Bidirectional integrin αIIbβ3 signalling regulating platelet adhesion under flow: contribution of protein kinase C

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Platelet adhesion on von Willebrand factor (vWf) requires the co-ordinated adhesive function of glycoprotein Ib/V/IX and integrin αIIbβ3. Recent evidence [Nesbitt, Kulkarni, Giuliano, Gonclaves, Dopheide, Yap, Harper, Salem and Jackson (2002) J. Biol. Chem. **277**, 2965–2972] suggests that outside-in signals from both receptors play important roles in regulating platelet-adhesion dynamics under flow. In the present study, we have examined the mechanisms utilized by protein kinase C (PKC) to promote irreversible platelet adhesion on vWf. We demonstrate that PKC is primarily activated downstream of integrin αIIbβ3, not glycoprotein Ib, during platelet adhesion on vWf. This integrin αIIbβ3-dependent PKC activation establishes a positive-feedback loop that promotes further integrin αIIbβ3 activation, calcium mobilization and firm platelet adhesion. This feedback loop appears to be most relevant at relatively low cytosolic calcium concentrations (mean $\Delta[Ca^{2+}]_i \sim 100$ nM) as artificially elevating calcium (mean $\Delta[Ca^{2+}]_i > 500$ nM) induces integrin αIIbβ3 activation and irreversible platelet adhesion independent of PKC. Our studies demonstrate the existence of a complex signalling relationship operating between PKC, cytosolic calcium and integrin αIIbβ3 that serves to regulate platelet-adhesion dynamics under flow. Furthermore, we have established the existence of PKC-dependent and -independent pathways regulating integrin αIIbβ3 activation and stable platelet adhesion on vWf.

Key words: flow, platelet adhesion, protein kinase C.

INTRODUCTION

Platelet adhesion and aggregation at sites of vascular injury are essential for the cessation of bleeding in traumatized vessels and also for the development of pathological thrombi, precipitating diseases such as unstable angina, acute myocardial infarction and stroke. Platelet adhesion is a complex process involving the synergistic contribution of multiple receptor–ligand interactions. Under high shear conditions, such as those prevailing in the microcirculation or in areas of arterial narrowing, platelet adhesion to the injured vessel wall is initiated by the interaction between glycoproteins (GPs) Ib/V/IX and von Willebrand factor (vWf) [1–3]. This adhesive interaction supports both platelet tethering and translocation (rolling), whereas a second adhesive event, mediated by vWf engagement of integrin αIIbβ3 and/or collagen binding to integrin $\alpha2\beta1$ or GPVI, promotes platelet arrest. Once stably adherent, platelets provide a highly reactive surface for the recruitment of additional platelets, partially via the surface expression of vWf [4]. Thus under high shear conditions, vWf plays a major role in supporting both primary platelet adhesion and thrombus growth.

Recent analysis of thrombus formation *in vivo*, using intravital microscopy, has highlighted the dynamic nature of platelet adhesion and aggregation at sites of vascular injury. In particular, these studies have demonstrated that in the arterial circulation, platelets typically exhibit a variable period of surface translocation before forming firm adhesion contacts [4]. Factors regulating the transition from surface translocation to firm adhesion remain incompletely understood, although studies on a purified vWf substrate suggest important roles for calcium and protein kinase C (PKC) in this process. In particular, GPIb and integrin αIIbβ3-derived calcium signals appear to play important co-operative roles in promoting stable platelet adhesion on vWf [5]. We have recently demonstrated [5] that GPIb engagement of vWf elicits transient calcium spikes that initiate reversible integrin αIIbβ3 activation and platelet arrest, whereas subsequent integrin αIIbβ3 engagement of vWf induces sustained cytosolic calcium oscillations that are sufficient to maintain integrin αIIbβ3 activation, irreversible platelet adhesion and spreading. The precise mechanism by which PKC induces integrin αIIbβ3 activation under flow conditions has been less clearly defined, although one possibility is that PKC primarily signals downstream of GPIb, phosphorylating one or more substrates involved in affinity regulation of integrin αIIbβ3 [6–8].

To gain further insight into the role of PKC in promoting integrin αIIbβ3 activation and stable platelet adhesion on vWf, we have utilized a confocal-based flow assay *in vitro* that enables real-time assessment of cytosolic calcium flux in translocating platelets. These studies have defined an important role for PKC in integrin αIIbβ3 bidirectional signalling necessary for efficient platelet adhesion under flow. (The term bidirectional signalling refers to both inside-out and outside-in integrin αIIbβ3 signalling.) We demonstrate a major role for integrin αIIbβ3 in regulating PKC activation during platelet adhesion on vWf. Furthermore, we have established that this integrin αIIbβ3-dependent PKC activation participates in a positive-feedback loop to promote further integrin αIIbβ3 activation and calcium mobilization. By potentiating integrin αIIbβ3 activation, and indirectly calcium flux, PKC appears to play a pivotal role in regulating the transition from reversible to irreversible platelet adhesion.

Abbreviations used: AM, acetoxymethyl ester; AS, aggrastat; BAPTA/AM, bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetrakis(AM); DIC, differential interference contrast; DM, dimethyl; GP, glycoprotein; Hct, haematocrit; IP_3, D-*myo*-inositol 1,4,5-trisphosphate; mAb, monoclonal antibody; NP, *o*-nitrophenyl; PKC, protein kinase C; RBC, red blood cell; Tg, thapsigargin; vWf, von Willebrand factor.

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MATERIALS AND METHODS

Materials

Bisindolylmaleimide I, and the calcium chelators, EGTA–acetoxymethyl ester (AM) and bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetrakis(AM) (BAPTA/AM) were obtained from Calbiochem (San Diego, CA, U.S.A.). *o*-Nitrophenyl (NP)-EGTA, 5,5′-dimethyl-BAPTA/AM (DM-BAPTA/AM), Oregon Green 488 BAPTA-1/AM and Fura-Red/AM were purchased from Molecular Probes (Eugene, OR, U.S.A.). Apyrase was a gift from Dr Francois Lanza (Institut National de la Sante et de la Recherche Medicale Unite 311, Strasburg Cedex, France) and it was purified from potatoes by the method of Molnar and Lonard [9]. Human vWf (HvWf) was purified to homogeneity from plasma cryoprecipitate as described by Montgomery and Zimmerman [10]. All other reagents were obtained from sources described previously [11–13].

Antibodies

PAC-1, an activation-specific monoclonal antibody (mAb) directed at the activated form of human integrin αIIbβ3, was obtained from Beckton Dickinson (Dalmore Drive, Scoresby, Victoria, Australia) and FITC-conjugated anti-mouse IgM antibody (α-IgM) was from Southern Biotechnology Associates (Birmingham, AL, U.S.A.).

Washed platelet preparations

Human whole blood (15 mM trisodium citrate, pH 7.4) was collected from healthy volunteers who had not received any anti-platelet medication in the 2 weeks preceding sample collection. Washed platelets were prepared as described previously [12] and resuspended in Tyrode's buffer (10 mM Hepes/12 mM $NaHCO_3$/137 mM NaCl/2.7 mM KCl/5 mM glucose; pH 7.4) containing 1 mM $CaCl_2$. Autologous red blood cells (RBCs) were prepared as described previously [14]. For flow studies *in vitro*, washed platelets were reconstituted with autologous packed RBCs [50 % (v/v) haematocrit (Hct)] in the presence of 0.4 unit/ml apyrase (ADPase activity) and 1 unit/ml hirudin.

