Vascular Smooth Muscle Cell Phenotype-Dependent Phosphodiesterase 4D Short Form Expression: Role of Differential Histone Acetylation on cAMP-Regulated Function

Douglas G. Tilley and Donald H. Maurice

Department of Pharmacology & Toxicology, Queen’s University, Kingston, Ontario, Canada, K7L 3N6
Running Title: cAMP-Regulated PDE4D Expression in Smooth Muscle

Corresponding Author:

Dr. Donald H. Maurice, PhD,
Career Investigator, Heart & Stroke Foundation of Ontario,
Department of Pharmacology & Toxicology, Queen’s University,
Kingston, Ontario, Canada, K7L 3N6
Tel: 613-533-6000 (75089); Fax: 613-533-6412
Email: mauriced@post.queensu.ca

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 Abbreviations: VSMC, vascular smooth muscle cell; GPCR, G-protein coupled receptor; PDE, cyclic nucleotide phosphodiesterases; CRE, cAMP response element; CREB, CRE binding protein; ATF-1, activating transcription factor 1; EPAC, exchange protein activated by cAMP; ERK, extracellular signal-regulated protein kinase; pCPT, 8-pCPT-2’-O-Me-cAMP.
Abstract

Sustained activation of adenylyl cyclase in vascular smooth muscle cells (VSMCs) results in the activation of a series of complex regulatory systems designed to desensitize these cells to further cAMP-mediated events. While an increase in Phosphodiesterase (PDE) 4-mediated hydrolysis of cAMP forms an integral part of this desensitization program in both “contractile/quiescent” and “synthetic/activated” VSMC, distinct PDE4D gene variants coordinate these event in these phenotypically distinct cells. Using a combination of pharmacological, biochemical and molecular biological approaches, and both in vivo and in vitro systems, we have identified the molecular basis underlying this VSMC phenotype-selective expression of PDE4D in response to cAMP-elevating agents in these cells. Thus, while the PKA/CREB/CRE signaling cascade regulates PDE4D expression in each VSMC phenotype, elevated levels of histone acetylation of the intronic promoter regulating PDE4D1 and PDE4D2 expression allows selective cAMP-mediated induction of expression of these PDE4D variants in “synthetic/activated”. In contrast, the newly described EPAC1/Rap1A cAMP-dependent signaling cascade plays no role in regulating PDE4D expression in either VSMC phenotype. Our data are presented in the context of PDE4-mediated desensitization to cAMP-elevating agents in VSMCs and with the recognition that cAMP-elevating agents are being considered as adjunctive pharmacotherapy in percutaneous coronary interventions, including stenting.
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Introduction

Vascular smooth muscle cells (VSMCs) alter their phenotype in response to vascular injury, or when propagated in tissue culture (Indolfi et al., 2003; Owens et al., 2004). “Contractile/quiescent” VSMCs resident within healthy arteries are contractile but have limited proliferative, migratory and synthetic capabilities. In contrast, “synthetic/activated” VSMCs found within the intima of damaged blood vessels or generated by tissue culture, display a reduced contractile capacity and increased proliferative, migratory and synthetic capabilities (Indolfi et al., 2003; Owens et al., 2004). Migration of VSMCs into the intimal layer of damaged arteries, and their proliferation and deposition of extracellular matrix within the intima, allow “synthetic/activated” VSMCs to promote both post-angioplasty and “in-stent” restenosis. Restenosis remains the major factor limiting long-term success in these interventions and therapeutic approaches limiting VSMC proliferation, migration and extracellular matrix deposition and reduce restenosis are being sought.

While cAMP-elevating agents inhibit the proliferation, migration and synthesis of extracellular matrix proteins by “synthetic/activated” VSMCs (Indolfi et al., 2003; Maurice et al., 2003), and some of these agents reduce restenosis in animal models (Agata et al., 2000; Indolfi et al., 2000), two factors limit their effectiveness. First, agents that increase VSMC cAMP are also potent vasorelaxants and can cause systemic hypotension (Maurice et al., 2003). Second, the same regulatory systems which allow cAMP-elevating agents to impact VSMCs functions also act to desensitize these effects in
response to prolonged application. A protein kinase A (PKA)-dependent phosphorylation of components involved in hormone-regulated activation of adenyl cyclases contributes to desensitization of cAMP-mediated effects in VSMCs (Kohout and Lefkowitz, 2003). More recently, several reports have shown that increased cAMP hydrolysis by cyclic nucleotide phosphodiesterases (PDEs) also plays a central role in desensitizing signaling through the cAMP signaling axis in several cells, including VSMCs (Rose et al., 1997; Mehts et al., 1999; Tilley and Maurice, 2002; Rybalkin et al., 2003; Maurice et al., 2003).

The PDE4 family of enzymes represent the most extensively studied of the 11 distinct multigene families of PDEs expressed in mammals. In human, rat and mouse, four distinct PDE4 genes, namely PDE4A, PDE4B, PDE4C and PDE4D, encode numerous PDE4 enzymes variants (Conti et al., 2003; Houslay and Adams, 2003; Maurice et al., 2003). The dominant PDE4 expressed in rodent or human arterial VSMCs is PDE4D (Liu and Maurice, 1999; Palmer and Maurice, 2000). Through the use of alternate promoters, PDE4D can yield six PDE4D “long-forms” (PDE4D3-5 and PDE4D7-9) and two PDE4D “short-forms” (PDE4D1-2) (Conti et al., 2003; Houslay and Adams, 2003; Liu and Maurice, 1999; Maurice et al., 2003). Cellular PDE4D expression is regulated transcriptionally by PKA and the promoters regulating expression of one “long-form”, PDE4D5, and both “short form”, PDE4D1-2, contain cAMP-response elements binding protein (CREB) consensus response elements (CREs) (Vicini and Conti, 1997; Le Jeune et al., 2002). In rat aortic VSMCs PDE4D expression is also regulated at the translational levels by both PKA and the extracellular signal-regulated kinases (ERKs) (Liu et al.
In addition to impacting \textit{PDE4D} expression, PKA and ERKs also regulate PDE4D catalytic activity (Houslay and Adams, 2003; Maurice \textit{et al.}, 2003). Thus, while phosphorylation of PDE4D long forms by PKA activates these enzymes, ERK-mediated phosphorylation of the long and short PDE4Ds has form-specific effects on their activities (Baillie \textit{et al.}, 2000; Houslay and Adams, 2003). PKA phosphorylation of some long form PDE4D variants also regulates their ability to associate with various scaffolding/anchoring proteins, (reviewed in Wong and Scott, 2004).

