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Isoenzymes of cyclic nucleotide phosphodiesterase in the human aorta: characterization and the effects of E4021

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Abstract

In extracts of the human aorta, five isoenzymes of cyclic nucleotide phosphodiesterase, namely, phosphodiesterase I, phosphodiesterase II, phosphodiesterase III, phosphodiesterase IV and phosphodiesterase V, were identified exclusively in the cytosolic fraction, and no phosphodiesterase activity was detected in the particulate fraction. Phosphodiesterase V and phosphodiesterase I were the major cGMP-hydrolyzing enzymes in the human aorta. A novel vasorelaxant, sodium 1-[6-chloro-4-(3,4-methylenedioxybenzyl)aminoquinazolin-2-yl]piperidine-4-carboxylate sesquihydrate (E4021), relaxed prostaglandin $F_{2\alpha}$-precontracted strips of human pulmonary artery with an ED_{50} value of 0.5 μM. E4021 potently and highly selectively inhibited the activity of phosphodiesterase V from human aorta with a K_i value of 2.4 nM. These results suggest that there is a unique distribution of phosphodiesterase isoenzymes in the human aorta and that inhibitors of phosphodiesterase V might be useful as a new type of vasodilator in the treatment of clinical disorders.

Keywords: Phosphodiesterase; cGMP-specific phosphodiesterase (phosphodiesterase V); E4021; Vasorelaxant; Aorta, human

1. Introduction

cAMP and cGMP play important roles in the regulation of vascular smooth muscle tone, as do Ca^{2+} ions. The intracellular concentrations of these cyclic nucleotides appear to be regulated by the balance between the activities of adenylate and guanylate cyclases and those of cyclic nucleotide phosphodiesterases. Phosphodiesterases exist as multiple isoenzymes in a variety of tissues and cell types. Recent information obtained from cloning, primary-sequencing and drug-selectivity studies makes it clear that there exists a large superfamily of phosphodiesterases, which consists of at least seven distinct families of phosphodiesterases, as follows: Ca^{2+}-calmodulin-dependent phosphodiesterases (phosphodiesterase I); cGMP-stimulated phosphodiesterases (phosphodiesterase II); cGMP-inhibited phosphodiesterases (phosphodiesterase III); cAMP-specific phosphodiesterases (phosphodiesterase IV); cGMP-specific phosphodiesterases (phosphodiesterase V); photoreceptor phosphodiesterases (phosphodiesterase VI); and new high-affinity cAMP-specific phosphodiesterases in yeast, designated HCP1 (phosphodiesterase VII) (Beavo and Reifsnyder, 1990; Beavo et al., 1994). It is widely accepted that there are not only tissue-related but also species-specific differences in the distributions of phosphodiesterase isoenzymes. Furthermore, it is likely that the intracellular distribution of phosphodiesterase isoenzymes is an important factor in determining their respective regulatory roles (Weishaar et al., 1987).

Phosphodiesterase inhibitors are being investigated for use as cardiotonic agents, vasodilators, anticoagulants, bronchodilators and antidepressants for the treatment of a wide range of clinical disorders, such as heart failure, asthma, depression and dementia (Beavo

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and Reifsnyder, 1990; Nicholson et al., 1991). Recently, E4021, a novel vasorelaxant, was newly synthesized and was reported to be a potent and selective inhibitor of phosphodiesterase V isolated from porcine aorta (Saeki et al., 1995). Such a potent and selective inhibitor of phosphodiesterase V is expected to be useful for clinical application in the treatment of patients with angina pectoris, pulmonary hypertension and heart failure (Saeki et al., 1995). However, for the appropriate therapeutic application of this drug, it is necessary to characterize the effects of E4021 in human tissues and the effects on each of the isoenzymes of phosphodiesterase isolated from human vascular smooth muscle. Accordingly, the objectives of the present study were as follows: (1) to identify the isoenzymes of phosphodiesterase and to determine their intracellular distribution in the human aorta; (2) to investigate the effects of E4021 on strips of human pulmonary artery and on the activity of each isoenzyme of phosphodiesterase isolated from human aorta.

