTH treatment for a time-course (Fig. 3.8B). Interestingly, there was a very slight increase in phosphorylation of Akt over a relatively high basal level by 5 min, however this was not significant with repeated experiments.

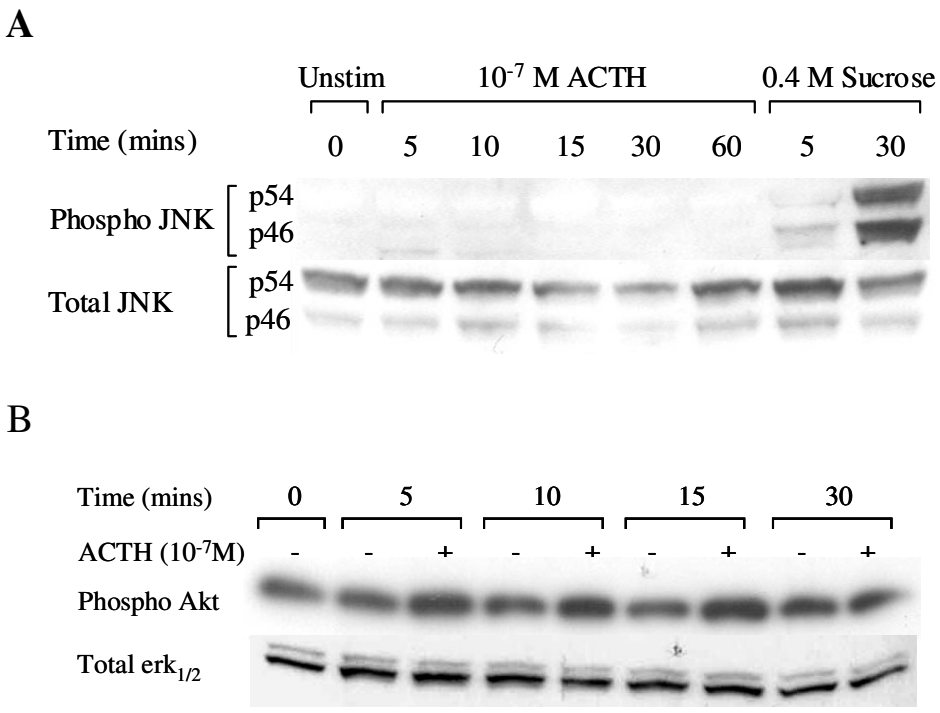


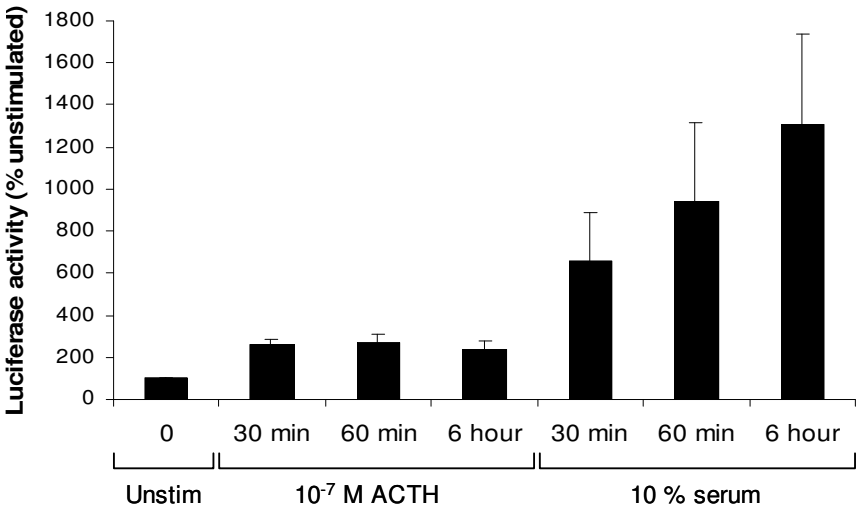
Fig 3.8 JNK and Akt activation by ACTH*. H295R cells were left in SFM overnight prior to stimulation with 10^{-7} M ACTH, 0.4 M sucrose or an equivalent volume of SFM for the indicated times. A, lysates were analysed for phospho JNK levels by Western blotting (upper panel). Blots were stripped and re-probed for total JNK (lower panel). B, lysates were analysed for phospho Akt levels (upper panel). Blots were then stripped and re-probed for total $Erk_{1/2}$ (lower panel).*

3.3.5 ACTH effect on nuclear translocation of $Erk_{1/2}$

An important question to answer was whether the activation of the $Erk_{1/2}$ pathway by ACTH in H295R cells would actually allow translocation of $Erk_{1/2}$ to the nucleus where it could upregulate target genes, or whether $Erk_{1/2}$ was retained in the cytoplasm following phosphorylation. If $Erk_{1/2}$ was retained in the cytoplasm, this would indicate that a proliferative response would not follow (Smith et al., 2004). In Y1 cells, ACTH was previously shown to induce upregulation of the immediate early genes, c-fos and c-jun (Kimura et al., 1993) thus indicating that $Erk_{1/2}$ was translocated to the nucleus to bring about this effect. In order to investigate this, a luciferase assay system was utilised whereby co-transfection of a Gal4-Elk, Gal4-luc and Renilla constructs allowed the measurement of MAPK ability to induce target gene expression following treatments with ACTH. This assay was used to assess both the effect of long-term treatment (6 h) as well as short-term treatment (30 min) of ACTH compared to 10 % serum (Fig. 3.9A).

Fig. 3.9A demonstrates around a 3-fold activation of transcription following treatment with ACTH for 30 mins compared with that of serum which caused a 6-fold increase in luciferase activity. Interestingly though, a more prolonged treatment with serum caused a cumulative increase in luciferase activity with time, however ACTH induced luciferase activity did not increase above that of the 30 min time-point. This suggests that although ACTH does induce the translocation of phospho $Erk_{1/2}$ to the nucleus and the subsequent transcription of MAPK target genes, it is a relatively short-lived effect that may be regulated by feedback mechanisms. The upregulation of Elk-1 was shown to be a MEK dependent process since the effect could be inhibited by pre-treatment with the inhibitor U0126 (Fig. 3.9B).

A



B

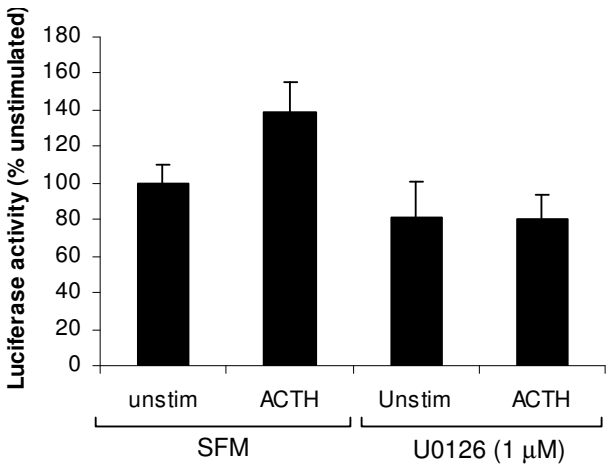


Fig. 3.9 **Transcriptional activation of Elk-1**. *H295R cells were plated into 6 well plates and when at around 50 % confluence were transfected with Gal4-Elk, Gal4-Luc and Renilla plasmids using a 6:1 ratio of fugene:DNA. The next day cells were washed once in SFM and left in SFM overnight. A, the following day, cells were stimulated for the indicated times with 10^{-7} M ACTH, 10 % serum or SFM alone and then transferred back to SFM for the remaining time. Cells were harvested after 6 hours of total treatment time and luciferase and renilla activity measured using the dual luciferase assay kit (Promega) and results expressed as a percentage of unstimulated. The experiment was performed on three separate occasions in duplicate. B, cells were pre-treated with 1 μM U0126 for 1 h or SFM followed by stimulation with 10^{-7} M ACTH or SFM for 30 min. Cells were transferred back to SFM for the remaining time (6 h in total) and then analysed for luciferase and renilla activity. The experiment was performed three times in duplicate.*

3.3.6 ACTH effect on Proliferation

Having demonstrated that ACTH induces the activation of $Erk_{1/2}$, a classically proliferation inducing pathway, and that short treatments with ACTH induce the upregulation of $Erk_{1/2}$ target genes, it was necessary to investigate whether ACTH would lead to proliferation of H295R cells.

The MTS proliferation assay (Promega) was used to quantify proliferation. Initial experiments suggested that an overnight period of serum starvation was not sufficient to enable the measurement of an effect of a 24h 10% serum treatment compared to SFM in H295R cells (data not shown). Consequently, a period of 48h of serum starvation was preferred to ensure that cells had stopped dividing prior to treatment. To investigate the effect of short-term treatment with ACTH on proliferation of H295R cells, cells were first serum-starved for 48 hours prior to treating with 10^{-7} M ACTH or 10 % serum for 5 min to 1 h. Cells were then transferred to SFM and left for a further 24, 48 and 72 h and the MTS proliferation assay subsequently performed. Results demonstrated no difference between untreated cells and those treated with either ACTH or 10 % serum for all of the time-points studied suggesting that the MTS assay was not sensitive enough to look at these short stimulations or that these particular cells do not respond to such short treatments in terms of proliferation. However, it was decided to use this method to look at the effect of long-term treatments with ACTH compared to serum alone and also the effect of ACTH treatment in the presence of serum. Fig. 3.10 shows a 3-fold increase in cell number after treatment for 72 h with 10 % serum compared to untreated (unstim). On the other hand, treatment with various concentrations of ACTH in SFM consistently had no effect on proliferation of cells.

ACTH at various concentrations in the presence of serum appeared to show a trend towards a dose-dependent effect, but this was not significantly different from that obtained with serum alone.

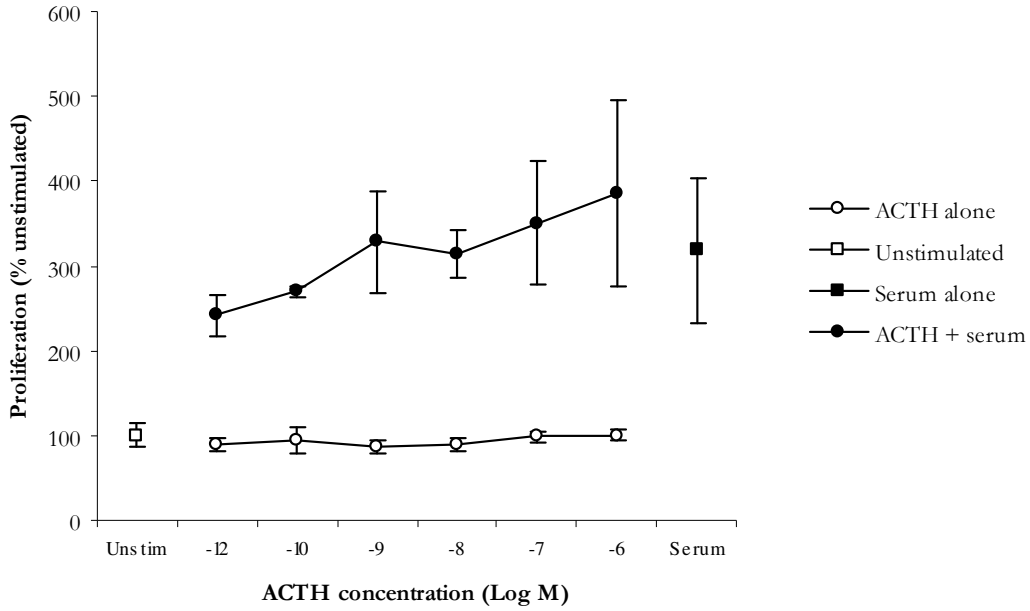


Fig. 3.10 Proliferative response to ACTH*. H295R cells were plated into 12 well plates and the next day left in SFM for 48 hours. Medium was replaced with SFM containing the indicated concentrations of ACTH or medium containing 10 % serum with various concentrations of ACTH for a further 72 h. The MTS assay was then performed. N=3 with 6 replicates per treatment. Error bars represent SEM.*

3.4 DISCUSSION

3.4.1 The Y1 adrenocortical tumour cell line

The use of the mouse Y1 adrenocortical cell line to look into the mitogenic signalling responses to ACTH was initially sought for a number of reasons. This was primarily because Y1 cells express the MC2R and respond to ACTH in terms of a potent increase in levels of cAMP and in terms of synthesizing and secreting steroids (Yasamura et al., 1966, Schimmer, 1979), Y1 cells resemble the human adrenal cortex in that the major melanocortin receptor expressed is the MC2R thus allowing the study of the effects of ACTH MC2R interactions, and ruling out the complications of lower affinity ACTH binding to alternative receptors. Secondly,

this model has been used extensively by a number of groups to investigate signalling mechanisms other than the cAMP/PKA pathway, initiated by ACTH, and its effect on mitogenesis (Masui and Garren, 1971, Lotfi et al., 1997, Forti et al., 2002, Watanabe et al., 1997).

The aim of this thesis was to characterise the mitogenic mechanisms initiated by ACTH in the adrenal using this mouse adrenal cell line, Y1. Previously published data described an increase in $Erk_{1/2}$ phosphorylation in response to ACTH following a 72 h period of serum starvation in order to growth arrest cells in G_1 phase of the cell cycle (Lotfi et al., 1997). Initial experiments to gauge the viability of cells following increasing times in SFM, indicated that at 72 h cells were no longer in a healthy state and could not be used. However, at 48 h in SFM there was no reproducible and significant effect of ACTH on $Erk_{1/2}$ phosphorylation over that of untreated cells (Fig.3.2B). Attempts to try to reduce the constitutive activation of this pathway were unsuccessful; long-term treatment with dexamethasone, which is known to upregulate MAPK phosphatase expression (Lasa et al., 2002), did not reduce basal activation of $Erk_{1/2}$ and, pre-treatment with U0126 only temporarily abolished constitutive $Erk_{1/2}$ phosphorylation, an effect that was reversed immediately by removal of the inhibitor.

Explanations for the lack of responsiveness to ACTH in terms of $Erk_{1/2}$ phosphorylation could be explained by a change in phenotype of the cells over time. Y1 cells have been previously shown to change with passage number, for example, they lost their capacity to produce corticosterone over time, probably due a deficiency of the 21-hydroxylase enzyme (CYP 21) (Parker et al., 1985). The current passage of the Y1 clone present at the ATCC has lost responsiveness to ACTH although are still able to produce steroids. Aside from a lack of $Erk_{1/2}$ activation in response to ACTH, cells also did not respond to FGF (Fig. 3.3A), a known adrenal mitogen and activator of this pathway (Viard et al., 1993, Hornsby et al., 1983, Lotfi et al., 1997) thus to look at the effect of ACTH, a so-called weak mitogen, was not possible using this model.

The explanation for the varied findings in ACTH responses in Y1 cells are probably the result of different groups investigating alternative strains of cells. Many strains of Y1 cells have been previously described as expressing a large amplification of c-Ki-ras protooncogene thus leading to over activation of the $Erk_{1/2}$ pathway (Schwab et al., 1983). One report has summarized previously published data on the amplification, chromosomal location, expression, and activity of c-Ki-ras proto-oncogene in Y1 cells (Rocha et al., 2003). In their particular Y1 cells they found a 10 to 20-fold increase in both mRNA expression and protein levels of Ki-ras compared to mouse 3T3 fibroblasts in addition to higher constitutive levels of c-Ki-ras in its active, GTP bound form. It may be that in our strain of Y1 cells, this enhanced c-Ki-ras proto-oncogene expression and activity is even more pronounced leading to loss of responsiveness even to FGF.

