Cytosolic Calcium Changes in a Process of Platelet Adhesion and Cohesion on a von Willebrand Factor-Coated Surface Under Flow Conditions

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Recent flow studies indicated that platelets are transiently captured onto and then translocated along the surface through interaction of glycoprotein (GP) Ib with surface-immobilized von Willebrand factor (vWF). During translocation, platelets are assumed to be activated, thereafter becoming firmly adhered and cohered on the surface. In exploring the mechanisms by which platelets become activated during this process, we observed changes in platelet cytosolic calcium concentrations ([Ca^{2+}]_i) concomitantly with the real-time platelet adhesive and cohesive process on a vWF-coated surface under flow conditions. Reconstituted blood containing platelets loaded with the Ca^{2+} indicator Fura Red and Calcium Green-1 was perfused over a vWF-coated glass surface in a flow chamber, and changes in [Ca^{2+}]_i were evaluated by fluorescence microscopy based on platelet color changes from red (low [Ca^{2+}]_i) to green (high [Ca^{2+}]_i) during the platelet adhesive and cohesive process. Under flow conditions with a shear rate of 1,500 s^{-1}, no change in [Ca^{2+}]_i was observed during translocation of platelets, but [Ca^{2+}]_i became elevated apparently after platelets firmly adhered to the surface. Platelets preincubated with anti-GP Ib-IIIa antibody c7E3 showed no firm adhesion and no [Ca^{2+}]_i elevation. The intracellular Ca^{2+} chelator dimethyl BAPTA did not inhibit firm platelet adhesion but completely abolished platelet cohesion. Although both firm adhesion and cohesion of platelets have been thought to require activation of GP Ib-IIIa, our results indicate that [Ca^{2+}]_i elevation is a downstream phenomenon and not a prerequisite for firm platelet adhesion to a vWF-coated surface. After platelets firmly adhere to the surface, [Ca^{2+}]_i elevation might occur through the outside-in signaling from GP Ib-IIIa occupied by an adhesive ligand, thereby leading to platelet cohesion on the surface.

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be generated through the interaction between surface-immobilized vWF and GP Ib by translocation and is necessary for firm platelet adhesion to the vWF-coated surface, is independent of [Ca\(^{2+}\)] elevation. However, platelet [Ca\(^{2+}\)] elevation, presumably triggered by binding of activated GP Ib-IIIa to immobilized vWF (firm adhesion), is a prerequisite for the second phase of GP Ib-IIIa activation necessary for subsequent platelet cohesion.

**MATERIALS AND METHODS**

**Materials.** Visible light-excitable Ca\(^{2+}\) indicator Fura Red acetoxymethyl ester (AM), Calcium Green-1 AM, and intracellular Ca\(^{2+}\) chelator 5,5'-dithiobis(2-nitroflavone) ethane-N,N',N''-tetraacetic acid (dimethyl BAPTA) AM were purchased from Molecular Probes, Inc (Eugene, OR). The Fab fragment of human/mouse chimeric anti–GP Ib-IIIa monoclonal antibody c7E3, which totally inhibits the ligand-binding functions of GP Ib-IIIa at concentrations up to 0.3 \(\mu\)mol/L,\(^{11,12}\) was purchased from Eli Lilly and Co (Indianapolis, IN). AP1, an anti-GP Ib monoclonal antibody that completely blocks the vWF-GP Ib interaction at concentrations up to 0.1 \(\mu\)mol/L, was a kind gift from Dr Thomas Kunicki (The Scripps Research Institute, La Jolla, CA).\(^{8,13}\) The Fab\(\gamma\)2 fraction of AP1 was prepared by papain digestion of IgG at low pH and the collection of flow-through fractions in protein A-Sepharose (Pharmacia-LKB Japan, Tokyo, Japan) column chromatography as described.\(^{8,14}\) The antithrombin agent argatroban was supplied by Mitsubishi Chemical Corp (Tokyo, Japan). Apyrase (grade VIII) was from Sigma-Aldrich Japan Co (Tokyo, Japan), and bovine serum albumin (fraction V) was from Calbiochem (La Jolla, CA). Human native vWF containing the highest molecular weight multimers, as judged by sodium dodecyl sulfate (SDS)-1.5% agarose gel electrophoresis,\(^{15}\) was purified from cryoprecipitates, as described.\(^{16-18}\)

