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EFFECTS OF SELECTIVE INHIBITORS ON CYCLIC NUCLEOTIDE PHOSPHODIESTERASES OF RABBIT AORTA

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Abstract—In this study three forms of cyclic nucleotide phosphodiesterase (PDE) isolated from rabbit aorta were pharmacologically characterized, and the consequence of selective inhibition of calmodulin-stimulated PDE (CaM-PDE) and cGMP specific PDE (cG-PDE) was evaluated using PDE inhibitors. The cG-PDE (F1) was selectively inhibited by M&B 22948 (IC_{50} = 0.5 μM) and dipyridamole (IC_{50} = 7 μM). The cAMP-PDE (cA-PDE, F3) was inhibited more effectively by the cA-PDE inhibitor milrinone than by other PDE inhibitors. The cA-PDE preparation appeared to contain both cG-inhibited PDE and cG-insensitive PDE based on an additive inhibition of the activity by milrinone and SQ 65442, respective inhibitors of these enzymes. Vinpocetine, 8-methoxymethyl isobutylmethylxanthine (8-MeOMeMIX) and M&B 22948 effectively inhibited CaM-PDE (F2). Vinpocetine was a more selective inhibitor of CaM-PDE than M&B 22948 or 8-MeOMeMIX. CaM-PDEs isolated from rabbit aorta and bovine brain exhibited a similar sensitivity to these inhibitors. Seventy-two percent of the cGMP-hydrolyzing activity of this rabbit aortic CaM-PDE preparation was immunoadsorbed to monoclonal antibody (ACC-1) against CaM bound to brain CaM-PDE. Vinpocetine, 8-MeOMeMIX and M&B 22948 at concentrations (30 and 100 μM) which inhibit CaM-PDE greater than 60% increased cGMP but not cAMP levels in *l*-norepinephrine (NE) preincubated rabbit aortic slices. At concentrations selectively inhibiting cG-PDE, dipyridamole and M&B 22948 increased cGMP levels in untreated slices but failed to increase cGMP levels significantly in NE-treated slices. By contrast, vinpocetine failed to increase cGMP significantly in untreated slices, although it increased cGMP levels in NE or KCl preincubated slices. These data indicate that, in activated (precontracted) aorta, CaM-PDE is a major enzyme, whereas in untreated aorta cG-PDE is a predominant enzyme for the hydrolysis of cGMP. This study also shows a usefulness of selective inhibitors in identifying different forms of PDE and similar drug sensitivities and immunoadsorption of aortic and brain CaM-PDEs by a monoclonal antibody.

cAMP and cGMP play an important regulatory role in the relaxation of vascular smooth muscles [1–6]. The intracellular concentrations of these cyclic nucleotides, in turn, appear to be regulated by cyclic nucleotide phosphodiesterases (PDEs) [7–9]. Multiple forms of PDEs have been isolated from many tissues and species [10–13]. In the case of vascular smooth muscle, three forms (cG-PDE, CaM-PDE and cA-PDE) of PDE were isolated from most species, including rabbit, by DEAE ion exchange chromatography although there were some exceptions [14–21]. In many studies, different forms of PDE are identified as F1, F2 or F3 according to their order of elution from a DEAE ion exchange column. However, this method sometimes can lead to confusion. For example, under the same isolation conditions F1 was identified as cG-PDE in human aorta but as CaM-PDE in rat and bovine aorta [16]. Further, cA-PDE was eluted as F2 in bovine coronary arteries but as F3 in most other vascular preparations [15–19]. Recently, characterization of each PDE fraction was attempted using PDE inhibitors. One study showed that multiple forms of bovine aortic PDE were inhibited differently by some of the PDE inhibitors tested [16]. Rolipram and Ro 20-1724 showed a 20- to 50-fold selectivity for cA-PDE over other PDEs. M&B 22948 and dipyridamole exhibited a 40- to 50-fold selectivity for cG-PDE over CaM-PDE. In other studies with PDEs from bovine coronary arteries and rabbit aorta, vinpocetine and 8-MeOMeMIX exhibited a more than 20-fold selectivity for CaM-PDE over cA-PDE [15, 19]. The cA-PDEs from bovine coronary arteries and aorta were selectively inhibited by 1-isoamyl-3-isobutylxanthine (IIX), milrinone or rolipram [16, 17, 19]. Bovine aortic cG-PDE was inhibited most effectively by M&B 22948 and dipyridamole [16]. However, it is not known from these limited studies whether each of these PDE forms exhibits a similar sensitivity or selectivity to these inhibitors in different species or tissues [9, 16, 17]. For instance, drug sensitivity of cA-PDE in non-vascular tissues appeared to differ markedly among different species or tissues [9, 16, 17]. Recently, cA-PDE was subdivided into at least two subclasses, a cGMP-insensitive form and a cGMP-sensitive form [12, 13]. The type(s) of Ca-PDE present in vascular tissues has not been determined.

