Distinct phosphodiesterase-4D variants integrate into protein kinase A-based signaling complexes in cardiac and vascular myocytes

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Submitted 23 April 2008; accepted in final form 2 December 2008

Raymond DR, Carter RL, Ward CA, Maurice DH. Distinct phosphodiesterase-4D variants integrate into protein kinase A-based signaling complexes in cardiac and vascular myocytes. Am J Physiol Heart Circ Physiol 296: H263–H271, 2009; doi:10.1152/ajpheart.00425.2008.—Numerous cAMP-elevating agents regulate events required for efficient migration of arterial vascular smooth muscle cells (VSMCs). Interestingly, when the impact of cAMP-elevating agents on individual migration-related events is studied, these agents have been shown to have distinct, and sometimes unexpected, effects. For example, although cAMP-elevating agents inhibit overall migration, they promote VSMC adhesion to extracellular matrix proteins and the formation of membrane extensions, which are both events that are essential for and promote migration. Herein, we extend previous observations that identified phosphodiesterase-4D3 (PDE4D3) as an integral component of a PKA/A kinase-anchoring protein (AKAP) complex in cultured/hypertrophied rat cardiac myocytes to the case for nonhypertrophied cardiac myocytes. Moreover, we show that while rat aortic VSMCs also express PDE4D3, this protein is not detected in PKA/AKAP complexes isolated from these cells. In contrast, we show that another PDE4D splice variant expressed in arterial vascular myocytes, namely, PDE4D8, integrates into PKA/AKAP-based signaling complexes in VSMCs. Consistent with the idea that a PDE4D8/PKA/AKAP complex regulates specific VSMC functions, PKA and PDE4D8 were each recruited to leading-edge structures in migrating VSMCs, and inhibition of PDE4D8 recruitment to pseudopodia of migrating VSMCs would stimulate the expression of PDE4D variants with distinct promoters, the PDE4D gene encodes at least 10 distinct variants (4, 7, 17, 37, 39, 40, 41). Based on the size of their amino-terminal regulatory/targeting domains, distinct PDE4D variants are defined as “long” (PDE4D3, PDE4D4, PDE4D5, PDE4D7, PDE4D8, and PDE4D9), “short” (PDE4D1), or “supershort” (PDE4D2, PDE4D6, and PDE4D10) (4, 7, 17, 37, 39, 40, 41). Importantly, recent reports have shown that the unique regulatory/targeting domains of the distinct long PDE4D isoenzyme variants likely represents the mechanism by which they have selective actions in cells (4, 7, 17, 37, 39, 40, 41). Indeed, these domains allow specific recruitment of these enzymes into defined signaling complexes as well as their differential sensitivity to activation subsequent to phosphorylation by several kinases, including PKAs and ERK1/2 (16, 19, 21, 35). Human and rodent contractile VSMCs each express PDE4D isoenzyme variants with electrophoretic and immunological characteristics consistent with each of the long PDE4D forms, except for PDE4D4. In addition, synthetic VSMCs can be stimulated to induce the expression of PDE4D variants with electrophoretic and immunological characteristics consistent with both short (PDE4D1) and supershort (PDE4D2) variants (22, 39, 40).

The impact of cAMP-elevating agents in VSMC migration is complex. Thus, although cAMP-elevating agents inhibit VSMC migration, several individual events required for cell migration, including integrin-based adhesions and the formation of VSMC membrane extensions, are each stimulated by cAMP-elevating agents (5, 13, 29). Recent findings indicating that intracellular cAMP levels are nonuniform and that these distinct “pools” of cAMP allow localized cAMP-mediated effects likely explain these seemingly dichotomous findings (1, 2, 11, 12, 38, 44). Although tethering of PKA to selected intracellular sites by virtue of binding to A kinase-anchoring proteins (AKAPs) is acknowledged to allow the compartmentation of PKA-dependent effects (1, 2, 11, 12, 38, 44), only recently have the functional consequences of similarly tethering PDEs in cells been analyzed systematically (9, 17, 23). In the context of localizing cAMP signaling through tethering of PDE4 enzymes

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in cells, several recent reports have described how unique amino-terminal sequences in individual PDE4 variants allows tethering of these enzymes to Src family kinases, β-arrestins, receptors for activated kinase C, or AKAPs in cells (17). For example, tethered PDE4D3 to a striated muscle AKAP (mAKAP)-based complex was purported to allow coordinated local regulation of cAMP signaling in these cells (11, 12). More recently, similar signaling complexes have been reported to allow localized subcellular cAMP signaling in several cell types (1, 2, 38). However, limited information describes the role of tethered PDE4s in coordinating important functions in VSMCs, including their migration.

