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SELECTIVE INHIBITION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASES OF HUMAN, BOVINE AND RAT AORTA

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Abstract—Cyclic nucleotide phosphodiesterase (PDE) activity from the 105,000 *g* supernatant of human, bovine and rat aorta smooth muscle cells was resolved by DEAE-trisacryl chromatography into three major forms showing similar properties in each species. In addition to the two PDE forms previously characterized in vascular tissues (a cAMP-PDE and a calmodulin-dependent PDE), a cGMP-PDE, insensitive to calmodulin, was isolated and characterized in the aorta of the three species. Each isolated PDE form was differently inhibited by various chemical compounds, and these compounds produced effects on cyclic nucleotide levels in isolated rat aorta which could be expected from their inhibitory effect on isolated PDE forms. At concentrations non-selectively inhibiting the three isolated PDE forms (including the calmodulin-dependent one), IBMX (3-isobutyl-1-methylxanthine) and trequinsin markedly and dose-dependently increased both cAMP and cGMP aorta levels (up to 7-fold, in presence of 500 μM IBMX). By contrast selective inhibitors of cGMP-PDE or cAMP-PDE could only induce a moderate elevation (by 1.5–3-fold) in cGMP or cAMP levels, respectively. In the case of M&B 22,948, a highly specific and potent inhibitor of cGMP-PDE, a concentration-dependent increase in tissue cGMP levels was produced by concentrations (in the μM range) active in inhibiting the isolated enzyme. In the case of selective cAMP-PDE inhibitors (rolipram and Ro 20-1724), however, a significant increase in aorta cAMP content was induced only in the presence of drug concentrations which were much higher (200 and 500 μM, respectively) than those inhibiting the isolated enzyme (IC_{50}: 5 and 18 μM, respectively). Inhibitors of both cGMP-PDE and cAMP-PDE (dipyridamole, cilostamide and its derivative AAL 05) produced the same moderate effects as did the combination of a selective cGMP-PDE inhibitor and a selective cAMP-PDE inhibitor on the levels of both cGMP and cAMP. These results show that the three forms of PDE isolated from aortic smooth muscle retain properties that they exhibit in the tissue and which are similar in the three species examined, including man. They suggest that each form participates in a specific manner to the regulation of cAMP and cGMP concentrations in aorta smooth muscle cells.

Cyclic nucleotide phosphodiesterases (PDEs)† play a major part in the regulation of intracellular concentrations of adenosine and guanosine 3′:5′-cyclic monophosphates (cAMP and cGMP, respectively) [1]. Multiple forms of PDEs have been separated from many tissues [2–4]. It would appear that different isoenzymes are involved in the regulation of intracellular concentrations of cAMP and cGMP. However, the relationships between the properties of the purified forms and PDE activity within cells remains to be established. The use of selective inhibitors may provide a means to characterize isolated PDE forms and to investigate the roles of the PDEs in regulating the activity and the tissue content of cAMP and cGMP.

In the case of vascular smooth muscle, two PDE forms have been isolated from porcine coronary arteries [5–9]: one form is relatively specific for cAMP and the other is activated by calmodulin (CaM) and hydrolyzes both cAMP and cGMP. In previous studies in our laboratory, however, one additional form of PDE that is specific for cGMP but insensitive to CaM has been isolated from bovine aorta, using a different technique [10]. Recently Hagiwara *et al.* [11] have also chromatographically resolved PDE activity from rabbit aorta into three peaks, but these peaks did not include a form of PDE that selectively hydrolyses cAMP. These different results could be related to differences either in tissue origin or in the method of fractionation. We were able to show in a previous work [12] that platelet and aorta PDEs were different, and thus a possible difference between tissues was not excluded. Therefore in the present paper fractionation of aortic PDE activity was performed in three species (bovine, human and rat) under identical conditions.

The effects of xanthine derivatives and papaverine have been previously studied on the two forms of PDE isolated from porcine coronary arteries and on the cyclic nucleotide contents of this tissue [6, 9, 13]. A number of other compounds have been proved to inhibit potently or selectively the different PDE isolated from various non vascular tissues [14–17]. We report here the ability of some of these various and structurally different compounds to: (1) inhibit

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† Abbreviations: cAMP, adenosine 3′:5′-cyclic monophosphate; cGMP, guanosine 3′:5′-cyclic monophosphate; PDE, 3′:5′-cyclic nucleotide phosphodiesterase; EGTA, ethylene glycol bis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′tetraacetic acid.

the PDEs isolated from aortic smooth muscle, and (2) increase cAMP and/or cGMP content in the same tissue in relation to the substrate specificity of the inhibited PDE form.

The results show no species differences: three PDE forms displaying similar properties in each species could be isolated. These three enzymes, including one form that specifically hydrolyses cGMP and is insensitive to CaM, seem to be representative of the PDEs contained in aorta smooth muscle cells, since their respective inhibitors produced increases in the tissue contents of cyclic nucleotides which would be expected from their known inhibition of the isolated forms.

