Activation of Protein Kinase C Is Required for the Stable Attachment of Adherent Platelets to Collagen but Is Not Needed for the Initial Rapid Adhesion Under Flow Conditions

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Abstract—We have investigated the role of protein kinase C (PKC) in the initial events of $\alpha$IIb$\beta$3-integrin–mediated platelet adhesion to collagen under flow conditions. Although adhesion caused activation of PKC, as evidenced by pleckstrin phosphorylation, the PKC inhibitors GF 109203X and Gö 6976 had no effect on adhesion, even though they prevented pleckstrin phosphorylation. The initial kinetics and extent of platelet adhesion to collagen (<5 seconds) and tyrosine phosphorylation of $p125^{Fak}$ and $p72^{syk}$ were not influenced by the PKC inhibitors, whereas adhesion to polylysine was prevented. These results indicate that adhesion to collagen and polylysine involve different mechanisms and requirements for PKC activation. Pretreatment with GF 109203X destabilized collagen-adherent platelets, accelerating their detachment, which was associated with tyrosine dephosphorylation of $p125^{Fak}$. Thus, although PKC activation was not required for rapid platelet adhesion to collagen, it appears to play an important role in stabilizing the attachment of adherent platelets to collagen. We also examined the effect of PKC activation by the phorbol ester phorbol 12-myristate 13-acetate (PMA) on platelet adhesion to collagen. PMA at 100 nmol/L strongly potentiated adhesion and tyrosine phosphorylation of $p125^{Fak}$ and $p72^{syk}$ and activated $\beta$-integrins, as determined by increased exposure of the 15/7 epitope. The PMA-stimulated adhesion was partially blocked by an anti-$\alpha$2$\beta$3 antibody, was completely inhibited by GF 109203X, and was not correlated with the extent of pleckstrin phosphorylation. Therefore, strong PKC activation may lead to inside-out signaling, enhancing the role of $\beta$-integrins in adhesion. Pleckstrin phosphorylation does not appear to be involved in the initial phase of basic or PMA-stimulated adhesion but may help stabilize the adherent platelets. (Arterioscler Thromb Vasc Biol. 1999;19:3044-3054.)

Key Words: platelets ▪ adhesion ▪ collagen ▪ PKC ▪ $p125^{Fak}$ ▪ $p72^{syk}$

Protein kinase C (PKC) was initially identified as a threonine-serine protein kinase dependent on calcium and phospholipids and known to be activated during the earliest events of signal transduction, tumor promotion, and cell regulation. Activation of PKC has been linked to reorganization of the actin cytoskeleton, cell motility, adhesion, and the formation of focal contacts. The involvement of PKC in cell adhesion to extracellular matrix (ECM) proteins has long been proposed from the observations that direct stimulation of PKC by phorbol esters can activate integrins, potentiating adhesion and spreading, and that inhibitors of PKC prevent adhesion. There is also evidence that direct integrin binding to ECM proteins leads to PKC activation. Although stimulation of PKC by phorbol esters can mediate integrin activation and enhance adhesion, there is little evidence that PKC directly regulates these events.

Platelets are a good model to study signal transduction events during attachment of cells to ECM proteins or for cell-cell interactions. Several studies suggest that PKC plays an important role in blood platelet activation associated with granule secretion and aggregation. In particular, prior activation of PKC with PMA desensitizes platelets to thrombin stimulation, as indicated by an accelerated inositol trisphosphate metabolism and decreased internal Ca2+ mobilization. Much interest has focused on a protein of apparent molecular mass of 47 kDa, called pleckstrin, which is phosphorylated when platelets are activated by agonists that directly or indirectly activate PKC. Phosphorylation of pleckstrin correlates closely with serotonin secretion from platelet-dense granules and is frequently used as a marker of PKC activation in human platelets. More recently, it was shown that pleckstrin phosphorylation in platelets exposed to thrombin or to the thrombin receptor–activating peptide (TRAP) is also partially dependent on activation of phosphoinositide (PI) 3-kinase. In platelets, as in other cell types, adhesion to collagen causes tyrosine phosphorylation of a 125-kDa cytosolic tyrosine kinase, known as a focal adhesion kinase $p125^{Fak}$. A
process strongly correlated with the formation of focal contacts.\textsuperscript{25,26} Signaling molecules that have been shown to be activated during collagen stimulation include the p72\textsuperscript{tyr} tyrosine kinase.\textsuperscript{27} The initial phase of protein tyrosine phosphorylation of several tyrosine kinases depends on prior PKC activation,\textsuperscript{28} and it has been proposed that protein tyrosine phosphorylation in platelets occurs after activation of PKC.\textsuperscript{29} This suggests that PKC activation may also influence the initial stage of tyrosine phosphorylation of p125\textsuperscript{FAK} and p72\textsuperscript{tyr} after platelet adhesion. Tyrosine phosphorylation of p125\textsuperscript{FAK} in platelets stimulated with a mixture of epinephrine and anti-LIBS6 antibody\textsuperscript{30} or in platelets adherent either to fibrinogen or to immobilized immunoglobulin IgG\textsuperscript{31,32} is blocked when PKC activity was inhibited with a specific PKC inhibitor, bisindolylmaleimide GF 109203X, suggesting that p125\textsuperscript{FAK} is activated downstream of PKC. PKC-induced serine phosphorylation of p125\textsuperscript{FAK} has been suggested to regulate the intracellular stability of focal adhesion kinase in mouse 3T3 cells.\textsuperscript{33}

