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ISOLATION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ISOZYMES FROM PIG AORTA

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Abstract—Five phosphodiesterase isozymes were separated from the supernatant of pig aortic smooth muscle homogenates, using DEAE-Toyopearl 650S chromatography in the presence of 0.1 mM Ca^{2+} followed by re-chromatography in the absence of Ca^{2+} and affinity chromatography on immobilized rolipram or cGMP. Type I (calmodulin-dependent family) preferentially hydrolysed cGMP and its activity was stimulated by calmodulin. Type II (cGMP-stimulated family), which had not yet been identified in aortic smooth muscle, hydrolysed both cGMP and cAMP. Its cAMP hydrolysis was stimulated by 10 μM cGMP. Type III (cGMP-inhibited family) and IV (cAMP-specific family) preferentially hydrolysed cAMP. The cAMP hydrolytic activity of Type III was inhibited by cGMP, but that of Type IV was not. Type V (cGMP-specific family) preferentially hydrolysed cGMP and its activity did not depend on calmodulin. The inhibition of all five phosphodiesterase isozymes by various phosphodiesterase inhibitors was investigated, and the potency and selectivity of each phosphodiesterase inhibitor discussed.

Cyclic nucleotide phosphodiesterases (PDEs†) play a major role in the regulation of intracellular concentrations of cAMP and cGMP [1]. Multiple forms of PDEs have been isolated from many tissues [2–7]. Numerous investigators have defined different 'forms' on the basis of the order of elution from diethylaminoethyl (DEAE) resin, using names such as peaks I, II, III or IV, often modified by descriptions of regulatory or kinetic properties. The emergence of different nomenclatures for the identification of PDE isozymes has caused some confusion. Recently, many of the 'peaks' of activity have been shown to contain multiple isozymes. Moreover, the order of elution of isozymes from DEAE varies with species, tissue, pH, eluting salt and in some cases the physiological status of tissue before fractionation. However, recent data suggest that at least five different isozymes exist, and Beavo and Reifsnyder [8] classified PDEs into five isozyme families, based largely on primary protein and cDNA sequence information, as follows: Ca^{2+}-calmodulin (CaM)-dependent family (Type I); cGMP-stimulated family (Type II); cGMP-inhibited family, selectively inhibited by a large number of positive inotropic agents such as milrinone (Type III); cAMP-specific family, selectively inhibited by rolipram (Type IV); cGMP-specific family, selectively inhibited by zaprinast (Type V).

In the case of vascular smooth muscle, three PDE forms have been isolated from human, bovine and rat aorta [9]; (1) zaprinast-inhibited, cGMP-PDE (Type V), (2) CaM-dependent PDE (Type I), (3) cAMP-specific PDE (probably Type III + Type IV). Subsequently, the presence of Type III and Type IV in vascular smooth muscle has been suggested [9–11]. Also, Lindgren *et al.* [12] separately isolated Type III and IV from rat aorta, using CIT-agarose affinity column, and demonstrated the inhibition of both Type III and IV isozymes by various cAMP-PDE inhibitors. Thus, it has been suggested that four PDE isozymes (I, III, IV and V) exist in vascular smooth muscle. However, there is no reported study that simultaneously investigated the inhibition of all four PDE isozymes from the same tissue by various PDE inhibitors.

In the present study, we isolated five PDE isozymes (I, II, III, IV and V) from porcine aorta using anion-exchange chromatography in the presence or absence of Ca^{2+} and affinity chromatography on immobilized rolipram (AAL 115-AH sepharose conjugate) [13] or cGMP [14], and investigated the inhibition of all five PDE isozymes by various PDE inhibitors.

MATERIALS AND METHODS

PDE preparation. During tissue homogenization, all the manipulations were conducted at 4°, unless otherwise stated.

