

Effect of PP1D-1, a Synthetic Antiplatelet Compound, on Rabbit Platelets

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ABSTRACT—The antiplatelet mechanism of a synthetic compound, 2-chloro-3-methoxycarbonylpropionamido-1,4-naphthoquinone (PP1D-1), was studied by employing washed rabbit platelets *in vitro*. PP1D-1 concentration-dependently inhibited thrombin (0.1 U/ml)-, platelet-activating factor (2 ng/ml)-, collagen (10 μ g/ml)-, arachidonic acid (100 μ M)- and U46619 (1 μ M)-induced aggregation and ATP release in washed rabbit platelets. The IC_{50} values of PP1D-1 for aggregation induced by the above inducers are 17.9 ± 1.7 , 9.8 ± 1.1 , 3.9 ± 0.4 , 1.8 ± 0.3 and 1.7 ± 0.3 μ M, respectively. PP1D-1 did not affect platelet thromboxane B₂ or prostaglandin D₂ formation induced by arachidonic acid, indicating that it did not affect cyclooxygenase and thromboxane synthase activities. PP1D-1 significantly inhibited the formation of inositol 1,4,5-trisphosphate caused by these five platelet stimulators. Moreover, PP1D-1 inhibited the increase in intracellular calcium concentration induced by these agents. On the contrary, PP1D-1 did not inhibit thapsigargin-elevated intracellular calcium concentration in indomethacin-pretreated platelets, indicating it did not influence the effect of thapsigargin. According to these data, PP1D-1 exerts antiplatelet effects mainly by inhibiting phosphoinositide turnover.

Keywords: 2-Chloro-3-methoxycarbonylpropionamido-1,4-naphthoquinone (PP1D-1), Platelet (rabbit), Inositol 1,4,5-trisphosphate (IP₃), Intracellular calcium concentration, Thapsigargin

It is generally accepted that platelets play an important role in the progress and development of thrombotic disorders (1), especially cerebral vascular diseases, e.g., transient ischemic attack (2), ischemic heart diseases such as myocardial infarction (3) and peripheral vascular diseases (4). Consequently, the inhibition of platelet functions by drugs is thought to be a useful method for the prophylaxis and treatment of these diseases (5). For this reason, many antiplatelet drugs (e.g., aspirin, sulfipyrazone, dipyridamole and ticlopidine) have been used clinically. Among these antiplatelet agents, aspirin is the first drug of choice for long term oral treatment of these diseases (6). Aspirin irreversibly inhibits the cyclooxygenase activity, thereby suppressing thromboxane A₂ (TXA₂) synthesis. Aspirin merely partly inhibits platelet release reaction and aggregation. Conversely, aspirin does not inhibit platelet adhesion or spreading. Aspirin also fails to entirely prevent coronary artery reocclusion following thrombotic therapy, which suggests that more specific and potent inhibitors of platelet function may be of clinical use (7). Therefore, searching for agents that interfere

with the platelet activation mechanisms, including G-protein, phospholipase C, protein kinase C or calcium mobilization by platelet agonists is very important.

In our laboratory, we have been studied and developed antiplatelet drugs for years. The major source of compounds have come from plant components (8) and also some chemical synthetic compounds. PP1D-1, a synthetic compound (2-chloro-3-methoxycarbonylpropionamide-1,4-naphthoquinone), was prepared in a study of the cytotoxicity of 1,2-disubstitutednaphth[2,3-*d*]imidazole-4,9-diones and related compounds (9), and we found that it could prevent mouse edema by protecting the microvasculature from the challenge of inflammatory mediators (10). Recently, PP1D-1 was found to have a potent anti-aggregatory activity. The purpose of the present study is to assess the mechanism of its inhibitory activity on platelet aggregation.

MATERIALS AND METHODS

Preparation of washed rabbit platelets

Washed platelets were prepared from blood withdrawn with a siliconized syringe from the marginal vein of New

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Zealand rabbits. Each rabbit was phlebotomized every month, and a 20-ml blood sample was collected each time. Blood from rabbits was mixed with EDTA (final concentration of 6 mM) and centrifuged at $90\times g$ at room temperature (25°C); platelet-rich plasma (PRP) was obtained from the upper portion. The platelet suspension was obtained from EDTA-anticoagulated PRP according to the washing procedure described previously (11). Platelet number was counted by a cell counter (Hemalaser 2; Sebia, Moulineaux, France) and adjusted to 3.0×10^8 cells/ml. The platelet pellets were suspended in Tyrode's solution containing calcium (1 mM) and bovine serum albumin (BSA) (0.35%). All glassware was siliconized.

