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Vascular Smooth Muscle Cell Phosphodiesterase (PDE) 3 and PDE4 Activities and Levels are Regulated by Cyclic AMP in Vivo

DOUGLAS G. TILLEY and DONALD H. MAURICE

Department of Pharmacology and Toxicology, Queen's University, Kingston, Ontario, Canada

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ABSTRACT

Prolonged incubation of several cell types, including cultured vascular smooth muscle cells (VSMC), with cyclic AMP-elevating agents increases cAMP phosphodiesterase (PDE) activity and levels. In this work, we describe for the first time an increase in arterial VSMC cAMP PDE activity and levels caused by cAMP-elevating agents when these agents are administered to rats in vivo. Injections of rats with dibutyryl cAMP (dbcAMP) or forskolin increased both PDE3 and PDE4 activities in aortic and femoral artery VSMC. Consistent with the idea that cAMP-elevating agents increased PDE3 and PDE4 activities by acting directly on VSMC, local delivery of dbcAMP or forskolin to femoral arteries using a pluronic gel-based approach increased femoral artery VSMC PDE3 and PDE4 activities to levels similar to those observed after injection of these agents. Consistent with a role for de novo mRNA and protein synthesis in the cAMP-elevating agent induced increase in PDE3 and PDE4, 1) systemic administration of forskolin increased PDE3A, PDE3B, and PDE4D mRNA levels in aortic VSMC and femoral artery VSMC, 2) local delivery of dbcAMP increased PDE3A, PDE3B, and PDE4D3 protein levels in femoral artery VSMC, and 3) local administration of either actinomycin D or cycloheximide attenuated the effect of dbcAMP. In addition, our results indicate that the PDE3 and PDE4 variants increased by cAMP-elevating agents in arterial VSMC in situ were distinct from those elevated by these agents in cultured arterial VSMC. Consistent with the effect of increased VSMC cAMP PDE on blood vessel function, inhibition of PDE3 and PDE4 activities potentiated the relaxant effect of forskolin in dbcAMP-treated femoral artery rings to a greater extent than in untreated control blood vessels. We propose that our findings are consistent with the concept that cAMP regulates VSMC cAMP PDE activity and levels in vivo and that VSMC phenotype influences the choice of cAMP PDE variant that is elevated. Our findings are discussed in the context that agents aimed at specific PDE3 or PDE4 variants could perhaps allow greater control of cAMP-mediated regulation of VSMC behaviors that are phenotype-dependent.

Vascular smooth muscle cells (VSMC) in situ express contractile proteins, control the contractility of blood vessels, and are said to express a contractile phenotype. However, in response to vascular damage, or when cultured, VSMC can display a more proliferative and migratory phenotype that is referred to as synthetic (Owens, 1998; Thyberg, 1998). Numerous physiological and pharmacological agents increase VSMC cAMP and relax contractile VSMC or inhibit proliferation and migration of synthetic VSMC (Rybalkin and Bornfeldt, 1999; Koyama et al., 2001). Intracellular levels of cAMP are dynamically regulated by the concerted actions of adenylyl cyclases and cyclic nucleotide phosphodiesterases (PDE). Presently, at least 11 distinct PDE families, each containing several genes, encode more than 50 different PDE enzyme variants (Manganiello and Degerman, 1999; Soderling and Beavo, 2000). Profiles of contractile arterial VSMC cAMP PDE activities in several species identify PDE3 and PDE4 family members as the major cAMP PDE activities in these vessels (Polson and Strada, 1996).

Two PDE3 genes (*PDE3A and PDE3B*) encoding distinct proteins with similar overall structures have been described previously (Miki et al., 1996; Degerman et al., 1997; Manganiello and Degerman, 1999; Movsesian, 1999; Kasuya et al., 2000; Liu and Maurice, 1998). mRNA encoding PDE3A is abundant in heart, vascular, airway, and gastrointestinal smooth muscle, whereas PDE3B mRNA is most abundant in white and brown fat (Reinhardt et al., 1995). Although some cells, including blood platelets, express only one member of the PDE3 family (Haslam, et al., 1999; Manganiello and Degerman, 1999), others, including human and rat aortic

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ABBREVIATIONS: VSMC, vascular smooth muscle cells; PDE, phosphodiesterase; dbcAMP, dibutyryl cAMP; fsk, forskolin; IBMX, 3-isobutyl-1-methylxanthine; BCA, bicinchoninic acid; DAPI, 4′,6′-diamidino-2-phenylindole dihydrochloride hydrate; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; PCR, polymerase chain reaction.

VSMC, express both PDE3A and PDE3B (Liu and Maurice, 1998; Palmer and Maurice, 2000). PDE3s bind cAMP and cGMP with high affinity (K_m, 0.1–1 μM), but a 10-fold lower V_{max} for cGMP allows this cyclic nucleotide to act as a potent competitive inhibitor of cAMP hydrolysis by these enzymes in cells (Maurice and Haslam, 1990a,b; Verde et al., 1999; Choi et al., 2001). Inhibition of PDE3 by cGMP, or drugs (e.g., cilostamide, milrinone, lixazinone) stimulates myocardial contractility, relaxes vascular and airway smooth muscle, and inhibits platelet aggregation (Haslam et al., 1999; Manganiello and Degerman, 1999). PDE3s are activated when phosphorylated by either cAMP-dependent protein kinase (PKA), or phosphatidyl inositol-3-phosphate–dependent protein kinase (Manganiello and Degerman, 1999). In addition, cAMP-elevating agents increase PDE3 activity and levels in several cell types (Degerman et al., 1997), including cultured rat and human aortic VSMC (Rose et al., 1997; Liu and Maurice, 1998; Palmer and Maurice, 2000).

Four PDE4 genes have been identified (*PDE4A*, *PDE4B*, *PDE4C*, and PDE4D); PDE4 activity is detected in most mammalian cells, except blood platelets (Houslay, 1998; Conti and Jin, 1999; Haslam et al., 1999). Perhaps owing to the weak vasorelaxation caused by PDE4 inhibitors (Polson and Strada, 1996), expression of PDE4s in blood vessels has not been studied extensively. Recently, we demonstrated that two PDE4D "long forms" (PDE4D3, PDE4D5) were expressed in rat and human VSMC (Liu and Maurice, 1999; Palmer and Maurice, 2000). PKA-dependent phosphorylation of each PDE4D3 and PDE4D5 activates these enzymes (Houslay, 1998; Conti and Jin, 1999; Liu and Maurice, 1999), whereas activation of the MAPK cascade can activate, or inhibit, these enzymes, perhaps in a cell-type- or targeting-dependent manner (Hoffman et al., 1999; Liu and Maurice, 1999; MacKenzie et al., 2000; Baillie et al., 2001). In cultured rat and human aortic VSMC, incubation with cAMP-elevating agents induces expression of two PDE4D "short forms": PDE4D1 and PDE4D2 (Liu et al., 2000). In addition to its effect on PDE4D3 activity (Liu and Maurice, 1999; MacKenzie et al., 2000; Baillie et al., 2001), the mitogen-activated protein kinase cascade also regulates *PDE4D* expression, inhibiting cAMP-induced increases in the PDE4D "short-forms" through a mechanism involving mRNA destabilization (Liu et al., 2000). Selective and regulated targeting of PDE4s also regulates the impact of these enzymes on cell function (Beard et al., 1999; Liu and Maurice, 1999; McPhee et al., 1999; Yarwood et al., 1999; Grange et al., 2000).

