Essential Role of Protein Kinase Cδ in Platelet Signaling, αIIbβ3 Activation, and Thromboxane A2 Release*

Received for publication, May 10, 2006, and in revised form, July 28, 2006. Published, JBC Papers in Press, August 8, 2006, DOI 10.1074/jbc.M604504200

Daniel Yacoub, Jean-François Théorêt, Louis Villeneuve, Haissam Abou-Saleh, Walid Mourad, Bruce G. Allen, and Yahye Merhi

From the Research Center, Montreal Heart Institute and University of Montreal, Montreal, Quebec H1T 1C8, Canada

The protein kinase C (PKC) family is an essential signaling mediator in platelet activation and aggregation. However, the relative importance of the major platelet PKC isoforms and their downstream effectors in platelet signaling and function remain unclear. Using isolated human platelets, we report that PKCδ, but not PKCα or PKCβ, is required for collagen-induced phospholipase C-dependent signaling, activation of αIIbβ3, and platelet aggregation. Analysis of PKCδ phosphorylation and translocation to the membrane following activation by both collagen and thrombin indicates that it is positively regulated by αIIbβ3, outside-in signaling. Moreover, PKCδ triggers activation of the mitogen-activated protein kinase-kinase (MEK)/extracellular-signal regulated kinase (ERK) and the p38 MAPK signaling. This leads to the subsequent release of thromboxane A2, which is essential for collagen-induced but not thrombin-induced platelet activation and aggregation. This study adds new insight to the role of PKCs in platelet function, where PKCδ signaling, via the MEK/ERK and p38 MAPK pathways, is required for the secretion of thromboxane A2.

Platelets are required for normal hemostasis but are also actively involved in thrombosis, restenosis, and inflammatory reactions. Platelet interactions with other components of the blood and with the vascular wall represent a fundamental aspect of platelet function. These interactions are primarily mediated by specific cell adhesion molecules that induce adhesion to other platelets, leukocytes, endothelial cells, and the extracellular matrix, leading to platelet activation and amplification of the thrombo-inflammatory reactions (1–5).

Platelet adhesion represents the first step in thrombogenesis. The initial binding of platelets to damaged blood vessels, which does not necessitate activation, is predominantly mediated by the interactions of platelet glycoprotein (GP) IIb-IX-V (lecintin-rich) with von Willebrand Factor (6) at high shear and αIIbβ3 with collagen at low shear (7). This in turn induces platelet signaling and activation, which lead to aggregation through αIIbβ3 binding to fibrinogen (8).

Platelet signaling may be induced by the adhesion process itself or by several agonists leading to granule secretion and activation (9–11). One of the most physiologic and potent agonists, thrombin, activates platelets through proteinase-activated receptors (12). Stimulation of proteinase-activated receptors 1 and 4 in human platelets induces inside-out signaling (13) through the activation of phospholipase C (PLCβ). Furthermore, collagen, the most abundant protein of the extracellular matrix, promotes the adhesion and activation of platelets through its binding to platelet integrin αIIbβ3, and GPVI (14, 15). Binding of collagen to GPVI stimulates a non-receptor protein-tyrosine kinase that phosphorylates and thereby activates PLCγ (16–18). Activated PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate, generating the second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate. Inositol 1,4,5-trisphosphate, in turn, mediates the release of Ca2+ from intracellular stores, whereas DAG activates PKCs.

The PKC family comprises 12 related isoforms, classified into three structurally and functionally distinguished subgroups (19–22). The conventional PKC isoforms (PKCα, PKCβI, PKCβII, and PKCγ) are regulated by both DAG and Ca2+, the novel isoforms (PKCδ, PKCe, PKCθ, and PKCη) are insensitive to Ca2+, and the atypical isoforms (PKCζ and PKCc/λ) are Ca2+- and DAG-insensitive (23). Even though PKCs share a high degree of homology, it is believed that particular PKC isoforms have unique and specific functions within different cell types (24). In platelets, multiple PKC isoforms are expressed (25–28), and it is presumed that each individual isoform plays one or more distinct roles. Indeed, several studies have underlined a role for PKCs in platelet function, such as aggregation and secretion of granular contents (29–31). However, the role of individual PKC isoforms in platelet function remains to be elucidated. For instance, although it is well established that collagen-induced platelet activation and aggregation through GPVI is mediated by tyrosine kinase Syk and activation of PLCγ2 (32, 33), there is limited information concerning the roles of specific PKC isoforms and their downstream effectors in this process.