Platelet aggregation and ATP release

Platelet aggregation was measured by the turbidimetric method (12). The absorbance of the platelet suspension was taken as 0% aggregation and that of Tyrode's solution as 100% aggregation. ATP released from platelets was measured by bioluminescence (13). Both aggregation and ATP release were measured by a Lumi-aggregometer (Model 1020; Payton, Scarborough, Canada) connected to two dual-channel recorders.

Thromboxane B_2 (TXB₂) and prostaglandin D_2 (PGD₂) assay

After incubation of washed platelets with the inducer for 6 min, EDTA (2 mM) and indomethacin (50 μ M) were added to halt the formation of TXB₂ and PGD₂. After 2 min of centrifugation in an Eppendorf microcentrifuge (Model 5414C; Eppendorf, Hamburg, Germany), the supernatant was obtained, and the TXB₂ and PGD₂ were assayed by EIA (enzyme immunoassay) kits.

cAMP and cGMP assay

The platelet suspension (1×10^9 cells/ml) was warmed at 37°C for 1 min in a Lumi-aggregometer. The drug, prostaglandin E₁ (PGE₁, 1 μ M), sodium nitroprusside (SNP, 10 μ M), 3-isobutyl-1-methyl-xanthine (IBMX, 300 μ M) or PP1D-1, was added and incubated for 45 sec. The reaction was stopped by adding EDTA (10 mM) followed by immediately heating at 100°C for 2 min. On cooling to 4°C, the precipitated protein was isolated by centrifugation in an Eppendorf microcentrifuge for 5 min. The concentrations of cAMP and cGMP in the supernatant were measured by EIA kits.

Measurement of intracellular calcium concentration in platelets

The intracellular calcium concentration was measured by the method of Pollock and Rink (14). Platelets (3×10^8 cells/ml) were incubated with fura-2-acetoxymethyl ester (fura-2/AM, 5 μ M) at 37°C for 40 min and then

centrifuged at $500\times g$. The pellet was collected and then washed with Tyrode's solution containing EDTA (1 mM). After centrifugation, platelets were resuspended in the Tyrode's solution containing CaCl₂ (1 mM). Fluorescence (excitation wavelength 339 nm, emission wavelength 500 nm) was measured with a fluorescence spectrophotometer (Model F4000; Hitachi, Tokyo). At the end of the experiment, the cells were treated with 0.1% Triton X-100 followed by the addition of EGTA (10 mM) to obtain the maximal and minimal fluorescence, respectively. The intracellular calcium concentration was calculated as described for fura-2 using the Ca²⁺-dye dissociation constant of 224 nM (15).

Assay of inositol 1,4,5-trisphosphate (IP₃) mass content

Platelets (10^9 cells/ml, 500 μ l) were prepared as described above, and then incubated with dimethylsulfoxide (DMSO, 0.5%) and various concentrations of PP1D-1 at 37°C for 3 min and stimulated with the aggregation inducers. The reactions were terminated by adding 100 μ l of ice-cold perchloric acid (20%) followed by 20 min of incubation in an ice bath. The mixture was centrifuged at $2000\times g$ for 15 min at 4°C, and the supernatant was recovered and its pH was adjusted to 7.5 with 10 N KOH solution. KClO₄ was precipitated for 30 min at 4°C and then sedimented by centrifugation at $2000\times g$ for 15 min at 4°C. The amount of IP₃ in the subsequent supernatant was determined by radioimmunoassay (RIA) kits.

Drugs

PP1D-1 (Fig. 1) was prepared as described previously (9) and was dissolved in DMSO. Collagen (type I, bovine Achilles tendon) was homogenized in 25 mM acetic acid and stored at -20°C at the concentration of 1 mg/ml. Platelet-activating factor (PAF) was dissolved in chloroform and diluted with 0.1% BSA/saline solution immediately before use. Arachidonic acid (AA), EDTA (disodium salt), luciferin-luciferase, DMSO, BSA, prostaglandin E₁ (PGE₁), SNP, IBMX, indomethacin, 9,11-dideoxy-9 α ,11 α -methanoepoxy PGF_{2 α} (U46619, a TXA₂ analogue), thapsigargin and trichloroacetic acid were purchased from Sigma Chemical Co. (St. Louis,

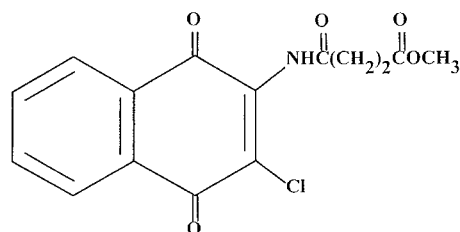


Fig. 1. Chemical structure of PP1D-1.

