Platelet Aggregation and Exposure of Fibrinogen Receptors by Prostaglandin Endoperoxide Analogues

By Thomas A. Morinelli, Stefan Niewiarowski, Elizabeth Kornecki, William R. Figures, Yanina Wachtfolg, and Robert W. Colman

Two stable analogues of prostaglandin endoperoxides—9,11-azo-prostanoid III (9-11-azo PGH2) and 9,11-methanoepoxy PGH2 (U46619 compound)—caused aggregation of washed human platelets and release of α-granule contents. Platelet aggregation induced by these compounds was potentiated by the addition of purified human fibrinogen in a dose-dependent manner. Incubation of prostaglandin endoperoxide analogues with washed platelets in a nonstirred system exposed fibrinogen receptors. Enzymes that remove adenosine diphosphate (ADP) [apyrase and creatine phosphate in the presence of creatine phosphokinase (CP/CPK)], adenosine triphosphate (ATP), a competitive antagonist of ADP, and 5′ parafluorosulfonylbenzoyl adenosine (FSBA), an affinity label for ADP binding sites, all blocked platelet aggregation and binding of 125I-fibrinogen to platelets stimulated by prostaglandin endoperoxide analogues. In contrast, these compounds did not prevent platelet shape change induced by prostaglandin endoperoxide analogues. Apyrase, CP/CPK, ATP, and FSBA reversed platelet aggregation stimulated by ADP or prostaglandin endoperoxide analogues. Carbocyclic thromboxane-A2 (CTA2), a stable analogue of thromboxane-A2, blocked shape change and platelet aggregation and caused disaggregation of platelets stimulated by prostaglandin endoperoxide analogues. However, CTA2 did not inhibit ADP-induced platelet shape change and aggregation, and it did not disaggregate platelets that were previously aggregated by ADP. The mechanism of platelet aggregation induced by analogues of prostaglandin endoperoxides appears to involve (A) initial release of ADP and fibrinogen, (B) ADP-induced exposure of platelet membrane fibrinogen receptors, and (C) fibrinogen binding to the exposed receptors resulting in platelet aggregation. The occupation of prostaglandin endoperoxide receptors is required during all stages of this process.

FOUR ARACHIDONATE metabolites formed by the platelets can induce platelet aggregation: PGG2, PGH2 (prostaglandin endoperoxides), thromboxane-A2, and 15-hydroxyperoxy thromboxane-A2. The mechanism by which these compounds cause platelet aggregation is not well understood. On the basis of experiments with enzymes depleting ADP, Malmsten et al.,3 Claesson and Malmsten,4 and Bressler et al.5 suggested that the aggregation of platelets produced by prostaglandin endoperoxides is mediated by the secretion of platelet ADP. On the other hand, Charo et al.6 found that platelets in platelet-rich plasma (PRP) were aggregated by low concentrations of PGH2 (approximately 0.5 μM) without any release of platelet constituents, suggesting that the aggregation and the secretion induced by PGH2 were separate events and that PGH2 could cause aggregation of platelets directly.

Ingerman et al.7 and Minkes et al.8 have reported that arachidonic acid or PGG2 induced the aggregation of platelets from patients with storage pool disease even in the absence of enzymatically detectable ADP. Similar results were obtained using PRP or gel-filtered platelets.8 On the other hand, Weiss and Lages9 studied 12 patients with storage pool disease and they suggested that granule-derived ADP mediated platelet aggregation by arachidonic acid and PGG2.

Recently, Bennett et al.10 observed that PGH2 exposed fibrinogen receptors on gel-filtered human platelets. Since fibrinogen binding to PGH2-stimulated platelets was only slightly inhibited by ATP, a specific ADP blocker, they concluded that the majority of the platelet fibrinogen binding stimulated by PGH2 was directly due to the interaction of PGH2 with platelets.

