Glycoprotein Ib Cross-linking/Ligation on Echicetin-coated Surfaces or Echicetin-IgM in Stirred Suspension Activates Platelets by Cytoskeleton Modulated Calcium Release*

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Cross-linking platelet GPIb with the snake C-type lectin echicetin provides a specific technique for activation via this receptor. This allows GPIb-dependent mechanisms to be studied without the necessity for shear stress-induced binding of von Willebrand factor or primary αIIbβ3 involvement. We already showed that platelets are activated, including tyrosine phosphorylation, by echicetin-IgM-induced GPIb cross-linking. We now investigate the mechanism further and demonstrate that platelets, without modulator reagents, spread directly on an echicetin-coated surface, by a GPIb-specific mechanism, causing exocytosis of α-granule markers (P-selectin) and activation of αIIbβ3. This spreading requires actin polymerization and release of internal calcium stores but is not dependent on external calcium nor on src family tyrosine kinases. Cross-linking of GPIb complex molecules on platelets, either in suspension or via specific surface attachment, is sufficient to induce platelet activation.

Binding of platelets to damaged vessel wall is the first step in sealing a wound to prevent bleeding. Under the high shear conditions encountered in smaller vessels, this involves glycoprotein (GP) Ib on platelets binding to von Willebrand factor (VWF) on the subendothelium, to slow the platelets down via a rolling interaction, so that other platelet receptors can be activated and engaged to gain stable adhesion. This is a complex process and many aspects are still not understood. In particular, the mechanism regulating the shear-dependent binding between GPIb and VWF is still unknown, as well as the way in which this binding activates platelets. Several recent studies showed that the cytoskeleton has a critical role in this process. Although about 75% of GPIb is bound to membrane cytoskeleton through filamin, another 25% appears to be free (1). Increasing the amount of free GPIb by preventing actin polymerization with cytoskeleton D also enhances platelet aggregation responses to VWF. Similar results were also obtained in model systems where GPIb/IX was transfected into CHO cells (2, 3). CHO cells expressing a truncated form of GPIb, where the filamin binding domain was deleted, also show a higher agglutination response to VWF than those expressing wild-type GPIb/IX. However, Dong et al. (4) found a decrease in ristocetin-induced VWF binding to CHO cells expressing a filamin binding domain deleted form of GPIb, compared with cells expressing the wild type.

In CHO cells expressing wild type GPIb, cytochalasin D also enhanced agglutination responses to VWF (3). On the other hand, cytochalasin D decreased the size of agglutinates of CHO cells expressing a filamin-binding domain-deleted form of GPIb in response to VWF/ristocetin, a still unexplained phenomenon (3). Stable binding of platelets to a VWF-coated surface involves both GPIb and αIIbβ3. Binding of VWF to GPIb on platelets enhances the amount of actin associated with GPIb (5). Signaling from GPIb to activation of αIIbβ3 is a controversial area with many postulated mechanisms. A number of authors have suggested that tyrosine phosphorylation plays an important role, and several possible mechanisms for induction of tyrosine phosphorylation have been proposed involving roles for the Fcγ receptor and/or the FcγRIIA receptor on platelets, possibly via association with GPIb. Recently, clustering of GPIb complex expressed in CHO cells via cytoplasmic cross-linking of modified GPIX was shown to activate αIIbβ3 (6). It is known that a small part of the population of the GPIb complex molecules GPIbα and GPIbβ is palmitoylated (7). Palmitoylation may be important for localizing these molecules in lipid rafts. The presence of palmitoylated GPIb complex in rafts may provide a possible mechanism to incorporate other GPIb complexes into these signaling structures via clustering with VWF. Platelet rolling on VWF was shown to be accompanied by internal calcium signaling as a necessary step in causing stable adhesion and involves cytoskeleton (8). The interaction with VWF includes GPIb complex forming “tethers,” which both slow the platelet and trigger the activation necessary for stable binding (9). The absence of GPIb-cytoskeleton links results in the truncated GPIb being pulled out of the platelet membrane during rolling and in a reduction in platelet adhesion (3). Other mechanisms regulating the interaction of the GPIb complex with the cytoskeleton and platelet activation via GPIb complex are thought to involve phosphorylation of GPIbα and GPIbβ, as well as cytoplasmic components such as filamin, in particular via cAMP-dependent protein kinase, although they remain poorly understood (10, 11). It was also shown that mitogen-activated protein kinase (12) and phosphatidylinositol 3-kinase (13, 14) are involved in GPIb signaling.

