DIFFERENTIAL CYCLIC NUCLEOTIDE PHOSPHODIESTERASE 3 EXPRESSION IN CONTRACTILE AND SYNTHETIC VASCULAR SMOOTH MUSCLE CELLS

by

HEATHER ANNE DUNKERLEY

A thesis submitted to the Department of Pharmacology & Toxicology in conformity with the requirements for the degree of Master of Science

Queen's University

Kingston, Ontario, Canada

November, 2001

QUEEN'S UNIVERSITY AT KINGSTON

SCHOOL OF GRADUATE STUDIES AND RESEARCH

PERMISSION OF CO-AUTHORS

I/we, the undersigned, hereby grant permission to microfilm any material designated as being co-authored by me/us in the thesis copyrighted to the person named below:

Name of copyrighted author	Signature of copyrighted author
Heather Dunkerley	H Dunkerley

Name(s) of co-author(s)	Signatures of co-author(s)
Sandra Jimmo	S Jimmo
DANIEL PALMER	
Doug Tilley	Doug Tilley
Donald Maurice.	Donald Maurice
H. Liu.	H. Liu

DATE: Dec. 21, '01

ABSTRACT

Reocclusion of coronary arteries after angioplasty is a severe complication of ballooning in which migration and proliferation of vascular smooth muscle cells (VSMC) results in neointimal formation. This event occurs despite the fact that the primary function of VSMC in healthy arteries is contraction, not proliferation. It is well established that cyclic nucleotide levels affect migration and proliferation of VSMC. Consequently, it was hypothesized that the pattern of expression of cyclic nucleotide phosphodiesterases (PDE), which hydrolyze cyclic nucleotides, differ between proliferating (synthetic), and non-proliferating (contractile) VSMC. To determine the role of PDE in neointimal formation, I investigated the expression of the cAMP PDE that account for the majority of PDE activity in rat vasculature. Phosphodiesterase 3 (PDE3) and phosphodiesterase 4 (PDE4) expression was determined during *in vitro* culturing of primary rat aortic VSMC, and in rat neointimal VSMC, formed in response to balloon catheter-induced injury. Our results identify changes in the expression of PDE3A, a PDE3 variant, in both models. Thus, while PDE3 activity in rat aorta was approximately 65% of the total PDE activity, it was significantly less in cultured VSMC (approximately 25% of total PDE activity). This decrease in PDE3 activity correlated with a decrease in PDE3 mRNA and protein levels. Similarly, in intimal VSMC formed in response to balloon injury, PDE3 activity was 25% of total PDE activity, compared to 65% of total activity in medial VSMC. Again, PDE3A expression was reduced in intimal VSMC relative to levels in medial VSMC. Thus, similar changes in PDE3 expression occurred during culturing and *in vivo* activation of VSMC. Of potential therapeutic interest was the fact that human VSMC in culture displayed a parallel loss of PDE3A expression,

compared to freshly isolated human VSMC. These findings are significant in the context of appropriate targeting of PDE variants in attempts to inhibit neointimal formation following vascular injury.

CO-AUTHORSHIP

I would like to acknowledge Dr. Donald H. Maurice for his participation in writing and in designing this work. In addition, I would like to acknowledge Daniel Palmer for providing data used in the generation of Table 2.1 and Figure 2.3, and Hangaun Liu for providing immunoblotting data used in the generation of 2.1C. Also, I would like to acknowledge Douglas Tilley and Sandra Jimmo for their contribution to experiments involving balloon-catheterization surgeries and isolation of primary VSMC, respectively.

ACKNOWLEDGEMENTS

Human aortic VSMC were derived from isolated explants from thoracic aorta that were generously provided by Drs. J. G. Pickering (The John P. Robarts Research Institute, London, Ontario, Canada) and C. Graham (Queen's University, Kingston, Ontario, Canada), respectively.

I am also grateful to Drs. S. Wolda and Cathy Farrell for providing the PDE4D specific monoclonal antibody used in these studies.

In addition, the selective anti-PDE3 antisera used in this study were a generous gift of Dr. J.A. Beavo, Department of Pharmacology, University of Washington, Seattle, WA.

Finally, I would like to thank my supervisor, Dr. Donald H. Maurice for his guidance and his encouragement throughout this project. I would also like to thank members of our laboratory who make the lab an enjoyable place to be, and who are always willing to offer assistance. Thank you to Sandra Jimmo, Dan Palmer, Doug Tilley, Stu Netherton and Dan Raymond. Finally, I want to thank my family and friends for their support in all I do.

TABLE OF CONTENTS

Chapter 2 – Manuscript

Chapter 3 - General Discussion

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AC	adenylyl cyclase
AKAP	A-kinase anchoring proteins
ANP	atrial natriuretic peptide
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
COX-2	cycloosygenase-2
CRE	cyclic AMP-response element
CREB	cyclic AMP-response element binding protein
DAPI	4',6'-Diamidino-2-phenylindole Dihydrochloride, hydrate
DMEM	Dulbecco's modified Eagle's medium
DTAF	Dichlorotriazinyl Amino Fluorescein
ECL	enhanced chemiluminescence
EDRF	endothelium derived relaxing factor
EGTA	ethylene-bis (oxyethylenenitrilo) tetraacetic acid
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
FSK	forskolin
GC	guanylyl cyclase

G-protein	GTP-binding protein
GPCR	G-protein coupled receptor
H & E	hematoxylin and eosin
HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid
IP3	inositol trisphosphate
K_m	Michaelis constant
MAPK	mitogen-activated protein kinase
MEK	ERK kinase
MLCK	myosin light chain kinase
mRNA	messenger ribonucleic acid
NO	nitric oxide
PDE	phosphodiesterase
PDGF	platelet-derived growth factor
PKA	protein kinase A, or cAMP-dependent protein kinase
PKG	cGMP-dependent protein kinase
PMSF	phenylmethylsulfonyl fluoride
ROM	reactive oxygen-metabolites
RT-PCR	reverse transcriptase – polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
TEMED	N,N,N',N'-tetramethylethylenediamine
TTBS	Tween-supplemented Tris-buffered saline
VSMC	vascular smooth muscle cell

Chapter 1-Introduction

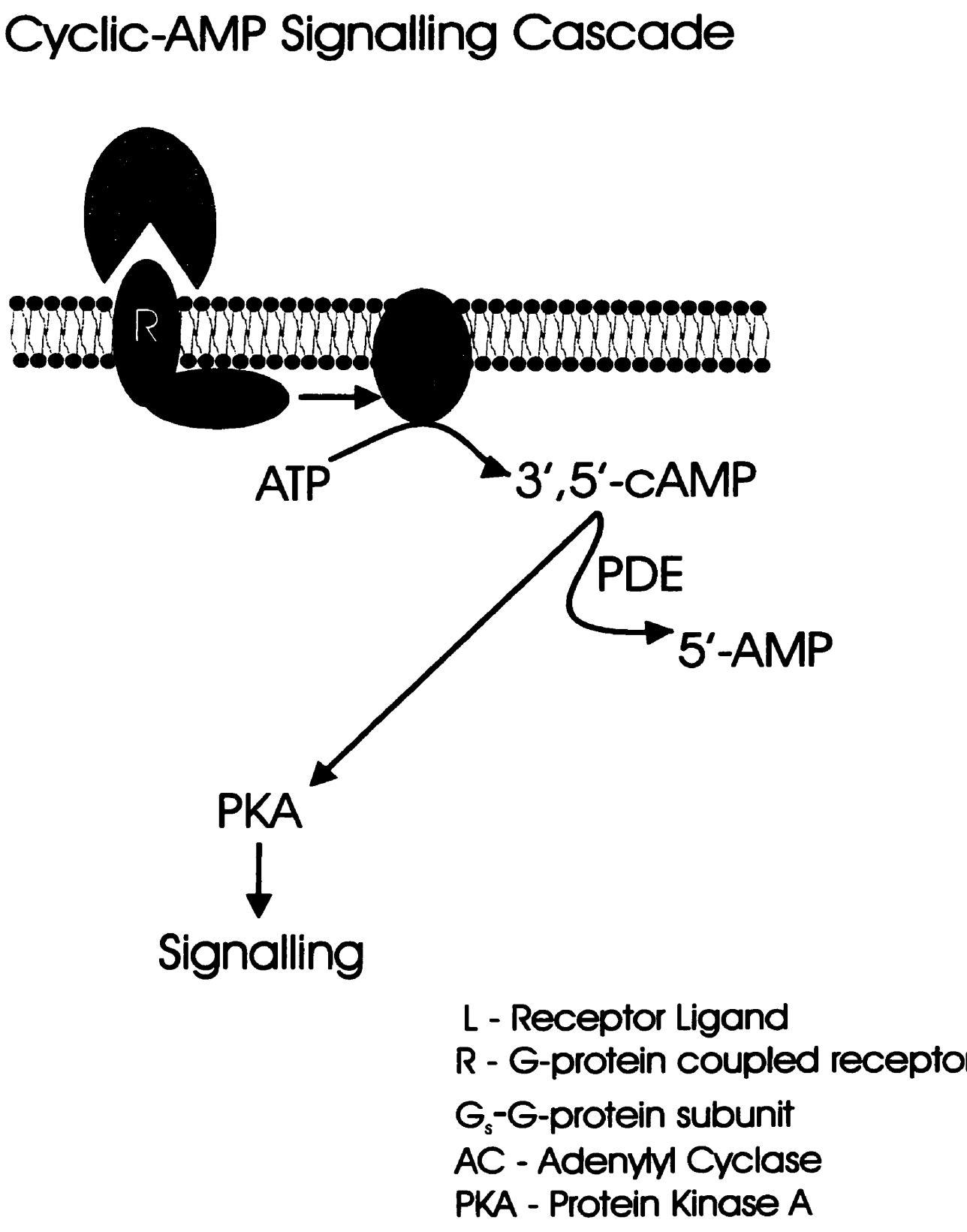
1.1 Cyclic Nucleotide Intracellular Signalling

Second messengers play a central role in eliciting intracellular responses by extracellularly applied agents such as hormones, neurotransmitters, and drugs. Many of these responses are mediated through the cyclic nucleotides, cAMP and cGMP, which have been shown to play a crucial role in cell growth and differentiation as well as in transcriptional regulation. cAMP and cGMP are synthesized by adenylyl cyclases and guanylyl cyclases, respectively, following initiation by the appropriate extracellular signals. The resulting signalling cascade involving cAMP or cGMP, is terminated by cyclic nucleotide phosphodiesterases (PDE), which hydrolyze the cyclic nucleotides. PDE hydrolyze the 3'-phosphodiester bond of cAMP or cGMP to form the corresponding biologically inactive noncyclic products, 5'-AMP and 5'-GMP, respectively (Dousa, 1999). PDE are the only intracellular enzymes that catalyze this reaction in mammalian cells. A general overview of the cAMP signalling cascade is illustrated in Figure 1.1.

1.1.1 Synthesis of cAMP

G protein-coupled receptors (GPCR) are integral to the process of cAMP production. The diversity of these plasma membrane bound receptors enables intracellular translation of extracellular signalling molecules such as nucleotides, peptides, amines, Ca^{2+} and photons resulting in cAMP formation (Rana *et al.*, 2001). Structurally, GPCR consist of an N-terminal extracellular domain, a C-terminal intracellular domain, and seven transmembrane spanning domains, that are involved in binding agonists and antagonists, and interaction with heterotrimeric GTP binding proteins. Once bound, a ligand such as epinephrine causes a conformational change in

Figure 1.1


Cyclic-AMP Signalling Cascade
R
ATP
3',5'-cAMP
PDE
5'-AMP
PKA
Signalling
L - Receptor Ligand
R - G-protein coupled receptor
G_s-G-protein subunit
AC - Adenylyl Cyclase
PKA - Protein Kinase A

the receptor that leads to activation of the dominant adenylyl cyclase activating heterotrimeric G protein (G_s), following exchange of GDP for GTP. Dissociation of the GTP-bound α subunit from the dimeric β_γ subunit allows the α_s to activate adenylyl cyclase, a major effector molecule of some G proteins, including G_s. Intrinsic GTPase activity of the α_s subunit causes hydrolysis of GTP to GDP and leads to reassociation of the G protein subunits, resulting in termination of its effector activity (Radhika and Dhanasekaran, 2001). Although other mechanisms can lead to activation of adenylyl cyclases, this heterotrimeric G protein mechanism dominates in many cells including vascular smooth muscle cells (VSMC), the major cell type studied in my research.

1.1.2 Adenylyl Cyclases

Adenylyl cyclases (AC) are the proteins responsible for the synthesis of cAMP from ATP. Currently, there are ten known isoforms, nine membrane-bound and one soluble form (Patel *et al.*, 2001). Similar to GPCRs, their isoform multiplicity and tissue diversity facilitates differential intracellular signaling from a single extracellular event. The isoforms share significant sequence homology, as well as overall three-dimensional structure. All membrance-bound ACs possess two hydrophobic domains, each consisting of six transmembrane spans. Two large cytoplasmic domains constitute the catalytic site, one between the two hydrophobic domains, and one at the C terminus (Patel *et al.*, 2001). Although the $G\alpha_s$ subunit is known to stimulate activity of all AC isoforms, several AC isoforms are also regulated by other G proteins such as $G\alpha_i$ and $G\beta_\gamma$, by protein kinases, phosphatases, calcium and Ca^{2+}/CaM (Hanoune, 2001). The response to these modulators varies depending on the specific AC isoform. As such, isoform multiplicity

enables integration of several signal transduction pathways through cross-talk (Hanoune, 2001).

A principal pharmacological tool for studying cAMP-mediated effects in cells has been forskolin (FSK), since it activates all known isoforms of AC. FSK is purported to activate AC by binding to the catalytic site of the enzyme and stabilize the active conformation. In this way, FSK augments intrinsic AC activity and increases the production of cAMP.

1.1.3 Cyclic AMP-Dependent Protein Kinase

Most well documented effects of cAMP are mediated through the activation of cyclic AMP-dependent protein kinases (PKA). PKA are heterotetramers consisting of two regulatory (R) subunits and two catalytic (C) subunits (Steinberg and Brunton, 2001). Two isoforms of PKA are known, and these are classified as type I (PKA-RI) or type II (PKA-RII), based on their different R subunits. Both of these isoforms have been identified in vascular smooth muscle, where PKA-RI is primarily cytosolic, and PKA-II is particulate (Edwards and Scott, 2000). In the absence of cAMP, PKA exists in an inactive state. This stringent control is maintained by the regulatory subunits that prevent the catalytic site from establishing its active form (Murray, 1990). When cAMP levels increase to a significant level, they bind to the R subunit. Binding of cAMP to the R-subunits is a highly coordinated process in which the two cAMP binding sites influence one another's affinity for cAMP. Since the cAMP-ligand subunits have lower affinity for the C subunit, cAMP binding brings about dissociation of the heterotetramer, and release of the active catalytic subunits (Murray, 1990). For as long as cAMP levels remain elevated, catalytic subunits phosphorylate cellular protein substrates, and thus modulate

cell function. Once cAMP levels are reduced, the R and C subunits reassociate and render PKA inactive. Differential subcellular targeting of PKA-RII is achieved through interaction of the R subunit with specialized anchoring proteins called A-kinase anchoring proteins (AKAPS). These proteins are members of a large family that are produced in a cell-specific manner, including in vascular smooth muscle (Edwards and Scott, 2000). Because of their various cellular locations and their cell specific expression, AKAPS play an essential role in differential intracellular targeting and compartmentalization of cAMP signalling (Houslay and Milligan, 1997).

In VSMC, PKA activation has been associated with relaxation (Stull *et al.*, 1982). Intracellular targets for PKA in smooth muscle include phospholamban and a sarcolemmal Ca^{2+}-pumping ATPase, which both serve to reduce intracellular Ca^{2+}, resulting in relaxation (Pitari, 2001). Another significant target for PKA is a transcription factor known as cAMP-response element –binding factor (CREB). PKA activates CREB by phosphorylation, with the serine residue at position 133 playing a central role. Transcription of targeted genes is then modulated at the promoter site by binding of CREB to the cAMP-response element (CRE) (Brindle and Montminy, 1992).

1.1.4 Guanylyl Cyclases

The second messenger cGMP is an intracellular second messenger that regulates cellular function through events such as protein kinase activation, cyclic nucleotide phosphodiesterase activation or inhibition, and direct gating of ion channels (Lucas *et al.*, 2000). Guanylyl cyclases (GC), the enzymes responsible for the synthesis of cGMP from GTP are present in most tissues. There are two forms of guanylyl cyclases, the soluble

GC and the particulate GC forms. These two families of GC differ in their kinetic properties, structure, cellular location, and regulation.

Soluble guanylyl cyclases (sGC) are heterodimers composed of two subunits, an α and a β subunit. Each subunit contains a heme-binding domain as well as dimerization, and catalytic domains. In vascular smooth muscle, nitric oxide (NO) is a significant endogenous activator of sGC. NO activates sGC by binding to the heme prosthetic group. Although much less potent, carbon monoxide (CO) is also an endogenous ligand that binds in a manner similar to NO (Chinkers and Garbers, 1989).

Particulate guanylyl cyclases (pGC) are membrane bound proteins, consisting of a single transmembrane domain, an extracellular domain and an intracellular domain. Binding of peptide ligands, such as atrial natriuretic peptide (ANP), to the extracellular domain results in activation of pGC, thereby increasing cGMP synthesis. The intracellular domain is a regulatory domain that possesses dimerization properties, which is necessary for catalytic function (Chinkers and Garbers, 1989). The catalytic domain, also located on the intracellular domain of pGC, is highly homologous with the catalytic domain of sGC and those of AC.

