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Characterisation of cyclic nucleotide phosphodiesterase isoforms in the media layer of the main pulmonary artery

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Abstract

Cyclic nucleotides are involved in the control of pulmonary vascular tone. In the present study, we measured the cyclic nucleotide specific phosphodiesterase (PDE) activity in the media of bovine isolated main pulmonary artery (MPA). Total cAMP- and cGMP-PDE activities were measured in microsomal and cytosolic fractions. Both cyclic nucleotides were hydrolysed in these subcellular fractions at consistently higher rate in the cytosolic than in the microsomal fraction. Using different classes of PDE modulator, at least four PDE isoforms (PDE1, 3, 4 and 5) were identified in these fractions. PDE3 (cilostamide-sensitive), PDE4 (rolipram-sensitive) and PDE5 (zaprinast- and DMPPO-sensitive) isoforms appeared as the main isozymes implicated in the cAMP and cGMP hydrolytic activities. Calcium–camodulin stimulated PDE activity (PDE1) was mainly present in the cytosolic fraction. PDE2, although present, had a lower hydrolytic activity since addition of its specific inhibitor, erythro-9-(2-hydroxy-3nonyl)adenine (EHNA), to a combination of inhibitors of PDE3, 4 and 5 produced no further significant reduction in the enzymatic activity. Resolution of PDE activities from the cytosolic fraction using anion exchange chromatography confirmed this finding. Functional experiments performed in endothelium-denuded rings of rat MPA revealed that all specific PDE inhibitors used relaxed precontracted vascular smooth muscle preparations in a concentration-dependent manner. The rank order of potency was cilostamide > zaprinast > rolipram >> EHNA. The present study demonstrates the presence in the smooth muscle cells-containing layer of MPA of PDE1, 3, 4 and 5 isoforms and suggests that PDE3, 4 and 5 are the main enzymes involved in the control of vascular tone.

Keywords: Smooth muscle; Vascular tone; Phosphodiesterase; Cyclic nucleotide; Subcellular fractions

1. Introduction

Preliminary results of this study were at the 2000 Meeting of the GRRC Meeting [1]. Pulmonary vascular tone is controlled by a variety of circulating and locally released mediators some of which are known to modulate cyclic nucleotide (cAMP and cGMP) levels in the vascular smooth muscle cell [2]. Cyclic nucletotides are synthesised by adenylyl and guanylyl cyclases [3,4]. Agents elevating cAMP or cGMP concentration lead to relaxation of precontracted pulmonary arteries [5–7]. For instance, nitric oxide (NO) induces a dose-dependent increase in cGMP concentration and a step-wise relaxation of pulmonary artery [8,9]. The family of cyclic nucleotide PDE which hydrolyses cAMP and cGMP represents the unique degradation pathway for these intracellular compounds [10]. As a consequence, PDE activity is implicated in the control of vascular tone and the pharmacological modulation of this activity (e.g. using PDE inhibitors) can also control vascular tone.

Knowledge of the cellular role played by the PDE is complicated by the fact that up to 11 PDE isoenzyme families have been characterised by molecular biological techniques in different mammalian tissues [11–13]. In vascular tissues, at least five PDE isozymes have been identified, the activity of which is both differentially controlled

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Abbreviations: CCRC, cumulative concentration-response curve; EHNA, erythro-9-(2-hydroxy-3nonyl)adenine; IBMX, 3-isobutyl-1-methylxanthine; DMPPO, 1.3 dimethyl-6-(2-propoxy-5-methane sulphonylamidophenyl)-pyrazolo[3,4-d]pyrimidin-4-(5H)-one; MPA, main pulmonary artery; PDE, cyclic nucleotide phosphodiesterase.

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by cAMP, cGMP or Ca^{2+}–calmodulin and sensitive to selective pharmacological inhibitors [10,14]. Moreover, the distribution of PDE isoforms is variable depending on (1) subcellular location (microsomal vs. cytosolic); (2) portions of the arterial bed (e.g. extra vs. intrapulmonary arteries); (3) distribution within a specific layer in the same portion of artery (e.g. media vs. adventitia or intima) [6,15–17]. Interestingly, in the pulmonary circulation, it has been shown that some PDE inhibitors relax vascular smooth muscle under physiological conditions [6]. In addition, PDE content and specific PDE activity are altered in some physiological (e.g. switch from foetal to new-born stage) [18,19] and pathophysiological conditions (e.g. chronic hypoxia) [17,20]. Moreover, treatment with PDE inhibitors counteracts pulmonary hypertension development both in animals and human [21–23]. Therefore, precise knowledge of distribution, biochemical and pharmacological characteristics of PDEs in pulmonary vessels is necessary for a better understanding of the regulation of pulmonary vascular tone under both physiological and pathophysiological conditions.

Previous studies have examined PDEs activity in the whole pulmonary artery [6,17]. However, since vasoreactivity is mainly mediated by the contractile properties of vascular smooth muscle cells, the present work was dedicated to the characterisation of the PDE activity in the pulmonary artery layer containing smooth muscle cells, i.e. media. The objectives were thus: (1) to estimate the specific cAMP and cGMP hydrolytic activity of PDEs; (2) to pharmacologically characterise the various PDE isoforms in two MPA subcellular fractions (microsomal/cytosolic); (3) to isolate cytosolic PDE isoforms by anion exchange chromatography; and (4) to assess the implication of the different characterised PDE isoforms in the control of vascular tone using endothelium-denuded rings of MPA. Selective PDE isoform inhibitors were used in pharmacological and functional studies.

