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

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Multiple families of cyclic nucleotide phosphodiesterases (PDE) have been described, and the regulated expression of these genes in cells is complex. Although cAMP is known to control the expression of certain PDE in cells, presumably reflecting a system of feedback on cAMP signaling, relatively little is known about the influence of non-cAMP signaling systems on PDE expression. In this study, we describe a novel mechanism by which activators of the protein kinase C (PKC)-Raf-MEK-ERK cascade regulate phosphodiesterase 4D (PDE4D) expression in vascular smooth muscle cells (VSMC) and assess the functional consequences of this effect. Whereas a prolonged elevation of cAMP in VSMC resulted in a protein kinase A (PKA)-dependent induction of expression of two PDE4D variants (PDE4D1 and PDE4D2), simultaneous activation of both the cAMP-PKA and PKC-Raf-MEK-ERK signaling cascades blunted this cAMP-mediated increase in PDE4D expression. By using biochemical, molecular biological, and pharmacological approaches, we demonstrate that this PDE4D-selective effect of activators of the PKC-Raf-MEK-ERK cascade was mediated through a mechanism involving altered PDE4D mRNA stability and markedly attenuated the cAMP-mediated desensitization that results from prolonged activation of the cAMP signaling system in cells. The data are presented in the context of activators of the PKC-Raf-MEK-ERK cascade having both short and long term effects on PDE4D activity and expression in cells that may influence cAMP signaling.

The cyclic nucleotides, cAMP and cGMP, control numerous physiological processes including intermediary metabolism and cellular proliferation, motility, or contractility. Cyclic nucleotide-mediated signaling is terminated either when the stimulus for generation of cAMP or cGMP is removed or when cyclic nucleotides are hydrolyzed. Hydrolysis of cAMP or cGMP is catalyzed by members of a multigene family of enzymes called cyclic nucleotide phosphodiesterases (PDE). In mammals, 10 individual families of PDE have been described with each classified on the basis of molecular sequence, substrate selectivity, and inhibitor sensitivity (1–4). Each PDE family has been shown to comprise several individual genes, each of which can be alternatively processed to yield several different mRNA. In most instances members of several PDE families are expressed in individual cells, with differential subcellular targeting, and selective regulation by phosphorylation by various protein kinases emerging as important factors in determining their individual roles (5–8).

Due to the important role played by both cAMP and cGMP in the regulation of myocardial contractility and blood vessel relaxation, the PDE present in these tissues have received a considerable amount of attention as therapeutic targets (9, 10). Members of at least four PDE families (PDE1, PDE3, PDE4, and PDE5) have been shown to contribute to termination of cyclic nucleotide signaling in vascular smooth muscle cells (VSMC) (10). Whereas enzymes of the PDE1 and PDE5 families hydrolyze cGMP, members of the PDE1, PDE3, and PDE4 families catalyze the breakdown of cAMP. A large body of evidence supports important roles for PDE3 and PDE4 in the regulation of cAMP-mediated effects in VSMC, and much of this evidence is consistent with the idea that these enzymes regulate separate, although overlapping, cAMP pools in cells (10–26). Thus, a strong correlation exists between the increase in cAMP which occurs as a result of the pharmacological inhibition of PDE3 activity and inhibition of VSMC contractions (10, 11, 14, 15). In contrast, although pharmacological inhibition of PDE4 raises VSMC cAMP levels, and PDE4 inhibitors inhibit VSMC proliferation (16, 20) and migration (21) of these cells, these agents are generally poor relaxants (10, 11, 14). Interestingly, synergistic effects of simultaneous inhibition of PDE3 and PDE4 activities have been reported (11, 14, 18). In this context, dual-selectivity PDE3/PDE4 inhibitors may prove useful agents in cells expressing members of both these enzyme families (9, 10, 27).

The regulation of PDE4 activity and expression in cells is...
complex (7–8). In both human and rat four distinct genes encode PDE4 enzymes (PDE4A, PDE4B, PDE4C, and PDE4D), with each, as a result of alternate splicing or the use of alternate promoters, giving rise to multiple variants (7–8). Recently, a significant amount has been elucidated concerning the regulation of PDE4 activity and expression in cells. Indeed, it is now clear that selective PDE4 variants can be regulated by transcriptional and/or by post-translational mechanisms (7–8). Presumably reflecting a form of negative feedback for cAMP signaling, phosphorylation and activation of certain PDE4 variants by the cAMP-dependent protein kinase (PKA) have been reported (7, 8, 28–32). Although a mitogen-activated protein kinase-mediated phosphorylation of a PDE4B variant had no functional consequences (34), activation of the mitogen-activated protein kinase cascade has recently been shown to affect PDE4D3 activity, perhaps in a cell-dependent manner (32–33). While in HEK293 cells, ERK2-mediated phosphorylation led to a transient inhibition of PDE4D3 activity (33) and activation of the PKC-Raf-MEK-ERK cascade in rat aortic VSMC activated and translocated particulate PDE4D3 to the cytosol of these cells (32). In addition to this acute effect of cAMP elevation, more prolonged increases in cellular cAMP have been shown to bring about marked increases in PDE4 activity in several cell types (18, 35–41). Interestingly, although these treatments always result in elevated levels of PDE4 activity, the PDE4 gene responsible appears to be cell type-specific. For example, while incubation with forskolin decreased the expression level of PDE4A and increased that of PDE4D in Jurkat cells (40), a similar treatment of selected monocyte cell lines resulted in marked elevations in PDE4A expression and decreases, in PDE4D expression (38–39). In addition, changes in PDE4B expression have also been reported following treatment with cAMP-elevating agents. Thus, treatment of rats with a prostacyclin analogue caused a marked increase, and decrease, in myocardial PDE4B and PDE4D, respectively (42).

