Integrin $\alpha_{IIb}\beta_3$-dependent Calcium Signals Regulate Platelet-Fibrinogen Interactions under Flow

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Platelet adhesion to fibrinogen is important for platelet aggregation and thrombus growth. In this study we have examined the mechanisms regulating platelet adhesion on immobilized fibrinogen under static and shear conditions. We demonstrate that integrin $\alpha_{IIb}\beta_3$ engagement of immobilized fibrinogen is sufficient to induce an oscillatory calcium response, necessary for lamellipodial formation and platelet spreading. Released ADP increases the proportion of platelets exhibiting a cytosolic calcium response but is not essential for calcium signaling or lamellipodial extension. Pretreating platelets with the Src kinase inhibitor PP2, the inositol 1,4,5-trisphosphate (IP$_3$) receptor antagonist 2-aminoethoxydiphenyl borate (APB-2), or the phospholipase C (PLC) inhibitor U73122 abolished calcium signaling and platelet spreading, suggesting a major role for Src kinase-regulated PLC isoforms in these processes. Analysis of PLC$\gamma^2$+$\gamma^3$ mouse platelets revealed a major role for this isoform in regulating cytosolic calcium flux and platelet spreading on fibrinogen. Under flow conditions, platelets derived from PLC$\gamma^2$+$\gamma^3$ mice formed less stable adhesive interactions with fibrinogen, particularly in the presence of ADP antagonists. Our studies define an important role for PLC$\gamma^2$ in integrin $\alpha_{IIb}\beta_3$-dependent calcium flux, necessary for stable platelet adhesion and spreading on fibrinogen. Furthermore, they establish an important cooperative signaling role for PLC$\gamma^2$ and ADP in regulating platelet adhesion efficiency on fibrinogen.

Platelet adhesion and aggregation at sites of vascular injury are critical for the arrest of bleeding in traumatized vessels and also for the development of arterial thrombi, precipitating diseases such as acute myocardial infarction and stroke. The two principle adhesive ligands promoting platelet aggregation are von Willebrand factor (vWF) and fibrinogen, with each having distinct, complementary roles in this process (1–5). Fibrinogen acts as a bridging molecule between adjacent activated platelets and is the principle adhesive ligand promoting platelet aggregation under low and intermediate shear flow conditions (1, 2). In addition, it also contributes to stable aggregate formation under high shear (1, 5, 6). It is well established that the binding of fluid-phase fibrinogen to the platelet surface is dependent on the activation of integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) through intracellular signaling processes linked to various G protein-coupled and tyrosine kinase-linked receptors (7).

Platelets can also adhere to immobilized fibrinogen, a process that is important for primary platelet adhesion onto artificial surfaces, including vascular prostheses (8, 9), and for normal thrombus development (5, 6). In the case of thrombus formation, active integrin $\alpha_{IIb}\beta_3$ on the surface of firmly adherent platelets adsorbs soluble fibrinogen to the thrombus surface, thereby providing a reactive substrate for the recruitment of additional platelets. In contrast to soluble fibrinogen, the adhesion of platelets onto immobilized fibrinogen does not require affinity modulation of integrin $\alpha_{IIb}\beta_3$ (10). As such, surface-adsorbed fibrinogen can potentially promote platelet-thrombus interactions independent of initial platelet activation. This concept is supported by experimental findings demonstrating that platelet activation inhibitors have no effect on the ability of platelets to form stable adhesive interactions with a purified fibrinogen matrix under flow (11–13). This contrasts with all other platelet adhesive interactions, involving substrates such as vWF, collagen, fibronectin, and vitronectin, in which the formation of stable adhesive bonds with these surfaces is considered activation-dependent (10, 12–17).

Once adherent to fibrinogen, platelets become activated and undergo substantial cytoskeletal remodeling, leading to platelet shape change and spreading. Integrin $\alpha_{IIb}\beta_3$ outside-in signals have been demonstrated to play a major role in this process (18, 19), although signaling processes downstream of this receptor per se do not appear to be sufficient for platelet spreading independent of co-stimuli such as ADP. Current evidence suggests that integrin $\alpha_{IIb}\beta_3$ engagement of fibrinogen induces activation of Src kinases and Syk, which promote cytoskeletal remodeling, leading to shape change and filopodial extension (20–22). During this process, platelets secrete their granule contents, and the release of ADP promotes cytosolic calcium flux and lamellipodial extension via signaling pathways linked
the activation of phosphoinositide 3-kinase (23–25).

