Synergistic Adhesive Interactions and Signaling Mechanisms Operating between Platelet Glycoprotein Ib/IX and Integrin \( \alpha_{IIb}\beta_3 \)

STUDIES IN HUMAN PLATELETS AND TRANSFECTED CHINESE HAMSTER OVARY CELLS*

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This study investigates three aspects of the adhesive interaction operating between platelet glycoprotein Ib/IX and integrin \( \alpha_{IIb}\beta_3 \). These include the following: 1) examining the sufficiency of GPIb/IX and integrin \( \alpha_{IIb}\beta_3 \) to mediate irreversible cell adhesion on immobilized von Willebrand factor (vWF) under flow; 2) the ability of the vWF-GPIb interaction to induce integrin \( \alpha_{IIb}\beta_3 \) activation independent of endogenous platelet stimuli; and 3) the identification of key second messengers linking the vWF-GPIb/IX interaction to integrin \( \alpha_{IIb}\beta_3 \) activation. By using Chinese hamster ovary cells transfected with GPIb/IX and integrin \( \alpha_{IIb}\beta_3 \), we demonstrate that these receptors are both necessary and sufficient to mediate irreversible cell adhesion under flow, wherein GPIb/IX mediates cell tethering and rolling on immobilized vWF, and integrin \( \alpha_{IIb}\beta_3 \) mediates cell arrest. Moreover, we demonstrate direct signaling between GPIb/IX and integrin \( \alpha_{IIb}\beta_3 \). Studies on human platelets demonstrated that vWF binding to GPIb/IX is able to induce integrin \( \alpha_{IIb}\beta_3 \) activation independent of endogenous platelet stimuli under both static and physiological flow conditions (150–1800 s⁻¹). Analysis of the key second messengers linking the vWF-GPIb interaction to integrin \( \alpha_{IIb}\beta_3 \) activation demonstrated that the first step in the activation process involves calcium release from internal stores, whereas transmembrane calcium influx is a secondary event potentiating integrin \( \alpha_{IIb}\beta_3 \) activation.

The integrin family of cell surface adhesion receptors mediates cell-cell and cell-matrix interactions responsible for mammalian development, inflammation, immunity, and hemostasis (1, 2). For circulating cells such as platelets and leukocytes, integrins play an indispensable role in anchoring these cells to the luminal surface of blood vessels at sites of vascular injury and inflammation. However, a limitation of integrin function is their relative inefficiency at forming adhesion contacts under conditions of blood flow. This has been most clearly demonstrated from studies of leukocyte adhesion to post-capillary venules, in which the formation of integrin adhesion contacts requires an initial cell-tethering step dependent on one or more selectin family members (3–5).

There is now strong evidence that platelets also utilize a multistep adhesion mechanism, involving glycoprotein (GP) Iβ/IX and one or more surface integrins, to mediate stable adhesion at sites of vascular injury (6, 7). Platelet tethering involves the binding of the GPIb/IX complex to subendothelial von Willebrand factor (vWF). This multivalent adhesive interaction is unique in that it can tether platelets at high shear stresses (6), a key requirement for the ability of platelets to secure hemostasis throughout the arterial circulation. vWF binding to GPIb/IX also induces platelet activation, converting the major platelet integrin, \( \alpha_{IIb}\beta_3 \), from a low affinity to a high affinity receptor capable of engaging the C1 domain of vWF (8–10). This latter adhesive interaction is essential for stable platelet adhesion on vWF and also for subsequent cytoskeletal reorganization leading to platelet spreading (11, 12).

A major unresolved issue is the mechanism by which the vWF-GPIb interaction induces activation of integrin \( \alpha_{IIb}\beta_3 \) under physiological flow conditions. In particular, it is unclear whether GPIb/IX induces integrin \( \alpha_{IIb}\beta_3 \) activation directly, through the generation of intracellular second messengers, or involves an indirect pathway dependent on the release of ADP and/or the generation of thromboxane A₂ (TXA₂). Evidence favoring the latter mechanism has been suggested from studies of shear-induced platelet aggregation using a cone-and-plate viscometer (13, 14), in which the exposure of platelets in suspension to pathological levels of shear induces platelet activation in an ADP-dependent manner. According to this model (Fig. 1), shear-induced binding of soluble vWF to GPIb induces transmembrane calcium influx through an unidentified surface channel functionally linked to the GPIb/IX complex. The subsequent rise in intracellular calcium promotes secretion of dense granule ADP, which in turn engages one or more purinergic receptors (15–18) on the cell surface leading to integrin \( \alpha_{IIb}\beta_3 \) activation (19). Thus under pathological levels of shear, \( \alpha_{IIb}\beta_3 \) function may be compromised, leading to platelet dysfunction.

