Distinct Glycoprotein Ib/V/IX and Integrin αIIbβ3-dependent Calcium Signals Cooperatively Regulate Platelet Adhesion under Flow*  

Warwick S. Nesbitt, Suhasini Kulkarni, Simon Giuliano, Isaac Goncalves, Sacha M. Dopheide, Cindy L. Yap, Ian S. Harper, Hatem H. Salem, and Shaun P. Jackson‡  

From the Australian Centre for Blood Diseases, Monash University, Box Hill Hospital, Box Hill, Victoria 3127, Australia  

We have investigated the calcium signaling relationship between the two major platelet adhesion receptors, glycoprotein Ib/V/IX (GPIb/V/IX) and integrin αIIbβ3, involved in regulating platelet adhesion on von Willebrand factor (vWF) under flow. Our studies demonstrate that GPIb engagement of immobilized vWF elicits a transient calcium spike that may function to promote reversible arrest of translocating platelets. Subsequent integrin αIIbβ3 engagement of vWF promotes sustained calcium oscillations that are essential for the maintenance of irreversible adhesion. GPIb-induced calcium spikes appear distinct from those initiated by integrin αIIbβ3, in that the former are exclusively mediated through release of intracellular calcium stores via a signaling mechanism independent of PI 3-kinase. GPIb engagement of vWF under flow initiates a transient calcium spike that elicits calcium influx following platelet activation (15, 16). Understanding the mechanisms regulating platelet translocation is potentially important, since recent studies have demonstrated that the majority of platelets adhering to the injured vessel wall and to the surface of thrombi in vivo undergo a variable period of surface translocation prior to forming stationary adhesion contacts (4, 5).

There is a considerable body of evidence demonstrating that in addition to mediating platelet tethering and translocation, GPIb transduces signals necessary for integrin αIIbβ3 activation. The mechanism by which GPIb transduces signals remains controversial and appears to be significantly influenced by the experimental conditions used to study this process. For example, based on studies of shear-induced platelet aggregation using a cone-and-plate viscometer, GPIb has been proposed to initiate platelet activation by inducing transmembrane calcium influx, leading to integrin αIIbβ3 activation through an indirect mechanism dependent on released ADP (6). In contrast, recent studies examining platelet adhesion to an immobilized vWF matrix have demonstrated the existence of a distinct GPIb signaling mechanism linked to intracellular calcium mobilization (7). GPIb-dependent calcium mobilization is a shear-sensitive signaling process promoting integrin αIIbβ3 activation directly, independent of released ADP.

An important unresolved issue is the relative contribution of GPIb and integrin αIIbβ3 outside-in signaling to cytosolic calcium flux initiated by the platelet-vWF interaction. For example, a recent study by Kuwahara et al. has suggested that calcium flux during shear-dependent platelet adhesion on vWF is exclusively mediated through integrin αIIbβ3 (8). The involvement of integrins in the regulation of cytosolic calcium levels has been well established in a range of cell types, including platelets, leukocytes, endothelial cells, fibroblasts, and osteoclasts (9). In general, calcium signaling is utilized by β2 leukocyte integrins as well as integrins that engage ligands containing the tripeptide sequence Arg-Gly-Asp (RGD), including platelets, leukocytes, endothelial cells, fibroblasts, and osteoclasts (9). In contrast, transient calcium spikes that are generally inefficient at initiating platelet adhesion under conditions of rapid blood flow, due to their slow intrinsic binding kinetics. In contrast, the rapid formation and dissolution of bonds between the vWF A1 domain and GP Ib supports efficient platelet tethering and translocation (rolling) under conditions of high shear (3). Understanding the mechanisms regulating platelet translocation is potentially important, since recent studies have demonstrated that the majority of platelets adhering to the injured vessel wall and to the surface of thrombi in vivo undergo a variable period of surface translocation prior to forming stationary adhesion contacts (4, 5).

