0026-895X/05/6803-596–605$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 68:596–605, 2005

Vol. 68, No. 3
14126/3046942
Printed in U.S.A.

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 and Toxicology, Queen's University, Kingston, Ontario, Canada

Received April 22, 2005; accepted June 2, 2005

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. Although 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" VSMCs, distinct *PDE4D* gene variants coordinate these events 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, whereas the protein kinase A/cAMP response element-binding protein/cAMP response element 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 VSMCs. 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.

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 postangioplasty 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.

Although 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 that allow cAMP-elevating agents to affect VSMC 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 adenylyl cyclases contrib-

This study was supported by both the Heart and Stroke Foundation of Ontario (HSFO; grant T5426) and the Canadian Institute for Health Research (grant MOP-57699). D.H.M. is an HSFO Career Investigator, and D.G.T. is the recipient of an Ontario Graduate Scholarship.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.105.014126.

ABBREVIATIONS: VSMC, vascular smooth muscle cell; PKA, protein kinase A; PDE, cyclic nucleotide phosphodiesterase; CREB, cAMP-response element-binding protein; CRE, cAMP response element; ERK, extracellular signal-regulated protein kinase; EPAC, exchange protein activated by cAMP; 8-pCPT-2′-*O*-Me-cAMP, 8-(4-chlorophenylthio)-2′-*O*-methyl-cAMP; DMSO, dimethyl sulfoxide; H89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; PBS, phosphate-buffered saline; ODN, oligodeoxynucleotide; bp, base pair(s); Ro 20-1724, 4-[(3-butoxy-4-methoxyphenyl)-methyl]-2-imidazolidinone.

utes 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; Mehats et al., 1999; Tilley and Maurice, 2002; Rybalkin et al., 2002; 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 (*PDE4A*, *PDE4B*, *PDE4C*, and *PDE4D*) encode numerous PDE4 enzyme 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) (Liu and Maurice, 1999; Conti et al., 2003; Houslay and Adams, 2003; 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 forms, PDE4D1 and -2, contain cAMP-response element-binding protein (CREB) cAMP 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., 2000). In addition to affecting PDE4D expression, PKA and ERKs also regulate PDE4D catalytic activity (Houslay and Adams, 2003; Maurice et al., 2003). Thus, although 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 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 (for review, see Wong and Scott, 2004).

Previous studies in our laboratory (Liu and Maurice, 1999; Liu et al., 2000; Tilley and Maurice, 2002) have shown that cAMP-elevating agents increased PDE4 activity and up-regulated expression of *PDE4D* in both contractile/quiescent and synthetic/activated arterial VSMCs. It is interesting, however, that different *PDE4D* gene products were increased by cAMP-elevating agents in these two phenotypically distinct VSMC populations. Thus, whereas 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 *PDE4D* gene products not expressed in contractile/quiescent VSMCs (Tilley and Maurice, 2002). Because 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. Because the effect of cAMP activation of the novel cAMP effector known as exchange protein 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 Laboratories (Constance, QC, 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: PKI (Dr. Rudolph Juliano, University of North Carolina at Chapel Hill, Chapel Hill, NC), ACREB (Dr. Charles Vinson, National Cancer Institute, National Institutes of Health, Bethesda, MD), Flag-EPAC1 (Dr. Xiaodong Cheng, University of Texas Medical Branch, Galveston, TX), and [EE-Rap1A(63E); Dr. Lawrence Quilliam, Indiana University School of Medicine, Indianapolis, Indiana].

Pharmacological Treatment of Rats. Vehicle (saline) or dibutyryl-cAMP (15 mg/kg) were administered via i.p. injection to six male Wistar rats (three each condition) every hour for 5 h, followed by euthanization (1.7 ml/kg euthanyl), the aortae were removed and processed, and protein concentrations were determined as described previously (Tilley and Maurice, 2002).

Pharmacological Treatment of Cells. Primary cultures of rat aortic 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 μM), H89 (10 μM), cycloheximide (100 μM), or 8-pCPT-2′-*O*-Me-cAMP (10 μM) for predetermined periods. After 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, 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 phosphorothioate CRE (5′-TGACGTCATGACGTCATGACGTCA-3′) or mismatch (5′-TGTGGTCATGTGGTCATGTGGTCA-3′) oligodeoxynucleotide (ODN), both of 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 μM).

Immunoblotting. Equivalent aliquots (10 μg) of NIH 3T3 cells, VSMCs, or aortic tissues were subjected to SDS-polyacrylamide gel electrophoresis 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, Bothell, WA), phospho-CREB (1:1000; Affinity Bioreagents, Golden, CO), FLAG-M2 (1:40,000; Sigma-Aldrich, St. Louis, MO), EPAC1 (1:500; Upstate Biotechnology, Lake Placid, NY), Rap1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), or β-actin (1:40,000; Sigma-Aldrich), respectively, and 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 tempera-

