Evidence for Interaction Between Platelet Fibrinogen Receptors

By Ellinor I. B. Peerschke

Previous analysis of fibrinogen binding to human aspirin-treated gel-filtered platelets yielded upwardly concave Scatchard plots. To ascertain whether this was due to the presence of independent heterogeneous receptor populations binding fibrinogen with different affinities, the dissociation of purified $^{125}$I-fibrinogen from ADP-treated gel-filtered platelets was evaluated as a function of receptor occupancy. Dissociation of bound labeled fibrinogen was measured after 50-fold dilution with buffer containing 0, 0.2, 0.8, and 2.0 mg/ml unlabeled fibrinogen. Dissociation of labeled fibrinogen increased with increasing receptor occupancy and was biphasic. With buffer alone, 76.0% ± 5.8% (SD) of labeled fibrinogen dissociated in 30 min, with an initial rate of $0.392 \pm 0.175$ min$^{-1}$; with 0.2 mg/ml fibrinogen, 83.7% ± 3.9% dissociated, with an initial rate of $0.589 \pm 0.044$ min$^{-1}$; with 0.8 mg/ml, 91.8% ± 1.3% of the labeled fibrinogen dissociated, with an initial rate of $0.910 \pm 0.028$ min$^{-1}$; and with 2.0 mg/ml fibrinogen, 97.3% ± 2.3% of label dissociated, with an initial rate of $1.06 \pm 0.257$ min$^{-1}$ (n = 5). The final rates of fibrinogen dissociation were unaffected by unlabeled fibrinogen in the dilution buffer and were not statistically different from the final dissociation rate of $0.015 \pm 0.10$ min$^{-1}$ observed following dilution with buffer alone. These results were neither an artifact of aspirin treatment or gel filtration, as similar observations were made using non-aspirin-treated washed platelets, nor were they an artifact of the purified fibrinogen preparations, because binding studies using whole plasma as the major source of fibrinogen also yielded upwardly concave Scatchard plots. Since the data demonstrate that the initial rate and extent of fibrinogen dissociation are dependent on fibrinogen receptor occupancy, they suggest receptor interactions possibly resulting from receptor clustering or crosslinking. Because the dissociation was biphasic, the results also suggest some heterogeneity among platelet–fibrinogen interactions.

During the last few years, evidence has accumulated to suggest that fibrinogen acts as a cofactor for adenosine diphosphate (ADP) induced human platelet aggregation.1,2 Its specific binding to platelets has been demonstrated under different conditions by several investigators,3,7 but different results have been obtained for several binding parameters, including (1) the total number of fibrinogen receptors, (2) the fibrinogen concentration necessary to saturate these receptors, and (3) the length of time needed to reach equilibrium. It is not surprising, therefore, that the geometry of the Scatchard analyses reported in the literature has varied, as well, from straight lines3,4 to upwardly concave plots.6,7

These two geometries have very different implications for ligand–receptor interactions: straight line plots suggest the presence of a single class of receptors, whereas curvilinear plots can indicate a number of ligand–receptor interactions, such as receptor heterogeneity, and a group of interactions resulting in a decrease in the apparent affinity of receptors for ligand when fractional saturation of the receptor increases, including receptor clustering and crosslinking by divalent or multivalent ligand.8,9 The present study was designed to distinguish among some of these possibilities. One approach to this problem relevant to platelet–fibrinogen interactions is to measure the dissociation rate of bound labeled fibrinogen as a function of receptor occupancy.10,11 If, for example, the receptor sites are heterogeneous and independent, dissociation of labeled fibrinogen would be unaffected by the extent of receptor saturation. In contrast, if fibrinogen–receptor interactions occurred that resulted in decreased affinity of receptors for ligand with increasing receptor occupancy, then one would predict increases in the rate of fibrinogen dissociation as a function of increased receptor saturation.

MATERIALS AND METHODS

Preparation of Gel-Filtered Platelets (GFP)

Blood was obtained from healthy volunteers, collected into 1/10 vol of 0.11 M sodium citrate, and incubated with 1/20 vol of 1 mM acetylsalicylic acid (Merek and Co., West Point, Pa.) to prevent release mediated by thromboxane-A$_2$.12 Platelets were concentrated and filtered through a column of Sepharose 2B (Pharmacia Fine Chemicals, Piscataway, N.J.) equilibrated with 0.01 M HEPES-buffered (N-2-hydroxyethyl-piperazine-N’2-ethane sulfonic acid; Calbiochem, San Diego, Calif.) modified Tyrode’s solution, pH 7.5, containing 2 mg/ml bovine serum albumin (HBMT).6

Preparation of Washed Platelets (WP)

Blood was collected into sodium citrate as described above. Platelet-rich-plasma (PRP) was obtained by centrifugation (280 g, 15 min) and was acidified to pH 6.5 with 0.11 M citric acid. Platelets were separated from the PRP by centrifugation (1000 g, 20 min) and resuspended in 0.15 M NaCl. The pH of this suspension was adjusted to pH 6.5 with citric acid, the platelets were centrifuged again (1000 g, 20 min), and then resuspended in 1/10 vol of 0.15 M NaCl and diluted to the desired final platelet count with HBMT.