1.1.5 Cyclic GMP-Dependent Protein Kinase

The principle mediator of cGMP signaling is PKG. Two genes for PKG have been identified in mammals, PKGI and PKGII, with only PKGI being expressed in VSMC (Lucas *et al.*, 2000). PKGI is a cytosolic protein with an overall structure similar to that of PKA. As with PKA, in the presence of cGMP, PKG is activated and phosphorylates target proteins. However, activation of PKG does not involve the dissociation of R and C subunits. PKGI is hypothesized to regulate $[Ca^{2+}]_i$ by

phosphorylating several targets including the IP3 receptor and phospholamban which are involved in smooth muscle relaxation (Raeymaekers *et al.*, 1990). Other targets are L-type Ca^{2+} channels and the Ca^{2+} activated K^{+} channel, which are both involved in tone maintenance, and myosin light chain phosphatase that mediates vasodilation (Surks *et al.*, 1999). Many of the peptide substrates for PKG are also substrates for PKA, implying a cross-over of function. However, co-localization studies indicate that subcellular compartmentalization may dictate kinase activity (Pfeifer *et al.*, 1999). Therefore an increase in one cyclic nucleotide (eg cAMP) may not compensate for a decrease in the other cyclic nucleotide (eg cGMP). In this way, PKA and PKG may serve similar, yet independent physiological functions in vascular smooth muscle.

1.2 Cyclic Nucleotide Phosphodiesterases (PDE)

Maintaining balance between synthesizing enzymes and catabolizing enzymes is essential in any signalling cascade. Therefore, cyclic nucleotide PDE, which are responsible for the catalysis of cAMP and cGMP to their respective 5' noncyclic nucleotides, play a key role in cyclic nucleotide signalling. In addition, it is noteworthy that the maximal capacity for cyclic nucleotide hydrolysis by PDE is ten fold greater than the maximal synthetic capacity of cyclases (Dousa, 1999). This imbalance in catalytic capacity accentuates the relative importance of PDEs in affecting physiological functions within the cell.

Ten different gene families encompassing at least 30 isoforms of PDE have been identified in mammalian tissues (Beavo, 1995; Conti *et al.*,1995; Manganiello *et al.* 1995; Degerman *et al.* 1997; Houslay *et al.* 1998; Soderling *et al.* 1998; Fisher *et al.* 1998; Hayashi *et al.* 1998; Fujishige *et al.* 1999; Soderling *et al.* 1999).

The classification of gene families is based on the enzyme's primary amino acid sequence. Although the catalytic domain situated in the carboxy-terminal region is highly homologous in all PDE, individual family members differentiate with respect to their amino and COOH-terminal region sequences, and regulatory sites (Beavo, 1995). An overview of PDE family characteristics is presented in Table 1.

Table 1.1 Cyclic Nucleotide Phosphodiesterase Families of Isoenzymes

Family	K_m (μM)	Selective Inhibitors	Number of Genes	Number of Splice Variants	Ref
PDE1 Ca^{2+}-calmodulin stimulated	cAMP: 10-50 cGMP: 1-3	Vinpocetine	3	1A: 2 1C: 5	a
PDE2 cGMP-stimulated	cAMP: 30 cGMP: 15	EHNA	1	2A: 2	a
PDE3 cGMP- inhibited	cAMP: 0.1-0.3 cGMP:0.03-0.3	Cilostamide Cilostazol Milrinone	2	3A: 2	a
PDE4 cAMP-specific	cAMP: 1-3 cGMP: >300	Ro 20-1724 Rolipram	4	4A: 5 4B: 3 4C: 3 4D: 5	a
PDE5 cGMP-specific	cAMP: >100 cGMP: 4 -20	Sildenafil Zaprinast	1	5A: 2	a
PDE6 cGMP-specific in re	cAMP: >100 cGMP: 4 -20	Dipyridamole IBMX	3	6A: 2 6B: 2	a
PDE7 cAMP-specific	cAMP: 0.2		2	7A: 2	a
PDE8 cAMP-specific	cAMP: 0.15	Dipyridamole	2		b
PDE9 cGMP-specific	cAMP: 230 cGMP: <0.15	Zaprinast	1		b

PDE10 cAMP-inhibited	cAMP: 0.26 cGMP: 7.2	Dipyridamole	1		b
PDE11	cAMP: 1 cGMP: 0.5	Zaprinast Dipyridamole	1	11A: 3	b

Reference: (a) Beavo, 1995; (b) Francis *et al.* 2001

1.2.1 Cyclic Nucleotide Phosphodiesterases in Vascular Smooth Muscle

The development of PDE isoform selective inhibitors, combined with the use of PDE regulators such as calmodulin and cGMP, has facilitated the development of a PDE activity profiles in vascular smooth muscle of many species including human, rat, dog, rabbit, bovine and pig (Polson and Strada, 1996). The cGMP hydrolysing PDE in vascular smooth muscle are PDE1 and PDE5, while the cAMP hydrolysing PDE are PDE3, PDE4.

1.2.2 PDE1

PDE1 is a Ca^{2+}/calmodulin stimulated PDE that exists in smooth muscle of many species. However, its activity, as a percentage of total cGMP hydrolysing activity varies among species (Polson and Strada, 1996). Three different gene products are expressed differentially in various blood vessels. PDE1A and PDE1B both have a higher affinity for cGMP than for cAMP; however, PDE1C has an equal affinity for cGMP and cAMP (Polson and Strada, 1996). Vinpocetine is an effective selective inhibitor of PDE1A and PDE1B, but is a poor inhibitor of PDE1C. In keeping with its inhibition of PDE1 activity, vinpocetine potentiates GC activation.

1.2.3 PDE5

PDE5 is a calmodulin-independent cGMP specific PDE found in several species. Its activity in human artery accounts for approximately 70% of the total cGMP specific

hydrolyzing activity (Polson and Strada, 1996). Several inhibitors specific for PDE5 exist, including zaprinast, dipyridamole, and the novel anti-impotence therapeutic, sildenafil. Similar to PDE1 inhibitors, PDE5 inhibitors also potentiate the effects of GC activators, such as increased vascular relaxation (Pitari, 2001).

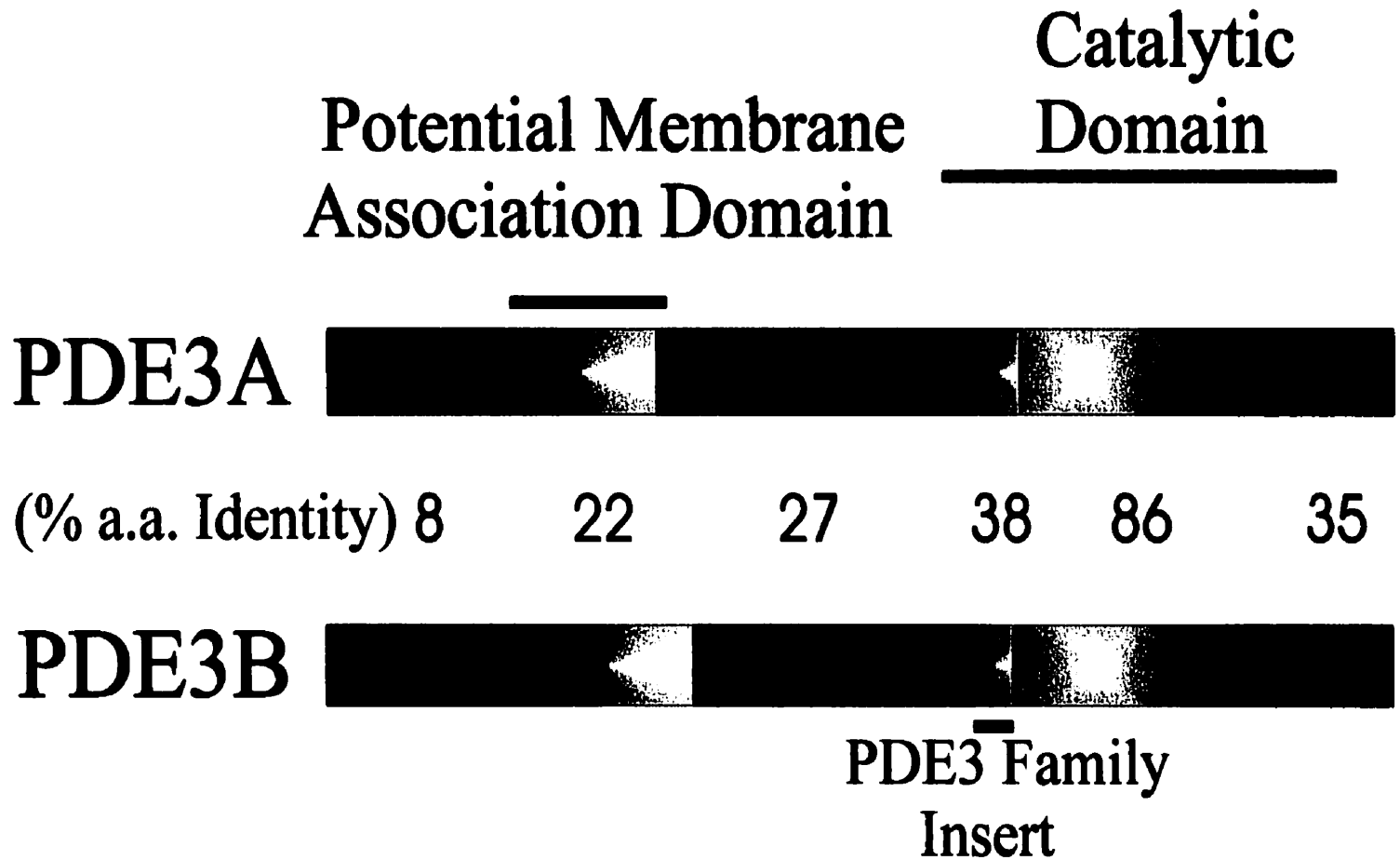
1.2.4 PDE2

Although PDE2 is abundant in vascular endothelial cells, its expression in VSMC is very low (Polson and Strada, 1996). PDE2 hydrolyzes both cGMP and cAMP. Due to its low abundance in vascular smooth muscle, PDE2 inhibitors have no potentiating effects when used in conjunction with activators of either adenylyl or guanylyl cyclases.

1.2.5 PDE3

PDE3 has a low K_m value (0.1-1 μM) for both cAMP and cGMP, although PDE3 is considered a cAMP PDE, because of its 10 fold greater V_{max} for cAMP (Beavo, 1995). In fact, cGMP (due to its affinity for PDE3) acts as a competitive inhibitor of cAMP hydrolysis by PDE3. Therefore, PDE3 is often referred to as the cGMP-inhibited PDE (Manganiello *et al.*, 1995). Because of the inhibitory effects of cGMP, PDE3 is an important enzyme in cross-talk between cAMP and cGMP signalling. There are two genes encoding PDE3 enzymes, PDE3A and PDE3B. Figure 1.2 illustrates the domain structure of PDE3A and PDE3B. Initially it was thought that only PDE3A was expressed in cardiovascular tissues, such as vascular smooth muscle and cardiomyocytes, while PDE3B was expressed in adipose tissue. It is now clear that both are present in vascular smooth muscle and cardiac muscle (Liu and Maurice, 1998; Palmer and Maurice, 2000). Although both gene products are expressed in vascular smooth muscle, PDE3A is considerably more abundant than PDE3B. Agents such as milrinone that have been

Domain structure of PDE3



Adapted from J. Biol. Chem. 1997 272: 6823-6826.

Figure 1.2

previously used clinically to stimulate myocardial contractility, inhibit platelet aggregation, and relax vascular smooth muscle, elicit their effects, at least in part, through inhibition of PDE3. Other PDE3 inhibitors include cilostamide and cilostazol.

Since PDE3 accounts for 60% of the total cAMP hydrolysis in vascular smooth muscle, it is reasonable to speculate that PDE3 is an attractive pharmacological target. Among other things, its significant role in vascular smooth muscle is demonstrated by the fact that combined use of an AC activator and a PDE3 inhibitor results in synergistic relaxation of rat aorta (Maurice and Haslam, 1990).

1.2.6 PDE4

PDE4 is a cAMP specific enzyme that is insensitive to cGMP inhibition. It has a K_m value between 1 and 4 μM, which is 10-fold higher than that of PDE3. In vascular smooth muscle, PDE4 activity is lower than PDE3 activity, but it still contributes 20-30% of the total cellular cAMP hydrolytic activity (Polson and Strada, 1996). Due to the existence of four different genes that undergo alternate splicing, and possess alternate promoters, there are many PDE4 isoforms (Burns *et al.*, 1996). The four genes are PDE4A, PDE4B, PDE4C, and PDE4D. Although PDE4A, PDE4B, and PDE4D have all been detected in vascular smooth muscle, PDE4D isoforms are the most abundant (Liu *et al.*, 2000).

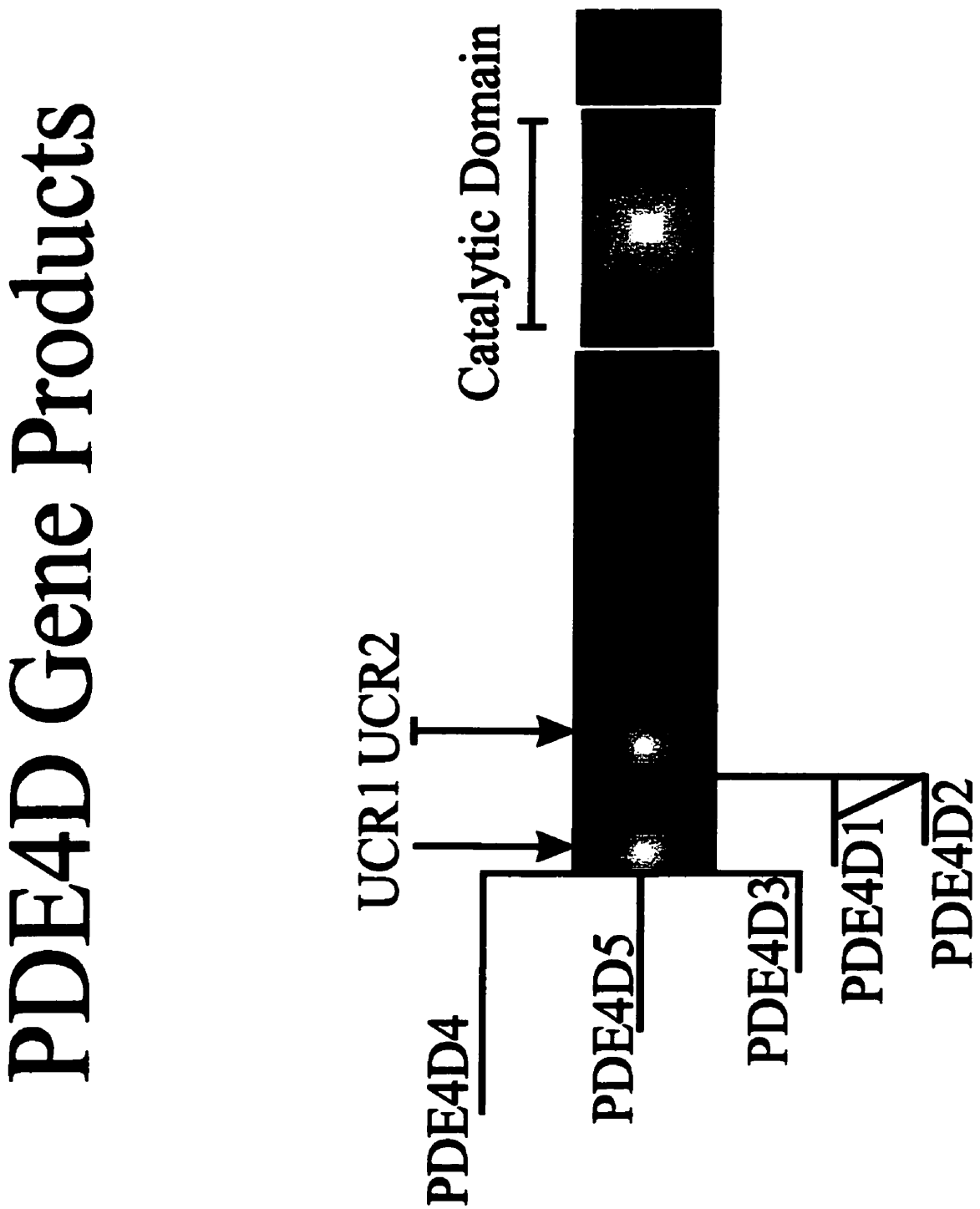
The PDE4D gene gives rise to 5 mRNAs due to alternate splicing and the use of alternate intronic promoters. The protein products of these mRNAs have been designated PDE4D1, PDE4D2, PDE4D3, PDE4D4, and PDE4D5 (Houslay *et al.*, 1998). Based on the use of two different promoters, these isozymes are either short forms (PDE4D1, PDE4D2), or long forms (PDE4D3, PDE4D4, PDE4D5). Two groups of sequences with

high levels of homology are found in PDE4D long isozymes that are not found in other PDE. Lying upstream of the catalytic domain, they are called upstream conserved regions (UCR1 and UCR2) (Bolger *et al.*, 1993) and are separated by a less conserved linker region. While long forms of PDE4D contain both UCRs, the short form contain only UCR2. The PDE4D gene products are shown in Figure 1.3. It has been suggested that these regions are involved in conformational changes necessary for modulating catalytic activity (Houslay *et al.*, 1998). Regulation of the PDE4D isozymes by phosphorylation has been demonstrated through activation of cAMP-PKA signalling, and through activation of the PKC-Raf-MEK-ERK signalling cascade (Conti *et al.*, 1995; Liu *et al.*, 2000).