ture. Cross-linking was stopped by adding glycine to a final concentration of 0.125 M and mixing for 5 min. Cross-linked medial fragments were centrifuged at 1000*g*, rinsed twice with ice-cold PBS mix (PBS; 10 mM NaBu, 100 μg/ml phenylmethylsulfonyl fluoride, and 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 were then 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. Nonimmune rabbit IgG was also used in preliminary experiments as a negative control for chromation immunoprecipitation (data not shown). Samples and input controls underwent reverse cross-linking by incubation with 200 mM NaCl and 10 μg of RNase A at 65°C for 4 h, and the DNA was purified by phenol/chloroform extraction and resuspended in 50 μl of Tris-EDTA 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 described previously (Tilley and Maurice, 2002) for cycle numbers indicated, with gene-specific oligonucleotide 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 −240 bp from the transcription start site (sense, 5′-CGGAATTCGCCCCTGCTCTCACCCTCT-3′; antisense, 5′-GCGAATTCGAGGTTGGATTAGTGGGG-3′), as described by 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 5 μg/ml fibronectin-coated coverslips were treated with vehicle (0.1% DMSO) or forskolin (10 μM) for 4 h, rinsed in serum-free media for 30 min, and then challenged with either vehicle, isoproterenol (100 nM), Ro 20-1724 (10 μM), or both isoproterenol and Ro 20-1724 for 20 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 (1:500; Sigma-Aldrich) and phalloidin-tetramethylrhodamine B isothiocyanate (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., Denver, CO). Each experiment was performed in triplicate and repeated four times for 12 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 reflect similar results obtained in at least three separate experiments.

Results

Differential Effect 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 (Rose et al., 1997; Liu and Maurice, 1999) 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-analog caused a 2-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 dibutyryl-cAMP (30 mg/kg) further increase PDE4D3 expression but did not induce expression of the *PDE4D* short-form variants in these cells (data 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

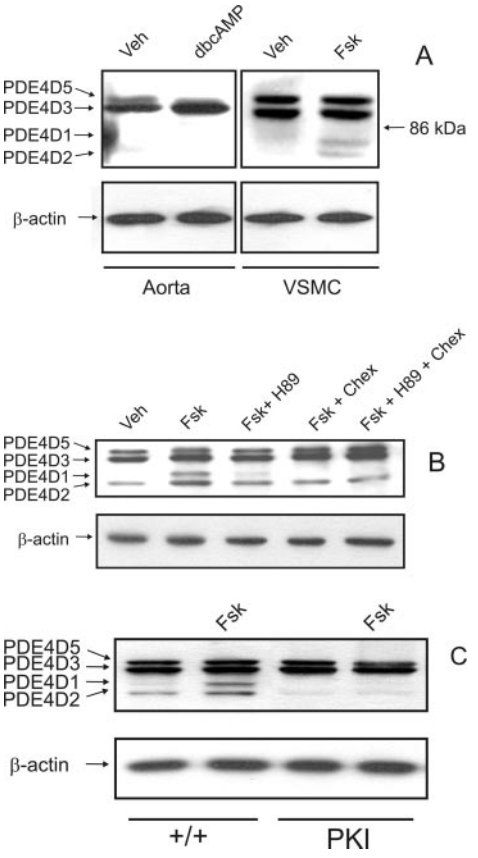

Fig. 1. Prolonged cAMP signaling effects on PDE4D variant expression in contractile/quiescent VSMC, synthetic/activated VSMC, and NIH 3T3 cells. A, left, rats received an i.p. injection of vehicle (Veh, saline) or 15 mg/kg dibutyryl cAMP (dbcAMP) every hour for 5 h, after which the aortae were harvested, processed, and subjected to immunoblot analysis as described under *Materials and Methods* ($n = 3$). Aorta represents contractile/quiescent VSMCs from the whole aortae of the rats. A, right, confluent primary cultures of synthetic/activated VSMCs were incubated with fresh culture medium supplemented with either vehicle [Veh, 0.1% (v/v) DMSO] or forskolin (Fsk, 10 μM) for 4 h, after which they were processed and subjected to immunoblot analysis as described under *Materials and Methods* ($n = 3$). Because PDE4D3 and PDE4D5 have been shown previously to not undergo induction by cAMP signaling in synthetic/activated VSMCs (Liu and Maurice, 1999; Liu et al., 2000), blots were consistently exposed to allow reliable detection of PDE4D1 and PDE4D2. B, NIH 3T3 cells were incubated with vehicle ($n = 6$), forskolin (10 μM; $n = 6$), forskolin, and H89 (10 μM; $n = 3$), forskolin, and cycloheximide (Chex, 100 μM, $n = 3$) or forskolin and both H89 and cycloheximide ($n = 3$) for 4 h. After these incubations, cells were processed and immunoblotted as described in A. C, naive (+/+) or PKI-expressing NIH 3T3 cells were incubated with vehicle or forskolin (10 μM; $n = 3$) for 4 h. After these incubations, cells were processed and immunoblotted as described in A.

VSMC lysates with PDE4D-specific antisera showed that *PDE4D* accounted for ≥80% of total PDE4 activity in these cells. It is interesting that no changes in the residual non-PDE4D activity after 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. Because 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. It is noteworthy that PDE4 activity was high in NIH 3T3 cells (Table 1), and these cells expressed both PDE4D3 and PDE4D5 under basal conditions (Fig. 1B). In addition, 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), although 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 (Rose et al., 1997; Liu et al., 2000) 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-fold above basal values ($P < 0.05$, $n = 3$ each), reductions of 75% and 65%, respectively (Fig. 1B). Likewise, heterologous PKI expression reduced forskolin-mediated increases in PDE4D1 and PDE4D2 expression to levels 4.2 ± 0.5- and 2.1 ± 0.5-fold 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 VSMC as well as in NIH 3T3 cells. Because 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. Although PKA represents the best studied cAMP-effector enzyme, several reports have shown that EPAC, a recently described guanine nucleotide exchange factor 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 whether 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 analog, 8-pCPT-2′-*O*-Me-cAMP (Enserink et al., 2002), did not alter basal PDE4 activity levels, did not significantly affect the ability of forskolin to increase this activity in these cells (Table 2), and did not cause phosphorylation of CREB (data not shown). However, we and others (Rangarajan et al., 2003) have shown that at 10 μM, 8-pCPT-2′-*O*-Me-cAMP was sufficient to induce previously reported EPAC1-Rap1A–mediated cellular adhesion in other cell types, such as ovarian carcinoma cells (OVCAR) or NIH 3T3 after their transfection with FLAG-tagged EPAC1 (D. Tilley and D. Maurice, unpublished observations). Consistent with the phosphodiesterase activity mea-