Platelet adhesion to an exposed subendothelium is a critical physiological process that must occur rapidly at the site of vessel-wall injury.\textsuperscript{34} Previously, we have studied the mechanisms of platelet adhesion to collagen under flow conditions, with shear parameters similar to those in microcirculation.\textsuperscript{35} Platelet adhesion under these conditions is very efficient, occurring within seconds, and in a plasma-free, Mg\textsuperscript{2+}-containing buffer it is mediated primarily by the \(\alpha_\text{IIb}\beta_3\)-integrin,\textsuperscript{36} although other receptors or associated proteins may participate.\textsuperscript{37–40}

In the present study, we investigated the involvement of PKC in platelet adhesion to collagen under flow conditions by using several PKC inhibitors. In cases of vessel-wall injury, not only must platelet adhesion to the exposed adhesive proteins of the ECM be rapid (seconds to minutes), but also, the stability of the attached platelets must be sufficient to prevent blood loss and contribute to the healing process. Our continuous-flow approach\textsuperscript{36} for following adhesion kinetics as well as biochemical changes provides an opportunity to study separately the roles of PKC during the initial attachment of platelets to collagen and in stabilizing the collagen-platelet interaction. Because adhesion can activate PKC and cause tyrosine phosphorylation of p125\textsuperscript{FAK} and p72\textsuperscript{tyr} and because these kinases are present in focal contacts,\textsuperscript{41–43} we also examined the influence of PKC activation by a phorbol ester on adhesion and the tyrosine phosphorylation of these kinases.

## Methods

### Materials

Type I collagen from rat skin was a gift from Dr G. Balian (University of Virginia, Charlottesville). Polylysine, BSA, indomethacin, prostaglandin I\(_1\) (PGL), apyrase (grade VII), phorbol 12-myristate 13-acetate (PMA), and bovine thrombin were from Sigma Chemical Co. The PKC inhibitors indolocarbazole Gø 6976 and bisindolylmaleimide I (GF 109203X) were from Calbiochem-Novabiochem Corp. \([\text{32P}]\)orthophosphoric acid was from DuPont NEN. A monoclonal antibody (mAb) 4D10 against p72\textsuperscript{tyr} was purchased from Santa Cruz Biotechnology, Inc. The mAbs 2A7 and BC3 against p125\textsuperscript{FAK} were gifts from Dr T. Parsons (University of Virginia, Charlottesville). The mAbs 4G10 and PY20 against phosphotyrosine were purchased from Upstate Biotechnology Inc and Transduction Laboratories, respectively. An mAb 15/7 that recognizes a high-affinity or ligand-occupied conformation of \(\beta_3\)-integrin\textsuperscript{44} was a gift from Dr T. Yednock (Athena Neurosciences Inc). FITC-conjugated goat anti-mouse IgG1 and purified mouse IgG1 were from Zymed Laboratories Inc. Anti–human integrin-\(\alpha_\text{IIb}\) and anti–human integrin-\(\alpha_\text{IIb}\) mAbs, P1E6 and P1D6, respectively, were from Gibco/BRL. An anti–\(\beta_3\)-integrin mAb 1959 was from Chemicon. GRGDSP peptide was from Peninsula Laboratories. The alkaline phosphatase–conjugated goat anti-mouse secondary antibody and the chemiluminescence-based Western blot detection system were from Bio-Rad.

### Platelet Preparation

After informed consent had been obtained, platelet-rich plasma was obtained from freshly drawn blood anticoagulated with acid-citrate-dextrose (ACD: 120 mmol/L sodium citrate, 110 mmol/L glucose, 80 mmol/L citric acid) by centrifugation as described.\textsuperscript{45} Platelets were isolated from platelet-rich plasma by centrifugation at 620g for 20 minutes in the presence of 0.05 volumes of ACD, apyrase (7.5 U/mL ADPase activity), indomethacin (1 \(\mu\)g/mL), and PGI\(_2\) (0.3 \(\mu\)g/mL). For labeling, 10\textsuperscript{9} platelets were resuspended in 9 mL of phosphate-free buffer (140 mmol/L NaCl, 5 mmol/L KCl, 0.05 mmol/L CaCl\(_2\), 0.1 mmol/L MgCl\(_2\), 0.01 g/mL BSA, 16.5 mmol/L glucose, 15 mmol/L HEPES, pH 7.4) containing 1 \(\mu\)g/mL indomethacin and 7.5 U/mL apyrase. The platelet suspension was incubated for 20 minutes at room temperature, and the labeling process was performed by incubating platelets with 1 mCi carrier-free \([\text{32P}]\)orthophosphate for 90 minutes at 37°C. Subsequently, platelets were centrifuged, washed again, and resuspended at a concentration of 4\times10\textsuperscript{9} to 6\times10\textsuperscript{9}/mL in a modified Tyrode’s buffer (140 mmol/L NaCl, 0.34 mmol/L NaH\(_2\)PO\(_4\), 2.9 mmol/L KCl, 10 mmol/L HEPES, 12 mmol/L NaHCO\(_3\), 5 mmol/L glucose, pH 7.4) containing 2 mmol/L MgCl\(_2\). In selected experiments, inhibitors of platelet activation apyrase (7.5 U/mL), indomethacin (1 \(\mu\)g/mL), and GRGDSP peptide (500 \(\mu\)mol/L) were included before adhesion assays were performed. Platelet suspensions were used for experiments warmed at 37°C for 10 minutes before addition of compounds as appropriate.

