Sequential cytoplasmic calcium signals in a 2-stage platelet activation process induced by the glycoprotein Ibα mechanoreceptor

Mario Mazzuccato, Paola Pradella, Maria Rita Cozzi, Luigi De Marco, and Zaverio M. Ruggeri

We found that the interaction of platelets with immobilized von Willebrand factor (VWF) under flow induces distinct elevations of cytosolic Ca²⁺ concentration ([Ca²⁺]) that are associated with sequential stages of integrin αIIbβ3 activation. Fluid-dynamic conditions that are compatible with the existence of tensile stress on the bonds between glycoprotein Ibα (GPIbα) and the VWF A1 domain led to Ca²⁺ release from intracellular stores (type γ peaks), which preceded stationary platelet adhesion. Raised levels of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate, as well as membrane-permeable calcium chelators, inhibited these [Ca²⁺] oscillations and prevented stable adhesion without affecting the dynamic characteristics of the typical platelet translocation on VWF mediated by GPIbα. Once adhesion was established through the integrin αIIbβ3, new [Ca²⁺] oscillations (type γ) of greater amplitude and duration, and involving a transmembrane ion flux, developed in association with the recruitment of additional platelets into aggregates. Degradation of released adenosine diphosphate (ADP) to AMP or inhibition of phosphatidylinositol 3-kinase (PI3-K) prevented this response without affecting stationary adhesion and blocked aggregation. These findings indicate that an initial signal induced by stressed GPIbα-VWF bonds leads to αIIbβ3 activation sufficient to support localized platelet adhesion. Then, additional signals from ADP receptors and possibly ligand-occupied αIIbβ3, with the contribution of a pathway involving PI3-K, amplify platelet activation to the level required for aggregation. Our conclusions modify those proposed by others regarding the mechanisms that regulate signaling between GPIbα and αIIbβ3 and lead to platelet adhesion and aggregation on immobilized VWF. (Blood. 2002;100:2793-2800)

Introduction

Platelets adhere at sites of vascular injury to prevent hemorrhage but may also form occluding arterial thrombi that cause disease. In vessels in which rapid blood flow creates high wall-shear rates, such as arterioles in the normal circulation or atherosclerotic matrix components, particularly collagens. Binding of glycoprotein Ibα (GPIbα), a constituent of the GPIb-IX-V complex, to the VWF A1 domain (A1VWF) initiates platelet tethering to these surfaces but by itself only support translocation with stop-and-go motion. Once tethered, however, platelets rapidly achieve irreversible adhesion mediated by different integrins, including αIIbβ3 bound to the Arg-Gly-Asp (RGD) motif in the VWF C1 domain. Activated αIIbβ3 serves also to immobilize on the surface of adherent platelets the plasma proteins, mainly VWF and fibrinogen, that mediate the recruitment of additional platelets into the forming thrombus.

Platelet activation, necessary to promote the ligand-binding function of αIIbβ3, is coupled to the interactions that establish initial platelet-surface contacts, as shown by the fact that VWF binding to GPIbα leads to aggregation. Sustained elevations of intracellular calcium concentration ([Ca²⁺]), a marker of activation, occur in association with shear-induced platelet aggregation dependent on VWF and GPIbα and may be the consequence of a transmembrane ion flux. Oscillations of [Ca²⁺], have also been observed to accompany platelet adhesion to VWF, but this finding has been given discordant interpretations. Some results suggest that the VWF-GPIbα interaction may induce transient elevations (spikes) of [Ca²⁺], that activate αIIbβ3, in an initially reversible manner and influence the dynamic aspect of platelet-surface contacts before stable adhesion is established. This is in contrast to the idea that transient tethering to immobilized VWF depends only on GPIbα, whereas activation of αIIbβ3, like that of other integrins on leukocytes, leads to irreversible adhesion. Moreover, it has been proposed that phosphatidylinositol 3-kinase (PI3-K) plays an essential role in the activation of αIIbβ3 required for stable platelet adhesion. To evaluate these conclusions, we concurrently analyzed the instantaneous velocity and [Ca²⁺], in single platelets interacting with immobilized VWF. We identified a sequence of distinct cytosolic Ca²⁺ elevations associated with a 2-step process.
of αIIbβ3 activation. The first signal involves release from intracellular stores and always precedes stationary adhesion. The second signal, which is coupled to adenosine diphosphate (ADP)–receptor stimulation and is inhibited by wortmannin, follows stationary adhesion but precedes the initiation of platelet aggregation on the surface. These results challenge the current interpretation of the mechanisms leading to the intracytoplasmic Ca2+ transients linked to stable platelet adhesion and aggregation.