Studies using the PDE4 specific inhibitors rolipram or Ro 20-1724, have shown that unlike PDE3, PDE4 inhibitors are poor relaxants of endothelium-denuded isolated blood vessels (Polson and Strada, 1996). However, in preparations where endothelium is present, PDE4 inhibition is effective in vasorelaxation (Lugnier and Komas, 1993). This may be due to the presence of the endothelium derived relaxing factor (EDRF) that results in NO formation and subsequent activation of GC. Since cGMP has inhibitory effects on PDE3, cAMP concentrations increase (Maurice and Haslam, 1990). This combined PDE3 inhibition and cAMP increase renders PDE4 the major cAMP hydrolysing enzyme, resulting in greater physiological effects following pharmacological inhibition (Lugnier and Komas, 1993).

Other functional differences in PDE3 and PDE4 are evident from studies on migration and proliferation. Unlike PDE3 inhibition, PDE4 inhibition potentiates the anti-migratory and anti-proliferative effects of activation of AC in cultured VSMC.

Figure 1.3


PDE4D Gene Products
Catalytic Domain
UCR1 UCR2
PDE4D4
PDE4D5
PDE4D3
PDE4D1
PDE4D2

PDE3 inhibitors acted synergistically with PDE4 inhibitors, but did not elicit significant effects on their own (Palmer *et al.*, 1998). This synergistic effect of PDE3 and PDE4 simultaneous inhibition is consistent with evidence showing a super-additive increase in intracellular cAMP concentrations (Rose *et al.*, 1997).

1.3 Blood Vessel Structure

Large arteries such as the aorta are composed of three basic layers, including the intima, media, and adventitia (Burkitt, 1993). In healthy vessels, the intima surrounds the lumen and consists of a single layer of flattened endothelial cells supported by a layer of elastin, called the internal elastic lamina. Several endothelial derived molecules, including prostacyclin (PGI-2), endothelial cell-derived relaxing factor (EDRF-NO), endothelin (ET-1), and platelet-derived growth factor (PDGF) have significant effects on blood vessel function (Banai, 2001). The internal elastic lamina lies next to the media, and is composed of VSMC, enclosed within concentric elastin and collagen fibres. The VSMC within this layer control the contractility of the blood vessel. Proper tone maintenance within individual blood vessels is significant because it can have an impact on blood pressure, and therefore the entire systemic circulation. Furthest from the lumen, and separated from the media by the external elastic lamina, is the tunica adventitia, which is composed of loose collagen and sparse adventitial fibroblasts.

1.3.1 Vascular Pathologies

Atherosclerosis of the coronary arteries is considered the primary cause of ischemic heart disease. Formation of a fibrous plaque is generally considered to be the type of lesion involved in well-developed atherosclerosis. Plaque formation occurs over a period of time, after accumulation of lipid, multiple cell types and extracellular matrix

protein. Generally, it is believed that lesions progress following localization of macrophages and lipid droplets. The lesion advances as macrophage cells and VSMC accumulate. Accumulation of extracellular matrix proteins, lipids and minerals causes progression of a lesion to an advanced stage, leading to significant blood vessel narrowing, stenosis.

Currently, vascular occlusions are treated most commonly by balloon angioplasty. This procedure involves insertion of a balloon catheter into the occluded artery, followed by its inflation such that the area of narrowing within the blood vessel is dilated. The success of this procedure however, is limited by the development of restenosis in 30-50% of patients within six months of undergoing this treatment (Indolfi *et al.*, 1998).

Endothelial denudation and damage to medial VSMC that results from use of the balloon catheter is a significant initiating event in the progression of restenosis, or re-narrowing of the blood vessel. Endothelium is a potential regulator of arterial wall homeostasis. Once lost, its role as a barrier to the underlying VSMC is eliminated. In addition, endothelial secretion of factors that regulate VSMC proliferation, growth, migration and death is affected (Allaire and Clowes, 1997). An initial event in restenosis is platelet accumulation directly at the site of damage. PDGF released by platelets is freely accessible to the underlying VSMC, and triggers release of basic fibroblast growth factor (bFGF) from damaged endothelial and arterial smooth muscle cells. These two growth factors stimulate proliferation of vascular smooth muscle cells within the media. The second phase of the response is mediated by VSMC that migrate toward the lumen by traversing the extracellular matrix and cross the internal elastic lamina (Libby and Tanaka, 1997). Once in the intima, VSMC accumulate by proliferating, forming a

neointimal layer. Although endothelial cells recover and grow over the originally denuded surface, their critical ability to synthesize endothelial derived relaxing factor-NO is diminished after mechanical injury.

1.4 Differentiation of Vascular Smooth Muscle Cells

The principal function of VSMC in a mature and healthy blood vessel is contraction. Under these conditions, VSMC exist in a differentiated state in which proliferation and migration occur at very low rates (Owens, 1995). However, VSMC demonstrate phenotypic plasticity. During embryonic development, smooth muscle cells that resemble fibroblasts are involved in vasculogenesis, the de novo formation of blood vessels (Walsh and Takahashi, 2001). Angiogenesis, the process in which new blood vessels are extended from existing blood vessels, such as in leiomyogenic tumorigenicity, is also facilitated by proliferative SMC resembling fibroblasts. Furthermore, vascular pathologies such as atherosclerosis and restenosis, involve VSMC of a dedifferentiated phenotype in which proliferation and migration are central functions (Walsh and Takahashi, 2001). This reversible process of conversion between 'contractile' and 'synthetic' VSMC has been termed 'phenotypic modulation' (Sobue *et al.*, 1999). In addition to rates of proliferation and migration, characteristics such as morphology, protein expression and transcriptional regulation have been established for these VSMC phenotypes. Generally, modulation from a contractile to a synthetic phenotype is associated with a loss of differentiation markers such as contractile proteins, and an increase in the expression of extracellular matrix proteins and cytokines (Denger *et al.*, 1999). Several contractile proteins are specialized to smooth muscle. These include specific isoforms of contractile proteins such as actin (α-actin), myosin heavy chains

(SM1, SM2), myosin light chains (LC17a, LC17b), as well as regulators of contraction such as calponin, caldesmon, vinculin, and tropomyosin (Owens, 1995). Their expression during this time of development is often used as an indication of VSMC differentiation. Although they are not expressed simultaneously, the appearance of one is often considered a marker for differentiation. One of six actin isomers, α-actin is often the earliest expressed contractile proteins, and is therefore often associated with the initiation of a differentiated VSMC (Owens, 1995). In addition to a decrease in contractile proteins, dedifferentiated cells also exhibit a decrease in levels and activities of proteins that facilitate contraction. Integrins that are critical for appropriate interactions between cells and between the cell and the extracellular matrix differ in their subunit make-up after maturation. Although these correlations exist between phenotype and protein expression, little is known regarding the underlying molecular events that affect transcriptional regulation of differentially expressed proteins. It is also unclear, pertaining to atherosclerosis and restenosis, whether changes in the state of diffentiation are the cause or the consequence of the pathology.

An opposing theory to 'phenotypic modulation' of blood vessels is that of a sub-population expansion. It is argued that within every blood vessel, smooth muscle cells lie in a wide range of differentiation states. In the case of atherosclerosis and restenosis, proliferating VSMC result from an expansion of pre-existing dedifferentiated VSMC, rather than a modulation of an existing differentiated VSMC. However, irrespective of the initial identity and origin of the lumen occluding VSMC, its removal is of paramount clinical importance.

1.5 Mitogen-Activated Protein Kinases

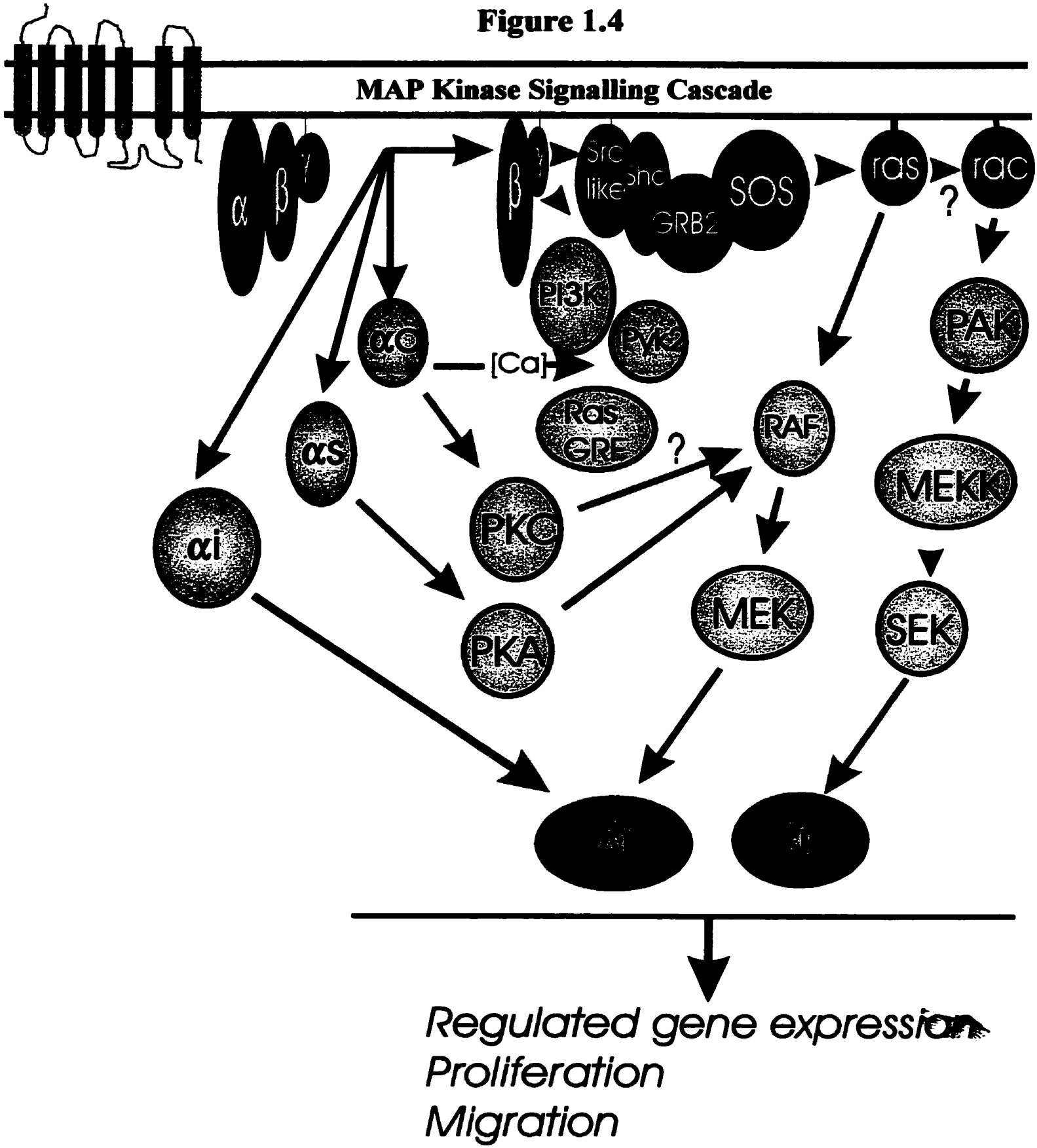
Mitogen-activated protein kinases (MAPK) are serine-threonine protein kinases that mediate cell growth, differentiation, and apoptosis. Several extracellular stimuli such as stress, cytokines, neurotransmitters and growth factors are initiators of sequential phosphorylation of proteins that ultimately lead to MAPK activation (Mii *et al.*, 1996). These upstream MAPK kinases are called MAPKK and MAPKKK. The three families of MAPK include extracellular signal-regulated kinases (ERKS), stress-activated protein kinases (SAPKS) or c-Jun NH2-terminal kinases (JNKs), and p38/RK. With regard to mitogenic responses, growth factor stimulation leading to ERK activation has been most widely studied. In this signal transduction cascade, binding of growth factor such as PDGF to membrane-associated receptors leads to activation of Ras and Raf, which in turn activate MEK and ERK (Zou *et al.*, 1998). An overview of the MAPK signalling cascade is shown in Figure 1.4. Beyond playing a significant role in proliferation and regulation of cell cycle progression, MAPK may regulate other biological effects such as differentiation, cell attachment, SMC contraction, and protein synthesis, depending on the stimuli (Bornfeldt *et al.*, 1997).

1.6 Cyclic-AMP Mediation of VSMC Migration and Proliferation

Previous studies involving VSMC in culture have demonstrated that increasing cAMP levels through AC activation, or PDE inhibition markedly inhibits proliferation (Indolfi *et al.*, 1997).

Studies focused on the signaling events leading to inhibition of proliferation, following cAMP increases, have reported crosstalk between PKA and several points in mitogenic signal transduction pathways. Some of these points of convergence are mediated through inhibition of Ras-Raf, inhibition of phosphatidyl inosital-3-kinases

Figure 1.4


MAP Kinase Signalling Cascade
α
β
γ
β
Src
like
Shc
GRB2
SOS
ras
?
rac
PI3K
PAK
αq
[Ca]
PKC
Ras
GRF
?
RAF
αs
MEKK
αi
PKC
MEK
SEK
PKA
Regulated gene expression
Proliferation
Migration

(PI3K), inhibition of cyclin-dependent kinase (cdk), and induction/repression of gene responses.

Activation of the MAPK/ERK cascade in VSMC can occur by several growth factors, including PDGF. PKA can inhibit this cascade, although the point of inhibition is unclear. Several studies showing decreased Raf-1 activity following increased cAMP levels suggest that PKA inhibits the MAPK cascade through phosphorylation of Raf-1 (Bornfeldt *et al.*, 1997).

It has been shown that cAMP levels vary during the cell cycle, which consists of four phases including G1, S (DNA synthesis), G2, and M (mitosis). Low levels of cAMP are required during the transitions from late G1 to S, and from late G2 to M in order for the cycle to progress (Rybalkin and Bornfeldt, 1999). Therefore, increased levels of cAMP inhibit VSMC proliferation by arresting them primarily in the G1/S phase, but also in the G2/M phases of the cell cycle (Bornfeldt and Krebs, 1999). cAMP may elicit its effects through cyclin dependent kinases (cdk), that play a significant role in regulating the progression of the cell cycle. Following stimulation by growth factors, cyclins increase and form complexes with cdks, allowing the transition from G1 to S in the cell cycle. Increased cAMP, resulting in PKA activation may arrest the cell cycle by causing a down-regulation of the cyclins necessary to activate cdks. PKA phosphorylates Raf-1 and inhibits its kinase activity, therefore inhibiting the MAPK cascade and results in attenuation of proliferation (Inoue *et al.*, 2000). Finally, PKA may mediate its inhibitory effects through promoter elements such as CRE. CRE binding proteins such as CREB are directly phosphorylated by PKA, and therefore may elicit its effects by differentially

repressing or enhancing transcription of genes related to cell proliferation (Bornfeldt and Krebs, 1999).

Although it is generally thought that MAPK activation leads proliferation, studies have shown that this response may vary, depending on the downstream enzymes expressed by a particular cell. For instance, in SMC expressing COX-2, such as in selected newborn strains, MAPK activation by PDGF-BB leads to significant synthesis of PDE2, and a resulting activation of PKA. This then leads to inhibition of proliferation through the same mechanisms previously described. This growth inhibitory effect of MAPK activation was not observed in SMC not expressing COX-2 (Bornfeldt *et al.*, 1997). Thus, SMC cell type is an important factor in determining the effects of MAPK activation on growth responses.

1.7 Animal Models of Balloon Angioplasty

Animal models of balloon angioplasty have become the standard method to study synthetic VSMC *in vivo*. This model has been used extensively to study factors involved in VSMC proliferation following injury, as well as to test the therapeutic potential of various agents following balloon injury.

Specifically, the rat carotid artery injury model has been well-characterized, and results obtained with this procedure are reproducible. The standard time point at which arteries are removed from the rat following injury is 14 days. This time duration is required in order to generate a significant amount of neointima within the lumen of the injured arteries. A modification of this "single-injury balloon model" is the "double-injury balloon model" in which 14 days following the first injury, the artery is ballooned again, and removed after another 14 day period. This model is advantageous because

VSMC proliferation is greatly enhanced following the second ballooning, and therefore increases the amount of neointima. Both of these rat balloon-catheter models have been used extensively to test various drugs, in an attempt to prevent neointimal formation.

In terms of cyclic nucleotides and their effects on VSMC proliferation *in vivo*, several studies have shown that cAMP analogues, and/or PDE3 inhibitors such as milrinone and cilostazol elicit some suppression of neointimal formation (Indolfi *et al.*, 1997; Inoue *et al.*, 2000; Ishizaka *et al.*, 1999). Further, this suppression of neointimal formation was reversed upon inhibition of PKA, clearly attributing the anti-prolifaterive effects of the cAMP elevating agents to an increase in cAMP signalling (Indolfi *et al.*, 1997).