In this study we have examined the mechanisms regulating platelet adhesion and activation on a fibrinogen matrix. In contrast to previous reports, our studies do not demonstrate an absolute requirement for ADP for lamellipodial extension and platelet spreading on fibrinogen. Rather, they suggest that integrin αIIbβ3 outside-in signaling linked to Src kinase-mediated phospholipase Cγ2 (PLCγ2) activation is critical for platelet spreading, whereas ADP release serves a secondary role, potentiating platelet activation. Furthermore, we demonstrate that integrin αIIbβ3-dependent calcium flux, combined with ADP release, plays an important role in sustaining platelet-fibrinogen interactions under flow. These findings challenge previous concepts of the mechanisms regulating platelet adhesion and activation on fibrinogen, defining a pivotal role for integrin αIIbβ3-dependent calcium flux in these processes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Apyrase was purified as described previously (26). Human fibrinogen was purified from fresh frozen plasma according to Jakobsen et al. (27). The Src kinase inhibitor PP2 was purchased from Calbiochem-Novabiochem. Probenecid, 2-aminoethoxydiphenyl borate (APB-2), and the P2Y1 antagonist A3P5PS were obtained from Sigma. The P2Y12 antagonist AR-C69931MX was obtained from AstraZeneca. The anti-phosphotyrosine monoclonal antibody (PY20) was from ICN. PAC-1 was from BD Biosciences. The anti-PLCγ2 polyclonal antibody was obtained from Santa Cruz Biotechnology, and the anti-phosphotyrosine monoclonal antibody (PY20) was from ICN.

**Platelet Preparation**—Washed human and murine platelets were prepared as described previously (26, 30). For adhesion studies, washed platelets were 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).

**Static Adhesion Assays**—Static adhesion assays were performed using a modified method of Yuan et al. (28). Briefly, glass coverslips (12 mm in diameter; Lomb Scientific) were coated with fibrinogen (100 μg/ml) for 2 h at room temperature and then blocked with 10% heat-inactivated human serum pretreated with phenylmethylsulfonyl fluoride (25 μg/ml). Platelets in Tyrode’s buffer (1–3 × 107/ml) supplemented with 1 mM CaCl2 were allowed to adhere to the fibrinogen matrix for the indicated time periods. Adherent platelets were fixed with 3.7% formaldehyde for 15 min, mounted onto glass slides, and imaged using differential interference contrast (DIC) microscopy or phase contrast microscopy for surface area analysis as described by Yap et al. (26). Where indicated, platelets were preincubated with vehicle alone (Me2SO, 0.25% (v/v)), the Src kinase inhibitor (PP2, 1–10 μM), apyrase (1.5 units/ml, ADPase activity), the ADP receptor antagonists against P2Y1 (A3P5PS, 200 μM) or P2Y12 (AR-C69931MX, 100 nM), the IP3 receptor antagonist (APB-2, 20 μM), or the PLC inhibitor (U73122, 5–10 μM) for 10 min at 37 °C prior to the performance of adhesion assays. In other experiments, the role of TXA2 was assessed by pretreating platelets with aspirin (1 mM) for 30 min at 37 °C prior to the performance of adhesion assays. The pharmacological activity of these inhibitor(s) was confirmed as follows: PP2 abolished collagen-induced platelet aggregation (31); apyrase abolished ADP (25 μM)-induced platelet aggregation; aspirin blocked arachidonic acid (1.2 mM)-induced platelet aggregation; APB-2 inhibited thrombin (0.5–1.0 units/ml)-induced platelet aggregation.

**Measurement of Integrin αIIbβ3 Activation**—Integrin αIIbβ3 activation during platelet adhesion to fibrinogen under static conditions was assessed using the integrin αIIbβ3 activation-specific antibody PAC-1.

![Figure 1](image-url)

**Figure 1.** Platelet spreading on immobilized fibrinogen occurs independent of endogenous ADP or TXA2. Washed platelets (3 × 107/ml) were incubated with buffer (Control) or apyrase (1.5 units/ml ADPase activity) either alone (Apyrase) or in combination with aspirin (1.5 mM, Apyrase/Aspirin) for 30 min at 37 °C. Platelets were then allowed to adhere to immobilized fibrinogen (100 μg/ml) for 50 min at 37 °C. A, adhesion and spreading were performed in the presence of the integrin αIIbβ3 activation-specific antibody, PAC-1 (2 μg/ml). Adherent platelets were fixed, labeled with FITC-conjugated secondary antibody, and imaged using either DIC or confocal fluorescence microscopy (PAC-1). Images were taken from one experiment representative of four. B, PAC-1 binding was quantified using confocal software as described under “Experimental Procedures.” These data were taken from two experiments, representative of four. The bar represents the mean PAC-1 fluorescence. C, in time course studies, control or apyrase/aspirin-treated platelets were allowed to adhere and spread on fibrinogen for 10, 20, 30 or 50 min, prior to fixation. The mean surface area of the adherent platelets at each time point was determined as described under “Experimental Procedures.” These results represent the mean ± S.E. of three individual experiments performed in duplicate.
Platelets were allowed to adhere to fibrinogen in the presence of PAC-1 (2 μg/ml) followed by fixation (3.7% formaldehyde) and incubation with a FITC-conjugated anti-mouse IgG F(ab')2 fragment. PAC-1 immunofluorescence was visualized using confocal fluorescence microscopy (100× magnification, TCS-SP, Leica) and quantified using the Leica TCS NT software as described previously (26). In some studies, platelets were preincubated with the indicated concentrations of APB-2, U73122, PP2, or apyrase alone or in combination with aspirin prior to the performance of adhesion studies.