MATERIALS AND METHODS

Phosphodiesterase preparations. All manipulations were conducted at +4°. Tissues were minced and homogenized using an Ultra Turrax (excepted for rat aorta) and a glass pestle homogenizer in 6 vol. (w/v) of buffer A (20 mM Tris–HCl, 2 mM Mg acetate, 1 mM dithiothreitol, 5 mM EDTA, 2000 U/ml aprotinin, pH 7.5). In this buffer, phosphodiesterase preparations did not hydrolyze ^{14}C-methylcasein. The homogenate was centrifuged at 105,000 *g* for 60 min. The supernatant was applied to a DEAE-trisacryl (IBF) column and first eluted by buffer B (20 mM Tris–HCl, 2 mM Mg acetate, 1 mM dithiothreitol, pH 7.5) until no more absorbance was detected in the eluate at 280 nm. Elution was then continued at a flow rate of 50 ml/hr with a linear NaCl gradient in buffer B. Above 90% of the supernatant PDE activity was recovered after elution in the different fractions. The different peaks collected were dialysed overnight with 3 changes of buffer C (20 mM Tris–HCl, 2 mM Mg acetate, pH 7.5), fractionated into aliquots and stored at −80°. Bovine serum albumin (1 mg/ml) was added to the aliquots devoted to inhibition studies.

Phosphodiesterase assay. Phosphodiesterase activities were determimed according to Wells *et al.* [5], in the presence of calcium ($CaCl_2$ 10 μM) and an excess of CaM (15 nM), or in absence of CaM and calcium (in presence of 1 mM EGTA). CaM was obtained from rat brain [18] or bovine brain [19]. Kinetic studies were carried out with substrate concentrations ranging from 0.1 to 100 μM. Apparent Michaelis constant (K_m) and maximal velocity (V_m) values were derived by computer analysis according to Cleland [20]. Both incubation time and enzyme concentration in the assay medium were adjusted so that no more than 15% of the substrate was hydrolyzed under the assay conditions. The concentration of each drug which inhibited 50% of the enzymatic activity (IC_{50}) was determined at 1 μM substrate concentration. The curve obtained by plotting % enzymatic activity vs the logarithmic concentration of the inhibitor included 6 concentrations of inhibitors in its linear portion. The IC_{50} was calculated by linear regression (correlation coefficient >0.95).

Measurement of cyclic nucleotide levels. Rat aortic cyclic nucleotide levels were determined as previously described [21]. Briefly, the rat thoracic aorta was removed, cleaned of adjacent tissues after introducing a metal probe into the lumen of the vessel (thus destroying the endothelium; [22]), cut transversally into 4 or 5 rings and incubated in 1 ml of a modified Krebs bicarbonate solution (37°, pH 7.4, bubbled with 95% O_2–5% CO_2). Groups of aortic rings, selected so that each contained a section from each level of a different aorta, were preincubated for a 120-min period, during which the incubation medium was changed twice. After this initial incubation a PDE inhibitor, or equal volume of solvent, was added for a period of 5–30 min (during this time the effects of the drugs did not vary significantly, as shown by preliminary experiments), then the pieces of aorta were quickly frozen using an aluminium clamp precooled in liquid N_2 and stored at −80° for a few days, before being thawed and homogenized in 400 μl of 1 N $HC10_4$ cAMP and cGMP were assayed in the 10,000 *g* (5 min) supernatants by radioimmunological methods [23, 24]. Cyclic nucleotide levels were expressed with respect to DNA, which was extracted from the 10,000 *g* (5 min) pellets and assayed fluorometrically by a previously described adaptation [21] of the published procedures [25, 26]. In all cases control tissues were exposed to solvents at the same final concentration as was present in the final drug containing solutions used. The one-tailed Student's *t*-test was used when comparing cyclic nucleotide levels in absence and presence of PDE inhibitors.

Materials. 4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724, gift of Hoffman-Laroche), 4-(3-cyclopentyloxy-4-methoxyphenyl)2-pyrrolidone (rolipram or ZK 62711, gift of Schering), *N*-cyclohexyl-*N*-methyl-4-(1,2-dihydro-2-oxo-6-quinolyloxy) butyramide (cilostamide, synthesized by C. Lugnier; [27]) and *N*-cyclohexyl-*N*-methyl-4-(1,2-dihydro-2-oxo-6-quinolyloxy) valeramide (AAL 05; [27]) were dissolved in dimethyl sulfoxide or ethanol. Papaverine (Sigma), 3-isobutyl-1-methylxanthine (IBMX, Sigma) and 9,10-dimethoxy-2-mesitylimino-3-methyl-3,4,6,7-tetrahydro-2*H*-pyrimodo (6,1-*a*) isoquinolin-4-one hydrochloride (trequinsin, gift of Hoechst) were dissolved in water or physiological solution. 2-*O*-propoxyphenyl-8-azapurin-6-one (M&B 22,948, gift of May & Baker) and dipyridamole (gift of Boehringer Ingelheim) were dissolved in 1 N NaOH and 1 N HCl, respectively, then neutralized and diluted at least 25 times in water to provide stock solutions. Aprotinin (Iniprol) was obtained from Laboratoires Choay, Paris and 2′-deoxy-cAMP and 2′-deoxy-cGMP from Sigma. All reagents were of analytical grade.