Porcine aorta was excised and after removal of adhering fat, adventitia and endothelial cells, the media was cut into small pieces and stored at −80°. The tissue (20 g) was homogenized using Hiscotron in 10 vol. (w/v) of buffer A (20 mM Tris–HCl, 0.1 mM $CaCl_2$, 2 mM Mg acetate, 1 mM dithiothreitol, 0.02 mM leupeptin, 1.3 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM NaN_3, 50 mg/L aprotinin, 10 mg/L trypsin

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† Abbreviations: PDE, phosphodiesterase; CaM, calmodulin; Type I, CaM-activated PDE; Type II, cGMP-stimulated PDE; Type III, cGMP-inhibited PDE; Type IV, cAMP-specific PDE; Type V, cGMP-specific PDE; DMSO, dimethyl sulfoxide; EGTA, ethyleneglycol-bis-(β-aminoethylether)*N*,*N*'-tetraacetic acid; IBMX, 3-isobutyl-1-methylxanthine; CIT, cilostamide.

inhibitor, pH 7.4). The homogenate was centrifuged at 105,000 *g* for 60 min.

The supernatant was applied to a DEAE-Toyopearl 650S column (2 × 33 cm) pre-equilibrated with buffer A. After application, the column was washed with 1.5 bed volumes of buffer A until no more absorbance was detected in the elute at 280 nm. Then a linear gradient of 0.05–0.4 M NaCl (500 mL) was started. Elution was performed at a flow rate of 1 mL/min, and 3.3 mL fractions were collected and 10 μL of 200 mM (EGTA) ethyleneglycol-bis-(β-aminoethylether)*N*,*N*′-tetraacetic acid was immediately added to each fraction.

All fractions containing Type V activity were pooled and diluted 2-fold with buffer B (20 mM Tris–HCl, 0.1 mM EGTA, 2 mM Mg acetate, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, pH 7.4). The diluted Type V fraction was applied to a Blue sepharose CL-6B column (2 × 30 cm) pre-equilibrated with buffer B. After application, the column was washed with 1 bed volume of buffer B and then 1.5 bed volumes of buffer B containing 0.5 M NaCl. Type V was eluted with 1.5 bed volumes of buffer B containing 0.5 M NaCl and 10 mM cGMP and used as the enzyme source of Type V in PDE assay.

All fractions containing PDE activity, but not Type V activity, in the first DEAE chromatography were re-applied to DEAE-Toyopearl 650S column (2 × 33 cm) pre-equilibrated with buffer B. After application, the column was washed with 1 bed volume of buffer B, and a linear gradient of 0.1–0.3 M NaCl (500 mL) was started.

All fractions containing Type I activity in the second DEAE chromatography were pooled and used as the enzyme source of Type I in PDE assay.

Type II fractions contaminating Type III in the second DEAE chromatography were applied to DEAE-Toyopearl 650S column (2 × 18 cm) pre-equilibrated with buffer B or cGMP affinity column prepared as previously described by Martins *et al.* [14]. In the case of DEAE-Toyopearl column, the column was washed with 1 bed volume of buffer B, and a linear gradient of 0.15–0.2 M NaCl (250 mL) was started. In the case of cGMP affinity column, Type II was isolated according to the method of Martins *et al.* [14] with some modifications. Fractions containing Type II and Type III were applied to cGMP affinity column (bed volume = 5 mL), pre-equilibrated with buffer B. The column was washed with 30 mL of buffer B (Fraction G-1) followed by 30 mL of 0.5 M NaCl in buffer B (Fraction G-2). Type II was eluted with 30 mL of 10 mM cGMP and 0.3 M NaCl in buffer B (Fraction G-3).

The part of fractions containing both Type III and Type IV in the second DEAE chromatography was applied to AAL 115-affinity column [Sepharose 4B linked to 4-(3-carboxypropyloxy-4-methoxyphenyl)-2-pyrrolidone, a derivative of rolipram] (bed volume = 1 mL), prepared as described previously by Fougier *et al.* [13], pre-equilibrated with buffer B. The column was washed with 30 mL of buffer B (Fraction R-1) followed by 20 mL of 0.5 M KCl in buffer B (Fraction R-2). Then the column was washed with 10 mL of 2 M KCl in buffer B (Fraction R-3). The column was equilibrated with 10 mL of 0.5 M KCl in buffer B at room temperature (Fraction R-4). Type IV was eluted with 20 mL of 2 mM cAMP and 0.5 M KCl in buffer B (Fraction R-5). Fraction R-2 and R-5 were used as the enzyme sources of Type III and Type IV, respectively.

