Interaction of coagulation Factor Xₐ with human platelets
(hemostasis/thrombin/platelet factor 3/Factor V)

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ABSTRACT When human ¹²⁵I-labeled Factor Xₐ is incubated with washed platelets, prothrombin, and Ca²⁺, a small amount of thrombin is formed which causes the platelet release reaction after a period of time that decreases as the Xₐ concentration is increased from 0.9 to 19 ng/ml. After a further lag period, the Xₐ binds reversibly to receptors on the platelet surface and rapid thrombin formation follows (3 units or 1 µg of thrombin formed per min per ng of Xₐ bound to 10⁶ platelets). When platelets are treated with either thrombin (0.5 units/ml) or calcium ionophore A23187 prior to addition of Xₐ, binding begins immediately. Thrombin formation occurs at the platelet surface at rates that correlate with the amount of Xₐ bound. Dibutyryl cyclic AMP inhibits the release reaction, Xₐ binding, and rate of thrombin generation in parallel. The platelet Xₐ receptor is distinct from the previously described thrombin receptor and appears to be a protein because treatment of platelets with thrombin at 50 units/ml destroys Xₐ binding sites. The results suggest that specific receptors for Xₐ appear on the platelet surface after the release reaction occurs. The bound Xₐ catalyzes thrombin formation 1000-fold faster than does Xₐ added to reactions in which phospholipids are substituted for platelets.

The first stage in hemostasis is thought to be adhesion of platelets to subendothelial collagen or other substances at sites of injury. The platelet release reaction then occurs and secretion of numerous substances, including ADP and products of arachidonic acid transformation, is believed to promote further platelet aggregation with the eventual formation of a primary hemostatic plug. Later, fibrin accumulates to provide a more permanent arrest of bleeding. Understanding of the interactions between platelets and plasma coagulation factors in generating a fibrin clot is incomplete. Numerous investigators have observed that platelets accelerate the clotting of plasma (for review, see ref. 1). The term "platelet factor 3" historically has been used to describe this procoagulant activity, although neither its subcellular localization nor its chemical nature has been clearly defined (2). Factor Xₐ catalyzes the conversion of prothrombin to thrombin. Factor V, Ca²⁺, and phospholipid increase the rate of the reaction by organizing the enzyme and substrate into a lipoprotein complex (3). Milstone (4) showed that phospholipid and Factor V can replace platelets in prothrombin activation. Phospholipid can substitute for platelets in various assays that measure the clotting time of plasma as well, and it has been proposed that platelets may promote clotting by providing a phospholipid surface (5). Marcus et al. (6) suggested that more than phospholipid is involved because 20 times more extracted platelet phospholipid is required to achieve the same clot-promoting activity as that present in the intact membranes. Experiments to measure the interaction of individual plasma coagulation factors with platelets require purified factors and platelets washed free of plasma. We have previously described the interaction of thrombin with washed human platelets (7). Numerous experiments suggest that the first step in the action of thrombin on platelets is binding of thrombin to a specific cell surface receptor (7–10). We now report the first of our studies to determine if thrombin is actually formed on the platelet surface. We have measured the interaction of homogeneous ¹²⁵I-labeled Factor Xₐ (¹²⁵I-Xₐ) with platelets and the effect of bound Xₐ on thrombin generation. Jevons and Bartoin (11) demonstrated that bovine Xₐ caused platelet aggregation and release when the platelets were not washed free of plasma prothrombin. Undoubtedly this reaction was due to thrombin that was generated and then bound to its receptor. Our experiments demonstrate that platelets also have a distinct receptor for Xₐ that only appears on the platelet surface after the release reaction occurs.

MATERIALS AND METHODS

Platelets were isolated from human blood and washed to remove plasma as described previously (12). Human peripheral blood lymphocytes, provided by John Rogers, were stimulated with phytohemagglutinin, cultured for 5 days, and harvested in logarithmic growth (13). They were 97% viable by trypsin blue exclusion.