MO, USA). Thrombin (bovine) was purchased from Parke Davis & Co. (Detroit, MI, USA) and dissolved in 50% glycerol to give a stock solution of 100 NIH units/ml. TXB₂, PGD₂, cAMP and cGMP EIA kits were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). The D-myo-IP₃ [³H] assay kit was obtained from Amersham (Buckinghamshire, UK).

RESULTS

The effect of PP1D-1 on platelet aggregation and ATP release

Thrombin (0.1 U/ml), PAF (2 ng/ml), collagen (10 µg/ml), AA (100 µM) and U46619 (1 µM) all caused approximately 80–90% aggregation in washed rabbit platelets. These aggregations were concentration-dependently inhibited by PP1D-1 with IC₅₀ values of 17.9±1.7, 9.8±1.1, 3.9±0.4, 1.8±0.3 and 1.7±0.3 µM, respectively (Fig. 2). In addition to the inhibition of platelet aggregation, PP1D-1 also inhibited ATP release from the platelets activated by these five stimulators in concentration-dependent manners. The inhibitory effect on ATP release paralleled the inhibitory effect on platelet aggregation (Fig. 3, for thrombin).

Effect of PP1D-1 on TXB₂ and PGD₂ formation

The TXB₂ level was extremely low in resting platelets (0.2±0.1 ng/ml). After challenging with AA (100 µM),

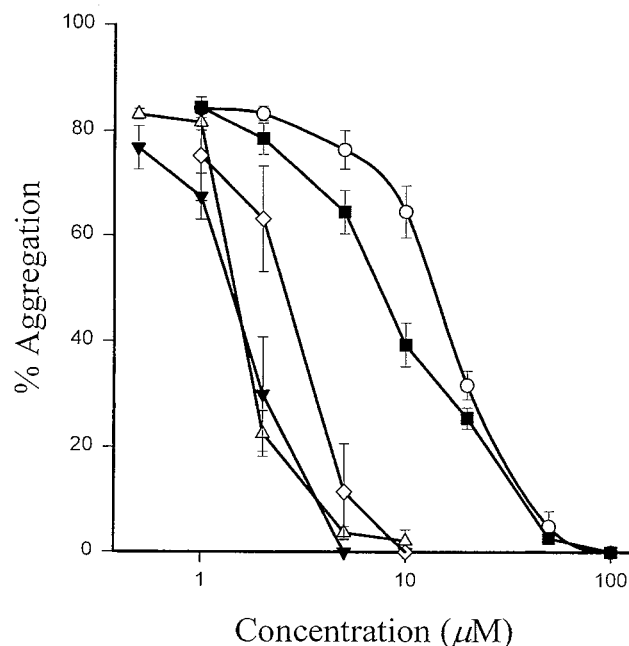


Fig. 2. Concentration-dependent inhibition by PP1D-1 of the platelet aggregation induced by thrombin (0.1 U/ml, ○), PAF (2 ng/ml, ■), collagen (10 µg/ml, ◇), U46619 (1 µM, ▼) and arachidonic acid (100 µM, △). Washed rabbit platelets were incubated with DMSO (0.5%) or various concentrations of PP1D-1 at 37°C for 3 min, and then the inducer was added to trigger the aggregation. Values are presented as means±S.E.M. (n=6).