Our laboratory has reported previously that in cultured (synthetic) rat or human aortic VSMC, PDE3 and PDE4 activities are elevated after incubation of these cells with cAMP-elevating agents and that this increase attenuated responses of these cells to further stimulation with cAMP-elevating agents (Rose et al., 1997; Liu and Maurice, 1999; Liu et al., 2000; Palmer and Maurice, 2000). So far, no information is available concerning the cAMP-mediated regulation of cAMP PDE activity or levels in arterial VSMC incubated in vivo with cAMP-elevating agents. We report here that cAMP-elevating agents increased PDE3 and PDE4 activities in contractile VSMC in vivo and we identify potentially important differences in the PDE3 and PDE4 variants increased by these agents in vivo compared with those increased in synthetic VSMC. The data are consistent with the idea that prolonged incubations of contractile and synthetic VSMC with cAMP-elevating agents increase different cAMP PDEs in these cells. This knowledge could prove important in situations in which selective cAMP-dependent effects were desired in either synthetic, or contractile, VSMC phenotypes, such as, for example, inhibition of vascular restenosis post angioplasty in which selective effects on synthetic VSMC would be desired.

Materials and Methods

General Reagents. Radioactive chemicals, [^{3}H]cAMP and 5′-[^{14}C]AMP, were obtained from PerkinElmer Life Sciences (Boston, MA). Superscript II reverse transcriptase and *Taq* DNA polymerase were from (Invitrogen, Carlsbad, CA). Ro 20-1724, cilostamide, forskolin (fsk) and dibutyryl-cAMP (dbcAMP) were purchased from Calbiochem-Novachem Corporation (San Diego, CA), sodium fluoride (NaF), phenylephrine, cycloheximide, and actinomycin D from Sigma-Aldrich (Oakville, ON, Canada), zardaverine from Biomol (Plymouth Meeting, PA) and 3-isobutyl-1-methylxanthine (IBMX) from Sigma-Aldrich. Tris-HCl, benzamidine, EDTA, EGTA, dithiothreitol, phenylmethylsulfonyl fluoride, and Triton X-100 were obtained from ICN Biomedicals (Costa Mesa, CA), whereas leupeptin was from Roche Applied Science (Mississauga, ON, Canada). Potassium chloride (KCl), calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$), magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$) and potassium dihydrogen orthophosphate (KH_2PO_4) were purchased from BDH Incorporated (Ontario, Canada). Pluronic F127 NF (poloxamer 407 NF) surfactant was a gift from BASF Corporation (Mt. Olive, NJ) and Affi-gel 601 and the column supports were from Bio-Rad (Mississauga, ON, Canada). The bicinchoninic acid (BCA) protein assay and bovine serum albumin were from Pierce (Rockford, IL). All other chemicals were of reagent grade and purchased from Fisher Scientific (Nepean, ON, Canada). Male Wistar rats were obtained from Charles River (Constance, PQ, Canada). The anesthetics ketamine, xylazine, and euthanyl (pentobarbital sodium), were obtained via Animal Care (Queen's University at Kingston, ON, Canada) from Rogar/STB Inc. (London, ON, Canada), Bayer (Ontario, Canada) and Bimeda-MTC (Cambridge, ON, Canada), respectively.

Systemic Delivery of cAMP-Elevating Agents in Vivo. Two cAMP agents, dbcAMP (15 mg/kg) or fsk (1 mg/kg), supplemented with 0.01 mg/ml of IBMX, a broad-spectrum cyclic nucleotide phosphodiesterase inhibitor, were administered to male Wistar rats (300–350 g) in 0.5-ml (dbcAMP) or 30-μl (fsk) i.p. injections. For control injections, rats were administered either saline (vehicle for dbcAMP) and IBMX or 0.02% dimethyl sulfoxide (vehicle for forskolin) and IBMX. Doses of dbcAMP, fsk, and IBMX used in this study were obtained from a review of relevant previous literature (Palmer and Doukas, 1984; Maeda et al., 1997; Tumer et al., 1997) and from preliminary experiments. Using this approach, rats were administered treatments once every hour for 5 h. One hour after the final injection (6 h total) rats were euthanized (1.7 ml/kg euthanyl), and the aorta and femoral arteries were removed and processed as described below.

Pluronic Gel-Based Local Administration of cAMP-Elevating Agents in Vivo. After administration of ketamine (70 mg/kg)-xylazine (5 mg/kg) to male Wistar rats (300–350 g) and isolation of femoral arteries through hind-leg incisions, the perivascular space surrounding each femoral artery was filled with vehicle or a 500-μl volume of chilled pluronic gel solution [20% (w/v)] supplemented with test agents. Because the chemical characteristics of solutions of pluronic gel allow them to form semisolid gels at temperatures above 10°C once applied to the perivascular spaces, the 500-μl solution encased the femoral artery. In the experiments described in this report, effects of two cAMP-elevating agents fsk (100 μM) or dbcAMP (1 mM) on the femoral artery were tested, as well as the impact of a transcriptional inhibitor (actinomycin D, 4.0 μM) or an inhibitor of translation (cycloheximide, 100 μM). In an early experiment, we

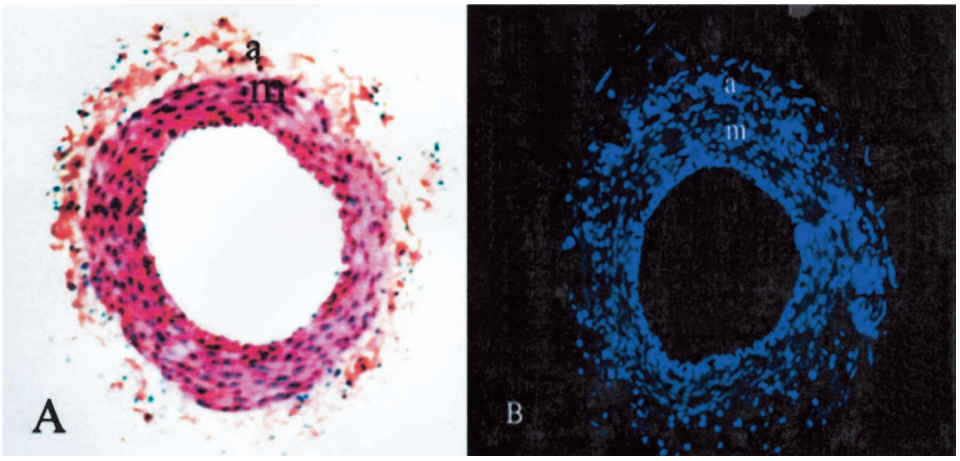


Fig. 1. A 500-μl aliquot of a 20% (w/v) solution of pluronic gel was added to one femoral artery, whereas a similar aliquot of this solution, supplemented with 1 mg/ml DAPI, was applied to the other. After the solutions had polymerized (1–2 min) the rat was allowed to recover and kept in a clean cage for 4 h. At the end of this period, the animal was euthanized and the femoral arteries were removed, cleaned of adherent fat and connective tissue, fixed and sectioned for histological examination (see *Materials and Methods*). The adventitial (a) and medial (m) layers of these rat femoral artery rings are indicated in a hematoxylin-and-eosin–stained section of the femoral artery treated with only Pluronic gel (A), whereas a fluorescence image of the DAPI-stained nuclei in both these layers is shown (B).

validated that a small molecule [4′,6′-diamidino-2-phenylindole dihydrochloride hydrate (DAPI), 1 mg/kg] would diffuse from the pluronic gel to the medial VSMC (Fig. 1). At the end of the treatment period (4 or 16 h), rats were euthanized (1.7 ml/kg euthanyl) and femoral arteries excised and processed as described below.

Excision and Processing of Rat Arteries and Protein Determinations. Rat aorta and femoral arteries removed from euthanized rats were processed surgically to allow isolation of predominantly medial layer-VSMC tissue by removal of adherent fat using fine instruments. Previous studies have shown that this approach allows isolation of blood vessel segments comprising intact medial layers, which contain VSMC, and a small amount of adventitial layer-derived tissue containing few fibroblasts (Fig. 1). Isolated blood vessels processed in this manner were homogenized in a lysis buffer containing 52.5 mM Tris-HCl, pH 7.4, 5.25 mM $MgCl_2$, 5 mM benzamidine, 1 mM EDTA, 100 mM dithiothreitol, 200 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1% Triton X-100, and 50 mM NaF. Cellular debris and unlysed cells were removed by centrifugation at 1000*g* (3000 rpm) for 3 min. The 1000*g* supernatants were stored at 4°C until cAMP PDE activity assays were conducted. Protein concentrations of lysates were determined using the BCA protein assay system from Pierce, according to the manufacturer's recommendations, using bovine serum albumin as the standard.