Although neointimal formation was partially suppressed by local delivery of cAMP elevating agents, namely cilostazol, complete neointimal suppression was not achieved. The most notable effect was observed using cilostazol in the context of the double-injury balloon model, which elicited 83% inhibition of neonintimal formation. However, in the context of the single-injury balloon, inhibition was markedly less (Inoue *et al.*, 2000; Ishizaka *et al.*, 1999; Indolfi *et al.*, 2000). Generally, cilostazol, the most commonly tested PDE3 inhibitor has proven ineffective in preventing VSMC proliferation *in vivo*.

1.8 Research Rationale

Since PDE3 and PDE4 account for more than 75% of the total PDE activity in rat vascular smooth muscle, their expression profile is highly significant in the context of regulation of cyclic nucleotide signalling. A significant amount of work in our laboratory using rat VSMC in culture led to the observation that PDE activity profiles were different

in cultured VSMC than in VSMC from whole isolated aorta. While PDE3 activity in whole aortic VSMC accounted for more than 60% of total PDE activity, this activity in cultured VSMC was less than 30% of the total. Similarly, there was also a noticeable difference in PDE4 activity profiles between VSMC in culture, and from whole aorta. In VSMC from freshly isolated aortic VSMC, PDE4 activity was less than 40% of the total, while in cultured VSMC PDE4 activity was upward of 50% of the total PDE activity. Thus, while the total PDE3 and PDE4 activity in both freshly isolated aortic VSMC, and in cultured VSMC was 80%, the individual contribution of PDE3 and PDE4 was noticeably different in the two tissue samples. The PDE3:PDE4 activity ratio in freshly isolated aortic VSMC was 1.5:1, while the PDE3:PDE4 ratio in cultured VSMC was markedly lower at 0.8:1.

Given that cyclic nucleotides are known to play a significant role in the regulation of migration and proliferation, and that inhibition of PDE3 and PDE4 individually results in differential effects on migration and proliferation, it was reasonable to associate the difference in PDE profiles with the phenotypic difference between cultured VSMC (synthetic) and freshly isolated VSMC (contractile).

Although the comparison of PDE3:PDE4 ratios in contractile and synthetic VSMC PDE profiles did highlight a potentially important difference in these cells, the ratio did not characterize the nature of the change in activity. The change in the activity ratio may have been due to decreased PDE3 activity, increased PDE4 activity, or a combination of both. Therefore, it was my objective to more fully characterize the change that occurs through passaging of VSMC.

Cultured VSMC are often used as a model for the *in vivo* situation where, following balloon-induced injury, VSMC change from a contractile to a synthetic phenotype, resulting in neointimal formation. We chose to use this rat balloon catheter-induced injury model as a relevant *in vivo* paradigm for the changes in PDE observed in culture.

Based on this rationale, the following research hypothesis was proposed:

The markedly lower PDE3 to PDE4 activity ratio observed in synthetic VSMC, compared to contractile VSMC, is due to a decrease in PDE3 expression, an increase in PDE4 expression, or a combination of both.

1.9 Research Objectives

In order to test our research hypothesis, the following two objectives were proposed:

1. Using PDE activity assays, RT-PCR and immunoblotting, to determine PDE3 and PDE4 activity, RNA and protein expression in freshly isolated aortic cells, and in primary culture of rat VSMC at increasing passage number between 0 and 5.
2. To determine PDE3 and PDE4 expression levels in isolated neointimal and media vascular smooth muscle cells following vascular injury to the rat aorta, induced by balloon catheterization

1.10 Clinical Significance of the Study

Since PDE3 and PDE4 are major cAMP hydrolyzing enzymes in mammalian vasculature, and have been shown to play a role in regulating migration and proliferation of VSMC, it may be of great significance to determine their expression in proliferating VSMC. These 'synthetic' cells are often associated with vascular pathologies such as restenosis, and are currently targets for several classes of drugs, including PDE inhibitors. In order to most effectively target PDE in 'synthetic' VSMC, it is first necessary to

characterize their expression. The appropriate use of PDE inhibitors in a clinical setting may lead to suppression of restenosis with fewer negative side-effects on the cardiovascular system as a whole.

Chapter 2

Differential Phosphodiesterase 3A Expression in Contractile and Synthetic Vascular Smooth Muscle Cells: Significance for Use of Phosphodiesterase 3 Inhibitors in Cardiovascular Tissues

Heather A. Dunkerley, Hanguan Liu, Douglas G. Tilley, Daniel Palmer, Sandra L. Jimmo and Donald H. Maurice

Department of Pharmacology & Toxicology, Queen's University at Kingston, Kingston, Ontario, Canada, K7L 3N6

2.1 Abstract

Arterial VSMC *in situ* normally express a contractile phenotype but can adopt a 'synthetic' phenotype in response to vascular damage, or when cultured. A comparison of literature values for phosphodiesterase 3 (PDE3) and phosphodiesterase 4 (PDE4) activities in 'contractile' and 'synthetic' VSMC identifies a marked difference in the PDE3:PDE4 activity ratio between these two VSMC phenotypes. While 'contractile' VSMC express a PDE3:PDE4 activity ratio of around 1.5, cultured VSMC consistently express a much lower PDE3:PDE4 activity ratio, usually about 0.8. By measuring PDE3 and PDE4 activities in 'contractile' and 'synthetic' VSMC, as well as measuring the expression level of these proteins in these cells, we demonstrate that the PDE3:PDE4 activity ratio is decreased in 'synthetic' VSMC due to a phenotype-associated reduction in PDE3A expression. This finding is discussed in the context of PDE3 inhibitors being used to inhibit VSMC functions including contraction, proliferation and migration. In addition, since the reduced expression of PDE3A in 'synthetic' VSMC also occurs in

human VSMC, the impact of this phenomenon on the potential of using PDE3 inhibitors in treatments of human cardiovascular diseases is discussed.

2.2 Introduction

In healthy blood vessels, VSMC function primarily to control contraction, and as such, are said to express a contractile phenotype (Owens, 1998). In contrast, during development, in response to vascular damage or during angiogenesis, VSMC alter their expression profile, and adopt a more 'synthetic' phenotype (Owens, 1998). While contractile VSMC express a specific set contractile proteins and exhibit very low proliferative or migratory capacities, synthetic VSMC express significant extracellular matrix and are highly proliferative and migratory in response to growth factors (Owens, 1998; Thyberg, 1998). Though not identical, a similar alteration in the phenotype of arterial VSMC occurs when these cells are placed in culture (Li *et al.*, 1999; Worth *et al.*, 2001). For this reason, cultured VSMC are commonly used as a model of 'synthetic' VSMC.

Increases in 3':5'-cyclic adenosine monophosphate (cAMP), or 3':5'-cyclic guanosine monophosphate (cGMP) influence VSMC relaxation-contraction coupling, proliferation, migration and cellular metabolism (Rybalkin and Bornfeldt, 1999; Lucas *et al.*, 2000; Koyama *et al.*, 2001). While cAMP and cGMP can have selective effects on some VSMC functions, several points of convergence between cAMP and cGMP signalling contribute to an overall similarity in their effects (Koyama *et al.*, 2001). The synthesis of cAMP and cGMP in cells is catalyzed by adenylyl cyclases and guanylyl cyclases, respectively. Once formed, the cyclic nucleotides are selectively hydrolysed by cyclic nucleotide PDE (Dousa, 1999; Soderling and Beavo, 2000). Presently, 11 distinct

families of mammalian PDE have been described with individual families being distinguished based on their substrate selectivity, inhibitor sensitivity and amino acid sequence. Several different PDE activities are detected in homogenates of blood vessels or cultured arterial VSMC (Polson and Strada, 1996; Rybalkin and Bornfeldt, 1999). Thus, members of the phosphodiesterase 1 (PDE1), phosphodiesterase 3 (PDE3), phosphodiesterase 4 (PDE4) and phosphodiesterase 5 (PDE5) families (Kakkar *et al.*, 1999; Degerman *et al.*, 1997; Houslay *et al.*, 1998; Corbin and Francis, 1999) have been shown to be expressed in bovine, porcine, rodent and human VSMC (Polson and Strada, 1996). While PDE1 and PDE5 hydrolyse cGMP in all VSMC studied, cAMP is hydrolyzed by PDE1, PDE3 and PDE4, with the role for PDE1 in this process being species specific (Polson and Strada, 1996; Rose *et al.*, 1997; Rybalkin *et al.*, 1997; Palmer and Maurice, 2000).

Although PDE3 and PDE4 activities together account for the majority of the cAMP hydrolysing capacity in freshly isolated arterial VSMC and cultured arterial VSMC, potentially important differences in the relative abundance of PDE3 and PDE4 in these two VSMC populations have been noted (Polson and Strada, 1996). Thus, while PDE3 activity is more abundant in freshly isolated arterial VSMC, this activity accounts for less than 20% of cAMP hydrolysis in cultured arterial VSMC. In contrast, in cultured VSMC, PDE4, not PDE3, is the most abundant cAMP PDE activity, accounting for greater than 75% of the total. Although a phenotype-dependent change in VSMC PDE3 and PDE4 activities could significantly influence the ability of PDE3 and/or PDE4 inhibitors to affect VSMC functions, the molecular basis and potential functional consequence of this difference have not been previously investigated.

In this report, we observed that a marked reduction in the expression of the major cardiovascular PDE3 gene, PDE3A, accompanies the change in phenotype that occurs during culturing of rat aortic VSMC. In addition, using a balloon-induced endothelial denudation method to induce vascular damage, we demonstrate that the intimal VSMC that accumulate in response to vascular damage similarly express markedly less PDE3A than the medial VSMC in this same artery. Interestingly, a similar loss of PDE3A is observed when human aortic VSMC undergo phenotypic modulation. Our data is presented in the context of continuing efforts to use PDE3 inhibitors to limit cardiovascular diseases (Haslam *et al.*, 1999; Movsesian, 1999; Park *et al.*, 2000; Osinski and Schror, 2000; El Beyrouty and Spinler, 2001; Tanabe *et al.*, 2001).

2.3 Materials and Methods

2.3.1 *Cell Culture*

Primary cultures of rat aortic VSMC were established using the enzymatic digestion of Wistar rat aorta as described previously (Rose *et al.*, 1997). VSMC were maintained in culture in Dulbecco's Modified Eagle's medium (DMEM) supplemented with calf serum (10 % v/v) or bovine serum (10 % v/v), penicillin-streptomycin (100 ug/ml) at 37° C in a humidified atmosphere saturated with 95% air-5% CO_2. The VSMC were sub-cultured by trypsinization and dilution at a ratio of 1:3. The VSMC used in these experiments were obtained between passages 0 and 7.

2.3.2 *Cyclic Nucleotide Phosphodiesterase Activity Assay*

cAMP PDE activity was assayed by a modification of Davis & Daly (1979), as previously described (Rose et al., 1997), using 1-5 μg protein from cultured VSMC

homogenate, or from Wistar rat aortic homogenate. The cAMP PDE activity was expressed as pmol/min/mg of protein.

2.3.3 *Immunoblotting*

VSMC homogenates were boiled in sample buffer and aliquots (5-30 μg) were subjected to SDS-PAGE, transferred to nitrocellulose membrane (Bio-Rad) and probed with selective antisera as described previously (Liu and Maurice, 1999). Primary antibodies used in this study were directed against β-actin (Sigma) or murine PDE3A and PDE3B; generous gift of Dr. J.A.Beavo, Department of Pharmacology, University of Washington, Seattle, WA (Zhao *et al.*, 1997). Immunoreactive protein bands were visualized using horseradish peroxidase-conjugated secondary antisera and an enhanced chemiluminescence kit (Amersham, Canada).

2.3.4 *Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)*

RNA isolation and RT-PCR were carried out as described previously (Liu and Maurice, 1998). Sense and anti-sense primers for rat PDE3A (5'-CCGAATTCCCTTATCATAACAGAATCCACGC -CACT-3', 5'-GGGAATTCGTGTTTCTTCAGGTCAGTAGCC-3') and rat PDE3B (5'-CCGAATTCTATCACAATCGTGTGCATGCCACAGA-3', 5'-CCGAATTCTTTGAG ATCTGTAGCAAGGATTGC-3') were used for PCR. These primers, spanning two introns gave rise to PCR products encoding PDE3A (508 bp) and PDE3B (499 bp). Conditions used for RT-PCR were optimized to allow linear amplification of all products (Liu and Maurice, 1998), and these were visualized and quantified following agarose gel

electrophoresis, digital photography and analysis of digital images as described (Liu and Maurice, 1998).

2.3.5 *Balloon-Catheter Induced Aortic Damage*

Male Wistar rats (300g) were anesthetized using ketamine/water/xylazine cocktail (1:2:1). An abdominal incision was made, and a 2F-Fogarty balloon catheter (Baxter) was inserted into the left iliac artery. The balloon was inflated and passed three times through the abdominal portion of the aorta. Following removal of the balloon catheter, the left iliac artery was sutured closed. After 14 days, rats were sacrificed and abdominal aortae were removed. The isolated aortae were rinsed free of blood, cleared of fat and connective tissue and cut longitudinally into three equal portions using a sharp blade. The three aortic segments were separated and used either for PDE activity determinations, immunoblot analysis or immunohistochemical studies. PDE activity assays and immunoblotting were carried out as described previously (Liu and Maurice, 1998).

2.3.6 Immunohistochemistry

Blood vessel segments were fixed with 4% paraformaldehyde and embedded in paraffin. Successive aortic slices were placed on slides, all of which was carried out in the core laboratory for the department of Pathology, Queen's University, Kingston, ON. Paraffin was removed from the slide by successive incubation of aortic segments in toluol, absolute ethanol, distilled water, and 0.1 M Tris. Antigens were exposed on de-paraffinized aortic segments by rinsing in water, followed by heating to boiling, for 2x 5

min each time, in a 0.1 mM EDTA solution (pH 8). Following a cooling period (30 min), aortic segments were rinsed with 0.1M Tris, incubated with BSA (10 % w/v, 1 h) and incubated with the primary antibody of interest (30 μg/ml, 48h, ambient temp), or DAPI to stain nuclei. Antisera used included rabbit anti-PDE3 (polyclonal). Following this incubation aortic segments were rinsed with 0.1 M Tris and incubated with 1:200 dilutions of biotin-conjugated goat anti-rabbit antisera. Aortic segments were then rinsed with 0.1 M Tris, and allowed to incubate with streptavidin conjugated DTAF (Jackson Immuno Research), (5.6 μl/ml in BSA solution) for 30 min. Following rinses in 0.1 M Tris, aortic segments were rinsed with anti-fade reagent (Molecular Probes, Oregon USA) and covered with cover slips. Sections were viewed on a Zeiss Axiovert S100 fluorescent microscope, equipped with a SensiCam high performance CCD camera.

2.3.7 *Isolation of Neointimal VSMC Following Balloon Injury*

Following removal of balloon-injured aorta, intimal tissue was isolated from the remainder of the aorta by incubating the lumen of the intact vessel with 1x trypsin-EDTA in Ca^{2+} and Mg^{2+} free HBSS (4 times, 50 μl volumes) (Life Technologies). The four fractions of intimal tissue were collected by centrifugation and pooled prior to further processing.

2.3.8 *Protein Assay*

Protein concentrations were determined using the BCA protein assay (Pierce) according to the manufacturer's protocol. BSA was used as a protein standard.

2.4 Results and Discussion

2.4.1 *cAMP PDE activity in contractile and synthetic rat aortic VSMC*

As described in Section 2.2, arterial VSMC *in situ*, which normally express a contractile phenotype, can adopt a synthetic phenotype in response to vascular damage, or when cultured (Owens, 1998; Thyberg, 1998; Li *et al.*, 1999; Worth *et al.*, 2001). Interestingly, a comparison of literature values for PDE3 and PDE4 activities in arterial VSMC (contractile) and cultured (synthetic) VSMC was presented in a review by Polson and Strada (1996) and identifies a marked difference in the PDE3:PDE4 activity ratio between these two VSMC phenotypes. Indeed, while contractile VSMC express a PDE3:PDE4 activity ratio of around 1.5, cultured VSMC consistently express a much lower PDE3:PDE4 activity ratio, usually about 0.8 (Polson and Strada, 1996; Rose *et al.*, 1997). Based on these data, we hypothesized that the expression of PDE3 or PDE4, or both, might be different in these two distinct VSMC phenotypes. Secondly, we proposed that these differences could significantly alter the ability of PDE3 or PDE4 inhibitors to affect VSMC functions such as contraction or migration. Since the PDE3:PDE4 activity ratio in contractile and synthetic VSMC could be different due to changes in the expression of either PDE3 or PDE4, or both of these activities, we measured PDE3 and PDE4 activities in rat aortic VSMC prior to culture (contractile) and after culture (synthetic), investigated if these differences occurred *in vivo* in rats and determined if a similar difference existed in human aortic VSMC.

In our experiments, synthetic rat aortic VSMC were shown to express significantly less cAMP PDE activity than contractile rat aortic VSMC (Fig. 2.1A). In addition, based on the effects of PDE3- or PDE4-selective inhibitors, synthetic VSMC

were shown to have significantly less PDE3 activity than contractile VSMC (Fig. 2.1A). In fact, PDE3 activity decreased linearly with sub-culture until passage 4, at which time it was reduced by more than 50%, when compared to PDE3 activity in VSMC not submitted to culture. In contrast, no difference in PDE4, or in the residual non-PDE3/PDE4 activity, as assessed using the broad-selectivity inhibitor (IBMX), were detected. In fact, when IBMX-mediated inhibition was taken as a measure of total cAMP PDE activity, the difference in PDE3 activity observed between contractile and synthetic VSMC accounted for the entire difference between these two phenotypically distinct VSMC (Fig. 2.1A).