Previous studies in our laboratory (Tilley and Maurice, 2002; Liu \textit{et al.}, 2000; Liu and Maurice, 1999) have shown that cAMP-elevating agents increased PDE4 activity and up-regulated expression of \textit{PDE4D} in both "contractile/quiescent" and "synthetic/activated" arterial VSMCs. Interestingly, however, different \textit{PDE4D} gene products were increased by cAMP-elevating agents in these two phenotypically-distinct VSMCs populations. Thus, while prolonged incubation with cAMP-elevating agents in "contractile/quiescent" VSMCs stimulated their expression of PDE4D3, similar treatments of "synthetic/activated" VSMCs resulted in the induction of PDE4D1 and PDE4D2, the two short-form \textit{PDE4D} gene products not expressed in "contractile/quiescent" VSMCs (Tilley and Maurice, 2002). Since the different responses of these phenotypically distinct VSMCs might be predicted to differentially alter their responses to future cAMP-elevating agents, we undertook to elucidate the molecular basis for selective cAMP-mediated induction of the short PDE4D variants in "synthetic/activated" VSMCs. Since the impact of cAMP activation of the novel cAMP
effector, Exchange Factor Activated by cAMP (EPAC), on PDE4D activity and expression had not been studied previously, we also assessed its role on these events in this study.

**Materials and Methods**

**General Reagents.** Male Wistar rats were obtained from Charles River (Constance, PQ, Canada). All materials and reagents used for cell culture, pharmacological treatment of rats and cells, cAMP PDE activity measurements, immunoblotting, and PCR are described previously (Tilley and Maurice, 2002; Rose et al., 1997). All other chemicals of reagent grade, glass slides and coverslips were purchased from Fisher Scientific (Nepean, ON, Canada). 8-pCPT-2’-O-Me-cAMP was purchased from Biolog Life Sciences Institute (Bremen, Germany). Plasmids used were generous gifts from Dr. Rudolph Juliano (PKI; University of North Carolina), Dr. Charles Vinson (ACREB; NIH), Dr. Xiaodong Cheng (Flag-EPAC1; University of Texas) and Dr. Lawrence Quilliam (EE-Rap1A(63E); Indiana University).

**Pharmacological Treatment of Rats.** Vehicle (saline) or dbcAMP (15 mg/kg) were administered via i.p. injection to 6 male Wistar rats (3 each condition) every hour for 5 h, followed by euthanization (1.7 mL/kg euthanyl) and the aortae removed, processed and protein concentrations determined as described previously (Tilley and Maurice, 2002).
Pharmacological Treatment of Cells. Primary cultures of rat aortic vascular smooth muscle cells (VSMCs) and NIH 3T3 fibroblasts (cells) were cultured as described previously (Liu and Maurice, 1999). Culture media was removed and replaced with fresh media supplemented with vehicle (0.1% dimethyl sulfoxide (DMSO)) or combinations of forskolin (0.1-10 µmol/L), H89 (10 µmol/L), cycloheximide (100 µmol/L), or 8-pCPT-2’-O-Me-cAMP (10 µmol/L) for pre-determined periods. Following this incubation, treated cells were washed with PBS (pH 7.4), harvested and processed in a lysis buffer described previously (Tilley and Maurice, 2002).

Transfections. NIH 3T3 cells were transiently transfected with plasmids encoding either PKI, ACREB, Flag-tagged EPAC1 or EE-tagged Rap1A(63E), or appropriate control plasmids, using the FuGENE 6 transfection reagent as recommended (Roche Diagnostics Corporation, Laval, Qc, Canada). NIH 3T3 cells were also transfected with either a phosphothiorate CRE (5’-TGACGTCATGACGTCATGACGTCA-3’) or mismatch (5’-TGTTGGTCATGTGGTCATGTGGTCA-3’) oligodeoxynucleotide (ODN) which were synthesized by Cortec (Kingston, ON).

cAMP PDE Activities. Levels of cAMP PDE activity in NIH 3T3 cell or VSMC lysates were determined as described previously by us (Tilley and Maurice, 2002), with 3 µg of cellular lysate protein. PDE4 activity levels were determined using a maximally effective selective concentration of Ro 20-1724 (10 µmol/L).
Immunoblotting. Equivalent aliquots (10 µg) of NIH 3T3 cells, VSMCs or aortic tissues, were subjected to SDS-PAGE and immunoblotted as described previously (Tilley and Maurice, 2002). PDE4D, phospho-CREB, Flag-EPAC1, EPAC1, Rap1A or β-actin were detected and quantitated by chemiluminescence using selective antisera against PDE4D (1:4000, ICOS corporation), phospho-CREB (1:1000, Affinity Bioreagents), FLAG-M2 (1:40,000, Sigma-Aldrich), EPAC1 (1:500, Upstate), Rap1 (1:200, Santa Cruz Biotechnology Inc.) or β-actin (1:40,000, Sigma-Aldrich), respectively, and an appropriate secondary horseradish peroxidase-conjugated antisera. Individual PDE4D variants, Flag-EPAC1, Rap1A, EE-Rap1A(63E), phosphor-CREB and β-actin are indicated. Representative immunoblots shown were obtained in at least three independent experiments.

Preparation of Aortae, VSMCs and NIH 3T3 Cells for Chromatin

Immunoprecipitation. Aortic medial tissues were prepared by incubating aortae in an enzyme solution described previously (Rose et al., 1997) to remove adventitial and intimal tissues. Minced medial tissues were cross-linked in tissue culture media supplemented with formaldehyde (1% v/v) for 15 min at room temperature. Cross-linking was stopped by adding glycine to 0.125 mol/L final and mixing for 5 min. Cross-linked medial fragments were centrifuged at 1000 x g, rinsed twice with ice cold PBS mix (PBS, 10 mmol/L NaBu, 100 µg/mL PMSF, 1 µg/mL leupeptin), and then subsequently homogenized in PBS mix. NIH 3T3 cells or VSMCs were incubated with media
supplemented with formaldehyde (1 % v/v) at 37°C for 10 min. Cross-linked cells then were rinsed and homogenized in PBS mix as described above.