Fractions containing each PDE isozyme were dried by dialysing against 3 L of buffer B containing 20% polyethylene glycol and then resuspended with buffer B containing 30% ethylene glycol and stored at −20°.

Measurement of PDE activity. PDE activity was determined by a modification of a previously described two-step radioisotopic procedure [15]. Samples were incubated at 30° for 10 or 20 min in a total volume of 0.2 mL containing 50 mM Tris–HCl, pH 8.0, 0.1 mM EGTA or 0.9 mM $CaCl_2$ and 27.8 U/mL CaM, 0.1 mM $MgCl_2$, 1 μM [^{3}H]-cGMP (100,000 dpm/tube) or 1 μM [^{3}H]cAMP (100,000 dpm/tube), with or without drug. The reaction was stopped by incubation of 95° for 1.5 min and then the assay tube was cooled in ice-water. After addition of 50 μL of snake venom solution (1 mg/mL), the assay mixture was incubated at 30°. After 10 min, 550 μL of AG X-1 resin slurry (resin : H_2O = 1 : 2) was added, the assay tube was stood in ice-water. After 10 min, the assay tube was centrifuged at 10,000 rpm for 2.5 min, and then radioactivity in 450 μL of the supernatant was measured. Hydrolysis of substrate did not exceed 10%; under these conditions PDE activity was proportional to time and enzyme concentration. The IC_{50} (concentration of drug causing 50% inhibition of the enzymatic activity) values of several reference PDE inhibitors were calculated by plotting the percentage of enzymatic activity determined at 1 μM substrate concentrations vs the logarithmic concentration of the inhibitor. The tested compounds were dissolved in dimethyl sulfoxide (DMSO) and then diluted with assay buffer, at concentrations ranging from 10^{-8} to 10^{-4} M. The final concentration of DMSO was less than 0.4% (v/v). Control measurements with vehicle were performed in each case. Results are the means of three or nine determinations.

Protein analysis. Protein concentration was determined by BCA method (Pierce, IL, U.S.A.), after precipitation with 10% (final concentration) trichloroacetic acid.

Materials. Leupeptin, benzamidine, aprotinin, trypsin inhibitor from soybean type I-S, EGTA, cAMP, cGAMP, CaM from bovine brain (>40,000 units/mg protein), snake venom from *Ophiophagus hannah*, dipyridamole, nicardipine, nifedipine, papaverine and 3-isobutyl-1-methylxanthine (IBMX) were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). DEAE-Toyopearl 650S was from Toyo Soda MFG, Co., Ltd (Tokyo, Japan). Dithiothreitol was from Nacalai Tesque Inc. (Kyoto, Japan). Polyethylene glycol and ethylene glycol were from Kishida Kagaku Co., Ltd (Osaka, Japan) and Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan), respectively. DMSO was from Junsei Chemical Co., Ltd (Tokyo, Japan). M & B 22948, MY-5445, milrinone and 4-(3-carboxypropyloxy-4-methoxyphenyl)-2-pyrrolidone were synthesized by Eisai Co. Vinpocetine was extracted from Calan®

