ADP and Epinephrine-Induced Release of Platelet Fibrinogen

By Karen L. Kaplan, Michele J. Dauzier, and Suzanne Rose

Human platelets gel-filtered into Tyrode's buffer containing 1 mM Mg$^{2+}$ and 0.35% bovine serum albumin were studied to determine whether they would undergo biphasic aggregation and release of α-granule proteins in response to adenosine diphosphate (ADP) or epinephrine without addition of exogenous fibrinogen. Fibrinogen concentration in the supernatant of unaggregated gel-filtered platelets was less than 1 pmole/ml. With addition of ADP or epinephrine, biphasic aggregation was seen, with release of platelet fibrinogen, β-thromboglobulin, and platelet factor 4. Fibrinogen concentration in the supernatant after aggregation ranged from 15 to 70 pmole/ml. Release of the α-granule proteins by epinephrine was coincidental with release of the dense granule adenine nucleotides. Aggregation and α-granule protein release by both ADP and epinephrine were inhibited by added Ca$^{2+}$ at 1–2 mM. The ability of gel-filtered platelets to undergo ADP- and epinephrine-induced aggregation and release in the absence of exogenous fibrinogen suggests that released platelet fibrinogen may be able to fulfill the requirement for fibrinogen in ADP- and epinephrine-induced platelet aggregation and release.

MATERIALS AND METHODS

Venous blood was collected from normal volunteers into 1/10 volume 3.8% citrate and centrifuged at 150 g for 15 min to prepare platelet-rich plasma. Platelets were separated from plasma by chromatography on Sepharose 2B or Biogel A. 50 M equilibrated with Tyrode's buffer$^{17}$ that contained 1 mM Mg$^{2+}$ and 0.35% bovine serum albumin or 0.35% human serum albumin (Sigma, St. Louis, Mo.) but no added Ca$^{2+}$. Gel-filtered platelets were used immediately after collection from the column. Aggregation studies were performed at 37°C with stirring at 900 rpm in a Payton dual channel aggregometer with Riken Denshi recorder. A quantity of 0.1 ml of aggregating agent was added to 0.9 ml of gel-filtered platelets (platelet count 160,000-300,000) in the aggregometer cuvette. In most experiments, aggregation was stopped by transferring the cuvettes rapidly to a melting ice bath. In some experiments, aggregation was stopped with addition of 1/4 volume 5% formaldehyde-EDTA$^{20}$ and then transferring the cuvettes to a melting ice bath. The cuvettes were centrifuged at 4°C for 15 min and the supernatants decanted for radioimmunoassay as described below. For the samples to which formaldehyde-EDTA was added, 200 μl 2M Tris hydroxymethylaminomethane was added to 800 μl of the supernatant before storage for assay in order to neutralize the formaldehyde.$^{22}$ ADP (Nutritional Biochemical Corp., Cleveland, Ohio) was dissolved in 0.9% saline in 10 μl and stored frozen. Epinephrine was either epinephrine-HCl for injection (Parke-Davis Co., Morris Plains, N.J.) or epinephrine bitartrate (Sigma Chemical Co., St. Louis, Mo.) dissolved in 0.9% saline for each experiment. All other chemicals were reagent grade. Release of β-thromboglobulin and platelet factor 4 was quantitated by radioimmunoassay.$^{15}$ Fibrinogen release was quantitated by radioimmunoassay for fibrinopeptide A$^{24}$ after treatment of the samples with thrombin 1 U/ml for 1 hr at 37°C and in one experiment fibrinopeptide was assayed also.$^{15}$ The β-thromboglobulin assay was not affected by treatment of samples with formaldehyde-EDTA and

From the Columbia University College of Physicians and Surgeons, Department of Medicine, New York, N.Y.

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Address reprint requests to Karen L. Kaplan, M.D., Columbia University College of Physicians and Surgeons, Department of Medicine, 630 West 168th Street, New York, N.Y. 10032.

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neutralization with Tris, but in the platelet factor 4 and fibrinopeptide A assays, the immunoreactivity was markedly decreased by this treatment. To assay fibrinogen or fibrin monomer that might have become associated with the platelets during the gel-filtration, a radioimmunoassay for fibrinogen was developed. The antibody for this assay was a rabbit antifibrinogen antibody kindly provided by Dr. Clarence Merskey. This antibody does not distinguish between fibrinogen and its proteolysis products.

