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Effects of Depogen on Various Isozymes of Cyclic Nucleotide Phosphodiesterase Isolated from Porcine Aorta

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Summary. **Cyclic nucleotide phosphodiesterase (PDE) isozymes were separated from the soluble fraction of porcine aortic smooth muscle by DEAE-Sepharose Fast Flow ion exchange chromatography, and the effects of depogen (a theophilline-7-acetice acid salt of drotaverine) on the isozymes were investigated in comparison with those of various selective or nonselective PDE inhibitors. Five PDE isozymes (Types I-V) were identified on the basis of their regulatory and kinetic properties and their sensitivities to selective PDE inhibitors. Type I (calmodulin-stimulated) preferentially hydrolyzed cGMP. The presence of Type II was demonstrated by a marked stimulation of activity by cGMP. Because of an insufficient amount of the isozyme and the unavailability of specific inhibitors, no further study of the Type II PDE was conducted. The Type III (cGMP-inhibited) and Type IV(cAMP-specific) isozymes preferentially hydrolyzed cAMP. The Type V PDE (cGMP-specific) hydrolyzed cGMP with a high selectivity. Depogen inhibited Types I, III and V PDE isozymes with potencies much weaker than selective inhibitors of each PDE isozyme as well as a nonselective PDE inhibitor, 3-isobutyl-1-methylxanthine. In contrast, the inhibitory action of depogen was most potent toward Type IV PDE ($IC_{50}=17\ \mu M$), being more potent than that of RO 20-1724, a Type IV-specific inhibitor ($IC_{50}=32\ \mu M$). Theophilline-7-acetic acid produces only a minimal inhibition while the inhibitory activities of drotaverine were very much like those of depogen. These results suggest that depogen is a selective inhibitor of Type IV PDE isozyme and that the inhibitory activity of depogen is mainly attributable to drotaverine.**

Key words—depogen, PDE, vascular smooth muscle, cAMP, cGMP.

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INTRODUCTION

Concentrations of cAMP and cGMP, two important intracellular messengers responsible for the relaxation of vascular smooth muscle by various agents, are controlled by the balance between their synthesis and breakdown. In mammalian cells, cyclic nucleotide phosphodiesterase (PDEs) are the sole enzymes that subserve the breakdown of cAMP and cGMP.[1]

Findings that multiple forms of PDEs exist in almost all tissues or cell types[2] aroused a renewed interest in modulators of this enzyme as therapeutic agents, and selective inhibitors of certain types of PDEs are now being eagerly searched for. The approach proved to be promising especially in the cardiovascular research area: numerous agents that selectively inhibited myocardial cGMP-inhibited PDE (Type III) were found to be potent inotropic agents.[3-5]

Depogen is a theophylline-7-acetic acid salt of drotaverine (Fig. 1), synthesized by Szentmiklosi et al.[6] to improve the pharmacokinetical parameters of drotaverine (the absorption of the substance is extremely rapid), a congener of papaverine which was shown to have more potent spasmolytic, cardiovascular and coronary dilatory effects than papaverine.[7] Preclinical and clinical investigations with depogen showed a profound decrease in vascular resistance in the extremities.[8] Disturbance of peripheral circulation produced by laurate in the extremities of the rat was demonstrated to be improved by depogen.[9]

In the present study an attempt was made to identify and characterize PDE isozymes present in porcine aortic smooth muscle, and the effects of depogen on these isozymes were studied in comparison with those of various representative PDE inhibitors such

as 3-isobutyl-1-metylxanthine (IBMX), zaprinast (a selective Type V PDE inhibitor,[10]) cilostazol (a selective Type III PDE inhibitor[11]) and RO 20-1724 (a selective Type IV PDE inhibitor[12]). As the compound was found to be an active inhibitor of Type IV PDE, the effects of its two components were also studied in order to identify the component responsible for the inhibitory action.

MATERIALS AND METHODS

Isolation of PDE isozymes with a DEAE-Sepharose Fast Flow ion exchange chromatographic method

Unless otherwise stated, all manipulations necessary for the preparation of PDE isozymes were conducted at 4℃. Fresh pig aortas were obtained from a local slaughterhouse. After rapid removal from the animals, the materials were transported to the laboratory in an ice-cold Krebs-Henseleit solution. Adhering fat, connective tissue and endothelial cells were removed and the media was cut into small pieces and frozen in liquid nitrogen. The frozen tissues were pulverized with a stainless-steel percussion mortar at liquid nitrogen temperature and were stored at −80℃ until further treatment.