In this report, we confirm that PDE4D3 can be integrated into a PKA-based signaling complex in rat cardiac myocytes. In contrast, we report that even though vascular myocytes also express this PDE4D variant, it was not integrated into PKA-based complexes in these cells. In contrast, we show that a distinct PDE4D variant, namely, PDE4D8, integrated into PKA-based signaling complexes in VSMCs. Of functional importance, we show that both PKA and PDE4D8 are enriched in leading-edge structures of migrating VSMCs and that antagonizing the recruitment of PDE4D8 into these structures caused marked alterations in these actin-rich structures. These data are consistent with the idea that distinct PDE4D variants integrate into PKA-based complexes in cardiac and vascular myocytes and identify a potentially important role for disruption of these complexes to achieve cell-specific regulation of cAMP signaling in cells of the cardiovascular system.

MATERIALS AND METHODS

Materials. Expression plasmids encoding PDE4D3 or hexahistidine-tagged RIIβ regulatory subunit of PKA were gifts from Drs. M. D. Houslay (University of Glasgow, Glasgow, Scotland) and C. S. Rubin (Albert Einstein College of Medicine, Bronx, NY), respectively. While the PDE4D3-specific antibody used in our study was purchased from Abcam (Cambridge, MA), previously validated (33) PDE4D8- and PDE4D9-specific antisera used here were gifts from Dr. M. Conti (Stanford University, Palo Alto, CA). All FALCON cell culture inserts were purchased from BD Biosciences (Mississauga, ON, Canada), and all other reagents were obtained as previously described (28, 32).

Cell culture and transient transfections. Transient transfection of HEK-293T or rat aortic VSMCs was as previously described (28, 32). Protocols for arterial and cardiac tissue harvesting from male Sprague-Dawley rats (~200 g) were approved by the Queen’s University Animal Care Committee and followed the guidelines established by the Canadian Council on Animal Care. Methodologies used to generate arterial tissue-derived cell lysates were as previously described (39, 40). Right ventricular myocytes (herein termed “cardiac myocytes”) were isolated as previously described (15). Briefly, on the day of the experiment, an adult male Sprague-Dawley rat (200–225 g body wt) was killed by cervical dislocation. The heart was rapidly removed, mounted on a Langendorff apparatus via aortic cannulation, and perfused with O2-saturated 1 mM Ca2+-containing Tyrode solution at a perfusion rate of 10 ml/min (37°C, 5 min). Subsequent to this initial perfusion, the perfusate was switched to Ca2+-free Tyrode solution for 5 min, after which the heart was then perfused for 7 min in Ca2+-free Tyrode solution supplemented with proteases [collagenase (0.02 mg/ml, Yakult) and protease type XIV (0.004 mg/ml, Sigma Chemicals)]. After this ultimate perfusion, the right ventricular free wall was removed, minced in 10 ml of protease-supplemented Tyrode solution [collagenase (0.5 mg/ml), protease XIV (0.1 mg/ml), and BSA (3 mg/ml, Sigma)], and gently agitated for 20–40 min at 37°C. Suspended rod-shaped cells (cardiac myocytes) were used directly for analysis without subculture and without exposure to culture media supplemented with bovine growth factors.

