The Effect of Aggregation and Release on Platelet Prothrombin-Converted Activity

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Platelets provide a procoagulant activity for the conversion of prothrombin to thrombin during normal hemostasis. This activity designated as platelet prothrombin-converting activity (PPCA) was monitored as rate of thrombin production in a two-stage assay using gel-filtered bovine platelets, factor Xa, and prothrombin. Expression of PPCA was not associated with ADP-induced release or platelet shape change but was associated with aggregation. Release of the contents of dense bodies, measured by release of [14C]-5-hydroxytryptamine, was not required for expression of PPCA during platelet aggregation. During the PPCA assay, 5-hydroxytryptamine was released, but only after onset of thrombin production. Furthermore, the release of 5-hydroxytryptamine was retarded during the assay by the addition of 2 mM theophylline and 100 nM prostaglandin E1, without a comparable reduction in PPCA. In addition, [125I]-factor-Xa was bound in greater amounts to platelets (aspirin-treated) after ADP-induced aggregation (without detectable release) than to unactivated control platelets. Finally, the PPCA of the ADP-activated platelets was saturated with respect to factors Xa and Va at less than 1 nM concentrations, indicating that the aggregation induced by ADP leads to the exposure of specific procoagulant sites by some process other than dense body secretion.

At least two reactions in the overall process of clot formation are accelerated by platelets: activation of factor X by factor IXa and factor VIIIa, and activation of prothrombin by factor Xa and factor Va. Phospholipids can substitute for platelets in these reactions, suggesting that the platelet contribution involves expression of platelet phospholipid. However, Walsh has theorized that separate specific sites on the platelet contribute to the two different activation steps described above. This would require a more complex platelet contribution than that supplied by phospholipids alone. Furthermore, Miletich et al. has proposed that prothrombin activation is dependent on platelet factor V and on the active phospholipids that are associated with the vesicular release of 5-hydroxytryptamine.

The difference between the earlier view of the importance of phospholipids, indicated above, and that of the later view of specific sites arose from the difference in assay systems. The earlier work employed an assay in which the clotting process was initiated by the addition of the venom from Vipera russelli, which coupled platelet contribution with formation of clots. This assay discriminated between native and activated platelets but yielded similar results for certain phospholipids and activated platelets. Later studies investigated prothrombin activation using...
washed platelets and purified factor Xa and prothrombin. Using this assay, platelets were observed to be more effective than phospholipid and to provide a factor-V-like activity referred to as platelet factor V.

Although the systems using purified components have added support to the hypothesis of multiple sites, the precise details of the mechanism of conversion from inactive to active sites on platelets remain unknown. In our studies we have employed an assay using purified reagents and have investigated at what stage of platelet activation—shape change, aggregation or release (secretion)—that catalytic activity for prothrombin activation appears.

MATERIALS AND METHODS

Reagents

Bovine serum albumin (3x recrystallized), bovine fibrinogen (95% clottable), benzamidine/HCl, N-2 hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), Echis carinatus venom, disodium ethylenedinitriolotetraacetate (EDTA), theophylline, prostaglandin E1, NADH, acetylsalicylic acid (ASA), sodium dodecylsulfate (SDS), and tris-hydroxmethylaminomethane (Tis) were obtained from Sigma Chemical Company. Imipramine was a gift from Ciba. Aspirin was purchased from Bayer Company; Handifluor for radioactivity detection from Mallinckrodt; Siliclad from Clay Adams; Sepharose-2B from Pharmacia Fine Chemicals Company; 5(2I4C)hydroxytryptamine ([I4C]-5-HT) from New England Nuclear; and Human Sensor i251 fibrinogen (85% of the radioactivity was clottable) from Abbott Laboratories.

Stock solutions of reagents were prepared as follows: aspirin was stirred for 1 hr in distilled water containing a predetermined amount of KOH to yield a final pH of 7.4. At the end of the stirring the solution was cleared of carrier by filtration and adjusted to 18 mM. Alternatively, ASA was dissolved in ethanol, and this solution was diluted with water, adjusted to pH 7.4, and adjusted to a final concentration of 100 mM. Prostaglandin E1 (PGE1) was dissolved, 1 mg/ml, in dimethyl-sulfoxide-containing butylated hydroxytoluene of 100 Mg/ml, final concentration. This stock solution was serially diluted into saline before addition to platelets.

