Phosphodiesterase 4 Expression and Proliferation Rates in a Cellular Model of Pulmonary Hypertension

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by

Jennifer Elena Millen

Division of Neuroscience & Biomedical Systems
Institute of Biomedical and Life Sciences
University of Glasgow

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Declaration

I declare that the work described in this thesis has been carried out by myself unless otherwise cited or acknowledged. It is entirely of my own composition and has not, in whole or in part, been submitted for any other degree.

Jennifer E. Millen

October 2004

Abstract

Pulmonary arterial hypertension (PAH) is characterised by increased vascular resistance which leads to pulmonary artery remodelling and increased smooth muscle cell proliferation (Fishman 2004; Humbert et al., 2004). Chronic hypoxia treated rats (to induce PAH) display alterations in cyclic nucleotide signalling pathways. Both cAMP and cGMP levels are reduced (MacLean et al., 1997), with a corresponding increase in PDE3A/B and PDE5A2 in pulmonary arteries of the chronic hypoxic rat (Murray et al., 2002). The objective of this study was to investigate the expression of PDE4 isoforms in the cellular model of PAH as the PDE4 family represents the major cAMP-hydrolysing activity within these cells (Palmer et al., 1998).

It was shown that in human pulmonary artery smooth muscle cells (hPASMC), the PDE4A10, PDE4A11, PDE4B2 and PDE4D5 isoforms all show significant increases in expression after 14 days exposure of chronic hypoxia (10% O_2). The increase in PDE4 expression did not correspond to an increase in PDE4 activity levels however. Levels of cAMP were tripled and PKA activity was doubled after 7 days in 10% O_2. The hypoxia induced increase in cAMP was determined to be ERK dependant as use of the MEK inhibitor, UO126, could reduce hypoxic cAMP levels to that of normoxic cells.

The autocrine loop shown previously in aortic smooth muscle cells (Baillie et al., 2001), was demonstrated to be in effect in hPASMC. In this loop, active ERK led to the production of PGE_2 through PLA_2. This increase in PGE_2 stimulated adenylyl cyclase and increased the generation of cAMP. Using the COX-2 inhibitor, indomethacin, completely ablated the rise in cAMP levels in hypoxia. Exogenous PGE_2 mimicked the hypoxia induced rise in cAMP in normoxic cells. Inhibition of ERK also led to a decrease in PDE4 activity through the reduced level of cAMP generated and thus decreased the amount of PKA able to activate PDE4. This effect was not witnessed in hypoxia, suggesting desensitisation to cAMP stimulation had occurred. Indeed, it was observed that hypoxic cells produced less cAMP in response to cAMP agents such as rolipram or PGE_2 than normoxic cells.

Vascular smooth muscle cells have been reported to proliferate in response to hypoxia. Indeed, it was demonstrated in this study that hPASMC do increase their proliferation in response to hypoxia in both serum starved cells and cells cultured in serum. The PKA RII subunit was demonstrated to be essential for normal proliferation of these cells. Both PKA

RI and PKA RII can significantly reduce proliferation in both normoxic and hypoxic cells. Also, the recently discovered cAMP substrate Epac (de Rooij et al., 1998), elicits anti-proliferative effects on serum induced proliferation in hPASMC.

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Abbreviations

AC	adenylyl cyclase
AKAP	A kinase anchoring protein
ASMC	Airway smooth muscle cell
ATP	adenosine trisphosphate
BMPR	Bone morphogenetic protein receptor
Ca^{2+}/CaM	calcium/calmodulin
cAMP	cyclic 3'5' adenosine monophosphate
CHO	Chinese hamster ovary
CRE	cAMP response element
CREB	cAMP response element binding protein
Cdk	Cyclin dependent kinase
COPD	chronic obstructive pulmonary disorder
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide trisphosphate
DTT	dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Diaminoethanetetra-acetic acid
EGF	epidermal growth factor
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
Epac	Exchange protein directly activated by cAMP
ERK	Extracellular regulated kinase
ET-1	Endothelin-1
FCS	foetal calf serum
GEF	Guanine nucleotide exchange factor
GPCR	G-protein coupled receptor
G-protein	guanine nucleotide binding regulatory protein
GRK	G-protein receptor specific kinase
HIF	hypoxia inducible factor
HRE	hypoxia response element
hr	hour
IBMX	isobutylmethylxanthine

IC_{50}	Concentration of inhibitor required to inhibit half the specific activity
IL	interleukin
IP_3	inositol-1, 4, 5-triphosphate
K_m	Michealis-Menton constant
kb	kilobase
kDa	kiloDalton
KHEM	potassium (K), HEPES, EGTA, Magnesium
l	litre
LB	Luria-Bertoni
LR	linker region
M	molar
mg	milligram
MAP kinase	mitogen activated protein kinase
MEK	MAPK kinase
min	minute
mRNA	messenger RNA
NO	nitric oxide
NOS	nitric oxide synthase
PA	phosphatidic acid
PAEC	pulmonary arterial endothelial cell
PAF	Pulmonary arterial fibroblast
PAGE	Polyacrylamide gel electrophoresis
PAH	pulmonary arterial hypertension
PASMC	Pulmonary arterial smooth muscle cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDE	phosphodiesterase
PDGF	Platelet derived growth factor
PGE_2	prostaglandin 2
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PLA_2	phospholipase A_2
PMA	Phorbol myristate
PPH	primary pulmonary hypertension

RACK	receptor for activated C kinase
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcription
SAPU	Scottish antibody production unit
SDS	sodium dodecyl sulphate
sec	second
SH3 domain	Src homology 3 domain
SmBm	smooth muscle basal medium
SmGm	smooth muscle growth medium
TBE	tris/borate/EDTA
TBS	tris buffered saline
TEMED	N,N,N',N'-Tetramethyl-ethylenediamine
TFA	Trifluoroaceatic acid
TGF	Transforming growth factor
VEGF	vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
5-HT	serotonin

Chapter 1

General Introduction

1.1 Cyclic nucleotide signalling pathways

1.1.1 Cyclic nucleotides

The cyclic nucleotides, cyclic 3'5' adenosine monophosphate (cAMP) and cyclic 3'5'-guanosine monophosphate (cGMP) are intracellular second messengers involved in a myriad of cellular events including metabolism, cell growth and proliferation, gene regulation and vascular tone (Pellegrino & Wang 1997; Dousa 1999). cAMP was discovered in 1958 (Sutherland & Rall 1958) and the second messenger concept was proposed whereby binding of hormones or neurotransmitters, so called 'first messengers', to cell-surface receptors elicited a signal which is transduced via an intracellular mediator (second messenger) such as cAMP.

Adenylyl cyclase catalyses the synthesis of cAMP from ATP and cAMP-phosphodiesterases (PDE), which are the only entities able to degrade cAMP, hydrolyse cAMP to the inactive form, 5'AMP. The cognate synthesis and degradation of cGMP exists with guanylyl cyclase providing the synthetic activity of cGMP and cGMP-PDEs being responsible for the degradation and inactivation of cGMP.

The well-characterised target for cAMP action within a cell is the cAMP-dependant kinase, protein kinase A (PKA). Stimulation of PKA results in the phosphorylation of a number of downstream targets leading to alterations in gene expression and cellular processes (Cho-Chung et al., 1995; Shabb 2001). Similarly, cGMP mediates the majority of its effects through cGMP dependant protein kinase (Lohmann et al., 1997). Mammalian cGMP kinase exists in two isoforms, cGMP kinase I and cGMP kinase II which show cell specific distribution and different regulatory roles (Ruth 1999).

1.1.1.1 G-Protein Coupled Receptors

Adenylyl cyclase can be activated directly by forskolin or indirectly through interaction with an activated G-protein coupled receptor (GPCR). These receptors are seven transmembrane spanning proteins that form an α–helix with an extracellular N-terminus, three extracellular loops, three intracellular loops and an intracellular C-terminal tail. The superfamily of GPCRs include receptors for many different ligands and are therefore able to respond to numerous extracellular stimuli including taste, light, odour, neurotransmitters and hormones. GPCRs are responsible for the transduction of these signals into intracellular second messengers able to carry the message inside cells, thus achieving the correct response. Upon ligand binding, the GPCR undergoes a conformational change

allowing interaction and activation of a heterotrimeric guanine nucleotide binding protein (G-protein). This conformational change allows the exchange of GDP for GTP on the G-protein α-subunit which activates the G-protein and promotes the dissociation of the Gα- and $G_{\beta\gamma}$- subunits of the G-protein. The dissociated subunits are then free to act upon their effector protein, e.g. adenylyl cyclase, in a positive or negative fashion. The cAMP signalling pathway is activated by one of the $G\alpha_s$ isoforms and can be inhibited by the $G\alpha_i$ isoforms. Activation of adenylyl cyclase is sustained until the GTP bound to the $G\alpha_s$ is hydrolysed to GDP and the G-protein subunits reassociate.

GPCR signalling is regulated by mechanisms at the level of the ligand, the receptor, the G-protein and at numerous stages in the signalling pathway. The earliest method of regulation is removal of the agonist from the extracellular fluid by transporters or through degradation. The receptor itself is regulated by feedback mechanisms initiated by activation (reviewed in Bohm et al., 1997, Ferguson 2001). This receptor desensitisation is caused by the uncoupling of the G-protein from the receptor in response and can occur within seconds of activation. The most immediate cause of desensitisation is phosphorylation of the receptor by intracellular serine/threonine kinases (GRKs), which phosphorylate the intracellular loop and C-terminal tail of the GPCR. The G- protein receptor kinases (GRKs) selectively phosphorylate agonist occupied receptors promoting the recruitment of cytosolic binding proteins known as arrestins, which sterically uncouple the receptor from the G-protein (reviewed in Luttrell & Lefkowitz 2002). Arrestin binding is also thought to aid receptor endocytosis. Second messenger dependant kinases such as PKA or PKC can phosphorylate various GPCRs including those without agonist bound, whereupon they also serve to inhibit the interaction between the G-protein and receptor.

A growing number of proteins have been identified (Bohm et al., 1997) that both bind GPCRs and couple GPCRs to G-protein independent signal transduction pathways or affect G-protein specificity and agonist selectivity. These include GRKs, arrestins and A-kinase anchoring proteins (AKAPS).

1.1.1.2 Adenylyl Cyclases

Adenylyl cyclases (ACs) are encoded by multiple genes and are transmembrane proteins. Nine different isoforms have been cloned to date which show tissue-specific expression and differential regulation. AC isoforms from all families are highly expressed in brain tissue, although individual isoforms show distinct patterns of localisation within the brain. AC isoform expression in tissues outside of the brain shows a highly specialised

distribution pattern (Antoni et al., 2000, Hanoune & Defer 2001; summarised in table 1.1). For example, AC2, 3, 5-8 are all expressed in rat pulmonary vascular tissue with AC2, 5 and 8 being of functional importance (Jourdan et al., 2001).

Each AC isoform consists of two hydrophobic domains composed of six transmembrane helices (M1 and M2) and two cytoplasmic domains (C1 and C2) which contain the catalytic core. C1 and C2 share homology between different isoforms whereas the helices do not (Hanoune et al., 1997, Houslay & Milligan 1997).

All AC isoforms are regulated by G-protein subunits with type-specific effects. Group1 isoforms (AC1, 3, 8) are stimulated by calcium and calmodulin. Group2 (AC2, 4, 7) are activated by βγ subunit and protein kinase C (PKC) phosphorylation. Group 3 (AC5, 6) are inhibited by low concentrations of calcium and Group 4 (AC9) is insensitive to calcium or the βγ subunit. All isoforms are activated by the $G\alpha_s$ subunit of the G protein (reviewed in Hanoune & Defer 2001, Cooper 2003; for a summary of AC regulation, see table 1.2). Even within these general subclasses, the cyclases are so different to one another that they have other regulators that act upon each of them in a specific fashion. For example, Ca^{2+}/calmodulin can regulate AC in a positive (AC1 and AC8) or negative (AC3, 5 and 6) manner. PKA phosphorylation has also been shown to reduce AC activity by direct phosphorylation (Iwami et al., 1995). PKA phosphorylation affects AC5 and AC6 and causes a 'switching' of receptor coupling from Gαs to Gαi which exerts an inhibitory effect on AC activity and reduces cAMP synthesis. The diterpene, forskolin, can directly activate all AC isoforms with the exception of AC9.

1.1.2 Cellular Targets of cAMP

The range of action of cAMP is extremely wide with effects reaching cell differentiation, ion channel conductivity and gene transcription amongst others. Targets of cAMP action are cyclic nucleotide gated channels (Kraus-Friedmann 2000) and the Rap-GEFs Epac1 and Epac 2 (exchange protein directly activated by cAMP). The first identified target of cAMP within cells was the cAMP dependant protein kinase A, PKA.

1.1.2.1 Protein Kinase A

The classically known target of cAMP within cells is the cAMP dependant kinase, PKA. PKA is a heterodimeric enzyme consisting of two regulatory (R) and two catalytic (C) subunits. cAMP binds to the R subunit of PKA inducing conformational changes that dissociate the enzyme into an R subunit dimer with four molecules of cAMP bound and

two, free, active C subunits able to phosphorylate target serine/threonine residues (reviewed in Skalhegg & Tasken 2000, PKA targets reviewed in Shabb 2001).

There are two types of PKA which differ in their regulatory subunits, RI or RII, which interact with an identical C subunit. As with AC, PKA exists in several isoforms. Four isoforms of the R subunit have been identified; RIα, RIβ, RIIα and RIIβ, with several splice variants of RIα reported. The RI isoforms share high homology with each other, are ubiquitously expressed and are activated transiently by low cAMP levels. The RII isoforms also share a high homology with each other, are predominantly expressed in brain, fat, endocrine and reproductive tissues and require persistent levels of high cAMP stimulation for activation (Feliciello et al., 2001). The C subunit also exists in multiple forms; Cα, Cβ and Cγ, with splice variants of both Cα and Cβ reported (Uhler et al., 1986; Showers et al., 1986). A large number of PKA holoenzymes therefore exist with different biochemical properties (Skalhegg & Tasken 2000). Indeed it is known that the PKAI complex ($RI\alpha_2C_2$ or $RI\beta_2C_2$) is generally cytoplasmic whereas the PKAII complex is usually membrane-associated (Cho-Chung et al., 1995, Houslay & Milligan 1997, Skalhegg & Tasken 2000; Murphy & Scott 1998). PKAII is associated to membranes through tethering of the R subunit to A-kinase anchoring proteins (AKAPs) which are found throughout the cell tethered to various subcellular components, thus providing localisation of the PKA activity. PKAII is also known to be subject to autophosphorylation by the C subunit (Skalhegg & Tasken 2000, Shabb 2001; reviewed in Michel & Scott 2002).

1.1.2.2 A-kinase anchoring proteins

PKAII exists associated with a variety of subcellular structures including the plasma membrane, endoplasmic reticulum, microtubules, Golgi and nucleus (summarised in Murphy & Scott 1998, Feliciello et al., 2001). This association exists through an interaction of PKARII with an anchoring protein tethered to the membrane. These AKAPs bind an N-terminal region of the RII protein, at its dimerisation (RII: RII) interface, and contain a unique targeting domain responsible for localising the AKAP/PKAII to a specific subcellular structure (Murphy & Scott 1998, Michel & Scott 2002). A new group of AKAPs has also been identified that can bind both RII and RI (Huang et al., 1997). This anchoring of PKA provides tight regulation of cAMP levels in specific cellular locations. In addition, some AKAPs also act as scaffolding proteins by associating with other kinases and phosphatases. This is true in the case of AKAP79 which scaffolds PKA, PKC and the calcium/calmodulin dependant phosphatase PP2B to regions near the plasma membrane (Dodge & Scott 2000).

1.1.2.3 Exchange Protein Directly Activated by cAMP

PKA-independent actions of cAMP involve an exchange protein that is directly activated by cAMP, Epac (de Rooij et al., 1998). Epac1 and Epac2 are guanine exchange factors for the small Ras like proteins, Rap1 and Rap2 (de Rooij et al., 2000). A third Epac family member, Repac, lacks the regulatory sequences of Epac 1 and 2 but is still able to activate Rap1 and 2 (de Rooij et al., 2000). Epac contains a cAMP binding domain (Epac 2 has two cAMP binding domains (Rehrmann et al., 2002)), a Dishevelled, Egl-10, Pleckstrin (DEP) domain shown to be responsible for membrane targeting of Epac (de Rooij et al., 2000), a Ras-exchanger motif, a Ras-association domain and a guanine nucleotide exchange factor (GEF) domain (Bos 2003). Epac 1 is ubiquitously expressed and displays a cell-cycle dependant localisation. At interphase, Epac1 has been shown to be located at both the mitochondria and nuclear membrane and translocates to the mitotic spindle and centrosomes during metaphase (Qiao et al., 2002). This translocation is not mediated by cAMP as Epac is membrane bound in the presence and absence of cAMP. Epac 2 is predominantly expressed in the brain and adrenal gland.

The catalytic domain of Epac is regulated by a direct interaction between the cAMP binding domain and the GEF domain which acts in an inhibitory fashion. Upon binding cAMP, this inhibition is released by a conformational change in the protein which exposes the GEF domain and allows Epac to activate downstream pathways (de Rooij et al., 2000, Rehrmann et al., 2003).

It has been demonstrated that cAMP activation of Rap1 is through Epac, whereas the cAMP induced inhibition of Raf1 is mediated by PKA (Enserink et al., 2002). Raf1 activates MEK which in turn activates ERK. cAMP has been demonstrated to have both stimulatory and inhibitory effects on the ERK pathway, but it is thought this occurs through PKA activation of Raf1 through Ras and the direct inhibitory effect of PKA on Raf1. As such, the roles of Rap1 only in cell signalling have been attributed to Epac. Epac activation of Rap1 plays a major role in cell adhesion by regulating integrin signalling (Rangarajan et al., 2003). Epac2 has also been reported to be involved in insulin secretion in β-cells (Holz 2004).

1.2 The Phosphodiesterase Superfamily

Hydrolysis of cyclic nucleotides is the only method of inactivation in cells. Cyclic nucleotide hydrolysis is mediated by a large superfamily of phosphodiesterase enzymes

(PDEs). PDEs catalyse the hydrolysis of cAMP and cGMP at their 3'-phosphoester bond to form the corresponding inactive 5' nucleoside monophosphate (5' AMP and 5' GMP). There are currently eleven known families of PDEs with differential tissue distribution, regulatory properties and sensitivity to inhibitors (summarised in table 1.3; reviewed in Francis et al., 1999; Dousa 1999; Soderling & Beavo 2000; Yuasa et al., 2000; Conti 2000; Mehats et al., 2002; Maurice et al., 2003). Each PDE family is encoded for by one to four genes, with multiple protein products arising from alternative splicing and multiple promoters resulting in over 50 mammalian PDE isoforms. Each isoform is expressed in a tissue and cell-specific manner.

All PDEs share the same modular structure, with three functional domains; a conserved catalytic domain of ~270 amino acids near the C-terminus, regulatory regions located at the N-terminus and the C-terminus (*Figure 1.1*). The catalytic domain contains sequences essential for cyclic nucleotide hydrolysis including the common PDE motif; $HD(X_2)\ H(X_4)\ N$ which contains consensus metal binding domains (Zn^{2+} and Mg^{2+}). The N-termini of PDEs are highly divergent and are conserved within a family, showing the N-terminus contains each family's specific functional motifs. Regions in this domain include regulatory sequences such as phosphorylation domains and targeting regions. It has also been proposed that the N-terminal region of a PDE contains regions for dimerisation.

PDE inhibitors have been reported as being clinically useful in many disorders (table 1.4 for summary of diseases PDEs are involved in; Torphy & Page 2000). These include disease states such as depression, asthma, pulmonary arterial hypertension (PAH) and erectile dysfunction. Family specific PDE inhibitors have shown promising results in clinical trials and as such, many are attractive targets for drug development (Schmidt et al., 1999; Torphy 1998; Burnouf & Pruniax 2002; Spina 2003; Michelakis et al., 2003; Eddahibi et al., 1998).

1.2.1 The PDE1 Family

The PDE1 family is encoded for by three genes; *PDE1A*, *PDE1B* and *PDE1C* with splice variants of each, and is known to hydrolyse both cAMP and cGMP (reviewed in Sonnenburg et al., 1998; Kakkar et al., 1999). PDE1A and 1B isoforms have a higher affinity for cGMP than cAMP and PDE1C hydrolyses both with equal efficiency. PDE1 is referred to as the calcium/calmodulin (Ca^{2+}/CaM) PDE due to the presence of Ca^{2+}/CaM binding sites within the PDE1 N-terminal region. Activity of the PDE1 family can be regulated by both Ca^{2+}/CaM binding and phosphorylation/dephosphorylation by PKA and

CaM-dependant kinase II. Activation of PDE1 enzymes occurs upon Ca^{2+}/CaM binding and thus reduces cAMP. As PDE1A is inhibited by PKA phosphorylation, PDE1 provides a "cross-talk" between calcium and cAMP. PDE1B is inhibited by CaM-dependant kinase II phosphorylation. Both types of phosphorylation reduce the affinity of PDE1 for CaM. It has also been reported that protein kinase C can induce PDE1B. PDE1C has been demonstrated to be essential for human smooth muscle cell (SMC) proliferation (Rybalkin et al., 2002). PDE1C is absent from quiescent arterial SMC and human aorta, but is induced in proliferating arterial SMC. Inhibition of PDE1 by vinpocetine reduces SMC proliferation (Rybalkin et al., 2002).

1.2.2 The PDE2 Family

The PDE2 family are known as the cGMP-stimulated PDEs and hydrolyse both cAMP and cGMP with similar V_{max}, although the affinity for cGMP is two-fold greater. There are three PDE2 isoforms arising from one gene, each containing two specific, noncatalytic cGMP binding domains in their N-terminus. Binding of either cyclic nucleotide induces a conformational change in the PDE2 enzyme, converting it to a more active form. As such, PDE2 provides a means of crosstalk between the two cyclic nucleotide signalling pathways.

PDE2 is predominantly expressed in the adrenal cortex (PDE2A1) and brain and cardiac tissue (PDE2A2). PDE2A3 contains a putative membrane targeting domain. PDE2 activity has been shown to have a role in Ca^{2+} channel regulation, olfactory signalling pathways and aldosterone secretion. Low levels of PDE2 have been detected in vascular smooth muscle cells, and it has been reported that PDE2 activity is involved in hypoxic pulmonary vasoconstriction yet a distinct function has not yet been identified (Haynes et al., 1996). The commonly used inhibitor of PDE2 is *erythro*-9-(2-hydroxyl-3-nonyl) adenine, EHNA (de Jong et al., 1992).

1.2.3 The PDE3 Family

The PDE3 family also hydrolyses both cAMP and cGMP with high affinity (K_m values range from 0.1 to 0.8μM) but with a 2-10 fold higher V_{max} for cAMP (reviewed in Degerman et al., 1997). cGMP in micromolar concentrations binds to the catalytic site of PDE3 and competes with cAMP hydrolysis, hence the PDE3 family being dubbed the cGMP-inhibited PDEs. As the V_{max} for cGMP is so low, cGMP hydrolysis is negligible and PDE3 behaves as a cAMP-specific PDE. PDE3A and PDE3B are also activated by PKA or PKB phosphorylation.

PDE3 is encoded for by two genes; *PDE3A* and *PDE3B* with a higher similarity of PDE3A between human and rat than between human PDE3A and B. This is also true for PDE3B. Alternative start codons give rise to three PDE3A isoforms. PDE3 is widely expressed. In tissues expressing both PDE3A and PDE3B, PDE3A levels are usually dominant. PDE3A is abundant in the cardiovascular system, including vascular smooth muscle, and PDE3B is found predominantly in adipose tissue, hepatocytes and the renal collecting duct epithelium. PDE3 is found in both cytosolic and particulate fractions of cells (Liu & Maurice et al., 1998; Palmer & Maurice 2000). PDE3 has been implicated in a variety of roles including myocardial contractility, smooth muscle relaxation and proliferation of T-lymphocytes and vascular smooth muscle cells. In addition, PDE3B plays a key role in insulin signalling in adipocytes. In response to insulin, PDE3B is phosphorylated and activated by Akt (Kasuga et al., 1999).

In vascular smooth muscle cells, both PDE3A and PDE3B are expressed. Sustained exposure to cAMP elevating agents in vivo increased both PDE3A2 and PDE3B in rat aortic quiescent SMC (Tilley & Maurice 2002). In cultured rat and human aortic SMC, prolonged cAMP elevation increases only PDE3B (Palmer & Maurice 2000). The reasons behind this difference are unclear. PDE3 inhibitors such as cilostamide or milrinone have also been demonstrated to relax arterial tissues and inhibit vascular SMC proliferation (Lindgren et al., 1989; Inoue et al., 2000).

1.2.4 The PDE4 Family

The PDE4 family of enzymes are the most diverse and extensively studied of all PDE families (reviewed in Muller et al., 1996; Salanova et al., 1998; Houslay 2001; Houslay & Adams 2003; for further details, see section 1.3). Four genes encode for PDE4; *PDE4A*, *PDE4B*, *PDE4C* and *PDE4D* which are located on three chromosomes. Alternative mRNA splicing and multiple promoters have led to the discovery of over 20 different PDE4 mammalian isozymes. PDE4 isoforms share 80% sequence identify of their catalytic domain with unique regions shared among PDE4 isoforms of a subfamily. Splice variants differ in their N-terminal region which confers regulatory properties and capability for interactions with other cell structures. PDE4 isoforms are characterised by the presence of two conserved domains within their N-terminus known as Upstream Conserved Region 1 (UCR1) and Upstream Conserved Region 2 (UCR2). PDE4 isoforms can exist as a "long form" with both UCR 1 and UCR2 intact, a "short form" with only UCR2, and "super short forms" with a truncated UCR2 only (*see figure 1.2).* PDE4 is expressed in almost all

cells, with the exception of blood platelets, and displays differential subcellular localisation between isoforms.

Regulation of PDE4 activity can occur through phosphorylation by PKA (Sette & Conti 1996; Ekholm et al., 1997; Hoffmann et al., 1998; Liu & Maurice 1999; MacKenzie et al., 2002) where PKA phosphorylation at Ser^{54} of PDE4D3 and the equivalent residue in the UCR1 of other long forms induces activation of these enzymes. This provides a feedback mechanism for regulating the levels of cAMP.

The p42 MAP kinase, ERK2 also phosphorylates PDE4 isoforms resulting in activation of short forms and inhibition of long forms (Hoffmann et al., 1999; MacKenzie et al., 2000). This is with exception of PDE4A members, which do not act as a substrate for ERK2 (Baillie et al., 2000).

PDE4 is a cAMP-specific enzyme, with a K_m of 1-3μM for cAMP. It is insensitive to cGMP and Ca^{2+}/CaM and is specifically inhibited by Rolipram and Ro 20-1724. PDE4 inhibitors are of great interest due to the role PDE4 activity has been shown to play in disease states including asthma, chronic obstructive pulmonary disease (COPD) and depression (reviewed in Spina et al., 1998, Spina 2003 and Burnouf & Pruniaux 2002).

1.2.5 The PDE5 Family

PDE5 enzymes are known as the cGMP binding, cGMP specific PDEs and are characterised by the presence of two cGMP binding domains located N-terminally to the catalytic unit. PDE5 is encoded for by a single gene that gives rise to four splice variants; PDE5A1, PDE5A2, PDE5A3 and PDE5A4, which have differential tissue distribution but share identical catalytic domains. PDE5 is expressed in several tissues including vascular smooth muscle cells, heart and lung (Loughney et al., 1998; Giordano 2001).

Binding of cGMP to both PDE5 cGMP binding domains does not affect PDE5 activity, but does induce a conformational change that allows phosphorylation by PKG, the cGMP dependant kinase, and PKA at Ser^{92} (Thomas et al., 1990; Corbin & Frances 1999). Phosphorylation leads to an increase in PDE5 activity. PDE5 is also activated by Zn^{2+} ions which are essential for catalysis. Specific inhibitors for PDE5 include DMPPO; a compound currently under investigation in models of hypoxia induced pulmonary hypertension due to its vasodilatory properties (Eddahibi et al., 1998); and Sildenafil, a treatment for male erectile dysfunction that has also been shown to reduce the hypoxic

increase in pulmonary arterial pressure (PAP) in models of hypoxia induced pulmonary hypertension (Zhao et al., 2001; Michelakis et al., 2003). PDE5 levels and activity have been reported to increase in hypoxia induced models of pulmonary hypertension and the selective dilation of the pulmonary vasculature by Sildenafil provides a new target for treatment of pulmonary hypertension (Murray et al., 2002).

1.2.6 The PDE6 Family

The PDE6 family are expressed in retinal rods and cones and play a key role in the visual transduction cascade. Due to this, PDE6 are more often referred to as the photoreceptor PDE family. PDE6 is highly specific for cGMP and is the only known family of PDE to be regulated by G-proteins. They exist as hetero- or homotetramers comprised of catalytic subunits (α, α', β) and inhibitory subunits (γ and δ). The two types of photoreceptor cells, rod and cone, express different forms of PDEγ. The visual cascade is initiated by photons that interact with rhodopsin, resulting in the activation of the G-protein transducin (Tαβγ). Active transducin (Tα-GTP) dissociates from Tβγ and binds to the inhibitory γ subunits of membrane bound PDE6. This relieves inhibition of PDE6 and decreases cGMP, closing the cGMP-gated channel and resulting in hyperpolarisation of the membrane.

Rod PDEγ is also expressed in lung, kidney, heart, pulmonary smooth muscle and airways. In these systems, Rod PDEγ is phosphorylated by the G-protein receptor kinase (GRK) 2 which regulates the epidermal growth factor (EGF) stimulation of ERK1/2 in human embryonic kidney 293 cells (HEK 293; Wan et al., 2001) This regulation also involves the Src kinase which exists in a complex with PDEγ and upon stimulation with EGF, GRK2 associates with this complex. Src inhibitors block activation of ERK1/2 by EGF as does mutation of the Thr^{62} residue of PDEγ (Wan et al., 2003).

1.2.7 The PDE7 Family

The PDE7 family are high affinity cAMP PDEs, similar to that of PDE4, but are rolipram insensitive (Michaeli et al., 1993). The PDE7 family are comprised of two genes and three individual PDE7 isoforms have been identified, PDE7A1, PDE7A2 and PDE7B1. PDE7A1 is expressed in the immune system, PDE7A2 is highly expressed in skeletal muscle and heart and PDE7B is abundant in the pancreas, brain, heart and skeletal muscle (Hetman et al., 1999). PDE7A was initially suggested to play a role in T-cell activation and proliferation (Li et al., 1999), however studies using PDE7 knockout mice have disproved this (Yang et al., 2003). Recently, it has been discovered that PDE7 activity can be inhibited by spiroquinazolinones (Lorthiois et al., 2004; Bernardelli et al., 2004).

1.2.8 The PDE8 Family

The PDE8 family are cAMP specific with an affinity for cAMP to rival that of PDE4 and were discovered screening a database of ESTs (expressed sequence tags) to identify PDE ESTs (Soderling et al., 1998; Soderling & Beavo 2000; Hayashi et al., 2002). Two variants of PDE8 exist, PDE8A and PDE8B. PDE8A is highly expressed in testis, eye, liver, kidney, skeletal muscle, embryo, ovary and brain (Soderling et al., 1998). PDE8B is expressed highest in the human thyroid gland, brain and kidney.

PDE8 is unique in that it contains a single PAS domain (Per, Arnt, and Sim proteins)at the N-terminus which regulates protein:protein interactions. Specific functions for the PDE8 isoforms remain to be discovered. PDE8 is, unusually, insensitive to inhibition by the so-called general PDE inhibitor, 3-Isobutyl-1-methylxanthine, (IBMX).

1.2.9 The PDE9 Family

PDE9 is a cGMP specific PDE expressed in many tissues including spleen, small intestine, kidney, heart and brain tissue (Fisher et al., 1998). Unlike other cGMP PDEs, PDE9 lacks a non catalytic cGMP binding domain. Four variants of PDE9 have been identified but their function is as yet unclear. The only compounds known to date to inhibit PDE9 are the PDE5 and PDE1 inhibiting compounds, zaprinast and SCH518866 (Dousa, 1999).

1.2.10 The PDE10 Family

PDE10 is a dual substrate family with K_m of 0.05μM for cAMP and 3μM for cGMP. The V_{max} for cGMP hydrolysis is approximately five times greater than that for cAMP. This suggests cGMP hydrolysis by PDE10 can be inhibited by cAMP, therefore PDE10 has been referred to as the cAMP-inhibited, cGMP specific PDE (Soderling et al., 1999; Fujishige et al., 1999). Two splice variants of PDE10 have been identified with PDE10A1 and PDE10A2 containing two N-terminally located domains that are similar to the cGMP binding domains of PDE2, PDE5 and PDE6. Unlike the other cGMP specific PDEs however, the cGMP binding domains in PDE10 are not thought to primarily function as such. PDE10 is sensitive to inhibition by IBMX (Soderling & Beavo 2000).

1.2.11The PDE11 Family

The PDE11 family also catalyse the hydrolysis of both cAMP and cGMP and contain a GAF (so named for cGMP binding and stimulated phosphodiesterase, *Anabaena* adenylyl cyclases, and *Escherichia coli* FhlA) domain at their N-terminus, relating them to the other GAF containing PDEs, PDE2, 5, 6 and 10. There have been four isoforms of PDE11 identified to date (Yuasa et al., 2000) with each containing variations of the GAF domain.

PDE11A1, PDE11A2 and PDE11A3 contain one intact GAF domain and one incomplete GAF domain. PDE11A4 contains two complete GAF domains (Yuasa et al., 2001). PDE11 isoforms also display differential tissue expression with PDE11A3 being specifically expressed in testis and PDE11A4 are predominantly expressed in the prostate. PDE11A1 is abundant in skeletal muscle (Yuasa et al., 2001). No physical functions have been related to any of the PDE11 isoforms as yet.

1.3 Organisation and Regulation of the PDE 4 Family

The *Drosophila melanogaster dunce* gene was the first gene to be discovered that specifically affected behaviour. Mutations in the *dunce* gene led to defects in learning and memory (Davis & Dauwalder 1991). The mammalian PDE4 family are homologues of the *dunce* gene. Cloning of the *dunce* PDE led to discoveries surrounding the regulation and kinetics of cAMP PDEs and the subsequent cloning of mammalian PDE4 genes. Four mammalian PDE4 genes were discovered located on three chromosomes with a high conservation between species. These four genes each give rise to multiple splice variants with unique N-terminals that are all characterised by their ability to be specifically inhibited by rolipram.

1.3.1 Modular structure of PDE4

The PDE4 family are further characterised by the presence of upstream conserved regions known as UCR1 and UCR2 at their N-terminus. The region that links UCR1 and UCR2 together is called linker region 1 (LR1), and linker region 2 (LR2) connects UCR2 to the catalytic domain (*see figure 1.2*). In contrast to UCR1 and UCR2, the linker regions differ between the different PDE4 families, perhaps conferring isoform-specific functional properties. The alternative mRNA splicing of members of the PDE4 family occurs at two major splice sites, one at the extreme N-termini and the other at the beginning of UCR2. This means both "long" isoforms containing both UCR1 and UCR2 and "short" isoforms with only UCR2 are produced. An additional splice site also generates "super-short" isoforms which have an N-terminally truncated UCR2. UCR1 and UCR2 have no homology to one another, yet are conserved in PDE4 homologs, suggesting they are of functional importance.

Studies have revealed that the C-terminal of UCR1 interacts specifically with the N-terminal of UCR2 (Beard et al., 2000; Lim et al., 1999; MacKenzie et al., 2000). PKA phosphorylation is known to affect this interaction, but some investigators suggest it

strengthens the interaction (Lim et al., 1999), whilst others have shown that it can weaken it (Beard et al. 2000). UCR2 has also been shown to act as a regulatory domain on the catalytic region (Beard et al., 2000; Lim et al., 1999). N-terminally truncated PDE4, with the N-terminus of UCR2 removed, causes an increase in PDE4 activity indicating the role of the UCR2 as an inhibitory domain on the catalytic unit of PDE4. This has been demonstrated to be the case with the PDE4D and PDE4A5 isoforms (Lim et al., 1999; Beard 2002). UCR2 has also been implicated in the targeting of PDE4 isoforms.

1.3.2 Regulation of PDE4 activity

The PDE4 family is regulated through the actions of protein kinases. Long forms of PDE4 contain two PKA phosphorylation sites. In PDE4D3, these are Ser^{13} and Ser^{54}. Ser^{13} is located at the unique N-terminal of the isoform and Ser^{54} is located within UCR1. PKA phosphorylation elicits activation of the PDE4 enzyme (Alvarez et al., 1995; Sette & Conti 1997; Hoffmann et al., 1998; Beard et al., 2000; Oki et al., 2000). Only the Ser^{54} residue has been shown to be essential for activation by PKA phosphorylation in the PDE4D3 isoform by mutation of Ser^{54} to Ala^{54} (Sette & Conti 1996). Replacing Ser^{54} with Asp^{54} or Glu^{54} can mimic enzyme activation (Hoffmann et al., 1998). The PKA consensus sequence, RRESF, is also found within the UCR1 region of other PDE4 long forms, suggesting all can be activated by PKA phosphorylation. Other work has shown that this is the case for PDE4A8, PDE4B1, PDE4C2 and PDE4D5 as well as PDE4D3 (MacKenzie et al., 2002). PKA phosphorylation induces a conformational change in the PDE4 enzyme which increases the affinity of UCR1 for UCR2. This relieves the auto-inhibitory actions of UCR2 on the catalytic domain and allows PDE4 to bind cAMP (Lim et al., 1999). Although PKA does phosphorylate Ser^{13} in PDE4D3 unlike other PDE4 long forms, the functional consequences of this are still unclear (MacKenzie et al., 2002).

The extracellular kinase (p42), ERK2 is activated by dual phosphorylation (Prowse & Lew 2001) and phosphorylates the long form PDE4D3 at Ser^{579}. This leads to a reversible 75% reduction in PDE4D3 enzyme activity (Hoffmann et al., 1999). More recent work has revealed that although isoforms encoded by the PDE4A, 4B, 4C and 4D genes all contain the ERK2 docking sites, KIM and FQF, (MacKenzie et al., 2000), only PDE4B, 4C and 4D act as substrates for ERK2 (Baillie et al. 2000). ERK2 phosphorylation of PDE4 long forms is transient, as it causes a localised increase in cAMP and thus activation of PKA. PKA phosphorylation can negate the ERK2 inhibition of PDE4 long forms, (Baillie et al., 2000; Hoffmann et al., 1999) introducing feedback regulation. Studies have revealed that whereas ERK2 phosphorylation of PDE4 long forms causes inhibition of enzyme

activation, ERK2 phosphorylation of PDE4 short forms surprisingly induces activation (Baillie et al., 2000). Thus there are opposing effects on ERK by cAMP. Inhibition of ERK is achieved through the Raf-1 isoform whereas ERK activation by cAMP is achieved through the B-Raf isoform. The cAMP signalling pathway is subject to many points of "cross-talk" between itself and the ERK pathway (Houslay & Kolch 2000). In human aortic smooth muscle cells, ERK stimulation feeds into an autocrine loop that results in activation of a PDE4 long form (Baillie et al., 2001, figure 1.3). Activated ERK caused an increase in PGE_2 which was released from the cell and activated adenylyl cyclase. This induced a rise in cAMP levels with a concomitant increase in PKA activity and resultant PDE4D5 phosphorylation. This phosphorylation ablated the effect of ERK phosphorylation and a net activation of PDE4D5 was observed.

1.3.3 The PDE4A subfamily

The PDE4A gene is located at chr19p13.1 and gives rise to seven different isoforms (Houslay et al., 1995; reviewed in Houslay 2001). It was originally thought that the inactivation of cyclic nucleotides was the only function of phosphodiesterases, but research suggests PDEs also play a role in signal compartmentalisation. The 'super-short' PDE4A isoform, PDE4A1, (also known as RD1), is exclusively membrane-associated, a feature unique among the PDE4 isoforms. It was initially revealed that the first twenty-five residues of the N-terminus of PDE4A1 are essential in membrane targeting (Pooley et al., 1997; Houslay et al., 1995; Houslay et al., 1998). N-terminally truncated isoforms were found solely in the cytosol. Further work revealed that PDE4A1 was specifically targeted towards the Golgi complex in FTC cell lines (Pooley et al., 1997). Displacing PDE4A1 from the membrane requires non-ionic detergents indicating that the binding involved hydrophobic interactions. Investigation by Baillie et al., 2002 revealed that PDE4A1 is inserted into the membrane in a manner due solely to an 11-residue helical module within the N-terminus that also confers selectivity for interaction with phosphatidic acid (PA). Interaction with PA has previously been shown to alter PDE4 activity. The module identified has been named the tryptophan anchoring phosphatidic acid selective binding domain 1, (TAPAS-1). Insertion is Ca^{2+} dependant and irreversible.

The human PDE4A4B, initially known as pde46, is a homologue of the rat PDE4A5 and is a 'long form' PDE. PDE4A4B selectively binds SH3 domains e.g. of Src family kinases and this binding alters PDE4A4B sensitivity to rolipram through a conformational change in the catalytic unit (McPhee et al., 1999; MacKenzie & Houslay 2000). The SH3 binding domains located in the N-terminus and UCR2 region also act to target the PDE4A4B/4A5

isoform to the plasma membrane where it localises in membrane ruffles (Beard et al., 2002). Perinuclear localisation of PDE4A4B/4A5 has been attributed to the N-terminal of UCR2. During apoptosis in Rat-1 fibroblasts and PC12 cells, the SH3 domain is cleaved by caspase 3. PDE4A4B/4A5 loses its targeting ability and is found uniformly throughout the cell (Huston et al., 2000). Binding of rolipram to PDE4A4/4A5 also affects intracellular distribution, inducing foci formation (Terry et al., 2003). Another 'long form' PDE4A, rat PDE4A8 (also known as rpde39), does not have a human homologue and has been shown to be exclusively expressed in the testes and hepatocytes (Bolger et al., 1996). PDE4A7 is a catalytically inactive isoform which is N- and C-terminally truncated (Johnston et al., 2004). The inactivity of 4A7 is not through inhibitory actions of the N- or C-terminal domains as removal of either of these does not allow PDE4A7 to hydrolyse cAMP. The PDE4A10 isoform was originally cloned from a rat olfactory lobe cDNA library (Rena et al., 2001). PDE4A10 is another PDE4A 'long form' which migrates at approximately the same size as PDE4A4 and the newly characterised, PDE4A11 (Tm3) on SDS-PAGE (~121kDa) PDE4A10 is conserved between human, mouse and rat and displays a predominantly perinuclear localisation in COS7 cells (Rena et al., 2001).

1.3.4 The PDE4B subfamily

The human PDE4B gene is located at chr1 and it provides four PDE4B isoforms. PDE4B2 is a short form PDE, subject to activation by ERK2, and PDE4B1, 4B3 and 4B4 are all long forms (reviewed in Houslay 2001). All PDE4Bs are found in both particulate and cytosolic fractions of cells. It is well established that PDE4 is the predominant PDE in leukocytes and has been attributed with a role in inflammation as it is involved in the activation and proliferation of inflammatory cells (Banner et al., 1999; Banner et al., 2000; Ogawa et al., 2000). In addition, PDE4 inhibitors significantly reduce TNF-α and IL-β release from LPS-stimulated macrophages (Kambayashi et al., 1995, Timmer et al., 2002). PDE4B has been singled out in inflammation as it is the predominant PDE4 in neutrophils and monocytes. In particular, the PDE4B2 isoform is the sole PDE4B variant expressed in these cells and is induced by lipopolysaccharide (LPS) in a manner inhibited by interleukin (IL)-10 and IL-14 (Wang et al., 1999).

Induction of PDE4B2 has also been observed in cells exposed to IL-1β (Oger et al., 2002) or a combination of IL-1β and TNF-α (Hakonarson et al., 2001). The increase in PDE4B2 has been reported to be due to the raised cAMP levels in response to IL-1β treatment. Exposing myometrial cells to IL-1β raised PGE_2 prior to induction of PDE4B2 and this induced an increase in PDE4 activity. IL-1β caused an initial increase in cAMP levels,

peaking at twelve hours, before PDE4 activity increased to a peak at eighteen hours and cAMP levels were reduced. The non-specific inhibitor of PGE_2 synthesis, indomethacin, was observed to completely block the rise in cAMP and the increased PDE4 activity (Oger et al., 2002). PDE4B2 induction in myometrial cells has also been observed directly in response to cAMP raising agents (Mehats et al., 1999) or naturally in near pregnant and pregnant myometrium (Mehats et al., 2000). PDE4B2 has also been implicated in the contraction of myometrial strips as PDE4 inhibitors can block spontaneous myometrial contraction (Mehats et al., 2002).

1.3.5 The PDE4C subfamily

The PDE4C gene is located at chr19p13.2 and is reported to give rise to three long forms of PDE4C (Obernolte et al., 1997). There is little research on the PDE4C subfamily, although it is known that the PDE4C2 isoform is subject to regulation by ERK2 phosphorylation. Upon ERK2 phosphorylation at Ser^{535}, activity of PDE4C is inhibited (Baillie et al., 2000).

1.3.6 The PDE4D subfamily

The PDE4D gene is located at chromosome 5 and through two major and one minor splice points (*see figure 1.2*), nine splice variants have been reported so far (Bolger et al., 1997; Wang et al., 2003; Gretarsdottir et al., 2003). Only one of these is a short form, PDE4D1 with a weight of 68 kDa. The super-short form PDE4D2 has an N-terminal truncation and is the same weight as PDE4D1. PDE4D6 is also another super short form with a predicted weight of 59kDa. PDE4D3, PDE4D4, PD4D5, PDE4D7, PDE4D8 and PDE4D9 are all long form PDE4Ds with weights of 95, 119, 105, 103, 96 and 95kDa respectively (weights of PDE7, 8 and 9 are predicted). The similarity in sizes on SDS-PAGE of PDE4D1 with PDE4D2; PDE4D3 with PDE4D8, 9; PDE4D5 with PDE4D7 and indeed PDE4A4 with PDE4A10 and PDE4A11 highlights the necessity of correct identification of PDE4 isoforms by means other than SDS-PAGE.

The function of PDE4D has been investigated in knock-out mice models. Studies on $PDE4D^{-/-}$ mice have shown that PDE4D plays a major role in growth and contraction. These transgenic mice display a lower than normal weight due to a reduction in muscle and bone mass and decreased weight of internal organs (Jin et al., 1999). The airways of mice deficient in PDE4D are also refractory to muscarinic cholinergic stimulation (Hansen et al., 2000). Further studies revealed this was due to a five-fold increase in sensitivity to prostanoid stimulation and an enhanced cAMP synthesis. The tracheal response to the contractile agonist carbachol was reduced in transgenic mice and could be mimicked by

treating wild-type mice with rolipram. Inactivation of PDE4B had no effect on tracheal contraction. The PDE4D$^{-/-}$ phenotype could be reversed by indomethacin (Mehats et al., 2003).

1.3.6.1 Interactions and Targeting of the PDE4D subfamily

In COS7 cells, PDE4D1 and 4D2 were found solely in the cytosol while PDE4D3, 4D4 and 4D5 were located in both the cytosolic and particulate fractions (Bolger et al. 1997). This confirms the involvement of the unique N-terminal in targeting as both 4D1 and 4D2 are short forms and therefore do not possess it. Translocation of the 4D3 isoform from the particulate fraction of cells to the soluble fraction has been seen in vascular smooth muscle cells upon stimulation of both the ERK and PKA pathways (Liu & Maurice 1999).

Temporal compartmentalisation of the PDE signal has been shown in FRTL-5 cells (Jin et al. 1998). Upon stimulation with thyroid stimulating hormone (TSH), PDE4D long forms were activated at membranous structures almost immediately while 4D2 was activated in the cytosol after a period of time. PDE4D long forms are targeted towards the Golgi/centrosome and filamentous structures within these cells.

In addition to PKA and ERK phosphorylation, PDE4D3 is also subject to regulation by PA. Accumulation of endogenous PA, through inhibiting the actions of PA degraders, induced an increase in PDE4 activity with a resultant decrease in cAMP and PKA activity (Nemoz et al., 1997). In FRTL5 cells, PDE4D3 was demonstrated to specifically bind PA in a unique binding site in the N-terminal region which contains the PKA phosphorylation site (Grange et al., 2000).

As with PDE4A4B/4A5, PDE4D4 has also been shown to contain an SH3 domain that predisposes PDE4D4 to bind selective SH3 domain proteins (Beard et al., 1999). Binding of Src or Lyn SH3 domains are prevalent, with no detectable effect on the catalytic activity of PDE4D. It is postulated that this interaction is involved in targeting of the PDE4D isoform.

PDE4D5 specifically has been demonstrated to interact with the receptor for activated kinase (RACK1). This interaction does not affect activity of the enzyme, but does affect sensitivity to rolipram (Yarwood et al., 1999). No physiological role for this interaction has yet been uncovered.

It has been recently shown that all PDE4 isoforms can interact with the scaffolding proteins, β-Arrestin1/2. Indeed, it has been demonstrated that PDE4D3 and PDE4D5 are targeted to activated GPCR along with β-Arrestin, aiding the localised degradation of cAMP (Perry et al., 2002) and receptor desensitisation. The interaction of PDE4 and β-Arrestin is also involved in the "switching" of GPCR from the G_s subunit to the G_i subunit of the G-protein. This occurs upon PKA phosphorylation of the receptor and leads to ERK activation within the cell. By using a catalytically inactive mutant of PDE4D5 which displaces active PDE4D5 from β-Arrestin, it was demonstrated that with inactive PDE4D, β_2-adrenoceptor can induce higher levels of PKA and increase the levels of receptor activated ERK (Baillie et al., 2003). PDE4 thus plays a key role in β_2-adrenoceptor regulation.

The scaffold protein myomegalin was identified as an interacting protein for PDE4D through yeast two hybrid analyses using PDE4D3 as bait (Verde et al., 2001). This interaction was demonstrated to localise PDE4D3 to Golgi/centrosomal areas and occurs through the N-terminus of UCR2 in PDE4D (Verde et al., 2001). As UCR2 is conserved in PDE4 long forms, it is possible that other PDE4 isoforms also interact with myomegalin. The UCR2 of PDE4D3 has also been shown to interact with AKAP450, targeting PDE4D3 to the centrosomal region in Sertoli cells (Tasken et al., 2001). UCR2 is not the only putative targeting mechanism of PDE4. As each isoform differs only at their N-terminus and LR, it was proposed that these regions conferred isoform-specific targeting. Indeed, it has been demonstrated that the muscle-selective AKAP, mAKAP, interacts with the unique N-terminus of PDE4D3 and not PDE4D5, targeting it to perinuclear regions in cardiac cells (Dodge et al., 2001).

1.3.6.2 Induction of PDE4D by cAMP

Intronic promoters have been identified controlling the expression of the PDE4D1 and PDE4D2 isoforms. Within these promoters, cAMP response elements (CRE) have been identified that allow cAMP response element binding protein (CREB; reviewed in Montminy 1997; Shaywitz & Greenberg 1999; Mayr & Montminy 2001) to bind and induce expression. This was demonstrated to be the case in Sertoli cells, where raising cAMP levels upregulated the expression of PDE4D1 and PDE4D2 (Vicini & Conti 1997). A CRE site has also been uncovered in the PDE4D5 promoter and cAMP-dependant induction of PDE4D5 has been observed in human airway smooth muscle cells (Le Jeune et al., 2002).

1.4 PDE4 Inhibitors and Disease

It has long been known than an increase in cAMP levels can suppress the immune response and induce smooth muscle cell relaxation (Schudt et al., 1999; Torphy 1998; Torphy et al., 1999; Wong & Koh 2000; Essayan 2001). Studies revealed that the PDE4 family is responsible for the majority of cAMP hydrolysis in immune and inflammatory cells as well as pulmonary and airway smooth muscle cells.

For these reasons, there has been interest in the development of PDE4 inhibitors for use in such diseases as asthma and chronic obstructive pulmonary disorder, (COPD; Spina et al., 1998; Landells et al., 2001; Spina 2003; Grootendorst et al., 2003; PDE4 inhibitors reviewed in Burnouf & Pruniaux 2002). Indeed, theophylline, a non-specific PDE inhibitor, has been used in the treatment of asthma for over 70 years. Unfortunately, non-specific PDE inhibitors exhibit emetic properties due to raising cAMP levels in the emetic centres within the central nervous system. For this reason, PDE4 isozyme specific inhibitors are being developed and tested. These inhibitors demonstrated that PDE4A/4B inhibitors inhibited TNFα release from monocytes and attenuated the proliferation of T lymphocytes while PDE4D inhibitors had no effect on these responses (Manning et al., 1999). PDE4C is absent in cells involved in the immune response. Other disease states for which PDE4 inhibitors have been considered include depression, rheumatoid arthritis, eczema and multiple sclerosis (Doherty 1999; Essayan 1999; Houslay et al., 1998; Schmidt et al. 1999; Schudt et al. 1999; Souness & Rao 1997; Torphy 1998; Torphy et al., 1999; Wong & Koh 2000). A new PDE4 inhibitor-cilomilast, is in development as a treatment for COPD and asthma (Giembycz 2001).

Recent research has implicated PDE4D7 and PDE4D9 isoforms in ischemic stroke (Gretarsdottir et al., 2003). A decreased level of PDE4D7 and PDE4D9 was observed in EBV transformed B-cell lines from affected patients which correlated to an increased risk for the disease. The authors proposed that PDE4D is involved in atherosclerosis underlying ischemic stroke due to the previous research on PDE4 inhibitors. In vascular smooth muscle cells, PDE4 inhibitors have been demonstrated to significantly potentiate the antimigratory effects of forskolin (Palmer et al., 1998; Goncharova et al., 2003) and attenuate proliferation (Pan et al., 1994; Johnson-Mills et al., 1998). PDE3 and PDE4 inhibitors used together act synergistically to dramatically reduce proliferation (Pan et al., 1994; Johnson-Mills et al., 1998).

1.4.1 Relaxant effects of PDE4 inhibitors

It is well known that raising cAMP levels in vascular smooth muscle cells initiates relaxation (Murray 1990), an effect also seen with PDE inhibition. Studies have indicated that PDE4 and PDE3 inhibition exert the most potent relaxant effect. Indeed, in human intralobar pulmonary arteries, all PDE inhibitors tested induced a concentration dependant relaxation of which rolipram produced the most potent (Bardou et al., 2002). Other studies have confirmed the relaxant effect of PDE4 inhibitors, and the synergistic effect when used together with PDE3 inhibitors (Wagner et al., 1997; Eckly-Michel et al., 1997; Goirand et al., 2001; Pauvert et al., 2002). PDE4 inhibitors also potentiate the effect of cAMP elevating agents in rat aortic smooth muscle cell (Tilley & Maurice 2002). The vasorelaxant effect of PDE4 inhibitors has been demonstrated to require a functional endothelium present (Komas et al., 1991).

The vasorelaxant properties of PDE3 and PDE4 inhibitors are of considerable interest to researchers regarding therapies for cardiovascular disease. In contrast with the systemic circulation, the pulmonary circulation contracts in response to hypoxia. Chronic hypoxia is associated with several pulmonary diseases such as COPD and can be caused by vascular injuries, thrombosis and ischemic cardiovascular disease. Sustained chronic hypoxia leads to the development of pulmonary arterial hypertension (PAH, see section 1.5 for further details). The pulmonary vasoconstriction witnessed in hypoxia can be opposed by the use of PDE4 and PDE3 inhibitors (Pauvert et al., 2002; Goirand et al., 2001). In PAH, pulmonary artery relaxation is significantly attenuated in response to vasodilators. Studies investigating the responses of isolated pulmonary artery rings to agonists that induce relaxation in rat models of PAH, have demonstrated that PDE4 and PDE3 inhibitors can improve the response to vasodilators in the PAH rat (Wagner et al., 1997).

1.5 Pulmonary Arterial Hypertension

Pulmonary hypertension (PH) was previously grouped into two categories; primary pulmonary hypertension (PPH) or secondary pulmonary hypertension. Classification of the disease depended on the absence or presence of identifiable causes or risk factors. In 2003, this classification was revised to take into account the identification of genetic factors and is now grouped into three categories, idiopathic PAH, familial PAH and PAH related to risk factors or associated conditions *(see figure 1.4 for 'Venice classification').*

Pulmonary arterial hypertension is a progressive and usually fatal disorder characterized by a maintained increase of pulmonary arterial pressure (PAP) caused by occlusion of the small pulmonary arteries (reviewed in Humbert et al., 2004). When the mean PAP exceeds 25mmHg at rest and 30mmHg during exercise, this is diagnosed as PAH. Idiopathic and familial PAH is an uncommon disorder with an estimated occurrence of 1-2 cases per million people and is more commonly seen in females of 20-40 years of age. The symptoms of PAH are extremely variable and the age of onset differs dramatically between individuals. This generally leads to the disease being diagnosed in its latter stages. The variable age of onset and reduced penetrance of the disease also indicates environmental factors play a role in the development of PAH. Current treatment strategies include prostanoids such as beraprost, (Badesch et al., 2004), nitric oxide, endothelin receptor antagonists like bosentan (Channick et al., 2004; see section 1.5.5.1 for further details), vasodilators and in extreme cases, lung transplantation. PDE inhibitors are also included in the list of emerging therapies for pulmonary hypertension (Ghofrani et al., 2004).

In 1999, candidate gene mapping revealed a gene locus linked to familial PPH on chromosome 2q33 (Deng et al., 2000). Through further studies on this locus, known as PPH1, it has been indicated that mutations within a gene at this locus causes familial PPH (The International PPH Consortium, 2000; Machado et al., 2001). This gene is the bone morphogenetic protein type II receptor (BMPR-II). Germline mutations in *BMPR2* are also found within at least 26% cases of sporadic PAH (Thomson et al., 2000; Machado et al., 2001). To date, 46 unique mutations within the *BMPR2* gene have been identified in cases of PAH (Machado et al., 2001). The mutations are dispersed throughout the gene except for in three of the thirteen exons, 5, 10 and 13. The nature of the mutations range from frameshift to nonsense mutations, resulting in truncated transcripts and variable nucleotide changes. It has been suggested that the inheritance mechanism of PPH is haploinsufficiency (Machado et al., 2001). This would indicate the pathway mediated through BMPR-II to be of critical importance in maintenance and repair of the pulmonary vasculature. *BMPR2* encodes the transforming growth factor-β (TGF-β) type II receptor BMPR-II which is expressed in the endothelium and smooth muscle cells of the pulmonary and systemic vasculature.

Hypoxia-induced pulmonary hypertension is aggravated by the increased amount of 5-HT delivered to the lung in rats and this can be blocked with a 5-HT antagonist (Eddahibi et al., 1997). This occurs through a hypoxia-induced increase in 5-HT transporter (5-HTT)

levels (Eddahibi et al., 1999). A study of patients with PAH revealed a link between the variant of 5-HTT expressed and susceptibility to PAH (Eddahibi et al., 2003).

PAH occurs more commonly as a consequence of serious disorders such as COPD, HIV infection or left ventricular failure. Additionally, secondary PAH can develop due to the use of appetite suppressants such as fenfluramine or aminorex phentermine and in response to sustained hypoxia caused by cardiovascular disease, vascular injury or due to high altitude. The prevalence of secondary PAH is difficult to estimate due to the large number of causes overlapping with symptoms of more common disease states preventing a clear diagnosis.

The most frequent initial stimuli of PAH are mechanical obstruction to pulmonary flow and hypoxia. Acute hypoxia causes a reversible PAP increase whereas chronic exposure to hypoxia induces alterations in the small pulmonary vessels and renders the resultant increase in PAP irreversible.

1.5.1 Models used in research of PAH

The most common animal model of PAH is the chronic hypoxic rat (Rabinovitch et al., 1979). Male Wistar rats are exposed to low oxygen (10%O_2) by reducing the atmospheric pressure in a hypobaric chamber. A rise in PAP is observed after three days of hypoxia as well as right ventricular hypertrophy. After two weeks in hypoxia, hypertrophy of the pulmonary artery occurs (Vender 1994). A more recently utilised rat model is the Fawn hooded rat. The fawn hooded rats are used for their inherited sensitivity to the development of idiopathic PAH (Stelzner et al., 1992). Recently, other transgenic mice have provided valuable new models for study. For example, mice expressing a dominant-negative form of the BMPRII gene, dnBMPRII mice, display elevated PAP and pulmonary arterial muscularisation when the mutation is activated after birth (West et al., 2004). These mice confirm that a loss of function of the BMPRII gene is sufficient to produce a pulmonary hypertensive phenotype. Also, mice that overexpress the 5-HTT transporter, 5-HTT+ mice (see section 1.5.7 for further details), exhibit increased right ventricular pressure compared with wild-type mice (MacLean et al., 2004). Under conditions of hypoxia, the 5-HTT+ mice display increased remodelling compared to wild-type.

Cells can easily be grown in a hypoxic environment, mimicking conditions that lead to the development of PAH. Pulmonary vasoconstriction is an inherent property of pulmonary vascular smooth muscle cells (Voelkel & Tuder 1997) and a direct effect of hypoxia on

smooth muscle cells has been witnessed. After 1 week of hypoxia, the proliferation rates of PASMC from distal human pulmonary arteries were observed to increase (Yang et al., 2002) and isolated PASMC cells have also been observed to contract in response to hypoxia (Murray 1990). Human PASMC (hPASMC) have therefore been utilised as a cellular model of PAH (Murray et al., 2002; Murray et al., 2003).

