Studies on Antiplatelet Effects of OP-41483, a Prostaglandin I\(_2\) Analog, in Experimental Animals

II. Mechanism of Its Antiplatelet Effect

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Abstract—The mechanism for the inhibition of platelet functions by a prostaglandin I\(_2\) analog, OP-41483, was studied with guinea pig platelets. OP-41483, and PGI\(_2\) as well, inhibited aggregation, ADP release and thromboxane formation of platelets with IC\(_{50}\) values of 4.3–5.8 ng/ml and 0.6–0.9 ng/ml, respectively. The ligand binding study using [\(^3\)H]-OP-41483 suggested that OP-41483 bound with different affinities to two classes of binding sites on platelets. The dissociation constant of OP-41483 for the higher affinity site corresponded to the IC\(_{50}\) values of its antiplatelet effect. PGI\(_2\) as well as OP-41483 displaced [\(^3\)H]-OP-41483 previously bound to platelets, thus indicating that both agents exerted their antiplatelet effects by binding to the same site on platelets. OP-41483 and PGI\(_2\) activated adenylate cyclase and raised cyclic AMP levels in platelets. However, their inhibitory effect on platelet aggregation was not fully antagonized by an adenylate cyclase inhibitor, 2',5'-dideoxyadenosine (DDA), at a concentration completely inhibiting the increase of cyclic AMP. Moreover, this DDA-resistant effect of OP-41483 disappeared in the presence of calcium chloride (10\(^{-4}\)-10\(^{-3}\) M). OP-41483 and PGI\(_2\) inhibited thrombin-induced Ca\(^{++}\) influx into platelets. The inhibition of Ca\(^{++}\) influx was not reversed by DDA. Based on these results, we speculate that the inhibitory effects of OP-41483 and PGI\(_2\) on platelet functions are produced through dual mechanisms: one mediated by activation of adenylate cyclase and the other by an inhibition of Ca\(^{++}\) influx; and these two mechanisms seem to be independent of each other.

Our previous study has shown that OP-41483 \([E(E) - 6,9\alpha - \text{methylene} - 15 - \text{cyclopentyl} - 16,17,18,19,20 - \text{pentanor} - \text{PGI}_2]\), a stable analog of prostaglandin I\(_2\) (PGI\(_2\)), exhibits antiplatelet and antithrombotic effects in experimental animals by the oral, as well as parenteral, route of administration (1).

PGI\(_2\) is believed to inhibit platelet functions such as platelet aggregation, adhesiveness, ADP release and thromboxane formation by activating adenylate cyclase and increasing cyclic AMP levels in platelets. The increase of cyclic AMP in platelets is known to stimulate calcium uptake into calcium storage vesicles (2), causing a reduction in cytosolic free Ca\(^{++}\) ion (3). Besides the involvement of intracellular Ca\(^{++}\), there may be some contribution of extracellular Ca\(^{++}\) to the platelet functions and hence the antiplatelet effects of PGI\(_2\). There has been no previous report, however, on the effect of PGI\(_2\) on transmembrane calcium influx into platelets.

This led us to examine the effects of PGI\(_2\) and OP-41483 on cyclic AMP metabolism and Ca\(^{++}\) influx in platelets. The present paper describes the effects of OP-41483 on various functions of guinea pig platelets and refers to its possible mechanisms of action.

Materials and Methods

Preparation of platelet fractions: Male guinea pigs of the Hartley strain weighing...
350–800 g were anesthetized with pentobarbital sodium, and blood was taken by cardiac puncture into a plastic syringe containing 1 volume of 3.8% trisodium citrate or 1% EDTA-saline to 9 volumes of blood. Platelet-rich plasma (PRP) was prepared by centrifugation of the blood at 120×g for 10 min. Gel-filtered platelets (GFP) suspended in Tangen's buffer (0.140 M NaCl, 5.4×10^{-3} M KCl, 9.8×10^{-4} M MgCl₂, 5×10^{-5} M CaCl₂, 0.1% glucose, 0.35% bovine serum albumin, pH 7.6) was prepared from PRP as previously described (4). The washed platelet pellet was obtained by centrifugation of the PRP containing 0.1% EDTA at 1780 g for 15 min at 0–5°C. It was suspended in Tris-buffered EDTA-saline solution (0.145 M NaCl, 0.01 M Tris, 0.05 M EDTA, pH 7.4) and washed 3 times by re-centrifugation.

