Bird, Rebecca Jane (2010) *Novel areas of crosstalk between the cyclic AMP and PKC signalling pathways.* PhD thesis.

Novel areas of crosstalk between the cyclic AMP and PKC signalling pathways

Rebecca Jane Bird, M.Sci

Submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy

Division of Molecular and Cellular Biology
Faculty of Biomedical and Life Sciences
University of Glasgow

September 2010

DECLARATION

I hereby declare that the thesis, which follows, is my own composition, that it is a record of the work done by myself and that it has not been presented in any previous application for a higher degree.

Rebecca Jane Bird

ABSTRACT

Mediation of biological functions occurs via tightly regulated signal transduction pathways. These complex cascades often employ crosstalk with other signalling pathways to exert strict control to allow for correct cellular responses. The cyclic AMP signalling pathway is involved in a wide range of cellular processes which require tight control, including cell proliferation and differentiation, metabolism and inflammation. Protein Kinase C (PKC) signalling is also involved in the regulation of many biological functions, due to the wide range of PKC isoforms, and there is emerging evidence that there are critical points of crosstalk between these two central signalling pathways. The aims of this research, therefore, are to identify the molecular basis underlying this pivotal cross-communication.

The identification of the complex formed by Receptor for activated C Kinase 1 (RACK1), a scaffold protein for PKC, and the cyclic AMP-specific phosphodiesterase PDE4D5 demonstrated a potential area of crosstalk between the cyclic AMP and PKC signalling pathways although the function of the complex remained largely unknown. In this thesis I have outlined a role for RACK1 binding to PDE4D5 to control the enzymatic activity of the phosphodiesterase. Although RACK1 does not affect the intracellular localisation of PDE4D5, it does afford structural stability to PDE4D5, providing protection against denaturation. Furthermore, interaction with RACK1 facilitates high affinity binding of PDE4D5 to cyclic AMP and increases phosphodiesterase sensitivity to inhibition by rolipram, a PDE4-specific inhibitor that is a therapeutic treatment for depression and Alzheimer's disease. Additionally, RACK1-bound PDE4D5 was found to be activated by PKCα, providing a route of negative regulation by PKC on cyclic AMP in HEK293 cells.

The discovery of EPAC (Exchange Protein directly Activated by Cyclic AMP) has opened up the field of cyclic AMP research, providing an alternative route for the cyclic AMP signalling originally thought to occur solely through Protein Kinase A (PKA). Recent investigations have linked cyclic AMP signalling via EPAC to the control of inflammation, through the induction of Suppressor of Cytokine Signalling 3 (SOCS-3) to inhibit IL-6 signalling. Here I have further delineated this pathway in COS1 to show that induction of SOCS-3 by EPAC requires phospholipase C (PLC) ε. Investigation into downstream effectors of PLC action lead to the identification of PKCα and PKCδ as essential components of this pathway, further elucidating a mechanism by which cyclic AMP can

affect inflammation and revealing a point of crosstalk between the two signalling pathways.

Further elaborating on the identification of PKC isoforms α and δ as crucial components in the control of cytokine signalling by cyclic AMP via EPAC, investigations into the effect of cyclic AMP on PKC α and δ activation and autophosphorylation, and on downstream effectors, were carried out. It was revealed that cyclic AMP had no influence on PKCδ activity, although a role for cyclic AMP signalling through EPAC on the activation and autophosphorylation of PKCα was identified. Additionally, phosphorylation of the downstream kinase ERK was found to occur independently of PKC activation and required the presence of EPAC1 in COS1 cells.

The work presented in this thesis therefore begins to delineate a novel pathway in which the cyclic AMP and PKC pathways work together to afford cell regulation, including the regulation of gene expression, through novel areas of crosstalk.

ACKNOWLEDGEMENTS

I would like to gratefully acknowledge the guidance and support provided by my supervisor, Dr. Stephen Yarwood.

Further thanks to everyone in Lab 241, particularly Dr. Gillian Borland for her help and encouragement.

Finally, I would like to express my gratitude for the continual love and support of my parents, Geoff and Christine, and that of my friends and family, particularly Rebecca Keenan. Without these people, my whanau, this thesis would not have been possible. Kia ora.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 4

CHAPTER 5

CHAPTER 6

LIST OF ABBREVIATIONS

8Me	8-(4-chlorophenylthio)-2'-O-methyladenosine-3', 5'-cyclic monophosphate (8-pCPT-2'-O-Me-cyclic AMP)
AC	Adenylyl Cyclase
AKAP	A Kinase Anchoring Protein
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
cAMP	cyclic 3'5' Adenosine Monophosphate
CBD	cAMP Binding Domain
C/EBP	CCAAT/enhancer binding protein
CNG	Cyclic Nucleotide-Gated
COS	CV-1 (simian) in Origin, carrying the SV40 genetic material
CREB	cAMP Response Element Binding Protein
DEP	Dishevelled, Egl-10, Pleckstrin
dH2O	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Diaminoethanetetra-acetic acid
EPAC	Exchange Protein directly Activated by cAMP
ERK	Extracellular signal-Regulated Kinase/MAPK
FBS	Foetal Bovine Serum
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GEF	Guanine nucleotide Exchange Factor
GPCR	G-Protein Coupled Receptor
G-protein	Guanine nucleotide binding regulatory protein
HEK	Human Embryonic Kidney
HUVEC	Human Umbilical Vein Endothelial Cell
IC50	Concentration of inhibitor required to inhibit half the specific activity

Ig	Immunoglobulin
IP	Immunoprecipitate
JAK	Janus Kinase
kDa	Kilodalton
MAPK	Mitogen Activated Protein Kinase/ERK
MAPKK	MAPK Kinase/MEK
MAPKKK	MAPK Kinase Kinase
MEF	Mouse Embryonic Fibroblast
MEK	MAPK Kinase/ERK Kinase
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PDE	Phosphodiesterase
PDE4	cyclic AMP specific phosphodiesterase
PKA	Protein Kinase A
PKB	Protein Kinase B/Akt
PKC	Protein Kinase C
PLC	Phospholipase C
PMA	Phorbol 2-Myristate 3-Acetate
PP2A	Protein Phosphatase 2A
Ra	Ras Association Domain
RACK1	Receptor for Activated C Kinase
REM	Ras Exchange Motif
RNA	Ribonucleic Acid
Rolipram	4-[3-(cyclopentoxyl)-4-methoxyphenyl]-2-pyrrolidinone
SDS	Sodium Dodecyl Sulphate
SEM	standard error of mean
SH domain	Src Homology domain
siRNA	Small Interfering RNA
SOCS-3	Suppressor of Cytokine Signalling 3
STAT	Signal Transducers and Activators of Transcription
TBS	Tris Buffered Saline
TBST	TBS plus Tween 20
Tris	Tris (hydroxymethyl) aminomethane
Tween 20	Polyoxyethylene sorbitan monolaurate
UCR	Upstream Conserved Region

VSV	Vesicular Stomatitis Virus
WD-repeat	protein sequence motif containing repetitive sequences bounded by tryptophan and aspartic acid residues
WT	Wild type

Units

g	gram
l	litre
M	molar
mA	milliamp
mg	milligram
min	minute
ml	millilitre
mM	millimolar
nM	nanomolar
pM	picomolar
sec	second
µCi	microcurie
µg	microgram
µl	microlitre
µM	micromolar
^{o}C	degrees Celsius

CHAPTER 1

Introduction

1.1 Signal Transduction

The mediation of biological functions requires the use of first messengers, such as neurotransmitters, cytokines, hormones and growth factors, many of which are unable to permeate the cell membrane. To carry out their specific functions, they require the induction of signal transduction cascades, wherein a cell surface receptor is activated, eliciting an intracellular response. A second messenger is often employed in the conversion of the extra-cellular biological system to the intracellular response. The first point of contact for the extracellular stimuli is the wide range of cell surface receptors. The nature of signal transduction, and the affected pathways, is determined by which of these receptors are activated by the extracellular stimuli. These membrane associated receptors can be divided into three groups; ion-channel linked, enzyme-linked and G-protein linked (Berg *et al.*, 2002).

1.1.1 Ion-channel Linked Receptors

Ion channel linked receptors, such as the nicotinic acetylcholine receptor, are stimulated to open or close in response to external stimuli, particularly neurotransmitters or peptide hormones. A conformational change upon ligand binding creates an open channel, allowing molecules, generally ions specific to the receptor, to pass through. In the case of the nicotinic acetylcholine receptor, this is Na^+ ions (Alberts *et al.,* 2002). When the internal concentration of the ion is high enough, it causes a depolarisation of the membrane and initiates an action potential. Such receptors are common in neurons and other cells which require action potentials (Berg *et al.,*2002).

1.1.2 Enzyme-linked Receptors

Enzyme-linked receptors are activated by a wide range of hormones and growth factors and are important in signalling pathways that lead to cell growth, differentiation and proliferation. The cytosolic domains of these receptors are either associated with specific enzymes or possess its own enzymatic activity, giving this group of receptors their name (Berg *et al.,* 2002). There are four well documented classes of enzyme linked receptors found in mammalian systems: guanylyl cyclase receptors, serine/threonine kinase receptors, receptor tyrosine kinases and tyrosine kinase associated receptors.

Several signalling molecules, including natriuretic peptides, stimulate guanylyl cyclases. This stimulation catalyses the production of cyclic GMP, initiating a signalling pathway by activation of the serine/threonine kinase, cyclic GMP-dependent protein kinase (PKG) which is involved in neuronal function, smooth muscle relaxation and kidney and intestine ion flux (Berg *et al.,* 2002).

The serine/threonine kinase receptors are stimulated by the transforming growth factor β super-family which as well as having involvement in cell proliferation, differentiation and apoptosis, are involved in immune regulation and tissue repair. There are two structurally similar types of serine/threonine kinase receptors, designated type I and type II which work together to activate a signalling pathway. Upon ligand binding to a type II homodimer, a type I homodimer is activated and recruited to form a tetrameric complex. This complex then directly phosphorylates the gene regulating family of proteins called Smad, which translocates directly to the nucleus to activate its target genes (Souchelnytskyi *et al.,* 2001).

Receptor tyrosine kinases (RTKs) comprise the largest group of enzyme linked receptors and are stimulated by a wide range of growth factors and hormones including EGF (Epidermal Growth Factor), PDGF (Platelet derived growth factor) and VEGF (Vascular endothelial growth factor), as well as some cell surface bound signalling proteins such as ephrins that are involved in cell adhesion and migration (Gschwind *et al.,* 2004). Upon stimulation, RTKs are able to phosphorylate target proteins and have been indicated in a number of well studied pathways including MAPK cascades (detailed in section 1.5). Finally, tyrosine kinase associated receptors act via associated cytoplasmic tyrosine kinases, such as the Src family of proteins, which phosphorylate both target proteins and the receptors themselves (Hubbard and Till, 2000).

The cytokine receptors demonstrate one of the most direct routes of external signal transduction to the nucleus, through activation of the Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signalling pathway. JAKs are a group of cytoplasmic tyrosine kinases which associate with cytokine receptors. Once activated by the receptor, JAKs phosphorylate the gene regulatory STAT proteins, activating them and causing them to translocate to the nucleus where they induce transcription of target genes (Rawlings *et al.,* 2004). As with other enzyme-linked receptor pathways, this pathway is involved in cell proliferation, differentiation and apoptosis, and is also important in haematopoiesis (Rawlings *et al.,* 2004).

1.1.3 G-protein Coupled Receptors (GPCRs)

G-protein coupled receptors play a crucial role in cell signal transduction, mediating the majority of responses to neurotransmitters, hormones and external stimuli (Rosenbaum *et al.,* 2009). They comprise a large super-family of transmembrane receptor proteins which bind these ligands to transduce signals from outside the cell into an intracellular response. Binding of the ligand induces a conformational change, allowing the GPCR to act as a guanine exchange factor (GEF). It is then able to activate an associated G-protein through the exchange of GTP for its bound GDP. The subunits of the G protein are then able to regulate enzymatic effectors which in turn generate second messengers such as calcium and cyclic AMP (Rosenbaum *et al.,* 2009) (Figure 1.1). Over 800 GPCRs has been identified in the human genome, with around 460 of these believed to act as olfactory receptors and many more with as yet unknown functions (Deupi and Kobilka 2007). These 800 GPCRs are divided into 5 classes based on function and sequence homology. Together, these groups are known as the GRAFS (*g*lutamate, *r*hodopsin, *a*dhesion, *f*rizzled/taste, *s*ecretin) classification. Almost 90% of the known GPCRs fall into the Rhodopsin-like family, while the rest are divided between adhesion, glutamate, secretin and frizzled/taste groups. The Rhodopsin group is further divided into 4 subgroups, designated α, δ, γ and δ (Huang *et al.,* 2009). The α group includes opsins, melatonins and prostaglandins; β comprises peptide hormones; γ, chemokine receptors and somatostatins and opoids; and δ forms a group of purines, glycoproteins and the large number of olfactory receptors. The glutamate group includes calcium sensing receptors, $GABA_B$, and the Taste 1 cluster of receptors, while the Adhesion group is made up of GPCRs which are fused with an adhesion-like motif containing domains at their N-terminal (Bjarnadottir *et al.,* 2005). The Frizzled/Taste group contains, as the name suggests, the frizzled, and Taste 2 receptors, and the secretin group contains not only secretin receptors, but other large peptide receptors including those for calcitonin and glucagon (Deupi and Kobilka, 2007).

Currently, crystallography is only available for three GPCRs- Rhodopsin, Opsin and β2-Adrenergic receptor (Blois and Bowie, 2009). However, all GPCRs are believed to share a structural similarity of seven hydrophobic membrane spanning α-helical domains, connected by alternating intracellular and extracellular loops, an intracellular carboxy (C)-terminal end and an extracellular amino (N)-terminal end. Although homology is highly conserved between GPCRs with regard to the transmembrane domains, there is great variation of the intra- and extracellular areas with regard to complexity and size. The extracellular areas are involved in ligand binding, while intracellular regions are required

Figure 1.1
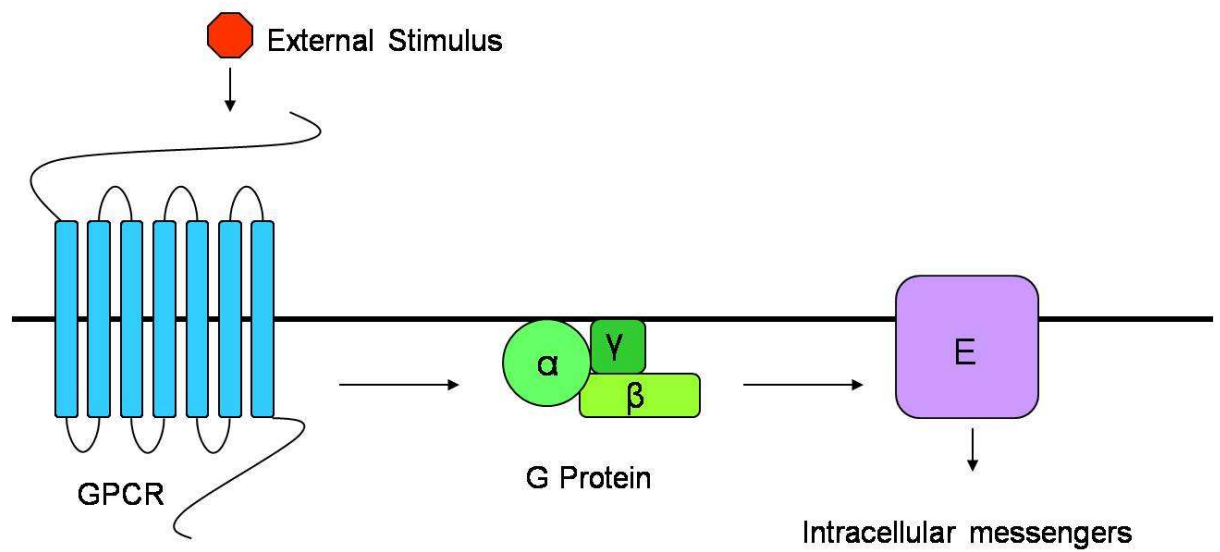
Figure 1.1 GPCR mediated signalling - Upon binding of an external stimulus to the outside of the transmembrane GPCR, a conformational change occurs to allow the GPCR to act as a GEF for an associated heterotrimeric G protein. Following this activation, the Gα subunit dissociates from the Gβγ subunits and acts on effector enzymes which generate second messengers such as cyclic AMP and calcium.

for signal transduction. The N-terminal and, in some GPCRs, extracellular loops, contain *N*-glycosylation sites and disulphide bridges between the extracellular loops to maintain correct protein folding and are conserved across the majority of the GPCRs. Conserved across the GPCRs in the C-terminal is a site for palmitoylation (Luttrell, 2008).

GPCRs may activate more than one G protein, of which there are few in number in mammalian genomes compared with the number of GPCRs encoded. G proteins comprise a heterotrimer consisting of the subunits α, β and γ. Structurally, Gα subunits share a homology consisting of an α-helical domain which inserts into its Ras-like GTP-GDP binding domain (Bridges and Lindsley, 2008). 12 Gγ and 5 Gβ subunit genes have been identified, and the majority of these form stable heterodimers which are, like the Gα subunit, able to regulate effector activity. The Gβ subunits share a seven bladed propeller structure made up of β-sheets, and a coiled-coil structure at the N-terminal that associates with the Gγ subunit. The Gγ subunits contain a Cys-Ala-Ala-x motif at the C-terminal of its α-helical structure which allows for membrane localisation by prenylation (Deupi and Kobilka, 2007). There are currently 16 identified Gα subunit genes, with splice variants giving rise to around 20 Gα subunit proteins, which are divided into 4 groups. These groups are $G\alpha_s$, which include the adenylyl cyclase stimulatory subunit $G\alpha_s$, and the olfactory subunit, $G\alpha_{olf}$; $G\alpha_i$ which comprises transducin, the retinal subunit, and gustducin, involved in taste, as well as $G\alpha_i$ 1, 2 and 3, which are adenylyl cyclase inhibitory subunits; $G\alpha_q$ which consists of the regulators of phospholipase C activity $G\alpha_q$ and Gα11, as well as Gα14 and Gα15; and finally Gα12 includes Gα12 and Gα13 which regulate cytoskeletal assembly and cell growth via RhoGEFs (Luttrell, 2008). As can be seen from the stimulatory and inhibitory functions of the $G\alpha_s$ and $G\alpha_i$ subunits, the control of adenylyl cyclase by G-proteins is an important component of signal transduction. Since the main thrust of the research in this study is centred around new mechanisms of cyclic AMP signal transduction, the next section will concentrate on cyclic AMP signal transduction.

1.2 Cyclic AMP Signal Transduction

The prototypical second messenger, 3'-5'-cyclic adenosine monophosphate, or cyclic AMP (cyclic AMP) (Figure 1.2) was first identified in the 1950s as a small intracellular mediator. Normal concentrations of cyclic AMP within cells are usually in the region of

Figure 1.2
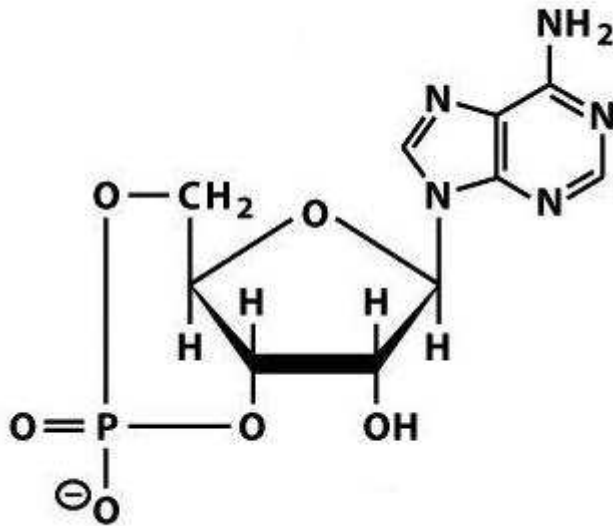
Figure 1.2 Structural representation of cyclic AMP - The second messenger 3'-5'cyclic adenosine monophosphate (cyclic AMP).

10^{-7}M (Cooper, 2003). However, extracellular signals, such as hormones that activate Gs-coupled GPCR, are able to increase levels by more than twenty fold through activation of a family of adenylyl cyclase enzymes (Cooper, 2003). Cyclic AMP is essential for a range of critical cellular processes, including the activation of protein kinases and the regulation of Ca^{2+} passage through ion channels (Cheng *et al.,* 2008). Classically, rises in intracellular cyclic AMP levels were thought to elicit cellular responses mainly through the activation of cyclic AMP-dependent Protein Kinase A (PKA) (Conti *et al.,* 2003), however it is now known that cyclic AMP can exert actions in the cell through the activation of other, recently discovered, cyclic AMP effectors, such as cyclic nucleotide gated (CNG) ion channels and exchange protein activated by cyclic AMP (EPAC) (Figure 1.3).

It is widely accepted that cyclic AMP is compartmentalised, with fluorescence-resonance-energy-transfer (FRET) investigations showing both temporal and spatial cyclic AMP gradients within cells (Houslay and Adams, 2003). This compartmentalisation is thought to be due to a number of factors, including the actions of GPCRs, adenylyl cyclase, and PKA. Cyclic nucleotide phosphodiesterases (PDEs), which hydrolyze cyclic AMP to 5'-AMP and are the only means of degradation of cyclic AMP, are thought to play an important role in cyclic AMP compartmentalisation and formation of cyclic AMP gradients throughout the cell (Houslay and Adams, 2003). Compartmentalisation allows differential activation of PKA pools in different locations where they are able to phosphorylate only the target proteins in that area. A-kinase Anchor Proteins (AKAPs) anchor PKA isoforms at specific sites within the cell, allowing a response of the spatially distinct PKA pools to cyclic AMP gradients, resulting in modification of localised target proteins (Baillie and Houslay, 2005). AKAPs target PKA to distinct subcellular locations and to specific substrates with activation status of PKA dependent on localised cyclic AMP concentrations. Regulation of Ca^{2+} channels also occurs via these localised PKA-AKAP complexes. Differing responses and activations from compartmentalised cyclic AMP level changes are probably linked to the stimulation of varying receptors resulting in different physiological responses. Here we will try to cast light on this complexity by summarising the major components of the cyclic AMP signal transduction cascade.

1.2.1 Adenylyl Cyclases

Cyclic AMP is generated by adenylyl cyclases (ACs), large transmembrane proteins ranging between 120 and 140 kDa (Cooper 2003), which catalyse the conversion of ATP

Figure 1.3
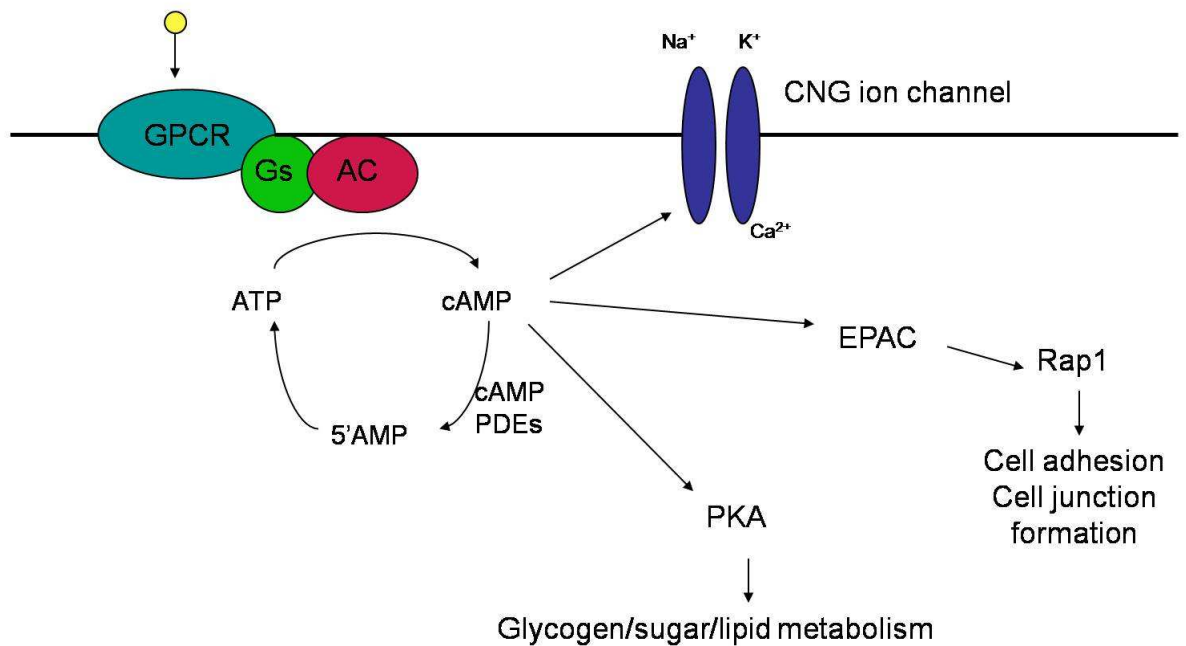
Figure 1.3 Cyclic AMP Signalling - Following GPCR mediated activation of adenylyl cyclase, cyclic AMP is synthesised by the enzyme from ATP, and is hydrolysed to 5'AMP by cyclic AMP specific phosphodiesterases (PDEs). Cyclic AMP levels control signalling through three main pathways- via cyclic nucleotide gated (CNG) ion channels to control the flux of ions, via EPAC (exchange protein directly activated by cyclic AMP) and Rap1 where cyclic AMP can control cell adhesion and junction formation, and via PKA where it influences the metabolism of glycogen, sugar and lipids, among other actions.

to cyclic AMP and phosphate. Mammalian systems contain ten ubiquitously expressed ACs, nine of which are membrane bound and one which is located in the cytosol (Luttrell, 2008). Each isoform possesses a differing tissue distribution and regulation requirement, allowing increases of cyclic AMP levels that are both specifically localised and occurring under specific conditions (Tabakoff et $al.,$ 2001). Structurally, ACs consist of a short, variable intracellular N-terminal region, two six-membrane-spanning cassettes termed M1 and M2, two 40 kDa cytosolic domains designated C1 and C2 which form the catalytic site, and an intracellular C-terminal. The catalytic site of ACs is formed between the C1 and C2 domains upon binding of $G\alpha_s$ or the drug forskolin to the outside of the C2 domain and the N-terminal of the C1 domain, causing a conformational change (Figure 1.4). Inhibition by G proteins can occur when $G\alpha_i$ binds to the C1 domain, preventing association of the two domains. This conformational change also forms a binding site at the opposing ends of the C1 and C2 domains. ATP binds to this interface where it is then catalysed into cyclic AMP (Cooper, 2003). The active site is highly conserved across the AC isoforms, which also share a high sequence and structural homology with the generators of cyclic GMP, the guanylyl cyclases which are discussed above. As well as activation by G proteins and forskolin, some AC isoforms are regulated by calcium, either in a calmodulin-dependent or -independent manner. Because of the importance of ACs in controlling cyclic AMP levels within the cell, they have become important targets for therapeutic treatment, for example forskolin analogues are successfully used in Japan for the treatment of asthma (Pierre et $al.,$ 2009).

1.2.2 Protein Kinase A (PKA)

Originally it was believed that all cyclic AMP signalling occurred via PKA. PKA is involved in the regulation of a wide range of physiological processes, ranging from metabolism to growth and memory (Cheng et $al.,$ 2008). There are two forms of PKA, PKAI and PKAII, which are determined by the presence of specific regulatory (R) subunits. Inactivated, PKA is a tetrameric complex of two R subunits and 2 catalytic subunits. While PKAI is found mainly in the cytoplasm of the cell, PKAII is located at organelles and cellular structures (Perkins et $al.,$ 2001). Unlike PKAI, Type II is generally not a soluble species, but is instead anchored within the cell by AKAPs at specific locations (Feliciello et $al.,$ 2001). There are four known PKA R subunits, two of which are generally expressed in a variety of tissues, and two that are found almost exclusively in the brain, adipose and adrenal tissues (Cheng et $al.,$ 2008). The three types of catalytic (C)

Figure 1.4
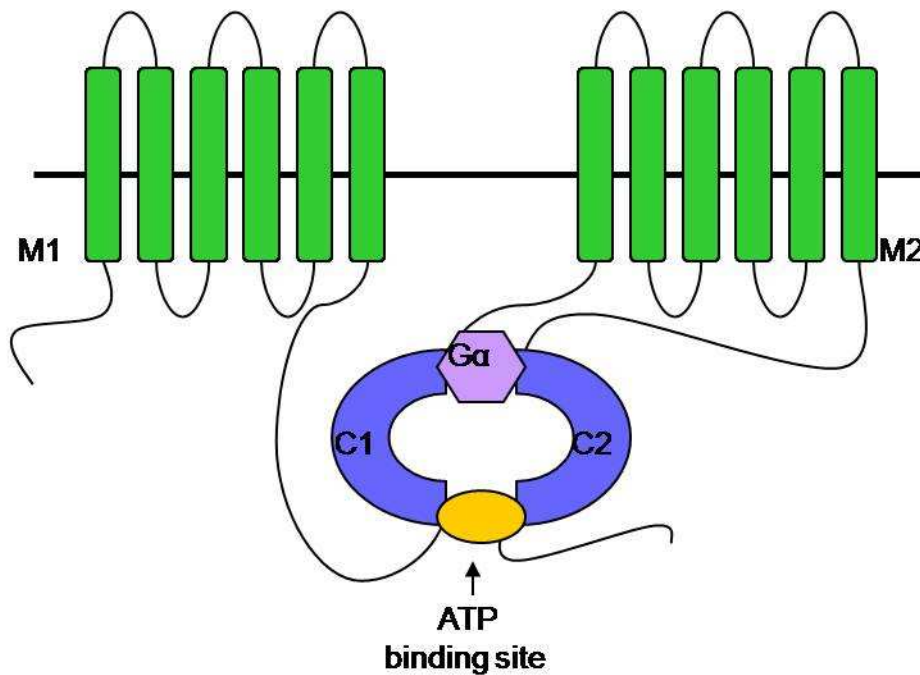
Figure 1.4 The structure of adenylyl cyclase - The adenylyl cyclase family of enzymes share a structure of two cassettes of six transmembrane spanning domains, termed M1 and M2 and two ~40 kDa cytoplasmic domains, C1 and C2. These cytoplasmic domains form a catalytic site at the C-terminal end of C1 and the N-terminal of C2, where Gα or forskolin bind. This binding causes a conformational change which creates an ATP binding site at the opposing ends of the cytoplasmic domains where cyclic AMP synthesis occurs.

subunits, α, β and γ, appear to have a broad tissue specificity, with C-α the most commonly found subunit of PKA (Dodge-Kafka and Kapiloff, 2006). Upon cyclic AMP binding to both sites on the R subunit, a conformational change occurs, lowering its affinity for the C subunits (Cheng *et al.,* 2008). The complex then dissociates, and the enzyme becomes active, allowing the C subunits to target a wide range of proteins at their PKA consensus phosphorylation sites (Chang *et al.,* 1993). These include ion channels, the GTPase Rho and transcription factors such as CREB (Cheng *et al.,* 2008). Cyclic AMP is also able to affect ERK activation (as discussed in section 1.5.4) through the inhibition of the protein kinase Raf1 by PKA phosphorylation (Houslay and Baillie, 2003) which is thought to play a role in inhibiting cell cycle progression in cells in which cyclic AMP is anti-mitogenic.

1.2.3 A-Kinase Anchor Proteins (AKAPs)

Because of its wide range of substrates, PKA signalling specificity is obtained through use of AKAPs, which anchor PKA at distinct subcellular locations to facilitate interaction with specific groups of substrates. In this way, AKAPs are able to control aspects of PKA signalling. There are more than 50 members of the AKAP family (McConnachie *et al.,* 2006), all structurally diverse but identical in three aspects- their ability to bind PKA through a PKA anchoring domain, their ability to form a multi-protein complex through binding other signalling enzymes, and the ability to target these complexes to specific sites within the cell (Beene and Scott, 2007). PKA anchoring occurs via one of its R subunits undergoing a hydrophobic interaction with an α-helical region comprised of between 14-18 amino acids, conserved across the AKAP family (McConnachie *et al.,* 2006). The N-terminal of the R subunit binds to this amphipathic region on the AKAP (Dodge-Kafka *et al.,* 2008). While the RII subunit of PKA exhibits a higher binding affinity for AKAP than RI (Dodge-Kafka *et al.,* 2006), some AKAPs show a preference for binding to the RI subunit, while others are dual specific and readily bind either subunit (Wong and Scott, 2004). Naming of AKAPs was originally based on the molecular weight of each protein based on where it rested when run on an SDS-PAGE gel (Wong and Scott, 2004) but due to the great and constantly increasing number of AKAP family members and splice variants sharing similar molecular weights as already identified AKAPs, proteins previously identified for other purposes now shown to act as AKAPs retain their original name, such as Gravin or Pericentrin (Smith *et al.,* 2006). The first AKAP to be discovered was MAP2 (Microtubule associated protein 2) (Wong and Scott, 2004), initially identified through its ability to co-purify with PKA, a means of identification still used today to identify other AKAPs (Smith *et al.,* 2006).

As well as binding and targeting PKA, one of the most important, defining actions of AKAPs is the ability to form multi-protein complexes. These complexes can include both enzymes involved in signal transduction, as well as enzymes involved in signal termination (McConnachie *et al.,* 2006) allowing both phosphorylation and dephosphorylation of a substrate, and both up- and down-regulation of pathways to occur through one AKAP multi-protein complex (Smith *et al.,* 2006). This also gives rise to the ability of crosstalk between pathways to occur via AKAPs. The first AKAP identified to bind another protein alongside PKA was AKAP 150/79, shown to bind not only PKA but also the phosphatase protein 2B (PP2B) (Smith *et al.,* 2006). Later, this same AKAP was identified to also bind Protein Kinase C (PKC) alongside these two proteins, acting as a multi-protein signalling complex (Smith *et al.,* 2006). Several other AKAPs bind PKC alongside PKA, including Pericentrin, AKAPLb2, which binds PKA, PKC and PKD with PKA and PKC activating PKD, and Gravin which binds PKA and PKC alongside the β2-adrenergic receptor (Wong and Scott, 2004). Additionally, AKAP350/450 binds PKA and PKC along with phosphatase proteins 1 and 2A and also the phosphodiesterase PDE4D3 (Smith *et al.,* 2006). Other AKAPs also bind components of cyclic AMP signalling, such as AKAP110 which binds PDE4A, and mAKAP which binds both PKA and PDE4D3, as well as PP2A, ERK5 and EPAC1 (McConnachie *et al.,* 2006).

The crucial role of AKAPs is the targeting of these multi-protein complexes to distinct subcellular locations, with the use of targeting motifs on the AKAP involved in protein-protein and protein-lipid interactions (Smith *et al.,* 2006). Ensuring signalling components are anchored in distinct sites within the cell allows greater control and specificity of signalling. It is important to note that splice variants of the same AKAP can target to completely different areas within the cell, while more than one AKAP may be targeted to a specific location (Wong and Scott, 2004). Of the complexes listed above, each displays a distinct and specific location of anchoring, with AKAP150/79 targeting to the plasma membrane, while AKAP350/450 anchors to the centrosomes and Golgi apparatus of a variety of cell types and Gravin targets the actin cytoskeleton (McConnachie *et al.,* 2006). mAKAP targets its complex components to the nuclear membrane of myocytes, brain and skeletal muscle cells, and demonstrates a negative feedback loop for control of cyclic AMP signalling, wherein several components including the downstream effectors PKA and EPAC 1 acting alongside a phosphodiesterase responsible for reducing cyclic AMP concentration (McConnachie *et al.,* 2006). This ability of AKAPs to bring signalling

components together at distinct sites within the cell not only provides a useful method of signal transduction specificity control through compartmentalisation, but also allows signalling pathways to converge.

1.2.4 Cyclic nucleotide-gated (CNG) Ion Channels

CNG ion channels are non-selective cation channels that are members of the voltage activated ion channel super-family, despite displaying a low voltage dependence (Kaupp and Seifert, 2002). CNG channels were first identified in retinal rod and cone photoreceptors. Cyclic AMP-dependent CNG ion channels are most commonly located in olfactory sensory neurons (OSN), although recently they have been identified in other non-neural tissues, including heart and liver (Kaupp and Seifert, 2002). These heterotetrameric complexes contain two or three different subunits and are made up of six segments which span the membrane, a pore region located between two of the segments, and a C-terminal cyclic nucleotide binding domain, to which cyclic AMP is able to bind directly (Schulte and Levy, 2007). It is these differing subunits which determine the functions and features of each channel, including ligand sensitivity. Upon cyclic AMP binding to the C-terminal domain, conformational changes to the CNG structure occur, resulting in the pore area opening and allowing the passage of K^+, Na^+ and Ca^{2+} ions. In particular, Ca^{2+} is important in the adaptation and excitation of sensory cells. CNG ion channel activity is also modified through both phosphorylation and Ca^{2+}/calmodulin action (Kaupp and Seifert, 2002).

1.2.5 Exchange protein directly activated by cyclic AMP (EPAC)

In 1998, de Rooij et al. reported that activation of Rap1 via cyclic AMP and Forskolin was independent of PKA. EPAC was then identified in a database search for proteins that contained sequence homology to cyclic AMP binding sites as well as to Ras and Rap1 GEFs. Radiolabelled cyclic AMP experiments were then used to determine that cyclic AMP binds directly to EPAC. The study also showed that EPAC and the PKA regulatory subunit Iα bind to cyclic AMP with a similar affinity. EPAC was then confirmed as a GEF for Rap1 through co-transfection experimentation in NIH3T3 cells with fluorescent labelled Rap1 used to show that it is directly activated by EPAC (de Rooij et al., 1998). Simultaneously, both isoforms were discovered during a screen for genes exhibiting second messenger binding motifs specific to the brain (Kawasaki et al., 1998). There are two EPAC isoforms, 1 and 2, encoded by distinct genes. EPAC1 is expressed abundantly throughout all cell types, particularly in the kidney, heart and thyroid glands (Roscioni et

al., 2008), while EPAC2 is mostly found within the brain, adrenal and pituitary glands (Kawasaki *et al.,* 1998). Identification of these new cyclic AMP receptors showed a complexity of cyclic AMP mediated signalling greater than previously thought, and raised the possibility that there may be a role for EPAC in some of the effects of cyclic AMP believed to act solely through PKA. The ubiquitous expression of both PKA and EPAC in all tissues allows for a greater precision of control of cyclic AMP signalling pathways, as a rise in intracellular cyclic AMP is able to activate PKA, EPAC as well as other cyclic AMP effectors present. This allows cyclic AMP to affect multiple pathways (Cheng *et al.,* 2008). Tight regulation of EPAC localisation to plasma and nuclear membrane, perinuclear regions and mitochondria, as well as subcellular distribution suggests cellular compartmentalisation is important for the wide range of signalling properties of EPAC (Roscioni *et al.,* 2008).

The discovery of EPAC proteins revolutionised the known cyclic AMP signalling pathway, opening up the field because of the wide range of downstream effects of EPAC, including extracellular signal related kinase (ERK), phospholipase C (PLC) and protein kinase B (PKB)/Akt (Roscioni *et al.,* 2008). This multi-domain family of proteins have a high affinity for cyclic AMP binding and also are able to activate the small GTPases Rap1 and Rap2, members of the Ras super-family (de Rooij *et al.,* 1998). EPAC has been found to be involved in the regulation of crucial processes within the lungs, brain, heart and immune systems. For example, EPAC has been shown to be involved in hypertrophy in myocardial cells (Morel *et al.,* 2005). In hypertrophy, cell size increases, causing an increase in tissue or organ volume. Although this occurs in the heart ventricles as a natural response to exercise, hypertrophy is also linked to diseases such as hypertension. Not only has increased expression of both EPAC isoforms been indicated in myocardial hypertrophy (Ulucan *et al.,* 2007) but activation of EPAC in cardiomyocytes has been shown to cause activation of cardiac hypertrophy associated genes via the involvement of the small GTPase Rac and NFAT (nuclear factor of activated T-cells), a transcription factor regulated by calcium (Morel *et al.,* 2005). Whether NFAT regulation occurs via EPAC mediated calcium signalling has yet to be determined. The involvement of cyclic AMP in cell proliferation is also well documented (Watson, 1975; Stork and Schmitt 2002; Takahashi *et al.,* 2004) and strong evidence links Rap1 to control of cell division (Knox and Brown, 2002). Current research suggests that EPAC is able to both stimulate and inhibit cell division depending on cell type. Studies in COS1 cells have shown that overexpression of EPAC is believed to play a role in stimulating mitosis (Qiao *et al.,* 2002)

while in Jurkat cells overexpression of EPAC has been demonstrated to inhibit cell proliferation (Boussiotis *et al.,* 1997).

1.2.5.1 EPAC structure

The EPAC1 and EPAC2 isoforms share sequence homology, including a C-terminal catalytic region, comprising a CDC25 homology domain for nucleotide exchange (CDC25HD) which is found in all eukaryotes, except plants, and is found in all guanine nucleotide exchange factors which regulate Ras-like G-proteins Ras, Rap and Ral (van Dam *et al.,* 2009). This region also contains a Ras association domain (RA) and a Ras exchange motif (REM). The regulatory region of both isoforms contains a Dishevelled/Egl-10/Pleckstrin domain (DEP) as well as a cyclic nucleotide binding (CNB) domain (Figure 1.5). This domain is similar to the domain found in both PKA and the cyclic AMP receptor protein found in bacteria (Cheng *et al.,* 2008). Additionally, EPAC2 possesses a second CNB domain at the N-terminal of the DEP domain. Functionally, the purpose of this second CNB domain is unknown as it is not required for cyclic AMP regulation by EPAC2 (Bos, 2006) and has a low affinity for cyclic AMP binding (de Rooij *et al.,* 2000). Interestingly, this is the only common structural aspect between EPAC and PKA.

This lack of structural similarity between EPAC and PKA meant that understanding how cyclic AMP binding affects the structure of EPAC, and whether binding causes a conformational change that allows EPAC to interact with its downstream effectors, could not be obtained via comparisons with other known cyclic AMP receptors. However, recently the crystal structure of EPAC2 was solved, in the absence of cyclic AMP, allowing visualization of the closed formation (Rehmann *et al.,* 2007). Additionally, Deuterium Exchange Mass Spectroscopy (DXMS) studies have shown that conformational changes in what is termed the 'switchboard' section, where the CNB C-terminal and the catalytic region of the protein meet, occur upon cyclic AMP binding (Brock *et al.,* 2007). This causes a rearrangement of the domains to expose the catalytic region and allow binding of effectors. Binding of cyclic AMP leaves the catalytic region relatively unaltered (Brock *et al.,* 2007). Residues on the CDC25HD and the N-terminal helical bundle form an ionic latch, and it is theorised that, upon cyclic AMP binding, the CDC25 domain is forced to move, breaking the interactions which form the ionic latch (Harper *et al.,* 2008). It is

Figure 1.5
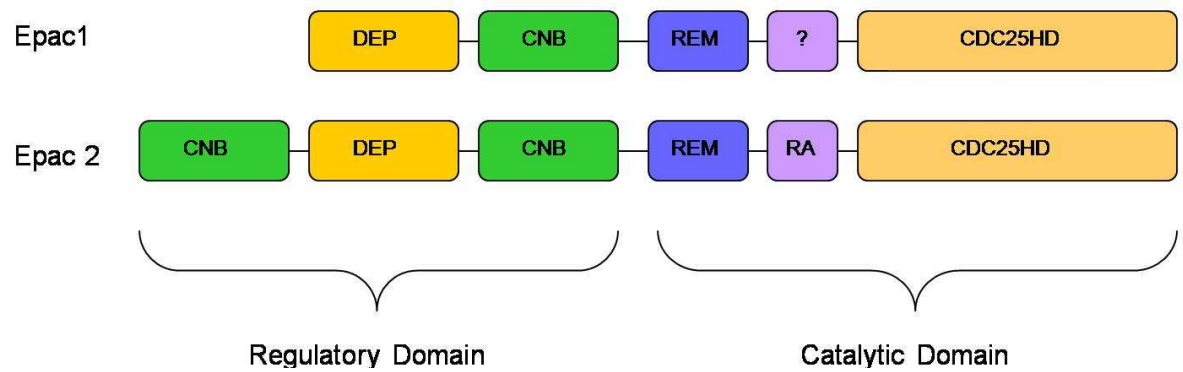
Figure 1.5 EPAC structure - EPAC1 and EPAC2 share a common domain structure. The catalytic domain at the C-terminal of the protein comprises a CDC25 homology domain (CDC25HD) for nucleotide exchange, a Ras association domain (RA) and a Ras exchange motif (REM). The regulatory region contains a Dishevelled/Egl-10/Plekstrin domain (DEP) as well as a cyclic nucleotide binding (CNB) domain. EPAC2 possesses two of these CNB domains, which has an as yet unknown purpose. (Adapted from Bos, 2006)

interesting to note that the cyclic AMP analogue 8-pCPT-2'O-Me-cyclic AMP (8Me), a highly selective EPAC agonist with an affinity for EPAC 10 times greater than cyclic AMP (Bos, 2006), causes similar, though not identical, conformational changes as cyclic AMP (Harper *et al.,* 2008).

1.2.5.2 EPAC signalling

Cyclic AMP is well characterised as promoting calcium mobilization and influx (Rubin and Adolf, 1994; Cheng *et al.,* 2008) although previously this ability has been solely linked to the actions of PKA. Recently, however, EPAC has been shown to regulate calcium signalling in three ways. Firstly, it has been suggested that EPAC directly interacts with intracellular calcium receptors such as ryanodine receptors and inositol 1, 4, 5-triphosphate (IP3) receptors (Roscioni *et al.,* 2008) to mediate calcium release in response to calcium mobilizing second messengers such as IP3 and NAADP (Holz *et al.,* 2006). Supportive evidence in the form of the identification of a macromolecular complex comprising EPAC1, a muscle specific AKAP (mAKAP), PDE4D3, PKA and RYR2, the type 2 isoform of the ryanodine receptor (Dodge-Kafka and Kapiloff, 2006) further links EPAC to calcium release. Secondly, PKA independent phosphorylation of ryanodine receptors and IP3 receptors to increase their sensitivity to both calcium mobilizing second messengers and to calcium itself has been shown to occur via Rap and ERK. It is possible that these actions may be mediated by EPAC (Holz *et al.,* 2006). Lastly, EPAC has been linked to activation of the PLC isoform, ε. PLCε is one of 6 isoforms of the PLC family of proteins, which hydrolyse phosphatidylinositol 4, 5- bisphosphate (PIP2) to the second messengers, IP3 and diacylglycerol (DAG). To date, little is known about the mechanisms of PLCε other than its unique ability for regulation by the direct binding of small G-proteins including Ras, at its CDC25 and Ras-associating domains. This allows PLCε to act not just as a PLC, activating PKC via mediation of calcium and DAG, but also as a component in GTPase function (Bunney and Katan, 2006). EPAC has been shown to activate PLCε in HEK293 cells via the GTPase Rap2B upon elevations of cyclic AMP (Schmidt *et al.,* 2001) and also to interact with PLCε in the β-adrenergic receptor stimulation of Ca^{2+}-induced Ca^{2+} release (CICR) in cardiac myocytes (Oestreich *et al.,* 2009). This interaction provides a link between two classical second messenger signalling pathways, those of cyclic AMP and calcium.

The importance of EPAC-regulated Ca^{2+} signalling is highlighted by the fact that EPAC2 has been shown to be involved in control of insulin exocytosis in pancreatic β-cells via cyclic AMP stimulation (Ozaki *et al.,* 2000). This is believed to occur through a Rab3 interacting protein, Rim2, and a subunit of the K^+-ATP channel, SUR1 (Kashima *et al.,* 2001). Additionally, as EPAC2 has been shown to couple pancreatic β cell cyclic AMP production to stimulate calcium dependent exocytosis, as well as evidence that down-regulation of EPAC2 reduces calcium signalling and insulin secretion by glucagon-like peptide 1 (GLP1) (Holz, 2004) strongly links EPAC2 to a role in diabetes. Exocytosis is also involved in the release of neurotransmitters (NTs) and recent research has shown a potential role for EPAC in NT release at excitatory central synapses (Gekel and Neher, 2008).

1.2.5.3 EPAC and inflammation

EPAC has been linked to regulation of inflammation in vascular endothelium cells, via both cytokine signalling down regulation and cell-cell adhesion. EPAC is believed to regulate endothelial cell-cell junction formation by redistribution of vascular endothelial cadherin (Kooistra *et al.,* 2005). This redistribution allows the cadherin to bind to binding partners located on neighbouring cells in vascular endothelial cells, forming a tight junction and reducing cell permeability (Kooistra *et al.,* 2005). This reduction of permeability is essential as increased permeability has been shown to be a characteristic of oedema (Cullere *et al.,* 2005). Recently, EPAC has also been linked to control of inflammatory response by mediation of IL-6 signalling via the cytokine suppressor, SOCS-3 (Sands *et al.,* 2006). Cytokine signalling involves the secreted cytokine molecules regulating a wide range of biological functions, including proliferation, differentiation and inflammation. Cytokines interact with cell surface receptors to trigger transmission of signals to the nucleus, which results in transcription (Starr and Hilton, 1998). A family of proteins called suppressors of cytokine signalling, SOCS, were identified to negatively regulate these cytokine signalling pathways. This family has eight members, SOCS-1-7 and CIS. All of the SOCS-3 proteins contain a unique N-terminal region, a middle SH2 domain and a domain termed SOCS box at the C-terminal. This area is conserved not only across the SOCS proteins, but also in several other proteins, including some GTPases (Starr and Hilton, 1998). SOCS-1 was first discovered in 1997 simultaneously by three groups during a screen for similarity for the STAT3 SH2 domain (Naka *et al.,* 1997), as an inhibitor of differentiation of myeloid cells in response to IL-6 (Starr *et al.,* 1997) and as a protein that interacts with the JAK2 kinase domain (Endo *et al.,* 1997). SOCS-3 was

identified shortly after and has been shown to display a greater similarity to SOCS-1 than to other SOCS proteins, with both SOCS-1 and SOCS-3 showing a kinase inhibitory region not conserved in other family members. Additionally, both SOCS-1 and SOCS-3 are able to be induced by several cytokines (Gisselbrecht, 1999). SOCS-3 is the major SOCS gene in several areas, including the hypothalamus, pituitary, mammary gland and liver (Gisselbrecht, 1999).

SOCS-3 interferes with the JAK-STAT pathway, a critical pathway in the signalling of a number of cytokines including IL-6. SOCS-3 directly binds to and inhibits JAKs, preventing the phosphorylation of STATs and thus preventing the transcription of target genes, inhibiting the IL-6 stimulated response. SOCS-3 was found to be induced by cyclic AMP via EPAC1 in HUVECs (Sands *et al.,* 2006) and COS1 cells (Borland *et al.,* 2009) through a pathway involving EPAC and Rap1 activation of CCAAT/enhancer-binding proteins (C/EBPs) β and δ. C/EBP transcription factors associate with the SOCS-3 promoter, thereby inducing increased transcription of the SOCS-3 gene (Yarwood *et al.,* 2008).

1.2.6 Cyclic AMP phosphodiesterases

Although intracellular levels of cyclic AMP may be increased through stimulation of multiple adenylyl cyclase enzymes (Cooper, 2003) through the action of many different G-protein coupled receptors, hydrolysis to 5'AMP, via the action of a large family of phosphodiesterase enzymes (PDE), is the only known means of cyclic AMP degradation in cells (Lugnier, 2006). While adenylyl cyclases are localized to the plasma membrane, PDE activity occurs throughout the cell in the cytosol, cytoskeleton, nucleus and membrane, and is therefore essential in the compartmentalisation of cyclic AMP gradients (Bolger *et al.,* 2003). PDEs are thus not only able to terminate cyclic AMP signals via degradation of cyclic AMP, but also have the ability to 'fine-tune' levels in specific areas to increase signal specificity, through exertion of a combination of intracellular localisation, regulatory mechanisms and enzyme kinetics (Terrin *et al.,* 2006).

It is theorised that the actions of PDEs on cyclic nucleotides is through a water molecule, induced by an OD2 atom in the Asp318 residue of the PDE, that is able to act as a nucleophile, attacking the cyclic AMP phosphorous atom (Kang *et al.,* 2006), thus hydrolyzing the 3' cyclic phosphate bond. The first PDE to be identified was an orthologue of the mammalian PDE4 isoforms (Richter *et al.,* 2005), the *Drosophila* dunce gene, which

has been shown to have a role in learning and memory. The number of genes encoding the various PDE families varies widely between species. For example, humans possess sixteen genes encoding PDE4, while *C. elegans* has only one (Conti *et al.,* 2003). To date, eleven PDE families have been identified, of which 8 contain isoforms with the ability to degrade cyclic AMP. This diversity of these isoforms, which, through alternative splicing (Bolger *et al.,* 1997), now number over thirty, suggests both a specificity of the isoforms to regulate distinct mechanisms within the cell, and the possibility of regulation of PDEs through alternative signalling pathways (Houslay and Adams, 2003). The members of the PDE families appear to share structural homology of their C-terminal domains, with N-terminals differing greatly between them (Conti *et al.,* 2003) (Figure 1.6).

Of the eight cyclic AMP degrading PDE families, PDE4 isoforms account for the majority of cyclic AMP hydrolysis (Conti *et al.,* 2003) and are widely expressed in a variety of tissues. More than twenty PDE4 variants have been characterised to be expressed in mammalian cells (Bender and Beavo, 2006) and are involved in a wide range of cellular functions, such as macrophage and monocyte activation, neutrophil infiltration, cardiac contractility and brain function (Bender and Beavo, 2006). PDE4 isoforms are distinguishable by their ability to be inhibited by the anti-depressant drug rolipram (Conti *et al.,* 2003). Inhibitors of PDE4 have also been found to have anti-inflammatory actions, and have potential in the treatment of a number of diseases, including chronic obstructive pulmonary disease (COPD), asthma and depression (Bolger *et al.,* 2006). This therefore makes PDE4 isoforms highly interesting because of their potential as therapeutic targets (Houslay *et al.,* 2005). PDE4 isoforms share a catalytic domain composed of 17 α-helices that form three subdomains. Located between the subdomains are two metal-ion binding sites, with the centre of the domain forming a binuclear motif containing a Zn^{2+} and Mg^{2+} ion (Castro *et al.,* 2005). Complete understanding of this domain is important for the synthesis of PDE4 inhibitors, as well as determining the mechanism of cyclic AMP binding to the PDE4 isoforms for degradation (Bolger *et al.,* 2003). The majority of the catalytic domain, particularly the metal ion binding sites, is conserved across all PDE4 isoforms, with 75% sequence homology between PDE4 isoforms (Bender and Beavo, 2006).