2. Materials and methods

2.1. Preparation of phosphodiesterases

Human aorta and kidney were obtained within 1 h of autopsy from subjects who had not had any pathological condition prior to death that might have contributed to a variation in the distribution of phosphodiesterases, for example, hypertension or heart failure. These organs were quickly frozen in liquid nitrogen and stored at −80°C until use. Written informed consent was given by each subject's family. Cytosolic and particulate phosphodiesterase isoenzymes from human aorta were extracted and separated by a modified version of the previously described procedures (Saeki and Saito, 1993; Sugioka et al., 1994). All subsequent procedures were performed at 4°C. In brief, 20 g of the aorta were minced, suspended in 10 volumes of buffer A that contained 10 mM tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl; pH 7.8), 0.25 M sucrose, 5 mM $MgCl_2$, 0.2 mM ethylene-glycol-bis-(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 10 mg/l leupeptin, 1 μM (p-amidinophenyl) methanesulfonyl fluoride hydrochloride (p-APMSF), 2 mM benzamidine hydrochloride and 1 mM dithiothreitol. The tissue was homogenized with a hand-held Potter-Elvehjem homogenizer fitted with a serrated Teflon pestle (three passes, ten strokes per pass). The homogenate was centrifuged at 100 000 × g for 60 min and then the supernatant was dialyzed for 4 h against 2 l of 20 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, 0.2 mM dithiothreitol, 1.3 mM benzamidine and 1 μM p-APMSF (buffer B). Dialysis was repeated twice. After the addition of $CaCl_2$ to a final concentration of 0.5 mM, the dialyzed supernatant was applied to a column (16 × 1.6 cm i.d.) of DEAE-Toyopearl 650S which had been equilibrated with buffer B that contained 0.1 mM $CaCl_2$ (buffer C). The column was then washed with several bed volumes of buffer C, after which the phosphodiesterase isoenzymes were eluted at a flow rate of 1.0 ml/min with a 500-ml linear gradient of NaCl from 0.05 to 0.4 M in buffer C. Fractions of 3.3 ml were collected, with immediate addition of 15 μl of 0.4 M EGTA to each fraction. Fractions containing phosphodiesterase activity, apart from those with cGMP-specific phosphodiesterase (phosphodiesterase V) activity, were collected and dialyzed against buffer D that contained 20 mM bis(2-hydroxyethyl)iminotris-(hydroxymethyl)-methane (Bis-tris) acetate (pH 6.5), 2 mM ethylenediaminetetraacetic acid (EDTA), 50 mM sodium acetate, 0.2 mM dithiothreitol, 1 mM benzamidine and 1 μM p-APMSF. After the addition of EGTA to a final concentration of 1.5 mM, the dialyzed supernatant was reapplied to a column of DEAE-Toyopearl 650S that had been equilibrated with buffer D. After application, the column was washed with several bed volumes of buffer D. The elution was performed at a flow rate of 3.0 ml/min with a continuous 600-ml gradient of 0.05–0.8 M sodium acetate in buffer D and fractions of 6.4 ml were collected.

The further purification of Ca^{2+}-calmodulin-dependent phosphodiesterase (phosphodiesterase I) was carried out as follows. Fractions containing phosphodiesterase I from the second column of DEAE-Toyopearl 650S were pooled and dialyzed against buffer E that contained 30 mM Tris-HCl (pH 8.0), 50 mM NaCl, 3 mM $MgCl_2$ and 0.1 mM dithiothreitol. After the addition of $CaCl_2$ to a final concentration of 0.2 mM, the dialyzed product was applied to a column (6 × 1.5 cm i.d.) of calmodulin-Sepharose (see below) that had been equilibrated with buffer E plus 0.2 mM $CaCl_2$ (buffer F). The column was washed with several bed volumes of buffer F that had been supplemented with 0.2 M NaCl (buffer G). Then phosphodiesterase I activity was eluted with buffer G plus 2 mM EGTA instead of 0.2 mM $CaCl_2$. The calmodulin-Sepharose resin was prepared by coupling 40 mg of purified calmodulin to 3 g of cyanogen bromide-activated Sepharose 4B, as described previously by Klee et al. (1979). Calmodulin was purified to homogeneity from bovine brain by the previously described procedure (Gopalakrishna and Anderson, 1982).

The particulate phosphodiesterase isoenzymes from human aorta were extracted after centrifugation of the homogenate in buffer A at 100 000 × g for 60 min. The pellet was removed and suspended in 10 volumes of buffer A, with subsequent centrifugation as before. The resultant supernatant was discarded. This procedure was performed twice in all. The final pellet was

resuspended in 10 volumes of buffer A that contained 0.4 M NaCl, 1% Triton X-100 and 0.1% polyoxyethylene 4-lauryl ether (Brij 30) and was stirred overnight. The detergent-extracted proteins were subjected to centrifugation at $60000 \times g$ for 30 min. The resultant supernatant was dialyzed for 4 h against 2 l of buffer D and this dialysis step was repeated once. The dialyzed supernatant was applied to a column of DEAE-Toyopearl 650S that had been equilibrated with buffer D and phosphodiesterase activity was eluted with a linear gradient of sodium acetate from 0.05 to 0.8 M in buffer D.

Phosphodiesterase II from human kidney was purified as described previously (Sugioka et al., 1994).