Recent studies with PDE inhibitors indicated cGMP hydrolysis by CaM-PDE in intact vascular tissues. Vinpocetine produced a concentration-dependent elevation of cGMP levels as well as relaxation in isolated rabbit aorta [15]. 8-MeOMeMIX potentiated a sodium nitroprusside (SNP)-induced cGMP increase and vasorelaxation in bovine

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coronary arteries [19]. M&B 22948 increased cGMP levels and produced vasorelaxation in rat and rabbit aortas [19, 22]. The role of cG-PDE in cGMP hydrolysis is not clear from reported studies because the concentrations of M&B 22948 and dipyridamole used were high enough to inhibit both cG-PDE and CaM-PDE. In addition, conflicting results were reported on the effects of these inhibitors on cGMP levels at their selective cG-PDE inhibitory concentrations in vascular tissues [2, 16, 20, 22].

In the present study, selective inhibitors were used to characterize three forms of PDE isolated from rabbit aortic extracts by DEAE ion exchange chromatography. Also, rabbit aortic and homogeneous bovine brain CaM-PDEs were directly compared for their drug sensitivities and reactivities with a monoclonal antibody against calmodulin bound to bovine brain CaM-PDE. In addition, we assessed the relative roles of CaM-PDE and cG-PDE in regulating cyclic nucleotide levels in an isolated vascular tissue. Part of this study was presented previously in abstract form [23].

MATERIALS AND METHODS

Ion exchange chromatographic isolation of different forms of PDE. Three different forms of PDE were isolated from rabbit aorta using our modification [24] of the method of Hidaka *et al.* [20]. Frozen rabbit aortas (Pel-Freeze) were freed of fat and connective tissue. The minced rabbit aorta was homogenized in 3 vol. of 50 mM Tris–acetate buffer (pH 6.0) containing 3.75 mM 2-mercaptoethanol and 10 μM leupeptin (PDE isolation buffer) using a Brinkmann PT-10 Polytron (2 × 15 sec bursts at the maximum speed). The homogenate was sonicated (20 sec/ml homogenate) with a Branson Sonifier 200 at 70% output with an alternating 30-sec duration of sonication and cooling. The sonicated homogenate was centrifuged at 100,000 *g* for 60 min. The resulting supernatant fraction (20 ml) was applied to a 10 × 300 mm DEAE-Sephacel column (bed vol. 24 ml) equilibrated with PDE isolation buffer. The loaded column was washed with a three bed volume of PDE isolation buffer containing 70 mM sodium acetate. PDEs were eluted from the column using a step gradient of 30 ml each of 220, 350 and 700 mM sodium acetate in PDE isolation buffer. The flow rate was 0.5 ml/min. Fractions (3 ml) were collected and assayed for cAMP- and cGMP-degrading activity (substrate concentration, 1 μM; 20 μl aliquot of each fraction) in the presence and absence of 0.04 μM calmodulin (CaM) and 100 μM $CaCl_2$. Peak fractions of PDE activity were pooled and labeled F1, F2 and F3 according to the order of elution from the column. Each pooled fraction was concentrated 5- to 10-fold using an Amicon ultrafiltration cell fitted with a YM10 membrane. Unless otherwise indicated, the concentrated enzyme fraction was stored in 0.4 mg/ml bovine serum albumin (BSA) at −70°. Rabbit aortic CaM-PDE and cA-PDE activities were stable over several months under this condition. Freezing caused about 30% loss of activity of the CaM-PDE preparation.

Phosphodiesterase assay. Phosphodiesterase activity was measured by the method previously reported [24]. The reaction mixture (0.2 ml) contained 50 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$, [^{3}H]cyclic AMP or [^{3}H]cyclic GMP (about 50,000 cpm), unlabeled cyclic AMP or cyclic GMP, various concentrations of drug or vehicle, and appropriate amounts of enzyme (10–50 μl eluate). Enzyme was preincubated in the reaction mixture lacking substrate for 5 min at 30°. Enzyme reaction was initiated by adding a mixture (20 μl) of tracer and unlabeled substrate (1 μM substrate unless otherwise noted). After 15 min of incubation at 30°, the reaction was terminated by boiling for 3 min after which 100 μg (10 μl) of snake venom (*Crotalus atrox*, Sigma Chemical Co., St Louis, MO) was added. The mixture was incubated for 10 min at 30° to liberate nucleosides. AG1-X2 resin chloride form 200–400 mesh [0.8 ml of 1:3 (v/v) slurry of resin and water] was added to remove unhydrolyzed cyclic nucleotides and 5′-AMP or 5′-GMP. After vortexing and centrifugation at 1000 *g* for 10 min, the resulting supernatant fractions (0.4 ml) were transferred to counting vials with 10 ml of Biofluor (New England Nuclear, Boston, MA) or Universol (ICN Co., Irvine, CA) for the counting of radioactivity in a scintillation counter.