3.4.2 Bovine adrenocortical cells

Alternative adrenal derived cells were investigated to find an appropriate model for the study of ACTH induced mitogenesis. Bovine adrenocortical cells (SBAC) were purchased from ECACC and cultured according to the instructions given by the supplier. SBACs are primary cells that have been previously published to require the addition of IGF-1 and cAMP as well as extracellular matrix components, to maintain the expression of 11β hydroxylase and 21-hydroxylase (Cheng and Hornsby, 1992).

Experiments were performed to ascertain the signalling characteristics of this cell line in response to ACTH. Treating cells with increasing doses of ACTH caused no increase in cAMP production or $Erk_{1/2}$ phosphorylation compared to untreated cells, when stimulations were for 45 mins and 5 mins respectively (Fig. 3.4 and data not shown). This is in contrast to previous findings by a group who demonstrated potent stimulation of steroid production following treatment of ACTH derived from pituitary extracts (Shah and Murray, 2001). The lack of a functional MC2R therefore rules out these cells for further study.

We also investigated an immortalized bovine adrenocortical cell line derived from the forced expression of telomerase ribonucleoprotein complex (TERT) (Thomas et al., 2000, Thomas and Hornsby, 1999). When transplanted into SCID mice, these cells form fully functional tissues However, we found that when grown *in vitro* these cells do not respond to ACTH to produce cAMP and further do not increase $Erk_{1/2}$ phosphorylation in response to ACTH. This suggests either that cell culture conditions were not optimised to allow the cells to be fully functional or that these adrenal cells require other factors that are present *in vivo* to enable them to function normally.

3.4.3 NCI-H295R cells

Although H295R cells express all of the major adrenocortical enzyme systems, they have been primarily and most successfully used in the study of ZG responses. Investigations into the mechanisms regulating adrenal cell responses to angiotensin II have been carried out where the activation of PKC has been shown to be mediated through the AT1 receptor (Bird et al., 1993, Bird et al., 1994). However, the investigation into ZF responses i.e that of ACTH effects, have not been investigated fully since these cells have a low level of MC2R expression and therefore ACTH responsiveness (Mountjoy et al., 1994). Thus most experiments used to investigate cAMP dependent pathways have used the direct stimulation of adenylate cyclase with forskolin or cAMP analogues to allow a potent stimulation of cAMP production, PKA activation and cortisol synthesis and release (Rainey et al., 1993).

We however have characterised this cell line further in terms of it responses to ACTH to ascertain whether it could be used to study mitogenic signalling. Results from this chapter confirmed the expression of the MC2R in H295R cells that could be visualised at 30 cycles and furthermore, a small but significant response to ACTH in terms of cAMP production. This was in contrast to stimulation with

forskolin, which gave a 5-fold increase in cAMP production (at 10^{-5} M) compared to a maximum of a 1.5-fold increase with 10^{-7} M ACTH (Fig. 3.4). The lack of a potent response to ACTH may simply reflect the relatively low level expression of the MC2R, however it is possible that it may reflect a problem of coupling of the receptor to G_s proteins, which may not be limited to only this receptor. For example, H295R cells express the β_2 Adrenergic receptor (Kosti et al., 2002), however they do not respond to isoproterenol by accumulating cAMP (data not shown).

Aside from the cAMP response, homologous displacement binding experiments demonstrated specific binding of ^{125}I-ACTH to H295R cells with an IC_{50} of 4.68 x 10^{-7} M (Fig. 3.5). Furthermore, this curve spreads over only two log units suggesting binding to only one receptor sub-type. This affinity is not in accordance with that found previously in Y1 cells where the IC_{50} was 0.13 nM (Schioth et al., 1996). Although the ^{125}I-ACTH peptide is very difficult to use due to its "sticky" nature, the results were very reproducible and thus a difference in affinity probably reflects a difference in behaviour of the MC2R in this cell line. The possibility of cofactors present in either of the two cell lines that alter the affinity of the MC2R for ACTH cannot be ruled out however there is currently no evidence for this in the literature. Table 3.1 shows the affinities of ACTH (1-39) and α-MSH for the different melanocortin receptors, determined by transient transfection in COS-1 cells, or Y1 cells in the case of the MC2R (Schioth et al., 1996, Schioth et al., 1997). ACTH binds with various affinities to all of the melanocortin receptors however the closest affinity to that found in our experiments is that of the MC3R, however there is still a 3-fold discrepancy.

The possible binding of ACTH to alternative melanocortin receptors in order to mediate an $Erk_{1/2}$ response will be further investigated in Chapter 4.

Ligand	*Receptor*				
	MC1R	**MC2R**	**MC3R**	**MC4R**	**MC5R**
ACTH (1-39)	3.95	0.13	135	2170	4920
α-MSH	0.113	-	44.7	357	2440

Table 3.1 IC_{50} of POMC peptides for melanocortin receptors *(nM) adapted from (Schioth et al., 1997, Schioth et al., 1996).*

3.4.4 Signalling pathways activated by ACTH

With limited availability of adrenal cell lines for the study of ACTH effects on mitogenesis, we thought it a reasonable and novel course of action to study these effects in H295R cells, a cell line that expresses the MC2R and responds functionally to its stimulation with ACTH. Furthermore, growth characteristics of these cells have not been studied extensively.

There are many strains of H295R cells available that have been selected to grow in different media based on their ability to maintain their steroidogenic capacity. For example, the growth supplement Ultroser G (Biosepr SA, France) was found to allow cells to retain their steroidogenic capacity (Hornsby and McAllister, 1991). However, due to the problems with importation of Ultroser to North America, other growth media such as Nu-Serum (Collaborative Biomedical products) and Cosmic calf serum (Hyclone) have also been tested. Nu-Serum and Ultroser were required in order to maintain responses to Angiotensin II and K^+ however many groups have grown H295R cells in media supplemented with 10 % FBS (Montanaro et al., 2005, Li and Wang, 2005). Since the responses to Angiotensin II were not our primary focus, we initially grew cells in 10 % FBS. When investigating the $Erk_{1/2}$ response following ACTH treatment we found a potent increase in $Erk_{1/2}$ phosphorylation after 5 mins stimulation which decreased over 1 h back to

basal levels (Fig. 3.6). This was also a dose dependent effect with an EC_{50} of around 3 nM (Fig. 3.7). Responses in cells grown in Ultroser containing media were also investigated and shown not to be as potent (data not shown). The reason for this may be that of a differentiation of cells where they lose glomerulosa phenotype and gain a more fasciculata phenotype which may account for why these responses have not been previously investigated in this cell line. This is supported by previous observations where changes in phenotype of H295R cells occurred dependent on whether treated with angiotensin II or forskolin. Angiotensin treatment promoted glomerulosa phenotype and the associated expression of CYP11B2 and production of sterone and forskolin treatment promoted CYP11B1 expression and the production of cortisol (Cobb et al., 1996).

The effect of ACTH on other mitogenic and anti-apoptotic pathways was also investigated. The JNK (SAPK) and p38 MAPK pathways are generally activated by stress stimuli (Kyriakis et al., 1994). The JNK pathway has previously been demonstrated to be activated by ACTH *in vivo* in rat adrenals and in Y1 cells (Watanabe et al., 1997) whereas activation of the p38 MAPK pathways has not been reported. This paper however also showed a decrease in $Erk_{1/2}$ activity following ACTH treatment for 15 mins in contrast to that found by other groups (Lotfi et al., 1997). We found no activation of either of these stress-activated pathways pathways in H295R cells in response to ACTH.

In investigating the activity of the PI3K/Akt pathway, results showed a high constitutive activation of Akt however no significant change following ACTH stimulation (Fig. 3.8 B). This observation was in contrast to that seen in Y1 cells where a high constitutive activation of Akt was dephosphorylated by 5 mins of ACTH treatment, an effect that was maintained for at least 4 hours (Forti et al., 2002). Furthermore, that study demonstrated that inactivation of Akt by ACTH or wortmannin was responsible for increased levels of $p27^{Kip1}$ protein. These effects were shown to be mediated by a mechanism dependent on cAMP/PKA activation, and thus may account for the difference seen in the H295R cell line where there is no potent cAMP response.

In view of the data summarised above it was decided to investigate whether ACTH could stimulate the transcription of Erk½ target genes and cause proliferation of H295R cells.

3.4.5 Proliferative effect of ACTH on H295R cells

The increase in luciferase activity induced by ACTH in the MAPK luciferase reporter assay suggested that as well as inducing the phosphorylation of $Erk_{1/2}$, ACTH also allows the translocation of active $Erk_{1/2}$ to the nucleus to affect transcription of the target gene Elk-1 (Fig. 3.9A). This was a specific effect of $Erk_{1/2}$ activation because it was inhibited by the MEK1 inhibitor U0126 (Fig. 3.9B). Luciferase activity was increased 3-fold over untreated cells in contrast to a 6-fold activation of cells treated with 10 % serum (at the 30 min time-point). This is perhaps not surprising as serum is a potent mitogen that is likely to enhance other pathways driving Elk-1 transcription.

Although luciferase activity was induced with ACTH stimulation this effect was only seen up to 30 mins, at which point there was no further induction. This is in contrast to serum treatment which caused an increase in luciferase activity with time. This suggests that short periods of ACTH treatment have a positive effect on $Erk_{1/2}$ target genes, and further, that there is likely to be a regulatory feedback mechanism whereby uncontrolled transcription is prevented. This feedback mechanism may be the ACTH induced upregulation of MAPK phophatase 1 (MKP-1). This has been previously described in Y1 cells in response to ACTH where its protein levels are increased by 30 mins and act to depshophorylate $Erk_{1/2}$ (Bey et al., 2003). These observations are supportive of the literature describing the differential effects of short-term versus long-term treatment of ACTH on the proliferation of Y1 cells (Lotfi et al., 1997).

$Erk_{1/2}$ signals can lead to various outcomes such as proliferation, cell cycle arrest or differentiation and it is thought that signal duration is a crucial determinant of the signal potential (Assoian, 2002). Recent insights into the effect of $Erk_{1/2}$ signal duration on the stabilisation of c-fos have been investigated where it was demonstrated that although both transient and sustained $Erk_{1/2}$ activation lead to c-fos gene transcription, the c-fos protein is relatively unstable unless $Erk_{1/2}$ activity remains (Murphy et al., 2002). Sustained $Erk_{1/2}$ activation has been associated with a progression through the G1 phase of the cell cycle in a number of systems (Meloche et al., 1992, Cook and McCormick, 1996, Weber et al., 1997, Vouret-Craviari et al., 1993). The activation of $Erk_{1/2}$ in response to ACTH in H295R cells however is very much a transient response (Fig. 3.7B). Transient $Erk_{1/2}$ activation is often not associated with proliferation (Murphy et al., 2002, Cook and McCormick, 1996) however this is not always the case. For example it was demonstrated in mouse embryonic fibroblasts (MEFs) obtained from an A-raf, Raf-1 double knock-out mouse had delayed ability to enter the S-phase of the cell cycle which was associated with a reduction in levels of transiently induced MEK and $Erk_{1/2}$ activation with no alteration in sustained $Erk_{1/2}$ activation (Mercer et al., 2005).

Due to the probable dual effects of ACTH on adrenal cells i.e. that of growth promoting and growth inhibitory, the approach to investigating this was to look at both short-term treatments (5 min to 2 hours) and long-term treatments (72 h) on the proliferation of H295R cells. The MTS assay was used as a measure of cell number and thus was a late marker of proliferation. There was no effect of treatments from 5 mins to 2 h on proliferation of H295R cells for both ACTH and serum (data not shown). This probably reflects a lack of sensitivity of the MTS assay and does not necessarily suggest a pro or anti-proliferative effect of ACTH.

Pro-longed treatment of H295R cells with various concentrations of ACTH in SFM also showed no increase or decrease in proliferation compared with SFM alone (Fig. 3.10). However, during these experiments it was decided not to change the media to prevent following 72 hours in SFM, cells from detaching from the plate. This may

however have decreased any effects that may have been seen if the media was changed daily.

ACTH was also incubated at various concentrations in the presence of 10 % serum however there was no significant difference in these treatments compared with serum alone. Cells treated with serum showed only a 3-fold increase in proliferation compared to those in SFM, therefore, if ACTH was behaving as a weak mitogen or indeed an anti-proliferative agent, its effects may not necessarily be evident using this assay.

The proliferation experiments on H295R cells were therefore inconclusive. Although there was no significant increase or decrease in proliferation of cells treated with ACTH in the presence of serum, any small change may have been masked by the presence of serum. However without serum, effects may have been masked by loss of cells through peeling off. In order to gain more useful results, the study may be improved in the future by incubating cells in a lower concentration of serum in order to allow the cells to remain healthy at the same time as reducing any masking effects of the serum. Also, ideally, a more sensitive marker of proliferation could be used to determine any small changes which would allow the quantification of any short-term effects of ACTH.

3.4.6 Conclusions

One of the aims of this chapter was to find a suitable *in vitro* model for the analysis of classical mitogenic pathways in the adrenal gland. Initial studies in Y1 cells lead to results contradicting those which I was trying to repeat i.e. a complete lack of effect of ACTH on $Erk_{1/2}$ signalling. An alternative strategy using primary bovine adrenal cells (SBAC) was thought to be a good alternative mainly because the cells, if used at early passages, would not display cell cycle irregularities and would thus be a truer reflection of the normal adrenal cell. However, much has been published in the literature that primary bovine adrenal cells require a cocktail of growth factors to maintain their steroidogenic capacity *in vitro* and furthermore do not accumulate

cAMP following ACTH stimulation (Cheng and Hornsby, 1992). The bovine adrenal cells immortalised by TERT have yet to be studied extensively *in vitro* and may require optimisation of growth conditions in order for cells to respond to ACTH. In the H295R model, we found a cell line that although studied extensively for its glomerlosa like functions and angiotensin II responses, also displays a number of characteristics of performing fasciculata functions. Its weak cAMP and cortisol response to ACTH was already known however this is the first time this cell line has been shown to potently stimulate $Erk_{1/2}$ activity similar to that seen by other GPCRs. The fact that no effect of ACTH on the PI3 kinase/Akt pathway was seen is a very important observation because it was the dephosphorylation of Akt that was thought to be responsible for mediating the anti-proliferative effect of ACTH in Y1 cells (Forti et al., 2002). Although proliferation experiments were inconclusive, I would hypothesize that although the $Erk_{1/2}$ response to ACTH seems to be regulated to prevent over activation of the pathway, there would be no anti-proliferative effect of ACTH on this cell line due to the lack of a potent cAMP response. This characteristic also enables the closer study of the steps involved following the binding of ACTH to an adrenocortical cell through to the nuclear targeting of $Erk_{1/2}$, seemingly without the complication of cAMP mediated effects. It may be that the lack of regulation of the cell cycle by a cAMP/PKA dependent mechanism may have contributed to the malignancy of the original adrenal tumour in which H295R cells were derived.

4 MECHANISMS OF $ERK_{1/2}$ ACTIVATION

4.1 INTRODUCTION

The activation of the $Erk_{1/2}$ signalling cascade is a common feature of a range of cellular responses to a vast array of stimuli and is present in virtually all cell types. Since this is such a common feature, the specificity of each signal is thought to be dependent on the mechanisms by which this pathway is activated. For example, the final outcome and intracellular targets of the signal may be affected by the cellular location of activated $Erk_{1/2}$ and the components recruited into signalling complexes (Tohgo et al., 2003). There are numerous steps involved in the transduction of an $Erk_{1/2}$ signal from a GPCR agonist, many of which have been elucidated for other GPCRs. These pathways may be specific to certain cell types, certain receptors, or depend on the G-protein to which they are coupled. Some of these steps include PKA or cAMP dependent pathways; pathways dependent on the transactivation of a growth factor receptor; those dependent on an increase in PKC activity, sometimes by the switching of G protein coupling; and pathways dependent on the internalisation of a GPCR and/or the binding of β-arrestins which act as scaffolds for $Erk_{1/2}$ signalling (Ohtsuka et al., 1996, van Corven et al., 1993, Daaka et al., 1997, Luttrell et al., 1997). Such mechanisms have been discussed in detail in section 1.8.