2. Materials and methods

2.1. Preparation of microsomal and cytosolic fractions

The preparation of microsomal and cytosolic fractions derived from bovine pulmonary arteries was performed according to a protocol modified from a method described previously [24]. Briefly, bovine MPA obtained from a local slaughterhouse was dissected and washed in Krebs–Henseleit (KH) solution (4°). Adventitial and intimal layers were mechanically removed from MPA leading to the separation of the smooth muscle containing layer, i.e. media. Typically 80 g of tissue were weighed and promptly transferred in three volumes (w/v) of a buffer containing 0.3 M sucrose, 20 mM K-PIPES, 4 mM EGTA, and various protease inhibitors: 50 μM 4-(2-aminoethyl)-benzene-sulfonyl fluoride hydrochloride (Pefabloc), 1 μM pepstatin, 1 μM leupeptin, 0.24 unit/100 mL of trypsin inhibitor unit aprotinin/100 mL, and 2 mM dithiothreitol. Tissues were then homogenised 3×30 s on ice, and the mixture was centrifuged for 20 min at 6500 *g* (4°) to remove mitochondria, all nuclei and other cellular fragments. The supernatant was centrifuged again for 60 min at 86,000 *g* (4°) and the resulting pellet containing the microsomal fraction was then re-suspended in 0.3 M sucrose–5 M K-PIPES, pH 7.4. The supernatant which contained the diluted cytosolic enzymes, as well as the microsomal fraction were frozen in liquid nitrogen, and stored at −80°. The protein contents of the subcellular fractions were determined by the Lowry method [25] using bovine serum albumin as standard. Bovine arteries were used in these experiments since in this species, unlike the rat, the smooth muscle layer can be separated from the adventitial and intimal layers and sufficient microsomal and cytosolic material can be obtained to perform biochemical and pharmacological analysis.

2.2. Phosphodiesterase assay and chromatographic resolution of PDE activities

PDE activities were measured by a two-step assay as described previously [26]. The hydrolysis of substrate (1 μM [^{3}H]-cAMP or 1 μM [^{3}H]-cGMP) was assayed in the presence of either 1 mM EGTA or 10 μM Ca^{2+} plus an excess (20 nM) of calmodulin, with or without addition of PDE inhibitors. Determinations were made in duplicate. In order to detect and gauge the relative activity of specific PDE classes in our subcellular preparations, we took advantage of the selective inhibition or activation of the enzymes by a variety of substances [10,14]. Thus, PDE1 activity was determined as the fraction of PDE activity which could be either activated by the Ca^{2+}–calmodulin complex or inhibited by 10 μM nimodipine. PDE2 activity was assessed using the selective inhibitor EHNA (50 μM), in the presence of PDE3 and 4 inhibitors plus a stimulating concentration (5 μM) of cGMP. EHNA has been shown to be a more potent inhibitor of PDE2 isoform when the enzyme is stimulated by cGMP [27]. Either 1 μM cilostamide, 10 μM LY195115 or 5 μM cGMP was used to inhibit PDE3 activity, while 10 μM rolipram or 10 μM denbufylline was used to inhibit PDE4 activity. The compound 1.3 dimethyl-6-(2-propoxy-5-methane sulphonylamidophenyl)-pyrazolo[3,4-d]pyrimidin-4-(5H)-one (DMPPO) (1 μM) was used to inhibit selectively PDE5 cGMP activity, and zaprinast (10 μM) was used to inhibit both PDE5 and 1 activities. All concentrations used were derived from previous experiments on purified smooth muscle PDE isoforms [14,16]. Some experiments were also performed in the presence of non-specific PDEs inhibitors such as 3-isobutyl-1-methylxanthine (IBMX) (1 mM), theophylline (5 mM) or caffeine (5 mM).

For chromatographic resolution of PDE activities, the supernatant containing the cytosolic enzymes was applied to a Q2-MPLC column (Biorad). The elution was performed

at a flow rate of 0.7 mL/min (30 bar pressure) with a gradient of NaCl (0.05–0.45 M) in Tris–HCl buffer. Eluted fractions (0.7 mL) were collected and assayed for cAMP- and cGMP-specific PDE activities in the absence or the presence of various PDE inhibitors.

2.3. Isometric contraction measurements

Wistar male rats aged from 8 to 10 weeks, and weighing 280–340 g were anaesthetised by intraperitoneal injection of ethylcarbamate (400 mg/100 g of weight). The heart and lungs were removed *en-bloc* and MPA rings (3 mm in length) were dissected in KH solution. Isometric contraction was measured in endothelium-denuded rings mounted between two stainless steel clips in vertical 5 mL organ baths, part of a computerised isolated organ bath system (IOS, EMKA Technologies). Baths were filled with KH solution (composition in mM: 118.4 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 25 $NaHCO_3$, 1.2 KH_2PO_4, 11.1 D-glucose, pH 7.4) maintained at 37° and bubbled with a 95% O_2–5% CO_2 gas mixture. The upper stainless clip was connected to an isometric force transducer (EMKA Technologies). Tissues were set at optimal length by equilibration against a passive load of 10 mN as determined in previous experiments [9]. At the beginning of each experiment, a K^+-rich (80 mM) solution, obtained by substituting an equimolar amount of KCl for NaCl from KH solution, was repeatedly applied in order to obtain at least two contractions similar in both amplitude and kinetics as a control. To impair the functioning of endothelium, the MPA lumen was perfused with distilled water before mounting in the bath. Successful removal of the endothelium was confirmed by the inability of acetylcholine (1 μM) to induce more than 10% of relaxation in phenylephrine (1 μM) contracted rings.