Recently, we have begun to investigate the role of cAMP in controlling PDE4 activity and expression in VSMC (18–19, 21, 32). As a result of these studies, we recently reported that the two PDE4D variants expressed in rat aortic VSMC, PDE4D3 and PDE4D5, were activated following phosphorylation by protein kinase A (PKA) (32). Interestingly, a selective PKC-Raf-MEK-ERK cascade-mediated phosphorylation of the particulate fraction of PDE4D3 in these cells was shown to increase the efficiency with which this enzyme was phosphorylated by PKA and to result in the translocation of this particulate PDE4D3 (32). In the work described here, we have investigated the impact of the PKC-Raf-MEK-ERK cascade on the effects of prolonged increases in cAMP elevation on PDE4D activity and expression in VSMC. We show that a prolonged increase in cAMP results in a marked increase in the expression of two short variants of the PDE4D gene (PDE4D1 and PDE4D2) and that activation of the PKC-Raf-MEK-ERK cascade blunts this increase. Our data are discussed in the context of the PKC-Raf-MEK-ERK cascade having a biphasic effect on PDE4D activity in VSMC, increasing it in the short term, but decreasing it in the long term, and are consistent with a paradigm in which PDE4D activity and expression are subject to regulation by multiple and overlapping signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Materials—Tissue culture reagents (Dulbecco's modified Eagle's medium, fetal serum, HEPES, penicillin/streptomycin, Hanks' balanced salt solution (HBSS), trypsin-EDTA), SuperScript reverse transcriptase, and [α-32P]dCTP.**

Radioactive products were from NEN Life Science Products, ([5-3H]AMP, [3H]cAMP, and [α-32P]dCTP). Ro 20-1724 and clotrimazole were from Calbiochem, and isobutylmethylxanthine, forskolin, and 8-Br-cAMP were from Research Biochemicals International (Natick MA). Tris-HCl, benzamide, EDTA, EGTA, dithiothreitol, phenylmethanesulfonyl fluoride, Triton X-100, and NaCl were from ICN Biomedicals (Costa Mesa, CA), and leupeptin, Afl-Gel 601, Dowex 50 (200–400 mesh), chloramphenicol, and the columns supports were from Bio-Rad. The BCA protein assay and bovine serum albumin were from Pierce. All other chemicals were of reagent-grade purchased from Fisher. PDE4D-specific monoclonal antibody was provided by ICOS Corp. (Bothell, WA).

**Cell Culture—**Primary cultures of rat aortic VSMC were prepared as described previously (18). VSMC were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 8 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 95% air, 5% CO2 humidified atmosphere. For all experiments, cells were used between passages 4 and 12.

**Treatment of VSMC with Pharmacological Agents—**Culture media were removed and replaced with fresh culture media supplemented with either (i) forskolin (0.1–100 μM), (ii) 8-B-cAMP (0.01–1 mM), (iii) PMA (0.1–100 nM), (iv) angiotensin II (0.1–1.0 μM), or (v) vehicle (0.1% dimethyl sulfoxide (Me2SO)). At the end of the incubation period, treated cells were washed with HBSS (with Ca2+ and Mg2+) and harvested in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl2, 5 mM benzamide, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 μl leupeptin, and 1% Triton X-100. Cells were removed from the flask by scraping. Cellular debris and unlysed cells were removed by centrifugation at 1,000 × g for 5 min. The 1,000 × g supernatant was transferred to microtubes and stored at 4 °C until assayed for cAMP PDE activity (see below). For some experiments cysolic and particulate fractions were prepared. In these instances, rat aortic VSMC were lysed as described above except that Triton X-100 was excluded from the lysis and the 1,000 × g supernatant subjected to a further centrifugation step (152,000 × g, 30 min).

**Assay of cAMP Phosphodiesterase Activity—**cAMP phosphodiesterase activity was assayed as described previously (18). Briefly, reactions were carried out in a total volume of 100 μl containing 5 μmol of Tris-HCl (pH 7.4), 0.5 μmol of MgCl2, 10 nM of EGTA, and 0.1 nM of [3H]cAMP containing 55,000–80,000 dpm for 30 min at 30 °C. Reactions were terminated by addition of 50 μl of 0.5 M ice-cold EDTA (pH 7.4) supplemented with 5 3H]AMP (1,800 dpm). Samples were diluted with 0.3 ml of HEPES-NaCl buffer (0.1 M NaCl, 0.1 M HEPES (pH 8.5)) prior to purification of the product of the reaction, 5 3H]AMP and 5 14C]AMP were recovered by chromatography using a polycarlylamide-borate gel column (Afl-Gel 601, 1 ml bed volume), and the purified 5 3H]AMP was quantified by liquid scintillation counting, corrected for recovery of 5 14C]AMP, normalized to the total protein content, and the total activity expressed as pmol min mg protein. Total protein concentration of each sample was determined using the BCA Protein Assay system from Pierce, according to the manufacturer's methodology using bovine serum albumin as the standard.

