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Biochemical investigation of Phosphodiesterase type IV post-translational modification, cellular localisation and interaction with associated binding proteins

A thesis presented by

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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.

Suryakiran Vadrevu

Abstract

cAMP is a secondary messenger that is involved in a variety of signalling pathways through its effectors including EPAC, PKA and ion channels. cAMP signalling regulates processes such as memory, muscle contraction and inflammatory responses. PDE enzymes offer a mechanism to negatively regulate elevated cAMP levels elicted by activators of adenylyl cyclase. Studies have shown that cAMP signalling is compartmentalised through binding of PDEs to A-kinase anchoring proteins (AKAPs) that scaffold PKA regulatory subunits.

In this study post-translational-modification of PDE4 isoforms is investigated. SUMOylation is a relatively newly identified post-translational modification that is known to regulate the structure and function of its substrates. PDE4 isoforms of the PDE4A and 4D subfamilies are SUMOylated by an E3 ligase, PIASy. SUMOylation alters the rolipram sensitivity and potentiates the PKA mediated activation of the isoforms whilst it confers protection from ERK-mediated inhibition of PDE4 activity. SUMOylation alters the association of PDE4 isoforms with binding partners like β-Arrestin, AKAP18 δ and UBC9.

Rolipram is an archetypal PDE4 specific inhibitor. In this study it is shown that in cells expressing a GFP tagged form of PDE4A4 undergoes redistribution into accretion foci upon chronic treatment with rolipram. Data suggests that foci formation requires protein turnover and is regulated by signalling pathways such as PI3 kinase pathway, p38 MAP kinase pathway and PKC pathways. Further, the Immunomodulatory drug Thalidomide® also inhibits foci formation.

PDE4 isoforms have isoforms specific N-terminal regions, which play a crucial role in sub-cellular localisation and protein-protein interactions. It is shown here that PDE4D5 interacts with a novel RhoGAP called ARHGAP21 which has been previously reported to bind β-arrestins. This interaction is independent of GAP activity of ARHGAP as well as PDE4 activity. Previous

reports have indicated a role of β-Arrestin, PDE4 and ARHGAP21 in regulation of actin cytoskeleton dynamics. Hence complex β-Arrestin-PDE4-ARHGAP21 may play a crucial role in regulating actin dynamics.

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Abbreviations

7TM	Seven transmembrane
AC	Adenylyl cyclase
AKAP	A-kinase anchoring protein
AKIP	A-Kinase interacting protein
AMP	Adenosine monophosphate
ARB	B-arrestin
AR	Adrenergic receptor
Arf	ADP ribosylation factor
ATP	Adenosine Triphosphate
At1R	Angiotensin 1 receptor
Bp	base pair
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
Ca+2/CAM	Calcium/Calmodulin
CaMK	Calmodulin Kinase
Cd 23/28	Cluster of differentiation
CDK	Cell division Kinase
cDNA	Complementary DNA
cGMP	Cyclic Guanosine monophosphate
CHO	Chinese Hamster Ovary
CNG	Cyclic Nucleotide gated
COPD	Chronic obstructive pulmonary disease
CRE	cAMP response element
CREB	cAMP response element binder
dNTP	deoxy nucleotide tri phosphates
DiSC1	Disrupted in Schizoprenia 1
DEAE	Diethyl aminoethyl
DMEM	Dulbecco’s modified eagle’s medium
DMSO	Di methyl Sulfoxide
DNA	Deoxyribo nucleic acid
DTT	Dithiothreitol

ECL	Enahnced chemiluminiscence
E.coli	Escherichia coli
EDTA	Diamino ethane tetra acetic acid
EGTA	Ethyelene Glycol tetra acetic acid
EPAC	Exchange protein for activated cAMP
ERK	Extracellular regulatory kinase
EHNA	Erythro-9-(2-hydroxy-3-nonyl) adenine
FBS	foetal bovine serum
Frsk	Forskolin
FSH	Follicle stimulating hormone
GABA	Gama-Amino-Butyric-Acid
GAF	GTPase activating factor
GAP	GTPase activating protein
GDI	Guanine nucleotide dissociation inhibitors
GDP	Guanosine di phosphate
GEF	GTP exchange factor
GPCR	G-protein coupled receptor
GRK	G-protein receptor kinase
GST	Glutathione-s-transferase
GTP	Guanosine triphosphate
kD	Kilo Dalton
HA	Haemagglutinin
HARBS	High affinity rolipram binding site
HCP1	High affinity cAMP-specific PDE
IBMX	Iso-buty-lmethyl-Xanthine
LARBS	Low affinity rolipram binding site
LB	Luria-Bertini
LH	Leutinising Hormone
MAP	Mitogen Activate protein
MBP	Maltose binding protein
mRNA	messenger ribonucleic acid
MWt	Molecular Weight
NO	Nitric Oxide

PAGE	Polyacrylamide gel electrophoresis
PI3	Phosphotidyl inositol-3
PDE	Phosphodiesterase
PKA	Protein Kinase-A
PKB/AkT	Protein Kinase B
PKC	Protein Kinase C
PIAS	Protein Inhibitor of activated STAT
PM	Plasma membrane
PrBP	Prenyl binding protein
PTH	Parathyroid hormone
PVR	Perinatal vascular resistance
REM	Ras exchange motif
SDS	Sodium-dodecyl-sulfate
STAT	Signal Transducer and activator of Transcription
TBE	Tris Buffered EDTA
TBST	Tris Bufferes Saline-Tween
TCR	T-Cell receptor
TE	Tris-EDTA
TNF	Tumor necrosis factor
UCR	Upstream conserved region
VSV	Vesicular Stomatitis Virus
RACK	receptor of activated C-kinase
SAE	SUMO activating enzyme
SUMO	Small Ubiquitin like modifier

Chapter 1 General introduction

1.1 Cell Signalling

In multi-cellular organisms various organ systems rely on complex cell-cell communication to attain co-ordination. Biological mediators such as peptide hormones, cytokines and growth factors, also known as first messengers, carry out this communication. First messengers cannot penetrate the cells so they need an effective messenger to communicate their signal from the plasma membrane through to the cell interior. The signals can be transduced to the interior of the cell by plasma membrane receptors and ion channels which elicit a specific and complex signalling response leading to the production of second messengers, which generate the desired intracellular response (Houslay and Milligan, 1997).

1.2 G-protein coupled receptors

G-protein coupled receptors (GPCRs) are the largest family of plasma membrane receptors and comprise nearly 1000 receptor variants which relay the signal from plasma membrane to the interior of the cell. Binding of ligands such as peptide hormones initiates conformational changes in the receptor which leads to activation of the one or more members of the guanine-nucleotide-binding signal transducing proteins (G-proteins) which consequently generate an intracellular signal affecting a complex network of pathways within the cell (Luttrell, 2006 and Hollmann et al; 2005).

GPCRs possess hepta-helical transmembrane structures (7 TM). The transmembrane (TM) domains are connected to each other by alternating extracellular and intracellular loops. GPCRs have an extra-cellular N-terminal tail and an intra-cellular C-terminal tail. The N-terminal region can vary in length from 4-50 amino acids and most GPCRs exhibit at least one consensus sequence (Asn-X-Ser/Thr) for N-linked glycosylation within this region. The C-terminal region varies considerably in length (12-359 amino

acids) with the exception of the mammalian GnRH receptor, which completely lacks an intracellular C-terminal domain (Sealfon et al., 1997). The C-tail is usually rich in serine and threonine residues that are potential sites for phosphorylation by GRKs and second messenger kinases for receptor desensitisation (Freedman and Lefkowitz, 1996). The C-terminal tail may also possess consensus sequences for palmitoylation and ubiquitination, which regulate the signalling of GPCRs (Milligan et al; 2004, Hollmann et al, 2005, Yin et al; 2004). Each TM domain ranges from 20-27 amino acids in length and the orientation of TM domains in the plasma membrane is highly conserved. Various amino acids in TM domains are highly conserved and they specify the ligand specificity and receptor activation of the GPCR. The TM III region contains a crucial amino acid residue immediately after a conserved cysteine residue, which determines the nature of the ligand binding to the GPCR. If the amino acid is acidic then the ligand is more likely to be a biogenic amine. However, if the amino acid is basic then a peptide ligand can be expected (Hollmann et al; 2005 and Yin et al; 2004). The binding of biogenic amines to their receptors is characterised by a complex of interactions involving key residues in TMs III, V and VI (Strader et al., 1987). In these receptors, the amine of the ligand interacts with the carboxyl group of an aspartate residue in TM III, whereas the catechol ring interacts with residues in TMs V and VI. Interactions of the ligand with TMIII are important for binding, while interactions with TMs V and VI are more important for receptor activation (Strader et al., 1997).

Various mechanisms have evolved to terminate the signalling cascades through GPCRs. Agonist activation of GPCRs initiates a series of reactions which result in the “turn off” of the GPCR signal, a process known as desensitisation, thus preventing continuous receptor-mediated signalling through chronic ligand stimulation. Receptor level regulation, also known as homologous desensitisation, is mediated by phosphorylation of the receptor within its intracellular domains, via G-protein coupled receptor kinases (GRK) and second messenger kinases such as cAMP-activated Protein Kinase A (PKA) (Vaughn et al; 2006). GRK phosphorylation marks the receptor for β-arrestin-mediated internalisation, which can lead to long-term desensitisation

if the receptor is degraded and thereby down-regulated. β-arrestin is recruited to the preferentially GRK-phosphorylated receptors and acts as a physical barrier thereby preventing the interaction between the GPCR and coupling G-protein. The N-terminus of β-arrestin binds to phosphate groups of the phosphorylated receptors (Vaughn et al, 2006) and aids in internalisation by associating with endocytotic machinery including proteins like clathrin with the help of an α-helical motif present on arrestin (Wolfe and Trejo, 2007). Endocytosed receptors can either be returned to the plasma membrane in a resensitised state or targeted for degradation. The mechanisms of GPCR resensitisation are thought to involve the internalisation of agonist-activated receptors into endosomal compartments which contain a GPCR-specific phosphatase. Endosomal acidification promotes the association of the receptor with the GPCR phosphatase and dephosphorylation of the receptor. Dephosphorylated GPCRs are subsequently recycled back to the cell surface where they can be again activated by agonist (Simaan et al; 2005). Alternatively, activated receptors can be targeted for degradation via a process known as down regulation, which will lead to sorting of receptors for degradation by proteasomal/lysosomal pathway. Receptors are 'marked' for proteasomal degradation following ubiquitination, which is mediated through the action of an E3-ligase that interacts with β-arrestin 2 thereby ubiquitinating the receptor (Shenoy and Lefkowitz, 2005, Shenoy and Lefkotwitz, 2005).

Heterotrimeric G proteins are guanosine nucleotide binding proteins and couple to agonist-occupied GPCRs. The complex comprises α, β and γ subunits which are 40-46kDa, 35kDa and 8kDa, respectively. Gα subunits contain two domains, a domain involved in binding and hydrolysis of GTP that is structurally homologous to the GTPases of monomeric G proteins and elongation factors, and a unique helical domain, which buries the bound GTP in the protein core. Gα subunits undergo lipid modification, which allows for the attachment to the intracellular membrane and also free movement along the membrane. Four distinct classes of Gα proteins have been identified namely, G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$ (Hollmann et al; 2005 and Javitch et al; 1997). Each class of Gα subunit shows varying affinity for a wide variety of

GPCRs and G-proteins have been shown to lose or gain affinity due to various post-translational modifications such as phosphorylation and palmitoylation. When inactive, the guanosine diphosphate (GDP)-bound α-subunit is tightly bound to $\beta\gamma$ subunits and is not associated with the GPCR. Upon ligand binding to the GPCR, the transmembrane loops of receptors undergo conformational changes, thereby projecting out the sites that show high affinity for G-proteins. A GDP/GTP exchange factor (GEF) converts GDP on the α subunit to a GTP (guanosine triphosphate) there by activating the G-protein complex. The activated heterotimeric complex then dissociates into α-GTP and $\beta\gamma$ subunits (Javitch et al; 1997). These two active complexes alter the activity of various effectors thereby initiating a range of downstream signaling events (Li et al; 2006). The self-regulatory GTPase activity within the α subunit hydrolyses the GTP into GDP and leads to re-association of the $\alpha\beta\gamma$ subunits, and thus terminating signalling (Brink et al; 2004 and Li et al; 2006).

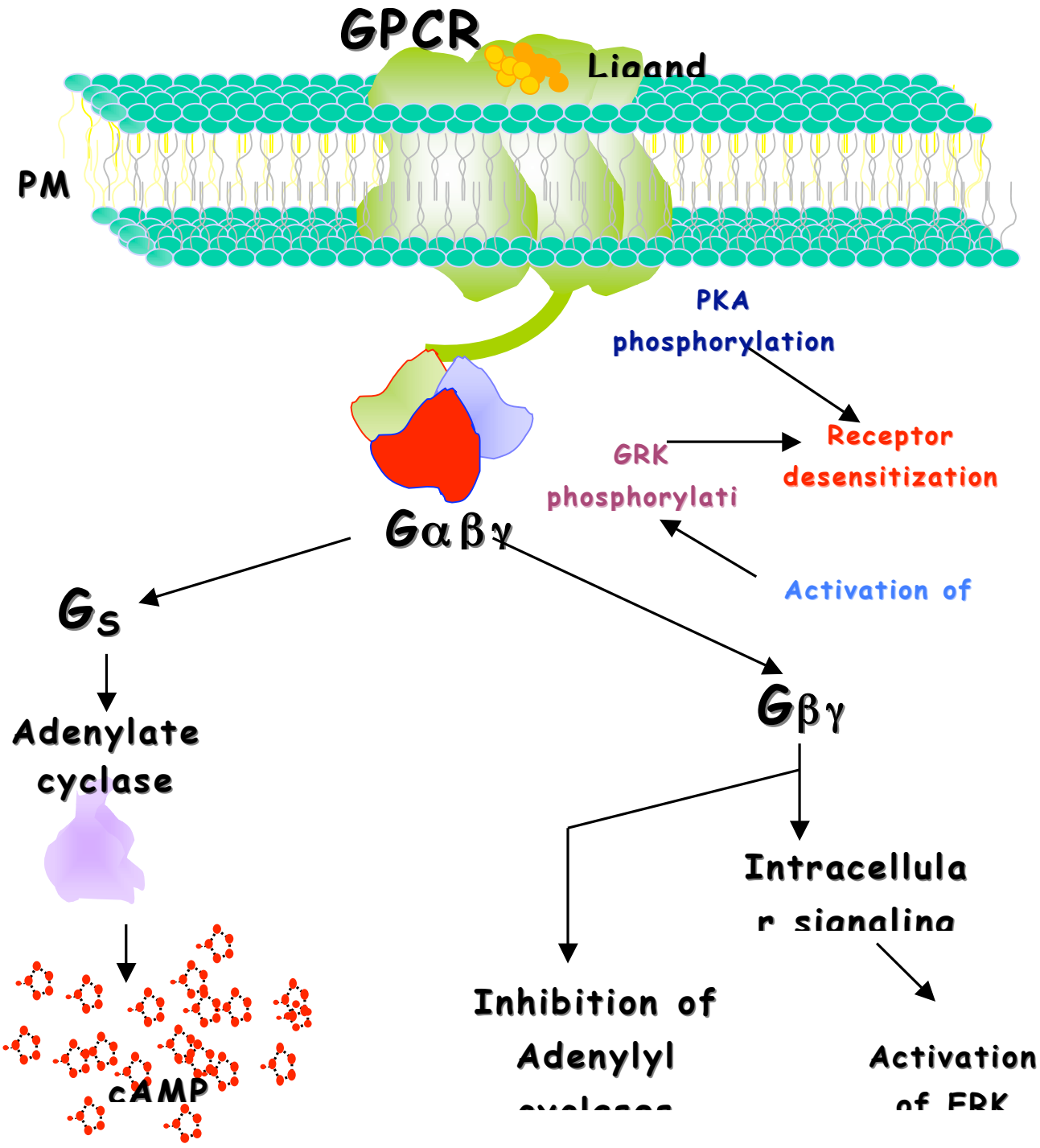


Figure 1.1-Schematic representation of G-protein coupled receptor signaling

1.3 Adenylate Cyclases

Adenylate cyclases (AC) are cAMP producing enzymes. They convert adenosine tri-phosphate (ATP) into 3'5'-cyclic adenosine monophosphate (cAMP). Ten isoforms of AC have been identified and the isoforms have distinct distribution and are differentially regulated, as listed in table 1.1 (Bundey and Insel, 2004). Nine of the isoforms are membrane bound while the other is a soluble cytosolic form (Sun et al; 2004). The membrane bound isoforms show distinct structural homology similar to DNA polymerase and possess a cyclase homology domain that plays a vital role in the catalytic activity of the isoforms (Cooper and Crosswaite, 2006). Regulation of adenylate cyclase isoforms is achieved by various mechanisms, which include binding of di-valent cations e.g. calcium, magnesium and manganese, binding of G-proteins and phosphorylation. Ca^{+2}/calmodulin binding regulates the activity of isoforms 1, 3 and 8. Ca^{2+} inhibits AC5 and AC6 activity, whereas AC2, AC4 and AC7 are insensitive to Ca^{2+} (Cooper, 2003). Manganese-dependent cytosolic AC10 is uniquely activated by bicarbonate ions (Sun et al; 2004).

Phosphorylationn is another major regulator of adenylate cyclase activity. Various kinases are known to phosphorylate adenylate cyclases. The activity of AC1, AC2, AC3, AC5 and AC7 is enhanced by PKC phosphorylation whereas PKC action inhibits the activity of AC4 and AC6. Phosphorylation of AC5, AC6 by PKA, and AC3, AC1 by calmodulin kinases (CaMK) II and IV are known to have down-regulatory actions (Beazley and Watts, 2006). Nitric Oxide (NO) has also been reported to have regulatory affects on AC activity in that NO donors are known to inhibit the activity of AC5 and AC6 (Cooper, 2003).

Forskolin is a labdane diterpene from the plant *Plectranthus barbatus* and is known to augment the levels of cAMP in the cells by allosterically activating ACs in a mechanism similar to that achieved by activated $G_s\alpha$ (Hurley, 1999 and Hurley 1998).

Isoform	Cellular distribution	Regulation	
AC1, 3 and 8	Lipid rafts	Stimulated by calcium	Inhibited by both $G\beta\gamma$ and $G_i\alpha$. Stimulated by $G_s\alpha$
AC2, 4 and 7	Non-rafts	Calcium insensitive	Stimulated by $G\beta\gamma$ and by $G_s\alpha$ Not effected by $G_i\alpha$
AC5 and 6	Lipid rafts	Inhibited by Calcium	Stimulated by $G_s\alpha$. Inhibited by $G_i\alpha$. Not affected by $G\beta\gamma$
AC9	Not known	Inhibited by Calcineurin	------
Soluble Isoform	Cytosol	Not known	------

Table 1.1- The adenylate cyclase isoforms, their cellular distribution and regulatory mechanisms.

1.4 cAMP

The discovery of the signal functioning of 3', 5' cyclic adenosine mono-phosphate (cAMP) led to the now widely accepted concept of a secondary messenger system (Beavo and Brunton, 2002). Cyclic AMP has been implicated in various metabolic activities, such as ion-channel regulation, cell growth and differentiation (Beavo and Brunton, 2003, Houslay and Milligan, 1997).

The levels of cAMP within cells are under stringent control, which if not maintained can lead to erroneous signalling events and subsequently disease e.g. cholera. Cholera toxin catalyses the ADP ribosylation of α-Gs leading to constitutive adenylyl cyclase activation (Houslay and Milligan, 1997). Various signalling complexes as discussed below tightly regulate cAMP levels.

1.5 Effectors of cAMP signalling

1.5.1 Protein kinase A (PKA)

Protein phosphorylation is one of the most important post-translational modifications. Protein kinases are a class of enzymes that phosphorylate their target proteins at Ser, Thr or Tyr residues (Krebs and Beavo, 1979). Protein kinase A provides the classic downstream effector of cAMP action (Taylor et al; 2005, Taylor et al; 1990, Kim et al; 2006).

Protein kinase A is a tetrameric holoenzyme complex (R_2C_2) in its inactive state comprises of two catalytic(C) subunits and two regulatory (R) subunits (R_2C_2).

The binding of two cAMP molecules to each of the R (regulatory) subunits leads to a conformational change in the tetrameric holoenzyme complex subsequently allowing the separation of the regulatory subunits from the catalytic subunits (Daniel et al; 1998, Johnson et al; 2001, Tasken and Aandahl, 2004) (figure 1.2).

There are four genes RIα, RIβ, RIIα, and RIIβ encoding the regulatory subunits. Similarly, there are three genes for encoding the catalytic subunit, namely, Cα, Cβ, and Cγ (Tasken and Aandhal, 2004). A further C-subunit has been identified recently, known as PrKX and is encoded by human X-chromosome. This subunit binds RI regulatory subunits but not RII subunits (Zimmermann et al; 2000).

The C-subunit contains a core catalytic site flanked by amino-terminal and carboxyl-terminal tails varying between 39-50 aminoacids in length. These two tail regions maintain the stability of the core (Taylor et al; 2005). The N-terminal tail of C-subunit is known to undergo post-translational modifications including phosphorylation and myristoylation. The amino-terminus of C-subunit is also known to interact with AKIP (A-kinase interacting protein), which promotes the transportation of C-subunit into nucleus (Taylor et al; 2005)

Regulatory subunits (R-subunits) play a key role in interacting with A-kinase anchoring proteins (AKAPs). AKAPs interact with R-subunits through their N-terminal region, which has a dimerization/docking domain whereas the C-terminus portions of the R-subunits contain the sites for cAMP binding (Taylor et al, 2005). Dimerization of RII subunits is a prerequisite for their interaction with AKAPs (Tasken and Aandhal 2004).

Two different isoforms of PKA are reported to exist in cells. Isoform I, comprises RIα and RIβ subunits whereas isoform II possesses RIIα, and RIIβ subunits. PKA-I is mostly cytosolic while PKA-II is known to associate with AKAPs and is predominantly localized within sub-cellular compartments (McConnachie et al; 2006, Michel and Scott, 2002, Taylor et al, 1990). PKA phosphorylates Ser/Thr residues within various consensus sites. The most stringent PKA consensus motif is Arg-Arg-X-Ser/Thr but motifs including Arg-Lys-X-Ser/Thr or Arg-Lys-X-X-Ser/Thr where X is a hydrophobic amino acid can also serve as PKA substrates (Kennelly and Krebs, 1991, Zetterqvist et al; 1976).

Protein Kinase I is an endogenous inhibitor of PKA. Isoforms of PKI bind to and inhibits the activity of various catalytic subunits of PKA.

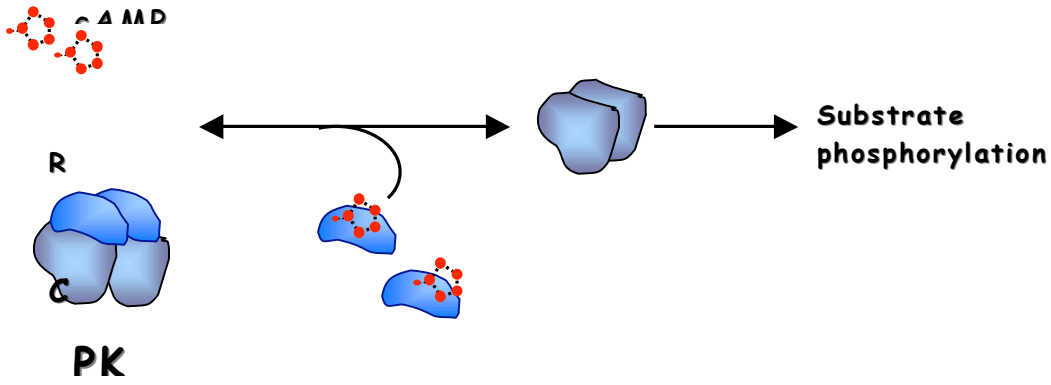


Figure 1.2: Schematic representation of activation of Protein Kinase A

1.5.2 Exchange Protein Activated by cAMP (EPAC)

In 1998, De Rooij and coworkers (De Rooij et al, 1998) identified an 881 amino acid protein with distinct regions for cAMP binding and GEF activity naming it Exchange Protein Activated by cAMP (EPAC) thus identifying another effector of the cAMP signaling cascade (Kraemer et al; 2001, Kawasaki et al; 1998).

Two isoforms of EPAC have been identified, namely EPAC1 and EPAC2. Both the isoforms activate the Ras-like G-protein Rap, which in turn has two isoforms, namely Rap1 and Rap2.

EPAC has an N-terminal DEP (Dishevelled, Egl-10, Pleckstrin) domain, a cyclic nucleotide-binding domain, a Ras exchange motif (REM) and catalytic domains from the N-terminal towards C terminal regions in the same order. The DEP domain is known to be involved determining localization of EPAC within the cell. EPAC is generally associated with mitochondrial and peri-nuclear regions of the cell (Magiera et al; 2004). EPAC2 differs from EPAC1 in that it has an extra cAMP-binding region in its extreme N-terminus and a Ras binding domain between REM and catalytic regions (Rehmann et al; 2003). Cyclic AMP binds EPAC and induces conformational changes in the N-terminal region eventually leading to the activation of GEF domain. The EPAC GEF activity promotes the exchange of GDP for GTP on Rap. EPAC-cAMP complex therefore leads to activation of Rap1 and Rap 2 in a PKA-independent manner.

EPAC plays a major role in integrin-mediated cell adhesion by interacting with Rap1 and light chain 2 of Microtubule associated Protein 1A (MAP1A-LC2) (Yarwood et al; 2005, Borland et al; 2006), Rangarajan et al; 2003). It has also been reported that EPAC is able to interact with AKAP6, which suggests the role of EPAC in spatial and temporal aspects of cAMP signaling (McConnachie et al, 2006).

EPAC has also been shown to be involved in elevating intracellular calcium levels via Rap2B activation subsequently leading to the activation of Phospholipase Cε (PLCε) (Keiper et al; 2004, Vom et al; 2004). EPAC has a similar role in activation of PKCε, through PLC and PKD. This pathway is suggested to be involved in inflammatory pain (Hucho et al; 2005). The elevation of intracellular calcium by EPAC has been shown to have a role in regulating the secretory activity of pancreatic acinar cells (Chaudhari et al; 2007).

1.5.3 *Cyclic nucleotide gated ion channels (CNG ion channels)*

CNG ion channels are regulated by cyclic nucleotides like cAMP and/or cGMP. They were first discovered in vertebrate retinal rods and olfactory sensory neurons (Bradley et al; 2005, Pifferi et al; 2006). There are six genes that encode four A subunits and two B subunits of CNG. They exist in either homo- or hetero-tetrameric states and the cyclic nucleotide binding occurs within their C-terminal region (Young and Krougliak, 2004, Bradley et al, 2005). Activation of a GPCR coupled adenylyl or guanylyl cyclase leads to increase in levels of cyclic nucleotides, which can then bind and regulate CNG ions channel activity. For example, CNG $(A2)_2$-A4-B1b is present in olfactory sensory neurons and is sensitive to cAMP where by elevated levels of cAMP increase the activity of the CNG. However, CNGs are not inactivated by cyclic nucleotides. CNG ions channels are non-specific cation channels and their activity is regulated by calcium-calmodulin cascade (Bradley et al; 2005).

1.6 The cyclic Nucleotide phosphodiesterase (PDE) superfamily

Cyclic Nucleotide phosphodiesterases (PDEs) are a class of enzymes that catalyse the hydrolysis of cyclic nucleotides into their respective 5` mononucleotides. PDEs are localised in various cellular compartments and they regulate the basal levels of cyclic nucleotides in the cells (Houslay and Adams; 2003). The PDE superfamily constitutes 11 subfamilies that differ in their sequence homology, substrate specificity, regulation and selective inhibition (Lynch et al; 2006 Houslay and Adams; 2003, Houslay; 2001 and Houslay and Milligan 1997). Despite their differences, all PDE subfamilies share a highly conserved catalytic region with some structural differences generated by the substrate specificity. In the nomenclature of PDE superfamily, an Arabic numeral is used to indicate the family, then a capital letter indicating the gene within the family followed by a numeral indicating the splice variant are used. For example, PDE4B1 indicates subfamily 4, gene B and splice variant 1 (Lynch et al; 2006). Isobutylmethylxanitine (IBMX) inhibits all PDE enzymes except isoenzymes of PDE subfamilies 8 and 9, which are insensitive to it. Table 1.2 depicts various features of PDE subfamilies.

PDE	Genes	Substrate	Regulatory domains	Selective inhibitors
PDE1	A, B, C	cAMP and cGMP	CaM binding	Nicarpidine and Vinopectine
PDE2	A	cAMP and cGMP	GAF	EHNA
PDE3	A and B	cAMP		Cilostimide
PDE4	A, B, C and D	cAMP	UCR	Rolipram and Ariflo®
PDE5	A	cGMP	GAF	Sildenafil
PDE6	A, B, and C	cGMP	GAF	Zaprinast and Sildenafil
PDE7	A and B	cAMP	___	BRL 50481 and ICI 242
PDE8	A	cAMP	___	Unknown and IBMX insensitive
PDE9	A	cGMP	___	**Zaprinast and SCH51866.IBMX insensitive**
PDE10	A	cAMP and cGMP	GAF	Dipyridamole and Zaprinast
PDE11	A	cAMP and cGMP	GAF	Dipyridamole and Zaprinast

Table 1.2 Classification of Cyclic nucleotide PDE superfamilies.

1.6.1 Phosphodiesterase-1

Phosphodiestarase1 family of enzymes are calcium-calmodulin dependent dual specificity phosphodiesterases. Three genes A, B and C encode both 5' and 3' mRNA splice variants of PDE1 isoenzymes (Lugnier; 2006, Goraya and Cooper; 2005, Snyder et al; 1999). PDE1A and PDE1B genes encode two splice variants PDE1A1, 2 and PDE1B1, 2 respectively and preferentially hydrolyse cGMP whereas PDE1C gene encodes five splice variants 1-5 and hydrolyses both cAMP and cGMP (Giembycz; 2005)

PDE1 isoforms are dimers and although they have been found in the cytosolic, some studies suggest that they can exist in particulate fractions of olfactory neurons and plasma membrane (Goraya and Cooper; 2005, Francis 2001). PDE1 activity can be inhibited by vinopectine and nicardipine (Houslay 2001). PDE1 activity can be regulated by PKA and Cam Kinase II phosphorylation (Lugnier; 2006).

1.6.2 *Phosphodiesterase-2*

Beavo and coworkers (Beavo et al, 1971) first discovered phosphodiesterase 2 in 1971 in rat liver preparations where they showed that the enzyme hydrolysed cAMP more effectively in the presence of cGMP. Three splice variants; PDE2A1 PDE2A2 and PDE2A3 respectively are generated from PDE2 gene. PDE2A2 is more hydrophobic than the other two splice variants of the family. PDE2 enzymes, like PDE1, are dual-specific and hydrolyse cGMP and cAMP with K_m values 10µmol/ L and 30µmol/ L respectively (Zaccolo and Movsesian; 2007).

cGMP plays an important role in the regulation of PDE2 activity. cGMP activates PDE2 in all or none fashion by binding to its GAF domain located between the N-terminal region and catalytic region of the enzymes. At higher concentrations cGMP inhibits cAMP hydrolysis of PDE2 by competitive effects at the catalytic site (Wu et al; 2004). PDE2 isoforms have been shown to localise to sub-cellular membrane structures such as Golgi and perinuclear

membranes. Phosphorylation by PKC α-isoform has been shown to activate the PDE2 enzymes in live rat Golgi endosomal fractions under PMA stimulation (Geoffroy et al; 1999). PDE2 enzymes can be selectively inhibited by EHNA and non-selectively by IBMX (Lugniere et al, 1986).

1.6.3 *Phosphodiesterase-3*

PDE3 isoforms show high affinity to both cAMP and cGMP and are inhibited by cilostamide and milrinone. Two genes A and B encode different PDE3 isoforms which are different from other PDEs in that they have a 44 amino acid insert in their catalytic region and determine their affinity to cGMP (Manganiello et al; 1995). PDE3 isoforms show high affinity to cGMP but hydrolyse cAMP at a 10-fold greater rate than cGMP and are therefore classed as being inhibited by cGMP (Beavo; 1995).

PDE3A isoforms are expressed widely in muscle tissue, myocardium and platelets. However, PDE3B isoforms are expressed in adipocytes and hepatocytes (Beavo, 1995). PDE3 enzymes are regulated by various kinases as well as by hormones. They are phosphorylated by both PKA and PKB/Akt. Phosphorylation by either kinase has been shown to activate the enzymes. Also, hormones such as insulin and glucagon are known to activate the enzyme in Golgi-Endosomal fractions of rat liver tissue by the action of PKB and PKA, respectively (Zaccolo; 2007, Geoffroy et al, 2001, Kitamura et al; 1999, Beavo; 1995). PDE3B is expressed in pancreatic islets where it is known to mediate the effects of leptin on insulin release. Furthermore, PDE3B inhibitors have been shown to increase glucose-mediated insulin release. This effect is attributed to the function of cGMP in islet cells. Inhibition of PDE3B in beta cells leads to elevation of cGMP levels which in turn leads to elevation of cAMP levels and Ca^{+2}-mediated exocytosis. However, PDE3B-mediated cross talk between cAMP-cGMP pathways in islet beta cells has not been well documented (Pyne and Furman, 2003).

1.6.4 *Phosphodiesterase-5*

PDE5 is a cGMP-specific, rolipram-insensitive phosphodiesterase that can be inhibited by zaprinast (Hamet and Coquil; 1978, Lugnier et al; 1986). PDE5 gene encodes three splice variants from two alternate promoters (Lin et al; 2002, Loughney et al; 1998). PDE5 isoforms are expressed in lung , kidney, brain and platelets where they contribute to regulation of cGMP levels (Pyne and Burns, 1993, Giordano et al; 2001, Kameni Tcheudji et al; 2001). Expression of PDE5A1 and A2 in pulmonary artery smooth muscle cells is elevated by hypoxic conditions (Murray et al; 2002).

PDE5 activity has a vaso-relaxing effect that is mediated through nitric oxide (NO) and cGMP (martin et al; 1986, Schoeffter et al; 1987, Prickaerts et al; 2004). PDE5 activity is shown regulate object and spatial memory in rodents. Elevation of cGMP levels by inhibiting PDE5 isoforms led to enhanced memory performance which was mediated through hippocampal regions in rodents (Prickaerts et al; 2004).

Cellular cGMP levels and PKA phosphorylation regulate PDE5 activity. Binding of cGMP to PDE5 is required for its phosphorylation by PKA, which in turn leads to increased affinity for cGMP (Burns et al; 1992 and Francis et al; 2002). Hanson et al; 1998, have shown that PDE5 plays a crucial role in perinatal vascular resistance (PVR) in ovine and murine prenatal development by regulating the cGMP levels. They have also shown that mRNA and protein levels of PDE5 decrease within the one hour of birth when PVR decreases. However, mRNA and protein levels of PDE5 undergo a secondary increase between the 4-7days after birth. PDE5 has also been shown to be involved in fluid transport in malphigian tubules that form a part of arthropod excretory system by regulating cGMP levels (Broderick et al, 2004).

PDE5A1 is known to interact with the pro-apoptotic protease caspase-3 upon an apoptotic stimulus both *in vitro* and *in vivo*. This interaction leads to cleavage and subsequent inactivation of the enzyme (Frame et al, 2003). In the presence of the gamma subunit of PDE6 the proteolysis of PDE5A1 by

caspase-3 is increased, contrary to the action of caspase on PDE6 isoforms in the presence of its gamma subunit (Frame et al; 2001).

1.6.5 *Phosphodiesterase-6*

Phosphodiesterase 6 was first discovered in retinal rods. It hydrolyses cGMP specifically and is zaprinast inhibited (Beavo, 1995, Goridis and Viramaux, 1974). PDE6 is a heterodimer complex containing two catalytic subunits (α in rods and ά in cones and a β subunit), two inhibitory subunits (γ in rods and γ' in cones), and a δ prenyl binding protein (PrBP) subunit, all of which are encoded by separate genes, A for α subunit, B for β subunit, C for ά, D for δ subunit, G for γ and H for γ' subunits (Lugnier, 2006). As well as binding to prenylated photoreceptor proteins such as GRK7 and Rab8, PrBP binds to the catalytic subunits of membrane bound PDE6 and has been suggested to reduce transducin-mediated activation of the phosphodiesterase in frogs (Norton et al; 2005).

PDE plays a major in visual cycle. Briefly, activated rhodopsin activates transducin molecules, which are G protein heterotrimers, which exist in GDP-bound inactive and GTP-bound active states. Activated, GTP-bound transducin binds to catalytic subunits of PDE6 and increases affinity of the enzyme for cGMP and leads to hydrolysis of the cyclic nucleotide. A decrease in cGMP concentration activates the CNG, which leads to sensory responses through the optic nerve (Ionita and Pittler, 2007). PDE6 is negatively regulated by individual phosphorylation of PKC and CDK-5 (Hayashi et al; 2002, Udovichenko et al; 1994, Udovichenko et al, 1996). The degradation of PDE6 by caspase-3 is modulated by gamma subunit of the PDE (Frame et al. 2001). In the presence of PDE6 rod gamma subunit casapse-3 was shown to exhibit reduced PDE6 proteolysis.

1.6.6 *Phosphodiesterase-7*

In 1993, Michael and workers (Michaeli et al; 1993) isolated and characterised a cAMP phosphodiesterase different from both PDE3 and PDE4 isoforms and called it HCP1 (High affinity cAMP-specific PDE). It was later re-named as PDE7 (Beavo, 1995). PDE7 is not sensitive to cGMP and is not inhibited by the PDE3 and/or PDE4 selective inhibitors like milrinone and rolipram.

The PDE7 family comprises two different subfamilies of isoforms, 7A and 7B (Han et al 1997, Gardner et al; 2000, Sasaki et al; 2000). PDE7A was initially thought to be crucial in T-cell function. T-Cell receptors, Cd23 and CD28 when stimulated increase the expression of PDE7A and thus decrease cAMP levels and PKA activity in T-cells (Li et al, 1999). PDE7A is also known to be involved in T-cell proliferation and regulating the production of chemokines such as IL-2, 4 and 7 (Li et al; 1999 and Nakata et al; 2002). However, further studies (Yang et al 2003) showed that PDE7A is not necessary for T-cell function. Indeed, transgenic mice lacking PDE7A gene were found to have functional T-cells. The absence of PDE7A did not affect the IL-2 production driven by Cd23 and cd28 or T-cell proliferation suggesting that T-cell function is not regulated by PDE7A (Yang et al; 2003)

PDE7B is implicated in dopaminergic signalling. Primary striatal cells treated with dopamine showed elevated levels of PDE7B, which is believed to mediate through a cAMP-PKA-CREB cascade, which promotes PDE7B transcription (Sasaki et al; 2004).

1.6.7 *Phosphodiesterase-8*

Two different subfamilies of PDE8, namely A and B, were first identified and cloned by three different groups from both mouse and human (Fisher et al; 1998, Hayashi et al; 1998 and Soderling et al; 1999).

PDE8A and PDE8B have 5 and 4 splice variants, respectively (Hayashi et al; 2002, Wang et al, 2001). The PDE8 family is insensitive to the PDE4 cAMP

specific selective inhibitor, rolipram and, surprisingly, the non-selective PDE inhibitor, IBMX. However, PDE8B subfamily is sensitive to EHNA and inhibitors of PDE5 (Fisher et al, 1998). PDE8 isoforms have an N-terminus that is homologous to PAS domain of many signalling protein (Soderling et al 1998). The role of the PAS domain is not known but it has been suggested it may be involved in either PDE dimer formation or promote protein-protein interactions.

1.6.8 *Phosphodiesterase-9*

In 1998, Fisher and coworkers (Fisher et al 1998) identified Phosphodiesterase 9. Although the PDE9A family was originally thought to have only 4-splice variants (Guipponi et al; 1998, Rentero et al; 2003) at least 20 mRNA splice variants have identified have since been identified. PDE9A1 specifically hydrolyses cGMP and is insensitive to IBMX and the PDE5 selective inhibitor, sildenafil. However, it is sensitive to the PDE5 inhibitor SH51866, which is also a PDE1 inhibitor (Soderling et al; 1998). PDE9 isoforms show varied localisation within the cell (Renetero et al; 2006). PDE9A1 isoform is exclusively localised to the nucleus whereas PDE9A5 isoform is cytoplasmic (Wang et al, 2003).

1.6.9 *Phosphodiesterase –10*

PDE10 is dual-substrate specific enzyme family. It has two mRNA alternative splice variants, PDE10A1 and PDE10A2 (Kotera et al; 1999). PDE10A contains two GAF domains although its structure is significantly different from other cGMP-specific PDEs like PDE5 and PDE6. PDE10 hydrolyses both cGMP and cAMP. However, it shows more affinity to cAMP (Fujishige et al; 2000). PDE10A is inhibited by IBMX, zaprinast, SCH51866 but is not inhibited by sildenafil and EHNA (Soderling et al; 1999). PDE10A2 is known to localise to intracellular membrane fractions like Golgi apparatus (Kotera et al; 2004). However upon PKA phosphorylation at Thr16, PDE10A2 was shown to localise to cytosol suggesting the role of PKA in determining the sub-cellular localisation of the isoform (Kotera et al; 2004).

1.6.10 *Phosphodiesterase-11*

PDE11 is also a dual-substrate specific family of PDEs and is sensitive to IBMX, dipyridamole and zaprinast (Fawcett et al; 2000). Despite the dual substrate specificity and presence of GAF domains these enzymes are not activated by either of the cyclic nucleotides. (Yuasa et al, 2000). So far, four splice variants have been identified of which PDE11A3 and PDE11A4 are well characterised. Interestingly, PDE11A4 contains two complete GAF domains whereas PDE11A3 contains one complete and one incomplete GAF domains. Also, PDE11A4 contains putative phosphorylation sites for both PKA and PKG. However, PDE11A3 lacks phosphorylation sites for both the kinases suggesting that these isoforms differ in their N-terminal regions, which is a common phenomenon observed on PDE superfamily (Hetman et al; 2000 and Yuasa et al, 2000).

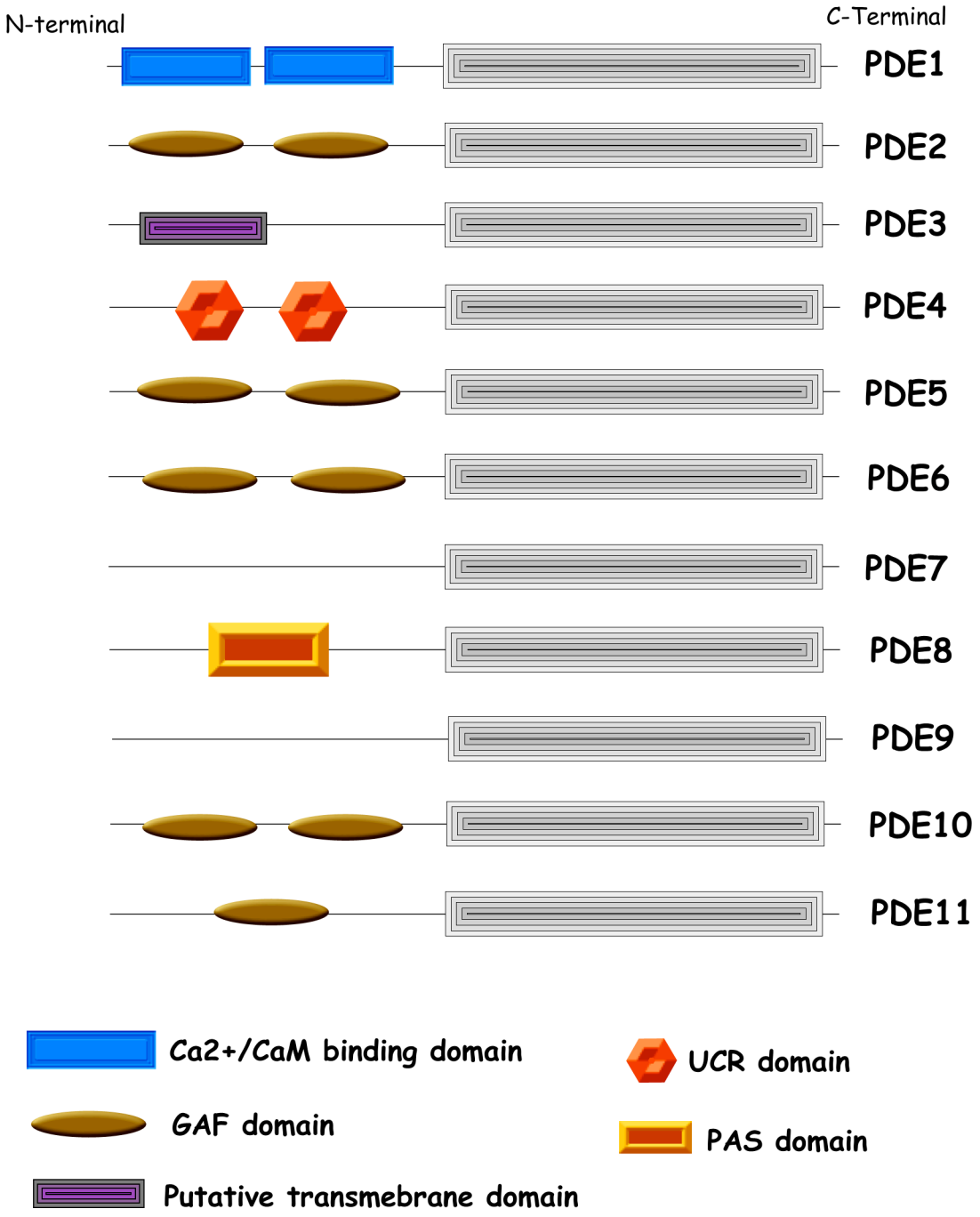


Figure 1.3 Modular structures of PDE enzyme-superfamily (Adapted from Lynch et al, 2006)

1.7 cAMP Phosphodiesterase 4

1.7.1 Phosphodiesterase gene and structure

Phosphodiesterase-4 (PDE4) enzymes are a family of more than 20 isoforms encoded by four genes A, B, C and D (Conti et al 2003, Houslay et al; 2005, Houslay et al, 2004). The *dunce*$^{+}$ PDE4 gene was cloned from *Drosophila melanogaster* (Davis et al; 1989) and was used to identify the mammalian homologues by Swinnen and coworkers (Swinnen et al; 1989). They also showed that the different PDE4 isoforms arise by alternative mRNA splicing which was confirmed and extended in studies of the human forms (Bolger et al; 1996).

Genes encoding PDE4 isoforms are located on various chromosomes. PDE4D subfamily is located on chromosome 5 in humans and chromosome 2 in rats, whereas PDE4B subfamily is located on human chromosome 1 and rat chromosome 5 (Szpirer et al, 1995). PDE4A and PDE4C are located on chromosome 19 at 19p13.2 and p13.1 respectively (Engels et al; 1995, Horton et al; 1995, Sullivan et al; 1994). The mRNA splice variation generates PDE4 isoforms each possessing a unique N-terminal region with two upstream conserved regions (UCR1 and UCR2) and a highly conserved catalytic unit (Bolger et al; 1996). The residues of the extreme C-terminal region of the catalytic unit are unique to each of the four sub families (Bolger et al; 1996). The UCR1 is 60 amino acids in length and the UCR2 has 80 amino acids long. The UCR1 domain is linked to the UCR2 by linker region 1(LR1) which is 24 amino acids long and UCR2 is linked to the catalytic unit (320-350 amino acids in length) by LR2, which is 10-28 amino acids long (Conti and Jin; 1999, Houslay and Adams, 2003, Lynch et al 2006).

PDE4 isoforms can be classified into three groups; Long, short and super-short forms. Long forms have a unique N-terminal region, complete UCR1 and UCR2 regions, catalytic region and a C-terminal region. Short forms lack the UCR1 region while super-short forms lack UCR1 and have an N-terminally truncated UCR2. A representation of PDE4 structure is shown in Figure 1.4.

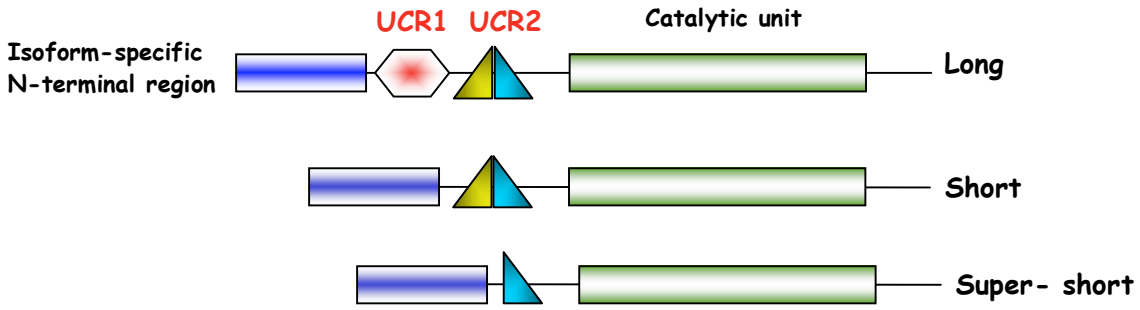


Figure 1.4 Schematic of modular structures of PDE4 isoforms

1.7.2 *Catalytic Domain*

Interpreting the mechanisms involving the catalytic activity of PDE4 isoforms requires knowledge of PDE4 structure. Xu and coworkers (Xu et al; 2000) were the first to determine the structure of a PDE catalytic domain, namely the catalytic unit of PDE4B. It was determined that catalytic domain of the PDE4 has 17α helices, which form 3 distinct sub domains. The first sub-domain contains four α helices connected by two inter-connecting loops. The second sub-domain contains two α helices that are oriented perpendicular to two anti-parallel α helices. The third sub-domain is composed of five of α helices and an extended β-hairpin loop (Xu *et al* 2000).

A deep pocket of 21 residues in the COOH-terminal sub-domain extends into the junction of the three sub-domains. All 21 residues are highly conserved in all the 11 PDE families. Twenty of these residues lie near the pocket and 12 lie within the pocket. The pocket accommodates a molecule of either cAMP substrate or 5'AMP product and is lined with negatively charged and hydrophobic amino acids (Xu *et al* 2000). The deep pocket serves as a substrate-binding site and has two divalent cation binding sites. Metal binding site 1 (Me1) is the deeper set and holds the ion by four direct interactions with His^{238}, His^{274}, Asp^{275}, and Asp^{392}. Metal binding site 2 is slightly superficial in location and is close to the Me1. The Me1 is usually occupied by a Zn^{+2} ion and is very tightly held. The Me2 can bind Mn^{+2}, Mg^{+2} or Zn^{+2}. However, the

physiologically relevant ion is thought to be Mg^{+2} (Houslay MD and Adams DR; 2003).

Depending upon the kinetics of rolipram binding to the catalytic unit, the PDE4 isoforms can exist in two conformational states namely a high affinity rolipram binding site (HARBS) and or a low affinity rolipram binding site (LARBS) (Souness and Rao; 1997). The LARBS conformation is seen in the inactive enzyme and the HARBS is seen in catalytically active enzyme. Divalent cation binding plays an important role in releasing the product of cAMP hydrolysis from the catalytic site (Laliberte et al; 2002, Liu et al; 2001). PDE4 isoforms upon phosphorylation and activation by PKA change their affinity to rolipram and convert to a HARBS conformation (Hoffmann et al, 1998). PDE4 interaction with various binding partners such as SRC tyrosyl kinases, XAP2, RACK1 has has also been shown to change the rolipram affinity of the isoforms (Bolger et al; 2003 and McPhee et al; 1996, Yarwood et al 1999). (Please refer to appendix II for an Image of catalytic subunit)

1.7.3 PDE4 isoforms

1.7.3.1 Phosphodiesterase- 4A

PDE4A isoforms differ from other PDE4 subfamilies in that they their C terminus is not conserved and that they are not inhibited by ERK phosphorylation (Mackenzie et al, 2000). PDE4A1 is a super-short form (Sullivan et al; 1998). PDE4A4 is human homologue of rat PDE4A5 both of which are long forms. PDE4A7 is the only PDE4 isoform that is spliced both on the 3' and 5' ends of the mRNA transcript (Sullivan, 1998) and it lacks the residues that confer catalytic activity (Johnston et al, 2004). PDE4A10 and 4A11 are also long forms (Wallace et al; 2005 and Rena et al 2001). PDE4A10 is known to be upregulated in differentiated monocytes and macrophages (Shepherd et al; 2004). PDE4A11 is localised to both particulate and soluble cellular fractions (Wallace et al; 2005).

1.7.3.2 Phosphodiesterase -4B

PDE4B1, 4B3 and 4B4 are long forms so far reported from PDE4B subfamily. PDE4B1 is a human isoform whereas B3 and B4 are expressed in rat (Shepherd, 2003 and Huston et al; 1997). PDE4B2 is a short form that is expressed in both humans and rat. PDEB5 is a super-short form expressed only in brain and its N-terminal region is identical to that of PDE4D6 (Cheung et al; 2007), the first time, to date, this phenomenon has been noted.

1.7.3.3 Phosphodiesterase-4C

Three isoforms have been reported from PDE4C family by various groups (Engels, et al; 1995 Owens et al, 1997 and Obernolte et al, 1997), all of which are long isoforms. It has been argued that it is unlikely for a PDE4C short form to exist because of the lack of space between long form exons (Sullivan et al; 1999). However, other studies (Obernolte et al; 1997) have identified four splice variants. Isoforms PDE4C-791 and 426 were isolated from foetal lung whereas PDE4C delta-54 and delta 109 were isolated from testis mRNA.

1.7.3.4 Phosphodiesterase-4D

PDE4D encodes 9 isoforms 4D1-4D9 (Bolger et al; 1997). PDE4D1 is a short form and PDE4D2 is super-short form (Nemoz et al; 1996). PDE4D3, 4D4 and 4D5 are long forms (Bolger et al; 1997). PDE4D6 is a brain-specific super-short form and PDE4D7 is a long form (Wang et al; 2003). PDE4D8 and PDE4D9 are long forms that are closely related to PDE4D3 (Richter et al; 2005).

1.7.4 Regulation of PDE4 isoforms

1.7.4.1 *Transcriptional* r*egulation of PDE4 isoforms*

Cyclic AMP sensitive promoters regulate cellular PDE4 activity at a transcriptional level. Follicle stimulating hormone (FSH)-mediated elevation of

cAMP levels in sertoli cells were shown to elevate the mRNA transcripts and protein levels of PDE4D family enzymes particularly PDE4D1 and PDE4D2 (Vicini and Conti; 1997) This elevation in protein levels was due to cAMP-mediated activation of promoters of PDE4D1 and 4D2. Similarly, Jurkart-T cells pre-treated with 8-bromo-cAMP or with Prostaglandin E2 increased the protein levels of PDE4D1-D3, PDE4A4 and PDE3 in a protein synthesis sensitive manner as confirmed by using Actinomycin D to inhibit transcription and Cycoheximide to inhibit translation suggesting that elevated cAMP levels directly impact cellular PDE4 expression (Seybold et al; 1998). Furthermore, Oxo-7-prostacyclin-treated rats exhibited a marked change in expression profile of the PDE4 isoforms in heart tissue. Forty-eight hours post-treatment, levels of mRNA transcripts of PDE4B3 were elevated while the levels of mRNA transcript levels of isoforms D1, D2 and D3 Kosti et al; 1997). The 5` flanking sequence of PDE4A10 exon-1 is shown to have cell type-specific promoter-like activity (Rena et al; 2001). Furthermore, two regions upstream to exon-1^{4A10} identified in mouse, namely A and B, were shown to bind transcription factors. Region B has binding sites for transcription factors CREB, GATA, Nuclear factor-κB and H_2O_2-activated USF suggesting that PDE4A10 might be involved in inflammatory response (Rena et al; 2001). Similar studies on PDE4A11 (Wallace et al; 2005) of a region 1 Kb upstream of exon-1^{4A11} identified a potential transcription binding site as identified by an increase in luciferase activity in reporter construct assays The PDE4D subfamily has also been shown to be subject to transcriptional regulation. Exons encoding isoform-specific N-terminal regions of PDE4D5 were shown to exhibit promoter activity (Le Jeune et al; 2002). This transcriptional regulation is reported to be driven by cAMP due to the presence of a CRE site upstream of the exon (Le Jeune et al; 2002). Transcriptional regulation of PDE4D1 and PDE4D2 has also been noted (Vicini and Conti, 1997).

1.7.4.2 *Regulation by Protein Kinase A*

In 1996, Sette and Conti reported that PDE4 isoforms are activated upon phosphorylation by cAMP dependent protein Kinase (PKA) from observations of rat thyroid cells stimulated with TSH. PDE4D3 was the first studied isoform

for PKA phosphorylation. The target sites were mapped within two PKA consensus sites at Ser^{13} and Ser^{54}. Ser^{13} is located with PDE4D3's unique N-terminal region while Ser^{54} is in the UCR1 region (Alvarez et al; 1995, Hoffmann et al; 1998, Sette and Conti, 1996). Although there are two PKA phosphorylation sites on PDE4D3 only Ser^{54} is required for the activation of the enzyme (Sette and Conti 1996).

PKA phosphorylation occurs at the R-R-E-S-F motif in UCR1 region, which is conserved in long PDE4 isoforms. PKA phosphorylation at this site activates the enzymes (MacKenzie et al 2002). The glutamate residue present in PKA consensus site is highly conserved in all PDE4 long forms and has an inhibitory affect on PKA phosphorylation (Hoffman et al; 1998). Mutational analysis of the glutamine preceding the Ser in PKA consensus site was shown to increase PDE4 activity, which was further increased by PKA phosphorylation (Hoffmann et al; 1998).

UCR1 and UCR2 bind with each other by electrostatic interactions between positively charged amino acids in UCR1 and negatively charged amino acids in UCR2, an interaction which is modulated by PKA phosphorylation (Beard et al; 2000). The intramolecular association of UCR1 and UCR2 is known to form a module that has an inhibitory affect on catalytic unit. This inhibitory effect is abolished by phosphorylation of PKA on UCR1 region, which opens the module and thereby activates the enzyme (Lim et al, 1999).

PKA phosphorylation is also known to have an affect on rolipram sensitivity of PDE4 long forms. PKA phosphorylation increases the rolipram-binding affinity of certain long-forms by switching on the HARBS conformation (Mackenzie et al; 2002 and Hoffman et al; 1999).

1.7.4.3 *Regulation by Extra-cellular signal regulated kinase*

The ERK-MAP kinase family is a family of serine-threonine kinases and is one of the most important signalling cascades that can regulate the activity of the cAMP messenger system through crosstalk (Mackenzie 2000).

ERK1 and ERK2 are members of MAP Kinase family. Phosphorylation by ERK requires three motifs, a kinase interaction motif (KIM), FQF domain and a proline-directed serine consensus motif found in all authentic ERK substrates. The KIM domain is located upstream of the target Ser/Thr and has a consensus sequence [Leu/Val]-Xaa-Xaa- [Arg/Lys]-[Arg/Lys]-Xaa $_{(6)}$-Leu-Xaa- [Leu/Ser]. FQF domain is downstream of the target Ser/Thr and has a consensus of Phe-Glu-Phe (Lynch et al; 2006, Houslay and Adams; 2003 and Mackenzie et al, 2000).

It has been shown that PDE4 isoforms are substrates for the ERK-MAP kinases. ERK2 is known to associate with the PDE4 catalytic subunit by binding to the KIM domain and FQF site on an exposed β hairpin loop and α helix respectively. These two sites flank an ERK phosphorylation site. Phosphorylation of long, short and super short forms of PDE4 enzymes by ERK leads to either the activation or inhibition of the particular isoform depending upon the presence or absence of UCR1/2 regulatory modules (Hoffmann et al 1999 Mackenzie et al; 2000, Baillie et al; 2000, Baillie et al; 2001)

Ser^{579} in PDE4D3 isoform is a target site of phosphorylation by ERK, which leads to the inhibition of the activity of PDE4D3. Interestingly, isoforms of the PDE4B, 4C and 4D subfamilies, but not the PDE4A subfamilies, are ERK substrates. This is due the presence of a target Ser/Thr in a P-x-ser/Thr-P motif in the PDE4B, 4C and 4D families. In the PDE4A subfamily, one of the proline residues in the motif is replaced by and arginine. PDE4A subfamily of isoforms are therefore not inhibited by ERK2 (Baillie et al; 2000, Baillie et al; 2001).

As mentioned, PDE4D3 is also regulated by the PKA-mediated phosphorylation at Ser^{54} within UCR1, which leads to activation of the enzyme (Mackenzie et al; 2000). The phosphorylation events regulating PDE4D3 counteract each other as PKA-mediated activation is ablated by the ERK-mediated inhibition of PDE4D3. This provides a regulatory system whereby ERK-mediated inhibition leads to an increase of cAMP levels which triggers

the subsequent PKA-mediated phosphorylation and activation of PDE4D3 (Hoffmann R, 1999). PDE4D3 therefore provides a point of cross talk between the ERK MAP kinase pathway and cAMP pathway.

Hill and coworkers (Hill et al, 2006) showed that PDE4D3 is activated rapidly and phosphorylated at Ser^{579} and Ser^{239} upon challenging RAW macrophages with hydrogen peroxide (H_2O_2). Phosphorylation at Ser^{579} was revealed to be mediated by ERK whereas phosphorylation at Ser^{239} occurred by as yet unidentified kinase downstream of PI3 kinase. Phosphorylation at Ser^{239} reverses the inhibitory effect caused by phosphorylation at Ser^{579} by ERK. It is proposed that phosphorylation at Ser^{239} might reverse the inhibitory effect of ERK phosphorylation by inhibiting the interaction of UCR1-UCR2-catalytic region.

1.7.4.4 *Regulation by Phosphatidyl Inositol Kinase-3*

PDE4A5, a rat homologue of human PDE4A4, is activated and phosphorylated by an unknown kinase downstream to PI3 kinase in F442-pre-adipocytes activated by Growth hormone stimulation and contributed to differentiation of the pre-adipocytes into adipocytes (Mackenzie et al, 1998). Activation and differentiation were not seen when the cells were treated with PI3-kinase inhibitors such as wortmannin and Ly294002. In further studies (Yarwood et al; 1999) differentiation was reported to be transduced through a Janus Kinase–2 and Signal Transducer and activator of Transcription-5 (STAT5) pathway.

1.7.4.5 *The action of Phospholipids*

It has been suggested that certain PDE4 long forms can be activated by acidic phospholipids. This activation is not seen in short and super-short forms. Lysophosphatidic acid and phosphatidyl-serine, but not phosphatidyl choline, increase the activity of PDE4D3 activity up to 3-fold (Disanto et al, 1995 and Nemoz et al, 1997). Subsequent work (Garange et al; 2000)

showed that phosphatidic acid failed to activate a UCR1 deletion mutant of PDE4D3.