An important unresolved issue is the relative contribution of GPIb and integrin αIIbβ3 outside-in signaling to cytosolic calcium flux initiated by the platelet-vWF interaction. For example, a recent study by Kuwahara et al. has suggested that calcium flux during shear-dependent platelet adhesion on vWF is exclusively mediated through integrin αIIbβ3 (8). The involvement of integrins in the regulation of cytosolic calcium levels has been well established in a range of cell types, including platelets, leukocytes, endothelial cells, fibroblasts, and osteoclasts (9). In general, calcium signaling is utilized by β2 leukocyte integrins as well as integrins that engage ligands containing the tripeptide sequence Arg-Gly-Asp (RGD), including platelets, leukocytes, endothelial cells, fibroblasts, and osteoclasts (9).

The platelet is a specialized adhesive cell that plays a central role in the normal blood clotting process through its ability to rapidly adhere to subendothelial matrix proteins and to other activated platelets at sites of vascular injury. Platelet adhesion is a multistep process requiring an initial cell-tethering step, involving interaction between the glycoprotein Ib/V/IX (GPIb/V/IX) receptor complex with subendothelial von Willebrand factor (vWF) and a firm adhesion step mediated by one or more platelet integrins (1, 2). Integrins themselves are generally inefficient at initiating platelet adhesion under conditions of rapid blood flow, due to their slow intrinsic binding kinetics. In contrast, the rapid formation and dissolution of bonds between the vWF A1 domain and GP Ib supports efficient platelet tethering and translocation (rolling) under conditions of high shear (3). Understanding the mechanisms regulating platelet translocation is potentially important, since recent studies have demonstrated that the majority of platelets adhering to the injured vessel wall and to the surface of thrombi in vivo undergo a variable period of surface translocation prior to forming stationary adhesion contacts (4, 5).
α_{IIbβ3} that serves to regulate platelet translocation dynamics and firm platelet adhesion under flow.

EXPERIMENTAL PROCEDURES

Materials—5,5′-Dimethyl-BAPTA; AM; Oregon Green 488 BAPTA-1; AM; Fura Red; AM; and NP-EGTA were from Molecular Probes, Inc. (Eugene, OR). Apparatus was purified from potatoes according to the method of Molnar and Lorand (20) and was a generous gift from Dr. Francois Lanza. Human vWF was purified to homogeneity from plasma cryoprecipitate according to the methods of Montgomery and Zimmerman (21). LY294002 was from Calbiochem. Wortmannin was purchased from Sapphire Bioscience P/L (New South Wales, Australia). All other reagents were obtained from sources described previously (22, 23).

Platelet Isolation and Reconstitution with Red Blood Cells—Anticoagulated whole blood (15 ml trisodium citrate, pH 7.4) was collected from healthy volunteers who had not received any anti-platelet medication in the preceding 2 weeks. Platelet isolation was carried out according to Yuan et al. (22), and washed platelets were finally resuspended in modified Tyrode’s buffer (10 mM HEPES, 12 mM NaHCO3, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 5 mM glucose) supplemented with 1 mM CaCl2/MgCl2 or 1 mM EGTA/MgCl2 where indicated. Autolysed red blood cells were removed according to Yap et al. (24) prior to reconstitution with isolated platelets (50% (v/v) hematocrit) in the presence of 0.4 units/ml appyrase (ADPase activity) and 1 unit/ml hirudin.