### Platelet Adhesion Assay

The continuous-flow adhesion approach was essentially as described previously.\textsuperscript{46} BrCN-activated Sepharose beads are coated with native soluble collagen type I from rat skin or in control experiments with polylysine or BSA and used as adhesive substrates. One syringe containing washed platelets and a second syringe containing isotonic saline are connected via Teflon tubing to the microadhesion column. Their contents are mixed and pumped through the protein-coated beads. The flow rates through the beads are regulated by a variable syringe pump and give shear rates at the bead surface from 850 to 3400 s\(^{-1}\). Adhesion to collagen is determined by counting of single platelets in the suspension before and after exposure to the beads with a resistive particle counter and is expressed as the percentage of platelets bound to collagen. To determine the changes in protein phosphorylation, we usually used platelets that had adhered to 50 \(\mu\)L of protein-coated beads at a pumping speed of 3.4 mL/s, which yields a shear rate of 1700 s\(^{-1}\). Platelets (250 \(\mu\)L; \(1\times10\textsuperscript{8}\) to \(1.5\times10\textsuperscript{8}\)) were pumped at this shear rate for \(\sim\)90 seconds. To obtain sufficient amount of proteins for immunoprecipitations (1 to 2 mg), we used \(10\times\) platelets per immunoprecipitation. PMA and PKC inhibitors were prepared in 0.1% (vol/vol) DMSO. When the effect of PKC inhibitors was studied, platelets were pretreated with these inhibitors or with 0.1% DMSO to serve as controls for 10 minutes at 37°C. In some experiments, adhesion studies were performed after an initial precubation for 10 minutes with apyrase (7.5 U/mL) and indomethacin (1 \(\mu\)g/mL), followed by 3 minutes of treatment with 20 or 100 mmol/L PMA without stirring. To investigate the PMA-stimulated adhesion to collagen, we used beads coated with half the amount of collagen compared with the original beads used for our other experiments.\textsuperscript{47}

### Platelet Detachment Assay

Washed platelets preincubated either with the PKC inhibitor GF 109203X (12 \(\mu\)mol/L) or with 0.1% DMSO (control) were pumped through the adhesion column for \(\sim\)90 seconds as described above.

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\(\text{Polanowska-Grabowska and Gear PKC and Platelet Adhesion to Collagen 3045}\)
Two different approaches were used to study detachment. In the first, beads with adherent platelets were immediately washed with saline, suspended in a Mg\(^{2+}\)-containing medium, and shaken for 10, 30, 60, 120, and 240 minutes at 37°C in a shaking water bath at 60 strokes/min and with a stroke length of 30 mm. In the second approach, platelets adherent to the collagen-coated beads were left in the column for 10, 30, 60, 120, and 240 minutes in the presence of physiological buffer, and subsequently, buffer was pumped through the column for 6 minutes under constant shear. In both approaches, detachment of platelets was determined by counting of the released platelets in the suspending buffer or in the effluent from the column, respectively. The phosphorylation of p125\(^{FAK}\) was determined at the same time points. Lysis buffer was added to every tube containing collagen beads with platelets and buffer. Platelet proteins were concentrated and then used for immunoprecipitation with anti-p125\(^{FAK}\) mAb BC3, followed by Western blotting with anti-phosphotyrosine and anti-p125\(^{FAK}\) antibodies. The protein bands were visualized by chemiluminescence. The phosphorylation of p125\(^{FAK}\) determined by densitometry at defined time points is expressed as a percentage of the tyrosine phosphorylation measured at time 0, when all platelets were attached to the beads.

Gel Electrophoresis and Western Blotting

The same amounts of adherent, aggregated, and control platelets were lysed in SDS-containing buffer (2% SDS [wt/vol], 5% 2-mercaptoethanol [vol/vol], 10% glycerol [vol/vol], 0.002% bromphenol blue [vol/vol], and 62.5 mmol/L Tris, pH 6.8) and heated for 5 minutes at 95°C. Platelet lysates (from 1.1×10\(^7\) /lane) were separated by 8% or 12% SDS-PAGE and electrotransferred from the gels onto nitrocellulose membranes. To block residual protein-binding sites, membranes were incubated overnight with 5% BSA (vol/vol) in TBS-T buffer (150 mmol/L NaCl, 50 mmol/L Tris [pH 7.5], and 0.1% [vol/vol] Tween 20). The blots were then washed and incubated for 2 hours with anti-p125\(^{FAK}\), anti-p72\(^{Syk}\), or anti-phosphotyrosine antibodies. Membranes were washed 4 times in TBS-T, and bound primary antibody was detected by incubation with alkaline phosphatase–conjugated goat anti-mouse IgG as a secondary antibody diluted 1:3000. Membranes were washed and treated with ECL reagents before exposure to ECL-Hyperfilm (Amersham).

Immunoprecipitations

Adherent platelets (5×10\(^9\)/500 μL) were lysed in an ice-cold lysis buffer as described.23 Insoluble cell debris was removed by centrifugation at 3000g for 5 minutes. The supernatant was pre cleared with protein A-Sepharose CL-4B, removed, and incubated for 18 hours at 4°C with 5 μg of anti-p125\(^{FAK}\) (2A7), with 5 μg of anti-p72\(^{Syk}\) (4D10), or with 5 μg of an appropriate control antibody. Anti-mouse IgG–adsorbed protein A beads were then added, and the immune complexes were washed twice with lysis buffer and twice with ice-cold PBS buffer, pH 7.2, before resuspension in SDS sample buffer. Immunoprecipitated proteins were subjected to 8% SDS-PAGE, transferred to nitrocellulose, and probed as described above.

Fluorescence-Activated Cell Sorting Analysis of Platelets

Washed platelets were resuspended in a modified Tyrode’s buffer containing 0.1% BSA, 2 mmol/L Mg\(^{2+}\), 10 μmol/L indomethacin, and 7.5 U/mL apyrase. They were incubated with PMA (20 or 100 nmol/L) or with 0.1% DMSO for 3 minutes without stirring at 37°C. The platelet stimulations were stopped by the addition of 100 volumes of PBS buffer, pH 7.2, containing aggregation inhibitors and centrifugation. Then the platelets were resuspended in a Tyrode’s buffer, followed by the addition of the appropriate mAb, such as 15/7, P1E6, 1959, or control mouse IgG1. Subsequently, anti-mouse FITC-conjugated antibody was added, and after 30 minutes, the platelets were fixed by the addition of an equal volume of 2% (wt/vol) paraformaldehyde in PBS buffer, pH 7.2, followed by centrifugation. Finally, platelets were washed and resuspended in Tyrode’s buffer at 5×10\(^6\)/mL and analyzed for FITC fluorescence on a Becton-Dickinson FACStar. In some experiments, before stimulation with PMA, platelets were pretreated with the PKC inhibitor GF 109203X (12 μmol/L) for 10 minutes.

