A Critical Role for Phospholipase Cγ2 in αIβ3-mediated Platelet Spreading*

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The interaction of fibrinogen with the integrin αIβ3 plays a crucial role in platelet adhesion and platelet activation leading to the generation of intracellular signals that nucleate the reorganization of the cytoskeleton. Presently, we have only a limited understanding of the signaling cascades and effector proteins through which changes in the cytoskeletal architecture are mediated. The present study identifies phospholipase Cγ2 (PLCγ2) as an important target of the Src-dependent signaling cascade regulated by αIβ3. Real-time phase-contrast microscopy is used to show that formation of filopodia and lamellapodia in murine platelets on a fibrinogen surface is dramatically inhibited in the absence of PLCγ2. Significantly, the formation of these structures is mediated by Ca2+ elevation and activation of protein kinase C, both directly regulated by PLC activity. With the involvement of Syk, SLP-76, and Btk, αIβ3-induced PLCγ2 activation partly overlaps with the pathway used by the collagen receptor glycoprotein VI. Important differences, however, exist between the two signaling cascades in that activation of PLCγ2 by αIβ3 is unaltered in murine platelets, which lack the FeR γ-chain or the adaptor LAT, but is abolished in the presence of cytochalasin D. Therefore, PLCγ2 plays not only a crucial role in activation of αIβ3 by collagen receptors but also in αIβ3-mediated responses.

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The abbreviations used are: vWF, von Willebrand factor; 2-ABP, 2-aminoethoxydiphenylborate; BSA, bovine serum albumin; FAK, focal adhesion kinase; FcR γ-chain, Fc receptor γ-chain; Fura 2-AM, Fura 2-acetoxyethyl ester; FRP, platelet-rich plasma; GPVI, glycoprotein VI; PKC, protein kinase C; PBS, phosphate-buffered saline; PLCγ2, phospholipase Cγ2; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N',N'-tetraacetic acid.

αIβ3 induces outside-in signals that lead to reorganization of the cytoskeleton and synergize with other agonists to mediate activation. The central role of the integrin αIβ3 in thrombosis and hemostasis is highlighted by the severe bleeding disorders in patients with Glanzmann thrombasthenia, which lack functional integrin.

One of the earlier events to occur following ligation of αIβ3 is the activation of the tyrosine kinase Syk via one or more Src kinases (1, 2). This leads to tyrosine phosphorylation of the adaptor molecule SLP-76, which is constitutively associated with a second adaptor SLAP-130 (3, 4), also known as Fyn-binding protein or adhesion- and degranulation-promoting adapter protein (5, 6). Together with proteins of the Vav GTPase exchange family, the adapter Nck, and the actin-binding protein VASP, this cascade has been shown to lead to activation of phosphoinositide 3-kinase and phosphorylation of FAK, and subsequent reorganization of the cytoskeleton in αIβ3-transfected Chinese hamster ovary cells (7–9). Evidence that this cascade mediates reorganization of the cytoskeleton in platelets by αIβ3 has been provided using kinase inhibitors and murine cells deficient in Src kinases, Syk and SLP-76 (2, 3, 10).

Recently, we have shown that αIβ3 as well as the receptor for vWF, the glycoprotein (GP) Ib-IX-V complex, stimulate tyrosine phosphorylation of PLCγ2 (11, 12). PLCγ2 is known to be a major target of signaling by the collagen receptor GPVI in platelets. GPVI exists in a complex with the Fc receptor γ-chain (FeR γ-chain), which contains one copy of an immunoreceptor-tyrosine-based activation motif (13). GPVI activates platelets through tyrosine phosphorylation of the Feγ receptor tyrosine kinase-mediated Syk and Fyn and recruitment of Syk (14–16). Syk regulates a cascade that involves the phospholipase suggesting that PLCγ2 activity is not required for GPVI-mediated signals (12).

The role of PLCγ2 downstream of the fibrinogen receptor αIβ3 is not known. In the present study, we show that PLCγ2 is activated downstream of the fibrinogen receptor αIβ3 and that this plays a critical role in spreading through the mobilization of calcium and activation of protein kinase C. In addition, we also identify a number of additional proteins that are regulated downstream of the integrin-regulated signaling cascade but demonstrate important differences with the cascade used by GPVI. The present study expands the role of the PLCγ2 in platelet activation by demonstrating a central role in remod-
eling of the cytoskeleton by immunoreceptor tyrosine-based activation motif and integrin receptors.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents—**PLC-γ2 and anti-Syk polyclonal antibodies were from commercial sources (16). Polyclonal rabbit anti-FAK antibody was from Santa Cruz Biotechnology Inc., Santa Cruz, CA. Fibrinogen depleted of plasminogen and vWF were from Kordia Labortory Supplies, Leiden, NL. 2-aminoethoxydiphenylborate (2-ABP) was from Tocris (Ellisville, MO), the PLC inhibitor U71322 and the PLC inhibitor M-50 and 60 kDa co-migrate with Src family kinases. There is also a constitutively phosphorylated band of 50 kDa. The constitutively phosphorylated bands between 50