RESULTS

Isolation of aorta PDEs from different species

As illustrated in Fig. 1, PDE activity of the 105,000 *g* supernatant from the aorta of each species studied (human, Fig. 1A; rat, Fig. 1B; bovine, Fig. 1C) could be resolved by DEAE-trisacryl column chromatography into 3 peaks:

one peak hydrolyzed both cyclic nucleotides (but preferentially cGMP at low substrate concentrations)

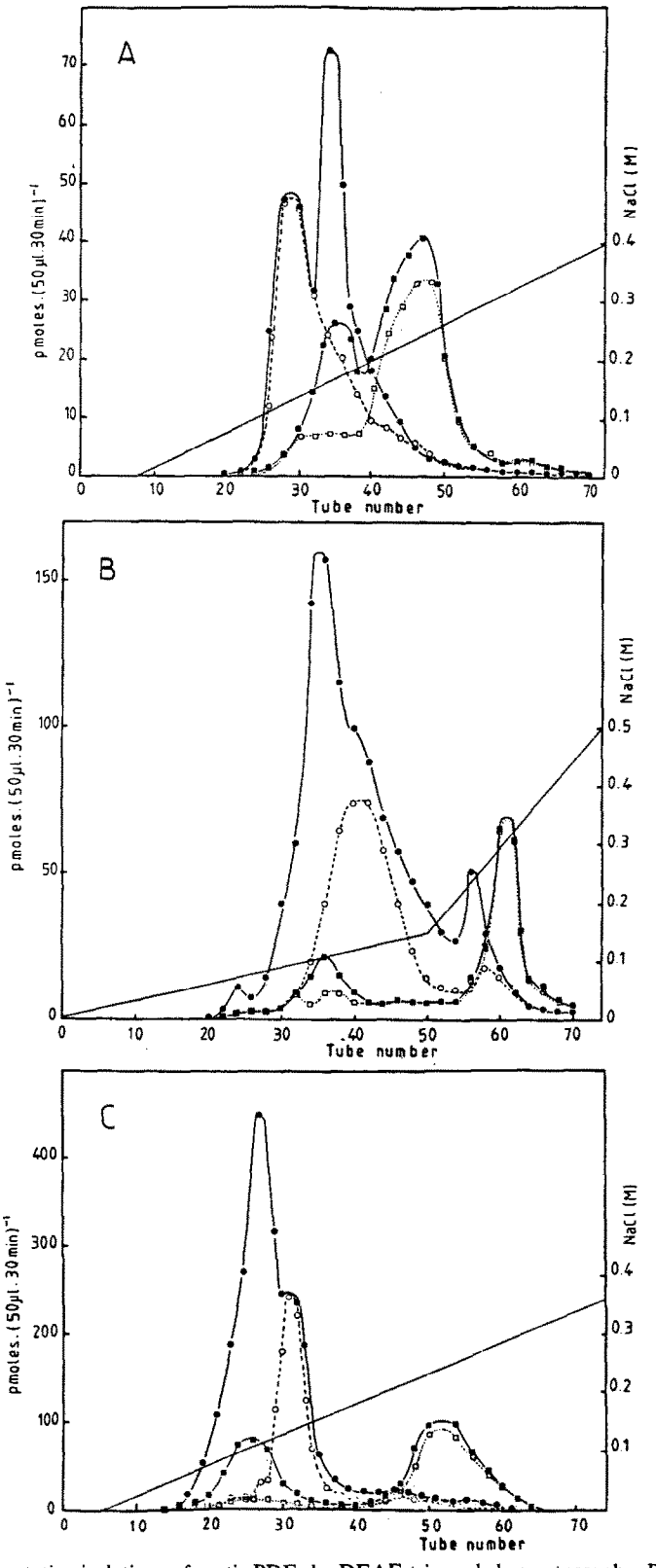

Fig. 1. Representative isolations of aortic PDEs by DEAE-trisacryl chromatography. PDE activity was determined using 1 μM cGMP (circles) or 1 μM cAMP (squares) as substrate, in presence of EGTA (open symbols) or of CaM (closed symbols). (A) A piece (2 cm long) of human aorta was obtained during surgical hepatic transplantation. The media layer (0.246 g) was freed from adventitial and intimal layers, homogenized and centrifuged. The supernatant was deposited on a 10 × 0.9 cm column and eluted with 200 ml of a linear NaCl gradient (0–0.4 M). Fractions of 2.5 ml were collected. (B) The aortae (0.62 g) from 13 male Wistar rats weighing 400–500 g were collected. The supernatant was submitted to DEAE-trisacryl chromatography on a 10 × 0.9 cm column and eluted by 2 successive linear NaCl gradients (0–0.15 M, 300 ml, and 0.15–0.5 M, 200 ml). Fractions of 6 ml were collected. (C) The bovine aorta was obtained from a local slaughterhouse. The medial layer (10 g) was freed of adventitia and intima. The supernatant was chromatographed on a DEAE-trisacryl column (15 × 2.4 cm), eluted by a linear NaCl gradient (0–0.4 M, 600 ml) and 7 ml fractions were collected.

and was activated 6–15-fold by an excess of CaM; it was named CaM-PDE;

one peak hydrolyzed cGMP with a high degree of selectivity and was insensitive to CaM; it was called cGMP-PDE:

the third peak hydrolyzed preferentially cAMP and was not stimulated by CaM; it can be called cAMP-PDE.