Pleckstrin phosphorylation assays

Platelets were resuspended in phosphate-free Tyrode buffer, supplemented with 10 mM theophylline and loaded for 2 h with 0.5 μCi of [^{32}P]P_i. They were then washed three times to remove any unincorporated radioactive label. [^{32}P]P_i-labelled platelets were then incubated with the indicated concentrations of the mAb c7E3 Fab, PKC inhibitors or apyrase, before exposure to the soluble agonists PMA or thrombin for 5 min as indicated, or to a purified vWf matrix for 60 min as described below under static adhesion assays. Whole platelet lysates were prepared by directly lysing the platelets in reducing buffer (supplemented with 2 mM EDTA), and subjected to SDS/PAGE and autoradiography for detection of [^{32}P]P_i incorporation.

Flow-based adhesion assays *in vitro*

Flow assays were performed using glass microcapillary tubes (Microslides, Vitro Dynamics, NJ, U.S.A.) coated with HvWf (100 μg/ml). Microcapillary tubes were coated overnight at 4 °C before blocking with 10 % (v/v) heat-inactivated human serum (containing 50 μg/ml PMSF). Washed platelets reconstituted with RBCs (50 % Hct) and Tyrode's buffer (containing 1 mM $CaCl_2$/1 mM $MgCl_2$ or 2 mM EGTA/1 mM $MgCl_2$) were perfused through HvWf-coated microcapillary tubes at a shear rate of 1800 s^{-1} [13]. Platelet adhesion was visualized in real time using differential interference contrast (DIC) microscopy (DMIRB Leica microscope; Leica, Lasertechnik, Heidelberg, Germany) and video-recorded for off-line analysis. For platelet calcium studies, washed platelets were loaded with 1 μM Oregon Green BAPTA-1 and 1.25 μM Fura-Red as described by Yap et al. [14]. In the indicated experiments, washed platelets were incubated with 100 μM DM-BAPTA/AM or 0.2–0.5 μM GF109203X for 30 min at 37 °C, before reconstitution with RBCs. In experiments examining the effect of transient calcium release on platelet adhesion, Oregon Green BAPTA-1/Fura Red-loaded platelets were incubated for a further 30 min at 37 °C with the UV-sensitive caged calcium chelator, NP-EGTA (10 μM). These platelets were perfused through vWf-coated microcapillary tubes as described above, and the release of caged calcium was induced by transient exposure to near-UV light (300–400 nm) generated by a 100 W Hg lamp for 0.6 s as described by Nesbitt et al. [5].

Analysis of cell-adhesion contacts under flow

In all experiments, any cell forming an adhesion contact with immobilized HvWf for longer than 40 ms was recorded as a tethered cell. Translocation was defined as platelet movement more than 1 cell diameter from the point of initial attachment, and stationary adhesion was defined as cells not moving more than 1 cell diameter over a 10 s period. The number of irreversibly adherent platelets was quantified from five random fields of view and the results presented represent the means $\pm$ S.E.M. from four independent experiments.

Static adhesion assays

Static adhesion assays were performed using a modified method of Yuan et al. [12]. Briefly, glass coverslips were coated with 10 μg/ml HvWf overnight at 4 °C. Washed platelets were allowed to adhere and spread on the matrix for 30 min under stationary conditions in Tyrode's buffer, supplemented with 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 μg/ml PAC-1. At the end of the incubation period, adherent platelets were fixed for 1 h in 1 % (w/v) paraformaldehyde, stained for 30 min with FITC-conjugated secondary antibody and then mounted in Permafluor. Adherent platelets were then imaged by confocal microscopy as described above. For static adhesion assays involving calcium measurement, platelets were loaded with the calcium indicator dyes Oregon Green 488 BAPTA-1/AM (1 μM) and Fura-Red/AM (1.25 μM), for 30 min at 37 °C, and monitored by confocal microscopy as described above.

Immunofluorescence studies

In flow assays, washed platelets reconstituted with RBCs were perfused through microcapillary tubes for 5 min in the presence of 1 μg/ml PAC-1. Adherent platelets were fixed, incubated with an FITC-conjugated secondary antibody and visualized using confocal microscopy (Leica TCS SP; Leica, Lasertechnik, Heidelberg, Germany).

FACS analysis

Washed platelets were resuspended in Tyrode's buffer containing 1 mM $CaCl_2$ and 1 μg/ml PAC-1, before stimulation for up to 5 min with the indicated soluble agonists. At the end of the incubation period, platelets were fixed with 1 % paraformaldehyde for 1 h. Platelets were washed twice with Tyrode's

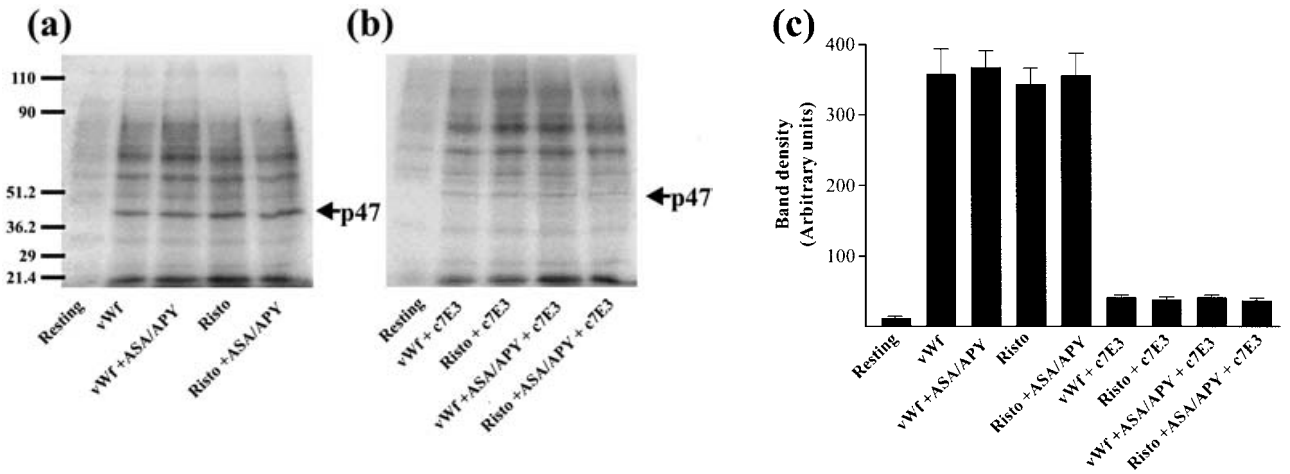

Figure 1 PKC activation and pleckstrin phosphorylation during platelet adhesion to vWf

(**a**, **b**) Washed platelets loaded with [^{32}P]P_i were allowed to spread under static conditions on vWf-coated coverslips for 60 min as described in the Materials and methods section, in the presence of ristocetin (Risto) and/or c7E3 where indicated. Treatment of platelets with aspirin (ASA) or apyrase (APY) (**a**) does not significantly affect pleckstrin phosphorylation (labelled p47) on vWf. (**b**) A significant decrease in the level of pleckstrin phosphorylation in the presence of c7E3 Fab. (**c**) Densitometric analysis was performed on p47 bands to quantify the level of pleckstrin phosphorylation; the histogram represents means ± S.E.M. ($n = 3$). Results are represented as band density in arbitrary units.

buffer to remove excess PAC-1 and then incubated for 30 min with FITC-conjugated secondary antibody. Again the platelets were washed twice with Tyrode's buffer and finally resuspended in PBS and 1 % BSA before being subjected to FACS analysis using a Beckton Dickinson FACScalibur™ flow cytometer (BD Immunocytometry Systems, San Jose, CA, U.S.A.).

