Pig aortic endothelial-cell cyclic nucleotide phosphodiesterases

Use of phosphodiesterase inhibitors to evaluate their roles in regulating cyclic nucleotide levels in intact cells

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Two cyclic nucleotide phosphodiesterase (PDE) activities were identified in pig aortic endothelial cells, a cyclic GMP-stimulated PDE and a cyclic AMP PDE. Cyclic GMP-stimulated PDE had K_m values of 367 μM for cyclic AMP and 24 μM for cyclic GMP, and low concentrations (1 μM) of cyclic GMP increased the affinity of the enzyme for cyclic AMP ($K_m = 13 \mu\text{M}$) without changing the V_{max} . This isoenzyme was inhibited by trequinsin [IC_{50} (concn. giving 50% inhibition of substrate hydrolysis) = 0.6 μM for cyclic AMP hydrolysis in the presence of cyclic GMP; $\text{IC}_{50} = 0.6 \mu\text{M}$ for cyclic GMP hydrolysis] and dipyrindamole ($\text{IC}_{50} = 5 \mu\text{M}$ for cyclic AMP hydrolysis in the presence of cyclic GMP; $\text{IC}_{50} = 3 \mu\text{M}$ for cyclic GMP hydrolysis). Cyclic AMP PDE exhibited a K_m of 2 μM for cyclic AMP and did not hydrolyse cyclic GMP. This activity was inhibited by trequinsin ($\text{IC}_{50} = 0.2 \mu\text{M}$), dipyrindamole ($\text{IC}_{50} = 6 \mu\text{M}$) and, selectively, by rolipram ($\text{IC}_{50} = 3 \mu\text{M}$). Inhibitors of cyclic GMP PDE (M&B 22948) and of low K_m (Type III) cyclic AMP PDE (SK&F 94120) only weakly inhibited the two endothelial PDEs. Incubation of intact cells with trequinsin and dipyrindamole induced large increases in cyclic GMP, which were completely blocked by LY-83583. Rolipram, SK&F 94120 and M&B 22948 did not significantly influence cyclic GMP accumulation. Dipyrindamole enhanced the increase in cyclic GMP induced by sodium nitroprusside. Cyclic AMP accumulation was stimulated by dipyrindamole and trequinsin with and without forskolin. Rolipram, although without effect alone, increased cyclic AMP in the presence of forskolin, whereas M&B 22948 and SK&F 94120 had no effects on resting or forskolin-stimulated levels. These results suggest that cyclic GMP-stimulated PDE regulates cyclic GMP levels and that both endothelial PDE isoenzymes contribute to the control of cyclic AMP.

INTRODUCTION

The cyclic nucleotide cascades of endothelial cells are targets for a number of endogenous substances which may modify endothelial function. Endothelium-derived relaxing factor (EDRF) and atrial natriuretic factors increase cyclic GMP synthesis through activation of the soluble and particulate guanylate cyclases, respectively (Martin *et al.*, 1988). Activation of the cyclic AMP cascade has also been demonstrated in intact cells, and a number of substances, including adenosine and calcitonin-gene-related peptide, stimulate adenylate cyclase in isolated endothelial-cell membrane preparations (Dembinska-Kiec *et al.*, 1980; Makarski, 1981; Goldman *et al.*, 1983; Crossman *et al.*, 1987).

Little is known of the processes in endothelial cells responsible for the catabolism of cyclic AMP and cyclic GMP. In the studies reported herein, we have partially purified the phosphodiesterases (PDEs) from primary cultures of pig aortic endothelial cells and attempted to characterize them by employing kinetic analysis, responsiveness to intracellular regulators and susceptibility to the actions of selective inhibitors as the criteria for categorization. Furthermore, we have attempted to evaluate the relevance of the PDE isoenzymes in intact

cells by studying the effects of selective inhibitors on cyclic AMP and cyclic GMP accumulation.