The two first peaks were eluted by similar NaCl concentrations. This may explain the permutation of these peaks in the elution order of human aortic PDEs as compared to bovine and rat aorta PDEs. cAMP-PDE was always eluted at a NaCl concentration higher than 0.2 M.

Properties of bovine aorta PDEs

Since separation profiles of PDEs from the three species studied showed similar pattterns, and for reasons of supply convenience, bovine aorta PDE was chosen for further studies.

The question arose whether a better separation of the first two peaks of PDE could be obtained. The mixture of CaM-PDE and cGMP-PDE from bovine aorta (fractions 27–31 from Fig. 1C) was resolved by a second chromatography into two clearly separated forms (Fig. 2). These two forms were eluted at the same NaCl concentration as in the first chromatography (CaM-PDE: 0.07 to 0.1, cGMP-PDE: 0.1 to 0.14 M NaCl), keeping their substrate specificity and CaM sensitivity, without reassociation phenomenon.

Study of the hydrolytic properties of the three PDE forms showed that CaM-PDE and cAMP-PDE exhibited properties which were comparable to those of the same forms isolated from other tissues [2–10]. With the exception of the hydrolysis of cGMP by CaM-PDE activated by CaM (Hill [28] coefficient = 1.02), the enzymatic activity of all PDE forms exhibited a non michaelian behaviour (data not shown) with one low K_m value (K_{mL}) less than 1 μM, one high K_m value (K_{mH}) ranging between 1 and 100 μM, and corresponding low and high V_m values (V_{mL} and V_{mH}, respectively; see legend to Table 1).

As is apparent in Fig. 1C, cGMP-PDE exhibited a minor hydrolytic activity towards cAMP as a substrate (10–20-fold less than for cGMP). This PDE form might therefore be identical to the cGMP-sensitive PDE, found in some tissues to hydrolyze cAMP when stimulated by cGMP [29–31]. However, addition of cGMP (1–100 μM) did not stimulate, and even inhibited, cAMP hydrolysis by this PDE form, indicating that it did not present the properties of the so-called cGMP-sensitive PDE.

2′-Deoxy derivatives of cAMP and cGMP (which are not hydrolysed by PDE) were also used to assess the selectivity of each separated PDE form for cyclic nucleotides. As shown in Table 1, cAMP-PDE and cGMP-PDE were respectively inhibited by the corresponding 2′-deoxy derivative with a high degree of selectivity, the other derivative being practically inactive. By contrast CaM-PDE could be inhibited by the two 2′-deoxy cyclic nucleotides, although in the experimental conditions 2′-deoxy-cGMP was much more potent than 2′-deoxy-cAMP, when using either 1 μM cGMP as a substrate (Table 1) or 1 μM cAMP (IC_{50} values: 50 μM for 2′-deoxy-cAMP and 4 μM for 2′-deoxy-cGMP). Thus it can be said that: (1) cAMP-PDE has the best specificity towards cAMP as substrate; (2) cGMP-PDE specifically recognizes cGMP; and (3) CaM-PDE interacts with both cAMP and cGMP, but preferentially with cGMP, using 1 μM substrate concentration.

Inhibition of isolated PDE forms by various compounds

The non-michaelian kinetics obtained with the three PDE forms separated here did not allow us to determine K_i values for the different inhibitors against the different enzyme forms, so that IC_{50}

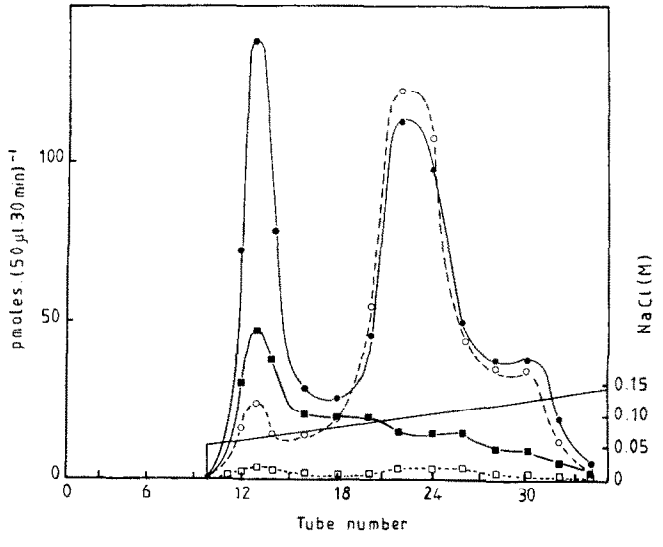

Fig. 2. Re-chromatography of a mixture of CaM-PDE and cGMP-PDE from bovine aorta. PDE activity was determined using 1 μM cGMP (circles) or 1 μM cAMP (squares) as substrate, in presence of EGTA (open symbols) or of CaM (closed symbols). Fractions 27–31 from Fig. 1C were pooled and eluted on a 10 × 0.9 cm DEAE-trisacryl column by a linear NaCl gradient (0.05–0.15 M, 240 ml).