Statistical Analysis

When appropriate, evidence for significant differences between control and test situations was assessed by Student’s t test for paired samples. Data are usually presented as mean±SD.

Results

Pleckstrin Phosphorylation After Platelet Adhesion to Collagen

One-dimensional gel electrophoresis of \(^{32}\)P-labeled human platelets showed that platelet adhesion to collagen under flow conditions stimulated a marked increase in pleckstrin phosphorylation of 3.2±0.8-fold within 90 seconds of perfusion (Figure 1A). Under the same flow conditions, phosphorylation of pleckstrin also occurred in platelets adherent to the nonspecific substrate polylysine, albeit to a greater extent (6.5±0.5-fold) than that observed in platelets adherent to collagen (Figure 1A). As expected, platelets stimulated either with the G protein–coupled receptor agonist thrombin (1 U/mL) or by a direct activator of PKC, PMA (100 nmol/L), also revealed strong pleckstrin phosphorylation (7.0±0.7- and 6.7±0.8-fold, respectively). Analysis of platelets in the effluent from the microcolumn showed that neither shear forces acting alone nor possible brief platelet-collagen con-
tact during flow through the beads caused pleckstrin phosphorylation (Figure 1A). Nonspecific adhesion to BSA was <5% and did not result in pleckstrin phosphorylation (data not shown). Because the stimulation of pleckstrin phosphorylation might be enhanced by secondary events, such as formation of arachidonate metabolites, release of ADP, or \( \alpha_\text{IIb}\beta_3 \)-dependent aggregation, we also evaluated pleckstrin phosphorylation in adherent platelets in the presence of the cyclooxygenase inhibitor indomethacin apyrase, which removes ADP that might be inadvertently released from the platelets, and the GRGDSP peptide, which blocks fibrinogen binding and aggregation. None of these inhibitors used alone or in combination influenced pleckstrin phosphorylation, indicating that adhesion to collagen activated PKC independently of these factors (Figure 1B).

PKC Inhibitors Do Not Influence Rapid Platelet Adhesion to Collagen: Effects on Pleckstrin Phosphorylation

To investigate the role of PKC in platelet adhesion, we examined whether a prior exposure to PKC-specific inhibitors modified the adhesion kinetics and pleckstrin phosphorylation. We tested adhesion in the presence of 2 frequently used specific PKC inhibitors: the indolocarbazole Gö 6976, which preferentially inhibits Ca\(^{2+}\)-dependent isozymes and bisindolylmaleimide GF 109203X, which interacts with the catalytic subunit of PKC and blocks both Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent isozymes. We found that pretreatment of platelets with 0.5 to 20 \( \mu \text{mol/L} \) Gö 6976 did not influence efficiency of adhesion to collagen (within 90 seconds of perfusion) (Figure 2A). Under the same conditions, adhesion to polylysine was reduced in a dose-dependent manner, reaching a maximal level of inhibition (85%) at 10 \( \mu \text{mol/L} \) GF 109203X (Figure 2A). Similar behavior was found for pretreatment with Gö 6976 at 1 to 15 \( \mu \text{mol/L} \); adhesion to collagen was not affected, whereas inhibition of adhesion to polylysine reached maximum (80%) at 10 \( \mu \text{mol/L} \). In most subsequent studies, we used single concentrations of inhibitors, 12 \( \mu \text{mol/L} \) GF 109203X and 10 \( \mu \text{mol/L} \) Gö 6976, which are known to inhibit PKC activity and have often been used in the literature. Pretreatment with these inhibitor concentrations did not affect the initial kinetics (<5 seconds) of platelet adhesion to collagen (Figure 2B), whereas adhesion to polylysine was almost completely blocked. Preincubation with GF 109203X in the range of 0.5 to 20 \( \mu \text{mol/L} \) inhibited pleckstrin phosphorylation in collagen-adherent platelets in a dose-dependent manner, reaching almost complete inhibition at 5 \( \mu \text{mol/L} \) (data not shown). At 12 \( \mu \text{mol/L} \), GF 109203X inhibition was 98±7%, and in polylysine-adherent platelets it was 95±8% (data not shown). Gö 6976 at 10 \( \mu \text{mol/L} \) partially blocked pleckstrin phosphorylation induced by adhesion to collagen (65±4%, Figure 3), whereas it strongly blocked phosphorylation caused by polylysine (89±6%, data not shown).

PKC Inhibition Does Not Influence Adhesion-Induced Tyrosine Phosphorylation of p125\(^{\text{FAK}} \) and p72\(^{\text{Syk}} \)

Because GF 109203X was the most potent and specific inhibitor of PKC and it effectively blocked pleckstrin phosphorylation in collagen-adherent platelets, we chose this compound to study tyrosine phosphorylation of p125\(^{\text{FAK}} \) and p72\(^{\text{Syk}} \). We previously showed that rapid adhesion to collagen but not to polylysine under flow conditions causes tyrosine phosphorylation of p125\(^{\text{FAK}} \). In the present study under similar flow conditions, p72\(^{\text{Syk}} \) was also tyrosine phosphorylated in collagen-adherent platelets (Figure 4). Platelet preincubation either with DMSO or with GF 109203X had no effect on overall protein tyrosine phosphorylation in either control or adherent cells (data not shown). As illustrated in Figure 4, GF 109203X did not influence tyrosine phosphorylation of p125\(^{\text{FAK}} \) (Figure 4A) and p72\(^{\text{Syk}} \) (Figure 4B) induced by platelet adhesion to collagen. Western blotting revealed similar protein levels of p125\(^{\text{FAK}} \) and p72\(^{\text{Syk}} \) before and after platelet exposure to the inhibitor.