**Preparation of Human Platelets—**Human blood was taken from drug-free volunteers on the day of the experiment and drawn into sodium citrate. Platelet-rich plasma (PRP) was obtained by centrifugation of the blood samples at 4°C overnight to a concentration of 3 × 10^8/ml. PRP was centrifuged at 1000 g for 6 min at room temperature. The supernatant was discarded and the platelet pellet was resuspended in Tyrode’s-HEPES buffer to a concentration of 5 × 10^8 cells/ml in 2 units/ml apyrase and 10 μM indomethacin. Dishes coated with fibrinogen were washed twice with PBS to remove non-adherent cells. Platelets adherent to fibrinogen or in suspension over BSA were lysed in a buffer containing 100 mM EDTA, 5 mM HCl, and 1% Nonidet P-40. Phospholipids were extracted by addition of 400 μl of chloroform-methanol-HCl (100/200/1, v/v/v), and [32P]phosphatidic acid was separated by thin-layer chromatography. Thin-layer chromatography plates were exposed to Kodak PhosphorScreen and phosphatidic acid signals were quantified using Molecular Imager FX and Quantity One Software version 4 for Macintosh (Bio-Rad). Statistical analyses were performed using Student’s t test.

**RESULTS**

**Differential Effects of Cytochalasin D on α_{IIb}β_{3} and GPVI-induced Phosphorylation—**It has been shown previously that tyrosine phosphorylation by α_{IIb}β_{3} is regulated through activation of a Src family kinase and that it is modulated by disruption of actin polymerization using cytochalasin D (2). Thus α_{IIb}β_{3}-mediated tyrosine phosphorylation events can be divided into those that are mediated upstream and downstream of actin polymerization. We have compared the effect of the actin polymerization inhibitor cytochalasin D and the Src kinase inhibitor PP2 on signals mediated by α_{IIb}β_{3} and GPVI. Stimulation of α_{IIb}β_{3} was achieved by incubation of platelets over a fibrinogen-coated matrix for 30 min. GPVI was activated with the snake toxin convulxin under stirring conditions in suspension using an incubation time of 30 s, a time point known to be the peak for tyrosine phosphorylation induced by this agonist. α_{IIb}β_{3} and GPVI induce overlapping patterns of increases in tyrosine phosphorylation as measured using whole cell lysates. Both stimuli induced marked increases in tyrosine-phosphorylated bands of 70–80 kDa, which co-migrate with Syk, Btk, and SLP-76. The constitutively phosphorylated bands between 50 and 60 kDa co-migrate with Src family kinases. There is also a marked increase in a band of 130 kDa and a number of minor bands around this molecular mass in response to the two agonists. In contrast, a doublet of tyrosine-phosphorylated proteins with a molecular mass between 28 kDa and 32 kDa is regulated downstream of α_{IIb}β_{3} but not by GPVI (Fig. 1A, arrows). Convulxin stimulates marked tyrosine phosphorylation of proteins of 36 and 12 kDa, which co-migrate with LAT and the FcR γ-chain, respectively (Fig. 1A, arrows). Both signaling cascades are strongly dependent on the presence of functionally active Src kinases. Incubation of platelets with 20 μM PP2 causes a complete loss of inducible tyrosine phosphorylation following convulxin stimulation and markedly inhibits the response to fibrinogen, although tyrosine phosphorylation of a 130-kDa band is preserved (Fig. 1A, arrows). Cytochalasin D has a distinct effect on the degree of phosphorylation by fibrinogen-coated coverslip and real time calcium imaging was performed using Openlab software (Imprison, Coventry, UK).

**Microscopy—**Platelets (1.5 × 10^6 in 0.5 ml of Tyrode’s-HEPES buffer) were added to fibrinogen-coated coverslips and incubated for 30 min at 37°C. Non-adherent platelets were washed away and attached platelets were fixed with 3% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100 in PBS. Platelets were stained for F-actin with rhodamine-conjugated phalloidin and viewed under an inverted fluorescence microscope using Openlab imaging software (Imprison). Platelet spreading was observed in real time at 37°C and recorded using time-lapse laser scanning phase-contrast microscopy. The system consisted of a Bio-Rad Microradiance laser scanning imaging system (Bio-Rad) attached to a Zeiss Axiosvert inverted microscope equipped with a Solent Scientific environmental chamber; a Ph3 Plan-apochromat 63 × 1.4 NA objective was used and the images were acquired using Laser-sharp 2000 (version 4.2) software.