The powder (10 g) was homogenized with a Polytron homogenizer (PT 10/35, Kinematica, Switzerland) at a setting of 8 in 5 vol. (w/v) of 0.25 M sucrose, 10 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 0.2 mM EGTA/Tris (buffer A) containing 0.1 mM phenylmethyl sulfonyl-fluoride, 0.5 μg/ml leupeptin, and 0.5 μg/ml pepstatin A for five 15-s periods with 45-s intervals. The homogenate was centrifuged at 1,000× g for 20 min. The supernatant was further centrifuged at 100,000× g for 60 min. The resulting supernatant was filtered through four layers of gauze and dialyzed overnight against two changes of 100 vol. of buffer B (70 mM sodium acetate, pH 6.7, 5 mM 2-mercaptoethanol and protease inhibitors). The rest was applied to a DEAE-Sepharose Fast Flow ion exchange chromatographic column (1.5×27cm) pre-equilibrated with buffer B. After washing the column with three column-vol. of buffer B from which protease inhibitors were omitted, PDE isozymes were eluted at a rate of 48 ml/hr using a linear gradient of 0.07 to 1.0 M sodium acetate, pH 6.8, containing 5 mM 2-mercaptoethanol (600 ml). The fractions were collected every 10 min and cAMP- and cGMP-hydrolysing activity was assayed with 20 μl aliquot as described below. Peak fractions were collected as 1 ml-aliquots and stored at −80℃. PDE isozyme activity continued stable over several months under these conditions.

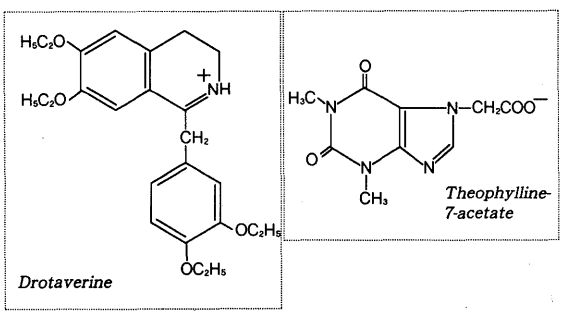

Fig. 1. Chemical structure of depogen, a theophylline-7-acetic acid salt of drotaverine.

Phosphodiesterase assay

Cyclic AMP- and cGMP-hydrolysing activity was determined by a modification of the radiochemical method by Arch and Newsholme.[14] Samples (1–5 μg protein) were pre-incubated at 30℃ for 10 min in a total volume of 0.11 ml of 50 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 1 mM ethyleneglycol bis (β-aminoethyl-ether)-N, N, N′, N′-tetraacetic acid (EGTA) /Tris, 2.5 mM dithiothreitol, 2.5% dimethylsulfoxide (DMSO), 25 μg/ml 5′-nucleotidase (from *Crotalus atrox* venom), 0.23 mg/ml bovine serum albumin and various concentrations of drugs or vehicle. The reaction was initiated by adding 20 μl of [^{3}H]-cAMP or [^{3}H]-cGMP (2.5 Ci/mmol) to make a final concentration of 1 μM. After 10 min incubation at 30℃, the reaction was terminated by adding 1.2 ml of 50% (w/v) AGl-X8 anion-exchange resin slurry to adsorb unresponsive cAMP or cGMP. The radioactivity remaining in the 1,000×g supernatant was measured using a liquid scintillation counter and corrected for recovery on the basis of the recovery of [^{14}C]-adenosine or [^{14}C]-guanosine as a standard (in the range of 0.05 to 1 μM, the recovery of either of these two ^{14}C-labeled nucleosides was 27.6% irrespective of concentration). The PDE assays were conducted in the linear reaction range, where less than 10% of the initial substrate was hydrolyzed. The IC_{50} (concentrations of inhibitors which inhibited 50% of the enzyme activity) of several drugs was calculated by plotting the percentage of enzymatic activity determined at 1 μM substrate concentration against the logarithmic concentration of the inhibitors. The test compounds were dissolved in DMSO. The final concentration of DMSO in the reaction mixture was adjusted to 2.5% (v/v). Control measurements with a vehicle were performed in each case. Data presented are the means±S.E.M. Protein concentration was determined by using the