This study was therefore undertaken to examine the role of PKCs, in particular PKCδ, in platelet activation and aggregation. Using thrombin and collagen, two physiological and potent platelet activators, we were able to show that PKCδ is essential for collagen-induced but not thrombin-induced platelet aggregation and activation of integrin αIIbβ3. This study also

* This work was supported by the Canadian Institutes of Health Research and the Heart and Stroke Foundation of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Laboratory of Experimental Pathology, Research Center, Montreal Heart Institute, 5000 Belanger St., Montreal, PQ H1T 1C8, Canada. Tel.: 514-376-3330 (ext. 3035); Fax: 514-376-1355; E-mail: yahye.merhi@cmh-hic.org.

2 The abbreviations used are: GP, glycoprotein; PKC, protein kinase C; PLC, phospholipase C; TxA2, thromboxane A2; DAG, diacylglycerol; ERK, extracellular-signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase.
show that PKCδ is positively regulated by αIIbβ3 outside-in signaling and is involved in the release of TxA2 through the MEK/ERK and p38 MAPK signaling pathways, which is required for collagen-induced but not thrombin-induced platelet activation and aggregation.

**EXPERIMENTAL PROCEDURES**

**Materials**—All of the cell-permeable signaling inhibitors were from Calbiochem. The TP receptor (TxA2 receptor) antagonist SQ29548 was from Cayman Chemical (Ann Arbor, MI). The αIIbβ3 receptor antagonists Reopro and Aggrastat came from Centocor (Horsham, PA) and Merck (Whitehouse, NJ), respectively. Phospho-specific antibodies for PKCδ, MEK1/2, ERK1/2, and p38 MAPK came from Cell Signaling Technologies (Beverly, MA) and Santa Cruz Biotechnologies (Santa Cruz, CA). The PKCδ isoform-selective antibody was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY), whereas the anti-MEK1/2, ERK1/2, and p38 antibodies were from cell signaling. Rottlerin (Biomol, Plymouth Meeting, PA) is a cell-permeable PKC inhibitor that selectively inhibits PKCδ (IC50 = 3–6 μM) 5-10-fold more potently than α and β and 13–33-fold more potently than ζ, ξ, and η. In platelets, its widely used at 5–10 μM as a specific inhibitor of the novel PKCδ (25, 34).

**Preparation of Human Platelets**—Venous blood was drawn from healthy volunteers, free from medication known to interfere with platelet function for at least 10 days before the experiment, in accordance with the guideline of the ethical committee of the Montreal Heart Institute. The platelets were prepared as previously described (35, 36). Briefly, the platelets were obtained by series of differential centrifugations and adjusted to a final concentration of 250 × 10⁶/ml using an automated cell counter in which purity exceeded 99%. The platelets were allowed to stand at 37 °C for 30 min before further experiments.

**Platelet Aggregation**—Aggregation of washed platelets was monitored on a four-channel optical aggregometer (Chronolog Corp., Havertown, PA) (35, 36). The platelets were preincubated with inhibitors for 5 min at 37 °C. The samples were then

**FIGURE 1. Phospholipase C signaling is required for platelet activation and aggregation: role of PLC signaling in αIIbβ3 activation, P-selectin translocation, and platelet aggregation.** Isolated human platelets (250 × 10⁶/ml) were preincubated with the PLC inhibitor, U73122 (1–5 μM), prior to stimulation by collagen (2 μg/ml) or thrombin (0.1 unit/ml). αIIbβ3 activation and P-selectin translocation to the membrane (left and middle panels, respectively) were assessed by flow cytometry. Overlay plots (gray, base line; white, control; black, U73122 at 5 μM) shown are presented as the number of events over the log of associated fluorescence and are representative of mean data presented in the lower panels (n = 3; *, p < 0.05 versus base line). Collagen- and thrombin-induced platelet aggregation (right panel) was monitored in the presence or absence of increasing doses of U73122 (1–5 μM) and traces shown are representative of mean data presented in the bottom panels (n = 4; *, p < 0.05 versus base line).
stimulated under continuous stirring (1000 rpm) at 37 °C. Platelet aggregation was recorded and measured 5 min after the addition of the agonists. 

