CONCISE REPORT

Participation of ADP in the Binding of Fibrinogen to Thrombin-Stimulated Platelets

By Edward F. Plow and Gerard A. Marguerie

Thrombin and adenosine diphosphate (ADP) supported the binding of $^{125}$I-fibrinogen to washed human platelets with similar kinetics and affinity. Platelet secretion, as measured by $^{14}$C-serotonin release, and fibrinogen binding exhibited an identical dependence on thrombin concentration. Enzymatic removal of ADP with apyrase or creatine phosphate/creatine phosphokinase (CP/CPK) from thrombin-stimulated platelets markedly inhibited $^{125}$I-fibrinogen binding, but pretreatment of platelets with CP/CPK prior to thrombin stimulation was without effect. Thus, ADP, released from the platelet, participates in the binding of fibrinogen to thrombin-stimulated platelets.

FIBRINOGEN is an essential cofactor for the aggregation of washed human platelets by ADP. The molecule binds directly to platelets stimulated with ADP but not to unstimulated cells, and the binding sites for fibrinogen exhibit many of the characteristics of a discrete and saturable receptor system. From the analysis of the specific binding of fibrinogen to ADP-stimulated platelets, linear Scatchard plots are derived indicative of a single class of binding sites with an affinity of 1.5-2.3 x $10^6$ M$^{-1}$, and approximately 40,000 molecules are bound per platelet in the presence of 1 mM calcium. Additional features of fibrinogen binding to ADP-stimulated platelets include a requirement for divalent ions (calcium or magnesium) and the complete inhibition of binding by the enzymatic removal of ADP. Recently, Hawiger et al. have shown that thrombin also supports the binding of fibrinogen to washed human platelets. The affinity and number of molecules bound were similar to values that they derived with ADP-stimulated platelets. However, ADP resides within platelet storage granules, and thrombin induces the nonlytic release of ADP. In this study, we have assessed if platelet ADP participates in the binding of fibrinogen to thrombin-stimulated platelets.

MATERIALS AND METHODS

Platelets were isolated from fresh human blood drawn into acid-citrate-dextrose (ACD) by differential centrifugation followed by gel filtration. The sepharose 2B-CL column (40 ml bed volume) was equilibrated in Tyrode's buffer containing 2% bovine serum albumin at pH 7.2, freed of divalent ions with Chelex 100. The characteristics of the labeled and unlabeled fibrinogen utilized in this study have been previously reported in detail. The binding of $^{125}$I-fibrinogen to the washed human platelets was measured as follows: platelets were diluted in Tyrode's-albumin buffer to a final concentration of $10^6$/ml with 1 mM calcium, and the stimulus, thrombin or ADP, was added. After 10 min at 22°C, hirudin was added in a tenfold excess of the concentration required to inhibit the clotting activity of thrombin. (The hirudin concentrations also completely inhibited thrombin-induced platelet aggregation.) Subsequently, $^{125}$I-fibrinogen and potential inhibitors were added. The samples were gently mixed and incubated at 22°C without stirring. Incubation times were measured relative to the addition of $^{125}$I-fibrinogen at time 0. At selected times, duplicate or triplicate 50μl aliquots were removed and layered onto 400 μl of 20% sucrose in a 500-μl conical tube and spun for 2.5 min at 11,750 rpm in a Beckman microfuge. The $^{125}$I-fibrinogen bound per platelet was determined by counting the radioactivity in the centrifuge tube tip. All results are reported as the specific binding of $^{125}$I-fibrinogen, which was derived by subtracting binding observed in a 50-fold molar excess of unlabeled fibrinogen (nonspecific) from the total $^{125}$I-fibrinogen binding. The nonspecific binding represented ≤5% of total binding at all concentrations of $^{125}$I-fibrinogen utilized. Statistical analyses were performed using a Texas Instruments Model TI-55 calculator.

Platelet recovery and the release reaction were measured by isotopically labeling the platelets in platelet-rich plasma with either $^{51}$Cr (Amersham, Arlington Heights, Ill.) or $^{2-} ^{14}$C-serotonin (New England Nuclear, Boston, Mass.) as previously described. After isolation, the labeled platelets were utilized as in binding assays, substituting unlabeled fibrinogen for $^{125}$I-fibrinogen. Percent recovery and serotonin release were calculated relative to the radioactivity of unstimulated platelets treated in an identical manner.