1.5.2 Pathology of PAH

PAH is associated with right ventricular hypertrophy and changes in the structure and function of smooth muscle cells, fibroblasts and endothelial cells leading to a sustained increase in PAP and vasoconstriction. These changes, along with the heterogeneity among cell phenotypes, contribute to the vascular remodelling (reviewed in Mandegar et al., 2004; Humbert et al., 2004), altered tone, thrombosis and vasoreactivity witnessed in PAH. Although the entire pulmonary vascular tree undergoes histological alterations, it is the smaller vessels which are primarily affected. Vascular smooth muscle cells proliferation rate increases, resulting in medial hypertrophy and arteriolar muscularisation, fibrosis, thrombosis. Endothelial cells increase in proliferation causes intimal thickening and the formation of plexiform lesions. The dysfunction of inflammatory cells is also considered to play a role in the development of PAH. All of these problems contribute to the remodelling of the pulmonary vascular tree. Medial hypertrophy is mainly due to abnormalities observed in pulmonary artery smooth muscle cell (PASMC) function, namely an increase in proliferation and migration (Yuan & Rubin 2001). Another characteristic of some forms of PAH is the formation of plexiform lesions arising from the concentric endothelial cell proliferation and smooth muscle cells migration. These lesions occur distally to obliterative intimal lesions.

Pathophysiological studies have highlighted the importance of several mediators in the development of PAH, namely, Endothelin-1 (section 1.5.5.1; Giaid et al., 1993), nitric oxide (section 1.5.5.2; Giaid et al., 1995), prostacyclin (1.5.5.3; Christman et al., 1992; Tuder et al., 1999) and serotonin (5-HT, section 1.5.7; Herve et al., 1995; MacLean et al., 2000).

1.5.3 Pulmonary artery smooth muscle cells

Increased smooth muscle cell proliferation and hypertrophy have been implicated in the development of PAH (Yuan & Rubin 2001) and leads to muscularisation of the pulmonary resistance vessels. Hyperplasia is characteristic of the larger vessels, whereas the small vessels undergo more extensive remodelling (Humbert et al., 2004). The pulmonary artery smooth muscle cells are normally present in a quiescent and contractile state, but injury to

the vessel wall activates cells into a proliferative, migratory and secretory phenotype. This phenotypical change has been reported to be dependant upon the growth factors transforming growth factor β (TGF-β), and basic fibroblast growth factor (bFGF; Boudreau et al., 1991). The smooth muscle cells then migrate through to the intimal layer and can invade the lumen of the vessel forming concentric lesions. Thrombin induced hypertrophy has been demonstrated to occur through activation of a set of immediate early genes (IEG), whereas PDGF induced proliferation occurs through an alternative set of IEG (Rothman et al., 1994). IEG expression in both instances appears to be regulated by extracellular calcium, PKC and sodium (see below for further details).

In cultured PASMC, hypoxia inhibits the voltage gated potassium channels in PASMC causing a depolarisation of the membrane and a resultant increase in intracellular calcium (Platoshyn et al., 2001; Limsuwan et al., 2001; reviewed in Strange et al., 2002). The rise in Ca^{2+} occurs through many different mechanisms including activation of voltage dependant calcium channels, the production of inositol 1,4,5-triphosphate (IP_3) thus facilitating the release of Ca^{2+} from intracellular stores, and the increase of Ca^{2+} levels by reversing Na^+/ Ca^{2+} exchange (Madden et al., 2001). It has also been reported that the maintenance of Ca^{2+} levels is aided by the inhibition of Na^+/ Ca^{2+} exchange (Wang et al., 2000). A reduction in K^+ channel expression and function has been observed in PASMC from PAH patients (Yuan et al., 1998a; Yuan et al., 1998b; review of hypoxic effects on K^+ channels, Raj & Shimoda 2002) as well as in PASMC cultured in hypoxia (Wang et al., 1997) or exposed to serum from patients with PAH (Limsuwan et al., 2001). The rise in intracellular calcium also leads to vasoconstriction and activation of PKC. PKC is a calcium dependant enzyme that stimulates DNA synthesis in systemic artery smooth muscle cells and PASMC will not proliferate in response to hypoxia unless PKC is activated. Recent studies using a PKC-ε knockout mouse model confirmed the role of PKC in hypoxic vasoconstriction as the transgenic mice displayed reduced hypoxic pulmonary vasoconstriction (Littler et al., 2002). As well as increased proliferation and hypertrophy, hypoxia also decreases apoptosis of PASMC.

PASMC exposed to hypoxia also display an increase in Rho-kinase activity that has been reported to contribute to hypoxia-induced pulmonary vasoconstriction (Wang et al., 2001). This is mediated by Rho-kinase increasing myosin light chain phosphorylation. Use of the Rho-kinase inhibitor Y-27632 was able to ablate this effect. In addition, Rho-kinase activation causes an inhibition of myosin phosphatase, enabling PASMC to sustain constriction (Wang et al., 2003).

It has been shown that vascular smooth muscle cells display marked heterogeneity with several functionally distinct phenotypes (Frid et al., 1997). Each distinct population of cells expresses different cytoskeletal and contractile proteins and channels and display different growth rates and responses to growth factors. In hypoxia, there is a redistribution of these cell types, altering the phenotype and responses of each pulmonary artery. This heterogeneity of smooth muscle cells adds complexity to an already complex interplay of many different factors involved in the response to chronic hypoxia and the development of PAH.

1.5.4 Pulmonary artery fibroblasts

An increase in extracellular matrix proteins and pulmonary arterial fibroblasts (PAF) is observed in PAH due to sustained hypoxia. This causes vascular hardening maintaining pulmonary vasoconstriction. Increased fibroblast proliferation has been shown to exceed that of PASMC and pulmonary artery endothelial cells (PAEC) in hypoxic animal models (Belknap et al., 1997). Hypoxia acts through $G\alpha_{i/o}$ and G_q coupled P_{2Y} activation to promote fibroblast proliferation (Stenmark et al., 2002). Molecular studies have revealed that constitutive p38 MAPK (section 1.5.6) activity enhances PAF proliferation in remodelled vessels (Welsh et al., 2001).

Similar to PASMC, PAF also display marked heterogeneity (Stenmark et al., 2002). Responses to hypoxia are unique among the sub populations of cells, with some even displaying a reduction in DNA synthesis. Populations of PAF with hypoxia induced changes in proliferative and matrix-producing phenotypes also display the expression of smooth muscle α-actin, suggesting that some PAF transdifferentiate into myofibroblasts (Stenmark et al., 1995).

1.5.5 Pulmonary artery endothelial cells

Endothelium-dependant mediators have been implicated in the modulation of hypoxic pulmonary vasoconstriction. Indeed endothelial dysfunction has long been considered to have a key role in the development of PAH. The endothelium releases vasoconstrictors such as endothelin and vasodilators including prostacyclins and NO. An imbalance between vasodilators and vasoconstrictors and an increase in PAEC proliferation is apparent in PAH.

1.5.5.1 Endothelin-1

Endothelin-1 (ET-1; Yanigasawa et al., 1988; Masaki et al., 1991) is a potent vasoconstrictor produced by both pulmonary and systemic endothelial cells. It is released upon stimulation by a range of agents including epinephrine, TGF-β and IL-1 and also by shear stress or hypoxia. Once released, ET-1 binds to type A receptors (ET_A) found mainly in smooth muscle cells or type B receptors (ET_B) found on endothelial cells. ET_A receptors are generally thought to regulate vasoconstriction by facilitating the production of IP_3, while ET_B receptors of endothelial cells lead to vasodilation through the release of nitric oxide (NO; Eddahibi et al., 1991). However, the distribution of receptors displays anatomical heterogeneity with ET_A mediating vasoconstriction in the large arteries, while in the smaller resistance arteries atypical ET_B receptors regulate vasoconstriction (McCulloch et al., 1998). No effect has been observed through the binding of ET-1 to endothelial cells, hence the contributory effect of ET-1 to sustained vasoconstriction. ET-1 also contributes to vascular remodelling through increasing the proliferation of PASMC via ET_A receptors (Janakideva et al., 1992; Zamora et al., 1993).

ET-1 can also indirectly increase cGMP levels through NO release and directly stimulate or inhibit the synthesis of cAMP in pulmonary arteries. The ET-1 induced increase in cGMP occurs in the larger pulmonary arteries alone through the ET_A receptor. The increased synthesis of cAMP by ET-1 is observed in the small resistance arteries and could be blocked by an ET_B receptor antagonist. ET-1 mediated reduction in cAMP levels is mediated in part by the ET_A receptor and occurs in the main pulmonary arteries. All these effects were endothelium independent (Mullaney et al., 2000). Under chronic hypoxic conditions, basal levels of cAMP are reduced in all but the small resistance arteries of the rat where cAMP levels are mildly increased (Mullaney et al., 1998). ET-1 induced cAMP responses were lost in the hypoxic rat. Reduction of cAMP in the large pulmonary arteries was no longer observed in hypoxic rats, instead, a significant increase of cAMP was observed. In the resistance arteries of the hypoxic rat, ET-1 was no longer able to increase cAMP (Mullaney et al., 1998).

ET-1 levels have been shown to be raised in systemic and pulmonary circulation in patients with primary and secondary PAH to a level sufficient enough to induce vasoconstriction (Stewart et al., 1991; Cody et al., 1992; Cacoub et al., 1997). The role of ET-1 in PAH (reviewed in MacLean 1998; MacLean 1999) has been investigated further through the use of chronic hypoxic rat models. In lungs from the chronic hypoxic rat, ET-1 levels and ET_A receptor expression increase (Li et al., 1994). Further investigation into these alterations in

the lung uncovered an increase in vasoconstriction mediated by the ET_A receptor in the large and small pulmonary arteries (MacLean et al., 1995; McCulloch et al., 1998). ET receptor antagonists have thus been developed to investigate their therapeutic potential in PAH. To date, the ET_A and mixed ET_A /ET_B receptor antagonists tested have proven to be effective at hindering the development of and even reversing PAH in chronic hypoxic rat models (DiCarlo et al., 1995; Chen et al., 1997; Underwood et al., 1998; reviewed in MacLean 1999). Currently, a mixed ET-1 receptor antagonist, bosentan, is in use as a treatment for PAH patients (Channick et al., 2004).

1.5.5.2 Nitric Oxide

Nitric oxide (NO) is a well characterised molecule with functions including inhibition of smooth muscle growth and constriction (Singh & Evans 1997). The effects of NO are mediated partly through the soluble guanylyl cyclase receptor which increases cGMP in cells and, in turn, increases PKG activity. PKG reduces the intracellular concentration of Ca^{2+} and hyperpolarises the membrane through inhibition of voltage and receptor Ca^{2+} channels and activation of K^+ channels respectively. Smooth muscle relaxation is also induced through the activation of myosin light chain phosphatase by cGMP. Independently of cGMP, NO signalling in hypoxic conditions has been demonstrated to induce modifications in enzymatic activity and, more recently, activate the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) causing a decrease in intracellular calcium (Mingone et al., 2003).

NO is produced in endothelial cells during the conversion of L-arginine to L-citrulline which is catalysed by nitric oxide synthase (NOS). There are three known types of NOS, nNOS or type I NOS which is secreted by neurones. Type II NOS or iNOS is inducible and secreted by various cells in response to cytokine stimulation. Type III NOS or eNOS is a Ca^{2+}-dependant, constituitively expressed isoform secreted by endothelial cells. eNOS deficient mice display mild PAH in a normal atmosphere and have an increased susceptibility to hypoxia induced pulmonary hypertension (Fagan et al., 1999). Similar studies in mice with disrupted iNOS or nNOS genes suggest these isoforms have a minor role in determining PAP in mice.

Results from studies investigating NO levels in hypoxic conditions and in PAH have produced conflicting results. In hypoxic rat lungs, eNOS and iNOS mRNA and protein levels have been reported to significantly increase (Igari et al., 1998). Immunocytochemical staining revealed the de novo eNOS and iNOS proteins appear in the small and medium sized arteries of hypoxic rats whereas these proteins appear limited to

large arteries in normoxic rats (LeCras et al., 1996). Hypoxia has also been shown to induce iNOS in a HIF-1 dependant manner in PAEC and PASMC (Palmer et al., 1998). Lungs from patients with PAH also display an increased amount of eNOS, especially in the plexiform lesions (Mason et al., 1998). Lung NO production in PAH patients has been reported by several groups to increase (Archer et al., 1998; Forrest et al., 1999).

A number of studies have reported a reduction in NO levels in PAH. Both acute and chronic hypoxia were observed to attenuate NO production in the main pulmonary artery of the rat with a resultant decrease in intracellular levels of cGMP (Shaul et al., 1993; MacLean et al., 1996). In addition, pulmonary arteries from patients with PAH have been observed to exhibit reduced expression of eNOS (Giaid & Saleh 1995). More recent work on pulmonary arteries from the chronic hypoxic rat have shown that although endothelial NO production in response to carbachol was reduced, total NO protein was unaltered, indicating hypoxia affects eNOS activity at the post-translational level (Murata et al., 2002) Such results were the reason for use of inhaled NO therapy in PAH.

NO has been shown to inhibit ET-1 induced pulmonary vasoconstriction (Lang & Lewis 1991) and attenuate vascular remodelling (Horstman et al., 1998). Inhaled NO has proven beneficial to patients with short-term and reversible PAH, but not for patients requiring to use it over long periods due to its irritant properties and short action. Among patients treated with inhaled NO, the effects are varied and it is not always successful as a treatment. Underlying this could be the cause for opposing results from different research groups. As such, the definitive role of NO in PAH remains to be determined.

1.5.5.3 Prostaglandins

Arachodonic acid (AA) is metabolised through the cyclooxygenase and lipooxygenase pathways to form prostaglandins and leukotrines. Metabolites of arachodonic acid such as prostacyclin (PGI_2) and thromboxane (T_xA_2) elicit vasoactive effects in the pulmonary circulation (reviewed in Christman, 1998). PGI_2 is produced by endothelial cells and is a potent vasodilator and an inhibitor of platelet aggregation. PGI_2 activation of prostacyclin receptors stimulates synthesis of cAMP, leading to vasodilation and reduced DNA synthesis in vascular smooth muscle cells. The net effect of this is a reduction in pulmonary vascular resistance. T_xA_2 is produced by platelets and is a vasoconstrictor.

Depending on experimental conditions, hypoxia has been reported to both increase and decrease prostacyclin release. Patients with severe PAH have an imbalance in production

of PGI_2 and T_xA_2 and a reduction in expression of PGI_2 synthase (Christman et al., 1992; Tuder et al., 1999). An overexpression of PGI_2 synthase has been reported to protect mice against hypoxia induced pulmonary hypertension (Geraci et al., 1999). Conversely, PGI_2 has been reported to increase in response to seven days hypoxia (Shaul et al., 1991). ET-1 can also induce PGI_2 production in rat lung (Barnard et al., 1991).

In most instances, the effects of PGI_2 are mediated by the production of cAMP as responses can be mimicked in cultured cells using isoprenaline and forskolin, and potentiated using the general PDE inhibitor, IBMX.

Clinical studies investigating the use of prostacyclin analogues as a treatment in PAH revealed they increase survival rate (reviewed in Badesch et al 2004). However, short half-lifes and side effects compromise the benefits, therefore new prostacyclin analogues are under development and testing. Subcutaneous delivery of Treprostinil has beneficial effects on exercise and hemodynamics, however side effects including nausea, headaches and pain and erythema at the site of delivery. In Europe, another prostacyclin analogue, Iloprost, has recently been approved for the treatment of PAH and is delivered through inhalation. Inhaled iloprost has little effect on the systemic circulation, providing selective vasodilation of the pulmonary circulation as does inhaled nitric oxide, but only iloprost studies indicate an increased survival rate.

1.5.5.4 Vascular Growth Factors

Many vascular growth factors are secreted by PAEC and a number of these have been observed to be increased in PAH. The most well characterised example is the vascular endothelial growth factor (VEGF). Although VEGF is not secreted by endothelial cells under physiological conditions, it is present in plexiform lesions of PAH patients (Archer & Rich 2000). The only known target of VEGF is the endothelial cells themselves. In hypoxic conditions, the VEGF gene is stimulated almost instantly due to binding of the hypoxia inducible factor -1 (HIF-1; reviewed in Semenza 2000) to a hypoxia response element (HRE) in the promoter region (Gerber et al., 1997). VEGF is also known to be upregulated in PAH (Tuder et al., 1995).

Endothelial cells under hypoxic conditions are also known to release a platelet-derived growth factor (PDGF) which induces vasoconstriction and stimulates PAF migration and proliferation (Peacock et al., 1993; Faller et al., 1999). PDGF is also capable of inducing VEGF. Another growth factor upregulated in PAH is TGF-β (Acrot et al., 1993). In

vascular cells, the predominant effects of TGF-β are growth inhibition, cell differentiation and stimulation of collagen synthesis. Other growth factors elevated in PAH include; bFGF (Arcot et al., 1995); IGF-1 (Perkett et al., 1992) and EGF (Gillespie et al., 1989). The increase in secreted growth factors from endothelial cells can lead to increased proliferation of PASMC through feeding into the mitogen activated protein kinases (MAPK) pathways (Xiao 1993).

1.5.6 Mitogen Activated Protein Kinases

Mitogen activated protein kinases (MAPK) are activated by a wide range of stimuli including growth factors, hormones, extracellular matrix components, GPCR agonists, cellular stress and cytokines (reviewed in Gutkind 2000; Tibbles & Woodgett 1999; Wildman et al., 1999). MAPK are split into groups according to their function. The best characterised MAPK are the extracellular regulated kinases 1 and 2, also known as p44 and p42 respectively. The functions of the ERK1/2 are extremely diverse and include regulation of cell proliferation (reviewed in Stork & Schmitt 2002), differentiation and tumorogenesis. Other well characterised MAPK are; the c-Jun NH_2-terminal kinases also known as JNK/SAPK, and the p38 MAPK family. JNK and p38 MAPK are involved in the cellular response to stressors. MAPK are activated through phosphorylation by a MAPK kinase (MAPK) which is activated through phosphorylation by a MAPKK kinase (MAPKKK). Each family of MAPK have specific MAPKK and MAPKKK and upstream activators (*figure 1.4*).

1.5.6.1 Activation of MAPK

The classical MAPK pathway of ERK1/2 is now well characterised (*figure 1.5*). Binding of epidermal growth factor (EGF) to its tyrosine kinase receptor (RTK) leads to the phosphorylation of the receptor itself and other substrates. Phosphorylated sites act as docking sites for adaptor proteins such as Grb2 which contains a Src homology 2 (SH2) domain and two SH3 domains or Shc which possesses a phosphotyrosine binding (PTB) domain, one SH2 and one SH3 domain. Shc acts as a substrate for the EGF receptor and upon phosphorylation binds the SH2 domain of Grb2, resulting in the recruitment of SOS. SOS induces the exchange of GDP bound to Ras for GTP and initiated the MAPKKK cascade. Once activated, ERK1/2 translocates to the nucleus and phosphorylates key transcription factors that ultimately regulate the expression of genes essential for processes such as cell proliferation. GPCRs can also influence cell growth and differentiation through activation of the ERK1/2 pathway (reviewed in Gutkind 2000). This occurs in the same manner as RTKs. In contrast, activation of the JNK pathway occurs through GPCRs

and not RTKs (Coso et al., 1995). How this occurs is as yet unclear as is the activation of the p38 pathway by GPCRs.

1.5.6.2 Hypoxia-induced activation of MAPK

Numerous studies have revealed hypoxia induced activation of the MAPK cascades in many different cell types (reviewed in Minet et al., 2000a). PAF and PAEC have been reported by several groups to display a marked activation of the ERK1/2 pathway under hypoxic conditions (Minet et al., 2000b; Scott et al., 1998; Welsh et al., 2001). In addition, pulmonary arteries from chronic hypoxic rats also show an activation of ERK1 and 2 that peaks at day seven (Jin et al., 2000). Increased phospho-ERK1/2 was observed in both large and small pulmonary arteries along with the activation of JNK and p38.

The p38 family and JNKs have also been reported to display an increased activity in PAF exposed to acute hypoxia for up to 30 hours (Scott et al., 1998). Activation of p38 peaked at 6 hours and 24 hours while JNK activation peaked at 6 hours before returning to basal levels. p38 activity is also increased in PAF, but not in aortic fibroblasts, under chronic hypoxic conditions (Welsh et al., 2001). The transient increase in activation can be explained by the induction of MAPK phosphatases in hypoxia or in response to MAPK activation (Laderoute et al., 1999; Ward et al., 1994). This feedback control could be to avoid apoptosis which has been shown to occur with sustained MAPK activation.

1.5.6.3 HIF-1 and ERK1/2

Hypoxia inducible factor -1 (HIF-1) is a transcription factor responsible for the induction of expression of glycolytic enzymes, metabolic enzymes, proteins involved in proliferation and proteins involved in vascular biology (reviewed in Semenza 1999; Huang & Bunn 2003). HIF-1 can be induced by various hormones, serum and hypoxia. HIF-1 is composed of two subunits, HIF-1α and HIF-1β. Both subunits are constitutively expressed, however HIF-1α is rapidly degraded under normal conditions by proteasomal degradation. Under hypoxic conditions however, degradation of the HIF-1α subunit is attenuated allowing a higher amount of functional dimers to form (Sutter et al., 2000). Thus, research has focused on HIF-1α as the regulator of the hypoxic response. HIF-1 activates target genes by translocating to the nucleus and binding to a hypoxia response element (HRE) in the promoter region. HIF-1α and HIF-1β knockout mice models results in embryonic lethality. Partially HIF-1α deficient mice display a marked reduction in vascular remodelling in response to hypoxia (Yu et al., 1999).

HIF-1α is highly phosphorylated *in vivo* which allows the stabilisation of the protein. Phosphorylation of HIF-1α is mediated by an ERK1/2 dependant pathway (Minet et al., 2000b). The other well known MAPK, JNK and p38 MAPK, do not phosphorylate HIF-1α (Richard et al., 1999). ERK1/2 phosphorylation has been shown to promote the transcriptional activity of HIF-1α (Richard et al., 1999). The effect of HIF-1α on gene expression is also regulated by p53 which inhibits HIF-1 stimulated transcription (Blagosklonny et al., 1998). Other regulators of HIF-1 include Jab1 and p300/CBP (Bae et al., 2002; Kallio et al., 1998).

1.5.7 Role of 5-Hydroxytryptamine in PAH

The use of appetite suppressants such as aminorex has been determined to be a significant risk factor for the development of PAH (Fishman 1999). These drugs belong to a vast class of amphetamine and epinephrine like drugs that act by increasing local and circulating 5-HT levels. This increase is achieved by the inhibition of 5-HT transporters (5-HTT), release of platelet 5-HT and preventing 5-HT clearance by inhibiting monoamine oxidase (MAO).

5-HT is a vasoconstrictor of the pulmonary circulation and a co-mitogen of vascular smooth muscle cells in culture. 5-HTT is also involved in 5-HT signalling through activation of NAD(P)H oxidase to produce reactive oxygen species (ROS) and ERK1/2 activation which is involved in 5-HT induced smooth muscle cells hyperplasia or hypertrophy (Lee et al., 1999). 5-HT levels have been reported to increase under several conditions that lead to the development of PAH (reviewed in MacLean et al., 2000). Levels of the 5-HTT are also observed to increase in lung tissue and pulmonary arteries from PAH patients with a resultant enhancement of PASMC proliferation in response to 5-HT (Eddahibi et al., 2001). This has also been observed in hypoxic PASMCs (Eddahibi et al., 1999). The increased expression of the 5-HTT is also associated with a polymorphism of the 5-HTT promoter (Eddahibi et al., 2001). It has previously been shown that mice with targeted 5-HTT gene disruption are less prone to develop PAH than control mice (Eddahibi et al., 2000). 5-HTT inhibitors have been observed to attenuate hypoxia induced PAH.

Vasoconstriction induced by 5-HT occurs through the $5\text{-HT}_{1B/1D}$ and 5-HT_{2A} receptors depending on vascular tone and in a species specific manner. In humans, vasoconstriction is mediated by the 5-HT_{1B} receptor (MacLean 1999). In rat pulmonary arteries, vasoconstriction usually occurs through the 5-HT_{2A} receptor, however in the chronic hypoxic rat model, vasoconstriction is mediated by the 5-HT_{1B} and 5-HT_{2A} receptors

(MacLean et al., 1996). 5-$HT_{1B/1D}$ receptors are coupled to the $G\alpha_i$ protein and induce contraction by decreasing cAMP levels. Contraction through the 5-HT_{2A} receptors occurs through stimulating the $G\alpha_q$ pathway, leading to an increase in intracellular calcium and activation of PKC.

1.5.8 Cyclic nucleotide pathways in PAH

1.5.8.1 Cyclases

Functionally important isoforms of adenylyl cyclase found in the rat lung are AC2, AC-6 and AC-8 (Jourdan et al., 2001). AC-2 is potently stimulated by PKC (Jacobowitz & Iyengar 1994) and is insensitive to calcium. AC-6 is inhibited by both PKA and PKC, as well as by calcium (Lai et al., 1999; Iwami et al., 1995; Cooper et al., 1995). AC-8 is stimulated by calcium binding and PKC has no effect (Cali et al., 1994). Taken together, these regulatory properties of the AC isoforms found in the lung allows for a tight regulation of cAMP levels, able to tailor stimulation or inhibition when required in response to specific effectors. Under chronic hypoxic conditions, it has been demonstrated that AC activity measured under GTP, forskolin or isoproterenol stimulation is reduced in rat hearts (Pei et al., 2000; Hrbasova et al., 2003). Hypoxia has also been demonstrated to affect guanylyl cyclase. Soluble GC levels and activity have been reported to increase in chronic hypoxic rats (Li et al., 1999).

1.5.8.2 Cyclic nucleotides

Many of the effects of the pathways mentioned exert their effects through cyclic nucleotides. Stimulation of the cAMP or cGMP pathways in smooth muscle leads to relaxation while inhibition of these nucleotides would lead to contraction of smooth muscle. This is mediated by the cAMP- and cGMP-dependant kinases, PKA and PKG.

An increase in either cAMP or cGMP is known to inhibit vascular smooth muscle cell proliferation (reviewed by Koyama et al., 2000; Hayashi et al., 2000). Through the actions of PKA, cAMP attenuates proliferation by antagonising mitogenic pathways and causes a cell cycle block by arresting cells in the G_1 phase and also in the G_2/M phase (Kronemann et al., 1999; Stewart et al., 1999; Van Oirschot et al., 2001). Growth factors such as PDGF induce the ERK pathway in early G_1 phase of the cell cycle. Several studies have indicated the regulation of ERK activation by cAMP (reviewed in Schmitt & Stork 2001). cAMP can inhibit growth factor mediated activation of ERK and also suppress activity of the ERK pathway (Bornfeldt & Krebs 1999; Yu et al., 1997). It is believed this is achieved through the antagonism of Raf activation. However, inhibition of the ERK1/2 pathway is not

always required for cAMP induced growth arrest (Balmanno et al., 2003). Inhibition of ERK by cAMP is cell-specific, in certain cell types cAMP can lead to the activation of ERK. In cells expressing COX-2, activation of the ERK cascade acts as a negative regulator of proliferation through the production of cAMP (Bornfeldt and Krebs 1999).

Raising cGMP levels also attenuates smooth muscle cell proliferation, although not as potently as cAMP (Yu et al., 1997). In contrast to the cAMP-induced block of the cell cycle, it has been observed that cGMP delays G_1/S transition in smooth muscle cells (Fukumoto et al., 1999). This indicates the pathways governed by cAMP and cGMP involved in proliferation are distinct.

In addition to its inhibitory effects on proliferation, cAMP also inhibits migration of smooth muscle cells (Goncharova et al., 2003; Newman et al., 2003). It is unclear how this is achieved, although a link between PKA and inhibition of cell migration is clear in several studies, although this effect is again cell-specific (Sun et al., 2002; Kaufman et al., 2002). In addition, cAMP inhibits the synthesis of some extracellular matrix proteins (Kaji et al., 1996) and also, as mentioned previously is involved in the role of smooth muscle cell contraction/relaxation. It appears that raising cAMP levels is able to inhibit most of the processes involved in the response to vascular injury in smooth muscle cells, making cAMP signalling pathways an attractive therapeutic target in cardiovascular disease such as PAH. Indeed it has been shown that the chronic hypoxic rat displays reduced levels of both cyclic nucleotides in all pulmonary arteries with the exception of the resistance arteries (MacLean et al., 1996). The differing effects of hypoxia on the cyclic nucleotide levels in separate arteries can be explained by the heterogeneity of the smooth muscle cells in each branch. It should be noted that other studies have indicated a rise in cGMP levels in chronic hypoxic rats compared to controls (Cohen et al., 1996). An increase in cyclic nucleotides would be beneficial under hypoxia, aiding to restore the normal tone of the pulmonary circulation. In fact, it has been suggested that hypoxia initially leads to an increase in cAMP through PGI_2 activation, yet after prolonged exposure to hypoxia, desensitisation occurs and pathways are initiated to remove cAMP. In addition, it has been shown that PKA can activate PDE3A and PDE4 in smooth muscle whilst inhibiting AC6, altogether reducing cAMP levels (Murthy et al., 2002).

1.5.8.3 CREB

The cAMP response element binding protein (CREB) is activated by PKA and is responsible for cAMP effects on gene expression (reviewed in Mayr & Montminy 2001; Shaywitz & Greenberg 1999). PKA activates CREB through phosphorylation on Ser^{133}. Phosphorylated CREB is then able to bind to cAMP responsive elements (CRE) in the promoter region of cAMP inducible genes.

CREB has also been reported to be phosphorylated at Ser^{133} by PKG (Pilz et al., 2003), Akt (Shaywitz et al., 1999), and PKC (Saeki et al., 1999) amongst other kinases (reviewed in Johannessen et al., 2004). Other sites in CREB also act as phosphoacceptor sites including Ser^{117} and Ser^{121} including hypoxia induced kinases (Taylor et al., 2000).

Hypoxia has been shown to lead to a rapid phosphorylation of CREB at Ser^{133} in a manner more potent than that of forskolin in PC12 cells (Beitner-Johnson & Millhorn 1998). CREB phosphorylation was not mediated through PKA or any of the pathways known to activate CREB. In addition, the phosphorylation of CREB was sustained for 24 hours which is in contrast with the typical rapid phosphorylation/dephosphorylation of CREB. The authors of this study suggested that the maintained CREB phosphorylation implicated a role for CREB in the cells adaptive response to hypoxia. Indeed, it is already known that the CREB coactivator CBP/p300 interacts with HIF-1α and participates in the transcriptional regulation of hypoxia induced genes (Arany et al., 1996). In smooth muscle cells, active CREB is apparent in proliferating cells and reduced in those that are proliferation-resistant (Klemm et al., 2001). Indeed, it was reported that CREB levels were reduced in hypoxia where smooth muscle cells are known to exhibit increased proliferation. The activity of CREB was not measured however. These studies implicate CREB in the hypoxic response and a further investigation of CREB regulation and function could aid in the understanding of hypoxia regulated gene expression.

1.5.8.4 Phosphodiesterases

Phosphodiesterase (PDE) activity is critical for the tight regulation of cyclic nucleotide levels. An increase in PDE activity reduces the levels of cyclic nucleotides and thus the effect of cAMP or cGMP on smooth muscle functioning. The lung is known to express all PDEs except the retinal specific PDE6 (Polson & Strada 1996; Soderling & Beavo 2000; Yuasa et al., 2000; Koyama et al., 2001). This presents the possibility that the actions of cyclic nucleotides in the pulmonary circulation are dependant on the activities of the PDE families. Indeed, it has been demonstrated that both cAMP-PDE and cGMP-PDE activity

increase in the pulmonary arteries of the chronic hypoxic rat (MacLean et al., 1997). Within the pulmonary artery, PDE1, PDE2, PDE3, PDE4 and PDE5 activities have all been demonstrated (Pauvert et al., 2002). Inhibitors of each of these families were also demonstrated to relax precontracted vascular smooth muscle preparations (Pauvert et al., 2002).

A role for PDE1C in vascular smooth muscle cells has already been mentioned (Rybalkin et al., 2002) and it is also recognised that PDE1C isoform expression in vascular smooth cells differs between species (Palmer & Maurice 2000). This is also thought to be the case with PDE2 expression in vascular smooth muscle cells.

PDE2 displays low activity levels and although PDE2 inhibitor use uncovered a role for PDE2 in hypoxic pulmonary constriction in rat lung in one study (Haynes et al., 1996), a regulatory function for PDE2 in vascular smooth muscle cell function has not been reported.

Both genes of the PDE3 family are expressed in vascular smooth muscle cells and have been demonstrated to be induced in response to cAMP-elevating agents in vivo (Tilley & Maurice 2002). However, maintained increases in cAMP levels in cultured rat and human aortic SMC only increased levels of PDE3B (Palmer & Maurice 2000). Chronic hypoxia has been observed to increase PDE3 activity in rat pulmonary vessels (MacLean et al., 1997). This increase can be explained by the induction of both PDE3A and PDE3B in chronic hypoxic rat pulmonary arteries (Murray et al., 2002). In the same study, a cAMP-mediated increase of the PDE3A isoform in hypoxic hPASMC was demonstrated.

The use of PDE3 inhibitors on vascular SMC has been demonstrated to inhibit proliferation (Johnson-Mills 1998), migration (Palmer et al., 1998) and act as a vasodilator (Bardou et al., 2001). Indeed, cilostamide attenuates hypoxia induced PAH (Phillips et al., 2000). Unfortunately, PDE3 inhibitors also affect the systemic circulation and could lead to harmful side effects if employed as a treatment. Thus, newly developed PDE3 inhibitors are currently under investigation as putative therapies for cardiovascular diseases. Milrinone and SCA40 have been shown to be potent relaxants of preconstricted pulmonary arteries (Jeffrey & Wallstall 1998). Milrinone has also been reported to significantly decrease PAP in the hypoxic dog and heart failure patients (Jaski et al., 1985; Kato et al., 1998).

In rat and human vascular SMC, PDE5A1 and PDE5A2 are expressed (Murray et al., 2002; Rybalkin et al., 2002). PDE5 is well known to be involved in the regulation of smooth muscle cell relaxation. Under conditions of chronic hypoxia, PDE5 expression and activity are observed to increase in rat pulmonary arteries and hPASMC (Murray et al., 2002), specifically the PDE5A2 isoform. In other animal models of PAH, this result is also observed (Hansen et al., 1998; Black et al., 2001). Consistent with these studies, inhibitors of PDE5 are observed to inhibit proliferation and migration of vascular SMC, enhance NO-protection against vascular leakage and act in a pulmonary specific manner (Osinski et al., 2001; Schutte et al., 2000; Eddahibi et al., 1998). All of these mechanisms can attenuate the development of PAH. Indeed, the PDE5 inhibitor sildenafil has been shown to be effective at improving the prognosis for PAH patients with no effect on the systemic pressure (Zhao et al., 2001; Michelakis et al., 2003). In the chronic hypoxic rat, treatment with sildenafil prior to hypoxic exposure protects against the rise in PAP and inhibits vascular remodelling (Sebki et al., 2003).

PDE4 inhibitors have been developed for use in inflammatory conditions such as COPD due to their ability to suppress the activity of immune and inflammatory cells and reduce airway smooth muscle hypertrophy and hyperplasia (Vignola 2004; Schmidt et al., 1999; Essayan 1999; Spina 2003). Recently, new PDE4 inhibitors, cilomilast (Ariflo) and roflumilast, have been developed for use in the treatment of COPD and asthma (Giembcyz 2001; Compton et al 2001; Underwood et al., 1998). Investigation into the role of PDE4 identified PDE4A and PDE4B as the major regulators of inflammatory cell functions with 4D also mediating in part (Manning et al., 1999). PDE4 and PDE3 inhibitors are able to cause relaxation of bronchial rings and when used in tandem, a much greater bronchorelaxant effect is seen than when used separately. Airway SMC proliferation has also been shown to be regulated by cAMP levels (Tomlinson et al., 1995). PDE4 inhibitors are known to display similar results in vascular SMC and therefore have been considered for use in pulmonary disorders. Indeed PDE4 inhibitors are also capable of inhibiting SMC migration (Palmer et al., 1998) and proliferation (Chen et al 2002, Ogawa et al 2002). In both proliferation and migration studies, PDE4 inhibitors or PDE3 inhibitors used alone have mild effects, when used together they synergise to potentiate their effect (Palmer et al., 1998). Due to the success of clinical trials using PDE4 inhibitors as a treatment for COPD, the potential for PDE4 inhibitors in modulating the response to hypoxia is apparent. It is as yet unknown what effect, if any, hypoxia has on PDE4 in vascular SMC. However, a differential expression of PDE4 in cases of COPD has been observed (Barber et al., 2004). It would therefore be beneficial to uncover the PDE4 isoforms expressed in

pulmonary arterial cells and to distinguish isoform-specific functions. In this study, I set out to identify the PDE4 isoforms expressed in human pulmonary artery smooth muscle cells and investigate their role in the altered cyclic nucleotide levels apparent in hypoxia. In addition, the cAMP regulation of proliferation was investigated.

Adenylyl Cyclase Isoform	High Expression	Potential Function
I	Brain	Circadian rhythm, learning, memory
II	Brain, lung	Synaptic plasticity, cell proliferation arrest
III	Olfactory epithelium, pancreas	Odorant stimulation
IV	Widespread	
V	Brain, heart,	
VI	Heart, kidney, widespread	Cell proliferation
VII	Widespread	Ethanol dependancy
VIII	Brain, pancreas	Synaptic plasticity
IX	Pituitary, widespread	

Table 1.1 Summary of adenylyl cyclase isoform distribution.
This table summarises the current knowledge of specific adenylyl cyclase isoform distribution throughout the body and potential associated functions.

Adenylyl Cyclase Isoform	Gsα	Gsβγ	Ca2+	PKC
I	+	-	+	0
II	+	?	0	+
III	+	0	?	?
IV	+	+	0	?
V	+	0	-	+ (ξ)
VI	+	0	-	?
VII	+	+	0	+
VIII	+	?	+	?
IX	+	?	0 (inhibited by calcineurin)	?

Table 1.2 Summary of adenylyl cyclase regulation

This table summarises the regulation of each adenylyl cyclase isoform. Question marks represent modes of regulation that have not been fully investigated for that isoform. Stimulation is represented by + and inhibition by -. 0 signifies no effect on that isoform.

PDE family	Nucleotide specificity	Inhibitors	Regulation
PDE1	cAMP/cGMP	Nicardipine, vinpocentine, zaprinast	(+) Ca^{2+}/calmodulin (-)PKA/PKG, CamKII
PDE2	cAMP/cGMP	EHNA	(+) cGMP, PKC
PDE3	cAMP	Cilostamide, cilostazol, milrinone, amrinone	(+) PKA, PKB (-) cGMP
PDE4	cAMP	Roflumilast, Rolipram, Ro-20-1724, cilomilast, denbufylline, BAY 19-8004	(+)PKA, ERK, PA (-)ERK, caspases
PDE5	cGMP	Sildenafil, DMPPO, vardenafil, tadalafil Zaprinast	(+) cGMP, PKA, PKG (-) caspases
PDE6	cGMP	Zaprinast, dipyridamole, sildenafil, DMPPO	(+)Transducin (-) cGMP
PDE7	cAMP	Dipyrimidamole	(+/-) PKA
PDE8	cAMP	Dipyridamole	PAS domain
PDE9	cGMP	Zaprinast	
PDE10	cAMP/cGMP	Dipyramidole	(-) cAMP
PDE11	cAMP/cGMP	Dipyramidole, zaprinast	

Table 1.3 Summary of phosphodiesterase family regulation

This table summarises substrate specificity and regulatory mechanisms for each of the phosphodiesterase families. Compounds known to inhibit each family are also shown, not all are selective. Stimulation is represented by +, and inhibition by -.

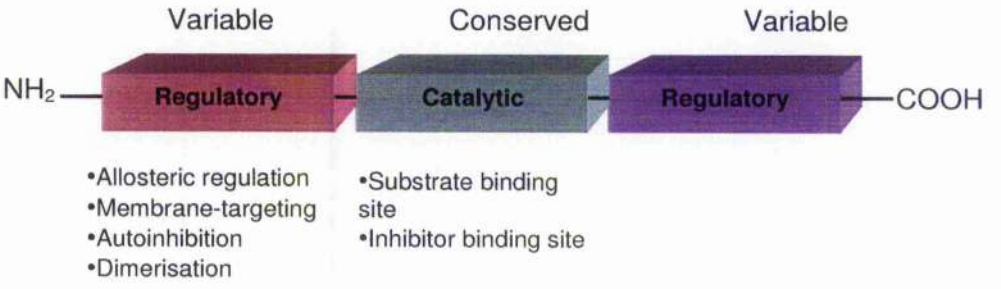


Figure 1.1 Modular structure of phosphodiesterases

All phosphodiesterase families share a common modular structure with a unique N-terminal involved in regulation, a catalytic domain responsible for nucleotide hydrolysis which is conserved throughout all PDEs and a unique C-terminal domain.

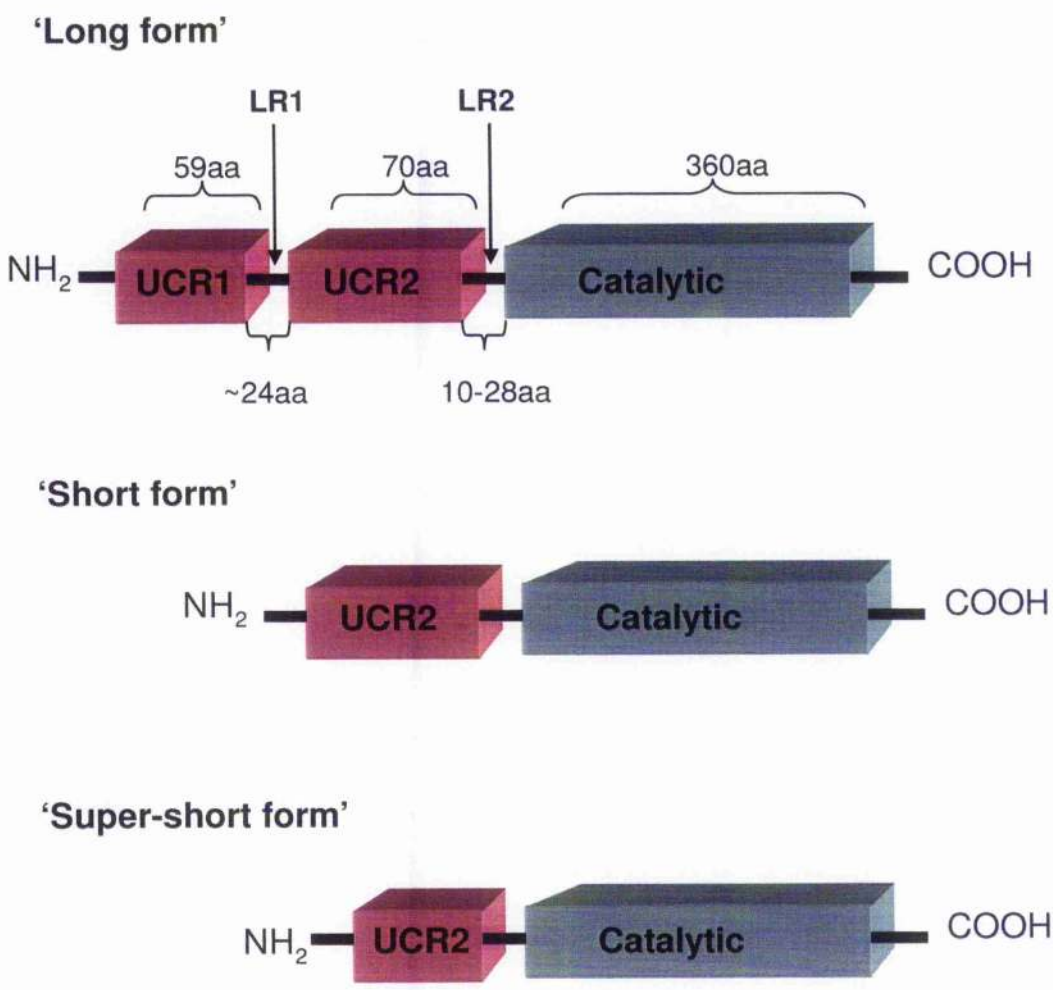


Figure 1.2 Splice variants of the PDE4 family

The PDE4 family are characterised by the presence of upstream conserved regions known as UCR1 and UCR2 at their N-terminus. The region that links UCR1 and UCR2 together is called linker region 1 (LR1), and linker region 2 (LR2) connects UCR2 to the catalytic domain. The linker regions differ between the different PDE4 families. Alternative mRNA splicing of members of the PDE4 family gives rise to "long" isoforms containing both UCR1 and UCR2 and "short" isoforms with only UCR2 are produced. An additional splice site also generates "super-short" isoforms which have an N-terminally truncated UCR2.

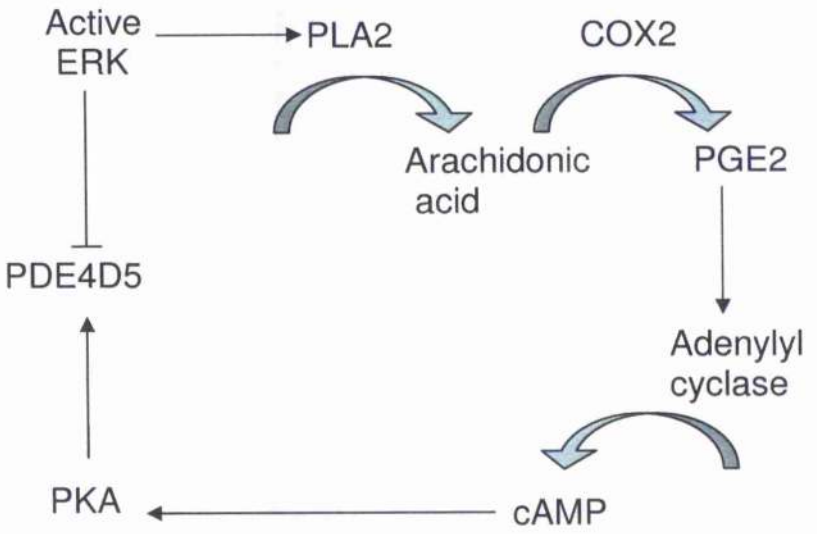


Figure 1.3 Autocrine activation of PDE4D5 by ERK

ERK activation inhibits PDE4D5 activity through direct phosphorylation. In human aortic smooth muscle cells however, ERK leads to the autocrine production of PGE_2 which stimulates adenylyl cyclase. cAMP levels then rise and PKA is activated and can then phosphorylate PDE4D5. Due to the rapidity and magnitude of ERK dependant PKA phosphorylation of ERK, activation overall is achieved (Baillie et al., 2001).

PDE Family	Disease states in which PDE plays a role	References
PDE1	Parkinsons's disease Tumorigenesis	Kakkar et al., 1999; Kakkar et al., 1997
PDE3	PAH	Wagner et al., 1997; MacLean et al., 1997; Murray et al., 2002
PDE4	Asthma, COPD, depression, malignant gliomas, PAH, acute lymphoblastic leukemia, ischemic stroke	Torphy 1998; Schmidt et al., 1999; Schudt et al., 1999; Torphy et al., 1999; Spina 2003; Gale et al., 2002; Nemoz et al., 1985; Grootendorst et al., 2003; Chen et al., 2002; Ghoframi et al., 2004; Hatzelmann & Schudt 2001; Wagner et al., 1997;Goirand et al., 2001; Ogawa et al., 2002; Gretarsdottir et al., 2003
PDE5	Lung ischemia reperfusion injury, PAH	Schutte et al., 2000; Sebkhi et al., 2003; Michelakis et al., 2003; Eddahibi et al., 1998; Goirand et al., 2001; MacLean et al., 1997; Murray et al., 2002

Table 1.4 Summary of disease states PDEs are reported to play a role

This table summarises some of the diseases PDE inhibitors are therapeutic in or diseases in which the PDE family has been implicated. References for the named disorders are also included.

Revised Clinical Classification of Pulmonary Hypertension (Venice 2003)

1. Pulmonary arterial hypertension (PAH)

1.1 Idiopathic (IPAH)

1.2 Familial (FPAH)

1.3 Associated with (APAH):

1.3.1 Collagen vascular disease

1.3.2 Congenital systemic-to-pulmonary shunts

1.3.3 Portal hypertension

1.3.4 HIV infection

1.3.5 Drugs and toxins

1.3.6 Other

1.4 Associated with significant venous or capillary involvement

1.4.1 Pulmonary veno-occlusive disease (PVOD)

1.4.2 Pulmonary capillary hemangiomatosis (PCH)

1.5 Persistent pulmonary hypertension of the newborn

2. Pulmonary hypertension with left heart disease

2.1 Left sided atrial or ventricular heart disease

2.2 Left sided valvular heart disease

3. Pulmonary hypertension associated with lung diseases and/or hypoxemia

3.1 Chronic obstructive pulmonary disease

3.2 Interstitial lung disease

3.3 Sleep-disordered breathing

3.4 Alveolar hypoventilation disorders

3.5 Chronic exposure to high altitude

3.6 Developmental abnormalities

4. Pulmonary hypertension due to chronic thrombotic and/or embolic disease

4.1 Thromboembolic obstruction of proximal pulmonary arteries

4.2 Thromboembolic obstruction of distal pulmonary arteries

4.3 Non-thrombotic pulmonary embolism

5. Miscellaneous

Figure 1.4 Venice classification of pulmonary arterial hypertension.

With the variety of new disorders proven to lead to pulmonary arterial hypertension, the previous classification of PAH was considered to be outdated. Thus, in the Venice 2003 meeting, the classification was revised. Adapted from Simonneau et al., 2004.

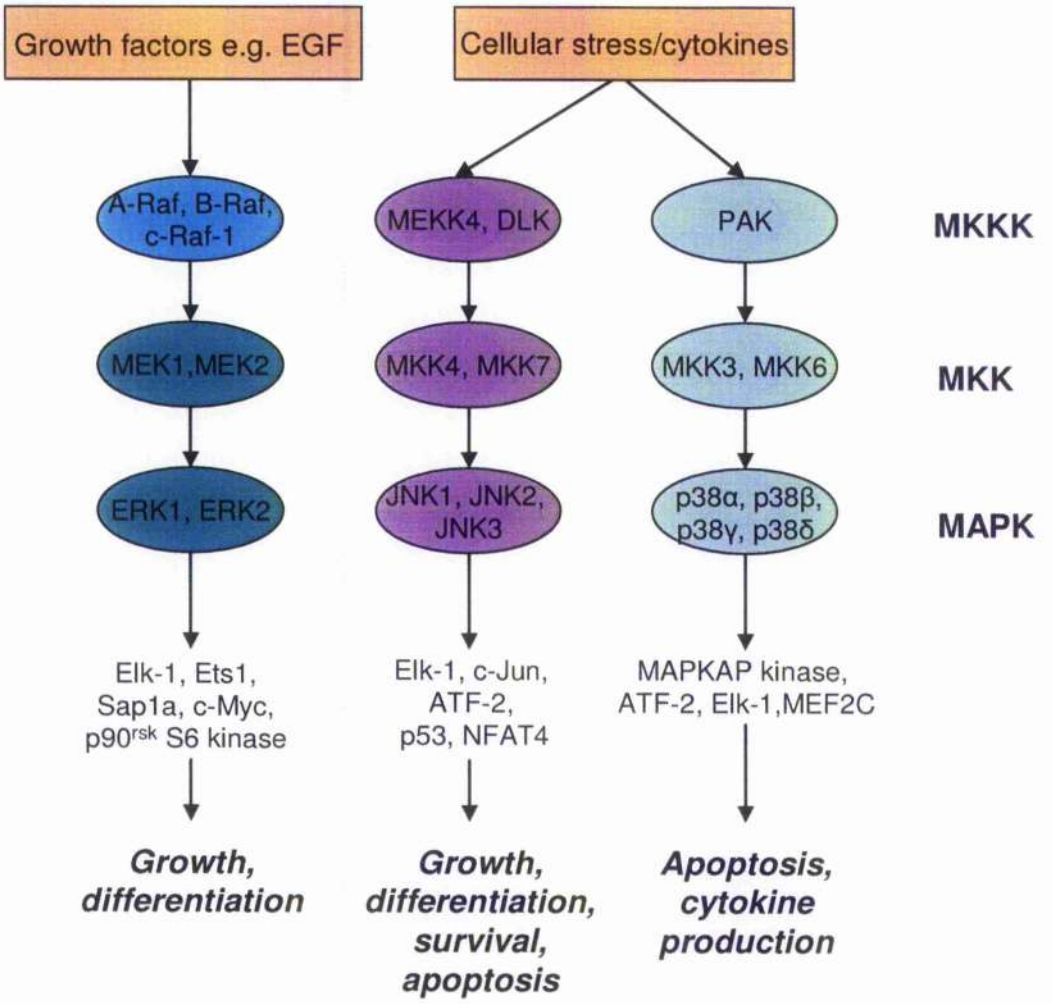


Figure 1.5 Activation of MAPK

The ERK, JNK and p38 cascades are the classic examples of MAPK cascades. Upon stimulus, a MAP kinase kinase kinase, MAPKKK, activates its MAP kinase kinase MAPKK. This leads to activation of the MAP kinase which can then relocate to the nucleus and regulate gene expression through its action on transcription factors.

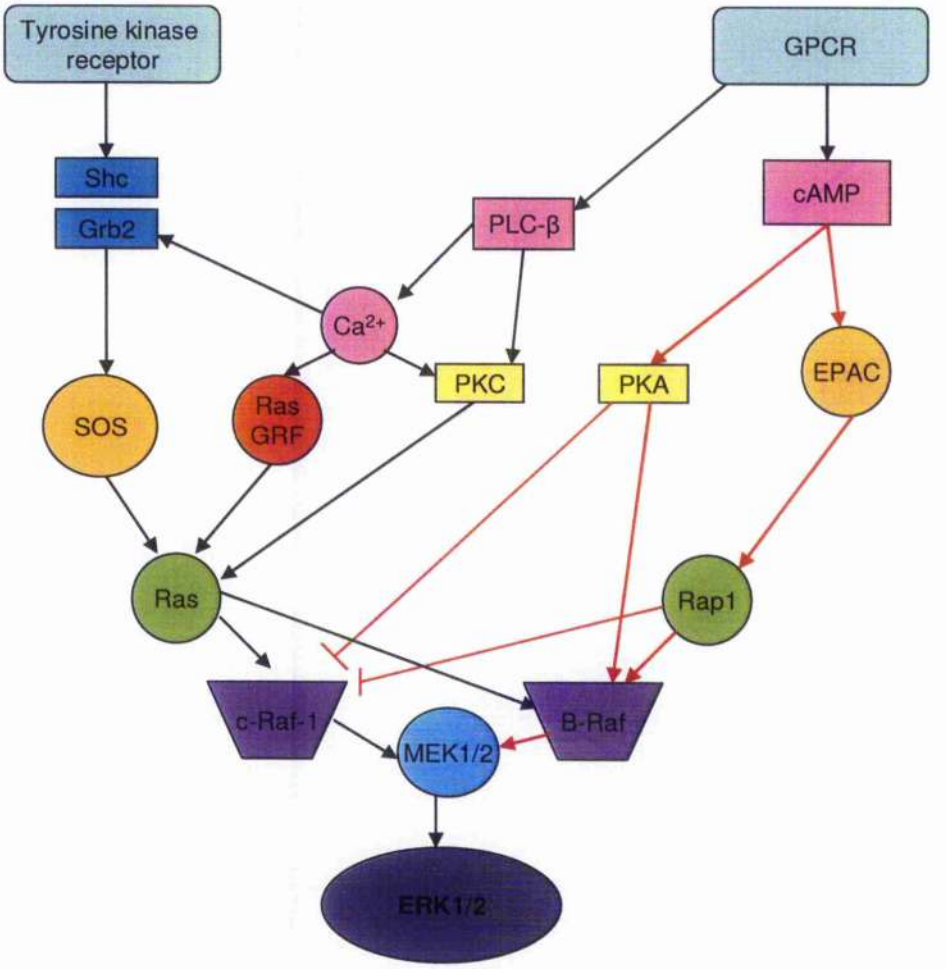


Figure 1.6 ERK pathway

The ERK pathway can be activated through the actions of a stimulated G-protein coupled receptor or activated tyrosine kinase receptor. The intracellular cascades have different points of cross talk where they can 'finely-tune' the ERK response following stimulation. Red arrows signify the cAMP pathway involved in ERK activation.

Chapter 2

Materials and Methods

2.1 Mammalian Cell Culture

2.1.1 Maintenance of hPASMC

2.1.1.1 Human Pulmonary Artery Smooth Muscle Cells

Human Pulmonary Artery Smooth Muscle Cells (hPASMC) are obtained commercially from Cambrex Biowhittaker and are derived from 1st and 2nd order pulmonary arteries. Each vial of cryopreserved cells came with a certificate of analysis showing guaranteed viability of cells, presence of smooth muscle cell markers and negative viral detection. hPASMC were maintained in Smooth Muscle Growth Medium (SmGm, Cambrex) which had been optimized for the growth of smooth muscle cells. The SmGm was prepared using Smooth Muscle Basal Medium (SmBm) supplemented with 5% Foetal Calf Serum (FCS), gentamycin GA-1000 (50µg/ml) penicillin/streptomycin (100 units/ml), human fibroblast growth factor-B (2ng/ml), human EGF (0.5 ng/ml) and insulin (5µg/ml). These were all available from Cambrex in single use aliquots as a SmGm-2 bullet kit. Cells were seeded into 25cm^2 flasks at the recommended seeding density of 3500cells/cm^2 using the following calculations:

Max. area that can be plated = No. of cells available/Recommended seeding density

Max. no. flasks prepared = Max. surface area that can be plated/Growth area of flask

Flasks were prepared with 1ml SmGm-2/5cm^2 growth area of flask at 37°C in a 5% CO_2 atmosphere for 30 minutes prior to seeding. The day after seeding, and every other day thereafter, the SmGm-2 was replenished to remove unattached cells and waste from the cells. As the cells became more confluent, 2ml SmGm-2/5cm^2 growth area of flask was used. Cells were maintained in a Heraues $C0_2$ incubator set at 37°C in an atmosphere of 5% CO_2. The cells were examined daily for any signs of stress, for example atypical morphology. Each vial of cells arrived at passage 3 and were used at passage 5-7 for all experiments. Cambrex guarantee normal morphology and proliferation rates of cells until passage 10.

2.1.1.2 Passaging of hPASMC

Cells were passaged at 85% confluency. This was achieved by rinsing the cells in SmBm to remove all traces of serum. To detach the cells from the culture vessel, 2mls/100mm plate of Trypsin/EDTA (145mM NaCl, 5.4mM KCl, 8.2mM Na_2HPO_4, 1.5mM KH_2PO_4, 42mM Trypsin, 10mM Glucose and 0.7mM EDTA) was added to the culture flask which

was then left at room temperature. After 5 minutes incubation with Trypsin, the culture vessel was rapped gently to detach any remaining adherent cells. Complete cell detachment was checked by microscopic examination. The cells were then collected in 2mls/100mm plate of fresh SmGm-2 and centrifuged at 700rpm for 3 min in the MSE Mistral 1000 swinging bucket centrifuge. The Trypsin/SmGm-2 solution was aspirated off the cells before resuspending them in prewarmed SmGm-2. The cells were then plated out at the optimal seeding density of 3500 cells per cm^3 of growth area. SmGm-2 was replaced every other day.

2.1.1.3 Hypoxic hPASMC Model

At the desired passage number and confluency, the culture vessels were split into two groups following replenishment of SmGm-2. One group was returned to the normoxic incubator whilst the other group were transferred to a hypoxic incubator (Wolf Laboratories – Galaxy CO_2 Galaxy incubator with 2-99% oxygen control) at an atmosphere of 5% CO_2 (550 bar), 10% O_2 balance N_2 (300 bar). Cells were initially maintained in the hypoxic incubator for 24 hours – 14 days to determine the optimal time point to investigate signalling. At all times, the hypoxic cells were treated identically to the control cells.

2.1.1.4 Treatment of hPASMC with Agonists/Inhibitors

Prior to treating hPASMC with various drugs, the cells were quiesced for 24 hours by replacing the growth medium with SmBm.

2.1.2 Transfection of hPASMC with the Amaxa Nucleofactor

hPASMC were transfected using a nucleofector and human aortic smooth muscle cell nucleofector kit (Amaxa) according to manufacturer's instructions. Briefly, hPASMC were trypsinsed as in section 2.1.1.2 and $1x10^6$ cells/transfection were resuspended in 100µl nucleofector solution. This was placed into a cuvette and inserted into the nucleofector that was set at programme U-25. Once the programme was finished, the cells were gently transferred into 3 wells of a 6-well culture plate containing SmGm and left at 37°C in an atmosphere of 5% CO_2 for 4-24 hours.

2.1.2.1 Transfection of siRNA constructs

Using the nucleofector as described in section 2.1.2, siRNA samples were transfected into hPASMC. The siRNA samples were diluted to 60nM prior to use and after nucleofection, the cells were gently transferred into 3 wells of a 6-well culture plate containing SmGm and left at 37°C in an atmosphere of 5% CO_2 for 24 hours.

2.2 Proliferation Analysis

2.2.1 DNA Synthesis Assays

2.2.1.1 Assay preparation

hPASMC were plated out into 96-well tissue culture plates at a seeding density of 1000 cells/well 5 days prior to the DNA synthesis assay. Cells were quiesced in SmBm for 24 hours prior to treatments. Smooth muscle growth medium containing 5% FCS was reintroduced after this time.

