INVESTIGATING THE ROLE OF PDE4D1/2 IN VASCULAR SMOOTH MUSCLE CELL DESENSITIZATION

by

Nathalie S.Y. Butler

A thesis submitted to the Graduate Program in the Department of Biomedical and Molecular Sciences in conformity with the requirements for the degree of Master of Science

Queen’s University
Kingston, Ontario, Canada
October 2016

Copyright © Nathalie S.Y. Butler, 2016
Abstract

Vascular smooth muscle cell (VSMC) behaviour and phenotypic modulation is critical to vessel repair following damage, and the progression of various cardiovascular diseases. The second messenger cyclic adenosine monophosphate (cAMP) plays a key role in VSMC function under the synthetic/activated phenotype, which is typically associated with unhealthy cell behaviour. Consequently, cAMP signaling is often targeted in attempts to impact several pathological diseases, including atherosclerosis, restenosis, and pulmonary arterial hypertension (PAH). The cyclic nucleotide phosphodiesterases (PDEs) catalyze hydrolysis of cAMP to an inactive form, and therefore directly regulate cAMP signaling. The PDE4D family dominates in synthetic VSMCs, and there is considerable interest in determining how distinct PDE4D isoforms affect cell function. Specifically, we are interested in the potential link between short isoforms of PDE4D and VSMC desensitization to pharmacological agents that impact cardiovascular disease via cAMP signaling. This study extends on previous work that assessed the expression of PDE4D splice variants in rat aortic VSMCs following prolonged challenge with cAMP-elevating agents. It was determined that PDE4D1 and PDE4D2 were uniquely expressed in synthetic VSMCs incubated with these agents, and that this upregulation impacted PDE activity and cAMP accumulation in these cells. Here, we report that PDE4D1 and PDE4D2 are markedly upregulated in synthetic human aortic smooth muscle cells (HASMCs) following prolonged challenge with cAMP-elevating agents. Using a combination of RNAi-based and pharmacological approaches, we establish that this upregulation is reflected in levels of cAMP PDE activity, and restricted to the cytosolic sub-cellular compartment. Our results suggest a role for localized PDE4D1 and PDE4D2 activity in regulating cAMP-mediated desensitization in HASMCs, and highlight their therapeutic potential in treating various cardiovascular diseases.
Co-Authorship

Nathalie Butler performed all experiments described in this thesis under the supervision and guidance of Dr. Donald H. Maurice. Assistance and co-authorship were provided by Bibiana Umana. Bibiana Umana provided guidance and assistance in cell culture, RNAi-based knockdowns, immunoblotting experiments and analysis, and assays of cAMP PDE activity.
Acknowledgements

My time in Dr. Maurice’s lab has been truly unforgettable, and an extremely rewarding experience. I’ve been very fortunate to be surrounded by such kind people, and this thesis could not have been written without their help and support.

Firstly, I would like to thank my parents for their patience, love, and guidance. You taught me the importance of perseverance and dedication, and most importantly, you showed me that your circumstances can’t stop you from achieving your goals if you’re willing to work for them. I would not have been able to reach this point in my academic career at this age without your unwavering support throughout every challenge we’ve faced as a family. I am so grateful for every sacrifice you’ve made to give me the opportunity to pursue my dreams.

I’ve spent the past two years surrounded by wonderful labmates and friends who have made my time in the Maurice Laboratory so enjoyable. Don, Bibi, Catherine, Fabien, Silja, Sarah, Jodi, Paulina, and Jonah, I am so grateful for your kindness and support. Your guidance in the lab has been indispensable to my project, and your friendship outside of the lab has made the past two years an incredible experience. Thank you so much for all the great memories.

I would especially like to thank Bibi for her kind words, advice, and encouragement. I will always be thankful for your patience and support during some very challenging times.

Finally, I would like to thank Don for accepting me into his lab, challenging me to think outside of the box, and encouraging me throughout my project. Working with you has been a pleasure, and your guidance and support is what makes the Maurice lab so incredible. I am so grateful for your patience and faith in my abilities.

I would like to dedicate this thesis to my Mother. No matter where we are, we’ll be together forever.
# Table of Contents

Abstract ................................................................................................................................. i  
Co-authorship ........................................................................................................................ ii  
Acknowledgements ............................................................................................................... iii  
List of Figures .......................................................................................................................... vi  
List of Tables .......................................................................................................................... vii  
List of Abbreviations .............................................................................................................. viii  
Chapter 1: Introduction ........................................................................................................ 1  
  1.1 VSMC Phenotypes ......................................................................................................... 1  
  1.2 The Role of VSMC Phenotype Modulation in Pathological Disease ......................... 3  
  1.3 Cyclic AMP Signaling .................................................................................................... 6  
  1.4 Compartmentalized cAMP Signaling ........................................................................... 9  
  1.5 Cyclic Nucleotide Phosphodiesterases and Compartmentalized cAMP Signaling .... 14  
  1.6 Cyclic Nucleotide Phosphodiesterases ...................................................................... 15  
  1.7 Cyclic Nucleotide Phosphodiesterase Isoforms and Compartmentalization ............ 16  
  1.8 The PDE4 Family ......................................................................................................... 21  
  1.9 PDE Isoforms and VSMC Phenotypes ........................................................................ 24  
  1.10 Molecular Mechanisms of Desensitization ............................................................... 27  
  1.11 Physiological and Clinical Significance of Desensitization ........................................ 30  
Research Rationale .............................................................................................................. 33  
Research Objectives and Hypothesis ................................................................................... 35  
Chapter 2: Materials and Methods ...................................................................................... 37  
  2.1 Materials ....................................................................................................................... 37  
  2.2 Cell Culture .................................................................................................................. 38  
  2.3 siRNA-based Knockdowns ......................................................................................... 38  
  2.4 RNA Isolation, Reverse Transcription, and Quantitative PCR .................................. 39  
  2.5 Immunoblotting .......................................................................................................... 40  
  2.6 cAMP PDE Activity Assay ........................................................................................ 41  
  2.8 Statistical Analysis ....................................................................................................... 42  
Chapter 3: Results ............................................................................................................... 43  
  3.1 RNAi-based Knockdown of HASMC PDE4D mRNAs ............................................... 43  
  3.2 RNAi-based Knockdown of PDE4D Protein Levels in HASMCs ............................... 46  
  3.3 Expression of PDE4D Isoforms in HASMCs Incubated with Forskolin and IBMX ..... 48  
  3.4 RNAi-based Knockdown of PDE4D and PDE4D1 in HASMCs Impacts PDE4 Activity ................................................................. 49  
  3.5 Incubation of HASMCs with Forskolin and IBMX Increases PDE4 Activity ............ 50  
  3.6 Sub-cellular Distribution of PDE4 Activity in HASMCs ............................................. 50  
Chapter 4: Discussion ......................................................................................................... 54  
  4.1 Summary of Findings ................................................................................................. 54  
  4.2 Forskolin and IBMX Induce the Expression of PDE4D1 and PDE4D2 in HASMCs .... 55  
  4.3 Incubation with Forskolin and IBMX Increases PDE4 Activity in HASMCs ............ 58  
  4.4 Forskolin and IBMX-Induced Changes in PDE4 Activity are Enriched in the Cytosol ........................................................................................................................................ 60
4.5 Future Directions

A. Improve selectivity and efficiency of PDE4D1 and PDE4D2 knockdown
B. Explore how PDE4D1 and PDE4D2 Impact cAMP Accumulation in HASMCs
C. Investigate the effects of prolonged challenge with forskolin and IBMX in contractile HASMCs
C. Examine PDE4D1 and PDE4D2 as potential therapeutic targets

References
List of Figures

Figure 1.1: Phenotypic switching by VSMCs.................................................................2
Figure 1.2: Stages in atherosclerotic plaque development...........................................5
Figure 1.3: cAMP signaling dynamics.................................................................10
Figure 1.4: Compartmentalized cAMP signaling.......................................................13
Figure 1.5: Structure and domain organization of mammalian PDE families.............17
Figure 1.6: Tethered PDEs are key mediators of compartmentalized cAMP signaling.20
Figure 1.7: PDE4 families and isoform structure.......................................................23
Figure 1.8: Differential expression of PDE3 and PDE4 isoforms in contractile and synthetic VSMCs..............................................................................................................26
Figure 1.9: Second messenger kinase-mediated desensitization of GPCRs..............29
Figure 1.10: G protein coupled receptor kinase- and arrestin-mediated GPCR desensitization....32
Figure 1.11: Proposed mechanism through which PDE4D1 upregulation mediates HASMC desensitization.............................................................................................................36
Figure 3.1: PDE4D mRNA levels in HASMCs transfected with a control siRNA, PDE4D-specific, or PDE4D1-specific siRNA .................................................................45
Figure 3.2: Anti-PDE4D immunoreactive species in HASMC lysates and expression in cells transfected with PDE4D- and PDE4D1-specific siRNAs.............................................47
Figure 4.1: Proposed mechanism through which PDE4D1 upregulation mediates HASMC desensitization.............................................................................................................68
List of Tables

Table 2.1: Drugs used to target PDE families or increase intracellular cAMP.........................38
Table 3.1: Effects of forskolin and IBMX or RO 20-1724 on cAMP PDE activity in HAMSCs....53
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’-AMP</td>
<td>5’-adenosine monophosphate</td>
</tr>
<tr>
<td>AC</td>
<td>adenyl cyclase</td>
</tr>
<tr>
<td>AKAP</td>
<td>A-kinase anchoring protein</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>β-AR</td>
<td>β-adrenergic receptor</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine 3’, 5’-monophosphate</td>
</tr>
<tr>
<td>CIAP</td>
<td>calf intestinal alkaline phosphatase</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding factor</td>
</tr>
<tr>
<td>EC</td>
<td>endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>Epac</td>
<td>exchange protein activated by cAMP</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescent resonance energy transfer</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine-nucleotide-exchange factor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GRK</td>
<td>G protein-coupled receptor kinase</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HASMC</td>
<td>human aortic smooth muscle cell</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney cell line</td>
</tr>
<tr>
<td>hIP</td>
<td>human prostacyclin receptor</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>mAKAP</td>
<td>muscle-selective A-kinase-anchoring protein</td>
</tr>
<tr>
<td>MMP</td>
<td>metalloproteinase</td>
</tr>
<tr>
<td>PAH</td>
<td>pulmonary arterial hypertension</td>
</tr>
<tr>
<td>PASMC</td>
<td>pulmonary artery smooth muscle cell</td>
</tr>
<tr>
<td>PCI</td>
<td>cyclic nucleotide phosphodiesterase</td>
</tr>
<tr>
<td>PDE</td>
<td>percutaneous coronary intervention</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PGE(_1)</td>
<td>prostaglandin E1</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
</tr>
<tr>
<td>RACK1</td>
<td>receptor for activated protein kinase C I</td>
</tr>
<tr>
<td>UCR</td>
<td>upstream conserved region</td>
</tr>
<tr>
<td>VASP</td>
<td>vasodilator-stimulated phosphoprotein</td>
</tr>
<tr>
<td>VSMC</td>
<td>vascular smooth muscle cell</td>
</tr>
</tbody>
</table>
Chapter 1. Introduction

1.1 VSMC Phenotypes

Vascular smooth muscle cells (VSMCs) are specialized cells within the tunica media of blood vessels. The principal function of VSMCs is contraction, allowing for regulation of blood flow, vascular tone, and pressure\(^1\). Fully differentiated VSMCs in mature blood vessels exhibit extremely low levels of proliferation and migration, and express a unique repertoire of signaling molecules and ion channels required for high contractile function\(^2\). In contrast to cardiac and skeletal myocytes and other terminally differentiated cells, VSMCs can exist in a diverse range of phenotypes.

VSMCs exhibit various distinct phenotypes at different stages of development\(^3\). Even in mature blood vessels, VSMCs are able to undergo rapid and reversible phenotypic changes in response to local biochemical and mechanical cues\(^4\). In adult, healthy blood vessels, smooth muscle cells predominantly exist in a quiescent state, termed the contractile phenotype, which is characterized by a low proliferative and migratory index\(^1\). Following vessel injury and under pathological conditions, however, cells undergo a phenotypic shift from a contractile to a synthetic state\(^4\) (Figure 1.1). These proliferative, synthetic cells can migrate from the media into the intimal region of blood vessels, and contribute to vascular and tissue repair following injury\(^3\). A similar change in phenotype is observed when VSMCs are isolated from their native environment and cultured in the absence of physiological signals required to maintain the normal contractile phenotype\(^4\).

VSMCs reside within a highly dynamic, complex microenvironment, in which many local signaling factors and pathways interact and influence cell differentiation, phenotype, and behaviour\(^3\). Several growth factors, including platelet-derived growth factor (PDGF), basic
Figure 1.1: Phenotypic switching by VSMCs. Mature VSMCs maintain phenotypic plasticity, and are able to switch between quiescent and activated states. In normal, healthy blood vessels, the contractile phenotype dominates, and is characterized by low cell proliferation and migration. In contrast, vascular injury leads to a switch to the synthetic phenotype, in which cells are highly proliferative and migratory. (Modified from Milewicz et al., 2010)
fibroblast growth factor (FGF), and epidermal growth factor (EGF), trigger signaling pathways that stimulate growth and migration, and are therefore implicated in the switch to a synthetic phenotype¹.

1.2 The Role of VSMC Phenotype Modulation in Pathological Disease

In response to injury, VSMCs produce several growth factors which exert a paracrine effect on neighbouring cells to stimulate phenotypic remodelling³. The transition from the contractile to the synthetic phenotype, and the resulting increase in cell proliferation and migration, is necessary for tissue repair following vessel injury⁶. While this phenotype change is required for normal vascular function and healing, failure to switch back to the quiescent, contractile phenotype after tissue repair can lead to several cardiovascular pathologies.