2.4.2 *PDE3A, but not PDE3B, mRNA and protein are decreased upon culturing of rat aortic VSMC*

In earlier reports we showed that contractile and synthetic rat and human aortic VSMC each expressed both PDE3A and PDE3B (Maurice *et al.*, 1995; Liu and Maurice, 1998; Palmer and Maurice, 2000). To establish if the reduced PDE3 activity found in synthetic VSMC was due to a general reduction in PDE3, or specific to one PDE3 gene, we determined the levels of expression of both PDE3A and PDE3B mRNA and protein in contractile rat aortic VSMC as well as in synthetic VSMC as generated by our sub-culture protocol. Our data are show a marked reduction in PDE3A expression in synthetic VSMC (Fig. 2.1B). Thus, while synthetic rat aortic VSMC showed a marked sub-culture-dependent reduction in PDE3A mRNA, no changes in the level of PDE3B mRNA were observed in these cells (Fig. 2.1B). Consistent with the lack of observed difference in PDE4 activity between contractile and synthetic VSMC, no differences in

the levels of PDE4D3 mRNA, the major PDE4 enzyme expressed in these cells (Liu and Maurice, 1999), were detected.

Consistent with these results, immunoblot analysis demonstrated that synthetic VSMC expressed markedly less PDE3A protein than contractile VSMC, while the levels of PDE3B in these homogenates were similar (Fig. 2.1C). Our observation that PDE4 activity and PDE4D3 mRNA levels were similar between contractile and synthetic VSMC followed with immunoblot analysis of these samples of VSMC in which PDE4D3 protein levels in contractile and synthetic VSMC were indistinguishable (Fig. 2.1D). Since our comparison of PDE3B mRNA and protein levels between contractile and synthetic VSMC found the level of expression of this PDE3 variant to be similar and that no change in PDE4 activity or PDE4D3 expression were noted, we conclude that the decrease in PDE3:PDE4 activity ratio observed in synthetic VSMC is most likely due to the decreased expression of PDE3A in synthetic VSMC. A similar decrease in expression of cardiac PDE3A was recently noted to associate with development of decompensated dilated cardiomyopathy (Smith *et al.*, 1997). In fact, the authors of this earlier report suggested that the reduced expression of PDE3A in cardiomyocytes could account in part for the ineffectiveness of PDE3 inhibitors such as milrinone in the treatment of severe congestive heart failure. Whether or not similar mechanisms are involved in reducing PDE3A expression in synthetic VSMC and diseased cardiomyocytes awaits further study.

2.4.3 *Rat aortic intimal VSMC exhibit reduced PDE3 activity and reduced PDE3A expression*

As stated in the Introduction, the synthetic phenotype expressed by cultured VSMC has often been used as a model of the altered phenotype expressed by intimal VSMC that accumulate following vascular damage (Owens, 1998). In fact, several pharmacological approaches designed to reduce the accumulation of intimal VSMC, and reduce the incidence of intimal lesions and cardiovascular disease, are based on results obtained with cultured synthetic VSMC. In order to assess if the reduced PDE3A expression that accompanied the development of a synthetic phenotype in sub-cultured VSMC also accompanied the phenotypic modulation of VSMC *in vivo*, the PDE3 activity of neo-intimal VSMC was measured. The results obtained agree with the idea that a similar loss of PDE3A expression accompanies the vascular damage-induced *in vivo* phenotypic modulation of rat aortic VSMC. Thus, intimal VSMC PDE3 activity was significantly lower ($60\%<P<0.05$) than that expressed by contractile VSMC isolated from either undamaged rat aorta, or from the medial layer of aorta following damage (Fig. 2.2A). Our finding that intimal VSMC expressed less PDE3 activity, compared to medial VSMC, was similar to that demonstrated by immunoblot analysis in which these tissues showed a marked reduction in the level of PDE3A in intimal VSMC (Fig. 2.2B). Immunoblotting results showing that PDE3A present in medial VSMC were similar whether this tissue was isolated from aorta that had been damaged, or from control aorta not subject to mechanical endothelial denudation (Fig. 2.2B) corresponded to our observation that the change in PDE3A was selective for intimal VSMC. Regrettably, since the amounts of tissue obtained in these types of experiments was very low, it was

not possible to determine the level of PDE3B present in the intimal layer-derived tissues. However, given that virtually no PDE3A was detected when intimal tissues were immunoblotted, we think it reasonable to conclude that much of the residual PDE3 activity present in intimal VSMC (Fig. 2.2A) could represent PDE3B expressed in these cells. Further studies will be needed to confirm this supposition. Consistent with the activity measurements (Fig. 2.2A), and with results obtained using synthetic rat aortic VSMC generated by our sub-culturing protocol, levels of PDE4 activity (Fig. 2.2A) and of PDE4D3 protein expressed (not shown) in intimal and medial derived VSMC were similar.

To confirm that PDE3A expression was specifically reduced in intimal VSMC, an immunohistochemical approach was used. Thus, while incubation of aortic sections isolated from ballooned aorta with a β-actin specific monoclonal antibody showed that both intimal and medial VSMC expressed similar amounts of this protein (not shown). Application of our PDE3 antisera identified a marked difference in staining for PDE3 between these layers of VSMC (Fig.2.2C). Virtually no specific staining for PDE3 was detected in the intimal layer of ballooned aorta in three replicate experiments. To further validate that similar numbers of VSMC were present in the intimal and medial layers, VSMC cell nuclei were stained with DAPI. Since both DAPI and β-actin staining clearly identified large numbers of cells in the intimal layers of these damaged aorta, we conclude that intimal VSMC express very low levels of PDE3 protein (Fig 2.2C). PDE3B expression could not be determined because of the lack of suitable antisera.

2.4.4 Human aortic contractile and synthetic VSMC express different levels of PDE3A

To determine if the marked decrease in PDE3A expression that accompanied the phenotypic modulation of rat aortic VSMC from contractile to synthetic also occurred with human VSMC, PDE3 and PDE4 activities were measured in 'contractile' and 'synthetic' human aortic VSMC. As shown in Table 2.1, synthetic human aortic VSMC express three PDE activities, namely PDE1, PDE3 and PDE4. Interestingly, as observed with rat aortic VSMC, 'synthetic' human aortic VSMC expressed significantly less PDE3 activity than 'contractile' human aortic VSMC (Table 1). Our finding of PDE1C expression in human 'synthetic' VSMC, and its absence in 'contractile' human aortic VSMC or 'synthetic' rat aortic VSMC, has been previously reported (Rybalkin *et al.*, 1997; Palmer and Maurice, 2000). Corroborating the loss of PDE3 activity observed in human 'synthetic' VSMC, immunoblot analysis of 'contractile' and 'synthetic' human aortic VSMC identifies a marked reduction in PDE3A expression in 'synthetic' human aortic VSMC, compared to 'contractile' VSMC (Fig. 2.3). Consistent with the modest difference in PDE4 activity recorded between 'synthetic' and 'contractile' rat VSMC (Table 2.1), no differences were observed in PDE4D3 expression in these two phenotypically distinct human aortic VSMC (not shown). Based on these data, we conclude that a similar reduction in PDE3A expression accompanies the 'contractile' to 'synthetic' phenotypic switch that occurs in human aortic VSMC when these cells are placed in culture. Immunohistochemical analysis similar to that carried out by us following balloon-induced vascular damage would support this conclusion; unfortunately, no vascular specimens with actively developing intimal layers are available to us. In fact, an earlier attempt to investigate expression of PDE1C in human

intimal VSMC demonstrated that at later times, the intimal VSMC express a phenotype more similar to that of VSMC in the medial layer (Rybalkin *et al.*, 1997).

2.4.4 *Pharmacological and Therapeutic Implications*

PDE3 has been a significant pharmacological target for the treatment of several cardiovascular disorders (Haslam *et al.*, 1999; Movsesian, 1999; Park *et al.*, 2000; Osinski and Schror, 2000; El Beyrouty and Spinler, 2001; Tanabe *et al.*, 2001). Therapeutic use of PDE3 inhibitors may result in stimulated myocardial contractility, inhibition of platelet aggregation, and relaxation of vascular and airway smooth muscle. Indeed, milrinone, and more recently cilostazol, have been shown to markedly affect the functioning of cardiovascular tissues (Haslam *et al.*, 1999; Movsesian, 1999; Park *et al.*, 2000; Osinski and Schror, 2000; El Beyrouty and Spinler, 2001; Tanabe *et al.*, 2001). Recently, cilostazol has been considered a promising candidate drug for prevention of restenosis due to its vasodilating and anti-platelet effects (Tanabe *et al.*, 2001). Indeed, several studies have indicated some degree of success in suppressing neointimal formation in animal models using cilostazol (Inoue *et al.*, 2000; Aoki *et al.*, 2001). Results of trials in which aspirin and cilostazol were delivered in combination following stenting showed comparable results to those obtained when aspirin and ticlopidine, an inhibitor of platelet aggregation, were used (Park *et al.*, 2000). Based on our present results, we suggest that cilostazol may act preferentially to inhibit platelet PDE3A when used in this situation. In fact, our data demonstrating a clear and marked reduction in the expression of the major PDE3 in the proliferative and migratory synthetic VSMC provides strong support for the idea that the effects of this agent are most likely not due to direct vascular effects.

Based on the results obtained here, it may be argued that rather than inhibiting PDE3 inhibition, a strategy of PDE4 inhibition could perhaps prove to be more effective in reducing accumulation of intimal VSMC following vascular injury. In fact, previous work identifying PDE4 inhibitors as more potent inhibitors of rat aortic VSMC migration than PDE3 inhibitors substantiates this idea (Palmer *et al.*, 1998). In addition, since PDE4 inhibitors are not potent vasodilators in blood vessels without intact endothelial function (Komas *et al.*, 1991), use of these agents might result in fewer undesirable cardiovascular effects such as vascular relaxation. Altered PDE expression in different endothelial phenotypes has also been reported (Keravis *et al.*, 2000). The impact of these findings on our data remains to be established.

On a more fundamental level, our data are also perhaps informative with respect to cAMP-mediated regulation of PDE3- *vs* PDE4-sensitive VSMC functions. Thus, PDE3 inhibitors are potent inhibitors of agonist-mediated contractions of arterial tissue, and potentiate the relaxant effects of activators of adenylyl cyclases (Maurice and Haslam, 1990), while PDE4 inhibitors are generally poor inhibitors of arterial contractions, and only have effects when used in combination with PDE3 inhibitors or in blood vessels with intact endothelium functions (Komas *et al.*, 1991). Regulation of contractility of VSMC clearly is a function more relevant to VSMC expressing a contractile phenotype. Interestingly, PDE4 inhibitors are more effective at inhibiting proliferation, or migration, of VSMC, activities that are more relevant to VSMC expressing a synthetic phenotype. Based on the data presented in this report, we hypothesize that the absolute amounts of PDE3 and PDE4 in these phenotypically distinct

VSMC populations is a determining factor of whether PDE3 or PDE4 inhibitors will be effective. Clearly further studies will be required to directly test this idea.

	Total cAMP PDE Activity (pmol/min/mg)	PDE1 Activity (pmol/min/mg)	PDE3 Activity (pmol/min/mg)	PDE4 Activity (pmol/min/mg)
Human Contractile Aortic VSMC	161 ± 9	None	116 ± 3**	23 ± 5**
Human Synthetic Aortic VSMC	124 ± 5*	531 ± 39*,**	31 ± 4*,**	17 ± 3**

Table 2.1 ***Differential PDE activity in contractile and synthetic human aortic VSMC***

Activities are means ● S.E. of a representative experiment carried out in triplicate using 1 μM cAMP as substrate. Similar values were obtained in four separate experiments. Values are significantly different *, $P < 0.05$; one-way ANOVA (Tukey post hoc test) relative to human contractile aortic VSMC; **, $P < 0.05$; one-way ANOVA (Tukey post hoc test) relative to total cAMP PDE activity of relevant human aortic VSMC phenotype.

Figure 2.1 ***Differential expression of PDE3A in contractile and synthetic rat aortic VSMC***

Rat aorta were lysed in PDE assay buffer or enzymatically dissociated to generate VSMC for culture (Material & Methods). Dissociated VSMC were plated on plastic tissue culture plates and cultured at 37°C until confluence (3-6 days). These VSMC were designated as passage 0 (P0). At confluence, P0 VSMC were isolated and separated into three aliquots. One third of the P0 VSMC was plated again and grown to confluence while the remaining two thirds were used for determinations of PDE activity, mRNA and protein levels, as described in Materials and Methods. (A) Total cAMP PDE activity, PDE3 activity (1 μM cilostamide) and PDE4 activity (10 μM Ro 20-1724) in homogenates of each VSMC population were determined as described in Material and Methods. Data are m ± S.E. of three separate experiments (n=3). (B) Levels of PDE3A or PDE3B mRNA in aorta and at each individual passage number were determined by RT-PCR (Materials & Methods). A representative photograph (i) of PDE3A reaction products is shown and (ii) an average of the changes in abundance of PDE3A and PDE3B during culturing is shown (n=3). (C) Representative immunoblot from three experiments in which levels of PDE3A and PDE3B in aorta and cultured VSMC were determined using a pan-reactive PDE3 antibody. Rat epididymal fat, which exclusively expresses PDE3B, and subcellular fractions of synthetic VSMC, which express both PDE3A and PDE3B are also shown as controls. (D) Representative immunoblot (n=3) in which levels of PDE4D3 in VSMC from each passage were determined using a PDE4D-specific antibody. No changes in PDE4D3 were observed when PDE4D3 amounts were corrected for by normalizing to β-actin using a selective monoclonal antibody.

Figure 2.1A

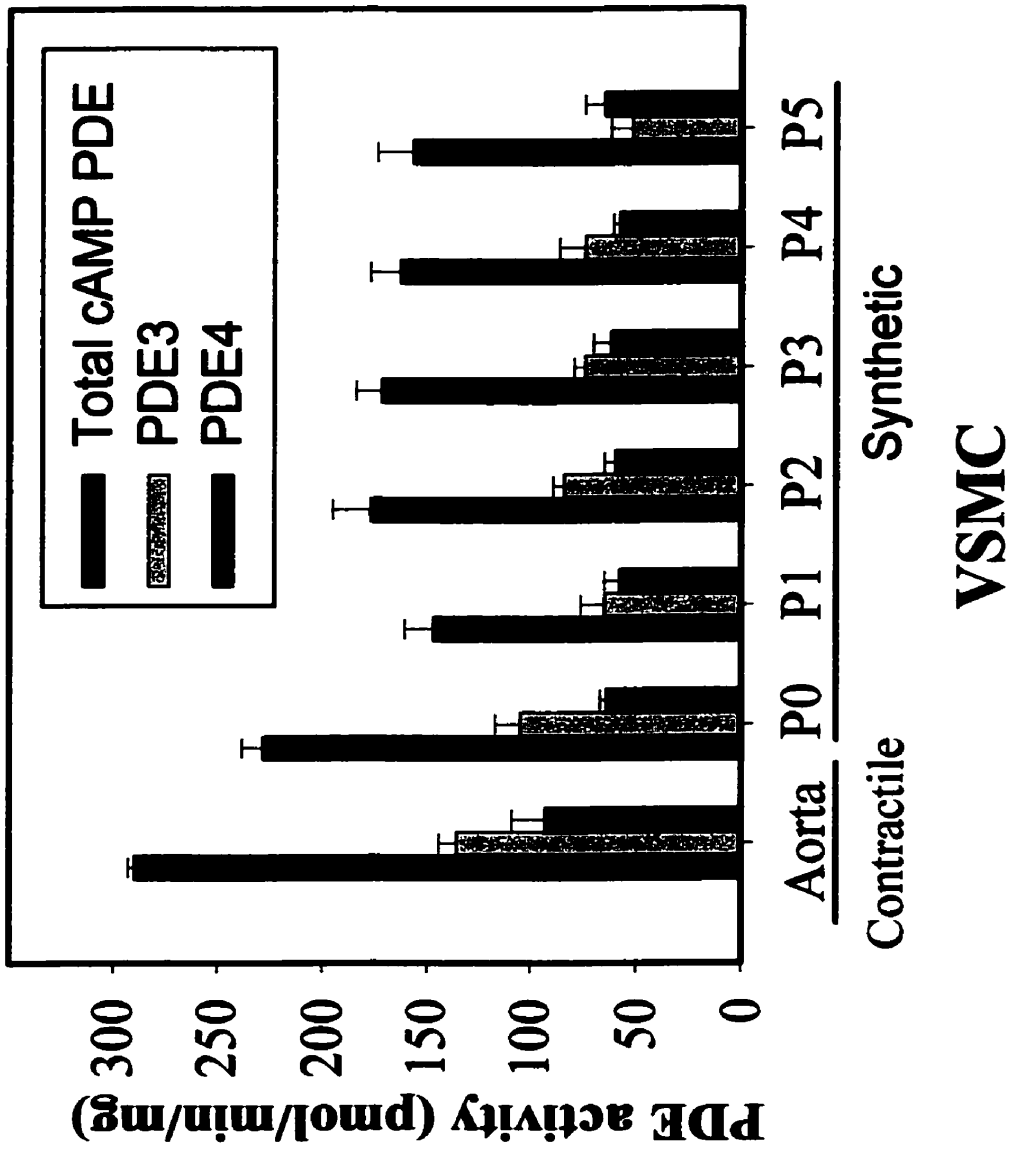
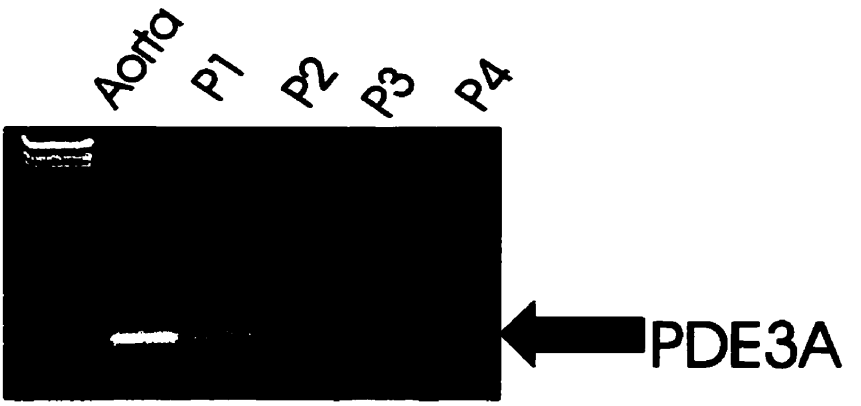