Factor Xa was radiolabeled with [125I] by the lactoperoxidase procedure, as modified by Dahlback and Stenflo, to a specific activity of 3200 cpm/ng. No biologic activity was lost during the iodination. SDS-polyacrylamide gel electrophoresis showed 78% of the label in factor Xa and about 8% in possibly unactivated factor X. The remaining 14% was fairly uniformly distributed throughout the gel.

Factor V was assayed according to the method of Kappeler; factor Va activity was expressed as bovine factor units, where 1 unit is the activity present in 1 ml of bovine plasma. Bovine factor Xa and prothrombin were prepared according to Owen et al. and factor Va according to Esmon. These factors were dialyzed into the gel-filtration buffer immediately before use, as described below.

Factor Va was prepared by activating purified factor V with thrombin and quantitatively separating the thrombin from the factor Va by ion-exchange chromatography. The preparation exhibited a single, somewhat diffuse, band when examined on alkaline disc-gel electrophoresis. The factor Va possessed two subunits, both of which are required for activity. The properties of the factor Va are exactly as described in a previous publication.

Prothrombin was purified as described previously. No detectable heterogeneity was observed following alkaline or SDS-polyacrylamide gel electrophoresis. All detectable protein was rapidly and completely cleaved by incubation with thrombin.

Factor X2 was purified by chromatography of the BaSO4 eluate on QAE-Sephadex. Trace contaminating proteins were removed by a combination of chromatography on meta-aminobenzamidine-agarose followed by gel filtration on Ultrogel 34. The factor X2 exhibited a single band on alkaline disc-gel electrophoresis, a single band on SDS-polyacrylamide gel electrophoresis prior to disulfide bond reduction, and 2 bands following disulfide bond reduction. The preparation had a specific activity of 130 U/mg. Factor X was converted to factor Xa by incubation with the purified factor X activator from Russell's viper venom. Factor Xa was isolated by chromatography on QAE-Sephadex. Fresh preparations exhibited a single band on alkaline disc-gel electrophoresis.
**Preparation of Platelet-Rich Plasma**

Blood was collected from the jugular vein of 1-12-mo-old cows into a syringe containing anticoagulant. One volume of 3.5% sodium citrate, pH 6.3, added to 9 volumes of blood, maintained the pH between 7.0 and 7.8. The blood was then centrifuged in 30-ml Corex centrifuge tubes (Corning) for 2 min at 750 g and for 5 min at 1200 g. The platelet-rich plasma (PRP) was harvested for gel filtration. Glass syringes, 15-gauge needles, and centrifuge tubes were all siliconized.

**Gel Filtration of Platelets**

PRP (5–10 ml) was filtered through Sepharose-2B (60–250 μm particle size) packed in a 50-ml plastic syringe barrel. The column was equilibrated with and platelets were gel-filtered into one of the buffered salt solutions developed by Tangen and Berman, which contained 145 mM KCl, 100 mM MgCl₂, 5.5 mM glucose, and 5 mM HEPES, pH 7.4 (HEPES was substituted for Tris). Bovine serum albumin was dialyzed extensively against this buffer and was present in concentrations of 0.1% (w/v) in the buffer used for column equilibration and elution of platelets. The agarose column (2.6 x 9 cm) was supported by a stainless-steel filter support (Millipore) held in place with a neoprene O-ring. The agarose was equilibrated by passing 2.4 bed volumes of buffer solution through the column before filtering the PRP (5–10 ml). Shortly after the platelets began to elute, 5–8 ml was collected. The final gel-filtered platelet (GFP) concentration was 60%-80% of the PRP concentration. Gel filtration was completed in less than 15 min, and experiments with GFP were begun 45-60 min following blood collection.