Analysis of Platelet Calcium Flux—Platelet calcium flux was monitored as previously described by Yap et al. (24). Isolated platelets (1.5 × 10^7/ml) suspended in platelet wash buffer (PWB) (4.3 mM KH2PO4, 4.3 mM NaH2PO4, 12.4 mM NaCl, 2.6 mM KCl, 1.3 mM NaHCO3, 0.5% (v/v) bovine serum albumin, 10 mM theophylline) were incubated for 30 min at 37 °C with Oregon Green BAPTA-1, AM (1 μM) and Fura Red, AM (1.25 μM). Unincorporated dye was removed by washing twice with PWB. Platelets were subsequently resuspended in Tyrode’s buffer containing either 1 mM CaCl2/MgCl2 or 1 mM EGTA/MgCl2. Real time platelet calcium flux was monitored via confocal microscopy using Oregon Green BAPTA-1 fluorescence and Fura Red fluorescence (with emission wavelengths of 500–570 and 600–710 nm, respectively). Fluorescence ratios were converted to relative cytosolic calcium concentration (Δ(Ca2^+)i) according to Equation 1,

$$\Delta [Ca^{2+}]_{i} = 170 \times (R - R_{min})/(R_{max} - R) \times F_{max}/F_{min}$$

where 170 is the Ks value of Oregon Green BAPTA-1 Ca2^+ binding; R represents the measured fluorescence ratio; R_{max} is the mean fluorescence ratio determined from 200–400 cells suspended in Tyrode’s buffer supplemented with 2 mM EGTA/Fura Red; R_{min} represents the fluorescence ratio determined from 200–400 cells preincubated with 70 μM DM-BAPTA, AM (incubated for 30 min at 37 °C) and reconstituted in Tyrode’s buffer supplemented with 2 mM EGTA/Fura Red, AM, representing the mean fluorescence values (arbitrary units) of Oregon Green BAPTA-1 for R_{max} and R_{min} respectively. The calculated calcium flux values are designated Δ[Ca2^+]i, to indicate that all calcium concentration estimates are relative to a zero point, set by DM-BAPTA calcium chelation.

Off-line Calcium Analysis—Calcium flux recordings were analyzed off-line using Leica Physiology Software (Leica TCS SP; Leica, Heidelberg, Germany), in the case of single cell recordings. Alternatively, platelet Δ(Ca2^+)i was analyzed at a population level using MCID® Image analysis software (Macro Imaging Research Inc., Ontario, Canada). The first 3 min of platelet flow was captured as sequential 37.5-s series (0.586 frames per second capture rate) via confocal microscopy, and individual frames were analyzed for fluorescence pixel intensities at 5.86-s intervals following background subtraction of small pixel targets of less than 10-pixel diameters. Data analyzed in this way were pooled over the entire 37.5-s scan.

RESULTS

Cytosolic Calcium Flux Regulates Platelet Translocation Behavior—Recent studies examining shear-dependent platelet adhesion on immobilized vWF under flow have demonstrated a role for both the GPIb/V/IX and integrin α_{IIbβ3} receptor complexes in regulating cytosolic calcium flux (7, 8). However, the temporal relationship and mechanisms by which these receptors regulate cytosolic calcium (Δ(Ca2^+)i) remain incompletely understood. In this study, we have employed a confocal-based dual dye ratiometric Ca2^+ assay to accurately quantify real-time cytosolic calcium flux during platelet adhesion under flow (24). In initial studies, Oregon Green BAPTA and Fura Red-loaded human platelets were perfused through human vWF-coated (100 μg/ml) microcapillary tubes at a shear rate of 1,800 s^−1, and the relationship between cytosolic calcium flux and platelet translocation behavior was examined. Fig. IA demonstrates that the platelet population exhibited a broad range of Δ(Ca2^+)i, ranging from 0 to 1,200 nM, and translocation velocities between 0 and 20 μm/s, similar to those reported by Suavage et al. (1). Detailed single-cell analysis revealed that platelets exhibit three broad calcium response subclasses that differ with respect to the magnitude and dynamics of their calcium flux. The first subclass consists of platelets with a relatively low cytosolic calcium content (Δ(Ca2^+)i, <20 nM). These platelets were characterized by a rapid rate of translocation across the vWF surface and minimal calcium oscillations (Fig. IB). The second subclass exhibited a moderately elevated (intermediate) Δ(Ca2^+)i, ranging from 20 to 65 nM, which underwent minor oscillations; these platelets displayed a reduced rate of translocation that was stop-start in nature (Fig. IB). The third and final subclass of platelets displayed elevated oscillatory Δ(Ca2^+)i, ranging from 65 up to 1200 nM. The defining quality of platelets falling into this high range calcium response subclass was the pulsatile nature of the calcium flux, with cells undergoing rapid base-line to peak oscillations. The translocation behavior of these platelets was characterized by extended periods of stationary adhesion on the matrix surface (Fig. IB). To examine in more detail the correlation between Δ(Ca2^+)i and translocation behavior, platelets were pretreated with the high affinity calcium chelator DM-BAPTA prior to perfusion through vWF-coated microcapillary tubes. DM-BAPTA treat-
Cooperative Calcium Signaling between GPIb and Integrin αIIbβ3