RESULTS

GPIb and integrin αIIbβ3 regulation of PKC

Previous studies have demonstrated that PKC activation in vWf-stimulated platelets primarily occurs downstream of GPIb and is not inhibited significantly by integrin αIIbβ3 antagonists [6,7]. However, these studies were performed in suspension-based assays in which soluble vWf was induced to bind GPIb by the application of high shear, or alternatively, by the use of an artificial modulator [6,7]. To investigate the relative roles of GPIb and integrin αIIbβ3 in promoting PKC activation under static adhesion conditions, [^{32}P]P_i-loaded platelets were applied to a HvWf matrix in the presence or absence of the integrin αIIbβ3 antagonist, c7E3 Fab. In these studies, PKC activation was monitored indirectly by examining phosphorylation of the PKC-specific substrate, pleckstrin, as described in the Materials and methods section. As shown in Figures 1(a) and 1(c), in the absence of c7E3 Fab, pleckstrin phosphorylation was readily observed in platelets spreading on vWf (means ± S.E.M. = 369.4 ± 22.74 arbitrary units). Increasing the affinity of vWf for GPIb by adding ristocetin to the adhesion assays did not show any significant effect on pleckstrin phosphorylation (means ± S.E.M. = 376.8 ± 7.09 arbitrary units), whereas pretreating platelets with c7E3 Fab inhibited pleckstrin phosphorylation by approx. 87 % (Figures 1b and 1c; means ± S.E.M. = 46.89 ± 12.47 arbitrary units), even when ristocetin was present in the assay (means ± S.E.M. = 47.44 ± 18.67 arbitrary units). The ability of integrin αIIbβ3 to promote PKC activation was not dependent on the release of endogenous agonists, such as ADP or thromboxane A_2, as similar levels of pleckstrin phosphorylation were also observed in aspirin- and apyrase-treated platelets (Figures 1a–1c; vWf+aspirin/apyrase). These studies suggest a major role for integrin αIIbβ3 in regulating PKC activation during platelet adhesion on vWf.

Role of PKC in promoting calcium mobilization and platelet adhesion under flow

We have demonstrated previously an important role for PKC in promoting integrin αIIbβ3 activation and firm platelet adhesion on a vWf matrix under static and flow conditions [14]. Under flow (1800 s^{-1}), we have consistently noted that up to 20–30 % of platelets treated with the PKC inhibitor, GF109203X [15], retained their capacity to form stationary adhesion contacts with the vWf matrix and bind the activation-specific antibody directed against integrin αIIbβ3, PAC-1 (Figures 2a and 2b) [14]. This partial inhibitory effect was unlikely to be due to incomplete inhibition of PKC, as GF109203X completely inhibited pleckstrin phosphorylation induced by multiple agonists, including thrombin and PMA (Figures 2c and 2d). Furthermore, two other PKC inhibitors, calphostin C and RO31–8220, also abolished integrin αIIbβ3 activation and platelet spreading under static conditions, but only partially inhibited stationary adhesion and integrin αIIbβ3 activation under flow (results not shown).

To gain further insight into the mechanism by which PKC regulates platelet adhesion under flow, we examined the effect of PKC inhibition on cytosolic calcium flux. We have recently demonstrated a strong correlation between cytosolic calcium levels and the translocation behaviour of platelets under flow [5]. Pretreating platelets with 500 nM GF109203X had no effect on the basal cytosolic calcium levels in resting platelets (Figure 3a). However, this inhibitor significantly reduced the proportion of translocating platelets undergoing a sustained oscillatory cytosolic calcium response, leading to a decrease in the mean calcium level (from approx. 100 to 60 nM; Figures 3a and 3b). The calculated calcium flux is designated $\Delta[Ca^{2+}]_i$ to indicate that all calcium concentration estimates are relative to a zero point set by DM-BAPTA calcium chelation. These data are represented in the present study as $\Delta[Ca^{2+}]_i$ versus time

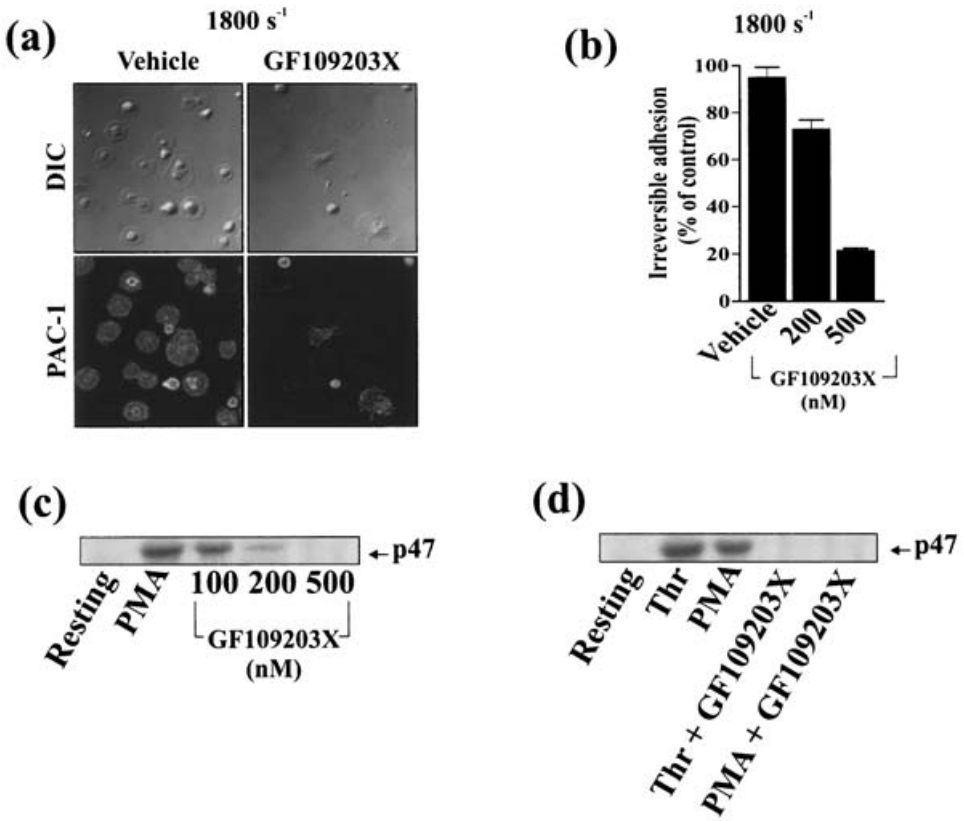

Figure 2 Effect of the PKC inhibitor GF109203X on pleckstrin phosphorylation and platelet adhesion

(**a**) Platelets were treated with vehicle (Me_2SO) or 500 nM GF109203X before perfusion over a purified vWf matrix at 1800 s^{-1} in the presence of PAC-1 mAb. Non-adherent platelets were washed away before fixation with 3.8 % paraformaldehyde and stably adherent platelets were imaged by DIC and fluorescence microscopy. The DIC images show platelets that were capable of forming stationary adhesion contacts on the vWf matrix under flow and the fluorescence images show PAC-1 binding to the adherent platelets. (**b**) A decrease in the level of irreversible adhesion on vWf at 1800 s^{-1} in response to 200 and 500 nM GF109203X (means $\pm$ S.E.M., $n = 4$). (**c**, **d**) Washed platelets loaded with [^{32}P]P_i were incubated with the indicated concentrations of GF109203X before exposure for 60 min to 200 nM PMA (**c**) or 1 unit/ml thrombin (Thr) as indicated (**d**). Whole platelet lysates were prepared as described in the Materials and methods section, and subjected to SDS/PAGE and autoradiography. (**c**) Dose-dependent effects of GF109203X on pleckstrin phosphorylation (labelled p47) induced by PMA. (**d**) Inhibition of PMA and Thr-induced pleckstrin phosphorylation by 500 nM GF109203X is shown.