**Measurements of VSMC cAMP—**In experiments in which the impact of forskolin, PMA, AngII, or a combination of these agents on VSMC cAMP activity was determined, the method of Maurice et al. (43) was used. Briefly, confluent cultures of VSMC were incubated for 16 h with fresh culture media supplemented with 10 μCi ml1 [3H]hypoxanthine. Radiolabeled cells were rinsed with fresh medium and incubated with either (i) forskolin (0.1–10 μM), (ii) PMA (100 μM), (iii) angiotensin II (100 μM), or combinations of forskolin (0.1–10 μM) and PMA (100 μM) or angiotensin II (100 μM) in 0.5 ml for 5 min. Similarly, when increases in VSMC cAMP levels caused by isoproterenol were measured, VSMC were incubated for 16 h with fresh culture media supplemented with 10 μCi ml1 [3H]hypoxanthine and forskolin (10 μM), PMA (100 μM), or both forskolin and PMA for 16 h. Radiolabeled cells were rinsed with fresh medium and incubated with isoproterenol (0.01 μM) for 5 min. In all cases, reactions were terminated by addition of 0.5 ml of ice-cold 10% trichloroacetic acid. Following addition of recovery markers, [14C]cAMP (700 dpm), [3H]cAMP was isolated and purified by column chromatography on alumina and Dowex 50 and quantitated by liquid scintillation. The corrected [3H]cAMP present in individual samples was expressed as a percentage of the total [3H] present in the cells in the same sample.

**Reverse Transcription and Amplification by Polymerase Chain Reaction (RT-PCR)—**RNA purification and RT-PCRs were carried out as described previously (19). Briefly, RNA was purified from cultured rat aortic VSMC (5–10 × 106 cells), using a single step procedure, Trizol Reagent (Life Technologies, Inc). First strand cDNA was generated from 5 to 10 μg of total RNA using oligo(dT)15 (Cortec, Cortong, Ontario) to prime the reverse transcription (SuperScript Moloney mu
Incubation of Rat Aortic VSMC with Forskolin Increases cAMP PDE Activity

Confluent monolayers of rat aortic VSMC were incubated with forskolin for 4, 8, or 16 h supplemented with forskolin (10 μM). At the end of the incubation, cells were washed with HBSS and lysed in ice-cold lysis buffer (without Triton X-100), and 1000 x g supernatant, 132,000 x g supernatant, and 132,000 x g particulate fractions were obtained by differential centrifugation as described under “Experimental Procedures.” Total cAMP PDE activity and PDE4 activity of each fraction were determined as described under “Experimental Procedures,” using Ro 20–1724 (10 μM) as a selective PDE4 inhibitor. Values are mean ± S.E. from three experiments, each carried out in triplicate.

| Fraction Time | cAMP PDE Activity |
|---------------|--------------------|
|                | Total PDE4         |
| 1,000 x g supernatant | 0.0 ± 0.0 |
| 0.1 ± 0.0     | 16 240.5 ± 12.4 |
| 1000000 x g supernatant | 0.0 ± 0.0 |
| 0.1 ± 0.0     | 16 165 ± 7.1 |
| 1000000 x g particulate | 0.0 ± 0.0 |
| 0.1 ± 0.0     | 16 131 ± 6.8 |

*p < 0.05 when compared with 0-h time point in the presence or absence of Ro 20–1724.

Effects of actinomycin D or cycloheximide on 8-Br-cAMP-mediated increases in rat aortic VSMC PDE4 activity

Confluent monolayers of rat aortic VSMC were incubated with fresh culture medium supplemented with 8-Br-cAMP (1 μM), actinomycin D (4 μM), cycloheximide (100 μM), or 8-Br-cAMP (1 μM) with either actinomycin D (4 μM) or cycloheximide (100 μM) for 16 h. At the end of the incubation, cells were washed with HBSS and lysed in ice-cold lysis buffer, and 1,000 x g supernatant fractions were obtained by differential centrifugation as described under “Experimental Procedures.” Total cAMP PDE activity and PDE4 activities were determined as described under “Experimental Procedures,” using Ro 20–1724 (10 μM) as a selective PDE4 inhibitor. Values are mean ± S.E. from three experiments, each carried out in triplicate.

| Treatments | Total PDE4 activity |
|------------|---------------------|
| None       | 56.3 ± 3.3          |
| Actinomycin D (4 μM) | 75.3 ± 2.9 |
| Cycloheximide (100 μM) | 78.2 ± 2.7 |
| 8-Br-cAMP (1 μM) + actinomycin D (4 μM) | 94.7 ± 5.5 |

*p < 0.05 compared with control value.

*p < 0.05 compared with 8-Br-cAMP-stimulated value.

RESULTS

Incubation of Rat Aortic VSMC with Forskolin or 8-Br-cAMP Increases PDE4 Activity—Consistent with our previous work (18–19, 32), treatment of rat aortic VSMC with forskolin or 8-Br-cAMP caused a time- and concentration-dependent increase in total cAMP PDE activity, with marked increases in both PDE3 (18–19) and PDE4 (Table I). By using a selective inhibitor of PDE3 (cisteamine, 1 μM) or of PDE4 (Ro 20-1724, 10 μM) to measure these activities, a 16-h incubation of VSMC with forskolin (10 μM) caused increases of 210 ± 22 and 108 ± 17% in PDE3 (not shown) and PDE4 (Table I), respectively. Recently, we reported that both the cytosolic PDE3A as well as the particulate PDE3B were increased in rat aortic VSMC following prolonged increases in cAMP (19). Changes in cytosolic and particulate PDE4 activities were also noted in our experiments (Table I). Although some of the increase in PDE4 at steady state could be due to PKA-mediated phosphorylation of PDE4D3 and PDE4D5 (32), the increase in cAMP activity using our treatment protocol were inhibited by inhibition of either actinomycin D or cycloheximide, a role for de novo mRNA and protein synthesis was established (Table II).