According to Statistics Canada (2011), cardiovascular disease is the second leading cause of death among men and women, accounting for approximately 20% of all deaths in Canada. Atherosclerosis, a progressive disease of the large arteries, is the primary underlying cause of heart disease and stroke⁷. Atherosclerotic lesions develop following vascular injury, which leads to endothelial dysfunction and triggers a chronic inflammatory response of the vessel wall⁸. The endothelium is in direct contact with circulating blood, and forms a selectively permeable barrier via intercellular tight junctions⁹. Low-density lipoprotein (LDL) diffuses freely through these endothelial cell (EC) junctions, and endothelial dysfunction leads to lipid accumulation in the subendothelial space¹⁰. Trapped LDL becomes oxidized, and stimulates production of pro-inflammatory adhesion molecules and growth factors that increase monocyte adhesiveness and endothelium permeability⁷. Monocytes recruited to the vessel wall differentiate into macrophages, which take up highly oxidized LDL particles and form cholesteryl-ester-loaded
foam cells. During the early stages of atherosclerosis, these foam cells cluster and form aggregates, creating an atheromatous core\textsuperscript{11}. As the atherosclerotic plaque grows and matures, the macrophage-derived foam cells die, releasing lipids and cell debris that accumulate extracellularly and produce a necrotic core\textsuperscript{12}. Macrophages and macrophage foam cells release metalloproteinases (MMPs) that facilitate basement membrane remodeling, allowing for synthetic VSMC migration towards the lesion\textsuperscript{13}.

Platelet adhesion following endothelial injury leads to VSMC proliferation within the tunica media, followed by migration into the intima\textsuperscript{14} (Figure 1.2a). Once in the intima, VSMCs proliferate further in response to mediators, including PDGF, and produce extracellular matrix (ECM) molecules which form a fibrous cap over the atherosclerotic plaque\textsuperscript{15}. While gradual vessel dilation can compensate for intermediate lesions and early arterial wall thickening, SMC proliferation and migration leads to development of an advanced lesion, which may protrude into the lumen and occlude blood flow\textsuperscript{8}. Physical damage to the atherosclerotic plaque disturbs the fibrous cap and exposes pro-coagulant factors within the lesion core, triggering thrombus formation, often leading to end-stage disease events\textsuperscript{11} (Figure 1.2b).

Smooth muscle cell behaviour also plays a crucial role in the pathogenesis of pulmonary arterial hypertension (PAH). In familial or idiopathic PAH, increased pulmonary arterial pressure and vascular resistance is caused by the presence of plexiform lesions, and increased muscularization of pulmonary and peripheral arteries\textsuperscript{16}. Pulmonary artery smooth muscle cell (PASMC) hypertrophy, proliferation, and migration contribute to vascular remodelling in PAH. Smooth muscle cells in the medial layer of proximal arteries undergo hypertrophy and proliferate as a result of sustained elevation in intraluminal pressure, thickening the vessel wall and reducing the lumen diameter\textsuperscript{17}. In severe cases of PAH, activated, proliferating PASMCs in the
Figure 1.2: *Stages in atherosclerotic plaque development.* (a) In atherosclerosis, VSMCs exist in an activated, synthetic state. These synthetic VSMCs migrate from the media to the intima, where they proliferate and produce ECM components. Cell proliferation and ECM deposition leads to plaque growth and eventual lumen narrowing. (b) Fibrous cap fracture exposes pro-coagulant factors in the plaque core to blood coagulation factors, leading to thrombus formation. (Modified from Libby et al., 2011)\textsuperscript{11}
media migrate to the subendothelial space, where they contribute to neointima formation between the ECs and the internal elastic lamina. In normal, healthy pulmonary blood vessels, the majority of PASMCs exist in a quiescent, nonmigratory state. However, as is the case with atherosclerotic plaque development, changes in local environmental cues in PAH trigger a switch to the activated, synthetic phenotype. This transition leads to dysregulation of cell function, and ultimately the process of vascular remodelling that is central to the pathogenesis of PAH.

Atherosclerotic plaques can develop in the coronary arteries, and cause hardening and narrowing of the vessels that supply blood to the heart muscle. Several techniques of percutaneous coronary intervention (PCI), including balloon angioplasty and coronary stenting, have been developed to restore blood flow though blocked arteries in the treatment of coronary artery disease. However, a high incidence of in-stent restenosis remains a significant clinical drawback, where vascular injury caused by stent implantation leads to neointimal hyperplasia and vessel re-narrowing. In-stent restenosis is a complex, excessive reparative response to injury, in which coagulation and inflammatory factors stimulate a switch from the contractile, to the synthetic VSMC phenotype; this switch leads to cell proliferation and ECM production, and ultimately neointimal lesion formation.

While similar changes in cell behaviour and vascular morphology occur in atherosclerosis, the inflammatory and reparative process that underpins restenosis occurs within weeks of stent implantation. In contrast, atherosclerotic disease develops over several years as a result of long-term vascular remodelling. Synthetic VSMC behaviour plays an important role in the pathophysiology of several diseases, thus modulation of smooth muscle cell phenotype represents a potential target through which to impact these vascular pathologies.
1.3 Cyclic AMP Signaling

Extracellular signals are transmitted to a cell’s interior via signal transduction, allowing the cell to modulate its functions according to stimuli in the microenvironment. Signaling molecules further relay that information from the cell membrane to the cytoplasm to trigger a response. Several biological processes in many different organisms and cell types, including cells of the heart and vasculature, are dependent on cyclic adenosine 3’, 5’-monophosphate (cAMP). cAMP plays a key role in regulating critical cellular processes, including proliferation, migration, and differentiation.

Adenylyl cyclases (AC) catalyze the conversion of adenosine triphosphate (ATP) to cAMP at the plasma membrane (Figure 1.3). The AC enzyme family consists of ten unique isoforms, nine of which possess distinct mechanisms of regulation by G proteins, Ca²⁺, and phosphorylation. The tenth, however, is regulated by bicarbonate and is largely cytosolic. The nine canonical ACs are large, integral polypeptides containing two transmembrane regions, each consisting of six membrane-spanning helices, and two cytosolic domains. Residues from both cytosolic domains contribute to an interface and form the active site; thus the association of the two domains is required for catalysis. All nine ACs are subject to multiple forms of regulation by G-protein subunits, Ca²⁺/calmodulin, and protein phosphorylation. Guanosine triphosphate (GTP)-bound G-protein α-subunit, Gαs, binding induces a conformational change, bringing the catalytic residues together and stimulating catalysis. In addition, some ACs are activated by Ca²⁺/calmodulin binding, or protein kinase phosphorylation.

cAMP exerts its effects on cell function through three groups of effectors: the cAMP-dependent protein kinases, which includes protein kinase A (PKA) and exchange proteins.
activated by cAMP (Epac)\textsuperscript{30}, and the cyclic nucleotide-gated ion channels (cNMP-regulated channels), which are not expressed in VSMCs\textsuperscript{31}.

The cyclic AMP-dependent protein kinases are inactive tetramers consisting of two regulatory (R) and two catalytic (C) subunits. The regulatory and catalytic subunits are encoded by seven different genes in total, giving rise to four regulatory subunit isoforms (RI\textalpha, RII\textalpha, RI\textbeta, and RII\textbeta), and three isoforms of the catalytic subunit (C\textalpha, C\textbeta, C\textgamma)\textsuperscript{32}; isozymes are designated type I or type II PKAs according to the regulatory subunits they are composed of\textsuperscript{33}. In the absence of cAMP, PKA exists in an inactive state where access to the active sites within the catalytic subunits is blocked by the regulatory subunits. Upon binding of two cAMP molecules to each of the regulatory subunits, the PKA holoenzyme dissociates and releases a complex of two active catalytic subunits which can then phosphorylate their substrates\textsuperscript{34} (Figure 1.3).

Substrates in close proximity to the holoenzyme or catalytic subunits are rapidly phosphorylated, thus subcellular targeting of PKA isoforms plays an important role in substrate selection\textsuperscript{32}. The free catalytic subunits phosphorylate serine or threonine residues on cytoplasmic protein substrates, or nuclear substrates following passive diffusion into the nucleus\textsuperscript{33}. Within the nucleus, PKA-dependent phosphorylation of cAMP response element binding factor (CREB) induces gene transcription\textsuperscript{32}.

Initially, PKA was thought to be the sole effector responsible for all cAMP signaling. However, inhibition of PKA failed to eliminate cAMP-induced activation of the small GTPase Rap1\textsuperscript{35}. Two proteins, Epac1 and Epac2, were later identified as mediators of cAMP responses, and are guanine-nucleotide-exchange factors (GEFs) for the small GTPases Rap1 and Rap2\textsuperscript{36} (Figure 1.3). Epac consists of a C-terminal catalytic domain, and an auto-inhibitory regulatory domain containing a cAMP-binding site. The regulatory domain sterically hinders access to the
catalytic region in the closed, inactive conformation; this inhibition is reversed upon cAMP binding\textsuperscript{37}. Active Epac promotes Rap1 release of guanosine diphosphate (GDP) and GTP binding. Through activation of Epac and consequent guanine nucleotide exchange and Rap1 acativation, cAMP impacts cell differentiation\textsuperscript{38} and proliferation\textsuperscript{39}.

Termination of cAMP-mediated signaling is required for proper cell function and homeostasis. The cyclic nucleotide phosphodiesterases (PDEs) catalyze cAMP breakdown into 5'-AMP, thus preventing over-activation of cAMP-dependent signaling pathways and cell saturation with cAMP following AC activation\textsuperscript{40} (Figure 1.3). In addition, PDEs regulate both amplitude and duration of cAMP-mediated signals\textsuperscript{41}.

1.4 Compartmentalized cAMP Signaling

External signals are communicated to a cell’s interior via intracellular signal transduction pathways, which require spatial and temporal regulation in order to trigger an appropriate response. cAMP and its effectors are implicated in a large variety of signaling cascades, which can have very different, and sometimes opposing effects on cell function. However, cAMP is freely diffusible, which is seemingly contradictory to its ability to induce specific, localized responses. How is a single second messenger molecule able to regulate a wide range of complex and distinct cellular processes simultaneously? This paradox can be resolved with the concept of compartmentalized cAMP signaling.

Elevations in cAMP followed by PKA activation triggered by different agonists leads to different physiological responses and effects on cell function, even within the same tissue. This was demonstrated in cardiac myocytes by Buxton and Brunton, where isoproterenol and prostaglandin E1 (PGE\textsubscript{1}) both increased intracellular cAMP to similar levels, but did not trigger
Figure 1.3 cAMP signaling dynamics. Upon agonist binding to a G protein-coupled receptor, the stimulatory G\(_s\alpha\) subunit dissociates, and then binds to and activates adenylyl cyclase. Membrane-bound adenylyl cyclase converts ATP into cAMP, which freely diffuses within the cell and activates two major effectors, PKA and Epac. cAMP binding to the regulatory subunits of PKA, causing a conformational change and release of the active catalytic subunits. cAMP binds to the guanine nucleotide exchange factor, Epac, which activates the small GTPase Rap1. The cyclic nucleotide PDEs terminate cAMP signaling by degrading cAMP into its inactive form, 5’-AMP. (Modified from Bailie et al., 2005)\(^{42}\)
the same downstream effectors: while isoproterenol activation of β-adrenergic receptors led to PKA phosphorylation of enzymes involved in glycogen metabolism and contractile activity, PGE₁ activation of PKA did not result in phosphorylation of those protein substrates. In addition, it was shown that isoproterenol and prostaglandin binding to distinct GPCRs led to selective activation of membrane-associated or cytosolic PKA, respectively. Cells are able to distinguish between elevations in cAMP that arise from activation of different receptors, and can then modulate their function accordingly.

Signaling molecules necessary for cAMP generation, including GPCRs, G-protein subunits, and some ACs, are restricted to sphingolipid- and cholesterol-rich lipid raft domains within the plasma membrane. The cytosolic domains of AC5, AC6, and AC8 likely interact with other raft-embedded signaling proteins, and selectively couple to certain GPCRs. Tethering AC isozymes and GPCRs to specific locations creates distinct points of cAMP generation at the plasma membrane, resulting in discrete “clouds” of cAMP which activate effectors in the immediate vicinity. Selective coupling to GPCRs and targeting to lipid rafts can partially explain the differential activation of downstream effectors, but additional mechanisms must exist to account for the spatiotemporal specificity of cAMP-mediated signaling.

Following synthesis at specific points across the plasma membrane, cAMP follows its concentration gradient and quickly diffuses outward. Subcellular targeting of cAMP effector molecules, including PKA and Epac, further contributes to signaling specificity and compartmentalization. Anchoring proteins represent the backbone of multi-protein signaling complexes, termed signalosomes, and bring cAMP effectors close to their downstream substrates. By placing certain signaling proteins in close proximity, anchoring proteins allow for signal integration and selective, local regulation of cellular processes.
Compartmentalization of PKA signaling is accomplished by A-kinase anchoring proteins (AKAPs). Over 50 unique AKAPs have been identified since their initial discovery, which are diverse in sequence and structure, but are functionally-related. All AKAPs share a PKA-anchoring domain consisting of an amphipathic helix which binds to a hydrophobic groove formed by an N-terminal four-helix crossing bundle within the regulatory subunit dimer (Figure 1.4). While AKAPs share this PKA-binding domain, and are therefore similar in function, diversity arises from a unique targeting sequence that directs the protein complex to a specific subcellular location. In addition, AKAPs also interact with several other signaling molecules, and form complexes that often include enzymes for both propagation and termination of a signal. The multivalence of these anchoring proteins sequesters cAMP effector proteins together with the appropriate downstream targets, and allows for precise control of signal duration. These AKAP-based signaling complexes respond to distinct pools of cAMP created at the plasma membrane, and contribute to compartmentalization of cAMP signaling.