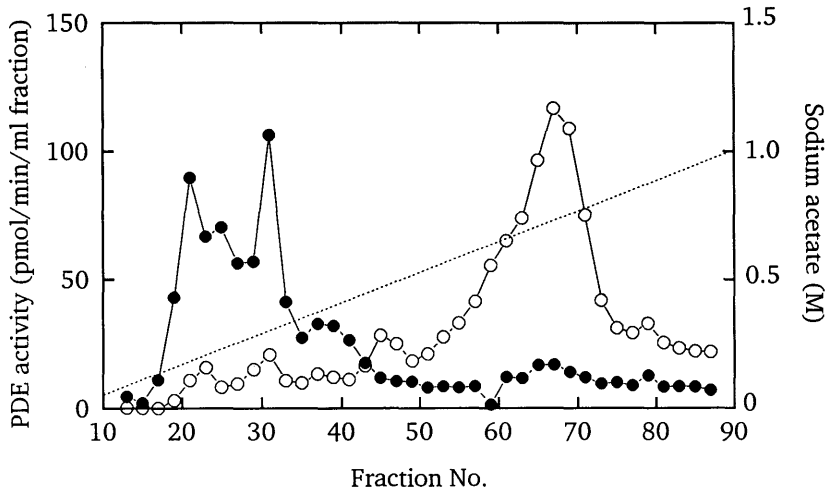

Fig. 2. A representative record showing the elution profile of PDE activities from DEAE-Sepharose Fast Flow ion exchange chromatographic colum. PDE activity was determined using 1 μM cAMP (○) or 1 μM cGMP (●) as a substrate. Three independent chromatographic runs gave essentially the same elution profiles.

Bio-Rad Protein Assay kit with bovine serum albumin as a standard.

Drugs and reagents

Leupeptin and pepstatin A were obtained from Peptide Institute (Osaka). Calmodulin, phenylmethylsulfonyl fluoride, 5′-nucleotidase, and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma Chemical Co. (St. Louis, MO, USA). [3-^{3}H]-cGMP and [8-^{3}H]-cAMP were from Amersham (Tokyo). cAMP and cGMP were from Yamasa (Choshi). DEAE-Sepharose Fast Flow anion exchange resin was from Pharmacia (Tokyo). AG1-X8 anion exchange resin (200-400 mesh) was from Bio-Rad (Tokyo). RO 20-1724 was from Funakoshi (Tokyo). Cilostarzol and depogen were the gifts from Nihon Zhoki Pharmaceutical Co. (Osaka). Zaprinast was the gift from Rhone-Poulenc Rorer (Dagenham, UK). Other chemicals used were of the highest purity grade commercially available.

RESULTS

Pig aorta PDE isozymes

Fig. 2 depicts the elution profile of PDE activities from the DEAE-Sepharose Fast Flow ion exchange chromatographic column. The PDE activities were assayed either for cAMP or cGMP as substrates. As this figure shows, the PDE activity in fractions 20-21 and 30-31 was preferentially exhibited towards cGMP, while the PDE activity in fractions 61-67 was preferentially exhibited towards cAMP.

Fig. 3 depicts the effects of calmodulin and cGMP on the cyclic nucleotide hydrolyzing activities of these fractions. The effects of several selective inhibitors of PDEs are also depicted in the figure. The activity of fraction 20-21 was not modified by calmodulin but was strongly inhibited by a Type V-selective inhibitor, zaprinast, suggesting the presence of PDE V in these fractions, while the activity of fraction 30-31 was markedly potentiated by calmodulin, suggesting the presence of Type I PDE. (Though slight, the activity was inhibited by zaprinast). Thus, the peak is designated as I in the figure. The PDE activity of fraction 29 and around was markedly potentiated by cGMP, a feature which is characteristic of Type II PDE. Thus, the peak is designated as II in the figure. The activity of fractions 61-67 was specific to cAMP and was not affected by calmodulin or zaprinast, but was inhibited by RO 20-1724, a specific inhibitor of Type IV PDE. In addition, a part of the activity of this peak was inhibited by inhibitors of Type III PDE (cilostazol and cGMP), indicating the presence of Types III and IV isozymes in this fraction.