Immunoadsorption of CaM-PDE activity by monoclonal antibody (Mab) against CaM bound to bovine brain CaM-PDE. Solid phase antibody preparation and immunoadsorption of CaM-PDE activity were carried out by a modification of the method of Hurwitz *et al.* [25]. Briefly, solid-phase *Staphylococcus aureus* (Staph A) CaM-PDE Mab was prepared as follows: 1 ml of 10% Staph A (heat-inactivated, formalin-fixed cells of the Cowan 1 strain of *S. aureus*) was washed twice with 25 mM Tris–HCl buffer, ph 7.5, containing 2 mM magnesium acetate (TM buffer), resuspended in 0.2 ml of TM buffer containing approximately 1 μg of bovine brain CaM-PDE Mab (ACC-1), 0.2 mg/ml BSA, 0.5 μg CaM and 0.2 mM $CaCl_2$, and incubated at 5° for 1.5 to 2 hr. ACC-1 is a monoclonal antibody directed against CaM bound to CaM-PDE and has a 100 times less affinity for free CaM than for CaM bound to CaM-PDE [26]. The resulting Staph A–CaM-PDE Mab complex was washed twice with TM buffer and stored at −20°. Aliquots (0.2 ml) of bovine brain and rabbit aortic CaM-PDEs were incubated with the native or boiled Staph A–PDE Mab complex in the presence of CaM and $CaCl_2$ at 5° for 2–3 hr, and centrifuged. The supernatant fractions were removed and the pellets were washed twice. Both the supernatant fractions and resuspended pellets were assayed for PDE activity using 2 μM cGMP as a substrate.

Determination of cyclic GMP and cyclic AMP levels in aorta. Male New Zealand white rabbits (2.5–3.5 kg) were anesthetized in an atmosphere of 50% CO_2/50% air and then killed in an atmosphere of 100% CO_2 in accordance with USDA guidelines for humane treatment of experimental animals. The thoracic aorta was removed and cleaned of extraneous tissues. Rabbit aorta was incubated at 37° for 60 min with a change of buffer after each 15 min of incubation in a Krebs–Ringer–bicarbonate (KRB) buffer (124 mM NaCl, 2.6 mM $NaHCO_3$, 1.25 mM KH_2PO_4, 5 mM KCl, 1.3 mM $MgSO_4$,

0.8 mM $CaCl_2$ and 10 mM dextrose), pH 7.5. The tissue was aerated constantly with 95% O_2 and 5% CO_2. Segments (approx. 20–30 mg wet weight) were cut from the aorta. The aortic segments were incubated at 37° for 15 min with 0.3 μM norepinephrine unless otherwise indicated and then for an additional 10 min with a drug or vehicle in the aerated buffer. Following the final incubation, segments were rapidly frozen in liquid nitrogen. The frozen segment was homogenized in 1 ml of 6% trichloroacetic acid (TCA) and centrifuged at 1600 *g* for 10 min. The supernatant TCA was extracted with 3 × 5 ml of ether. Separate aliquots (100 μl) of the aqueous phase (after removal of residual ether) were assayed by radioimmunoassays for cGMP and cAMP after acetylation. The TCA precipitates of tissue homogenates were solubilized by incubation in 2 ml of 1 N NaOH at 90° for 10 min. Protein concentration of the solubilized precipitates was determined by the method of Lowry *et al.* [27].

Materials. Cyclic GMP and cAMP were purchased from the Sigma Chemical Co. and used without further purification. Tritiated cyclic nucleotides, [^{3}H]cGMP (sp. act. 42.5 Ci/mmol) and [^{3}H]cAMP (sp. act. 31.2 Ci/mmol) obtained from New England Nuclear, were either lyophilized or purified on Bio-Rad AGIX-8 HCOOH columns (bed vol. 2.5 ml) in some cases. Briefly, 100–200 μl of ^{3}H-labeled cyclic nucleotide (250 μCi/250 μl) was applied to the column. The column was washed with 8 ml of reagent grade water, and [^{3}H]cAMP and [^{3}H]cGMP were successively eluted with 8 ml of 1 N formic acid and 8 ml of 4 N formic acid. Each eluate was evaporated in a Speed Vac concentrator and reconstituted with 250 μl of 50% ethanol. Dipyridamole and CaM were purchased from the Sigma Chemical Co. Vinpocetine was synthesized by Dr B. Bauer at the Schering-Plough Corp. 8-MeOMeMix was supplied by Dr J. Wells (Vanderbilt University). Milrinone and M&B 22948 were obtained from Dr A. Alousi of Sterling Winthrop and May & Baker Ltd respectively. Bovine brain CaM and CaM-PDE were supplied by Dr D. Wolff (Rutgers University) and Dr R. Kincaid (NIH), respectively. Bovine brain CaM-PDE was prepared as described by Kincaid *et al.* [28]. Monoclonal antibody to CaM bound to bovine brain CaM-PDE (ACC-1) was supplied by Dr J. Beavo (University of Washington). Frozen rabbit aortas were purchased from Pel-Freeze Biologicals. Reagents for RIA were obtained from Biomedical Technologies, Inc.. Other chemicals and reagents used were of the highest commercial grade.

Preparation of drug solutions and statistical analysis. Stock solutions (10 mM) of M&B 22,948, dipyridamole and IIX were prepared by dissolving these substances in 10% triethanolamine, 30% methanol and 0.5 N NaOH, respectively. Vinpocetine was dissolved in either 100% dimethyl sulfoxide (DMSO) or 0.05 N HCl. Subsequent dilution was made using reagent grade water. Final concentrations of vehicles were 1% or lower, and any vehicle effect (less than 10% inhibition) was subtracted from the effect of an agent. The IC_{50} (concentration required for 50% inhibition of the hydrolysis of cyclic nucleotide) values were obtained by linear regression. Student's *t*-test was used to analyze the statistical significance of drug effects.