Platelet Adhesion to Fibrinogen under Flow Conditions—Flow assays were performed as described previously (26). In studies examining the ability of platelets to adhere to the fibrinogen matrix, washed platelets (5 × 10^9/ml) were reconstituted with washed red blood cells (50% v/v; containing 0.04 units/ml apyrase and 1 unit/ml hirudin) as described previously (26). Platelets were perfused through fibrinogen (100 μg/ml)-coated microcapillary tubes at a wall shear rate of 150 s⁻¹ for 2 minutes at 37 °C. Platelet-matrix interactions were visualized using epifluorescence microscopy (Leica DMIRB) and video-recorded for off-line analysis. Platelet tethering was analyzed at 30, 60, and 90 s, and each platelet that interacted with the fibrinogen matrix for ≥2 frames (40 ms) was scored as “tethered.”

In studies examining cell displacement, DiOC₆-labeled platelets were perfused through fibrinogen-coated microcapillary tubes for 5 min at 150 s⁻¹. Platelets were considered as dislodged when exhibiting spatial displacement on the surface greater than 1 platelet diameter from their initial attachment point (12). Similar analysis was used for calcium-dye loaded human and murine platelets when perfused across a fibrinogen matrix (see below), except that platelet adhesion was monitored for 100 frames (0.586 frames/s) using confocal fluorescence microscopy (TCS-SP, Leica). Similar analysis was used to examine adhesion strength at high shear (1800 s⁻¹); however, in this case, platelets were first perfused at 150 s⁻¹ for 5 min followed by an increase in wall shear rate to 1800 s⁻¹ for a further 60 s.

Analysis of Cytosolic Calcium Flux under Static and Flow Conditions—Changes in cytosolic calcium levels were monitored according to published methods (14, 26). Briefly, washed platelets (1 × 10⁹/ml) were loaded with Oregon Green 488 BAPTA-AM (1 μM) and Fura Red-AM (1.25 μM) for 30 min at 37 °C. For mouse platelets, the calcium dyes were loaded at a platelet density of 2 × 10⁷/ml in the presence of 1.25 mM Probenecid. Dye-loaded platelets (1 × 10⁹/ml) were then either allowed to adhere to fibrinogen under static conditions or reconstituted with red blood cells (50%) prior to perfusion through fibrinogen-coated microcapillary tubes at 150 s⁻¹ for human platelets and 600 s⁻¹ for mouse platelets. To examine the changes in calcium flux, sequential confocal images of adherent platelets were captured at a scan rate of 0.586 frames/s for 37.5 s at the indicated time points. Real-time platelet calcium flux was calculated from ratiometric fluorescence measurements and converted to intracellular calcium concentrations as described previously (14, 26).

Assessment of PLC-γ2 Phosphorylation—Washed platelets (1 × 10⁹/ml), treated with vehicle alone (0.25% Me₂SO) or PP2 (10 μM) for 10 min, were then applied to fibrinogen-coated dishes (100 μg/ml) for 30 min at 37 °C. Adherent cells were lysed with radioimmunoprecipitation assay buffer (10 mM Tris, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 2 mM benzamidine). To examine the level of PLC-γ2 phosphorylation in resting cells, non-adherent platelets in suspension were lysed with radioimmunoprecipitation assay buffer. All lysates were centrifuged at 15,000 × g for 5 min, and the resulting supernatant was subjected to immunoprecipitation by mixing with an α-PLC-γ2 polyclonal antibody (2 μg/ml) and protein A-Sepharose beads (50% v/v slurry) for 2 h at 4 °C. The beads were washed, and immunoprecipitated proteins were separated on a 7.5% SDS-PAGE under reducing conditions and immunoblotted using either an anti-PLC-γ2 monoclonal antibody or an anti-phosphotyrosine monoclonal antibody (PY20).

Statistical Analysis—Statistical significance of results was deter-
mined using one-way analysis of variance. The p values are indicated where appropriate (*, p \leq 0.05; **, p \leq 0.01).

RESULTS

Role of ADP in Promoting Integrin \(\alpha_{IIb}\beta_3\) Activation and Platelet Spreading on Immobilized Fibrinogen—Immobilized fibrinogen supports the adhesion and activation of platelets through engagement of integrin \(\alpha_{IIb}\beta_3\). Previous studies have suggested an important role for ADP in promoting lamellipodial formation in fibrinogen-adherent platelets (18, 23). However, its role in promoting integrin \(\alpha_{IIb}\beta_3\) activation remains less clear. To investigate this role, we examined the effect of apyrase on integrin \(\alpha_{IIb}\beta_3\) activation during platelet adhesion to fibrinogen by performing indirect immunofluorescence studies using the activation-specific integrin \(\alpha_{IIb}\beta_3\) antibody, PAC-1. As demonstrated in Fig. 1A, robust PAC-1 binding and platelet spreading was observed in control and apyrase-treated platelets following adhesion to fibrinogen. Using confocal imaging, we demonstrated that PAC-1 staining occurred predominantly at the granulomere on the apical surface of spreading platelets (Fig. 1A). Quantification of PAC-1 fluorescence on the surface of spread platelets revealed no difference between control and apyrase-treated platelets (data not shown). These findings suggest that integrin \(\alpha_{IIb}\beta_3\) engagement of fibrinogen can modulate the affinity status of integrin \(\alpha_{IIb}\beta_3\) and induce lamellipodial extensions independent of ADP.