Fig. 1. Correlation of cytosolic Δ[Ca2+]i and translocation behavior on the surface of immobilized vWF. A, dot plot distribution showing the range of Δ[Ca2+]i, and single cell translocation velocities (μm s−1) recorded within a platelet population (150 × 10^6/liter) perfused over immobilized vWF at a shear rate of 1,800 s−1. Conditions were as follows. Resting, static platelet suspension in Tyrode’s buffer supplemented with Ca2+/Mg2+; Control, perfused over immobilized vWF at 1,800 s−1; DM-BAPTA, incubated with 70 μM of the calcium chelator, DM-BAPTA, AM for 30 min at 37 °C, washed human platelets were perfused through vWF-coated microcapillary tubes. Consistent with previous findings (24), none of these inhibitors, either alone or in combination, modified the distribution of calcium events initiated by vWF engagement of GPIb and integrin αIIbβ3 at 1,800 s−1 (data not shown).

The Relative Roles of GPIb/V/IX and Integrin αIIbβ3 in Regulating Cytosolic Calcium Flux—Our recent studies have demonstrated that platelets forming irreversible adhesion contacts on a vWF matrix, under static or flow conditions, exhibit a sustained oscillatory calcium response (24). To investigate the relative roles of GPIb/V/IX and integrin αIIbβ3 in regulating these calcium changes, platelets were pretreated with vehicle, Aggrastat, or c7E3 Fab prior to performing adhesion studies on vWF. As demonstrated in Fig. 2A, platelets forming irreversible adhesion contacts with vWF under static conditions elicited sustained oscillatory calcium responses. However, blocking ligand binding to integrin αIIbβ3 completely abolished these sustained oscillations, with all cells exhibiting distinct calcium spikes (Fig. 2A). Several lines of evidence demonstrate that these calcium spikes are elicited as a result of the vWF-GPIb interaction, rather than secondary to the release of ADP or thromboxane A2. First, pretreating platelets with apyrase or aspirin had no inhibitory effect on the generation of these calcium spikes (data not shown). Second, blocking ligand binding to GPIb abolished all calcium responses (data not shown). Third, pretreating platelets with ristocetin, a cationic modulator that increases the affinity of the vWF-GPIb interaction, increased the proportion of cells that elicited these isolated calcium spikes (Fig. 2C) but did not affect the amplitude of the Ca2+ transients (mean Δ[Ca2+]i = 305 ± 146 nm; n = 40) (Fig. 2B). These studies suggest that even in the absence of shear, GPIb binding to vWF is sufficient to induce a transient Ca2+ signal.