Epac proteins are similarly targeted to distinct subcellular locations by anchoring proteins. Intracellular localization of Epac proteins occurs via the membrane targeting disheveled/Egl-10/pleckstrin and Ras-association domains. Following cAMP binding and activation, Epac1 translocates to the plasma membrane, where it contributes to Rap-mediated cell-ECM adhesion. At the membrane, Epac1 interacts with members of the ezrin-radixin-moesin (ERM) family of anchoring proteins via its N-terminus.

Anchoring cAMP effectors with other signaling molecules and tethering ACs to lipid rafts allows for some spatiotemporal regulation; however, in the absence of mechanisms to terminate signaling, cAMP would saturate the entire cell and indiscriminately activate all
Figure 1.4 Compartmentalized cAMP signaling. Several mechanisms of compartmentalization contribute to spatiotemporal regulation of cAMP signaling. Specific GPCRs and ACs are tethered to distinct locations within the plasma membrane, and create pools of cAMP. These pools grow as cAMP freely diffuses and encounters its effectors. Anchoring proteins act as a scaffold for these effectors and other signaling molecules, allowing for signal propagation and generation of subcellular domains of cAMP signaling. PDEs degrade cAMP to restrict cAMP diffusion, and regulate the duration and extent of cAMP-mediated signaling. (Modified from Averaimo and Nicol, 2014)\textsuperscript{56}.  

effectors it encounters. Formation of distinct cAMP pools within the cell, and precise spatiotemporal regulation of signaling is therefore dependent on cAMP degradation (Figure 1.4).

1.5 Cyclic Nucleotide Phosphodiesterases and Compartmentalized cAMP Signaling

Due to its high diffusion constant (700 µm²/s), cAMP would saturate an entire cell lacking compartmentalization within a fraction of a second following synthesis at the plasma membrane. Phosphodiesterases play a critical role in creating distinct cAMP pools and generating stimulus-specific responses by degrading, and therefore restricting cAMP diffusion. The development of fluorescent resonance energy transfer (FRET)-based sensors allowed for detection of spatial and temporal changes in cAMP in living cells. PKA regulatory and catalytic subunit dissociation following cAMP binding can be monitored through changes in fluorescence resonance energy transfer between donor and acceptor moieties. Stimulation of β-adrenergic (β-AR) receptors generated localized increases in cAMP levels, within an approximate range of 1 µm. However, discrete cAMP gradients disappeared with addition of a broad spectrum PDE inhibitor, isobutyl-methyl-xanthine (IBMX). Therefore, PDE-catalyzed degradation of cAMP is necessary for formation of cAMP micro-domains and spatiotemporal regulation of signaling, allowing cells to respond appropriately to distinct stimuli.

Diverse isoforms of PDEs can be targeted to specific subcellular locations, where they are sometimes tethered by AKAPs. Rapid hydrolysis of cAMP in these PDE-gated compartments restricts diffusion of the second messenger, and shapes the intracellular pools required for precise regulation of stimulus-specific signaling.

While PDE-mediated hydrolysis restricts free diffusion of cAMP, it does not form an impenetrable barrier to cAMP movement. Instead, anchored PDEs create localized “sinks” as
they catalyze the conversion of cAMP into 5′-AMP, resulting in a localized concentration gradient of cAMP. Generation of these cAMP gradients produces a large range of microenvironments in the cytosol that are precisely regulated by PDE isozymes that are tethered to specific subcellular sites. Spatiotemporal regulation of local amounts of cAMP is controlled by the opposing activities of two enzyme families: adenylyl cyclases, which synthesize cAMP at the plasma membrane, and cyclic nucleotide PDEs, which catalyze cAMP hydrolysis.

1.6 Cyclic Nucleotide Phosphodiesterases

The cyclic nucleotide PDEs are a family of related phosphohydrolases that catalyze the hydrolysis of 3′ cyclic phosphate bonds, and are the only endogenous enzymes capable of degrading cAMP and cGMP. Multiple PDE genes, alternative mRNA splicing, and transcriptional processing give rise to nearly 100 isozymes which can be classified into 11 distinct gene families (Figure 1.5). Members of the PDE protein superfamily are similar in catalytic function, and the central catalytic domains are highly homologous across families. The N-terminal and C-terminal domains, however, are highly divergent between PDE families, and contain structural elements that target different isoforms to specific subcellular locations or signalosomes, and allow for unique mechanisms of regulation and post-translational modifications.

The PDEs represent attractive therapeutic targets, as impacting degradation is likely to have a more rapid and larger effect on global cAMP levels compared to targeting synthesis, and there is a multitude of different isoforms that are associated with specific cellular functions and pathological conditions. Several PDE inhibitors have been developed for use in treating several diseases and chronic conditions, including congestive heart failure, inflammatory airways
disease\textsuperscript{63}, and erectile dysfunction\textsuperscript{64}. The cyclic nucleotide PDEs are critical regulatory enzymes in cell signaling, and possess high therapeutic potential.

PDEs play a critical role in intracellular signaling through degradation of the second messengers cAMP and cGMP. Because there are only two substrates for PDEs, it is seemingly redundant that nearly 100 unique isoforms exist to hydrolyze cyclic nucleotides. Although the isozymes are almost identical in catalytic function, N-terminal regions unique to each enzyme may allow for precise intracellular targeting\textsuperscript{61}. Furthermore, these anchored PDEs control localized cAMP gradients and contribute to compartmentalized signaling.

1.7 Cyclic Nucleotide Phosphodiesterase Isoforms and Compartmentalization

The unique N-terminal domains of each PDE family determines subcellular targeting to membranes, organelles, anchoring proteins, and signalosomes. For instance, members of the PDE2, PDE3, and PDE4 families are found in specific sites according to relative hydrophobicity of the amino termini, and the presence of membrane-association domains. These differences between PDE isoforms result in distinct intracellular localization to membrane-associated, cytosolic, and microsomal fractions\textsuperscript{65}. Tethering PDEs within cellular microenvironments allows for formation and regulation of local cAMP gradients, and is often accomplished through interactions with scaffolding proteins, including AKAPs and β-arrestin\textsuperscript{66} (Figure 1.6).

Scaffolding proteins anchor several PDE isoforms to subcellular structures, where they degrade cAMP, and therefore create and shape distinct pools of the second messenger. These scaffolding proteins include the β-arrestins, which recruit PDE4D5 to the plasma membrane\textsuperscript{67}. Following stimulation of HEK293 cells with the β agonist isoproterenol, which binds to adenylyl
Figure 1.5 Structure and domain organization of mammalian PDE families. The cyclic nucleotide PDEs hydrolyze cAMP or cGMP. Nearly 100 PDE isoforms are classified into 11 enzyme families, which share a highly conserved catalytic domain. The N- and C-terminal regions contain unique domains which confer unique regulatory properties to each family. These divergent domains allow different isoforms to be regulated differently, and targeted to distinct subcellular locations and signaling complexes. (Modified from Maurice et al., 2014)
cyclase-coupled β2-ARs, PDE4D5 was targeted to plasma membrane. This recruitment is accomplished through interaction with β-arrestin, as PDE4D5 contains one β-arrestin binding site within the catalytic domain, and another within the unique N-terminal region. The interaction between β-arrestin at PDE4D5 allows for an agonist-dependent increase in cAMP degradation in close proximity to adrenergic receptors; increased cAMP breakdown also affects activity of PKA at the plasma membrane.

While β-arrestin-anchored PDE4D5 plays a critical role in limiting cAMP levels at the plasma membrane of isoproterenol-challenged cells, PDE recruitment has no effect on PKA activity in the cytosol, or in unstimulated cells. Anchoring PDE4D5 targets cAMP degradation to sites of localized PKA activity at the plasma membrane, and allows PDEs to restrict cAMP diffusion and quench PKA activity in β-agonist-activated cells.

Muscle-selective A-kinase-anchoring protein (mAKAP) interacts with both PKA and PDE4D3, and acts as a scaffold for a cAMP signaling complex at perinuclear regions of cardiomyocytes. Placing a cAMP effector in close proximity to a cAMP-metabolizing enzyme creates a negative feedback loop, in which PDE4D3 directly mediates PKA activity and cAMP levels. Under basal conditions, PDE4D3 is constitutively active, and catalyzes hydrolysis of cAMP and limits PKA activation. Upon AC activation, however, cAMP levels increase rapidly, and bind to the regulatory subunits of PKA and trigger release of the active catalytic subunits from the signaling complex. These catalytic subunits are then free to phosphorylate their protein substrates, including PDE4D3. Phosphorylation of PDE4D3 causes a two- to three-fold increase in its $V_{max}$, thus elevating the rate of cAMP breakdown and driving cAMP levels back to basal conditions. PKA phosphorylation of PDE4D3 also increases its affinity for mAKAP, preventing diffusion away from the local cAMP pool. Anchored, localized PDE activity allows
for more rapid termination of cAMP signaling\textsuperscript{70}. In addition to the cAMP-binding proteins PKA and PDE4D3, this signalosome also includes components of the ERK5 MAP kinase signaling pathway, and the calcium release channel ryanodine receptor; therefore, this mAKAP signaling complex coordinates signals from cAMP, Ca\textsuperscript{2+}, and MAP kinase pathways\textsuperscript{72}.

Experiments performed by Terrin \textit{et al.} (2006) demonstrate that PDE isoforms are restricted to distinct compartments in HEK293 cells, and highlight the importance of subcellular targeting in regulating cAMP signaling\textsuperscript{73}. It was observed that PGE\textsubscript{1} stimulation of transmembrane AC led to a higher cAMP response at the plasma membrane, as cytosolic PDE4D maintained low levels of cAMP within the bulk cytosol. While PDE4D is localized to the cytosolic pool, PDE4B is located within the membrane fraction, and the cooperative activity of PDE4B and PDE4D generates and shapes a cAMP gradient following PGE\textsubscript{1} binding to its GPCR. Displacement of PDE4D from its cytoplasmic anchors completely abolished the cAMP gradient, suggesting that compartmentalization of PDE4 isoforms is required to shape and spatially restrict pools of cAMP\textsuperscript{73}.

A model of cAMP compartmentalization in which PDEs serve as an enzymatic barrier to diffusion would predict that the concentration of the second messenger would be highest at the site of its synthesis, at the plasma membrane, and would progressively decrease towards the inner cytosol. Contradictory to this model, however, Terrin \textit{et al.} (2006) show that a compartment found within the deep cytosol accumulates higher levels of cAMP relative to the bulk cytosol. Instead, they propose that PDEs do not enzymatically gate distinct pools of cAMP, but instead form local “sinks” that draw the cAMP into distinct subcellular compartments\textsuperscript{73} (Figure 1.6).
Figure 1.6 Tethered PDEs are key mediators of compartmentalized cAMP signaling. PDEs are anchored at specific subcellular sites, within macromolecular signaling complexes. Tethered PDEs shape and regulate distinct, localized cAMP gradients, and therefore their activity directly controls the cAMP effectors PKA and Epac. The unique N-terminal region of PDE isoforms allows for selective interaction with other signaling factors, and anchoring proteins. Local cAMP pools are represented as red-shaded ovals. C = catalytic subunit of PKA; R = regulatory subunit of PKA; β-AR = β-adrenergic receptor. (Modified from Stangherlin and Zaccolo, 2012)
1.8 The PDE4 Family

The PDE enzyme superfamily is comprised of nearly 100 isozymes related in catalytic function, which are generated from 11 distinct gene families via mRNA splicing and transcriptional processing. Of these, the PDE4 family is the largest, and has been studied most extensively, and its members represent valuable therapeutic targets. Inhibitors of PDE4 activity, including roflumilast and rolipram, have been used in treating immunoinflammatory-related pulmonary diseases, including asthma, COPD, allergic rhinitis, and idiopathic pulmonary fibrosis.[74–76.

The PDE4 enzyme family is encoded by four complex genes of approximately 50 kb, giving rise to four subfamilies (PDE4A, PDE4B, PDE4C, and PDE4D). Multiple promoters within these genes and alternative splicing produce about 25 cAMP-specific isozymes.[77. These variants are classified as long, short, super-short, and dead-short according to the domains they contain. The highly conserved catalytic domain consists of 315 amino acid residues, and is shared by all PDE4s, but unique N- and C-terminal domains impart different structural and regulatory properties on each isoform.[77,78. The upstream conserved regions (UCR) 1 and 2 lie immediately N-terminally to this catalytic core in the long isoforms. The short isoforms, however, lack UCR1, while super-short isoforms lack UCR1 and also contain a truncated UCR2. Finally, each PDE4 subfamily contains a unique C-terminal region, whose function is currently unknown (Figure 1.7)[79.

The PDE superfamily includes almost 100 distinct enzyme members, despite performing only two related catalytic functions: hydrolysis of cAMP and cGMP. Thus, it follows that the catalytic core of these enzymes is highly conserved between isoforms. Each family and isozyme, however, is characterized by the divergent N- and C-terminal regions they contain. The unique
region N-terminal to the catalytic domain exert significant effects on PDE function, by determining mechanisms of regulation and subcellular targeting, which contribute to localized cyclic nucleotide degradation and therefore compartmentalization\textsuperscript{78}. For example, a small stretch of amino acids within the unique 88 residue N-terminal region of PDE4D5 is required for interaction with the scaffold protein receptor for activated protein kinase C (RACK1)\textsuperscript{80}. Residues 12 through 49 of PDE4D5 form an amphipathic helical RACK1 interaction domain which binds to the C-terminal region of RACK1. RACK1 has previously been shown to interact with protein kinase C (PKC) following activation by diacylglycerol, the Src protein tyrosyl kinase, and integrins\textsuperscript{81–83}. By binding multiple signaling factors, RACK1 acts as a scaffold and mediates the recruitment of PDE4D5 to a protein complex, and likely contributes to the creation of localized cAMP pools and compartmentalized signaling.