METHODS AND MATERIALS

Materials

Cyclic [2,8- ^3H]AMP (41 Ci/mmol) and cyclic [8- ^3H]GMP (13.8 Ci/mmol) were purchased from Amersham International (Amersham, Bucks., U.K.). DEAE-Trisacryl was supplied by IBF (Villeneuve La Garenne, France). M&B 22948 (2-*O*-propoxyphenyl-8-azapurin-6-one) was synthesized by the Department of Pharmaceutical Chemistry, Rhône-Poulenc Ltd. (Dagenham, Essex, U.K.). SK&F 94120 [5-(4-acetamidophenyl)pyrazin-2(1*H*)-one] was a gift from Smith Kline and French Research Ltd. (Welwyn, Herts., U.K.), and rolipram [ZK-62711; 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone] was generously provided by Schering A.G. (Hounslow, Middx., U.K.). Trequinsin (HL-725; 9,10-dimethoxy-2-mesitylimino-3-methyl-3,4,6,7-tetrahydro-2*H*-pyrimido[6,1-*a*]isoquinolin-4-one) was supplied by Hoechst Pharmaceuticals (Hounslow, Middx., U.K.). LY-83583 (6-anilino-5,8-quinolinedione) was generously provided by Ely Lilly and Co.

Abbreviations used: PDE, cyclic nucleotide phosphodiesterase (EC 3.1.4.17); EDRF, endothelium-derived relaxing factor; IC_{50} , concentration giving 50% inhibition of substrate hydrolysis.

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(Indianapolis, IN, U.S.A.). Acetylated low-density lipoprotein labelled with 1,1'-dioctadecyl-1,3,3',3'-tetramethylindocarbocyanine perchlorate was purchased from Biogenesis Ltd. (Bournemouth, Hants., U.K.). Forskolin was purchased from Cambridge Bioscience (Cambridge, U.K.). Collagenase (LC52) was purchased from Worthington Biochemicals Corp. (Freehold, NJ, U.S.A.). All other chemicals were obtained from Sigma Chemical Co., BDH Chemicals (both of Poole, Dorset, U.K.) and May & Baker Laboratory Chemicals (Eccles, Manchester, U.K.).

Endothelial-cell culture

The endothelial cells were cultured by a modification of the method previously described by Martin *et al.* (1988). The cells grew as a strict monolayer and exhibited the characteristic 'cobblestone' appearance. In randomly selected cultures, over 95% of the cells were observed to fluoresce after incubation with acetylated low-density lipoprotein labelled with 1,1'-dioctadecyl-1,3,3',3'-tetramethylindocarbocyanine perchlorate (Voyta *et al.*, 1984).

Partial purification of endothelial-cell PDEs

Cells (100–150 million) grown in 250 ml culture flasks (Nunc) were washed three times with phosphate-buffered saline (Dulbecco & Vogt, 1954) before being scraped from the surface and removed to a 50 ml polypropylene centrifuge tube. The cells were then centrifuged (2000 *g*, 5 min) and, after the phosphate-buffered saline had been removed, the pellet was stored at -70°C until required.

The cell pellet was homogenized in 6 vol. of 20 mM-Tris/HCl (pH 7.5) containing 2 mM-magnesium acetate, 1 mM-dithiothreitol, 5 mM-EDTA and 2000 units of aprotinin/ml with a Dounce homogenizer. The homogenate was then centrifuged at 105 000 *g* for 60 min and the supernatant (20 ml) applied to a DEAE-Trisacryl column (7 cm $\times$ 0.9 cm) pre-equilibrated with column buffer (20 mM-Tris/HCl, 2 mM-magnesium acetate, 1 mM-dithiothreitol, 20 μM -*N*^α-tosyl-L-lysylchloromethane hydrochloride, pH 7.5). The column was washed with 50 ml of column buffer, and PDE activities were eluted with two successive linear gradients of NaCl (0–150 mM in 80 ml and 150–400 mM in 70 ml) in column buffer; 2 ml fractions were collected, assayed, and for short-term storage at -20°C , ethylene glycol was added to a final concentration of 30% (v/v). Assays on the pooled peak fractions were performed within 48 h after homogenization of the cells.