Preparation of platelet microsome fraction (crude membrane fraction): The platelet pellet, suspended in 0.44 M sucrose at 16–30×10⁵ platelets/mm³, was sonicated for 20 sec at 20 kHz using an Ultrasonic disrupter™ (Tomy Seiko, Japan) and centrifuged at 12,000×g for 10 min. The supernatant was further centrifuged at 105,000 × g for 60 min. The precipitate was suspended in 0.25 M sucrose at the protein concentration of 1 mg/ml and used as the crude membrane fraction.

Platelet aggregation: Platelet aggregation was tested with citrated PRP (5–7×10⁵/platelet/mm³) or GFP (4–5×10⁵ platelets/mm³) by a turbidimetric method (5) using an aggregometer (Sienco, U.S.A.). PRP or GFP was preincubated with PGI₂ or OP-41483 for 5 min at 37°C, and then platelet aggregation was induced by ADP, collagen, arachidonic acid or thrombin. In some experiments, the adenylate cyclase inhibitor 2',5'-dideoxyadenosine (DDA, 30 μg/ml) was added 5 min prior to the addition of inducers. The extent of aggregation was assessed by the maximum change of light transmittance within 5 min after the addition of inducers.

Thromboxane formation and release of ADP: PRP was preincubated with OP-41483 or PGI₂ at 37°C for 10 min, followed by 5 min-incubation with collagen or arachidonic acid. In the experiment on thromboxane formation, the reaction was terminated with acetone. The mixture was washed with petroleum ether and adjusted to pH 3.4 with formic acid. Thromboxane B₂ in the mixture was extracted by ethylacetate and determined with a TXB₂ RIA kit (New England Nuclear, U.S.A.). In the experiment on ADP release, the reaction was terminated by adding 1% EDTA, and ADP was determined by the luciferase method (6).

Cyclic AMP formation: PRP or GFP was incubated with OP-41483 or PGI₂ for 1 min at 37°C. DDA (30 μg/ml) was added 5 min prior to the addition of prostaglandins. The reaction was stopped with trichloroacetic acid, and cyclic AMP contents were determined by a radioimmunoassay with a Yamasa cyclic AMP assay kit™ (7).

Adenylate cyclase activity: Adenylate cyclase activity was assayed by the method of Solomon (8). The incubation medium (total volume of 30 μl) consisted of 5×10^{-3} M creatine phosphate, 0.025 M Tris acetate (pH 7.6), 5×10^{-3} M magnesium acetate, 5×10^{-4} M ATP, 5×10^{-5} M cyclic AMP, 1×10^{-5} M GTP, 1×10^{-3} M dithiothreitol, 50 U/ml creatinephosphokinase, 0.1 mg/ml bovine serum albumin, approximately 2 μCi of γ-[³²P]-ATP, and OP-41483 or PGI₂. In some experiments, DDA (100 μg/ml) was also added in the medium. The suspension of a platelet crude membrane fraction (10 μl) was added into the mixture and incubated at 30°C for 15 min. The reaction was terminated by adding the mixture (100 μl) of 2% lauryl sulfate, 0.045 M ATP, 1.3×10^{-3} M cyclic AMP and 8-[³H]-cyclic AMP (0.005 μCi), and immediately heated at 100°C for 3 min. The cyclic AMP fraction in the mixture was separated by two step column chromatography with Dowex 50×4 and alumina and then mixed with RIAFLOW™. The radioactivity of [³²P]-cyclic AMP was counted by a liquid scintillation counter (Packard, U.S.A.).

Binding of 12-[³H]-OP-41483 to platelets and its displacement: The binding assay was performed according to the method of Siegel et al. (9). PRP was incubated with 12-[³H]-OP-41483 (1–300 ng/ml, 0.008–2.4 μCi/ml) and U-[¹⁴C]-sucrose (0.008–2.4 μCi/ml) for 5 min at room temperature, followed by 5 min incubation with or without unlabelled OP-41483 (3000 ng/ml). [¹⁴C]-Sucrose and unlabelled OP-41483 were added to assess the extent of extracellular space and non-
specific binding of \([^{3}H]\)-OP-41483, respectively. For the displacement experiment, PRP was incubated with \([^{3}H]\)-OP-41483 (10 ng/ml, 0.08 \(\mu\)Ci/ml) and \([^{14}C]\)-sucrose (0.08 \(\mu\)Ci/ml) for 5 min, followed by 5 min-incubation with PGI\(_{2}\) or OP-41483 at 1–3000 ng/ml. After the incubation, the reaction mixture was centrifuged at 12,000 \(\times g\) for 1 min. The precipitate and an aliquot of the supernatant were dissolved in Soluene\(^{TM}\) and their radioactivities were counted by a liquid scintillation counter.