Ca\(^{2+}\) indicator-loading on platelets. Blood was collected as described\(^{19-21}\) from nonsmoking healthy donors who had not taken any medication for at least 2 weeks using argatroban at a final concentration of 125 \(\mu\)g/mL as anticoagulant. Platelet-rich plasma (PRP) was obtained by centrifugation at 180g for 10 minutes. Apyrase was added (final concentration, 3 U/mL) to PRP and the mixture was centrifuged on a PRP with a density of 1.053–1.078 g/mL containing 2–3% hematocrit. After centrifugation, the supernatant was removed and the remaining platelet fraction was washed three times in HEPES buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 3 mmol/L MgCl2, 3 mmol/L NaH2PO4, 5.5 mmol/L glucose, 3.5% albumin, 3.5 mmol/L N-2-hydroxethylpiperazine N'-2-ethanesulfonic acid, pH 7.2) containing 3 U/mL apyrase, 30 \(\mu\)mol/L Fura Red AM, and 20 \(\mu\)mol/L Calcium Green-1 AM at 37°C for 30 minutes. Ca\(^{2+}\) indicator-loaded platelets were centrifuged again and resuspended in HEPES buffer (pH 7.4). Erythrocytes were washed 3 times in HEPES buffer. Ca\(^{2+}\) indicator-loaded platelets and washed erythrocytes were mixed together and adjusted to 2 \(\times\) 10\(^8\) platelets/mL and a 35% hematocrit.

**Perfusion chamber and epifluorescence videomicroscopy.** The perfusion chamber used varied the shear rate in a linear manner as originally described by Usami et al.\(^{21}\) In brief, this chamber was designed to reproduce shear rates starting from a predetermined maximum value at the entrance and decreasing to 0 at the exit.\(^{21}\) Nafion tape (Iuchi Inc, Osaka, Japan) was used to adjust flow path height to 0.1 mm. Glass coverslips (24 \(\times\) 50 mm; Matsunami Glass, Osaka, Japan) were coated with 200 \(\mu\)L of suspension of purified vWF (100 \(\mu\)g/mL), placed in a humid environment at room temperature for 60 minutes, and rinsed with 10 mL of HEPES buffer before perfusion. The perfusion chamber was assembled and mounted on BX60 Olympus epifluorescence microscope (Tokyo, Japan). The inner surface of the vWF-coated area was focused with a 100\(\times\) objective lens (UPlanApo 100\(\times\) oil; Olympus), the focus depth of which was 0.49 \(\mu\)m. Thus, the [Ca\(^{2+}\)]i changes as well as adhesive processes of platelets were observed preferentially in platelets adjacent to the vWF surface. Microscopy was equipped with an epifluorescent illumination apparatus (BX-FLA; Olympus; the excitation filter/dichroic mirror/barrier filter combination was MBP490/DM505/BA515IF, respectively) attached to a color charge-coupled device (CCD) camera (U-VPF-N; Olympus).

Before perfusion, Ca\(^{2+}\) (final concentration, 1 mmol/L) was added to cell suspensions containing erythrocytes and Ca\(^{2+}\) indicator-loaded platelets in HEPES buffer. Reconstituted blood containing platelets were aspirated through the chamber by a syringe pump (Model 935; Harvard Apparatus, South Natick, MA) at a constant flow rate of 0.285 mL/min and 37°C in a thermostatic air bath (Model U1-50; Iuchi Inc). In the present study, platelets were perfused through the chamber a single time and not recirculated. [Ca\(^{2+}\)]i changes and the entire adhesive and cohesive process were recorded with a Hi-8 video cassette recorder (VL-HL1; Sharp Inc, Osaka, Japan), with a video rate of 30 frames per second, ie, time resolution of 0.033 seconds for each frame. Only platelets interacting with the vWF-coated surface were analyzed in this study. Freely flowing platelets in perfused blood could be discriminated from platelets interacting with the vWF surface by (1) the effective focus depth directed to the vWF surface and (2) the limit of time resolution of videotapes. Therefore, the background signals (flowing platelets) that were usually less than 20 pixel values can be separated from platelets interacting with the vWF surface (>140 pixel values) and subtracted as background by a computer-assisted image analysis using Win ROOF software (Mitani Corp, Fukui, Japan), as described.\(^{12,19}\)

