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Potent Effects of Novel Anti-platelet Aggregatory Cilostamide Analogues on Recombinant Cyclic Nucleotide Phosphodiesterase Isozyme Activity

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ABSTRACT. The inhibitory potential of novel anti-platelet aggregatory cilostamide analogues on phosphodiesterase (PDE) isozyme activities was investigated with recombinant PDE isozymes expressed in a baculovirus/Sf9 expression system. The recombinant enzymes (PDE1–PDE5 and PDE7) showed K_m values and sensitivities to selective inhibitors similar to those reported previously for native enzymes purified from tissues. The cyclooctylurea derivative OPC-33540 (6-[3-[3-cyclooctyl-3-[(1*R**,2*R**)-2-hydroxycyclohexyl]ureido]-propoxy]-2(1*H*)-quinolinone) inhibited recombinant PDE3A (IC_{50} = 0.32 nM) more potently and selectively than the classical PDE3 inhibitors cilostamide, cilostazol, milrinone, and amrinone. The cyclopropylurea derivative OPC-33509 [(-)-6-[3-[3-cyclopropyl-3-[(1*R*,2*R*)-2-hydroxycyclohexyl]ureido]-propoxy]-2(1*H*)-quinolinone] was less potent (IC_{50} = 0.10 μM) than OPC-33540, demonstrating that the cyclooctyl moiety was important for a potent inhibitory effect. In platelets, OPC-33540 potentiated cyclic AMP accumulation concentration-dependently in both the absence and the presence of 3 nM prostaglandin E_1 (PGE_1) (doubling concentrations: 32.5 and 6.2 nM, respectively). OPC-33540 inhibited thrombin-induced platelet aggregation potently (IC_{50} = 27.8 nM). The anti-platelet aggregation effect also was stimulated in the presence of 3 nM PGE_1 (IC_{50} = 6.0 nM). There was a good correlation between the IC_{50} values of PDE3 inhibitors in this study for recombinant PDE3A activity and their IC_{50} values for thrombin-induced platelet aggregation (r = 0.998). These data demonstrated that OPC-33540 is a highly selective and potent PDE3 inhibitor and a useful probe for identification of the intracellular functions of PDE3. BIOCHEM PHARMACOL **59**;4:347–356, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. phosphodiesterase 3 inhibitor; cilostamide; OPC-33540; platelet aggregation; cyclic AMP; recombinant enzyme

In intracellular signal transduction, cyclic nucleotides are well-characterized second messengers. Cyclic nucleotide PDEs‖ are the major enzymes involved in the metabolism of intracellular cyclic nucleotides. In mammalian tissues, seven isozymes, PDE1 through PDE7, have been identified on the basis of primary structures and enzyme kinetics obtained by biochemical and molecular biological studies [1]. Recently, three additional PDE isozymes, PDE8, PDE9, and PDE10, were cloned by searching the Expressed Sequence Tag (EST) database [2–5], indicating that there are at least ten isozymes of PDE known at present. Each PDE isozyme has a C-terminal catalytic domain conserved throughout the family, and an N-terminal regulatory domain unique for each isozyme. They are expressed in tissue- and cell-specific distribution patterns, and they show different substrate affinities and inhibitor sensitivities [6]. Most cell types express one or more PDE isozymes, each regulating intracellular cAMP and/or cGMP concentrations in different cellular compartments and in different manners.

In cardiovascular tissues, PDE3 and PDE4 are well established as the dominant cAMP-hydrolysis isozymes. PDE1, PDE3, PDE4, and PDE5 are expressed in aortic smooth muscle cells [7, 8]; PDE1, PDE2, PDE3, and PDE4 are expressed in the heart [9], whereas PDE2, PDE3, and PDE5 are found in platelets [10–12]. PDE3 is thus a unique cAMP-regulating isozyme expressed in all of these tissues. These tissues contribute significantly to the pathogenesis of arteriosclerosis obliterans and restenosis after angioplasty. The inhibition of PDE3 activity in cardiovascular tissues results in increased levels of cAMP with consequent reduc-

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‖ *Abbreviations*: PDE, phosphodiesterase; IBMX, 3-isobutyl-1-methylxanthine; cAMP and cGMP, cyclic AMP and cyclic GMP, respectively; DMF, dimethylformamide; and PGE_1, prostaglandin E_1.

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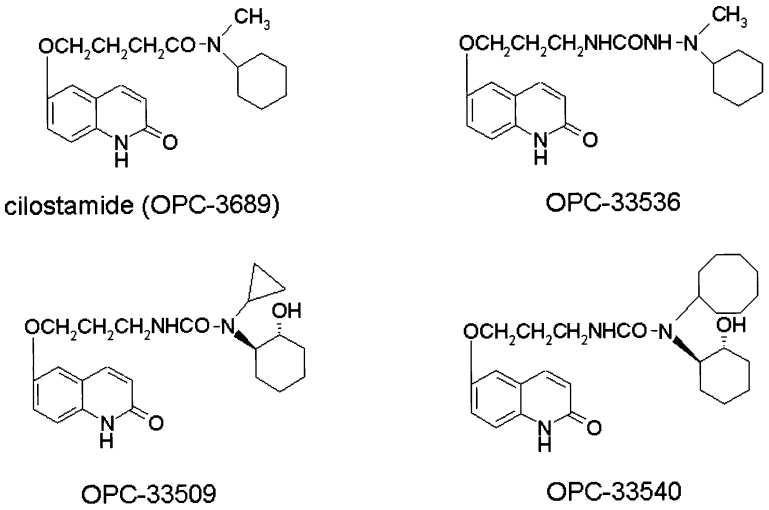

FIG. 1. Chemical structures of OPC compounds.

tion in platelet aggregation and smooth muscle cell proliferation *in vitro*, and induction of a cardiotonic effect [11, 13]. In a rat balloon-induced injury model of intimal hyperplasia, oral administration of cilostamide (OPC-3689), one of the most selective and potent PDE3 inhibitors, has been found to suppress the intimal proliferation [14]. Recently, it was reported that local administration of the cardiotonic PDE3 inhibitor amrinone also inhibits neointima formation in this model [15]. Therefore, PDE3 inhibitors may possess therapeutic potential for ischemic disease involving thrombosis and vascular smooth muscle cell proliferation. Indeed, cilostazol (OPC-13013) has been available clinically for a long time as a vasodilator and anti-platelet drug [16–18].