RESULTS

Isolation and properties of multiple forms of PDE from rabbit aorta. Three peaks of PDE activity were obtained by DEAE-Sephacel chromatography from 100,000 *g* supernatant fractions of rabbit aortic homogenates (data not shown) in agreement with a previous report [15]. The first peak of PDE activity selectively hydrolyzed cGMP and was not stimulated by CaM and Ca^{2+}. This form was designated as cG-PDE. The second peak hydrolyzed cGMP preferentially and was stimulated by CaM and Ca^{2+}; it was called CaM-PDE. The last peak selectively hydrolyzed cAMP and was not stimulated by CaM and Ca^{2+}. This peak was called cA-PDE. The CaM-PDE was the only form which, when stimulated with CaM and Ca^{2+}, exhibited a Michaelian kinetic behavior. cG-PDE and cA-PDE exhibited non-

Table 1. Immunoadsorption of rabbit aortic and bovine brain CaM-PDEs by monoclonal antibody to CaM bound to bovine brain CaM-PDE (ACC-1)

	cGMP-hydrolyzing activity (units × 10^{-6})			
	Rabbit aortic CaM-PDE		Bovine brain CaM-PDE	
Condition	Supernatant fraction	Pellet	Supernatant fraction	Pellet
Boiled ACC-1	0.82	0.15	1.86	0
Native ACC-1	0.24	0.76 (72%)	0.13	1.50 (92%)

CaM-PDE Mab (1 μg) conjugated to Staph A was incubated with 0.2 ml of CaM-PDE in the presence of 0.5 μg CaM/0.2 ml and 0.2 mM of $CaCl_2$ at 5° on a gyrotory shaker for 2–3 hr. At the end of incubation, the mixture was centrifuged at 10,000 *g* for 2 min. The cGMP-hydrolyzing activity in the supernatant fraction and the precipitate was assayed using 2 μM cGMP. One unit of activity was defined as activity hydrolyzing 1 μmol cGMP/min at 30°.

Numbers in parentheses represent the percent of total activity.

Table 2. Inhibition of separate PDE forms from rabbit aorta by selective inhibitors

Compound	IC_{50} (μM) F1 (cG-PDE)	F2* (CaM-PDE)	F3 (cA-PDE)
Vinpocetine	>300	19	>300
8-MeOMeMIX	10	8	240
M&B 22948	0.5	8	122
Dipyridamole	7	114	150
Milrinone	>300	>300	10

Unless otherwise indicated, IC_{50} values were obtained from one or two experiments. cGMP (0.5 μM) was used as a substrate for F1 and F2, and cAMP (0.5 μM) for F3. Calmodulin and Ca^{2+} were not added to assay tubes.

* Values are averages of two to four separate experiments.

Table 3. An additive inhibitory effect of milrinone and SQ 65442 on cAMP hydrolysis by rabbit aortic cA-PDE (F3)

Inhibitor	Concn (μM)	% Inhibition
Milrinone	10	37 ± 1
	20	50 ± 1
	40	58 ± 1
SQ 65442	10	46 ± 1
	20	51 ± 2
	40	54 ± 3
Milrinone	10	
+	+	
SQ 65442	10	82 ± 1*

Each value is the mean ± SE of three incubations. cA-PDE (F3) was incubated with 1 μM cAMP at 30° for 10 min. In a separate experiment 1 μM cGMP inhibited activity by 25 ± 3%, suggesting the presence of cG-inhibited PDE. All inhibitions were significant at $P < 0.05$ (df = 4), Student's t-test. A similar result was observed in another experiment.

* $P < 0.05$ (df = 4) vs milrinone or SQ 65442 alone, Student's t-test.

Table 4. Effects of inhibitors on CaM-PDEs of rabbit aorta and bovine brain

Inhibitor	K_i (μM) Rabbit aortic CaM-PDE	Bovine brain CaM-PDE
8-MeOMeMIX	6.5	4.3
M&B 22948	6.5	9.8
Vinpocetine	15	22.2

CaM-PDE (20 μg and 2.5 ng proteins for aortic and brain CaM-PDEs respectively) was incubated with 1 μM cGMP at 30° for 15 min. The activities of aortic and brain CaM-PDEs were 120 pmol/mg protein/min and 341 nmol/mg protein/min respectively. The activity of these enzymes were stimulated 3- to 5-fold by 0.04 μM CaM and 0.1 mM $CaCl_2$. K_i values were calculated from the relationship: $K_i = IC_{50}/(1 + S/K_m)$ where IC_{50} is the concentration of drug required for 50% inhibition and S the concentration of substrate used. The K_m values of cGMP were 2.2 and 1.6 μM, respectively, for rabbit aortic and bovine brain CaM-PDEs.

linear Lineweaver–Burk plots: the high and low K_m values of cGMP-PDE for cGMP were 2.6 and 13 μM. K_m values of cAMP-PDE for cAMP were 0.8 and 8 μM. The apparent K_m value of CaM-PDE for cGMP was 2.2 μM. The CaM-PDE showed a broad pH optimum between 7.5 and 8.0 and was stimulated 2- to 3-fold by CaM (0.04 μM) and $CaCl_2$ (0.1 mM).