**Platelet Prothrombin-Converting Activity (PPCA) Assay**

The assay was a classical two-stage assay composed of an activation stage and a clotting stage. The first stage contained the following components: approximately 8 x 10⁷ platelets, 0.17 U of factor Va, 260 ng of factor Xa, and 64 μg of prothrombin in 1 ml of gel-filtration buffer that contained 1% albumin and to which CaCl₂ was added to a final concentration of 10 mM. For the second stage, 0.1-ml samples were removed at intervals from the activation mix and added to 0.2 ml of cold 4.5 mM EDTA. Thrombin produced in the first stage was measured as the time required for 0.1-ml aliquots of this diluted sample to clot 0.1-ml volumes of 1% fibrinogen (w/v) at 37°C. A calibration curve was constructed by plotting the clotting time of a completely activated prothrombin sample versus the reciprocal of the unit of thrombin (assuming 1 200 U of thrombin can be generated from 1 mg of prothrombin). The clotting time of selected dilutions of the sample (in stage 2 of the PPCA assay) was converted to units of thrombin using this calibration curve.

**Assay of ¹⁴C-5-HT Release**

Prior to gel filtration, PRP was incubated with 0.6 μM ¹⁴C-5-HT for 10 min at room temperature. Gel filtration was performed after the 10-min incubation. The gel-filtered platelet sample for which release was to be determined was immediately centrifuged for 30 sec in a Microfuge (Beckman) and radioactivity in the supernatant counted. Radioactivity was also counted in the supernatant of a control sample of platelets, centrifuged at the same time as the experimental sample. Release was calculated by dividing the radioactivity of the supernatant of the experimental sample less that in the control supernatant by the radioactivity in the control sample less that in the control supernatant. This radio times 100 is percent release. Release measured in this manner is reported from the time the centrifugation began. Rarely did the radioactivity of the control supernatant exceed 1% of the total radioactivity in the control platelet preparation. EDTA added before centrifugation did not decrease this 1% value and, therefore, was not used in these experiments.

**Monitoring Lysis by Lactate Dehydrogenase Assay**

Lactate dehydrogenase was assayed by a modification of the method of Nisselbaum and Bodansky. The final assay solution of 30 μl contained 0.3 M sodium phosphate, 3 mM sodium pyruvate, 0.7 mM NADH, pH 7.4. One-hundred percent lysis was established by freeze-thawing (4x) a sample of the control GFP. The amount of sample lysis was assessed from aliquots of the supernatant from a 30-sec centrifugation (Beckman Microfuge), and lysis measured in this manner is reported from the time the centrifugation began.
Monitoring Aggregation of Platelets

Aggregation of platelets was monitored in a Chrono-Lab aggregometer according to the method of Born.\textsuperscript{15} Percent aggregation is defined as the percent change in light transmittance with the buffer set at 100\% and the sample of gel-filtered platelets set at 0\%.

Factor-Xa-Binding Assay

Factor Xa binding to platelets was measured by a modification of the method of Kenney et al.,\textsuperscript{16} which was an adaptation of the procedure of Feinbert et al.\textsuperscript{17} Gel-filtered platelets, whether activated or not, were treated as described in each particular experiment and were then incubated with radiolabeled factor Xa for 15 min at room temperature. A 0.5-ml aliquot of this solution was layered onto 0.5 ml of a silicone-oil mixture. This mixture was composed of Dow Chemical Company silicone-oil mixture (1.070 g/ml) adjusted to 1.030 g/ml with light mineral oil (Mallinckrodt Chemical Company). The sample was centrifuged 5 min in a Beckman Microfuge. The aqueous layer was withdrawn and the oil poured out and allowed to drain. The bottom tip of tube containing the platelet pellet was cut off and the radioactivity measured in a Beckman Biogamma Counter.

RESULTS

Characterization of GFP

These platelet suspensions were first examined for contaminating plasma proteins. Platelet supernatants (Beckman Microfuge, 30 sec) had 0.035\% of the plasma factor V activity. Unactivated GFP at concentrations comparable to those in PRP had factor V activities of 0.05\% of that in plasma, whereas platelets activated by freezing in a dry-ice–ethanol mixture and thawing at 37\degree C 4 times displayed significant factor V activity (0.0015 U/10\textsuperscript{8} platelets), or about 0.8\% of that in plasma. These results are qualitatively similar to those reported previously for human platelets.\textsuperscript{6} Unactivated GFP were also tested for factor Xa and prothrombin using the PPCA assay. No prothrombin was converted to thrombin in this assay if factor Xa was not added, and likewise, no thrombin was detected when factor Xa was added without prothrombin.

