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Cyclic AMP and cyclic GMP phosphodiesterase inhibition by an antiplatelet agent, 6-[(3-methylene-2-*oxo*-5-phenyl-5-tetrahydrofuranyl) methoxy]quinolinone (CCT-62)

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Abstract

The antiplatelet activity of (6-[(3-methylene-2-*oxo*-5-phenyl-5-tetrahydrofuranyl) methoxy]quinolinone) (CCT-62) was determined in vitro in rabbit platelets. CCT-62 inhibited rabbit platelet aggregation and ATP release caused by thrombin (0.1 U/ml), platelet-activating factor (2 ng/ml), collagen (10 μg/ml), arachidonic acid (100 μM), and 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$ (1 μM) in a concentration-dependent manner. The IC_{50} values for platelet aggregation were 18.4 ± 4.5, 10.1 ± 1.6, 3.0 ± 0.9, 1.5 ± 0.3 and 1.0 ± 0.3 μM, respectively. In addition, CCT-62 disaggregated the clumped platelets caused by these aggregation inducers. It also inhibited phosphoinositide breakdown and intracellular calcium elevation induced by the above platelet aggregation inducers. CCT-62 increased intracellular cyclic AMP and cyclic GMP levels in a concentration- and time-dependent manner. Furthermore, it potentiated cyclic AMP formation caused by prostaglandin E_1 but not that caused by 3-isobutyl-1-methylxanthine. CCT-62 did not affect adenylate or guanylate cyclase but inhibited cyclic AMP- and cyclic GMP-phosphodiesterase activities. The antiplatelet effect of CCT-62 was reversed by a protein kinase A inhibitor, *N*-[2-(*P*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89). This data clearly indicated that CCT-62 is an inhibitor of phosphodiesterases and that its antiplatelet effect is mainly mediated by elevation of cyclic AMP levels. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: CCT-62; cAMP; cGMP; Phosphodiesterase; Phosphoinositide breakdown

1. Introduction

Platelets play pivotal roles in the arrest of bleeding after vascular injury and subsequent tissue repair. Platelet activation is a result of a complex signal transduction cascade reaction brought about by stimulants. The process of platelet activation is regulated, in part, by levels of the second messengers, adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and guanosine 3′:5′-cyclic monophosphate (cyclic GMP). Increased intracellular cyclic AMP or cyclic GMP levels lead to inhibition of agonist-induced platelet activation–aggregation, adhesion and release of granule contents (Feinstein et al., 1985; Radomski et al., 1987). Cyclic AMP is synthesized when prostaglandin I_2 or prostaglandin E_1 binds to specific platelet receptors and activates adenylate cyclase (Schafer et al., 1979), and cyclic GMP can be synthesized after platelets have been stimulated by sodium nitroprusside, which is believed to activate guanylate cyclase by generating nitric oxide. The steady state levels of cyclic nucleotides are maintained by a balance between the rate of synthesis by adenylate (guanylate) cyclase and the rate of hydrolysis by cyclic nucleotide phosphodiesterase. Cyclic nucleotide phosphodiesterases catalyse the hydrolysis of 3′–5′-cyclic nucleotides to the corresponding nucleoside 5′-monophosphates and thereby play important roles in the regulation of the cyclic nucleotide concentration. For this reason, inhibition of phosphodiesterase activity increases intracellular cyclic AMP or cyclic GMP levels and regulates the function of platelets (Maurice and Haslam, 1990). Moreover, adenylate or guanylate cyclase agonists and phosphodi-

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esterase inhibitors synergize to elevate intracellular cyclic AMP or cyclic GMP levels, providing a good rationale for the therapeutic use of these agents alone or in combination with other agents.

In a preliminary study, we found that a synthetic compound, CCT-62, has strong antiplatelet effects. It inhibited platelet activation caused by distinct platelet stimulators at a low concentration. In the present study, we evaluated the mechanism of its inhibitory activity on platelet aggregation and showed it to be a phosphodiesterase inhibitor in rabbit platelets.

2. Materials and methods

2.1. Washed platelet preparation and aggregation measurement

Washed platelets were prepared from blood withdrawn with a siliconized syringe from the marginal vein of New Zealand rabbits. The platelet suspension was obtained from EDTA-anticoagulated platelet-rich plasma according to the washing procedure described previously (Teng et al., 1987). Platelet number was counted with a cell counter (Hemalaser 2, Sebia, France) and adjusted to 3.0×10^8 platelets/ml. The platelet pellets were suspended in Tyrode's solution containing Ca^{2+} (1 mM) and bovine serum albumin (0.35%). All glassware was siliconized.

Platelet aggregation was measured by the turbidimetric method (Born and Cross, 1963). ATP released from platelets was measured by the bioluminescence method (DeLuca and McElory, 1978). Both aggregation and ATP release were measured with a Lumi-aggregometer (Model 1020, Payton, Canada) connected to two dual-channel recorders.