TABLE 1

Effects of incubation of NIH 3T3 cells with forskolin, H89, and cycloheximide on PDE4 activity

Wild-type or PKI-transfected NIH 3T3 cells were incubated with forskolin with or without H89 and/or cycloheximide for 4 h. After the incubations, the cells were lysed and processed, and total cAMP PDE and PDE4 activity were determined as outlined under *Materials and Methods*. Calculation of % increases: [(PDE4 activity$_{treatment}$ − PDE activity$_{DMSO}$)/PDE activity$_{DMSO}$] × 100. Values are mean ± S.E.M. from three determinations.

Additions ($n = 3$)	cAMP PDE Activity	PDE4 Activity	PDE4 Activity
	pmol/min/mg		%
NIH 3T3			
DMSO [0.1% (v/v)]	111.4 ± 9.0	89.6 ± 6.5	
H89 (10 μM) + Chex (100 μM)	93.5 ± 3.5	77.2 ± 4.8	
Fsk (10 μM)	275.3 ± 2.2[a]	236.9 ± 1.4[a]	164
Fsk (10 μM) + H89 (10 μM)	146.8 ± 10.7[abc]	120.1 ± 8.6[abc]	34
Fsk (10 μM) + Chex (100 μM)	174.1 ± 5.2[ab]	154.0 ± 3.3[ab]	72
Fsk (10 μM) + H89 (10 μM) + Chex (100 μM)	115.6 ± 3.8[bc]	95.6 ± 4.3[bc]	
NIH 3T3			
DMSO [0.1% (v/v)]	257.1 ± 3.7	154.1 ± 20.6	
Fsk (1 μM)	412.2 ± 18.4[a]	307.7 ± 11.7[a]	100
Fsk (1 μM) + H89 (10 μM)	318.8 ± 5.5[ab]	228.0 ± 8.9[ab]	48
NIH 3T3/PKI			
DMSO [0.1% (v/v)]	273.2 ± 9.2	206.9 ± 9.4	
Fsk (1 μM)	319.6 ± 9.6[a]	237.3 ± 12.6	15

Fsk, forskolin; Chex, cycloheximide.
[a] $P < 0.05$ in comparison with DMSO.
[b] $P < 0.05$ in comparison with forskolin.
[c] $P < 0.05$ in comparison with forskolin + cycloheximide.

surements, immunoblot analysis of lysates of treated VSMCs or NIH 3T3 cells with PDE4D-selective antisera showed that 8-pCPT-2′-*O*-Me-cAMP did not affect levels of PDE4D3, PDE4D5, PDE4D1, or PDE4D2 (Fig. 2, A and C). Furthermore, 8-pCPT-2′-*O*-Me-cAMP did not affect 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. 2, B and C), obviating a problem in the initial studies caused by low levels of EPAC expression in our cells. Because several Rap1 guanine nucleotide exchange factors 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 whether Rap1 might directly affect *PDE4D* expression in these cells, independently of EPAC. NIH 3T3 expressing Rap1A(63E), a constitutively active form of Rap1A, 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 altered neither basal *PDE4D* expression nor the ability of forskolin to induce PDE4D1 and PDE4D2 (Table 2; Fig. 2, B and C). Taken together, these data are inconsistent with the notion that EPAC1- or Rap1A-mediated signaling plays 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 (data not shown); 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). To determine whether a cAMP-response element-binding protein (CREB)-CRE system might regulate cAMP-induced PDE4D1 and PDE4D2 expression in cells, the effect of forskolin on PDE4D1 and PDE4D2 expression was determined in cells expressing ACREB, a dominant-negative CREB variant. Although forskolin treatment of NIH 3T3 cells or those expressing ACREB resulted in a similar concentration- and time-dependent and H89-senstive phosphorylation of CREB at Ser^{133}, as well as the highly homologous ATF1 protein at its equivalent site Ser^{63} (Fig. 3, A and B), cAMP-mediated induction of PDE4D1 and PDE4D2 after treatment with forskolin for 4 h was markedly blunted in ACREB-expressing cells. Indeed, whereas ACREB expression did not significantly alter PDE4D3 or PDE4D5 expression in these cells, it markedly inhibited 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. To more directly test the hypothesis that a CRE within the PDE4D1 and PDE4D2 promoter was required for cAMP-mediated regulation of *PDE4D* expression, we also tested the effect 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. Although 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 *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, whereas forskolin increased PDE4D1 and PDE4D2 expression by factors of 11.9 ± 0.7 and 6.0 ± 1.2 compared with basal values, respectively ($P < 0.05$, $n = 4$),

TABLE 2

Effects of incubation of synthetic/activated VSMCs or NIH 3T3 cells with 8-pCPT-2′-*O*-Me-cAMP and/or forskolin on PDE4 activity

Synthetic/activated VSMCs and wild-type or Flag-EPAC1- or EE-Rap1A(63E)-transfected NIH 3T3 cells were incubated with 8-pCPT-2′-*O*-Me-cAMP with or without forskolin for 4 h. After the incubations, the cells were lysed and processed, and total cAMP PDE and PDE4 activity were determined as outlined under *Materials and Methods*. Values are mean ± S.E.M. from three determinations.