**Chromatin Immunoprecipitation.** NIH 3T3, VSMC or aortic samples underwent processing and chromatin immunoprecipitation as described by others (Forsberg et al., 2000), using 2.5 µL of 1 mg/mL anti-acetylated histone H3 polyclonal antibody per sample at 4°C overnight. Non-immune rabbit IgG was also used in preliminary experiments as a negative control for chromation immunoprecipitation (not shown). Samples and input controls underwent reverse crosslinking by incubation with 200 mmol/L NaCl and 10 µg RNase A at 65°C for 4 hours, and the DNA was purified by phenol:chloroform extraction and resuspended in 50 µL TE buffer.

**PCR Amplification of DNA Recovered From Chromatin Immunoprecipitation.** The level of immunoprecipitated PDE4D1/2 promoter DNA in medial aortic, VSMC and NIH 3T3 samples was determined by PCR analysis as previously described (Tilley and Maurice, 2002) for cycle numbers indicated, with gene-specific oligonucleotides primers targeted either to a 140 bp region of the rat PDE4D intronic promoter -1240 bp from the transcription start site (sense: 5’-CGCAATTCCACAGTGAGCAGAAATAGAC-3’; antisense: 5’-GCGAATTCTAAAGGAACAGCAATGGGA-3’), or a 210 bp region -240bp from the transcription start site (sense: 5’-CGGAATTCGCCCTGCTCTCACCCTCT-3’; antisense: 5’-GCGAATTCTCAGGATATTAGTGGGA-3’), as described by Vicini and Conti (Vicini and Conti, 1997). PCR products were separated by electrophoresis on 1%
agarose gels, visualized with ethidium bromide and quantitated by scanning densitometry. Products were sequenced to confirm their identity.

Visualization of actin dynamics in VSMCs. Serum-starved rat aortic VSMCs grown on 5µg/mL fibronectin-coated coverslips were treated with vehicle (0.1% DMSO), or forskolin (10 µmol/L) for 4 h, rinsed in serum-free media for 30 min, and then challenged with either vehicle, INE (100 nmol/L), Ro 20-1724 (10 µmol/L) or both INE and Ro 20-1724 for 15 min. Cells were rinsed in PBS, fixed with paraformaldehyde (4% v/v) for 20 min, rinsed in PBS and incubated for 1 h with fresh PBS containing 4’,6’-diamidino-2-phenylindole (DAPI, 1:500; Sigma-Aldrich) and phalloidin-tetramethylrhodamine B isothiocyanate (phalloidin-TRITC, 1:500; Sigma-Aldrich). Coverslips were mounted on glass slides and analyzed using a Zeiss Axiovert S100 microscope equipped with fluorescence capability. Images were handled using Slidebook 3.0.1 software (Intelligent Imaging Innovations, Inc.). Each experiment was performed in triplicate and repeated four times for a total of twelve samples per condition.

Statistical Analysis. Individual experiments were carried out in triplicate and data are presented as Means ± S.E.M. of at least three independent experiments. Statistically significant differences were determined using the unpaired Student’s t test method and a value of P < 0.05 was considered statistically significant. Representative immunoblots shown are reflective of similar results obtained in at least three separate experiments.
Results

Differential impact of cAMP-elevating agents on PDE4D expression in “contractile/quiescent” and “synthetic/activated” arterial VSMC

We previously reported that sustained exposure of rat aortic VSMCs in vivo (Tilley and Maurice, 2002), or of cultured rat aortic VSMCs in vitro (Liu and Maurice, 1999; Rose et al., 1997) to cAMP-elevating agents selectively increased levels of different PDE4D variants in these distinct VSMC phenotypes. Consistent with these earlier reports, a 4 h treatment of rats with a cAMP-analogue caused a two-fold increase in the expression of PDE4D3, the dominant long form PDE4D variant expressed in “contractile/quiescent” rat aortic VSMCs (Fig. 1A). Consistent with our previous work (Tilley and Maurice, 2002), longer treatments (8 h) with larger doses of dbcAMP (30 mg/Kg) further increase PDE4D3 expression but did not induce expression of the PDE4D short-form variants in these cells (not shown). In contrast, a 4 h treatment of cultured VSMC with a cAMP-elevating agent resulted in the induction of two PDE4D short forms, namely PDE4D1 and PDE4D2, but did not alter the level of expression of either PDE4D3 or PDE4D5 in these VSMCs (Fig. 1A). Consistent with an earlier report (Liu and Maurice, 1999), immunoprecipitation of VSMC lysates with PDE4D-specific antisera showed that PDE4D accounted for ≥ 80% of total PDE4 activity in these cells. Interestingly, no changes in the residual non-PDE4D activity following cAMP-based treatments in our experiments were noted (not shown).
Signaling systems involved in cAMP-mediated regulation of PDE4D expression in “contractile/quiescent” VSMCs, “synthetic/activated” VSMCs and NIH 3T3 fibroblasts

Since analysis of the molecular basis for PDE4D1 and PDE4D2 induction in “synthetic/activated” VSMCs necessitated the use of reagents encoded by plasmids, as well as synthetic oligonucleotides, and primary cultures of rat aortic VSMCs are very poorly transfected, we carried out our initial studies in both cultured rat aortic VSMCs as well as the more readily transfectable NIH 3T3 cells. Importantly, PDE4 activity was high in NIH 3T3 cells (Table 1) and these cells expressed both PDE4D3 and PDE4D5 under basal conditions (Fig. 1B). Also, a 4 h incubation with cAMP-elevating agents markedly upregulated expression of both PDE4D1 (12.8 ± 1 fold) and PDE4D2 (6.1 ± 1 fold) in these cells (P<0.05, n = 6 each), while not significantly affecting the levels of expression of either of the two long variants, PDE4D3 or PDE4D5 (Fig 1B).