The efficiency of gel filtration for removing fibrinogen was also tested, as indicated in Fig. 1. The results from this experiment indicated that free fibrinogen was almost entirely removed from the platelet suspension.

The platelets were tested for retention of functional activity (Fig. 2). GFP did
not aggregate as well as platelets in PRP when stimulated with ADP (45% of control) and, furthermore, they lost their capacity to aggregate much more rapidly than PRP. However, these platelets, when diluted 1:1 with plasma, would respond as well as the platelets in PRP diluted 1:1 with the gel-filtration buffer. Platelets, gel-filtered without albumin in the agarose gel equilibration buffer, were unable to regain their full ability to aggregate.

The ability of GFP to aggregate in response to ADP suggests that a plasma cofactor was not removed completely by gel filtration. The loss in aggregability of GFP with time suggests the cofactor was bound but was diffusing off the platelets. However, no permanent process was responsible for the loss in aggregation, since the platelets in dilute plasma at 37°C regain all their activity. This cofactor could have been fibrinogen, since the labeled fibrinogen used to test the effectiveness of the gel filtration may not have had sufficient time to exchange with a bound cofactor. We elected not to attempt removal of the possible cofactor that would require the addition of fibrinogen or another cofactor to regain response to ADP. Rather, we chose to use the retained aggregability for examining the mechanism by which this aggregation promotes PPCA.

**Experimental Procedure**

The experimental procedure for the studies described below (Fig. 3) involved three steps. Step 1 involved aggregation of washed platelets by addition of ADP. This step included appropriate controls and, in selected cases, pretreatment of platelets with aspirin to inhibit release. Platelets were observed for aggregation and \(^{14}\)C-5-HT release. Step 2 involved generation of thrombin following addition of factor Xa and prothrombin to the platelets, treated by one of a variety of protocols in step 1. This step included appropriate controls and, in selected cases, theophylline and PGE\(_1\) were added just prior to addition of factor Xa and prothrombin to
Fig. 3. Outline of experimental procedure used in the study of the response of gel-filtered washed platelets in ADP, employing the PPCA, $^{14}$C-5-HT release, and aggregation assays. ASA was added to most samples to inhibit release during ADP-induced aggregation (step 1). Theophylline and PGE, were used in one set of experiments to delay and reduce platelet release of $^{14}$C-5-HT (step 2). Platelet aggregation was observed following stirred ADP treatment (step 1). $^{14}$C-5-HT release was monitored during both the ADP-induced aggregation (step 1) and in one set of thrombin generation experiments (step 2). PPCA of platelets was monitored in both the initial and maximal phase of thrombin production in step 2 by moving samples into step 3 (addition of fibrinogen) at intervals after addition of prothrombin and factor Xa.

inhibit or retard thrombin-induced release from platelets. $^{14}$C-5-HT release was also measured in specific cases. Step 3 was simply a measure of the thrombin produced as outlined in description of the PPCA assay.

Characterization of the PPCA Assay System

In the first series of studies, platelets were aggregated with ADP and their expressed catalytic activity compared with that of native platelets using the PPCA assay system. This activity was expressed as the rate of thrombin production (step 2 in Fig. 3). The results are shown in Fig. 4. Aggregated platelets generated thrombin 2–4 times more rapidly than did native control platelets. In both the aggregated and control series, two rates of thrombin generation were seen. There was an initial low rate of thrombin production for the first 3–5 min, which was followed by a maximal rate of thrombin production. Both the initial and maximal rates were a function of the number of platelets in the reaction system (Fig. 5). This dependence permitted definition of the PPCA unit as: thrombin produced/min/10$^8$ platelets under conditions where platelet numbers are rate limiting.