Materials and methods
Preparation of blood samples
These studies were approved by an institutional review board. Venous blood from healthy volunteers who were not taking any medication and who gave informed consent according to the Declaration of Helsinki was collected into 1:6 final volume of citric acid/citrate/dextrose (pH 4.5) or 400 units/mL (final concentration) of the α-thrombin inhibitor hirudin (Iketon, Milan, Italy). Fifty milliliters of blood was centrifuged at 800g for 50 seconds, and the supernatant platelet-rich plasma (PRP) was collected. The procedure was repeated twice to obtain approximately 15 mL PRP. Platelets were incubated for 20 minutes at 37°C with the calcium fluorescent probe fluo-3 acetoxymethyl ester-AM (Fluo-3-AM; 80 μM final concentration; Molecular Probes, Eugene, OR). In selected experiments, platelets were loaded simultaneously with Fluo-3-AM and 1,2-bis(4-aminophenyl)ethane-N,N,N′,N′-tetraacetic acid (BAPTA-AM; 80 μM final concentration; Molecular Probes). Erythrocytes separated from the same blood were washed 3 times in a divalent cation-free HEPES (N,2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid)–Tyrode buffer (10 mM HEPES, 140 mM sodium chloride [NaCl], 2.7 mM potassium chloride [KCl], 0.4 mM monobasic sodium phosphate [NaH2PO4], 10 mM sodium bicarbonate, and 5 mM dextrose [pH 6.5]) and finally resuspended in the same buffer with 1.75 mM probenecid (Sigma, St Louis, MO). Probenecid was used to prevent leakage of Fluo-3-AM from cells. An adequate volume of PRP containing 2 × 108 to 8 × 108 platelets loaded with Fluo-3-AM was mixed with an aliquot of erythrocyte suspension (0.50 hematocrit) and 5 U/mL apyrase (grade 3; Sigma). The mixture was centrifuged at 800g for 15 minutes, the supernatant was discarded, and the cell pellet was suspended in HEPES-Tyrode buffer containing 1.75 mM probenecid to obtain a hematocrit value of 0.42 to 0.45. If needed, a suspension of erythrocytes in HEPES-Tyrode buffer containing 1.75 mM probenecid and with a hematocrit level of 0.42 to 0.45 was added to obtain a platelet count between 5000 and 20 000 μL. Divalent cations (2 mM Ca2+ and 1 or 2 mM magnesium [Mg2+]) were added before perfusion. In some experiments, autologous plasma containing 400 U/mL hirudin and 1.75 mM probenecid was used instead of HEPES-Tyrode buffer to prepare the final cell suspension. ADP plus epinephrine and collagen (Chromo-Log, Hawthorne, PA) induced normal aggregation of platelets loaded with Fluo-3-AM. The mean percentage (± 95% confidence interval [CI]) of platelets with surface expression of P-selectin, a marker of activation, was 4.84% ± 0.86% before and 6.66% ± 1.22% after labeling; the difference was not statistically significant.

Perfusion experiments
Human plasma VWF and a recombinant VWF fragment containing the A1 domain (residues 445 to 733 of the mature protein) were prepared as described previously.17 The 2 proteins were diluted in phosphate-buffered saline (20 mM dibasic sodium phosphate, 20 mM NaH2PO4, 2.7 mM KCl, and 0.15 M NaCl [pH 7.4]) to a final concentration of 100 μg/mL and 100 μL of the solution was used to coat glass coverslips for 60 minutes at 22 to 25°C. These were then washed with the coating buffer and kept in a moist environment until assembled in a modified Hele-Shaw flow chamber.43 The chamber was positioned in an inverted microscope equipped with epifluorescent illumination (Diaphot-TMD; Nikon Instech, Kanagawa, Japan), an intensified charge-coupled digital video camera (C-2400-87; Hamamatsu Photonics, Shizuoka, Japan), and appropriate filters. The total area of an optical field corresponded to approximately 0.007 mm2. Blood cells were aspirated through the chamber with a syringe pump (Harvard Apparatus, Boston, MA), at flow rates calculated to obtain wall-shear rates between 500 and 20 000 seconds−1. When indicated, various substances were added to the blood cell suspension before perfusion. These included prostaglandin E1 (PGE1), EGTA (ethyleneglycoltetraacetic acid), dibutyryl cyclic adenosine monophosphate (cAMP), 8Br-cyclic guanosine monophosphate (cGMP), theophylline, wortmannin (all from Sigma), sodium nitroprusside (Merck, Sharp and Dome, Whitehouse Station, NJ), and sildenafil (Pfizer, New York, NY). To prepare a solution of sildenafil, a tablet (50 mg) was stripped of the outside coating, crushed, and mixed with 0.5 mL water and 0.5 mL ethanol 95%; the suspension was centrifuged at 1000g for 5 minutes; and the clear supernatant was centrifuged again at 11 000g for 5 minutes to eliminate insoluble particles. Monoclonal IgGs, used when indicated, were prepared and characterized as described previously; LJ-B1 blocks the VWF-binding function of GPIba18 and LJ-CP8 blocks the ligand-binding function of αIIbβ3.19 Experiments were recorded in real time on videotape at the rate of 25 frames/second, which resulted in a time resolution of 0.08 second. Selected sequences were also digitized in real time by using a TARGA-2000 Plus board (Truevision, Indianapolis, IN).

Measurement of motion and Ca2+ mobilization in platelets
Image analysis was performed either on recorded experiments or online with custom-made software (Casti Imaging, Venice, Italy). The program tracked the area of single platelets and determined the position of the corresponding centroid on all the frames collected at a sampling rate of 25/second, then calculated instant velocity and variations of light intensity measured as the total of all the pixels in a platelet. Thus, information on the measured variables was obtained every 0.04 second. Instantaneous velocity was calculated according to the general equation v = s/t, where s is the distance separating the centroid of a platelet in 2 successive frames, and t is the time interval between the 2 frames (0.04 second). To minimize the effects of variations in centroid position resulting from changes in platelet shape or orientation, the instantaneous velocity at time t (vt) was obtained by applying a smoothing algorithm according to the following equation:

\[ V_{t \text{instantaneous}} = \frac{(v_{t-1}) + (v_t) + (v_{t+1})}{3} \]