**Determination of Phosphatidic Acid Production—**Platelets were suspended in Tyrode’s-HEPES without phosphate and were labeled with [32P]orthophosphate (0.5 mCi/ml) for 1 h at 30°C. Platelets were washed twice and resuspended in Tyrode’s buffer at a concentration of 5 × 10^8/ml. Platelets were incubated for 30 min in 6-well plates (5 × 10^8/well) coated with fibrinogen or over BSA in the presence of 2 units/ml apyrase and 10 μM indomethacin. Dishes coated with fibrinogen were washed twice with PBS to remove non-adherent cells. Platelets adherent to fibrinogen or in suspension over BSA were lysed in a buffer containing 100 mM EDTA, 5 mM HCl and 1% Nonidet P-40. Phospholipids were extracted by addition of 400 μl of chloroform-methanol-HCl (100/200/1, v/v/v). LPS and [32P]phosphatidic acid was separated by thin-layer chromatography. Thin-layer chromatography plates were exposed to Kodak PhosphorScreen and phosphatidic acid signals were quantified using Molecular Imager FX and Quantity One Software version 4 for Macintosh (Bio-Rad). Statistical analyses were performed using Student’s t test.

**Measurement of Platelet Cytosolic Ca2+ Concentration—**Platelets isolated from PRP were resuspended in modified Tyrode’s-HEPES buffer to a concentration of 3 × 10^8 cells/ml and incubated with Fura 2-ace- toxymethyl ether (Fura 2-AM, 3 μM, 1 h, 30°C) (Molecular Probes, Eugene, OR). After being washed in Tyrode’s-HEPES buffer, platelets were resuspended at 2 × 10^8 cells/ml in the presence of 2 units/ml apyrase and 10 μM indomethacin. Platelets were allowed to spread on a fibrinogen-coated coverslip and real time calcium imaging was performed using Openlab software (Imprison, Coventry, UK).

**Preparation of Mouse Platelets—**Blood (750–1000 μl) was taken from drug-free volunteers on the day of the experiment and drawn into sodium citrate. Platelet-rich plasma (PRP) was obtained by centrifugation of the blood samples at 300 × g for 10 min at room temperature. PRP was centrifuged at 1000 × g in the presence of prostacyclin (0.1 μg/ml) for 6 min at room temperature. The cell pellet was resuspended in a modified Tyrode’s-HEPES buffer to a concentration of 5 × 10^8 cells/ml in Tyrode’s-HEPES buffer.

**Preparation of Mouse Platelets—**Blood (750–1000 μl) was taken from drug-free volunteers on the day of the experiment and drawn into sodium citrate. Platelet-rich plasma (PRP) was obtained by centrifugation of the blood samples at 300 × g for 10 min at room temperature. PRP was centrifuged at 1000 × g in the presence of prostacyclin (0.1 μg/ml) for 6 min at room temperature. The cell pellet was resuspended in a modified Tyrode’s-HEPES buffer to a concentration of 5 × 10^8 cells/ml in Tyrode’s-HEPES buffer.
Whereas the response to GPVI is only slightly inhibited by 10 μM cytochalasin D, there is a marked reduction in tyrosine phosphorylation of all bands by αIIbβ3, showing that it has a much greater dependence on the reorganization of the cytoskeleton (Fig. 1A).