Figure 4 demonstrates not only that washed platelets were rate limiting in this system, but also that they limited the absolute amount of prothrombin converted to thrombin. All of the prothrombin was converted to thrombin by the platelets pretreated with ADP, whereas only a limited amount was converted by native platelets. This limited conversion almost certainly was due to the limit of substrate that resulted when the remainder of the prothrombin not converted to thrombin was converted to prethrombin-1 by thrombin (not by factor Xa) generated in the assay system. This explanation was supported by the fact that 80 µg/ml of prothrombin-activating protein from Echis carinatus venom (which converts prethrombin-1 to thrombin) raised the thrombin concentration of the assay system with native platelets to that expected if all the prothrombin present had been activated.
Fig. 4. The time course of production of thrombin in the PPCA assay. Gel-filtered platelets were prepared and handled as described in Materials and Methods and in Fig. 1, respectively. The step 2 reaction mixture contained 8 x 10^7 platelets, 0.17 U of factor Va (when present), 260 ng of factor Xa, and 64 μg of prothrombin in 1 ml of gel-filtration buffer containing 1% (w/v) albumin and 10 mM CaCl2. The reaction was quenched at designated times by adding 1 part of the reaction mixture to 2 parts of 4.5 mM EDTA, pH 7.4. After rewarming to 37°C, step 3 of the study was initiated by adding 1 part of the reaction mixture in EDTA to 1 part of 1% (w/v) clottable fibrinogen, and the time of clot formation measured. The clotting time was converted to thrombin units by use of a standard curve, and the thrombin units produced were plotted versus the time of incubation in step 2. The solid circles indicate platelet samples pretreated with 12 μM ADP and allowed to aggregate maximally, and the open circles indicate platelets that were untreated. In cases where factor Va was added, the rate of thrombin production was doubled.

Fig. 5. The dependence of initial and maximal thrombin production on platelet concentration. The conditions were the same as specified in Fig. 2 for the PPCA assay, but the platelet concentration was varied. The initial rates (solid circles) were calculated from the slope of a line drawn tangentially to the initial thrombin production curve (0–5 min period depicted in Fig. 2) and the maximal rates (open circles) were obtained from the slope of a line drawn tangentially to the thrombin production curve just before the substrate was exhausted in step 2 of the study. The rates varied linearly with the number of platelets. Therefore, it was possible to define the PPCA unit as thrombin units produced/min/10^7 platelets.
Correlation of PPCA Expression With Events in Step I (Fig. 3)

In the second series of studies, the initial rate of PPCA development was correlated with platelet aggregation and \(^{14}\text{C}\)-5-HT secretion. The assumption was that the initial rate of PPCA development was influenced by prior manipulation in step 1 (see Fig. 3), whereas the maximal rate of PPCA development reflects a thrombin feedback in step 2 (see Fig. 3). The rate of initial PPCA development over time against the extent of aggregation (see Fig. 6) showed that PPCA formation was dependent on aggregation and not simply on the presence of ADP. PPCA development was not observed in platelet systems to which ADP was added unless these platelets were stirred. Platelets that were not stirred neither aggregated nor expressed PPCA, although shape change did occur. Finally, Fig. 5 shows that, though platelet aggregation was reversed with time, PPCA once developed was not reversed.

Secretion occurred during the ADP-induced aggregation of platelets described above (see Fig. 3), as well as during the generation of thrombin (step 2, Fig. 3). Therefore, in the third series of studies, inhibition of platelet secretion, measured by release of \(^{14}\text{C}\)-5-HT was attempted. Figure 7 (A and B) compares the PPCA expression of platelets exposed to ADP, which aggregated and secreted, with those platelets treated with ASA, which were aggregated but secreted reduced amounts of \(^{14}\text{C}\)-5-HT (step 1, Fig. 3).