Cyclic AMP is able to self regulate its own levels by activating PDE4 isoforms in a number of ways. PDE4 isoforms are regulated by both phosphorylation as well as at the genetic level via alterations in PDE expression. PKA phosphorylation has been shown to

Figure 1.6
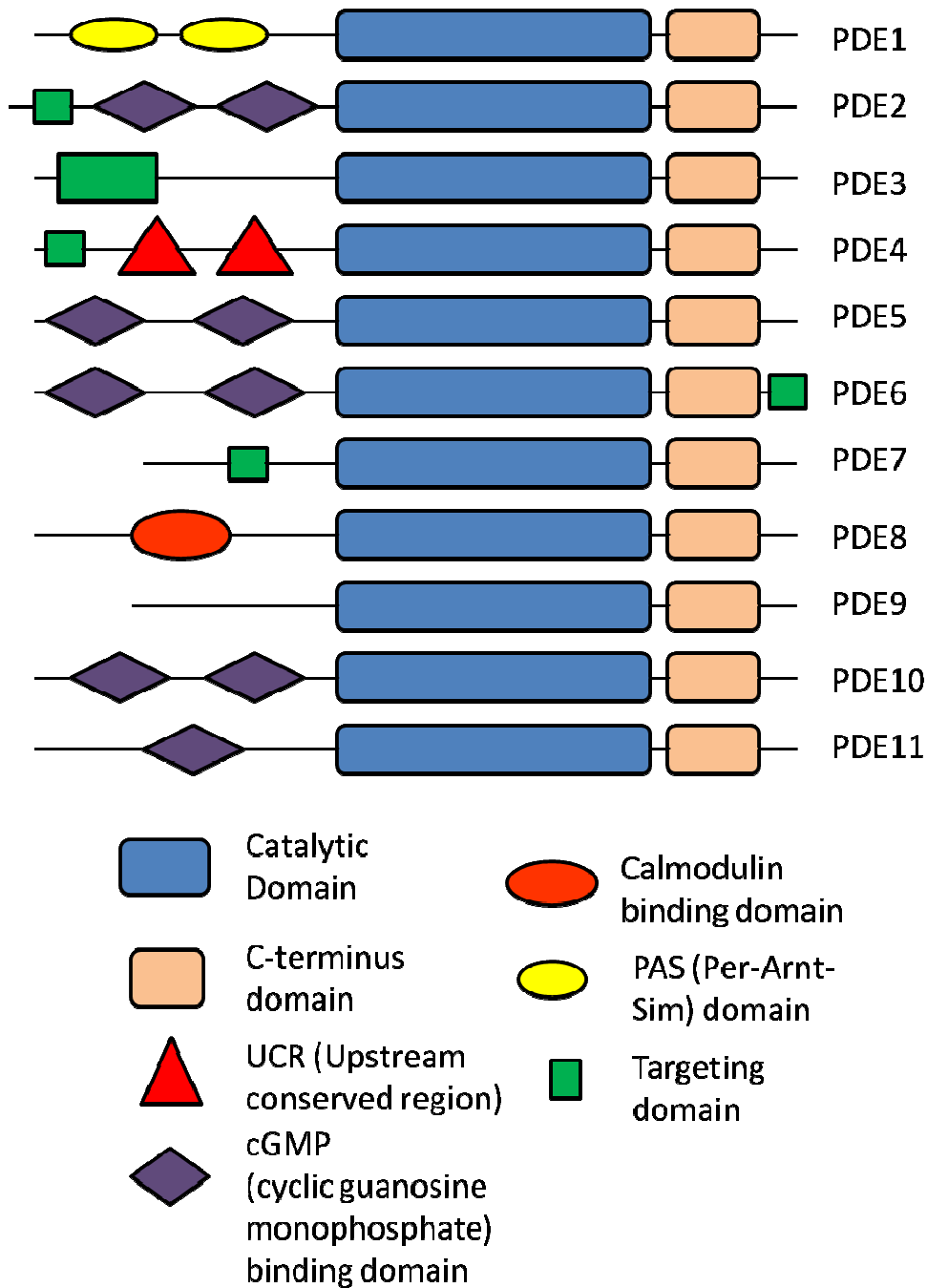
Figure 1.6 The phosphodiesterase family - The eleven known phosphodiesterase (PDE) families. Eight of these (PDE1, PDE2, PDE3, PDE4, PDE7, PDE8, PDE10 and PDE11) hydrolyse cyclic AMP, while PDE5, PDE6 and PDE9 are cyclic GMP specific. All isoforms share a structural homology at their C-terminal, while possessing different, family specific, N-terminal regions (Amended from Conti *et al.,* 2000).

regulate long forms of PDE4 isoforms. Cyclic AMP feedback regulation has been demonstrated in T cells, monocytes, neurons, Sertoli and thyroid cells, where increased cyclic AMP levels result in increased transcription of the PDE4 genes (Park *et al.,* 2003). A rise in cyclic AMP levels within the cell leads to increased PDE activity, potentially to desensitise processes within the cell to the higher cyclic AMP levels. A sustained, long-term increase of levels of cyclic AMP results in a rise in levels of mRNA and protein expression of PDE4 isoforms. In addition to this, cyclic AMP is able to cause both PKA and ERK MAP kinase phosphorylation, resulting in increased kinase activity, by targeting specific residues on both their N-terminal and in the UCRs, as shown in PDE4D3 studies (MacKenzie *et al.,* 2002). The beginning of this study focuses on novel regulation of a member of the type 4D family of cyclic AMP PDEs and so we will concentrate on this family in the next section.

1.3 Cyclic AMP-specific Phosphodiesterases, Type 4 PDEs

In humans, there are 4 PDE4 genes, encoding more than twenty isoforms in the subfamilies PDE4A, PDE4B, PDE4C and PDE4D, distinguishable by their unique N-terminal regions (Figure 1.7). The genes themselves are over 18 exons long with a size of approximately 50kb, but despite this obvious complexity, there is almost complete conservation between mouse and human PDE4 genes, indicating a likely strong evolutionary selective pressure to maintain concurrence of the genes between species (Conti *et al.,* 2003). The isoforms are encoded by alternatively spliced mRNA transcripts (Bolger *et al.,* 1997). The unique N-terminal domains, by which each isoform can be easily identified, are thought to play an important role in intracellular targeting, where they are involved in protein-membrane and protein-protein interactions, such as those between PDE4D3 and AKAPs (Wong and Scott, 2004), and PDE4D5 with receptor for activated C-kinase 1 (RACK1) (Le Jeune *et al.,* 2002). In addition, members of the PDE4 family can be categorised in three ways based on the presence or absence of two domains called upstream conserved regions (UCRs). Those termed 'short' only have UCR2, and 'super-short' lack both UCR1 and possess only a truncated UCR2 domain (MacKenzie *et al.,* 2002) (Figure 1.8). UCR 1 and 2 are located between the N-terminal and the catalytic site, and have important regulatory roles for the catalytic domain, where they are able to influence PDE4 activity. UCRs appear to influence the differential functions of the isoforms upon catalytic domain phosphorylation,

Figure 1.7
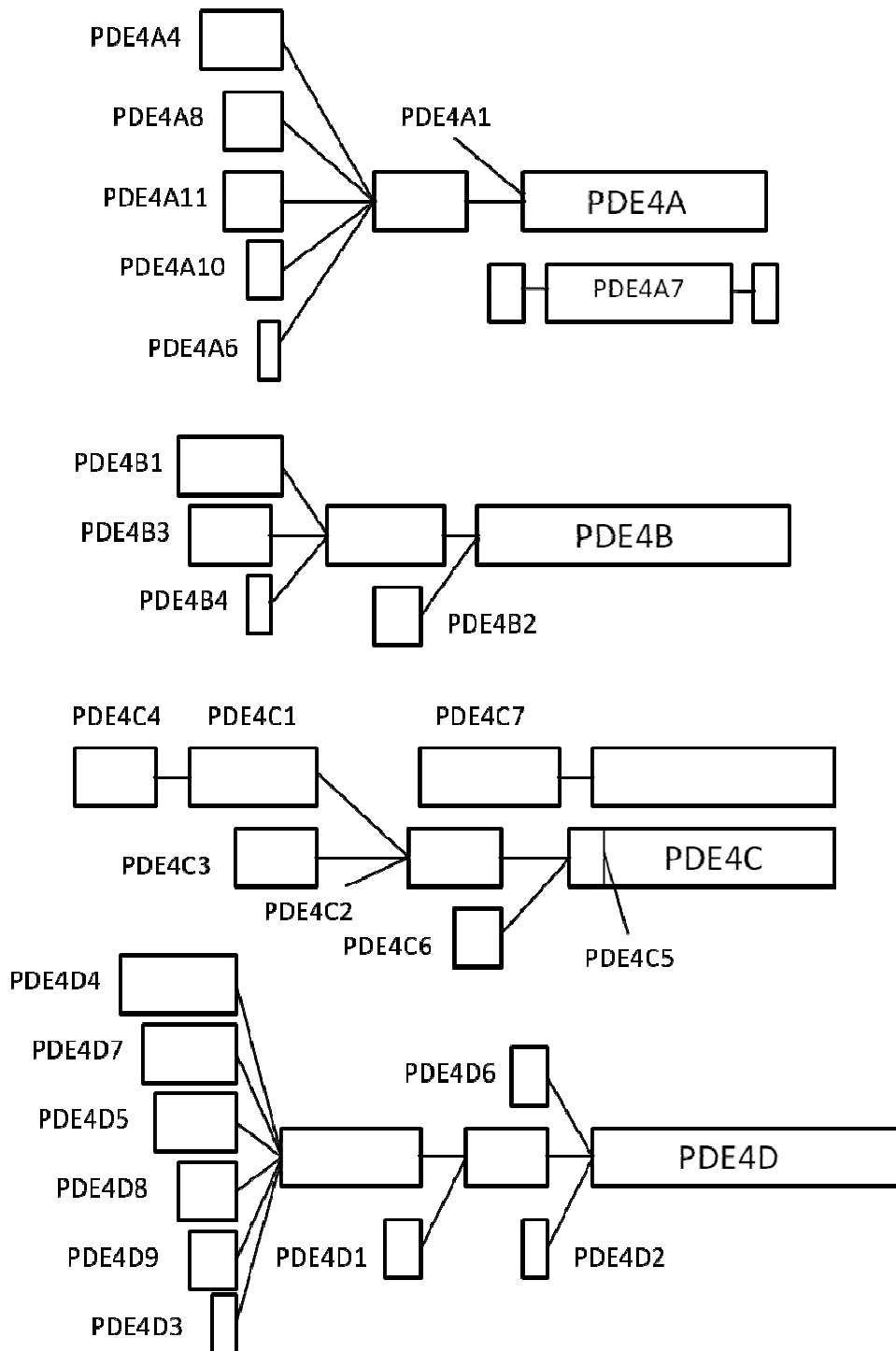
Figure 1.7 Phosophodiesterase 4 - The PDE4 family is responsible for the majority of cyclic AMP hydrolysis. Having more than 20 isoforms, this family is encoded by four genes, A-D. The isoforms share a basic structural similarity, yet are distinguished by their isoform specific N-terminal regions (Amended from Bender and Beavo, 2006).

Figure 1.8
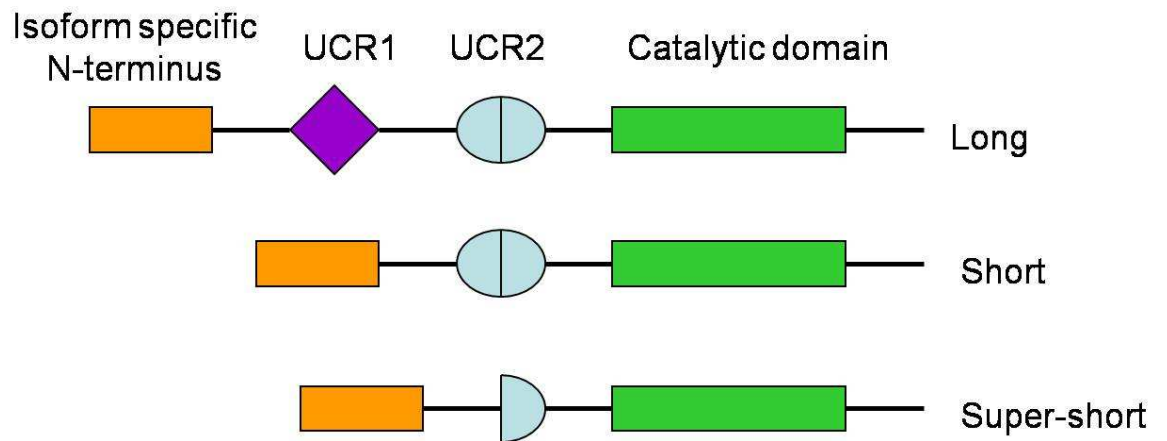
Figure 1.8 The structure of UCRs in long, short and supershort PDE4 isoforms -The PDE4 family can be categorised based on the presence or absence of the UCR1 and UCR2 domains which influence PDE activity. Long PDE4 isoforms contain both of these domains and short PDE4 isoforms possess only the UCR2 domain, while super-short PDE4 isoforms lack UCR1 and possess a truncated UCR2 domain.

with ERK phosphorylation effects differing depending on the UCRs present (Houslay and Baillie, 2003). With UCR1 and 2 domains interacting with each other to form a regulatory domain in long isoforms, ERK phosphorylation results in inhibition, while in the short isoforms, with only UCR2 present, phosphorylation results in activation (Lim *et al.,* 1999). In super-short isoforms, ERK phosphorylation does not result in any activity, indicating ERK phosphorylation requires a section of the UCR domain not present in the truncated domains found in super-short isoforms (Le Jeune *et al.,* 2002). PKA activation of long isoforms results in a V_{max} increase of two to three times. Studies using PDE4D3 have shown the regulatory module of the two regions is involved in phosphorylation of the PDE4 by PKA and ERK MAP kinase (MacKenzie *et al.,* 2000).

1.3.1 PDE4D

PDE4D mRNA is found in a wide variety of cells, with protein levels being particularly high in the brain, liver and heart, and also in inflammatory cells (Bender and Beavo, 2006). Given this wide tissue distribution it is perhaps not surprising that PDE4D isoforms have been implicated in a number of diseases, including ischemic stroke, atherosclerosis and osteoporosis (Bender and Beavo, 2006). PDE4 activity is the most dominant of PDE activity in inflammation, and is found in eosinophils, neutrophils and CD4+ lymphocytes, suggesting important roles for PDE4 isoforms in diseases such as COPD and asthma. This places PDE4 isoforms as an ideal potential therapeutic target (Le Jeune *et al.,* 2002). In particular, PDE4D isoforms are of interest due to their hypothesised ability to mediate the effects of rolipram and other PDE4 inhibitors (Richter *et al.,* 2005). Located on chromosome 5q12, the gene encoding PDE4D has a complex genomic arrangement, composed of two major exon clusters which encode the catalytic and regulatory regions which are highly conserved, and three exons which encode the specific N-terminals. Nine splice variants of the PDE4D gene have been identified, six of which are long isoform and each can be distinguished by the unique N-terminal domain (Richter *et al.,* 2005). While PDE4D1 and PDE4D2 are found only in the cytosol, PDE4D3, PDE4D4 and PDE4D5 can be located in either the cytosol or membranes of the cell, with the majority of PDE4D isoforms present in the membranes being PDE4D4 (60%), and only 30% being made up of PDE4D5 and PDE4D3 (Bolger *et al.,* 1997). While the V_{max} of PDE4D3 appears to be the same in both the cytosolic and membrane fractions, those of PDE4D4 and PDE4D5 are around 3 times higher in the membrane fraction than the cytosolic (Bolger *et al.,* 1997). The range of locations, abundance and activities of the PDE4D isoforms suggests that each splice variant has both a highly specific and unique role to play within the cell. It also

appears that localisation to the particulate fraction of the cell has an influence on the PDE4D isoform enzyme properties, such as sensitivity to rolipram, with the adoption of conformations different to the cytosolic isoform counterpart (Bolger *et al.,* 1997).

1.3.2 Interactions between PDE4D5 and receptor for activated C-kinase 1 (RACK1)

PDE4D isoforms have been shown to interact with a number of other proteins. For example, PDE4D3 binds to myomegalin, a large protein found in cardiac and skeletal muscle (Verde *et al.,* 2001), PDE4A5 forms a complex with immunophilin XAP2, an associated protein of the Hepatitis B virus X protein (Bolger *et al.,* 2003) and PDE4A5, PDE4A4 and PDE4D4 all bind with the Src family of tyrosine kinases (McPhee *et al.,* 1999). Another such interaction is the protein-protein interaction occurring between the splice variant PDE4D5 and RACK1 (Yarwood *et al.,* 1999), a protein which has been shown to act as both an anchor and shuttle for a number of proteins within the cell (McCahill *et al.,* 2002). PDE4D5 is distinguishable from other PDE isoforms by 88 amino acids at the N-terminal of the enzyme, which is unique to PDE4D5 and highly conserved between mammalian species, and is responsible for recruitment of PDE4D5 to RACK1 (Bolger *et al.,* 2002). One attractive hypothesis is that interaction with RACK1 through this unique N-terminal region serves to recruit and anchor PDE4D5 into a position where it is able to regulate local cyclic AMP levels at the plasma membrane (Le Jeune *et al.,* 2002).

RACK1 is a 36 kDa protein, was originally cloned from a chicken liver cDNA library (McCahill *et al.,* 2002) and was identified as a homologue of the β subunit of G proteins. Both Gβ and RACK1 are members of the large WD repeat family, which consists of regulatory proteins, all sharing the Trp-Asp40 (WD40) motif. In RACK1, the WD40 motif is highly conserved between species with the primary structure of RACK1 having 100% homology between human, mouse, chicken, rat and cow (Berns *et al.,* 2000). RACK1 was first discovered to bind PKC serine/threonine kinases following activation of PKC isoforms by phorbol esters or diacylglycerol (McCahill *et al.,* 2002). RACK1 binds activated PKC isoforms through a non-substrate binding site and controls specificity of PKC-mediated signalling by translocating and anchoring the activated protein to particular cell fractions (Schechtman and Mochly-Rosen, 2001). As such, RACK1 is mainly recognised as an anchoring protein for the PKC family although it has been shown to bind a number of proteins, acting as an adaptor or anchoring protein in several protein-protein and protein-membrane interactions (McCahill *et al.,* 2002).

Like Gβ, RACK1 has a seven bladed β propeller structure. Arranged in a rigid ring formation, it is the multiple binding sites of this formation that allows RACK1 to act as an adaptor or scaffold protein for a range of proteins (Figure 1.9). RACK1 is believed to recruit and anchor these proteins into a signalling cascade by shuttling and anchoring the proteins to their appropriate subcellular locations, suggesting that RACK1 plays an important role in intracellular signalling (Liedtke *et al.,* 2002). While PKCβII appears to be its preferred binding partner, RACK1 has been shown to interact with numerous proteins, such as Src, PLC γ, Dynamin-1, ras-GAP, integrins and several viral proteins, for example BZLF1, the EBV activation protein, the Adenovirus E1A protein and the Influenza M1 protein (McCahill *et al.,* 2002). Src is a tyrosine kinase proto-oncogene activated in a number of cancers including breast and colon. Phorbol ester stimulation of PKC activates Src which causes Src to bind to RACK1 by the SH2 domain independently of PKC activation. However, binding of both kinases to RACK1 can result in cross-talk between the serine/threonine kinase and tyrosine kinase pathways (Mamidipudi *et al.,* 2004). The signalling enzyme, Phospholipase Cγ (PLCγ), generates diacylglycerol upon activation of growth factor receptors and contains a C2 domain where RACK1 binds when cells are stimulated with epidermal growth factor (Schechtman and Mochly-Rosen, 2001), while Dynamin-1 binds to RACK1 via the PH (Pleckstrin Homology) domain. Dynamin-1 is involved in vesicle recycling and its intrinsic GTPase activity is increased 6 times *in vitro* when bound to RACK1 and, at high concentration, also competes with Gβγ to bind RACK1 (Dell *et al.* 2002). Ras is an important protein in cell growth. Its activation is terminated by GAP, a GTPase activating protein which binds RACK1 through both the C2 and SH2 domains. GAP is also a PKC substrate, and RACK1, GAP and PKC are believed to form a trimolecular complex in order to increase the proximity of GAP to PKCIIB (Schechtman and Mochly-Rosen, 2001).

There is also some evidence that RACK1 can affect signalling from GPCRs through direct protein interactions. For example RACK1 binds to the Gβγ subunit (Dell *et al.,* 2002) and this binding has been shown to result in RACK1 translocating from the cytosol to the membrane of the cell. Although RACK1 has no effect on Gβγ functions, such as chemotaxis and MAPK signalling, it inhibits activation of both phospholipase C β2 and adenylyl cyclase II (Chen *et al.,* 2005). This inhibition of ACII prevents cyclic AMP from being generated, thus having an effect on the control of cyclic AMP levels within the cell. Interestingly, although Gβγ mediated ACII activation is inhibited, it has no effect on cyclic

Figure 1.9
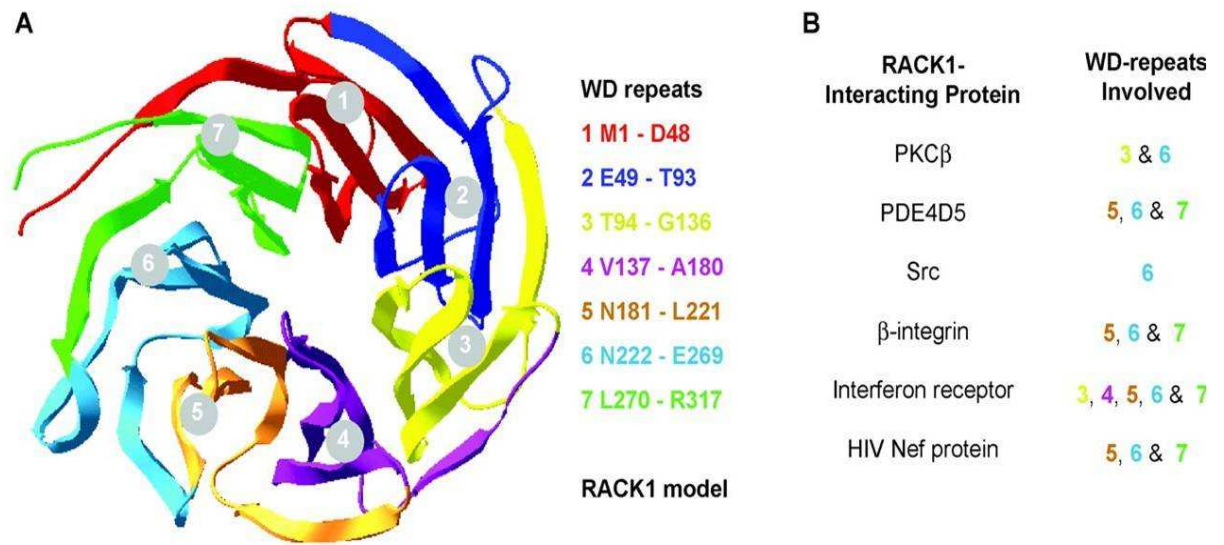
Figure 1.9 Structure of Receptor for Activated Protein Kinase C - The structure of RACK1 shows its seven blade propeller structure, composed of WD repeats, and the blades involved in interaction with various proteins, including PDE4D5 which interacts with WD repeats 5, 6 and 7 (McCahill *et al.,* 2002).

AMP signalling controlled by Gαi and Gαs acting receptors. It may also be possible that other RACK1 binding proteins maybe also be involved in the regulation of the Gβγ functions inhibited by RACK1 (Chen *et al.,* 2004).

The PDE4D5/RACK1 signalling complex is of particular interest as it spans both the cyclic AMP signalling pathway, and the PKC signalling pathway, and may be an area of cross-talk between the two pathways. RACK1 and PDE4D5 are believed to interact similarly to Gβ, a β propeller protein, and Gγ, a helical signal transduction molecule, which exhibit a coiled-coil interaction between their N-terminal regions, and via an interaction between blades 5, 6 and 7 of the Gβ C-terminal and helical and non-helical sections of the C-terminal of Gγ, with the helical segment of Gγ fitting into the groove created by the blades of Gβ (McCahill *et al.,* 2002). The C-terminal domain of the WD-repeat protein is clearly important in this helix/groove-like interaction, and this is believed to be the case within the RACK1/PDE4D5 complex also (Bolger *et al.,*2006) (Figure 1.10). A subdomain within the 88 amino acids of the unique N-terminal region of PDE4D5 was found to be essential in binding to RACK1 (Figure 1.11). The RACK1 interaction domain (RAID1) consists of a segment of charged amino acids, and a second section of hydrophobic amino acids. The hydrophobic amino acids, Leu 29 to Leu 38, form a ridge along one side of the RAID1 helical structure. In addition, the Arg34 residue in the charged amino acid segment of RAID1 was found to be essential in binding to RACK1, with mutations of this residue preventing binding between the proteins (Bolger *et al.,*2006). As with Gβ in its interaction with Gγ, it is blades 5, 6 and 7 of RACK1 that interact with PDE4D5, forming a trough that the N-terminal of PDE4D5 fits into (McCahill *et al.,* 2002). In fact, only the presence of these blades is required for binding to occur, with truncated RACK1 proteins still binding when these three propellers were present (McCahill *et al.,* 2002). Comparisons with other proteins which interact with these blades of RACK1, such as β-integrin, show little homology between the binding proteins, indicating that PDE4D5 binds to an area within these blades distinct from that which β-integrin binds to (Steele *et al.,* 2001).

PDE4D5 binds readily to both RACK1 and β-arrestin (Bolger *et al.,* 2003). However, it appears binding to both proteins cannot occur at the same time, due to overlapping binding sites within the PDE4D5 N-terminal. This suggests that these proteins compete to bind to PDE4D5, with studies in HEK293 cells showing that binding to RACK1 is double that of β-arrestin, suggesting a preference of PDE4D5 towards RACK1 (Bolger *et al.,* 2006). As

Figure 1.10
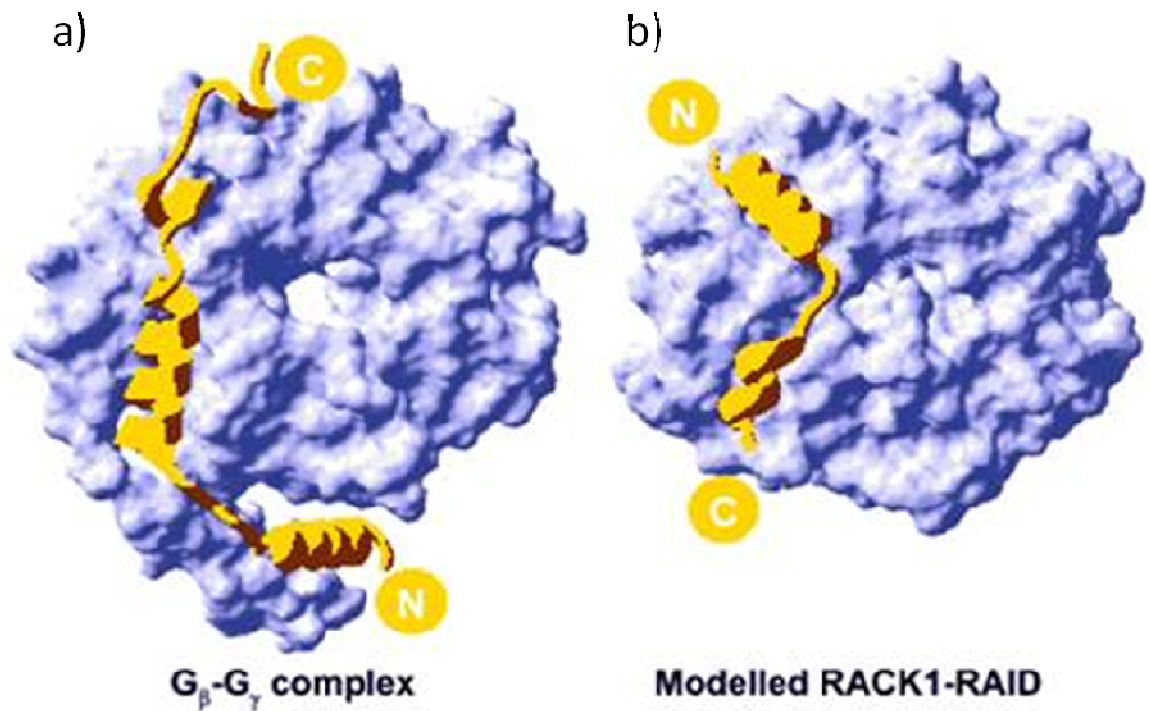
Figure 1.10 Modelling of the theorised RACK1/PDE4D5 interaction based on the Gβ/Gγ subunit interaction - The Gβ protein has a seven bladed beta propeller structure similar to that of RACK1. It interacts with the helical protein Gγ through formation of a cleft created by blades 5, 6 and 7 (a). It is likely that RACK1/PDE4D5 interact in a similar manner (b) via the RACK1 interaction domain (RAID) on PDE4D5 (McCahill *et al.*, 2002).

Figure 1.11
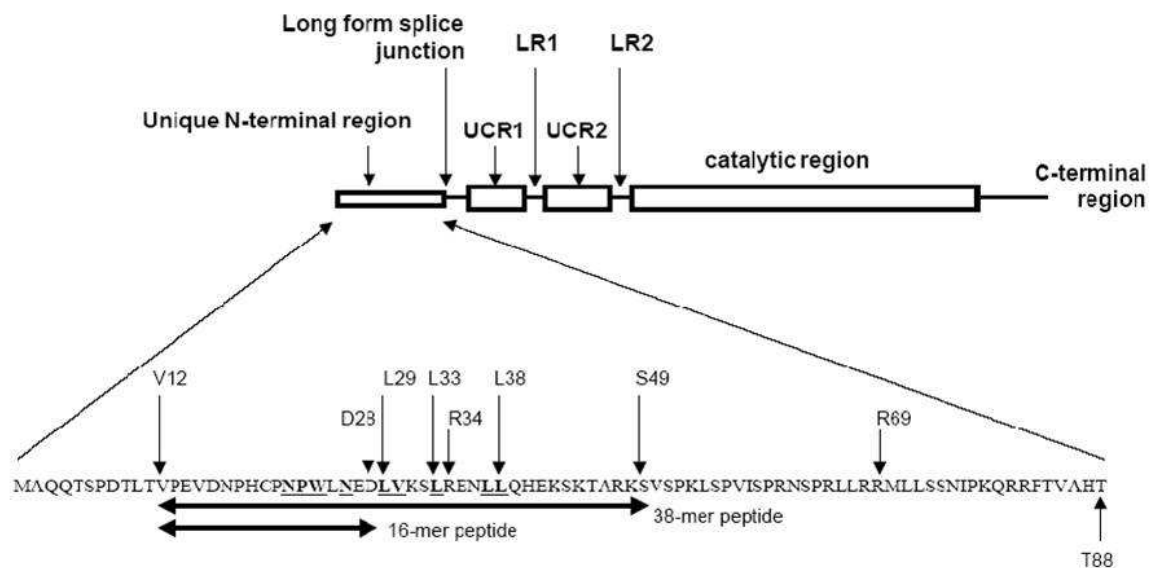
Figure 1.11 The PDE4D5 RACK1 interacting domain - The RACK1 interaction domain (RAID1) is located at the N-terminal region of the PDE4D5 protein. Comprising an 88 amino acid region, the hydrophobic segment between Leu29 and Leu38 forms a ridge, while Arg34, located within the charged region of the domain, is essential in RACK1 binding. The 16 and 38-mer peptides indicated with horizontal arrows are those tested for binding to RACK1, leading to the identification of the RAID1 domain (Bolger *et al.,* 2002).

both RACK1 and β-arrestin also bind to sites within the catalytic region, the proteins appear to span across PDE4D5, thus physically blocking the other protein from interacting with PDE4D5 (Bolger *et al.,* 2006). That PDE4D5 is only able to exclusively bind one protein at a time, but RACK1 may possibly have the ability to bind to other proteins at the same time is both interesting and also raises questions as to whether this has an effect on the action and purpose of the binding complex, and whether additional proteins may bind to RACK1 and influence the function of the complex. PDE4D5 was first identified as a RACK1 interacting protein in a yeast two-hybrid screen, and the interaction between the two proteins was found to be highly specific (Yarwood *et al.,* 1999). PDE4D5 was not found to bind other WD40 repeat proteins, and RACK1 has not been found to interact with any other members of the PDE4 family. The interaction of these two proteins is therefore both specific and unique, suggesting an important functional role for the complex. While different proteins interact with different RACK1 WD40 repeats, repeats 5, 6 and 7 are essential in the interaction between PDE4D5 and RACK1, although it is not these repeats alone that are necessary for binding,. This was demonstrated by Steele *et al.* (2001), where a RACK1 construct composed of these three repeats was found to be 25% less effective at binding PDE4D5 than wild type RACK1, suggesting the propeller structure of RACK1 is needed for optimal binding. While PDE4D5 has been shown to bind to RACK1, the effects of this anchoring on cellular functions has yet to be determined, although this interaction has been shown to increase sensitivity to the inhibitor rolipram. PDE4D5 recruitment by RACK1 may also result in modulation of local cyclic AMP levels, thus regulating localised effector molecules that have also been recruited to the specific location.

RACK1 is thought to have numerous roles in cellular physiology and, by extension, several diseases. One such disease is cancer, where concentrations have been found to be four times higher in non-small cell lung carcinoma than in normal human lung tissue, and almost twenty times higher in colon cancer. Its presence in the maturation of *D. melanogaster* zygotes (McCahill *et al.,* 2002) suggests an involvement in the growth factor and cell proliferation aspects of the regulation of cell development, as well as cell adhesion, where RACK1 is believed to act as a scaffold protein for proteins involved in adhesion complexes, such as β integrins (Liliental and Chang, 1998). Over expression of RACK1 has been found to increase spreading and actin stress fibres in cells, thus reducing their growth rate in anchorage-dependent and -independent conditions. This indicates an involvement of RACK1 in both cell growth and movement. Within the cell, as well as forming protein-protein interactions, RACK1 has been suggested to be involved in cell

migration and cell-cell interactions, prolongation of the G0/G1 phase of the cell cycle, inhibition of c-Src kinase activity and NIH3T3 cell growth, as well has having a function in both growth factor dependent and apoptotic intracellular signalling pathways (Mamidipudi *et al.,*2004). In addition to this, PKCβII shuttling of RACK1 may increase or decrease phosphorylation of neighbouring substrates to activate proliferation or growth arrest respectively (Berns *et al.,* 2000).

RACK1 has also been found to be highly upregulated in angiogenesis (Berns *et al.,* 2000) and has also been linked to cystic fibrosis through the discovery of its role in epithelial chloride channel function, where it binds to the Na^+/H^+ exchange regulatory factor (NHERF1), a binding partner of the cystic fibrosis transmembrane regulator (CFTR). PKC regulates cyclic AMP-dependent CFTR chloride function by binding to RACK1 which in turn binds NHERF1. RACK1 potentially acts as a scaffold protein, holding the enzyme in the locale of CFTR (Liedtke *et al.,* 2002). Evidence also suggests a role for RACK1 in the immune response, particularly in the generation of phagocyte produced superoxide anions. A ligand-initiated activation, superoxide anions are released by the immune system to destroy invading pathogens and it is likely that RACK1 acts through sequestration of PKCβII in order to negatively regulate generation of the anions. It is interesting to note that PDE4 inhibitors are able to interfere with the inflammatory functions of neutrophils, where PDE4 inhibition leads to blockage of oxygen radical release from ligand stimulated neutrophils, possibly indicating a potential complimentary mechanism between RACK1 and PDE4 in immune responses (McCahill *et al.,* 2002).

1.4 Protein Kinase C Signalling

The PKC family of Serine/Threonine kinases comprises around 10 isoforms and is involved in a number of signalling pathways by altering protein function by phosphorylation. The isoforms can be divided into three categories based on their activation requirements- conventional, novel and atypical. Conventional PKC isoforms (α, βI, βII and γ) and novel isoforms (δ, ε, η and θ) both require DAG for activation, with conventional PKCs also requiring calcium. Atypical (ζ and λ/ι) require neither of these second messengers to be activated (Parker and Murray-Rust, 2004). This variety of PKC isoforms is an integral part of PKC signalling, with each isoform expressing differing characteristics, activity and tissue distribution, resulting in a wide range of effectors

downstream of PKC. Various isoforms have been linked to a number of cell functions, including cell proliferation and differentiation, cell adhesion and migration, barrier function and apoptosis. A vast number of PKC targets have currently been identified, ranging from growth factor receptors and ion channel regulators, to calcium and calmodulin binding proteins, cytoskeletal components and transcriptional factors. This range of biological functions means that disruption of PKC regulation can be linked to a number of disease states (Mackay and Twelves, 2007).

1.4.1 PKC isoforms

Activation of conventional and novel PKCs occurs when a GPCR, activated by external stimuli, activates a G protein which in turn activates PLC. PLC hydrolyses PIP2 into diacylglycerol (DAG) which stays at the plasma membrane where hydrolysis occurs, and inositol 1,4,5-triphosphate (IP3) which is freed into the cytoplasm. At sufficiently high levels, DAG activates novel PKCs, while conventional PKCs also require the interaction of IP3 and calcium channels located on the endoplasmic reticulum to release calcium stores into the cytosol, before they can become activated. Upon stimulation, PKC translocates to the plasma membrane, where this activation occurs. When inactive, the pseudosubstrate domain at the N-terminal of the PKC is bound to the catalytic domain. This holds the PKC in a folded conformation, effectively blocking the catalytic site from interaction with substrates. Activation upon translocation by the binding of C1 to DAG or phorbol esters, and/or binding of C2 to calcium or phosphatidylserine, causes a conformational change. This facilitates the disassociation of the pseudosubstrate from the catalytic domain, unfolding the protein and allowing PKC to act on its substrates.

1.4.2 PKC structure

Structurally, all PKC isoforms possess a conserved catalytic domain comprising ATP/substrate binding and catalysis motifs and a regulatory domain at the N-terminal, joined by a hinge region (Figure 1.12). The catalytic domain, a ~45kDa region at the C-terminal of the protein, is responsible for ATP binding and substrate docking, sometimes termed as C3 and C4 domains respectively (Amadio *et al.,* 2006). PKC isoforms contain three conserved sites in the catalytic domain where phosphorylation is required before catalytic activity is obtained (Cameron *et al.,* 2007). These sites are located in the activation loop, the turn motif, and the hydrophobic motif, although atypical PKCs lack the

Figure 1.12
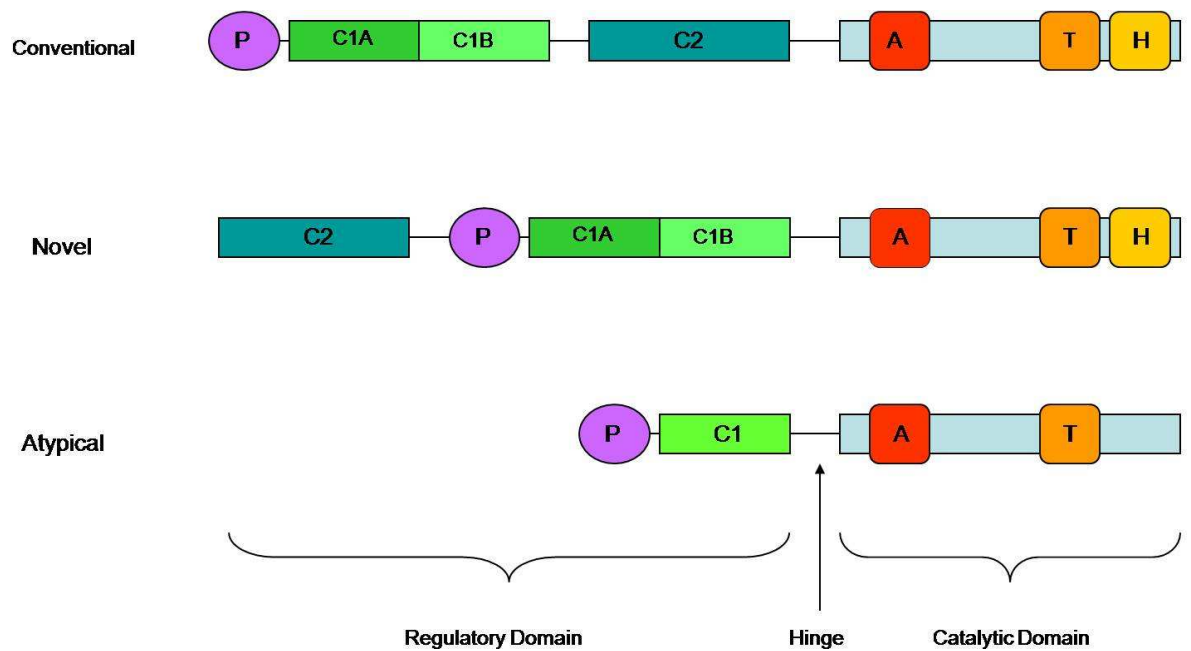
Figure 1.12 Structural representation of Protein Kinase C isotype structure and classification - All PKC isoforms contain a conserved catalytic domain that contains an ATP binding site (A) and a turn motif (T) and in conventional and novel isoforms, a hydrophobic motif (H) at the C-terminal of the catalytic domain. This region is attached to the regulatory region by a hinge. The regulatory domain is comprised of two membrane targeting modules, C1 and C2. The C2 domain is involved in calcium binding and is absent from atypical PKC structure, and unable to bind calcium in novel PKCs. All three groups of PKC isoforms contain the C1 domain of which two repeats, C1A and C1B, are resent in conventional and novel PKCs where it acts as a DAG/phorbol ester binding site, while atypical PKCs possess a single repeat. All isoforms contain a psuedosubstrate domain upstream of the N-terminal of the C1 domain, which holds the isoform in a folded conformation until activation through binding to the C-terminal of the PKC protein.

latter (Corbalan-Garcia and Gomez-Fernandez, 2006). This phosphorylation rearranges PKC into a more stable conformation (Newton, 2003).

The regulatory domain comprises two membrane targeting modules, C1 and C2, acting as a DAG and phorbol ester binding site, and a calcium sensor respectively. The C1 domain is an 8 kDa cysteine rich motif (Corbalan-Garcia and Gomez-Fernandez, 2006) of which there are two repeats in conventional and novel PKCs, termed C1A and C1B, and just one in atypical PKCs. Each repeat forms a zinc finger-like motif (Brose and Rosenmund, 2002) that forms a single ligand binding site between two β sheets (Newton, 2003). In atypical PKCs this DAG binding site is disrupted, although the single C1 domain found in these isoforms still allows ceramide and PIP3 binding, and other protein-protein interactions through a Phox and Bern 1 (PB1) domain in this catalytic region, involved in mediation of interaction with scaffolding proteins that also contain PB1 domains (Mellor and Parker, 1998). It is this protein-protein interaction that regulates atypical PKCs, in addition to phosphorylation by phosphoinositide-dependent kinase 1 (PDK1) (Scott, 2003). All PKC isoforms possess an autoinhibitory pseudosubstrate that rests just upstream of the C1 domain, which is involved in holding the PKC in a folded conformation until activation by binding to the C-terminal region of the protein (Corbalan-Garcia and Gomez-Fernandez, 2006). This pseudosubstrate domain shares similarity with PKC phosphorylation sites, with the exception of an alanine residue present at the serine/threonine phosphorylation site (Mellor and Parker, 1998).

The C2 domain, present in conventional and novel PKCs but absent from atypical isoforms, is a 12 kDa region (Newton, 2003) that, in conventional PKCs, acts as a calcium activated membrane targeting motif (Corbalan-Garcia and Gomez-Fernandez, 2006). This domain consists of a β sandwich fold containing three distinct binding sites for calcium, termed Ca1-3. Calcium binding causes a conformational change, forming a cleft for binding of the acidic head of membrane phospholipids, such as phosphatidylserine, which targets and anchors PKC to the plasma membrane (Brose and Rosenmund 2002). Novel PKCs lack the crucial calcium binding residues so are unable to utilise this mechanism (Newton, 2003), but the C2 domain in these isoforms still serves an important function of mediating protein-protein interactions such as acting as a phosphotyrosine binding domain in PKCδ (Benes *et al.,* 2005).

1.4.3 Compartmentalisation of PKC signalling

Compartmentalisation of PKC isoforms, as in other signalling pathways, is a useful mechanism to ensure specificity of the signalling response. Many of the PKC isoforms have their own distinct subcellular locations, with a study in NIH3T3 cells showing isoforms to translocate upon activation to specific and diverse locations within the cell. PKCs α, βI, βII and η were observed to translocate to cytoskeletal areas, while PKCα also translocated to ER. PKCε also, along with γ and δ, translocated to the Golgi apparatus (Goodnight *et al.,* 1995) and this spatial distribution of isoforms is often obtained through the use of scaffold proteins. Some scaffold proteins such as RACK1 bind activated PKC. RACK1, as described above, is involved in controlling the PKC signal specificity by translocating and anchoring activated PKC to specific subcellular locations (Schechtman and Mochly-Rosen, 2001). The range of scaffold proteins shown to interact with PKC range from those which are specific to one isoform, such as the PKCα scaffold plakophilin2 (Bass-Zubek *et al.,* 2008) and some binding to many, like AKAPs. AKAPs are well documented as facilitating compartmentalisation of PKC and have been shown, in some cases, to also bind PKC. Two examples of this are AKAP12, also known as Gravin, which targets both PKC and PKA to the cytoskeleton (Nauert *et al.,* 1997) and is particularly shown to bind PKCα and PKC βII (Piontek and Brandt, 2003), and AKAP79, which forms a complex with PKA, PKC and the protein phosphatase 2B (Perkins*, Wang et al.* 2001). AKAP79 has been shown to bind a number of the PKC isoforms, including α, βII, δ, ε and ζ (Faux *et al.,* 1999).

PKCδ has been shown to be involved in differentiation and cell growth, and has been linked to apoptosis and tumour development (Gschwendt, 1999). PKCγ, found in high concentrations in the brain, has been indicated as important in learning and memory, while PKCε is believed to be involved in GABA$_A$ receptor function (Brose and Rosenmund, 2002). PKC θ and ζ have been linked to T cell and B cell receptor signalling respectively, and PKCβ has been indicated in some immunodeficiency disorders and in lung, gastric and breast cancers, as has PKCα (Mackay and Twelves, 2007). PKCα differs from other isotypes as it is found in all tissue types. Additionally, PKCα requires phosphorylation at 3 residues located within its kinase domain before activation can occur (Nakashima, 2002). Like other isoforms, PKCα is involved in a multitude of biological processes including proliferation, cell migration and adhesion, and cell differentiation and has also been linked to haemopoeisis and apoptosis through its ability to phosphorylate Bcl-2 (Ruvolo *et al.,* 1998). Additionally PKCα has been indicated to play a crucial role in several disease

states, including cardiac hypertrophy, cancer, and immune diseases. Within cells, PKCα has been linked to several key functions including the alteration of tight junctions which act as barriers between cells to protect the cells internal balance. An increased concentration of PKCα activity alters the ability of the cells to form these tight junctions properly, allowing foreign molecules into the cell, which has been linked to some cancers. Supporting this evidence is that several endothelial cancers have been shown to exhibit higher than normal levels of PKCα activity (Rosson *et al.,* 1997). PKCα has also been linked to hypertrophy in myocardial tissue (Dorn and Force, 2005) and increased levels of PKCα have been shown to interfere with contractility. Additionally PKCα is thought to be involved in regulation of erythrocyte development, aiding the differentiation of erythroid progenitor cells (Myklebust *et al.,* 2000). Several of the isoforms have been shown to have an effect on cell function, such as cell division and migration, through the ERK cascade. PKC phosphorylates, or mediates the phosphorylation of Raf, a mitogen activated protein kinase kinase kinase (MAP3K), an upstream component of the ERK1/2 signalling cascade (Carroll and May, 1994).

1.4.4 Interaction between RACK1 and PKC

The expression of RACK1 throughout a variety of tissues indicates an important functional role (McCahill *et al.,* 2002). In addition because of its importance in PKC signalling, it is likely that RACK1 plays a crucial role in areas where PKC has a vital function. Investigations into the presence of RACK1 in rat brains has found a reduction in levels by almost half in aged rats, compared to their younger counterparts as well as PKCβ translocation loss (Pascale *et al.,* 1996). PKC isozymes are believed to have an important role in learning and memory function due to their high levels in the brain. PKC is also involved in other brain functions such as synaptic efficiency and neurotransmitter release (Matthies *et al.,* 1987). In some diseases of the brain, such as Alzheimer's disease, PKC activity and translocation is attenuated. Interestingly, PKCβII levels remain unchanged in both cytosolic and membrane fractions, while RACK1 levels are decreased in both fractions, indicating a cause for the impaired PKC signalling pathway (McCahill *et al.,* 2002). Another potential role for RACK1 in the brain is in drug dependence mechanisms. For example, ethanol interferes with PKC action on a number of neurotransmitters such as glycine, glutamate and GABA (McCahill *et al.,* 2002). As ethanol is able to translocate RACK1 to the nucleus, it is possible that this is associated with the altered action of PKC. Brain levels of RACK1 have also been shown to be modulated in morphine treated rats

alongside PKCα and β levels, where the drug is able to control expression levels of PKC isozymes.

1.5 Mitogen Activated Kinase Signalling

A common mechanism of signal transduction involves the sequential activation of protein kinases in a cascade. One such cascade is the Mitogen Activated Protein Kinase (MAPK) cascade (Figure 1.13). MAPKs were first identified as being activated in response to growth factor stimulation of the cell, giving the kinase its name (Seger and Krebs, 1995). MAPK cascades are initiated by either a small GTP-binding protein, such as a Ras family protein, or via an adaptor protein (Rubinfeld and Seger, 2005). There are four MAPK cascades, each distinct and named for the subgroup which their MAPK components fall into. These are the p38 MAPK cascade and the c-Jun N-terminal kinase (JNK) cascade, both involved in the stress response and apoptosis; the big mitogen activated protein kinase (BMK) cascade involved in mitogenic and stress signals; and the ERK 1/2 cascade, involved in cell proliferation and differentiation (Shaul and Seger, 2007).

1.5.1 The extracellular regulated kinase (ERK) cascade

The first MAPK cascade identified and greatly studied is the ERK1/2 cascade (Seger and Krebs, 1995), which comprises between three and six levels of protein kinases activated sequentially to form the cascade (Figure 1.14). Activation of the ERK cascade can occur through the action of extracellular stimuli, including neurotransmitters, hormones and growth factors acting via several mechanisms such as GPCRs, ion channels and tyrosine kinase receptors (Rubinfeld and Seger, 2005). Adaptor proteins such as Crk and GRB2 (Growth Factor Receptor Bound Protein 2) link the receptor to a GEF, transducing the signal to small GTP-binding proteins such as Ras and Rap1 which in turn activate the main cascade (Shaul and Seger, 2007). This protein then transmits the signal to the next level of the cascade, known as MAP3K, which includes the Raf family of Raf-1, B-Raf and A-Raf. Raf is then able to activate the MAPKKs, MEK1 and 2 via serine phosphorylation at two residues, Ser218 and Ser222 at the activation loop (Alessi *et al.,* 1994). Although phosphorylation of one residue allows activation, this is only partial and phosphorylation of both residues is required for full activation (Seger *et al.,* 1994). MEKs can phosphorylate both Tyr and Thr residues on ERK, a unique ability that classes them as dual

Figure 1.13
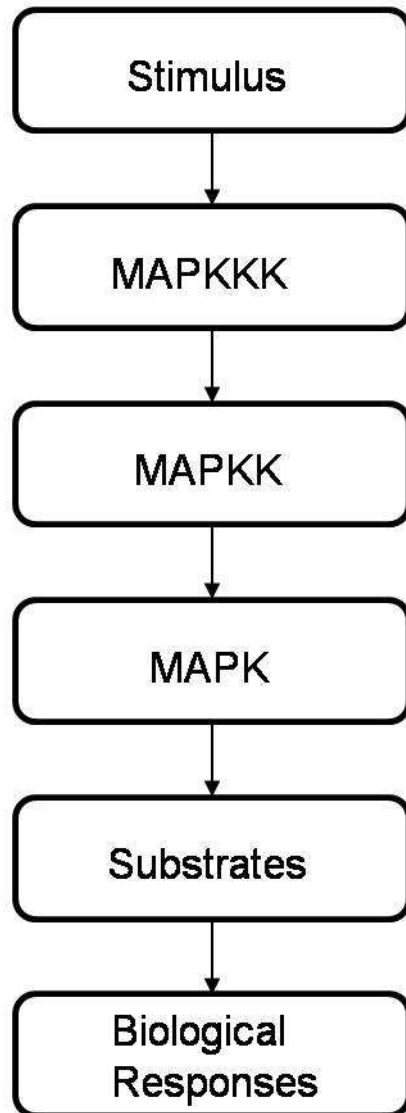
Figure 1.13 The MAPK cascade - All four MAPK pathways follow a similar cascade-MAPK kinase kinase (MAPKKK) is activated by a stimulus to activate a MAPK kinase (MAPKK) which in turn activates the MAPK for which the cascade is named. This MAPK can then act on specific substrates to illicit a biological response.

Figure 1.14
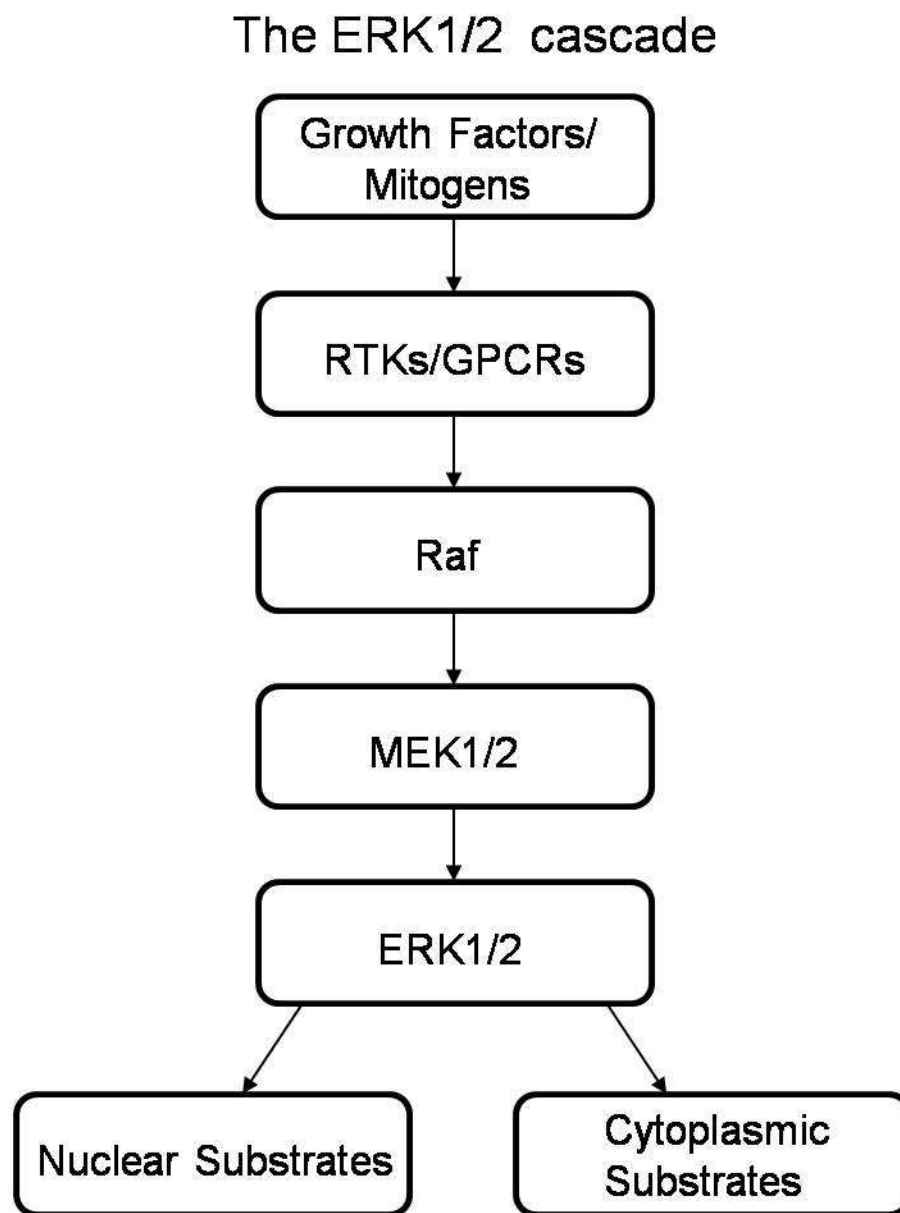
Figure 1.14 The ERK cascade - The ERK cascade is initiated by growth factors and mitogens activating receptor tyrosine kinases (RTKs) or G-protein coupled receptors (GPCRs) to activate Raf isoforms. Raf activates MEK1/2 which activates ERK1/2. ERK is then able to interact with cytoplasmic substrates, or to translocate to the nucleus to interact with substrates there and initiate transcription.

specificity protein kinases. Activation of ERK occurs when MEKs phosphorylate first Tyr185, followed by Thr183 (Rubinfeld and Seger, 2005).

There are 3 MEKs- MEK1, a 45 kDa protein, MEK2 (46 kDa) and MEK1b (43 kDa), an inactive, alternately spliced variant of MEK1 with unknown physiological roles and lacking the ability to activate ERK or undergo autophosphorylation. MEKs contain a regulatory N-terminal domain surrounding a catalytic kinase domain, followed by a short C-terminal domain (Shaul and Seger, 2007). As well as activating ERK, they act as cytoplasmic anchors, holding ERKs at specific locations within the cell (Rubinfeld et al., 1999). Downregulation of MEKs mainly occurs by dephosphorylation at Ser218 and Ser222 by PP2A, a phosphoserine/threonine phosphatase (Sontag et al., 1993).

MEKs are highly selective for ERKs, which they activate by phosphorylation at the residues Tyr185 and Thr183 at the activation loop. ERK1/2 are serine/threonine kinases of the MAPK group of proteins, which possess the ability to phosphorylate a wide range of proteins, the majority of which are regulatory proteins. There are two genes encoding ERKs, ERK1 which gives a 44 kDa protein, and ERK2, a 42 kDA protein, as well as alternative spliced variants, all of which share the Thr-X-Tyr motif at the activation site (Boulton et al., 1991). ERK1/2 are proline-directed protein kinases, phosphorylating serine or threonine residues that are adjacent to proline resides. ERKs possess a catalytic domain surrounded by regulatory regions containing the activation motif, and a C-terminal area important in interaction with MEK (Rubinfeld et al., 1999) and also with other proteins, termed the common docking (CD) domain. (Tanoue et al., 2000). ERKs are inactivated by dephosphorylation at either one or both of the Thr/Tyr residues, mediated by MAPK phosphatases, Ser/Thr phosphatases such as PP2A, or protein-Tyrosine phosphatases such as PTP-S2 (Shaul and Seger, 2007). While the components of the ERK cascade are located within the cytosol, activation by external stimuli causes translocation of Raf-1 to the membrane (Leevers et al., 1994), and of MEKs and ERKs to the nucleus (Jaaro et al., 1997) where they accumulate for extensive periods, remaining even when inactive. This suggests the nucleus as a potential site of ERK signal termination (Volmat et al., 2001).