Additions ($n = 3$)	cAMP PDE Activity	PDE4 Activity	PDE4 Activity
	pmol/min/mg		%
VSMC			
DMSO [0.1% (v/v)]	155.2 ± 6.8	48.1 ± 2.0	
pCPT (10 μM)	168.0 ± 9.4	48.4 ± 6.2	0
Fsk (10 μM)	234.8 ± 24.9[a]	108.5 ± 14.7[a]	126
pCPT (10 μM) + Fsk (10 μM)	236.2 ± 23.4[a]	106.0 ± 13.1[a]	120
NIH 3T3			
DMSO [0.1% (v/v)]	218.0 ± 8.6	165.0 ± 5.3	
pCPT (10 μM)	233.3 ± 5.0	178.6 ± 6.6	8
Fsk (10 μM)	375.1 ± 2.8[a]	295.9 ± 1.5[a]	79
pCPT (10 μM) + Fsk (10 μM)	381.4 ± 2.8[a]	298.6 ± 3.3[a]	81
NIH 3T3/EPAC1			
DMSO [0.1% (v/v)]	302.3 ± 13.1	214.3 ± 20.3	
pCPT (10 μM)	295.5 ± 18.3	225.8 ± 23.4	5
Fsk (10 μM)	477.6 ± 18.7[a]	374.4 ± 19.9[a]	75
pCPT (10 μM) + Fsk (10 μM)	444.7 ± 19.6[a]	364.7 ± 20.7[a]	70
NIH 3T3/Raqp1A(63E)			
DMSO [0.1% (v/v)]	200.1 ± 6.4	142.7 ± 6.8	
pCPT (10 μM)	189.6 ± 15.6	145.1 ± 14.9	2
Fsk (10 μM)	394.7 ± 10.3[a]	315.7 ± 8.7[a]	121
pCPT (10 μM) + Fsk (10 μM)	393.1 ± 3.9[a]	310.6 ± 3.2[a]	118

pCPT, 8-pCPT-2′-*O*-Me-cAMP; Fsk, forskolin.
[a] $P < 0.05$ in comparison with DMSO

levels of induction in the presence of the CRE decoy were significantly reduced. Indeed, in the presence of 1.5 μM CRE decoy, forskolin-induced increases in PDE4D1 and PDE4D2 were reduced to 4.77 $\pm$ 0.49 and 1.77 $\pm$ 1.43, respectively ($P < 0.05$, $n = 3$ each). The concentration-dependence 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 1 and 2 μM CRE decoy ($P < 0.05$, $n = 3$ each). At 2 μM CRE, the endogenous levels of PDE4D3 and PDE4D5 expression also seem 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.

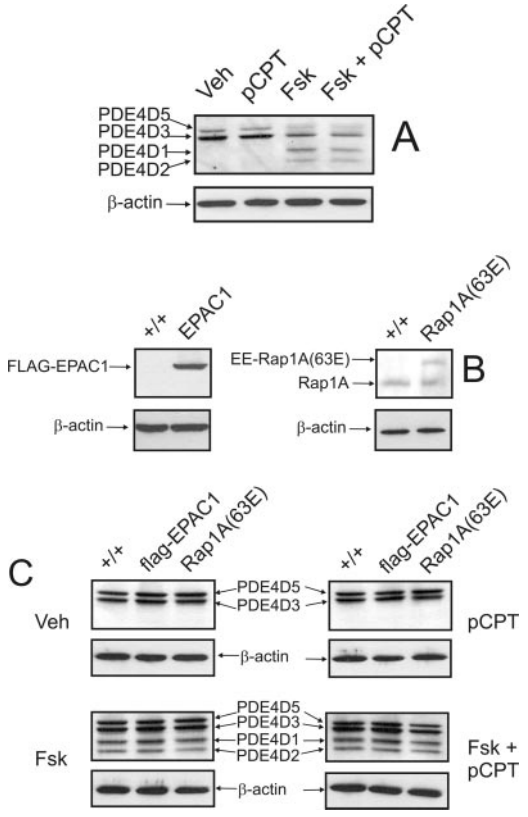

Fig. 2. Role of EPAC1 and Rap1A in PDE4D regulation by cAMP in VSMC and NIH 3T3 cells. A, synthetic/activated VSMC were incubated with vehicle [Veh, 0.1% (v/v) DMSO], 8-pCPT-2′-*O*-Me-cAMP (pCPT, 10 μM), forskolin (Fsk, 10 μM), or 8-pCPT-2′-*O*-Me-cAMP and forskolin for 4 h ($n = 3$). After these incubations, PDE4D immunoreactive proteins were investigated by immunoblot analysis as described under *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 Biotechnology) 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 (data 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).