2.2. Intracellular Ca^{2+} concentration

The method of Pollock and Rink (1986) was followed. Platelets (3×10^8 platelets/ml) were incubated with fura-2/AM (5 μM, ester form). Fluorescence (excitation wavelength 339 nm, emission wavelength 500 nm) was measured with a Hitachi fluorescence spectrophotometer (Model F4000, Hitachi, Tokyo, Japan). At the end of the experiment, the cells were treated with 0.1% Triton X-100 followed by the addition of 10 mM EGTA to obtain the maximal and minimal fluorescences, respectively. Intracellular Ca^{2+} was calculated as described for fura-2, using the intracellular calcium-dye dissociation constant of 224 nM (Grynkiewicz et al., 1985).

2.3. Phosphoinositide breakdown assay

This method was modified from those of Huang and Detwiler (1986), and Neylon and Summers (1987). EDTA-platelet rich plasma was centrifuged at $500 \times g$ for 10 min and the platelet pellets were suspended in 1 ml calcium-free and bovine serum albumin-free Tyrode's solution containing 75 μCi/ml [^{3}H]inositol and 1 mM EDTA. After incubation at 37°C for 2 h, the platelet pellets were collected by centrifugation ($500 \times g$, 4 min) and resuspended in Tyrode's solution. In the presence of 5 mM LiCl, which inhibits inositol monophosphate phosphatases, the reaction was carried out at 37°C for 6 min with a stirring bar driven at 900 rev/min. An equal volume of 10% (w/v) trichloroacetic acid was added to stop the reaction. After centrifugation at $1000 \times g$ for 10 min, 1 ml volume of supernatant were pooled and trichloroacetic acid was removed by extraction with 5×2 volume diethyl ether. The aqueous phase, containing the inositol phosphates, was adjusted to pH 7–8 and diluted to 4 ml with distilled water before its application to a Dowex-1 ion-exchange column for separation of the inositol phosphates, as described previously by Neylon and Summers (1987). Radioactivity levels ([^{3}H]inositol monophosphate) were counted by a liquid scintillation counter.

2.4. Cyclic AMP and cyclic GMP measurements

After a 1-min incubation at 37°C in a Lumi-aggregometer, platelet suspension (1×10^9 platelets/ml) was challenged with prostaglandin E_1 (1 μM), sodium nitroprusside (10 μM) or various concentrations of CCT-62. The reaction was stopped by addition of EDTA (10 mM) followed by immediate boiling for 2 min. After the mixture was cooled to 4°C, the precipitated protein was centrifuged in an Eppendorf microcentrifuge for 5 min. Cyclic AMP and cyclic GMP contents in the supernatant were measured with enzyme immunoassay kits.

2.5. Isolation of guanylate cyclase and enzyme activity assay

Washed platelets were resuspended in a Tris–HCl buffer (50 mM, pH 7.4). The platelets were sonicated in ice for 10 s each for 4 to 6 times at setting 5 (Vibra cell, Sonics and Materials, Danbury, CT) and centrifuged at $39\,000 \times g$ at 4°C for 20 min. The supernatant was used as a source of soluble guanylate cyclase. The pellet (platelet membrane) was washed twice with the same Tris–HCl buffer. Protein content was determined with Bio-Rad assay and adjusted to 1 mg/ml. Guanylate cyclase activity was determined by the method described by Gerzer et al. (1983). The enzyme preparation (50 μl) was incubated with dimethylsulfoxide, sodium nitroprusside, or CCT-62 in a final volume of 200 μl with the following reactants: guanosine 5′-triphosphate (GTP, 0.2 mM containing 1×10^6 cpm [α-^{32}P]GTP), $MgCl_2$ (5 mM), cyclic GMP (2.5 mM), creatine phosphate (15 mM), and creatine phosphokinase (30 μg). The reaction was initiated by addition of the enzyme preparation to the reaction mixture and, after incubation at 30°C for 10 min, terminated by addition of 200 μl of 0.5 M HCl. The

reaction mixture was then heated to 100°C for 6 min, cooled in an ice bath, and added to 200 μl of 1 mM imidazole. GTP and cyclic GMP were separated on neutral alumina (White and Zenser, 1971) and radioactivity levels ([^{32}P]cyclic GMP) were determined in a liquid scintillation counter.

2.6. Determination of adenylate cyclase activities

The platelet membrane fraction prepared as described above was resuspended in a Tris–HCl buffer containing 5 mM EDTA. Adenylate cyclase activity was measured as described by Insel et al. (1982). The membrane fraction (20 μl) was added to a final volume of 60 μl reaction mixture consisting of 50 mM Tris–HCl, pH 7.4, 2.6 mM EDTA, 13 mM $MgCl_2$, 25 mM creatine phosphate, 1 mg/ml creatine phosphokinase, 1 mM cyclic AMP, 0.5 mM ATP (containing 1×10^6 cpm [α-^{32}P]ATP), and other reactants as indicated in the text. The reaction was initiated by addition of platelet protein and allowed to proceed at 30°C for 20 min. The reaction was terminated by addition of 0.2 ml of 0.5 M HCl and the mixture was boiled for 6 min. After the mixture was cooled in an ice bath, imidazole (1 mM, 200 μl) was added. ATP and cyclic AMP were separated on neutral alumina and the radioactivity levels ([^{32}P]cyclic AMP) were determined with a liquid scintillation counter.