Forskolin or 8-Br-cAMP Induces the Expression of PDE4D1 and PDE4D2 in Rat Aortic VSMC—A strategy of selective immunoblotting with PDE4-selective antisera was used to determine the identity of the PDE4 variant being affected by forskolin, or 8-Br-cAMP, treatment of rat aortic VSMC. By using this approach, we determined that the major effect of prolonged increases in cAMP in VSMC was related to the induction of expression of two PDE4 variants not expressed in untreated control cells. While untreated control VSMC expressed both 95 ± 2 and 105 ± 3 kDa anti-PDE4D-immunoreactive proteins, which we have previously shown to be PDE4D3 and PDE4D5 (32), respectively, three novel PDE4D-immunoreactive species were detected in VSMC lysates following prolonged incubations with either forskolin or 8-Br-cAMP (Fig. 1A). One of the anti-PDE4D-immunoreactive proteins detected in treated VSMC migrated slightly more slowly than PDE4D3 and was shown previously to be the PKA-phosphorylated...
PDE4D1 and PDE4D2 in cultured rat aortic VSMC are inhibited by forskolin (10 or 100 μM) or vehicle (Me2SO) for 2 h. Anti-PDE4D immunoreactive species were visualized as in A. D, confluent rat aortic VSMC were incubated with fresh culture medium supplemented with vehicle (Me2SO) or forskolin (10 μM) for 4 h. RT-PCR reactions using primers selective for PDE4D3, PDE4D2, PDE4D1, or GAPDH were carried out as described under “Experimental Procedures.” Products of these reactions were separated on 1% agarose gels, stained with ethidium bromide, and visualized under UV light, excised, and counted by liquid scintillation.

FIG. 1. Forskolin- or 8-Br-cAMP-induced expression of PDE4D1 and PDE4D2 in cultured rat aortic VSMC is inhibited by actinomycin, cycloheximide, or H89. A, confluent rat aortic VSMC were incubated with fresh culture medium supplemented with forskolin (10 or 100 μM), 8-Br-cAMP (100 μM or 1 μM), or vehicle (Me2SO) for 16 h. At the end of the incubation period, cells were washed with HBSS and lysed in ice-cold lysis buffer. VSMC lysates were centrifuged at 1,000 g for 10 min at 4 °C, and the supernatant fractions were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed by immunoblot with a PDE4D-specific monoclonal antibody (61D10E). Anti-PDE4D immunoreactive species were visualized as in B. B, confluent rat aortic VSMC were incubated with fresh culture medium supplemented with forskolin (10 μM) alone or in the presence of forskolin (10 μM) and cycloheximide (100 μM) or actinomycin D (4 μM) or vehicle (Me2SO) for 8 h. At the end of the incubation period, cells were washed with HBSS and lysed in ice-cold lysis buffer. Anti-PDE4D immunoreactive species were visualized as in A. C, confluent rat aortic VSMC were incubated with forskolin (10 μM) or forskolin (10 μM) and H89 (10 μM) for 2 h. Anti-PDE4D immunoreactive species were visualized as in A. D, confluent rat aortic VSMC were incubated with fresh culture medium supplemented with vehicle (Me2SO) or forskolin (10 μM) for 4 h. RT-PCR reactions using primers selective for PDE4D3, PDE4D2, PDE4D1, or GAPDH were carried out as described under “Experimental Procedures.” Products of these reactions were separated on 1% agarose gels, stained with ethidium bromide, and visualized under UV light, excised, and counted by liquid scintillation.
Confluent monolayers of rat aortic VSMC were incubated with fresh culture medium supplemented with forskolin (10 μM), 8-Br-cAMP (1 mM), PMA (100 nM), forskolin (10 μM) and PMA (100 nM), or 8-Br-cAMP (1 mM) and PMA (100 nM) for 16 h. At the end of the incubation, cells were washed with HBSS and lysed in ice-cold lysis buffer, and 1,000 μg of VSMC lysates were determined as described under “Experimental Procedures.” PDE3 and PDE4 activities of VSMC lysates were determined as described under “Experimental Procedures,” using cistolamide (1 μM) or Ro 20–1724 (10 μM) as selective PDE3 or PDE4 inhibitors, respectively. Values are mean ± S.E. from three experiments, each carried out in triplicate.

| Treatments                  | cAMP PDE activity | pmol/min/mg of protein |
|-----------------------------|-------------------|------------------------|
| None                        | PDE3              | 14.1 ± 3.8             |
|                            | PDE4              | 95.2 ± 2.1             |
| Forskolin (100 μM)          | 44.4 ± 7.5a       | 159.5 ± 4.9a           |
| 8-Br-cAMP (1 mM)            | 37.0 ± 4.9a       | 138.1 ± 4.8a           |
| PMA (100 nM)                | 31.1 ± 3.0        | 82.5 ± 2.1             |
| Forskolin (10 μM) + PMA (100 nM) | 39.0 ± 4.4a   | 105.5 ± 4.5a           |
| 8-Br-cAMP (1 mM) + PMA (100 nM) | 41.5 ± 5.6a   | 104.2 ± 4.6a           |

*p < 0.05 compared with control values.