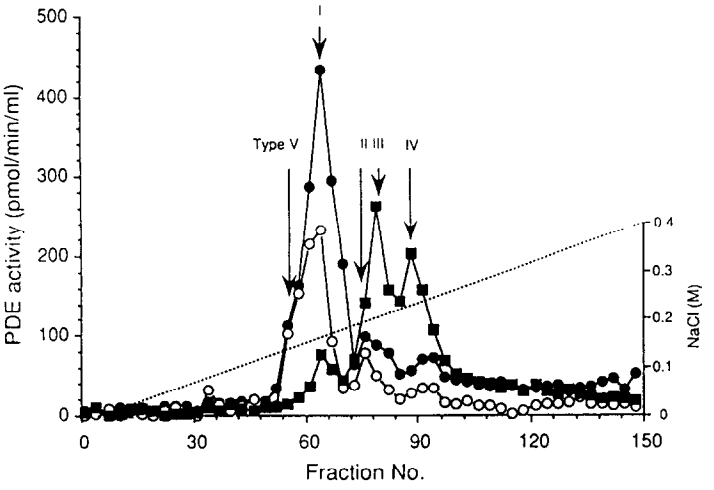

Fig. 1. Elution profile from DEAE-Toyopearl 650S chromatography of porcine aortic extract in the absence of Ca^{2+}. PDE activity was determined using 1 μM cGMP (circles) or 1 μM cAMP (squares) as substrate, in the presence of EGTA (open symbols) or CaM (closed symbols). Porcine aorta (10 g) was homogenized in 10 vol. (w/v) of buffer C (20 mM Tris–HCl, 5 mM EDTA, 2 mM Mg acetate, 1 mM dithiothreitol, 125 mg/L aprotinin, 10 mg/L leupeptin, 200 mg/L benzamidine, pH 7.4). The homogenate was centrifuged at 105,000 *g* for 60 min. The supernatant was applied to a DEAE-Toyopearl 650S column (2 × 33 cm) pre-equilibrated with buffer B as described in Materials and Methods. After application, the column was washed with buffer B. Then a linear gradient of 0–0.5 M NaCl (500 mL) was started.

(Takeda Chemical Industries Ltd, Osaka, Japan). [8,5′-^{3}H]cGMP (33.9 Ci/mmol, 1 mCi) and [2,8-^{3}H]-cAMP (33.5 Ci/mmol, 1 mCi) were taken from NEN (Boston, MA, U.S.A.). EAH-sepharose 4B and AG 1-X2 anion exchange resin were from Pharmacia (Uppsala, Sweden) and Bio-Rad (Richmond, CA, U.S.A.), respectively.

RESULTS

Isolation of porcine aorta PDEs

Figure 1 shows the elution profile from DEAE-Toyopearl 650S chromatography of porcine aortic extract in the absence of calcium, using three different PDE assay conditions. Fractions 52–58 hydrolysed cGMP with a high degree of selectivity and were insensitive to CaM (Type V). Fractions 59–73 preferentially hydrolysed cGMP and were activated by CaM and Ca^{2+} (Type I). Cyclic GMP hydrolytic activity in fractions 73–85 was eluted at the same NaCl concentration to that of Type II from porcine heart extract (data not shown). Two peaks (fraction 79 and 88) preferentially hydrolysed cAMP, and cAMP hydrolytic activities in both fractions were inhibited by milrinone at IC_{50} of 0.2 and 2.5 μM, respectively (Type III and Type IV).

Both Type V and Type I were eluted at a similar NaCl concentration (0.15 M NaCl), in the absence of calcium. However, these isozymes were separately isolated by DEAE-Toyopearl 650S column chromatography in the presence of 0.1 mM calcium. Under this condition, the second peak was eluted at a higher concentration (0.2 M) of NaCl than in the presence of EGTA (Fig. 2). The first peak was pooled as Type V fraction and furthermore purified 6-fold by blue sepharose column (Table 1). Cyclic nucleotide hydrolytic activity of Type V was not dependent on CaM (Table 1).