Immunoadsorption of rabbit aortic and bovine brain CaM-PDE. As expected, most of the cGMP hydrolyzing activity (in 0.2 ml) of bovine brain CaM-PDE was immunoadsorbed by 1 μg of bovine brain CaM-PDE Mab (ACC-1) conjugated to Staph A (Table 1). Seventy-two percent of rabbit aortic CaM-PDE (F2) activity was adsorbed to this excess amount of immobilized antibody (Table 1), indicating the presence of a small amount of other contaminating PDE. The cGMP-hydrolyzing activity remaining in the supernatant fraction after removal of CaM-PDE was not stimulated significantly by CaM and Ca^{2+}, whereas the immunoadsorbed activity in the precipitate was stimulated 4.5-fold by CaM and Ca^{2+} (results not shown).

Inhibition of different PDE fractions by various inhibitors. We examined the effects of PDE inhibitors on each form of rabbit aortic PDE isolated by the ion exchange chromatography. Table 2 summarizes the results showing the IC_{50} values of inhibitors. CaM-PDE was inhibited most effectively by CaM-PDE inhibitors 8-MeOMeMIX, M&B 22948, and vinpocetine with respective IC_{50} values of 8, 8 and 19 μM. Their IC_{50} values were not affected by the presence or absence of CaM and Ca^{2+} in the reaction mixture. cG-PDE was inhibited by dipyridamole (IC_{50} = 7 μM), M&B 22948 (IC_{50} = 0.5 μM) and 8-MeOMeMIX (IC_{50} = 10 μM). Unlike M&B 22948 and 8-MeOMeMIX, vinpocetine had minimal inhibitory activity against cG-PDE. cA-PDE was inhibited by the selective cA-PDE inhibitor milrinone most effectively as found previously in other tissues [17, 24, 29].

Additive inhibitory effect of milrinone and SQ 65442 on rabbit aortic cA-PDE activity. Milrinone, a selective inhibitor of cGMP inhibited PDE [30], and SQ 65442, a selective cGMP-insensitive PDE inhibitor [31], inhibited the rabbit aortic cA-PDE activity with the same IC_{50} value of 20 μM (Table 3). Although each agent at 10 μM inhibited activity less than 50%, addition of both agents resulted in an additive (80%) inhibition. Additionally, cGMP (1 μM) inhibited activity 25–30%. These results indicate the presence of both cGMP-inhibited PDE and cGMP-insensitive PDE in the ion exchange column purified rabbit aortic cA-PDE preparation (F3).

Comparison of drug sensitivity of aortic and brain CaM-PDEs. To examine whether CaM-PDE shows species/tissue differences, drug sensitivities of rabbit aorta and bovine brain CaM-PDEs were compared. Respective K_i values of 8-MeOMeMIX, M&B 22948 and vinpocetine for inhibition of cGMP hydrolysis were similar for aortic and brain CaM-PDEs (Table 4).

Effects of PDE inhibitors on cyclic nucleotide levels in NE-treated rabbit aorta. To evaluate relative con-