We established in the previous chapter that the H295R cell line is a good alternative model to Y1 cells for studying the effects of ACTH on $Erk_{1/2}$ signalling. These cells express the MC2R, show specific binding to I^{125}-ACTH and furthermore, respond to ACTH to produce cAMP. H295R cells also have a potent response in terms of activation of the $Erk_{1/2}$ cascade following ACTH treatment. The aim of this chapter is to look in greater detail at the processes involved in ACTH signalling to $Erk_{1/2}$, from the binding of ACTH to a receptor, coupling of the receptor to a G protein and the subsequent pathways which lead to an increase in the phosphorylation of

$Erk_{1/2}$. The aim is to gain a greater understanding of the factors involved in these signalling mechanisms both for the MC2R and GPCRs in general.

In order to look more closely at the intermediate steps involved, it was decided to use a strategy that would utilise many of the very specific inhibitors that are now available, to try to modulate the activities of important components in such signalling pathways and to look at the effect of these on $Erk_{1/2}$ phosphorylation using western blotting. These inhibitors and their mechanisms of action are discussed below as follows:

4.1.1 Inhibitors of PKA pathway

H89 is a widely used cell-permeable inhibitor of PKA activity and although it is thought to be very selective at a concentration of 10^{-7} M, at higher concentrations it is thought to inhibit other protein kinases such as PKC (Cohen, 2002). Use of this inhibitor will allow us to investigate the role of PKA in ACTH induced $Erk_{1/2}$ activation. Another inhibitor, SQ22536 (9-(tetrahydro-2-furyl)-adenine), is a cell permeable inhibitor of adenylate cyclase activity. This inhibitor, an analogue of adenosine, was originally found to inhibit adenylate cyclase activity in human blood platelets and has been used previously in investigating the role of cAMP in $Erk_{1/2}$ signalling (Harris et al., 1979, Li et al., 2004). SQ22536 will allow us to investigate $Erk_{1/2}$ activation independently of PKA.

4.1.2 Pertussis toxin

Pertussis toxin is secreted by the bacterium causing whooping cough, *Bordetella pertussis*. The S_1 subunit of the toxin catalyzes the addition of ADP-ribose to the α subunit of G_i proteins irreversibly. This prevents the release of GDP thus locking it in the GDP bound state and abrogating its inhibitory effect on adenylate cyclase (Hsia et al., 1984). Experimental conditions for the use of this inhibitor vary from a few hours pre-treatment to 2 days. In order to be consistent with previous

conditions an overnight pre-treatment in SFM is deemed sufficient to investigate the role of G_i proteins in the transduction of the $Erk_{1/2}$ signal by ACTH.

4.1.3 Tyrosine kinase inhibitors

To investigate the role of transactivation of tyrosine kinases, a number of general and specific chemical inhibitors are available. Genistein is a soy isoflavone with a structure similar to that of estradiol (Borras et al., 2006). It has been used widely as a potent inhibitor tyrosine kinase activity affecting the activity of all growth factor receptors and non-receptor tyrosine kinases such as *c-src* and may therefore be used to assess the general involvement of tyrosine kinases in the $Erk_{1/2}$ pathway (Akiyama et al., 1987). AG1478 on the other hand is one of a range of AG compounds available that specifically and potently inhibits EGF receptor activity, a receptor commonly involved in the transduction of $Erk_{1/2}$ signals by GPCRs. It has been used in a variety of studies including for example determining the role of EGF receptor transactivation following stimulation of endothelin-1, LPA and thrombin receptors in rat-1 fibroblasts (Kranenburg and Moolenaar, 2001, Xu et al., 2007).

In investigating specifically the role of *src* family kinases various chemical inhibitors are available. PP1 and PP2 are related compounds, both found to be potent, selective inhibitors of *src* family kinases at nanomolar ranges (Hanke et al., 1996, Karni et al., 2003). However one study reported the inhibition of the PDGF receptor by both chemicals therefore use of another inhibitor, SU6656, which is also a cell permeable, potent *src* inhibitor that does not inhibit PDGF receptor activity is a further alternative in investigating the role of *src* family kinases in $Erk_{1/2}$ acitivation (Blake et al., 2000).

4.1.4 PKC and Ca^{2+} pathway inhibitors

Several inhibitors of the PKC pathway are available for investigation of these components in ACTH induced $Erk_{1/2}$ activation. GF109203X is a highly selective, cell permeable PKC inhibitor that competitively binds to the ATP-binding site of

many of the PKC isoforms (Toullec et al., 1991). Calphostin C is also a highly specific inhibitor of PKC however it binds to its regulatory domain and therefore competes for the binding of DAG and phorbol esters (Kobayashi et al., 1989).

To investigate whether an influx of Ca^{2+} following ACTH binding to the MC2R is involved in mediating an $Erk_{1/2}$ response, the L-type Ca^{2+} channel blocker, nifedipine, may be used as one of the most extensively studied inhibitors of this process. Nifedipine is also used clinically as a vasodilator. In addition however, BAPTA-AM is a cell permeable form of BAPTA, a Ca^{2+} chelator, which will allow an investigation into the involvement of intracellular Ca^{2+} release in mediating the $Erk_{1/2}$ response (Tsien, 1980).

4.1.5 Inhibitors of Akt pathway

The cell permeable fungal metabolite, wortmannin, is a potent, selective and irreversible inhibitor of PI3-K catalytic activity that has been widely used in investigating the role of this component in various signalling mechanisms (Yano et al., 1993). Although other inhibitors such as LY294002 have been developed, wortmannin is still the inhibitor of choice for most researchers.

4.1.6 Inhibitors of internalisation

Two widely used inhibitors of receptor internalisation are sucrose and mono dansylcadaverine (MDC). Sucrose has been shown previously in our laboratory to inhibit internalisation of ACTH, possibly by causing abnormal polymerization of clathrin into empty microcages thus rendering clathrin unavailable to form coated pits (Heuser and Anderson, 1989, Baig et al., 2002). MDC is also a widely used inhibitor of receptor endocytosis, which functions by preventing clathrin coated pit formation through covalent coupling to the membrane (Davies et al., 1984).

4.2 METHODS

4.2.1 Pre-treatment of H295R cells with inhibitors and stimulators

Pertussis toxin, SQ22536, genistein, MDC, AG1478, PP2, SU6656, GM6001, Calphostin C and GF109203X were obtained from Calbiochem (Nottingham, UK), and used at the concentrations indicated. H89, nifedipine, Phorbol 12-myristate 13-acetate (PMA) and human recombinant epidermal growth factor (EGF) were obtained from Sigma-Aldrich (Poole, UK). BAPTA-AM was obtained from AG Scientific (San Diego, CA). NDP-αMSH and 11-24 ACTH was obtained from Bachem (Merseyside, UK). The anti phospho tyr-417 *src* antibody was purchased from Cell Signaling Technology (Danvers, MA, US) and the monoclonal anti-*src* clone GD11 antibody was from Upstate Cell Signaling Solutions (Lake Placid, NY).

Table 4.1 demonstrates the vehicle in which chemicals were dissolved and concentrations at which inhibitors were reconstituted, stored and used.

Inhibitor	Form	Final concentration (M)	Details
H89	10^{-2} M in DMSO	10^{-5}	Aliquot and store at -20°C. Dilute in SFM.
SQ22536	Lyophilised	10^{-4} and 10^{-5}	Reconstitute in H_2O to 0.1 M, aliquot and store at -20°C. Dilute in SFM.
PTX	Lyophilised	100 ng/ml	Reconstitute in sterile dH_2O to 50μg/ml, aliquot and freeze. Dilute in SFM.
Genistein	Lyophilised	5×10^{-6}	Reconstitute in DMSO to 0.01 M, aliquot and store at -20°C. Dilute in SFM.
AG1478	Lyophilised	10^{-6}	Reconstitute in DMSO to 0.01M, aliquot and store at -20°C. Dilute in SFM.
PP2	10^{-2} M in DMSO	10^{-6}	Aliquot and store at -20°C. Dilute in SFM
SU6656	10^{-2} M in DMSO	10^{-6}	Aliquot and store at -20°C. Dilute in SFM

GF109203X	Lyophilised	5×10^{-6}	Reconstitute in DMSO to 0.01 M, aliquot and store at -20°C. Dilute in SFM.
Calphostin C	Lyophilised	10^{-6}	Reconstitute in DMSO to 10^{-3} M, aliquot and store at -20°C. Dilute in SFM.
Nifedipine	Lyophilised	10^{-6}	Prepare a stock solution of 10^{-3} M in DMSO. Aliquot and store at -20°C. Dilute in SFM.
BAPTA-AM	Lyophilised	5×10^{-5}	Reconstitute in DMSO to 0.01 M, aliquot and store at -20°C. Dilute in SFM.
Wortmannin	Lyophilised	10^{-7}	Reconstitute in DMSO to 10^{-2} M, aliquot and store at -20°C. Dilute in SFM.
Sucrose	Solid	0.45	Prepare fresh in SFM
MDC	Lyophilised	3×10^{-4}	Reconstitute in DMSO to 10 mg/ml, aliquot and store at –20°C. Dilute in SFM.

Table 4.1 *Summary of inhibitors and their preparation.*

4.3 RESULTS

4.3.1 Investigating the role of the MC2R in $Erk_{1/2}$ activation

Previous reports demonstrate that ACTH binds with by far the highest affinity to the MC2R (see table 1.1), and is therefore the most likely receptor to mediate the action of ACTH in terms of the $Erk_{1/2}$ response. However, ACTH also binds with a lower affinity to other melanocortin receptors raising the possibility of ACTH acting through a different receptor to produce this response. In addition, data from binding studies in the previous chapter imply that binding of ^{125}I ACTH to H295R cells is a very low affinity interaction with an IC_{50} of 1.11×10^{-6} M in contrast to a previously described IC_{50} for ACTH binding in Y1 cells of 0.3 nM (Schioth et al., 1996). This suggests that ACTH may bind to an alternative melanocortin receptor in the H295R cell line, which could in turn mediate activation of the $Erk_{1/2}$ pathway. To test this hypothesis we used an analogue of α-MSH. α-MSH and γ-MSH are natural endogenous hormones for the human melanocortin-1, 3, 4 and 5

receptors. 4-Norleucine, 7-D-phenylalanine-α-melanocyte-stimulating hormone (NDP-MSH) is a synthetic analogue of α-MSH and was initially found to act as a highly potent melanotropin with long bioactivity, and also acts as a potent agonist at these four receptors but has no affinity for the MC2R (Sawyer et al., 1980, Joseph et al., 2003). This peptide was used in a time-course of activation of H295R cells to investigate whether NDP-MSH could mimic the $Erk_{1/2}$ response given by that of ACTH (Fig. 4.1).

Fig. 4.1 demonstrates that there is no significant activation of $Erk_{1/2}$ along a time-course of 30 mins after treatment with 10^{-7} M NDP-MSH compared with unstimulated cells and with 10^{-7} M ACTH stimulated cells at 5 min.

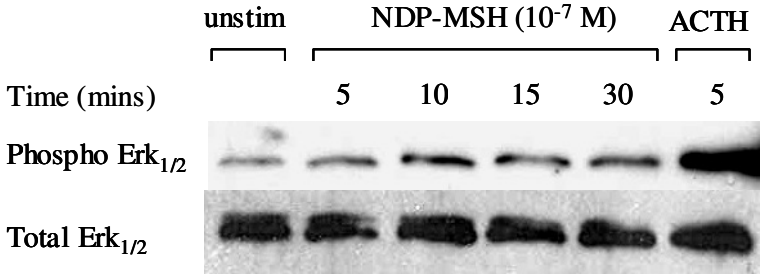


Fig 4.1 *H295R cells were plated into 6 well plates and when 70-90 % confluent left in SFM overnight. The next day cells were treated with either 10^{-7} M NDP-MSH or 10^{-7} M ACTH for the indicated times. Lysates were harvested and analysed for phospho $Erk_{1/2}$ (upper panel). Blots were stripped and re-probed for total $Erk_{1/2}$. This is representative of two independent experiments.*

4.3.2 Involvement of cAMP and PKA in $Erk_{1/2}$ activation by ACTH

The most common mechanism by which G_s coupled GPCRs mediate $Erk_{1/2}$ responses is through cAMP and PKA dependent processes. Although the cAMP response to ACTH was modest, we attempted to mimic this elevation of cAMP

using a submaximal dose of forskolin. At 10^{-6} M forskolin a comparatively similar cAMP stimulation to that obtained with 10^{-7} M ACTH was seen (Fig. 4.2A). This induced $Erk_{1/2}$ phosphorylation almost as effectively as ACTH implying that forskolin and ACTH are acting through the same pathway (Fig. 4.2B).

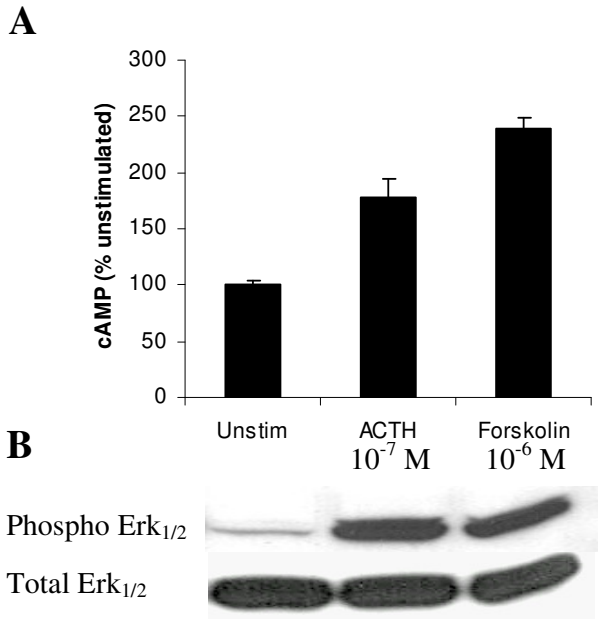


Fig. 4.2 *Forskolin activation of cAMP production and $Erk_{1/2}$. H295R cells were left in SFM for either 1 h (A) or overnight (B). Cells were then treated for 30 min (A) or 5 min (B) with SFM, 10^{-7} M ACTH or 10^{-6} M forskolin. Measurement of cAMP production (A) and Erk activation (B) were then analysed. For A, n=3 and error bars represent the SEM. For B, n=2.*

However, when a combination of ACTH and forskolin was investigated, it was apparent that ACTH was additive to a maximal dose of forskolin implying the presence of an alternative ACTH dependent pathway (Fig. 4.3A). The PKA inhibitor, H89 (10^{-5} M) was only partially effective at reducing the forskolin response however it was ineffective at reducing the ACTH induced $Erk_{1/2}$ activation (Fig. 4.3B). An inhibitor of adenylate cyclase activity, SQ22536, was used to investigate the involvement of cAMP in the ACTH induced $Erk_{1/2}$ response. At the two concentrations used no significant effect on $Erk_{1/2}$ activation was seen (Fig. 4.3C).