Determination of cAMP PDE Activities. Levels of cAMP PDE activity in blood vessel lysates were determined by a modification of the method of Davis and Daly (1979), as we described previously (Rose et al., 1997), using equivalent amounts of tissues derived from rat aortas or femoral arteries, and with 1 μM [^{3}H]cAMP as substrate (containing approximately 100,000 d.p.m). Contributions of PDE3 or PDE4 to total VSMC cAMP PDE activities were determined pharmacologically using maximally effective selective concentrations of inhibitors of PDE3 (cilostamide, 1 μM) or PDE4 (Ro 20-1724, 10 μM). Maximum cAMP hydrolysis catalyzed by cAMP PDE was determined using the broad-spectrum PDE inhibitor IBMX at 500 μM.

TABLE 1

Primers used for reverse transcription PCR of RNA from rat aorta and femoral arteries

Underlined nucleotides are designed for introducing an *Eco*RI site in the PCR products.

cDNA	Primer Pairs
Rat PDE3A	5′-CCGAATTCCCTTATCATAACAGAATCCACGCCACT-3′
	5′-CCGAATTCGTGTTTCTTCAGGTCAGTAGCC-3′
Rat PDE3B	5′-CCGAATTCTATCACAATCGTGTGCATGCCACAGA-3′
	5′-CCGAATTCTTTGAGATCTGTAGCAAGGATTGC-3′
Rat PDE4D	5′-GGTGGGCTTCATAGACTACAT-3′
	5′-TGGCTTTCCTCTTCTGCTACA-3′
Rat 18S	5′-GGACCAGAGGCAAAGCATTTGCC-3′
	5′-TCAATCTCGGGTGGCTGAACGC-3′

Reverse-Transcription-Polymerase Chain Reaction Amplification of mRNA Encoding VSMC PDE3 and PDE4 Variants. Levels of RNA encoding PDE3A, PDE3B, PDE4D, or 18S RNAs were quantified as we described previously (Liu and Maurice, 1998; Liu et al., 2000). Briefly, RNA was isolated and purified from arterial tissue by TRIzol (Invitrogen). For each experiment, a constant amount of RNA (10 μg) was reverse-transcribed using random hexamers (NNN NNN) (Cortec, Kingston, ON, Canada) to prime the reverse transcription in a total volume of 100 μl with Superscript II reverse transcriptase (Invitrogen). Amplification reactions (PCR) for 18S RNA were performed using *Taq* DNA polymerase (Invitrogen) and several volumes (1–10 μl) of the first strand reaction. Differences in amounts of 18S RNA amplified from control or treated samples (usually $<$10%) were used to correct the volume of first-strand reaction product used in the amplification reactions for the PDE RNAs. Optimal conditions for PCR were determined previously and involved a 1-min denaturing step at 95°C, followed by 1 min each of annealing at 55–58°C and extension at 72°C, with gene-specific oligonucleotide primers, respectively (Table 1). PCR products generated by these reactions were separated by electrophoresis on 1% agarose gels, visualized with ethidium bromide, digitally photographed and the images transferred to an IBM personal computer. Relative amounts of PDE3A, PDE3B, and PDE4D RNA amplified in each reaction were determined by scanning densitometry using Corel Photo-Paint 8.0 software (Corel Corporation, Ottawa, ON, Canada) per manufacturer's recommendation and described previously (Liu and Maurice, 1998, 1999).

Immunoblotting of Vascular Tissue Lysates. Equivalent amounts of protein from samples of rat aorta and femoral artery tissues, as determined by the BCA protein assay system from Pierce (see above), were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (Bio-Rad), and incubated with a blocking solution containing 20 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween 20 supplemented with 5% powdered nonfat milk for 1 h. PDE3A, PDE3B, PDE4D, and β-actin were each detected by incubation of nitrocellulose membranes with PDE3-, PDE4D-, or β-actin–selective antisera and appropriate secondary horseradish peroxidase-conjugated antisera by chemical luminescence as described previously (Liu and Maurice, 1998). For our experiments, three PDE3-selective antisera, one PDE4D-selective

antiserum, and one β-actin–selective antiserum were used. For PDE3, a rabbit polyclonal antiserum generated against a murine PDE3B carboxyl-terminal fragment (1:1,000 dilution, generously provided by Dr. J. Beavo, University of Washington), and two commercially available PDE3 antisera (1:1000 dilutions; Santa Cruz Biotechnology, Santa Cruz, CA) were used. PDE4D was detected using a 1:4,000-fold dilution of a PDE4D-selective monoclonal antiserum generously provided by ICOS Corporation (Bothell, WA). The β-actin–selective antiserum (Sigma-Aldrich, 1:10,000) allowed detection of this protein. Immunoreactive proteins, detected by chemical luminescence, were quantified by scanning densitometry using Corel Photo-Paint 8.0 software as per manufacturer's recommendations and described previously (Liu and Maurice, 1998). Amounts of PDE3A, PDE3B or PDE4D3 were normalized to amounts of β-actin present in these same tissue samples before sample comparisons.

Inhibition of Contraction Protocol. After pluronic gel-mediated administration of dbcAMP (1 mM) or vehicle (saline) to rat femoral arteries, rings of these arteries were generated (3–4 mm) and attached to a multimyograph (model 610M; Danish Myo Technology, Aarhus, Denmark) and equilibrated with a 5% CO_2/95% O_2-saturated Krebs buffer (118 mM NaCl, 4.74 mM KCl, 1.18 mM $MgSO_4 \cdot 7H_2O$, 1.25 mM $CaCl_2 \cdot 2H_2O$, 1.18 mM KH_2PO_4, 24.9 mM $NaHCO_3$, and 10 mM dextrose). After application of a tension equivalent to 0.5 g to each ring, these were subject to three to four rounds of KCl-mediated contractions over a period of 1 h. Once stable KCl-induced contractions were achieved, an ascending concentration-response curve of phenylephrine-induced contractions was generated for each ring. The impact of dbcAMP-treatment on cAMP-mediated relaxations of femoral artery rings was then determined by subjecting reequilibrated rings to a sub-maximal phenylephrine-induced contraction (E_{70}) after a prior 1-min challenge with forskolin (10 or 100 nM), isoproterenol (1–10 nM), or zardaverine (30 or 300 nM) or combinations of these agents. This abbreviated protocol, rather than one that would have allowed full concentration-response curves for each agent to have been determined for each ring, was adopted because the latter would require a minimum of 5 h, a period of time significantly longer than the half-life of PDE4D proteins in VSMC (Liu et al., 2000). Tension development was monitored and analyzed using the myodaq acquisition and myodata systems (Danish Myo Technology), expressed in units of milliNewtons, and presented as percentages after normalization to control values.

