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Cyclic AMP-mediated regulation of vascular smooth muscle cell cyclic AMP phosphodiesterase activity

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1 Rat cultured aortic vascular smooth muscle cells (VSMC) express both cyclic GMP-inhibited cyclic AMP phosphodiesterase (PDE3) and Ro 20-1724-inhibited cyclic AMP phosphodiesterase (PDE4) activities. By utilizing either cilostamide, a PDE3-selective inhibitor, or Ro 20-1724, a PDE4-selective inhibitor, PDE3 and PDE4 activities were shown to account for 15% and 55% of total VSMC cyclic AMP phosphodiesterase (PDE) activity.

2 Treatment of VSMC with either forskolin or 8-bromo-cyclic AMP caused significant concentration- and time-dependent increases in total cellular cyclic AMP PDE activity. Using cilostamide or Ro 20-1724, we demonstrated that both PDE3 and PDE4 activities were increased following forskolin or 8-bromo-cyclic AMP treatment, with a relatively larger effect observed on PDE3 activity. The increase in cyclic AMP PDE activity induced by forskolin or 8-bromo-cyclic AMP was inhibited by actinomycin D or cycloheximide, demonstrating that new mRNA synthesis and protein synthesis were required. An analogue of forskolin which does not activate adenylyl cyclase (1,9-dideoxyforskolin) or an analogue of cyclic GMP (8-bromo-cyclic GMP) did not affect total cyclic AMP PDE activity.

3 Incubation of VSMC with 8-bromo-cyclic AMP for 16 h caused a marked rightward shift in the concentration-response curves for both isoprenaline- and forskolin-mediated activation of adenylyl cyclase. A role for up-regulated cyclic AMP PDE activity in this reduced potency is supported by our observation that cyclic AMP PDE inhibitors (IBMX, cilostamide or Ro 20-1724) partially normalized the effects of isoprenaline or forskolin in treated cells to those in untreated cells.

4 We conclude that VSMC cyclic AMP PDE activity is increased following long-term elevation of cyclic AMP and that increases in PDE3 and PDE4 activities account for more than 70% of this effect. Furthermore, we conclude that increases in cyclic AMP PDE activity contribute to the reduced potency of isoprenaline or forskolin in treated VSMC. These results have implications for long-term use of cyclic AMP PDE inhibitors as therapeutic agents.

Keywords: Cyclic nucleotide phosphodiesterase; cyclic AMP; vascular smooth muscle cells; heterologous desensitization

Introduction

A number of studies in vascular smooth muscle cells (VSMC) have correlated increases in adenosine 3′:5′-cyclic monophosphate (cyclic AMP) or guanosine 3′:5′-cyclic monophosphate (cyclic GMP) with relaxation (Lincoln, 1989; Murray, 1990). While the synthesis of cyclic AMP or cyclic GMP in cells is catalyzed by the adenylyl or guanylyl cyclase enzymes, respectively (McDonald & Murad, 1996; Sunahara *et al*., 1996), the metabolic inactivation of these cyclic nucleotides is catalyzed by cyclic nucleotide phosphodiesterases (PDE) (Beavo & Reifsnyder, 1990). PDE are subdivided into seven distinct families based on substrate selectivity, inhibitor sensitivity and molecular sequence (Beavo & Reifsnyder, 1990; Bolger *et al*., 1993; Manganiello *et al*., 1995).

In VSMC, a role for PDE3 and PDE4 families in regulating cyclic AMP breakdown has been demonstrated (Lugnier *et al*., 1986; Weishaar *et al*., 1986; Schoeffter *et al*., 1987; Maurice *et al*., 1991). Members of these two PDE families selectively hydrolyze cyclic AMP and several selective pharmacological inhibitors for each family are known (reviewed in Beavo & Reifsnyder, 1990; Bolger *et al*., 1993; Manganiello *et al*., 1995). Thus, inhibition of cyclic AMP breakdown by the selective inhibitors of either PDE3 or PDE4 increases VSMC cyclic AMP levels and relaxes vascular smooth muscle (Lugnier *et al*., 1986; Weishaar *et al*., 1986; Schoeffter *et al*., 1987; Maurice *et al*., 1991; Bolger *et al*., 1993; Manganiello *et al*., 1995).

There are several mechanisms by which cells can regulate cyclic AMP PDE activity. Within the PDE3 family, metabolism of cyclic AMP is inhibited by cyclic GMP (Grant & Colman, 1984; Manganiello *et al*., 1990). This effect of cyclic GMP on cyclic AMP metabolism can have important functional consequences in tissues in which PDE3 activity is an important component of the overall cyclic AMP hydrolytic activity (Maurice & Haslam, 1990a). The functional importance of PDE3 has been clearly demonstrated in blood platelets and in some vascular and cardiac muscle (Maurice & Haslam, 1990a,b; Maurice *et al*., 1991; Delpy *et al*., 1996). The activities of certain isozymes of PDE3 and PDE4 are also regulated via phosphorylation by protein kinases (Grant *et al*., 1988; Sette *et al*., 1994; Alvarez *et al*., 1995; Rahn *et al*., 1996; Sette & Conti, 1996).

Transcriptional regulation of members of the PDE3 and PDE4 families has been described. PDE3B expression is increased upon differentiation of 3T3-L1 fibroblasts to 3T3-L1 adipocytes (Elks *et al*., 1983). Transcriptional regulation of PDE4 activity has been studied in Sertoli cells and most recently in monocytes (Swinnen *et al*., 1989; Torphy *et al*., 1992; 1995; Verghese *et al*., 1995; Manning *et al*., 1996). Incubation of these cells for several hours with activators of adenylyl cyclase, or with cyclic AMP analogues, was shown to increase significantly PDE4 activity and up-regulate selected variants of PDE4. In Sertoli cells, prolonged elevation of intracellular cyclic AMP increased the expression of selected PDE4D isozymes (Swinnen *et al*., 1989), whereas in monocytes, these incubations increased mRNA for PDE4A and PDE4B but decreased mRNA for PDE4D (Torphy *et al*., 1992; 1995; Verghese *et al*., 1995; Manning *et al*., 1996). Although PDE3 activity is present in these cell types, no change in PDE3 activity was found in these studies.