Cilostamide possesses both antithrombotic and anti-intimal hyperplastic actions [11, 14, 19]. However, its clinical use is limited by tachycardiac side-effects, due to the inhibition of cardiac PDE activity. Therefore, we have screened compounds with structural similarity to cilostamide (Fig. 1) in a search for a novel agent with enhanced antithrombotic and anti-intimal hyperplastic actions and reduced circulatory effects. A cyclopropylurea derivative, OPC-33509 [(-)-6-[3-[3-cyclopropyl-3-[(1*R*,2*R*)-2-hydroxycyclohexyl]ureido]-propoxy]-2(1*H*)-quinolinone], has been discovered as a novel agent [19]. In addition, a cyclooctylurea derivative, OPC-33540 (6-[3-[3-cyclooctyl-3-[(1*R**,2*R**)-2-hydroxycyclohexyl]ureido]-propoxy]-2(1*H*)-quinolinone), and a semicarbazide derivative, OPC-33536 (2-cyclohexyl-2-methyl-N^1- [3-[(2-oxo-1,2-dihydro-6-quinolinyl,oxy]propyl]-1-hydrazinecarboxamide), also have been identified as extremely potent inhibitors of platelet aggregation. In the present study, we determined the effects of these agents on recombinant PDE isozyme activity as well as platelet aggregation and cAMP concentrations. To our knowledge, this is the first report of effects on activities of all PDE isozymes using recombinant proteins derived from single genes.

MATERIALS AND METHODS

Materials

OPC-33540, OPC-33536, OPC-33509, cilostamide, and cilostazol were synthesized by Drs. Takao Nishi and Yasuo Koga, Mr. Yoshito Kihara, and Mr. Minoru Okada of the Third Tokushima Institute of New Drug Research, Otsuka Pharmaceutical Co., Ltd. [19]. Amrinone, milrinone, cAMP, and cGMP were purchased from the Sigma Chemical Co. Vinpocetine and rolipram were obtained from BIOMOL Research Labs., Inc. Erythro-9-(2-hydroxyl-3-nonyl)adenine (EHNA) was purchased from Research Biochemicals International. Dipyridamole, IBMX, and DMF were obtained from the WAKO Pure Chemical Co. [^{3}H]AMP and [^{3}H]GMP were purchased from Amersham. cDNAs for bovine PDE1B1 [20], bovine PDE2A1 [21], bovine PDE5A1 [22], and mouse PDE7A2 [23] were gifts of Dr. J. A. Beavo (University of Washington). Purified recombinant rat PDE4B2 enzyme [24] was provided by Dr. M. Conti (Stanford University). Calmodulin protein was isolated from bovine brain as previously reported [25].

Construction of Recombinant Baculoviruses

Construction of recombinant PDE1, PDE2, PDE5, and PDE7 baculoviruses was performed according to methods previously reported [26]. Briefly, cDNAs for bovine PDE1B1 in pcDNA3, bovine PDE2A1 in pVL1393, and bovine PDE5A1 in pBluescript SK(−) were subcloned, respectively, into the *Bam*HI, *Eco*RI, or *Sac*I sites of the pFastBac donor plasmid vector. cDNA for mouse PDE7A2 in pVL1393 was subcloned into the *Bam*HI/*Not*I site. pFastBac-PDE1, PDE2, PDE5, and PDE7 were transformed into DH10Bac competent cells for transposition into bacmids. Bacmid DNAs for these PDE isozymes isolated from the transformed cells were transfected into Sf9 insect cells. High titer recombinant viral stocks encoding these PDE isozymes were obtained and used for subsequent infection of

TABLE 1. Kinetic and pharmacological characterization of baculovirus-expressed recombinant PDE isozymes

	K_m (μM)		V_{max} (nmol/min/mg)	IC_{50} (μM) for inhibitor	
	cAMP	cGMP			
PDE1		2.9 ± 0.3	73.9 ± 12.0	Vinpocetine	23.2 (20.5–26.2)
PDE2	46.4 ± 3.4	ND*	107.9 ± 30.9	EHNA	9.2 (5.2–16.2)
PDE3A	0.24†	0.094 ± 0.022	0.80 ± 0.10	Cilostamide	0.027 (0.016–0.049)
				cGMP	0.075 (0.062–0.091)
PDE3B	0.47 ± 0.08	0.29 ± 0.03	0.17 ± 0.03	Cilostamide	0.050 (0.034–0.093)
				cGMP	0.32 (0.28–0.38)
PDE4	2.5 ± 0.6		128.3 ± 19.0	Rolipram	0.45 (0.30–0.69)
PDE5		0.98 ± 0.15	84.7 ± 7.3	Dipyridamole	0.26 (0.20–0.34)
PDE7	0.25 ± 0.02		0.23 ± 0.00	IBMX	7.0 (2.7–14.0)

Substrate concentrations for V_{max} and IC_{50} were as follows: PDE1, 0.4 μM cGMP; PDE2, 3 μM cAMP; PDE3, 0.1 μM cAMP; PDE4, 0.4 μM cAMP; PDE5, 0.4 μM cGMP; and PDE7, 0.1 μM cAMP. The IC_{50} values are the means (95% confidence limits) of three or four separate experiments performed in duplicate and are expressed as the concentrations of inhibitors producing 50% inhibition of enzyme activity. The K_m and V_{max} values are means ± SEM of three or four experiments.