We have previously demonstrated that shear increases the proportion of platelets exhibiting a GPIb-dependent calcium signal (7). To investigate the effects of shear on the magnitude and duration of calcium signals initiated by GPIb/V/IX and integrin αIIbβ3, control or Aggrastat-treated platelets were perfused through vWF-coated microcapillary tubes at a shear rate of 1,800 s−1. Platelets forming stationary adhesion contacts under high shear exhibited a sustained oscillatory calcium response similar in magnitude and duration to that observed under static conditions (data not shown). Integrin αIIbβ3 blockade under these conditions resulted in almost complete inhibition of high range Δ[Ca2+]i (Fig. 3A), with a concomitant (5-fold) increase in platelet translocation velocity, from a mean of 6.2 μm s−1 in control platelets up to 34.1 μm s−1 in Aggrastat-treated platelets (data not shown). To examine specifically the effects of shear on GPIb-derived calcium signals, independent...
of platelet translocation, Aggrastat-treated platelets were perfused through vWF-coated microcapillary tubes in the presence of ristocetin. Under these experimental conditions, the vWF-GPIb interaction sustains stationary platelet adhesion under shear flow (1800 s⁻¹), independent of integrin α₁β₃ engagement (data not shown). In control studies, we demonstrated that the presence of ristocetin did not have any significant effect on the overall translocation of platelets independent of integrin α₁β₃ engagement (compare Fig. 3C with Fig. 2, A and B). Analysis of Aggrastat-treated platelets at 1800 s⁻¹ revealed that 36% of adherent cells displayed discrete calcium transients or "spikes," that exhibited a mean Δ[C₅²⁺]₀ of 391 ± 182.9 nm (maximum Δ[C₅²⁺]₀ = 896 nm; n = 41) (Fig. 3, B and C). Thus, regardless of the experimental conditions, the vWF-GPIb interaction appears to elicit transient Ca²⁺ spikes that are distinct from the complex oscillatory response initiated by integrin α₁β₃ engagement of vWF.

**The Role of Transient Calcium Spikes in Regulating Platelet Adhesion under Flow**—To investigate the potential importance of transient calcium spikes in regulating platelet adhesion dynamics under flow, we examined calcium response profiles of individual translocating platelets. Detailed analysis of platelets undergoing high range Ca²⁺ responses under shear conditions revealed a subset of cells that exhibited elevated but transient calcium responses (Fig. 4A). As demonstrated in Fig. 4A, these transient calcium spikes coincided with a brief period of stationary adhesion, which was followed by a return to surface translocation following a decline in Δ[C₅²⁺], toward 100 nm. In contrast, all platelets exhibiting sustained oscillatory spikes in the absence of integrin α₁β₃ engagement (+200 nM Aggrastat) at the surface of immobilized vWF (n = 3). C. platelets were allowed to adhere to immobilized vWF in the presence of mg/ml ristocetin; +Ristocetin, control platelets; +Ristocetin +Aggrastat, platelets treated with 200 nM Aggrastat prior to adhesion in the presence of 1 mg/ml ristocetin. Regardless of the experimental conditions, the vWF-GPIb interaction appears to elicit transient Ca²⁺ spikes that are distinct from the complex oscillatory response initiated by integrin α₁β₃ engagement of vWF.
ever, following brief exposure to UV irradiation, these cells rapidly released caged calcium (data not shown). To investigate the effects of transient calcium spikes on platelet adhesion under flow, NP-EGTA-loaded platelets were perfused through vWF-coated microcapillary tubes at a shear rate of 1,800 s⁻¹. Exposure of these cells to UV light for a period of 0.6 s resulted in the generation of a rapid spike in ∆[Ca²⁺]ᵢ, approaching 700–1200 nm (Fig. 4B). Examination of the translocation properties of these platelets demonstrated that stationary adhesion was tightly controlled by the onset of the calcium spikes (Fig. 4B). Platelet adhesion under these experimental conditions was mediated through integrin αIβ₃, since it was completely prevented by pretreating platelets with c7E3 Fab or Aggrastat (Fig. 4B). In control studies, we demonstrated that the effects of uncaged calcium on stationary platelet adhesion were likely to be direct rather than secondary to release of ADP or thromboxane A₂, since pretreating platelets with apyrase or aspirin had no effect on UV light-induced platelet adhesion (data not shown).