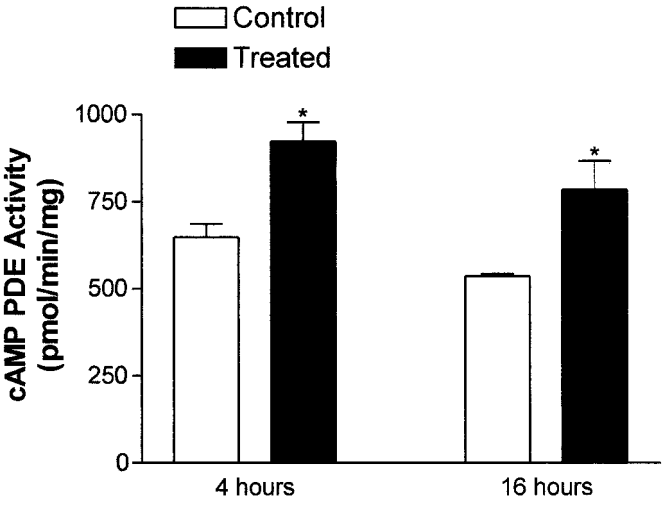


Fig. 2. Effect of local administration of dbcAMP on rat femoral artery cAMP PDE activity. A 500-μl volume of a 20% (w/v) solution of pluronic gel (control) or this volume of pluronic gel supplemented with 1 mM dbcAMP (treated) was applied perivascularly to the two femoral arteries of an anesthetized rat and allowed to polymerize (1–2 min). Rats were allowed to recover and kept in clean cages for either 4 or 16 h. At the end of these periods, animals were euthanized and the femoral arteries were removed, cleaned of adherent fat and connective tissue, and homogenized in a lysis buffer (see *Materials and Methods*). cAMP PDE activity in lysates of treated or contra-lateral control femoral arteries were determined as described under *Materials and Methods* and expressed as mean ± S.E.M. of seven sets of femoral arteries, performed in triplicate (4-h time point), or two experiments performed in triplicate (16-h time point). ⋆, $P < 0.05$ compared with activity in lysates of femoral arteries treated with pluronic gel alone.

Statistical Analysis. Data are presented as means ± S.E.M. of n independent experiments (as indicated). Within each experiment, values are means of three individual determinations for each experimental condition. Statistically significant differences were deter-

TABLE 2
The effect of systemic administration of dbcAMP on cilostamide-, Ro 20-1724- and IBMX-inhibited cAMP PDE activity in the rat aorta and femoral artery

	Control Activity[a]	Inhibited Activity[b]	Treated Activity (15 mg/kg dbcAMP)[a]	Inhibited Activity[b]	Activity Increase[c]
	pmol/min/mg				%
Aortas ($n = 6$)					
Basal	1130.0 ± 101.4		1628.3 ± 100.0		47 ± 9
Cilostamide-inhibited	607.3 ± 56.1	522.7 ± 55.5 [46%]	842.0 ± 116.0	786.3 ± 40.8* [48%]	60 ± 20
Ro 20-1724-inhibited	689.5 ± 60.1	440.5 ± 60.5 [39%]	967.3 ± 23.8	661.0 ± 93.6* [41%]	53 ± 15
IBMX-inhibited	57.5 ± 7.3	1072.5 ± 94.9 [95%]	90.2 ± 19.6	1538.2 ± 81.9* [94%]	47 ± 9
Femoral Arteries ($n = 3$)					
Basal	1331.3 ± 51.2		1727.0 ± 98.7		30 ± 8
Cilostamide-inhibited	655.7 ± 52.7	675.7 ± 20.0 [51%]	801.7 ± 50.3	925.3 ± 68.7* [54%]	37 ± 8
Ro 20-1724-inhibited	885.7 ± 63.5	445.7 ± 15.9 [33%]	1042.0 ± 60.5	685.0 ± 40.4* [40%]	55 ± 13
IBMX-inhibited	66.0 ± 7.0	1265.3 ± 55.7 [95%]	59.7 ± 9.9	1667.3 ± 101.2* [97%]	31 ± 7

cAMP PDE activities are expressed as:
[a] Mean ± S.E.M. for aorta (six experiments carried out in triplicate) and femoral arteries (three experiments carried out in triplicate) in the presence or absence of a PDE3 inhibitor (cilostamide, 1 μM), a PDE4 inhibitor (Ro 20-1724, 10 μM), or a broadly selective PDE inhibitor (IBMX, 500 μM).
[b] Mean ± S.E.M of amount of cAMP PDE activity inhibited by the selective PDE inhibiting agents, or the percentage that this activity represents of the total cAMP PDE (square brackets).
[c] Increases in total cAMP PDE activity or of the activities inhibited by cilostamide, Ro 20-1724, or IBMX in the aorta or femoral arteries treated with 15 mg/kg dbcAMP supplemented with 0.01 mg/kg IBMX compared with rats injected with IBMX alone.
* $P < 0.05$ between treated and control inhibited activities.

mined using the unpaired (systemic injection experiments) or paired (Pluronic gel experiments) Student's t test method. A value of $p < 0.05$ was considered statistically significant.

Results

Impact of Systemic Administration of cAMP-Elevating Agents on Rat Aortic and Femoral Artery VSMC cAMP PDE. Previous published work by our group (Liu and Maurice, 1998; Palmer and Maurice, 2000; Dunkerley et al., 2002), and results shown here (Fig. 1), demonstrate that the vast majority (>90%) of cells in rat aorta, or rat femoral artery, once cleaned of adherent connective tissue and fat, are medial VSMC. For this reason, samples of rat aorta or femoral artery isolated and processed for our studies were defined as aortic VSMC or femoral artery VSMC, respectively. Using a strategy of selective cAMP-PDE inhibition, aortic and femoral artery VSMC were shown to express predominantly PDE3 and PDE4 activities (Table 2). Thus, whereas the PDE3-selective inhibitor cilostamide (1 μM) reduced aortic or femoral artery VSMC cAMP PDE activity by about 50%, the PDE4-selective inhibitor Ro 20-1724 (10 μM) caused approximately 35% inhibition of cAMP hydrolysis in each of these vascular tissues (Table 2). Also, in other studies (Rose et al., 1997; Liu and Maurice, 1998), cilostamide and Ro 20-1724 inhibited rat aortic VSMC cAMP PDE activity by about 85%, a value consistent with their individual effects. Also, when a dual PDE3 and PDE4 inhibitor, zardaverine, was used, a similar extent of inhibition was obtained (not shown). The broad-spectrum PDE inhibitor IBMX inhibited slightly more cAMP PDE activity (~95%).

Administration of dibutyryl cAMP (dbcAMP), or forskolin, increased aortic and femoral artery VSMC cAMP PDE activity in the rat. Thus, intraperitoneal injections of dbcAMP (15 mg/kg) increased rat aortic and femoral artery VSMC cAMP PDE activities by 47 ± 9 and 30 ± 8%, respectively (Table 2). Forskolin (1 mg/kg), when similarly injected, increased aortic and femoral artery VSMC cAMP PDE activities by 56 ± 7 and 55 ± 7%, respectively. Based on the effects of cilostamide or Ro 20-1724, dbcAMP increased aortic VSMC PDE3 and PDE4 activities by 60 ± 20 and 53 ± 15%, respectively, and femoral artery VSMC PDE3 and PDE4 activities by 37 ± 8 and 55 ± 13%, respectively (Table 2). PDE3 and PDE4 activities of aortic or femoral artery VSMC treated with only 0.01 mg/ml of IBMX, but not the dbcAMP or forskolin, were not different from values obtained from these blood vessels isolated from naive rats (not shown).

Impact of Local Delivery of cAMP-Elevating Agents on Femoral Artery VSMC PDE3 and PDE4 Activities. To more directly test the effects of cAMP-elevating agents on femoral artery VSMC PDE3 and PDE4 activities, without the

TABLE 3

The effect of local administration of dbcAMP on cilostamide-, Ro 20-1724- and IBMX-inhibited cAMP PDE activity in the rat femoral artery

Data in the first section are expressed as described in Table 2. Data in the other sections depict the influence of coapplication of actinomycin D or cycloheximide, respectively.