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1 The abbreviations used are: GP, glycoprotein; vWF, von Willebrand factor; HvWF, human von Willebrand factor; HvWF, bovine von Willebrand factor; PKC, protein kinase C; ATPαS, adenosine 5′-O-(4-thiotriphosphate); AM, acetylcytisine; BAPA-AM, 1,2-bis(o-aminophen-oxo)ethane-N,N,N′,N′-tetraacetic acid tetra(acetylcysteine) ester; mAb, monoclonal antibody; Ab, antibody; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; CHO, Chinese hamster ovary; DIC, differential interference contrast; A835PS, adenosine 3′-phosphate 5′-phosphosulfate; BIM, bisindolylmaleimide I; TXA₂, thromboxane A₂.
the vWF-GPIb interaction has been postulated to induce integrin αIIbβ3 activation by an indirect mechanism critically dependent on transmembrane calcium influx and the release of ADP. The mechanism by which platelets become activated on an immobilized vWF matrix under physiological flow conditions has been less clearly defined. This is a potentially important issue given the critical role played by vWF in promoting platelet-vessel wall and platelet-platelet adhesion contacts under flow. A recent study by Kuwahara et al. (20) has raised the interesting possibility that integrin αIIbβ3 activation on the surface of platelets adhering to immobilized vWF occurs in a calcium-independent manner. In their studies, adhesion of platelets to vWF under physiological flow conditions was not associated with detectable changes in the cytosolic concentration of calcium, and furthermore, chelating intracellular calcium did not inhibit integrin αIIbβ3-dependent stationary platelet adhesion. Several other reports have begun to challenge the hypothesis that calcium influx is indispensable for GPIb/IX-dependent signaling. For example, Francesconi et al. (21, 22) failed to detect transmembrane calcium influx following vWF binding to GPIb/IX. Moreover, Kermode et al. (23) have suggested that previously observed changes in cytosolic calcium initiated by vWF binding to GPIb/IX may be artifactual, resulting from extrusion of the indicator dyes from loaded platelets. Our recent studies also do not support an indispensable role for calcium influx in GPIb/IX-dependent signaling, as pretreating platelets or GPIb/IX-transfected CHO cells with extracellular calcium chelators did not prevent GPIb/IX-induced cytoskeletal remodeling (24). The reason for the apparent discrepancies between different studies in terms of GPIb/IX signaling is not immediately evident but may reflect methodological differences that influence the platelet αIIbβ3 activation process. For example, studies in a cone-and-plate viscometer primarily examine the effects of pathological levels of shear on the formation of platelet-platelet adhesion contacts (aggregation) in suspension, whereas studies in flow chambers principally examine platelet adhesion onto reactive protein surfaces.

In this study we have examined several aspects of the adhesive and signaling relationship operating between GPIb/IX and integrin αIIbβ3 using an in vitro flow-based adhesion assay. First, we have examined whether vWF engagement of GPIb/IX and integrin αIIbβ3 is sufficient to mediate irreversible cell adhesion under flow. Second, we have examined whether the vWF-GPIb interaction can transduce signals directly to regulate the ligand binding status of integrin αIIbβ3. Third, we have examined whether there is a second messenger role for calcium in linking the vWF-GPIb interaction to integrin αIIbβ3 activation. Our studies indicate that the sequential binding of vWF to GPIb/IX and integrin αIIbβ3 is sufficient to mediate irreversible cell adhesion under flow and that GPIb/IX can transduce signals directly to regulate the ligand binding function of integrin αIIbβ3. In addition, we have demonstrated that intracellular calcium mobilization and activation of protein kinase C (PKC) are two key signaling events linking the vWF-GPIb interaction to integrin αIIbβ3 activation over the full range of shear forces experienced by platelets in vivo.

EXPERIMENTAL PROCEDURES

Materials—FITC-conjugated phalloidin, adenosine 3′-phosphosulfate 5′-phosphosulfate (A3P5PS), and acetylserineic acid were purchased from Sigma. ATP-αS, calphostin C, bisindolylmaleimide I (BIM), and the calcium chelators, EGTA-AM and 1,2-bis(o-aminophenoxy)ethane-N,N,N,N′-tetraacetic acid tetra(acetoxyethyl) ester (BAPTA-AM), were from Calbiochem. 5,5′-Dimethyl-BAPTA, AM, Oregon Green 488 BAPTA-1, AM, and Fura Red, AM, were from Molecular Probes Inc. (Eugene, OR). Aggrastat was from Merck & Co. Inc. (Whitehouse Station, NJ). Apyrase was purified from potatoes according to the method of Molnar and Lorand (25). Human von Willebrand factor (HvWF) and bovine von Willebrand factor (BvWF) were purified to homogeneity from plasma cryoprecipitate according to the method of Montgomery and Zimmerman (26). Botrocetin was a generous gift from Prof. Michael Berndt (Baker Medical Research Institute, Melbourne, Australia). AR-C69931MX was generously supplied by AstraZeneca R & D Charnwood (Leicestershire, UK) (27). All other reagents were obtained from sources described previously (28–30).

Antibodies and DNA Constructs—The anti-β3 chimeric Fab fragment of the monoclonal antibody (mAb) 7E3 (c7E3 Fab abciximab) was from Eli-Lilly (Centocor, Leiden, Netherlands). The complex-specific anti-αIIbβ3 antibody, P2, was from Coulter/Immunotech (Marseille, France). The anti-human integrin αIIbβ3 antibody, LM609, was from Chemicon.
International, Inc. (Temecula, CA). Anti-GPIbα mAb, AK2, was generously donated by Prof. Michael Berndt (Baker Medical Research Institute, Melbourne, Australia). PAC-1 mAb was from Becton Dickinson (Victoria, Australia), and FITC-conjugated anti-mouse IgM (μ) or IgG (adsorbed with sheep and goat sera, respectively) was obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL). CHO-pDIX-1 cells were kindly provided by Dr. Peter Newman (Blood Research Institute, Milwaukuee, WI). The pZeoSV vector and Zeo+ were from Invitrogen (San Diego, CA).