**SDS-PAGE and Immunoblotting**—The platelets were stimulated for the appropriate time with stirring at 37 °C in the presence or absence of inhibitors; the reaction was terminated by the addition of a 4 × SDS-Laemmli sample buffer and then boiled for 10 min. The proteins were resolved by electrophoresis in 8–10% SDS-polyacrylamide gels and subsequently transferred to nitrocellulose. The membranes were blocked with 5% bovine serum albumin at room temperature, incubated overnight at 4 °C with the primary antibody, washed, and labeled with horseradish peroxidase-conjugated secondary antibody at room temperature. Following additional washing steps, proteins were detected by enhanced chemiluminescence (PerkinElmer Life Sciences).

**Subcellular Fractionation of Platelets**—The platelet reactions were terminated by the addition of an equi-volume of ice-cold Triton X-100 lysis buffer (2% Triton X-100, 10 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 20 μg/ml benzamidine, and 100 mM Tris-HCl, pH 7.4), and the samples were allowed to stand on ice for 30 min. The platelet lysates were subsequently centrifuged at 100,000 × g for 2 h at 4 °C to separate the soluble from the insoluble membrane fractions. The pellets (insoluble Triton X-100 membrane fractions) were solubilized in SDS sample buffer, boiled for 5 min, and both fractions were analyzed by SDS-PAGE immunoblotting as described above.

**Flow Cytometry**—αtβ3 activation and P-selectin translocation were assessed by flow cytometry, as described previously (35, 36), using monoclonal antibodies against P-selectin (clone AK4 phycoerythrin-conjugated; Santa Cruz) and the active form of αtβ3 (clone PAC-1 fluorescein isothiocyanate-conjugated; Becton Dickinson, Mississauga, Canada). The platelets (250 × 10^6/ml) were preincubated with inhibitors for 5 min at room temperature prior to cell stimulation. The samples were then fixed with 1% paraformaldehyde for 1 h at 4 °C, washed, and labeled with saturating concentration of monoclonal antibody for 30 min. αtβ3 activation was determined by incubating PAC-1 antibody in platelet suspension prior to activation. All of the samples were analyzed on an Altra flow cytometer (Beckman Coulter, Mississauga, Canada). Nonspecific labeling was excluded using appropriately labeled isotype-matched control IgG. The platelets were identified and gated by their characteristic forward and side scatter properties, and 20,000 platelets were analyzed from each sample.

**Confocal Immunofluorescence and Imaging**—The platelets from the above experiments were fixed with 2% (v/v) paraformaldehyde in phosphate-buffered saline, washed twice, and allowed to immobilize on poly-l-lysine-coated coverslips overnight. Adhered platelets were subsequently blocked with 2% bovine serum albumin and incubated with wheat germ agglutinin with Alexa 488 in 1% bovine serum albumin for 1 h. The coverslips were then permeabilized with 0.1% Triton X-100 in 2% normal donkey serum, incubated with polyclonal anti-PKCα for 3 h, washed, labeled with anti-rabbit IgG-Alexa 555 secondary antibody for 1 h, and mounted on microscopic slides. A series of fluorescent confocal images (Z stacks) were acquired with a LSM 510 confocal microscope (Zeiss, Oberkochen, Germany). Anti-rabbit (donkey) Alexa 555 (Molecular Probes, Eugene, OR) and wheat germ agglutinin with Alexa 488 were visualized using a 543-nm helium-neon laser line and a 488-nm argon laser line, respectively. A 100 × 1.3 Plan-Neofluar objective (Zeiss) was used for magnification. Voxel size is 30 × 30 × 150 nm (X, Y, and Z). Z stacks were deconvolved with the Huygens Pro 2.6.5a software (Scientific Volume Imaging). Deconvolved Z stacks were saved in.tif image file format series and transferred to the LSM510 software.

**Thromboxane A2 Measurement**—The platelets were prepared as described above, pretreated with antagonists or with vehicle solution, and stimulated under stirring conditions at 37 °C. The reaction was terminated by the addition of an equi-volume of ice-cold EDTA solution (10 mM) containing 10 μM of indomethacin, and the samples were centrifuged at 3000 × g for 5 min at 4 °C. Thromboxane B2, the stable metabolite of TxA2, was measured using a correlate-EIA thromboxane B2 enzyme immunoassay kit (Assay Designs, Inc, Ann Arbor, MI), according to the manufacturer’s instructions.

**Data Analysis**—The experiments were performed at least three times on different donors. The results are presented as the means ± S.E. Comparisons of multiple treatments in the same individuals were analyzed by repeated measures analysis of variance with Bonferroni t test for multiple comparisons, whereas treatment groups composed of different individuals were analyzed by one-way analysis of variance. The values with p < 0.05 were considered statistically significant.