	Control Activity[a]	Inhibited Activity[b]	Treated Activity[a]	Inhibited Activity[b]	Activity Increase[c]
		pmol/min/mg			%
	No Addition		1 mM dbcAMP		
Effect of dbcAMP Alone (n = 4)					
Basal	663.9 ± 31.1		888.2 ± 47.9		35 ± 11
Cilostamide-inhibited	294.9 ± 17.4	369.0 ± 23.4 [55%]	345.1 ± 28.5	543.2 ± 24.7* [61%]	51 ± 18
Ro 20-1724-inhibited	511.0 ± 19.2	152.9 ± 15.5 [23%]	661.8 ± 52.0	226.5 ± 2.3* [26%]	53 ± 16
IBMX-inhibited	33.3 ± 6.4	630.7 ± 32.3 [95%]	37.0 ± 3.4	851.2 ± 54.1* [96%]	35 ± 10
	4 mM Actinomycin D		4 mM Actinomycin D + 1 mM dbcAMP		
Effect of Actinomycin D Addition (n = 4)					
Basal	596.1 ± 38.2		692.7 ± 30.4		18 ± 11
Cilostamide-inhibited	271.0 ± 12.4	325.1 ± 27.2 [55%]	307.3 ± 12.1	385.4 ± 19.4 [56%]	22 ± 14
Ro 20-1724-inhibited	491.9 ± 24.1	104.2 ± 14.9 [17%]	565.8 ± 24.2	126.9 ± 7.9 [22%]	30 ± 19
IBMX-inhibited	22.7 ± 2.4	573.5 ± 36.2 [96%]	23.5 ± 2.2	669.2 ± 31.1 [97%]	18 ± 11
	100 mM Cycloheximide		100 mM Cycloheximide + 1 mM dbcAMP		
Effect of Cycloheximide Addition (n = 4)					
Basal	750.5 ± 29.2		800.4 ± 48.3		7 ± 7%
Cilostamide-inhibited	405.1 ± 18.4	345.5 ± 20.3 [46%]	426.9 ± 25.7	373.5 ± 38.6 [47%]	9 ± 11%
Ro 20-1724-inhibited	597.7 ± 25.3	152.9 ± 16.6 [20%]	618.3 ± 40.3	182.3 ± 12.9 [23%]	24 ± 16%
IBMX-inhibited	37.3 ± 3.4	713.3 ± 29.6 [95%]	37.5 ± 4.6	762.0 ± 46.1 [95%]	7 ± 6%

* $P < 0.05$ between treated and control inhibited activities.

potential complications intrinsic to systemic administration of such drugs [increased heart rate, vasorelaxation (Movsesian, 1999)], a strategy of local drug administration using a pluronic gel-based approach was adopted (Indolfi et al., 1997; Fig. 1). Our data from these experiments are consistent with dbcAMP, or forskolin, acting locally to increase PDE3 and PDE4 activities in femoral artery VSMC. Thus, after 4 h of dbcAMP (1 mM) or forskolin (100 μM) administration, femoral artery VSMC cAMP PDE activity was elevated 45 $\pm$ 11% (n = 7) or 40 $\pm$ 13% (n = 6), respectively. Inclusion of IBMX with cAMP-elevating agents had no impact on the cAMP PDE activity increases caused by the former (data not shown). Because our previous work with cultured rat and human VSMC (Rose et al., 1997; Liu and Maurice, 1998; Palmer and Maurice, 2000) had showed that cAMP-elevating agents increased cAMP PDE activities to a greater extent after 16 h than after 4 h, we also determined the impact of a 16-h dbcAMP treatment on femoral artery VSMC cAMP PDE activity. Because no significant difference between the increases at 4 and 16 h were detected (Fig. 2), all subsequent experiments were carried out using the 4-h time point. As observed when dbcAMP was administered systemically, locally applied dbcAMP also increased each PDE3 and PDE4 activities in femoral artery VSMC (Table 3).

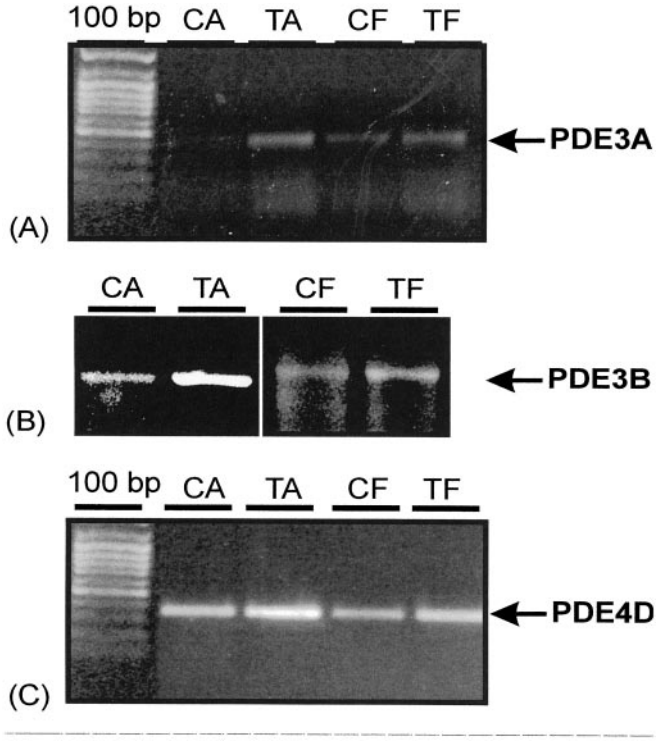


Fig. 3. Effects of systemic injections of forskolin on levels of PDE3 or PDE4 mRNA in rat aortic or femoral artery VSMC. Rats were subjected to five hourly injections of 1 mg/kg forskolin supplemented with 0.01 mg/ml IBMX, or 0.01 mg/ml of IBMX alone (see *Materials and Methods*). One hour after the final injection (6 h total time), rats were euthanized and their aortae and femoral arteries were removed, cleaned of adherent fat and connective tissue and homogenized in TRIzol reagent (about 1 ml of TRIzol per gram of tissue). Total RNA (10 μg) purified in this fashion was reverse transcribed using SuperScript Moloney murine leukemia virus reverse transcriptase and random hexanucleotide oligonucleotide primers. Amplification was routinely carried out using the Moloney murine leukemia virus-catalyzed first strand reaction, *Taq*DNA polymerase, and 20 pmol each of gene-selective sense and antisense oligonucleotide primer pairs (Table 1), as described previously (Liu and Maurice, 1998; see *Materials and Methods*). Results of representative amplification reactions of PDE3A (A), PDE3B (B), and PDE4D (C) in control or treated rat aorta (CA, TA), or femoral artery (CF, TF), are shown. To ensure that identical amounts of first-strand reaction volumes were used to amplify PDE3A, PDE3B, or PDE4D from control or treated samples, and as such that any differences between these would be obviated, volumes of first strand used were determined based on the amounts of 18S amplified from these samples. Similar results were obtained in several reverse transcription-PCR reactions using aortic or femoral artery-derived RNA isolated from rats in two independent experiments.

Effect of cAMP-Elevating Agents on Levels of PDE3 and PDE4 Expressed in Aortic and Femoral Artery VSMC. In work published previously, our laboratory has shown that levels of specific PDE3 and PDE4 variants were increased after incubation of rat, or human, aortic VSMC with cAMP-elevating agents (Liu and Maurice, 1998; Palmer and Maurice, 2000). In this earlier work, PDE3B levels were increased in a time- and concentration-dependent manner in both rat and human VSMC, whereas PDE3A levels were either unaffected [cultured human aortic VSMC (Palmer and Maurice, 2000)] or regulated in a biphasic manner [cultured rat aortic VSMC (Liu and Maurice, 1998)]. In cultured rat aortic VSMC, cAMP-elevating agents also caused a PKA-dependent phosphorylation and activation of the two PDE4D "long-forms" (i.e., PDE4D3 and PDE4D5) and a marked increase in the levels of two PDE4D "short forms" (i.e., PDE4D1 and PDE4D2) (Liu et al., 2000). In cultured rat aortic VSMC, cAMP-elevating agents did not increase levels of the PDE4D "long forms" (Liu et al., 2000). In marked contrast, data presented here showed that cAMP-elevating agents increased different PDE3 and PDE4 variants when administered in vivo. Thus, levels of mRNA encoding PDE3A and PDE3B were both elevated in rat aortic VSMC and femoral artery VSMC after injections with cAMP-elevating agents (Fig. 3, A and B). Consistent with this, immunoblot analysis revealed that levels of each PDE3A and PDE3B were increased in femoral artery VSMC after local application of dbcAMP to this artery (Fig. 4, A and E). Similarly, injection of forskolin or local application of dbcAMP each increased PDE4D mRNA (Fig. 3C) and PDE4D3 protein amounts (Fig. 4, C and E). In the six experiments in which this was measured, compared with levels in contralateral control femoral arteries, dbcAMP increased femoral artery VSMC PDE3A, PDE3B, and PDE4D3 protein levels by 302 $\pm$ 97, 106 $\pm$ 23, and 159 $\pm$ 26% (n = 6), respectively, when normalized to levels of β-actin. In marked contrast to results obtained in cultured rat aortic VSMC, no evidence of the "short-forms" of PDE4D (i.e., PDE4D1 and PDE4D2) was observed in the in vivo context, whether the cAMP-elevating agents were administered for 4 or 16 h (Fig. 4, C and F).