The study of the activation of platelets via GPIb/VWF is complicated by the shear stress requirement so that most in vitro studies have been done using modulators such as ristocetin and botrocetin. We recently showed that cross-linking GPIb by the snake C-type lectin echicetin using IgM is a practical...
approach to the specific activation of platelets via GPIb (15). The use of echicetin as ligand avoids two complications of VWF, the requirement for shear to induce (VWF)/GPIb binding as well as VWF/αIPIβ3 interactions following platelet activation. We asked the question whether binding between platelet GPIb and a surface-bound substrate is enough in itself to induce platelet activation without other, possible shear-induced changes that might facilitate this activation. We thus showed that platelets bind and spread on an echicetin-coated surface, without a requirement for other molecules, and that they are activated, as demonstrated by P-selectin expression and fibrinogen binding. This primary interaction involves the cytoskeleton and internal calcium release but not tyrosine phosphorylation, which plays a role at a later stage in the activation process. Activation of platelets on an echicetin-coated surface provides a practical approach to the study of the early stages of platelet activation induced by cross-linking GPIb.

EXPERIMENTAL PROCEDURES

Materials—Lyophilized *Echis carinatus* sochureki venom was from Latoxan (Rosans, France), bovine serum albumin, ristocetin, cytocalasin D, peroxidase-conjugated rabbit anti-mouse antibodies, fluorescein isothiocyanate, Dabcyl (1,4-diazabicyclo[2.2.2]octane), and xestospongin C were from Sigma. N-Ethylmaleimide was from Fluka (Buchs, Switzerland). Latrunculin B was from Calbiochem (Juno, Lucerne, Switzerland). Human fibrinogen (VWF and plasminogen-free) was from Enzyme Research Laboratories (South Bend, IN). FITC-conjugated fibrinogen was prepared as previously described (16). Alboaggregin A (17) and convulxin (18) were prepared as previously described. FITC-coupled chicken anti-P-selectin (CD62P) and FITC-coupled chicken IgY as control were from WAK-Chemie Medical GmbH (Bad Soden, Germany). Sephadex G-10, Sepharose 2B, and Sepharose 4B were from Amersham Biosciences (Uppsala, Sweden). Autoradiography films were from Fujifilm (Dielsdorf AG, Switzerland). Anti-phosphotyrosine monoclonal antibody (4G10) was from Upstate Biotechnology Inc. (Lake Placid, NY). The GPIb/IIa inhibitor EMD132338 (19) was a kind gift from Dr. Sabine Bernatat-Danielovsky, Merck (Darmstadt, Germany). The GPIb/IIa inhibitor Ro44-9883 (20) was a kind gift from Dr. Beat Steiner, Hoffmann-La Roche (Basel, Switzerland). VM16a monoclonal antibody against GPIbα was a kind gift from Dr. Alexey Mazurov. Human von Willebrand factor was a kind gift from Dr. Sergey Domogatsky (Moscow, Russia). PolyScreen PVDF membranes were from PerkinElmer Life Sciences (Zaventem, Belgium). Echicetin-specific IgM was prepared as previously described (15). Polyvinyl alcohol (30–70 kDa, Sigma)-based microscope slide mounting medium was prepared by adding 2.4 g to 6 ml of water and stirring for a few hours. Then 6 ml of 200 mM Tris, pH 8.5, buffer was added and stirring continued overnight. After warming to 50 °C the mixture was centrifuged for 10 min at 3000 × g. The supernatant was carefully removed from any insoluble material, 0.6 g of Dabcyl was added, and the mixture was stirred until Dabcyl was dissolved.