Because of the inherent instability of the prostaglandin endoperoxides and thromboxane-A2, it has proved difficult to evaluate their mechanism of action. This problem can be overcome to some extent by the use of their stable analogues.1 The azo analogue of PGH2 (9,11-azo-prostanoid III; 9,11-azo PGH2) has been synthesized by Corey et al.11 This compound caused irreversible platelet aggregation and release of serotonin. It was found to be 8.0 times more potent than PGG2. The methanoepoxy analogue of PGH2 (U46619 compound) was synthesized by Bundy.12 This compound caused platelet aggregation and release of serotonin and ATP from platelet-dense granules.13,14 Carbocyclic thromboxane-A2 (CTA2) is a stable analogue for the platelets can induce platelet aggregation: PGG2, PGH2 (prostaglandin endoperoxides), thromboxane-A2, and 15-hydroxyperoxy thromboxane-A2. The mechanism by which these compounds cause platelet aggregation is not well understood. On the basis of experiments with enzymes depleting ADP, Malmsten et al.,3 Claesson and Malmsten,4 and Bressler et al.5 suggested that the aggregation of platelets produced by prostaglandin endoperoxides is mediated by the secretion of platelet ADP. On the other hand, Charo et al.6 found that platelets in platelet-rich plasma (PRP) were aggregated by low concentrations of PGH2 (approximately 0.5 μM) without any release of platelet constituents, suggesting that the aggregation and the secretion induced by PGH2 were separate events and that PGH2 could cause aggregation of platelets directly.

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of thromboxane-\(\Lambda_2\) that blocks platelet aggregation induced by arachidonic acid and by U46619.\(^{13}\)

The purpose of our study was to investigate the mechanism by which two stable prostaglandin endoperoxide analogues—9,11-azo PGH\(_2\) and the U46619 compound—cause aggregation of human platelets. Our data suggest that this aggregation is mediated through release from platelets of ADP and fibrinogen, followed by the exposure of fibrinogen receptors and subsequent platelet–fibrinogen interactions. Presence of ADP was required for the exposure of platelet fibrinogen receptors. However, the occupation of prostaglandin endoperoxide receptors on the platelet surface was also critical for all stages of this process.

**MATERIALS AND METHODS**

**Reagents**

The stable analogue of the prostaglandin endoperoxide PGH\(_2\), 9,11-azo PGH\(_2\) and carbocyclic thromboxane-\(\Lambda_2\) (CTA\(_2\)) were obtained through the generosity of Dr. K. C. Nicolau (Department of Chemistry, University of Pennsylvania, Philadelphia, Pa.). 15(S)-hydroxy-9\(,\)11-1-azo-9,11-methano-epoxyprostadienoic acid (U46619) was donated by Dr. J. Pike (Upjohn Company, Kalamazoo, Mich.). 9,11-azo PGH\(_2\), U46619, and CTA\(_2\) were dissolved in 10% ethanol at a concentration of 10 mM and further diluted in 0.05 M Tris-HCl, 5\(\times\)10\(^{-3}\) M NaCl buffer, pH 7.4.

The ADP antagonist, 5\(\times\) parafuorosulfonylbenzoyladenosine (FSBA), was a gift of Dr. Roberta F. Colman, (Department of Chemistry, University of Delaware, Newark, Del.). ADP, ATP, creatine phosphate, creatine phosphokinase, bovine serum albumin, and glucose were purchased from Sigma Chemical Co., St. Louis, Mo. Human fibrinogen was obtained from Kabi (Stockholm, Sweden). The silicone oils DC200 and DC500 used in the binding experiments were purchased from William F. Nye, Inc. of New Bedford, Mass.

**Human Washed Platelets**

Human platelets were isolated from whole blood by the method of Mustard et al.\(^{13}\) The apyrase used in the platelet washing and aggregation experiments was prepared by the method of Molnar and Lorand.\(^{15}\)

**Platelet Aggregation**

Washed platelets (5 \(\times\) 10\(^8\)/ml) were suspended in Tyrode's solution containing bovine serum albumin.\(^{16}\) Aggregations were performed in a Payton aggregometer (Scarborough, Ontario). The rate of aggregation was measured from the slope of the initial increase in light transmission, recorded as light transmission units (LTU) per minute. The instrument was calibrated for zero LTU with a platelet suspension and with Tyrode's buffer for 100% transmittance (100 LTU). Aggregation was carried out in the following manner: 400 \(\mu\)l of platelet suspensions were incubated at 37\(^{\circ}\)C with stirring for 1 min, and various concentrations of the aggregation-inducing agent were added. The final volume of the incubation mixture was adjusted to 500 \(\mu\)l with Tyrode's solution prior to the addition of aggregating agents. Aggregation was followed over a 3-min interval. Platelet disaggregating agents were added 1 min after the addition of stimulatory compound.