To investigate further the relationship between sustained calcium oscillations and firm cell adhesion, NP-EGTA-loaded platelets were pretreated with a suboptimal concentration of Tg (1 nM) 2 min prior to perfusion through vWF-coated microcapillary tubes. Pretreatment with 1 nM Tg had minimal effect on platelet translocation behavior at a shear rate of 1,800 s⁻¹ prior to UV photolysis (Fig. 4C). Triggering of a Ca²⁺ spike by UV photolysis of NP-EGTA led to an equivalent transient Ca²⁺ elevation as that observed for control platelets not incubated with Tg (Fig. 4C). Tg pretreatment partially blocked the reuptake of the NP-EGTA-elicted Ca²⁺ spikes and led to the onset of an oscillatory Ca²⁺ response (∆[Ca²⁺]ᵢ >100 nm) (Fig. 4C) in the entire platelet population (data not shown). The prolonged oscillatory Ca²⁺ response in the presence of Tg following UV exposure directly correlated with an increased duration of stationary adhesion (Fig. 4C). This stationary adhesion was mediated by integrin αIβ₃, since it was completely blocked by pretreating platelets with c7E3 or Aggrastat (Fig. 4C). Taken together, these data suggest that transient Ca²⁺ spikes may serve to initiate integrin αIβ₃ activation and transient cell arrest; however, irreversible adhesion appears to be dependent on sustained integrin αIβ₃-dependent calcium oscillations.

**Extracellular Calcium and PI3-Kinase Requirement for GPIb and Integrin αIβ₃-derived Calcium Signals—**Our recent studies have defined an important role for transmembrane calcium influx and PI3-kinase in potentiating sustained Ca²⁺ oscillations and integrin αIβ₃ activation during platelet adhesion to immobilized vWF under flow (24, 26). To investigate the contribution of transmembrane calcium influx to GPIb and integrin αIβ₃-dependent calcium signaling, in vitro flow studies were performed in the presence or absence of EGTA. As demonstrated in Fig. 5A, EGTA markedly reduced integrin αIβ₃-dependent calcium signals at 1,800 s⁻¹, resulting in an 86% reduction in the frequency of ∆[Ca²⁺]ᵢ events occurring above 100 nm in the platelet population. In contrast, chelating extracellular calcium had no inhibitory effect on the magnitude or duration of GPIb-dependent calcium spikes (n = 40) (Fig. 5B). Significantly, even in the absence of extracellular calcium, integrin αIβ₃ engagement of vWF was still able to induce a sustained oscillatory calcium response (data not shown), indicating that ligand binding to this receptor can initiate calcium release from internal stores.

To investigate the role of PI3-kinase for GPIb and integrin αIβ₃-dependent calcium signaling, platelets were pretreated with concentrations of wortmannin (100 nM) or LY294002 (25 μM) that effectively abolish PI3-kinase activation in vWF-stim-
ulated platelets (26). As demonstrated in Fig. 6A, both inhibitors significantly inhibited integrin αIIbβ3-dependent high range calcium oscillations, resulting in a 98% reduction in the frequency of calcium events occurring above 100 nM in the platelet population. In contrast, neither wortmannin nor LY294002 had any significant effect on transient calcium spikes initiated by the vWF-GPIb interaction (Fig. 6B). Consistent with our previous findings, inhibition of sustained calcium oscillations resulted in an inability of the platelets to form irreversible adhesion contacts under flow (data not shown). These findings suggest that PI 3-kinase involvement in shear-dependent platelet adhesion is primarily linked to integrin αIIbβ3, not GPIb-dependent calcium signals.

DISCUSSION

The studies reported here have demonstrated the existence of two distinct, cooperative calcium signaling mechanisms utilized by GPIb/V/IX and integrin αIIbβ3 to regulate platelet-
adhesive behavior under flow (Fig. 7). A significant finding from these studies is that GPIb/V/IX engagement of vWF elicits a transient calcium response that may serve to initiate integrin αⅠbβ3 activation; however, this calcium signal appears insufficient to maintain sustained platelet adhesion in the shear field. Our studies suggest that subsequent integrin αⅠbβ3 engagement of vWF triggers outside-in signaling events linked to the initiation and propagation of sustained oscillatory calcium flux that is necessary for stable platelet adhesion.