The extreme N-terminal region which is responsible for subcellular targeting of PDE4 isoforms is followed by stretches of unique amino acid sequence, called UCR1 and UCR2. The C-terminal region of UCR1 interacts directly with the N-terminal region of UCR2 via electrostatic interaction\textsuperscript{85}. The interaction between these independent domains is attenuated by PKA-mediated phosphorylation of a serine residue at the extreme N-terminal end of UCR1, S\textsubscript{54}\textsuperscript{71}. In long PDE4 isoforms, phosphorylation of this residue disrupts an interaction between S\textsubscript{54} and an adjacent glutamate residue, releasing the enzyme from a low activity state\textsuperscript{77}. Phosphorylation and activation of PDEs follows AC-catalyzed generation of cAMP and subsequent PKA activation; this pathway may maintain intracellular cAMP levels, contribute to termination of signal transduction, and produce cell desensitization.

The PDE4D subfamily consists of nine distinct isoforms produced via alternative splicing and multiple transcription start sites: PDE4D1-PDE4D9. The long PDE4D isoforms include
Figure 1.7 PDE4 families and isoform structure. The four PDE4 genes give rise to four enzyme subfamilies, consisting of 25 isozymes in total. All variants share a highly conserved catalytic core, but diverge at the amino-terminal regulatory regions. The PDE4 isoforms are classified as long, short, super-short, or dead-short depending on the presence of the regulatory domains UCR1 and UCR2. PKA-mediated phosphorylation of UCR1 (red circle) and ERK-mediated phosphorylation of the catalytic core (black circle) are shown. The N-terminal region, NTR (red), is unique to each isoform, and plays a key role in the regulation of catalytic activity and subcellular localization. (Modified from Houslay, 2009)
PDE4D3, PDE4D4, PDE4D5, PDE4D7, PDE4D8, and PDE4D9, and contain both UCR1 and UCR2. The short isoform, PDE4D1, and the super-short isoforms, PDE4D2 and PDE4D6, only contain the complete or partial UCR2 module, respectively. Each of the nine PDE4D isoforms can be distinguished by their unique N-terminal regions, which contain amino acid sequences that influence subcellular localization and catalytic activity.

1.9 PDE Isoforms and VSMC Phenotypes

Individual PDE variants are unique in enzymatic regulation, subcellular targeting, and also tissue-expression patterns; their relative proportions can vary between VSMCs isolated from different vascular structures, or even the same vessel. The phenotypic switch that is triggered by vascular injury is accompanied by a change in the PDE expression profiles exhibited by contractile and synthetic VSMCs: while both activated and quiescent SMCs use PDE3 and PDE4 to hydrolyze cAMP, PDE3 activity is greater in contractile cells, and PDE4 activity dominates in the synthetic VSMC phenotype (Figure 1.8). The change in the PDE3/PDE4 activity ratio that accompanies the transition in phenotype is largely dependent on downregulation of PDE3 isoforms, as PDE4 activity is approximately equal in both cell types. This results in increased dependence on PDE4-mediated regulation of signaling, and therefore cAMP-driven signaling in synthetic SMCs. The switch in dependence on the cGMP-hydrolyzing PDE3 family to the cGMP-insensitive PDE4 family is one of several mechanisms through which VSMCs become more reliant on cAMP. Furthermore, expression of distinct PDE3 and PDE4 variants is increased by cAMP-elevating agents in VSMCs in situ, which are considered contractile, vs cultured, or synthetic, arterial VSMCs. Both PDE3 and PDE4 inhibitors have been used to treat vascular pathologies. Although PDE3 inhibition has proven to impact blood pressure by relaxing
contractile SMCs and reduce intimal accumulation of synthetic SMCs following angioplasty, the pro-arrhythmic side effects of these agents diminish their therapeutic potential. In contrast, due to the increased dependence on PDE4s in synthetic VSMCs, PDE4 inhibition may allow for greater phenotype- and tissue-specific selectivity.

In addition to differences in PDE3/PDE4 activity ratios between VSMC phenotypes, distinct PDE4 variants are selectively upregulated following prolonged challenge with cAMP-elevating agents in contractile vs synthetic cells. Sustained cAMP elevation triggers regulatory mechanisms that serve to desensitize cells to further cAMP-mediated events, including PDE4 upregulation in both quiescent and activated VSMCs. However, distinct PDE4D variants are responsible for this desensitization in each phenotype. Phenotype-dependent differences in PDE4 variant expression can be traced back to chromatin remodeling and transcriptional activation.

The histone proteins that interact directly with genomic DNA can undergo reversible post-translational modifications, including methylation, phosphorylation, and acetylation. These covalent modifications alter chromatin structure, and therefore impact relative gene expression by determining which promoters are accessible for transcription. Levels of histone H3 acetylation within the intronic promoter regulating PDE4D short isoform expression are significantly higher in synthetic VSMCs compared with that from contractile VSMCs. Histone acetylation has been previously shown to release genome elements from a repressed state and cause increased gene transcription, which is consistent with PDE4D1 and PDE4D2 induction after cAMP-elevation in activated VSMCs, and not quiescent VSMCs. Prolonged challenge of synthetic VSMCs with cAMP-elevating agents activates a PKA/CREB/CRE signaling cascade, leading to upregulation of the PDE4D short-form variants. These isoforms are not expressed in
Figure 1.8 Differential expression of PDE3 and PDE4 isoforms in contractile and synthetic VSMCs. Catalytic activity of PDE3 and PDE4 is approximately equal in contractile VSMCs within the medial layer of blood vessels. Downregulation of PDE3 isoforms in synthetic VSMCs, however, results in increased dependence of PDE4-catalyzed cAMP hydrolysis. While prolonged cAMP elevation induces short PDE4 isoform (PDE4D1/4D2) expression in activated VSMCs, this is not observed in quiescent VSMCs. (Modified from Houslay et al., 2007)
both synthetic and contractile cells under basal conditions, nor are they induced in contractile VSMCs following sustained cAMP-elevation (Figure 1.8).

The cAMP-mediated induction of PDE4D1 and PDE4D2 expression may have therapeutic potential in limiting vascular disease progression and vessel stenosis. Agents that elevate cAMP have been shown to reduce intimal hyperplasia by reducing VSMC proliferation and migration, but their therapeutic value is diminished due to their undesired effects on systemic blood pressure. Due to the higher PDE4/PDE3 activity ratio in synthetic VSMCs, PDE4-selective inhibitors may have a greater impact on neointimal, activated SMCs in stenotic lesions compared to the quiescent cells within the medial vessel layers that control vascular tone and blood pressure. Furthermore, upregulation of the short PDE4D variants may play a significant role in desensitization to prolonged cAMP signaling in synthetic VSMCs. Inhibition of these PDE4D isoforms may increase the sensitivity of activated VSMCs to cAMP-elevating agents used to treat vascular pathologies.

1.10 Molecular Mechanisms of Desensitization

Prolonged, persistent agonist stimulation of G protein-coupled receptors (GPCRs) lead to reduced receptor responsiveness via mechanisms of desensitization at the transcriptional, translational, and protein levels. Changes in transcription and translation of signaling molecules contribute to down-regulation of receptors, which occurs after long-term agonist activation. Rapid desensitization, however, is achieved through regulation of receptor degradation, covalent post-translational modification, protein-protein interaction, and subcellular localization.

The most rapid mechanism of desensitization involves phosphorylation of GPCRs and subsequent uncoupling from associated G proteins. Following agonist stimulation, GPCRs
produce cAMP and diacylglycerol, which activate the second messenger kinases PKA and PKC. These second messenger kinases then participate in a negative feedback loop, in which they phosphorylate serine residues within the cytoplasmic loop or C-terminal tail of GPCRs. This covalent modification triggers a conformational change which blocks interaction with the G protein, therefore preventing further signal transduction (Figure 1.9)\(^95\). This mechanism of desensitization is termed heterologous, as it is not agonist-specific: any agent that causes a global increase in cAMP or diacylglycerol will activate the second messenger kinases which phosphorylate and desensitize any GPCR containing a PKA or PKC consensus site. In contrast, homologous desensitization occurs when activated GPCRs become less responsive to the specific agonists they are capable of binding to\(^96\).

The major mechanism of rapid, homologous desensitization is mediated by G protein-coupled receptor kinases (GRKs) and the β-arrestins. Upon agonist binding to a GPCR, the α subunit dissociates from the βγ subunit complex, which remains membrane-bound. Together, G\(_{βγ}\) and phosphatidylinositol bisphosphate within the plasma membrane bind to the C-terminal pleckstrin homology domain of GRK. This interaction places the kinase in a position that is conducive to receptor phosphorylation, and therefore selectively targets GRKs to agonist-occupied GPCRs (Figure 1.10)\(^97\). Once phosphorylated, the receptor’s affinity for arrestin binding greatly increases: for instance, GRK-phosphorylation of β-ARs causes a 10 to 30-fold increase in affinity for β-arrestin\(^93\). Arrestins are capable of terminating further signaling by sterically inhibiting the receptor-G protein interaction that is required for signal transduction.

The arrestins have a dual function in regulating receptor activity, as they also contribute to receptor sequestration and therefore long-term desensitization induced by sustained agonist exposure. After binding to phosphorylated GPCRs, β-arrestins interact directly with clathrin and
Figure 1.9 Second messenger kinase-mediated desensitization of GPCRs. Activation of PKA and PKC by the second messengers cAMP or diacylglycerol leads to phosphorylation of GPCRs. Serine residues within the C-terminal region or cytoplasmic loop of receptors become phosphorylated, preventing the interaction with the G protein that is required for signaling. (Modified from Hausdorff et al., 1990)
act as adaptor-like molecules which promote receptor internalization (Figure 1.10)\(^98\). This interaction and subsequent sequestration is viewed as an early step in receptor “down-regulation,” as some internalized receptors are degraded within lysosomes. Long-term challenge with β-adrenergic agonists eventually causes a decline in the levels of the mRNA encoding β-ARs, due to reduced transcript stability\(^94\).

Regulation of agonist-induced signaling and desensitization also occurs at the level of cAMP degradation by PDEs. Elevated intracellular cAMP gives rise to greater PDE activity through increased gene transcription\(^100\). While changes in transcription levels cannot account for rapid functional uncoupling of receptors, PDE upregulation may contribute to the long-term changes in receptor responsiveness that cells experience as a result of sustained agonist exposure.

### 1.11 Physiological and Clinical Significance of Desensitization

Desensitization following prolonged agonist challenge results in reduced responsiveness to these agonists, and is relevant in the pharmacotherapy of cardiovascular disease. Dobutamine is a derivative of isoproterenol which acts directly on β adrenergic receptors to increase cardiac contractility and output. Its positive inotropic effects are typically used to increase cardiac output in cases of acute heart failure following surgery or cardiogenic shock\(^101\). However, decreased sensitivity to β-AR agonists, such as dobutamine, often occurs while treating chronic heart failure. This loss in responsiveness is a consequence of receptor downregulation, and increased activity of a GRK, β-adrenergic receptor kinase. GRK-catalyzed receptor phosphorylation plays a key role in the desensitization of agonist-occupied receptors\(^102\).
A similar phenomenon occurs following continued presence of the prostacyclin analogue iloprost. While inhaled iloprost has been found to produce a longer duration of vasodilation and is an effective therapy for severe pulmonary hypertension, the required dose must be progressively increased due to tolerance as a result of receptor desensitization. PKC-mediated phosphorylation of the human prostacyclin receptor (hIP) is critical to the iloprost tolerance that is observed in treating PAH. Targeting the mechanisms responsible for desensitization may enhance the therapeutic value of drugs that are currently used to treat cardiovascular disorders.
Figure 1.10 *G* protein coupled receptor kinase- and arrestin-mediated GPCR desensitization. Following agonist binding to receptors, the α subunit dissociates and the free βγ subunit can interact directly with GRKs. GRK-catalyzed phosphorylation promotes interaction with arrestins, which sterically uncouple the G protein from its receptor, terminating further signaling. Arrestins act as adaptor molecules which interact with clathrin, and drive receptor internalization into endosomes, and eventual lysosomal degradation. (Modified from Ritter et al., 2009)
**Research Rationale**

The PDE4 enzyme family is the largest and most extensively studied among the 11 PDE families encoded within the human genome. Approximately 25 PDE4 isozymes are generated from four distinct genes, *PDE4A*, *PDE4B*, *PDE4C*, and *PDE4D*. Each of these variants are classified as long, short, or super-short isoforms on the basis of the domains they contain: long isoforms possess complete N-terminal upstream conserved regions UCR1 and UCR2, followed by a highly conserved catalytic core. In contrast, short and super-short isoforms lack UCR1, and also contain a truncated UCR2 domain, respectively. The UCR1-UCR2 module interacts directly with the catalytic core, and plays a key role in regulating catalytic activity. The extreme N-terminal region is unique to each variant, and impacts the mechanisms of regulation that control its activity and subcellular location\(^8^4\). While all PDE isozymes perform nearly identical catalytic functions, the divergent N-terminal region imparts unique features that allow PDEs to shape distinct pools of cAMP and contribute to compartmentalized signaling.