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The aim of the present study was to determine if prolonged elevations in cyclic AMP could regulate the level of cyclic AMP PDE activity in VSMC, and to investigate the effect of this treatment on PDE3 and PDE4 activity. We incubated rat cultured aortic VSMC for 4, 8 or 16 h with either (i) forskolin, an activator of adenylyl cyclase, (ii) 1,9-dideoxyforskolin, a structural analogue of forskolin which does not activate adenylyl cyclase, (iii) 8-bromo-cyclic AMP or 8-bromo-cyclic GMP, lipophilic analogues of cyclic AMP and cyclic GMP, and then determined the cyclic AMP PDE activity in cell lysates. To determine the PDE3 and PDE4 activity in these cell lysates, the extent of inhibition of total cyclic AMP PDE activity caused by either a PDE3-selective inhibitor (cilostamide, 1 μM), a PDE4-selective inhibitor (Ro 20-1724, 10 μM), or a combination of these two agents was measured. In addition, to assess the possible functional significance of increased cyclic AMP PDE activity, cyclic AMP levels were measured in response to incubation with isoprenaline or forskolin, in control and treated cells.

Methods

Cell culture

Primary cultures of rat aortic VSMC were a generous gift from Dr S. Pang (Department of Anatomy and Cell Biology, Queen's University) following isolation from rat aortae as described previously (Pang & Venance, 1992). The identity of the VSMC was confirmed by α-actin staining. VSMC were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 8 mM HEPES buffer, 100 units ml^{-1} penicillin and 100 $\mu g\ ml^{-1}$ streptomycin at 37°C in a 95% air-5% CO_2 humidified atmosphere. Cells were passaged by washing once with Hanks' balanced salt solution (HBSS) (without Ca^{2+} or Mg^{2+}) treated with 1% trypsin-ethylenediamine tetraacetic acid (EDTA) for 2 min to detach cells and resuspended in growth medium. To maintain cell stocks, 75 cm^2 flasks were seeded with 10^6 cells and 20 ml of medium per flask. For all experiments, cells were used between passages 6 and 12.

Treatment of rat cultured aortic VSMC with pharmacological agents

In our studies of cyclic AMP PDE activity, 3×10^5 cells were seeded in 25 cm^2 flasks and the experiments initiated when the cells reached confluence (3–4 days). Culture medium was removed and replaced with 5.0 ml of fresh culture medium supplemented with either (i) forskolin (1.0–100 μM), (ii) 1,9-dideoxyforskolin (1.0–100 μM), (iii) 8-bromo-cyclic AMP (0.01–1 mM), (iv) 8-bromo-cyclic GMP (0.01–1 mM) or (v) vehicle (0.1% dimethylsulphoxide, (DMSO)). After 4, 8 or 16 h, the treated cells were washed once with 6 ml of HBSS (with Ca^{2+} and Mg^{2+}) and harvested in 1 ml of lysis buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 5 mM benzamidine, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μM leupeptin and 1% Triton X-100. Cells were removed from the flask by scraping. Cellular debris and unlyzed cells were removed by centrifugation at $10\,000 \times g$ for 5 min at 4°C. The $10\,000 \times g$ supernatants were transferred to microtubes and stored at 4°C until assayed for cyclic AMP PDE activity (see below).

In experiments which determined VSMC cyclic AMP and cyclic GMP levels, VSMC were seeded in 24 well plates in 0.5 ml culture medium at 5×10^4 cells ml^{-1}. At confluence, the cells were incubated overnight (16 h) with fresh culture medium supplemented with 20 $\mu Ci\ ml^{-1}$ [^{3}H]-hypoxanthine and either (i) forskolin (1–100 μM), (ii) 1,9-dideoxyforskolin (1–100 μM), (iii) 8-bromo-cyclic AMP (0.01–1 mM), (iv) 8-bromo-cyclic GMP (0.01–1 mM) or (v) drug vehicle (0.1% DMSO). As described previously, (Maurice *et al*., 1993), incubation of cultured VSMC with [^{3}H]-hypoxanthine allows homogeneous labelling of both the ATP and GTP metabolic pools. The presence of forskolin, or any other agent used in these studies to increase cellular cyclic AMP, did not alter the uptake of [^{3}H]-hypoxanthine by the VSMC, a parameter which is directly correlated to the specific activity of both the ATP and GTP metabolic pools in these cells (Maurice *et al*., 1993). Indeed, whether the VSMC were treated with drug or not, a 1% increase in cellular [^{3}H]-cyclic AMP is equivalent to 260 ± 38 pmol mg^{-1} protein (Maurice *et al*., 1993).

Following this drug treatment and metabolic ATP and GTP labelling, the VSMC were washed with HBSS (with Ca^{2+} and Mg^{2+}), covered with fresh HBSS (with Ca^{2+} and Mg^{2+}) and the temperature maintained at 37°C throughout each experiment. In these experiments, adenylyl cyclase was stimulated by addition of either isoprenaline (0.1 nM–10 μM) or forskolin (0.1–10 μM) to individual wells for 1 min. The reactions were terminated by addition of 0.5 ml of 10% trichloroacetic acid. Following addition of recovery markers, ([^{14}C]-cyclic AMP and [^{14}C]-cyclic GMP (1000 d.p.m. each)), the cyclic nucleotides were isolated and purified by column chromatography on alumina and Dowex 50 resin and quantitated by liquid scintillation as described previously (Maurice *et al*., 1993). The [^{3}H]-cyclic AMP and [^{3}H]-cyclic GMP present in individual wells of VSMC is expressed as a percentage of the total ^{3}H in each well.