The correlation between aggregation and PPCA was preserved in both experimental settings, regardless of whether or not secretion occurred. Furthermore, the correlation between \(^{14}\text{C}\)-5-HT release and PPCA was not preserved because there was twice as much PPCA developed in platelets that released only half as much \(^{14}\text{C}\)-5-HT. Thus, the enhancement in initial PPCA correlated more consistently with the extent of aggregation of platelets than with the amount of \(^{14}\text{C}\)-5-HT release. The appearance of \(^{14}\text{C}\)-5-HT in plasma would not necessarily indicate secretion, since lysis would lead to the same results. However, in an aggregation experiment using 12 \(\mu\text{M}\) ADP, untreated GFP released 35% and aspirin-treated GFP released 5% of \(^{14}\text{C}\)-5-HT, whereas lactate dehydrogenase was not released in measurable amounts in either sample.
Fig. 7. Correlation of initial PPCA with percent release of $^{14}$C-5-HT and percent aggregation induced by ADP. The methods for measuring platelet release of $^{14}$C-5-HT and platelet aggregation and the manner in which samples were handled are described in Materials and Methods and Fig. 3, respectively. The methods for measuring initial PPCA are outlined in Figs. 4 and 5. Half of the gel-filtered platelet samples were incubated 10 min at 37°C with 100 μM aspirin prior to assay (step 1, Fig. 3). Aggregation induced by ADP was monitored as outlined in Materials and Methods. When each sample had reached its maximum aggregation, aliquots were assayed for initial PPCA and release of $^{14}$C-5-HT. (A) The relation of PPCA to release of $^{14}$C-5-HT (□ aspirin-treated; ○ untreated). The dashed lines connect the increasing release obtained at final concentrations of ADP of 6, 12, and 24 μM, respectively. (B) The relation of PPCA to platelet aggregation (■ aspirin-treated; ● untreated). The initial point on the curve is from unactivated GFP, and the following 3 pairs of points were obtained at 6, 12, and 24 μM ADP, respectively.

Lack of Correlation Between Initial PPCA and Release During Step 2 (Fig. 3)

The above data suggest that aggregation, not $^{14}$C-5-HT release or lysis (in step 1, Fig. 3), is the determining factor in PPCA development. However, a simple statement that aggregation and not release results in PPCA formation (step 1, Fig. 3) cannot be made since significant release can occur during thrombin generation (step 2, Fig. 2). Table 1 shows results of a fourth series of studies in which PPCA and release during thrombin generation were both increased in the ADP-treated sample compared to the unaggregated sample. However, PGE$_1$ and theophylline depressed release in both aggregated and unaggregated samples but did not affect initial PPCA. More specifically, release was reduced in the ADP-treated sample by PGE$_1$ and theophylline to the same level as release by the control, whereas initial PPCA was not reduced at all. Although release could not be as completely inhibited during step 2, as it is during addition of ADP in step 1, these studies showed that the development of initial PPCA did not correlate with the timing or extent of $^{14}$C-5-HT released during the assay.

Correlation of PPCA With Factor Xa Binding

Finally, in an effort to show in physical terms that platelets treated with ADP develop PPCA as a function of aggregation and not of dense body secretion, the
Table 1. Effect of PGE, and Theophylline Treatment on Initial PPCA and on Release of ³⁵C-5-HT Induced by Thrombin Produced During the PPCA Assay

<table>
<thead>
<tr>
<th>Platelet Treatment*</th>
<th>Initial PPCA (units)</th>
<th>1 min</th>
<th>2 min</th>
<th>3 min</th>
<th>5 min</th>
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<tr>
<td>ASA</td>
<td>1.1</td>
<td>13</td>
<td>25</td>
<td>34</td>
<td>51</td>
</tr>
<tr>
<td>ASA, PGE, and theophylline</td>
<td>1.1</td>
<td>3</td>
<td>4</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>ASA, ADP</td>
<td>2.5</td>
<td>52</td>
<td>58</td>
<td>60</td>
<td>61</td>
</tr>
<tr>
<td>ASA, ADP, PGE, and theophylline</td>
<td>2.5</td>
<td>10</td>
<td>23</td>
<td>31</td>
<td>32</td>
</tr>
</tbody>
</table>

