Effect of Calcium Ion Concentration on the Ability of Fibrinogen and von Willebrand Factor to Support the ADP-induced Aggregation of Human Platelets

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To investigate the suggestion that von Willebrand factor (vWF) can substitute for fibrinogen in supporting ADP-induced aggregation of human platelets, we studied platelet reactions in two media: (1) a high calcium medium, Tyrode-albumin solution containing calcium ions in the physiological range of 2 mmol/L, and (2) a low calcium medium, modified Tyrode-albumin solution from which calcium salt was omitted (calcium ion concentration approximately 20 μmol/L). In the high calcium medium vWF even at concentrations up to six times as high as physiological, showed little or no potentiation of ADP-induced platelet aggregation, whereas fibrinogen strongly potentiated reversible aggregation without thromboxane formation or release of granule contents. In the low calcium medium, either vWF or fibrinogen supported biphasic aggregation in response to ADP, with thromboxane formation and release of granule contents. Aspirin and the thromboxane receptor blocker BM 13.177 inhibited these secondary responses to vWF, indicating that they require thromboxane A2 formation and feedback amplification by thromboxane A2. A monoclonal antibody, 10E5, to the platelet glycoprotein Ib/IIa complex inhibited both primary and secondary aggregation. Although vWF supports ADP-induced aggregation when the concentration of ionized calcium is in the micromolar range, it does not support ADP-induced aggregation in the presence of a concentration of ionized calcium in the physiological range, indicating that vWF probably cannot substitute for fibrinogen in supporting ADP-induced aggregation in vivo.

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Preparation of vWF. vWF was prepared from factor VIII concentrates (generously provided by Po Wah, Connaught Laboratories, Downsview, Ontario) by adsorption to bentonite and polyethylene glycol precipitations followed by chromatography on Bio-Gel A-15m, as described by McKee and colleagues. Characterization by gel electrophoresis in the presence of sodium dodecyl sulfate, using 4% acrylamide (reduced samples) or 1% agarose (nonreduced samples) indicated a monomer with a molecular weight of approximately 2.1 x 10^6 existing as multimers ranging in size from 2 to 3 x 10^6 to 8 to 10 x 10^6, similar to those in normal plasma. Ristocetin cofactor activities were determined using formaldehyde-treated platelets or lysophosphatidyl platelets; activities were in the range of 24 to 100 U/mg. The vWF, dissolved in phosphate-buffered saline, pH 7.4, was stored in small aliquots at -70°C, and samples were centrifuged for 2 minutes at 12,000 x g (Eppendorf centrifuge, Brinkmann, Rexdale, Ontario) to remove aggregates immediately before use.

Preparation of citrated PRP from afibrinogenemic patient. Citrated PRP was prepared from blood anticoagulated with 3.8% trisodium citrate, one part to nine parts blood. The platelet count was 4 x 10^10/mL.

Preparation of platelet suspensions. Suspensions of washed platelets from human blood were prepared as described previously. Two final suspending media were used; both were essentially Tyrode solutions containing 0.35% albumin and apyrase (Tyrode-albumin). The first medium (hereafter referred to as the high Ca^{2+} medium) contained approximately physiological concentrations of divalent cations: 2 mmol/L Ca^{2+} and 1 mmol/L Mg^{2+}; the second medium (hereafter referred to as the low Ca^{2+} medium) was a modified Tyrode-albumin solution from which Ca^{2+} was omitted. The Ca^{2+} concentration was approximately 20 zmol/L as measured by atomic absorption spectrometry. The platelets were prelabelled with ^14C-serotonin (0.2 μCi/mL of platelet suspension) in the first washing fluid to permit the measurement of the release of granule contents, as described previously. Imipramine at a concentration of 5 μmol/L was added at the time of ADP addition to prevent reuptake of released serotonin.