Members of the PDE4 family are critical cAMP-hydrolyzing enzymes, and together with PDE3, they represent the dominant isoforms within VSMCs. The activity ratio between PDE3 and PDE4, and the expression of individual PDE4 variants is highly dependent on VSMC phenotype. The synthetic phenotype, in which PDE4 activity dominates, is associated with unhealthy cell behaviour, and various cardiovascular pathologies\(^5^9\). Drugs used to treat these disorders, including dobutamine and iloprost, often exert their effects by increasing intracellular cAMP levels. While these agents impact the dysregulated cell migration and proliferation that contributes to disease progression, cells quickly desensitize and doses must be gradually increased in order to maintain their effects. Several mechanisms account for this phenomenon, including receptor phosphorylation, β-arrestin recruitment, and receptor internalization\(^1^0^2,1^0^4\).
Previously, our laboratory reported that sustained AC activation in VSMCs triggers several downstream regulatory mechanisms, including an increase in PDE4 activity. Both contractile and synthetic VSMCs are more dependent on PDE4-mediated hydrolysis following prolonged intracellular cAMP elevation, but distinct variants are responsible for these changes in each phenotype\(^9\). Cultured rat aortic VSMCs exist in the synthetic phenotype that is characteristic of the SMCs found within the intima of diseased or damaged blood vessels; when challenged with a cAMP-elevating agent for four hours, the two PDE4D short isoforms, PDE4D1 and PDE4D2, are exclusively upregulated. In contrast, treatment of contractile rat aortic VSMCs \textit{in vivo} with a cAMP analog did not result in short isoform upregulation, but caused increased expression of PDE4D3, the dominant long PDE4D variant. Activation of PKA triggers a signaling cascade involving CREB and cAMP response element (CRE)-mediated induction of gene expression in both quiescent and activated VSMCs. However, differences in histone acetylation of the intronic promoter controlling PDE4D short isoform expression give rise to the selective induction of PDE4D1 and PDE4D2\(^89\).

This data suggests that PDE4 upregulation in synthetic VSMCs plays a critical role in desensitizing these cells to prolonged exposure to cAMP-elevating agents, and terminating further cAMP signaling. Selective inhibition of the PDE4D variants that are responsible for desensitization may increase the therapeutic value of drugs that are currently used to treat various cardiovascular diseases. Furthermore, the phenotype-dependent expression of distinct isoforms may be useful in situations in which synthetic cells should be targeted without affecting healthy, contractile VSMCs.
Research Objectives and Hypothesis

We hypothesize that a) the short PDE4D variants, PDE4D1 and PDE4D2, are highly upregulated in human synthetic VSMCs following sustained challenge with cAMP-elevating agents, b) expression of the long PDE4D isoforms is unaffected by prolonged elevation of intracellular cAMP levels, c) following upregulation induced by cAMP elevation, PDE4D1 and PDE4D2 are located within the cytosol, and are not membrane-associated, and d) induction of PDE4D1 and PDE4D2 expression is responsible for the desensitization to cAMP-elevating drugs that synthetic VSMCs experience. We suggest that selectively inhibiting the upregulation or activity of PDE4D1 and PDE4D2 in human aortic smooth muscle cells (HASMCs) would prevent cell desensitization following prolonged treatment with cAMP-elevating drugs.

Here, we expand on Dr. Hanguan Liu’s work, where he investigated the effects of prolonged cAMP elevation on PDE4D expression in rat aortic VSMCs: we aim to confirm his observations and observe the effects of prolonged cAMP-elevation in human aortic smooth muscle cells (HASMCs). The main objectives in this project are to determine the effects of cAMP elevation on the expression of PDE4D isoforms, and to investigate the role that the short isoforms play in desensitization. Using a combination of pharmacological, biochemical, and molecular biological approaches, we will identify the molecular basis underlying VSMC desensitization to cAMP-elevating drugs. Specifically, we will use an siRNA-based approach to selectively knockdown PDE4D1 expression in HASMCs. We will then observe the effects of cAMP elevation in combination with PDE4D1 knockdown on mRNA levels, protein expression, and PDE activity. Lastly, we will use cell fractionation through differential centrifugation to investigate the subcellular localization of the short isoforms of PDE4D.
Figure 1.11 Proposed mechanism through which PDE4D1 upregulation mediates HASMC desensitization. (a) We hypothesize that prolonged activation of GPCRs and ACs with cAMP-elevating agents triggers a signaling pathway involving cAMP generation at the plasma membrane, and subsequent PKA activation. (b) PKA-mediated phosphorylation of CREB leads to activation and high levels of transcription of PDE genes, including the PDE4D1 gene. High levels of PDE4D1 activity in the cytosol participate in a negative feedback mechanism, where they hydrolyze cAMP and terminate further signaling.
Chapter 2. Materials and Methods

2.1 Materials

Human aortic smooth muscle cells (HASMCs), smooth muscle basal media (SmBM-2), and smooth muscle growth media bullet kits (SmGM-2) were obtained from Lonza Group Ltd. BioLite tissue culture flasks and BioLite Multidishes were purchased from Thermo Scientific (Fischer). Bovine serum albumin (BSA) was supplied by Calbiochem. Anti-PDE4D antibody was provided by ICOS Corporation (Bothell, WA, USA). Anti-β-actin antibody was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Lipofectamine 2000, and siRNA specific for PDE4D (5’-GAC AAG CAC AAU GCU UCC GUG GAA A-3’) were obtained from Invitrogen™ by Life Technologies. Silencer® Select Negative Control siRNA #1 was purchased from Ambion® by Life Technologies, and PDE4D1-specific siRNA (5’-UUC UCA AGC GCU UCA CGG GUC CGC U-3’) was bought from Life Technologies Inc. The PGK1, GAPDH, PDE4D, and PDE4D1 primers were purchased from Sigma-Aldrich Life Science. Forskolin was ordered from Sigma-Aldrich, and 3-isobutyl-1-methylxanthine (IBMX), cilostamide, and RO 20-1724 were all obtained from Calbiochem. Immobilized Boronic Acid Gel was ordered from Thermo Scientific. [2,8-3H] Adenosine 3’, 5’-Cyclic Phosphate, Ammonium Salt was purchased from PerkinElmer, and [8-14C] Adenosine 5’ monophosphate, Ammonium Salt were purchased from Amersham Biosciences.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Effect</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fsk</td>
<td>Adenylyl cyclase agonist, increases global cAMP</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>IBMX</td>
<td>Pan- PDE inhibitor</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Cil</td>
<td>Selective PDE3 inhibitor</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>RO 20-1724</td>
<td>Selective PDE4 inhibitor</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>DMSO</td>
<td>Vehicle control</td>
<td>Fisher Scientific</td>
</tr>
</tbody>
</table>

**Table 2.1** *Drugs used to target PDE families or increase intracellular cAMP.*

### 2.2 Cell Culture

HASMCs isolated from human donors were grown in SmGM-2, consisting of smooth muscle basal media (SmBM-2) supplemented with 5% fetal bovine serum (FBS), 1% human Epidermal Growth Factor (hEGF), insulin, human Fibroblast Growth Factor-B (hFGF-B), and Gentamicin/Amphotericin B (GA-1000). The HASMCs were kept in a 37 °C incubator containing 5% CO₂/95% air. SMCs were cultured on BioLite tissue culture-treated flasks and Multidishes. At approximately 80-90% confluency, HASMCs were passaged and sub-cultured using Trypsin 0.5% EDTA. Cells used in experiments had been passaged between four to 11 times.

### 2.3 siRNA-based Knockdowns

HASMCs were plated in T-25 tissue culture flasks or in 6- or 24-well culture plates, and then grown in SmGM-2 until they reached approximately 80% confluency. At this point, cells were transfected using a mixture of lipofectamine 2000 and PDE4D, PDE4D1, or negative control siRNA. Immediately before adding transfection reagents, the SmGM-2 in which the HAMSCs were maintained, was removed and replaced with basal media, SmBM-2.
Transfections with the PDE4D, PDE4D1, and negative control siRNA were performed with a 1:1 mixture of siRNA and lipofectamine 2000, where were allowed to form a transfection complex during a 20min incubation at room temperature. Following this incubation period, the transfection complex was applied to HASMCs in SmBM-2. After a 5hr incubation at 37 °C, the SmBM-2 was removed and replaced with SmGM-2 lacking antibiotics, GA-1000. In all experiments, HASMCs were transfected twice, 24hrs apart. The knockdown efficiency of siRNA transfection was assessed using quantitative PCR (qPCR) following RNA isolation.

### 2.4 RNA Isolation, Reverse Transcription, and Quantitative PCR

Approximately 16hrs after the second siRNA transfection, RNA was isolated from the transfected HASMC monolayers and purified using the Qiagen RNeasy® Mini Kit according to manufacturer’s instructions. Reverse transcription was used to produce cDNA from purified RNA using oligo(dT)18 (Sigma-Aldrich) and the Quagen Omniscript RT kit, according to the manufacturer’s instructions. The concentration and purity of the isolated RNA was assessed using a NanoDrop 1000 (Thermo Scientific).

Quantitative real-time PCR (qPCR) amplifications were performed using Bio-Rad iTaq™ Universal SYBR® Green Supermix with 2.5 ng of cDNA template and the following primers:

- PGK (Fwd: 5’-CTGTGGGGGTATTTGAATGG-3’, Rev: 5’-CTTCCAGGAGCTCCAAACTG-3’), GAPDH (Fwd: 5’-GAGTCAACGGATTTGGTCGT-3’, Rev: 5’-TTGATTTTGAGGGATCTCG-3’), PDE4D (Fwd: 5’-ACGTGGCATGGAGATAAGCC-3’, Rev: 5’-TTGCTCTGCTTACCATTACGA-3’), and PDE4D1 (Fwd: 5’-GAAGGAGCAGCCCTCATGT-3’, Rev: 5’-TCTAGCTGGTCCAGACACCA-3’).

Thermocycler settings for qPCR amplification with the PGK, GAPDH, and PDE4D primers
were: initial denaturation step: 95 °C for 30s, followed by 40 cycles of 95 °C for 5s, annealing temperature ($T_a$) for 30s (PGK $T_a = 60$ °C, GAPDH $T_a = 60$ °C, PDE4D $T_a = 64$ °C), and then 72 °C for 30s.

2.5 Immunoblotting

Cell lysates were produced from homogenization of confluent HASMC monolayers. Cells were lysed using a triple-detergent lysis buffer, consisting of a Tris (50 mM, pH 7.4) buffer supplemented with 1% Ipegal CA-630, 10 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, 5 mM benzamidine, 10 mM sodium orthovanadate, 150 mM sodium chloride, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 10 mM sodium fluoride, 0.1 mg/mL phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL pepstatin A, 1 μg/mL E-64, 5 μg/mL bestatin, 1 μg/mL aprotinin, and 1 μg/mL leupeptin. After adding triple-detergent lysis buffer, and scraping and homogenizing the cells, the homogenate was centrifuged at 10 000 rpm for 5min to collect cell debris and membranes within the pellet fraction. The detergent-soluble fraction, or supernatant, was collected, and the protein concentration was quantified using a bicinchoninic acid (BCA) protein assay normalized to a standard curve using a pre-diluted bovine serum albumin (BSA) set (Thermo Scientific).

Protein lysates were combined with 2x or 9x SDS-PAGE loading sample buffer, consisting of a Tris (1M, pH 6.8) buffer, supplemented with 10% SDS, glycerol, β-mercaptoethanol, and 1% bromophenol blue. After boiling at 100 °C, samples were resolved by SDS-PAGE (7.5% acrylamide). Resolved lysates were transferred to PVDF membranes (0.45 μm pore size), blocked with 5% BSA in Tween 20 (TBST) buffer (Fisher Scientific) for 1hr, then immunoblotted with target-specific antibodies. The following antisera were used: anti-
PDE4D (1:4000), and anti-phospho PKA (1:5000), anti-β actin (1:10 000). To visualize immunoreactive bands, specific binding of primary antibodies was detected by chemiluminescence with horseradish peroxidase conjugated secondary antisera for 1hr.

2.6 cAMP PDE Activity Assay

HASMCs were transfected twice with appropriate siRNAs, and incubated at 37 °C for 16hrs with 10 µM forskolin and 100 µM IBMX. Cells were then lysed with triple-detergent lysis buffer in the presence or absence of 1% Ipegal CA-630, 0.1% sodium dodecyl sulfate, and 0.5% sodium deoxycholate. In subcellular fractionation experiments, the cytosolic proteins were separated from the membrane-associated proteins during cell homogenization. HASMCs were lysed using triple-detergent lysis buffer as described above, but lacking 1% Ipegal CA-630, 0.1% sodium dodecyl sulfate, and 0.5% sodium deoxycholate. Following homogenization, cells were centrifuged at 1000 rpm for 10mins at 4 °C to pellet whole cells, organelles, and debris. The supernatant was then centrifuged at 20 000 x g for 30mins at 4 °C, to produce a new supernatant (cytosolic protein) and pellet (membranes and associated proteins). The pellet was resuspended in triple detergent buffer containing 1% Ipegal CA-630, 0.1% sodium dodecyl sulfate, and 0.5% sodium deoxycholate. The three detergents were also added to the cytosolic fraction to the same final concentrations. Protein concentrations in these fractions was determined using the BCA protein assay, as described above. Cytosolic and particulate fractions were brought to the same protein concentration, not volume, in order to add an equal amount of protein by mass to each reaction in the cAMP PDE activity assay.

After protein concentrations in the lysates were determined, reactions were carried out in a total volume of 100 µL consisting of PDE assay buffer (50 mM Tris-HCl pH 7.4, 5 mM...
magnesium chloride, 0.1 mM EGTA pH 7.4), 5 µM [2,8-3H] Adenosine 3’, 5’-Cyclic Phosphate, Ammonium Salt, 1.5 or 2.5 µg of protein, 5 µM cilostamide, and 10 µM RO 20-1724. Reactions were allowed to proceed for 30-60mins at 37 °C, then stopped by boiling samples at 100 °C. [8-14C] Adenosine 5’ monophosphate (0.02 µCi/mL) was added to each sample as a recovery marker.