The relaxant effect of PDE inhibitors (0.001–100 μM) was studied by constructing a cumulative concentration-response curve (CCRC) in rings precontracted with a submaximal concentration (0.5 μM) of phenylephrine inducing 83% of the maximum contractile response as determined in control experiments. The lowest PDE inhibitor concentration was added to the bath 5 min after establishment of the maximal tension. A concentration increment was made once the maximal relaxant effect to the preceding concentration had been recorded (generally 3–6 min). PDE inhibitors were administered to one-half of the rings. The unexposed rings served as temporal control.

2.4. Statistical analysis

Results are expressed as the mean ± SEM with N the sample size. Significance was tested by means of Student's *t*-test at a *P*-value of 0.05. In relaxation experiments, IC_{50} the concentration of PDE inhibitors inducing 50% of the maximal relaxation was graphically determined from the mean CCRC.

2.5. Chemicals and drugs

Acetylcholine, caffeine, EGTA, EHNA, protease inhibitors (except Pefabloc), IBMX, phenylephrine, theophylline, zaprinast, were purchased from Sigma. Pefabloc [4-(aminoethyl)-benzenesulfonyl fluoride] was ordered from Boehringer–Mannheim. Nimodipine was from Calbiochem. Cilostamide, and rolipram were purchased from Tocris. All other materials were of reagent grade. All buffer solutions were prepared with deionized water from Millipore Milli Ro-Milli-Q-UF system (18 ± 0.2 MΩ/cm^2). [8-^{3}H]-cAMP (30–50 Ci/mmol) and [8-^{3}H]-cGMP (5–15 Ci/mmol) were purchased from New England Nuclear and purified by thin layer chromatography on silica gel, using isopropanol:NH_4OH:H_2O (70:15:15) as a solvent. Calmodulin was purified from bovine brain as previously reported [16]. The following compounds were kindly provided by the indicated company: denbufylline (Beecham-Wulfing), LY193115 (1,3-dihydro-3,3-dimethyl-1-5-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)-2H-indol-2-one, (Eli-Lilly), DMPPO (Glaxo-Wellcome). PDE inhibitors were dissolved in either DMSO or water. The final solvent concentrations in the assay were either 0.5 or 0.1% in organ bath and did not significantly affect hydrolytic or contractile activities.

3. Results

3.1. Measurement of PDE activities in bovine pulmonary artery media

The cAMP- and cGMP-specific PDE activities were assayed in the microsomal and cytosolic fractions as illustrated in Fig. 1. Both specific hydrolytic activities

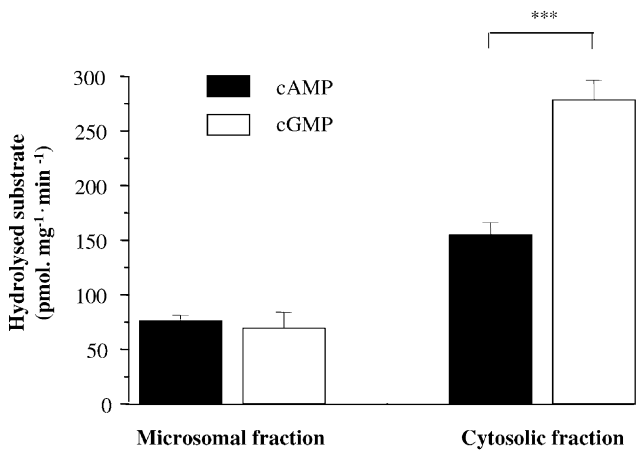

Fig. 1. Total cAMP- and cGMP-specific PDE activities in the microsomal and cytosolic fractions derived from smooth muscle cells of the bovine main pulmonary artery. The hydrolytic activities were determined in the presence of 1 μM [^{3}H]-cAMP or 1 μM [^{3}H]-cGMP. The total hydrolytic activity is higher in cytosolic than in the microsomal fractions. Data are shown as mean ± SEM (N = 9–13); $^{***}P < 0.001$.

Table 1
Inhibitory effect of selective and non-selective PDE inhibitors in both subcellular fractions isolated from smooth muscle of the bovine main pulmonary artery