Role of de Novo RNA and Protein Synthesis in cAMP-Elevating Agent-Induced Increases in PDE3 and PDE4 Activities and Levels in Vivo. In our previous work in cultured rat and human aortic VSMC, we identified a role for de novo RNA and protein synthesis in mediating cAMP-induced increases in cAMP PDE activity (Rose et al., 1997; Liu and Maurice, 1999; Palmer and Maurice, 2000). To determine whether each was required for cAMP-induced increases in cAMP PDE activity in vivo, the effects of an inhibitor of transcription (actinomycin D, 4 μM) or translation (cycloheximide, 100 μM) on dbcAMP-mediated increases in cAMP-PDE activity were measured. Our data is consistent with an important role for de novo RNA and protein synthesis in mediating the effects of dbcAMP on femoral artery

VSMC cAMP PDE activity. Thus, while administration of dbcAMP alone markedly increased femoral artery VSMC cAMP PDE activity, addition of either actinomycin D or cycloheximide to the dbcAMP-containing pluronic gel, markedly attenuated these increases (Table 3). Similar results were obtained when a paradigm in which each femoral artery received dbcAMP and only one received the inhibitor was used (not shown). Because neither actinomycin D nor cycloheximide altered basal femoral artery cAMP PDE activity, but each inhibited the dbcAMP-induced increase in this activity, we conclude that de novo RNA and protein synthesis were required for dbcAMP-induced increases in femoral artery VSMC cAMP PDE activity in vivo. When several experiments were compared, actinomycin D was shown to inhibit dbcAMP-induced increases in femoral artery VSMC PDE3A, PDE3B, and PDE4D3 levels by 66, 25, and 63%, respectively (Fig. 5, A, B, and D), whereas cycloheximide was shown to inhibit these effects of dbcAMP on PDE3A, PDE3B, and PDE4D3 levels by 78, 65, and 81%, respectively (Fig. 5, E, F, and H). The observation that cycloheximide was slightly more effective in this setting may indicate that under these conditions, utilization of endogenous RNA in VSMC in vivo, especially for PDE3B, was more important than its synthesis.

Impact of dbcAMP-Treatment of Rat Femoral Arteries on Zardaverine-Induced Relaxation. In previous work, prolonged incubations of cultured rat aortic VSMC with cAMP-elevating agents resulted in a marked desensitization of these cells to further effects of activators of adenylyl cyclase (Rose et al., 1997). A role for increased cAMP PDE activity in this effect was revealed when it was partially reversed by IBMX (Rose et al., 1997). By comparing the impact of cAMP PDE inhibition on the ex vivo relaxant effects of forskolin, data obtained in these studies are consistent with a similarly increased role for PDE3 and PDE4 in dbcAMP-treated femoral arteries compared with control arteries. Thus, although the percentage forskolin-induced inhibition of contraction of control and dbcAMP-treated femoral artery rings was were not different, 11 ± 4 versus 11 ± 8% (control versus treated, 10 nM forskolin) and 49 ± 19 versus 55 ± 12% (control versus treated, 100 nM forskolin), results obtained when zardaverine, a dual-specificity PDE3/PDE4 inhibitor, was included with forskolin were different. Thus, zardaverine potentiated the forskolin-mediated relaxation of dbcAMP-treated femoral arteries to a larger extent than in the contra-lateral untreated arteries. Indeed, in the three independent experiments in which this was measured, inhibition of phenylephrine-induced contractions by the com-

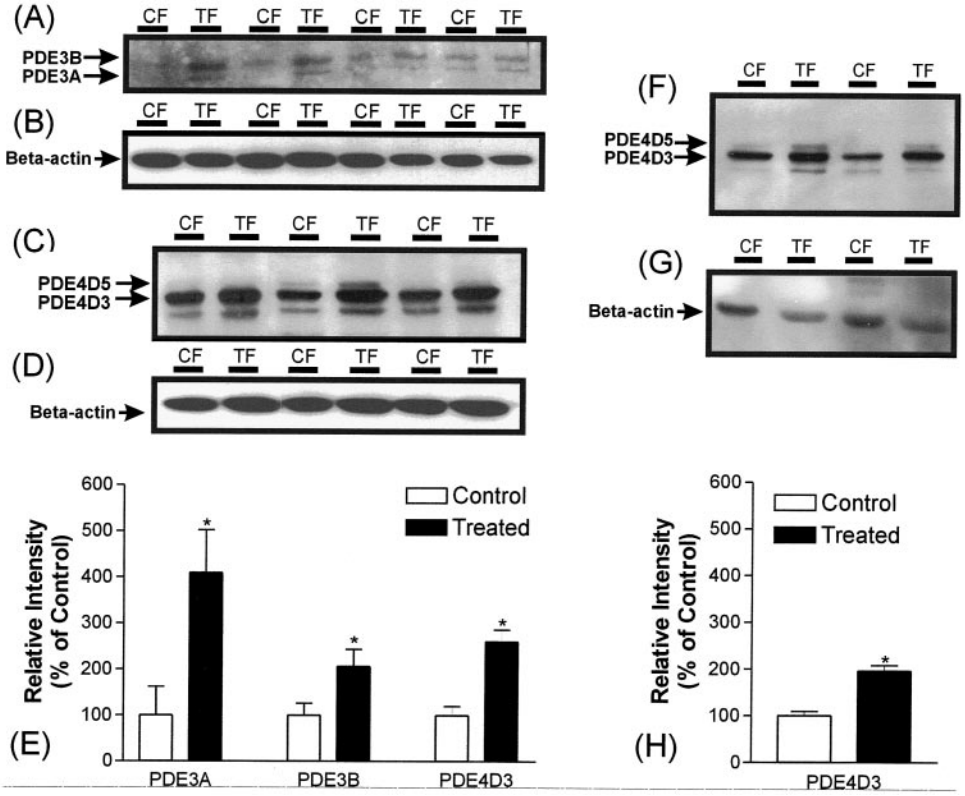


Fig. 4. Impact of local application of dbcAMP on femoral artery PDE3 and PDE4 levels. A 500-μl volume of a 20% (w/v) solution of pluronic gel (control) or this volume of pluronic gel supplemented with 1 mM dbcAMP (treated) was applied perivascularly to femoral arteries of anesthetized rats and allowed to polymerize (1–2 min). Rats were allowed to recover and kept in clean cages for either 4 or 16 h after which they were euthanized and their femoral arteries removed, cleaned of adherent fat and connective tissue, and homogenized in a lysis buffer (see *Materials and Methods*). Homogenates of rat femoral arteries (10 μg of protein) were resolved electrophoretically, transferred to nitrocellulose and blotted with PDE3-, PDE4D-, or β-actin–selective antisera. Top (A-D, F-G), representative immunoblots of the analysis of several rat femoral arteries incubated with pluronic gel (control, CF) or pluronic gel supplemented with 1 mM dbcAMP (treated, TF) for 4 h (A-D) or 16 h (F-G). Also shown are histograms in which the mean ± S.E.M. of the increases in PDE3A (E), PDE3B (E), or PDE4D3 (E and H) were quantified. Values are from six experiments in which levels of PDE3A, PDE3B, and PDE4D3 in rat femoral arteries were compared after 4-h incubations with dbcAMP (E) or from two experiments after a 16-h treatment (H), after normalization to levels of β-actin in these samples. ⋆, $P < 0.05$ differences in immunoreactivity detected between treated and control femoral arteries.