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Supported in part by Biomedical Research Support Grant RR05736-08.

Submitted March 4, 1982; accepted June 7, 1982.

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0006-4971/82/6005-0024$01.00/0

Blood, Vol. 60, No. 4 (October), 1982

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**Fibrinogen Purification and Iodination**

Fibrinogen was purified from fresh frozen plasma and iodinated with $^{125}$I-NaI (New England Nuclear, Boston, Mass.) using chloramine-T (Fisher Scientific) as described. This iodinated fibrinogen (3000 cpm/ng) was between 93% and 95% clottable with thrombin, and the radioactivity was 99% precipitated by 10% trichloroacetic acid (Mallincrodt Chem, N.Y., N.Y.).

**Fibrinogen Binding**

To ascertain whether the curvilinear Scatchard plots were an artifact of the purified, low solubility fibrinogen preparations, binding was measured at equilibrium as previously described using whole plasma as the major source of fibrinogen with iodinated fibrinogen added as a trace to give a specific activity of 2000 cpm/µg. Plasma fibrinogen concentrations were determined using Dade Diagnostics DATA F1 reagent systems.

**Fibrinogen Dissociation**

All experiments were performed at room temperature. Concentrated GPF or WP (3-4 x 10⁹/ml) were incubated with 10 μM ADP (Sigma Chemical Co., St. Louis, Mo.) and 1-2 µg/ml $^{125}$I-labeled fibrinogen (low initial receptor occupancy). After 1 min, samples were (A) centrifuged through silicone oil to measure total binding, (B) diluted 50-fold with HBMT containing 10 μM ADP, or (C) diluted 50-fold with HBMT containing 10 μM ADP plus 0.2, 0.8, and 2.0 mg/ml unlabeled fibrinogen to increase fibrinogen receptor occupancy. After 2, 5, 10, 20, and 30 min intervals, aliquots of (B) and (C) were centrifuged through silicone oil to measure the amount of remaining bound fibrinogen.

The microfuge tubes containing HBMT with unbound fibrinogen in a layer above the silicone oil and platelet pellets below the oil were inverted and allowed to drain. The tips containing the pellets were sliced off and counted in a gamma counter (Micromedic Systems, Horsham, Pa.). The percent of bound fibrinogen remaining at each time point was calculated and plotted on a semilog plot. The dissociation rates were then determined by linear regression.

Similar studies were performed using platelet suspensions initially exposed to 0.2 and 0.8 mg/ml iodinated fibrinogen (higher initial receptor occupancy). Dissociation studies were done as described except that before dilution, platelets with bound fibrinogen were centrifuged (10,000 g, 1 min) through 30% sucrose and onto a 40% albumin cushion to separate them from the large amount of unbound fibrinogen.

**Control for Nonspecific Binding**

Nonspecific binding was assessed by performing binding studies in the presence of excess unlabeled fibrinogen (5 mg/ml). Dilution experiments were performed as described. Nonspecific binding at each time interval was subtracted from the total binding observed. Dissociation of nonspecifically bound label was 90% complete in 2 min and was unaffected by the presence of unlabeled fibrinogen in the diluting buffer, as expected.

**RESULTS**

To confirm that purified fibrinogen used previously to evaluate platelet fibrinogen interactions was (1) binding to platelets in a manner similar to fibrinogen in plasma, and (2) that it was not the cause of the upward curvature of the Scatchard plots, binding studies were repeated using whole plasma as the major source of fibrinogen with $^{125}$I-fibrinogen added as tracer. Upwardly concave plots were again seen (Fig. 1).

Thus, possible explanations for the geometry of these Scatchard plots were sought. To determine whether heterogeneous fibrinogen receptor populations, or interactions among fibrinogen receptors, or some combination was responsible, the dissociation of fibrinogen from ADP-treated platelets was measured as a function of receptor occupancy. This was done by comparing the rates and extents of dissociation of labeled fibrinogen from platelets following dilution in