Table 1. Inhibition (IC_{50}, μM) of separate PDE forms from bovine aorta by various compounds

Compound	cAMP-PDE	cGMP-PDE	CaM-PDE
2'-Deoxy-cAMP	7 ± 2	>1000	165 ± 9
2'-Deoxy-cGMP	>1000	3 ± 1	6 ± 1
Trequinsin	0.4 ± 0.1	5 ± 1	13 ± 2
Papaverine	3 ± 1	6 ± 2	62 ± 5
Rolipram	5 ± 1	246 ± 15	2700 ± 153
IBMX	9 ± 2	5 ± 0.4	6 ± 1
AAL 05	12 ± 2	4 ± 1	122 ± 4
Dipyridamole	13 ± 3	0.3 ± 0.1	103 ± 8
Cilostamide	19 ± 3	28 ± 5	256 ± 16
Ro 20-1724	18 ± 3	384 ± 21	940 ± 37
M&B 22,948	247 ± 11	0.4 ± 0.1	21 ± 3

Results are the means ± S.E.M. of at least 3 determinations obtained on 2 or 3 different enzymatic preparations.

IC_{50} values were determined on cAMP-PDE in presence of 1 μM cAMP + EGTA, on cGMP-PDE in presence of 1 μM cGMP + EGTA and on CaM-PDE in presence of 1 μM cGMP and an excess CaM.

cAMP-PDE: $K_{mL} = 0.4\,\mu M$, $k_{mH} = 50\,\mu M$, $V_{mL} = 0.76\,nmol \cdot mg^{-1} \cdot min^{-1}$, $V_{mH} = 1.35\,nmol \cdot mg^{-1} \cdot min^{-1}$.

cGMP-PDE: $K_{mL} = 0.1\,\mu M$, $K_{mH} = 2.6\,\mu M$, $V_{mL} = 0.33\,nmol \cdot mg^{-1} \cdot min^{-1}$, $V_{mH} = 2.17\,nmol \cdot mg^{-1} \cdot min^{-1}$.

CaM-PDE: $K_m = 0.8\,\mu M$, $V_m = 2.32\,nmol \cdot mg^{-1} \cdot min^{-1}$.

values of the inhibitors were determined at a substrate concentration near to the apparent low K_m value, which is also in the physiological range (1 μM; [32]). Table 1 shows the IC_{50} values of different compounds on the three PDE forms obtained from bovine aorta. The order of potency of the different compounds as inhibitors was clearly different for each form. Trequinsin, papaverine and rolipram were the most potent inhibitors of cAMP-PDE, with IC_{50} values in the micromolar range or less. IBMX, AAL 05, dipyridamole, cilostamide and Ro 20-1724 all inhibited cAMP-PDE with IC_{50} values in the 10 μM range. M&B 22,948 was the least potent inhibitor of this form, showing an IC_{50} value of at least one order of magnitude higher than that of the other compounds.

By contrast M&B 22,948 and dipyridamole were the most potent inhibitors of cGMP-PDE, with IC_{50} values less than 1 μM. Trequinsin, papaverine, IBMX and AAL 05 inhibited this PDE form in the micromolar range, whereas cilostamide was a little less potent and rolipram and Ro 20-1724 were only active in the 100 μM range.

IBMX and trequinsin exhibited the most potent inhibitory effect on CaM-PDE, with IC_{50} values of about 10 μM, M&B 22,948 was active at a two-fold higher concentration, and all other compounds were only active in the 100 μM range and even in the 1 mM range in the case of rolipram and Ro 20-1724. The order of potency of the tested compounds as inhibitors of CaM-PDE was unchanged using either cAMP or cGMP as a substrate, and in either the presence or the absence of CaM (data not shown).

cAMP-PDE from human aorta was inhibited to a similar extent as was cAMP-PDE from bovine aorta by rolipram, Ro 20-1724 and IBMX (respective IC_{50} values: 6, 43 and 12 μM). Rolipram and M&B 22,948 inhibited respectively cAMP-PDE and cGMP-PDE with about the same potency in rat aorta (respective IC_{50} values: 4 and 1 μM) as in bovine aorta.

Effects of PDE inhibitors on the levels of cAMP and cGMP in rat isolated aorta

Table 2 shows the effects of most of these PDE inhibitors on the levels of cAMP and cGMP in rat isolated aorta. Only papaverine is not shown since its effects on cyclic nucleotide contents in rat isolated aorta have already been reported [33]. The variability of cAMP and cGMP basal levels in vascular tissue is a general observation (see for example Lorenz and Wells [34]). Such a variability led us to include control groups in all experiments. Nevertheless, it constitutes an obstacle for detecting small effects of drugs.

Increasing concentrations of PDE inhibitors were tested until a significant effect was found on cAMP or cGMP levels. The lowest concentration tested was near the IC_{50} value obtained on isolated cAMP- or cGMP-PDE forms. The effect of this concentration, when active, is indicated in Table 2; in other cases the effect of the lowest active concentration is given, the highest inactive concentration is also mentioned. In most cases, the maximal effect of the drugs could not be determined because of their limited solubility.

IBMX 10 μM was able to increase moderately but significantly both cAMP and cGMP levels. At a concentration of 500 μM IBMX produced a marked increase (by 7-fold) in these levels.

At concentrations inhibiting the three forms of PDE isolated, trequinsin concentration-dependently increased both cAMP and cGMP levels. However, trequinsin 1 μM, a concentration close to the IC_{50} on cAMP-PDE, did not increase the cyclic nucleotide levels.