Fractions containing PDE activity except for Type V, in DEAE-Toyopearl 650S chromatography in the presence of 0.1 mM calcium, were re-applied to a DEAE-Toyopearl 650S chromatography column in the absence of calcium (Fig. 3). Type I was eluted at 0.15 M NaCl. Type I preferentially hydrolysed cGMP and it hydrolytic activity was stimulated 4-fold by CaM (Table 1). Type II, Type III and Type IV were eluted at 0.18, 0.2 and 0.22 M NaCl, respectively. However, these PDE isozymes were not resolved separately. The Type II fraction containing Type III was therefore re-applied to DEAE chromatography or cGMP affinity column. Type II was purified 100-fold by cGMP affinity column and hydrolysed both cGMP and cAMP (Table 1). cAMP hydrolysis by Type II was stimulated 3.5-fold by 10 μM cGMP (data not shown). The part of fractions containing Type III and Type IV was applied to AAL 115-affinity column. Type III was eluted by 0.5 M KCl at 4° and Type IV was eluted by 2 mM cAMP at 25°. Both Type III and Type IV preferentially hydrolysed cAMP, and the cAMP hydrolytic activity of Type III was inhibited by cGMP, but that of Type IV was not (Table 2). Type IV was purified 30-fold by AAL 115-affinity column (Table 1).

The apparent K_m values of the five PDE isozymes were: Type I, 8.9 μM for cGMP in the presence of CaM; Type II, 17.7 μM for cAMP in the presence

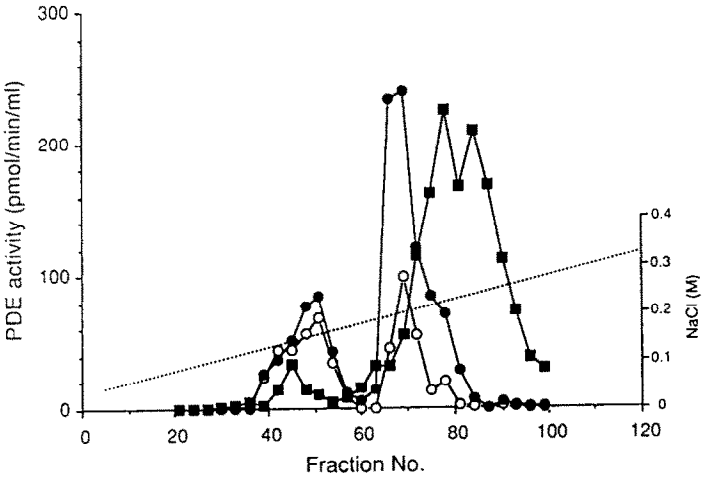

Fig. 2. Elution profile from DEAE-Toyopearl 650S chromatography of porcine aortic extract in the presence of Ca^{2+}. PDE activity was determined using 1 μM cGMP (circles) or 1 μM cAMP (squares) in the presence of EGTA (open symbols) or CaM (closed symbols). Porcine aorta was homogenized in 10 vol. (w/v) of buffer A as described in Materials and Methods. Then, the 10,500 *g* supernatant was applied to DEAE column pre-equilibrated with buffer A, washed with buffer A, and eluted by a linear gradient of 0.05–0.4 M NaCl as described in Materials and Methods.

Table 1. Specific activities of five PDE isozymes

	Specific activity (nmol/min/mg of protein)			
	cGMP		cAMP	
	EGTA	CaM	EGTA	CaM
105,000 *g* supernatant	0.07	0.13	0.11	0.12
A-1	0.18	0.11	0.01	0.03
BS (Type V)	1.14	1.15	0.15	0.12
B-1 (Type I)	0.35	1.29	0.14	0.53
B-2	0.05	0.06	0.19	0.23
G-3 (Type II)	5.21	4.33	3.00	2.56
B-3	0.04	0.04	0.96	0.98
R-2 (Type III)	0.08	0.08	0.34	0.31
R-5 (Type IV)	0.18	0.08	28.88	27.26

Each value was obtained from one experiment.

PDE activity was determined using 1 μM cGMP as substrate, in the presence of EGTA or CaM, or 1 μM cAMP as substrate, in the presence of EGTA or CaM.