*Platelets were incubated with 100 µM ASA for 10 min, then gel-filtered, and the suspension adjusted to 2.5 µM imipramine. An aliquot of gel-filtered platelets, stirred with 12 µM ADP, reached maximum aggregation and exhibited a baseline 1% release of ³⁵C-5-HT as compared to the non-ADP-treated, gel-filtered control platelets. Aliquots of the non-ADP, treated GFP, and the aggregated GFP were incubated for 5 min at 37°C with 2 mM theophylline and 100 nM PGE, . Two other aliquots were incubated as controls. During step 2, Fig. 3, all four of these samples were incubated in PPCA reaction mixtures, and aliquots were withdrawn at minute intervals and assayed for ³⁵C-5-HT released and thrombin produced (Step 3, Fig. 3).

formation of factor-Xa-binding sites on the platelet was assessed in a fifth series of studies in a manner comparable to that of a Miletich et al, and Dahlback and Stenflo. According to their observations and to our studies with PPCA, factor-Xa-binding sites on platelets should develop coincident with the development of PPCA. Thus, without addition of prothrombin and the resultant complications arising from thrombin formation, ASA-treated platelets can be aggregated with ADP and, if PPCA is generated, factor-Xa-binding sites should also be generated. Therefore, we initiated a series of experiments whereby the relative binding of ¹²⁵I-labeled factor Xa to platelets (i.e., thrombin-activated and ADP-activated) was measured. Aliquots of these same suspensions of platelets also were assayed for PPCA. The results indicated that factor Xa binding to platelets was approximately proportional to their PPCA (Fig. 8) for the ADP-activated platelets. When the gel-filtered platelets were preincubated with 100 µM ASA for 10 min prior to gel filtration, ADP-induced aggregated platelets did not release ³⁵C-5-HT during aggregation nor after the addition of factor Xa.

These binding results can be compared with those of Dahlback and Stenflo. For GFP fully activated with thrombin, 1.5 ng factor Xa was bound at equilibrium to 10⁸ platelets, and the equilibrium constant was 2.8 × 10¹⁰ M⁻¹. The corresponding values reported by Dahlback and Stenflo were 2–3 ng/10⁸ and 2.8 × 10⁸–1.0 × 10¹⁰ M⁻¹. If the 1.5 ng is corrected for the radiolabeled impurities, as was probably done by Dahlback and Stenflo, about 2 ng of factor Xa was bound per 10⁸ platelets, but the equilibrium constant would also increase. The results reported in Fig. 8 show less factor Xa was bound in 15 min than at equilibrium (30 min), but the ratio between the amount bound at 15 min to the total in these experiments was the same as that calculated from the results of Dahlback and Stenflo. Less factor Xa was bound in 15 min to the ADP-activated platelets than to thrombin-activated platelets, but both samples were approaching saturation at the same free factor Xa concentrations. Therefore, ³⁵C-5-HT release was not necessary for binding, nor did its absence appear to effect affinity of binding. The saturation of these sites at low concentrations of factor Xa was also demonstrable by the PPCA assay.

We determined the concentration of factor Xa required to saturate the
Fig. 8. Binding of factor Xa to platelets as a function of the concentration of unbound factor Xa. The procedure for the measurement of binding is described in Materials and Methods. Platelets were activated either by thrombin (1 U/ml) or 6 μM ADP after adjusting the gel-filtered platelet suspension to 100 μM ASA, 2.5 μM imipramine, and 2.5 mM calcium chloride. Factor Xa was added to GFP 2 min after addition of thrombin or 5 min after the addition of ADP. Binding was assessed 15 min after the addition of 125I-factor-Xa or a mixture of 125I-factor-Xa and factor Xa at a molar ratio of 1:140. Aliquots that were treated identically, except only unlabeled factor Xa was added, were tested for release after the same 15-min incubation. Release was also measured in aliquots, before factor Xa had been added, 4 min after thrombin activation and 5 min after addition of ADP. Factor Xa, designated on the abscissa, represents only the concentration of unbound 125I-factor-Xa. The binding results as depicted were obtained from thrombin-activated platelets with 125I-factor-Xa alone (○) and with mixed factor Xa (□) and from ADP-induced aggregated platelets with 125I-factor-Xa alone (□) and with mixed factor Xa (■). The upper dashed line represents specific binding of factor Xa to thrombin-activated platelets and is the difference between the results obtained for 125I-factor-Xa alone and that when excess unlabeled factor Xa was present. The lower dashed curve designates specific binding for platelets activated during ADP-induced aggregation.