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*Fig. 1. Typical Scatchard analysis of fibrinogen binding to human ADP-treated GFP using purified fibrinogen (○) and plasma (□) as the major sources of fibrinogen. Binding studies were performed on separate occasions using platelets from different volunteers.*
the presence and absence of increasing concentrations of unlabeled fibrinogen. As shown (Fig. 2A, Table 1), the dissociation of labeled fibrinogen bound to ADP-treated platelets at low initial receptor occupancy increased with increasing fibrinogen in the diluting buffer and was biphasic. Dilution alone resulted in the dissociation of 76.0% ± 5.8% (SD) of labeled fibrinogen in 30 min with an initial rate of 0.392 ± 0.175 min⁻¹. In the presence of 0.2 mg/ml unlabeled fibrinogen, 83.7% ± 3.9% of label dissociated with an initial rate of 0.589 ± 0.044 min⁻¹. Adding 0.8 mg/ml unlabeled fibrinogen to the diluting buffer resulted in dissociation of 91.8% ± 1.3% of the labeled fibrinogen with an initial rate of 0.910 ± 0.028 min⁻¹. Dilution in the presence of 2.0 mg/ml unlabeled fibrinogen resulted in dissociation of 97.3% ± 2.3% of label with an initial rate of 1.06 ± 0.257 min⁻¹ (n = 5). The difference in both the initial rates and final extents of dissociation between each group were statistically significant (p < 0.02).

Similar increases in the rate of fibrinogen dissociation with increased receptor occupancy were noted in
Table 1. Comparison of Dissociation Kinetics of Fibrinogen Bound to Aspirin-Treated Gel-Filtered Platelets (ASA-GFP) and Washed Platelets (WP)

<table>
<thead>
<tr>
<th>Fibrinogen in Diluting Buffer (mg/ml)</th>
<th>Initial Rate of Dissociation (min⁻¹) (Mean ± SD)</th>
<th>Percent Fibrinogen Remaining Bound (t = 30 min) (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ASA-GFP</td>
<td>WP</td>
</tr>
<tr>
<td></td>
<td>0.392 ± 0.175</td>
<td>0.414 ± 0.160</td>
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<tr>
<td></td>
<td>0.589 ± 0.044</td>
<td>0.615 ± 0.160</td>
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<tr>
<td></td>
<td>0.910 ± 0.028</td>
<td>0.885 ± 0.056</td>
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<tr>
<td></td>
<td>1.060 ± 0.257</td>
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<tr>
<td></td>
<td>24.0 ± 5.8</td>
<td>24.5 ± 6.4</td>
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<td></td>
<td>16.3 ± 3.9</td>
<td>12.8 ± 2.6</td>
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<td></td>
<td>8.2 ± 1.3</td>
<td>5.3 ± 1.1</td>
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<td>2.7 ± 2.3</td>
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The initial dissociation rate of fibrinogen bound at 2.0 mg/ml was 0.828 ± 0.296 min⁻¹ (SD, n = 3) compared to an initial dissociation rate of 0.488 ± 0.126 min⁻¹ for fibrinogen bound to platelets at 0.2 mg/ml. These dissociation rates are, of course, an average of the constantly decreasing rate of fibrinogen dissociation as a function of decreasing receptor occupancy.

The final rates of fibrinogen dissociation (t = 10–30 min) were unaffected by increases in receptor occupancy resulting from the presence of unlabeled fibrinogen in the diluting buffer. Following dilution in the absence of unlabeled fibrinogen, the final dissociation rate was 0.015 ± 0.010 min⁻¹. With 0.2 mg/ml unlabeled fibrinogen in the buffer, it was 0.022 ± 0.010 min⁻¹, and in the presence of 0.8 mg/ml unlabeled fibrinogen, the final dissociation rate was 0.026 ± 0.010 min⁻¹. These rates were not significantly different from each other and suggest that there may be more than one component to platelet fibrinogen interactions, only the major one of which is dependent on receptor occupancy.

Implicit in the interpretation of these results is the assumption that the receptor occupancy upon dilution immediately reflects the higher fibrinogen concentrations in the diluting buffer. To check this, dissociation studies were performed using GFP that had bound labeled fibrinogen at 0.2 and 0.8 mg/ml and were diluted with buffer containing the same respective fibrinogen concentrations. A typical experiment illustrates that, in a system using 0.2 mg/ml labeled and unlabeled fibrinogen, 87% of the labeled fibrinogen dissociated with an initial rate of 0.585 min⁻¹. With 0.8 mg/ml labeled and unlabeled fibrinogen, 92% of the label dissociated from platelets with an initial rate of 0.930 min⁻¹. These values are in agreement with those reported above for the dissociation of labeled fibrinogen bound at trace initial concentrations (1–2 μg/ml) and confirm that receptor occupancy changes rapidly after addition of unlabeled fibrinogen with the diluting buffer.