2.2. Measurement of phosphodiesterase activities

Phosphodiesterase activities were determined by the two-step method of Hidaka and Asano (1976). Unless otherwise noted, the enzymatic reaction was performed in a total volume of 0.5 ml. The reaction mixture consisted of a buffer that contained 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 50 μg of bovine serum albumin, 1 mM EGTA, substrate (1.0 μM cyclic [^{3}H]AMP or 1.0 μM cyclic [^{3}H]GMP) and enzyme. Each reaction was incubated at 30°C for 15 min. 5'-[^{3}H]AMP or 5'-[^{3}H]GMP formed by phosphodiesterase was converted to [^{3}H]adenosine or [^{3}H]guanosine by the action of nucleotidase (snake venom). The radioactivity of the product, which was isolated by chromatography on a cation-exchange resin, was measured in a liquid scintillation counter. The drugs E4021, zaprinast, MS-857, dipyridamole and rolipram were dissolved in dimethylsulfoxide at a final concentration of 1%. At this concentration dimethylsulfoxide inhibited enzymatic activities by less than 5%.

To overcome possible contamination by cAMP-specific phosphodiesterase (phosphodiesterase IV), the activity of cGMP-inhibited phosphodiesterase (phosphodiesterase III) were assayed in the presence of 30 μM rolipram. At this concentration, rolipram almost completely inhibited phosphodiesterase IV and did not significantly inhibit phosphodiesterase III. The activity of phosphodiesterase IV was assayed in the presence of 15 μM cGMP to rule out possible contamination by phosphodiesterase III. The reactions catalyzed by phosphodiesterase III and phosphodiesterase IV were monitored with 1.0 μM [^{3}H]cAMP as substrate. Ca^{2+}-calmodulin-dependent phosphodiesterase (phosphodiesterase I) and cGMP-specific phosphodiesterase (phosphodiesterase V) were assayed with 1.0 μM [^{3}H]cGMP as substrate in the absence of Ca^{2+}-calmodulin, and cGMP-stimulated phosphodiesterase (phosphodiesterase II) was assayed with 1.0 μM [^{3}H]cAMP as substrate in the presence of 15 μM cGMP.

2.3. Preparation of strips of human pulmonary artery and recording of contractile activity

The pulmonary artery (outer diameter, 2–3 mm) was dissected out from human lung, with subsequent removal of connective tissues. Helically cut strips with endothelium, 1 mm in width and 12 mm in length, were prepared and were suspended vertically in a 5-ml organ bath under a tension of 1.2 g in Krebs-Henseleit solution that had the following composition: 115 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4 and 10 mM dextrose. The medium in the organ bath was maintained at 37°C and bubbled with a mixture of 95% O_2 and 5% CO_2. The strips of tissue were allowed to equilibrate for 90 min before the start of experiments. During the equilibration period, the solution in the organ bath was replaced every 20 min. The presence of a functional endothelium was confirmed by relaxation response to 10^{-6} M acetylcholine in 10^{-7} M norepinephrine-precontracted strips. Changes in muscle tension were recorded isometrically with force-displacement transducers (TB-651T; Nihon Kohden, Tokyo, Japan).

2.4. Reagents

Cyclic [^{3}H]AMP and cyclic [^{3}H]GMP were obtained from Du Pont-New England Nuclear (Wilmington, DE, USA). Unlabelled cAMP, cGMP, 3-isobutyl-1-methylxanthine (IBMX), snake venom (*Crotalus atrox*), dipyridamole, zaprinast and prostaglandin $F_{2\alpha}$ were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DEAE-Toyopearl 650S was purchased from Toyo Soda MFG Co. (Tokyo, Japan). E4021 (sodium 1-[6-chloro-4-(3,4-methylenedioxybenzyl) aminoquinazolin-2-yl]piperidine-4-carboxylate sesquihydrate) was kindly supplied by Eisai Co. (Tokyo, Japan). MS-857 (4-acetyl-1-methyl-7-(4-pyridyl)-5,6,7,8-tetrahydro-3-(2*H*)-iso-quinolinone) and rolipram (4-(3'-cyclopentyloxy-4'-methoxyphenyl)-2-pyrrolidone) were gifts from Mitsui Pharmaceuticals (Tokyo, Japan) and Nihon Schering (Tokyo, Japan), respectively. All other chemicals were of reagent grade or of the highest grade commercially available.