Inhibition of PKA, or of translation, in cultured rat aortic VSMCs (Liu et al., 2000; Rose et al., 1997), or NIH 3T3 cells (Table 1), significantly blunted cAMP-elevating agent induced increases in PDE4 activity in these cells. Thus, inhibition of PKA with H89, or by the heterologous expression of the PKA inhibitory peptide, PKI, and the addition of cycloheximide, each inhibited cAMP-mediated increases in PDE4 activity in NIH 3T3 cells (Table 1). In keeping with our previous work, inhibition of PKA or of translation in these cells also inhibited forskolin-induced increases in PDE4D1 and PDE4D2 expression. Thus, treatment of NIH 3T3 cells with H89 blunted the forskolin-
mediated increase in PDE4D1 and PDE4D2 expression to levels 3.4 ± 1.5 and 2.9 ± 1.1 above basal values (P<0.05, n = 3 each); reductions of 75% and 65%, respectively (Fig. 1B). Similarly, heterologous PKI expression reduced forskolin-mediated increases in PDE4D1 and PDE4D2 expression to levels 4.2 ± 0.5 and 2.1 ± 0.5 above basal values (P<0.05, n = 3 each); reductions corresponding to 75% and 80%, respectively (Fig. 1C).

In each cell type addition of cycloheximide all but abolished forskolin-mediated increases in PDE4D1 and PDE4D2 expression (Fig. 1B). These data are consistent with a major role for PKA in the cAMP-mediated induction of PDE4D1 and PDE4D2 in each “synthetic/activated” rat aortic VSMCs as well as NIH 3T3 cells. Since our previous work also identified a dominant role for PKA and de novo protein synthesis in mediating cAMP-induced increases in PDE4D3 and PDE4D5 expression in “contractile/quiescent” rat VSMCs in vivo (Tilley and Maurice, 2002), taken together these data are inconsistent with the idea that differential regulation of PDE4D expression in “synthetic/activated” and “contractile/quiescent” VSMCs resides at the levels of cAMP-mediated activation of PKA.

Assessment of the role of EPAC1 and Rap1 on PDE4 activity and PDE4D expression

While PKA represents the best studied cAMP-effector enzyme, several reports have shown that the Exchange Protein Activated by cAMP (EPAC), a recently described guanine nucleotide exchange factor (GEF) for the low molecular weight G-protein, Rap1, was activated by cAMP and could allow cAMP to signal independently of PKA (Bos, 2003). In this context, we sought to determine if EPAC was also involved in
coordinating the effects of cAMP on PDE4D expression in VSMCs. Incubation of “synthetic/activated” VSMCs, or NIH 3T3 cells, with several concentrations of the recently described EPAC-selective cAMP analogue, 8-pCPT-2’-O-Me-cAMP (Enserink et al., 2002), did not alter basal PDE4 activity levels, nor did this agent significantly impact the ability of forskolin to increase this activity in these cells (Table 2), or cause phosphorylation of CREB (not shown). However, we and others (Rangarajan et al., 2003) have shown that at 10µM 8-pCPT-2’-O-Me-cAMP was sufficient to induce previously reported EPAC1-Rap1A-mediated cellular adhesion in other cell types such as ovarian carcinomas cells (OVCAR) or NIH 3T3 following their transfection with FLAG-tagged EPAC1 (unpublished observations). Consistent with the phosphodiesterase activity measurements, immunoblot analysis of lysates of treated VSMCs, or NIH 3T3 cells, with PDE4D-selective antisera showed 8-pCPT-2’-O-Me-cAMP did not impact levels of PDE4D3, PDE4D5, PDE4D1 or PDE4D2 (Fig. 2A, 2C). Furthermore, 8-pCPT-2’-O-Me-cAMP did not impact forskolin-induced increases in PDE4D1 and PDE4D2 in either of these cells. Similar results were obtained when NIH 3T3 cells expressing a FLAG-tagged EPAC1 construct were treated with 8-pCPT-2’-O-Me-cAMP (Table 2, Fig. 2B, 2C), obviating a problem in the initial studies caused by low levels of EPAC expression in our cells. Since several Rap1-GEFs have been described, and some of these have been implicated in regulating PKA-mediated activation of Rap1 independently of EPAC, we also sought to determine if Rap1 might impact directly PDE4D expression in these cells, independently of EPAC. NIH 3T3 expressing a constitutively active form of Rap1A, Rap1A(63E) were used for these studies. Again, as was the case in
untransfected NIH 3T3 cells, or the EPAC1-expressing NIH 3T3 cells, Rap1A(63E) expression did not alter either basal PDE4D expression, nor the ability of forskolin to induce PDE4D1 and PDE4D2 (Table 2, Fig. 2B, 2C). Taken together, these data are inconsistent with EPAC1- or Rap1A-mediated signaling playing a role in cAMP-regulated expression of PDE4D expression in VSMCs, or NIH 3T3 cells. Local application of 8-pCPT-2’-O-Me-cAMP to blood vessels in vivo using our recently developed assay system (Tilley and Maurice, 2002) did not result in elevated levels of activated Rap1 in “contractile/quiescent” VSMCs (not shown) and as such it is unlikely that EPAC participates in PDE4D expression in “contractile/quiescent” VSMC.

