Arachidonate-Induced Fibrinogen Binding to Thrombin-Degranulated Rabbit Platelets is Independent of Released ADP

By Elizabeth J. Harfenist, Maria A. Guccione, Marian A. Packham, Raelene L. Kinlough-Rathbone, and J. Fraser Mustard

It has been established that fibrinogen binding occurs during adenosine diphosphate (ADP)-induced platelet aggregation, but studies of fibrinogen binding to platelets stimulated with arachidonate or most other aggregating agents are complicated by the release of platelet granule contents, which include both ADP and fibrinogen. Therefore, thrombin–degranulated rabbit platelets that have released more than 90% of their amine storage granule contents have been prepared for studies of the binding of 125I-fibrinogen during aggregation induced by agents that cause the release reaction with untreated platelets. To establish that thrombin–degranulated platelets are suitable for investigations of fibrinogen binding, the responses of these platelets to ADP were studied before other aggregating agents were used. The patterns of ADP-induced aggregation, deaggregation, fibrinogen binding and loss of bound fibrinogen, as well as the effects of inhibitors of platelet aggregation, were similar to those exhibited by untreated control platelets. The fibrinogen binding during ADP-induced aggregation was specific, saturable and reversible, and the apparent number of fibrinogen receptors and their association constant were in the same range as those calculated for control platelets. These thrombin–degranulated platelets have been used to study arachidonate-induced aggregation and 125I-fibrinogen binding. Responses to arachidonate were similar to ADP-induced responses except for the effects of creatine phosphate/creatine phosphokinase (CP/CPK), which converts ADP to ATP, and of indomethacin, which prevents the conversion of arachidonate to prostaglandin endoperoxides and thromboxane A2. CP/CPK abolished ADP-induced aggregation and fibrinogen binding of thrombin-degranulated platelets, but had much less effect on the responses to arachidonate. On the other hand, indomethacin had no effect on responses to ADP, but blocked aggregation and fibrinogen binding caused by arachidonate. We observed no evidence of synergism between arachidonate and the low residual concentration of ADP (less than 0.06 µM) that might have been released from the thrombin–degranulated platelets. Thus, arachidonate, through its products, appears to cause aggregation and fibrinogen binding to thrombin–degranulated platelets by a mechanism independent of ADP, although ADP may enhance the reactions. These products probably act by exposing fibrinogen receptors on the platelet surface in a manner similar to ADP.

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Pro), to prevent polymerization of fibrin, formed from released fibrinogen, into an insoluble clot. Thrombin-degranulated platelets prepared by this method were shown to aggregate in response to thrombin, ADP, or arachidonate.

The purpose of the present study was to answer the following two questions: (1) Are ADP-induced aggregation and fibrinogen binding of thrombin–degranulated rabbit platelets similar to those of untreated platelets? (2) Is fibrinogen binding involved in the aggregation of thrombin–degranulated platelets by arachidonate, an agent that can act independently of ADP?

We chose to test arachidonate, since it is liberated from platelets in response to a variety of agents, and it is the precursor of the prostaglandin endoperoxides and the potent aggregating agent, thromboxane A2. When rabbit platelets are stimulated with arachidonate they aggregate and release 40%–50% of their amine storage granule contents. Kinlough-Rathbone and her coworkers have shown, however, that thrombin-degranulated platelets can aggregate in response to arachidonate by a mechanism that is independent of ADP. Other investigators, using different approaches, have also concluded that arachidonate and its products can cause platelet shape change and aggregation by an ADP-independent pathway.

MATERIALS AND METHODS

Human fibrinogen (Grade L) was from AB Kabi, Stockholm, Sweden; factor XIII was removed by chromatography on DEAE cellulose. Human α-thrombin (lot H-T) was kindly supplied by Dr. D.L. Aronson, Bureau of Biologies, FDA, Bethesda, Md. It was dissolved at 75 U/ml in 50% glycerol and stored at −20°C. Gly-Pro-Arg-Pro was from Vega Biochemicals, Tucson, Ariz. Arachidonic acid (grade I), creatine phosphate (CP), creatine phosphokinase (CPK), indomethacin, and N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) were from Sigma Chemical Co., St. Louis, Mo. Prostaglandin E1 (PGE1) was a generous gift from the Upjohn Company, Kalamazoo, Mich. Bovine albumin (Pentex, fraction V) was from Miles Laboratories, Inc., Elkhart, Ind. Na51Cr was from New England Nuclear, Boston, Mass. 14C-Serotonin was obtained as 5-hydroxytryptamine-3-14C-creatinine sulfate (54 mCi/mmol) from Amersham Corp., Arlington Heights Ill. 125I-albumin was from Charles E. Frost and Co., Montreal, Canada. Apyrase, prepared by the method of Molnar and Lorand, was dissolved in 0.15 M NaCl and stored at −20°C; at a concentration of 9 μl/ml, it caused 91% conversion of 9 μM ADP to AMP and adenosine in 2 min at 37°C, at concentrations of 1 μl/ml and lower, it caused no observable degradation of 9 μM ADP in 10 min at 37°C. All other chemicals were reagent grade. Except where indicated, all reagents were dissolved in 0.15 M NaCl before use, and all concentrations are expressed as final values after all additions.