Assay of cyclic AMP phosphodiesterase activity

Cyclic nucleotide phosphodiesterase activity was assayed by a modification of the method of Davis & Daly (1979). Reactions were carried out in a total volume of 100 μl containing 5 μmol Tris-HCl (pH 7.4), 0.5 μmol $MgCl_2$, 10 nmol ethylene glycol-bis-(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid (EGTA) and 0.1 nmol [^{3}H]-cyclic AMP containing 55 000 to 60 000 d.p.m. Following a preincubation period of 2 min at 30°C, a sample of VSMC homogenate (5 μg of protein) was added and the reaction allowed to proceed at 30°C for 30 min. The reaction was terminated by addition of 50 μl of 0.5 M ice-cold EDTA (pH 7.4). Recovery marker (0.1 ml of [^{14}C]-AMP, 1800 d.p.m.) and 0.3 ml of N-2-hydroxyethyl piperazine-N′-2-ethanesulphonic acid, (HEPES)-NaCl buffer (0.1 M NaCl, 0.1 M HEPES, pH 8.5) was added to each sample before purification of the product of the reaction, [^{3}H]-AMP. [^{3}H]-AMP and [^{14}C]-AMP were recovered by chromatography by use of a polyacrylamide-boronate gel column (Affi-gel 601 Bio-Rad, 1 ml bed volume). Samples were applied following prewashing of the columns with 8 ml of HEPES-NaCl buffer. After 4 additional washes of the columns with 2 ml of HEPES-NaCl and equilibration of the columns with 1 ml of 0.05 M Na-acetate (pH 4.8), the 5′ AMP was eluted with 4 ml of 0.05 M Na-acetate. The recovered [^{3}H]-AMP was quantified by liquid scintillation counting, corrected for recovery of [^{14}C]-AMP, normalized to the total protein used in the assay and the total activity expressed as pmol min^{-1} mg^{-1} protein.

Protein assays

The total protein concentration of each sample was determined by the BCA Protein Assay system from Pierce, according to the manufacturer's methodology with bovine serum albumin as the standard.

Statistical analysis

Data are presented as means ± s.e.mean of at least three independent experiments. Within each experiment, values were means from three individual determinations for each experimental condition. Statistical differences between cyclic AMP PDE activities or between cyclic AMP levels were determined by Student's t test for either paired or unpaired samples with $P < 0.05$ considered significant.

Materials

Tissue culture reagents (DMEM, calf serum, HEPES, penicillin/streptomycin, HBSS, trypsin-EDTA) were from GIBCO BRL (Ontario, Canada). Radioactive products were from NEN Life Science Products (Massachusetts, U.S.A.) ([^{3}H]-hypoxanthine, [^{14}C]-cyclic AMP, [^{3}H]-cyclic AMP, [^{3}H]-cyclic GMP), Amersham Life Science (Ontario, Canada). ([^{14}C]-cyclic AMP) or Moravek Biochemicals (California, U.S.A.) ([^{14}C]-cyclic GMP). Ro 20-1724 (4-[(3-butoxy-4-methoxyphenyl)methyl]-2-imidazolidinone) was from Calbiochem-Novachem Corporation (Ontario, Canada) and isobutyl methylxanthine (IBMX) was from the Aldrich Chemical Company (Ontario, Canada). Forskolin, 1,9-dideoxyforskolin, 8-bromo-cyclic AMP and 8-bromo-cyclic GMP were from Research Biochemicals International (Massachusetts, U.S.A.), while isoprenaline, Tris-HCl, benzamidine, EDTA, EGTA, DTT, PMSF, Triton X-100 and NaCl were from ICN Biomedicals Incorporated (Quebec, Canada). Leupeptin was from Boehringer Mannheim (Quebec, Canada). Affi-gel 601, Dowex 50 (200–400 mesh), alumina and the column supports were from BioRad (Ontario, Canada). The BCA protein assay and bovine serum albumin were from Pierce (Ontario, Canada). All other chemicals were of reagent grade and purchased from Fisher Scientific (Ontario, Canada).

Results

Cyclic AMP PDE activity in rat cultured aortic VSMC

Total cyclic AMP PDE activity and the effects of selected cyclic AMP PDE inhibitors on this activity, were determined in primary cultures of rat aortic VSMC (Table 1). To define whether IBMX-insensitive cyclic AMP PDEs were present in the VSMC, the fraction of total cyclic AMP PDE activity which could be inhibited by this broad-spectrum PDE inhibitor, was determined. Also, the % cyclic AMP PDE activity attributable to PDE2, PDE3 or PDE4 was determined by use of erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA, 10 μM), cilostamide (1 μM) or Ro 20-1724 (10 μM), respectively. The concentrations of IBMX and EHNA were taken from the literature (Beavo & Reifsnyder, 1990), while those for cilostamide and Ro 20-1724 were determined to be selective with partially purified rat aortic PDE3 and PDE4, respectively (unpublished observations). In our assays, the cyclic AMP PDE activity remaining after inhibition with both cilostamide and Ro 20-1724 was approximately 30% of the total (Table 1). This cyclic AMP PDE activity was not inhibited by addition of 10 μM EHNA, or higher concentrations of either cilostamide or Ro 20-1724, but was totally inhibited by incubation with IBMX (Table 1).

Effect of forskolin on cyclic AMP levels in rat cultured aortic VSMC

Incubation of VSMC with 100 μM forskolin caused a marked and sustained increase in cyclic AMP. Thus, [^{3}H]-cyclic AMP levels were increased from a basal level of 0.080 ± 0.008% of total ^{3}H to 2.23 ± 0.24% of total ^{3}H in 5 min and further to 4.09 ± 0.18% of total ^{3}H in 30 min (mean $\pm$ s.e.mean, $n = 3$). This high level of cyclic AMP was sustained for at least 6 h, the longest time point studied.