3. Results

3.1. Isolation and characterization of the cytosolic and particulate isoenzymes of phosphodiesterase from the human aorta

Typical chromatograms demonstrating the isolation of the different isoenzymes of phosphodiesterase in cytosolic and particulate fractions from the human aorta are shown in Fig. 1. Fig. 1A shows the profile of

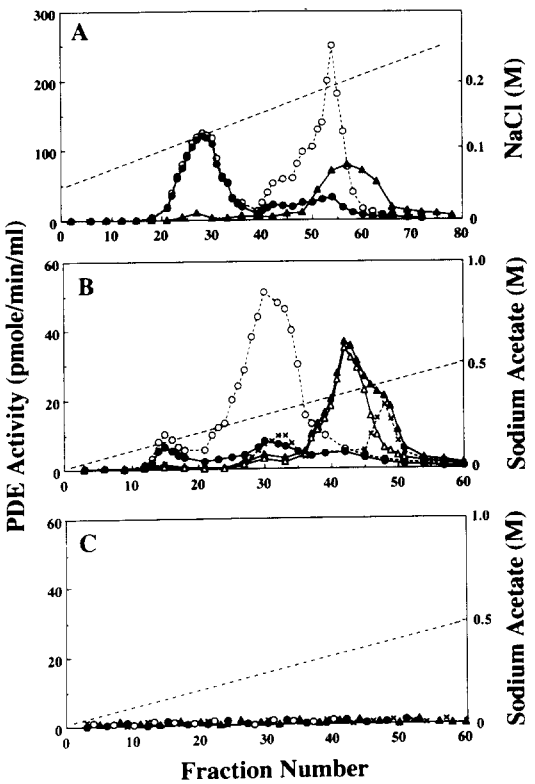

Fig. 1. Profiles of the elution of phosphodiesterase isoenzymes in extracts of human aorta from columns of DEAE-Toyopearl 650S. (A) Chromatography of cytosolic isoenzymes of phosphodiesterase in the presence of Ca^{2+} ions. (B) Further separation of cytosolic isoenzymes of phosphodiesterase in the absence of Ca^{2+} ions. (C) Separation of particulate phosphodiesterase isoenzymes. A linear gradient of NaCl from 0.05 to 0.4 M (A) or of sodium acetate from 0.05 to 0.8 M (B,C) was applied. Aliquots of each fraction were directly assayed as described in Materials and methods: ▲ hydrolysis of cAMP (1.0 μM); × hydrolysis of cAMP (1.0 μM) with 15 μM cGMP; △ hydrolysis of cAMP (1.0 μM) with 30 μM rolipram; ● hydrolysis of cGMP (1.0 μM); ○ hydrolysis of cGMP (1.0 μM) with Ca^{2+}-calmodulin. Each profile is representative of elution profiles from three different specimens.

elution from a column of DEAE-Toyopearl 650S of the isoenzymes in the cytosolic fraction from human aorta. This column was equilibrated and eluted with a buffer that contained Ca^{2+} ions. There were two distinct peaks of cGMP-hydrolytic activity. The enzyme in the first peak (fractions 21–38) hydrolyzed cGMP with a high degree of selectivity and was insensitive to Ca^{2+}-calmodulin. The K_m value for cGMP was 1.1 μM but that for cAMP was not determined because of the enzyme's low affinity for cAMP. These results suggested that this isoenzyme was a cGMP-specific phosphodiesterase (phosphodiesterase V). The activity in the second peak (fractions 40–60) preferentially hydrolyzed cGMP and this activity was potentiated by Ca^{2+}-calmodulin, suggesting that Ca^{2+}-calmodulin-dependent phosphodiesterase (phosphodiesterase I) was present in this peak. These results indicated that phosphodiesterase I and phosphodiesterase V could be clearly separated by chromatography on DEAE-Toyopearl 650S in the presence of Ca^{2+} ions. In addition, cAMP-hydrolyzing activity was detected in fractions 48–69.