By examining real time changes in cytosolic calcium during platelet translocation, we have been able to establish a close correlation between cytosolic calcium fluctuations and the stop-start nature of platelet translocation. In particular, we have been able to identify platelets that exhibit transient calcium oscillations that are associated with intermittent phases of stationary adhesion, often lasting 10–20 s or more. In all cells, the transition from stationary adhesion to surface translocation corresponded to a drop in the cytosolic calcium, approaching 100 nM. These studies suggest that a critical calcium threshold must be reached and maintained for platelets to sustain stable adhesion contacts in a shear field. Furthermore, we have demonstrated that this calcium-dependent regulation of platelet translocation behavior is mediated through the reversible activation of integrin αⅠbβ3, establishing for the first time an important role for this receptor in regulating platelet translocation dynamics under flow.

Our studies have also provided new insight into the relative contribution of GPIb and integrin αⅠbβ3 outside-in signaling toward vWF-induced calcium flux. The traditional model for platelet activation by vWF, based primarily on studies of shear-induced platelet aggregation using a cone-and-plate viscometer, had suggested that GPIb-induced transmembrane calcium influx represented the critical proximal signaling step for subsequent ADP release and integrin αⅠbβ3 activation. However, recent studies from our laboratory have demonstrated that vWF binding to GPIb is sufficient to induce intracellular calcium mobilization, independent of transmembrane calcium influx and ADP release, as a necessary event for subsequent integrin αⅠbβ3 activation (7, 24). In this report, we demonstrate the cooperative relationship between GPIb and integrin αⅠbβ3 in regulating cytosolic calcium flux and, furthermore, demonstrate that the calcium signaling mechanisms operating downstream of GPIb/V/IX and integrin αⅠbβ3 are distinct, in that the former are primarily due to calcium release from internal stores, whereas the latter are dependent on both intracellular calcium mobilization and transmembrane calcium influx. The reason for this difference remains to be established but may be related to differences in the duration and magnitude of calcium mobilization. Calcium store emptying is a major stimulus for transmembrane calcium influx, and it is conceivable that integrin αⅠbβ3-dependent sustained calcium flux leads to more extensive depletion of intracellular calcium stores in comparison with the transient calcium spikes initiated by GPIb binding. Alternatively, integrin αⅠbβ3 engagement may directly activate an associated calcium influx pathway, as proposed by Brass (15).

This study demonstrates for the first time a role for PI 3-kinase in regulating integrin αⅠbβ3 calcium signaling and, somewhat unexpectedly, does not support an important role for this kinase in GPIb signaling, at least under high shear conditions. There are two potential mechanisms by which PI 3-kinase may promote integrin αⅠbβ3 activation and sustained calcium oscillations. First, while not essential for GPIb-dependent calcium flux, PI 3-kinase may function downstream of cytosolic calcium to initiate integrin αⅠbβ3 activation. Alternatively, PI 3-kinase may participate in integrin αⅠbβ3 outside-in signaling events associated with the initiation and propagation of intracellular calcium oscillations, necessary for sustained integrin αⅠbβ3 activation. While we have not formally excluded the former hypothesis, several lines of evidence suggest that the latter hypothesis is more likely. First, to our knowledge there are no precedents for PI 3-kinase signaling downstream of calcium. Second, there is a considerable body of evidence demonstrating PI 3-kinase involvement in integrin αⅠbβ3 outside-in signaling. For example, the production of 3-phosphorylated phosphoinositides in thrombin-stimulated platelets is regulated downstream of integrin αⅠbβ3 (27). Furthermore, direct ligand binding to integrin αⅠbβ3 is sufficient to induce PI 3-kinase activation and a selective increase in the cellular levels of phosphatidylinositol 3,4-bisphosphate (28). This integrin αⅠbβ3-dependent activation of PI 3-kinase has been proposed to sustain integrin αⅠbβ3 activation as an important event for cytoskeletal reorganization, platelet spreading, and irreversible aggregation (28, 29).