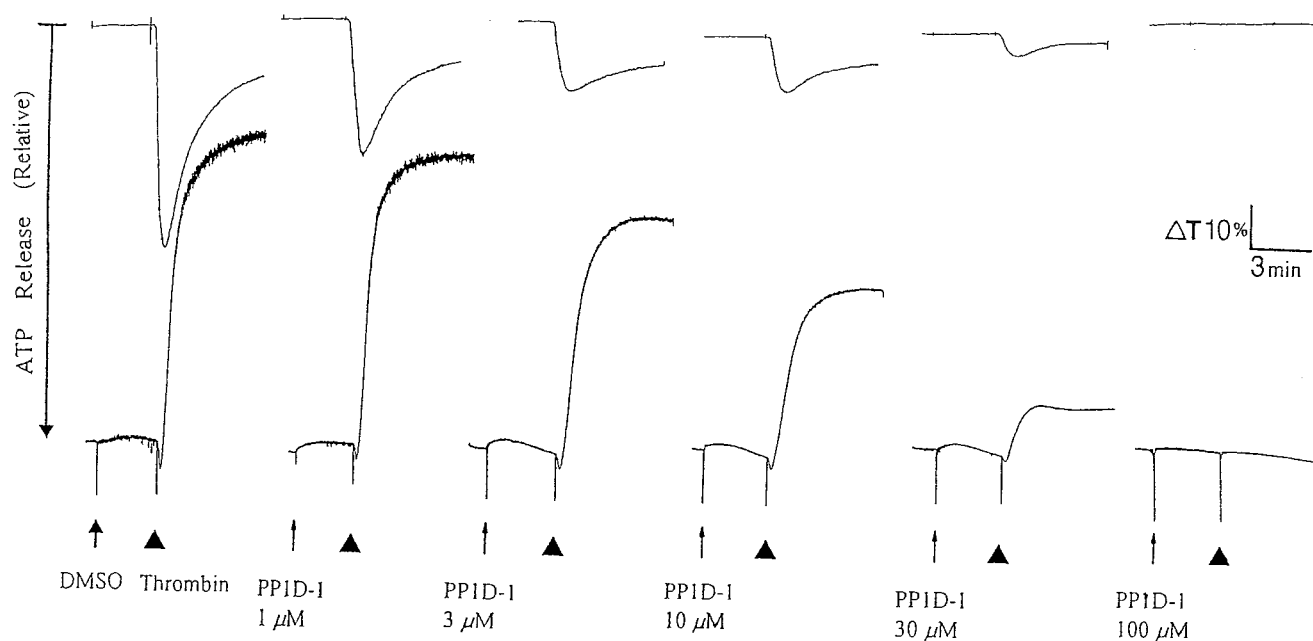


Fig. 3. PP1D-1 inhibition of the platelet aggregation and ATP release induced by thrombin. Washed rabbit platelets were preincubated with various concentrations of PP1D-1 or DMSO (0.5%) at 37°C for 3 min, and then thrombin (0.1 U/ml) was added to trigger the aggregation (upward tracings) and ATP release (downward tracings).

Table 1. Effects of PP1D-1 on thromboxane B₂ formation of washed rabbit platelets caused by arachidonic acid, collagen, U46619, thrombin and PAF

	Thromboxane B ₂ formation (ng/ml)				
	AA	Collagen	U46619	Thrombin	PAF
Control	430.7±96.5	232.0±2.0	3.8±2.0	18.1±2.3	160.3±28.7
PP1D-1 50 µM	—	—	—	6.2±0.8***	17.0±4.3***
20 µM	—	—	—	20±2.0	53.6±11.1**
10 µM	393.2±128.3	4.5±1.3***	6.5±1.4	26.6±5.2	90.5±19.3*
Imidazole 1 mM	52.5±6.1***	—	—	—	—
Indomethacin 0.5 µM	17.9±3.3***	—	—	—	—

The thromboxane B₂ level of resting platelets is 0.2±0.1 ng/ml. Values are presented as means±S.E.M. (n=8).

*P<0.05, **P<0.01, ***P<0.001, as compared with the respective control. Final concentration: arachidonic acid (AA), 100 µM; collagen, 10 µg/ml; U46619, 1 µM; thrombin, 0.1 U/ml; PAF, 2 ng/ml.

collagen (10 µg/ml), thrombin (0.1 U/ml) and PAF (2 ng/ml), TXB₂ levels were increased to 430.7±96.5, 232.0±2.0, 18.1±2.3 and 160.3±28.7 ng/ml, respectively (Table 1). PP1D-1 (10 µM) did not inhibit AA-induced TXB₂ formation (Table 1). In the contrast, PP1D-1 (10 or 50 µM) markedly inhibited collagen-, thrombin- and PAF-induced TXB₂ formation (4.5±1.3, 6.2±0.8, 17.0±4.3 ng/ml). AA also induced the formation of PGD₂ in washed rabbit platelets (Table 2). PP1D-1 did not affect the formation of AA-induced PGD₂. Imidazole (1 mM), an inhibitor of thromboxane synthase, inhibited the formation of TXB₂ caused by AA. Complementary to inhibition of the formation of TXB₂, imidazole also enhanced the PGD₂ formation caused by AA. Indomethacin, an inhibitor of cyclooxygenase, suppressed the formation of AA-induced TXB₂ and PGD₂ significantly (Table 2).