where \(v_t\) is the instantaneous velocity at time \(t\) calculated from the change in position between frames \(x - 1\) and \(x\), \(v_{t-1}\) is the instantaneous velocity at time \(t-1\) calculated from the change in position between frames \(x - 2\) and \(x - 1\), and \(v_{t+1}\) is the instantaneous velocity at time \(t+1\) calculated from the change in position between frames \(x\) and \(x + 1\). When the instantaneous velocity was less than 0.5 mm/second, the platelet was considered to be transiently arrested (zero velocity), provided that any changes in centroid position between frames \(x - 2\) and \(x + 1\) occurred in aberrant directions (as determined by a test of binomial correspondence) and the distance between the initial and final position was less than a platelet diameter. Arrest times were calculated as the sum of all frames in which a platelet had zero velocity. Stable adhesion was defined as zero velocity for 30 seconds or more. Thus, in this case, the surface imprint of a platelet in the first frame of the observation period overlapped at least partly with the imprints in all subsequent 749 frames. The variations in intensity of the Flu-3 AM fluorescence were converted into \([Ca^{2+}]_i\) by using the following equation: \([Ca^{2+}]_i = K_d - F_{max}F_{min} - F\), where \(K_d\) is the dissociation constant of Flu-3 AM for the interaction with \([Ca^{2+}]_i\), corresponding to 864 nM at 37°C; F is the measured fluorescence intensity of a single platelet; \(F_{max}\) is the fluorescence intensity of a single platelet treated with the \([Ca^{2+}]_i\) ionophore A23187 (10 μM; Sigma) in the presence of 2 mM calcium chloride; and \(F_{min}\) is the fluorescence intensity of an unstimulated single platelet. The baseline \([Ca^{2+}]_i\) of the resting state was calculated in single platelets that translocated on the VWF surface without showing changes in fluorescence for at least 10 consecutive frames (that is, the fluorescence intensity in each frame was within 15% of the value in the first frame and < 200 nM). The baseline \([Ca^{2+}]_i\) was also determined in platelets treated with PGE1 and sodium nitroprusside, which did not undergo any change in cytosolic Ca2+ on

From www.bloodjournal.org by guest on November 13, 2017. For personal use only.
interacting with immobilized VWF (Figure 5). The mean (± SD) baseline value was calculated for each platelet examined in detail. A platelet was considered activated when all of the following conditions were met: a change of [Ca$^{2+}$], was more than 3 SDs above the resting state value in at least 3 consecutive frames, and at least 200 nM, and the [Ca$^{2+}$] oscillation showed an identifiable peak and returned to baseline in a discrete period of time.

Results

**Two types of [Ca$^{2+}$] elevations occur in platelets interacting with immobilized VWF under flow**

Platelets loaded with Fluo-3 AM and suspended with red cells in plasma were perfused over immobilized VWF at different wall-shear rates. Use of a low platelet count (2 × 10$^7$/mL) reduced the number of interactions on the surface and facilitated the analysis of single-cell events while still permitting the formation of small thrombi. We found that all aggregates formed around single platelets that displayed transient [Ca$^{2+}$] elevations, first during translocation and then after stationary adhesion to immobilized VWF (Figure 1). We identified 2 types of peaks that differed in the [Ca$^{2+}$] level reached, the duration of the elevation, and the relation to motion on the surface. One type, designated α/β, appeared while platelets were translocating on the VWF surface and was characterized by a rapid increase to concentrations as high as 1 to 2 μM (Figure 1). This peak was arbitrarily designated α when the [Ca$^{2+}$] level was above 0.4 μM and β when it was lower. In experiments performed with a wall-shear rate of 3000 seconds$^{-1}$, approximately 20% of the translocating platelets showed at least one α/β Ca$^{2+}$ peak, and 9% established stationary adhesion within the limited observation period of 30 seconds (Table 1). Approximately 30% of the firmly adherent platelets had a distinct type of Ca$^{2+}$ elevation, designated γ, which reached levels higher than 2 to 3 μM in most cases, had a total duration of several seconds, and showed a pulsing behavior (Figure 1 and Table 1). After a type γ Ca$^{2+}$ increase, platelets were likely to promote the arrest of additional platelets that translocated in their vicinity, and these in turn showed pronounced cytosolic Ca$^{2+}$ elevations and started to form aggregates (Figure 1). Once established, these aggregates could grow quickly, displaying periodical and synchronous [Ca$^{2+}$] pulses (Figure 1).

To evaluate more extensively the signals elicited during initiation of platelet adhesion to VWF, we performed experiments with platelets suspended in buffer at a count of 5 × 10$^7$/mL to minimize aggregate formation. On perfusion over immobilized VWF at a wall-shear rate of 3000 seconds$^{-1}$, surface-interacting platelets showed all 3 types of cytosolic Ca$^{2+}$ peaks, with typical magnitude and duration (Figure 2, left panel). Additional to the cell suspension of a function-blocking anti-α/β, monoclonal antibody had no effect on the appearance of α/β peaks but obliterated γ peaks (Figure 2, middle panel; and Table 1). Moreover, when A1VWF was used as the immobilized substrate instead of native multimeric VWF, only α/β peaks occurred, even when platelets were perfused without α/β inhibition (Figure 2, right panel). Platelets interacting with A1VWF, which lacks the RGD sequence, and those with blocked α/β, interacting with native VWF could roll but could neither adhere irreversibly nor aggregate; the absence of prolonged arrest of motion was reflected in an increase in the average translocation velocity (Table 1).