To examine in further detail the relationship between ADP release and platelet spreading, time course adhesion assays were performed. As demonstrated in Fig. 1C, platelets pretreated with apyrase spread significantly slower than control platelets, with half-maximal spreading of control platelets occurring within <10 min compared with 20–30 min for apyrase-
Involvement of PLC \(_2\) in Integrin \(\alpha_{IIb}\beta_3\) Signaling

Platelet Adhesion on Fibrinogen—Resting platelets (data not shown). In all studies, combining aspirin with apyrase had no further inhibitory effect beyond that observed with apyrase alone, which excludes a major role for TXA\(_2\) in promoting cytosolic calcium flux (data not shown). Overall, these studies define an important, albeit non-essential role for ADP in promoting cytosolic calcium flux during platelet adhesion on fibrinogen.

Src Kinases are Essential for Integrin \(\alpha_{IIb}\beta_3\) Activation and Calcium Mobilization following Platelet Adhesion to Fibrinogen—The demonstration that ADP antagonists did not eliminate platelet activation induced by immobilized fibrinogen raised the possibility that fibrinogen engagement of integrin \(\alpha_{IIb}\beta_3\) was sufficient to induce cytosolic calcium flux through outside-in signaling processes. Src kinases play a central role in integrin signaling, and a recent study (22) has demonstrated an important role for Src kinases in integrin \(\alpha_{IIb}\beta_3\)-dependent cytoskeletal remodeling. To examine the role of Src kinases in promoting integrin \(\alpha_{IIb}\beta_3\) activation and calcium flux, platelets were pretreated with the Src kinase inhibitor, PP2 (34). As demonstrated in Fig. 3, A and B, treatment of platelets with PP2 completely eliminated calcium responses in all platelets adhering to fibrinogen. This reduction in calcium flux was associated with an inhibition of PAC-1 binding and platelet spreading (Fig. 3C). Dose-response studies demonstrated a strong correlation between inhibition of PAC-1 binding and platelet spreading over a concentration range previously demonstrated to be selective for Src kinase inhibition (Fig. 3D and data not shown) (34). These findings suggest an important role for Src kinases in promoting cytosolic calcium flux.

PLC and IP\(_3\) Contribute to Integrin \(\alpha_{IIb}\beta_3\) Activation and Platelet Spreading on Immobilized Fibrinogen—The demonstration that Src kinases promote calcium signals on fibrinogen raised the possibility that one or more PLC isoforms may be regulated downstream of integrin \(\alpha_{IIb}\beta_3\). Src kinases phosphorylate and activate PLC\(_1\) and PLC\(_2\) (35); however, PLC\(_2\) is the predominant isoform present in platelets (36). To investigate changes in tyrosine phosphorylation of PLC\(_2\), platelets were allowed to adhere to fibrinogen in the presence or absence of PP2, and the phosphorylation status of PLC\(_2\) was assessed by performing anti-phosphotyrosine immunoblots on PLC\(_2\) immunoprecipitates. As shown in Fig. 4A, adhesion of platelets to fibrinogen evoked tyrosine phosphorylation of PLC\(_2\), whereas PP2 treatment completely abolished this phosphorylation event. To investigate the functional importance of PLC for integrin \(\alpha_{IIb}\beta_3\) activation and platelet spreading on fibrinogen, platelets were treated with the PLC inhibitor U73122 or the IP\(_3\) antagonist APB-2. As demonstrated in Fig. 4B, both of these pharmacological inhibitors completely blocked PAC-1 binding and platelet spreading on fibrinogen and eliminated cytosolic calcium flux (data not shown). Taken together, these studies suggest a potentially important role for one or more Src kinase-regulated PLC isoforms in integrin \(\alpha_{IIb}\beta_3\) outside-in signaling.

Role of PLC\(_2\) in Promoting Cytosolic Calcium Flux during Platelet Adhesion on Fibrinogen—The mobilization of intracellular calcium during platelet adhesion to fibrinogen is important for cytoskeletal remodeling and has been demonstrated to be ADP-dependent (32). To investigate the absolute requirement for ADP for fibrinogen-induced cytosolic calcium flux, confocal imaging studies were performed on adherent platelets labeled with the calcium-indicator dyes Oregon Green BAPTA-AM and Fura Red. Consistent with previous reports (26, 33), platelets firmly adherent to fibrinogen elicited a sustained, oscillatory calcium response that coincided with lamel- lipodial extension and platelet spreading. As demonstrated in Fig. 2A, pretreating platelets with apyrase and aspirin markedly delayed the onset of the calcium response; however, by 50 min the majority of platelets exhibited a sustained calcium response and spread. Furthermore, analysis of the pattern of the cytosolic calcium response in individual platelets (Fig. 2B) revealed no difference in the frequency or magnitude of calcium oscillations between control and apyrase/aspirin-treated platelets. However, by 50 min there was no difference in spreading between control and apyrase-treated platelets. In both the fixed end point and time course adhesion assays, there was no further decrease in the rate or extent of platelet spreading when aspirin was combined with apyrase, suggesting that TXA\(_2\) did not make a significant contribution to this response.