Effect of PP1D-1 on cAMP and cGMP formation

There were 1.8±0.5 pmol/ml cAMP and 1.9±0.2 pmol/ml cGMP formed in resting platelets, respectively. PGE₁ (1 µM) and IBMX (300 µM) both significantly increased cAMP formation in washed rabbit platelets

Table 2. Effects of PP1D-1 on prostaglandin D₂ formation of washed rabbit platelets caused by arachidonic acid (100 µM)

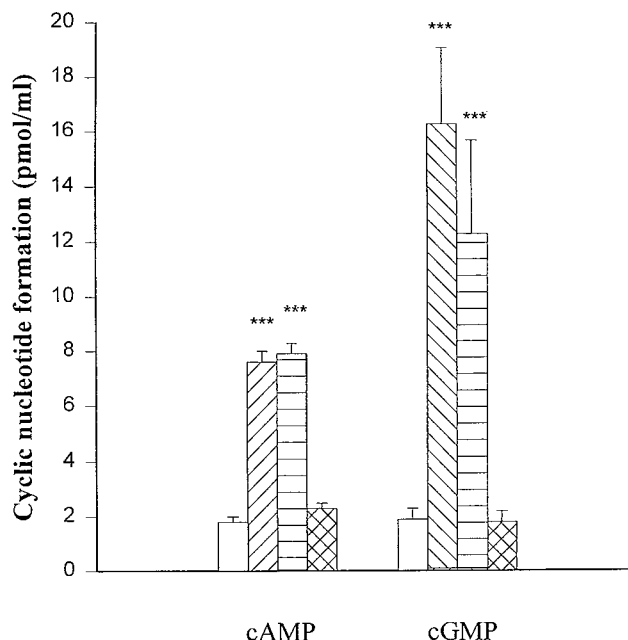
Treatment	Prostaglandin D ₂ (ng/ml)
Control	4.7±1.3
PP1D-1 (10 µM)	6.5±1.7
Indomethacin (0.5 µM)	0.5±0.1*
Imidazole (1 µM)	447.3±102.1***

The prostaglandin D₂ level of resting platelets is 0.1±0.0 ng/ml. Values are presented as means±S.E.M. (n=6). *P<0.05, ***P<0.001, as compared with the control (100 µM arachidonic acid).

(7.8±0.4 and 7.9±0.5 pmol/ml, respectively, in Fig. 4). cGMP levels were also increased in washed rabbit platelets stimulated with SNP (10 µM) and IBMX (300 µM) (16.2±3.2 and 12.1±3.5 pmol/ml, respectively, Fig. 4). However, PP1D-1 did not increase cAMP or cGMP levels in platelets.

Effect of PP1D-1 on the intracellular calcium concentration in washed rabbit platelets

In the presence of thrombin (0.1 U/ml), PAF (2 ng/ml), AA (100 µM), collagen (10 µg/ml) and U46619

**Fig. 4.** Effects of prostaglandin E₁ (1 µM, ▨), sodium nitroprusside (10 µM, ▩), IBMX (300 µM, □) and PP1D-1 (100 µM, ▩) on cAMP and cGMP level of washed rabbit platelets. Data are presented as means±S.E.M. (n=6). ***P<0.001, as compared with resting (DMSO 0.5%, □).