Preparation of Washed Platelets and Reconstitution with Washed Red Blood Cells—Whole blood (anticoagulated with 15 mM trisodium citrate, pH 7.4) was collected from healthy volunteers (20–30 s of age) who had not received any anti-platelet medication in the preceding 2 weeks. Washed platelets were prepared as described previously (29) and resuspended in Tyrode’s buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 5 mM glucose) prior to reconstitution with washed platelets (50% (v/v) autologous packed red blood cells), in the presence of 0.4 units/ml apyrase, ATP g/ml), according to a modified method of Yuan et al. (29). Cells were washed three times with washing buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 5 mM glucose) prior to reconstitution with washed platelets (50% (v/v) autologous packed red cells), in the presence of 0.4 units/ml apyrase and 10 mM ATP, in the absence of calcium (1 mM EGTA-AM (50 μM) or AR-C69931MX with A3P5PS (100 nM and 200 μM each), EGTA-AM (50 μM) for 30 min at 22 °C, prior to reconstitution with DM-BAPTA, AM (70 μM). Changes in cytosolic calcium concentration were associated with increased or decreased fluorescence emission of Oregon Green and Fura Red, respectively (emission wavelengths of 500–570 nm for Oregon Green and 500–510 nm for Fura Red). These changes were analyzed by confocal microscopy and based on a ratio signal intensity in the Oregon Green and Fura Red channels. Fluorescence ratios were then converted to calcium concentration units according to Equation 1.

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

where 170 = \( K_C \) of Oregon Green Ca^{2+} binding; \( R = \) fluorescence ratio; \( R_{\text{max}} \) and \( R_{\text{min}} \) represent the fluorescence ratio of platelets that have been incubated with 50 μM A23187 + 10 μM Ca^{2+} or 70 μM DM-BAPTA, AM, and 2 μM EGTA, respectively; \( F_{\text{max}} \) and \( F_{\text{min}} \) represent the Oregon Green fluorescence of \( R_{\text{max}} \) and \( R_{\text{min}} \). The calcium concentrations for minimal and maximal calcium fluorescence ratios are calculated based on established methods, and the values obtained are in close agreement with that previously published (20, 32, 33).

Basil cytosolic calcium concentrations were determined in platelets in suspension and compared with calcium levels obtained in platelets adherent to HvWf under static or flow conditions. For population analysis of calcium response of platelets adherent to HvWf under static conditions, the cytosolic calcium concentration was determined over a 37.5-s time interval, 10 min after adhesion. The calcium concentration in pulsing platelets was determined using MCID software (Imaging Research Inc., Canada) at 7-s intervals over the 37.5-s period. For single cell analysis, the calcium dynamics in individual platelets was monitored every 0.586 s over a 37.5-s time interval (Leica TCS NT software). Under flow conditions, the frequency of a calcium “event” (where event is defined as the recording of a particular calcium concentration) was determined at 7-s intervals over a 37.5-s period. Statistical Analysis—Significant differences were detected using Student’s t test and one-way analysis of variance, using the Prism software package (GraphPAD Software for Science, San Diego, CA).

RESULTS

The necessity of GPIb/IX and integrin αIβ3 for irreversible platelet adhesion on vWf has been clearly established through the use of specific inhibitors against either receptor (6, 12, 34, 35) and from the study of platelets with qualitative or quantitative deficiency of GPIb/IX or integrin αIβ3 (37–41). However, it has yet to be established definitively that both of these two receptors are sufficient to mediate irreversible cell adhesion under flow, independent of other platelet adhesion receptors or endogenous stimuli. To investigate this possibility, we performed studies on CHO cells co-transfected with GPIb/IX and integrin αIβ3. We chose this cell line as it does not express GPIb/IX or integrin αIβ3, does not contain storage granules...
for ADP or other activating stimuli, or generate TXA2 following cell stimulation. We have recently demonstrated that CHO cells transfected with the GPIb/IX complex are able to tether and translocate on a vWF matrix but are unable to form irreversible adhesion contacts (30). To investigate the ability of integrin αIIbβ3 to support irreversible adhesion contacts under flow, we transfected GPIb/IX-expressing CHO cells with integrin αIIbβ3. As demonstrated in Fig. 2A, FACS analysis of CHO-Ib/IX cells with an antibody against GPIbα or GPIIX revealed efficient expression of the receptor complex on the cell surface, whereas an antibody against integrin αIIbβ3 failed to bind CHO-Ib/IX. In contrast, CHO-Ib/IX cells transfected with integrin αIIbβ3 (CHO-Ib/IX-αIIbβ3) were recognized by antibodies against GPIbα, GPIIX, and the integrin αIIbβ3 complex (Fig. 2A).