As a prelude to comparing the signaling cascades regulated by αIIbβ3 and GPVI, Figure 1 illustrates fibrinogen-induced tyrosine phosphorylation and effects of cytochalasin D and PP2. A, influence of cytD and PP2 on whole-cell tyrosine phosphorylation of platelets stimulated with convulxin or fibrinogen. B, influence of cytD on tyrosine phosphorylation of indicated proteins. C, fibrinogen-induced tyrosine phosphorylation of signaling proteins in platelets. Platelets were placed in dishes coated with fibrinogen or BSA in the presence of 2 units/ml apyrase and 10 μM indomethacin with or without 20 μM cytochalasin D (cytD) or PP2 for 30 min at 37 °C. Dishes coated with fibrinogen were washed twice with PBS to remove non-adherent cells. Platelets adherent to fibrinogen or in suspension over BSA were lysed in ice-cold immunoprecipitation buffer and subjected to immunoprecipitation for the indicated proteins or used directly for SDS-PAGE and immunoblotted for tyrosine-phosphorylated proteins. Samples were adjusted prior to immunoprecipitations to a similar number of cells in each group. For convulxin (14 nM) platelets in solution were stimulated in the presence of the indicated inhibitors for 30 s and lysates were subjected to immunoprecipitation for the indicated proteins. Results are representative of three experiments.
by αIIbβ3 and GPVI, we investigated some of the proteins that undergo increases in tyrosine phosphorylation following incubation of platelets over a fibrinogen-coated matrix for 30 min through immunoprecipitation with specific antibodies and Western blotting with the antiphosphotyrosine antibody, 4G10. In agreement with studies from other groups, platelet spreading over fibrinogen leads to a substantial phosphorylation of the integrin αIIbβ3 subunit (Fig. 1C), tyrosine kinase Syk, focal adhesion kinase (FAK) (Fig. 1B), adapter proteins SLP-76 (Fig. 1C) and SLAP-130 as well as the guanine nucleotide exchange factor Vav1 and the ubiquitin-regulator c-Cbl (not shown). In addition, we identified a number of proteins that have been previously shown to be phosphorylated downstream of GPVI, namely the FcRγ-chain, the Tec family tyrosine kinase Btk (Fig. 1C), as well as PLCγ2 (Fig. 1B). The adapter molecules Grb2 and Gads were also recruited to signaling complexes downstream of αIIbβ3 and formed associations with unidentified tyrosine-phosphorylated proteins of 32, 55, and 150 kDa, and 50, 76, and 130 kDa, respectively (not shown). There was, however, one important omission in the proteins that undergo tyrosine phosphorylation downstream of αIIbβ3 relative to GPVI, namely the adapter LAT. Whereas LAT is one of the major tyrosine-phosphorylated proteins downstream of GPVI we were not able to detect an increase in LAT phosphorylation after αIIbβ3 stimulation (Fig. 1B). These data demonstrate that platelet adhesion to fibrinogen leads to tyrosine phosphorylation of PLCγ2 and identifies many similarities but also important differences with the events regulated downstream of GPVI.

The effect of cytochalasin D and PP2 on phosphorylation of Syk, LAT, FAK, and PLCγ2 by αIIbβ3 and GPVI was explored following their immunoprecipitation and Western blotting for phosphotyrosine. Tyrosine phosphorylation of Syk induced by αIIbβ3 and convulxin was reduced in the presence of cytochalasin D (Fig. 1B) and abolished in the presence of PP2 (not shown). In contrast, cytochalasin D had a differential effect on tyrosine phosphorylation of a band of 12 kDa, which co-precipitates with Syk and was shown through immunoprecipitation studies to be the FcRγ-chain (not shown). Tyrosine phosphorylation of FcRγ-chain by αIIbβ3 was abolished by treatment with cytochalasin D, whereas it was only slightly inhibited in convulxin-stimulated cells (Fig. 1B, arrows). This demonstrates that tyrosine phosphorylation of FcRγ-chain is dependent on actin polymerization in response to αIIbβ3. As already discussed, αIIbβ3 does not cause phosphorylation of LAT. In contrast LAT is strongly phosphorylated downstream of GPVI and this phosphorylation is reduced following cytochalasin D treatment (Fig. 1B) and abolished after inhibition of Src kinases with PP2 (not shown). In contrast αIIbβ3 stimulation induces a robust, cytochalasin D-sensitive phosphorylation of FAK, whereas convulxin stimulation only causes minimal tyrosine phosphorylation of FAK (Fig. 1B) even at times up to 30 min (not shown). Cytochalasin D had a differential effect on the regulation of tyrosine phosphorylation of PLCγ2 by αIIbβ3 and

**Fig. 2.** Fibrinogen-induced PLCγ2 phosphorylation is not altered in LAT and FcRγ-chain-deficient platelets. Murine platelets were placed in dishes coated with fibrinogen or BSA in the presence of 2 units/ml apyrase and 10 μM indomethacin for 30 min at 37 °C. Dishes coated with fibrinogen were washed twice with PBS to remove non-adherent cells. Platelets adherent to fibrinogen or in suspension over BSA were lysed in ice-cold immunoprecipitation (IP) buffer and subjected to immunoprecipitation for the kinase Syk (A), the adaptor protein SLP-76 (B), or PLCγ2 (C) and immunoblotted for tyrosine-phosphorylated proteins. Results are representative of two experiments. WB, Western blot.
GPVI. Tyrosine phosphorylation of PLCγ2 by αIIbβ3 was almost abolished in the presence of cytochalasin D, whereas the response to convulxin was inhibited by ~50% (Fig. 1B). PP2 completely blocked tyrosine phosphorylation of PLCγ2 by both receptors (not shown).