**CREB-CRE signaling is involved in the cAMP-induced, PKA-dependent, induction of PDE4D1 and PDE4D2 in “synthetic/activated” VSMCs and NIH 3T3 cells**

Previous work has shown that the minimal promoter regulating PDE4D1 and PDE4D2 expression in both murine and human cells contains a cAMP-response element (CRE) (Vicini and Conti, 1997). In order to determine if a cAMP-response element binding protein (CREB)-CRE system might regulate cAMP-induced PDE4D1 and PDE4D2 expression in cells, the impact of forskolin on PDE4D1 and PDE4D2 expression was determined in cells expressing a dominant negative CREB variant, namely ACREB. While forskolin treatment of NIH 3T3 cells, or those expressing ACREB, resulted in a similar concentration- and time-dependent and H89-sensitive phosphorylation of CREB at S\(^{133}\), as well as the highly homologous ATF1 protein at its
equivalent site S\(^{63}\) (Fig. 3A, 3B), cAMP-mediated induction of PDE4D1 and PDE4D2 after treatment with forskolin for 4 h was markedly blunted in ACREB expressing cells. Indeed, while ACREB expression did not significantly alter PDE4D3 or PDE4D5 expression in these cells, it did markedly inhibit forskolin-mediated induction of both PDE4D1 and PDE4D2 by an average of 52 ± 19% and 51 ± 18%, respectively (P<0.05, n = 3 each; Fig. 3C). This effect of ACREB was consistent with a necessary role for CREB in regulating the cAMP-dependent induction of PDE4D1 and PDE4D2 caused by forskolin. In order to more directly test the hypothesis that a CRE-element within the PDE4D1 and PDE4D2 promoter was required for cAMP-mediated regulation of \(\text{PDE4D} \) expression, we also tested the impact of introducing a “decoy” CRE phosphorothioate oligonucleotide, or a mismatched control (see Materials and Methods) on forskolin-mediated increases in PDE4D short forms in these cells. While forskolin caused a significant increase in PDE4 activity in cells containing the control oligonucleotide, cells treated with the authentic CRE decoy oligonucleotide were shown to have a blunted response (Fig. 4A). Consistent with a role in \(\text{PDE4D} \) expression, immunoblot analysis with a PDE4D-specific antiserum confirmed that the CRE decoy caused a concentration-dependent reduction in forskolin-stimulated PDE4D1 and PDE4D2 induction (Fig. 4B). In these studies while forskolin increased PDE4D1 and PDE4D2 expression by factors 11.9 ± 0.7 and 6.0 ± 1.2 compared to basal values, respectively (P<0.05, n = 4), levels of induction in the presence of the CRE decoy were significantly reduced. Indeed, in the presence of 1.5\(\mu\)M or 2\(\mu\)M CRE decoy, forskolin-induced increases in PDE4D1 and PDE4D2 were reduced to 4.77 ± 0.49, 1.77 ± 1.43, and 3.21 ± 1.55, 0.92 ± 0.71,
respectively (P<0.05, n = 3 each). The concentration-dependency of CRE decoy inhibition of the induction of the short PDE4D variants is confirmed by the significant differences in inhibition of PDE4D1 and PDE4D2 induction between 1µM and 2µM CRE decoy (P<0.05, n = 3 each). At 2µM CRE, the endogenous levels of PDE4D3 and PDE4D5 expression also appear to be decreased, which may implicate CRE site availability in regulating the basal expression of long PDE4D variants, a notion supported by the existence of CRE sequences in the promoter of PDE4D5 as described by others (Le Jeune et al., 2002). Thus, taken together these results establish a role for a CREB/CRE mechanism regulating cAMP-mediated induction of PDE4D1 and PDE4D2 in response to prolonged cAMP signaling in these cells.

Phenotype-dependent differential histone acetylation of the PDE4D intronic promoter controlling PDE4D1 and PDE4D2 induction in VSMCs

Reversible post-translational modifications of histones, including ADP-ribosylation, methylation, glycosylation, phosphorylation, and acetylation have been shown to play a critical role in regulating gene transcription in many cell types. Among these, histone acetylation is the best studied and has been shown to associate with chromatin remodeling and subsequent transcriptional activation of several genes in VSMCs (Kumar and Owens, 2003; Manabe and Owens, 2001). Since our data identified selective induction of PDE4D1 and PDE4D2 in “synthetic/activated” VSMCs, and the absence of this event in “contractile/quiescent” VSMCs, we sought to determine if
selective short PDE4D variant induction in “synthetic/activated” VSMCs might be reflective of a phenotype-dependent difference in levels of histone acetylation at the intronic PDE4D1 and PDE4D2 promoter in these cells. Overall, our data are consistent with an important role for phenotype-dependent histone-3 (H3)-acetylation in controlling cAMP-mediated induction of PDE4D1 and PDE4D2 expression in “contractile/quiescent” and “synthetic/activated” VSMCs. Thus, utilizing a chromatin immunoprecipitation-based approach and two individual pairs of PDE4D intronic promoter sequence-derived oligonucleotide primers (see “Materials and Methods”), PDE4D intronic promoter DNA sequences were readily amplified from acetylated H3-immunoprecipitates from “synthetic/activated” VSMCs, but not from H3-immunoprecipitates derived from “contractile/quiescent” VSMCs (Fig. 5A). Intermediate amounts of PDE4D intronic DNA sequences were amplified when acetylated-H3 immunoprecipitates from NIH 3T3 cells were analyzed. Thus, while the amount of intronic PDE4D promoter which could be amplified from total DNA prior to immunoprecipitation (input samples) from these three cell types were similar, that which was amplified from acetylated H3-immunoprecipitates from these cell types was significantly greater in samples of “synthetic/activated” VSMCs, and NIH 3T3 cells, when compared to that from “contractile/quiescent” VSMCs (Fig. 5B). Since acetylation of H3 has been shown previously in other systems to indicate a relative increase in transcriptional activity, our data from these studies are consistent with the hypothesis that the PDE4D intronic promoter regulating PDE4D1 and PDE4D2 expression is activated in “synthetic/activated” VSMCs relative to that present in “contractile/quiescent” VSMCs.
This difference could, at least in part, account for the lack of PDE4D1 and PDE4D2 induction following cAMP-elevation in the “contractile/quiescent” VSMCs in vivo.