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Role of PLC\(_2\) in Promoting Integrin \(\alpha_{IIb}\beta_3\)-dependent Cytosolic Calcium Flux—To investigate the potential role for PLC\(_2\) in platelet spreading on fibrinogen and integrin \(\alpha_{IIb}\beta_3\) calcium signaling, adhesion studies were performed with platelets derived from PLC\(_2^{-/-}\) mice. These platelets have been demonstrated to have a major defect in their activation response to soluble and fibrillar collagen, although their responsiveness to a variety of soluble agonists, including ADP, appears intact (37). PLC\(_2^{-/-}\) platelets adhered normally to immobilized fibrinogen; however, spreading was significantly delayed relative to PLC\(_2^{+/+}\) platelets (Fig. 5A). Maximal spreading was observed within 30 min for PLC\(_2^{+/+}\) platelets compared with 60 min for PLC\(_2^{-/-}\) platelets. As demonstrated in Fig. 5B,
this reduction in the rate of platelet spreading correlated with a delay in the proportion of platelets exhibiting a cytosolic calcium response. Within 30–40 min of adhesion, 80–100% of PLC\(_{\gamma2}\) platelets elicited an oscillatory calcium response compared with <15% of the PLC\(_{\gamma2}\) platelets. However, by 60–70 min, essentially all PLC\(_{\gamma2}\) platelets had undergone a sustained calcium response (Fig. 5, B and C) and had adopted a spread morphology.

Examination of the role of ADP in promoting mouse platelet cytosolic calcium flux and spreading on a fibrinogen matrix revealed findings similar to those observed with human platelets, in that both the rate of spreading and the proportion of platelets exhibiting a cytosolic calcium response were markedly lower in apyrase-treated platelets (compare Fig. 5 with Fig. 6, and data not shown). Significantly, PLC\(_{\gamma2}\) platelets treated with apyrase failed to extend lamellipodia and to spread; however, these platelets still underwent morphological changes following adhesion, with the majority of platelets becoming spherical and extending filopodia (Fig. 6A). Consistent with their morphological defects, these PLC\(_{\gamma2}\) platelets also exhibited minimal calcium flux (Fig. 6, B and C). These studies suggest an important role for PLC\(_{\gamma2}\) in integrin \(\alpha_{\text{IIb}}\beta_3\) outside-in signaling relevant to platelet spreading on fibrinogen.

Investigation of the Role of ADP in Promoting Platelet-Fibrinogen Interactions under Flow—Our studies to date have demonstrated an important cooperative signaling role for integrin \(\alpha_{\text{IIb}}\beta_3\) and ADP in promoting platelet activation on fibrinogen under static adhesion conditions. To investigate the significance of these findings with respect to platelet adhesion in a shear field, flow-based adhesion assays were performed on fibrinogen-coated microcapillaries. In preliminary studies, we confirmed that adhesion of platelets to fibrinogen inversely correlated with shear rate, with maximal adhesion at low shear (150 s\(^{-1}\)) and progressively fewer platelet-matrix interactions at higher shears (600–1800 s\(^{-1}\)) (data not shown). Analysis of the cytosolic calcium response under low shear conditions (150 s\(^{-1}\)) demonstrated that adherent platelets exhibited an oscillatory calcium response that was associated with firm platelet-matrix contacts with the fibrinogen matrix. However, there was a small but significant reduction (~20%) in the proportion of tethered platelets exhibiting a sustained calcium response. Consistent with our static adhesion data, the reduced calcium signaling in apyrase-treated platelets was the result of a delay in the onset of the calcium response. However, at later time points the percentage of platelets exhibiting a sustained calcium response was not significantly different between control and apyrase-treated platelets (Fig. 7B). The decrease in calcium flux at the earlier time points was associated with a reduction in the number of platelets forming sustained adhesion contacts with the fibrinogen matrix. As demonstrated in Fig. 7C, up to 12% of platelets tethering to the matrix at 150 s\(^{-1}\) were displaced within 10 s of tethering. Pretreating platelets with apyrase approximately doubled the proportion of