PKC Inhibitors Stimulate Platelet Detachment From Collagen

To investigate a possible role of PKC in maintaining stable attachment of platelets to collagen, we studied the effect of...
GF 109203X on spontaneous detachment of adherent platelets from the collagen surface. Platelets preincubated with the PKC inhibitor that had adhered to the collagen-coated beads revealed enhanced detachment from the collagen-coated beads compared with DMSO-treated controls both after 6 minutes of flushing of the column at a shear rate of 850 s⁻¹ (Figure 5A) and after time spent in a shaking bath (Figure 5B). Detachment was correlated with decreased tyrosine phosphorylation of p125⁵⁵FAK (Figure 5C), and it was not associated with changes in FAK amount, as determined by an immunoblot using anti-p125⁵⁵FAK antibody (data not shown). These results suggest involvement of PKC in maintaining platelet attachment after the initial binding to collagen.

Effects of PMA Stimulation

Several reports indicate that phorbol esters enhance adhesion of different cell types to adhesive proteins without having any effect on the adhesion to nonspecific substrates.⁴⁸–⁵⁰ In addition, phorbol esters may desensitize receptor-mediated responses, such as thrombin-stimulated internal Ca²⁺ mobilization and formation of inositol phosphates.¹⁹ In the present study, platelets in the presence of indomethacin and apyrase were preincubated with 20 nmol/L PMA for 3 minutes without stirring and used for adhesion. Treatment with this low dose of PMA caused strong phosphorylation of pleckstrin (Figure 6A) and a weak tyrosine phosphorylation of p72⁷²syk (Figure 6B) and p125⁵⁵FAK (Figure 6C), without having any effect on adhesion (Figure 7). PMA at 100 nmol/L and 1 μmol/L (data not shown) caused an increase in phosphorylation of pleckstrin similar to that observed for 20 nmol/L (Figure 6A). However, tyrosine phosphorylation of p125⁵⁵FAK and p72⁷²syk was much stronger at 100 nmol/L PMA than at the lower dose of PMA (Figure 6B and 6C). Adhesion was significantly accelerated and potentiated by this PMA treatment (Figure 7). Preincubation with GF 109203X reversed the effect of PMA on the extra level of adhesion, bringing it down to control levels (Figure 7). To determine whether the

Figure 3. Effects of PKC inhibitors on adhesion-induced phosphorylation of pleckstrin (p47). Washed platelets labeled with ³²P and incubated with DMSO vehicle or with PKC inhibitors GF 109203X (0.5 to 20 μmol/L) and Gö 6976 (10 μmol/L) were pumped for 90 seconds through the collagen-coated beads as in Figure 2. The adherent cells were immediately lysed in SDS sample buffer. Proteins from 1.1×10⁷ platelets were analyzed by 12% SDS-PAGE and autoradiography. Autoradiogram is representative of 3 platelet preparations. Collagen represents collagen-adherent platelets; effluent, platelets that failed to adhere to collagen and had passed through the column.

Figure 4. Effect of the PKC inhibitor GF 109203X on adhesion-induced tyrosine phosphorylation of p125⁵⁵FAK (A) and p72⁷²syk (B). Washed platelets were preincubated either with the specific protein kinase inhibitor GF 109203X (12 μmol/L) or with 0.1% DMSO and used for adhesion experiments. The same amounts of control (resting platelets) and adherent platelets (5×10⁷) were solubilized in lysis buffer and incubated with appropriate antibody: anti-p125⁵⁵FAK (2A7) or anti-p72⁷²syk (4D10), followed by the addition of rabbit anti-mouse IgG bound to protein A-Sepharose. Immune complexes were solubilized, separated on 8% SDS-PAGE, transferred to nitrocellulose membrane, and probed with a mixture of mAbs 4G10 and PY20 specific for phosphotyrosine residues or with anti-p125FAK and anti-p72syk antibodies. Blots are representative of 3 platelet preparations.
stimulatory effects of PMA would result from PMA-induced aggregation, PMA-dependent adhesion was studied in the presence of 500 μmol/L GRGDSP peptide. The RGD-containing peptide had no effect on PMA-stimulated adhesion to collagen. We also examined whether PMA could stimulate platelet adhesion to the nonspecific substrate BSA and did not observe any significant adhesion to BSA-coated beads.

Inhibition of PMA-Stimulated Adhesion to Collagen With Anti-α5β1 Antibody
We have previously shown that the antibody 6F1 against the α5β1-integrin inhibits up to 80% platelet adhesion to collagen in the presence of Mg2+ in a plasma-free buffer.25,36 In contrast, 6F1 antibody did not prevent platelet adhesion to polylysine (data not shown). In the presence of the 6F1 antibody, PMA-stimulated adhesion (after 90 seconds of perfusion at a contact time of 1.35 seconds) dropped from 70±4% to 21±3%. In comparison, “basic” adhesion of untreated platelets dropped from 36±5% to 7±2% under the same conditions. Therefore, the extra adhesion due to PMA stimulation was blocked by ~60% by the anti-α5β1 antibody. In contrast, PMA-dependent adhesion was not blocked by an antibody (P1D6) against the α5β1-integrin.