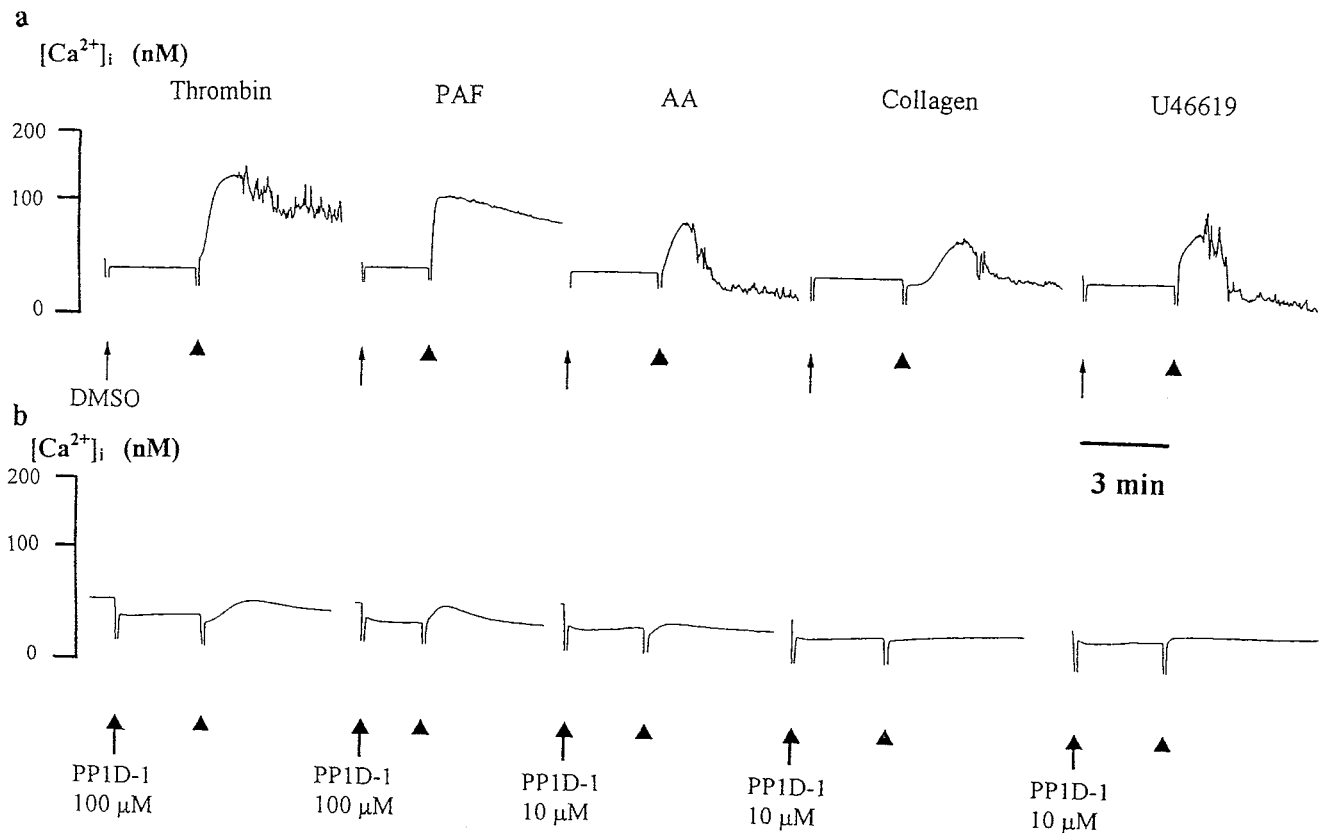


Fig. 5. Effects of PP1D-1 on the increase of intracellular calcium concentration ($[Ca^{2+}]_i$) of platelets caused by some aggregation inducers. Fura-2-loaded platelets were preincubated with DMSO (0.5%, panel a) or PP1D-1 (panel b) at 37°C for 3 min, and then thrombin (0.1 U/ml), PAF (2 ng/ml), arachidonic acid (AA, 100 μ M), collagen (10 μ g/ml) or U46619 (1 μ M) was added.

(1 μ M), platelet intracellular calcium concentration was increased (Fig. 5). The increment was only short-lived and decreased towards the resting level within a few minutes because the aggregation interfered with the fluorescence signal, which is a limitation of the technique. As shown in Fig. 5, PP1D-1 (100 μ M for thrombin and PAF; 10 μ M for AA, collagen and U46619) markedly inhibited the intracellular calcium concentration caused by these inducers.

As shown in Table 3, thapsigargin (1 μ M) significantly increased the intracellular calcium concentration to 198.9 ± 16.9 nM ($n=5$). Pretreatment of platelets with indomethacin (3 μ M) plus thapsigargin increased intracellular calcium concentration to 135.9 ± 17.6 nM ($P < 0.05$ as compared with thapsigargin alone, $n=5$). In the presence of PP1D-1 (100 μ M), thapsigargin increased intracellular calcium concentration to 126.1 ± 12.7 nM (no statistical difference with indomethacin-treated platelets, $n=5$), but PP1D-1 had no inhibitory effect on thapsigargin/indomethacin-treated platelets (thapsigargin increased it to 114.5 ± 12.4 nM, no statistical difference compared to that in indomethacin-treated platelets, $n=5$).