Studies from our group (Baillie et al; 2002) identified a novel phosphatidic acid binding domain in the N-terminus of PDE4A1. This domain was named TAPAS1 (tryptophan anchoring phosphatidic acid selective domain). The sequence of the TAPAS domain is LVX_{aa}WWD$X_{aa}X_{aa}$ K/R, where residues LVXWW are needed for membrane insertion and residues DXXK/R are needed for calcium binding. The TAPAS domain uses submicromolar free calcium ions to bind to Asp21 in PDE4A1, allowing insertion of this isoform into membranes. This feature is analogous to that of annexin whereby calcium binding switches on a conformation that exposes the tryptophan residues in the protein such that they can be inserted into the membrane (Campos et al; 1998).

1.8 *Targeting of PDE4 isoforms to signalling complexes*

It is now widely appreciated that cAMP signalling is compartmentalised within cells (Houslay et al; 2007, Zaccolo 2006, Zaccolo et al; 2006, Lissandron et al; 2006, Terrin et al; 2006, Houslay and Adams, 2003). Buxton and Brunton, in 1983, for the first time showed that ventricular myocytes challenged with isoprenaline and prostaglandin E1 produced different responses on glycogen phosphorylase activity suggesting that their cAMP responses are compartmentalised. Recently, Zaccolo and Pozzan; 2002 have demonstrated the presence of distinct pools of cAMP in live neonatal cardiomyocytes using fluorescence resonance energy tranfer (FRET) technology.

PDE4 isoforms have been shown to contain regions that confer a high degree of specificity in allowing them to interact with a wide variety of signalling complexes. This allows PDE4 isoforms to regulate the cAMP levels in specific intracellular compartments (Lynch et al; 2006, Houslay and Adams, 2003). The following section discusses the various signalling PDE4 binding proteins which have been shown to regulate compartmentalisation of cAMP signalling. A schematic of cAMP signalling is shown in Figure 1.5.

1.8.1 *β-arrestins*

Arrestins are ubiquitously expressed scaffold proteins and are of two types, visual arrestins and β-arrestins. Visual arrestins are expressed only in retinal tissue (Yamaki et al, 1987 and Shinohara et al 1987), whereas the β-arrestins are expressed in all tissues particularly in the brain and spleen (Attramadal et al; 1992). Visual arrestins play an important mechanistic role in the visual responses whereas β-arrestins are involved in complex signalling pathways including GPCR desensitisation (Lefkowitz and Shenoy; 2005 and Luttrell and Lefkowitz, 2002).

Recently, it has been demonstrated that PDE4D5 interacts with β-arrestins (Bolger G. B *et al*, 2003). PDE4D isoforms are ubiquitously expressed in a wide range of cell types and PDE4D3 and PDE4D5 are the most predominant isoforms (Conti M; 2003). Specifically, the interaction appears to involve amino acid residues 70-88 within the unique N-terminal region of PDE4D5 (Bolger et al, 2006). Two hybrid assays show that both the unique N-terminal region and a portion of catalytic region are required for the interaction PDE4D5 with β-arrestins. Most probably the two different domains of βarrestins interact with the two interaction sites of PDE4D5. Indeed, the site within the conserved PDE4 catalytic region allows PDE4s from all subfamilies to interact with β-arrestin (Baillie et al; 2007 and Bolger G. B et al; 2003). β-arrestins have two domains, N- and C- domains each of which are composed of seven-stranded β helix loops. Truncation experiments show that the amino terminal regions of both domains are required to interact with PDE4 isoforms. Also, residues K25 and R26 within a β helix loop of the N-terminus of arrestin have been shown to be crucial for interaction with the FXFELXL and FQFELTLEED motifs present in the catalytic site of the PDE4 isoforms (Baillie et al; 2007 and Bolger et al 2003). It is through this site in the catalytic unit that all the PDE4 isoforms can potentially interact with β-arrestin. In case of PDE4D5, an additional β-arrestin binding site is present in the N-terminal region of the isoform. Whether the site within the specific amino terminal region of PDE4D5 facilitates the binding of common binding site in catalytic

site, or allows other proteins that interact with β-arrestin to bind is unknown. However, it is known that β-arrestin does not affect the enzymatic activity of the PDE4 enzymes (Bolger G. B et al, 2003).

It has been recently reported that desensitisation of β_2 adrenergic receptor involves the β-arrestin mediated recruitment of PDE4D5, thus leading to decrease in cAMP levels around the receptor and eventually leading to re-coupling of G proteins to the receptor. In contrast, the β_1 adrenergic receptor shows very low affinity to β-arrestins. This is in accordance with the observation that stimulation of cardiac contraction by β_2-adrenergic receptor is transient and that the receptor undergoes desensitisation very quickly. However, the stimulation of cardiac contraction by β_1 adrenergic receptor recovers very slowly. Furthermore, the activity of β_1 adrenergic receptor is not affected by inhibiting PDE4D in β_2-AR knockout murine myocytes, indicating that PDE4D is involved in regulating the activity of β_2 AR, but not that of the β_1-AR, thereby contributing to the differential regulation of cardiac contraction by adrenergic agonists (Xiang Y *et al*, 2005 and Lynch et al; 2005).

Agonist-induced β_2-AR desensitisation is also mediated by second messenger responses. PKA tethered to AKAP5 is constitutively bound to the receptor and subsequently phosphorylates the receptor upon ligand activation. Similarly, an agonist-mediated PKA-AKAP79 complex is recruited to the receptor inducing receptor phosphorylation eventually leading to receptor desensitisation (Willoughby et al; 2006, Houslay and Baillie; 2005, Lynch et al; 2005, Fraser et al; 2000, Tao et al; 2003). PKA-mediated phosphorylation of the receptor leads switching of receptor coupling from G_s to G_i. This G_s to G_i switching now allows for ERK1/2 activation through Src recruitment to the complex (Baillie et al; 2003).

PKA (Cong et al; 2001) or PKC (Krasel et al; 2001) -mediated phosphorylation and activation of GRK2 leads to its increased interaction with $G_{\alpha\beta\gamma}$ subunits and desensitisation of the β_2-AR receptor by GRK2 phosphorylation (Li et al; 2006). This activation and recruitment of GRK2 to the β_2 AR is reversed by

ERK phosphorylation of GRK2, which decreases its affinity for Gαβγ. Li and coworkers (Li et al. 2006) showed that PKA phosphorylation of the receptor and its subsequent switching from G_s to G_i signalling is controlled by recruitment of a β-arrestin-PDE4D5 complex to the receptor where PDE4D5 regulates the levels of cAMP and thereby PKA activity and prevents inappropriate recruitment of GRK2 to the membrane.

Abrahamsen and coworkers (Abrahamsen *et al;* 2004) showed that T cell activation is initiated in lipid rich membrane micro-domains known as lipid rafts and leads to the production of cAMP within these lipid rafts. Concomitant stimulation of CD-28 (cluster of differentiation), induces β-arrestin dependent recruitment of PDE4 enzymes to the lipid rafts The long isoform PDE4A4, the short isoforms PDE4B2, and PDE4D1/2 were all found to rapidly recruited to lipid rafts in a β-arrestin dependent process. This recruitment of PDE4 enzymes to lipid rafts offers a way to downregulate the inhibitory affects of cAMP elevation on T-cell activation, which is mediated by PKA through a Csk-Lck pathway (vang *et al,* 2003). The process of cAMP elevation in lipid rafts and PDE4 recruitment achieved only by activation of the T-cell receptor (TCR) and concomitant activation of CD28 as upon TCR stimulation alone, there is insufficient PDE4 to reduce the cAMP levels and therefore maximal T- cell activation cannot be achieved due to the inhibitory effects of cAMP signalling (Abrahamsen et al; 2004).

Apart from GPCR desensitisation, β-arrestins are also involved in receptor internalisation. After receptor activation and desensitisation, β-arrestins binds to adaptor protein-2 (AP-2), which concentrates the receptor–βarrestin complex to clathrin-coated pits (Laporte et al; 1999 and Goodman et al; 1996). Once the receptor-arrestin complex reaches the clathrin-coated pits, the pits will be pinched off by the Src-phosphorylated G protein dynamin (Sever, 2002). Arrestin then dissociates from the internalised receptor complex which will be either degraded via proteasomal/lysosomal complex or recycled back to membrane after dephosphorylation by phoshphatase PP2A (Vaughan et al; 2006 and Oakley et al; 1999).

1.8.2 *RACK1*

RACK1-Receptor of Activated C-kinase is a 36 KDa protein with a seven bladed propeller structure, each blade consisting of β sheets. RACK1 has Trp-Asp 40 (WD40) repeats. These repeats are highly conserved in a range of species. RACK1 serves as a binding protein for Protein kinase C. However, other proteins like Src, integrin β subunits, and γ-aminobutyric acid (GABA) are also known to interact with RACK1 (McCahill *et al*, 2002). RACK1 associates with proteins in either a constitutive or stimulus-dependent manner. RACK1 has multiple independent protein binding sites, which suggest the individual blades of RACK1 may be able to interact with specific protein classes. Similar to its interaction with the Gβ and Gα subunits, the RAID1 (RACK1 Interaction Domain) within the N-terminal region of PDE4D5 interacts with WD repeats on RACK1 (Bolger et al; 2006 and Yarwood et al; 1999). Recent work (Bolger et al. 2006) showed that RACK1 has a further PDE4 binding site within the catalytic unit suggesting that RACK1 might interact with other isoforms of other PDE4 subfamilies. The functional consequences of RACK1-PDE4 interaction are not yet known but it is predicted that PDE4D5 interacting with RACK1 controls the cAMP levels within its immediate surroundings and alter the sensitivity of the RACK1-interacting proteins to PKA phosphorylation (McCahill *et al,* 2002). In support of this, it has been reported that RACK1 has a role in regulating the PKA activity of the defective CFTR chloride channel in cystic fibrosis. However, The regulatory mechanisms of this process are still unknown. Its been showed that activation of adenylyl cyclase and subsequent production of cAMP by forskolin caused the translocation of RACK1 to nucleus without altering the location of PKC βII (McCahill et al; 2002). This indicates that RACK1 might be involved in shuttling of proteins into nucleus. It has also been shown that inhibition of ethanol-induced compartmentalisation of RACK1 is inhibited by cAMP analogues that prevent activation of PKA by cAMP (McCahill et al; 2002). Thus PDE4-RACK1 interaction may have a role in regulating the PKA activity within the environment of this scaffold.

1.8.3 *AKAPs*

A Kinase Anchoring Proteins, known as AKAPs for short, are a family of proteins that can bind directly to regulatory subunit II of PKA (McConnachie et al; 2006 and Newlon M. G. *et al*, 1999) and thus generate a compartmentalized PKA response to various intracellular gradients of cAMP. Although all most all AKAPs have been shown to bind R_{II} subunit of PKA, some AKAPs like $AKAP_{CE}$ bind to R_I subunit (Angelo et al; 2000). The interaction of AKAPs with PKA is disrupted by peptide likes Ht31 (McConnachie et al; 2006, Carr et al; 1992). Interestingly, some AKAPs like AKAP250, also known as gravin, are PKA phosphorylated. PKA phosphorylation is crucial for association of gravin to β_2 AR and subsequent resensitisation of the receptor (Tao et al; 2003). To date more than 50 AKAPs have been identified and they show sparse homology. However, they share some a common R_{II} subunit-binding domain (Wong and Scott; 2004).

PDE4 isoforms have been shown to interact with certain AKAPs (Dodge K. L., *et al* 2001 and Asirvatham A. L *et al*. 2004). PDE4D3 has been reported to bind mAKAP (muscle–selective) and AKAP 450. AKAP 450 interacts with the UCR2 of PDE4D3, whereas mAKAP interacts with the N-terminal region of PDE4D3 (Dodge K. L., *et al* 2001).

As mentioned previously, PDE4D3 can be phosphorylated by PKA at two sites, namely Ser^{13} and Ser^{54} (Sette and Conti 1996). Phosphorylation of PDE4D3 at Ser^{54} by PKA bound to mAKAP leads to activation of the enzyme. This results in increased activity of PDE4D3, which decreases the local cAMP levels. This in turn, leads to deactivation of the mAKAP bound PKA, leading to the dephosphorylation and deactivation of PDE4D3 itself. Thus these components form a signalling complex with unique feedback control (Dodge K. L., *et al* 2001). PKA phosphorylation at Ser^{13} of PDE4D3 increases the affinity of the isoform for mAKAP (Carlisle Michel et al; 2004) but does not alter the activity of PDE4D3.

It has been shown that mAKAP recruits PDE4D3 to the nuclear envelope where the isoform is shown to be in a complex with PKA, the ryanodine receptor (RyR) and protein phosphatase 2A. This complex is believed to regulate the function of RyR by modulating PKA phosphorylation of the receptor as shown by elevated PKA phosphorylation of the receptor in PDE4D deficient-mice cardiac tissue (Lehnart et al; 2005 and Kapiloff et al; 2001).

PDE4A interacts with AKAP95 and AKAP149 in T lymphocytes (Asirvatham et al. 2004). AKAP95 has been identified in the nucleus of T lymphocytes and is known to interact with c-Myc binding protein. AKAP149 is known to be present in cytoplasm of the T cells and binds to phosphatase PP1 (Asiravatham *et al*. 2004). Since these AKAPs are organelle-specific and they bind to PKA and specific forms of PDE4s, it is likely these interactions play an important role in regulating cAMP homeostasis in T cells.

AKAPs have been shown to be involved in a wide range of functions. They are involved in sperm mobility; aquaporin-mediated water reabsorption in renal collecting ducts; cell cycle progression and gene regulation (Bajpai et al; 2006, McSorley et al; 2006, Tasken and Aandhal; 2004).

1.8.4 *XAP2 immunophilin*

Immunophilins are key targets for immunosuppressive drugs including cyclosporin and rapamycin. Most immunophilins are known to have a characteristic amino acid sequence known as the Immunophilin Domain (Bolger et al; 2003). This domain in many immunophilins has a rotamase activity, which is targeted by the drugs. Rotamases are those enzymes that enhance the rate of protein folding (Avramut and Achim, 2002). Immunophilins are also known to have protein chaperone function (Bolger et al; 2003). XAP2 (AIP/Ara9) is an immunophilin that does not have these general functions (Bolger, G.B. *et al* 2003).

XAP2 or Hepatitis B virus X-associated protein, also known as ARA9 or Aryl hydrocarbon receptor (AhR) Interacting Protein (AIP), has been shown to form

a complex with Hsp90 and the dioxin receptor regulating the stability of the complex (Meyer and Perdew; 1999 and Kuzhandaivelu et al; 1996). XAP2 is also associated with regulation of transcriptional activity of both X-HBV and AhR (Meyer et al; 2000 and Meyer et al; 1998). XAP2, along with Hsp90, regulates the transcription of other proteins such as the peroxisome proliferator in association with peroxisome proliferator-activated receptor alpha (PPARalpha) (Sumanasekera et al; 2003).

PDE4A5 is targeted to both insoluble and soluble fractions of the cell (Beard, M.B. *et al*, 2002). Bolger and coworkers (Bolger et al 2003) showed that XAP2 specifically interacts with PDE4A5. This interaction requires the tetratricopeptide repeat (TPR) within the N-terminal region of XAP2, which interacts, with the unique N-terminal region of PDE4A5 and a motif within UCR2. Arg^{271} within XAP2 TPR interacts with an EELD motif in UCR2 of PDE4A5. The functional significance of this interaction is inhibition of PDE4A5 activity by XAP2 in a non-competitive way. This is mediated through a regulatory region extended to the cAMP-binding site in PDE4A5, presumably UCR2. This interaction also evokes a decrease in sensitivity of the isoform towards inhibition by the specific PDE4 inhibitor, rolipram. Furthermore, binding of XAP2 acts to inhibit PKA phosphorylation of PDE4A5 at Ser^{140} in UCR1. This indicates that XAP2 and UCR2 bind to similar regions within UCR1 of PDE4A5. This suggests that XAP2 blocking of PKA phosphorylation of UCR1 leads to changes in the conformation in UCR1 thereby preventing it from interacting with UCR2. The functional significance this interaction and subsequent effects on PDE4A5 activity are yet to be fully appreciated. However, given the expression profiles of PDE4 isoforms, particularly the PDE4A subfamily in inflammatory cells and the role of AhR and complexes containing AhR in inflammatory diseases like COPD, it could be argued that PDE4A and XAP2 might play an important role in mediating inflammatory responses (Wallace et al; 2005, Teske et al; 2005, Matinez et al; 2002). In support of this, the PDE4A4 isoform has been reported to be significantly elevated in monocytes from smokers (Barber et al; 2004).

1.8.5 *SH3 binding domains*

Src, Lyn and Fyn are Src homology 3 (SH3) domain containing tyrosine kinases that regulate the function of a wide variety of cellular proteins. They are globular proteins and can be divided into two classes depending upon their binding consensus motifs. Class I SH3 binding domains have a P-X-X-P motif, and confer specificity to interaction with Src Kinases (Ramos-Morales et al; 1994). Class II SH3 binding sites are variable and bind to motifs such as X-P-X-X-P-R and confer specificity to interactions with proteins including Crk and abl (Alexandropoulos et al; 1995). In some instances, SH3 binding domains are known to bind proteins that do not contain this consensus sequence but have positively charged sequences (Jia et al; 2005 and Pawson; 1994).

PDE4A5 and PDE4D4 have been shown to be able to bind to SH3 domains of Lyn, Fyn and Src kinases. PDE4D4 has also been identified to bind the SH3 domains of fodrin and abl tyrosine kinases. PDE4A5 has a P-X-X-P motif whereas PDE4D4 has polyproline stretches (Beard M.B *et al*, 1999, McPhee et al; 1999 and O'Connell, 1996). The interaction was mapped to the N-terminal region PDE4A5, the truncation of which prevented the tyrosyl kinase interaction. The probable function of such an association may be is to provide a means of regulating the affinity of PDE4A5 for cAMP and PDE4 inhibitors. It is suggested that SH3 domains bound to PDE4A5 initiate conformational changes in LR2 region of the enzyme thus increasing its affinity to rolipram thereby converting it to the HARBS conformation (McPhee et al; 1999). The interaction of PDE4A5 with SH3 binding domains is also known to change the subcellular localisation of PDE4A5 (Beard et al; 2002 and Huston et al; 2000).

Caspases are enzymes involved in initiating apoptotic cell death and their substrates have a consensus motif Asp-Xaa-Xaa-Asp within which cleavage by caspase occurs. Caspase-3 cleavage of PDE4A5 N-terminal region prevents its interaction with Lyn and ablates the ability of the isoform to localise to the perinuclear region (Huston et al; 2000). Caspase cleavage is

specific to PDE4A5 and is not seen in other PDE4A isoforms (Wallace et al; 2005).

Since PDE4A5 is localised to membranes, it is also possible that PDE4A5-Src interactions might contribute to a reduction of local concentrations of cAMP surrounding the tyrosyl kinase and other proteins recruited to complexes at the plasma membrane. This would provide local and specific control of actions of cAMP, possibly mediated through PKA action, on such tyrosyl kinases and their associated complexes. The association of PDE4A5 N-terminal region with SH3 domains might also help in regulating the activity of the PDE4A5 and targeting PDE4A5 to membrane and cytoskeletal fractions of the cell (O'Connell, 1996).

1.8.6 *Myomegalin*

Myomegalin is a scaffold protein related to *Drosohphila centrosomin* protein. The C-terminus of Myomegalin interacts with UCR2 region of PDE4D3 in yeast-two hybrid assays (Verde et al; 2001). Myomegalin is composed of α-helical and coiled-coil structures and shares domains with microtubule associated proteins. It co-localises with PDE4D3 at the sarcoplasmic reticulum in cultured skeletal muscle cells and is though to anchor the PDE4D3 to golgi/centrosomal regions in Cos cells (Verde et al; 2001).

1.8.7 *Disrupted in Schizophrenia-1*

Disrupted-in-Schizophrenia 1(DISC1) was first reported to be linked to schizophrenia upon the observation that the DISC1 was disrupted by translocation, leading to DISC1 haploinsufficiency in cases of familial schizophrenia (Millar et al; 2000). More recently, a translocation within the PDE4B gene has been linked to instances of inherited schizophrenia (Millar et al; 2005).

DISC1 is thought have as many as 5-7 isoforms that differ from each other in their C-termini and share a common N-terminal region although some

isoforms like c-75kDa lack N-terminal regions that are shared among the isoforms and is thought to exist as homodimer. DISC1 expression is cytoplasmic but can also be detected at the mitochondria and nucleus (James et al; 2004).

The N-terminal region of DISC1 has been shown to interact with the UCR2 region of isoforms from all PDE4 subfamilies with subfamily-specific differences in sensitivity to dissociation from DISC1 in the presence of elevated cAMP (Millar et al; 2005; Murdoch et al; 2007). An N-terminal truncated DISC1 was found to interact with PDE4B1 dynamically whereby its interaction with PDE4B1 was attenuated by elevating cellular cAMP levels or by PKA activation (Murdoch et al; 2007). However, this dynamic interaction was not observed for full-length DISC1, where cAMP elevation failed to dissociate the PDE4B1-DISC1 whilst allowing for the release of isoforms PDE4D3 and PDE4C2. The possible reason for this is that the full length DISC1 isoform has extra binding sites for PDE4B1 within its N-terminal region which is not present in the shorter–isoform, thus making it insensitive to the actions of cAMP/PKA (Murdoch et al. 2007).

Missense mutations within in the DISC1 gene, which were found to elicit schizophrenia-like phenotype in mice, were revealed to be located within DISC1's PDE4B1-specific binding regions (Clapcote et al. 2007). This would suggest that alterations in PDE4B1-DISC1 interactions, and associated cAMP signalling, might confer susceptibility to schizophrenia (Millar et al; 2007).

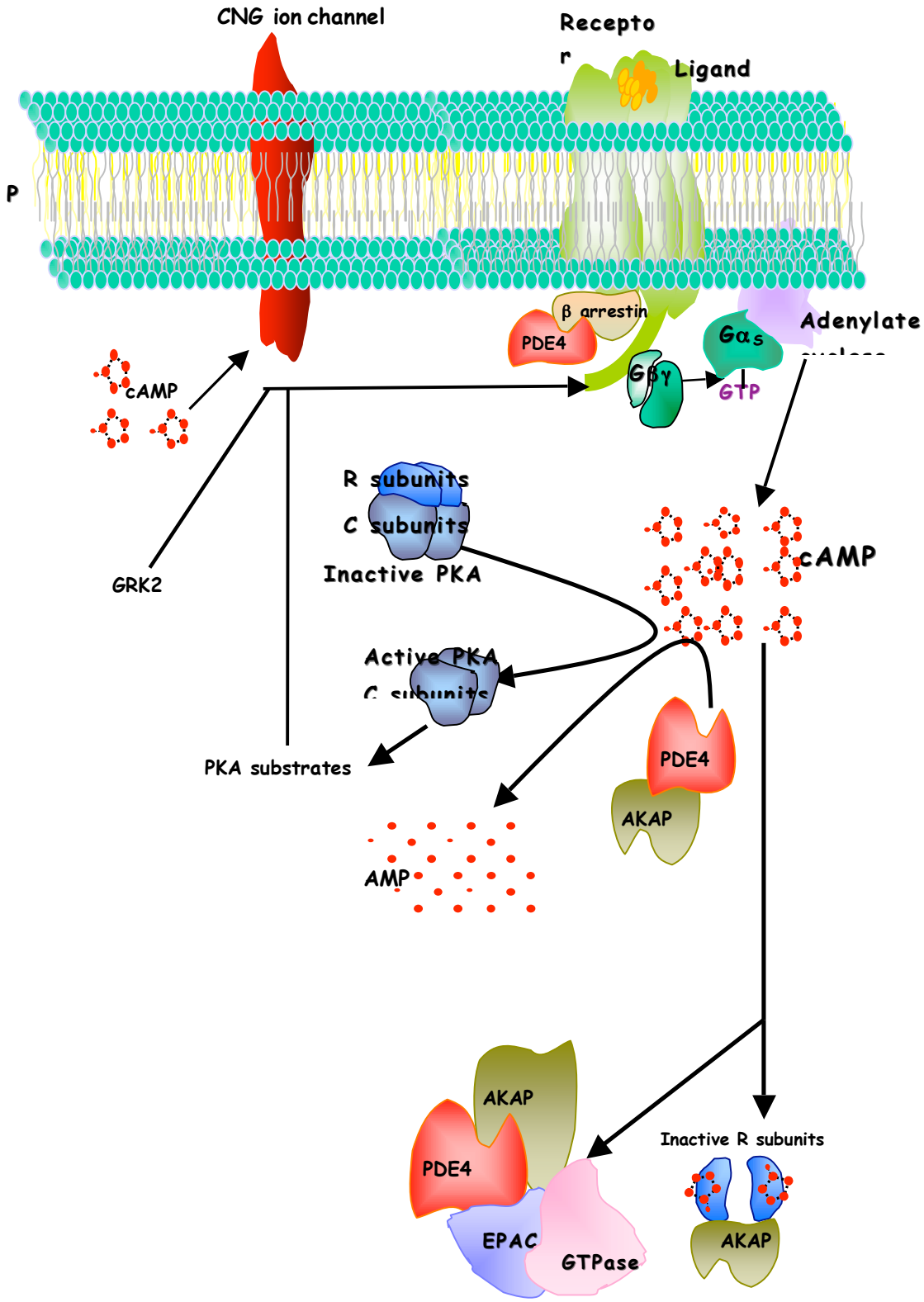


Figure 1.5 - Schematic presentation of the cAMP signalling system

1.8.8 PDE4 oligomerisaton

The concept of PDE4 oligo/dimerisation has been proposed by various studies (Richter and Conti, 2004, Richter and Conti; 2002 and Lee et al; 2002, Rocque et al; 1997). Rocque and coworkers suggested that purified PDE4B2 exists as a dimer and showed that it exhibited different affinity for rolipram compared to its monomeric form. Mutating the N-terminal region the isoform drastically reduced its ability to dimerise.

PDE4 oligomerisation was further characterised by Richter and Conti who showed that UCR1 and UCR2 might also have a role in oligomerisation of PDE4 long forms. Studies from our group (Beard et al 2000) originally showed that the C-terminal half of UCR1 and the N-terminal half of UCR2 of PDE4D3 interact with each other internally. Richter and Conti suggested that this interaction might be switched from internal to external allowing UCR1 to interact with UCR2 on a dimer and *vice versa*. Considering positively charged residues Arg-98 and 101 contained within the UCR1 domain of PDE4D3 interact with negatively charged residues Glu^{146}, Glu^{147}, and Asp^{149} present within the UCR2 domain of the same molecule (Beard et al 2000), Richter and Conti proposed that these interactions may also occur between two molecules of PDE4 isoforms thus leading to dimerisation (Richter and Conti, 2004). Richter and Conti demonstrated that PDE4D3 can dimerise and that disruption of dimerisation ablates the PDE activity of the isoform mediated by either PKA phosphorylation or by phosphatidic acid binding (Richter and Conti; 2004).

Others (Lee et al; 2002) have suggested that the PDE4 C-terminal region is necessary for oligomerisation. It was shown that C-terminal truncates of PDE4D failed to oligomerise whereas full-length PDE4D and the Catalytic domain were able to oligomerise. Arg^{358} was identified as a crucial residue in dimer interface which when mutated, prevented dimerisation. This finding was also supported by gel filtration chromatography analysis, where full-length isoforms existing as oligomers eluted first, followed by the Arg^{358} mutant, which was present as a monomer. Mutational analysis identified key residues

putatively involved in dimerisation, namely Glu^{315}, Asn^{321}, Asp^{322}, Asn^{328}, Arg^{358}, and Ile^{362}. These residues are highly conserved in PDE 1, 3, 4, 8 and 9 subfamilies. These observations are contradictory to the model for PDE4 dimerisation as suggested by Richter and colleagues (Richter and Conti; 2002) in which it is argued that the upstream conserved regions are involved in dimerisation. Although further investigation is needed to confirm the sites of dimerisation, it is likely that UCR1, UCR2 and catalytic regions contribute to dimerisation of PDE4 isoforms.

Functional significance of PDE4 dimerisation is yet unknown. Beard and coworkers (Beard *et al*, 2000) showed that UCR1 and UCR2 interact with each other to form a regulatory module, which has a role in the activation of PDE4 long forms upon PKA phosphorylation. The long form splice variants of PDE4 as discussed above are proposed to exist as dimers due to inter-molecular interaction of UCR1-UCR2 regions; they show higher sensitivity to inhibitors like rolipram after phosphorylation by PKA. This is in agreement with the previous findings that purified PDE4B2 and PDE4A isoforms show a higher affinity to rolipram when present in oligomeric state (Rocque et al, 1997 and Jacobitz et al 1996). The formation of this high affinity state for rolipram binding has been shown to involve residues outside the catalytic region towards the N-terminal region of the protein (Rocque et al, 1997). Dimerisation helps in stabilising the PDE4 long forms, so that they exhibit a high affinity for rolipram binding state. However, it has been suggested that although dimerisation stabilises the long forms and helps in maintaining the high affinity rolipram binding state, it is not required for the HARBS conformation as there are fewer rolipram binding sites in the monomeric state compared to the dimeric form (Richter and Conti 2004).

It has been shown that dimerisation modulates the activity of long PDE4 forms upon modifications including phosphorylation, ligand binding, and protein-protein interaction (Richter and Conti 2004) and that dimerisation may be a requirement for enzyme activation.

1.8.9 Foci formation

PDE4 isoforms PDE4A4B and to some extent PDE4A5, have been shown to undergo dynamic redistribution and form accretion foci after chronic treatment with the PDE4 selective inhibitor rolipram (Terry et al; 2003). This process is triggered by HARBS-sensing PDE4 inhibitors such as rolipram and RO 20-1724 but not by LARBS-sensing inhibitors such as Ariflo®. Protein synthesis is crucial for formation and maintenance of foci (Terry et al; 2003). It is thought PDE4A4 foci formation is mediated by conformational changes produced upon binding of the inhibitor. The unique N-terminal region of PDE4A4 is also thought to contribute to the process, as other isoforms of the PDE4A subfamily are not known to form foci.

It has been noted that the triggering of HARBS conformation upon PDE4 inhibitor binding is associated with the side effects produced upon treatment with PDE4 inhibitors (Martin et al; 2002 and Souness et al; 1997). The ability of a PDE4 inhibitor to induce PDE4A4 foci formation may therefore serve as an important screen for identifying potentially therapeutic PDE4 inhibitors.

1.9 *PDE4 isoforms in diseases- PDE4 inhibitors as therapeutics agents*

PDE4 inhibitors may serve as therapeutic agents in diseased states. PDE4 inhibitors are known to elicit anti-inflammatory effects (Cheng and Grande; 2007) and have also been used in the treatment of many CNS disorders such as depression (Cheng and Grande; 2007, Dastidar et al; 2007, Houslay et al; 2005). The potential therapeutic value of selective PDE4 inhibitors has been assessed in PDE4 knockout studies. In mice the knock down of the *PDE4B* gene decreased production of tumour necrosis factor (TNF) upon exposure to lipopolysaccharide (LPS) (Jin and Conti; 2002). However, knockdown of PDE4D in mice did not alter TNF production. However, PDE4D knockdown has been shown to attenuate the muscarinic-cholinergic response leading to the loss of hyper-reactivity of the airways (Hansen *et al in* 2000). These studies indicate that there is little or no redundancy in the roles of the PDE4 subfamilies and pave the way for development of PDE4 inhibitors that can

selectively inhibit the activity of the isoforms in various inflammatory tissues thus offering a promising treatment for inflammatory diseases including arthritis and asthma. The sequence similarity of the catalytic sites for these enzymes will make it difficult to design sub-family or isoform specific inhibitors successfully. However, a recent study (Wang et al; 2007) has shown the PDE4 inhibitor NVP {4-[8-(3-nitrophenyl)-[1, 7] napthyridin6-yl]benzoic acid} binds the same conformation in the cAMP and inhibitor binding pocket of all PDE4 subfamilies but the PDE4A and PDE4C subfamilies show significant differences in key residues that are displaced by inhibitor binding. This indicates that there is a possibility to design subfamily-specific PDE4 inhibitors.

It has been reported that the PDE4B2 isoform inhibits the cAMP-induced apoptotic cell death in B cells, thus leading to Diffuse Large Cell B Cell lymphoma (Smith et al; 2005). It is therefore possible that inhibition of this isoform may induce death of the malignant cells and serve as a therapeutic agent in the treatment of lymphomas.

Follicle stimulating hormone (FSH), Human chorionic Gonadotropin (hCG) and Luteinizing hormone (LH) are the principal hormones that regulate ovulation. It was noted that PDE4 inhibition in FSH-primed rats, untreated with LH and hCG, stimulated ovulation indicating that PDE4 function modulates the signalling mechanisms involved in the ovulation (McKenna et al, 2005). The rat oocytes produced thus were found to be as equally fertile as oocytes produced by untreated rats. PDE4 inhibitors may therefore be useful as therapeutic agents in infertility conditions with hCG and LH defects (McKenna *et al* 2005).

It is now well established that smooth muscle contraction and relaxation is regulated by cyclic nucleotides cAMP and cGMP which act as secondary messengers and regulate many downstream pathways and are associated with many physiological responses (Abdel-latif AA 2001). Myometrial smooth muscle cells express five different types of PDEs (1-5), of which, PDE4 family is the most predominant with PDE4 activity comprising 75% of the cAMP -

specific PDE activity in late pregnancy. PDE4D and PDE4B are the most predominant PDE4 isoforms expressed in human myometrium; especially PDE4B2 is selectively induced in the myometrium at the late stage of pregnancy (Mehats et al; 2005). Elevation of cAMP levels led to increase of PDE4 activity due to induction of PDE4B2, PDE4D1 and PDE4D2. β-agonists can relax a non-pregnant myometrium but not that of a pregnant myometrium, indicating the β adrenergic receptors of pregnant myometrium are desensitized. However, PDE4 inhibitors can reverse this effect and potentiate the relaxant functions of β-agonists. It is therefore likely that selective PDE4 isoforms are involved in regulating the contractile functions of myometrium and altered PDE4 activity/expression within the myometrium may induce pre-term labour (Méhats et al. 2004).

Prostaglandin E_2 (PGE_2) induces PDE4B2, PDE4D1 and PDE4D2 expression in a cAMP-dependent manner. PGE_2 also mediates heterologous desensitisation of β-adrenergic receptors inhibiting their relaxant functions, an effect which is reversed by the presence of PDE inhibitors. PDE4 inhibitors may therefore be of use to increase the tocolytic efficiency with β-mimetics so as to prevent premature births (Méhats et al, 2004).

Bones are formed through the action of three important cell types, osteoblasts, osetoclasts and osetocytes respectively. Osteoblasts are involved in bone formation. Osteoclasts are involved resorption and osteocytes comprise the ossified part of the bone. Osteoblasts when stimulated by factors such as 1, 25$(OH)_2$D3, parathyroid hormone, interleukin-6, prostaglandin E_2 stimulate production of a cytokine known as TNF-Related Activation Induced Cytokine (TRANCE). TRANCE then activates the signalling pathways leading to differentiation of osteoblasts to osteoclasts. This process is inhibited by osteoprotergerin (OPG), a decoy receptor for TRANCE, which is also produced in osteoblasts (Takami M et al, 2005). Recent studies have revealed that PDE4 enzymes are involved in regulating the expression of TRANCE, as determined by increased levels of TRANCE after treatment of osteoblasts with PDE4 inhibitor rolipram (Takami et al;

2005). The effect of PDE4 inhibition appears to be mediated through a PKA-dependent pathway as PKA inhibitors were shown to attenuate IBMX-induced TRANCE expression in osteoblasts (Takami et al; 2005). Furthermore, it was shown that the PDE inhibitor-treated osteoblasts elicited increased activity of ERK and p38 MAPKand that treatment with ERK inhibitor PD98059 and p38 MAPK inhibitor SB 203580 reduced the rolipram-induced TRANCE expression in osteoblasts. These findings are indicative of a cAMP-PKA-PDE4 pathway regulating the expression of TRANCE via ERK and p38 activation (Takami M *et al*, 2005). PDE4 activity may therefore regulate bone cell differentiation induced by TRANCE.

PDE4 genes have also been implicated in osteoporosis, a disease associated with a decrease in osteoblast levels (Reneland et al; 2005). Rolipram-mediated inhibition of PDE4 leads to elevation of alkaline phosphatase activity, a marker for the differentiation of osteoblasts to osteoclasts (Reneland et al; 2005). Inhibition of PDE4 also induces increased expression of osteopontin, osteocalcin and collagen type I which mark osteoblast differentiation suggesting a role of PDE4 enzymes in bone disorders including osteoporosis (Reneland et al; 2005).

Glucocorticoids and Parathyroid hormone (PTH) play a crucial role in regulating bone metabolism. PTH is known to decrease bone resorption (loss) by reducing osteoblastic (bone forming cells) apoptosis via a cAMP-dependent pathway (Ahlstorm, M. et al, 2005). Glucocorticoids, in contrast, decrease osteoblast levels, potentially leading to osteoporosis (Ahlstorm, M. et al, 2005).

Evidence also suggests that PDE4 may mediate the effects of dexamethasone-regulated cAMP signalling in the osteoblasts. Synthetic glucocorticoid Dexamethasone is known to a decrease cAMP-specific PDE activity in MG-63 and SaOS-2 osteosarcoma cells (Ahlstorm, M. *et al*, 2005). Dexamethasone treatment also leads to reduced rolipram sensitivity of the total cAMP-PDE activity (Ahlstorm, M. *et al*, 2005). The reduction in cAMP response and the decrease in sensitivity to rolipram indicate an altered PDE

profile in the osteosarcoma cells. In support of this, it was noted that there is a massive decrease in the expression of PDE4A4 and PDE4B1 isoforms in these cells (Ahlstorm, M. *et al*, 2005).

Glucocorticoids act as anti-inflammatory and immuno-suppressive agents by inhibiting prostaglandin biosynthesis (Ahlstorm, M. *et al*, 2005). They mainly inhibit the cyclo-oxygenase activity thereby reducing the levels of PGE_2 whose functions are mainly anabolic. However, PDE4 inhibitors rolipram and denbufylline have been shown to increase the PGE_2-mediated anabolic effects and subsequent bone formation in rat bone marrow cells (Ahlstorm, M. *et al*, 2005). All these studies indicate glucocorticoid-induced bone loss is mediated through a PDE4-dependent pathway (Ahlstorm, M. *et al*, 2005).

Rolipram is the first generation PDE4 inhibitor to be used as an anti-depressive agent (Scott et al; 1991). However, the side effects (e.g. nausea) associated with usage of rolipram have limited its role as a therapeutic agent (Scott et al; 1991). However, with the advent of second-generation PDE4 inhibitors such as roflumilast and cilomilast, which exhibit anti-inflammatory and CNS effects, PDE4 inhibitors are emerging as potentially useful therapeutic agents (Houslay et al; 2005). However, neither of these compounds successfully passed phase-II clinical trials, revealing side effects and efficacy problems still need to be resolved in order to market PDE4 inhibitors successfully for therapeutic use (Geimbycz, 2006).

1.10 *SUMOylation*

Ubiquitylation is a post-translational modification involving the attachment of the 76-amino acid protein ubiquitin to its substrate protein, which will then mark its substrate for proteasomal-degradation (Zhao et al; 2007).

Small Ubiquitin-related Modifier (SUMO) belongs to a family of ubiquitin-like proteins that are closely related to ubiquitin and are covalently attached to substrates as a part of their post-translational modification (Kerscher; 2007 and Johnson; 2004). SUMO was first discovered in mammalian cells and has

since been reported in all eukaryotic organisms where it is known to be involved in a wide range of functions. SUMO is also known as, Pmt2p, PIC-1, GMP-1, Ubl1 and sentrin (Johnson, 2004).

1.10.1 *Components of SUMO conjugation pathway*

SUMO conjugation pathway is very similar to ubiquitin pathway both in the process and in the components. However, many functional differences do exist between the ubiquitin and SUMO (Kerscher, 2006). Please refer to figure 3.1

1.10.1.1 *SUMO genes and isoforms*

In mammals SUMO is encoded by four SUMO genes, which encode SUMO molecules 1-4 (Geiss-Friedlander and Melchior, 2007). SUMO-2 and 3 share 95% sequence homology and 50% sequence homology with SUMO-1. SUMO-4 differs from the other three SUMOs in that it can form covalent bonds with its substrate. (Owerbach et al 2005, Bohren et al; 2004, Johnson; 2004, Melchior et al; 2003, Hayashi et al; 2002, Apionishev et al; 2001). SUMO-1 differs from SUMO-2 and SUMO-3 in that the latter two form poly SUMOylation chains whereas SUMO1 does not form poly SUMO chains. This unique ability of SUMO-2 and 3 is attributed to the presence of a consensus on the target molecule SUMO modification site ΨKXXE where Ψ is an aliphatic residue and X is any amino acid. This consensus sequence is not present in SUMO-1 suggesting that the differences within their primary structures contribute to their distinct functions (Tatham et al; 2001). In support of this, it was shown that in plant cells SUMO-2 and SUMO-3 levels were elevated in response to stress whereas SUMO-1 levels were unaltered under similar conditions (Lois et al; 2003).

1.10.1.2 SUMO-activating enzyme (E1)

SUMO molecules exist in an inactive state until processed by SUMO-activating enzyme. SUMO-activating enzyme is a heterodimer consisting of two Aos1 (SAE1) and Uba2 (SAE2) units. Aos1 is structurally analogous with

the N-terminus of ubiquitin activating enzyme Ub E1, whereas Uba2 is structurally similar to the C-terminus of Ub E1, which contains the enzyme's active site (Johnson et al; 1997).

1.10.1.3 SUMO-conjugating enzyme (E2)

UBC9 is the universal SUMO-conjugating enzyme in all organisms with a SUMO conjugation pathway (Hayashi et al; 2002). It is structurally and functionally similar to Ub conjugation enzyme Ub-E2 (Johnson and Blobel, 1997).

1.10.1.4 SUMO ligases (E3)

There are three classes of E3 ligases, namely Protein inhibitor of Activated STAT (PIAS), RNBP2/NUP358 and polycomb group protein PC2 (Johnson; 2004).

PIAS proteins are inhibitors of the Signal Transducer and Activator Transcription (STAT) class of proteins, which are involved in the JAK-STAT signalling pathway. There are four different PIAS proteins PIAS1, PIAS3, PIASx and PIASy (Jackson; 2001 and Gross et al; 2001). All PIAS proteins have been shown to possess SUMOylation (E3 ligase) activity and show substrate-specificity. (Chun *et al*; 2003 and Miyauchi *et al*; 2001). All PIAS proteins have a conserved N-terminus with a variable C-terminus. These proteins contain SAP, SP-ring and SIM domains from the N-terminus to the C-terminus. The SAP domain (SAR, Acinus and PIAS domain), is involved in chromosomal organisation by interacting with DNA at AT-rich regions (Aravind et al; 2000). The SP-ring domain contains the E3 ligase activity. The SIM domain (SUMO interaction motif) contains an S/T-X-X-D/E consensus motif, which is thought to mediate interaction with SUMO. This SIM consensus sequence is also seen in SAE-2 and RanBP2 (Minty et al; 2000).

RanBP2, also known as Nup358, is a nuclear GTPase that forms a complex with chromatin-bound guanosine nucleotide release factor RCC1. This

RanBP2-RCC1 complex pore protein localises to the cytoplasmic side of nuclear membrane. It possesses a leucine-rich N-terminal region and eight zinc finger motifs. The C-terminus contains regions homologous to cyclophilins (Yokoyama et al; 1995). RanBP2 was shown to have SUMO E3 ligase activity similar to PIAS proteins. RANBP2 interacts with UBC9 bound SUMO-1 and transfers the SUMO to SP100. E3 ligase activity of the protein is evoked by two repeats of a unique 50-amino acid region known as internal repeat domain, IR0) which is not seen in any other E3 ligases (Pichler et al; 2004 and Pichler et al; 2002).

Pc2 is recently discovered E3 ligase and belongs to a class of proteins called polycomb groups that are known to be involved in gene repression. Pc2 does not share any homology with other E3 ligases (Kagey et al; 2003).

1.10.1.5 *SUMO deconjugating enzymes*

Seven genes encode various SUMO deconjugating enzymes that are known to cleave SUMO from SUMOylated proteins in mammals. Some cleave the SUMO precursor as well as SUMO from SUMOylated proteins (e.g.; Ulp1) while other members only function in latter process (Melchior et al; 2003). All SUMO cleavage enzymes have a conserved C-terminal domain named the Ulp (Ubiquitin like protease) domain, which has isopeptidase activity. The enzymes have a variable N-terminal domain that is regulatory in function and targets the enzyme to specific sub-cellular compartments (Johnson et al 2004).

1.10.2 *SUMO conjugation pathway*

SUMOylation conjugation involves three enzymatic steps. In summary, a SUMO molecule is activated in an ATP-dependent manner by SUMO activating enzyme 1, with the SUMO deconjugating enzymes contributing to this process. This activation leads to maturation of the SUMO molecule. The SAE1/SAE2 complex then transfers the matured SUMO to conjugating enzyme E2, UBC9. It is then transferred from UBC9 to its substrate, a

process, which requires the substrate-specific E3 ligases (Kerscher; 2006). A more detailed explanation of this pathway is described in Chapter 3.

1.10.3 Regulation *of SUMO conjugation pathway*

The mechanisms regulating SUMO conjugation are poorly understood. There is no evidence for a common signalling pathway for SUMOylation of substrates. Most of SUMO consensus motifs in a vast number of proteins are sites for other post-translational mechanisms such as acetylation and ubiquitinylation. For example, the acetylation sites of Sp3, a transcription factor, are also a part of SUMO consensus motif thus acetylation and SUMOylation compete for the same sites and may have an inhibitory affect on each other (Braun et al; 2001). Similarly, ERK phosphorylation and SUMOylation of ELK-1 seems to elicit opposing effects on each other as well as on the activity of the transcription factor (Yang et al; 2003).

Gam1, an adenoviral gene product, is a unique protein that the inhibits SAE1/SAE2 heterodimer complex both *in vivo* and *in vitro* thus elevating unprocessed SUMO molecules and consequently inhibiting the SUMO conjugation pathway and promoting loss of SUMO-1 from the nucleus (Boggio et al; 2004 and Colombo et al; 2002). Gam1 forms a complex with SAE1/SAE2 in a dose-dependent manner and inhibits the *de novo* SUMO conjugation of substrates. Furthermore, it has been shown to decrease the levels of both SAE1/SAE2 and UBC9 proteins through regulation of transcription. Gam1 was found not to affect the conjugation pathway in the presence of preformed SAE1/SAE2 heterodimer complexes suggesting its inhibitory function is partially mediated through its ability to prevent the formation of heterodimer complexes in cells (Boggio et al; 2004).

1.11 Small GTPases

Rho GTPases belong to Ras superfamily of GTPases along with other subfamilies including Ras, Rab, Ran and Arf. Rho GTPases are small GTPases that regulate actin cytoskeletal system in all eukaryotes (Aspenstrom et al; 2004). So far, 22 different Rho GTPases have been identified that can be divided into 8 groups, of which Rho, Cdc42 and Rac1 are the most studied (Jaffe and Hall; 2005). Classification and groups of Ras and Rho GTPases are shown in Table 1.3.

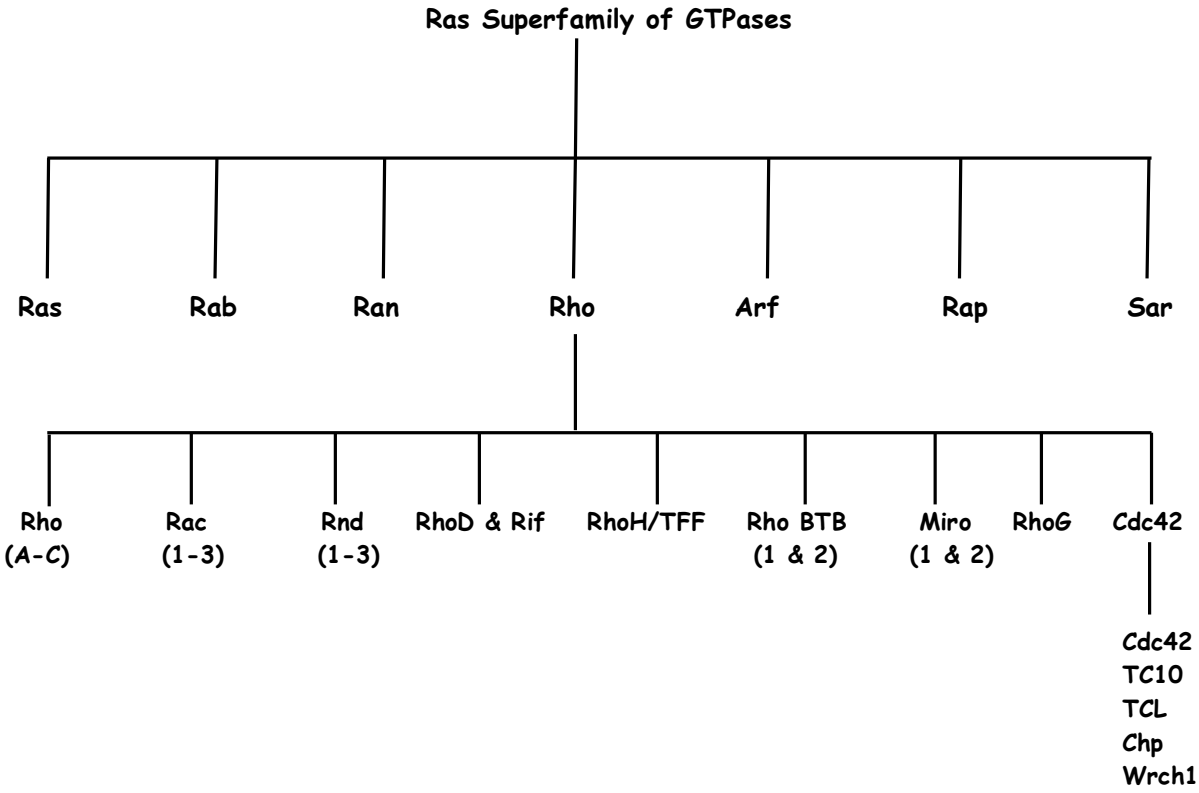


Table 1.3 Groups of Ras and Ras like GTPases

1.11.1 *GTPase cycle and regulation of Rho GTPases*

Rho GTPases cycle between a GTP-bound active and GDP-bound inactive state. Rho GTPase cycle is regulated by various proteins including GEFs (Guanine exchange factors), GAPs (GTPase activating proteins) and GDIs (Guanine nucleotide dissociation inhibitors). GEFs exchange GDP for GTP thereby activating Rho; GAPs switch on the intrinsic GTPase activity of Rho thereby inactivating Rho. GDIs block the dissociation of GDP from Rho thus blocking their activation (Jaffe and Hall; 2005, Aspenstrom; 2004, Luo; 2000 and Olofsson; 1999). In resting cells, Rho exists in a complex with GDI, which can be dissociated from Rho upon appropriate stimulation (Saski and Takai; 1998). Two regions called switch-I and switch-II play a crucial role in activation, inactivation and protein interactions of Rho GTPases (Dvorsky and Ahmadian, 2004). Figure 1.5 is a diagrammatical representation of the GTPase cycle of RhoGTPases.

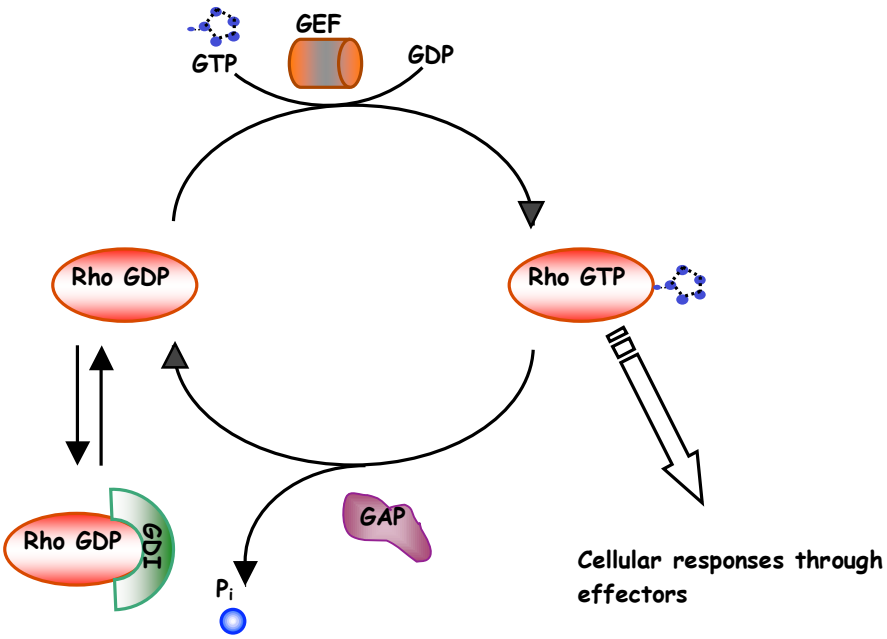


Figure 1.6 Schematic of GTPase cycle. Rho GTPase cycle is regulated by GAPs, GEFs and GDIs.

1.11.2 *Guanine exchange factors (GEF)*

Guanine exchange factors or GEFs, act by nucleotide exchange and GTPases are subsequently activated by the virtue of the higher levels of cellular GTP. GEFs lack preference for GDP or GTP and modify the nucleotide-binding site of G-proteins, thereby accelerating the release of the nucleotide from the G-protein so that GTP can replace it. The guanine nucleotide and GEF also compete for binding to the G protein thereby promoting the binding of the nucleotide to the G-protein (Bos et al; 2007).

GEF activity is regulated by various mechanisms including protein-protein/lipid interactions, secondary messengers and Post-translational modifications (Garcia-Mata and Burridge; 2006, Bos et al; 2007 and Ebinu et al; 1998). Most Rho-GEFs have been shown to contain a putative PDZ domain (Postsynaptic density protein PSD95; the *Drosophila* septate junction protein Discs-large; tight junction protein, ZO-1). PDZ domains mediate protein targeting and allow for the formation of multi-protein complexes thus providing a mechanism to limit individual GEF activity to various sub-cellular compartments. This has been reported for GEFs including Kalirin-7 and betaPIX exchange factor (Liu and Horowitz; 2006, Garcia-Mata and Burridge; 2006).

Second messengers including cAMP, calcium and DAG have all been shown to modulate the function of various GEFs as discussed previously in 1.5.2, cAMP is a regulator of EPAC; Ca^{+2} and DAG regulate Ras GEFs, Ras GRP1 and 2, which activate golgi-localised Ras in response to elevated levels of either of these second messengers (Bos et al; 2007 and Ebinu et al; 1998).

Tyrosine phosphorylation of the RhoGEF Vav by Lck and Src kinases within its Dbl homology (DH) domain is shown to disrupt the auto-inhibitory effect of the intra-molecular interaction of the Ac-DH domain leading to activation of the GEF (Aghazadeh et al; 2000).

1.11.3 *GTPase activating proteins*

GTPase activating proteins (GAPs) are regulators of G-protein signalling (RGS) and contain a 120 amino acid homologous RGS domain (Willars; 2006 and Popov et al; 1997). Approximately, 70 putative GAPs specific for RhoGTPases have been identified, 30 for Ras GTPases, five for Rap and one for Ran-GTPases (Bernards and Settleman; 2005).

The catalytic domains of GAPs from any two families are very different although GAPs of same subfamily share similar sequences (Boguski and McCormick; 1993). GTP hydrolysis by GTPases is very slow and requires a GAP to hasten the process. GAP-assisted GTP hydrolysis has been explained by two different models; in the first model, GAPs help stabilise the catalytic machinery of a GTPase that otherwise performs the hydrolysis at a slower rate (Moore et al; 1993 and Eccleston et al; 1991). In the second model, GAPs provide GTPases with an arginine finger residue (Arg^{789}), which stabilises the transition states of the active site (Scheffezek and Ahmadian, 2005 and Scheffezek et al; 1997).

RasGAPs contain a catalytic domain, PH domain and a C-2 domain (Conserved domain 2 in PKC). Some RasGAPs, like P120GAP, also contain SH2 and SH3 domains (Bernards and Settleman; 2004). Various regulatory mechanisms have been reported for individual RasGAPs. Serth and coworkers (Serth et al; 1991) showed that p21 is inhibited by lipid micelles in *vitro*. The PH domain of some RasGAPs, including GAP1m and GAPIP4BP, has been reported to induce translocation of the GAPs to plasma membrane (Lockyer et al; 1997).

RhoGAPs possess a conserved catalytic domain. Different RhoGAPs have various regulatory domains such as a BCR (Breakpoint Cluster Region) domain. RhoGAPs including RasGAPs can be regulated by phospholipids such as phosphotidylserine. In case of p190, a RhoGAP as well as a RacGAP, phospholipid binding has been shown to switch the GTPase preference with phospholipid binding inhibiting theRhoGAP activity of p190

and subsequently activating its RacGAP activity (Ligeti and Settlemen, 2006 and Ligeti et al; 2004).

RhoGAPs and other Rho proteins have been shown to play a significant role in pathogen invasion. Bacterial toxins pumped into host cell cytoplasm modify the Rho proteins and alter the dynamics of actin cytoskeleton to aid pathogen uptake into host cells (Scheffzek and Ahmadian; 2005).

RapGAPs contain RapGAP domain and differ from GAPs of other families in that they do not use arginine finger residue in regulating GTPase activity and utilise two lysine residues (Daumke et al; 2004 and Brinkmann et al; 2002). Some RapGAPs contain PDZ domains, which may serve to target this protein to specific subcellular locations like cytoskeleton as well as to other signalling molecules (Schefzek and Ahmadian, 2005)

RabGAPs are involved in vesicle trafficking. All Rabs identified share similar primary sequence but exhibit different substrate specificities. They function to regulate the activity of Rab GTPases within the same sub-cellular location (Bernards; 2002).

Arf GAPs are GAPs for ADP ribosylation factors (ARF). One of the major roles of ARFs is in regulating vesicle/endosomal trafficking in conjunction with the Rab proteins (Gaynor et al; 1998). ArfGAP activity is regulated by phosphorylation and phospholipid interaction (Menetrey et al; 2007).

RanGAP is a GAP for the Ran G protein, which is involved in nucleocytoplasmic shuttling of proteins. RanGAP1 is one of the first proteins identified to become SUMOylated. RANGAP1 associates with the nuclear-pore complex upon SUMOylation (Mahajan et al; 1998).

1.11. 4 *Guanine dissociation inhibitors (GDI)*

Guanine dissociation inhibitors (GDI) bind to GTPases and prevent the dissociation of GDP from the inactive GTPase. GDIs in turn, are regulated by

GDI dissociation factors (GDFs) (Dermardrossian and Bokoch; 2005, Olofsson; 1999). So far, three Rho GDIs have been identified that function to regulate the activity of Rho GTPases (Olofsson; 1999), RabGDIs function in a similar manner in regulating the RabGTPase cycle (Olofsson; 1999). It has been shown that RhoGDI is a substrate for phosphorylation by p21-activated kinase 1 (Pak1) leading to the dissociation of RhoGDI from RacGTPase. Pak1 action therefore indirectly promotes Rac activation (Dermardrossian et al; 2004).

1.12 Thesis Aims

The data presented in this thesis is divided into three distinct chapters. Each chapter focuses on different aspects of PDE4 function. Previous unpublished work from our group has shown that certain PDE4 isoforms can be SUMOylated *in vitro.* Chapter 3 aims to characterise the SUMOylation of PDE4 isoforms both *in vitro* and *in vivo* and to examine the effects of SUMOylation on PDE4 signalling and compartmentalisation of PDE4 signalling.

PDE4A4 has been shown to form accretion foci reversibly only in the presence of rolipram and its analogues and that this process is dependent upon protein synthesis (Terry et al; 2003). Considering that only PDE4 inhibitors that cause emesis lead to PDE4A4 foci formation and not the non emetic ones, PDE4A4 foci formation may be important in understanding the side-effects elicited upon treatment with PDE4 inhibitors. Chapter four therefore aims to indentify the signalling pathways involved in foci formation and maintenance.

PDE4 isoforms and their interacting partners like β-arrestins have been shown to play an important role in cell migration and stress fibre formation (Flemming et al; 2004 and Barnes et al; 2005). Chapter 5 describes the preliminary work in characterising the interaction of novel RhoGAP ARHGAP21/10 with PDE4D5 and β-arrestin.

Chapter 2 Materials and Methods

2.1 Materials

Throughout the following methods the names of the companies used for the purchase of specific reagents are given in parentheses. Where no company is mentioned, the reagent was purchased from Sigma-Aldrich. All reagents were of analytical grade.

2.2 Methods

All molecular biology techniques were undertaken in DNAase and RNAase free environment using sterilised equipment and aseptic technique. Buffers were of molecular biology grade and were sterilised by autoclaving or sterilised filtration.

2.2.1 Large scale production of plasmid DNA

A sample of *E.coli* bacteria carrying a plasmid containing the DNA of interest was taken from a frozen glycerol stock and transferred to a 2 L flask of culture medium (LB broth- 1% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast extract, 1% (w/v) NaCl distilled H_20) supplemented with antibiotic ampicillin or kanamycin (100µg/ml final concentration) depending upon the antibiotic resistance of the plasmid by means of a sterile pipette. The culture was incubated at 37ºC overnight in an orbital shaking incubator. The cells were harvested the next day by centrifugation at 6000 x g for 15 minutes using the JA-14 rotor in a Beckman refrigerated centrifuge. The plasmid DNA was extracted from the cell pellet using the Wizard maxi-prep kit (Promega®, UK) according to manufacturer's instructions. The principle of the method involves precipitating of DNA from cell lysates by isopropanol treatment followed by its purification using a silica resin.

DNA was precipitated from the eluted solution by addition of room temperature isopropanol followed by centrifugation at 15000 x g for 30 minutes at 4ºC. DNA pellet was then washed with 5 ml of room temperature 70% ethanol and air dried for 5-10 minutes before resuspended in 500 µl of distilled H_2O.

2.2.2 Small-scale production of DNA

QIAprep® Spin miniprep kit was used for this procedure. The principle of this method involves alkaline lysis of bacteria followed by adsorption onto a silica resin from which the DNA will be extracted by water. A sample of E.coli bacteria carrying a plasmid containing the DNA of interest was taken from a frozen glycerol stock was cultured in 5 ml of LB broth containing appropriate antibiotic was incubated at 37ºC overnight in an orbital shaking incubator. The cells were harvested the next day by centrifugation at 3000 x g for 10 minutes using a Jouan tabletop centrifuge. Pelleted cells were resuspended and subjected to alkaline lysis by lysis buffer provided by the manufacturer. DNA was adsorbed onto a silica spin column followed by a wash with ethanol. The column was air-dried and the DNA was eluted into sterile distilled H_2O.