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 (Manabe and Owens, 2001; Kumar and Owens, 2003). Because 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 whether 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, using a chromatin immunoprecipitation-based approach and two individual pairs of PDE4D intronic promoter sequence-derived oligonucleotide primers (see *Ma-*

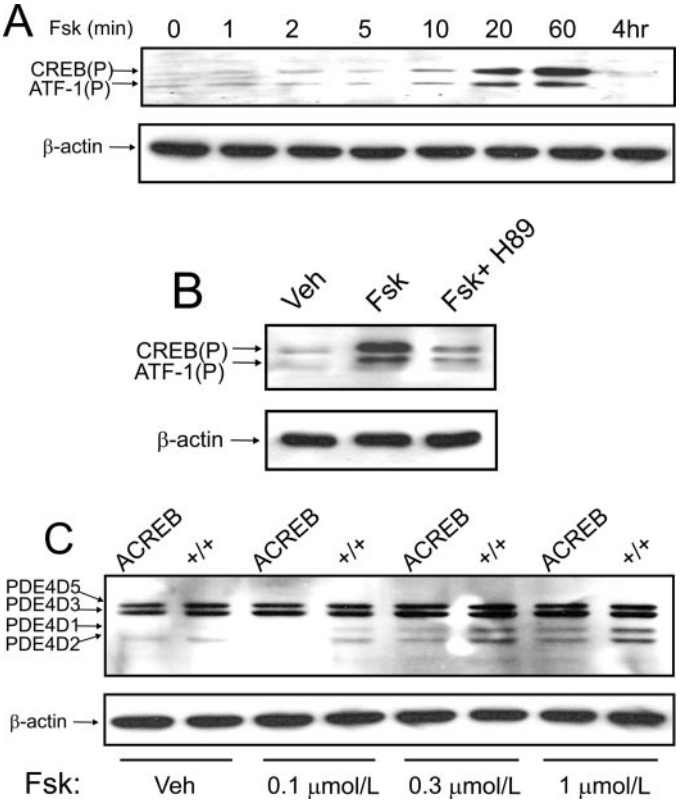

Fig. 3. Involvement of PKA and CREB in forskolin-induced PDE4D1 and PDE4D2 expression. A, NIH 3T3 cells were incubated with forskolin (Fsk, 10 μM) for 1 min to 4 h. After these incubations, lysates were probed for phospho-CREB and ATF1 as described under *Materials and Methods* ($n = 3$). B, NIH 3T3 cells were incubated with vehicle [Veh, 0.1% (v/v) DMSO], forskolin (10 μM), or forskolin with H89 (10 μM) for 5 min ($n = 3$). Phosphorylated CREB and ATF1 were visualized as in A. C, wild-type (+/+) or ACREB-transfected NIH 3T3 cells were incubated with vehicle or forskolin (0.1 to 1 μM) for 4 h and probed for PDE4D as described under *Materials and Methods* ($n = 3$).

terials 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, whereas the amount of intronic PDE4D promoter that could be amplified from total DNA before immunoprecipitation (input samples) from these three cell types were similar, the amount amplified from acetylated H3-immunoprecipitates from these cell types was significantly greater in samples of synthetic/activated VSMCs and NIH 3T3 cells compared with that from contractile/quiescent VSMCs (Fig. 5B). Because 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 account, at least in part, for the lack of PDE4D1 and PDE4D2 induction after 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 (Maurice et al., 2003; Owens et al., 2004). 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 effect of cAMP-mediated induction of PDE4D1 and PDE4D2 activity in synthetic/activated VSMCs on cAMP-induced inhibition of cellular processes necessary for VSMC migration. Therefore, we determined whether prior induction of PDE4D1 and PDE4D2 in synthetic/activated VSMCs would limit a future cAMP-induced cytoskeletal rearrangement in these cells (Fig. 6). Thus, although incubation of naive [0.1% (v/v) DMSO, 4 h] 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 μM) were virtually insensitive to isoproterenol (Fig. 6G). It is noteworthy that levels of PKA were not reduced after forskolin treatment (data 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 μM) normalized the effect of isoproterenol in these cells to that seen with the combination

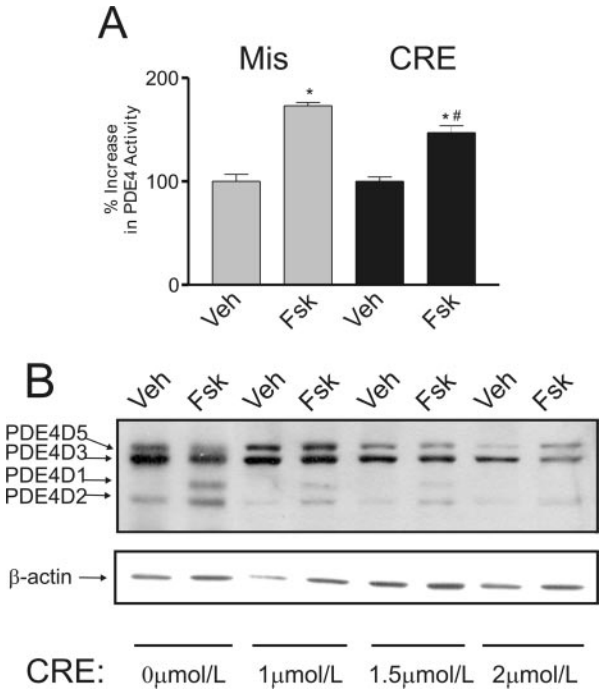

Fig. 4. Effect of CRE ODNs on forskolin-induced induction of PDE4D1 and PDE4D2. A, NIH 3T3 cells were transfected with either 1 μM CRE or mismatch (Mis) ODN as described under *Materials and Methods*. Transfected cells were incubated with vehicle [Veh, 0.1% (v/v) DMSO] or forskolin (Fsk, 1 μM) for 4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