**Activity of cAMP-elevating agents.** Sin PMA selective inhibits forskolin- or 8-Br-cAMP-induced increases in PDE4 activity that occurs in response to incubation of VSMC with cAMP-elevating agents (not shown). The reduced effect of PMA at longer times is consistent with the increased importance of changes in PDE3 activity at these time points (18, 19).

**PMA or AngII Inhibits the cAMP-mediated Induction of PDE4D1 and PDE4D2 Expression**—Since our data showed that a cAMP-dependent induction of PDE4D1 and PDE4D2 contributed to the forskolin-mediated increased PDE4 activity in VSMC and that PMA or AngII could blunt these increases, we determined the effects of PMA or of AngII on PDE4D1 and PDE4D2 expression in these cells. Thus, either PMA or AngII added simultaneously with cAMP-elevating agents markedly inhibited the cAMP-induced increase in PDE4D1 and PDE4D2 expression (Fig. 2A). Since the phosphorylation-mediated short-term activation of PDE4D3 caused by PMA or AngII was shown previously to involve the PKC-Raf-1-MEK-ERK cascade (32), experiments were undertaken to address the involvement of this pathway in the inhibition of PDE4D1 and PDE4D2 expression caused by these agents. Results consistent with a role for the PKC-Raf-MEK-ERK cascade in mediating the effects of PMA or AngII on rat aortic VSMC PDE4 expression were obtained using selective inhibitors. Since results of preliminary experiments with some of the agents used (Fig. 2B and PD98059) indicated that their selectivity was reduced following prolonged incubations (>2 h) in cells (not shown), these experiments were carried out over the shortest incubation periods possible (<2 h). Addition of the PKC-selective inhibitor, Bis-1, completely inhibited the phosphorylation of PDE4D3 caused by either PMA or AngII (32) and reversed the inhibitory effect of these agents on the forskolin-induced increase in PDE4D1 and PDE4D2 expression (Fig. 2B). A role for MEK-ERK involvement was addressed using the MEK-specific inhibitor PD98059. Addition of PD98059 completely inhibited the effects of PMA and of AngII on PDE4D2 phosphorylation (Fig. 2C and Ref. 32) and reversed the inhibitory effects of PMA (Fig. 2C) or AngII (not shown) on PDE4D1 and PDE4D2 expression. Since in certain cells PMA had been shown to bring about cellular effects by ultimately increasing intracellular Ca<sup>2+</sup> concentrations, the effects of a Ca<sup>2+</sup>-ionophore (ionomycin) were also tested in some experiments. In our experiments ionomycin (1–100 nM) had no effect on basal PDE4 activity nor on the increase in this activity caused by cAMP-elevating agents (not shown).

**FIG. 2.** PMA or angiotensin II inhibition of forskolin-induced expression of PDE4D1 in rat aortic VSMC, and effects of Bis-1 or PD98059. A, confluent rat aortic VSMC were incubated with fresh culture medium supplemented with vehicle (MeSO), forskolin (10 μM), or forskolin (10 μM) and PMA (100 nM), or angiotensin II (100 nM) for 2 h. At the end of the incubation period, cells were washed with HBSS and lysed in ice-cold lysis buffer. VMSC lysates were centrifuged at 1,000 × g for 10 min at 4°C, the supernatant fractions were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed by immunoblot with a PDE4D-specific monoclonal antibody (61D10E). Anti-PDE4D immunoreactive species were visualized by ECL as per the supplier’s recommendation using a horseradish peroxidase-conjugated goat anti-mouse IgG, as described under “Experimental Procedures.” B, confluent rat aortic VSMC were incubated with fresh culture medium supplemented with PMA (100 nM), angiotensin II (100 nM), forskolin (10 μM), forskolin (10 μM) and PMA (100 nM), or forskolin (10 μM) and angiotensin II (100 nM) for 1 h in the presence of Bis-1 (2.5 μM). At the end of the incubation, samples were processed, and anti-PDE4D immunoreactive proteins were visualized as in A. C, confluent rat aortic VSMC were incubated with fresh culture medium supplemented with vehicle (MeSO), forskolin (10 μM), PMA (100 nM), or forskolin (10 μM) and PMA (100 nM) in the presence or absence of PD98059 (1 μM) for 2.5 h. At the end of the incubation, samples were processed and anti-PDE4D immunoreactive proteins visualized as in A.

**Effect of PMA on PDE4 Expression Alters cAMP Signaling in VSMC**—Since incubation of VSMC with PMA or AngII reduced basal PDE4 activity and since PDE4 activity is dominant in these cells, we hypothesized that activators of adenylyl cyclase should more effectively increase cAMP in PMA-pretreated cells. Consistent with this, incubation of PMA-pretreated cells with a β-adrenoceptor agonist, isoproterenol, resulted in a more marked increase in cAMP than was caused by this agent in untreated control cells (Fig. 3A). Indeed, in the three experiments in which this effect was assessed, PMA potentiated the increase in cAMP caused by isoproterenol by approximately 70%. In addition, since we had previously reported that a marked desensitization to isoproterenol accompanies a pro-