*ND, not determined.

†Ref. 29.

Sf9 cells. Baculoviruses of human PDE3A1 [27] and rat PDE3B1 [28] were gifts of Dr. V. C. Manganiello (National Heart, Lung, and Blood Institute, United States National Institutes of Health) [29].

Expression of Recombinant PDE Isozymes in Sf9 Cells

Sf9 cells were maintained in Grace's insect medium containing 10% fetal bovine serum in tissue culture or spinner flasks at 27°. For the expression of recombinant PDE1B1, PDE2A1, PDE3A1, PDE3B1, PDE5A1, and PDE7A2 enzymes, Sf9 cells were infected with recombinant baculoviruses. Recombinant PDE enzymes were prepared as described elsewhere [30]. Briefly, at 60–70 hr post-inoculation, the Sf9 cells were harvested and suspended in 50 mM Tris–HCl, pH 7.4, 1 mM $MgCl_2$, 1 mM EDTA, 0.1 mM EGTA, 40% glycerol, 3 mM sodium benzamidine, 10 μg/mL of leupeptin, 10 μg/mL of pepstatin, and 1 mM phenylmethylsulfonyl fluoride. After sonication three times for 20 sec, the suspensions were centrifuged at 18,000 *g* for 30 min, and the supernatants were used for PDE assays, since PDE activity (hydrolysis activity of cAMP and cGMP) was negligible in the homogenates of normal (non-infected) Sf9 cells under the experimental conditions of the present study (see Discussion).

PDE Assay

PDE activity was measured by a slight modification of a method previously described [10], with the exception of PDE1. Reactions were carried out at 30° for 10 min in a buffer containing 50 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 2 mM EGTA, 0.1 mg/mL of BSA, and [^{3}H]cAMP or [^{3}H]cGMP (100,000 cpm) at the concentrations indicated in Table 1, and the PDE enzyme in the presence or absence of inhibitor in a total volume of 200 μL. Assays were stopped by incubating the tubes at 100° for 5 min. After cooling, a 20-μL aliquot of 1 mg/mL of snake venom was added to each assay tube, followed by incubation at 30° for 10 min. The samples were diluted with 1 mL of water and applied to 1-mL Bio-Rad AG 50W-X8 resin (100–20 mesh) columns. After washing two times with 10 mL of water, the columns were eluted with 3 mL of 1 N NH_4OH, and the eluate was collected in scintillation vials. The radioactivity of [^{3}H]adenosine or [^{3}H]guanosine was counted by liquid scintillation. The reaction for PDE1 activity was carried out at 30° for 10 min in a buffer containing 20 mM Tris–HCl, pH 8.0, 3 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.2 mg/mL of BSA, 0.1 μM calmodulin, and 0.4 μM [^{3}H]cGMP (100,000 cpm). All compounds were dissolved in DMF at 6×10^{-2} M and diluted subsequently in DMF or the reaction mixture. The final concentration of DMF was 0.5%.

cAMP Assay

cAMP was determined in washed human platelets in the presence or absence of different concentrations of OPC-33540, OPC-33536, and cilostamide alone, or with 3 nM PGE_1 (Ono Pharmaceutical Co., Ltd.). Washed platelets (200 μL of a suspension containing 3×10^8 cells/mL in Tyrode HEPES buffer, pH 7.4) were incubated for 3 min at

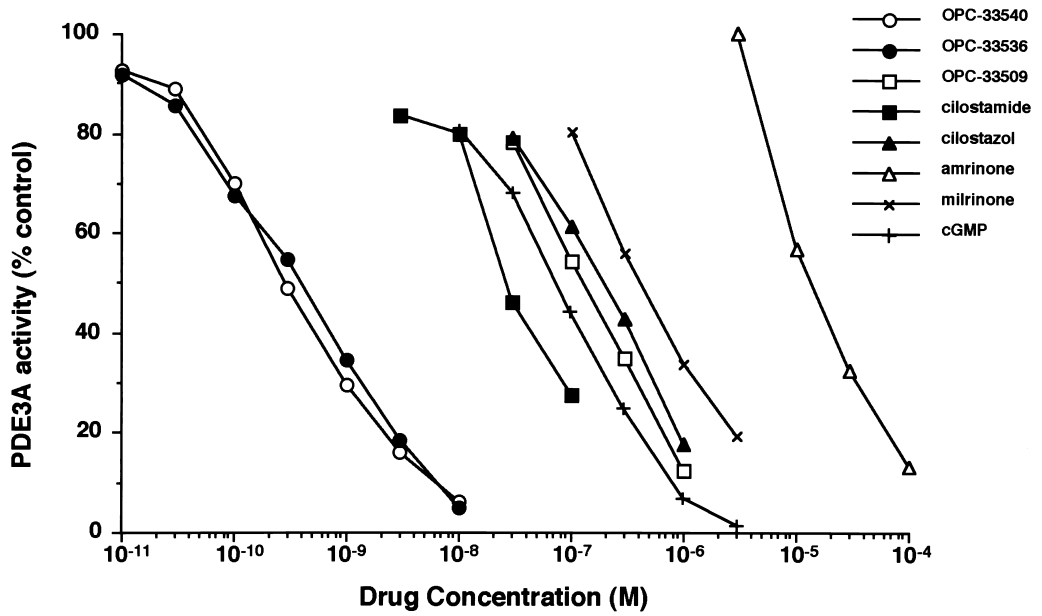

FIG. 2. Effects of OPC-33540, OPC-33536, OPC-33509, and PDE3 inhibitors on recombinant PDE3A activity. Enzyme concentration and incubation time were optimized to give about 10% hydrolysis of cAMP in the absence of inhibitors. The cAMP concentration was 0.1 μM. Control PDE3A activity was approximately 0.5 pmol/min. Each point is the mean of three or four separate experiments performed in duplicate, and data are expressed as percent of control.