**Purification of Echicetin**—Lyophilized *E. carinatus* sochureki venom was dissolved at 50 mg/ml in 50 mM sodium acetate, pH 5.0 (buffer A). Insoluble components were removed by centrifugation, and supernatant was loaded on a Fractogel EMD SO, 650/50 (column 10 × 150 mm, Merck, Darmstadt, Germany) equilibrated with buffer A. Elution of echicetin was performed by a 0 to 1 M gradient of NaCl in buffer A. Fractions (5 ml) were collected at a 3 ml/min flow rate. Echicetin activity was determined by its ability to block alboaggregin A-induced agglutination of fixed platelets. The fractions containing echicetin were pooled and concentrated by a SpeedVac. Fractions containing echicetin were further purified by affinity chromatography on an anti-echicetin rabbit polyclonal antibody-Sepharose 4B column.

**Coupling of FITC to Echicetin**—Purified echicetin was diazoylated in aqueous bovine serum albumin (9.5 μmol/ml) using 16 μmol/ml of 3.5% trifluoracetic acid. After dialysis, 1% glutaraldehyde was added and the mixture was dialyzed for 12 h. The solution was used immediately for in vitro experiments. FITC was coupled to echicetin at a molar ratio of 5:1. The mixture was incubated at room temperature for 2 h in darkness. FITC-echicetin conjugate was separated from free FITC by gel filtration on a Sephadex G-10 column equilibrated with 10 mM Tris buffer, pH 7.4.

**Protein Determination and SDS-PAGE**—Protein determination was performed with bovine serum albumin as standard. SDS-PAGE was performed by the method of Laemmli (21).

**Preparation of Washed Platelets and Platelet Aggregation**—Human platelets were isolated, less than 2 h after blood collection, fromuffy coat obtained from the Swiss Red Cross Blood Transfusion Service. To remove the buffy coat was added 30 ml of 100 mM citrate, pH 6.5. Platelet-rich plasma and the platelet pellet were isolated by successive centrifugation steps. Platelets were resuspended in 113 mM NaCl, 4.3 mM K2HPO4, 4.3 mM NaH2PO4, 24.4 mM NaH2PO4, 5.5 mM glucose, pH 6.5 (buffer B) and centrifuged at 250 × g for 5 min. The platelet-rich supernatant was centrifuged at 1000 × g for 10 min, and platelets were washed with buffer B once more. Washed platelets were resuspended in 10 mM Hepes, 140 mM NaCl, 4 mM KCl, 5.5 mM glucose, pH 7.4 (buffer C), and the platelet count was adjusted to 5 × 108 platelets/ml by dilution with buffer C. Samples were kept at room temperature until used for aggregation studies. Platelet aggregation was monitored by light transmission in an aggregometer (Lumitec, France) with continuous stirring at 1100 rpm at 37 °C. Platelets were washed with buffer C containing 2 mM CaCl2 and 2 mM MgCl2 at 37 °C for 2 min before starting the measurement by adding the samples for analysis.

**Flow Cytometry**—Samples were analyzed using a Becton Dickinson FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany). Excitation was with an argon laser at 488 nm. The FACScan was used in a standard configuration with a 530-nm bandpass filter. Standard beads containing specific amounts of “mean equivalent soluble fluorescein molecules” were used for calibration. Standard beads or platelets were gated, and data were obtained from fluorescence channels in a logarithmic mode. A total of 5000 events was analyzed. Specific binding of FITC-labeled echicetin was calculated by subtracting unspecific binding as determined with a 100-fold excess of unlabeled echicetin.