**Evaluation of platelet [Ca\(^{2+}\)]i changes during the platelet adhesive and cohesive process under flow conditions.** The emission peaks of Calcium Green-1 and Fura Red were 531 nm and 637 to 657 nm, respectively.\(^{22}\) Elevated [Ca\(^{2+}\)]i results in increased fluorescence intensity of Calcium Green-1 and decreased fluorescence intensity of Fura Red at an excitation wavelength of 490 nm. Thus, the combined use of these 2 Ca\(^{2+}\) indicators allows colorimetric analysis of cytosolic calcium changes, with ordinal fluorescence in which platelets glow from red (→orange→yellow-green→) to green with increasing platelet [Ca\(^{2+}\)]i.\(^{22}\) Analogue records of video tape images were digitized by a frame grabber (DIG98; Detect, Tokyo, Japan) and analyzed by an image processing application (Win ROOF), as described.\(^{15,19}\) Changes in platelet [Ca\(^{2+}\)]i were evaluated based on a ratio of signal intensity at G-channel (sensitive peak at 534 nm, green) relative to R-channel (sensitive peak at 594 nm, red) from digitized images. In this regard, we used in this study considerably higher concentrations of Ca\(^{2+}\) indicators (30 \(\mu\)mol/L Fura red AM and 20 \(\mu\)mol/L Calcium Green-1 AM) than usual to obtain sufficient signal intensity, which is crucial for precise colorimetric evaluation of [Ca\(^{2+}\)]i changes during observation periods. These concentrations of Ca\(^{2+}\) indicators were selected based on our preliminary experiments, in which various concentrations of Ca\(^{2+}\) indicators on [Ca\(^{2+}\)]i changes under flow on a collagen- or vWF-coated glass surface were tested. In addition, to address the possibility that the combination loading of such high concentrations of Ca\(^{2+}\) indicators seriously alters platelet functions, we compared the entire adhesive and cohesive process of Ca\(^{2+}\) indicator-loaded platelets with that of platelets labeled with mepacrine at a concentration (10 \(\mu\)mol/L) assumed to preserve normal platelet functions.\(^{5,7}\) No significant differences between these 2 approaches were observed in the extent of platelet translocation or final platelet surface coverage (see below). Next, to minimize the effect of photo-activation of platelets or photo-bleaching of fluorescence, our observation of platelet adhesive processes was performed by limiting the continuous photo-exposure time. In fact, preliminary experiments confirmed that 3-second consecutive illumination did not induce significant photo-activation of platelets and that any photo-bleaching had no effect on evaluation of platelet [Ca\(^{2+}\)]i.

**Evaluation of the extent of firm platelet adhesion and cohesion.** During the platelet adhesive process on a vWF-coated surface under flow conditions, platelets that initially translocate along the surface gradually become firmly adhered to the surface. The extent of firm
Platelet adhesion was determined by counting translocating platelets, defined as those moving along the surface at a distance greater than their diameters in 1 second as evaluated based on the logical OR algorithm of superimposed images (10 frames in 1 second) and Win ROOF computer software, within a defined area. Platelet adherence to the surface was evaluated using Win ROOF software to determine the percentage of the area covered by adhering platelets in a defined area after background subtraction and binarization of each image.