37° followed by incubation with 5 μL of 2 units/mL of thrombin (Mochida Pharmaceutical Co.) for 5 min at 37°. Platelet reactions were stopped with 400 μL of ice-cold ethanol. Precipitated proteins were removed by centrifugation at 1500 *g* for 10 min at 4°, and the supernatant cAMP level was determined with a cAMP enzyme immunoassay system (Amersham).

Platelet Aggregation Study

Platelet aggregation was investigated as detailed earlier with slight modifications [17]. Washed platelets (200 μL of a suspension containing 3×10^8 cells/mL in Tyrode HEPES buffer, pH 7.4) were incubated for 3 min at 37° in the presence or absence of different concentrations of OPC-33540, OPC-33536, and cilostamide alone, or in combination with 3 nM PGE_1, followed by incubation with 5 μL of 2 units/mL of thrombin for 5 min at 37°. The intensity of light transmitted over 5 min was measured using a PAM-8C aggregometer (Mebanix Co., Ltd.). The inhibition rate was calculated by comparison of maximum aggregation rates with the control value.

Statistical Analysis

Data are expressed as means ± SEM. The drug concentrations and PDE inhibitory ratios were transformed into logarithms and logits, respectively, and IC_{50} values and 95% confidence limits were calculated by log–logit regression analysis. The concentrations of drugs able to increase the level of cAMP by 2-fold were calculated by a regression model with power transformation. The statistical significance of differences between multiple groups was evaluated using one-way ANOVA followed by Dunnett's test (two-tailed). Correlation coefficients were calculated with Pearson's method. In all cases, $P < 0.05$ was considered significant.

RESULTS

Expression and Characterization of Recombinant PDE Isozymes

To investigate the effects of the compounds on the activities of PDE isozymes, baculovirus-expressed recombinant enzymes were prepared. Table 1 shows the K_m values for the substrates cAMP or cGMP, the V_{max} values, and the half-maximal inhibitory concentrations (IC_{50}) of selective or nonselective inhibitors for each PDE isozyme. Recombinant PDE1 and PDE5 hydrolyzed cGMP with K_m values of 2.9 and 0.98 μM, respectively. PDE1 activity was activated about 3-fold by Ca^{2+}/calmodulin. Recombinant PDE2 hydrolyzed cAMP with a high K_m, 46.4 μM. The hydrolysis of cAMP exhibited positive cooperativity, with a Hill coefficient of 1.5. cAMP hydrolytic activity of PDE2 was stimulated 2.5-fold by 3 μM cGMP. Recombinant PDE3A, PDE3B, and PDE7 hydrolyzed cAMP or cGMP with low K_m values. Enzyme activities were inhibited by the respective PDE isozyme-selective inhibitors, with IC_{50} values similar to those reported previously [6, 29, 31, 32]. PDE3A and PDE3B activities were inhibited completely by 3 and 10 μM cGMP, respectively (Fig. 2). As no selective inhibitor for PDE7 has been designed, the nonselective PDE inhibitor IBMX was chosen for examination and shown to inhibit PDE7 activity with an IC_{50} of 7.0 μM. The IC_{50} of

TABLE 2. Inhibition of recombinant PDE isozymes by OPC33540, OPC33536, OPC33509, and PDE3 selective inhibitors

	IC_{50} (μM)						
	PDE1	PDE2	PDE3A	PDE3B	PDE4	PDE5	PDE7
OPC-33540	42.9 (20.0–82.0)	52.3 (37.3–85.1)	0.00032 (0.00023–0.00043)	0.0015 (0.0008–0.0059)	100.8 (48.4–222.1)	2.5 (2.0–3.1)	51.3 (36.0–85.9)
OPC-33536	112.9 (92.7–137.6)	20.9 (13.1–29.5)	0.00034 (0.00022–0.00054)	0.0019 (0.0009–0.0120)	45.3 (19.4–436.0)	2.7 (2.3–3.2)	20.3 (13.1–28.1)
OPC-33509	> 300	> 300	0.10 (0.05–0.20)	0.28 (0.14–0.82)	> 300	12.1 (6.4–24.9)	119.5 (93.2–153.3)
Cilostamide	> 300	12.5 (9.1–18.4)	0.027 (0.016–0.049)	0.050 (0.034–0.093)	88.8 (51.7–160.0)	15.2 (8.1–58.0)	22.0 (16.3–29.8)
Cilostazol	> 100	45.2 (29.3–85.1)	0.20 (0.12–0.34)	0.38 (0.18–1.75)	88.0 (52.1–308.1)	4.4 (3.7–5.1)	21.4 (9.0–36.4)
Amrinone	> 300	> 300	16.7 (10.6–28.0)	31.2 (18.5–56.9)	> 300	229.6 (157.3–436.7)	> 300
Milrinone	262.7 (209.0–330.1)	> 300	0.45 (0.26–0.78)	1.0 (0.7–1.9)	17.5 (3.2–34.8)	49.1 (36.8–65.3)	58.3 (9.5–230.2)

Values are the means (95% confidence limits) of three or four separate experiments performed in duplicate and are expressed as the concentrations of inhibitors producing 50% inhibition of enzyme activity.

rolipram against PDE7 was approximately 300 μM. The data demonstrated that these baculovirus-expressed recombinant PDE isozymes are appropriate for examination of the effects of PDE inhibitors on PDE isozyme activities.