**Ca$^{2+}$ signals induced by GPIbα binding to VWF differentially depend on release from intracellular stores or transmembrane ion flux and are initiated under shear stress**

Platelets translocating on VWF in the presence of the extracellular Ca$^{2+}$ chelator EGTA (2 or 10 mM) showed only α/β peaks (no γ peaks; Figure 3A). In contrast, when cytoplasmic Ca$^{2+}$ was chelated with BAPTA-AM, all Ca$^{2+}$ oscillations in translocating platelets were obliterated (Figure 3A). These results indicate that α/β peaks involve release from intracellular stores, whereas γ peaks depend on a transmembrane ion flux. The latter idea was also supported by the demonstration that platelets resuspended in the absence of added extracellular Ca$^{2+}$ and with 2 mM Mg$^{2+}$ did not show γ peaks but could still establish irreversible adhesion, thereby
approximately 50% lower at 20,000 seconds$^{-1}$.

BAPTA-AM, in which case 135 platelets were examined in 3 separate experiments. Stable adhesion represents the number of platelets that arrested in the same position for at least 30 seconds relative to the total number of platelets analyzed in the field of view. The frequency of the different types of [Ca$^{2+}$] peaks is expressed as the percentage of platelets showing at least one Ca$^{2+}$ oscillation of a given type relative to the total number analyzed in the field of view during a 30-second observation period. Apyrase was used at a final concentration of 10 U/mL; the anti-vWF antibody LJ-CP8 at 100 ng/mL; BAPTA-AM at 80 mM; and sodium nitroprusside at 5 μM.

Results were obtained by using the perfusion conditions described in the legend for Figure 2, with a wall-shear rate of 3000 seconds$^{-1}$. The values shown are the mean ± 95% CI, except for aggregation, which was assessed qualitatively. Between 200 and 250 platelets were examined in 5 separate experiments for all conditions except BAPTA-AM, in which case 135 platelets were examined in 3 separate experiments. Stable adhesion represents the number of platelets that arrested in the same position for at least 30 seconds relative to the total number of platelets analyzed in the field of view. The frequency of the different types of [Ca$^{2+}$] peaks is expressed as the percentage of platelets showing at least one Ca$^{2+}$ oscillation of a given type relative to the total number analyzed in the field of view during a 30-second observation period. Apyrase was used at a final concentration of 10 U/mL; the anti-vWF antibody LJ-CP8 at 100 ng/mL; BAPTA-AM at 80 mM; and sodium nitroprusside at 5 μM.

*P < .05 compared with control.
†P < .01 compared with control.

indicating that αMβ3 function was preserved under these conditions (not shown). The number of platelets tethered to VWF was influenced by hydrodynamic flow conditions and reached a plateau at 3000 to 6000 seconds$^{-1}$ wall-shear rate. The interaction was approximately 50% lower at 20,000 seconds$^{-1}$ and 80% lower at 500 seconds$^{-1}$ (Figure 3B). Type α/β cytosolic Ca$^{2+}$ oscillations occurred in an increasing proportion of translocating platelets as the shear rate increased, reaching a maximum at 6000 seconds$^{-1}$ that was unchanged up to 20,000 seconds$^{-1}$. In contrast, less than 2% of the surface-translocating platelets showed cytosolic Ca$^{2+}$ oscillations at the shear rate of 500 seconds$^{-1}$ (Figure 3B). The peak [Ca$^{2+}$], of a type α elevation was also directly related to the shear rate, reaching an average value of 1.8 μM at 6000 seconds$^{-1}$ (Figure 3C).

Measurements of the instantaneous velocity of individual platelets revealed a stop-and-go motion, with alternating rapid deceleration and acceleration (Figure 4A). The peak of all type α [Ca$^{2+}$], elevations (by definition > 0.4 μM) was coincident with a temporary arrest that occurred, on average, 2.06 seconds (range, 0.2 to 8.12 seconds) after the preceding velocity peak and 0.61 seconds (range, 0.24 to 1.12 seconds) before the subsequent one (n = 30; Figure 4A). Therefore, the arrest during which a platelet could generate a signal through GPIb$\alpha$ activation or aggregation was observed on the surface (Table 1). In previous studies, we showed that PGE$_1$, which increases cAMP levels, interferes with αMβ3 activation and blocks the irreversible adhesion of flowing platelets interacting with immobilized VWF, as well as thrombus formation. Sodium nitroprusside, which acts as a caged nitric oxide and activates cGMP-dependent protein kinases, has the same effect (data not shown). Both inhibitors, as exemplified here by sodium nitroprusside (Figure 5), completely prevented both α/β and γ Ca$^{2+}$ oscillations in platelets interacting with immobilized VWF under shear stress. In this case, the average translocation velocity was increased and platelets could not establish irreversible adhesion (Table 1). Two distinct phosphodiesterase (PDE) inhibitors, the nonspecific theophylline and the PDES-specific sildenafil, also inhibited in a dose-dependent manner all cytoplasmic Ca$^{2+}$