**RESULTS**

**Role of PLC in Platelet Activation and Aggregation**—Collagen activates platelets via a tyrosine kinase-dependent cascade that culminates in tyrosine phosphorylation of PLCγ. Activation of PLCγ leads, in turn, to the generation of second messenger inositol 1,4,5-triphosphate and DAG, which mobilize intracellular Ca2+ and activate certain PKC isoforms, respectively (14, 15). Thus, it was important that we first confirm the relative role of PLC in collagen- and thrombin-induced platelet activation and aggregation. As depicted in Fig. 1, collagen- and thrombin-induced platelet activation (αtβ3 activation and P-selectin translocation) and aggregation were inhibited, in a dose-dependent manner, by the PLC inhibitor U73122. The

**FIGURE 2.** Collagen-induced but not thrombin-induced platelet aggregation and activation is PKCα dependent. A, impact of PKC-selective isoform inhibitors on collagen-induced (2 μg/ml) and thrombin-induced (0.1 unit/ml) platelet aggregation. Washed platelets were pretreated with the pan-specific PKC inhibitor (Chelerythrin, 5 μM); specific inhibitors of PKCa (HBDE, 100 μM) and PKCb (Hispidin, 10 μM); the conventional PKCα and β inhibitor (Go-6976, 100 nM); or the specific PKCα inhibitor (Rottlerin, 10 μM) prior to stimulation. Aggregation traces shown are representative of at least three independent experiments. B, role of PKCα in collagen-induced (2 μg/ml) and thrombin-induced (0.1 unit/ml) αtβ3 activation and translocation of P-selectin to the cell surface. Washed platelets were pretreated with the selective PKCα inhibitor, Rottlerin (10 μM) prior to stimulation, and the cells were analyzed by flow cytometry. Overlay plots (gray, base line; white, control; black, Rottlerin) shown are presented as the number of events over the log of associated fluorescence and are representative of mean data presented in the bottom panels (n = 4; *, p < 0.05 versus control).
PKCδ in Platelet Function

**A**

Collagen → PMA → Thrombin → PMA

| Time (s) | 0 | 15 | 30 | 60 | 120 | 300 | 300 |
|----------|---|----|----|----|-----|-----|-----|
| p-PKCδ   |   |    |    |    |     |     |     |
| PKCδ     |   |    |    |    |     |     |     |

**B**

CTL Rott U Reo Agg

|                | B                |                |
|----------------|------------------|----------------|
| Collagen       | B                |                |
| Thrombin       | B                |                |
| p-PKCδ         |                  |                |
| PKCδ           |                  |                |

FIGURE 3. Phosphorylation of PKCδ requires the engagement of αIIbβ3. A, time-dependent phosphorylation of PKCδ. Washed platelets were stimulated with collagen (2 μg/ml) or thrombin (0.1 unit/ml) for the appropriate time; the lysates were subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an anti-phospho-PKCδ antibody. Total kinase levels were measured using a selective PKCδ antibody. The blots shown are representative of three independent experiments. PMA, phorbol myristate acetate; U, effect of Rottlerin (Rott, 10 μM), U73122 (U, 5 μM), Reopro (Reo, 100 nM), and Aggrastat (Agg, 100 nM) on the phosphorylation of PKCδ. The blots were analyzed by densitometry, and the results are depicted as the relative percentages of phosphorylation (n = 4; *, p < 0.05 versus control). B, base line; CTL, control.

PKCδ is essential for collagen-induced but not thrombin-induced platelet function.

Phosphorylation of PKCδ and Regulation by αIIbβ3—Having shown that PKCδ is essential for collagen-induced but not thrombin-induced platelet activation and aggregation, we next evaluated its phosphorylation as an index of its activation. As expected, both collagen and thrombin were able in a time-dependent manner to phosphorylate PKCδ (Fig. 3A). However, PKCδ appears to be phosphorylated more rapidly by thrombin than by collagen, that is, 30 s after stimulation by thrombin as compared with 60 s by collagen. As shown in Fig. 3B, pretreatment of platelets with U73122 completely prevented the phosphorylation of PKCδ by both collagen and thrombin, indicating that PLC is responsible for the activation of PKCδ. Surprisingly, Rottlerin, which selectively inhibits PKCδ, prevented its phosphorylation by collagen but not by thrombin.