To examine the roles of GPIb/IX and integrin αIIbβ3 in mediating adhesion under flow conditions, we perfused CHO-Ib/IX-αIIbβ3 cells through bovine vWF (BvWF)-coated micropipette tubes. In initial flow assays, we compared the ability of CHO-Ib/IX cells and CHO-Ib/IX-αIIbβ3 to tether to BvWF at 150 s⁻¹. As demonstrated in Fig. 2B, the presence of integrin αIIbβ3 had no significant effect on the efficiency of CHO-Ib/IX-αIIbβ3 tethering to vWF. Cell tethering was mediated by GPIb/IX as it was completely prevented by pretreating CHO-Ib/IX (data not shown) and CHO-Ib/IX-αIIbβ3 cells with a blocking antibody against GPIbα. The number of cells adhering to vWF under shear conditions reflects the sum of translocating and irreversibly adherent cells (6). Cell translocation is mediated by the reversible binding of GPIb/IX to the A1 domain of vWF, whereas irreversible adhesion is dependent on integrin αIIbβ3 engagement of the C1 domain of vWF (10). Thus the formation of irreversible adhesion contacts on a vWF matrix under flow is an indirect marker of integrin αIIbβ3 activation. Analysis of the percentage of cells forming stationary adhesion contacts (Fig. 2C) revealed a significant increase in the ability of CHO-Ib/IX-αIIbβ3 cells to form stationary adhesion contacts. In contrast to CHO-Ib/IX cells that were previously demonstrated to tether and roll but not to irreversibly adhere to immobilized vWF (30), 97.3 ± 1% of CHO-Ib/IX-αIIbβ3 cells (p < 0.001) formed stationary adhesion contacts at low shear (150 s⁻¹) (Fig. 2C). Increasing wall shear rate to 3000 s⁻¹ was associated with 7.3% of CHO-Ib/IX-αIIbβ3 cells adopting a rolling type adhesion. However, even at shear rates as high as 6000 s⁻¹, 86.3 ± 2.2% of CHO-Ib/IX-αIIbβ3 cells maintained stable adhesion contacts. In contrast, in the presence of c7E3 Fab (α-IIbβ3) the percentage of CHO-Ib/IX-αIIbβ3 cells forming stationary adhesion decreased by 45.53, 61.81, 63.91, and 65.50% at 150, 750, 3000, and 6000 s⁻¹, respectively. (Fig. 2C, α-IIbβ3). The proportion of stationary c7E3 Fab-treated CHO-Ib/IX-αIIbβ3 cells at each shear rate examined was similar to that observed with CHO cells expressing GPIb/IX alone (data not shown) and is consistent with the ability of the vWF-GPIb interaction to support stationary cell adhesion at lower shear rates. Previous studies have demonstrated that transfection of αIIbβ3 or the β3 subunit alone on the surface of CHO cells leads to the formation of the αβ₃ receptor complex on the cell surface (42, 43). FACS analysis of CHO-Ib/IX-αIIbβ3 cells with an antibody that specifically recognizes αβ₃ demonstrated the presence of this receptor on the cell surface (data not shown). To investigate the contribution of αβ₃ to stationary cell adhesion, we pretreated CHO-Ib/IX-αIIbβ3 cells with a blocking antibody against the αβ₃ complex (LM609). As demonstrated in Fig. 2C, inhibition of integrin αβ₃ showed minimal effect on stationary adhesion relative to control levels over the range of shear conditions examined. Combining LM609 with a specific inhibitor of integrin αIIbβ3 (Aggrastat) resulted in a similar inhibition of stationary cell adhesion to that observed with c7E3 Fab alone (Fig. 2C), confirming the critical role for αIIbβ3 in mediating stationary cell adhesion under these experimental conditions. Taken together, these studies confirm that GPIb/IX and integrin αIIbβ3 are both necessary and sufficient for the formation of stable adhesion contacts on a vWF matrix under flow. Moreover, they raise the interesting possibility that the vWF-GPIb/IX interaction can transduce signals directly to regulate the ligand binding affinity of integrin αIIbβ3 independent of endogenous platelet stimuli.
To investigate the possibility of direct signaling between GPIb/IX and integrin αIIbβ3 in human platelets, we examined the importance of endogenous platelet agonists such as ADP and TXA2 in promoting vWF-induced integrin αIIbβ3 activation in platelet adhesion assays. Our initial focus was on the role of ADP in regulating vWF-induced activation of integrin αIIbβ3. In preliminary studies, we examined the effect of apyrase on vWF-induced integrin αIIbβ3 activation on the surface of platelets adherent to immobilized vWF under static conditions. Washed platelet static adhesion assays were performed in the presence of apyrase (16.5 units/ml), as described under “Experimental Procedures.” In these experiments the activation status of integrin αIIbβ3 was monitored using two assays as follows: first, by a direct method involving binding of PAC-1 (a well characterized activation-specific anti-αIIbβ3 mAb) to the surface of adherent platelets; and second, by monitoring platelet spreading (an indirect marker of integrin αIIbβ3 activation). In control studies, we demonstrated that platelets adherent to immobilized vWF at pH 6.5, in the presence of the platelet activation inhibitor, theophylline (5 mM) (Resting), retained their resting discoid shape, and did not bind PAC-1, indicating that under these experimental conditions there was minimal platelet activation. In contrast, platelets adherent to vWF at pH 7.4 (in the absence of theophylline) underwent rapid morphological conversion from flat discs to round dendritic forms and ultimately to fully spread cells exhibiting a “fried egg” appearance (Fig. 3, A and B (Control)). These cells stained strongly with PAC-1, confirming integrin αIIbβ3 activation under these experimental conditions. Adding apyrase (16.5 units/ml) to the adhesion assay did not inhibit the binding of PAC-1 to the surface of platelets or the ability of these cells to extend numerous filopodial extensions and spread, suggesting that vWF-induced integrin αIIbβ3 activation and cytoskeletal reorganization under static conditions can occur independent of ADP. Similarly, pretreating platelets with ADP receptor antagonists, AR-C69931MX (α-P2TAC) and A3P5PS (α-P2TPLC) or ATPaS (α-P2TAC and α-P2TPLC), did not prevent platelet spreading on vWF (data not shown). In control studies, we demonstrated that the concentrations of apyrase, ATPaS, and AR-C69931MX used in our experimental assays abolished platelet aggregation induced by 10 μM exogenous ADP, whereas A3P5PS was used at concentrations known to inhibit platelet shape change induced by exogenous ADP (17). To investigate the potential importance of TXA2 in promoting vWF-induced integrin αIIbβ3 activation, PAC-1 binding was assessed on aspirin-treated washed platelets. As demonstrated in Fig. 3A, pretreatment of platelets with aspirin alone, or aspirin in combination with apyrase, did not prevent integrin αIIbβ3 activation or the ability of platelets to spread on vWF. In control studies, we demonstrated that aspirin completely inhibited platelet aggregation.
induced by 1.5 mM arachidonic acid. Taken together, these studies suggest that the vWF-GPIb interaction can induce integrin αIIbβ3 activation and platelet cytoskeletal reorganization independent of endogenous ADP and TXA2.