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**Fig. 5.** An important role for the PLC\(_{\gamma2}\) isoform in promoting platelet spreading and calcium mobilization during adhesion to immobilized fibrinogen. Platelets from PLC\(_{\gamma2}\) or PLC\(_{\gamma2}\)-derived mice were isolated and calcium dye-loaded as described under "Experimental Procedures." Platelets were allowed to adhere to human fibrinogen-coated (100 \(\mu\)g/ml) coverslips for 70 min at 37 °C in the presence of 1 mM calcium. A, adherent platelets were fixed at the indicated time points and visualized using DIC microscopy. Images are taken from one experiment representative of four. B, cytosolic calcium flux in adherent platelets was monitored as described under "Experimental Procedures." The proportion of adherent platelets undergoing a sustained oscillatory calcium response over a 70-min period was quantified and expressed as a percentage of the total number of adherent cells. These data represent the mean ± S.E. from four individual experiments. C, representative calcium traces from individual PLC\(_{\gamma2}\)- and PLC\(_{\gamma2}\)-derived platelets demonstrating calcium flux in fibrinogen-adherent cells at the indicated time points.
platelets detaching from the point of initial contact. Furthermore, increasing the tensile stress on formed bonds by exposing platelets initially adherent at 150 s⁻¹ to rapid increases in shear up to 1800 s⁻¹ resulted in up to 60% of apyrase-treated platelets detaching from their point of initial contact as compared with 20% of control platelets (Fig. 7D).

**Role of Src Kinases in Promoting Firm Platelet Adhesion under Flow**—To investigate the functional importance of Src kinases in regulating platelet adhesion to fibrinogen under flow conditions, platelets were pretreated with PP2 prior to perfusion through fibrinogen-coated microcapillary tubes. As demonstrated in Fig. 8A, PP2 had no effect on the ability of platelets to tether to fibrinogen; however, it completely abolished cytosolic calcium flux in all adherent platelets (Fig. 8B), resulting in defective platelet spreading (data not shown). Analysis of the stability of platelet adhesion contacts at 150 s⁻¹, revealed that inhibition of calcium flux by PP2 resulted in 30% of platelets displacing from the point of initial attachment (Fig. 8C), whereas >90% of platelets were displaced when exposed to sudden increases in shear (Fig. 8D). Several lines of evidence suggest that the defect in the stability of platelet adhesion following PP2 or apyrase treatment was primarily a result of reduced calcium signaling. First, analysis of control and apyrase-treated platelets following exposure to sudden increases in shear revealed that all platelets exhibiting a sustained oscillatory calcium response resisted the detaching effects of increased shear, whereas platelets without a detectable calcium response were easily detached (Fig. 9A). Second, pretreating platelets with intracellular calcium chelators resulted in the formation of unstable adhesion contacts in 100% of platelets (Fig. 9B). Finally, pretreating platelets with the PLC inhibitor U73122 (Fig. 9B) or the IP₃ receptor antagonist APB-2 abolished stable platelet-fibrinogen interactions.

**Role of PLCγ2 in Promoting Sustained Platelet Adhesion under Flow**—To investigate the role of PLCγ2 in regulating platelet adhesion on fibrinogen under flow, PLCγ2⁻/⁻ platelets were perfused through fibrinogen-coated microcapillary tubes. In initial studies we demonstrated that adhesion of wild-type murine platelets to fibrinogen was also shear rate-dependent. However, in contrast to human platelets, maximal adhesion occurred at 600 s⁻¹ instead of 150 s⁻¹, with progressively less adhesion at higher shears (data not shown). The ability of murine platelets to adhere efficiently at higher shear rates may reflect the smaller dimensions of these cells relative to human platelets, leading to reduced tensile stress on adhesive bonds. Alternatively, these differences may reflect distinct binding kinetics between murine integrin αᵢ₃β₂ and human fibrinogen. As observed with human platelets, pretreating PLCγ2⁻/⁻ murine platelets with apyrase had no effect on the initial adhesion of these cells to fibrinogen (data not shown); however, it reduced the capacity of these platelets to maintain sustained adhesion contacts in response to rapid increases in shear (Fig. 10A). This reduction in stable adhesion was associated with a decreased proportion of platelets exhibiting sustained calcium oscillations during the early stages of adhesion (Fig. 10B). It should be noted that displacement of mouse platelets was distinct from that observed with human platelets in that the latter typically detached from the matrix with sudden increases in shear, whereas mouse platelets translocated slowly over the fibrinogen matrix. This difference may be attributable to the species variation outlined above.

Analysis of PLCγ2⁺/+ platelets demonstrated a small, non-
significant reduction in the proportion of platelets exhibiting a sustained oscillatory calcium response in the absence of apyrase (Fig. 10B). Thus, >85% of adhesion contacts formed by PLCγ2−/− platelets remained stable following exposure to a sudden increase in tensile stress (Fig. 10A). In the presence of apyrase, <5% of PLCγ2−/− platelets elicited a sustained calcium flux following adhesion to fibrinogen (Fig. 10B), and exposure of these platelets to sudden increases in shear resulted in a high proportion of these platelets (65%) becoming displaced from the point of initial contact (Fig. 10A). Together, these findings demonstrate an important role for PLCγ2 in integrin αIIbβ3 outside-in signaling relevant to stable platelet adhesion on fibrinogen. Furthermore, similar to findings in human platelets, they suggest an important role for ADP in stabilizing platelet-fibrinogen interactions under flow.