**Thrombin-stimulated calcium influx into platelets:** Calcium influx was examined by a modified method of Lee et al. (10). GFP suspended in the medium containing 5\(\times\)10\(^{-5}\) M CaCl\(_{2}\) was incubated with or without DDA (30 \(\mu\)g/ml) for 5 min at 37°C, followed by 1 min-incubation with prostaglandins (OP-41483 or PGI\(_{2}\)), \(^{45}\)CaCl\(_{2}\) (0.75 \(\mu\)Ci/ml) and an indicator of extracellular space, \([^{3}H]\)-inulin (0.75 \(\mu\)Ci/ml). Then the mixture was incubated with thrombin for 1–5 min. The reaction was terminated with 5\(\times\)10\(^{-3}\) M EGTA and immediately centrifuged at 12,000 \(\times g\) for 1 min. The radioactivities of the precipitate and an aliquot of the supernatant were counted as described in the binding assay.

**Protein determination:** Protein concentration was determined by the method of Lowry et al. (11).

**Test compounds:** PGI\(_{2}\) was dissolved in a small amount of ethanol and diluted with 0.1 M glycine-sodium hydroxide buffer (pH 10.0). OP-41483 was dissolved in ethanol and diluted with 0.9% sodium chloride.

**Reagents:** \([^{3}H]\)-OP-41483 (9.7 Ci/mmoles), PGI\(_{2}\) and OP-41483 were synthesized by Ono Pharmaceutical Co., Ltd., Japan. ATP, ADP, collagen (bovine achilles tendon), bovine thrombin, arachidonic acid and bovine serum albumin were obtained from Sigma Chem. Co., U.S.A. Cyclic AMP, 2',5'-dideoxyadenosine, firefly lantern extract and Soluene\(^{TM}\) were from Kohjin Chem. Co., Japan; PL Biochem. Co., U.S.A; Calbiochem. Co., U.S.A; and Packard Co., U.S.A, respectively. \(\gamma\)-[\(^{32}\)P]-ATP was obtained from Radiochem. Centre Amersham U.S.A. Other radiolabeled compounds and RIAFLOW\(^{TM}\) were obtained from New England Nuclear, U.S.A.

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**Results**

**Effects on platelet function:** As shown in Fig. 1A, OP-41483 as well as PGI\(_{2}\) inhibited ADP- and collagen-induced platelet aggregation in PRP in a concentration-dependent...
manner. OP-41483 at similar concentrations inhibited platelet aggregation induced by arachidonic acid. OP-41483 and PGI₂ also inhibited the release of ADP from platelets induced by collagen (Fig. 1B) and thromboxane B₂ formation induced by collagen (Fig. 1C), but both agents failed to inhibit thromboxane B₂ formation induced by arachidonic acid (Fig. 1C). IC₅₀ values for OP-41483 on these platelet functions were 4.3–6.2 ng/ml, and those for PGI₂ were 0.6–0.9 ng/ml (Table 1). OP-41483 is 0.14–0.16 times more potent than PGI₂.

**Binding of [³H]-OP-41483 to platelets:** [³H]-OP-41483 selectively bound to platelets, and Scatchard analysis revealed two classes of binding sites (Fig. 2). Dissociation constants of OP-41483 to high and low affinity binding sites were calculated to be 17.7 nM (6.3 ng/ml) and 158.8 nM (56.9 ng/ml), respectively. The dissociation constant of OP-41483 for the high affinity site corresponded to its IC₅₀ values of the inhibitory effect on platelet functions.

PGI₂ (10–300 ng/ml) displaced previously bound [³H]-OP-41483, and the effect of PGI₂ was 2–3 times more potent than that of unlabelled OP-41483 (Fig. 3).