Because the results obtained with the phosphorylation state of PKCδ mainly correspond with those obtained with the activation and aggregation of platelets, we presumed that αIIbβ3 might regulate or be involved in the phosphorylation of PKCδ. To support this possibility, we showed that the blockade of αIIbβ3 using Reopro (a monoclonal antibody that specifically binds to αIIbβ3) or Aggrastat (a non-peptide antagonist of αIIbβ3) was able to completely abolish the phosphorylation of PKCδ (Fig. 3B) induced by both collagen and thrombin. These results indicate that αIIbβ3 outside-in signaling may positively modulate the phosphorylation of PKCδ.

Translocation of PKCδ to Cell Membrane and Regulation by αIIbβ3—One particular and important aspect of PKC activation is the intracellular redistribution of the enzyme from the cytosol to the cell membrane. We studied this mechanism using confocal microscopy and subcellular fractionation of platelets into soluble and membrane fractions (Fig. 4). We found that in resting platelets, PKCδ is distributed across the cytosol of the cell and redistributed to the membrane after activation by collagen and thrombin. We also found that in platelets pretreated with Rottlerin, U73122, or Reopro, PKCδ was incapable of translocating to the platelet membrane upon activation by collagen. In contrast, thrombin-induced translocation of PKCδ was not affected by Rottlerin but was inhibited by U73122 and Reopro (Fig. 4). Thus, the phosphorylation and translocation of PKCδ to platelet responses to collagen, but not to thrombin, were completely blocked by PP1 and Piceatannol, which selectively inhibit Src and Syk, respectively (data not shown). These results indicate that platelet activation and aggregation, in response to both collagen and thrombin, occur in a PLC-dependent manner.

Implication of PKCs in Platelet Activation and Aggregation—Because PLC is involved in platelet function and PKCs are downstream effectors, we sought to evaluate the specific role of individual PKC isoforms in platelet function. As illustrated in Fig. 2A, aggregation of platelets induced by collagen and thrombin was completely inhibited by the wide spectrum PKC inhibitor, chelerythrin. However, platelet aggregation was not affected by the PKCa and PKCb inhibitors HBDDE and Hipidin, respectively, or by Gö6976, an inhibitor of both the conventional PKCa and β isoforms. In contrast, collagen-induced platelet aggregation was completely blocked by a selective PKCδ inhibitor (Rottlerin), whereas aggregation of platelets by thrombin remained unaffected by Rottlerin. In addition, activation of αIIbβ3 in response to collagen was completely prevented by PKCδ inhibition, whereas the response to thrombin was less affected (Fig. 2B). On the other hand, P-selectin translocation was slightly reduced by Rottlerin but only in response to thrombin. These results clearly indicate that activation of PKCδ is essential for collagen-induced but not thrombin-induced platelet function.



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Upon platelet activation. In platelets, both thrombin and collagen can activate MAPK signaling pathways, and it is presumed that the PKC family may be involved in this process (37–39). Thus, the MEK/ERK and p38 signal- ing pathways are important downstream effectors of PKCδ in platelets.

**Role of Secondary Mediators in PKCδ Signaling**—It is well established that collagen induces platelet aggregation through a pathway that is primarily mediated by the release of TxA2 and ADP (40–42) and that thrombin-induced platelet aggregation seems to be less dependent upon these mediators. It was therefore important that we determine the role of TxA2 and ADP secretion in collagen-and thrombin-induced PKCδ signaling. As expected, we were first able to confirm the importance of TxA2 in collagen-induced but not thrombin-induced platelet aggregation (Fig. 7A) and activation of integrin αIIbβ3 (Fig. 7B), using the TxA2 receptor antagonist SQ29548 and the COX inhibitor indomethacin. Moreover, the ADP scavenger apyrase also diminished collagen-induced but not thrombin-induced platelet aggregation (Fig. 7A).

In platelets, the PKC family is an important signaling mediator required for activation, secretion of granule contents, and aggregation. In the current study, we examined the role of individual PKC isoforms in platelet activation and aggregation induced by physiological agonists such as colla-

**DISCUSSION**

![Image](image_url)
PKCα in Platelet Function

A

Collagen

Thrombin

U0126

SB202190

B

Control

U0126

SB202190

Collagen

Thrombin
PKCα in Platelet Function

It is well established that collagen supports the adhesion and activation of platelets at sites of vascular injury via αIIbβ3 and GPVI (43, 44). The binding of collagen to GPVI results in receptor clustering and phosphorylation of the tyrosine kinase Syk and the activation of several signaling pathways including PLC. Phospholipase C can then hydrolyze phosphatidylinositol 4,5-bisphosphate and generate DAG, which in turn, activates certain PKC isoforms. In platelets, many PLC isoforms have been identified; all of the evidence suggests that thrombin activates a G-protein-coupled PLCβ pathway, whereas signaling through collagen is mediated by receptor-bound tyrosine kinases and activation of PLCγ (45, 46). Indeed, PLC inhibition using U73122 completely prevented platelet aggregation, αIIbβ3 activation, and P-selectin translocation following platelet activation.