**Platelet Adhesion**—Glass coverslips (13-mm diameter, Assistent, Germany) were washed with acetone and ethanol and placed in the wells of 24-well culture plates. Echicetin, VWF, or convulxin at 10 μg/ml in 10 mM Tris buffer, pH 7.4, containing 150 mM NaCl (TBS) were placed on the surface of the coverslips and incubated at 4 °C overnight. After incubation the coverslips were washed with the same buffer. BSA at 2 mg/ml in TBS was placed on the surface of the coverslips either coated with echicetin, VWF, or convulxin or untreated for making a BSA-coated surface and were incubated for 2 h at room temperature. Coverslips were washed with TBS and kept at 4 °C until used. Fresh gel-filtered platelets (control or treated with inhibitor, 109 platelets/ml) were placed on the coated coverslips. Coverslips were washed with TBS, and adherent platelets were fixed with 1% formaldehyde for 30 min. For examination of P-selectin expression, adherent fixed platelets were stained with FITC-coupled anti-P-selectin antibodies (CD62P) for 30 min. For examination of GPIb/IIa activation FITC-labeled fibrinogen at 150 μg/ml in TBS was added to platelet suspension for 30 min before fixing. After incubation the coverslips with adherent platelets were washed three times with TBS, removed from the wells, and mounted upside down onto glass microscope slides using a polyvinyl alcohol-based mounting agent (20%) containing 1% of Dabcyl as an anti-bleaching reagent. The adherent platelets were between the glass slide and the coverslip.

The morphology of the adherent platelets was studied under the microscope (Nikon E600) at 1000× magnification, and images were saved using a video camera (Hamamatsu C5810) and FrameGrabber software. Average surface area per platelet was calculated in microns-square by measuring the surface area covered by platelets in each field and dividing by the number of platelets.

**RESULTS**

**Platelet Adhesion and Spreading to Echicetin-, VWF-, and BSA-covered Surfaces under Static Conditions**—Platelet adhesion to echicetin-coated surfaces was studied under static conditions using human VWF- or BSA-coated surfaces as controls. In the experiments with VWF-coated surfaces, platelet adhesion was performed in the presence of ristocetin. Morphological changes as well as P-selectin expression on the surface of adherent platelets were examined. Platelets adhered to echicetin-coated surfaces and were strongly activated, first extending pseudopods and finally being fully spread with release of P-selectin from storage granules (Fig. 1, A and B). On the surfaces coated with VWF, platelets were fully spread and P-selectin was expressed as well (Fig. 1, C and D). In the absence of ristocetin, platelets did not spread on the VWF-coated surface (data not shown). On the BSA-coated surfaces platelets were resting or very slightly activated, with small pseudopods. Only a very low level of P-selectin expression was detected in this case (Fig. 1, E and F). All data shown are representative of...
at least three experiments with platelets from different donors.

Platelet Activation and Spreading on Echicetin-coated Surfaces Is a GPIb-dependent Process—Pretreating platelets with an antibody to GPIb (VM16d) that blocks GPIb-echicetin interactions (15) completely prevented activation and spreading on echicetin-coated surfaces (Fig. 1, G and H). We showed previously that platelets agglutinate in response to echicetin followed by IgMx. This agglutination was also prevented by pretreating the platelet suspension with VM16d (15).

Platelet Adhesion/Activation to VWF/Ristocetin but Not Echicetin Requires αIIbβ3—It is known that VWF can bind to both GPIb and αIIbβ3 on the platelet surface. The involvement of αIIbβ3 in platelet adhesion/activation was studied using specific inhibitors EMD132338 (19) and Ro44-9883 (20) (data shown only for EMD132338). EMD132338 and Ro44-9883 had no effect on platelet spreading or on P-selectin expression on an echicetin-coated surface (Fig. 2, C and D, compared with A and B). No differences were found on BSA-coated surfaces either (both not activated, data not shown). However, EMD132338 and Ro44-9883 strongly reduced platelet spreading on VWF-coated surfaces (Fig. 2, compare G with E) while having less effect on P-selectin expression (Fig. 2, compare H with F).