Measurement of PDE activity

PDE activity was determined by the two-step radioisotope method of Thompson *et al.* (1979). The reaction mixture contained 20 mM-Tris/HCl (pH 8.0), 10 mM-MgCl₂, 4 mM-2-mercaptoethanol and 0.05 mg of bovine serum albumin/ml. Unless otherwise stated, the concentration of substrate (cyclic [³H]AMP or cyclic [³H]GMP) was 1 μM .

The IC₅₀ values (concentration which produced 50% inhibition of substrate hydrolysis) for the compounds examined were determined from concentration–response curves, in which concentrations ranged from 0.1 μM to 1 mM. Three concentration–response curves were generated for each agent.

For the determination of V_{max} and K_m values, the concentration of cyclic AMP or cyclic GMP was varied

while the amount of ³H-labelled cyclic nucleotide remained constant. The Lineweaver–Burk plots were analysed by linear least-squares regression analysis to obtain K_m values.

Protein was determined as described by Lowry *et al.* (1951), with bovine serum albumin as the standard.

Categorization of PDE isoenzymes

The nomenclature for the different cyclic nucleotide PDEs adopted in this paper is based on that of Beavo (1988) and Torphy (1988).

Measurement of cyclic nucleotides

Cyclic nucleotide studies were performed as previously described (Martin *et al.*, 1988). Drugs were added to the cells at the concentrations and times indicated in the Results section. The DNA content of the cell pellets was measured by an automated procedure employing the fluorochrome Hoechst 33258 (Sterzel *et al.*, 1985).

Statistical analysis

Data are presented as means $\pm$ S.E.M. and analysed by Student's *t* test or, where stated, the Mann–Whitney test. Values were considered to be statistically significant when *P* was less than 0.05.

RESULTS

Endothelial-cell cyclic nucleotide PDEs

The present studies demonstrated the presence of two PDE isoenzymes in pig aortic endothelial cells, a cyclic GMP-stimulated isoform and a cyclic AMP PDE exhibiting properties similar to those of the Type IV PDE identified in cardiac muscle by Reeves *et al.* (1987).

The cytosolic fraction of pig aortic endothelial cells contained 58% of the cyclic GMP hydrolytic activity (assayed at 1 μM substrate concentration) and 58% of the cyclic AMP hydrolytic activity (assayed at 2 μM substrate concentration) (Table 1). Addition of Ca²⁺/calmodulin had no effect on either cyclic GMP or cyclic AMP hydrolysis in the particulate and soluble fractions, indicating that the calmodulin-stimulated (Type 1b) PDE is absent in endothelial cells (Table 1). Addition of cyclic GMP (1 μM) to the cytosol and pellet increased cyclic AMP hydrolysis (2 μM substrate concn.) approx. 2-fold and did not alter the percentage of total cyclic AMP PDE activity associated with each fraction (Table 2).

Two peaks of cytosolic PDE activity were resolved by DEAE-Trisacryl ion-exchange chromatography (Fig. 1). The first peak, eluted at 0.12 M-NaCl, exhibited activity against both cyclic GMP and cyclic AMP, and addition of 1 μM -cyclic GMP stimulated cyclic AMP hydrolysis (measured with 2 μM substrate) 4-fold. The second peak of activity was eluted with 0.19 M-NaCl and selectively hydrolysed cyclic AMP, displaying little or no activity against cyclic GMP. Over 90% of cyclic GMP PDE and cyclic AMP PDE activities applied to the DEAE-Trisacryl column were recovered after elution with the NaCl gradient.

Kinetic analysis revealed that the peak-1 enzyme exhibited values of $>200 \mu\text{M}$ for cyclic AMP and 24 μM for cyclic GMP (results not shown). The kinetics suggested positive co-operativity. Addition of 1 μM -cyclic GMP decreased the apparent K_m for cyclic AMP to 13 μM without affecting the V_{max} . Lineweaver–Burk plots

Table 1. Effects of Ca^{2+} + calmodulin on cyclic GMP PDE and cyclic AMP PDE in cytosolic and particulate fractions of endothelial cells

Cells were homogenized and subcellular fractions prepared as described in the Methods and materials section. Assays were performed at 30 °C in the presence of 200 μM -EGTA with or without CaCl_2 (2 mM) plus calmodulin (calm) (10 units/ml). The substrate concentration was 1 μM for cyclic GMP and 2 μM for cyclic AMP. Results represent means $\pm$ S.E.M. of three experiments. Values in parentheses show % of total cellular activity.