2.2.1.2 Addition of [^{3}H]-Thymidine

Twenty hours after additions, 0.5µCi/well [^{3}H]-Thymidine (Amersham) was added to the plates. The cells were placed in their respective incubators for a further four hours to allow the tritiated Thymidine to incorporate before the plates were placed at -20°C to stop proliferation.

2.2.1.3 Measuring radioactivity

The 96-well tissue culture plates were allowed to thaw at room temperature for two hours before using a Betaplate 96-well harvester (Wallac) to harvest the plates onto glass fibre filter mats (Wallac). These were then placed into sample bags (Wallac) before being placed into a 1205 Betaplate Liquid Scintillation Counter (Wallac) and [^{3}H]-Thymidine incorporation, expressed as counts per minute (cpm), was measured.

2.3 Biochemical Analysis

2.3.1 Harvesting Cell Lysate

2.3.1.1 Whole cell lysate production

Cells were harvested on ice and all buffers were pre-chilled. Cell media was removed from plates and the cells were rinsed in ice-cold PBS. The cells were drained before scraping into a 1.5ml eppendorf in 350µls / 100mm plate complete KHEM buffer (50mM KCl, 10mM EGTA, 1.92mM EGTA, 59mM HEPES-KOH pH 7.2 plus complete, EDTA-free protease inhibitor cocktail tablets (Roche) and 1% Triton X-100. The cells were homogenized by passing them through a 26 gauge needle 12 times whilst on ice. The cells were then centrifuged at 13000rpm at 4°C for 3 minutes (Biofuge fresco, Heraeus) to remove cellullar debris. The supernatant was then removed, aliquoted and used for analysis or snap frozen in dry ice before being stored at -80°C.

2.3.2 Determination of protein concentration (Bradford assay)

Protein assays were carried out in 96 well microtitre plates. 2µl of sample was added to 48µl dH_2O and 200µl Bio-rad reagent (diluted 1/5 with distilled water). Each sample was analysed in triplicate. Protein concentration of samples was calculated by measuring the absorbances and constructing a standard curve using known concentrations of Bovine Serum Albumin (BSA). This was achieved by using the Revelation package on the computer, connected to an MRX microtitre plate reader, which read absorbance at a wavelength of 590nm. Protein concentrations were determined by plotting the standard curve and using least squared regression analysis to obtain the line of best fit. The equation of the line was used to determine the protein concentration of the samples.

2.3.3 SDS-PAGE Electrophoresis

2.3.3.1 Sample preparation for SDS-PAGE electrophoresis

Lysates were obtained from 100mm dishes scraped into 350µl of cKHEM + 1% Triton X-100 buffer and spun at 13000 rpm for 3 mins at 4°C. The supernatant was kept and used for protein measurement before boiling in Hannah sample buffer (260 mM Tris/HCl (pH6.7), 55.5% Glycerol, 8.8% SDS, 0.007% Bromophenol blue, 11.1% 2-mercaptoethanol, Laemmli et al., 1970), for 5 minutes to denature the proteins.

2.3.3.2 Casting and running an acrylamide gel

When using over 50µg protein, a Bio-Rad Protean II system was used. 16cm thick plates were rinsed with distilled water and ethanol prior to use. The apparatus was assembled according to the manufacturer's instructions. A resolving gel containing the appropriate percentage of acrylamide (determined by the molecular weight of the protein of interest), usually 10% unless otherwise stated, was cast between the two plates of the gel apparatus (10% 29:1 acrylamide:N,N'-methylenebisacrylamide mix, 375mM Tris/HCl (pH8.8), 0.1% SDS, 0.1% Ammonium persulphate, 0.06% N,N,N',N',-tetramethylethylenediamine (TEMED)).This was overlaid with water to ensure an even gel and allowed to polymerise at room temperature for one hour. The water was then removed and a 5% stacking gel was poured, (5% 29 :1 acrylamide:N,N'-methylenebisacrylamide mix, 125mM Tris/HCl (pH6.8), 0.1% SDS, 0.1% Ammonium persulphate, 0.1% TEMED). A comb was carefully inserted immediately between the plates. The stacking gel was allowed to polymerise for 30 minutes before the comb was removed and the wells were washed with tank buffer (192mM Glycine, 25mM Tris, 0.15% SDS) to remove any unpolymerised acrylamide. The gels were then placed in a running tank containing tank buffer in both upper and lower reservoirs. Bio-Rad prestained broad range precision protein markers were loaded into the

first well to allow determination of protein weight by gel electrophoresis migration. Prepared samples were loaded into the wells and the gels were run at the appropriate current (8mA overnight) until the dye front reached the bottom of the gel.

2.3.3.3 Running a NuPAGE gel

When using less than 50µg protein, the Novex NuPAGE gel systems were used with Invitrogen pre-cast 4-12% Tris-Glycine gels. Samples were prepared as in 2.3.3.1. Tank buffer used was either MOPS buffer or MES buffer depending on the size of the protein wishing to visualize. Gels were run for 45 minutes at 200V.

2.3.4 Western Blotting

2.3.4.1 Protein transfer from acrylamide gel to nitrocellulose membrane

Following electrophoresis, the gels were placed into a transfer cassette. Under transfer buffer (192mM Glycine, 25mM Tris, 20 % Methanol), a piece of foam was overlaid with a piece of Whatmann 3MM filter paper. The gel was placed on top of this and overlaid with a piece of Protran nitrocellulose paper (Schleicher & Schuell). The nitrocellulose was covered with another piece of Whatmann filter paper and finally another piece of foam. During assembly, care was taken to ensure air bubbles were excluded from all layers. The cassette was closed and placed into a Hoefer transfer tank filled with transfer buffer with the nitrocellulose side of the cassette to the positive electrode. The proteins were transferred for 0.06 amps overnight or 0.6 amps for 1.5 hours.

2.3.4.2 Blocking of nitrocellulose

Once the proteins had been transferred onto the nitrocellulose as in section 2.3.4.1., the membrane was washed with distilled water followed by visualisation of the transferred proteins with Ponceau S stain (0.1% Ponceau S, 3% Trichloroaceatic acid). The Ponceau S stain was added to the nitrocellulose for a few minutes until the protein became stained, the nitrocellulose membrane was then washed with water and rinsed with Tris-buffered saline (TBS-tween 20) (137mM NaCl, 20mM Tris/HCl (pH7.6), 0.1% tween20) before immunological detection of protein. The unoccupied protein binding sites on the nitrocellulose were blocked with 5% skimmed milk powder (Marvel) in TBS-tween20 for 1 hour at room temperature with gentle agitation.

2.3.4.3 Immunoblotting

After blocking, the appropriate primary antibody was added at the relevant dilution (see table 2.1) in 2% skimmed milk powder in TBS-tween20. This incubation was carried out for 1-2h at room temperature or overnight at 4°C with vigorous agitation. The membrane

was washed three times in TBS-tween20 for 5 minutes before being incubated with the appropriate diluted secondary antibody mix for 1 hour at room temperature with vigorous agitation. The secondary antibody (Sigma) was a horse-radish peroxidase (HRP) conjugated anti-immunoglobulin (IgG) antibody directed against the primary antibody. This antibody was diluted 1:5000 in 2 % skimmed milk powder in TBS-tween20. The nitrocellulose was finally washed 3-5 times in TBS-tween20 for 5 minutes. To detect immunoreactive bands, the membrane was incubated with ECL reagents (Amersham) for a minute with gentle rocking. A piece of x-ray film (Kodak) was exposed to the membrane in a darkroom for the appropriate amount of time and then developed using a Kodak X-omat.

2.3.5 Phosphodiesterase 4 Activity Assay

Phosphodiesterase activity was measured using a modification of the two step procedure of Thompson and Appleman [Thompson and Appleman., 1971], as described previously by Marchmont and Houslay [Marchmont and Houslay, 1980]. The first step is the hydrolysis of the ^{3}H-cyclic nucleotide (8 position of the adenine or guanine ring) by the phosphodiesterases, which generates labelled nucleotide monophosphate. In the second step, incubation with snake venom, (which has 5'-nucleotidase activity), converts the mono-phosphate ring to the corresponding labelled nucleoside. The unhydrolysed cyclic nucleotide was separated from the nucleoside by batch binding of the mixture to Dowex 1X8-400 anion exchange resin. This binds only the charged nucleotides and leaves behind the uncharged nucleosides.

2.3.5.1 Activation of Dowex

To activate the Dowex 1X8 -400, 4l of 1M NaOH was added to 400g of the resin, the mixture was stirred for 15 min at room temperature. The resin was allowed to settle and the supernatant was poured off. The resin was then washed 30 times with 4l distilled water. The resin was then resuspended in 4l 1M HCl and stirred for 15 min at room temperature before being allowed to settle by gravity. The resin was then washed 5 times with 4l distilled water. The activated resin was stored at 4°C as a 1:1 slurry with distilled water until required.

2.3.5.2 Sample preparation for assay

Into 1.5ml eppendorf tubes an appropriate amount of cell lysate was placed (30-60 µg protein depending on the concentration of the sample) and made up to a volume of 50µl with PDE assay diluting buffer (20mM Tris/HCl, pH7.4). Each sample tube was repeated in triplicate. All tubes were set up on ice, and remained on ice until all components for the

assay had been added. To each tube 50µl of 2µM cAMP containing 3µCi [^{3}H]cAMP in 20mM Tris/HCl /10mM $MgCl_2$ pH7.4 was added, the tubes vortexed and incubated at 30°C for 10 min. After this time, the tubes were boiled for 2 min to inactivate any PDE present and then cooled on ice for 15 minutes. 25µl of 1mg/ml snake venom in 20mM Tris/HCl, pH 7.4 was added to each tube, mixed by vortexing and incubated at 30°C for 10 minutes. The tubes were then cooled on ice for 15 minutes and 400µl Dowex/ethanol/water, in a 1:1:1 ratio, was added to the tubes, vortexed and incubated on ice for at least 20 min. The tubes were vortexed again, the dowex removed by centrifugation at 13000 rpm for 3 min (Heraeus bench top centrifuge) and 150µl of the clear supernatant added to 1ml Opti-scint scintillation fluid and counted on a Wallac 1409 liquid scintillation counter.

2.3.5.3 Determination of PDE3 and PDE4 activity

The PDE3 family is specifically inhibited by the drug cilostamide (Hidaka et al., 1979) and the PDE4 family by rolipram (Wachtel, 1982). PDE inhibitors were dissolved in 100% DMSO as 10mM stocks and diluted in PDE assay dilution buffer for use in assay. The residual levels of DMSO do not affect PDE activity at the concentrations used (Spence et al., 1995). Measurement of PDE activity with and without cilostamide (10µM) and with and without rolipram (10µM) present gave the contribution of PDE3 and PDE4 respectively.

2.3.6 Immunoprecipitation

2.3.6.1 Pre-clearing agarose beads

Protein G beads were used when the target protein was immuno-precipitated with a monoclonal antibody or a polyclonal antibody raised in a sheep and protein A beads were used when the target protein was immuno-precipitated with any other polyclonal antibodies. 25 µl of the appropriate beads were washed in 200 µl cKHEM buffer and recovered by centrifugation at 13000 rpm for 2 min at 4 °C in a refrigerated bench-top centrifuge. 300 µg of protein from cell lysate, diluted to a total of 500 µl with lysis buffer supplemented with protease inhibitors, was added to the washed beads and incubated with end-over-end rotation at 4 °C, for 30 min.

2.3.6.2 Binding target protein to antibody

After pre-clearing the beads were collected by centrifugation at 13000 rpm at 4 °C for 2 min (Heraeus refrigerated bench-top centrifuge), the supernatant was removed and placed

into a fresh eppendorf tube. To this fresh tube 7 µl of the polyclonal antibody or 3 µl monoclonal antibody was added and the tube rotated end-over-end at 4 °C for at least 2 h, to enable the protein and antibody to bind. The solution was then transferred into a tube containing 60 µl pre-washed protein beads (A or G as required) and rotated end-over-end overnight at 4 °C. The beads were isolated from solution by centrifugation at 13000 rpm at 4 °C for 2 min (Heraeus refrigerated bench-top centrifuge) and washed to remove any non-specifically bound protein. The beads were washed firstly in lysis buffer supplemented with 500 mM NaCl, secondly with lysis buffer supplemented with 0.1 % SDS and then washed with 0.1 % NP-40 in 10 mM Tris pH 7.4. The final wash was carried out in 20 mM Tris (pH 7.4) to prepare samples for use in a PDE assay.

2.3.7 Cyclic AMP Assay

Intracellular cAMP levels were measured using a modification of the procedure of Savage. (Savage, 1995). The assay involves the measurement of cAMP by the use of the cAMP-binding protein, cyclic AMP-dependant protein kinase. (Brown, 1972) Briefly, following stimulation of the cells, they were lysed to release the cytosolic cylic nucleotides. After neutralization, the lysate was incubated with [5', 8' -^{3}H] cyclic AMP and cyclic AMP binding protein, allowing competition of labelled and unlabelled cyclic AMP for a limited number of binding sites on the binding protein. Activated charcoal was then added to the sample to bind any free cyclic nucleotide and the charcoal pelleted by centrifugation. The radioactivity of the supernatant was then measured. A standard curve was generated by incubating a range of known concentrations of cAMP with the fixed amounts of binding protein and radioactive cyclic AMP. It was then possible to determine cAMP concentrations from the radioactivity counted by reference to the standard curve.

2.3.7.1 Agonist Treatment

Cells were plated out in 6-well plates at the optical seeding density of 3500cells/cm^2 four days prior to treatment. Cells were serum starved overnight prior to agonist stimulation. Agonists were usually made to a stock solution of 10mM in 100% DMSO and were diluted in SmBm to a desired concentration prior to addition to cells. Each treatment was performed in triplicate in each assay.

2.3.7.2 Lysate extraction for assay

To halt agonist treatment, the media was aspirated from the cells and 100µl of 2% Perchloric acid was added directly onto the cells. The media was kept to be used for measuring extracellular cAMP levels. Following incubation on ice for 15 minutes, the cells were scraped into eppendorfs and the precipitated protein pelleted by centrifuging the

samples in a microfuge at 13000rpm for 3 mins. Two wells from both normoxic and hypoxic plates were lysed into 100µl cKHEM + 1% Triton X-100 as in section 2.3.3.1. to be used for protein quantification.

2.3.7.3 Neutralisation of the sample

The supernatant from section 2.3.6.2 was added to 5µl universal indicator solution (BDH) and it turned pink. The samples were neutralised by gradual addition of 2M KOH, 0.5M Triethanolamine until the solution turned light green, indicating a pH of 7-7.5. Centrifugation at 13000rpm for 3 minutes pelleted the potassium perchlorate precipitate.

2.3.7.4 Reagent preparation for assay

cAMP assay buffer: 50mM Tris/4mM EDTA kept at 4°C.

The following solutions were prepared fresh on the day of the assay.

cAMP Standards: Stock of 1mM cAMP in cAMP assay buffer diluted 1:100 and an initial dilution of 32pmol/50µl generated. A series of two-fold dilutions from this concentrated standard generated the dilutions used in the assay.

[5', 8' -^{3}H] cyclic AMP: 8.7µl [5', 8' -^{3}H] cAMP
10mls cAMP assay buffer.

Binding Protein: 10mg Bovine Crude Fraction
125mg BSA
25mls cAMP assay buffer.

Diluted 1:5 in cAMP assay buffer for use on day of assay.

Charcoal Solution: 2% w/v activated charcoal
1% w/v BSA
30mls cAMP assay buffer.

Charcoal solution was stirred on ice for 20 minutes prior to use in assay.

2.3.7.5 Sample preparation for assay

A standard curve was obtained for each assay by including a set of tubes containing known quantities of cAMP ranging from 0.00625 and 16pmol. The standards tubes were set up in duplicate on ice with the unknown samples in the order as follows (with binding protein being added last):

Tube No	cAMP (pmol/50µl)	Buffer (µl)	[^{3}H]-cAMP (µl)	Binding Protein (µl)
1-2	Blank	200	100	0
3-4	Zero	100	100	100
5-6	0.0625	50	100	100
7-8	0.125	50	100	100
9-10	0.25	50	100	100
11-12	0.5	50	100	100
13-14	1	50	100	100
15-16	2	50	100	100
17-18	4	50	100	100
19-20	8	50	100	100
21-22	16	50	100	100
23 onwards	Unknown	50	100	100

The first two tubes with no binding protein were included as a blank to determine the amount of cAMP that remained after nucleotide binding by charcoal. Tubes 3 and 4 indicated the maximum cAMP bound in the absence of unlabelled cAMP and tubes 5 to 22 were prepared with known concentrations of unlabelled cAMP to aid in the construction of a standard curve to allow calculation of unknown cAMP values.

Tubes 23 onwards were set up as shown using samples prepared as in 2.3.6.1 or media from the cells. The tubes were vortexed and incubated on ice for 2 hours. After this time, 25µl charcoal solution was added to each tube. The tubes were vortexed and centrifuged at 13000rpm for 3 minutes. 300µl supernatant from each tube was added to 1ml Opti-scint scintillation fluid, vortexed until clear and counted on a Wallac 1409 liquid scintillation counter.

2.3.7.6 Construction of standard curve

Microsoft Excel was used to plot standards (pmol cAMP vs 1/DPM-mean blank) and generate a formula for the best fit curve.

2.3.7.7 Calculation of cAMP levels

After generating the best fit formula for the standard curve, this was used with the unknown samples to calculate their cAMP concentrations. Results were calculated as pmol cAMP/ng protein and expressed as mean percentage normoxic basal ± S.E., with normoxic basal = 100%.

2.3.8 PKA Assay

2.3.8.1 Cell lysate extraction for PKA assay

Cells were grown in both normoxia and hypoxia for seven days, after which time the cell media was removed, the cells washed with PBS and drained thoroughly. The monolayer of cells was scraped into 500µl extraction buffer (5mM EDTA, 50mM Tris, pH 7.5) and homogenized by drawing through a 13 gauge needle 12 times. The cell debris was then removed by centrifugation for 2 mins at 13000 rpm at 4°C (Heraeus refrigerated bench-top centrifuge) and the supernatant was used for a PKA assay.

2.3.8.2 PKA assay tube pre-incubation

For each cell lysate, the following were set up on ice in 1.5 ml eppendorf tubes. The cell extract was added last and the samples were incubated on ice for 20 min to allow the inhibitor to bind PKA.

Tube	Cell Extract	Diluent	4x PKA Inhibitor	4x PKA Activator
A	10µl	20µl	0µl	0µl
B	10µl	10µl	10µl	0µl
C	10µl	10µl	0µl	10µl
D	10µl	0µl	10µl	10µl

PKA Assay Diluent: 50mM Tris, pH7.5.

4 x PKA Inhibitor: 4µM PKI(6 -22) amide, 50mM Tris, pH7.5.

4 x PKA Activator: 40µM cAMP, 50mM Tris, pH7.5.

4 x PKA substrate: 200µM Kemptide, 400µM ATP, 40mM $MgCl_2$, 1 mg/ml BSA, 50mM Tris, pH7.5.

2.3.8.3 PKA assay reaction

To 1ml of the 4 x PKA substrate, 6000 μCi/mmol of [γ-^{32}P]ATP was added whilst maintained on ice. To the first assay tube, (1A), 10μl of radioactive 4 x PKA substrate solution was added. The sample was mixed gently before being placed in a waterbath at 30°C for 10 minutes. 20 seconds after substrate addition to the first tube, 10 μl of the radioactive substrate was added to the next tube, (1B), which was then mixed and incubated. This method of addition of substrate to tubes every 20 seconds continued until substrate had been added to all samples and they were incubating at 30 °C. After the 10 min incubation of the first tube, 20μl of the reaction mix was removed and spotted onto a pre-marked piece of ion exchange phosphocellulose paper P81 (Whatman). This was carried out for all tubes after they had each undergone the 10 min incubation with the substrate. The phosphocellulose pieces were then placed into a large beaker containing 1 %(v/v) phosphoric acid (H_3PO_4) and washed for 3 min with slight agitation. The waste acid was removed and the acid wash repeated. The phosphocellulose was then washed twice in dH_2O before being placed in 1.5 ml eppendorf tubes, to which 1 ml scintillation fluid was added. The ^{32}P incorporated into the peptide bound to the phosphocellulose was counted on the Wallac 1409 liquid scintillation counter. Two separate vials were set up with 10μl of the radioactive 4 x PKA substrate added to 1ml scintillation fluid to enable the determination of the total counts from the substrate solution.

2.3.8.4 Calculations for PKA assay data

Step 1.

$$\frac{Total\ counts}{4nmolATP} x \frac{1nmol}{1000pmol} = cpm/pmol\ phosphate$$

Step 2.

$$\frac{(cpm/phosphocellulose)x2}{STEP1} = total\ pmol\ peptide - incorporated\ phosphate$$

Step 3.

$$\frac{STEP\ 2}{10\ \text{min}} = pmol/\ \text{min}\ /assay\ tube$$

Step 4.

$$\frac{STEP\ 3}{0.01ml\ extract} = pmol/\min/ml$$

Step 5.

*Tube A – Tube B = pmol/*min *activated PKA*

*Tube C - Tube D = pmol/*min *total PKA*

Step 6.

$$\frac{pmol/\min\ activated\ PKA}{pmol/\min\ total\ PKA} x100 = \%\ activated\ PKA$$

2.4 Molecular Biology

To prevent any contamination all glassware, tubes, tips, buffers and media used for molecular biology were sterilised.

2.4.1 DNA Production

2.4.1.1 Small scale production of DNA

5ml LB growth media (580mM NaCl, 0.5 % (w/v) BactoYeast Extract, 1 % (w/v) Bacto-Tryptone pH7.5) supplemented with antibiotics in a sterile 25 ml universal tube was spiked with a pipette tip from a glycerol stock of transformed cells containing the DNA of interest. The culture was incubated at 37 °C overnight with agitation and the cells harvested the next day by centrifugation at 3000 rpm for 5 min (Heraeus bench-top refrigerated centrifuge). The DNA was extracted from the cells using the QIAprep spin miniprep kit according to the manufacturer's instructions. Briefly: The bacterial pellet was resuspended in 250µl Buffer P1 (resuspension buffer), to which 250µl Buffer P2 (lysis buffer) was added. The solutions were mixed and incubated at room temperature for 5 minutes. 350µl of Buffer N3 (neutralisation buffer) was then mixed with the lysed cells and the solution was centrifuged for 10 min at 13000 rpm. The supernatant was added to the QIAprep spin column and centrifuged at 13000 rpm for 1 min. The flow through was discarded and the column washed with 750µl Buffer PE. The column was centrifuged for 1 min prior to the elution of the bound DNA to ensure removal of all buffer. 50µl dH_2O was placed onto the resin in the column and incubated for 5 minutes and the DNA was eluted

from the column by centrifugation for 1 min at 13000 rpm (Heraeus bench-top refrigerated centrifuge).

2.4.1.2 Large scale production of DNA

400ml LB growth media supplemented with antibiotics in a 2l flask was spiked with a pipette tip from a glycerol stock of transformed cells containing the DNA of interest. The culture was incubated at 37°C overnight with agitation and harvested the next day by centrifugation at 5000g for 10 minutes using the JA-14 rotor in the Beckman refrigerated centrifuge. The DNA was extracted from the cell pellet using the Promega Wizard Maxiprep kit according to the manufacturer's instructions. Briefly: The bacterial pellet was resuspended in 15ml resuspension solution, to which 15ml lysis buffer was added. The solutions were mixed gently and inverted for 15 minutes. 15ml of neutralisation buffer was then added to the lysed cells and the mixture was gently inverted three times. The cell lysate was clarified by centrifugation of the solution at 13000 g for 15 minutes at room temperature and then passed through muslin to separate out clumps. 0.6 x volume Isopropanol was added and mixed before centrifugation at 13000g for 1 hour at room temperature. The DNA pellet was then washed and resuspended in 2mls TE buffer. 10mls DNA purification resin was added and mixed with the resuspended DNA pellet. The resin/DNA mix was then passed through a Maxicolumn by attached to a vacuum manifold and applying a vacuum. To wash the columns, 25mls column wash solution was added and drawn through by vacuum. The resin was rinsed with 5mls of 80% ethanol. To ensure all ethanol was removed, the maxicolumn was centrifuged at 1300g for 5 minutes and the resin was then dried by applying a vacuum for 5 minutes. Following this, 1.5mls preheated (70°C) nuclease-free water was added to the maxicolumn to elute the DNA into a 50ml screw cap tube. The water was left on the column for 5 minutes at room temperature before centrifugation at 1300g for 5 minutes.

2.4.1.3. Quantification of DNA & RNA

DNA and RNA concentrations were quantified by a WPA Lightwave spectrophotometer blanked with distilled water. 5µl DNA or RNA was diluted to 1ml with distilled water and absorbance measurements were taken at 260nM and 280nM. The concentration of nucleic acid was then calculated using the following approximations:

An absorbance reading of 1 at 260 nM corresponds to;

50µg/ml double stranded DNA

40µg/ml single stranded RNA

Therefore, [DNA] (µg/ml) = A_{260} x 50 x 200 (Dilution Factor)

[RNA] (µg/ml) = A_{260} x 40 x 200 (Dilution Factor).

The ratio between the absorbance measurements at 260nM and 280nM provided an indication of the purity of the nucleic acid. In solution, pure DNA or RNA typically have A_{260} :A_{280} ratios of between 1.8 and 2.

2.4.2 Glycerol Stock Production

A 500µl sample from an overnight culture of transformed cells was taken aseptically and placed into a sterile screw top cryovial to which 500µl sterile 80% glycerol was added. The sample was mixed well and stored at –80°C for further use.

2.4.3 Reverse Transcription PCR

2.4.3.1 Total RNA extraction

For isolation of total RNA from hPASMC, a Qiagen RNeasy kit was used. Cells were trypsinised as described in section 2.1.1.2, pelleted and the medium aspirated. The cell pellet was then either frozen at -80°C for use at a later date or resuspended in 600µl Buffer RLT containing 10µl/ml of 14.5M β-Mercaptoethanol. Lysates were then loaded onto QIA shredders to ensure maximum homogenization of sample and the manufacturer's instructions for the kit were followed.

In order to prevent potential contamination from genomic DNA, an incubation step with 4 units DNase at room temperature for 15 minutes was included. The clean up protocol was also used to ensure maximal purity. Total RNA was eluted in RNase free H_2O and stored at -20°C.

To determine the concentration and purity of RNA, the absorbency of the sample was measured at 260nm and 280nm in a spectrophotometer. (WPA, Lightwave).

2.4.3.2 cDNA synthesis

First strand synthesis was carried out in each reaction using 2µg total RNA catalysed by the enzyme Superscript II reverse transcriptase (Invitrogen). The reaction was primed using 500ng of Oligo(dT)15 (Promega), in a final volume of 20µl. The reverse transcription mixture contained 1µl of 10mM stock dNTP mix, 4µl 5X First strand buffer, 2µl 0.1M DTT, 40 units of RNasin Ribonuclease inhibitor and 200 units of Superscript II RNase H⁻ Reverse Transcriptase or 1µl H_2O(molecular biology grade) as a negative

control for PCR. The mixture was incubated at 42°C for 50 minutes before heating to 70°C for 15 minutes to terminate the reaction. Control reactions were carried out without enzyme.

2.4.3.3 PCR reaction

The PCR reaction was set up using Platinum *Pfx* DNA Polymerase (Invitrogen). 1µg cDNA (generated in 2.4.3.2) was added to 2.5µl of 10X *Pfx* amplification buffer, 0.75µl of 10mM dNTPs, 0.5µl 50mM $MgSO_4$, 0.75µl 10µM of each of sense and antisense primers for detection of the transcript of interest, 0.75µl 10µM of sense and antisense primers for cyclophylin, 0.25µl of Platinum Pfx DNA Polymerase and made up to a total volume of 25µl with RNase- free water. Tubes were also set up as blanks using the control reactions without enzyme from section 2.4.3.2.

The PCR conditions used were as follows:

95°C for 2 minutes,
94°C for 30 secs,
*58/60°C for 30 secs,
72°C for 1 minute,
} 40 cycles
72°C for 10 minutes,
4°C forever.

*The annealing temperature for 4D5 was 58°C whereas it was 60°C for all other isoforms.

2.4.3.4 Semi-Quantitative RT-PCR

The PCR reaction was set out as in section 2.4.3.2 in triplicate. One tube per sample was removed after 30 cycles, one after 35 cycles and another was left to undergo another 5 cycles. To ensure even comparison, there was no final extension step of 72°C for 10 minutes.

2.3.4.5 Visualisation of PCR Products

DNA was visualised using agarose gel electrophoresis, the percentage of agarose used in the gel was dependent on the size of DNA fragment to be identified, usually 2 %. For a 2 % agarose gel 2 % agarose was dissolved in 1 x TBE (45mM Tris/HCl, 1mM EDTA, 0.9M Boric Acid) by heating until the agarose dissolved. To this, 0.01 % ethidium bromide was added, which enabled visualisation of the DNA under a UV light source. The molten agar was poured into the gel apparatus, set up according to the manufacturer's instructions and allowed to set completely. The comb and end stoppers were removed, the gel tank filled with 1x TBE and the samples loaded into the lanes. All DNA samples were diluted 6:1 in 6x sample buffer (0.25 % Bromophenol Blue, 0.25 % xylene cyanol blue, 30 % glycerol in

H_2O). In order to size the DNA fragments, the molecular size marker XIV (Promega) was loaded alongside the samples. The gel was run at 100 volts until the dye front moved sufficiently through the gel. The gel was removed from the tank and the DNA observed under UV light.

The range of separation of linear DNA molecules according to agarose concentration;

Percentage Gel	Size of Fragment (kb)
0.9	0.5 -7
1.2	0.4 -6
1.5	0.2 -3
2	0.1 -2

2.5 Microarray Analysis

2.5.1 RNA Extraction

2.5.1.1 Extraction of total RNA

Total RNA from both normoxic and hypoxic samples was isolated as in section 2.4.3.1 using the Qiagen RNeasy kit. RNA concentration was determined and samples were stored at -80°C until needed.

2.5.1.2 Clean up of total RNA

RNA was cleaned up following the add-on protocol of the Qiagen RNeasy kit to increase purity and remove potential contaminants.

2.5.2 Incorporation of Fluorophores

2.5.2.1 cDNA synthesis

Two reaction tubes were set up together, one with either normoxic or hypoxic RNA and Cy3 or Cy5 –dCTP. cDNA was synthesised using 25µg of total RNA mixed with 12µg random hexamers and incubated at 70°C for 10 minutes to allow the primers to anneal. First strand cDNA synthesis was performed using 400U Superscript II (Invitrogen) and 40 µM of one of Cy-3 or Cy-5. This reaction was mixed and incubated at 25°C for 10 minutes then 42°C for five hours before stopping the reaction then cleaning up the cDNA using a QIAquick column.

2.5.3 Hybridisation

2.5.3.1 Incubation of microarray with sample

Both sets of cDNA were mixed together with a hybridisation buffer and incubated at 95°C for 3 minutes before being placed on ice for 1 minute. This mixture was then placed on the microarray and covered with a coverslip. The microarray was then rotated gently at 42°C for 24 hours.

2.5.3.2 Washing of microarray

The microarray was washed gently with SSC buffer three times by being placed inside a 50ml centrifuge tube with buffer and placed horizontal on a rotator set at the lowest setting. The microarray was then dried by brief centrifugation.

2.5.4 Analysis

Analysis of microarray was carried out by Dr. Ian McPhee. A Genetic Microsystems 418 array scanner was used to obtain the data with Imagene 5.0 to determine the values. Gene sight light 3.2 was used to normalise the data and calculate ratios.

2.6 Statistical Analyses

Data was analysed by two-way ANOVA with Bonferroni's post test using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, to analyse raw data. Where only two groups of data were to be analysed, an unpaired t-test was performed, and $^{*}p<0.05$ was considered to be significant.

Antibody	Company	Dilution	Incubation Time	Secondary
PDE4A,B,C,D	ICOS	1:10000	1 hour	Anti-mouse IgG
PDE4A,B,C,D	SAPU	1:5000	1 hour	Anti-goat IgG
4A4	Genosys	1:5000	2 hours	Anti-rabbit IgG
4A10	Genosys	1:1000	2 hours	Anti-rabbit IgG
pERK1/2	Cell Signalling	1:1000	Overnight	Anti-mouse IgG
ERK1/2	Cell Signalling	1:1000	Overnight	Anti-mouse IgG
PKA RIα	BD Transduction	1:1000	1 hour	Anti-mouse IgG
PKA RIIα	BD Transduction	1:1000	1 hour	Anti-mouse IgG
PKA RIIβ	BD Transduction	1:2000	1 hour	Anti-mouse IgG
α - tubulin	Sigma	1:10000	1 hour	Anti-mouse IgG

Table 2.1 Antibodies and Dilutions

This table shows the antibodies used for western blotting throughout this study and the conditions determined to get an optimal immunoreactive signal.

Chapter 3

Effect of Chronic Hypoxia on PDE4 in hPASMC

3.1 Introduction

3.1.1 Pulmonary Arterial Hypertension

3.1.1.1 Pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is characterised by increased vascular resistance which leads to pulmonary artery remodelling and increased smooth muscle cell proliferation (Fishman 2004; Humbert et al., 2004). Normal mean adult pulmonary arterial pressure is ~14mm Hg whereas patients with pulmonary hypertension have a mean PAP exceeding 25mmHg at rest and 30mmHg during exercise. Pulmonary hypertension can occur without apparent cause, previously known as Primary Pulmonary Arterial Hypertension (PPAH) It is now known that PAH can be due to mutations in the bone morphogenetic protein receptor II, *BMPR2,* or 5-HT transporter gene (The International PPH Consortium et al., 2000; Thomson et al., 2000; Machado et al., 2001; Eddahibi et al., 2003). Hence these forms have been classified as familial pulmonary arterial hypertension (FPAH; *table 1.5*). These cases only account for 6% of all PPAH patients. The occurrence of PPAH in the population is rare, with only two cases per million. PPAH can occur in men, women and children of all ages though it is most commonly seen in females between 20 and 40 years old. PAH occurs more often secondary to pulmonary vascular disorders, such as chronic obstructive pulmonary disorder (COPD), and exposure to hypoxic conditions. This form of PAH is now classified as PAH'PAH with lung disease and /or hypoxemia' and is relatively common but is underdiagnosed due to the large number of causes. As such, an estimate of the prevalence of secondary PAH is difficult to obtain. PAH is usually progressive and fatal.

There is currently no cure for PAH. Current treatments for PAH include anticoagulants, diuretics, calcium channel blockers and vasodilators. Vasodilators used include inhaled nitric oxide (NO), ACE inhibitors or prostacyclin. So far, only treatment with prostacyclin analogues such as iloprost have improved survival. Heart and/or lung transplantation is considered in extreme cases. Promising results have been found with sildenafil, a PDE5 inhibitor, which has been shown to significantly reduce pressure in severe PPAH patients (Michelakis et al., 2003; Prasad et al., 2000). It has even been shown to be more effective than inhaled NO in decreasing PAP and reducing pulmonary vascular resistance (Michelakis et al., 2002). The responses to medication are varied between patients so it can take time before a successful drug therapy is achieved. If a cure is to be found for PAH,

then the underlying cellular and molecular changes, along with the physiology of the disease, must be determined.

3.1.1.2 Hypoxia-induced Pulmonary Hypertension

Hypoxia causes high altitude-induced PAH and is also thought to play a role in PAH associated with COPD. The mechanism underlying this is currently unclear. Animal and cellular models have been utilised to investigate PAH, and to suggest possible treatment targets.

Chronic hypoxia can lead to the structural remodelling of pulmonary arteries. In contrast to systemic arteries, the pulmonary arteries are distinct in that they constrict in response to hypoxia (Voelkel & Tuder, 1997). This is required for ventilation-perfusion matching in which pulmonary capillary blood flow is automatically adjusted to match alveolar ventilation.

It is known that vasoconstriction is an intrinsic property of pulmonary vascular smooth muscle cells (Voelkel and Tuder 1997) and it has been shown that hypoxia can have a direct effect on smooth muscle cells. After 1 week of hypoxia, proliferation rates of PASMC from distal human pulmonary arteries were observed to increase (Yang et al 2002). The cells were also observed to contract in response to hypoxia (Murray 1990). Due to this, hypoxic PASMC have been used as a cellular model of PAH.

3.1.2 Cyclic Nucleotide Signalling in Models of Hypoxia-Induced Pulmonary Hypertension

3.1.2.1 Cyclic Nucleotides in Hypoxia

The second messengers' cAMP and cGMP have been shown to play vital roles in the regulation of vascular smooth muscle tone (Murray et al., 1990b). cAMP and cGMP relax smooth muscle through the activation of protein kinase A (PKA) and protein kinase G (PKG) respectively. PKA and PKG can induce relaxation by acting on downstream targets of Ca2+ mobilisation (Shabb 2001; Marin et al., 1998). Activation of these kinases is achieved by either stimulating adenylyl or guanylyl cyclase or through inhibiting the action of cyclic nucleotide phosphodiesterases (PDEs). These pathways have been of interest to researchers to uncover the mechanisms underlying PAH (MacLean et al., 1996). Investigation of the hypoxic Wistar rat model uncovered that the levels of both cyclic nucleotides are seen to decrease, with a corresponding increase in PDE activity, in response to two weeks of 10% O_2 (MacLean et al., 1997).

3.1.2.2 Phosphodiesterases in Hypoxia

In the rat pulmonary arterial circulation, the predominant cyclic nucleotide phosphodiesterases are from the PDE1, PDE3, PDE4 and PDE5 families (Rabe et al., 1994), although PDE2 is also present (MacLean et al., 1997).

Investigations into the expression of these PDEs in hypoxia have revealed that their profiles are altered. PDE activity was examined in pulmonary arteries from control and hypoxia-induced pulmonary hypertensive rats (MacLean et al., 1997). cAMP-PDE activity was increased in first-branch and intrapulmonary arteries from the hypoxic rats. cGMP-PDE activity was also increased in the first, intrapulmonary and main pulmonary arteries of the hypoxic rat. Investigation of these increases attributed it to an increase in PDE3, PDE1 and PDE5 activities (MacLean et al 1997).

Using human PASMC and rat pulmonary artery homogenates, it was discovered that PDE3 and PDE5 expression levels and activity were increased after two weeks exposure at 10% O_2 (Murray et al., 2002). The increase in the PDE3A isoform in hPASMCs was observed to be PKA-dependant and could be mimicked by cAMP analogs (Murray et al., 2002). There was also an increase in PDE3 and PDE5A2 mRNA and protein levels in pulmonary arteries from the chronic hypoxic rat model (Murray et al., 2002).

The role of PDE inhibitors as a therapy for PHT is under investigation. PDE inhibitors have been shown to reduce smooth muscle cell proliferation, migration and induce vasodilation of the pulmonary circulation (Koyama et al., 2001; Ghofrani et al., 2004; Wagner et al., 1997; Bardou et al., 2002; Goirand et al., 2001; Palmer et al., 1998; Osinski & Shror 2000). Zaprinast, rolipram, cilostamide and theophylline can all oppose pulmonary vasoconstriction seen in hypoxia (Pauvert et al., 2002, Goirand et al., 2001). PDE3 or 4 inhibitors can also improve responses to β-adrenergic agents that are attenuated in hypoxia. When both PDE3 and PDE4 inhibitors are used, they act synergistically to potentiate this effect (Wagner et al., 1997). Rolipram has been observed to be more potent than PDE3 or PDE5 inhibitors at relaxing human intralobar pulmonary arteries (Bardou et al., 2002).

3.1.2.3 PDE4 in Hypoxia

PDE4 is a cAMP-specific, rolipram-inhibited PDE that is known to be the major hydrolysing enzyme of cAMP in vascular smooth muscle cells. There are eighteen known human PDE4 isoforms with differing subcellular locations and modes of regulation

(reviewed in Houslay 2001; further details in general introduction, sections 1.2.4 and 1.3). General PDE4 inhibitors are currently being tested as a putative therapy for such diseases as asthma and COPD (Giembycz 2001; Spina 2003). However, little is known about the effect hypoxia has on PDE4 isoforms in vascular smooth muscle cells. In this chapter, I set out to investigate PDE4 expression and activity in hPASMC under normoxic and hypoxic conditions.

Results

3.2 Characterisation of PDE4 Profile in hPASMC

All methods used in this chapter are described in full in section 2, materials and methods.

3.2.1 Analysis of PDE4 expression

To determine the PDE4 profile in hPASMC, western blotting was employed as described in section 2.3.4. PDE4A, 4B, 4C and PDE4D antibodies have been raised against peptide sequences from the C-terminal of each PDE4 family. This has generated subfamily-specific isoforms as the C-terminal is only conserved among subfamily members, i.e. all PDE4A isoforms but not any PDE4B, PDE4C or PDE4D isoforms (Huston et al, 1996; Mackenzie et al., 1998). These PDE4 subfamily specific antibodies were used to probe hPASMC lysates to identify the PDE4 family isoforms present within these cells. The results gained were further specified and confirmed by RT-PCR analysis.

3.2.2. PDE4A profile in hPASMC

3.2.2.1 Western blot analysis of PDE4A isoforms in hPASMC

To identify whether PDE4A isoforms were expressed in hPASMC, cell lysates were immunoblotted with a 4A monoclonal antibody (Materials and Methods 2.3.4) raised against the sequence EEFVVAVSHSS, which is found at the C-termini of all known active human PDE4A isoforms. This detected a single PDE4 splice variant that co-migrated with recombinant PDE4A4 (Genbank accession L20965) and PDE4A10 (Genbank accession AF073745) isoforms, having an apparent Mwt of 120kDa when analysed by SDS-PAGE *(Figure 3.1)*. PDE4A11 (Genbank accession AF069488) is also known to migrate at this weight. Thus, isoform specific antibodies were employed to identify this band. The PDE4A4 specific antibody did not react with hPASMC lysate indicating the absence of PDE4A4 in these cells. The specificity of this PDE4A4 specific antibody was confirmed by detecting PDE4A4 recombinant protein, but not PDE4A10 or PDE4A1. The specificity of the PDE4A10 antibody was confirmed by the detection of the PDE4A10 recombinant protein and not PDE4A4 or PDE4A1. This PDE4A10 specific antibody identified a band at 120kDa, confirming the presence of the PDE4A10 isoform in hPASMC. A PDE4A11 antibody has not yet been successfully generated so it was impossible to confirm the absence or presence of PDE4A11 by western blotting.

3.2.2.2 RT-PCR analysis of PDE4A isoforms in hPASMC

RT-PCR was employed to identify the120kDa immunoreactive species detected through western blotting (Materials and Methods 2.4.3). As the long human PDE4A isoforms, PDE4A4, PDE4A10 and PDE4A11, all migrate at this weight on an SDS-PAGE gel, isoform specific primers were used to investigate transcript expression of these three splice variants. Primers to PDE4A1 and PDE4A7 were also used to investigate if transcripts for these isoforms were present or not. This was because PDE4A7 is truncated at the C-terminus and there is no antibody available to detect it. Also, in the case of PDE4A1, it is possible that it could be expressed at such low levels that western blotting would be unable to detect it. RT-PCR analysis of total RNA identified the presence of PDE4A7, PDE4A10 and PDE4A11 transcripts in hPASMC but not those for PDE4A4 (*Figure 3.3*).

3.2.3 PDE4B profile in hPASMC

3.2.3.1Western blot analysis of PDE4B isoforms in hPASMC

To identify if any PDE4B isoforms are expressed in hPASMC, cellular lysates were immunoblotted with a PDE4B specific polyclonal antibody (Materials and Methods 2.3.4) raised against the sequence DPENRDSLGETDIDIATED, which is found at the C-terminus of all known, active PDE4B isoforms. This antisera detected a single PDE4B splice variant that co-migrated with the recombinant PDE4B2 standard (Genbank accession L20971) This PDE4B2 standard recombinant protein migrated at 80 kDa on SDS-PAGE (*Figure 3.4*).

3.2.3.2 RT-PCR analysis of PDE4B isoforms in hPASMC

As a means of confirming the presence of the PDE4B2 isoform, RT-PCR was employed to identify transcripts for the single immunoreactive species observed at 80kDa through western blotting (Materials and Methods 2.4.3). Primers specific to PDE4B1, PDE4B2 and PDE4B3 were also used to investigate transcript expression of these three known human PDE4B splice variants. RT-PCR analysis of total RNA using such isoform specific primers identified the presence of PDE4B2 transcripts but failed to detect those for PDE4B1 and PDE4B3, thus confirming the absence of expression of the PDE4B1 and PDE4B3 long isoforms in hPASMC *(Figure 3.6).*

3.2.4 PDE4C profile in hPASMC

3.2.4.1Western blot analysis of PDE4C isoforms in hPASMC

To identify whether PDE4C is expressed in hPASMC, cellular lysates were immunoblotted (Materials and Methods 2.3.4) with a polyclonal PDE4C specific antibody, which detects a sequence located in the extreme C-termini of all known active PDE4C isoforms. This detected a single reactive species which co-migrated with recombinant PDE4C2 (Genbank accession U66346), which migrated with an apparent molecular weight of 80kDa on SDS-PAGE *(Figure 3.7)*

3.2.4.2 RT-PCR analysis of PDE4C isoforms in hPASMC

As a means of confirming the presence of the PDE4C2 isoform, RT-PCR was employed to identify the reactive species observed at 75kDa through western blotting (Materials and Methods 2.4.3). Primers specific to PDE4C2 were used to investigate transcript expression. RT-PCR analysis of total RNA using isoform specific primers identified the presence of PDE4C2 transcripts in hPASMC, thus confirming the western blot data that identified PDE4C2 expression *(Figure 3.8)*.

3.2.5 PDE4D profile in hPASMC

3.2.5.1Western blot analysis of PDE4D isoforms in hPASMC

To identify if any PDE4D isoforms are expressed in hPASMC, cellular lysates were immunoblotted with a PDE4D specific monoclonal antibody (Materials and Methods 2.3.4) raised to a peptide corresponding to the sequence; TQDSESTEIPLDEQVEE, located at the C-termini of all known PDE4D isoforms. This detected three immunoreactive bands. These co-migrated with recombinant PDE4D1/2 (Genbank accession U50157, U50158 respectively), PDE4D3 (Genbank accession L20970) and PDE4D5 (Genbank accession S:1059276), whose molecular weights are 68 kDa, 95kDa and 105kDa, respectively, on SDS-PAGE (*Figure 3.9*).

3.2.5.2 RT-PCR analysis of PDE4D isoforms in hPASMC

To confirm the presence of the PDE4D1/2, PDE4D3 and PDE4D5 in hPASMC, RT-PCR was employed to identify the reactive species observed through western blotting (Materials and Methods 2.4.3). Primers specific to PDE4D1, PDE4D2, PDE4D3 and PDE4D5 were used to identify transcript expression. Primers to PDE4D4 were also used to determine if this isoform was present at such low levels that western blotting would be unable to detect it. RT-PCR analysis of total RNA using isoform specific primers identified transcripts for

PDE4D1, PDE4D2, PDE4D3 and PDE4D5, thus confirming their expression in hPASMC, but failed to identify transcripts for PDE4D4, confirming the inability to detect this isoform immunologically in hPASMC *(Figure 3.11)*.

3.2.6 cAMP-PDE activity in hPASMC

PDE3 and PDE4 are known to be the main cAMP hydrolysing enzymes expressed in vascular smooth muscle cells (Palmer et al., 1998). In order to determine how much of this is attributable to PDE4 within hPASMC, a cAMP-PDE activity analysis (Materials and Methods 2.3.5) was carried out in the presence and absence of either the PDE4 selective inhibitor rolipram (Nemoz et al., 1985) or the PDE3 selective inhibitor, cilostamide. Briefly, the PDE activity of cell lysates was assayed in the presence of 1μM cAMP in either the absence of presence of 10μM of the PDE4-specific inhibitor rolipram or the PDE3-specific inhibitor cilostamide. Analysis revealed that the main cAMP hydrolysing enzyme in hPASMC, contributing 53 ± 7% (SEM; n = 6) of the total cAMP-PDE activity, is the rolipram-sensitive fraction, namely PDE4 activity. It was found that a further 25 ± 8% (SEM; n=3) of the activity is due to PDE3 activity *(Figure 3.12)*. Total cAMP-PDE activity was 66.5 ± 4.7 pmol/min/mg protein (SEM; n=6), PDE4 activity was 37.1 ± 5.3 pmol/min/mg protein (SEM; n=6) and PDE3 activity was 16.9 ± 0.7 pmol/min/mg protein (SEM; n=3).

3.3 Effect of chronic hypoxia on PDE4 in hPASMC

3.3.1 Examining the effect of chronic hypoxia on PDE4

Previous research into models of pulmonary hypertension has indicated that cAMP signalling is altered. Thus, once the PDE4 profile and activity had been determined in hPASMC, I set out to investigate whether these were altered by exposing the cells to hypoxic conditions. Initially, the cells were maintained in a 10% oxygen (balance N_2) environment for a period of two weeks as done before (Murray et al 2002) and to determine the optimal time point to study the cells.

3.3.2 Analysis of PDE4 isoforms expression in chronic hypoxia

Cells were placed under hypoxic conditions (Materials and Methods 2.1.1.3) with a corresponding set of normoxic controls. Cells were harvested for lysate or total RNA as required and examined for PDE4 expression compared to normoxic.

3.3.2.1 PDE4A

3.3.2.1.1Western blot analysis of PDE4A

Cells were harvested and probed with the PDE4A subfamily antibody as described in materials and methods 2.3.4. PDE4A expression was seen to alter throughout the two weeks of hypoxia *(Figure 3.13)*. The PDE4A isoform which migrated at 120 kDa on SDS-PAGE, and was thus the combined PDE4A10+PDE4A11 band, was seen to increase to 209 ± 58% (SEM; n=3) of basal levels after 24 hours exposure to hypoxia and this increase was maintained throughout the 14 days of incubation. Normoxic PDE4A10/4A11 only increased to 102 ± 63% (SEM; n=3) of basal levels.

3.3.2.1.2 Semi-quantitative RT-PCR analysis of PDE4A

In order to gain insight into whether the increase in PDE4A was due to either PDE4A10 or PDE4A11, I undertook semi-quantitative RT-PCR analysis on total RNA isolated from cells maintained under hypoxic conditions for seven days and the corresponding normoxic controls (Materials and Methods 2.4.3.3). Primers specific for PDE4A10 and PDE4A11 were used and PCR was carried out on total RNA isolated from seven day normoxic and hypoxic cells. Primers for cyclophilin were also included to use as a control. It was seen that an increase in both PDE4A10 and PDE4A11 *(Figure 3.14a)* after seven days of hypoxia was confirmed. PDE4A10 levels were seen to increase to 138 ± 15.9% (SEM; n=3) as compared with normoxic. PDE4A11 increased to 124 ± 8% (SEM; n=3) of normoxic levels *(Figure 3.14b)*.

3.3.2.2 PDE4B

3.3.2.2.1Western blot analysis of PDE4B

The PDE4B subfamily specific antibody was used to probe cell lysates (Materials and Methods 2.3.4) collected over a two week period of exposure to 10% oxygen to examine any effect on the expression of the PDE4B2 isoform. The expression of PDE4B2 changed throughout the two weeks exposure with a transient increase at day 5-7 to 250 ± 64 % (SEM; n=3) of basal levels and then this gradually decreased to 120 ± 2% over a further seven days of hypoxia *(Figure 3.15)*.

3.3.2.2.2 Semi-quantitative RT-PCR analysis of PDE4B

Total RNA from cells collected after seven days hypoxia and normoxia was used for semi-quantitative RT-PCR with PDE4B2 specific and cyclophilin primers to examine the increase observed in PDE4B2 seen through western blotting (Materials and Methods

2.4.3.3). Transcript levels of PDE4B2 at day seven were seen to be 125 ± 16% (SEM; n=3) that of normoxic controls *(Figure 3.16)*. This increase was considered insignificant in an unpaired t-test.

3.3.2.3 PDE4C

3.3.2.3.1 Western blot analysis of PDE4C

Lysates from hypoxic and normoxic cells were collected over the two week period and probed with the PDE4C specific antibody (Materials and Methods 2.3.4). PDE4C protein expression was observed to remain at basal levels throughout the two weeks with only a 104 ± 3% (SEM; n=3) increase detected at day 7 *(Figure 3.17)*.

3.3.2.4 PDE4D

3.3.2.4.1 Western blot analysis of PDE4D

To examine the protein levels of PDE4D within hypoxic and normoxic cells, the cells were harvested and the cell lysate probed with the PDE4D specific antibody (Materials and Methods 2.3.4). A gradual increase in PDE4D5 protein levels, peaking at day five to seven, was observed *(Figure 3.18)*. This increase was maintained for the duration of the fortnight. Levels of PDE4D5 were seen to rise to 172 ± 19 % (SEM; n=3) at day 7 and 177 ± 17% (SEM; n=3) at day 14 of basal levels. PDE4D1/4D2 and PDE4D3 were not observed to change.

3.3.2.4.2 Semi-quantitative RT-PCR analysis of PDE4D expression

Total RNA from seven day hypoxic and normoxic cells was used for RT-PCR with PDE4D1/2, PDE4D3 and PDE4D5 specific primers (Materials and Methods 2.4.3.3). PDE4D1/2 levels were seen to alter slightly, with an increase of 115 ± 8% of PDE4D1 and 116 ± 7 % of PDE4D2 (SEM; n=3) of normoxic levels. PDE4D5 was confirmed to significantly increase after seven days hypoxia to 115 ± 1% of basal levels *(Figure 3.19a, b)*.

3.3.3 Western blot analysis of HIF-1α Expression

Cells were placed under hypoxic conditions (Materials and Methods 2.1.1.3) with a corresponding set of normoxic controls for seven days to reach the time point witnessed with maximal change in PDE expression. Cell lysate was used to perform western blotting with an anti-HIF-1α antibody to identify a key example of the expression of hypoxia regulated genes at this time point *(Figure 3.20)*. An anti-human monoclonal HIF-1α antibody used in previous studies was procured from BD Pharmingen (Hanze et al., 2003)

and identified an immunoreactive band at 120kDa in hypoxic samples only. This concurs with the known molecular weight for HIF-1α.

3.3.4 Microarray Analysis of normoxic and hypoxic hPASMC

Total RNA from normoxic and seven day hypoxic cells was used for hybridisation onto custom made cell-signalling microarrays from MWG Biotech. RNA was hybridised onto the microarray chips and analysis was carried out by Dr. Ian McPhee of Scottish Biomedical. The chips contained 'spots' of hybridisation for specific PDE4 isoforms and other signalling effectors including PKA isoforms, Rap and ERK. Spots for general PDE4 families were also included. As the normoxic and hypoxic cDNA samples had been synthesised from RNA, they had incorporated different fluorophores. In different experiments, this labelling of samples was reversed to allow for naturally differing intensities of the fluorophores. After hybridisation, fluorophores were excited and intensity values were taken for each fluorophore for each spot on the chip. After normalisation, this data was then interpreted as a ratio of normoxic over hypoxic. By corresponding these values to a 'map' of the chip, changes in gene expression could be identified.

The changes are listed in figure 3.21. It was observed that the increase seen through western blotting and RT-PCR in PDE4A10/4A11, PDE4B2 and PDE4D5 could also be seen by microarray analysis as the general PDE4A and PDE4B, along with the specific PDE4D5 spots produced values indicative of an increase in expression in hypoxia. Other PDE isoforms observed to increase were the PDE5A3, PDE8A, PDE9A and PDE3. Strangely however, the general PDE5A spot indicated an overall decrease in PDE5A isoforms. The largest increase in expression of a PDE was of the PDE3B isoform, this isoform has previously been reported to increase in hypoxic hPASMC (Murray et al., 2002). Modulation of the cAMP pathway appears to occur in the hypoxic samples as the PKA isoforms and Rap1b also show a change in expression. PKARI expression is altered with a shift in the RIα to RIβ ratio as PKARIβ expression is increased in hypoxia. As there was no indication of an RIα isoform decrease in hypoxia, this indicates an overall increase in the PKARI subunit of PKA. Also noteworthy were increases in p53, NFκB and iNOS, all of which are already known to increase in hypoxia (Graeber et al., 1994; Chiarugi et al., 1999; Igari et al., 1998; Palmer et al., 1998).

3.3.5 Effect of chronic hypoxia on cAMP-PDE activity

With the observed increase in PDE4 expression in hypoxic hPASMC, I then set out to determine cAMP-PDE activity in normoxic and hypoxic cells to see if this was altered in hypoxic cells by the change in PDE4 expression.

3.3.5.1 Total cAMP-PDE activity

Lysates from hypoxic and normoxic cells were assayed for cAMP PDE activity with 1μM cAMP as substrate as described in section 2.3.5. Surprisingly, there appeared to be no significant increase in total cAMP-PDE activity *(Figure 3.22)*. Normoxic total cAMP-PDE activity was 66.5 ± 4.7 pmol/min/mg protein (SEM; n=6), whilst a value of 70.8 ± 4.7 pmol/min/mg protein (SEM; n=6) was noted after 7 days hypoxia.

3.3.5.2 PDE3

PDE enzymatic activity of seven day hypoxic cell lysates was assayed in the presence of 10μM of the PDE3-specific inhibitor cilostamide in order to determine PDE3 activity (Materials and Methods 2.3.5). As can be seen in figure 3.22 no evident change in PDE3 activity was observed. Normoxic PDE3 activity was 16.9 ± 0.7 pmol/min/mg protein (SEM; n=3) whilst a value of 19.6 ± 6.2 pmol/min/mg (SEM; n=3) was observed after 7 days hypoxia.

3.3.5.3 PDE4

Cell lysates from seven day hypoxic and normoxic cells were assayed for PDE4 enzymatic activity. This was achieved by measuring total cAMP-PDE activity at 1μM cAMP substrate concentration in the presence and absence of 10μM of the PDE4-specific inhibitor, rolipram (Materials and Methods 2.3.5). Again, little difference in PDE4 activity was noted *(Figure 3.22)*. Normoxic PDE4 activity was 37.2 ± 5.3 pmol/min/mg protein (SEM; n=6), which increased to 44.3 ± 3.4 pmol/min/mg protein after 7 days hypoxia (SEM; n=3).

3.3.5.4 Immunoprecipitated PDE4 subfamilies

The total PDE4 activity is represented by four subfamilies within hPASMC. As each subfamily, and indeed individual isoforms, are subject to different modes of regulation, measuring total PDE4 activity would not reveal any change in specific PDE4 subfamily activities. Therefore, I used cell lysates from seven day hypoxic and normoxic cells to assay specifically for the different PDE4 subfamily enzymatic activities. This was achieved by selectively immunopurifying the respective PDE4 subfamilies, as described in section 2.3.6, and using the resulting samples in a cAMP-PDE assay. An increase in the PDE activity for all four PDE4 subfamilies, including PDE4C, was observed *(Figure 3.23)*. However, whilst the apparent increase in PDE4A, PDE4B and PDE4C activities was not significant that for the PDE4D subfamily was significantly increased ($p<0.01$) to 172% of normoxic PDE4D activity. Thus the key target for hypoxia-induced increase in PDE4 activity is due to the PDE4D sub-family, but such an effect can be partially masked due to

the activities of the three other PDE4 subfamilies, which are little if at all changed in hypoxia.

3.4 Discussion and Conclusions

Exposing vascular smooth muscle cells to hypoxia has been shown to alter many cell signalling pathways including cAMP and cGMP signalling (MacLean et al., 1997; Murray et al., 2002). The decrease observed in cyclic nucleotides in hypoxia has been attributed to increased hydrolysis by PDEs as cAMP-PDE and cGMP-PDE activities have been reported to increase in the pulmonary arteries of the hypoxic rat (MacLean et al., 1997). The cGMP-PDE PDE5 and the cAMP-PDE PDE3 have previously been reported to increase during chronic hypoxia in the pulmonary arteries of the rat and in hPASMC (Murray et al., 2002). Although PDE4 activity represents a major cAMP-hydrolysing activity within these cells, their expression and activity have not been fully investigated.

Each cell type has a specific profile of cAMP-PDE isoforms, which is presumed to enable differential regulation of cAMP signalling (Houslay & Milligan 1997; Houslay 1998). It has previously been established that the major cAMP-PDEs present within vascular smooth muscle cells are PDE3 and PDE4 (Maurice et al., 2003). Prior research has investigated PDE3 expression and shown that the PDE3A and PDE3B isoforms are present within hPASMC (Murray et al 2002). Investigation into the PDE4 profile showed conclusively that PDE4A7, PDE4A10, PDE4A11, PDE4B2, PDE4C, PDE4D1, PDE4D2, PDE4D3 and PDE4D5 are expressed in hPASMC. PDE4 activity contributes approximately 53% of the total cAMP-PDE activity within hPASMC with PDE3 being responsible for another 25% under basal/resting conditions.

Once I had established the PDE4 profile within hPASMC, I set out to investigate if this profile was altered in chronic hypoxia, initially through western blotting and then confirmation by RT-PCR. This enabled me to discover that the level of expression of the PDE4 isoforms, PDE4A10, PDE4A11, PDE4B2 and PDE4D5 were all increased to varying degrees over 14d of exposure to 10% O_2. The increase in both PDE4A10 and PDE4A11 was due to increased transcription with RT-PCR identifying a 138% and 124% increase in PDE4A10 and PDE4A11 respectively. This was the largest increase observed of the PDE4 isoforms increased in hypoxia. The increased transcription of PDE4A10/PDE4A11 is suggestive of a hypoxic transcription factor such as HIF-1 being responsible for the upregulation of these PDE4A isoforms. As PDE4A10 and PDE4A11 are recently characterised PDE4A isoforms, their functional attributes have not yet been determined. It has been hypothesised that PDE4A10 could be involved in the inflammatory

response due to the presence of possible sites for GATA within its promoter. This site is known to interact with the H_2O_2-activated USF (Andrews 2000) as well as CREB and NFκB. Indeed, acute hypoxia has been observed to increase GATA DNA binding activities (Jamali et al., 2004). It is known however that the PDE4A subfamily is not subject to regulation by ERK (Baillie et al. 2000). This means PDE4A10 and PDE4A11 activities are not susceptible to inhibition by the increased active ERK reported in hypoxic vascular smooth muscle cells (Minet et al., 2000b; Scott et al., 1998; Welsh et al., 2001).