---

Table 1. Selected variables characteristic of platelet interaction with immobilized VWF and Ca$^{2+}$ signaling under different conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>Translocation velocity (μm/s)</th>
<th>Mean arrest time in seconds (range)</th>
<th>Stable adhesion, %</th>
<th>Aggregation</th>
<th>Results according to type of [Ca$^{2+}$] peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.9 ± 0.4</td>
<td>5.51 ± 2.6 (0.2-30)</td>
<td>9.2 ± 2.6</td>
<td>+</td>
<td>20.3 ± 6.2</td>
</tr>
<tr>
<td>Apyrase</td>
<td>2.4 ± 0.4</td>
<td>5.70 ± 2.5 (0.3-30)</td>
<td>8.5 ± 3.1</td>
<td>–</td>
<td>22.4 ± 4.5</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>10 nM</td>
<td>2.2 ± 0.3</td>
<td>5.50 ± 2.2</td>
<td>–</td>
<td>21.3 ± 3.6</td>
</tr>
<tr>
<td>100 nM</td>
<td>2.9 ± 0.2†</td>
<td>5.21 ± 2.1 (0.2-30)</td>
<td>7.5 ± 2.8</td>
<td>–</td>
<td>19.8 ± 4.2</td>
</tr>
<tr>
<td>Anti-vWF</td>
<td>4.6 ± 0.5†</td>
<td>1.19 ± 0.56 (0.2-3.78)†</td>
<td>0</td>
<td>–</td>
<td>23.7 ± 3.2</td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td>5.2 ± 0.3†</td>
<td>1.08 ± 0.49 (0.2-3.08)†</td>
<td>0</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>Sodium nitroprusside</td>
<td>5.6 ± 0.3†</td>
<td>0.98 ± 0.44 (0.2-4.48)†</td>
<td>0</td>
<td>–</td>
<td>0</td>
</tr>
</tbody>
</table>

Frequency, % Concentration, nM

<table>
<thead>
<tr>
<th>α/β</th>
<th>γ</th>
<th>α</th>
<th>γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.03</td>
<td>0.01</td>
<td>0.0</td>
</tr>
</tbody>
</table>

---

Of note, the relation between type α [Ca$^{2+}$], elevations and platelet motion was similar regardless of whether αMβ3 was functionally blocked (Figure 4A) or not. The type α/β pattern of cytosolic Ca$^{2+}$ elevations was distinctly different from type γ, which developed during several seconds only in a motionless platelet that had already established irreversible adhesion to VWF (Figure 4B). The observation of Ca$^{2+}$ elevations in relation to platelet motion allowed a reproducible discrimination between type α/β and γ peaks by different observers. Moreover, the shape of all type α peaks (by definition > 0.4 μM) was represented with good statistical parameters ($R^2 = 0.71$: coefficient of variation, < 6%) by a mathematical formula that yielded the value of 1.5 second for the typical duration of the oscillation, with a lag time of 0.2 second from beginning to maximum value of the [Ca$^{2+}$], increase.

**Stimulation through GPIb$\alpha$ leads to sequential stages of αMβ3 activation that distinctly mediate stable platelet adhesion and aggregation**

When blood cells were perfused over immobilized VWF with a wall-shear rate of 3000 seconds$^{-1}$; apyrase, which contains an adenosine diphosphatase activity, and wortmannin, at concentrations that inhibit PI3-K, had no effect on the frequency and amplitude of type α/β [Ca$^{2+}$] elevations in platelets but completely prevented the appearance of type γ peaks (Figure 5). Apyrase and wortmannin did not significantly affect the average translocation velocity, and a normal proportion of platelets achieved stationary adhesion, but no platelet aggregation was observed on the surface (Table 1). In previous studies, we showed that PGE$_1$, which increases cAMP levels, interferes with αMβ3 activation and blocks the irreversible adhesion of flowing platelets interacting with immobilized VWF, as well as thrombus formation. Sodium nitroprusside, which acts as a caged nitric oxide and activates cGMP-dependent protein kinases, has the same effect (data not shown). Both inhibitors, as exemplified here by sodium nitroprusside (Figure 5), completely prevented both α/β and γ Ca$^{2+}$ oscillations in platelets interacting with immobilized VWF under shear stress. In this case, the average translocation velocity was increased and platelets could not establish irreversible adhesion (Table 1). Two distinct phosphodiesterase (PDE) inhibitors, the nonspecific theophylline and the PDES-specific sildenafil, also inhibited in a dose-dependent manner all cytoplasmic Ca$^{2+}$

---

![Figure 2](image-url)

**Figure 2.** Distinct [Ca$^{2+}$], elevations mediated by GPIb$\alpha$ or αMβ3, in platelets interacting with immobilized VWF. Platelets loaded with Fluo-3 AM were suspended at a count of 5 × 10^5/mL with homologous washed red cells in a buffer containing 2 mM Ca$^{2+}$ and 1 mM Mg$^{2+}$ (the same results were obtained with 2 mM Mg$^{2+}$). The suspension was perfused over immobilized multimeric VWF or A1VWF at the indicated shear rates for 90 seconds, after which the [Ca$^{2+}$] of all surface-interacting platelets was measured in real time for the next 30 seconds during translocation or stationary adhesion. In some experiments, the anti-vWF, monoclonal antibody LJ-CP8 was added at the final concentration of 100 μg/mL. Experiments with A1VWF were performed at the shear rate of 2000 seconds$^{-1}$ to obtain comparable numbers of surface-tethered platelets under all conditions. Arrows indicate typical γ Ca$^{2+}$ peaks, which were present only in untreated platelets interacting with VWF. Comparable results were obtained in 4 different experiments.
are the mean was measured as a function of the shear rate during perfusion. Each point shows the mean from different experiments using the same conditions described for panel B.