Hundreds of substrates for ERK have been identified, and as well as targeting a number of kinases which are further involved in signal transduction to target proteins, such as Ribosomal S6 Kinases (RSKs), Mitogen- and stress-activated kinases (MSKs), and

MAPK-interacting kinases (MPKs), ERK also activates the cytoskeletal proteins paxillin and synapsin1; cytosolic phospholipase A2 which is involved in regulation of arachidonic acid release; and transcription factors such as ELK1, c-Fos and p53. ERKs have also been shown to act on MAPK cascade proteins upstream of itself, including MEKs, Raf-1 and growth factor receptors, creating a feedback mechanism to control its own activity (Dougherty *et al.,* 2005).

1.5.2 Functions of the ERK cascade

Due to the large number of proteins identified as being phosphorylated by ERK, ERK has been linked to a number of physiological roles and disease states. The most extensively studied role of ERK is its involvement in cell proliferation and, by extension, its role in tumour development. ERKs are rapidly activated by mitogenic signals, the transduction of which lead to proliferation at an accelerated level. If unchecked, this accelerated proliferation can lead to oncogenesis (Hoshino *et al.,* 1999) and a number of oncogenes have been shown to encode proteins which are involved in signalling via the ERK cascade (Zhang and Lodish, 2004). Additionally, a specific MEK inhibitor has been shown to inhibit mouse tumour growth (Sebolt-Leopold *et al.,* 1999). Elevated levels of ERK activation have been observed in a number of cell lines derived from a wide range of organs (Rubinfeld and Seger, 2005). ERKs located in the brain have also been implicated in learning and memory function (Berman *et al.,* 1998), and activation of ERK has been shown to be required for cell cycle switch from G1 to S phases in a number of cells, allowing DNA replication (Pages *et al.,* 1993). ERK has also been implicated in monocyte differentiation (Kharbanda *et al.,* 1994) and T cell maturation (Alberola-Ila *et al.,* 1995), as well as having anti-apoptotic ability wherein reduction in ERK activation is required for the apoptotic response. Upstream of ERK itself, Raf-1 has been shown to prevent apoptosis through phosphorylation of Bad, which prevents its interaction with Bcl-2 (Blagosklonny *et al.,* 1997).

As with other signalling pathways, specificity of ERK cascade effects is important, as is determined through a number of factors. These include control of the length and intensity of the signal, localisation within the cell and the use of scaffold proteins, and crosstalk with other signalling pathways. Duration and strength of the ERK cascade signal is a useful mechanism of controlling specificity as transient and prolonged ERK activation causes

varying cellular effects, through regulation by phosphatases or feedback loops leading to downregulation of upstream components (Marshall, 1995).

1.5.3 Compartmentalisation of ERK signalling

Compartmentalisation of the ERK cascade to specific subcellular locations also allows for control of specificity of the cascade effects (Harding *et al.,* 2005), with cytoplasmic distribution of ERK controlled by a variety of scaffold proteins (Chuderland and Seger, 2005). ERK is released from these proteins upon extracellular stimulation and can then translocate to the nucleus via a mechanism still largely unknown, although homodimerisation of ERKs (Khokhlatchev *et al.,* 1998) and interaction with nuclear pore proteins (Matsubayashi *et al.,* 2001) have been proposed as possible mechanisms of translocation. Scaffold proteins are utilised to bring ERK cascade components to specific subcellular locations and into the vicinity of other components and effectors. There are a number of scaffolds shown to be involved in the ERK cascade, each having distinct localisations and effects on the cascade. Some serve as spatial regulators of the ERK cascade. An example of this is Sef, a transmembrane protein located on the Golgi apparatus which is involved of activated MEKs, which allows ERK phosphorylation. This interaction prevents ERK translocating to the nucleus, allowing ERK to phosphorylate proteins in the cytosol (Torii *et al.,* 2004). MEK Partner 1 (MP1) acts in a similar way, localising to endosomes where it specifically promotes activation of ERK1, and shows no interaction with ERK2 or MEK2 (Teis *et al.,* 2002), while paxillin is involved in ERK activation at focal adhesion sites, binding inactivate ERK, MEK and active Raf. Activated ERK phosphorylates paxillin, allowing binding of focal adhesion kinases, resulting in focal adhesion complex formation and epithelial morphogenesis (Ishibe *et al.,* 2004). Other scaffolds, such as the multi-domain protein KSR (Kinase Suppressor of Ras), are involved in facilitating the activation of ERK at the plasma membrane, where it acts as a scaffold for ERK, MEK and Raf (McKay and Morrison, 2007).

1.5.4 Crosstalk between the ERK and cyclic AMP signalling pathways

Crosstalk with other pathways is also an important method of specificity and control of the ERK cascade. ERK has been shown to exhibit crosstalk with several other pathways, perhaps most importantly the cyclic AMP signalling pathway (Figure 1.15). The mechanisms by which crosstalk occurs between the cyclic AMP and ERK signalling pathways is both complex and controversial. In 1993, three studies were published

Figure 1.15
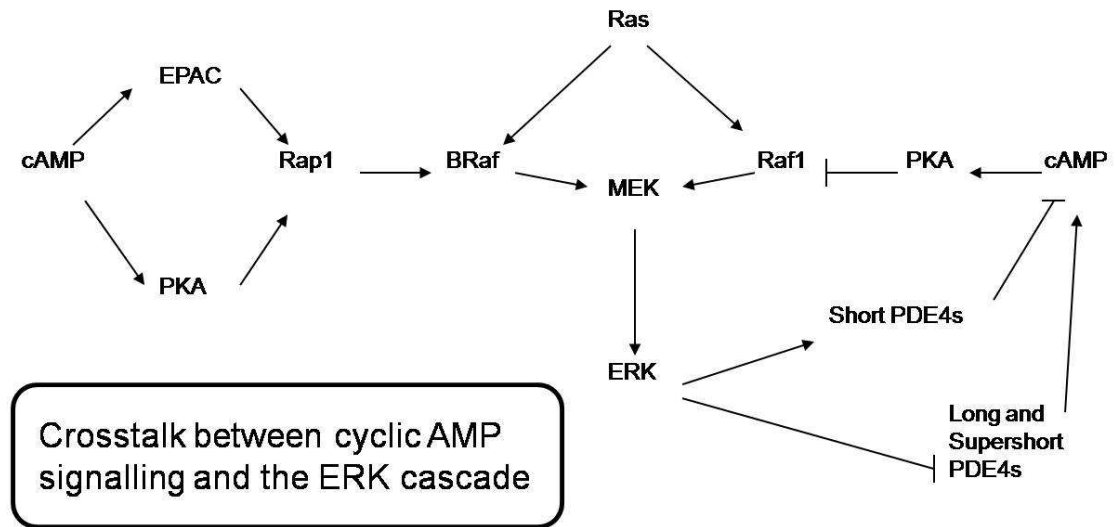
Figure 1.15 Crosstalk between the cyclic AMP and ERK pathways - The cyclic AMP and ERK pathways have been shown to have cell specific points of crosstalk. Cyclic AMP has been shown to activate ERK via Rap1 action on B-Raf either via PKA or in a PKA-independent manner through EPAC. PKA has also been shown to inhibit ERK activation in response to elevated cyclic AMP levels by inhibition of Raf-1 action. ERK itself is able to act on cyclic AMP specific phosphodiesterases, activating short forms isoforms to increase cyclic AMP levels, or inhibiting long and super-short forms to reduce cyclic AMP concentrations, forming a feedback loop.

identifying the ability of cyclic AMP to block ERK activation stimulated by growth factors in Rat1 (Wu *et al.,* 1993; Cook and McCormick, 1993) and NIH3T3 fibroblasts (Burgering *et al.,* 1993). Since then, investigations in other cell types have shown several instances of crosstalk between the pathways.

There are a number of distinct points of crosstalk between the cyclic AMP and ERK signalling pathways. The most closely examined of these is the Raf family of protein kinases, which in an isoform specific manner is able to effect ERK activation in both a positive and negative manner in response to cyclic AMP (Houslay and Baillie, 2003). Three genes encode for the isoforms A-Raf, B-Raf and C-Raf, also known as Raf-1. All three share a common structure and all activate MEK. Rafs are activated at the plasma membrane with phosphorylation of different sites for each isoform a requirement of activation (Dumaz and Marais, 2005). Raf1 has been shown, in several cell types including vascular muscle cells and fibroblasts, to be involved in blocking ERK activation (Stork and Schmitt, 2002). Phosphorylation of Raf1 at residue Ser259 by either PKA or Akt/PKB blocks activation of Raf1 by Ras, effectively blocking the ERK cascade. PKA has also been suggested to phosphorylate Raf1 at Ser621 to inhibit its action and block the cascade (Houslay and Kolch, 2000). Another model of cyclic AMP influence on ERK via Raf1 is theorised in Dumaz and Marais (2005) wherein the binding of the regulatory protein 14-3-3 to phosphorylated sides on Raf1 holds the Raf isoform in a closed and inactive conformation. Ras activation would then cause translocation of Raf1 to the plasma membrane, displacing 14-3-3 and allowing activation of Raf1. However, in the presence of high cyclic AMP levels, PKA phosphorylation of Raf1 would create an additional binding site for 14-3-3, where Ras activation couldn't cause its dissociation, thus inhibiting both Raf1 activation and translocation to the plasma membrane (Dumaz and Marais, 2005).

Another Raf isoform, B-Raf, has also been linked to the involvement of cyclic AMP in ERK signalling, causing activation of ERK in PC12 and other neuronal cells through the small GTPase Rap1 (Houslay and Kolch, 2000). B-Raf is found in abundance in the brain, and in a range of other cells from endothelial to endocrine, as well as in various organs (Stork and Schmitt, 2002). There is much debate as to the mechanism by which activation of B-Raf leads to ERK activation and whether this occurs in a PKA dependent or independent manner via EPAC, which is well documented as a GEF for Rap1 (Houslay and Kolch, 2000). The Src family of kinases and PP1 have also been suggested to be involved in this mechanism (Dumaz and Marais, 2005). While PKA has been shown to phosphorylate B-Raf in PC12 cells, experiments in FRTL-5 cells showed EPAC to mediate

Rap1 activation of B-Raf in response to elevated cyclic AMP levels (Dumaz and Marais, 2005).

The outcome and mechanisms of crosstalk between ERK and cyclic AMP appears to be a cell type specific occurrence, as demonstrated by the ability of ERK to interact with PDE4 isoforms. ERK has been shown to inactivate long form PDE4s by phosphorylation, thus causing an increase in cyclic AMP levels which in turn inhibits ERK activation, creating a negative feedback loop that allows ERK to suppress its own activation. Conversely, ERK has also been shown to activate short form PDE4s, promoted the breakdown and therefore reduction in cyclic AMP levels within the cell (Houslay and Kolch, 2000). The actions of ERK on controlling cyclic AMP levels therefore depends on the relative amounts of long and short form PDE4s within the cell, showing that self control of ERK signalling via cyclic AMP depends on the cell type and PDE4 distribution. Indeed, even within the same cell, the effects of cyclic AMP on ERK activation can differ depending on culture conditions, as demonstrated in PC12 cells. In response to nerve growth factor (NGF) PC12 cells display cyclic AMP activation of Rap1 through EPAC, which causes a sustained activation of B-Raf and ERK by Rap1. However, in response to epidermal growth factor (EGF) a transient ERK activation by cyclic AMP via activation of Raf-1 mediated by Ras can be seen in PC12 cells (as reviewed in Dumaz and Marais, 2005). These different results suggest that the control exhibited by cyclic AMP over the ERK cascade, and vice versa, is clearly cell type specific.

1.6 Thesis aims

The aim of this research is to identify novel mechanisms of crosstalk between the cyclic AMP and PKC signalling pathways. In Chapter 3, we elucidate on the RACK1/PDE4D5 complex and its role as a point of crosstalk between the cyclic AMP and PKC pathways by testing the hypothesis that binding to RACK1 affects PDE4D5 targeting, activity and regulation by PKC. In Chapter 4 the role of both cyclic AMP and PKC in the control of cytokine activity by the SOCS-3 protein is outlined, investigating the hypothesis that PKC isoforms are involved in the induction of SOCS-3 by cyclic AMP via EPAC. Finally, in Chapter 5, the theory that cyclic AMP via EPAC influences PKC autophosphorylation in COS1 cells is investigated.

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 General reagents

All reagents were ordered from Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK or Sigma-Aldrich Company Ltd, Gillingham, Dorset, UK unless stated below.

Reagents	Supplier
Syringes	BD Biosciences, Oxford, UK
26½ gauge needles	BD Biosciences, Oxford, UK
Thinwall Ultra-Clear™ 5 ml tubes, 13 x 51 mm	Beckman Coulter (UK) Ltd, High Wycombe, Buckinghamshire, UK
Protease inhibitor cocktail	Boehringer Ingelheim Ltd., Bracknell, Berkshire, UK
Bio-Rad Bradford protein assay reagent	Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK
All horseradish peroxidase (HRP)-conjugated secondary antibodies	GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK
Enhanced Chemiluminescence (ECL)	GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK
X-ray film	Kodak Ltd, Hemel Hempstead, Hertfordshire, UK
Pre-stained broad range protein marker (6-175 kDa)	New England Biolabs (UK) Ltd, Hitchin Hertfordshire, UK
Marvel low-fat milk powder	Premier Brands UK, Knighton, Adbaston, Staffordshire, UK
Complete and complete EDTA-free protease inhibitor tablets	Roche Diagnostics Ltd, Burgess Hill, UK
30% acrylamide/bisacrylamide	Severn Biotech Ltd, Kidderminster, Worcestershire, UK
Protran® nitrocellulose	Whatman plc, Maidstone, Kent, UK

2.1.2 Cell culture materials

Reagents	Supplier
BD Falcon™ 10 cm plates, 6-well plates, 12-well plates and 96-well plates	BD Biosciences, Oxford, UK
75 cm^2 flasks	Corning B.V. Life Sciences, Schiphol-Rijk, The Netherlands
DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulphate) Liposomal Transfection Reagent	Roche Diagnostics Ltd, West Sussex, UK
Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen Ltd, Paisley, UK
Foetal bovine serum (FBS, US origin)	Invitrogen Ltd, Paisley, UK
Lipofectamine™ 2000	Invitrogen Ltd, Paisley, UK
Newborn calf serum (NCS, New Zealand origin)	Invitrogen Ltd, Paisley, UK
Penicillin and streptomycin	Invitrogen Ltd, Paisley, UK
Phosphate Buffered Saline (PBS)	Invitrogen Ltd, Paisley, UK
0.25 % (w/v) Trypsin-EDTA	Invitrogen Ltd, Paisley, UK

2.1.3 Chemical reagents

Reagent	Properties	Working Concentration	Supplier
1, 2-diaoctanoyl-sn-glycerol	DAG analogue and PKC activator	10μM, 50μM, 100μM	Sigma-Aldrich
8-pCPT- 2'0-Me-cyclic AMP	Activator of EPAC	10μM	Biolog Life Science Institute
8-pCPT-cyclic AMP	Activator of EPAC and PKA	10μM	Biolog Life Science Institute
BAPTA-AM	Calcium chelator	10μM	Merck Biosciences
Forskolin	Activator of Adenylyl Cyclase	10μM	Merck Biosciences
GF 109203X	PKC inhibitor, with specificity towards α and βI isoforms	50μM	Merck Biosciences
Isoproterenol	β-Adrenoreceptor agonist, with specificity towards β1 and β2	10μM	Merck Biosciences
MG-132	Proteasome inhibitor	6μM	Merck Biosciences
Phorbol 12-myristate 13-acetate	PKC activator	10μM	Merck Biosciences
Ro31-7549	PKC inhibitor, with specificity towards α, βI and βII, γ and ε.	50μM	Merck Biosciences
Rolipram	PDE4 inhibitor	10μM	Merck Biosciences
U73122	PLC inhibitor	10μM, 50μM	Merck Biosciences
U73343	Negative control for U73122	10μM, 50μM	Merck Biosciences

2.1.4 Antibodies

Protein	Type	Dilution	Source	Supplier
EPAC1	Polyclonal	1:1000	Rabbit	generously provided by Professor Johannes Bos, University of Utrecht, The Netherlands
EPAC2	Polyclonal	1:1000	Goat	Santa Cruz
p42/44 MAP Kinase (ERK)	Polyclonal	1:1000	Rabbit	Cell Signaling Technology
Phospho-p42/44 MAP Kinase (ERK)	Polyclonal	1:1000	Rabbit	Cell Signaling Technology
GAPDH	Monoclonal	1:100000	Mouse	Ambion
Anti-HA	Polyclonal	1:1000	Rabbit	Sigma-Aldrich
PDZ-GEF1	Polyclonal	1:1000	Rabbit	Generously provided by Dr. Daniela Rotin, University of Toronto, Canada
PKCα	Polyclonal	1:1000	Rabbit	Cell Signaling Technology
Phospho-PKCα/βII	Polyclonal	1:1000	Rabbit	Cell Signaling Technology
PKCδ	Polyclonal	1:1000	Rabbit	Cell Signaling Technology
Phospho-PKCδ/θ	Polyclonal	1:1000	Rabbit	Cell Signaling Technology
PLC ε	Polyclonal	1:1000	Rabbit	Upstate Cell Signaling solutions
RACK1	Monoclonal	1:1000	Mouse	BD Transduction Laboratories
SOCS-3	Polyclonal	1:1000	Goat	Santa Cruz Biotechnology Inc
Anti Vesicular Stomatitis Viris (VSV)	Polyclonal	1:1000	Rabbit	Sigma-Aldrich

2.1.5 siRNA

siRNA	Target Sequence	Supplier	Cat #	Product Name
EPAC1	GCCCGGAACUUGCC UGUUU	Dharmacon	D-00 7676-03	siGenome siRNA Human RAPGEF3
PLC ε	CAGGGTCTTGCCAGT CGACTA	Qiagen	SI 00115521	HP GenomeWide siRNA Hs_PLCE1_1
PKCα	CGCAGTGGAATGAG TCCTTTA	Qiagen	SI 00605927	HP Validated siRNA Hs_PRKCA_6
PKCδ	AACTCTACCGTGCCA CGTTTT	Qiagen	SI 00301329	HP GenomeWide siRNA Hs_PRKCD_7

2.1.6 General Buffers

Laemmli Electrophoresis Sample Buffer 5x

10% (w/v) SDS, 300mM Tris HCl, pH 6.8, 0.05% (w/v) bromophenol blue, 50% (v/v) glycerol, 10% (v/v) β-mercaptoethanol

Lysis Buffer

10mM Tris HCl, pH 7.5, 0.1mM EDTA, containing protease inhibitor cocktail (Boehringer)

PBS

137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.47mM KH_2PO_4, pH 7.4

PDE Assay Buffer

40mM Tris HCl, 5mM $MgCl_2$, 3.75mM β-mercaptoethanol

Running Buffer 5x for SDS-PAGE

25mM Tris, 250mM glycine, 0.1% (w/v) SDS

TBS

20mM Tris HCl, pH 7.6, 137mM NaCl

TBST

20mM Tris HCl, pH 7.6, 137mM NaCl, 1% (v/v) Tween 20

Transfer Buffer for Western blotting

25mM Tris, 192 mM glycine, 20% (v/v) methanol

2.2 Methods

2.2.1 Mammalian Cell Culture

Cell culture techniques were performed using standard aseptic techniques.

2.2.1.1 Maintenance of cell lines

Established and confluent cells lines were resuspended in cell freezing medium (9ml cell growth medium, 1ml DMSO) in 1ml aliquots, frozen at -80°C and then transferred to liquid nitrogen storage. Cells were stored long term in liquid nitrogen, ensuring the integrity of the cell lines. Cells were revived by individual vials being quickly thawed and directly added to 10ml of pre-warmed growth medium. Cells were sedimented by centrifugation at 1000 x g_{max} for 5 minutes, the supernatant removed and the cell pellet resuspended in complete medium.

2.2.1.2 COS1 cells

The COS1 cell line is derived from African green monkey kidney cells, wherein the CV-1 cell line was immortalized via transformation with the SV40 virus (Jensen *et al.,* 1964).

Cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) foetal bovine serum, 2mM L-glutamine, 100U/ml Penicillin and 100μg/ml Streptomycin, at 37°C, 5% (v/v) CO_2. Cells were passaged at approximately 90% confluence by removing growth media and washing cells in 10ml of pre-warmed sterile phosphate buffered saline (PBS; Invitrogen). 2ml of trypsin-EDTA solution was added and the cells

were incubated at 37°C for 5 minutes. Cells were examined microscopically to ensure efficient cell detachment, and then 10ml of growth media was added to inactivate the trypsin-EDTA solution. Cells were centrifuged at 1000 x g_{max} for 5 minutes, the supernatant removed and the cell pellet resuspended in the required volume of growth media. 1ml of resuspended cells was added to 9ml of fresh grown media in sterile flasks. Cells were then incubated as above until required.

2.2.1.3 HEK293 cells

The human embryonic kidney 293 (HEK293) cell line were derived by transformation of human embryonic kidney cells with DNA from human adenovirus type 5 (Graham *et al.,* 1977). This cell line was cultured as described above for COS1 cells.

2.2.1.4 DOTAP liposomal transfection

Fresh medium was added to 100mm plates and a DOTAP/DNA mixture was prepared by diluting 7.5µg DNA in DMEM to 75µl in a microcentrifuge tube while 45µl of DOTAP was diluted in DMEM to 140µl in a separate microcentrifuge tube. The DNA solution was then transferred to the DOTAP solution and mixed by pipetting. The mixture was then incubated at room temperature for 15-30 minutes, before being added to the fresh medium in the appropriate plates. Cells were incubated overnight at 37°C, 5% (v/v) CO_2.

2.2.1.5 siRNA transfection

COS1 cells were seeded into 6 well plates at approximately $1x10^5$ cells/cm^2. Cells were then transfected with the appropriate siRNA at concentrations noted in the figure legends, using Lipofectamine 2000 (Invitrogen) transfection reagent according to the manufacturer's instructions. Transfection was repeated the following day, and experiments carried out 48 hours later.

2.2.1.6 Preparation of whole cell lysates

Confluent cells were harvested on ice into 1x Laemmli Electrophoresis Sample Buffer (Laemmli, 1970). The growth media was removed and cells were washed in chilled PBS. The PBS was then aspirated and an appropriate volume of the buffer was added. Cells were

then scraped into a 1.5ml microcentrifuge tube. Lysates were either used immediately, or frozen at -20°C until required.

2.2.1.7 Sub-cellular fractionation

Confluent cells were harvested on ice into an appropriate buffer. The growth media was removed and cells were washed in chilled PBS. The PBS was then aspirated and an appropriate volume of the required buffer was added. Cells were then scraped into a 1.5ml microcentrifuge tube. Lysates were either used immediately, or frozen at -80°C until required. Cells were then scraped into a 1.5ml microcentrifuge tube. Cells were homogenised by 7 strokes of a 26½ G needle and 1ml syringe, and then centrifuged at 1000 x g_{max} in a bench-top, refrigerated centrifuge for 5 minutes at 4°C. The supernatant was transferred to an ultra-centrifuge tube and the pellet (containing the P1 fraction: nuclei and unbroken cells) discarded. The supernatant was centrifuged in a bench top ultrafuge at 100,000 x g_{max} for 30 minutes at 4°C. The supernatant (SN fraction: enriched cytosolic proteins) was retained and the pellet (P2 fraction: plasma membrane, endoplasmic reticulum, Golgi vesicles, endosomes and lysosomes) resuspended in lysis buffer and centrifuged again at 100,000 x g_{max} for 30 minutes at 4°C. The resulting supernatant was discarded and the pellet resuspended in cell lysis buffer and placed in a 1.5ml microcentrifuge tube. SN and P2 fractions were either used immediately or frozen until required.

2.2.1.8 Protein concentration determination

To determine the protein concentration of cell lysates, a Bradford Assay was carried out, using bovine serum albumin (BSA) as the standard in this spectroscopic assay. In a clear, 96 well plate, a standard curve of known BSA concentrations between 0 and 10 µg was diluted to a final volume of 50µl with distilled water. The protein samples were diluted in distilled water at a 1:10 ratio, also to a final volume of 50µl. Bradford reagent from Bio-Rad was diluted 1:5 with distilled water and 200µl added to each well. This reagent causes a colour change from brown to blue, the intensity of which is directly proportionate to the concentration of the protein. The 96 well plate was read at 560nm using a microplate reader, which provided protein concentrations of each sample.

2.2.2 Biochemistry techniques

2.2.2.1 SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a common method of separating proteins based on their molecular weight. Protein samples at required concentrations were denatured in 1x Laemmli electrophoresis sample buffer (Laemmli, 1970). Samples were boiled for 5 minutes and then loaded into wells in either 10% (w/v) (8.4ml dH$_2$O, 5ml 1.5M Tris-HCl pH 8.8, 6.6ml 30% (w/v) acrylamide mix, 0.1ml 10% (w/v) SDS, 0.1ml 10% (w/v) ammonium persulphate, 0.01 TEMED) or 12% (w/v) (6.6ml dH$_2$O, 5ml 1.5M Tris-HCl pH 8.8, 8.4ml 30% (w/v) acrylamide mix, 0.1ml 10% (w/v) SDS, 0.1ml 10% (w/v) ammonium persulphate, 0.01 TEMED) polyacrylamide gel with a 5% stacking gel (6.8ml dH$_2$O, 1.25ml 0.5M Tris-HCl, 1.7ml 30% (w/v) acrylamide mix, 0.1ml 10% (w/v) SDS, 0.1ml 10% (w/v) ammonium persulphate, 0.01ml TEMED). 10μl of pre-stained molecular weight protein marker was also loaded into a well as a guide for determining the molecular weight of the sample proteins. Electrophoresis was carried out overnight at 14mA.

2.2.2.2 Western immunoblotting

Western immuno-blotting is a method of visualising proteins separated by SDS-PAGE. It allows the detection of the individual proteins using specific anti-sera. Proteins separated by SDS-PAGE were transferred to Whatman nitrocellulose membrane in transfer buffer at 300mA for 4 hours. Following transfer, Ponceau staining (0.1% (w/v) Ponceau S, 5% (v/v) acetic acid) was used to confirm effective transfection and protein loading. The nitrocellulose was then washed in 1x TBST for half an hour, then blocked in 5% (w/v) milk powder (Marvel) or 5% (w/v) BSA in TBST for 30 minutes at room temperature. Primary antibody was added at required dilution (see section 2.1.4) in a 1% (w/v) milk powder/TBST or BSA/TBST solution. The nitrocellulose and primary antibody were sealed in a plastic container and incubated either overnight at 4°C or for 1 hour at room temperature. The nitrocellulose was then washed with TBST for 3x 10 minutes washes, and an appropriate horseradish peroxidase (HRP) conjugated anti-immunoglobulin G (IgG) secondary antibody added, diluted 1:2500 in 1% (w/v) milk powder/TBST or BSA/TBST solution. Nitrocellulose and secondary antibody were incubated for 1 hour at room temperature in a sealed plastic container. The nitrocellulose was then washed with TBST

and the enhanced chemiluminescence (ECL) western immuno-blotting kit was used to visually detect the bound antibodies. After being treated with ECL solution the membrane was exposed to blue-light sensitive autoradiography film, which was developed using the Kodak X-Omat processor.

2.2.2.3 Phosphodiesterase activity assay

Measurement of PDE activity was carried out using a radioactive cyclic AMP hydrolysis assay as previously described (Bolger *et al.,* 1997). Hydrolysis of cAMP by PDE isoforms results in 5'AMP being formed. In this assay, cyclic AMP is hydrolysed along with $[8-^3H]$ adenosine 3', 5'-cyclic phosphate (Amersham). Snake venom from *Crotalus atrox* (Sigma-Aldrich) is added to prevent the recirculation of hydrolysed cyclic AMP by further hydrolysis to adenosine, while the Dowex (Sigma-Aldrich) binds the non-hydrolysed cyclic AMP (Bolger *et al.,* 1997).

Preparation and activation of Dowex-1

Dowex slurry was prepared by washing Dowex in 1M NaOH for 15 minutes. The resin was allowed to settle and the fines decanted. Distilled water was added and left overnight, and then the resin was washed with distilled water until pH 9.0. The resin was then washed for 10 minutes in 1M HCl, and allowed to settle. The resin was washed with distilled water until pH 4.0 and then made up with distilled water at a 1:1 ratio and stored at 4°C until required. This slurry was used in the assay after the addition of 100% ethanol at a 2:1 ratio.

Assay Procedure

PDE activity was assayed as a measurement of cyclic AMP hydrolysed by PDE. The assay used a 0.2µM cAMP solution containing 5µCi $[^3H]$ cAMP (Amersham) per ml prepared in PDE assay buffer. 50µl of this substrate solution was placed in a 1.5ml microcentrifuge tube with an appropriate amount of cell extract diluted to 50µl in assay buffer. This mixture was incubated for 10 minutes at 30°C and then boiled for 2 minutes to stop the reaction by inactivating the PDE. Tubes were transferred to watery ice and left to cool completely. 25µl of 1mg/ml snake venom from *Crotalus atrox* was then added and the tubes incubated for 10 minutes at 30°C. The tubes were then returned to ice and 400µl of previously prepared Dowex/ethanol slurry was added to each tube. The tubes were left on ice for 15 minutes, with occasional vortexing, and then centrifuged at 13000rpm for 2 minutes at 4°C in a refrigerated bench top centrifuge. 150µl of supernatant from the

reaction tubes was added to scintillation tubes containing 4ml Ecoscint scintillant fluid, with total counts being obtained by the addition of 50µl cAMP substrate solution added to 4ml of scintillant and then assayed in a scintillation counter.

Determination of Phosphodiesterase Activity

The resulting data was processed using the following calculation to give the cyclic AMP activity for each sample:

(Net counts per minute/total counts per minute) x (392/150) x (total cyclic AMP (pmol)/time (min)) x (100/47), wherein 392/150 is the volume correction for the volume of supernatant measured in scintillant fluid and 100/47 provides the reaction correction factor as 47% of the reaction binds to the Dowex slurry (Bolger *et al.,* 1997).

2.2.2.4 RACK1 Co-immunoprecipitation experiments

Equal amounts of cell pellet or supernatant protein were prepared in equal amounts of lysis buffer. Following this 0.1% (v/v) Triton X-100 was added to each sample and incubated on ice for 15 minutes. At this stage 50µl per sample was retained and boiled in Laemmli electrophoresis sample buffer (Laemmli, 1970) as input controls. Samples were pre-cleared by adding 5µg of mouse IgM (Sigma-Aldrich) and 50µl anti-mouse IgM agarose (Sigma-Aldrich) to each sample. Samples were then incubated on an orbital shaker for 30 minutes at 4 ºC. Beads were then pelleted and the supernatant transferred to fresh microcentrifuge tubes. Duplicates of each sample were then treated with either 5µg of anti-RACK1 antibody (IgM clone) or 5µg mouse IgM and incubated on a wheel in cold room for 30 min. 50µl of anti-mouse IgM-agarose was then added to each microcentrifuge tube and samples incubated at 4 ºC for a further 30 minutes. The agarose beads were then pelleted for 30 seconds at 13000 x g_{max} and washed 3 times with 500µl lysis buffer. Beads were then boiled in electrophoresis buffer and separated by SDS-PAGE along with the retained input controls.

2.2.2.5 Densitometry and statistical analysis

Non-saturating exposures from multiple experiments were quantified densitometrically using ImageJ software. Densitometric values were normalized to the expression of either GAPDH or RACK1 protein levels in the same sample. Statistical significance was determined using Student's two-tailed t-test.

CHAPTER 3

Coordination of Regulatory Crosstalk Between the Cyclic AMP and Protein Kinase C (PKC) Signalling Pathways by the Receptor for Activated C-Kinase 1 (RACK1)/ Cyclic AMP-specific Phosphodiesterase 4D5 (PDE4D5) Signalling Complex

3.1 INTRODUCTION

Cyclic AMP-specific phosphodiesterases (PDEs) are crucial components of the cyclic AMP signalling system and represent the only means of reducing cyclic AMP levels in cells through hydrolysis to 5'AMP (Lugnier, 2006). Of the 11 known classes of cyclic nucleotide PDEs, eight are able to hydrolyse cyclic AMP (Bolger *et al.,* 1997; Houslay and Adams, 2003). The PDE4 cyclic AMP-specific group is widely expressed in a variety of tissues and accounts for the majority of cyclic AMP hydrolysis activity in cells (Conti *et al.,* 2003). PDE4 isoforms are encoded by four genes, termed A-D, that through complex alternate splicing of cognate mRNAs, give rise to around twenty PDE isoforms (Conti *et al.,* 2003). The PDE4D group is distinguishable by unique N-terminal regions that have been found to readily interact with other cellular proteins (Conti *et al.,* 2003). For example, the phosphodiesterase PDE4D5 has been found to interact with the ubiquitously expressed, WD40 signalling scaffold protein, RACK1 (Yarwood *et al.,* 1999; McCahill *et al.,* 2002) although to date the precise function of this signalling complex is largely unknown. Whether RACK1 binding to PDE4D5 effects the intracellular localisation of the complex or a conformational change of the bound PDE, or has any influence on PDE activity, has yet to be determined. However, it has been demonstrated that interaction with RACK1 *in vitro* has been shown to increase the sensitivity of PDE4D5 to the PDE4-selective inhibitor, rolipram, which suggests that RACK1 may have some influence on the conformation of bound PDE4D5 (Yarwood *et al.,* 1999). In addition, the influence of RACK1-binding partners, other than PDE4D5, on the status of PDE4D5 is largely unknown and may reveal key areas of novel signalling regulation and crosstalk.

The interaction between PDE4D5 and RACK1 is highly specific since, to date, RACK1 has not been shown to bind to any other members of the cyclic AMP specific PDE4 family, nor does PDE4D5 bind to other WD40 repeat proteins (Yarwood *et al.,* 1999). RACK1 and PDE4D5 are believed to interact in a manner similar to the heterotrimeric G-protein subunits, Gβ and Gγ (McCahill *et al.,* 2002). In this model blades 5, 6 and 7 of the RACK1 β-propeller form a groove to which the N-terminal of PDE4D5 binds (McCahill *et al.,* 2002). An 88 amino acid sub-domain, termed the RACK1 interaction domain (RAID1), located in the N-terminal region of PDE4D5 was identified as an essential requirement for RACK1 binding (Bolger 2002). In addition, a low affinity RACK1 binding site has also been located in the PDE4D5 catalytic domain (Bolger *et al.,* 2006). Further studies have shown that while PDE4D5 is only able to bind one protein at a time (Bolger *et al.,* 2006), it

is likely that RACK1 is able to bind other proteins at the same time as PDE4D5 such as Src (Mamidipudi *et al.,* 2004), integrin β-subunits (Liliental and Chang, 1998) and PKC isoforms (McCahill *et al.,* 2002). This ability raises questions as to whether additional proteins binding to RACK1 influence the function and specificity of the complex, with possible impact on the function of RACK1-bound PDE4D5. This chapter aims to investigate this eventuality by exploring whether RACK1 binding affects PDE4D5 targeting, activity and regulation by the classical RACK1-interacting partner, PKCα (see section 1.4.4).

3.2 MATERIALS AND METHODS

All Materials and Methods as outlined in Chapter 2 with variations described below.

Thermodenaturation

Microcentrifuge tubes containing equal amounts of protein from fractionated samples were placed in a 45ºC water bath at 1 minute intervals. After a total time of 10 minutes, all samples were placed on ice and used in a phosphodiesterase enzyme assay.

Densitometry and Statistical Analysis

Non-saturating exposures from multiple experiments were quantified densitometrically using ImageJ software. Densitometric values of PDE4, anti-VSV and PKCα levels were usually normalised to the expression of RACK1 protein levels, as a loading control, in the same sample. Statistical significance was determined using Student's two-tailed t-test.

3.3 RESULTS

3.3.1 Isoproterenol promotes PDE activation in membrane and cytosolic fractions of HEK293 cells - As a first step to elucidating whether or not interactions with RACK1 affect the activity and intracellular localisation of PDE4D5 we first sought to determine the impact of elevating intracellular cyclic AMP on the activation of PDE4 isoforms in HEK293 cells. HEK293 is a well characterised cell line that has been used previously in the study of cyclic AMP signalling and in particular the interaction of RACK1 and PDE4D5 (Lynch *et al.,* 2007). PDE4 isoforms are responsible for 68% of PDE activity in HEK293 cells, with PDE4B2, PDE4D3 and PDE4D5 providing the majority of this activity (Lynch *et al.,* 2005). Cells were treated for various times in the presence or absence of 10μM isoproterenol, fractionated into high speed pellet and cytosolic S/N fractions and assessed for PDE activity in the presence or absence of the PDE4 specific inhibitor, rolipram, as described in Materials and Methods. Although the fractionation technique is crude, it is sufficient to show differences between cytosolic and membrane activity and is advantageous over use of whole cell lysates for this reason. Isoproterenol is a β2-adrenoreceptor agonist which increases production of cyclic AMP in cells through the activation of adenylyl cyclases by $G_{s\alpha}$ (Higgins and David, 1976) and has been shown to affect cyclic AMP levels in HEK293 cells up to a 13-fold increase (Jones *et al.,* 2003). Protein levels were assessed by Bradford assay to ensure equal amounts of protein were analysed. Analysis of total PDE activity in the pellet fraction showed a significant increase in PDE activity following 15 minutes isoproterenol stimulation, which was ablated by inclusion of rolipram in the assay (Figure 3.1). This indicates that isoproterenol promotes activation of PDE4 isoforms in this fraction of HEK293 cells. Analysis of the supernatant fraction demonstrated a similar trend, with isoproterenol stimulating cell PDE activity, peaking at 15 minutes with an approximate 1.6 fold increase when compared to non-stimulated cells. Again, inclusion of rolipram in PDE assays completely ablated isoproterenol-stimulated PDE activity in supernatant fractions indicating that isoproterenol specifically activates PDE4 isoforms in HEK293 cells. These results show that an increase in cyclic AMP levels following isoproterenol stimulation causes a significant increase in PDE4 activity in soluble and particulate fractions of HEK293 cells.

3.3.2 RACK1 and PDE4D5 do not undergo activation-coupled translocation in HEK293 cells - Although RACK1 and PDE4D5 have been established to form a stable complex in

Figure 3.1
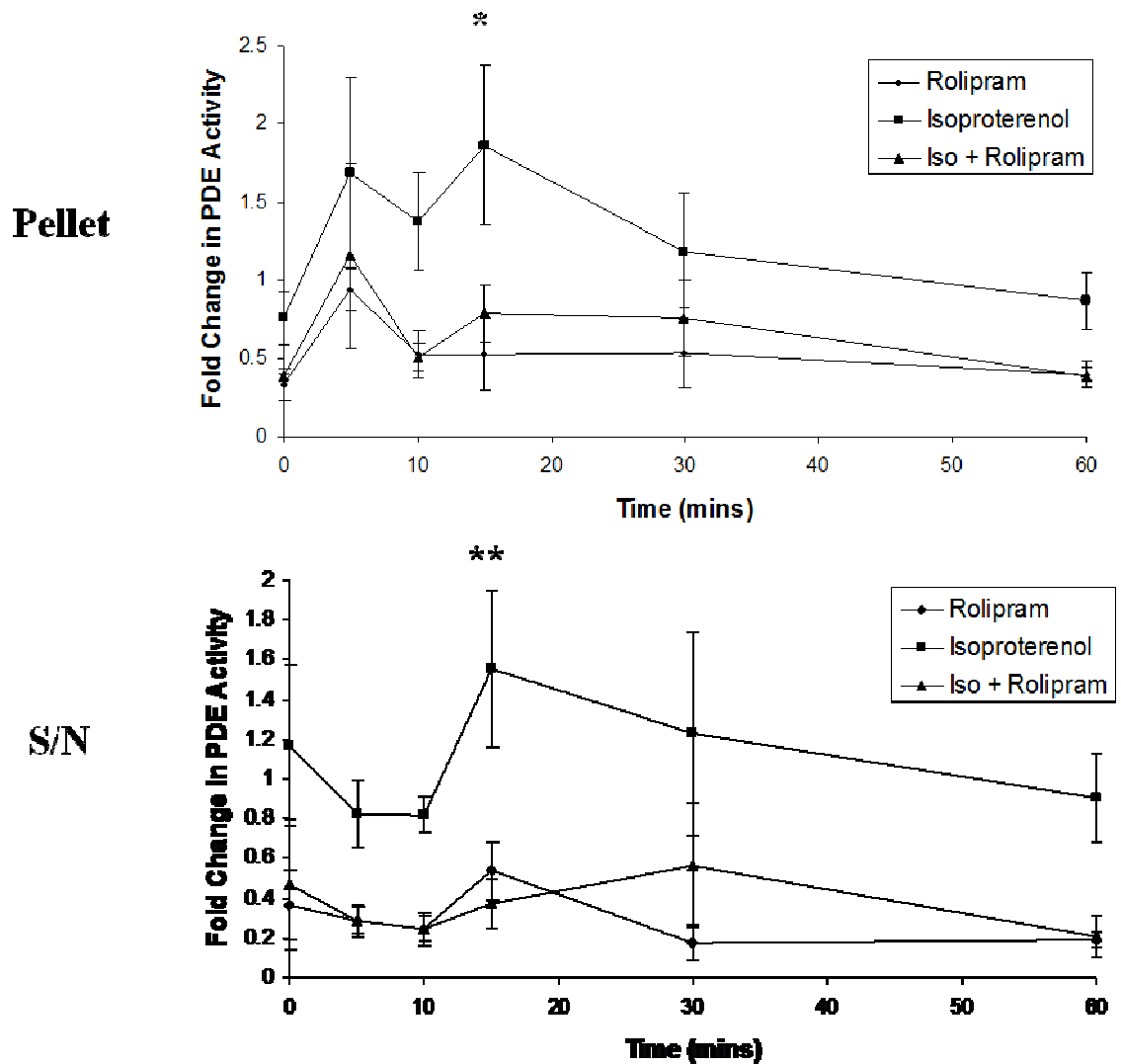
Figure 3.1 Enhanced PDE4 activity following stimulation of HEK293 cells with isoproterenol - HEK293 cells were stimulated with 10μM isoproterenol for the indicated times and then harvested and fractionated into high speed pellet and supernatant fractions. Samples of pellet and supernatant fractions were then assayed for PDE hydrolytic activity in the presence or absence of the type 4 PDE inhibitor, rolipram (10μM). Values obtained from three separate experiments were normalised against control and plotted as means ± SEM for each cellular fraction. Significant differences relative to control (t=0) are indicated by * , $p<0.01$ and **, $p<0.001$.

cells (Yarwood *et al.,* 1999), the effects of PDE4D5 binding to RACK1 on the cellular localisation and activity of PDE4D5 have yet to be determined. We therefore sought to determine whether translocation of PDE4D5/RACK1, as previously reported for the PDE4D5/β-arrestin signalling complex (Perry *et al.,* 2002), contributed to the increases in particulate PDE activity following isoproterenol stimulation described in Figure 3.1. Firstly, to investigate whether the PDE4D5/RACK1 complex undergoes activation-coupled translocation, HEK293 cells were stimulated with or without 10μM isoproterenol, at a range of time points between 0 and 60 minutes. Following stimulation, cells were harvested and fractionated by high-speed centrifugation into crude membrane (pellet or P2) and cytosolic (supernatant or S/N) fractions. Equal amounts of protein from each fraction were then separated by SDS-PAGE and immunoblotted with antibodies to RACK1 and PDE4D, which recognises all PDE4D isoforms, as PDE4D5 is known to interact with RACK1 (Figure 3.2). The immunoblots demonstrate that although RACK1 immunoreactivity is detectable in both pellet and supernatant fractions, it is clearly enriched in the pellet fraction of HEK293 cells (Figure 3.2). Immunoblotting with the anti-PDE4D antibody also demonstrated the presence of the PDE4D isoforms, PDE4D3 and PDE4D5, as previously described (Yarwood *et al.,* 1999). Immunoblots also demonstrated that although PDE4D3 and PDE4D5 are enriched in the supernatant fraction of cells, immunoreactivity for both isoforms is clearly detectable in the pellet fraction (Figure 3.2).

Stimulation with isoproterenol however seemed to have little effect on the distribution of PDE4D3, PDE4D5 or RACK1 between pellet and supernatant fractions at any of the time points tested (Figure 3.2). To test that isoproterenol stimulation does indeed cause activation of the cyclic AMP signalling pathway in HEK293 cells, experiments were carried out to confirm that the isoproterenol could stimulate the phosphorylation of the PKA-substrate <u>c</u>yclic AMP <u>r</u>esponse <u>e</u>lement <u>b</u>inding protein (CREB), which is phosphorylated in response to increased cyclic AMP levels in a wide variety of cell types (Yamamoto *et al.,* 1988). As CREB activation occurs through PKA action in response to an elevation in cyclic AMP levels, and PKA is also able to activate PDE4D isoforms, measurement of CREB phosphorylation is a reasonable assay for determining that isoproterenol stimulation can cause activation of the cyclic AMP signalling pathway in HEK293 cells. In this experiment the dephosphorylation and inactivation of CREB coincides with activation of PDEs as demonstrated in Figure 3.1, indicating that following PDE activation cyclic AMP levels become lowered and CREB is inactivated. Immunoblots using an antibody that recognises the phosphorylated and active form of CREB show a

Figure 3.2 RACK1 and PDE4D5 do not undergo activation-coupled translocation following isoproterenol stimulation of HEK293 cells

HEK293 cells were stimulated with 10μM isoproterenol at the indicated time points ranging from 0 to 60 min. Following stimulation, cells were harvested and fractionated by high-speed centrifugation into pellet and supernatant fractions. Equal amounts of protein were separated by SDS-PAGE and immunoblotted with anti-PDE4D and anti-RACK1 specific antibodies. The immunoblots show an enrichment of RACK1 (36kDa) in the pellet fraction, while PDE4D5 (105kDa, upper) and PDE4D3 (95 kDa, lower) are enriched in the supernatant fraction. Densitometry was carried out on immunoblots from three separate experiments and the results plotted as means on the graph.

Figure 3.2

Antibody

Anti-PDE4D

RACK1

Anti-PDE4D

RACK1

Fraction

Pellet

Supernatant

Isoproterenol (10μM)   - + - + - + - + - + - +
Time (mins)   0   5   10   15   30   60
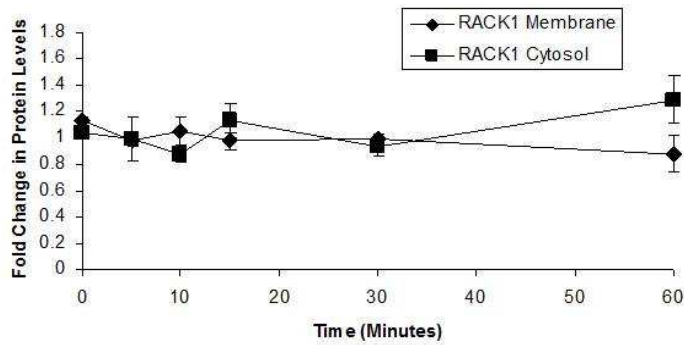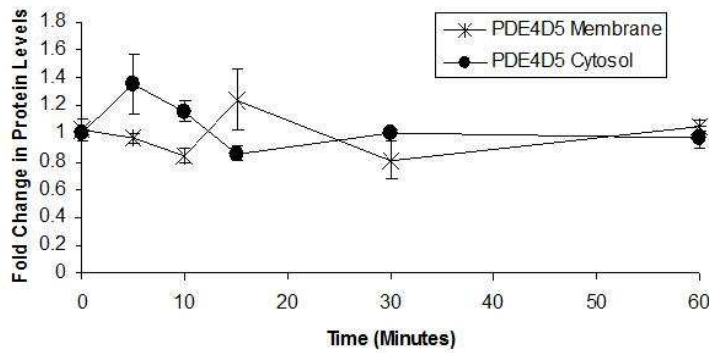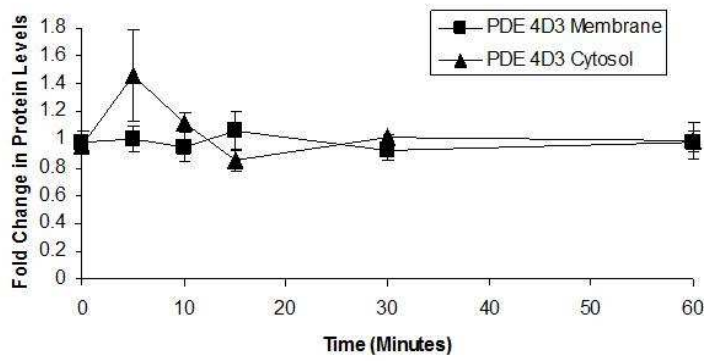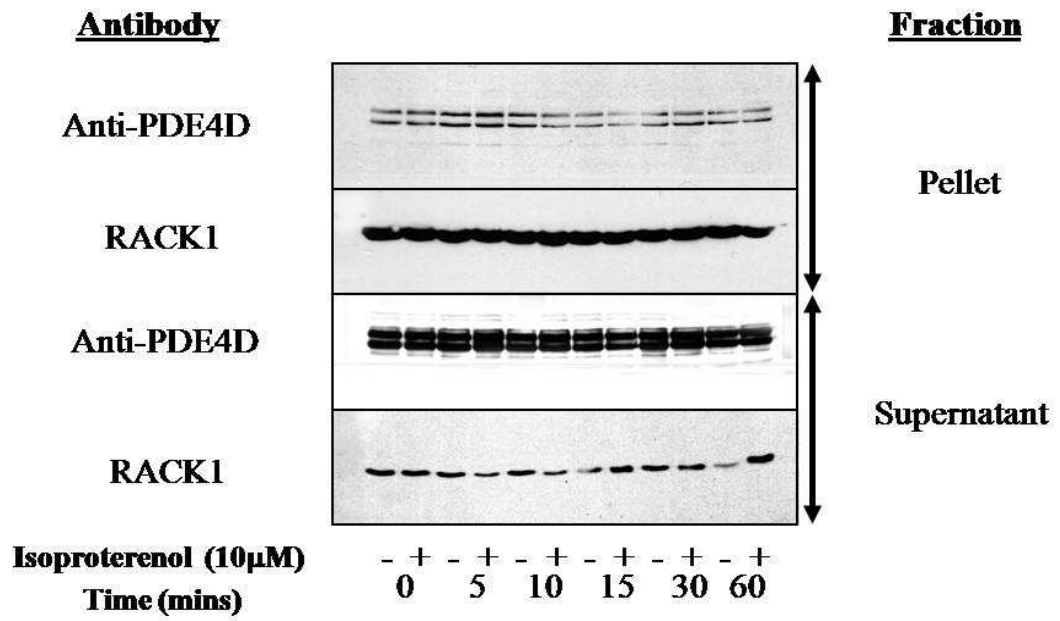
significant increase in phospho-CREB levels following 15 minutes isoproterenol stimulation (Figure 3.3), with no concomitant translocation of PDE4D3, PDE4D5 or RACK1. These results indicate that isoproterenol is able to stimulate elevations in intracellular cyclic AMP levels in HEK293 cells; however, the RACK1/PDE4D5 does not appear to undergo activation-coupled translocation in response to elevated levels of intracellular cyclic AMP in this cell type.

3.3.3 The effect of RACK1 on PDE4D5 subcellular localisation - Having established that RACK1 and PDE4D5 do not undergo activation coupled translocation in response to elevations in intracellular cyclic AMP, the role of RACK1 in the intracellular targeting of PDE4D5 was investigated. HEK293 cells were transfected with VSV-tagged cDNA encoding wild type PDE4D5 (PDE4D5-WT) or with VSV-tagged PDE4D5 where the leucine at position 33 in the unique N-terminal had been mutated to aspartate (PDE4D5-L33D). The PDE4D5-L33D mutation in PDE4D5 has previously been shown to prevent interaction with RACK1 (Bolger *et al.,* 2006). Although this mutation may also interfere with β-arrestin binding the binding of both RACK1 and β-arrestin are mutually exclusive. The PDE4D5-L33D mutant is therefore a useful tool for investigating the interaction between RACK1 and PDE4D5. To confirm that this mutation does in fact prevent interaction with RACK1 in HEK293 cells immunoprecipitation experiments were carried out from transfected cells using an anti-RACK1 antibody. Results demonstrated that whereas PDE4D5-WT precipitated with RACK1, there was no significant association detected between RACK1 and PDE4D5-L33D (Figure 3.4). To check whether association with RACK1 affected the intracellular distribution of PDE4D5, HEK293 cells transfected with PDE4D5-WT or PDE4D5-L33D were harvested and fractionated by high-speed centrifugation. Equal amounts of protein from both PDE4D5-WT– and PDE4D5-L33D-transfected cells and untransfected fractions were separated by SDS-PAGE and then immunoblotted with anti-RACK1 and anti-VSV specific antibodies. Immunoblots demonstrated (Figure 3.5) that RACK1 immunoreactivity was enriched in the membrane fractions of untransfected, PDE4D5-WT and PDE4D5-L33D transfected cells, while PDE4D5 protein levels, indicated by the anti-VSV reactive bands in the PDE4D5-WT and PDE4D5-L33D transfected cell fractions are enriched in the cytosolic fraction with lower levels of expression in the particulate fractions. Densitometric analysis of immunoblots demonstrated that the relative distributions of PDE4D5-WT and PDE4D5-L33D were approximately equal between the two fractions (Figure 3.5) and that while the majority of RACK1 is found in the pellet fraction, binding with PDE4D5 has little effect on its

Figure 3.3
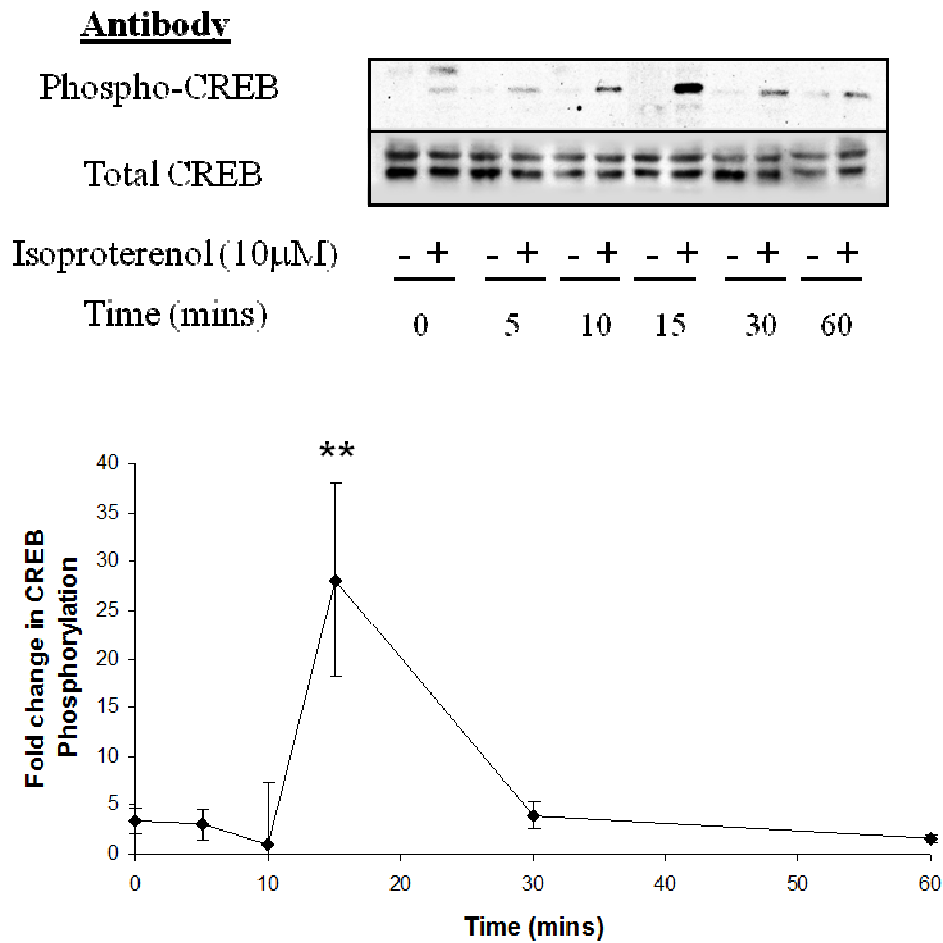
Figure 3.3 Isoproterenol stimulation of HEK293 cells induces phosphorylation of CREB on Ser 133 - HEK293 cells were stimulated with 10μM isoproterenol for the indicated times between 0 and 60 min. Cells were then harvested and equal amounts of protein from whole cell extracts were separated by SDS-PAGE and immunoblotted with anti-CREB and anti-phosphoCREB (Ser133) specific antibodies. Densitometric values taken from three separate experiments were plotted as means with significant differences relative to control (t=0) indicated by **, $p < 0.001$.