RESULTS

Shear-dependency of [Ca\(^{2+}\)]i elevation in a platelet adhesive and cohesive process on a vWF-coated surface. Platelet [Ca\(^{2+}\)]i changes during the adhesive process on a vWF-coated surface were evaluated at shear rates of 50 s\(^{-1}\), 750 s\(^{-1}\), or 1,500 s\(^{-1}\), representing low, intermediate, or high shear rate, respectively (Fig 1). After 8 minutes of blood perfusion at 50 s\(^{-1}\), platelet adhesion was sparse and platelets appeared mostly reddish, indicating only slight [Ca\(^{2+}\)]i elevation. Even at a shear rate of 750 s\(^{-1}\), most platelets remained red, although a larger number of platelets, some of which certainly cohered, adhered to the surface. At 1,500 s\(^{-1}\), the surface area covered by platelets was greatly increased, and green-colored platelets (high [Ca\(^{2+}\)]i), most of which cohered, were more abundant. These observations indicate the shear-dependency of [Ca\(^{2+}\)]i elevation as well as platelet adhesion and cohesion on a vWF-coated surface. Thus, all further experiments to examine real-time changes in [Ca\(^{2+}\)]i during the platelet adhesive and cohesive process were performed at a wall shear rate of 1,500 s\(^{-1}\).

Time course of [Ca\(^{2+}\)]i changes during a platelet adhesive and cohesive process on a vWF-coated surface at a shear rate of 1,500 s\(^{-1}\). The platelet adhesive and cohesive process on a vWF-coated surface involves (1) an initial platelet attachment to the vWF-coated surface; (2) translocation of platelets along the surface mediated by interaction between immobilized vWF and GP Ib; (3) firm adhesion to the surface mediated by the
Fig 2. Time-course of $[Ca^{2+}]_i$ changes during a platelet adhesive and cohesive process on a vWF-coated surface under a shear rate of 1,500 s$^{-1}$. Reconstituted blood containing platelets loaded with $Ca^{2+}$ indicators was perfused over a vWF-coated surface with a shear rate of 1,500 s$^{-1}$. (Upper panels) Images obtained at 10 seconds, 1 minute, 4 minutes, and 8 minutes of perfusion (original magnification $\times$ 1,000). To distinguish translocating platelets from those firmly adhering to the surface, images were reconstructed by superimposition of 10 frames (2,380 $\mu m^2$ each) obtained every 0.1 seconds (total, 1 seconds) at each time point indicated. Thus, individual platelets translocating along the surface are seen as multiple images in a line (arrow 1), linked with a motion trace that appears as an artifact in a digitized video image when platelets are moving in a manner beyond the limit of time resolution of the videotape, whereas noncohered platelets not moving for at least 1 second are seen as single entities (arrows 2 and 3). Translocating platelets are reddish, whereas cohering platelets preferentially seen at late stages of platelet adhesive and cohesive processes (4 and 8 minutes of perfusion) are green (arrow 4). Single platelets firmly adhering to but not yet cohering on the surface are heterogeneous in color, with some appearing reddish (arrow 2) and others greenish (arrow 3). These time-course images were taken from the different locations in the same perfusion to minimize a possible photo-activation of platelets. (Lower panels) Time-course changes of G/R ratio (left) and platelet surface coverage (right). Data represent the mean and SD of results obtained from 5 independent perfusions at a shear rate of 1,500 s$^{-1}$ (see Fig 1 legend). Note that the G/R ratio and surface coverage of platelets increase as a function of time.