**Platelet Release Studies**

Following platelet aggregation (at 37\(^{\circ}\)C), these samples were centrifuged at 7500 g for 3 min in an Eppendorf centrifuge (Brinkman Instrument) for the measurement of released low-affinity platelet factor 4/\(\beta\)-thromboglobulin antigen (LA-PF\(_4\)/\(\beta\)TG). LA-PF\(_4\)/\(\beta\)TG antigen release was measured by radioimmunoassay as described by Rucinski et al.\(^{15}\) The level of LA-PF\(_4\)/\(\beta\)TG in samples of 1% Triton-solubilized platelet suspensions were considered as 100% of the total platelet LA-PF\(_4\)/\(\beta\)TG. Effects of prostaglandin endoperoxide analogues on the release of platelet constituents in a nonstirring system were measured at 22\(^{\circ}\)C.

**Iodination of Human Fibrinogen**

Human fibrinogen was labeled by the iodine monochloride method of MacFarlane\(^{16}\) with Na\(^{125}\)I (NEN, Boston, Mass.). The specific activity of the labeled fibrinogen ranged from 2.03 \(\times\) 10\(^6\) cpm/nmole to 6.7 \(\times\) 10\(^5\) cpm/nmole. Iodinated fibrinogen had a clottability ranging between 89% and 95%, and it aggregated ADP-stimulated platelets to the same extent as unlabeled fibrinogen. Aliquots, usually 10–25 \(\mu\)l, of 125I-fibrinogen were stored at –80\(^{\circ}\)C until used. The preparations were stable for 30 days.

**Binding of 125I-Fibrinogen to Human Platelets**

125I-fibrinogen binding to isolated human platelets was performed essentially as previously described.\(^{20,21}\) Platelets (10\(^8\)/ml) (400 \(\mu\)l) were incubated with 25 \(\mu\)l of various concentrations of 125I-fibrinogen and 25 \(\mu\)l of either ADP or prostaglandin endoperoxide analogues. Incubations were carried out for 10 min at 22\(^{\circ}\)C. Nonspecific binding was measured by incubating stimulated platelets for 10 min with 25 \(\mu\)l of unlabeled fibrinogen (20 mg/ml) followed by a 10-min incubation period with labeled fibrinogen. Specific fibrinogen binding was calculated by subtracting values of 125I-fibrinogen bound nonspecifically (i.e., in the presence of an excess of cold fibrinogen) from the values of total 125I-fibrinogen bound.

At the end of the 10-min incubation period, aliquots of the platelet suspension were placed over silicone oil and centrifuged for 3 min at 7500 g. Both the platelet pellet and aliquots of the supernatant were counted in an Intertechnique \(\gamma\)-counter to determine the percent 125I-fibrinogen bound. The values obtained for specific binding of fibrinogen to platelets were plotted according to the method of Scatchard\(^{22}\) and analyzed by least-square fitting of the data.\(^{20,21}\)

**RESULTS**

We first studied the aggregation of washed human platelets by the stable analogues of prostaglandin endoperoxides (Fig. 1A) and by ADP (Fig. 1B). The release of LA-PF\(_4\)/\(\beta\)TG by these agents was also studied. Both compounds, 9,11-azo PGH\(_2\) (0.6 \(\mu\)M) and U46619 (1.2 \(\mu\)M), in the absence of added fibrinogen, caused platelet shape change and aggregation comparable to that brought about by ADP (46 \(\mu\)M) in the presence of fibrinogen (1 mg/ml). Platelet aggregation induced by the endoperoxides was accompanied by appreciable release of the \(\alpha\)-granule constituent, LA-PF\(_4\)/\(\beta\)TG antigen, while ADP (46 \(\mu\)M) induced platelet stimulation occurred with only minimal release. Addition of exogenous fibrinogen was essential for ADP-induced platelet aggregation, but it was not necessary for platelet aggregation by the endoperoxide...
analогues. A release of LA-PF₄/βTG antigen from platelets also occurred in response to U46619 or 9,11-azo PGH₂ in a nonstirred system at 22°C (Table 1). ADP (10 µM) (Table 1) did not cause any significant release in a nonstirred system; 100 µM ADP in the same system, likewise had no effect on the release of LA-PF₄/βTG (not shown).