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**TABLE III**

| Treatments                  | PDE3 PDE4 | pmol/min/mg of protein |
|-----------------------------|-----------|------------------------|
| None                        | PDE3      | 14.1 ± 3.8             |
|                            | PDE4      | 95.2 ± 2.1             |
| Forskolin (100 μM)          | 44.4 ± 7.5a| 159.5 ± 4.9a           |
| 8-Br-cAMP (1 mM)            | 37.0 ± 4.9a| 138.1 ± 4.8a           |
| PMA (100 nM)                | 31.1 ± 3.0| 82.5 ± 2.1             |
| Forskolin (10 μM) + PMA (100 nM) | 39.0 ± 4.4a| 105.5 ± 4.5a           |
| 8-Br-cAMP (1 mM) + PMA (100 nM) | 41.5 ± 5.6a| 104.2 ± 4.6a           |

*p < 0.05 compared with control values.

a *p < 0.05 compared with effects of either forskolin or 8-Br-cAMP alone.
Confluent VSMC were incubated with [3H]hypoxanthine (2 μM final concentration, 10 μCi/ml) for 16 h supplemented with vehicle (Me2SO), forskolin (10 μM), PMA (100 nM), or forskolin (10 μM) and PMA (100 nM). Following this metabolic labeling and treatment, cells were rinsed with HBSS and incubated with isoproterenol (0.01–1 μM) for 5 min. At the end of this incubation, cells were lysed with trichloroacetic acid (10% w/v final) and [3H]cAMP isolated as under “Experimental Procedures.” Values are mean ± S.E. of triplicate determinations from a representative experiment. A, confluent VSMC were incubated with [3H]hypoxanthine (2 μM final concentration, 10 μCi/ml) for 16 h supplemented with vehicle (Me2SO), forskolin (10 μM), PMA (100 nM), or forskolin (10 μM) and PMA (100 nM). Following this metabolic labeling and treatment, cells were rinsed with HBSS and incubated with isoproterenol (0.01–1 μM) for 5 min. At the end of this incubation, cells were lysed with trichloroacetic acid (10% w/v final) and [3H]cAMP isolated as under “Experimental Procedures.” Values are mean ± S.E. of three experiments carried out in triplicate. B, confluent VSMC were incubated with [3H]hypoxanthine (2 μM final concentration, 10 μCi/ml) for 16 h supplemented with vehicle (Me2SO), forskolin (10 μM), PMA (100 nM), or forskolin (10 μM) and PMA (100 nM). Following this metabolic labeling and treatment, cells were rinsed with HBSS and incubated with isoproterenol (0.10 μM) with or without Ro 20-1724 (10 μM) or zardaverine (10 μM) for 5 min. Following the incubations, cellular protein was precipitated with trichloroacetic acid (10% w/v final) and [3H]cAMP isolated as under “Experimental Procedures.” Values are mean ± S.E. of three experiments carried out in triplicate.

A, PMA treatment of VSMC potentiates isoproterenol-induced increases in cAMP and attenuates cAMP-mediated heterogeneous desensitization. B, following this metabolic labeling and treatment, cells were rinsed with HBSS and incubated with isoproterenol (0.01–1 μM) for 5 min. At the end of this incubation, cells were lysed with trichloroacetic acid (10% w/v final) and [3H]cAMP isolated as under “Experimental Procedures.” Values are mean ± S.E. of three experiments carried out in triplicate.

Inhibition of cAMP-induced Increases in PDE4D Expression by PMA or Ang II Is Mediated by a Mechanism Involving PDE4D mRNA Stability—Neither PMA nor AngII had any effect on forskolin- or isoproterenol-induced increases in cAMP in untreated control cells (not shown). However, each PMA and AngII inhibited the induction of PDE4D1 and PDE4D2 expression by a direct activator of adenylyl cyclases (forskolin) and a direct activator of PKA (8-Br-cAMP). Based on these findings, PMA or AngII could, in principle, have altered the cAMP-dependent accumulation of PDE4D1 and PDE4D2 by affecting the expression or the stability of either the mRNA or the protein for these PDE4D variants. In an attempt to assess which of these potential mechanisms was at play in VSMC, we initially examined the influence of incubation of rat aortic VSMC with PMA on the rate of accumulation and clearance of PDE4D proteins in these cells. Our data from these studies was consistent with an effect of PMA on the rate of accumulation of PDE4D1 and PDE4D2 and inconsistent with an effect on the rate of clearance of these proteins from cells. Thus, addition of PMA to cultures in which expression of PDE4D1 and PDE4D2 had been induced by prior treatment with forskolin, followed by removal of the adenylyl cyclase activator, caused an accumulation of phosphorylated PDE4D3 (32) but had no effect on the amount of time required for PDE4D1 or PDE4D2 to return to basal levels (Fig. 4A). Indeed, as determined by densitometric analysis, the amount of time required for PDE4D1 to decrease below immunodetectable levels was about 2 h, whether or not PMA had been added. Although the amount of time required for PDE4D2 to return to control values was significantly longer (approximately 5 h), again PMA had no effect (Fig. 4A). The molecular basis for the different rates of PDE4D1 and PDE4D2 clearance in VSMC is not known. In contrast to the lack of effect of PMA on the rate of clearance of PDE4D1 and PDE4D2, PMA markedly prolonged the amount of time required for PDE4D1 and PDE4D2 to accumulate in forskolin-treated cells (Fig. 4B). Thus, while PDE4D1 was readily detected after 90 min of incubation with 10 μM forskolin, simultaneous addition of PMA delayed the accumulation of PDE4D1 such that this protein was only detected after 150 min of treatment (Fig. 4B). Moreover, the level of PDE4D1 present in cells incubated with both forskolin and PMA was consistently lower than that obtained in cells incubated with forskolin alone (Fig. 4B). Data consistent with an effect of PMA on expression of PDE4D gene products were also reflected in the effects of this agent on the cAMP-induced changes in PDE4D mRNA levels. Thus, addition of PMA to cells inhibited the marked increases in PDE4D1 and PDE4D2 mRNA as well as the very modest increase in PDE4D3 mRNA (Fig. 5, A and B). Indeed, in the five experiments in which this was measured, PMA inhibited the forskolin-induced increase in PDE4D1, PDE4D2, and PDE4D3 mRNA by 75 ± 10, 79 ± 12, and 85 ± 19%, respectively (Fig. 5A). Since PMA or AngII inhibited the cAMP-dependent, PKA-mediated increase in PDE4 activity in rat aortic VSMC but had no effect on the cAMP-dependent, PKA-mediated increase in PDE3 activity in these cells (Table III), we hypothesized that
in three separate experiments the treatment, cells were washed three times with fresh culture medium and incubated with vehicle (Me2SO) or PMA (100 nM) for 0, 45, 90, 150, or 180 min. At the end of these incubations, cells were lysed in ice-cold lysis buffer, and lysates were centrifuged at 1,000 g for 10 min at 4 °C. The supernatant fractions were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed by immunoblot with a PDE4-specific monoclonal antibody (61D10E). Anti-PDE4 immunoreactive species were visualized by ECL as per the supplier’s recommendation using a horseradish peroxidase-conjugated goat anti-mouse IgG as described under “Experimental Procedures.” Immunoblots were quantitated by scanning densitometry using Corel Photo-Paint 8.0, and the immunoreactive bands at 0 min were designated a relative score of 1.0. B, VSMC were incubated with fresh culture medium supplemented with forskolin (10 μM) or forskolin (10 μM) and PMA (100 nM) for the indicated period. At the end of the incubation, cells were processed and anti-PDE4D-immunoreactive proteins were visualized as described in A.