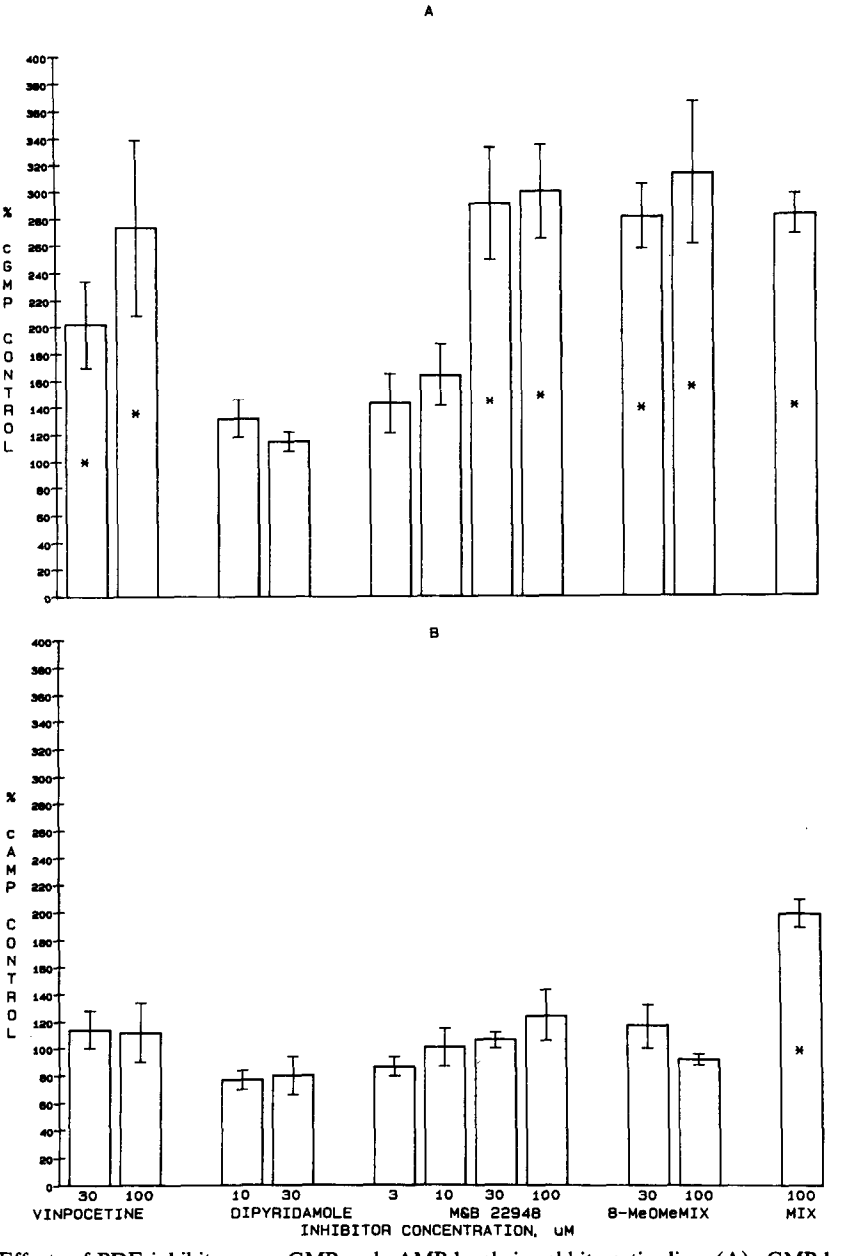

Fig. 1. Effects of PDE inhibitors on cGMP and cAMP levels in rabbit aortic slices (A) cGMP levels; (B) cAMP levels. Each value is the mean ± SE of four to eight incubations. The basal cGMP and cAMP levels were 0.092 ± 0.012 (four experiments) and 0.84 ± 0.04 pmol/mg protein (three experiments) respectively. Key: * $P < 0.05$ vs basal levels ($df > 5$), Student's t-test.

tributions of CaM-PDE and cG-PDE to cyclic nucleotide hydrolysis in the intact cells, effects of selective PDE inhibitors on cyclic nucleotide levels were examined in NE-treated rabbit aortic slices. A time course study showed that an addition of 8-MeOMeMIX (100 μM) resulted in an elevated cGMP level within 1 min, which was maintained for over 20 min (data not shown). Therefore, aortic slices were incubated with a test drug for 10 min in subsequent studies. Figure 1 summarizes the effects of various concentrations of selective PDE inhibitors on cGMP and cAMP levels in NE-preincubated rabbit aortic slices. MIX, a non-selective inhibitor, at 100 μM increased both cGMP (3-fold) and cAMP (2-fold) levels. By contrast, vinpocetine, 8-MeOMeMIX and M&B 22948 selectively increased cGMP levels without elevating cAMP levels. The selective CaM-PDE inhibitor vinpocetine increased cGMP levels in a concentration-dependent manner. 8-MeOMeMIX and M&B 22948, inhibitors of both cG-PDE and CaM-PDE, also increased rabbit aortic GMP levels in a concentration-dependent manner. M&B 22948, however, failed to increase cGMP levels at a concentration (3 μM) above its cG-PDE

Table 5. Effects of dipyridamole and M&B 22948 on cGMP levels in NE-treated and untreated rabbit aortic slices

Expt.		Concn (μM)	cGMP (pmol/mg protein) NE-treated	Vehicle-treated
1	None (control)		0.18 ± 0.02 (100%)	0.12 ± 0.03 (100%)
	Dipyridamole	10	0.16 ± 0.04 (89%)	0.47 ± 0.11* (392%)
	Dipyridamole	30	0.20 ± 0.03 (111%)	0.66 ± 0.04* (550%)
2	None (control)		0.14 ± 0.01 (100%)	0.11 ± 0.02 (100%)
	M&B 22948	1	0.16 ± 0.03 (114%)	0.23 ± 0.05 (210%)
	M&B 22948	3	0.18 ± 0.03 (129%)	0.23 ± 0.02* (210%)

Each value is the mean ± SE of three to four separate incubations. Slices were preincubated with or without 0.3 μM NE for 15 min, followed by a 10-min incubation with an inhibitor or vehicle.

* $P < 0.05$ vs control (df = 6), Student's t-test.

IC_{50} but below its CaM-PDE IC_{50}. Further, dipyridamole, a selective inhibitor of cG-PDE, at concentrations (10 and 30 μM) 1.4 to 4.3 times its cG-PDE IC_{50} value failed to increase either cGMP or cAMP levels. These data suggest that in NE-stimulated rabbit aortic slices inhibition of CaM-PDE but not cG-PDE increases cGMP levels.