Functional consequences of PDE4D1 and PDE4D2 induction on “synthetic/activated” VSMCs

As presented in the Introduction, de-differentiation of “contractile/quiescent” VSMCs into “synthetic/activated” VSMCs, occurs in blood vessels in response to vascular damage and numerous studies have shown that the increased proliferative and migratory index of the “synthetic/activated” VSMCs contributes to blood vessel damage-associated narrowing, or stenosis (Owens et al., 2004; Maurice et al., 2003). In this context, we have shown previously that PDE4 inhibitors are efficient at limiting “synthetic/activated” VSMC migration and, in fact, have proposed that these drugs might be useful in limiting vascular events such as in-stent restenosis (Palmer et al., 1998). In light of results obtained here, we chose to assess the impact of cAMP-mediated induction of PDE4 activity, namely PDE4D1 and PDE4D2, in “synthetic/activated” VSMCs on cAMP-induced inhibition of cellular processes necessary for VSMC migration. Therefore, we determined if prior induction of PDE4D1 and PDE4D2 in “synthetic/activated” VSMCs would limit a future cAMP-induced cytoskeletal rearrangement in these cells (Fig. 6). Thus, while incubation of naïve (0.1% v/v DMSO, 4h) “synthetic/activated” VSMCs with isoproterenol caused a rapid (<20 min) disassembly of the actin cytoskeleton in these cells (Fig. 6C), VSMCs in which PDE4D1 and PDE4D2 had been induced by a prior 4 h treatment with forskolin (10 µmol/L) were
virtually insensitive to isoproterenol (Fig. 6G). Importantly, levels of PKA were not reduced following forskolin treatment (not shown). Consistent with a role for increased PDE4 activity in blunting the effects of isoproterenol in forskolin-pretreated cells, addition of the PDE4 inhibitor Ro, 20-1724 (10 µmol/L) normalized the effect of isoproterenol in these cells to that seen with the combination in naïve cells (Fig. 6H). In each of the 12 experiments in which this response was studied, Ro,20-1724 “normalized” the responses of forskolin-pretreated cells to those of naïve cells. These results were entirely consistent with those previously reported by us implicating increased PDE4 activity in the desensitization of VSMCs to the effects of cAMP-elevating agents on cAMP-mediated cellular effects (Tilley and Maurice, 2002; Rose et al., 1997).
Discussion

Two novel findings concerning cAMP signaling and the impact of cAMP-elevating agents on PDE4D expression in VSMCs emerge from this study. First, we report that both “contractile/quiescent” and “synthetic/activated” VSMCs differentially increase PDE4D variant expression in response to prolonged challenge with cAMP-elevating agents. Each VSMC phenotype utilizes PKA-dependent signaling in this process, without involvement of the recently discovered EPAC-Rap1 cAMP effector pathway. Second, we show that a phenotypically-dependent altered level of histone acetylation at the intronic PDE4D promoter controlling expression of PDE4D1 and PDE4D2 in “contractile/quiescent” and “synthetic/activated” VSMCs likely represents the molecular basis for differential expression of these short PDE4D variants in these cells in response to prolonged treatment with cAMP-elevating agents.

Tissue and cell type selective regulation of PDE expression: Our data are consistent with numerous published reports which suggest that specific PDE4 gene products mediate cAMP-induced desensitization to cAMP-elevating agents in individual cell types and, in fact, expand on this paradigm (Liu et al., 2000; Erdogan and Houslay, 1997; Mehats et al., 1999; Shepherd et al., 2004; Seybold et al., 1998; Verghese et al., 1995; D'Sa et al., 2002). Thus, in addition to confirming that PDE4D is a dominant PDE expressed in VSMCs and that prolonged cAMP elevation increases PDE4D activity, our data unequivocally demonstrate that different VSMC phenotypes respond to this
challenge by inducing the expression of distinct \textit{PDE4D} gene-derived variants. The observation that the long PDE4D variants are not transcriptionally regulated by prolonged cAMP signaling in “synthetic/activated” VSMCs while the short PDE4D variants are may imply a mechanism to decrease VSMC responsiveness to certain cAMP effects. To our knowledge, these data are the first to report a phenotype-dependent differential regulation of individual \textit{PDE4D} variants, and that this phenomenon may rely on the histone acetylation status of their promoters. The argument in favor of chromatin remodeling directing promoter availability of PDE4 variants is supported by the observation by others that PDE4A10 contains a CRE sequence which can be activated by cAMP in HEK 293 cells in culture, but undergoes a reduction in activity following serum withdrawal (Rena \textit{et al.}, 2001). Serum starvation has been shown to modulate the phenotype of VSMCs from a synthetic/activated to a contractile/quiescent state (Li \textit{et al.}, 1999), a process known to involve chromatin remodeling, therefore adding credence to the notion that increased acetylation of CREs in the promoters of \textit{PDE4} genes can direct their transcription. Interestingly, the data presented here, in combination with our previous report demonstrating loss of Phosphodiesterase 3A (\textit{PDE3A}) expression in rat “synthetic/activated” VSMCs (Dunkerley \textit{et al.}, 2002) and earlier reports of reduced soluble guanylyl cyclase, and protein kinase G expression in “synthetic/activated” VSMCs (Anderson \textit{et al.}, 2000), may point to a more general phenotype-mediated reorganization of cyclic nucleotide signaling in these cells. While provocative, the overall role that differential histone acetylation of the promoters controlling the expression of
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PDE3A, soluble guanylyl cyclase and protein kinase G may play in these events remains to be assessed.

While to date unique, our data may be of relevance in other situations in which phenotypic modulation of smooth muscle cells occurs. In this context, two recent reports identify marked changes in PDE4 expression during changes in cellular phenotype. Thus, Leroy and colleagues have reported that marked changes in PDE4 variant expression occurs at parturition and that this event correlates with reduced effectiveness of several pharmacological agents used to limit pre-term labour (Mehats et al., 1999; Mehats et al., 2001; Oger et al., 2002). Similarly, Shepherd et al., (2004) recently described a profound change in PDE4D isoform expression during differentiation of monocytes into macrophage which may account for differential effects of PDE4 inhibitors between these cell types. In addition, a marked differences in the PDE4 variants up-regulated in specific neurons following prolonged cAMP challenges may be related to events similar to those described here (D'Sa et al., 2002). Clearly, while similar to our findings, further work will be required to determine if similar factors regulate these other systems. Most intriguingly, an Icelandic study recently showed that decreased expression of long PDE4D variant mRNA correlates with an increase in susceptibility to carotid stroke brought on by the development of atherosclerosis, a disease marked by phenotypic modulation of VSMCs from a “contractile/quiescent” to a “synthetic/activated” state (Gretarsdottir et al., 2003). The disease-associated haplotype extends over regions of the
promoters of the PDE4D gene, which suggests an alteration in transcriptional regulation of PDE4D variants, a phenomenon that would support our observation of differential transcriptional control of PDE4D variants in phenotypically distinct VSMCs.