The inability of apyrase to prevent platelet spreading on vWF was somewhat surprising given that a number of previous studies have suggested that platelet spreading on a fibrinogen matrix is ADP-dependent (44, 45). To investigate further whether ADP or TXA2 play a role in promoting platelet spreading on vWF, we examined the effects of apyrase and/or aspirin on the rate and extent of platelet spreading. As demonstrated in Fig. 3, B and C, apyrase-treated platelets spread at a slower rate than control or aspirin-treated platelets. Eliminating the cellular effects of ADP reduced the rate of platelet spreading by 54% at 7.5 min (p < 0.001), 48% at 15 min (p < 0.01), and by 39% at 30 min (p > 0.05). After 60 min, untreated and apyrase-treated platelets spread to a similar extent. The ability of platelets to spread fully after 60 min is not likely to be due to inactivation of apyrase, as adding additional fresh apyrase at 15, 30, and 45 min did not prevent platelet spreading (data not shown). Similarly, AR-C69931MX/A3P5PS treatment reduced the rate of platelet spreading at the early time points of 7.5, 15, and 30 min but had no effect at 60 min (data not shown). Combining aspirin with apyrase had no greater inhibitory effect on the rate of spreading than treating platelets with apyrase alone (Fig. 3C), excluding an important role for TXA2 in promoting vWF-induced cytoskeletal reorganization under static conditions.

To investigate the potential importance of ADP and TXA2 in promoting vWF-induced integrin αIIbβ3 activation under flow conditions, platelet adhesion studies were performed using an in vitro flow-based adhesion assay. In these experiments, anticoagulated whole blood was used in preference to washed platelets as efficient platelet tethering in this assay is dependent on the presence of red blood cells (24). The major advantage of whole blood is that it simulates more closely the platelet adhesion process as it occurs in vivo. In the following experiments, anticoagulated whole blood was perfused through HvWf-coated microcapillary tubes in the presence of apyrase alone or apyrase with aspirin. We initially examined the effect of these inhibitors on the number of platelets tethering to the vWF matrix at low (150 s⁻¹), intermediate (600 s⁻¹), or high shear rates (1800 s⁻¹). As demonstrated in Fig. 4A, apyrase alone, or in combination with aspirin, had no significant effect on the number of platelets tethering to vWF over the first 60 s of whole blood perfusion. It was not possible to examine accurately platelet adhesion beyond 60 s due to the formation of platelet thrombi, particularly at elevated shear rates. In control studies, using a sensitive single platelet detection assay, we confirmed that ADP (10 μM) was unable to induce platelet aggregation in apyrase-treated blood and that aspirin eliminated arachidonic acid-induced platelet aggregation (data not shown). As demonstrated in Fig. 4B, apyrase alone reduced the number of platelets forming stationary adhesion contacts at low shear (150 s⁻¹) by 57% at 5 s (p < 0.01), 41% at 30 s (p < 0.05), and by 30% after 60 s (p < 0.05). In contrast, at 600 and 1800 s⁻¹, apyrase had no significant inhibitory effect on the number of platelets forming stationary adhesion contacts. Similar findings were apparent using the ADP receptor antagonists ATPoS and AR-C69931MX/A3P5PS (data not shown). Under all shear conditions, combining aspirin with apyrase had no further inhibitory effect on irreversible platelet adhesion compared with apyrase alone (Fig. 4B), excluding an important role for the prostaglandin pathway under these experimental conditions. It should also be noted that under all shear conditions, platelets were able to spread fully in the presence or absence of apyrase and the other inhibitors (data not shown).