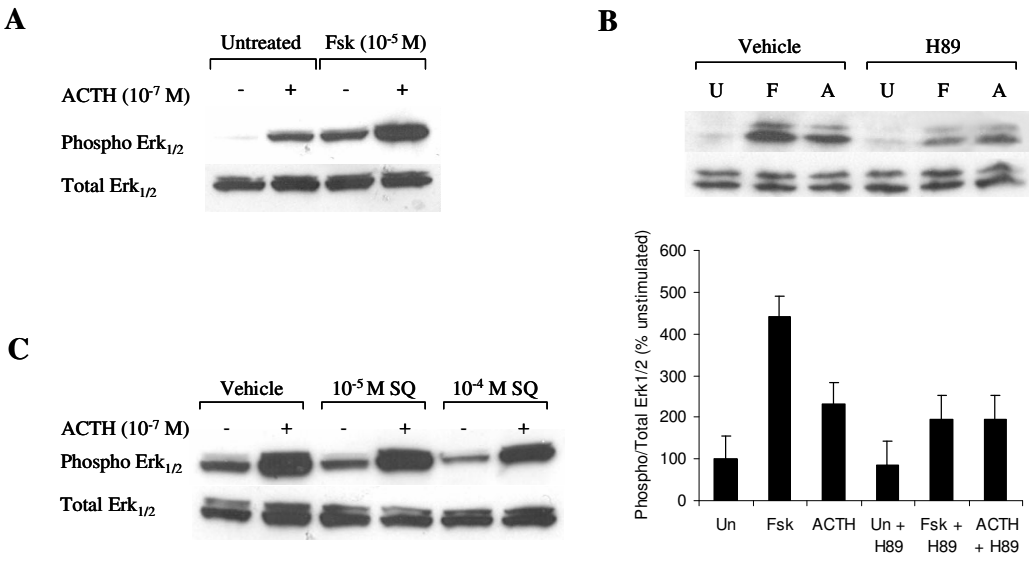


Fig 4.3 The role of the cAMP pathway in transducing the ACTH induced Erk$_{1/2}$ signal*. A, H295R cells were pre-treated with forskolin for 1 hour followed by the addition for 5 minutes of the indicated reagents and analysed for phospho Erk$_{1/2}$ levels. B, Cells were pre-treated for 1 hour with SFM or 10^{-5} M H89 prior to the addition of either SFM, forskolin (10^{-6} M) or ACTH (10^{-7} M). The graph shows densitometry on the results of 3 independent experiments with error bars representing SEM. C, Cells were pretreated for 1 hour with vehicle or SQ22536 (at the indicated concentrations) prior to the addition of SFM or ACTH at 10^{-7} M. The above results are representative of three experiments in each case.*

4.3.3 Involvement of alternative G_i protein subunits

We investigated the possibility that the Erk$_{1/2}$ signal is mediated by the switching of coupling of the MC2R from G_s to G_i by use of pertussis toxin (PTX). Fig. 4.4 shows the result of a 24 hour pre-treatment with pertussis toxin followed by a 5 minute treatment with either SFM or 10^{-7} M ACTH. No effect on Erk$_{1/2}$ activation is seen suggesting that ACTH induced Erk$_{1/2}$ activation is not dependent on G_i proteins.

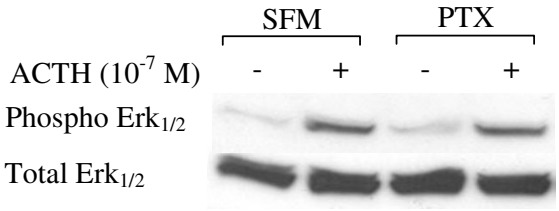


Fig. 4.4 *Effect of PTX on $Erk_{1/2}$ activation. H295R cells were plated into 6 well plates and when around 70 % confluent were serum-starved over-night in the presence or absence of 100 ng/ml PTX. Cells were treated for 5 min with ACTH or SFM and harvested for analysis of $Erk_{1/2}$ phosphorylation. This is representative of three independent experiments.*

4.3.4 Transactivation of a receptor or non-receptor tyrosine kinase

Common intermediary components involved in the transduction of $Erk_{1/2}$ signals, from agonist binding at GPCRs, are growth factor tyrosine kinases. Genistein, a general inhibitor of tyrosine kinases, was used initially to assess their involvement (Fig. 4.5).

Fig. 4.5A demonstrates partial inhibition of EGF induced $Erk_{1/2}$ activation by 50 μM genistein however it was unable to modulate ACTH induced $Erk_{1/2}$ phosphorylation. This suggests that tyrosine kinase activity is not involved in the ACTH induced $Erk_{1/2}$ response. To further confirm this a number of other, more specific, inhibitors of tyrosine kinase activity were also tested. AG1478, a potent and specific inhibitor of EGF receptor activity was used to confirm that $Erk_{1/2}$ phosphorylation was not mediated through transactivation of the EGF receptor, as the most commonly activated receptor in the mediation of the $Erk_{1/2}$ cascade from GPCRs. Fig 4.5B shows the potent inhibition of EGF induced $Erk_{1/2}$ by this compound but the lack of effect on $Erk_{1/2}$ activation by ACTH, further suggesting that the EGF receptor has no role in this particular response.

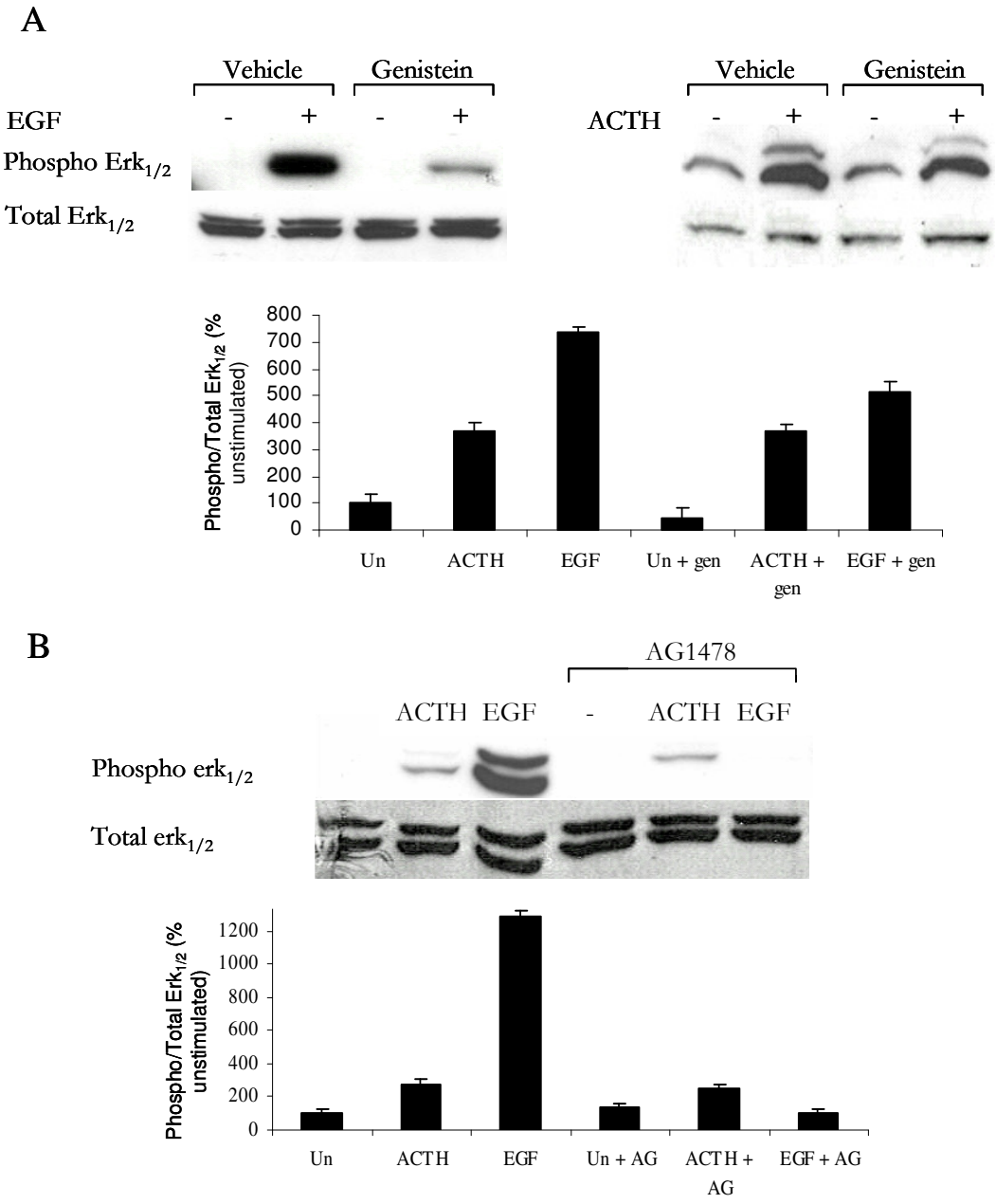


Fig. 4.5 *Transactivation of a tyrosine kinase receptor does not mediate Erk activation. H295R cells were plated into 6 well plates and when around 70 % confluent were left in SFM overnight. The next day cells were pre-treated for 1 h with either vehicle alone, 50 μM Genistein (A) or 1 μM AG1478 (B) prior to the addition of ACTH (to a final concentration of 10^{-7} M), EGF (to a final concentration of 100 ng/ml) or SFM (-) for 5 mins. Cells were harvested and analysed for phospho and total $Erk_{1/2}$ levels. Densitometry of results from 3 independent experiments are shown with error bars representing SEM.*

Src family members are often involved in transducing an $Erk_{1/2}$ signal from a GPCR agonist therefore it was decided to look at the role of these tyrosine kinases in the ACTH response. Firstly, specific inhibitors of *Src* family kinases, PP2 and SU6656, were investigated (Fig. 4.6A).

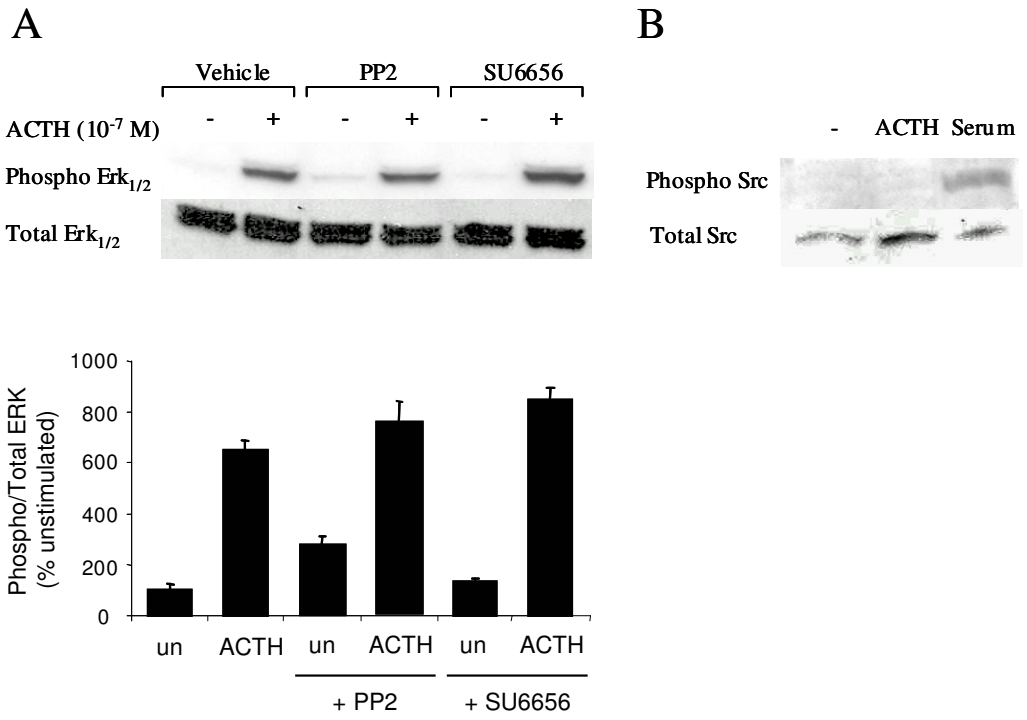


Fig. 4.6 *A, H295R cells were pre-treated for 1 h with either, vehicle alone, PP2 (10^{-6} M) or SU6656 (10^{-6} M) prior to addition of ACTH to the medium (final concentration of 10^{-7} M) and lysates were analysed for phosphorylation of $Erk_{1/2}$. Densitometry on 3 independent experiments was performed with error bars representing SEM. B, H295R cells were treated for 5 mins with SFM, ACTH or 10 % serum and lysates were analysed for phospho src. The blot was stripped and re-probed with anti-Src (Clone GD11).*

No difference was seen between ACTH stimulation alone versus pre-treatment with either PP2 or SU6656 suggesting that, as implied previously with genistein pre-treatment, the *Src* family tyrosine kinases are not involved in this response. However, to confirm this, it was decided to look at whether members of this family

were phosphorylated following ACTH stimulation. Using a phospho Tyr-417 specific Src family kinase antibody, H295R cells treated with SFM, ACTH (10^{-7} M) or 10 % serum for 5 mins were analysed by Western blotting for *Src* phosphorylation and for total *Src* protein (Fig. 4.6B). No phosphorylation of *Src* was seen with ACTH treatment and furthermore, treatment with serum produced only a faint band that could only be seen after a long exposure. This may be explained by low expression of *Src* in this cell line as evidenced by the total *Src* antibody.

4.3.5 Investigation into the role of the PKC pathway

As previously mentioned, a possible role for Ca^{2+} in the binding of ACTH to its receptor and the influx of Ca^{2+} after ACTH stimulation has been demonstrated (Cheitlin et al., 1985, Enyeart and Enyeart, 1998), thus the involvement of Ca^{2+} and the possible activation of the PKC pathway in the $Erk_{1/2}$ cascade were investigated. PKC inhibitory compounds were tested for their ability to inhibit PKC activation using the phorbol ester PMA, a potent activator of this pathway, as a positive control (Fig. 4.7B). PMA activated $Erk_{1/2}$ at 10^{-7} M and this was inhibited using 5 x 10^{-6} M GF109203X. Calphostin C, used at 10^{-6} M did not inhibit this activation (data not shown), however this is not surprising since this inhibitor competes for binding of DAG but has no effect on Ca^{2+} induced stimulation of PKC activity. These inhibitors were then used at the same concentrations to look at the effect on $Erk_{1/2}$ activation after ACTH treatment of H295R cells for 5 mins (Fig. 4.7B). No inhibition of $Erk_{1/2}$ was evident (calphostin C – data not shown) and since these inhibitors cover a broad spectrum of PKC isoforms, this suggests that the activation of PKC is not required for $Erk_{1/2}$ activity.