PDE isozymes	Inhibitors	Percentage of inhibition of the cAMP-specific PDE activity	
		Microsomal	Cytosolic
PDE1	Nimodipine (10 μM)	28.5 ± 8.1 (2)	36.4 ± 2.5 (2)
PDE2	EHNA (50 μM)	17.0 ± 5.6 (6)	19.6 ± 5.7 (6)
PDE2[a], 3	EHNA (50 μM) + cGMP (5 μM)	60.5 ± 3.2 (3)	56.3 ± 2.5 (5)
PDE3	Cilostamide (1 μM)	45.3 ± 4.5 (3)	37.6 ± 2.9 (3)
PDE3	LY195115 (10 μM)	54.3 ± 2.0 (6)	45.7 ± 0.9 (6)*
PDE3	cGMP (5 μM)	54.2 ± 2.6 (6)	44.4 ± 5.4 (3)
PDE4	Rolipram (10 μM)	34.6 ± 4.1 (6)	36.9 ± 2.1 (7)
PDE4	Denbufylline (10 μM)	28.7 ± 5.1 (3)	43.5 ± 1.1 (3)*
PDE5, 1	Zaprinast (10 μM)	15.1 ± 4.2 (7)	26.4 ± 1.9 (7)*
PDE5	DMPPO (1 μM)	5.7 ± 2.3 (4)	0.2 ± 3.6 (3)
PDE4, 3	Rolipram + LY195115 + cGMP	80.5 ± 0.8 (3)	72.5 ± 0.4 (3)*
PDE4, 3	Rolipram + LY195115 + cGMP	83.0 ± 2.2 (3)	79.9 ± 1.1 (3)
PDE4, 3, 2[a]	Rolipram + LY195115 + cGMP + EHNA	87.3 ± 2.6 (3)	86.7 ± 1.3 (3)
PDE4, 3, 2[a], 1	Rolipram + LY195115 + cGMP + EHNA + zaprinast	88.6 ± 2.1 (3)	88.4 ± 0.5 (3)
	IBMX (1 mM)	95.7 ± 0.2 (3)	96.7 ± 0.2 (3
	Caffeine (5 mM)	75.5 ± 0.8 (4)	83.0 ± 2.4 (4)*
	Theophylline (5 mM)	90.8 ± 0.3 (2)	93.7 ± 2.1 (2)

Data are shown as mean ± SEM (N). Statistical comparisons were carried out using Student's *t*-test; $^{*}P < 0.05$, when comparing the inhibition in the microsomal membrane and cytosolic fractions. When combined, the PDE inhibitors were used at the following concentrations: rolipram: 10 μM, LY195115: 10 μM, cGMP: 5 μM, EHNA: 50 μM, zaprinast: 10 μM.

[a] PDE2 stimulated by cGMP.

were determined in the presence of 1 μM substrate. They were similar in the microsomal fraction (75 pmol/mg/min), while the cGMP activity was 1.8-fold higher than the cAMP activity in the cytosolic fraction. The cAMP- and cGMP-specific activities were, respectively, 2- and 3.8-fold higher in the cytosolic fraction than in the microsomal membrane, suggesting that 2/3 of cAMP- and 4/5 of cGMP-PDE activities were localised in the cytoplasm.

3.2. Pharmacological characterisation of PDEs in bovine MPA media

The first set of pharmacological tests was performed in the presence of various PDE inhibitors, as reported in Table 1. The total cAMP hydrolytic activity in control conditions was estimated in the presence of 1 μM cAMP and 1 mM EGTA. In the cytosolic fraction, nimodipine, a potent inhibitor of PDE1 [16] inhibited cAMP hydrolytic activity by 36%. EHNA decreased cAMP-dependent PDE activity by 20 and 56% in the absence and in the presence of cGMP, respectively. Total cAMP-PDE activity was decreased by 37–45% in the presence of PDE3 inhibitors (cilostamide, LY195115, cGMP). PDE4 inhibitors (rolipram and denbufylline) reduced the cAMP hydrolytic activity to the same extent as PDE3 inhibitors. Zaprinast, but not DMPPO, inhibited PDE cAMP hydrolytic activity by 26%. In another set of experiments, several combinations of PDE inhibitors were used to discriminate between the effective targets of these inhibitors. The mixture of rolipram and LY195115 inhibited cAMP hydrolytic activity by 72% suggesting the presence of another isozyme in addition to that of PDE3 and 4 isozymes. The addition of cGMP (5 μM) to the combination of rolipram and LY195115 produced only a further 5% inhibition of the cAMP-PDE activity, suggesting a minor participation of the PDE2 in cAMP hydrolytic activity. Moreover, in the presence of rolipram, LY195115 and cGMP, the PDE2 inhibitor EHNA did not produce a significant additional inhibitory effect (compare lines 12 and 13 in Table 1). Hence, in the presence of PDE2, 3, 4 inhibitors, zaprinast did not have a significant inhibitory effect (compare lines 13 and 14 in Table 1). The combination of PDE1, 2, 3, 4, 5 inhibitors (Table 1, line 14) decreased total cAMP-PDE activity by 88%, suggesting the presence of a cAMP-PDE isoform insensitive to the inhibitors used. Finally, non-specific PDE inhibitors such as IMBX, caffeine and theophylline were used to complete the pharmacological profile. In both subcellular fractions IBMX decreased total hydrolytic activity by 96%, confirming the presence of a residual PDE activity. The level of inhibition of cAMP hydrolytic activities induced by the various PDE inhibitors was similar in both subcellular fractions, except for LY195115, denbufylline, zaprinast and caffeine (Table 1).