ADP, hirudin, apyrase, CP, and CPK were purchased from Sigma Chemical Co. (St. Louis, Mo.) and were of the highest grade available. As utilized, apyrase and CP/CPK did not degrade fibrinogen (as judged by polyacrylamide gel electrophoresis), did not cause $^{51}$Cr or $^{14}$C-serotonin release from platelets, and did not block platelet aggregation induced by thrombin. The α-thrombin utilized was the kind gift of Dr. John Fenton.

RESULTS

The binding of fibrinogen to thrombin-stimulated platelets was initially assessed as a function of time and compared with ADP-stimulated platelets. To restrict the activity of the thrombin to the platelet as opposed to the fibrinogen, the platelets were stimu-

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lated with thrombin for 10 min and excess hirudin was added prior to the addition of \( ^{125}\text{I} \)-fibrinogen. ADP-stimulated platelets were treated in a similar manner, including the addition of hirudin. As shown in Fig. 1A, both thrombin and ADP supported the binding of \( ^{125}\text{I} \)-fibrinogen to platelets, whereas less than 1000 molecules bound to the unstimulated cells. With both stimuli, one-half maximal fibrinogen binding was attained at 4.2 min, and maximal binding, achieved within 15 min, did not change during the subsequent 30 min. The capacity to achieve saturable binding of fibrinogen to thrombin-stimulated platelets was assessed after a 30-min incubation (Fig. 1B). The binding of \( ^{125}\text{I} \)-fibrinogen to the cell was linear in the range of 0.1–0.7 \( \mu M \) \( ^{125}\text{I} \)-fibrinogen added, and saturation was attained at higher concentrations indicative of a discrete number of binding sites. The Scatchard plot derived from this data (see insert to Fig. 1B) was linear \((r = 0.998)\), suggesting a single class of binding sites. The affinity of binding was \( 2.0 \times 10^6 M^{-1} \), and 37,000 molecules were bound per platelet. ADP stimulation of the same platelet preparation supported the binding of 28,000 molecules/platelet with an affinity of \( 2.4 \times 10^6 M^{-1} \).

The relationship between \( ^{125}\text{I} \)-fibrinogen binding and \(^{14}\text{C} \) serotonin release was measured as a function of thrombin concentration. As shown in Fig. 2, these two events were very closely correlated. Thrombin, at concentrations of \(<0.25 \text{ mU/ml}\), failed to induce fibrinogen binding or serotonin release, whereas above 0.5 mU/ml, fibrinogen binding and serotonin release occurred in a parallel fashion. When fibrinogen binding was expressed as a percent of the maximum binding at 25 mU thrombin/ml and correlated with the percent serotonin release, a linear correlation coefficient of \( r = 0.993 \) was derived.

Since fibrinogen binding and the release reaction exhibited an identical dependence on thrombin concentration, the role of released ADP in supporting binding of fibrinogen to thrombin-stimulated platelets was considered. Two enzymatic systems, apyrase and CP/CPK, were utilized to effect the removal of ADP, and both markedly inhibited the binding of fibrinogen to thrombin-stimulated platelets (Table 1). When ADP was added to the platelets, these enzymes inhibited fibrinogen binding by \( \approx 98\% \); and, with thrombin-stimulated platelets, inhibition exceeded 86%. Huang and Detwiler\(^9\) have recently reported that nonsecreted ADP in plasma influences platelet aggregation. To remove such nonsecreted ADP from our washed human platelets, the cells were isolated by differential centrifugation and incubated with CP/CPK for 10 min prior to gel filtration. As shown in Table 1, pretreatment of the platelets with CP/CPK resulted in

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**Fig. 1.** Binding of \( ^{125}\text{I} \)-fibrinogen to thrombin-stimulated platelets. (A) Final concentration of \( ^{125}\text{I} \)-fibrinogen, platelets, thrombin and ADP were 0.3 \( \mu M \), 10\(^6 \) platelets, 2.5 mU/ml, and 12.5 \( \mu M \), respectively. (B) The specific binding curve shown was derived by subtracting the binding observed in a 100-fold \( M \) excess of unlabeled fibrinogen from the total \( ^{125}\text{I} \)-fibrinogen binding after a 30-min incubation with \( 10^5 \) platelets stimulated with 2.5 mU/ml thrombin. The insert is the Scatchard plot of the data in Fig. 1B.