RNA isolation, RT-PCR, and generation of expression constructs. RNA was purified from cultured rat aortic VSMCs, freshly isolated rat aortic VSMCs, or freshly isolated cardiac ventricular tissue and subsequently reverse transcribed and amplified by PCR as previously described (28, 32). Oligonucleotides used for PCR-based amplification of oligo-dT-primered cDNAs were as follows (restriction sites are italicized): PDE4D3 (Accession No. RNU09457), sense 5′-CAGGATC- CATGATGCTGATGAACTTCT-3′; PDE4D5 (Accession No. E1F20484), sense 5′-AGGGATCTCATGCTGATGAACTTCT-3′; PDE4D7 (Accession No. AF536979), sense 5′-GTGATATGATGCAGGAAACCATTG-3′; PDE4D8 (Accession No. E121818), sense 5′-GGGAATTCTACGGCTTTTCGGGATCTC-3′; PDE4D9 (Accession No. AY388961), sense 5′-GGGGATCTCATGATGAACTTCT-3′; and PDE4D (common), antisense 5′-GGCGCTGAGTGTTGTGACCAAC-3′. Constructs designed to allow the expression of PDE4D3, PDE4D8, or PDE4D9 amino-terminal fragments (NT-PDE4D3, NT-PDE4D8, and NT-PDE4D9, respectively) were generated using the primers listed above, and construct expressing green fluorescent protein [GFP (PDE4D3-GFP, PDE4D8-GFP, and PDE4D9-GFP)] were generated after digestion of PCR-derived fragments and ligation into pEGFP-C3 expression vectors.

Cell fractionation and isolation of PKA-based signaling complexes. Generation of cell, arterial tissue, or cardiac tissue lysates and their use in cAMP-agarose pulldowns were performed as previously described by us (28, 31, 32) using the antisera listed in Materials. Briefly, lysates were generated by homogenization in Tris (50 mM, pH 7.4)-based buffer supplemented with 5 mM magnesium chloride, 5 mM benzamidine, 1% Triton X-100, 150 mM sodium chloride, 5 mM sodium pyrophosphate, 10 mM sodium B-glycerophosphate, 1 μg/ml pepstatin A, 2 μg/ml E-64, 100 μg/ml PMSF, 2 μg/ml leupeptin, and 0.1 mM DTT and the subsequent centrifugation of this homogenate at 10,000 g. To reduce nonspecific protein binding in cAMP-agarose pulldown assays, cleared lysates were incubated with protein A/G agarose beads (bed volume: 25 μl) for 4 h at 4°C. After the removal of the protein A/G beads by centrifugation (1,000 g), several cellular lysates were incubated with cAMP-agarose (bed volume: 50 μl) for 16 h at 4°C. cAMP-agarose-associated signaling complexes containing PKA-RII, EPAC, PDEs, and associated proteins were isolated by centrifugation at 1,000 g, and the remaining cleared lysate was kept for the analysis of “unbound” cell lysate proteins. After an extensive wash of cAMP-agarose pellets with ice-cold lysis buffer, specifically adsorbed proteins were selectively eluted using cAMP (10 mM). PDE3 and PDE4 activities were measured as previously described (28, 32). PDE3 and PDE4 activities were determined by assessing the amount of PDE activity inhibited by cilostamide or Ro 20-1724, respectively (28, 32).

Visualization and harvesting of VSMC pseudopodial structures. Leading-edge pseudopodial structures were isolated by a modification of the method originally described by Cho and Klemke (8) and applied to NIH 3T3 fibroblasts (18). Briefly, for our work, VSMCs in 0.5% FBS-supplemented DMEM were placed on the upper level of fibronectin-coated (20 μg/ml) 23-cm2-diameter BD Falcon cell culture inserts or 6.5-cm2 BD Falcon Fluoroblok inserts (3-μm pores). Chemotactic factors were subsequently added to the underside of the inserts, and cells were allowed to extend projections for 2 h. For pseudopodia visualization, cells on 6.5-cm2 inserts were rinsed in PBS, fixed with paraformaldehyde [4% (vol/vol), and incubated for 1 h in phallolidin-tetramethylrhodamine B isothiocyanate (1.500, Sigma-Aldrich)-PBS. Inserts were mounted on glass slides, and pseudopodia structures were visualized with a Zeiss Axiovert S100 microscope with fluorescence capability and imaging (Slidebook 3.0.1 software, 3i, Denver, CO). For pseudopodia harvesting, after a wash of the 23-cm2-diameter inserts with PBS (4°C), pseudopodia were
collected in lysis buffer by scraping. Cell bodies from the upper chamber were similarly collected into lysis buffer.