PMA Stimulation May Cause a Conformational Change in β1-Integrins
Because PMA-stimulated adhesion to collagen was partially inhibited by the anti-α5β1 antibody 6F1 and because PMA may stimulate inside-out signaling events, resulting in activation of integrins,51 we investigated whether PMA directly affects platelet β1-integrins. We used the monoclonal antibody 15/7, which recognizes an activation-dependent epitope on the β1-subunit of integrins.44 Fluorescence-activated cell sorting (FACS) analysis showed that treatment with 100 nmol/L PMA (but not with 20 nmol/L PMA or with DMSO) markedly increased (up to 30%) the binding of the 15/7 antibody to platelets. In contrast, platelet stimulation with 100 nmol/L PMA did not increase the binding of the control antibody, purified mouse myeloma IgG1, or anti-β1 mAb 1959. Similarly, the binding of the anti-α5 mAb P1E6 was unchanged by this treatment. The increased binding of the 15/7 antibody to platelets was inhibited by pretreatment with GF 109203X.

Downregulation of PKC by PMA
Because prolonged treatment of cells with PMA downregulates PKC,4 we examined how this treatment influences platelet adhesion to collagen. Platelets treated with the combination of the GRGDSP peptide, indomethacin, and apyrase were incubated without stirring with 100 nmol/L PMA or DMSO (control) for 3 and 30 minutes and 2, 4, and 6 hours at 37°C. DMSO-treated platelets exhibited only basic adhesion, identical to untreated platelets. Preincubation with PMA for up to 2 hours potentiated platelet adhesion to the same degree (~30% to 40%) as pretreatment for shorter times (3 to 30 minutes). Protein phosphorylation patterns, specifically pleckstrin phosphorylation, were not altered during 2 hours of incubation compared with 3- and 30-minute incubations (Figure 8). However, pretreatment with PMA for 4 hours no longer potentiated adhesion and did not influence initial adhesion kinetics, even though it caused higher phosphorylation of pleckstrin in DMSO-treated controls. A 6-hour incubation with PMA caused an additional decrease in pleckstrin phosphorylation compared with 4-hour treatment,
and the initial adhesion rate was the same as in basic adhesion (DMSO-treated controls or untreated platelets). The extent of adhesion after this treatment was significantly decreased (up to 50%) compared with control platelets when perfusion times were lengthened to 90 seconds. In addition, the platelet detachment assay using the shaking bath showed that the platelets that had adhered to collagen beads after 6-hour treatment with PMA were also rapidly detached from collagen, with an initial rate (5.5%/min) that was faster than that observed for GF 109203X–treated platelets (3.5%/min) or controls (1%/min). Almost complete detachment of PMA-treated platelets from collagen-coated beads was observed after 1 hour of shaking. Protein levels visualized with Coomassie stain did not indicate any proteolytic degradation up to 6 hours, and the amount of p125FAK determined by Western blotting with anti-p125FAK antibody was constant (data not shown).

Discussion

Platelet Adhesion to Collagen: Activation of PKC and Effects of PKC Inhibitors

We have shown that platelet adhesion to collagen under flow conditions induced rapid PKC activation, evidenced by pleckstrin phosphorylation, and the initial rate was the same as in basic adhesion (DMSO-treated controls or untreated platelets). The extent of adhesion after this treatment was significantly decreased (up to 50%) compared with control platelets when perfusion times were lengthened to 90 seconds. In addition, the platelet detachment assay using the shaking bath showed that the platelets that had adhered to collagen beads after 6-hour treatment with PMA were also rapidly detached from collagen, with an initial rate (5.5%/min) that was faster than that observed for GF 109203X–treated platelets (3.5%/min) or controls (1%/min). Almost complete detachment of PMA-treated platelets from collagen-coated beads was observed after 1 hour of shaking. Protein levels visualized with Coomassie stain did not indicate any proteolytic degradation up to 6 hours, and the amount of p125FAK determined by Western blotting with anti-p125FAK antibody was constant (data not shown).
the initial platelet-collagen interaction (Figures 5A and 5B). This method was less harsh than the shaking bath (Figure 5B)—after 1 hour, ~10% detachment occurred in the control compared with 45% due to shaking—and may better simulate conditions in the microcirculation. Detachment caused by the more gentle flushing conditions did not significantly increase with time, in contrast to the results from the shaking bath, in which the effects of continuous mechanical stress accumulated over time. The accelerated platelet detachment was associated with decreased tyrosine phosphorylation of p125FAK (Figure 5C).

The prolonged exposure of platelets to PMA (4 and 6 hours), which downregulates PKC, had no effect on initial adhesion rates (<1 second) and is consistent with our findings with PKC inhibitors. This treatment caused accelerated detachment of adherent platelets similar to that observed for GF 109203X–treated platelets. Interestingly, a 6-hour treatment with PMA also caused a partial (up to 50%) decrease in adhesion efficiency within 90 seconds of perfusion, compared with the 4-hour PMA treatment (which yielded the same adhesion as untreated controls). This is most likely due to higher detachment rates of initially adherent platelets during 90 seconds of perfusion, in contrast to the 4-hour PMA treatment. It appears that a significant portion of platelets pretreated with PMA for 6 hours, which initially adhere to collagen, are only weakly attached and are unable to resist shear forces and maintain stable attachment under flow for 90 seconds.

Our results suggest that PKC activation directly or indirectly plays an important role in maintaining platelet attachment to collagen and that adhesion-induced PKC activation, although not required in the initial phase of adhesion, helps stabilize the platelet-collagen interaction. This effect could well be important in hemostasis for maintaining effective platelet adherence to exposed adhesive proteins at sites of vessel-wall injury. Possibly, a sustained PKC stimulation downstream of the αβ1-integrin binding to collagen may provide positive feedback required to maintain the receptor in an active conformation. PKC may also play a role in the stabilizing function of other platelet collagen receptors.