**Effects on cyclic AMP level and adenylate cyclase activity in platelets:** Addition of OP-41483 and PGI₂ into PRP elevated cyclic AMP levels in platelets in a concentration-dependent manner (Fig. 4). OP-41483 and PGI₂ gave five fold-increase in cyclic AMP at 10.9 and 2.6 ng/ml, respectively. OP-41483 (0.1–100 ng/ml) and PGI₂ (0.03–30 ng/ml) also activated adenylate cyclase in a platelet crude membrane fraction (Fig. 5). The stimu-

| Table 1. In vitro effects of PGI₂ and OP-41483 on platelet functions in guinea pig PRP |
|-----------------------------------------------|-----------------|-----------------|-----------------|
| Platelet function                            | Inducer         | IC₅₀ (ng/ml)    | Relative IC₅₀   |
|                                              |                 | PGI₂            | OP-41483        |
| Platelet aggregation                         | ADP (2×10⁻⁶ M)  | 0.9             | 6.2             | 0.15 |
|                                              | Collagen (1.4 μg/ml) | 0.8            | 5.0             | 0.16 |
|                                              | Arachidonic acid (5×10⁻⁴ M) | —            | 6.2             | —    |
| ADP release                                  | Collagen (1.0 μg/ml) | 0.9            | 5.8             | 0.16 |
| Thromboxane B₂                               | Collagen (1.0 μg/ml) | 0.6            | 4.3             | 0.14 |
|                                              | Arachidonic acid (5×10⁻⁴ M) | 10<           | 30<             | —    |

Figures in the table are the mean of three experiments.

![Fig. 2. Scatchard plot of [³H]-OP-41483 binding to guinea pig platelets. Each point represents the mean of three experiments.](image-url)
lated activity was almost completely inhibited by the adenylate cyclase inhibitor 2',5'-dideoxyadenosine (DDA) at a concentration of 100 μg/ml.

Effects of 2',5'-dideoxyadenosine and calcium chloride on antiplatelet and cyclic AMP elevating effects of OP-41483: OP-41483 (1–30 ng/ml) and PGI₂ (0.3–10 ng/ml) elevated platelet cyclic AMP and inhibited thrombin-induced aggregation in GFP suspension (Figs. 6 and 7). DDA (30 μg/ml) almost completely inhibited the increase of cyclic AMP by OP-41483 and PGI₂. However, their inhibitory effect on thrombin-induced aggregation was only partially reversed by DDA (Figs. 6 and 7). Furthermore, calcium chloride at 10⁻⁴–10⁻³ M diminished the inhibitory effect of OP-41483 (30 ng/ml) on thrombin-induced aggregation in DDA-treated GFP which contained 5×10⁻⁵ M CaCl₂ (Table 2). Calcium chloride (10⁻³ M) itself neither induced platelet aggregation nor enhanced thrombin-induced platelet aggregation.

Effect on calcium influx: Thrombin at 0.5 U/ml stimulated 5–6-fold calcium influx (Ca²⁺ influx) into platelets in GFP (Fig. 8). OP-41483 (1–30 ng/ml) inhibited the thrombin-induced Ca²⁺ influx (Figs. 8 and 9). PGI₂ (0.3–10 ng/ml) also inhibited the Ca²⁺ influx (Fig. 9). DDA (30 μg/ml) did not inhibit the effect of OP-41483 and PGI₂ on the Ca²⁺ influx.

Discussion

As shown in Fig. 1, PGI₂ and its synthetic analog OP-41483 inhibited platelet aggrega-
tion induced by a variety of inducers, the ADP release induced by collagen and thromboxane formation induced by collagen, but they did not inhibit arachidonic acid-induced thromboxane formation. Failure of both compounds to inhibit arachidonic acid-induced thromboxane formation suggests that they inhibit thromboxane formation at the step of arachidonic acid release from phospholipids, directly or indirectly.

The difference in potencies of OP-41483 in inhibiting the above three different platelet

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**Fig. 5.** Activation of adenylate cyclase by OP-41483 (—) and PGI₂ (---) and its inhibition by 2',5'-dideoxyadenosine (100 µg/ml) in a crude membrane fraction of guinea pig platelets. Each point represents the mean±S.E. of three experiments. ●: with dideoxyadenosine, ○: without dideoxyadenosine.