2.7. Purification of phosphodiesterase isozyme and measurement of enzyme activities

All procedures were performed at 4°C. Washed platelets (11.5 ml) were homogenized in 35 ml of buffer (20 mM Tris–HCl, 5 mM 2-mercaptoethanol, 2 mM benzamidine, 2 mM EDTA and 50 mM sodium acetate, pH 6.5) with a sonicator (Vibra Cell, Sonics and Materials) for 10 s. The homogenate (40 ml) was centrifuged for 20 min at 25 000 $\times g$ and the supernatant was applied to the DEAE-Sepharose CL-6B column (17 × 1.5 cm) (Pharmacia Biotech, Uppsala, Sweden) that had been pre-equilibrated with 170 ml of homogenization buffer. A flow rate of 30 ml/h was used throughout the ion-exchange chromatography procedure. The phosphodiesterases were eluted with a linear 400 ml, 50 to 1000 mM sodium acetate gradient. Fractions, 7.5 ml each, were collected and stored at 4°C until assayed for phosphodiesterase activity.

Phosphodiesterase activity was determined according to Wells et al. (1975). The enzyme (1 mg/ml, 0.1 ml) was incubated with Tris–HCl (0.2 ml) at 37°C for 5 min, and then 0.1 ml cyclic AMP (final concentration 1 μM containing 0.1 μCi [^{3}H]cyclic AMP and 1 mM EGTA) or cyclic GMP (final concentration 1 μM containing 0.1 μCi [^{3}H]cyclic GMP and 1 mM EGTA) was added. After 30 min at 37°C, the sample was heated to 100°C for 1 min. After the samples were cooled to room temperature, *Ophiophagus hannah* snake venom (1 mg/ml, 0.1 ml) was added and incubated at 25°C for 30 min for the conversion of the 5′-AMP or 5′-GMP to the uncharged nucleoside, adenosine or guanosine, respectively. An ion-exchange resin slurry (1.0 ml Dowex-1, 100 to 200 mesh) was added to adsorb all unconverted cyclic AMP or cyclic GMP and the mixture was centrifuged. A sample of 0.5 ml of the supernatant was taken for the determination of [^{3}H] counts in a liquid scintillation counter.

2.8. Materials

CCT-62 (Fig. 1) was chemically synthesized and dissolved in dimethylsulfoxide. 6-Hydroxyquinolin-2(1H)-one (1.61 g, 10 mmol), K_2CO_3 (1.38 g, 10 mmol), and dry *N,N*-dimethlyformamide (50 ml) were stirred at room temperature for 30 min. To this solution, 2-bromoacetophenone (1.99 g, 10 mmol) was added to dry *N,N*-Dimethlyformamide (10 ml) in one portion. The resulting mixture was stirred at room temperature for 24 h (TLC monitoring) and then poured into ice-water (100 ml). The pale-yellow solid thus obtained was collected and crystallized from CH_2Cl_2/Et_2O 1:10 (2.15 g, 77%), and was finally dissolved in dry tetrahydrofuran (0.84 g in 60 ml). To this solution was added activated Zn powder (0.26 g, 3.9 mmol), hydroquinone (6 mg), and ethyl 2-(bromomethyl)acrylate (0.78 g, 4 mmol). The mixture was refluxed under N_2 atmosphere for 6 h (TLC monitoring). After being cooled, it was poured into an ice-cold 5% HCl solution (300 ml), and extracted with CH_2Cl_2 (3 × 60 ml). The CH_2Cl_2 extracts were combined and washed with H_2O, dried (Na_2SO_4), and then evaporated to give a residual solid which was crystallized from a mixed solvent of CH_2Cl_2/Et_2O 1:10 to give CCT-62. Collagen (type I, bovine Achilles tendon) was homogenized in 25 mM acetic acid and stored at −20°C at a concentration of 1 mg/ml. Platelet-activating factor was dissolved in chloroform and diluted in 0.1% bovine serum albumin/saline solution immediately before use. Arachidonic acid, EDTA (disodium salt), luciferin-luciferase, dimethylsulfoxide, Dowex-1 (100–200 mesh: X8, chloride) resin, alumina (type WN-3: neutral), DEAE-Sepharose (CL-6B), myoinositol, bovine serum albumin, prostaglandin E_1, sodium nitroprusside, indomethacin, 3-isobutyl-1-methylxanthine, 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$, trichloroacetic acid, ATP, GTP, cyclic AMP and cyclic GMP were purchased from Sigma Chemical (St. Louis,

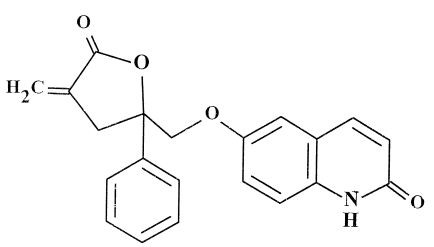

Fig. 1. Chemical structure of CCT-62.