these agents were acting by altering the stability of the PDE4D mRNA rather than through a transcriptionally based mechanism. Based on this we reasoned that the t½ of PDE4D mRNA should be shorter when determined in cells incubated with both forskolin and PMA, compared with that obtained in cells incubated with forskolin alone. By using an experimental approach based on measuring the decay of mRNA following inhibition of transcription with actinomycin D, our data are consistent with a PMA-mediated destabilizing effect on PDE4D mRNA (Fig. 6). Thus, in the four experiments in which this was measured, addition of 100 nM PMA reduced the t½ of PDE4D1 mRNA by about 60% (79 ± 3 to 34 ± 3 min) (Fig. 6, A and B). Similarly, in three separate experiments the t½ of mRNA encoding PDE4D2 and PDE4D3 was shortened by approximately 50% by addition of PMA to forskolin-treated cells. Thus, the t½ of PDE4D2 was reduced from 96.3 ± 7 to 51 ± 4 min following addition of PMA to the cells, while this agent reduced the t½ of PDE4D3 mRNA 73 ± 10 to 39 ± 9 min. PMA did not alter the rate of decay of GAPDH mRNA in this study (12% decay in 4 h).

DISCUSSION

In a previous report, we identified PDE4D as the major PDE4 variant expressed in cultured rat aortic VSMC, and we presented evidence for the expression of both PDE4D3 and PDE4D5 variants in these cells (32). In addition, we reported that incubation of rat aortic VSMC with cAMP-elevating agents, or with structural analogues of cAMP, caused a rapid phosphorylation-dependent activation of both these proteins. In this previous work (32) we also defined a novel role for the PKC-Raf-MEK-ERK cascade in the regulation of PDE4D activity whereby activation of this cascade led to a phosphorylation-dependent activation of particulate PDE4D3 that was additive with the effects of PKA. Interestingly, stimulation of both the cAMP-PKA and PKC-Raf-MEK-ERK cascades in VSMC led to the translocation of particulate PDE4D3 to the cytosol of these cells. Clearly, this form of regulation could potentially allow for the rapid regulation of PDE4D activity at selected regions within cells and impact on cellular functions requiring local PDE4D-mediated control of cAMP levels. In the studies detailed here, we have investigated the effect of prolonged elevations in cAMP on PDE4D expression in VSMC, and we have characterized the impact of activation of the PKC-Raf-MEK-ERK cascade on this cAMP-mediated regulation of PDE4D expression. These studies demonstrate that the regulation of PDE4D expression in cells is controlled by both these signaling cascades and that the coordinated regulation of PDE4D expression could significantly impact cAMP-mediated signaling.