2.2.3 Quantification of DNA

Both DNA and RNA concentrations were measured using a WPA lightwave spectrophotometer. 5 µl of DNA was diluted into 1ml distilled H_2O and the absorbance measurements were taken at 260 and 280 nM against distilled water as blank.

The concentration of nucleic acid was calculated using the following approximations:
An absorbance of 1 at 260 nM corresponds to 50 µg/ml double stranded DNA, 37 µg/ml single stranded DNA, 40 µg/ml single stranded RNA.

The ratio between the absorbance measurements at 260nM and 280nM provided an indication of the purity of the nucleic acid. In solution RNA and

DNA have A260: A280 ratios of 1.8 or 2.0 respectively. If the absorbance ratio is significantly less than this it indicates that the nucleic acid may be impure.

2.3 Site-directed mutagenesis

2.3.1 Primer designs

Site-directed mutagenesis is altering parental DNA by incorporating designed changes using engineered primers and polymerase chain reaction. While designing the 5'-primer the nucleotide bases that encode new amino acid were kept as close to the original sequence as possible. Depending upon the length of each primer varied between 45-50 nucleotides in length containing both desired mutation and flanked unmodified sequence in total. A melting temperature of 75ºC was considered good. 3' primer is reverse complement of 5' primer to amplify the other strand. G or C was always chosen for the first and last nucleotide of the primer as it ensures efficient bonding.

The following formula is commonly used for estimating the melting temperature Tm of the primers:

$$Tm\ (°C) \approx 2\ (N_A+N_T) + 4\ (N_G+N_C)$$

N equals the number of adenine (A), thymidine (T), guanidine (G), or cytosine (C) bases in the primer.

The 5' and 3'primers are designed for PDE4D5 SUMO K mutant (PDE4D5K323R) are shown below.

5'-primer CCCAAGGTTTGGAGTTCGAACTGAACAAGAAGATGTCC
3'-primer GGACATCTTTCTTGTTCAGTTCGAACTCCAAACCTTGGG

The base substitutions made to the K323R mutation are shown underlined.

2.3.2 Mutant strand synthesis reaction

Site directed mutagenesis was performed using the Quickchange site-directed mutagenesis kit according to manufacturer's instructions (Stratagene, UK). Primers designed in section 2.3.1 were custom synthesised and purified by HPLC by Thermo scientific®. The sample reactions then prepared in thermo-tubes (Abgene, UK) as below:

5 μl of 10x reaction buffer (supplied with the kit);
x μl (5-50 ng)of dsDNA template (See below);
x μl (125 ng) of oliconucleotide primer #1 (sense primer);
x μl (125 ng) of oligonucleotide primer #2 (antisense primer);
1 μl of dNTP mix (supplied with the kit);
Sterile, deionised H_2O to a final volume of 50μl

A series of sample reactions were set up with various amounts of dsDNA template while the primer concentration was kept constant. Once the reactions were set up 1μl of *pfu* turbo DNA polymerase (2.5U/μl) was added into each tube and mixed by vortexing. The reactions were cycled in a PTC-200 Peltier thermo cycler PCR machine using the parameters indicated below in table 2.1

Segment	**Cycles**	**Temperature**	**Time**
1	1	95°C	30 seconds
2	12	95°C	30 seconds
		55°C	1 minute
		68°C	2 minutes/kb of plasmid length

Table 2.1 Cycling Parameters for the QuickChange Site-Directed Mutagenesis Method.

Following temperature cycling, the reactions were placed on ice for 2minutes to cool the reaction to below 37°C.

2.3.3 Dpn I digestion of parental plasmid

1μl of the *Dpn I* restriction enzyme (1U/μl) (Stratagene, UK) directly was added to each amplification reaction and the samples were thoroughly mixed by pipetting the solution up and down. Then the reaction mixtures were centrifuged at 18,000 x g in a Heraeus microcentrifuge for 1 minutes and immediately incubated at 37°C for 1 hour to digest the wild type parental super coiled ds DNA.

2.3.4 Transformation of XL1-Blue supercompetent cells

The XL1-Blue supercompetent cells (Stratagene, UK) were gently thawed on ice. For each transformation 50μl of the supercompetent cells were aliquoted to a pre-chilled Falcon® polypropylene tube. 1μl of each *DpnI* treated DNA was then added to separate aliquots of the supercompetent cells, swirled gently to mix and were incubated on ice for 30 minutes. Transformation reactions were then heat shocked by incubating the samples at 42°C for 45 seconds in a water bath with and the samples were immediately transferred on to ice for 2 minutes. 0.5 ml of LB broth preheated to 37°C was added to transformation reactions and then incubated at 37°C for 1 hour. 250μl of each transformation reaction was spread onto an agar plate containing 100 ug/ml final concentration of ampicillin and incubated at 37°C for 16 hours. A number of single colonies were selected for analysis by plasmid preparation followed by DNA sequencing to verify successful mutagenesis.

2.3.5 Sequence analysis

Samples of DNA obtained from mutagenesis were sent to Baseclear® sequencing centre, The Netherlands. Routine DNA and deduced aminoacid sequence analysis were performed on the Genejockey II programme.

2.3.6 Glycerol stocks

A single colony was picked from an agar plate and for long term storage of *E.coli* containing a plasmid of interest was used to inoculate 5 ml of LB broth supplemented with appropriate antibiotic. The culture was grown overnight at 37°C overnight in an orbital shaking incubator. 1 ml of the overnight culture was transferred into a sterile eppendorff® tube with 500µl of sterile glycerol (Riedel-DeHaën, Germany). The glycerol stock was immediately frozen to -70°C using dry ice and then stored at -80°C until required. To revive the cells, cells from glycerol stock were picked using a nichrome wire near a Bunsen flame and were streaked onto an LB plate and incubated at 37°C over night. Single colonies from the picked plate were used in further experiments.

2.3.7 Agarose Gel analysis of DNA

DNA was visualised using agarose gel electrophoresis. 1% agarose was dissolved in 1 x TBE buffer (9M Tris base, 20mm EDTA, 0.9M Boric acid) by heating in the microwave with occasional swirling to reduce air bubbles in the solution. To agarose solution, 0.01% Ethidium bromide was added to enable the visualisation the DNA under UV light. A comb and end stoppers were set up into the gel apparatus and the agarose solution was poured into it. Once the gel was set, the comb and end stoppers were removed and the gel tank was filled with 1 x TBE buffer until the gel was immersed in the buffer. 5µl of 1 Kb DNA ladder (Roche®, UK) was added to the first lane to enable prediction of size of the DNA fragments. All the samples were then diluted 5:1 by adding 6 x DNA blue/orange loading dye (Promega®, UK). The samples were then loaded onto the gel which was then run at 50V until the dye front migrated along the gel to ensure sufficient separation of the fragments. The gel was then carefully removed from the tank and examined under UV light.

2.4 Expression and purification of proteins

2.4.1 Maltose Binding Proteins (MBP) fusion proteins

E.coli BL21 pLys (DE3) cells transformed with the appropriate pMAL plasmid, as described in Table 2.2 were grown in 30ml of LB broth supplemented with 100 ug/ ml ampicillin, overnight in an orbital shaking incubator at 37°C. 450ml of sterilised LB broth supplemented with 100 ug/ ml ampicillin, was inoculated with overnight culture and grown in the orbital shaker at 37°C for 1-2h. A 1 ml sample of culture was removed to a plastic cuvette and the optical density measured at 600nm (OD_{600}) against a rich media control. Expression of the fusion protein was induced with 0.2mM isopropyal-β-D-thiogalactopyranoside (IPTG) when an OD_{600} of 0.6-1 was achieved. An OD_{600} at this level ensured that the culture was in the logarithmic phase, where bacteria will grow exponentially. Expression of the target protein was undertaken for 4h in an orbital shaker set at 30°C. 1 ml samples of culture were removed hourly to monitor protein expression. These samples, including the sample used for OD_{600} measurement were centrifuged at 13000 x g and the pellet was re-suspended in 100 µl 1x SDS sample buffer for analysis by SDS-PAGE and Coomassie® staining, as described in section 2.10. Four hrs post-induction the cells were harvested by centrifugation at 4000 x g for 10 minutes. The cells were re-suspended in resuspension buffer (50mm Tris-HCl; pH 8.0, 10mM NaCl and 10mm β-mercaptoethanol). The re-suspended cells were then frozen at -80°C and thawed on ice. Lysozyme was added at a final concentration of 1mg/ml and the cell suspension was subjected to sonication three times for 30 seconds each with an interval of 30 seconds in between to achieve cell lysis. NP-40 was added to a final concentration of 0.05% to aid cell lysis and the subsequent cell debris removed by centrifugation at 13000 x g for 15 minutes. 1 ml of amylose resin (New England Biolabs, UK) was pre-equilibrated with re-suspension buffer containing 0.05% NP-40. The fusion protein was eluted from the amylose resin with 500 µl of 10mM maltose, 50mM Tris-HCl; pH 8.0 by incubating end-over-end for 20 minutes at 4°C.

This was repeated up to three times, if necessary. The eluted fractions were pooled and dialysed using a slide-A-lyser dialysis cassettes (Pierce Rockford, USA) against three 650ml volumes of dialysis buffer (100mM NaCl, 50mM Tris-HCl, pH 8.0, 5% glycerol) for 1h each at 4°C. The purified fusion protein was frozen in aliquots on dry ice and stored at -80°C. The expression time course and final purification to homogeneity was analysed by SDS-PAGE and Coomassie® staining, described in section 2.10.

2.4.2 Glutathione-S-Transferase (GST) Fusion proteins

Recombinant GST fusions proteins were expressed and purified from *E.coli* essentially as described for MBP fusion proteins (section 2.4.1). Competent *E.coli* BL21 pLys (DE3) cells were transformed with appropriate pGEX plasmid (Table 2.2). GST fusions proteins were purified using glutathione sepharose resin (Amersham Biosciences, UK) and eluted using 10mM glutathione following the sample protocol as described in section 2.4.1.

2.4.3 In vitro transcription and translation (TnT®) of recombinant proteins

TnT®, Promega kit is a system designed for expression of proteins through *in vitro* transcription and translation of PCR generated templates using rabbit reticulocyte lysates. The reactions were performed as described in manufacturer's instructions (Promega® TB126). Briefly, 40µl of rabbit reticulocyte TnT Sp6® quick master mix, 0.5ug of DNA template, 2µl of amino acid mixture were mixed in an eppendorff® tube and the total volume was made upto 50µl with nuclease free water. Reaction tubes were incubated at 30°C for 2 hours. Transferring the tubes to 4°C terminated the reactions and the resulting recombinant proteins were stored at -80°C.

2.4.4 Purification of TnT® yields

Reaction samples from TnT® were mixed with equal volumes of 4M saturated ammonium sulphate in TE buffer (10mM Tris, pH 7.5, 1mM EDTA) at room

temperature. Samples were then incubated in ice water for 20 minutes and then centrifuged for 20 minutes at 14,000 x g at 4°C. The supernatant was removed and the pellets were washed with ice-cold TE buffer containing 2M ammonium sulphate and 2mM Dithiothreitol. The samples were centrifuged and the pellets were resuspended in modified TE buffer.

2.5 Maintenance of cell lines

2.5.1 COS1 and COS7 cell lines

Cos1 and Cos7 cell lines are derived from African green monkey kidney cells and have been transformed with SV40 virus. The cells were propagated in complete DMEM (DMEM supplemented with 10% FBS, 0.1% Penicillin and Streptomycin (10000U/ml), 2mM Glutamine). The cell line was maintained at 37°C in an atmosphere of 95% air and 5% CO_2. The cells were passaged when approximately 90% confluence was reached. This was carried out in a laminar flow hood to maintain sterility. To passage the cells the growth media was removed and 5 ml of pre-warmed sterile phosphate buffered saline (PBS) was added. The cells were washed by gentle agitation with sterile PBS, which was then aspirated. 1 or 2ml of Trypsin-EDTA solution was added and cells were incubated for 5min at 37°C. The cells were vigorously agitated and then analysed under a microscope to check for efficient cell detachment. Once this state was achieved, 10ml of growth media was added to inactivate the trypsin-EDTA solution. The cells were collected by centrifugation at 1000 x g for 3 minutes. The growth media supernatant was removed and the cell pellet re-suspended in the appropriate volume of fresh media. Cells were routinely split 1:5 into fresh media in sterile flask. The cells were returned for incubation at 37°C in atmosphere of 95% air and 5% CO_2 until confluent.

2.5.2 HEK293 cells

The human embryonic kidney-293 cell line has epithelial cell morphology. These cells were maintained as described in section 2.5.1 for COS1 and COS7 cells

2.5.3 CHO cells

CHO cells were cultured in HAM's F12 nutrient mix with Glutamax-1 10% foetal bovine serum (FBS) plus 0.1% Penicillin / streptomycin (10,000U/ml).

2.5.4 HEK293 cells with inducible (Flp-In-T-Rex) At1a receptor

HEK293 cells stably expressing At1a receptor were maintained in complete DMEM supplemented with 200 ug/ml Hygromycin (Calbiochem, UK). Cells were treated with 1 ug/ml doxycycline 24-96 hours before assays to induce expression of receptors cloned into Flp-In- locus.

2.5.5 Freezing and Thawing of cell lines

The storage of cells with a low passage number at -200°C in liquid nitrogen vats ensured the long-term integrity of the specific cell line. To revive cells from this temperature the individual vial was quickly thawed and added directly to 10ml of pre-warmed fresh growth media, under sterile conditions. Once the cell line was established and confluent, further 1ml aliquots of cells were re-suspended in freezing media. These were initially frozen at -80°C before being transferred to -200°C for long-term storage.

2.6 Transfection of mammalian cell with plasmid DNA

Transfection is a process of introducing foreign DNA into mammalian cells to allow the expression of recombinant proteins.

A list of DNA plasmids used for the transfection of mammalian cell lines is described in table 2.2

2.6.1 DEAE-dextran transient transfection

This method of mammalian cell transfection was used for the transfection of COS1 and COS7 cell lines only. Flasks of confluent cells were passaged the day prior to transfection and plated to ensure 70% confluence on the day of transfection. These plates were incubated overnight at 37°C in an atmosphere of 95% air and 5% CO_2. The amount of DNA required for each transfection plate is dependent on the number of cells to be transfected and should be scaled accordingly. This protocol is accurate for 100mm plates of 70% confluent cells in 10ml of growth media. 10ug of desired plasmid DNA was diluted in 10mM Tris-Cl, pH 7.6, 0.1mM EDTA to a final volume of 250 μl. 200μl of 10mg/ml DEAE-Dextran was added to the DNA solution, mixed and incubated for 15 min at room temperature. Where co-transfection of two DNA plasmids was required 10ug of each plasmid in final 250 μl of 10mM Tris-Cl, pH 7.6, 0.1mm EDTA was added to 200μl of 10mg/ml DEAE-Dextran. During the incubation period the growth media was removed from the plates and replaced with 10ml of fresh growth media supplemented with 100μM sterile chloroquine. The DNA-DEAE-Dextran solution was added directly to the appropriate transfection plate and incubated for 3-4hrs at 37°C. The growth medium was removed from the plates and 10ml of sterile PBS containing 10% Di-methyl sulphoxide (DMSO) added and aspirated immediately. The plates were washed twice with 10ml of sterile PBS before the introduction of 10ml of fresh growth media. The plates were incubated for approximately 2 days at 37°C in an atmosphere of 95% air and 5% CO_2 prior to any cell treatments and harvesting.

2.6.2 Polyfect® transient transfection

The Polyfect® method of mammalian cell transfection from QIAGEN® U.K, was used for transfection of COS1, COS7, and HEK293 cell lines. Cells were

cultured and prepared for transfection essentially as described in section 2.6.1. 4ug of the desired plasmid DNA for the transfection was mixed with 25μl of Polyfect for HEK 293 cells (80μl for COS cells) and the final volume of the mixture was made up to 300 μl with serum free growth media. The reaction mixture was incubated at room temperature for 5-10 minutes to allow complex formation. During incubation growth medium was removed from the plates and replaced with 7ml of fresh complete DMEM. Post-incubation, 1 ml of complete DMEM was added to reaction mixture, which was then gently mixed and added to the appropriate trasnfection plate. The plates were then incubated for approximately 2 days at 37°C as described in section 2.6.1

2.6.3 FuGENE® 6 transient transfection

The FuGENE®6 method of mammalian cell transfection, from Roche® diagnostics, Germany was used for the trasnfection of COS1, COS7 and HEK293 cell lines that were used for confocal microscopy analyses for better morphology. Cells were grown and prepared as described in section 2.6.1. This protocol is accurate for one well of a 6-well plate containing 70% confluent cells in 2ml of growth media. 5μl of FUGENE®6 transfection reagent was added to 95μl of serum free growth media and incubated at room temperature for 5minutes. 1ug of the desired plasmid DNA for cell transfection was added to reaction mixture and were further incubated for 15-45 minutes allowing the complex formation. The transfection reagents were then added directly to the appropriate well of the transfection plate. The plates were incubated as described in section 2.6.1 prior to harvesting.

2.6.4 Generation of stable cell lines

Chinese hamster ovarian cells (CHO) and HEK293 cells were transfected with plasmids as described in Table 2.2 using the transfection agent FUGENE® 6 (Roche® diagnostics, USA). Cells were allowed to grow for further 48-72 hours before starting the selection system.

After transfection, cells were split to 1:10, 1:20, and 1:50 into 15 cm dishes and were selected by growing them in media containing selection antibiotic. The choice of selection antibiotic was determined by the plasmid DNA used for transfection. For example, G418 was used for all plasmid DNAs cloned into pcDNA. An appropriate concentration (lowest concentration) of drug that gave massive cell death within 3 days was used. Cells were left in the selection medium for a week with occasional topping of selection medium. After the first week, any dead floating cells were removed, fresh selection media was added and the cells were replaced in the incubator for further selection. The process was repeated for 2-4 weeks until single isolated colonies started appearing. Single colonies were scraped carefully into 1ml of fresh medium with a micropipette. Cells were then plated into a 24-well plate and 1 ml of fresh selection media was added to them. Once the cells reached 70% confluency, they were trypsinised and then they were plated into a 6-well plate. Once the cells were confluent, they were split into a 75-cm^2 flask. Once confluent, cells were tested for protein expression and were stored at -200°C.

2.7 Immunocytochemistry

COS1 or COS7 cells were plated on to cell culture treated cover slips and transfected in 6-well plates with the appropriate plasmid DNA using FUGENE® 6 (Roche® diagnostics, USA) as described in 2.6.3. Following protein expression for 24h and any subsequent cell treatments the cells were fixed in sterile PBS containing paraformaldehyde (4% Paraformaldehyde with 10mM $MgCl_2$ and 150mM NaOH pH 7.5). The cells were then washed three times with 2ml of sterile PBS. Cells were then permeabilised with 200μl of 0.2% TritonX-100. This was repeated three times and the excess TritonX-100 removed by blotting with napkins. The proteins were then blocked using 10% goat serum (dependent upon the primary antibody) and 2% BSA diluted in 20mM Tris-HCl, pH 7.5 and 150mM NaCl. The protein of interest was detected using specific primary anti-serum. If staining for two different proteins, the cells were first stained for the protein with monoclonal anti-sera followed by polyclonal. Appropriate dilution of 200μl of primary anti-serum in 1:1 TBS

and blocking solution was added to cover slips for 2h at room temperature. The cover slips were washed three times with 200μl of blocking solution and incubated with 200μl of secondary antibody conjugated to Alexa® 594/495 from Molecular Probes (Eugene, OR, USA). The cells were then fixed to a microscopic slide using immuno-mount and observed using Zeiss® Pascal laser-scanning microscope (Jena, Germany).

2.8 Preparation of cell extracts

2.8.1 Preparation of nuclear extracts

Cells were grown and transfected using appropriate methods as described in section 2.6. Forty-eight hours post-transfection, cells were washed with cold sterile PBS and they were scraped into 500μl of hypotonic buffer (1M HEPES pH 7.0, 1M KCl, 1M $MgCl_2$, 1M DTT, 10% Triton X-100, 100% Glycerol, 500mM PMSF, 1mg/ml Aprotinin, 1mg/ml, Leupeptin). The cells were disrupted with 10-20 strokes of a Dounce homogeniser (Wheaton #357542, USA) and centrifuged at 3000-x g for 5 minutes. The supernatant was the cytoplasmic fraction. The pellet was resuspended in 100μl cold extraction buffer (Hypotonic buffer with 5M NaCl). Resuspended pellets were then incubated on an end over end rotating wheel for 20 minutes at 4°C. Cells were then centrifuged at 13,000 x g, for 10 minutes. The supernatant was used as nuclear extract and the pellet or nuclear matrix was resuspended in 100μl extraction buffer. The fractions were all subjected to SDS-PAGE and immunoblotting as described in section 2.10

2.8.2 Sub-cellular fractionation

Cells were grown and transfected using appropriate methods as described in section 2.6. Post-transfection, cells were washed with sterile PBS and they were scraped into 500μl of KHEM buffer (50mM KCl, 50mM HEPEs; pH7.2, 10mM EGTA, 1.92mM $MgCl_2$, and 1mM DTT) and 1x protease inhibitors (Roche® Diagnostics, Germany). Cells were then passed through brown

gauge needle for 10 minutes and were centrifuged at 2000 x g for 10 minutes. The pellet was P1 fraction and supernatant was removed into another eppendorff® tube and was centrifuged at 75000 x g for 30 minutes at 4°C. The supernatant obtained was S fraction and the pellet was resuspended in 750μl of KHEM buffer and centrifuged at 75000 x g for 30 minutes at 4°C. The pellet was retained as P2 fraction. P1 and P2 fractions were resuspended in the same volume of buffer as that of S fraction. The fractions were all subjected to SDS-PAGE and immunoblotting as described in section 2.10.

2.8.3 Whole cell lysate

Confluent cells were harvested at temperatures less than 4°C using buffers that had been previously chilled to minimise protein degradation in the whole cell extract. The cell culture media was aspirated and the cells were washed twice with ice cold sterile PBS. The cell plates were drained thoroughly and the appropriate volume of cell lysis buffer added. For a 6-well plate 100µl of cell lysis buffer was added whereas a 100mm plate received 500µl of cell lysis buffer. For the production of whole cell lysate, 3T3 lysis buffer (25mM HEPES, pH 7.5, 50mM NaCl, 10% glycerol, 1% triton, 50mM NaF, 30mM Na pyrophosphate, 5mM EDTA) and 1x protease inhibitor cocktail (Roche® diagnostics, Germany) was used.

2.9 Determination of protein concentrations

The protein concentration of purified recombinant proteins or cell lysates was determined using bovine serum albumin (BSA) as a standard in a spectrophotometric assay. The assays were undertaken in a clear 96-well micro-titre plate. A standard curve of known proteins concentrations was generated using a series of BSA standards of 0-5 ug in a final volume of 50μl. Protein assay reagent (BioRad®, UK) was diluted 1:5 with sterile water and 200μl of diluted reagent was added to each well of 96-well micro-titre plate. The intensity of the colour change (Brown to blue) was directly proportional to the protein concentration of the sample. The 96-well plate was analysed with

a 590nm test filter using Dynex MRX micro-titre plate reader controlled through Dynex Revelation, version 3.04-computer software. A standard curve was generated by plotting A590 of the BSA standards against their protein concentrations, with least squares regression used to obtain the best-fit line. The protein concentration of cell lysates was determined by comparison of their A590 with the standard curve.

2.10 Protein analysis

2.10.1 SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a method that is routinely employed to separate proteins by virtue of their molecular weight (Schägger and von Jagow, 1987). Protein samples of 1-100ug were denatured and reduced by dilution in 1x SDS sample buffer (10% SDS, 300mM Tris-HCl; pH 6.8, 0.05% bromophenolblue, 50% glycerol and 10% β-mercaptoethanol). The samples were boiled for 5 minutes and the loaded directly to an appropriate well of an Invitrogen™ UK protein gel. NuPAGE® 4.12% Bis-Tris polyacrylamide gel immersed in Invitrogen™ NuPAGE® MES or MOPS SDS running buffer. 5μl of Bio-Rad®, UK pre-stained molecular weight protein marker was also loaded to an appropriate well of the gel to allow the molecular weight of the proteins within the sample to be estimated. The gels were run at 200V for 1 hr. For more detailed description of Invitrogen NUPAGE® pre-cast gels and associated Xcell II™ apparatus please consult the manufacturer's instructions.

2.10.2 Visualisation of electrophoreses proteins by Coomassie® staining

Proteins separated by SDS-PAGE can be visualised by a variety of methods. The limit of coomassie® staining is approximately 0.1-0.5ug of proteins per band on a polyacrylamide gel and detects all proteins within a sample. Gels that required Coomassie® staining were removed from the pre-cast gel cassette and washed with sterile water to remove residual running buffer.

Coomassie® stain (1.25g (w/v) of Brilliant blue R_{250}, 44.4% Methanol, 5.6% acetic acid) was added to the gel with gentle shaking for 2h at room temperature. The gel was transferred from Coomassie® stain into de-stain (44.4% Methanol, 5.6% acetic acid). De-stain removed all background staining with Coomassie® stain remaining bound to the proteins resulting in the detection of all proteins present in the sample. The gels was then washed with sterile water and incubated with sterile water plus 10% glycerol to prevent the gel cracking during drying.

2.10.3 Western Immuno-blotting

Proteins separated by SDS-PAGE can be visualised by a variety of methods. Western immunoblotting allows the detection of the individual proteins with specific anti-sera. The proteins separated by SDS-PAGE were transferred to nitrocellulose membrane using the XCell™ blotting apparatus and NuPAGE® transfer buffer containing 20% methanol. The proteins were transferred with an applied voltage of 30V for 1h. For a more detailed description of XCell™ blotting apparatus setup and buffer compositions please consult the manufacturer's instructions. Following the transfer of the sample proteins, as indicated by successful transfer of the pre-stained molecular weight markers, the nitrocellulose membrane was incubated or blocked in 5% milk (Marvel®, UK), reconstituted in TBST (20mM Tris-Cl pH 7.6, 150mM, NaCl and 0.1% Tween20) for 1h at room temperature with gentle agitation. The primary antibody was added at the appropriate dilution to a solution of 1% milk powder in TBST. Details of the primary antibodies used in the experiments are indicated throughout the text where they have been used. The blocked nitrocellulose membrane was then sealed in an airtight hybridisation bag containing the primary antibody solution and this was incubated for 1h at room temperature, or overnight at 4°C, with vigorous agitation. The membrane was then washed several times with TBST before the application of the appropriate horseradish peroxidase (HRP) conjugated anti immunoglobulin secondary antibody diluted 1:5000 in 1% milk powder in TBST in a sealed bag as before. Similarly, this was incubated for 1h at room temperature, or

overnight at 4°C, with vigorous agitation. The membrane was again washed several times with TBST before employing the Amersham Biosciences, UK enhanced chemiluminiscence (ECL) western blotting-kit as the visualisation protocol for detecting bound antibodies. Briefly, the bound antibodies were detected by exposure of the membrane to lumionol, a substrate for HRP that oxidises luminol in the presence of hydrogen peroxide. Luminol is converted to an excited stated which emits light that is received by blue-light sensitive X-ray film and developed using the Kodak® X-Omat model 2000 processor.

2.11 Fusion protein interactions

2.11.1 GST-MBP pull-downs assays

Appropriate amounts of MBP fusion proteins were mixed with 100µl of slurry 50% (v/v) PBS-washed amylose resin (Amersham Biosciences, UK) for 2h at 4°C. The beads were pelleted by centrifugation at 14,000 x g for 1 min and washed twice with PBS containing 1% Triton X-100 before the addition of 50 ug of the required GST fusion protein in a 1ml solution of PBS and Triton x-100 containing 5mM Dithiothreitol. After 2h incubation at 4°C, the beads were collected and washed three times with PBS and Triton x-100 solution and eluted in 100 µl of 1 x SDS sample buffer. Eluates were resolved by SDS-PAGE and the bound GST fusion proteins were detected by immunoblotting as described in section 2.10.

2.11.2 Peptide arrays

Peptide arrays are Whatmann®50 cellulose membranes on which peptide sequences are directly synthesised (Reineke et al; 2001 and Frank; 2002). Each spot on a peptide array contains an overlapping 25mers spanning the entire protein sequence. These peptide arrays are able to bind purified recombinant proteins and provide evidence for direct protein interaction and the elucidation of the critical domains and the residues involved (Espanel and Hooft van Huijsduijnen; 2005 and Bolger et al; 2006). Substitution alanine

scans were also made where a particular amino acid is substituted by alanine to identify the critical residues involved in binding. The peptide arrays used in the experiments detailed in the thesis were kindly produced by Dr. E. Klussmann (Forschungsinstiut FÜr Molekulare Pharmakologie, Berlin, Germany) using the Intavis Bioanalytical instruments (Köhn, Germany) Autospot-Robot ASS 222 and utilising Fmoc-Chemistry. Recombinant GST fusion proteins were produced homogeneity as described in section 2.4.2. The peptide arrays were activated by immersion in 100% ethanol and then washed in TBST for 10min at room temperature on an orbital shaker. The peptide arrays were then incubated or blocked with 5% milk powder (Marvel®), reconstituted in TBST for 1h at room temperature with vigorous agitation. Recombinant GST fusion protein or GST alone as control was then added to 1% milk powder in TBST to a final concentration of between 3-10ug/ml and incubated with the peptide array in a hybridisation bag overnight at 4°C with vigorous agitation. The peptide array was then subjected to three 10 min washes in TBST. Binding of the GST fusion protein to the peptide array was detected by incubation of the membrane with protein or GST specific primary antibody, followed by a HRP conjugated secondary antibody followed by ECL as described in section 2.10.3. As a general rule the primary antibody was used at two-fold less than the recommended dilution for immuno-blotting. The resolution of spots, distinct from the GST control peptide array, on the blue light sensitive X-ray film were indicative of positive interaction of the recombinant fusion protein with the peptide array and the critical sequences were analysed for putative consensus or binding motifs using sites like Scansite (Scansite.mit.edu).

2.11.3 GST-Rhotekin pulldown assays

GST-Rhotekin beads were prepared as per manufacturer's instructions (Millipore, UK) and as described by Barnes et al; 2005. Following the necessary cell treatments, cells were lysed in 1x ice-cold Mg^{2+} lysis buffer (125mM HEPES, pH 7.5, 750mM NaCl, 5%Igepal CA-630, 50mM $MgCl_2$, 5mM EDTA, 10% glycerol, 10ug/ml aprotinin and 10ug/ml leupeptin). Lysed cells were then incubated on ice for 10 min and then scraped into prechilled

1.5 ml eppendorff® tube. Lysates were centrifuged at 15000 x g for 5 min. Supernatant was then removed into a fresh tube and protein concentration was determined as described in section 2.9. A volume of lysate containing 70ug of protein was then pipetted into a fresh prechilled 1.5 ml eppendorff® tube and mixed with 30ug of GST Rhotekin beads in a final volume of 300µl. Lysates with beads were allowed to rotate for 1h at 4°C before the beads were washed 3 times with 1 x Mg^{2+} lysis buffer. Following the last wash the majority of the supernatant was removed by pipette and the beads were aspirated to dryness with a flat gel-loading tip followed by SDS-PAGE and immuno-blotting to determine the Rho activity of the samples.

2.12 Co-immunoprecipitation

Mammalian cell lines were co-transfected, as described above in section 2.6 and cell lysates were produced by sub-cellular fractionation as described in 2.8. The protein concentrations of the samples were determined as described in section 2.9 and the concentrations equalised for all samples to contain approximately 250ug of protein in a 500µl volume of ice-cold KHEM. A 30µl sample of the diluted lysate was removed for western immunoblotting to determine he relative immuno-reactive inputs of the co-expressed proteins for the co-immunoprecipitation experiment. Anti-Flag or anti-VSV agarose beads pre-equilibrated in ice-cold KHEM to produce 50% slurry. 60µl of the slurry was added to each 500µl protein sample and these were incubated end-over-end wheel for 2 h at 4°C. The samples were centrifuged at 13,000 x g for 1 min at 4°C. Bound proteins were then eluted in 1x SDS sample buffer and subjected to SDS-PAGE and western immunoblotting as described in section 2.10.2 and 2.10.3 respectively. The quantification by densitometry, of the immuno-reactive amounts of the co-expressed proteins, in both the initial cell lysate and following co-immunoprecipitation, was determined using the Discovery series Quantity one® software, version 4.4.0. These data were used to compare the interaction efficiency of the two proteins and assess conditions that may facilitate the specific interaction. Control immuno-precipitations were undertaken in a similar manner with cell lysates produced from cells singly

transfected with protein that was co-immunoprecipitated to screen for non-specific binding to the chosen agarose bead conjugate.

2.13 Phosphodiesterase activity assay

To measure PDE activity a radioactive cAMP hydrolysis assay was employed. This procedure has been described previously (Marchmont and Houslay, 1980) and is a modification of a historical two-step procedure (Thomson and Appleman, 1971). PDE enzymes hydrolyse cAMP, which results in the formation of 5'AMP. In this assay, both [8-^{3}H] adenosine 3', 5'-cyclic mono-phosphate from Amersham Biosciences, UK and adenosine 3', 5'-cyclic mono-phosphate are hydrolysed.

2.13.1 Activation of Dowex 1x8-400 anion exchange resin

Dowex 1x8-400 was prepared and activated by dissolving 400g of dowex resin in 4 litres of 1M NaOH. The solution was stirred for 15 min at room temperature and the resin was allowed to settle down. The supernatant was removed and the dowex resin extensively washed 30 times with 4 litres of distilled water and allowed to settle after each wash. After thirty washes the resin was washed with 4 litres of 1M HCl for 15 min at room temperature and allowed to settle. The resin was then washed a further 5 times with distilled water and stored at 4°C as 1:1 slurry with distilled water. This procedure generally produced approximately 1 litre of Dowex slurry. This Dowex slurry was utilised in the PDE assay as a 2:1 solution of slurry to 100% ethanol.

2.13.2 Assay procedure

The assay was performed 1.5ml eppendorff® tubes. The cAMP substrate solution for the assay was composed of 2µl of 1mM 3', 5' cyclic adenosine mono-phosphate 3µl of 740GBq/mM [8-^{3}H] adenosine 3', 5'-cyclic mono-phosphate per millilitre of PDE substrate buffer (20mM Tris Cl; pH 7.4, 10mM $MgCl_2$). The appropriate volume of purified protein or cell extract was diluted to a final volume of 50µl in PDE assay buffer (20mM Tris Cl; pH 7.4). 50µl of PDE assay buffer was used as a blank control. The exact volume of purified

protein or cell extract required in the assay was predetermined in a pilot assay using increasing concentrations of protein samples to ensure activity measurements were within detection range. 50µl of cAMP substrate was added to 50µl of PDE containing sample, mixed and these were then incubated in a water bath at 30°C for 10 min to allow hydrolysis. The samples were then placed in a boiling bath for 2 min to inactivate the PDE and stop the reaction. The tubes were then cooled on ice for a minimum of 15 min. 25µl of 1mg/ml snake venom of *Ophiphagus hannah* was then added to the reaction tubes, mixed and incubated for a further 10min at 30°C. 400µl of Dowex-ethanol mixture was added to each reaction tube, mixed and incubated on ice for a further 15min. Snake venom prevents re-circularisation of uncharged 5' AMP by further hydrolysis to adenosine and the dowex slurry binds charged, unhydrolysed cAMP. Following incubation the tubes were then mixed again and centrifuged at 13000 x g for 3 minutes at 4°C in a refrigerated bench-top centrifuge. 150µl of supernatant from each reaction was added to 1ml of Opti-Flow SAFE1 scintillant in a fresh 1.5ml eppendorff® tube and mixed well. The extent of cAMP hydrolysis by PDE was determined by measuring the 8-^{3}H 5'-AMP in 150µl of each supernatant using a Wallac® 1409 liquid scintillation counter. 50µl of cAMP substrate solution was also added to 1ml of Scintillant and counted to provide a total count for the calculation of PDE activity.

2.13.3 Determination of phosphodiesterase activity

To determine specific PDE activity contained within any reaction tube the following formula was applied 2.61x (value-blank/average total) x10^{11}X10^{12} x (1000/ug protein) resulting PDE activity in pmoles/min/mg protein. To assess the effect of PDE inhibition, the activities of samples containing inhibitor were directly compared to an uninhibited control reaction and were expressed as the percentage of the aforementioned uninhibited control.

2.14 *In vitro* phosphorylation assays

2.14.1 In vitro PKA phosphorylation assay

All PKA phosphorylation assays were undertaken using Promega® purified PKA catalytic subunit. The expression and purification of MBP or GST fusion proteins in *E.coli* was undertaken as described in section 2.4 and the concentration of the purified protein determined as described in 2.9. 1ug of fusion protein and 10 units of Promega® recombinant protein kinase A were diluted in phosphorylation buffer (100mM Tris-HCl pH 7.5, 0.2mM ATP, 10mM $MgCl_2$, 30mM β-mercaptoethanol, 20% glycerol, 10mM Calyculin A) (One unit of PKA is defined as an amount of enzyme required to incorporate 1pmol phosphate into casein in one minute). The reaction was incubated at 30°C for 30 min to allow phoshphorylation. A control was set up without PKA catalytic unit. The reaction stopped by the addition 1 x SDS sample buffer. The samples were then analysed by SDS-PAGE and immunoblotting. The extent of PKA phosphorylation was determined by using a PKA phosphosubstrate antibody that specifically recognises phosphorylated PKA substrates.

2.14.2 In vitro ERK phosphorylation assay

All ERK phosphorylation assays were undertaken using purified recombinant MAPK-2 from Upstate cellular signalling®. The procedure was same as described in section 2.14.1.

2.15 Foci-Plate reader assays

This procedure was followed as described in Terry et al. 2003

CHO cells stably transfected with PDE4A4GFP were plated overnight onto 96-well plates (Costar, UK) at a density of $1x10^6$ cells/ml. Cells were then treated for 1-24h with a range of signalling inhibitors either with or without rolipram. PDE4A4-GFP accretion foci were quantified by measuring GFP fluorescence using a Packard Fusion Plate Reader equipped with appropriate filter set (GFP: exc 485nm, emi 527nm; Hoescht: exc 355nm, emi 460 nm).

Total GFP per well was measured first from live cells in full growth media, then cells were treated with an extraction buffer (0.4% formaldehyde in PBS with 1% tritonX-100) for 10 min at room temp. Full fixation and nuclear staining was completed with fixation buffer (4% formaldehyde with 10 uM Hoescht in PBS) and the cells were washed three times in PBS. PDE4A4 accretion foci were quantified by calculating immobile GFP as percentage of the total GFP (mobile and immobile). Hoescht values were used to monitor cell numbers.

2.16 *In vitro* SUMOylation assay

GST or MBP fusion proteins were expressed and purified as described in section 2.4. The purified proteins were used in the *in vitro* SUMOylation assays (Biomol, UK) according to the manufacturer's instructions. Briefly, the SUMOylation buffer, SUMO ligases, Mg-ATP mixture and 5ug of purified recombinant proteins were mixed in an eppendorff® tube and were incubated at 30°C for an hour. The SUMOylation assays were quenched using 25µl of 1 x SDS sample buffer and the samples were analysed by SDS-PAGE and immunoblotting as described in section 2.10. The extent of SUMOylation was determined using a SUMO specific antibody as well as by band shift caused by SUMOylation.

Plasmid	Antiobiotic resistance	Expression	Description
pcDNA 3.1 UBC9	Ampicillin	Mammalian	UBC9
PcDNA3.0His SUMO	Ampicillin	Mammalian	SUMO-1
pKW HA PIASy	Ampicillin	Mammalian	PIASy
pcDNA3.1VSV 4D5	Ampicillin	Mammalian	PDE4D5 VSV tag
pcDNA3.1VSV 4D5 FQF mutant	Ampicillin	Mammalian	PDE4D5 VSV tag-FQF mutant
pcDNA3.1VSV 4D5 K323R mutant	Ampicillin	Mammalian	PDE4D5 VSV tag-K323R mutant
pcDNA3.1FLAG βarrestin1	Ampicillin	Mammalian	β-arrestin-1 Flag tag
pEGFP RACK1	Kanamycin	Mammalian	RACK1 GFP fusion
MycEXV3 ERK2	Ampicillin	Mammalian	ERK2 myc tag
pEGFP AKAP18δ	Kanamycin	Mammalian	AKAP18δ GFP
pS462 4A4GFP	Kanamycin	Mammalian	GFP PDE4A4
pGEX3 UBC9	Ampicillin	*E.coli*	GST UBC9
pGEX4 4D5	Ampicillin	*E.coli*	PDE4D5 with GST
pGEX-4D5 K323R mutant	Ampicillin	*E.coli*	K323R mutant of PDE4D5
pGEX6 Arrestin	Ampicillin	*E.coli*	β-arrestin-1 GST
pGEX4T3 AKAP18δ	Ampicillin	*E.coli*	AKAP18δ GST
pGEX4T3 RACK1	Ampicillin	*E.coli*	RACK1 GST
pGEXEV3 ERK2	Ampicillin	*E.coli*	ERK2 GST
pGEX4T ARHGAP21 1064-1346	Ampicillin	*E.coli*	ARHGAP21(1064-1346 a.acids) GST
pGEX4T ARHGAP21 1042-1346	Ampicillin	*E.coli*	ARHGAP21(1042-1346 a.acids) GST
pGEX4T ARHGAP21 929-1346	Ampicillin	*E.coli*	GST of ARHGAP21 fragment 929-1346 a.acids
pGEX4T ARHGAP21 929-1096	Ampicillin	*E.coli*	ARHGAP21(929-1096 a.acids) GST
pGEX4T ARHGAP21 885-1096	Ampicillin	*E.coli*	ARHGAP21(885-1096 a.acids) GST
pGEX4T ARHGAP21 885-1052	Ampicillin	*E.coli*	ARHGAP21(885-1052) GST
pGEX4T ARHGAP21 929-1052	Ampicillin	*E.coli*	ARHGAP21(929-1052 acids) GST

pGEX4.0 UCR1	Ampicillin	*E.coli*	PDE4D UCR1 GST
pGEX4.0 UCR2	Ampicillin	*E.coli*	PDE4D UCR2GST
pGEX4.0 Catalytic region	Ampicillin	*E.coli*	PDE4D Catalytic region GST
pMALN Arrestin	Ampicillin	*E.coli*	β-arrestin-1 MBP
pMAL 4D5	Ampicillin	*E.coli*	PDE4D5 MBP
pMAL 4A4B	Ampicillin	*E.coli*	PDE4A4 MBP
pMALC2 4B1	Ampicillin	*E.coli*	PDE4B1 MBP

Table 2.2 cDNA plasmid constructs used for protein over-expression in either *E.coli* or mammalian cells.

Chapter 3 SUMOylation of PDE4 isoforms in HEK293 cells

3.1 Introduction

3.1.1 SUMO modification

Small Ubiquitin-like Modifier proteins modulate the structure and subsequently the function of its target protein by covalently attaching to a lysine residue within the consensus motif ΩKXE/D, where Ω is a hydrophobic amino acid and X is any residue (Johnson, 2004).

SUMO modification of proteins is known to regulate various cellular processes ranging from transcriptional activity (Motter and Castronovo, 2007, Yang et al; 2003 and Zhao et al; 2005) to cell signalling such as regulation of mitotic check points (Bischof and Dejean; 2007, Gutierrez and Ronai 2007), Kerscher et al; 2006).

3.1.2 SUMO conjugation pathway

The SUMO conjugation pathway is very similar to ubiquitin pathway both in structure and function (summarized in Figure 3.1). The components of SUMO pathway are highly conserved in all eukaryotes. There are four different kinds of SUMO molecules 1-4, which are conjugated to various substrates by specific ligases (Johnson, 2004).

Initiation of the SUMO conjugation pathway involves activation of a SUMO molecule by SUMO proteases. So far, six mammalian SUMO proteases have been identified and they have all been classed under ulp (Ubiquitin like protease) family. Human SUMO proteases SENP 1, 2, 3 and 5 are closely related to ulp1 in yeast whereas SENP 6 and 7 are related to ulp2 (Hay, 2007). SUMO proteases have dual functions. Firstly, their C-terminal hydrolase activity processes the SUMO molecule leading to its maturation such that the two C-terminal diglycine residues are exposed. They also

function to cleave SUMO molecules from SUMO-conjugated proteins by their isopeptidase activity thus contributing to SUMO turnover in the cells (Li and Hochstrasser, 2003).

SUMO maturation also involves the enzyme complex, SUMO activating enzyme E1 complex. Unlike ubiquitin E1, SUMO E1 enzyme is a heterodimer complex comprising the Aos1 and Uba2 proteins also known as SAE1 (SUMO Activating enzyme and SAE2 respectively. Both E1 subunits possess a nucleotide-binding motif, G*X*G*XX*G, which is able to form a thio-ester bond with the C-terminal glycine residue in SUMO molecules. This process is ATP-dependent and is inherent for activation of the enzyme complex (Desterro et al; 1999). Activated SUMO-E1 enzyme subsequently transfers the SUMO molecule to a C-terminal cysteine residue of UBC9, a SUMO conjugating enzyme; this transfer involves the formation of thio-ester bond between the SUMO molecule and UBC9. To date, UBC9 is the only SUMO conjugating enzyme to be identified (Hayashi et al; 2002).

Transfer of SUMO molecule from UBC9 to the target protein is achieved by E3 ligases. There are three different classes of E3 ligases as described in section 1.10.1.4. All classes of E3 ligases share three functional properties. Firstly, they all bind to E2 enzyme(UBC9). Secondly, they all bind to the SUMOylating target protein and thirdly, they all transfer SUMO from SUMO-UBC9 complex to the ε-amino group of the lysine residue within SUMO consensus motif present on the substrate (Johnson, 2004). Interestingly, only the E1 and E2, but not E3, enzymes are required for SUMO conjugation *in vitro*. However, E3 enzymes have been shown to enhance the SUMOylation efficiency of their substrates both *in vitro* and *in vivo* (Okuma et al, 1999). Furthermore, the E3 SUMO ligase is essential for the SUMO conjugation pathway in yeast (Takahashi et al; 2003).

Availability of SUMO molecules for conjugation is under stringent control as almost all of SUMO is present as protein conjugates (Desterro et al; 1999). The pathway is summarized in fig 3.1 and in the first paragraph of section 3.1.2.

3.1.3 PDE4 isoforms and SUMO pathway

Research within our group has shown that PDE4 enzymes can be ubiquitylated (unpublished data). Given the similarity of SUMO and Ubiquitin conjugation pathways a SUMOPLOT® analysis of PDE4 isoform sequences identified potential SUMOylation sites within members of the PDE4 family (www.abgent.com.cn/doc/sumoplot). This chapter focuses on the SUMOylation of PDE4 sub-familes.

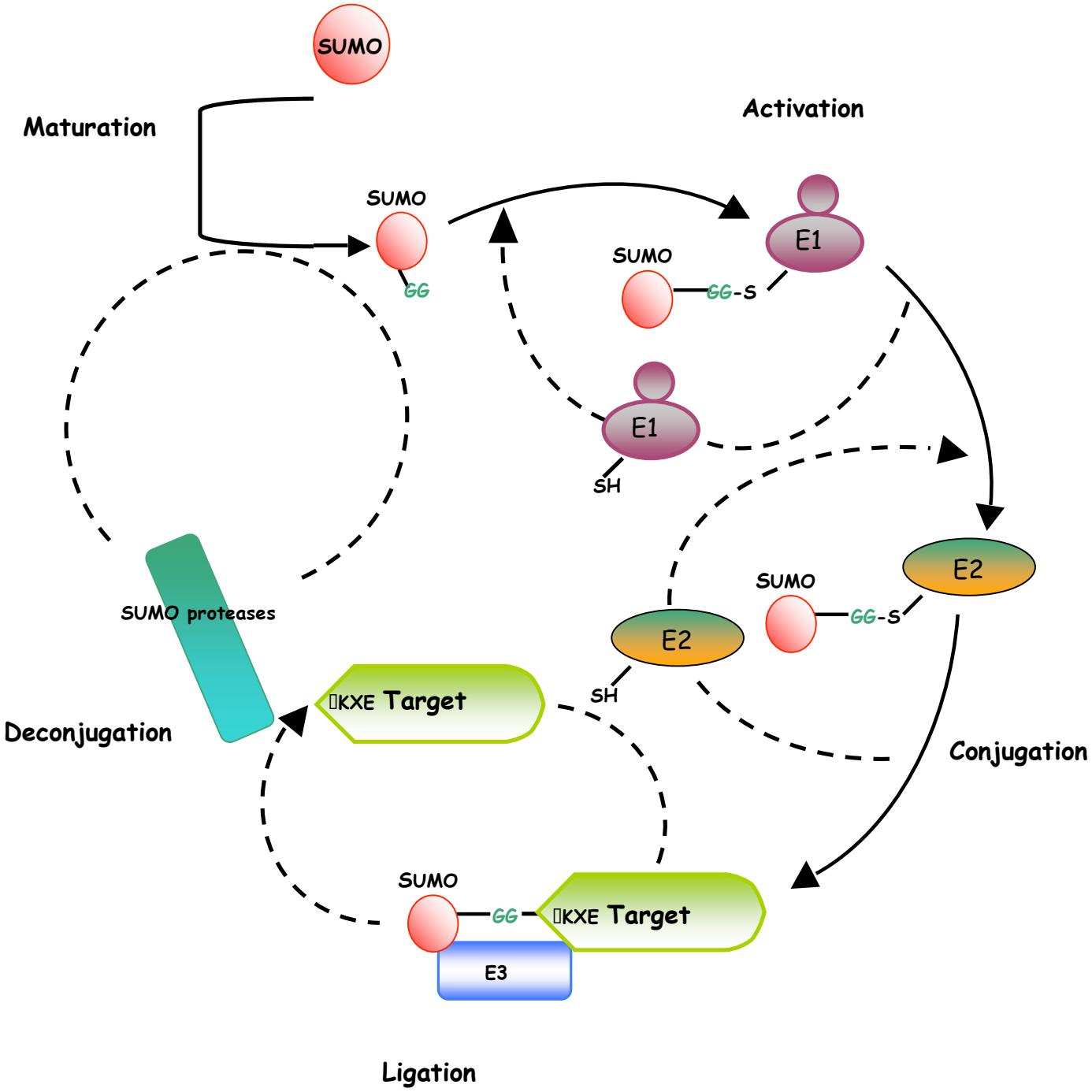


Figure 3.1 Schematic of SUMO conjugation pathway

3.2 Results

3.2.1 SUMOylation of PDE4 isoforms in HEK 293 cells

Earlier work from our group and collaborators (unpublished data) had shown that PDE4D5 is SUMOylated on lysine 323 *in vitro* (unpublished results). In order to examine the effects of SUMOylation of PDE4D5 *in vivo,* HEK293 cells were co-transfected with plasmids encoding an HA epitope-tagged PIASy E3 ligase and a VSV epitope tagged wild type PDE4D5 or a mutant of PDE4D5 in which residue K323 had been mutated to arginine by site-directed mutagenesis as described in section 2.3.. Expression of the transfected constructs in lysates was confirmed by SDS-PAGE and Western blotting. In figure 3.2 a 1, immunoblotting with anti-VSV antibody identified two species of wild-type PDE4D5 migrating at ~100 and 125 kDa corresponding to unmodified PDE4D5 and SUMOylated PDE4D5 respectively (upper panel). However, the PDE4D5 K323R mutant migrated as a single species of ~100kDa. Co-expression of PIASy E3 ligase was confirmed by probing with anti-HA antisera (lower panel). In a separate experiment, probing for SUMO-1 using an antibody against SUMO-1 identified a single band of ~125 kDa in the lysates expressing wild type PDE4D5 but not the K323R mutant (figure 3.2 a 2).

Since the lysine of PDE4D5 which undergoes SUMO modification is located within a region of the catalytic domain of PDE4D which is moderately conserved between the PDE4 subfamilies, sequence alignment of each PDE4 subfamilies was performed which revealed the SUMO consensus site to be present in only the PDE4A and 4D sub-families but not the PDE4B and PDE4C subfamilies (Figure 3.2 b).To test the ability of other PDE4 sub-families to serve as SUMOylation substrates plasmids expressing PDE4 isoforms from the other three sub-families namely, PDE4A4, PDE4B1 and PDE4C2 were co-expressed with or without PIASy E3 ligase in HEK293 cells. Immunodetection of transfected lysates showed, as predicted, PDE4A4 migrated as two species when co-transfected with PIASy E3 ligase with the higher molecular weight band corresponding to SUMOylated PDE4A4 (Figure 3.2 C 1). In contrast, the migration of immunodetected PDE4B1 and PDE4C2

isoforms was unaltered by PIASy co-expression (Figure 3.2 C 2, 3) indicative of their inability to serve as SUMOylation substrates.

3.2.2 Interaction of UBC9 with PDE4D5

Work from our group has indicated that PDE4D5 interacts with UBC9 SUMO conjugating enzyme E2 (unpublished data). To identify putative sites of interaction, a peptide array strategy was used. Indeed our group has exploited this technique to identify interaction sites that define the interaction of the PDE4D5 isoform with the signalling scaffold proteins, □arrestin and RACK1 (Bolger et al., 2006; Baillie et al., 2007). PDE4D5 peptides (25-mers) sequentially shifted by 5 amino acids across the entire sequence of PDE4D5, were immobilized on cellulose membranes. This was then probed with GST-UBC9, a recombinant fusion protein of UBC9 with Glutathione-S-transferase and expressed in *E. coli* prior to purification for use as a probe. Positive interactions were evident as dark spots on the PDE4D5 array. The probe identified an interaction site within the C-terminal portion of the PDE4D5 catalytic domain. This site is called FQF ERK docking site (Mackenzie et al; 2000) and is present in the catalytic region that maps to amino acids 661-685 (Spot 133, figure 3.3 a). Each amino acid of the interaction peptide was then sequentially mutated to alanine to identify key residues for interaction. On the alanine scanning substitution array mutation of either phenylalanine residue within the sequence “F670A/Q671/F672A” severely ablated the interaction of UBC9 with PDE4D5 (Figure 3.3 a). To further support of these findings, a peptide with N-terminal stearoyl group representing the PDE4D5 25-mer (Spot 133, figure 3.3a) shown to interact with UBC9 was generated in an effort to disrupt this interaction *in vivo*. Stearated peptides have been shown to enter cells and specific species have been used to disrupt protein-protein complexes by competing for binding partners (Hundsrucker et al; 2006, Baillie et al; 2007, Murdoch et al; 2007). HEK293 cells transiently expressing PDE4D5-VSV and UBC9 were treated with a non-related control stearated peptide or the PDE4D5 peptide containing “FQF” motif for 3 hours. PDE4D5-VSV was immunoprecipitated from lysates using VSV-conjugated agarose beads and co-precipitating UBC9 was detected using anti-UBC9 antisera.

PDE4D5 interaction with UBC9 in cells treated with the control peptide was unaltered from non-treated cells. However, in the cells treated with PDE4D5 “FQF” containing peptide, interaction of UBC9 with PDE4D5 was then abolished (Figure 3.3 b).

3.2.3 Interaction of PIASy SUMO E3 ligase with PDE4D5

Having determined an interaction of PDE4D5 with UBC9, interaction of PIASy E3 ligase with PDE4D5 was also studied. HEK293 cells transiently expressing wild type PDE4D5-VSV and PIASy-HA were harvested and the complexes were immunoprecipitated using VSV- and HA-conjugated agarose respectively. Control immunoprecipitation experiments were performed with Protein G-sepharose. Non-SUMOylated PDE4D5-VSV species showed interaction with immunoprecipitated PIASy (Figure 3.4 a1). In order to determine if PIASy interacts within the same region as UBC9, an “F670A/Q671/F672A” mutant of PDE4D5 was used. The F670A/Q671/F672A mutant of PDE4D5, which is unable to interact with UBC9, was still able to co-precipitate with PIASy E3 ligase. However, the PDE4D5-VSV “F670A/Q671/F672A” mutant migrated as a single band indicative the PDE4D5 was not SUMOylated (Figure 3.4 a2). Furthermore, the PDE4D5 mutant K323R, which is not a SUMOylation substrate, was also able to co-precipitate with PIASy (Figure 3.4 a3).

To further characterise the interaction of PDE4D5 with PIASy, purified GST-fusion proteins of PDE4D5 and its individual domains (UCR1, UCR2 and catalytic regions) were used in pull-down assays to test their ability to interact with PIASy-HA from transfected HEK293 cell lysates. The purified PDE4D GST fusion proteins were immobilised on glutathione sepharose beads and then incubated with PIASy-HA lysates for 2 hours. The complexes immobilised were then analysed for pull-down of PIASy. PIASy was shown to interact with only full-length PDE4D5 and not with any of the individual PDE4D domains of PDE4D5 (Figure 3.4 b). Expression and pull-down of the GST fusion proteins was visualized by Ponceau S staining of the membrane (Figure 3.4 b)

To study this interaction in greater detail, a scanning peptide array analysis was used. A peptide array spanning the full length PDE4D5 was incubated with PIASy-HA purified from an *in vitro* protein expression system as described in sections 2.4.3 and 2.4.4 and probed with a monoclonal anti-HA antibody. PIASy showed interaction with spots B8-10 and G2-G5, which correspond to PDE4D5 residues 111-140, which lie within the UCR1 region, and residues 486-530 within the catalytic domain (Figure 3.4 C2).

3.2.4 Affect of signalling pathways on SUMOylation of PDE4D5

In an attempt to determine the stimulus for SUMOylation of PDE4D5, HEK293 cells expressing PDE4D5-VSV and PIASy-HA were subjected to various treatments as indicated that would modulate various cellular signalling cascades including cAMP signalling (forskolin/IBMX, EPAC, Rolipram), oxidative stress pathway (H_2O_2)and ERK signalling (EGF, PMA, Anisomycin). SUMOylated PDE4D5 was immunoprecipitated using the SUMO-1 antibody and immunodetected with VSV antisera. SUMOylation of PDE4D5 did not appear to be affected by any of these stimuli (Figure 3.5).

3.2.5 Affect of SUMOylation on Sub-cellular localisation of PDE4D5

SUMO modification of various substrate proteins is shown to alter their sub-cellular localisation (Wies, 2003 and Zhao 2007). Nuclear extraction analysis was used to elucidate the effect of SUMOylation on sub-cellular localisation of PDE4D5. HEK293 cells transiently expressing PDE4D5 VSV with and without PIASy were treated with hypotonic buffer and extraction buffer to separate cytoplasmic and nuclear fractions respectively. Lysates were then analyzed by SDS-PAGE and immunoblotted for PDE4D5-VSV. SUMOylated and unmodified forms of PDE4D5 localised exclusively to cytoplasmic fractions and not to nuclear fractions of the cell (Figure 3.6). Lysates were re-probed for C-Jun, a nuclear protein, and p70S6 Kinase, a cytoplasmic protein as positive controls. To further evaluate the role of SUMOylation in determining sub-cellular localisation, fraction studies were carried out by differential

centrifugation. HEK293 cells expressing PDE4D5 before and after SUMOylation were subjected to sub-cellular fraction as described in section 2.9. The fractions were analysed by SDS-PAGE and immunoblotting (figure 3.6).

3.2.6 Affect of SUMOylation on PDE4 activity

Various interacting partners have been shown to change the activity and rolipram sensitivity of PDE4 isoforms (Houslay and Adams; 2003). A PDE assay was performed to determine the potential role of SUMOylation in regulating PDE4 activity. Purified GST-PDE4D5 was *in vitro* SUMOylated as described in section 2.16. The SUMOylated pool of GST-PDE4D5 was selectively isolated upon incubation with SUMO-1 antibody complexed with Protein G-sepharose beads. The precipitates were then used for PDE assays as described in section 2.13. SUMOylation did not affect the PDE4 activity in comparison to nonSUMOylated PDE4D5. A similar assay was performed with GST alone and GST incubated with SUMO ligases as controls (Figure 3.7 a).

PKA phosphorylation is known to increase PDE4 activity by activating the enzyme (Sette and Conti; 1996, Hoffmann et al; 1998, Mackenzie et al, 2002, Ekholm et al; 1997). In order to evaluate the role of SUMOylation in regulating the PKA-mediated activation of PDE4D5, SUMOylated and un-SUMOylated PDE4D5-GST was *in vitro* PKA phosphorylated as described in 2.14.1. Samples then immobilised on glutathione sepharose were washed and subjected to PDE assay as described in 2.13. PKA phosphorylated PDE4D5 showed a 2.5-fold increase in PDE activity compared to non-phosphorylated PDE4D5 whereas PKA phosphorylated SUMO PDE4D5 showed a five-fold increase in activity compared to its non-phosphorylated form (Figure 3.7 b). In similar experiments looking at the effects of ERK2 phosphorylation, un-SUMOylated PDE4D5 showed a decrease in activity by ~60% whereas ERK phosphorylation did not have any effect on SUMOylated PDE4D5 activity (Figure 3.7 c) with the PDE activity the same as the non-GRK phosphorylated PDE4D5-SUMO protein.

3.2.7 Affect of SUMOylation on rolipram sensitivity of PDE4D5

Interaction of SRC tyrosyl kinases through its SH3 domains is known to affect the rolipram sensitivity of PDE4 isoforms (McPhee et al, 1999). A rolipram dose response curve of *in vitro* SUMOylated and nonSUMOylated PDE4D5 was therefore performed to determine the effect of SUMOylation on rolipram sensitivity. PDE4D5 had an IC_{50} of 3.8 +/- 0.2uM for rolipram whereas SUMOylated PDE4D5 had an IC_{50} of 0.45 +/- 0.03uM, thus showing more sensitivity to rolipram. As anticipated, a mixed population of nonSUMOylated PDE4D5 and SUMOylated PDE4D5 exhibited an IC_{50} of 1.5 +/- 0.01uM for rolipram, a value intermediary with the two previous observations (figure 3.8 top panel). A further dose response assay was performed with the K323R SUMO mutant of PDE4D5, which similar to findings with the nonSUMOylated wild-type form had an IC_{50} of 3.9 +/- 0.027uM for rolipram *in vitro* (Figure 3.8 bottom panel).