MO). Thrombin (bovine) was purchased from Parke Davis (Detroit, MI) and dissolved in 50% glycerol to give a stock solution of 100 NIH units/ml. H89 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Cyclic AMP and cyclic GMP enzyme immunoassay kits and all radioactive materials, myo-[2-^{3}H]inositol (10 ~ 20 Ci/mmol), [α-^{32}P]adenosine triphosphate (3000 Ci/mmol), [α-^{32}P]guanosine triphosphate (3000 Ci/mmol), [^{3}H]cyclic AMP (24.0 Ci/mmol) and [^{3}H]cyclic GMP (14.8 Ci/mmol) were obtained from Amersham (Amersham, UK). 3′-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1) was a generous gift from Yong-Shin Pharmaceutical, Taiwan.

2.9. Statistical analysis

Results are expressed as the means ± S.E.M. for the indicated number of separate experiments. Statistical significance between drug-treated and untreated groups was evaluated by Student's *t*-test and *P* values of less than 0.05 were considered significant.

3. Results

3.1. Effects of CCT-62 on platelet aggregation and ATP release

Thrombin (0.1 U/ml), platelet-activating factor (2 ng/ml), collagen (10 μg/ml), arachidonic acid (100 μM) and 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$ (1 μM) all caused 80 ~ 90% aggregation of washed rabbit platelets. CCT-62 inhibited platelet aggregation and ATP release caused by the above platelet stimulators in a concentration-dependent manner. The IC_{50} values of CCT-62 for the aggregation caused by the above stimulators were 18.4 ± 4.5, 10.1 ± 1.6, 3.0 ± 0.9, 1.5 ± 0.3 and 1.0 ± 0.3 μM, respectively. Fig. 2 presents the typical inhibitory effect of CCT-62 on thrombin-induced aggregation and ATP release.

Table 1
Inhibitory effects of CCT-62 and prostaglandin E_1 (PGE_1) on thrombin-induced aggregation in washed rabbit platelets

	Inhibition (%)
PGE_1 1 μ	80.3 ± 2.5
+ H89 5 μM	38.6 ± 17.8[a]
+ H89 10 μM	12.9 ± 3.7[b]
+ H89 20 μM	7.0 ± 1.4[b]
CCT-62 60 μM	96.2 ± 3.7
+ H89 5 μM	96.8 ± 3.0
+ H89 10 μM	58.5 ± 8.8[c]
+ H89 20 μM	35.5 ± 4.8[c]
PGE_1 0.01 μM	6.4 ± 1.1
IBMX 20 μM	5.5 ± 1.2
CCT-62 1.5 μM	6.9 ± 0.9
IBMX 20 μM + CCT-62 1.5 μM	9.3 ± 2.0
PGE_1 0.01 μM + CCT-62 1.5 μM	69.2 ± 9.6

Washed rabbit platelets were incubated with CCT-62 and/or prostaglandin E_1 at 37°C for 3 min, then thrombin (0.1 U/ml) was used to trigger the aggregation. *N*-[2-(*P*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89) was added 1 min before the application of CCT-62 or prostaglandin E_1. Thrombin caused 90.4 ± 1.2% aggregation in the control experiments. Values are presented as means ± S.E.M. ($n = 6$).
[a] $P < 0.05$.
[b] $P < 0.001$ as compared with the control (prostaglandin E_1 + thrombin).
[c] $P < 0.001$ as compared with the control (CCT-62 + thrombin).
IBMX: 3-isobutyl-1-methylxanthine.

Prostaglandin E_1 (1 μM) almost completely inhibited (> 80%) platelet aggregation caused by these aggregation inducers. In contrast, sodium nitroprusside (10 μM) markedly inhibited platelet aggregation caused by arachidonic acid, collagen and 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$ (39.2 ± 12.3, 70.7 ± 10.8 and 92.2 ± 5.6% inhibition, respectively; $n = 6$) but only slightly inhibited platelet aggregation caused by platelet-activating factor and thrombin (13.7 ± 1.8 and 10.1 ± 1.6% inhibition, respectively; $n = 6$). The inhibitory effects of CCT-62 and prostaglandin E_1 on thrombin-induced platelet aggregation were attenuated by H89, a protein kinase A inhibitor, in a concentration-dependent manner (Table 1). However, 1-*H*-(1,2,4)-oxadiazolo-(4,3-α)-quinoxalin-1-