Figure 3.4
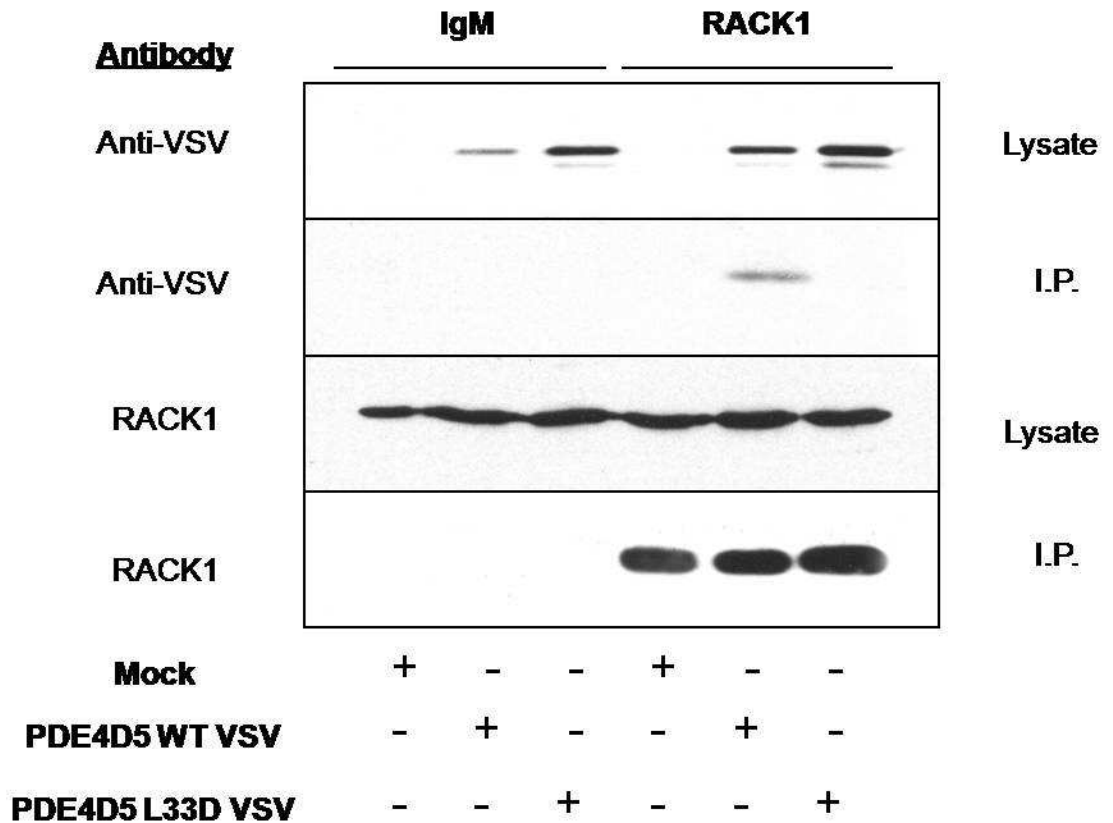
Figure 3.4 Wild-type PDE4D5, but not PDE4D5-L33D, associates with RACK1 in HEK293 cells - HEK293 cells were transfected with VSV-tagged PDE4D5 wild type (PDE4D5-WT) or VSV-tagged PDE4D5 L33D mutant (PDE4D5-L33D). Whole cell lysates were then prepared and immunoprecipitated with control (IgM) antibody or anti-RACK1 (IgM clone) antibodies. Immunoprecipitates and whole cell lysates were then separated by SDS-PAGE and immunoblotted with anti-VSV antibodies to detect PDE4D5 forms (upper bands), or anti-RACK1 antibody. The immunoblot shown here is representative of an experiment carried out on three separate occasions with similar levels of expression across all transfections.

Figure 3.5 Distribution PDE4D5-WT and PDE4D5-L33D activity and immunoreactivity between soluble and particulate fractions of HEK293 cells

HEK293 cells were transfected with VSV-tagged, PDE4D5 wild type (PDE4D5-WT) or VSV-tagged, PDE4D5 L33D (PDE4D5-L33D) non-RACK1 binding mutant DNA, and then fractionated to give pellet (P2) and cytosol (S/N) fractions. Equal amounts of protein from transfected and untransfected cell fractions were separated by SDS-PAGE and immunoblotted with anti-RACK1 and anti-VSV specific antibodies. The blot shows an enrichment of RACK1 in the membrane fraction, while the PDE4D5 protein, as indicated by the VSV bands on the blot, are enriched in the cytosolic fractions of both the WT and mutant. Densitometric values were obtained from non-saturating immunoblots from three separate experiments and plotted on the upper graph to show the relative distributions of PDE4D5-WT and PDE4D5-L33D between fractions, while the middle graph shows the proportional distribution of RACK1 in the pellet and supernatant fractions when in association with and without PDE4D5. PDE assays were also carried out on fractions from transfected cells, displayed on the lower graph.

Figure 3.5
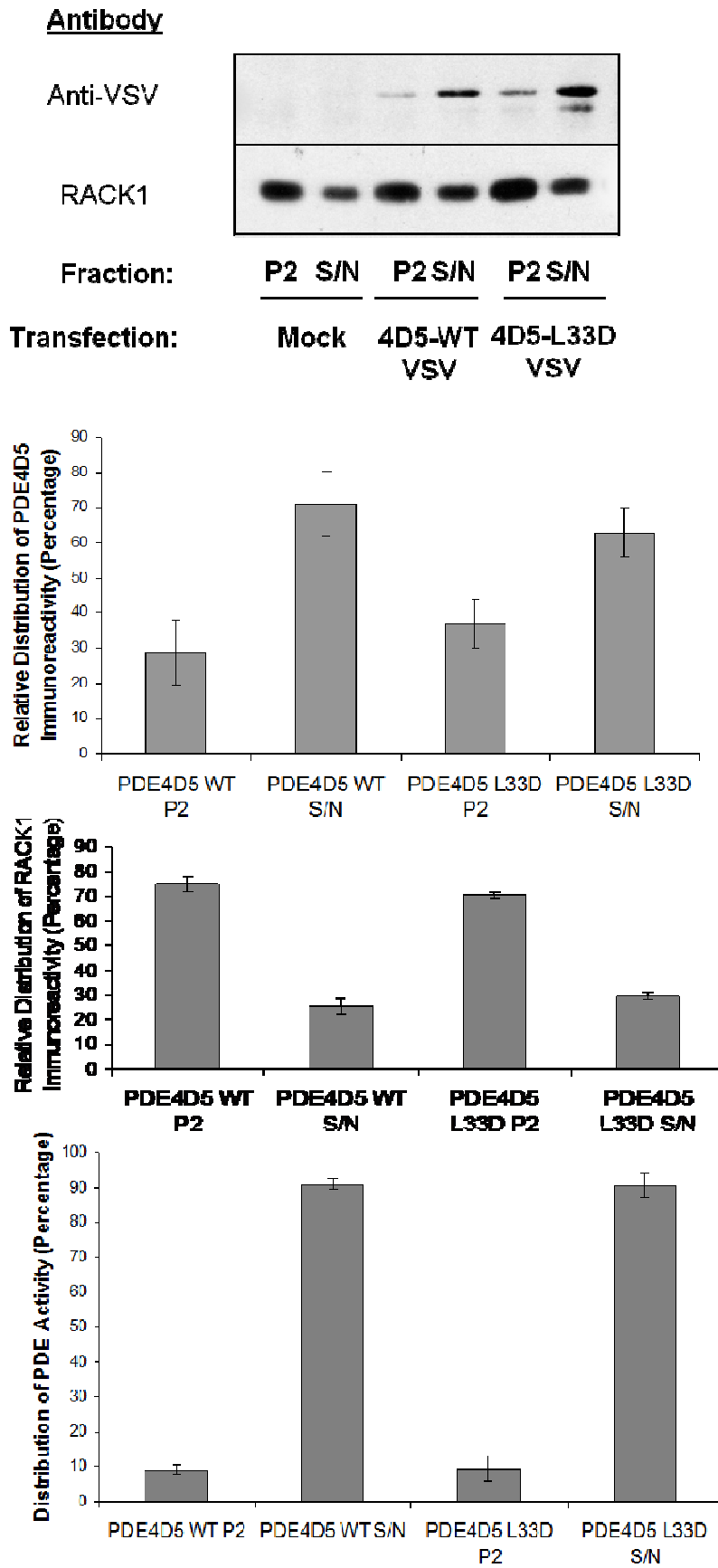
distribution within the cell. Together this data suggests that association with RACK1 does not play a significant role in determining the subcellular distribution of PDE4D5 in HEK293 cells.

3.3.4 The effect of interaction with RACK1 on the conformation of PDE4D5 - Having determined that there was little difference in terms of intracellular distribution between PDE4D5-WT and PDE4D5-L33D, denaturation experiments were carried out to determine whether binding to RACK1 affected the structural stability of PDE4D5. Denaturation of an enzyme reduces substrate binding and prevents enzyme activity. The use of complex formation to prevent denaturation can be seen in examples such as the Arp2/3 complex (Le Clainche *et al.,* 2001) which provides protection against enzyme denaturation when the complex components are bound in complex. The effects of thermodenaturation on PDE4D5 activity was examined following transfection of HEK293 cells with cDNA encoding VSV-tagged PDE4D5-WT and PDE4D5-L33D. Cells were harvested and fractionated into high-speed pellet and supernatant fractions which were then heated for various times at 45°C. This temperature was chosen after initial optimisation experiments were carried at 40°C, 45°C and 50°C to determine the appropriate temperature. The results from this initial experiment showed that 40°C resulted in little denaturation after the longest time point, while 50°C resulted in high denaturation at the earliest time point to a degree that enzymatic activity was negligible. 45°C, having resulted in a linear decay, was therefore chosen for this experiment. Samples were then assayed for PDE activity (Figure 3.6). Results demonstrated that all samples decayed with a single exponential; however there was a pronounced difference between the activity decay between PDE4D5-WT and PDE4D5-L33D expressed in the pellet fraction, with PDE4D5-L33D decaying more rapidly (Figure 3.6). In contrast, the rate of decay in PDE activity of PDE4D5-WT in the supernatant fraction was found to be slightly greater than PDE4D5-L33D. The supernatant fraction shows a decline in PDE activity of PDE4D5-WT falling by 50% by 10 minutes heat treatment, and PDE4D5-L33D falling by 20 minutes. The pellet fraction displays a decline of only 10% of PDE activity over 10 minutes in PDE4D5-WT PDE4D5 transfected cells, while PDE4D5-L33D mutant PDE4D5 transfected cells exhibit a decline in PDE activity of almost 70% at the same time point. A decay of pellet PDE4D5-WT was observed at higher temperatures, but as the decay was non- linear the results were not meaningful and are not shown. While only 10% of PDE4D5-WT activity is found in the pellet fraction of HEK293 cells (Figure 3.5), inhibition with rolipram occurs with a similar IC50 to that seen in the supernatant fraction (Figure 3.1). This indicates that these

Figure 3.6
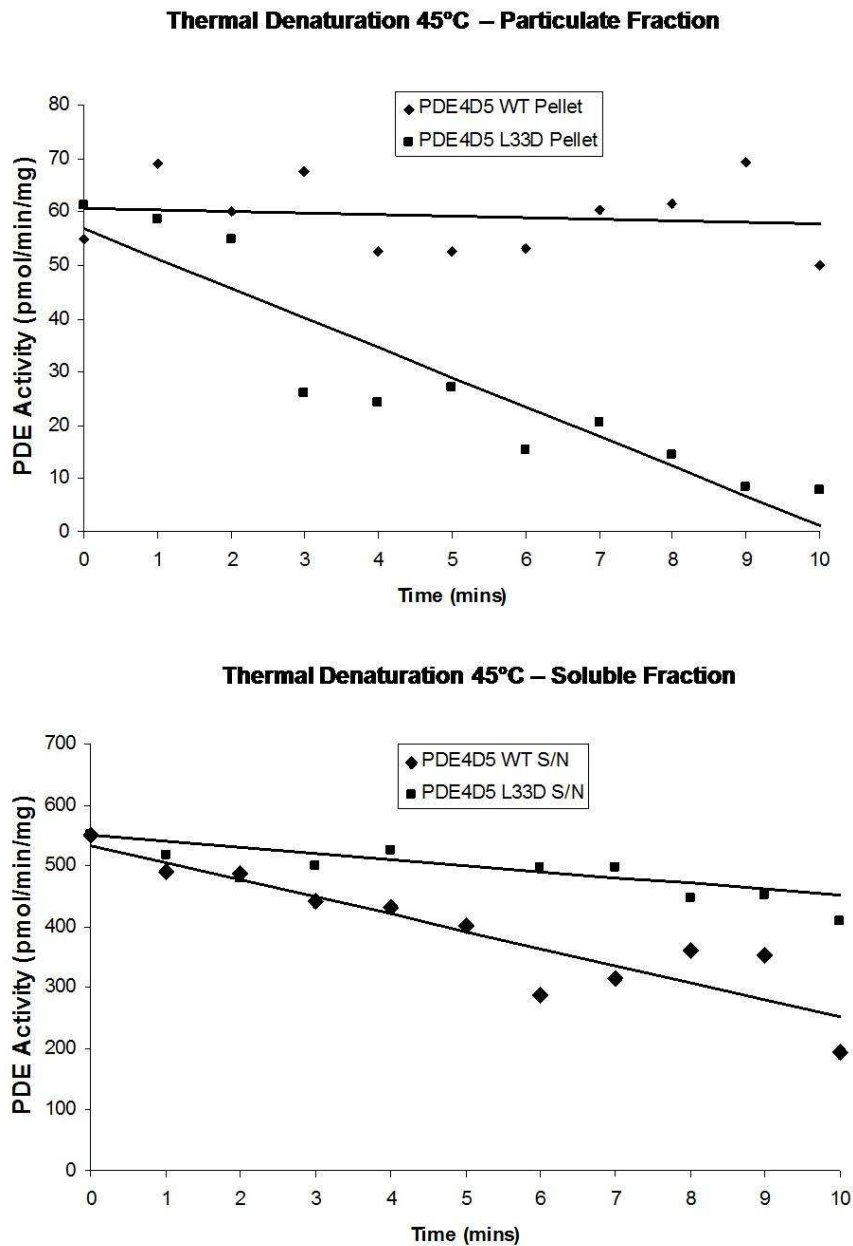
Figure 3.6 Thermal denaturation of particulate and soluble PDE4D5-WT and PDE4D5-L33D - HEK293 cells were transfected with cDNAs encoding either PDE4D5-WT or PDE4D5-L33D. Cells were then harvested and fractionated into soluble (S/N) and pellet (P2) fractions. Equal amounts of protein from each fraction were then heated in a water bath heated to $45^{o}C$ and removed at minute intervals, ranging between 1 and 10 min, and placed on ice. PDE assays were then carried out and mean enzyme activities from three separate experiments normalised to non-heat treated control at t=0 (100% PDE activity).

experiments were carried out within the linear range of the assay. Additionally, despite only possessing 10% of PDE4D5-WT activity, the pellet fraction has been shown to be sufficient to regulate activity of the β2-adrenoreceptor (Perry *et al.,* 2002) indicating functionality at the plasma membrane.

These results show that PDE4D5 that is able to bind RACK1 has a lower rate of decay of activity in response to high temperature than PDE4D5 incapable of binding RACK1. As PDE4D5 activity is based on its ability to interact with its substrate cyclic AMP, the lessened activity decay seen here in PDE4D5 bound to RACK1 suggests that it is this binding that may be responsible for providing some structural stability to the catalytic site of PDE4D5 and allowing PDE4D5 to hydrolyse cyclic AMP even under high temperature conditions. This supports the hypothesis that interaction with RACK1 may play a role in altering the conformation of PDE4D5 in particulate and soluble fractions of HEK293 cells.

To investigate this further we next determined whether interaction with RACK1 influences the enzymatic activity of PDE4D5. HEK293 cells were transfected with either PDE4D5-WT or PDE4D5-L33D cDNAs, harvested and fractionated into soluble and particulate fractions by high-speed centrifugation. Experiments were then carried out to determine the maximum rate of catalysis (V_{max}) of PDE4D5-WT or PDE4D5-L33D and the affinity of PDE4D5-WT or PDE4D5-L33D for cyclic AMP (Michaelis-Menton constant or K_m) by varying the amount of substrate (ranging between 1μM and 25μM). The PDE activity of each fraction was plotted using GraphPad Prism4 to give a Lineweaver-Burk, double reciprocal plot (1/PDE activity against 1/cyclic AMP concentration; Figure 3.7). The y intercept of plots provided the value of $1/V_{max}$, while the x intercept provided $-1/K_m$. V_{max} values determined for pellet fractions of both PDE4D5-WT and PDE4D5-L33D were low, at 0.32 ± 0.07 and 0.87 ± 0.09 pmol/min/mg respectively, while the supernatant fractions displayed higher rates of catalytic activity, with PDE4D5-WT possessing a V_{max} of 9.71 ± 2.09 pmol/min/mg, greater than the PDE4D5-L33D V_{max} value of 7.14 ± 1.62 pmol/min/mg. The higher rate of catalysis observed in the supernatant fractions of PDE4D5-WT and PDE4D5-L33D transfected HEK293 cells, contrasted with the relatively low K_m values of 16.7 ± 1.3 and 10.0 ± 1.5 μM respectively. The pellet fraction of PDE4D5-WT, however, shows a comparatively lower K_m value of 6.3 ± 0.9 μM, indicating a higher affinity for its substrate. Conversely, the non-RACK1 binding PDE4D5-L33D mutant pellet fraction has a K_m value of 20.0 ± 0.9 μM, demonstrating a loss of affinity for cyclic AMP when compared to the other PDE4D5 forms in the other fractions. As

Figure 3.7
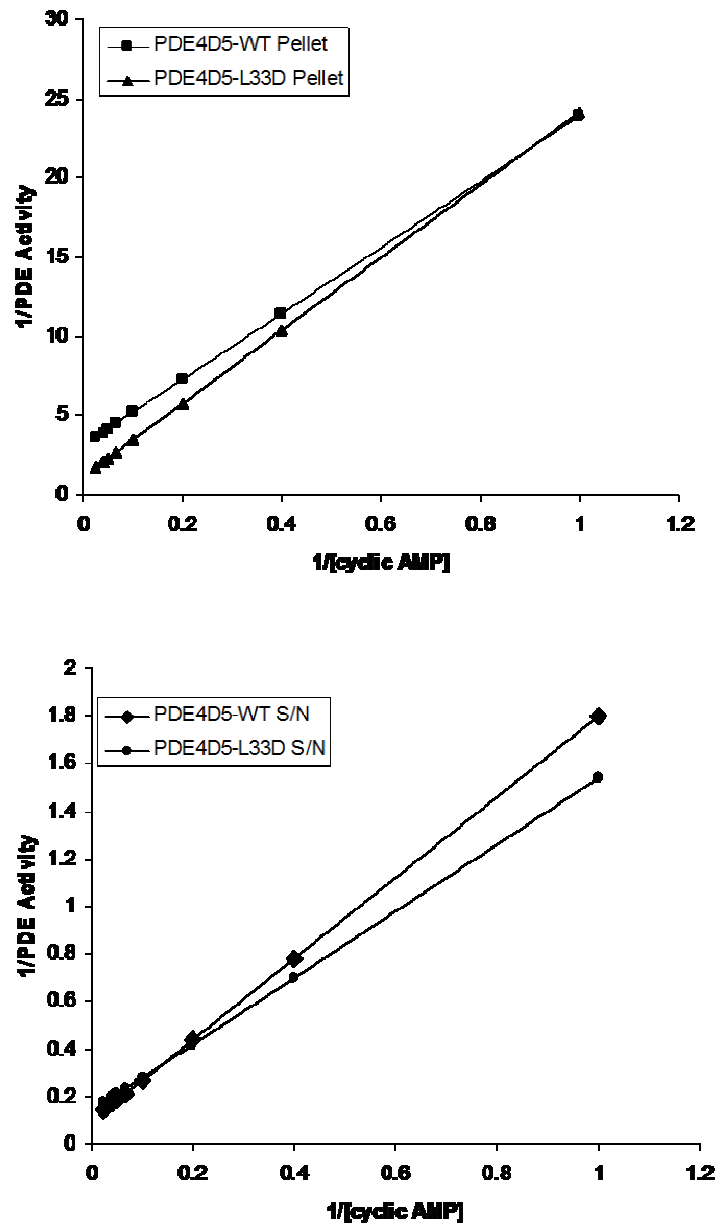
Figure 3.7 Determinations of V_{max} and K_m for PDE4D5-WT and PDE4D5-L33D
HEK293 cells were transfected with cDNAs encoding PDE4D5-WT DNA or PDE4D5-L33D. Cells were then harvested into assay buffer and fractionated into particulate (P2) and soluble fractions (S/N) by high-speed centrifugation. PDE assays were then carried out on equal amounts of fraction protein in the presence of cyclic AMP at concentrations ranging between 1μM and 25μM. Mean PDE activities from three separate experiments were then used to produce Lineweaver-Burk plots for each fraction and Vmax and Km values were calculated.

Lineweaver-Burk plots are prone to error as the reciprocals used in this plot distort experimental errors, these values were confirmed by fitting to the hyperbolic form of the Michaelis-Menten equation. Although there appears to be little difference in the catalytic rates between PDE4D5-WT and PDE4D5-L33D mutant PDE4D5, the significant difference in Km values indicates that the binding of RACK1 to PDE4D5 in particulate fractions is essential for maintaining high affinity of PDE4D5 for cyclic AMP in the membrane fractions of cells. Next we determined the effect of RACK1 binding on susceptibility of PDE4D5 to a specific enzyme inhibitor was investigated by measuring the dose response of PDE4D5-WT and PDE4D5-L33D to the PDE4 inhibitor, rolipram (Yarwood *et al.*,1999). HEK293 cells were transfected with either PDE4D5-WT or PDE4D5-L33D and then fractionated by high-speed centrifugation. Equal amounts of protein were treated with a range of rolipram concentrations, ranging from 0.01nM to 1mM, and then PDE activity was determined. The dose response curves (Figure 3.8) depicting the responses of supernatant fractions to rolipram show that the response of PDE4D5-WT and PDE4D5-L33D to inhibition by rolipram are very similar, while the pellet fraction graph shows a clear difference in the dose response of PDE4D5-WT and PDE4D5-L33D. The PDE4D5-L33D mutant curve is clearly to the left of the PDE4D5-WT curve and shows that the PDE4D5-L33D mutant in the pellet fraction is less susceptible to inhibition by rolipram. As outlined above, despite the pellet fraction of HEK293 cells only containing 10% of PDE4D5-WT activity, the IC50 of rolipram inhibition in this fraction is similar to that of the supernatant fraction (Table 3.1), and therefore the effects seen in response to rolipram are significant. In the pellet fraction, PDE4D5 not bound to RACK1 is less susceptible to rolipram inhibition than PDE4D5 that can bind RACK1. This suggests that RACK1 may allow easier interaction between rolipram and PDE4D5. As rolipram also interacts with the catalytic site of PDE4D5, it is likely that RACK1 binding holds PDE4D5 in a conformation where rolipram can readily access the catalytic site. These results (summarised in Table 3.1) and those from Figure 3.6 suggest that interaction of PDE4D5 with RACK1 is important to allow high affinity binding of PDE4D5 to its substrate cyclic AMP and for effective inhibition by rolipram in the membrane fraction of HEK293 cells. Moreover, the rolipram inhibition experiments suggest that RACK1 binding to PDE4D5 is important for allowing effective inhibition by low concentrations of rolipram in this fraction.

3.3.5 The effect of interaction with RACK1 on the activation of PDE4D5 - Given these strong influences on the conformation of particulate PDE4D5 we next sought to investigate

Figure 3.8
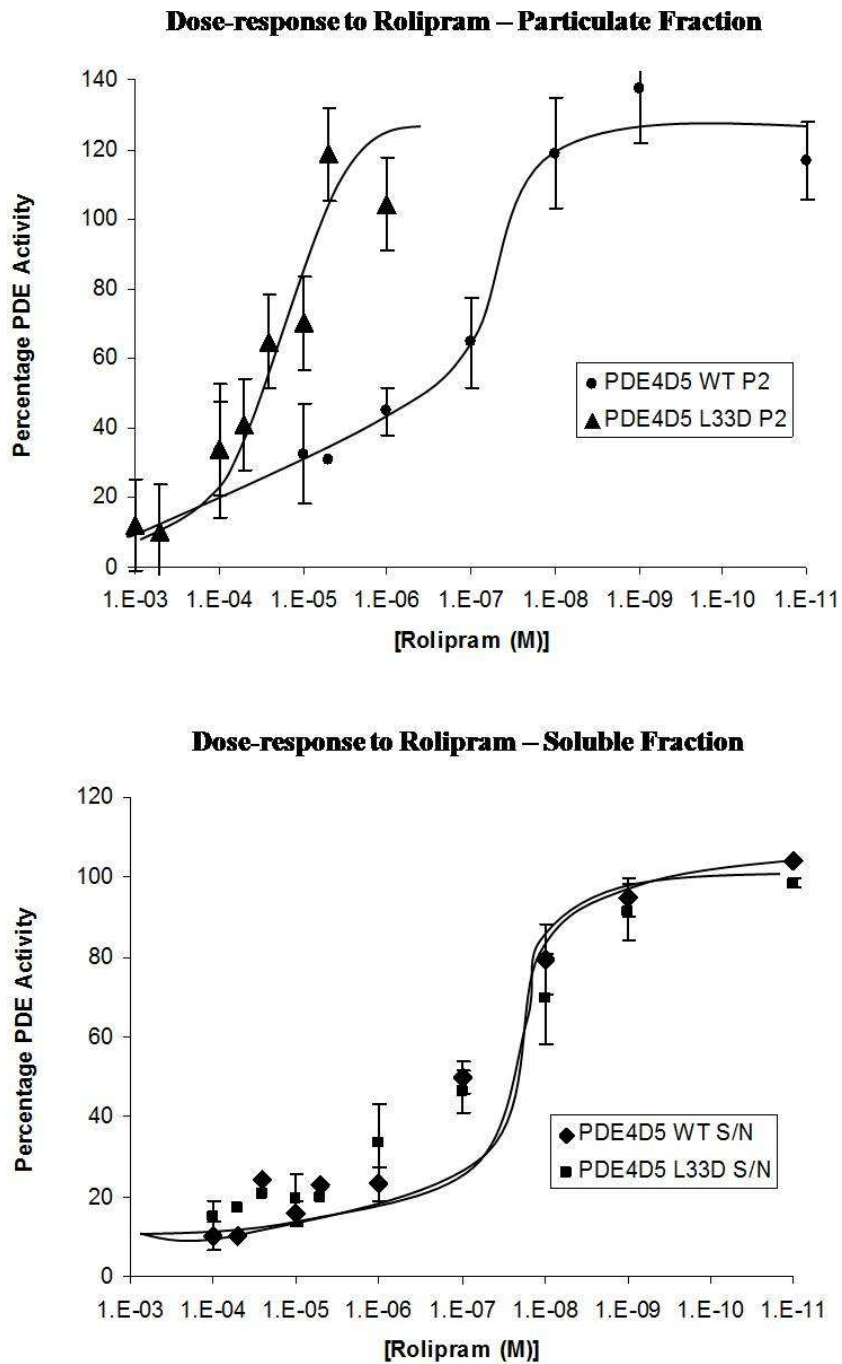
Figure 3.8 Dose-response of PDE4D5-WT and PDE4D5-L33D to inhibition by rolipram - HEK293 cells were transfected with cDNAs encoding either PDE4D5-WT or PDE4D5-L33D. Cells were fractionated and equal amounts of protein were then treated with various concentrations of the PDE4 inhibitor, rolipram, ranging from 0 to 1mM, for 15 minutes and then assayed for PDE activity. PDE activities obtained from three separate experiments were then plotted using GraphPad Prism4 as means ± SEM.

Table 3.1

Transfection	Fraction	Km (μM)	Vmax (pmol/min/mg)	IC50 Rolipram (μM)
PDE 4D5- WT	Pellet	6.3 ± 0.9	0.32 ± 0.07	0.10 ± 0.01
PDE 4D5- WT	S/N	$16.7 \pm 1.3**$	$9.71 \pm 2.09**^{,\,\#\#}$	$0.08 \pm 0.02^{\#\#\#}$
PDE 4D5- L33D	Pellet	20.0 ± 0.9 ***	0.87 ± 0.09	$10 \pm 0.90***$
PDE 4D5- L33D	S/N	$10.0 \pm 1.5^{\#\#}$	$7.14 \pm 1.62*^{,\,\#}$	$0.09 \pm 0.02^{\#\#\#}$

Table 1 K$_{m}$, V$_{max}$ and IC$_{50}$ values for PDE4D5-WT and PDE4D5-L33D Expressed in HEK293 Cells Km and Vmax determinations were done over a range of substrate concentrations between 1μM and 40μM cyclic AMP, defined experimentally and confirmed by fitting to the hyperbolic form of the Michaelis-Menten equation using GraphPad InStat 3. Dose-dependent inhibition by rolipram was determined in the presence of 0.2μM cyclic AMP over a range of rolipram concentrations between 0.01nM and 1mM. Significant differences relative to pellet-associated PDE4D5-WT (*,** and ***) or PDE4D5-L33D (#, ## and ###) are indicated (p<0.05, p<0.01 and p<0.001, respectively).

whether interaction with RACK1 has an effect on the ability of particulate PDE4D5 to be activated by isoproterenol, which is a potent regulator of PDE4 activity in HEK293 cells (Figure 3.1). RACK1 was immunoprecipitated from the pellet and soluble fractions of HEK293 cells that had been stimulated with isoproterenol (10μM) for 15 minutes and PDE4D5 activity associated with the immunoprecipitates determined by PDE assay (Figure 3.9). Results demonstrated that isoproterenol stimulation provoked a significant 1.6 fold increase in PDE4D5 activity associated with RACK1 immunoprecipitates from solubilised particulate fractions, but not from soluble fractions (Figure 3.9). This indicates that association with RACK1 causes a conformational change in PDE4D5 associated with particulate fractions that enables PDE4D5 activation by the cyclic AMP signalling cascade.

Given that RACK1 was originally identified as a scaffold protein for activated conventional PKC isoforms (McCahill *et al.,* 2002), and that cyclic AMP elevation has recently been linked to the activation of PKC in a number of cell types (Borland *et al.,* 2009), we next investigated whether conventional PKC isoforms could be involved in mediating the actions of isoproterenol on RACK1-bound PDE4D5. Firstly we found that stimulation of cells with the PKC activator, phorbol myristate acetate (PMA; 10μM), provoked similar levels of PDE4D5 activation as isoproterenol in the particulate fraction, but not soluble fraction, of HEK293 cells (Figure 3.9). PMA was also found to stimulate rapid activation and translocation of the PKCα isoform from the supernatant to the pellet fractions of HEK293 cells (Figure 3.10). Cells were stimulated with 10μM PMA over a 0 – 60 minute time course and showed a maximal association of activated PKCα in response to PMA after 5 minutes. No effect on RACK1 distribution within the cell was seen in response to PMA stimulation across the time course. Although PMA provokes a fast degradation of PKC, the time course of PDE4D5 activation in response to PMA (Figure 3.9) occurs before disappearance of PKC and therefore this does not affect the results.

Next, in order to determine whether isoproterenol can provoke PKC activation, HEK293 cells were stimulated with PMA and then cells were harvested and fractionated into particulate and soluble fractions which were then immunoprecipitated with anti-RACK1 antibodies (Figure 3.11). Equal amounts of protein from fractions together with RACK1 immunoprecipitates were separated by SDS-PAGE and then immunoblotted with anti-RACK1 and anti-PKCα antibodies (Figure 3.11). PKCα was clearly found to translocate to the particulate fraction in response to PMA stimulation. Translocation from the cytosol to

Figure 3.9
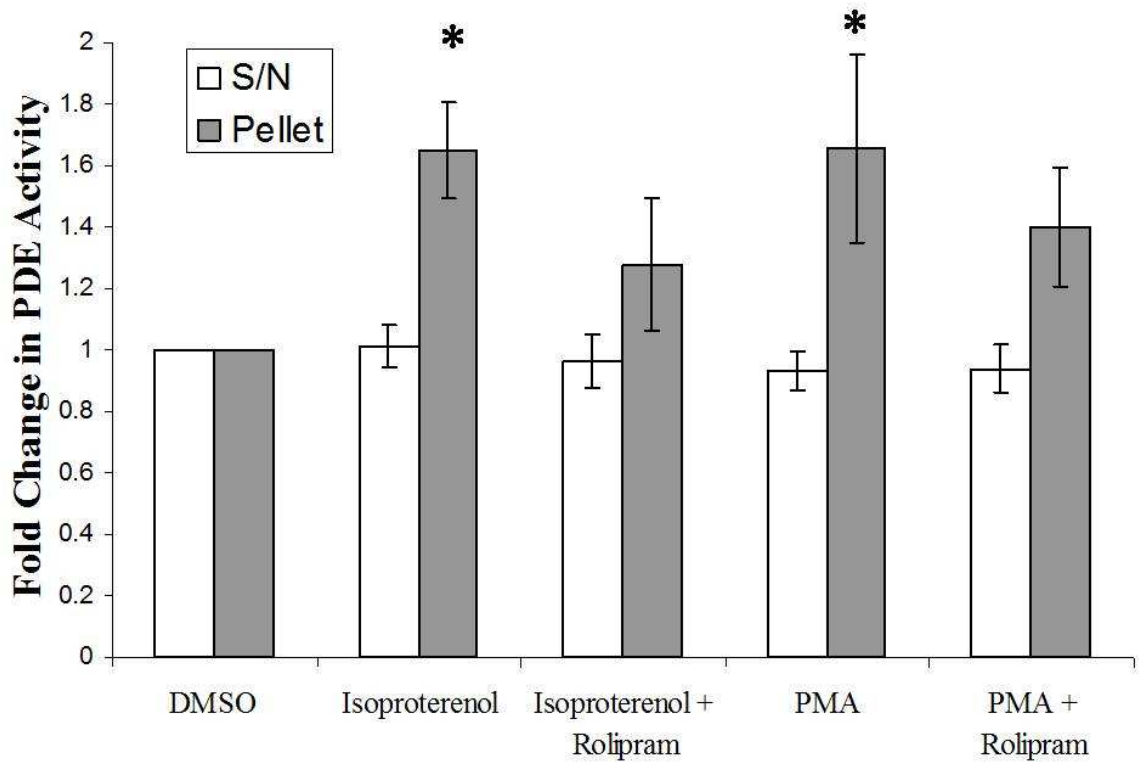
Figure 3.9 Isoproterenol and PMA stimulate PDE4 activity associated with RACK1 immunoprecipitates from HEK293 cells - HEK293 cells were stimulated with either 10μM isoproterenol or 10μM PMA for 15 minutes and then fractionated and immunoprecipitated with anti-RACK1 antibodies. RACK1 immunoprecipitates were then assayed for associated PDE activity in the presence or absence of rolipram as indicated in the histogram. Means ± SEM from three separate experiments were plotted as fold change relative to non-stimulated cells. Significant differences relative to control are indicated, * = $p<0.01$.

Figure 3.10 Stimulation of HEK293 cells with PMA triggers intracellular translocation of PKCα but not RACK1

HEK293 cells were stimulated with 10μM PMA at time points between 0 and 60 min following which cells were fractionated into soluble (S/N) and particulate (pellet) fractions. Equal protein amounts of cell fractions were separated by SDS-PAGE and then immunoblotted with anti-RACK1 and anti-PKCα antibodies. The immunoblots show that PKCα translocates from the supernatant to the pellet fraction within 5 min of PMA stimulation. Densitometry was carried out on immunoblots from three separate experiments and plotted as means ± SEM. Significant differences relative to non-stimulated control are indicated, * = $p<0.05$ and ** = $p<0.01$. Results are representative of an experiment carried out on three separate occasions.

Figure 3.10
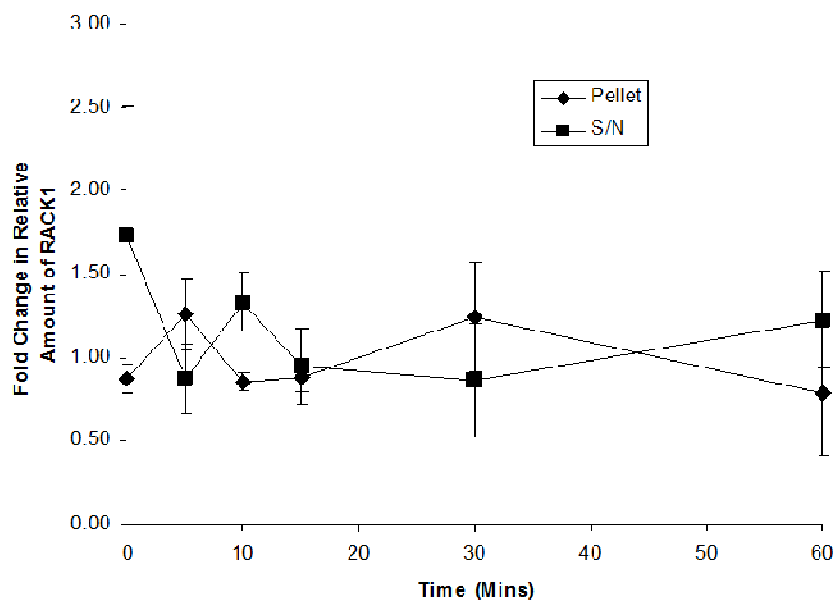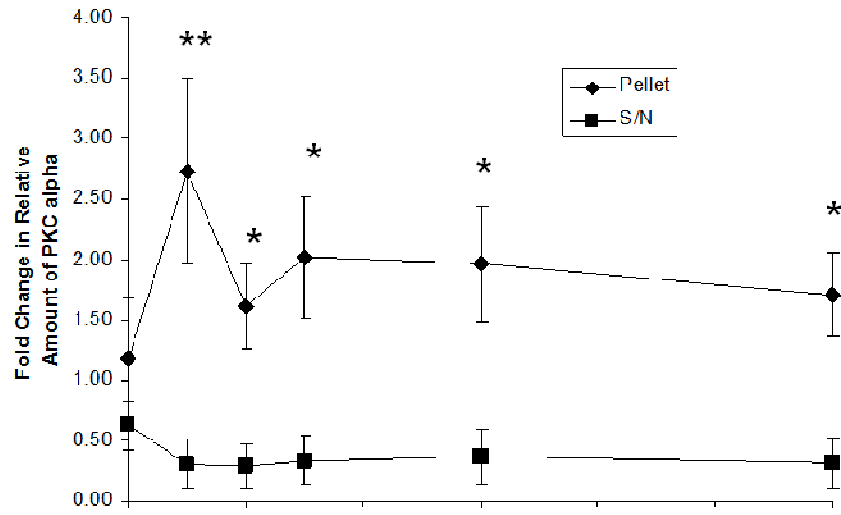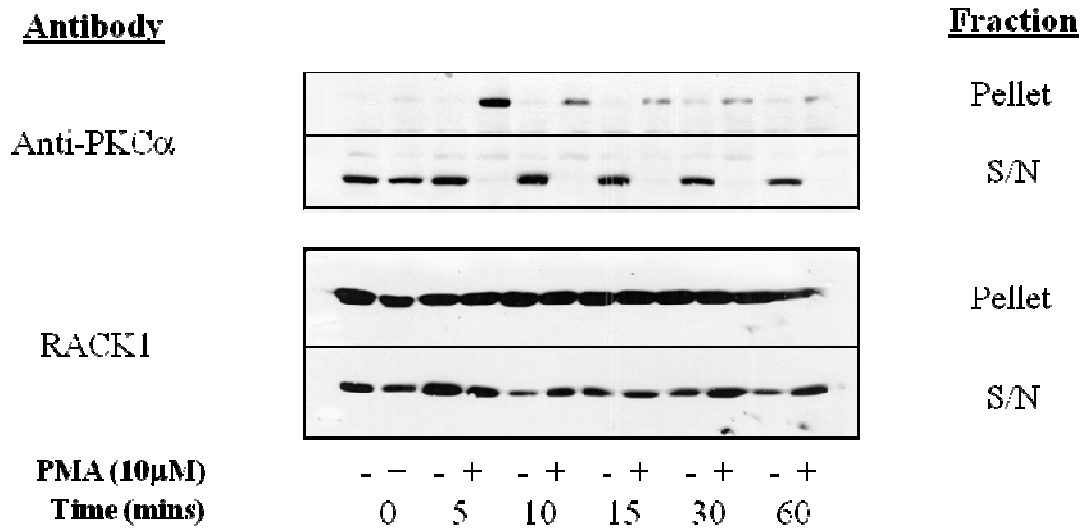
Figure 3.11
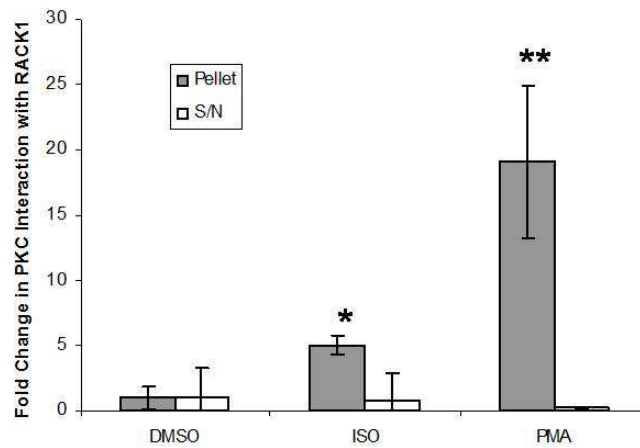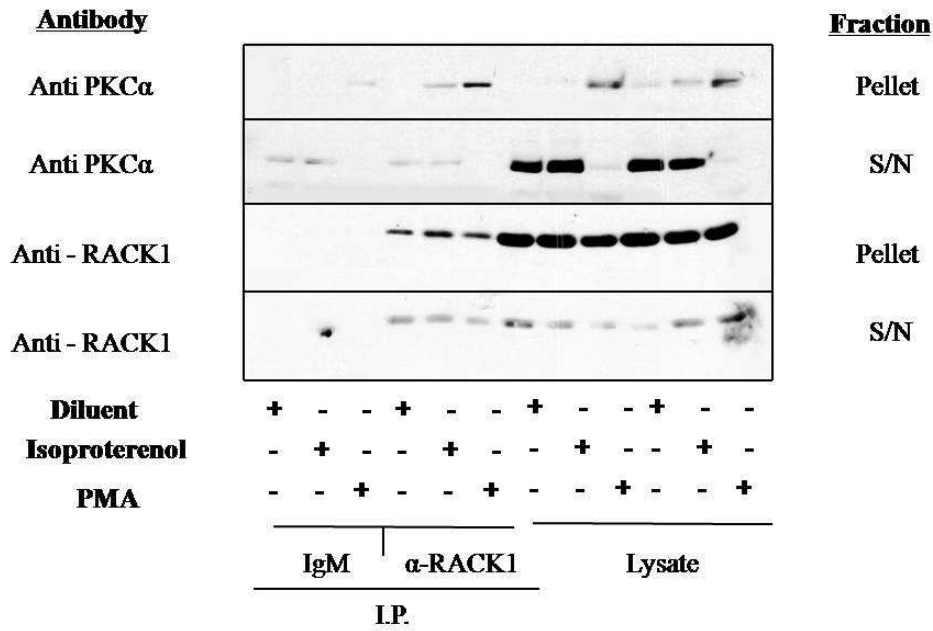
Figure 3.11 PMA or isoproterenol stimulation promotes the association of PKCα with RACK1 in the particulate fraction of HEK293 cells - HEK293 cells were stimulated with diluent (DMSO), isoproterenol or PMA for 15 minutes and cells were harvested and fractionated. Fractions were solubilised with 0.1% (v/v) Triton X-100 and then samples were pre-cleared with mouse IgM. Following this, samples were immunoprecipitated with either mouse IgM (negative control) or anti-RACK1 (IgM clone) antibody. Immunoprecipitates and lysate samples were separated by SDS-PAGE and then immunoblotted with anti-RACK1 and anti-PKCα antibodies. Densitometric values from the RACK1-immunoprecipitated samples were plotted as means ± SEM and represent the fold change in PKCα interaction with RACK1 in each fraction. Significant differences in PKCα immunoreactivity are indicated, * = $p<0.05$ and ** = $p<0.01$. Results are representative of an experiment carried out on three separate occasions.

the plasma membrane is a well documented indicator of PKC activation (Lin *et al.,* 2007) in response to PMA stimulation (Lim *et al.,* 1999) allowing for a clear indication of PKCα activation in these experiments, and it was found to associate with RACK1 as determined by immunoprecipitation (Figure 3.11). Surprisingly, isoproterenol stimulation also provoked an increase in PKCα immunoreactivity in pellet fractions and in RACK1 immunoprecipitates from pellet fractions (Figure 3.11). These results demonstrate that isoproterenol stimulation provokes a significant activation of RACK1-associated PKCα in HEK293 cells.

The establishment of an interaction between RACK1 and PKCα in the particulate fraction of cells leads to the question as to whether the interaction between PKCα, and RACK1 has any effect on RACK1-bound PDE4D5 activity. Our results from PDE assays carried out on RACK1 immunoprecipitates (Figure 3.9) suggest that this might be the case, however to test this idea further the effect of PMA was tested on HEK293 cells transfected with PDE4D5-WT or the PDE4D5-L33D non-RACK1 binding mutant. Transfected cells were stimulated in the presence or absence of PMA for 15 minutes, before being harvested, fractionated and the PDE4D5 activity in fractions assayed by PDE assay. Results demonstrated an almost 3.5 fold increase in PDE4D5 activity in the pellet fraction from PDE4D5-WT transfected cells stimulated with PMA, in comparison with non-stimulated PDE4D5-WT pellet fraction (Figure 3.12). In contrast, the pellet fraction from PMA-stimulated PDE4D5-L33D cells shows a PDE activity fold change of around only 1.5 (Figure 3.12). These results show that interaction with RACK1 in the particulate fraction of HEK293 cells causes a greater increase in PDE activity in the presence of PMA than when PDE4D5 is not bound to RACK1, suggesting that interaction with RACK1 is important for high levels of PDE activity in response to PKC activation.

Finally, to demonstrate that it is PKC activation *per se*, and not the effects of downstream effectors, that is responsible for the activation of PDE4D5 in the particulate fraction of HEK293 cells, cells were treated with general PKC and MEK specific inhibitors and PDE activity levels determined (Figure 3.13). HEK293 cells were pre-treated with U0126, a specific inhibitor of MEK1 and 2, a known downstream effector of PKC signalling (Favata *et al.,* 1998), or with the PKC specific inhibitors Ro-31-7549 or GF109203X (GFX) for 30 minutes, then stimulated for 15 minutes with or without PMA. Cells were then harvested and equal amounts of lysate protein were separated by SDS-PAGE and then

Figure 3.12
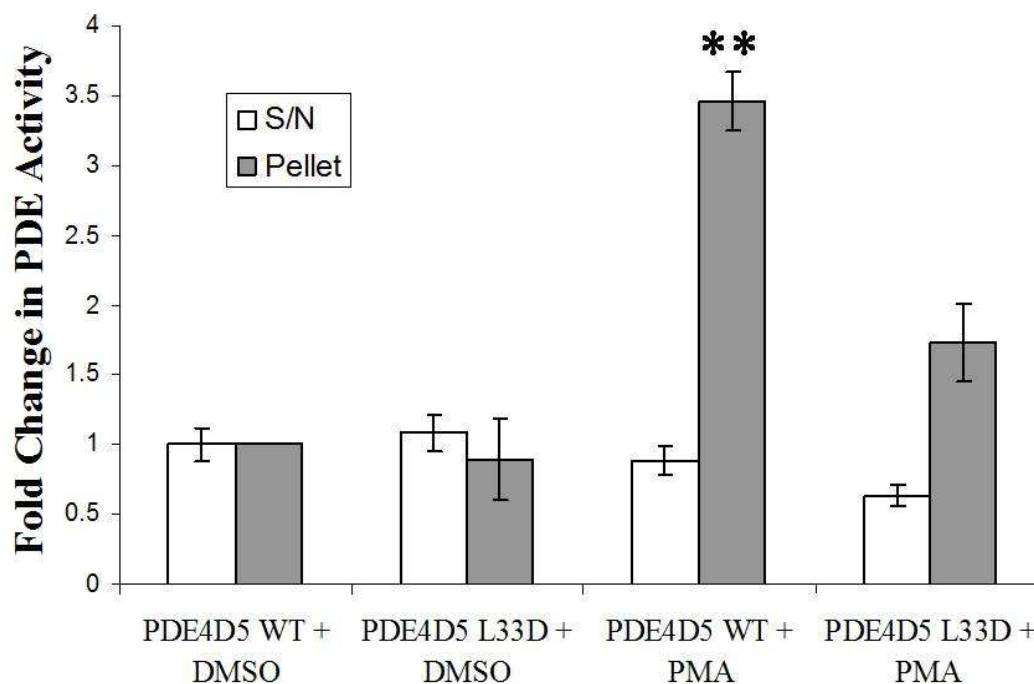
Figure 3.12 Association with RACK1 is required for PDE4D5 to be activated by PMA in the particulate fraction of HEK293 cells - HEK293 cells were transfected with either PDE4D5-WT or PDE4D5-L33D and then stimulated with PMA for 15 minutes. Following this, cells were harvested and fractionated by high-speed centrifugation PDE assays were carried out and activities were then plotted as means ± SEM and represent fold change relative to non-stimulated, pellet sample. Significant differences relative to control are indicated, ** = p<0.01. Results are representative of an experiment carried out on three separate occasions.

Figure 3.13
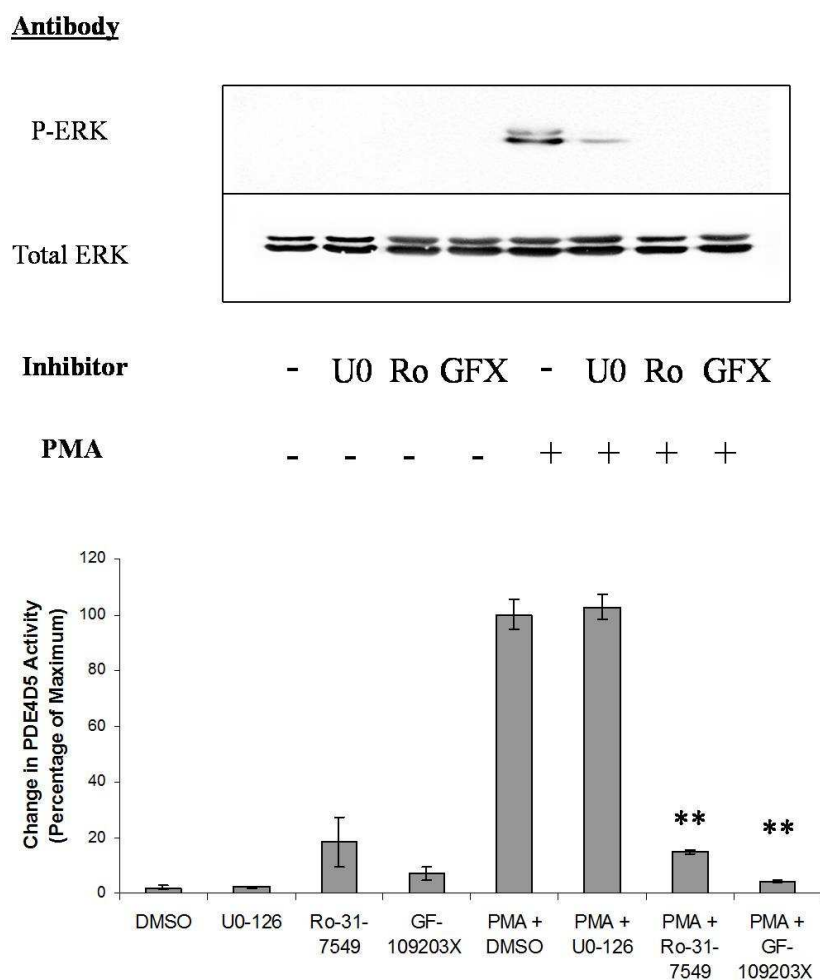
**Figure 3.13 Activation of RACK1-associated PDE4D5 by PMA requires PKC and not ERK**

a) HEK293 cells were transfected with cDNA encoding PDE4D5-WT and then pre-treated for 30 minutes with either DMSO or inhibitors U0126, Ro-31-7549 or GF109203X (GFX) and then stimulated with or without PMA for 15 minutes. Cells were then harvested and equal amounts of whole cell extract were separated by SDS-PAGE and immunoblotted with total ERK and phospho-ERK antibodies. Results represent a single experiment carried out on three separate occasions with similar results.

b) PDE4D5-WT-transfected cells were pre-treated with U0126, Ro-31-7549 or GFX prior to 15 minutes PMA stimulation. Cells were then fractionated and PDE4D5 activity associated with the particulate fraction of cells was assessed by PDE assay. Results are expressed as percentage of maximum (PMA-treated cells) and are means $\pm$ SEM for three separate experiments. Significant differences relative to PMA-treated cells are indicated, **, $p<0.01$.

immunoblotted for total ERK and phospho-ERK to determine the efficacy of the inhibitors (Figure 3.13a). Immunoblots demonstrate a robust activation of phospho-ERK following PMA stimulation, which is completely ablated following U0126, Ro-31-7549 or GFX treatment. In contrast, treatment with U0126 failed to block particulate PDE4D5 activation following PMA stimulation, whereas treatment of PMA stimulated cells with Ro-31-7549 and GFX caused a significant reduction in PDE activity (Figure 3.13b). These results show that activation of PDE4D5 by PMA-stimulated PKC isoforms is a direct effect of PKC on PDE4D5 and not through activation of the downstream effector, MEK.

3.4 DISCUSSION

The regulation of cyclic AMP levels within the cell allows control of numerous cellular processes (Cheng *et al.,* 2008). To fully appreciate these pleiotropic actions of cyclic AMP a thorough investigation into the role of specific cyclic AMP PDEs, their interaction with cellular proteins and the effects of this interaction on PDE activity is important to fully understand the complex regulation of cellular cyclic AMP levels. To date our knowledge of this level of sophisticated regulation is far from complete. Progress is starting to be made in this direction, particularly in the characterisation of protein interactions with the cyclic AMP-specific phosphodiesterase, PDE4D5 and the scaffold protein, β-arrestin (Bolger *et al.,* 2003; Lynch *et al.,* 2005). This complex is recruited from the cytosol to the plasma membrane in response to activation of β2-adrenergic receptor (Bolger *et al.,* 2003). The recruitment of the β-arrestin/PDE4D5 complex not only blocks the synthesis of cyclic AMP, but allows PDE4D5 to specifically hydrolyse cyclic AMP in the vicinity of the β2-adrenergic receptor, contributing to the control of compartmentalisation of cyclic AMP. In the current investigation we examined whether the RACK1/PDE4D5 was similarly recruited to the plasma membrane in response to β2-adrenergic receptor activation. While we found that isoproterenol stimulation was sufficient to activate PDE4 activity in general in HEK293 cells (Figure 3.1), and PDE4D5 specifically associated with RACK1 in the particulate fraction of cells (Figure 3.9), we did not see any translocation of the RACK1/PDE4D5 complex. This may not be surprising since the binding of RACK1 and β-arrestin to PDE4D5 appears to be mutually exclusive and, furthermore, interactions between PDE4D5 and RACK1 appear to inhibit interaction with β-arrestin (Bolger *et al.,* 2006). The RACK1/PDE4D5 complex may therefore perform different cellular functions than the β-arrestin/PDE4D5 complex. Indeed, the translocation of PDE4D5 and its cognate binding partners in response to β2-adrenergic receptor activation may depend on the cell context. In this respect β-arrestin/PDE4D5 translocation seems to occur in cells that express β2-adrenergic receptors to a high level, for example in cardiomyocytes (Lynch *et al.,* 2007) or HEK293 cells stably transfected to express high levels of receptor (Baillie *et al.,* 2003), and may reflect the ability of β-arrestin to develop high affinity interactions with this receptor type. Indeed, little translocation of β-arrestin/PDE4D5 is seen in wild type HEK293 cells, with no overexpression of the β2-adrenergic receptor (Dr. George Baillie, personal communication). The β2-adrenergic receptor may therefore present an effective recruitment site for β-arrestin/PDE4D5 signalling complexes, but not for RACK1/PDE4D5 complexes. It therefore remains to be determined what structural

influences direct the recruitment of RACK1/PDE4D5 complexes at the particulate fraction of cells.