RESULTS

Isolation of PKA-based, PDE4D-containing signaling complexes from cardiac myocytes and aortic VSMCs. Integration of a PDE4D variant, identified as PDE4D3, into a mAKAP/PKA-based signaling complex in hypertrophied-cultured rat cardiac myocytes has been reported previously by others (11, 12). Using a previously validated cAMP-agarose pulldown-based approach (28, 32), we report here that a PDE4D3-containing, PKA-based complex could also be isolated from freshly isolated, nonhypertrophied, noncultured rat cardiac myocytes. Indeed, using this approach, we isolated a PKA-based complex containing a 98-kDa anti-PDE4D (pan)-reactive protein from lysates generated from freshly isolated rat cardiac myocytes (Fig. 1A). Although mAKAP has been specifically identified as a PKA anchor in earlier studies (11, 12) using hypertrophied, cultured rat cardiac myocytes, immunoblot analysis did not reveal the presence of mAKAP in isolates from freshly isolated cardiac cells (not shown). This finding is consistent with the fact that mAKAP expression is increased upon treatment of cardiac myocytes with hypertrophic stimuli (11).

Immunoblot analysis of rat aortic VSMC lysates (Fig. 1, B and C) identified PDE4D3 as well as a 115-kDa PDE4D variant, likely representing either PDE4D5 or PDE4D7 or a mixture of both these comigrating species (17, 23). As with cardiac myocytes, a 98-kDa anti-PDE4D (pan)-reactive protein was also readily isolated from either cultured rat aortic VSMCs (Fig. 1B) or freshly isolated, noncultured rat aortic VSMCs (Fig. 1C). Interestingly, the anti-PDE4D (pan) protein isolated from these aortic VSMC lysates was not PDE4D3. Indeed, although these cells expressed significant PDE4D3 (Fig. 1, B and C), and even though the anti-PDE4D (pan)–reactive protein isolated from these cells behaved identically to PDE4D3 by SDS-PAGE analysis, it was immunologically distinct and was not recognized by the anti-PDE4D3 antiserum used in our study (Fig. 1, B and C). Taken together, these data support the idea that, although rat cardiac myocytes and rat aortic VSMCs each express PDE4D3, a distinct PDE4D variant with chromatographic characteristics indistinguishable from those of PDE4D3 was integrated into PKA-based complexes in VSMCs (Fig. 1, B and C). In addition to the 98-kDa PDE4D variant, PKA-containing complexes isolated from cultured aortic VSMCs also contained a 115-kDa PDE4D species (Fig. 1B). Due to the lack of PDE4D5- or PDE4D7-selective antisera, the identity of this PDE4D variant was not confirmed. Consistent with this idea is the finding that, using an antibody specific to AKAP5 (anti-AKAP5; middle), or an antibody specific for AKAP12 (anti-AKAP12; bottom), Samples identified as Unbound (+cAMP) and Beads (+cAMP) were derived from samples in which cAMP-agarose pulldowns were carried out in the presence of a saturating concentration of cAMP (10 mM) to compete for RIIβ binding to cAMP-agarose binding. Representative immunoblots are of at least 4 independent experiments in which similar data were obtained.
with our previous work (39, 40), noncultured rat aortic VSMCs expressed significantly less of the 115-kDa PDE4D species, and none was detected in cAMP-agarose pulldowns from these cells (Fig. 1C).

Two dominant AKAPs were detected in cultured aortic VSMCs, and each of these was isolated with PKA and PDE4D in our cAMP-agarose-based isolation procedures. Thus, AKAP79 (AKAP5) and gravin (AKAP12) were both isolated with PKA and the PDE4D variant from lysates of VSMCs (Fig. 1D). Levels of AKAP proteins in lysates of noncultured rat cardiac myocytes were below resolution using our approach (not shown).