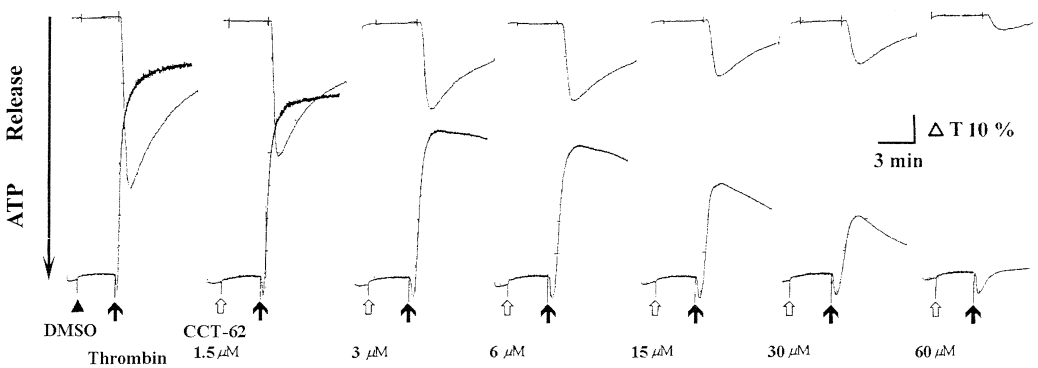

Fig. 2. Inhibitory effects of CCT-62 on the platelet aggregation and ATP release induced by thrombin. Washed rabbit platelets were preincubated with various concentrations of CCT-62 or dimethylsulfoxide (DMSO, 0.5%, control) at 37°C for 3 min, and then thrombin (0.1 U/ml) was added to trigger aggregation (upward tracings) and ATP release (downward tracings).

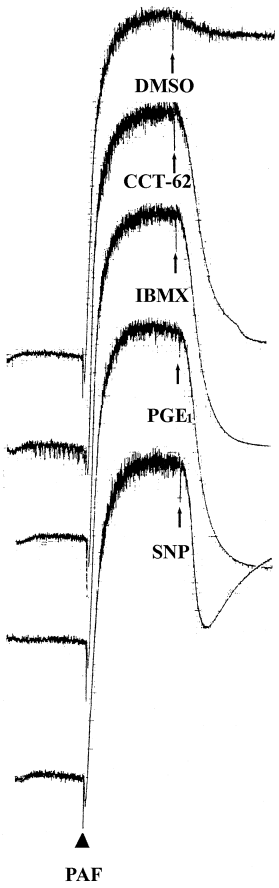

Fig. 3. Effects of CCT-62, 3-isobutyl-1-methylxanthine, prostaglandin E_1 and sodium nitroprusside on platelet disaggregation. Platelet aggregation was induced by platelet-activating factor (2 ng/ml) at 37°C for 4 min, and then dimethylsulfoxide (DMSO, 0.5%), CCT-62 (30 μM), 3-isobutyl-1-methylxanthine (IBMX, 300 μM), prostaglandin E_1 (PGE_1, 1 μM) or sodium nitroprusside (SNP, 1 mM) was added for another 5 min.

one (ODQ, 10 μM), a soluble guanylate cyclase inhibitor, had no effect on the antiaggregatory activity of CCT-62 and prostaglandin E_1 in the presence or absence of H89 (data not shown). Furthermore, the antiplatelet effect of a low concentration of prostaglandin E_1 (0.01 μM) was markedly potentiated by CCT-62. In contrast, CCT-62 did not potentiate the antiplatelet activity of 3-isobutyl-1-methylxanthine (Table 1).

CCT-62, prostaglandin E_1, sodium nitroprusside and 3-isobutyl-1-methylxanthine also markedly disaggregated clumped platelets that had been previously aggregated by the aggregation inducers (Fig. 3 for platelet-activating factor-induced platelet aggregation). Again, sodium nitroprusside was much less potent than CCT-62 and prostaglandin E_1 in disaggregating platelets.

3.2. Effect of CCT-62 on intracellular Ca^{2+} concentration

The intracellular calcium concentration of resting platelets was 12.3 ± 1.2 nM ($n = 6$). Thrombin (0.1 U/ml), platelet-activating factor (2 ng/ml), collagen (10 μg/ml), arachidonic acid (100 μM) and 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$ (1 μM) increased the intracellular calcium concentrations to 179.5 ± 22.2, 171.6 ± 27.4, 64.5 ± 7.0, 103.0 ± 21.7 and 107.3 ± 14.8 nM over the resting level, respectively. In contrast, in the presence of CCT-62 (60 μM), thrombin and platelet-activating factor only increased the intracellular calcium concentration to 95.9 ± 14.9 and 41.7 ± 12.0 nM over the resting level, respectively. Collagen, arachidonic acid and 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$ increased the intracellular calcium concentration to 8.1 ± 2.6, 26.8 ± 5.5 and 29.7 ± 8.8 nM, respectively, over the resting level in the presence of CCT-62 (3 μM).