**Fig. 2.** Relationship between \( ^{125}\text{I} \)-fibrinogen binding and \(^{14}\text{C} \) serotonin release. Parameters were measured 30 min after addition of fibrinogen at a 0.3 \( \mu M \) final concentration. Serotonin release was measured relative to the radioactivity within nonstimulated platelets. \(^{51}\text{Cr} \) recovery was greater than 95% at all thrombin concentrations.
FIBRINOGEN BINDING TO PLATELETS

Table 1. Effect of the Enzymatic Removal of ADP on the Binding of Fibrinogen to Thrombin or ADP-Stimulated Platelets

<table>
<thead>
<tr>
<th>Stimulus*</th>
<th>ADP Removal†</th>
<th>125I-Fibrinogen Bound‡ (Molecules/Platelet)</th>
<th>Percent Inhibition of 125I-Fibrinogen Binding§</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>None</td>
<td>31,714</td>
<td>0.0</td>
</tr>
<tr>
<td>ADP</td>
<td>Apyrase</td>
<td>676</td>
<td>97.9</td>
</tr>
<tr>
<td>ADP</td>
<td>CP/CPK</td>
<td>509</td>
<td>98.4</td>
</tr>
<tr>
<td>Thrombin</td>
<td>None</td>
<td>38,690</td>
<td>0.0</td>
</tr>
<tr>
<td>Thrombin</td>
<td>Apyrase</td>
<td>5,353</td>
<td>86.2</td>
</tr>
<tr>
<td>Thrombin</td>
<td>CP/CPK</td>
<td>4,776</td>
<td>87.7</td>
</tr>
<tr>
<td>Thrombin</td>
<td>Pretreated with CP/CPK</td>
<td>37,877</td>
<td>2.1</td>
</tr>
</tbody>
</table>

*Platelets (10^9/ml) were stimulated with 12.5 μM ADP or thrombin at 2.5 μl/ml.
†Final concentrations of apyrase, CP, and CPK were: 80 μg/ml, 25.5 mg/ml, and 0.5 mg/ml, respectively. In pretreatment with CP/CPK, platelets were isolated by differential centrifugation and preincubated for 10 min with CP/CPK prior to gel filtration.
‡0.3 μM 125I-fibrinogen was added and binding was measured after 30 min.
§Calculated relative to ADP or thrombin-stimulated platelets without enzymatic treatment.

only a 2% inhibition of fibrinogen binding. Thus, secreted ADP is implicated in the binding of fibrinogen to thrombin-stimulated platelets.

DISCUSSION

Following ADP or thrombin stimulation, washed human platelets exhibit the capacity to bind 125I-fibrinogen. The kinetics and affinity of fibrinogen binding were very similar, consistent with the induction of the same class of fibrinogen receptors by thrombin and ADP. ADP is apparently present within the serotonin-containing dense granules released by thrombin. Not only was the capacity of thrombin to induce the nonlytic release of serotonin and to support fibrinogen binding very closely correlated, but also, the enzymatic removal of ADP markedly inhibited the binding of 125I-fibrinogen to thrombin-stimulated platelets. Thus, apyrase (which converts ADP to AMP) and CP/CPK (which converts ADP to ATP) inhibited fibrinogen binding to thrombin-stimulated platelets by greater than 86%. Furthermore, the participating ADP was not initially accessible to CP/CPK, but arose as a result of thrombin stimulation. Thrombin releases approximately 6 nM ADP/10^8 platelets, whereas 1 μM exogenously added ADP is required to support fibrinogen binding. This increased effectiveness of released ADP could reflect (1) its high local concentration, close proximity, and/or presentation to the platelet membrane; (2) its increased reactivity with thrombin-stimulated platelets; or (3) its synergism with thrombin or other released constituents. The latter possibility is suggested since low doses of ADP and epinephrine, which act synergistically to induce platelet aggregation, also synergistically support fibrinogen binding. Any of these mechanisms would increase the effective concentration of released ADP and explain its capacity to participate in the binding of fibrinogen to thrombin-stimulated platelets.

REFERENCES

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