Radiolabeled tracer was prepared by incubating 2.5 mCi $^{125}$I with 30 μl 1.0M phosphate buffer, pH 7.5, 250 μg fibrinogen (Kabi, grade L, Stockholm) in 25 μl 0.15M NaCl, and 3.75 μg chloramine T in 10 μl phosphate buffer for 1 min at room temperature. The reaction was stopped by addition of 3.75 μg sodium metabisulfite in 10 μl phosphate buffer and then 100 μl 0.1M Tris-0.05M NaCl, pH 7.5, containing 0.1% ovalbumin. $^{125}$I-fibrinogen was separated from iodine by gel filtration on Sephadex G-75 and stored at 4°C. The radioimmunoassay buffer was 0.10M NaCl-0.05M Tris, 0.02% NaN₃, pH 7.4, which contained 1 U/ml heparin and 3.5% bovine serum albumin. The assay was performed in 12 x 75 mm polypropylene tubes. Buffer was added to the tubes before other reagents, and the tubes were inverted to coat the walls with albumin. All reagents were diluted in the assay buffer. The final volume in the assay was 650 μl, of which 100 μl was antiserum, 100 μl was standard or sample, and 50 μl was tracer with the remainder buffer. Tubes were incubated overnight at 37°C and then bound $^{125}$I-fibrinogen was separated from free by precipitation of the bound tracer with Staphylococcal protein A (IgGisor Code ISGL 10, the Enzyme Center, Boston, Mass.). The protein A was reconstituted to 10 ml (10% suspension) and then diluted 1/10 with deionized water. One-hundred microliters were added to each tube and the tubes were incubated for 20 min at room temperature and then centrifuged for 10 min at 2200 g. The supernatant was decanted and the precipitate was counted. In this system 80% of the radiolabeled fibrinogen was bound at an antisera dilution of 1/8000 and 42% was bound at a dilution of 1/800,000 as used in the assay. Fifty percent displacement of binding of tracer was obtained with 90 ng/ml fibrinogen and the lower limit of detection of the assay (20% displacement) was obtained with 20 ng/ml. ADP and ATP were measured by the firefly luciferase method of Holmesen et al. on ethanol-EDTA extracts of gel-filtered platelets, using an Aminco microphotometer (American Instruments Co., Silver Spring, Md.).

RESULTS

The first question to be addressed in this study was whether gel-filtered platelets would aggregate in response to ADP or epinephrine in the absence of added fibrinogen. Figure 1 shows representative tracings of biphasic aggregation in the absence of added fibrinogen. Tracings obtained with 5 μM ADP and 6.6 μM epinephrine are shown. Fibrinogen release occurred during aggregation with both agents, with release of 15 pmole/ml with ADP and 24 pmole/ml with epinephrine in the experiments shown in this figure. In some experiments, biphasic aggregation and release of α-granule-associated proteins occurred at ADP concentrations as low as 1 μM and at epinephrine concentrations as low as 0.2 μM, but in other experiments higher concentrations of ADP or epinephrine were required and with some preparations of gel-filtered platelets no aggregation was seen even with high concentrations of ADP or epinephrine, although the platelets aggregated in response to collagen. In experiments in which aggregation was weak, addition of fibrinogen at 0.1 or 0.5 mg/ml enhanced the aggregation response (not shown). The supernatant fibrinogen concentration in gel-filtered platelets to which saline instead of aggregating agent
was added was 0.8 pmole/ml in the ADP experiment in Fig. 1 and 0.9 pmole/ml in the epinephrine experiment in Fig. 1. Because of the possibility that platelets were activated during gel filtration and were therefore able to bind plasma fibrinogen, released platelet fibrinogen, or fibrin monomer during the filtration, platelets were gel-filtered in the presence and absence of 1% EDTA, which should prevent fibrinogen or fibrin monomer binding. Aliquots of platelets were separated from buffer immediately after gel filtration and at intervals up to 1 hr after gel filtration and the supernatant assayed for fibrinogen using a radioimmunoassay for fibrinogen rather than thrombin treatment and radioimmunoassay for fibrinopeptide A because the radioimmunoassay for fibrinogen detects fibrin as well as fibrinogen. The average fibrinogen concentrations in Tyrode's buffer were 0.7 and 0.5 pmole/ml, and the average fibrinogen concentrations with EDTA were 1.3 and 1.3 pmole/ml in the two experiments. The amount of supernatant fibrinogen approximately doubled over the hour of incubation. In another experiment, EDTA was added after gel filtration and the supernatant was assayed for fibrinogen using the fibrinopeptide A and B assays and again there was not a significant increase in fibrinogen concentration with EDTA.