Materials were obtained from the following sources: carrier-free [¹²⁵I]iodide, [¹³¹I]iodide, and [¹⁴C]serotonin biodoxalate (44 µCi/µmol) from New England Nuclear; bovine fibrinogen ("90% clottable") from Miles Laboratories; reagents for determination of lactic dehydrogenase (14), bovine serum albumin, ovalbumin, Factors VII- and X-deficient plasma, and rabbit brain cephalin from Sigma Chemical Co.; Gi-TROL coagulation control human plasma, level 1, from Dade; Apiezon oil from J. B. Biddle Co.; all other chemicals were reagent-grade products of Sigma, Fisher, or Mallinckrodt. Fat-free bovine serum albumin was prepared (15). Calcium ionophore A23187 was provided by Robert L. Hamill of Lilly Research Laboratories.

Preparation of Coagulation Factors. Prothrombin and Factor X were prepared from cryo-poor fresh-frozen human plasma, kindly provided by William Miller of the Missouri-Illinois Regional Red Cross Blood Program. The basic scheme involves barium citrate adsorption, ammonium sulfate elution, and chromatography on DEAE-cellulose and heparin-agarose. The prothrombin had a specific activity of 1500 thrombin units/mg of protein in a two-stage assay (16) and was free of contamination by Factor X or Xₐ; the Factor X preparation had a specific activity of 225 units/mg relative to control plasma and was initially free of Xₐ activity. Both proteins were homogeneous as judged by polyacrylamide gel electrophoresis in the absence (17) and presence (18) of sodium dodecyl sulfate.

Abbreviation: ¹²⁵I-Xₐ, ¹²⁵I-labeled Factor Xₐ.
The X-coagulant protein was prepared from Russell's viper venom (19). Human thrombin was isolated as described by Fenton et al. (20) after activation of the highly purified thrombin by incubation with either Taipan snake venom or Xa in the presence of Ca²⁺ and rabbit brain ephalin.

Xₐ was formed by incubating Factor X with 0.1% (wt/wt) of the X-coagulant protein at 37° for 15 min in 0.15 M sodium chloride/0.01 M calcium chloride/0.02 M Tris, pH 7.4. Xₐ was isolated by chromatography on QAE-A50 developed with a linear sodium chloride gradient from 0.15–0.40 M. When 0.1 ml of 0.025 M calcium chloride, 0.1 ml of rabbit brain ephalin (400 µg/ml), 0.1 ml of Xₐ (10 ng/ml) in 0.15 M sodium chloride/5.1 mM sodium citrate/7.1 mM sodium acetate/7.1 mM sodium diethyl barbiturate/bovine serum albumin, 10 mg/ml, adjusted to pH 7.4 with hydrochloric acid, and 0.1 ml of Factors VII- and X-deficient plasma were incubated at 37°, the clotting time was 30 sec.

Factors X and Xₐ were labeled with a chloramine-T procedure (21) to a specific activity of 5,000–10,000 cpm/pg. No change in the clotting activity was detectable initially, although some loss of activity relative to unlabeled preparations was noted after 2–3 weeks.

Coagulation Assays. All assays were done in a Fibrometer (BBL). The method of Fenton and Fasco (22) for determining thrombin activity was modified to make the assay volume 0.3 ml. Log-log plots were linear from final thrombin concentrations of 0.04 to 1.0 unit/ml, corresponding to clotting times of 160 to 15 sec.

Prothrombin and Factor X were assayed by standard methods (16, 23).

Isotope Assay. 125I-Iodide activity was determined in a Beckman Biogamma II gamma counter and 14C-serotonin, in a Beckman LS-230 liquid scintillation circuit. The ratio of isotopes was adjusted to minimize the correction of 125I activity in the liquid scintillation counter.

Interaction of Xₐ with Platelets. Immediately before use, washed platelets (1 × 10⁸/ml) were incubated with 5 µM 14C-serotonin for 15 min at 37°, centrifuged for 10 min at 2000 × g, and resuspended in a buffer containing fat-free albumin (5 mg/ml), glucose (1 mg/ml), 0.14 M sodium chloride, 2.5 mM calcium chloride, and 0.02 M Tris at pH 7.4. Reactions mixtures contained 1 × 10⁶ platelets per ml and 73 µg of prothrombin per ml (when added). After incubation in 12 × 75 mm Falcon tubes at 22°, the platelet suspensions (0.5 ml) were carefully layered onto 0.5 ml of an oil mixture (1 part Apiezon, 9 parts n-butyl phthalate, specific gravity 1.029) in 1.5-ml Eppendorf conical centrifuge tubes. After centrifugation at 12,000 × g in a Brinkmann microcentrifuge for 2 min, 0.2 ml of supernatant was removed for determination of unbound 125I activity or release of 14C-serotonin or both. The remaining liquid was aspirated and the tube (containing the platelet pellet) was cut off and its radioactivity was measured.