Effect of forskolin, 1,9-dideoxyforskolin, 8-bromo-cyclic AMP or 8-bromo-cyclic GMP on cyclic AMP PDE activity in rat cultured aortic VSMC

Figure 1 shows the effects of a 16 h incubation of VSMC with forskolin, 8-bromo-cyclic AMP or 1,9-dideoxyforskolin. Incubation of VSMC with either 10 μM or 100 μM forskolin or with 1 mM 8-bromo-cyclic AMP caused marked increases in total cyclic AMP PDE activity (Figure 1). However, incubation with 10 μM or 100 μM 1,9-dideoxyforskolin (Figure 1), a structural analogue of forskolin which does not activate adenylyl cyclase, with 1 mM 8-bromo-cyclic GMP (data not shown) or with 0.1% DMSO, the drug vehicle, did not result in any change in VSMC cyclic AMP PDE activity (Figure 1).

Inclusion of either 4 μM actinomycin D or 100 μM cycloheximide during a 16 h incubation with 8-bromo-cyclic AMP inhibited the increase in VSMC cyclic AMP PDE activity caused by this agent (Table 2). Thus, *de novo* mRNA and protein synthesis are necessary for 8-bromo-cyclic AMP-induced up-regulation of cyclic AMP PDE activity in VSMC. Incubation of VSMC with either actinomycin or cycloheximide alone for 16 h did not significantly alter the cyclic AMP PDE activity.

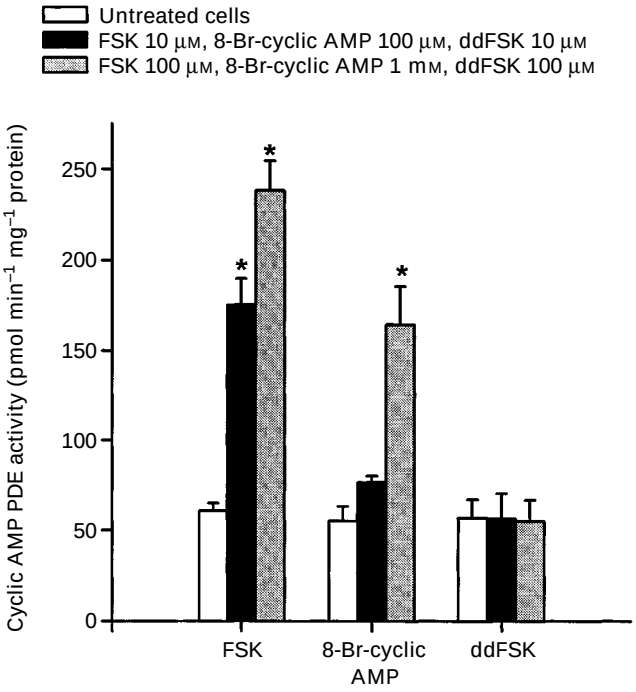

Figure 1 Effect of forskolin, 8-bromo-cyclic AMP or 1,9-dideoxyforskolin on cyclic AMP-PDE activity in rat cultured aortic VSMC homogenates. Basal cyclic AMP-PDE activity with 1 μM cyclic AMP substrate is shown for untreated cells as well as in cells treated with forskolin (FSK), 8-bromo-cyclic AMP (8-Br-cyclic AMP) or 1,9-dideoxyforskolin (ddFSK) for 16 h. Results are expressed as the mean $\pm$ s.e.mean for three independent determinations. $^*P < 0.05$ between untreated and treated cell homogenates.

Table 1 Inhibition of rat cultured aortic VSMC cyclic AMP PDE activity

Additions	*VSMC* (pmol min^{-1} mg^{-1})	
None (total activity)	77.1 ± 5.2	($n = 16$)
Cilostamide (1 μM)	65.8 ± 5.3*	($n = 16$)
Ro 20-1724 (10 μM)	37.5 ± 3.2*	($n = 16$)
Cilostamide (1 μM) + Ro 20-1724 (10 μM)	27.6 ± 1.7*,**	($n = 16$)
EHNA (10 μM)	78.4 ± 3.7	($n = 4$)
IBMX (500 μM)	5.3 ± 2.7*	($n = 3$)

Activities are the mean $\pm$ s.e.mean for several independent determinations in rat cultured aortic VSMC. $^*P < 0.05$ in comparison to total cyclic AMP PDE activity; $^{**}P < 0.05$ in comparison to cyclic AMP PDE activity in the presence of Ro 20-1724 or cilostamide alone.

Time course of forskolin-or 8-bromo-cyclic AMP-induced increases in rat cultured aortic VSMC cyclic AMP PDE activity

Both forskolin (Figure 2a) and 8-bromo-cyclic AMP (Figure 2b) caused a time-dependent increase in VSMC cyclic AMP PDE activity. While incubation of VSMC for 4 h with either agent resulted in a small increase in cyclic AMP PDE activity, in both cases total cyclic AMP PDE activity was more than doubled after 16 h.

Effects of forskolin or 8-bromo-cyclic AMP on rat cultured aortic VSMC PDE3 and PDE4 activities