The total cGMP activity in control conditions was also estimated in the presence of 1 μM cGMP and 1 mM EGTA. In the cytosolic fraction (Table 2), total cGMP-PDE activity was inhibited by 62% in the presence of DMPPO and by 69% in the presence of zaprinast, a compound probably acting on PDE5 and 1. Nimodipine only inhibited cGMP hydrolytic activity by 17 and 7% in the microsomal and cytosolic fractions, respectively, confirming a minor participation of PDE1 to the total cGMP activity under these

Table 2
Selective inhibition of the cGMP-specific PDE activity in the subcellular preparations isolated from smooth muscle of the bovine main pulmonary artery

PDE isozymes	Inhibitors	Percentage of inhibition of the cGMP-specific activity	
		Microsomal	Cytosolic
PDE1	Nimodipine (10 μM)	17.7 ± 1.4 (3)	9.7 ± 2.5 (3)*
PDE2[a]	EHNA (50 μM)	10.9 ± 3.4 (5)	14.7 ± 7.7 (5)
PDE5	DMPPO (1 μM)	59.8 ± 1.7 (4)	62.3 ± 2.9 (4)
PDE5	Zaprinast (10 μM)	64.4 ± 1.2 (5)	68.9 ± 0.9 (5)*
PDE5, 2[a]	Zaprinast (10 μM) + EHNA (50 μM)	67.2 ± 0.3 (2)	72.5 ± 1.2 (2)*
	IBMX (1 mM)	97.1 ± 0.7 (3)	97.6 ± 0.6 (3)
	Caffeine (5 mM)	56.4 ± 2.5 (4)	45.8 ± 1.8 (4)*
	Theophylline (5 mM)	81.0 ± 1.2 (3)	79.1 ± 1.7 (3)

Data are shown as mean ± SEM (N). Statistical comparisons were carried out using Student's *t*-test; $^{*}P < 0.05$ comparing the inhibition in the microsomal membrane and cytosolic fraction.

[a] PDE2 stimulated by cGMP.

conditions, i.e. in the absence of calcium. EHNA in combination with zaprinast decreased cGMP-PDE activity suggesting the presence of PDE2 hydrolysing cGMP (Table 2). IBMX induced a large inhibition (97%) of the cGMP hydrolytic activity. Contrary to IBMX, caffeine as well as theophylline were less effective on cGMP hydrolytic activity than on cAMP activity (Tables 1 and 2, last two rows).

To further assess the presence of the PDE1 in smooth muscle subcellular fractions, we tested the effect of Ca^{2+}–calmodulin complex on both cAMP and cGMP hydrolytic activities. We observed a stimulation of each specific activity in the presence of 20 nM calmodulin. This effect was especially pronounced (+177%) for the cGMP-specific activity in the cytosolic fraction (Table 3).

3.3. Chromatographic separation of PDE activities from cytosolic fraction

Fig. 2 illustrates the separation by MPLC of PDE activities from the cytosolic fraction of bovine MPA smooth muscle cells. Several peaks of cAMP hydrolytic activity were obtained, in the presence of 1 μM cAMP and 1 mM EGTA, the latter taken as the reference condition (Fig. 2A). Two major peaks of hydrolytic activity were obtained. The first peak (in fraction 23–29) was attenuated by either cGMP or LY195115 (not shown) suggesting the presence of PDE3 and was insensitive to rolipram suggesting the absence of PDE4. Furthermore, the lack of cGMP stimulation of PDE2 (Table 1) suggests the absence of PDE2 contribution to this peak. The second peak (in fraction 33–43) was reduced by 58 and 75% in the presence of 10 μM rolipram and cGMP, respectively, attesting the presence of both PDE4 and 3. PDE3 was also present in the shoulder and tail of the main peak (fraction 45–50), as indicated by the complete inhibition induced by cGMP (Fig. 2A) and LY195115 (not shown).

Fig. 2B shows the separation of cGMP hydrolytic activities. A single peak was obtained (in fraction 23–29), which was reduced by 90% in the presence of zaprinast, suggesting the presence of PDE5. This result is consistent with [the percentage of inhibition induced by either 1 μM DMPPO and 10 μM zaprinast on the cGMP-specific activity (Table 2).

Table 3
Activation of cAMP- and cGMP-specific PDE activity by calmodulin in the subcellular fractions isolated from smooth muscle of the bovine main pulmonary artery

	Percentage of activation	
	Microsomal	Cytosolic
cAMP-specific PDE activity	53.4 ± 4.5 (4)	97.8 ± 5.4 (4)*
cGMP-specific PDE activity	81.7 ± 6.5 (4)	177.6 ± 42.1 (4)*

The control experiments were performed in the presence of 1 mM EGTA while test experiments were performed in the presence of 10 μM Ca^{2+} and 20 nM calmodulin. Data are shown as mean ± SEM (N). Statistical comparisons were carried out using Student's *t*-test; $^{*}P < 0.05$ comparing the inhibition in the microsomal membrane and cytosolic fraction.

3.4. Relaxant activity of PDE inhibitors on precontracted artery rings

We tested the effects of the specific PDE inhibitors EHNA, cilostamide, rolipram and zaprinast for PDE types 2, 3, 4 and 5, respectively, as well as non-specific PDE inhibitors such as IBMX, theophylline, caffeine on rat endothelium-denuded MPA rings, precontracted with phenylephrine. All drugs tested induced concentration-dependent relaxations (Figs. 3 and 4). Based on the IC_{50} values

Table 4
Potency of PDE inhibitors on rat main pulmonary artery rings

Inhibitors	IC_{50} (μM)
EHNA	12.3
Cilostamide	0.16
Rolipram	1.6
Zaprinast	0.40
IBMX	0.58

MPA rings were precontracted with phenylephrine (0.5 μM); N = 4 for each inhibitor.