**Tables 1.** Effect of Stirring of Platelet Suspension on the Release of LA-PF₄/βTG Antigen

<table>
<thead>
<tr>
<th>Agent Added to Platelets</th>
<th>Stirring</th>
<th>LA-PF₄/βTG in the Supernatant (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>+</td>
<td>2.68 ± 1.56</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>1.99 ± 1.34</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>+</td>
<td>31.6 ± 10.4</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>33.5 ± 13.1</td>
</tr>
<tr>
<td>ADP (10 µM)</td>
<td>+</td>
<td>2.9 ± 1.27</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>2.9 ± 0.95</td>
</tr>
<tr>
<td>U46619 (2.1 µM)</td>
<td>+</td>
<td>10.63 ± 5.0</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>7.8 ± 3.6</td>
</tr>
<tr>
<td>9,11-azo PGH₂ † (2.0 µM)</td>
<td>+</td>
<td>13.6 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>15.0 ± 7.7</td>
</tr>
</tbody>
</table>

*Aliquots of 450 µl of platelets (5 x 10⁸/ml) were incubated at room temperature with various concentrations of stimulating agents. Final volume of the suspension was 0.5 ml. The incubations were carried out for 3 min in either stirred (+) or nonstirred (−) samples. After the incubation time, the suspensions were centrifuged in an Eppendorf centrifuge for 2 min. The supernatant was saved for LA-PF₄/βTG determination by radioimmunoassay. Values are an average from 5 experiments (± SD). As evaluated by t test, there was no statistical difference between values of LA-PF₄/βTG antigen found in the supernatants of stirred and nonstirred samples. Values of LA-PF₄/βTG antigen found in the supernatants of platelets incubated with U46619 or 9,11-azo PGH₂ were significantly higher than values of this antigen found in the supernatants of intact or ADP-treated platelets (p < 0.05).†Mean values from 3 experiments.

Figures 2 and 3 describe platelet aggregation and LA-PF₄/βTG release in response to various concentrations of 9,11-azo PGH₂ and U46619, respectively. Both platelet aggregation and LA-PF₄/βTG release were dose dependent; saturation occurred at approximately 1.0 µM 9,11-azo PGH₂ or 2.0 µM U46619. The lowest concentration of 9,11-azo PGH₂ and U46619 required to produce detectable platelet aggregation and release in this experiment were 0.3 µM and 0.5 µM.
S. U46619, 0.15 B/F, respectively. The addition of exogenous fibrinogen to platelets stimulated by low concentrations of 9,11-azo PGH₂ (0.13 μM), U46619 (0.03 μM), and ADP (10 μM) enhanced aggregation in a dose-dependent manner (Fig. 4).

The addition of U46619 or 9,11-azo PGH₂ to platelets resulted in ¹²⁵I-fibrinogen binding (Fig. 5A). Non-stirred platelets stimulated with low concentrations of

Fig. 3. Effect of various concentrations of U46619 on platelet aggregation and release of LA-PF₄/βTG antigen. For other explanations see Fig. 2.

Fig. 4. Effect of various concentrations of fibrinogen on platelet aggregation induced by 9,11-azo PGH₂, U46619, and ADP. Aliquots of 400 μl platelet suspension were incubated for 1 min with 50 μl of various amounts of fibrinogen. Then 50 μl of the following agents were added: 9,11-azo PGH₂, 0.13 μM (O—O—O), U46619 0.03 μM (△—△—△), or ADP 10 μM (●—●—●); concentrations listed are final. The rate of platelet aggregation was calculated from the slope of the initial change in light transmission units per minute (LTU/min). In the absence of exogenous fibrinogen, the addition of these low concentrations of ADP or prostaglandin endoperoxide analogues to platelets did not result in their aggregation. As calculated by Lineweaver-Burk reciprocal plot, Kₘ values for fibrinogen were 4.4 x 10⁻⁷ M for ADP-stimulated platelets, 1.55 x 10⁻⁷ M for 9,11-azo PGH₂-stimulated platelets, and 6.8 x 10⁻⁶ M for U46619-stimulated platelets.