Effects of Cilostamide Analogues and PDE3 Inhibitors on PDE3A Isozyme Activity

In cardiovascular tissues including smooth muscle cells and platelets, PDE3A is the predominant PDE3 subtype [27, 33]. Therefore, the effects of OPC-33540, OPC-33536, OPC-33509, and classical PDE3 selective inhibitors on recombinant PDE3A activity were examined. Cilostamide, cilostazol, milrinone, and amrinone all demonstrated concentration-dependent inhibition (Fig. 2). OPC-33540 and OPC-33536 also were found to inhibit recombinant PDE3A activity potently in a concentration-dependent manner, and with IC_{50} values of 0.32 and 0.34 nM, respectively (Table 2). The activity of OPC-33540 was about 100-fold more potent than that of cilostamide, one of the most selective and potent PDE3 inhibitors. OPC-33509, structurally similar to OPC-33540, demonstrated 330-fold less activity. Lineweaver–Burk plots for inhibition of PDE3A cAMP hydrolytic activity by OPC-33540 and OPC-33509 (Fig. 3) and OPC-33536 (data not shown) were indicative of a competitive mechanism of inhibition. The K_i values of OPC-33540, OPC-33536, and OPC-33509 for PDE3A were 0.22, 0.30, and 62 nM, respectively.

Selectivity of OPC-33540 and OPC-33536 for the PDE3 Isozyme

To evaluate the isozyme selectivity of the PDE3A inhibitory activity of OPC-33540, OPC-33536, OPC-33509, and

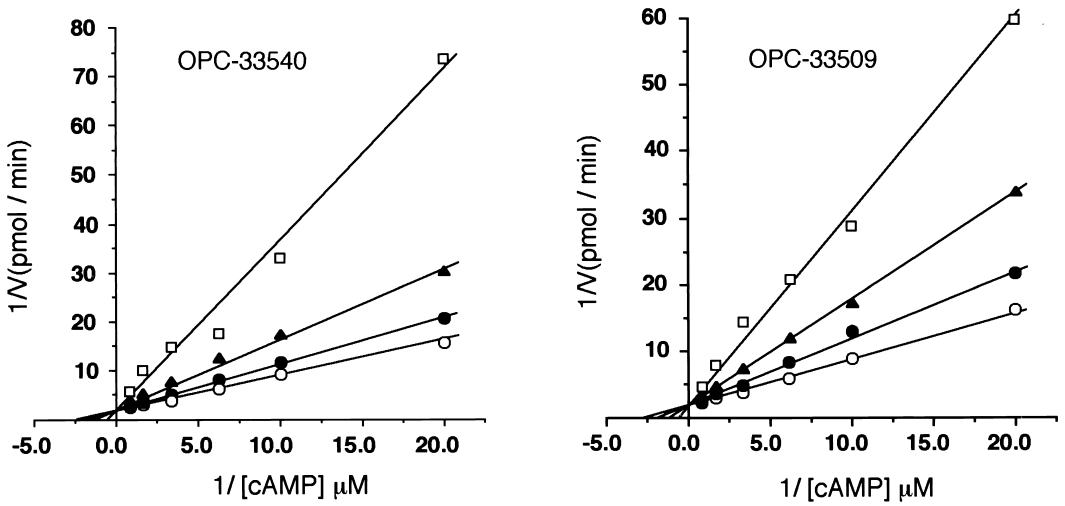

FIG. 3. Kinetic analysis of the inhibitory effects of OPC-33540 and OPC-33509 on recombinant PDE3A. Lineweaver-Burk plots are represented for different concentrations of OPC-33540 or OPC-33509. OPC-33540: (○) control; (●) 0.1 nM; (▲) 0.3 nM; and (□) 1 nM. OPC-33509: (○) control; (●) 30 nM; (▲) 100 nM; and (□) 300 nM.

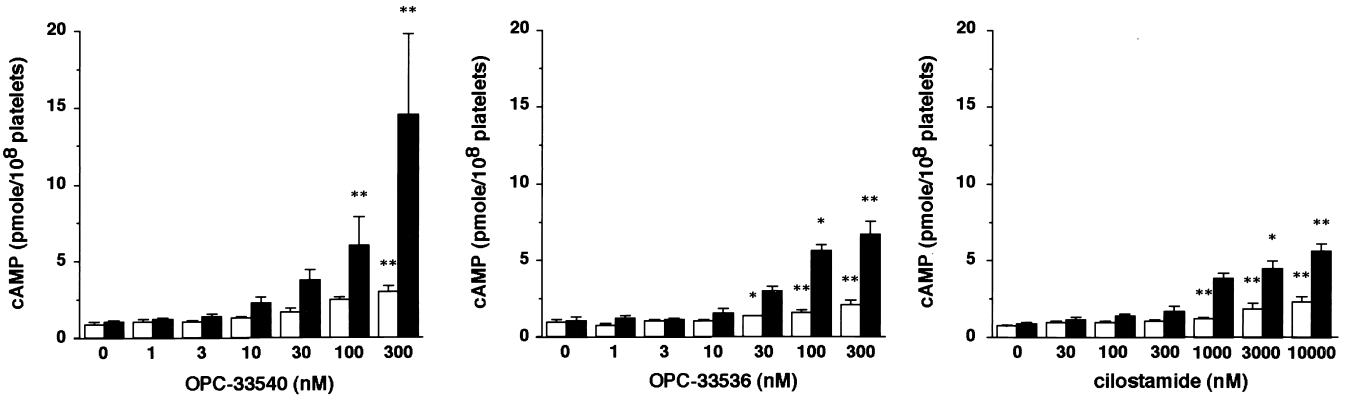

FIG. 4. Effects of OPC-33540, OPC-33536, and cilostamide on cAMP accumulation in human platelets. Washed human platelets were incubated with the PDE3 inhibitors alone (open columns) or with 3 nM PGE_1 (closed columns). PGE_1 itself at 3 nM did not affect cAMP accumulation significantly. Data shown are means ± SEM (N = 3). Key: (*) $P < 0.05$, and (**) $P < 0.01$ vs control in the absence or presence of PGE_1.