Characterization of PDE4D variants in cardiac myocytes and VSMCs. Since the data described above were consistent with the idea that the 98-kDa anti-PDE4D (pan) variant that integrated into PKA-based signaling complexes in VSMCs was not PDE4D3, we used a combination of RT-PCR and selective immunoblot analysis to identify the PDE4D variants expressed in this cell type. Overall, our data are consistent with the idea that several similarly sized PDE4D variants are expressed in both rat cardiac myocytes and aortic VSMCs. Thus, RT-PCR-based amplification and sequencing yielded products encoding the predicted fragments of PDE4D3 (382 bp), PDE4D5 (591 bp), PDE4D7 (606 bp), PDE4D8 (430 bp), and PDE4D9 (432 bp) in both freshly isolated and cultured aortic VSMCs and of PDE4D3 (382 bp), PDE4D8 (430 bp), and PDE4D9 (432 bp) in rat cardiac myocytes (Fig. 2A). Although previous work has reported that PDE4D5 was expressed in cultured murine and rat neonatal cardiac myocytes, under our experimental conditions, using noncultured cardiac myocytes, these transcripts were not detected. In fact, the results of ongoing studies, not directly related to the study described here, are consistent with the idea that PDE4D5 expression in cardiac myocytes is markedly affected by cell culture in the presence of bovine plasma-derived growth factors (not shown). Similar data have been previously reported by us for the expression of PDE3 and PDE4 genes in aortic VSMCs (39, 40).

Since we reported previously that there was no necessary correlation between mRNA and protein levels for PDE4D in rat cardiovascular cells (39, 40), we used a coupled immunoprecipitation- and immunoblot-based approach to determine the relative levels of expression of these multiple PDE4D isozymes in these cells. For these analyses, PDE4D variants were first immunoprecipitated using an anti-PDE4D (pan) antiserum and subsequently immunoblotted with PDE4D variant-selective antisera (Fig. 2B). Results from these experiments were consistent with the expression of each PDE4D3, PDE4D8, and PDE4D9 in both rat cardiac myocytes and rat aortic VSMCs, albeit at different levels (Fig. 2B). Regrettably, reagents that could have allowed the specific identification of the 115-kDa PDE4D variants expressed in rat aortic VSMCs were not available to us for the experiments described herein.

Characterization of PDE4D variants interacting with PKA in VSMCs. Since PDE4D3, PDE4D8, and PDE4D9 were each detected in lysates of aortic VSMCs (Fig. 2), but PDE4D3 was not detected in PKA-based complexes in these cells (Fig. 1), our data raised the intriguing possibility that PDE4D8, or PDE4D9, represented the dominant 98-kDa PDE4D variant that was detected in the vascular complexes isolated in our study. The PDE4D8- and PDE4D9-selective antisera available to us for this study (33) had low avidity for their respective antigens and did not allow us to determine whether PDE4D8 or PDE4D9 was present in PKA-based complexes isolated from rat aortic VSMCs (not shown). To overcome this technical limitation, aortic VSMCs were transiently transfected to express constructs encoding PDE4D-variant specific chimeras in which the amino-terminal fragments of PDE4D8 (NT-PDE4D8, amino acids 2–100) or PDE4D9 (NT-PDE4D9, amino acids 2–101), respectively, were fused to GFP. When aortic VSMCs individually expressing these chimeras were used in cAMP-agarose pulldown experiments, only
least 3 separate experiments. Indeed, while NT-PDE4D8/GFP protein was retained with PKA in cAMP-agarose pulldowns of transfected aortic VSMCs (Fig. 3), neither NT-PDE4D9-GFP (not shown) nor native GFP (Fig. 3) were isolated. These data are consistent with the idea that PDE4D8 was the dominant PDE4D variant that integrated into PKA-based complexes in aortic VSMCs.