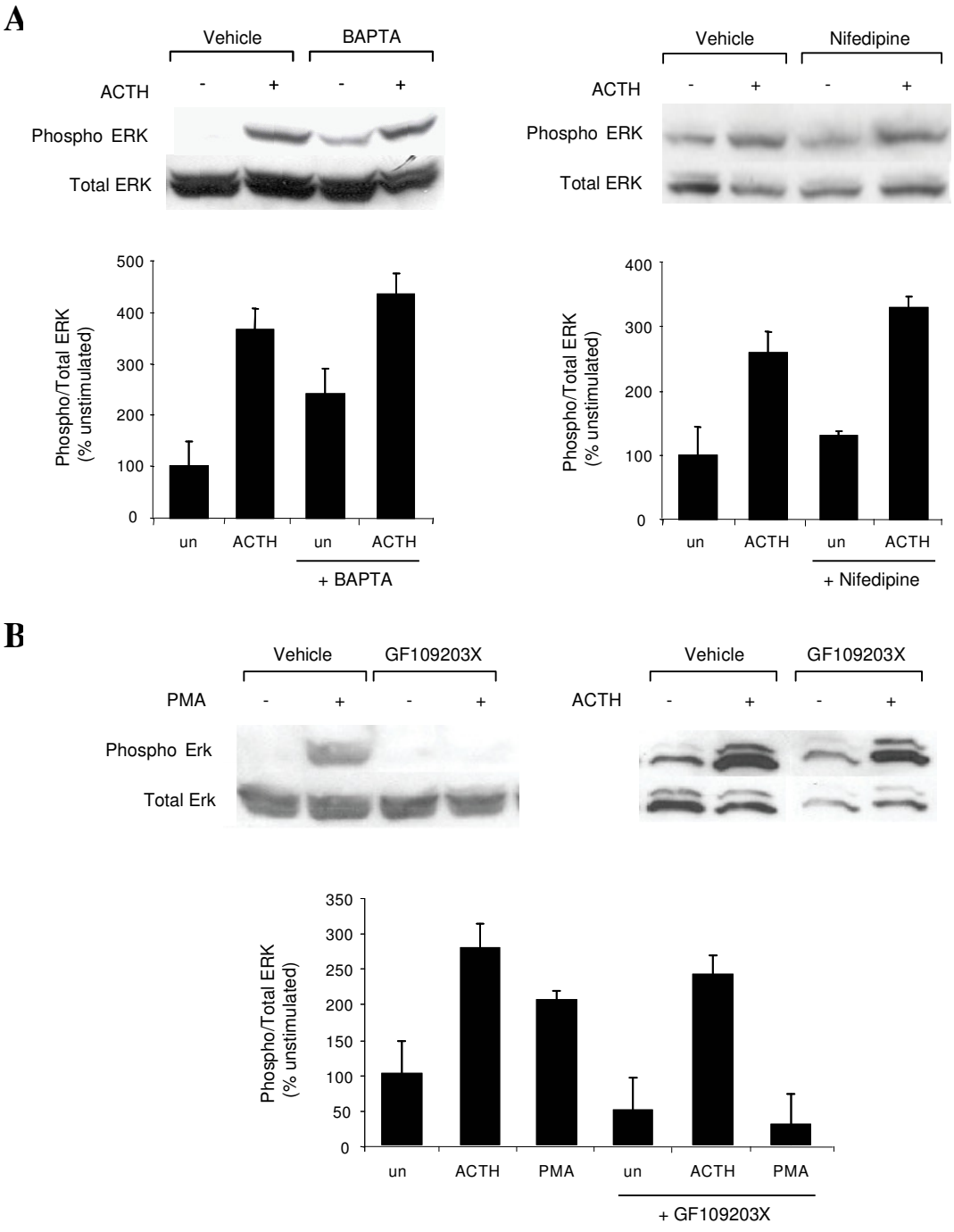


Fig. 4.7 *A, H295R cells were pre-treated with* 5 x 10^{-5} M *BAPTA-AM or* 10^{-6} *M nifedipine prior to stimulation with ACTH* (10^{-7} *M) and analysed for phospho and total* $Erk_{1/2}$. *Densitometry was performed on results of 3 independent experiments with error bars representing SEM. B, cells were treated with* 10^{-6} *M calphostin C or* 5 x 10^{-6} M *GF109203X prior to the addition of PMA* (10^{-7} *M) or ACTH* (10^{-7} *M) for 5 mins and analysed for phospho* $Erk_{1/2}$. *Densitometry was performed on results of 3 independent experiments with error bars representing SEM.*

To investigate whether the influx of Ca^{2+} following ACTH binding to the MC2R was mediating an $Erk_{1/2}$ response, the L-type Ca^{2+} channel blocker, nifedipine was used at 10^{-6} M. In addition, to look at the involvement of intracellular Ca^{2+} release in mediating the response, BAPTA-AM, a Ca^{2+} chelator, was used at 5×10^{-5} M. Fig. 4.7A shows that pre-treatment with these inhibitors also had no effect on the activation of $Erk_{1/2}$ by ACTH suggesting that the activation of this pathway is not mediated by a mechanism involving Ca^{2+}.

4.3.6 Interaction with the PI3-Kinase/Akt pathway

As discussed previously, many studies of GPCRs have demonstrated the upstream activation of the PI3-K/Akt pathway prior to $Erk_{1/2}$ activation. Furthermore the activity of this pathway has been previously shown in Y1 cells to be altered following ACTH exposure although its levels are not significantly altered in H295R cells (see Chapter 3). It was therefore decided to look at the effect of inhibition of this pathway on ACTH induced $Erk_{1/2}$ activation. Fig. 4.8A demonstrates the potent inhibition of constitutive Akt phosphorylation in H295R cells following a 1 h pre-incubation with 0.1 μM wortmannin. In contrast to the effect on Akt phosphorylation, the same concentration of wortmannin did not modulate $Erk_{1/2}$ activation induced by ACTH treatment (Fig. 4.8B) suggesting that the PI3-Kinase/Akt pathway does not act upstream of $Erk_{1/2}$ activation in this case.

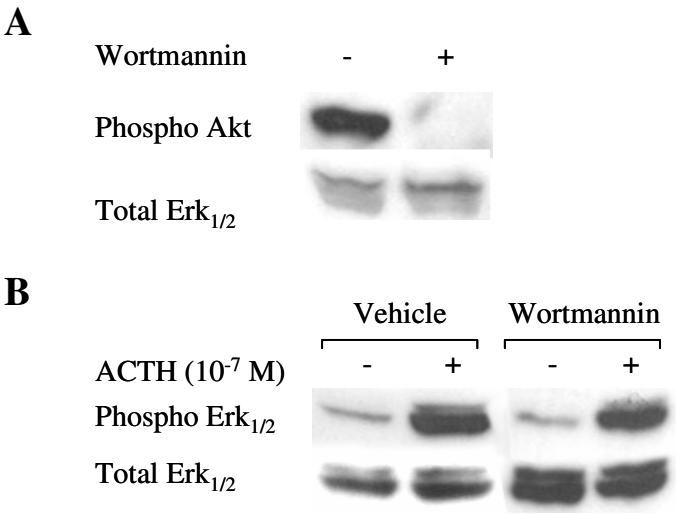


Fig. 4.8 *Akt phosphorylation is not a pre-requisite for Erk$_{1/2}$ activation. A, H295R cells were treated with 0.1 μM Wortmannin or SFM for 1 h and lysates analysed for levels of phospho Akt and total Erk$_{1/2}$. B, H295R cells were pre-treated for 1 h with 10^{-7} M Wortmannin or SFM prior to adding ACTH to a final concentration of 10^{-7} M ACTH or SFM for 5 mins. Lysates were analysed for phospho and total Erk$_{1/2}$.*

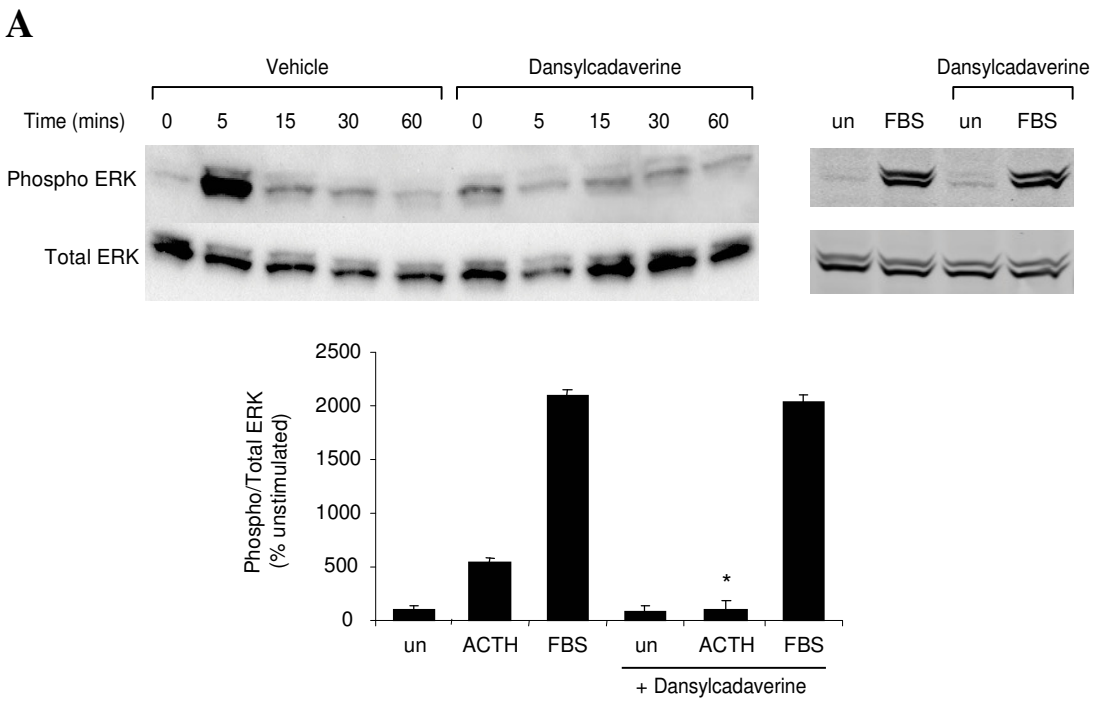
4.3.7 Involvement of Internalisation in signalling to Erk$_{1/2}$

Although there are various approaches to modulating internalisation including the use of dominant negative expression of dynamin, GRKs and β-arrestin, we first used inhibitors of clathrin-mediated internalisation to investigate the effects on ACTH induced Erk$_{1/2}$ activation. Fig. 4.9 demonstrates the effects of pre-treatment with two widely used inhibitors of clathrin-mediated internalisation, sucrose (Fig. 4.9B) and dansylcadaverine (Fig. 4.9A), on ACTH stimulated Erk$_{1/2}$ activation compared to vehicle alone.

Fig. 4.9A shows a normal time-course of Erk$_{1/2}$ activation, peaking at 5 mins following ACTH treatment and reducing back to basal levels by 60 mins when cells were treated with vehicle alone. However, in the presence of 0.3 mM

dansylcadaverine, the potent $Erk_{1/2}$ activation at 5 mins is completely abolished. In contrast H295R cells treated with 10% FBS for showed potent $Erk_{1/2}$ activation after 5 mins that was unaffected by pre-treatment for 1 hour with dansylcadaverine.

On the other hand, cells pre-treated for 1 h with 0.45 M sucrose, showed a large in increase in $Erk_{1/2}$ activation before stimulation with ACTH compared to vehicle alone (Fig. 4.9B). This would suggest a very non-specific effect that is presumably the result of osmotic shock induced by such a high concentration of sucrose. Treatment with ACTH caused no further increase in $Erk_{1/2}$ phosphorylation, however it cannot be suggested that this was due to inhibition of internalisation of the MC2R because the system was saturated.



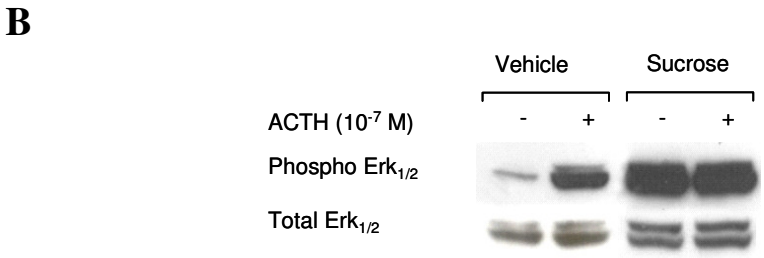


Fig. 4.9 *H295R cells were serum starved overnight then treated with either vehicle alone or 0.3 mM dansylcadaverine (A,) or vehicle alone or 0.45 M sucrose (B) for 1 h prior to the addition of 10^{-7} M ACTH for the indicated times (A) or for 5 mins (B). Cell lysates were analysed for phospho and total Erk levels. Densitometry of results from 3 independent experiments was performed. Error bars represent SEM. The * denotes a significant difference between the two ACTH treatments where $p<0.05$.*

4.4 DISCUSSION

4.4.1 The role of the MC2R

This chapter aimed to elucidate some of the mechanisms involved in the activation of the $Erk_{1/2}$ cascade by ACTH. The response would be presumed to be mediated though the binding of ACTH to the MC2R as the highest affinity receptor and as the mediator of steroid synthesis in the adrenal. However, since binding data in the previous chapter demonstrated a low affinity interaction of ACTH with the H295R cell surface, it was therefore necessary to investigate whether the $Erk_{1/2}$ response was being mediated by an alternative melanocortin receptor. NDP-MSH provided a useful tool since it acts as a potent agonist at melanocortin receptors other than the MC2R, for which it has no affinity. This peptide however had no stimulatory effect of the $Erk_{1/2}$ cascade in contrast to ACTH (Fig. 4.2) suggesting therefore that ACTH is unlikely to bind to another melanocortin receptor to bring about this response. Attempts to further investigate the central role of the MC2R in mediating the ACTH induced $Erk_{1/2}$ response were hampered by a lack of availability of an effective MC2R receptor antagonist. An N-terminally truncated fragment of ACTH (11-24) has been previously shown to act as an antagonist at the MC2R where cAMP production was abrogated (Seelig et al., 1971). However, when H295R cells were pre-incubated with this peptide prior to ACTH stimulation, no inhibitory effect was seen (data not shown). However, this is not surprising since others have reported a steroidogenic effect of this peptide and further, one study showed an additive effect on a maximal dose of ACTH (1-39) (Szalay et al., 1989).

These findings therefore do provide evidence to support a role for the MC2R in mediating the $Erk_{1/2}$ response to ACTH in these cells, but further highlights the difficulties in studying responses through this receptor.

4.4.2 Role of G_i proteins

A review by Rashid et al (Rashid et al., 2004) discussed the complexity of GPCR signalling and highlighted the promiscuity of many GPCRs which couple to alternative G-proteins. In view of this and the fact that several GPCRs have been shown to induce $Erk_{1/2}$ activation through alternative G-protein coupling (Daaka et al., 1997), it was decided to investigate whether this could occur in response to ACTH in H295R cells. Coupling of the MC2R to a G_i protein may have explained the dampened cAMP response to ACTH in these cells. Results from a single experiment however (triplicate wells), showed an additive effect of ACTH and forskolin on cAMP production suggesting that ACTH does not couple to G_i to inhibit this response (data not shown). However, better evidence is the lack of effect of PTX on ACTH induced $Erk_{1/2}$. For many receptors, alternative coupling to different G proteins most likely occurs due to different functional roles in heterologous cell types (Wu et al., 1997, Holst et al., 2001). Given that the main site of expression of MC2R is the adrenal gland, it would be surprising to find alternative coupling in adrenal cells such as H295Rs.

4.4.3 Involvement of G_s coupling and the cAMP/PKA pathway

Previous studies of G_s coupled receptors have demonstrated a direct role of the α_s subunit in mediating $Erk_{1/2}$ responses through the direct binding to *Src* as in the case of the β-adrenergic receptor (Ma and Huang, 2002). In addition, many other G_s coupled GPCRs also involve $G\alpha_s$ in the activation of $Erk_{1/2}$ where the increase in cytosolic cAMP and/or the activation of PKA results in the activation of an upstream kinase of $Erk_{1/2}$ (Norum et al., 2003, Klinger et al., 2002).

Attempts at RNAi mediated knock down of the $G\alpha_s$ gene were unsuccessful due to low transfection efficiency seen in these cells and therefore a role for the α_s subunit acting as a direct effector upstream of $Erk_{1/2}$ activation cannot be ruled out. In the future, use of a stably transfected RNAi hairpin may over come this problem.

Alternatively, one study outlined a method for elucidating the role of G_s in $Erk_{1/2}$ signalling where expression of an 83 amino acid polypeptide derived from the c-terminus of $G\alpha_s$ disrupted cAMP signalling of the dopamine 1A receptor and the β_2 AR (Feldman et al., 2002).