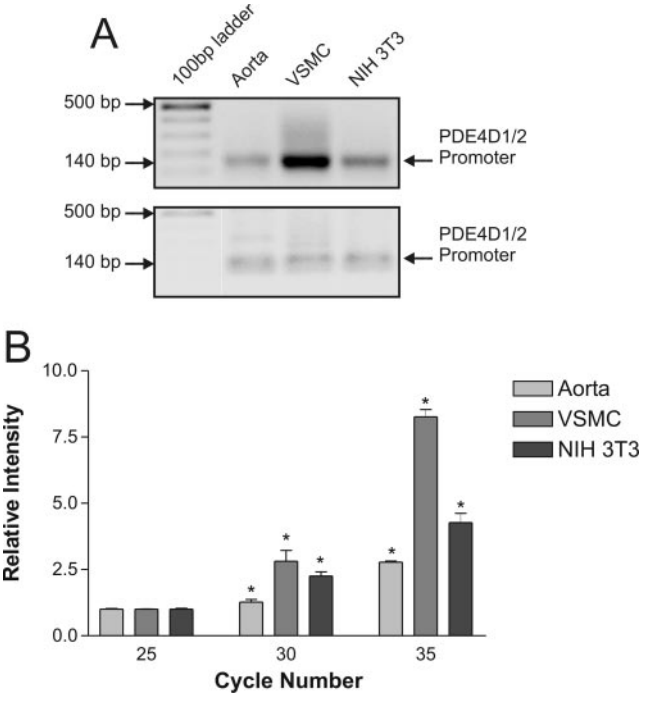

Fig. 5. Availability of the intronic promoter for PDE4D1/2 is greater in synthetic/activated VSMC and NIH 3T3 cells than contractile aortic VSMC. Contractile/quiescent VSMCs (Aorta), synthetic/activated VSMCs (VSMC), or NIH 3T3 cells (NIH 3T3) were cross-linked, lysed, processed, and PCR-amplified for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

in naive 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 naive 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 (Rose et al., 1997; Tilley and Maurice, 2002).

Discussion

Two novel findings concerning cAMP signaling and the effect 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 uses 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 probably 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 suggesting that specific *PDE4* gene products mediate cAMP-induced desensitization to cAMP-elevating agents in individual cell types and, in fact, expand on this paradigm (Verghese et al., 1995; Erdogan and Houslay, 1997; Seybold et al., 1998; Mehats et al., 1999; Liu et al., 2000; D'Sa et al., 2002; Shepherd et al., 2004). 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 *PDE4D* gene-derived variants. The observation that the long PDE4D variants are not transcriptionally regulated by prolonged cAMP signaling in synthetic/activated VSMCs, whereas 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 *PDE4D* variants and that this phenomenon might 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 that can by activated by cAMP in human embryonic kidney 293 cells in culture but undergoes a reduction in activity after serum withdrawal (Rena et al., 2001). Serum starvation has been shown to modulate the phenotype of VSMCs from a synthetic/activated to a contractile/quiescent state (Li et al., 1999), a process known to involve chromatin remodeling, which adds credence to the notion that increased acetylation of CREs in the promoters of *PDE4* genes can direct their transcription. It is interesting that the data presented here, in combination with our previous report demonstrating loss of *PDE3A* expression in rat synthetic/activated VSMCs (Dunkerley et al., 2002) and earlier reports of reduced soluble guanylyl cyclase and protein kinase G expression in synthetic/activated VSMCs (Anderson et al., 2000), may point to a more general phenotype-mediated reorganization of cyclic nucleotide signaling in these cells. Although provocative, the overall role that differential histone acetylation of the promoters controlling the expression of PDE3A, soluble guanylyl cyclase, and protein kinase G may play in these events remains to be assessed.

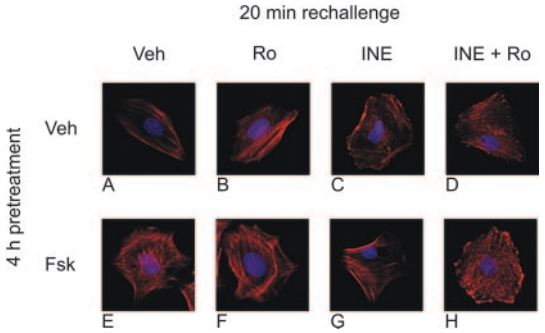

Fig. 6. PDE4 inhibition can restore normal cAMP function in desensitized synthetic/activated VSMC. Primary cultures of VSMCs were treated, rinsed, fixed, and stained as described under *Materials and Methods*. Four-hour pretreatment: A–D, vehicle [Veh, 0.1% (v/v) DMSO]; E–H, forskolin (Fsk, 10 μM). Twenty-minute rechallenge: A and E, vehicle; B and F, Ro 20-1724 (Ro; 10 μM); C and G, isoproterenol (100 nM); D and H, isoproterenol + Ro 20-1724. Shown are representative of 12 independent experiments.