RESULTS

Binding of Factors X and Xₐ to Platelets. In preliminary experiments, we attempted to measure the binding of 125I-labeled Factor X and its activated derivative, 125I-Xₐ, to washed human platelets by using both Millipore filtration and oil centrifugation, methods that have been demonstrated to be effective in the binding of thrombin to platelets (7, 24). No significant binding of either was observed in the absence of Ca²⁺ at concentrations ranging from 1 ng/ml to 10 µg/ml. Approximately 0.2–0.3% of the total radioactivity was found associated with the platelet pellet, which corresponds to values of trapped water determined independently by using 131I-labeled ovalbumin. In the presence of Ca²⁺, small increases in binding at low concentrations, particularly of Xₐ (see below), were detected but represented less than 3% of total radioactivity. No release of 14C-serotonin occurred in any of these experiments.

When prothrombin was included in reaction mixtures of platelets in buffer with Ca²⁺, addition of 125I-Xₐ resulted in slow formation of small amounts of thrombin which then caused the platelets to release 14C-serotonin but not lactic dehydrogenase. The time interval to obtain 50% of maximal release depended on the amount of 125I-Xₐ added, with values of 21 min at 0.9 ng of 125I-Xₐ per ml, 10 min at 4.7 ng, and 4.5 min at 19 ng (Fig. 1). After a further lag, 125I-Xₐ binding was observed, ranging from 0.18 ng of 125I-Xₐ bound per 10⁶ platelets at the lowest level to 1.5 ng at the highest level. The percentage of added 125I-Xₐ that bound to platelets varied from 20% to 8%. An increase in the rate of thrombin formation accompanied 125I-Xₐ binding, and the maximal rate obtained ranged from 0.86 to 3.6 units of thrombin per ml/min. Addition of 125I-Xₐ at concentrations of up to 100 ng/ml resulted in only a small further increase in binding (2.9 ng of 125I-Xₐ per 10⁶ platelets) and in rate of thrombin generation (4.6 units/ml per min), suggesting that Xₐ binding sites were saturated.

When the experiment shown in Fig. 1B was repeated in the presence of 200-fold excess (1.0 µg/ml) of unlabeled Factor X (zymogen), there was similar binding of 125I-Xₐ (0.7 ng per 10⁶ platelets) and the rate of thrombin formation was also unaffected (2.4 units/ml per min). This result suggests that the zymogen, Factor X, does not bind to the platelet Xₐ receptor.

The 125I-Xₐ bound to platelets in these experiments appeared to be unaltered as determined by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (Fig. 2). The stained gel shows the Xₐ used for these experiments and the radiochromatogram shows the 125I radioactivity in the platelet pellet after Xₐ binding occurred. The peaks of radioactivity corresponded to the stained heavy and light chains of Xₐ. The small shoulder of radioactivity in the latter is a radioactive contaminant in the 125I-Xₐ that migrates with the tracking dye. When the same amount of 125I-Xₐ was applied directly to control gels, the recovery of radioactivity in the peaks and the pattern of migration were the same as in Fig. 2.

Xₐ Binds to Platelets Only after the Release Reaction Has Occurred. When platelets were treated with thrombin (0.5 units/ml), Xₐ binding sites were available (Fig. 3A). In this experiment, the binding began immediately after addition of 125I-Xₐ (10 ng/ml) and reached 1.3 ng per 10⁶ platelets, which is similar to the binding measured in experiments shown in Fig.
1. This result indicates that prothrombin is not essential for $X_\alpha$ binding to platelets. Ca$^{2+}$ is required, and the optimal concentration of calcium chloride was 2.5 mM. Addition of prothrombin to the reaction mixtures after the $X_\alpha$ was bound resulted in immediate thrombin generation at high rates.