The differential effect of cytochalasin D on tyrosine phosphorylation of FcRγ-chain and PLCγ2 by αIIbβ3 and GPVI further distinguishes the two signaling cascades. Thus, activation of Syk by αIIbβ3 is independent of phosphorylation of FcRγ-chain and signals from the kinase are not mediated through tyrosine phosphorylation downstream of FcRγ-chain (25) and signals are associated with phospholipid extraction and thin layer chromatography. Results are representative of three human, A or two mice, B) experiments.

Phosphorylation of PLCγ2 Downstream of αIIbβ3 Is Independent of the Adapter LAT and Fc Receptor γ-Chain—Previously, we have reported that tyrosine phosphorylation of Syk and PLCγ2 through GPVI is abolished in FcRγ-chain-deficient murine platelets, whereas tyrosine phosphorylation of PLCγ2 is markedly reduced in LAT-deficient cells (21, 25). We have now investigated the role of these two proteins in tyrosine phosphorylation of Syk and PLCγ2 by αIIbβ3. Adhesion of murine platelets to fibrinogen induced a marked increase in tyrosine phosphorylation of Syk and PLCγ2, similar to that seen in human platelets. This increase in phosphorylation was not altered in platelets deficient in FcRγ-chain or LAT (Fig. 2). Similarly, tyrosine phosphorylation of the adapter SLP-76 by fibrinogen was unaltered in platelets deficient in FcRγ-chain or LAT (Fig. 2B). As with human platelets, FcRγ-chain co-purified with Syk upon stimulation by αIIbβ3. This association was preserved in LAT-deficient platelets (Fig. 2A, arrow). These results confirm that neither FcRγ-chain nor LAT are required for tyrosine phosphorylation of Syk and PLCγ2 by αIIbβ3 in contrast to signals from GPVI.

αIIbβ3 Stimulates Activation of PLCγ2, Calcium Elevation, and Spreading—The functional consequence of PLCγ2 phosphorylation downstream of αIIbβ3 was investigated by measurement of phosphatidic acid and intracellular calcium, two indirect markers of PLC activity. Platelets spread on a fibrinogen-coated surface had a 2.4 ± 0.1-fold increase in the production of phosphatidic acid relative to cells exposed to a BSA-coated surface (p = 0.005, Fig. 3A). The increase in phosphatidic acid was inhibited strongly in the presence of cytochalasin D (1.5 ± 0.1-fold) and reduced to basal levels using the PLC inhibitor U-73122 (0.96 ± 0.13-fold). PP2 also caused a complete inhibition of phosphatidic acid accumulation, reducing the level below that of cells exposed to BSA (0.57 ± 0.11-fold), strongly suggesting that the increase is mediated via PLCγ rather than PLCβ isoforms (Fig. 3A).

To further clarify the role of PLCγ2 downstream of αIIbβ3, we determined the production of phosphatidic acid in PLCγ2-deficient murine platelets. As seen in human platelets, spreading of murine platelets on fibrinogen caused an increase in the production of phosphatidic acid (1.5 ± 0.08-fold) relative to cells exposed to a BSA-coated surface (1.0 ± 0.05-fold). This increase was inhibited following cytochalasin D treatment (0.90 ± 0.12-fold) and in PLCγ2-deficient platelets (Fig. 3B). These data suggest that PLCγ2 is the main enzyme responsible for the increase in phosphatidic acid following spreading of platelets on fibrinogen.

The increase in formation of phosphatidic acid was associated with a sustained increase in intracellular calcium, as measured using the calcium reporter Fura 2 (delivered as Fura 2-AM). All of the platelets that had undergone partial or complete spreading on fibrinogen had elevated levels of intracellular calcium. Moreover, the increase in calcium and spreading (measured by monitoring fluorescence at 380 nm) occurred in parallel as illustrated in Fig. 4B suggesting that they are causally related. The calcium increase was observed 2–3 min after the initial contact with the fibrinogen-coated surface and is accompanied by the formation of filopodia and lamellipodia. The increase in intracellular calcium and spreading were substantially inhibited by cytochalasin D and PP2 although they had only minimal effects on attachment of the platelets to the fibrinogen-coated surfaces (Fig. 4A). Calcium elevation and spreading was also strongly inhibited by 2-ABP an inositol 1,4,5-trisphosphate receptor antagonist. The increase in intracellular calcium was specific for αIIbβ3 because it was absent in platelets from a patient diagnosed with Type III Glanzmann thrombasthenia, which express a non-functional αIIbβ3 (Fig. 4A). Importantly, the platelets from this patient were able to adhere to the fibrinogen-coated surface but spreading and calcium mobilization were almost completely inhibited (Fig. 4C). In contrast, these platelets spread and flux calcium normally on a collagen-coated surface (not shown).