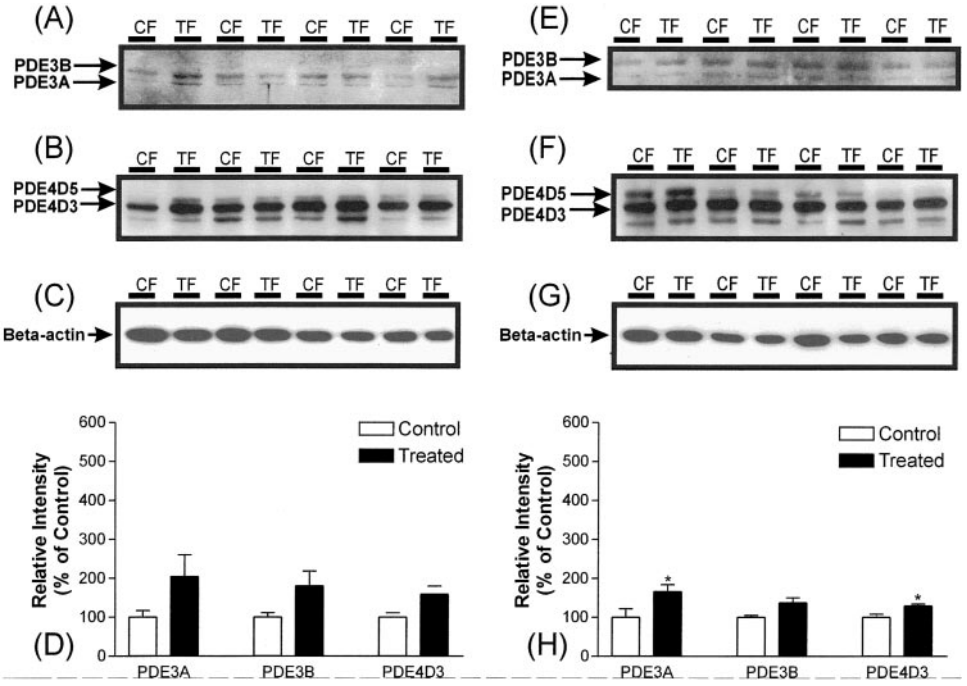


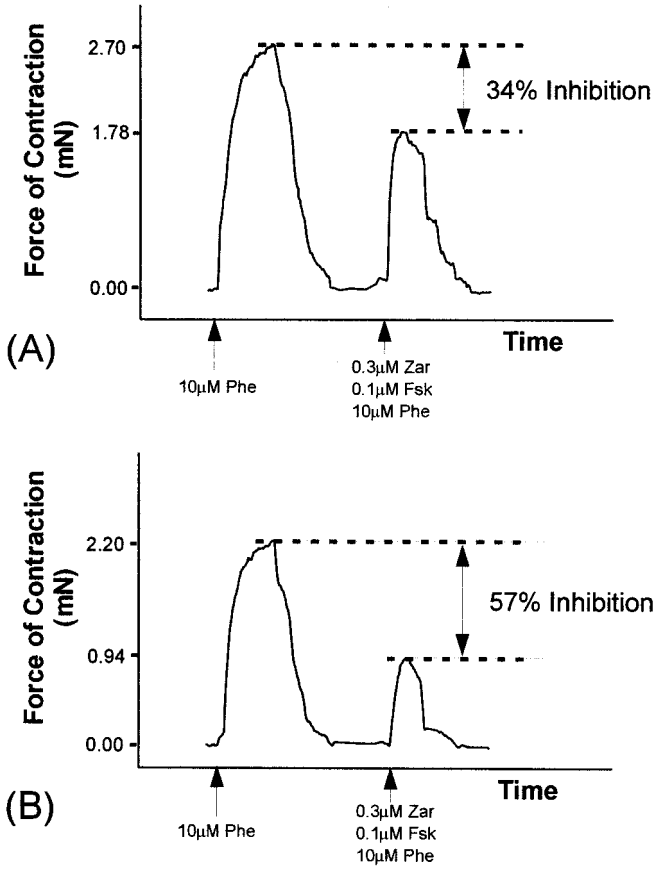
Fig. 5. Impact of actinomycin D or cycloheximide on dbcAMP-induced increases in femoral artery PDE3 and PDE4. A 500-μl volume of a 20% (w/v) solution of pluronic gel supplemented with 4 μM actinomycin D or 100 μM cycloheximide in the presence or absence of 1 mM dbcAMP was applied perivascularly to femoral arteries of anesthetized rats and allowed to polymerize (1–2 min). Rats were allowed to recover and kept in clean cages for 4 h, after which they were euthanized and their femoral arteries removed, cleaned of adherent fat and connective tissue, and homogenized in a lysis buffer (see *Materials and Methods*). Homogenates of rat femoral arteries (10 μg of protein) were resolved electrophoretically, transferred to nitrocellulose, and blotted with PDE3-, PDE4D-, or β-actin–selective antisera. Top (A-C, E-G), representative immunoblots of the analysis of several rat femoral arteries incubated with pluronic gel containing actinomycin D, cycloheximide (control, CF), or pluronic gel supplemented with 1 mM dbcAMP and either actinomycin D (A-C) or cycloheximide (E-G) (treated, TF) for 4 h. Also shown are histograms representing the mean ± S.E.M. of the impact of actinomycin D (D) or cycloheximide (H) on 1 mM dbcAMP-induced increases in PDE3A, PDE3B, and PDE4D3 levels from four separate experiments. ⋆, $P < 0.05$ comparison of immunoreactivity detected between treated and untreated femoral arteries after normalization for levels of β-actin in these samples.

bined actions of forskolin (100 nM) and zardaverine (300 nM) were on average 35% larger in the dbcAMP-treated femoral arteries (Fig. 6) than in contralateral control femoral artery-derived rings. Because the contractions of control and db-cAMP-treated femoral arteries in response to phenylephrine or inhibition of contraction to in response to phenylephrine by either forskolin (above) or isoproterenol (not shown) were unaltered by dbcAMP treatment, the increased effectiveness of zardaverine in treated arteries is consistent with an increased role for PDE3 and PDE4 in these arteries, compared with untreated control arteries (Fig. 6).