Effect of PP1D-1 on IP_3 formation

The amount of IP_3 in resting platelets was 1.1 ± 0.1 pmol of $IP_3/10^9$ cells. Thrombin (0.1 U/ml), PAF (2 ng/ml), AA (100 μ M) and U46619 (1 μ M) induced a rapid and transient formation of IP_3 , which peaked within 5 sec. Collagen (10 μ g/ml) also caused IP_3 formation in platelets with a slower rate, which peaked within 30 sec. The maximum production of IP_3 at 5 sec after thrombin, PAF, AA and U46619 was 4.1 ± 0.3 , 4.4 ± 0.6 ,

Table 3. Effects of PP1D-1 on thapsigargin-induced intracellular calcium concentration of washed rabbit platelets

	Increased intracellular calcium concentration (nM)
Thapsigargin (1 μ M)	198.9 ± 16.5
Indomethacin (3 μ M) + Thapsigargin (1 μ M)	$135.9 \pm 17.6^*$
PP1D-1 (100 μ M) + Thapsigargin (1 μ M)	$126.1 \pm 12.7^*$
Indomethacin (3 μ M) + PP1D-1 (100 μ M) + Thapsigargin (1 μ M)	$114.5 \pm 12.4^*$

Values are expressed as means \pm S.E.M. ($n=5$). * $P < 0.05$, as compared with thapsigargin (1 μ M) alone.

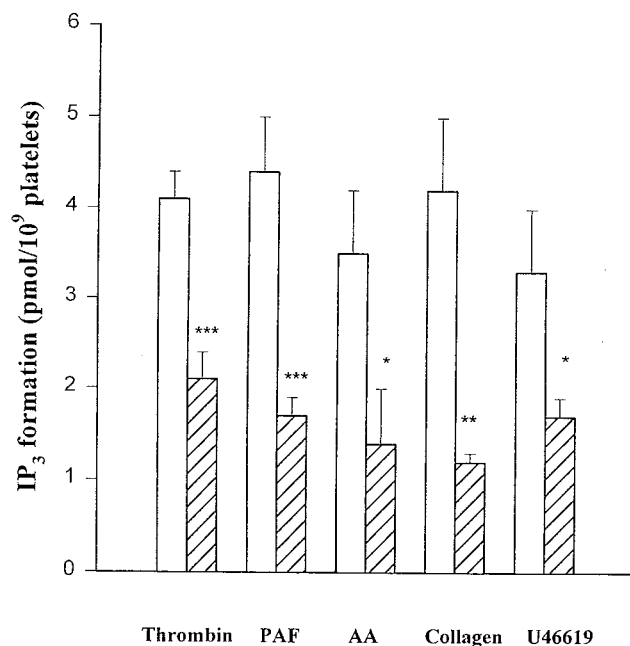


Fig. 6. PP1D-1 inhibition of the IP_3 formation in washed platelets caused by some aggregation inducers. Platelets were preincubated with DMSO (0.5%, □) or various concentrations of PP1D-1 (100 μ M for 0.1 U/ml thrombin and 2 ng/ml PAF; 10 μ M for 100 μ M AA, 10 μ g/ml collagen and 1 μ M U46619; ▨) at 37°C for 3 min, and then stimulators were added for another 5 sec, whereas collagen was added for another 30 sec. Increases in IP_3 are presented as means \pm S.E.M. ($n=5$). * $P<0.05$, ** $P<0.01$, *** $P<0.001$, as compared with the respective control.

3.5 ± 0.7 and 3.3 ± 0.7 pmol/ 10^9 cells, respectively, whereas the maximum production of IP_3 at 30 sec after collagen stimulation was 4.2 ± 0.8 pmol/ 10^9 cells. PP1D-1 (10 or 100 μ M) at the concentrations that markedly inhibited platelet aggregation also suppressed the IP_3 formation caused by these five inducers (Fig. 6).

DISCUSSION

Until now, no drugs could displace the position of aspirin in preventing strokes and ischemic heart diseases. Aspirin remains the gold standard for preventing platelet activation clinically but nevertheless it has some gastrointestinal side effects (16). Therefore, it is important to find drugs that have a similar or more potent effect on preventing platelet activation than aspirin without the gastrointestinal bleeding side effects.