M&B 22,948 (1 μM), a compound that selectively inhibits cGMP-PDE at this concentration, sig-

Table 2. cAMP and cGMP levels in rat isolated aorta in absence (control) and in presence (treated) of various PDE inhibitors

Compound		cAMP			cGMP		
		Control	Treated	Fold increase	Control	Treated	Fold increase
IBMX	10 μM	71 ± 5 (21)	106** ± 10 (20)	1.5	12 ± 1 (15)	20** ± 3 (15)	1.7
	500 μM	67 ± 7 (17)	493*** ± 64 (17)	7.4	14 ± 2 (12)	102*** ± 15 (12)	7.3
Trequinsin	1 μM	70 ± 9 (5)	74 ± 7 (5)	—	9 ± 2 (4)	9 ± 2 (5)	—
	10 μM	70 ± 9 (5)	103* ± 7 (5)	1.5	9 ± 2 (4)	19* ± 2 (5)	2.1
	500 μM	44 ± 5 (5)	148*** ± 10 (5)	3.4	6 ± 2 (5)	32** ± 6 (5)	5.3
M&B 22,948	1 μM	66 ± 7 (5)	54 ± 5 (5)	—	21 ± 2 (5)	36* ± 5 (5)	1.7
	10 μM	62 ± 5 (9)	63 ± 4 (9)	—	15 ± 2 (9)	37** ± 6 (10)	2.5
	50 μM	56 ± 4 (9)	65 ± 5 (15)	—	17 ± 2 (14)	41** ± 6 (15)	2.4
	500 μM	48 ± 3 (31)	55 ± 3 (30)	—	14 ± 2 (25)	44*** ± 3 (24)	3.1
	1 mM	47 ± 7 (5)	45 ± 6 (5)	—	21 ± 3 (5)	60* ± 12 (5)	2.9
Rolipram	100 μM	57 ± 5 (4)	84 ± 19 (4)	—	11 ± 1 (4)	13 ± 2 (4)	—
	200 μM	74 ± 3 (8)	107** ± 7 (8)	1.5	14 ± 1 (8)	13 ± 2 (8)	—
Ro 20-1724	100 μM	37 ± 7 (5)	44 ± 5 (5)	—	24 ± 6 (5)	17 ± 4 (5)	—
	500 μM	46 ± 6 (9)	91** ± 13 (9)	2.0	11 ± 2 (4)	15 ± 3 (4)	—
Ro 20-1724 +M&B 22,948	500 μM 500 μM	45 ± 3 (4)	89** ± 8 (4)	2.0	13 ± 3 (4)	39*** ± 6 (4)	3.0
Cilostamide	100 μM	38 ± 4 (5)	43 ± 5 (5)	—	21 ± 6 (5)	23 ± 2 (5)	—
	500 μM	30 ± 4 (4)	51** ± 5 (4)	1.7	9 ± 2 (4)	20** ± 1 (4)	2.2
AAL 05	500 μM	45 ± 5 (8)	73** ± 8 (8)	1.6	10 ± 1 (8)	29*** ± 2 (8)	2.9
Dipyridamole	10 μM	38 ± 1 (5)	44 ± 4 (5)	—	6 ± 2 (5)	8 ± 3 (5)	—
	100 μM	38 ± 1 (5)	51** ± 3 (5)	1.3	6 ± 2 (5)	27 ± 10* (5)	4.5

Data are means ± S.E. of N (in parenthesis) values assayed in duplicate, expressed in pmole/mg DNA. The fold increase, with respect to control, is indicated when significant. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Rats were male 7-week-old Wistar, except in the case of rolipram where female 16-week-old Wistar were used.

nificantly increased the cGMP level. This effect was concentration-dependent, a maximal increase of about 3-fold being reached using 500 μM. cAMP levels were not significantly affected by M&B 22,948 concentrations of 1 μM to 1 mM.

The other PDE inhibitors tested did not significantly alter the levels of cAMP and/or cGMP when used at concentrations inhibiting half-maximally the activity of the corresponding isolated PDE forms. Rolipram and Ro 20-1724, compounds that were relatively highly selective for cAMP-PDE, produced only modest, although significant, increases in cAMP levels (by 1.5- and 2.0-fold, respectively) without significant alteration in cGMP levels, at concentrations as high as 200 μM and 500 μM, respectively. At lower concentrations (up to 100 μM) no significant effect could be observed. The association of high concentrations (500 μM) of a cAMP-PDE inhibitor (Ro 20-1724) and a cGMP-PDE inhibitor (M&B 22,948) did not cause any further increase in cyclic nucleotide levels than did either drug alone. Compounds inhibiting both cAMP-PDE and cGMP-PDE with similar potencies (cilostamide and its derivative AAL 05) significantly increased both cAMP and cGMP levels. These increases, however, did not exceed 2–3-fold, using 500 μM concentrations of both compounds, thus reproducing the effect of the association of Ro 20-1724 with M&B 22,948. Dipyridamole, a compound which also inhibited both cAMP-PDE and cGMP-PDE, with a preference for the latter, also elevated the levels of both nucleotides. This effect was more marked on the cGMP level (by 4.5-fold) than on the cAMP level (by 1.3-fold). Nevertheless, a concentration as high as 100 μM was required to produce this effect.