The spreading of platelets on fibrinogen was further investigated by staining of the actin cytoskeleton using rhodamine-labeled phalloidin. In agreement with previous reports, human platelets are able to spread on fibrinogen with the formation of filopodia, lamellipodia, and limited stress fibers (Fig. 5A). Spreading is enhanced by the addition of ADP resulting in most
of the platelets forming stress fibers (Fig. 5B). Spreading and formation of stress fibers was completely inhibited in the presence of cytochalasin D, BAPTA-AM (which chelates intracellular calcium), U71322, and 2-ABP (Fig. 5, E–G and I). PP2 and the PKC inhibitor Ro 31-8220 also markedly inhibited spreading although a limited formation of filopodia was preserved (Fig. 5, C and K). The partial spreading in the presence of PP2 does not appear to be because of an incomplete inhibition of Src kinases as
three experiments. Platelets were placed on coverslips coated with fibrinogen in the presence of 2 units/ml apyrase and 10 μM indomethacin and the indicated inhibitors/agonists for 30 min at 37°C. Platelets were fixed, permeabilized, and stained for F-actin using rhodamine-conjugated phalloidin. Results are representative of at least three experiments. A, no inhibitors/agonist, 0.1% Me_SO; B, 0.1% Me_SO + 100 μM ADP; C, 20 μM PP2; D, 20 μM PP2 and 100 μM ADP; E, 20 μM cytochalasin; F, 10 μM BAPTA-AM; G, 10 μM U71322; H, 10 μM U71322 and 100 μM ADP; I, 100 μM 2-ABP; J, 15 mM methyl-β-cyclodextrin; (K) 10 μM Ro 31-8220; L, 10 μM Ro 31-8220 and 100 μM ADP.

higher concentrations of PP2 (60 μM) had a similar effect (not shown). The inhibitory action of PP2 and Ro 31-8220 was overcome by the addition of ADP (Fig. 5, D and L). Interestingly, this is not the case for inhibition by the general PLC inhibitor U71322 (Fig. 5H), indicating that activation of at least one PLC isoform is crucial for spreading on fibrinogen. Consistent with our previous studies on αIIbβ3 signaling (11) depletion of cholesterol using methyl-β-cyclodextrin had no effect on spreading (Fig. 5J).

To investigate the role of PLCγ2 in spreading on fibrinogen we used murine platelets deficient in the phospholipase. Murine platelets, however, undergo a more limited degree of spreading on fibrinogen relative to human platelets. The average surface area of a murine platelet on fibrinogen, as measured with the actin stain phalloidin, increases by 55% from 2.00 ± 0.04 μm² (n = 318) in the presence of BAPTA-AM to 3.11 ± 0.06 μm² (n = 378). In contrast human platelets increase their size by 335% from 2.6 ± 0.13 μm² (n = 83) in the presence of BAPTA-AM to 8.56 ± 0.60 μm² (n = 103) under the same conditions. Importantly, a larger proportion of the PLCγ2-deficient platelets had not spread on fibrinogen relative to control cells, although the difference in surface area of the population was not statistical significant possibly because of the relative small change in overall size (Fig. 6). Nevertheless, the ability of ADP to induce full spreading of the wild type platelets was reduced by around 10% in the PLCγ2 −/− cells relative to the controls (surface area: 7.91 ± 0.55 μm², n = 209; versus 8.77 ± 0.66 μm², n = 177; p = 0.008).

Interestingly, during the course of this work, we noticed that the proportion of non-spread platelets in the PLCγ2 −/− population relative to the controls increased with time suggesting a kinetic difference between the two populations (not shown). To investigate this further, we measured spreading on fibrinogen using time-lapse laser scanning phase-contrast microscopy. With this method we were able to observe spreading over time and to visualize structures that could not be readily detected with the actin-based stain. Control platelets were seen to rapidly (within 1 min) form elongated filopodia on fibrinogen, which are far greater in length than those observed in phalloidin-stained platelets (Fig. 7C). This was accompanied by the partial formation of lamellipodia. The change in morphology is dynamic with a clear synthesis and retraction of filopodia and lamellapodia continuing to occur over a course of 15–30 min before reaching an end stage consisting of a partial spread central core and a wide network of filopodia (Fig. 7). In comparison, the majority of the PLCγ2 −/− platelets underwent a far less pronounced change in morphology. A far smaller number of filopodia was seen at early times and this was accompanied by a very limited formation of lamellipodia in ~50% of the population. Moreover, in contrast to the wild type platelets, these structures were not maintained and the lamellipodia disappeared within a few minutes. The filopodia were also seen to either disappear within a few minutes or to change to a small network of very thin fibers that could barely be detected at the level of the light microscope (Fig. 7C). In comparison, spreading
of cells deficient in the adapter LAT on fibrinogen was indistinctable from that of wild type platelets (not shown). These results demonstrate a critical role for PLCγ2 but not LAT in spreading on fibrinogen.