These results were in agreement with those of Barnette et al.[15] and suggest the presence of at least five isozymes of PDE in the soluble fractions obtained from the pig aortic smooth muscle, i.e., calmodulin-dependent Type I, cGMP-stimulated Type II, cGMP-inhibited Type III, cAMP-specific Type IV and

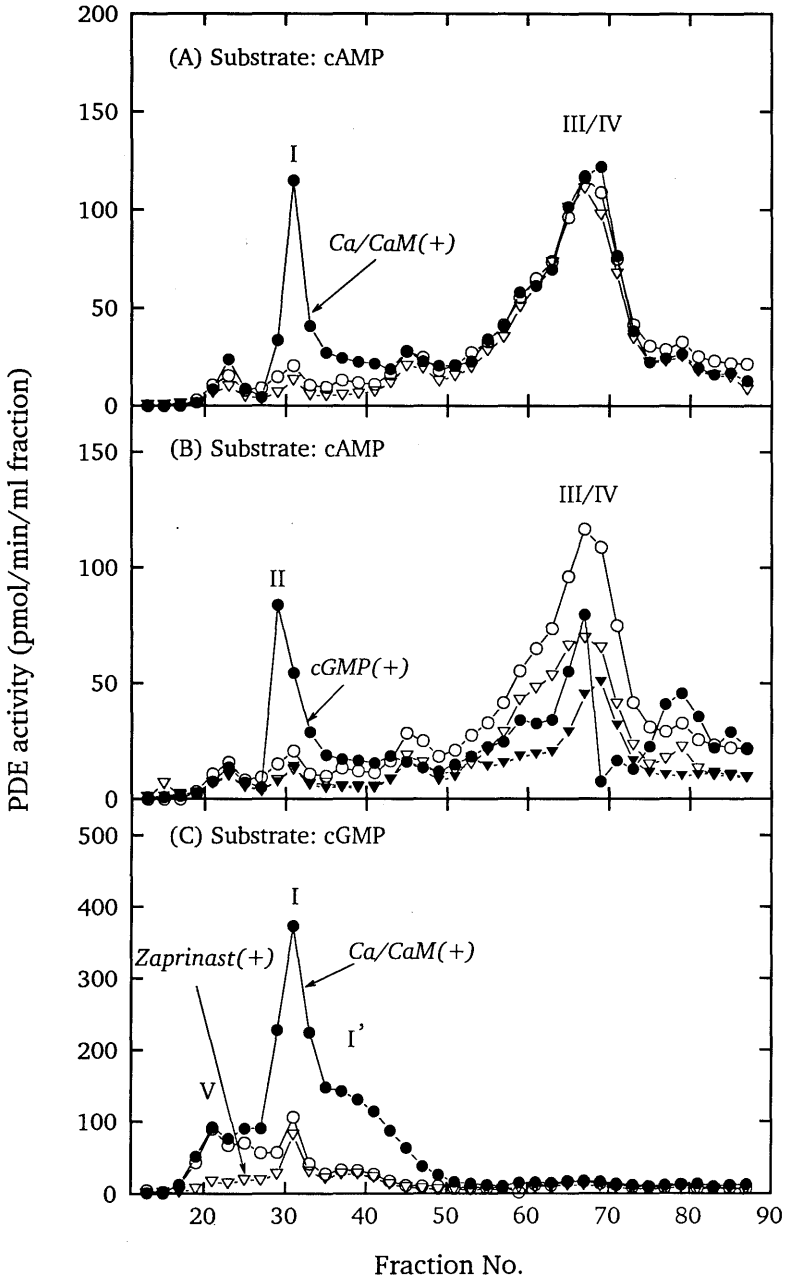

Fig. 3. Effects of calmodulin (CaM), cGMP and several representative PDE inhibitors on the PDE elution profile from DEAE-Sepharose Fast Flow ion exchange column. PDE activity was determined with 1 μM cAMP or cGMP as a substrate. (**A**) PDE activity with cAMP as a substrate. Symbols: ○, control; ●, 5 μg/ml Ca/CaM; ▽, 10 μM zaprinast. (**B**) PDE activity with cAMP as a substrate. Symbols: ○, control; ●, 10 μM cGMP; ▽, 10 μM RO 20-1724; ▼, 20 μM cilostazol. (**C**) PDE activity with cGMP as a substrate. Symbols: ○, control; ●, 5 μg/ml Ca/CaM; ▽, 10 μM zaprinast.

cGMP-specific Type V.

Inhibitory actions of depogen and PDE inhibitors on PDE activities

Table 1 and Fig. 4 show the results of preliminary experiments in which the inhibitory action of depogen and related compounds on PDE was assessed using a soluble fraction obtained from the pig aorta, in comparison with those of several representative PDE inhibitors. PDE activities were assayed with cAMP as a substrate and under conditions under which Types III and IV isozymes were mainly responsible for the observed hydrolyzing activities.