Fraction	Cyclic nucleotide hydrolysis (pmol/min per mg)			
	Cyclic GMP		Cyclic AMP	
	No addition	+ Ca^{2+} /calm	No addition	+ Ca^{2+} /calm
Homogenate	97 $\pm$ 9	99 $\pm$ 17	101 $\pm$ 20	104 $\pm$ 27
Cytosol	61 $\pm$ 13 (58 %)	59 $\pm$ 7 (58 %)	58 $\pm$ 16 (58 %)	58 $\pm$ 17 (55 %)
Pellet	126 $\pm$ 58 (42 %)	129 $\pm$ 64 (42 %)	164 $\pm$ 63 (42 %)	206 $\pm$ 95 (45 %)

Table 2. Effect of cyclic GMP on cyclic AMP PDE activity in subcellular fractions of pig aortic endothelial cells

Assays were performed at 30 °C in the presence of EGTA (200 μM) with or without cyclic GMP (1 μM). Results represent the means $\pm$ S.E.M. of three experiments. Values in parentheses show % of total activity.

Fraction	[Substrate]...	Cyclic AMP hydrolysis (pmol/min per mg)			
		No addition		+ cyclic GMP (1 μM)	
		0.25 μM	2 μM	0.25 μM	2 μM
Homogenate		27 $\pm$ 1	105 $\pm$ 5	32 $\pm$ 4	242 $\pm$ 28
Cytosol		21 $\pm$ 4 (73 %)	64 $\pm$ 17 (58 %)	25 $\pm$ 4 (70 %)	149 $\pm$ 15 (62 %)
Pellet		41 $\pm$ 16 (27 %)	147 $\pm$ 67 (42 %)	53 $\pm$ 17 (30 %)	311 $\pm$ 127 (38 %)

Fig. 1. DEAE-Trisacryl chromatography of PDE activity of the 100000 g supernatant fraction from pig aortic endothelial cells

Preparation of a 100000 g cytosolic fraction from pig aortic endothelial cells and its chromatography on DEAE-Trisacryl is described in the Methods and materials section. Fractions were assayed for cyclic AMP PDE activity (2 μM substrate) in the absence ($\blacklozenge$) and presence ($\blacktriangledown$) of 1 μM -cyclic GMP, and for cyclic GMP PDE activity (1 μM substrate) in the absence ($\blacksquare$) and presence ($\bullet$) of 2 mM- CaCl_2 plus 0.5 unit of calmodulin. All assays were performed in the presence of 200 μM -EGTA.

revealed linear kinetics and a K_m of 2 μM for cyclic AMP hydrolysis by peak-2 isoenzyme.

The activity of selective cyclic nucleotide PDE inhibitors against endothelial-cell cyclic GMP-stimulated and cyclic AMP PDEs is presented in Table 3. M&B 22948, a selective inhibitor of the Ca^{2+} -dependent and

Ca^{2+} -independent cyclic GMP PDEs (Types 1b and 1a respectively) in smooth muscle (Lugnier *et al.*, 1986), was only weakly effective in inhibiting the peak 1 and peak 2 PDEs. Similarly, SK&F 94120, a selective inhibitor of the low- K_m cyclic GMP-inhibited Type III cyclic AMP PDE (Reeves *et al.*, 1987), also exhibited only weak

Table 3. Inhibition of separate PDE activities by various compounds

IC₅₀ values were determined on cyclic GMP-stimulated PDE with 2 μ M-cyclic AMP as substrate in the presence or absence of 1 μ M-cyclic GMP, or with 1 μ M-cyclic GMP as substrate. Cyclic AMP PDE was assayed with 1 μ M-cyclic AMP as substrate. The results represent the means of duplicate determinations performed on two different batches of the enzymes.