Suspensions of washed rabbit platelets were prepared essentially as described by Ardlie et al. The final suspending medium was Tyrode solution containing 5 mM HEPES and albumin at 3.5 mg/ml (Tyrode-albumin). For studying untreated platelets, the platelet count was adjusted to approximately 7 × 107/μl and apyrase was added to the suspension at 0.25 μl/ml. For the preparation of thrombin-degranulated platelets, the platelet count was adjusted to 1.2 × 107/μl and apyrase was added at 1 μl/ml. The platelets were labeled in the first washing fluid with 14C-serotonin and 51Cr to measure release and lysis. The preparation of thrombin-degranulated platelets, using Gly-Pro-Arg-Pro to prevent fibrin polymerization, has already been described. The thrombin-degranulated platelets were suspended at 7 × 107/μl in Tyrode-albumin containing 0.2μl/ml. Platelet suspensions were kept at room temperature and incubated at 37°C for 3 min in aggregometer cuvettes immediately before use. Fibrinogen was iodinated with 125I by the ICI method of McFarlane. Aggregation and the binding of the 125I-fibrinogen with control or thrombin-degranulated platelets were studied as described previously. For aggregation measurements the suspensions were stirred at 1100 rpm; for binding studies they were stirred at 85 rpm, and 125I-albumin was used as a marker for trapped fluid.

RESULTS

By using Gly-Pro-Arg-Pro to prevent fibrin clot formation as previously described, thrombin-degranulated platelets were obtained in 70.9% ± 4.3% yield from washed rabbit platelets (mean ± SD of 8 experiments). They had released 92.1% ± 1.8% of their dense granule contents while undergoing less than 5% loss of 51Cr. The platelet suspensions were stable for many hours, provided 5 mM HEPES was included in the Tyrode-albumin medium to maintain a pH of 7.2–7.4. Thrombin-degranulated platelets aggregated in response to ADP (10 μM), thrombin (0.75 U/ml), or arachidonate (50 μM). When they were studied in an aggregometer, the amplitude of the oscillations in light transmission characteristic of disc-shaped platelets was slightly less than that seen with untreated platelets.

ADP-induced aggregation of thrombin-degranulated platelets was similar to that of control platelets (Fig. 1) except that their ability to deaggregate was somewhat impaired. There was no significant release of 14C-serotonin or loss of 51Cr upon stimulation with ADP. It can be seen in Fig. 1 that at both low (0.22 μl/ml) and high (9 μl/ml) concentrations of apyrase, the thrombin-degranulated platelets (panel B) deaggregated more slowly than control platelets (panel A) after aggregation with ADP in the presence of fibrinogen. Aggregation of thrombin-degranulated platelets with arachidonate in the presence of fibrinogen (Fig. 1, panel C) was similar to aggregation by ADP, except that deaggregation was slightly slower at the lower apyrase concentration, and the higher concentration of apyrase had no effect on the rate of deaggregation. The extent of aggregation was similar in all cases. Arachidonate-induced aggregation of thrombin-degranulated platelets was accompanied by
the loss of 2%–3% of $^{51}$Cr and no more than 9% of any residual $^{14}$C. This corresponds to the release of less than 0.06 $\mu M$ ADP. This calculation is based on previous observations$^{30}$ that the concentration of ADP in a suspension of untreated rabbit platelets would be 13.3 $\mu M$ at this platelet count ($6 \times 10^5/\mu l$); if approximately 60% is releasable, only 0.64 $\mu M$ releasable ADP would remain after the 92% release caused by thrombin (9% of 0.64 $\mu M = 0.06 \mu M$). With either ADP or arachidonate as aggregating agent, thrombin-degranulated rabbit platelets aggregated in the absence of added fibrinogen, as do untreated rabbit platelets,$^{3}$ but the extent of aggregation was enhanced by the addition of fibrinogen.