Having demonstrated that the vWF-GPIb/IX interaction was sufficient to induce integrin αIIbβ3 activation independent of ADP and TXA2, we proceeded to identify the key second messengers involved in this process. Initially, we focused on the role of calcium influx in promoting integrin αIIbβ3 activation, as the vWF-GPIb interaction has been proposed to induce transmembrane calcium influx (46, 47). Washed platelets were incubated with the membrane-impermeable calcium chelator (EGTA), prior to performing adhesion studies on immobilized HvWF. In all experiments 1 mM Mg²⁺ was added as a replacement divalent cation for calcium to enable ligand binding to integrin αIIbβ3. As demonstrated in Fig. 5A, chelating extracellular calcium did not prevent PAC-1 binding to the surface of platelets or the ability of platelets to spread fully on immobilized vWF, confirming that activation of integrin αIIbβ3 is not critically dependent on transmembrane calcium influx under these conditions. To investigate the potential importance of
calcium influx in promoting integrin α<sub>IIbβ<sub>3</sub></sub> activation under shear conditions, flow-based platelet adhesion studies were performed on untreated (Control) and EGTA-treated platelets (Fig. 5, B and D). In all studies, washed platelets were reconstituted with washed red blood cells to a hematocrit of 50% to enable performance of adhesion studies at high shear. In initial studies, we confirmed that pretreating platelets with EGTA had no inhibitory effect on the ability of platelets to tether or roll on vWF at 1800 s<sup>-1</sup> (data not shown). As demonstrated in Fig. 5B, chelating extracellular calcium with EGTA did not prevent platelet spreading under all conditions examined. Furthermore, EGTA treatment did not affect stable adhesion at low shear (150 s<sup>-1</sup>); however, stationary adhesion was reduced by 48.8 and 56.0% under higher shear (600 and 1800 s<sup>-1</sup>) (Fig. 5C). To investigate more directly whether chelating extracellular calcium was reducing integrin α<sub>IIbβ<sub>3</sub></sub> activation at higher shears or inhibiting some other calcium-dependent event required for stable platelet adhesion, PAC-1 binding studies were performed as described under “Experimental Procedures”. As demonstrated in Fig. 5D, chelating extracellular calcium consistently resulted in reduced PAC-1 binding to the surface of adherent platelets at all shear rates examined. Relative to control platelets, there was a 68.5, 63.4, 65.1, and 44.3% reduction in PAC-1 fluorescence at 0, 150, 600, and 1800 s<sup>-1</sup>, respectively (Fig. 5D). In control studies, we confirmed that the inhibitory effect of EGTA on stationary platelet adhesion and PAC-1 binding was not due to disruption of the integrin α<sub>IIbβ<sub>3</sub></sub> complex, as the complex-specific antibody, P2, bound EGTA-treated platelets to the same extent as control platelets (data not shown). Taken together, these studies demonstrate that calcium influx is not essential for vWF-induced integrin α<sub>IIbβ<sub>3</sub></sub> activation and irreversible platelet adhesion but is important for potentiating this process.

We have recently established that vWF binding to GPIb/IX is sufficient to induce the release of calcium from internal stores (24). To investigate the importance of calcium release in vWF-induced integrin α<sub>IIbβ<sub>3</sub></sub> activation, washed platelets were incubated with the membrane-permeable calcium chelators, EGTA-AM or BAPTA-AM. Under static conditions, EGTA-AM (Fig. 6A) or BAPTA-AM (data not shown) completely abolished PAC-1 binding and platelet spreading on immobilized vWF. In control studies, we demonstrated that platelets remained viable as pretreating these cells with ionophore A23187 restored PAC-1 binding and platelet spreading on immobilized vWF. Perfusion of EGTA-AM or BAPTA-AM-treated platelets through vWF-coated capillary tubes (1800 s<sup>-1</sup>) had no inhibitory effect on their ability to tether; however, >90% of the cells exhibited a rolling type adhesion and failed to form stationary adhesion contacts (Fig. 6B). Moreover, none of the adherent cells bound PAC-1 (Fig. 6C) or spread on the matrix (Fig. 6D). These studies demonstrate an absolute requirement for cytosolic calcium in vWF-induced integrin α<sub>IIbβ<sub>3</sub></sub> activation under static and flow conditions.

To examine more directly the relative contribution of calcium...
influx versus intracellular calcium mobilization to overall changes in cytosolic calcium, we established a quantitative dual dye ratiometric assay, as described under “Experimental Procedures.” Analysis of the cytosolic calcium concentration in resting platelets in suspension revealed that >95% of the cells exhibited a non-pulsatile cytosolic calcium level <50 nM, which was not significantly lowered by chelating extracellular calcium with EGTA (Fig. 7A, Resting, control versus EGTA/Mg). Following adhesion to immobilized vWf under static conditions, platelets exhibited a pulsatile calcium response that fluctuated between 150 and 800 nM (Fig. 7, A and B, Control). A typical pulsatile calcium response of a single platelet is demonstrated in Fig. 7B. After 10 min of adhesion, ~50% of the cells exhibited a pulsatile calcium response, and by 30 min all cells had undergone calcium oscillations and spread (data not shown). Chelation of extracellular calcium did not inhibit the ability of platelets to undergo a pulsatile calcium response, as demonstrated through single cell analysis (Fig. 7B, EGTA), but significantly reduced the cytosolic calcium range of individual cells down to 50–300 nM (Fig. 7, A and B, EGTA) (p < 0.0001).

Analysis of platelet calcium responses under shear conditions (Fig. 7, C and D, Resting versus Control) revealed an increase in cytosolic calcium levels in individual platelets adherent to the vWf matrix at each of the shear rates examined (150, 600, and 1800 s⁻¹). Overall, between 40 and 50% of adherent platelets exhibited elevated cytosolic calcium (data not shown) ranging between 100 and 800 nM at each shear rate examined (Fig. 7D). In the presence of EGTA, the percentage of platelets with an elevated cytosolic calcium concentration was reduced by ~50–70% (Fig. 7D, p < 0.0001), and the range of calcium concentrations decreased to 70–300 nM (Fig. 7D). In control studies we confirmed that the increase in vWf-induced cell fluorescence was due to increased mobilization of intracellular calcium, as pretreating platelets with DM-BAPTA (70 μM) completely abolished increases in cell fluorescence under static conditions (data not shown) and flow (Fig. 7, C and D, DM-BAPTA). Furthermore, stimulating platelets with thrombin induced a similar increase in cytosolic calcium to that previously reported (32, 33) (data not shown). Taken together, these studies demonstrate that platelet adhesion to vWf under static or flow conditions is associated with an increase in the cytosolic calcium level. This increase is due in part to the release of calcium from internal stores and is significantly potentiated by the influx of extracellular calcium.