Compound	IC ₅₀ (μ M)			
	Cyclic GMP-stimulated PDE			Cyclic AMP PDE
	Cyclic AMP	Cyclic AMP (+cyclic GMP)	Cyclic GMP	
M&B 22948	708	45	50	93
Trequinsin	4	0.6	0.6	0.2
Dipyridamole	17	5	3	6
SK&F 94120	643	485	558	> 1000
Rolipram	435	448	417	3

inhibitory activity against the two cytosolic endothelial isoenzymes. In contrast, trequinsin and dipyridamole, two non-selective PDE inhibitors, potentially inhibited both isoenzymes, whereas rolipram, a selective Type IV cyclic AMP PDE inhibitor (Reeves *et al.*, 1987), selectively inhibited the peak 2 PDE isoenzyme. Cyclic GMP only weakly inhibited (IC₅₀ > 100 μ M) the peak 2 cyclic AMP PDE.

Effects of selective PDE inhibitors on cyclic nucleotide accumulation

Dipyridamole (100 μ M) and trequinsin (10 μ M) induced large (1000 % and 1500 % respectively) increases in cyclic GMP (Table 4). These actions of the non-selective PDE inhibitors were dose-dependent over the concentration range 8–200 μ M (results not shown). Rolipram, M&B 22948 and SK&F 94120 did not significantly influence cyclic GMP levels (Table 4). The stimulatory actions of dipyridamole and trequinsin on cyclic GMP were abolished by LY-83583 (10 μ M), which interferes with the release of EDRF and inhibits guanylate cyclase (Mulsch *et al.*, 1988) (Table 5). Methylene Blue (10 μ M), an inhibitor of guanylate cyclase, and haemoglobin (10 μ M), which blocks the actions of EDRF (Martin *et al.*, 1985), were similarly effective in blocking these effects of the two PDE inhibitors (results not shown).

The increase in intracellular cyclic GMP induced by

dipyridamole (50 μ M) reached a peak 5–10 min after the initial exposure and then declined rapidly, to a level 2–3-fold above basal after 30 min, which then remained constant for up to 60 min. Leakage of cyclic GMP into the medium was greatly increased by dipyridamole, reaching a plateau after 15 min and remaining constantly elevated for up to 60 min (results not shown).

Dipyridamole (25 μ M) enhanced (3-fold) the increase in cyclic GMP induced by sodium nitroprusside (1 μ M), a stimulator of soluble guanylate cyclase (Waldman & Murad, 1987) (Table 6).

The effects of PDE inhibitors on cyclic AMP levels were tested with and without the activator of adenylate cyclase, forskolin (Table 7). Dipyridamole (100 μ M) and trequinsin (10 μ M) both significantly increased resting cyclic AMP (52 % and 100 % respectively) and increased accumulation in the presence of 10 μ M-forskolin (179 % and 246 % respectively), which, alone, elicited a slight but non-significant effect. Rolipram (50 μ M) had no effect alone, but significantly increased cyclic AMP accumulation in the presence of forskolin (72 %). Neither M&B 22948 nor SK&F 94120 significantly influenced cyclic AMP levels in either the absence or the presence of forskolin. As with cyclic GMP, endothelial cells extruded cyclic AMP into the extracellular medium. This leakage was increased by PDE inhibitors that elevated intracellular cyclic AMP levels (results not shown).

Table 4. Effects of PDE inhibitors on cyclic GMP accumulation

Cells were exposed to PDE inhibitors for 10 min before the incubation was terminated and cyclic GMP quantified as described in the Methods and materials section. The results represent means $\pm$ S.E.M. ($n = 6$); ** $P < 0.01$ for significant difference from control.