Reactions of Thrombin-Degranulated Platelets With ADP

The binding of $^{125}$I-fibrinogen to thrombin-degranulated platelets stimulated with ADP (10 $\mu M$) was characterized, and the results are summarized in Figs. 2–5 and in Table 1. There was no aggregation or significant specific binding of fibrinogen in the absence of ADP. The addition of 10 $\mu M$ ADP caused specific binding of fibrinogen followed by slow dissociation of bound fibrinogen during deaggregation (Fig. 2). The amount of fibrinogen that bound to thrombin-degranulated platelets (1.7%–2.8%) was similar to the amount that bound to control platelets (1.7%–2.7%) at a platelet count of approximately $6 \times 10^5/\mu l$ and $^{125}$I-fibrinogen concentrations of 7–20 $\mu g/ml$. The rate of binding was lower than with control platelets; with untreated platelets, maximum binding is reached within 0.2 min after ADP addition,$^{1,3}$ whereas with thrombin-degranulated platelets, 1–2 min was...
required before maximum binding occurred. The binding followed the aggregation curve more closely with thrombin-degranulated platelets. Specific fibrinogen binding was saturable at higher concentrations of $^{125}$I-fibrinogen (Fig. 3), and although the binding was not strictly equilibrium binding, it can be calculated from a Scatchard-type plot that the binding was consistent with the exposure of approximately 14,000 fibrinogen receptors per platelet with an association constant ($K_a$) of $1.8 \times 10^6 M^{-1}$. We observed no evidence for more than one class of receptors. Aggregation and $^{125}$I-fibrinogen binding were unchanged as the ADP concentration was increased from 10 $\mu M$ to 160 $\mu M$ (Fig. 4). Fig. 5 shows that approximately 80% of the $^{125}$I-fibrinogen could be prevented from binding or displaced by a large excess of unlabeled fibrinogen added before or immediately after the ADP. This represents the specific binding. However, an excess of fibrinogen added 3 min after the ADP was able to displace only 40% of the bound $^{125}$I-fibrinogen.

The inhibitors, EDTA and PGE$_1$, added in concentrations that block fibrinogen binding to untreated platelets, prevented ADP-induced aggregation and fibrinogen binding to thrombin-degranulated platelets (Table I). Table I also shows that CP/CPK, added before the ADP, prevented aggregation and fibrinogen binding, while indomethacin had no effect. Addition of CP/CPK 1 min after the ADP resulted in 80% ± 11% deaggregation within 3 min and 77% ± 8% dissociation of bound fibrinogen within 1 min.

Reactions of Thrombin-Degranulated Platelets With Arachidonate

Since all the responses of thrombin-degranulated platelets to ADP were similar to those of untreated platelets, the binding of $^{125}$I-fibrinogen to thrombin-
degranulated platelets stimulated with 50 μM arachidonate was characterized and the results are shown in Figs. 2–5 and Table 1 with those for ADP stimulation. Thrombin-degranulated platelets bound 125I-fibrinogen specifically in response to 50 μM arachidonate and the fibrinogen was lost as the platelets deaggregated (Fig. 2). The amount of fibrinogen bound was similar to that bound in response to ADP (1.5%–2.6%) for a platelet count of 6 × 10⁸/μl and 125I-fibrinogen concentrations of 11–20 μg/ml. Figure 3 shows that the 125I-fibrinogen binding curve for arachidonate is almost superimposable on the ADP curve, and Scatchard analysis gave approximately 1,360 fibrinogen receptors per platelet with an association constant of 1.6 × 10⁸ M⁻¹. We observed no evidence for a second class of receptors. Aggregation and fibrinogen binding were shown not to increase as the arachidonate concentration was increased above 50 μM (Fig. 4). In fact, at the highest arachidonate concentration (800 μM), aggregation and fibrinogen binding were less than at lower concentrations. Figure 5 shows that initially, unlabeled fibrinogen could displace or prevent the binding of approximately 80% of the 125I-fibrinogen but after 3 min, an excess of unlabeled fibrinogen displaced less than 40% of the 125I-fibrinogen. This pattern is almost identical to the pattern shown for ADP stimulation. EDTA and PGE₁ prevented aggregation and greatly reduced fibrinogen binding of thrombin-degranulated platelets stimulated with arachidonate, but the effects of CP/CPK and indomethacin were different than for ADP-induced responses (Table 1). CP/CPK, at the concentrations used, had much less effect on both aggregation and fibrinogen binding than with ADP, whereas indomethacin blocked arachidonate-induced aggregation and greatly reduced 125I-fibrinogen binding.