**Recruitment of PKA-PDE4D signaling complexes to leading-edge structures in aortic VSMCs.** As described in the Introduction, cAMP-elevating agents have a complex series of effects on the adhesion and migration of VSMCs, inhibiting migration but stimulating adhesion and the formation of leading-edge membrane extensions in migrating cells (8, 18). Having established that a NT-PDE4D8/GFP chimera could integrate into PKA-based complexes in VSMCs, we next chose to determine if there was any regioselectivity to this association in migrating aortic VSMCs. For these experiments, we used a modification of a method that allows the isolation and study of leading-edge structures (“pseudopodia”) of migrating cells (8, 18). This approach has been recently used to demonstrate that components of the cAMP signaling system, importantly including PKA, were preferentially recruited to leading-edge structures in migrating NIH 3T3 cells (18). Overall, our data are consistent with the idea that components of the cAMP signaling system are also recruited to leading-edge structures of migrating rat aortic VSMCs and, importantly, show that recruitment of PDE4D8, but not PDE4D3, to these structures may play an important role in regulating localized cAMP-mediated effects.

Aortic VSMCs subjected to a gradient of lysophosphatidic acid (LPA; 200 μg/ml; Fig. 4) or PDGF (50 ng/ml; not shown) extended leading-edge, actin-rich pseudopodia in the direction of the gradient, which could readily be visualized on the underside of the porous membranes (Fig. 4, A and B). Consistent with the dynamic nature of these structures (8), removal of the gradient allowed their rapid retraction to the upper chamber of these inserts (Fig. 4B, right). Similar to a previous report using NIH 3T3 cells (18), immunoblot analysis of the components present in aortic VSMC pseudopodia showed that while certain proteins were more abundant in the pseudopodia, most were not (Fig. 4C). For its part, levels of β-actin present in pseudopodia were similar to those present in the cell body (Fig. 4C), even though the migratory leading-edge structures were clearly actin based (Fig. 4B). In contrast, but also consistent with earlier work (18), levels of PKA-RIIβ, but not PKA-C, were enriched in pseudopodia of migrating aortic VSMCs (Fig. 4C). Consistent with the higher PKA-RII-to-PKA-C subunit ratio present in pseudopodia compared with the cell body, PKA-mediated phosphorylation of several PKA substrates was lower in the pseudopodia than in the cell body. For example, immunoblot analysis revealed that levels of phosphorylated vasodilator-stimulated phosphoprotein (phospho-VASP; Fig. 4C, VASP blot, top band) or of PKA-RIIβ itself (not shown) were lower in the pseudopodia than in the cell body. Although an established RII-based overlay assay allowed the detection of known AKAPs in these cells, including AKAP5 and AKAP12 (Fig. 1D), their levels in pseudopodia preparations were below detection with our reagents. While a higher level of tetrameric, and thus inactive, PKA in pseudopodia undoubtedly accounts for some reduction of PKA-mediated phosphorylation within these structures, our data are also consistent with a potential role for enrichment of certain PDE4D variants in these structures. Thus, the dominant cAMP-PDE activity of these cells, namely, PDE4, was enriched in pseudopodia compared with that present in the cell body (Fig. 4D). Moreover, consistent with these activity measurements, both 115- and 98-kDa anti-PDE4D (pan)–immunoreactive species were enriched in pseudopodia (Fig. 4C). Provocatively, the lack of PDE4D3 enrichment in pseudopodia combined with the enrichment of RIIβ in these structures was consistent with the idea that PDE4D enrichment in the pseudopodia of aortic VSMCs was coordinated through integration into the PKA-based complexes isolated previously from lysates of these cells (Fig. 1, B and C). Overall, these data are consistent with the idea that PDE4D3 was not integrated into PKA-based signaling structures in nonmigrating and migrating VSMCs and with the idea that PDE4D8 might be integrated into PKA-based structures enriched in pseudopodia of migrating VSMCs. To directly test this novel idea, we adopted a dominant negative, isoform-specific displacement strategy, as initially used by Houslay and colleagues (24), to compete targeting of specific PDE4 variants from their tethers in cells. In this earlier work, Houslay and colleagues (24) reported that this approach antagonized tethering of endogenous PDE4 enzymes in cells and effectively reduced the ability of the otherwise tethered enzyme to spatially regulate cellular function. Although heterologous expression of native GFP in aortic VSMCs resulted in its expression both in the bulk cytosol and in the nucleus, the NT-PDE4D8/GFP fusion was detected primarily in the bulk cytosol and was largely excluded from the nucleus of transfected cells (Fig. 5A). When used in the pseudopodia assay, VSMCs expressing GFP formed pseudopodia similar to those formed by nontransfected cells, and these contained GFP (Fig. 5B). In marked contrast, when VSMCs expressing the NT-PDE4D8/GFP chimera were studied, significant amounts of this chimera was detected on the underside of the filter in pseudopodial structures (Fig. 5B). Interestingly, while cells expressing the NT-PDE4D8/GFP chimera displayed cell body cytoskeleton structures indistinguishable from those in cells expressing GFP (Fig. 5A)
were able to extend pseudopodial structures, the actin in pseudopodia of NT-PDE4D8/GFP-expressing cells was completely disorganized and was unable to form stress fibers (Fig. 5B). These data are interesting in two respects. First, they show that recruitment of NT-PDE4D8/GFP to pseudopodia of migrating VSMC caused disassembly of pseudopodial actin-based structures. Second, they are consistent with the idea that recruitment of endogenous PDE4D8 to pseudopodia structures in migrating rat aortic VSMCs may be required for the proper control of actin cytoskeletal dynamics in these dynamic structures. Although the molecular basis for this effect is currently unknown, it is perhaps noteworthy that the actin cytoskeleton present in pseudopodia of NT-PDE4D8/GFP-expressing cells is similarly disorganized compared with that present in rat aortic VSMCs incubated with the cAMP-elevating agents forskolin and Ro 20-1724 (29). Clearly, future studies will be required to fully assess the molecular basis of these findings and to fully determine their impact on rat aortic VSMC migration.