Inhibitor	Cyclic GMP (pmol/ μ g of DNA)
None	0.10 $\pm$ 0.02
M&B 22948 (50 μ M)	0.10 $\pm$ 0.02
Dipyridamole (100 μ M)	1.11 $\pm$ 0.28**
SK&F 94120 (100 μ M)	0.11 $\pm$ 0.02
Trequinsin (10 μ M)	1.59 $\pm$ 0.46**
Rolipram (50 μ M)	0.15 $\pm$ 0.05

Table 5. Effects of dipyridamole and trequinsin on the cyclic GMP content of pig aortic endothelial cells in the presence and absence of LY-83583

Cells were preincubated with or without LY-83583 (10 μ M) for 10 min before exposure to dipyridamole and trequinsin for a further 10 min. The results represent means $\pm$ S.E.M. ($n = 4$); ** $P < 0.01$ for significant differences from control.

Inhibitor	Cyclic GMP (pmol/ μ g of DNA)	
	No LY-83583	+ LY-83583
None	0.07 $\pm$ 0.03	0.04 $\pm$ 0.02
Dipyridamole (100 μ M)	0.52 $\pm$ 0.07**	0.05 $\pm$ 0.01
Trequinsin (10 μ M)	0.29 $\pm$ 0.05**	0.04 $\pm$ 0.01

Table 5. Potentiation of sodium nitroprusside-induced cyclic GMP accumulation by dipyridamole

Cells were preincubated for 35 min with or without dipyridamole (25 μM), during which time the rapid rise in intracellular cyclic GMP in response to the PDE inhibitor had diminished from a peak value of 0.31 ± 0.09 pmol/ μg of DNA after 5 min to the value indicated. Cells were then incubated for a further 2 min with or without sodium nitroprusside (SNP; 1 μM). The results represent means $\pm$ S.E.M. Since some data in this table are significantly non-normal (Wilk-Shapiro test), statistical significance was assessed by the non-parametric Mann-Whitney test; $**P < 0.01$, $*P < 0.05$ denotes significant difference from control or a significant difference between two groups joined by a brace.

Addition	Cyclic GMP (pmol/ μg of DNA)	n
Control	0.05 ± 0.01	6
Dipyridamole	$0.11 \pm 0.03^{**}$	5
SNP	$0.51 \pm 0.17^{**}$	5
Dipyridamole + SNP	$1.16 \pm 0.26^{**}$ *	6

DISCUSSION

The important regulatory role which the vascular endothelium plays as an interface between blood and extravascular tissue, controlling interactions between leucocytes and the vessel wall, vascular tone, coagulation and passage of fluids, proteins and cells into and out of blood vessels has only recently been appreciated. The role of cyclic nucleotides in these processes is unclear, but the coupling of endothelial receptors to both the cyclic AMP and cyclic GMP cascades indicates that they serve a second-messenger role. Indeed, preliminary observations suggest that permeability (Stelzner *et al.*, 1988; Mizuno-Yagyu *et al.*, 1987) and release of humoral agents (Doni *et al.*, 1988; Shimokawa *et al.*, 1988) are under the influence of cyclic nucleotides. The demonstration of two cyclic nucleotide PDEs in pig aortic endothelial cells (Fig. 1) suggests that the degradation of cyclic AMP and cyclic GMP is sensitively controlled, lending further weight to the contention that they serve an important regulatory function.

Previous studies (Lugnier & Schini, 1988) also demonstrated the presence of a rolipram-inhibited cyclic AMP

PDE and a cyclic GMP-stimulated PDE in bovine aortic endothelial cells. This latter isoenzyme comprised only a small percentage of the total activity and, curiously, was only detected in 50% of the endothelial-cell cytosolic preparations. In contrast, in our pig aortic endothelial cell-free extracts, cyclic GMP-stimulated PDE accounted for all the cyclic GMP-hydrolytic activity and, in the presence of 1 μM -cyclic GMP, well over 50% of the total cyclic AMP-hydrolytic activity. The presence of the cyclic GMP-stimulated PDE suggests that elevation of cyclic GMP could decrease cyclic AMP concentrations in endothelial cells and, consequently, that these two cyclic nucleotides would have opposing effects. Evidence for this has been obtained in fibroblasts where the decrease in the content of cyclic AMP in response to atrial natriuretic factors has been ascribed to activation of PDE (Lee *et al.*, 1988). Thus far, we have been unable to demonstrate a decrease in endothelial cyclic AMP levels with agents that stimulate cyclic GMP accumulation (results not shown).