Fig 3. Effect of intracellular $Ca^{2+}$ chelator dimethyl BAPTA, anti-GP Ib, or anti–GP IIb-IIIa on $[Ca^{2+}]_i$ changes in the platelet adhesive and cohesive process on a vWF-coated surface under a shear rate of 1,500 s$^{-1}$. Experimental conditions were identical to those described in the Fig 2 legend, except that platelets were preincubated with 15 $\mu$mol/L of dimethyl BAPTA AM, 0.1 $\mu$mol/L of F(ab)$_2$ of AP1 (anti-GP Ib), 1 $\mu$mol/L of c7E3 (anti–GP IIb-IIIa) for 30 minutes at 37°C before perfusion. Superimposed images (total 30 frames of 3 seconds) were obtained at 8 minutes of perfusion. Unlike the control without blockers, platelets preincubated with dimethyl BAPTA evidenced no $[Ca^{2+}]_i$ elevation or no platelet cohesion. However, most platelets firmly adhered to the vWF surface to an extent comparable to that in the control experiment. Note the highly impaired firm adhesion (and no $[Ca^{2+}]_i$ elevation or coherion) in the presence of anti–GP IIb-IIIa antibody. In the presence of inhibitory anti-GP Ib, no significant platelet-surface interaction was observed at this shear rate.
interaction between immobilized vWF and activated GP IIb-IIIa; and (4) platelet cohesion mediated by binding of soluble adhesive proteins to activated GP IIb-IIIa, as determined under experimental conditions involving direct fluorescence-labeling of platelets in whole blood. All of these events were recapitulated in the present system using reconstituted blood containing washed platelets labeled with fluorescent Ca\textsuperscript{2+} indicators (Fig 2). Our approach also enabled evaluation of the platelet [Ca\textsuperscript{2+}]i changes in the real-time platelet adhesive and cohesive process. Under flow conditions at a shear rate of 1,500 s\textsuperscript{-1}, no elevation in [Ca\textsuperscript{2+}]i was observed during translocation of platelets, but [Ca\textsuperscript{2+}]i increased apparently after platelets firmly adhered to the surface (Fig 2). However, the time-lag from firm adhesion to the [Ca\textsuperscript{2+}]i elevation was highly variable among adhering platelets, with some firmly adhering platelets turning to green in a few seconds, whereas others remained red throughout the observation period (results not shown). A similar heterogeneity was also reported in the case of platelet adhesion on a fibrinogen-coated surface under low shear rate conditions. Platelet cohesion then occurred, using green platelets with fully elevated [Ca\textsuperscript{2+}]i as a base (Fig 2). In this regard, adhesive proteins, vWF, or fibrinogen released from platelets were thought to play a role, because reconstituted blood free of plasma components was used in this study.

**Effect of intracellular Ca\textsuperscript{2+} chelator dimethyl BAPTA, anti-GP Ib, or anti–GP IIb-IIIa on [Ca\textsuperscript{2+}]i changes during the platelet adhesive and cohesive process on a vWF-coated surface under a shear rate of 1,500 s\textsuperscript{-1}**. To confirm the time point and significance of the platelet [Ca\textsuperscript{2+}]i elevation in the platelet adhesive process on a vWF-coated surface, we evaluated the effect of the intracellular Ca\textsuperscript{2+} chelator dimethyl BAPTA, an inhibitory anti-GP Ib, or anti–GP IIb-IIIa antibody on [Ca\textsuperscript{2+}]i in our system. As judged by the visual observation at 8 minutes after perfusion, [Ca\textsuperscript{2+}]i chelation did not block firm platelet adhesion after platelet translocation, but it did abrogate [Ca\textsuperscript{2+}]i elevation and subsequent cohesion (Fig 3). This phenomenon was also confirmed by the time point observation at 30 seconds, 1 minute, 2 minutes, 4 minutes, and 6 minutes after the beginning of perfusion (results not shown). Neither [Ca\textsuperscript{2+}]i elevation nor firm platelet adhesion was observed by the blockage of functional sites on GP IIb-IIIa (Fig 3). Indeed, statistical image analysis (Fig 4) supported the visual findings.

**DISCUSSION**

Both firm adhesion of individual platelets and subsequent platelet cohesion on a vWF-coated surface have been thought to require activation of GP IIb-IIIa. Our results demonstrate that platelet [Ca\textsuperscript{2+}]i elevation is a downstream phenomenon and not a prerequisite for firm platelet adhesion to a vWF-coated surface. Although intraplatelet signaling plays a role in firm platelet adhesion, as evidenced by previous observations that prostaglandin E\textsubscript{1} (PGE\textsubscript{1})-induced elevation of intraplatelet cAMP levels blocks the firm adhesion, our results show that the first step in GP IIb-IIIa activation, which occurs during platelet translocation by the inside-out signaling from interac-
tion between immobilized vWF and GP Ib, is independent of platelet [Ca\(^{2+}\)] elevation. In contrast, platelet [Ca\(^{2+}\)] elevation appears to be necessary for subsequent platelet cohesion on the surface, in which platelet-platelet engagement is achieved by binding of soluble adhesive ligands to activated GP Ib-IIIa.