Fractions 40–69 from the column of DEAE-Toyopearl 650S, which contained all the phosphodiesterase activity except that of phosphodiesterase V, were pooled and reapplied to the same column, which was equilibrated and eluted in the presence of 1.5 mM EGTA instead of Ca^{2+} ions (Fig. 1B). Three distinct peaks (fractions 13–21, 23–35 and 37–48) of cGMP-hydrolytic activity were obtained. The cGMP-hydrolytic activity in fractions 23–35 was stimulated several-fold by Ca^{2+}-calmodulin. In addition, the cAMP-hydrolytic activity in fractions 27–35 was stimulated several-fold in the presence of a low concentration of cGMP (up to 15 μM). These findings suggested that two isoenzymes, Ca^{2+}-calmodulin-dependent phosphodiesterase (phosphodiesterase I) and cGMP-stimulated phosphodiesterase (phosphodiesterase II), were present in fractions 23–35 and fractions 27–35, respectively. Phosphodiesterase I was further purified by chromatography on calmodulin-Sepharose (data not shown). The hydrolysis of cGMP by the purified phosphodiesterase I was enhanced 3.5-fold by Ca^{2+}-calmodulin but the cAMP-hydrolytic activity was not stimulated by 15 μM cGMP. The K_m values for cAMP and cGMP in the absence of Ca^{2+}-calmodulin were 5.6 and 0.95 μM, respectively. The K_m values for hydrolysis of cAMP and cGMP by phosphodiesterase II were not determined because of the low level of its activity, as well as possible contamination by phosphodiesterase I. The phosphodiesterase activity in fractions 36–54 preferentially catalyzed the hydrolysis of cAMP. The cAMP-hydrolytic activity in the first half of the peak, fractions 36–45, was strongly inhibited by 15 μM cGMP but was not inhibited by 30 μM rolipram. This rolipram-insensitive cAMP-hydrolyzing activity eluted in parallel with the low cGMP-hydrolyzing activity (fractions 37–48). The K_m values for cAMP and cGMP for the enzyme in these fractions were 0.58 and 0.38 μM, respectively, in the presence of 30 μM rolipram. These findings demonstrated that this isoenzyme was a cGMP-inhibited phosphodiesterase (phosphodiesterase III). The cAMP-hydrolyzing activity in the latter half of the peak, fractions 46–54, was strongly inhibited by 30 μM rolipram but was not inhibited by 15 μM cGMP. The phosphodiesterase had a K_m value of 1.1 μM for cAMP but that for cGMP was not determined because of the isoenzyme's low affinity for cGMP. These results suggested that this isoenzyme was a cAMP-specific phosphodiesterase (phosphodiesterase IV). The cGMP-hydrolytic activity in fractions 13–21 was increased about 1.5-fold by Ca^{2+}-calmodulin and was inhibited by approximately 50% by 0.1 μM E4021,

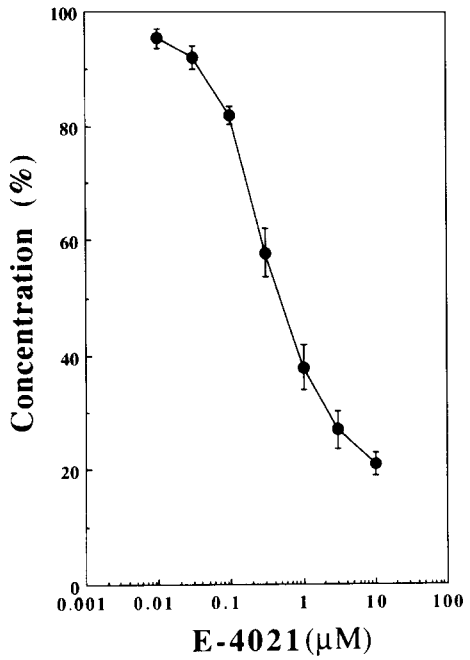

Fig. 2. Concentration-response curve demonstrating the effect of E4021 on strips of human pulmonary artery that had been precontracted with prostaglandin $F_{2\alpha}$ (10^{-6} M). The maximum contraction induced by 10^{-6} M prostaglandin $F_{2\alpha}$ in the absence of the agent was taken as 100%. Relaxation induced by 0.1 mM papaverine was taken as 0%. Vertical bars indicate S.E. ($n = 4$).

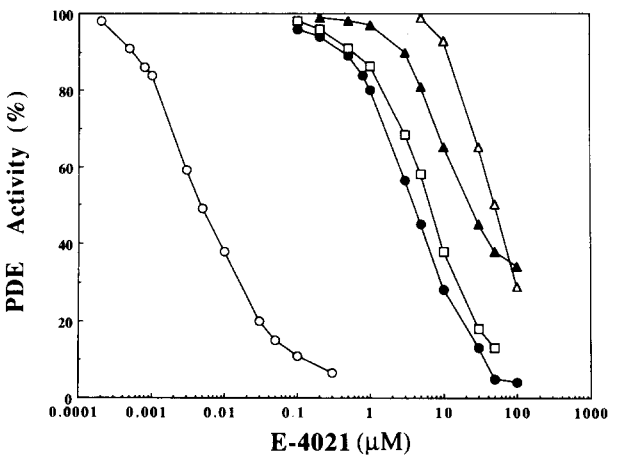

Fig. 3. Inhibition of human phosphodiesterases by E4021: Ca^{2+}-calmodulin-dependent phosphodiesterase (phosphodiesterase I) from aorta (▲); cGMP-inhibited phosphodiesterase (phosphodiesterase III) from aorta (△); cAMP-specific phosphodiesterase (phosphodiesterase IV) from aorta (●); cGMP-specific phosphodiesterase (phosphodiesterase V) from aorta (○); cGMP-stimulated phosphodiesterase (phosphodiesterase II) from kidney (□). Each point represents the mean value from the results of eight assays.

which is a selective inhibitor of phosphodiesterase V, as described below. These results suggested that this peak consisted of phosphodiesterase I and phosphodiesterase V and that the latter had not been completely separated from the former during the first chromatography on DEAE-Toyopearl 650S and was contaminant in the initial fractions used for chromatography on the second column of DEAE-Toyopearl 650S.