The increase in the PDE4B2 isoform represented the largest increase in protein levels among the altered PDE4 isoforms with an increase to 250% of basal levels. Although RT-PCR identified an increase in PDE4B2 transcription to 125 % of basal, this increase was not deemed significant by an unpaired t-test due to the large standard error of the mean. PDE4B2 has been established as having a key role in the regulation of inflammatory responses (Ma et al., 1999; Wang et al., 1999; Oger et al., 2002). Myometrial contractility occurs due to intrauterine infections which induce an inflammatory response involving an increase in the levels of IL-1β. An increase in PDE4B2 expression has been reported to occur in human smooth muscle myometrial cells exposed to IL-1β due to the increased cAMP levels produced through induction of PGE_2 (Oger et al., 2002), thereby acting in a feedback loop to regulate cAMP levels. IL-1β increased cAMP to maximal levels after 12 hours and this induced an increase in PDE4 activity, increasing the hydrolysis of cAMP and returning cAMP levels to normal after a further 12 hours. An increase in PDE4B2 has also been observed directly in response to cAMP raising agents in human myometrial cells (Mehats et al., 1999). This suggests that the increase in PDE4B2 could be due to an increase of cAMP in hypoxia. In hypoxic rat pulmonary arteries however, a decrease in cAMP levels is observed (MacLean et al. 1997). This occurred after 14 days of hypoxic exposure. The increase in PDE4B2 protein levels observed here was transient as protein levels returned to normal after 14 days, suggesting a similar feedback loop to that shown in the myometrial cells *(Figure 3.24)*. Hypoxia could initially induce an increase in cAMP levels through inflammatory mediators and cause an increase in PDE4B2 activity, reducing cAMP levels to below that of normal and alleviate the induction of PDE4B2 expression.

Although previous studies have shown cAMP prevents the induction and maintenance of contraction in smooth muscle cells (Silver 1985), PDE4 inhibitors can block spontaneous myometrial contraction (Mehats et al., 2002). During this spontaneous contraction, PDE4B2 levels increase, suggesting a role for PDE4B2 in this response (Mehats et al.,

2002). Therefore, this is suggestive of a remodulation of cAMP signalling during this contraction and infers PDE4B2 could be initially involved in the hypoxic pulmonary vasoconstrictive response seen in models of PAH.

PDE4D isoforms are involved in the maintenance of airway smooth muscle tone (Mehats et al., 2003). PDE4D knockout mice are refractory to muscarinic cholinergic stimulation and show increased sensitivity to PGE_2 (Hansen et al., 2000). Therefore an alteration in PDE4D could affect smooth muscle contraction and relaxation, suggesting the increased expression of PDE4D5 could also be involved in the hypoxic pulmonary vasoconstrictive response. As PDE4D5 increased levels are maintained whereas the PDE4B2 levels return to normal, it is possible that PDE4D5 is involved in the maintenance of contraction, while PDE4B2 is involved in the initial phase of contraction.

PDE4D5 levels have been reported to increase in human airway smooth muscle in response to cAMP-elevating agents (Le Jeune et al., 2002). This response was shown to be due to a cAMP-response element (CRE) within the promoter region of PDE4D5. This again is suggestive of an initial rise in cAMP levels in hypoxia which cause an increase in PDE4D5 expression. Indeed, the PDE4D5 increase was shown to be due, at least in part, to increased transcription as RT-PCR identified a 115% increase in transcript levels after 7 days hypoxia. PDE4D1 and PDE4D2 also contain a CRE site within their promoters. It was observed that their transcript levels increased to 115% and 116% respectively, although no notable increase in PDE4D1/PDE4D2 protein levels was observed. The effect of hypoxia on these PDE4D, cAMP-inducible isoforms, is again suggestive of an increase in cAMP levels in hypoxia.

In this study, the increase in PDE4 expression was not mirrored by a significant increase in total PDE4 activity after seven days of hypoxic exposure. Examining PDE4 subfamily activities, an increase in PDE4D activity to 172% of normoxic levels was observed. Total PDE3 activity was also unaffected, but has previously been reported to be increased after 14 days hypoxia in hPASMCs in a manner mimicked by cAMP-raising agents and blocked by a PKA inhibitor (Murray et al., 2002). In the aforementioned study (Mehats et al., 2002), the observed increase in PDE4 activity was not an immediate effect and required 18 hours exposure to IL-1β, not reaching maximal levels until after 24 hours. These combined results lend credence to the hypothesis that cAMP signalling undergoes a gradual remodulation during chronic hypoxia, with cAMP levels initially rising and a concomitant increase in PDE activity. After prolonged exposure to high cAMP levels however, the PDE

activities increase to a sufficient level to reduce cAMP and attenuate the effect of cAMP-raising agents (Wagner et al., 1997) alleviating the induction of PDEs by cAMP *(Figure 3.24)*. As many other pathways are altered in hypoxia, it is possible that after cAMP no longer regulates the expression of PDEs, other factors can affect their regulation and indeed maintain the increase in some PDEs.

To confirm the activation of hypoxia induced pathways in the seven day hypoxic cells, the presence of the transcription factor HIF-1α was investigated. HIF-1α is constituitively expressed within cells, but is degraded within minutes under normal conditions (Sutter et al., 2000). Under hypoxic conditions however, degradation of the HIF-1α subunit is attenuated allowing HIF-1 to act upon hypoxia inducible genes containing HIF-1 binding sites. Using a commercial HIF-1α antibody, the presence of HIF-1α was confirmed in hypoxic hPASMC, indicating the cellular responses to hypoxia were activated.

To further confirm the increase in PDE4 protein levels and investigate other signalling pathways, microarray analysis was utilised to examine hypoxia dependant changes in expression. Increases in p53, NFκB and iNOS, all of which are already known to increase in hypoxia, were observed (Graeber et al., 1994; Chiarugi et al., 1999; Igari et al., 1998; Palmer et al., 1998). Microarray analysis indeed confirmed an increase in PDE4D5, PDE4A10 and PDE4A11 expression in hypoxia. The increase observed in the PDE3B isoform has previously been identified in hypoxic hPASMCs (Murray et al., 2002).

The increase in the RIβ subunit of PKA also indicated an alteration in response to cAMP within hypoxic cells. PKARI has previously been shown to be a little overexpressed in normal cells upon stimulation of proliferation, but is constituitively expressed in tumours (McDaid., et al 1999). Vascular smooth muscle cells are known to proliferate in response to hypoxia (Yuan & Rubin 2001). This indicates upregulation of PKARIβ could be involved in the proliferative response of vascular smooth muscle cells to hypoxia.

However, the results from the microarray analysis are only indicative of possible alterations in gene expression as the analysis was only performed twice and problems with microarray analysis have to be taken into account (Watson et al., 2000). Variations between chip to chip, even spot-to spot, allow for problems when considering background levels to normalise data. With tightly regulated genes, this problem is emphasised and small, significant changes are difficult to confirm. Thus, enough replicate microarray hybridisations require to be performed to confirm the data collected.

The data presented in this chapter indicates that the cellular response to chronic hypoxia is a gradual process, with certain signalling pathways being altered immediately, and others occurring over an extended period due to alterations in gene expression and activity. The results suggest cAMP levels are increased immediately, with CRE-regulated genes being induced including PDE4B2, PDE4D1, PDE4D2 and PDE4D5. This increase in cAMP-PDE isoforms reduces cAMP over time and is responsible for the desensitisation of the cAMP pathway in hypoxia *(Figure 3.24)*. Unfortunately, as cAMP-PDE activity was not observed to increase after seven days and a time point of fourteen days was not examined, this would need further work to explore in detail.

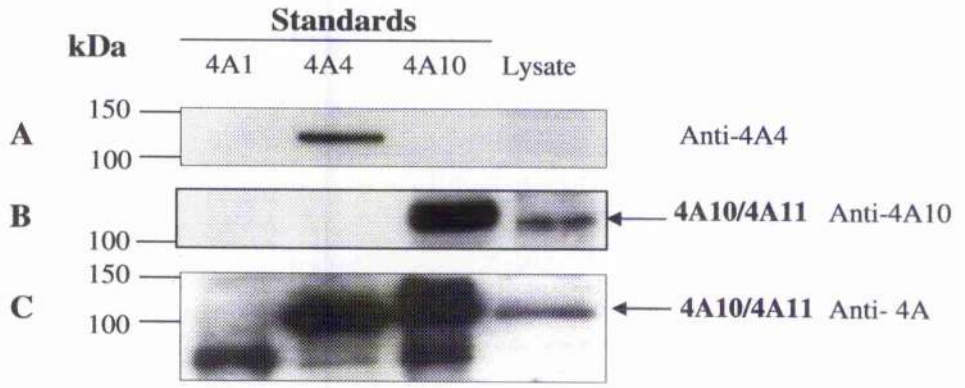


Figure 3.1 Western blot analysis of PDE4A isoforms expressed in hPASMC.

hPASMC cells were harvested in cKHEM buffer and cell lysate was made as described in section 2.3.1.1. 40µg protein was ran out on an 8% SDS-PAGE alongside 5µg recombinant protein standards of PDE4A isoforms PDE4A1 (79kDa), PDE4A4 (125kDa) and PDE4A10 (121kDa). This was then transferred to nitrocellulose and probed with an antibody directed against the c-terminus of PDE4A. As PDE4A4, PDE4A10 and PDE4A11 all run at similar weights on a gel, (125, 121 and 126 kDa respectively), isoform specific antibodies directed against the n-terminus of PDE4A4 *(Panel A)*, PDE4A10 *(Panel B)*, and the c-terminus of PDE4A to produce a general 4A antibody *(Panel C)* were used to distinguish which isoform was being expressed in hPASMC. The molecular weight markers are indicated. A band of approximately 120kDa (indicated in bold), thus corresponding to the PDE4A10/4A11 isoforms, was detectable in hPASMC lysate.

	PRIMER NAME	PRIMER SEQUENCES (5′ - 3′)	FRAGMENT LENGTH (bp)
4A1	4A1 F	TTCTTCTGCG AGACCTGCTC TAAGC	344
	4A1 R	GGT CTT CAC CCC AAA TCG GGG AAT	
4A4	4A4 F	CGG AAA GGA GCC TGT CTC TG	257
	4A4 R	AGT GCC ATG GAA GGA CGA GG	
4A7	4A7 F	GAT AAT GGT GCT TCC TTC AGA CCA AGG C	348
	4A7 R	CCA GCA GTA CGT GGG TGG ACT GCA GCA	
4A10	4A10 F	AGA TCT GTC AGC TTC GAG GCA G	281
	4A10 R	AGT GAG AAG TTG CTA CGG ACG C	
4A11	4A11 F	GCC TAG GCC GCA TCC CGG AGC TGC AAC T	350

Figure 3.2 Primer sequences used for analysis of PDE4A isoforms in hPASMC.

A table indicating primer sequences and fragment size expected for of human PDE4A splice variants, hsPDE4A1 (Genbank accession U97584), hsPDE4A4 (Genbank accession L20965), hsPDE4A7 (Genbank accession U18088), hsPDE4A10 (Genbank accession AF073745), and hsPDE4A11 (Genbank accession AF069488).

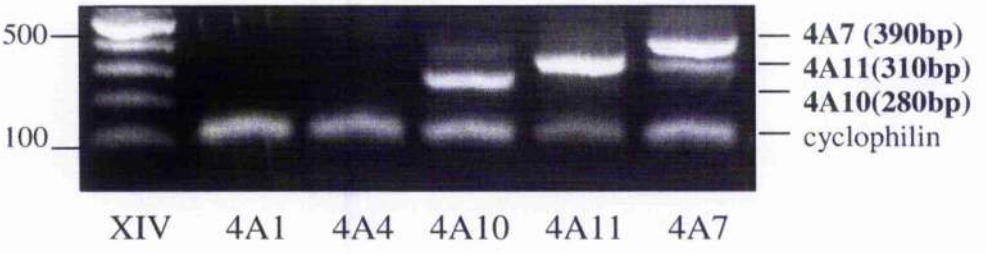


Figure 3.3 RT-PCR analysis of hPASMC using PDE4A specific primers.

RNA was isolated from hPASMC and subjected to first strand cDNA synthesis as described in section 2.4.3.2., followed by RT-PCR using primers specific for the PDE4A isoforms, hsPDE4A1, hsPDE4A4, hsPDE4A7, hsPDE4A10 and hsPDE4A11 *(see figure 3.2 for primer sequences and accession numbers).* The PCR products were ran out on a 2% agarose gel along side a negative control. When the negative control indicated contamination, the PCR result was discarded. DNA marker sizes are indicated. Primer products of approximately 280bp, 310bp and 390bp were detected with PDE4A10, PDE4A11 and PDE4A7 primer sets respectively (indicated in bold).

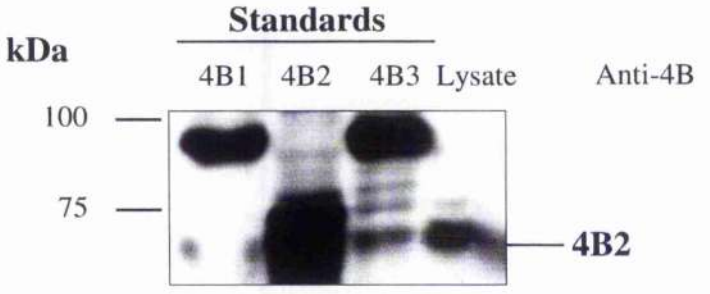


Figure 3.4 Western blot analysis of PDE4B isoforms expressed in hPASMC.

hPASMC cells were harvested in cKHEM buffer and cell lysate was made as described in section 2.3.1.1. 40µg protein was ran out on an 8% SDS-PAGE alongside 5µg recombinant protein of PDE4B isoforms PDE4B1 (104kDa), PDE4B2 (80kDa) and PDE4B3 (103kDa). This was then transferred to nitrocellulose and probed with an antibody directed against the common PDE4B c-terminus region. A band of approximately 70kDa (indicated in bold) could be detected. As this was the same weight as the PDE4B2 protein standard ran on the gel, this band was assumed to be PDE4B2 in hPASMC lysate.

	PRIMER NAME	PRIMER SEQUENCES (5′ - 3′)	FRAGMENT LENGTH (bp)
4B1	4B1 F	AAA GCA GGA GTG TGA TGA CGG	236
	4B1 R	CGT TGT CAA AGG CAG TGT GGT	
4B2	4B2 F	CCT TGA GAT GGC AAA GCA CTC	264
	4B2 R	AAT CAC AGT GGT GCT CTG CCT	
4B3	4B3 F	AAA AGC ATT CGG CAG CGT C	214
	4B3 R	TCG ACA TCG CCT TTG GTG A	

Figure 3.5 Primer sequences used for analysis of PDE4B isoforms in hPASMC.

A table indicating primer sequences and fragment size expected for the human PDE4B splice variants, hsPDE4B1 (Genbank accession L20966), hsPDE4B2 (Genbank accession L20971), and hsPDE4B3 (Genbank accession U85048).

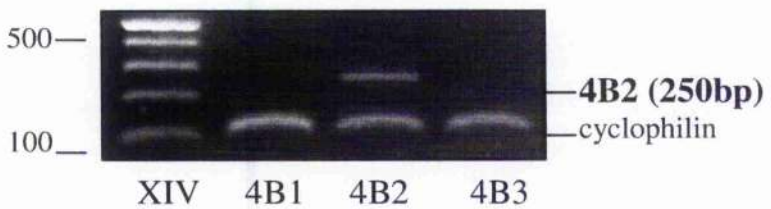


Figure 3.6 RT-PCR analysis of hPASMC using PDE4B specific primers.

RNA was isolated from hPASMC and subjected to first strand cDNA synthesis as described in section 2.4.3.2, followed by RT-PCR using primers specific for the PDE4B isoforms, hsPDE4B1, hsPDE4B2 and hsPDE4B3 *(see figure 3.5 for primer sequences and accession numbers).* The PCR products were ran out on a 2% agarose gel along side a negative control. When the negative control indicated contamination, the PCR result was discarded. DNA marker sizes are indicated. A primer product of approximately 250bp was detected with the PDE4B2 primer set (indicated in bold).

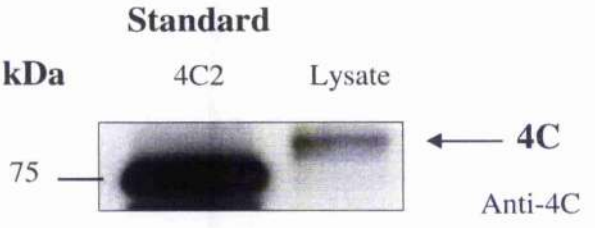


Figure 3.7 Western blot analysis of PDE4C isoforms expressed in hPASMC.

hPASMC cells were harvested in cKHEM buffer and cell lysate was made as described in section 2. 3.1.1. 40µg protein was run out on 8% SDS-PAGE alongside 5µg recombinant protein of the PDE4C isoform PDE4C2 (80kDa). This was then transferred to nitrocellulose and probed with an antibody directed against the c-terminus of PDE4C. A band of approximately 85kDa (indicated in bold) could be detected.

A

	PRIMER NAME	PRIMER SEQUENCES (5′ - 3′)	FRAGMENT LENGTH (bp)
4C	4C F	ATG GAT GGT AAA GCC CTT TGG CTC TTG G	95
	4C R	GTC TCC CTA AAT GGG TGG GAA AGT GAA G	

B

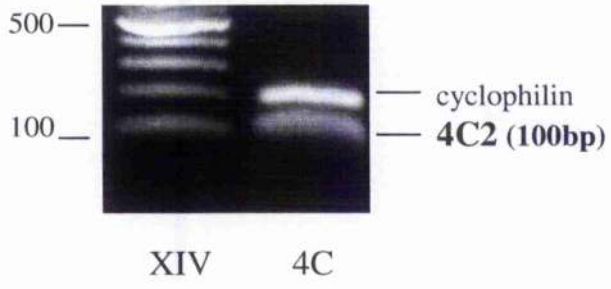


Figure 3.8 RT-PCR analysis of hPASMC using PDE4C specific primers.

Panel A, a table indicating primer sequences and fragment size expected of the transcript of the human PDE4C splice variant, hsPDE4C2 (Genbank accession U66346).

Panel B, RNA was isolated from hPASMC, subjected to first strand cDNA synthesis as described in section 2.4.3.2 and followed by RT-PCR using primers specific for the PDE4C isoform, hsPDE4C2. The PCR products were ran out on a 2% agarose gel along side a negative control. When the negative control indicated contamination, the PCR result was discarded. DNA marker sizes are indicated. A primer product of approximately 110bp was detected with the PDE4C2 primer set (indicated in bold).

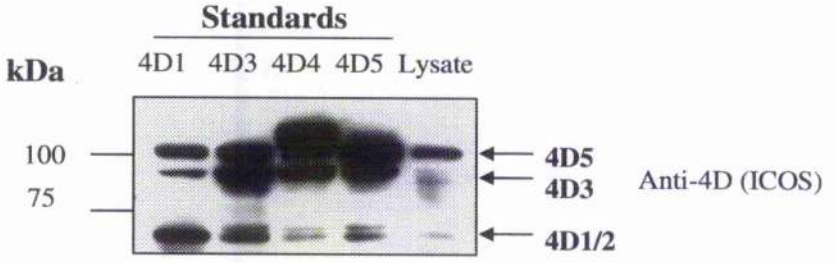


Figure 3.9 Western blot analysis of PDE4D isoforms expressed in hPASMC.

hPASMC cells were harvested in cKHEM buffer and cell lysate was made as described in section 2.3.1.1. 40µg protein was ran out on an 8% SDS-PAGE alongside 5µg recombinant protein of PDE4D isoforms PDE4D1 (68kDa, PDE4D2 runs at a similar weight, 67kDa), PDE4D3 (95kDa), PDE4D4 (119kDa) and PDE4D5 (105kDa). This was then transferred to nitrocellulose and probed with an antibody directed against the c-terminus of PDE4D. The molecular weight markers are indicated. Bands of approximately 100kDa, 89kDa and 65kDa (indicated in bold) could be detected in hPASMC lysate.

B	PRIMER NAME	PRIMER SEQUENCES (5′ - 3′)	FRAGMENT LENGTH (bp)
4D1/2	4D1/2 F	ATA TGA AGG AGC AGC CCT CAT G	221 & 307
	4D1/2 R	CCA GAC CGA CTC ATT TCA GAG A	
4D3	4D3 F	GCG AAC ATG ATG CAC GTG AA	292
	4D3 R	TGG CCA AGA CCT GAG CAA AT	
4D4	4D4 F	AGA AAT CCA GGA TGT CCT GGC	347
	4D4 R	TGC TAG GTG CTC GAT CTT GCA	
4D5	4D5 F	TGC CAG CTG TAC AAA GTT GAC C	212
	4D5 R	TTC TCG GAG AGA TCA CTG GAG A	

Figure 3.10 Primer sequences used for analysis of PDE4D isoforms in hPASMC.

A table indicating primer sequences and fragment size expected for the human PDE4D splice variants, hsPDE4D1/2 (Genbank accession U50157, U50158 respectively), hsPDE4D3 (Genbank accession L20970), hsPDE4D4 (Genbank accession L20969), and hsPDE4D5 (Genbank accession S:1059276).

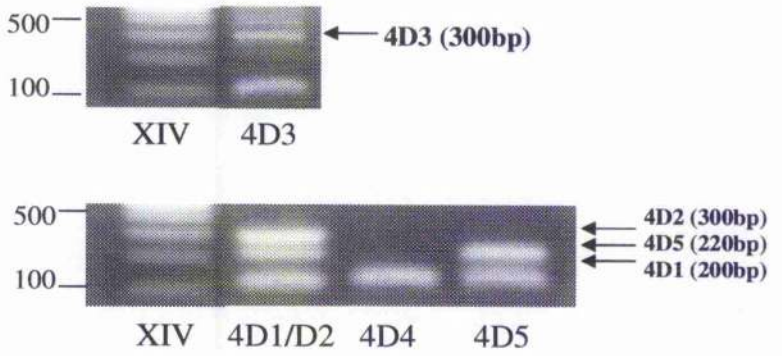


Figure 3.11 RT-PCR analysis of hPASMC using PDE4D specific primers.

RNA was isolated from hPASMC and subjected to first strand cDNA synthesis as described in section 2.4.3.2., followed by RT-PCR using primers specific for the PDE4D isoforms, hsPDE4D1, hsPDE4D2, hsPDE4D3, hsPDE4D4 and hsPDE4D5 *(see figure 3.11 for primer sequences and accession numbers).* The PCR products were ran out on a 2% agarose gel along side a negative control. When the negative control indicated contamination, the PCR result was discarded. DNA marker sizes are indicated. Primer products of approximately 200bp, 220bp, 300bp and 300bp were detected with the PDE4D1, PDE4D5, PDE4D3 and PDE4D2 primer sets respectively (indicated in bold).

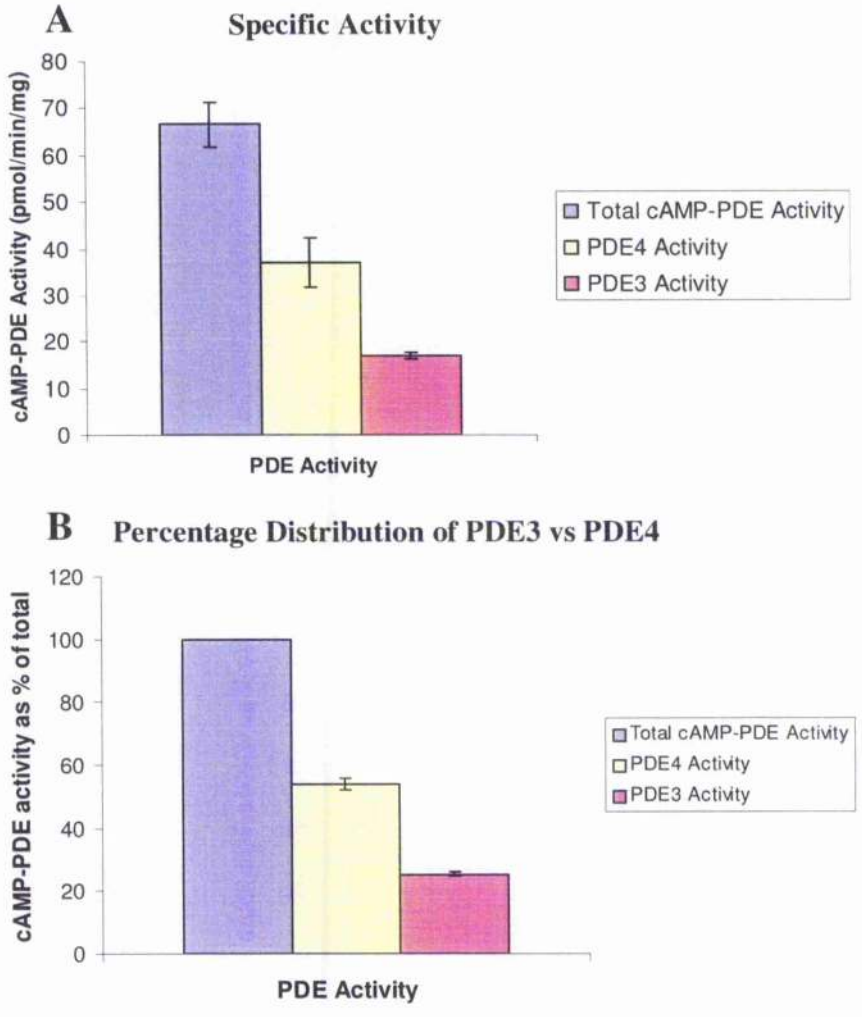


Figure 3.12 cAMP-PDE activity in hPASMC.

Cells were harvested in cKHEM + 1% Triton and the lysates were assayed for total cAMP-PDE Activity, PDE4 Activity, determined as the rolipram sensitive fraction, and PDE3 Activity, determined as the cilostamide sensitive fraction. Phosphodiesterase assay method was described in section 2.3.5. Results are expressed as *panel A,* mean +/- S.E. of 3 independent experiments in pmol/min/mg protein and *panel B,* percentage of total cAMP-PDE activity.

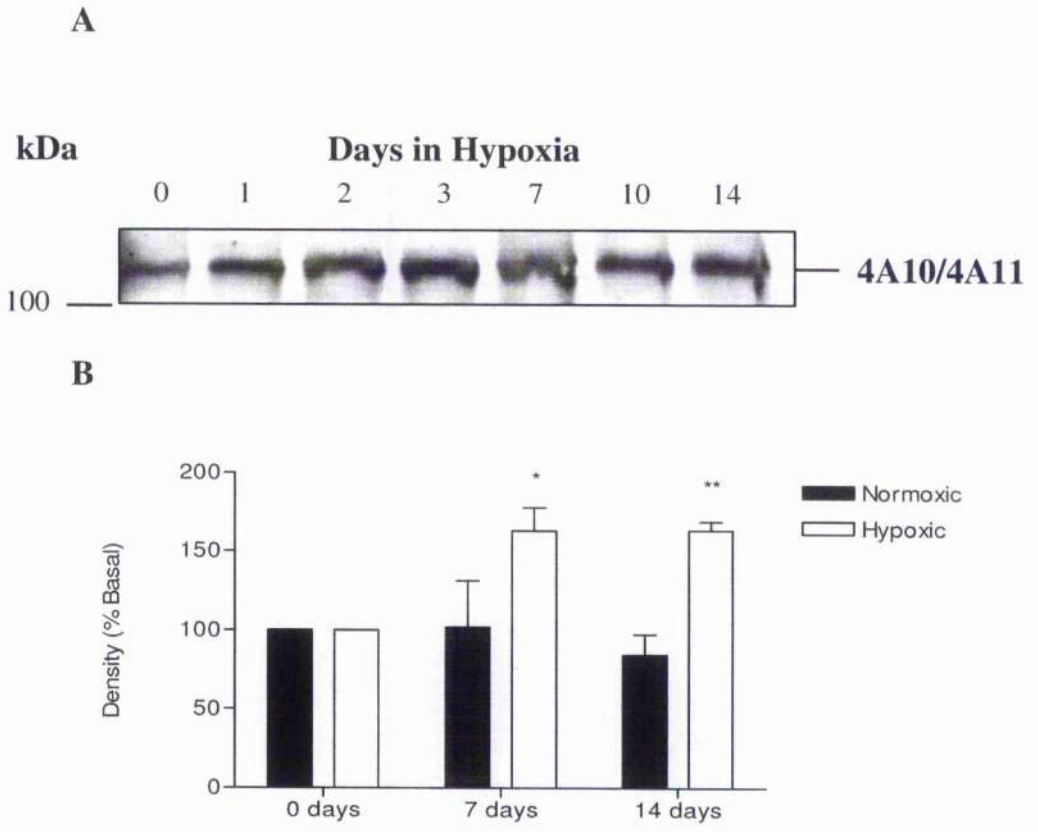


Figure 3.13 Western blot analysis of the effect of chronic hypoxia on PDE4A isoform expression.

hPASMC were split into two groups, one was treated as normal and the other was maintained in a 10% O_2 environment for a period of two weeks. Both sets were treated identically throughout. Cells were harvested from both groups at days throughout the time course and 40µg protein from each time point was used for western blotting.

Panel A, blots were probed with a pan PDE4A antibody. Results shown are hypoxic samples. *Panel B,* densitometry results with normoxic and hypoxic time points compared directly expressed as a percentage of the respective basal density.

Blot shown is representative of three independent experiments and *, ** denotes significance ($p<0.05$, $p<0.01$ respectively)

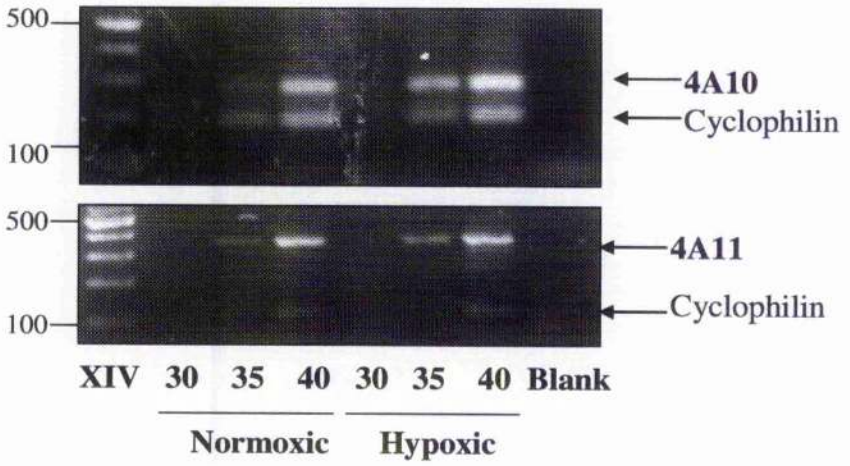


Figure 3.14a RT-PCR analysis of the effect of chronic hypoxia on PDE4A isoform expression.

RNA was isolated from both normoxic and 7 day hypoxic hPASMC and subjected to first strand cDNA synthesis as described in section 2.4.3.2. This was followed by RT-PCR using Superscript II and primers specific for the PDE4A isoforms *(see figure 3.2)*. The PCR products were ran out on a 2% agarose gel along side negative controls. When the negative control indicated contamination, the PCR result was discarded. DNA marker sizes are indicated. The PDE4A PCR products identified are shown and are representative of three independent experiments.

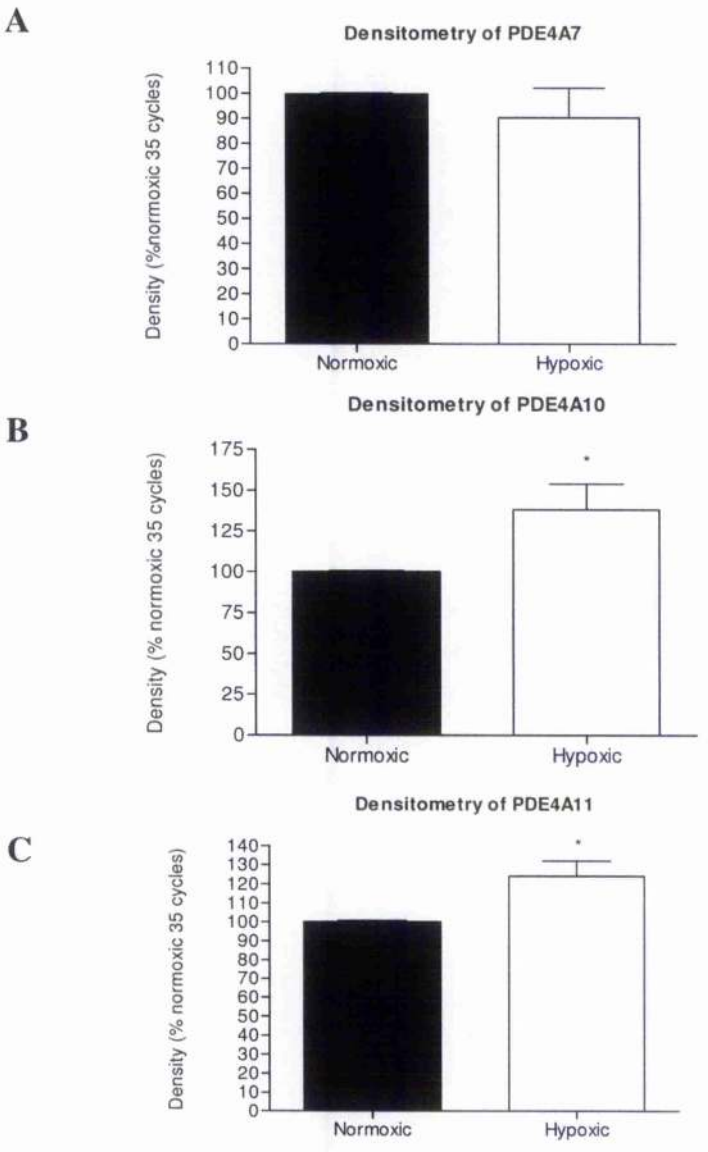


Figure 3.14b Densitometry of RT-PCR analysis of the effect of chronic hypoxia on PDE4A isoform expression.

Quantity-one software was used to measure the density of each band which was then normalised against the density of the corresponding cyclophilin band. *Panel A;* densitometric analysis of PDE4A7 band expressed as a percentage of the normoxic band present at 35 cycles. *Panel B;* densitometric analysis of PDE4A10 band expressed as a percentage of the normoxic band present at 35 cycles. *Panel C;* densitometric analysis of PDE4A11 band expressed as a percentage of the normoxic band present at 35 cycles. Results shown are mean ± S.E. of three independent PCR reactions and * denotes significance ($p<0.05$).

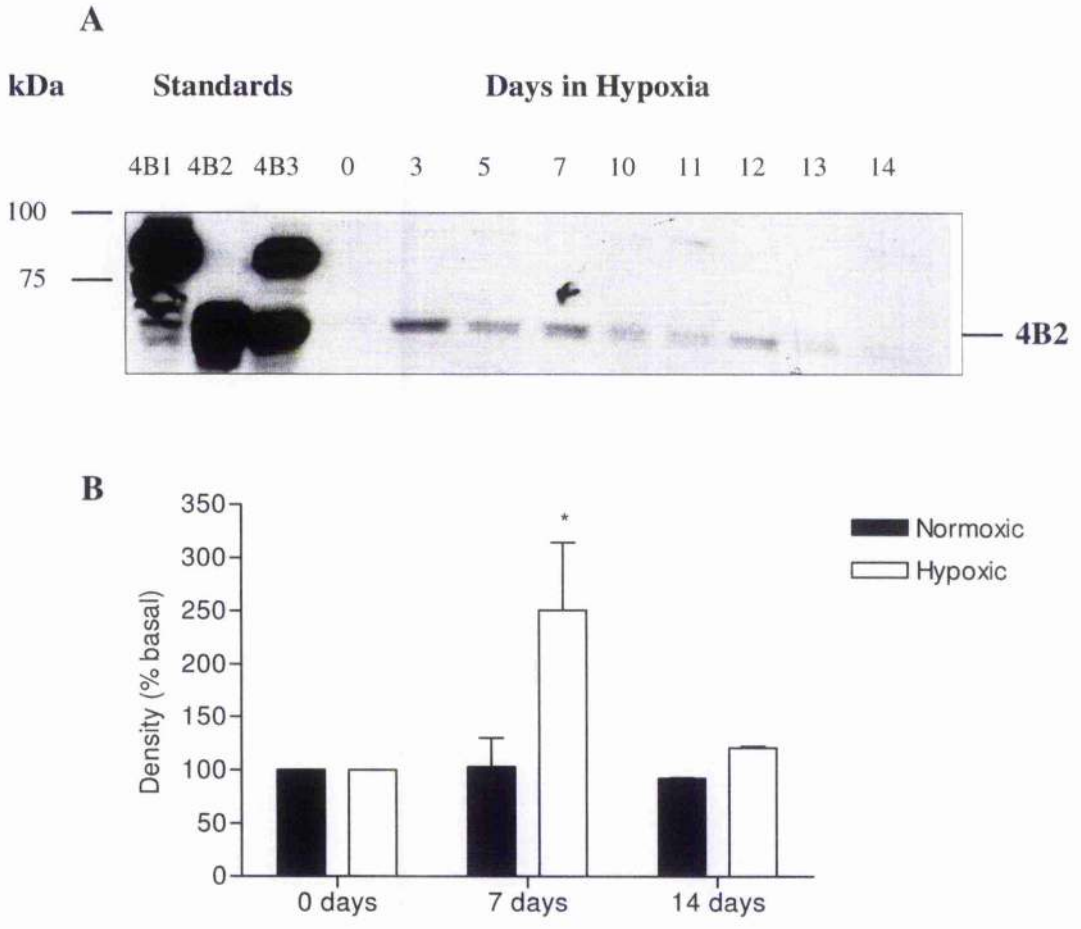
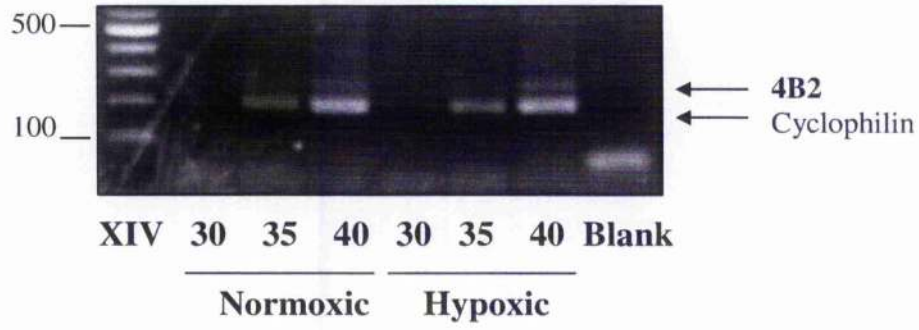


Figure 3.15 Western blot analysis of the effect of chronic hypoxia on PDE4B isoform expression.

hPASMC were split into two groups, one was treated as normal and the other was maintained in a 10% O_2 environment for a period of two weeks. Both sets were treated identically throughout. Cells were harvested from both groups at days throughout the time course and 40µg protein from each time point was used for western blotting.

Panel A, blots were probed with a pan PDE4B antibody. Results shown are hypoxic samples. *Panel B,* densitometry results with normoxic and hypoxic time points compared directly expressed as a percentage of the respective basal density. Blot shown is representative of three independent experiments and * denotes significance ($p<0.05$).

A



B

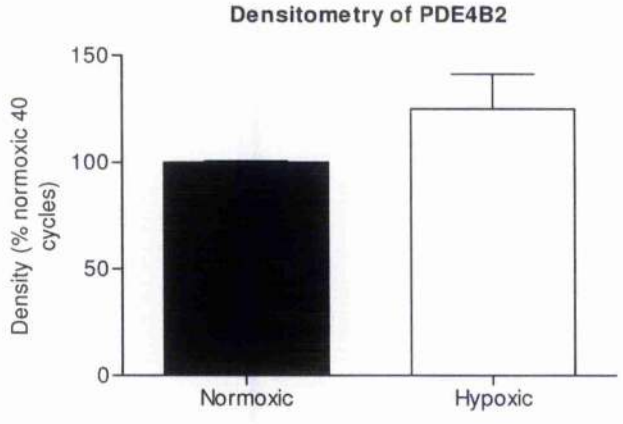


Figure 3.16 RT-PCR analysis of the effect of chronic hypoxia on PDE4B isoform expression.

RNA was isolated from both normoxic and 7 day hypoxic hPASMC and subjected to first strand cDNA synthesis as described in section 2.4.3.2. This was followed by RT-PCR using Superscript II and primers specific for the individual PDE4B isoforms *(see figure 3.5)*. The PCR products were ran out on a 2% agarose gel along side negative controls. When the negative control indicated contamination, the PCR result was discarded. DNA marker sizes are indicated. *Panel A,* PDE4B PCR products, *panel B*, densitometric analysis of PDE4B2 band expressed as a percentage of the normoxic band present at 40 cycles. The results shown are indicative of three independent experiments.

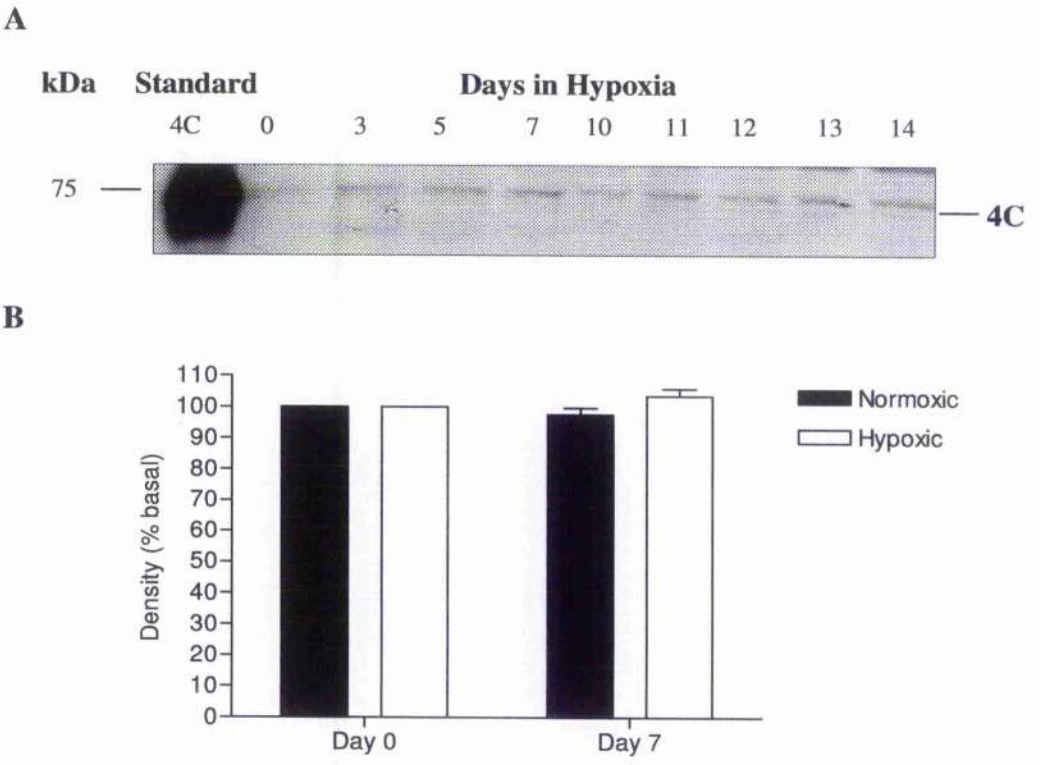


Figure 3.17 Western blot analysis of the effect of chronic hypoxia on PDE4C isoform expression.

hPASMC were split into two groups, one was treated as normal and the other was maintained in a 10% O_2 environment for a period of two weeks. Both sets were treated identically throughout. Cells were harvested from both groups at days throughout the time course and 40µg protein from each time point was used for western blotting.

Panel A, blots were probed with a pan PDE4C antibody. Results shown are hypoxic samples. *Panel B,* densitometry results with normoxic and hypoxic time points compared directly expressed as a percentage of the respective basal density.

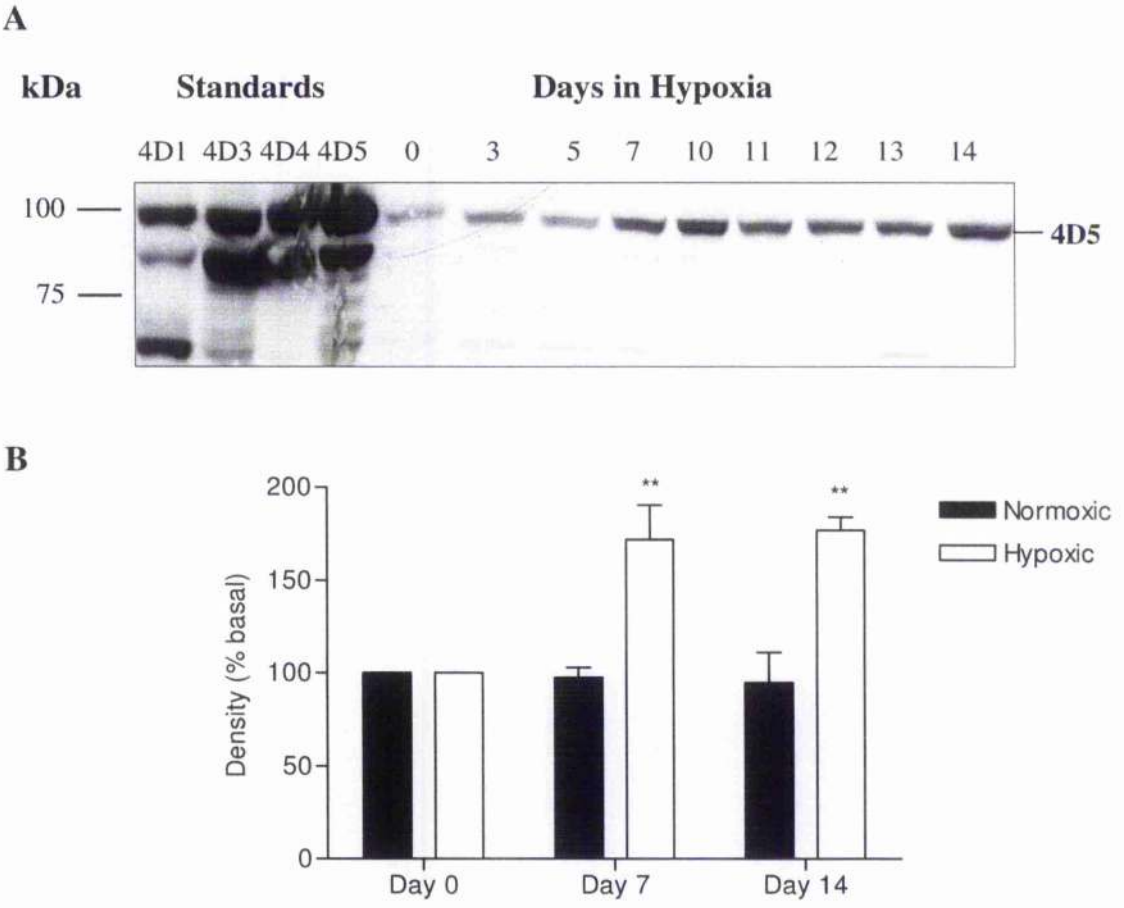


Figure 3.18 Western blot analysis of the effect of chronic hypoxia on PDE4D isoform expression.

hPASMC were split into two groups, one was treated as normal and the other was maintained in a 10% O_2 environment for a period of two weeks. Both sets were treated identically throughout. Cells were harvested from both groups at days throughout the time course and 40µg protein from each time point was used for western blotting.

Panel A, blots were probed with a pan PDE4D antibody. Results shown are hypoxic samples. *Panel B,* densitometry results with normoxic and hypoxic time points compared directly expressed as a percentage of the respective basal density.

Blot shown is representative of three independent experiments and ** denotes significance ($p<0.01$).

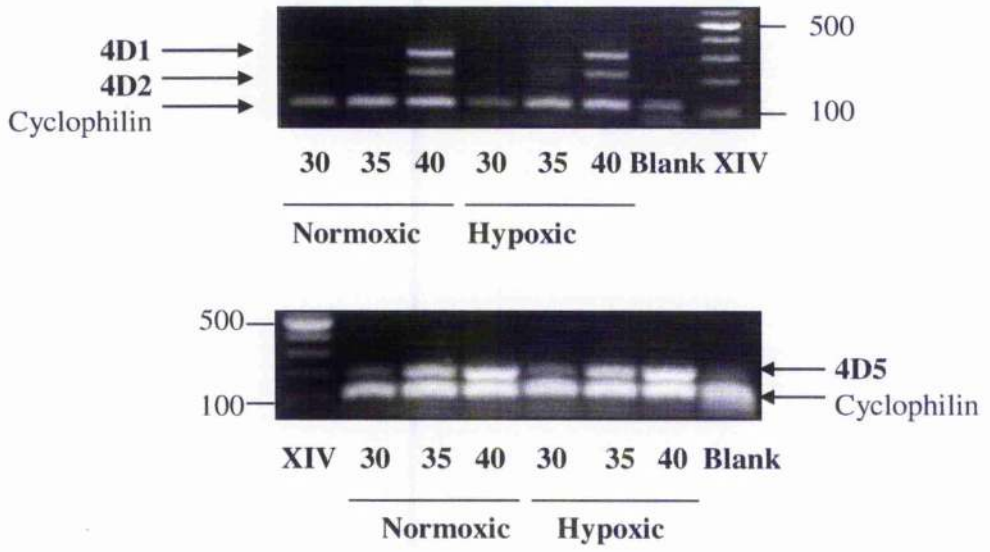


Figure 3.19a RT-PCR analysis of the effect of chronic hypoxia on PDE4D isoform expression.

RNA was isolated from both normoxic and 7 day hypoxic hPASMC and subjected to first strand cDNA synthesis as described in section 2.4.3.2. This was followed by RT-PCR using Superscript II and primers specific for the PDE4D isoforms *(see figure 3.11)*. The PCR products were ran out on a 2% agarose gel along side negative controls. When the negative control indicated contamination, the PCR result was discarded. DNA marker sizes are indicated. The PDE4D PCR products identified are shown and are representative of three independent experiments. PDE4D3 results are not shown due to only n=1 without contamination.

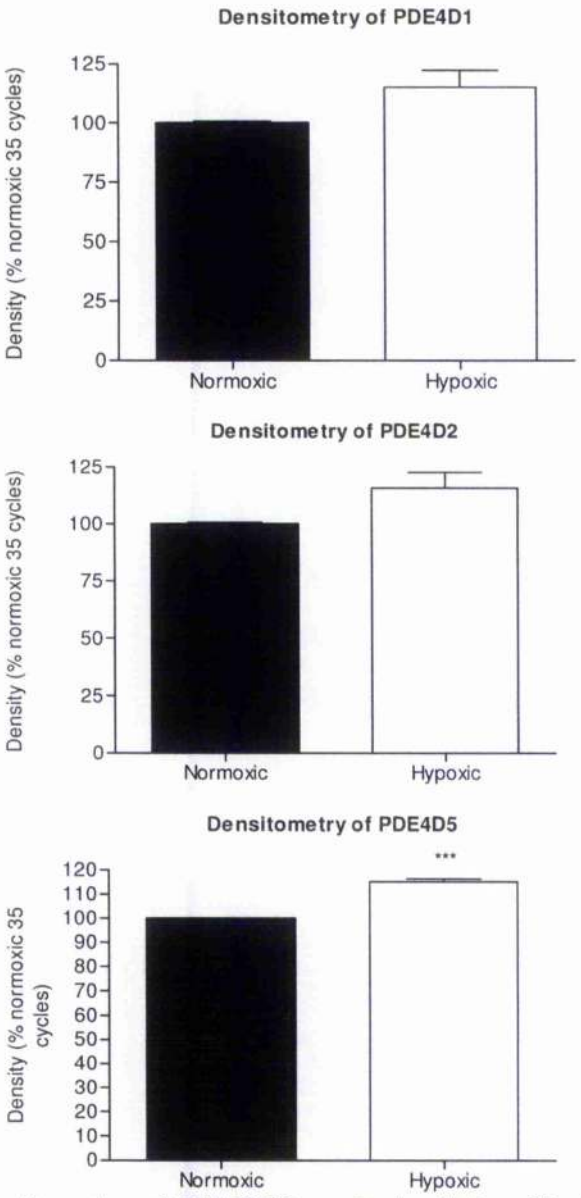


Figure 3.19b Densitometry of RT-PCR analysis of the effect of chronic hypoxia on PDE4D isoform expression. Densitometric analysis of results shown in figure 3.19a. Quantity-one software was used to measure the density of each band which was then normalised against the density of the corresponding cyclophilin band. *Panel A;* densitometric analysis of PDE4D1 band expressed as a percentage of the normoxic band present at 35 cycles. *Panel B;* densitometric analysis of PDE4D2 band expressed as a percentage of the normoxic band present at 35 cycles. *Panel C;* densitometric analysis of PDE4D5 band expressed as a percentage of the normoxic band present at 35 cycles. Results shown are mean ± S.E. of three independent PCR reactions and *** denotes significance ($p<0.001$).

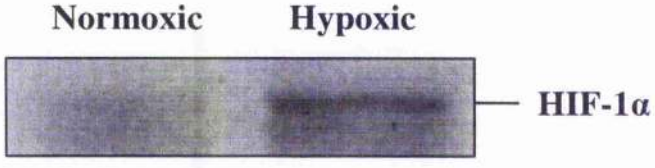


Figure 3.20 HIF-1α expression in normoxic and hypoxic hPASMC.

Seven day hypoxic and normoxic hPASMC were harvested as described in section 2.3.1.1 and used for western blotting with an anti-HIF-1α antibody. HIF-1α is 120kDa (Hanze et al., 2003), and a band was detected at 121kDa in hPASMC exposed to seven days hypoxia, but not in normoxic cells. Blot shown is representative of three independent experiments.

Gene	Ratio= norm/hyp	Number of spots changed/number of spots analysed
PDE5A	1.5	4/4
PDE4A	0.9	4/6
PDE4A11	0.9	3/4
PDE5A3	0.9	5/6
PDE8A	0.9	4/6
PDE4B	0.8	6/6
ERK1	0.8	4/6
ERK2	0.8	4/4
PKAR1B	0.8	5/6
iNOS	0.8	3/6
NFκB	0.8	6/6
p53	0.8	6/6
PDE4D5	0.7	4/6
PDE9A	0.7	2/2
PDE3B	0.7	5/6
Rap1B	0.7	5/6

Figure 3.21 Microarray analysis of RNA from normoxic and hypoxic hPASMC. Total RNA from normoxic and seven day hypoxic cells was used for hybridisation on a custom microarray chip (MWG Biotech). Results shown are from two separate chips, with reverse labelling of samples.

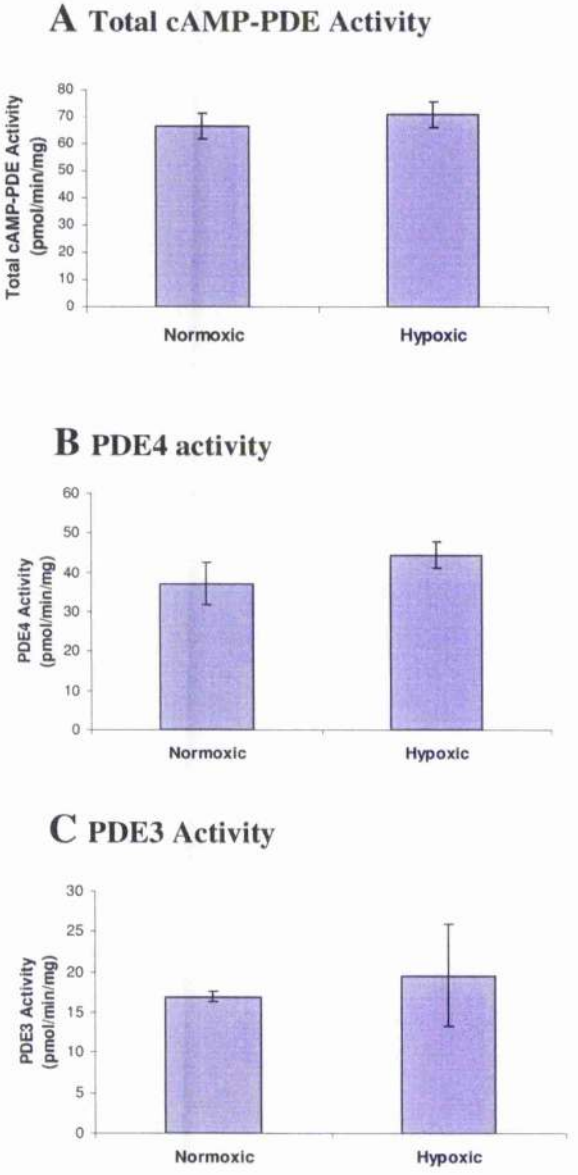


Figure 3.22 Effect of chronic hypoxia on cAMP-PDE activity.

Normoxic and seven day hypoxic cells were harvested in cKHEM + 1% Triton and the lysate was assayed for cAMP-PDE activity as in section 2.3.5. *Panel A,* total cAMP-PDE activity, means ± S.E. of 6 independent experiments. *Panel B,* PDE4 activity, means ± S.E. of 6 independent experiments. *Panel C,* PDE3 activity, means ± S.E. of 3 independent experiments. All results are expressed in pmol/min/mg protein.

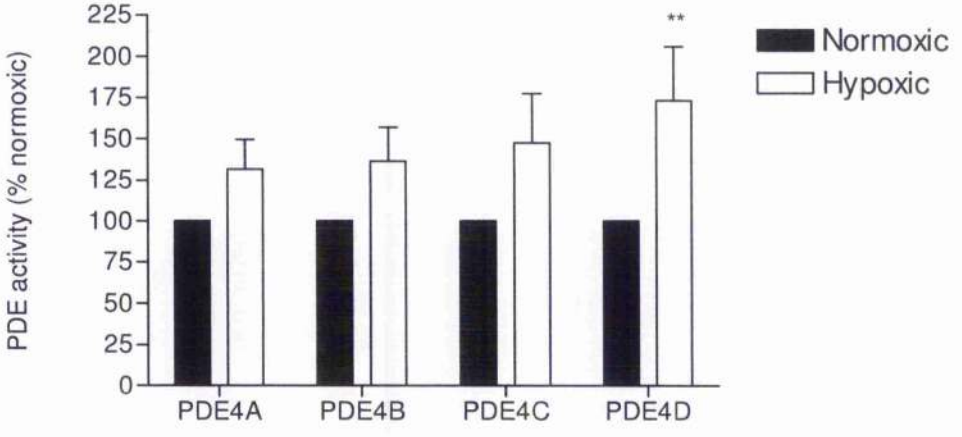


Figure 3.23 Effect of chronic hypoxia on PDE4 subfamily activity.

PDE4A, PDE4B, PDE4C and PDE4D activities were immunoprecipitated as described in section 2.3.6. Samples were then used in a cAMP-PDE assay as described in section 2.3.5. Results shown are mean ± S.E. of 6 independent experiments. Results were calculated in pmol/min/mg protein and converted to a percentage of normoxic activity for each PDE4 subfamily. Significance is denoted by ** ($p<0.01$).