Although RACK1 does not appear to act on targeting of PDE4D5 to specific cellular localisations, it does appear to act upon PDE4D5 structural stability. Enzymes are readily denatured when exposed to extreme or unfavourable conditions, and the stability of the protein effects how rapidly this denaturation occurs. To date, little is known as to the purpose of the RACK1/PDE4D5 complex. However, in this study, binding to RACK1 was found to afford PDE4D5 some protection against denaturation in thermal denaturation studies (Figure 3.6), with plasma membrane localisation non-RACK1 binding PDE4D5 showing a greater rate of denaturation when exposed to a temperature of $45^{o}C$ compared to RACK1 binding PDE4D5 under the same conditions, implying that the inability to bind RACK1 affects the stability of PDE4D5 and increases its susceptibility to denaturation. If RACK1 affects the conformational stability of PDE4D5, this is predicted to exert some effect on the enzymatic activity of RACK1-bound PDE4D5. Indeed, we found that RACK1 binding was necessary to maintain high affinity binding of PDE4D5 for its substrate, cyclic AMP (Figure 3.7), implying that the conformational change in PDE4D5 exerted by RACK1 binding to PDE4D5 in the particulate fraction of cells sensitises PDE4D5 toward its substrate, perhaps priming PDE4D5 for activation in this fraction. As RACK1 appears to cause a conformational change that aids interaction of PDE4D5 with cyclic AMP, this change is also likely to affect the interaction of PDE4D5 with the PDE4 specific inhibitor rolipram, which also interacts with the active site of the enzyme (Huai *et al.,* 2003). Indeed, dose-response experiments (Figure 3.8) revealed that that while cytosolic PDE4D5 exhibited a similar response to rolipram concentrations regardless of its RACK1 binding ability, PDE4D5 located in the particulate fraction of cells was more susceptible to inhibition by rolipram if bound to RACK1. These results are particularly intriguing as binding of RACK1 to PDE4D5 appears to allow lower concentrations of rolipram to inhibit the phosphodiesterase activity. Rolipram, with its anti-depressant and anti-inflammatory properties is a drug used to treat a variety of diseases, ranging from depression (Wachtel, 1983) to Alzheimer's (Gong *et al.,* 2004). Unfortunately, the drug has a number of side effects, the most prominent being its emetic effect (Houslay *et al.,* 2005). The implication of RACK1 binding to PDE4D5 requiring a lower concentration of rolipram to produce the same levels of inhibition of non-RACK1 bound PDE4D5 provides the possibility of RACK1 or a synthetic analogue being therapeutically taken alongside a

lower dose of rolipram to produce the same biological effect with potentially lessened side effects.

RACK1 is a multi-domain protein, possessing different sites for interaction with other proteins (Yarwood *et al.,* 1999). It is likely, therefore, to be able to form multi-protein complexes. As RACK1 was originally identified by its ability to bind activated conventional PKC isoforms, the RACK1/PDE4D5 complex forms a point of crosstalk between the cyclic AMP and PKC signalling pathways. The effect of activation of the PKC isoform α on the PDE4D5/RACK1 complex was investigated to determine its effect on localisation and on RACK1-bound PDE4D5 activity. PKCα and RACK1 were determined to interact in the particulate fraction of HEK293 cells (Figure 3.11) and the potent phorbol ester PMA, which causes PKC activation and translocation from the cytosol to the plasma membrane was found to cause similar levels of PDE4D5 activity in the membrane fraction of HEK293 cells to those induced by isoproterenol stimulations (Figure 3.9). Furthermore, PDE activity assays on RACK1-bound PDE4D5 and non-RACK1 binding mutant PDE4D5 expressing cells stimulated with PMA showed that the RACK1 interaction is essential to allow efficient activation of PDE4D5 by PKCα (Figure 3.12). That PKCα was the cause of the PDE4D5 activation and not downstream effectors of PKC was confirmed by treatment with various PKC and MEK specific inhibitors prior to determination of PDE activity (Figure 3.13). Further experiments to investigate the importance of RACK1 in anchoring PKCα at the plasma membrane, and its effects on PKC function and activation of PDE4D5 activity, by stable over-expression and anti-sense depletion of RACK1 would provide insight into whether RACK1 is required for PKC translocation and by investigating the effects of variation of RACK1 levels on the phosphorylation of ERK, a downstream effector of PKC would further demonstrate the requirement of RACK1 binding on PKC action on PDE4D5. Additionally, the use of peptide inhibitors of the PKCα/RACK1 interaction would further determine the importance of PKCα anchoring to the membrane on PKCα function. Additionally, PDE4D5 binds both RACK1 and β-arrestin in a mutually exclusive way although the affinity of PDE4D5 to each of these has yet to be measured. Further experimentation to determine whether PKCα can promote PDE4D5 activity when PDE4D5 is bound to β-arrestin would expand on the importance of RACK1 on the ability of PKCα to influence PDE4D5 activity.

The complex formed between PDE4D5 and RACK1 provides a point of crosstalk between the cyclic AMP and PKC signalling pathways as well as a potential point of feedback as

increased cyclic AMP levels have been shown to activate PKC (Borland *et al.,* 2009), and PKC activation increases PDE activity (Figure 3.12) which in turn reduces cyclic AMP levels. The results outlined in this chapter show the importance of RACK1 binding by PDE4D5 in its enzymatic activity and the effects of PKC activation on the phosphodiesterase activity of PDE4D5. To conclude, therefore, this chapter outlines a role for the RACK1/PDE4D5 complex in causing a conformational change to affect the enzymatic activity of the phosphodiesterase, and its response to the inhibitor rolipram. Furthermore, the complex forms a point of crosstalk between the cyclic AMP and PKC signalling pathways, with PKCα interacting with RACK1 at the plasma membrane and causing increases upon activation of RACK1 bound PDE4D5 activity, thus showing that the activation of PKC can negatively regulate cyclic AMP levels in HEK293 cells. As regulation of cyclic AMP levels allows control of a wide range of cellular processes, negative control of cyclic AMP signalling by PKC activation not only goes some way towards elucidating how cyclic AMP levels are lowered within the cell but may also provide a therapeutic target with which to control cyclic AMP signalling in disease associated with elevated cyclic AMP levels.

In conclusion, this chapter shows a novel point of crosstalk between the cyclic AMP and PKC signalling pathways wherein PKC activation leads to activation of RACK-1 bound PDE4D5, thus forming a negative feedback loop to control intracellular cyclic AMP levels in HEK293 cells.

CHAPTER 4

The Roles of <u>E</u>xchange <u>P</u>rotein <u>A</u>ctivated by <u>C</u>yclic AMP (EPAC) 1 and Protein Kinase C (PKC) Isoforms in the Induction of the <u>S</u>uppressor <u>of</u> <u>C</u>ytokine <u>S</u>ignalling (SOCS) 3 gene by cyclic AMP

4.1 INTRODUCTION

The EPAC (exchange protein activated by cyclic AMP) proteins, EPAC1 and EPAC2, are multi-domain proteins that act as guanine nucleotide exchange factors (GEFs) for the small GTPase Rap1, in response to elevations in intracellular cyclic AMP (de Rooij *et al.,* 1998). EPAC proteins have a high affinity for cyclic AMP and have been found to be involved in the regulation of a number of important physiological processes in the brain, lungs, heart and immune system, including cell differentiation and proliferation, apoptosis, muscle contraction, calcium handling and inflammation (as reviewed in Borland *et al.,* 2009). Due to these roles, EPAC activity has been linked with the development of a number of metabolic, neurodegenerative and inflammatory diseases; for example EPAC2 has been linked to autoimmune diseases, inflammatory disorders such as chronic asthma, cardiovascular disorders and Alzheimer's (McPhee *et al.,* 2005). Its downstream effector, Rap1, is involved in the regulation of several crucial cell functions, including cell proliferation, integrin-mediated cell adhesion and leukocyte migration (Hattori and Minato, 2003). Many of these functions have been found to be disrupted in some cancers such as leukaemia (Hattori and Minato, 2003), prostate (Bailey *et al.,* 2009) and breast cancers (Itoh *et al.,* 2007), while the reported ability of EPAC2 to promote exocytosis of insulin-containing granules from pancreatic beta cells suggests a role in combating diabetes (Holz, 2004). While traditionally the majority of cyclic AMP signalling was believed to occur via phosphorylation of intracellular proteins by PKA (Conti *et al.,* 2003), the discovery of EPAC has provided a novel mechanism for the mediation of cyclic AMP signalling in a variety of tissues, although these mechanisms are largely unknown.

Recent studies have outlined a PKA-independent pathway in which EPAC mediates the induction of the anti-inflammatory Suppressor of Cytokine Signalling (SOCS-3) gene by cyclic AMP (Sands *et al.,* 2006). SOCS proteins are an eight member family of proteins that act as negative regulators of cytokine signalling (Starr and Hilton, 1998). SOCS proteins usually have a low level expression in cells but are rapidly induced by cytokines. This allows the inhibition of JAK-STAT signalling, which forms a negative feedback loop (Shuai and Liu, 2003). SOCS-3 interferes with the JAK-STAT pathway by directly binding to Tyr1007 and inhibiting Janus Kinase 2 (JAK2), preventing the tyrosine phosphorylation and activation of Signal Transducers and Activators of Transcription (STATs) 1 and 3 (Auernhammer and Melmed, 2001). The JAK-STAT pathway is critical for the propagation of intracellular signalling from a range of cytokine receptors and is therefore

involved in a number of cellular functions, such as the immune response, haematopoiesis and cell growth and proliferation (Rawlings *et al.,* 2004). The JAK-STAT pathway is activated by a range of cytokines, including interleukin-6 (IL-6), a class I cytokine that is involved in the activation of a range of genes involved in cell proliferation, apoptosis and differentiation (Heinrich *et al.,* 2003). IL-6 is involved in both initiation and maintenance of immune and inflammatory responses and strict regulation is therefore required. Disruption of IL-6 signalling has been linked to a number of diseases, particularly those linked to the immune response such as rheumatoid arthritis, Castleman disease and Crohn's disease (Ishihara and Hirano, 2002). Increased levels of IL-6 have been implicated in a range of disorders, including osteoporosis, psoriasis, (Jones *et al.,* 2001) multiple myeloma (Ishihara and Hirano, 2002) and renal cancer (Takenawa *et al.,* 1991). IL-6 signalling involves the binding of the IL-6 receptor (IL6R) to the transmembrane gp130, a glycoprotein also employed by other members of the IL-6 family (Carroll *et al.,* 1998). These IL-6 family members are structurally distinct but share the gp130 subunit and include Interleukin-11 (IL-11), Cardiotrophin-1 (CT-1), Oncostatin M (OSM) and Leukaemia Inhibitory Factor (LIF) (Carroll *et al.,* 1998). A complex is formed, activating the receptor and initiating the signal transduction cascade through activation of the JAK-STAT pathway (Jones *et al.,* 2005). JAKs bind to the cell surface cytokine receptor where activation of JAKs creates phosphotyrosine residues on the receptor. This triggers recruitment of STAT3 to the receptor, where the residues act as docking sites for SH2 domains located on STAT3 and mediates the homodimerisation of STAT3 by tyrosine phosphorylation (Schindler *et al.,* 2007). The homodimerised complex then translocates to the nucleus where it recruits transcriptional co-activators, binds specific DNA elements and then is able to initiate transcription of genes responsive to IL-6 (Borland *et al.,* 2009). The induction of SOCS-3 by cyclic AMP, however, prevents this, thus inhibiting the IL-6 stimulated response, as SOCS-3 binds directly to the phosphorylated receptors by its SH2 domain, also inhibiting JAKs and preventing both recruitment and phosphorylation of STAT3, thus inhibiting IL-6 stimulated gene expression (Sands *et al.,* 2006) (Figure 4.1). As SOCS-3 induction by cyclic AMP occurs after 5 hours, elevations in cyclic AMP attenuate STAT activation. In this way, SOCS-3 is able to interfere with cytokine signalling, providing a means of negative control as well as a potential target for influence of IL-6 signalling. Expression of the SOCS-3 gene can occur in response to cytokines as well as other proteins. The minimal functional promoter of SOC-3 consists of two STAT-responsive elements (SREs), a GC-rich region and an A/T-rich region, and an AP1 (activation protein 1 element, with STATs acting at the proximal SRE and the GC- and

Figure 4.1

a)

b)
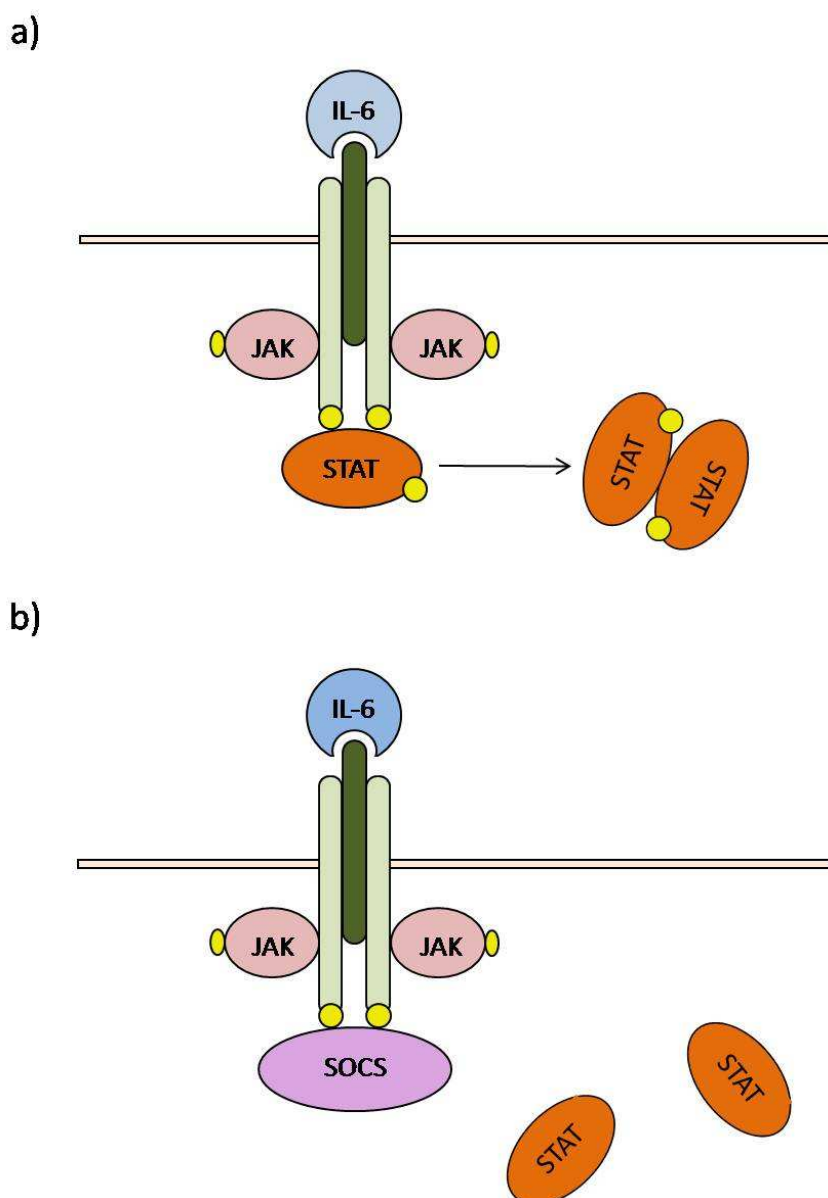
Adapted from Rønn *et al*. 2007

Figure 4.1 SOCS proteins inhibit the JAK-STAT pathway

a) Following IL-6 binding, JAKs bind to the cell surface cytokine receptor. Activation of JAKs creates phosphotyrosine residues on the receptor which act as docking sites for the SH2 domain of STATs and mediate homodimerisation of STATs. Dimerised STATs translocate to the nucleus where they activate transcription of target genes.

b) SOCS proteins inhibit the JAK-STAT pathway by binding to the phosphorylated receptors, preventing STAT binding and homodimerisation.

A/T- rich regions (Barclay *et al.,* 2007). No functional CREs (cyclic AMP response elements) are contained within the SOCS-3 promoter, as evidenced by the inability of PKA activation to induce SOCS-3 (Yarwood *et al.,* 2008). While the ability of cyclic AMP to affect gene transcription was thought to occur via PKA activation of the transcription factor CREB, recent studies have found that cyclic AMP is able to influence gene transcription independently of PKA through CCAAT/enhancer-binding proteins (C/EBPs) (Yarwood *et al.,* 2008). In COS1 cells, a role has been found for C/EBPβ in the activation of SOCS-3 by cyclic AMP (Borland *et al.,* 2009).

As the inflammatory response requires tight regulation, it is important to have a complete understanding of the mechanisms controlling SOCS-3 induction, a major mechanism in the control of the JAK-STAT pathway. While it has been shown that EPAC1 plays an important role in SOCS-3 induction by cyclic AMP in vascular endothelial cells (Sands *et al.,* 2006), MEFs (Yarwood *et al.,* 2008) and COS1 cell lines (Borland *et al.,* 2009), the pathway between these two points has not been fully expounded upon. While there are a multitude of possible downstream effects of EPAC1 in cells including other Ras family GTPases, PKB/Akt, Phospholipase D, ion channel activation and MAP kinases (Cheng *et al.,* 2008; Roscioni *et al.,* 2008), reports of an interaction between EPAC1 and the PLC isoform ε (Oestreich *et al.,* 2009), provided an avenue of investigation, both into the role of PLC itself, the products of its intracellular activity and their downstream effects, on SOCS-3 induction by cyclic AMP. Prior to this investigation nothing was known as to the effects of PLC ε on SOCS-3 induction. This chapter aims to investigate the downstream components of an EPAC1-activated signalling pathway leading from cyclic AMP generation to SOCS-3 induction in COS1 cells. This elucidated pathway shows a critical point of regulatory crosstalk between the cyclic AMP and PKC signalling pathways, forming part of a pathway which results in SOCS-3 gene induction, and the potential to regulate IL-6 signalling. The previous results chapter demonstrated that PKC activation leads to activation of RACK1-bound PDE4D5. This chapter investigates the hypothesis that the PKC signalling pathway is involved in the EPAC1 mediated induction of SOCS-3 by cyclic AMP, thus forming a point of crosstalk between the two pathways. The findings from Chapter 3, together with the findings presented in the current chapter, go some way towards defining a novel cyclic AMP-regulated signalling system with the ability to control key, disease-associated, transcriptional events in human cells.

4.2 MATERIALS AND METHODS

All Materials and Methods as outlined in Chapter 2 with variations described below.

Inhibitor treatment of COS1 cells

For SOCS-3 induction experiments, confluent COS1 cells were treated for 5 hours with PKC inhibitors Ro31-7549 or GF 109203X, or the PLC inhibitor U73122 in the presence or absence of cyclic AMP stimulating forskolin (10µM) plus rolipram (10µM) or 8Me (50µM). Samples were lysed directly into Laemmli electrophoresis buffer (Laemmli, 1970) and analysed by SDS-PAGE followed by Western blotting with anti-SOCS-3 and anti- Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific antibodies. For PKC down-regulation experiments, COS1 cells were stimulated with 1µM phorbol 12-myristate 13-acetate (PMA) or dimethyl sulphoxide (DMSO) for 48 hours. Cells were then stimulated for 5 hours with 10µM PMA, F/R, or 8Me. Cell lysates were then immunoblotted using anti-PanPKC, anti-SOCS-3 and anti-GAPDH specific antibodies. Experiments were all carried out in the presence of MG132 (6µM), a proteasome inhibitor, to prevent SOCS-3 degradation after synthesis.

Densitometry and Statistical Analysis

Non-saturating exposures from multiple experiments were quantified densitometrically using ImageJ software. Densitometric values for SOCS-3 were usually normalized to the levels of GAPDH protein expression found in the same sample, while PKC levels were normalized to the expression of RACK1 protein levels in the same sample. Statistical significance was determined using Student's two-tailed t-test.

4.3 RESULTS

4.3.1 EPAC1 mediates SOCS-3 induction in COS1 cells - The link between EPAC1 activation and SOCS-3 induction originally identified in human umbilical vascular endothelial cells (HUVECs) provides a novel pathway, independent of PKA, by which cyclic AMP is able to negatively control cytokine signalling and provides a new pathway of potential therapeutic targets (Sands *et al.,* 2006). In order to demonstrate that this pathway occurs in other cell types, it was decided to investigate its components in COS1 cells, which are highly amenable to transfection with siRNA.

In 1998, double stranded RNA (dsRNA) was found to be able to silence gene expression, a process called RNA interference (RNAi). RNAi uses the RNA-induced silencing complex (RISC) to guide dsRNA that has been cleaved into short interfering RNA (siRNA) fragments by the enzyme Dicer, to the target mRNA. Synthetically made siRNA, that is usually 21-23 nucleotides long, bypasses the need for cleavage by Dicer. RISC unwinds the siRNA and guides it to bind and degrade the complementary mRNA, thus silencing the gene (reviewed in Whitehead *et al.,* 2009). This allows an effect experimental method to investigate the effects of knockdown of a gene within a cell line. To show that EPAC1 is necessary for SOCS-3 induction in COS1 cells, knockdown of EPAC1 was achieved via transfection of COS1 cells with EPAC1 specific siRNA (GCCCGGAACUUGCCUGUUU, Dharmacon) or non-targeting control siRNA (Qiagen). Although sequences for this negative control siRNA are unavailable, the siRNA has no homology to any known mammalian gene and has been confirmed to enter RISC. In this experiment mock transfected cells were checked, but as there was no effect of control siRNA all comparisons were made against control siRNA treated cells.

Cells were then stimulated for 5 hours with a combination of forskolin and rolipram (F/R) or 8-pCPT-2'-O-Me-cyclic AMP (8Me), a cell permeable cyclic AMP analogue that is specific for EPAC, which it activates independently of PKA (Holz *et al.,* 2008). Forskolin elevates cyclic AMP levels within the cell by activation of adenylyl cyclase isoforms to increase cyclic AMP production within cells (Insel and Ostrom, 2003). Rolipram is a cell permeable phosphodiesterase 4 (PDE4) inhibitor, with documented anti-inflammatory and anti-depressant properties (Wachtel, 1983; Kehr *et al.,* 1985). As a specific PDE4 inhibitor, rolipram prevents breakdown of cyclic AMP and is preferable in these experiments to general PDE inhibitors such as isobutyl-methylxanthine (IBMX) which may give non-

specific effects due to its ability to act as an adenosine receptor antagonist (Schultz *et al.,* 1982). The use of forskolin and rolipram together is a common means of causing an increase of intracellular cyclic AMP levels through increased production and reduced breakdown and although Gs coupled receptors are present in COS-1 cells, the combination of F/R was chosen to maximally activate the system. The proteasome inhibitor MG132 was used in these experiments to prevent degradation of SOCS-3 upon synthesis. Cell lysates were prepared from stimulated cells and immunoblotted with anti-SOCS-3 and anti-GAPDH antibodies (Figure 4.2). GAPDH acts as a loading control due to its stable and high expression in these cells, making it a useful housekeeping gene (Barber *et al.,* 2005). The immunoblot clearly shows that SOCS-3 is induced in control siRNA treated cells, with the greatest induction seen in F/R treated cells. Additionally the immunoblots shows that treatment with 8Me is sufficient to induce SOCS-3, suggesting a role for EPAC. There is a significant reduction in SOCS-3 induction in EPAC1 siRNA treated cells stimulated with both 8Me and F/R compared to control siRNA treated cells stimulated with F/R, indicating that EPAC1 is required for SOCS-3 induction, as has been previously shown in human umbilical vein endothelial cells (HUVECs) and mouse embryonal fibroblasts MEFs (Sands *et al.,* 2006; Yarwood *et al.,* 2008).

4.3.2 Induction of the SOCS-3 gene in COS1 cells is dependent on PLCε - Having confirmed the involvement of EPAC1 in SOCS-3 induction by cyclic AMP in COS1 cells, efforts were made to identify the down-stream signalling components in this new pathway. Given the reported potential involvement of PLC ε as a novel downstream target of cyclic AMP and Rap2B (Schmidt *et al.,* 2001), this was considered as a potential starting point. Traditionally PLC signalling was thought to occur through two routes of activation- by tyrosine kinases via binding of the Src homology 2 (SH2) domain on PLCγ to tyrosine autophosphorylation sites found on activated tyrosine kinases, or through activation by G protein coupled receptors that activate Gq proteins, which in turn causes activation of PLCβ isoforms mediated by its Gqα subunits (Schlessinger, 1997). However, the identification of 6 PLC isoform families has expanded the range of PLC signalling as each isoform has unique requirements for regulation (Harden and Sondek, 2006). Recent studies have indicated an interaction between one such PLC isoform, PLCε, with EPAC, demonstrating that this interaction is involved in the β-adrenergic receptor stimulation of Ca^{2+}-induced Ca^{2+} release (CICR) in cardiac myocytes (Oestreich *et al.,* 2009), and that in HEK293 cells the activation of PLCε, via the GTPase Rap2B, occurs through elevations in intracellular cyclic AMP that stimulates EPAC (Schmidt *et al.,* 2001). This link between

Figure 4.2
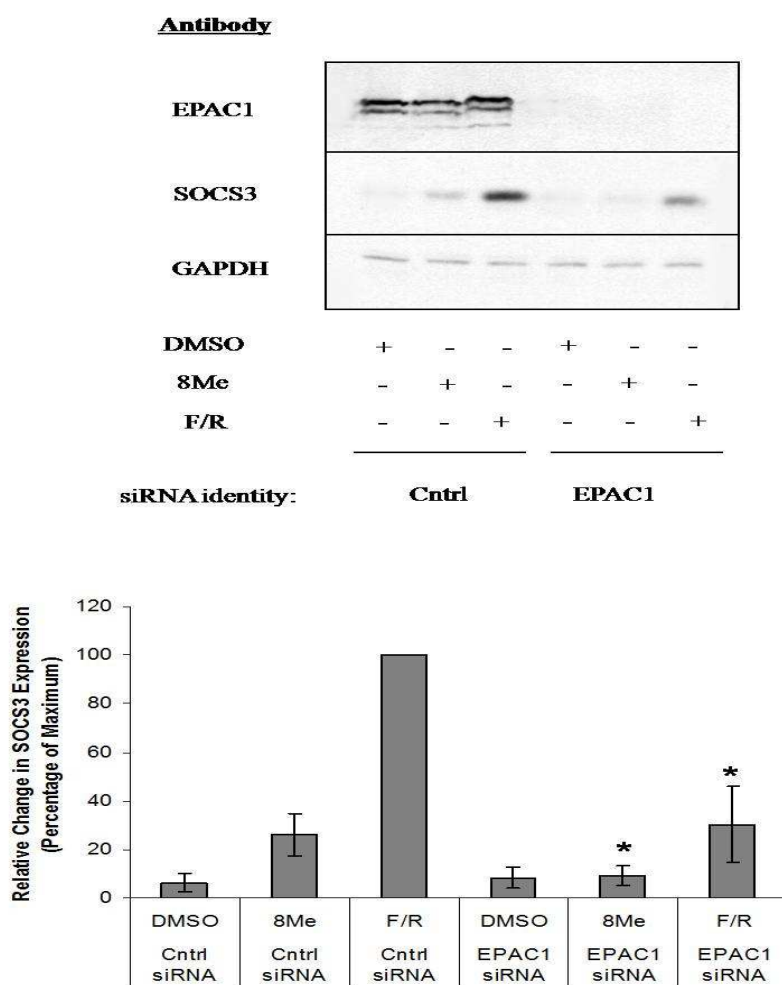
Figure 4.2 EPAC is necessary for SOCS-3 induction in COS1 cells.

COS1 cells were transfected with control siRNA or EPAC1 siRNA using Lipofectamine 2000 according to the manufacturer's instructions. Cells were then incubated for 5 hours with MG132 and either 10μM F/R or 50μM 8Me. Cell lysates were immunoblotted with EPAC1, SOCS-3 or GAPDH specific antibodies. Densitometry was carried out and the values plotted on a histogram with control siRNA treated F/R stimulated cells treated as 100% and all other values expressed relative to this. The blots show a clear knockdown of EPAC in cells treated with the EPAC-specific siRNA. Densitometry was carried out on immunoblots from three separate experiments and the results plotted as means on the graph with significant differences between SOCS-3 levels in EPAC specific siRNA treated cells compared to control siRNA treated cells, indicated by $*$ = $p < 0.05$ against F/R stimulation in control siRNA cells.

PLC signalling and EPAC raises the possibility of a role for PLCε in the EPAC mediated SOCS-3 induction pathway.

To investigate an involvement of PLC isoforms downstream of EPAC1, endogenous PLC activity in COS1 cells was inhibited using U73122, an aminosteroid that specifically inhibits PLC, at 10μM and 50μM. Although the precise mechanisms of action of this inhibitor are unknown, it has been shown to inhibit PLC in numerous cell types (Muto *et al.,* 1997). While little is known of its actions on PLCε itself, U73122 inhibits the phosphatidylinositol-specific PLC isoform family, of which PLCε is a member. The inactive analogue U73343 was used as a negative control at the same concentrations of U73122. COS1 cells were treated with these PLC inhibitors in the presence and absence of F/R for five hours. Cells were then harvested and immunoblotting carried out using anti-SOCS-3 and anti-GAPDH antibodies (Figure 4.3). Immunoblots demonstrated that there was a clear reduction in SOCS-3 induction by F/R in cells treated with 50μM U73122 resulting in a reduction of SOCS-3 expression by approximately 90%, in comparison to both the untreated and negative control treated cells (Figure 4.3). As U73122 appears to block SOCS-3 induction, this indicates a potential role for one or more PLC isoforms in SOCS-3 induction by cyclic AMP in these cells.

To further confirm a role for PLC in SOCS-3 induction and the role of PLCε in particular, COS1 cells were treated with various concentrations of PLCε-specific siRNA (CAGGGTCTTGCCAGTCGACTA, Qiagen) (Figure 4.4a) and non-targeting siRNA (Qiagen) as a specificity control, in the presence and absence of F/R (Figure 4.4b). PLCε siRNA treatment clearly reduced PLCε expression in the cells and inhibited induction of SOCS-3 when stimulated with F/R. This suggests that PLCε is required for SOCS-3 induction by cyclic AMP in COS1 cells. To determine whether PLCε is involved in SOCS-3 induction by EPAC1, cells were treated with PLCε siRNA as above, in the presence of 8Me, F/R or PMA, a phorbol ester which activates conventional and novel PKC isoforms (Figure 4.4c). 200nM of PLCε siRNA was chosen for this experiment as this concentration was shown in the previous experiments to be sufficient to knockdown PLCε expression in COS-1 cells. While 8Me, F/R and PMA all induce SOCS-3 in control cells, knockdown of PLCε expression clearly inhibited the ability of 8Me and F/R to induce SOCS-3 in these cells. This indicates that PLCε is found downstream of EPAC1 and that therefore PLCε is located within the EPAC1 induced SOCS-3 induction pathway in COS1 cells. As expected, SOCS-3 levels induced by PMA shows little reduction in cells treated with

Figure 4.3
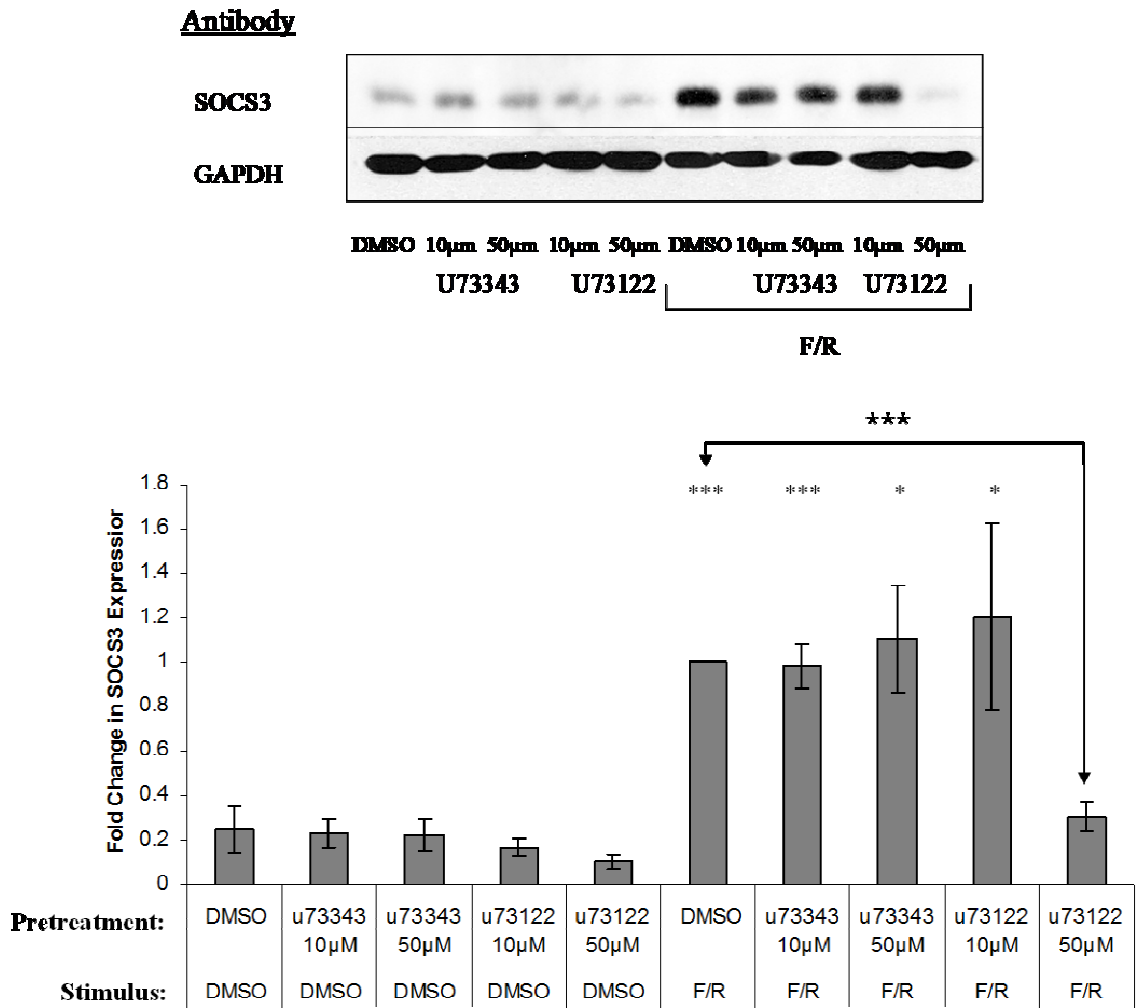
Figure 4.3 Effect of Phospholipase C inhibitors on SOCS-3 induction

COS1 cells were stimulated for 5 hours with 6μM of the proteosome inhibitor MG132, and 10μM F/R in the presence or absence of 10μM or 50 μM of the PLC inhibitor U73122 or 10μM or 50 μM of the inactive control inhibitor U73343. Cell lysates were then immunoblotted with SOCS-3 and GAPDH specific antibodies. The SOCS-3 blot shows endogenous SOCS-3 induction in cells not stimulated with F/R, which is slightly reduced by the presence of U73122 at both concentrations. Densitometry was carried out on immunoblots from three separate experiments and normalised to DMSO, F/R treated cells. The results were then plotted, with significant differences, relative to non-stimulated cells, indicated by ** = p<0.01 and *** = p<0.001.

Figure 4.4
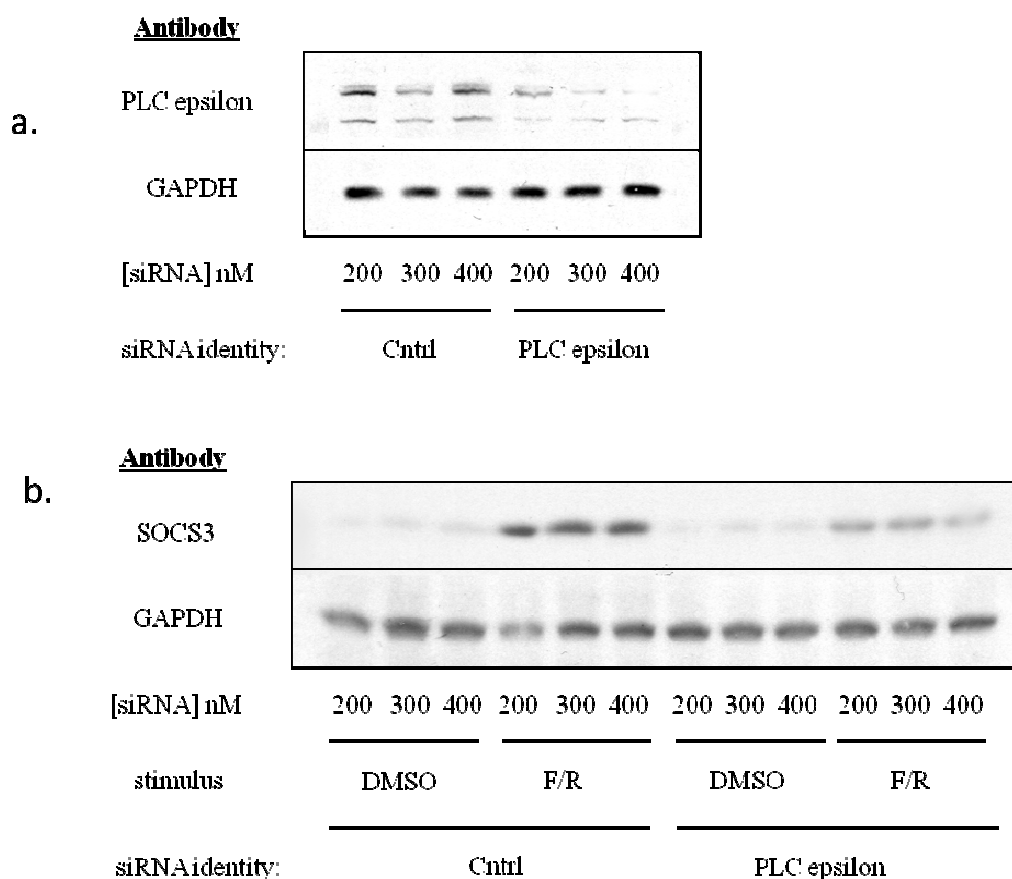
Figure 4.4 Effect of Phospholipase C epsilon siRNA on SOCS-3 induction

a) COS1 cells were transfected with control siRNA or PLCε specific siRNA using Lipofectamine 2000 according to the manufacturer's instructions at 200, 300 and 400 nM. Cells were harvested and lysates immunoblotted with PLCε and GAPDH specific antibodies. The PLCε blot shows a clear knockdown of PLCε in COS1 cells, with the 300nm and 400nm concentrations showing the greatest levels of knockdown.

b) COS1 cells were transfected with control siRNA or PLCε specific siRNA using Lipofectamine 2000 according to the manufacturer's instructions at 200, 300 and 400nm. Cells were then incubated for 5 hours with 6μM MG132, in the presence or absence of 10μM F/R. Cell lysates were then immunoblotted with SOCS-3 and GAPDH specific antibodies. Cells treated with PLCε specific siRNA show almost no SOCS-3 induction in cells in which F/R is absent, but in cells stimulated with F/R there is a clear reduction in the levels of SOCS-3, at all 3 concentrations of PLCε siRNA.

Figure 4.4 continued.
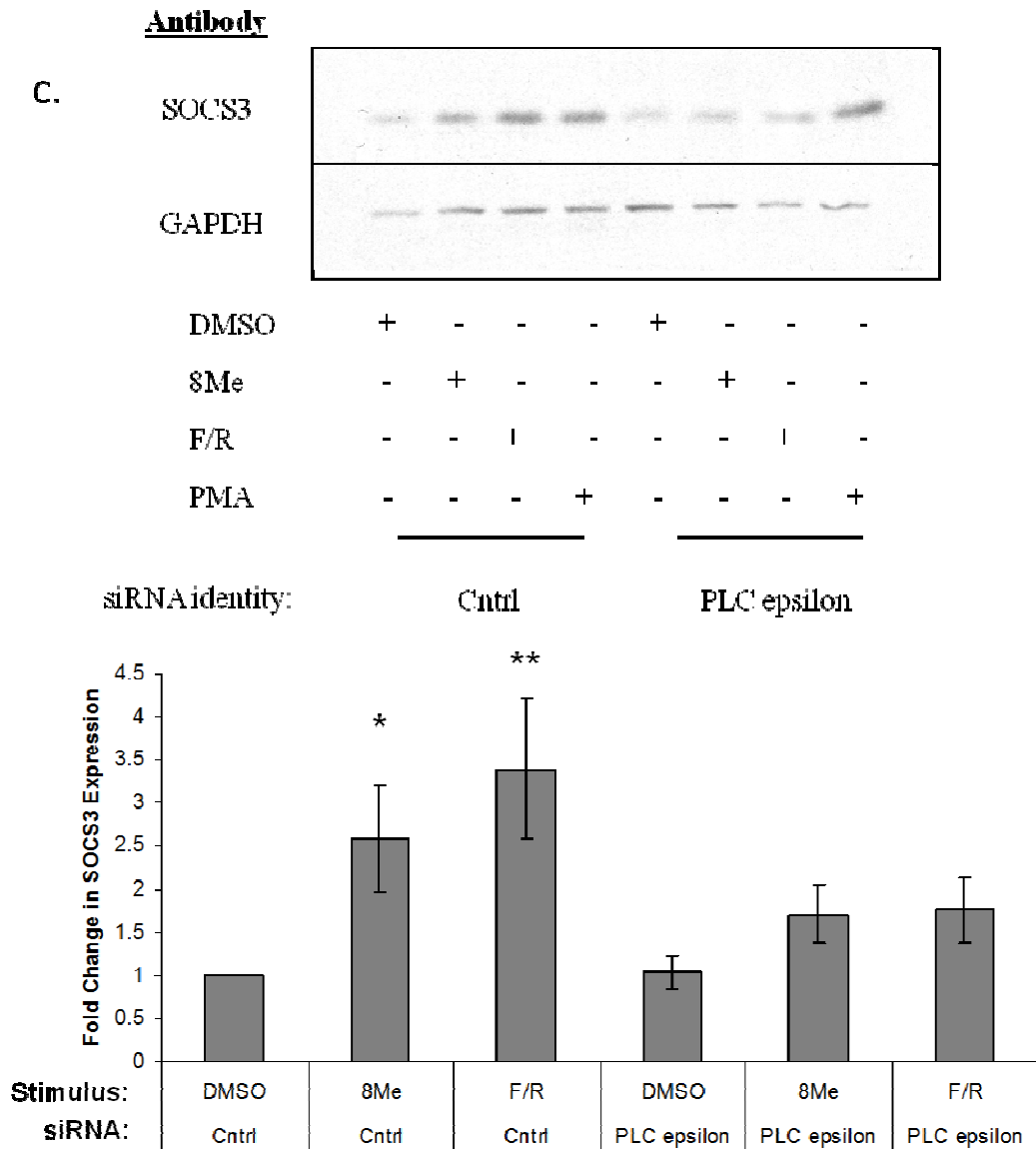
c) COS1 cells were transfected with 200nM of control siRNA or PLCε specific siRNA using Lipofectamine 2000 according to the manufacturer's instructions. Cells were then stimulated with either 10μM F/R, 50μM 8-pCPT-2'-O-Me-cyclic AMP (8Me) or 10μM PMA for 5 hours. Cell lysates were then immunoblotted with either SOCS-3 or GAPDH specific antibodies. In cells treated with the control siRNA, SOCS-3 is induced by 8Me, F/R and PMA, with F/R and PMA giving the highest levels. In cells treated with the PLCε siRNA, SOCS-3 induction by 8Me and F/R is clearly reduced. Densitometry was carried out on immunoblots from three separate experiments and normalised to DMSO, control siRNA treated cells. The results were then plotted, with significant differences, relative to non-stimulated cells, indicated by * = p<0.05 and ** = p<0.01.

PLCε siRNA as PKC activation occurs downstream of PLC. However the reduction in SOCS-3 induction in response to PLCε knockdown suggests a role for downstream effectors of PLC, such as PKC isoforms in the SOCS-3 signalling pathway.

4.3.3 Involvement of PKC isoforms in SOCS-3 induction - The actions of PLC isoforms in cells is well documented. Hydrolysis of PIP2 results in cleavage into the second messengers IP3 and diacylglycerol (DAG). DAG production causes activation of PKC isoforms, while IP_3 production causes mobilisation of Ca^{2+} by binding to IP3 receptors on endoplasmic and sarcoplasmic reticulum. These IP3 receptors control the influx of internal Ca^{2+} stores through the opening and closing of calcium channels on the membranes of the organelles (Mikoshiba, 2007). Having established a potential role for a PLC isoform in the SOCS-3 induction pathway, the potential roles of PLC products on the induction of the SOCS-3 by cyclic AMP and EPAC1 was investigated in COS1 cells.

Firstly, the involvement of Ca^{2+} was tested using a cell-permeable calcium chelator, BAPTA-AM (1,2-bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid, tetracetoxymethyl ester) that acts as an effective intracellular chelator of cytosolic calcium. COS1 cells were stimulated with various concentrations of 8Me (Figure 4.5a) or F/R (Figure 4.5b) in the presence or absence of this chelator to test whether the removal of endogenous Ca^{2+} levels affected SOCS-3 expression, therefore determining whether the presence of Ca^{2+} was required for SOCS-3 induction. Cell lysates were prepared and separated by SDS-PAGE and immunoblotted with SOCS-3 or GAPDH specific antibodies. These immunoblots clearly show an increase in SOCS-3 induction upon stimulation with either 8Me or F/R and a significant reduction in both stimulations in the presence of BAPTA-AM. From these results, it appears that endogenous intracellular Ca^{2+} is required for SOCS-3 induction by cyclic AMP and EPAC1 in COS1 cells. To determine the effects of the other PLC product DAG, a cell permeable DAG analogue, 1, 2-diaoctanoyl-sn-glycerol (OAG) that activates PKC was used at varying concentrations to test its ability to stimulate SOCS-3 in COS1 cells. Cells were incubated for 5 hours with either F/R or OAG in the presence or absence of BAPTA-AM. Cell lysates were immunoblotted with SOCS-3 or GAPDH antibodies (Figure 4.5c). The immunoblot shows that OAG treatment is sufficient to induce SOCS-3 expression, while co-stimulation with BAPTA-AM results in an ablation of this induction. This suggests that OAG action requires the presence of Ca^{2+}, indicating that DAG production in COS1 cells is sufficient for Ca^{2+} dependent induction of

Figure 4.5
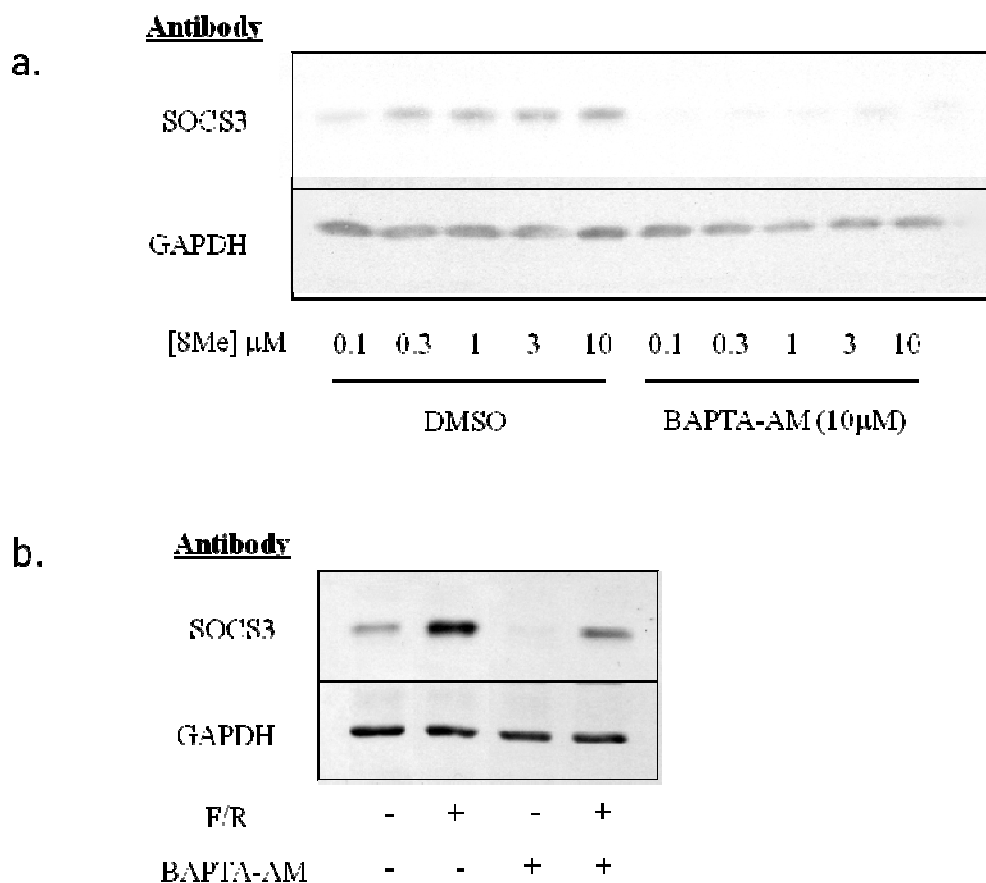
Figure 4.5 Calcium is required for both EPAC and DAG stimulated SOCS-3 induction in COS1 cells.

a) Effect of the presence of calcium on EPAC stimulated SOCS-3 induction.

COS1 cells were stimulated with 8Me at five concentrations ranging from 0.1µM to 10µM, in the presence or absence of the calcium chelator BAPTA-AM (10µM) for 5 hours. Cell lysates were then immunoblotted with SOCS-3 or GAPDH specific antibodies. The blots show that chelation of calcium prevents SOCS-3 induction in response to 8Me. Although untreated control cells were omitted from this experiment, no difference between 0 and 0.1µM 8Me was observed in other experiments.

b) Effect of presence of calcium on F/R stimulated SOCS-3 induction.

COS1 cells were stimulated for 5 hours with 10µM F/R in the presence or absence of 10µM BAPTA-AM. Cells lysates were immunoblotted with SOCS-3 or GAPDH specific antibodies. The blots indicate that the presence of BAPTA-AM is sufficient to reduce SOCS-3 induction by F/R.

Figure 4.5 continued.

c.

Antibody

SOCS3

GAPDH

[OAG] µM   0 10 50 100 0 10 50 100 0

DMSO   BAPTA-AM   F/R
(10µM)

d.
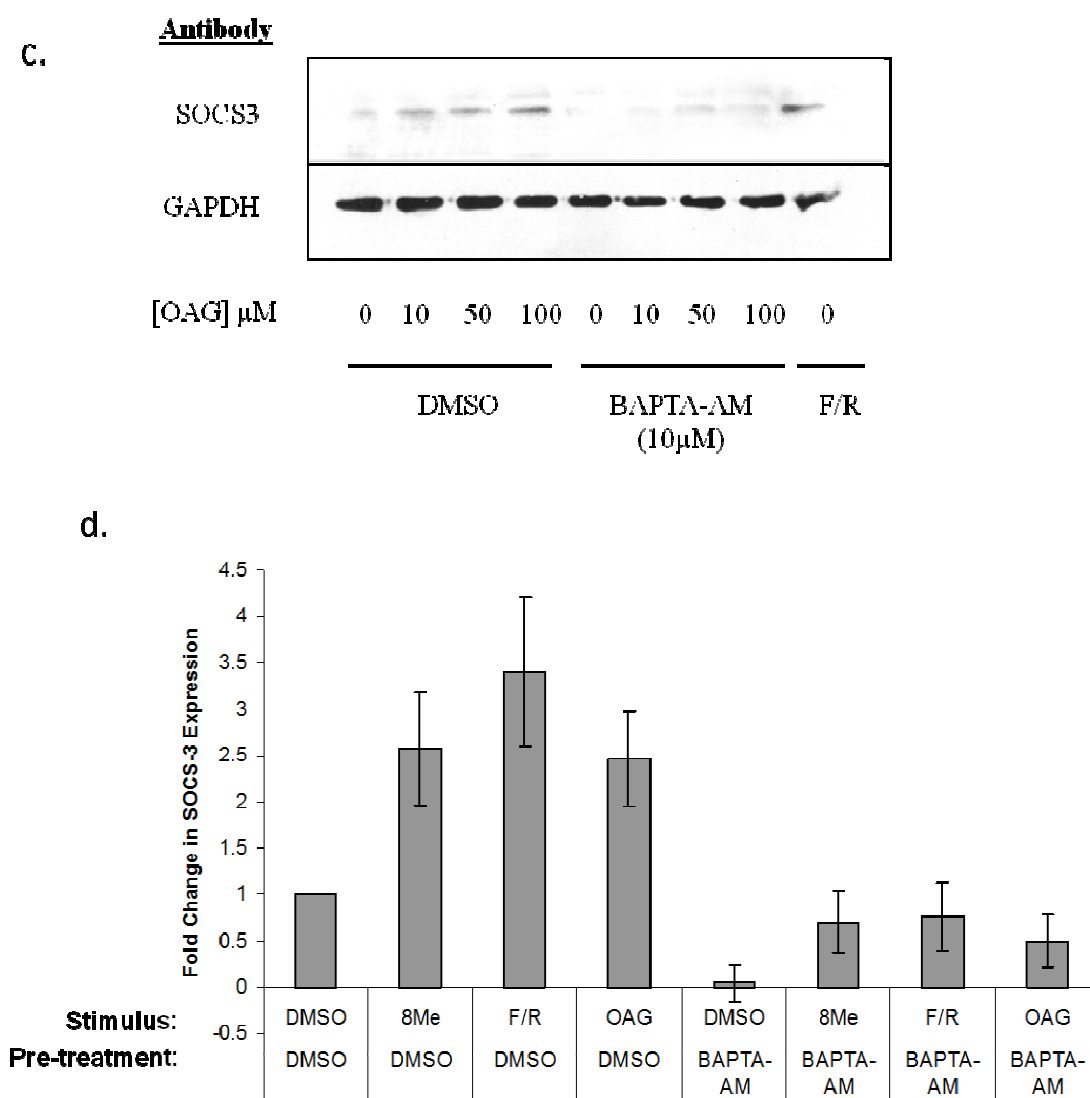
c) Effect of presence of calcium and OAG on SOCS-3 induction.

COS1 cells were stimulated for 5 hours with either 10µM F/R, or 10, 50 or 100µM of the cell permeable DAG analogue OAG in the presence or absence of 10µM BAPTA-AM. Cell lysates were then immunoblotted with SOCS-3 or GAPDH specific antibodies. Immunoblots reveal that expression of SOCS-3 in response to OAG stimulation is ablated by the presence of BAPTA-AM.

d) Densitometric values were obtained from figures 4.4 a-c, and combined to plot as a histogram, with control cells pre-treated with DMSO and then stimulated with DMSO expressed as 100% and all other values expressed relative to this.

SOCS-3. This implies a role for downstream signalling of PLC in the SOCS-3 regulatory pathway.

The main intracellular targets for DAG and Ca^{2+} in cells are the PKC family of signalling proteins. A number of PKC isoforms are activated by the products of PLC activation. Conventional PKC (α, β and γ) activation requires both DAG and Ca^{2+}, while novel PKC (δ, ε, η and θ) activation requires DAG but not Ca^{2+}. Atypical PKCs (ζ, ι and λ) require neither (Parker and Murray-Rust, 2004). The above results indicate a potential role for either conventional or novel PKCs in SOCS-3 induction as it appears their activators DAG and Ca^{2+} are required for SOCS-3 induction. The involvement of PKC in SOCS-3 induction was therefore investigated by stimulating COS1 cells with 8Me, F/R or PMA, a cell permeable activator of PKC, in the presence and absence of 50μM cell permeable PKC inhibitors Ro 31-7549 or GF 109203X (GFX). Ro-31-7549 acts on the ATP binding site of PKC, making it a selective PKC inhibitor (Wilkinson *et al.,* 1993) while GFX is also believed to interact with the ATP binding site, acting as a competitive inhibitor of ATP and making it a potent selective PKC inhibitor (Toullec *et al.,* 1991). Cells were then harvested and immunoblotting carried out using anti-SOCS-3 and anti-GAPDH antibodies (Figure 4.6). Results demonstrated that PMA is sufficient to induce expression of SOCS-3. SOCS-3 induction is clearly inhibited in cells stimulated with 8Me, F/R and PMA and treated with Ro 31-7549 or GFX, while stimulated cells absent of inhibitor treatment display SOCS-3 induction at varying levels, with F/R showing the greatest induction. GFX inhibits SOCS-3 induction in cells stimulated with 8Me and PMA, and significantly lowers SOCS-3 induction in F/R-stimulated cells, while Ro 31-7549 completely inhibits SOCS-3 induction with all stimulations. As the PKC inhibitors block SOCS-3 induction, this suggests that one or more PKC isoforms are required for SOCS-3 induction by cyclic AMP in COS1 cells.