In the particulate fraction, no detectable phosphodiesterase activity was observed with either cAMP or cGMP as substrate (Fig. 1C), suggesting that the particulate form of phosphodiesterase was not present in the human aorta. The K_m values of phosphodiesterase I, phosphodiesterase III, phosphodiesterase IV and phosphodiesterase V from human aorta for cAMP and cGMP are summarized in Table 1. The K_m values of phosphodiesterase II for cAMP and cGMP were not determined because the activity of this isoenzyme was low and it was hard to purify.

3.2. Effects of E4021, a novel vasorelaxant, on the prostaglandin $F_{2\alpha}$-induced contraction of helical strips isolated from human pulmonary artery

The effects of E4021, which produces vasorelaxation in the pig, on human pulmonary artery were examined. E4021 relaxed prostaglandin $F_{2\alpha}$-precontracted strips of human pulmonary artery with endothelium in a dose-dependent manner. The concentration-response curve for E4021 in this system is shown in Fig. 2. The apparent concentration of E4021 that produced 50% relaxation (ED_{50}) was 0.5 μM.

3.3. Effects of E4021 on isolated phosphodiesterase isoenzymes from human aorta

The effects of E4021 on human aortic phosphodiesterase I, phosphodiesterase III, phosphodiesterase IV and phosphodiesterase V and on phosphodiesterase II from kidney were investigated. As shown in Fig. 3, E4021 potently and very selectively inhibited the activity of phosphodiesterase V with an IC_{50} value of 4.0 nM. The inhibitory effects of E4021 on phosphodi-

Table 1
K_m values of phosphodiesterase isoenzymes from human aorta for cAMP and cGMP

Substrate	K_m value (μM)			
	Phosphodiesterase I	Phosphodiesterase III	Phosphodiesterase IV	Phosphodiesterase V
cAMP	5.6	0.58	1.1	ND
cGMP	0.95	0.38	ND	1.1

ND: Not determined.

Table 2
The effects of various inhibitors on the phosphodiesterase isoenzymes from human aorta

Agent	IC_{50} (μM) [a]				
	Phosphodiesterase I	Phosphodiesterase II	Phosphodiesterase III	Phosphodiesterase IV	Phosphodiesterase V
E4021	24.2 ± 3.0	6.7 ± 0.3	40.5 ± 3.4	3.0 ± 0.1	0.004 ± 0.001
Zaprinast	25.8 ± 1.0	68.2 ± 7.2	> 100	32.7 ± 3.4	0.34 ± 0.01
MS-857	> 100	90.2 ± 10.3	1.76 ± 0.38	13.5 ± 2.2	20.5 ± 0.3
Dipyridamole	89.3 ± 9.2	2.7 ± 0.5	90.8 ± 12.3	2.2 ± 0.1	0.30 ± 0.03
Rolipram	> 100	> 100	> 100	9.9 ± 0.2	> 100

[a] The IC_{50} value is defined as the concentration of drug required for 50% inhibition of the enzymatic activity. Phosphodiesterase I, phosphodiesterase III, phosphodiesterase IV and phosphodiesterase V were isolated from human aorta and phosphodiesterase II was isolated from human kidney. Each activity was assayed with 1.0 μM [^{3}H]cAMP or 1.0 μM [^{3}H]cGMP, as described in Materials and methods. Each value is the mean ± S.E. of the results from six experiments, which were performed using different preparations of enzyme isolated from three separate specimens.

esterase I, phosphodiesterase II, phosphodiesterase III and phosphodiesterase IV were weak and IC_{50} values were three orders of magnitude higher than that for phosphodiesterase V. Table 2 summarizes the IC_{50} values of E4021 in a comparison with other inhibitors of phosphodiesterase. Zaprinast was also a selective inhibitor of phosphodiesterase V but E4021 had a more potent inhibitory effect on phosphodiesterase V with an IC_{50} value that was two orders of magnitude lower than that of zaprinast. MS-857 was an inhibitor of phosphodiesterase III, and it also inhibited phosphodiesterase IV and phosphodiesterase V with slightly higher IC_{50} values. Rolipram was a selective inhibitor of phosphodiesterase IV. The rank order of the effective inhibition by dipyridamole, which is well known as a non-selective inhibitor of phosphodiesterase, was phosphodiesterase V > phosphodiesterase IV and phosphodiesterase II > phosphodiesterase I and phosphodiesterase III.