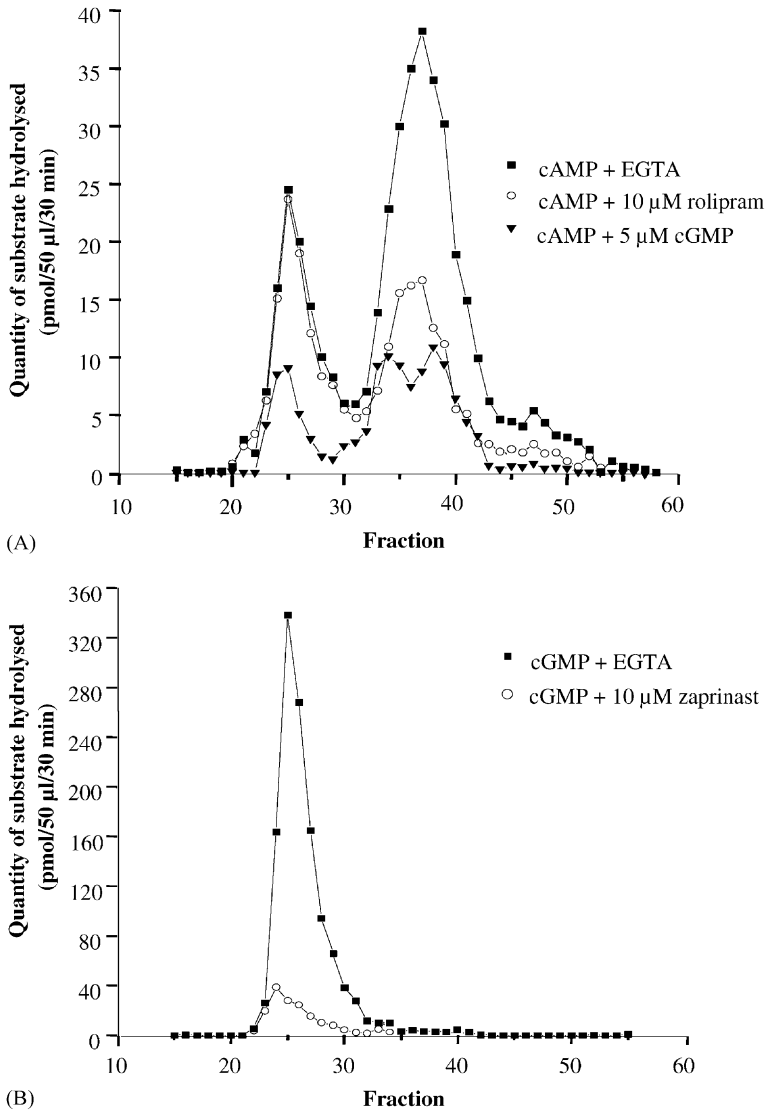

Fig. 2. Resolution of PDE activity following anion exchange chromatography of cytosoluble PDE isozymes found in smooth muscle from the bovine main pulmonary artery. (A) cAMP-specific PDE activities were determined using 1 µM [^{3}H]-cAMP in the presence of 1 mM EGTA (■), and in the additional presence of 10 µM rolipram (○) or 5 µM cGMP (▼). (B) cGMP-specific PDE activities with 1 µM [^{3}H]-cGMP in the presence of 1 mM EGTA (■) and in the additional presence of 10 µM zaprinast (○).

reported in Table 4, the rank order of potency was cilostamide > zaprinast > IBMX > rolipram >> EHNA. High concentrations (100 µM) of either cilostamide, rolipram, zaprinast or IBMX fully inhibited the phenylephrine-induced contraction, whereas EHNA produced only a modest inhibitory effect (30%). Caffeine and theophylline were also potent inhibitors of phenylephrine-induced contraction but at millimolar concentrations (Fig. 4).

4. Discussion

The present study has examined the presence of specific cyclic nucleotide-PDE activities in the media of the MPA and has revealed the major role of PDE1, 3, 4 and 5 isoforms in the control of pulmonary vascular tone.

Since vasodilatation is mainly mediated by relaxation of vascular smooth muscle cells, we paid special attention to the characterisation of the PDE activity in the pulmonary artery layer containing smooth muscle cells, i.e. media. In bovine MPA media, both cAMP and cGMP hydrolytic activities are higher in the cytosolic than in the microsomal fraction, a result in agreement with data reported for human pulmonary artery [6]. In the microsomal fraction, specific cAMP and cGMP hydrolytic activities appeared almost identical (75 pmol/mg/min), a situation different from that in tracheal smooth muscle where the cGMP hydrolytic activity is higher [28] or from that in cardiac muscle where the cAMP-specific PDE activity is the predominant hydrolytic activity [26,28]. In the present study, the total (including both subcellular fractions) cGMP-specific PDE activity appears greater than cAMP-specific one, whereas the two