Platelet aggregation. Aggregation was studied by recording light transmission through 1-mL samples of platelet suspension (5 x 10^9/μL) stirred at 1100 rpm at 37°C in a cuvette in an aggregation module (Payton Associates, Scarborough, Ontario). For measurement of TXB_{2}, formation and release of granule contents, reactions were stopped by centrifugation at 12,000 x g for 1 minute; the supernatant solutions were removed immediately, and aliquots were used for ^14C determination or stored at -70°C until assayed for TXB_{2} by radioimmunoassay. Concentrations of all materials added to platelet suspensions are given as final concentrations after all additions. In a few experiments, platelets were fixed by the addition of 1% formaldehyde and examined microscopically.

Patients with vWF deficiency. Two patients with von Willebrand disease subtype III, with nondetectable or minute amounts of vWF in plasma and in platelets, were B.B. and B.Z., described previously.

Patient with afibrinogenemia. The afibrinogenemic patient had plasma and platelet fibrinogen concentrations (measured by means of a fibrinopeptide A radioimmunoassay) of 3 μg/mL and 8.6 μg/10^10 platelets, respectively.

RESULTS

Comparison of effects of fibrinogen and vWF on ADP-induced platelet aggregation in the high Ca^{2+} medium (2 mmol/L). Addition of ADP (1 to 20 μmol/L) to suspensions of washed platelets containing 2 mmol/L Ca^{2+} caused a weak, reversible aggregation response (Fig 1). The extent of the change in light transmission was proportional to the concentration of ADP, and microscopic examination of formaldehyde-fixed platelets showed small aggregates at the peak of the response. That this response was not caused by traces of fibrinogen or vWF was indicated by the lack of inhibition by F(ab')2 fragments of antifibrinogen (225 μg/mL) and by the fact that a similar weak response was observed with platelets from two patients with a severe deficiency of vWF, a response also not inhibited by F(ab')2 fragments of antifibrinogen. (These F(ab')2 fragments did inhibit potentiation of this response by fibrinogen, whereas nonimmune F(ab')2 had no effect.) In this high Ca^{2+} medium, the addition of fibrinogen potentiated the ADP-induced aggregation of human platelets, but only the primary phase occurred, and it was followed by deaggregation (Fig 1A). Previously it has been shown that when only the primary phase of ADP-induced aggregation occurs upon the addition of fibrinogen, there is no appreciable formation of thromboxane or release of granule contents. In contrast, in seven out of nine experiments, the addition of vWF, in concentrations ranging from 30 to 90 μg/mL, had no effect or caused barely detectable potentiation of the aggregation induced by ADP alone; slight potentiation was observed in two experiments (Fig 1B). In none of these experiments was there appreciable TXB_{2} formation or release of granule contents.

Comparison of effects of fibrinogen and vWF on ADP-induced platelet aggregation in the low Ca^{2+} medium (20 μmol/L). In the low Ca^{2+} medium, addition of ADP to suspensions of washed platelets caused a weak, reversible aggregation response similar to that observed in the presence of 2 mmol/L Ca^{2+} (Fig 2A). As shown in previous studies, the effect of added fibrinogen on the response of platelets to ADP depended on the concentration of Ca^{2+} in the suspending medium. Thus, in the low Ca^{2+} medium, fibrinogen-potentiated aggregation occurred in two phases, a primary, reversible phase followed by a secondary irreversible...
Fig 2. Responses of washed human platelets to ADP (10 μmol/L) in the low Ca²⁺ medium (modified Tyrode-albumin from which Ca²⁺ was omitted). (A) No additions. (B) Fibrinogen (Fbg, 64 μg/mL) added before ADP. (C) vWF (60 μg/mL) added before ADP. Formation of TXB₂ (ng/10⁹ platelets) and release of ¹⁴C-serotonin (%) are shown beside the aggregation curves. Similar results were obtained in six experiments with fibrinogen at 11-400 μg/mL and vWF at 24-98 μg/mL.

Fig 3. Inhibition of potentiation by vWF (60 μg/mL) of aggregation of washed human platelets stimulated by ADP (10 μmol/L) in the low Ca²⁺ medium. (A) No additions. (B) vWF only added. (C) Ca²⁺ (2 mmol/L) added before vWF and ADP. (D) Aspirin (ASA, 100 μmol/L) added before vWF and ADP. (E) BM 13.177 (100 μmol/L) added before vWF and ADP. (F) 10E5 (10 μg/mL) added before vWF and ADP. Similar results were obtained for inhibition by Ca²⁺, aspirin, or BM 13.177 when tested with vWF at 30-90 μg/mL (10E5 was tested with vWF at 30 μg/mL). Formation of TXB₂ (ng/10⁹ platelets) and release of ¹⁴C-serotonin (%) are shown beside the aggregation curves. The aggregation curves from Figs 2A and 2C have been reproduced in Figs 3A and 3B, respectively, to facilitate estimations of inhibition.