ACTH is only a weak activator of cAMP accumulation in H295R cells, however, interestingly, using a submaximal dose of forskolin to mimic the level of cAMP production to that of 10^{-7} M ACTH lead to very similar $Erk_{1/2}$ responses. This is highly suggestive of a common mechanism of $Erk_{1/2}$ activation by ACTH and forskolin by a cAMP/PKA mediated mechanism. However, in contrast to this were three further pieces of information. Firstly, when added in combination, ACTH and forskolin produced an additive $Erk_{1/2}$ induction implying that alternative pathways are utilised. Secondly, the PKA inhibitor, H89, was effective (albeit not completely) at reducing forskolin mediated $Erk_{1/2}$ activation, however it had little or no effect on the ACTH induced effect. Thirdly, the adenylate cyclase inhibitor SQ22536 had no effect on ACTH induced $Erk_{1/2}$ activity further suggesting that a cAMP/EPAC mediated mechanism is unlikely to be important in this response. We therefore conclude that whilst cAMP production and activation of PKA, and possibly EPAC, is the probable mechanism of forskolin induced $Erk_{1/2}$ activation, ACTH action largely by-passes this mechanism. This is consistent with results seen in PKA-deficient Y1 cells (Le and Schimmer, 2001, Lotfi et al., 1997).

An interesting further experiment would be to look at the effect of H89 on ACTH induced $Erk_{1/2}$ activation over a time-course of ACTH treatment. It has been suggested previously that PKA can exert a negative feedback on $Erk_{1/2}$ activity through the phosphorylation of *src* (Schmitt and Stork, 2002) and indeed it was previously shown in Y1 cells that the anti-mitogenic effect of ACTH was mediated through a mechanism dependent on the cAMP/PKA pathway (Lotfi et al., 1997) therefore it would be interesting to see whether $Erk_{1/2}$ activity would be prolonged in H295R cells in the presence of H89.

4.4.4 Involvement of G_q/PKC pathway

A number of G_s coupled GPCRs have been shown to couple to both G_s and G_q proteins, one example of which is the glucagon-like peptide-1 receptor in CHO cells (Montrose-Rafizadeh et al., 1999). In addition, constituents of G_q coupled pathways may also be activated by alternative means other than through G_q coupling itself. Activation of Ca^{2+} channels within the plasma membrane can allow an influx of Ca^{2+} and result in the activation of PKC. There is evidence, largely from primary cultures of adrenal cells, that ACTH can induce Ca^{2+} influx via the opening of plasma membrane Ca^{2+} channels (Enyeart, 2005) which might in turn activate PKC and subsequently the $Erk_{1/2}$ pathway. Indeed, studies in Y1 cells would support the involvement of PKC in ACTH induced $Erk_{1/2}$ activation where PMA treatment mimicked that of ACTH in activating $Erk_{1/2}$ (Lotfi et al., 1997). Our results also confirmed that PMA mimics ACTH treatment in H295R cells, however, the use of inhibitors of PKC activity did not show that PKC acts upstream of $Erk_{1/2}$ activation. The use of two PKC inhibitors ensured coverage of a broad range of isoforms as these inhibitors have different mechanisms of actions (Salamanca and Khalil, 2005) and neither had any effect on the level of $Erk_{1/2}$ activation induced by ACTH. Therefore it can be concluded that these signalling molecules are unlikely to be involved in this response.

Neither Ca^{2+} influx nor release from intracellular stores was also shown to be likely to play a role in $Erk_{1/2}$ activation since neither the Ca^{2+} channel blocker (nifedipine) nor the Ca^{2+} chelator (BAPTA-AM) had any effect on $Erk_{1/2}$ activation in response to ACTH.

4.4.5 Growth factor transactivation and src

The transactivation of EGF receptors is a common feature in the transduction of mitogenic signalling pathways initiated in response to GPCR agonists. Inhibition of EGF receptor activity using the EGF specific inhibitor AG1478, has been shown to abolish downstream $Erk_{1/2}$ activation and proliferation in response to EGF and

GPCR agonists (Yin et al., 2003, Shah and Catt, 2002). A time course of EGF treatment showed potent stimulation of $Erk_{1/2}$ activation in a manner similar to ACTH (data not shown). Although a similar pattern of activation was seen, the EGF induced response did not reduce back to basal over the 1 hour investigated. However, since there was a similarity in the time-course, transactivation of the EGF receptor was thought to be a good candidate for mediating the ACTH response, albeit a weaker activation. However, even though results from this chapter have demonstrated the complete abolition of EGF induced $Erk_{1/2}$ activity by AG1478, this inhibitor had no effect on ACTH induced $Erk_{1/2}$ activity. It appears, therefore, that this common mechanism of activation of $Erk_{1/2}$ by GPCRs is not involved with this particular response.

Many of the studies implicating a role for growth factor transactivation by GPCRs have demonstrated the autophosphorylation of the EGF receptor, however, other tyrosine kinase receptors such as the PDGF receptor have also been implicated (Wang et al., 2005). Furthermore, one study has described the transactivation of the fibroblast growth factor (FGF) receptor-1 with agonist stimulation of the μ opioid receptor in rat C6 glioma cells (Belcheva et al., 2002). They demonstrated that a dominant negative FGF receptor-1 attenuated μ opioid receptor induced $Erk_{1/2}$ activation and found the response to be mediated through $G_{\beta\gamma}$, Ca^{2+}, PKC and matrix metalloprotease (MMP) dependent release of FGF from glioma cells. FGF has also been shown to stimulate potent proliferation of Y1 cells suggesting that this receptor could be a candidate for transactivation by ACTH. However, this is unlikely for two reasons. Firstly, the use of genistein, a non-specific tyrosine kinase inhibitor, abrogated EGF induced $Erk_{1/2}$ activation and, although not tested, would be expected to have the same effect with FGF; Secondly, studies in Y1 cells suggest that ACTH antagonises the proliferative effect of FGF through a process dependent on cAMP/PKA and the inactivation of PI3-K which results in destabilisation of c-Myc rather than transactivating this receptor (Lepique et al., 2004).

Investigation into the role of the non-receptor tyrosine kinase *src* showed that this protein was unlikely to be involved in ACTH induced $Erk_{1/2}$ activation. As previously mentioned, genistein had no effect on $Erk_{1/2}$ activity following ACTH treatment and this inhibitor would be expected to inhibit *src* activity. In addition, the specific *src* inhibitors PP2 and SU6656 had no effect on ACTH induced $Erk_{1/2}$ activation and further, *src* was not phosphorylated on Tyr 416 to its active form following ACTH treatment (Fig. 4.8). The phospho *src* antibody cross reacts with various members of the *src* family kinases including Lyn, Fyn, Yes, Lck and Hck, thus it seems that no other family member was substituting for *src* in this pathway. In these experiments the phosphorylation of the tyrosine 527 residue was not investigated. Dephosphorylation of this residue leads to an increase in *src* activity and therefore this could lead to an increase in its activity in H295R cells (Roskoski, 2005). However, this seems unlikely since total levels of *src* were almost undetectable. The fact that *src* has been shown to be involved in GPCR signalling to $Erk_{1/2}$ for such a wide range of GPCRs, and via a number of different pathways, raises the possibility that other, as yet unidentified, proteins may play similar roles in the $Erk_{1/2}$ response to ACTH.

4.4.6 PI3-K/Akt pathway

The observation in Y1 cells that Akt is dephosphorylated by 5 mins following ACTH treatment and that the upstream activator of Akt, PI3-K, is often involved in GPCR mediated activation of $Erk_{1/2}$ lead us to look at the involvement of the PI3-K/Akt pathway in ACTH induced $Erk_{1/2}$ activation in H295R cells. Results from the previous chapter showed high constitutive activation of Akt in H295R cells similar to that seen in Y1 cells (Forti et al., 2002). However, the activity of Akt generally remained unchanged following ACTH treatment. It was therefore hypothesised that inhibition of the PI3-K/Akt pathway would have no effect on ACTH induced $Erk_{1/2}$ activation and, indeed, this was the case where wortmannin predictably abolished constitutive Akt phosphorylation but had no effect on ACTH induced $Erk_{1/2}$ activation (Fig. 4.10). This difference between cell lines may be due to the weak cAMP response to ACTH in H295R cells. The dephosphoylation of

Akt seen in Y1 cells was thought to be mediated through a cAMP/PKA dependent mechanism since the effect could not be seen in a PKA defective derivative cell line (Forti and Armelin, 2000). Therefore, it is possible that in an adrenal cell in which ACTH is able to bring about both cAMP accumulation and $Erk_{1/2}$ activation, that dephosphorylation of Akt via a cAMP dependent mechanism serves to regulate the activity of the $Erk_{1/2}$ pathway.

4.4.7 Receptor internalisation

Finally, we have identified clathrin-mediated endocytosis as a possible pre-requisite for ACTH induced $Erk_{1/2}$ activation using chemical inhibition of this process. MDC is thought to function by preventing clathrin coated pit formation (Davies et al., 1984). This inhibitor reduced the 5 min stimulation of $Erk_{1/2}$ activity reproducibly back to basal levels (Fig. 4.11A) suggesting that either the recruitment of signalling components into the coated pit is necessary for $Erk_{1/2}$ signalling and/or internalisation of the receptor is necessary to bring about the activation of this pathway. Conversely, pre-treatment with hypertonic sucrose caused a potent activation of the $Erk_{1/2}$ cascade, presumably due to an osmotic shock effect, and therefore the effects of ACTH could not be analysed. The sucrose effect has been seen before in a previous study looking at the mechanisms of Ang II internalisation (Olivares-Reyes et al., 2005). Internalisation has been shown to be a pre-requisite for GPCR mediated $Erk_{1/2}$ activation in many previous studies, however, in some cases, the effects were thought to be due to internalisation of a down-stream effector such as the EGF receptor (Vieira et al., 1996). In the case of the ACTH response, however, we have shown that internalisation of a growth factor tyrosine kinase is unlikely due to a lack of sensitivity of the $Erk_{1/2}$ response to tyrosine kinase inhibitors.

In view of this data and given previous findings from our laboratory that demonstrate the internalisation of the ACTH receptor, the following chapter investigates in greater detail the role of internalisation of the MC2R in mediating the $Erk_{1/2}$ response.

5 THE ROLE OF INTERNALISATION OF THE MC2R IN SIGNALLING TO ERK

5.1 INTRODUCTION

Having demonstrated in the previous chapter a potential requirement for receptor internalisation in the mediation of the $Erk_{1/2}$ signal by ACTH, further investigation was necessary to confirm this finding. Internalisation of ACTH in H295R cells has not been previously investigated however, in Y1 cells, acid wash techniques have shown specific internalisation of I^{125} ACTH which could be inhibited by sucrose (Baig et al., 2002). It was therefore intended to investigate the ability of H295R cells to internalise ACTH and, if so, to attempt to modulate this effect with sucrose (as a positive control) and MDC. This evidence will ascertain whether or not the inhibition of $Erk_{1/2}$ signalling by MDC is through specific inhibition of receptor internalisation or a non-specific effect of the chemical.

Receptor internalisation is measured most commonly using the acid wash technique (Hunyady et al., 1994), which was the primary method used in this chapter. An additional biotin method of visualising internalisation was used to confirm some of the results (see chapter 2.16 for a description of this technique).

A further aim of the chapter is to investigate the role of the desensitisation/internalisation machinery in the mediation of the $Erk_{1/2}$ response. Many studies have used stable and transiently transfected cell lines expressing dominant negative dynamin, GRK2 and ß-Arrestin (Ferguson et al., 1995, Damke et al., 1994, Ferguson et al., 1996a). However, more recently the use of RNAi has allowed the study of the role of such proteins.

5.2 METHODS

The production of stable cell lines was chosen over transient transfection of the constructs as H295R cells have low transfection efficiency. In addition, by producing monoclonal lines, those lines showing the highest expression of dominant negatives could be selected for further experiments.

All plasmids were kind gifts from Laszlo Hunyadi. The GRK2 (K220M) has a mutation in the kinase domain leaving the protein kinase inactive (Ferguson et al., 1995). By competing with wild type GRK for receptors it exerts its dominant negative effect. The dominant negative dynamin plasmid (K44A) has a defect in GTP binding and hydrolysis and thus inhibits the budding of clathrin coated vesicles (Damke et al., 1994). Dominant negative ß-Arrestin 1 (V53D) binds well to clathrin but weakly to phosphorylated agonist-activated GPCRs thus mediating its dominant negative effect by competing with endogenous ß-Arrestin for clathrin. (Ferguson et al., 1996b, Krupnick et al., 1997, Orsini and Benovic, 1998).

5.3 RESULTS

5.3.1 Internalisation of the MC2R

Having seen the effect of MDC on ACTH induced $Erk_{1/2}$ activation, it was first necessary to confirm that the MC2R does internalise in response to ACTH treatment in H295R cells. Fig. 5.1 demonstrates specific internalisation of ACTH over 60 mins with around 16 % of cell surface ACTH receptors internalising over this period. Having demonstrated that the MC2R does internalise, a similar experiment was performed to determine whether this could be abrogated by treatment with MDC and also sucrose since this has been previously been shown to inhibit internalisation of the MC2R in Y1 cells. Fig. 5.2 demonstrates the results of these experiments.

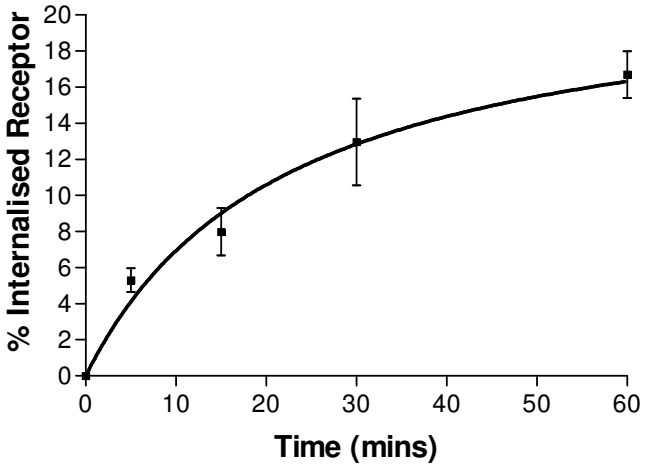


Fig. 5.1 *Internalisation of I^{125} ACTH in H295R cells. I^{125} ACTH was added and incubated for the indicated times. Internalisation was measured using the acid wash technique. Samples were analysed in duplicate in 5 independent experiments. Error bars represent SEM.*

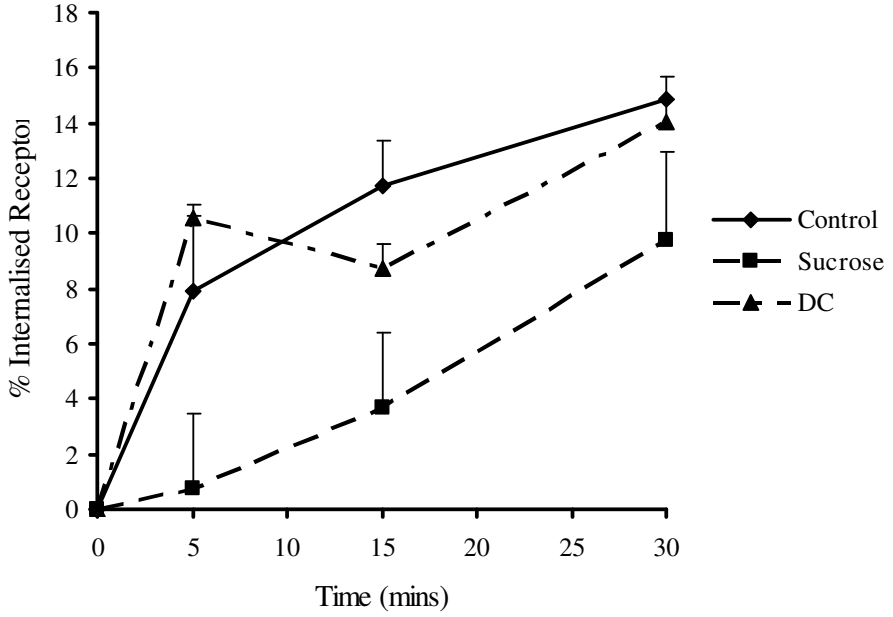


Fig. 5.2 *Effect of inhibitors on internalisation of MC2R. Cells were pretreated for 1 hour with either SFM, 0.4 M Sucrose or 0.3 mM MDC (DC) I^{125} ACTH was added and incubated for the indicated times and the amount of internalised ACTH analysed using the acid wash technique. Samples were analysed in duplicate in 3 independent experiments. Error bars represent SEM.*

Although it appears that sucrose effectively inhibited internalisation, no significant difference was found between that of untreated and MDC treated cells. The acid wash technique however caused significant variance between experiments thus making it difficult to see small differences between treatments. It was therefore decided to use an alternative approach to confirm the effect of MDC, that of the biotin method of internalisation. In essence, the protocol firstly labels surface proteins with biotin. Cells are then treated with either SFM or MDC followed by ACTH accordingly. Cells are then treated with a non-cell-permeable reducing agent which removes all surface biotin. Subsequent immunoprecipitation with an antibody to the desired protein and immunoblotting with an anti-bitotin antibody allows detection of internalised proteins. Although this is not a quantitative method, a difference in intensities of the bands could be visualised (Fig. 5.3).