Having confirmed the involvement of PLC in collagen- and thrombin-induced platelet activation and aggregation, we next evaluated the role of individual PKC isoforms in platelet function. First, the implication of PKCs in general was assessed with Chelerytin, a wide spectrum PKC inhibitor that dose-dependently inhibited collagen- and thrombin-induced platelet aggregation, thus supporting a role for PKCs in this process. We were thereafter able to demonstrate that collagen-induced platelet aggregation was mainly mediated by PKCα and not by the conventional PKCα or PKCβ isoforms, whereas thrombin-mediated platelet aggregation appeared to be dependent on multiple PKC isoforms. Indeed, inhibition of thrombin-induced platelet aggregation was only observed with the wide spectrum inhibitor Chelerytin or when the conventional PKC isoforms (PKCα and PKCβ) and the novel PKCα isoform were blocked (data not shown). In contrast, it has been shown that PKCα can interact with the tyrosine kinase Fyn to down modulate platelet function (34).
PKCκ in Platelet Function

A

B

C

JOURNAL OF BIOLOGICAL CHEMISTRY
VOLUME 281 • NUMBER 40 • OCTOBER 6, 2006
the snake venom alboaggregin-A, which is able to activate both GPIb-V-IX and GPVI on platelets. In our study, collagen, a physiological agonist, was employed to achieve platelet activation, and thus only signaling events mediated through GPVI and possibly αIIbβ3 were studied. It seems, therefore, that PKCδ may play dichotomous roles in platelet aggregation, possibly involving differential regulatory mechanisms related to the signaling pathways triggered by various specific receptors.

Platelet aggregation is a process that is accomplished by the adhesive interaction of two active αIIbβ3 receptors on adjacent platelets, via a fibrinogen bridge. Given that PKCδ is required to achieve this process, we next evaluated its involvement in the activation of integrin αIIbβ3 and in the translocation of P-selectin to the platelet surface upon activation. As expected, collagen-induced activation of αIIbβ3 was totally dependent on PKCδ, which explains the incapacity of platelets to aggregate in the presence of Rottlerin. On the other hand, PKCδ inhibition had no effect on thrombin-induced platelet aggregation while slightly reducing αIIbβ3 activation. These observations suggest the presence of a αIIbβ3 receptor threshold level required to mediate platelet aggregation. P-selectin translocation to the platelet surface, a marker of α granule secretion, can also be used as an index of platelet activation. The PKC family appears to be involved in this process through its capacity to phosphorylate a number of platelet proteins involved in the secretion of granule contents, such as platelet Sec 1 and syntaxin 4 (47–49).

Here, we have demonstrated that PKCδ does not appear to be required for collagen-induced P-selectin translocation and seems to have only a minor impact in this process in thrombin signaling. These results add new insights to the mechanisms of platelet P-selectin translocation; they also confirm our previous findings showing that the novel α granule secretion, can also be used as an index of platelet activation. The PKC family appears to be involved in this process through its capacity to phosphorylate a number of platelet proteins involved in the secretion of granule contents, such as platelet Sec 1 and syntaxin 4 (47–49).