Although there have been no studies of PKC involvement in platelet adhesion under flow conditions, several reports indicate that PKC inhibitors block adhesion under static conditions. In particular, Vuori and Ruoslahti9 reported that calphostin C inhibits fibroblast adhesion to fibronectin. PKC inhibitors also block platelet adhesion to von Willebrand factor and immunoglobulins.32,58 At first sight, these observations might seem to contradict our results. In these studies, however, adhesion is performed under static conditions and over time scales longer than half an hour. Under such conditions, not only would the initial cell attachment be observed, but also, the subsequent detachment could be accelerated by PKC inhibitors, leading to poorer retention of the initially adherent cells. The fact that adhesion in our system was not markedly blocked by PKC inhibitors could also be explained by a rapid adhesion under flow conditions, causing only limited activation of PKC, seen as a relatively low phosphorylation of pleckstrin and lack of secretion and aggregation, which are known to be closely associated with PKC activation. In contrast, most of the traditional platelet adhesion assays discussed in the literature involve a degree of platelet aggregation and secretion. In such assays, a combination of platelet adhesion, secretion, and aggregation might strongly activate PKC and is likely to be more sensitive to PKC inhibitors. This could help explain the difference between our results and the other findings32,58 and may reflect different situations in vivo: monolayer adhesion to vessel walls in the microcirculation (our system55) and multilayer adhesion/aggregation occurring during thrombus formation when methods used are based on the Baumgartner approach.34
There are several other assay systems for studying platelet adhesion under flow, and although adhesion characteristics can be followed, they do not provide the ready ability to carry out biochemical analyses on the adherent platelets, which is an important aspect of our system.\textsuperscript{25,35,36} Sixma et al\textsuperscript{59} recently described a microperfusion chamber based on earlier designs of this group, which was used for following adhesion kinetics at shear rates of 1600 s\textsuperscript{−1}. These conditions are very similar to our flow-through adhesion column containing collagen-coated Sepharose beads.\textsuperscript{35} “Stagnation-point” flow systems represent yet another approach\textsuperscript{60,61} and involve directing a jet of cells onto a surface where adhesion can be monitored. However, as indicated above, biochemical studies of the adherent platelets are not feasible.

PKC activation has been linked to tyrosine phosphorylation of p125\textsuperscript{FAK}.\textsuperscript{15} There are several distinct types of receptors that might signal tyrosine phosphorylation of p125\textsuperscript{FAK} and p72\textsuperscript{syk}. These include integrins,\textsuperscript{62} Fc\textsuperscript{y} receptors,\textsuperscript{63} various bioactive lipids such as lysophosphatidic acid, the G protein–linked neuropeptide receptors, and growth factor receptors.\textsuperscript{64} PKC inhibitors block tyrosine phosphorylation of p125\textsuperscript{FAK} in platelets bound to immobilized IgG via a low-affinity receptor, Fc\textsuperscript{y}RII, or when the \(\alpha_{\text{IIb}}\beta_{\text{3}}\)-integrin receptor is engaged.\textsuperscript{30} No such inhibition was found after stimulation of the G protein–linked neuropeptide receptors.\textsuperscript{65,66} PKC-independent tyrosine phosphorylation of p72\textsuperscript{syk} has also been observed during platelet aggregation stimulated by fibrillar collagen.\textsuperscript{27} Previously, we found that rapid adhesion to collagen under flow conditions primarily mediated by the \(\alpha_{\text{IIb}}\beta_{\text{3}}\)-integrin and in the absence of \(\alpha_{\text{m}}\beta_{\text{3}}\)-dependent aggregation was associated with tyrosine phosphorylation of p125\textsuperscript{FAK}.\textsuperscript{25} In the present study, we show that rapid adhesion also resulted in tyrosine phosphorylation of p72\textsuperscript{syk}. We also investigated a possible role of PKC activation in tyrosine phosphorylation of p125\textsuperscript{FAK} and p72\textsuperscript{syk}. PKC inhibitors did not modify the adhesion-induced tyrosine phosphorylation of p125\textsuperscript{FAK} and p72\textsuperscript{syk}. PKC inhibitors did not modify the adhesion-induced tyrosine phosphorylation of p125\textsuperscript{FAK} and p72\textsuperscript{syk}. PKC inhibitors did not modify the adhesion-induced tyrosine phosphorylation of p125\textsuperscript{FAK} and p72\textsuperscript{syk} (Figure 4), indicating that PKC is not required for phosphorylation of these tyrosine kinases during \(\alpha_{\text{IIb}}\beta_{\text{3}}\)-integrin–mediated adhesion. This result also indicates that different signaling pathways are involved in tyrosine phosphorylation of p125\textsuperscript{FAK} during \(\alpha_{\text{m}}\beta_{\text{3}}\)-mediated aggregation, which is dependent on PKC activation,\textsuperscript{15} and in the \(\alpha_{\text{IIb}}\beta_{\text{3}}\)-mediated adhesion.

Because adhesion to polylysine caused strong phosphorylation of pleckstrin and soluble polylysine initiates a distinctive signal transduction via a specific receptor,\textsuperscript{67} it appears that polylysine might not provide an appropriate “nonspecific” control in studies of adhesion to ECM proteins. However, as far as the PKC dependence is concerned, mechanisms of adhesion to collagen and polylysine seem to be very different, and polylysine experiments provide a useful comparison to results with collagen. This may not be true for other ECM proteins.