It remains to be established what the molecular mechanism is by which PI 3-kinase promotes integrin αⅠbβ3-dependent cytosolic calcium flux. Previous studies in platelets and a range of other cells have demonstrated that the PI 3-kinase lipid
product, phosphatidylinositol 3,4,5-trisphosphate, plays a significant role in the regulation of both intracellular calcium mobilization and transmembrane calcium flux (30–34). However, given previous observations that direct ligation of integrin αIIbβ3 leads to a selective increase in phosphatidylinositol 3,4-bisphosphate, it remains to be determined whether phosphatidylinositol 3,4,5-trisphosphate plays a significant role in integrin αIIbβ3-dependent calcium signaling.

Finally, much of our current conceptual understanding of integrin αIIbβ3 signaling in platelets is based on studies of platelets in suspension, in which the addition of soluble stimuli is required to initially induce integrin αIIbβ3 activation (inside-out signaling). In this assay system, subsequent ligand binding events are required to generate outside-in signals, necessary to promote relatively “late” platelet functional responses, including irreversible platelet aggregation, clot retraction, and the shedding of procoagulant-rich microvesicles. Our studies suggest that the establishment of integrin αIIbβ3 outside-in calcium signaling plays a critical “early” role in the initial activation of platelets on vWF. These studies define a previously unrecognized role for integrin αIIbβ3 in driving a calcium-dependent positive feedback mechanism that plays an important role in regulating the affinity status of the integrin itself.

Acknowledgments—We thank Dr. Steve Watson, Prof. Leslie Parise, and Dr. Yuping Yuan for helpful discussions and constructive advice during the preparation of the manuscript.

REFERENCES

1. Savage, B., Saldivar, E., and Ruggeri, Z. M (1996) Cell 84, 289–297
2. Savage, B., Almus-Jacobs, F., and Ruggeri, Z. (1998) Cell 94, 657–666
3. Ruggeri, Z. M (1997) Thromb. Haemost. 78, 611–616
4. Denis, C., Methia, N., Frenette, P. S., Rayburn, H., Ullman-Cullere, M., Hynes, R. O., and Wagner, D. D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9524–9529
5. Kulkarni, S., Dopheide, S. M., Yap, C. L., Ravant, C., Freund, M., Mangin, P., Heel, K. A., Street, A., Harper, I. S., Lanza, F., and Jackson, S. P (2000) J. Clin. Invest. 105, 21847–21854
6. Cooke, B. M., Usami, S., Perry, I., and Nash, G. B. (1993) Microvasc. Res. 45, 33–55
7. Yuan, Y., Depheide, S. M., Ivanidis, C., Salem, H. H., and Jackson, S. P (1997) J. Biol. Chem. 272, 21847–21854
8. Kovalcovic, T. J., Bachelot, C., Toker, A., Vlahos, C. J., Duckworth, B., Ambrosio, D., Nakamura, K., Persin, C., and Cambier, J. C. (1997) Immunity 7, 49–58
Distinct Glycoprotein Ib/V/IX and Integrin $\alpha_{\text{IIb}}\beta_3$-dependent Calcium Signals Cooperatively Regulate Platelet Adhesion under Flow

Warwick S. Nesbitt, Suhasini Kulkarni, Simon Giuliano, Isaac Goncalves, Sacha M. Dopheide, Cindy L. Yap, Ian S. Harper, Hatem H. Salem and Shaun P. Jackson

J. Biol. Chem. 2002, 277:2965-2972.
doi: 10.1074/jbc.M110070200 originally published online November 16, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M110070200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 33 references, 22 of which can be accessed free at http://www.jbc.org/content/277/4/2965.full.html#ref-list-1