The inhibitory effect (around 1/3-1/4 of that of papaverine) was found with depogen and drotaverine, while theophylline-7-acetic acid was without effect. As expected, the inhibition by cilostazol, a specific inhibitor of Type III isozyme, of the enzyme activity of soluble fraction was partial. Its potency was a little less than that of papaverine and about 1/4 of that of depogen.

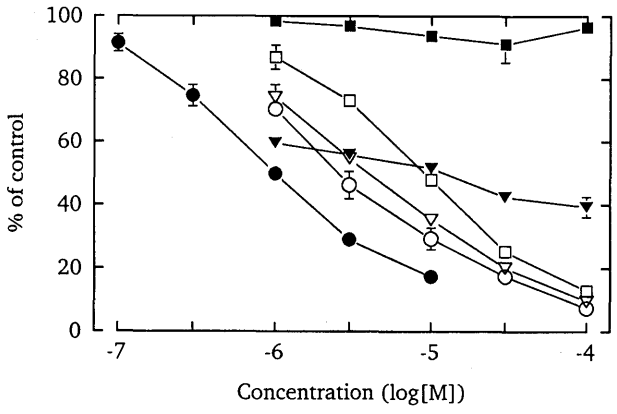

Fig. 4. The effects of depogen, its related compounds, and several representative PDE inhibitors on the cAMP (1 μM) hydrolyzing activity of soluble fraction isolated from the pig aorta. PDE activities were determined with 1 μM cAMP as a substrate. Symbols: ●, papaverine; ○, depogen; ▽, drotaverine; □, IBMX; ▼, cilostazol; ■, theophylline-7-acetic acid. The value represents the mean±S.E.M. of 4-6 experiments.

We next examined the effects of depogen and various representative PDE inhibitors on PDE isozymes using the fractions from DEAE-Sepharose Fast Flow ion exchange chromatography. As described above, Types I and V isozymes were well separated by one-step chromatography. However, the separation of Types III and IV was impossible with this method. We therefore used specific inhibitors of respective isozymes in order to determine separately the activities of these two types of isozymes. As a specific inhibitor of the Type III isozyme we used cilostazol. As a specific inhibitor of the Type IV isozyme we used RO 20-1724.

Fig. 5 shows the concentration-effect relationship of inhibition of PDE isozymes isolated the pig aortic smooth muscle produced by depogen. The effects of several representative inhibitors of PDE have also been depicted in the figure. Table 2 is a summary of IC_{50} values determined at a substrate concentration assumed to lie within physiological range.[10] Because of the unavailability of specific inhibitors, the inhibition of Type II isozyme was not assessed.

Depogen, drotaverine and RO 20-1724 exerted only a minimal effect on the Type I isozyme. The isozyme was inhibited by IBMX ($IC_{50}=7.3$ μM) and zaprinast ($IC_{50}=14$ μM). IBMX ($IC_{50}=48$ μM) and cilostazol ($IC_{50}=3.5$ μM) effectively inhibited the Type III isozyme, while the inhibition by depogen and drotaverine was weak ($IC_{50}=240$ and 267 μM, respectively). Among the compounds studied, depogen was the strongest inhibitor of the Type IV isozyme ($IC_{50}=17$ μM), its effect being stronger than a representative selective inhibitor of this type of isozyme, RO 20-1724 ($IC_{50}=32$ μM). As expected, the Type V isozyme was most strongly inhibited by zaprinast ($IC_{50}=1.7$ μM).

Table 1. IC_{50} values of depogen, its related compounds, and PDE inhibitors of the cAMP-hydrolysing activity in the soluble fraction from the pig aorta

Drug	IC_{50}[a] (μM)
Papaverine	1.1±0.2
Depogen	2.7±0.5
Drotaverine	4.0±0.4
IBMX	9.0±0.7
Cilostazol	10.8±1.9
Theophylline-7-acetic acid	>100

[a] IC_{50} values were obtained from inhibition curves of Fig. 4. The values represent the mean ± S.E.M. of 4-6 experiments.