Although unique so far, our data may be of relevance in other situations in which phenotypic modulation of smooth muscle cells occurs. In this context, several reports have identified marked changes in PDE4 expression during changes in cellular phenotype. Thus, Leroy and colleagues (Mehats et al., 1999, 2001; Oger et al., 2002) 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 preterm labor. Likewise, Shepherd et al. (2004) recently described a profound change in PDE4D isoform expression during differentiation of monocytes into macrophages that may account for differential effects of PDE4 inhibitors between these cell types. In addition, marked differences in the PDE4 variants up-regulated in specific neurons after prolonged cAMP challenges may be related to events similar to those described here (D'Sa et al., 2002). It is clear that although these findings are similar to ours, further work will be required to determine whether 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.

Effect 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-dependent and non–cAMP-dependent agents (Hoffmann et al., 1999; Liu and Maurice, 1999; Baillie et al., 2000; Baillie et al., 2001) 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 PDE4D variant over long PDE4D 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, because of the the robust increase in PDE4D1 and PDE4D2 that 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. There is now substantial evidence that some PDE4 inhibitors, such as rolipram, bind to long and short PDE4 variants with different affinities, whereas 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 that 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.

Acknowledgments

We are grateful to Daniel Raymond and Rhonda Carter (Department of Pharmacology and Toxicology, Queen's University, Kingston, Canada) for their instruction in plasmid purification and immunofluorescence microscopy, respectively.

References

Agata J, Miao RQ, Yayama K, Chao L, and Chao J (2000) Bradykinin B_1 receptor mediates inhibition of neointima formation in rat artery after balloon angioplasty. *Hypertension* **36:**364–370.

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.

Baillie G, MacKenzie SJ, and Houslay MD (2001) Phorbol 12-myristate 13-acetate triggers the protein kinase A-mediated phosphorylation and activation of the PDE4D5 camp phosphodiesterase in human aortic smooth muscle cells through a route involving extracellular signal regulated kinase (ERK). *Mol Pharmacol* **60:** 1100–1111.

Baillie GS, MacKenzie SJ, McPhee I, and Houslay MD (2000) Sub-family selective actions in the ability of Erk2 MAP kinase to phosphorylate and regulate the activity of PDE4 cyclic AMP-specific phosphodiesterases. *Br J Pharmacol* **131:** 811–819.

Bos JL (2003) Epac: a new cAMP target and new avenues in cAMP research. *Nat Rev Mol Cell Biol* **4:**733–738.

Conti M, Richter W, Mehats C, Livera G, Park JY, and Jin C (2003) Cyclic AMP-specific PDE4 phosphodiesterases as critical components of cyclic AMP signaling. *J Biol Chem* **278:**5493–5496.

D'Sa C, Tolbert LM, Conti M, and Duman RS (2002) Regulation of cAMP-specific phosphodiesterases type 4B and 4D (PDE4) splice variants by cAMP signaling in primary cortical neurons. *J Neurochem* **81:**745–757.

Dunkerley HA, Tilley DG, Palmer D, Liu H, Jimmo SL, and Maurice DH (2002) Reduced phosphodiesterase 3 activity and phosphodiesterase 3A level in synthetic vascular smooth muscle cells: implications for use of phosphodiesterase 3 inhibitors in cardiovascular tissues. *Mol Pharmacol* **61:**1033–1040.

Enserink JM, Christensen AE, de Rooij J, van Triest M, Schwede F, Genieser HG, Doskeland SO, Blank JL, and Bos JL (2002) A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. *Nat Cell Biol* **4:**901–906.

Erdogan S and Houslay MD (1997) Challenge of human jurkat T-cells with the adenylate cyclase activator forskolin elicits major changes in camp phosphodiesterase (PDE) expression by up-regulating PDE3 and inducing PDE4D1 and PDE4D2 splice variants as well as down-regulating a novel PDE4A splice variant. *Biochem J* **321:**165–175.

Forsberg EC, Downs KM, Christensen HM, Im H, Nuzzi PA, and Bresnick EH (2000) Developmentally dynamic histone acetylation pattern of a tissue-specific chromatin domain. *Proc Natl Acad Sci USA* **97:**14494–14499.

Gretarsdottir S, Thorleifsson G, Reynisdottir ST, Manolescu A, Jonsdottir S, Jonsdottir T, Gudmundsdottir T, Bjarnadottir SM, Einarsson OB, Gudjonsdottir HM, et al. (2003) The gene encoding phosphodiesterase 4D confers risk of ischemic stroke. *Nat Genet* **35:**131–138.

Hoffmann R, Baillie GS, MacKenzie SJ, Yarwood SJ, and Houslay MD (1999) The MAP kinase ERK2 inhibits the cyclic AMP-specific phosphodiesterase HSPDE4D3 by phosphorylating it at Ser579. *EMBO (Eur Mol Biol Organ) J* **18:**893–903.

Houslay MD and Adams DR (2003) PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization. *Biochem J* **370:**1–18

Indolfi C, Di Lorenzo E, Rapacciuolo A, Stingone AM, Stabile E, Leccia A, Torella D, Caputo R, Ciardiello F, Tortora G, et al. (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, Mongiardo A, Curcio A, and Torella D (2003) Molecular mechanisms of in-stent restenosis and approach to therapy with eluting stents. *Trends Cardiovasc Med* **13:**142–148.

Kohout TA and Lefkowitz RJ (2003) Regulation of G protein-coupled receptor kinases and arrestins during receptor desensitization. *Mol Pharmacol* **63:**9–18.