Columns containing Immobilized Boronic Acid Gel were washed and neutralized with PDE assay loading buffer (0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.1 magnesium chloride, pH 8.0). Samples were centrifuged at 10 000 rpm for 2mins, and then applied to columns. Columns were then washed with PDE assay loading buffer, and 5’-[3H]AMP and 5’-[14C]AMP were recovered with sodium acetate (0.05 M, pH 4.8). Scintisafe scintillation cocktail was added to a final concentration of 33%, and radiation was detected and quantified by a liquid scintillation counter, and corrected for recovery of 5’-[14C]AMP. Total cAMP phosphodiesterase activity was expressed as pmol min⁻¹ mg⁻¹ of protein. Cyclic AMP PDE activity assay data presented is obtained from three or four separate experiments.

2.7 Statistical Analysis

Statistical comparisons between two experimental groups were performed with an unpaired Student’s t-test, and presented as mean ± S.E.M. A value of p < 0.05 was considered statistically significant. Representative immunoblots and PDE assay data reflect similar results obtained in at least 2-3 separate experiments.
Chapter 3. Results

3.1 RNAi-based Knockdown of HASMC PDE4D mRNAs

Levels of the total PDE4D-related “transcriptome” (i.e. the combination of all the PDE4D transcripts) in human arterial smooth muscle cells (HASMCs) was assessed using quantitative RT-PCR with validated primer sets designed to amplify target sequences encoding the common catalytic domain of the all PDE4D mRNA transcripts (Figure 3.1). Further information on the reagents used and experimental procedures can be found in Chapter 2, Materials and Methods.

We observed that the basal level of PDE4D expression in HASMCs can be reduced significantly using a RNAi-based approach (p = 0.01). Specifically, double transfection (2 x 24hrs apart) of HASMCs with pan-PDE4D targeted siRNA designed to bind with unique PDE4D sequences within the catalytic core resulted in a marked RNAi-based knockdown of PDE4D. Thus, the most efficient of our pan-PDE4D siRNAs (Invitrogen™ HSS107723, Catalogue Number: 1299001) reduce PDE4D mRNA levels by 57% ± 10.1%, as determined through quantitative RT-PCR (Figure 3.1). Furthermore, transfection with either control or pan-PDE4D siRNAs had no significant effects on mRNA levels of the housekeeping genes analyzed in our work, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or phosphoglycerate kinase (PGK) (data not shown).

HASMCs were similarly transfected with an siRNA designed to allow knockdown of a unique PDE4D transcript (PDE4D1). The only unique sequence in PDE4D1 consists of a 45-amino acid stretch within the N-terminal region of the protein. Thus, this siRNA was designed to bind this region within the very short unique N-terminal domain-encoding sequence. Double transfection with this PDE4D1-specific siRNA reduced PDE4D mRNA levels by 35.5% ± 10.1% relative to control in HASMCs (p = 0.04), an effect less dramatic than that obtained using the
pan-PDE4D siRNA described above (Figure 3.1). Transfection with the PDE4D1-specific siRNA had no significant impact on mRNA levels of the housekeeping genes GAPDH or PGK (data not shown).
Figure 3.1 PDE4D mRNA levels in HASMCs transfected with a control siRNA, PDE4D-specific, or PDE4D1-specific siRNA.

Quantitative PCR was performed using cDNA produced from RNA isolated from HASMCs transfected twice with either a control siRNA or an equal amount of PDE4D- or PDE4D1-specific siRNA. The level of PDE4D mRNA in HASMCs transfected with PDE4D- or PDE4D1-specific siRNA is shown relative to that in cells transfected with control siRNA. Levels of PDE4D mRNA were compared to levels in HASMCs transfected with a control siRNA and treated with vehicle. Data was quantified using Bio-Rad CFX Manager software, and values were normalized to levels of the two housekeeping genes GAPDH and PGK (n = 4; p ≤ 0.05).
3.2 RNAi-based Knockdown of PDE4D Protein Levels in HASMCs

Levels of expression of PDE4D isoforms were also assessed at the protein level through immunoblotting with a pan-PDE4D antibody (Figure 3.2a). Consistent with previous work from our laboratory, and with the fact that HASMCs are known to express several PDE4D variants, immunoblot analysis of HASMC lysates with this anti-PDE4D antibody identified several anti-PDE4D proteins with molecular weights ranging between 70-105 kDa (Figure 3.2). The sizes of these proteins was consistent with expression of PDE4D3, PDE4D8, PDE4D9 (which are all approximately 93 kDa in size), PDE4D5 or PDE4D7 (which are both approximately 105 kDa), and PDE4D1 and PDE4D2 (which are known to be approximately 67-72 kDa in size)\(^{61,105}\). When lysates generated from HASMCs transfected with the pan-PDE4D siRNA were similarly analyzed, levels of the immunoreactive species with molecular weights of 70 kDa were reduced \((p = 0.03)\). Similarly, anti-PDE4D reactive proteins with molecular weight ~95 kDa and ~105 kDa, corresponding to the larger PDE4D splice variants, were also reduced in cells so-treated (Figure 3.2a)\(^{89,90}\).

Immunoblot analysis was also performed on lysates produced from HASMCs transfected with the PDE4D1-specific siRNA. This treatment did not significantly alter levels of the ~95 kDa and ~105 kDa immunoreactive species, nor those corresponding to the smaller ~70 kDa species, PDE4D1 or PDE4D2 (Figure 3.2a). This latter result was unexpected, given that the PDE4D1-targeted siRNA did significantly reduce levels of PDE4D mRNA. As predicted, levels of the loading control, β actin, were unaffected by any of the transfections performed in our studies.
Figure 3.2 Anti-PDE4D immunoreactive species in HASMC lysates and expression in cells transfected with PDE4D- and PDE4D1-specific siRNAs. (A) Lysates from HASMCs transfected with control, PDE4D-, or PDE4D1-specific siRNAs were immunoblotted with an anti-pan-PDE4D or anti-β actin antibody. (B) Densitometry analysis of the ~70 kDa immunoreactive species from immunoblots of transfected HASMCs with an anti-pan-PDE4D antibody. Levels of PDE4D protein expression were compared to levels in HASMCs transfected with a control siRNA and treated with vehicle. Data is representative of findings from 4 independent experiments.
3.3 Expression of PDE4D Isoforms in HASMCs Incubated with Forskolin and IBMX

Previously, our laboratory reported that incubation of cultured rat aortic VSMCs with a combination of the pan-AC activator, forskolin, or a metabolically stable and cell-permeable analogue of cAMP, 8-Br-cAMP, for periods exceeding 4 hours increased the of expression of PDE4D1 and PDE4D2 above their basal levels. In contrast, such treatments did not alter levels of the longer anti-PDE4D immunoreactive proteins. Consistent with this previous work, levels of the ~95 kDa and ~105 kDa anti-PDE4D-immunoreactive proteins in HASMCs used in my studies were not increased when these cells were incubated with forskolin and IBMX (Figure 3.2a). In contrast to the case with rat aortic VSMCs, levels of the ~75 kDa PDE4D-immunoreactive species were detected in control, untreated cells. However, consistent with the earlier report, prolonged incubations of these cells with the combination of forskolin and IBMX did increase the level of the ~75 kDa species, which we identify as PDE4D1. Indeed, densitometry analysis of this immunoreactive species revealed that this treatment caused a ~600% increase in expression compared to the untreated control HASMCs (Figure 3.2b).

Transfection of HASMCs with either the pan-PDE4D- or the PDE4D1-specific siRNAs prior to the treatment of these cells with forskolin and IBMX altered the impact of this cAMP-challenge and increased expression of anti-PDE4D immunoreactive proteins in these cells. In contrast, prior transfection with a control siRNA did not. Thus, transfection with the pan-PDE4D siRNA prior to treatment virtually abolished this dramatic upregulation in PDE4D1 observed in those transfected with the control siRNA (Figure 3.2b). Transfection of these cells with the PDE4D1-specific siRNA more modestly antagonized the increased expression of PDE4D1 caused by forskolin and IBMX treatments (Figure 3.2b).
The effects of forskolin/IBMX treatment on HASMC expression of PDE4D was also investigated at the mRNA level. Prolonged cAMP elevation caused a significant increase (~200%, p = 0.01) in PDE4D transcript levels compared to untreated control cells (Figure 3.2b). Furthermore, the effects of transfection with PDE4D- and PDE4D1-specific siRNA on PDE4D upregulation observed at the protein level were also reflected in the mRNA levels determined through quantitative PCR. While pan-PDE4D-specific siRNA nearly abolished the increase in PDE4D mRNA that was observed in control cells, PDE4D1-specific siRNA had a more moderate impact on the forskolin and IBMX-induced increase (Figure 3.1).

3.4 RNAi-based Knockdown of PDE4D and PDE4D1 in HASMCs Impacts PDE4 Activity

Since forskolin/IBMX increased expression of PDE4D1 and each siRNA used in our studies reduced this increase, we determined if these RNAi-based knockdowns had a similar effect on PDE4 PDE activity. This was investigated by measuring cAMP phosphodiesterase activities in these cells. To ensure that only PDE4 activity was measured, the PDE assay buffer was supplemented with a selective PDE3 inhibitor, cilostamide.

Consistent with the modest effects of both the pan-PDE4D and the PDE4D1-specific siRNAs on PDE4D protein expression in control cells (above), these siRNAs only modestly reduced basal cAMP-PDE activity and that fraction contributed by PDE4-encoded enzymes (Table 3.1). Thus, while transfection with pan-PDE4D siRNA caused a modest reduction in total cAMP-PDE4 activity, the PDE4D1-specific siRNA did not. As seen, the non-PDE4 and PDE3 activity, which is represented by the activity present in the assays conducted with RO 20-1724, was small and unchanged by transfection with these reagents (Table 3.1).
3.5 Incubation of HASMCs with Forskolin and IBMX Increases PDE4 Activity

As discussed above, treatment of HASMCs and rat aortic VSMCs with forskolin and/or IBMX increased the expression of the ~75 kDa PDE4D variant. As was the case in the amounts of mRNA and protein expression, forskolin and IBMX treatment markedly increased HASMC PDE4 activity. Thus, forskolin/IBMX treatment increased total PDE4 activity from 26.4 ± 6.32 pmol/mg/min to 41.6 ± 7.64 pmol/mg/min (Table 3.1). Consistent with its effects on untreated cells, the PDE4 inhibitor RO 20-1724 markedly reduced the rate of cAMP hydrolysis post-treatment in all cells. However, this effect was modestly reduced in cells that had been previously transfected with the pan-PDE4D siRNA. As was the case in cells not incubated with forskolin/IBMX, the ability of the PDE4D1-specific siRNA to blunt the increase in PDE4 activity was not significant (Table 3.1). Also, while there was a slight increase in non-PDE3 and PDE4 activity in cells treated with forskolin/IBMX, these values were indistinguishable in the three groups.

3.6 Sub-cellular Distribution of PDE4 Activity in HASMCs

Previous studies in the Maurice laboratory have shown that a fraction of the longer PDE4D variants (i.e. PDE4D3, PDE4D8, PDE4D9, PDE4D5, and PDE4D7) are particulate while the shorter ones, PDE4D1 and PDE4D2, are largely if not exclusively cytosolic. To investigate if the PDE4D siRNAs had a disproportionate effect on cytosolic PDE4 activity, we measured the PDE activity in isolated particulate and cytosolic fractions.

The cAMP PDE activity was determined in supernatant produced from a 20 000 x g centrifugation of HASMC lysate, and the effects of forskolin and IBMX treatment, RNAi-based knockdown, and RO 20-1724 addition were assessed. In cells transfected with control siRNA, a
16 hr incubation with the cAMP-elevating agents forskolin and IBMX caused a marked increase in enzymatic activity from 43.7 ± 11.0 pmol/mg/min to 78.9 ± 32.3 pmol/mg/min (Table 3.1). While an increase was also observed in whole cell lysates following similar treatment, the enzyme activity increased by 80.5% in the cytosolic fraction, compared to 57.6% in the whole cell fraction (Table 3.1). Furthermore, the presence of RO 20-1724 reduced the forskolin and IBMX-induced increase in activity to 34.4%, compared to the 80.5% increase that was observed in its absence (Table 3.1).

The impact of RNAi-based knockdown of PDE4D splice variants was also investigated in the cytosolic fraction. cAMP was hydrolyzed at 31.2 ± 10.8 pmol/mg/min in the cytosolic fraction isolated from HASMCs transfected with PDE4D-specific siRNA, which is much lower than the rate observed in control cells (Table 3.1). This effect of PDE4D knockdown on cytosolic enzyme activity is consistent with its impact on cAMP PDE activity in whole cell lysates. As was the case in both whole cell lysates and in the cytosolic fraction from control HASMCs, incubation with forskolin and IBMX caused a marked increase in cytosolic enzyme activity in cells transfected with PDE4D-specific siRNA (Table 3.1). While PDE4D knockdown failed to attenuate the treatment-induced increase in activity in whole cell lysates, a 65.7% increase was observed in the cytosolic fraction of transfected cells compared to the 80.5% increase exhibited by control cells (Table 3.1). The effect of RO 20-1724 addition was also consistent between subcellular fractions: the PDE4 inhibitor greatly reduced the rate of cAMP hydrolysis in both whole cell lysates and in the cytosolic fraction (Table 3.1).