Effect of Cytochalasin D on Platelet Adhesion and Activation—Platelets were treated with cytochalasin D (20 μM, from 4 mM stock in Me2SO), and their adhesion/activation to surfaces coated with echicetin, VWF, or BSA was examined. Cytochalasin D-treated platelets were not activated on any surface. No pseudopods or spreading were observed. Data are shown for echicetin only because there was no difference between the results with echicetin, VWF, and BSA. On binding to an echicetin surface αIIbβ3 was activated and bound FITC-labeled fibronectin (Fig. 3B), although not as strongly as on a convulxin-coated surface (Fig. 3F). Fibrinogen did not bind to cytochalasin D-treated platelets on an echicetin surface (Fig. 3D), indicating that αIIbβ3 was not activated. P-selectin expression was strongly reduced in cytochalasin D-treated platelets compared with controls regardless of the surface (Fig. 4, compare D with B). The cytochalasin D-treated platelets have a more pronounced discoid form (longer equilateral/axial ratio) than untreated and therefore appear larger than untreated/unactivated (Figs. 3C and 4C versus Fig. 1, E and G) (see also Fig. 5 for quantitation of this difference).

Effect of Latrunculin B on Platelet Adhesion and Activation—Platelets were treated with latrunculin B (20 μM, from 4 mM stock in Me2SO), and their adhesion/activation to surfaces were coated with echicetin, or BSA was examined. Latrunculin B-treated platelets were not activated on either surface. No pseudopods or spreading were observed. Data are shown for echicetin only because there was no difference between the results with echicetin, VWF, and BSA. On binding to an echicetin surface αIIbβ3 was activated and bound FITC-labeled fibronectin (Fig. 3B), although not as strongly as on a convulxin-coated surface (Fig. 3F). Fibrinogen did not bind to echicetin-coated surfaces (Fig. 3). P-selectin expression was strongly reduced in latrunculin B-treated platelets on echicetin-coated surfaces (Fig. 3D), indicating that αIIbβ3 was not activated. P-selectin expression was strongly reduced in latrunculin B-treated platelets compared with controls (Fig. 4, compare F with B). The latrunculin B-treated platelets have a more pronounced discoid form (longer equilateral/axial ratio) than untreated and therefore appear larger than untreated/unactivated (Fig. 4E versus Fig. 1, E and G) (see also Fig. 5 for quantitation of this difference).
Fig. 3. Activation of GPIb/IIa on the surface of platelets adherent to an echicetin-coated surface. Washed human platelets (1 × 10⁶ plts/ml) were placed on echicetin-coated surfaces for 30 min. After incubation, adherent platelets were fixed with formaldehyde and stained with FITC-labeled human fibrinogen. Cytochalasin D (20 μM) was added to platelet suspension 10 min before the start of the experiment. Platelet spreading on a convulxin-coated surface was used as a positive control. Adherent platelets were studied by light microscopy at 1000× magnification. On the left side are platelets in visible light and on the right side is the FITC-fibrinogen binding detected as fluorescence in UV light. A and B, control platelets on echicetin-coated surfaces; C and D, cytochalasin D-treated platelets on an echicetin-coated surface; E and F, control platelets on a convulxin-coated surface.