Finally, we examined the importance of PKC in GPIb/IX-mediated activation of integrin αIIbβ3, as the activation of one or more conventional forms of PKC is postulated to be an important calcium-dependent signaling event utilized by soluble agonists to induce integrin αIIbβ3 activation (47). For these studies, static and flow-based platelet adhesion assays were performed in the presence of the PKC inhibitors, BIM or calphostin. As demonstrated in Fig. 8A, pretreating washed platelets with BIM resulted in a concentration-dependent (0.5–10 μM) inhibition of PAC-1 binding to the surface of platelets adherent to vWf under static conditions. Moreover, these same concentrations of BIM, which have previously been demonstrated to inhibit specifically PKC (48, 49), also prevented platelet spreading. Similar findings were apparent with calphostin-treated platelets (data not shown), suggesting an important role for PKC in GPIb/IX-mediated integrin αIIbβ3 activation. To investigate the importance of PKC in promoting irreversible platelet adhesion and spreading under flow conditions, washed platelets reconstituted with red blood cells were perfused through vWf-coated microcapillary tubes (1800 s⁻¹) in the presence of either BIM or calphostin. As demonstrated in Fig. 8B, pretreating platelets with BIM at concentrations as low as 0.2–0.5 μM inhibited the ability of platelets to form irreversible adhesion contacts and spread. Similarly, calphostin (1 μM) inhibited stable platelet adhesion by 85% and prevented platelet spreading (data not shown), confirming an important role for PKC in regulating the ligand binding function of integrin αIIbβ3 under both static and physiologically relevant flow conditions.

**DISCUSSION**

The adhesion of platelets to vWf immobilized on the subendothelium or on the surface of adherent platelets is critical for the initiation of platelet-vessel wall and platelet-platelet adhe-
Signaling between Platelet GPIb/IX and Integrin $\alpha_{IIb}\beta_3$

The presence of extracellular calcium (EGTA/Mg) was representative of three. These results are from one experiment. The results presented in A demonstrate the range of cytosolic calcium concentrations observed in the platelet population after 10 min of adhesion to vWF, and B demonstrates a typical calcium oscillation profile of individual platelets. Note: the thin solid line in B represents the mean cytosolic calcium concentration around which calcium levels fluctuate. C and D, in these experiments, dye-loaded platelets (3 x 10^5/ml) were reconstituted with red blood cells prior to perfusion through vWF-coated microcapillary tubes at the indicated shear rates. The images in C contrast the low calcium levels in resting platelets (Resting) with that observed following platelet adhesion to vWF (Control). Chelating extracellular calcium reduced the calcium level in individual cells (EGTA/Mg) but did not prevent calcium oscillations, whereas pretreating platelets with dimethyl-BAPTA completely abolished vWF-induced calcium increases (DM-BAPTA). D, quantitation of fluorescence ratios in individual cells was performed as described under “Experimental Procedures.” These results demonstrate the distribution of cytosolic calcium concentrations of platelets in suspension (Resting), following adhesion to vWF in the presence of extracellular calcium (Control) or in the presence of EGTA/Mg (EGTA/Mg) or dimethyl-BAPTA (DM-BAPTA). These results are from one experiment representative of three.

sion contacts, especially under conditions of rapid blood flow (6, 50–52). A key outstanding issue is the mechanism by which the vWF-GPIb interaction induces integrin $\alpha_{IIb}\beta_3$ activation, thereby promoting the formation of irreversible adhesion contacts. This study has examined several aspects of the adhesive and signaling functions of GPIb/IX and integrin $\alpha_{IIb}\beta_3$. First, by using transfected CHO cells we have demonstrated that these two receptors are both necessary and sufficient for the formation of irreversible cell adhesion contacts on a vWF matrix under flow. Second, vWF binding to GPIb/IX can induce integrin $\alpha_{IIb}\beta_3$ activation directly, independent of endogenous platelet stimuli, such as ADP and TXA2. Third, we have defined a key role for cytoplasmic calcium and PKC in linking the vWF-GPIb/IX interaction to integrin $\alpha_{IIb}\beta_3$ activation. vWF-induced calcium release from intracellular stores appears to be the primary signaling event initiating integrin $\alpha_{IIb}\beta_3$ activation, whereas transmembrane influx of extracellular calcium appears to be a secondary event participating in the activation process. Finally, increases in the cytosolic concentration of calcium, combined with the activation of PKC, appear to be important for vWF-induced integrin $\alpha_{IIb}\beta_3$ activation over the full range of shear forces experienced by platelets in vivo.