Consistent with our previous work (18, 19, 21, 32), and previous reports by others (35–42) using non-vascular cell types, incubation of rat aortic VSMC with either forskolin or 8-Br-cAMP caused a time- and concentration-dependent increase in PDE4 activity. Whereas at lower levels of stimulation the increases in PDE4 were relatively modest, as much as 4-fold increases were achieved when cells were incubated for 16 h with 100 μM forskolin. The effects of the PKA inhibitor H89, as well as those of actinomycin D or cycloheximide, demonstrated that the forskolin- or 8-Br-cAMP-mediated increases in PDE4 activity occurred as a result of activation of PKA and that new mRNA and protein synthesis were both necessary.
Confluent rat aortic VSMC were incubated with fresh culture medium supplemented with forskolin (10 μM) or forskolin (10 μM) and PMA (100 nM) for 4 h. Following these incubations, actinomycin D (4 μM) was added and RNA isolated at 15-min intervals for 2 h. RT-PCR was used for PDE4D1- or GAPDH-specific primers were carried out under "Experimental Procedures" in the presence of 2 μCi of [α-32P]dCTP. PCR products for PDE4D1 or GAPDH were separated on 1% agarose gels, excised, and molecular weights consistent with PDE4D1 and PDE4D2 (7, 8), are present only in treated VSMC. Although in some reports PDE4D1 and PDE4D2 have been shown to co-migrate with identical molecular weights (40, 42), well resolved doublets of these PDE4D variants were shown to appear in mononuclear cells following prolonged incubation of these cells with a lipophilic analogue of cAMP (36, 37). Our data are consistent with previous reports of the cAMP induction of the short variants of the PDE4D gene (35–42) and show that the longer variants, PDE4D3 and PDE4D5, are not subject to cAMP regulation in rat aortic VSMC. The lack of an effect of cAMP on PDE4D3 expression is consistent with modest effects of cAMP on the expression of this variant in other cell types (40, 41, 45, 46).

A novel finding of our work is that addition of the phorbol ester, PMA, or of the vasoactive agent, AngII, markedly attenuated the cAMP induction of expression of PDE4D1 and PDE4D2 in rat aortic VSMC. Although in previous reports PMA was shown to cause a PKC-dependent, rapid and transient induction of PDE1C in some cells (47, 48), there have been no previous reports identifying a role for this agent in the regulated expression of any PDE4 gene, in any cell type. In addition to identifying a role for PMA, or AngII, in the expression of PDE4D1 and PDE4D2, our studies also identify the PKC-Raf-MEK-ERK cascade as potentially playing a signaling role in this phenomenon. Thus, the selective PKC inhibitor, Bis-1, or the MEK inhibitor, PD98059, both reversed the inhibitory effect of PMA on the cAMP-induced increase in PDE4D1 expression. Although we are extrapolating this mechanism to account for the effects of PMA, or AngII, on PDE4D2 expression, the time course of the induction of this splice variant (>2 h) and the reduced selectivity of both Bis-1 and PD98059 after incubation for periods greater than 2 h precluded our direct measurement of this effect.

Whereas up-regulation of PDE4 activity played a more important role in the cytosolic fraction, accounting for virtually all of the increase in total cAMP PDE activity, the relative role of increased PDE4 activity in the particulate fraction was less dominant, accounting for less than 50% of this increase. In recent work conducted in our laboratory (19), a role for increased expression of particulate PDE3B in rat aortic VSMC incubated with 8-Br-cAMP in the residual effects in the particulate fraction was elucidated.

By using both molecular biological (RT-PCR) as well as immunological methodologies (immunoblotting), our studies identify PDE4D1 and PDE4D2 as the major PDE4D variants increased following prolonged periods of incubation with cAMP-elevating agents. Thus, low levels of mRNA encoding PDE4D2 and PDE4D3 could be amplified from untreated control cells, and proteins previously identified as PDE4D3 and PDE4D5 (32) were detected in homogenates of untreated VSMC using a PDE4D-specific antiserum. Consistent with a cAMP-induced increase in VSMC expression of PDE4D1 and PDE4D2, a marked increase in the amount of PDE4D1 and PDE4D2 mRNA which could be amplified from treated cells was observed. In addition, whereas untreated control cells expressed PDE4D3 and PDE4D5 (32), immunobLOTS of treated cells allowed the detection of four new anti-PDE4D immunoreactive proteins. Whereas two of these were phosphorylated PDE4D3 and PDE4D5 (32), the other two PDE4D immunoreactive proteins present in treated cells are represented as PDE4D1 and PDE4D2, the two smallest known PDE4D splice variants (7, 8). Consistent with these assignments are our findings that these PDE4D-immunoreactive proteins, which migrate at molecular weights consistent with PDE4D1 and PDE4D2 (7, 8), are present...
marked effects on PDE4 activity and expression.2 In addition to identifying that the PKC-Raf-MEK-ERK cascade may play a role in mediating the effects of PMA- or AngII-mediated inhibition of cAMP-induced increases in PDE4D gene expression, another novel finding of our work was the identification of a role for destabilization of PDE4D mRNA as a potential molecular basis for this effect. Although this effect could, in principal, have been related to alteration of the synthesis or the stability of either PDE4D mRNA or PDE4D proteins, our data indicate that a mechanism involving a destabilizing of PDE4D mRNA was most likely. Thus, although addition of PMA to cells in which PDE4D1 and PDE4D2 had been induced by a prior incubation with forskolin did not affect the rate at which these proteins were cleared from cells, a co-incubation of both agents markedly reduced the rate at which these proteins accumulated when compared with the effects of forskolin alone. These data are inconsistent with a role for increased clearance of PDE4D proteins in these cells but are rather consistent with a role for an effects mediated by a decreased rate of synthesis. Although a role for PMA in reducing the rate of transcription of the PDE4D gene was theoretically possible, our data and previously reported observations (44) were inconsistent with such a mechanism and, rather, indicated that a mechanism involving altered stability of PDE4D mRNA was more likely. Thus, although PMA reduced the level of PDE4D mRNA was more likely. Thus, although PMA re-

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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

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