Data presented herein suggest that cyclic GMP-stimulated PDE plays a predominant role in regulating endothelial cyclic GMP: it is the only endothelial PDE which hydrolyses cyclic GMP (Fig. 1) and, furthermore, non-selective PDE inhibitors induced a large increase (>10-fold) in cyclic GMP, whereas rolipram, a selective inhibitor of Type IV cyclic AMP PDE, was without significant effect (Table 4). The sizes of the responses to dipyridamole and trequinsin suggest a rapid intracellular turnover of cyclic GMP, with synthesis being dependent on activation of soluble guanylate cyclase by EDRF, as demonstrated by the complete abolition of the effects of the PDE inhibitors by LY-83583 (Table 5), Methylene Blue and haemoglobin (results not shown). That PDE inhibitors potentiate the actions of stimulants of soluble guanylate cyclase is reinforced by the enhancement of the sodium nitroprusside-induced rise in cyclic GMP by dipyridamole (Table 6).

Neither calmodulin-independent cyclic GMP PDE (Type 1a) nor calmodulin-dependent PDE (Type 1b) reside in pig aortic endothelial cells. Not surprisingly, M&B 22948, a selective cyclic GMP PDE inhibitor (Lugnier *et al.*, 1986) exerted no influence on cyclic GMP levels (Table 4). This later result contrasts with previous reports (Martin *et al.*, 1988) in which slightly elevated cyclic GMP levels were observed in response to this compound; however, in that study, higher concentrations

Table 7. Effects of PDE inhibitors on cyclic AMP accumulation in the presence and absence of forskolin

Cells were exposed to PDE inhibitors for 10 min before being incubated with or without sub-maximal concentration of forskolin (10 μM) for a further 2 min. The results represent means $\pm$ S.E.M.; $*P < 0.05$ denotes significant difference of PDE inhibitor alone from control; $\dagger P < 0.05$, $\dagger\dagger P < 0.01$, denotes significant effect of PDE inhibitor + forskolin from forskolin alone.

Inhibitor	Cyclic AMP (pmol/ μg of DNA)			
	No forskolin	n	+Forskolin	n
None	1.08 ± 0.13	10	1.39 ± 0.21	9
M&B 22948 (50 μM)	1.08 ± 0.17	6	1.43 ± 0.25	5
Dipyridamole (100 μM)	$1.64 \pm 0.20^*$	10	$3.88 \pm 0.85^\dagger$	5
SK&F 94120 (100 μM)	1.14 ± 0.20	6	1.84 ± 0.36	5
Trequinsin (10 μM)	$2.18 \pm 0.38^*$	10	$4.81 \pm 0.90^\dagger\dagger$	5
Rolipram (50 μM)	1.27 ± 0.13	10	$2.39 \pm 0.35^\dagger$	9

of M&B 22948 (100 μ M) were employed, which may have been sufficient to inhibit cyclic GMP-stimulated PDE, leading to an increase in cyclic GMP. In tissues where the calmodulin-independent cyclic GMP PDE (Type 1a) is present, such as smooth muscle, concentrations of M&B 22948 as low as 1 μ M can elevate cyclic GMP (Lugnier *et al.*, 1986).

The importance of Type IV cyclic AMP PDE in the regulation of endothelial cyclic AMP was demonstrated by the increase observed when cells were incubated with rolipram in the presence of the adenylate cyclase activator, forskolin (Table 7). The contribution of cyclic GMP-stimulated PDE to cyclic AMP metabolism is uncertain; however, involvement is suggested, since non-selective PDE inhibitors increased cyclic AMP in the absence of forskolin, whereas, under these conditions, rolipram was ineffective.

In conclusion, pig aortic endothelial cells contain two major cyclic nucleotide PDE species, a cyclic AMP-specific PDE and a cyclic GMP-stimulated PDE. Cyclic GMP-stimulated PDE plays a dominant role in regulating intracellular cyclic GMP, whereas both isoenzymes are probably involved in controlling cyclic AMP levels.

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