3.3. Effects of CCT-62 on phosphoinostide breakdown

In the absence of CCT-62, thrombin (0.1 U/ml), platelet-activating factor (2 ng/ml), collagen (10 μg/ml), arachidonic acid (100 μM) and 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$ (1 μM) increased [^{3}H]in-

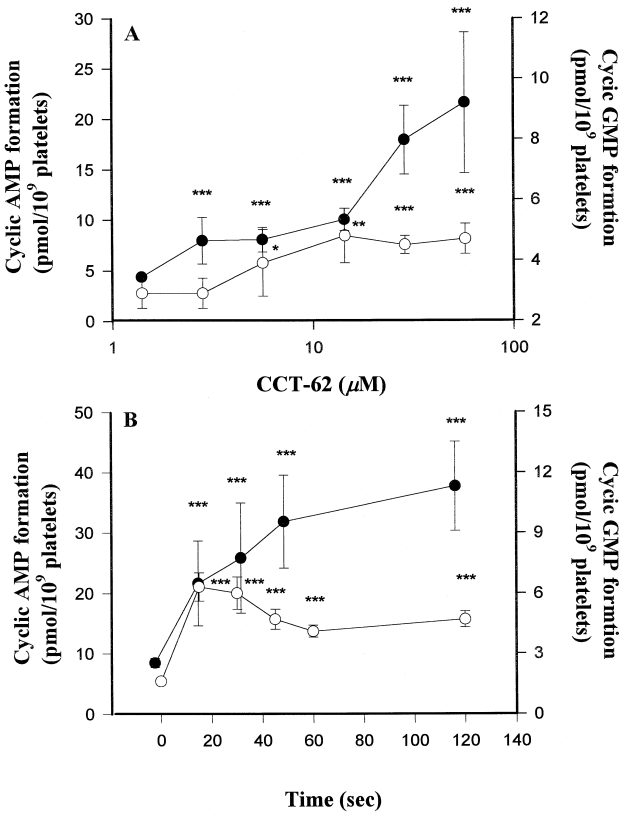

Fig. 4. Concentration- and time-dependent increase of platelet cAMP (●) and cGMP (○) levels caused by CCT-62. Washed rabbit platelets were preincubated at 37°C for 1 min; various concentrations of CCT-62 were added for 45 s (A) or CCT-62 (60 μM) was added for various time periods (B). The reactions were terminated by addition of EDTA (10 mM) followed by immediate boiling for 5 min. The cAMP and cGMP contents were determined by enzyme immunoassay. Values are presented as means ± S.E.M. ($n = 6$). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ as compared with the respective control.

Table 2
Effect of CCT-62, forskolin, sodium nitroprusside and YC-1 on platelet adenylate and guanylate cyclase activities

	Adenylate cyclase activity (pmol cAMP/min per mg protein)	Guanylate cyclase activity (pmol cGMP/min per mg protein)	
	Homogenate	Cytosol	Homogenate
Resting	7.1 ± 2.1	16.0 ± 1.2	17.5 ± 0.9
CCT-6 60 μM	5.0 ± 0.6	15.9 ± 1.7	13.2 ± 2.0
140 μM	4.9 ± 0.5	14.5 ± 0.8	16.5 ± 3.1
Forskolin 10 μM	29.0 ± 3.5[b]	–	–
Sodium nitroprusside 10 μM	–	27.9 ± 1.9[b]	28.8 ± 4.4[a]
100 μM	–	41.4 ± 5.5[b]	36.4 ± 7.1[a]
YC-1 100 μM	–	42.4 ± 7.6[b]	28.2 ± 0.6[b]

Adenylate cyclase and guanylate cyclase activities were determined as outlined in Section 2. Values are presented as means ± S.E.M. ($n = 6$).
[a] $P < 0.01$.
[b] $P < 0.001$ as compared with the respective resting values.

ositol monophosphate formation in washed rabbit platelets by 3.0 ± 0.2, 2.1 ± 0.1, 1.5 ± 0.1, 1.5 ± 0.1 and 1.4 ± 0.1 folds, respectively. CCT-62 completely inhibited the [^{3}H]inositol monophosphate formation caused by these five stimulators. Prostaglandin E_1 (1 μM) and 3-isobutyl-1-methylxanthine (300 μM) also completely inhibited [^{3}H]inositol monophosphate formation caused by the above agents (data not shown).

3.4. Effects of CCT-62 on cyclic AMP and cyclic GMP levels in washed rabbit platelets

The cyclic AMP and cyclic GMP levels of resting platelets were 2.5 ± 0.2 and 2.0 ± 0.3 pmol/10^9 platelets, respectively. CCT-62 (1.5 to 60 μM) increased both cyclic nucleotide levels in a concentration- and time-dependent manner (Fig. 4). Clearly, the increase in cyclic AMP levels was more pronounced than the increase in cyclic GMP levels. CCT-62 (60 μM) increased the cyclic AMP and cyclic GMP levels to 21.7 ± 7.0 and 4.7 ± 0.5 pmol/10^9 platelets, respectively. Prostaglandin E_1 (1 μM) and 3-isobutyl-1-methylxanthine (300 μM) also increased the cyclic AMP levels (31.3 ± 9.0 and 10.7 ± 2.1 pmol/10^9 platelets, respectively; $n = 6$) in rabbit platelets. The increase elicited by CCT-62 (60 μM) was not potentiated by 3-isobutyl-1-methylxanthine. However, CCT-62 and 3-isobutyl-1-methylxanthine markedly potentiated the formation of cyclic AMP caused by prostaglandin E_1 (799.0 ± 139.8 and 1623.2 ± 327.3 pmol/10^9 platelets, respectively; $n = 6$).