**Discussion**

In this study, we showed that the interaction of platelet GPIbα with VWF leads to 2 distinct types of \([\text{Ca}^{2+}]\) elevations linked to sequential stages of integrin αIIbβ3 activation (Figure 7). The first \([\text{Ca}^{2+}]\) peaks in the temporal sequence appear to have been initiated by mechanical stimulation, since their frequency increased as a function of shear stress above 2 Pa (the value in blood flowing with a wall-shear rate of 500 seconds\(^{-1}\)). Within this group, the distinction between type α and β peaks was based solely on intensity and the fact that α peaks, which by definition reach a \([\text{Ca}^{2+}]\) level above 0.4 mM, have a reproducible shape that facilitates the analysis of their relation to platelet motion. Indeed, type α peaks a reached maximum level while platelets were transiently arrested but at a predictably short time before detachment from the surface, when the tensile stress on the GPIbα-VWF bonds may be greatest. These findings support the concept that GPIbα has a mechanoreceptor function, although the proximal events responsible for transducing force into a biochemical signal remain unknown. Linkage of the GPIbα cytoplasmic tail to the membrane skeleton through filamin-a\(^{24,25}\) and to the ζ isoform of 14.3.3,26 a regulatory molecule in cellular signaling,27 may be relevant in this regard. Neither association is needed for the GPIbα-dependent induction of αIIbβ3 activation in heterologous cells,28 but a role in flowing platelets cannot be excluded. These uncertainties notwithstanding, it is clear that type α/β peaks are the consequence of rapid \([\text{Ca}^{2+}]\) release from intracellular stores. Such cytoplasmic \([\text{Ca}^{2+}]\) elevations are likely mediated by inositol-1,4,5-trisphosphate29 generated with diacylglycerol through the action of phosphatidylinositol-specific phospholipase C (Figure 7); a similar response has also been observed in endothelial cells30,31 and osteoblasts32 subjected to shear stress.

The first level of αIIbβ3 activation induced by platelet interaction with VWF under shear stress leads from transient to stable adhesion and is regulated by the cellular levels of cAMP and cGMP that control type α/β \([\text{Ca}^{2+}]\) signals. However, thrombus formation cannot progress at this stage, possibly because \([\text{Ca}^{2+}]\) release from cytoplasmic stores is susceptible to regulation by cAMP and cGMP levels.

**Figure 3. Effect of chelating agents on \([\text{Ca}^{2+}]\) elevations dependent on the interaction between GPIbα and A1VWF.** A blood cell suspension prepared as described in the legend for Figure 2 was perfused over immobilized VWF at the indicated shear rates for 90 seconds. Interacting platelets were identified and analyzed as described in the legend for Figure 2. (A) After addition of the membrane-impermeable \([\text{Ca}^{2+}]\) chelator EGTA (2 mM), no type α \([\text{Ca}^{2+}]\) elevations (Figure 1) occurred, but \(\omega\) β peaks were unchanged. In contrast, after addition of membrane-permeable BAPTA-AM, all \([\text{Ca}^{2+}]\) oscillations were obliterated. The same results were obtained in 3 different experiments. (B) After addition of EGTA (2 mM) and the anti-αIIbβ3 monoclonal antibody LJ-CP8 (100 μg/mL), all the platelets interacting with the surface in a 30-second period after an initial 90-second perfusion were enumerated (i); the proportion of these showing \(\omega\)/β \([\text{Ca}^{2+}]\) elevations was calculated (II). There were no γ peaks under these conditions. Results are the mean ± 95% CI from 3 separate experiments performed at the indicated shear rates between 500 and 20 000 seconds\(^{-1}\). (C) The peak [\([\text{Ca}^{2+}]\)] of type α oscillations was measured as a function of the shear rate during perfusion. Each point shows the mean ± 95% CI of at least 10 peaks and is representative of the results obtained in 3 different experiments using the same conditions described for panel B.