Phosphorylation of PKC is an essential regulatory mechanism required for complete kinase activity. Hence, having showed the importance of PKCδ in platelet function, it was important that we study the phosphorylation state of PKCδ after activation of platelets by collagen and thrombin. Some investigators have reported PKCδ to be phosphorylated upon stimulation by platelet activators such as thrombin, convulxin, and alboaggregin-A (25, 34, 51). Activation of PKCδ by collagen, however, has yet to be demonstrated. As expected, both collagen and thrombin were able to time-dependently phosphorylate PKCδ, as soon as 30 s for thrombin and 60 s for collagen. These times happen to coincide with the onset of aggregation induced by both these activators. When evaluating the impact of PLC (U73122) and PKCδ (Rottlerin) inhibition on the phosphorylation activity of PKCδ, as compared with the impact of these inhibitors on platelet aggregation, we found these two phenomena to correlate with one another. These observations lead us to believe that αIIbβ3, the main platelet integrin responsible for the formation of a platelet aggregate, might be participating in the regulation of the phosphorylation activity of PKCδ. This hypothesis was further demonstrated with functional αIIbβ3 antagonists (Reopro and Aggrestat), which inhibit platelet aggregation, binding to fibrinogen and subsequent outside-in signaling. In the presence of Reopro and Aggrestat, we were unable to detect any given phosphorylation of PKCδ after stimulation by either collagen or thrombin, thus supporting a role for αIIbβ3 outside-in signaling in the regulation of PKCδ. This regulatory mechanism most likely involves a positive feedback loop that maintains PKCδ phosphorylated, as recently demonstrated for the platelet PKCδ isoform (52). Platelet function is normally accompanied by phosphorylation and dephosphorylation of many proteins. Phosphatase activities are important regulators of signal transduction in platelets. Indeed, a protein-tyrosine phosphatase inhibitor (vanadate) has been shown to stimulate protein tyrosine phosphorylation and to increase all platelet functions (53). Blockade of secondary αIIbβ3 outside-in signaling prevents PKCδ phosphorylation, suggesting that such a signal is involved in the regulation of PKCδ, possibly via phosphatase inhibition. On the other hand, it has been reported that thrombin-induced activation of PKCδ is achieved by a αIIbβ3-independent pathway (51). However, the investigators in that study worked with very high thrombin concentrations (5 units/ml); it is likely that at these concentrations, PKCδ dephosphorylates much more slowly, thereby allowing the detection of a phosphorylation even in the absence of αIIbβ3 outside-in signaling. In the present study, we presume that PKCδ is activated independently of αIIbβ3 (the initial activation of PKCδ precedes the activation of αIIbβ3) but that it may be regulated by the latter. αIIbβ3 outside-in signaling contributes to the adhesive strengthening of the platelet plug and allows irreversible aggregation, both mechanisms being essential for the formation of stable and adequate platelet aggregates (54). The importance of αIIbβ3 outside-in signaling in the regulation of PKCδ was further pointed out when studying the translocation of the enzyme from the cytosol to the cell membrane upon activation (another important aspect of PKC activation). In resting platelets, PKCδ appeared to be randomly distributed in the cytosol and to rapidly translocate to the cell surface upon activation by collagen and thrombin. In experiments in which αIIbβ3 outside-in signaling was blocked (Repro), no translocation of PKCδ to the cell membrane upon activation was observed. Thus, the regulation of PKCδ by αIIbβ3 could be of physiological significance in platelet function.

Having established the role of PKCδ in platelet activation and aggregation, we then evaluated the involvement of the MAPK

**FIGURE 7. PKCδ is involved in TxA2 generation by platelets.** A, role of secondary mediators in collagen- and thrombin-induced platelet aggregation. Washed platelets were isolated and preincubated with vehicle solution or with the indicated inhibitors at 37°C for 5 min prior to stimulation with collagen (2 μg/ml) or thrombin (0.1 unit/ml). The traces shown are representative of three independent experiments. B, implication of TxA2 release by platelets in the activation of αIIbβ3. The Platelets were treated with the selective TxA2 receptor antagonist SQ9548 (50 μM), stimulated with collagen or thrombin, and analyzed by flow cytometry. The results are presented as dot plots of the log of αIIbβ3 fluorescence (fluorescein isothiocyanate) versus forward scattering and are representative of three independent experiments. C, role of PKCδ in the release of TxA2 by platelets. The platelets were incubated with vehicle solution (control) or with the indicated signaling inhibitors (U73122 (5 μM), Rottlerin (10 μM), U0126 (50 μM), SB202190 (50 μM), Reopro (100 nM), and indomethacin (10 μM)) prior to stimulation with collagen or thrombin. The supernatants were then analyzed for thromboxane B2, the stable metabolite of TxA2, by EIA (n = 3; *p < 0.05 versus control).
FIGURE 8. Diagram summarizing the role of PKCδ in platelet signaling. Collagen induces platelet signaling through its binding to GPVI and αIIbβ3, which represent the two main collagen receptors on the platelet surface. This leads, in turn, to sequential activation of the tyrosine kinases Src and Syk, which then phosphorylate PLCγ. The latter activates PKCδ and subsequently the MEK/ERK and p38 MAPK signaling pathways, which are ultimately responsible for the synthesis and secretion of TxA2. Released TxA2 can, in turn, bind to its receptor (TP) on the cell surface and activate the αIIbβ3 integrin through a pathway still to be elucidated. Finally, binding of fibrinogen to activated αIIbβ3 induces outside-in signaling that leads, in part, to a regulatory positive feedback loop, which maintains PKCδ phosphorylated. In thrombin-induced platelet signaling, PKCδ is also responsible for the synthesis and release of TxA2, primarily through activation of the MEK/ERK/p38 pathway. However, secretion of TxA2 does not appear to be required for the activation of αIIbβ3 or the aggregation process. Activation of αIIbβ3 appears to be regulated by more than one PKC isoform (Fig. 2A). In thrombin-stimulated platelets, outside-in signaling via fibrinogen binding to αIIbβ3 also contributes to maintain PKCδ phosphorylated.