DISCUSSION

This work reports that the PDE activity of aortic smooth muscle of three different species, including man, can be chromatographically resolved into three forms which differ from each other by their substrate specificity, their sensitivity to CaM and to inhibitors, but which display identical properties in the three species.

The number and the properties of the forms of PDE isolated from tissues vary from one tissue to the other and with the isolation procedure. Two PDE forms called peak I and peak II PDEs have been isolated from porcine coronary arteries [5–7]. The properties of CaM-PDE and cAMP-PDE described in the present paper are respectively identical to those of peak I and peak II PDEs. These two forms have been described in various other (non vascular) tissues, where they represent two major PDE forms [2–4]. They have also been found in bovine aorta smooth muscle, using isolation techniques identical to those applied to chromatographic resolution of PDE activity from porcine coronary arteries [27]. In a previous study from this laboratory, however, the

use of a different procedure (addition of 0.2 mM EGTA to homogenizing and elution buffer, and two successive DEAE-cellulose column chromatographies) allowed us to isolate from bovine aorta a third PDE form selectively hydrolyzing cGMP and insensitive to CaM [10]. Recently, PDE activity from rabbit aorta was also chromatographically resolved into three peaks, one of which selectively hydrolyzed cGMP and was not activated by CaM [11]. However, the PDEs isolated in the latter study differed from those obtained in others in that there was no form that selectively hydrolyzed cAMP.

In the present work, isolation of the PDEs was routinely performed using a buffer supplemented with aprotinin (protease inhibitor). Similar results were also obtained with a cocktail of protease inhibitors (including soybean trypsin inhibitor, leupeptin, chymostatin and aprotinin). Thus the fractionation procedure was conducted to preserve the PDE forms from proteolysis as much as possible, especially CaM-PDE which is reportedly altered by proteolysis and could subsequently loose its sensitivity to CaM [3]. Furthermore the buffer was supplemented with EDTA (5 mM). Under these conditions CaM-PDE was greatly activated by CaM (6–15-fold), suggesting that this form was relatively well preserved. It is known that in some cases the addition of a chelator to the fractionation buffer facilitates the dissociation of CaM from CaM-PDE [35], resulting in a change in the isoelectric point of this enzyme form. This may explain the dissociation of cGMP-PDE from CaM-PDE which is thus eluted earlier (0.07–0.1 M NaCl) than in absence of chelator 0.1–0.14 M NaCl [27]). Re-chromatography of a mixture of CaM-PDE and cGMP-PDE permitted a good separation of the two forms and no reassociation phenomenon was apparent.

The present work confirms the existence of a cGMP-PDE in the aorta and characterizes this enzyme form, the properties (sensitivity to inhibitors, kinetic properties) of which have never been reported. This PDE is quite different from CaM-PDE: it is insensitive to CaM and to inhibition by 2′-deoxy-cAMP. It seems also to be different from a cGMP-sensitive PDE which hydrolyzes cAMP and which has been described in various other tissues [29–31]. In addition, the order of potency of PDE inhibitors as antagonists of this form is clearly discordant with that found on CaM-PDE. M&B 22,948 and dipyridamole appear to be much more potent inhibitors of cGMP-PDE, than of CaM-PDE. Due to this difference, the selectivity of M&B 22,948 is found greater here than in previous reports [12, 15]. Isolation of cGMP-PDE also led us to reconsider the selectivity of other PDE inhibitors towards cAMP or cGMP hydrolysis: cilostamide, which has been previously reported as a highly selective inhibitor of cAMP-PDE in platelets [12, 16], inhibits equipotently aortic cGMP-PDE and cAMP-PDE, so that it does not behave as a selective cAMP-PDE inhibitor in this tissue; by contrast, trequinsin, recently reported to be a selective and potent inhibitor of cAMP breakdown in platelets [17] and an antihypertensive agent [36], is also a potent and rather selective inhibitor of aortic cAMP-PDE.

In order to investigate whether the three forms of PDE isolated were really present in the tissue, we measured cyclic nucleotide levels in rat isolated aorta incubated in the presence of various inhibitors.

IBMX and trequinsin, non selective inhibitors of the three forms, including CaM-PDE, are able to markedly increase (by up to 7-fold) the levels of both cAMP and cGMP. IBMX could induce significant changes in the levels of cyclic nucleotides at a concentration (10 μM) consistent with its effect on the three isolated PDE forms. Trequinsin is able to increase cAMP and cGMP levels at a concentration (10 μM) that inhibits both cGMP-PDE and CaM-PDE, but is markedly higher than the IC_{50} for cAMP-PDE inhibition. By contrast, even high or very high concentrations (>100 μM) of the drugs which selectively inhibit cGMP-PDE, cAMP-PDE or both could only produce weaker elevations in aorta cyclic nucleotide levels than those induced by compounds which also inhibit CaM-PDE. It seems that CaM-PDE, which used cGMP as prefered substrate when its activity was assayed in an acellular medium, could participate not only in the regulation of cGMP, but also of cAMP levels within the smooth muscle cell. It should be noted that CaM-PDE, which represents the major proportion of PDE activity when fully activated by Ca-CaM (see Fig. 1), could also be of significance, in an activated form, in resting arteries [37].