105,000 *g* supernatant: the supernatant applied to a DEAE-Toyopearl 650S column pre-equilibrated with buffer A. A-1: sample applied to Blue sepharose column. BS: fraction eluted with 10 mM cGMP in Blue sepharose chromatography. B-1: fraction containing Type I in the second DEAE chromatography. B-2: sample applied to cGMP affinity column. G-3: fraction eluted with 0.3 M NaCl and 10 mM cGMP in cGMP affinity chromatography. B-3: sample applied to AAL 115-affinity chromatography. R-2 and R-5: fraction eluted with 0.5 M KCl and 2 mM cAMP in AAL 115-affinity chromatography, respectively.

of 10 μM cGMP; Type III, 0.41 μM for cAMP; Type IV, 2.8 μM for cAMP; Type V, 4.8 μM for cGMP. These five PDE isozymes followed Michaelis–Menten kinetics.

Inhibition of isolated PDE isozymes by various PDE inhibitors

IC_{50} values of the inhibitors were determined at a substrate concentration within the physiological range (1 μM [9]). Table 1 shows the IC_{50} values of different compounds for the five PDE isozymes obtained from porcine aorta. The potency of the different compounds as inhibitors was clearly different for each isozyme. Type V was inhibited most effectively by zaprinast and dipyridamole (IC_{50} = 0.45 and 0.52 μM, respectively), which are well known to be selective inhibitors of Type V, and less effectively by nonselective inhibitors, papaverine and IBMX (IC_{50} = 8.8 and 2.9 μM, respectively). The calcium channel blocker nicardipine and the selective Type IV inhibitor rolipram had minimal inhibitory activity against Type V. Type I was inhibited by vinpocetine (IC_{50} = 32.0 μM), calcium channel blocking agents nicardipine and nifedipine (IC_{50} = 3.0 and 5.5 μM, respectively) and the nonselective inhibitor IBMX (IC_{50} = 7.1 μM). Zaprinast inhibited Type I at an IC_{50} of 37.6 μM, but dipyridamole did not. Type II was inhibited by dipyridamole and papaverine (IC_{50} = 3.6 and 2.3 μM, respectively). Zaprinast, nicardipine and nifedipine inhibited Type II in the 10 μM range. Type III was effectively inhibited by the selective Type III inhibitor milrinone and nonselective inhibitor papaverine. Also, cGMP inhibited cAMP hydrolysis of Type III at an IC_{50} of 0.62 μM. Zaprinast, dipyridamole and rolipram had minimal inhibitory activity against Type III. Type IV was effectively inhibited by the selective Type IV inhibitor rolipram. Papaverine and dipyridamole inhibited Type IV at IC_{50}s of 1.7 and 6.4 μM, respectively. cGMP had no effects on cAMP hydrolysis of Type IV.

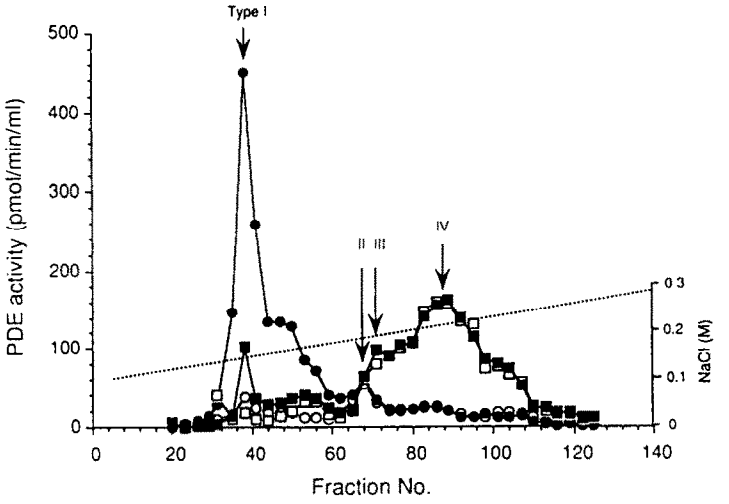

Fig. 3. Elution profile from DEAE-Toyopearl 650S re-chromatography of fractions containing PDE activity except for Type V in the first DEAE chromatography. PDE activity was determined using 1 μM cGMP (circles) or 1 μM cAMP (squares) in the presence of EGTA (open symbols) or CaM (closed symbols). Fractions containing PDE activity except for Type V (Fig. 2) were applied, washed with buffer B, and eluted by a linear gradient of 0.1–0.3 M NaCl as described in Materials and Methods.