3.2.8 Affect of SUMOylation and PKA phosphorylation on rolipram sensitivity of PDE4D5

Since SUMOylation was found to augment the increase in activity of PKA phosphorylated PDE4D5, whilst not having a direct effect itself on activity, a dose response assay for rolipram was carried out to examine the conjoint effects of PKA phosphorylation and SUMOylation on rolipram sensitivity of PDE4D5. PKA-phosphorylated PDE4D5 was shown to have an IC_{50} of 0.5 +/- 0.01uM whereas the non-phosphorylated PDE4D5 exhibited an IC_{50} of 3.8 +/- 0.2uM. The PKA phosphorylated and SUMOylated PDE4D5 form had an IC_{50} of 0.15 +/-0.009uM compared to 0.47 +/- 0.03uM for the non-PKA phosphorylated SUMOylated PDE4D5 protein (figure 3.9).

3.2.9 *Affect of SUMOylation and PKA phosphorylation on rolipram sensitivity of PDE4D5*

Mackenzie et al, 2000 showed that ERK2 phosphorylation has an inhibitory effect on PDE4D5 activity. To analyse the effect of SUMOylation on ERK-mediated inhibition of PDE4D5 activity a PDE assay was performed as shown in figure 3.7. To investigate the subsequent role of SUMOylation and ERK inhibition on rolipram sensitivity, a dose response curve was performed on SUMOylated and non-SUMOylated PDE4D5 proteins after *in vitro* ERK phosphorylation as described in 2.14.2. Non-SUMOylated PDE4D5 was totally inhibited by ERK phosphorylation. In contrast to this, ERK phosphorylation of SUMOylated PDE4D5 did not alter its rolipram sensitivity as compared to non-phosphorylated, SUMOylated PDE4D5 (figure 3.10).

3.2.10 Affect of SUMOylation and RACK1 binding on rolipram sensitivity of PDE4D5

RACK1 is an identified binding partner of PDE4D5 and is shown to change the sensitivity of PDE4D5 for rolipram by 3-4 fold (Yarwood et al; 1999). Given these findings, a dose response curve was performed to ascertain the effect of RACK1 binding on the rolipram sensitivity of SUMOylated and non-SUMOylated PDE4D5 *in vitro*. As the only fusion protein we have for RACK1 is a GST species, here I investigated the interaction of RACK1-GST with PDE4D5-MBP, instead of PDE4D5-GST. Purified RACK1-GST was incubated with immobilised SUMOylated and non-SUMOylated PDE4D5-MBP recombinant proteins and assayed for PDE activity. RACK1-bound PDE4D5 showed a 1.5 fold decrease in rolipram sensitivity with a shift in IC_{50} from 3.8 +/- 0.2uM (wild type) to 5.6 +/- 0.03uM. Similarly, the IC_{50} of rolipram for RACK1-bound, SUMOylated PDE4D5 increased from 0.45 +/- 0.03uM to 1.2 +/- 0.009uM. A control experiment was performed concomitantly in which GST-RACK1 was substituted with GST alone. GST alone did not affect the rolipram sensitivity of either SUMOylated or non-SUMOylated species of PDE4D5 (Figure 3.11).

3.2.11 Affect of SUMOylation on affinity of PDE4 isoforms for β-arrestin

All PDE4 isoforms are known to bind to β-arrestin at a site within the catalytic region (Bolger et al; 2003), an interaction which is critical for the recruitment of PDE4 isoforms to the β_2-AR and mediating receptor desensitisation (Perry et al; 2002). As SUMOylation of proteins is known to change the affinity of proteins for their interacting partners (Zhao, 2007), the role of SUMOylation in regulating the affinity of PDE4 isoforms for their interacting partners was assessed using *in vitro* pull-down assays. Purified MBP- PDE4A4 and 4B1 were incubated with β-arrestin-GST before and after SUMOylation. The complexes immobilised on amylose resin were washed and analysed by SDS-PAGE and subsequent immuno-detection for bound β-arrestin was carried using a polyclonal GST antibody. The amount of β-arrestin bound to SUMOylated 4A4 and 4B1 was determined by densitometric analysis using Quantity one software®. It was shown that the level of immonodetected β-arrestin bound to SUMOylated PDE4A4 was ~50% more than the β-arrestin bound to non-SUMOylated PDE4A4. However, in assays with recombinant PDE4B1 no change in affinity for β-arrestin binding was detected upon incubation with SUMO ligases (Figure 3.12 b). A control experiment to rule out any non-specific interactions was performed by incubating PDE4A4 and PDE4B1 with GST alone. Neither PDE4A4 nor PDE4B1 showed any interaction with GST alone (Figure 3.12 a).

3.2.12 Affect of SUMOylation on interaction of PDE4D5 with its partners in vitro

PDE4 isoforms interact with a plethora of signalling proteins that have multifarious functions (Lynch et al 2006). Pull-down assays were performed in order to understand the effects of SUMOylation on PDE4D5 interactions with partner proteins. Purified GST fusion proteins of PDE4 interacting partners including β-arrestin-1; AKAP18 δ, ERK2, UBC9 and RACK1 were incubated with PDE4D5-MBP or MBP alone. Complexes immobilised on amylose resin were analysed by SDS-PAGE and immunodetected using a GST antibody. All the partners showed an interaction with PDE4D5 MBP but not with MBP alone (Figure 3.13 I). Purified GST- partner proteins (β-arrestin-1; AKAP18 δ, ERK2,

UBC9 and RACK1) were incubated with SUMOylated and non-SUMOylated PDE4D5-MBP and also with the K323R PDE4D5-MBP SUMO mutant. It was found that all partner proteins examined exhibited an increased association for SUMOylated PDE4D5 compared to the non-SUMOylated form with association for β-arrestin-1, AKAP18δ, ERK2, RACK1 and UBC9 increased by 2.5-, 1.5-, 2.5-, 1.5- and 1.5-fold respectively. As anticipated, there was no change in partner association upon incubation with the K323R PDE4D5-MBP mutant even in the presence of SUMO ligases (Figure 3.13 ii a-e).

3.2.13 Affect of SUMOylation on interaction of PDE4D5 with its partners in HEK293 cells

The effect of SUMOylation of PDE4D5 on its interactions with binding partners was further assessed *in vivo*. HEK293 cells were transfected with plasmids encoding PDE4D5 partner proteins as described along with PDE4D5-VSV or the K323R PDE4D5-VSV SUMO mutant construct. Cells were also transfected with or without the PIASy expression vector. PDE4D5-VSV was immunoprecipitated using VSV-agarose and probed for individual partners proteins as indicated in figure 3.14 (a-e). Blots were then re-probed with a VSV monoclonal antibody. The association for SUMOylated PDE4D5 compared to the non-SUMOylated form for AKAP18δ, β-arrestin 1, ERK2, RACK1 and UBC9 was increased 1.5-, 1.5-, 5-, 5-, and 2-fold respectively. No change in association was detected in cells expressing the K323R PDE4D5 mutant with or without PIASy overexpression (Figure 3.14 a-e lanes three and four bottom and top panels). Membranes were re-probed for tubulin expression as a loading control.

3.3 Discussion

Post-translational modifications have long been known to regulate the function of proteins in distinct ways (Hasselgren, 2007). Modifications like myristoylation are known to be involved in membrane targeting (Frame, 2002) whereas ubiquitylation is known to recruit proteins for proteasomal degradation (Kaiser et al; 2000).

SUMO modification of proteins is a relatively newly identified and poorly characterised post-translational modification (Johnson et al; 2004). Components of SUMO modification are closely related to the Ubiquitin family in structure (Zha, 2007 and Krescher 2006). Of the PDE4 family of enzymes, sub-families 4A and 4D, but not 4B and 4C, have a conserved SUMO consensus site near their catalytic region (Figure 3.2 b). SUMO plot analysis of PDE4D5 sequence (www.abgent.com.cn/doc/sumoplot), using high stringency parameters, revealed a SUMOylation site at lysine 323. Previous work from our group has shown that PDE4D5 is SUMOylated *in vitro*. It was shown that only E1 and E2 components of the SUMO conjugation pathway but not E3 ligases were required for SUMOylation *in vitro* (Okuma et al; 1999). In similar studies in HEK293 cells which were co-transfected with UBC9 and PDE4D5, no SUMOylated PDE4D5 species were immunodetected in cell lysates. Furthermore, in HEK293 cells overexpressing PDE4D5 and E3 ligases, PIAS1 and PIAS2, no detectable levels of SUMOylated PDE4D5 were noted. However, in cells co-transfected with PIASy E3 ligase, SUMOylation of PDE4D5 (migrating at ~125KD) that could be readily detected by both SUMO-1 and PDE4D5 antibodies (Figure 3.2 a). Immunblot analysis revealed that not all the PDE4D5 cellular pool was SUMOylated as shown by two distinct PDE4D5 migration bands on the gel. Non-SUMOylated PDE4D5 migrated at ~100 KD as opposed to SUMOylated PDE4D5 that migrated at ~125 KD. Consequent analysis of PDE4A4 transfected into HEK293 cells with PIASy E3 ligase yielded similar results. SUMOylated PDE4A4 migrated at ~150KD and the non-SUMOylated species at 125KD (Figure 3.2 c).

PIASy E3 ligase is a complex molecule possessing a Chromatin binding domain, SUMO ligase domain and an acidic domain from its N-terminus to variable C-terminus in the same order (Sachdev et al; 2001). PIASy is known

to conjugate SUMO substrates such as LEF1 with SUMO-2 molecules and exhibits a preference for SUMO-2 over SUMO1 (Sachdev et al; 2001). In this study, PDE4D5 conjugates with SUMO-1 as confirmed by probing lysates transfected with PDE4D5 and PIASy for SUMO-1. However, probing same lysates for other SUMO species did not show any SUMOylated forms of PDE4D5. Overexpression of SUMO-2 in HEK293 cells in the presence of PIASy and PDE4D5 did not result in conjugation of PDE4D5 with SUMO-2 nor was PDE4D5 conjugation with SUMO-2 detected *in vitro*. The underlying preference of PIASy for SUMO-1 in conjugation with PDE4 isoforms is unclear. Mutation of the predicted SUMO site, Lys^{323} to Arg^{323} ablated SUMOylation of PDE4D5 (Figure 3.2 a).

Having confirmed SUMOylation of PDE4 isoforms *in vivo*, PDE4D5 was chosen for further characterisation due to its high expression levels and relatively known biochemistry. However, where appropriate, other isoforms were are also analysed in this study. To further understand the mechanism of SUMOylation of PDE4D5, interaction of the isoform with various components of SUMO conjugation pathway was investigated.

Although UBC9 did not directly lead to SUMOylation of PDE4D5 *in vivo*, it interacted with PDE4D5 both *in vitro* and *in vivo* (figure 3.3 and 3.13 ii e). UBC9 interacts at a multi-functional docking site on PDE4D5. This region is the FQF region located near the catalytic region of all PDE4s and is well known for it ability to act as a docking site for ERK2 for all isoforms (Mackenzie et al 2000). Other PDE4 binding partners such as β-arrestin and AKAP18δ are also known to interact with the FQF region (McSorley et al, 2006 and Bolger et al 2003). Although the FQF region plays an important role as a docking site for many PDE4-interacting partners, most partners bind PDE4s at multiple sites; for β-arrestin interaction at N-terminal region of PDE4D5 as well as the FQF region has been shown (Bolger et al; 2006)
Alanine mutagenesis of the F670A/Q671/F672A region severely ablated the interaction of UBC9 with PDE4D5 in peptide array analysis and incubation with a cell permeable disruption peptide comprising the FQF region produced

similar effects on PDE4D5:UBC9 interaction in HEK293 cells (Figure 3.3). Since UBC9 interacts at FQF region of PDE4D5, which conserved in all PDE4 isoforms, it could be postulated that UBC9 can potentially interact with members from all PDE4 subfamilies. However, the functional outcome of UBC9 interaction with the PDE4 subfamilies which cannot be SUMOylated remains unclear, as UBC9 is required for recognition of the SUMO consensus motif in substrates of SUMO conjugation pathway (Johnson and Blobel, 1997). The roles of UBC9 in functions other than SUMOylation have not been determined.

PDE4D5 was shown to interact with PIASy E3 ligase both *in vitro* and *in vivo* (Figure 3.4). E3 SUMO ligases exert functions that are independent of their ligase activity. For example, Pc2 is an E3 ligase that is known to regulate histone methylation (Kagey et al; 2003). Similarly, the PIAS class of E3 ligases are inhibitors of transcription factors STATs (Jackson 2001, Chung et al; 2003 and Chun et al; 2003). PIASy interaction with PDE4D5 in HEK293 cells led to SUMOylation of the isoform. In order to confirm the role of UBC9 in PIASy-mediated SUMOylation of PDE4D5, an F670A/Q671/F672A mutant of PDE4D5 was co-transfected with PIASy-HA into HEK293 cells. The F670A/Q671/F672A mutant immunoprecipitated with PIASy but PIASy did not SUMOylate the F670A/Q671/F672A mutant (Figure 3.4 a2). It could therefore be suggested that since UBC9 cannot be recruited to the FQF mutant, PIASy is unable SUMOylate the isoforms. In support of this; previous studies have shown that UBC9 is critical in identifying the SUMO consensus site on the substrate (Okuma et al, 1999, Johnson and Blobel, 1997).

Experiments showed that PIASy was able to interact with the K323R PDE4D5 mutant although it did not SUMOylate PDE4D5 due to the loss of lysine residue within the SUMO consensus site. These results also confirmed the prediction that only one consensus site for SUMOylation in PDE4 isoforms is present (Figure 3.4 a3). Partial pull-down assays were used to identify the binding regions of PIASy on PDE4D5. PIASy from HEK293 cell lysates failed to interact with purified GST fusion proteins of the UCR1, UCR2 and catalytic regions of PDE4D. PIASy only interacted with purified full length GST-

PDE4D5 (Figure 3.4 b). Further investigation of the interaction sites using scanning peptide array analysis identified two potential binding regions for PIASy on PDE4D5 (Figure 3.4 c). Region 1 included amino acids 111-140, which correspond to beginning of UCR1 region of PDE4D5. Region 2 included amino acids 486-530 that correspond to catalytic region of PDE4D5 (Figure 3.4 C). From these results it could be inferred that PIASy requires binding to more than one region on PDE4D5 for interaction and subsequent SUMOylation of the isoform *in vivo*.

Cell conditions like oxidative stress are known to activate certain post-translational modifications including phosphorylation of PDE4 isoforms (Hill et al; 2006). Similarly, various stress conditions such as heat shock, oxidative stress and ethanol are known to upregulate SUMOylation of target proteins (Kurepa et al; 2003). Furthermore, ERK phosphorylation is known to modulate SUMOylation of transcription factors including Elk-1 (Yang et al, 2003). On the basis of these observations, various signalling pathways which modulate PDE4 activity were examined to determine their effect on SUMOylation of PDE4D5. SUMOylation of PDE4D5 in HEK293 cells was unaltered by activation of ERK, elevation of cAMP, or oxidative stress (Figure 3.5). It may be that SUMOylation of PDE4D5 in this cellular environment is regulated by factors that are independent of the pathways studied in this investigation.
SUMOylation sites of certain substrates also act as sites for ubiquitylation as is reported for I_KB_α (Hay et al; 1999). In some instances SUMOylation is known to protect the substrate from being ubiquitylated and consequently targeted for proteasomal-degradation as has been shown for smad4 (Lin et al; 2003). Research from our group has shown that PDE4D5 is ubiquitylated within its N-terminus and that SUMOylation has no affect on ubiquitylation of PDE4D5 (unpublished findings).

SUMOylation is known to regulate or alter the sub-cellular localisation of substrates and most of the known SUMO substrates are nuclear proteins (Zhao, 2007 and Rodriguez et al; 2001). Analyzing PDE4D5 sequence reveals that the isoform does not have any nuclear localisation sequence. However, to confirm the role of SUMOylation and subsequent modulation of

PDE4D5 structure and function on sub-cellular localisation, nuclear translocation studies were carried out. Nuclear extracts were prepared and probed for the presence of PDE4D5. The purity of the subcellular fractions were confirmed using nuclear protein C-jun and cytosolic p70S6 Kinase as controls. In resting cells transiently expressing PDE4D5, the isoform was found to be exclusively localised to cytoplasmic fractions and excluded from the nucleus in both its non-SUMOylated and SUMOylated states (Figure 3.6). However, it has yet to be determined if modulators of cellular signalling would induce redistribution of SUMOylated PDE4D5 with cell. Furthermore, the possible role of SUMOylation in altering the localisation of PDE4D5 to various subcellular organelles including golgi and endoplasmic reticulum is yet to be determined. This would involve extensive microscopic analysis using organelle markers.

PDE activity is critical in regulating the cAMP levels in various compartments of cell. PKA and ERK phosphorylation are known to activate and inhibit the cellular PDE4 activity respectively (Mackenzie et al, 2000, Baillie et al 2001, Baillie et al; 2000, Sette and Conti, 1996 and Ekholm et al; 1997). It was therefore of interest to examine the effect of SUMOylation on PDE4 activity before and after PKA/ERK phosphorylation of the enzyme (Figure 3.7). SUMOylation did not affect non-PKA phosphorylated PDE4 activity. However, SUMOylation had an additive effect on PKA phosphorylation. Non-SUMOylated PDE4D5 showed a 2-fold increase in its activity after PKA phosphorylation whereas PKA phosphorylation of SUMOylated PDE4D5 exhibited a 4-fold increase in its activity. In contrast to this, ERK phosphorylation of non-SUMOylated PDE4D5 led to a decrease in activity by 75% whilst the activity of SUMOylated PDE4D5 was, rather remarkably, unaffected by ERK phosphorylation (Figure 3.7).

Upstream conserved regions (UCR) 1 and 2 as the name indicates are regulatory regions of PDE4 isoforms and associate with each other forming a regulatory module that interacts with catalytic region thereby regulating the catalytic activity of the PDE4 isoforms (Beard et al; 2000) . It is proposed that PKA phosphorylation at UCR1 region of PDE4 isoforms causes a

conformational change that leads to the disruption of binding between UCR1 and UCR2 region which in turn activates the enzyme activity (Richter and Conti 2002). ERK phosphorylation within the catalytic region of PDE4 long-isoforms is thought to promote the interaction of UCR1 and UCR2 regions and stabilise the isoform in a conformation that leads to inhibition of the PDE4 enzymes (Mackenzie et al; 2000). It is possible that SUMOylation stabilises the conformational changes initiated by PKA phosphorylation in PDE4D5 thus leading to elevated catalytic activity of the isoform. At the same time these conformational changes induced and or stabilised by SUMOylation of PDE4D5 may have an antagonistic effect on the conformational changes initiated by ERK phosphorylation thus keeping the isoform catalytically active. Considering the data so far, it could be postulated that since only a small fraction of the total intracellular pool of PDE4D5 is SUMOylated, SUMOylated PDE4D5 might contribute to differential regulation of the cAMP levels within the sub-cellular regions to which it is localised due to its inability to be inhibited in response to ERK signalling. SUMOylation of PDE4 enzymes may therefore serve to tailor cAMP signalling within distinct intracellular compartments.

To further elucidate the role of SUMOylation on affecting PDE4 catalytic activity, rolipram dose-response curves were carried out to study the kinetics of inhibition. Rolipram is a competitive inhibitor specific for PDE4 isoforms. Non-SUMOylated PDE4D5 elicited a 50% inhibition (IC_{50}) in its activity in response to 3.8+/-0.2 μM rolipram whereas purified SUMOylated PDE4D5 had an IC_{50} of 0.45 +/- 0.03μM. When an equimolar amount of SUMOylated and non-SUMOylated PDE4D5 were used the mixed population exhibited an IC_{50} of 1.5 +/- 0.01 μM whereas the K323R SUMO-null PDE4D5 mutant had an IC_{50} of 3.9 +/- 0.027 uM, a concentration similar to that of non-SUMOylated PDE4D5 suggesting that the change in rolipram sensitivity is a direct result of SUMOylation (Figure 3.8). PDE4 isoforms are known to exist in two different affinity states for rolipram, HARBS (High affinity rolipram binding state) and LARBS (Low affinity rolipram binding state) (Rocque et al; 1997). HARBS is promoted by PKA phosphorylation and the binding of interacting partner proteins including SRC family tyrosyl kinase LYN (Sette and Conti, 1996 and

McPhee et al, 1999). Given the observed effect of SUMOylation on rolipram sensitivity of PDE4D5, it could be argued that SUMOylation, like PKA phosphorylation, promotes the HARBS conformation in PDE4D5 thereby increasing its sensitivity to rolipram. This is evident from the unaltered rolipram sensitivity of PDE4D5 SUMO-null mutant as compared to the wild-type. Furthermore, a mixed population of PDE4D5 and SUMOylated-PDE4D5 exhibited a rolipram sensitivity that could be roughly placed between the values of the non-SUMOylated and SUMOylated PDE4D5 species.

Having determined the effect of PKA phosphorylation on PDE4 activity, the effect of the same on rolipram sensitivity of SUMOylated and non-SUMOylated PDE4D5 was determined. PKA phosphorylation increased the rolipram sensitivity of PDE4D5 by 7-fold whilst that of SUMOylated PDE4D5 was elevated only 3-fold (Figure 3.9). Increase in rolipram sensitivity after PKA phosphorylation is reflective of a switch to the HARBS conformation. It is likely most of the SUMOylated PDE4D5 pool exists in a HARBS conformation and therefore the effects of PKA phosphorylation on rolipram sensitivity are not as significant as those observed in non-SUMOylated PDE4D5. In order to evaluate the inhibitory effect of ERK phosphorylation on PDE4D5 activity and its rolipram sensitivity, dose response curves were performed with *in vitro* ERK-phosphorylated recombinant PDE4D5. As anticipated, wild type PDE4D5 activity was inhibited by ERK and rolipram sensitivity was lost whereas SUMOylated PDE4D5 exhibited no change in rolipram sensitivity in response to ERK phosphorylation as compared to the non-phosphorylated form. It is possible that SUMOylated PDE4D5 is able to counterbalance the conformational switches initiated by the ERK phosphorylation within the catalytic site thus overriding ERK's inhibitory effects on PDE4D5 (Figure 3.10). The SUMO consensus site on PDE4 isoforms is located proximal to the catalytic region and the presence of a SUMO molecule near the catalytic region may hinder the conformational changes induced by ERK phosphorylation. To confirm that the activity of SUMOylated PDE4D5 is non-responsive to ERK action, *in vitro* ERK phosphorylated purified fractions of both PDE4D5 and SUMOylated PDE4D5 were immuno-blotted using a phosphor-serine antibody to show that SUMOylated PDE4D5 is able to

undergo ERK phosphorylation (Figure 3.7 c). Similar analysis was performed using a phospho-PKA antibody to show that the recombinant proteins are able to serve as PKA substrates (Figure 3.7 b). The mechanism by which SUMO exerts these changes in structure and function of PDE4D5 is not understood. These structural effects would be best studied by X-ray crystallography analysis of the SUMOylated and non-SUMOylated PDE4D5 proteins in both the absence and presence of rolipram.

RACK1 is a scaffolding protein known to recruit activated PKC to different locations in cells. It has been reported that RACK1 selectively interacts with PDE4D5 through its unique N-terminal region thus preventing RACK1 binding other PDE4 isoforms (Yarwood et al; 1999). As it had also been shown that an *in vitro* PDE4D5-RACK1 complex displayed a decreased affinity for rolipram compared to PDE4D5 alone similar experiments were performed to determine the effect of RACK1 on rolipram sensitivity of PDE4D5 and SUMOylated-PDE4D5. In accord with the findings of Yarwood and co-workers, a recombinant MBP fusion of PDE4D5 protein incubated with a RACK1-GST fusion protein showed a slight increase in its rolipram IC_{50} value (5.6μM for the complex compared to 3.8μM for PDE4D5 alone). SUMOylated recombinant PDE4D5 incubated with RACK1-GST showed a similar shift in IC_{50} value with an increase from 0.45 μM to 1.2 μM. To rule out any effect of the GST moiety, both SUMOylated and non-SUMOylated species of PDE4D5 were incubated with GST alone. This had no effect on the rolipram sensitivity of either PDE4D45 species (Figure 3.11). The effect of RACK1 on rolipram sensitivity of PDE4D5 may be due to the conformational changes elicited by RACK1 interaction with the N-terminal region of PDE4D5, leading to a slight destabilisation of the catalytic region thereby affecting its rolipram sensitivity. SUMO-1 is an 11-KD molecule and is conjugated to a region close to catalytic domain at the C-terminal region of PDE4D5 whereas 37-KD RACK1 binds at the N-terminal region as well as the catalytic region (Bolger et al, 2006). The binding of these two molecules at either end of the PDE4D5 protein could restrict it and induce a conformation change that is somewhat between the conformational states produced by binding of either of the these molecules

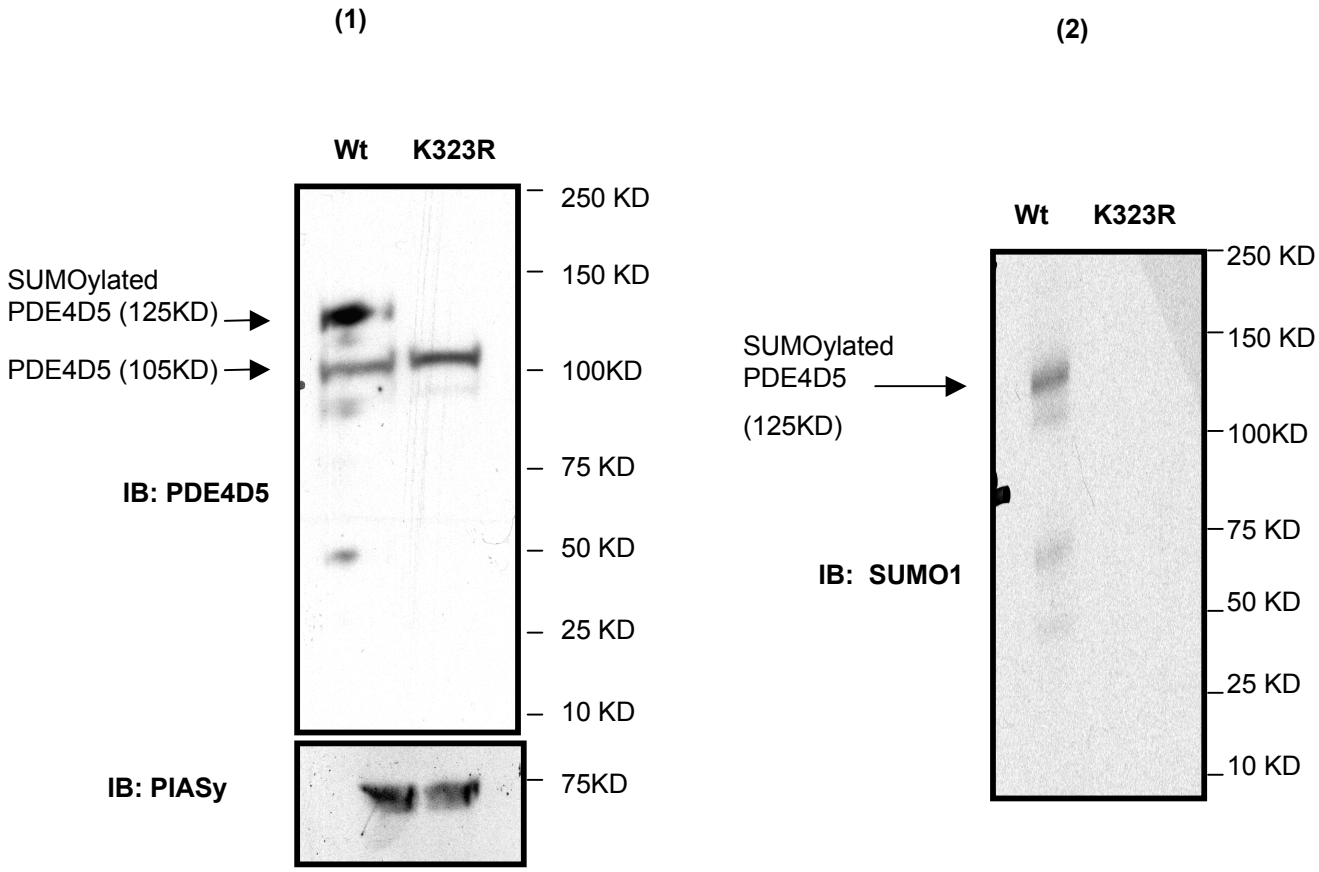
individually. This “new” conformation may change the rolipram sensitivity of the isoform.

SUMOylation of several substrates is known to change their binding affinity for their various interacting partners. However the underlying mechanism for this phenomenon is not known (Zhao, 2007). The modifications of histone acetylation and de-acetylation are processes which have been shown to regulate transcription with acetylation promoting and de-acetylation repressing transcriptional activity (Motter and Castronovo, 2007). SUMOylation of transcription factors including ELK1 and MEF2 inhibits their transcriptional activity by altering their affinity for their binding partners. However, ERK phosphorylation of ELK-1 and deSUMOylation of MEF2 by SENP3 leads to their eventual activation and prevents further SUMOylation (Yang et al; 2003 and Zhao et al; 2005). In case of p53, Arf targeted to nucleus increases the SUMOylation of p53 and its interaction with mdm2, which further promotes SUMOylation of p53 and prevents it from being ubiquitylated (Chen and Chen 2003). In light of these studies, similar experiments were performed to evaluate the affinity of PDE4 binding partners for SUMOylated PDE4 isoforms compared to non-SUMOylated forms. Beta arrestin 1 interacts with all PDE4 isoforms at site within their catalytic region (Figure 3.12 a) although it shows a preferred binding to PDE4D5 due to an extra interaction site present within the unique N-terminal region of isoform (Bolger et al, 2003 and Perry et al; 2002). For *in vitro* β-arrestin binding assays, recombinant PDE4D5 was not used as its β-arrestin binding is supplemented by this extra binding site. Instead, PDE4A4 and PDE4B1 were selected for the competition assays. SUMOylated and non-SUMOylated purified MBP-PDE4A4 and PDE4B1 were incubated with purified equal amounts of β-arrestin (Figure 3.12 b). As discussed earlier, PDE4B1 cannot be SUMOylated due to the lack of conserved lysine within the SUMO consensus motif (Figure 3.2) SUMOylated PDE4A4 showed an increased binding to β-arrestin compared to the non-SUMOylated form. However, PDE4B1 did not show any difference in its ability to bind β-arrestin before and after incubation with SUMO ligases. This observation also eliminates the possiblity of SUMO ligases in mediating any

increase in partner association directly. SUMOylated PDE4A4 showed a 50% increase in binding to β-arrestin1 compared to non-SUMOylated PDE4A4 and PDE4B1 (Figure 3.12). Similar *in vitro* and *in vivo* binding assays with PDE4 binding partners, AKAP18δ, ERK, RACK1 and UBC9, were performed. In all instances, the binding partners of PDE4D5 showed varying levels of increased association to SUMOylated PDE4D5 compared to the non-SUMOylated form in both *in vitro* and *in vivo* assays whereas association of partner proteins to the K323R SUMO mutant of PDE4D5 were unchanged in the presence of the SUMO ligases; There were significant differences in the association observed for each PDE4D5 partner proteins between *in vitro* and *in vivo* analyses. The binding affinities determined from *in vivo* experiments may differ from those of *in vitro* studies as other cellular components are known to influence SUMOylation. Factors including the SUMO cycle of PDE4 isoforms and the cell cycle which is known to be affected by the SUMO (Bischof and Dejean; 2007, Gutierrez and Ronai 2007) may contribute to the observed disparities. It could also be suggested that the competition between binding partners for SUMOylated species of PDE4D5 might also affect the partners' binding affinities for PDE4D5. The functional outcome of such increased binding can only be interpreted based on the existing evidence of function of their interaction. Increased binding of scaffolding proteins such as AKAPs, β-arrestins and RACK1 to SUMOylated PDE4D5 might result in sequestration of the isoform to various sub-cellular locations where the isoform would play a key role in regulating the cAMP levels in and around various protein complexes whose function is under stringent control of local cAMP levels. Such functioning of SUMOylated PDE4D5, which cannot be inhibited by ERK and is extremely sensitive to PKA phosphorylation, would only add more to the complexity of compartmentalised cAMP signalling. Furthermore, the ability of only certain PDE4 sub-families to become SUMOylated, a modification which would subsequently alter enzyme activity would allow a further means for regulating cAMP-associated signalling pathways within cells. SUMOylation of PDE4 isoforms may help maintain the cAMP levels within various cellular compartments of cells thereby specifically regulating the localised functions of various PDE4 associated proteins.

In conclusion, SUMOylation is novel post-translational modification of PDE4 isoforms, a discovery which increases the complexity of compartmentalised cAMP signalling. Further investigations are required to identify the pathways involved in regulating the SUMOylation of PDE4 isoforms. Also, considering the SUMO consensus site is semi-conserved among the PDE4 families, and the consequences of SUMOylation on PDE4 structure and function deserve further study.

a) SUMOylation of PDE4D5 at K323 in HEK 293 cells



b) **SUMOylation consensus site is semi-conserved in PDE4 subfamilies**

PDE4A: IPFRGV **K** TD QEELL
PDE4B: ISFRGV **N** TE NEDH
PDE4C: VPFRGV**Q** TD QEEQ
PDE4D: IPFRGV **K** TE QEDVL

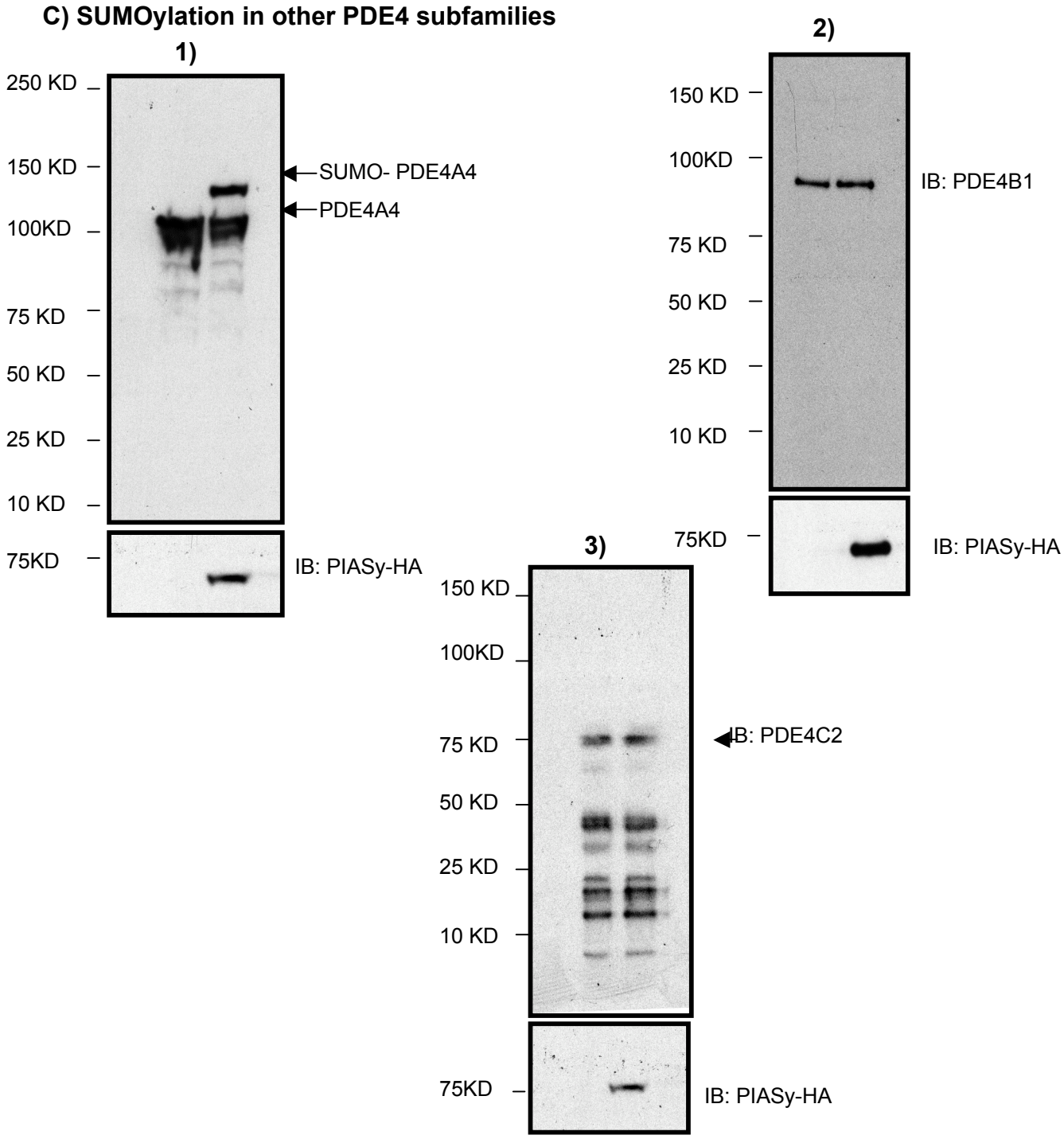


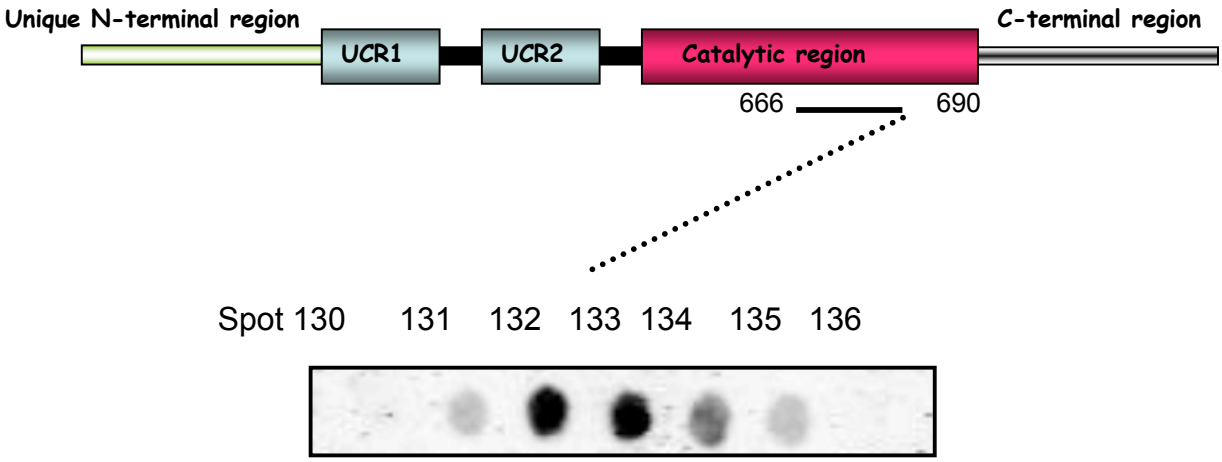
Figure 3.2 SUMOylation of PDE4 isoforms in HEK293 cells co-transfected with PIASy E3 ligase.

a) Wild type and K323R mutant of PDE4D5 VSV were co-transfected with PIASy-HA E3 ligase and blotted for 1)PDE4D5 2)SUMO1(top panel) and PIASy HA (bottom panel) respectively.

b) Sequence alignment near the semi-conserved SUMOylation site of PDE4 subfamilies A, B, C and D respectively.

c) PDE4A4, 4B1 and 4C2 were co-transfected with PIASy-HA E3 ligase and the lysates were blotted for 1)PDE4A4 2) PDE4B1 and 3)PDE4C2 respectively. All the blots were then reprobed for PIASy-E3 ligase with an HA antibody. The results shown are examples of experiments done from three separate transfections

a) Interaction of UBC9 on PDE4D5 Peptide array



Spot 130: STIPQSPSPADDPEEGRQGQTEKF (646-670)

Spot 131: SPSPADDPEEGRQGQTEKFQFELT (651-675)

Spot 132: PDDPEEGRQGQTEKFQFELTLEEDG (656-680)

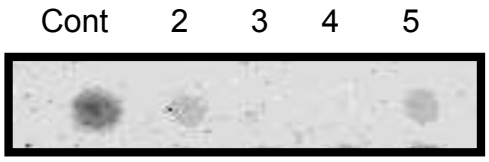
Spot 133: EGRQGQTEKFQFELTLEEDGESDTE (661-685)

Spot 134: QTEKFQFELTLEEDGESDTEKDSGS (666-690)

Spot 135: QFELTLEEDGESDTEKDSGSQVEED (671-695)

Spot136: LEEDGESDTEKDSGSQVEEDTSCSD (676-700)

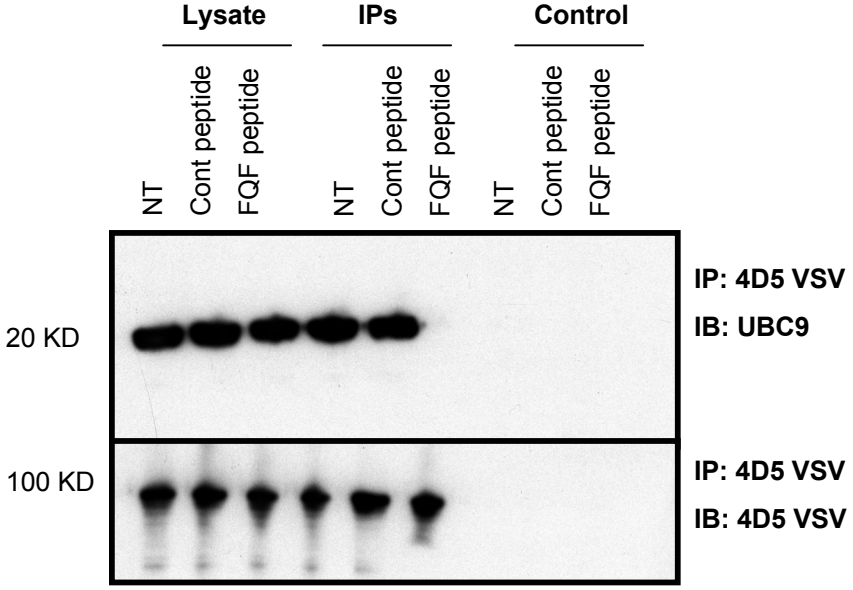
b) Alanine scan of key residues from the parent peptide



UBC9 GST

Control: E-G-R-Q-G-Q-T-E-K-F-Q-F-E-L-T-L-E-E-D-G-E-S-D-T-E
Spot2: E-G-R-Q-G-Q-T-E-K-A-Q-F-E-L-T-L-E-E-D-G-E-S-D-T-E
Spot3: E-G-R-Q-G-Q-T-E-K-F-Q-A-E-L-T-L-E-E-D-G-E-S-D-T-E
Spot4: E-G-R-Q-G-Q-T-E-K-A-Q-A-E-L-T-L-E-E-D-G-E-S-D-T-E
Spot 5: E-G-R-Q-G-Q-T-E-K-F-A-F-E-L-T-L-E-E-D-G-E-S-D-T-E

b) Interaction of UBC9 at FQF region of PDE4D5 in HEK293 cells



FQF peptide: Stearate- EGRQGQTEKFQFELTLEEDGESDTE

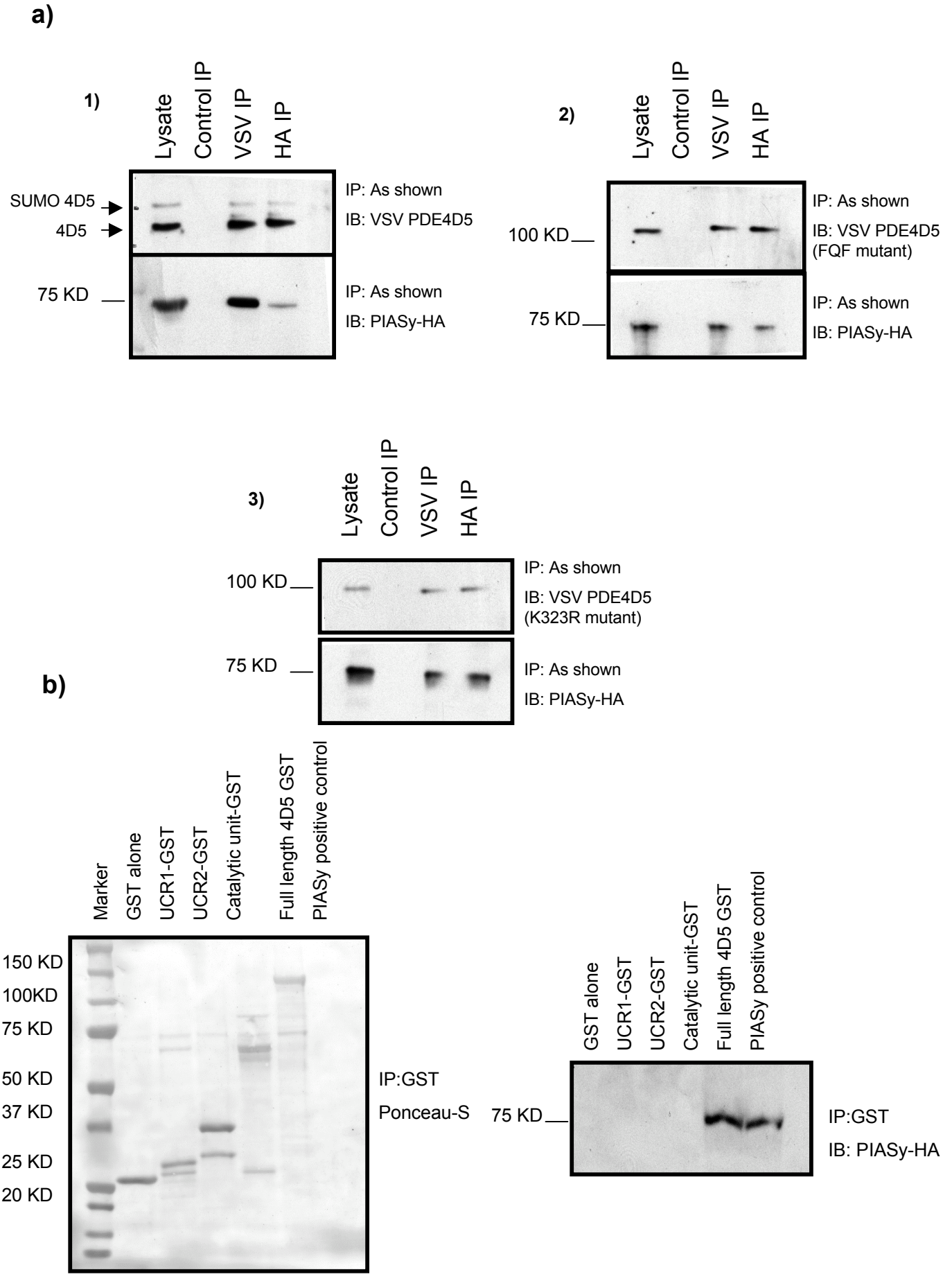
Control peptide: Stearate-
EGRQGQTEKAQAELTLEEDGESDTE

Figure 3.3 Interaction of UBC9 SUMO E2 ligase with PDE4D5 *in vitro* and *in vivo*.

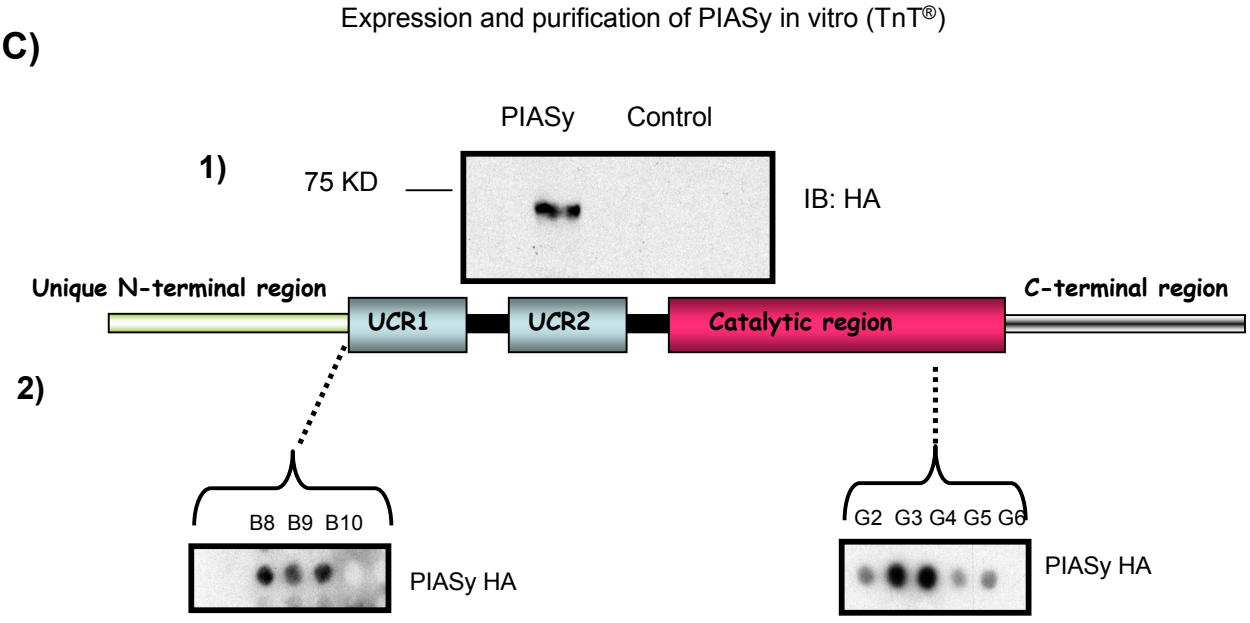
A) PDE4D5 is shown schematically as unique N-terminal region, Upstream conserved regions, catalytic domain and C-terminal region. Results show immobilized peptide 'spots' of overlapping 25-mer peptides each shifted along by five amino acids in the entire PDE4D5 sequence probed for interaction with either UBC9 GST or GST and detection by immunoblotting. Positively interacting peptides generate dark spots, while those that do not interact leave white (blank) spots. In all other sections of the array, spots were blank with either probe. Spot numbers relate to peptides in the scanned array and whose sequence is given as indicated. Arrays probed with purified GST did not yield any positively interacting spots..

b) PDE4D5peptide arrays were probed for UBC9-GST binding based upon the indicated 25-mer 'parent' PDE4D5 peptide, where the indicated amino acids were sequentially and individually replaced by alanine. Ct refers to the native peptide and all other spots reflect alanine substitutions of individual amino acids in the native spot. GST alone did not give any spots.

c) HEK293 cells were co-transfected with PDE4D5 VSV and UBC9. Cells were subjected to no treatment, control peptide treatment or FQF peptide treatment for 3hrs in an incubator and the lysates were used to immunoprecipitate PDE4D5 and the precipitate were blotted for UBC9 using an UBC antibody (Santa cruz) and VSV. All western blots shown are representative of the results obtained from three separate experiments. The peptide arrays interaction data are from experiments done once.


a)
1)
Lysate
Control IP
VSV IP
HA IP
SUMO 4D5
4D5
75 KD
IP: As shown
IB: VSV PDE4D5
IP: As shown
IB: PIASy-HA
2)
Lysate
Control IP
VSV IP
HA IP
100 KD
75 KD
IP: As shown
IB: VSV PDE4D5
(FQF mutant)
IP: As shown
IB: PIASy-HA
3)
Lysate
Control IP
VSV IP
HA IP
100 KD
75 KD
IP: As shown
IB: VSV PDE4D5
(K323R mutant)
IP: As shown
IB: PIASy-HA
b)
Marker
GST alone
UCR1-GST
UCR2-GST
Catalytic unit-GST
Full length 4D5 GST
PIASy positive control
150 KD
100KD
75 KD
50 KD
37 KD
25 KD
20 KD
IP:GST
Ponceau-S
GST alone
UCR1-GST
UCR2-GST
Catalytic unit-GST
Full length 4D5 GST
PIASy positive control
75 KD
IP:GST
IB: PIASy-HA

C)



Spot B8: GLILQANFVHSQRRESFLYRSDSDY (111-135)
Spot B9: ANFVHSQRRESFLYRSDSDYDLSPK (116-140)
Spot B10:SQRRESFLYRSDSDYDLSPKSMSRN (121-145)

Spot G2:IFQNLTKKQRQSLRKMVIDIVLATD (486-510)
Spot G3:TKKQRQSLRKMVIDIVLATDMSKHM (491-515)
Spot G4:QSLRKMVIDIVLATDMSKHMNLLAD (496-520)
Spot G5:MVIDIVLATDMSKHMNLLADLKTMV (501-525)
Spot G6:VLATDMSKHMNLLADLKTMVETKKV (506-530)

Figure 3.4 Interaction of PIASy SUMO E3 ligase with PDE4D5 *in vitro* and *in vivo*

a) HEK 293 cells were co-transfected with wt PDE4D5-VSV, FQF mutant of PDE4D5 VSV, K323R mutant of PDE4D5-VSV and PIASy-HA respectively. Lysates were used to immunoprecipitate using HA and VSV tags and Detection of bound proteins was performed by immunoblotting using monoclonal antibodies for VSV and HA tags. Protein G sepharose was used as a control.

b) HEK293 cell lysates transiently expressing PIASy-HA were incubated with purified GST (lane 1) or various truncates of purified GST PDE4D5 (UCR1 region, UCR2 region, catalytic region and full length 4D5). Complexes were immobilised on glutathione sepharose beads washed and analysed by SDS-PAGE. Detection of bound proteins was carried by immuno-blotting with monoclonal HA antibody. Later the membranes were ponceau-S stained to show the presence of GST proteins. Cell lysates expressing PIASy were loaded on to the gel as a standard.

c) Mapping the binding sites of PIASy interaction on PDE4D5 using peptide array analysis. PIASy-HA DNA was used to express the protein in vitro TnT ® kit as described in section 2.4.3 and 2.4.4. PDE4D5 is shown schematically as unique N-terminal region, UCR1 and UCR2, conserved catalytic region and sub-family specific C-terminal region. Results show immobilized peptide 'spots' of overlapping 25-mer peptides each shifted along by five amino acids in the entire PDE4D5 sequence probed for interaction with purified PIASy and detection by immunoblotting. Positively interacting peptides generate dark spots, while those that do not interact leave white (blank) spots. In all other sections of the array, spots were blank with either probe. Spot numbers relate to peptides in the scanned array and whose sequence with a.acids in parentheses is given as indicated. Arrays probed with lysates without PIASy did not yield any positively interacting spots. All western blots shown are representative of the results obtained from three separate experiments. The peptide arrays interaction data are from experiments done once.

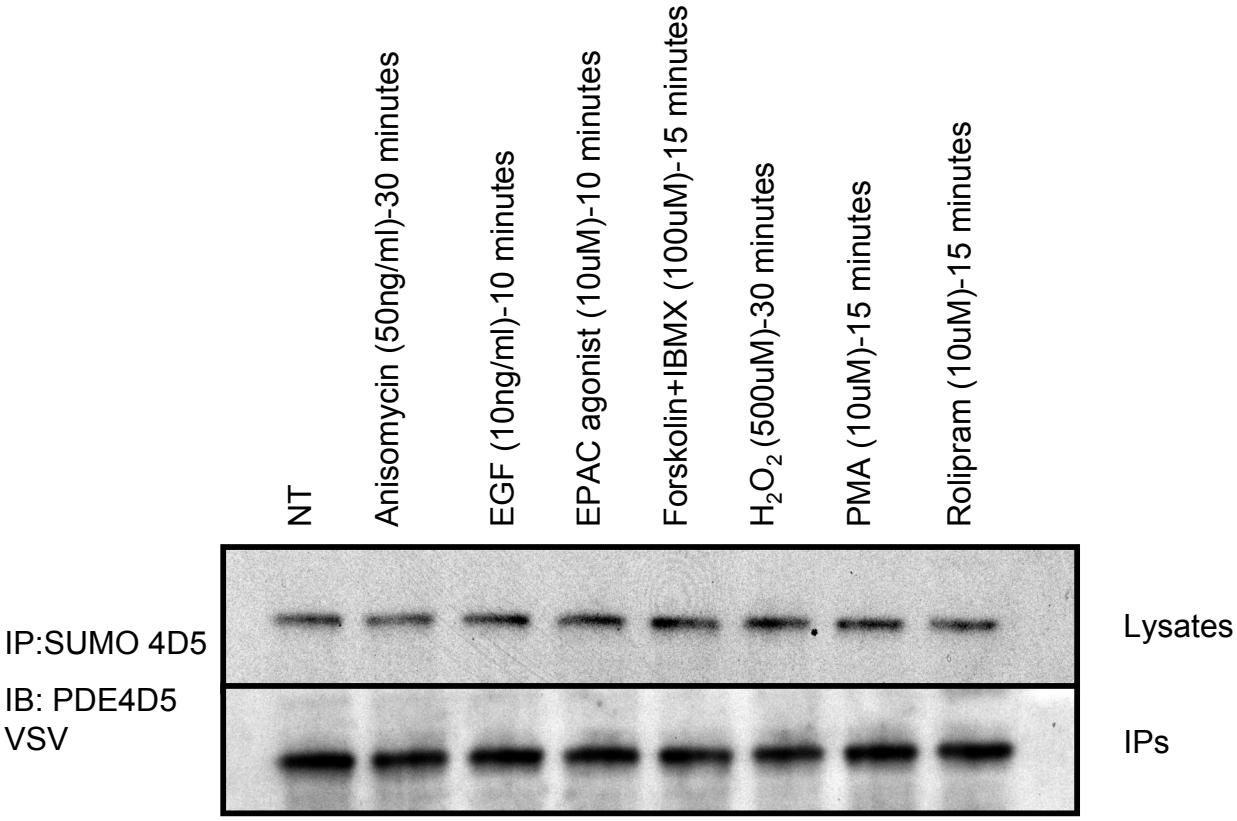


Figure 3.5 Effect of PDE4 activity on SUMOylation of PDE4D5

HEK293 cells co-transfected with PDE4D5 VSV and PIASy-HA were treated as indicated above and the lysates were then incubated with SUMO1 antibody (Cellular signalling). The complexes immobilised on to protein G sepharose were washed and analysed by SDS-PAGE. Detection of bound proteins was carried out by immunoblotting using a monoclonal VSV antibody. All western blots shown are representative of the results obtained from three separate experiments.

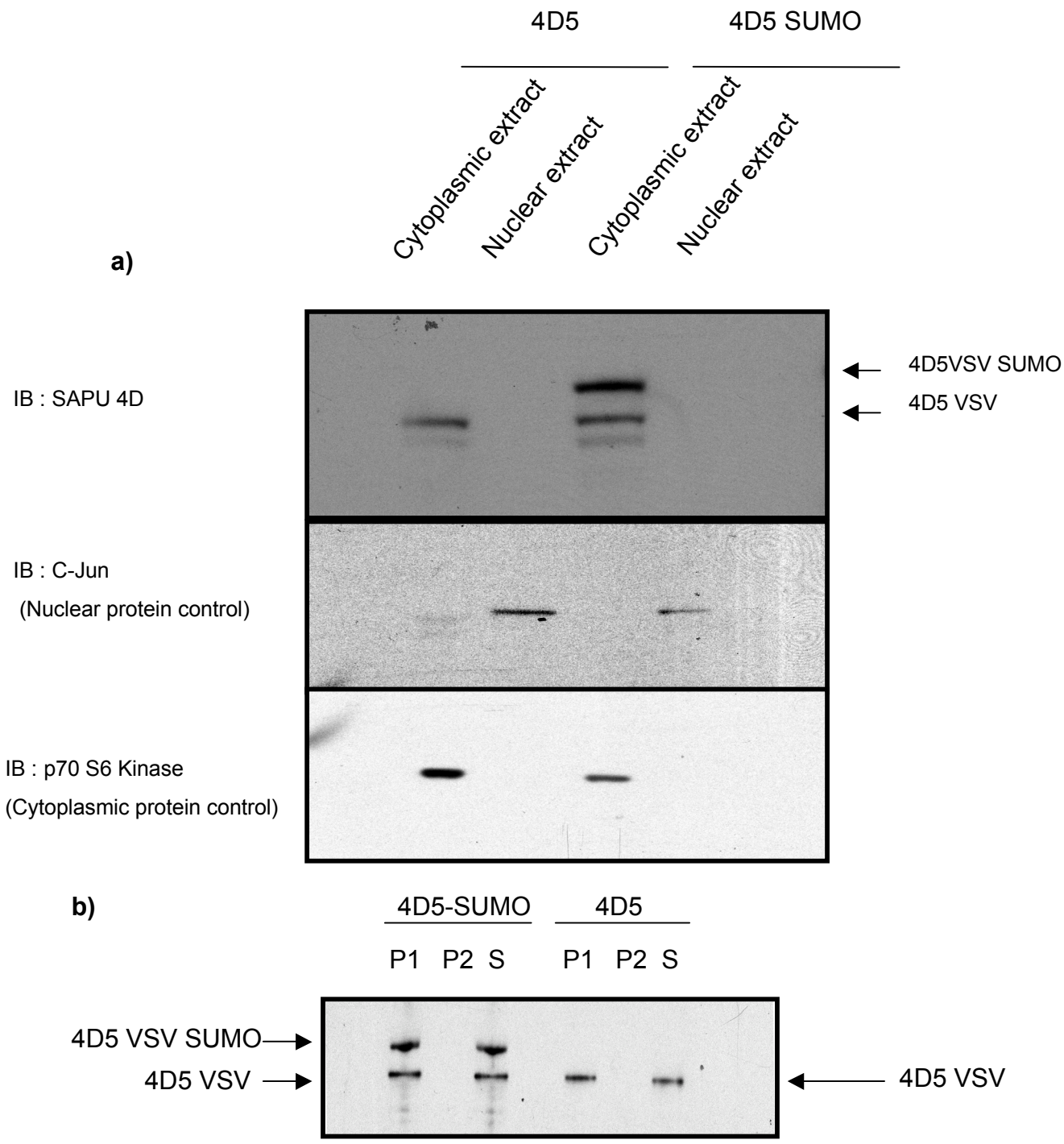


Figure 3.6 Effect of SUMOylation on sub-cellular localisation and of PDE4D5

a) HEK293 cells co-transfected with PDE4D5-VSV with and without PIASy-HA were subjected to nuclear extraction as described in section 2.8.3. The extracts were then normalised for protein by Bradford's assay and were analysed by SDS-PAGE. Detection of the proteins was performed by immunoblotting. C-jun and p70 S6 kinase were used as positive controls for nuclear and cytosolic proteins respectively.

b) HEK293 cells co-transfected with PDE4D5-VSV with and without PIASy-HA were subjected to sub-cellular fractionation. The extracts were the normalised for protein by bradford's assay and were analysed by SDS-PAGE followed by immunoblotting. All western blots shown are representative of the results obtained from three separate experiments.