Tables 3 and 4 show the forskolin- or 8-bromo-cyclic AMP-induced increases in total cyclic AMP PDE activity, respectively, as well as the relative effects of these agents on VSMC PDE3 and PDE4 activities. Following a 16 h incubation with either 100 μM forskolin (Table 3) or 1 mM 8-bromo-cyclic AMP (Table 4), the PDE3 component of the total activity was significantly increased. Indeed, PDE3 activity in treated VSMC was roughly doubled following incubation with either agent (Tables 3 and 4). Similarly, incubation of the cultured VSMC with either forskolin or 8-bromo-cyclic AMP also increased VSMC PDE4 activity. However, in contrast to the effects of these treatments on PDE3 activity, the percentage of the total cyclic AMP PDE activity due to PDE4 was not significantly different before and after treatment (Tables 3 and 4). The time-dependence of the 8-bromo-cyclic AMP-induced increases in PDE3 and PDE4 activities was also examined (Table 5). Although the amount of activity attributable to either PDE3 or PDE4 was increased at the 8 and 16 h time points, and PDE4 activity was increased linearly over this period, a larger increase in PDE3 activity occurred between 4 and 8 h of incubation. The cyclic AMP PDE activity not susceptible to inhibition by cilostamide and Ro 20-1724, but inhibited by IBMX, was also elevated following incubation with either forskolin or 8-bromo-cyclic AMP (Tables 3 and 4). While this residual activity was inhibited by more than 95% by IBMX in both treated and control VSMC, EHNA was equally without effect before or after treatment (Table 6). In parallel studies, we have determined that the level of expression of several VSMC cyclic AMP PDE are affected by prolonged incubations with either forskolin or 8-bromo-cyclic AMP. Thus, both PDE3s (PDE3A and PDE3B) as well as PDE4B and PDE4D are increased upon incubation of rat cultured aortic VSMC with agents that increase VSMC cyclic AMP (data not shown, manuscripts in preparation).

Table 2 Effects of actinomycin or cycloheximide on 8-bromo-cyclic AMP-mediated increases in rat cultured aortic VSMC cyclic AMP PDE activity

Treatments	*Activity* (pmol min^{-1} mg^{-1})
None	75.3±5.1
Actinomycin (4 μM)	70.0±3.7
Cycloheximide (100 μM)	69.3±5.9
8-Bromo-cyclic AMP (1 mM)	144.9±11.7*
8-Bromo-cyclic AMP (1 mM)+ actinomycin (4 μM)	68.4±5.0
8-Bromo-cyclic AMP (1 mM)+ cycloheximide (100 μM)	73.1±4.8

Activities represent the mean±s.e.mean of three independent determinations. *$P<0.05$ in comparison to cyclic AMP PDE activity in untreated rat cultured aortic VSMC.

Effects of forskolin or 8-bromo-cyclic AMP on isoprenaline- or forskolin-mediated increases in rat cultured aortic VSMC cyclic AMP

In untreated VSMC, isoprenaline (0.1 nM – 1 μM; Figure 3), or forskolin (1 μM – 100 μM; not shown), caused concentration-dependent increases in cyclic AMP which were potentiated by the addition of IBMX, cilostamide or Ro 20-1724 (Figure 3). In our experiments, all three cyclic AMP PDE inhibitors potentiated the isoprenaline-induced increases in cyclic AMP at all concentrations of isoprenaline tested (Figure 3). For example, when tested with 10 nM isoprenaline, the effects of cilostamide or Ro 20-1724 were consistent with PDE3 and PDE4 representing roughly 15% and 55% of the IBMX-inhibitable cyclic AMP PDE present in these cells (Figure 3).

Both isoprenaline (Figure 3) or forskolin (not shown) were much less potent in VSMC which had been incubated for 16 h with 1 mM 8-bromo-cyclic AMP; as reflected by a large rightward shift in the concentration-response curve (Figure 3). For example, the increase in cyclic AMP caused by 1 μM isoprenaline in the treated VSMC was only 11% of the increase in

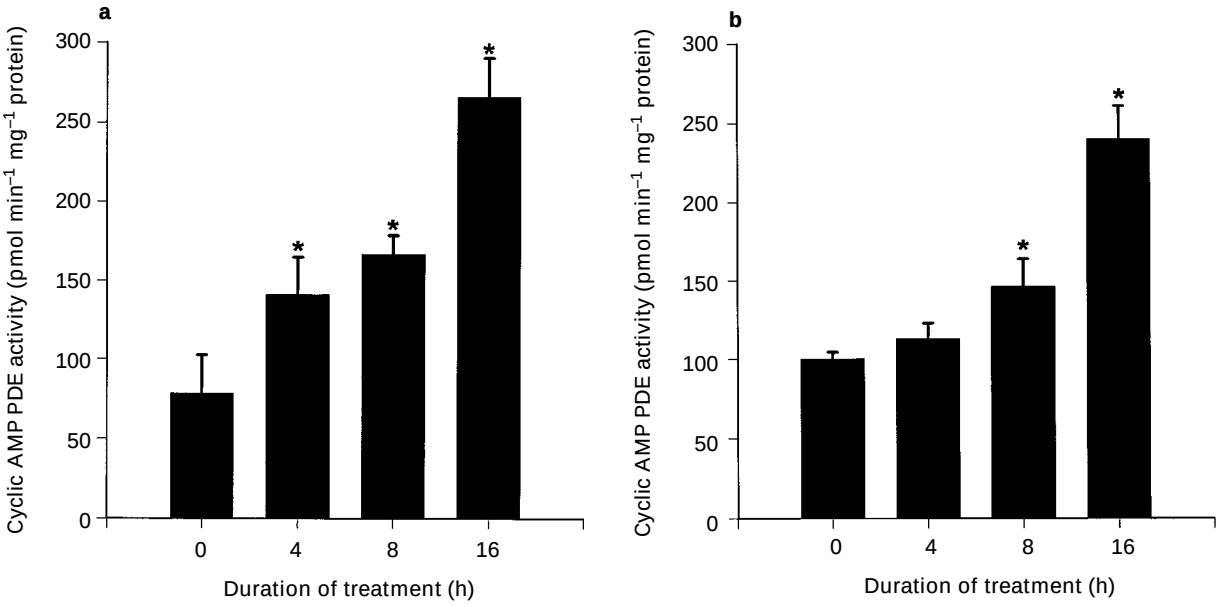

Figure 2 Effect of 4, 8 and 16 h incubations with 100 μM forskolin (a), or 1 mM 8-bromo-cyclic AMP (b) on cyclic AMP PDE activity in rat cultured aortic VSMC homogenates. Results are expressed as the mean±s.e.mean of three independent determinations. *$P<0.05$ between untreated and treated cell homogenates.