Fig. 4. Effect of various inhibitors on platelet spreading and P-selectin expression on echicetin-coated surfaces. Washed human platelets (1 × 10⁶ plts/ml) were placed on echicetin-coated surfaces for 30 min. After incubation adherent platelets were fixed with formaldehyde and stained with FITC-labeled anti P-selectin antibodies. Inhibitors were added to platelets for 5–10 min before the experiment. Adherent platelets were studied with light microscopy at 1000× magnification. On the left side platelets are shown in visible light, and on the right side shows P-selectin expression detected as fluorescence in UV light. A and B, control platelets; C and D, cytochalasin D (20 μM)-treated platelets; E and F, latrunculin B (20 μM)-treated platelets; G and H, BAPTA (50 μM)-treated platelets; I and J, xestospongin C (0.5 μM)-treated platelets; K and L, apyrase (1 unit/ml)-treated platelets; M and N, PGE1 (20 nM)-treated platelets; O and P, sodium nitroprusside (100 μM)-treated platelets; R and S, PP1 (8 μM)-treated platelets.

Cytochalasin D and Latrunculin B Enhance Platelet Agglutination but Inhibit Protein Tyrosine Phosphorylation Induced by Echicetin-IgMx Complex—Echicetin-IgMx complex binds to the surface of washed platelets and induces agglutination. Platelet agglutination by echicetin-IgMx complex increases in protein tyrosine phosphorylation.

To see the effect of preventing actin polymerization on platelet agglutination induced via echicetin-IgMx complex, platelets were treated with cytochalasin D (20 μM) or latrunculin B (20 μM) for 10 min before adding echicetin and IgMx. Both the cytochalasin D- and latrunculin B-treated platelets gave larger agglutinates compared with untreated platelets (Fig. 6, A and C). Levels of FITC-labeled echicetin binding to the surface of cytochalasin D-treated, latrunculin B-treated, and control platelets were examined by flow cytometry and are completely identical (data not shown). Therefore, the increased size of platelet agglutinates is not due to increased echicetin binding to the surface of cytochalasin D- or latrunculin B-treated platelets.

Comparison of protein tyrosine phosphorylation patterns shows a marked decrease of phosphorylation in platelets treated with cytochalasin D or latrunculin B. The decrease of phosphorylation covered most phosphorylated bands (Fig. 6B).

Protein Tyrosine Phosphorylation in Platelets Activated by Echicetin-IgMx Requires Internal Ca²⁺ Release—To study the involvement of Ca²⁺ release in platelet activation via echicetin-IgMx complex, platelets were treated either with BAPTA or with xestospongin C. Two concentrations of BAPTA were used. Inhibition of platelet aggregation to a low dose of thrombin after BAPTA treatment was used as control. At a low dose of BAPTA (10 μM) platelet aggregation to thrombin was strongly but not completely inhibited. At 50 μM (high dose) BAPTA completely inhibited platelet aggregation to thrombin. Neither concentration of BAPTA affected platelet agglutination induced by echicetin-IgMx complex. However, protein tyrosine phosphorylation in platelets treated with BAPTA (at 10 and 50 μM) was strongly suppressed (Fig. 7B) in comparison to control platelets (Fig. 7A). Xestospongin C (0.5 μM) did not affect platelet agglutination but also strongly inhibited tyrosine phosphorylation (Fig. 7, compare C with A).

Effect of Chelation of Intracellular Calcium on Platelet Adhesion/Activation—Platelets were pretreated with BAPTA-AM (50 μM) for 20 min before they were placed on the surfaces coated with echicetin, VWF, or BSA. BAPTA strongly inhibited platelet spreading and P-selectin expression on echicetin (Fig. 4, G and H) and VWF (data not shown) coated surfaces, although there were a few thin elongated pseudopods. There were no significant differences in platelet adhesion to BSA-coated surfaces (data not shown).

Effect of Blocking IP3 Receptors with Xestospongin C on Platelet Adhesion/Activation—Platelets were pretreated with xestospongin C (0.5 μM) for 20 min before they were placed on the surfaces coated with echicetin or BSA. Xestospongin C strongly inhibited platelet spreading and P-selectin expression on echicetin (Fig. 4, I and J)-coated surfaces, although there were a few thin elongated pseudopods.