The results reported here demonstrate that the development of PPCA is not linked to secretion of 5-HT (that is, secretion of dense body contents). Although
Fig. 9. PPCA as a function of variation in factor Va and factor Xa concentration. Gel-filtered platelets were activated by aggregation induced by 12 μM ADP, and when aggregation had reached a plateau, aliquots were tested for PPCA as outlined in Figs. 4 and 5, except factor Va was added in varied concentrations to the PPCA assay in one experiment (open circles) and the factor Xa concentration was varied in the absence of factor Va in another experiment (closed circles).

Complete separation of PPCA expression from 5-HT secretion was not obtained, neither initial PPCA nor factor Xa binding correlated with this secretion. In some samples of GFP treated with ASA, no 5-HT was released during ADP-induced aggregation, but initial PPCA was expressed. These experiments were complicated by secretion of 5-HT caused by thrombin generated during the measurement of PPCA. Attempts to block 5-HT release with PGE1 and theophylline during the assay were only partly successful. However, factor Xa binding after platelet aggregation was the clearest demonstration of PPCA development without 5-HT release. In this case, the problem of thrombin feedback was eliminated, and no release of 5-HT occurred during the 15-min incubation with factor Xa.

Previous reports were divided on whether secretion was necessary for platelet factor 3 activity. Spaet and Cintron first reported that aggregation of human platelets in PRP was necessary but not 5-HT release\textsuperscript{18} when ADP was the activating agent,\textsuperscript{17} and Hardisty and Hutton confirmed this.\textsuperscript{19} Joist et al.\textsuperscript{20} have questioned these results obtained in PRP and reported that platelets in a washed system aggregated without 5-HT release in the presence of 100 μM ADP. In this system, the platelets did not develop platelet factor 3 activity. Only when 5-HT was secreted along with small amounts of lactate dehydrogenase or chromium-51 (both markers of the release of cytoplasm), was significant platelet factor 3 expressed. They concluded that either release or lysis was necessary for platelet factor 3 activity.\textsuperscript{20} More recently, using a washed platelet system, Walsh also found simultaneous release of 5-HT and appearance of platelet factor 3 activity in ADP-activated washed platelets.\textsuperscript{21} As mentioned before, Miletich et al.\textsuperscript{1} reported factor Xa binding on thrombin-activated platelets correlated with 5-HT secretion.

Our results, indicating that 5-HT secretion is not related to PPCA development, are not necessarily in conflict with any of the previous findings. Separation of PPCA expression from 5-HT secretion in the bovine washed platelet system
suggests that some process that occurs before this release, but during or shortly after aggregation, is necessary for PPCA. The report by Kaplan et al. on secretion of α-granules before dense bodies or lysosomal-type granules is one such possible process that would be consistent with all the present results. Other unidentified processes could be involved, or for that matter, more than one process might be necessary. PGE, and theophylline added after aggregation, but before PPCA assay, partially blocked the activity in some of our experiments, a fact consistent with two required processes.

Factor-Xa-binding results reported here support and expand upon those reported by Dahlback and Stenflo. The fully activated bovine platelets bound about 300 molecules of factor Xa. Platelets, less well activated by ADP-induced aggregation, bound correspondingly less factor Xa. Factor-Xa-binding sites exposed by either thrombin or ADP were essentially saturated at a concentration of 10 ng of free factor Xa/ml of platelet suspension. These results are very similar to those obtained with human platelets.

These specific sites of PPCA differ in several respects to phospholipids in general. Miletich et al. reported the presence of a limited number of sites, each having the same high affinity for factor Xa. They later reported that the catalytic activity of these sites for prothrombin conversion per bound factor Xa was 15 times greater than were phospholipids. These same sites are saturated at low concentrations of factor V, also unlike phospholipid dispersions in general. These results certainly raise the possibility that another factor besides phospholipids might be involved in these sites.

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