signaling pathway in these platelet responses. It has been reported that physiological agonists such as collagen and thrombin can activate ERK1/2 and p38 in platelets; it also appears that the PKC family may be involved in this process (37–39). However, the exact role of the MAPK signaling cascade in platelet function remains unclear. Here, we have shown that collagen-induced but not thrombin-induced platelet aggregation and activation of αIIbβ3 are dependent on the MAPK signaling pathway, as demonstrated by the MAPK kinase inhibitors U0126 and SB202109. These results are in agreement with a previous study indicating that MEK1 is not involved in thrombin nor U46619 platelet response, although it plays an important role in collagen-induced platelet aggregation (55). In addition, we demonstrated that MEK1/2, ERK1/2, and p38 are activated by collagen and thrombin, and more importantly, established the requirement for PKCδ and PLC activation in this process. Moreover, we found that inhibition of αIIbβ3 slightly affected the phosphorylation activity of MEK1/2, ERK1/2, and p38, which indicates that the regulation of PKCδ phosphorylation exerted by αIIbβ3 outside-in signaling is not required for the activation and phosphorylation of PKCδ substrates and downstream effectors, such as the MAPK signaling cascade. These results indicate, too, that the αIIbβ3 outside-in signaling cascade involved in the regulation of PKCδ most likely involves a signaling pathway, downstream of PKCδ, other than that MEK/ERK and p38.

Secondary mediators, such as ADP and TxA2, have been reported to be involved in collagen-induced platelet aggregation; it was therefore important for us to establish the role of these mediators in PKCδ signaling. As expected, we were first able to confirm the importance of ADP and most importantly, TxA2 in collagen-induced but not in thrombin-induced platelet aggregation and activation of αIIbβ3. Furthermore, we showed that both collagen- and thrombin-induced TxA2 release by platelets strongly depends on PKCδ signaling, in addition to its upstream (PLC) and downstream (MEK/ERK/p38) effectors. These data suggest that the PKCδ/MEK/ERK/p38 signaling cascade is intimately involved in the generation and release of TxA2 in platelets, which appears to be essential for collagen-induced but not thrombin-induced platelet function. Also, because blockage of αIIbβ3 showed little or no effect on the release of TxA2, we can again conclude that the αIIbβ3 outside-in signaling cascade involved in the regulation of PKCδ does not lead to the activation of TxA2. In this connection, it has been recently shown that thrombin-mediated (via proteinase-activated receptor activation) TxA2 release by platelets was dramatically blocked by PKCδ inhibition (56). Here we confirm these observations and further elucidate the mechanisms by which this platelet phenomenon occurs, not only in thrombin-mediated but also in collagen-mediated signaling.

In conclusion, this study adds new insights to the role of PKCδ in platelet signaling and function (Fig. 8). After the binding of platelet receptors to collagen, phosphorylation of the tyrosine kinases Syk and Src lead in part to the activation of PKCδ, through the action of PLCγ. PKCδ then triggers activation of the MEK/ERK and p38 signaling pathways, which ultimately result in the generation and release of TxA2. Secretion of TxA2 in platelets is well known to cause recruitment and activation of platelets at sites of vascular injury. In this study, we have shown that the release of TxA2 is essential for collagen-induced αIIbβ3 activation and platelet aggregation. In thrombin-mediated platelet signaling, PKCδ is also required for the release of TxA2, and this platelet response occurs in a MEK/ERK/p38-dependent manner. In contrast to collagen-induced platelet signaling, this release does not appear to be essential for platelet function. Finally, binding of fibrinogen to αIIbβ3 trig-
ACKNOWLEDGMENT—We thank Dr. Danielle Libersan for technical assistance and help with the manuscript.

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