histograms (dotted lines), plotted together with displacement versus time histograms (solid lines) to demonstrate how the translocation of single representative platelets is affected by their cytosolic calcium oscillations. Alternatively, the bar charts show the average calcium concentrations recorded in a population of platelets undergoing non-synchronized oscillatory calcium responses during adhesion to immobilized matrices. Detailed analysis of the calcium responses in individual platelets revealed that inhibiting PKC significantly increased the proportion of rapidly translocating platelets (11.4–70 %), exhibiting low cytosolic calcium levels ($\Delta[Ca^{2+}]_i < 20$ nM; Figure 3c). This increase in rapidly translocating platelets was associated with a proportional decrease in the percentage of platelets exhibiting intermediate ($\Delta[Ca^{2+}]_i = 20$–65 nM) and high calcium levels ($\Delta[Ca^{2+}]_i$ approaching 1200 nM), resulting in a decrease in the percentage of stop–start translocating platelets (58.7–12.6 %) and firmly adherent platelets (29.9–17.4 %; Figures 3a and 3c). Although most GF109203X-treated platelets failed to form stationary adhesion contacts and showed very low cytosolic calcium levels (Figure 3d) of the platelets forming firm adhesion contacts, all cells displayed high oscillatory calcium levels (peak levels > 800 nM; Figure 3e). These studies raise the possibility that PKC regulation of platelet adhesion under flow, is at least partially due to an effect on cytosolic calcium levels. Furthermore, these studies also suggest that beyond a threshold calcium concentration ($\Delta[Ca^{2+}]_i > 100$ nM), PKC activation is no longer absolutely necessary for integrin αIIbβ3 activation.

The level of cytosolic calcium dictates the PKC requirement for firm platelet adhesion under flow

To investigate the possibility that high levels of cytosolic calcium induce integrin αIIbβ3 activation independent of PKC, the effects of artificially elevating cytosolic calcium levels on platelet adhesion was examined. In initial studies, cytosolic calcium levels were elevated by treating translocating platelets with 100 nM thapsigargin (Tg) (an inhibitor of the sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase), thereby dramatically enhancing the calcium response in all cells adhering to vWf (mean population calcium levels approx. 500 nM). This increase in cytosolic calcium was associated with all the translocating cells (100 %) forming stationary adhesion contacts with the vWf matrix (Figure 4a, vehicle versus Tg). Pretreating platelets with 500 nM GF109203X had minimal inhibitory effect on the platelet calcium response and on the ability of Tg-treated platelets to form stationary adhesion contacts with the vWf surface (Figure 4a). Identical results were obtained with platelets pretreated with

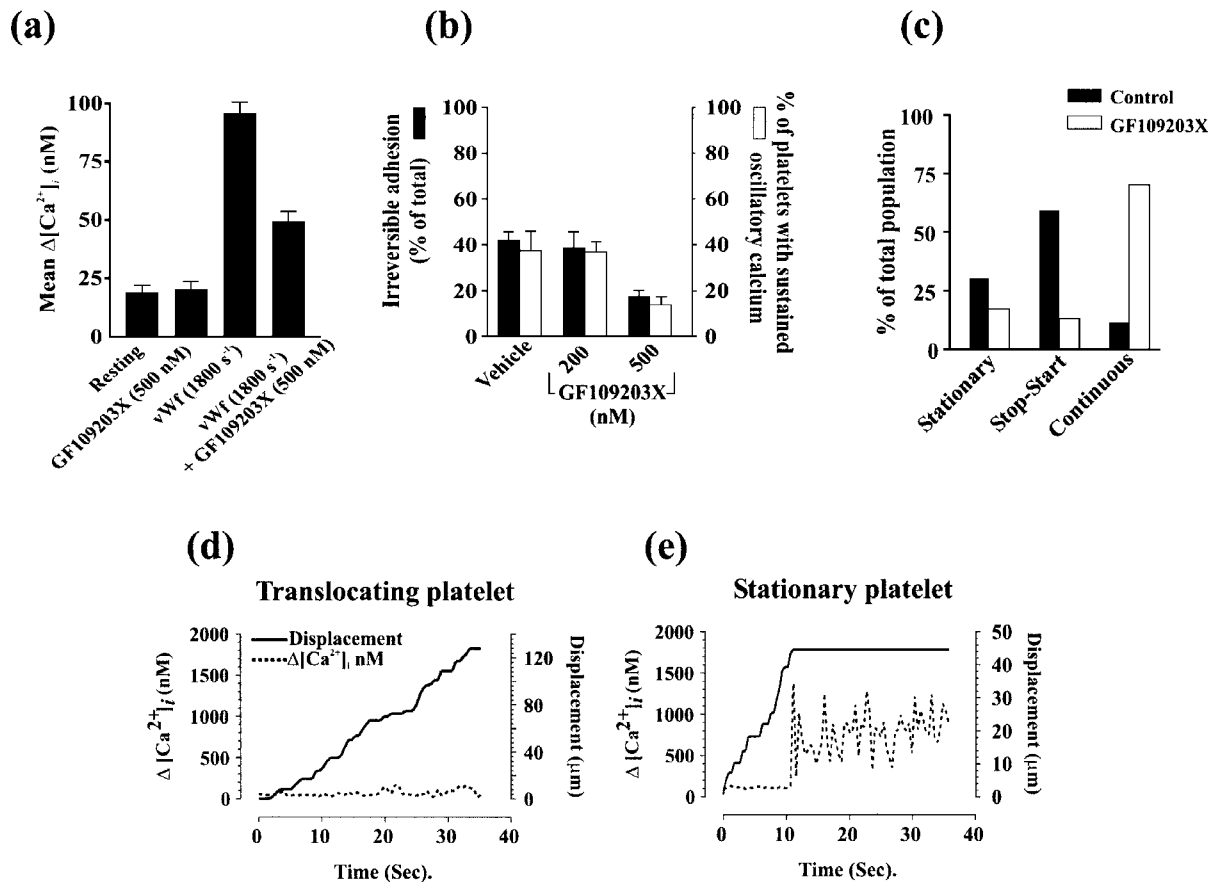

Figure 3 Effect of the PKC inhibitor GF109203X on platelet adhesion and calcium mobilization on immobilized vWf

Washed platelets (1×10^7 cells/ml) reconstituted with RBCs (50 % Hct) were treated with vehicle (0.25 % Me_2SO) or GF109203X (200 or 500 nM) before perfusion through vWf-coated microcapillary tubes at 1800 s^{-1}. (**a**) This Figure illustrates mean cytosolic calcium concentrations around which calcium levels oscillate in a population of platelets undergoing non-synchronized calcium flux in the presence or absence of GF109203X, either in suspension in Tyrode's buffer (bars 1 and 2) or during adhesion to vWf under flow at 1800 s^{-1} (bars 3 and 4). The data presented are from one experiment representative of five individual experiments. (**b**) In a dose-dependent manner, GF109203X reduces the number of platelets undergoing sustained calcium oscillations (right axis) and also the level of irreversible adhesion (left axis) on vWf under flow (means $\pm$ S.E.M., $n = 4$). (**c**) The proportion of vehicle (Me_2SO) and GF109203X-treated platelets (500 nM) displaying stationary adhesion, continuous or stop–start translocation behaviours are shown. (**d**, **e**) Typical calcium profiles of single platelets adherent to vWf either translocating rapidly across the vWf matrix (**d**), or forming stationary adhesion contacts (**e**), in the presence of GF109203X. The solid line shows platelet displacement as a function of time whereas the dotted line demonstrates cytosolic calcium flux profiles in these cells.

calphostin C and RO31–8220 (results not shown). This increase in stationary adhesion was probably due to the activation of integrin αIIbβ3, as it was completely inhibited by pretreating platelets with the integrin αIIbβ3 antagonist, c7E3 Fab (results not shown). Furthermore, all platelets forming stationary adhesion contacts with the vWf matrix bound PAC-1 (results not shown).