Effects of agents on cGMP levels in untreated versus NE- or KCl-treated rabbit aortic slices. To examine whether the state of activation of the aorta influences cyclic nucleotide response to selective cG-PDE inhibitors, effects of dipyridamole and M&B 22948 on cyclic nucleotide levels were compared between NE-treated and untreated rabbit aortic slices. Dipyridamole caused no significant or a small increase in cGMP levels in NE-preincubated slices, but it markedly increased cGMP levels (392–550% of control value) in untreated slices (Table 5). Similarly, M&B 22948 at concentrations for selective inhibition of cG-PDE failed to increase cGMP levels in NE-treated slices, whereas it produced a 2.1-fold increase in cGMP levels in untreated slices (Table 5). In contrast to these inhibitors, vinpocetine failed to increase cGMP levels in untreated slices, although it increased cGMP levels 2.7-fold in NE-preincubated slices (Table 6). Additionally, vinpocetine increased cGMP levels by 2-fold in KCl-preincubated aortic rings but not in untreated rings (Table 6).

DISCUSSION

In the present study multiple forms of rabbit aortic PDE were reliably identified using rather selective inhibitors. CaM-PDE was inhibited specifically by vinpocetine and cA-PDE by milrinone in line with previous studies on rabbit or bovine aorta [16, 17]. cG-PDE was inhibited selectively by dipyridamole and low concentrations of M&B 22948 in agreement with a previous study on the rat aortic enzyme [16]. Vinpocetine was the only selective CaM-PDE inhibitor among the inhibitors tested since M&B 22948 and 8-MeOMeMIX which effectively inhibited CaM-PDE also inhibited cG-PDE with an equal or greater potency. The IC_{50} of M&B 22948 for inhibition of cG-PDE in the rabbit aorta was comparable to its reported values in bovine aorta and human lung [16, 32]. By contrast, the cG-PDE inhibitory potency of dipyridamole in the rabbit (our data) was weaker than its potency in the bovine aorta where dipyridamole and M&B 22948 were equipotent [16].

Although all previous studies on drug sensitivity of vascular tissue CaM-PDE were conducted using ion exchange column-purified preparations [15, 16, 19], their functional purity was never examined. Our present immunoadsorption study indicates that the ion exchange column-purified rabbit aortic CaM-PDE (F2) contained about 28% of non-CaM-PDE activity. All CaM-PDE appeared to be absorbed by the monoclonal antibody since the unadsorbed PDE activity was not stimulated by CaM and Ca^{2+}. Since this non-Cam-PDE activity was weakly or minimally inhibited by dipyridamole and M&B 22948, the nature of this PDE is not known.

This study, when compared with previous results [9, 15, 16, 19, 23], indicates that CaM-PDEs from different tissues/species have similar drug sensitivities. Vinpocetine was shown previously to inhibit ion exchange column-purified rabbit aortic and bovine brain CaM-PDE with similar IC_{50} values [9, 15]. The inhibitory potencies of 8-MeOMeMIX and M&B 22948 against rabbit aortic CaM-PDE were comparable to their reported potencies against CaM-PDE of bovine aorta or coronary arteries [15, 16, 19]. In our earlier study, vinpocetine, 8-MeOMeMIX and M&B 22948 inhibited rabbit and porcine aortic CaM-PDEs in a similar rank order [23]. These agents again inhibited, in a similar manner, activity of both pure bovine brain CaM-PDE and rabbit aortic CaM-PDE (F2) in this study. In addition, rabbit aortic CaM-PDE cross-reacted with the anti-CaM monoclonal antibody which selectively recognizes conformation of CaM bound to

Table 6. Effect of vinpocetine on cGMP levels in NE-treated and untreated rabbit aortic slices

	cGMP (pmol/mg protein)	
Expt. 1	NE-treated	Vehicle-treated
None	0.15 ± 0.03 (100%)	0.32 ± 0.03 (100%)
Vinpocetine, 30 μM	0.20 ± 0.03 (133%)	0.42 ± 0.08 (131%)
Vinpocetine, 100 μM	0.41 ± 0.08* (273%)	0.41 ± 0.02 (128%)
Expt. 2		
None	0.41 ± 0.09 (100%)	0.52 ± 0.19 (100%)
Vinpocetine, 30 μM	0.93 (0.85, 1.01)† (227%)	0.50 ± 0.02 (96%)
Vinpocetine, 100 μM	1.10 ± 0.03† (268%)	0.73 (0.72, 0.74) (140%)
Expt. 3	KCl-treated	Vehicle-treated
None	0.25 ± 0.05 (100%)	0.16 ± 0.01 (100%)
Vinpocetine, 30 μM	0.51 ± 0.04* (204%)	0.19 ± 0.03 (119%)

Each value is the mean ± SE of three to four incubations unless otherwise indicated. The concentrations of NE and KCl were 0.3 μM and 75 mM respectively.

* $P < 0.05$ vs none (df = 6), Student's t-test.

† $P < 0.05$ vs none (df = 4), Student's t-test.