**Impact of differential PDE4D variant expression on non-cAMP-mediated signaling:**

In addition to an obvious effect on cAMP-signaling, our data identifying differential up-regulation of long and short forms of PDE4D may also have broader consequences on VSMC signaling. Indeed, an important role for “cross-talk” between ERK and PDE4D activities was recently reported to markedly influence cellular responses to both cAMP and non-cAMP-dependent agents (Liu and Maurice, 1999; Baillie et al., 2001; Baillie et al., 2000; Hoffmann et al., 1999) and expression (Liu et al., 2000). Indeed, taken together these reports are consistent with the idea that ERK-mediated phosphorylation inhibits PDE4D long forms (PDE4D3 and PDE4D5), and in fact can translocate particulate PDE4D3 away from its targeted membrane fraction to the cytosol, but activates PDE4D short forms (PDE4D1 and PDE4D2). Given the important role of ERK-mediated effects on VSMC migration and proliferation and the negative influence of cAMP on these events, differential PDE4D induction in “contractile/quiescent” and “synthetic/activated” VSMCs may allow for selective signaling between agents requiring ERK- or cAMP-dependent signaling in these two phenotypes of VSMCs. In fact, others have shown that monocyte differentiation into macrophages alters the profile of PDE4 variants to favor short PDE4 variant over long PDE4 variant expression, leading to increased growth factor stimulus (Shepherd et al., 2004). Thus, inducing the expression of short PDE4D
variants instead of long PDE4D variants might provide a mechanism to increase cAMP degradation and exacerbate the positive effects of growth factor stimulus on synthetic/activated VSMC migration and proliferation. In addition, these interactions may be all the more relevant if one considers that PKA-mediated effects at cSrc allow EPAC1-independent Rap1 activation and cell-type specific regulation of ERK, which then could again act to alter cAMP-signaling through PDE4D activity (Obara et al., 2004).

**Therapeutic implications:** Two potentially important therapeutic implications flow from our findings. First, due to the robust increase in PDE4D1 and PDE4D2 which occurs upon cAMP elevation in “synthetic/activated” VSMCs, we predict that these cells may more readily desensitize to the effects of prolonged cAMP-elevating agents than “contractile/quiescent” VSMCs through both increased cytosolic expression of these variants and activation of them by mitogenic stimuli via ERK. Second, we propose that development of *PDE4D* splice-variant selective inhibitors may provide greater specificity when PDE enzymes are targeted therapeutically in situations in which different effects in “contractile/quiescent” and “synthetic/activated” VSMCs are desired, such as in attempts to limit in-stent restenosis. Currently there is substantial evidence that some PDE4 inhibitors, such as Rolipram, bind to long and short PDE4 variants with different affinities, while other PDE4 inhibitors, such as piclamilast, do not possess this ability (Zhao et al., 2003). Thus, based on these differences in rolipram-binding to long and short forms of PDE4D, studies are currently underway to identify PDE4 inhibitors which
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might selectively inhibit PDE4D short forms, and thus selectively augment cAMP-signaling in “synthetic/activated” VSMC but not “contractile/quiescent” VSMC in the same artery in animal models of in-stent restenosis.

Acknowledgements

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References


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Footnotes

This study was supported by both a Heart and Stroke Foundation of Ontario (HSFO) grant (#T5426) and a Canadian Institute for Health Research (CIHR) grant (#MOP-57699).
Figure Legends

Fig. 1. Prolonged cAMP signaling effects on PDE4D variant expression in “contractile/quiescent” VSMC, “synthetic/activated” VSMC and NIH 3T3 cells –

(A, left panels) Rats received an i.p. injection of vehicle (Veh, saline) or 15 mg/kg dbcAMP every hour for 5 h, following which the aortae were harvested, processed and underwent immunoblot analysis as described in “Materials and Methods” (n = 3). “Aorta” represents contractile/quiescent VSMCs from the whole aortae of the rats. (A, right panels) Confluent primary cultures of “synthetic/activated” VSMCs (“VSMC”) were incubated with fresh culture medium supplemented with either vehicle (Veh, 0.1% v/v DMSO) or forskolin (Fsk, 10 µmol/L) for 4 h, following which they were processed and underwent immunoblot analysis as described in “Materials and Methods” (n = 3). As PDE4D3 and PDE4D5 have been shown previously to not undergo induction by cAMP signaling in synthetic/activated VSMCs (Liu et al., 2000; Liu and Maurice, 1999), blots were consistently exposed to allow reliable detection of PDE4D1 and PDE4D2. (B) NIH 3T3 cells were incubated with Veh (n = 6), Fsk (10 µmol/L; n = 6)), Fsk and H89 (10 µmol/L; n = 3), Fsk and cycloheximide (Chex, 100 µmol/L, n = 3) or Fsk and both H89 and Chex (n = 3) for 4 h. Following these incubations, cells were processed and immunoblotted as described in (A). (C) Naïve (+/+ or PKI-expressing NIH 3T3 cells were incubated with Veh or Fsk (10 µmol/L; n = 3) for 4 h. Following these incubations, cells were processed and immunoblotted as described in (A).
Fig. 2. Role of EPAC1 and Rap1A on PDE4D regulation by cAMP in VSMC and NIH 3T3 cells - (A) “Synthetic/activated” VSMC were incubated with vehicle (Veh, 0.1% v/v DMSO), 8-pCPT-2’-O-Me-cAMP (pCPT, 10 µmol/L), forskolin (Fsk, 10 µmol/L), or pCPT and Fsk for 4 h (n = 3). Following these incubations PDE4D immunoreactive proteins were investigated by immunoblot analysis as described in “Materials and Methods”. (B) Wild-type (+/+), FLAG-tagged EPAC1-transfected (EPAC1) or EE-tagged Rap1A(63E)-transfected (Rap1A(63E)) NIH 3T3 cells underwent immunoblot analysis as described above to verify the expression of FLAG-EPAC1 and EE-Rap1A(63E) (n = 3 each). An EPAC1-specific antibody (polyclonal, 1:500; Upstate) was also used to verify the expression of FLAG-EPAC1 with a much lower detection capacity, and could not detect endogenous EPAC1 expression in +/+ cells (not shown). (C) Wild-type (+/+), FLAG-tagged EPAC1-transfected (EPAC1) or EE-tagged Rap1A(63E)-transfected (Rap1A(63E)) NIH 3T3 cells were incubated with pharmacological agents and underwent immunoblot analysis as described in (A) (n = 3 each).