Table 3 Effect of forskolin on cilostamide- and Ro 20-1724-inhibited cyclic AMP PDE activity in rat cultured aortic VSMC

	Untreated		*Forskolin treated*	
Addition	*Activity* (pmol min^{-1} mg^{-1})	*Inhibition* (%)	*Activity* (pmol min^{-1} mg^{-1})	*Inhibition* (%)
None	74.0±5.4	0 (basal)	279.3±11.1	0 (basal)
Cilostamide (1 μM)	66.6±4.6*	10±2*	207.0±10.9*	26±3*,***
Ro 20-1724 (10 μM)	32.5±1.2*	56±2*	118.5±11.6*	58±3*
Cilostamide (1 μM)+ Ro 20-1724 (10 μM)	24.8±1.5*,**	66±1*,**	86.4±11.0*,**	69±4*,**

Activities represent the mean±s.e.mean of three independent experiments. Inhibition of activity in the presence of 1 μM cilostamide and/or 10 μM Ro 20-1724 is expressed as percentages of the basal value in the absence of the inhibitors. *$P<0.05$ in comparison to cyclic AMP PDE activity, or % inhibition, in the absence of PDE inhibitors: **$P<0.05$ in comparison to cyclic AMP PDE activity, or % inhibition, in the presence of either Ro 20-1724 or cilostamide alone; ***$P<0.05$ in comparison to % inhibition in untreated cells.

Table 4 Effect of 8-bromo-cyclic AMP on cilostamide- or Ro 20-1724-inhibited cyclic AMP PDE activity in rat cultured aortic VSMC

	Untreated		*8-Bromo-cyclic AMP treated*	
Addition	*Activity* (pmol min^{-1} mg^{-1})	*Inhibition* (%)	*Activity* (pmol min^{-1} mg^{-1})	*Inhibition* (%)
None	87.5±2.5	0 (basal)	203.7±7.0*	0 (basal)
Cilostamide (1 μM)	75.1±1.9*	14±5*	159.1±3.9*	22±3*,**
Ro 20-1724 (10 μM)	38.8±1.6*	56±4*	86.1±3.3*	58±3*
Cilostamide (1 μM)+ Ro 20-1724 (10 μM)	30.2±2.1*,***	65±1*,***	66.8±3.1*,***	67±1*,***

Activities represent the mean±s.e.mean of three independent experiments. Inhibition of activity in the presence of 1 μM cilostamide and/or 10 μM Ro 20-1724 is expressed as percentages of the basal value in the absence of the inhibitors. *$P<0.05$ in comparison to cyclic AMP PDE activity, or % inhibition, in the absence of PDE inhibitors: **$P<0.05$ in comparison to cyclic AMP PDE activity, or % inhibition, in the presence of either Ro 20-1724 or cilostamide alone; ***$P<0.05$ in comparison to % inhibition in untreated cells.

Table 5 Time-dependence of 8-bromo-cyclic AMP-induced increase in PDE3 and PDE4 activities in rat cultured aortic VSMC

Time	*PDE3*		*PDE4*	
(h)	(pmol min^{-1} mg^{-1})	(%)	(pmol min^{-1} mg^{-1})	(%)
0	14.1±1.1	14±2	66.8±2.9	67±3
4	19.6±3.3	18±2	70.9±5.8	65±3
8	36.5±2.2*	25±3*	90.6±4.3*	62±5
16	57.6±4.9*	24±3*	156.1±7.0*	65±5

Cyclic AMP PDE activity was 100.2±3.1, 109.1±4.9, 146.1±7.3 and 240.1±8.5 pmol min^{-1} mg^{-1} at 0, 4, 8 and 16 h, respectively. Values represent changes in cilostamide- (PDE3) or Ro 20-1724- (PDE4) inhibited cyclic AMP PDE activity (pmol $min^{-1}mg^{-1}$) or % inhibition of cyclic AMP PDE activity caused by cilostamide (PDE3) or Ro 20-1724 (PDE4) at each time point. *$P<0.05$ in comparison to cyclic AMP PDE activity at 0 h; **$P<0.05$ in comparison to % inhibition in 0 h VSMC.

Table 6 Effect of EHNA or IBMX on total cyclic AMP PDE activity in control and 8-bromo-cyclic AMP treated rat cultured aortic VSMC

	Untreated	*8-Bromo-cyclic AMP treated*
Addition	*Activity* (pmol $min^{-1}mg^{-1}$)	*Activity* (pmol min^{-1} mg^{-1})
None	81.2±3.1	209.1±4.4*
EHNA (10 μM)	78.4±3.7	212.3±7.4*
IBMX (500 μM)	5.3±2.7*	11.3±9.3*,**

Data represent the mean±s.e.mean of three independent determinations. *$P<0.05$ in comparison to cyclic AMP activity in untreated VSMC. **$P<0.05$ in comparison to cyclic AMP PDE activity in 8-bromo-cyclic AMP treated VSMC.

control cells (Figure 3). Consistent with a role for upregulated cyclic AMP PDE activity in the reduced potency of these agents in the treated VSMC, the effects of the cyclic AMP PDE inhibitors were more marked in the treated cells. For example, addition of IBMX potentiated the effect of 10 nM isoprenaline to a level approximately 3.3 fold larger in treated cells than was achieved in the untreated VSMC (Figure 3). This increased effectiveness of IBMX in treated VSMC was consistent with the overall increase in cyclic AMP PDE activity in VSMC following 8-bromo-cyclic AMP treatment (Tables 4 and 6). Consistent with up-regulated PDE3 and PDE4 activities in 8-bromo-cyclic AMP-treated VSMC, both cilostamide and Ro 20-1724, also potentiated the isoprenaline-induced increase in cyclic AMP to a greater extent in treated cells than in control cells (Figure 3). Indeed, both cilostamide and Ro 20-1724 increased the 10 nM isoprenaline-induced increase in cyclic AMP to levels 1.8 and 2.5 fold larger in treated VSMC than were obtained in control untreated VSMC, respectively. Again, these effects were consistent with the 8-bromo-cyclic AMP-mediated increases in PDE3 and PDE4 activities observed in treated VSMC (Tables 4 and 6).