The cAMP PDE activity in the particulate, or membrane, fraction produced from the 20 000 x g centrifugation was also determined. Interestingly, while forskolin and IBMX-treatment caused a marked increase in enzyme activity in both whole cell lysates and cytosolic fractions
across all RNAi-based knockdowns, the rate of cAMP hydrolysis in the membrane fraction post-treatment was not significantly different from that observed in untreated cells (Table 3.1). Furthermore, PDE4D knockdown had a much more modest effect on enzyme activity in the particulate fraction compared to the other sub-cellular fractions: in untreated cells, membrane-associated enzyme activity in cells transfected with PDE4D-specific siRNA was only 20.0% lower than that in treated HASMCs (Table 3.1).
<table>
<thead>
<tr>
<th>Condition</th>
<th>Whole Cell</th>
<th>Cytosol</th>
<th>Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>26.4 ± 6.32 (n=3)</td>
<td>43.7 ± 11.0 (n=2)</td>
<td>12.6 ± 0.796 (n=2)</td>
</tr>
<tr>
<td>PDE4D siRNA</td>
<td>22.1 ± 5.77 (n=3)</td>
<td>31.2 ± 10.8 (n=2)</td>
<td>10.5 ± 3.59 (n=2)</td>
</tr>
<tr>
<td>PDE4D1 siRNA</td>
<td>25.6 ± 11.9 (n=2)</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Control + RO 20-1724</td>
<td>7.74 ± 4.12 (n=3)</td>
<td>16.3 ± 5.74 (n=2)</td>
<td>5.44 ± 0.043 (n=2)</td>
</tr>
<tr>
<td>PDE4D siRNA + RO 20-1724</td>
<td>8.43 ± 2.61 (n=3)</td>
<td>18.0 ± 9.85 (n=2)</td>
<td>3.21 ± 1.56 (n=2)</td>
</tr>
<tr>
<td>PDE4D1 siRNA + RO 20-1724</td>
<td>7.34 ± 1.34 (n=2)</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td><strong>+ Fsk/IBMX</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>41.6 ± 7.64 (n=3)</td>
<td>78.9 ± 32.3 (n=2)</td>
<td>11.4 ± 1.99 (n=2)</td>
</tr>
<tr>
<td>PDE4D siRNA</td>
<td>38.0 ± 9.29 (n=3)</td>
<td>51.7 ± 22.2 (n=2)</td>
<td>8.73 ± 2.06 (n=2)</td>
</tr>
<tr>
<td>PDE4D1 siRNA</td>
<td>43.6 ± 10.7 (n=2)</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Control + RO 20-1724</td>
<td>15.0 ± 4.44 (n=3)</td>
<td>21.9 ± 8.90 (n=2)</td>
<td>3.61 ± 1.60 (n=2)</td>
</tr>
<tr>
<td>PDE4D siRNA + RO 20-1724</td>
<td>10.1 ± 2.77 (n=3)</td>
<td>17.3 ± 8.13 (n=2)</td>
<td>5.22 ± 1.55 (n=2)</td>
</tr>
<tr>
<td>PDE4D1 siRNA + RO 20-1724</td>
<td>11.2 ± 2.38 (n=2)</td>
<td>----</td>
<td>----</td>
</tr>
</tbody>
</table>

**Table 3.1** Effects of forskolin and IBMX or RO 20-1724 on cAMP PDE activity in HAMSCs
Chapter 4. Discussion

4.1 Summary of Findings

The cyclic nucleotide phosphodiesterases hydrolyze the second messengers cAMP and cGMP, and therefore play a key role in regulating their signaling. There is considerable interest in determining how these PDEs contribute to compartmentalized cAMP signaling, and furthermore, how they are involved in normal physiological processes and disease progression. Specifically, we are interested in the members of the PDE4D subfamily, and their impact on vascular smooth muscle cell function and cardiovascular disease. My findings provide evidence of cAMP-regulated expression of short isoforms of PDE4D, and shed light on a potential link between PDE4D1 upregulation and HASMC desensitization to therapeutically relevant drugs.

This study is an extension of the earlier work of Dr. Hanguan Liu, a past member of the Maurice Laboratory, who investigated the mechanisms of regulation of PDE4D expression in rat aortic VSMCs. Dr. Liu observed the effects of prolonged cAMP-elevation on the expression and enzymatic activity of PDE4D splice variants in rat aortic VSMCs, and using a combination of pharmacological, biochemical, and molecular biological approaches, identified key signaling cascades involved in mediating this induction of expression of PDE4D variants. Interestingly, Liu noted that activators of these signaling cascades attenuates the cAMP-mediated desensitization to prolonged activation of cAMP effectors. Further work by Dr. Douglas Tilley identified elevated levels of histone acetylation of the intronic promoter regulating PDE4D1 expression, which underlies the phenotype-dependent induction of PDE4D expression following prolonged cAMP-elevation in cultured rat aortic VSMCs.

In my studies, cell biological, pharmacological, and biochemical techniques were applied in experiments with HASMCs in order to confirm the effects of cAMP-elevation that were
previously observed in rat aortic VSMCs. We determined that PDE4D isoforms are upregulated following treatment with forskolin and IBMX at the protein and mRNA levels: specifically, PDE4D1 is uniquely up-regulated. This induction of expression is also seen at the level of cAMP PDE activity, through large increases in enzymatic activity post-treatment. Our findings also show that the treatment-induced increase in PDE4 activity is enriched in the cytosolic subcellular fraction. Furthermore, cAMP accumulation brought about by forskolin addition is impacted by RNAi-based knockdown of PDE4D.

Based on this work, I conclude that PDE4D1 expression is upregulated in HASMCs treated with forskolin and IBMX, and propose that this upregulation could lead to desensitization of these cells to further cAMP-elevating challenges. Moreover, I submit that one could exploit these events to improve the therapeutic value of existing drugs used to treat cardiovascular disease.

4.2 Forskolin and IBMX Induce the Expression of PDE4D1 and PDE4D2 in HASMCs

Previously, it was reported that prolonged treatment of rat aortic VSMCs with either forskolin or 8-Br-cAMP induced upregulation of PDE4D1 and PDE4D2. Immunoblot analysis of lysates from treated cells revealed four anti-PDE4D immunoreactive species, including the shortest PDE4D splice variants, which were not markedly less abundant in untreated control cell homogenates. Using a combination of molecular biological and immunological techniques, we observed similar effects on PDE4D expression following similar treatment of HASMCs, and identified PDE4D1 as the major PDE4D variant upregulated.

In both rat and human aortic SMCs, the “long-form” PDE4D variants PDE4D3/8/9 and PDE4D5/7 are expressed under basal conditions, as demonstrated by Liu and Maurice.105
through immunoblotting as described above. While expression levels of these splice variants is unaffected by prolonged increases in cAMP in these earlier studies, these enzymes were activated following PKA-mediated phosphorylation in rat aortic VSMCs. Immunoblot analysis of lysates of rat aortic VSMCs allowed for confirmation of PDE4D3 and PDE4D5 expression under basal conditions, on the basis of molecular weight derived from electrophoretic mobility. Phosphorylation of PDE4D3 and PDE4D5 occurs following incubation with forskolin or 8-Br-cAMP, as evidenced by an upward shift in the electrophoretic mobility of these isoforms. Treatment with calf intestinal alkaline phosphatase (CIAP) and pre-incubation with the selective PKA inhibitor, H89, both attenuated or completely abolished the forskolin-induced shift in the mobility of PDE4D3. The inhibitory effects of CIAP and H89 suggests that the upward shift in the electrophoretic mobility of PDE4D3 is the consequence of PKA-mediated phosphorylation\textsuperscript{105}. Our immunoblot studies did not allow us to detect changes in the mobility of any of the PDE4D variants investigated here. We are of the opinion that this is perhaps due to the modest immunoreactive signals obtained in our studies compared to those which were obtained in earlier studies.

However, it is also important to note that the effects of forskolin treatment on rat aortic VSMCs were rapid in onset, and maximal PDE4D3 phosphorylation was observed after only 20min incubation\textsuperscript{105}. Lysis and immunoblot analysis of HASMCs, however, was performed following 16hr incubation with forskolin and IBMX. Thus, differences observed could also be due to time-related differences between the two studies.

Two immunoreactive species of 75 kDa and 65 kDa were detected in both rat and human aortic SMCs following treatment with cAMP-elevating agents\textsuperscript{90}. These anti-PDE4D immunoreactive proteins were identified as PDE4D1 and PDE4D2 on the basis of molecular
weight. Interestingly, these isoforms only appear in rat aortic VSMC lysates following prolonged incubation with cAMP-elevating agents. In contrast, phosphorylation of PDE4D3 occurs rapidly, and the shift to the slower migrating phosphorylated species saturates after only 20min incubation\textsuperscript{105}. Addition of the inhibitors of transcription and protein synthesis, actinomycin D\textsuperscript{108} and cycloheximide\textsuperscript{109}, abolished the treatment-induced upregulation of PDE4D1 and PDE4D2 in rat aortic VSMCs, suggesting that changes in gene expression are responsible for the appearance of these splice variants.

Further work by Tilley and Maurice identifies epigenetic changes that contribute to PDE4D short form expression in rat aortic VSMCs. The intronic promoter controlling PDE4D1 and PDE4D2 expression contains a CRE, which together with CREB, plays a critical role in regulating expression of these splice variants in response to prolonged cAMP signaling\textsuperscript{89}. Post-translational modifications of the histones directly interacting with the PDE4D short-form promoter further contributes to regulation of gene transcription. Consistent with previous data demonstrating that histone acetylation is associated with transcriptional activation in VSMCs\textsuperscript{110}, the intronic promoter controlling PDE4D1 and PDE4D2 was found to be highly acetylated in rat aortic VSMCs\textsuperscript{89}. Furthermore, differences in levels of H3-acetylation within the short-form promoter were observed between VSMC phenotypes: the intronic promoter was hyperacetylated in synthetic/activated SMCs, relative to contractile/quiescent cells\textsuperscript{89}. This epigenetic discrepancy is likely responsible for the selective induction of PDE4D1 and PDE4D2 that is uniquely observed in synthetic rat aortic VSMCs, compared to contractile cells. Further studies to investigate the levels of histone modifications within the PDE4D1/PDE4D2 promoter are required to determine if a similar mechanism of regulating transcriptional availability causes selective short-form upregulation following forskolin and IBMX treatment in HASMCs.
4.3 Incubation with Forskolin and IBMX Increases PDE4 Activity in HASMCs

As described above, assays of cyclic nucleotide PDE activity revealed that 16hr incubation of HASMCs with forskolin and IBMX caused a marked increase in activity. To identify which variants are responsible for this increase, RNAi-based knockdown and pharmacological inhibition of PDE4 were used. Since our cAMP-PDE activity measurements were conducted in reaction buffers supplemented with the PDE3 inhibitor cilostamide, our studies assessed non-PDE3, cAMP PDE activity in HASMCs transfected with PDE4D-specific siRNA, and the treatment-induced increase in activity was further attenuated with addition of the PDE4 inhibitor, RO 20-1724. The effects of PDE4D knockdown and addition of RO 20-1724 are consistent with a role for the upregulation of PDE4D1 and PDE4D2, which was observed through immunoblotting and quantitative PCR.

Similar treatment of rat aortic VSMCs with forskolin or 8-Br-cAMP for 16hrs produces a significant increase in total cAMP PDE activity. This increase was attributed to increased cytosolic PDE3A and particulate PDE3B activity, and notably, altered cytosolic and particulate PDE4 activities\(^{90,111}\). Short-term incubation with the same cAMP-elevating agents, however, impact total cAMP PDE activity through distinct PDE isoforms. Liu and Maurice described a PKA- and PKC-mediated mechanism of phosphorylation and activation of PDE4D3, which occurs only 20mins after treatment with forskolin or 8-Br-cAMP.

Long PDE4D isoforms, including PDE4D3, are activated by PKA-mediated phosphorylation of a serine residue within the extreme N-terminal end of the upstream conserved region, UCR1\(^77\). The effects of PKA-phosphorylation can be mimicked through mutation of the target serine residue to aspartate or glutamate, or replacement of an adjacent glutamate residue.
with a neutral amino acid; thus, the interaction between this target serine residue and a glutamate residue is likely critical to this mechanism of PDE4 activation. Under basal conditions, the serine and glutamate residues participate in an ion-pair interaction, which keeps the PDE in a low activity state. PKA-catalyzed phosphorylation of the serine residue then disrupts this interaction and activates the enzyme\textsuperscript{77}.

PKA-mediated phosphorylation and consequent activation of long isoforms of PDE4 is a critical component of a feedback regulatory system, which also involves an extracellular-signal-regulated kinase (ERK). PDE4D3 activity is markedly inhibited by ERK-catalyzed phosphorylation of a serine residue within the C-terminal region of the catalytic core\textsuperscript{112}. Inhibition of PDE4D3 activity then causes an increase in intracellular cAMP levels, ultimately leading to activation of PKA. PKA-mediated phosphorylation of a target serine residue within UCR1 then negates the inhibitory effects of ERK-phosphorylation, and leads to a marked activation of PDE4D3 through disruption of an ion-pair interaction, as discussed above\textsuperscript{113}. Overall, ERK activation and subsequent phosphorylation and activation of PDE4D3 causes a transient increase in cAMP levels, which is quickly overturned through the action of PKA phosphorylation. Thus, regulation of PDE4D3 represents a point of cross-talk between the PKA and ERK signaling cascades. Immunoblotting analysis of rat aortic VSMC lysates, as described above, indicate that this mechanism of activation of PDE4D3 is likely responsible for the increase in PDE4 activity observed after short-term challenge with cAMP-elevating agents.