To further investigate the role of PKC isoforms in SOCS-3 induction by EPAC in COS1 cells, PKC down-regulation was achieved using prolonged PMA stimulation. Chronic stimulation with PMA results in down-regulation of conventional and novel PKC isoforms, leading to both a loss of responsiveness to PMA stimulation as well as a loss of PKC activity in the cells (Blumberg, 1988). This allows investigation into the requirement of activity of PKC isoforms on SOCS-3 induction. Cells were chronically stimulated with PMA for 48 hours before further stimulation with 8Me, F/R or PMA for 5 hours. Immunoblotting using anti-panPKC, anti-SOCS-3 and anti-GAPDH antibodies (Figure

Figure 4.6

Antibody
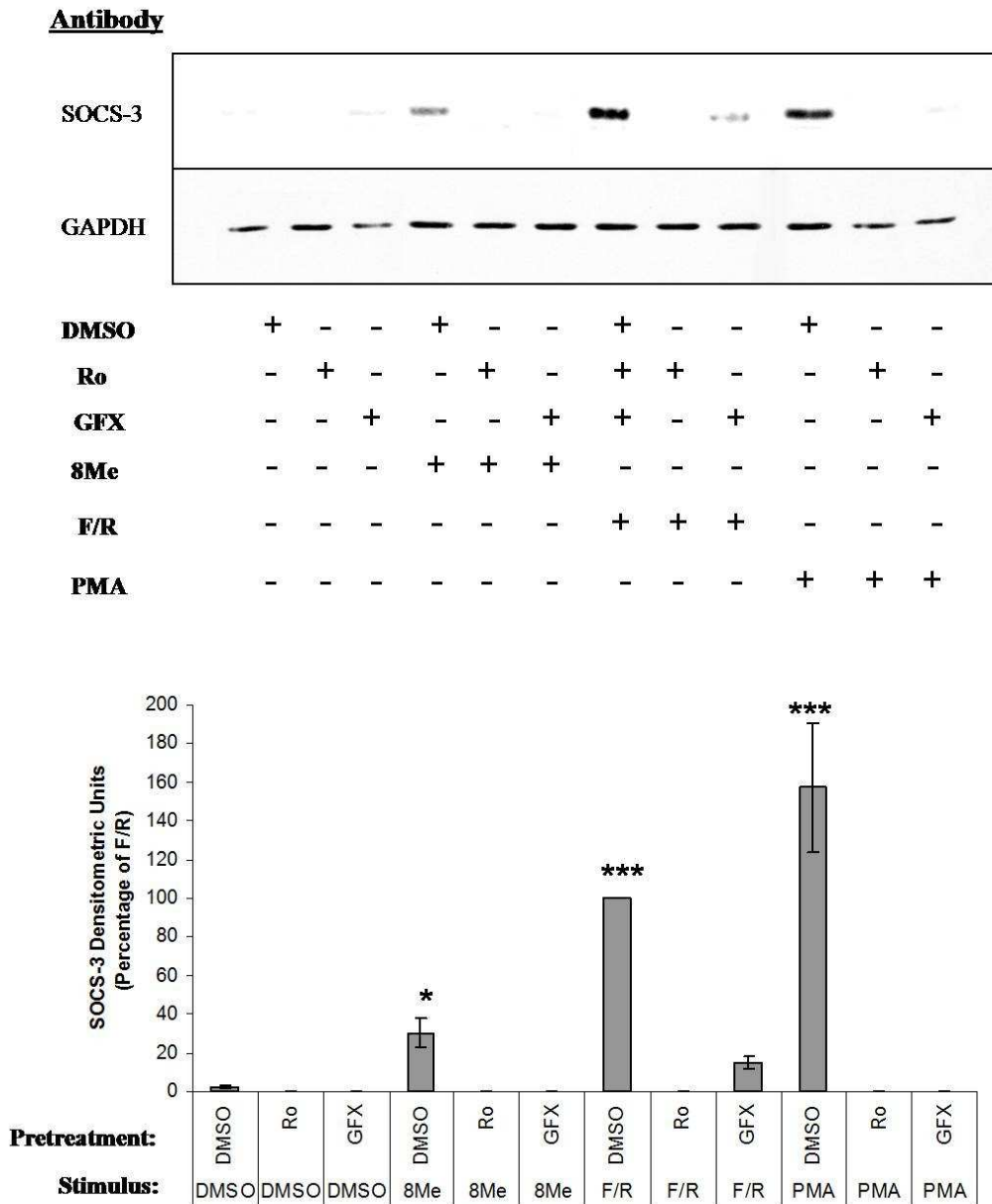
Figure 4.6 Effect of PKC inhibitors on SOCS-3 induction by cAMP

COS1 cells were stimulated for 5 hours with 6μM MG132 plus DMSO, 50μM 8Me, 10μM F/R, or 10μM PMA in the presence or absence of the PKC inhibitors 50μM Ro-31-7549 (Ro) or 50μM GF-109203X (GFX). Cell lysates were immunoblotted with SOCS-3 or GAPDH specific antibodies. The blot shows that Ro and GFX inhibit SOCS-3 induction in response to PMA and F/R stimulation. Densitometry was carried out and the values plotted on a histogram with DMSO pre-treated treated F/R stimulated cells treated as 100% and all other values expressed relative to this. Significant differences relative to non-stimulated are indicated by $* = p<0.05$ and $*** = p<0.001$.

4.7) showed that the prolonged stimulation by PMA ablated PKC levels and inhibited the ability of 8Me, F/R and PMA to induce SOCS-3. It is important to note that chronic stimulation with PMA may have other consequences besides the down-regulation of PKC and that down-regulation of PKC itself for that length of time would likely affect the health of the cells. However, the reduction in SOCS-3 expression seen in these results does provide another piece of evidence that PKC isoforms are involved in the induction of SOCS-3 by EPAC1 in COS1 cells and that due to the ability of PMA to influence this, the isoforms involved are likely to be conventional or novel PKCs.

4.3.4 The role of PKC isoforms α and δ in SOCS-3 induction by EPAC1 in COS1 cells -

As not all PKC isoforms are found in every cell type I aimed to identify the particular PKC isoforms that are involved in SOCS-3 induction in COS1 cells. Firstly, cell lysates from COS1, HeLa and Jurkat cells were separated by SDS-PAGE and immunoblotted with panPKC, RACK1 and a range of PKC isoform specific antibodies (Figure 4.8). The immunoblots clearly show the presence of PKC isoforms α, δ, ι and λ in COS1 cells. Cells were also screened for PKCγ or PKCη but expression of these isoforms were not found in any of the cell types investigated.

Both the PKC down regulation (Figure 4.7) and Ca^{2+} and DAG requirement (Figure 4.5) experiments above imply that conventional or novel PKC isoforms are involved in SOCS-3 induction downstream of EPAC1. The isoform screen above (Figure 4.8) implicates the involvement of either, or both, PKCα and PKCδ. Of these two isoforms, PKCα is known to be abundantly found in COS1 cells and because of this, knockdown of this isoform using siRNA was carried out to test the isoform's involvement in SOCS-3 induction. COS1 cells were transfected with either PKCα specific (CGCAGTGGAATGAGTCCTTTA, Qiagen) or non-targeting control siRNA (Qiagen) and then stimulated in the presence and absence of 8Me, F/R or PMA. Cell lysates were then immunoblotted with anti-PKCα, anti-SOCS-3 or anti-GAPDH antibodies (Figure 4.9) The immunoblots show that siRNA-mediated knockdown of PKCα caused a significant depletion of PKCα and that there is a significant reduction in SOCS-3 induction upon stimulation with both 8Me and F/R, which supports the hypothesis that PKCα is involved in SOCS-3 induction downstream of EPAC in these cells.

Figure 4.7
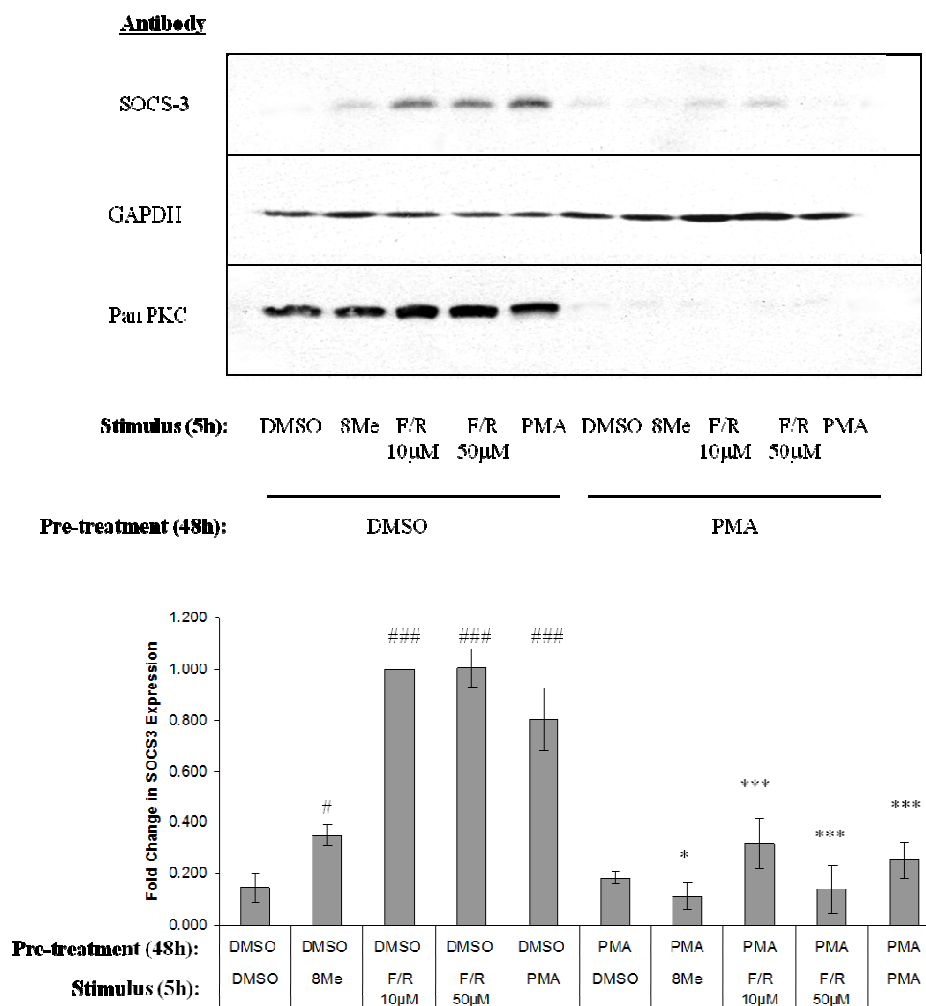
Figure 4.7 Effect of down-regulation of PKC expression on SOCS-3 induction

COS1 cells were stimulated with 1µM PMA for 48 hours to down-regulate endogenous PKC expression. Cells were then stimulated for 5 hours with either 50µM 8Me, a combination of either 10µM or 50µM F/R, or 10µM PMA in the presence of 6µM MG132. Cell lysates were then immunoblotted with SOCS-3, panPKC or GAPDH specific antibodies. The blots show that downregulation of PMA ablates SOCS-3 expression in response to 8Me and PMA and significantly reduces SOCS-3 induction by F/R. Densitometric values were obtained from immunoblots from three separate experiments and plotted as a histogram with cells pre-treated with DMSO and stimulated with F/R treated as 100% and all other values expressed relative to this.. Significant increases in SOCS-3 induction relative to control cells are indicated by # = p<0.05 and ### = p<0.001, and significant inhibition of SOCS-3 induction in cells pre-treated with PMA are indicated by * = p<0.05 and *** = p<0.001.

Figure 4.8
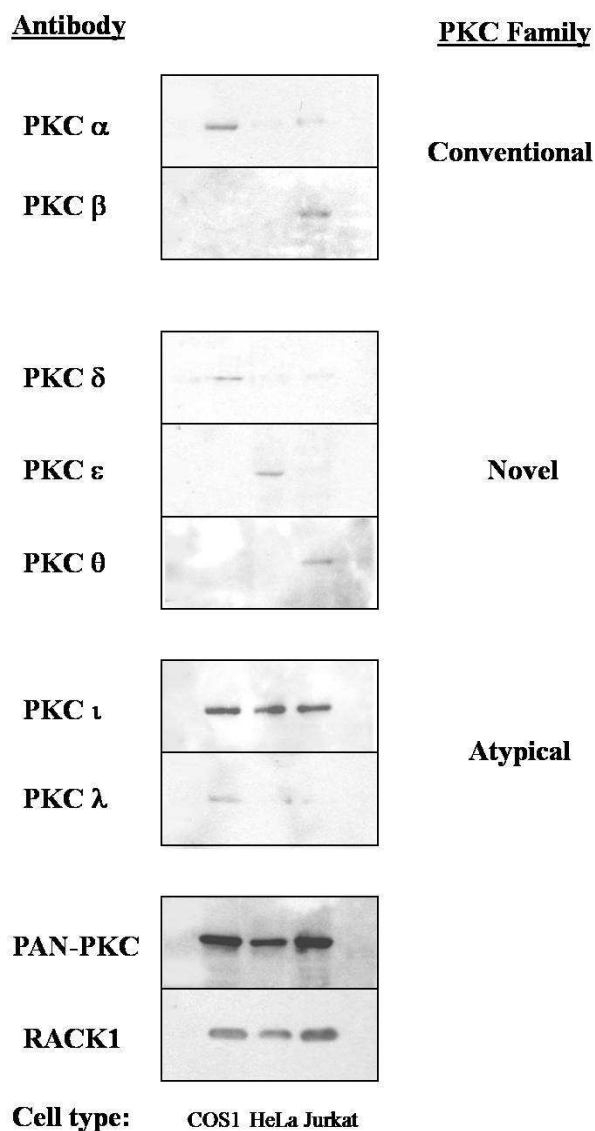
Figure 4.8 Screen of COS1 cells for PKC isoforms.

Equal amounts of protein from COS1, HeLa and Jurkat cell extracts were separated by SDS-PAGE and immunoblotted with panPKC, RACK1 or PKC isoform-specific antibodies. The blot for PKCα indicated this isoform's presence in COS1 cells, but not in HeLa or Jurkat, while probing for PKCβ showed that it was not present in either COS1 or HeLa cells, but was found in Jurkat cells. Of the Novel PKCs tested, PKCδ was found to be present in COS1 cells, but not HeLa or Jurkat, PKCε was identified in HeLa cells, but not in either COS1 or Jurkat, and PKCθ was only shown to be in found in Jurkat cells. The blot for PKCι shows it to be present in all three cell types tested, while PKCλ was neither in HeLa nor Jurkat, but present in COS1 cells.

Figure 4.9 The role of PKCα in SOCS-3 induction

COS1 cells were transfected with control siRNA or PKCα specific siRNA using Lipofectamine 2000 according to the manufacturer's instructions. Cells were then stimulated for 5 hours with 50μM 8Me, 10μM F/R, or 10μM PMA in the presence of 6μM MG132. Cell lysates were then immunoblotted with PKCα, SOCS-3 or GAPDH specific antibodies. Knockdown of PKCα by specific siRNA results in ablation of SOCS-3 expression by 8Me stimulation, and significant reduction of SOCS-3 induction by PMA stimulation and, to a slightly lesser extent by F/R. Densitometric values were obtained for the SOCS-3 immunoblots and were plotted with control siRNA treated, F/R stimulated as 100% and all other values relative to this. Significant differences between SOCS-3 expression in PKCα siRNA treated cells and control siRNA treated cells are indicated by * = p<0/05 and ** = p<0.01. Results are representative of an experiment carried out on three separate occasions.

Figure 4.9
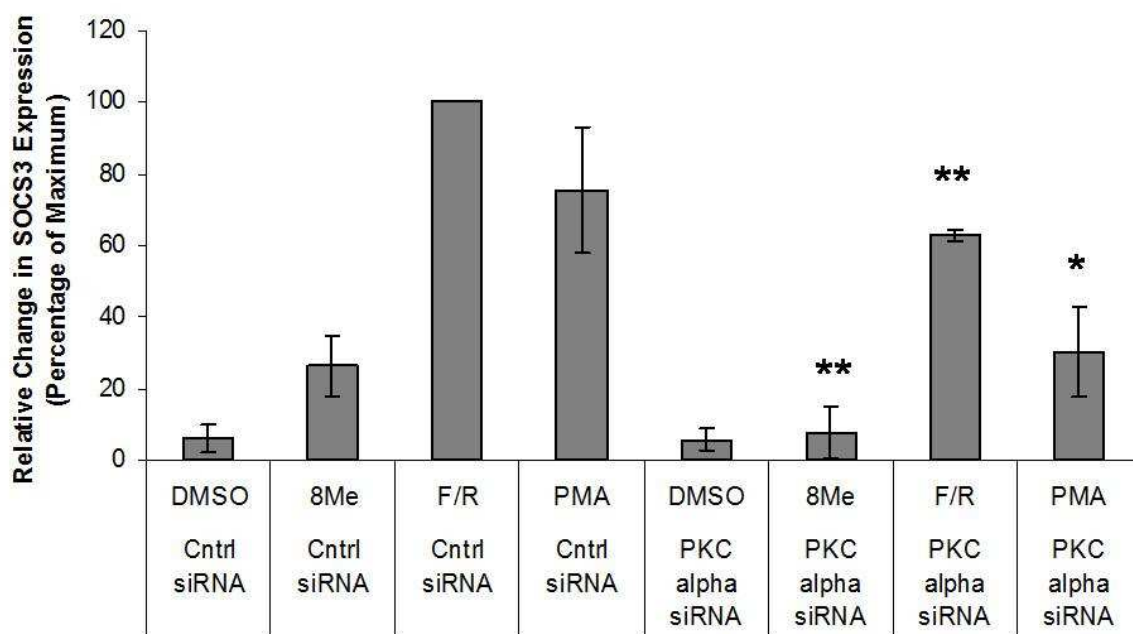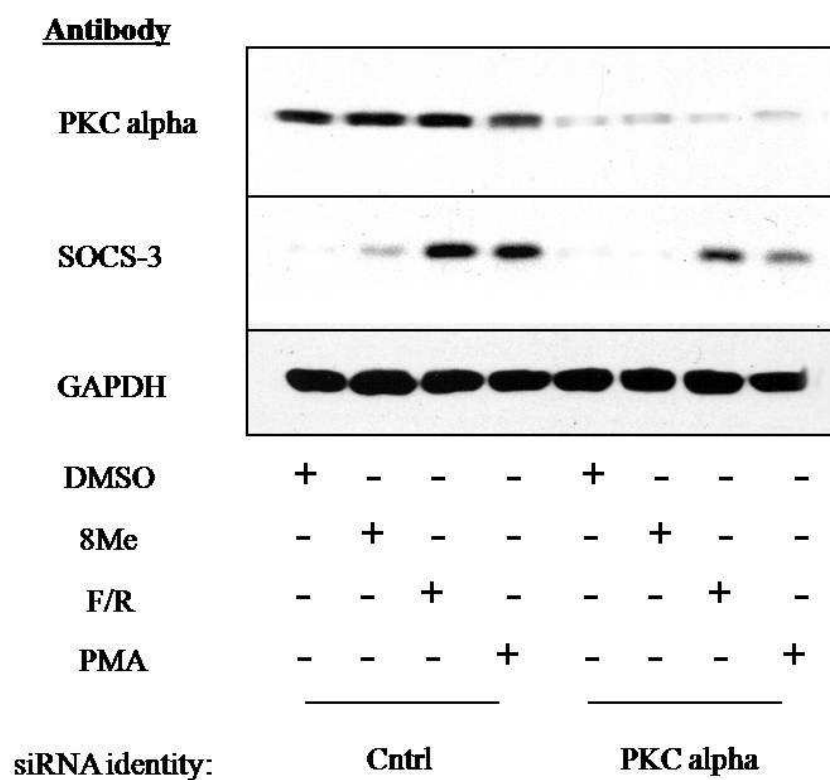
Although there is a significant reduction in stimulated SOCS-3 expression in cells treated with PKCα siRNA, the ablation was not complete. While there may be sufficient PKCα remaining following siRNA knockdown to cause some induction of SOCS-3 it is also possible that an additional PKC isoform activation or presence may be necessary for complete SOCS-3 induction by cyclic AMP. As PKCδ was the only other isoform shown to be present in COS1 cells (Figure 4.8) that could potentially be activated or down-regulated by PMA, or products of PLCε activity, a transfection with control siRNA, PKCα-specific siRNA, PKCδ-specific siRNA (AACTCTACCGTGCCACGTTTT, Qiagen) and a combination of PKCα- and PKCδ-specific siRNA was carried out. Cells were once again stimulated with 8Me, F/R or PMA and cell lysates were immunoblotted with SOCS-3, GAPDH, PKCα, PKCδ and phospho-ERK specific antibodies (Figure 4.10). Specificity of the PKC isoform siRNAs can be seen by the ability of PKCα siRNA to knockdown PKCα levels but not PKCδ and vice versa. ERK activity was measured since it has been previously shown that ERK is activated by PKCα (Kolch *et al.,* 1993) and therefore serves as a further control for the effects of PKCα knockdown. Results show a similar level of PKCα expression upon F/R and PMA stimulation in control cells as seen in the previous experiment, while the PKCδ signal in response to PMA is low. Although MG132 is present in the experiment to prevent protein degradation it is possible that the concentration used is sufficient to prevent degradation of the other proteins investigated but not PKCδ or that the time point used for this experiment is incompatible with visualisation of PKCδ activation by PMA in whole cell lysates. Results indicate that knockdown of PKCδ shows a similar reduction in SOCS-3 induction to cells treated with PKCα siRNA, with reductions of SOCS-3 expression of approximately 40% and 45% respectively in F/R stimulated cells, and approximately 60% for both in PMA stimulated cells. However, in cells treated with a combination of these siRNAs, an almost complete inhibition of SOCS-3 induction upon F/R and PMA stimulation can be seen with approximate reductions of SOCS-3 expression by 80% in F/R stimulated cells and 90% in PMA stimulated cells. These results indicate that both PKCα and PKCδ are required for SOCS-3 induction by cyclic AMP in COS1 cells.

Figure 4.10 The role of PKCα and PKCδ in SOCS-3 induction

COS1 cells were transfected with non-targeting control siRNA, PKCα-specific siRNA, PKCδ-specific siRNA or a combination of PKCα- and PKCδ-specific siRNA using Lipofectamine 2000 according to the manufacturer's instructions. Cells were then stimulated for 5 hours with 10μM F/R, or 10μM PMA in the presence of 6μM MG132. Cell lysates were then immunoblotted with SOCS-3, GAPDH, PKCα and PKCδ specific antibodies. The PKCα blot shows high levels of PKCα expression in cells treated with control siRNA and also in cells treated with PKCδ specific siRNA. The blots show that treatment with PKCα siRNA shows a reduction of SOCS-3 induction with both PMA and F/R stimulations, as does treatment with PKCδ siRNA. Cells treated with a combination of PKCα and PKCδ siRNA show an almost complete ablation of SOCS-3 expression upon PMA stimulation, and a significant reduction of SOCS-3 induction by F/R stimulation. SOCS-3 immunoblots from three separate experiments were quantified and the densitometric units plotted with control siRNA treated, F/R stimulated cells taken as 100% and all other values expressed relative to this. Significant differences between SOCS-3 expression in specific siRNA treated cells and cells treated with control siRNA are indicated by ** = p<0/01 and *** = p<0.001.

Figure 4.10
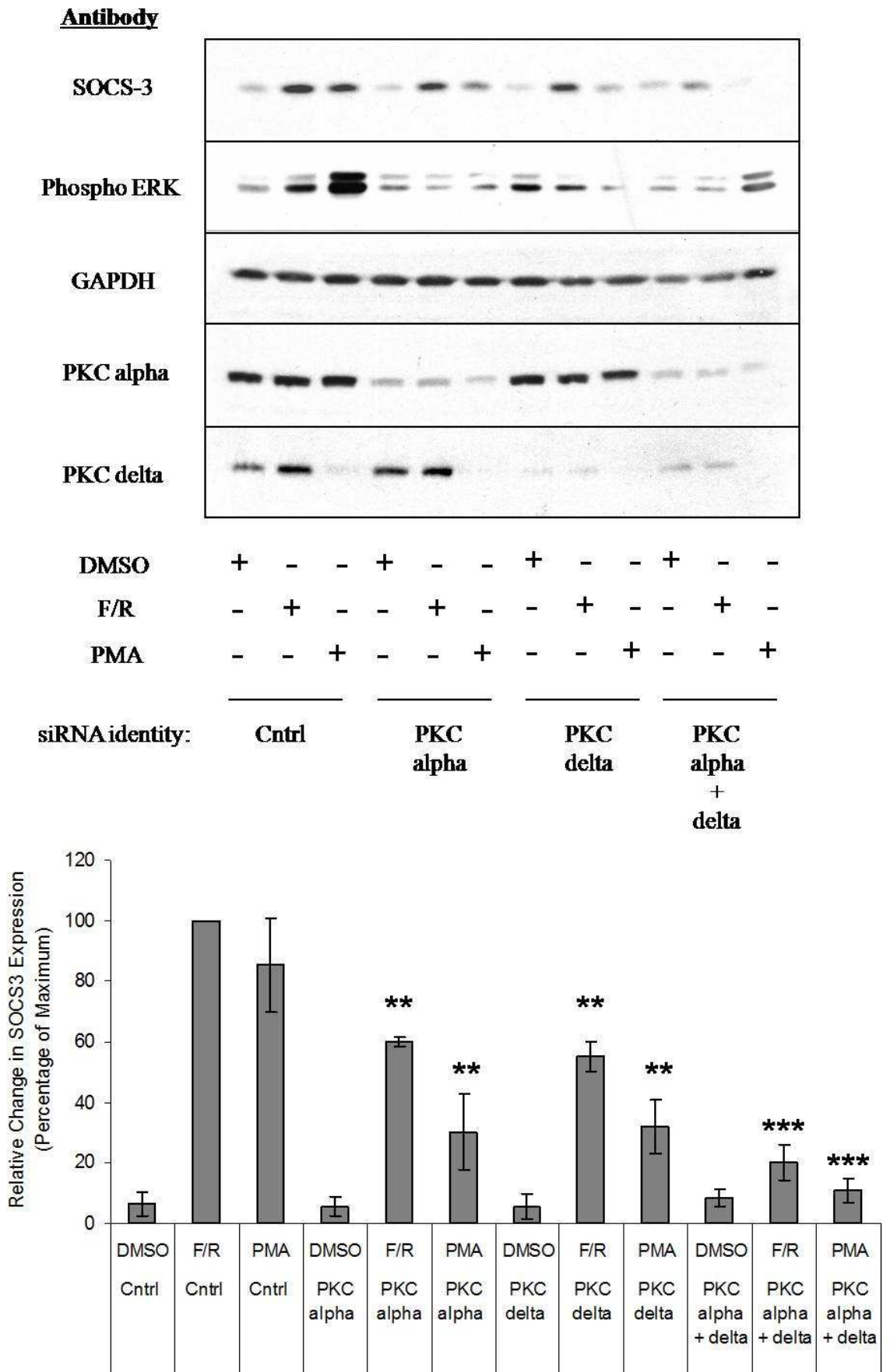
4.4 DISCUSSION

The role of EPAC, and its downstream effects are part of a novel pathway by which SOCS-3 expression is regulated, thus mediating IL-6 signalling. The induction of SOCS-3 by cyclic AMP elevations occurs independent of PKA, as F/R stimulated induction of SOCS-3 has previously been shown to be unaffected by the PKA inhibitor H-89 (Sands *et al.,* 2006; Yarwood *et al.,* 2008). The results outlined in this chapter reiterate that EPAC1 is involved in the control of SOCS-3 induction in COS1 cells, as previously shown in literature (Sands *et al.,* 2006; Yarwood *et al.,* 2008), as well as expounding on aspects of signalling downstream of EPAC1 that are involved in this pathway of SOCS-3 induction. Although the involvement of EPAC1 had been established within this pathway, the mechanisms through which EPAC induces SOCS-3 expression and therefore mediates control of IL-6 signalling were largely unknown.

While it was possible for there to be other downstream effects of EPAC involved in SOCS-3 induction, due to EPACs interaction with other Ras family members, phospholipase D, MAP kinases and PKB/Akt (Cheng *et al.,* 2008; Roscioni *et al.,* 2008), published data from other laboratories had indicated a connection between EPAC and PLCε through the small GTPase Rap2B (Schmidt *et al.,* 2001; Bunney and Katan, 2006; Oestreich *et al.,* 2009) and thus this provided a starting point for investigations into what aspects of EPAC activation downstream might be involved in control of SOCS-3 expression. The use of the general PLC inhibitor U73122 to block induction of SOCS-3 by elevation of cyclic AMP levels (Figure 4.3) shows not only that one or more PLC isoforms are involved, but that their presence is essential to ensure SOCS-3 induction via this pathway. Use of PLCε specific siRNA confirmed this necessary role for PLCε in SOCS-3 induction, not only via increase in intracellular cyclic AMP by F/R stimulations but by EPAC specific induction via stimulations with 8Me. This places PLCε directly downstream of EPAC1 in this pathway opening up the possibility that PLCε could therefore act as a therapeutic target to stimulate the induction of SOCS-3. Further experimentation with over expression of PLCε in cells would determine whether an increase in PLCε concentration would boost SOCS-3 induction, and therefore whether the addition of PLCε could be used to increase SOCS-3 expression in cells. To date, little else is known about the relationship between PLCε and EPAC, nor on the effect of PLCε on proteins downstream of EPAC.

Although no PLC isoform screen was carried out to determine which PLC isoforms are present in COS1 cells, the degree to which knockdown of PLCε by specific siRNA leaves no doubt as to the requirement of this isoform for SOCS-3 induction. However, this does not rule out the possibility of involvement of other PLC isoforms in SOCS-3 induction in COS1 cells and further screening may determine whether or not other isoforms may have a role in this pathway. However, it is important to note that as PLCε is the only PLC isoform that contains a RA domain as well as a CDC25 homology domain that functions as a GEF for Rap1 but not other Ras family members (Jin *et al.,* 2001). This unique ability among PLC isoforms to interact with Rap1 strongly suggests that PLCε alone is the PLC isoform acting in this pathway. The well-documented downstream effects of PLC activation, namely the production of DAG and the increase in intracellular Ca^{2+} via IP3, are part of the classic PLC signalling pathway, and investigation into the effects of these on SOCS-3 induction was the next logical step. Use of the potent calcium chelator, BAPTA-AM was used to remove Ca^{2+} from COS1 cells to determine the importance of calcium in SOCS-3 induction. It was found to be essential in both cyclic AMP and EPAC induced SOCS-3 expression. Further investigation into the effects of PLC activation products on this pathway using the DAG analogue OAG indicated the presence of DAG was also required for SOCS-3 induction in COS1 cells. Besides its activation of PLCε, EPAC has also been shown to modulate intracellular calcium release in cardiac myocytes via calcium-calmodulin-dependent-protein kinase (Pereira *et al.,* 2007). Additionally, EPAC has been linked to calcium mobilisation via its ability to directly bind and activate R-Ras, which has been shown to have a role in calcium handling by activating PLCδ (Lopez de Jesus *et al.,* 2006), and by directly activating SERCA (sarcoplasmic reticulum Ca^{2+} ATPase) and the ryanodine II receptor (Schmidt *et al.,* 2007).

A group of proteins that require both DAG and Ca^{2+} are the conventional isoforms of PKC. Additionally, the novel PKC isoforms are activated by DAG and so determination as to what, if any, involvement of these proteins followed. The use of the PKC inhibitors GFX and Ro provided a way to initially investigate the potential role of PKCs in SOCS-3 induction. Cells were stimulated with not only F/R and 8Me, which had already been shown to induce SOCS-3 expression in COS1 cells via increases in intracellular cyclic AMP and through EPAC respectively, but also with PMA, a potent phorbol ester known to specifically activate conventional and novel PKC isoforms by mimicking DAG. The level of induction of SOCS-3 upon PMA stimulation in these cells was almost as high as stimulation by F/R, indicating that activation of one or more PKC isoforms from the

conventional or novel families was able to induce SOCS-3 expression in COS1 cells. Treatment of the cells with specific PKC inhibitors Ro-31-7549 and GFX completely ablated SOCS-3 expression by all stimulations, with the exception of inhibition by GFX on F/R stimulation, although the SOCS-3 levels were significantly reduced. This inhibition of SOCS-3 induction by these inhibitors indicates the requirement of one or more conventional or novel PKC isoforms for SOCS-3 induction. It is important to note that the concentrations of Ro-31-7549 and GFX required to inhibit PKC in these cells are high. It has been suggested that PKC isoforms bound to AKAPs display insensitivity to PKC inhibitors (Dr. John Scott, personal communication). Therefore this requirement of higher than normal levels of PKC inhibitors suggests a potential compartmentalised PKC response with the isoforms anchored at specific locations by PKC-binding AKAPs (Faux *et al.,* 1999).

Confirmation of these findings was carried out by down-regulation of PKC due to chronic stimulation with PMA. Treatment by all three stimulations used above on cells where PKC had been ablated by prolonged exposure to PMA was unable to significantly induce SOCS-3 expression, further indicating a crucial role of PKC isoforms in this pathway. It was therefore necessary to narrow down which isoforms may be involved in COS1 cells. Screening for isoforms indicated only two possibilities in COS1 cells that fit the requirements outlined by both the PLC product experiments and by the PMA and PKC inhibitor experiments. These isoforms were PKCα and PKCδ. PKCs are integral within cell signalling with each isoform expressing different characteristics, activities and tissue distribution. PKCα differs from the other isotypes as it is found in all tissue types. Additionally, PKCα requires phosphorylation at residues located within its kinase domain before activation can occur (Nakashima, 2002). Like other isoforms, PKCα is involved in numerous biological processes including proliferation, apoptosis, cell migration and adhesion, and differentiation (Nakashima, 2002). PKCα has also been linked to erythrocyte production (Myklebust *et al.,* 2000). Additionally, PKCα has been implicated in several disease states including inflammatory disorders, endothelial cancers whereby an increase in PKCα activity has been linked to disruption of tight junction formation between cells, allowing foreign molecules such as growth factors to enter cells and cause abnormal cell growth (Rosson *et al.,* 1997), and in cardiac hypertrophy where increased PKCα levels have been shown to inhibit contractility (Dorn and Force, 2005). PKCδ has been shown to be involved in differentiation and cell growth, and has also been linked to tumour development as well as apoptosis (Gschwendt, 1999). This novel PKC isoform is thought

to be found compartmentalised in cells, with research indicating association with cytoskeletal and nuclear cell structures. Further investigation into possible interaction between these two isoforms and their interaction, would further elucidate the involvement of these isoforms in this pathway. Together these results have delineated a section of the pathway through which EPAC is able to mediate SOCS-3 induction in COS1 cells.

As both PKC and cyclic AMP can both activate ERK, it is likely that it is involved in SOCS-3 induction. This suggests a role for downstream effectors of ERK signalling, such as the C/EBP transcription factors, in linking both PKC and cyclic AMP signalling pathways to the induction of the SOCS-3 gene. One of these transcription factors, C/EBPβ, is known to be activated by ERK and has been recently shown to be required for SOCS-3 induction mediated by cyclic AMP and PKC (Borland *et al.,* 2009).

Compartmentalisation of cyclic AMP signalling to create temporal and spatial intracellular cyclic AMP gradients is controlled by several factors including elevations in cyclic AMP production by adenylyl cyclases, degradation by PDEs and the actions of the effectors downstream of cyclic AMP (Houslay and Adams, 2003). As previously mentioned, F/R stimulated induction of SOCS-3 occurs independently of PKA (Sands *et al.,* 2006;, Yarwood *et al.,* 2008), thus showing a compartmentalised effect of cyclic AMP signalling away from classic PKA activity within the cell. As SOCS-3 induction by F/R occurs after 5 hours stimulation, this also demonstrates a temporal compartmentalisation of cyclic AMP signalling. Additionally, the findings from Chapter 3, indicating the ability of PKCα to induce PDE4D5 activity, shows the possible control of cyclic AMP gradients within this pathway, with activation of EPAC signalling in response to cyclic AMP elevation activating PKCα which in turn is able to lower cyclic AMP levels through PDE action.

In conclusion, this chapter outlines a novel point of crosstalk between the cyclic AMP and PKC signalling pathways. We have shown that EPAC is activated by cyclic AMP and it in turn activates PLCε which cleaves PIP2 to form DAG and IP3. The downstream effect of IP3 production, increased intracellular calcium, and DAG activate the conventional PKCα, while DAG may activate the novel PKCδ. In turn, these PKC isoforms together induce SOCS-3 expression in COS1 cells. Conclusively, these results have not only indicated an area of cross-talk between cyclic AMP and PKC signalling pathways, but have outlined a potentially useful pathway with which to target and control SOCS-3 expression and therefore have an effect on IL-6 signalling. Having identified PLCε, and PKC α and δ as

key points in the EPAC induced SOCS-3 pathway, this provides areas for potential therapeutic targets, as inhibition of these proteins can block SOCS-3 induction as shown, while over-expression or the presence of protein mimics may increase SOCS-3 expression in COS1 cells, and thus inhibit IL-6 signalling.

CHAPTER 5

Phosphorylation and Activation of Protein Kinase C (PKC) Isoforms by Cyclic AMP in COS1 Cells

5.1 INTRODUCTION

Evidence is presented in the previous two results chapters that elevation of intracellular cyclic AMP levels may lead to the activation of the conventional protein kinase C isoforms, PKCα and PKCδ. In the following chapter attempts are made to formally test this hypothesis by determining the mechanisms controlling activation and phosphorylation of these conventional and novel PKC isoforms, in response to cyclic AMP in COS1 cells.

Control of PKC activity occurs initially via three priming sites which are phosphorylated by kinases such as 3-phosphoinositide-dependent protein kinase-1 (PDK1) (Parekh *et al.,* 2000) following ligand binding at the cell membrane. These sites, conserved across all PKC isoforms, are Thr505 in the activation loop, Ser643 in the turn motif and Ser662 in the hydrophobic motif (Keranen *et al.,* 1995). Phosphorylation at these sites affords higher specific activity and allows for autophosphorylation to further control PKC isoforms. Autophosphorylation of PKC isoforms is an important component of activity control and previous studies have shown, for example, that PKCα autophosphorylation is closely linked to its enzymatic activity (Hu and Exton, 2004).

PKCα possesses several autophosphorylation sites which control important aspects of PKCα activity. Autophosphorylation of Thr638 has been shown to be critical for the regulation of the rate of dephosphorylation of PKCα and its inactivation (Bornancin and Parker, 1996). This controls the duration of PKCα influence on its downstream effectors, allowing temporal regulation of PKCα activity (Garcia-Paramio *et al.,* 1998). Additionally, the phosphorylation of Ser657 in this isoform has been shown to control the accumulation of phosphate at other sites, maintaining resistance to phosphatases to prevent rapid dephosphorylation (Bornancin and Parker, 1997). Autophosphorylation at these residues, and at Thr497 and Tyr658, have also been linked to thermal stability of the isoform and targeting to specific subcellular localisations (Kitatani *et al.,* 2007).

For PKCδ isoforms, autophosphorylation of Ser643 is crucial to the catalytic maturation of the isoform (Parekh *et al.,* 2000), and following activation by DAG or PMA PKCδ is also able to be autophosphorylated at Ser229, Ser302 and Ser304. The autophosphorylation of these sites may play a role in the translocation of PKCδ to plasma and nuclear membranes (Durgan *et al.,* 2007).

The role of autophosphorylation in PKC subcellular localisation is of particular interest as numerous cellular processes, including differentiation and proliferation, require transduction of signals to specific intracellular regions, and it is thought that autophosphorylation of PKC isoforms induced by differing stimuli may allow specific targeting to these areas (Feng *et al.,* 2000).

PKC signal transduction is also closely regulated by the subcellular localisation of the protein. Upon cell-surface receptor activation, conventional and novel PKC isoforms are frequently induced to translocate to cell membranes by their membrane targeting regions, the C1 and C2 domains (Goodnight *et al.,* 1995). PKC isoforms are believed to be localised to the cytosol where there are held in place by scaffold interactions for example via AKAP interaction (Perkins *et al.,* 2001) or as in the case of PKCε binding to the phosphoserine/threonine binding protein 14-3-3 (as reviewed in Newton, 2010). Conventional PKC isoforms are targeted to the plasma membrane by calcium (as outlined in section 1.4.2). Novel PKC isoforms, such as PKCδ, which lack the calcium binding residues are activated by interaction at the C1 domain by endogenous lipid cofactors such as DAG and ceramide. This interaction not only causes the conformational change that removes the pseudosubstrate from the catalytic site, but also promotes PKCδ translocation to cell membranes (Steinberg, 2004).

Recent studies have indicated that PKC isoform autophosphorylation may be required for prevention of the C2 domain anchoring the protein to lipid membranes (Feng and Hannun, 1998). The means by which PKC is induced to translocate to the plasma membrane may also effect the rate of PKC autophosphorylation (Feng and Hannun, 1998). While calcium and the DAG analogue PMA induce a sustained association of PKC at the plasma membrane before autophosphorylation, other inducers of PKC translocation to the membrane, such as GPCR activation may cause a more transient association to the membrane, followed by a swift relocalisation to other subcellular regions (Feng and Hannun, 1998). This suggests that different effectors on PKC isoforms are able to elicit different rates of PKC activity within the cell, allowing appropriate timing and duration of the signal transduction, and providing both localised and temporal compartmentalisation of the PKC pathway.

In Chapter 3, a role for PKC was established in the control of activity of the cyclic AMP-specific phosphodiesterase 4D5 in response to elevations in intracellular cyclic AMP

following β-adrenoceptor stimulation. In addition, an obligate role for PKCα and PKCδ, in transducing the induction of the SOCS-3 gene in response to elevations in intracellular cyclic AMP levels, was also demonstrated in Chapter 4. The aims of the current chapter therefore are to investigate the effect of cyclic AMP on PKC activation and regulation of down-stream signalling through the ERK, MAP kinase cascade.

5.2 MATERIALS AND METHODS

All Materials and Methods as outlined in Chapter 2 with variations described below.

Densitometry and Statistical Analysis

Non-saturating exposures from multiple experiments were quantified densitometrically using ImageJ software. Densitometric values of PKC levels were usually normalized to the expression of RACK1 or GAPDH protein levels in the same sample. Statistical significance was determined using the Student's two-tailed t-test.

5.3 RESULTS

5.3.1 Elevation of cyclic AMP in COS cells stimulates autophosphorylation of PKCα -

To initially investigate the effects of cyclic AMP stimulation on PKC phosphorylation, and that of its downstream effectors, we tested the effect of elevated cyclic AMP levels on the phosphorylation of PKCα. COS1 cells were stimulated with or without the cyclic AMP-elevating agents, the adenylyl cyclase agonist forskolin (10μM) and the phosphodiesterase 4 inhibitor rolipram (10μM) (F/R) or the conventional/novel PKC-activator, phorbol myristate acetate (PMA; 10μM), over a range of time points between 0 and 960 minutes. Cell lysates were then immunoblotted for total PKCα protein or an antibody that recognises the Thr638 autophosphorylation site in PKCα (Phospho-PKCα) (Figure 5.1). Stimulation with PMA resulted in a reduction in the levels of PKCα and Phospho-PKCα after 300 minutes, while stimulations with (F/R) showed similar levels of PKCα and Phospho-PKCα across all time points tested. In this case it appears that preparation of whole cell lysates is insufficient to detect the phosphorylation and activation of PKCα in COS1 cells and are only suitable for examining the proteolytic reduction in expression of PKCα (Schmitz-Peiffer *et al.,* 1996) following PMA stimulation. It is important to note that the absence of the proteasome inhibitor MG132, used in experiments in Chapter 4, may cause degradation of PKCα following PMA stimulation.

As the immunoblots from lysates did not allow for visualisation of the well documented (Feng and Hannun, 1998) translocation of PKCα in response to PMA stimulation, nor for any effects F/R may have on differing localisations of PKCα, cells were fractionated by high-speed centrifugation to give crude membrane pellets and supernatant fractions which were then immunoblotted with total PKCα and Phospho-PKCα antibodies. It was decided to try this approach to detect active PKCα since the plasma membrane is the normal site for autophosphorylation (Feng *et al.,* 2000). In fact the fractionation technique revealed a marked difference in the effects of both stimulants on PKCα phosphorylation between the fractions. Following this protocol it was found that stimulation of cells with either F/R or PMA provoked a physical translocation of PKCα from the S/N to the pellet fraction, which was concomitant with an increase in Phospho-PKCα immunoreactivity in the same fraction (Figure 5.2), indicating activation of PKCα in this fraction. To show that the effects of F/R and PMA on PKCα activation was due to specific activation of this isoform, general PKC

Figure 5.1

Antibody   **Lysate**

Phospho PKC alpha

PKC alpha

Time (minutes)   0 30 60 300 480 960   0 30 60 300 480 960

                           F/R   PMA
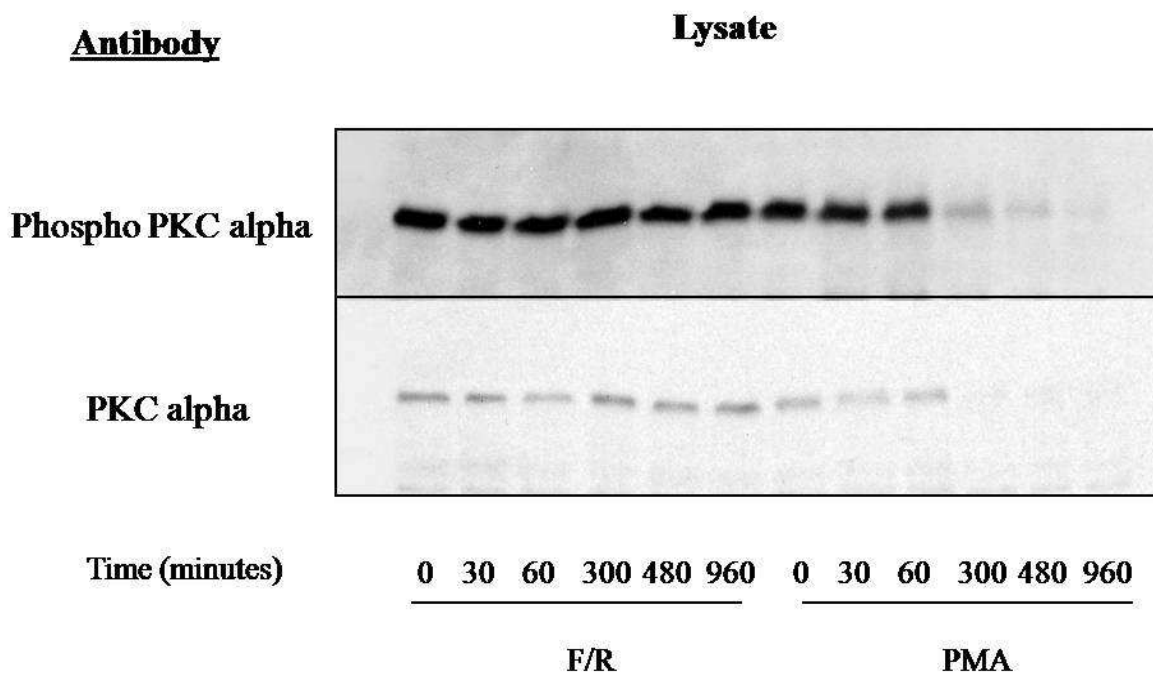
Figure 5.1 Cyclic AMP effects PKCα phosphorylation in COS1 cells

COS1 cells were stimulated with either 10µM forskolin and rolipram (F/R) or PMA over a series of time points ranging from 0 to 960 min. Following stimulation, cells were harvested into sample buffer. Equal amounts of protein from whole cell lysates were separated by SDS-PAGE and then immunoblotted with PKCα and Phospho-PKCα specific antibodies. Results are representative of an experiment carried out on three separate occasions.

Figure 5.2

Antibody		Fraction
Phospho PKC alpha		Pellet
Phospho PKC alpha		S/N
RACK1		

DMSO	+	-	-	+	-	-	+	-	-
Ro	-	+	-	-	+	-	-	+	-
GFX	-	-	+	-	-	+	-	-	+
F/R	-	-	-	+	+	+	-	-	-
PMA	-	-	-	-	-	-	+	+	+
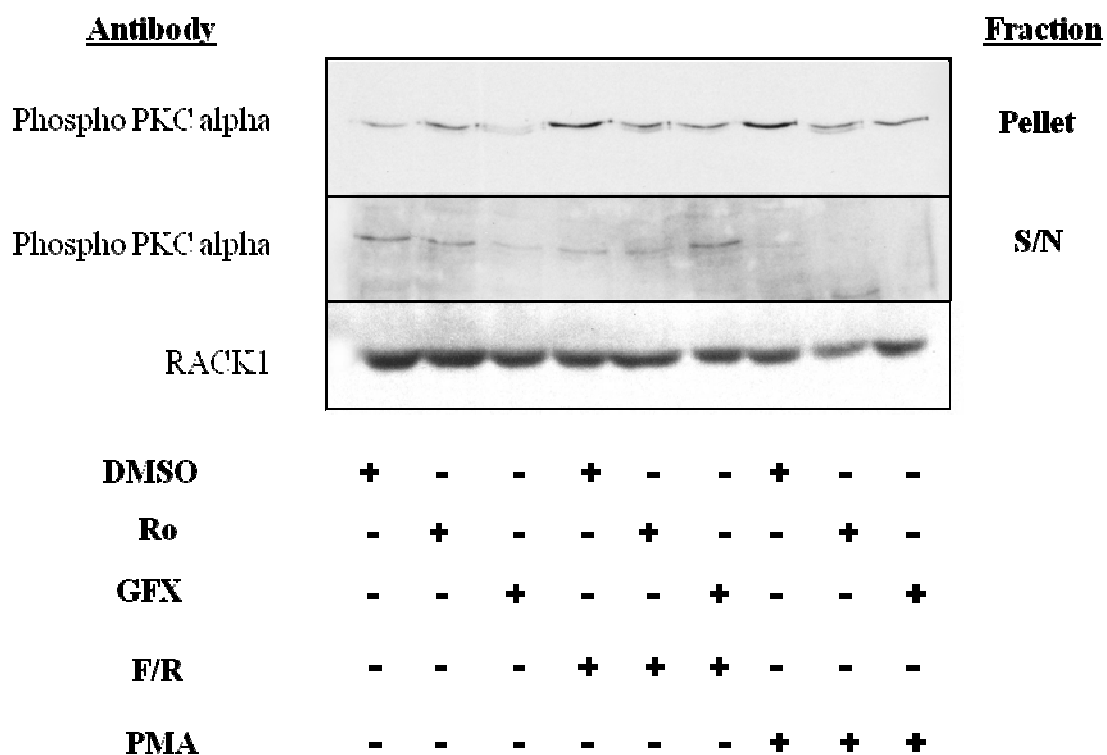
Figure 5.2 PKC inhibitors prevent phosphorylation of PKCα in response to cyclic AMP elevation

COS1 cells were treated with the PKC inhibitors Ro-31-7549 or GF109203X (GFX) in the presence or absence of F/R or PMA for 5 hours. Following stimulation, cells were harvested and fractionated by high-speed centrifugation into pellet and supernatant fractions. Equal amounts of protein were separated by SDS-PAGE and immunoblotted with Phospho-PKCα specific antibody and RACK1 specific antibody as a loading control. The immunoblot shows both F/R and PMA cause Phospho-PKCα expression in COS1 cells, while treatment with GFX reduces Phospho-PKCα levels in both the supernatant and pellet fractions. Results are representative of an experiment carried out on three separate occasions.

inhibitors Ro-31-7549 and GF109203X (GFX) were added to cells prior to stimulation with PMA or F/R. While both F/R and PMA stimulations caused an increase in Phospho-PKCα immunoreactivity in pellet fractions, Phospho-PKCα expression was reduced following the addition of either Ro-31-7549 or GFX (Figure 5.2). These results indicate that the influence of F/R and PMA on PKCα autophosphorylation is largely confined to the plasma membrane and that elevations in cyclic AMP levels not only affect PKCα autophosphorylation but also translocation in COS1 cells. The fractionation technique for determining PKC activation was therefore used in subsequent experiments.

As this experiment was carried out with stimulations at the 30 minute time point only, the effects of F/R and PMA stimulation on PKCα expression and phosphorylation across a range of time points was investigated. Cells were stimulated with either F/R or PMA for a range of time points between 0 and 300 minutes, fractionated by high-speed centrifugation and immunoblotted with PKCα, Phospho-PKCα and RACK1 specific antibodies, with RACK1 acting as a loading control. The resulting immunoblots for the pellet fraction (Figure 5.3a) shows that in response to F/R stimulation, PKCα expression in the S/N fraction remains relatively constant until around the 300 minute time point, while expression of Phospho-PKCα begins to decrease in the S/N fraction and increase in the pellet fraction from around the 60 minute time point. The PMA stimulation shows a rise in PKCα levels in the pellet fraction, peaking at 60 minutes before falling to below basal levels, probably due to activation-coupled proteolysis, as previously described (Schmitz-Peiffer *et al.,* 1996). The differing effects over time on PKCα autophosphorylation and activation in response to F/R or PMA can be clearly seen in the graph, Figure 5.4, where PKCα autophosphorylation is expressed as a fold change. This graph demonstrates a difference in temporal activity of PKCα in response to PMA and F/R. While PMA stimulation, which causes almost immediate translocation of PKCα to the pellet fraction, results in a greater fold change in PKCα activation, its influence is immediate and quickly diminished, peaking at 60 minutes after stimulation. The rapid signal decay may be a result of the absence of MG132 in these experiments, as discussed above, causing degradation of PKCα following PMA stimulation. Conversely, F/R stimulation shows a delayed effect on PKCα with significant PKCα levels occurring at 300 minutes. This difference in a temporal effect on PKCα depending on the stimulant may be an important contributor in the compartmentalised activation of PKCα, whereby the localisation and temporal activation of a protein affects its downstream targets and therefore its influence within the cell.

Figure 5.3

a)

b)
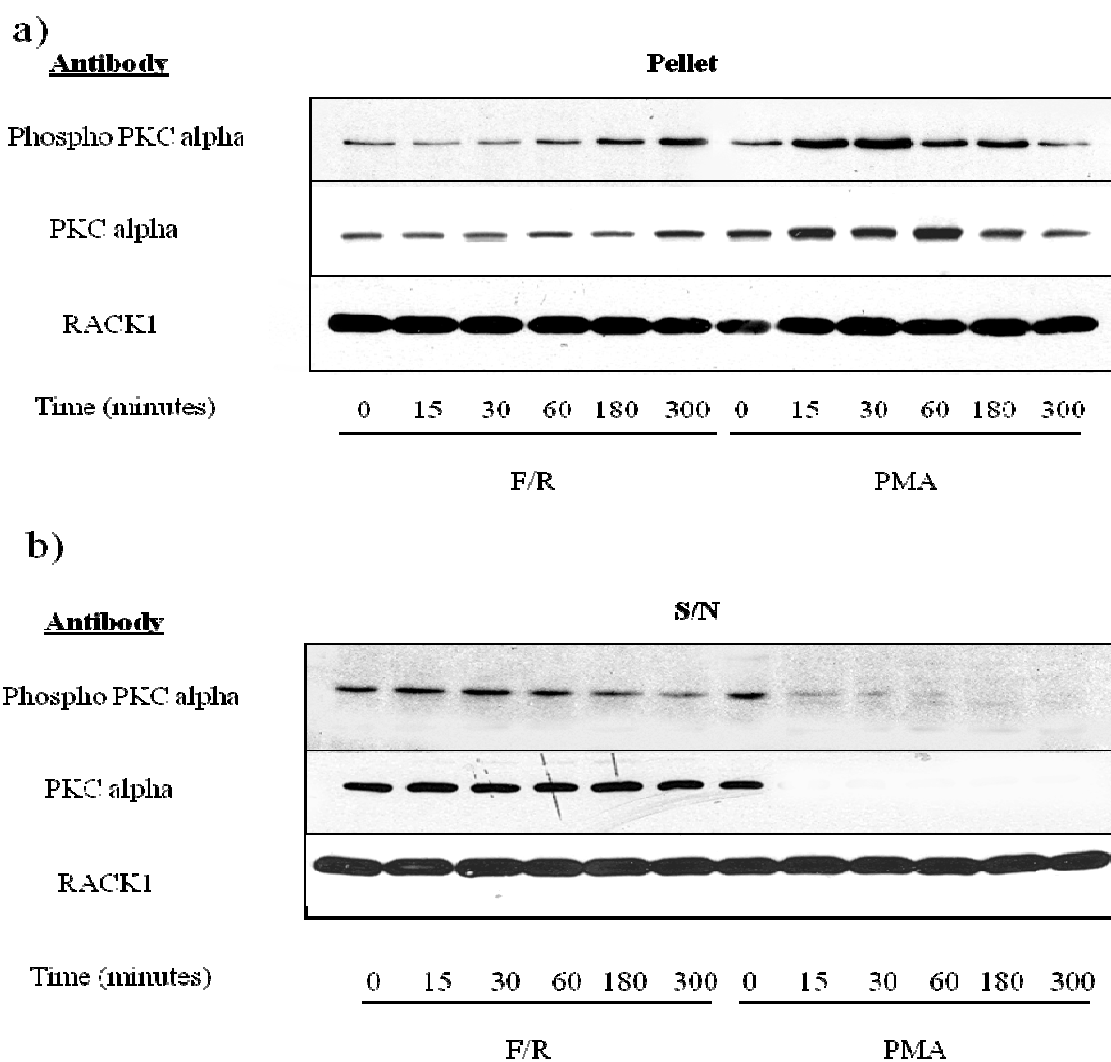
Figure 5.3 Elevation in cyclic AMP induces phosphorylation of PKCα in the supernatant fraction of COS1 cells

COS1 cells were stimulated with 10μM F/R or PMA at time points ranging from 0 – 300 min. Following stimulation, cells were harvested and fractionated by high-speed centrifugation into pellet and supernatant fractions. Equal amounts of protein were separated by SDS-PAGE and immunoblotted with PKC-α, Phospho-PKCα and RACK1 specific antibodies. The immunoblot shows an immediate induction of Phospho-PKCα in the S/N fraction in response to F/R, while the pellet fraction shows phosphorylation of PKCα only at the end of the time course. In response to PMA stimulation, PKCα shows an immediate translocation from the S/N to the pellet fraction. Results are representative of an experiment carried out on three separate occasions.