Fig. 5. Specific binding of ¹²⁵I-fibrinogen to platelets stimulated by U46619 and by 9,11-azo-PGH₂. (A) Amount of ¹²⁵I-fibrinogen bound to platelets stimulated by 2.1 μM U46619 (●—●—●) and by 1 μM 9,11-azo PGH₂ (O—O—O) as a function of the amount of ¹²⁵I-fibrinogen added. Experiment was performed as described in Materials and Methods. Incubation time was 5 min at 22°C. (B) Scatchard plot of the isotherm of fibrinogen binding to U46619-stimulated platelets. (C) Scatchard plot of the isotherm of fibrinogen binding to 9,11-azo PGH₂-stimulated platelets. The line has been drawn using least-squares method. Pfts, platelets; B/F, bound/free.
both endoperoxide analogues bound \(^{125}\)I-fibrinogen in a concentration-dependent manner. Scatchard analysis of these data indicated one class of binding sites (Fig. 5 B and C). These experiments therefore suggest that the platelet aggregation induced by 9,11-azo PGH\(_2\) or U46619 was mediated through the interaction of fibrinogen (either endogenous or exogenous) with its receptor on the platelet surface.

We next sought to determine whether \(^{125}\)I-fibrinogen binding and platelet aggregation were brought about by a direct effect of the endoperoxide analogues on platelets or by an indirect effect due to the ADP released from the platelet granules. Two enzymes that remove ADP (apyrase and creatine phosphokinase with creatine phosphate) and two ADP antagonists (ATP and FSBA) were tested to determine the role of ADP. These four inhibitors blocked platelet shape change induced by ADP (as indicated by a minus sign in Table 2); however, they had no effect on platelet shape change occurring during incubation of platelets with prostaglandin endoperoxide analogues. On the other hand, CTA\(_2\), an analogue of thromboxane-A\(_2\), inhibited 9,11-azo PGH\(_2\)-induced platelet shape change, but it had no inhibitory effect on shape change induced by ADP. Apyrase and CP/CPK were able to completely inhibit platelet aggregation brought about by ADP (in the presence of fibrinogen), 9,11-azo PGH\(_2\), and U46619. Similarly, FSBA and ATP completely inhibited platelet aggregation induced by ADP in the presence of fibrinogen as well as that induced by the prostaglandin endoperoxides. CTA\(_2\) had no effect on ADP-induced platelet aggregation; however, it completely blocked the aggregations induced by both prostaglandin endoperoxide analogues. These experiments presented in Table 2 suggest that platelet shape change caused by endoperoxide analogues was not mediated by the released ADP. On the other hand, platelet aggregation induced by these endoperoxide analogues appeared to be mediated by the released ADP.

The importance of the occupancy of specific receptors for the maintenance of platelet aggregation was evaluated in a series of experiments on platelet disaggregation (Fig. 6). Platelet aggregates were formed by ADP, in the presence of added fibrinogen or by the endoperoxide analogues prior to the addition of specific inhibitors. Apyrase, CP/CPK, FSBA, and ATP disaggregated platelet aggregates formed by either ADP or by the endoperoxide analogues. CTA\(_2\) reversed platelet aggregation induced by the endoperoxide analogues, but it did not reverse platelet aggregation brought about by ADP. These experiments indicate that prostaglandin endoperoxide-induced platelet aggregation requires a continuous occupancy of both ADP and prostaglandin endoperoxide receptors on the platelet surface. Persistence of platelet aggregates formed upon addition of ADP to platelets requires occupancy of ADP receptors, while prostaglandin endoperoxides are not critical in this process. Our data are consistent with other reports showing that FSBA\(^{23}\) and apyrase\(^{24}\) cause rapid disaggregation of platelet aggregates formed by ADP and dissociation of fibrinogen bound to platelets.

Table 3 shows that all blockers of ADP significantly decreased the amount of \(^{125}\)I-fibrinogen bound to platelets stimulated by ADP or by endoperoxide analogues. This experiment suggests that ADP is required for the exposure of fibrinogen receptors on platelets stimulated by endoperoxide analogues. CTA\(_2\) inhibited binding of \(^{125}\)I-fibrinogen to platelets stimulated by endoperoxide analogues but increased the amount of \(^{125}\)I-fibrinogen bound to ADP-stimulated platelets for reasons that are not clear at the present time. CTA\(_2\) alone did not cause platelet aggregation and fibrinogen binding to platelets.