**PMA Stimulated Adhesion to Collagen**

To complement our studies with PKC inhibitors, we examined the effect of direct PKC stimulation with phorbol esters on platelet adhesion and signaling events. We used 2 concentrations of PMA, 20 and 100 nmol/L, which caused nearly equal pleckstrin phosphorylation. This is consistent with reports that pleckstrin phosphorylation reaches saturation level (100%) at PMA concentrations >20 nmol/L.\textsuperscript{68} The lower concentration of PMA (20 nmol/L) had no effect on rapid adhesion kinetics (Figure 7) and adhesion-induced tyrosine phosphorylation of p125\textsuperscript{FAK} and p72\textsuperscript{syk}. This agrees with our findings with PKC inhibitors that PKC activation evidenced by pleckstrin phosphorylation was not required in the initial events of platelet adhesion to collagen. However, the higher dose of PMA caused a significant potentiation of adhesion as well as stimulation of tyrosine phosphorylation of p125\textsuperscript{FAK} and p72\textsuperscript{syk}. This PMA-stimulated adhesion was independent of the formation of arachidonate metabolites, released ADP, or RGD-dependent aggregation. The PKC inhibitor GF 109203X completely blocked the PMA effect, reducing enhanced adhesion.

These results show that PKC activation induced by 100 nmol/L PMA may regulate platelet adhesion, which at first sight seems to contradict our findings with PKC inhibitors, which exclude a regulatory role of PKC in initial rapid adhesion. However, it must be noted that PKC was activated much more by 100 nmol/L PMA than by adhesion alone (seen as increased pleckstrin phosphorylation) or by 20 nmol/L PMA. Different sensitivities to PKC inhibitors of control and 100 nmol/L PMA–stimulated adherent platelets also suggest that although PKC is activated in both situations, different degrees of activation (or different PKC isozymes) may be involved. Although we found that at higher PMA levels, PKC activation may regulate adhesion, such levels were not reached for unstimulated platelets in our adhesion assay (in the absence of secretion and aggregation). The results with unstimulated platelets and with platelets treated with 20 nmol/L PMA indicate that lower levels of PKC activity are not sufficient to influence rapid attachment. Therefore, it appears that adhesion of untreated platelets and platelets pretreated with 20 nmol/L PMA on the one hand and adhesion potentiation by 100 nmol/L PMA on the other involve 2 different mechanisms.

The precise mechanism by which PKC activation by 100 nmol/L PMA may regulate adhesion is not yet known. It is unlikely that pleckstrin is involved, because phosphorylation of pleckstrin did not correlate with stimulation of adhesion (both 20 and 100 nmol/L PMA induced the same level of pleckstrin phosphorylation, yet adhesion was enhanced only at the higher dose). We found that 100 nmol/L (but not 20 nmol/L) PMA caused partial activation of platelet \(\beta_{\text{3}}\)-integrin, as judged by an increased exposure of the 15/7 epitope. The increased binding of the 15/7 antibody to platelets was not associated with upregulation of the \(\alpha_{\text{IIb}}\beta_{\text{3}}\)-integrin, because FACS analysis showed unchanged binding of the anti-\(\alpha_{\text{IIb}}\) mAb P1E6 and anti-\(\beta_{\text{3}}\) mAb 1959 after PMA treatment. The PMA-induced activation of platelet \(\beta_{\text{3}}\)-integrin provides evidence of inside-out signaling to the \(\alpha_{\text{IIb}}\beta_{\text{3}}\)-receptor and may be directly responsible for the potentiation of adhesion. The anti-\(\alpha_{\text{IIb}}\beta_{\text{3}}\) antibody 6F1 caused partial (60%) inhibition of the PMA-induced potentiation of adhesion, most likely due to 6F1 sterically blocking the collagen-binding sites on the \(\beta_{\text{3}}\)-integrin. This suggests that the PMA stimulation is partially mediated by the \(\alpha_{\text{IIb}}\beta_{\text{3}}\)-receptor (most likely as a result of \(\beta_{\text{3}}\)-activation), and the remainder may be mediated via other receptors, such as GPVI\textsuperscript{69} or GPIV.\textsuperscript{20}

It is also possible that highly activated PKC, at levels greater than needed for saturation of pleckstrin phosphorylation, phosphorylates other regulators of adhesion. Indeed,
PKC at higher concentrations (200 nmol/L) was found to stimulate PI3 kinase in platelets, and potentiates arachidonic acid release in platelets activated with the ionophore A23187. We also observed that 100 nmol/L (but not 20 nmol/L) PKC induced phosphorylation of myosin light chain. This suggests that when PKC is highly activated, an increase in cytosolic calcium may influence adhesion.

**Conclusions**

We have found that platelet adhesion to collagen under flow conditions causes rapid activation of PKC and tyrosine phosphorylation of p125Fak and p72Syk. PKC inhibitors had no effect on basic adhesion kinetics and adhesion-induced tyrosine phosphorylation of p125Fak and p72Syk, suggesting that PKC activity is not required in the initial events of adhesion and tyrosine phosphorylations. In contrast, the PKC inhibitor GF 109203X destabilized adherent platelets after initial attachment. This resulted in accelerated detachment of the adherent platelets from the collagen surface, indicating that PKC activity is necessary for stabilizing focal contacts between platelet collagen receptors and cytoskeletal structures in the later stages of platelet adhesion. In addition, strong PKC activation induced by 100 nmol/L PMA markedly potentiated the rapid early phase of adhesion to collagen and was associated with a conformational change in β1-integrins detected as an increased exposure of the 15/7 epitope on platelets. This suggests that a high level of PKC activation in platelets initiates “inside-out” signaling, leading to activation of β1-integrins and to subsequent stimulation of the αβ3-integrin-dependent adhesion.

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Activation of Protein Kinase C Is Required for the Stable Attachment of Adherent Platelets to Collagen but Is Not Needed for the Initial Rapid Adhesion Under Flow Conditions

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