Aspirin Has No Effect on Platelet Agglutination or Protein Tyrosine Phosphorylation via Echicetin-IgMx Complex—To see if thromboxane formation is involved in the process of signaling...
through GPIb, platelets were treated with aspirin (100 μM) 10 min before adding echicetin IgMx. Platelets treated with aspirin show the same agglutination response via echicetin-IgMx as untreated (data not shown). Protein tyrosine phosphorylation is unaffected in aspirin-treated platelets compared with untreated (data not shown). Platelets treated with aspirin spread in the same way as controls on echicetin-coated surfaces (data not shown).

**Influence of Apyrase on Platelet Activation via Echicetin IgMx—**To investigate the role of ADP signaling in the process of platelet agglutination/activation induced by echicetin-IgMx complex, the platelets were treated with apyrase (1 unit/ml) for 5 min before adding the complex. There was no difference between the agglutination level (not shown) or protein tyrosine phosphorylation (Fig. 7D) of platelets treated with apyrase and controls (Fig. 7A). Platelets spread normally on echicetin-coated surfaces and expressed P-selectin in the presence of apyrase (Fig. 4, K and L).

**Activation of cAMP-dependent Kinase by PGE<sub>2</sub> Affects Platelet Responses to GPIb—**Platelets were treated with sodium nitroprusside (100 μM) and agglutinated with echicetin-IgMx in stirred suspension in an aggregometer. The treated platelets gave the same size of agglutinates as the control platelets. Protein tyrosine phosphorylation in platelets treated with PGE<sub>2</sub> was partly suppressed compared with control platelets (Fig. 7, E versus A). When PGE<sub>2</sub>-treated platelets were placed on an echicetin-coated surface spreading was strongly inhibited and P-selectin expression was reduced but not completely inhibited (Fig. 4, M and N).

**Activation of cGMP-dependent Kinase by NO (Sodium Nitroprusside) Affects Platelet Responses to GPIb—**Platelets were treated with sodium nitroprusside (100 μM) and agglutinated with echicetin-IgMx in stirred suspension in an aggregometer. The treated platelets gave the same size of agglutinates as the control platelets. Protein tyrosine phosphorylation in platelets treated with sodium nitroprusside was more strongly inhibited than with PGE<sub>2</sub>-treated platelets (Fig. 7F). Platelets treated with sodium nitroprusside spread normally on an echicetin-coated surface, and P-selectin expression was also like controls (Fig. 4, O and P).

**Effects of Inhibition of src Kinases by PP1 on Platelet Responses to GPIb—**Platelets treated with the src kinase family inhibitor PP1 (8 μM) showed a clear inhibition of tyrosine phosphorylation even before adding echicetin-IgMx and a nearly complete inhibition after agglutination (Fig. 7G). Platelets treated with PP1 gave larger agglutinates than controls (about 20%) but not as large as with cytochalasin D or latrunculin B treatment. Platelet spreading and P-selectin expression on echicetin-coated surfaces were slightly lower than controls (Fig. 4, R and S) (see also Fig. 5 for quantitation of this difference).

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to be necessary for shear stress activation. However, with VWF, αIIBβ3 inhibitors as well as anti-GPIb antibodies blocked platelet adhesion, spreading, and activation, indicating that both platelet receptors are essential. This agrees with results from previous studies with surfaces coated with mutant VWF, where either the A1 domain or the RGD site was altered (22). This might indicate that the density of GPIb-binding sites (A1 domains) on a VWF-coated surface is insufficient to trigger platelet activation. The BSA-coated surface gave low platelet adhesion, and these platelets were not activated.