**DISCUSSION**

The following mechanism of platelet aggregation induced by prostaglandin endoperoxide analogues is consistent with our data: stimulated platelets release
ADP and fibrinogen; subsequently, ADP acts on platelet membranes to expose fibrinogen receptors. Fibrinogen then binds to the exposed receptors, resulting in platelet aggregation. The receptors for the endoperoxides must be occupied during this entire process.

Fibrinogen is a constituent of the α-granules and it is released in parallel with LA-PF4/βTG antigen. The level of platelet fibrinogen amounts to about 100 μg/10⁹ platelets. During platelet aggregation by the endoperoxide analogues, about 70% of the platelet

Fig. 6. Disaggregation of platelet aggregates formed by ADP, U46619, and 9,11-azo PGH₂. Aliquots of 400 μl platelet suspensions (5 x 10⁹ platelets) were incubated for 1 min with 50 μl of stimulating agent. One minute later, 50 μl of inhibitor was added. (A) Aggregation induced by ADP (10 μM) in the presence of fibrinogen (50 μg/ml). Effect of CTA₂ (50 μM) and apyrase (100 U/ml) on platelet disaggregation. (B) Aggregation induced by ADP (10 μM) in the presence of fibrinogen (25 μg/ml). FSBA (100 μM) and ATP (500 μM) were added at maximal platelet aggregation. (C) Aggregation induced by 0.8 μM 9,11-azo PGH₂, CTA₂ (50 μM) and apyrase (100 U/ml) were added at maximal platelet aggregation. (D) Aggregation induced by 0.25 μM U46619. FSBA (100 μM) and ATP (500 μM) were added at maximal platelet aggregation. Final concentrations are given. Arrows indicate points of addition of the various reagents. In each control sample, volume was adjusted with 50 μl of 0.15 M NaCl. Each experiment presented in panels A, B, C, and D has been performed with a different preparation of platelets; for this reason, control aggregation tracings are different. In the experiment presented in panel A, the process of platelet disaggregation began prior to the addition of 0.9% NaCl.
LA-PF₄/βTG antigen is released (Figs. 1–3). Similar observations have been made by Kaplan et al., who studied the effect of U46619 on the release of α-granule contents of human platelets. This would correspond to 35 µg of platelet fibrogen in per 1 ml in the suspension containing 5 x 10⁶ platelets per 1 ml. This concentration of fibrogen was adequate to support platelet aggregation in our experimental system (Fig. 1). At low concentrations of the prostaglandin endoperoxide analogues, only a fraction of the α-granule constituent was released (Figs. 2 and 3). Under these conditions, addition of increasing concentrations of fibrogen to platelets stimulated by either 9,11-azo PGH₂, U46619, or ADP resulted in a similar dose-dependent increase in platelet aggregation (Fig. 4).

Interaction of platelets with the endoperoxide analogues resulted in the exposure of fibrogen receptors, as shown by ¹²⁵I-fibrogen binding data (Fig. 5). Scatchard plot analysis of the data indicated that only one class of fibrogen receptor became exposed on platelets. In our previous experiments with ADP-stimulated platelets, two classes of fibrogen receptors (i.e., low and high affinity receptors) were characterized on these platelets. The fibrogen binding sites exposed by these two prostaglandin endoperoxide analogues have some characteristics that are similar to the low affinity fibrogen receptors exposed by ADP. The experiment presented in Table 1 suggests that low concentrations of α-granule proteins were released by U46619 and 9,11-azo PGH₂, even in a nonstirred system. It is possible that low concentrations of platelet fibrogen released by the endoperoxide analogues would compete with ¹²⁵I-fibrogen for high affinity fibrogen binding sites. The release of endogenous fibrogen, in this case, would prevent our detection of the high affinity sites that are exposed by ADP. It has been shown that platelet fibrogen released during platelet stimulation may fulfill the requirement for plasma fibrogen in the aggregation process, and it may prevent detection of high affinity fibrogen binding sites on ADP-stimulated platelets. The dilution of exogenous ¹²⁵I-fibrogen with endogenous fibrogen makes the estimation of the number of binding sites and dissociation constant difficult.