Total cAMP PDE
PDE3
PDE4
PDE activity (pmol/min/mg)
300
250
200
150
100
50
0
Aorta
P0
P1
P2
P3
P4
P5
Contractile
Synthetic
VSMC

Figure 2.1B

(i)



(ii)

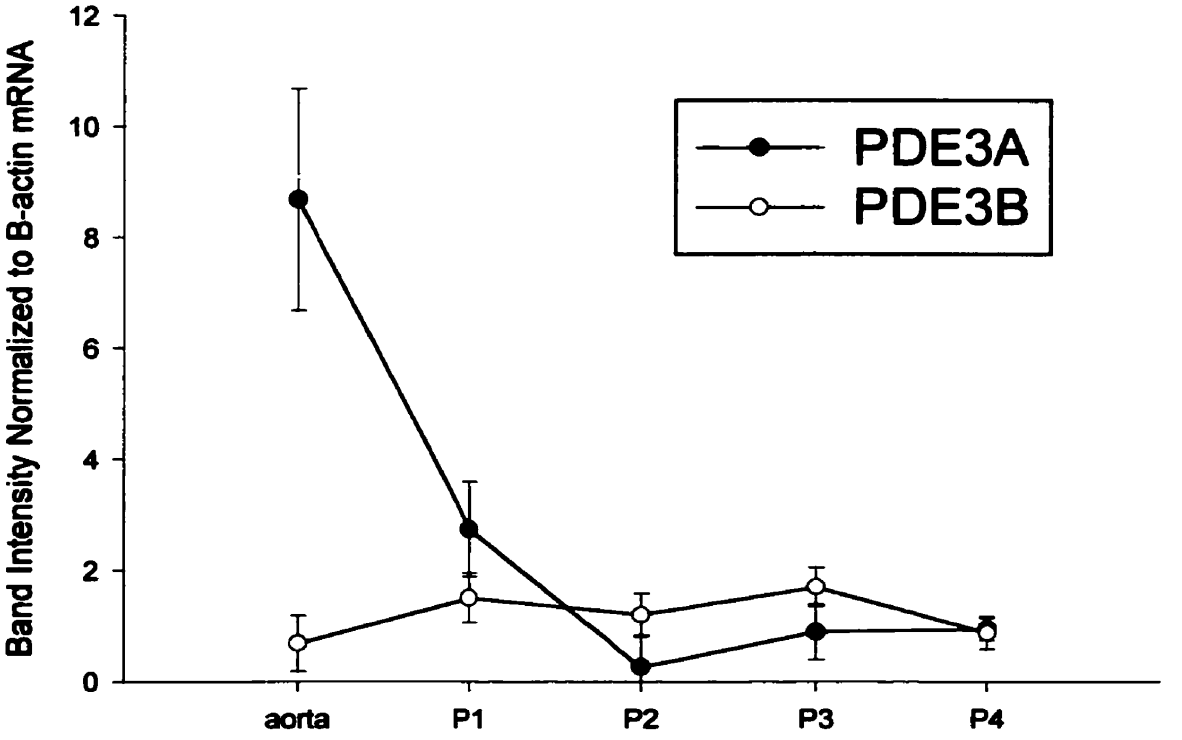


Figure 2.1C

Homogenate (ug per lane)
25
25
25
50
50
PDE3B
PDE3A
Rat Aorta
Rat epididymal fat
Rat cultured VSMC (P5)
Rat cultured VSMC (P5)
particulate fraction
Rat cultured VSMC (P5)
cytosolic fraction

Figure 2.1D

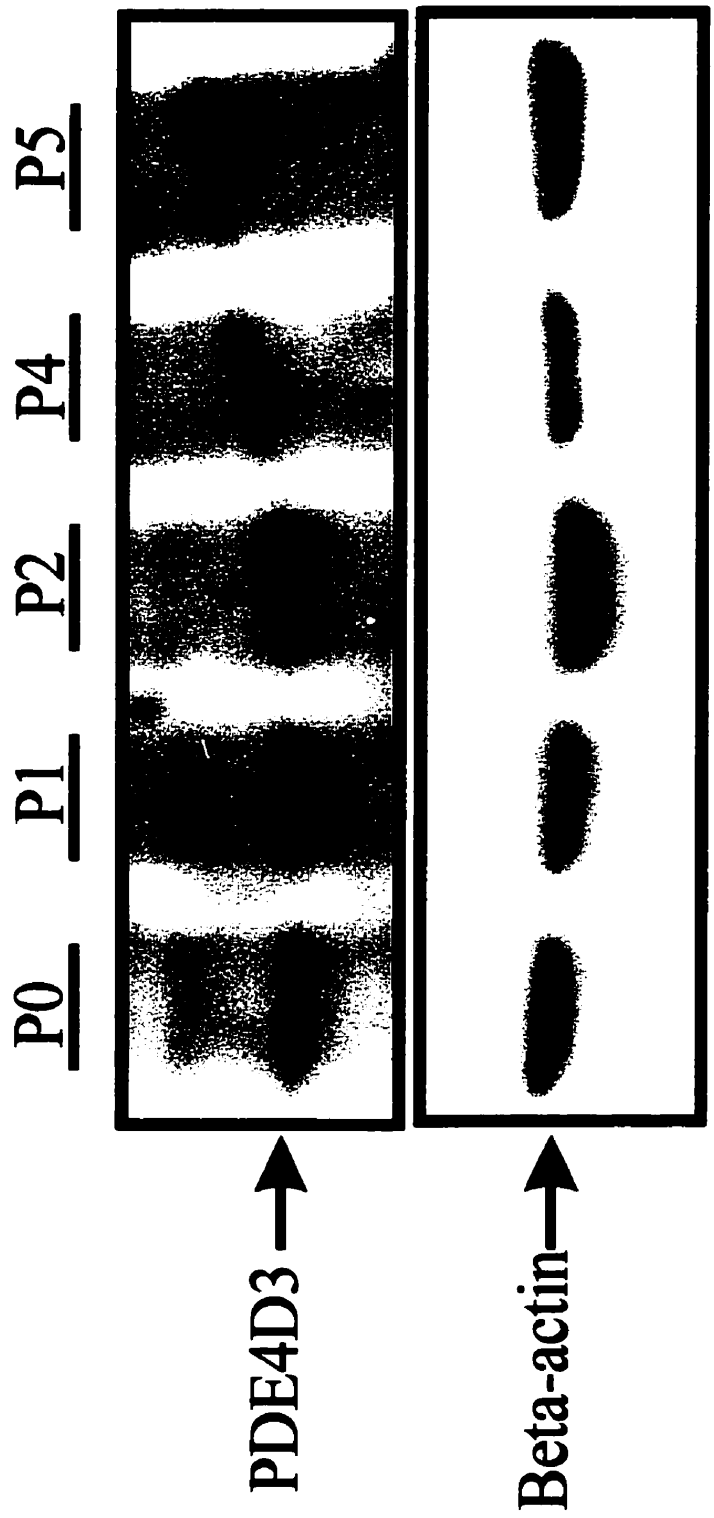


Figure 2.2 ***PDE3A expression in rat aortic neo-intimal VSMC formed in response to balloon-induced vascular damage***

2F-Fogarty balloons were used to induce vascular injury to the abdominal aorta of male Wistar rats, as described in Materials and Methods. Following this procedure, rats were returned to their cages and remained there for two weeks. After 14 days, aorta were removed and analysed. (A) Percent of total cAMP PDE activity, percent PDE3 (1 μM cilostamide), or combined percent PDE3 and PDE4 (10 μM zardaverine) were determined in homogenates of intimal and medial tissues following isolation of intimal tissue from medial tissue by digestion with trypsin (Materials and Methods). (B) Immunoblot analysis of rat aorta and of isolated intimal and medial tissues from ballooned aorta using a PDE3 selective antibody. Significant percentage decreases in intimal versus medial VSMC PDE3 activity was detected ($p < 0.05$). (C) Control or ballooned rat aorta were processed for immunohistochemical analysis as described in Materials and Methods. **A** A control rat aorta (not ballooned) stained with H & E is shown with the medial and endothelial layers identified. **B** Ballooned rat aorta stained with H & E, identifying medial and neo-intimal layers. **C** Control aorta (not ballooned) using immunohistochemistry with anti-PDE3 antisera. Neointimal VSMC are not present. **D** DAPI staining identifies an abundance of VSMC nuclei within the intima, and media, as well as sparce adventitial fibroblasts. **E** Imunohistochemical stained section of ballooned rat aorta using anti-β-actin antisera. Equal staining is observed in the medial and neo-intimal layers. **F** Immunohistochemical stained section using anti-PDE3 antisera showing ample staining in the media, while little or none is observed in the neo-intimal or adventitial layer.

Figure 2.2A

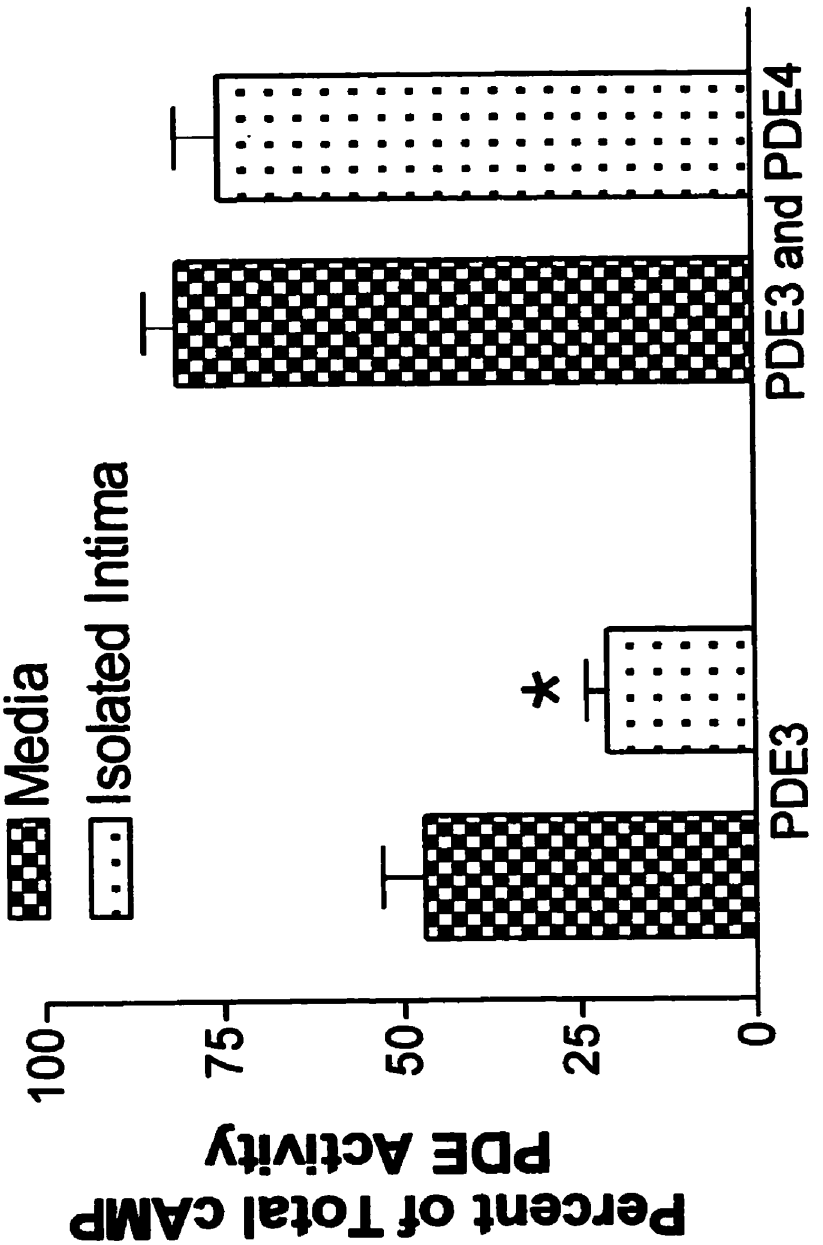

Percent of Total cAMP
PDE Activity
100
75
50
25
0
Media
Isolated Intima
*
PDE3
PDE3 and PDE4

Figure 2.2B

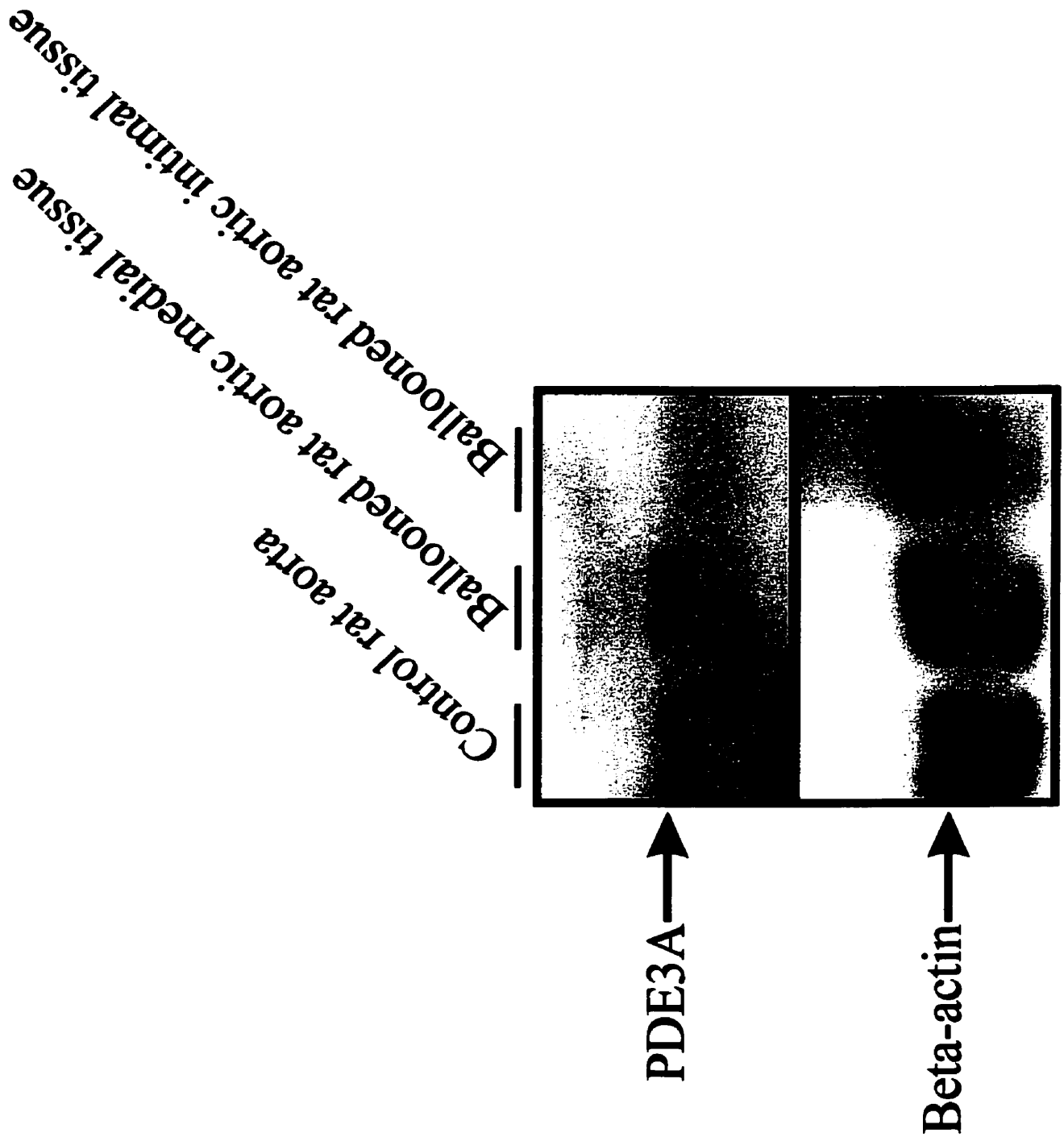
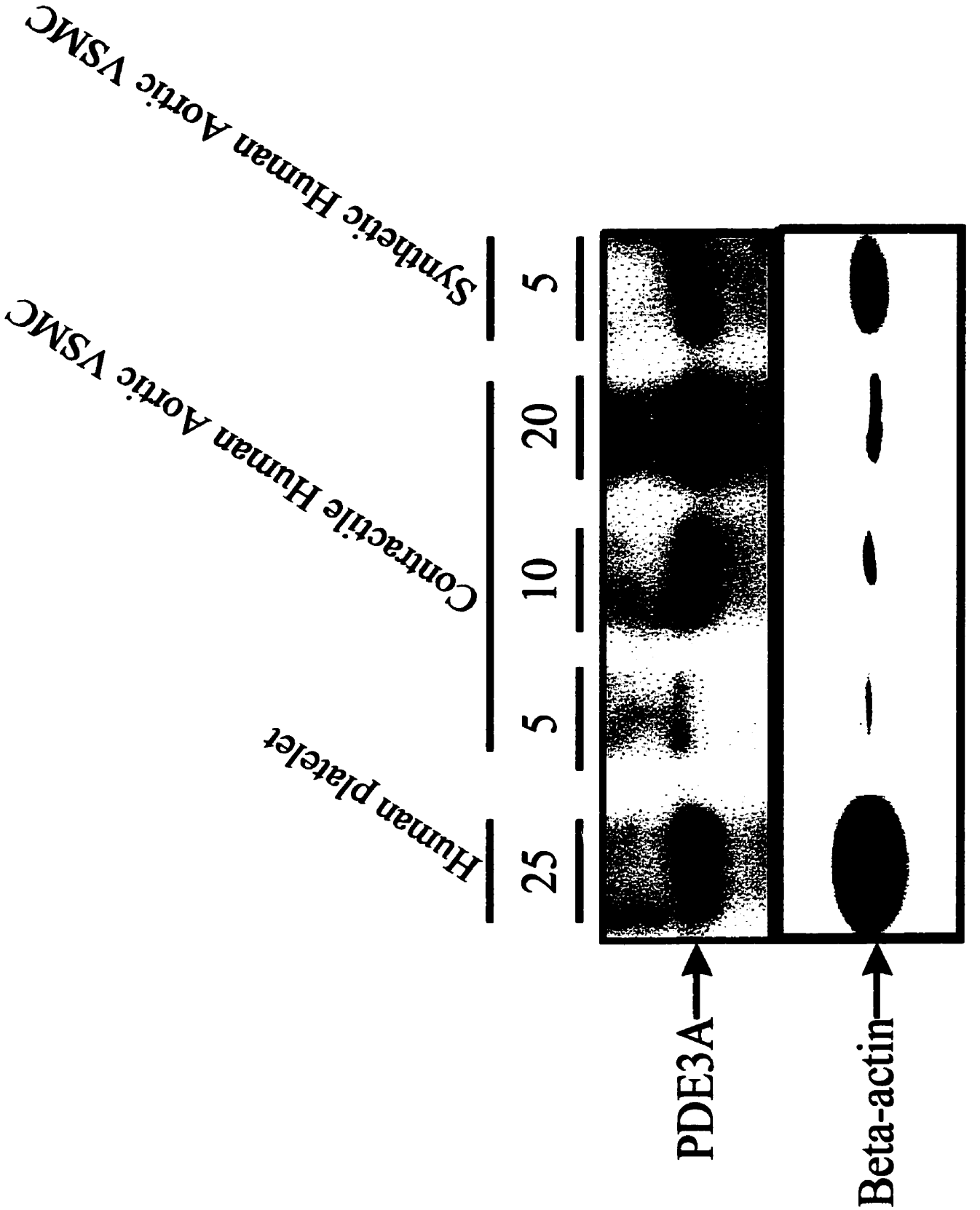