Table 2. Inhibitory effects of various PDE inhibitors on five PDE isozymes from porcine aorta

	IC_{50} (μM)				
PDE isozyme	V	I	II	III	IV
Zaprinast	0.45 ± 0.12 (N = 3)	32.2 ± 5.3 (N = 6)	46.8 ± 1.7 (N = 3)	9 ± 3* (N = 3)	79.6 ± 20.2 (N = 3)
Dipyridamole	0.52 ± 0.17 (N = 4)	31 ± 7* (N = 3)	3.6 ± 0.2 (N = 5)	49 ± 10* (N = 3)	6.4 ± 1.1 (N = 3)
Vinpocetine	94.9 ± 23.7 (N = 5)	32.0 ± 6.6 (N = 5)	69.2 ± 10.7 (N = 3)	7 ± 3* (N = 4)	41.3 ± 8.0 (N = 5)
Nicardipine	48 ± 1* (N = 3)	3.0 ± 0.5 (N = 3)	31.8 ± 5.6 (N = 3)	38.9 ± 5.8 (N = 4)	12.1 ± 3.5 (N = 3)
Nifedipine	15.8 ± 2.8 (N = 3)	5.5 ± 2.3 (N = 5)	48.5 ± 0.8 (N = 3)	16 ± 8* (N = 4)	45.1 ± 19.8 (N = 3)
Milrinone	88.5 ± 10.4 (N = 3)	52 ± 5* (N = 3)	73.7 ± 2.8 (N = 3)	0.74 ± 0.10 (N = 9)	22.2 ± 4.9 (N = 4)
Rolipram	39 ± 4* (N = 3)	13 ± 7* (N = 3)	48 ± 2* (N = 5)	11 ± 3* (N = 4)	0.72 ± 0.17 (N = 7)
Papaverine	8.8 ± 4.2 (N = 4)	21.6 ± 3.8 (N = 5)	2.3 ± 0.2 (N = 3)	0.62 ± 0.07 (N = 3)	1.7 ± 0.7 (N = 3)
IBMX	2.9 ± 0.8 (N = 3)	7.1 ± 2.8 (N = 4)	18.6 ± 0.8 (N = 3)	7.1 ± 0.9 (N = 3)	11.4 ± 0.9 (N = 3)
cGMP				0.62 ± 0.08 (N = 4)	10 ± 5*† (N = 3)

Values are means ± SE.
* Percent inhibition at 100 μM.
† Percent inhibition at 10 μM.

DISCUSSION

In the present study, five PDE isozymes were identified in porcine aorta, on the basis of their regulatory and kinetic properties and the effects of selective PDE inhibitors.

Type I preferentially hydrolysed cGMP, its activity was stimulated by CaM and its K_m value for cGMP was 8.9 μM. Type I was inhibited by vinpocetine and zaprinast in a similar rank order (IC_{50} = 32.0 and 32.2 μM, respectively). These results are comparable to those of a previous study on porcine aorta by Ahn *et al.* [16]. As shown in Fig. 3, however, the right shoulder of the first peak (Type I) was always

recognized. The shoulder could be due to a partially proteolysed isozyme or a different subclass of Type I, as described by Beavo and Reifsnyder [8].