Discussion

In this study, we describe the results of experiments which begin to assess the effects of long-term treatment of VSMC with agents that increase cyclic AMP on total cyclic AMP PDE

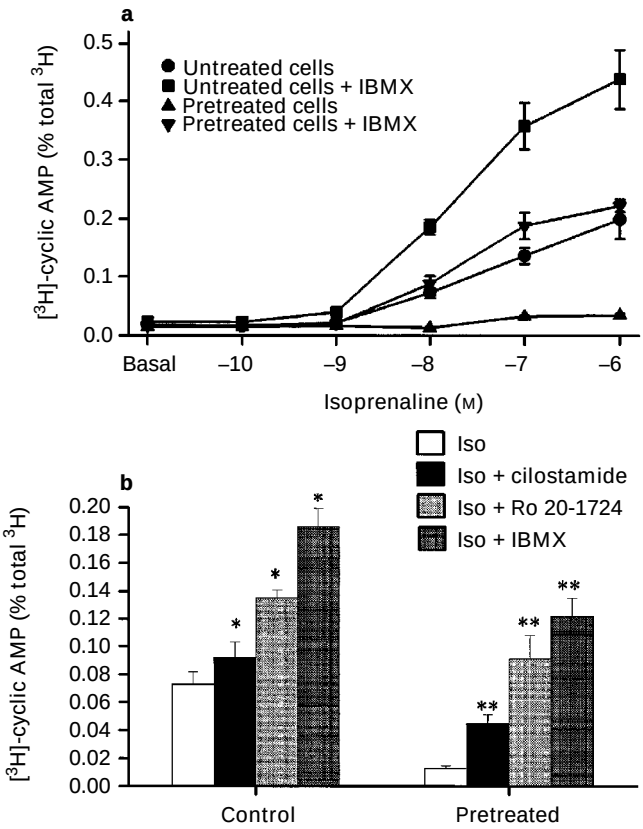

Figure 3 Effect of 8-bromo-cyclic AMP pretreatment on potentiation of isoprenaline-induced increases in cyclic AMP by PDE inhibitors. (a) Cells were pretreated with 1 mM 8-bromo-cyclic AMP for 16 h, challenged for 1 min with isoprenaline (0.1 nM to 1 μM) in the presence or absence of 500 μM IBMX and then cyclic AMP levels were measured. (b) Cells were pretreated with 1 mM 8-bromo-cyclic AMP for 16 h, challenged for 1 min with 10 nM isoprenaline (Iso) in the presence or absence of 1 μM cilostamide, 10 μM Ro 20-1724 or 500 μM IBMX and then cyclic AMP levels determined. Results are expressed as the mean$\pm$s.e.mean from three independent determinations. *$P<0.05$ in comparison to cyclic AMP levels following a 1 min incubation of control VSMC with 10 nM isoprenaline in the absence of other compounds. **$P<0.05$ in comparison to cyclic AMP levels following a 1 min incubation of treated VSMC with 10 nM isoprenaline in the absence of other compounds.

activity and, more specifically, on the levels of PDE3 and PDE4 activities. By use of selective pharmacological inhibitors, (EHNA (PDE2), cilostamide (PDE3) or Ro 20-1724 (PDE4)), homogenates of rat cultured aortic VSMC were shown to contain both PDE3 and PDE4 activities, but not PDE2 activity. Under our experimental conditions, PDE3 and PDE4 activities accounted for approximately 15% and 55% of the total cyclic AMP PDE hydrolytic activity measured. The remaining cyclic AMP PDE activity (approximately 30%) in VSMC homogenates was not inhibited by increasing the concentrations of either cilostamide or Ro 20-1724, or by EHNA, but was totally inhibited by a non-specific PDE inhibitor, IBMX. Experiments are presently underway to determine the possibility that PDE1 or PDE5 participate in this cyclic AMP hydrolysis. However, since our assay conditions do not involve reconstitution with calmodulin, a role for PDE1 in our assays is unlikely. *In situ* hydrolysis of cyclic AMP by PDE5 has recently been demonstrated (Dickinson *et al*., 1997), but it was not directly tested in our experiments.