M&B 22,948, the most selective cGMP-PDE inhibitor, significantly increases the cGMP level in isolated aorta at a concentration (1 μM) near its IC_{50} for inhibition of cGMP-PDE without altering the cAMP level. In fact cAMP levels are unaffected by concentrations as high as 1 mM. This strongly suggests that the effect of this drug is due to the inhibition of cGMP-PDE present in this tissue and confirms that this PDE form is different from CaM-PDE. However the maximal increase in cGMP level induced by M&B 22,948 remains rather moderate (by 3-fold).

The other PDE inhibitors used are either selective inhibitors of cAMP-PDE (rolipram, Ro 20-1724), or inhibitors of both cGMP-PDE and cAMP-PDE (AAL 05, cilostamide). They increase the aortic cyclic nucleotide level(s) only when concentrations higher or much higher than those inhibiting the isolated enzymes are used. Such an observation has already been made with other PDE inhibitors in vascular smooth muscle [8, 13] and explains that a strict correlation between PDE inhibition and the effect on cyclic nucleotide levels is difficult to establish in this type of tissue. This could have at least one of the following explanations. Firstly, low concentrations of these PDE inhibitors could induce compartmentalized and functionally important alterations in the cyclic nucleotide levels, without necessarily inducing significant changes in the total tissue levels of these nucleotides. Secondly, the large dispersion in the basal levels of cyclic nucleotides, and the rather weak effect of most PDE inhibitors on these levels, may explain why only high concentrations of these compounds produced significant increases. Thirdly, the concentrations of the drugs within the cell might not be sufficient to inhibit PDE, possibly because of a poor permeability through the plasma membrane or because of degradation,

although the wide spectrum of chemical structures used in the present study is not in favour of such a possibility. Nevertheless, when sufficiently high concentrations of cAMP-PDE inhibitors are used, the expected selective effect on cAMP levels, although modest (by 1.5-fold), is observed. No further effect is seen by associating a cAMP-PDE inhibitor (Ro 20-1724) with a cGMP-PDE inhibitor (M&B 22,948) than is observed with each drug separately. Compounds inhibiting both cAMP- and cGMP-PDE (cilostamide, AAL 05 and, although it preferentially inhibits cGMP-PDE, dipyridamole) also elevate the levels of both cyclic nucleotides to a moderate extent.

Altogether our results show a good correlation between inhibition of isolated PDE forms from aorta and the effect of inhibitors of the different PDE forms on the tissue. Thus, isolated PDE forms from aorta apparently retain the properties that characterize them in the intact tissue.

The relaxing effect of some of the PDE inhibitors tested here has been previously studied in rat isolated aorta, and it has been suggested that relaxation parallels inhibition of aortic cAMP-PDE [12]. The present investigation shows that the two selective cAMP-PDE inhibitors, rolipram and Ro 20-1724, elevated the aortic cAMP content by about 2-fold (Table 2) at concentrations close to those producing half-maximal relaxation in previous experiments (150 and 240 μM, respectively [12]). By contrast, selective accumulation of cGMP in rat aorta was produced by concentrations of M&B 22,948 much lower (1 μM) than those reported to relax this tissue (in the 100–500 μM range). Correlations between cAMP or cGMP levels and vascular relaxation have been reported in bovine coronary artery [38, 39]. The elevations in cAMP content (by about 2-fold) associated with half-maximal relaxation in this tissue were modest, as were those obtained with cAMP-PDE inhibitors in rat aorta in the present study. Elevations in cGMP content associated with 50% relaxation of coronary artery preparations were larger (5–6 fold increases) than the maximal increase in aortic cGMP content produced by the selective cGMP-PDE inhibitor M&B 22,948 (3-fold increase). Even IBMX produced only moderate (less than 2-fold) increases in both aortic cAMP and cGMP levels when used at a concentration (10 μM) which relaxed rat aortic strips by 50% [12]. Further, a significant effect of cilostamide on cAMP and cGMP levels could be seen only in the presence of a concentration of this drug (500 μM) substantially higher than that necessary to provoke relaxation (<100 μM).

As a whole, the present and previous [12] results show that concentrations of PDE inhibitors which are able to elicit relaxation, induced relatively small variations in cyclic nucleotide contents of rat isolated aorta. They are consistent with other findings [40] showing that isoprenaline-induced relaxation is associated with a 2.5-fold increase in aortic cAMP content. Larger elevations in aortic cAMP level may not be related to or necessary for relaxation. They also suggest that the three different PDE forms may play different roles in the aorta with respect to smooth muscle relaxation. A more detailed investigation to confirm this hypothesis and to define relationships between relaxation and cyclic nucleotide levels induced by PDE inhibitors in isolated aorta is underway.

In conclusion, we have reported that three PDE forms could be isolated from human, bovine and rat aorta, showing similar properties in each species. Their inhibition is associated with corresponding increases in the appropriate tissue cyclic nucleotide levels, especially in the case of cGMP-PDE, the inhibition of which by M&B 22,948 resulted in a selective increase in cGMP level. This work may be useful for the design of new and more specific PDE inhibitors and will help to explain the pharmacological properties of these drugs. It strengthens the case for the use of rat and bovine aorta as models for the design of new drugs, since human aortic PDEs exhibit the same properties as aortic PDEs of these species.

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