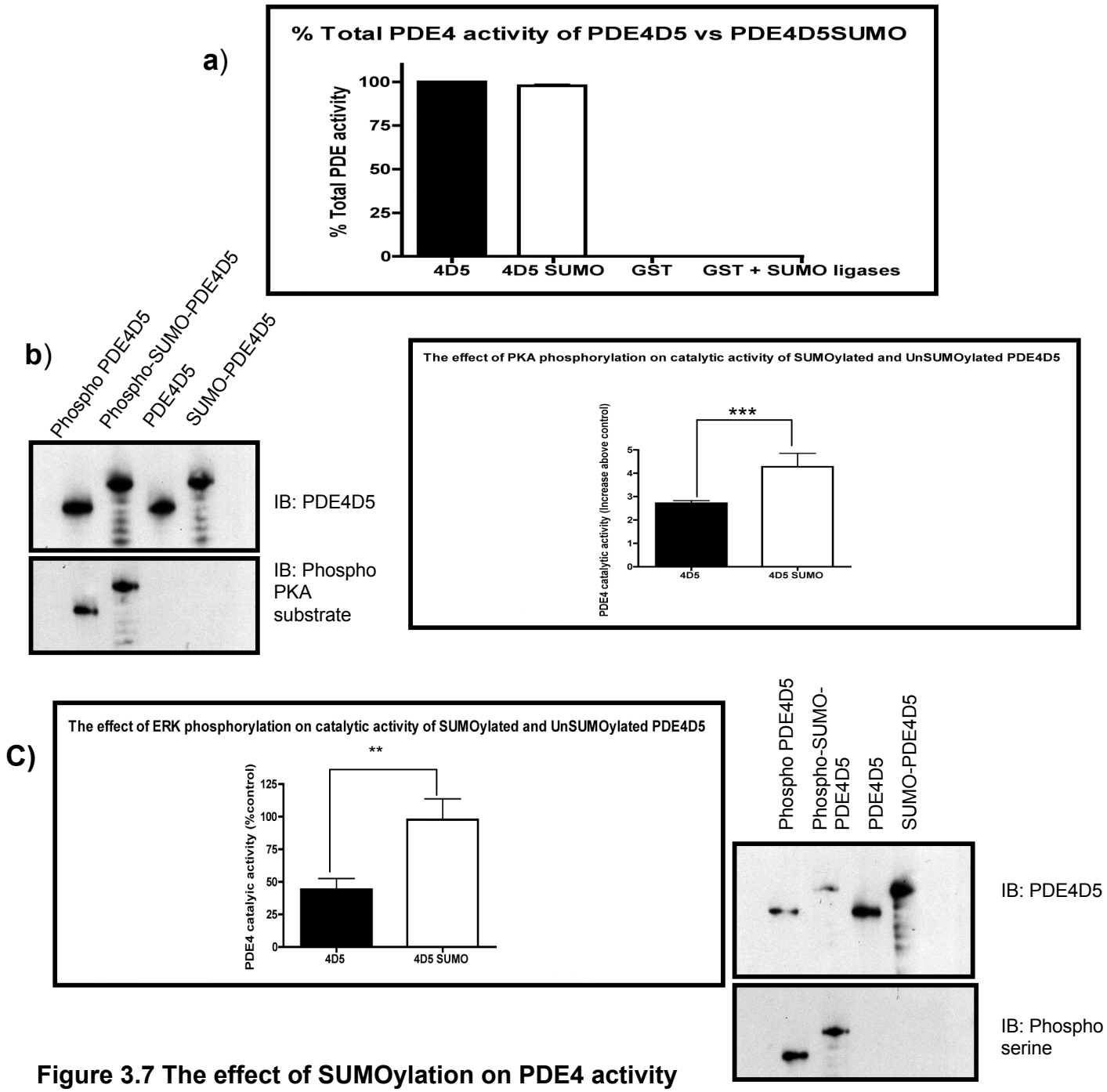
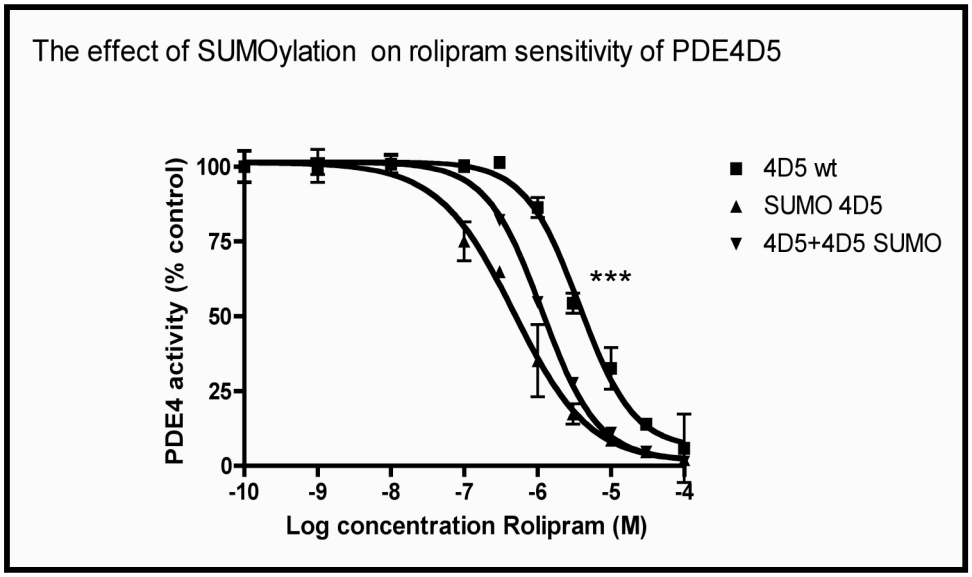


Figure 3.7 The effect of SUMOylation on PDE4 activity

a) Purified GST, GST PDE4D5, SUMOylated GST PDE4D5 and SUMOylated GST were used to perform PDE4 activity assay with 1μM cAMP.

b) Purified GST PDE4D5 and SUMOylated GST PDE4D5 were in vitro PKA phosphorylated and the samples immobilised on glutathione sepharose were washed and used for PDE4 activity assay with 1μM cAMP. A fraction of the samples were analyzed on SDS-PAGE and phosphorylation was detected by immunodetection using a monoclonal phospho PKA substrate antibody (Upstate).

C) Purified GST PDE4D5 and SUMOylated GST PDE4D5 were in vitro ERK2 phosphorylated and the samples immobilised on glutathione sepharose were washed and used for PDE4 activity assay with 1μM cAMP. A fraction of the samples were analyzed on SDS-PAGE and phosphorylation was detected by immunodetection using a conjugated phospho serine antibody (Abcam). All experiments are representative of the results obtained from three different experiments Paired t-test statistical analysis of the PDE activity data were undertaken where $p<0.05$ indicates significant difference in PDE activity



P<0.0001

IC50:

PDE4D5: 3.8+/-0.2uM

SUMO-PDE4D5: 0.45+/-0.03uM

PDE4D5+SUMO PDE4D5: 1.5 +/- 0.01uM

K323R PDE4D5: 3.9 +/- 0.027uM

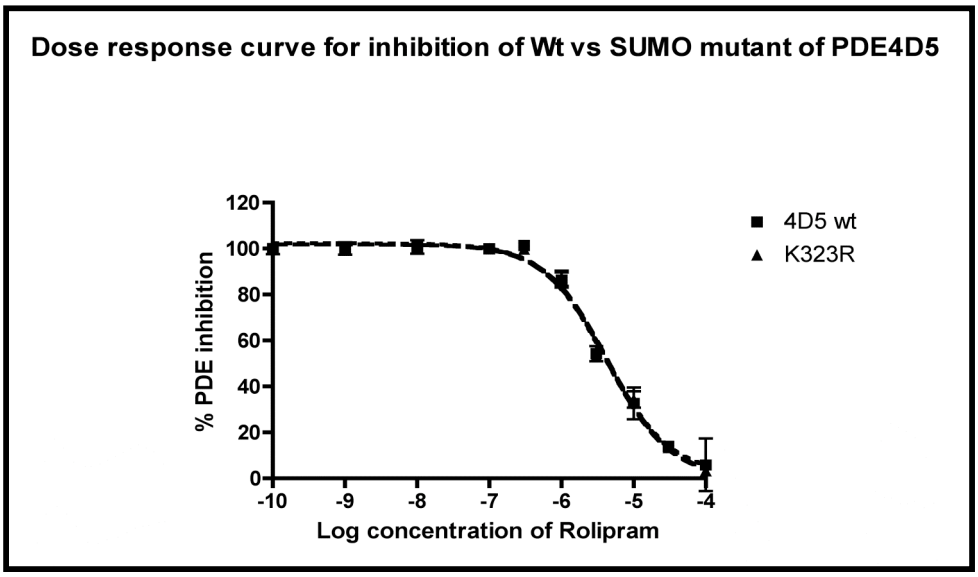
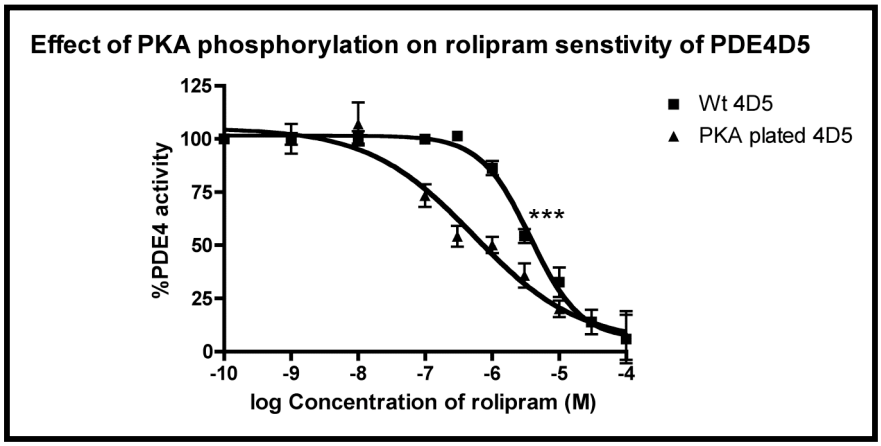


Figure 3.8 The effect of SUMOylation on rolipram sensitivity of PDE4D5

Inhibition of PDE4D5, SUMOylated PDE4D5, and an equimolar mixture of PDE4D5 and SUMOylated PDE4D5 by rolipram. Dose response curves for inhibition of purified recombinant PDE4D5 before and after SUMOylation and assayed with 1μM cAMP for rolipram. These data are given as IC_{50} values in micro-molar(μM) concentrations and are mean values +/- Standard deviation from three separate experiments. Paired t-test statistical analysis of the rolipram data were undertaken where $p<0.05$ indicates significant difference in rolipram sensitivity.

a)



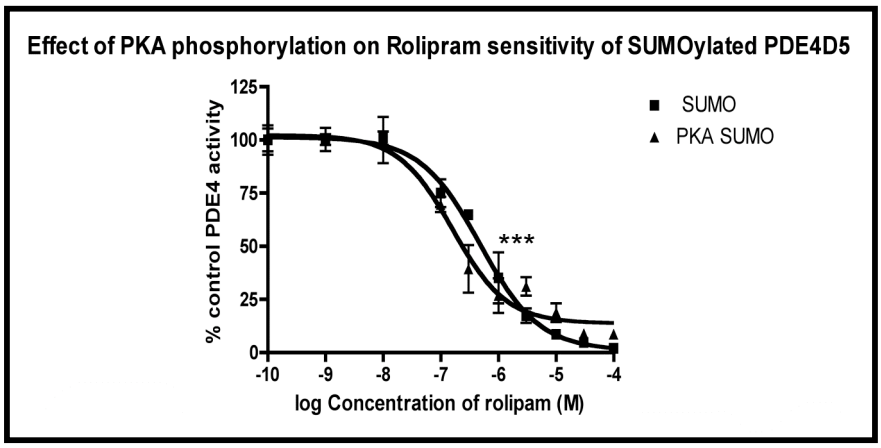
IC 50:

Wt: 3.8 +/- 0.2uM

Wt-PKA: 0.5 +/- 0.01uM

P < 0.0001

b)



IC 50:

SUMO: 0.47 +/- 0.03uM

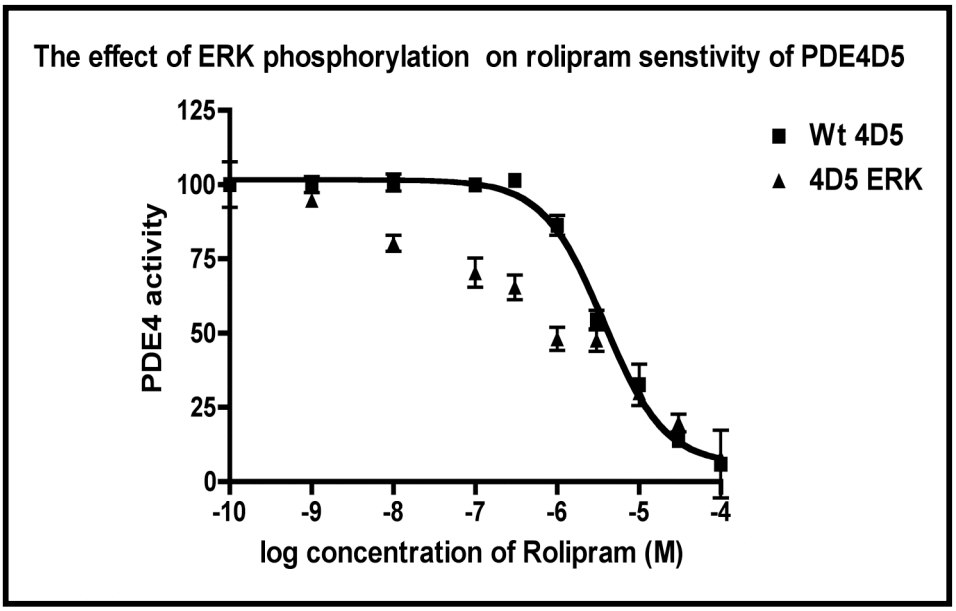
SUMO-PKA: 0.15+/- 0.009uM

P < 0.0001

Figure 3.9 The effect of SUMOylation and PKA phosphorylation on rolipram sensitivity of PDE4D5

Dose response curves for inhibition of purified recombinant PDE4D5 and SUMOylated PDE4D5 before and after PKA phosphorylation assayed with 1μM cAMP for rolipram. These data are given as IC50 values in micro-molar(μM) concentrations and are mean values +/- Standard deviation from three separate experiments. Paired t-test statistical analysis of the rolipram data were undertaken where $p<0.05$ indicates significant difference in rolipram sensitivity.

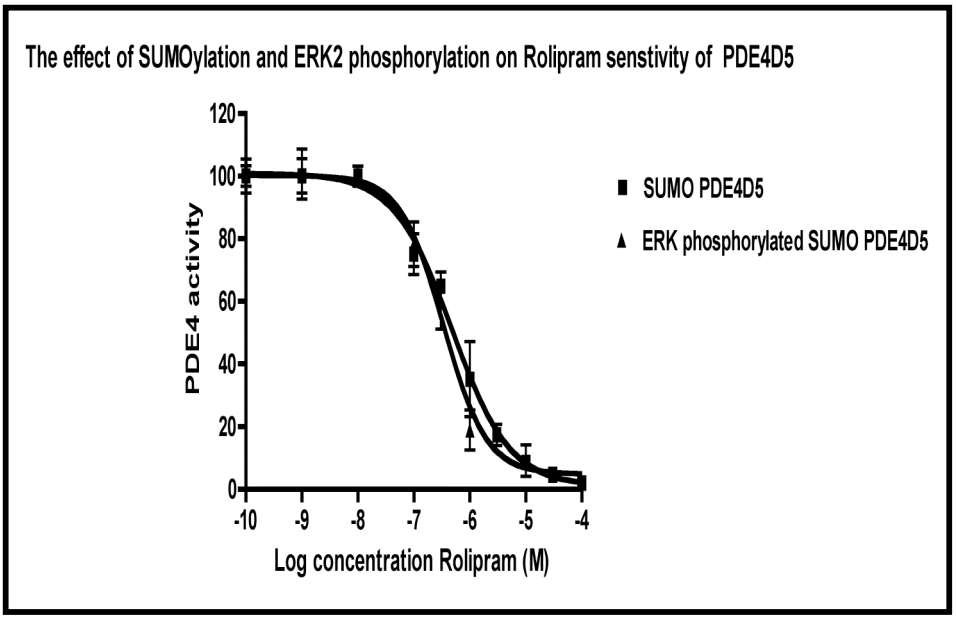
a)



IC 50:

PDE4D5: 3.8 +/- 0.2uM

b)



IC 50:

SUMO PDE4D5:

0.45 +/- 0.03uM

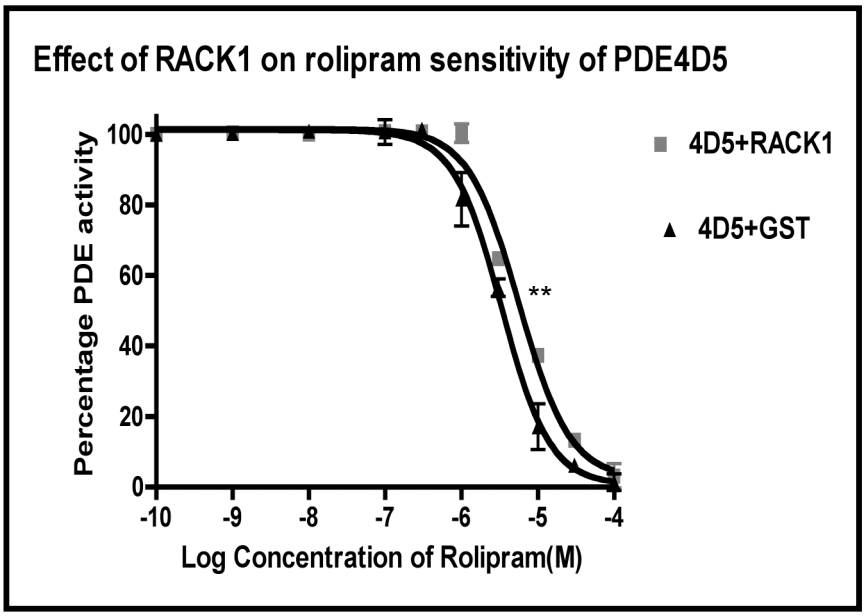
ERK SUMO PDE4D5:

0.45+/-0.01uM

Figure 3.10 The effect of SUMOylation and ERK phosphorylation on rolipram sensitivity of PDE4D5

Dose response curves for inhibition of purified recombinant PDE4D5 and SUMOylated PDE4D5 before and after ERK2 phosphorylation assayed with 1μM cAMP for rolipram. These data are given as IC50 values in micro-molar(μM) concentrations and are mean values +/- Standard deviation from three separate experiments. Paired t-test statistical analysis of the rolipram data were undertaken where $p<0.05$ indicates significant difference in rolipram sensitivity.

a)



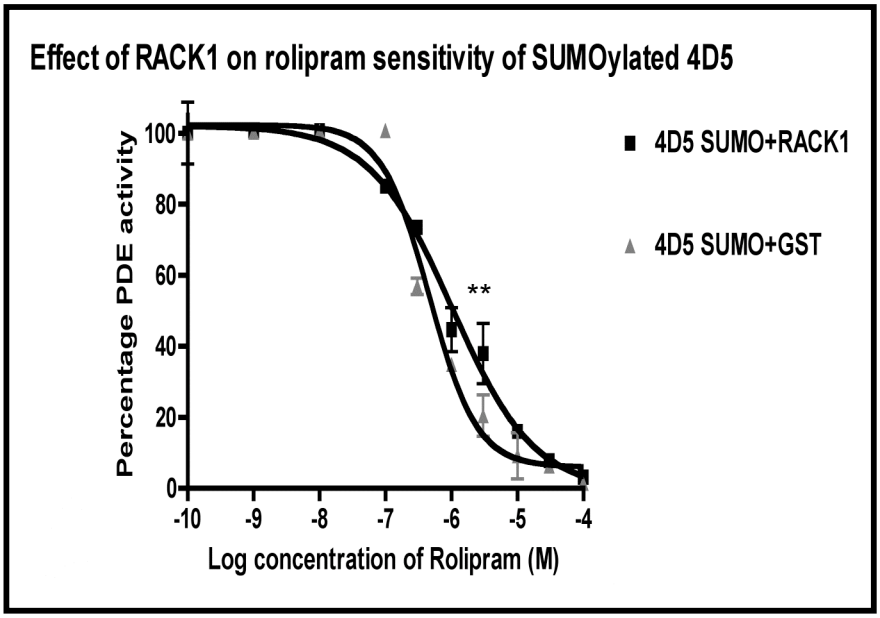
IC50:

PDE4D5 MBP+RACK1 GST: 5.6+/- 0.03uM

PDE4D5 MBP+GST: 3.8 +/- 0.19uM

P<0.001

b)



IC50:

SUMO PDE4D5 MBP+RACK1 GST: 1.2 +/- 0.009 uM

SUMO PDE4D5 MBP+GST: 0.45 +/- 0.03 uM

P<0.001

Figure 3.11 The effect of RACK1 binding on rolipram sensitivity of SUMOylated and unSUMOylated PDE4D5

Dose response curves assayed with 1μM cAMP for rolipram inhibition of purified, recombinant PDE4D5 MBP and SUMOylated PDE4D5-MBP after RACK1GST binding. Same dose response curves were carried out on PDE4D5-MBP and SUMO-PDE4D5-MBP mixed with GST alone as control. These data are given as IC50 values in micro-molar(μM) concentrations and are mean values +/- Standard deviation from three separate experiments. Paired t-test statistical analysis of the rolipram data were undertaken where $p<0.05$ indicates significant difference in rolipram sensitivity.

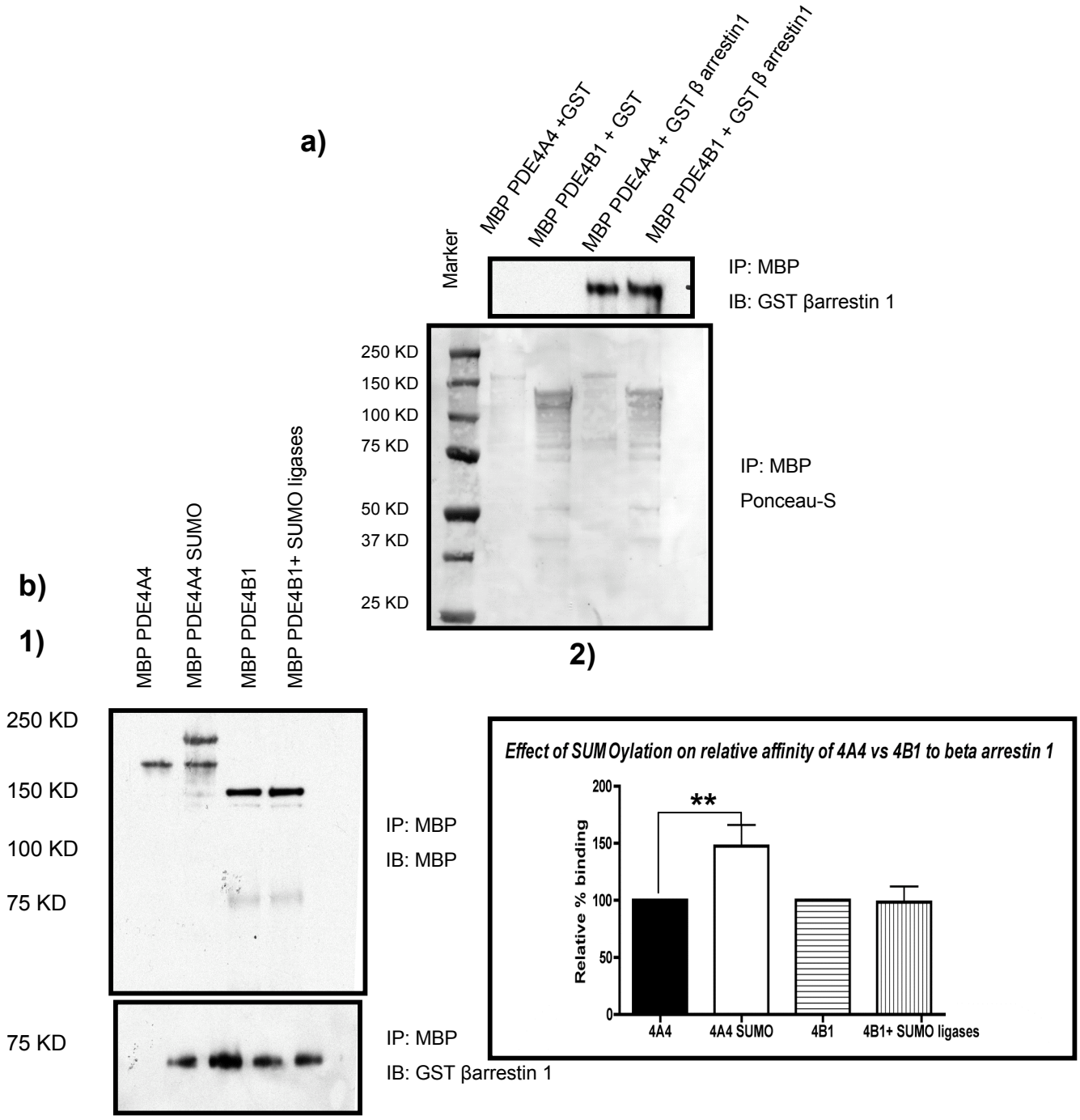
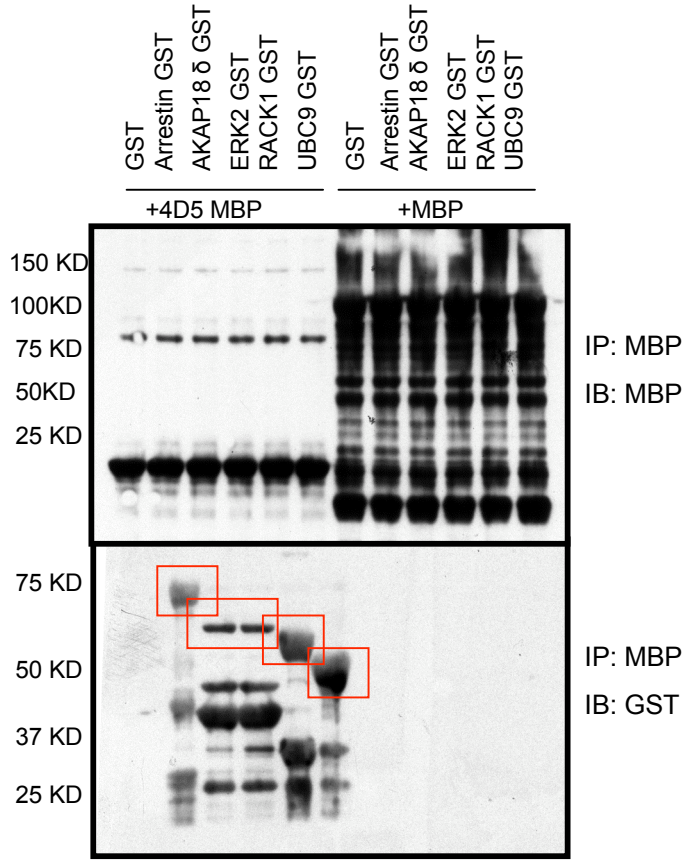


Figure 3.12 Effect of SUMOylation on affinity of PDE4 isoforms for arrestin

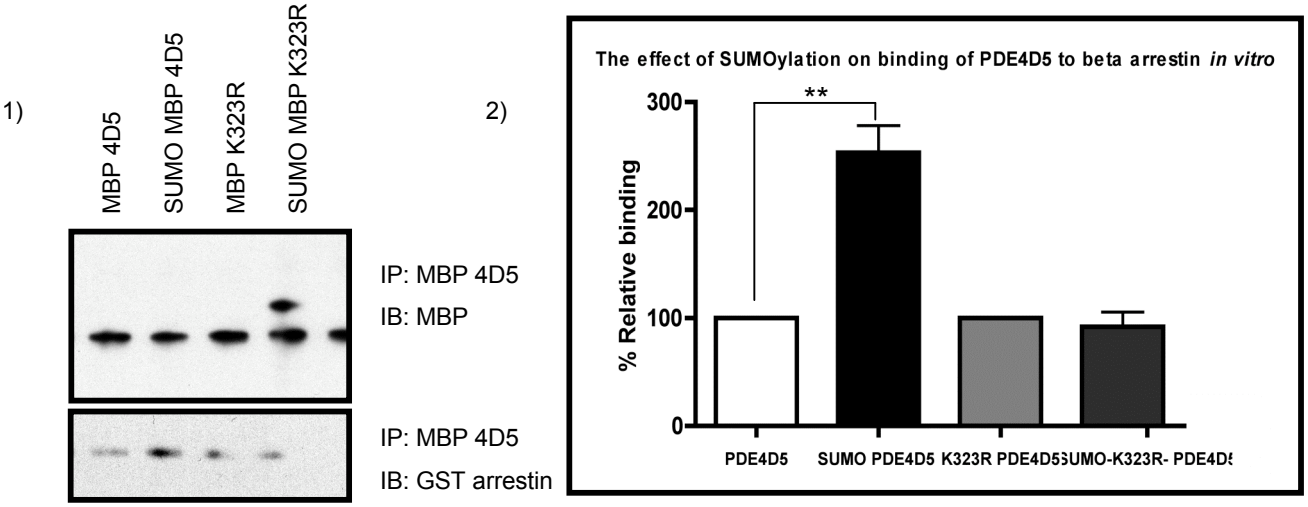
A) Purified MBP PDE4A4 and PDE4B1 were incubated with GST βarrestin 1 or GST alone. Complexes immobilised on amylose resin were washed and analysed by SDS-PAGE. Detection of bound proteins was carried by immuno blotting using a polyclonal antibody against beta arrestin 1.

B) Purified MBP PDE4A4 and PDE4B1 were SUMOylated in vitro and incubated with GST βarrestin 1 or GST alone. Complexes immobilised on amylose resin were washed and analysed by SDS-PAGE. Detection of bound proteins was carried by immuno blotting using a polyclonal antibody against beta arrestin 1. The amount of arrestin bound to each of the sample was was analysed by densitometry using Quantityone® software. These results shown were typical of experiments done on three different occasions. Paired t-test statistical analysis of the densitometry data were undertaken where $p<0.05$ indicates significant difference in beta arrestin binding.

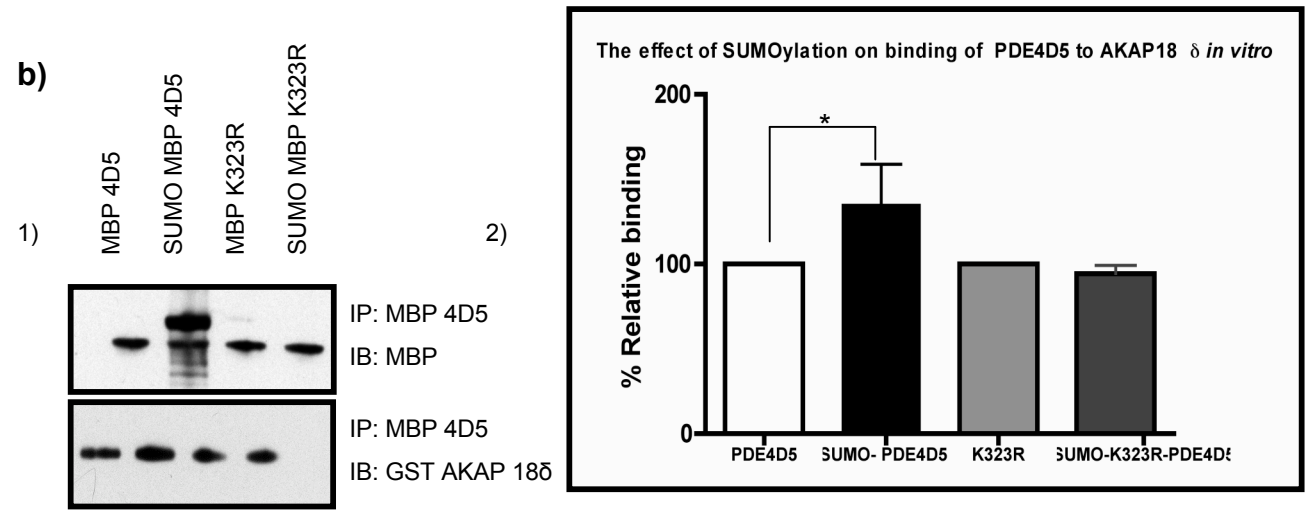
i)


GST
Arrestin GST
AKAP18 δ GST
ERK2 GST
RACK1 GST
UBC9 GST
GST
Arrestin GST
AKAP18 δ GST
ERK2 GST
RACK1 GST
UBC9 GST
+4D5 MBP
+MBP
150 KD
100KD
75 KD
50KD
25 KD
IP: MBP
IB: MBP
75 KD
50 KD
37 KD
25 KD
IP: MBP
IB: GST

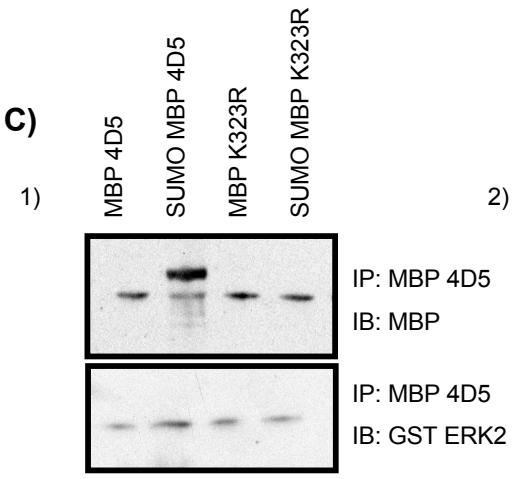
ii)a)


1)
MBP 4D5
SUMO MBP 4D5
MBP K323R
SUMO MBP K323R
IP: MBP 4D5
IB: MBP
IP: MBP 4D5
IB: GST arrestin
2)
The effect of SUMOylation on binding of PDE4D5 to beta arrestin in vitro
% Relative binding
300
200
100
0
**
PDE4D5
SUMO PDE4D5
K323R PDE4D5
SUMO-K323R- PDE4D5

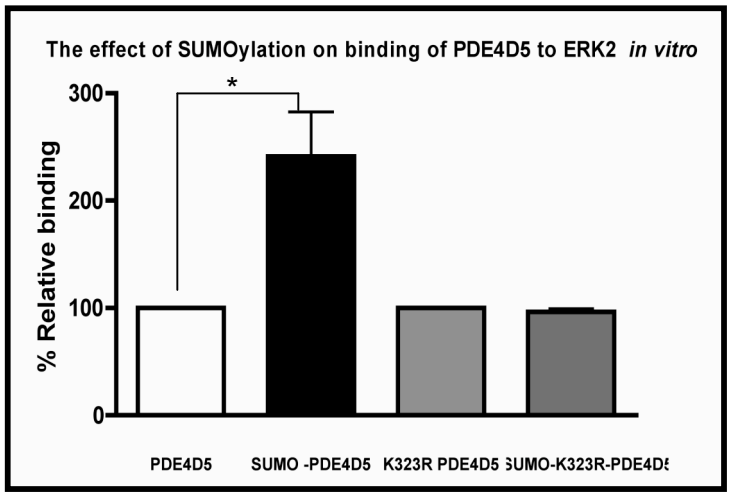
b)


1)
MBP 4D5
SUMO MBP 4D5
MBP K323R
SUMO MBP K323R
IP: MBP 4D5
IB: MBP
IP: MBP 4D5
IB: GST AKAP 18δ
2)
The effect of SUMOylation on binding of PDE4D5 to AKAP18 δ in vitro
% Relative binding
200
100
0
*
PDE4D5
SUMO- PDE4D5
K323R
SUMO-K323R-PDE4D5

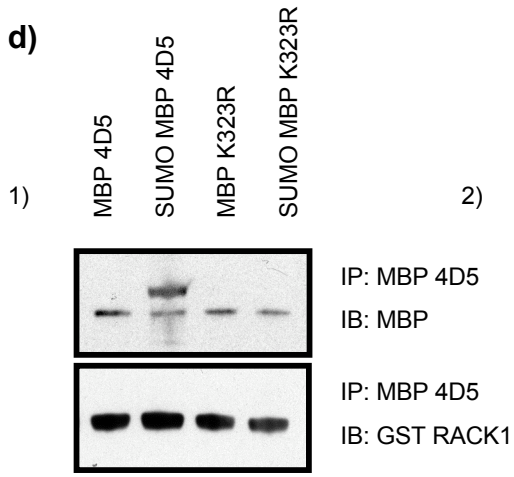
C)


1)
MBP 4D5
SUMO MBP 4D5
MBP K323R
SUMO MBP K323R
IP: MBP 4D5
IB: MBP
IP: MBP 4D5
IB: GST ERK2

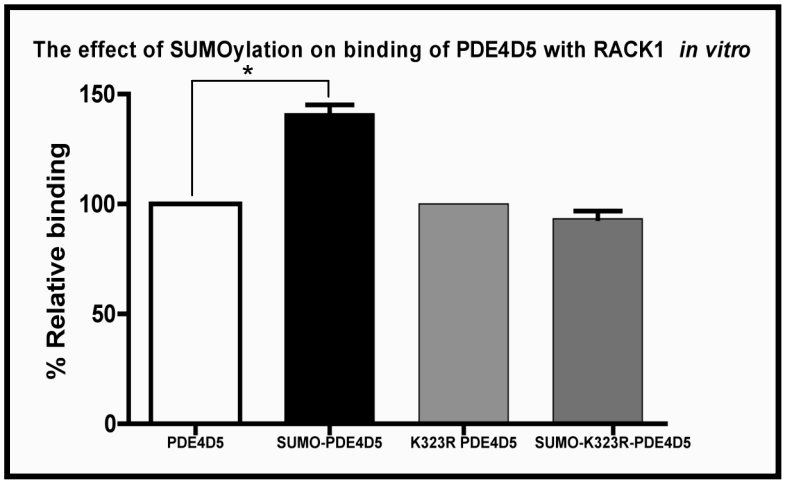
2)


The effect of SUMOylation on binding of PDE4D5 to ERK2 in vitro
% Relative binding
300
200
100
0
*
PDE4D5
SUMO -PDE4D5
K323R PDE4D5
SUMO-K323R-PDE4D5

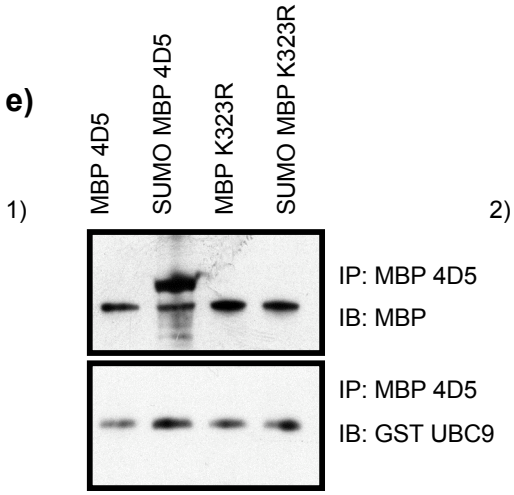
d)


1)
MBP 4D5
SUMO MBP 4D5
MBP K323R
SUMO MBP K323R
IP: MBP 4D5
IB: MBP
IP: MBP 4D5
IB: GST RACK1

2)


The effect of SUMOylation on binding of PDE4D5 with RACK1 in vitro
% Relative binding
150
100
50
0
*
PDE4D5
SUMO-PDE4D5
K323R PDE4D5
SUMO-K323R-PDE4D5

e)


1)
MBP 4D5
SUMO MBP 4D5
MBP K323R
SUMO MBP K323R
IP: MBP 4D5
IB: MBP
IP: MBP 4D5
IB: GST UBC9

2)

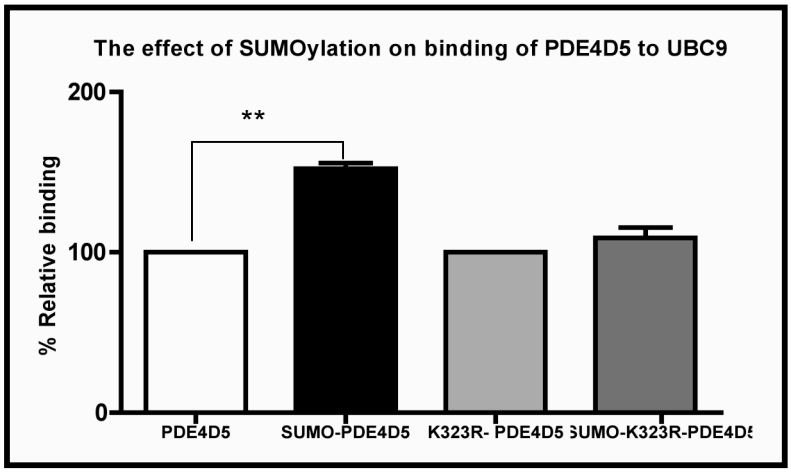
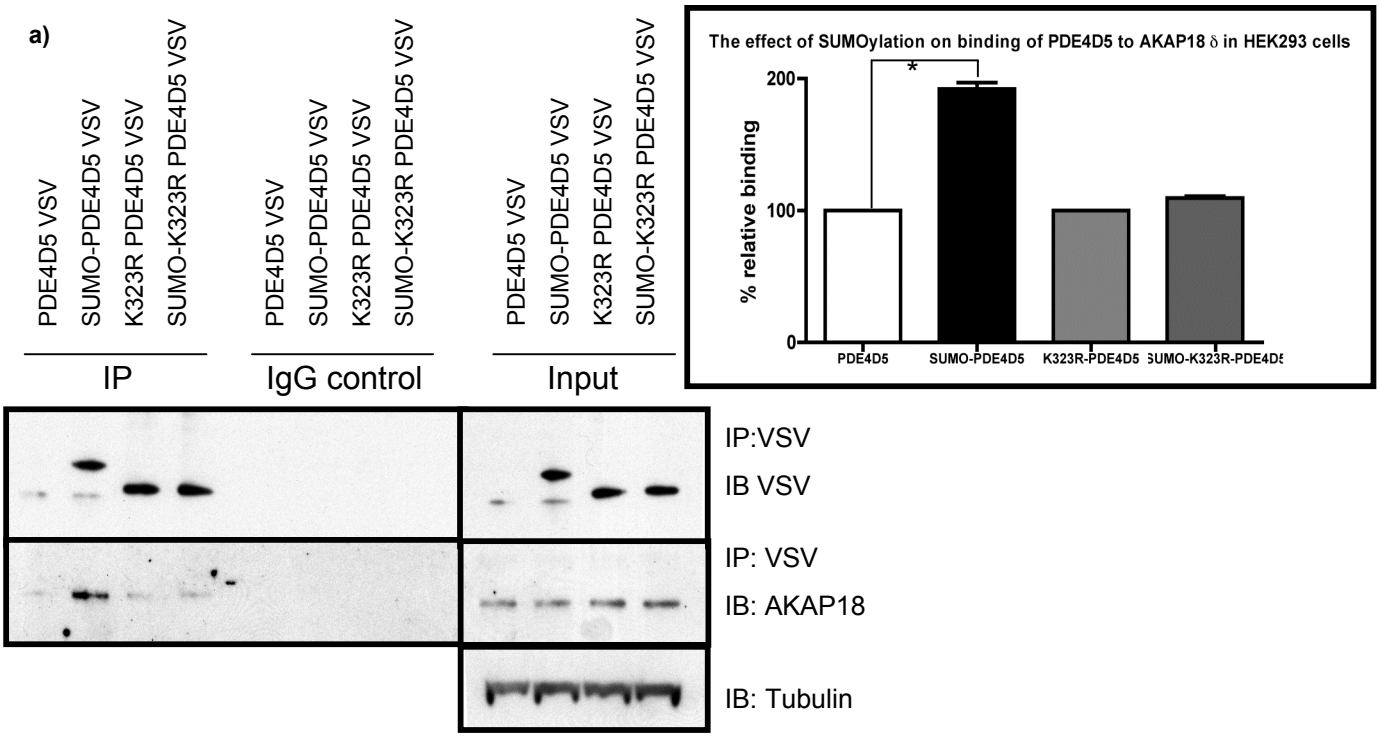

The effect of SUMOylation on binding of PDE4D5 to UBC9
% Relative binding
200
100
0
**
PDE4D5
SUMO-PDE4D5
K323R- PDE4D5
SUMO-K323R-PDE4D5

Figure 3.13 The effect of SUMOylation on interaction of PDE4D5 with its partners *in vitro*

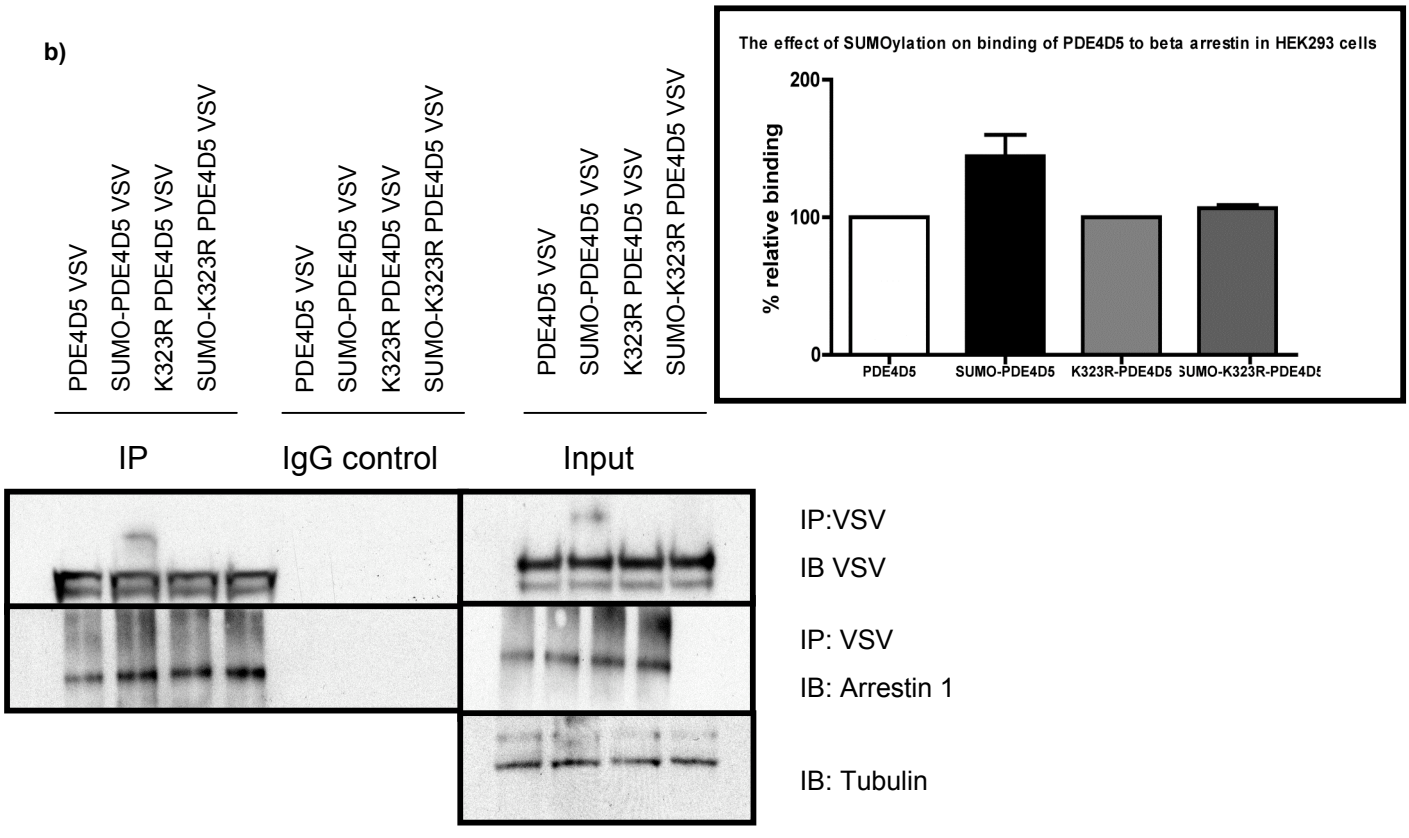
i) Purified MBP and PDE4D5 were incubated with purified GST and GST fusion proteins of indicated partner proteins respectively. Complexes were immobilised on amylose resin, washed and analysed by SDS-PAGE. Detection of bound proteins was carried out by immunoblotting using a monoclonal GST and MBP antibodies.

ii) Equimolar amounts of MBP fusion proteins of SUMOylated and non-SUMOylated PDE4D5 and K323R SUMO mutant of PDE4D5 were incubated with purified GST fusion proteins of indicated proteins. Complexes were immobilised on amylose resin, washed and analysed by SDS-PAGE. Detection of bound proteins was carried out by immunoblotting using a monoclonal GST and MBP antibodies. Quantification of bound proteins were carried out by densitometric analysis using Quantityone software ®. The results shown are examples of experiments done from three separate pull down assays performed by using purified proteins obtained from a single successful expression experiment undertaken for all the proteins in *E.coli*. Paired t-test statistical analysis of the densitometry data were undertaken where $p<0.05$ indicates significant difference in interacting partner binding as indicated.

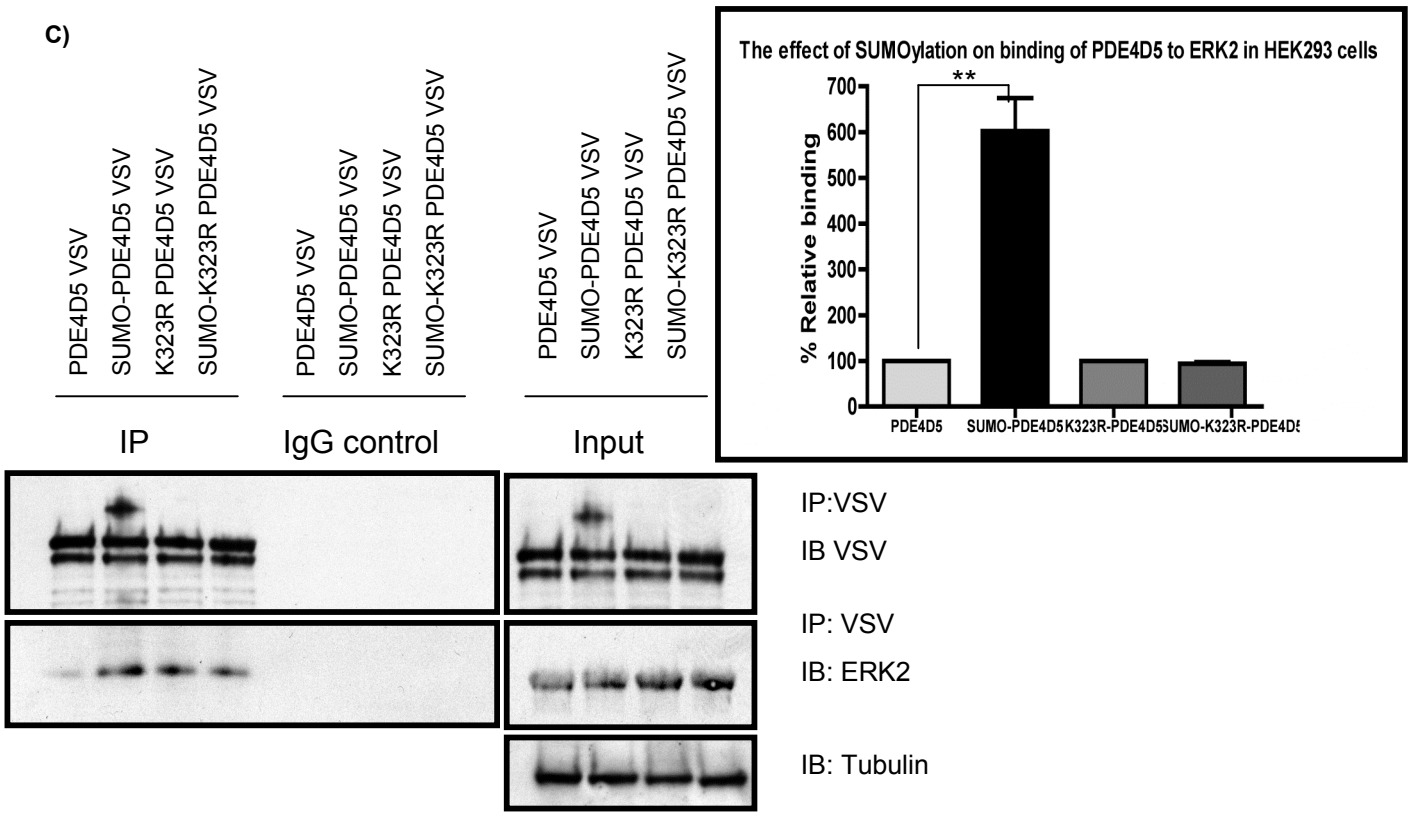
a)


PDE4D5 VSV
SUMO-PDE4D5 VSV
K323R PDE4D5 VSV
SUMO-K323R PDE4D5 VSV
IP
IgG control
Input
The effect of SUMOylation on binding of PDE4D5 to AKAP18 δ in HEK293 cells
% relative binding
200
100
0
*
PDE4D5
SUMO-PDE4D5
K323R-PDE4D5
SUMO-K323R-PDE4D5
IP:VSV
IB VSV
IP: VSV
IB: AKAP18
IB: Tubulin

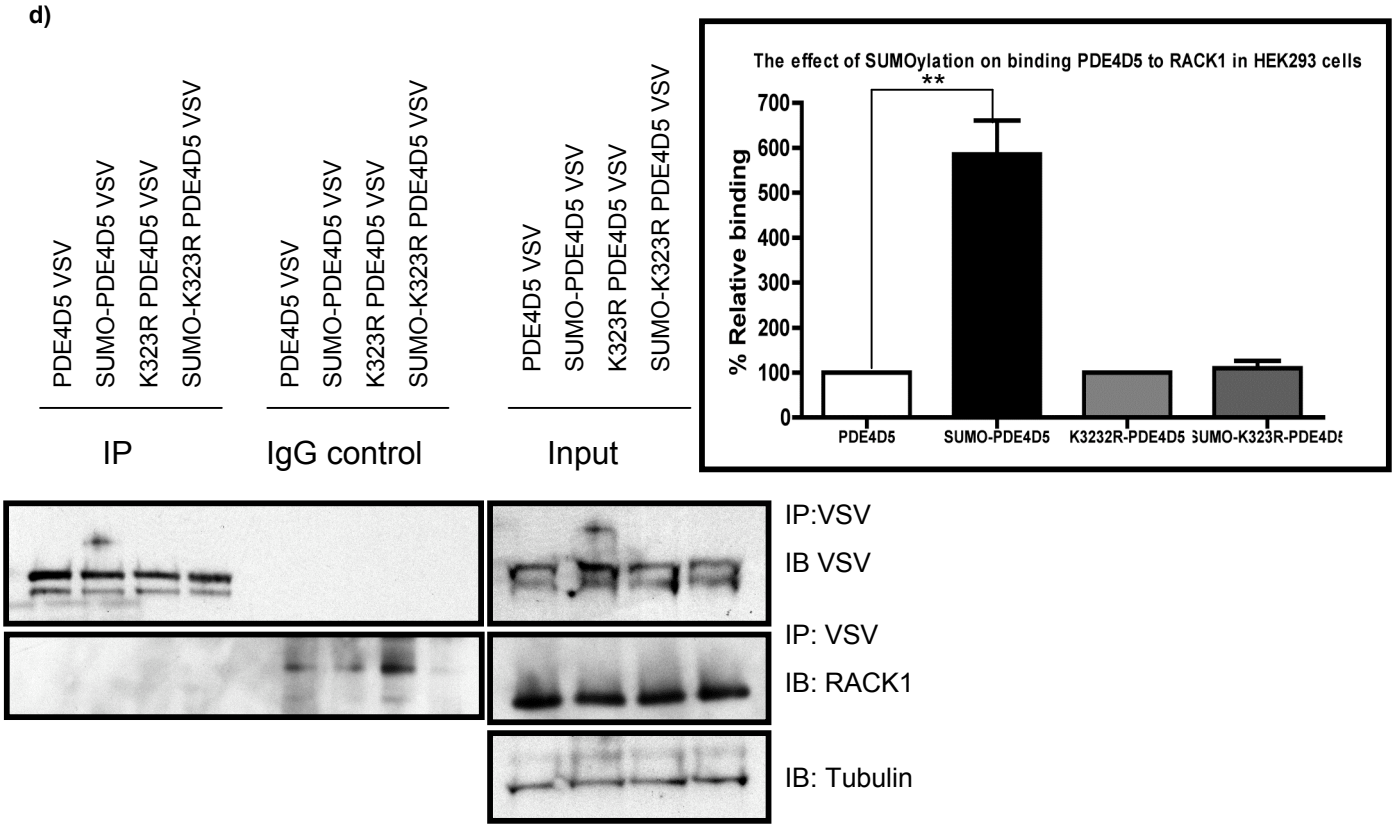
b)


PDE4D5 VSV
SUMO-PDE4D5 VSV
K323R PDE4D5 VSV
SUMO-K323R PDE4D5 VSV
IP
IgG control
Input
The effect of SUMOylation on binding of PDE4D5 to beta arrestin in HEK293 cells
% relative binding
200
100
0
PDE4D5
SUMO-PDE4D5
K323R-PDE4D5
SUMO-K323R-PDE4D5
IP:VSV
IB VSV
IP: VSV
IB: Arrestin 1
IB: Tubulin

c)


PDE4D5 VSV
SUMO-PDE4D5 VSV
K323R PDE4D5 VSV
SUMO-K323R PDE4D5 VSV
IP
IgG control
Input
IP:VSV
IB VSV
IP: VSV
IB: ERK2
IB: Tubulin
The effect of SUMOylation on binding of PDE4D5 to ERK2 in HEK293 cells
% Relative binding
PDE4D5
SUMO-PDE4D5
K323R-PDE4D5
SUMO-K323R-PDE4D5

d)


PDE4D5 VSV
SUMO-PDE4D5 VSV
K323R PDE4D5 VSV
SUMO-K323R PDE4D5 VSV
IP
IgG control
Input
IP:VSV
IB VSV
IP: VSV
IB: RACK1
IB: Tubulin
The effect of SUMOylation on binding PDE4D5 to RACK1 in HEK293 cells
% Relative binding
PDE4D5
SUMO-PDE4D5
K3232R-PDE4D5
SUMO-K323R-PDE4D5

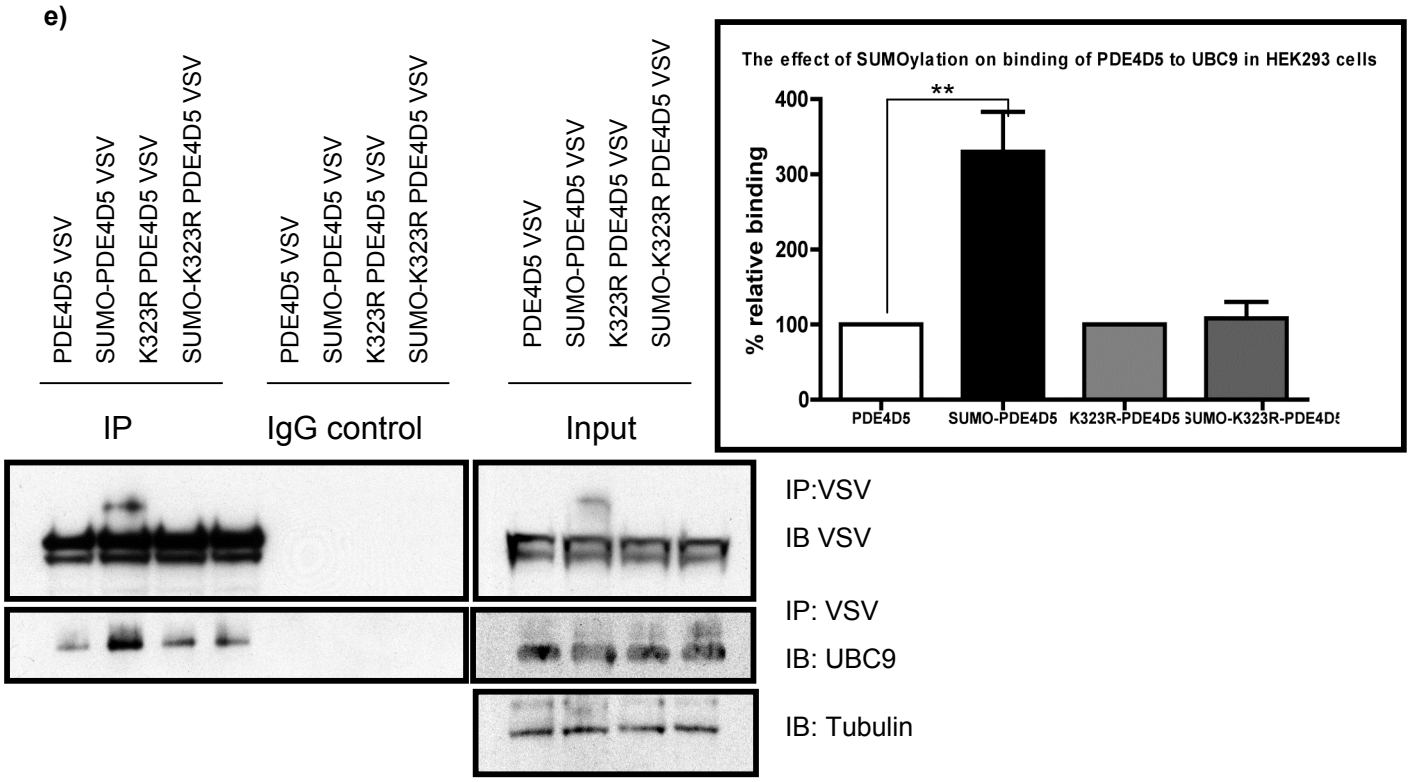


Figure 3.14 The effect of SUMOylation on interaction of PDE4D5 with its partners in HEK293 cells

HEK 293 cells were triple transfected with PDE4D5 VSV or K323R mutant of PDE4D5 VSV with PDE4D5 interacting partners indicated above and with or without PIASy respectively. Bound complexes were immunoprecipitated by VSV agarose, washed and analyzed by SDS-PAGE. Detection of bound complexes was performed by antibody to the respective partner protein. Non-specific IgG was used as a control and tubulin was immunoblotted as a loading control. Quantification of bound proteins was carried out by densitometric analysis using Quantityone software®. The results shown are examples of experiments done from three separate transfections and immunoprecipitation assays. Paired t-test statistical analysis of the densitometry data were undertaken where $p<0.05$ indicates significant difference in interacting partner binding as indicated.