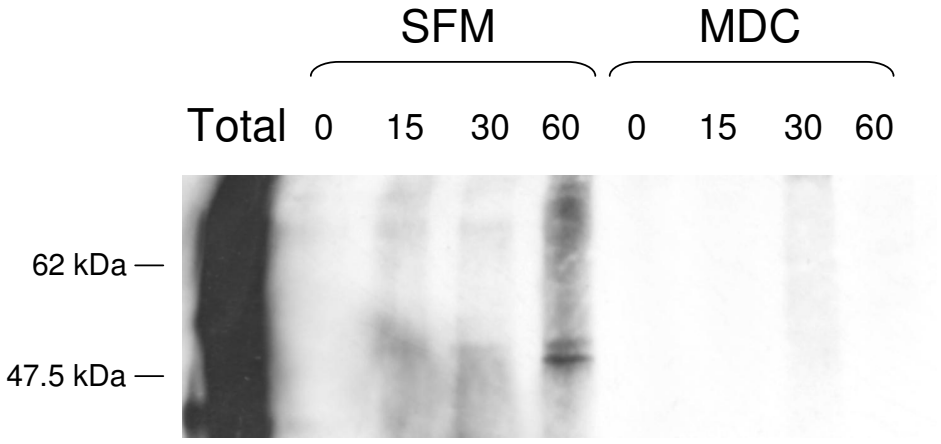


Fig. 5.3 *Effect of MDC on MC2R internalisation. H295R cells were treated with 10^{-7} M ACTH for the indicated times with or without MDC pretreatment. Internalisation was visualized using the Biotin method. This is representative of two independent experiments.*

This experiment demonstrates internalisation of the MC2R over 1 hr as shown with the acid wash technique. In addition it demonstrates the blocking of internalisation with MDC treatment over the full 1 hr period.

5.3.2 Effects of dominant negative dynamin, GRK2 and ß-Arrestin on $Erk_{1/2}$ activation

5.3.2.1 Expression of GRKs, dynamins and ß-Arrestins in H295R cells

Using RT-PCR for the different isoforms, H295R cells were analysed for GRK, Dynamin and ß-Arrestin expression (Fig. 5.4). These results demonsrate that H295R cells express only GRK 2 and 3, Dynamin 1 and 2 and ß-Arrestin 1 and 2.

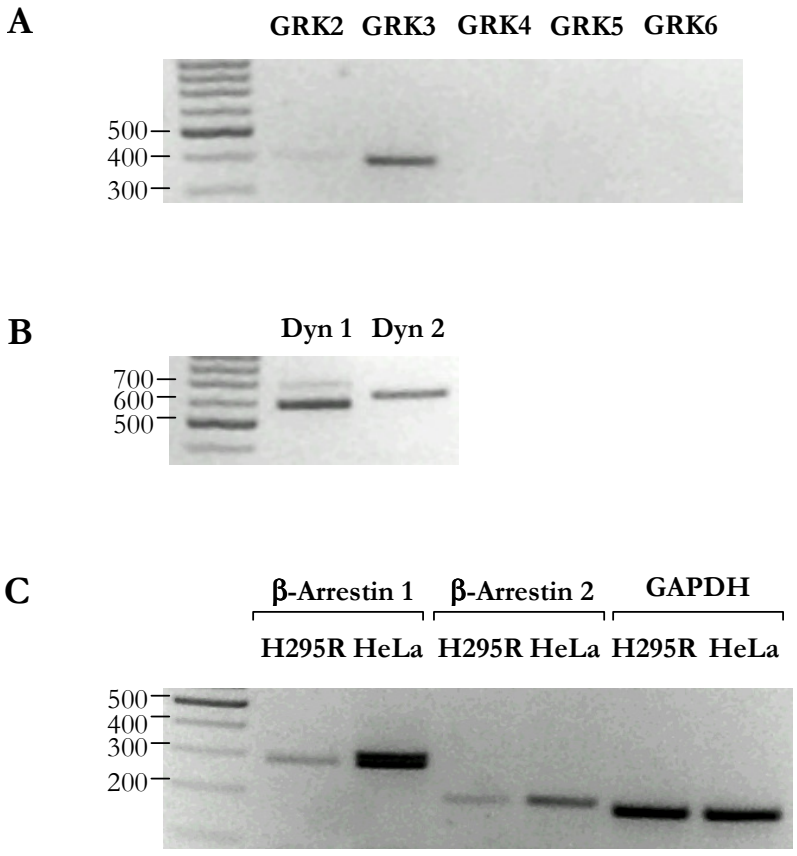


Fig. 5.4 *Expression of GRK, Dynamin and ß-Arrestin. PCRs were performed on H295R cDNA for 30 cycles. Markers are shown in base pairs (bp).* ***A****, GRK2 (407 bp), GRK3 (401 bp), GRK4 (310 bp), GRK5 (667 bp), GRK6 (547 bp).* ***B****, Dyn 1 (589 bp), Dyn 2 (635 bp).* ***C****, HeLa cDNA was used as a positive control for expression of ß-Arrestins. ß-Arrestin 1 (286 bp), ß-Arrestin 2 (193 bp), GAPDH (198 bp).*

5.3.2.2 GRK2 dominant negative

Monoclonal cell lines were produced using the method described in chapter 2.3.2. Fig. 5.5 shows the expression of the construct in G418 selected cells lines in clone 1, 5 and 6 (A). These lines were therefore used to look at the effect of dominant negative inhibition of GRK on $Erk_{1/2}$ activation by ACTH compared to cells expressing vector alone (B). It can be seen that the $Erk_{1/2}$ response to ACTH is maintained in all three clones however it may be slightly enhanced compared vector alone.

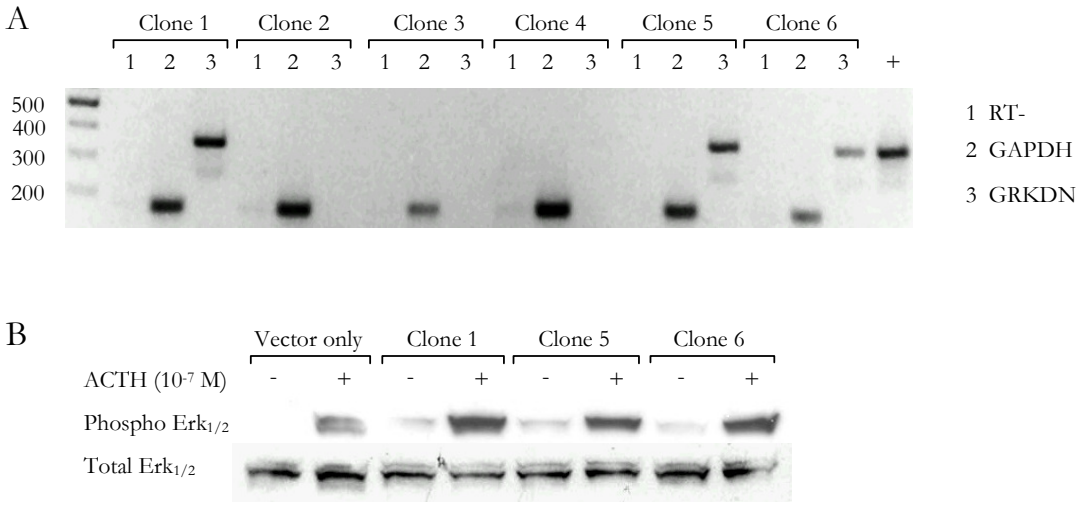


Fig. 5.5 *GRK dominant negative expression. A, Several monoclonal cell lines were selected in media containing G418 and screened by RT-PCR (30 cycles) for expression of the plasmid. Lanes 1 and 2 in each case are controls for genomic DNA contamination and GAPDH respectively. Lane 3 for each clone represents expression of GRK dominant negative (GRKDN, 362 bp). + represents a positive control for the GRK PCR (plasmid DNA). B, Immunoblot for phospho $Erk_{1/2}$ and total Erk on GRK clones (or vector alone clone) after treatment for 5 minutes with 10^{-7} M ACTH or SFM alone. This is representative of two experiments.*

5.3.2.3 Dynamin dominant negative

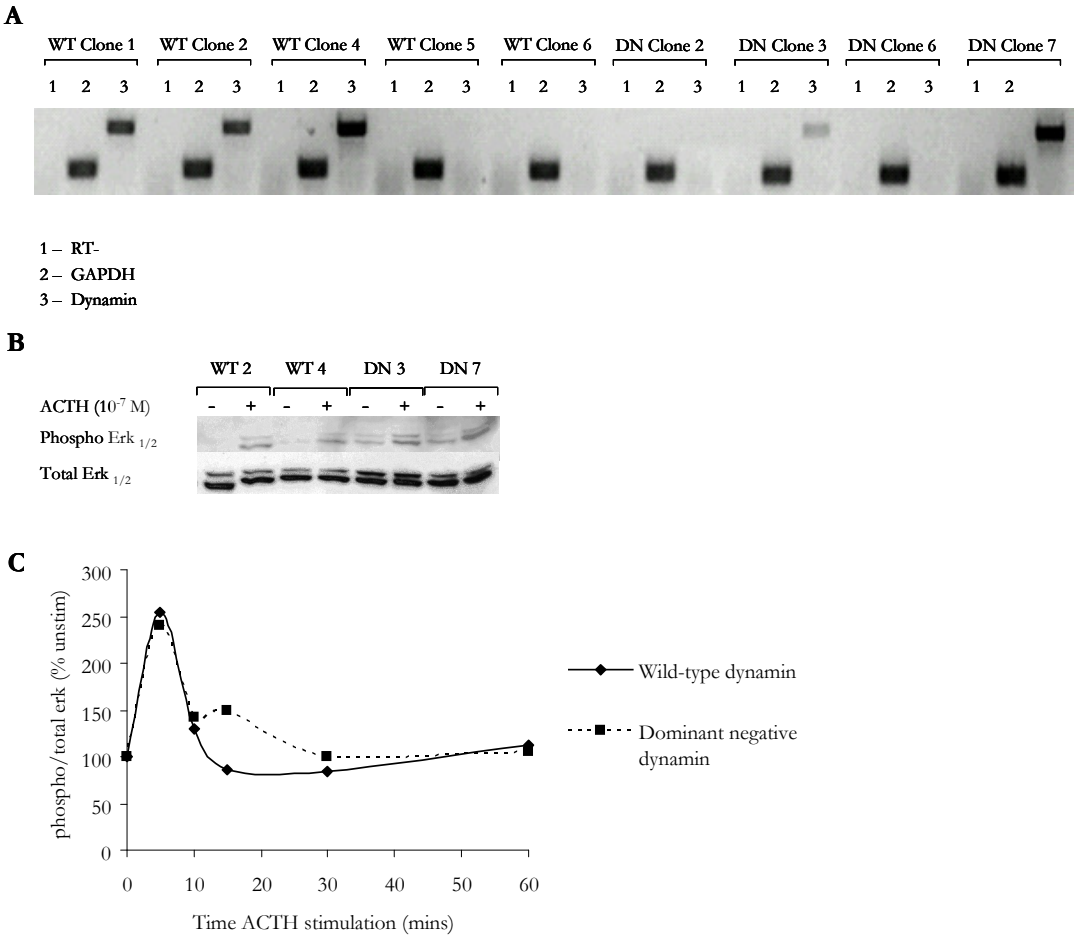


Fig 5.6 *Dynamin dominant negative. A, Several monoclonal cell lines were selected in media containing G418 and screened by RT-PCR (30 cycles) for expression of the plasmid. Lanes 1 and 2 in each case are controls for genomic DNA contamination and GAPDH respectively. Lane 3 for each clone represents expression of Dynamin dominant negative (400 bp). + represents a positive control for the Dynamin PCR (plasmid DNA). B, Monoclonal wild type and dominant negative dynamin clonal cells were treated with either SFM (-) or ACTH (+) for 5 mins and immunoblotted for phospho $Erk_{1/2}$ and total $Erk_{1/2}$. C, Dynamin polyclonal cells were treated with ACTH or SFM (zero point) for the indicated times and immunoblotted for phospho $Erk_{1/2}$ and total $Erk_{1/2}$. Densitometry was performed on the resulting blot as a ratio of phopsho:total $Erk_{1/2}$. Responses were normalised to % of unstimulated cells. This data is from two experiments.*

Monoclonal cell lines containing either wild type or dominant negative dynamin were produced and expression confirmed by RT-PCR (Fig.5.6A). Those cell lines with highest expression of the plasmids were analysed as above for their effect on ACTH induced activation of $Erk_{1/2}$. Fig. 5.6B shows the results from two experiments.

From this it can be seen that there is no significant difference between wild type and dominant negative clones. A time-course of ACTH treatment on polyclonal wild type and dominant negative dynamin expressing cells was also undertaken. Densitometry of the resulting blots was performed since there were differences in total $Erk_{1/2}$ (Fig. 5.6C). This again demonstrated no significant differences between wild type and dominant negative dynamin cells, indicating that dynamin is unlikely to be involved in the ACTH induced activation of $Erk_{1/2}$.

5.3.2.4 ß-Arrestin dominant negative

Finally, monoclonal H295R cell lines expressing wild type or dominant negative ß-Arrestin were produced (Fig. 5.7A). Wild type clone 2 and dominant negative clone 2 were compared for their ability to stimulate $Erk_{1/2}$ in response to ACTH (B). No significant difference was found between the two. Additionally, polyclonal cell lines expressing each of the plasmids were treated similarly. Again, no differences could be seen in the size of the $Erk_{1/2}$ response. Taken together, these results suggest no involvement of ß-Arrestins in the $Erk_{1/2}$ response to ACTH.