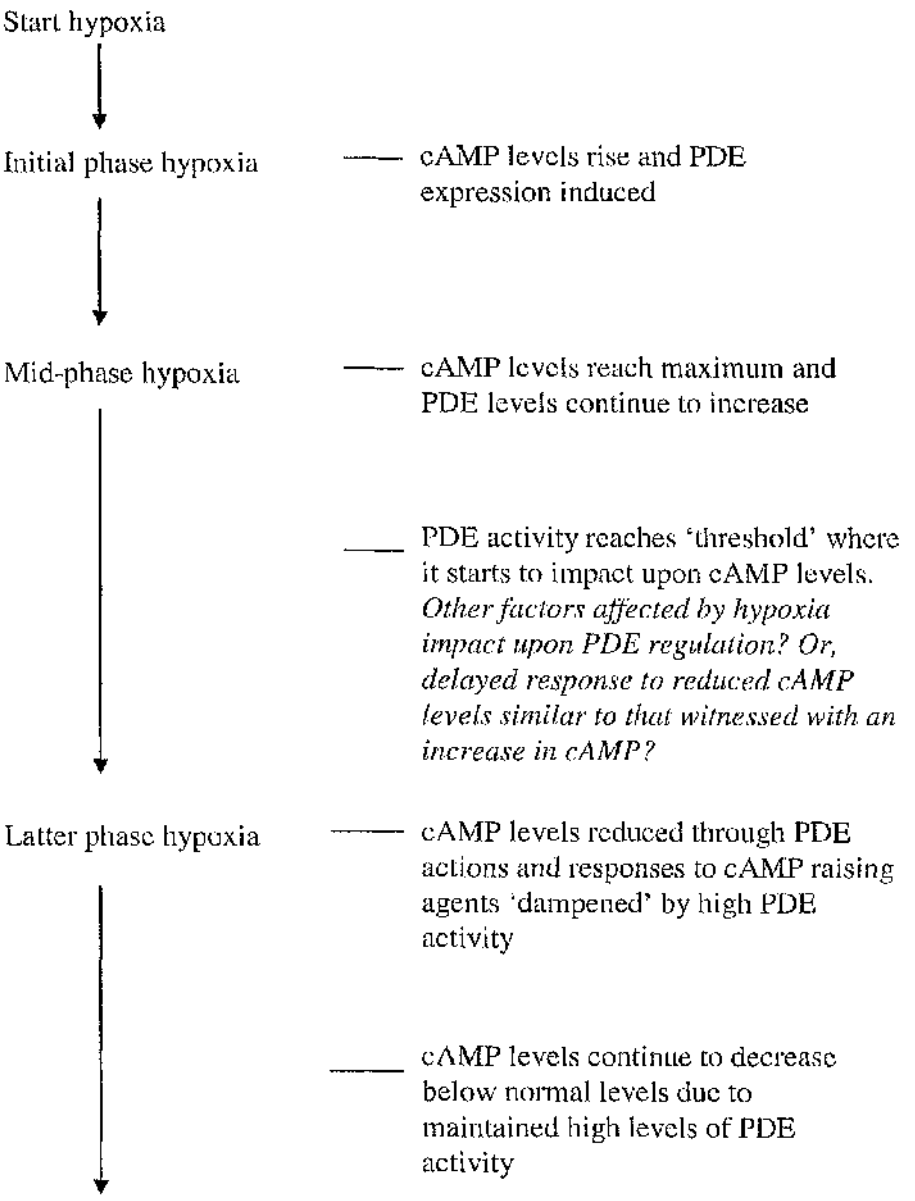


Figure 3.24 Proposed model of cAMP regulation during chronic hypoxia

Above is a model of a proposed theory to explain the desensitisation of the cAMP pathway in hypoxia and during prolonged increased cAMP. Hypoxia causes a rise in cAMP levels, possibly through prostacyclins which are known to increase in hypoxic conditions. This rise in cAMP levels induces PDE expression. cAMP levels continue to rise to maximum rapidly, preventing increases in PDE to have an impact. Once at maximum, cAMP levels remain steady until PDE expression and activity reaches a 'threshold' point where the amount of cAMP hydrolysed is more than the amount generated. This causes a reduction in cAMP levels and attenuates cAMP generation in response to exogenous cAMP elevating agents.

Chapter 4

Effect of Chronic Hypoxia on cAMP Levels in hPASMC

4.1 Introduction

4.1.1 Cyclic Nucleotide Signalling in Models of Hypoxia-Induced Pulmonary Hypertension

4.1.1.1 Cyclic Nucleotides in Hypoxia

It is well established that raising intracellular levels of cAMP and cGMP can lead to smooth muscle relaxation and inhibit VSMC proliferation (Koyama et al., 2001; Rybalkin & Bornfeldt 1999; Osinski & Shror 2000; Indolfi et al., 2001; Boyton & Whitfield 1983, Hayashi et al., 2000; Kronemann 1999; Hofmann 2000; Hakonarson & Grunstein 1998). Raising cAMP levels is known to be more effective at inhibiting VSMC proliferation than elevating cGMP (Murray 1990; Koyama et al 2000). The effects of these cyclic nucleotides are thought to occur through the activation of protein kinase A (PKA) and protein kinase G (PKG) which phosphorylate contractile proteins and channels. For example, PKA acts on the vasodilator-stimulated phosphoprotein, or VASP, which promotes actin nucleation and binds to actin filaments *in vitro* and associates with stress fibres in cells (Shabb 2001). PKA phosphorylation has a negative effect on VASP interaction with actin filaments (Harbeck et al., 2000). PKA and PKG can induce relaxation by acting on downstream targets of Ca2+ mobilisation (Shabb 2001; Marin et al., 1998).

It has been demonstrated that the chronic hypoxic rat displays reduced levels of both cyclic nucleotides in all pulmonary arteries with the exception of the resistance arteries (MacLean et al., 1996). It should be noted that other studies have indicated a rise in cGMP levels in chronic hypoxic rats compared to controls (Cohen et al., 1996). An increase in cyclic nucleotides would be beneficial under hypoxia, aiding to restore the normal tone of the pulmonary circulation. In fact, it has been suggested that hypoxia initially leads to an increase in cAMP through PGI_2 activation, yet after prolonged exposure to hypoxia, desensitisation occurs and pathways are initiated to remove cAMP.

4.1.1.2 Cyclases in Hypoxia

Studies of guanylyl cyclase activity have observed that soluble guanylyl cyclase activity is increased in hypoxia with a corresponding increase in cGMP levels (Li et al., 1999). In the rat lung, the adenylyl cyclase isoforms AC2, AC3 and AC5/6 predominate in pulmonary VSMC, although the isoforms AC 2, AC5 and AC8 are the most functionally important. Looking at these isoforms in hypoxia in the rat, there was no change of expression observed, (Jourdan et al 2001) although there are reduced responses to adenylyl cyclase activators such as forskolin. Under chronic hypoxic conditions, it has been demonstrated

that adenylyl cyclase activity measured under GTP, forskolin or isoproterenol stimulation is reduced in rat hearts (Pei et al., 2000; Hrbasova et al., 2003). Other studies have demonstrated no effect of hypoxia on AC activity however (Pei et al., 2000).

4.1.1.3 Attenuated responses in Hypoxia

It is well established that responses to agents known to stimulate cAMP production such as β-adrenergic agonists are diminished in hypoxia (Hrbasova et al., 2003). Hearts from chronic hypoxic animals indeed display a reduction in β-adrenoceptors and a desensitisation of adenylyl cyclase (Leon-Velarde et al., 2001; Voelkel et al., 1981) However, other studies have demonstrated an increase in β-adrenoceptors and unchanged adenylyl cyclase activity in isolated cardiac myocytes in response to chronic hypoxia (Li et al., 1996), highlighting the different effects of chronic hypoxia due to experimental differences. This is also apparent on studies investigating the effect of hypoxia on G_s and G_i signalling. G_s protein levels have been reported to be decreased or unchanged and G_i protein levels have reported to be increased or unaltered (Hrbasova et al., 2003; Leon-Velarde et al., 2001; Pei et al., 2000).

Precontracted pulmonary arteries relaxed by the addition of the β-adrenoceptor agonist, isoproterenol, demonstrated β-adrenergic relaxation is significantly attenuated in chronic hypoxic rat pulmonary arteries compared (Wagner et al., 1997). In addition, forskolin-stimulated relaxation is also attenuated in the chronic hypoxic rat (Wagner et al., 1997). As no change in adenylyl cyclase activity has been observed in hypoxia (Shaul et al., 1990), it was hypothesised that there was an increased hydrolysis of cAMP. Indeed, this attenuation was alleviated by the addition of either PDE3 or PDE4 inhibitors (Wagner et al., 1997).

As I observed PDE4 levels to increase in hypoxia without any marked increase in total PDE4 activity in the previous chapter, I set out to determine cAMP levels in both normoxic and hypoxic cells and the response of these levels to various agonist and inhibitors. All assays were carried out after seven days of hypoxia. This was in order to gain insight into why there was so little change in PDE4 activity in hypoxia at this time point by uncovering other pathways that can affect upon cAMP and also regulate PDE4 expression and activity in hPASMC. Also, the response to cAMP-elevating agents in hypoxic hPASMC has not yet been investigated.

Results

4.1 cAMP signalling in normoxic and hypoxic hPASMC

All methods used in this chapter are described in full in section 2, materials and methods. Results are calculated as pmol cAMP per ng protein and expressed as a percentage of basal, with basal figures equal to 100%. This normalisation was done due to the variability in absolute amounts of cAMP noted in the various preparations of smooth muscle cells. This could be due to a wide variety of factors including differences caused by serum, PGE1-mediated autocrine stimulation of adenylyl cyclase, cell numbers and cell cycle. However, I have also given an approximate value for cAMP levels, which gives an indication of the magnitude of the level experienced.

4.1.1 cAMP levels in normoxic and hypoxic hPASMC

Cells were maintained in hypoxia for seven days before being harvested, whilst the corresponding controls were maintained under normoxic conditions. These were then used for analysis of intracellular cAMP levels. cAMP levels were measured using a modification of the procedure of Savage, (Savage, 1995), as described in section 2.3.7. Intracellular levels of cAMP in hypoxia were observed to be elevated to three times that of normoxic levels with normoxic cAMP levels = 5.5 ± 2.2 pmol/ng protein and hypoxic cAMP levels = 15.8 ± 1.3 pmol/ng protein, n=3 *(Figure 4.1)*.

4.1.2 PKA Activity in hypoxic hPASMC

With this unexpected increase in cAMP levels in hypoxia, other methods were employed to confirm this observation independently. In doing this I chose to evaluate protein kinase A (PKA) activity as this enzyme provides a prime downstream signalling system that is activated by elevation of intracellular cAMP. Seven day hypoxic and normoxic hPASMC were collected and used for analysis of PKA activity levels as described in section 2.3.7. As can be seen in figure 4.2, under normoxic conditions the percentage of PKA activity activated was 5.5 ± 4.5 % of the total. In marked contrast to this, under hypoxic conditions the percentage of the total PKA activity that was activated had increased to 23.4 ± 6.8 % of the total (n=2). These data are entirely consistent with an increase in cAMP levels occurring in hypoxia.

4.1.3 PKA Expression in hypoxic hPASMC

Seven day hypoxic and normoxic cells were harvested and used for western blotting to identify if PKA subunit expression was altered along with cAMP signalling in hypoxia. Commercial antibodies for PKA RIα, PKA RIIα and PKA RIIβ were employed to detect protein levels of the different PKA subunits. No antibodies are available for the detection of PKA RIβ as yet. Comparing normoxic and hypoxic cell lysates, there was no change in the observed PKA subunits expression levels.*(Figure 4.3).*

4.2 Regulation of cAMP levels in hypoxic hPASMC

After identifying an increase in intracellular cAMP levels, the effect of cAMP pathway effectors was investigated to study the regulation of cAMP with an altered basal level. Previously, cAMP responses have been reported to be attenuated in models of chronic hypoxia (Wagner et al., 1997).

4.2.1 cAMP levels in normoxic and hypoxic hPASMC in response to PDE inhibitors

PDE inhibitors were employed to determine PDE regulation of cAMP levels in normoxic and hypoxic hPASMC. Thus 7d hypoxic and normoxic cells were treated with PDE inhibitors for 20 min prior to being harvested for analysis of intracellular cAMP levels. Rolipram and cilostamide were used to inhibit PDE4 and PDE3 activity, respectively and the non-specific PDE inhibitor IBMX was also used. After this treatment, not only were cells harvested for assay of intracellular cAMP, but the growth medium that the cells were maintained in was aspirated to examine extracellular cAMP levels. cAMP levels were measured as described in section 2.3.7.

4.2.1.1 Intracellular cAMP levels

Intracellular levels of cAMP were seen to increase significantly in response to rolipram ($p<0.001$), cilostamide ($p<0.001$) and IBMX treatment ($p<0.001$) in normoxic cells only *(Figure 4.4a).* Treating hypoxic cells with the inhibitors for the same length of time was observed to have no effect over hypoxic basal *(Figure 4.4a).* Rolipram increased normoxic cAMP levels to 16.4 pmol cAMP/ng protein, cilostamide increased cAMP levels to 14.5 pmol cAMP/ng protein and IBMX increased cAMP levels to 24.7 pmol cAMP/ng protein *(Figure 4.4b).* The increased levels of cAMP that occurred in normoxic cells in response to PDE4 or PDE3 inhibition was therefore approximately equal to that of hypoxic basal levels; 15.8 pmol/ng protein.

Comparing the effect of PDE inhibitors on normoxic samples against hypoxic samples, there was no significant difference between the cAMP levels reached in normoxia or hypoxia *(Figure 4.4b)*.

4.2.1.2 Extracellular cAMP levels

As intracellular cAMP levels in hypoxic hPASMC were not witnessed to rise in response to PDE inhibition, it was hypothesised that the basal levels of cAMP in hypoxia were maximal and extra cAMP generated might have been extruded from the cell. Thus, I set out to determine extracellular levels of cAMP in response to PDE inhibition also.

Extracellular cAMP levels in response to hypoxia were seen to rise to approximately double that of the normoxic extracellular cAMP levels *(Figure 4.5a, b)*. Basal normoxic extracellular cAMP levels were 12 pmol cAMP /ng protein, and hypoxic extracellular cAMP levels were 25.3 pmol cAMP /ng protein *(Figure 4.5a)*. In normoxic cells, both cilostamide and IBMX were observed to increase the extracellular cAMP levels to three times that of basal with an increase to 25.9 pmol cAMP /ng protein and 24.7 pmol cAMP /ng protein respectively *(Figure 4.5b)*, reaching approximately the same levels as hypoxic cells under the same treatment. Rolipram appeared to have no effect on normoxic extracellular cAMP levels over basal. In hypoxic cells, rolipram, cilostamide and IBMX appeared to have no effect on the extracellular cAMP levels compared to basal *(Figure 4.5a)*.

Comparing the effects of rolipram, cilostamide and IBMX on hPASMC cultured in normal conditions on cAMP extracellular levels against the equivalent hypoxic samples showed no significant difference in cAMP levels between normoxic and hypoxic cells, regardless of treatment. This indicates that the hypoxic cells were not generating any extra cAMP in response to PDE inhibition.

4.2.2 cAMP levels in normoxic and hypoxic hPASMC in response to cAMP effectors

Seven day hypoxic and normoxic cells were pre-treated with the non-selective PDE inhibitor, IBMX, for 20 min prior to being treated with either the β-adrenoceptor agonist isoproterenol or the adenylyl cyclase stimulator forskolin for 5 mins or isoproterenol only for 5 min. The cells were then harvested for analysis of intracellular cAMP levels. The growth medium the cells were maintained in was also aspirated to examine extracellular cAMP levels. cAMP levels were measured as described in section 2.3.7.

4.2.2.1 Intracellular cAMP levels

Intracellular levels of cAMP in normoxic cells were seen to increase in response to isoproterenol, IBMX and isoproterenol and IBMX and forskolin treatment ($p<0.001$) to 3.6 times, 4.4 times and 13.3 times that of normoxic basal levels respectively *(Figure 4.6a)*. Treating hypoxic cells did not have the same effect *(Figure 4.6a)*.Instead; isoproterenol only increased hypoxic cAMP levels to 1.6 times that of hypoxic basal. Using both isoproterenol and IBMX together raised hypoxic cAMP levels to 1.7 times that of hypoxic basal levels and IBMX used in conjunction with forskolin raised cAMP levels to only 2.9 times that of hypoxic basal *(Figure 4.6a)*.

Isoproterenol increased normoxic cAMP levels to 20.2 pmol cAMP /ng protein and hypoxic cAMP levels to 22.4 pmol cAMP /ng protein. IBMX and isoproterenol increased normoxic cAMP levels to 25.8 pmol cAMP /ng protein and hypoxic cAMP levels to 27.6 pmol cAMP /ng protein. However, IBMX and forskolin increased normoxic cAMP levels to 81.8 pmol cAMP /ng protein whereas they increased hypoxic cAMP levels to only 59.3 pmol cAMP /ng protein *(Figure 4.6b)*.

The increased levels of cAMP in response to β-adrenergic stimulation was therefore approximately equal in both normoxic and hypoxic cells with no significant difference between them *(Figure 4.6b)*, although hypoxic basal levels are higher than that of normoxic basal levels. Inhibition of PDE activity potentiated the effect of isoproterenol, but, again, there was no significant difference between the normoxic and hypoxic cAMP levels. Maximally raising cAMP levels with the adenylyl cyclase stimulator, forskolin, and at the same time inhibiting PDE activity, served to raise normoxic cAMP levels to approximately 81.8 pmol cAMP/ng protein. In hypoxic cells, cAMP was only elevated to 59.3 pmol cAMP/ng protein in response to IBMX and forskolin treatment. Therefore, the previously reported attenuation of the cAMP response in the chronic hypoxic rat is also apparent in chronically hypoxic hPASMC (Wagner et al., 1997).

Comparing the effect of isoproterenol, isoproterenol and IBMX, and forskolin with IBMX, there was no significant difference between the cAMP levels reached in normoxic or hypoxic cells *(Figure 4.6b)*.

4.2.2.2 Extracellular cAMP levels

Treating normoxic and hypoxic hPASMC with isoproterenol had no significant effect on extracellular cAMP levels compared to their respective basal cAMP levels *(Figure 4.7a, b)*

This was also the case for treatment with IBMX and isoproterenol or IBMX or forskolin. Although these treatments did induce a rise in normoxic extracellular cAMP levels, this increase was not significant over basal. Comparing the extracellular cAMP levels in response to these stimulations, it was also observed that there was no significant difference between normoxic and hypoxic extracellular cAMP levels.

4.2.3 cAMP levels in normoxic and hypoxic hPASMC in response to U0126

It had been previously reported that active ERK within smooth muscle cells can feed into the PGE_2 pathway and lead to an increase of cAMP levels through activating adenylate cyclase (Baillie et al., 2001; see figure 1.3). ERK inhibitors were then used to determine if this pathway was in effect in hPASMC. U0126 is an ERK inhibitor (Duncia et al., 1998) that works by inhibiting MEK1 (IC_{50} = 72nM) and MEK2 (IC_{50} = 58nM). As it was used at 10μM, both MEK1 and MEK2 were fully inhibited. Normoxic and hypoxic cells were treated with U0126 for a period of either two or forty-eight hours and cells immediately assayed for levels of intracellular cAMP. In this set of assays, basal normoxic cAMP levels were 192.7 pmol cAMP/ng protein and untreated hypoxic cAMP levels were 1531.8 pmol cAMP/ng protein (893% of basal; *Figure 4.8a).*

It was discovered that U0126 had no significant effect on cAMP levels in normoxic cells. In hypoxic cells however, cAMP levels were significantly reduced to 434 ± 138% of basal levels ($p<0.05$). This effect was further potentiated after 48 hrs with a reduction in cAMP to 315 ± 135 % of basal ($p<0.01$; *figure 4.8a, b*). By comparing normoxic and hypoxic values against each other, it was observed that there was no longer any significant difference in cAMP levels following treatment with U0126 *(Figure 4.8b).* In addition, when comparing the hypoxic cAMP levels in response to U0126 with normoxic basal cAMP levels, there was no significant difference *(Figure 4.8c).*

4.2.4 cAMP levels in normoxic and hypoxic hPASMC in response to PD980589

PD980589 is another ERK inhibitor that works by inhibiting MEK1 (IC50 = 4μM) and MEK2 (IC_{50} = 50μM) and as such was also used to investigate the effect of inhibiting ERK on cAMP levels to confirm the results seen with U0126 (Alessi et al., 1995). By using PD980589 at 20μM, only MEK1 was fully inhibited. In this set of assays, basal normoxic cAMP levels were 192.7 pmol/ng protein and basal hypoxic cAMP levels were 1531.8 pmol/ng protein (893% of basal; *Figure 4.8a*).

It was discovered that PD980589 had no effect on cAMP levels in normoxic cells after 2 hrs and 48 hours treatment *(Figure 4.8a,b)* In hypoxic cells, PD980589 reduced cAMP levels significantly to 319 ± 96% of basal levels ($p<0.01$) and this reduction was maintained after 48 hours at 293 ± 83% ($p<0.01$; *figure 4.8a,b).* By comparing normoxic and hypoxic values against each other, it was observed that as with U0126 treatment, there was no longer any significant difference following treatment with PD980589 *(Figure 4.8b).* In addition, when comparing the hypoxic cAMP levels in response to PD980589 with normoxic basal cAMP levels, there was no significant difference *(Figure 4.8c).*

4.2.5 Effect of PGE_2 and indomethacin on cAMP levels in normoxic and hypoxic hPASMC

As inhibition of the ERK pathway reduced cAMP levels in hypoxic cells to the equivalent of that in normoxia, it was proposed that the autocrine loop was indeed in effect in these cells. In order to address this experimentally, hPASMC were exposed to either PGE_2 or indomethacin to directly stimulate adenylyl cyclase or inhibit the actions of COX-2 and thus reduce production of endogenous PGE_2. Normoxic and hypoxic cells were treated with 1μM PGE_2 and 10μM indomethacin for a period of 10 mins or 2 hours respectively and then intracellular cAMP levels were assayed. In this set of assays, basal normoxic cAMP levels were 81.5 pmol/ng protein and basal hypoxic cAMP levels were 1416.9 pmol/ng protein (1738% of basal; *Figure 4.9b*).

It was discovered that PGE_2 treatment raised normoxic cAMP levels to 1787 ± 131% of basal ($p<0.001$; *figure 4.9a, b*). In hypoxic cells however, PGE_2 had no significant effect on cAMP levels with only a reduction to 1538 ± 29% of basal *(Figure 4.9a, b)*, suggesting AC function is compromised in hypoxic cells. Treatment with the COX inhibitor, indomethacin, reduced cAMP levels to 212 ± 5% that of basal levels in normoxic cells. cAMP levels in hypoxic indomethacin treated cells were reduced to 304 ± 38 % of basal, a level at which there was no significant difference between normoxic and hypoxic indomethacin treated samples *(Figure 4.9b).*

4.3 Discussion and Conclusions

It was observed in the previous chapter that exposing hPASMC to hypoxic conditions altered their PDE4 profile when compared to normoxic hPASMC. The increased expression of PDE4 did not result in higher levels of cAMP hydrolysis however as no change in PDE4 activity or indeed total cAMP-PDE activity was observed in response to hypoxia. I then chose to investigate cAMP levels in response to cAMP effectors to see if they could induce different effects within hypoxia and normoxia and investigate if desensitisation was apparent in hypoxic hPASMC.

Hypoxia was observed to induce a three-fold increase in basal levels of cAMP in hypoxic hPASMC. This is in contrast with previous studies that have reported a reduction in cAMP in hypoxic rat lung (MacLean et al., 1997). However, the increase in cAMP levels in hypoxia was confirmed by an approximate two-fold increase in the activity of the cAMP-dependant kinase, PKA, after 7 days hypoxia. This increased activity was not due to an increase in PKA RIα, PKA RIIα or PKA RIIβ expression. It is conceivable that as PKA RIβ protein levels were not investigated, then this PKA isoform might possibly be increased in hypoxia. It has also been indicated in studies in mice with PKA RI or RII 'knocked-out' that adaptive changes occur in the other subunit to compensate, thus it may be difficult to see any change of expression (Veugelers et al., 2003). The difference between these studies can be explained by the different time point of hypoxia studied. MacLean et al. measured cAMP levels after 14 days of hypoxia and it is possible that cAMP levels are modulated throughout this time course. Indeed, the effect of hypoxia on PDE3 is suggestive of an initial increase in cAMP levels as the increase in the PDE3A isoform observed in hPASMCs is PKA-dependant and can be mimicked by cAMP analogs (Murray et al., 2002). Also, results in chapter 3 demonstrate that the cAMP-inducible PDE4 isoforms present within hPASMC display an increased expression after 7 days hypoxia.

PDE inhibitors have been previously shown to reduce smooth muscle cell proliferation and induce vasodilation of the pulmonary circulation (Koyama et al., 2001; Ghofrani et al., 2004; Wagner et al., 1997; Bardou et al., 2002; Goirand et al., 2001; Palmer et al., 1998; Osinski & Shror 2000), both of which are characteristic responses to elevated cAMP. The

response to the inhibition of each PDE family differs in magnitude. For example, rolipram has been observed to be more potent than PDE3 or PDE5 inhibitors at relaxing human intralobar pulmonary arteries (Bardou et al., 2002). Thus, the levels of cAMP generated in response to PDE inhibition were measured. In normoxic hPASMC, PDE4, PDE3 and general PDE inhibition elevated cAMP levels to approximately three times that of basal. Surprisingly, PDE inhibition did not appear to elevate cAMP over hypoxic basal in hypoxic cells. As responses to PDE inhibition are still apparent in hypoxic models (Wagner et al., 1997), it was hypothesised that the basal levels of cAMP in hypoxic cells represented a threshold level above which, excess cAMP was extruded from the cells. Measuring extracellular cAMP in response to PDE inhibition revealed this was not the case however as extracellular cAMP levels were equal in basal hypoxic cells and hypoxic cells with PDE inhibition. Intriguingly however, it was demonstrated that whereas the extracellular levels of cAMP in normoxic cells in response to cilostamide and IBMX were increased, extracellular cAMP generated in response to rolipram was equal to that of basal normoxic extracellular cAMP levels.

The levels of cAMP in response to isoproterenol and forskolin was measured in normoxic and hypoxic hPASMC to determine if the attenuated responses to these agents in hypoxic models (Leon-Velarde et al., 2001; Voelkel et al., 1981; Wagner et al., 1997) is due to a reduced production of cAMP. Indeed, this was demonstrated to be the case. In normoxic cells, isoproterenol alone and isoproterenol with IBMX, increased cAMP levels to approximately four times that of basal. IBMX and forskolin treated cells maximally increased cAMP to thirteen times basal levels. In hypoxic cells however, isoproterenol with and without IBMX only managed to increase cAMP levels to approximately double that of hypoxic basal cells. The amount of cAMP generated in response to IBMX and forskolin was also reduced in hypoxic cells as it was only increased to three times that of hypoxic basal levels. Although hypoxic basal levels are higher than that of normoxic basal cAMP levels, the increased cAMP levels in response to isoproterenol and forskolin stimulation was greater in normoxic cells. The reduced cAMP in response to isoproterenol and forskolin stimulation in hypoxic cells was not due to excess cAMP being extruded from the cells as extracellular cAMP levels were approximately equal in normoxic and hypoxic cells.

ERK is known to regulate PDE4 enzymatic activity. It has been previously shown that ERK inhibits the activity of the long forms PDE4B1, 4C2 and 4D5 as they have an ERK docking motif within their catalytic domain (MacKenzie et al., 2000). PDE4A enzymes are

unaffected as they lack the ERK phosphorylation motif (Baillie et al. 2000). As PDE4D5 is seen to increase too in hypoxia, I hypothesised that due to the known increase in pERK in hypoxia it may become inhibited constitutively by being phosphorylated by ERK. I thus investigated the effects of ERK inhibitors on cAMP levels to see if this could perhaps release the PDE4D5 from inhibition and allow it to hydrolyse cAMP within hypoxia. It was observed that the ERK inhibitors PD985089 and U0126 reduced hypoxic cAMP levels to half of hypoxic basal levels. This reduced level of cAMP was not significantly different to normoxic basal levels. This indicates ERK regulates the increased level of cAMP witnessed initially in hypoxia. However, ERK inhibition of PDE4D5 is transient due to the resultant PKA phosphorylation of PDE4D5 overriding any effect of ERK, indicating another mechanism for ERK regulation of cAMP levels.

Previous research into PDE4 in smooth muscle cells revealed an autocrine loop whereby active ERK fed into the PGE_2 pathway leading to a rise in cAMP, activating PKA thus activating PDE4D5 and decreasing cAMP levels (Baillie et al 2001; see figure 1.3). It is known that levels of PGE_2 and active ERK are increased in hypoxia (Shaul et al., 1991; Jin et al., 2000), also COX-2 expression is observed to increase in response to sustained hypoxia (Bradbury et al., 2002; Yang et al., 2002). Thus, I set out to investigate if this is how ERK affects cAMP levels in hypoxia by examining cAMP levels in response to PGE_2 and indomethacin. I observed that PGE_2 increased normoxic cAMP levels to that of basal hypoxic cells and indomethacin reduced hypoxic cAMP levels to that of basal normoxic cells. In hypoxic cells, the response to exogenous PGE_2 was desensitised, with no increase in cAMP levels over basal. This indicates that the autocrine effect observed previously in human aortic smooth muscle cells (Baillie et al., 2001) was in effect within my cells. Thus an increased level of cAMP could be due to an increase in endogenous PGE observed previously in hypoxia (Bradbury et al., 2002).

The results shown in this chapter demonstrate desensitisation to cAMP-elevating agents in hypoxia is due to reduced cAMP production in response to G_s stimulation. Desensitisation was most likely caused by the prolonged exposure to increased cAMP induced by 7 days of hypoxia as maintained exposure of cells to cAMP analogs or AC stimulators has previously been shown to cause desensitisation (Moon et al., 2002). In addition, the known elevation of ERK in hypoxia (Jin et al., 2000) I have shown here is intimately involved in mediating the hypoxia-induced increase of cAMP. This presumably occurs through the actions of ERK on PLA_2 and the generation of PGE_2, with consequent autocrine effect

(Pinelli et al., 1999), coupled perhaps with the ability (Baillie et al., 2000) of ERK to modify the activity of members of the PDE4 family.

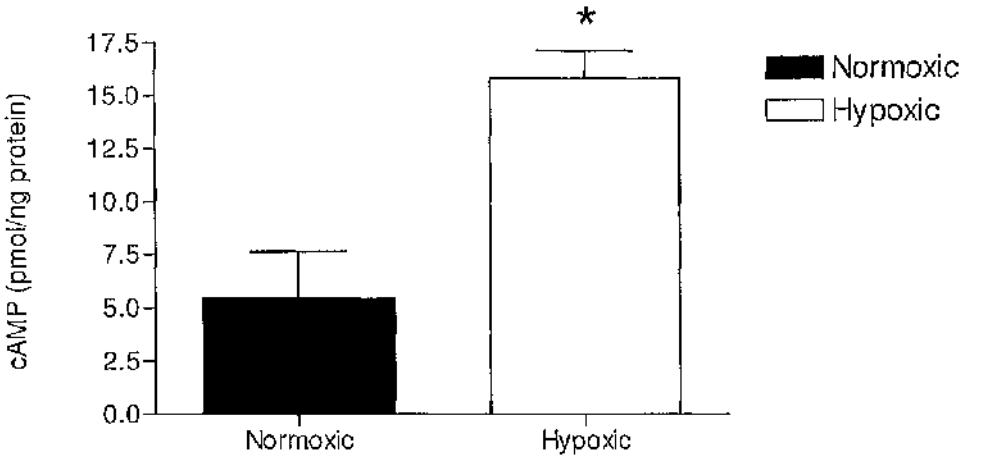


Figure 4.1 Effect of chronic hypoxia on intracellular cAMP levels.

Cell lysates were assayed for cAMP levels in normoxic and seven day hypoxic hPASMC as described in section 2.3.7. Normoxic and hypoxic samples show 5.5 ± 2.2 and 15.8 ± 1.3 pmol cAMP/ng protein respectively. Results are expressed as mean +/- S.E. of 3 independent experiments as pmol cAMP per ng protein and * denotes significance ($p<0.05$) between normoxic and hypoxic cAMP levels.

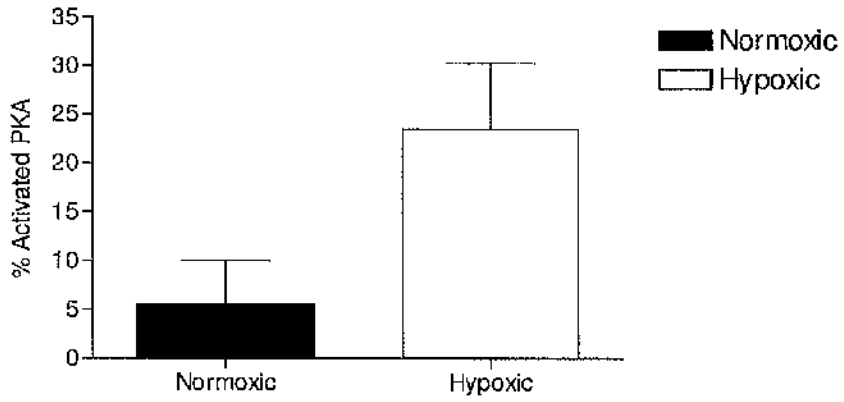
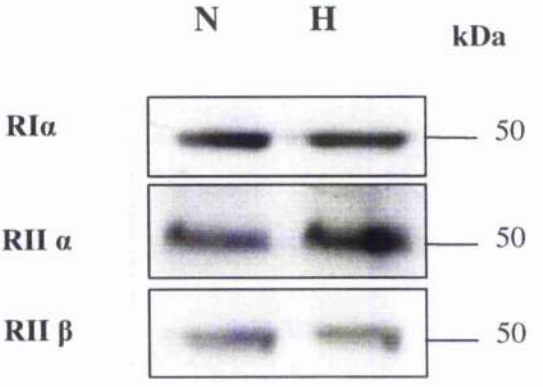


Figure 4.2 Effect of chronic hypoxia on PKA activity.

Cell lysates were assayed for PKA activity in normoxic and seven day hypoxic hPASMC as described in section 2.3.7. Normoxic and hypoxic samples show 5.5 ± 4.5 % and 23.4 ± 6.8 % activated PKA respectively. Results are expressed as mean +/- S.E. of 2 independent experiments as percentage of activated PKA.

A



B

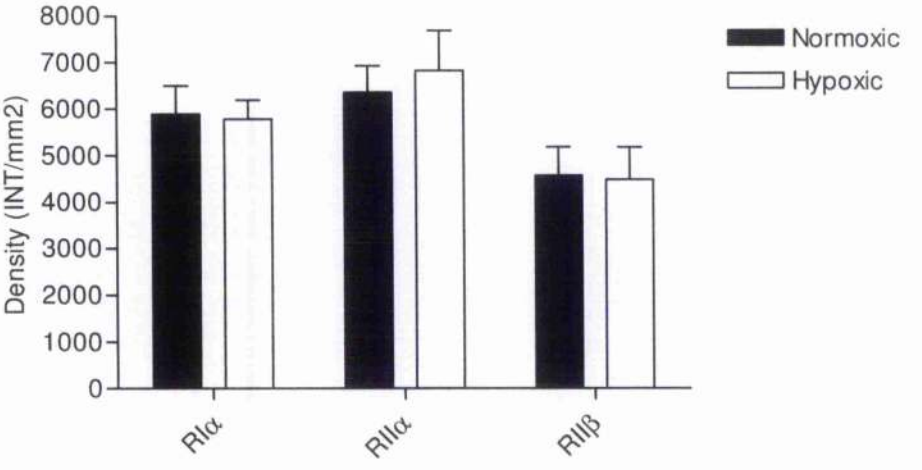
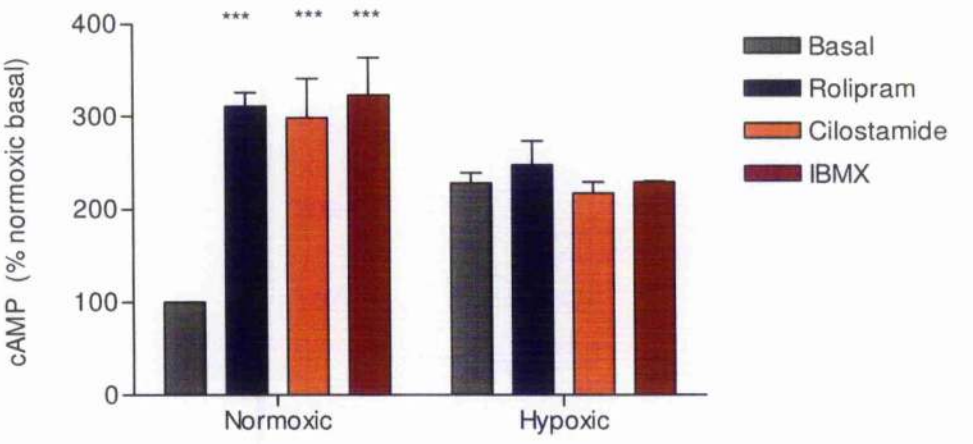


Figure 4.3 Effect of chronic hypoxia on PKA subunit expression.

Panel A, seven day hypoxic and corresponding normoxic cells were harvested as described in section 2.3.1.1 and used for western blotting with antibodies raised against the specific subunits of PKA. All isoforms are 50kDa in weight so blots were done separately. The blots shown are representative of three individual experiments.

Panel B, densitometry analysis of bands for each subunit expressed as mean ± S.E. of 3 independent blots as INT/mm^2.

A



B

	Normoxic	Hypoxic
Basal	1	1
Rolipram	3.1 +/- 0.1	1.1 +/- 0.1
Cilostamide	3 +/- 0.4	1 +/- 0.1
IBMX	3.2 +/- 0.4	1.0 +/- 0.06

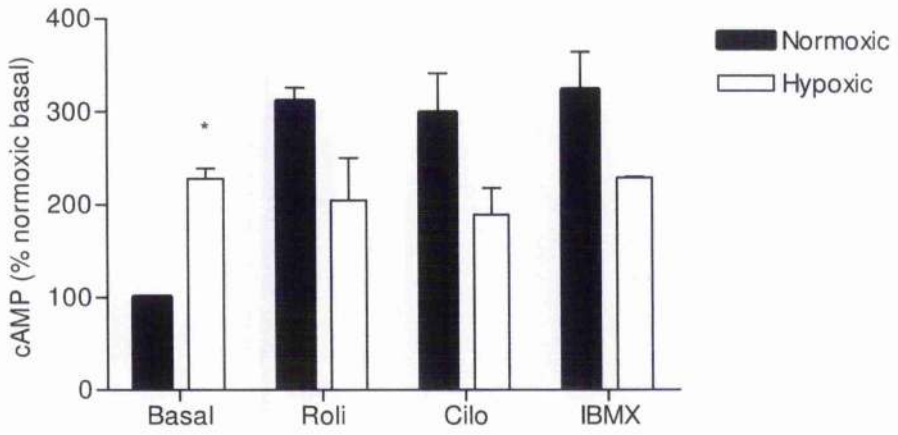
Figure 4.4a Effect of PDE inhibitors in chronic hypoxia compared with normoxic levels on intracellular cAMP levels.

Cells were pre-treated with 10μM rolipram, 10μM cilostamide or 10μM IBMX for 20 min and were immediately assayed for cAMP levels in normoxic and seven day hypoxic hPASMC as described in section 2.3.7.

Panel A, results graphed as mean cAMP ± S.E. of fold normoxic basal. *Panel B,* results expressed as mean ± S.E. of fold basal for both normoxic and hypoxic samples.

Results shown are mean ± S.E. of three independent experiments and *** denotes significance ($p<0.001$).

A



B

	Normoxic	Hypoxic
Basal	5.5	15.8
Rolipram	16.4	18.3
Cilostamide	14.5	15.7
IBMX	24.7	19.3

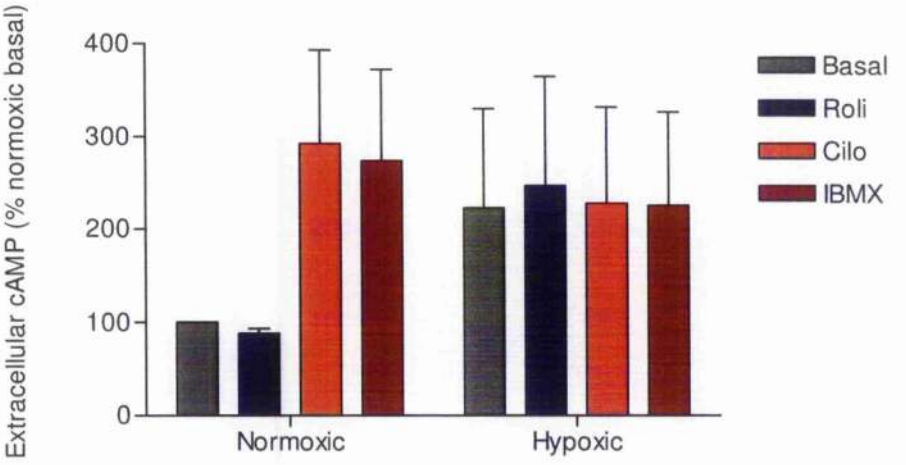
Figure 4.4b Effect of PDE inhibitors in chronic hypoxia compared with normoxic levels on intracellular cAMP levels.

Cells were pre-treated with 10μM rolipram, 10μM cilostamide or 10μM IBMX for 20 min and were immediately assayed for cAMP levels in normoxic and seven day hypoxic hPASMC as described in section 2.3.7.

Panel A, results graphed as mean cAMP ± S.E of fold normoxic basal with normoxic against hypoxic analysis. *Panel B,* approximate pmol cAMP/ng protein

Results shown are mean ± S.E. of three independent experiments and * denotes significance ($p<0.05$).

A



B

	Normoxic	Hypoxic
Basal	1	1
Roli	0.8 +/- 0.1	1.1 +/- 0.1
Cilo	2.9 +/- 1	1 +/- 0.03
IBMX	2.7 +/- 1	1 +/- 0.07

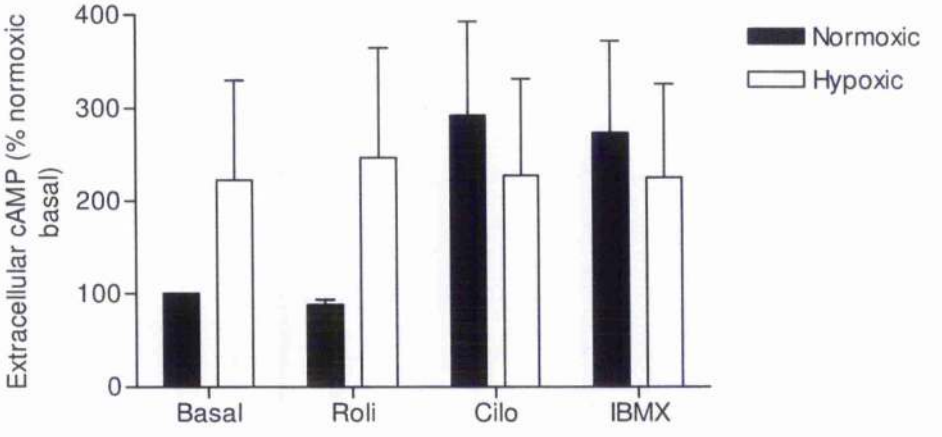
Figure 4.5a Effect of PDE inhibitors in chronic hypoxia compared with normoxic levels on extracellular cAMP levels.

Cells were pre-treated with 10μM rolipram, 10μM cilostamide or 10μM IBMX for 20 minutes and were immediately assayed for cAMP levels in normoxic and seven day hypoxic hPASMC as described in section 2.3.7.

Panel A, results graphed as mean cAMP +/- S.E. of fold normoxic basal. *Panel B,* results expressed as mean ± S.E. of fold basal for both normoxic and hypoxic samples.

Results shown are mean +/- S.E. of three independent experiments.

A



B

	Normoxic	Hypoxic
Basal	12	25.3
Roli	11.4	27
Cilo	18.5	25.9
IBMX	16.9	24.7

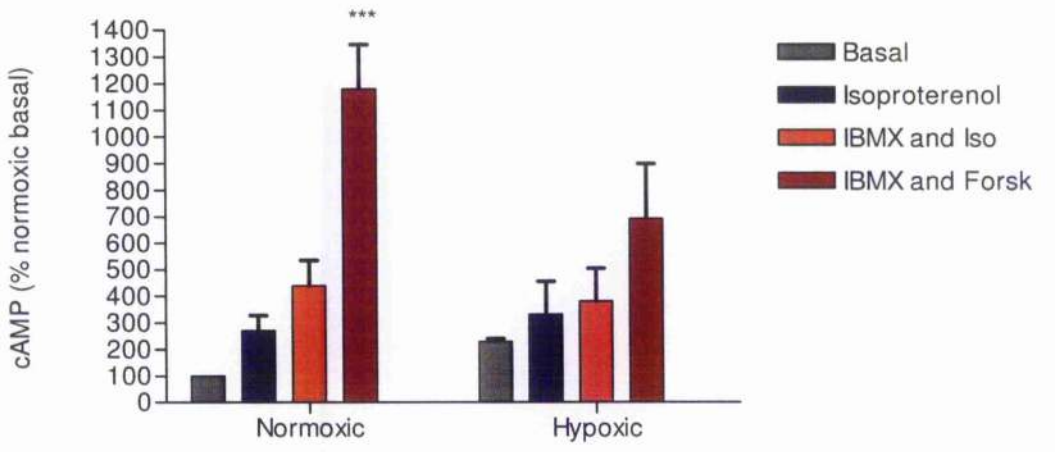
Figure 4.5b Effect of PDE inhibitors in chronic hypoxia compared with normoxic levels on extracellular cAMP levels.

Cells were pre-treated with 10μM rolipram, 10μM cilostamide or 10μM IBMX for 20 min and were immediately assayed for cAMP levels in normoxic and seven day hypoxic hPASMC as described in section 2.3.7.

Panel A, results graphed as mean cAMP ± S.E of fold normoxic basal with normoxic against hypoxic analysis. *Panel B,* approximate pmol cAMP/ng protein

Results shown are mean +/- S.E. of three independent experiments.

A



B

	Normoxic	Hypoxic
Basal	1	1
Isoproterenol	3.6 +/- 1	1.6 +/- 0.6
IBMX + Iso	4.4 +/- 1	1.7 +/- 0.4
IBMX + Forsk	13.3 +/- 1.6	2.9 +/- 0.5

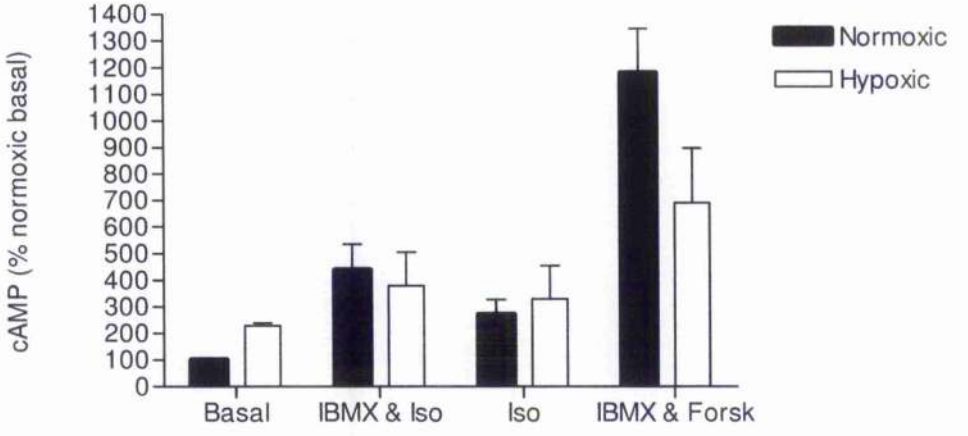
Figure 4.6a Effect of cAMP effectors in chronic hypoxia compared with normoxic levels on intracellular cAMP levels.

Cells were pre-treated with 10μM IBMX for 20 minutes prior to stimulation with 10μM forskolin or 10μM isoproterenol for 5 minutes, or only stimulated for 5 minutes with isoproterenol and were immediately assayed for cAMP levels in normoxic and seven day hypoxic hPASMC as described in section 2.3.7.

Panel A, results graphed as mean cAMP ± S.E. of fold normoxic basal.

Panel B, results expressed as mean ± S.E. of fold basal for both normoxic and hypoxic samples. Results shown are mean ± S.E. of three independent experiments and *** denotes significance ($p<0.001$).

A



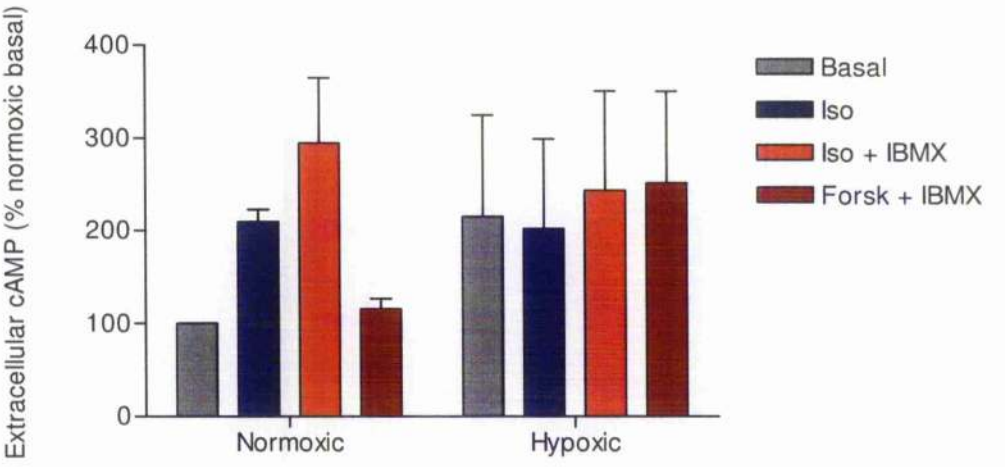
B

	Normoxic	Hypoxic
Basal	5.5	15.8
Isoproterenol	20.2	22.4
IBMX + Iso	25.8	27.6
IBMX + Forsk	81.8	59.3

Figure 4.6b Effect of cAMP effectors in chronic hypoxia compared with normoxic levels on intracellular cAMP levels. Cells were pre-treated with 10μM IBMX for 20 minutes prior to stimulation with 10μM forskolin or 10μM isoproterenol for 5 minutes, or only stimulated for 5 minutes with isoproterenol and were immediately assayed for cAMP levels in normoxic and seven day hypoxic hPASMC as described in section 2.3.7.
Panel A, results graphed as mean cAMP ± S.E of fold normoxic basal with normoxic against hypoxic analysis. *Panel B, approximate* pmol cAMP/ng protein.

Results shown are mean ± S.E. of three independent experiments.

A



B

	Normoxic	Hypoxic
Basal	1	1
Iso	2 +/- 0.1	1 +/- 0.1
Iso + IBMX	2.9 +/- 0.7	1.1 +/- 0.1
Forsk + IBMX	2.1 +/- 0.8	1 +/- 0.1

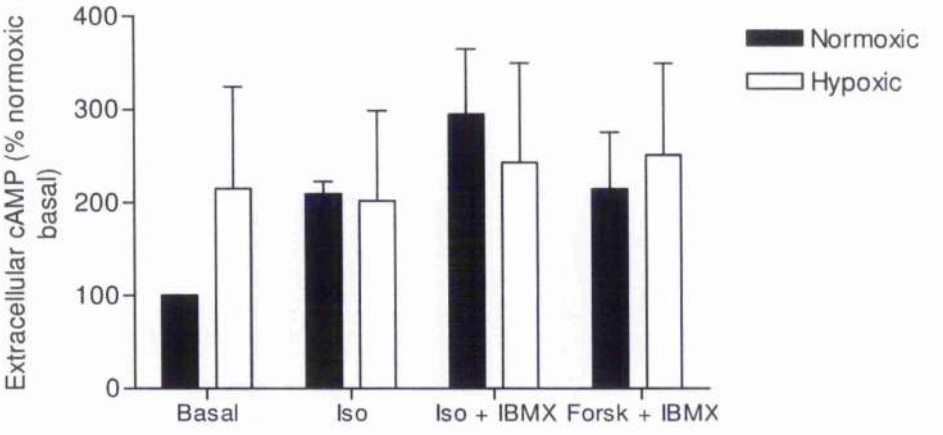
Figure 4.7a Effect of cAMP effectors in chronic hypoxia compared with normoxic levels on extracellular cAMP levels.

Cells were pre-treated with 10μM IBMX for 20 minutes prior to stimulation with 10μM forskolin or 10μM isoproterenol for 5 minutes, or only stimulated for 5 minutes with isoproterenol and were immediately assayed for extracellular cAMP levels in normoxic and seven day hypoxic hPASMC as described in section 2.3.7.

Panel A, results graphed as mean cAMP +/- S.E. of fold normoxic basal. *Panel B,* results expressed as mean ± S.E. of fold basal for both normoxic and hypoxic samples.

Results shown are mean +/- S.E. of three independent experiments.

A



B

	Normoxic	Hypoxic
Basal	12	25.3
Iso	33	21
Iso + IBMX	24	27.6
Forsk + IBMX	30.7	47

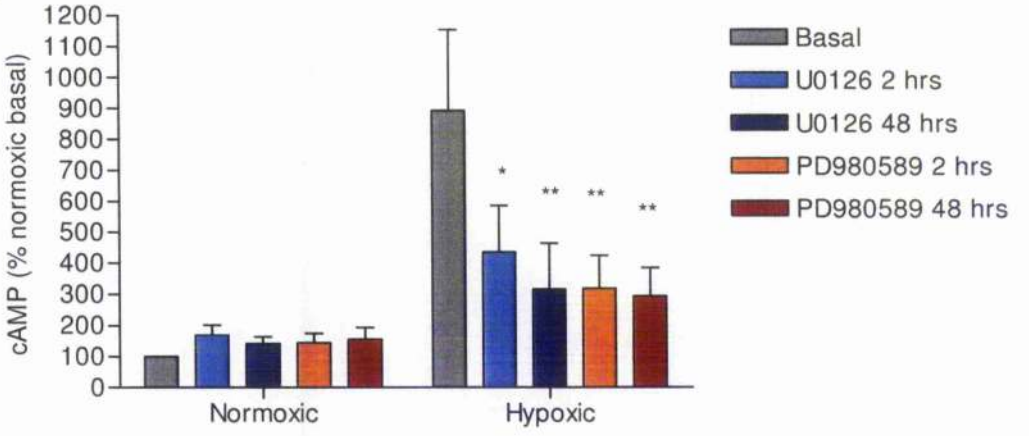
Figure 4.7b Effect of cAMP effectors in chronic hypoxia compared with normoxic levels on extracellular cAMP levels.

Cells were pre-treated with 10μM IBMX for 20 minutes prior to stimulation with 10μM forskolin or 10μM isoproterenol for 5 minutes, or only stimulated for 5 minutes with isoproterenol and were immediately assayed for extracellular cAMP levels in normoxic and seven day hypoxic hPASMC as described in section 2.3.7.

Panel A, results graphed as mean cAMP ± S.E of fold normoxic basal with normoxic against hypoxic analysis. *Panel B,* approximate pmol cAMP/ng protein.

Results shown are mean +/- S.E. of three independent experiments.

A



B

	Normoxic	Hypoxic
Basal	1	1
U0126 2 hrs	1.6 +/- 0.5	0.5 +/- 0.2
UO126 48 hrs	1.4 +/- 0.4	0.4 +/- 0.2
PD980589 2 hrs	1.4 +/- 0.5	0.4 +/- 0.1
PD980589 48 hrs	1.6 +/- 0.6	0.4 +/- 0.1

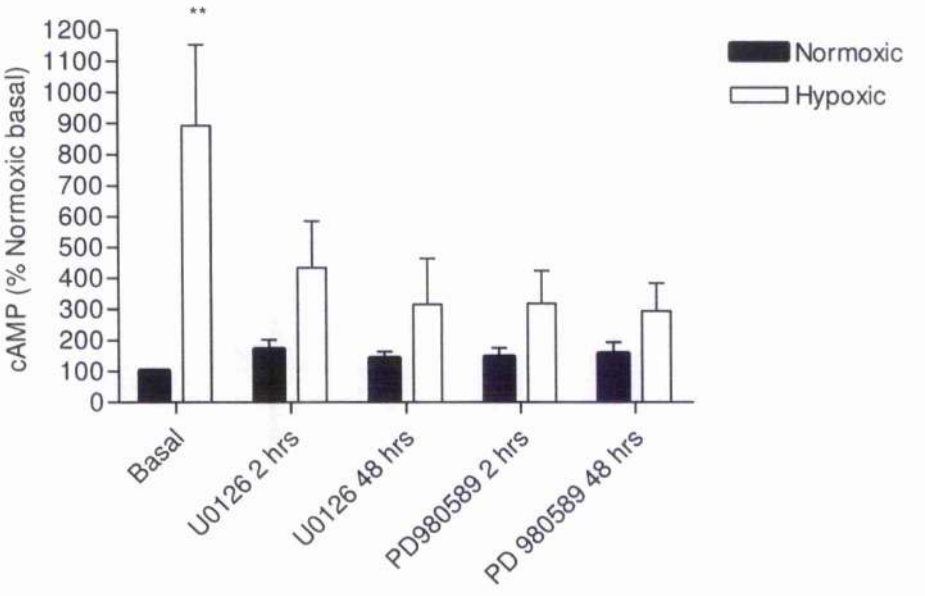
Figure 4.8a Effect of MEK inhibitors on cAMP levels in hypoxic and normoxic hPASMC.

Cells were treated with 10μM U0126 or 20μM PD980589 for 2 and 48 hours and were immediately assayed for cAMP levels in normoxic and seven day hypoxic hPASMC as described in section 2.3.7.

Panel A, results graphed as mean cAMP +/- S.E. of fold normoxic basal. *Panel B,* results expressed as mean ± S.E. of fold basal for both normoxic and hypoxic samples.

Results shown are mean +/- S.E. of four independent experiments and *, ** denotes significance ($p<0.05$, $p<0.01$ respectively).

A



B

	Normoxic	Hypoxic
Basal	192.7	1531.8
U0126 2 hrs	268.5	555.8
UO126 48 hrs	235.6	396.1
PD980589 2 hrs	190.3	470.7
PD980589 48 hrs	188.5	475.6

Figure 4.8b Effect of MEK inhibitors on cAMP levels in hypoxic and normoxic hPASMC.

Cells were treated with 10μM U0126 or 20μM PD980589 for 2 and 48 hours and were immediately assayed for cAMP levels in normoxic and seven day hypoxic hPASMC as described in section 2.3.7.

Panel A, results graphed as mean cAMP ± S.E of fold normoxic basal with normoxic against hypoxic analysis. *Panel B,* approximate pmol cAMP/ng protein.

Results shown are mean +/- S.E. of four independent experiments and ** denotes significance ($p<0.01$).

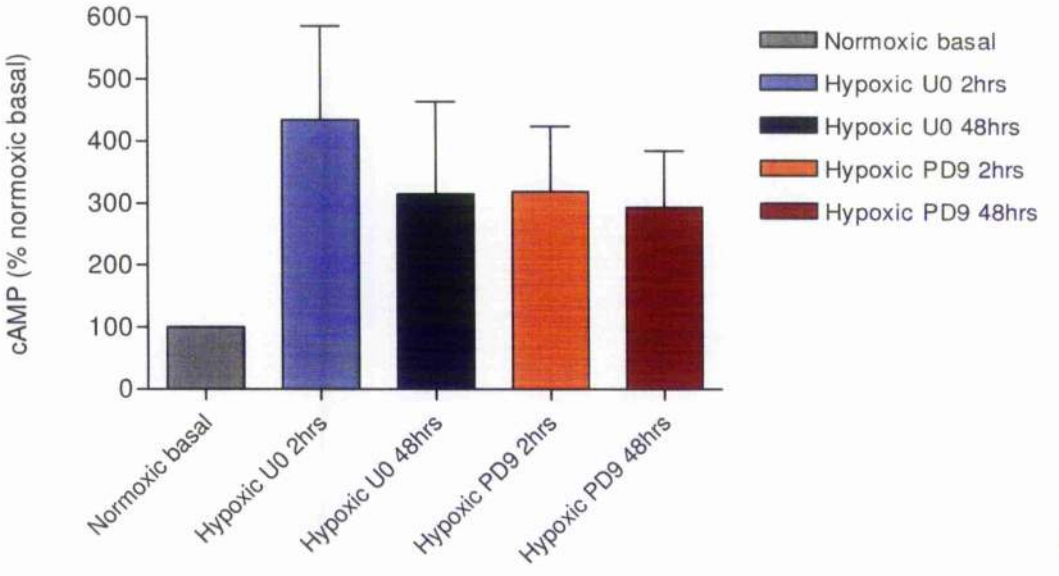


Figure 4.8c Effect of MEK inhibitors on cAMP levels in hypoxic and normoxic hPASMC.

Cells were treated with 10μM U0126 or 20μM PD980589 for 2 and 48 hours and were immediately assayed for cAMP levels in normoxic and seven day hypoxic hPASMC as described in section 2.3.7. This graphs shows hypoxic levels of cAMP in response to ERK inhibition compared to normoxic basal cAMP levels.

Results shown are mean +/- S.E. of four independent experiments.