Chapter 4 Rolipram induced foci formation of PDE4A4

4.1 Introduction

4.1.1 Altered localisation of proteins is regulated by multiple factors

The localisation of a protein and its function are often inter-dependent. Changes in a protein's sub-cellular localisation are affected by various factors including post-translational modifications, alterations in expression/degradation rates and sequestration into complex aggregates. Protein localisation may also alter between various states of disease and health (Chu et al, 2007). For instance, Elk-1 transcription factor is a key component of ERK-MAPKinase pathway (Iwata et al; 2001). Elk-1 along with alpha-synuclein is known to form cytoplasmic inclusion bodies where it is known to cause MAPKinase attenuation in glial cells in Parkinson's disease and multiple system atrophy (Chu et al, 2007 and Iwata et al; 2001). Similarly, importin α1 is involved in transport of proteins across the nuclear membrane into nucleus. In conditions like Alzheimer's disease, the function of importin α1 is disrupted and the protein is shown to localise to aggregates called Hirano bodies which are also seen in conditions associated with oxidative stress mediated injury (Chu et al; 2007 and Lee et al, 2006).

4.1.2 PDE4A4 redistributes into foci

It has been shown that the PDE4 isoform, PDE4A4 undergoes redistribution when subjected to chronic rolipram treatment (Terry et al; 2003). This redistribution is specific for PDE4A4 and its rat ortholog PDE4A5. It was demonstrated that the process occurs over 16 hours and is dependent upon *de novo* protein synthesis as inhibition of protein synthesis by cycloheximide or puromycin prevents redistribution of the isoform

Rolipram, as well as a few other PDE4-specific inhibitors such as RO20-1724 and RS25344, induce a profound redistribution of the PDE4A4 isoform. This

occurs in a dose-dependent, reversible manner and leads to the formation of accretion foci within the cell (Figure 4.1). Foci formation confers memory in that, cells having been pre-exposed to rolipram form foci, which are then dispersed upon the removal of rolipram, re-form foci upon further rolipram challenge at a faster rate than naïve cells being exposed to rolipram for the first time. This is likely due to rolipram-induced initiation of *de novo* synthesis of protein(s) required for foci formation (Terry et al, 2003).

Accretion foci formation is PDE4 isoform-specific thus suggesting a contribution of the N-terminal regions of human PDE4A4 rat PDE4A5 in foci formation. In support of this, foci formation is not seen with other long isoforms of PDE4A subfamily such as PDE4A10 and PDE4A11 (Wallace et al; 2005). Foci formation has not been demonstrated by isoforms of the PDE4B, PDE4C and PDE4D subfamilies. However, it has not been investigated whether a chimeric PDE4B, 4C or 4D isoform expressing the unique N-terminal region of PDE4A4 would form foci in response to prolonged rolipram treatment. Foci formation is known to be blocked by PDE4 inhibitors such as Ariflo® which inhibit the foci formation by displacing rolipram from the catalytic site.

4.1.3 Molecular mechanisms of foci formation and inside-out signalling

Since foci formation is only shown by PDE4A4 and PDE4A5, It was proposed that the isoform specific N-terminal region along with catalytic region mediates conformational changes upon rolipram binding thus leading to foci formation (Terry et al; 2003). These changes along with other factors like protein synthesis are collectively called ‘inside-out’ signalling as foci formation is a cAMP-independent action and is thought to be triggered by an altered conformation of PDE4A4 (Figure 4.1e). This model proposes that when rolipram binds the catalytic region, conformational changes are induced which are exposed to the surface of the PDE4 molecule (Appendix II for image). Knowledge of the 3-D structure of the catalytic unit has led to a model in which a conformational change emanating from the catalytic pocket is relayed through the loop connecting helix-7 with helix-8 to the surface of the catalytic

unit via interactions with the N- and C-termini of helix 10 and helix-11. Two residues, His^{505} and His^{506}, of loop 7/8 are thought to interact with Val^{475} of helix-10. These residues play a crucial role in foci formation as mutation at any of these sites leads to disruption in foci formation. The 7/8-loop, along with helix-10 and helix-11, forms a groove-like structure that could potentially serve as a binding pocket for other proteins or PDE4A4's N-terminal region. These rolipram-induced conformational changes, may subsequently allow for PDE4A4 partner protein(s), or PDE4A4 itself to interact thus triggering redistribution of the isoform into foci.

4.1.4 Foci formation and signalling pathways

The aim of this investigation was to identify the signalling pathways mediating foci formation, maintenance and dispersal. It was also of interest to determine if PDE4A4 interacting proteins are components of foci.

4.2 Results

4.2.1 Formation of rolipram-induced foci of PDE4A4

CHO cells stably expressing a GFP fusion of PDE4A4 were analysed by confocal microscopy following incubation with rolipram or DMSO for 10-16hrs. Foci could be seen as tiny green aggregates in cells treated with rolipram. Cells treated with DMSO showed no foci (Figure 4.1). The amount of foci formation was quantified by a fluorescence based plate reader assay as described previously (Terry et al; 2003). A similar experiment was carried out with CHO cells transiently expressing GFP alone. Rolipram treatment did not lead to any aggregation of GFP in CHO cells (Figure 4.1d).

4.2.2 The effect of cAMP signalling on foci formation

Terry and co-workers (Terry et al; 2003) showed that foci formation was independent of PKA action and that cAMP had inhibitory effect on foci formation by displacing rolipram from the active site. In order to identify the

component(s) of cAMP signalling through which these effects are mediated, cells were treated with various cAMP effectors. Elevation of intracellular cAMP upon challenge with forskolin alone or forskolin plus the non-selective PDE inhibitor, IBMX, in the presence or absence of PKA inhibitor, H89, inhibited foci formation. However, augmentation of cAMP levels through activation of the G_s-coupled β_2-AR with isoprenaline treatment had no effect on foci formation. Similarly, treatment with EPAC agonist (8-CPT-2'-O-Me-cAMP) did not alter foci formation (figure 4.2).

4.2.3 The effect of MAP kinase pathway on foci formation

Previous work has shown that ERK MAP kinases are able to regulate PDE4 activity by phosphorylating isoforms from the PDE4B/C/D sub-families, which possess a KIM domain, a proline-directed serine consensus and FQF domain in the same order (Mackenzie et al, 2000). Isoforms of the PDE4A subfamily have KIM and FQF domains but cannot be phosphorylated and regulated by ERK due to the presence of an arginine residue in the proline-directed serine consensus making the sequence R-x-Ser-P instead of P-x-Ser-P (Baillie et al; 2000, Baillie et al; 2001, Hoffmann et al 1999 Mackenzie et al; 2000).

To assess any potential role of ERK and other MAP kinases in regulating the foci formation of PDE4A4-GFP, various inhibitors and activators of MAP kinases were evaluated. Cells were incubated with MEK inhibitors (UO126 and PD98059), p38 MAP kinase activator, anisomycin, p38 MAP kinase inhibitor, SB203580, and a JNK inhibitor (Anthra [1, 9-*cd*] pyrazol-6(2*H*)-one 1, 9-pyrazoloanthrone). The compounds were added to cells individually as well as in various combinations in the presence or absence of rolipram. Inhibition of MEK enhanced foci formation by 50 ± 17 % as determined by the fluorescent plate reader assay whereas Activation of p38 MAP kinase completely inhibited foci formation. Addition of either the p38 MAP kinase inhibitor SB203580 (Davies et al; 2000) or the JNK inhibitor did not have any effect on foci formation. Cells treated with both anisomycin and SB203580

inhibited foci formation. Similarly, cells treated with anisomycin and JNK inhibitor did not form foci (figure 4.3).

4.2.4 The effect of MAP kinase pathway on foci formation

In order to investigate the role of PKC and calcium signalling in foci formation of PDE4A4-GFP, cells were challenged with various PKC inhibitors and calcium modulators. PMA, a PKC activator, did not affect foci formation nor did EGCG (Epigallocathecingallate) Rottlerin, a PKCδ inhibitor, completely inhibited foci formation. PMA treatment reversed the effects of rottlerin on foci formation and led to 80% ± 10.1% increase in foci formation compared to incubation with rottlerin alone. PKC inhibitors, RO320432 (PKCα,β1,ε) and GO6983 (PKCα,β,γ,δ) elicited a 70-90% enhancement in the fluorescence immobilised in PDE4A4-GFP accretion foci as compared to treatment with rolipram alone (Figure 4.4a).

To further ascertain the role of PKC-calcium signalling, various calcium modulators such as thapsigargin and ionomycin were used. Both compounds completely inhibited foci formation as evident by a 98% decrease in immobile GFP. Inhibiting CaMKII with KN62 had no effect, nor did inhibition of calcineurin with cyclosporin A (Figure 4.4b), whilst inhibition of calmodulin with W-7 and ophiobolin-A treatments led to cell loss/death.

4.2.5 PI3 kinase inhibition enhances foci formation

To assess the possible role of PI3 kinase signalling in foci formation, cells were incubated with PI3-K inhibitor wortmannin. This increased the percentage of immobile GFP in accretion foci to 175% ± 15.5 % (Figure 4.5). Furthermore, PI3-K-specific inhibitor LY294002 also increased the percentage immobile GFP by a similar extent (196% ± 19.2, 5). However, inhibiting the downstream effectors of PI3 kinase, p70S6K and GSK3β, with rapamycin and GSK3β Inhibitor respectively, had no effect on rolipram-induced foci formation.

4.2.6 The effect of tyrosine kinases on foci formation

Inhibition of cellular tyrosine kinase activity using Genistein ablated foci formation (Figure 4.6). To identify the role of individual tyrosine kinase families in foci formation, cells were challenged with Src tyrosyl kinase inhibitors, SU66656 and PD163893. Blockade of SRC tyrosyl kinase signalling elicited no effect on foci formation. However, inhibition of the PDGF receptor tyrosine kinase inhibited rolipram-induced foci formation (figure 4.6).

4.2.7 Role of cdk5 in foci formation

To evaluate the role of cdk5 on foci formation, cells stably expressing PDE4A4-GFP were treated with Roscovitine. At a concentration of 10μM, Roscovitine inhibited foci formation but did not have any effect at 1uM concentration.

4.2.8 The role of thalidomide in foci formation

PDE4 isoforms, of the PDE4A and PDE4B subfamilies are known to be involved in regulating the functioning of immune cells. PDE4 inhibition blocked antigen-stimulated T-cell proliferation and bacterial lipopolysaccharide (LPS)-stimulated tumour necrosis factor alpha (TNFalpha) release from peripheral blood monocytes (Manning et al; 1999). The ability of thalidomide to inhibit TNF production and acts as an immunomodulator in conditions including Erythema nudosum leprosum is well established (Melchert and list; 2007, Okafor et al. 2003). Considering the possible link between thalidomide and PDE4 signalling, the role of thalidomide in affecting foci formation of PDE4A4GFP was examined. It was found that thalidomide was able to inhibit foci formation at a concentration of 400μM±3.6uM.

4.2.9 PDE4 oligomerisation and foci formation

It has been suggested that PDE4 isoforms may oligomerise by interactions involving their UCR1 and UCR2 domains (Richter et al; 2002). In order to determine if PDE4A4 is able to oligomerise, CHO cells stably expressing PDE4A4-GFP were transfected with a V5-tagged PDE4A4. Post-transfection, cells were treated with and without rolipram for 16hours to allow foci formation. GFP- and V5- immunoprecipitates were blotted for both with both GFP and V5 antisera (Figure 4.9). Similar experiments were carried out with VSV- and Flag-tagged forms of PDE4D3 in transfected CHO cells treated with or without rolipram. PDE4A4 oligomerisation was not detected (Figure 4.9b) under these conditions used. However, PDE4D3 oligomerisation was detected (Figure 4.9a).

4.2.10 Role of protein turnover on foci formation

As reported previously, protein synthesis is required for foci formation (Terry et al: 2003). Assays were therefore carried out in an effort to better understand the roles of protein synthesis and degradation on foci formation. CHO cells expressing PDE4A4-GFP treated with rolipram were also treated with either cycloheximide (CHX) or puromycin to inhibit protein synthesis or with MG132 to inhibit proteasomal degradation of proteins. Cells were also treated with MG132 and CHX at the same time in order to inhibit protein turnover. In all instances, foci formation was blocked (Figure 4.10).

4.2.11 Role of protein turnover on stability of preformed foci

To evaluate the role of protein turnover on the stability and dispersal of preformed foci, cells expressing PDE4A4-GFP were pre-treated with rolipram for 16hours, followed by treatment with CHX, MG132, or both in the presence of rolipram over time. Cells treated with rolipram alone were used as a positive control and untreated cells as a negative control. Inhibition of protein synthesis by CHX started to disperse rolipram-induced foci within 30 minutes of its addition to the cells and completely dispersed foci after 5 hours.

Inhibition of proteasomal protein degradation with MG132 yielded similar effects on foci dispersal. The rate of foci dispersal did not change when both protein synthesis and degradation were inhibited simultaneously (figure 4.11). The half life ($t_{1/2}$) of foci dispersal after inhibition of protein turn over was approximately 75 minutes.

4.2.12 The role of MAP kinases in dispersal of preformed foci of PDE4A4

Having evaluated any potential role of various MAP kinases in formation of foci, the effects of MAP kinases on dispersal and stabilisation of foci were examined. Cells pre-treated with rolipram were challenged with anisomycin or anisomycin/MG132/cyloheximide in the continued presence of rolipram (Figure 4.12). Similar treatments were carried out with UO126, which is a MEK inhibitor. Cells treated with anisomycin with and without cycloheximide+MG132 dispersed foci completely within 30minutes after the addition of the drug. In a similar experiment, cells treated with UO126, incubation with UO126 alone initially led to an increase in foci formation within 30 minutes of drug exposure by 60% ± 0.06%. After 150 mins, foci levels had returned to levels prior to UO126 addition and within 300 min foci were completely dispersed. In cells treated with UO126 in the presence of both cycloheximide and MG132, foci dispersal was observed as early as 30 mins after UO126 addition and all foci had dispersed by 300 mins (Figure 4.12). The $t_{1/2}$ of foci dispersal in the presence of UO126 and rolipram was 240 mins while in cells pre-treated with CHX and MG132 this was dramatically reduced to 90 minutes.

4.2.13 The role of calcium and PKC signalling on stability and dispersal of foci

In order to evaluate the effects of calcium and PKC signalling in stabilising pre-formed foci and foci dispersal, cells pre-exposed to rolipram for 16 hr were treated with thapsigargin which elevates intracellular calcium levels by triggering Ca^{2+} release from intracellular stores from endoplasmic reticulum (Won and Orth, 1995). Thapsigargin was added to the cells in the presence or absence of MG132 and CHX. Similar treatments were carried out with rottlerin

and RO320432, which is a PKC inhibitor. Thapsigargin initially dispersed the foci at a slightly slower rate than rottlerin and RO320432 treatments, both of which dispersed all foci within 30 mins. However, all the three compounds dispersed foci within 30 mins in cells treated with MG132 and CHX. The $t_{1/2}$ for foci dispersal by thapsigargin in the presence of rolipram was approximately 45 mins. An accurate measure of the $t_{1/2}$ of foci dispersal in the presence of thapsigargin, rolipram, CHX and MG132 could not be made using the time points analysed. A shorter time course with measurements taken at earlier time points would be required to evaluate this.

4.2.14 The role of tyrosine kinases in foci dispersal

To evaluate the role of tyrosine kinase action in dispersal of pre-formed foci, genistein was added to cells in the presence or absence of MG132 and CHX. Genistein treatment alone, as well as in the presence of MG132 and CHX, quickly dispersed pre-formed foci within 30 mins (Figure 4.14). The $t_{1/2}$ of foci dispersal upon tyrosine kinase inhibition could not be determined from the existing data and would require measurements over a shorter time course.

4.2.15 The role of PI3 kinases in maintenance of foci

The PI3 kinase inhibitor, LY294002 dispersed pre-formed foci in cells within 30 mins of inhibitor treatment, both in the presence and absence of MG132 and CHX (Figure 4.15).

4.2.16 Co-localisation studies

To identify components and localisation of foci, co-localisation studies were carried using confocal microscopy to examine the localisation of foci with intracellular compartments and various known PDE4 interacting proteins. Table 4.1 lists some of the proteins and treatments that were performed. Proteins that showed minimum interaction or co-localisation with foci were represented by (+) and those with the strongest interaction/co-localisation with foci by (+++). Foci were not found to localise to any of the cellular organelles

examined including mitochondria, Golgi apparatus or lysosomes. CHO cells treated with rolipram for 16hrs showed an up regulation of PYK2. Endosomal proteins like Rab9 and Rab11, Ubiquitin ligases like mdm2 and cbl, various PKCs and cytoskeletal proteins like actin, tubulin and vimentin were also stained in CHO cells stably expressing PDE4A4-GFP and treated with rolipram for induction of foci. (See appendix II for images).

4.3 Discussion

The conformational changes and folding of a protein almost always dictate the ability of a protein to function properly. Mis-folding of a protein might lead to aggregate formation that may eventually lead to refolding to its native functional conformation state or to its degradation (Chu et al, 2007 and Murphy et al, 2007). Proteins aggregates also occur in disease states such as Huntington's disease, which is characterised by poly-glutamine aggregates (Nagaoka et al; 2004).

Previous work has shown that wild-type PDE4A4 as well as PDE4A4 chimeras with various epitope tags undergo reversible redistribution into accretion foci upon chronic rolipram treatment (Terry et al; 2003). This redistribution is isoform-specific as only PDE4A4 and rat PDE4A5, which share the same N-terminal sequence, form foci and not any other isoforms from other PDE4 sub-family. In order to establish the signalling components in promoting and maintaining rolipram-induced foci, CHO cells stably expressing a PDE4A4-GFP chimera were subjected to treatments with various inhibitors and activators of signalling pathways.

PDE4 isoforms play a pivotal role in controlling cAMP levels within cells. It is widely appreciated that PKA phosphorylation activates these isoforms thus making them key regulators of the cAMP signalling cascade. To determine to the role of cAMP in rolipram-induced foci formation, cells were challenged with various effectors of this pathway. Challenge with 8-CPT-2'-Me-cAMP which activates EPAC, did not affect foci formation or dispersal. To elevate cAMP levels, cells were treated with forskolin, to activate adenylate cyclase

independently of G_s-proteins, (Seamon and Daly, 1981) and also in concert with IBMX to inhibit PDE activity simultaneously, thus maximising cAMP production (Turner et al, 1993 and Leemhuis et al; 2002). Under these conditions, foci formation was inhibited. Inhibition of PKA activity by H89 treatment did not affect foci formation suggesting the effects of cAMP on foci formation were not mediated through PKA, or at least through a PKA isoform which was H89-sensitive. Increasing cAMP levels via activation of G_s signalling of the β_2-adrenoceptor with isoproterenol challenge did not affect rolipram-induced foci formation thus indicating that foci formation is not driven via a G_s-adenylyl cyclase pathway. Taken together, these data show that inhibition of foci formation occurs only with agents, which maximise intracellular cAMP levels. It is still unclear which cAMP signalling pathways inhibiting foci formation. It is possible that cAMP actions are being mediated through a PKA isoform which is H89-insensitive or through other effectors including ion channels.

Isoforms of the PDE4A subfamily are not phosphorylated by ERK due to the substitution of an arginine for a proline in the proline directed serine consensus (Baillie et al; 2000, Baillie et al; 2001, Hoffmann et al 1999 Mackenzie et al; 2000). However, in order to determine any indirect effects of ERK, as well as effects of other members of MAP kinases, on foci formation, various inhibitors were tested. Inhibition of MEK using UO126 and PD98059 did not affect foci formation. Activation of p38 MAP kinase by anisomycin (Hazzalin et al; 2002) ablated foci formation. To test the specificity of anisomycin treatment on foci formation, SB203580, a p38 MAP kinase inhibitor, (Davies et al; 2000) was used. However, SB203580 did not reverse the effects of anisomycin. As anisomycin is also known to activate JNK (c-Jun N-terminal kinase) along with p38 MAP kinase (Sampieri et al; 2007), a JNK inhibitor (Anthra [1,9-*cd*] pyrazol-6 (2*H*)-one1, 9-pyrazoloanthrone) was also used to study if the effects of anisomycin were being mediated through a JNK pathway. However, JNK inhibition failed to reverse the effects of anisomycin treatment (Figure 4.3). The role of downstream targets of p38 MAPK was therefore studied to determine their influence on PDE4A4-GFP foci formation.

Rottlerin was used to inhibit both MAPKAPK2 and PKCδ (Blanquet et al, 2003). Rottlerin treatment abolished PDE4A4-GFP foci formation. Signalling crosstalk between the PKC and MAPK pathways is well documented (Blanquet et al, 2003). Considering the potential role for MAPK action in PDE4A4-GFP foci formation and its ablation by rottlerin, the possible involvement of PKCδ in this process was investigated using PMA (Powell et al; 2003). PMA did not trigger PDE4A4-GFP foci formation alone, nor did PMA affect rolipram-induction of foci. However, most strikingly, PMA reversed the inhibitory effect of rottlerin on foci formation, restoring the percentage immobile GFP to ~80% (figure 4.4). This indicated the possible involvement of PKC signalling, possibly PKCδ, in foci formation. PKC inhibitor RO320432 which inhibits isoforms PKCα, β1,ε (Birchall et al; 1994) and GO6983 which inhibits PKCα,β,γ, δ isoforms (Wang et al; 2004) elicited a 70-90% enhancement in the fluorescence immobilised in PDE4A4-GFP accretion foci when compared to treatment with rolipram alone (Figure 4.4a). These data suggest that signalling of these PKC isoforms has a restraining influence on foci formation.

PKC isoforms can be divided into three classes; conventional, novel and atypical. Conventional isoforms includeα, β_1, β_2 andγ; ε, η, θ, μ are novel forms and ζ, λ are atypical forms (Mackay and Twelves, 2007). Conventional and novel forms are regulated by diacylglycerol and phosphatidylserine while atypical isoforms are regulated by phosphatidylserine only. Activation of conventional PKC forms is calcium dependent whereas signalling of PKC isoforms of the other two groups is calcium independent (Mackay and Twelves, 2007). To investigate the involvement of the calcium-dependent PKC isoforms in foci formation, intracellular calcium modulators including thapsigargin and ionomycin were used. Thapsigargin elevates intracellular calcium levels by inhibiting calcium/ATPase-mediated reuptake of calcium into endoplasmic reticulum (Won and Orth, 1995) whereas ionomycin mobilises calcium across the cell membrane (Aagaard-tillery and Jelinek, 1995). Raising intracellular calcium with these agents blocked rolipram-induced of PDE4A4-GFP foci formation (Figure 4.4b) suggesting that Ca^{2+} ions exert an

inhibitory influence on foci formation. These data are also further suggestive of a role of a Ca^{2+}-dependent PKC isoform in foci formation. To identify the downstream targets responsible for the effect of calcium on foci formation, various inhibitors of calcium signalling were tested. Inhibiting CaMKII with KN62 (Minami et al; 1994) had no effect, nor did inhibition of calcineurin with cyclosporin A (Figure 4.4b), while inhibition of Calmodulin with W-7 (Caulfield et al; 1991) and ophiobolin-A led to cell loss/death. It is possible that Ca^{2+}/PKC activates other, as yet unkown, downstream targets or that Ca^{2+}/PKC inhibits foci formation directly.

Earlier studies showed that challenging 3T3-F442 fibroblasts with growth hormone activates PDE4A5, the mouse orthologue of human PDE4A4, through a PI3 kinase and p70S6 kinase pathway (Mackenzie et al 1998). As inositol phosphates and PI3 kinase signalling are critical in regulating intracellular calcium levels (Balla et al, 2006), various PI3-K inhibitors were assessed in their abilities to mediate PDE4A4-GFP foci formation. Blockade of PI3-K with wortmannin increased the percentage immobile GFP in accretion foci to 175% ± 8.32 %, indicating an inhibitory input for PI3-K (Figure 4). However, wortmannin has also been shown to function as an inhibitor of MAPK. To address this, the same experiment was performed with the PI3-K-specific inhibitor LY294002 (Baumann and West, 1998), which also increased the percentage immobile GFP further to a similar extent. However inhibiting PI3-K downstream targets, p70S6K, with rapamycin (Powell et al; 2003) and GSK3β with GSK3β Inhibitor, (Calbiochem, UK) had no effect on rolipram-induced foci formation (Figure 4.5). It is therefore likely the effects of PI3-K signalling on foci formation are mediated through other downstream effectors.

It is known that PDE4A5/PDE4A4 interact with the SH3 domains of Src, lyn and fyn kinases (Beard et al, 1999 and Beard et al, 2002). Hence the role of tyrosine kinases in foci formation was determined using general tyrosine kinase inhibitor, genistein (Constantinou and Huberman; 1995). Interestingly, genistein ablated rolipram-induced foci formation implicating tyrosines kinase activity in foci formation. However, the Src family inhibitors PD168393 and

Su6656 (Fry et al; 1998, Bowman et al; 2001) had no effect on rolipram-induced foci formation. Inhibitors for JAK2 (AG490) and IR (HNMPA-(AM) $_3$) led to cell loss/death. Inhibition of the tyrosine kinase receptors VEGF-R (oxindole) and EGF-R (PD168393) had no effect on foci formation. However, the PDGF-R inhibitor AG17 blocked foci formation (Figure 4.6), indicating that this receptor kinase may promote rolipram induction of PDE4A4-GFP foci.

Cyclin-dependent kinase 5 is activated by p35/p39 and is known to regulate the MEK-ERK pathway and calcium signalling of CAMKII and the Ca^{2+}-sensitive adenylate cyclase isoform IX (Pareek and Kulkarni, 2006). The role of cdk5 signalling in foci formation was examined using its inhibitor roscovitine. Cells treated with 1μM roscovitine (Cdk5 IC_{50} -0.2uM) elicited no effect on foci formation. However, at a concentration of 10μM, foci formation was inhibited by 40% (Figure 4.7). At this higher concentration, roscovitine can also inhibit Cdk1, Cdk2 and ERK1/2 (Dangi and Shapiro, 2005). It is probable, therefore, that the inhibitory effect of roscovitine on foci formation is mediated via a target other than Cdk5 which can be explained on the basis that cdk5 is a brain-specific kinase so it wouldn't be expressed in CHO cells (Wei and Tomizawa, 2007); hence the only effects of roscovitine at higher concentrations were seen when it can inhibit other cdks and erk. The presence of cdk5 in CHO cells can also be confirmed by immunoblotting the CHO cell lysates for the kinase.

PDE4A4 activity has been shown to be of importance in initiation of the immune response by T-cells (Manning et al; 1999). Thalidomide® has been reported to have immunomodulatory properties as it is known to have a variety of effects including decreasing the production of monocyte derived TNF-alpha, and regulates the T-helper cell response by regulating the production of IL-4, 5 and IFN-γ *in vitro* (Melchert and List, 2007, Okafor, M.C, 2003). In order to investigate a potential link between foci formation and PDE4A4's immunoregulatory properties, cells were challenged with thalidomide to examine its effects on foci formation (Figure 4.8). At a concentration of 400μM, thalidomide completely inhibited foci formation of

PDE4A4-GFP (IC50: 150μM). Thalidomide action is proposed to be mediated a through Wnt signalling pathway. Gsk3 is known to be a component of Bmp/DKK1/Wnt signalling and inhibition of any of the three components reverses the effects of Thalidomide treatment (Knobloch et al; 2007). It should be noted that although Thalidomide inhibited foci formation, inhibition of GSK3 had no effect. However, the role of GSK3 inhibition in reversing the Thalidomide-induced effects on foci formation is yet to be determined. It is possible that the effect of Thalidomide treatment on PDE4A4 foci formation is mediated through other components of the Wnt signalling pathway.

In some instances protein aggregates are due to oligomerisation of misfolded proteins. For instance chicken Hsp25 is known to form inclusion bodies when over expressed in HeLa cells (Katoh et al; 2004). It has been suggested that PDE4 isoforms oligomerise by a process that involves their UCR1 and UCR2 domains (Richter al, 2004, Richter and Conti, 2002). In order to evaluate whether PDE4A can form dimers in these cells, CHO cells stably expressing PDE4A4-GFP were transfected with a V5 epitope-tagged form of PDE4A4. In parallel, oligomerisation experiments were carried out with differentially epitope-tagged forms of PDE4D3 (FLAG and VSV tags). PDE4A4 did not show oligomerisation/dimerisation prior to, and after rolipram treatment in co-immunoprecipitation experiments. However, PDE4D3 oligomerisation was evident in co-immunoprecipitation assays (Figure 4.9). The lack of observed oligomerisation of PDE4A4 is curious. However, there are a number of possible explanations to support these findings. Although PDE4A4 is able to form foci, the isoform may require other extraneous stabilising factors, which are lost upon cell disruption, i.e. a dynamic process stabilises foci and upon cell lysis, foci disaggregate. It is also possible that the tagged PDE4A4 is excluded from binding the GFP-tagged species. Immunocytochemical analyses and FRET/BRET would of interest to examine whether the differentially tagged forms of PDE4A4 co-localise, and potentially dimerise in foci in live cells.

Terry and coworkers have shown that protein synthesis is required for foci formation (Terry et al; 2003). To further characterise the role of protein

turnover in PDE4A4 foci formation, cycloheximide (CHX) and MG132 were used to inhibit protein synthesis and proteasomal degradation of proteins respectively (Christner et al; 1999, Steinhilb et al; 2001). Both CHX and MG132 ablated foci formation (Figure 4.10). Furthermore, when both protein synthesis and degradation were inhibited simultaneously, foci formation was totally blocked suggesting that protein turnover is crucial for foci formation. Similar results were obtained when puromycin was used to inhibit protein synthesis (Chow et al; 1995). To further investigate the role of protein turnover in continued presence of foci, a time course was carried out of cycloheximide and MG132 treatments. Cells pre-treated with rolipram were incubated with cycloheximide, MG132 or both over a time course of 5 hours in the continued presence of rolipram. In all the cases, foci started to disperse within first 30 mins of treatment with the $t_{1/2}$ for dispersal approximately 75 mins. After 5 hours the total immobile GFP levels was virtually zero (Figure 4.11). This data indicated that protein turnover is crucial both for formation and maintenance of foci.

As protein turnover had shown to be of significance in foci formation and stability, compounds activating/inhibiting various signalling pathways were tested in conjunction with CHX and MG132 treatments. The principle behind this investigation was to identify and distinguish between signalling pathways regulating foci formation from those influencing the stability of the pre-formed foci during inhibition of protein turnover. Any compounds that enhance foci dispersal presumably do so by a mechanism independent of the effects of protein turnover on foci stability. Activation of p38 MAPK with anisomycin in the continued presence of rolipram, after a 16 hr pre-treatment with rolipram, dispersed the foci within 30 mins. Addition of CHX and MG132 did not alter the effects of anisomycin on foci dispersal. However, inhibition of MEK with UO126 delayed the dispersal of pre-formed foci for 4 hrs although all foci eventually dispersed by the 5 hr timepoint. However, UO126 enhanced the dispersal of foci induced by MG132 and CHX. The $t_{1/2}$ for foci dispersal by MEK inhibitor UO126 in the presence of CHX and MG132 was ~120 mins while in cells treated with UO126 and rolipram only, the $t_{1/2}$ was ~240 mins suggesting that UO126-induced dispersal of foci occurs via a mechanism

which is enhanced by protein turnover (Figure 4.12). Similarly, raising intracellular calcium levels using thapsigargin induced foci dispersal with a $t_{1/2}$ of ~45 mins, which was shortened to ~ 30 in the presences of MG132 and CHX. Rottlerin and RO320432 treatments also dispersed foci within 30 mins of agonist exposure. The presence of MG132 and CHX enhanced the dispersal in both cases (Figure 4.13). Similar effects were noted with inhibitors of tyrosine kinases and PI3 kinases using genistein and Ly294002 respectively where both inhibitors dispersed preformed foci within 30 minutes and the presence of CHX and MG132 did not affect the rate of foci dispersal. As foci dispersal with these compounds was rapid, a shorter time course of treatments would be required to accurately determine the rates of foci dispersal with these agents. However, these data do suggest that the stability of foci is dependent upon phosphorylation events mediated by tyrosine kinase and/or PI3-K signalling.

A potential role for PDE4A4 ubiquitination in the turnover of PDE4A4 was assessed using confocal microscopy and pulse-chase experiments. However, in accord to work from our lab (unpublished data) neither ubiquitination nor turnover of PDE4A4 was detected in pulse chase experiments. This was confirmed by immunocytochemical analysis (Appendix II for images).

In order to identify components of foci, co-localisation studies were carried out by confocal microscopy. The proteins studied and the treatments used in the process are listed in Table 4.1 (Please refer to Appendix II for images).

Proteins such as ubiquitin did not co-localise with foci. Similarly, no localisation of foci was observed with sub-cellular organelles including lysosomes and mitochondria. The role of SUMOylation in foci formation was also assessed. CHO cells stably expressing PDE4A-GFP were immuno-stained for SUMO modification. However, SUMOylation did not affect foci formation and SUMO did not co-localise with foci.

The inhibitor/activator studies implicated multiple signalling pathways in foci formation/dispersal with the potential for cross-talk between pathways, a

model of which is depicted in the figure 4.16. Foci formation appears to be independent of a cAMP/EPAC, and possibly a cAMP/PKA, pathway as shown from figure 4.2. However, elevated cAMP levels were able it inhibit foci formation and induce dispersal of pre-formed foci (Figure 4.2 and Terry et al. 2003). Inhibition of PI3 kinase signalling increased foci formation (fig 4.5). However, whether the effects of PI3 kinase action are direct or mediated by its downstream targets or via cross talk with other signalling pathways is yet unknown. The PI3 kinase pathway can activate PKC isoforms including PKC θ (Theta), ζ (Zeta), δ (Delta) and ε (Epsilon) upon insulin signalling (Sampson and cooper, 2006, Campbell et al; 2004, Hirai and Chida; 2003). Inhibition of PKC-ε (Epsilon) by RO320432 enhanced foci formation. RO320432, however, is not a PKC-ε specific inhibitor and can inhibit other PKC isoforms such as β1 and α. Hence the effects of other PKC isoforms cannot be ruled out. Similarly, PKC θ is also activated by PI3 kinases and its effects on foci are still unknown as there are no specific inhibitor compounds available to assess its effects. Inhibition of PKC-δ by rottlerin ablated foci formation suggesting a role of PKC-δ in foci formation. However, the pathway through which PKC-δ inhibitor rottlerin inhibits foci, as well as the mechanisms of PKC-δ regulation, has yet to be determined. PKCs might act directly or influence other pathways to regulate foci formation. It is possible that PKC signalling cross-talks with the MAP kinase pathway. MAP kinase pathway is a canonical pathway that is activated by various growth factors and controls cell growth and division (Pimienta and Pascual; 2006). Cross-talk between MAP kinase signalling and pathways including PKC signalling (Rozengurt; 2007, Baillie et al 2001), PI3 kinase signalling (Kim et al; 2007, Xu and Yu, 2007) and cAMP signalling is well documented (Baillie et al; 2001, Pyne et al; 1997, Pyne et al; 1994). It would be of interest to assess the MAP kinase activity in response to inhibition or activation of PI3 kinase signalling in the PDE4A4-GFP cell line to determine if there is cross-talk between these pathways. Unpublished work from our group indicates PDE4A4 can be phosphorylated within its N-terminus by MAKAP kinase-2 after stimulation of the PDE4A4-GFP cells with anysomycin, a p38 MAP kinase activator. It was therefore of relevance to examine the effects of anisomycin on foci formation. Inhibition and activation of MAP kinase signalling had conflicting effects on foci formation. Activation of p38

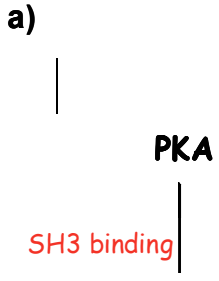
MAPK led to scattering of foci whereas MEK inhibition enhanced foci formation (Figure 4.3). It has yet to be assessed whether these differential effects of MAP-K signalling are directly influencing foci formation or if their actions are being mediated through other downstream signalling complexes. It would be of interest to establish whether mutation of PDE4A4's MAPKAP kinase-2 phosphorylation site altered the effects of anisomycin/MEK inhibition on foci formation.

PI3 kinase has also been reported to influence cAMP levels in the cells through its downstream effector Akt (Hans et al; 2006). Hans and co-workers (Hans et al; 2006) have shown that Akt phosphorylates PDE3A which leads to activation of the isoform. PDE3A is a cAMP-specific PDE (Beavo; 1995). Since cAMP has an inhibitory effect on foci, it could be suggested that PI3 kinase-Akt-PDE3A pathway might affect foci formation by modulating cAMP levels.

Thalidomide® is an anti-inflammatory agent and is known to have teratogenic properties (Okafor; 2003) Thalidomide inhibited foci formation. However, the mechanism for this action is unknown and requires further investigation. Thalidomide® is known to affect various pathways including Wnt signalling (Knobloch, 2007), PI3 kinase/Akt pathway (Gockel et al; 2004) and signalling of some PKC isoforms including PKC-θ (Payvandi et al; 2005). It is probable that the effects of thalidomide on foci formation are transduced via one or more of these pathways.

The involvement of multitude of signalling pathways renders understanding foci formation rather complex as it hinders identifying the signalling pathway involved in the process. Furthermore, the lability of foci in cell lysates complicates the identification of components by proteomic analyses, where preliminary studies were not of sufficient quality to be useful. In conclusion, foci formation of PDE4A4 is a very unique phenomenon not noticed with any other PDE4 isoforms. The ability of PDE4A4 to form foci reversibly only in the presence of rolipram and its analogues is a very novel finding. Also, the ability of only those PDE4 inhibitors that cause emesis to form foci and not the non-emetic ones suggest that PDE4A4 foci formation might be a key to understanding the mechanism of the side-effects caused by PDE4 inhibitor usage as therapeutics. Although Terry and coworkers (Terry et al 2003) have

identified PDE4 domains that could potentially lead to foci formation, extensive truncation mutational analyses is needed to identify the signalling complexes and components involved in the process.

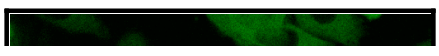


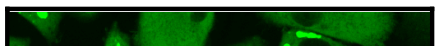
SH3 binding
domain

c)

Vehicle

10μM Rolipram (10-16hrs)

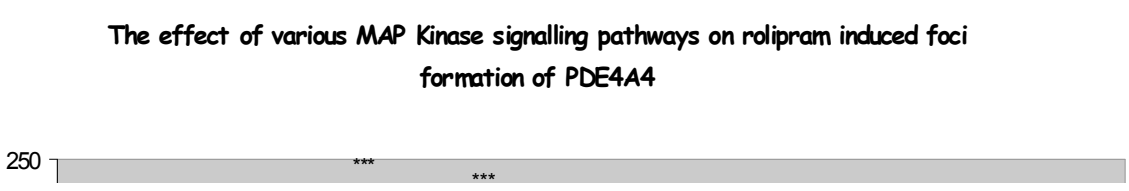




e) **Protein synthesis required to allow foci formation**

Figure 4.2 The effect of cAMP signalling components on rolipram induced foci formation of PDE4A4

CHO cells stably expressing PDE4A4-GFP were plated 1×10^5 per well in a cell culture treated 96-well plate for a quantitative plate reader assay. The following day, cells were then treated with inhibitors as indicated above for 16hrs. Post-treatment, total GFP was measured from live cells in Packard Bell Fusion® plate reader. Cells were then subjcted to extraction of mobile GFP using an extraction buffer for 10 minutes at room temperature. Cells were then fixed in 4% formaldehyde buffer and Hoescht33258 for further 10 minutes followed by three washes in PBS. Once the extraction, fixation and Hoescht staining were finished, immobile GFP was measured in the plate reader and corrected for cell number using Hoescht signal. All the data was background corrected. Untreated cells were used as negative control and rolipram alone treated cells as positive control. All the results are represented as a percentage of rolipram induced foci. All the data shown are representative of results obtained from three separate experiments. Paired t-test statistical analysis of the quantified immobile GFP data were undertaken, where $p<0.05$ indicates a statistically significant difference level of inhibition.



a)

The effect of PKC pathway on rolipram induced foci formation of PDE4A4

The effect of PI3 kinase pathway on rolipram induced foci formation of PDE4A4 GFP

P<0.0001

The effect of tyrosine kinases on rolipram induced foci formation of PDE4A4GFP

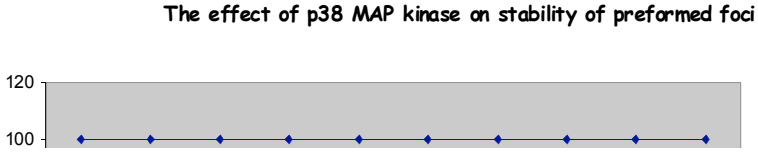
The effect of cdk5 on rolipram induced foci formation of PDE4A4

The effect of thalidomide on rolipram induced foci formation of PDE4A4

a)

The effect of protein turnover on rolipram induced foci formation by PDE4A4

The effect of protein turnover on stability of pre-formed foci


The effect of p38 MAP kinase on stability of preformed foci of PDE4A4
120
100

The effect of thapsigargin (Calcium levels) on stability of preformed foci of PDE4A4

The effect of Ro320432 on stability of preformed foci of PDE4A4

The effect of Tyrosine kinases on stability of preformed foci of PDE4A4

The effect of Ly294002 on stability of preformed foci of PDE4A4

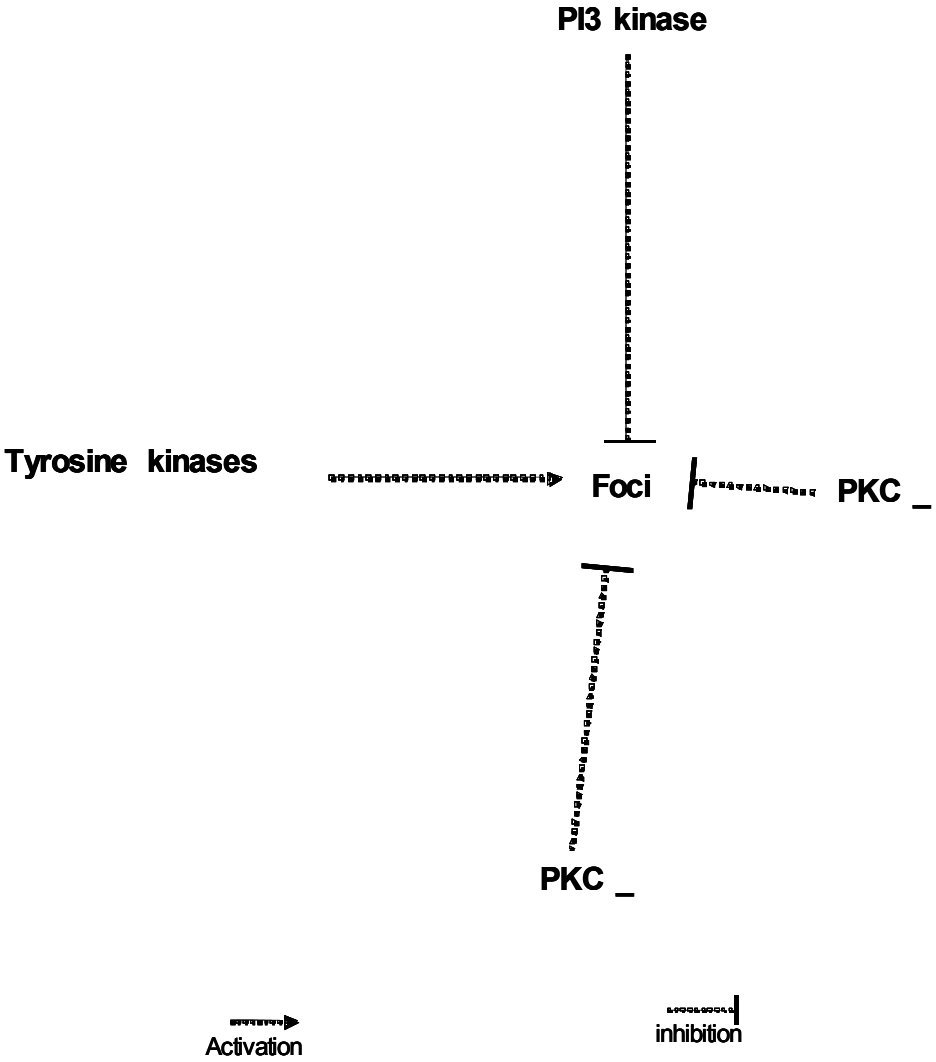


Figure 4.16 A schematic model of depict the effects of various s ignalling pathways on PDE4A4GFP - Foci formation.

Antibody/probe/Treatment	*Target*	*Interaction with foci*
MG132	proteasome inhibitor	Inhibits foci formation
Brefeldin A	protein transport inhibitor	No effect
Leptomycin B	nuclear export inhibitor	No effect
Ubiquitin	ubiquitinated proteins	+
P27kip1	ubiquitinated Cdk inhibitor	+

Chapter 5 RhoGAPs, Arrestins and PDE4 isoforms

5.1 Introduction

5.1.1 Rho Signalling

Guanine nucleotide-binding proteins (GNBP) regulate a plethora of vital cellular processes including cytoskeletal reorganisation, cell adhesion and cell cycle progression (Lundquist; 2006, Bishop and Hall; 2000, Jaffe and Hall, 2005, Luo, 2000). Small GTPases of the Ras superfamily are the most studied GNBPs with the monomeric 20-30 KDa Rho GTPases being the best characterised among the GTPases of the Ras super family (Bishop and Hall; 2000).

It is now well appreciated that Rho GTPases are involved in actin-dynamics (Aspenstrom, 2004 and Luo, 2000). However, Rho-GTPases have also been shown to interact with a wide range of effector proteins, modulating their activity and thus participating in various signalling pathways (Jaffe and Hall; 2005). Rho GTPases interact with their effectors through a so-called “switch region” that is also necessary for their GTPase activity. Point mutations within this region have been shown to ablate the interaction of Rho GTPases, such as cdc42, Rho and Rac, with most of their effectors (Joneson et al; 1996 and Lamarche et al; 1996).

The functioning of GNBPs is activated by the binding of GTP and is terminated by the hydrolysis of GTP to GDP by its intrinsic GTPase activity. The GTP-GDP cycle is regulated by three different classes of proteins. In resting cellular conditions GNBPs exist in a complex with guanine dissociation inhibitors (GDIs), which stabilise GDP-bound Rho proteins. GEFs are guanine exchange factors that facilitate the exchange of GDP to GTP in the catalytic region of Rho proteins. The third class of proteins, GAPs, are GTPase activating proteins, which interact with and enhance the otherwise slow

intrinsic GTPase activity of species such as Rho proteins (Scheffzek and Ahmadian; 2005).

5.1.2 RhoGAPs

RhoGAPs act as negative regulators of GNBPs by enhancing the GTPase activity of Rho, consequently shifting Rho into an inactive state (Willars; 2006). Almost all members of Rho family have been shown to have specific GAPs. Rho-GAPs of different subfamilies possess subfamily-specific structural domains that play an important role in regulation and targeting of GAPs (Bernards and Settleman; 2005).

GAPs play an important role in brain development. The NR2B subunit of the NMDA (N-methyl-D-aspartate) receptor has been shown to interact with p250GAP, a GAP for Rho-cdc42. This promotes the dephosphorylation of NR2B as well as altering its sub-cellular localisation thereby regulating synaptic plasticity by reorganising actin in dendritic spines from hippocampal slices stimulated with NMDA (Nakazawa et al; 2003). Similarly, p250GAP is phosphorylated by src kinase, Fyn, and leads to differentiation of oligodendrocytes (Taniguchi et al; 2003). A detailed description of RhoGAPs is discussed in section 1.11.1.3.

5.1.3 *Role of cAMP signalling components in Rho transduction pathways*

A role for cAMP and its signalling components in Rho signalling pathways has been suggested by various studies (Appert-collin et al; 2007, Flemming et al; 2004, Diviani et al; 2004, Diviani et al; 2006, Howe et al; 2004). In particular, elevation of PKA has been shown to both activate and inactivate Rho signalling pathways (Howe A, 2004).

In REF52 cells, elevation of cAMP levels by rolipram inhibition of PDE4 activity was found to downregulate integrin-dependent actin adhesion microspikes. It was shown that these effects were mediated by PKA regulation of the Rho A-ROCK-Myosin pathway (Fleming et al; 2004).

α1 adrenergic receptors have been shown to be involved in cardiac hypertrophy (O'Connell et al; 2003) which was showed by double knockout of α1A/C and α1B adrenoceptors in male mice where the heart size was smaller than wild type and the heart function was affected by the knock out of the receptors (O'Connell et al; 2003). In further support to this, AKAP-Lbc has been shown to possess GEF activity aside from its PKA anchoring function. In cardiomyocytes, activation of its GEF activity has been reported to mediate through its interaction with heterotrimeric G protein 12 (G_{12}) downstream to α1 adrenergic receptors, which subsequently promote Rho-A activation, by AKAP-LBc. PKA-mediated phosphorylation and recruitment of 14-3-3 protein leads to inactivation of the AKAP-GEF. The GEF activity of AKAP-Lbc has been shown to play a significant role in cardiac hypertrophy, which was evident by hypertrophic responses of heart cells subjected to lentiviral suppression of AKAP-Lbc (Appert-collin et al; 2007, Diviani et al; 2006, Diviani et al; 2004). Furthermore, anchored PKA has been shown to have a key role in the formation of pseudopodia and chemotactic cell migration by activating Rac GTPase through phosphorylation of vasodilator stimulated phosphoprotein (VASP) and protein tyrosine phosphatase localised to pseudopodia (Howe et al; 2005).

The studies mentioned above indicate the potential of cAMP signalling, PKA action and PDE activity in cytoskeletal rearrangement and cell migration. The aim of this study is to try and elucidate the role of the cAMP signalling in Rho transduction pathways through PDE4 interactions with ARHGAP21/10.

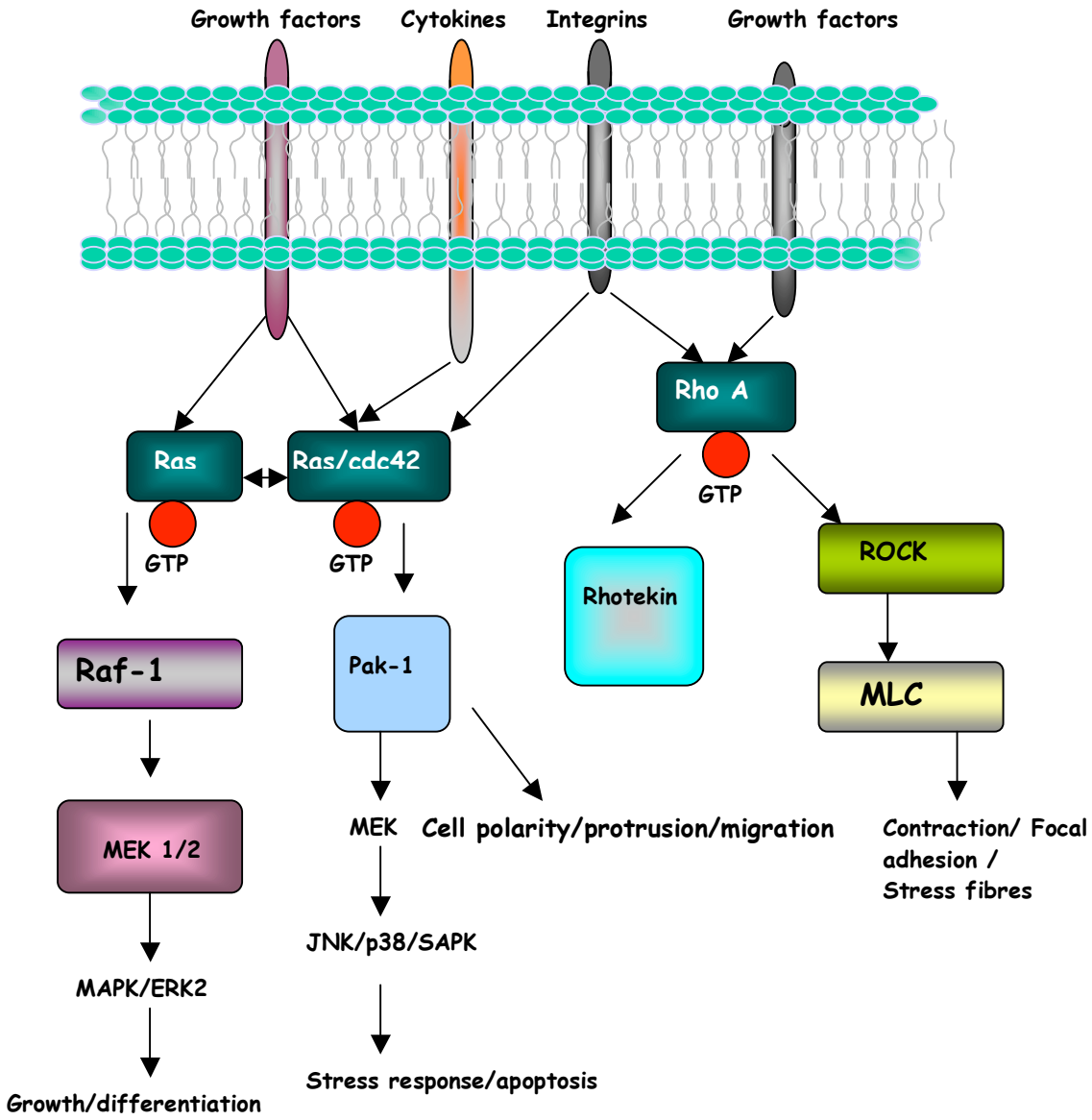


Figure 5.1 Schematic representation of Rho signalling cascade.

5.2 Results

5.2.1 *Expression and purification of ARHGAP21/10*

ARHGAP21, also known as ARHGAP 10 is a novel Rho/Rac/cdc42-like GTPase activating protein expressed in chondrosarcoma, breast cancer, kidney and brain tumours (Katoh and Katoh; 2004).

A yeast two hybrid analysis by our group, in collaboration with Hybrigenics, France, to look for novel binding partners for βarrestin identified ARHGAP21 as a potential interactor (Please refer to appendix II for list of constructs used in yeast two hybrid analyses). Assays were performed to validate these findings. However, ARHGAP 21 is a huge 1957 amino acid protein and no as yet, a cDNA construct encoding the full-length protein is still to be generated. However, specific ARHGAP21 antisera, allowed for pull-down studies of the endogenous protein to be performed. Furthermore seven truncates covering amino acids 885-1346 (Figure 5.2), which were gifted from Philippe Chavrier's group at the Curie institute, Paris as previously (Dubois et al, 2005) were used to assess ARHGAP21: PDE4 interactions.

In order to examine the potential interaction of ARHGAP21 with β-arrestin, various GST truncates of ARHGAP21 were expressed as soluble proteins in *E. coli* and purified on glutathione sepharose beads. These purified proteins were analysed by SDS-PAGE and expression was visualised by coommassie staining. Truncate-1 corresponds to ARHGAP21 residues 885-1096 (75 KD) which includes the ARF binding domain of ARHGAP21 (Dubois et al; 2005). Truncate 2 comprises amino acids 929-1052 (37KD); truncate 3 includes residues 885-1052 (45 KD); truncate 4 is of amino acid region 929-1096 (45 KD); truncate 5 incorporates amino acids 929-1346 (160 KD); truncate 6, which comprises residues 1064-1346, contains both the ARF binding domain and RhoGAP domain, and truncate 7, which is 150 KD, includes amino acids 1042-1346. A schematic of full-length ARHGAP21 and truncates described here are depicted in figure 5.2 along with a coomassie stained gel analysis of the same purified GST fusion proteins.

5.2.2 *Mapping the binding sites of β-arrestin-1 on ARHGAP21 using peptide array analysis*

A peptide array analysis was used to identify the potential binding sites of β-arrestins on ARHGAP21. For this analysis a full-length peptide array of ARHGAP21 was incubated with a purified recombinant GST fusion protein of β-arrestin1 and probed with a GST antibody. GST alone was used as a control to determine non-specific interactions (Figure 5.3). This process identified a binding site mapped to amino acids 1321-1360 within the RhoGAP region of ARHGAP 21. This potential binding site is consistent with the residues of the ARHGAP21 fragment identified in yeast-two hybrid analysis carried out by Hyrbigenics, France as well as with previously published data by (Xiao et al; 2007), where both RhoGAP10 and RanGAP1 were identified as potential β-arrestin interaction proteins in a proteomics analysis study of β-arrestin immunoprecipitates.

5.2.3 β-arrestin 1-GST binding to an ARHGAP21 alanine-scanning substitution array

To determine key amino acid residues within the identified β-arrestin 1 binding domain of ARHGAP21, an alanine scanning substitution array of the ARHGAP21 parent peptides was used. Each amino acid from the parent peptide identified within the binding site (figure 5.3) was sequentially mutated to alanine while alanine residues in the parent peptide were substituted with aspartic acid (Figure 5.4).

In this study it was found that substitution of Thr1329Ala reduced the interaction severely. Similar substitution of Thr1348Ala and Thr1349Ala reduced the interaction to a lesser extent. Substitution of Glu1344Ala and Glu1345Ala also ablated the interaction. However, a double mutation of Glu1344Ala and Glu1345Ala actually served to increase the binding of β-arrestin to ARHGAP21 compared to individual substitutions of Glu1344Ala and Glu1345Ala. Furthermore, substitution of Glu1340Ala, Glu341Ala, Glu1344Ala and Glu1345Ala drastically reduced the interaction. Double substitutions of Phe1337Ala and Phe1338Ala decreased the binding.

Individual single substitutions of Leu1330Ala, Leu1347Ala and Pro1346Ala decreased the binding. No effect was seen upon substituting Histidine residues at 1334 and 1334 with alanine (Figure 5.4).

5.2.4 A *RhoGAP fragment of ARHGAP21 binds to the N-domain of β-arrestin1*

To identify the sites of interaction of ARHGAP21 on βarrestin1, a β-arrestin 1 peptide array was incubated with a purified GST-RhoGAP fragment (amino acids 1064-1346) of ARHGAP21 and probed with a GST antibody. GST alone was used as control to identify any non-specific interactions (Figure 5.5). Probing with the RhoGAP fragment yielded only three positive spots. These peptide spots correspond to β-arrestin 1 residues 96-130 which are located within the protein's N-domain (Figure 5.5). Peptide spots of residues 96-125 showed a stronger interaction than the spot of amino acids 106-130. Probing the peptide array with purified ARHGAP21 fragments, which did not include the RhoGAP domain did not yield any positive spots.

5.2.5 Binding of ARHGAP21 RhoGAP domain to *β-arrestin1 alanine substitution scans*

To gain further insight into ARHGAP21 binding regions of βarrestin1, an alanine substitution-scanning array was generated. The purified RhoGAP domain of ARHGAP21 (amino acids 1064-1346) was overlaid on an alanine substitution scan of parent peptides (amino acids 96-130) from the previously identified binding region (figure 5.6). Substitution of any of the amino acids within the stretch from Lys102 to His106 (sequence KLGEH) to alanine ablated the binding of ARHGAP21's RhoGAP domain. Similarly, substitutions within the stretch Pro109 to Glu133 (sequence PFTFE) partially reduced the binding of the RhoGAP domain of ARHGAP21 (figure 5.6).

5.2.6 *The interaction of β-arrestin1 with ARHGAP21 in pull-down assays*

Equimolar amounts of various purified GST fusions of ARHGAP21 truncates were used in pull-down assays to determine their ability to interact with a purified recombinant MBP fusion of β-arrestin 1. As shown in Figure 5.7, MBP-β-arrestin1 was able to interact directly with the RhoGAP domain-containing fragments 5, 6 and 7 (lanes 6-8; bottom panel, figure 5.7) but with fragments 1-4 of ARHGAP21 (lanes 2-5, fig 5.7). MBP-β-arrestin1 did not bind to GST alone (lane 1, figure 5.7).

5.2.7 Activation of Rho in HEK 293-AT1aR cells.

Rho GTPases are involved in regulation of F-actin dynamics in cells (Jaffe and Hall; 2005, Aspenstrom et al; 2004). HEK293 cells expressing the angiotensin AT1a receptor have been shown exhibit stress fibre formation in response to angiotensin II challenge through a $G_{\alpha q/11}$/Rho-mediated pathway (Barnes et al; 2005). In order to evaluate the Rho activation profiles in these cells, a Rho activity assay was performed. Figure 5.8(a) shows that in resting conditions (0 minutes) there is minimal Rho activity. However, in response to angiotensin II challenge this activity is increased transiently for up to 10 minutes after which activity returns to basal levels within 20 minutes of agonist exposure. Confocal analysis (Figure 5.8(b) of the HEK293 cell line stably expressing the AT1a receptor demonstrates the formation of stress fibres upon stimulation with Angiotensin II.

5.2.8 *Interaction of β-arrestin 1 with ARHGAP21 in HEK293-At1aR cells*

In order to determine whether full length ARHGAP21 interacts with β-arrestin1, a co-immunoprecipitation assay was performed. HEK293-AT1aR cells were transfected with β-arrestin 1-GFP and then treated with angiotensin II for 10 minutes in order to activate the Rho signalling. Panel 1 of Figure 5.9 showed that β-arrestin 1 interacted with full-length ARHGAP21 in both resting and stimulated cells. However, agonist stimulation did not appear to have an effect on the interaction. Panel 2 of figure 5.9 shows the expression of ARHAGP21, immuno-detected as a ~250 KD species.

5.2.9 PDE4D5 interacts with ARHAGP21 in HEK293-AT1aR cells

Both β-arrestin 1 and β-arrestin 2 have been shown to interact with PDE4 isoforms and recruit them to G-protein coupled receptors at the plasma membrane in response to agonist challenge (Perry et al; 2002 and Baillie et al; 2003). β-arrestins can interact with all PDE4 isoforms through a common β-arrestin binding site located within the catalytic subunit. However, β-arrestin shows preference for interaction with the PDE4D5 isoform due the presence of an additional binding site within PDE4D5's unique N-terminal region (Baillie et al; 2003).

In order to assess whether PDE4D5 can also be sequestered to the ARHGAP21-β-arrestin 1 complex, immunoprecipitation experiments were carried out. Endogenous ARHGAP21 was immunoprecipitated from the HEK293-AT1aR cells using an ARHGAP21 antibody and precipitates were then probed for endogenous PDE4D5 using a PDE4D5 N-terminal-specific antibody. Panel 1) of figure 5.10 shows the endogenous PDE4D5 from HEK293-AT1aR cells and its pull-down in ARHGAP21 precipitates as detected by immunoblotting with the PDE4D5 N-terminal-specific antibody. Panel 2) of figure 5.10 shows the immuno-detected ARHGAP21 from the cell lysate as well as IP using ARHGAP21 antisera.