Figure 5.4
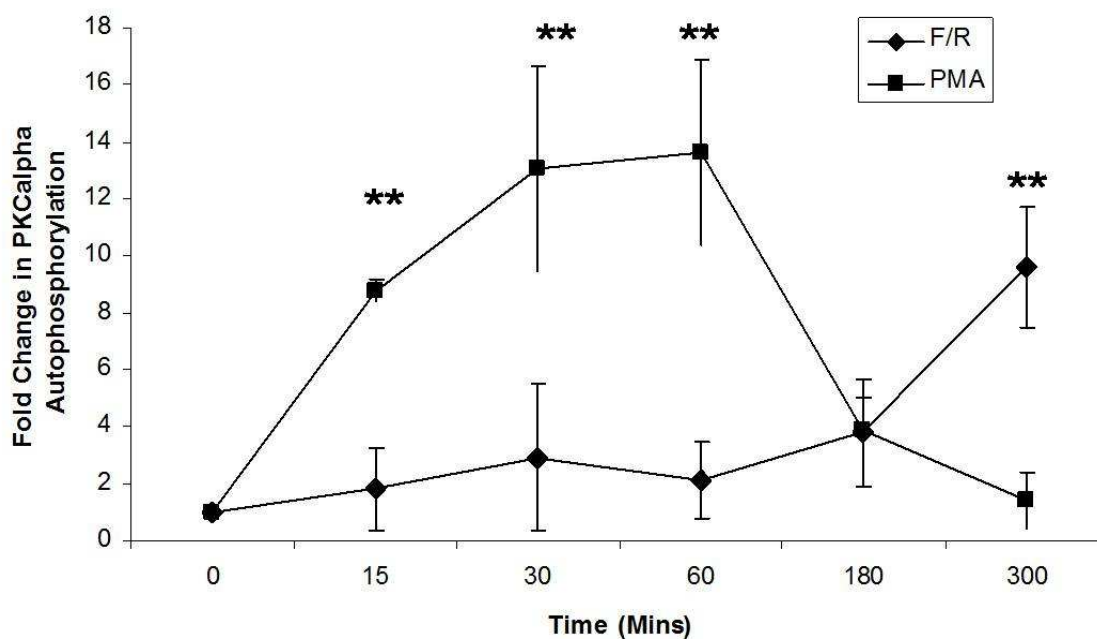
Figure 5.4 Cyclic AMP levels have a temporal effect of PKCα autophosphorylation
Densitometric values obtained from three repeats of the previous experiment were plotted on a graph, with values expressed as a total fold change of PKCα autophosphorylation. The graph shows a different temporal response of PKCα to PMA and F/R, with F/R displaying a delayed effect on PKCα phosphorylation. Significant differences relative to non-stimulated are indicated by ** = p<0.01.

5.3.2 Cyclic AMP causes PKCα autophosphorylation via EPAC - Given that PKCα and PKCδ appear to play a role in SOCS-3 induction in response to EPAC activation in COS1 cells (Chapter 4), it was decided to determine whether the above effects of cyclic AMP elevation in response to F/R treatment on PKCα autophosphorylation occurred via activation of EPAC. In these experiments 8-pCPT-2'0-Me-cyclic AMP (8Me) was used as a stimulant on the cells. 8Me is a specific activator of EPAC that, at the concentrations used in experiments, does not activate PKA (Holz *et al.,* 2008). COS1 cells were stimulated with varying concentrations of 8Me ranging from 0-10μM. Cells were then harvested and fractionated by high-speed centrifugation into pellet and supernatant fractions and immunoblotted with Phospho-PKCα specific antibody. The immunoblot (Figure 5.5a) shows an increase in Phospho-PKCα expression in response to increasing levels of 8Me starting at 0.3μM, indicating that cyclic AMP is able to act via EPAC to induce autophosphorylation of PKCα.

Next, the time course of PKCα autophosphorylation in response to EPAC activation was investigated. COS1 cells were stimulated with either 10μM 8Me or 10μM 8-pCPT-cAMP, a non-hydrolysable cyclic AMP analogue that activates both EPAC and cyclic AMP dependent PKA, at various time points ranging from 0 to 960 min. Cells were then harvested and fractionated by high-speed centrifugation into pellet and supernatant fractions and immunoblotted for PKCα, Phospho-PKCα and RACK1. The resulting immunoblots (Figure 5.5b) show that PKCα translocation to the particulate fraction peaks after 30 minutes of 8Me stimulation and 300 minutes of 8-pCPT-cAMP stimulation, while Phospho-PKCα levels remain fairly constant following 8Me stimulation until the 480 minute time point where it peaks and then is ablated by 960 minutes (Figure 5.5b). In response to 8-pCPT-cAMP stimulation the immunoblot shows a steady rise in Phospho-PKCα immunoreactivity until 480 minutes, following which it is also ablated. These results show that EPAC activation is sufficient to induce rapid, autophosphorylation and activation of PKCα in COS1 cells.

To determine that it is EPAC1 and not EPAC2 that is involved in the regulation of PKCα activity in these cells, COS1 and Rat Cerebellum (as a positive control) lysates were separated by SDS-PAGE and immunoblotted with an EPAC2 specific antibody (Figure 5.6a). Immunoblots demonstrated that while EPAC2 is enriched in samples of Rat Cerebellum, it is investigate the importance of EPAC1 in this pathway, COS1 cells were transfected with cDNAs encoding either HA-tagged PDZ-GEF1, a Rap1 guanine

Figure 5.5 Cyclic AMP induces PKCα autophosphorylation via EPAC

a) COS1 cells were stimulated with varying concentrations of 8-pCPT-2'-O-Me-cAMP (8Me) ranging from 0-10μM for 5 hours. Cells were then harvested and fractionated by high-speed centrifugation into pellet and supernatant fractions. Equal amounts of protein from the pellet fractions were separated by SDS-PAGE and immunoblotted with Phospho-PKCα specific antibody. The immunoblot shows an increase in Phospho-PKCα expression in response to increasing levels of 8Me starting at 0.3μM.

b) COS1 cells were stimulated with 10μM 8Me or 8-pCPT-cAMP at various time points ranging from 0-960 min. Cells were then harvested and fractionated by high-speed centrifugation into pellet and supernatant fractions and equal amounts of protein from the pellet fractions were separated by SDS-PAGE. Immunoblotting with PKCα, Phospho-PKCα and RACK1 specific antibodies was then carried out. The resulting immunoblots show that PKCα expression peaks after 30 min of 8Me stimulation and 300 min of 8-pCPT-cAMP stimulation, while Phospho-PKCα levels remain fairly equal upon 8Me stimulation until the 480min time point where it peaks and then is ablated by 960 min. In response to 8-pCPT-cAMP stimulation the immunoblot shows a steady rise in expression of Phospho-PKCα until 480min, following which it is also ablated. This immunoblot is a result of a single experiment carried out.

Figure 5.5

a)

Antibody

Pellet

Phospho PKC alpha

| 0   0.1   0.3   1.0   3.0   10 |

8-pCPT-2'-O-Me-cyclic AMP (µM)

b)

Antibody

Pellet

Phospho PKC alpha

PKC alpha

RACK1

Time (minutes)   0   30   60   300 480 960   0   30   60   300 480 960

8-pCPT-2'-O-Me
cyclic AMP   8-pCPT-cyclic AMP
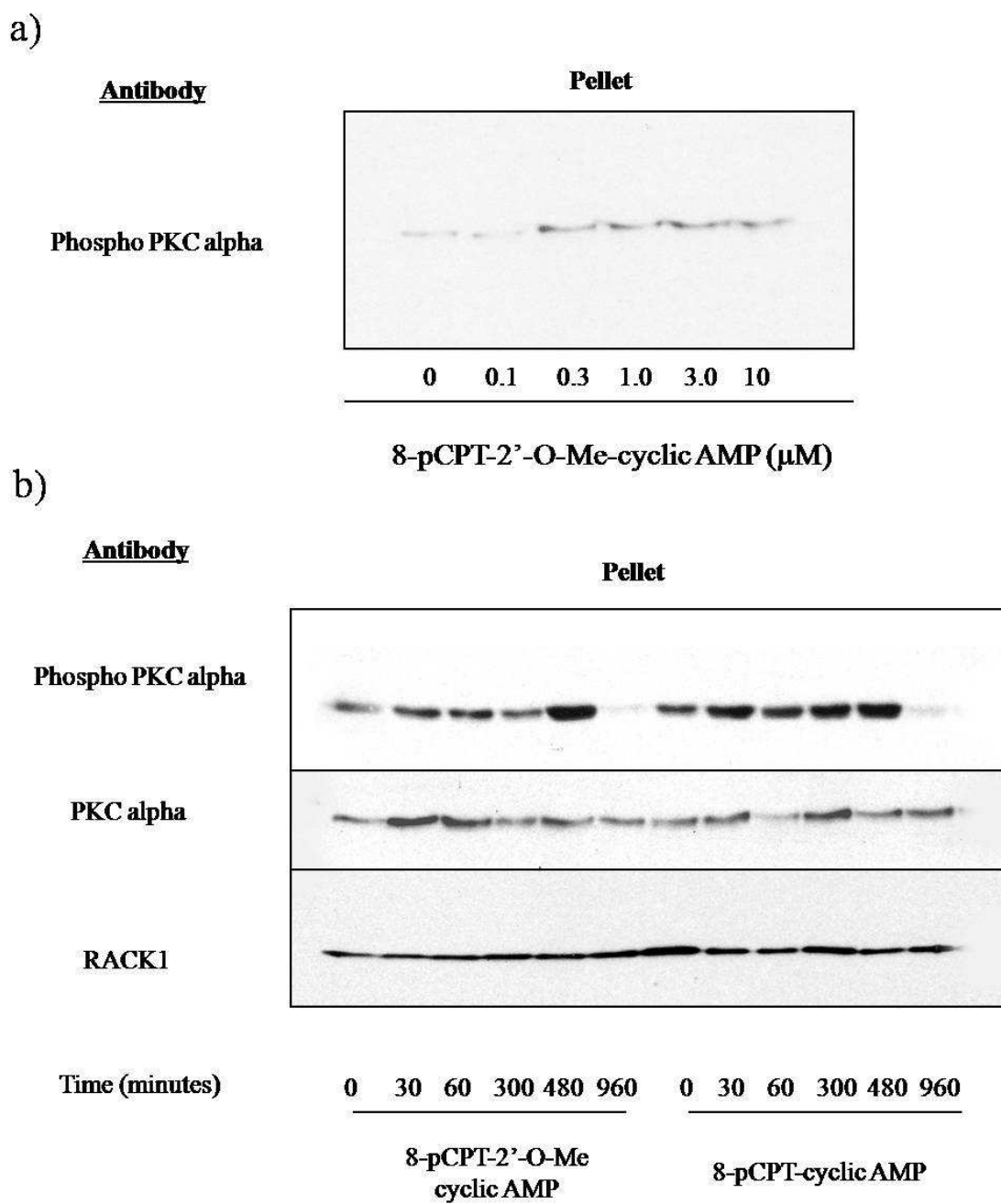
Figure 5.6 ERK phosphorylation by cyclic AMP in COS1 cells does not involve EPAC2 but may involve EPAC1

a) COS1 and Rat Cerebellum lysates were separated by SDS-PAGE and immunoblotted with EPAC2 specific antibody. The immunoblot demonstrates that while EPAC2 is enriched in the Rat Cerebellum, it is not present in COS1 cells.

b) COS1 cells were transfected with HA-tagged PDZ-GEF1 or EPAC1 using DOTAP according to the manufacturer's instructions, and stimulated in the presence or absence of 10μM PMA. Cells were harvested and lysates separated by SDS-PAGE, then immunoblotted using Phospho-ERK, PDZ-GEF1, HA and GAPDH specific antibodies. While the PDZ-GEF1 and HA blots show that the transfection is successful, the Phospho-ERK blots show that ERK phosphorylation occurs in response to 5 hours PMA stimulation in mock transfected cells. In PDZ-GEF1 transfected cells Phospho-ERK levels are greatly reduced in comparison with mock transfected cells while cells transfected with EPAC1 and stimulated with PMA show an increase in ERK phosphorylation above levels shown in mock transfected cells.

Results are representative of an experiment carried out on three separate occasions.

Figure 5.6

a)

b)
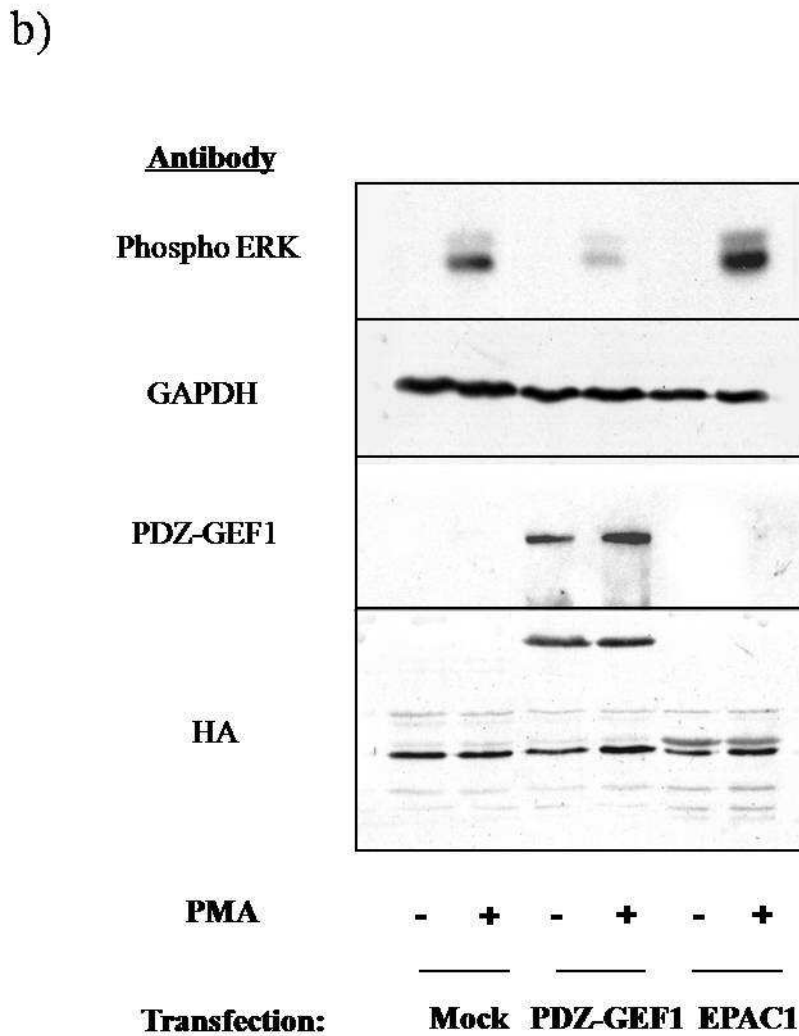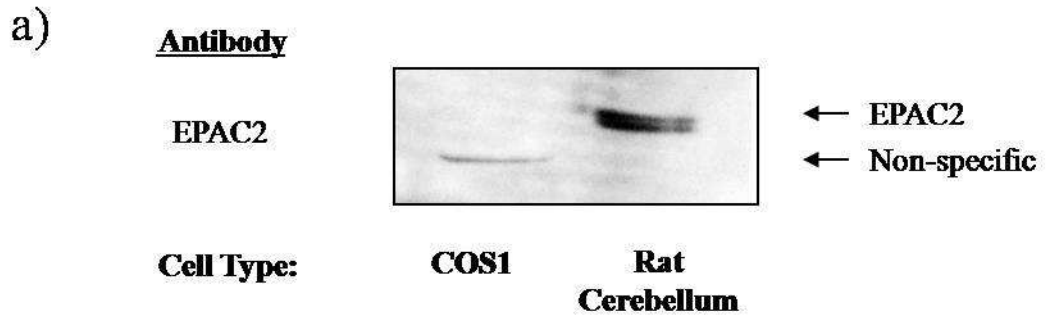
not present in COS1 cells, indicating that any effects of cyclic AMP via EPAC on PKCα

autophosphorylation and activation are occurring through the EPAC1 isoform. To further

nucleotide exchange factor, or EPAC1 and then stimulated in the presence or absence of 10μM PMA. Cell lysates were immunoblotted using Phospho-ERK, PDZ-GEF1, HA and GAPDH specific antibodies (Figure 5.6b). While the PDZ-GEF1 and HA blots show that the transfection is successful, the Phospho-ERK blots show that ERK phosphorylation occurs in response to PMA stimulation in mock transfected cells. In PDZ-GEF1 transfected cells Phospho-ERK levels are greatly reduced in comparison with mock transfected cells, while cells transfected with EPAC1 and stimulated with PMA show significant increase in ERK phosphorylation. These results show that signalling through PDZ-GEF1 is not required for ERK phosphorylation in response to PKC activation, and may in fact inhibit ERK phosphorylation which is unsurprising considering Rap1 has been shown to have both a positive and negative effect on ERK signalling (Stork and Schmitt, 2003).

5.3.3 Elevation of intracellular cyclic AMP levels in COS1 cells does not affect autophosphorylation and activation of PKCδ

- In the previous chapter, siRNA experiments demonstrated an obligatory role for both PKCα and PKCδ isoforms in mediating cyclic AMP-induced SOCS-3 expression in COS1 cells. Having established that cyclic AMP promotes autophosphorylation, and hence activation of PKCα, the effect of cyclic AMP on PKCδ activation was next investigated. COS1 cells were therefore transiently transfected with a cDNA encoding PKCδ to boost the low endogenous levels of the protein and to allow easier visualisation of PKCδ response to stimulations. Protein overexpression is a common technique employed to investigate affects of the protein which may normally be difficult to measure. The inclusion of mock transfected cells as a control allows for detectable results to be influenced by the overexpression of the protein and not by the transfection itself. Cells were then stimulated with F/R or PMA as previously described, harvested and fractioned by high-speed centrifugation before being immunoblotted with Phospho-PKCδ, ERK and Phospho-ERK (Thr202/Tyr204) specific antibodies (Figure 5.7). The Phospho-PKCδ antibody is known to recognise the Ser643 autophosphorylation site. Immunoblots demonstrated that transfection with PKCδ increased basal levels of Phospho-PKCδ immunoreactivity in the pellet fraction of COS1 cells and stimulation with PMA, but not F/R, further enhanced Phospho-PKCδ immunoreactivity in this fraction (Figure 5.7a). Phospho-PKCδ immunoreactivity from the supernatant fraction corresponds with activation-coupled translocation to the pellet fraction (Figure 5.7b). Again F/R treatment had little effect on the phosphorylation and translocation of PKCδ (Figure 5.7b). To demonstrate that F/R was in fact active in these

Figure 5.7

a)

Antibody   **Pellet**

Phospho PKC delta

b)

S/N

Phospho PKC delta

c)

S/N

Phospho ERK

Total ERK

Diluent	+	-	-	+	-	-
F/R	-	+	-	-	+	-
PMA	-	-	+	-	-	+

Transfection: Mock   PKC delta
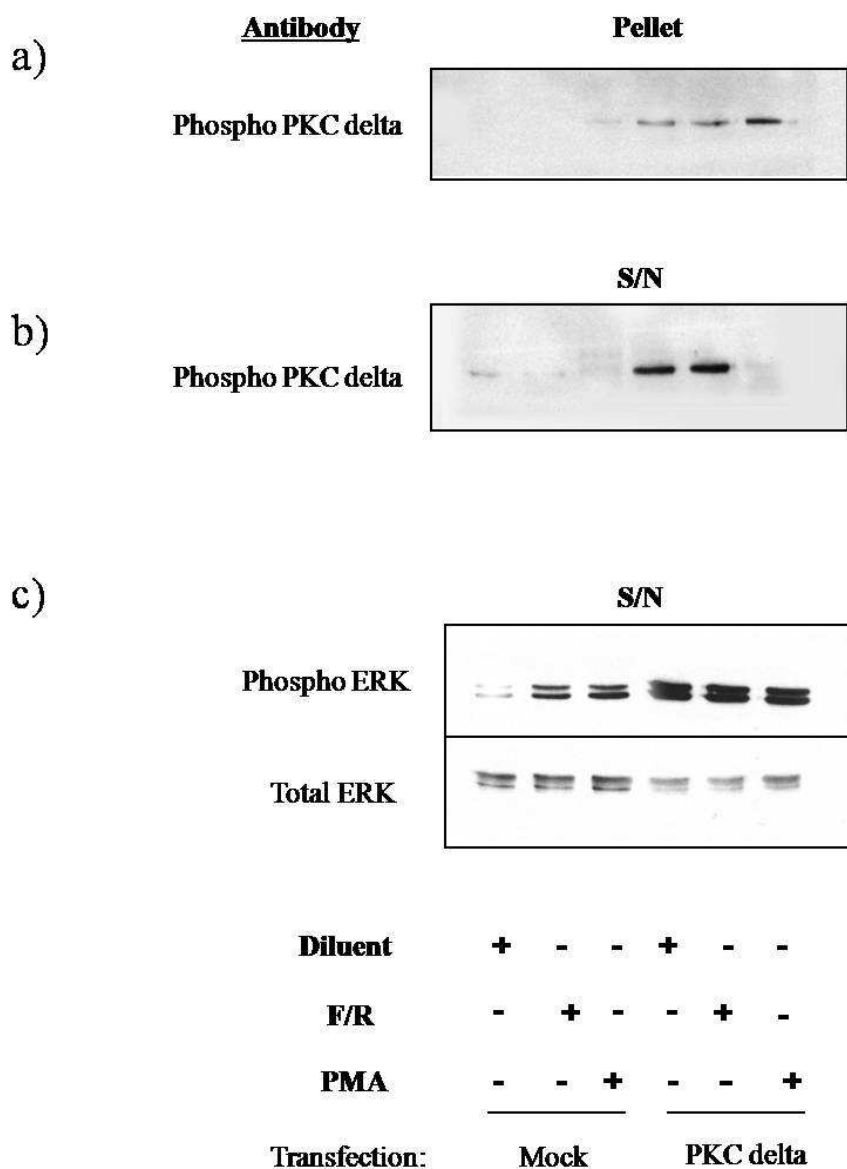
Figure 5.7 Cyclic AMP does not induce autophosphorylation of PKCδ

COS1 cells were transfected with PKCδ using DOTAP according to the manufacturer's instructions. Cells were then stimulated with 10μM F/R or PMA for 5 hours, harvested and fractioned by high-speed centrifugation. Equal amounts of the pellet and supernatant fractions were separated by SDS-PAGE and immunoblotted with Phospho-PKCδ, ERK and Phospho-ERK specific antibodies. The blots show expression of Phospho-PKCδ in response to PMA stimulation, but not in response to F/R in the pellet fraction. The Phospho-ERK blots show that ERK is phosphorylated by F/R and PMA stimulation in untransfected cells, while transfection with PKCδ as no effect on Phospho-ERK expression. Results are representative of an experiment carried out on three separate occasions.

experiments, supernatant fractions were probed with Phospho-ERK antibodies which demonstrated that transfection with PKCδ and F/R and PMA all caused an increase in ERK activation as determined by immunoblotting with Phospho-ERK antibodies (Figure 5.7c). These results indicate that while overexpression of PKCδ is sufficient to induce phosphorylation and activation of the downstream effector, ERK, and that stimulation with PMA produces an increase in levels of Phospho-PKCδ immunoreactivity in the pellet fraction of COS1 cells, elevation of cyclic AMP by F/R appears to have no significant influence on PKCδ autophosphorylation and activation in COS1 cells.

5.3.4 Cyclic AMP induced ERK phosphorylation in COS1 cells - As both F/R and PMA stimulations caused increases in Phospho-ERK levels in untransfected COS1 cells (Figure 5.7), that appear to be unrelated to PKCδ activation, we therefore tested an involvement of PKCα in this signalling cascade. COS1 cells were stimulated with F/R or PMA at various time points ranging from 0 to 960 minutes. Cells were then harvested, fractionated and immunoblotted with Phospho-PKCα, RACK1, ERK and Phospho-ERK specific antibodies. The pellet fraction (Figure 5.8a) shows F/R induced Phospho-PKCα immunoreactivity peaking at 300 minutes, coinciding with in increases in Phospho-ERK immunoreactivity in the same fraction. PMA stimulation caused a peak of Phospho-PKCα immunoreactivity in this fraction at 30 minutes while ERK phosphorylation levels remain steady between 30 and 300 minutes before peaking at 480 minutes. In the supernatant fraction (Figure 5.8b) small increases in Phospho-PKCα levels were observed in response to F/R stimulation, with ERK phosphorylation levels significantly increased following 300 minutes of stimulation. However, it is possible that this increase in ERK phosphorylation is a result of other effects of cyclic AMP elevation rather than as a direct result due to the long time period between F/R stimulation and detectable ERK phosphorylation. Long-term stimulation with PMA caused activation-coupled translocation of Phospho-PKCα from this fraction, while Phospho-ERK levels were observed to peak at 30 minutes stimulation before steadily declining. The immunoblots show that ERK phosphorylation in response to F/R occurs in a similar fashion to that of PKCα, indicating that phosphorylation of PKCα in response to cyclic AMP may lead to localised phosphorylation and activation of its downstream effector ERK in COS1 cells.

Finally, the effect of inhibition of PKC on ERK phosphorylation in response to cyclic AMP elevation and PMA stimulation was investigated by pre-treatment of COS1 cells in the presence or absence of 50μM Ro-31-7549 or 50μM GFX before being stimulated by

Figure 5.8 Cyclic AMP induces ERK phosphorylation in COS1 cells

COS1 cells were transfected with 10μM F/R or PMA at various time points ranging from 0-960 min, harvested and fractionated by high-speed centrifugation into pellet and supernatant fractions. Equal amounts of protein from both fractions were separated by SDS-PAGE and immunoblotted with Phospho-PKCα, RACK1, ERK and Phospho-ERK specific antibodies.

a) The pellet fraction shows F/R induced Phospho-PKCα expression peaking at 300 min, which is mimicked in the Phospho-ERK blot. PMA stimulation in this fraction causes a peak of Phospho-PKCα expression at 30 min while ERK phosphorylation levels remain steady at 30-300min before peaking at 480 min.

b) The supernatant fraction shows small increases of Phospho-PKCα levels are shown in response to the F/R stimulation time course, with ERK phosphorylation levels significantly observed from 300 min of stimulation. Stimulation with PMA shows an ablation of Phospho-PKCα in this fraction, while Phospho-ERK levels peak at 30 min stimulation before steadily declining. Results are representative of an experiment carried out on three separate occasions.

Figure 5.8

a)
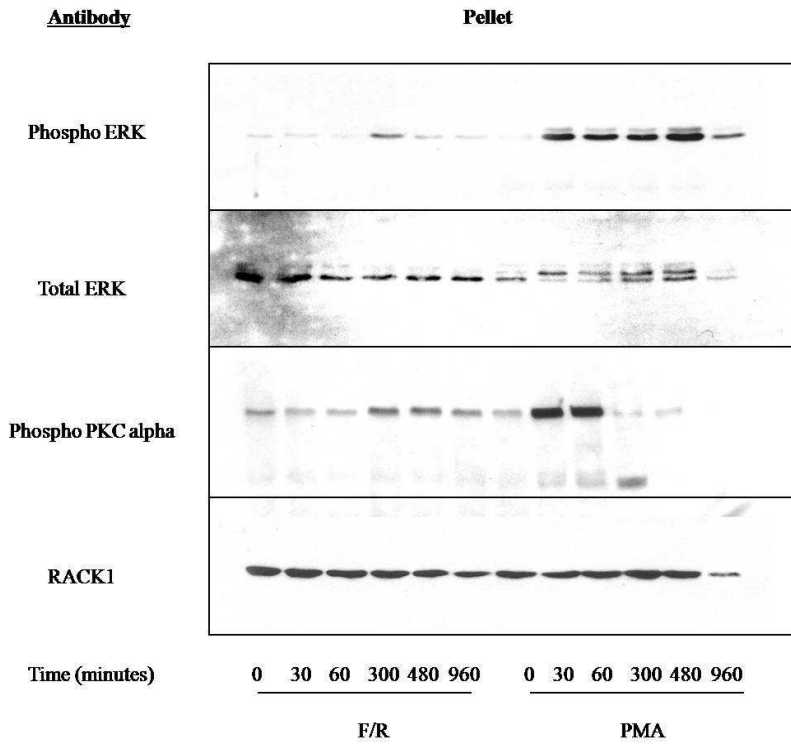
b)
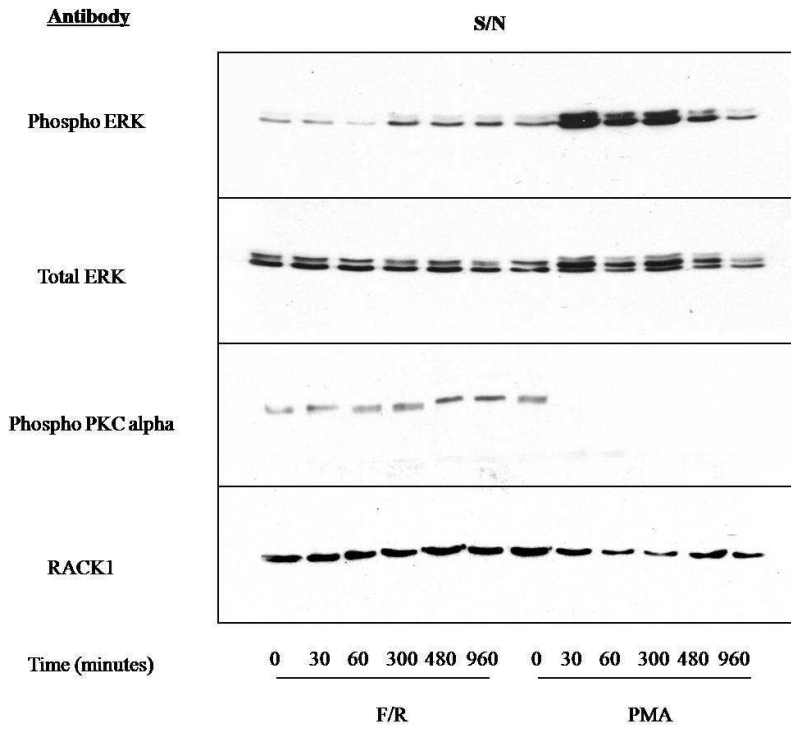
the presence or absence of F/R or PMA. Cell extracts were immunoblotted with ERK and Phospho-ERK specific antibodies (Figure 5.9) to show that F/R stimulation alone significantly increases Phospho-ERK levels in comparison to unstimulated cells, while the addition of Ro-31-7549 or GFX had little effect on this activation. Stimulation with PMA alone greatly increased ERK phosphorylation levels which were reduced to near that of the unstimulated cells following addition of Ro-31-7549 or GFX. These results indicate that while PMA induced phosphorylation of ERK is greatly reduced by the inhibition of PKC, the inhibition of PKC does not reduce ERK phosphorylation levels in response to F/R. This suggests that although the time-course of ERK activation in response to F/R stimulation is compatible to that of PKCα activation, the stimulation of ERK in response to elevations in intracellular cyclic AMP occurs independently of EPAC1-activated PKCα and likely involves PKA or EPAC1 independent of PKCα. A similar experiment utilising 8Me and PKA inhibitors such as H89, as well as experimentation to determine via which Raf isoform ERK phosphorylation occurs would further elucidate this pathway.

Figure 5.9 Cyclic AMP induction of ERK phosphorylation can occur independently of EPAC1 activated PKCα

COS1 cells were pre-treated in the presence or absence of 50μM Ro-31-7549 or GFX before being stimulated by the presence or absence of 10μM F/R or PMA for 5 hours. Cells were then harvested and fractionated by high-speed centrifugation into pellet and supernatant fractions. Equal amounts of protein from the supernatant fractions were separated by SDS-PAGE and immunoblotted with ERK and Phospho-ERK specific antibodies. Densitometry was carried out and the values plotted on a histogram. The blots show expression of Phospho-ERK in response to F/R stimulation that is not inhibited by the addition of Ro-31-7549 or GFX. Densitometric values were obtained from the Phospho-ERK immunoblots and plotted as a percent of maximum, with significant changes in ERK phosphorylation indicated by ** = p<0.01 and *** = p<0.001. Results are representative of an experiment carried out on three separate occasions.

Figure 5.9
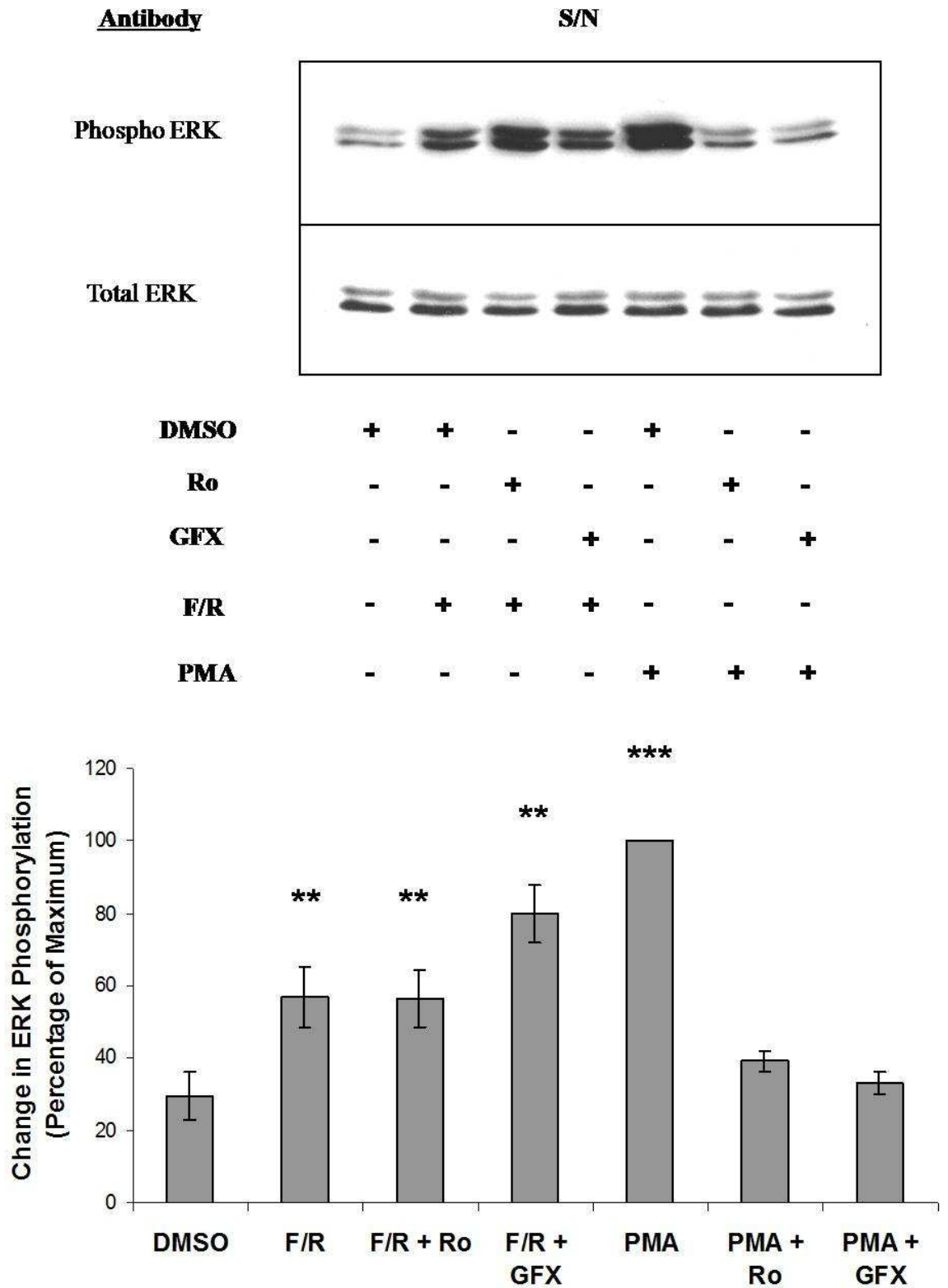
5.4 DISCUSSION

Autophosphorylation of PKC isoforms has been shown to be important in the regulation of enzyme activity (Hu and Exton, 2004). It has also been linked to effective compartmentalisation of the PKC signalling pathway by both localisation and temporal activity, in response to differing stimuli (Kitatani *et al.,* 2007). Such compartmentalisation of PKC signal transduction is important in several important cellular processes. Having established a role for PKC in the cyclic AMP-induced activation of the cyclic AMP-specific phosphodiesterase 4D5, through which levels of cyclic AMP can be regulated, and in the induction of the SOCS-3 gene by cyclic AMP, an investigation into the effect of cyclic AMP levels on PKC isoform activation was important to fully understand the interactions between these two pathways.

Currently, little is known about the role of cyclic AMP in PKC regulation, although its affects on PKC appear to be cell specific. In skeletal myoblasts, cyclic AMP has been shown to reduce PKC activity (Nishizuka, 1986), and to inhibit PKC activity in human platelets (Kroll *et al.,* 1988), while in PC12 cells cyclic AMP has been found to activate atypical PKC isoforms (Wooten *et al.,* 1996) and cause translocation of PKCε (Graness *et al.,* 1997). Similarly, the effect of cyclic AMP on upstream effectors of PKC, such as calcium, appears to differ between cell types, with cyclic AMP inhibiting the hydrolysis of PLC to IP3 in neuroblastoma/glioma hybrid cells (Campbell *et al.,* 1990) but increasing intracellular calcium levels in other cell lines (Chase and Wong, 1988). In light of this, the evidence presented in this chapter may only be representative of the actions of cyclic AMP in specifically COS1 cells.

Our investigations in this chapter have identified a role for cyclic AMP in the autophosphorylation and activation of the PKCα isoform, but not PKCδ. Results show that elevation of cyclic AMP induces PKCα autophosphorylation differently than stimulation with the phorbol ester PMA (Figure 5.3), with PMA inducing a more immediate translocation to the plasma membrane and subsequent phosphorylation of the isoform which is sustained for an extended time period. Cyclic AMP stimulation does not appear to induce a gross translocation of PKCα however levels of autophosphorylated forms of the enzyme accumulate slowly in the pellet fraction of COS1 cells. Such differences in temporal phasing may play an important role in the compartmentalised effect of this PKC isoform.

Further experiments established that cyclic AMP is able to affect PKCα autophosphorylation via activation of EPAC1 (Figure 5.5a). Although PKA can also be seen to effect PKCα autophosphorylation (Figure 5.5b), the majority of the effect of cyclic AMP appears to occur via EPAC, with PKA influence occurring, as with PMA, as a more immediate but transient effect in lysate samples. Furthermore, this effect by EPAC1 in COS1 cells appears to be limited to PKCα, as cyclic AMP elevation in cells expressing PKCδ, the other PKC isoform shown to be abundantly present in COS1 cells and linked to SOCS-3 induction, does not induce autophosphorylation of PKCδ (Figure 5.7).

Although ERK phosphorylation in response to cyclic AMP elevation appears to follow a similar trend to PKCα autophosphorylation by F/R stimulation, PMA stimulation induces ERK phosphorylation at time points differing from its induction of PKCα phosphorylation (Figure 5.8). However, PMA stimulation can be seen to induce ERK phosphorylation at a greater extent in cells overexpressing EPAC1, suggesting the presence of EPAC1 in COS1 cells is required for phosphorylation of ERK. EPAC has been reported previously to affect ERK phosphorylation and activity. EPAC mediated activation of Rap GTPase has been shown to stimulate ERK (Wang *et al.,* 2006) and has also been shown to influence ERK phosphorylation by blocking the cyclic AMP elevating hormone calcitonin (Holz *et al.,* 2006). Here we have shown that the action of EPAC on ERK phosphorylation appears to occur independently of PKC, as use of specific PKC inhibitors does not reduce ERK phosphorylation by F/R and in some cases can be seen to increase Phospho-ERK levels (Figure 5.9). Further work to determine which Raf isoform cyclic AMP exerts this effect on ERK phosphorylation would confirm whether EPAC is able to cause effects downstream of PKC without directly affecting PKC itself, or if its effects are on PKC itself. As EPAC acts on B-Raf (Roscioni *et al.,* 2008), and PKC on Raf-1 (Kolch *et al.,* 1993), activation assays or siRNA on these isoforms would provide greater understanding into the route by which cyclic AMP is able to induce phosphorylation of ERK via EPAC1. Although cyclic AMP is known to activate ERK, evidence indicating that it is able to do so via EPAC1 shows that cyclic AMP can control ERK activation in a PKA independent manner. This allows for ERK signalling and the mediation of transcription to occur separately from activation of PKA and other components of the PKA signalling pathway.

In conclusion, this chapter outlines a role not only for cyclic AMP influence by EPAC on PKC autophosphorylation, but also a potential role for EPAC in the phosphorylation of ERK independent of PKC activation.

CHAPTER 6

General Discussion

6.1 Overview

Biological functions are mediated by complex signal transduction pathways. Such pathways require tight regulation to elicit the correct cellular response and this regulation is often a result of crosstalk between different signalling pathways (Stork and Schmitt, 2002). Investigation of mechanisms of action and control of signalling pathways, and in particular the control exerted by other pathways is important not only to gain a more complete understanding into the workings of the pathway, but also to provide potential areas of therapeutic drug targeting. The pathway outlined in this thesis not only combines two separate signalling pathways which have been shown to influence and control the action of the other, but also provides understanding and access into the potential control of several biological functions and disease states.

6.2 The role of cyclic AMP in inflammation

The cyclic AMP signalling pathway is a well documented, compartmentalised signal transduction cascade that is involved in numerous cellular processes ranging from cell differentiation and growth to the regulation of glycogen and lipid metabolism (Cheng *et al.,* 2008). Although PKA was once believed to be the sole route for cyclic AMP signalling, the recent discovery of EPAC (Exchange Protein Activated by Cyclic AMP) provided a new avenue of research in cyclic AMP signalling (Roscioni *et al.,* 2008). EPAC was identified in a database search for proteins containing sequence homology to both cyclic AMP binding sites and Ras and Rap1 GEFs following the discovery that Rap1 activation by cyclic AMP occurred independently of PKA (de Rooij *et al.,* 1998). The two EPAC isoforms identified were hypothesised to be involved in cyclic AMP effects previously believed to act solely through PKA action and as, like PKA, EPAC isoforms are found ubiquitously in tissue, they provide an additional means for precise control of cyclic AMP signalling. This allows cyclic AMP to affect multiple pathways (Cheng *et al.,* 2008), with regulation of EPAC localisation and specific subcellular locations suggesting that compartmentalisation of EPAC plays an important role in its signalling properties (Roscioni *et al.,* 2008). There are a wide range of downstream effectors of EPAC, PLC and ERK (Roscioni *et al.,* 2008).

To date, the effects of EPAC and its regulation are not fully understood, although it has been shown to play important roles in calcium signalling through both direct interaction with intracellular calcium receptors and through control of exocytosis, which alongside insulin secretion links EPAC to a role in diabetes (Holz *et al.,* 2006; Roscioni *et al.,* 2008) and in anti-inflammatory signalling. In 2006, Sands et al, identified the ability of cyclic AMP level elevations in vascular endothelial cells to inhibit IL-6 signalling. This inhibition involved the induction of SOCS-3, which was found to occur independently of PKA and to instead require the activation of EPAC. From this data, a pathway was outlined, through which an increase in intracellular cyclic AMP activated Rap1 via EPAC to cause induction of SOCS-3 and mediate the pro-inflammatory effect of IL-6 receptor signalling (Sands *et al.,* 2006).

As IL-6 initiates and mediates immune and inflammatory responses, disruption of this tightly controlled signalling has been linked to diseases such as Crohn's and rheumatoid arthritis (Ishihara and Hirano, 2002), while increased IL-6 levels have been linked to diseases including osteoporosis (Jones *et al.,* 2001) and renal cancer (Takenawa *et al.,* 1991). The pathway outlined in this study may therefore provide drug targets by which control of IL-6 signalling can be asserted via SOCS-3. Additionally, EPAC has been linked to several important cellular functions, including exocytosis, cell differentiation and proliferation, and apoptosis (as reviewed in Borland *et al.,* 2009) that need tight regulation. EPAC has also been identified as having a role in a range of neurodegenerative, inflammatory and metabolic disorders such as Alzheimer's, diabetes and asthma (McPhee *et al.,* 2005). The wide ranging cellular functions and diseases linked to the components of the pathway outlined in this work demonstrates that elucidation of these areas of crosstalk provides important understanding into the importance of the control exhibited by this pathway. EPAC has also been shown to mediate the induction of SOCS-3 in several cell types (Sands *et al.,* 2006; Yarwood *et al.,* 2008; Borland *et al.,* 2009). SOCS-3 is capable of inhibiting the JAK-STAT pathway, allowing control of IL-6 signalling, a cytokine involved in the maintenance of the inflammatory and immune responses. However the pathway by which cyclic AMP is able to induce SOCS-3 has not been fully outlined.

The pathway elucidated here between EPAC and SOCS-3 expands upon the identification of the involvement of EPAC1 in the control of SOCS-3 induction (Sands *et al.,* 2006; Yarwood *et al.,* 2008; Borland *et al.,* 2009). A link between EPAC and PKCε via the small GTPase Rap2B has been well established (Schmidt *et al.,* 2001; Bunney and Katan, 2006;

Oestreich *et al.,* 2009), providing a starting point for investigations, although no known effects of PLCε on SOCS-3 had been reported. PLC inhibitor treatment and PLCε specific siRNA experiments indicated that PLCε is necessary for SOCS-3 induction by EPAC in COS1 cells. Further investigation into downstream effectors of PLC action led to the identification of PKCα and PKCδ as essential components of this pathway by which EPAC is able to induce SOCS-3 in COS1 cells. As further investigation within our group also recently identified that this route of induction of SOCS-3 by cyclic AMP via EPAC and PKC is dependent on activation of ERK and the transcription factor C/EBPβ (Borland *et al.,* 2009), this work goes some way towards fully delineating a mechanism by which cyclic AMP can affect inflammation. Identification of these key points in the pathway may provide areas for potential therapeutic targeting as elevating cyclic AMP levels or activating components in the pathway would increase SOCS-3 induction, thereby inhibiting IL-6 signalling.

6.3 Activation of PKC by cyclic AMP

As outlined above, we have identified a role for PKC isoforms in a pathway induced by cyclic AMP. This discovery led to further investigation into the effect of cyclic AMP, via EPAC, on PKCα and δ activation and autophosphorylation. Autophosphorylation of PKC isoforms has been shown to be important not only for PKC activity but also for subcellular localisation and compartmentalisation, indicating it plays an important role in tight enzyme control and disruption of this activity can effect biological functions which in turn can lead to disease pathologies (Feng and Hannun, 1998). As activity of PKC isoforms effects biological processes ranging from cell proliferation and adhesion to apoptosis, tight regulation of PKC activity and signalling is crucial as disruption can be linked to several disease states ranging from immunodeficiency disorders to cardiac hypertrophy to a range of cancers (Mackay and Twelves, 2007). To date, little is understood about the effects of cyclic AMP on PKC regulation. What is known suggests that any effect occurs in a cell specific manner, with cyclic AMP causing inhibition of PKC activity in platelets (Kroll et all 1988) but inhibiting activity in skeletal myoblats (Nishizuka, 1986).

Activation of PKC occurs following ligand binding at the cell membrane. Three priming sites, conserved across PKC isoforms are phosphorylated, allowing for control by

autophosphorylation (Parekh *et al.,* 2000). For PKCα, autophosphorylation has been identified as important in targeting of the protein to specific subcellular locations (Kitatani *et al.,* 2007). Similarly, autophosphorylation in PKCδ has been linked to its ability to translocate to cell membranes (Durgan *et al.,* 2007).

The role of autophosphorylation in PKC subcellular localisation is of particular interest as numerous cellular processes, including differentiation and proliferation, require transduction of signals to specific intracellular regions, and it is thought that autophosphorylation of PKC isoforms induced by differing stimuli may allow specific targeting to these areas (Feng *et al.,* 2000). There is therefore a clear importance of understanding the mechanisms by which PKC isoforms can be autophosphorylated, and in particular how effectors outside of the PKC signalling pathway may exert control over PKC activation.

In this study, a role for cyclic AMP was found in the activation and autophosphorylation of PKCα, but not for PKCδ, occurring largely via EPAC. As results show that PKCα is activated by EPAC stimulation while PKCδ is not, it is likely that PKCδ plays a supportive role in the induction of SOCS-3 by EPAC. It is possible that PKCδ is involved in targeting of PKCα or in some other facilitator role, and further investigation is required to determine the precise role of PKCδ in this pathway. Further investigation would also expand on to what degree cyclic AMP exerts control on PKC and on its downstream effectors.

6.4 Control of cyclic AMP by PKC

In this work we have outlined a role for cyclic AMP in control of PKC signalling. This forms part of a negative feedback loop, as we have also identified a point of crosstalk between the two signalling pathways whereby PKC is able to exert control over cyclic AMP. In 1999, Yarwood *et al.* used a yeast two-hybrid screen to identify the interaction between the scaffold protein RACK1 and the cyclic AMP-specific phosphodiesterase PDE4D5, demonstrating a potential area of crosstalk between the PKC and cyclic AMP signalling pathways. As phosphodiesterases are the only means of reducing intracellular cyclic AMP levels, and an understanding of their control would provide a mechanism to artificially control cyclic AMP signalling. Although binding between these two proteins was shown to be unique and specific (Yarwood *et al.,* 1999) with RACK1 as the only

WD40 protein bound by PDE4D5 and RACK1 showing no ability to bind other phosphodiesterases, the function of this complex remains largely unknown.

Here we have outlined evidence that binding of RACK1 to PDE4D5 is important in control of its enzymatic activity. Unlike the β-arrestin/PDE4D5 complex which blocks cyclic AMP synthesis and allows hydrolysis of cyclic AMP in the vicinity of the β2-Adrenergic receptor (Bolger *et al.,* 2003), the RACK1/PDE4D5 complex did not appear to translocate, indicating that RACK1 does not target PDE4D5 to specific cellular localisations. It does however affect the structural stability of the phosphodiesterase, providing PDE4D5 protection against denaturation as well as affording high affinity binding of PDE4D5 to cyclic AMP. Additionally this study confirmed the results of Yarwood *et al.* (1999) which showed that binding to RACK1 increased the susceptibility of PDE4D5 to inhibition by the PDE4-specific inhibitor rolipram. Rolipram is used therapeutically to treat diseases such as depression and Alzheimer's, it has an emetic effect on patients (Houslay *et al.,* 2005). It is possible therefore that treatment with RACK1 or a synthetic analogue could allow a lower dosage of rolipram to be administered to obtain the same results with lessened side effects.

In the initial study identifying the RACK1/PDE4D5 complex (Yarwood *et al.,* 1999) it was hypothesised that RACK1 could potentially act as a scaffold protein, recruiting not only PDE4D5 but also other proteins to form a signalling complex. In this investigation we have identified the ability of activated PKCα to activate RACK1-bound PDE4D5, increasing cyclic AMP degradation as a result. This indicates a role of negative regulation by PKC on cyclic AMP in HEK293 cells, whereby through activating PDE4D5 to hydrolyse cyclic AMP, PKC is able to cause a reduction in intracellular cyclic AMP levels.

Both cyclic AMP and PKC signalling have been implicated in similar biological functions and disease states. For example, both cyclic AMP and PKC signalling have recently been implicated in control of the mammalian circadian clock, involving EPAC (O'Neill *et al.,* 2008) and the PKCα isoform in complex with RACK1 (Receptor for Activated Protein Kinase C 1) (Robles *et al.,* 2010) respectively. As components between both pathways form integral parts of the negative feedback loops which control the circadian pacemaker, the work presented here indicates that it is likely that these pathways are working in conjunction with each other.

6.5 Future Directions

Although a clear pathway containing points of crosstalk between cyclic AMP and PKC signalling pathways has been outlined, there are several directions in which this investigation could be carried forward in the future. There is the potential to provide therapeutic targets for the control of inflammation due to the effect of SOCS-3 on IL-6 signalling as well as other biological roles and diseases associated with this cytokine, and the potential role of the PDE4D5/RACK1 complex in treatment of disorders with the anti-depressant and anti-inflammatory drug rolipram, as well as potential control over other functions and disease states associated with components of this pathway. Several areas of investigation would therefore provide a deeper understanding into the control and effects of the pathway.

Other areas in which this study could be taken forward include conclusive determination as to whether ERK phosphorylation in this pathway is a consequence of EPAC or PKC activation, by investigating which Raf isoform is involved in the pathway. Further elucidation into the supportive role of PKCδ in the pathway, and whether any other proteins associated with components of the pathway, such as AKAPs assert any level of control, or if they themselves are affected by the pathway would expand on the work laid out in this thesis.

Additionally, many of the components of the pathway are found in almost all cell types, so determination as to whether there is a role for this pathway in cells other than COS1 or HEK293, both of which are kidney cells, would provide further evidence for the role of the pathway in cellular function and the suitability of its areas of crosstalk as therapeutic targets. This is particularly important as the pathway may not be viable in all cell types, which may further determine the suitability of its use in therapeutic control of cell functions, due to components which have been shown to have different effects in different cell types. For example EPAC has been identified as stimulating cell division in COS1 cells (Qiao *et al.,* 2002), while in Jurkat cells the opposite effect has been shown (Boussiotis *et al.,* 1997).

6.6 Conclusions

To conclude, the evidence presented in this work goes some way towards outlining a pathway through which the cyclic AMP and PKC pathways act together to control and regulate each other and their activity via novel areas of crosstalk (Figure 6.1). In this pathway, cyclic AMP activity via EPAC is able to induce SOCS-3 and influence IL-6 control through the activation of PKC isoforms. PKC is also able to effect negative feedback on cyclic AMP through the ability to increase cyclic AMP degradation through activation of PDE4D5 when bound to RACK1. The discoveries made in this thesis may also provide avenues for drug therapies for a range of disorders linked with components of the pathway.
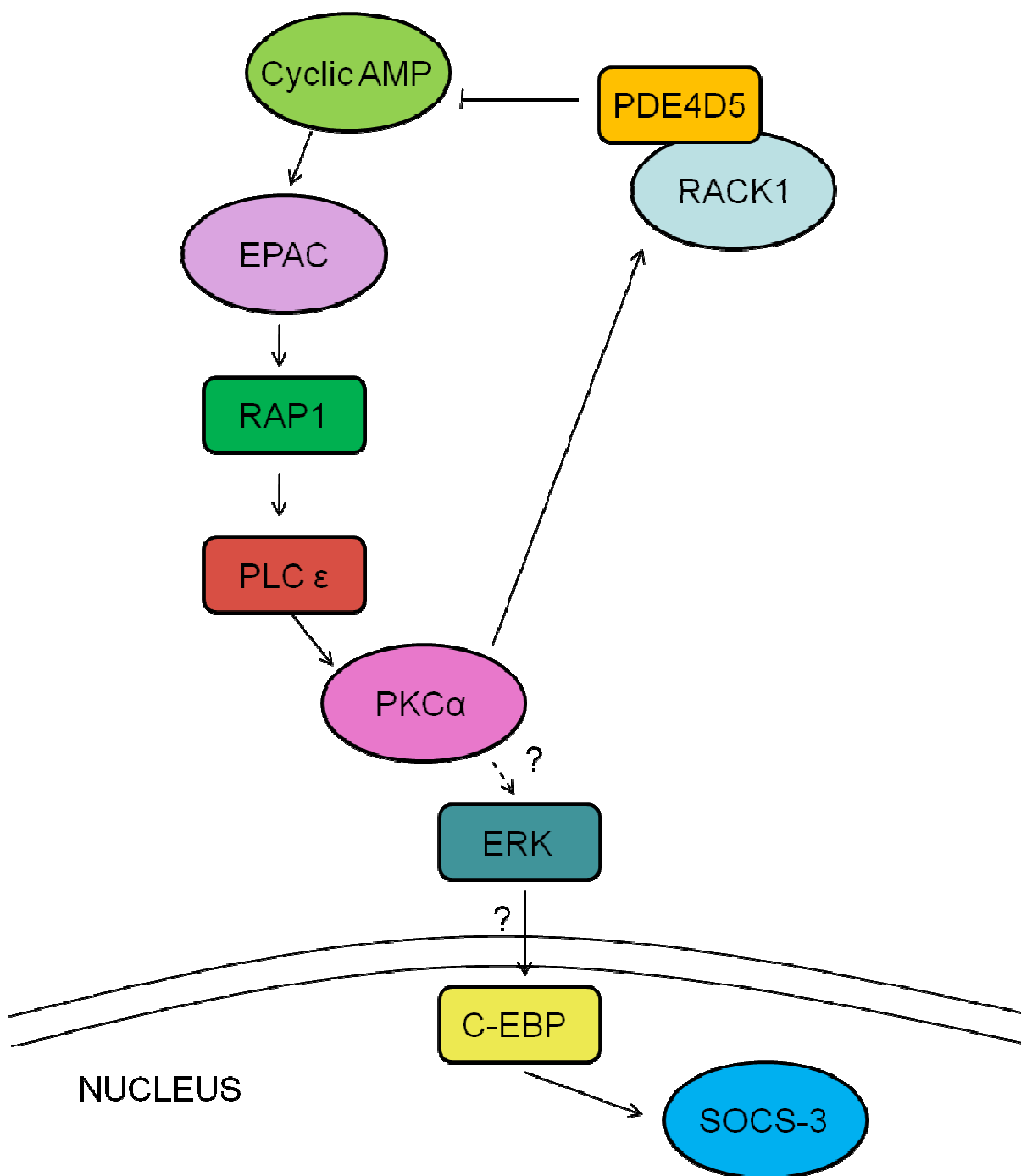
Figure 6.1

Figure 6.1 Proposed pathway of crosstalk between cyclic AMP and PKC signalling pathways

In response to cyclic AMP elevation, EPAC is able to induce SOCS-3 expression via PLCε and activation of PKCα. PKCα negatively regulates cyclic AMP levels by activating PDE4D5 while in complex with RACK1. PKCα may also influence ERK activation of C/EBP to induce SOCS-3 expression.