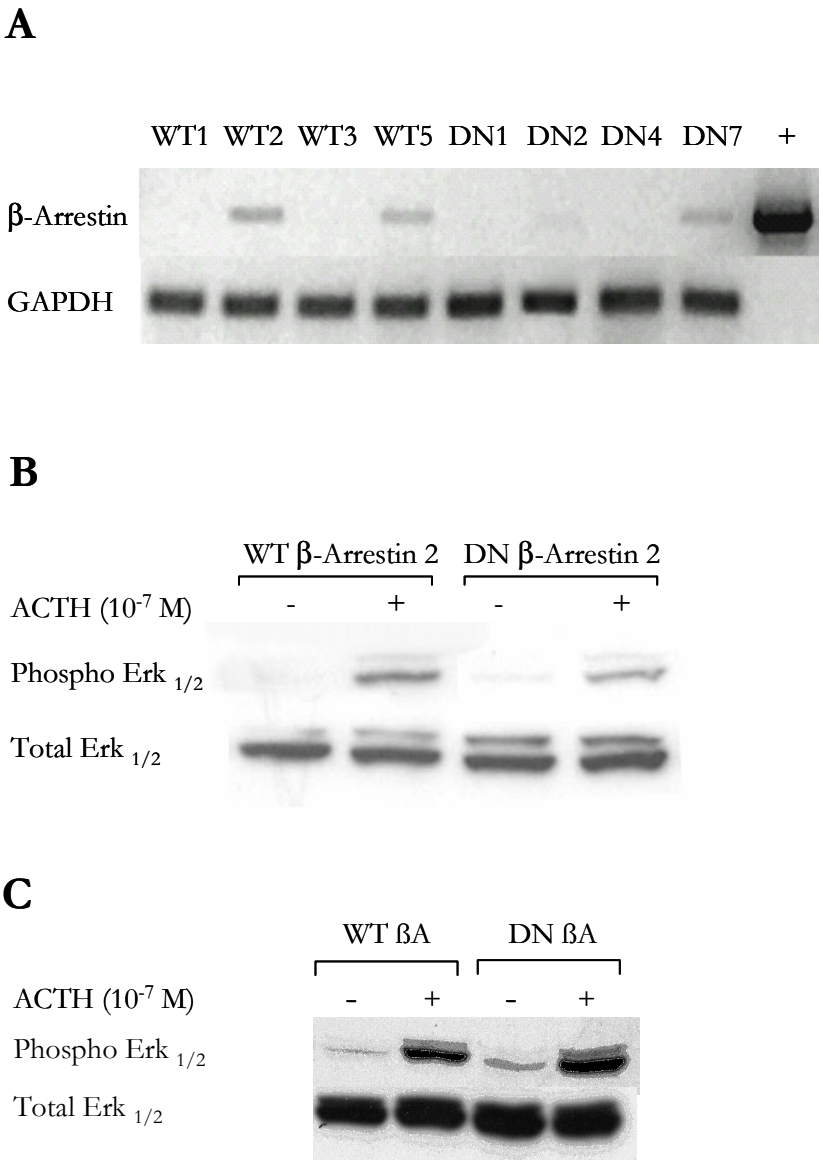


Fig. 5.7 *Dominant negative ß-Arrestin. A, Several monoclonal cell lines were selected in media containing G418 and screened by RT-PCR (30 cycles) for expression of GAPDH (198 bp) and the ß-Arrestin (482 bp) plasmids. + represents a positive control for the ß-Arrestin PCR (plasmid DNA). B, ß-Arrestin clones (WT 2 and DN 2) were treated with ACTH (+) or SFM (-) for the 5 mins and immunoblotted for phospho Erk$_{1/2}$ and total Erk$_{1/2}$. C, ß-Arrestin (βA) polyclonal cells were treated with ACTH (+) or SFM (-) for 5 mins and immunoblotted for phospho Erk$_{1/2}$ and total Erk$_{1/2}$. This is representative of two experiments.*

5.4 DISCUSSION

In the previous chapter it was demonstrated that $Erk_{1/2}$ activation induced by ACTH in H295R cells could be inhibited by MDC. MDC was first shown to inhibit internalisation of I^{125}-EGF in acid wash internalisation studies (Haigler et al., 1980). Although the exact mechanism of interference in endocytosis is not known, it is thought to be due to its covalent coupling to cellular membranes mediated by the enzyme transglutaminase (Davies et al., 1984). This coupling appears to prevent clathrin mediated internalisation of a host of receptors in a number of cell types. Despite its wide use in similar studies, we wanted to confirm that MDC was mediating its $Erk_{1/2}$ inhibitory effect through the modulation of internalisation of the MC2R. Therefore in this chapter we have verified through use of the biotin assay that MDC prevents internalisation of the MC2R over 1 hour implying that it is through such a mechanism that ACTH mediates $Erk_{1/2}$ activation. It is difficult to quantify the amount of internalisation that has taken place in the first 5 minutes when the $Erk_{1/2}$ response is at its strongest, however, results from the acid wash method (Fig. 5.1) would suggest that only around 4% of MC2-receptors have internalised at this point, suggesting that few receptors are required to cause this rapid response.

These data are not comparable with those from any other GPCR since most internalisation dependent $Erk_{1/2}$ activation mechanisms are seen with receptors which rapidly endocytose and have been discovered to be dependent on the internalisation of a transactivated growth factor receptor rather than the GPCR itself (Pierce et al., 2000). Given that the MC2R is demonstrated to internalise over 1 hour in contrast to the 5 minute activation of $Erk_{1/2}$, it suggests that there are regulatory mechanisms in place which quickly modulate the $Erk_{1/2}$ response to allow only transient activity of this pathway. MAPKs are negatively regulated by MKPs and previous studies in H295R cells have demonstrated that stimulation of cells with dibutyryl-cAMP induces MKP-1 mRNA (within 30 minutes) and protein (within 1 hr). It also promoted increased ^{32}P associated with MKP-1 (Sewer and Waterman, 2003). Thus the $Erk_{1/2}$ response may be regulated through a cAMP

mechanism allowing only transient activation of this pathway. Although it was not done in this investigation, an interesting experiment would be to look at the effect of H89 on the timecourse of $Erk_{1/2}$ activation to investigate whether $Erk_{1/2}$ activation was prolonged in the absence of cAMP regulation.

5.4.1 Dominant negative Dynamin

The fact that expression of a dominant negative dynamin in H295R cells did not effect $Erk_{1/2}$ activation was surprising. Dynamin is directly required for the budding of clathrin coated vesicles and thus internalisation and since MDC was able to block this response, dominant negative dynamin would also be thought to have the same effect. Although the expression of the construct was confirmed by PCR analysis, protein expression was not determined thus we cannot rule out that the protein was functionally inactive. Due to time constraints, establishment of the effect of dominant negative dynamin expression on MC2R internalisation using the biotin method was not performed. Experiments using the acid wash technique however were not conclusive (data not shown).

Alternatively, the lack of effect may be explained by the differing actions of MDC and the dominant negative dynamin. MDC is thought to prevent the formation of clathrin coated pits by covalent coupling to the membrane whereas the dominant negative dynamin blocks vesicle budding due to a defect in GTP binding and hydrolysis (Davies et al., 1984, Damke et al., 1994). It is possible therefore, that the formation of a vesicle is enough to allow recruitment of the necessary components to enable the activation of the $Erk_{1/2}$ pathway.

5.4.2 Dominant negative GRK and ß-arrestin

Previous studies in our laboratory have demonstrated a possible role for GRKs in the desensitisation of the MC2R since the cAMP response induced by ACTH in Y1 cells could not be desensitised by either forskolin or isoproterenol, and more importantly that expression of a dominant negative GRK inhibited internalisation

of the MC2R in Y1 cells (Baig et al., 2002). Y1 cells express both GRK2 and 5. Since H295R cells also expressed only two of the GRKs (2 and 3) it was thought that a dominant negative GRK2 would be sufficient to block the activity of both endogenous enzymes. The fact that the GRK mutant had no effect on $Erk_{1/2}$ activation was therefore surprising since it was assumed that GRK is involved in internalisation of the MC2R in H295R cells as it is in Y1 cells. This would seem reasonable as internalisation dynamics appear to be similar in both cell types, occurring over 1 hour with around 20% total internalisation in Y1 cells and 16% in H295R cells. Again, due to time constraints, it was not possible to analyse the effect of the mutant on ACTH internalisation by the biotin method, and therefore we cannot rule out the possibility that the dominant negative protein was not having its desired effect.

Alternatively, GRK may mediate its effect by a mechanism independent of its kinase activity. The dominant negative mutant is still capable of binding to an activated receptor however, it is not able to phosphorylate it and recruit ß-arrestin. Therefore it is possible that GRK functions by recruiting $Erk_{1/2}$ to receptor-GRK complexes within clathrin coated pits independently of receptor phosphorylation. This would concur with the apparent lack of effect of ß-arrestin mutants on $Erk_{1/2}$ activation in H295R cells.

The GRK expression pattern in H295R cells is interesting in that studies utilising RNAi knock down of GRK isoforms have shown GRK 5 and 6 to be important in ß-arrestin 2 mediated $Erk_{1/2}$ signalling for both the Angiotensin 1A receptor and the V_2 Vasopressin receptor whereas GRK2 and 3 were not capable of mediating the response (Kim et al., 2005, Ren et al., 2005). Only GRK2 and 3 are present in H295R cells suggesting that the ACTH induced $Erk_{1/2}$ response may be unlikely to occur via a ß-arrestin mediated mechanism. This is supported by the fact that those $Erk_{1/2}$ responses mediated through ß-arrestin are usually prolonged in contrast to the transient response seen with ACTH treatment (Murphy and Blenis, 2006). These observations may explain why the expression of the ß-arrestin dominant

negative construct also appeared to have no effect on ACTH induced $Erk_{1/2}$ activation.

Although this preliminary data using dominant negative forms of dynamin, GRK and ß-arrestin indicate no involvement of either internalisation or GRK/ß-arrestin mediated signalling, these mechanisms cannot be ruled out. In each case the dominant negative used is for one isoform whereas H295R cells express at least one other isoform. For this reason the dominant negative proteins may be insufficient to compete with all of the relevant proteins present. For example it has been shown for the m3 muscarinic acetylcholine receptor that, out of dominant negative mutants to isoforms 2, 3, 5 and 6, only 6 was effective at blocking desensitisation (Willets et al., 2003). In addition, catalytically inactive GRK mutants impaired phosphorylation and internalisation of m2 mAChR in COS 7 cells but had no effect in BHK-1 or HEK 293 cells (Tsuga et al., 1994, Pals-Rylaarsdam et al., 1995). It was subsequently found that varied levels of GRK2 existed in the different cell types therefore the effectiveness of the dominant negative may be dependent on levels of GRK expressed in the cell line (Aramori et al., 1997).

A study comparing the effectiveness of ß-arrestin dominant negative V53D and 319-418, found that the V53D mutant was only modestly effective at inhibiting $ß_2$AR internalisation in COS-1 cells since it still has some ability to bind to phosphorylated GPCRs (Krupnick et al., 1997). The 319-418 mutant, on the other hand, was more effective since it completely lacked the ability to bind to receptors but still had a high affinity to clathrin. If there were small differences in the $Erk_{1/2}$ response to ACTH using the V53D mutant, an immunoblot may not have been able to demonstrate this effectively due to a lack of sensitivity. Future studies to investigate the role of these mechanisms further could make use of RNAi to knock down the relevant proteins and again use the phospho $Erk_{1/2}$ immunoblot to determine the effect. Successful RNAi knockdowns within the H295R cell line have since been carried out in our laboratory for other genes using stable expression systems.

Finally, a requirement for endocytosis in the $Erk_{1/2}$ response to ACTH may be a mechanism through which signal specificity is conferred. This model fits with the suggestion that multiple mechanisms exist to spatially regulate the MAPK signalling pathways (Murphy and Blenis, 2006).

FINAL DISCUSSION

In summary, this thesis has investigated, in a cell line not previously used for such studies, the potential mitogenic signalling mechanisms arising from the action of ACTH on adrenal cells. The H295R model has been shown to express a functional MC2R, expressed at low levels, evidenced by a weak but nonetheless significant cAMP response to ACTH. Furthermore, it has shown a strong and reproducible $Erk_{1/2}$ activation in a manner similar to that shown in certain Y1 cell strains. This cell line however, although originating from a carcinoma, does not contain the mutant Ras oncogene present in Y1 cells and thus enables a more effective look at the $Erk_{1/2}$ pathway. In addition, because there is only a weak cAMP response, this has allowed us to dissect out the $Erk_{1/2}$ pathway without the interference of a robust cAMP/PKA response.

Using the H295R model we have established that $Erk_{1/2}$ activation is transient, peaking at 5 minutes and returning to basal levels by around 15 minutes. Studies as to the mitogenic effect of this transient response have so far produced conflicting results. Although short treatments of ACTH have shown an increase in Elk-1 transcriptional activity as measured by luciferase activity, as yet a proliferative response has yet to be shown in terms of increasing cell number, although there did appear to be a dose dependent effect of ACTH in the presence of serum. These studies however, were hampered because H295R cells have a low rate of division and could not be maintained in SFM for periods needed to register a measurable response. An alternative method would be to use 2% serum in the presence of ACTH in order to maintain basal metabolism of the cells. In addition, other markers of proliferation such as BrdU labelling could be used as an earlier marker. There has been no recent advancement in this field in terms of establishing whether or not ACTH is involved in maintenance of growth of the adrenal. Clinical and animal evidence is highly suggestive of a mitogenic role for ACTH, however, it is likely to be a complex process involving the interplay between a number of factors and signalling pathways.

Whilst a proliferative role for ACTH has not been established in these studies, an alternative role is perhaps the requirement of $Erk_{1/2}$ activation for steroidogenesis. The main controlling step in steroid production is thought to be the cAMP-dependent expression of the steroidogenic acute regulatory (StAR) protein, a mitochondrial protein essential for cholesterol transport to the inner mitochondrial membrane where it is converted to pregnenolone by the cytochrome P450 side chain cleavage complex (Stocco, 2000). Steroidogenic factor 1 (SF-1) is an orphan member of the nuclear receptor family and has been shown to contribute to the regulation of steroid synthesis by activating a variety of genes involved in steroid biosythesis (Lala et al., 1992). One study demonstrated that cAMP induced steroid synthesis is dependent on the phosphorylation of $Erk_{1/2}$ and that $Erk_{1/2}$ activation resulted in enhanced phosphorylation of SF-1 and a concomitant increase in steroid production through increased transcription of the StAR gene (Gyles et al., 2001).

Having established the presence of an $Erk_{1/2}$ response to ACTH, our attention focused on the mechanisms by which this signal is activated. In general, investigating signalling mechanisms involving the MC2R has been hampered due to a lack of an effective antibody to it and the difficulty in expressing functional receptor in non adrenal cell lines. Despite this, using the endogenously expressed MC2R in H295R cells, we have been able to investigate some of the mechanisms by which ACTH induces an $Erk_{1/2}$ response. It appears that cAMP signalling may provide a partial explanation for the increase in $Erk_{1/2}$ activity probably through the activation of PKA, however this does not account for the entire response. Thus it seems likely that another less well-recognised $Erk_{1/2}$ signalling pathway, possibly dependent on an internalisation complex, is involved. That MDC reduces the $Erk_{1/2}$ response significantly (and the cell surface expression of MC2R) but had no effect on serum induced $Erk_{1/2}$ activation implies the requirement for internalisation of the MC2R. This, however, has not been confirmed by the expression of dominant negative dynamin I. Furthermore, use of GRK2 and ß-arrestin dominant negatives also showed a lack of involvement of GRK and ß-arrestin which often accompany such mechanisms of $Erk_{1/2}$ activation. However, this may be consistent with recent

studies demonstrating arrestin-independent but clathrin-dependent mechanisms of GPCR internalisation (Wolfe and Trejo, 2007).

Consistent with these atypical features of the internalisation of the MC2R is the recent study from our laboratory that the MC2R within the H295R cell interacts with nucleoporin 50, one of a family of nuclear transport proteins, and translocates to the nucleus with a time course similar to that of receptor internalisation (Doufexis et al., 2007). The study of intracellular trafficking of the MC2R may therefore present a future avenue of investigation to fully understand the signalling pathways elicited by this receptor.

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