The inhibition of phosphodiesterase V from human aorta by E4021 was further analyzed by construction of a Lineweaver-Burk plot (Fig. 4A) and a Dixon plot (Fig. 4B). As shown in Fig. 4A, E4021 inhibited phosphodiesterase V competitively with respect to cGMP. The K_i value of E4021 for phosphodiesterase V was 2.4 nM.

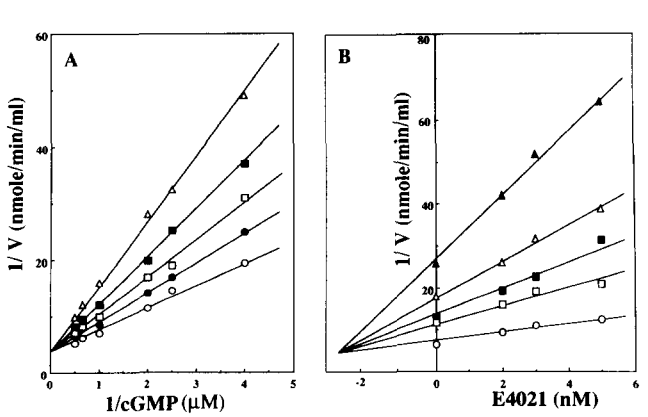

Fig. 4. Kinetic analysis of the effects of E4021 on cGMP-specific phosphodiesterase (phosphodiesterase V) from human aorta. (A) Lineweaver-Burk plots for E4021 at concentrations of 10 nM (△), 5 nM (■), 3 nM (□), 2 nM (●) and 0 nM (○). (B) Dixon plots for substrate (cGMP) concentrations of 0.25 μM (▲), 0.3 μM (△), 0.4 μM (■), 0.5 μM (□) and 1.0 μM (○).

4. Discussion

The major goals of this study were to identify the isoenzymes of phosphodiesterase, to determine their intracellular distribution in the human aorta, and to compare this distribution with phosphodiesterase profiles from other human tissues. Fractionation of the cytosolic phosphodiesterase activity from the human aorta yielded five distinct fractions. The elution characteristics, substrate specificities and selectivities with respect to regulation by effectors led us to designate these isoenzymes phosphodiesterase I, phosphodiesterase II, phosphodiesterase III, phosphodiesterase IV and phosphodiesterase V. Of the five phosphodiesterase isoenzymes, phosphodiesterase I and phosphodiesterase V were the main contributors to the hydrolysis of cGMP in the presence of Ca^{2+}-calmodulin; the former represented about 55% of the total cGMP-hydrolyzing activity and the latter about 40%. Recently, we showed that four isoenzymes of phosphodiesterase, namely, phosphodiesterase I, phosphodiesterase II, phosphodiesterase III and phosphodiesterase IV, are present in human kidney and heart (Sugioka et al., 1994). In the cytosolic fractions from kidney and heart, the activity of phosphodiesterase I was the major contributor to the cGMP-hydrolyzing activity in the presence of Ca^{2+}-calmodulin, whereas phosphodiesterase V was a very minor component or was not detected. The results of the kinetic characterization of each of the phosphodiesterase isoenzymes from human aorta were almost the same as those from the human kidney or heart. However, the activity of phosphodiesterase I from the aorta had a K_m value for cAMP of 5.6 μM, which was slightly higher than that

from kidney and heart (Sugioka et al., 1994). The discrepancy might be due to differences among the isoforms in the phosphodiesterase I family (Wang et al., 1990). Our results verified that phosphodiesterase V was characteristically present in the cytosolic fraction of the aorta.

Multiple isoenzymes of phosphodiesterase have been isolated from the vascular smooth muscles of a variety of mammals including bovine (Lugnier et al., 1986), rat (Lindgren et al., 1991), pig (Wells et al., 1981; Reeves et al., 1987; Saeki and Saito, 1993) and human (Hidaka et al., 1977; Lugnier et al., 1986). A more recent study, using more selective inhibitors of phosphodiesterase and improved resolving procedures, showed that the tissues actually contain more isoenzymes of phosphodiesterase than previously reported. Five isoenzymes of phosphodiesterase, designated phosphodiesterase I through phosphodiesterase V, have been identified in extracts of porcine aorta (Saeki and Saito, 1993). There were few differences in the kinetic properties of phosphodiesterase isoenzymes between those in the human aorta and those in the porcine aorta. Although phosphodiesterase I and phosphodiesterase V accounted for a significant proportion of the cGMP-hydrolyzing activity in both human and porcine aorta, the proportion of cGMP hydrolyzed by phosphodiesterase V was apparently higher in human aorta than in porcine aorta. Thus, only minor differences in the distribution of phosphodiesterase isoenzymes were found between the human aorta and the porcine aorta.