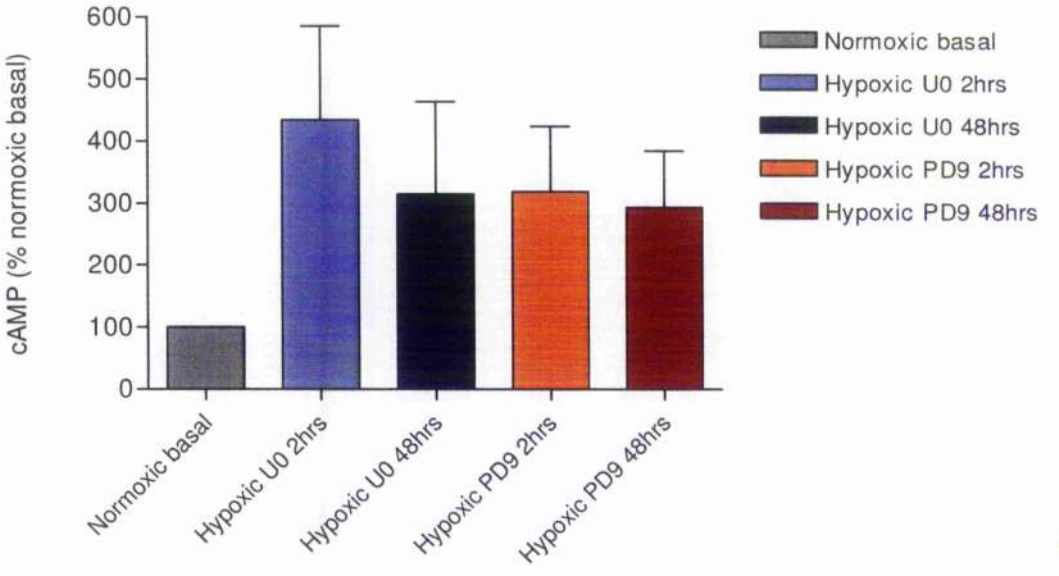
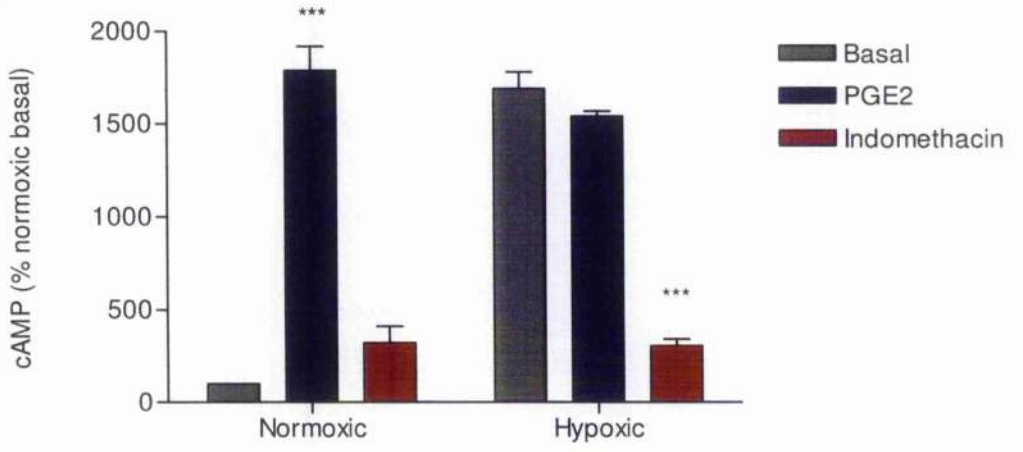


Figure 4.8c Effect of MEK inhibitors on cAMP levels in hypoxic and normoxic hPASMC.

Cells were treated with 10μM U0126 or 20μM PD980589 for 2 and 48 hours and were immediately assayed for cAMP levels in normoxic and seven day hypoxic hPASMC as described in section 2.3.7. This graphs shows hypoxic levels of cAMP in response to ERK inhibition compared to normoxic basal cAMP levels.

Results shown are mean +/- S.E. of four independent experiments.

A



B

	Normoxic	Hypoxic
Basal	1	1
PGE2	17.9 +/- 1.3	0.9 +/- 0.03
Indomethacin	2.1 +/- 0.05	0.2 +/- 0.03

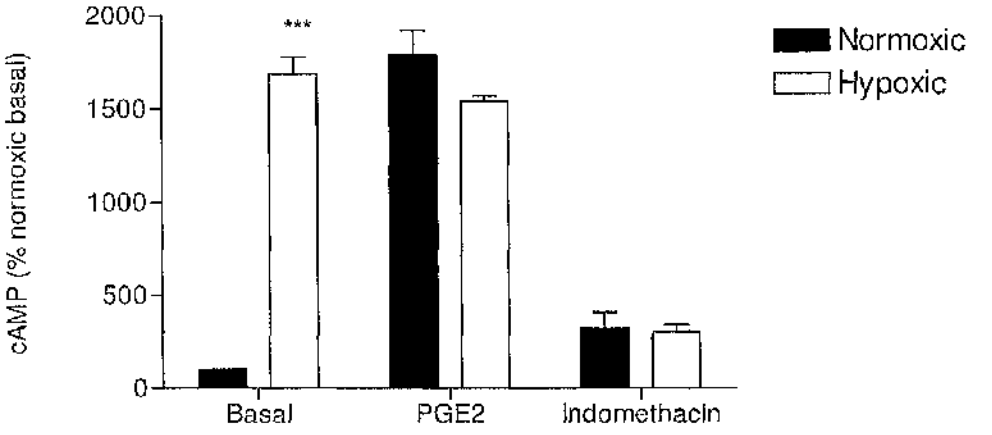
Figure 4.9a Effect of PGE2 and indomethacin on cAMP levels in hypoxic and normoxic hPASMC.

Cells were treated with 1μM PGE_2 for 10 mins and 10μM indomethacin for 2 hours and were immediately assayed for cAMP levels in normoxic and seven day hypoxic hPASMC as described in section 2.3.7.

Panel A, results graphed as mean cAMP +/- S.E. of fold normoxic basal. *Panel B,* results expressed as mean ± S.E. of fold basal for both normoxic and hypoxic samples.

Results shown are mean +/- S.E. of two independent experiments and *** denotes significance ($p<0.001$).

A



B

	Normoxic	Hypoxic
Basal	81.5	1416.9
PGE2	1456.8	1253.2
Indomethacin	261.5	247.8

Figure 4.9b Effect of PGE2 and indomethacin on cAMP levels in hypoxic and normoxic hPASMC.

Cells were treated with 1μM PGE_2 for 10 mins and 10μM indomethacin for 2 hours and were immediately assayed for cAMP levels in normoxic and seven day hypoxic hPASMC as described in section 2.3.7.

Panel A, results graphed as mean cAMP ± S.E of fold normoxic basal with normoxic against hypoxic analysis. *Panel B,* approximate pmol cAMP/ng protein.

Results shown are mean +/- S.E. of two independent experiments.

Chapter 5

ERK in Hypoxic hPASMC

5.1 Introduction

5.1.1 The ERK pathway

The mitogen activated protein kinases (MAPK) are activated by a wide range of stimuli including growth factors, hormones, extracellular matrix components, GPCR agonists, cellular stress and cytokines (reviewed in Gutkind 2000; Tibbles & Woodgett 1999; Wildman et al., 1999; see general introduction for further details). The best characterised MAPK are the extracellular regulated kinases 1 and 2, also known as p44 and p42 respectively. The involvement of ERK1/2 in cell proliferation is well established (reviewed in Stork & Schmitt 2002).

ERK1/2 is activated by the binding of e.g. EGF to its tyrosine kinase receptor (RTK) which results in the activation of the Ras – Raf – MEK1/2 – ERK1/2 pathway (reviewed in Kolch 2000). It was originally thought that ERK1/2 could also be activated by cAMP through Epac activating Rap1 and thus B-Raf, leading to MEK1/2 activation and finally the activation of ERK *(figure 1.5)*. However, recent work has demonstrated that Rap1 activation is PKA-independent and does not act through B-Raf to lead to the activation of ERK, instead Rap1 is now known to activate 'inside-out' signalling to integrins (Bos et al., 2003). This presents a model whereby cAMP activation of ERK via Raf is mediated through PKA and activation of Epac leads to integrin activation which has also been demonstrated to activate ERK *(figure 5.1)*. ERK is activated specifically by dual phosphorylation (Prowse & Lew 2001) and, upon activation, moves to the nucleus to act upon its transcription factors and becomes dephosphorylated (Volmat et al., 2001). ERK remains inactive in the nucleus until it translocates back to the activating MEK in the cytoplasm (Peyssonnaux, et al., 2001).

5.1.1.1 Regulation of ERK activation by cAMP

Regulation of the ERK cascade by the cAMP pathway provides vital crosstalk between hormone and growth factor signalling (reviewed in Houslay & Kolch 2000). ERK can be inhibited or activated by cAMP in a cell specific manner (reviewed in Stork & Schmitt 2002).

Inhibition occurs through the actions of PKA. PKA blocks Raf-1 activation and inhibits Raf-1 activity (Cook & McCormick 1993; Mischak et al., 1996), thus blocking Ras-dependant signals to ERK. PKA has been demonstrated to act indirectly through

phosphorylation of Src upon Rap1 to inhibit ERK (Ribeiro-Neto et al., 2002; Schmitt & Stork 2002b). Another PKA-mediated inhibition of ERK is the activation of Akt (PKB) which inhibits Raf-1 (Zimmerman & Moelling 1999) and thus prevents activation of ERK. cAMP can also inhibit ERK through activation of MAP kinase phosphatases (MKP; Burgun et al., 2000)

cAMP can lead to the activation of ERK. In cell types that express B-Raf, for example endothelial cells (Wojnowski et al., 1997), PKA activates B-Raf, leading to activation of ERK. PKA activation of Rap1 through Src can therefore lead to activation as well as inhibition of ERK. Indeed, ERK activation by PKA has been shown to require members of the Src family of kinases (Lindquist et al., 2000). The recently discovered cAMP substrate, Epac (de Rooij et al., 1998) may also activate Rap1 independently of PKA.

5.1.1.2 Regulation of cAMP levels by ERK

ERK is also capable of regulating cAMP levels through the actions of the cAMP-specific PDE4 family. Although isoforms encoded by the PDE4A, PDE4B, PDE4C and PDE4D genes contain the ERK2 docking sites, KIM and FQF (MacKenzie et al., 2000), PDE4A does not act as a substrate for ERK (Baillie et al., 2000). Phosphorylation of PDE4 long forms by ERK2 has been demonstrated to lead to a 75 % reduction in enzyme activity (Hoffmann et al., 1999). This ERK-mediated inhibition of PDE4 is transient as the result is a rise in cAMP levels, and the consequential activation of PKA. PKA phosphorylation of PDE4 negates the ERK2 phosphorylation (Baillie et al., 2000; Hoffmann et al., 1999), hence the reversal of inhibition of PDE4 activity.

ERK can also lead to activation of the long form, PDE4D5 (Baillie et al., 2001, figure 1.3). This is apparent in human aortic smooth muscle cells whereby activated ERK caused an increase in PGE_2 which was released from the cell and activated adenylyl cyclase. This induced a rise in cAMP levels with a concomitant increase in PKA activity and resultant PDE4D5 phosphorylation. This phosphorylation ablated the effect of ERK phosphorylation and a net activation of PDE4D5 was observed.

ERK2 phosphorylation of PDE4 short forms results in their activation (Baillie et al., 2000). This differential regulation of PDE4 activity by ERK2 is due to the upstream conserved region 1 (UCR1) which is only found in PDE4 long forms and contains the PKA phosphorylation site. Intriguingly, the super short form PDE4D2, which lacks UCR1 and

has a truncated UCR2, is inhibited by ERK2 phosphorylation, indicating the requirement of the N-terminal portion of UCR2 for activation of PDE4 short forms (MacKenzie et al., 2000). As PDE4D2 also lacks the PKA phosphorylation site, it is not subject to feedback regulation by PKA.

5.1.1.3 ERK in hypoxia

The activation of ERK in hypoxia has been characterised in pulmonary artery fibroblasts, pulmonary artery endothelial cells and in pulmonary arteries from the chronic hypoxic rat (Minet et al., 2000b; Scott et al., 1998; Welsh et al., 2001; Jin et al., 2000). ERK1/2 phosphorylation has been shown to promote the transcriptional activity of HIF-1α (Richard et al., 1999) and therefore the transcription of the vascular endothelial growth factor, VEGF.

In this chapter, I set out to investigate ERK expression specifically in hypoxic hPASMC. In chapter 4, I observed ERK to play a key role in the regulation of hypoxic cAMP levels in hPASMC. As ERK is known to achieve this regulation through the PDE4 family, I thus also set out to investigate the effect of ERK on PDE4 activity and expression in an attempt to uncover the hypoxia mediated rise in cAMP. Also, I investigated the effect of PDE4 on active ERK expression by employing siRNA technology.

Results

5.2 The autocrine loop and PDE activity in hypoxic hPASMC

Results from the previous chapter indicated an autocrine loop whereby active ERK can lead to an increase in cAMP levels through the production of PGE_2 and stimulation of adenylyl cyclase. In a previous study, this mechanism has been shown to override inhibition of PDE4D5 by ERK and lead to net activation of PDE4D5 in human aortic smooth muscle cells due to the rapidity of the response and the magnitude of the resultant PKA phosphorylation of PDE4D5 (Baillie et al., 2001).

5.2.1 Effect of ERK inhibitors on PDE4 activity

In order to determine the effect of ERK on overall PDE4 activity in these cells, cells were treated with the MEK inhibitors, 20μM PD980589 and 10μM U0126 for either 30 min or 48 h and then harvested for use in a cAMP-PDE activity assay.

5.2.1.1 Effect of PD980589 on PDE4 activity

Treating cells with 20μM PD980589 for 30 mins or 48 hours had no significant effect on total cAMP-PDE activity or PDE4 activity in normoxic or hypoxic hPASMC *(Figure 5.2)*.Normoxic basal PDE4 activity was 9.0 ± 1.3 pmol/min/mg protein, 30 minutes treatment reduced this to 8.0 ± 0.5 pmol/min/mg protein and 48 hours treatment reduced this to 6.4 ± 0.8 pmol/min/mg protein (SEM; n=2). Hypoxic cells displayed a similar trend with basal PDE4 activity being 9.0 ± 0.2 pmol/min/mg protein, 30 mins treatment reduced this to 7.5 ± 1.8 pmol/min/mg protein and 48 hours reduced PDE4 activity to 6.7 ± 0.1 pmol/min/mg protein (SEM; n=2).

5.2.1.2 Effect of U0126 on PDE4 activity

Treating cells with 10μM U0126 for 30 min or 48 hours again had no significant effect on either total cAMP-PDE activity or PDE4 activity in either normoxic or hypoxic hPASMC *(Figure 5.3)*. Normoxic basal PDE4 activity was 9.6 ± 0.5 pmol/min/mg protein , 30 minutes treatment with 10μM U0126 had no significant effect on PDE4 activity, whereas 48 hours treatment with 10μM U0126 significantly reduced PDE4 activity to 6 ± 0.5 pmol/min/mg protein (SEM; n=4; $p<0.05$). Hypoxic basal PDE4 activity was 7.7 ± 1.1 pmol/min/mg protein and 30 mins treatment with 10μM U0126 had no significant effect on

this. 48 hours of treatment with 10μM U0126 induced a small, but insignificant increase in PDE4 activity to 9 ± 0.3 pmol/min/mg protein (SEM; n=4).

These results suggest little effect of ERK on total PDE4 activity. However, ERK differentially regulates long, short and super short forms which are all present in hPASMC. PDE4B2 and PDE4D1, whose expression increases in hypoxia, can potentially be activated by ERK phosphorylation, so inhibition of ERK might be expected to reduce their activity if they were so ERK phosphorylated. PDE4D2 and PDE4D5, whose expression is also increased in hypoxia, can be inhibited upon phosphorylation by ERK. Thus inhibiting ERK might be expected to alleviate this and thereby activate these isoforms if they were so ERK phosphorylated. Hence, ERK inhibition might be expected to result in contrasting effects on the activity of various PDE4 isoforms in hPASMC. Indeed, this may be complicated further if the actions of ERK are compartmentalised and only certain PDE4 isoforms are available for phosphorylation by ERK.

5.2.1.3 Effect of U0126 on ERK phosphorylation

To ensure that ERK was fully inhibited for the full 48 h, cells were treated with U0126 for 30 min and 48 h and prepared for gel electrophoresis. The proteins were transferred onto nitrocellulose and probed with anti-pERK to detect levels of active ERK. Figure 5.3 shows that U0126 does inhibit ERK activity after 30 minutes and 48 hours treatment as the densitometry results compared with basal levels show. *(Figure 5.4)*

5.2.2 PDE Activity in response to PGE_2 and indomethacin in hypoxic hPASMC

As the cAMP assay results in the previous chapter indicate that active ERK feeds into an autocrine pathway involving generation of PGE_2, I set out to investigate the effect of PGE_2 and the COX inhibitor, indomethacin on cAMP-PDE activity in normoxic and hypoxic hPASMC. PGE_2 directly stimulates adenylyl cyclase to increase cAMP while indomethacin inhibits the actions of COX and thus reduces production of endogenous PGE_2 in response to elevated ERK. In order to determine the effect of PGE_2 and indomethacin on overall PDE4 activity in these cells, cells were treated with 1μM PGE_2 for 10 mins and 10μM indomethacin for 2 hours and then harvested for use in a cAMP-PDE activity assay.

5.2.2.1 Total cAMP-PDE activity

As can be seen in panel A of fig 5.5, both PGE_2 and indomethacin had no effect on hypoxic or normoxic total cAMP-PDE activity.

5.2.2.2 PDE4 activity

Seven day hypoxic and normoxic cells were treated with 1μM PGE_2 for 10 min or 10μM indomethacin for 2 hours. In figure 5.5, panel B, the results are shown. Basal PDE4 activity was 5.7 ± 0.2 pmol/min/mg protein and 6 ± 0.2 pmol/min/mg protein in normoxic and hypoxic cells respectively. Addition of PGE_2 significantly reduced PDE4 activity in normoxic cells to 4.6 ± pmol/min/mg protein ($p<0.05$). In hypoxic cells however, an insignificant increase in PDE4 activity to 7.2 ± pmol/min/mg protein was observed (SEM; n=2). An increase in PDE4 activity was expected as PGE_2 activates adenylyl cyclase. The COX-2 inhibitor indomethacin caused a decrease in both normoxic and hypoxic PDE4 activity. This reduction was only significant in hypoxic cells, with PDE4 activity being reduced to 2.2 ± 1.5 pmol/min/mg protein (SEM; n=2). These results are indicative of an autocrine effect in hPASMC, and suggest that this loop is enhanced in hypoxia through increased PGE_2 and ERK. However, it was observed that PGE_2 fails to increase PDE4 activity in normoxic cells, suggesting further analyses are required.

5.2.3 Effect of U0126 and Indomethacin on PDE4 expression levels

As U0126 and indomethacin were both observed to normalise hypoxic intracellular cAMP levels, their effect on the expression levels of the PDE4 isoforms previously observed to increase in hypoxia was investigated. Hypoxic and normoxic cells were treated with 10μM U0126 or 10μM indomethacin for 24 hours and harvested as described in section 2.3.1.1 for use in western blotting.

5.2.3.1 PDE4A10/4A11

Lysates from hypoxic and normoxic cells treated with 10μM U0126 and 10μM indomethacin for 24 hours and were run out on gels alongside basal controls and a PDE4A10 protein standard, then transferred to nitrocellulose and probed with an anti-PDE4A antibody to investigate PDE4A10/4A11 expression levels. The resulting blots were scanned for densitometry analysis. As can be seen in figure 5.6, panel A and B, U0126 and indomethacin had no significant effect on normoxic or hypoxic protein levels of PDE4A10/4A11, although U0126 did increase normoxic PDE4A levels slightly.

5.2.3.2 PDE4B2

Normoxic and hypoxic lysates from cells treated with 10μM U0126 or 10μM indomethacin, as in section 5.2.3.1, were used for gel electrophoresis alongside a 4B2 protein standard and basal controls. The resulting membranes after transfer were probed with an anti-4B antibody. The blots were used for a densitometry analysis. U0126 and indomethacin were found to have no significant effect on normoxic or hypoxic PDE4B2 protein levels after 24 hours *(Figure 5.7).*

5.2.3.3 PDE4D5

Cells maintained in hypoxia for seven days and their corresponding normoxic controls were treated with 10μM U0126 or 10μM indomethacin for 24 hr sand used for gel electrophoresis as in section 5.2.3.1. After transfer, the membranes were probed with an anti-PDE4D specific antibody and it was revealed that U0126 and indomethacin had no significant effect on PDE4D5 protein levels. There was a slight decrease in PDE4D5 levels, but this was apparent in both normoxic and hypoxic cells *(Figure 5.8).*

5.3 ERK in normoxic and hypoxic hPASMC

ERK is known to inhibit PDE4B, PDE4C and PDE4D long form activities within cells by causing the phosphorylation of their catalytic unit (Baillie et al., 2000). In contrast to this, ERK is known to activate the PDE4B2 short form by such phosphorylation (Baillie et al., 2000). Numerous studies have revealed a hypoxia-dependant induction of the ERK1/2 pathway in many cell types (Minet et al 2000a). In the chronic hypoxic rat, ERK1 and 2 are shown to be increased in pulmonary arteries (Jin et al., 2000). Due to this and the results from the previous chapter which indicate the involvement of ERK in the regulation of cAMP levels, the effect of activating ERK directly through the use of the epidermal growth factor (EGF) was investigated to determine the effect of ERK activation in hPASMC on PDE4 activity and also the effect of raising cAMP on ERK.

5.3.1 ERK in normoxic and hypoxic hPASMC

5.3.1.1 Effect of EGF on pERK levels

Seven day hypoxic and normoxic cells were treated with 50pg/μl EGF for up to 20 min and then harvested for analysis by gel electrophoresis. After transferring, the membranes were probed with anti-pERK. This revealed that basal levels of pERK in hypoxic cells were not significantly increased compared to normoxic levels although levels of pERK2 were slightly elevated in hypoxia. EGF stimulation increased normoxic levels of pERK1

and 2 to approximately 6 times that of basal and continued to increase until they reached approximately 8 times that of basal levels after 10 minutes of EGF stimulation *(Figure 5.9).* In hypoxic cells, phosphorylation of ERK1 and 2 peaked at approximately 17 times that of hypoxic basal levels at 5 mins stimulation. After 10 mins, ERK phosphorylation was reduced, although at approximately 10 times that of hypoxic basal levels *(Figure 5.9).* This demonstrates that a more rapid and potentiated phosphorylation of ERK occurs in hypoxia than in normoxia.

5.3.1.2 Effect of EGF on PDE4 activity levels

Cell lysates of normoxic cells treated with 50pg/μl EGF for both 4 min and 10 min were used in a cAMP-PDE activity assay in the presence of 10μM rolipram to determine the effect of raising active ERK within normoxic hPASMC on PDE4 activity levels. Assay results showed that PDE4 activity reached 2.5 times that of basal levels after 4min of EGF stimulation and this increased level was maintained up to 10min *(Figure 5.10).* This indicates ERK acts to increase PDE4 activity in hPASMC, presumably through the autocrine loop whereby active ERK leads to active PKA which can phosphorylate and negate any inhibition of ERK on PDE4D5. Also, ERK would directly activate 4B2 and 4D1 present in hPASMC.

5.4 Knockdown of PDE4D5 in normoxic and hypoxic hPASMC

To investigate the relationship determined previously between 4D5 and pERK (Baillie et al., 2001), siRNA technology was employed (Schutze et al., 2004). The discovery of a natural method of gene silencing has provided an invaluable tool in gene functioning studies (Dykxhoorn et al., 2003), although the mechanism by which siRNA works is still unclear.

5.4.1 Effect of PDE4D5 siRNA on PDE4D5 expression

Normoxic hPASMC were transfected with 60nM of the PDE4D5 siRNA construct and harvested after 24 hours incubation as in section 2.3.1.1 for western blotting to examine levels of PDE4D5 expression. This construct worked successfully to knock down levels of endogenous PDE4D5 as can be seen in figure 5.11 with over 80% reduction in PDE4D5 protein levels.

5.4.2 Effect of PDE4D5 siRNA on pERK expression

Seven day hypoxic and normoxic cells transfected with the PDE4D5 siRNA construct were also harvested for analysis of basal pERK expression. It was observed that knocking out PDE4D5 resulted in an increase of basal pERK expression within normoxic cells to approximately double and a decrease in hypoxic cells to 20% of basal hypoxic levels *(Figure 5.12)*. This demonstrates that the cross-talk between cAMP and ERK is modified in hypoxia with a reversal of effect.

Many thanks to Dr. M. Lynch, Gardiner Lab who created siRNA constructs.

5.5 Discussion and Conclusions

The results in chapter 4 demonstrate the regulation of cAMP levels through the MAPK, ERK. This has previously been hypothesised to occur through the production of PGE_2 and the consequential AC stimulation (Baillie et al., 2001). Indeed, it is known that ERK, COX-2 and PGE_2 display increased levels in hypoxia (Jin et al., 2000; Scott et al., 1998; Bradbury et al., 2002), contributing to the increased cAMP observed in hPASMC in chapter 4. PDE4 is also involved in this loop as ERK and the cAMP-dependant kinase, PKA, both act upon PDE4 with opposing effects and thus impact upon the regulation of cAMP levels. The increased PDE4D5 discussed in chapter 3 is subject to inhibition by the increased ERK levels in hypoxia. The increased levels of ERK are transient however (Jin et al., 2000; Scott et al., 1998), as hypoxia has also been observed to stimulate MAPK phosphatase 1 (MKP-1; Laderoute et al., 1999). ERK phosphorylation peaks at day 7 (Jin et al., 2000; Scott et al., 1998), the time point studied throughout this investigation. Maximal levels of active ERK would therefore inhibit PDE4D5 activity (Baillie et al., 2000; Hoffmann et al., 1999) and result in a maintained increase of cAMP levels generated by PGE_2. This increase would lead to increased levels of active PKA and activation of PDE4D5 as PKA phosphorylation negates the inhibitory effect of ERK (Baillie et al., 2000). Thus cAMP levels would be reduced and, along with the hypoxia-induced activation of MKP-1 and resultant decrease in ERK activity, PDE4D5 would continue to reduce cAMP levels below that of normal cells.

I thus initially set out to investigate the effect ERK has on PDE4 activity in hPASMC by employing two different ERK inhibitors. Both U0126 and PD980589 inhibit ERK activation by inhibiting its upstream kinases, MEK. U0126 was used at a concentration that inhibited both MEK1 and MEK2, whereas PD980589 was used at a concentration that inhibited MEK1 only. Examining the effects of ERK inhibition on PDE4 activity, inhibiting MEK1 only had no significant effect in either normoxic or hypoxic cells. However, inhibition of both MEK1 and MEK2 induced a significant reduction in PDE4 activity after 48 hours in normoxic cells. This could possibly be due to a reduced generation of PGE_2, and thus reduced cAMP levels, decreased PKA activity and therefore decreased PKA activation of PDE4. This indicates ERK is required to maintain a balanced effect with PKA on PDE4 activity. In hypoxic cells, this effect was not observed. Instead, there was no significant effect on PDE4 activity after 48 hours of U0126 treatment. This could occur due to the desensitised response to PGE_2 on cAMP levels in hypoxic hPASMC observed in chapter 4. This would result in reduced activation of PKA compared to

normoxic cells and thus ERK inhibition of PDE4 activity could balance the reduced level of PKA activation of PDE4. This would mean that in normoxic cells, ERK would lead to the activation of PDE4 by PKA and reduce cAMP levels. Indeed activation of ERK by EGF was observed to elevate PDE4 activity to three times that of basal in hPASMC. Raised cAMP levels would remain increased in hypoxic cells due to the inhibition of PDE4 by ERK. This could also be responsible for the lack of increased PDE4 activity in hypoxia demonstrated in chapter 3.

To further examine this effect on PDE4 activity, the effect of either PGE_2 or indomethacin treatment on PDE4 activity was measured. Exogenous PGE_2 had no significant effect on PDE4 activity in hypoxic cells, but induced a small reduction in PDE4 activity in normoxic cells ($p<0.05$). This contrasts with the earlier results indicating ERK leads to activation of PDE4 through PGE_2 in normoxic cells. However, the amount of PGE_2 added to the cells is far greater than the amount of endogenous PGE_2 produced though ERK activity. As a result, the effects of exogenous PGE_2 will be greatly enhanced compared to that witnessed normally in the cell through ERK. Therefore it is possible that the increased PKA activity caused by this amount of PGE_2 has a greater effect on its substrates. As well as PDE4, the vast range of PKA substrates includes GPCRs. Upon PKA phosphorylation, the "switching" of GPCR from the G_s subunit to the G_i subunit of the G-protein occurs and this leads to increased levels of receptor activated ERK and reduced cAMP. This increased ERK could then override PKA phosphorylation of PDE4 and result in net inhibition. In hypoxic cells, the lack of effect of PGE_2 on PDE4 activity could be explained by the desensitisation of AC mentioned above. Indeed, it was observed in chapter 4 that PGE_2 failed to increase cAMP levels over hypoxic basal levels.

Treating hPASMC with the COX-2 inhibitor indomethacin induced a significant decrease in PDE4 activity in both normoxic and hypoxic cells. This is due to the lack of PGE_2 generated by ERK because of COX-2 inhibition and therefore a decrease in cAMP (as observed in chapter 4) and PKA activity. This would mean PDE4 would be regulated solely by basal levels of ERK and thus an inhibition of PDE4 activity would be apparent. With the increased levels of active ERK in hypoxia, indomethacin would then be expected to have a greater inhibitory effect on PDE4 activity than in normoxic cells. However, the increased basal levels of PKA activity could affect this, and reduce the effect of ERK on PDE4.

As ERK and COX-2 inhibition by U0126 and indomethacin reduce cAMP levels in hPASMC, it was hypothesised that this could affect the increase in PDE4 expression in hypoxia. As such, normoxic and hypoxic hPASMC were treated with U0126 or indomethacin and examined for levels of PDE4A, PDE4B and PDE4D expression. U0126 and indomethacin had no effect on PDE4A, PDE4B or PDE4D expression, however, treatment with these compounds was only for 24 hours and a more chronic treatment could display an alteration in PDE4 expression.

ERK activation can occur through a number of direct and indirect pathways (see general introduction for more details; reviewed in Peyssonnaux & Eychene 2001). As such, the activation of ERK by EGF in normoxic and hypoxic hPASMC was investigated. In normoxic cells, EGF induced a gradual activation of ERK, reaching an increase of eight times that of basal levels after ten minutes. In hypoxic cells, the activation of ERK is potentiated and more rapid, peaking after 5 minutes at 17 times that of hypoxic basal levels, and falls again to 10 times that of hypoxic basal levels after 10 minutes.

Following on from the effect of ERK on cAMP and PDE4 activity, siRNA constructs to effectively 'knock-out' PDE4D5 in normoxic and hypoxic hPASMC were used to investigate any effect of PDE4D5 on active ERK. It was observed that a successful reduction of PDE4D5 expression induced increased levels of activated ERK in normoxic hPASMC. This could be due to the increased PKA mediated phosphorylation of the β-adrenoceptor and the resulting increase in receptor activated ERK by G_s to G_i switching. Indeed, it has been observed in cardiomyocytes that rolipram increases levels of ERK through this mechanism (Baillie et al., 2002), although this effect was witnessed with β-adrenoceptor stimulation.

In hypoxic cells, silencing PDE4D5 expression resulted in a significant reduction of pERK levels ($p<0.01$). This indicates PDE4D5 is essential for phosphorylation of ERK in hypoxic hPASMC and suggests the mode of activation of ERK is altered in hypoxia. ERK can be either inhibited or activated by elevated cAMP levels, usually in a cell-specific manner (reviewed in Houslay & Kolch 2000; Stork & Schmitt 2002). PKA is capable of acting upon Raf, which results in an inhibition of ERK activation in some cell types (Mischak et al., 1996; Cook et al., 1993) and an activation in others. Therefore the effect of cAMP on the ERK pathway is dependant upon the predominant Raf isoform within a cell. The results presented here suggest cAMP activates the ERK pathway in normoxic hPASMC and inhibits ERK activation in hypoxic hPASMC, possibly by switching from

signalling through B-Raf signalling in normoxia to Raf-1 signalling in hypoxia. However, this would contrast with the increase of ERK witnessed in hypoxia (Jin et al., 2000; Scott et al., 1998).

Other studies have demonstrated a switch from cAMP inhibition of proliferation to cAMP stimulated proliferation. In normal human kidney cells, cAMP acts in an anti-proliferative manner. However, in cells derived from polycystic kidney patients, cAMP stimulates proliferation (Yamaguchi et al., 2004). This was due to a disruption in calcium mobilisation that alleviated B-Raf/ERK inhibition through the reduction of Akt.

It has been demonstrated that Rap1 does not always activate B-Raf, (Zwartkruis 1998) and this is dependant on the B-Raf isoform expressed. Thus, if B-Raf activity is altered in hypoxia, this could explain the activation of ERK observed. It could be that PDE4D5 aids the activation of B-Raf by Rap1 and knocking out PDE4D5 prevents this, by increasing cAMP and resulting in an inhibition of ERK, possibly through PKA mediated activation of Src.

The results presented in this chapter show a delicate balance of ERK and PKA phosphorylation exists to regulate PDE4 activity within the autocrine loop demonstrated in smooth muscle cells (Baillie et al., 2001) and indicates a complex method of cAMP regulation, dependant on various factors and tailored by the cell in a concentration dependant manner. In hypoxia, modifications to this pathway appear to result in a confusion of signals attempting perhaps to return to normal signalling.

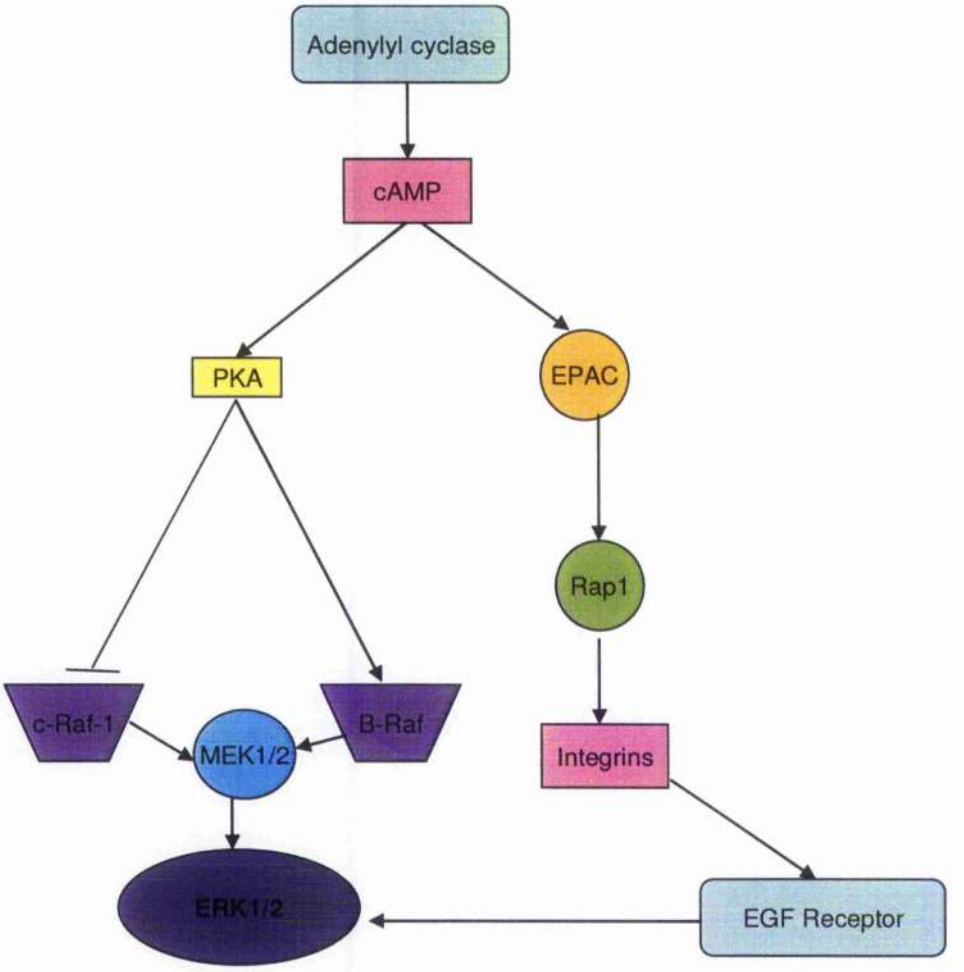
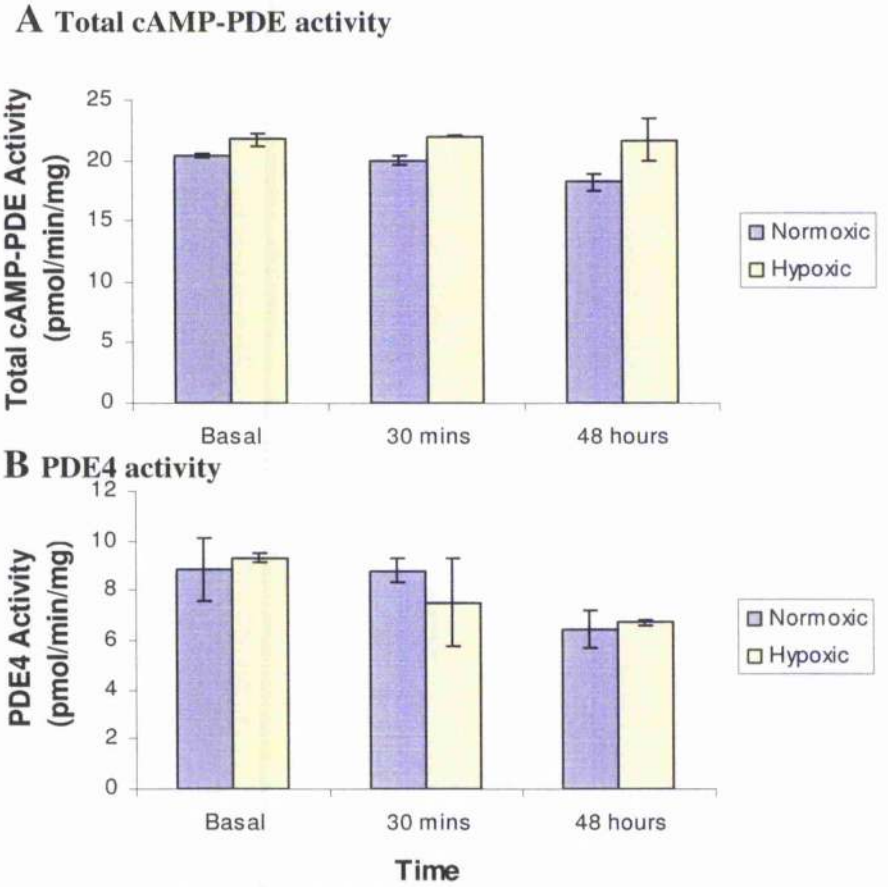


Figure 5.1 cAMP activation of ERK

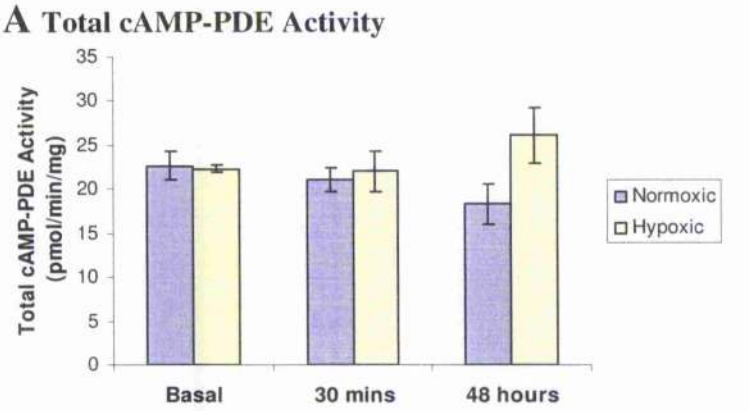
Following generation of cAMP, PKA and Epac are activated. PKA can lead to the activation of ERK through B-Raf, or inhibition of ERK through c-Raf-1. Thus, ERK responses to cAMP are dependant on Raf isoforms present within the cell. Activation of Epac has no impact upon PKA signalling to ERK, instead it leads to the activation of integrin signalling (Bos et al., 2003). Integrin-mediated adhesion has been demonstrated to lead to the partial activation of the epidermal growth factor receptor, resulting in the activation of ERK (Bill et al., 2004).

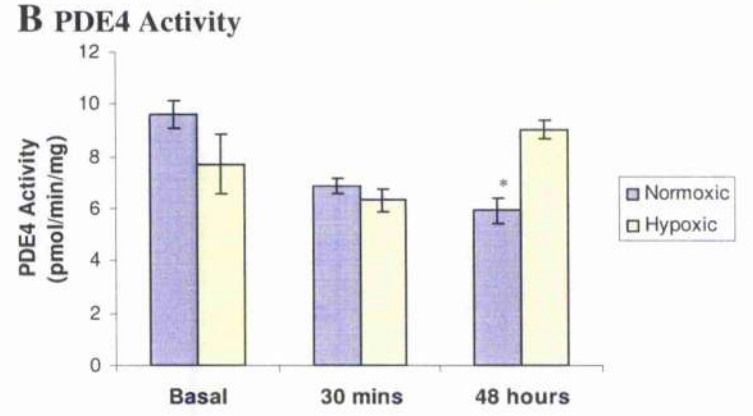


C

Total cAMP-PDE Activity	Normoxic	Hypoxic
Basal	20 +/- 0.1	22 +/- 0.5
30 mins	20 +/- 0.4	22 +/- 0.06
48 hours	18 +/- 0.7	22 +/- 0.2
PDE4 Activity	Normoxic	Hypoxic
Basal	9 +/- 1.3	9 +/- 0.2
30 mins	8 +/- 0.5	7.5 +/- 1.8
48 hours	6.4 +/- 0.8	6.7 +/- 0.1

Figure 5.2 Effect of PD980589 on PDE Activity in normoxic and hypoxic hPASMC. Cells were treated with 20μM PD980589 for 30 mins or 48 hours and were immediately harvested to be used in a cAMP-PDE activity assay for cAMP levels in normoxic and seven day hypoxic hPASMC as described in section 2.3.5. *Panel* A, total cAMP-PDE activity results graphed as mean cAMP ± S.E. *Panel B,* PDE4 activity results graphed as mean ± S.E. *Panel C*, results expressed as mean ± S.E. in pmol/min/mg in tabular form. Means ± S.E. of 2 independent experiments





C

Total cAMP-PDE Activity	Normoxic	Hypoxic
Basal	22.6 +/- 1.6	22.3 +/- 0.4
30 mins	21.1 +/- 1.4	22 +/- 2.3
48 hours	18.3 +/- 2.3	26 +/- 3.2
PDE4 Activity	Normoxic	Hypoxic
Basal	9.6 +/- 0.5	7.7 +/- 1.1
30 mins	6.9 +/- 0.3	6.3 +/- 0.5
48 hours	6 +/- 0.5	9 +/- 0.3

Figure 5.3 Effect of U0126 on PDE Activity in hypoxic and normoxic hPASMC.

Cells were treated with 10μM U0126 for 30 mins or 48 hours and were immediately harvested to be used in a cAMP-PDE activity assay for PDE activity in normoxic and seven day hypoxic hPASMC as described in section 2.3.5. *Panel* A, total cAMP-PDE activity results graphed as mean cAMP ± S.E. *Panel B,* PDE4 activity results graphed as mean ± S.E. *Panel C*, results expressed as mean ± S.E. in pmol/min/mg in tabular form. Means ±S.E. of 4 independent experiments.

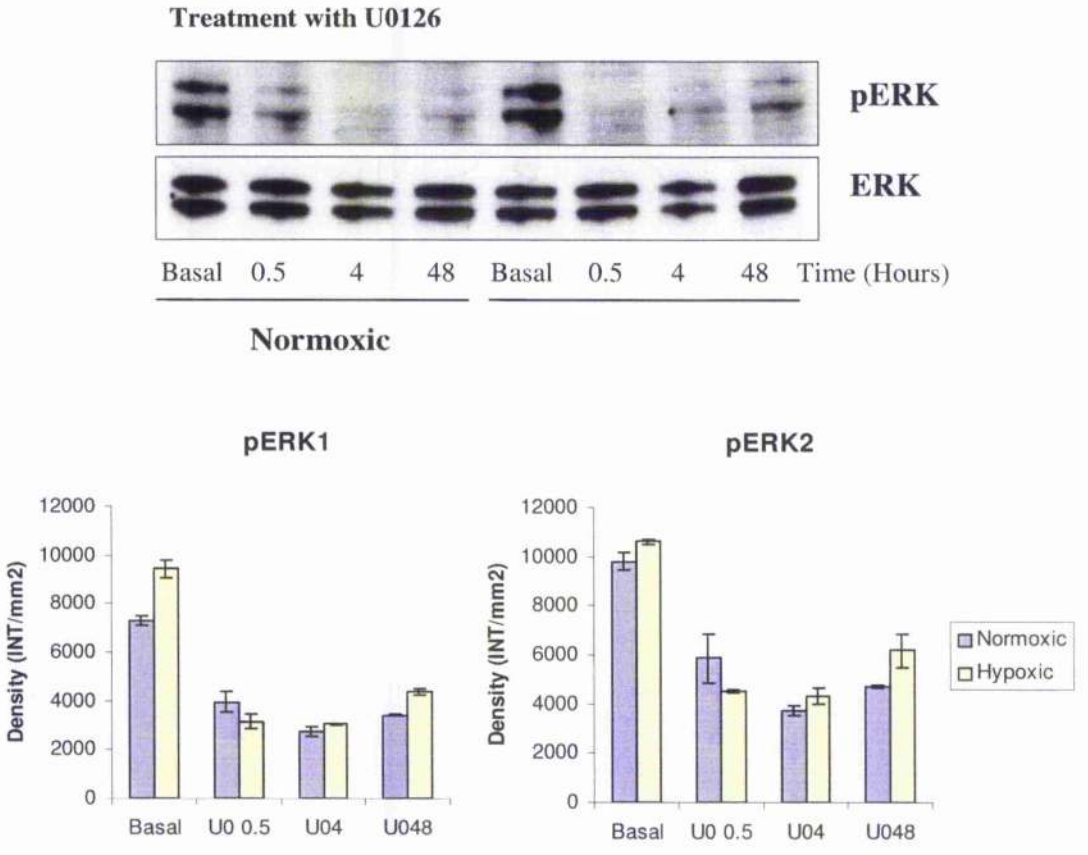
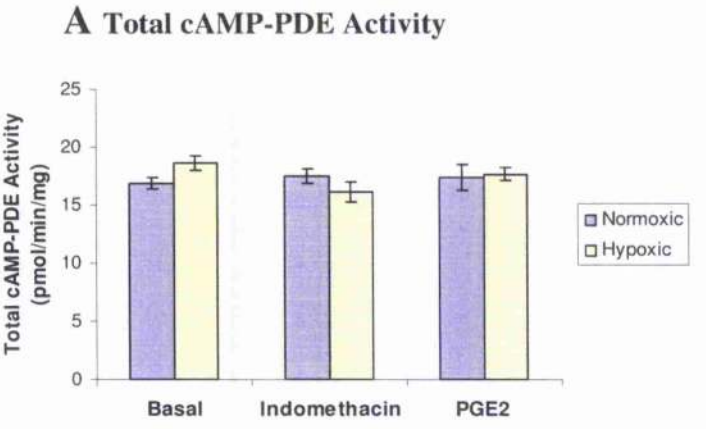
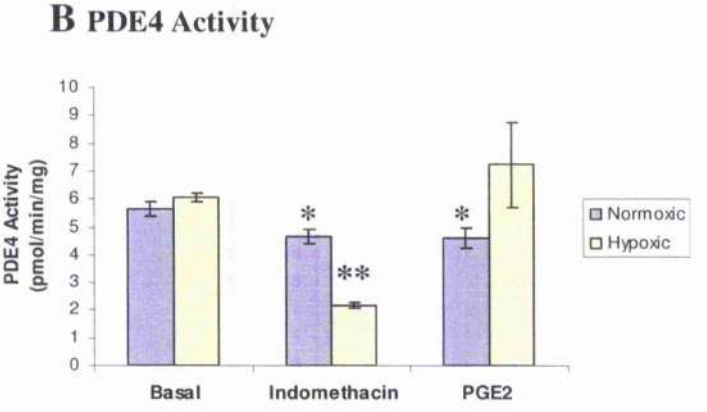


Figure 5.4 Effect of U0126 treatment on ERK1/2 phosphorylation.

Seven day normoxic and hypoxic hPASMC were treated with 10μM of the MEK inhibitor, U0126, for differing lengths of time to ensure ERK1/2 phosphorylation was inhibited. Cells were harvested as in section 2.3.1.1 and subjected to gel electrophoresis before being transferred onto a nitrocellulose membrane and probed with and anti-phospho-ERK1/2 antibody. The membrane was then stripped prior to probing with total ERK2 antibody. The blot shown is indicative of three separate experiments. Densitometry analysis of the three experiments is shown below the blot.





C

PDE4 Activity	Normoxic	Hypoxic
Basal	5.7 +/- 0.2	6 +/- 0.2
Indomethacin	4.7 +/- 0.3	2.2 +/- 0.1
PGE_2	4.6 +/- 0.4	7.2 +/- 1.5

Figure 5.5 Effect of PGE_2 and indomethacin on PDE Activity in hypoxic and normoxic hPASMC

Cells were treated with 1μM PGE_2 for 10 mins and 10μM indomethacin for 2 hours and were harvested to assay for cAMP-PDE activity levels in normoxic and seven day hypoxic hPASMC as described in section 2.3.5. *Panel* A, total cAMP-PDE activity results graphed as mean cAMP ± S.E. *Panel B,* PDE4 activity results graphed as mean ± S.E. *Panel C,* results expressed as mean ± S.E. in pmol/min/mg in tabular form. Means ± S.E. of 2 independent experiments. Significance is as compared with basal and is denoted by *, ** ($p<0.05$, $p<0.01$ respectively.)

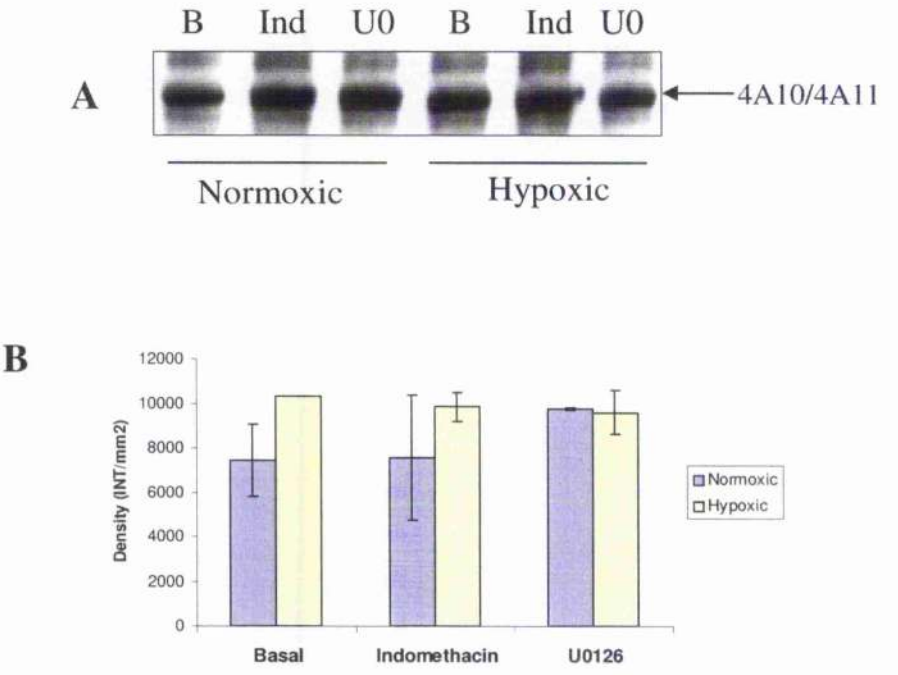


Figure 5.6 Effect of U0126 and indomethacin on PDE4A10/11 expression levels in hypoxia and normoxia.

Cells were treated with 10μM U0126 and 10μM indomethacin for 24 hours and were harvested as described in section 2.3.1.1 20μg protein of each sample was used for SDS-PAGE alongside a basal control sample on a 4-12% gel. *Panel A,* The proteins were transferred to nitrocellulose and probed with a PDE4A antibody. *Panel B,* the densitometry results as compared to normoxic samples expressed in INT/mm^2. Blots shown are representative of two independent experiments.

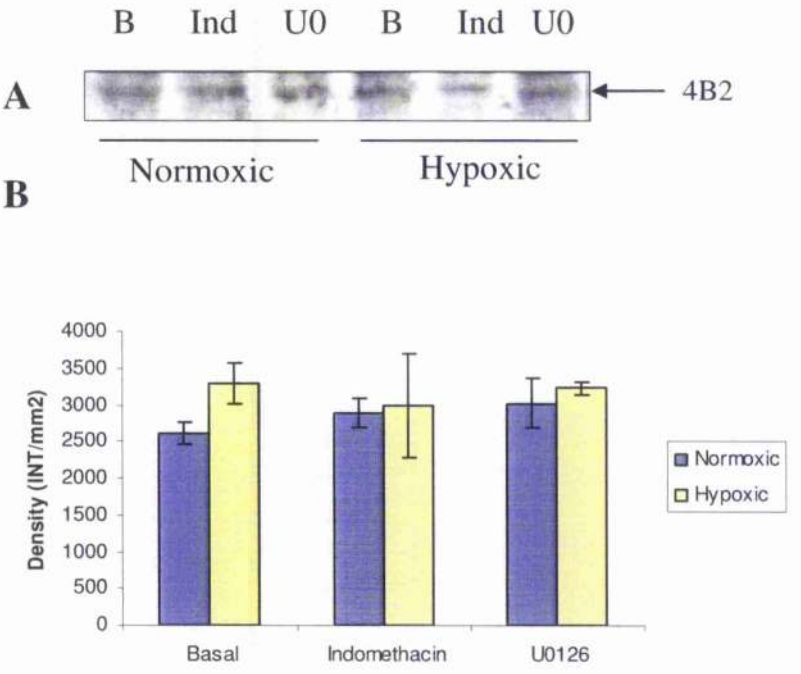


Figure 5.7 Effect of U0126 and indomethacin on PDE4B2 expression levels in hypoxia and normoxia.

Cells were treated with 10μM U0126 and 10μM indomethacin for 24 hours and were harvested as described in section 2.3.1.1 20μg protein of each sample was used for SDS-PAGE alongside a basal control sample on a 4-12% gel. *Panel A,* the proteins were transferred to nitrocellulose and probed with a PDE4B antibody. *Panel B,* the densitometry results as compared to normoxic samples expressed in INT/mm^2. Blots shown are representative of two independent experiments.

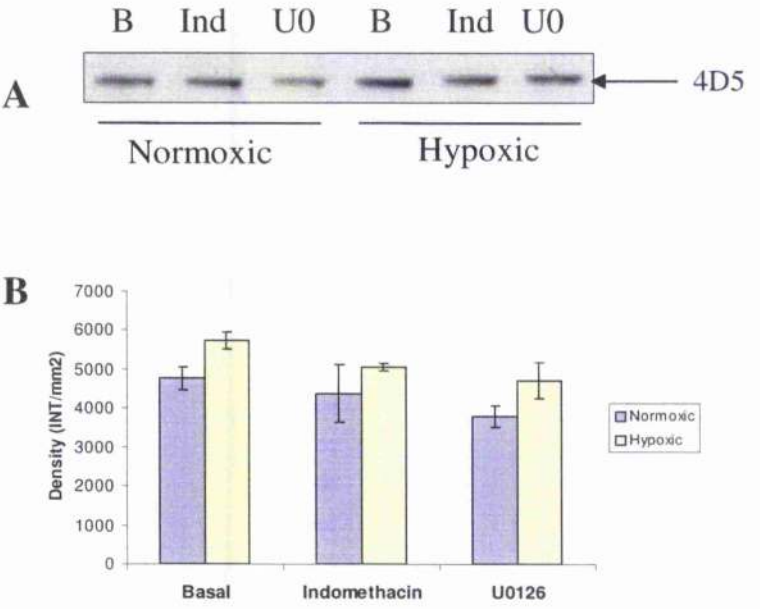


Figure 5.8 Effect of U0126 and indomethacin on PDE4D5 expression levels in hypoxia and normoxia.

Cells were treated with 10μM U0126 and μM indomethacin for 24 hours and were harvested as described in section 2.3.1.1. 20μg protein of each sample was used for SDS-PAGE alongside a basal control sample on a 4-12% gel. *Panel A,* the proteins were transferred to nitrocellulose and probed with a PDE4D antibody. *Panel B,* the densitometry results as compared to normoxic samples expressed in INT/mm2. Blots shown are representative of two independent experiments.

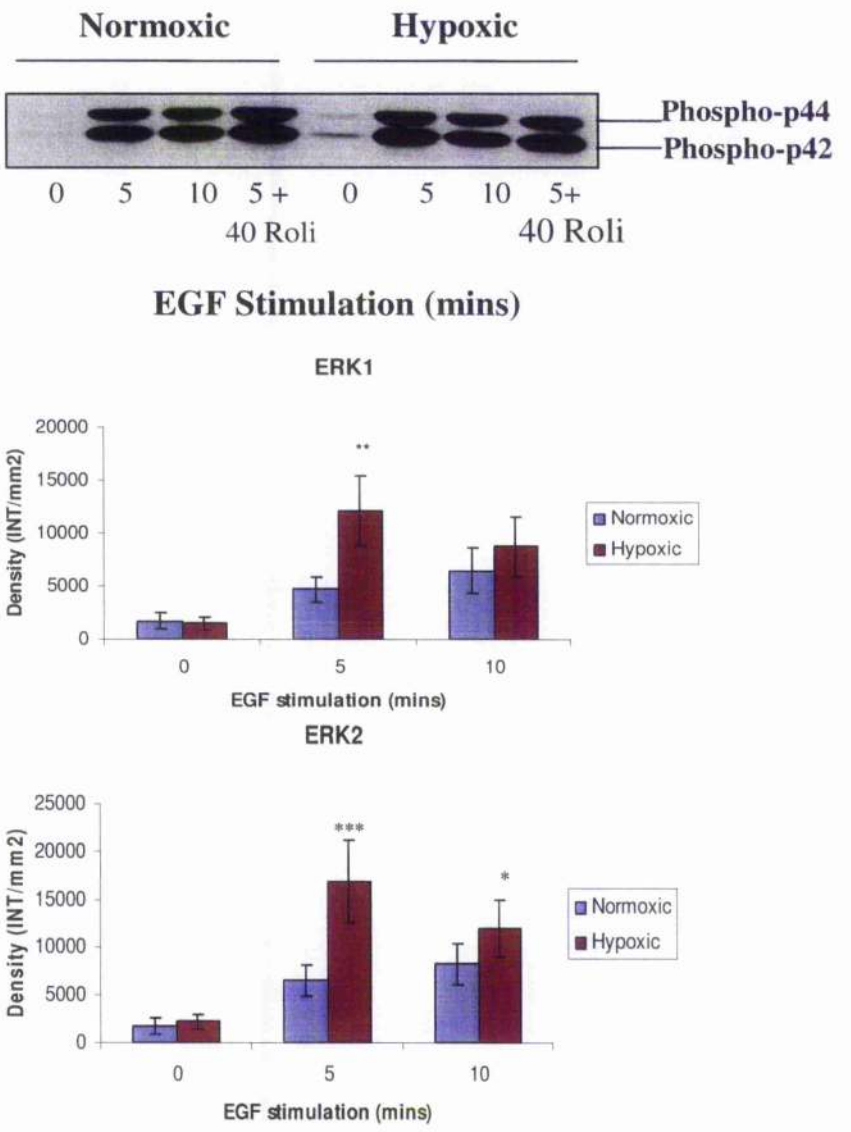


Fig 5.9 Effect of EGF on pERK in normoxic and hypoxic hPASMC.

Seven day hypoxic and normoxic hPASMC were treated with 50ng/μl EGF for five or ten minutes and also pre-treated with 10μM rolipram for 40 min prior to EGF treatment for five minutes. These cells were then collected as described in section 2.3.1.1 and 30μg lysate used on a 10% agarose gel. *Panel A,* After transferring onto nitrocellulose, an anti-phospho ERK1/2 antibody was used to identify the phosphorylated forms of ERK. *Panel B,* the densitometry results as compared to normoxic samples expressed in INT/mm^2. Results not shown for rolipram due to differences of n=2. Levels of pERK shown are representative of four independent experiments.

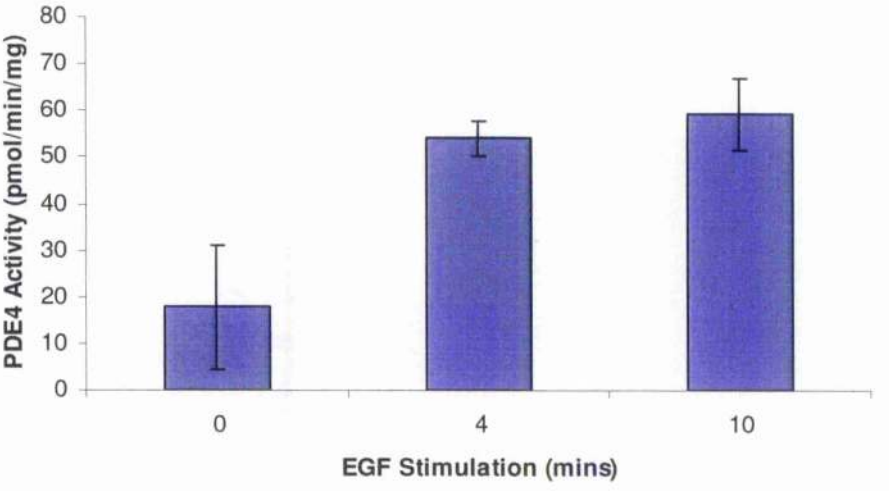


Figure 5.10 Effect of EGF on PDE4 activity levels. Cells were stimulated with 50ng/μl EGF for four and ten minutes prior to being immediately harvested for use in a cAMP-PDE activity assay as described in section 2.3.5. Results shown are mean ± S.E. of three independent assays.