We demonstrated that long-term incubation of the VSMC with either forskolin, or 8-bromo-cyclic AMP, caused both a time- and concentration-dependent increase in total cyclic AMP PDE activity. The dependence of this effect on cyclic AMP was tested by incubating rat cultured aortic VSMC with 1,9-dideoxyforskolin, an analogue of forskolin which does not activate adenylyl cyclase, or with 8-bromo-cyclic GMP. No increase in cyclic AMP PDE activity was detected when 1,9-dideoxyforskolin or 8-bromo-cyclic GMP were used in our experiments. With both forskolin- or 8-bromo-cyclic AMP, total cyclic AMP PDE activity continued to increase for 16 h, the longest time point studied. Since no significant increase in total cyclic AMP PDE activity was present following a 4 h incubation with 8-bromo-cyclic AMP, the shortest time point used in our studies, it is unlikely that increases in cyclic AMP PDE activity in VSMC are related to direct cyclic AMP-dependent protein kinase (PKA)-induced phosphorylation of the VSMC cyclic AMP PDEs (Grant *et al*., 1988; Sette & Conti, 1996). However, a significant increase in cyclic AMP PDE activity had occurred by 4 h when VSMC were treated with forskolin. We hypothesize that this difference is related to the rate of increase in intracellular cyclic AMP, rather than to differences in the mechanisms by which these agents increase cyclic AMP PDE activity. This hypothesis is consistent with the very rapid increase in cyclic AMP which occurs in VSMC treated with forskolin (<5 min), compared to the slower rate of diffusion of 8-bromo-cyclic AMP into cells. Moreover, cyclic AMP-dependent increases in cyclic AMP PDE activity in the VSMC were completely inhibited by actinomycin D or by cycloheximide, indicating that these increases were the result of synthesis of new mRNA and protein. In previously published studies carried out with either Sertoli, U937 or Mono Mac 6 cells (Torphy *et al*., 1995; Verghese *et al*., 1995; Manning *et al*., 1996), a cyclic AMP-mediated up-regulation of PDE4 activity was found following incubation of these cell types with forskolin, or with membrane-permeable cyclic AMP analogues. In our studies, we demonstrated that long-term treatment of rat aortic VSMC with either forskolin or with 8-bromo-cyclic AMP also increased VSMC PDE4 activity. However, in contrast to results obtained in these other cell types, both PDE4 and PDE3 activities were up-regulated by cyclic AMP in VSMC. To our knowledge this is the first time a significant increase in PDE3 activity has been shown following pharmacological treatments which elevate cellular cyclic AMP levels. Interestingly, although the amount of PDE4 activity in VSMC was always more abundant than that for PDE3, the effect of forskolin or 8-bromo-cyclic AMP were always relatively more marked on PDE3 than PDE4 activity. Thus, whereas the amount of PDE4 activity in control and 8-bromo-cyclic AMP-treated VSMC was a constant percentage of the total cyclic AMP PDE activity, roughly twice as much PDE3 activity was present in treated cells than in the untreated controls. The molecular basis for the different levels of up-regulation for PDE3 and PDE4 activities in VSMC in response to cyclic AMP is not known. However, in parallel studies in our laboratory we have started to determine the expression pattern of PDE3 and PDE4 isozymes in VSMC, and to determine the effect of our treatment protocol on this expression. In relation to this, we have shown previously that mRNA for both PDE3A and PDE3B are present in vascular smooth muscle tissue isolated from rat aorta (Maurice *et al*., 1995). More recently, we have obtained evidence that both PDE3A and PDE3B are expressed in rat cultured aortic VSMC and that the level of expression of both are regulated by cyclic AMP (manuscript in preparation). Also, we have identified PDE4A, PDE4B and PDE4D gene products in cultured VSMC and have begun to assess the effect of cyclic AMP generation on the regulation of the expression of these proteins in these cells (manuscript in preparation).

As demonstrated in concentration-response studies, pretreatment of VSMC with forskolin or 8-bromo-cyclic AMP decreased the potency of isoprenaline. Although it is clear that other factors were also at work, our data are consistent with a role for up-regulation of total cyclic AMP PDE activity in this phenomenon. In order to assess the role of up-regulated cyclic AMP PDE activity in the reduced responses to isoprenaline of treated VSMC, we attempted to normalize the isoprenaline-induced increases in cyclic AMP with inhibitors of cyclic AMP

PDE activity. For these studies, we used the broad spectrum cyclic AMP PDE inhibitor, IBMX, as well as the PDE3 and PDE4 selective agents, cilostamide and Ro 20-1724, respectively. Thus, using each of these cyclic AMP PDE inhibitors, we were able to reduce markedly the differences between isoprenaline-mediated increases in cyclic AMP in control and in treated cells. In fact, inhibition of total cyclic AMP PDE activity with IBMX resulted in a 3 fold larger potentiation of the effects of isoprenaline in treated, as compared to control, VSMC. Similarly, the effects of cilostamide and Ro 20-1724 were consistent with a role for both PDE3 and PDE4 up-regulation in this phenomenon. Thus, while inhibition of PDE3 or PDE4 activities in control VSMC increased isoprenaline-induced accumulations of cyclic AMP, the effects of either of these agents were consistently more marked in treated cells. In fact, the effects of cilostamide or Ro 20-1724 on the magnitude of the isoprenaline-induced increases in cyclic AMP were found to correlate with the level of PDE3 or PDE4 activity present in the VSMC. This was true both for control and for 8-bromo-cyclic AMP-treated cells.

In our experiments, we estimate the fraction of the reduced potency of isoprenaline which could not be reversed by inhibition of cyclic AMP PDE activity at approximately 40%. In this context, PKA-mediated phosphorylation of the VSMC β-adrenoceptors, as well as post-receptor effects at either heterotrimeric GTP-binding proteins G_s or G_i, or at adenylyl cyclase itself, will most likely have an important role. Although a significant amount of progress aimed at delineating the relative importance of each of these potential sites of regulation in the process of desensitization has been made recently by use of heterologous expression systems (reviewed in Perkins *et al*., 1991), the relative impact of each on desensitization in non-transfected cells is more limited (Hausdorff *et al*., 1990; Bylund *et al*., 1994). However, in studies with Sertoli cells, mononuclear cells as well as adipocytes, combined effects of long-term increases in cyclic AMP on both the synthesis and catabolism of cyclic AMP similar to those presented here by us have been described (Swinnen *et al*., 1989; Torphy *et al*., 1992; 1995; Bousquet-Mélou *et al*., 1995; Verghese *et al*., 1995; Manning *et al*., 1996). In an attempt to assess directly the importance of these events in VSMC, the role of changes in the cyclic AMP-generating system in VSMC following treatment with either forskolin or 8-bromo-cyclic AMP will be the subject of future studies in our laboratory.

The physiological role of the phenomenon described here is unknown. However, given the dynamic regulation of cyclic AMP levels which occurs in response to hormones in most tissues, it is reasonable to propose that cyclic AMP-mediated expression of cyclic AMP PDEs might form part of normal homeostatic mechanisms.

In conclusion, long-term treatments which elevate intracellular levels of cyclic AMP cause an increase in cyclic AMP PDE activity in rat cultured aortic VSMC. Although these treatments increased total cyclic AMP PDE activity, a relatively more marked effect was associated with PDE3. This cyclic AMP-induced up-regulation of cyclic AMP PDE activity, in part, causes a reduced potency of activators of adenylyl cyclase.

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