To date, Type II has not been identified in vascular smooth muscle [6, 7]. In the present study, Type II was firstly identified in the porcine aorta without endothelium. The cGMP hydrolytic activity in the sample applied to cGMP affinity column accounted for only 4% of that of 105,000 *g* supernatant (data not shown), showing that Type II is present at very low levels. This may explain why others have been unable to identify it in vascular smooth muscle. Type II hydrolysed both cAMP and cGMP and its cAMP hydrolytic activity was stimulated by cGMP. Type II had a K_m value of 17.7 μM for cAMP in the presence of cGMP and its cAMP hydrolytic activity was inhibited by dipyridamole (IC_{50} = 3.6 μM). These results are comparable to those of Type II in pig aortic endothelial cells reported by Souness *et al.* [17]. Souness *et al.* furthermore identified four PDE isozymes, but not Type II, in pig aortic smooth muscle cells [11]. However, in our preliminary studies, we identified Type II in the cultured smooth muscle cells of porcine aorta. Therefore, Type II in endothelial cell-removed porcine aorta may be derived from aortic smooth muscle cells.

Type III and Type IV were separately isolated from porcine aorta, using AAL 115-affinity column [13]. Type III and Type IV preferentially hydrolysed cAMP. The cAMP hydrolytic activity of Type III was inhibited by cGMP and milrinone (IC_{50} = 0.62 and 0.74 μM, respectively). On the other hand, the cAMP hydrolytic activity of Type IV was not inhibited by cGMP and was inhibited by rolipram (IC_{50} = 0.72 μM). The inhibitory potencies of milrinone for Type III and of rolipram for Type IV were identical to those observed with purified Type III from dog cardiac membranes [18, 19] and aorta [19] and with purified Type IV from rat heart cytosol [13]. The K_m values of Type III and Type IV were 0.41 and 2.8 μM for cAMP, respectively. These values were comparable to those observed with purified Type III from dog heart [18] and purified Type IV from rat brain and heart [20]. These results suggest that Type III and Type IV were separately isolated.

Type V hydrolysed cGMP with a high degree of selectivity and was insensitive to CaM. Type V was inhibited by zaprinast and dipyridamole (IC_{50} = 0.45 and 0.52 μM, respectively). The inhibitory potencies of zaprinast and dipyridamole were identical with those in Type V from bovine aorta [16]. Although anion-exchange chromatography has been used to isolate PDE isozymes, the isozymes could not be separately resolved into each peak of PDE activity [21]. In the presence of EGTA, Type V and Type I were eluted at a similar NaCl concentration (Fig. 1). However, in the presence of 0.1 mM Ca^{2+}, Type V was dissociated from Type I, which was eluted later than in the presence of EGTA (Fig. 2). It is known that in some cases the addition of a chelator to the fractionation buffer facilitates the dissociation of endogenous CaM from Type I, resulting in a change of the isoelectric point [6]. This may explain the dissociation of Type V from Type I. The one step isolation method of Type V is useful for screening of selective inhibitors of Type V.

Many selective PDE inhibitors are known, but there is no information about the inhibitory profiles of these inhibitors for the five PDE isozymes in tissue. In the present studies, the inhibition of all five PDE isozymes by various PDE inhibitors was simultaneously investigated (Table 1). Rolipram is a selective inhibitor of Type IV, and its inhibitory selectivity for Type IV was more than 200 times that of other isozymes in pig aorta. On the other hand, although zaprinast and vinpocetine are well known as selective inhibitors of Type V and Type I, respectively, these inhibitors did not have adequate selectivity and potency to investigate the physiological functions of the both Type V and I. Dipyridamole, which is well known as a selective inhibitor of Type V, inhibited not only Type V in the 0.1 μM range, but also Type II and Type IV at 1 μM. Thus, there are no selective inhibitors of Type V or Type I as well as Type II. More potent selective inhibitors of Type V or Type I are needed to investigate the roles of Type V and Type I isozymes in aortic smooth muscle cells and to determine whether selective inhibition of Type V or Type I is responsible for effects such as cGMP elevation and relaxation of smooth muscle.

Our isolation methods of PDE isozymes from the porcine aorta, which contains five PDE isozymes, are useful for investigation of the activity and potency of PDE inhibitors and for the screening of selective inhibitors of PDE isozymes.

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