When platelets were incubated with 10 mM dibutyryl cyclic AMP for 20 min prior to addition of thrombin (0.5 unit/ml), the release reaction was inhibited by 50% as determined by measuring $[^{14}C]$serotonin release. We then added $^{125}$I-$X_\alpha$ and prothrombin and found that $^{125}$I-$X_\alpha$ binding to these platelets was decreased by approximately 50% and thrombin formation was also 50% of the rate obtained with control platelets. This finding suggests that platelet binding sites for $X_\alpha$ appear as a result of the release reaction rather than from some other action of thrombin on the platelet surface. When $[^{14}C]$serotonin release from platelets was stimulated by using calcium ionophore A23187, $X_\alpha$ binding sites appeared. In this experiment, platelet suspensions were incubated with either thrombin (0.5 unit/ml) or 1.0 $\mu$M A23187 for 15 min. $^{125}$I-$X_\alpha$ (10 ng/ml) was then added and the binding of $X_\alpha$ was measured. Both treatments resulted in 1.4 ng of $^{125}$I-$X_\alpha$ bound per 10$^8$ platelets in 20 min. Addition of prothrombin also resulted in immediate and rapid thrombin generation in both cases, indicating that thrombin itself is not specifically required.

**Characteristics of $X_\alpha$ Receptor Sites.** When 1000-fold excess unlabeled $X_\alpha$ was added (Fig. 3A) to platelets that had bound $^{125}$I-$X_\alpha$, the labeled $X_\alpha$ was rapidly displaced, indicating that binding is reversible. This result suggests that $X_\alpha$ binds at the platelet surface, but direct demonstration of this point by electron microscopy/autoradiography, as was done in studies of the thrombin receptor (7), was impossible due to the small number of $X_\alpha$ binding sites per platelet (e.g., in Fig. 3, 1.3 ng of $^{125}$I-$X_\alpha$ bound per 10$^8$ platelets corresponds to only 170 $X_\alpha$ molecules per platelet).

When platelets were treated with thrombin at 50 units/ml for 0–50 min and then 10 ng of $^{125}$I-$X_\alpha$ per ml was added for 10 min, there was a progressive decrease in $^{125}$I-$X_\alpha$ bound, from 0.9 ng per 10$^8$ platelets to 0.15 platelets (Fig. 3B). The latter value is similar to the amount of $^{125}$I-$X_\alpha$ not displaced by excess unlabeled $X_\alpha$ or bound in 10 min to platelets not treated with thrombin (Fig. 3A) and therefore may reflect binding not functionally related to the interaction investigated here. The decrease in $X_\alpha$ binding is not a result of platelet lysis. Less than 4% of the total lactic dehydrogenase activity of sonicated platelets was released from platelets treated with thrombin at 50 units/ml for 60 min. A likely explanation for this time-dependent effect of thrombin is proteolysis of the $X_\alpha$ receptor, although another possibility is a conformational change in the membrane that masks $X_\alpha$ receptor sites. Prolonged treatment of platelets with thrombin at 0.5 unit/ml did not affect $^{125}$I-$X_\alpha$ binding. This experiment also shows that thrombin does not compete with $X_\alpha$ for binding because the same amount of $^{125}$I-$X_\alpha$ was initially bound in the presence of thrombin at 0.5 or 50 units/ml (Fig. 3B, 0 time). In the latter case, the thrombin concentration was 0.5 $\mu$M, a 2500-fold molar excess over $^{125}$I-$X_\alpha$ (0.2 nM).