Long-term incubation with cAMP-elevating agents, however, leads to upregulation of PDE4D short-forms in both rat and human aortic SMCs, as evidenced by quantitative PCR and immunoblotting data. Prolonged exposure to forskolin or 8-Br-cAMP was also accompanied by a significant increase in total PDE4 activity in rat cells. This increase was attenuated by addition of
actinomycin D or cycloheximide, indicating that *de novo* mRNA and protein synthesis is required for this treatment-induced change in cyclic AMP PDE activity\textsuperscript{90}. Therefore, post-translational PKA- and ERK-mediated phosphorylation and subsequent activation of PDE4 long isoforms cannot account for increased cAMP hydrolysis in response to sustained treatment with cAMP-elevating agents. Together, the absence of an upward shift in PDE4D3 electrophoretic mobility and the upregulation of PDE4D1/PDE4D2 strongly suggest that the short isoforms of PDE4 are responsible for increased cAMP PDE activity in HASMCs following 16hr incubation with forskolin and IBMX.

The activities of the long- and short-isoforms of PDE4D are regulated through distinct mechanisms. Whereas ERK-phosphorylation of a target serine residue at the C-terminal end of the catalytic core has an inhibitory effect on long PDE4 isoforms, short isoforms are activated\textsuperscript{114}. This discrepancy is the result of differences in the modular structure of long splice variants compared to short isoforms. Specifically, the absence of UCR1 in the short PDE4 isoforms, including PDE4D1 and PDE4D2, significantly impacts how these variants are regulated. The intact UCR1/UCR2 module is required to coordinate the actions of both ERK- and PKA-phosphorylation to produce a transient increase in cAMP, followed by PDE4D and eventual cAMP hydrolysis\textsuperscript{114}. In PDE4D1, however, the bare UCR2 unit redirects ERK-phosphorylation to cause enzyme activation\textsuperscript{115}. Further work must be done to determine if these mechanisms of regulation contribute to increased cAMP PDE activity in HASMCs following treatment-induced upregulation of PDE4D1 and PDE4D2.

### 4.4 Forskolin and IBMX-Induced Changes in PDE4 Activity are Enriched in the Cytosol
Prolonged incubation of rat aortic VSMCs with forskolin or 8-Br-cAMP produced significant increases in total cAMP PDE activity. Cell fractionation through differential centrifugation revealed that this treatment-induced increase in cAMP hydrolysis is the result of increased activity of cytosolic PDE3A, particulate PDE3B, and PDE4 in both fractions. As described above, HASMCs were similarly subjected to differential centrifugation, to determine which subcellular compartment is most impacted by forskolin and IBMX treatment. Marked increases in both total and cytosolic cAMP PDE activity were observed following exposure to these cAMP-elevating agents. While treatment-induced increases in total PDE activity in rat aortic SMCs were the result of PDE3-catalyzed cAMP hydrolysis, all experiments performed in HASMCs were performed in the presence of the selective PDE3 inhibitor, cilostamide; therefore, changes in the rates of cAMP hydrolysis that were observed following prolonged challenge with forskolin and IBMX must be the product of a different PDE family.

The dominant PDE families responsible for cAMP hydrolysis in VSMCs are PDE3 and PDE4. Despite pharmacological inhibition of PDE3, forskolin and IBMX treatment produced a large increase in cAMP PDE activity in HASMCs. Quantitative PCR data indicates that incubation of HASMCs with these cAMP-elevating drugs causes a marked increase in expression of PDE4D mRNA. Furthermore, treatment results in expression of two anti-PDE4D immunoreactive species, PDE4D1 and PDE4D2, which are not present in untreated control cells. Together, this data strongly suggests that upregulation of these short PDE4D isoforms is responsible for the increase in cAMP hydrolysis that is observed following prolonged challenge with forskolin and IBMX.

To determine which subcellular compartments contain short PDE4D isoforms, we used cell fractionation and differential centrifugation of HASMC lysates before assaying cAMP PDE
activity. As was the case in the whole cell lysates, the rate of cAMP hydrolysis in the cytosolic fraction was markedly increased in HASMCs treated with forskolin and IBMX. Consistent with a role for PDE4D1 and PDE4D2 upregulation, this increase was attenuated with either transfection with PDE4D-specific siRNA, and addition of RO 20-1724. However, in the particulate, or membrane-associated, fraction, prolonged exposure to these cAMP-elevating agents failed to produce a significant increase in cAMP PDE activity: in cells transfected with either control or PDE4D-specific siRNA, the rate of cAMP hydrolysis within the particulate fraction was approximately equal, and in some cases slightly lower, in treated HASMCs compared to their untreated, control counterparts. This data indicates that the short PDE4D isoforms which are upregulated in response to cAMP elevation are restricted to the cytosolic fraction within HASMCs.

The unique N-terminal region of several PDE isoforms target specific variants to subcellular structures. For example, a β-arrestin binding site within the N-terminal region of PDE4D5 tethers this enzyme to the plasma membrane, where it creates and shapes distinct pools of cAMP. PKA-catalyzed phosphorylation within the N-terminal UCR1 unit of PDE4D3 not only increases enzymatic activity, as discussed above, but also causes translocation of this PDE4D splice variant from the particulate to the cytosolic fraction of rat aortic VSMCs. The coordinated phosphorylation of PDE4D3 that occurs following short-term exposure to forskolin, and subsequent translocation, is dependent on coordinated PKA- and ERK-mediated phosphorylation. Because the PKA consensus site and target serine residue lie within the UCR1 region which the PDE4D short isoforms lack, PDE4D1 and PDE4D2 cannot be targeted to the cytosolic fraction through this mechanism. However, the absence of a unique N-terminal region in short isoforms still plays a role in determining the subcellular compartments these splice
variants are targeted to. Several PDE isoforms contain subcellular targeting sequences within this N-terminal region, which PDE4D1 and PDE4D2 lack. Therefore, we hypothesize that the short PDE4D variants are restricted to the cytosolic fraction of HASMCs. This is supported by the cAMP PDE activity assay data described above: increases in total cAMP PDE activity caused by forskolin and IBMX-induced upregulation of PDE4D1 and PDE4D2 were also observed in the cytosolic fraction, but not in the particulate fraction of HASMCs.

4.5 Future Directions

A. Improve selectivity and efficiency of PDE4D1 and PDE4D2 knockdown

Future work is needed to improve the effectiveness of the RNAi-based approach that was used to selectively knock down PDE4D1 in HASMCs. Quantitative PCR data described above demonstrated that whereas PDE4D-specific siRNA was able to nearly abolish the forskolin and IBMX-induced increase in PDE4D mRNA observed in control cells, PDE4D1-specific siRNA had a much more modest effect. Immunoblotting analysis reveals that incubation with these cAMP-elevating drugs only alters the expression of two immunoreactive species, which were identified as PDE4D1 and PDE4D2 on the basis of molecular weight. If the RNAi-based knockdown of the short PDE4D isoforms was highly efficient, its impact on treatment-induced increases in mRNA expression should be similar in magnitude to that observed in pan-PDE4D knockdown with siRNA. Selective knockdown of these splice variants using a RNAi-based approach has proven challenging, as the unique N-terminal region in PDE4D1 consists of a mere 45-amino acid stretch. Furthermore, this amino acid sequence is absent in the super-short isoform, PDE4D2. In future studies, where highly efficient, selective knockdown of these splice variants is required, an RNAi-based approach may not be sufficient. Instead, targeted genome
editing methods, such as CRISPR/Cas9 knockout screening, may be employed to investigate the effects of PDE4D1 and PDE4D2 on cAMP signaling, and HASMC desensitization\textsuperscript{116}.

**B. Explore how PDE4D1 and PDE4D2 Impact cAMP Accumulation in HASMCs**

Future work is required to investigate the effects of prolonged challenge with cAMP-elevating agents, and subsequent upregulation of PDE4D1 and PDE4D2 on desensitization to prolonged cAMP signaling. This could be accomplished using assays of cAMP accumulation in HASMCs, in which cells pre-incubated with forskolin and IBMX are treated with increasing concentrations of a cAMP-elevating agent. We would predict that increasing concentrations would generate greater levels of intracellular cAMP, and that control cells would quickly desensitize to its effects, as evidenced by a plateau in the dose-response curve at higher concentrations. To determine if forskolin and IBMX-induced upregulation of short PDE4D isoforms plays a key role in this desensitization, we would assess the effects of RNAi-based and pharmacological knockdown of PDE4D1 and PDE4D2 on cAMP accumulation.

RNAi-based knockdown of PDE4D reduced basal PDE4 mRNA and protein expression, and enzyme activity; therefore, we predict that activators of adenylyl cyclase should effect a greater increase in intracellular cAMP in HASMCs transfected with PDE4D-specific siRNA. Data collected from one experiment was consistent with a role for PDE4D1 and PDE4D2 in mediating cAMP accumulation in HASMCs: combined treatment with forskolin and transfection with PDE4D-specific siRNA reduced the level of desensitization observed. RNAi-based knockdown caused a statistically significant, albeit modest, increase in intracellular cAMP levels at each concentration of forskolin added, compared to control cells. Thus, it would seem that PDE4D knockdown in HASMCs potentiated forskolin-induced increases in cAMP, and reduced the cAMP-mediated desensitization that occurs at higher concentrations.
Similar experiments were conducted in rat aortic VMSCs, as described by Liu and Maurice\textsuperscript{90}. Rat aortic SMCs were incubated with cAMP-elevating agents for 16hrs prior to assays of intracellular cAMP accumulation in cells treated with isoproterenol, a β-AR agonist capable of producing increases in intracellular cAMP levels. Combined treatment of cells with forskolin and phorbol myristate acetate (PMA), which reduces PDE4 activity and attenuates the cAMP-induced increase in cAMP PDE activity, improved the effectiveness of isoproterenol addition\textsuperscript{90}. Together, this data is consistent with a role for PDE4D1 and PDE4D2 expression, in desensitization to cAMP-elevating agents in both rat and human aortic SMCs.

While the data obtained is internally consistent with the effects of PDE4D knockdown and inhibition which were observed at the mRNA, protein, and enzyme activity levels, the cAMP accumulation assay needs to be repeated with multiple biological replicates and with a diverse range of cAMP-elevating agents. In the future, addition of cilostamide or the PDE3/PDE4 dual selectivity inhibitor zardaverine would provide insight on the PDE4-specific effects on cAMP-mediated desensitization of HASMCs. Cells that were not incubated with forskolin and IBMX should also be included as an additional control to investigate the effects of increased expression of short PDE4D isoforms.

C. Investigate the effects of prolonged challenge with forskolin and IBMX in contractile HAMSCs

Tilley and Maurice explored the effects of prolonged incubation with forskolin or 8-Br-cAMP on PDE4D expression in both synthetic/activated and contractile/quiescent rat aortic VSMCs. Long-term challenge of rat aortic VSMCs caused selective upregulation of distinct PDE4D splice variants in each phenotype: while incubation of contractile/quiescent VSMCs with a cAMP analog \textit{in vivo} produced a significant increase in PDE4D3 expression, similar treatment
of cultured synthetic/activated VSMCs induced expression of the short PDE4D isoforms. Consistent with this previous work, we observed induction of PDE4D1 and PDE4D2 expression, with no change in levels of PDE4D3 or PDE4D5, in cultured HASMCs following 16hr incubation with forskolin and IBMX. To confirm our hypothesis that short PDE4D isoforms are uniquely upregulated in synthetic HASMCs, the use of an in vivo model would provide insight on the effect that sustained challenge with cAMP-elevating agents has on contractile HASMCs.

Tilley and Maurice also describe phenotype-dependent epigenetic discrepancies that contribute to selective PDE4D isoform induction: higher levels of histone acetylation at the intronic PDE4D1/PDE4D2 promoter in synthetic VSMCs promotes their expression in response to prolonged exposure to cAMP-elevating drugs. Chromatin immunoprecipitation using anti-acetylated histone H3 antibody, followed by PCR amplification of recovered DNA would prove useful in exploring the underlying cause behind selective PDE4D isoform expression in HASMCs.

Further work is also needed to confirm whether de novo mRNA and protein synthesis is required for forskolin and IBMX-induced upregulation of PDE4D1 and PDE4D2 in HASMCs. We hypothesize that treatment-induced upregulation of these PDE4D variants will be prevented through inhibition of transcription and protein synthesis with actinomycin D and cycloheximide, respectively.

**D. Examine PDE4D1 and PDE4D2 as potential therapeutic targets**

Due to their role in cAMP-mediated desensitization, and phenotype-dependent expression in response to prolonged challenge with cAMP-elevating agents, the short PDE4D isoforms represent attractive therapeutic targets. In treating various cardiovascular diseases, including cardiac failure and pulmonary arterial hypertension, cell desensitization negatively impacts the
efficacy of pharmacological intervention\textsuperscript{102,103}. Selectively targeting and inhibiting the molecular mechanism behind this desensitization would raise the therapeutic value of these drugs, by maintaining desired effects on cell behaviour and therefore disease progression at lower doses. In the future, development of highly selective inhibitors of PDE4D1 and PDE4D2 would impact HASMC desensitization to agents that exert their effects through cAMP signaling, and may prove useful in treating a wide range of cardiovascular pathologies.
Figure 4.1 Proposed mechanism through which PDE4D1 upregulation mediates HASMC desensitization. We propose that (a) prolonged activation of GPCRs and ACs with cAMP-elevating agents triggers a signaling pathway involving cAMP generation at the plasma membrane, and subsequent activation of cAMP effectors. (b) cAMP signaling induces expression of PDE4D1 and PDE4D2, which are found in the cytosol. High levels of PDE4D1 activity in the cytosol participate in a negative feedback mechanism, where they hydrolyze cAMP and terminate further signaling and contribute to HASMC desensitization.
References


59. Houslay MD, Baillie GS, Maurice DH. cAMP-Specific Phosphodiesterase-4 Enzymes in the Cardiovascular System: A Molecular Toolbox for Generating Compartmentalized
cAMP Signaling. Circ Res. 2007;100:950-967. doi:10.1161/01.RES.0000261934.56938.38.


102. Baliga BS, Pronczuk AW, Munro HN. Mechanism in a Cell-free of Cycloheximide...