REFERENCES

Alberola-Ila, J., Forbush, K. A., Seger, R., Krebs, E. G. and Perlmutter, R. M. (1995). "Selective requirement for MAP kinase activation in thymocyte differentiation." Nature **373**(6515): 620-623.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. (2002). "Molecular Biology of the Cell, 4th Ed." Chapter 15, p842

Alessi, D. R., Saito, Y., Campbell, D. G., Cohen, P., Sithanandam, G., Rapp, U., Ashworth, A., Marshall, C. J. and Cowley, S. (1994). "Identification of the sites in MAP kinase kinase-1 phosphorylated by p74raf-1." Embo J **13**(7): 1610-1619.

Amadio, M., Battaini, F. and Pascale, A (2006). "The different facets of protein kinases C: old and new players in neuronal signal transduction pathways." Pharmacol Res **54**(5):317-325.

Auernhammer, C. J. and S. Melmed (2001). "The central role of SOCS-3 in integrating the neuro-immunoendocrine interface." J Clin Invest **108**(12): 1735-1740.

Bailey, C. L., Kelly, P. and Casey, P.J.(2009). "Activation of Rap1 promotes prostate cancer metastasis." Cancer Res **69**(12): 4962-4968.

Baillie, G. S., Sood, A., McPhee, I., Gall, I., Perry, S.J., Lefkowitz, R.J. and Houslay, M.D. (2003). "beta-Arrestin-mediated PDE4 cAMP phosphodiesterase recruitment regulates beta-adrenoceptor switching from Gs to Gi." Proceedings for the National Academy of Sciences of the United States of America **100**: 940-945.

Baillie, G. S. and Houslay, M.D. (2005). "Arrestin times for compartmentalised cAMP signalling and phosphodiesterase-4 enzymes." Current Opinion in Cell Biology **17**: 129-134.

Barber, R. D., Harmer, D. W., Coleman, R. A. and Clark, B. J. (2005). "GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues." Physiol Genomics **21**(3): 389-395.

Barclay, J. L., Anderson, S.T., Waters, M.J. and Curlewis, J.D. (2007). "Characterization of the SOCS3 promoter response to prostaglandin E2 in T47D cells." Mol Endocrinol **21**(10): 2516-2528.

Bass-Zubek, A. E., Amargo, E. V., Garcia, N. J., Hsieh, S. N., Chen, X., Wahl, J. K., Denning, M. F. and Green, K. J. (2008). "Plakophilin 2: a critical scaffold for PKC alpha that regulates intercellular junction assembly." J Cell Biol **181**(4): 605-613.

Beene, D. L. and J. D. Scott (2007). "A-kinase anchoring proteins take shape." Curr Opin Cell Biol **19**(2): 192-198.

Bender, A. T. and Beavo, J.A. (2006). "Cyclic Nucleotide Phosphodiesterases: Molecular Regulation to Clinical Use." Pharmacological Reviews **58**: 488-520.

Benes, C. H., Wu, N., Elia, A. E., Dharia, T., Cantley, L. C. and Soltoff, S. P. (2005). "The C2 domain of PKCdelta is a phosphotyrosine binding domain." Cell **121**(2): 271-280.

Berg, J., Tymoczko, JL. and Stryer, L. (2002). "Biochemistry, 5th ed." Chapter 15 p395

Berman, D. E., Hazvi, S., Rosenblum, K., Seger, R. and Dudai, Y. (1998). "Specific and differential activation of mitogen-activated protein kinase cascades by unfamiliar taste in the insular cortex of the behaving rat." J Neurosci **18**(23): 10037-10044.

Berns, H., Humar, R., Hengerer, B., Kiefer, F.N. and Battegay, E.J. (2000). "RACK1 is up-regulated in angiogenesis and human carcinomas." The FASEB Journal **14**(2549-2558).

Bjarnadottir, T. K., Fredriksson, R. and Schioth, H. B. (2005). "The gene repertoire and the common evolutionary history of glutamate, pheromone (V2R), taste(1) and other related G protein-coupled receptors." Gene **362**: 70-84.

Blagosklonny, M. V., Giannakakou, P., el-Deiry, W. S., Kingston, D. G., Higgs, P. I., Neckers, L. and Fojo, T. (1997). "Raf-1/bcl-2 phosphorylation: a step from microtubule damage to cell death." Cancer Res **57**(1): 130-135.

Blois, T. M. and J. U. Bowie (2009). "G-protein-coupled receptor structures were not built in a day." Protein Sci **18**(7): 1335-1342.

Blumberg, P. M. (1988). "Protein kinase C as the receptor for the phorbol ester tumor promoters: sixth Rhoads memorial award lecture." Cancer Res **48**(1): 1-8.

Bolger, G. B., Erdogan, S., Jones, R.E., Loughney, K., Scotland, G., Hoffmann, R., Wilkinson, I., Farrell, C. and Houslay, M.D. (1997). "Characterization of five different proteins produced by alternatively spliced mRNAs from the human cAMP-specific phosphodiesterase PDE4D gene." Biochemical Journal **328**(539-548).

Bolger, G. B., McCahill, A., Huston, E., Cheung, Y., McSorley, T., Baillie, G.S.& Le Jeune, I.R., Shepherd, M., Van Heeke, G., Houslay, M.D. and Hall, I.P. (2002). "Cyclic AMP-dependent Transcriptional Up-regulation of Phosphodiesterase 4D5 in Human Airway Smooth Muscle Cells: Identification and Characterization of a Novel PDE4D5 Promoter." The Journal of Biological Chemistry **277**(39): 35980-35989.

Bolger, G. B., McCahill, A., Huston, E., Cheung, Y., McSorley, T., Baillie, G.S. and Houslay, M.D. (2003). "The Unique Amino-terminal Region of the PDE4D5 cAMP Phosphodiesterase Isoform Confers Preferential Interaction with B-Arrestins." The Journal of Biological Chemistry **278**(49): 49230-49238. .

Bolger, G. B., Peden, A.H., Steele, M.R., MacKenzie, C., McEwan, D.G., Wallace, D.A., Huston, E., Baillie, G.S. & Houslay, M.D. (2003). "Attenuation of the Activity of the cAMP-specific Phosphodiesterase PDE4A5 by Interaction with the Immunophilin XAP2." Journal of Biological Chemistry **278**(35): 33351-33363.

Bolger, G. B., Baillie, G.S., Li, X., Lynch, M.J., Herzyk, P., Mohamed, A., High Mitchell, L., McCahill, A., Hundsrucker, C., Klussmann, E., Adams, D.R. and Houslay, M.D. (2006). "Scanning peptide array analyses identify overlapping binding sites for the signalling scaffold proteins, β-arrestin, and RACK1, in cAMP-specific phosphodiesterase PDE4D5." Biochemical Journal **398**: 23-36.

Borland, G., Bird, R.J., Palmer, T.M and Yarwood, S.J. (2009). "Activation of protein kinase Calpha by EPAC1 is required for the ERK- and CCAAT/enhancer-binding protein beta-dependent induction of the SOCS-3 gene by cyclic AMP in COS1 cells." J Biol Chem **284**(26): 17391-17403.

Borland, G., Smith, BO. & Yarwood, SJ (2009). "EPAC proteins transducer diverse cellular actions of cAMP." Br J Pharmacol.

Bornancin, F. and P. J. Parker (1996). "Phosphorylation of threonine 638 critically controls the dephosphorylation and inactivation of protein kinase Calpha." Curr Biol **6**(9): 1114-1123.

Bornancin, F. and P. J. Parker (1997). "Phosphorylation of protein kinase C-alpha on serine 657 controls the accumulation of active enzyme and contributes to its phosphatase-resistant state." J Biol Chem **272**(6): 3544-3549.

Bos, J. L. (2006). "Epac proteins: multi-purpose cAMP targets." Trends in Biochemical Sciences **31**(12): 680-686.

Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., DePinho, R. A., Panayotatos, N., Cobb, M. H., Yancopoulos, G. D. (1991). "ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF." Cell **65**(4): 663-675.

Boussiotis, V. A., Freeman, G. J., Berezovskaya, A., Barber, D. L. and Nadler, L. M. (1997). "Maintenance of human T cell anergy: blocking of IL-2 gene transcription by activated Rap1." Science **278**(5335): 124-128.

Bridges, T. M. and C. W. Lindsley (2008). "G-protein-coupled receptors: from classical modes of modulation to allosteric mechanisms." ACS Chem Biol **3**(9): 530-541.

Brock, M., Fan, F., Mei, F. C., Li, S., Gessner, C., Woods, V. L., Jr.and Cheng, X. (2007). "Conformational analysis of Epac activation using amide hydrogen/deuterium exchange mass spectrometry." J Biol Chem **282**(44): 32256-32263.

Brose, N. and C. Rosenmund (2002). "Move over protein kinase C, you've got company: alternative cellular effectors of diacylglycerol and phorbol esters." J Cell Sci **115**(Pt 23): 4399-4411.

Bunney, T. D. and M. Katan (2006). "Phospholipase C epsilon: linking second messengers and small GTPases." Trends Cell Biol **16**(12): 640-648.

Burgering, B. M., Pronk, G.J., van Weeren, P.C., Chardin, P. and Bos, J.L. (1993). "cAMP antagonizes p21ras-directed activation of extracellular signal-regulated kinase 2 and phosphorylation of mSos nucleotide exchange factor." EMBO J **12**(11): 4211-4220.

Cameron, A. J., De Rycker, M., Calleja, V., Alcor, D., Kjaer, S., Kostelecky, B., Saurin, A., Faisal, A., Laguerre, M., Hemmings, B. A., McDonald, N., Larijani, B. and Parker, P. J. (2007). "Protein kinases, from B to C." Biochem Soc Trans **35**(Pt 5): 1013-1017.

Campbell, M. D., Subramaniam, S., Kotlikoff, M.I., Williamson, J.R. and Fluharty, S.J. (1990). "Cyclic AMP inhibits inositol polyphosphate production and calcium mobilization in neuroblastoma X glioma NG108-15 cells." Mol Pharmacol **38**(2): 282-288.

Carroll, G., Bell, M., Wang, H., Chapman, H. and Mills, J. (1998). "Antagonism of the IL-6 cytokine subfamily--a potential strategy for more effective therapy in rheumatoid arthritis." Inflamm Res **47**(1): 1-7.

Carroll, M. P. and W. S. May (1994). "Protein kinase C-mediated serine phosphorylation directly activates Raf-1 in murine hematopoietic cells." J Biol Chem **269**(2): 1249-1256.

Castro, A., Jose Jerez, M., Gil, C. and Martinez, A. (2005). "Cyclic Nucleotide Phosphodiesterases and Their Role in Immunomodulatory Responses: Advances in the Development of Specific Phosphodiesterase Inhibitors." Medicinal Research Reviews **25**(2): 229-244.

Chang, X. B., Tabcharani, J. A., Hou, Y. X., Jensen, T. J., Kartner, N., Alon, N., Hanrahan, J. W. and Riordan, J. R. (1993). "Protein kinase A (PKA) still activates CFTR chloride channel after mutagenesis of all 10 PKA consensus phosphorylation sites." J Biol Chem **268**(15): 11304-11311.

Chase, H. S., Jr. and S. M. Wong (1988). "Isoproterenol and cyclic AMP increase intracellular free [Ca] in MDCK cells." Am J Physiol **254**(3 Pt 2): F374-384.

Chen, S., Dell, E.J., Lin, F., Sai., J. and Hamm, H.E (2004). "RACK1 Regulates Specific Functions of Gβγ." The Journal of Biological Chemistry **279**(17): 17861-17868.

Chen, S., Lin, F. and Hamm, H.E. (2005). "RACK1 Binds to a Signal Transfer Region of Gβγ and Inhibits Phospholipase C β2 Activation." The Journal of Biological Chemistry **280**(39): 33445-33452.

Cheng, X., Ji, Z., Tsalkova, T. and Mei, F.. (2008). "Epac and PKA: a tale of two intracellular cAMP receptors." Acta Biochim Biophys Sin (Shanghai) **40**(7): 651-662.

Chuderland, D. and R. Seger (2005). "Protein-protein interactions in the regulation of the extracellular signal-regulated kinase." Mol Biotechnol **29**(1): 57-74.

Conti, M., Richter, W., Mehats, C., Livera, G., Park, J. and Jin, C. (2003). " Cyclic AMP-specific PDE4 Phosphodiesterases as Critical Components of Cyclic AMP Signaling. ." The Journal of Biological Chemistry **278**(8): 5493-5496.

Cook, S. J. and F. McCormick (1993). "Inhibition by cAMP of Ras-dependent activation of Raf." Science **262**(5136): 1069-1072.

Cooper, D. M. F. (2003). "Regulation and organization of adenylyl cyclases and cAMP." Biochemical Journal **375**: 517-529.

Corbalan-Garcia, S. and J. C. Gomez-Fernandez (2006). "Protein kinase C regulatory domains: the art of decoding many different signals in membranes." Biochim Biophys Acta **1761**(7): 633-654.

Cullere, X., Shaw, S. K., Andersson, L., Hirahashi, J., Luscinskas, F. W. and Mayadas, T. N. (2005). "Regulation of vascular endothelial barrier function by Epac, a cAMP-activated exchange factor for Rap GTPase." Blood **105**(5): 1950-1955.

de Rooij, J., Zwartkruis, F. J., Verheijen, M. H., Cool, R. H., Nijman, S. M., Wittinghofer, A. and Bos, J. L. (1998). "Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP." Nature **396**(6710): 474-477.

de Rooij, J., Rehmann, H., van Triest, M., Cool, R. H., Wittinghofer, A. and Bos, J. L. (2000). "Mechanism of regulation of the Epac family of cAMP-dependent RapGEFs." J Biol Chem **275**(27): 20829-20836.

Dell, E. J., Connor, J., Chen, S., Stebbins, E.G., Skiba, N.P., Mochly-Rosen, D. and Hamm, H.E. (2002). "The βγ Subunit of Heterotrimeric G Proteins Interacts with RACK1 and Two Other WD Repeat Proteins." The Journal of Biological Chemistry **277**(51): 49888-49895.

Deupi, X. and B. Kobilka (2007). "Activation of G protein-coupled receptors." Adv Protein Chem **74**: 137-166.

Dodge-Kafka, K. L. and M. S. Kapiloff (2006). "The mAKAP signaling complex: integration of cAMP, calcium, and MAP kinase signaling pathways." Eur J Cell Biol **85**(7): 593-602.

Dodge-Kafka, K. L., Langeberg, L. and Scott, J. D. (2006). "Compartmentation of cyclic nucleotide signaling in the heart: the role of A-kinase anchoring proteins." Circ Res **98**(8): 993-1001.

Dodge-Kafka, Bauman, A. and Kapiloff, M. S. (2008). "A-kinase anchoring proteins as the basis for cAMP signaling." Handb Exp Pharmacol(186): 3-14.

Dorn, G. W. and T. Force (2005). "Protein kinase cascades in the regulation of cardiac hypertrophy." J Clin Invest **115**(3): 527-537.

Dougherty, M. K., Muller, J., Ritt, D. A., Zhou, M., Zhou, X. Z., Copeland, T. D., Conrads, T. P., Veenstra, T. D., Lu, K. P. and Morrison, D. K. (2005). "Regulation of Raf-1 by direct feedback phosphorylation." Mol Cell **17**(2): 215-224.

Dumaz, N. and R. Marais (2005). "Integrating signals between cAMP and the RAS/RAF/MEK/ERK signalling pathways. Based on the anniversary prize of the Gesellschaft fur Biochemie und Molekularbiologie Lecture delivered on 5 July 2003 at the Special FEBS Meeting in Brussels." FEBS J **272**(14): 3491-3504.

Durgan, J., Michael, N., Totty, N. and Parker, P. J. (2007). "Novel phosphorylation site markers of protein kinase C delta activation." <u>FEBS Lett</u> **581**(18): 3377-3381.

Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Matsumoto, A., Tanimura, S., Ohtsubo, M., Misawa, H., Miyazaki, T., Leonor, N., Taniguchi, T., Fujita, T., Kanakura, Y., Komiya, S., Yoshimura, A. (1997). "A new protein containing an SH2 domain that inhibits JAK kinases." <u>Nature</u> **387**(6636): 921-924.

Faux, M. C., Rollins, E. N., Edwards, A. S.. Langeberg, L. K.. Newton, A. C. and Scott, J. D. (1999). "Mechanism of A-kinase-anchoring protein 79 (AKAP79) and protein kinase C interaction." <u>Biochem J</u> **343 Pt 2**: 443-452.

Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feeser, W. S., Van Dyk, D. E., Pitts, W. J., Earl, R. A., Hobbs, F., Copeland, R. A., Magolda, R. L., Scherle, P. A., Trzaskos, J. M. (1998). "Identification of a novel inhibitor of mitogen-activated protein kinase kinase." <u>J Biol Chem</u> **273**(29): 18623-18632.

Feliciello, A., Gottesman, M. E. and Avvedimento, E. V. (2001). "The biological functions of A-kinase anchor proteins." <u>J Mol Biol</u> **308**(2): 99-114.

Feng, X. and Y. A. Hannun (1998). "An essential role for autophosphorylation in the dissociation of activated protein kinase C from the plasma membrane." <u>J Biol Chem</u> **273**(41): 26870-26874.

Feng, X., Becker, K. P., Stribling, S. D., Peters, K. G. and Hannun, Y. A. (2000). "Regulation of receptor-mediated protein kinase C membrane trafficking by autophosphorylation." <u>J Biol Chem</u> **275**(22): 17024-17034.

Garcia-Paramio, P., Cabrerizo, Y., Bornancin, F. and Parker, P. J. (1998). "The broad specificity of dominant inhibitory protein kinase C mutants infers a common step in phosphorylation." <u>Biochem J</u> **333 (Pt 3)**: 631-636.

Gekel, I. and E. Neher (2008). "Application of an Epac activator enhances neurotransmitter release at excitatory central synapses." <u>J Neurosci</u> **28**(32): 7991-8002.

Gisselbrecht, S. (1999). "The CIS/SOCS proteins: a family of cytokine-inducible regulators of signaling." <u>Eur Cytokine Netw</u> **10**(4): 463-470.

Gong, B., Vitolo, O. V., Trinchese, F., Liu, S., Shelanski, M. and Arancio, O. (2004). "Persistent improvement in synaptic and cognitive functions in an Alzheimer mouse model after rolipram treatment." <u>J Clin Invest</u> **114**(11): 1624-1634.

Goodnight, J. A., Mischak, H., Kolch, W. and Mushinski, J. F (1995). "Immunocytochemical localization of eight protein kinase C isozymes overexpressed in NIH 3T3 fibroblasts. Isoform-specific association with microfilaments, Golgi, endoplasmic reticulum, and nuclear and cell membranes." <u>J Biol Chem</u> **270**(17): 9991-10001.

Graham, F. L., Smiley,J., Russell, W.C. and Nairn, R.. (1977). "Characteristics of a human cell line transformed by DNA from human adenovirus type 5." <u>J Gen Virol</u> **36**(1): 59-74.

Graness, A., Adomeit, A., Ludwig, B., Müller, WD., Kaufmann, R. and Liebmann, C. (1997). "Novel bradykinin signalling events in PC-12 cells: stimulation of the cAMP pathway leads to cAMP-mediated translocation of protein kinase Cepsilon." Biochem J **327 (Pt 1)**: 147-154.

Gschwendt, M. (1999). "Protein kinase C delta." Eur J Biochem **259**(3): 555-564.

Gschwind, A., Fischer, O. M. and Ullrich, A. (2004). "The discovery of receptor tyrosine kinases: targets for cancer therapy." Nat Rev Cancer **4**(5): 361-370.

Harden, T. K. and J. Sondek (2006). "Regulation of phospholipase C isozymes by ras superfamily GTPases." Annu Rev Pharmacol Toxicol **46**: 355-379.

Harding, A., Tian, T., Westbury, E., Frische, E. and Hancock, J. F. (2005). "Subcellular localization determines MAP kinase signal output." Curr Biol **15**(9): 869-873.

Harper, S. M., Wienk, H., Wechselberger, R. W., Bos, J. L., Boelens, R. and Rehmann, H. (2008). "Structural dynamics in the activation of Epac." J Biol Chem **283**(10): 6501-6508.

Hattori, M. and N. Minato (2003). "Rap1 GTPase: functions, regulation, and malignancy." J Biochem **134**(4): 479-484.

Heinrich, P., Behrmann, I., Haan, S., Hermanns, HM., Muller-Newen, G., and Schaper, F. (2003). "Principles of interleukin (IL)-6-type cytokine signalling and its regulation." Biochem. J. **371**: 1-20. .

Higgins, T. J. and J. R. David (1976). "Effect of isoproterernol and aminiphylline on cyclic AMP levels of guinea pig macrophages." Cell Immunol **27**(1): 1-10.

Holz, G. G. (2004). "Epac: A new cAMP-binding protein in support of glucagon-like peptide-1 receptor-mediated signal transduction in the pancreatic beta-cell." Diabetes **53**(1): 5-13.

Holz, G. G., Kang, G., Harbeck, M., Roe, M. W. and Chepurny, O. G. (2006). "Cell physiology of cAMP sensor Epac." J Physiol **577**(Pt 1): 5-15.

Holz, G. G., Chepurny, O. G. and Schwede, F. (2008). "Epac-selective cAMP analogs: new tools with which to evaluate the signal transduction properties of cAMP-regulated guanine nucleotide exchange factors." Cell Signal **20**(1): 10-20.

Hoshino, R., Chatani, Y., Yamori, T., Tsuruo, T., Oka, H., Yoshida, O., Shimada, Y., Ari-i, S., Wada, H., Fujimoto, J. and Kohno, M. (1999). "Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors." Oncogene **18**(3): 813-822.

Houslay, M. D. and W. Kolch (2000). "Cell-type specific integration of cross-talk between extracellular signal-regulated kinase and cAMP signaling." Mol Pharmacol **58**(4): 659-668.

Houslay, M. D. and D. R. Adams (2003). "PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization." Biochem J. **370**(Pt 1): 1-18.

Houslay, M. D. and Baillie, G.S. (2003). "The role of ERK2 docking and phosphorylation of PDE4 cAMP phosphodiesterase isoforms in mediating cross-talk between the cAMP and ERK signalling pathways." Biochemical Society Transactions **31**(6): 1186-1190.

Houslay, M. D., Schafer, P. and Zhang, K.Y.J. (2005). "Phosphodiesterase-4 as a therapeutic target." Drug Discovery Today: Keynote Review **10**(22): 1503-1519.

Hu, T. and J. H. Exton (2004). "Protein kinase Calpha translocates to the perinuclear region to activate phospholipase D1." J Biol Chem **279**(34): 35702-35708.

Huai, Q., Wang, H., Sun, Y., Kim, H. Y., Liu, Y. and Ke, H. (2003). "Three-dimensional structures of PDE4D in complex with roliprams and implication on inhibitor selectivity." Structure **11**(7): 865-873.

Huang, Y., Zheng, Y., Su, Z. and Gu, X. (2009). "Differences in duplication age distributions between human GPCRs and their downstream genes from a network prospective." BMC Genomics **10 Suppl 1**: S14.

Hubbard, S. R. and J. H. Till (2000). "Protein tyrosine kinase structure and function." Annu Rev Biochem **69**: 373-398.

Insel, P. A. and R. S. Ostrom (2003). "Forskolin as a tool for examining adenylyl cyclase expression, regulation, and G protein signaling." Cell Mol Neurobiol **23**(3): 305-314.

Ishibe, S., Joly, D., Liu, Z. X. and Cantley, L. G. (2004). "Paxillin serves as an ERK-regulated scaffold for coordinating FAK and Rac activation in epithelial morphogenesis." Mol Cell **16**(2): 257-267.

Ishihara, K. and T. Hirano (2002). "IL-6 in autoimmune disease and chronic inflammatory proliferative disease." Cytokine Growth Factor Rev **13**(4-5): 357-368.

Itoh, M., Nelson, C. M., Myers, C. A. and Bissell, M. J. (2007). "Rap1 integrates tissue polarity, lumen formation, and tumorigenic potential in human breast epithelial cells." Cancer Res **67**(10): 4759-4766.

Jaaro, H., Rubinfeld, H., Hanoch, T. and Seger, R. (1997). "Nuclear translocation of mitogen-activated protein kinase kinase (MEK1) in response to mitogenic stimulation." Proc Natl Acad Sci U S A **94**(8): 3742-3747.

Jensen, F. C., Girardi, A.J., Gilden, R.V. and Koprowski, H. (1964). "Infection of human and simian tissue cultures with rous sarcoma virus." Proc Natl Acad Sci U S A **52**: 53-59.

Jin, T. G., Satoh, T., Liao, Y., Song, C., Gao, X., Kariya, K., Hu, C. D. and Kataoka, T. (2001). "Role of the CDC25 homology domain of phospholipase Cepsilon in amplification of Rap1-dependent signaling." J Biol Chem **276**(32): 30301-30307.

Jones, S. A., Horiuchi, S., Topley, N., Yamamoto, N. and Fuller, G. M. (2001). "The soluble interleukin 6 receptor: mechanisms of production and implications in disease." Faseb J **15**(1): 43-58.

Jones, S. M., Hiller, F.C., Jacobi, S.E., Foreman, S.K., Pittman, L.M. and Cornett, L.E. (2003). "Enhanced beta2-adrenergic receptor (beta2AR) signaling by adeno-associated viral (AAV)-mediated gene transfer." BMC Pharmacol **3**: 15.

Jones, S. A., Richards, P. J., Scheller, J. and Rose-John, S. (2005). "IL-6 transsignaling: the in vivo consequences." J Interferon Cytokine Res **25**(5): 241-253.

Kang, N. S., Chae, C.H. and Yoo, S.E. (2006). "Study on the hydrolysis mechanism of phosphodiesterase 4 using molecular dynamics simulations." Molecular Simulation **32**(5): 369-374.

Kashima, Y., Miki, T., Shibasaki, T., Ozaki, N., Miyazaki, M., Yano, H. and Seino, S. (2001). "Critical role of cAMP-GEFII--Rim2 complex in incretin-potentiated insulin secretion." J Biol Chem **276**(49): 46046-46053.

Kaupp, U. B. S. and Seifert, R. (2002). "Cyclic nucleotide-gates ion channels." Physiological Reviews **82**(3): 769-824.

Kawasaki, H., Springett, G. M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D. E. and Graybiel, A. M. (1998). "A family of cAMP-binding proteins that directly activate Rap1." Science **282**(5397): 2275-2279.

Kehr, W., Debus, G. and Neumeister, R. (1985). "Effects of rolipram, a novel antidepressant, on monoamine metabolism in rat brain." J Neural Transm **63**(1): 1-12.

Keranen, L. M., Dutil, E. M. and Newton, A. C. (1995). "Protein kinase C is regulated in vivo by three functionally distinct phosphorylations." Curr Biol **5**(12): 1394-1403.

Kharbanda, S., Saleem, A., Hirano, M., Emoto, Y., Sukhatme, V., Blenis, J. and Kufe, D. (1994). "Activation of early growth response 1 gene transcription and pp90rsk during induction of monocytic differentiation." Cell Growth Differ **5**(3): 259-265.

Khokhlatchev, A. V., Canagarajah, B., Wilsbacher, J., Robinson, M., Atkinson, M., Goldsmith, E. and Cobb, M. H. (1998). "Phosphorylation of the MAP kinase ERK2 promotes its homodimerization and nuclear translocation." Cell **93**(4): 605-615.

Kitatani, K., Idkowiak-Baldys, J. and Hannun, Y. A. (2007). "Mechanism of inhibition of sequestration of protein kinase C alpha/betaII by ceramide. Roles of ceramide-activated protein phosphatases and phosphorylation/dephosphorylation of protein kinase C alpha/betaII on threonine 638/641." J Biol Chem **282**(28): 20647-20656.

Knox, A. L. and N. H. Brown (2002). "Rap1 GTPase regulation of adherens junction positioning and cell adhesion." Science **295**(5558): 1285-1288.

Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marme, D. and Rapp, U. R. (1993). "Protein kinase C alpha activates RAF-1 by direct phosphorylation." Nature **364**(6434): 249-252.

Kooistra, M. R., Corada, M., Dejana, E. and Bos, J. L. (2005). "Epac1 regulates integrity of endothelial cell junctions through VE-cadherin." FEBS Lett **579**(22): 4966-4972.

Kroll, M. H., Zavoico, G.B. and Schafer, A.I. (1988). "Control of platelet protein kinase C activation by cyclic AMP." Biochim Biophys Acta **970**(1): 61-67.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature **227** (5259): 680-685.

Le Clainche, C., Didry, D., Carlier, M.F. and Pantaloni, D. (2001). "Activation of Arp2/3 complex by Wiskott-Aldrich Syndrome protein is linked to enhanced binding of ATP to Arp2." J Biol Chem **276**(50): 46689-46692.

Le Jeune, I. R., Shepherd, M., Van Heeke, G., Houslay, M.D. and Hall, I.P. (2002). "Cyclic AMP-dependent Transcriptional Up-regulation of Phosphodiesterase 4D5 in Human Airway Smooth Muscle Cells." The Journal of Biological Chemistry **277**(39): 35980-35989.

Leevers, S. J., Paterson, H. F. and Marshall, C. J. (1994). "Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane." Nature **369**(6479): 411-414.

Liedtke, C. M., Yun, C.H.C., Kyle, N. and Wang, D. (2002). "Protein Kinase Cε-dependent Regulation of Cystic Fibrosis Transmembrane Regulator Involves Binding to a Receptor for Activated C Kinase (RACK1) and RACK1 Binding to a Na+/H+ Exchange Regulatory Factor." The Journal of Biological Chemistry **277**(25): 22925-22933.

Liliental, J. C. and Chang, D.D. (1998). "RACK1, a Receptor for Activated Protein Kinase C, Interacts with Integrin β Subunit." The Journal of Biological Chemistry **273**(4): 2379-2383.

Lim, J., Pahlke, G. and Conti, M. (1999). "Activation of the cAMP-specific phosphodiesterase PDE4D3 by phosphorylation. Identification and function of an inhibitory domain." J Biol Chem **274**(28): 19677-19685.

Lin, Y. F., Lee, H. M., Leu, S. J. and Tsai, Y. H (2007). "The essentiality of PKCalpha and PKCbetaI translocation for CD14+monocyte differentiation towards macrophages and dendritic cells, respectively." J Cell Biochem **102**(2): 429-441.

Lopez De Jesus, M., Stope, M. B., Oude Weernink, P. A., Mahlke, Y., Borgermann, C., Ananaba, V. N., Rimmbach, C., Rosskopf, D., Michel, M. C, Jakobs, K. H. and Schmidt, M. (2006). "Cyclic AMP-dependent and Epac-mediated activation of R-Ras by G protein-coupled receptors leads to phospholipase D stimulation." J Biol Chem **281**(31): 21837-21847.

Lugnier, C. (2006). "Cyclic nucleotide phosphodiesterase (PDE) superfamily: A new target for the development of specific therapeutic agents." Pharmacology & Therapeutics **109**: 366 – 398.

Luttrell, L. M. (2008). "Reviews in molecular biology and biotechnology: transmembrane signaling by G protein-coupled receptors." Mol Biotechnol **39**(3): 239-264.

Lynch, M. J., Baillie, G. S. and Houslay, M. D. (2007). "cAMP-specific phosphodiesterase-4D5 (PDE4D5) provides a paradigm for understanding the unique non-redundant roles that PDE4 isoforms play in shaping compartmentalized cAMP cell signalling." Biochem Soc Trans **35**(Pt 5): 938-941.

Lynch, M. J., Baillie, G.S., Mohamed, A., Li, X., Maisonneuve, C., Klussmann, E., van Heeke, G. and Houslay, M.D. (2005). "RNA Silencing Identifies PDE4D5 as the Functionally Relevant cAMP Phosphodiesterase Interacting with Barrestin to Control the Protein Kinase A/AKAP79-mediated Switching of the B2-Adrenergic Receptor to Activation of ERK in HEK293B2 Cells." The Journal of Biological Chemistry **280**(39): 33178-33189.

Mackay, H. J. and C. J. Twelves (2007). "Targeting the protein kinase C family: are we there yet?" Nat Rev Cancer **7**(7): 554-562.

MacKenzie, S. J., Baillie, G.S., McPhee, I., MacKenzie, C., Seamons, R., McSorley, T., Millen, J., Beard, M.B., van Heeke, G. and Houslay, M.D. (2002). "Long PDE4 cAMP specific phosphodiesterases are activated by protein kinase A-mediated phosphorylation of a single serine residue in Upstream Conserved Region 1." British Journal of Pharmacology **136**: 421-433.

MacKenzie, S. J. and Houslay, M.D. (2000). "Action of rolipram on specific PDE4 cAMP phosphodiesterase isoforms and on the phosphorylation of cAMP-response-binding protein (CREB) and p38 mitogen-activated protein (MAP) kinase in U937 monocytic cells." Biochemical Journal **347**: 571-578.

Mamidipudi, V., Chang, B.Y., Harte, R.A., Lee, K.C. and Cartwright, C.A. (2004). "RACK1 inhibits the serum- and anchorage-independent growth of v-Src transformed cells." FEBS letters **567**: 321-326.

Marshall, C. J. (1995). "Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation." Cell **80**(2): 179-185.

Matsubayashi, Y., Fukuda, M. and Nishida, E. (2001). "Evidence for existence of a nuclear pore complex-mediated, cytosol-independent pathway of nuclear translocation of ERK MAP kinase in permeabilized cells." J Biol Chem **276**(45): 41755-41760.

Matthies, H. J., Palfrey, H. C., Hirning, L. D. and Miller, R. J. (1987). "Down regulation of protein kinase C in neuronal cells: effects on neurotransmitter release." J Neurosci **7**(4): 1198-1206.

McCahill, A., Warwicker, J., Bolger, G.B., Houslay, M.D. and Yarwood, S.J. (2002). "The RACK1 Scaffold Protein: A Dynamic Cog in Cell Response Mechanisms." Molecular Pharmacology **62**(6): 1261-1273.

McConnachie, G., Langeberg, L. K. and Scott, J. D. (2006). "AKAP signaling complexes: getting to the heart of the matter." Trends Mol Med **12**(7): 317-323.

McKay, M. M. and D. K. Morrison (2007). "Integrating signals from RTKs to ERK/MAPK." Oncogene **26**(22): 3113-3121.

McPhee, I., Scotland, G., Huston, E., Beard, M. B., Ross, A. H., Houslay, E. S. and Houslay, M. D. (1999). "Association with the SRC family tyrosyl kinase LYN triggers a conformational change in the catalytic region of human cAMP-specific phosphodiesterase HSPDE4A4B. Consequences for rolipram inhibition." J Biol Chem **274**(17): 11796-11810.

McPhee, I., Gibson, L. C., Kewney, J., Darroch, C., Stevens, P. A., Spinks, D.,Cooreman, A. and MacKenzie, S. J. (2005). "Cyclic nucleotide signalling: a molecular approach to drug discovery for Alzheimer's disease." Biochem Soc Trans **33**(Pt 6): 1330-1332.

Mellor, H., and Parker, PJ. (1998). "The extended protein kinase C superfamily." Biochem J. **332**(2): 281-292.

Mikoshiba, K. (2007). "IP3 receptor/Ca2+ channel: from discovery to new signaling concepts." J Neurochem **102**(5): 1426-1446.

Morel, E., Marcantoni, A., Gastineau, M., Birkedal, R., Rochais, F., Garnier, A., Lompre, A. M., Vandecasteele, G. and Lezoualc'h, F. (2005). "cAMP-binding protein Epac induces cardiomyocyte hypertrophy." Circ Res **97**(12): 1296-1304.

Muto, Y., Nagao, T. and Urushidani, T. (1997). "The putative phospholipase C inhibitor U73122 and its negative control, U73343, elicit unexpected effects on the rabbit parietal cell." J Pharmacol Exp Ther **282**(3): 1379-1388.

Myklebust, J. H., Smeland, E. B., Josefsen, D. and Sioud, M. (2000). "Protein kinase C-alpha isoform is involved in erythropoietin-induced erythroid differentiation of CD34(+) progenitor cells from human bone marrow." Blood **95**(2): 510-518.

Naka, T., Narazaki, M., Hirata, M., Matsumoto, T., Minamoto, S., Aono, A., Nishimoto, N., Kajita, T., Taga, T.. Yoshizaki, K.. Akira, S. and Kishimoto, T. (1997). "Structure and function of a new STAT-induced STAT inhibitor." Nature **387**(6636): 924-929.

Nakashima, S. (2002). "Protein kinase C alpha (PKC alpha): regulation and biological function." J Biochem **132**(5): 669-675.

Nauert, J. B., Klauck, T. M., Langeberg, L. K. and Scott, J. D. (1997). "Gravin, an autoantigen recognized by serum from myasthenia gravis patients, is a kinase scaffold protein." Curr Biol **7**(1): 52-62.

Newton, A. C. (2003). "Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm." Biochem J **370**(Pt 2): 361-371.

Newton, A. C. (2010). "Protein kinase C: poised to signal." Am J Physiol Endocrinol Metab **298**(3): E395-402.

Nishizuka, Y. (1986). "Perspectives on the role of protein kinase C in stimulus-response coupling." J Natl Cancer Inst **76**(3): 363-370.

O'Neill, J. S., Maywood, E. S., Chesham, J., Takahashi, J. S. and Hastings, M. H. (2008). "cAMP-dependent signaling as a core component of the mammalian circadian pacemaker." Science **320**(5878): 949-953.

Oestreich, E., Malik, S., Goonasekera, SA., Blaxall, BC., Kelley, GG., Dirksen, RT. and Smrcka, AV (2009). "Epac and phospholipase Cepsilon regulate Ca2+ release in the heart by activation of protein kinase Cepsilon and calcium-calmodulin kinase I." J Biol Chem **284**(3): 1514-1522.

Ozaki, N., Shibasaki, T., Kashima, Y., Miki, T., Takahashi, K., Ueno, H., Sunaga, Y.,Yano, H., Matsuura, Y., Iwanaga, T., Takai, Y. and Seino, S. (2000). "cAMP-GEFII is a direct target of cAMP in regulated exocytosis." Nat Cell Biol **2**(11): 805-811.

Pages, G., Lenormand, P., L'Allemain, G., Chambard, J. C., Meloche, S. and Pouyssegur, J. (1993). "Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation." Proc Natl Acad Sci U S A **90**(18): 8319-8323.

Parekh, D. B., Ziegler, W. and Parker, P. J. (2000). "Multiple pathways control protein kinase C phosphorylation." Embo J **19**(4): 496-503.

Park, J., Richard, F., Chun, S., Park, J., Law, E., Horner, K., Jin, S.C. and Conti, M (2003). "Phosphodiesterase Regulation is Critical for the Differentiation and Pattern of Gene Expression in Granulosa Cells of the Ovarian Follicle." Molecular Endocrinology **17**(6): 1117-1130.

Parker, P. J. and J. Murray-Rust (2004). "PKC at a glance." J Cell Sci **117**(Pt 2): 131-132.

Pascale, A., Fortino, I., Govoni, S., Trabucchi, M., Wetsel, W. C. and Battaini, F. (1996). "Functional impairment in protein kinase C by RACK1 (receptor for activated C kinase 1) deficiency in aged rat brain cortex." J Neurochem **67**(6): 2471-2477.

Pereira, L., Metrich, M., Fernandez-Velasco, M., Lucas, A., Leroy, J., Perrier, R., Morel, E., Fischmeister, R., Richard, S., Benitah, J. P., Lezoualc'h, F. and Gomez, A. M. (2007). "The cAMP binding protein Epac modulates Ca2+ sparks by a Ca2+/calmodulin kinase signalling pathway in rat cardiac myocytes." J Physiol **583**(Pt 2): 685-694.

Perkins, G. A., Wang, L., Huang, L. J., Humphries, K., Yao, V. J., Martone, M., Deerinck, T. J., Barraclough, D. M., Violin, J. D.. Smith, D., Newton, A., Scott, J. D., Taylor, S. S. and Ellisman, M. H. (2001). "PKA, PKC, and AKAP localization in and around the neuromuscular junction." BMC Neurosci **2**: 17.

Perry, S. J., Baillie, G. S., Kohout, T. A., McPhee, I., Magiera, M. M., Ang, K. L., Miller, W. E., McLean, A. J., Conti, M., Houslay, M. D. and Lefkowitz, R. J. (2002). "Targeting of cyclic AMP degradation to beta 2-adrenergic receptors by beta-arrestins." Science **298**(5594): 834-836.

Pierre, S., Eschenhagen, T., Geisslinger, G. and Scholich, K. (2009). "Capturing adenylyl cyclases as potential drug targets." Nat Rev Drug Discov **8**(4): 321-335.

Piontek, J. and R. Brandt (2003). "Differential and regulated binding of cAMP-dependent protein kinase and protein kinase C isoenzymes to gravin in human model neurons: Evidence that gravin provides a dynamic platform for the localization for kinases during neuronal development." J Biol Chem **278**(40): 38970-38979.

Qiao, J., Mei, F. C., Popov, V. L., Vergara, L. A. and Cheng, X. (2002). "Cell cycle-dependent subcellular localization of exchange factor directly activated by cAMP." J Biol Chem **277**(29): 26581-26586.

Rawlings, J. S., Rosler, K. M. and Harrison, D. A (2004). "The JAK/STAT signaling pathway." J Cell Sci **117**(Pt 8): 1281-1283.

Rehmann, H., Wittinghofer, A. and Bos, J. L. (2007). "Capturing cyclic nucleotides in action: snapshots from crystallographic studies." Nat Rev Mol Cell Biol **8**(1): 63-73.

Richter, W., Jin, S-L. C. and Conti, M. (2005). "Splice variants of the cyclic nucleotide phosphodiesterase PDE4D are differentially expressed and regulated in rat tissue." Biochemical Journal **388**: 803-811.

Robles, M. S., Boyault, C., Knutti, D., Padmanabhan, K. and Weitz, C. J. (2010). "Identification of RACK1 and protein kinase Calpha as integral components of the mammalian circadian clock." Science **327**(5964): 463-466.

Ronn, S. G., Billestrup, N. and Mandrup-Poulsen, T. (2007). "Diabetes and suppressors of cytokine signaling proteins." Diabetes **56**(2): 541-548.

Roscioni, S. S., Elzinga, C. R. and Schmidt, M. (2008). "Epac: effectors and biological functions." Naunyn Schmiedebergs Arch Pharmacol **377**(4-6): 345-357.

Rosenbaum, D. M., Rasmussen, S. G. and Kobilka, B. K. (2009). "The structure and function of G-protein-coupled receptors." Nature **459**(7245): 356-363.

Rosson, D., O'Brien, T. G., Kampherstein, J. A., Szallasi, Z., Bogi, K., Blumberg, P. M. and Mullin, J. M. (1997). "Protein kinase C-alpha activity modulates transepithelial permeability and cell junctions in the LLC-PK1 epithelial cell line." J Biol Chem **272**(23): 14950-14953.

Rubin, R. P. and M. A. Adolf (1994). "Cyclic AMP regulation of calcium mobilization and amylase release from isolated permeabilized rat parotid cells." J Pharmacol Exp Ther **268**(2): 600-606.

Rubinfeld, H., Hanoch, T., Seger, R. (1999). "Identification of a cytoplasmic-retention sequence in ERK2." J Biol Chem **274**(43): 30349-30352.

Rubinfeld, H. and R. Seger (2005). "The ERK cascade: a prototype of MAPK signaling." Mol Biotechnol **31**(2): 151-174.

Ruvolo, P. P., Deng, X., Carr, B. K. and May, W. S. (1998). "A functional role for mitochondrial protein kinase Calpha in Bcl2 phosphorylation and suppression of apoptosis." J Biol Chem **273**(39): 25436-25442.

Sands, W., Woolson, HD., Milne, GR., Rutherford, C., and Palmer, TM. (2006). "Exchange Protein Activated by Cyclic AMP (Epac)-Mediated Induction of Suppressor of Cytokine Signaling 3 (SOCS-3) in Vascular Endothelial Cells." Mol Cell Biol **26**(17): 6333-6346.

Schechtman, D. and D. Mochly-Rosen (2001). "Adaptor proteins in protein kinase C-mediated signal transduction." Oncogene **20**(44): 6339-6347.

Schindler, C., Levy, D. E. and Decker, T. (2007). "JAK-STAT signaling: from interferons to cytokines." J Biol Chem **282**(28): 20059-20063.

Schlessinger, J. (1997). "Phospholipase Cγ activation and phosphoinositide hydrolysis are essential for embryonal?development." Proc Natl Acad Sci U S A **94**(7): 2798-2799.

Schmidt, M., Evellin, S., Weernink, PA., von Dorp, F., Rehmann, H., Lomasney, JW. and Jakobs, KH. (2001). "A new phospholipase-C-calcium signalling pathway mediated by cyclic AMP and a Rap GTPase." Nat Cell Biol **3**(11): 1020-1024.

Schmidt, M., Sand, C., Jakobs, K. H., Michel, M. C. and Weernink, P. A. (2007). "Epac and the cardiovascular system." Curr Opin Pharmacol **7**(2): 193-200.

Schmitz-Peiffer, C., Browne, C. L. and Biden, T. J. (1996). "Characterization of two forms of protein kinase C alpha, with different substrate specificities, from skeletal muscle." Biochem J **320 (Pt 1)**: 207-214.

Schulte, G. and F. O. Levy (2007). "Novel aspects of G-protein-coupled receptor signalling--different ways to achieve specificity." Acta Physiol (Oxf) **190**(1): 33-38.

Schutz, W., Tuisl, E. and Kraupp, O. (1982). "Adenosine receptor agonists: binding and adenylate cyclase stimulation in rat liver plasma membranes." Naunyn Schmiedebergs Arch Pharmacol **319**(1): 34-39.

Scott, J. D. (2003). "A-kinase-anchoring proteins and cytoskeletal signalling events." Biochem Soc Trans **31**(Pt 1): 87-89.

Sebolt-Leopold, J. S., Dudley, D. T., Herrera, R., Van Becelaere, K., Wiland, A., Gowan, R. C., Tecle, H., Barrett, S. D., Bridges, A., Przybranowski, S.. Leopold, W. R. and Saltiel, A. R. (1999). "Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo." Nat Med **5**(7): 810-816.

Seger, R., Seger, D., Reszka, A. A., Munar, E. S., Eldar-Finkelman, H., Dobrowolska, G., Jensen, A. M., Campbell, J. S., Fischer, E. H. and Krebs, E. G. (1994). "Overexpression of mitogen-activated protein kinase kinase (MAPKK) and its mutants in NIH 3T3 cells. Evidence that MAPKK involvement in cellular proliferation is regulated by phosphorylation of serine residues in its kinase subdomains VII and VIII." J Biol Chem **269**(41): 25699-25709.

Seger, R. and E. G. Krebs (1995). "The MAPK signaling cascade." Faseb J **9**(9): 726-735.

Shaul, Y. D. and R. Seger (2007). "The MEK/ERK cascade: from signaling specificity to diverse functions." Biochim Biophys Acta **1773**(8): 1213-1226.

Shuai, K. and B. Liu (2003). "Regulation of JAK-STAT signalling in the immune system." Nat Rev Immunol **3**(11): 900-911.

Smith, F. D., Langeberg, L. K. and Scott, J. D. (2006). "The where's and when's of kinase anchoring." Trends Biochem Sci **31**(6): 316-323.

Sontag, E., Fedorov, S., Kamibayashi, C., Robbins, D., Cobb, M. and Mumby, M. (1993). "The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the map kinase pathway and induces cell proliferation." Cell **75**(5): 887-897.

Souchelnytskyi, S., Ronnstrand, L., Heldin, C. H. and ten Dijke, P. (2001). "Phosphorylation of Smad signaling proteins by receptor serine/threonine kinases." Methods Mol Biol **124**: 107-120.

Starr, R., Willson, T. A., Viney, E. M., Murray, L. J., Rayner, J. R., Jenkins, B. J., Gonda, T. J., Alexander, W. S., Metcalf, D., Nicola, N. A. and Hilton, D. J. (1997). "A family of cytokine-inducible inhibitors of signalling." Nature **387**(6636): 917-921.

Starr, R. and D. J. Hilton (1998). "SOCS: suppressors of cytokine signalling." Int J Biochem Cell Biol **30**(10): 1081-1085.

Steele, M. R., McCahill, A., Thompson, D.S., MacKenzie, C., Isaacs, N.W., Houslay, M.D. and Bolger, G.B. (2001). "Identification of a surface on the β-propeller protein RACK1 that interacts with the cAMP-specific phosphodiesterase PDE4D5." Cellular Signalling **13**: 507-513.

Steinberg, S. F. (2004). "Distinctive activation mechanisms and functions for protein kinase Cdelta." Biochem J **384**(Pt 3): 449-459.

Stork, P. J. and J. M. Schmitt (2002). "Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation." Trends Cell Biol **12**(6): 258-266.

Tabakoff, B., Nelson, E., Yoshimura, M., Hellevuo, K. and Hoffman, P.L. (2001). "Phosphorylation Cascades Control the Actions of Ethanol on Cell cAMP Signalling." Journal of Biomedical Science **8**: 44-51.

Takahashi, H., Honma, M., Miyauchi, Y., Nakamura, S., Ishida-Yamamoto, A. and Iizuka, H. (2004). "Cyclic AMP differentially regulates cell proliferation of normal human keratinocytes through ERK activation depending on the expression pattern of B-Raf." Arch Dermatol Res **296**(2): 74-82.

Takenawa, J., Kaneko, Y., Fukumoto, M., Fukatsu, A., Hirano, T., Fukuyama, H., Nakayama, H., Fujita, J. and Yoshida, O. (1991). "Enhanced expression of interleukin-6 in primary human renal cell carcinomas." J Natl Cancer Inst **83**(22): 1668-1672.

Tanoue, T., Adachi, M., Moriguchi, T. and Nishida, E. (2000). "A conserved docking motif in MAP kinases common to substrates, activators and regulators." <u>Nat Cell Biol</u> **2**(2): 110-116.

Teis, D., Wunderlich, W. and Huber, L. A. (2002). "Localization of the MP1-MAPK scaffold complex to endosomes is mediated by p14 and required for signal transduction." <u>Dev Cell</u> **3**(6): 803-814.

Terrin, A., Di Benedetto, G., Pertegato, V., Cheung, Y., Baillie, G., Lynch, M.J., Elvassore, N., Prinz, A., Herberg, F.W., Houslay, M.D. and Zaccolo, M. (2006). "PGE1 stimulation of HEK293 cells generates multiple contiguous domains with different [cAMP]: role of compartmentalized phosphodiesterases." <u>Journal of Cell Biology</u> **175**(3): 441-451.

Torii, S., Kusakabe, M., Yamamoto, T., Maekawa, M. and Nishida, E. (2004). "Sef is a spatial regulator for Ras/MAP kinase signaling." <u>Dev Cell</u> **7**(1): 33-44.

Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E. and Loriolle, F. (1991). "The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C." <u>J Biol Chem</u> **266**(24): 15771-15781.

Ulucan, C., Wang, X., Baljinnyam, E., Bai, Y., Okumura, S., Sato, M., Minamisawa, S., Hirotani, S. and Ishikawa, Y. (2007). "Developmental changes in gene expression of Epac and its upregulation in myocardial hypertrophy." <u>Am J Physiol Heart Circ Physiol</u> **293**(3): H1662-1672.

van Dam, T. J., Rehmann, H., Bos, J. L. and Snel, B. (2009). "Phylogeny of the CDC25 homology domain reveals rapid differentiation of Ras pathways between early animals and fungi." <u>Cell Signal</u> **21**(11): 1579-1585.

Verde, I., Pahlke, G., Salanova, M., Zhang, G., Wang, S., Coletti, D., Onuffer, J., Jin, S. L. and Conti, M. (2001). "Myomegalin is a novel protein of the golgi/centrosome that interacts with a cyclic nucleotide phosphodiesterase." <u>J Biol Chem</u> **276**(14): 11189-11198.

Volmat, V., M. Camps, M., Arkinstall, S., Pouyssegur, J. and Lenormand, P. (2001). "The nucleus, a site for signal termination by sequestration and inactivation of p42/p44 MAP kinases." <u>J Cell Sci</u> **114**(Pt 19): 3433-3443.

Wachtel, H. (1983). "Potential antidepressant activity of rolipram and other selective cyclic adenosine 3',5'-monophosphate phosphodiesterase inhibitors." <u>Neuropharmacology</u> **22**(3): 267-272.

Wang, Z., Dillon, T. J., Pokala, V., Mishra, S., Labudda, K., Hunter, B. and Stork, P. J. (2006). "Rap1-mediated activation of extracellular signal-regulated kinases by cyclic AMP is dependent on the mode of Rap1 activation." <u>Mol Cell Biol</u> **26**(6): 2130-2145.

Watson, J. (1975). "The influence of intracellular levels of cyclic nucleotides on cell proliferation and the induction of antibody synthesis." <u>J Exp Med</u> **141**(1): 97-111.

Whitehead, K. A., Langer, R. and Anderson, D.G. (2009). "Knocking down barriers: advances in siRNA delivery." Nat Rev Drug Discov **8**(2): 129-138.

Wilkinson, S. E., Parker, P. J. and Nixon, J. S. (1993). "Isoenzyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C." Biochem J **294 (Pt 2)**: 335-337.

Wong, W. and J. D. Scott (2004). "AKAP signalling complexes: focal points in space and time." Nat Rev Mol Cell Biol **5**(12): 959-970.

Wooten, M. W., Seibenhener, M.L., Matthews, L.H., Zhou, G. and Coleman, E.S. (1996). "Modulation of zeta-protein kinase C by cyclic AMP in PC12 cells occurs through phosphorylation by protein kinase A." J Neurochem **67**(3): 1023-1031.

Wu, J., Wu, J., Dent, P., Jelinek, T., Wolfman, A., Weber, M.J. and Sturgill, T.W. (1993). "Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3',5'-monophosphate." Science **262**(5136): 1065-1069.

Yamamoto, K. K., Gonzalez, G.A., Biggs, W.H. and Montminy M.R. (1988). "Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB." Nature **334**(6182): 494-498.

Yarwood, S. J., Steele, M.R., Scotland, G., Houslay, M.D. and Bolger, G.B. (1999). "The RACK1 Signaling Scaffold Protein Selectively Interacts with the cAMP-specific Phosphodiesterase PDE4D5 Isoform." The Journal of Biological Chemistry **274**(21): 14909-14917.

Yarwood, S. J., Borland, G., Sands, W. A. and Palmer, T. M. (2008). "Identification of CCAAT/enhancer-binding proteins as exchange protein activated by cAMP-activated transcription factors that mediate the induction of the SOCS-3 gene." J Biol Chem **283**(11): 6843-6853.

Zhang, J. and H. F. Lodish (2004). "Constitutive activation of the MEK/ERK pathway mediates all effects of oncogenic H-ras expression in primary erythroid progenitors." Blood **104**(6): 1679-1687.