Additionally, because of the possibility that the bovine serum albumin used in the Tyrode's buffer contained bovine fibrinogen, which might be supporting the aggregation, experiments were carried out with human serum albumin. Tyrode's buffer prepared with human serum albumin contained <0.15 pmole fibrinogen/ml. ADP- and epinephrine-induced platelet aggregation and release of platelet fibrinogen using platelets in Tyrode's buffer with human albumin were not different from aggregation and release when bovine albumin was used in the buffer.

The detailed time course of release of fibrinogen was then determined in response to 50 μM ADP (Fig. 2) and 212 μM epinephrine (Fig. 3). The higher concentrations of ADP and epinephrine were used because with the lower concentrations used in the experiments shown in Figs. 1, 4, and 5, platelet reactivity deteriorated rapidly so that only a small number of time points could be obtained. Release of two other alpha-granule proteins, β-thromboglobulin and platelet factor 4, was also measured and release of ADP and ATP by epinephrine was determined. With both ADP and epinephrine there was an initial phase with a small amount of release followed by a phase of rapid release of the three proteins. With epinephrine, it was found that release of the three alpha-granule proteins coincided with release of the dense granule nucleotides, ADP and ATP. In four experiments in which release was stopped with addition of 5% formaldehyde-100 mM EDTA, rather than just immersion in ice, adenine nucleotide release again coincided with β-thromboglobulin release (data not shown). Platelet factor 4 and fibrinopeptide A could not be assayed in these experiments because of marked loss of immuno-reactivity with the formaldehyde treatment.

Because Ca²⁺ ions have been shown to influence ADP-induced aggregation of washed platelets in the presence of fibrinogen as well as ADP and epinephrine-induced binding of fibrinogen to platelets, the effect of added Ca²⁺ on ADP and epinephrine-induced aggregation and release of fibrinogen, β-thromboglobulin, and platelet factor 4 using gel-
filtered platelets was examined. As shown in Figs. 4 and 5, increasing the Ca\(^{2+}\) concentration of the suspending buffer caused a progressive decrease in release of the proteins and in the extent of aggregation. With ADP (Fig. 4), second phase aggregation was seen with 0, 0.1, and 0.25 mM added Ca\(^{2+}\) but only primary aggregation occurred with 1 and 2 mM added Ca\(^{2+}\). As seen in Fig. 5 epinephrine-induced aggregation was much less sensitive to Ca\(^{2+}\) than was epinephrine-induced release. Second wave aggregation with epinephrine was inhibited above 2 mM added Ca\(^{2+}\).

**DISCUSSION**

The studies described in this article demonstrate that human platelets can aggregate in response to ADP and epinephrine in the presence of very low initial fibrinogen concentrations in the suspending fluid, i.e., <1 pmole/ml. The present studies also show that aggregation in the presence of low fibrinogen concentrations is biphasic and that the second phase of aggregation is accompanied by release of alpha-granule proteins, i.e., fibrinogen, \(\beta\)-thromboglobulin, and platelet factor 4.

If fibrinogen is required for ADP and epinephrine-induced aggregation, there are various possible sources for this fibrinogen. We would like to propose that a small amount of platelet fibrinogen released in the
initial period of stirring with ADP or epinephrine is rapidly bound to the surface of the platelets to serve as a cofactor for aggregation and the major part of α-granule release. Alternatively, a small amount of plasma fibrinogen could be bound to platelets activated during gel filtration or a small amount of platelet fibrinogen could be released during gel filtration and bound to the platelet surface. In the experiments described above, in which EDTA was added before or after gel filtration to prevent association of fibrinogen with the platelets, there was <1 pmole/ml more fibrinogen in the supernatant with EDTA. Whether the association of this amount of fibrinogen with the platelets is sufficient to permit subsequent aggregation and release is not known. A third possibility is that some other molecule from the plasma, presently unidentified, serves as a cofactor for ADP- and epinephrine-induced aggregation. The first of these alternatives is the one we favor, but the data do not allow exclusion of the other.