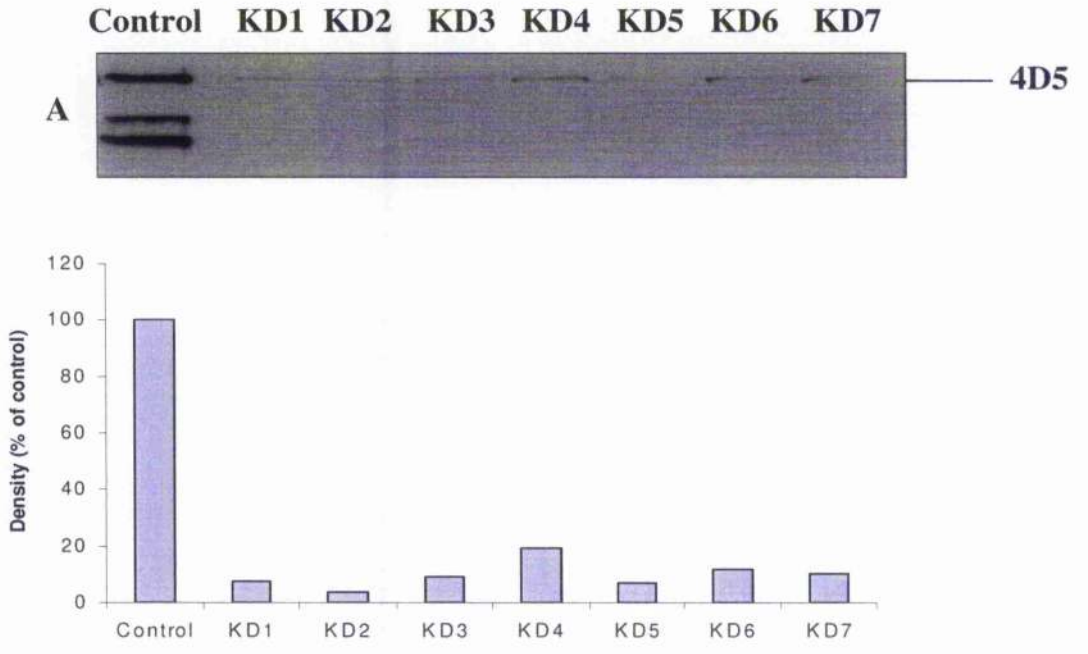


Figure 5.11 PDE4D5 knockdown in hPASMC

Normoxic and 7d hypoxic hPASMC were nucleofected with 60nM PDE4D5 siRNA. After nucleofection, the cells were transferred into 6 well plates and incubated for 24 hours. Cells were then harvested as described in section 2.3.1.1 20µg protein of each sample was used for SDS-PAGE alongside a basal control sample on a 4-12% gel. *Panel A,* the proteins were transferred to nitrocellulose and probed with an antibody directed against the c-terminus of PDE4D. *Panel B,* the densitometry results as compared to normoxic samples expressed as a percentage of the control PDE4D5 band.

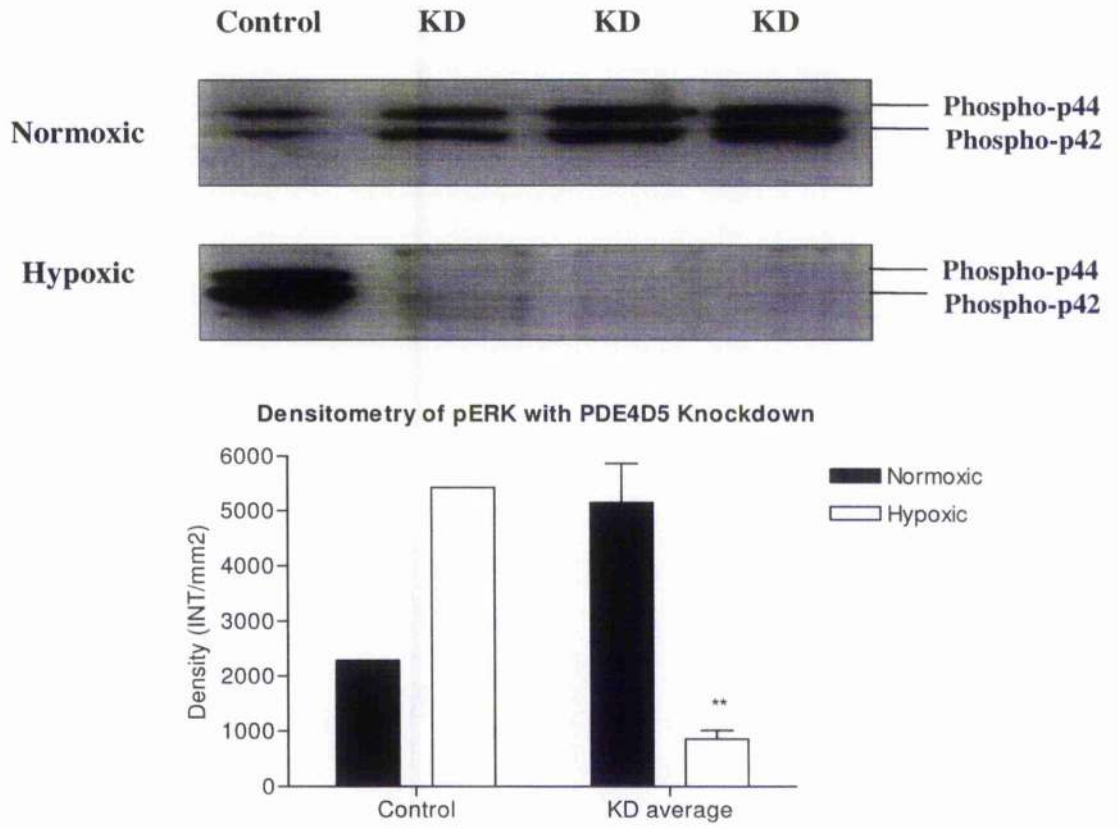


Figure 5.12 Effect of knockdown of 4D5 on pERK expression in normoxic and hypoxic hPASMC

Normoxic and 7d hypoxic hPASMC were nucleofected with 60nM PDE4D5 siRNA. After nucleofection, the cells were transferred into 6 well plates and incubated for 24 hours. Cells were then harvested as described in section 2.3.1.1 20µg protein of each sample was used for SDS-PAGE alongside a basal control sample on a 4-12% gel. *Panel A,* the proteins were transferred to nitrocellulose and probed with a phospho-ERK antibody. *Panel B,* the densitometry results as compared to control samples expressed in INT/mm^2 and ** denotes significance ($p<0.01$).

Chapter 6

Proliferation in hPASMC

6.1 Introduction

6.1.1 The Cell Cycle

The eukaryotic cell cycle is a tightly regulated process. It is divided into four phases; Gap1 (G_1) phase, S phase, Gap 2 (G_2) phase and M phase. The restriction point at which the cell becomes committed to completing the cell cycle is within the G_1 phase. During G_1 phase, the cell cycle is under the influence of extracellular stimuli which it requires for the induction of necessary proteins to pass the restriction point. Following this, and entering into the S phase, DNA synthesis occurs. The second Gap phase, G_2, is where the cell prepares for cell division by confirming DNA duplication is completed and the DNA is undamaged. Once this checkpoint is passed, the cell enters into the M phase where mitosis occurs. When cells have exited the cell cycle they are quiescent and are said to be in G_0.

The cell cycle is regulated by complexes of cyclin dependant kinases and cyclins. The cyclin dependant kinases (cdk) are expressed at similar levels throughout the cell cycle and cyclins are expressed and degraded throughout the cell cycle at appropriate points to allow the cycle to continue. The cyclin-dependant kinases therefore undergo a sequential activation and inactivation through the cell cycle, providing regulatory phosphorylation.

6.1.2 Cyclic AMP Inhibition of the cell cycle

The ability of cAMP to regulate the cell cycle was first reported 29 years ago (Pastan et al., 1975). Whilst it has been shown that raising the intracellular level of cAMP can promote the proliferation of certain cell types such as pituitary cells and thyrocytes, (Yonehara et al., 2001; Iacovelli et al., 2001) this action has also been noted to inhibit proliferation in most cell types, including endothelial cells, NIH 3T3 cells, Rat-1 fibroblasts and smooth muscle cells (D'Angelo 1997; Schmitt & Stork 2001; Osinki et al., 2000).

In vascular smooth muscle cells cAMP attenuates proliferation induced by vascular injury (Indolfi et al., 1997). It also prevents cells from entering S phase by arresting them within the G_1 phase of the cell cycle. This is achieved by various means. For example, cAMP has been shown to reduce levels of cyclin D1 and cyclin D3 by increasing their degradation (Kronemann et al., 1999; Stewart et al., 1999; Van Oirschot et al., 2001). This decrease in cyclin D levels results in a reduced phosphorylation of Rb (Boucher et al., 2001) and therefore reduces availability of proteins required for the G_1/S phase transition. Such a reduction of cyclin D also increases the amount of free $p27^{Kip1}$ protein which can then inhibit cyclin E/cdk2 (L'Allemain et al., 1997). An overexpression of cyclin D1 in cells

can result in abnormal cell cycle control as seen in cancer cells (Musat et al., 2004). This is seen to be the case in breast and lung cell carcinomas, as well as in tumour cell lines (Bos et al., 2004; Yuan et al., 2004).

$p27^{Kip1}$ binds to active cdk complexes resulting in their inactivation and a G1 block in the cell cycle (Kato et al., 1994, L'Allemain et al., 1997). Interestingly, increases in the cell cycle inhibitor proteins $p21^{Cip1}$ (Hayashi et al., 2000, Bauer et al., 2001) and $p27^{Kip1}$ (L'Allemain et al., 1997, Van Oirschot et al., 2001) have also been shown to be elicited by increased levels of cAMP.

cAMP can also in certain circumstances block growth factor activation of the extracellular regulated kinases, ERK 1 and 2. This can be achieved through PKA mediated inhibition of Raf-1 (Cook & McCormick 1993; Mischak et al., 1996). PKA has also been demonstrated to act indirectly through the phosphorylation of Src upon Rap1 to inhibit ERK (Ribeiro-Neto et al., 2002). However, the Src family of kinases have also been implicated in activating ERK1/2 through a process involving cAMP (Schmitt & Stork 2002). Activation of ERK1/2 promotes the expression of cyclin D1, cyclin E, cyclin A and the degradation of $p27^{Kip1}$ (Lavoie et al., 1996; Stork & Schmitt 2002). This allows the cell to pass through the G_1/S phase transition (Schmitt & Stork 2001). However, inhibition of the ERK1/2 pathway is not a prerequisite for cAMP induced growth arrest (Balmanno et al., 2003).

6.1.3 Epac

Until recently, the main target of cAMP within cells was thought to be protein kinase A (PKA). In 1998 however, an exchange protein directly activated by cAMP (Epac) was uncovered as another major target of cAMP within cells (deRooij et al., 1998). Epac was uncovered by investigating a PKA-insensitive activation of Rap1 by cAMP. Epac is a guanine nucleotide exchange factor for the Ras like small GTPases Rap1 and Rap2. Although cAMP is well established to play a role in cell cycle control, it has been previously attributed to act solely through the actions of PKA. Recent studies show that there are PKA independent mechanisms involved in cAMP regulation of the cell cycle (Cass et al., 1999, Fujita et al., 2002). It has been shown that endogenous Epac localisation is dependant on cell cycle. During interphase, Epac is mainly localised to the nuclear membrane and mitochondria in COS-7 cells. Epac disassociates from these membranes in metaphase and localises to the mitotic spindle and centrosomes. Once the cell cycle is completed, however, Epac returns to the nuclear membrane (Qiao et al., 2002). Initial work on the cAMP-ERK pathway suggested the Epac activation of Rap1 lead to the activation of

ERK through B-Raf; however, recent studies have shown that Rap1 does not activate B-Raf (Bos et al., 2003). Instead, Rap1 is involved in integrin signalling which can activate the EGF receptor, leading to ERK activation (Bill et al., 2004). Notwithstanding this, the effect of Epac-mediated cAMP effects on cellular proliferation has not been investigated to date.

6.1.4 Phosphodiesterases and proliferation

In many disease states where excessive cellular proliferation is a hallmark, an increase in PDE activity and consequential decrease in cAMP levels has been observed (Savini et al., 1995; Vignola 2004; Schmidt et al., 1999; Essayan 1999; Spina 2003). Indeed, cAMP-specific PDE inhibitors have been shown to attenuate proliferation in various cell types, including vascular smooth muscle cells and glioma cells by inducing the cell cycle inhibitor proteins, $p21^{Cip1}$ and $p27^{Kip1}$ (Chen et al., 2002, Ogawa et al., 2002). Stimulation of proliferation itself has also been shown to induce the expression of PDE1C in human smooth muscle cells (Rybalkin et al., 2003). Using umbilical cord blood mononuclear cells, the PDE4 selective inhibitor rolipram has been shown to have an inhibitory effect on proliferation whilst specific inhibitors of PDE3, PDE5 and PDE1 did not, indicating both compartmentalisation of cAMP actions and specificity for cAMP over cGMP (Banner et al., 2000).

In A-172 glioma cells, rolipram has been observed to induce $p21^{Cip1}$ and $p27^{Kip1}$ proteins and to decrease the activity of cdk2, leading to a G_1 block (Chen et al., 2002). In human acute lymphoblastic leukaemia cells, rolipram, but not selective inhibitors for either PDE3 or PDE1, suppressed growth. The resultant increase in cAMP caused by PDE4 inhibition led to an induction of p53 and $p21^{Cip1}$, G_1 and G_2/M cell cycle arrest and increased apoptosis (Ogawa et al., 2002).

In human aortic smooth muscle cells, both the PDE4 selective inhibitor, rolipram and the PDE3 selective inhibitor, CI-930 attenuated proliferation by reducing the number of cells entering S phase (Johnson-Mills et al., 1998). Indeed, using both PDE4 and PDE3 inhibitors together produced an additive inhibitory effect on proliferation. It was interesting to note that whilst using either inhibitor alone did not appear to raise cAMP levels significantly, it was only when they were used in tandem was there a substantial increase in intracellular cAMP.

6.1.5 Proliferation in Hypoxia Induced Pulmonary Hypertension

Vascular smooth muscle cell growth and hypertrophy are being intensely studied in an effort to uncover the mechanisms governing thickening of the pulmonary arteries in PHT and other chronic lung disorders (Voelkel & Tuder 1997). This thickening has been demonstrated (Heath 1993) to be due to progressive muscularisation of the pulmonary arteries due to smooth muscle cell hypertrophy and hyperplasia. Vascular smooth muscle is normally contractile and quiescent, but stress or injury to the vessel wall results in a conversion of the cells to a migratory, secretory and proliferative phenotype (Heath 1993). Hyperplasia plays an important part in hypertension as shown by a significant increase in smooth muscle cell proliferation in pulmonary vessels from chronic hypoxic animals (Meyrick & Reid 1978, Wohrley et al., 1995). The normal aortic smooth muscle cell growth in rat is 0.01%/day which increases to 1%/day in hypertensive rats. This thickening of the pulmonary artery contributes to the rise in pulmonary artery pressure witnessed in PHT (Rich et al., 1987).

The three cell types of the pulmonary artery; endothelial cells, smooth muscle cells and fibroblasts can all show increased proliferation when maintained in hypoxic conditions compared to those in normoxia (Stenmark et al., 2002; Voelkel et al., 1997; Cool et al., 1995; reviewed in Humbert et al., 2004). The mechanism by which hypoxia increases the proliferation of vascular cells is unknown, although it is known that many of the growth factors which induce proliferation in vascular smooth cells are upregulated in hypoxia e.g. ET-1, PDGF, VEGF and 5-HT (Brij & Peacock 1998).

In this chapter, I set out to investigate whether the increased rate of proliferation in hypoxic hPASM cells compared to normoxic hPASM cells could be affected by compounds known to interfere with cAMP signalling. Thus, for example, I used agonists and antagonists to allow for selective perturbation of signalling through the RI and RII isoforms of PKA as it has suggested from studies done on HL-60 cells that RI is growth stimulatory while RII is growth inhibitory (Cho-Chung et al., 1993). I also employed two novel Epac agonists, 8 -pCPT-2′-O-Me-cAMP and 8 -pMeOPT-2′-O-Me-cAMP so as to, for the first time, evaluate the putative role that Epac might play in mediating aspects of the cAMP regulation of the cell cycle. I also used selective inhibitors of PDE4 and PDE3, so as to appreciate any role of these key cAMP degrading enzymes. The methods employed in this chapter are described in full in section 2.2, with a table of drug concentrations at the end of this chapter.

6.2 Basal Proliferation Rates in Normoxic and Hypoxic hPASM cells

All proliferation assays were carried out after seven days of hypoxia to evaluate the effect of compounds in normoxic cells compared to cells with the PDE4 profile maximally altered.

To determine the basal proliferation rates in both seven day hypoxic and corresponding normoxic controls, DNA synthesis was measured by [^{3}H]-thymidine incorporation as described in section 2.2. Briefly, cells were cultured in 96-well plates in both normoxic and hypoxic conditions as normal until day five of hypoxic exposure. The cells were quiesced overnight in serum free medium and on day six, serum was reintroduced to the medium in one set of plates, while another was refreshed with serum free medium. After 20 hours, 0.5 μCi [^{3}H]-thymidine was added to each well and the cells were harvested after a further 4 hours to measure radioactivity.

In all results in this chapter, results are presented as a percentage with 100% being equal to mean normoxic basal proliferation rate. Two-way ANOVA with Bonferroni's post test was performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, to analyse raw data. Where only two groups of data were to be analysed, an unpaired t-test was performed, and *$p<0.05$ was considered to be significant.

6.2.1 Basal Proliferation Rates

It was observed that hypoxia induced a 0.5 fold increase in proliferation over basal in serum-free cells, however this increase was not significant. This increase was larger in cells with serum re-introduced. With serum, hypoxia caused a significant increase in proliferation ($p<0.001$). Serum induced proliferation was doubled in hypoxic cells compared to normoxic proliferation *(Figure 4.1, Panel A and B).*

6.3 Effect of Raising cAMP on Proliferation Rates in Normoxic and Hypoxic hPASMC

It has been previously reported that an increase in cAMP levels exerts an anti-proliferative effect in vascular smooth muscle cells (Kronemann et al., 1999; Koyama et al 2000;

Hayashi et al., 2000; Bornfeldt & Krebs 1999; Indolfi et al., 2001). The extent of this effect has not been measured in hypoxic hPASM cells specifically however.

6.3.1 Effect of PDE inhibitors

The effect of PDE inhibition on proliferation in normoxic and seven day hypoxic cells was investigated using the selective PDE4 inhibitor, rolipram; the selective PDE3 inhibitor, cilostamide; a mixed PDE1 and PDE5 inhibitor, zaprinast and also using both rolipram and cilostamide together as such actions have been observed to potentiate the effect of either inhibitor alone (Johnson-Mills et al., 1998).

6.3.1.1 Effect of Rolipram

Treating serum-starved cells with rolipram for 24 hours had no significant effect on normoxic or hypoxic cells in serum-free medium (*Figure 6.2, Panel A),* although it was observed to reduce the rate of proliferation in hypoxic cells to basal levels seen in normoxic cells.

Rolipram treatment of cells with serum re-introduced, again had no significant effect on proliferation in either normoxic or hypoxic cells.

6.3.1.2 Effect of Cilostamide

Cilostamide did not significantly reduce proliferation in normoxic serum starved cells. However, in contrast to this, cilostamide significantly reduced proliferation ($p<0.05$) in hypoxic serum starved cells, (62 % of hypoxic basal levels). This reduced rate was equivalent to 90% of normoxic basal levels *(Figure 6.2, Panel A).*

Cilostamide had a significant inhibitory effect on serum-induced proliferation in both normoxic and hypoxic cells. Proliferation was significantly reduced following cilostamide addition to 38% ($p<0.05$) in normoxic cells and 56% ($p<0.05$) in hypoxic cells of respective serum induced control rates. Thus, hypoxic proliferation was reduced to almost that of normoxic serum induced proliferation *(Figure 6.2, Panel B).* The increased effect of cilostamide was consistent with the data in chapter 4 which showed cilostamide increased cAMP levels to 3 times that of basal in normoxic cells, whereas it didn't significantly affect cAMP levels in hypoxia.

6.3.1.3 Effect of Zaprinast

Evaluating serum-starved cells treated with zaprinast, there was no significant effect on normoxic or hypoxic cells after 24 hours treatment *(Figure 6.2, Panel A).* With cells

grown in serum-containing medium for the same 24 hours, there was still no significant effect *(Figure 6.2, Panel B).*

6.3.1.4 Effect of Rolipram and Cilostamide

Using both PDE4 and PDE3 inhibitors together has previously been shown to potentiate the effect of either single inhibitor in studies on human coronary artery smooth muscle cells (Johnson-Mills et al., 1998). Indeed, treating normoxic and hypoxic serum starved cells with both rolipram and cilostamide together induced a larger inhibition of proliferation than with either inhibitor alone. This effect was significant in hypoxic cells ($p<0.01$) where proliferation was reduced to 50% of hypoxic basal proliferation rate *(Figure 6.2, Panel A).* In normoxic cells however, this effect was not significant.

The combined inhibition of PDE3 and PDE4 activity had the largest inhibitory effect of any of the inhibitors on the proliferation status of both normoxic and hypoxic cells grown with serum for the last 24 hours. Under conditions for serum-stimulated proliferation, it was observed that rolipram and cilostamide acted synergistically to reduce proliferation rates to 45% in normoxic cells and to 30% ($p<0.01$) in hypoxic cells of respective serum induced control rates *(Figure 6.2, Panel B).*

6.3.2 Effect of cAMP effectors

Another way of raising cAMP within the cell is through activating adenylyl cyclase directly with an adenylyl cyclase activator such as forskolin, or indirectly through a beta-adrenergic agent such as isoproterenol. As such, the effect of both isoproterenol and forskolin on proliferation was investigated in hPASM cells.

6.3.2.1 Effect of Isoproterenol

24h treatment with isoproterenol had no significant effect on proliferation in normoxic or hypoxic cells, whether in serum free or serum containing medium *(Figure 6.3, Panel A and B).*

6.3.2.2 Effect of Isoproterenol and Rolipram

In contrast to using either isoproterenol or rolipram alone, when used together for the 24h treatment, then a highly noticeable inhibition of proliferation was evident. This inhibition was significant in hypoxic serum induced proliferation where proliferation was reduced to 20% ($p<0.05$) in hypoxic cells of respective serum-induced control rates *(Figure 6.3, Panel*

B). The effect on serum starved normoxic and hypoxic cells and normoxic serum induced proliferation was not significant.

Rolipram and isoproterenol have no effect on proliferation when used alone, thus these results indicate that a 'threshold' level of cAMP activation is required to be breached in order for it to inhibit proliferation in hPASMC.

6.3.2.3 Effect of Forskolin

Introducing forskolin alone to the cells had no significant effect under either hypoxic or normoxic conditions in either serum starved cells or cells with serum re-introduced *(Figure 6.3, Panel A and B).* Furthermore, adding the non-selective PDE inhibitor, 3-isobutyl-1-methylxanthine, (IBMX), along with forskolin appeared to increase the rate of proliferation of control levels in serum-treated normoxic cells, although not significantly. In hypoxic serum-induced proliferation, the combination of forskolin together with IBMX acted in an anti-proliferative manner, where proliferation was reduced to 20% ($p<0.05$) of the hypoxic serum induced control rate *(Figure 6.3, Panel B).*

These results indicate that indeed a threshold level of cAMP is required for cAMP to act in an inhibitory fashion on proliferation in hPASMC. However, it was observed in the chapter 4 that the cAMP produced in hypoxic cells in response to IBMX and forskolin is less than that in normoxic cells. Thus, this is suggestive of IBMX acting through a different mechanism to override the proliferative mechanism induced in hypoxia.

6.4 Effect of Growth Factors on Proliferation Rates in Normoxic and Hypoxic hPASMC

Epidermal growth factor (EGF) has been thoroughly studied in a wide range of cell types and is well known to serve as a growth factor and mitogen. In chapter 5, EGF was shown to activate PDE4 in hPASMC, thus it was used in this study to investigate any effect on proliferation.

Transforming Growth Factor-β_1 (TGF-β_1) has been under the spotlight in PHT research since uncovering the role of inherited mutations in the *BMPR2* gene in the development in PAH (see general introduction section 1.5; Thomson et al., 2000; Machado et al., 2001). TGF-β is an important regulator of cellular differentiation and proliferation in the lung(Vicencio et al., 2002) and has been shown to be both a stimulator of cell growth and

an inhibitor, depending on cell type. In rat aortic vascular smooth muscle cells, TGF-β_1 decreased serum-induced proliferation (Grainger et al., 1994). Therefore, I used TGF-β_1 in these proliferation assays to see if it can impact upon hPASMC proliferation also.

6.4.1. Effect of EGF

EGF had no effect on serum starved proliferation in either normoxic or hypoxic cells (*Figure 6.4, Panel A).* In cells cultured in serum, 50 pg/µl medium EGF induced a 25% increase in proliferation compared to the normoxic serum induced control rate of proliferation. In hypoxic cells, this increase was even greater with an increase of 50% in proliferation ($p<0.01$) compared to the hypoxic serum induced control proliferation rate *(Figure 6.4, Panel B).* Therefore, EGF requires a serum-induced pathway to elicit a proliferative effect in hPASMC.

6.4.2 Effect of EGF and Rolipram

Using EGF or rolipram has been demonstrated to have no effect on serum starved proliferation in hPASMC. It was also shown here that when used together, EGF and rolipram do not affect proliferation of serum starved cells *(Figure 6.4, Panel A.)*

Serum-induced proliferation of normoxic hPASMC was not affected by the addition of EGF and rolipram. EGF alone has previously been shown to elicit a 25% increase in serum induced proliferation in normoxic cells and rolipram caused a 40% reduction in hPASMC proliferation in normoxic serum-induced proliferation (6.3.3.1). Thus, it appears here that when used together they negate the effect of each other. In hypoxic cells, rolipram alone was previously shown to reduce serum-induced proliferation by 28% and EGF caused a 50% increase. Again, when used together, EGF and rolipram appear to negate the effect of one another on hypoxic serum-induced proliferation as no effect is seen compared to hypoxic serum-induced control rate of proliferation *(Figure 6.4, Panel B).*

6.4.3 TGF-β_1

TGF-β_1 appeared to have no effect on proliferation in either normoxic or hypoxic hPASM cells that had been serum starved *(Figure6.5, Panel A.).* Serum-induced proliferation of normoxic and hypoxic hPASMC is also unaffected by TGF-β_1 *(Figure 6.5, Panel B).*

6.5 Effect of the Src Family Inhibitor, PP2, on Proliferation Rates in Normoxic and Hypoxic hPASMC

The Src family of kinases have previously been implicated in activating ERK1/2, and has also been shown to be required for cAMP activation of Rap1 in fibroblast cells(Schmitt & Stork 2002). PP2 is a potent inhibitor of the Src family kinases which also inhibits many other kinases including CSK, LCK and SAPK 2a/p38.

I also investigated here a compound called PP3, which inhibits selectively the EGF receptor tyrosyl kinase but not the activity of Src family tyrosyl kinases, which are inhibited selectively by PP2. These two compounds allow the potential to gain insight into the relative roles of the EGFR tyrosyl kinases and SRC family tyrosyl kinases in proliferation of the hPASM cells under normoxic and hypoxic conditions.

6.5.1 Effect of PP2

PP2 elicited no significant effect on proliferation in normoxic serum starved cells. In hypoxia however, PP2 reduced proliferation to 38% ($p<0.05$) of the basal proliferation rate in normoxic serum starved cells *(Figure 6.6, Panel A)*. PP2 had no effect on normoxic serum induced proliferation, but reduced hypoxic proliferation to 25% ($p<0.01$) of hypoxic serum-induced proliferation control rate *(Figure 6.6, Panel B)*.

6.5.2 Effect of PP3

PP3 had no significant effect on proliferation in normoxic or hypoxic serum starved cells *(Figure 6.6, Panel A)*. PP3 also had no significant effect on serum induced proliferation in normoxic or hypoxic cells *(Figure 6.6, Panel B)*.

These results indicate that Src is involved in the increased hypoxic proliferation rate of hPASMC. The Src family of kinases have been demonstrated to activate Rap1 (Schmitt and Stork 2002b) which can activate B-raf and activate ERK. Indeed, ERK activation by PKA has been demonstrated to require Src (Lindquist et al., 2000). This suggests the increased PKA activity shown in chapter 4 in hypoxic hPASMC acts on Src to activate the ERK pathway through Rap1-B-raf. This would also explain the increased levels of ERK witnessed in hypoxia (Jin et al., 2000; Scott et al., 1998).

6.6 The cAMP Pathway

As mentioned previously (see general introduction section 1.5.8.2; reviewed by Koyama et al., 2000; Hayashi et al., 2000), it is known that cAMP acts as an anti-proliferative agent within vascular smooth muscle cells. Although no significant effect was observed using the AC stimulator, forskolin alone or the beta-adrenergic agent, isoproterenol alone on proliferation, there was an inhibition of proliferation using the combined PDE inhibitors rolipram and cilostamide. It is well known that cAMP signalling is compartmentalised in cells and that for effects to be seen then increased cAMP levels must occur in a functionally relevant compartment. One way to achieve this and to evaluate whether actions are actually due to cAMP is to swamp the cell with a cAMP analogue. When cAMP elicited effects are observed, it can be determined if this is through specific PKA isoforms or EPAC, using selective agonists and antagonists. As PKA activity was observed to increase in hypoxic hPASMC in chapter 3, specific PKA isoform agonists and antagonists were employed to investigate the effect of this on hPASMC proliferation.

6.6.1 Effect of PKA Agonists

PKA is a heterodimer consisting of two regulatory and two catalytic subunits. Previous research has indicated differing roles in growth regulation effected by the RI and RII subunits. Specific agonist pairs for the PKA RI and PKA RII subunits were employed to investigate any differing effect on proliferation by cAMP signalling through these two PKA isoforms.

6.6.1.1 PKA RI Agonist

The cAMP analogs 8-PIP-cAMP and 8-HA-cAMP were used to activate PKA RI selectively. In serum starved cells, the PKA RI agonist had no significant effect on proliferation in normoxic or hypoxic cells *(Figure 6.7, Panel A).* The PKA RI agonist also had no effect on normoxic serum induced proliferation. However, it did reduce hypoxic serum induced proliferation to 50% ($p<0.001$) of the control hypoxic serum induced proliferation rate *(Figure 6.7, Panel B).*

6.6.1.2 PKA RII Agonist

The cAMP analogs 8-PIP-cAMP and 8-MBC-cAMP were used to activate PKA RII selectively. The PKA RII agonist had no significant effect on proliferation in normoxic serum starved cells *(Figure 6.7, Panel A).* It did reduce hypoxic proliferation to 47%

($p<0.05$) of the basal hypoxic proliferation rate in serum starved cells. Serum-induced proliferation was reduced to 57% ($p<0.001$) in hypoxic cells compared to the hypoxic control serum induced proliferation rate (*Figure 6.7, Panel B*). There was no effect on normoxic serum-induced proliferation however.

6.6.1.3 PKA RI and RII Agonists

Using both the PKA RI and PKA RII agonists together had no significant effect on normoxic or hypoxic proliferation in serum starved cells *(Figure 6.7, Panel A)*. Normoxic serum induced proliferation was reduced to 68% ($p<0.05$) of normoxic control serum induced proliferation rate. This effect was greater in hypoxic cells, where these agonists used together reduced serum induced proliferation to 35% ($p<0.001$) of the hypoxic control proliferation rate *(Figure 6.7, Panel B)*.

These results indicate PKA RI:RII exists in a balanced activation state within hPASMC, and if this is altered in favour of either subunit, then this elicits an inhibitory effect on proliferation. In particular, PKA RII appears to have a significant role in the increased hypoxic proliferation rate seen in hPASMC as activating this subunit reduces hypoxic proliferation to that of normoxic cells, in both serum starved cells and those with serum reintroduced. Agonising the PKA RI subunit was also capable of returning the hypoxic serum induced proliferation rate to that of normal, but only when the serum response pathways were in effect, similar to the treatment with forskolin and IBMX (6.3.2.3).

6.6.1.4 Effect of the PKA Agonist, 6-BnZ-cAMP

The non-selective PKA agonist, 6-BnZ-cAMP, which activates both RI and RII forms of PKA, was used to confirm the results witnessed using the RI and RII agonists in combination. In serum starved cells, 6-BnZ had no significant effect on proliferation in normoxic or hypoxic cells (*Figure 6.7, Panel A*). 6-BnZ also had no significant effect on normoxic serum induced proliferation, but did reduce hypoxic serum induced proliferation to 71% ($p<0.01$) of the hypoxic control serum induced proliferation rate *(Figure 6.7, Panel B)*.

This again confirms the role of PKA in the hypoxic induced proliferation witnessed in hPASMC as it has no effect on normoxic proliferation, whether in the presence or absence of serum. The magnitude of the effect induced by the PKA RI and RII agonists together compared to 6-BnZ suggests the separate agonist somehow synergise to enhance their effect whereas 6-BnZ perhaps has a more 'real' effect on PKA.

6.6.2 Effect of PKA Antagonists and Inhibitor

6.6.2.1 Effect of the PKA Inhibitor, H89

H89 has been used by various investigators to inhibit protein kinase A selectively. However it is now known that H89 is also capable of inhibiting various other kinases, including Rho kinase (Davies et al., 2000). H89 had no significant effect on normoxic serum induced proliferation or on proliferation in serum starved normoxic cells *(Figure 6.7, Panel A and B).* Intriguingly, H89 induced a similar reduction in proliferation in hypoxic cells to that of the PKA agonist, although with a more potent effect. In serum starved hypoxic cells, H89 reduced proliferation to 49% ($p<0.05$) of basal hypoxic proliferation, while it reduced hypoxic serum induced proliferation to 64% ($p<0.001$) of the hypoxic serum induced proliferative control rate *(Figure 6.7, Panel A and B).*

The results obtained using PKA agonists and the PKA inhibitor, H89, suggest PKA is involved in a balance to maintain the increased hypoxic proliferation as any alteration to PKA signalling ultimately leads to a reduction in proliferation, decreasing levels to that of normoxic proliferation or lower. Thus, antagonists of the PKA RI and PKA RII were employed to investigate if this also occurs with one of the subunits inhibited.

6.6.2.2 Effect of PKA RI Antagonist

The PKA RI antagonist had no significant effect on proliferation in hPASMC whether in normoxic, hypoxic cells or in the presence or absence of serum *(Figure 6.8, Panel A and B).*

6.6.2.3 Effect of the PKA RII Antagonist

The PKA RII antagonist reduced proliferation in serum starved cells, although this effect was only significant in hypoxic cells with proliferation reduced to 37% ($p<0.05$) of the hypoxic basal proliferation rate *(Figure 6.8, Panel A).* The PKA RII antagonist completely ablated serum induced proliferation in normoxic and hypoxic cells, reducing proliferation rates to < 5% in both normoxic and hypoxic cells *(Figure 6.8, Panel B).*This highly significant reduction in serum induced proliferation is indicative of the cells undergoing apoptosis when PKA RII is inhibited, thus PKA RII appears to be essential for the normal growth of hPASMC.

6.6.3 Effect of cAMP analogs

The majority of downstream effects of cAMP have been attributed to the cAMP dependant kinase, PKA. However, in 1998, another major cAMP target was uncovered in cells (de

Rooij et al., 1998). This cAMP-GEF was an Exchange Protein Activated by cAMP, (Epac), and has recently been shown to be involved in cell adhesion (Rangarajan et al., 2003). Localisation of Epac within cells is cell-cycle dependant. As such, two cAMP analogs were employed in this assay. Both of these analogs activate PKA and Epac (Enserink et al., 2002). 8-bromo-cAMP has a higher affinity for PKA than Epac, whilst another, CPT-cAMP, is known to have a higher affinity for Epac. These cAMP analogs were used to determine if PKA or EPAC had the larger role in regulation of proliferation in hPASMC under normoxic and hypoxic conditions.

6.6.3.1 Effect of 8-Br-cAMP

The cAMP analog 8-Br-cAMP was observed to have no significant effect on the proliferation of hPASMC *(Figure 6.9, Panel A and B).*

6.6.3.2 Effect of CPT-cAMP

In serum starved cells and cells cultured with serum, the cAMP analog, CPT-cAMP, induced a highly significant reduction in proliferation of normoxic and hypoxic cells *(Figure 6.9, Panel A and B).* Cells appeared morphologically normal after treatment in all cases. In normoxic and hypoxic serum starved cells, CPT-cAMP reduced proliferation to 44% and 10% ($p<0.01$) of basal rates respectively. Serum induced proliferation was reduced to approximately 17% ($p<0.001$) in both normoxic and hypoxic cells when compared to their respective serum induced proliferation control rate.

6.6.4 Specific EPAC agonist

Although CPT-cAMP has a higher affinity for EPAC over PKA, there is still some activation of PKA reported in the use of this analog. At the time of this study, there was originally one cAMP analog altered to specifically activate EPAC available. This was 8 -pCPT-2′-O-Me-cAMP (Enserink et al., 2002). This agonist was used in the proliferation assay to confirm the results observed with CPT-cAMP and to investigate this anti-proliferative effect further.

6.6.4.1 Effect of 8 -pCPT-2′-O-Me-cAMP

It was observed that the EPAC agonist, 8 -pCPT-2′-O-Me-cAMP, had no significant effect on proliferation in normoxic or hypoxic serum starved cells *(Figure 6.10, Panel A).*

8 -pCPT-2′-O-Me-cAMP reduced serum-induced proliferation to approximately 50% ($p<0.01$) in normoxic cells and 36% ($p<0.001$) in hypoxic cells when compared with their respective control rate of serum induced proliferation *(Figure6.10, Panel B).*

6.6.5 Effect of cAMP Effectors on the anti-proliferative capacity of 8 - pCPT-2′-O-Me-cAMP

In an attempt to understand the effect of the EPAC agonist on proliferation, PDE inhibitors and other cAMP effectors were used in conjunction with the agonist to see if they could recover the cells from this or further potentiate the effect.

6.6.5.1 Rolipram

Including rolipram with the EPAC agonist for 24 hours potentiated the effect seen with rolipram alone or the 8 -pCPT-2′-O-Me-cAMP alone. In serum starved hypoxic cells, this combined treatment reduced proliferation to 65% in normoxic cells and 56% ($p<0.01$) in hypoxic cells *(Figure 6.11, Panel A).*

Treatment with rolipram and 8 -pCPT-2′-O-Me-cAMP reduced serum induced proliferation to 45% in normoxic cells and 27% ($p<0.001$) in hypoxic cells *(Figure 6.11, Panel B).* Again this was greater than the effect seen with either compound used alone.

6.6.5.2 Cilostamide

Using the PDE3 inhibitor cilostamide with 8 -pCPT-2′-O-Me-cAMP again potentiated the effect of using either compound alone. Normoxic proliferation was reduced to 73% in serum starved cells, although this was not significant to treatment with either cilostamide or the Epac agonist alone. Hypoxic proliferation in serum starved cells was significantly affected with a decrease to 47% ($p<0.01$) of basal hypoxic proliferation *(Figure 6.11, Panel A).* Serum induced proliferation was reduced to 34% in normoxic cells and 34% ($p<0.001$) in hypoxic cells when compared to the respective control serum induced proliferation rates *(Figure 6.11, Panel B).*

6.6.5.3 Rolipram and Cilostamide

Using both rolipram and cilostamide with 8 -pCPT-2′-O-Me-cAMP potentiated the effect seen with 8 -pCPT-2′-O-Me-cAMP alone. However, this effect was not as great as that seen when using rolipram and cilostamide alone. In serum-starved cells, the combined treatment had no effect on normoxic proliferation, but reduced hypoxic proliferation to 62% ($p<0.05$) of hypoxic basal proliferation *(Figure 6.11, Panel A).* The combined treatment also reduced normoxic proliferation to 30% of control serum induced proliferation, although this was not significant. In hypoxic cells with serum reintroduced, this effect was greater, with a reduction to 22% ($p<0.001$) of hypoxic control serum induced proliferation *(Figure 6.11, Panel B).*

6.6.5.4 8-br-cAMP

8-bromo-cAMP used with 8 -pCPT-2′-O-Me-cAMP had no effect on the anti-proliferative capacity of 8 -pCPT-2′-O-Me-cAMP alone *(Figure 6.12, Panel A and B).*

6.6.5.5 6-BnZ

The PKA agonist, 6-BnZ, slightly potentiated the inhibitory effect of 8 -pCPT-2′-O-Me-cAMP when used together in all conditions *(Figure 6.12, Panel A and B).*

6.6.5.6 PKA RI agonist

Normoxic serum starved cells treated with both the EPAC agonist and a specific PKA RI agonist did not have any significant effect on proliferation when compared to 8 -pCPT-2′-O-Me-cAMP used alone. However, the combined effect was increased in hypoxic serum starved cells compared to using either compound alone *(Figure 6.13, Panel A).* Hypoxic serum starved proliferation was reduced to 55% ($p<0.05$) of basal hypoxic proliferation.

The combined effect of the Epac agonist and PKA RI agonist did not have any effect over that of 8 -pCPT-2′-O-Me-cAMP alone in serum induced proliferation in normoxic and hypoxic cells (*Figure 6.13, Panel B).*

6.6.5.7 PKA RII agonist

Using the PKA RII agonist in conjunction with the Epac agonist slightly reduced the anti-proliferative effect of using either agonist alone in serum starved normoxic and hypoxic cells, although not significantly *(Figure 6.13, Panel A).* There was no effect observed, however, in cells with serum reintroduced (*Figure 6.13, Panel B).*

6.6.5.8 PKA RI and RII agonists

Using both the RI and RII agonists, along with the EPAC agonist, potentiated the anti-proliferative effect of using either both the RI and RII agonists or the Epac agonist alone on proliferation in serum starved cells. This effect was significant ($p<0.01$) in hypoxic serum starved cells where proliferation was reduced to 50% of hypoxic basal (*Figure 6.13, Panel A.)*

Using both the RI and RII agonists along with the Epac agonist had no significant effect on normoxic serum induced proliferation when compared with the Epac agonist alone. However, it did potentiate the effect of the RI and RII agonist or the Epac agonist observed in hypoxic serum induced proliferation, further reducing it to 17% ($p<0.001$) of the hypoxic control serum induced proliferation rate *(Figure 4.13, Panel B).*

6.7 Effect of the EPAC Agonist, 8 -pMeOPT-2′-O-Me-cAMP, and a Rap1 Inhibitor on Proliferation compared to 8 -pCPT-2′-O-Me-cAMP

6.7.1 Arresting cell cycle using a Rap1 Inhibitor, GGTI-298

GGTI-298 (Calbiochem) is a GGTase I inhibitor that inhibits the processing of Rap1A without affecting H-Ras. It is also capable of arresting cells in the G_0/G_1 phase of the cells cycle, but it has previously been reported that using GGTI-298 can reduce the inhibitory effects on proliferation seen with cAMP-elevating agents in RPE cells (Hecquet et al., 2002). As can be seen in figure 6.14, the Rap1 inhibitor reduced both normoxic and hypoxic proliferation rates to 30% and 20% respectively of control levels. Including the EPAC agonist along with this inhibitor had no significant effect on normoxic or hypoxic proliferation over that of the Rap1 inhibitor alone *(Figure 6.14)*. Epac has been demonstrated to activate Rap1 (Enserink et al., 2002), therefore these results suggest the anti-proliferative effect of the Epac agonist is independent of Rap1 as the Rap1 inhibitor can not preserve the proliferative capacity of hPASM cells when used with the Epac agonist. However, as the Rap1 inhibitor reduces proliferation significantly when used alone, this can not be assumed.

6.7.2 A second EPAC agonist, 8 -pMeOPT-2′-O-Me-cAMP

As a second novel EPAC agonist became available towards the end of this study, I decided to use this to confirm the anti-proliferative effect of the first EPAC agonist and to compare the potency of both. Biolog reported this new agonist to have a higher activity and membrane permeability compared with 8 -pCPT-2′-O-Me-cAMP. Anti-proliferative activities were compared in cells with serum re-introduced.

In normoxic and hypoxic cells, 8 -pCPT-2′-O-Me-cAMP had an IC_{50} of approximately 50μM. The IC_{50} for 8 -pMeOPT-2′-O-Me-cAMP was different to this. In normoxic cells, it had an IC_{50} of 1mM and in hypoxic cells, an IC_{50} of 500μM. Although both agonists elicit an anti-proliferative effect on both normoxic and hypoxic cells, the original agonist, 8 -pCPT-2′-O-Me-cAMP was far more potent at inhibiting proliferation in normoxic and hypoxic hPASMC *(Figure 6.15)*.

6.8 Discussion and Conclusions

Exposing vascular smooth muscle cells to hypoxia has been shown to increase their proliferative capacity as witnessed in the rat model of hypoxia induced pulmonary hypertension(Meyrick & Reid 1978, Wohrley et al., 1995) I have shown that human pulmonary artery smooth muscle cells are also useful as cellular model of pulmonary hypertension for proliferation studies as they double their serum-induced proliferation rate when exposed to 10% O_2 for seven days.

Previous studies have established that raising cAMP within smooth muscle cells acts in an anti-proliferative manner (reviewed in Bornfeldt & Krebs 1999) The adenylyl cyclase stimulator, forskolin has been shown to achieve this, in part by reducing serum stimulated cyclin D1 and cyclin A levels in vascular smooth muscle cells (Kronemann et al., 1999) Raising cAMP through PDE4 inhibition can also elicit an antiproliferative effect as shown in A172 glioma cells and human acute lymphoblastic leukaemia cells (Chen et al., 2002, Ogawa et al., 2001) In human aortic and pulmonary arterial smooth muscle cells, PDE3 and PDE4 inhibitors have previously been shown to attenuate proliferation (Johnson-Mills et al., 1998, Indolfi et al., 1997). These effects have not been investigated in hypoxia however.

Rolipram did not have a significant effect on hPASMC proliferation, which contrasts with previous studies demonstrating rolipram can significantly reduce the proliferation of smooth muscle cells (Pan et al., 1994). However, other studies suggest there is no decrease in proliferation (Souness et al., 1992; Osinski and Schror, 2000). Cilostamide however, did significantly reduce proliferation of hPASMC as observed previously (Tsuchikane et al., 1999) with a greater effect observed in normoxic cells than in hypoxic cells. This can perhaps be explained by the desensitisation of the cAMP pathway demonstrated to occur in hypoxia in chapter 4. Using both of these inhibitors together, produced a supra-additive effect as observed in previous studies on smooth muscle cells (Johnson-Mills et al., 1998; Pan et al., 1994).

Raising cAMP levels with the β_2-adrenoceptor agonist, isoproterenol, appeared to have no effect on cell proliferation in hypoxia or normoxia. However, an action of isoproterenol was uncovered when cells were pre-treated with the PDE4 inhibitor, rolipram. Prior work with Albuterol, a β_2-adrenoceptor agonist has shown (Stewart et al., 1999) it inhibits

thrombin-stimulated proliferation when used prior to the restriction point. This it seemingly does by reducing cyclin D1 levels. Such inhibition could, however, be alleviated by treating cells with either the ERK inhibitor, PD98059 or the proteasome inhibitor MG132 (Stewart et al., 1999). Here I showed that treating cells with isoproterenol and rolipram together produced a highly significant inhibition of proliferation in both normoxic and hypoxic cells, indicating that the cells either immediately exited the cell cycle or underwent apoptosis. This suggests the pathway induced by β_2-adrenoceptor stimulation is under great influence by PDE4. Indeed, it has been shown that PDE4 is recruited in a complex with β-Arrestin to the β_2-adrenoceptor upon stimulation of the receptor and plays a role in the phosphorylation of the receptor, one consequence of which is to regulate G_s to G_i switching in HEK 293 cells (Baillie et al., 2002, Perry et al., 2002).

There has been no indication that the role of PDE4 in mediating G-protein switching affects cell proliferation, however it is known that G-protein coupled receptors (GPCRs) play a role in cell growth. Mutations affecting $G\alpha_s$ are known to be present in different types of tumours (Chen & Iyengar 1994). It is also possible that PDE4 plays a role in the interaction of β-Arrestin 2 with the oncoprotein Mdm2. Mdm2 associates with β-Arrestin 2 when a GPCR is stimulated, interfering with it's self ubiquitination and thus p53 degradation, allowing an increase in p53 activity (Wang et al., 2003). p53 is a tumour suppression protein capable of inducing growth arrest and apoptosis. An increase in p53 might help to explain the results seen using both rolipram and isoproterenol together suggesting that PDE4 perhaps enhances the binding of β-Arrestin 2 to Mdm2, thus reducing p53 ubiquitination.

Similarly, forskolin had no effect on cell proliferation unless the non-selective PDE inhibitor, IBMX, was first used. In normoxic cells, this treatment unexpectedly increased the rate of DNA synthesis, whereas in hypoxic cells, this rate was reduced to 20% of the control hypoxic serum induced proliferation rate. Stimulation of cAMP with forskolin alone has previously been reported to result in an attenuation of serum induced proliferation in vascular smooth muscle cells (Kronemann et al., 1999). This was not observed with the hPASMC. In certain conditions however, stimulation of cAMP levels can result in increased DNA synthesis in neonatal but not adult pulmonary SMC(Guldemeester et al., 1998). The use of forskolin and IBMX together has also been shown to inhibit serum-induced proliferation previously in pancreatic cancer cells (Boucher et al., 2001).

EGF is a well known mitogen and in this study was shown to induce a significant increase in serum induced proliferation but, interestingly, not in serum free cells. This suggests that EGF requires an additional input, supplied by a serum factor, to exert its effects on these cells. This increase caused by EGF in serum-treated cells was unaffected by rolipram in normoxic cells but was reduced to control levels in hypoxic cells, indicating the effects of EGF and rolipram can negate one another. EGF acts on the Ras/Raf/MEK/ERK pathway to activate cell growth and proliferation, therefore these results suggest rolipram exerts an inhibitory effect on this pathway. Another growth factor, TGF-β_1 has been used in studies of activation of ERK and cell proliferation as it is an important regulator of cellular proliferation in the lung (Vicencio et al., 2002) and has been shown to be both a stimulator of cell growth and an inhibitor, depending on cell type. The results are, however, conflicting. In epithelial cells, TGF-β_1 has been reported to both induce a G1 block in the cell cycle with a corresponding inhibition of ERK (Howe et al., 1993) and also to activate ERK and promote cell proliferation (Hartsough & Mulder 1995). In hPASMC, TGF-β_1 had no effect on proliferation.

Src has been demonstrated to be essential in both PC12 cells and NIH 3T3 fibroblasts to witness forskolin-stimulated ERK activation (Klinger et al., 2002). It has also been demonstrated in both NIH3T3 cells and mouse embryonic fibroblasts that Src phosphorylation by PKA is required for cAMP inhibition of ERK and cell proliferation (Schmitt & Stork 2002). Using the Src inhibitor, PP2, in hPASMC significantly reduced hypoxic serum induced proliferation and also hypoxic proliferation in serum starved cells. There was no significant effect on normoxic cells under either condition. This suggests there is a pathway not mediated by the serum response factor that is altered in hypoxic cells and requires Src for the increased proliferation observed in hypoxia.

The anti-proliferative actions of cAMP on cell proliferation have routinely been attributed to the cAMP-dependant kinase, PKA. PKA has been demonstrated to increase the levels of p27Kip1 and decrease the levels of cyclins D1 and D3 (L'Allemain et al., 1997, Kronemann et al., 1999, Stewart et al., 1999, Van Oirschot et al., 2001). This results in a G1 block in the cell cycle. As mentioned previously, PKA can also exert an effect upon ERK activation, which can lead to either cell proliferation or differentiation or apoptosis (Schmitt & Stork 2002). PKA is a heterodimer consisting of two regulatory and two catalytic subunits. Activation of PKA requires binding of cAMP to the regulatory subunits which releases the catalytic subunits. Opposing functions for the two regulatory isoforms of PKA in the cell cycle have been observed (reviewed in Cho-Chung et al., 1995). Indeed,

the balance of the RI/RII ratio is critical as alterations can lead to tumour formation (Stergiopoulos & Stratakis 2003). PKA RI is a little overexpressed in normal cells upon stimulation of proliferation, yet is constitutively overexpressed in cancer cells and is associated with a poor prognosis in different human cancers (Miller et al., 1993, McDaid et al., 1999). Inhibition of PKA results in a significant reduction of cellular proliferation in hypoxic hPASMCs only, whether serum starved or not. Activation of PKA is also capable of reducing cell proliferation, although only significantly in hypoxic serum induced proliferation. This effect appears to be predominantly due to the PKA RII subunit as the PKA RII agonist elicits a greater effect than the RI agonist. These effects are only significant in hypoxia, suggesting an alteration in PKA signalling in hypoxia. This is confirmed by the significant reduction of serum induced proliferation by the general PKA agonist, 6-BnZ, in hypoxia, but not in normoxic cells. As stimulation of total PKA has no effect on normoxic serum induced proliferation, this suggests an alteration of PKA RI/RII ratio in hypoxic cells. When the specific subunit agonists are used together however, it does not mirror the effect of a general PKA agonist. They appear to synergise and reduce serum induced proliferation more dramatically. PKA RI inhibition has no significant effect on hPASMC proliferation, although it does appear to cause a slight increase in normoxic serum starved cells. RII inhibition results in a highly significant reduction of cell proliferation in both normoxic and hypoxic cells. These results suggest that PKA RII is essential for normal cell proliferation and is involved in the hypoxia-induced increase of proliferation. It should be noted that the PKA RI subunit was also demonstrated to induce a small, albeit not statistically significant, increase in normoxic proliferation in serum starved cells. This suggests the PKA RI subunit can act in a growth inducing manner, which is regulated by the PKA RII subunit and, vice versa, the PKA RII subunit can act in an inhibitory manner which is regulated by PKA RI. This again is indicative of an alteration in the RI to RII ratio in hypoxic cells. In chapter 3 however, western blotting of the PKA RIα, RIIα and RIIβ subunits did not show any change in expression. PKA RIβ expression was not investigated.

PKA is not the only effector of cAMP signalling in cells. Epac is another target for cAMP signalling in mammalian cells, although its effects on proliferation have not yet been investigated. Using the cAMP analog, CPT-cAMP, it was clear this activated something different to the cAMP analog, 8-bromo-cAMP as it produced a more potent inhibition of proliferation. Indeed, it was later discovered that CPT-cAMP has a higher affinity for Epac than PKA. Using the novel Epac agonists, 8 -pCPT-2′-O-Me-cAMP and 8 -pMeOPT-2′-O-Me-cAMP, it was revealed for the first time that Epac is capable of potently inhibiting

proliferation. Epac appears to require the serum response to have an impact as this inhibition only occurs in serum induced proliferation. This is similar to the effect of the Src inhibitor PP2. Thus, this suggests that Epac affects proliferation in a manner similar to that of Src inhibition. It is known that both Epac and Src activate the GTPase Rap1. Activation of Rap1 has been associated with both the cAMP mediated activation of ERK1 and thus cell proliferation (Ribeiro-Neto et al., 2002) and the cAMP mediated inhibition of ERK and cell proliferation (Schmitt & Stork 2001). The Epac agonist used in this study has previously been shown to have no direct effect upon ERK activity in NIH3T3 cells stably transfected with Epac1 and CHO cells (Enserink et al., 2002). However, it is possible that Epac could act indirectly on ERK activity. The divergent roles of Rap1 in cAMP mediated cell proliferation are indicative of numerous stimuli acting on Rap1. As Epac is already known to activate Rap1, it seems possible that the levels of activation of Epac could regulate the PKA directed activation of Rap1. Indeed, increasing cAMP levels in the cell through PDE inhibition, raising PKA activation by addition of a cAMP analog or direct PKA stimulation, or even specific PKA regulatory subunit activation, showed only slight potentiation or alleviation of this effect. This is suggestive of a cAMP threshold level at which Epac is activated and elicits its effect rather than a concentration dependant effect. It also appears that the level of PKA cannot influence the effect of Epac once activated. Thus, Epac perhaps acts as an 'off' switch for PKA mediated signalling through Rap1.

In this chapter, I have presented results that suggest a role for PKA RI:RII imbalance in hypoxia-induced proliferation and demonstrate a new role for Epac in cell proliferation.

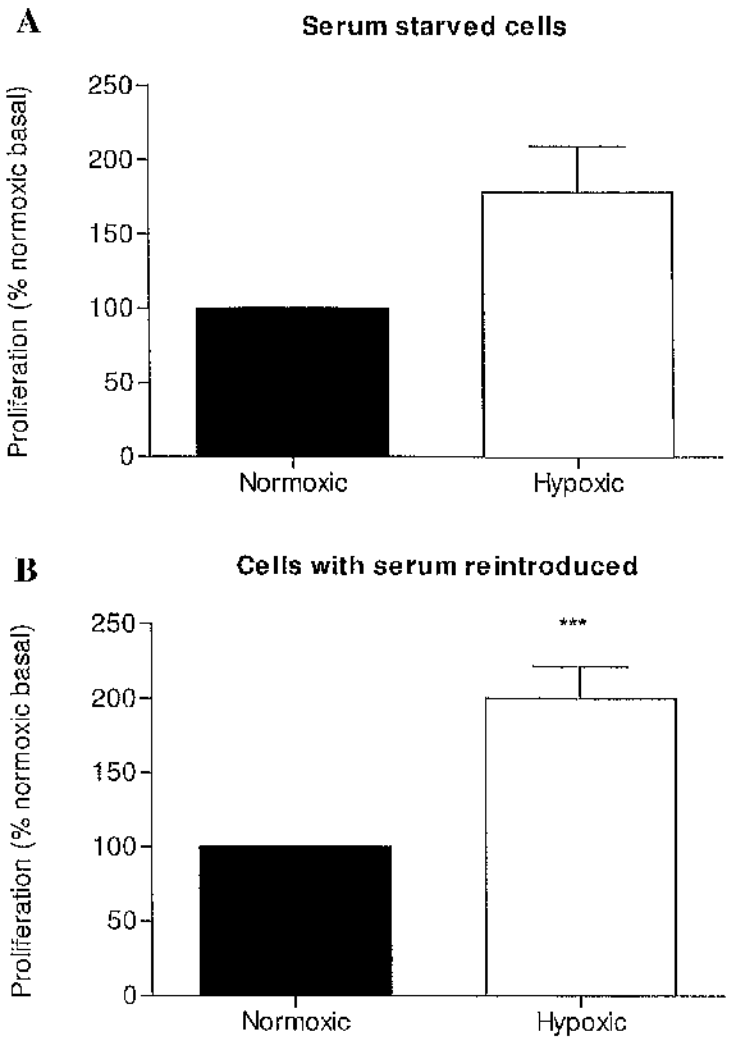


Figure 6.1 Effect of chronic hypoxia on proliferation in hPASMC.

Normoxic and hypoxic cells were cultured in 96-well plates for five days prior to serum starvation overnight. One set of cells was maintained in serum free medium, *(Panel A)*, and another had serum re-introduced *(Panel B)*. After twenty hours, [^{3}H]-thymidine was added to all plates for a further four hours before being harvested as described in section 2.2 to measure incorporation rates as a measure of DNA synthesis.

Results shown are means ± S.E. of 4 independent experiments expressed as a percentage of the normoxic basal proliferation rate. Significance is denoted by *** ($p<0.001$).

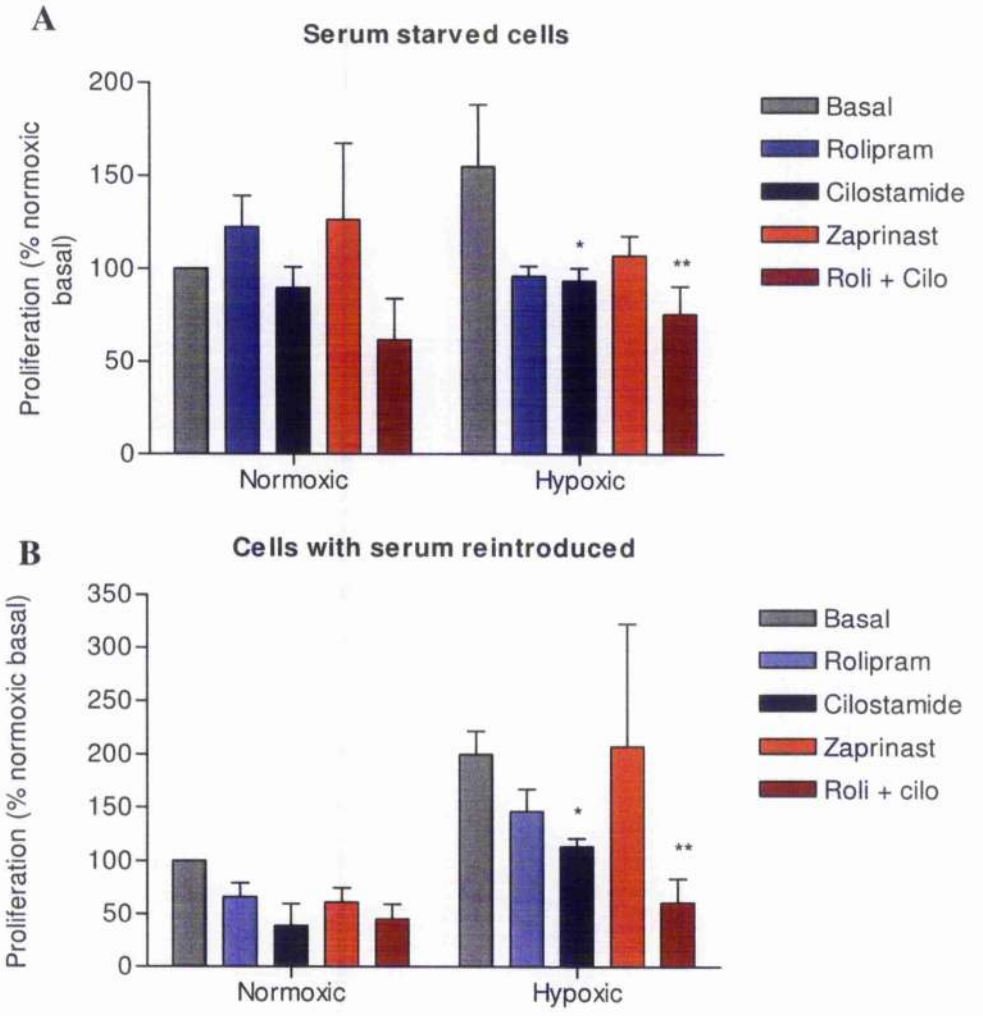


Figure 6.2 Effect of PDE inhibitors on proliferation in hPASMC.