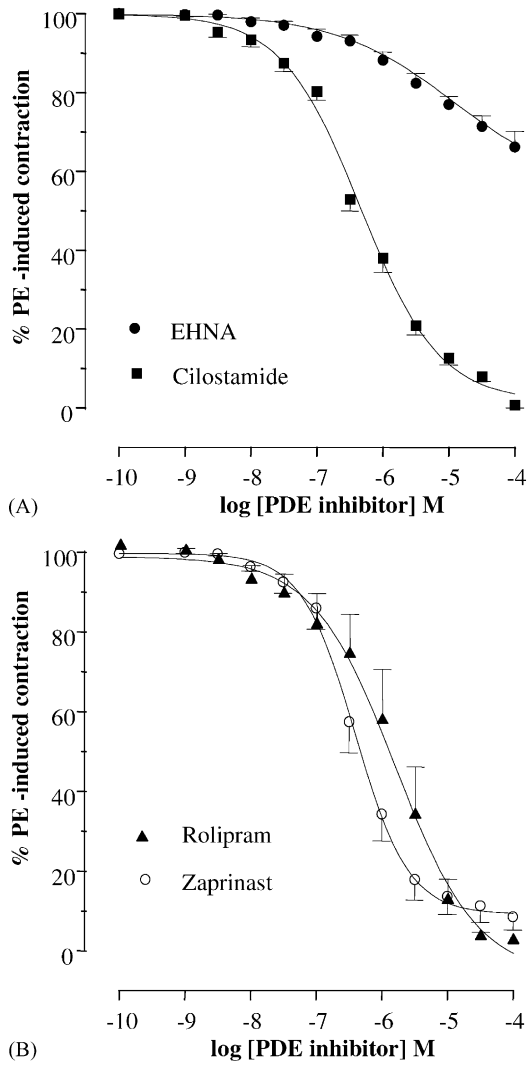

Fig. 3. Cumulative concentration-response curves for the effect of specific PDE inhibitors on contraction induced by 0.5 μM phenylephrine (PE). (A) Effect of EHNA (●), and cilostamide (■). (B) Effect of zaprinast (○) and rolipram (▲). Ordinate, contraction expressed as a percentage of the PE-induced response. Data points are mean ± SEM (N = 5–6). The IC_{50} values are summarised in Table 4.

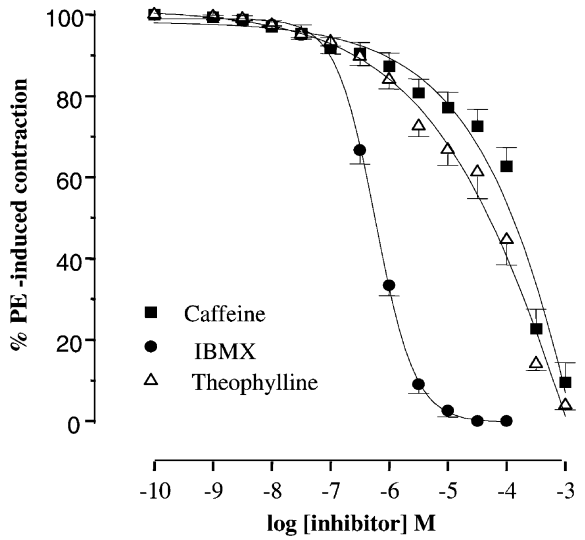

Fig. 4. Cumulative concentration-response curves for the effect of non-specific PDE inhibitors on contraction induced by 0.5 μM phenylephrine (PE). Effect of IBMX (●), theophylline (△) and caffeine (■). Ordinate, contraction expressed as a percentage of the PE-induced response. Data points are mean ± SEM (N = 5–6).

activities were equivalent in the whole MPA from the rat [17] confirming the variable quantitative distribution of PDEs isoforms in the different layers of the artery.

Study of the effect of pharmacological PDE inhibitors reveals the presence of at least five PDE isoforms in the cytosolic and microsomal fractions of pulmonary smooth muscle cells. In both subcellular fractions, cAMP-PDE activity was inhibited by 35–50%, on the one hand, by cilostamide, LY195115 and cGMP, and on the other hand, by rolipram and denbufylline. Moreover, a combination of PDE3 and 4 inhibitors further inhibited the enzymatic activity by 70–80% (Table 1) indicating that PDE3 and 4 effects were additive. In another way, analysis of chromatographic profile showed that both peaks of cAMP hydrolytic activity were inhibited by cGMP whereas only the second one was decreased by rolipram. PDE3 and 4 thus appear as the most important cAMP-dependent isozymes present in the media of bovine MPA as is the case in the aorta, another conduit artery [16]. In a similar way, cGMP-PDE activity was strongly inhibited (60–70%) by zaprinast or DMPPO, two recognised inhibitors of the type PDE5 [14]. Moreover, zaprinast inhibited by 90% the peak of cGMP hydrolytic activity (Fig. 2B). PDE5 thus appears as the main isozyme responsible for the hydrolysis of cGMP in both subcellular fractions of bovine MPA, which is consistent with the result obtained in human pulmonary artery [6]. In contrast, this activity was absent in the membrane-bound fraction of bovine aorta [15]. This difference can be ascribed to the fact that PDE5 is mainly a cytosolic enzyme [14] and, thus, the observed inhibitory effect in microsomal fractions could be due to the presence of additional zaprinast-sensitive PDEs. Such isozymes have been described in other tissues such as CaM-insensitive PDE1 [26] or PDE9A, 10A and 11A [29–31]. The present study also indicates the existence of two additional, non-specific, PDE isozymes: PDE1 and 2. cAMP hydrolytic activity was inhibited in the presence of nimodipine, a PDE1 inhibitor [16] and in the presence of 10 μM zaprinast a PDE1 and 5 inhibitor. In contrast, this activity was not altered in the presence of DMPPO, a specific PDE5 inhibitor. Both cAMP and cGMP hydrolytic activities were enhanced by calmodulin, with a marked effect for the cGMP hydrolytic activity in the cytosolic fraction, a result in accordance with the known Ca^{2+}–calmodulin-dependent regulation of PDE1, its specificity for cGMP and its preferential location in the cytosolic fraction [15]. A 15–20% inhibition of both cAMP- and cGMP-PDE activities were observed in the presence of EHNA. This effect may represent a non-specific inhibitory effect related to the high