3.5. Effects of CCT-62 on adenylate and guanylate cyclase activities

The basal activity of guanylate cyclase in cytosol and homogenate fractions of platelet lysate was 16.5 ± 1.2 and 17.5 ± 0.9 pmol cyclic GMP/min per mg protein, respectively. Sodium nitroprusside, a guanylate cyclase activator, increased the activity of guanylate cyclase in a concentration-dependent manner in both fractions (Table 2). 3′-(5′-

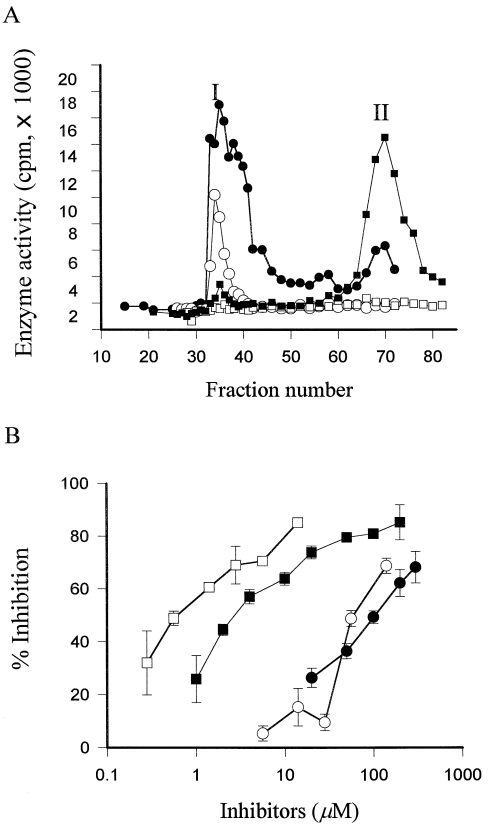

Fig. 5. Effect of CCT-62 on platelet phosphodiesterase isozymes. (A) Profile of platelet phosphodiesterase activity in the chromatographic fractions. An extract of platelet homogenate was prepared and applied to a column of DEAE-Sepharose pre-equilibrated with buffer as described in Section 2. The extract was eluted with a sodium acetate gradient (50 to 1000 mM), and fractions of 7.5 ml each were collected. Each fraction was assayed for phosphodiesterase activity by addition of cAMP (0.25 μM, ■) or cGMP (0.25 μM, ●) in the presence of EGTA (1 mM). Phosphodiesterase activity was also assayed in the presence of CCT-62 at 60 μM (○ for cGMP phosphodiesterase, □ for cAMP phosphodiesterase). (B) Effect of various concentrations of CCT-62 (0.6–60 μM) and 3-isobutyl-1-methylxanthine (IBMX, 10–300 μM) affected the phosphodiesterase activity of peak I and peak II. □, CCT-62 on peak II; ■, IBMX on peak II. ○, CCT-62 on peak I; ●, IBMX on peak I.

Hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1, 100 μM), a compound which stimulates guanylate cyclase activity via a NO-independent pathway (Wu et al., 1995), also increased the enzyme activity. However, CCT-62 even at the concentration of 140 μM did not affect guanylate cyclase activity (Table 2).

In the adenylate cyclase activity assay, forskolin (10 μM) increased the enzyme activity from 7.1 ± 2.1 to 29.0 ± 3.5 pmol cyclic AMP/min per mg protein. Again, CCT-62 (140 μM) did not affect the activity (Table 2).

3.6. Effects of CCT-62 on phosphodiesterase activity

By using DEAE-Sepharose column chromatography, we separated two isozymes of phosphodiesterase from rabbit platelets. As shown in Fig. 5A, peak I was a phosphodiesterase specific for cyclic GMP whereas peak II was a phosphodiesterase isozyme which hydrolyzes cyclic AMP. CCT-62 concentration dependently inhibited both enzymes, with IC_{50} values of 75.0 ± 2.3 and 0.6 ± 0.1 μM, respectively. 3-Isobutyl-1-methylxanthine also inhibited both enzymes but was less potent. The IC50 values of 3-isobutyl-1-methylxanthine were 122.3 ± 21.8 and 3.3 ± 0.4 μM, respectively (Fig. 5B).

4. Discussion

In this study, we found that CCT-62 inhibited aggregation, ATP release, phosphoinositide breakdown and intracellular calcium increase which were caused by the presence of thrombin, platelet-activating factor, collagen, arachidonic acid and 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$ in washed rabbit platelets. It also disaggregated platelets that had been aggregated by these agents. All of these effects of CCT-62 were paralleled by increased cyclic AMP levels and inhibition of platelet phosphodiesterase.