It was previously shown that treating platelets or CHO cells expressing GPIb complex with cytochalasin D, or similar reagents affecting actin polymerization, increases the size of platelet agglutinates formed in response to VWF/ristocetin or botrocetin (1, 2). Similarly, CHO cells, expressing a truncated form of GPIb lacking filamin attachment sites, were reported to show enhanced adhesion to VWF (slower rolling) in the presence of ristocetin or botrocetin (1). These authors also suggested that this may be due to decreased restrictions on GPIb lateral mobility in the platelet membrane because of weaker attachment to the membrane-associated cytoskeleton. On the other hand, Dong et al. (4) found a decrease in ristocetin-induced VWF binding to CHO cells expressing a filamin binding domain deleted form of GPIb, compared with cells expressing the wild type. Platelets treated with cytochalasin D or with latrunculin B, agglutinated with echicetin-IgM, also gave larger agglutinates than control platelets. Formaldehyde-fixed cytochalasin D-treated platelets also gave larger agglutinates than formaldehyde-fixed control platelets (data not shown), suggesting that the distribution of GPIb complexes on the platelet surface rather than their lateral mobility is critical for this phenomenon. Some data exist indicating that GPIb molecules are more rigidly distributed along actin fibers in resting platelets and have a more random distribution in activated platelets or cytochalasin D-treated platelets. Clustering GPIb by cytoplasmic cross-linking in CHO cells slowed rolling on VWF, also suggesting that distribution of GPIb has a critical role in VWF binding (6).

Cytochalasin D or latrunculin B treatment almost completely inhibited the tyrosine phosphorylation response of echicetin-IgMk-agglutinated platelets (Fig. 6). Cytochalasin D- or latrunculin B-treated platelets on an echicetin-coated surface did not spread or release, demonstrating that actin polymerization is a critical role in the early stages of the activation process (Fig. 4).

Cytoplastic calcium release is also thought to be involved in signaling from GPIb to platelet activation. Recent results indicate that there are two stages in calcium release, an early rapid signal derived from GPIb ligation, and a second longer signal, downstream of αIIBβ3 activation, in platelets rolling/adhering on a VWF-coated surface, measured as fluorescence signals from single platelets (8, 24). We were unable to demonstrate a calcium signal with stirred platelets agglutinated with echicetin-IgM (data not shown). However, in platelets loaded with the calcium chelator BAPTA via BAPTA-AM, tyrosine phosphorylation was strongly inhibited compared with control platelets. In addition, platelets loaded with BAPTA reacted very weakly to an echicetin-coated surface, but were not completely inhibited, developing a few long, thin pseudopods. Xestospongin C is thought to prevent calcium release in response to inositol 1,4,5-trisphosphate receptors (23). Tyrosine phosphorylation in response to echicetin-IgM, in platelets treated with xestospongin C, was strongly inhibited compared with control platelets. Platelets loaded with xestospongin C also reacted very weakly to an echicetin-coated surface and, as with BAPTA treatment,
developed a few long, thin pseudopods. Thus, although calcium release is important for further downstream signal transduction, it is less critical than actin polymerization for the first stages of platelet activation via GPIb cross-linking. BAPTA and xestospongin C also completely inhibited P-selectin release and αIIbβ3 activation in response to an echicetin-coated surface as well as to a VWF-coated surface in the presence of ristocetin. The BAPTA- and xestospongin C-treated platelets had lower levels of protein tyrosine phosphorylation than the untreated controls, thus possibly indicating that slight activation due to platelet washing was also inhibited.

Because autocrine activation via agonists released from platelets can be an important factor in platelet activation, the effects of inhibitors of these pathways was also tested. Neither apyrase, which degrades ADP released from dense granules and prevents activation via ADP receptors, nor aspirin, which inhibits cyclooxygenase and prevents formation of thromboxanes that feed back via their receptors, had any significant effect on platelet spreading or P-selectin expression, indicating that these processes are likely to be independent of feedback from the release reaction.

To distinguish which signaling pathways might be involved in the downstream events from GPIb-derived signals, the effects of several types of kinase inhibitors were examined. PP1, a general src kinase inhibitor, completely inhibited tyrosine phosphorylation and inhibits major tyrosine phosphorylation of most of the signaling molecules and blocks αIIbβ3 activation and P-selectin release.

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