It was essential to confirm that the observed arachidonate-induced aggregation and fibrinogen binding were due to arachidonate alone and not to a synergistic effect of 50 μM arachidonate and the very low amounts of ADP that might be released from thrombin-degranulated platelets. Table 2 shows that no synergy was detected with concentrations of ADP up to 0.10 μM, a concentration higher than the maximum (0.06 μM) that we calculated could be released from the thrombin-degranulated platelets by arachidonate. This was observed both with 50 μM arachidonate and with a low concentration, 15 μM, which is a threshold concentration for aggregation and fibrinogen binding (Fig. 4).

### DISCUSSION

The rationale for using thrombin-degranulated platelets to study the interactions of platelets with aggregating agents that normally cause the release reaction was that, since these platelets do not have granule contents to release, it would be possible to distinguish between those effects that are due solely to the aggregating agent under study and those that are caused at least in part by released ADP. Our aim in this study was to investigate arachidonate-induced binding of 125I-fibrinogen to platelets, but it was necessary to establish first that the properties of thrombin-degranulated platelets are similar to those of untreated control platelets. Since ADP-induced fibrinogen binding to untreated rabbit platelets has undergone exten-

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### Table 1. Effects of Inhibitors on Responses of Thrombin-Degranulated Platelets to ADP or Arachidonate

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Aggregation Peak Height (%)</th>
<th>125I-Fibrinogen Bound (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ADP</td>
<td>Arachidonate</td>
</tr>
<tr>
<td></td>
<td>ADP</td>
<td>Arachidonate</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>0</td>
<td>5 ± 4</td>
</tr>
<tr>
<td>PGE₁</td>
<td>0</td>
<td>87 ± 8</td>
</tr>
<tr>
<td>CP/CPK</td>
<td>97 ± 2</td>
<td>24 ± 4</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>101 ± 6</td>
<td>100</td>
</tr>
</tbody>
</table>

Aggregation peak height and 125I-fibrinogen bound are expressed as percentages of the values observed in the absence of inhibitor. Platelets were incubated with inhibitor for 3 min at 37°C before the addition of either ADP or arachidonate. Suspensions were centrifuged 2 or 3 min after addition of aggregating agent. Final concentrations: ADP, 10 μM; arachidonate, 50 μM; 125I-fibrinogen (for binding), 12–18 μg/ml; unlabeled fibrinogen (for aggregation), 60 μg/ml; EDTA, 9.7 mM; PGE₁, 0.57 μM; CP, 6.2 mM; CPK, 8.6 U/ml; indomethacin, 10 μM; platelet count, 6 × 10⁸/μl. Means ± SD of 3 or 4 experiments.

### Table 2. Investigation of Synergism Between Arachidonate and Low Concentrations of ADP in Stimulation of Thrombin-Degranulated Platelets

<table>
<thead>
<tr>
<th>Arachidonate (μM)</th>
<th>ADP (μM)</th>
<th>Aggregation Peak Height (%)</th>
<th>125I-Fibrinogen Bound (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>—</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>0.05</td>
<td>102 ± 3</td>
<td>107 ± 10</td>
</tr>
<tr>
<td>50</td>
<td>0.10</td>
<td>102 ± 3</td>
<td>107 ± 10</td>
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<tr>
<td>15</td>
<td>—</td>
<td>0</td>
<td>16 ± 6</td>
</tr>
<tr>
<td>15</td>
<td>0.05</td>
<td>0</td>
<td>16 ± 6</td>
</tr>
<tr>
<td>15</td>
<td>0.10</td>
<td>0</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>—</td>
<td>0.10</td>
<td>0</td>
<td>0 ± 5</td>
</tr>
</tbody>
</table>

Values for aggregation peak heights and for 125I-fibrinogen bound are expressed as percentages of values obtained with 50 μM arachidonate alone. ADP and arachidonate were added at the same time, and for binding measurements, the platelet suspensions were centrifuged 2 min later. Final concentrations: 125I-fibrinogen (for binding), 11–18 μg/ml; unlabeled fibrinogen (for aggregation), 60 μg/ml; platelet count, 6 × 10⁸/μl. Means ± SD of 3 or 4 experiments.
platelets, fibrinogen and arachidonate 961