Fig. 3. Involvement of PKA and CREB in forskolin-induced PDE4D1 and PDE4D2 expression - (A) NIH 3T3 cells were incubated with forskolin (Fsk, 10 µmol/L) for 1 min - 4 h. Following these incubations, lysates were probed for phospho-CREB and ATF1 as described in “Materials and Methods” (n = 3). (B) NIH 3T3 cells were incubated with vehicle (Veh, 0.1% v/v DMSO), Fsk (10 µmol/L) or Fsk with H89 (10 µmol/L) for 4 h.
µmol/L) for 5 min (n = 3). Phosphorylated CREB and ATF1 were visualized as in (A).

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(C) Wild-type (+/+) or ACREB-transfected NIH 3T3 cells were incubated with Veh, or Fsk (0.1 - 1 µmol/L) for 4 h and probed for PDE4D as described in “Materials and Methods” (n = 3).

Fig. 4. Impact of CRE ODNs on forskolin-induced induction of PDE4D1 and PDE4D2 - (A) NIH 3T3 cells were transfected with either 1µM CRE or mismatch (Mis) ODN as described in “Materials and Methods”. Transfected cells were incubated with vehicle (Veh, 0.1% v/v DMSO) or forskolin (Fsk, 1 µmol/L) for 4 h. Following treatment, cell lysates were generated and PDE4 activity assessed as described in “Materials and Methods”. * indicates P<0.05 when compared to DMSO of same ODN-transfected cells, while # indicates P<0.05 when compared to Fsk of mismatch ODN-transfected cells (n = 3 each). (B) NIH 3T3 cells were transfected with increasing amounts of CRE decoy. Transfected cells were incubated Veh or Fsk (1 µmol/L) for 4 h, lysed and PDE4D immunoreactive proteins were investigated by immunoblot analysis as described in “Materials and Methods” (n = 3 each).

Fig. 5. Availability of the intronic promoter for PDE4D1/2 is greater in synthetic/activated VSMC and NIH 3T3 cells than contractile aortic VSMC - “Contractile/quiescent” VSMCs (Aorta), “synthetic/activated” VSMCs (VSMC) or NIH 3T3 cells (NIH 3T3) were crosslinked, lysed, processed and PCR amplified for 30 cycles
as outlined in “Materials and Methods” (A, top panel). 1µg non-immunoprecipitated MOL14126 total DNA (input controls) were PCR-amplified for 30 cycles for comparison (A, bottom panel) (n = 3 each). (B) Increasing PCR cycle numbers were run to analyze the difference in amplification of the PDE4D1/2 promoter in the different cell types; * indicates P<0.05 when compared to the corresponding tissue/cell type in cycle 25 (n=3 each).

Fig. 6. PDE4 inhibition can restore normal cAMP function in desensitized “synthetic/activated” VSMC - Primary cultures of VSMCs were treated, rinsed, fixed and stained as described in “Materials and Methods”. 4 h pretreatment: vehicle (Veh, 0.1% v/v DMSO), A-D, forskolin (Fsk, 10 µmol/L), E-H. 15 min rechallenge: Veh (A, E), Ro 20-1724 (Ro, 10 µmol/L) (B, F), isoproterenol (INE, 100 nmol/L) (C, G), or INE + Ro (D, H). Panels shown are representative of twelve independent experiments.
Table 1. Effects of incubation of NIH 3T3 cells with forskolin (Fsk), H89 and cycloheximide (Chex) on PDE4 activity – Wild-type or PKI-transfected NIH 3T3 cells were incubated with Fsk with or without H89 and/or Chex for 4 h. Following the incubations, the cells were lysed, processed and total cAMP PDE and PDE4 activity were determined as outlined in “Materials and Methods”. Calculation of % increases: \[
\frac{\text{PDE4 activity}_{\text{treatment}} - \text{PDE activity}_{\text{DMSO}}}{\text{PDE activity}_{\text{DMSO}}} \times 100
\]
Values are mean ± S.E.M. from three determinations.

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<th>Additions</th>
<th>cAMP PDE (pmol/mg protein/min)</th>
<th>PDE4 (pmol/mg protein/min)</th>
<th>Δ PDE4 (%)</th>
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<td>DMSO (0.1% v/v)</td>
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<td>H89 (10 µmol/L) + Chex (100 µmol/L)</td>
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<td>237.3 ± 12.6</td>
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\(^a\) P < 0.05 in comparison to DMSO

\(^b\) P < 0.05 in comparison to Fsk

\(^c\) P < 0.05 in comparison to Fsk + Chex
Table 2. Effects of incubation of “synthetic/activated” VSMCs or NIH 3T3 cells with 8-pCPT-2'-O-Me-cAMP (pCPT) and/or forskolin (Fsk) on PDE4 activity -

“Synthetic/activated” VSMCs, and wild-type or Flag-EPAC1- or EE-Rap1A(63E)-transfected NIH 3T3 cells were incubated with pCPT with or without Fsk for 4 h.

Following the incubations, the cells were lysed, processed and total cAMP PDE and PDE4 activity were determined as outlined in “Materials and Methods”. Values are mean ± S.E.M. from three determinations.

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<th>Additions (n=3)</th>
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* P < 0.05 in comparison to DMSO
A

% Increase in PDE4 Activity

Veh  Fsk  Veh  Fsk

Mis  CRE

*  #
B

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β-actin →

CRE: 0 μmol/L  1 μmol/L  1.5 μmol/L  2 μmol/L