**DISCUSSION**

The studies presented here provide new insight into the mechanisms regulating platelet adhesion on immobilized fibrinogen. In contrast to previous reports (10–13), our studies demonstrate that firm platelet adhesion on fibrinogen is not an instantaneous irreversible event but is in fact activation-dependent. More specifically, our studies suggest that maintenance of integrin αIIbβ3-fibrinogen bonds is dependent on integrin αIIbβ3-derived calcium signals. These calcium signals appear to be regulated by one or more Src kinase family members linked to the activation of PLCγ2−/−. Our studies also define an important role for ADP in potentiating integrin αIIbβ3 calcium signals. The release of dense granule ADP, in concert with the activation of PLCγ2, appears to play a major role in promoting irreversible platelet adhesion and spreading on fibrinogen.

Our studies define an important role for integrin αIIbβ3 outside-in calcium signals in promoting firm platelet adhesion and spreading on fibrinogen. In contrast to previous reports demonstrating that cytosolic calcium flux on immobilized fibrinogen is dependent on released ADP (24, 25), we have provided several lines of evidence suggesting that integrin αIIbβ3 outside-in signaling per se is sufficient to induce calcium flux. First, a sustained oscillatory calcium response was observed in fibrinogen-adherent platelets under experimental conditions eliminating the platelet-activating effects of ADP and TXA2. Second, the magnitude, pattern, and duration of the cytosolic...
calcium response in these platelets were similar to those described previously for integrin \( \alpha_{IIb}\beta_3 \) calcium signals (14, 15) but distinct from soluble agonist-induced calcium flux (38, 39). Third, the cytosolic calcium response was strictly dependent on Src kinases. These non-receptor tyrosine kinases play a critical role in integrin signal transduction but are not essential for soluble agonist-induced calcium flux (21). Finally, the demonstration that PLC\(_2\) mouse platelets have a defective calcium response during platelet adhesion to fibrinogen is consistent with its previously defined role in adhesion receptor signal transduction (40).

**Fig. 8.** Src kinases promote cytosolic calcium flux and stable platelet adhesion on immobilized fibrinogen under low shear conditions. Washed platelets \((5 \times 10^7/ml)\) in Tyrode’s buffer were reconstituted with 50% red blood cells and labeled with DiOC\(_6\) (1 mM) or calcium dyes where indicated. Platelet suspensions were pretreated with vehicle alone (Control) or 10 \(\mu\)M PP2 for 10 min at 37 °C prior to perfusion through fibrinogen-coated microcapillary tubes at a wall shear rate of 150 s\(^{-1}\). A, the total number of DiOC\(_6\)-labeled platelets tethered to the fibrinogen matrix over a 90-s period was calculated as outlined under “Experimental Procedures.” B, the percentage of calcium dye-loaded platelets exhibiting a sustained oscillatory calcium response was determined using confocal software as outlined under “Experimental Procedures.” These data represent the mean ± S.E. of three experiments. C, the percentage of cells displaced was determined after 5 min of perfusion as described under “Experimental Procedures.” These data represent the mean ± S.E. from eight separate experiments. D, DiOC\(_6\)-labeled platelets treated with vehicle alone or PP2 (10 \(\mu\)M) were perfused through fibrinogen-coated microcapillary tubes at 150 s\(^{-1}\) for 5 min followed by an increase in shear rate to 1800 s\(^{-1}\). Platelet displacement was quantified as described in C. The results represent the percentage of fibrinogen-adherent platelets displaced following the shear increase (arrow).

**Fig. 9.** Cytosolic calcium flux maintains firm platelet adhesion on fibrinogen. A, calcium dye-loaded platelets were perfused for 5 min through fibrinogen-coated microcapillary slides at 150 s\(^{-1}\) followed by an increase in shear rate up to 1800 s\(^{-1}\) (see Fig. 7D). The cytosolic calcium levels in platelets that displaced or remained adherent were examined and divided into cells exhibiting Low \(Ca^{2+}\) (platelets with mean calcium levels \(<100 \text{nM}\)) or Elevated \(Ca^{2+}\) (platelets with mean calcium levels \(>100 \text{nM}\)). These data represent the percentage of platelets exhibiting low or elevated \(Ca^{2+}\) that became displaced following the shear increase (mean ± S.E. from three separate experiments). B, calcium dye-loaded platelets treated with vehicle (Control), U73122 (1 \(\mu\)M), or DM-BAPTA (70 \(\mu\)M) were initially perfused through fibrinogen-coated microcapillary slides at 150 s\(^{-1}\) followed by an increase in shear rate up to 1800 s\(^{-1}\). Calcium flux was monitored by capturing consecutive confocal images 2 s before and 28 s after the increase in shear. Results represent the mean ± S.E. of three individual experiments and depict the percentage of fibrinogen-adherent platelets displaced following the shear increase (arrow).

\(^2\) I. Goncalves, S. C. Hughan, S. M. Schoenwaelder, C. L. Yap, Y. Yuan, and S. P. Jackson, unpublished observations.
These observations differ from previous studies demonstrating stability of platelet-fibrinogen interactions in a shear field. Similarly, Syk, which binds to the cytoplasmic tails of ITAM motifs as well as the β3-tail of integrin α1β3, was determined. These data represent the mean ± S.E. of three individual experiments (**, p < 0.01). A, platelets initially perfused through fibrinogen-coated microcapillary tubes at 600 s⁻¹ were subsequently subjected to an increase in shear rate to 10,000 s⁻¹. Consecutive confocal images were taken 2 s before and 28 s after the increase in shear rate, and the percentage of fibrinogen-adherent platelets displaced following the shear increase (arrow) was quantified as described under “Experimental Procedures.” The results represent the mean ± S.E. of three individual experiments.