Further evidence that high levels of cytosolic calcium can induce integrin αIIbβ3 activation independent of PKC was derived from studies utilizing the caged calcium compound, NP-EGTA. Brief exposure of NP-EGTA-loaded platelets to UV light leads to the transient release of caged cytosolic calcium, resulting in a rapid increase in cytosolic calcium and reversible development of integrin αIIbβ3-dependent adhesion contacts [5]. As shown in Figure 4(b), initiation of transient calcium spikes (peak calcium levels approaching 2000 nM) in NP-EGTA-treated platelets on exposure to UV light, resulted in all cells forming transient stationary adhesion contacts with the vWf matrix. Moreover, NP-EGTA-loaded platelets pretreated with GF109203X were also found to form transient stationary adhesion contacts with the vWf matrix on calcium uncaging, despite PKC inhibition (Figure 4c). In control studies, we confirmed that stationary adhesion under these conditions was mediated by integrin αIIbβ3, as it was completely abolished by pretreating platelets with the αIIbβ3 antagonists, c7E3 Fab (Figure 4d) or aggrastat (AS; results not shown). These studies provide further evidence that the induction of elevated cytosolic calcium levels (mean cytosolic calcium levels >500 nM) promotes integrin αIIbβ3 activation independent of PKC.

PKC potentiates calcium mobilization via an integrin αIIbβ3-dependent mechanism

The finding that PKC inhibitors decrease cytosolic calcium flux during platelet translocation on immobilized vWf was somewhat unexpected in light of previous findings that PKC activators do not promote cytosolic calcium flux [8]. In fact, phorbol esters significantly increase the decay rate of calcium transients in activated platelets [16]. To investigate further the relationship among PKC activation, integrin αIIbβ3 activation and calcium mobilization, we compared the effects of PMA on platelet calcium transients in suspension-based assays with

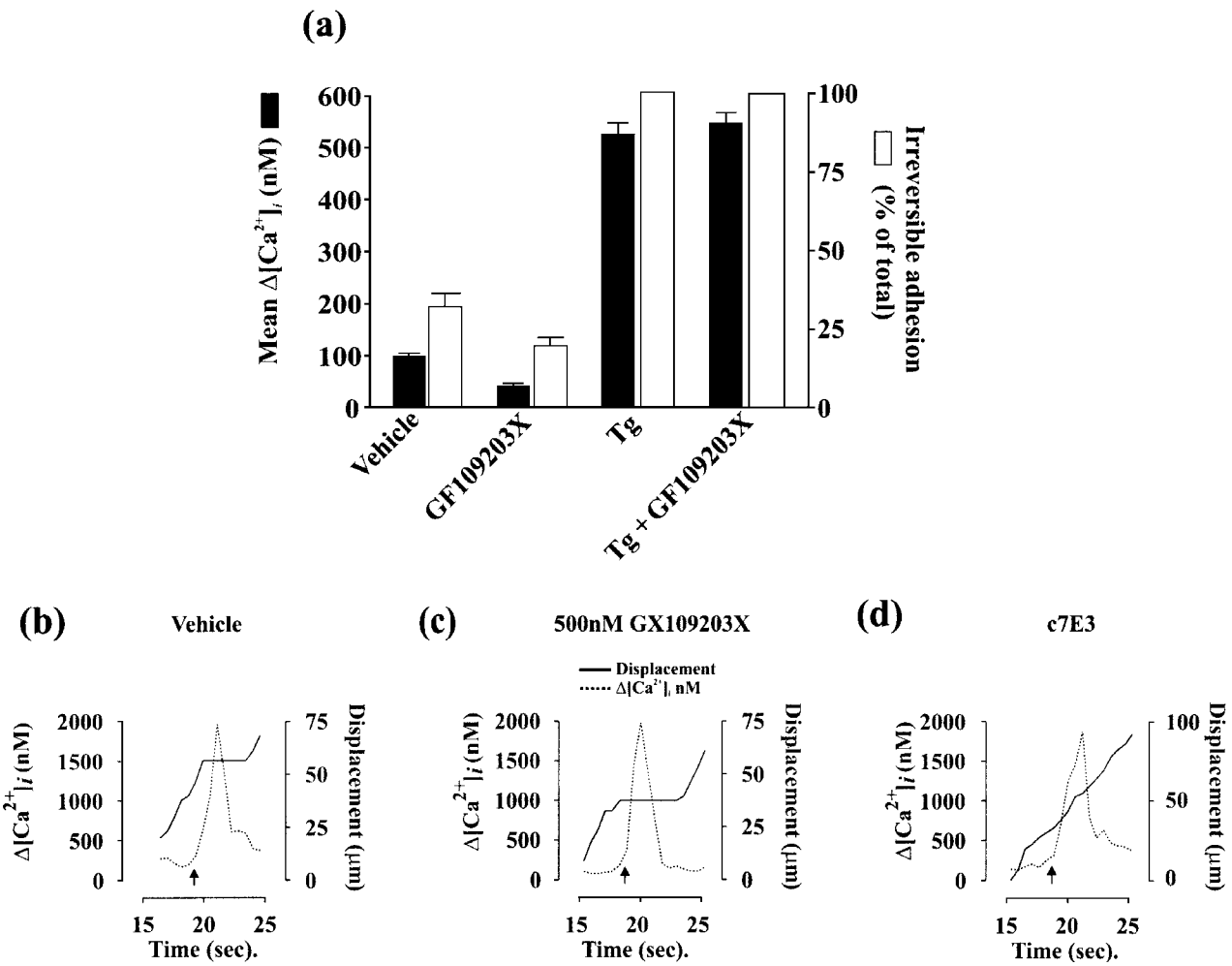

Figure 4 Effect of artificially elevating cytosolic calcium on platelet translocation and integrin αIIbβ3 activation, and the ability of GF109203X to inhibit platelet adhesion on vWf

Washed platelets (1×10^7 cells/ml) reconstituted with RBCs (50 % Hct) were treated with vehicle (0.25 % Me_2SO) or 500 nM GF109203X, before the addition of 100 nM Tg. Platelets were perfused immediately through vWf-coated microcapillary tubes at 1800 s^{-1}. (**a**) Mean cytosolic calcium concentrations expressed by the adherent platelet population (left axis) and the number of irreversibly adherent platelets (right axis) in both the presence or absence of GF109203X (means $\pm$ S.E.M., $n = 4$). (**b**–**d**) Platelets loaded with 10 μM of the caged calcium compound NP-EGTA were reconstituted at (150×10^9 l^{-1}) in 50 % RBCs and perfused at 1800 s^{-1} via vWf-coated microcapillary tubes. The platelets were allowed to translocate for approx. 18 s before being exposed to a near-UV light source for 0.6 s (marked by the arrow). (**b**) Displacement versus time graph of a representative control NP-EGTA-loaded platelet exposed to UV light. (**c**, **d**) Displacement versus time graphs of a representative NP-EGTA-loaded platelet treated with 500 nM GF109203X or c7E3 Fab respectively, before exposure to near-UV light.

those obtained from adhesion assays on vWf. For these studies, platelets in suspension were imaged by confocal microscopy and ratiometric calcium levels were determined on individual platelets while in suspension. Consistent with previous studies [8,16], stimulating platelets in suspension with 200 nM PMA did not result in a significant increase in the mean cytosolic calcium level (Figure 5a). However, it induced an approx. 3-fold increase in the level of PAC-1 binding to the surface of these cells (Figure 5b), suggesting that direct PKC stimulation can induce a low level of integrin αIIbβ3 activation in the absence of a detectable increase in calcium levels. It should be noted that the rate of increase and magnitude of PAC-1 binding induced by PMA under these experimental conditions was low relative to that induced by ionophore A23187 or thrombin (Figures 5a and 5b).