Table 2. IC_{50} values of depogen and various PDE inhibitors of pig aortic PDE isozymes

Inhibitor	IC_{50} (μM)			
	PDE isozyme			
	I	III	IV	V
Depogen	102±25	240±7.0	17±1.3	177±40
Drotaverine	180±15	267±17	23±1.3	132±14
IBMX	7.3±3.4	48±11	44±15	63±9.6
Cilostazol	N.D.[a]	3.5±1.3	N.D.[a]	N.D.[a]
RO 20-1724	N.I.[b]	N.D.[a]	32±9.0	N.I.[b]
Zaprinast	14±2.8	N.D.[a]	N.D.[a]	1.7±0.3

IC_{50} values were obtained from inhibition curves of Fig. 5. The values represent the mean ±S.E.M. of 3 experiments. [a]N.D., not determined. [b]N. I., no inhibition up to 100 μM.

DISCUSSION

From the present study conducted with several selective inhibitors or activators it became apparent that at least five isozymes of PDE are present in pig aortic smooth muscle. Type I was stimulated by calmodulin and inhibited by zaprinast and IBMX. The inhibitory potencies found were comparable to those of previous studies on the pig aorta.[10,16] The right shoulder of the Type I peak previously described by Beavo Reifsnyder[17] and Saeki and Saito[16] was also found in the present study (Fig. 3). Whether the shoulder represents a partial proteolytic product of the Type I isozyme or a subclass of Type I needs to be studied.