CaM-PDE. This result indicates that rabbit aortic CaM-PDE and bovine brain CaM-PDE induce a similar conformation of CaM when CaM is bound to them. Further, about 50% of cGMP-hydrolyzing activity in rabbit aortic CaM-PDE (F2) could be adsorbed by a monoclonal antibody [33] to bovine brain CaM-PDE which was linked to Sepharose (Ahn, unpublished observation), thus indicating a similar antigenic structure shared by aortic and brain CaM-PDE.

The inhibitory potencies of SQ 65442 and milrinone against rabbit aortic cA-PDE were much lower than their reported potencies against purified cA-PDEs [30, 31, 34] raising a possibility that the rabbit aortic cA-PDE (F3) contained a mixture of at least two low K_m cAMP-PDEs. Indeed, our additivity study using these inhibitors indicated that the F3 preparation contained both cGMP-inhibited PDE and cGMP-insensitive PDE. Both cGMP-inhibited and cGMP-insensitive PDEs were reported previously to occur in calf liver [35]. In marked contrast to rabbit aorta, guinea pig and canine aortas have been reported to be very sensitive to milrinone but not to rolipram, a cGMP-insensitive PDE inhibitor [36]. These data indicated that guinea pig and canine aortas contained mainly cGMP-inhibited PDE but rabbit aorta contained equal activities of cGMP-inhibited PDE and cGMP-insensitive PDE. The species-related differences in drug sensitivity appeared to result from the difference in the relative amounts of each low K_m cA-PDE present in various tissues or species.

The present study also addressed the relative importance of CaM-PDE and cG-PDE in regulating cyclic nucleotide levels in a vascular tissue. Selective inhibition of CaM-PDE by vinpocetine resulted in elevated cGMP but not cAMP levels in NE-preincubated rabbit aortic slices in agreement with a previous observation [15] with KCl-precontracted rabbit aortic strips. 8-MeOMeMIX and M&B 22948 at concentrations sufficiently high to inhibit the isolated CaM-PDE also raised cGMP but not cAMP levels in NE-preincubated rabbit aortic slices. These data indicate that CaM-PDE is a predominant enzyme for hydrolysis of cGMP but not cAMP in NE-contracted rabbit aorta. However, CaM-PDE was also reported to regulate cAMP levels in cultured human astrocytoma cells upon stimulation of muscarinic cholinergic receptors [37]. In contrast to the selective increase of cGMP levels by these inhibitors, isobutylmethylxanthine, a non-selective PDE inhibitor, increased both cGMP and cAMP levels in NE-treated rabbit aorta in agreement with previous reports on KCl-preincubated rabbit aorta [15] and bovine coronary arteries [19]. Elevation of cGMP levels by the above selective PDE inhibitors (vinpocetine and M&B 22948) is unlikely to be brought about through direct activation of guanylate cyclase by these inhibitors since their effects on cGMP level are dependent on the state of rabbit aorta. Also, M&B 22948 did not stimulate rabbit aortic soluble or particulate guanylate cyclase (Ahn, unpublished observation).

Although cG-PDE has become recognized as a distinct PDE form, its role in the regulation of cyclic nucleotide levels was not clear from previous studies. Dipyridamole or M&B 22948 increased cGMP levels in precontracted rat and rabbit aortas [16, 22] and bovine coronary arteries [2], but the concentrations of these agents used were high enough to inhibit CaM-PDE also. At concentrations for selective inhibition of cG-PDE, M&B 22948 and dipyridamole produced little or no increase in rat aorta [16]. In contrast to the above results, one study [38] with

bovine coronary arteries showed a potentiation of glyceryl trinitrate-induced elevation of cGMP by dipyridamole at 0.5 μM, an IC_{50} concentration for inhibition of cG-PDE.

In the present study, selective inhibition of cG-PDE by M&B 22948 (lower concentrations) and dipyridamole did not lead to a significant increase in cGMP levels in NE-treated rabbit aortic slices, but it led to a marked increase in cGMP levels in untreated slices. By contrast, selective inhibition of CaM-PDE by vinpocetine resulted in elevated cGMP levels in NE- or KCl-treated slices but not in untreated slices. These data suggest that the relative cGMP-hydrolyzing activity of cG-PDE and CaM-PDE may be influenced markedly by the presence or absence of an agent which opens up the receptor-operated or the voltage-operated Ca^{2+} channel. In fact, in rabbit aorta an alpha-adrenergic agonist (e.g. phenylephrine or NE) or KCl was found to increase intracellular calcium concentration [39] which may, in turn, activate CaM-PDE. Thus, the relative activity of these PDEs may be determined simply by the extent of CaM-PDE activation. In NE- or KCl-treated slices the activated CaM-PDE may become a predominant enzyme for cGMP hydrolysis, whereas in untreated slices cG-PDE rather than (unactivated) CaM-PDE may be a more active enzyme.

In summary, the present study together with earlier data showed that available PDE inhibitors are useful in identifying different forms of aortic PDE. CaM-PDEs from aorta and brain had similar drug sensitivities and immunoreactivities with monoclonal antibody to CaM bound to brain CaM-PDE. More importantly, results of this study suggest that the relative importance of CaM-PDE and cG-PDE in the hydrolysis of cGMP can vary in an intact vascular tissue depending on the state (relaxed vs contracted) of the vascular tissues.

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