In contrast with suspension-based assays, stimulating vWf-adherent platelets under static conditions with PMA, resulted in an increase in the overall level of platelet adhesion and spreading on this matrix, and this coincided with an enhanced cytosolic calcium response throughout the entire platelet population adherent to vWf (Figures 5c and 5d). Pretreating platelets with AS abolished stable adhesion and calcium flux in control and PMA-stimulated platelets, suggesting that the potentiating effects of PKC on cytosolic calcium flux occurs indirectly, as a result of the up-regulation of the adhesive and signalling function of integrin αIIbβ3 (Figures 5c and 5d). The effect of PKC inhibition was specific to integrin αIIbβ3-dependent calcium flux, as they had no effect on GPIb-derived calcium signals (Figure 6).

To examine the effect of direct PKC activation on cytosolic calcium mobilization and firm platelet adhesion in a shear field, platelets were perfused through vWf-coated microcapillary tubes in the presence or absence of PMA. In control studies, 37 % of washed platelets tethering to the vWf matrix formed stationary adhesion contacts (Figure 7a). Addition of PMA resulted in a dramatic increase in the proportion of cells forming firm adhesion contacts with the vWf matrix (> 98 %; Figure 7a). Single cell analysis of the cytosolic calcium levels in vehicle-treated

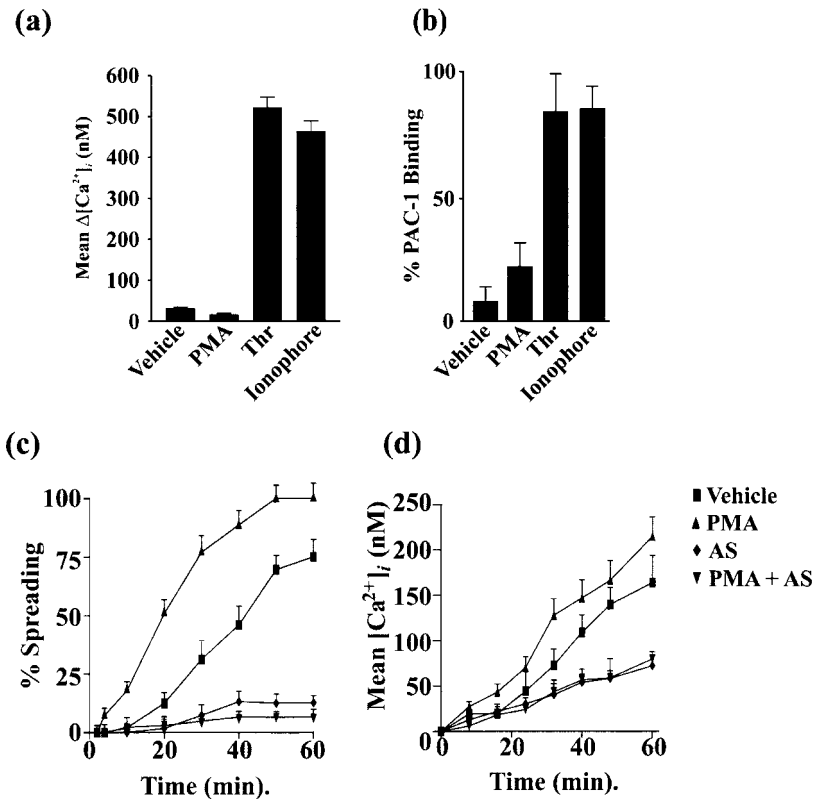

Figure 5 Relationship between PKC and integrin αIIbβ3 activation in regulating cytosolic calcium flux

Calcium dye-loaded platelets were stimulated in suspension under non-stirred conditions with 200 nM PMA, 1 unit/ml thrombin (Thr) or 100 nM ionophore A23187 (ionophore). (**a**) PMA stimulation of platelets in suspension does not induce a significant increase in the mean cytosolic calcium level in the platelet population, whereas a dramatic increase in calcium is induced by Thr or ionophore stimulation (means $\pm$ S.E.M., $n = 4$). (**b**) Illustrates the level of PAC-1 binding to platelets stimulated as described above. The level of PAC-1 binding is expressed as a percentage relative to the results obtained from Thr-stimulated platelets (means $\pm$ S.E.M., $n = 6$). (**c**, **d**) Calcium dye-loaded platelets pretreated with vehicle (0.25 % Me_2SO) or 200 nM PMA were allowed to settle on a vWf matrix under static conditions for 60 min at 37 °C, in the presence or absence of the anti-αIIbβ3 antagonist AS. (**c**) The percentage of cells spread on the vWf matrix over a 60 min time period. (**d**) Real-time changes in mean cytosolic calcium concentrations over 60 min. The results are expressed as means $\pm$ S.E.M. from four individual experiments.

cells revealed that all platelets translocating over the vWf surface typically exhibited relatively low basal calcium levels (< 100 nM), before forming stationary adhesion contacts (Figure 7c). Detailed analysis of > 100 platelets demonstrated that the onset of sustained calcium oscillations coincided with the formation of firm adhesion contacts (Figure 7c). In PMA-treated platelets, there was a considerable lag time between the onset of stationary adhesion and the subsequent oscillatory calcium response (Figures 7b and 7d). The initiation of stationary adhesion contacts, as well as the sustained oscillatory calcium response, in both vehicle and PMA-treated platelets was dependent on integrin αIIbβ3 engagement of vWf as both were abolished by pretreating platelets with AS (Figures 7a and 7b). The apparent temporal dissociation of integrin αIIbβ3 activation and oscillatory calcium flux after PMA exposure further supports our hypothesis that PKC serves to promote cytosolic calcium flux by an indirect signalling mechanism operating downstream of integrin αIIbβ3.

PKC potentiates integrin αIIbβ3-dependent adhesive and signalling functions independent of transmembrane calcium influx

To investigate whether PKC regulation of platelet adhesion was dependent on either calcium influx or intracellular calcium mobilization, flow studies were performed on platelets pretreated either with EGTA to chelate extracellular calcium or with the membrane-permeant calcium chelator DM-BAPTA/AM, to chelate cytosolic calcium. As shown in Figure 8(a), despite an approx. 70 % decrease in the level of platelet stationary adhesion to vWf in the presence of EGTA, PMA was capable of inducing stationary adhesion contact formation in 100 % of EGTA-treated platelets. Similar to our earlier findings, PMA elicited a delayed oscillatory calcium response in all EGTA-treated platelets adherent to vWf (results not shown). These latter findings provide further evidence that the elevation in intracellular calcium resulting from PMA stimulation of translocating platelets is due, at least partially, to calcium mobilization from intracellular stores. In contrast with our findings in EGTA-treated platelets, PMA was not capable of inducing stationary adhesion of DM-BAPTA/AM-treated platelets (Figure 8a), with all platelets translocating in a rapid continuous manner. Furthermore, PMA-induced PAC-1 binding to the surface of platelets was completely eliminated by pretreating platelets with DM-BAPTA/AM (Figure 8b). The inability of PMA to induce integrin αIIbβ3 activation under these experimental conditions was not due to the inhibition of pleckstrin phosphorylation (Figure 8c), as PMA induced robust pleckstrin phosphorylation in DM-BAPTA/AM-treated platelets, presumably by the activation of one or more calcium-independent (atypical and/or novel) isoforms of PKC. These studies support an essential role for intracellular calcium mobilization, not influx, for PKC-dependent integrin αIIbβ3 regulation.