Current studies on the activation of integrins have raised the possibility that there are at least 2 distinct phases in modulation of GP Ib-IIIa (also known as integrin \(\alpha\text{IIb}\beta3\)), namely an increase in affinity or an increase in avidity.24 An increase in affinity is caused by conformational changes in the heterodimer itself, resulting in a greater strength of ligand binding, whereas an increase in avidity is induced by clustering or multimerization of GP Ib-IIIa on the plasma membrane.24 Although alternative interpretations may be possible, it is interesting to assume that firm adhesion of individual platelets is a consequence of the affinity modulation of GP Ib-IIIa, independent of [Ca\(^{2+}\)] elevation, and that platelet cohesion requires the avidity modulation of GP Ib-IIIa, a more drastic GP Ib-IIIa activation. Platelet [Ca\(^{2+}\)] elevation may serve to trigger this avidity modulation of GP Ib-IIIa. Indeed, recent studies using cell types other than platelets suggested that [Ca\(^{2+}\)] elevation triggers clustering of several membrane receptors, including other members of integrin family.25-27 When [Ca\(^{2+}\)] is elevated, the Ca\(^{2+}\)-binding protein complex activates the myosin light chain kinase, which is known to activate actin-activated myosin ATPase.28-30 The contraction of actin fibers evoked by the myosin ATPase may play a role in clustering of membrane receptors on the cell surface.

With regard to mechanisms by which [Ca\(^{2+}\)] is elevated in platelets during platelet aggregation processes in a closed suspension system, Chow et al.31 using a cone-and-plate type viscometer, demonstrated that cooperative functions of GP Ib and GP Ib-IIIa are essential for platelet [Ca\(^{2+}\)] elevation in platelet aggregation mediated by high shear stress. On the other hand, Ikeda et al.32 in a similar experimental system, reported that platelet [Ca\(^{2+}\)] elevation is absolutely dependent on the vWF-GP Ib interaction without GP Ib-IIIa functions. The basis for these discrepant findings remains uncertain, but may rest in the different blocking agents used in the respective inhibition studies. Although our experimental system involving observation of platelet adhesiveness and cohesive process on a surface is quite different from systems that analyze platelet aggregation in the soluble phase, our findings that platelet [Ca\(^{2+}\)] elevation occurs only after firm adhesion indicates that the binding of GP Ib-IIIa to immobilized vWF is indispensable in this regard. An outside-in signaling from the GP Ib-IIIa complex, generated when occupied by ligands, is likely the trigger for platelet [Ca\(^{2+}\)] elevation. Indeed, earlier studies, albeit in static experimental conditions, indicated the critical involvement of GP Ib-IIIa functions in activation of platelet membrane calcium channels.33,34

Although the vWF-GP Ib interaction is also involved in [Ca\(^{2+}\)] elevation by capturing rapidly flowing platelets onto a surface under high shear rate conditions, our results confirmed that the contribution of this interaction is only indirect, indicating the lack of any substantial role for the inside-out signaling generated from GP Ib in [Ca\(^{2+}\)] elevation during platelet translocation. In conclusion, our approach involving observations of changes in platelet [Ca\(^{2+}\)] during a real-time platelet adhesive and cohesive process under flow conditions showed distinct phase-specific mechanisms of platelet activation (adhesion and cohesion) that have not been demonstrable in previous static or closed stirring experiments. Studies under physiologic flow conditions to determine in detail the process of platelet activation will further contribute to an understanding of the complex mechanisms involved in mural platelet thrombogenesis.

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