other classical PDE3 inhibitors, their effects on recombinant PDE1, PDE2, PDE4, PDE5, and PDE7 activities and PDE3B subtype activity were examined. PDE6 is exceptional in that it forms heterotetramers and is found specifically in photoreceptors. All of the PDE3 inhibitors tested in this study, including cGMP, exhibited 1.8- to 5.6-fold weaker activity against the PDE3B subtype than against PDE3A (Tables 1 and 2). The effects of OPC-33540 on activities of PDE isozymes other than PDE3 were very weak in comparison. OPC-33540 had the highest selectivity for PDE3A among these compounds. OPC-33540 displayed selectivity for PDE3A of about 8,000-fold greater than that for PDE5 and 320,000-fold greater than that for PDE4, with potencies against other PDE isozymes falling in between. It was shown that cilostazol and amrinone appeared to be somewhat effective against PDE5, and milrinone appeared to be somewhat effective against PDE4. These data demonstrated that OPC-33540 and OPC-33536 were highly selective PDE3 inhibitors.

Effects of OPC-33540 and OPC-33536 on cAMP Accumulation in Platelets

To evaluate the PDE inhibitory activities of OPC-33540 and OPC-33536 in cells, their effects on cAMP accumulation in human platelets in the absence or presence of PGE_1 were examined. OPC-33540 increased cAMP in platelets in a concentration-dependent manner, 2-fold at 32.5 nM (Fig. 4). OPC-33536 and cilostamide also increased cAMP 2-fold at 271 nM and 1.3 μM, respectively. In the presence of 3 nM PGE_1, which did not significantly affect cAMP alone, the cAMP increasing ability of these compounds was enhanced. The concentrations required to increase cAMP concentration by 2-fold were 6.2, 11.6, and 184 nM, respectively.

Anti-platelet Aggregation Effects of OPC-33540 and OPC-33536

The effects of OPC-33540, OPC-33536, and cilostamide on human platelet aggregation induced by thrombin were examined in the absence or presence of PGE_1 (Fig. 5). OPC-33540 potently inhibited thrombin-induced platelet aggregation, with an IC_{50} of 27.8 nM. OPC-33536 and cilostamide also inhibited platelet aggregation, with IC_{50} values of 71.3 nM and 1.1 μM, respectively. In the presence of 3 nM PGE_1, the anti-platelet aggregation effects of OPC-33540, OPC-33536, and cilostamide all were stimulated about 5-fold; their IC_{50} values were 6.0, 12.7, and 253 nM, respectively. The enhancement of the anti-platelet aggregation effects of these compounds by PGE_1 correlated well with the change in cAMP.

DISCUSSION

Since the existence of PDE isozymes was first reported [34], to confirm the isozyme selectivity of PDE inhibitors, the inhibitory effect of such compounds on PDE activity has been evaluated with enzymes isolated through multiple steps from tissues or organs. However, it is possible that native PDE enzymes isolated from tissues contain not only a single major isozyme but also some other minor or unknown isozymes. In addition, since the major isozyme might consist of multiple PDE subtypes, such as PDE3A and 3B, it is difficult to evaluate not only the isozyme selectivity but also its subtype specificity. Baculovirus-expressed recombinant enzymes allow this problem to be overcome, since they are derived from single genes and can be generated in large amounts. Therefore, in the present study, we evaluated the effects of novel anti-platelet aggregatory agents on recombinant PDE isozymes expressed in a baculovirus expression system. Contamination of cAMP and cGMP hydrolysis activities derived from endogenous proteins of Sf9 cells is very low. We compared the cAMP and cGMP hydrolysis activities of supernatants from homogenates of Sf9 cells that expressed recombinant PDE with activities in homogenate supernatants of non-infected Sf9 cells. In the assay for recombinant PDE3B, which indicates the lowest V_{max} among recombinant PDE isozymes in this study (Table 1), PDE activity (cAMP hydrolysis activity)