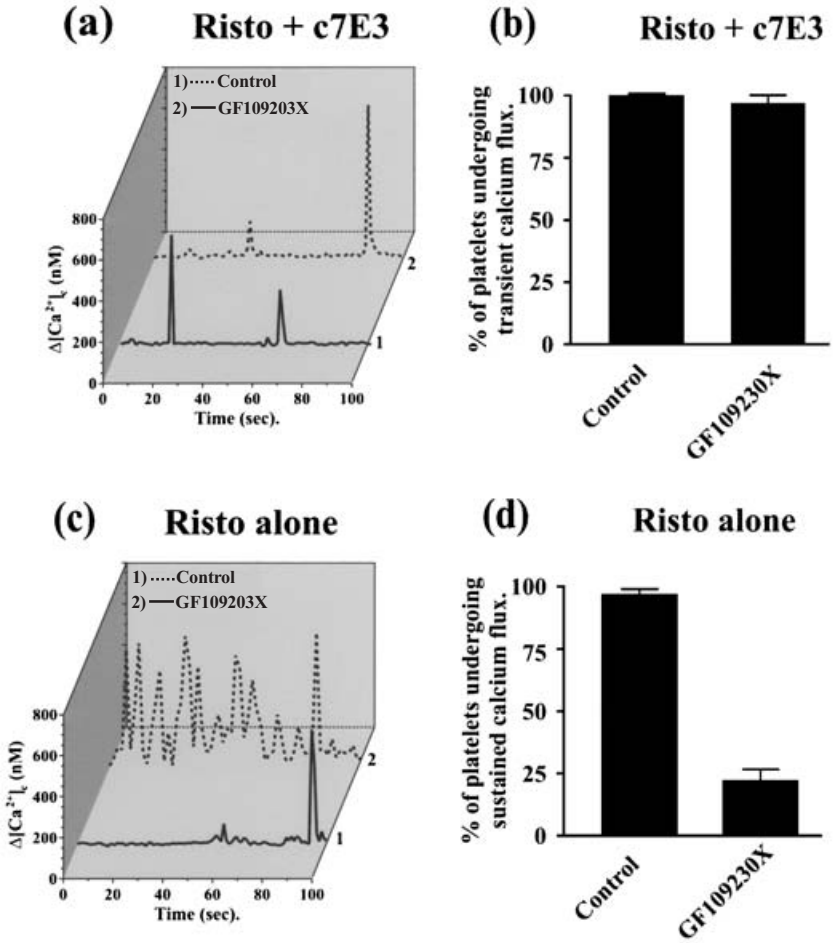

Figure 6 Effect of GF109203X on GPIb/V/IX and αIIbβ3-derived calcium mobilization on vWf under flow

Washed platelets (1×10^7 cells/ml) reconstituted with RBCs (50 % Hct) were treated with vehicle (0.25 % Me_2SO) or 500 nM GF109203X, before perfusion via vWf-coated microcapillary tubes at 1800 s^{-1} in the presence of ristocetin (Risto) and/or 20 μg/ml c7E3 Fab as indicated. (**a**) GPIb-derived cytosolic calcium transients in single adherent platelet are not affected by 500 nM GF109203X. (**b**) GF109203X treatment has no effect on the percentage of platelet population undergoing transient calcium spiking. (**c**) Sustained oscillatory calcium flux induced by αIIbβ3 is blocked by GF109203X treatment. (**d**) Decrease in the percentage of platelets undergoing a sustained calcium oscillation on vWf in the presence of GF109203X. (**a**, **c**) The calcium responses of single platelets are representative of 25 independent platelets per experiment ($n = 3$).

DISCUSSION

To date, the signalling processes regulating integrin αIIbβ3 activation during platelet adhesion on vWf have been very poorly defined. Recent studies from our laboratory have suggested a potentially important role for integrin αIIbβ3-derived calcium signals in this process [5]. In the present study, we have demonstrated the existence of a complex signalling relationship operating between PKC, calcium and integrin αIIbβ3, which serves to regulate the dynamics of platelet adhesion under flow. More specifically, we have demonstrated that bidirectional signalling via PKC is important for initiating and maintaining integrin αIIbβ3 activation and stationary platelet adhesion on vWf. Moreover, we have established that the level of cytosolic calcium dictates the requirement of PKC for integrin αIIbβ3 activation. Finally, our studies have demonstrated a potentially important role for PKC in promoting integrin αIIbβ3-dependent calcium flux, thereby establishing a positive-feedback loop that potentiates further integrin αIIbβ3 activation.

An unexpected finding from the present study was the major role played by integrin αIIbβ3 in regulating PKC activation during platelet adhesion on vWf. This is distinct from previous findings demonstrating that PKC activation primarily occurs downstream of GPIb [6,7]. This difference probably reflects technical differences in the experimental approaches of the two studies. For example, our studies were performed on immobilized vWf matrices, whereas all previous studies examining PKC activation were performed in suspension-based assays. Therefore a level of caution should be exercised when attempting to extrapolate directly findings based on aggregation studies to those obtained from adhesion experiments.

Defining the relative contribution of GPIb and integrin αIIbβ3 in regulating PKC activation and calcium flux is a critical issue with respect to the mechanism by which vWf induces integrin αIIbβ3 activation. For example, if PKC and calcium are primarily regulated downstream of GPIb, with little contribution of integrin αIIbβ3 outside-in signalling, this would support a model in which these molecules participate in a direct linear signalling pathway linking GPIb to integrin αIIbβ3 activation. However, this is not consistent with the current findings that PKC is primarily regulated downstream of integrin αIIbβ3 and that integrin αIIbβ3-dependent calcium flux is required for sustained integrin αIIbβ3 activation and firm platelet adhesion on vWf [5]. Moreover, our present studies have demonstrated that

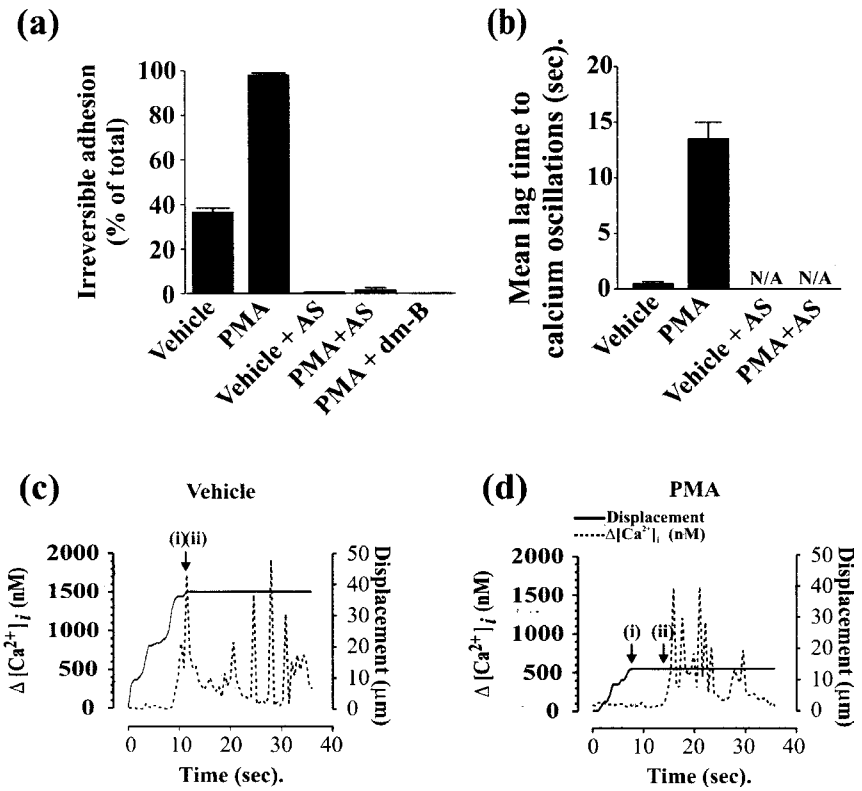

Figure 7 Relationship between PKC and calcium in regulating integrin αIIbβ3 activation under flow conditions

Calcium dye-loaded platelets reconstituted in RBCs (50 % Hct) were perfused via vWf-coated microcapillary tubes at 1800 s^{-1} in the presence of vehicle (0.25 % Me_2SO) or 200 nM PMA. Where indicated, platelets were also preincubated with either 500 nM AS or 100 μM DM-BAPTA/AM (dm-B) before perfusion. (**a**) The level of stationary platelet adhesion on immobilized vWf in response to PMA stimulation in the presence or absence of AS or dm-B (means $\pm$ S.E.M., $n = 4$). (**b**) The lag time between stationary adhesion contact formation and the onset of calcium oscillations in adherent platelets. N/A, not applicable. (**c**, **d**) Typical translocation behaviours and calcium profiles of single platelets adherent to vWf in the presence (**d**) or absence of PMA (**c**). The solid line shows platelet displacement as a function of time, whereas the dotted line demonstrates cytosolic calcium flux profiles in these cells. The arrows in (**c**, **d**) indicate the onset of stationary adhesion (i) and the subsequent onset of calcium mobilization (ii).