DISCUSSION

Efforts to elucidate the mechanisms by which cAMP-elevating agents selectively regulate multiple cellular events simultaneously in cardiac myocytes have recently borne fruit. Indeed, combined with biochemical data identifying AKAP-based, PKA- and/or EPAC-containing cAMP signaling complexes in cardiac myocytes (11, 12), cAMP biosensor-derived data have identified several distinct semiautonomously regulated cAMP pools in these cells (26, 31, 34, 42, 45). In marked contrast, little data are available describing the extent to which cAMP signaling is compartmented in arterial VSMCs or describing how this compartmentation might allow cAMP-mediated effects to be regulated in these cells. Indeed, to our knowledge, this study
represents one of the first to comprehensively approach these issues in VSMCs.

In this study, we extend on earlier work using cultured cardiac myocytes (11, 12) and show that a cAMP signaling complex, containing both PKA and PDE4D3, can be isolated from noncultured, freshly isolated rat cardiac myocytes. Moreover, while our data identified an analogous complex in both freshly isolated and cultured VSMCs, we showed that PDE4D3 was not integrated into PKA-based complexes in VSMCs. Indeed, while we report that cardiac myocytes and arterial VSMCs each expressed three distinct 98-kDa PDE4D variants, namely, PDE4D3, PDE4D8, and PDE4D9, we show that PDE4D3 was not integrated into PKA-based complexes in VSMCs. Indeed, while our data are consistent with the idea that PDE4D8, rather than PDE4D3 or PDE4D9, represents the dominant PDE4D integrated into PKA-based signaling complexes in VSMCs. Since our study specifically focused on VSMCs rather than cardiac myocytes, future work will be needed to determine if PDE4D8 or PDE4D9 also integrate into PKA-based complexes in cardiac myocytes.