**Figure 4. Relation between instantaneous velocity and \([\text{Ca}^{2+}]\) elevations during platelet interaction with immobilized VWF.** (A) A blood cell suspension was prepared as described in the legend for Figure 2. After addition of the anti-αIIbβ3 antibody LJ-CP8 (100 μg/mL) and 2 mM EGTA to chelate extracellular Ca\(^{2+}\), the suspension was perfused over immobilized VWF at a shear rate of 3000 seconds\(^{-1}\) for 90 seconds. Interacting platelets were analyzed during the successive 30 seconds. Panel AI shows movement and fluorescence changes of a representative platelet interacting with the surface during the indicated time interval. The diagrams on the right depict \([\text{Ca}^{2+}]\) elevations dependent on the interaction between GPIbα and A1VWF. After addition of the membrane-impermeable Ca\(^{2+}\) chelator EGTA (2 mM), no type α \([\text{Ca}^{2+}]\) elevations (Figure 1) occurred, but \(\omega\) β peaks were unchanged. In contrast, after addition of membrane-permeable BAPTA-AM, all \([\text{Ca}^{2+}]\) oscillations were obliterated. The same results were obtained in 3 different experiments. (B) After addition of EGTA (2 mM) and the anti-αIIbβ3 monoclonal antibody LJ-CP8 (100 μg/mL), all the platelets interacting with the surface in a 30-second period after an initial 90-second perfusion were enumerated (i); the proportion of these showing \(\omega\)/β \([\text{Ca}^{2+}]\) elevations was calculated (II). There were no γ peaks under these conditions. Results are the mean ± 95% CI from 3 separate experiments performed at the indicated shear rates between 500 and 20 000 seconds\(^{-1}\). (C) The peak [\([\text{Ca}^{2+}]\)] of type α oscillations was measured as a function of the shear rate during perfusion. Each point shows the mean ± 95% CI of at least 10 peaks and is representative of the results obtained in 3 different experiments using the same conditions described for panel B.
bind immobilized VWF but not soluble VWF and fibrinogen as required for aggregation. A second level of \( \alpha_{\text{IIb}}\beta_3 \) activation must be reached for aggregation to occur, and this appears to require signal amplification associated with type \( \gamma \) \( \text{Ca}^{2+} \) elevations induced by ADP released in response to the initial GPIb \( \alpha_{\text{IIb}}\beta_3 \) activation. Release of ADP in the microenvironment of firmly adherent platelets may also explain why other platelets that transiently arrest in their vicinity become rapidly activated (Figure 1). These conclusions are in agreement with the idea that secreted ADP is necessary for shear-induced platelet aggregation initiated by soluble VWF binding to GPIb \( \alpha_{\text{IIb}}\beta_3 \) and that inhibition of the P2Y1 and P2Y12 ADP receptors reduces platelet aggregation after adhesion to collagen-bound VWF. In other experiments, we found that concurrent blockage of P2Y1 and P2Y12 has the same effect as apyrase in preventing type \( \gamma \) \( \text{Ca}^{2+} \) elevations and platelet aggregation on immobilized VWF (data not shown). Progression to the second stage of \( \alpha_{\text{m}3}\beta_3 \) activation involves one or more signaling molecules inhibited by wortmannin, possibly including PI3-K. Activation of PI3-K may be linked directly to the VWF-GPIb \( \alpha_{\text{IIb}}\beta_3 \) interaction or follow further cytoplasmic \( \text{Ca}^{2+} \) mobilization induced by the P2Y1 receptor. Both ADP stimulation and activation of a wortmannin-sensitive pathway are needed for the appearance of a second \( \text{Ca}^{2+} \) signal, a type \( \gamma \) peak, which precedes the onset of platelet aggregation and is prevented by monoclonal antibodies that block ligand binding to activated \( \alpha_{\text{m}3}\beta_3 \). The effect of \( \alpha_{\text{m}3}\beta_3 \) blockage on the appearance of \( \gamma \) peaks may be explained by the fact that only firmly adherent platelets can be activated locally by ADP, or it may indicate a previously suggested role of \( \alpha_{\text{m}3}\beta_3 \) in mediating \( \text{Ca}^{2+} \) transport across the platelet membrane. The characteristics of type \( \gamma \) \( \text{Ca}^{2+} \) elevations are compatible with a mechanism of store-mediated \( \text{Ca}^{2+} \) entry, which is known to be inhibited by wortmannin.

Results similar to ours were reported previously, but some differences are notable both with respect to experimental findings and interpretation of the mechanisms that mediate stable platelet adhesion and aggregation on a VWF surface. A distinctive aspect of our studies was the discrimination between type \( \alpha/\beta \) and \( \gamma \) \( \text{Ca}^{2+} \) oscillations, which was supported by experiments performed with a recombinant VWF A1 domain fragment and may be explained by the methods used. Measurements performed every 0.04 second provide a sufficient time resolution to identify the rapid \( \text{Ca}^{2+} \) peaks related to GPIb \( \alpha_{\text{IIb}}\beta_3 \) function that, as type \( \gamma \) oscillations, have a 0.2-second average interval between onset and peak \( \text{Ca}^{2+} \) concentration. Monitoring of calcium dynamics in individual platelets every 0.586 second, as was done previously, may have concealed the occurrence of type \( \alpha/\beta \) \( \text{Ca}^{2+} \) oscillations, even though they appear in 6 times as many platelets as type \( \gamma \) peaks (Table 1). The biased overestimation of type \( \gamma \) peaks may explain the conclusion that a flux of extracellular \( \text{Ca}^{2+} \) contributes to all signals linked to the interaction of platelets with immobilized VWF. In fact, as shown here, type \( \alpha/\beta \) \( \text{Ca}^{2+} \) oscillations directly related to the engagement of GPIb \( \alpha_{\text{IIb}}\beta_3 \) by surface-bound VWF under shear stress are insensitive to extracellular \( \text{Ca}^{2+} \) concentration.