Discussion

In this study, the effects of in vivo administration of cAMP-elevating agents on rat arterial VSMC PDE3 and PDE4 activities, as well as the levels of these enzymes, were determined for the first time. Before this report, no studies directly addressed this issue in any blood vessel in any species. In one earlier report, Palmer and Doukas (1984) showed that systemic injections of dbcAMP in rats increase cAMP PDE activity in heart, although no attempt was made to distinguish the cAMP PDEs involved in these increases or the mechanism by which they were elevated. More recently, Kostic et al. (1997) reported that an intramuscular injection of 7-oxo-prostacyclin to rats had a complex effect on cardiac cAMP PDE levels, although a role for cAMP in these effects was discounted. The data presented in this report show that cAMP-elevating agents increase cAMP PDE activity in aortic VSMC and femoral artery VSMC, and determined the cAMP PDE variants involved. We have identified PDE3 and PDE4 as the dominant cAMP PDEs in aortic VSMC and femoral artery VSMC and demonstrated that each was increased after administration of cAMP-elevating agents. At a mechanistic level, our studies reveal cAMP-elevating agent-induced increases in levels of PDE3A, PDE3B, and PDE4D3 in both aortic VSMC and femoral artery VSMC. Moreover, consistent with a role for de novo synthesis of these enzymes in arterial VSMC in vivo, addition of actinomycin D, or cycloheximide, attenuated a significant proportion of the increases in PDE3 and PDE4 activities and reduced the dbcAMP-induced increases in levels of each PDE3A, PDE3B, and PDE4D3. Because both systemic and local delivery of dbcAMP, or forskolin, increased femoral artery VSMC PDE3 and PDE4 activities, and that local inhibition of transcription or translation attenuated these effects, we conclude that cAMP-elevating agents acted locally at the VSMC. In an attempt to determine whether increases in femoral artery VSMC cAMP PDE activity affected these arteries at a functional level, we compared the effect of zardaverine, a dual PDE3/PDE4 inhibitor, on forskolin-induced inhibition of contraction in db-cAMP-treated or untreated femoral arteries ex vivo. Our data indicated that simultaneous inhibition of both PDE3 and PDE4, using zardaverine, had a more marked effect in treated femoral arteries than in control arteries. Because the impact of zardaverine was increased in treated arteries, but the contractions of these vessels in response to phenyleph-

rine and their relaxation in response to forskolin or isoproterenol were unaltered by dbcAMP-treatment, we conclude that our treatment protocol selectively altered the contribution of cAMP PDE activity in these vessels. This effect was similar to that reported previously by us for cultured rat aortic VSMC (Rose et al., 1997) and by others for myometrial muscle (Mehats et al., 2001). Because cAMP levels in cells are determined based on the coordinated activities of adenylyl cyclases and cAMP PDE (Houslay, 1998), the increased sensitivity to zardaverine observed in these experiments is consistent with the notion that prolonged treatments with cAMP-elevating agents altered cAMP fluxes in these cells to emphasize a role for degradation. Further studies designed to directly assess the impact of such treatments on these blood vessels in situ will be required to more directly assess this hypothesis.



The effect of dbcAMP-induced increases in PDE3 and PDE4 on the inhibition of phenylephrine-induced contraction in the femoral artery

	Control *% Inhibition of Contraction*	Treated *% Inhibition of Contraction*	*% Increase in Inhibition of Contraction in dbcAMP-Treated Femoral Arteries*
	a	b	c
	34	57	68
	47	57	21
	65	75	15
Mean	49	63*	35
SEM	9	6	16

*P<0.05 between treated and control % inhibtion of contraction

Fig. 6. Functional impact of dbcAMP-induced increases in femoral artery PDE3 and PDE4 activity. A 500-μl volume of a 20% (w/v) solution of pluronic gel (control) or this volume of pluronic gel supplemented with 1 mM dbcAMP (treated) was applied perivascularly to femoral arteries of anesthetized rats and allowed to polymerize (1–2 min). Rats were allowed to recover and kept in clean cages for 4 h after which they were euthanized and their femoral arteries removed and cleaned of adherent fat and connective tissue (see *Materials and Methods*). Using an approach that allowed drug-mediated inhibition of phenylephrine-induced contractions to be measured (Maurice and Haslam, 1990), the impact of a 4-h incubation of femoral arteries with either pluronic gel (A) or pluronic gel supplemented with 1 mM dbcAMP (B) on forskolin and zardaverine-induced inhibition of contraction were measured. ⋆, $P < 0.05$ comparison between the impact of forskolin and zardaverine in control and dbcAMP-treated femoral arteries.

Although our studies indicate that in vivo administration of cAMP-elevating agents increased PDE3 and PDE4 activities and levels in rat aortic and femoral artery VSMC in a manner similar to that described previously by us in cultured rat or human aortic VSMC (Rose et al., 1997; Palmer and Maurice, 2000), some interesting and potentially important differences were observed. First, our finding that PDE3 and PDE4 activities accounted for 50 and 35% of cAMP PDE activity in rat arteries, respectively, was different from our earlier reports, in which we showed that PDE3 and PDE4 activities represented 20 and 65% of cAMP PDE activity, respectively, in cultured rat aortic VSMC (Rose et al., 1997; Liu and Maurice, 1999; Liu et al., 2000). Recently, we showed that these differences were caused by a marked decrease in PDE3A upon culturing of these cells and that a similar reduction occurred when VSMC were activated in response to vascular injury in vivo (Dunkerley et al., 2002). Second, although the results of our study provide evidence that cAMP-elevating agents increased aortic and femoral artery VSMC PDE3 and PDE4 activities, the overall magnitude of the increases in vivo were significantly smaller than those achieved in cultured rat or human aortic VSMC (Rose et al., 1997; Liu et al., 2000). Indeed, irrespective of whether dbcAMP was administered systemically or locally, this agent increased cAMP PDE activity by about 50% in blood vessel in vivo, whereas similar treatments increased these activities by 100 to 300% in cultured rat aortic VSMC (Rose et al., 1997; Liu et al., 2000). Although numerous factors might have contributed to the different responses of in vivo and in vitro arterial VSMC to cAMP-elevating agents, we suggest that our finding that the variants of PDE3 and PDE4D regulated in vivo were different from those increased in vitro could perhaps be important. In this context, our data shows that administration of cAMP-elevating agents to aortic or femoral artery VSMC in vivo significantly increased levels of PDE4D3, but did not result in induction of expression of either PDE4D1 or PDE4D2. This effect is completely different from the large increases in PDE4D1 and PDE4D2 reported previously by us for cultured VSMC (Liu et al., 2000). Although we have not presently determined the molecular basis of the difference between the response of in vivo (contractile) and in vitro (synthetic) VSMC to cAMP-elevating agents, one interesting possibility could be that regulation of PDE4D expression by cAMP, and perhaps other factors, was dependent on the VSMC phenotype. Indeed, in combination with our previous studies (Dunkerley et al., 2002), these data support the notion that synthetic and contractile VSMC could differentially regulate their responses to prolonged challenges with cAMP-elevating agents.

Although much of our work was directed at PDE4D, because the three other PDE4 genes (*PDE4A*, *PDE4B*, and *PDE4C*) are also differentially processed to yield "long" and "short" forms, our findings are perhaps of more general importance, and may be relevant in cell types that express other PDE4 and undergo

similar phenotypic changes. For example, the recently described changes in PDE4 inhibitor sensitivity of myometrial smooth muscle that occurs at parturition (Mehats et al., 2001) may be related to changes in the PDE4B variants expressed in contractile and synthetic smooth muscle cell phenotypes.

In conclusion, we describe results obtained in experiments aimed at determining the influence of cAMP-elevating agents on arterial VSMC in vivo. Our data present a picture in which cAMP-elevating agents bring about significant increases in cAMP PDE activities and in which the modes of regulation seemed to be PDE family-dependent. Indeed, although PDE3A levels were unaffected in previous experiments in which cultured rat aortic VSMC were incubated with cAMP-elevating agents, significant increases in PDE3A were reported in vivo. Also, the increased levels of PDE4D3 and the absence of PDE4D1 and PDE4D2 induction in VSMC in vivo lend further support to the notion that contractile and synthetic VSMC regulate cAMP PDE activity very differently. Although the molecular basis for these differences will require further experimentation, we suggest that they may be of pharmacological and therapeutic importance, especially when conditions in which selective cAMP-dependent effects are desired in either synthetic, or contractile, VSMC phenotypes, such as inhibition of restenosis after balloon angioplasty.

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Address correspondence to: Dr. D. H. Maurice, PhD, Career Investigator, Heart and Stroke Foundation of Ontario, Associate Professor, Department of Pharmacology and Toxicology, Botterell Hall, A221, Queen's University at Kingston, Kingston, Ontario, Canada, K7L 3N6. E-mail: mauriced@post.queensu.ca