Given that cAMP-elevating agents often can have mixed effects in the cardiovascular system, simultaneously increasing the frequency and force of cardiac contractions (14, 27, 30) while dilating blood vessels (5, 23, 29), our finding that...
distinct, and perhaps cell type-specific, PDE4D variants may define cAMP signaling complexes in individual cardiovascular cell types may be both physiologically and therapeutically important. In this context, our finding that several components of the cAMP signaling system were enriched in pseudopodia of migrating VSMCs myocytes, and, importantly, that PDE4D variants were differentially enriched in these structures, may help to explain the complex role of the cAMP-PKA signaling axis in regulating vascular myocytes functions, including migration. Thus, we report a significant enrichment of RIIB in pseudopodia of migrating VSMCs, similar to that previously reported for fibroblasts (18). In contrast to findings in fibroblasts, however, VSMC pseudopodial PKA activity, as assessed based on VASP phosphorylation, was decreased, not increased, relative to that of the cell body. Although several mechanisms are likely at play in bringing about this difference, our data are consistent with the idea that PDE4D8 enrichment in pseudopodia regulates pseudopodial cAMP levels and antagonizes PKA activation within these structures. While future studies using cAMP biosensors will be needed to formally test this hypothesis, our observations that VASP and RI phosphorylation levels were each reduced in pseudopodia are consistent with this hypothesis.

Previous reports have shown that PKA can have both pro- and antimitagulatory effects in cells, and some have suggested that this paradox might be due to the need for an “optimal” level of PKA activity in coordinating this complex cell function (8, 18, 37). Interestingly, our findings further refine this concept by showing that PKA within distinct compartments of cells may fulfill unique functions and that dysregulation of PKA activity in any of these compartments can impact cAMP-regulated functions within this domain. To this point, our data indicate that antagonism of PDE4D8 recruitment into pseudopodia in migrating VSMCs led to aberrant actin cytoskeletal dynamics in this structure. In contrast, PDE4D3 and PDE4D9 were not enriched in pseudopodia, and cells expressing GFP chimeras of the amino-terminal domains of these enzymes had no impact on actin dynamics. Although not strictly comparable, the impact of NT-PDE4D8/GFP pseudopodial accumulation on actin dynamics in these structures was reminiscent of our previous observations of the impact of cAMP-elevating agents on leading-edge structures in these cells (29). Since it is likely that the effect of NT-PDE4D8/GFP in these pseudopodial structures is cAMP dependent, we hypothesize that it is due to competing with the endogenous PDE4D8 present in these structures in migrating cells (Fig. 6). Obviously, in the future, more targeted studies will be needed to directly test this exciting hypothesis. In addition to being of potential significance to those with an interest in identifying mechanisms by which cAMP simultaneously regulates numerous cell functions selectively, we submit that our finding may also be important therapeutically. Thus, a significant overlap exists in the cAMP-sensitive systems that control the functions of cardiac and vascular myocytes. Due to this overlap, the use of several cAMP-elevating agents for the treatment of certain cardiovascular indications has had unexpected effects. For example, whereas β-adrenoreceptor agonists (i.e., dobutamine) or PDE3 inhibitors (i.e., milrinone) have positive effects on cardiac inotropy, lusitropy, chronotropy, and dromotropy and would be expected to increase cardiac output, their vasorelaxant effect in vascular myocytes limits their utility (27). Indeed, while these agents have been shown to have beneficial effects in the short term in patients with heart disease, their longer-term use was associated with treatment-associated increases in mortality (27). Similarly, while most reports have shown that PDE4 inhibitors have few, if any, effects on the heart, one recent report (20) very indirectly tested the idea that prolonged use of such agents might eventually have unwanted cardiac effects. Since all PDE4 inhibitors thus far tested in animal or human trials have been designed to compete for cAMP binding at the active site, and all PDE4s derived from individual PDE4 genes share identical catalytic domains, these agents will not have utility in obviating these side effects, should they occur. In contrast, we submit that our data are consistent with a distinct strategy for the inhibition of PDE4 activity, which might reduce the occurrence of off-tissue effects. Indeed, as suggested previously (see Ref. 17 and some sources cited therein), we propose that greater selectivity may be possible if approaches designed to disrupt PDE4D/PKA-based complex interactions, rather than compete for cAMP binding, were developed. Of course, while significant further work will be required to assess the overall feasibility of this approach, the data in this report, in combination with earlier reports to the same effect, establish that, when possible, selective disruption of PDE4D8 compartmentation in VSMCs may allow blood vessel-selective effects of certain cAMP-elevating agents within the cardiovascular system.

**REFERENCES**