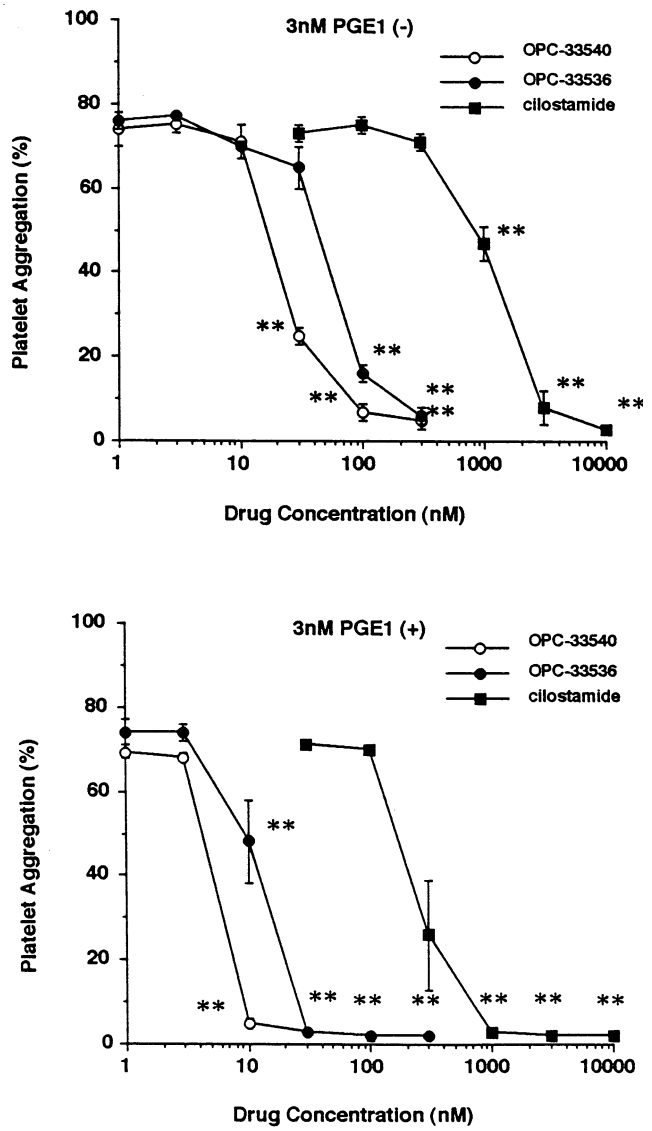

FIG. 5. Effects of OPC-33540, OPC-33536, and cilostamide on thrombin-induced human platelet aggregation. Washed human platelets were incubated with the PDE3 inhibitors {PGE_1 alone [PGE_1 (−)] or plus 3 nM PGE_1 [PGE_1 (+)]}. PGE_1 at 3 nM did not affect thrombin-induced human platelet aggregation significantly. Data shown are means ± SEM (N = 3). Key: (**) $P <$ 0.01 vs control in the absence or presence of PGE_1.

from normal Sf9 cells was equivalent to 7% of the total PDE3B activity. For other PDE isozymes, contamination was much less and was 0.1% for PDE1 and PDE5. Therefore, we did not partially purify the enzymes via an ion exchange chromatography column, except for PDE4.

The kinetic and pharmacological characteristics of the baculovirus-expressed recombinants PDE1 and PDE7 prepared in this study were very similar to those previously reported with COS-7 cells and Sf9 cells, respectively [23, 35]. In addition to the rolipram-insensitivity of PDE7, it has been reported that a novel IBMX-insensitive PDE might be PDE7 [36]. However, IC_{50} values of IBMX for recombinant PDE7 in this study and in another report [2] were 7.0 and 30 μM, respectively, suggesting that PDE7 seems to be an IBMX-sensitive PDE. Recently, PDE8 and PDE9, newly discovered PDE isozymes, have been reported as IBMX-insensitive PDEs (IC_{50} values greater than 100–200 μM) [2–4]. The activity of recombinant PDE5 expressed in COS-7 cells is inhibited by dipyridamole and zaprinast with IC_{50} values of 3.5 and 2.5 μM, respectively, several-fold higher than the values for native PDE5, a cGMP-specific PDE [22]. The IC_{50} values of dipyridamole and zaprinast for native PDE5 isolated from human platelets are 0.259 and 0.234 μM, respectively [32]. In addition, IC_{50} values for PDE5 from the human aorta are 0.30 and 0.34 μM, respectively [37]. The activity of baculovirus-expressed recombinant PDE5 in this study was inhibited by dipyridamole and zaprinast with IC_{50} values of 0.26 and 0.20 μM, respectively (Table 1, and data not shown). Thus, baculovirus-expressed recombinant PDE5 had an inhibitor sensitivity similar to native PDE5. The K_m value observed for recombinant PDE5 was also similar to that reported for human aorta PDE5 [37]. Characteristics of recombinant PDE2 have not been reported; the K_m for the cAMP of native PDE2 (cGMP-stimulated PDE) is high (36 μM), and the IC_{50} value for inhibition of its cAMP hydrolysis by EHNA is 14.6 μM [31, 38]. As shown in Table 1, these values are also similar to ours for recombinant PDE2. cAMP hydrolysis activity of recombinant PDE2 was stimulated by cGMP and exhibited positive cooperativity.

In biochemical and biophysical characterizations of PDE enzymes, PDE isozyme-selective inhibitors, such as the agents listed in Table 1, have played important roles as bioprobes [6]. We previously developed and characterized the PDE3 selective inhibitor cilostamide (OPC-3689 [11]), still widely used in pharmacological, biochemical, and cell biological studies. PDE3 can be purified from platelets and adipose tissue by affinity column chromatography when coupled with derivatives of cilostamide [16, 39]. We discovered OPC-33540 in the process of screening compounds for antithrombotic and anti-intimal hyperplastic actions. The present study demonstrated that OPC-33540 potently inhibited recombinant PDE3A activity with an IC_{50} value of 0.32 nM, 100 times more potently than cilostamide in this respect. OPC-33540 also demonstrated extreme selectivity for PDE3 when its inhibition of cAMP hydrolysis was compared with that of other PDE isozymes (PDE1, 2, 4.5, and 7), suggesting that this agent is one of the most selective and potent PDE3 inhibitors and could be extremely useful for investigation of intracellular cAMP signaling pathways. Recently, three additional PDE isozymes, PDE8, PDE9, and PDE10, were discovered by searching the EST database; these PDE isozymes are not inhibited by PDE3 selective inhibitors such as mirlinone, SKF94120, and enoximone [2–5]. Therefore, although it is speculated that OPC-33540 also may not inhibit the activities of PDE8, PDE9, and PDE10, it will be necessary to examine the effect of OPC-33540 on their activities in the future. OPC-33509, with a cyclopropyl moiety instead of the cyclooctyl moiety present in OPC-33540, was found to be far less potent, indicating that the cyclooctyl moiety