Control rat aorta
Ballooned rat aortic medial tissue
Ballooned rat aortic intimal tissue
PDE3A
Beta-actin

Figure 2.3 ***Differential expression of PDE3A protein in contractile and synthetic human aortic VSMC***

Segments of human aorta, and primary cultures of human aortic smooth muscle cells HVSMV derived from isolated explants from thoracic aorta were lysed and immunoblotting was carried out as described previously using a selective PDE3 polyclonal antibody (Palmer and Maurice, 2000). In addition a selective β-actin monoclonal antibody was used to normalize for intracellular protein within the total homogenate.

Figure 2.3


Synthetic Human Aortic VSMC
Contractile Human Aortic VSMC
Human platelet
5
20
10
5
25
PDE3A
Beta-actin

Chapter 3 – General Discussion

3.1 Regulation of Cyclic Nucleotide Signalling by PDE

The findings of this study are significant in several respects. In terms of the significance on a molecular level, we have identified a mechanism that could potentially account for the specificity of PDE regulation of cyclic nucleotide signalling throughout the cell. The way in which 11 different families of PDE differentially regulate cell function is somewhat unclear. The existence of multiple families of adenylyl cyclases, protein kinases, and especially protein kinase-anchoring proteins have led to the widely accepted hypothesis that cyclic nucleotide signalling is compartmentalized within the cell, and that different PDE gene families may be responsible for the hydrolysis of different pools of cAMP within these compartments. Compartmentalization of signalling events has been used to explain several observations that demonstrate a dissociation of cAMP accumulation from physiological response (Steinberg and Brunton, 2001). For example, in an isolated heart preparation, forskolin-induced cAMP elevation results in different protein phosphorylation events, and contractility, than isoproterenol-induced elevation of cAMP. Although both agents elevate cellular cAMP concentrations to similar levels, the downstream effects are more pronounced with isoproterenol (England and Shahid, 1987).

Similar studies have demonstrated that PDE may be involved in dissociating cyclic nucleotide levels and their effects downstream. Thus, it was observed in rat mesangial cells that with similar PKA activity, inhibition of either PDE3 with cilastozol, or PDE4 with rolipram resulted in markedly different cellular responses. There was a significant suppression of reactive oxygen-metabolites (ROM) in response to PDE4 inhibition, while

PDE3 inhibition showed no effect on ROM. Alternatively, PDE3 inhibition resulted in suppression of mitogenic DNA synthesis, while PDE4 inhibition did not. Based on these findings, the author hypothesized that in rat mesangial cells, which are phenotypically similar to VSMC, the cAMP-PKA signalling pathway that controls ROM generation is coupled to cAMP hydrolysis by PDE4, whereas the cAMP-PKA signalling pathway coupled to cAMP hydrolysis by PDE3 regulates proliferation (Chini *et al.*, 1997).

Our findings suggest a further mechanism for signalling regulation in synthetic VSMC. Instead, or in addition to compartmentalization of cyclic nucleotide signalling where PDE hydrolyze only specific pools of cAMP, signalling could be altered by a phenotype-dependent change in PDE expression. The loss of PDE3A expression as shown in our study is in itself a regulatory mechanism, and constitutes one of several explanations for the biological basis of the considerable multiplicity of PDE.

3.2 PDE Profile in Human Synthetic Vascular Smooth Muscle Cells

In terms of the clinical relevance of our findings, it is important to note that the majority of our research was carried out using rat vascular smooth muscle cells, not human synthetic VSMC. Although the loss of PDE3A expression observed in rat synthetic VSMC was also seen in human synthetic VSMC, the overall PDE expression profiles are different in rat and human VSMC. For example, human synthetic VSMC have been shown to express a form of the calmodulin-stimulated PDE1 gene family that is not expressed in human contractile VSMC, nor in most other mammalian synthetic VSMC (Rybalkin *et al.*, 1997). Therefore it is a uniquely human phenomenon that may not be studied in bovine, monkey or rat tissues. PDE1C, one of three PDE1 isoforms that primarily hydrolyzes cAMP, was the major cAMP hydrolyzing enzyme in primary

cultures of human VSMC, accounting for 80% to 85% of the total cAMP-hydrolyzing activity (Rybalkin and Bornfeldt, 1999). Interestingly, it was also observed that a cGMP hydrolyzing isoform of the PDE1 family, PDE1B, was detected in the VSMC isolated from the medial layer of human aorta, but was absent from primary cultures of human VSMC. This switch from PDE1B, a cGMP hydrolyzing enzyme in 'contractile' VSMC, to PDE1C expression, a cAMP hydrolyzing enzyme in 'synthetic' VSMC, is significant in the context of proliferation. Since cAMP is thought to be a more potent inhibitor of proliferation than cGMP, it is reasonable to hypothesize that the expression of PDE1C is necessary in order to keep the levels of cAMP low in these human synthetic VSMC (Rybalkin and Bornfeldt, 1999).

3.3 Cyclic-GMP Signalling in Synthetic Vascular Smooth Muscle Cells

It is widely recognized that cultured VSMC display a marked loss of several cGMP-regulated effector proteins, when compared to cells not subjected to cell culture. Our finding of a loss of PDE3A in cultured VSMC, and in neointimal VSMC, is consistent with this premise. In fact, cGMP-inhibition of PDE3A has been established to play a dominant role in the regulation of this enzyme both through inhibition of platelets, and inhibition of aortic contraction. However, the functional impact of this loss of PDE3A on cGMP mediated regulation of cAMP, is difficult in this context due to the previously published loss of the soluble GC under these conditions.

The disappearance of PDE1B, a cGMP hydrolyzing enzyme in human synthetic VSMC, is consistent with several studies that show a decrease in cGMP regulated proteins in these cells. It has been demonstrated numerous times that upon balloon catheter-induced injury, PKG expression is decreased in neointimal VSMC, but not in

medial smooth muscle cells (Anderson *et al.*, 2000). In fact, these enzymes have been implicated in determining the differentiation state of the VSMC. This was directly demonstrated by transfecting VSMC cell lines with PKG cDNA, which resulted in dedifferentiated 'synthetic' VSMC being transformed to 'contractile', differentiated VSMC (Boerth *et al.*, 1997).

With respect to the findings presented in this thesis, the loss of PDE3A in rat and human synthetic VSMC follows with these observations since PDE3 is a cGMP-inhibited enzyme. The absence of PKG and PDE3 in synthetic VSMC are potential indicators of a trend toward an overall decrease of cGMP sensitive proteins in synthetic VSMC. Combined with a loss of PDE1B observed in human VSMC, it appears that there is a general loss of cGMP regulation and cGMP sensitivity in synthetic VSMC. Thus, the relative importance of cAMP in these cells is enhanced, since it appears to be the major cyclic nucleotide responsible for downstream signalling events, including those events involved in inhibition of cell proliferation. Therefore, the cAMP hydrolyzing enzymes, namely PDE4, play an important regulatory role in these cells.

3.4 PDE3 Inhibitors for the Treatment of Restenosis

Most current strategies for prevention of restenosis related to inhibition of PDE, have focused on PDE3. Although the PDE3 inhibitor cilostazol has been shown to prevent neointimal formation in some animal models, the same degree of neointimal suppression has not been demonstrated in human trials. When cilostazol treatment was compared to treatment with ticlopidine, an anti-platelet agent, the results demonstrated that there was no significant difference in neointimal formation between the two groups (Nagaoka *et al.*, 2001). Since PDE3 is abundant in platelets, this observation suggests

that any positive effects achieved by cilostazol treatment are most likely a result of its effects on platelets, not on vascular smooth muscle cells. Although antiplatelet therapy has shown some success in reducing neointimal formation, based on the mechanism of neointimal formation, it is likely that using PDE inhibitors targeted specifically at VMSC is a more promising strategy for prevention of restenosis. Based on our findings in both rat and human synthetic VSMC, we believe that PDE3 is not the prime target for inhibition, since its expression is low in these cells.

3.5 PDE4 Inhibitors for the Treatment of Restenosis

The findings obtained from rat VSMC suggest that PDE4 inhibitors may prove more successful for preventing restenosis, since PDE4 is the most abundant cAMP hydrolyzing enzyme present in rat synthetic VSMC. Further, PDE4 may be a better therapeutic target than PDE3 because unlike PDE3 inhibitors, PDE4 inhibitors elicit few effects on the systemic circulation. Whereas PDE3 inhibitors stimulate myocardial contractility, vascular and airway smooth muscle relaxation, and inhibition of platelet aggregation, PDE4 inhibitors do not cause these potentially dangerous effects (Shakur *et al.*, 2001).

Currently PDE4 inhibitors are prospective agents for the treatment of chronic lung diseases such as asthma and chronic obstructive pulmonary disease (Barnette and Underwood, 2000). Their ability to suppress the activity of inflammatory cells renders them promising anti-inflammatory agents. PDE4 inhibitors achieve this result by inhibiting the generation of cytokines, reducing the production of pro-inflammatory mediators such as basophils, neutrophils and eosinophils, as well as attenuating de-granulation of these cells (Barnette and Underwood, 2000).

In this respect, PDE4 inhibitors are potentially useful in the context of restenosis. Since one of the principal initiating events of restenosis is inflammation, the ability of PDE4 inhibitors to suppress the immune response could be significant in their overall efficacy.

3.6 Clinical Significance of Our Findings

We have shown a loss of PDE3A in rat synthetic VSMC both in culture, and in neointima. Moreover, we have shown that the loss of PDE3A in cultured rat VSMC parallels the loss of PDE3A observed in human VSMC in culture. We did not however, examine human neointima because of our inability to obtain neointimal VSMC that are still of the synthetic phenotype. In most cases, reendothelialization of arterial lesions over time causes neointimal VSMC to lose their synthetic phenotype, and become contractile VSMC. Therefore, it is not possible to state with absolute certainty that PDE3A is absent in human neointimal VSMC. However, we can predict, based on our findings from cultured and neointimal rat VSMC, that human VSMC in culture are a valid paradigm for synthetic VSMC from human neointima. Unfortunately, PDE profiles in human synthetic VSMC are unique in that they express an abundance of PDE1C. Therefore, neointima from animal models are not an appropriate model for use as an accurate alternative for human neointima.

The fact that human synthetic VSMC have unique species-specific characteristics must be borne in mind when developing therapeutic strategies to treat restenosis. Although we have hypothesized that PDE4 inhibitors would be successful in preventing restenosis, this may not be the case since unlike the rat, PDE4 is not the primary cAMP-hydrolyzing enzyme in humans. There is such a high expression of PDE1C in human

synthetic VSMC, that its presence may make it difficult to detect any effects of PDE4 inhibition. Therefore a combination of PDE inhibitors, such as PDE3, PDE4 and/or PDE1 may prove to be more effective than inhibition of a single PDE isoform.

3.7 Future Directions

The findings presented in this study are relevant in many respects, and therefore may be pursued from several different perspectives. In terms of cyclic nucleotide signalling in synthetic VSMC, the loss of PDE3 activity could be correlated with the loss or no loss of specific PDE3 regulated cell functions. Determining whether the decrease in PDE3A does indeed alter cyclic nucleotide signalling regulation can be achieved by using PDE3 inhibitors in contractile VSMC and in synthetic VSMC and comparing aspects of cell function.

The reduction the cGMP inhibited PDE3 enzyme shown in our study serves to call attention to the fact that in synthetic VSMC, there appears to be a decrease of proteins normally sensitive to cGMP. Some of the proteins involved in the cGMP signalling cascade, such as sGC and PDE5, as well as some downstream cGMP effector proteins have not been studied in the context of an overall decrease of expression and/or sensitivity in synthetic VSMC. This may substantiate our hypothesis that in synthetic VSMC, there is a total cellular desensitization to cGMP.

A natural progression of our work, with respect to the clinical implications for treatment of restenosis is the use of PDE4 and/or combined PDE3/PDE4 inhibitors. Using the rat model of balloon catheter-induced injury, inhibitors used at various time points following balloon catheterization may be informative with regard to the point at which PDE3/4 inhibition is most effective. The route of inhibitor delivery may also be

relevant. Local drug delivery through pluronic gel compared to systemic drug delivery may present significantly differently results.

Reference List

Allaire E and Clowes AW (1997) Endothelial cell injury in cardiovascular surgery: the intimal hyperplastic response. *Ann.Thorac.Surg.* **63**:582-591.

Anderson PG, Boerth NJ, Liu M, McNamara DB, Cornwell TL, and Lincoln TM (2000) Cyclic GMP-dependent protein kinase expression in coronary arterial smooth muscle in response to balloon catheter injury. *Arterioscler.Thromb.Vasc.Biol.* **20**:2192-2197.

Aoki M, Morishita R, Hayashi S, Jo N, Matsumoto K, Nakamura T, Kaneda Y, and Ogihara T (2001) Inhibition of neointimal formation after balloon injury by cilostazol, accompanied by improvement of endothelial dysfunction and induction of hepatocyte growth factor in rat diabetes model. *Diabetologia* **44**:1034-1042.

Banai S KAGS (2001) Coronary Athersclerosis and Restenosis, in *Heart Physiology and Pathophysiology* (Sperelakis N KYTACM ed) pp 967-100, Academic Press, San Diego.

Barnette MS and Underwood DC (2000) New phosphodiesterase inhibitors as therapeutics for the treatment of chronic lung disease. *Curr.Opin.Pulm.Med.* **6**:164-169.

Beavo JA (1995) Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. *Physiol Rev.* **75**:725-748.

Boerth NJ, Dey NB, Cornwell TL, and Lincoln TM (1997) Cyclic GMP-dependent protein kinase regulates vascular smooth muscle cell phenotype. *J.Vasc.Res.* **34**:245-259.

Bolger G, Michaeli T, Martins T, St John T, Steiner B, Rodgers L, Riggs M, Wigler M, and Ferguson K (1993) A family of human phosphodiesterases homologous to the dunce learning and memory gene product of Drosophila melanogaster are potential targets for antidepressant drugs. *Mol.Cell Biol.* **13**:6558-6571.

Bornfeldt KE, Campbell JS, Koyama H, Argast GM, Leslie CC, Raines EW, Krebs EG, and Ross R (1997) The mitogen-activated protein kinase pathway can mediate growth inhibition and proliferation in smooth muscle cells. Dependence on the availability of downstream targets. *J.Clin.Invest* **100**:875-885.