In the present study, Types III and IV were not

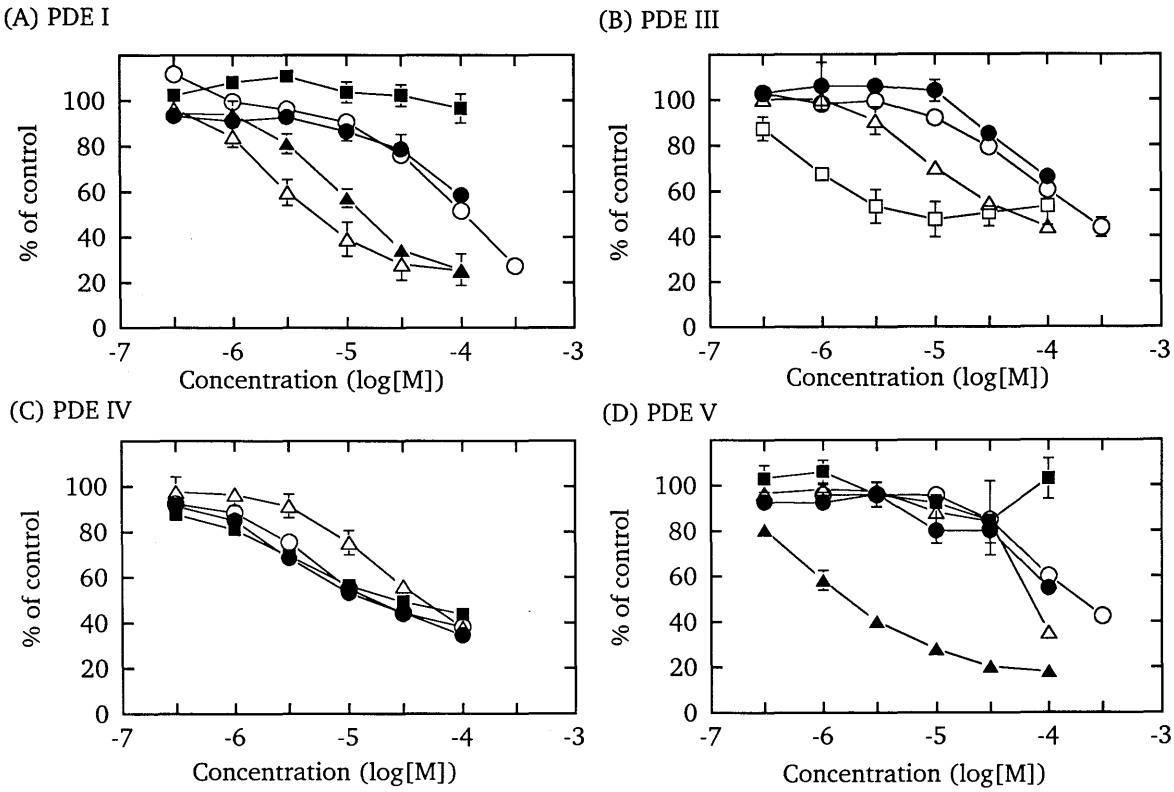

Fig. 5. Effects of depogen and various representative PDE inhibitors on the PDE isozymes. The activity of Type I PDE was determined in the presence of Ca/calmodulin (5 μg/ml). The activities of Type III and IV PDEs were assessed in the presence of 100 μM RO 20-1724 and 10 μM cilostazol, respectively. [^{3}H]-cAMP (1 μM) was used as a substrate in the assays for Types I, III, IV PDEs, and [^{3}H]-cGMP (1 μM) for Type V PDE. The fractions used for the respective PDE isozymes were: 30 and 31 for Type I; 61 for Type III; 67 for Type IV; 20 and 21 for Type V (for fraction number, see Fig. 3). The value represents the mean$\pm$S.E.M. of three experiments. Symbols: ○, depogen; ●, drotaverine; △, IBMX; ▲, zaprinast; □, cilostazol; ■, RO 20-1724.

completely separated. The fraction 61-67 appeared to contain two isozymes: a cGMP-inhibitable form or Type III and a cAMP-specific form or Type IV (Fig. 3). The occurrence of both Type III and Type IV in the same broad peak has been reported by many authors.[18-21] To differentiate the activities of the two forms of PDE present in this broad peak, we determined the activities of fractions 61-67 to hydrolyze cAMP in the presence of either a saturating concentration of cilostazol (10 μM), a selective inhibitor of Type III, or in the presence of RO 20-1724 (100 μM), a selective inhibitor of Type IV. The activity observed in the presence of RO 20-1724 was potently inhibited by cilostazol, and the activity seen in the presence of cilostazol was inhibited by RO 20-1724. Thus, it may be inferred that the broad peak (fraction 61-67) was composed of approximately equal amounts of Type III and Type IV isozymes. Type V hydrolyzed cGMP with a high degree of selectivity and was insensitive to calmodulin. Its activity was potently inhibited by zaprinast ($IC_{50}=1.7$ μM).

The existence of five PDE isozymes (I, II, III, IV and V) in the soluble fraction from the porcine aorta has recently been reported.[16] The elution profile of PDE isozymes from the DEAE-Toyoperal 650S chromatographic column is essentially similar to that of the present study. The presence of Types I, III, IV and V PDE isozymes has also been demonstrated in human, bovine, and rat aorta.[22] More recently it was demonstrated by Rabe et al.[23] that the human pulmonary artery expressed Types I, III, IV, and V PDEs. Type II PDE was not found either in the soluble fraction or in particulate fraction. The elution profile obtained with MonoQ HRP/5 anion exchange chromatography was similar to that of the present study except for the fact that Type V represented a major form of cGMP hydrolyzing activity. Substrate specificities determined for the PDE isozymes are

essentially the same in all these studies, including our own suggesting the uniformity in substrate specificities of PDE isozymes (I, III, IV and V) of vascular smooth muscles. Type I and/or V PDE are principally responsible for hydrolysis of cGMP, while Type III and/or IV PDEs are major cAMP hydrolyzing enzymes.

The most important finding of the present study is a significant inhibition of Type IV PDE by depogen ($IC_{50}=17\ \mu M$). This is the first report on the inhibitory action of depogen on Type IV PDE. It should be, however, noted that the inhibition of Type IV PDE by depogen was evaluated with a PDE preparation in which Type III and Type IV PDE co-existed. One should realize that the IC_{50} values of depogen obtained in the present study are only relative to other representative PDE inhibitors. Conclusive estimation of the potency of depogen in the inhibition of Type IV PDE will require complete separation of the Type IV PDE isozyme.

Depogen has two components in its structure, namely drotaverine and theophylline-7-acetic acid. The effects of drotaverine were very much like those of depogen, indicating that drotaverine was mainly responsible for the action of depogen. In preliminary experiments conducted with a crude enzyme fraction it was found that the inhibitory activity of theophylline-7acetic acid was very weak as compared with the strong inhibitory action of depogen and drotaverine (Fig. 4). We therefore did not include the substance in successive experiments (Fig. 5 and Table 2).

Even compared with that of RO 20-1724, the inhibitory effect of depogen on type IV PDE was stronger. As PDE IV inhibitors were reported to have antidepressant, tracheal smooth muscle relaxant and other effects,[24] depogen may prove to be a useful remedy for such physical conditions.

In conclusion, with DEAE-Sepharose Fast Flow ion exchange chromatography, five PDE isozymes could be identified in the pig aortic smooth muscle. It was found that depogen was a selective inhibitor of Type IV PDE. The action was mainly attributable to the presence of drotaverine.

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