Platelet aggregation is a result of complex signal transduction cascade reactions induced by stimulants. One of them is TXA_2 , which is an important mediator of the release reaction and aggregation in platelets. It takes a responsibility for amplifying platelet activation. Therefore, it is important to evaluate whether PP1D-1 affects

TXA_2 formation in stimulated-platelets. We measured TXB_2 , a stable metabolite of TXA_2 , instead of TXA_2 to evaluate the activity of cyclooxygenase. PP1D-1 at 10 μ M completely inhibited AA-induced aggregation but did not inhibit AA-induced TXB_2 formation in washed rabbit platelets. Furthermore, PP1D-1 did not affect the formation of PGD_2 caused by AA. These data indicated that PP1D-1 did not affect the activities of cyclooxygenase and thromboxane synthase. On the contrary, PP1D-1 inhibited TXB_2 formation caused by the other four stimulators. This result implied that PP1D-1 inhibits one of the platelet activation steps prior to cyclooxygenase. Therefore, we exclude the possibility that PP1D-1 dampens platelet aggregation by inhibiting cyclooxygenase or thromboxane synthase activities.

Platelet responses are executed according to the intracellular calcium concentration, causing shape change, aggregation and degranulation (17). PP1D-1 inhibited the increase in intracellular calcium concentration caused by these five inducers. This effect is parallel to its anti-aggregatory effect. This data suggested that PP1D-1 inhibited platelet aggregation may be accomplished by inhibiting an increase of intracellular calcium concentration. There are several mechanisms that affect intracellular calcium concentration in platelets. One of these mechanisms is increased intracellular cAMP or cGMP levels in platelets by intensifying adenylate or guanylate cyclase activities or by inhibiting phosphodiesterase activity. The levels of intracellular cAMP and cGMP were both significantly increased by PGE_1 , SNP or IBMX. These cyclic nucleotide elevators inhibit the increase in intracellular calcium concentrations caused by these five inducers (data not shown). These data correspond to the previous studies showing that cAMP or cGMP could inhibit intracellular calcium mobilization (18, 19). In the present study, PP1D-1 had no effect on cAMP or cGMP levels in washed rabbit platelets. It also did not potentiate the formation of cAMP or cGMP caused by PGE_1 , SNP or IBMX (data not show). These results indicated that PP1D-1 neither increases adenylate, guanylate cyclase activities nor inhibits phosphodiesterase activity. These data also excluded the possibility that PP1D-1 inhibited elevations of the intracellular calcium concentration by increasing cyclic nucleotides.

Another method to inhibit intracellular calcium mobilization is by disturbing the phosphoinositide breakdown when the platelets are activated by the platelet stimulators. As generally accepted, phosphoinositide breakdown and subsequent activation of calcium mobilization are involved in agonist-induced physiological responses of platelets including ATP release, TXA_2 production and aggregation (20). IP_3 is formed during phosphoinositide breakdown and this is mostly responsi-

ble for agonists-induced increases in intracellular calcium concentration (21–23). PP1D-1 inhibited the formation of IP_3 induced by these platelet activators. This result indicates that PP1D-1 may inhibit platelet activation through inhibiting phosphoinositide breakdown. Furthermore, it is necessary to evaluate whether PP1D-1 inhibited elevated intracellular calcium concentration by an IP_3 -independent pathway. Therefore, we took thapsigargin, a specific inhibitor of the endoplasmic reticulum Ca^{2+} -ATPase (24), which causes inhibition of resequestration of Ca^{2+} that has leaked from the intracellular Ca^{2+} stores, resulting in decreased Ca^{2+} within the stores (25) and increased intracellular Ca^{2+} concentration with an IP_3 -independent manner (26). Thapsigargin treatment of platelets may lead to the formation of TXA_2 and this eicosanoid contributes to part of the thapsigargin-induced increases in intracellular calcium concentrations (27, 28). In this study, the fact that indomethacin partially inhibited thapsigargin-induced increases in intracellular calcium concentrations could prove this point. PP1D-1 slightly affected thapsigargin-induced increase in intracellular calcium concentration, but it did not inhibit the effect of thapsigargin in the presence of indomethacin. This data indicated that PP1D-1 partly inhibited thapsigargin-evoked intracellular calcium concentrations by inhibiting the action of TXA_2 . This result is correlated with the inhibitory effect of PP1D-1 on a TXA_2 analogue, U-46619. These data also demonstrated that PP1D-1 did not affect intracellular calcium concentrations caused by another IP_3 -independent pathway.

In summary, PP1D-1 inhibits platelet activation by suppressing intracellular calcium concentration. This action may be due to its inhibitory effect on IP_3 formation. PP1D-1 may also possess antithrombotic potential in vivo because it prolongs the tail bleeding time in conscious mice (data not shown). Further investigation of its in vivo antithrombotic effect is warranted.

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