concentration of EHNA used. The inhibitory effect of EHNA on cAMP-PDE activity was potentiated (55–60%) in the presence of cGMP, which stimulates the PDE2 and inhibits PDE3. In fact, comparison of the effect, on the one hand, of EHNA in the presence of cGMP and, on the other hand, of cGMP alone (lines 3 and 6, Table 1) indicates only a 6–12% participation of PDE2 to the cAMP hydrolytic activity. These results confirm the existence of PDE2 isozyme in bovine MPA media as in whole rat MPA [17]. However, they contrast with a study stating the absence of PDE2 in the human pulmonary artery [6] although our present observation is in accordance with the chromatographic separation performed in this latter study showing an activation of cAMP hydrolytic activities in the presence of 5 μM cGMP. It must pointed out that in the study of Rabe *et al.* [6] experiments were performed in absence of EHNA. Finally, in the bovine MPA media, combination of multiple PDE inhibitors or use of the potent but non-specific inhibitor IBMX (Tables 1 and 2) did not fully inhibit the PDE activity suggesting the presence of other PDE isozymes such as PDE7, 8A, and 9A insensitive to classical inhibitors [12,13].

In the present study, we also assessed the functional role of PDEs. An increase in either cAMP or cGMP level is generally associated with a relaxation of smooth muscle cells *via* both calcium-dependent (i.e. simulation of $Ca^{2+}Mg^{2+}$ATPases) and calcium-independent (i.e. effect on contractile apparatus) processes [32–34]. As a consequence, inhibition of cyclic nucleotide PDEs should result in a decrease in tension. In pulmonary artery media, all substances which inhibited cyclic nucleotide hydrolytic activity relaxed precontracted pulmonary artery rings. At the same concentrations as those inhibiting PDE activity, cilostamide, rolipram and zaprinast exhibited potent relaxant effects confirming the implication of PDE3, 4 and 5 in the control of pulmonary artery tone. These results are in agreement with those of a recent work [35] showing that inhibitors of PDE3 and 5 are able to blunt the acute hypoxic vasoconstriction in the rat MPA. Interestingly, it must be noted that in rat MPA, rolipram and zaprinast exhibit a relaxant action for concentrations (IC_{50}) similar to their K_i value on the enzyme (0.7–1 μM) [16]. Also in agreement with our result on PDE activity, EHNA even at a high concentration (100 μM) only evoked a weak relaxant effect suggesting that PDE2 exerts a minor role in the control of tone in this preparation. In the present work, the functional role of PDE1 was not assess due to the lack of specific inhibitor for this enzyme. For example, vinpocetine, initially described as selective inhibitor of PDE1 [36], exhibits additional effects, on the one hand, on PDE4 isoform [37] and, on the other hand, on the activation of calcium-activated potassium channels, as recently demonstrated in a patch-clamp study [38].

There is a clear cross-talk between cGMP- and cAMP-PDE-dependent signalling pathways [39]. Such cross-talk is evidenced in the present study by the inhibitory effect of cGMP on the cAMP-dependent hydrolytic activity of PDE3 (Table 1). However, such cross-talk is more difficult to address in functional experiments. For example, PDE5 inhibitors increase the cGMP concentration which, secondary, inhibits PDE3 activity. The resulting increase in cAMP concentration could either account, by itself, for the relaxant effect or stimulate PDE4 activity leading to an inverse effect on the vascular tone [39]. Also from a functional point of view, our study does not rule out the possibility that either microsomal or cytosolic PDE control the cAMP and/or cGMP levels in vicinity of the nuclei and, thus could participate in the control of gene expression through the CREB cascade [40], i.e. the sequence of events linking the increase in cytosolic cyclic nucleotide concentration to the activation of gene expression *via* the binding of the cAMP response element binding protein (CREB) to the DNA sequenced called cAMP response element (CRE).

In the present study, we also measured the effect of classical methylxanthine-derivative as non-specific PDE inhibitors. IBMX inhibited by 95–97% both cAMP- and cGMP-specific PDEs whereas theophylline and caffeine were more active on cAMP- than on cGMP-dependent PDE activity. In vascular and visceral smooth muscles including pulmonary smooth muscles, caffeine induces both transient contractile and maintained relaxant effects [41–43]. Whereas the contractile effect is clearly due do the action of caffeine on intracellular calcium release *via* ryanodine receptors [43,44], the relaxant action is generally ascribed, at least in part, to its PDE inhibitory effect, although this latter has not been so far clearly demonstrated. Setting apart the effects of caffeine on the Ca^{2+} release through the ryanodine-sensitive Ca^{2+} release channels and, the ensuing activation of calcium-activated potassium channels (BK_{Ca}) which in turn could induce a hyperpolarisation and a concomitant vascular smooth muscle relaxation [45], our results show that caffeine at concentrations generally used for *in vitro* functional experiments (mM range) is an effective PDE inhibitor in pulmonary vascular smooth muscle.

In conclusion, the present study has shown the presence of at least five PDE isoforms in the MPA. These PDE isozymes are present in the smooth muscle cells-containing layer of the pulmonary artery wall, i.e. media. Combination of biochemical, pharmacological and functional experiments indicate that PDE1, 3, 4 and 5 are the main isoforms involved in the control of pulmonary vascular tone. Further studies should now assess the effect of a combination of specific inhibitors of these four isozymes under conditions of increased pulmonary vascular tone such as pulmonary arterial hypertension.

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