**Function of $X_\alpha$ Bound to Platelets.** The time course of increased thrombin formation shown in Fig. 1 suggested that the $^{125}$I-$X_\alpha$ bound to platelets accounts for the accelerated prothrombin activation. To determine if $X_\alpha$ must be associated with the platelets to convert prothrombin to thrombin at significant rates, the platelets had to be effectively removed from the reaction mixture. We treated platelets with thrombin at 0.5 unit/ml as outlined in Fig. 3 and then incubated them for 20 min with $^{125}$I-$X_\alpha$ (10 ng/ml). Platelets were separated from one portion of this reaction mixture by centrifugation through oil. The supernatant from this reaction mixture was further centrifuged for 10 min at 185,000 $\times$ g in a Beckman Airfuge to remove any platelet fragments. Another portion of the original
reaction mixture was centrifuged at $2400 \times g$ for 10 min, the supernatant was removed, and the pellet of aggregated platelets was resuspended in buffer. Prothrombin was then added to the high-speed supernatant solution, the resuspended pellet, and a portion of the original unspun reaction mixture. The maximal rate of thrombin generation was 2.5 units/ml per min in the original unspun mixture, 0.8 in the resuspended pellet, and 0.006 in the supernatant fraction. The $^{125}\text{I}$-Xa in the supernatant fraction (0.3 ng/ml) was assayed and found to be fully active. This experiment indicates that prothrombin activation is catalyzed by the Xa, that is bound to platelets.

The platelets do not serve merely as a source of phospholipid in these experiments. When rabbit brain cephalin (120 μg of phospholipid per ml) was used in place of platelets with prothrombin and 1–10 ng of Xa per ml, no detectable thrombin was formed in 60 min. When Xa was tested at 1 ng/ml, thrombin was initially formed at a rate (0.7 unit/ml per min) comparable to that observed when Xa at 1 ng/ml was added to platelets (Fig. 1A), although with the phospholipid the final thrombin yield was submaximal (10 units/ml). When Xa at 1 μg/ml was incubated with prothrombin alone or with prothrombin and human lymphocytes (5 $\times$ 10$^5$/ml), thrombin was formed at a rate of 0.014 unit/ml per min. Thus, 1 ng of Xa in a solution with platelets is at least as effective in initial rate of thrombin generation as 1 μg of Xa in a solution with phospholipid and 50-fold more effective than 1 μg of Xa in a solution with (or without) lymphocytes.

**DISCUSSION**

Our experiments enabled us to observe features of the interaction of Xa and platelets. Xa binds to the platelet surface after the release reaction has occurred. This association has the properties of a receptor–ligand interaction in that it is specific, saturable, reversible, and correlates with a physiological response (thrombin generation). The affinity of the receptors for Xa is high (less than 1% of the potential Xa concentration in plasma is saturating), and the activity of Xa is increased at least 50,000-fold when the enzyme is bound to the platelet. Of course, these experiments do not simulate in vivo hemostasis (e.g., lower temperature, absence of inhibitors and cofactors, and no opportunity for localization of platelets in high concentrations).

Papahadjopoulos and Hanahan (25) demonstrated that Xa can form a calcium-dependent complex with phospholipid. We believe that the receptor reported here cannot be solely phospholipid in nature because the number of sites per platelet is small (several hundred), the binding capacity of the receptor is destroyed by thrombin treatment, and only a 50-fold increase in Xa activity is observed when phospholipid is substituted for platelets.

The Xa receptor of platelets is similar to Factor V in a number of ways. Factor V also binds Xa (in the presence of Ca$^{2+}$) after treatment with low thrombin concentrations (26), accelerates prothrombin activation by Xa, and is inactivated by high concentrations of thrombin in a time-dependent manner (27). Factor V coagulant activity has been described in platelets (28, 29) and Österud, et al. (30) have shown that it is increased when the platelets are stimulated or broken by freezing and thawing. Whether the platelet Xa receptor is physically identical to plasma Factor V or Xa awaits further study. In preliminary experiments we found that an IgG fraction of plasma from a patient with an acquired inhibitor of Factor V blocked both Xa binding and rapid thrombin generation when added to thrombin-treated platelets. Control IgG had no effect. The Xa receptor is distinct from the thrombin receptor because Xa binding requires Ca$^{2+}$ and occurs only after the release reaction. Also, thrombin does not compete with Xa for binding. We have not yet evaluated the kinetics of the binding of thrombin generated by Xa at the platelet surface to the thrombin receptor. Whether Xa is also formed on the platelet surface is unknown, but future studies using factor IXa should resolve this question.

We speculate that the Xa receptor functions in hemostasis by localizing small amounts of Xa on the platelet surfaces at sites of platelet aggregates. This would allow for rapid local thrombin generation.

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