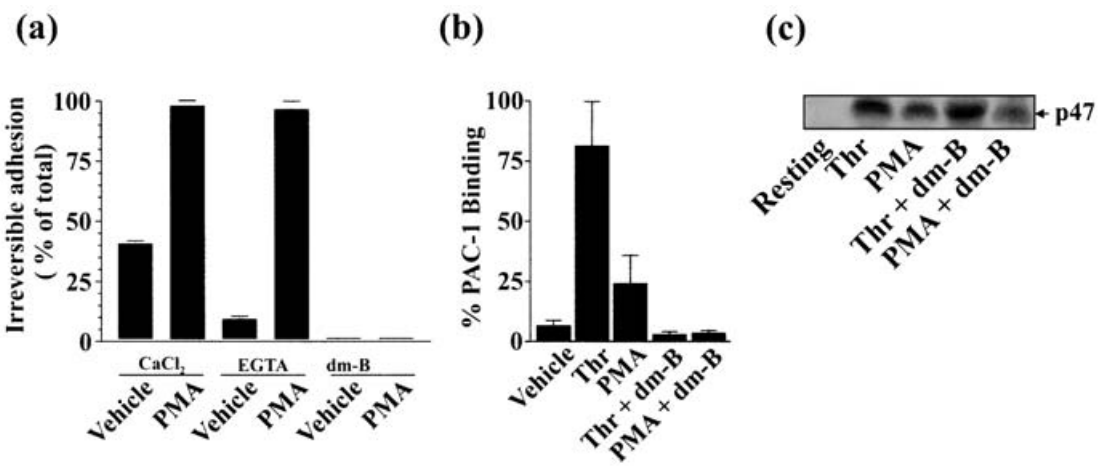

Figure 8 Effect of extracellular and intracellular calcium chelation on integrin αIIbβ3 activation and pleckstrin phosphorylation induced by PMA

Calcium dye-loaded platelets treated with vehicle (0.25 % Me_2SO) or 100 μM dm-B were reconstituted with RBCs (50 % Hct) in Tyrode's buffer supplemented with either 1 mM $CaCl_2$ or 2 mM EGTA and 2 mM $MgCl_2$, and perfused via vWf-coated microcapillary tubes at 1800 s^{-1}, in the presence or absence of 200 nM PMA. (**a**) A significant increase in the level of stationary platelet adhesion on immobilized vWf in response to PMA stimulation in the presence of both $CaCl_2$ and EGTA/$MgCl_2$. The data also show that PMA-induced stationary adhesion can be abolished by pretreating platelets with dm-B (means $\pm$ S.E.M., $n = 4$). Washed platelets pretreated with vehicle (0.25 % Me_2SO) or 100 μM dm-B were stimulated in suspension under non-stirred conditions with 200 nM PMA or 1 unit/ml Thr in the presence of PAC-1 mAb. (**b**) The level of PAC-1 binding to PMA or thrombin (Thr)-stimulated platelets in suspension. The level of PAC-1 binding is expressed as a percentage relative to the results obtained from Thr-stimulated platelets (means $\pm$ S.E.M., $n = 6$). (**c**) Washed platelets loaded with [^{32}P]P_i were incubated with or without 100 μM dm-B before exposure for 5 min to 200 nM PMA or 1 unit/ml Thr as indicated. The results demonstrate that 100 μM dm-B treatment does not inhibit Thr or PMA-induced pleckstrin phosphorylation (labelled p47).

whereas direct PKC activation with PMA results in a low level of integrin αIIbβ3 activation, sustained integrin αIIbβ3-dependent stationary adhesion does not require ongoing PKC activity if cytosolic calcium levels are sufficiently elevated. These findings support a model in which PKC inside-out signalling primarily serves to initiate integrin αIIbβ3 activation and transient stable adhesion, whereas subsequent outside-in signalling establishes a positive-feedback loop to promote further integrin αIIbβ3 activation and intracellular calcium mobilization.

Our studies also demonstrate that the absolute requirement of PKC for integrin αIIbβ3 activation is dictated by the cytosolic calcium concentration. In the presence of intracellular calcium chelators, direct activation of PKC is insufficient to induce integrin αIIbβ3 activation, despite robust pleckstrin phosphorylation under these experimental conditions. These findings are consistent with the possibility that the calcium-activated (conventional) isoforms of PKC are required for integrin αIIbβ3 activation. However, when the mean cytosolic calcium level is increased to more than 100 nM, PKC appears to be no longer required for integrin αIIbβ3 activation. These findings suggest that PKC regulation of integrin αIIbβ3 occurs within a defined calcium concentration range, a finding that may explain previous observations that PKC inhibitors do not prevent platelet aggregation induced by potent platelet-activating stimuli, including thrombin [18–20].

Finally, our studies have demonstrated that phorbol ester stimulation of platelets is sufficient to induce stationary platelet adhesion, independent of an initial calcium signal. This is distinct from untreated platelets in which firm platelet adhesion always coincided with an immediate calcium response. The reason for the delayed calcium response in PMA-treated platelets is unclear. Recent studies [21–24] have suggested that elementary transient calcium signals known as 'calcium puffs' provide a means for priming the D-*myo*-inositol 1,4,5-trisphosphate (IP_3)-dependent calcium signalling mechanism in many cell types, including HeLa cells, human umbilical-vein endothelial cells, and NIH-3T3 cells, which facilitates the generation of global calcium-signalling events in response to weak agonist stimulation. Although purely speculative, it is possible that the GPIb-derived calcium spikes act in a manner similar to calcium puffs, priming an IP_3-dependent calcium release mechanism in platelets, such that when integrin αIIbβ3 is engaged, the resulting outside-in signalling results in rapid calcium mobilization. Direct integrin αIIbβ3 activation induced by phorbol esters may bypass the initial calcium signalling events required to prime the IP_3 receptor, thereby explaining the delay in calcium flux downstream of integrin αIIbβ3 activation. Regardless of the exact mechanism, our studies support an important role for PKC in promoting integrin αIIbβ3 bidirectional signalling relevant to platelet adhesion under flow.

We thank Dr Sacha Dopheide, Dr Yuping Yuan and Dr Simone Schoenwaelder for their helpful discussions and advice. Many thanks to Suhasini Kulkarni and Cindy Yap for their technical assistance. We acknowledge Dr Francois Lanza for donating apyrase. This work was supported by grants from the National Health and Medical Research Council of Australia, National Heart Foundation of Australia and the Wellcome Trust.

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Received 5 June 2002/6 January 2003; accepted 13 February 2003
Published as BJ Immediate Publication 13 February 2003, DOI 10.1042/BJ20020868