Cells were treated as in figure 6.1 and all PDE inhibitors were added when the serum free medium was replenished, *(Panel A)*, or serum was re-introduced to the cells *(Panel B)*. After twenty hours, [^{3}H]-thymidine was added to all plates for a further four hours before being harvested as described in section 2.2 to measure incorporation rates as a measure of DNA synthesis.

Results shown are means ± S.E. of 3 independent experiments expressed as a percentage of the normoxic basal proliferation rate. Significance is denoted by *, ** ($p<0.05$, $p<0.01$ respectively).

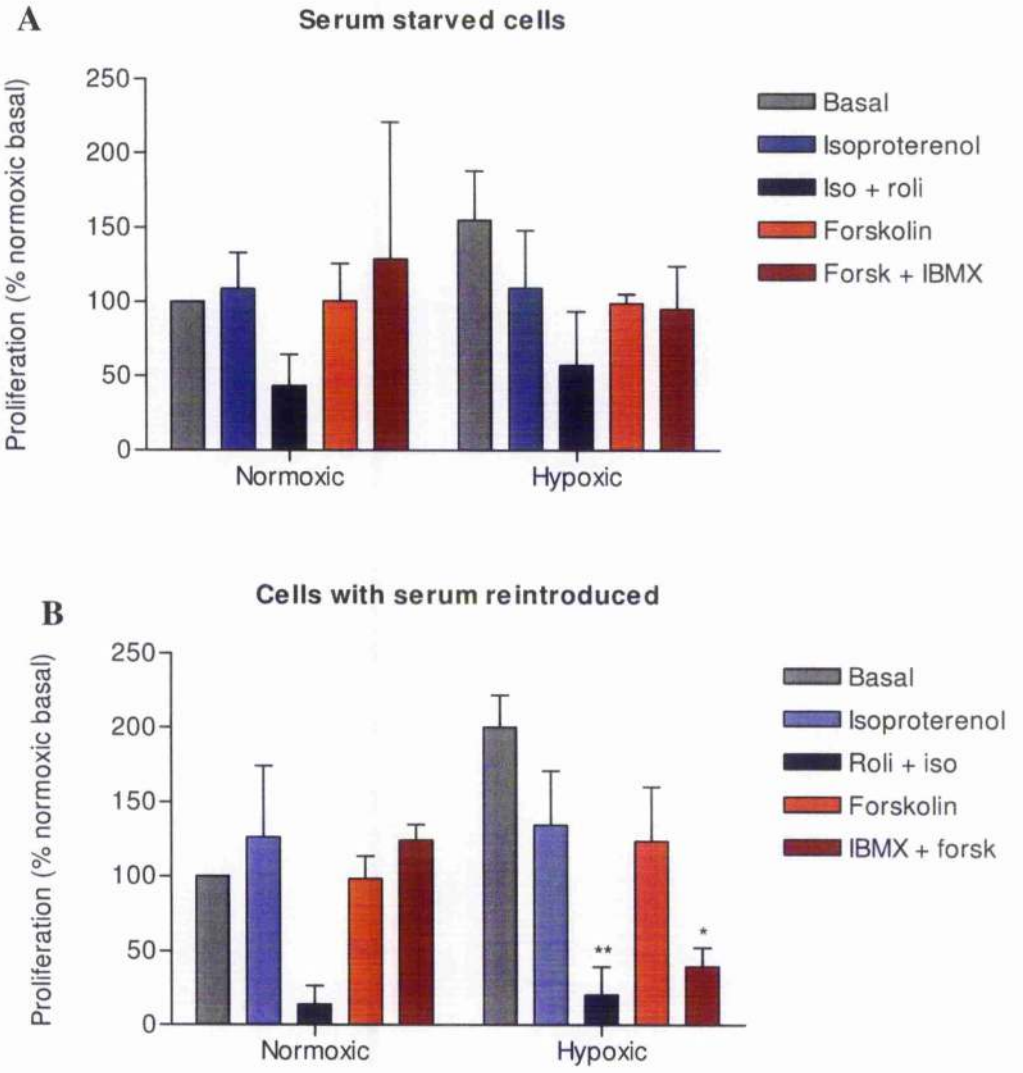


Figure 6.3 Effect of cAMP effectors on proliferation in hPASMC.

Cells were treated as in figure 6.1 and all additions were made when the serum free medium was replenished, *(Panel A)*, or serum was re-introduced to the cells *(Panel B)*. After twenty hours, [^{3}H]-thymidine was added to all plates for a further four hours before being harvested as described in section 2.2 to measure incorporation rates as a measure of DNA synthesis.

Results shown are means ± S.E. of 3 independent experiments expressed as a percentage of the normoxic basal proliferation rate. Significance is denoted by * , ** ($p<0.05$, $p<0.01$ respectively).

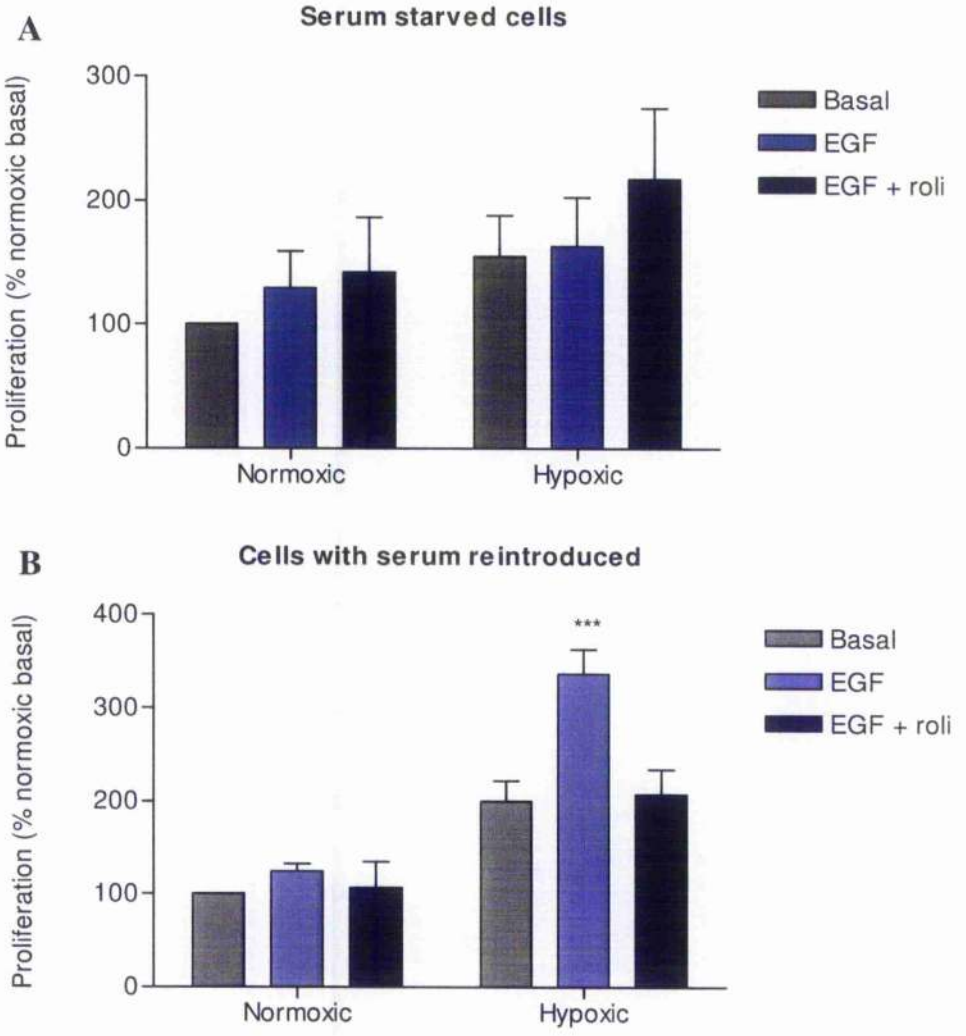


Figure 6.4 Effect of EGF on proliferation in hPASMC.

Cells were treated as in figure 6.1. 50pg/µl medium of EGF was added when the serum free medium was replenished, *(Panel A)*, or serum was re-introduced to the cells *(Panel B)*. After twenty hours, [^{3}H]-thymidine was added to all plates for a further four hours before being harvested as described in section 2.2 to measure incorporation rates as a measure of DNA synthesis.

Results shown are means ± S.E. of 3 independent experiments expressed as a percentage of the normoxic basal proliferation rate. Significance is denoted by *** ($p<0.001$).

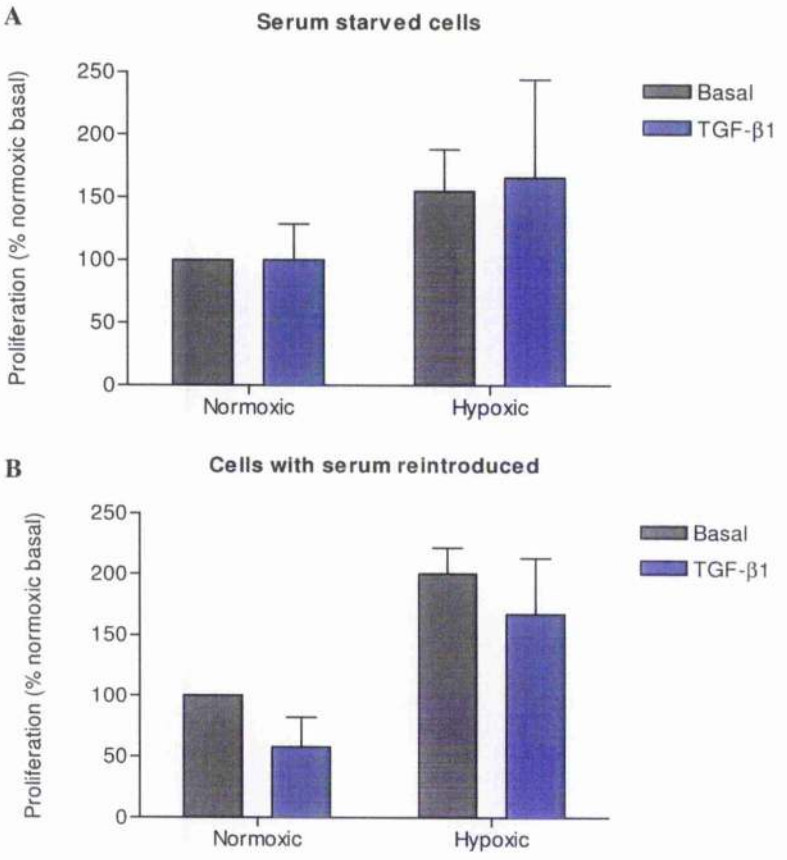


Figure 6.5 Effect of TGF-β_1 on proliferation in hPASMC.

Cells were treated as in figure 6.1. TGF-β_1 was added when the serum free medium was replenished, *(Panel A)*, or serum was re-introduced to the cells *(Panel B)*. After twenty hours, [^{3}H]-thymidine was added to all plates for a further four hours before being harvested as described in section 2.2 to measure incorporation rates as a measure of DNA synthesis.

Results shown are means ± S.E. of 3 independent experiments expressed as a percentage of the normoxic basal proliferation rate.

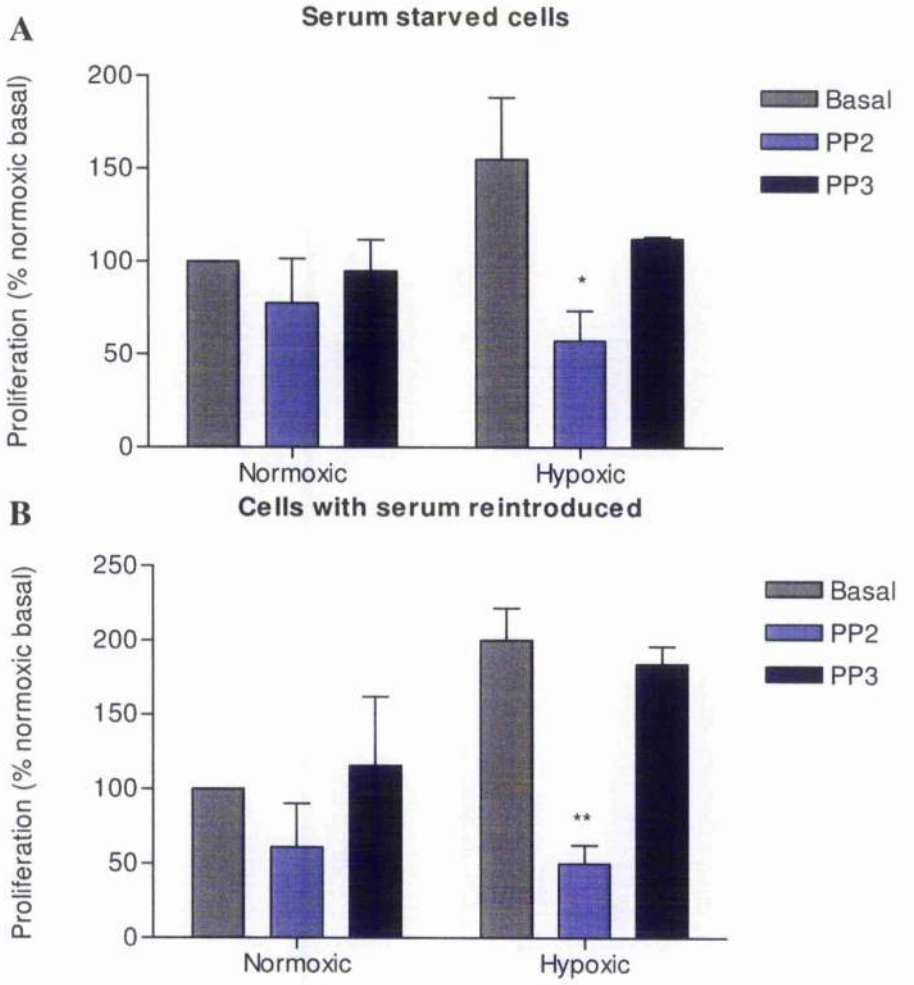


Figure 6.6 Effect of Src inhibitor on proliferation in hPASMC.

Cells were treated as in figure 6.1. PP2 and PP3 were added when the serum free medium was replenished, *(Panel A)*, or serum was re-introduced to the cells *(Panel B)*. After twenty hours, [^{3}H]-thymidine was added to all plates for a further four hours before being harvested as described in section 2.2 to measure incorporation rates as a measure of DNA synthesis.

Results shown are means ± S.E. of 4 independent experiments expressed as a percentage of the normoxic basal proliferation rate. Significance is denoted by *** ($p<0.001$).

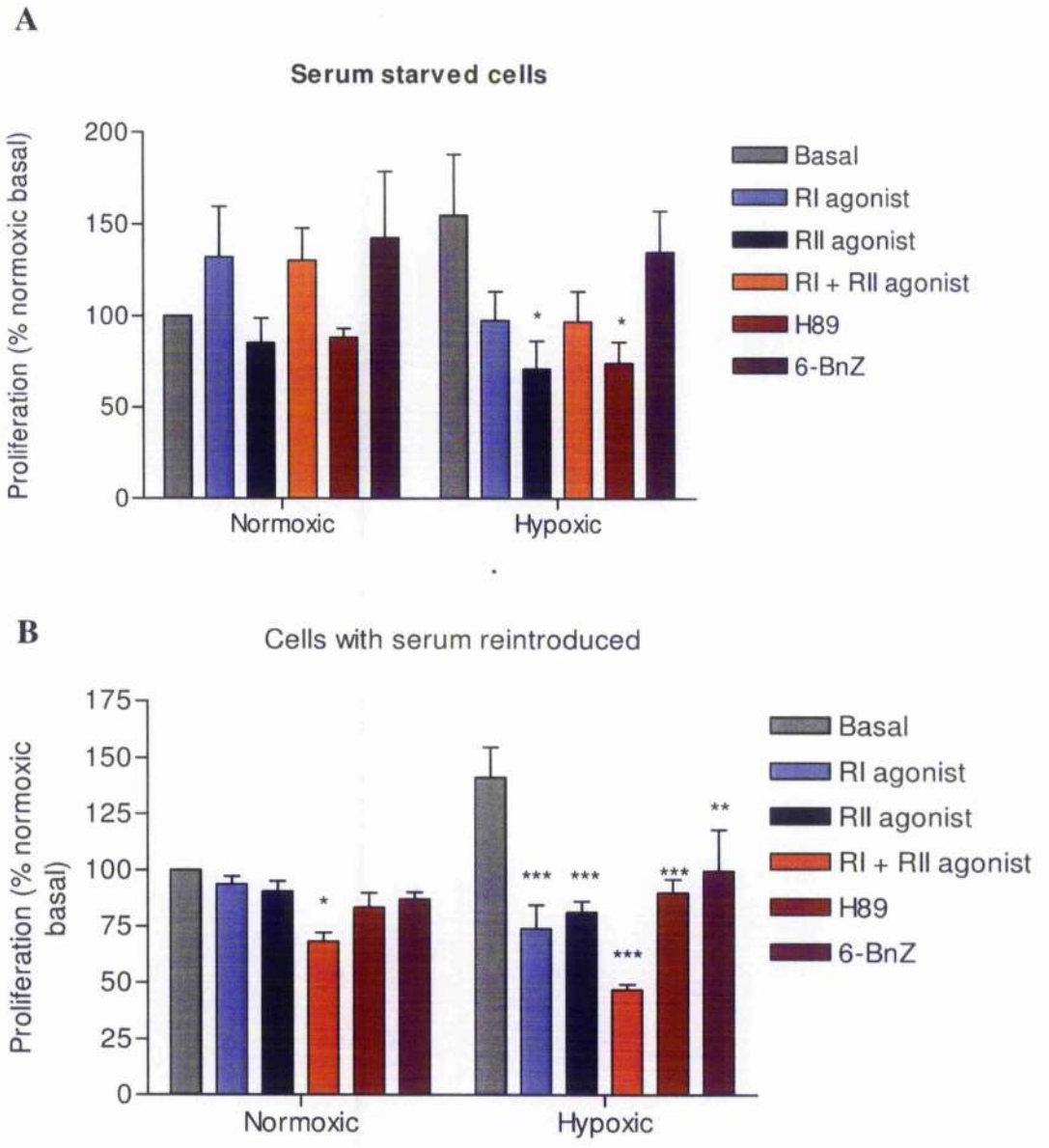


Figure 6.7 Effect of PKA agonists and inhibitor on proliferation in hPASMC.

As in figure 6.1, agonists and inhibitors were added when the serum free medium was replenished, *(Panel A)*, or serum was re-introduced to the cells *(Panel B)*. After twenty hours, [^{3}H]-thymidine was added to all plates for a further four hours before being harvested as described in section 2.2 to measure incorporation rates as a measure of DNA synthesis.

Results shown are means ± S.E. of 3 independent experiments expressed as a percentage of the normoxic basal proliferation rate. Significance is denoted by *** ($p<0.001$).

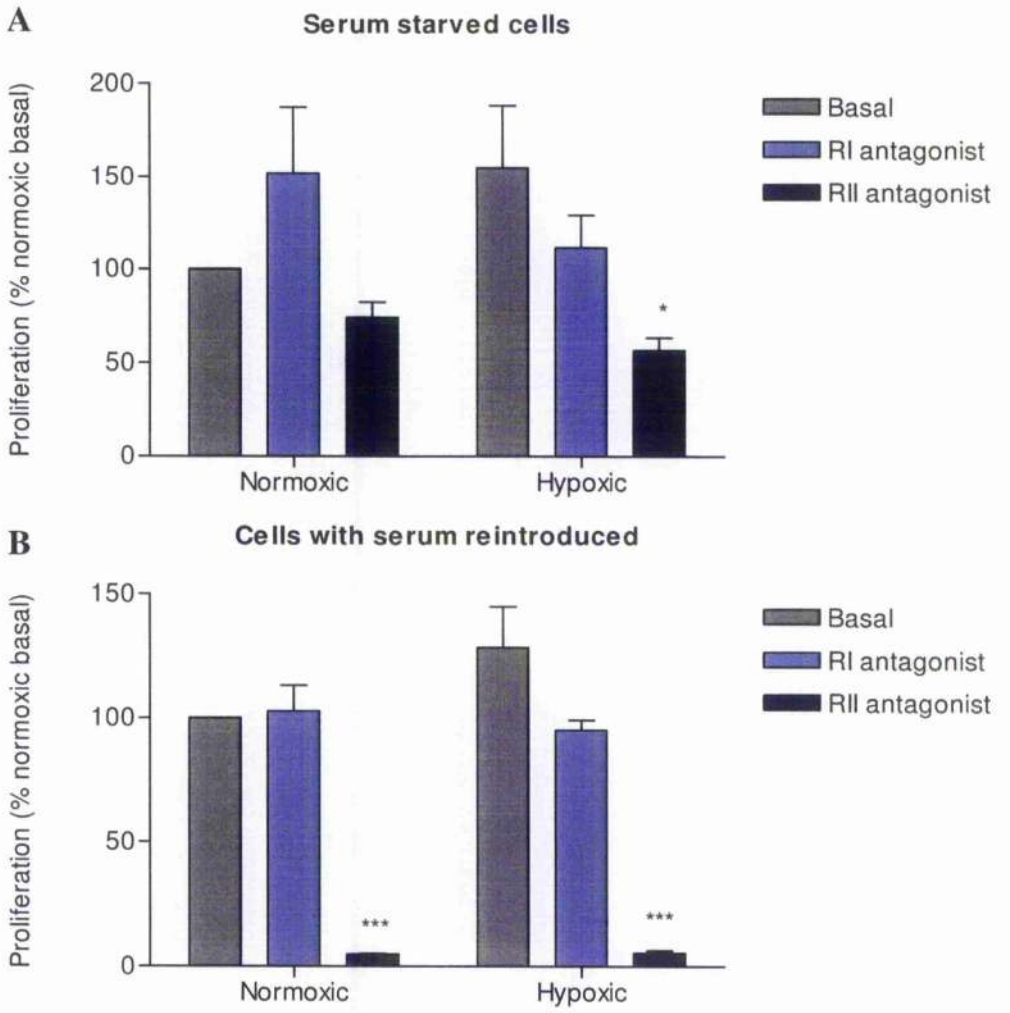


Figure 6.8 Effect of PKA antagonists on proliferation in hPASMC.

Cells were treated as in figure 6.1. Antagonists were added when the serum free medium was replenished, *(Panel A)*, or serum was re-introduced to the cells *(Panel B)*. After twenty hours, [^{3}H]-thymidine was added to all plates for a further four hours before being harvested as described in section 2.2 to measure incorporation rates as a measure of DNA synthesis.

Results shown are means ± S.E. of 3 independent experiments expressed as a percentage of the normoxic basal proliferation rate. Significance is denoted by *, *** ($p<0.05$, $p<0.001$ respectively).

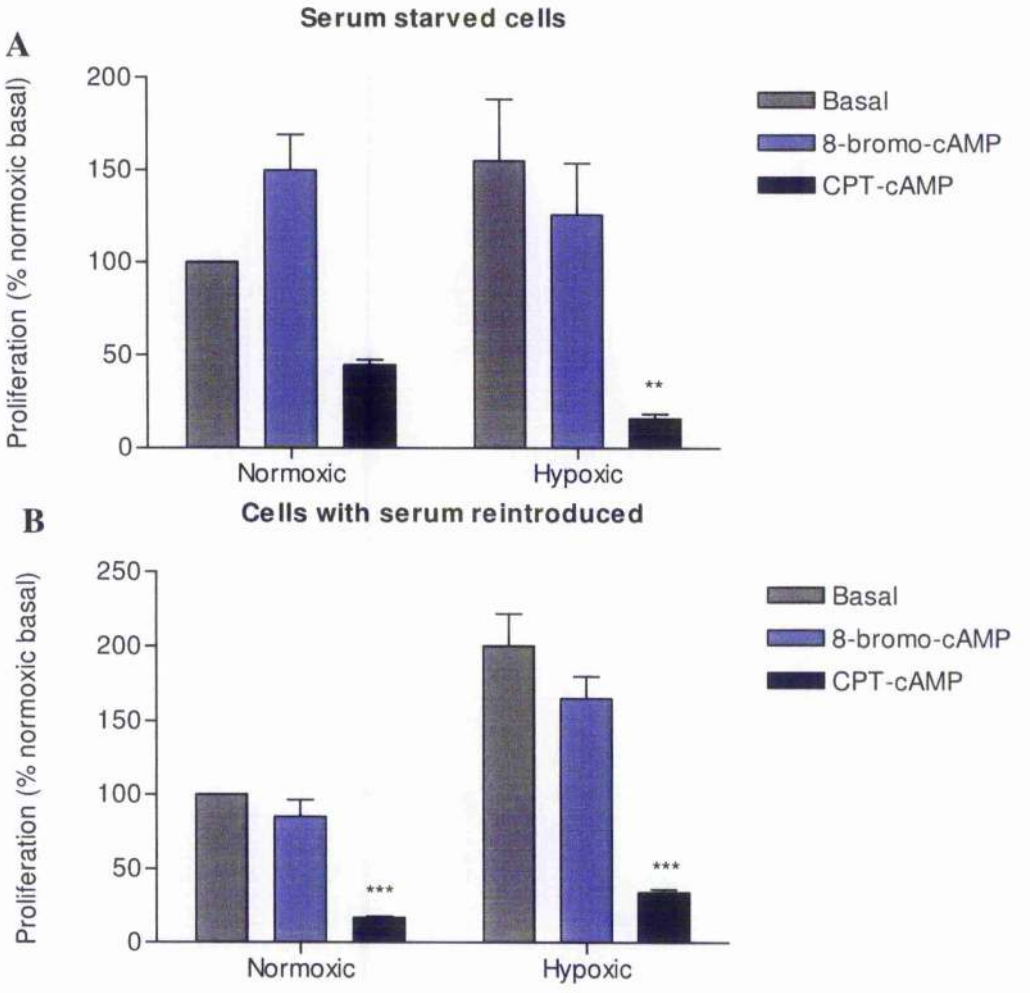


Figure 6.9 Effect of cAMP analogs on proliferation in hPASMC

Cells were treated as in figure 6.1. 8-bromo-cAMP or CPT-cAMP were added when the serum free medium was replenished, *(Panel A)*, or serum was re-introduced to the cells *(Panel B)*. After twenty hours, [^{3}H]-thymidine was added to all plates for a further four hours before being harvested as described in section 2.2 to measure incorporation rates as a measure of DNA synthesis.

Results shown are means ± S.E. of 3 independent experiments expressed as a percentage of the normoxic basal proliferation rate. Significance is denoted by **, *** ($p<0.01$, $p<0.001$ respectively).

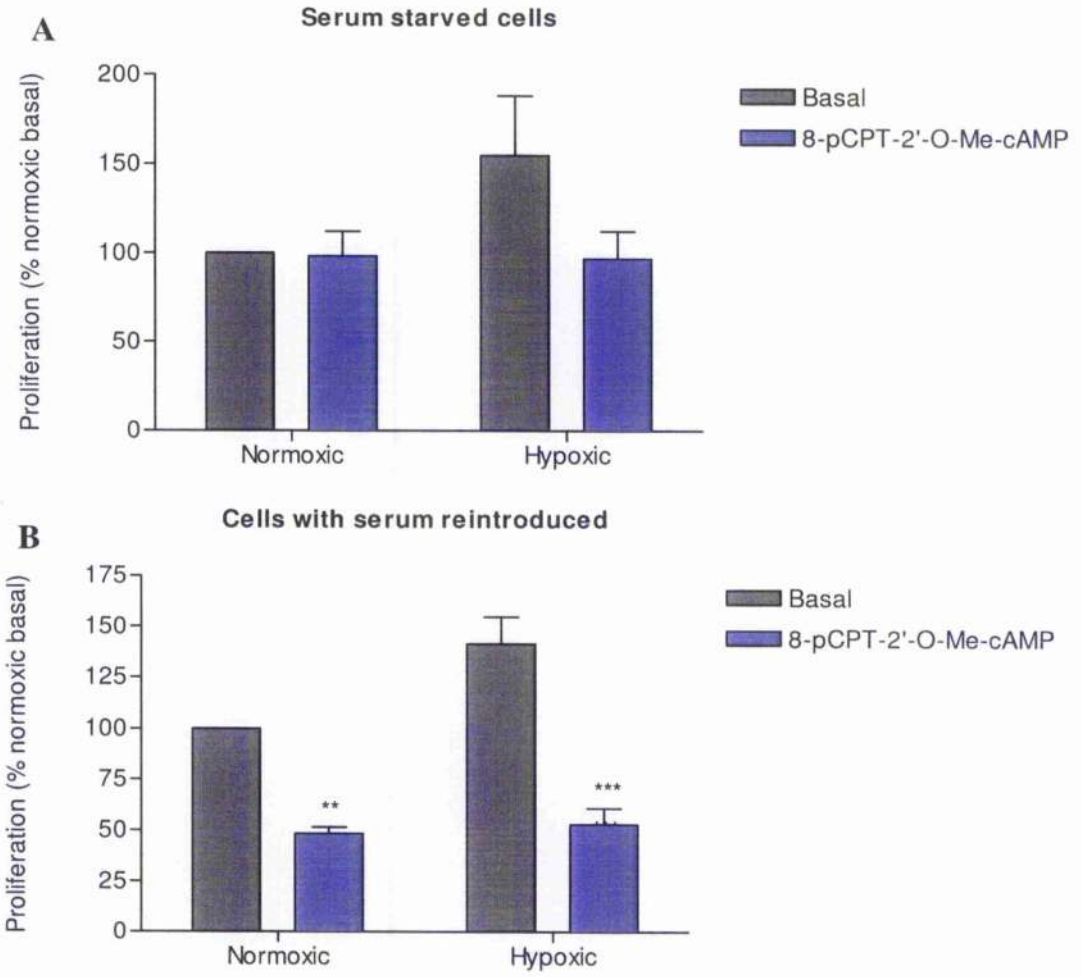


Figure 6.10 Effect of 8 -pCPT-2′-O-Me-cAMP on proliferation in hPASMC.

Cells were treated as in figure 6.1. 8 -pCPT-2′-O-Me-cAMP was added when the serum free medium was replenished, *(Panel A)*, or serum was re-introduced to the cells *(Panel B)* After twenty hours, [^{3}H]-thymidine was added to all plates for a further four hours before being harvested as described in section 2.2 to measure incorporation rates as a measure of DNA synthesis.

Results shown are means ± S.E. of 3 independent experiments expressed as a percentage of the normoxic basal proliferation rate. Significance is denoted by **, *** ($p<0.01$, $p<0.001$ respectively).

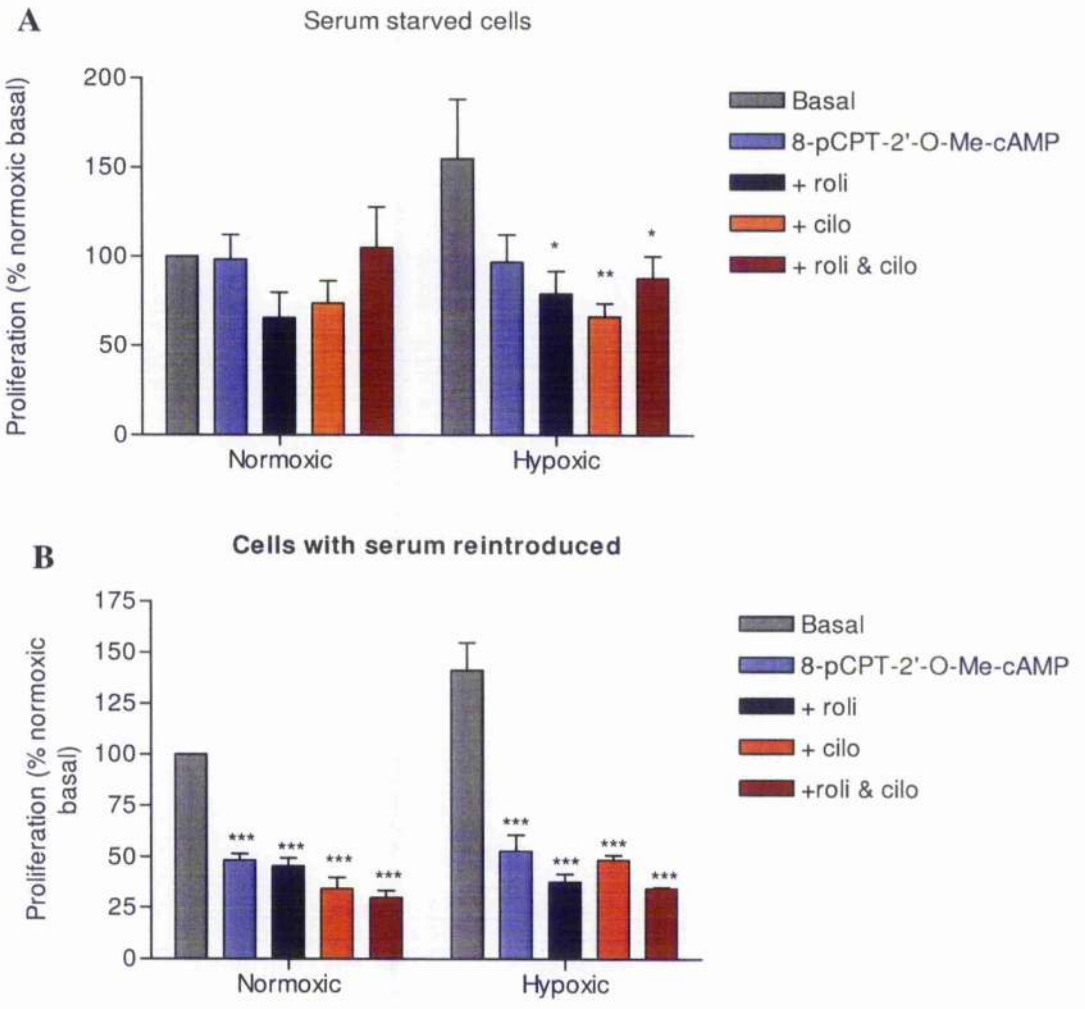


Figure 6.11 Effect of PDE inhibitors in combination with 8 -pCPT-2′-O-Me-cAMP on proliferation in hPASMC.

As in figure 6.1, 8 -pCPT-2′-O-Me-cAMP plus inhibitors were added when the serum free medium was replenished, *(Panel A)*, or serum was re-introduced to the cells *(Panel B)*. After twenty hours, [^{3}H]-thymidine was added to all plates for a further four hours before being harvested as described in section 2.2 to measure incorporation rates as a measure of DNA synthesis.

Results shown are means ± S.E. of 3 independent experiments expressed as a percentage of the normoxic basal proliferation rate. Significance is denoted by *, **, *** ($p<0.05$, $p<0.01$, $p<0.001$ respectively).

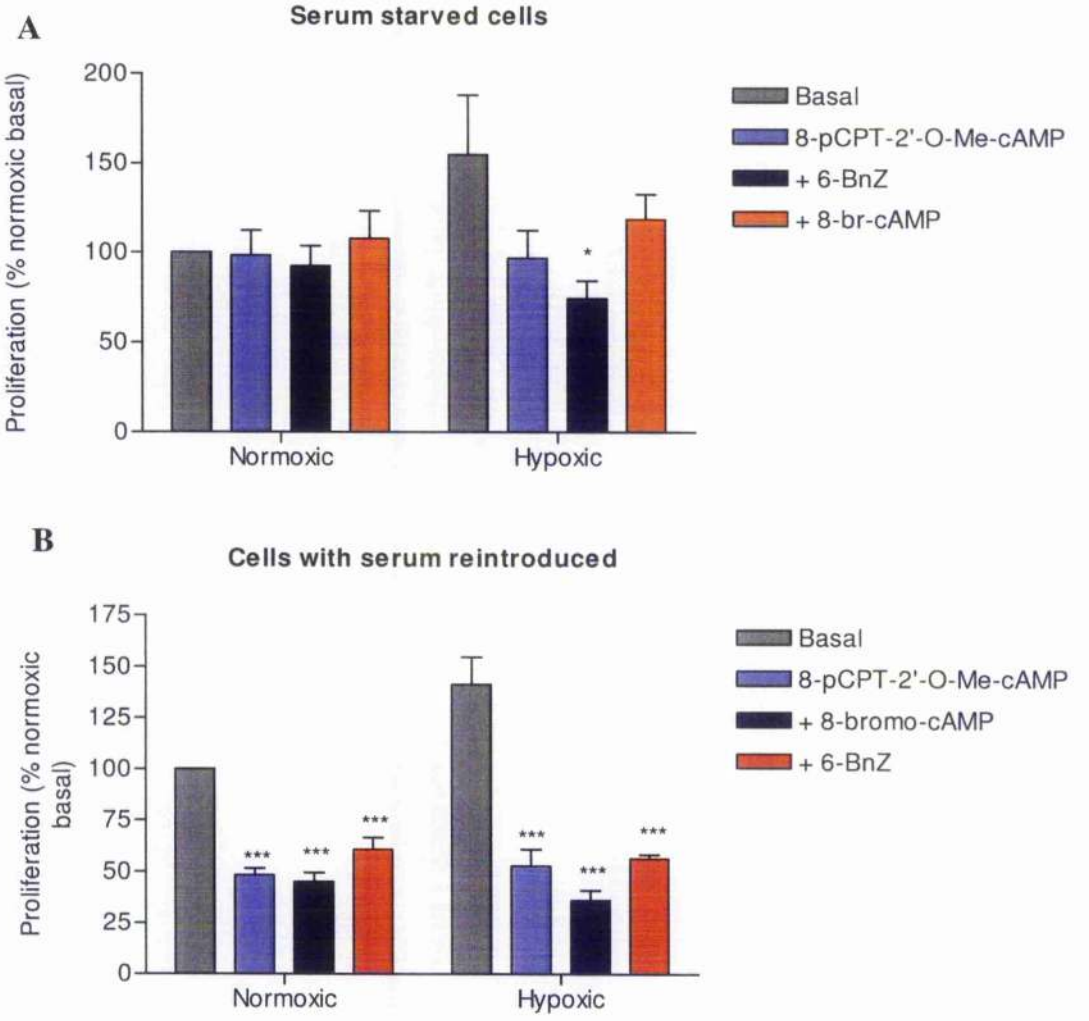


Figure 6.12 Effect of cAMP effectors in combination with 8 -pCPT-2′-O-Me-cAMP on proliferation in hPASMC.

As in figure 6.1, 8 -pCPT-2′-O-Me-cAMP and other additions were made when the serum free medium was replenished, *(Panel A)*, or serum was re-introduced to the cells *(Panel B)*. After twenty hours, [^{3}H]-thymidine was added to all plates for a further four hours before being harvested as described in section 2.2 to measure incorporation rates as a measure of DNA synthesis.

Results shown are means ± S.E. of 3 independent experiments expressed as a percentage of the normoxic basal proliferation rate. Significance is denoted by *, *** ($p<0.05$, $p<0.001$ respectively).

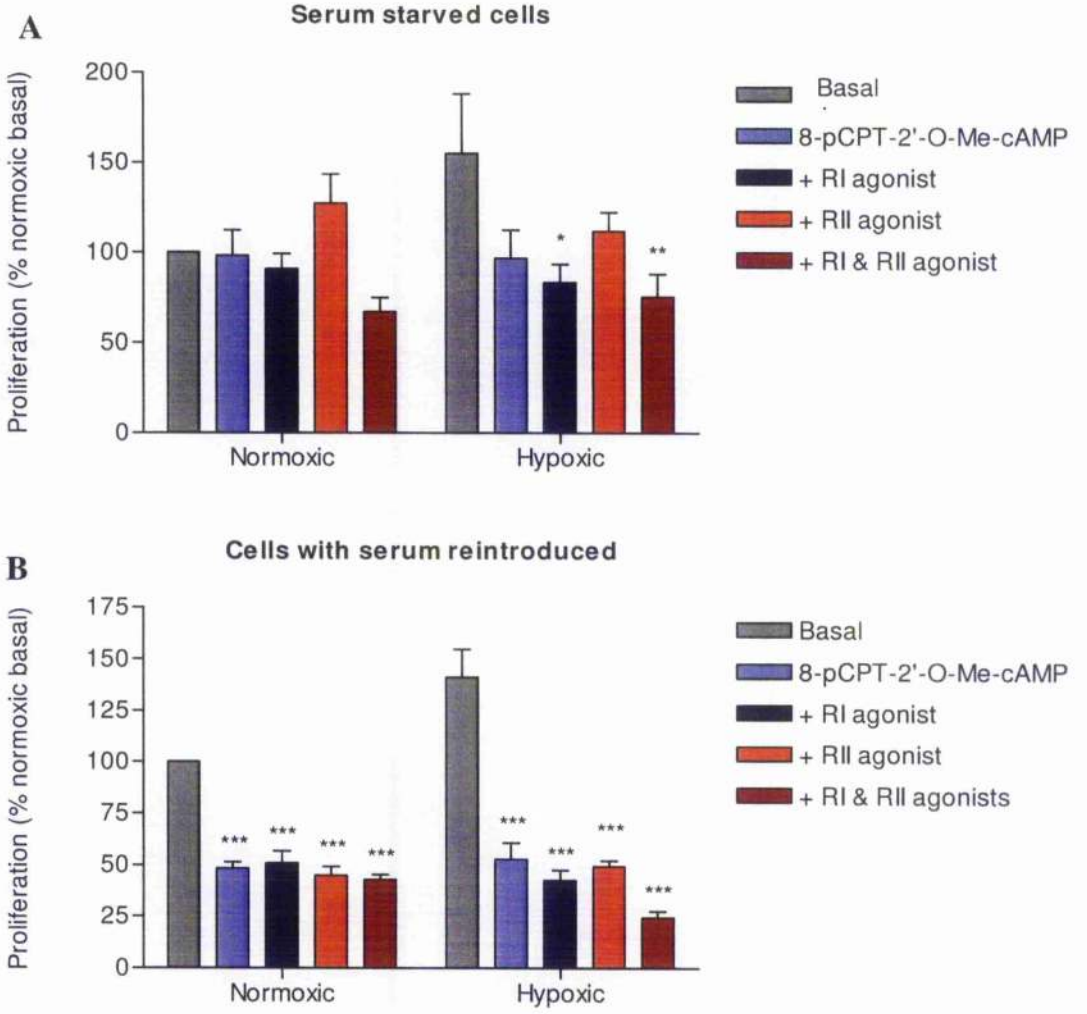


Figure 6.13 Effect of PKA agonists in combination with 8 -pCPT-2′-O-Me-cAMP on proliferation in hPASMC.

As in figure 6.1, 8 -pCPT-2′-O-Me-cAMP and agonists were added when the serum free medium was replenished, *(Panel A)*, or serum was re-introduced to the cells *(Panel B)*. After twenty hours, [^{3}H]-thymidine was added to all plates for a further four hours before being harvested as described in section 2.2 to measure incorporation rates as a measure of DNA synthesis.

Results shown are means ± S.E. of 3 independent experiments expressed as a percentage of the normoxic basal proliferation rate. Significance is denoted by *, **, *** ($p<0.05$, $P<0.01$, $p<0.001$ respectively).

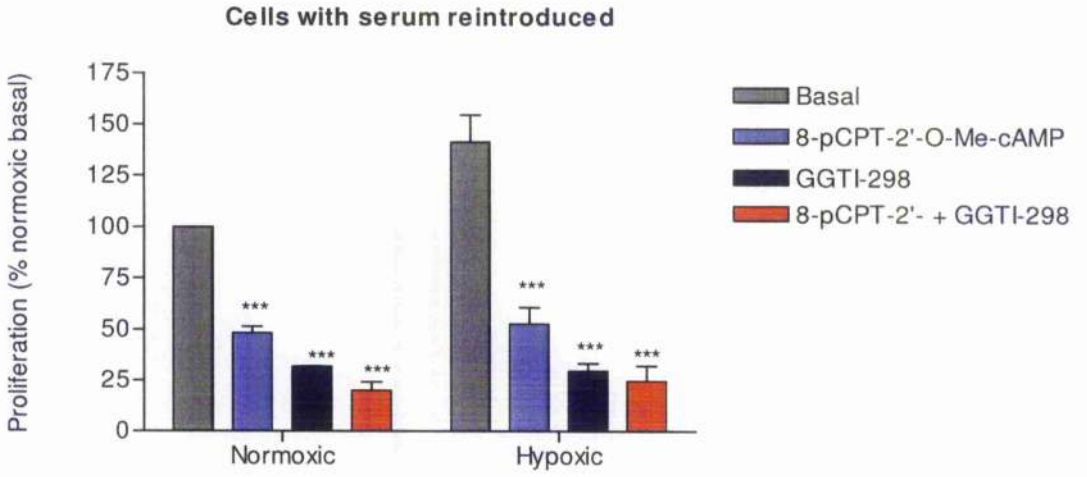


Figure 6.14 Effect of Rap1 inhibitor on proliferation in hPASMC.

Cells were treated as in figure 6.1. GGTI-298 and the EPAC agonists were added when the serum was re-introduced to the cells. After twenty hours, [^{3}H]-Thymidine was added to all plates for a further four hours before being harvested as described in section 2.2 to measure incorporation rates as a measure of DNA synthesis.

Results shown are means ± S.E. of 3 independent experiments expressed as a percentage of the normoxic basal proliferation rate. Significance is denoted by *** ($p<0.001$).

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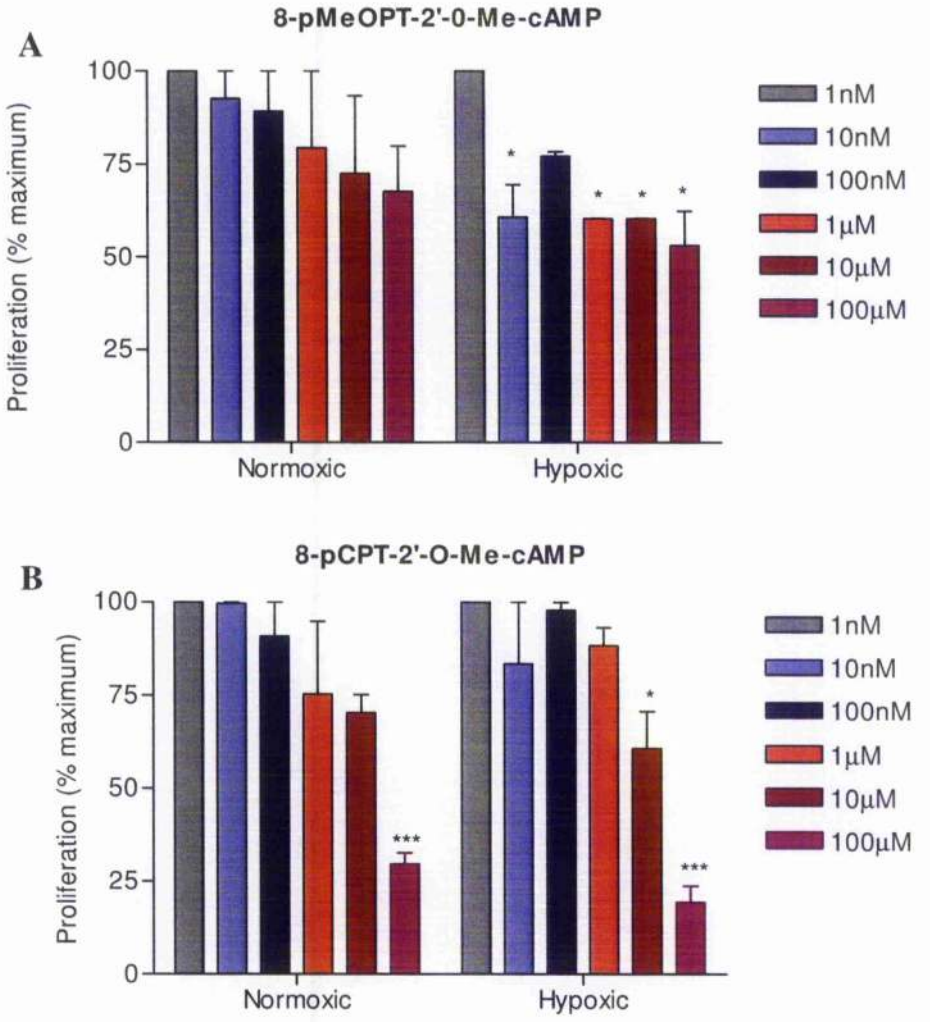

Figure 6.15 Inhibition of proliferation in response to EPAC agonists.

Cells were treated as in figure 6.1. 8-pCPT-2′-O-Me-cAMP, *(Panel A)*, or 8-pMeOPT-2′-O-Me-cAMP, *(Panel B)*, was added at varying concentrations when serum was re-introduced to the cells. After twenty hours, [^{3}H]-thymidine was added to all plates for a further four hours before being harvested as described in section 2.2 to measure incorporation rates as a measure of DNA synthesis.

Results shown are means +/- S.E. of 3 independent experiments as percentage inhibition compared to 1nM treatments which showed no effect on proliferation. Significance is denoted by *, *** ($p<0.05$, $p<0.001$ respectively).

Drug	Final Concentration
Rolipram	10μM
Cilostamide	10μM
Zaprinast	10μM
IBMX	10μM
Isoproterenol	10μM
Forskolin	10μM
EGF	50pg/μl
TGF $-\beta_1$	10ng/μl
PP2	10μM
PP3	10μM
PKA RI agonists; **8-PIP-cAMP** **8-HA-cAMP**	 10μM 5μM
PKA RII agonists; **8-PIP-cAMP** **8-MBC-cAMP**	 10μM 5μM
H89	10μM
PKA RI antagonist; **Rp-8-Cl-cAMPS**	 10μM
PKA RII antagonist; **Rp-8-CPT-cAMPS**	 10μM
6-BnZ-cAMP	10μM
8-bromo-cAMP	100μM
CPT-cAMP	10μM
8 -pCPT-2′-O-Me-cAMP	10μM
GGTI-298	10μM
8-pMeOPT-2′-O-Me-cAMP	10μM

Table 6.1 Drugs used and their final concentration in the proliferation assays

This table lists the compounds used throughout this chapter and the concentration used at in the proliferation assays.

Chapter 7

General Discussion and Future Directions

The cyclic nucleotides cAMP and cGMP have well characterised functions including roles in cell proliferation, differentiation, inflammation and maintenance of vascular tone (Murray 1990; Koyama et al., 2001; Pellegrino & Wang 1997; Dousa 1999; Torphy et al., 1999; Wong & Koh 2000; Essayan 2001). The cell tailors its response to these second messengers through a complex interplay of the synthesis and degradation mechanisms available for cAMP and cGMP. For example, there are nine forms of adenylyl cyclase (AC; Antoni et al., 2000, Hanoune & Defer 2001) capable of regulating the synthesis of cAMP and eleven families of phosphodiesterases, all able to efficiently degrade cAMP or cGMP (Francis et al., 1999; Dousa 1999; Soderling & Beavo 2000; Yuasa et al., 2000; Conti 2000; Mehats et al., 2002; Maurice et al., 2003).To add to this regulation, it is also well documented that compartmentalised responses to cAMP exist within a cell due to localised cAMP production, anchored cAMP substrates and targeted PDE isoforms. The net result of this is a highly specialised form of signalling, enabling the cell to tailor the response to cAMP and act in the desired manner.

The superfamily of phosphodiesterases is well established as a crucial component in the regulation of cAMP signalling. Differential tissue distribution and regulatory properties of PDEs add a further complexity to cAMP signalling, along with the generation of multiple splice variants. In addition, the expression of PDE isoforms has been demonstrated to be altered in response to hypoxia (Murray et al., 2002) and COPD (Barber et al., 2004). For example, in the pulmonary arteries of the chronic hypoxic rat model of pulmonary arterial hypertension (PAH), increased levels of the PDE3 and PDE5 expression and activity (Murray et al., 2002) have been demonstrated to be responsible for the reduction in cAMP and cGMP levels witnessed after 14 days exposure to 10% O_2 (MacLean et al., 1997). This alteration in PDE expression was also observed in the cellular model of PAH, hypoxic human pulmonary artery smooth muscle cells (Murray et al., 2002). Thus, the reduced levels of cyclic nucleotides have been attributed to an increased hydrolysis induced by hypoxia.

The PDE4 family of enzymes represent a major part of the cAMP hydrolysing activity within vascular smooth muscle cells (Palmer et al., 1998) but their activity and expression has not previously been investigated in hypoxia. PDE4 inhibitors are considered therapeutic targets for cardiovascular diseases such as COPD and asthma (Spina et al., 1998; Landells et al., 2001; Spina 2003). PDE4 inhibitors have also been considered for

use in treating PAH, as they are capable of synergising with PDE3 inhibitors to produce an enhanced relaxation of arteries and potentiated inhibition of smooth muscle cell proliferation (Pan et al., 1994; Palmer et al., 1998; Chen et al 2002, Ogawa et al 2002). I thus set out to determine if PDE4 has a role in the altered signalling apparent in models of PAH.

The main finding of the work presented here demonstrates that specific PDE4 isoforms are indeed affected by hypoxia. Increased PDE4 expression was detectable in hypoxic hPASMC after 7 days of hypoxic treatment, although there was no discernable affect on overall PDE4 activity. Indeed, it was also discovered that, in contrast with previous studies, cAMP levels are elevated following 7 days hypoxia treatment in hPASMC. Taken together, it was concluded from these results that the response to chronic hypoxia is a gradual one, with an initial increase in cAMP in an attempt to restore normal signalling, but instead causing the increased expression of the PDE3A isoform (Murray et al., 2002) and cAMP-inducible PDE4D1, PDE4D2, PDE4D5 isoforms. The elevated levels of cAMP-PDE isoforms possibly then leads to the marked reduction in cAMP and the desensitisation of further cAMP stimulation as observed previously (MacLean et al., 1997; Wagner et al., 1997). The elevated levels of PDE4B2 could also be due to the increased cAMP as an increase in PDE4B2 has been observed in human myometrial cells directly in response to cAMP raising agents (Mehats et al., 1999). The increased PDE4A10 and PDE4A11 expression highlights the importance of uncovering the regulatory properties and specific roles of these isoforms within a cell.

The increased expression of PDE4B2 and PDE4D5 also indicates a role for these isoforms in the hypoxic vasoconstrictive response in the pulmonary circulation. Previous studies have implicated increased levels of PDE4B2 in the contraction of myometrial strips (Mehats et al., 2002). In addition, PDE4D knockout mice display altered responses to muscarinic cholinergic stimulation (Hansen et al., 2000)

Further work in chapter 4 identified a role of the extracellular regulated kinase (ERK) in the increased levels of cAMP observed in hypoxia. ERK has previously been shown to act in an autocrine manner to stimulate production of PGE_2 and raise cAMP levels in human aortic smooth muscle cells (Baillie et al 2001; see figure 1.3). As cAMP has been demonstrated to regulate ERK activity within this loop (Pinelli et al., 1999), this provides a mechanism whereby cAMP levels and ERK activity can directly influence one another and themselves. Such a mechanism should enable the cell to regulate proliferative signals

correctly through inhibition of proliferation by cAMP and stimulation of proliferation through ERK activation, for example. In chapter 6, I demonstrated that hypoxic hPASMC have an increased proliferation rate, therefore the balance between cAMP signalling and ERK activation is altered. In fact, ERK was demonstrated to be directly involved in the hypoxia-induced increase in cAMP levels as MEK inhibitors used on hypoxic cells reduced cAMP levels to that of normoxic cells. It appears that the aforementioned components of the autocrine loop are increased in response to hypoxic stimulus. An increased ERK activity is well documented, and PGE_2 and COX-2 levels correspondingly have been noted to increase in hypoxia (Shaul et al., 1991; Jin et al., 2000; Bradbury et al., 2002; Yang et al., 2002). Interestingly, the increased ERK activity observed in hypoxia is transient (Jin et al., 2000; Scott et al., 1998). This is also suggestive of ERK regulation of cAMP levels in hypoxia as I have proposed that the increased cAMP observed is also temporary.

A recent study has shown that cAMP also regulates the autocrine loop albeit in a negative fashion. In human bronchial epithelial cells, stimulation of ERK leads to the increased production of prostaglandins in a manner which was potentiated by using a protein kinase A inhibitor (Pinelli et al., 1999). This indicates cAMP has an inhibitory effect on the ERK pathway in human bronchial epithelial cells thus providing a mechanism to down regulate the production of prostaglandins and thus cAMP.

In addition, the increased ERK activity in hypoxia regulates cAMP through its actions on the PDE4 family (Baillie et al., 2000). Dependant on isoform type, ERK can either inhibit or promote PDE4 activity (Houslay 2003; Baillie et al., 2000; Hoffmann et al., 1999), thus providing a cell specific ERK regulation of cAMP which is dependant on the PDE4 isoform profile within the cell. This is further complicated with the tight compartmentalisation of cAMP and ERK signalling within cells (Zaccolo et al., 2002; Pouyssegur et al., 2002). Pools of cAMP exist in regions of cells and can thus be localised away from the degrading action of ERK activated PDE4 short forms, or next to ERK inhibited PDE4 long forms within the cell and vice versa.

In combination, these results suggest the observed increase in cAMP levels in hypoxia may be an initial attempt by the cell to down regulate the enhanced ERK activity (Jin et al., 2000; Scott et al., 1998) and prostaglandin production (Shaul et al., 1991) witnessed in hypoxia that leads to increased smooth muscle cell proliferation (Yau & Zahradka 2003). Instead, the increased ERK maintains the increased level of cAMP through PGE_2

production and PDE4 inhibition, and levels continue to rise until a 'threshold' is breached whereby PKA phosphorylation of PDE4 overturns the inhibitory effect of ERK and cAMP is degraded at a faster rate than synthesised. ERK would then be reduced as a result. The decrease in ERK activity would completely alleviate inhibition of the PDE4 long forms present in hPASMC and increase the rate of cAMP hydrolysis even more, resulting in a desensitisation of the cell to cAMP raising agents. Indeed a negative feedback of cAMP has been demonstrated before and is thought to exist to protect against excessive accumulation of cAMP (Moon et al., 2002; Degerman et al., 1997).

Unfortunately, detecting the effect of ERK on PDE4 activity is compromised by the opposing modes of regulation ERK exerts on PDE4 long forms and short forms. To address this in future, immunoprecipitated PDE4 subfamilies should be assayed for PDE4 activity to determine the extent of inhibition. In addition, multiple time points must be used to gain a clearer understanding.

In conclusion, the first part of this study indicates a complex method of regulation of both cAMP and ERK. cAMP and ERK directly influence each other in a manner that has PDE4 at its centre. With the change in signalling that occurs in hypoxia, the balance that exists between cAMP and ERK is thrown into confusion and as a result, aids the development of pulmonary remodelling and desensitisation of the cAMP pathway.

The second part of this study examined the effect of hypoxia on the proliferative capacity of hPASMC. It was observed that hypoxia induces a significant increase in hPASMC proliferation, both in response to serum and in serum starved cells. This suggests the increased proliferation occurs in response to increased mitogenic signals produced in hypoxia. In addition, results gained using specific PKA agonists indicate the involvement of cAMP signalling. It appears that the increase in cAMP levels and proliferation witnessed in hypoxia contrast each other, suggesting the cAMP pathway is severely impaired in hypoxia.

Chapter 6 also revealed a novel role for the recently discovered cAMP substrate, Epac (de Rooij et al., 1998). Previous studies investigating the role of cAMP and cell proliferation have attributed all effects to PKA, although PKA-independent pathways are being uncovered (Cass et al., 1999). Surprisingly, the role of Epac in cAMP-mediated inhibition or stimulation of proliferation has not been investigated. Using an Epac-specific agonist, it was discovered that Epac selectively inhibits serum-induced proliferation. Following on

from this, identification of cell-cycle proteins altered in response to this agonist should be investigated to determine how Epac affects proliferation. In addition, utilising this agonist in cells with PKA RI or PKA RII knocked out could be useful as the results presented here show that Epac and PKA both serve the same function when overstimulated in these cells, indicating a balancing mechanism between PKA and Epac signalling. The result of this is either PKA or Epac inhibit cell proliferation in the absence of equal stimulation of the other. Also, the effect of stimulating Epac in other cells should be examined to determine if this is specific to smooth muscle cells.

The majority of the work presented here adds a further dimension to the role and regulation of cAMP in pulmonary arterial hypertension. It is obvious that PAH is a complex process, inducing the alteration of signalling pathways in an attempt to restore normal tone to the pulmonary circulation. Unfortunately, these alterations can directly and indirectly affect other pathways leading to enhanced signalling and the resultant remodelling of the pulmonary arterial tree. Due to the numerous signalling pathways involved in the hypoxic response, it is apparent that multiple drug therapies are required to attenuate the development of PAH. The results presented in this study further establish the potential of prostanoid therapy and PDE inhibition as a treatment for PAH. As both can impact upon cAMP and ERK pathways, it is possible these therapies can affect upon remodelling and hypoxia-induced transcription. Further studies examining the time points over the development of PAH should uncover a more precise characterisation of the effects of hypoxia on cAMP signalling and affected pathways, allowing the identification of specific therapeutic targets to potentiate the beneficial effects of such treatments and reduce the side effects associated with the non-specificity of the treatments used today.

The proliferation studies carried out here identify an exciting role for Epac in the regulation of proliferation. Although the Epac response is equal in both normoxic and hypoxic cells, a better understanding of the mechanisms controlling this response could help elucidate the alterations occurring in hypoxia to induce proliferation.

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