The discrimination between type \( \alpha/\beta \) and \( \gamma \) \( \text{Ca}^{2+} \) peaks may also explain a different interpretation of the proposed role of PI3-K in shear-dependent signaling between GPIb \( \alpha_{\text{IIb}}\beta_3 \) and \( \alpha_{\text{m}3}\beta_3 \). We found that wortmannin had no influence on the frequency and peak concentration of the EGTA-insensitive and GPIb \( \alpha_{\text{IIb}}\beta_3 \)-dependent type \( \alpha/\beta \) \( \text{Ca}^{2+} \) oscillations but that it obliterated the \( \alpha_{\text{m}3}\beta_3 \)-dependent type \( \gamma \) \( \text{Ca}^{2+} \) peaks also abolished by EGTA (Table 1). Thus, PI3-K, possibly with other signaling molecules inhibited by wortmannin, appears to have a role in shear-induced platelet activation by contributing to the ADP-dependent amplification phase required for aggregation but not to the initial response directly linked to GPIb \( \alpha_{\text{IIb}}\beta_3 \) (Figure 7). Our results did not confirm that stationary platelet adhesion to VWF is inhibited by wortmannin and occurs only after \( \alpha_{\text{m}3}\beta_3 \)-dependent sustained \( \text{Ca}^{2+} \) oscillations. To the contrary, we found that stable adhesion is a prerequisite for, rather than a consequence of such oscillations. Thus, although our data do support the conclusion that \( \text{Ca}^{2+} \) oscillations linked to \( \alpha_{\text{m}3}\beta_3 \) function (type \( \gamma \) peaks) are influenced by wortmannin, our findings indicate that the function of PI3-K, possibly dependent on signaling through ADP receptors, is required for platelet aggregation but not irreversible adhesion to VWF. In fact, removal of ADP or treatment with wortmannin obliterated the \( \alpha_{\text{m}3}\beta_3 \)-dependent and sustained type \( \gamma \) \( \text{Ca}^{2+} \) peaks without reducing the frequency of stable platelet adhesion to VWF and had only a small effect on the average translocation velocity of the entire platelet population (Table 1). It should be noted that our definition of stable adhesion was stringent,
brillar collagen type VI.23 The sequence RGD in the respective. The A1 domain also interacts with the nonfibrillar collagen type VI.23 The sequence RGD in the VWF C1 domain interacts with activated α\textsubscript{IIb}β\textsubscript{3}. PIP\textsubscript{2} indicates phosphatidylinositol-4,5-bisphosphate; IP\textsubscript{3}, inositol-1,4,5-trisphosphate; DG, diacylglycerol; PKC, protein kinase C; and PI3-K, phosphatidylinositol 3-kinase. ADP is shown interacting with two 7-transmembrane-domain, G-protein-coupled receptors: P2Y1, linked to Ca\textsuperscript{2+} release from internal stores, and P2Y12, linked to the regulation of adenyl cyclase activity. The position of α(β)/γ [Ca\textsuperscript{2+}] peaks relative to GPIb-IX-V engagement by A1VWF and signal amplification by ADP and PI3-K, respectively, differs from the previous interpretation by Yap et al. Also, an increase in cAMP and cGMP levels blocked the first Ca\textsuperscript{2+} response linked to GPIb-IX-V stimulation (that is, α(β)/γ peaks) and consequently other downstream activation events.

Our results indicate that the contribution of initial α\textsubscript{IIb}β\textsubscript{3} activation to the dynamic aspects of platelet interaction with immobilized VWF is more limited than suggested by others.3 In fact, inhibition of α/β and subsequent γ peaks by chelating cytoplasmic Ca\textsuperscript{2+} or raising cyclic AMP or cGMP levels had the blocking did not affect α/β Ca\textsuperscript{2+} peaks (Table 1). Thus, transient arrest times are the same regardless of whether platelets have no intracytoplasmic Ca\textsuperscript{2+} oscillations or have a normal frequency of type α/β peaks but α\textsubscript{IIb}β\textsubscript{3} is inhibited (Table 1). Such findings indicate that the interaction of GP\textsubscript{Ib}x with immobilized VWF is not directly influenced by cytoplasmic Ca\textsuperscript{2+} oscillations and is solely responsible for initiating transient platelet-arrest periods that can last several seconds, even under high shear stress. Subsequent activation of α\textsubscript{IIb}β\textsubscript{3}, linked to the occurrence of α/β Ca\textsuperscript{2+} peaks, undoubtedly permits prolongation of these contacts and establishment of stationary adhesion, in accordance with a previously suggested mechanism.5 It is worth noting that the average platelet translocation velocities of 30 to 60 μm/second\textsuperscript{-1} measured by Nesbitt et al\textsuperscript{13} after functional blockage of α\textsubscript{IIb}β\textsubscript{3} or chelation of intracellular Ca\textsuperscript{2+} were greatly in excess of the values measured in these (Table 1) or earlier\textsuperscript{5} studies under comparable flow conditions and VWF concentration on the surface. The discrepancy, barring differences in the qualitative multimeric composition of the VWF used, may be explained by the use of a low time resolution for motion analysis (0.576 second, that is, < 2 frames/second), which may have affected the individual tracking of multiple and identical objects moving rapidly on the surface.

In conclusion, our results support the definition of a mechanism that links shear-induced stimulation of GP\textsubscript{Ib}x to 2 sequential and distinct stages of α\textsubscript{IIb}β\textsubscript{3} activation characterized by specific cytosolic Ca\textsuperscript{2+} elevations (Figure 7). These findings provide the basis for a detailed definition of the signaling pathways initiated by the VWF-GP\textsubscript{Ib}x interaction that may regulate platelet participation in hemostasis and thrombosis. The functional importance of these signals in relation to those generated by other thrombogenic substrates, such as collagen, remains to be established. In this regard, the nature of the vascular lesion evoking a platelet response may be important in determining what pathway of platelet activation will be followed. For example, injured endothelial cells release VWF that, while bound to their surface, may initiate platelet adhesion and activation in the absence of subendothelial denudation.\textsuperscript{40} Clarification of this issue is one of the goals of future studies.

Acknowledgments

We thank Brian Savage and Marco Cattaneo for stimulating discussion on many aspects of platelet physiology; Jerry Ware, James R. Roberts, and Richard A. McCIntock for contributing to the preparation of recombinant A1VWF; and Rachel Braithwaite and Eileen Bristow for excellent secretarial assistance.

References


Sequential cytoplasmic calcium signals in a 2-stage platelet activation process induced by the glycoprotein Ibα mechanoreceptor

Mario Mazzucato, Paola Pradella, Maria Rita Cozzi, Luigi De Marco and Zaverio M. Ruggeri