An elevation of cyclic nucleotide levels, either by activation of adenylate and guanylate cyclase, or by inhibition of phosphodiesterase, is the most potent inhibitory pathway to regulate platelet activation (Rhee et al., 1993; Morgan and Newby, 1989; Geiger et al., 1992). Both cyclic nucleotides are involved in platelet responses, including aggregation, ATP release, protein phosphorylation, intracellular calcium mobilization and glycoprotein IIb/IIIa activation (Herstrup et al., 1994). Most of these effects are accomplished by cyclic AMP/cyclic AMP-dependent protein kinase or cyclic GMP/cyclic GMP-dependent protein kinase, respectively. Both cyclic nucleotide protein kinases phosphorylate their substrates, including myosin light chain kinase, calcium pump (Nishizuka, 1984), and exert their antiplatelet effects. In addition, it is reported that cyclic nucleotides disaggregate platelets (Gryglewski et al., 1978). Since CCT-62 also disaggregated platelets, this would imply that CCT-62 might increase cyclic nucleotides. Actually, CCT-62 increased both cyclic nucleotides in a concentration- and time-dependent manner that was parallel to its antiplatelet effect. The antiaggregatory effects of CCT-62 and prostaglandin E_1 were markedly reversed by H89, a cyclic AMP/cyclic AMP-dependent protein kinase inhibitor. This result indicates that cyclic AMP is a dominant factor in the mediation of the antiaggregatory effect of CCT-62. In the presence of H89, ODQ did not further reverse the antiaggregatory effect of CCT-62. This implies that the increase in cyclic GMP caused by CCT-62 adds little to its antiaggregatory effect. CCT-62 inhibited the phosphoinositol breakdown and intracellular calcium mobilization caused by platelet stimulators in rabbit platelets. These data also support those of a previous study showing that cyclic nucleotides affect the activity of phospholipase C and intracellular calcium concentrations (Benjamin et al., 1992). CCT-62 inhibited platelet aggregation caused by arachidonic acid; however, CCT-62 did not affect the formation of thromboxane B_2 caused by external applied arachidonic acid (data not shown), indicating that CCT-62 does not affect the activity of cyclooxygenase. These data support the findings of a previous study that cyclic nucleotides do not affect the activity of cyclooxygenase (Minkes et al., 1977).

CCT-62 specifically inhibited cyclic nucleotide phosphodiesterase in a concentration-dependent manner in lysates from washed rabbit platelets. This inhibitory effect may be the main explanation of how CCT-62 increased both cyclic nucleotides because it neither activated adenylate cyclase nor activated soluble or particulate guanylate cyclase activity in lysates from washed rabbit platelets. Furthermore, CCT-62 potentiated both the cyclic AMP-elevating and antiaggregatory effects caused by prostaglandin E_1 but not those caused by 3-isobutyl-1-methylxanthine. These results confirm that inhibition of the cyclic nucleotide phosphodiesterase by CCT-62 contributed to its antiaggregatory effect.

Two phosphodiesterase isozymes were separated from rabbit platelets by DEAE-Sepharose chromatography. One was cyclic GMP-specific phosphodiesterase (peak I) and the other was cyclic AMP-specific phosphodiesterase (peak II). CCT-62 inhibited both phosphodiesterases in a concentration-dependent manner. CCT-62 had a stronger inhibitory effect on cyclic AMP-specific phosphodiesterase (peak II) than on cyclic GMP-specific phosphodiesterase (peak I). Actually, CCT-62 was approximately 125 times more potent as an inhibitor of rabbit platelet cyclic AMP-specific phosphodiesterase (peak II) than as an inhibitor of cyclic GMP phosphodiesterase (peak I). This demonstrates that CCT-62 induced much higher cyclic AMP levels than cyclic GMP levels in rabbit platelets. It also explains why cyclic GMP did not play a role in the antiaggregatory effect of CCT-62. Thus, CCT-62 is a more specific inhibitor of cyclic AMP phosphodiesterase in rabbit platelets.

5. Conclusion

The recent discovery of the diversity of phosphodiesterase isoforms and subsequent realization that phosphodiesterase isoforms can be regulated by other agents has led to an extensive reevaluation of phosphodiesterases as possible therapeutic targets. Clearly, a detailed analysis of the cell type-specific expression of the phosphodiesterase isoforms and possible functional consequences associated with their different expression are the focus of current efforts by many investigators. It has been demonstrated that the cyclic AMP-specific phosphodiesterase in rabbit platelets belongs to type III phosphodiesterase (Maurice and Haslam, 1990). Therefore, we suggest that peak II in our study is the type III phosphodiesterase. For this reason, CCT-62 might be an inhibitor of type III phosphodiesterase. At present, seven phosphodiesterase isozymes have been identified in mammalian tissue. More experiments are needed to verify whether CCT-62 affects other phosphodiesterases.

CCT-62 increases cyclic nucleotides due to its inhibitory effect on phosphodiesterase. Obviously, the increased cyclic AMP contributes to its antiplatelet effect. Whether CCT-62 also possesses an antithrombotic effect in vivo needs to be investigated in other studies.

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