**Fig. 6.** Effect of OP-41483 on platelet cyclic AMP formation (left) and thrombin (0.5 U/ml)-induced aggregation (right) in gel-filtered guinea pig platelets under the presence (○) or absence (●) of 2',5'-dideoxyadenosine (30 µg/ml). Each point represents the mean±S.E. of three experiments.
functions (i.e., aggregation, thromboxane formation and release reaction) was small; and the relative potency of OP-41483 to PGI₂, expressed in terms of IC₅₀ ratio, was in the constant range of 0.14-0.16 in all parameters examined (Table 1). This suggests that OP-41483 may share similar mechanisms with PGI₂ in inhibiting platelet functions. Furthermore, the dissociation constant of OP-41483 for the higher affinity binding site on platelets corresponded to the IC₅₀ values of its inhibitory effects on platelet functions, and the displacement effect of PGI₂ on previously-bound [³H]-OP-41483 was more potent than that of OP-41483 (Figs. 2 and 3). From these results, we speculated that the PGI₂ and OP-41483 initially bound to the high affinity binding site and thereby inhibited a variety of platelet functions through a common intermediary process.

So, we studied intermediary mechanisms of OP-41483 and PGI₂ for antiplatelet effects. PGI₂ activates adenylate cyclase and increases cyclic AMP in platelets (12); and conversely, a variety of compounds that increase cyclic AMP level have been shown to inhibit platelet functions (13). Therefore, the antiplatelet effects of PGI₂ may at least partly
be caused through its effect to activate adenylate cyclase. A close relationship has been shown between the binding capacities of prostaglandins and their stimulating effect on adenylate cyclase in human platelets (14). As shown in Figs. 4 and 5, we showed that OP-41483 and PG12 can activate adenylate cyclase in a platelet crude membrane fraction and increase cyclic AMP in platelets. A parallel relationship was found between their antiplatelet and cyclic AMP-elevating effects. There is no doubt that OP-41483 and PG12 exert their antiplatelet effects, at least in part, by acting on the adenylate cyclase system.

Of particular interest is the fact, however, that an inhibitor of adenylate cyclase, 2'-5'-dideoxyadenosine (DDA), only partially diminished the inhibitory effect of OP-41483 and PG12 on thrombin-induced platelet aggregation, although it completely inhibited the increase of cyclic AMP levels (Figs. 6 and 7). The remaining inhibitory effect of OP-41483 on thrombin-induced aggregation after DDA treatment was antagonized by increasing extracellular calcium concentrations (Table 2), suggesting that the DDA-resistant inhibition is produced by inhibiting calcium mobilization (e.g., inhibition of Ca^2+ influx across

![Fig. 8. Time course of thrombin (0.5 U/ml)-stimulated calcium influx into platelets and its inhibition by OP-41483 (30 ng/ml) in gel-filtered guinea pig platelets containing 5 x 10^-5 M CaCl2. Each point represents the mean±S.E. of three experiments. •: thrombin, △: thrombin+OP-41483, ○: unstimulated control.](image)

![Fig. 9. Inhibitory effect of PG12 (left) and OP-41483 (right) on thrombin (0.5 U/ml)-stimulated calcium influx into platelets under the presence (●) or absence (○) of 2'-5'-dideoxyadenosine (30 μg/ml) in gel-filtered guinea pig platelets. Each point represents the mean±S.E. of three experiments. □: unstimulated control. Gel-filtered platelets were incubated with thrombin for 5 min.](image)
the membrane, inhibition of calcium release from calcium storage vesicles or facilitation of calcium uptake by the storage vesicles). We also found that DDA only partially diminished the inhibitory effect of OP-41483 and PGI₂ on ADP- and collagen-induced aggregation in guinea pig PRP (data are not shown).

Furthermore, OP-41483 and PGI₂ inhibited thrombin-stimulated Ca²⁺ influx into platelets, and the effect was not reversed by DDA (Figs. 8 and 9). From these results, we speculated that the antiplatelet effects of OP-41483 and PGI₂ were, at least partly, mediated by its inhibition of Ca²⁺ influx and that the inhibition is independent of its activation of adenylate cyclase. It is also possible that in platelets, Ca²⁺ influx is regulated by some minimal functional compartments of the adenylate cyclase-cyclic AMP system, which are not inhibited by DDA.

In summary, the inhibitory effect of OP-41483 and PGI₂ on platelet functions may be initiated by their binding to the same specific binding sites on platelets and mediated through dual mechanisms. One mechanism may be an activation of adenylate cyclase, causing an increase of cyclic AMP levels and subsequent facilitation of Ca²⁺ sequestration by calcium storage vesicles (2). The other may be an inhibition of Ca²⁺ influx into platelets. The latter mechanism appears to be independent of the increase in cyclic AMP. PGI₂ and OP-41483 may cause a decrease in cytoplasmic Ca²⁺ concentrations through both mechanisms, thereby inhibiting a variety of platelet functions.

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