Kumar MS and Owens GK (2003) Combinatorial control of smooth muscle-specific gene expression. *Arterioscler Thromb Vasc Biol* **23:**737–747.

Le Jeune IR, Shepherd M, Van Heeke G, Houslay MD, and Hall IP (2002) Cyclic AMP-dependent transcriptional up-regulation of phosphodiesterase 4D5 in human airway smooth muscle cells. Identification and characterization of a novel PDE4D5 promoter. *J Biol Chem* **277:**35980–35989.

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.

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.

Manabe I and Owens GK (2001) Recruitment of serum response factor and hyperacetylation of histones at smooth muscle-specific regulatory regions during differentiation of a novel p19-derived in vitro smooth muscle differentiation system. *Circ Res* **88:**1127–1134.

Maurice DH, Palmer D, Tilley DG, Dunkerley HA, Netherton SJ, Raymond DR, Elbatarny HS, and Jimmo SL (2003) Cyclic nucleotide phosphodiesterase activity, expression and targeting in cells of the cardiovascular system. *Mol Pharmacol* **64:**533–546.

Mehats C, Tanguy G, Dallot E, Cabrol D, Ferre F, and Leroy MJ (2001) Is up-regulation of phosphodiesterase 4 activity by PGE2 involved in the desensitization of beta-mimetics in late pregnancy human myometrium? *J Clin Endocrinol Metab* **86:**5358–5365.
Mehats C, Tanguy G, Dallot E, Robert B, Rebourcet R, Ferre F, and Leroy MJ (1999) Selective up-regulation of phosphodiesterase-4 cyclic adenosine 3′,5′-monophosphate (cAMP)-specific phosphodiesterase variants by elevated cAMP content in human myometrial cells in culture. *Endocrinology* **140:**3228–3237.
Obara Y, Labudda K, Dillon TJ, and Stork PJ (2004) PKA Phosphorylation of Src mediates Rap1 activation in NGF and cAMP signaling in PC12 cells. *J Cell Sci* **117:**6085–6094.
Oger S, Mehats C, Dallot E, Ferre F, and Leroy MJ (2002) Interleukin-1beta induces phosphodiesterase 4B2 expression in human myometrial cells through a prostaglandin E2- and cyclic adenosine 3′,5′-monophosphate-dependent pathway. *J Clin Endocrinol Metab* **87:**5524–5531.
Owens GK, Kumar MS, and Wamhoff BR (2004) Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev* **84:**767–801.
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.
Rangarajan S, Enserink JM, Kuiperij HB, de Rooij J, Price LS, Schwede F, and Bos JL (2003) Cyclic AMP induces integrin-mediated cell adhesion through Epac and Rap1 upon stimulation of the beta 2-adrenergic receptor. *J Cell Biol* **160:**487–493.
Rena G, Begg F, Ross A, MacKenzie C, McPhee I, Campbell L, Huston E, Sullivan M, and Houslay MD (2001) Molecular cloning, genomic positioning, promoter identification and characterization of the novel cyclic AMP-specific phosphodiesterase PDE4A10. *Mol Pharmacol* **59:**996–1011.
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, Rybalkina I, Beavo JA, and Bornfeldt KE (2002) Cyclic nucleotide phosphodiesterase 1C promotes human arterial smooth muscle cell proliferation. *Circ Res* **90:**151–157.
Seybold J, Newton R, Wright L, Finney PA, Suttorp N, Barnes PJ, Adcock IM, and Giembycz MA (1998) Induction of phosphodiesterases 3B, 4A4, 4D1, 4D2 and 4D3 in jurkat T-cells and in human peripheral blood T-lymphocytes by 8-bromo-cAMP and Gs-coupled receptor agonists. Potential role in β2-adrenoreceptor desensitization. *J Biol Chem* **273:**20575–20588.
Shepherd MC, Baillie GS, Stirling DI, and Houslay MD (2004) Remodelling of the PDE4 cAMP phosphodiesterase isoform profile upon monocyte-macrophage differentiation of human U937 Cells. *Br J Pharmacol* **142:**339–351.
Tilley DG and Maurice DH (2002) Vascular smooth muscle cell phosphodiesterase (PDE) 3 and PDE4 activities and levels are regulated by cyclic AMP in vivo. *Mol Pharmacol* **62:**497–506.
Verghese MW, McConnell RT, Lenhard JM, Hamacher L, and Jin SL (1995) regulation of distinct cyclic AMP-specific phosphodiesterase (phosphodiesterase type 4) isozymes in human monocytic cells. *Mol Pharmacol* **47:**1164–1171.
Vicini E and Conti M (1997) Characterization of an intronic promoter of a cyclic adenosine 3′,5′-monophosphate (cAMP)-specific phosphodiesterase gene that confers hormone and cAMP inducibility. *Mol Endocrinol* **11:**839–850.
Wong W and Scott JD (2004) AKAP signalling complexes: focal points in space and time. *Nat Rev Mol Cell Biol* **5:**959–970.
Zhao Y, Zhang HT, and O'Donnell JM (2003) Inhibitor binding to type 4 phosphodiesterase (PDE4) assessed using [^{3}H]piclamilast and [^{3}H]rolipram. *J Pharmacol Exp Ther* **305:**565–572.

Address correspondence to: Dr. Donald H. Maurice, Department of Pharmacology and Toxicology, Queen's University, Kingston, Ontario, Canada, K7L 3N6. E-mail: mauriced@post.queensu.ca