Bornfeldt KE and Krebs EG (1999) Crosstalk between protein kinase A and growth factor receptor signaling pathways in arterial smooth muscle. *Cell Signal.* **11**:465-477.

Brindle PK and Montminy MR (1992) The CREB family of transcription activators. *Curr.Opin.Genet.Dev.* **2**:199-204.

Burkitt HG YBHJ (1993) *Functional Histology*. Longman Group Limited, New York.

Burns F, Zhao AZ, and Beavo JA (1996) Cyclic nucleotide phosphodiesterases: gene complexity, regulation by phosphorylation, and physiological implications. *Adv.Pharmacol.* **36**:29-48.

Chini CC, Grande JP, Chini EN, and Dousa TP (1997) Compartmentalization of cAMP signaling in mesangial cells by phosphodiesterase isozymes PDE3 and PDE4. Regulation of superoxidation and mitogenesis. *J.Biol.Chem.* **272**:9854-9859.

Chinkers M and Garbers DL (1989) The protein kinase domain of the ANP receptor is required for signaling. *Science* **245**:1392-1394.

Conti M, Nemoz G, Sette C, and Vicini E (1995) Recent progress in understanding the hormonal regulation of phosphodiesterases. *Endocr.Rev.* **16**:370-389.

Corbin JD and Francis SH (1999) Cyclic GMP phosphodiesterase-5: target of sildenafil. *J.Biol.Chem.* **274**:13729-13732.

Davis CW and Daly JW (1979) A simple direct assay of 3',5'-cyclic nucleotide phosphodiesterase activity based on the use of polyacrylamide-bononate affinity gel chromatography. *J.Cyclic.Nucleotide.Res.* **5**:65-74.

Degerman E, Belfrage P, and Manganiello VC (1997) Structure, localization, and regulation of cGMP-inhibited phosphodiesterase (PDE3). *J.Biol.Chem.* **272**:6823-6826.

Denger S, Jahn L, Wende P, Watson L, Gerber SH, Kubler W, and Kreuzer J (1999) Expression of monocyte chemoattractant protein-1 cDNA in vascular smooth muscle cells: induction of the synthetic phenotype: a possible clue to SMC differentiation in the process of atherogenesis. *Atherosclerosis* **144**:15-23.

Dousa TP (1999) Cyclic-3',5'-nucleotide phosphodiesterase isozymes in cell biology and pathophysiology of the kidney. *Kidney Int.* **55**:29-62.

Edwards AS and Scott JD (2000) A-kinase anchoring proteins: protein kinase A and beyond. *Curr.Opin.Cell Biol.* **12**:217-221.

El Beyrouty C and Spinler SA (2001) Cilostazol for prevention of thrombosis and restenosis after intracoronary stenting. *Ann.Pharmacother.* **35**:1108-1113.

England PJ and Shahid M (1987) Effects of forskolin on contractile responses and protein phosphorylation in the isolated perfused rat heart. *Biochem.J.* **246**:687-695.

Fisher DA, Smith JF, Pillar JS, St Denis SH, and Cheng JB (1998) Isolation and characterization of PDE9A, a novel human cGMP-specific phosphodiesterase. *J.Biol.Chem.* **273**:15559-15564.

Francis SH, Turko IV, and Corbin JD (2001) Cyclic nucleotide phosphodiesterases: relating structure and function. *Prog.Nucleic Acid Res.Mol.Biol.* **65**:1-52.

Fujishige K, Kotera J, and Omori K (1999) Striatum- and testis-specific phosphodiesterase PDE10A isolation and characterization of a rat PDE10A. *Eur.J.Biochem.* **266**:1118-1127.

GM Pitari DMVBSW (2001) Cyclic Nucleotides and Protein Phosphorylation in Vascular Smooth Muscle Relaxation, in *Heart Physiology and Pathophysiology* (N Sperelakis YKATMC ed) pp 805-828, Adademic Press, San Diego.

Hanoune JDN (2001) Regulation and Role of Adenylyl Cyclase Isoforms. *Annu.Rev.Pharmacol.Toxicol.* **41**:145-174.

Haslam RJ, Dickinson NT, and Jang EK (1999) Cyclic nucleotides and phosphodiesterases in platelets. *Thromb.Haemost.* **82**:412-423.

Hayashi M, Matsushima K, Ohashi H, Tsunoda H, Murase S, Kawarada Y, and Tanaka T (1998) Molecular cloning and characterization of human PDE8B, a novel thyroid-specific isozyme of 3',5'-cyclic nucleotide phosphodiesterase. *Biochem.Biophys.Res.Commun.* **250**:751-756.

Houslay MD and Milligan G (1997) Tailoring cAMP-signalling responses through isoform multiplicity. *Trends Biochem.Sci.* **22**:217-224.

Houslay MD, Sullivan M, and Bolger GB (1998) The multienzyme PDE4 cyclic adenosine monophosphate-specific phosphodiesterase family: intracellular targeting, regulation, and selective inhibition by compounds exerting anti-inflammatory and antidepressant actions. *Adv.Pharmacol.* **44**:225-342.

Indolfi C, Avvedimento EV, Di Lorenzo E, Esposito G, Rapacciuolo A, Giuliano P, Grieco D, Cavuto L, Stingone AM, Ciullo I, Condorelli G, and Chiariello M (1997) Activation of cAMP-PKA signaling in vivo inhibits smooth muscle cell proliferation induced by vascular injury. *Nat.Med.* **3**:775-779.

Indolfi C, Di Lorenzo E, Rapacciuolo A, Stingone AM, Stabile E, Leccia A, Torella D, Caputo R, Ciardiello F, Tortora G, and Chiariello M (2000) 8-chloro-cAMP inhibits smooth muscle cell proliferation in vitro and neointima formation induced by balloon injury in vivo. *J.Am.Coll.Cardiol.* **36**:288-293.

Indolfi C, Stabile E, Perrino C, and Chiariello M (1998) Mechanisms of restenosis after angioplasty and approach to therapy (Review). *Int.J.Mol.Med.* **2**:143-148.

Inoue Y, Toga K, Sudo T, Tachibana K, Tochizawa S, Kimura Y, Yoshida Y, and Hidaka H (2000) Suppression of arterial intimal hyperplasia by cilostamide, a cyclic nucleotide phosphodiesterase 3 inhibitor, in a rat balloon double- injury model. *Br.J.Pharmacol.* **130**:231-241.

Ishizaka N, Taguchi J, Kimura Y, Ikari Y, Aizawa T, Togo M, Miki K, Kurokawa K, and Ohno M (1999) Effects of a single local administration of cilostazol on neointimal formation in balloon-injured rat carotid artery. *Atherosclerosis* **142**:41-46.

Kakkar R, Raju RV, and Sharma RK (1999) Calmodulin-dependent cyclic nucleotide phosphodiesterase (PDE1). *Cell Mol.Life Sci.* **55**:1164-1186.

Keravis T, Komas N, and Lugnier C (2000) Cyclic nucleotide hydrolysis in bovine aortic endothelial cells in culture: differential regulation in cobblestone and spindle phenotypes. *J.Vasc.Res.* **37**:235-249.

Komas N, Lugnier C, and Stoclet JC (1991) Endothelium-dependent and independent relaxation of the rat aorta by cyclic nucleotide phosphodiesterase inhibitors. *Br.J.Pharmacol.* **104** :495-503.

Koyama H, Bornfeldt KE, Fukumoto S, and Nishizawa Y (2001) Molecular pathways of cyclic nucleotide-induced inhibition of arterial smooth muscle cell proliferation. *J.Cell Physiol* **186**:1-10.

Li S, Sims S, Jiao Y, Chow LH, and Pickering JG (1999) Evidence from a novel human cell clone that adult vascular smooth muscle cells can convert reversibly between noncontractile and contractile phenotypes. *Circ.Res.* **85**:338-348.

Libby P and Tanaka H (1997) The molecular bases of restenosis. *Prog.Cardiovasc.Dis.* **40**:97-106.

Liu H and Maurice DH (1998) Expression of cyclic GMP-inhibited phosphodiesterases 3A and 3B (PDE3A and PDE3B) in rat tissues: differential subcellular localization and regulated expression by cyclic AMP. *Br.J.Pharmacol.* **125**:1501-1510.

Liu H and Maurice DH (1999) Phosphorylation-mediated activation and translocation of the cyclic AMP- specific phosphodiesterase PDE4D3 by cyclic AMP-dependent protein kinase and mitogen-activated protein kinases. A potential mechanism allowing for the coordinated regulation of PDE4D activity and targeting. *J.Biol.Chem.* **274**:10557-10565.

Liu H, Palmer D, Jimmo SL, Tilley DG, Dunkerley HA, Pang SC, and Maurice DH (2000) Expression of phosphodiesterase 4D (PDE4D) is regulated by both the cyclic AMP-dependent protein kinase and mitogen-activated protein kinase signaling pathways. A potential mechanism allowing for the coordinated regulation of PDE4D activity and expression in cells. *J.Biol.Chem.* **275**:26615-26624.

Lucas KA, Pitari GM, Kazerounian S, Ruiz-Stewart I, Park J, Schulz S, Chepenik KP, and Waldman SA (2000) Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol.Rev.* **52**:375-414.

Lugnier C and Komas N (1993) Modulation of vascular cyclic nucleotide phosphodiesterases by cyclic GMP: role in vasodilatation. *Eur.Heart J.* **14 Suppl I**:141-148.

Manganiello VC, Murata T, Taira M, Belfrage P, and Degerman E (1995) Diversity in cyclic nucleotide phosphodiesterase isoenzyme families. *Arch.Biochem.Biophys.* **322**:1-13.

Maurice DH and Haslam RJ (1990) Nitroprusside enhances isoprenaline-induced increases in cAMP in rat aortic smooth muscle. *Eur.J.Pharmacol.* **191**:471-475.

Maurice DH, Senis YA, Liu H, Giles AR, and Horn EH (1995) Cell layer-specific expression of cyclic nucleotide phosphodiesterases in rat arteries: molecular cloning using isolated cell layers from paraformaldehyde-fixed tissues. *J.Pharmacol.Toxicol.Methods* **34** :175-185.

Mii S, Khalil RA, Morgan KG, Ware JA, and Kent KC (1996) Mitogen-activated protein kinase and proliferation of human vascular smooth muscle cells. *Am.J.Physiol* **270**:H142-H150.

Movsesian MA (1999) Beta-adrenergic receptor agonists and cyclic nucleotide phosphodiesterase inhibitors: shifting the focus from inotropy to cyclic adenosine monophosphate. *J.Am.Coll.Cardiol.* **34**:318-324.

Murray KJ (1990) Cyclic AMP and mechanisms of vasodilation. *Pharmacol.Ther.* **47**:329-345.

Nagaoka N, Matsubara T, Okazaki K, Masuda N, Shikaura K, and Hotta A (2001) Comparison of ticlopidine and cilostazol for the prevention of restenosis after percutaneous transluminal coronary angioplasty. *Jpn.Heart J.* **42**:43-54.

Osinski MT and Schror K (2000) Inhibition of platelet-derived growth factor-induced mitogenesis by phosphodiesterase 3 inhibitors: role of protein kinase A in vascular smooth muscle cell mitogenesis. *Biochem.Pharmacol.* **60**:381-387.

Owens GK (1995) Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev.* **75**:487-517.

Owens GK (1998) Molecular control of vascular smooth muscle cell differentiation. *Acta Physiol Scand.* **164**:623-635.

Palmer D and Maurice DH (2000) Dual expression and differential regulation of phosphodiesterase 3A and phosphodiesterase 3B in human vascular smooth muscle: implications for phosphodiesterase 3 inhibition in human cardiovascular tissues. *Mol.Pharmacol.* **58**:247-252.

Palmer D, Tsoi K, and Maurice DH (1998) Synergistic inhibition of vascular smooth muscle cell migration by phosphodiesterase 3 and phosphodiesterase 4 inhibitors. *Circ.Res.* **82**:852-861.

Park SW, Lee CW, Kim HS, Lee NH, Nah DY, Hong MK, Kim JJ, and Park SJ (2000) Effects of cilostazol on angiographic restenosis after coronary stent placement. *Am.J.Cardiol.* **86**:499-503.

Patel TB, Du Z, Pierre S, Cartin L, and Scholich K (2001) Molecular biological approaches to unravel adenylyl cyclase signaling and function. *Gene* **269**:13-25.

Pfeifer A, Ruth P, Dostmann W, Sausbier M, Klatt P, and Hofmann F (1999) Structure and function of cGMP-dependent protein kinases. *Rev.Physiol Biochem.Pharmacol.* **135**:105-149.

Polson JB and Strada SJ (1996) Cyclic nucleotide phosphodiesterases and vascular smooth muscle. *Annu.Rev.Pharmacol.Toxicol.* **36**:403-427.

Radhika V and Dhanasekaran N (2001) Transforming G proteins. *Oncogene* **20**:1607-1614.

Raeymaekers L, Eggermont JA, Wuytack F, and Casteels R (1990) Effects of cyclic nucleotide dependent protein kinases on the endoplasmic reticulum Ca2+ pump of bovine pulmonary artery. *Cell Calcium* **11**:261-268.

Rana BK, Shiina T, and Insel PA (2001) Genetic variations and polymorphisms of G protein-coupled receptors: functional and therapeutic implications. *Annu.Rev.Pharmacol.Toxicol.* **41**:593-624.

Rose RJ, Liu H, Palmer D, and Maurice DH (1997) Cyclic AMP-mediated regulation of vascular smooth muscle cell cyclic AMP phosphodiesterase activity. *Br.J.Pharmacol.* **122**:233-240.

Rybalkin SD and Bornfeldt KE (1999b) Cyclic nucleotide phosphodiesterases and human arterial smooth muscle cell proliferation. *Thromb.Haemost.* **82**:424-434.

Rybalkin SD, Bornfeldt KE, Sonnenburg WK, Rybalkina IG, Kwak KS, Hanson K, Krebs EG, and Beavo JA (1997) Calmodulin-stimulated cyclic nucleotide phosphodiesterase (PDE1C) is induced in human arterial smooth muscle cells of the synthetic, proliferative phenotype. *J.Clin.Invest* **100**:2611-2621.

Shakur Y, Holst LS, Landstrom TR, Movsesian M, Degerman E, and Manganiello V (2001) Regulation and function of the cyclic nucleotide phosphodiesterase (PDE3) gene family. *Prog.Nucleic Acid Res.Mol.Biol.* **66**:241-277.

Smith CJ, Huang R, Sun D, Ricketts S, Hoegler C, Ding JZ, Moggio RA, and Hintze TH (1997) Development of decompensated dilated cardiomyopathy is associated with decreased gene expression and activity of the milrinone-sensitive cAMP phosphodiesterase PDE3A. *Circulation* **96**:3116-3123.

Sobue K, Hayashi K, and Nishida W (1999) Expressional regulation of smooth muscle cell-specific genes in association with phenotypic modulation. *Mol.Cell Biochem.* **190**:105-118.

Soderling SH, Bayuga SJ, and Beavo JA (1998) Identification and characterization of a novel family of cyclic nucleotide phosphodiesterases. *J.Biol.Chem.* **273**:15553-15558.

Soderling SH, Bayuga SJ, and Beavo JA (1999) Isolation and characterization of a dual-substrate phosphodiesterase gene family: PDE10A. *Proc.Natl.Acad.Sci.U.S.A* **96**:7071-7076.

Soderling SH and Beavo JA (2000) Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions. *Curr.Opin.Cell Biol.* **12**:174-179.

Steinberg SF and Brunton LL (2001) Compartmentation of G protein-coupled signaling pathways in cardiac myocytes. *Annu.Rev.Pharmacol.Toxicol.* **41**:751-773.

Stull JT, Blumenthal DK, Miller JR, and DiSalvo J (1982) Regulation of myosin phosphorylation. *J.Mol.Cell Cardiol.* **14 Suppl 3**:105-110.

Surks HK, Mochizuki N, Kasai Y, Georgescu SP, Tang KM, Ito M, Lincoln TM, and Mendelsohn ME (1999) Regulation of myosin phosphatase by a specific interaction with cGMP- dependent protein kinase Ialpha. *Science* **286**:1583-1587.

Tanabe Y, Ito E, Nakagawa I, and Suzuki K (2001) Effect of cilostazol on restenosis after coronary angioplasty and stenting in comparison to conventional coronary artery stenting with ticlopidine. *Int.J.Cardiol.* **78**:285-291.

Thyberg J (1998) Phenotypic modulation of smooth muscle cells during formation of neointimal thickenings following vascular injury. *Histol.Histopathol.* **13**:871-891.

Walsh K and Takahashi A (2001) Transcriptional regulation of vascular smooth muscle cell phenotype. *Z.Kardiol.* **90 Suppl 3**:12-16.

Worth NF, Rolfe BE, Song J, and Campbell GR (2001) Vascular smooth muscle cell phenotypic modulation in culture is associated with reorganisation of contractile and cytoskeletal proteins. *Cell Motil.Cytoskeleton* **49**:130-145.

Zhao AZ, Zhao H, Teague J, Fujimoto W, and Beavo JA (1997) Attenuation of insulin secretion by insulin-like growth factor 1 is mediated through activation of phosphodiesterase 3B. *Proc.Natl.Acad.Sci.U.S.A* **94**:3223-3228.

Zou Y, Hu Y, Metzler B, and Xu Q (1998) Signal transduction in arteriosclerosis: mechanical stress-activated MAP kinases in vascular smooth muscle cells (review). *Int.J.Mol.Med.* **1**:827-834.