The extent of binding was similar in both cases that ADP is not a cofactor in platelet aggregation. Fibrinogen binding with either type of platelet, showing both subsequently lost their bound fibrinogen as the bound '25I-fibrinogen in a specific, saturable, and reversible manner when stimulated with ADP, and both subsequently lost their bound fibrinogen as the platelets deaggregated even when there was sufficient ADP in the suspension to cause aggregation initially. The extent of binding was similar in both cases (1.7%–2.7% of the added fibrinogen when the platelet count was 6 x 10^9/μl, the ADP concentration was 9–10 μM, and the '25I-fibrinogen concentration was 10–20 μg/ml;32 the number of fibrinogen receptors (n) and binding affinities appear to be in the same range [n = 19.500 and K_a = 1.3 x 10^6 M^-1 for control platelets (calculated from data in reference 5, Fig. 5) and n = 14,000 and K_a = 1.8 x 10^6 M^-1 for thrombin-degranulated platelets]. Increasing the concentration of ADP from 10 μM to 160 μM did not affect the extent of fibrinogen binding with either type of platelet, showing that ADP is not a cofactor in platelet aggregation. With both types of platelets, most of the bound '25I-fibrinogen could be displaced initially by an excess of unlabeled fibrinogen, but part of the bound fibrinogen ceased to be exchangeable within a short time after the addition of ADP, each of the inhibitors, EDTA, PGE_1, and CP/CPK, prevented or greatly reduced fibrinogen binding at a concentration that is effective with untreated platelets.32 We concluded from this study that although the platelets had lost more than 90% of their granule contents, the fibrinogen receptors and the mechanisms controlling their availability had not been significantly affected by the thrombin treatment and that thrombin-degranulated platelets were suitable for studying reactions with other aggregating agents.

With arachidonate as aggregating agent, the patterns of aggregation and fibrinogen binding of thrombin-degranulated platelets were similar to those caused by ADP except for the effects of indomethacin, apyrase, and CP/CPK. Arachidonate appeared to cause specific, saturable, and reversible fibrinogen binding to thrombin-degranulated platelets, with no evidence for more than one class of fibrinogen receptor. Since the rate and extent of binding, the loss of bound fibrinogen during deaggregation, the number of receptors, the affinity of binding, the ability of unlabeled fibrinogen to displace bound labeled fibrinogen, and the inhibition by EDTA and PGE_1 were almost identical to the corresponding effects observed with ADP, we conclude that both aggregating agents act by exposing the same fibrinogen receptors on the platelet surface. The differences in their mechanisms of action must lie in the manner in which they or their products interact with the platelets to reveal the receptors. As with ADP, the binding did not increase as the arachidonate concentration was increased above 50 μM. At high arachidonate concentrations, the extents of aggregation and fibrinogen binding were actually decreased, possibly because of perturbation of the platelet membrane by arachidonate.21,35,36

Indomethacin, which blocks the conversion of arachidonate to prostaglandin endoperoxides and thromboxane A_2, had no effect on ADP-induced reactions. This finding is consistent with the concept that ADP acts by a mechanism that is independent of the arachidonate-thromboxane pathway. Indomethacin abolished arachidonate-induced aggregation and greatly reduced the extent of arachidonate-induced fibrinogen binding, indicating that arachidonate itself did not cause the responses, but acted through its metabolites. Both apyrase and CP/CPK remove added ADP from the platelet suspending medium and thus inhibit ADP-induced effects on either control or thrombin-degranulated platelets. Apyrase had no observable effect on arachidonate-induced aggregation of thrombin-degranulated platelets, but CP/CPK, which removes ADP more quickly than apyrase at the concentrations used, did cause slight inhibition of arachidonate-induced aggregation and fibrinogen binding. We conclude from this that although a small amount of released ADP may be partially responsible for the observed effects, arachidonate can cause fibrinogen binding to thrombin-degranulated platelets by a mechanism that is independent of ADP. The platelets had released 92% of their dense granule contents during thrombin pretreatment, and up to 9% of the residual ^14C was lost upon stimulation with arachidonate. This could result in a concentration of ADP in the platelet suspending medium of 0.06 μM. Thus, there was a possibility that observed fibrinogen binding was in part due to a small amount of released ADP or to the synergistic effect of this ADP with added arachidonate. Since addition of CP/CPK to thrombin-degranulated platelets after stimulation with 10 μM ADP caused extensive deaggregation and dissociation of bound fibrinogen, it can be concluded that even if any released ADP were to be bound to the platelets it would still be accessible to the scavenger, CP/CPK. We failed to demonstrate synergism between concentrations of added ADP up to 0.1 μM and either 50 μM or 15μM arachidonate. Therefore, synergism between released ADP and arachidonate is unlikely, but because of the small inhibitory effect of CP/CPK we cannot rule out a small contribution from released ADP.
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