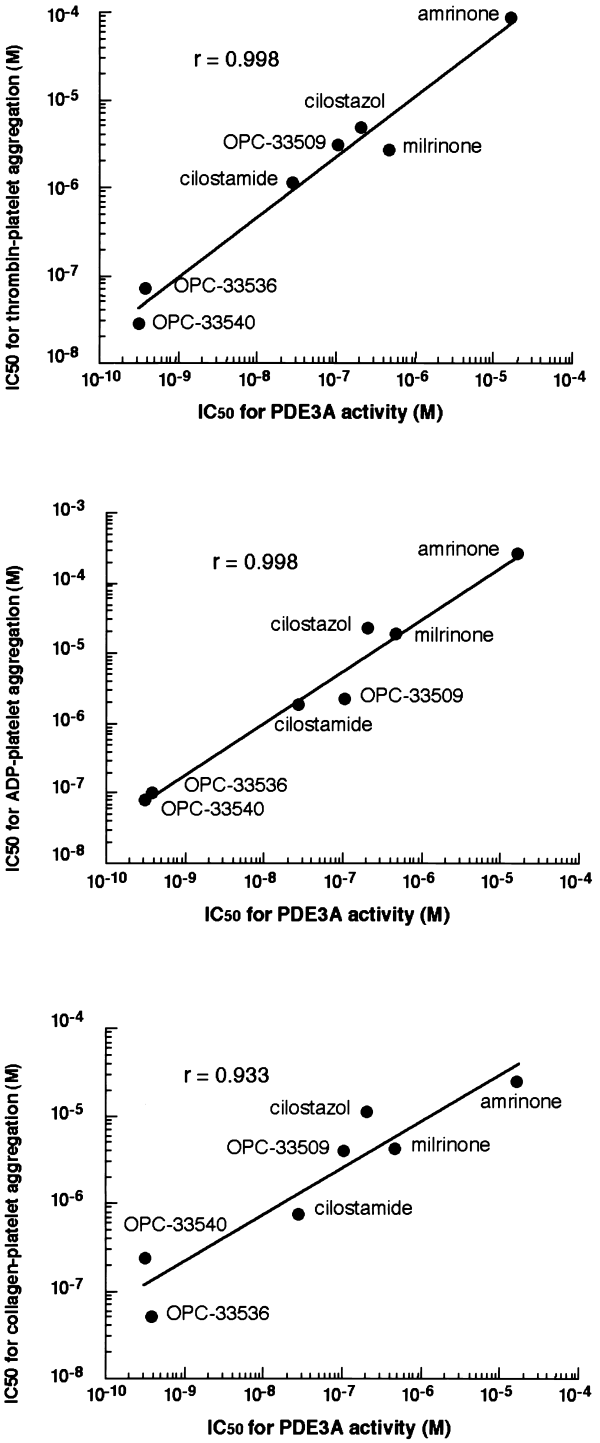

FIG. 6. Correlations between PDE3 inhibition and anti-platelet aggregation effects. Plots show IC_{50} values for PDE3A activity and platelet aggregation with OPC-33540, OPC-33536, cilostamide, OPC-33509, cilostazol, milrinone, and amrinone. The IC_{50} values for platelet aggregation were obtained with thrombin- and collagen-induced human platelet aggregation and ADP-induced rabbit platelet aggregation.

of OPC-33540 may be very important for potent inhibition of PDE3A. In another platelet aggregation study [19], the inhibitory potency of a series of *N*-cycloalkyl-*N*-2-hydroxycyclohexylurea derivatives was found to be dependent on the ring size, supporting the significance of the cyclooctyl moiety. Furthermore, comparison of OPC-33536 with cilostamide suggests that its semicarbazide moiety contributes more to PDE3A inhibition than the amide moiety of cilostamide.

OPC-33540 inhibits thrombin-, ADP-, and collagen-induced platelet aggregation (Figs. 5 and 6; [19]). As shown in Fig. 6, a good correlation between IC_{50} values for inhibition of recombinant PDE3A activity and inhibition of ADP-, thrombin-, or collagen-induced platelet aggregation was obtained in the present experiments (r = 0.998, 0.998, and 0.933, respectively). Ashida and Sakuma [40] have reported that the inhibition of cAMP-PDE, possibly PDE3, is important for the suppression of ADP-induced platelet aggregation, and that inhibition of cGMP-PDE, possibly PDE5, is linked to reduction of 5-hydroxytryptamine release.

PDE3 isozymes comprise at least two subtypes, PDE3A and PDE3B [41]. Tissue- and cell-distribution patterns of PDE3 subtypes are distinct; cardiovascular tissues express PDE3A, and adipose tissue and hepatocytes express PDE3B. Among the PDE3 inhibitors used in this study, including cGMP, none discriminated clearly between PDE3A and 3B. Since PDE3A is the PDE3 subtype in platelets [33], our results indicated that the anti-platelet aggregation action of the inhibitors in this study was due to inhibition of PDE3A activity, which consequently increased the cAMP content of platelets. From the point of view of PDE3 subtypes, even if a PDE3A subtype-specific inhibitor were developed, it appears that it will be difficult to increase cAMP in platelets without increasing cAMP in the heart, because PDE3A is also present in the heart.

In summary, studies using baculovirus-expressed recombinant PDE isozymes demonstrated that the novel anti-platelet aggregatory agent OPC-33540 is an extremely selective and potent PDE3 inhibitor, stimulating cAMP accumulation in platelets in both the presence and the absence of PGE_1. OPC-33509, an OPC-33540 derivative, which has been evaluated in clinical trials for treatment of ischemic diseases, also was shown to possess similar properties, but was somewhat less potent.

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