DISCUSSION

In this study we show that engagement of αIIbβ3 by fibrinogen causes activation of PLCγ2 and increase in intracellular Ca2+ and that these events are crucial for platelet spreading. We also show that although the regulation of PLCγ2 by αIIbβ3 has many similarities with the signaling events downstream of the collagen receptor GPVI, the two pathways can be distinguished on the role of key signaling proteins. Signaling by αIIbβ3 is dependent on Src and Syk family kinases and leads to cytoskeletal reorganization followed by a marked phosphorylation of FAK (2, 27), but is independent of the glycolipid-enriched membrane domain-associated adapter LAT and the FcR γ-chain (Figs. 1 and 2). In addition, αIIbβ3 can signal via a Src kinase-independent pathway, which is associated with tyrosine phosphorylation of an unidentified protein of about 130 kDa and limited spreading. The present study therefore identifies a novel pathway of activation of PLCγ2 by αIIbβ3 that is dependent on cytoskeletal reorganization and mediates platelet spreading. Dissecting out the full sequence of signaling events downstream of αIIbβ3 will be challenging in that not only does the integrin induce cytoskeletal reorganization, its signaling cascade is also strongly regulated by this event.

GPVI signaling is also dependent on Src and Syk family kinases, but its signaling cascade takes place mainly in glycolipid-enriched membrane domains and is mediated through a pathway that utilizes LAT and the FcR γ-chain (11, 13, 16, 21). In addition, GPVI signaling is associated with minimal FAK phosphorylation. It is important to note, however, that GPVI signaling is also partly dependent on cytoskeletal rearrangements in that it is inhibited partially in the presence of cytochalasin D. It is possible that cytoskeletal rearrangements contribute to GPVI clustering thereby enhancing the functional outcome or that they reinforce activation independent of clustering.

It has been shown that Src is constitutively associated with αIIbβ3 and plays a key role in the activation of Syk following fibrinogen binding (2). This initiates downstream processes such as phosphorylation of the adapter molecules SLP-76 and SLAP-130, activation of Vav family proteins, as well as phosphorylation of the kinase FAK (1, 3). Distal αIIbβ3 signaling events, e.g. FAK phosphorylation, have been shown to be heavily dependent on reorganization of the cytoskeleton and can be
prevented by inhibitors of actin polymerization such as cytochalasin D (2). Similar to FAK phosphorylation, PLC\(\gamma\)2 phosphorylation is also sensitive to cytochalasin D, identifying this as a late event in the \(\alpha_{IIb}\beta_3\) signaling cascade. Interestingly, FAK is implicated in the regulation of PLC\(\gamma\)1 in COS-7 cells and a mouse fibroblast cell line in response to integrin activation. In these cells FAK and PLC\(\gamma\)1 interact directly following autophosphorylation of Tyr-397 of the kinase and it is suggested that this interaction recruits PLC\(\gamma\)1 to the membrane and increases the enzymatic activity of the kinase (28). A similar interaction between FAK and PLC\(\gamma\)2 may take place in platelets following \(\alpha_{IIb}\beta_3\) activation, and would provide a route of recruitment to the membrane that is independent of LAT phosphorylation as FAK is associated with the \(\beta_3\) subunit of the integrin in platelets (2).

Although tyrosine phosphorylation of PLC\(\gamma\)2 does regulate activation of the enzyme (29), this does not necessarily lead to a functional activity as shown downstream of the platelet vWF receptor GPIb-IX-V (12). The activity is also dependent on substrate availability and membrane recruitment and it is therefore crucial to identify the functional activity of the phospholipase. Fibrinogen stimulation caused an increase in the production of phosphatidic acid and intracellular calcium through pathways that were inhibited strongly by cytochalasin D and completely by the Src kinase inhibitor PP2 and UT1322 (Figs. 3 and 4), an inhibitor of all PLC isoforms (30). The sensitivity to PP2 strongly suggests that the increase in phosphatidic acid is mediated by a PLC\(\gamma\) isoform rather than another isotype. This was supported by the observation that mice deficient in PLC\(\gamma\)2 also showed decreased production of phosphatidic acid. The lack of complete inhibition of phosphatidic acid formation in these studies could reflect the fact that mouse platelets express a low level of PLC\(\gamma\)1 (31). These data indicate that fibrinogen stimulation triggers functional activation of PLC\(\gamma\)2 downstream of the platelet integrin \(\alpha_{IIb}\beta_3\). Activation of PLC isoforms and its importance in integrin-mediated Ca\(^{2+}\) signaling is a well established phenomenon and has been observed in various cells expressing different integrins (32–36) including platelets activated by the \(\alpha_\beta_3\)-specific peptide GFOGER (37).