PLATELETS, von WILLEBRAND FACTOR, AND CALCIUM

The present results show that vWF, unlike fibrinogen, either fails to potentiate or causes only slight potentiation of ADP-induced aggregation in an artificial medium contain-
ing a concentration of Ca\(^{2+}\) within the physiological range of 1 to 2 mmol/L. These experiments were done with concentrations of vWF ranging from 30 to 90 \(\mu\)g/mL, which are much higher than the normal plasma concentration of approximately 10 \(\mu\)g/mL. In contrast, as had been shown previously, fibrinogen strongly potentiates ADP-induced aggregation in the presence of physiological concentrations of Ca\(^{2+}\), although aggregation is reversible regardless of the concentration of fibrinogen and does not involve thromboxane formation or release of granule contents. These observations lead one to question whether vWF plays a significant role in supporting ADP-induced platelet aggregation in vivo. It is possible, however, that in vivo there are other factors as yet not understood that enable this protein to contribute to the support of aggregation at physiological Ca\(^{2+}\) concentrations.

In accord with the findings of other investigators, vWF is similar to fibrinogen in its ability to potentiate ADP-induced platelet aggregation in media in which the concentration of Ca\(^{2+}\) is in the micromolar range; potentiation by either vWF or fibrinogen causes activation of the arachidonate pathway, leading to thromboxane formation, release of granule contents, and a biphasic aggregation response that is essentially irreversible. The low Ca\(^{2+}\) concentration in the plasma may have been responsible for the strong potentiation by vWF of ADP-induced aggregation in citrated PRP from the afibrinogenemic patient studied in the present investigation and from one of the afibrinogenemic patients (V.M.) studied by De Marco and colleagues. The inability of fibrinogen or vWF to support second phase aggregation in the low Ca\(^{2+}\) medium in the presence of aspirin or the thromboxane receptor blocker BM 13.177 confirms dependence on the arachidonate pathway that leads to thromboxane A\(_2\) (TXA\(_2\)) formation. Inhibition by 10E5, the monoclonal antibody directed against the glycoprotein IIb/IIIa complex, indicates that the binding of vWF to this complex is involved in potentiation of aggregation; 10E5 has previously been shown to inhibit fibrinogen support of ADP-induced aggregation and the binding of fibrinogen and vWF to activated platelets.

These experiments provide another example of the observation that treatments that cause close platelet contact in a medium containing a micromolar concentration of Ca\(^{2+}\) activate the arachidonate pathway, leading to thromboxane formation, release of granule contents, and secondary aggregation. This effect of micromolar concentrations of Ca\(^{2+}\) on platelet responsiveness has been shown for aggregation by ADP or epinephrine in the presence of fibrinogen, for polylysine-induced agglutination, and for aggregation of chymotrypsin-treated platelets by fibrinogen as well as for aggregation by ADP in the presence of vWF in the present experiments. This response of platelets to close platelet-to-platelet contact in a low Ca\(^{2+}\) medium requires feedback amplification by TXA\(_2\), since it is prevented by BM 13.177, an inhibitor that blocks the thromboxane receptor. Since none of these agonists causes the secondary phase of aggregation, thromboxane formation, or the release of granule contents in a medium containing a physiological concentration of Ca\(^{2+}\), this effect of close contact must be caused, at least in vitro, by low concentrations of Ca\(^{2+}\), and it must be considered in all experiments using citrated PRP or suspensions of washed platelets in artificial media without added Ca\(^{2+}\). Although some investigators have described the effect of a physiological concentration of Ca\(^{2+}\) as inhibition of ADP-induced aggregation, it is apparent that the truly abnormal situation is a Ca\(^{2+}\) concentration in the micromolar range.

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