The data also suggest that if platelet fibrinogen supports aggregation, it does so at lower concentrations than are required for exogenous fibrinogen, since even at maximal release the fibrinogen concentrations found in these experiments (from 15 to 70 pmole/ml) were less than the amounts that have been said to be required, but biphasic aggregation of 60% or more was still obtained. For example, Weiss and Rogers reported that 0.025 mg/ml fibrinogen (75 pmole/ml) added to citrated platelet-rich plasma of afibrinogenemic patients would bring aggregation by 2 μM ADP into the range seen with a group of normal controls and the extent of aggregation increased with increasing added fibrinogen up to 0.1–0.2 mg/ml (300–600 pmole/ml).12 Mustard et al. found that 0.5 mg fibrinogen/ml (1500 pmole/ml) added to washed platelets supported aggregation induced by 8.3 μM ADP, while no aggregation was seen without added fibrinogen.13 More recently, Marguerie and colleagues reported that 6 μM ADP caused no aggregation with 0.016 mg/ml fibrinogen (50 pmole/ml) with gradually increasing extent of aggregation up to 0.1 mg/ml (300 pmole/ml) added fibrinogen.17 Support of aggregation by released platelet fibrinogen could occur by binding of a fraction of the released fibrinogen to the platelet surface. There have been several reports of binding of fibrinogen to ADP or epinephrine-stimulated platelets.15–20 Some authors have found a single class of binding sites for fibrinogen on ADP-stimulated platelets, with \( K_d \) values for fibrinogen of 0.8 × 10⁻⁷ M,16 1.3 × 10⁻⁷ M,17 and 1.5 × 10⁻⁷ M.20 Peerschke and colleagues found two classes of fibrinogen binding sites on ADP-stimulated platelets, with \( K_d \) 1.3 × 10⁻⁷ for the high affinity site and 4.5 × 10⁻⁷ for the low affinity site.18 Niewiarowski and colleagues19 reported binding of fibrinogen to platelets stimulated by ADP or treated with chymotrypsin, and in each case found two classes of binding sites. For ADP stimulated platelets the \( K_d \) for the high affinity site was 3.2 × 10⁻⁸ M and for the low affinity site was 5.6 × 10⁻⁸ M, while for chymotrypsin-treated platelets the \( K_d \) for the high affinity site was 1.9 × 10⁻⁸ M and for the low affinity site was 3.8 × 10⁻⁸ M. In the experiments described in this report, supernatant fibrinogen concentrations after aggregation ranged from 15 to 70 pmole/ml (1.5–7 × 10⁻⁸ M); thus if Niewiarowski and colleagues' estimation of 1300 high affinity fibrinogen receptors on ADP-stimulated platelets, with \( K_d \) of 3.2 × 10⁻⁸ M is correct, then one could predict that released fibrinogen would be adequate for half-saturation of these receptors.

Calcium ions have previously been reported to inhibit second phase aggregation and ³⁵C-serotonin release from washed platelet suspensions containing Mg⁺⁺ and added fibrinogen.28 Both Ca⁺⁺ and Mg⁺⁺ support ADP-induced binding of fibrinogen to platelets.15,16,18,30 There is no binding in the absence of Ca⁺⁺ and Mg⁺⁺.8 Maximal binding occurs at 0.5 mM Ca⁺⁺ or 2.5 mM Mg⁺⁺, with decreasing binding at higher concentrations of either ion.10 Ca⁺⁺ and Mg⁺⁺ do not produce additive binding.15,30 In the present experiments, Ca⁺⁺ ions in the presence of 1 mM Mg⁺⁺ inhibited both ADP- and epinephrine-induced aggregation and release. Second wave aggregation in response to ADP was prevented by 1 and 2 mM Ca⁺⁺, and in response to epinephrine, it was inhibited at 2–2.5 mM and prevented at 5 mM. The residual aggregation seen at these Ca⁺⁺ concentrations is largely primary aggregation, which as shown in Figs. 2 and 3, is normally associated with little fibrinogen release. The Ca⁺⁺ inhibition appears to be related to the total cation concentration, since 2–5 mM Mg⁺⁺ without added Ca⁺⁺ was also inhibitory (data not shown). It is possible that inhibition of aggregation and release by high cation concentrations is related to the inhibition of fibrinogen binding demonstrated previously by Bennett and Vilaine.16

The ability of platelets to release endogenous fibrinogen with various stimuli, together with evidence that initial thrombin formation occurs in relation to the platelet surface,31 suggests that during formation of a hemostatic plug, fibrinogen will be released from the platelets and converted by thrombin generated on the platelet surface to fibrin. The fibrin strands then consolidate the platelet plug. If this sequence is correct, patients with plasma afibrinogenemia or dysfibrinogenemia who have normal platelet fibrinogen would be expected to have normal hemostasis.
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