5.2.10 PDE4D5 interacts with ARHAGP21 in vitro

Full-length purified PDE4D5-MBP and GST fragments 1 (residues 885-1096) and 6 (residues 1064-1346) of ARHGAP21 were used in pull-down assays to determine whether PDE4D5 interaction with ARHGAP21 is direct. Panel 2) of figure 5.11 shows that purified recombinant PDE4D5-MBP interacted directly with the fragments containing ARF binding domain of ARHGAP21 as well as the Rho binding domain of ARHGAP21 (figure 5.11). However there are some overlapping regions in both fragments. As a control, purified fragments of ARHGAP21-GST were also incubated with MBP alone (lanes 4 and 5, figure

5.11). A similar control was set up with PDE4D5 MBP and GST alone (lane 3, figure 5.11). No interaction was seen in the control lanes.

5.2.11 *Effect of PDE4 activity on interaction of ARHGAP21 with PDE4D5 and β -arrestin1*

For scaffolding proteins such as DISC1, its interaction with PDE4 isoforms can be altered by elevating intracellular cAMP levels (Murdoch et al; 2007). It was therefore of interest to determine whether the interaction of PDE4D5 with the ARHGAP21/β-arrestin1 complex is dynamically regulated by increasing cAMP levels, PDE4 activity or Rho activation in cells. ARHGAP21 was immunoprecipitated from HEK293-AT1aR cells under basal conditions and from cells, which had been treated with cAMP activators Forskolin/IBMX, PDE4 inhibitors like Rolipram and $G_{q/11}$/Rho activator, Angiotensin II either alone or in combination. The lysates and IPs were then immunoblotted for PDE4D5 and Arrestin. Panel 1 of figure 5.12 confirmed that neither elevated Rho activity nor cAMP levels had an impact on Arrestin-ARHGAP21-PDE4D5 complex. Panel 2 of figure 5.12 shows that increased intracellular cAMP levels and PDE4 activity do not alter PDE4-ARHGAP21-arrestin interaction. Similarly, Rho activation did not alter the dynamics of ARHGAP21-Arrestin-PDE4D5 complex. Both the blots were stripped with stripping buffer and were reblotted for a PKA phosphorylation using PKA phospho substrate antibody as an indicator of PKA activation upon elevation of cAMP levels.

5.3 Discussion

RhoGTPases along with their regulators are involved in important cytological functions including cytoskeletal rearrangement, cell migration, cytokinesis and cell cycle regulation (Jaffe and Hall 2005). The ability to participate and regulate in multiple signalling pathways requires co-ordination, with the recruitment signalling scaffolds through protein-protein interactions playing an important role (Pawson; 2007, Pawson and Warner, 2007).

RhoGTPases have been shown to interact with a wide variety of proteins, which they use as their effectors (Bishop and Hall; 2000, Boguski and McCormick; 1993). The dynamics of these interactions can be modified by conformational changes of the RhoGTPase induced by GDP/GTP binding and its intrinsic GTPase activity the activity of which can be regulated by GAFs and GAPs (Bishop and Hall 2000).

ARHGAP21 is a novel GTPase activating protein (GAP) for cdc42 (Khato et al; 2004, Basseres et al; 2002). Using a novel peptide array analysis technique, along with co-immunoprecipitation studies, it was shown that interaction of ARHGAP21 with β-arrestin1 and PDE4D5 occurs both *in vitro* and *in vivo*. ARHGAP21 contains a PDZ domain, a pleckstrin homology (PH) domain and a RhoGAP domain (Basseres et al; 2002). Recently it has been reported that ARHGAP21 region corresponding to amino acids 885-1096 interacts with ARF1 (Figure 5.2 for schematic) implicating it in the regulation of F-actin dynamics through the Arp2/3 complex (Dubois et al 2005). All identified domains of ARHGAP21 have individual functions. The pleckstrin homology domain is known to be involved in translocation of proteins to plasma membrane in response to PI3 kinase activity (Bernards and Settleman 2004, Bos et al; 2007). The Rho GAP domain is involved in regulating the activity of GTPases.

Yeast two-hybrid analysis of β-arrestin 1 performed commercially for our group by Hybrigenics, France, as well as a large-scale proteomic study (Xiao et al; 2007) have revealed ARHGAP21 as a potential binding partner for β-arrestin1 and β-arrestin2.

Here, a peptide array of full-length ARHGAP21/10 was overlaid with purified β-arrestin1-GST (Figure 5.3). β-arrestin 1 strongly interacted with residues of the RhoGAP domain (amino acids 1321-1360), supporting the yeast–two-hybrid analyses, and further indicating that the RhoGAP domain acts a interaction site for β-arrestin 1 (Figure 5.3).

In order to identify the key residues involved in β-arrestin/ARHGAP21 interaction, an alanine substitution peptide array scan was performed (Figure 5.4). The alanine substitution scan of the RhoGAP domain (amino acids 1321-1360) of ARHGAP21 demonstrated a strip of acidic and basic residues interspersed with aromatic and hydrophobic residues to be crucial for β-arrestin interaction. The Glu1328Ala substitution led to a decrease in β-arrestin binding by around 30% compared to control, whereas individual alanine substitutions of T1329 and L1330 reduced the interaction by 80 and 20% respectively (Figure 5.4 top panel). Individual alanine substitutions of Glu1344 and Glu1345 led to an 80% decrease in binding of β-arrestin. Combined alanine substitutions at Glu1340, Glu1341, Glu1344 and Glu1345 decreased the binding of β-arrestin by 60%. Double alanine substitutions at Glu1340 and Glu1341 elicited a 50% decrease in binding whereas the double alanine substitution at Glu1344 and Glu1345 produced only a 20% decrease in binding. A combined alanine substitution at His1333 and His1334 did alter binding (Figure 5.4 top panel). Single alanine substitutions at Pro1346, Leu1347, Thr1348 and Thr1349 reduced the binding of arrestin by ~ 30% respectively (Figure 5.4 bottom panel). Similarly, double alanine substitutions at Phe1337 and Phe1338, Thr1348 and Thr1349, Glu1352 and Glu1353 reduced the binding by ~20%. Multiple alanine substitution of glutamate residues at positions 1340, 1341, 1344, 1345, 1352 and 1353 together reduced the peptide's interaction with by β-arrestin only ~20% (Figure 5.4 bottom panel). Taken together, these data suggest that residues Thr1329, Glu1341, Gly1342, Glu1344, and Glu1345 are critical in determining the interaction of β-arrestin 1 with RhoGAP domain of ARHGAP21.

To further characterise the interaction, *in vitro* pull-down assays were performed using purified GST truncates of ARHGAP10/21 (Figure 5.7). A purified MBP fusion of β-arrestin 1 MBP exhibited an interaction with only truncates containing the RhoGAP domain (truncates 5, 6 and 7) of ARHGAP10/21 in pull-down assays but not with fragments containing comprising the ARF binding domain or the PH domain. These data suggested that the interaction of β–arrestin with ARHGAP21 is mediated by the RhoGAP domain of ARHGAP21/10 and that the interaction is direct (Figure 5.7).

In reciprocal binding studies, in which a full-length peptide array of β–arrestin 1 was overlaid with purified GST-RhoGAP domain (1064-1346) of ARHGAP21, an interaction site within the N-domain of β–arrestin 1 was identified (Figure 5.5 top panel). No interaction was observed with GST-ARHGAP21 fragments 1-4.

To further define the residues of β–arrestin involved in its interaction with ARHGAP21, an alanine substitution-scanning array was used. Individual mutations of any of residues within the region 102-106 (sequence KLGEH) abolished the interaction of β–arrestin with ARHGAP21 (Figure 5.6). Alanine substitution of residues Pro109 and Phe110 attenuated the interaction severely. A similar effect was observed with substitutions at Phe112 and Glu113 (Figure 5.6).

From the peptide array analyses, it could be suggested that there is a consensus site for ARHGAP21 binding to β-arrestin of E- $(X)_4$-KLGEH- $(X)_5$-E (Figure 5.6). However, analyses also showed that βarrestin-ARHGAP21 interaction appears to involve a strip of acidic amino acids on each protein. These observations are somewhat curious as it would be expected that if electrostatic interactions were involved in binding then the key acidic amino acids of β-arrestin would be complemented by a strip of basic residues on ARHGAP21 and *vice versa*. Mutagenesis of these identified residues in the full-length proteins would be required to define the sites of interaction more clearly. It is possible these stretches of acidic amino acids are involved in

presenting correctly other key amino acids on each peptide that are important for binding but are not affected upon alanine substitution. This needs further investigation.

A GAP domain is present in all GAPs of Ras GTPase family including Ras, Rho, Rac, Rap, Rab, Arf, Ran and RGS proteins which are G_{α}GAPs (Scheffzek and Ahmadian, 2005). Since the GAP domains of most GAPs share a high degree of homology, it would be of interest to study the interaction of β–arrestin with GAP domains of other Ras proteins. All GAP domains are involved in regulating the GTPase activity of GNBPs. GAPs enhance the intrinsic GTPase activity of GTPases and, consequently, inhibit the GTPase as described in section 1.11.1.3. In one model they either stabilise the catalytic domain of GTPases or provide a key arginine residue into the catalytic domain of the GTPase (Scheffzek and Ahmadian 1997, Moore and Eccleston, 1991). The significance of the second model is better explained in that the long side chain of the arginine residue can act as a bridge to bring intra-protein distances closer and its guanidium group can act like a stabiliser of the transition states involved in GTP hydrolysis by GNBPs. However, recent work by Fidyk and Cerione, 2002 has shown that GAPs enhance the GTPase activity by both stabilising the catalytic domain as well as providing the arginine residue. Given the initial role of GAPs in enhancing the GTPase activity of GNBPs and from the data presented on β–arrestin-ARHGAP21 interaction, it could be argued that binding of β–arrestins to the RhoGAP domain might influence GAP activity either by interrupting the inherent ability of GAP to enhance the catalytic activity of GTPases or by acting as a physical barrier by preventing the ability of GAP to bind its respective GTPase altogether, leaving the GTPase in a constitutively active state.

To understand the functional outcome of ARHGAP21-β–arrestin interaction, functional assays were carried out using a HEK293-AT1ar stable cell line. This cell line was chosen to study functional nature of these interactions as activation of Rho signalling is shown to be triggered by Angiotensin II

treatment of these cells (Barnes et al, 2005). Rho activation assays were carried out using these cells as described in section 2.11.3. A peak transient activation of Rho was seen after stimulation with Angiotensin II for 10 min with Rho activity returning to basal levels after 20 min (Figure 5.8 a). Confocal analyses revealed that the activation of Rho upon incubation of cells with Angiotnesin II, induced stress fibre formation (Figure 5.8 b). Rho activation by $G_{\alpha Q/11}$ is mediated either in a phoshpolipase C/PKC dependent manner or through activation of tyrosine kinases (Kuwahara and Kuwahara; 2002, Hirshman and Emala, 1999). It has been reported that β–arrestin–1-mediated activation of Rho, and subsequent actin reorganisation and stress fibre formation in angiotensin II-stimulated HEK293-AT1aR cells requires $G_{\alpha Q}/G_{11}$ signalling (Barnes et al; 2005). Recruitment of β-arrestin 1 to agonist-activated AT1a receptors not only activates Rho, but also regulates β-arrestin-mediated activation of the ERK MAP kinase pathway. This regulation is hypothesised to be due to competition between the βarrestin1 and 2 for binding sites on At1a receptor (Barnes et al; 2005). It has also been proposed that upon receptor stimulation, β-arrestin 1 and $G_{\alpha Q/11}$ co-ordinate to potentiate the activation of Rho, which, in turn, activates Rho kinase, ROCK, and thus leads to actin rearrangement for the formation of stress fibres (Barnes et al; 2005). Here, HEK293-AT1ar cells were transfected with a β-arrestin-1-GFP fusion protein and cells were stimulated to attain maximum Rho activation in the cells. Co-immunoprecipitation studies of cell lysates revealed that interactions were unaltered in response to Angiotensin II exposure as compared to non-stimulated cells (figure 5.9 top panels). Since β–arrestins activate Rho, and if they also bind to RhoGAPs, which have an inhibitory affect on Rho, it would be logical to assume binding of βarrestin to ARHGAP21 would be augmented under conditions of maximum Rho activation. The data suggests that Rho activation by β–arrestin is independent of its interaction with the RhoGAP and binding of β–arrestin to ARHGAP21 is not affected by Rho activity (Figure 5.9). The stable association of β–arrestin 1 with ARHGAP21 in both resting and agonist-stimulated cells is perhaps mediated by the RhoGAP domain, functioning independently of ARHGAP21. Studies of other ARHGAP21 interacting proteins has shown that ARHGAP10/21 is able to bind ARF1 (ADP

ribosylation factor 1), which allows for the regulation of cdc42 activity at Golgi membranes (Dubois et al; 2005). ARHGAP21 interacts with ARF1 through its PH domain plus a α-helix motif located towards its C-terminus with switch regions of ARF1. Eventhough the PH domain is necessary for this interaction, it cannot on its own bind to ARF1 independent of the C-terminal α-helix (Menetrey et al; 2007). ARHGAP21/10 acts as GAP for CDC42 recruited to Golgi in ARF1-dependent manner and regulates the function of its downstream effectors, Arp2/3, which are involved in F-actin dynamics in the proximity of the Golgi membranes. Arp2/3 is also implicated in the transport of clathrin-coated vesicles via the trans-Golgi network (Dubois et al; 2005).

It is known that β-arrestins play an important role in GPCR desensitisation, internalisation and resensitisation (Oakley et al, 1999, Laporte et al; 1999). When receptor is internalised in a complex with β-arrestin, dephosphorylation of the receptor by phosphatase PP2A, followed by dissociation of β-arrestin from the receptor promotes the recycling of receptor from endosomal vesicles back to the plasma membrane. If β–arrestin fails to dissociate from the receptor, then receptor is targeted to proteasomal degradation thus preventing receptor resensitisation (Simaan et al; 2004, Zhang et al; 1999, Zhang et al; 1997). β-arrestin dissociation from the lutenising hormone/Chorionic gonadotrophin receptor (LH/CGR) has been shown to be ARF6-dependent (Mukherjee et al; 2000). Although the role of β–arrestin in directly regulating the vesicle transport is poorly understood, given their role in receptor desensitisation/resensitisation, and their interaction with ARFs, it could be suggested that the β–arrestin-ARHGAP10 complex might function in regulating F-actin dynamics at the Golgi thereby regulating the transport of clathrin-coated vesicles to plasma membrane surface. β–arrestin has been shown to mediate PI3 kinase activation in MEF cells in a manner independent of ERK and G_i signalling (DeWire et al; 2007 and Povsic et al; 2003). Most RhoGAPs, including ARHGAP21, possess a PH domain, which binds phosphotidylinositides, thus altering substrate specificity of the RhoGAP and its translocation to plasma membrane (Ligeti and Settleman; 2006, Bernards and Settleman; 2004). Given these findings it could be argued that

ARHGAP21 might act as a chaperone in response to phosphatidylinositide signalling, recruiting β-arrestins to plasma membrane to mediate GPCR receptor desensitisation and internalisation.

Considering β–arrestins also function as scaffolds, anchoring proteins involved in many signalling pathways, the interaction with ARHGAP10/21 may be important for regulating Rho activity associated with these β-arrestin complexes in different subcellular locations. β-arrestin complexes have previously been shown to mediate β2-AR desensitisation by recruiting PDE4 isoforms to the plasma membrane. PDE4 binding to β-arrestin regulates PKA phosphorylation and consequent the G_s-G_i switching of the receptor (Baillie et al; 2003 and Perry et al; 2002). βarrestin recruitment of PDE4 has also been shown to occur within lipid rafts of T-cell membranes, regulates T-cell activation and cytokine production (Abrahamsen et al, 2004).

Of all PDE4 isoforms, PDE4D5 shows preference for binding to β–arrestin due to the presence of an extra site for βarrestin interaction located within its isoform-specific N-terminal region (Baillie et al, 2007, Bolger et al; 2003). The potential interaction of PDE4D5, with an ARHGAP21/β–arrestin 1 complex and its possible role in actin dynamics was therefore investigated. PDE4 has previously been shown to be involved in Rho-mediated cell migration (Fleming et al; 2004). In REF52 cells, rolipram inhibition of PDE4 resulted in suppression of the RhoA-ROCK-Myosin signalling pathway which is required for integrin-mediated actin-adhesion spike formation. Furthermore, PDE4 inhibition led to impaired cell migration (Fleming et al; 2004).
In this study the HEK293-AT1aR cell line was used to study PDE4D5 interaction with this complex. Immunoprecipitated ARHGAP21 was shown to interact with PDE4D5 both in resting and Angiotensin II stimulated cells (Figure 5.10 top panel). As noted previously in β-arrestin/ARHGAP21 co-immunoprecipitation experiments, interaction of PDE4D5 with ARHGAP21 was unaltered by activation of Rho. *In vitro* pull-down assays were performed to determine if PDE4D5 is able to bind directly to ARHGAP21 or is the interaction observed *in vivo* mediated by β-arrestin recruitment of the enzyme.

Purified recombinant PDE4D5 was found to interact directly with fragments RhoGAP domain and ARF binding domains of purified ARHGAP21-GST (Figure 5.11 bottom panel). However both the fragments have some overlapping regions. Interestingly, PDE4D5, but not β-arrestin, was shown to interact with the ARF binding domain of ARHGAP21 (Figure 5.7 top panel and Figure 5.11 bottom panel). It could be inferred that PDE4D5 and β-arrestin might interact with ARHGAP21 independent of each other. However, both proteins are able to interact with the RhoGAP domain of ARHGAP21. It is possible that β–arrestin and PDE4D5 compete for binding to the RhoGAP domain.

Interaction of PDE4s with some partner proteins can, in certain instances, be regulated by cAMP. Elevation of cAMP levels and subsequent activation of PKA has been shown to mediate the dissociation of PDE4D3 and PDE4C2 isoforms from DISC1, a scaffold protein implicated in schizophrenia (Murdoch et al; 2007). Furthermore, in neuronal SHSY5Y cells, the interaction of an endogenous 71KD DISC1 species with the PDE4B1 isoform is attenuated by increased cAMP levels (Millar et al; 2005).

Conversely, the interaction of PDE4D3 with mAKAP has been shown to increase in response to elevated cAMP levels, whereby cAMP-mediated PKA phosphorylation of PDE4D3 on the Ser13 within its unique N-terminal region, increases PDE4D3 affinity for mAKAP (Carlisle et al; 2004).
It was therefore of interest to assess the functional consequences of elevated cAMP levels in the protein-protein interactions of a β–arrestin-ARHGAP21-PDE4D5 complex. In HEK293-AT1aR cells treated with or without Angiotensin II, forskolin/IBMX-induced elevation of cAMP had no impact on the affinities of PDE4D5-ARHGAP21 and β–arrestin-ARHGAP21 interactions as revealed in co-immunprecipitation experiments (Figure 5.12). Similarly, inhibiting PDE4 activity with rolipram, in the presence or absence of elevated Rho activity, did not alter the interactions of the protein complexes (Figure 5.12). Small interfering (si) RNA to knock down ARHGAP21 expression in HEK293-AT1aR cells was used to further characterise the interactions of the β-

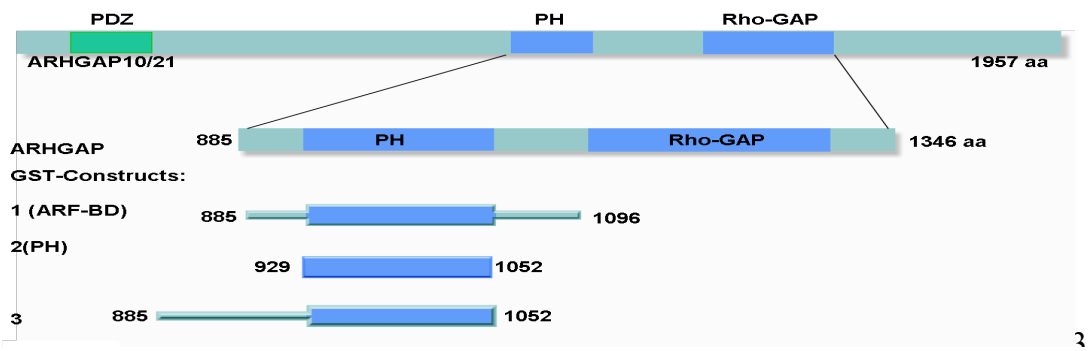
arrestin/ARHGAP21/PDE4D5 complex and its role in stress fibre formation. However treatment with ARHGAP21 siRNA resulted only in a 15% reduction in ARHGAP21 expression in cells, which was insufficient to exploit functionally. Further work is therefore required to optimise the transfection efficiency of ARHGAP21 siRNA with these cells.

ARHGAP21 is a poorly characterised RhoGAP. Its functional role in signalling complexes has yet to be elucidated. Although the cAMP-PKA pathway has been shown to be involved in regulation of cell migration and motility (Howe et al; 2005, Howe, 2004 and Fleming et al; 2004), the role of PDE4 isoforms in these processes is still unclear. As cAMP signalling in cells is compartmentalised (Lynch et al; 2006, Houslay and Adams, 2003, Zaccolo and Pozzan, 2002), it could be postulated that PDE4D and β–arrestin might co-ordinate Rho signalling pathways by interacting with and regulating the activity of ARHGAP21. The functional significance of ARHGAP21/β-arrestin, ARHGAP21/PDE4D5 and, potentially, ARHGAP21/β-arrestin/PDE4D5 interactions in these cells needs further investigation.

As it has been shown that both β–arrestin and PDE4D5 bind ARHGAP21 within its RhoGAP domain, it is possible the two proteins compete for binding to ARHGAP21. The amounts of ARHGAP21 complexing with β-arrestin, PDE4D5 or both may vary with cell type with the possibility that interactions are also altered in response to extracellular stimuli. Considering PDE4D5 also binds ARHGAP21 within its ARF binding region, a PDE4D5-ARHGAP21 complex may be sequestered to different sub-cellular compartments than β-arrestin-ARHGAP21 and β–arrestin-PDE4D5-ARHGAP21 complexes. Since the pull-down assays were not performed with full-length ARHGAP21 protein, it is possible that β–arrestin, as well as PDE4D5, might have additional binding sites on ARHGAP21 aside from those identified and discussed here.
Sequence analysis of ARHGAP21/10 using scansite (http://scansite.mit.edu/motifscanseq.phtml) identified a potential PKA consensus motif within the protein. However, as shown in Figure 5.12, when ARHGAP21 was immunoblotted with a PKA substrate-specific antiserum, no

phospho-band of the molecular weight of ARHGAP21 was observed in precipitates as well as in lysates. However, a 100kD phosphorylated species was detected (Fig 5.12) One possible PKA substrate, which migrates at this size is PDE4D5. Selective immunoprecipitation of PDE4D5 with PDE4D5-specific antisera followed by probing with a PKA substrate antibody would indicate whether or not the unknown phospho-protein is PDE4D5. Notwithstanding this, PDE4D5 is known to be phosphorylated and activated by PKA (Baillie et al, 2001).

In conclusion, these studies suggest ARHGAP21 is a binding partner for both β–arrestins and PDE4 isoforms. Investigating these interactions and their functional roles may be of importance in the development of therapeutic targets in disease states linked to changes in cell migration and cytoskeletal rearrangement including tumour metastasis, asthma and COPD.



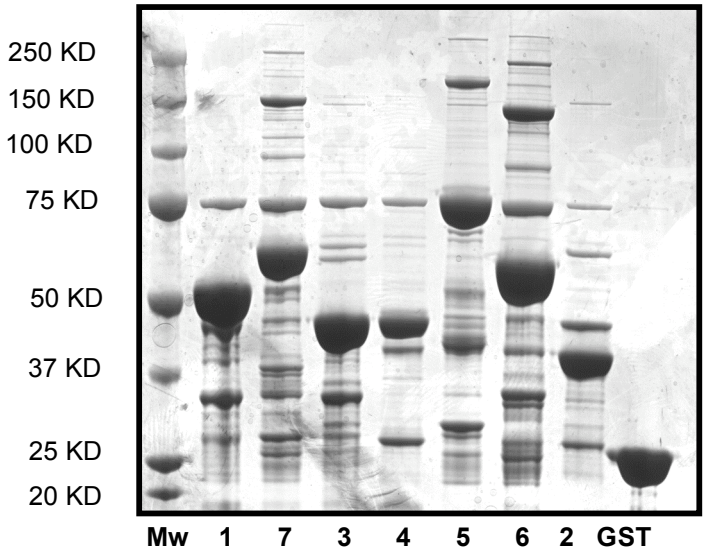
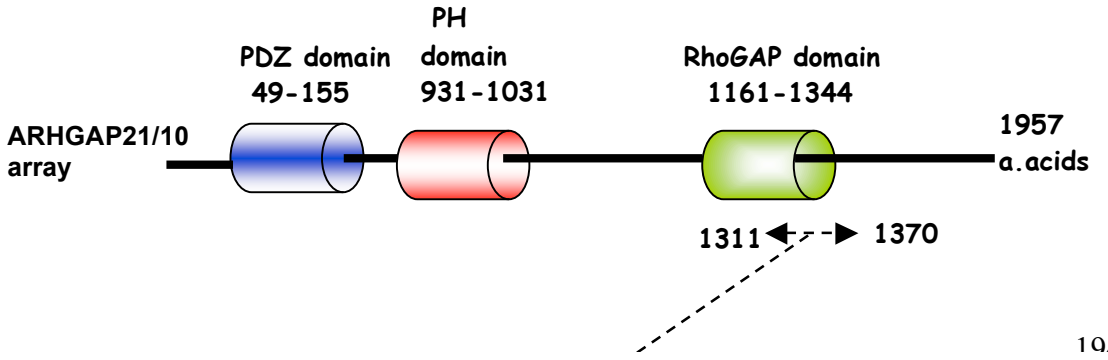


Figure 5.2 Expression and purification of GST-truncates of ARHAGP21 in *E coli*.

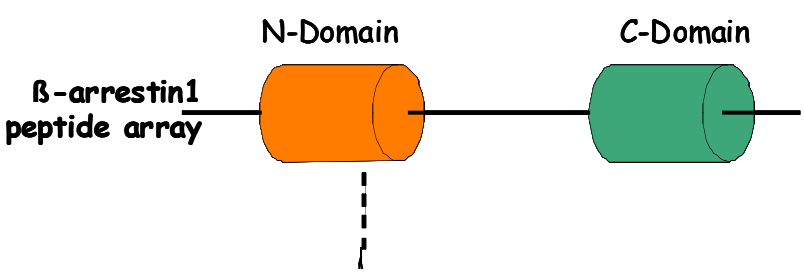
ARHGAP21 and different truncates used in this are schematically represented. ARHGAP21 contains an N-terminal PDZ domain, a PH domain and a conserved RhoGAP domain. GST and different ARHGAP constructs were expressed and purified from *E.coli* as described in chapter 2.4.2. Purified proteins were analysed by SDS-PAGE and Coommassie staining as described in section 2.10. The gels represent the single successful expression experiment undertaken for all the proteins in *E.coli*



Cont
P1321
D1322
Q1323
Y1324
K1325
I1326
V1327
E1328
T1329
L1330
I1331
Q1332
H1333
H1334
D1335
V1336
1337
1338
1339
E1340
E1341
1342
1343
1344
1345
E1341
E1345
E1345
334

Figure 5.4 Alanine-scanning substitution analyses to probe the binding sites for β-arrestin1-GST in the RhoGAP domain of ARHGAP10/21

ARHGAP21/10 peptide arrays were probed for β-arrestin1–GST binding based upon the indicated 25-mer 'parent' ARHGAP21/10 peptide, where the indicated amino acids were sequentially and individually replaced by alanine. Ct refers to the native peptide and all other spots reflect alanine substitutions of individual amino acids in the native spot. The peptide array interaction data are from experiments done once.



GST alone

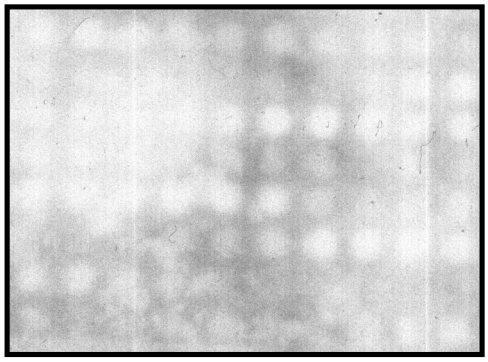


Figure 5.5 Probing a β-arrestin1 array with ARHGAP10/21 (1064-1346) –GST

Beta arrestin-1 is shown schematically with its N and C-domains. Results show immobilized peptide 'spots' of overlapping 25-mer peptides each shifted along by five amino acids in the entire Beta-arrestin-1 sequence probed for interaction with ARHGAP21 fragment with 1064-1346 amino acids and detection by immunoblotting. Positively interacting peptides generate dark spots, while those that do not interact leave white (blank) spots. In all other sections of the array, spots were blank with either probe. Spot numbers relate to peptides in the scanned array and whose sequence is given as indicated. Arrays probed with a. acids 885-1096 of purified ARHGAP21 GST did not yield any spots. Arrays probed with purified GST did not yield any positively interacting spots. The peptide array interaction data are from experiments done once.

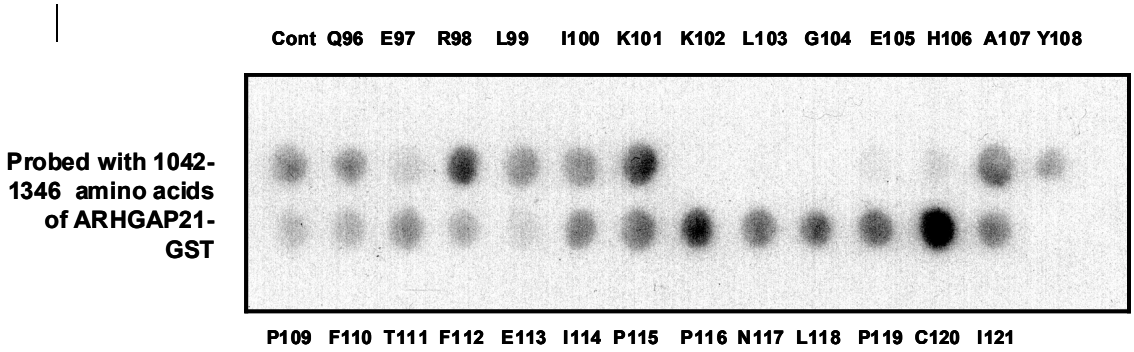
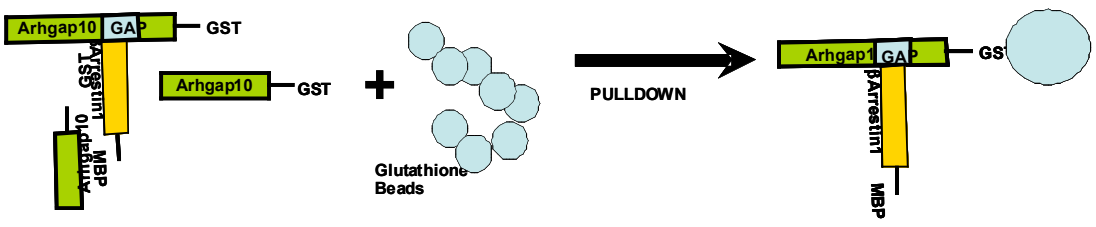
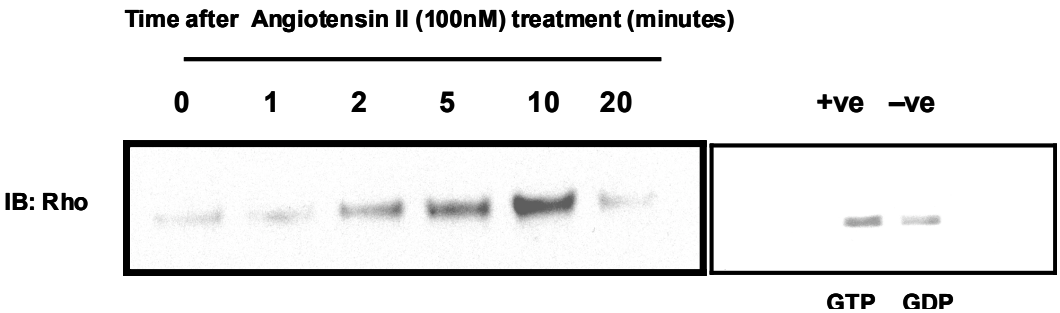
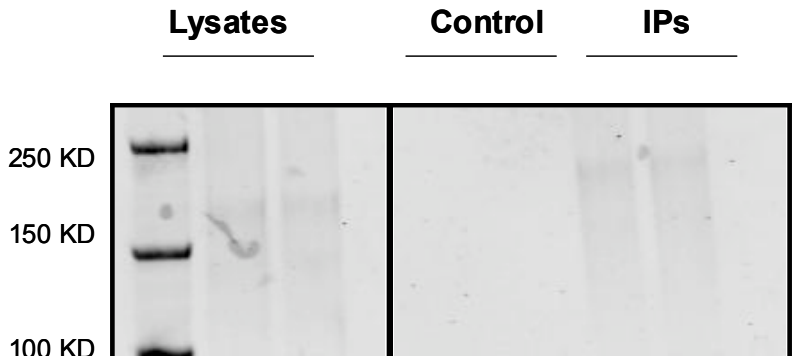


Figure 5.6 Alanine scanning substitution analyses to probe binding sites ARHGAP21-GST in the N-domain of β-arrestin 1

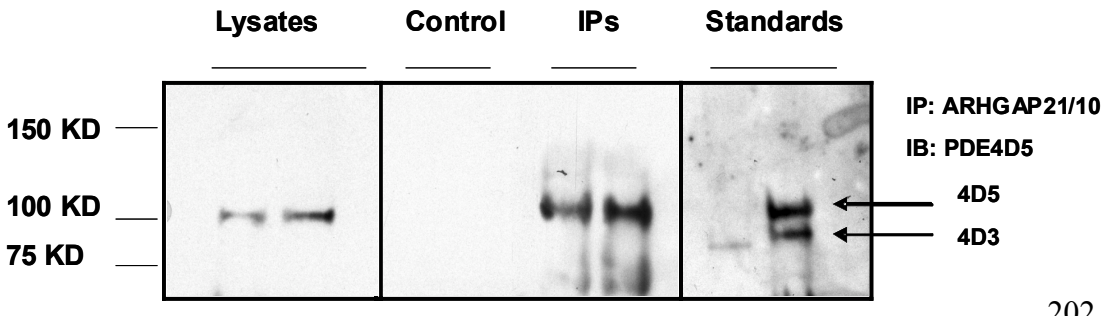
β-arrestin1peptide arrays were probed for ARHGAP21/10 binding based upon the indicated 25-mer 'parent' β-arrestin1 peptide, where the indicated amino acids were sequentially and individually replaced by alanine. Ct refers to the native peptide and all other spots reflect alanine substitutions of individual amino acids in the native

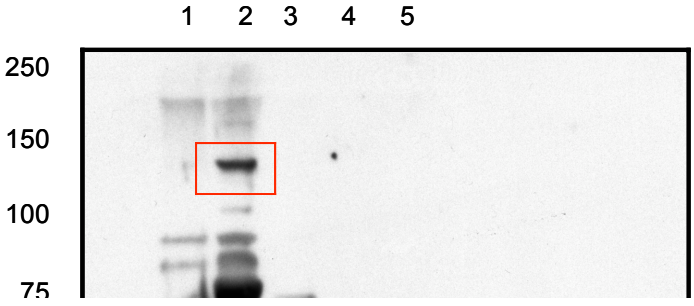


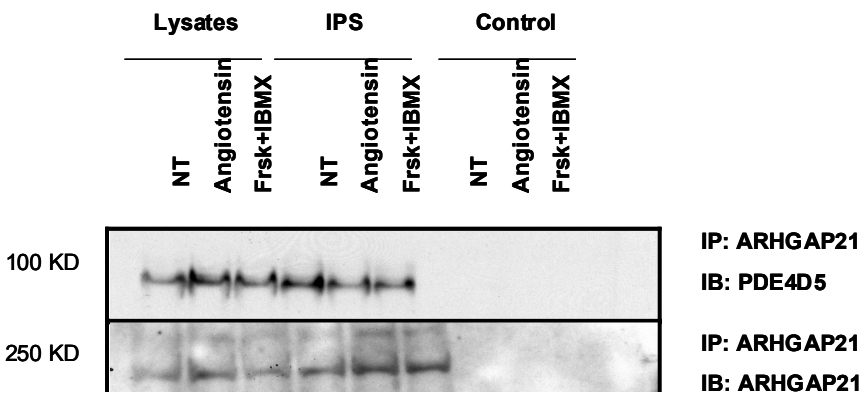

Time after Angiotensin II (100nM) treatment (minutes)
0
1
2
5
10
20
+ve
–ve
IB: Rho
GTP
GDP



IP: Arhgap21201


Lysates
Control
IPs
Standards
150 KD
100 KD
75 KD
IP: ARHGAP21/10
IB: PDE4D5
4D5
4D3


1 2 3 4 5
250
150
100
75
IB: GST


Lysates
IPS
Control
NT
Angiotensin
Frsk+IBMX
NT
Angiotensin
Frsk+IBMX
NT
Angiotensin
Frsk+IBMX
100 KD
250 KD
IP: ARHGAP21
IB: PDE4D5
IP: ARHGAP21
IB: ARHGAP21

Chapter 6 Final discussion

PDE4 enzymes play a crucial role in regulating cAMP levels within various sub-cellular compartments (Houslay et al; 2007, Zaccolo 2006, Zaccolo et al; 2006, Lissandron et al; 2006, Terrin et al; 2006, Houslay and Adams, 2003). Compartmentalization of cAMP signalling is now well documented due to the advent of genetically encoded sensors of cAMP that have allowed for the detection of cAMP gradients within live cells (Willoughby and Cooper, 2008, Tasken and Aandahl 2004, Zaccolo et al, 2002, Dunn et al; 2006, Dal molin et al; 2006, Willoughby and Cooper, 2007). Components of cAMP signalling including AKAPs (Dodge-Kafka et al; 2006), adenylate cyclases (Cooper and Crossthwaite, 2006) and PDEs (Houslay et al; 2007) are specifically recruited to intracellular micro-domains where they respond to and modulate cAMP pools. Proper targeting of these proteins is of critical importance for functional cAMP signalling within cells.

To date 21 PDE4 isoforms have been identified, each of which has an isoform specific N-terminal region that confers capability to interact with a variety of proteins that regulate various signalling molecules and their transduction pathways (Houslay et al; 2007, Houslay and Adams, 2003, Lynch et al; 2006). Many of these PDE4-signalling scaffold protein complexes are discussed in detail in section 1.8.

6.1 SUMOylation of PDE4 isoforms in HEK293 cells

Post-translational modifications of a protein play a major role in regulation of its structure, function and subcellular localisation. The most well established post-translational modification of PDE4s is phosphorylation, which is catalysed by kinases including PKA, ERK, and an as yet unknown kinase downstream of PI3 kinase, (Mackenzie et al, 2002, Mackenzie et al, 2000, Mackenzie et al; 1998 and Ekholm et al, 1997, Hill et al; 2006). Sections 1.7.4.2, 4.3 and 4.4 discuss the regulation of PDE4 isoforms by PKA and ERK in detail.

SUMOylation is relatively a newly identified post-translational modification and is known to affect the localisation of proteins, and their functioning (Kerscher, O; 2007 and Johnson, E; 2004). SUMO plot (www.abgent.com/doc/sumoplot) analysis of PDE4s identified two of the subfamilies as potential SUMO substrates. Accordingly, in chapter 3, analysis was performed to characterise the SUMO modification of PDE4 isoforms particularly, PDE4D5. Although the conditions in which PDE4D5 is SUMOylated were initially unknown. Co-transfection with PIASy E3 ligase led to conjugation of PDE4D5 with SUMO-1 whilst co-transfections with other E3 ligases such as PIAS1 and PIAS2 did not induce PDE4D5 SUMOylation (Figure 3.2). The SUMO conjugation motif is ΨKXE, where Ψ is an aliphatic residue and X is any amino acid (Hay 2005). This SUMO conjugation consensus motif is located within the catalytic region of PDE4D5. In confirmation of this being the site of SUMOylation within PDE4D5, mutation of the lysine residue within this sequence to arginine, abrogated SUMOylation of PDE4D5 (Figure 3.2a). Sequence alignment of the long isoforms from the four sub-families revealed that only the 4A and 4D sub-families possess a potential SUMOylation site within their primary structures. This was verified in SUMOylation assays whereby HEK293 cells were transfected with PDE4A4, 4B1, 4C2 and 4D5 isoforms plus PIASy E3 ligase. Only in cells expressing the PDE4D and PDE4A isoforms was SUMOylation detected (Figure 3.2 c). It would be of interest to determine whether PDE4 isoforms are differently conjugated to other SUMO molecules, such as SUMO2-4 and if this process is altered under different physiological conditions. Identification of the mechanisms regulating SUMOylation would offer means to modulate of functioning of the PDE4A and PDE4D subfamilies.

UBC9 is a SUMO E2 ligase (Johnson and Blobel 1997, Johnson et al, 1997). Figure 3.3 shows that UBC9 interacts at the FQF 'multi-functional' docking domain of PDE4D5. The FQF docking domain is conserved in all PDE4 isoforms. However, only 4A and 4D subfamilies were shown to be SUMOylation substrates thus demonstrating that UBC9 binding alone is not sufficient for SUMOylation to occur *in vivo*. The FQF multi-protein docking domain of PDE4 isoforms serves as a site for interaction for proteins including

β-arrestin (Bolger et al, 2006), ERK2 (Mackenzie et al, 2000) and AKAP18δ (McSorley et al, 2006). Further analysis is required to characterise the interactions of these various partners at the FQF and whether their interactions are competitive. If the interactions are dynamic, then, for example, would ERK binding to the FQF domain block interaction of UBC9 with the PDE4 isoform and subsequently preventing it from being SUMOylated. Changes in the level of expression of the scaffolds competing for binding to this site is likely to have functional consequences for PDE4 regulation and its subcellular localisation.

PIAS proteins are Protein inhibitors of activated STAT and possess SUMO E3 ligase activity (Takahashi et al, 2003). PIAS proteins interact with a variety of proteins and act as transcriptional repressors. This repressor activity can either be dependent or independent of their SUMO E3 ligase activity (Palmivo; 2007). There are four different PIAS proteins PIAS1, PIAS3, PIASx and PIASy (Jackson; 2001 and Gross et al; 2001). As co-transfection of PIASy and PDE4D5 was shown to induce SUMOylation of the isoform, the interaction of PIASy with PDE4D5 was examined in further detail (Figure 3.4). Wild type PDE4D5 interacted with PIASy and was SUMOylated. The F670A/Q671/F672A mutant of PDE4D5 which was unable to interact with UBC9, plus the K323R mutant of PDE4D5 (both of which were still able to bind E3 ligase) were not conjugated with SUMO suggesting that a functional FQF region as well as a conserved motif for SUMO are necessary for SUMOylation (Figure 3.4 a2 and a3). Truncation analyses to identify the PIASy binding site(s) on PDE4 revealed that PIASy bound only full-length PDE4D5 protein but not to any of the individual PDE4 domains (Figure 3.4 b) suggesting that interaction of PIASy with PDE4 is complex and requires more than one binding site on the molecule. In support of this, further characterisation of the interaction using scanning peptide array analysis identified (figure 3.4c) two potential PIASy interaction sites on a PDE4D5 peptide array (figure 3.4c); one site in close proximity to PKA phosphorylation site within the UCR1 region (amino acids 111-145) of PDE4D5 and another site within the PDE4D catalytic region (amino acids 486-530). Confirmation of these PDE4D5 regions as binding domains of PIASy, plus the identification of

the residues of PIASy required for this interaction, would require further mutagenesis studies.
Other members of the E3 ligase family include the RNBP2/NUP358 and polycomb group protein PC2 proteins (Johnson, 2006). The potential role of these E3 ligases in SUMOylation of PDE4D5 is still to be determined.

Treatments with various signalling activators and inhibitors were used to assess the influence of cell signal transduction on PDE4D5 SUMOylation. None of the treatments and associated pathways studied altered the SUMOylation status of PDE4D5 under experimental conditions. It therefore still remains to be determined which factors drive SUMOylation of PDE4 enzymes *in vivo*. SUMOylation patterns of proteins have been shown to be variable during different phases of cell cycle (Bischof and Dejean, 2007, Bischof et al, 2006). It would therefore be of interest to examine whether PDE4 SUMOylation varies during different phases of cell cycle.

SUMOylation of proteins is known to affect their localisation within cells. For PDGF-C, conjugation with SUMO-1 targets the protein to the nucleus (Reigstad et al; 2006). Nuclear localisation and subcellular fractionation studies of SUMOylated and non-SUMOylated PDE4D5 (Figure 3.6a) revealed that PDE4D5 remained in soluble fraction of the cells before and after SUMOylation. However, a small amount of SUMOylated and non-SUMOylated PDE4D5 species were present in the P1 cellular fraction, which includes both nuclear membranes and unbroken cells. As PDE4D5 expression appears to be cytosolic within HEK293 cells, the detection of PDE4D5 within the P1 fraction is likely due to the presence of unbroken cells within this fraction. Although SUMOylation did not effect localisation of PDE4D5 in HEK293 cells, it would be of importance to investigate whether the localisation PDE4 isoforms is altered upon SUMOylation in other cells types and if the localisation is affected by cellular stimuli.
PDE4 enzyme action is an integral part of cAMP signalling as it is the sole means of decreasing cAMP levels within cells. Hence any cellular effects, which mediate changes in PDE4 activity, would directly influence cAMP signalling. A potential role for SUMOylation in regulating the PDE4 activity

was therefore examined. SUMOylation did not affect the activation of PDE4D5 both in *in vitro* and *in vivo* assays. However, it was found that SUMOylation potentiated the effect of PKA phosphorylation in activating PDE4D5. Furthermore, and somewhat surprisingly, SUMOylation of PDE4D5 appeared to negate the inhibitory effect of ERK phosphorylation on PDE4D5 activity *in vitro* (Figure 3.7). A possible explanation for the loss of ERK effect is that conjugation of SUMO to the catalytic unit of PDE4D5 induces a conformational change affecting its interaction with the UCR1/UCR2 regulatory module. This is consistent with previous studies showing that PKA phosphorylation of UCR1 is able to override the inhibitory effect of ERK phosphorylation on PDE4 activity by attenuating the interactions of the UCR1/UCR2 module (Beard et al; 1999). Furthermore, the PDE activity of short PDE4 isoforms, which lack a UCR1 domain, is not affected by ERK phosphorylation. Further studies are required to assess the ERK response on SUMOylated PDE4 *in vivo*. Based on this novel finding from the *in vitro* studies, it could be suggested that in cells PDE4 SUMOylation functions to further compartmentalise cAMP signalling whereby subpopulations of PDE4s are able to remain active in the presence of an ERK stimulus.

Although SUMOylation did not affect the overall enzyme activity of PDE4D5, it was shown to increase its rolipram sensitivity. SUMOylated PDE4D5 was found to be 10 times more sensitive to inhibition by rolipram than its non-SUMOylated form. PDE4 isoforms exist in two different states of rolipram sensitivity, namely HARBS and LARBS (Souness and Rao, 1997). Binding of proteins such Src tyrosyl kinases and XAP2, as well as PKA phosphorylation, have been reported to induce switching between the LARBS and HARBS states (Bolger et al; 2003, McPhee et al, 1996). It is possible that the increased sensitivity of rolipram elicited by SUMOylated PDE4D5 is due to switching to the HARBS conformational state. Since SUMO is a ~11KD molecule, it is possible its conjugation to the PDE4 at the catalytic region stabilises the isoform in the HARBS configuration.

PKA phosphorylation of PDE4D5, which evokes a shift from LARBS to HARBS, was shown to increase the rolipram sensitivity of both non-

SUMOylated and SUMOylated PDE4D5 to a similar extent. SUMOylation did not appear to enhance the PKA-mediated increase in PDE4D5's rolipram sensitivity as it is likely the SUMOylated form already exists predominantly in a HARBS state.

SUMOylated PDE4D5 was completely unaffected by ERK action and its rolipram sensitivity was same as that of the non-phosphorylated species (Figures 3.8, 3.9 and 3.10). Similar experiments with PDE4D5 binding partner RACK1, following earlier findings of Yarwood and co workers (Yarwood et al 1999) who showed that binding of RACK1 to PDE4D5 N-terminus decreased the rolipram sensitivity of the isoform, revealed that both wild type and SUMOylated PDE4D5 exhibited a decreased sensitivity to rolipram in the presence of purified recombinant RACK1 (figure 3.11). As RACK 1 binds to the N-terminal region as well as to the catalytic region (Bolger et al; 2006) and SUMO conjugation occurs in the catalytic region, it is possible that the HARBS induced by linkage of SUMO is reversed to a certain extent by RACK1 binding to the N-terminus and catalytic region of PDE4D5.

The effects of SUMOylation on the binding of PDE4D5 to known interactors including RACK1, □arrestins, ERK, AKAP18δ and UBC9 was evaluated both *in vivo* and *in vitro*. All partners studied exhibited an increased binding to the SUMOylated species of PDE4D5 compared to its non-SUMOylated form (Figure 3.13 and 3.14). However, the extent by which binding affinities increased differed between *in vitro* and *in vivo* studies. In *in vivo* assays, it is likely many other proteins in addition to PDE4D5 are being SUMOylated and this may subsequently affect the affinity of PDE4D5 interactions with its binding partners.

As only the PDE4A and PDE4D sub-families are SUMOylated, it is possible that SUMOylation provides a means for subfamily-specific regulation of PDE4 signalling within cells. Since cAMP signalling is compartmentalised, SUMOylation might offer a way of modulating PDE4 activity within specific sub-cellular compartments without altering the PDE4 activity globally. Furthermore, the increased affinity of PDE4 interactors for the PDE4

SUMOylated species would offer a way of redistributing the cellular PDE4 population. Future work would entail identifying the processes which drive SUMOylation of PDE4 enzymes in an effort to further characterise its functional roles.

For the *in vivo* experiments performed in this study, both PDE4 isoforms and SUMO ligases were overexpressed in the cells. However, given the lability of SUMOylated proteins and the ability of SUMO isopeptidases to quickly deSUMOylate conjugated proteins, it would be necessary to repeat this study focussing on endogenous proteins so as to get a better understanding of SUMOylation of PDE4s under normal physiological conditions. It would also be of interest to carry out structural analysis to resolve the conformational state of SUMOylated PDE4 in an effort to further understand the effects of SUMOylation on rolipram sensitivity. As SUMOylation is likely to trigger the HARBS conformation of PDE4, PDE4 SUMOylation may eliminate the emetic effects caused by PDE4 inhibitors. Compounds or small molecules, which trigger PDE4 SUMOylation may therefore be of therapeutic use.

6.2 Rolipram induced foci formation of PDE4A4

PDE4 isoforms in a basal state are known to exist in the LARBS conformation. The formation of complexes with proteins such as SRC tyrosyl kinases is known to confer switching of PDE4A4 to the HARBS state as described in section 1.7.2 (McPhee et al; 1996). PDE4A4 is known to form accretion foci in cells subjected to chronic rolipram treatment (Terry et al; 2003). Chapter 4 describes the process in detail and the experiments carried out in an effort to identify the signalling pathways and associated proteins involved in the process of PDE4A4 foci formation. These accretion foci are independent of cytoskeletal proteins and sub-cellular organelles such as Golgi (Figure 4.1 and table 4.1). cAMP has an inhibitory effect on foci formation and quickly disperses any pre-formed foci by competing with rolipram for the binding site on PDE4. Similarly, non-foci forming PDE4 selective inhibitors similarly antagonise the action of rolipram by displacing it from the active site of PDE4A4. Activation/inhibition of the components of cAMP signalling

including PKA and EPAC did not have any clear effects on foci formation (Figure 4.2). It is therefore likely that the cAMP molecule binding directly to the active site of PDE4A4 mediates the effects of cAMP signalling on foci formation.

Earlier studies (Terry et al; 2003) proposed a model for foci formation known as “inside-out signalling” whereby binding of rolipram to PDE4A4 induces conformational changes within the catalytic site that are transferred through helix 10/11 and loops 7/8 to the surface of the catalytic unit. This, presumably together with the unique N-terminal region of PDE4A4, forms a new conformation that interacts with yet unidentified proteins to couple to the formation of foci in a process that depends upon active protein synthesis (Figure 4.1).

It is widely known that ERK phosphorylation inhibits activity of all long PDE4 isoforms except those of the PDE4A sub-family, which lack a proline in the consensus site for ERK phosphorylation within their catalytic unit (Mackenzie et al. 2000). In order to investigate the role of other MAP kinases in foci formation and maintenance, various MAP kinase inhibitors were used to treat cells stably expressing a GFP fusion protein of PDE4A4B. Inhibition of MEK did not affect rolipram-induced foci formation nor did the inhibition of MAPKAP kinase2. Inhibition of either kinase did not induce foci formation either.

Activation of MAPKAP kinase-2, via activation of p38 MAPK with anisomycin, abrogated rolipram-induced foci formation. However, strangely, the effects of anisomycin were not reversed by SB203580, a p38 inhibitor. Anisomycin is known to stimulate other effectors, such as JNK (Sampieri et al; 2007). However concomitant treatment with the JNK inhibitor did not reverse the effects of anisomycin on foci formation (Figure 4.3). Anisomycin can also inhibit protein synthesis at concentrations above 10ug/ml (Sampieri et al; 2007) and it is possible that this might be the process through which it attenuates foci formation. However in these studies anisomycin was used at a lower concentration concentration. It would be of interest to assess the action of anisomycin on protein synthesis in these cells at the concentrations used.

The cdk5 inhibitor roscovitine, when used at higher concentrations where it is known to affect both cdks and MAP kinases resulted in inhibition of foci thus reiterating a potential role of MAP kinases in foci formation (Figure 4.7).

MAPKAP kinase 2 can also be inhibited by rottlerin, which is also an inhibitor of PKC (Blanquet et al, 2003). Rottlerin was found to inhibit foci formation. In order to rule out PKC action in this process, treatments were performed with PMA and EGCG. Although PMA did not affect foci formation on its own, it reversed the effects of rottlerin by increasing the immobile fraction of PDE4A4-GFP foci. Inhibition of PKC isoforms PKCα, β1,ε with RO320432 (Birchall et al; 1994) and GO6983 to inhibit PKCα, β, γ, δ (Wang et al; 2004) increased the levels of foci in cells suggesting the signalling of PKC isoforms α, β, γ, δ, or ε is involved in foci formation. To confirm PKC involvement in foci formation future work would entail the selective siRNA-mediated knockdown of specific PKC isoforms.

Calcium is an important intracellular messenger regulating many signalling pathways. In order to evaluate its effects on foci formation/dispersal, treatments were performed with calcium modulators thapsigargin and ionomycin (Won and Orth, 1995, Aagaard-tillery and Jelinek, 1995). Both ionomycin and thapsigargin inhibited foci formation, indicating an involvement of calcium, derived either from intracellular stores or externally, in regulating this process.

One effector of calcium signalling is PKC. Only conventional but not novel and atypical isoforms of PKC is known to be activated by calcium (Mackay and Twelves, 2007). It is possible that such species are indeed involved in foci regulation, as PKC inhibition appears to facilitate foci formation. To further investigate possible downstream effectors of calcium signalling, inhibitors or calmodulin Kinase II (KN62) and calcineurin (Cyclosporin) were used. However, both of these had no effect on foci formation (Figure 4.4).

PDE4A4B can be activated upon stimulation of p70S6 kinase and PI3 Kinase in response to growth hormone stimulation in fibroblasts (Mackenzie et al, 1998). Hence to ascertain the potential role of PI3 kinase pathway in foci formation, CHO cells stably expressing PDE4A4GFP were treated with the PI3 kinase inhibitors, wortmanin and Ly294002, both of which enhanced the foci formation. However, inhibition of p70S6 kinase by rapamycin and GSK3 by the GSK3β inhibitor had no effect on foci formation thus ruling out the role of both of these kinases. PI3 kinase, however, triggers a complex pathway of events in cells whereupon it activates a myriad of kinases, including PKC (Hirai and Chida; 2003) and ERK (Kim et al; 2007, Xu and Yu, 2007), leaving a number of possible actions and thereby routes to pursue in further research, thus the downstream targets for PI3 kinase effects have still to be determined (Figure 4.5).

PDE4A4 is known to associate with Src tyrosyl kinases (Beard et al, 2002 and McPhee et al, 1999). In order to determine the possible involvement of tyrosyl kinases in foci formation the general tyrosyl kinase inhibitor genistein was used. Genistein treatment was found to abolish foci formation. In order to get further insight into a possible role of specific tyrosyl kinases in foci formation more inhibitors were assayed. These included inhibitors for Src tryosyl kinases (Su6656); EGFR tyrosyl kinases (PD168393) and PDGFR tyrosyl kinase (AG17). However, of these only the PDGF receptor Tyrosyl kinase inhibitor AG17 inhibited foci formation (Figure 4.6). This suggests that the PDGF receptor may be important in foci formation. In order to address this future studies should be done to see if the PDGR receptor accumulates in foci or if siRNA-mediated knockdown of the PDGFR inhibits foci formation. The PDGF receptor is well known to dimerise upon ligand binding. The bound ligand is known to induce transphosphorylation of the intracellular domains of the receptors. These phosphorylated intracellular regions are known to initiate various signalling responses. Downregulation of PDGF receptor is achieved by its internalisation upon ligand binding to the receptor. The receptor is either then subjected to proteasomal degradation or recycled back to the surface of the receptor (Chiarugi et al; 2002).

Researchers have showed that inhibition of PDE4 activity regulates immunomodulatory functions (Jin et al, 2005, Jin and Conti 2002). Similarly, immunomodulatory functions of thalidomide have been recently established (OKafor, 2003, Powell 1999); a potential role of thalidomide in regulating foci formation was therefore studied. Thalidomide treatment inhibited foci formation. Thalidomide is known to act as an anti-inflammatory agent by decreasing the levels of TNFα yet at the same time it is shown to increase the production of IFNγ and IL-5 in mononuclear cells (Direskeneli et al; 2007). Conversely, PDE4 isoforms have been shown to decrease the production of IFNγ and IL-5 in T-cells (Peter et al, 2007). Hence it could be hypothesised that foci formation might be a net result of the balance of these two affects, the confirmation of which requires further investigation.

It has been suggested that PDE4 isoforms may form oligomers by a process involving their regulatory domains UCR1 and UCR2 (Richter and Conti, 2002). However, work form our laboratory suggests that whilst this may occur in transfected cells overexpressing PDE4 isoforms it is less likely to occur *in vivo* due to the interaction of PDE4 isoforms with partner proteins, which would prevent dimerisation occurring. Furthermore, UCR1/2 is conserved amongst sub-families implying that heterodimers may occur between different isoforms. This very clearly does not occur *in vivo* as isoforms can be selectively immunoprecipitated. Thus dimerisation may only be a feature of overexpression systems or exist only under certain cellular conditions.

When it occurs, dimerization has been suggested to regulate the sensitivity of PDE4 isoforms to rolipram inhibition (Richter et al 2004). In order to evaluate any potential role of oligomerization in foci formation, co-immunoprecipitation studies were carried out using differently tagged PDE4A4 species and, in separate experiments with differently tagged PDE4D3 species (Figure 4.9). Doing this, it was found that overexpressed PDE4D3 oligomerized in cells whilst overexpressed PDE4A4 did not appear to show any dimerisation in the presence or absence of rolipram. It might be that the residues required for oligomerisation are not well conserved in PDE4A4 thus preventing the oligomerisation. Another explanation is that PDE4A4 foci formation perhaps

requires stabilising factors that are lost upon cell lysis thus disrupting foci. However, the role of experimental conditions cannot be ruled out. Further investigations are required to identify the key residues as well as their conservation in various PDE4 sub-families to understand the role of oligomerisation in foci formation. Although it is clear that foci PDE4A4 species must co-exist the interaction may not be direct but mediated by a linker protein. Perhaps this is the induced species that requires protein synthesis and this may be released upon cell disruption. It may then be useful to treat cells with a membrane permeable cross-linker to see if this causes the rolipram-induced association of species with PDE4A4. Indeed, this may be the route in future to analyse immunoprecipitates by proteomics.

Previous reports (Terry et al; 2003) established that protein synthesis is required for foci formation as well as maintenance. To further investigate the role of protein turnover in foci formation cells were treated with the protein synthesis inhibitor, cycloheximide as well as the protein degradation inhibitor MG132 respectively. Both cycloheximide and MG132 strongly inhibited foci formation. Also they started dispersing rolipram-preformed foci within 30 minutes and completely dispersed them by the end of 5 hours. Inhibition of total cell protein turnover yielded similar results (Figure 4.10 and 4.11).

PDE4A4 did not show any ubiquitination in the presence or absence of rolipram as determined by immunoblotting nor did it co-localise with ubiqutinylated proteins in confocal studies. Further studies are required to unravel the role of protein turnover in foci stability as well as in formation.

To further ascertain the role of protein turnover in conjunction with signalling pathways on foci stability, various inhibitors that were used in foci formation assays as described above were assayed. This was based upon the idea of trying to discriminate compounds that regulated foci formation from foci stabilisation. By blocking protein synthesis and degradation we can observe a rate of decay of foci. However, any compounds that accelerated the decay, presumably could exert an effect on the machinery that held foci together independent of any action of protein synthesis and turnover.

Thapsigargin caused complete dispersal of foci within 30 minutes in the absence of active protein turnover and started dispersing foci within 30 minutes in the presence of active protein turnover. However by the end of 5 hours all the foci were dispersed as measured by plate reader assay, whereas PKC inhibition with Rottlerin and RO320432 dispersed all foci within 30 minutes both in the presence and absence of active protein turnover.

Similarly, activation of p38 MAPK by anisomycin dispersed foci completely within 30 minutes in the presence or absence of active protein turnover. However, inhibition of MEK using UO126 although initially enhanced the immobile fraction of GFP eventually stimulated the dispersal of foci (figure 4.12). It should be noted that anisomycin and UO126 have inhibitory and promoting affects on foci formation respectively which reflects their effects on foci stability.

The general tyrosine kinase inhibitor, genistein and PI3 kinase inhibitor, Ly294002 yielded similar results as anisomycin suggesting an inhibitory effect on foci formation (figure 4.13 and 4.14).

In general it could be proposed that these signalling inhibitors and their respective signalling complexes could act in three different ways. Firstly, they might affect foci formation and stability directly. Secondly, they might alter the signals for protein turnover thereby influencing foci indirectly and finally they might act to affect foci formation as well as protein turnover thus enhancing or slowing foci formation as well as dispersal. Unravelling the components of foci formation and the signalling complexes will require further rigorous investigations using techniques such as truncation studies and proteomics analysis, plus electron microscopy to identify whether foci are confined to vesicles or other sub-cellular organelles etc.