To verify that the observations on the dissociation kinetics were not an artifact of the aspirin-treated GFP suspensions, studies were done with non-aspirin-treated washed platelets, as shown in Fig. 2 and Table 1. Again, the initial rates and extents of fibrinogen dissociation were increased by the presence of unlabeled fibrinogen in the diluting buffer, and the final dissociation rates remained the same. There were no significant differences noted between fibrinogen dissociating from GFP and WP.

That the observed differences in the rate and extent of fibrinogen dissociation were not attributable to rebinding of label in platelet suspensions diluted with buffer alone was shown by the following experiments. Four experiments were done in which (1) fibrinogen dissociation was measured over a range of dilutions (1:10, 1:25, 1:50, 1:75, and 1:100) and dilutions plus unlabeled fibrinogen at 2, 5, 10, 20, and 30 min intervals. One such experiment is shown in Fig. 3. A dilution sufficient to prevent rebinding was achieved above a 1:25 dilution: increasing the dilution beyond this level no longer affected the difference in the amount of fibrinogen dissociated by dilution alone or
dilution plus unlabeled fibrinogen. (2) An equal aliquot of ADP-treated platelets that had not been exposed to fibrinogen (labeled or unlabeled) was added to the ADP-treated platelet–fibrinogen suspensions immediately following 1:50 and 1:100 dilution with buffer containing 10 μM ADP or ADP plus unlabeled fibrinogen. Neither the rate nor the extent of fibrinogen dissociation was altered by the doubled population of free receptors.

**DISCUSSION**

In a previous study we showed that the binding of fibrinogen to ADP-treated platelets did not fit a simple hyperbola, since analysis by the method of Scatchard showed upwardly concave plots. The simplest explanation for this is different binding affinities for native and iodinated fibrinogen, but this has been discounted by isotope dilution experiments. The present investigation describes the study of the kinetics of fibrinogen dissociation as a function of receptor occupancy to determine whether fibrinogen was binding to heterogeneous platelet receptors or whether interactions among fibrinogen receptors were responsible for the curved Scatchard plots.

Fibrinogen dissociation from ADP-treated platelets was biphase, with only the initial component affected by the degree of receptor occupancy. Thus, enhanced initial dissociation rates were noted with increasing fibrinogen concentrations in the diluting buffer (Fig. 2, Table 1). The initial dissociation rate of 1.06 min⁻¹ reported here for the dissociation of labeled fibrinogen in the presence of 2.0 mg/ml unlabeled fibrinogen is the same as that reported by Bennett and Vilaire (1.08 min⁻¹). No change in the final dissociation rates was observed with increasing concentrations of unlabeled fibrinogen in the diluting buffer.

These results suggest that fibrinogen binding to ADP-treated platelets does not conform to predictions made for any single model describing ligand–receptor interactions, but rather that a combination of ligand–receptor interactions is operative. The increase in the initial dissociation rates with increasing fibrinogen concentration in the diluting buffer (increasing receptor occupancy) is consistent with the fibrinogen–receptor interactions resulting in a decrease in the apparent affinity of receptors for ligand when fractional saturation of the receptors increases. Such interactions may include receptor clustering and/or crosslinking, ligand polymerization, and/or binding of a flexible ligand where, for example, regions of the fibrinogen molecule would bind to platelet receptors with different affinity depending on their conformation.

Of these models, the last two are the least likely. Fibrinogen polymerization has not been described in the absence of proteases cleaving peptides A and/or B, and the highly ordered tertiary structure of the molecule imparts a rigidity that precludes flexibility except in portions of the carboxy-terminal of the Aα chain, whose role in fibrinogen binding and aggregation is doubtful. Fibrinogen receptor clustering, however, has been proposed and was recently demonstrated by electron microscopy.

As mentioned previously, the final component of fibrinogen dissociation from ADP-treated platelets was unaffected by the degree of receptor occupancy and may therefore suggest some heterogeneity among platelet fibrinogen receptors. Indeed, observations by Kornecki et al., demonstrating the presence of high but low affinity binding sites on chymotrypsin-treated thrombasthenic platelets, and a report by Hawiger et al. demonstrating high affinity binding of isolated fibrinogen γ-chains to platelets as well as low affinity binding α-chains are consistent with this observation.

The present study suggests that interactions among platelet fibrinogen receptors occur that result in decreases in the apparent affinity of the receptors for ligand with increased receptor occupancy and may thus be responsible in large part for yielding upwardly concave Scatchard plots. It should be noted that these interactions (1) occur at physiologic fibrinogen concentrations, (2) are independent of platelet washing techniques as similar results were obtained with both aspirin-treated GFP and non-aspirin-treated WP, and (3) are independent of the purified fibrinogen preparations used, since similar association kinetics were observed using whole plasma as the major source of fibrinogen.

**ACKNOWLEDGMENT**

The author is grateful to Dr. Jolyon Jesty and Dr. Barry Coller for helpful discussions and criticisms.

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