In a recent study, Weiss and Lages described a pronounced decrease of platelet aggregation responses to PGG₂ and to arachidonic acid in patients with α-granule deficiency. This observation is compatible with our data on the significance of the release of platelet fibrogen and ADP in platelet aggregation induced by the endoperoxide analogues.

Our data suggest that the endoperoxide analogues have both direct and indirect effects on platelets. In agreement with Parise et al., we observed that the endoperoxide analogues stimulated platelet shape change independently of secreted ADP (Table 2). On the other hand, our studies indicate that ADP is an essential mediator in endoperoxide-induced platelet aggregation. Enzymes that remove ADP (apyrase and creatine phosphokinase in the presence of creatine phosphate) and agents that block ADP receptors, either covalently such as FSBA or competitively such as ATP, inhibited aggregation of platelets stimulated by these endoperoxide analogues and inhibited binding of ¹²⁵I-fibrogen to the endoperoxide-stimulated platelets (Tables 2 and 3). Moreover, apyrase, ATP, and FSBA disaggregated platelets that were aggregated by the endoperoxide analogues (Fig. 6). The ADP scavengers and inhibitors appeared to act in a specific manner because they did not alter platelet shape change caused by endoperoxide analogues, but did show effects on aggregation initiated by the ana-
logues. On the other hand, CTA₂, an analogue of thromboxane-A₂, blocked platelet shape change and platelet aggregation induced by the endoperoxide analogues, but it did not inhibit the action of ADP on the platelets (Table 2).

A direct action of endoperoxide analogues in platelet aggregation was revealed by the experiments on platelet disaggregation (Fig. 6). These experiments suggest that when endoperoxides are used to stimulate platelets, receptor occupancy is required for platelet aggregation. Synergism between the actions of arachidonic acid and ADP on platelets has been reported previously. Synergism also may explain the effectiveness of very low concentrations of ADP in promoting fibrinogen receptor exposure on platelets stimulated by the prostaglandin endoperoxides. These concentrations of ADP might not be easily detectable by chemical methods. Recently, Bennett et al. concluded that exogenous PGH₂ stimulated fibrinogen receptor exposure independently of secreted platelet ADP. These authors found that 20 μM ATP inhibited the binding of fibrinogen to platelets by 90% when they were stimulated by 2 μM ADP. In contrast, the fibrinogen binding stimulated by 2–4 μM PGH₂ was only inhibited by 6% by the same concentration of ATP. It is possible that under conditions of synergistic action of ADP and prostaglandin endoperoxide, the concentration of ATP used by Bennett et al. was not adequate to block ADP action. In our experimental system, 1 mM ATP was necessary to block binding of 125I-fibrinogen to platelets stimulated by 10 μM ADP and by two endoperoxide analogues (Table 3).

There may be some similarity between the mechanisms by which prostaglandin endoperoxides and epinephrine stimulate platelet aggregation. Plow and Marguerie demonstrated that fibrinogen receptor exposure on platelets stimulated by epinephrine was inhibited by enzymes that remove ADP. This occurred in a system in which release of dense granule constituents was not detectable. It can be pointed out, however, that assays of the release of dense granule constituents (14C-serotonin or ATP release) are less sensitive and more subject to error than radioimmunoassay of secreted platelet proteins. In our experimental system, an increase of LA-PF₄/βTG antigen was detectable in supernatants of platelet suspensions incubated with stable endoperoxide analogues under nonstirring conditions (Table 1).

The mechanisms by which 9,11-azo PGH₂ and U46619 caused platelet aggregation appeared to be very similar. In most cases, the platelet aggregating effect of 9,11-azo PGH₂ was about two times stronger as compared with the same molar concentrations of U46619. However, in some experiments (Fig. 4 and Fig. 5D), platelet preparations were unusually sensitive to the U46619. We do not presently know how to explain this observation.

Experiments performed over the last several years in a number of laboratories demonstrated that fibrinogen receptor exposure and fibrinogen–receptor interaction are the most critical events in platelet aggregation induced by ADP, thrombin, thromboxane A₂, and epinephrine. The present experimental data show that the same is true for platelet aggregation induced by stable prostaglandin analogues.

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