By performing studies on CHO cells co-transfected with GPIb/IX and integrin $\alpha_{IIb}\beta_3$ we have demonstrated that these receptors are sufficient to mediate irreversible cell adhesion under flow conditions, independent of other platelet adhesion receptors. Moreover, these studies suggest that the vWF-GPIb interaction can transduce signals to regulate the ligand binding function of integrin $\alpha_{IIb}\beta_3$, independent of endogenous platelet stimuli. This conclusion is supported by our platelet experiments in which eliminating the cellular effects of endogenous ADP and TXA2 did not prevent GPIb/IX-mediated integrin $\alpha_{IIb}\beta_3$ activation. Furthermore, two reports published during preparation of this manuscript (53, 54) have also demonstrated that vWF binding to GPIb/IX is sufficient to induce activation of integrin $\alpha_{IIb}\beta_3$ on the surface of CHO cells. Thus the CHO cell system appears to be a potentially powerful model to investigate the structural elements of the GPIb/IX receptor complex required for signal transduction and integrin $\alpha_{IIb}\beta_3$ activation. Furthermore, by enabling genetic manipulation of various signaling pathways, this system should assist in delineating the important signaling processes linking the vWF-GPIb/IX interaction to integrin $\alpha_{IIb}\beta_3$ activation.

Our studies raise the interesting possibility that there may be important differences in the mechanism of shear-induced activation of platelets in suspension compared with their activation on immobilized vWF. Shear-induced activation of platelets in suspension is abolished by enzymes that metabolize ADP (14, 46, 55), suggesting that the major pathway for integrin $\alpha_{IIb}\beta_3$ activation under these experimental conditions is not via direct signaling between GPIb/IX and integrin $\alpha_{IIb}\beta_3$ but is through an indirect pathway involving released ADP.
Whereas our studies suggest a potentially important role for ADP in accelerating integrin $\alpha_{IIb}\beta_3$ activation under static and low shear conditions, under intermediate and high shear conditions vWF-induced integrin $\alpha_{IIb}\beta_3$ activation occurs independent of ADP. Another major difference between shear-induced activation of platelets in suspension compared with their activation on immobilized vWF is the requirement for extracellular calcium. Chelating extracellular calcium abolishes platelet aggregation in a cone-plate viscometer suggesting an indispensable role for transmembrane calcium influx under these experimental conditions (46, 47). In contrast, our studies of surface-activated platelets suggest that the vWF-GPIb interaction can induce integrin $\alpha_{IIb}\beta_3$ activation independent of calcium influx.

A consistent finding in our calcium imaging studies was the importance of calcium influx in maintaining an elevated cytosolic calcium level under both static and shear conditions. In the absence of extracellular calcium, the proportion of cells exhibiting elevated calcium was approximately halved, thereby explaining the reduction in the level of integrin $\alpha_{IIb}\beta_3$ activation under these conditions. Presumably, this lower level of integrin $\alpha_{IIb}\beta_3$ activation is sufficient for irreversible platelet adhesion under static and low shear conditions (150 s$^{-1}$) but is less effective in sustaining stationary adhesion contacts at elevated shear rates. Our current working model for vWF-induced platelet activation under shear conditions is presented in Fig. 9, in which vWF engagement of GPIb/IX and integrin $\alpha_{IIb}\beta_3$ induces calcium release from internal stores. This increase in cytosolic calcium may in turn induce transmembrane calcium influx through store-mediated calcium entry mechanisms. Once elevated, cytosolic calcium promotes the activation of a range of signaling enzymes including PKC (54), leading to the phosphorylation of one or more protein substrates involved in integrin $\alpha_{IIb}\beta_3$ activation. There are of course many aspects of this model that remain to be delineated, including the signaling processes utilized by GPIb/IX and integrin $\alpha_{IIb}\beta_3$ to induce calcium mobilization, and the relative contribution of GPIb/IX and integrin $\alpha_{IIb}\beta_3$-dependent signaling processes to overall changes in cytosolic calcium.

Our findings that calcium plays a key second messenger role in promoting integrin $\alpha_{IIb}\beta_3$ activation and irreversible platelet adhesion on vWF under flow contradicts a recent report by Kuwahara et al. (20). This latter study has suggested that the initial phase of stable platelet adhesion is not dependent on changes in the cytosolic concentration of calcium, whereas subsequent platelet aggregation and thrombus growth is calcium-dependent. The reason for this apparent discrepancy is not clear but may reflect methodological differences between the two studies. In our study we have defined stationary adhesion as cell movement of less than one cell diameter over a 10-s period, whereas the study of Kuwahara et al. (20) examined cell adhesion over a 1-s time interval. In our experience, many platelets remain stationary for periods greater than 1 s during surface translocation, due to the “stop-start” nature of the platelet translocation process (6). It is therefore possible that extending the period of individual platelet analysis in the study of Kuwahara et al. (20) would have resulted in a higher percentage of tethered platelets being scored as translocating platelets following chelation of intracellular calcium. Other
The ability of the vWF-GPIb interaction to induce integrin αIIbβ3 activation independent of endogenous platelet stimuli, such as ADP and TXA2, has potentially important clinical implications. Currently, the two major classes of orally active anti-platelet drugs used for the prevention of arterial thrombosis are aspirin and the thienopyridine class of ADP receptor antagonists. Our studies suggest that neither of these anti-platelet agents, either alone or in combination, are likely to prevent completely platelet activation following adhesion to immobilized vWF. Although our studies do not exclude a potentially important role for ADP and TXA2 in promoting subsequent platelet-platelet aggregation and thrombus formation under flow, they nonetheless raise the interesting possibility that inhibitors of GPIb/IX-mediated integrin αIIbβ3 activation may have important synergistic effects with other antithrombotic agents.

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