Recently, E4021, a novel vasorelaxant, was synthesized and was reported subsequently to be a potent and selective inhibitor of porcine phosphodiesterase V (Saeki et al., 1995). The effect of E4021 on strips of human pulmonary artery and its inhibitory effects on each of the phosphodiesterase isoenzymes isolated from human aorta were investigated. E4021 relaxed prostaglandin $F_{2\alpha}$-precontracted strips of human pulmonary artery in a dose-dependent manner, with an ED_{50} value of 0.5 μM, being just as potent as when strips of porcine coronary artery were used (Saeki et al., 1995). E4021 inhibited the activity of phosphodiesterase V isolated from human aorta with an IC_{50} value of 4 nM, which was three orders of magnitude lower than IC_{50} values for other phosphodiesterase isoenzymes. The inhibition of the activity of phosphodiesterase V from human aorta was competitive with respect to cGMP with a K_i value of 2.4 nM. Therefore, it seems likely that E4021 binds in the region of the catalytic site of phosphodiesterase V from human aorta. The IC_{50} values of E4021 for each isoenzyme of phosphodiesterase in human aorta were similar to values reported in porcine aorta (Saeki et al., 1995). The IC_{50} value of E4021 for the activity of phosphodiesterase V from human aorta was two orders of magnitude lower than the ED_{50} value for relaxation of strips of human pulmonary artery. This discrepancy was also observed in porcine coronary arteries (Saeki et al., 1995). In the study of porcine coronary arteries, it was concluded that the vasorelaxant effect of E4021 was mediated by an increase in the level of cGMP for the following reasons: (1) E4021 had a relaxant effect in prostaglandin $F_{2\alpha}$-precontracted porcine coronary artery, with a significant increase in the level of cGMP; and (2) E4021 potentiated the relaxant activity induced by h-ANP (human atrial natriuretic peptide) in isolated porcine coronary artery (Saeki et al., 1995). Although further studies are necessary to clarify the relationship between inhibition of phosphodiesterase V and the vasorelaxant activity of E4021 in human tissue, the various results suggest that the vasorelaxant efficacy of E4021 in human pulmonary artery might also be linked to the inhibition of phosphodiesterase V and to a subsequent increase in the intracellular level of cGMP. The difference between the effect of E4021 on the activity of phosphodiesterase V and the vasorelaxant activity of E4021 might be attributable to the low rate of uptake of E4021 by smooth muscle cells and, to some extent, to the high concentration of prostaglandin $F_{2\alpha}$ employed in these studies.

The vascular relaxing responses in vitro to inhibitors of phosphodiesterase V, such as zaprinast (Griffith et al., 1985; Lugnier et al., 1986; Martin et al., 1986; Souness et al., 1989; Schoeffer et al., 1987) and MY-5445 (Souness et al., 1989; Hidaka and Endo, 1984), have been reported. Saeki et al. (1995) showed recently that, in vivo, E4021 caused dilation of the large coronary artery and a selective reduction in mean pulmonary arterial pressure in the pig. Furthermore, it was reported that E4021 mitigated myocardial ischemia in the guinea pig (Kodama et al., 1994). Zaprinast was reported not only to produce a selective reduction in mean pulmonary arterial pressure, via an increase in the level of cGMP (Braner et al., 1993), but also to reverse vasorelaxant tolerance to nitrovasodilators via functional inhibition of phosphodiesterase V (Pagani et al., 1991; Silver et al., 1991; Merkel et al., 1992; Pagani et al., 1993). The selective pulmonary vasodilating effects of E4021 and zaprinast might be due to the high level of guanylate cyclase activity in the pulmonary vascular smooth muscle caused by mediators such as atrial natriuretic peptide and/or nitric oxide, an endothelium-derived relaxing factor.

Inhibitors of phosphodiesterase I and phosphodiesterase III are well known as vasorelaxants. Because of an increase in the intracellular levels of cAMP, inhibitors of phosphodiesterase III not only induce vasorelaxation but also increase the development of isometric force in cardiac muscle and the heart rate (Kauffman et al., 1987; Silver et al., 1988; Harris et al., 1989). The action of vinpocetine is thought to be linked to the inhibition of phosphodiesterase I and subse-

quent vasodilatation, via an increase in the intracellular level of cGMP (Hagiwara et al., 1984; Chiu et al., 1988). As compared to these inhibitors, E4021, which is an inhibitor of phosphodiesterase V and increases the intracellular level of cGMP without affecting that of cAMP, can be expected to be more useful for clinical applications to the treatment of patients with angina pectoris, pulmonary hypertension and heart failure. Furthermore, the availability of this potent and selective inhibitor of human phosphodiesterase V should allow us to investigate in further detail the functions of phosphodiesterase V in human tissues.

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