6.3 RhoGAPs, Arrestins and PDE4 isoforms

Rho family of GTPases regulate F-actin and microtubule dynamics. These GTPases are activated by guanine exchange factors (GEFs) and are inhibited by GTPase activating proteins (GAP) and guanine dissociation inhibitors (GDI) (Buchsbaum et al; 2007). ã-arrestins have been shown to be an integral part of wide variety of processes like chemotaxis, endocytosis and stress fibre formation by interacting or regulating Rho GTPases (Kim and Han, 2007, Barnes et al, 2005, Goodman et al, 1996). Similarly, PDE4 isoforms are known to regulate cell migration by regulating the activity of Rho GTPases (Fleming et al; 2004). Chapter 5 describes these interactions in detail and attempts to characterise their functional outcome.

ARHGAP21 is ~1957 amino acid protein and is a novel GTPase activating protein (GAP) for Cdc42 comprising PDZ, ARF binding, PH and Rho binding domains along with a GAP domain (Menetrey et al; 2007, Dubois et al, 2005, Basseres et al, 2002). ARHGAP21 was shown to interact with ã-arrestins in a yeast two-hybrid screen (DeWire et al 2007). In order to characterise the interaction of arrestin-ARHGAP21, various truncates of ARHGAP21 were used (Figure 5.2). To identify the binding site of ã-arrestin on ARHGAP21, a full length ARHGAP21 peptide array was probed with purified β-arrestin-1 GST. ã-arrestin showed strong interaction with the RhoGAP domain (1321-1360) of the ARHGAP21 peptide array (Figure 5.2). Alanine substitution analysis of selected residues within the region revealed key residues important for the interaction. In particular, alanine substitution of a series of glutamic acid residues, namely E1341, E1342, E1344, and E1345, either collectively or individually severely affected β-arrestin interaction (Figure 5.3). In reciprocal studies to further characterise the interaction sites of ARHGAP21 on ã-arrestins, a peptide array of β-arrestin 1 was probed with truncates containing the purified RhoGAP domain of ARHGAP21 (Figure 5.4). The RhoGAP domain of ARHGAP21 showed interaction with peptides containing residues 96-130, mapping to N-domain of β-arrestin 1. Chapter 5 attempted to characterise the interaction with ã-arrestin1. Understanding the interaction of ARHGAP21 with ã-arrestin 2 comprises a part of future work. Alanine substitution scan of binding regions revealed key residues on ã-arrestin that

interact with ARHGAP21. From peptide array analysis, it could be proposed that ARHGAP21 interacts with an E-$(X)_4$-KLGEH-$(X)_5$-E site on ã arrestin 1 (Figure 5.5 and Figure 5.6).

Pull down assays with purified fragments of ARHGAP21 and ã arrestin 1 were carried out to ascertain the nature of the interaction. Purified ã arrestin 1 interacted with fragments of ARHGAP21 containing the RhoGAP domain (figure 5.7). To further elucidate the interaction, HEK293 cells stably expressing the angiotensin AT1A receptor were used. The AT1A receptor activates Rho through the G_q/G_{11} pathway with β-arrestin (Figure 5.8, Barnes et al 2005). Hence these cells would prove valuable for understanding the interaction of ã arrestin with ARHGAP21, a Rho GTPase activating protein. ã arrestin interacted with ARHGAP21 in basal conditions and the interaction did not change even after G_q/G_{11} activation, suggesting that the interaction is governed by factors independent of G_q/G_{11} pathway (Figure 5.9).

All PDE4 isoforms are known to interact with ã arrestins through their conserved catalytic unit. In the case of PDE4D5, this interaction leads to translocation of ã arrestin/PDE4D5 complex to the β_2-adrenoceptor and leads to consequent desensitisation of the receptor (Perry et al, 2002). Of all the PDE4 isoforms studied so far, ã arrestins show preferential binding to PDE4D5 due an extra binding site present in its isoform specific N-terminal region (Bolger et al, 2003). Thus to determine the involvement of PDE4 isoforms, particularly PDE4D5, in ã arrestin/ARHGAP21 interaction, lysates the AT1A receptor stable cell line were used for co-immunoprecipitation studies. Endogenous ARHGAP21 was found to interact with PDE4D5 (Figure 5.10). PDE4D5 directly interacted with both the ARF binding domain as well as the RhoGAP domain of ARHGAP21 (Figure 5.11). Since only truncates were used in this study, it is still not clear if PDE4D5 has other binding sites on ARHGAP21. Further investigations are required to identify any extra binding sites if any and also the residues involved in the interaction by peptide array analysis and through further mutation and truncation analyses. Also, it would be of interest to establish whether isoforms from other PDE4 sub-families are able to interact with ARHGAP21. It should be noted that

arrestins only bind to RhoGAP domain whereas PDE4D5 binds to both ARF and RhoGAP domains of ARHGAP21. Whether arrestin and PDE4D5 compete with each other for binding to the RhoGAP domain of ARHGAP21 is yet to be determined.

PDE4 activation is sometimes known to alter the affinity of these enzymes to certain interacting partners. For instance, PKA phosphorylation and activation is shown to release PDE4D3 and PDE4C2, but not PDE4B1, from the full-length DISC1 isoform (Murdoch et al; 2007). Henceforth, the role of PDE4 activity and PKA phosphorylation in regulating the interaction of ARHGAP21 with PDE4D5 was determined in the presence or absence of G_q/G_{11} activation. Neither PDE4 inhibition in cells nor PKA phosphorylation (with subsequent activation of PDE4 activity) affected the interaction of PDE4D5 with ARHGAP21 (Figure 5.12). It could be inferred from the above results that ARHGAP21/PDE4D5/β-arrestin interaction is independent of G_q/G_{11} activity or PKA activity or PDE4 activity.

Future work would involve delineation of binding sites of PDE4s on ARHGAP and *vice versa* along with understanding the dynamics of the interaction including the role of signalling pathways, but most importantly, the functional outcome of the interaction. Since ARHGAP21 is a novel GAP it is poorly characterised and its functions are not fully understood. Dubois and coworkers have shown that ARHGAP21/10 acts as a GAP for cdc42 in Golgi and regulates the F-actin dynamics (Dubois et al; 2005). Given that arrestin and PDE4D5 bind to the RhoGAP domain of ARHGAP21, it could be speculated that either of these proteins, or both, would have an affect the GAP activity of ARHGAP21. PDE4 activity is required for cell migration as reported previously (Fleming et al. 2004). One possibility is that ARHGAP21/PDE4D5 along with arrestin might play a role in cell migration. Also, the PH domains of ARHGAP21 might play a crucial role in membrane translocation of this GAP, which might help recruit the arrestin/PDE4D5 complex to membrane receptors under the influence of appropriate stimulus such as PI3 kinase activity. Since the arrestin-PDE4D5 complex is involved

in receptor desensitisation, ARGAP21 might play a role in endocytosis and recycling of the complex to and from Golgi to the plasma membrane.

In conclusion, the arrestin/PDE4D5/ARHGAP21 interaction is a novel finding and needs extensive characterisation to understand the functional outcome. It still needs to be determined whether all the three proteins function together or in individual complexes.

6.4 Final Conclusion

These studies have given further insight into the complexity of PDE4 regulation. The identification of effects of SUMOylation on PDE4 function paves a way to understand the complex compartmentalisation of cAMP signalling. Foci studies attempted to shed light on the role of PDE4 inhibitors in regulating the localisation of the PDE4A4 isoform. Identification of β-arrestin/ARHGAP21/PDE4D5 interaction complex gives further insight into the role of cAMP signalling in regulating cell migration which is the basis for conditions such as tumour metastasis and wound repair.

Appendix I

List of constructs used for Yeast two hybrid screening by Hybrigenics for mapping arrestin-Arhagp21 interaction

	Clone name	Type Seq	Start	Stop
1	hgx1193v1_pB27C-48	5p 3p	4743	5274
2	hgx1193v1_pB27C-2	5p 3p	4737	5274
3	hgx1193v1_pB27C-37	5p 3p	4557	5274
4	hgx1193v1_pB27C-8	5p 3p	4557	5274
5	hgx1193v1_pB27C-63	5p 3p	4557	5274
6	hgx1193v1_pB27C-36	5p 3p	4557	5274
7	hgx1193v1_pB27C-27	5p 3p	4557	5274
8	hgx1193v1_pB27C-5	5p 3p	4530	5032
9	hgx1193v1_pB27C-68	5p 3p	4746	5627
10	hgx1193v1_pB27C-55	5p 3p	4746	5627
11	hgx1193v1_pB27C-19	5p 3p	4746	5627
12	hgx1193v1_pB27C-46	5p 3p	4626	5659
13	hgx1193v1_pB27C-56	5p 3p	4683	5668
14	hgx1193v1_pB27C-15	5p 3p	4749	5277
15	hgx1193v1_pB27C-9	5p 3p	4521	5312
16	hgx1193v1_pB27C-25	5p 3p	4521	5312
17	hgx1193v1_pB27C-69	5p 3p	4749	5277
18	hgx1193v1_pB27C-26	5p 3p	4626	5393
19	hgx1193v1_pB27C-65	5p 3p	4749	5277
20	hgx1193v1_pB27C-47	5p 3p	4749	5277

Clone 1- hgx1193v1_pB27C-48

5'CATCACCTCAGATTATTCCACCACATCGTCTGCTACATACTTGACTAGC
CTGGACTCCAGTCGACTGAGCCCTGAGGTGCAATCCGTGGCAGAGAGC
AAGGGGGACGAGGCAGATGACGAGAGAAGCGAACTCATCAGTGAAGGG
CGGCCTGTGGAAACCGACAGCGAGAGCGAGTTTCCCGTGTTCCCCACA
GCCTTGACTTCAGAGAGGCTTTTCCGAGGAAAACTGCAAGAAGTGACTA
AGAGCAGCCGGAGAAATTCTGAAGGAAGTGAATTAAGTTGCACCGAGGG
AAGTTTAACATCAAGTTTAGATAGCCGGAGACAGCTCTTCAGTTCCCATA
AACTCATCGAATGTGATACTCTTTCCAGGAAAAAATCAGCTAGATTCAAG
TCAGATAGTGGAAGTCTAGGAGATGCCAAGAATGAGAAAGAAGCACCTT
CGTTAACTAAAGTGTTTGATGTTATGAAAAAAGGAAAGTCAACTGGGAGT
TTACTGACACCCACCAGAGGCGAATCCGAAAAACAGGAACCC-3'

Clone 2- hgx1193v1_pB27C-2

5'CAGCACCATCACCTCAGATTATTCCACCACATCGTCTGCTACATACTTG
ACTAGCCTGGACTCCAGTCGACTGAGCCCTGAGGTGCAATCCGTGGCA
GAGAGCAAGGGGGACGAGGCAGATGACGAGAGAAGCGAACTCATCAGT
GAAGGGCGGCCTGTGGAAACCGACAGCGAGAGCGAGTTTCCCGTGTTC
CCCACAGCCTTGACTTCAGAGAGGCTTTTCCGAGGAAAACTGCAAGAAG
TGACTAAGAGCAGCCGGAGAAATTCTGAAGGAAGTGAATTAAGTTGCAC
CGAGGGAAGTTTAACATCAAGTTTAGATAGCCGGAGACAGCTCTTCAGTT
CCCATAAACTCATCGAATGTGATACTCTTTCCAGGAAAAAATCAGCTAGA
TTCAAGTCAGATAGTGGAAGTCTAGGAGATGCCAAGAATGAGAAAGAAG
CACCTTCGTTAACTAAAGTGTTTGATGTTATGAAAAAAGGAAAGTCAACT
GGGAGTTTACTGACACCCACCAGAGGCGAATCCGAAAAACAGGAACCC-
3'

Clone 3-hgx1193v1_pB27C-37

5'CTTTGCCATCCTGAAAGAGAGCCCCAGGTCACTTCTGGCACAGAAGTC
CTCCCACCTTGAAGAGACAGGCTCTGACTCTGGCACTTTGCTCAGCACG
TCTTCCCAGGCCTCCCTGGCAAGGTTTTCCATGAAGAAATCAACCAGTC
CAGAAACGAAACATAGCGAGTTTTTGGCCAACGTCAGCACCATCACCTC
AGATTATTCCACCACATCGTCTGCTACATACTTGACTAGCCTGGACTCCA
GTCGACTGAGCCCTGAGGTGCAATCCGTGGCAGAGAGCAAGGGGGACG
AGGCAGATGACGAGAGAAGCGAACTCATCAGTGAAGGGCGGCCTGTGG
AAACCGACAGCGAGAGCGAGTTTCCCGTGTTCCCCACAGCCTTGACTTC
AGAGAGGCTTTTCCGAGGAAAACTGCAAGAAGTGACTAAGAGCAGCCGG
AGAAATTCTGAAGGAAGTGAATTAAGTTGCACCGAGGGAAGTTTAACATC
AAGTTTAGATAGCCGGAGACAGCTCTTCAGTTCCCATAAACTCATCGAAT
GTGATACTCTTTCCAGGAAAAAATCAGCTAGATTCAAGTCAGATAGTGGA
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Clone 8-hgx1193v1_pB27C-5

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Clone 15- hgx1193v1_pB27C-9

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Clone 18-hgx1193v1_pB27C-26

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Appendix II

Section II.I

Structure-activity relationship of PDE4 inhibitors- A preliminary study

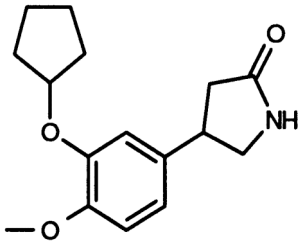
Rolipram is an active site directed inhibitor and is classified as first generation PDE4 selective inhibitor along with Ro 20-1724. Second generation of PDE4 inhibitors include Ariflo® and Roflumilast®. Third generation PDE4 inhibitors include Xanthine derivatives. Rolipram inhibits isoforms of all PDE4 subfamilies and is not subfamily specific (Houslay and Adams, 2003, Lugnier,et al 1986, Lugnier, 2006). It is widely known that the side effects like nausea and emesis caused by rolipram have limited the use of rolipram as a therapeutic agent (Houslay et al; 2005).

It has been shown (Terry et al, 2003) that PDE4A4 and its orthologue PDE4A5 when subjected to chronic rolipram treatment (10-18hrs) can undergo redistribution in cells and form accretion foci. In that study it was proposed that various of the side-effects caused by rolipram may be mediated through cAMP-independent foci formation due to the redistribution of PDE4A4 and associated proteins within the cell (Terry et al; 2003).

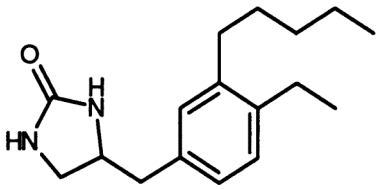
This concept was formulated from observing that various of the PDE4 inhibitors that are known to have pronounced side effects induce foci formation (example; Rolipram, RO 20-17240), whereas, other PDE4 inhibitors like Ariflo® do not induce foci formation of PDE4A4 and have lesser effects (Terry et al; 2003).

I set out to gain further insight into this by screening a large panel of PDE4 inhibitors using a plate reader assay to identify inhibitors that allowed foci formation and to quantify their action, as described in section 2.15.

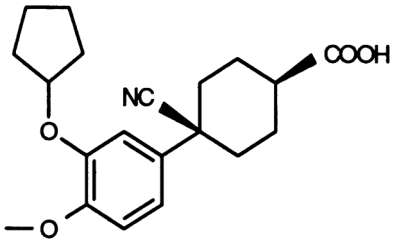
All PDE4 inhibitors that induce foci were then to be analysed by our collaborator, Dr David Adams (Herriot-Watt University, Dept Chemistry) so that their structures can be compared in order to try and identify features that might potentially contribute to foci formation. In so doing we hoped to gain novel insight into an approach that would allow us to design a novel PDE4 inhibitor that is devoid of side effects. This section of appendix presents various compounds that have been studied as a part of structure-activity relationship.



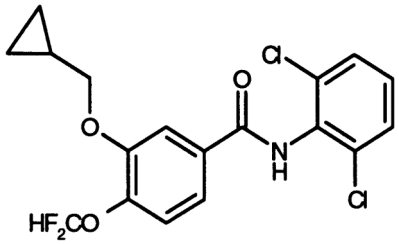
Rolipram



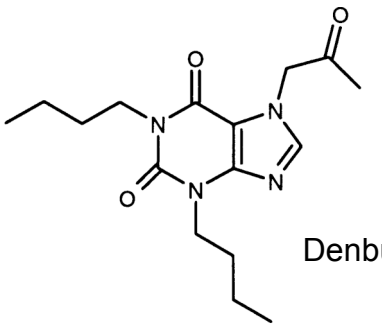
Ro 20-1724



Ariflo (Cilomilast)



Roflumilast



Denbufylline

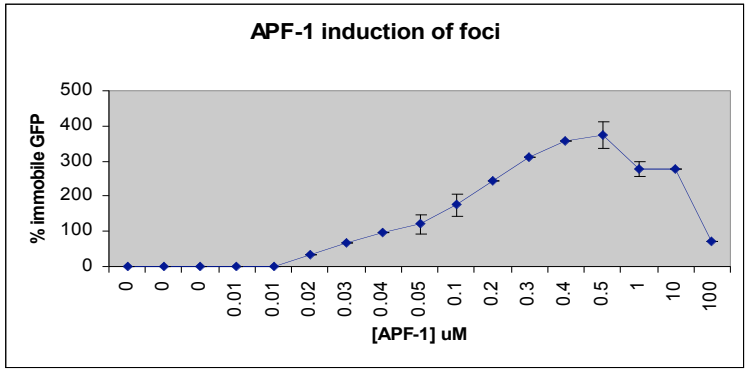
Figure II.I: Chemical structures of various PDE4 inhibitors. Chemical structures taken from Houslay et al; 2005

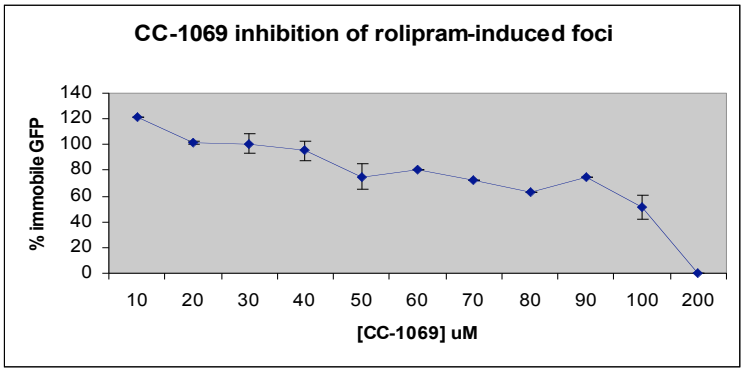
Compound	IC50	Induces foci	Inhibits foci
RS-33793 (10mg/ml)	---	Yes	No
YM-907 (10mg/ml)	---	No	Yes
YM-976 (20mM)	2.2nM	No	Yes
Roflumilast (50mM)	1nM	No	Yes
CDP-40 (50mM)	4nM	No	Yes
ICI-63,197 (1mM)	4.6uM	No	Yes
Denbufylline (50mM)	1uM	No	Yes
Zardaverine (5mM)	2.5uM	No	Yes
Trequensin (1mM)	500nM	No	Yes
Milrinone (50mM)	1uM	No	No
CC-1069 (50mM)	10uM	No	Yes
CC-1003 (50mM)	10uM	No	No
CC-1104 (2.5mM)	2.5uM	No	Yes
CC-1018 (1mM)	5uM	No	Yes

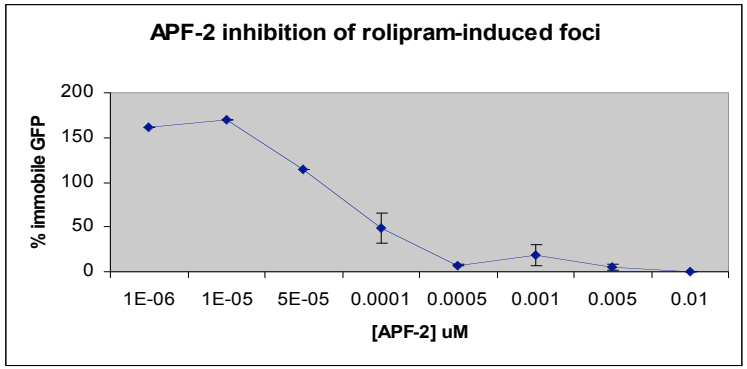
CC-1115 (50mM)	1uM	No	No
CC-9088 (50mM)	10uM	No	Yes
CC-7034 (50mM)	50nM	No	Yes
Enoximone (10mM)	0.25uM	No	Yes
Amrinone (10mM)	5uM	No	Yes
Papverine (10mM)	10uM	No	Yes
CP-80633 (20mg/ml)	1.5uM	No	Yes
AH-8883 (200mM)	8uM	Yes	No
Z-15370A (20mg/ml)	----	No	Yes
CH-4259 (50mm)	1uM	No	Yes
CT-2396 (20mM)	2uM	Yes	No
NCS-613 (100mM)	1uM	No	Yes
ICI-118,233 (20mg/ml)	----	No	No
PMNPQ (100mM)	1uM	Yes	No
Cinpanfylline (100mM)	1uM	Yes	No

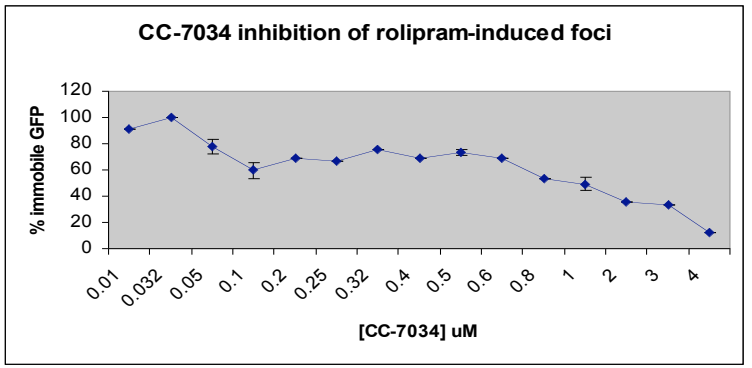
APF-1 (50mM)	5uM	Yes	No
APF-2 (20mM)	2uM	No	Yes
RO-1067146 (4mM)	0.5uM	Yes	No
Pyradizanone I (250mM)	2.0uM	No	Yes
Pyradizanone II (250mM)	2.0uM	No	Yes
RWJ-22867 (50mM)	1uM	No	No
RO-458, 2640 (10mM)	1uM	Yes	No

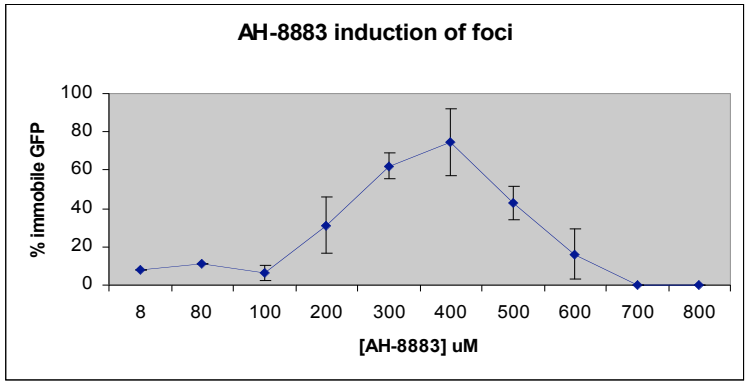
Table II.I: A table showing various PDE4 inhibitors, stock concentrations and their IC/EC_{50} values for foci induction and inhibition as determined in a foci plate reader assay described in section 2.15

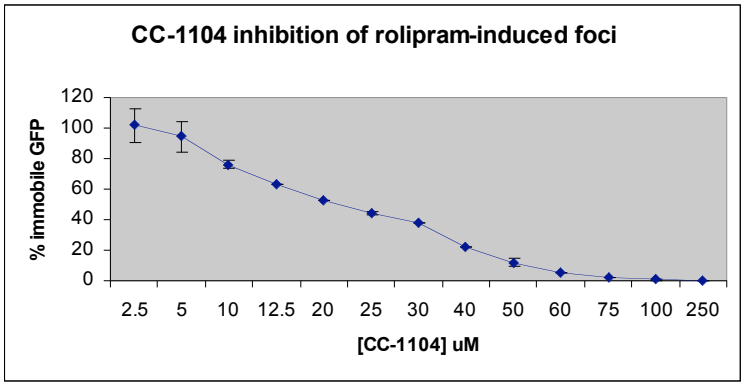

APF-1 induction of foci
% immobile GFP
500
400
300
200
100
0
0 0 0 0.01 0.01 0.02 0.03 0.04 0.05 0.1 0.2 0.3 0.4 0.5 1 10 100
[APF-1] uM

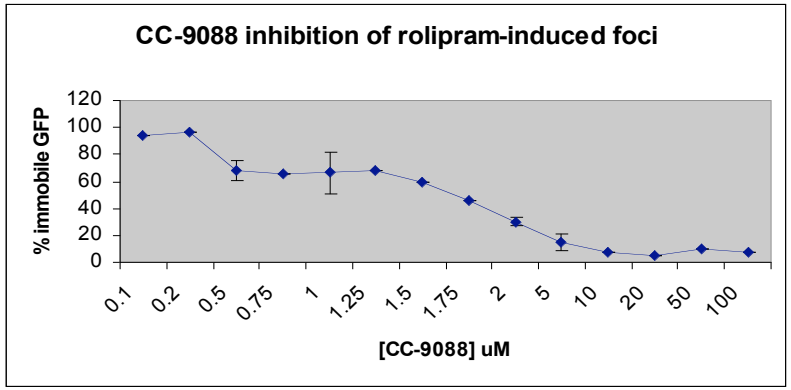

CC-1069 inhibition of rolipram-induced foci
% immobile GFP
140
120
100
80
60
40
20
0
10 20 30 40 50 60 70 80 90 100 200
[CC-1069] uM

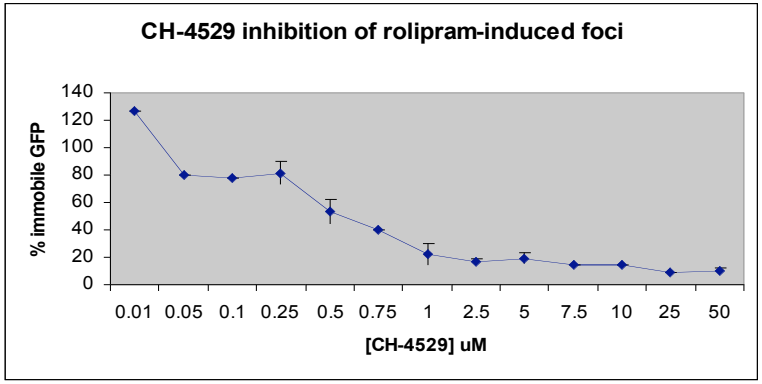

APF-2 inhibition of rolipram-induced foci
% immobile GFP
200
150
100
50
0
1E-06 1E-05 5E-05 0.0001 0.0005 0.001 0.005 0.01
[APF-2] uM

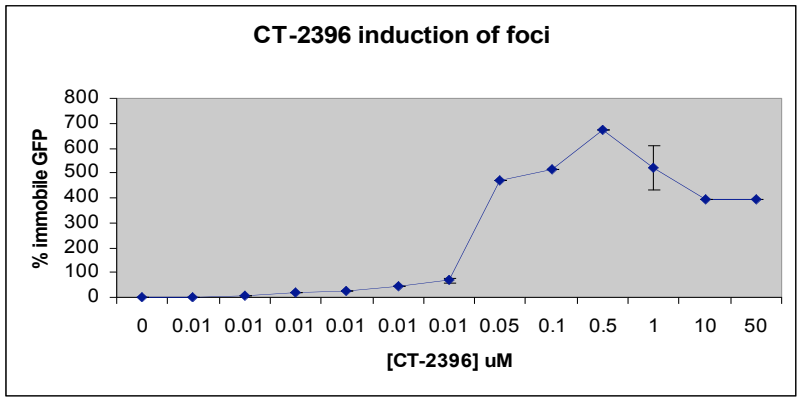

CC-7034 inhibition of rolipram-induced foci
% immobile GFP
120
100
80
60
40
20
0
0.01 0.032 0.05 0.1 0.2 0.25 0.32 0.4 0.5 0.6 0.8 1 2 3 4
[CC-7034] uM

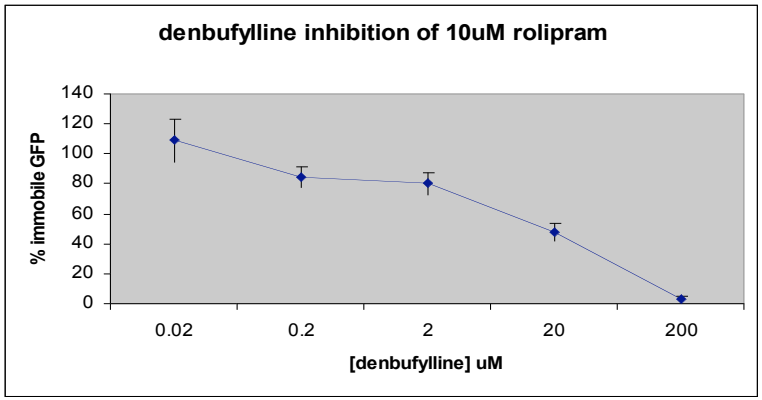

AH-8883 induction of foci
% immobile GFP
100
80
60
40
20
0
8 80 100 200 300 400 500 600 700 800
[AH-8883] uM

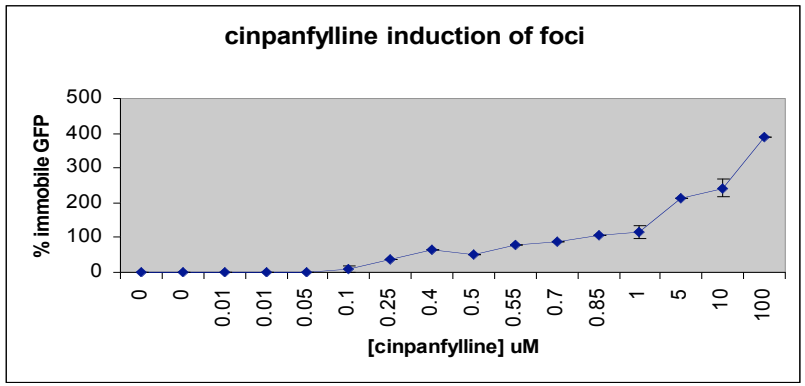

CC-1104 inhibition of rolipram-induced foci
% immobile GFP
120
100
80
60
40
20
0
2.5 5 10 12.5 20 25 30 40 50 60 75 100 250
[CC-1104] uM

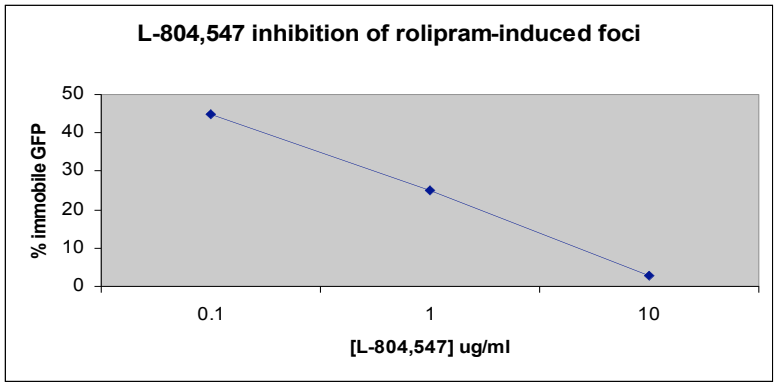

CC-9088 inhibition of rolipram-induced foci
% immobile GFP
120
100
80
60
40
20
0
0.1
0.2
0.5
0.75
1
1.25
1.5
1.75
2
5
10
20
50
100
[CC-9088] uM

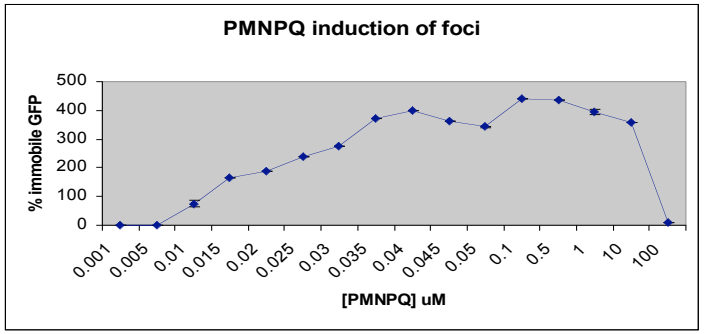

CH-4529 inhibition of rolipram-induced foci
% immobile GFP
140
120
100
80
60
40
20
0
0.01
0.05
0.1
0.25
0.5
0.75
1
2.5
5
7.5
10
25
50
[CH-4529] uM

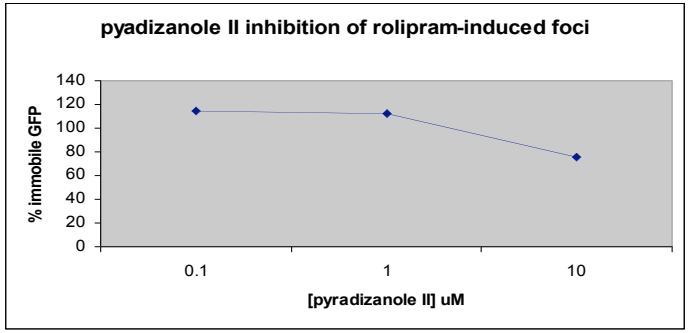

CT-2396 induction of foci
% immobile GFP
800
700
600
500
400
300
200
100
0
0
0.01
0.01
0.01
0.01
0.01
0.01
0.05
0.1
0.5
1
10
50
[CT-2396] uM

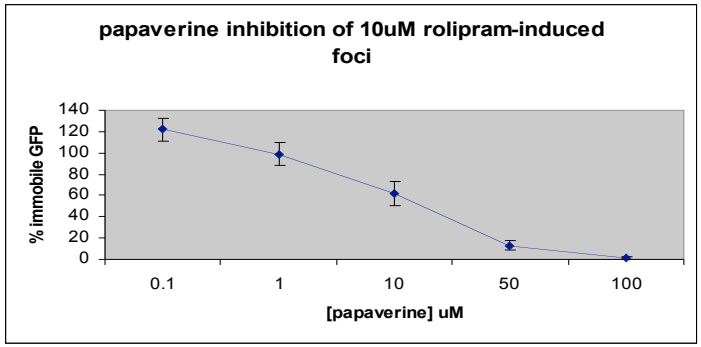

denbufylline inhibition of 10uM rolipram
% immobile GFP
140
120
100
80
60
40
20
0
0.02
0.2
2
20
200
[denbufylline] uM

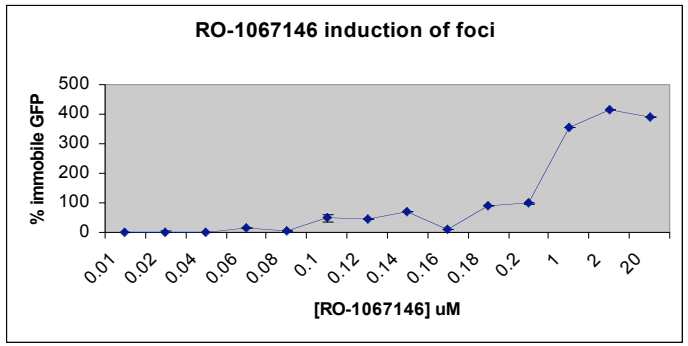

cinpanfylline induction of foci
% immobile GFP
500
400
300
200
100
0
0
0
0.01
0.01
0.05
0.1
0.25
0.4
0.5
0.55
0.7
0.85
1
5
10
100
[cinpanfylline] uM

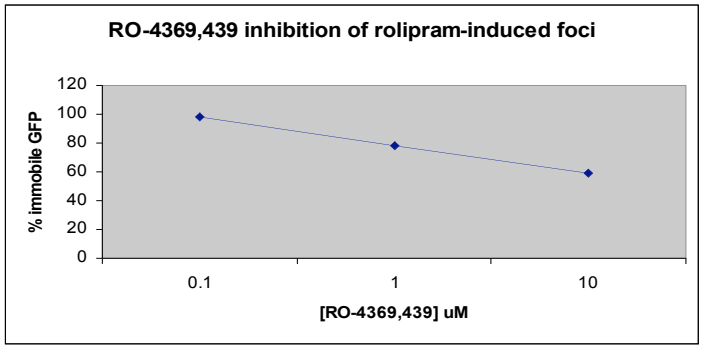

L-804,547 inhibition of rolipram-induced foci
% immobile GFP
50
40
30
20
10
0
0.1
1
10
[L-804,547] ug/ml

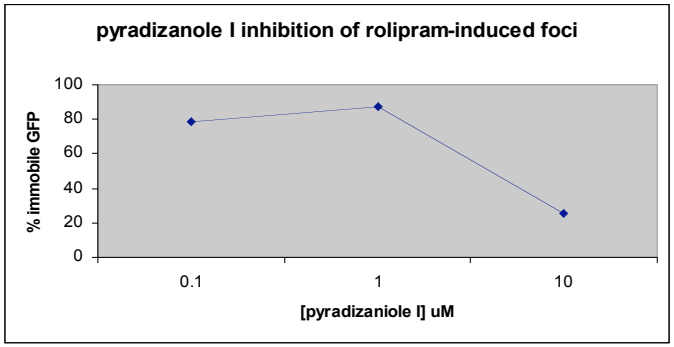

PMNPQ induction of foci
% immobile GFP
500
400
300
200
100
0
0.001
0.005
0.01
0.015
0.02
0.025
0.03
0.035
0.04
0.045
0.05
0.1
0.5
1
10
100
[PMNPQ] uM

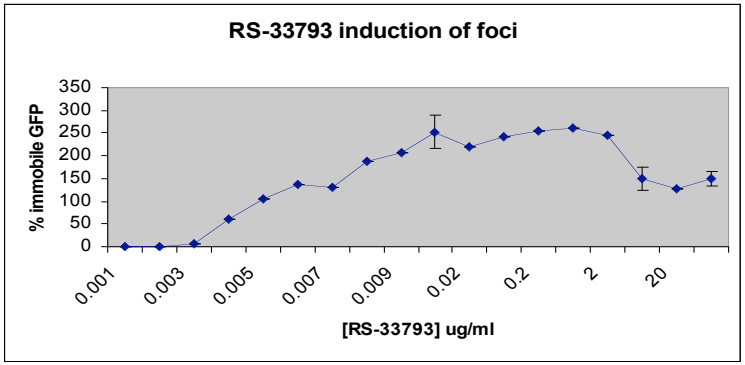

pyadizanole II inhibition of rolipram-induced foci
% immobile GFP
140
120
100
80
60
40
20
0
0.1
1
10
[pyradizanole II] uM

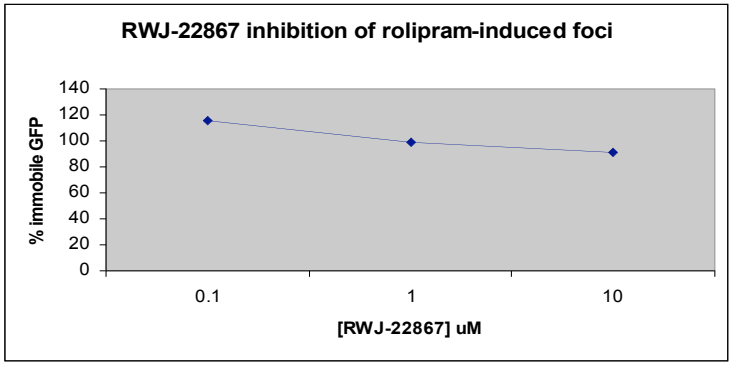

papaverine inhibition of 10uM rolipram-induced foci
% immobile GFP
140
120
100
80
60
40
20
0
0.1
1
10
50
100
[papaverine] uM

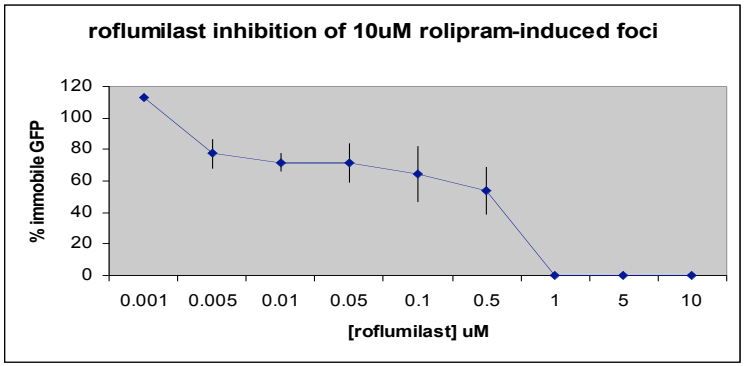

RO-1067146 induction of foci
% immobile GFP
500
400
300
200
100
0
0.01
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0.16
0.18
0.2
1
2
20
[RO-1067146] uM

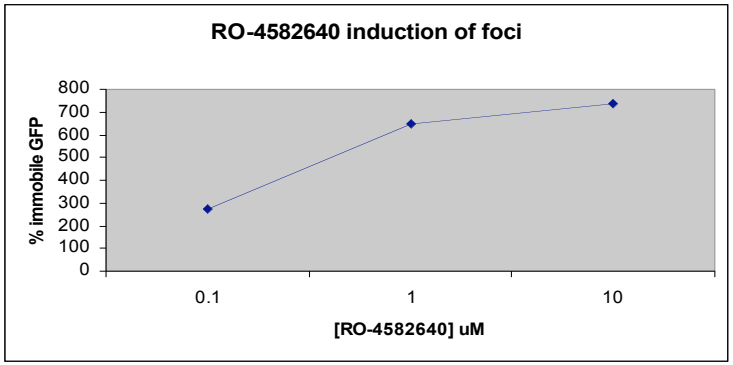

RO-4369,439 inhibition of rolipram-induced foci
% immobile GFP
120
100
80
60
40
20
0
0.1
1
10
[RO-4369,439] uM

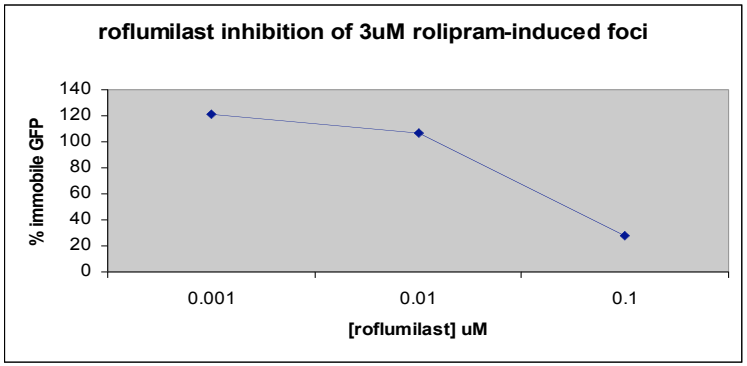

pyradizanole I inhibition of rolipram-induced foci
% immobile GFP
100
80
60
40
20
0
0.1
1
10
[pyradizaniole I] uM


RS-33793 induction of foci
% immobile GFP
350
300
250
200
150
100
50
0
0.001
0.003
0.005
0.007
0.009
0.02
0.2
2
20
[RS-33793] ug/ml


RWJ-22867 inhibition of rolipram-induced foci
% immobile GFP
140
120
100
80
60
40
20
0
0.1
1
10
[RWJ-22867] uM


roflumilast inhibition of 10uM rolipram-induced foci
% immobile GFP
120
100
80
60
40
20
0
0.001
0.005
0.01
0.05
0.1
0.5
1
5
10
[roflumilast] uM


RO-4582640 induction of foci
% immobile GFP
800
700
600
500
400
300
200
100
0
0.1
1
10
[RO-4582640] uM


roflumilast inhibition of 3uM rolipram-induced foci
% immobile GFP
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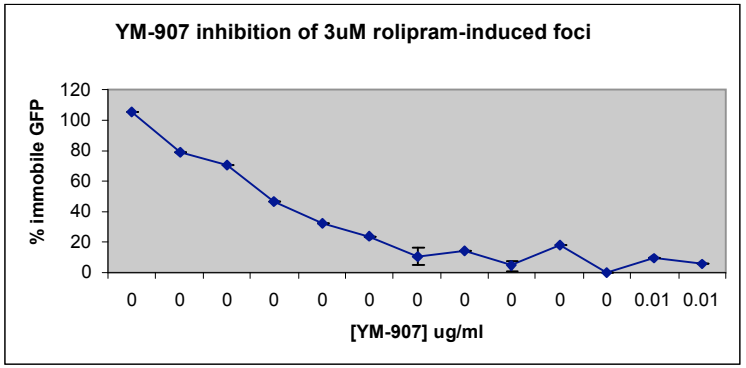
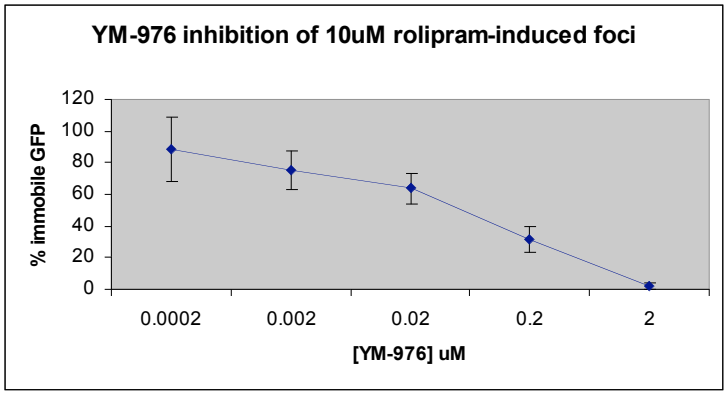
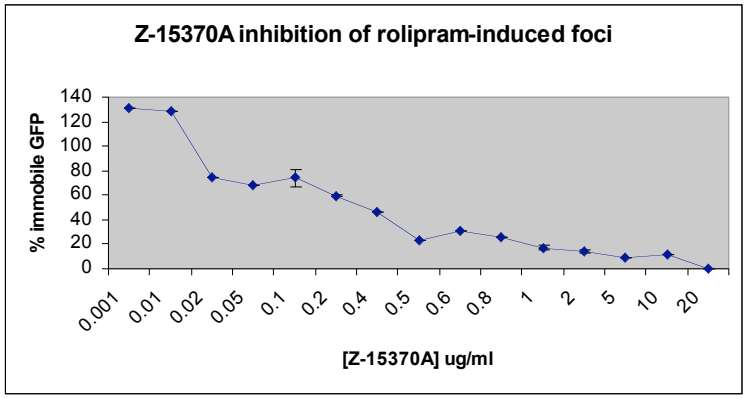
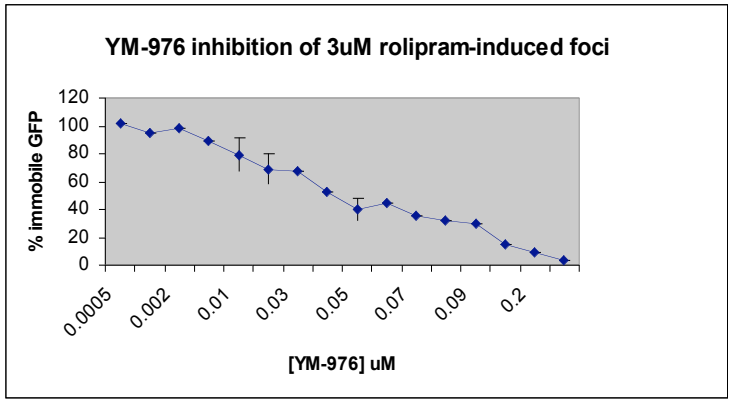
120
100
80
60
40
20
0
0.001
0.01
0.1
[roflumilast] uM


YM-907 inhibition of 3uM rolipram-induced foci
% immobile GFP
120
100
80
60
40
20
0
0
0
0
0
0
0
0
0
0
0
0.01
0.01
[YM-907] ug/ml







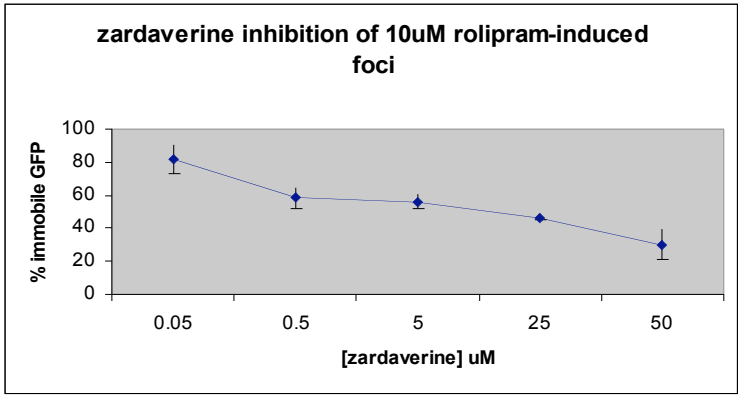


Figure II.II The effect of various PDE4 inhibitors on Rolipram induced foci of PDE4A4

Various PDE4 inhibitors were tested for their affects on rolipram (3uM) induced foci formation of GFP fusion protein of PDE4A4 in CHO cells using a florescent plate reader assay as described in section 2.15

Figure II.III Sequence alignment of human PDE4A4 and PDE4B2.

Sequence alignment was performed using ClustalW (Larkin et al; 2007).

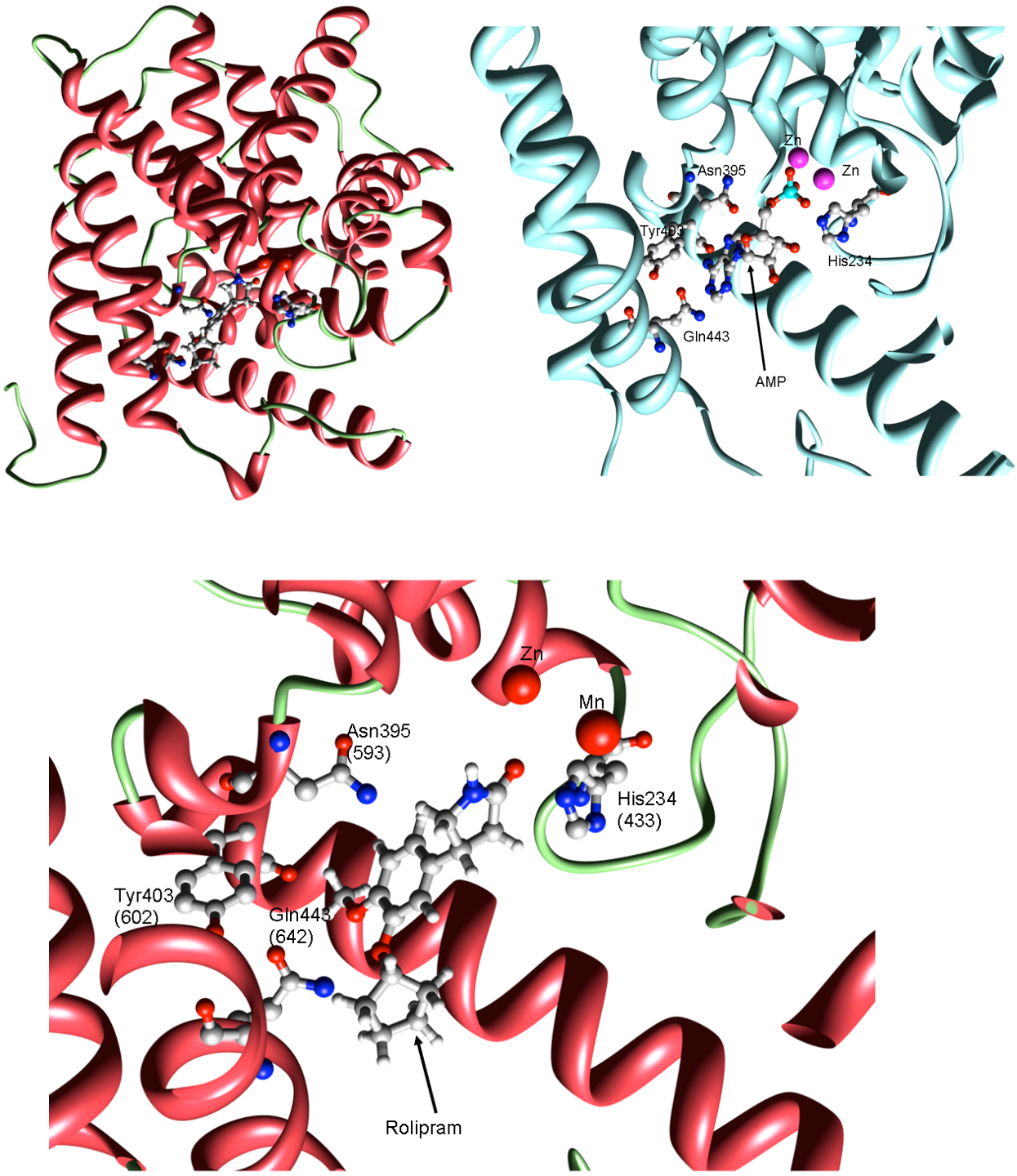
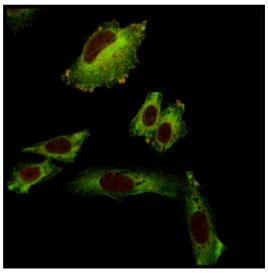


Figure II.IV: Crystal structure of catalytic domain of PDE4B2B

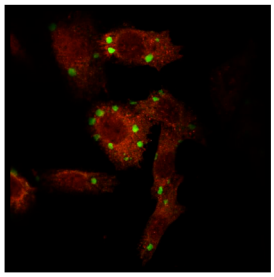
Image showing chain A of the dimeric PDE4B2B with rolipram bound. (1R06.pdb). Xu, R.X., *et al*. (2004) Crystal structures of the catalytic domain of phosphodiesterase 4B complexed with rolipram and AMP and the corresponding amino acids of PDE4A4 are shown in the parentheses. *JMB*. **337**. 355-365. Images were made using Chimera (Petterson et al; 2004).

Ubiquitin

No Rolipram

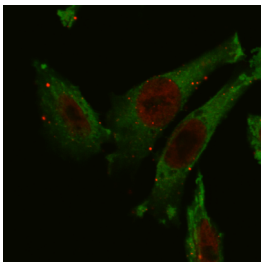


Rolipram

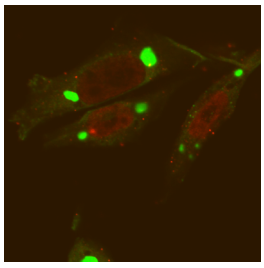


DISC1

No Rolipram

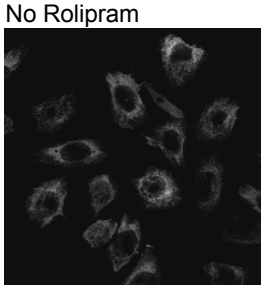


Rolipram

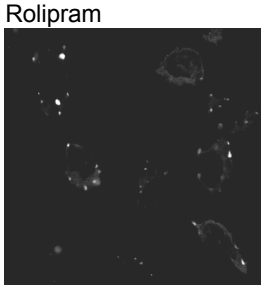


Leptomycin

No Rolipram

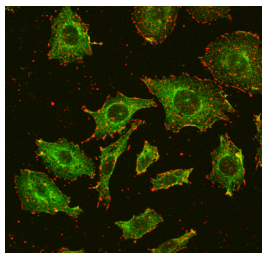


Rolipram

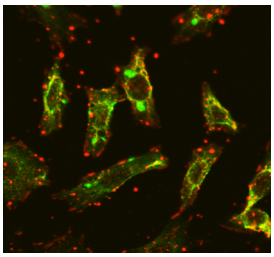


Phospho tyrosine

No Rolipram

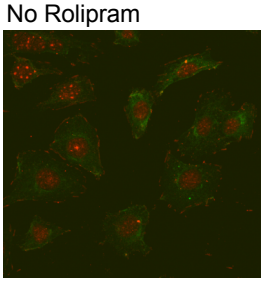


Rolipram

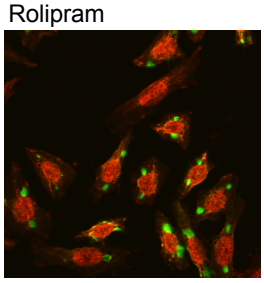


PYK2

No Rolipram

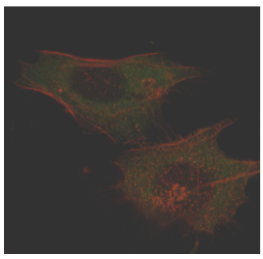


Rolipram

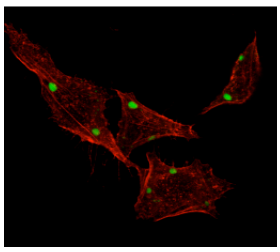


Actin

No Rolipram

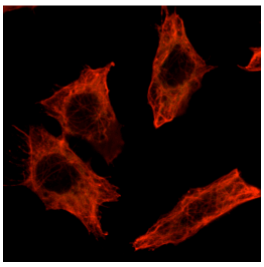


Rolipram

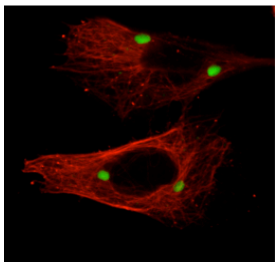


Tubulin

No Rolipram

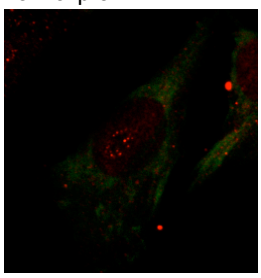


Rolipram

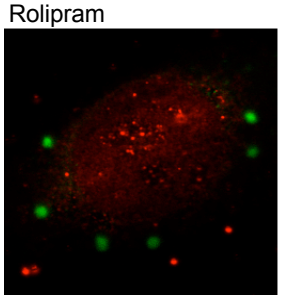


Rab 11

No Rolipram



Rolipram



Rab 9

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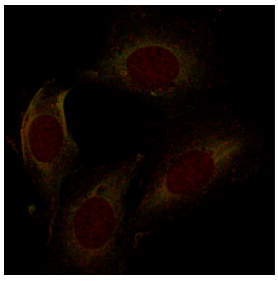
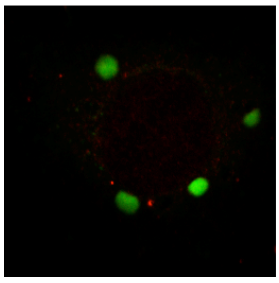
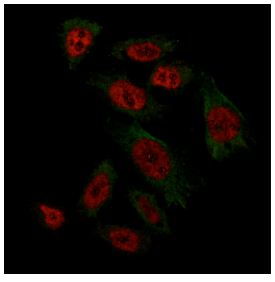


Rolipram

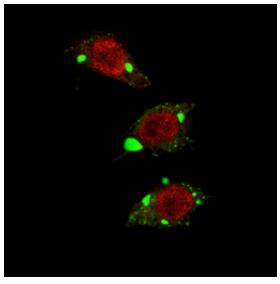


mdm2

No Rolipram



Rolipram



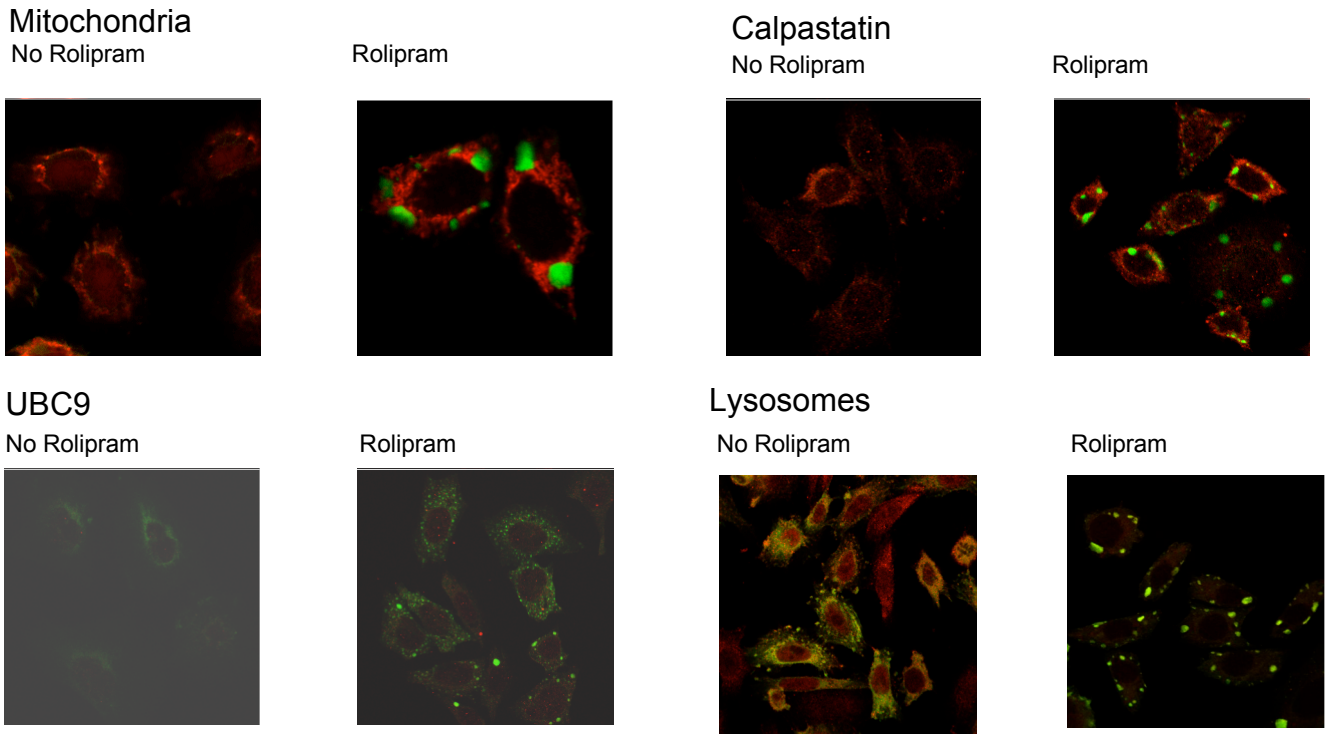


Figure II.V: Immunocytochemical studies of foci interacting proteins

Immunocytochemical analysis of CHO cells stably expressing PDE4A4-GFP treated with and without rolipram (10uM-16hrs) and stained for various proteins and various sub-cellular organelles. All data shown are representatives of the results obtained from three separate experiments.

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