The finding for an important role for PLCγ2⁺⁻ in integrin α1β3, outside-in signaling is consistent with recent findings that PLCγ2 becomes tyrosine-phosphorylated following integrin α1β3 ligation (41), a finding confirmed in the present study. It is also consistent with the proposed involvement of ITAM-like signaling processes in integrin α1β3 signaling. For example, there is strong evidence for an important role for the non-receptor tyrosine kinases, including Src family kinases and Syk, in initiating integrin α1β3 signaling. The Src kinase Fyn has been demonstrated to phosphorylate ITAM tyrosine motifs as well as the β3-tail of integrin α1β3 (22, 31, 42). Similarly, Syk, which binds to the cytoplasmic tails of ITAM-bearing receptors (22, 43), also binds integrin α1β3. Other similarities between integrin α1β3 and ITAM signaling include the recruitment of adaptor molecules to ligated receptors, such as LAT (linker for activator of T-cells) in the case of ITAM receptors (35, 44), and Shc with integrin α1β3 (45). These molecules have been demonstrated to facilitate the binding and activation of signaling molecules such as PLCγ2 and phosphoinositide 3-kinase, promoting phosphoinositide turnover and cytosolic calcium flux.

It is generally assumed that ADP release is essential for a sustained oscillatory calcium response and platelet spreading on fibrinogen (24, 25). Although our findings are consistent with an important role for ADP in promoting these platelet responses, they suggest that its role is not absolutely, at least under the experimental conditions employed in this study. The reasons for the apparent differences between our findings and others are not clear but may primarily reflect technical differences. For example, our studies have demonstrated that the time of onset of sustained calcium oscillations and the rate of platelet spreading is markedly slower in the presence of ADP antagonists, and hence differences in the time courses used to assess the effects of ADP inhibitors may have a significant bearing on the interpretation of results. Furthermore, we have found that subtle experimental differences, such as preparation and density of the adhesive matrix and the temperature at which the adhesion assays are performed, can significantly influence the platelet response. In this context, it is noteworthy that other investigators have reported cytosolic calcium flux and platelet spreading on fibrinogen independent of ADP (33).

Our studies have defined an important role for integrin α1β3 calcium signals and released ADP in maintaining the stability of platelet-fibrinogen interactions in a shear field. These observations differ from previous studies demonstrating that platelet activation inhibitors do not significantly inhibit platelet adhesion to fibrinogen under flow (12, 13). It is possible that at the lower shear rates (50 s⁻¹) sufficient tensile stress was not applied to adhesive bonds to significantly disrupt the majority of platelet-fibrinogen interactions. For example, we have demonstrated that at 150 s⁻¹ only 20% of PP2-treated platelets were displaced from the point of initial adhesion, despite complete elimination of calcium signals. Direct comparison of our studies with others is also complicated by the fact that previous studies have not assessed the effects of platelet activation inhibitors on the dynamics of the platelet-fibrinogen interaction under flow (15). Without information on the percentage of tethered cells forming immediate firm adhesion contacts or detaching from the matrix and with no information on the effects of increased tensile strength on adhesive interactions, it is difficult to reconcile the potential differences between our studies and previous results.

Two distinct models have been proposed to describe platelet adhesion on fibrinogen and vWF under flow conditions. Platelet adhesion to vWF is a multistep process involving GPIbα/V/IX and integrin α1β3. Adhesion is initiated by a reversible tethering step between the A1 domain of vWF and GPIbα followed by a secondary adhesive interaction between the C1 domain of vWF and integrin α1β3. This two-step model of platelet adhesion is critically dependent on the cooperative signaling function of both receptors, with GPIbα-derived calcium spikes initiating integrin α1β3 activation and transient bond formation with vWF, whereas subsequent integrin α1β3-dependent calcium signals sustain firm adhesion. An important difference between immobilized vWF and fibrinogen is that the latter ligand can engage integrin α1β3 in its low affinity state and, according to previous findings, maintain firm adhesion independent of platelet activation. However, our findings suggest a reinterpretation of this model in which the mechanisms regulating platelet adhesion on vWF are also relevant to fibrinogen. Thus, the initial bond formation between fibrinogen and integrin α1β3 is potentially transient and reversible, depending on the tensile stress applied to the formed bonds. Conversion of these bonds to firm adhesion contacts is dependent on integrin α1β3-dependent calcium flux and also on the release of ADP. These calcium changes are likely to support firm adhesion through multiple mechanisms, including effects on integrin α1β3 affinity and receptor clustering and through reorganization of the cytoskeleton. Elucidating the mechanisms coordinating integrin α1β3 calcium signals with ADP release will be important.
to the full understanding of processes regulating platelet adhesion efficiency on fibrinogen.

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