Functional activation of isoforms of phospholipase C results in the formation of the second messengers inositol 1,4,5-trisphosphate and 1,2-diacylglycerol leading to a mobilization of intracellular calcium and activation of PKC isoforms, respectively. Calcium and PKC have been shown to play major roles in mediating many of the responses associated with platelet activation including secretion and aggregation. Spreading on fibrinogen leads to an elevation of intracellular calcium and is inhibited by treatment with the calcium chelator BAPTA-AM, the inositol 1,4,5-trisphosphate receptor antagonist 2-ABP, and the PKC inhibitor Ro 31-8220 (Figs. 4 and 5). A role for calcium in \(\alpha_{IIb}\beta_3\)-mediated spreading under static conditions has previously been shown on a vWF-coated surface although it is likely that this reflects a role in integrin activation (38). Several studies have linked \(\alpha_{IIb}\beta_3\) engagement with calcium elevation at high shear. It is suggested that under flow conditions, the interaction of vWF with GPIb-IX-V induces transient calcium signals promoting \(\alpha_{IIb}\beta_3\) activation and platelet arrest. Subsequent \(\alpha_{IIb}\beta_3\) engagement induces intracellular calcium mobilization followed by transmembrane calcium influx. The integrin calcium response is sustained and necessary for irreversible adhesion (39–41). These results indicate a crucial role of both PLC-regulated second messengers for \(\alpha_{IIb}\beta_3\)-mediated responses.

Significantly, ADP is able to overcome the effect of the Src family kinase inhibitor PP2 on spreading of human platelets, whereas this is not the case in the presence of the non-specific inhibitor of PLC isoforms, UT1322 or the calcium chelator BAPTA-AM (Fig. 5). This indicates that activation of a PLC isoform is necessary for platelet spreading on fibrinogen but that the increase in lipase activity could be mediated through activation of PLC\(\gamma\)2 by fibrinogen or PLC\(\beta_3\) by the P2Y1 ADP receptor.

A dramatic reduction in spreading was observed in the PLC\(\gamma\)2\(-/-\) mice platelets using time-lapse laser scanning phase-contrast microscopy (Fig. 7). These results demonstrate that the activation of PLC\(\gamma\)2 is required for the formation of filopodia and lamellipodia. The observation that the filopodia and the limited formation of lamellipodia is a highly dynamic process and that this leads to continuous changes in morphology in wild type but not PLC\(\gamma\)2\(-/-\) platelets over a course of 15–30 min suggests that this is mediated by sustained activation of the phospholipase. We speculate that the transient formation of filopodia and spreading in PLC\(\gamma\)2\(-/-\) platelets is mediated by a limited and non-sustained mobilization of calcium, possibly mediated by activation of the limited amount of PLC\(\gamma\)1 that is also present in murine platelets (31) as spreading is completely inhibited following chelation of intracellular calcium or by inhibition of protein kinase C. Further work is required to compare the dynamics of calcium elevation and spreading under these conditions. Importantly, and consistent with the phosphorylation results, spreading on fibrinogen in LAT\(-/-\) platelets was not altered.

There is increasing evidence that PLC\(\gamma\)2 plays a central role in many aspects of platelet activation. It is well established that the phospholipase is the main target of signaling cascades downstream of Fc\(\gamma\)RIIA and the collagen receptor GPVI. PLC\(\gamma\)2 is necessary for the activating function of these receptors and ultimately causes activation of the integrin \(\alpha_{IIb}\beta_3\), which is crucial for platelet adhesion and thrombus formation. The present study shows that PLC\(\gamma\)2 also plays a critical role downstream of the integrin \(\alpha_{IIb}\beta_3\) thereby providing a new concept of regulation of the phospholipase in platelets. This activation is essential for functional responses mediated by the integrin. Spreading of platelets on fibrinogen is dependent on activation of PLC and mobilization of cytosolic calcium demonstrating a critical role for both arms of the lipase-based signaling cascade. Interestingly, despite substantial similarities between the pathways of GPVI and \(\alpha_{IIb}\beta_3\)-stimulated PLC\(\gamma\)2 activation, the two signaling cascades can be distinguished by their differential usage of specific signaling molecules, the requirement of cytoskeletal rearrangements, and the spatial organization. Further investigations will reveal if \(\alpha_{IIb}\beta_3\)-mediated PLC\(\gamma\)2 activation is involved in other late events in platelet function such as thrombus stability and clot retraction.

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