Protein Kinase Cδ Differentially Regulates Platelet Functional Responses

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Objective—Protein Kinase C delta (PKCδ) is expressed in platelets and activated downstream of protease-activated receptors (PARs) and glycoprotein VI (GPVI) receptors. The purpose of this study was to investigate the role of PKCδ in platelets.

Methods and Results—We evaluated the role of PKCδ in platelets using two approaches—pharmacological and molecular genetic approach. In human platelets pretreated with isoform selective antagonistic RACK peptide (δV1-1)TAT, and in the murine platelets lacking PKCδ, PAR4-mediated dense granule secretion was inhibited, whereas GPVI-mediated dense granule secretion was potentiated. These effects were statistically significant in the absence and presence of thromboxane A2 (TXA2). Furthermore, TXA2 generation was differentially regulated by PKCδ. However, PKCδ had a small effect on platelet P-selectin expression. Calcium- and PKC-dependent pathways independently activate fibrinogen receptor in platelets. When calcium pathways are blocked by dimethyl-BAPTA, AYPGKF-induced aggregation in PKCδ null mouse platelets and in human platelets pretreated with (δ V1-1)TAT, was inhibited. In a FeCl3-induced injury in vivo thrombosis model, PKCδ+/− mice occluded similar to their wild-type littermates.

Conclusions—Hence, we conclude that PKCδ differentially regulates platelet functional responses such as dense granule secretion and TXA2 generation downstream of PARs and GPVI receptors, but PKCδ deficiency does not affect the thrombus formation in vivo. (Arterioscler Thromb Vasc Biol. 2009;29:699-705.)

Key Words: platelets ■ thrombosis ■ protein kinase C ■ fibrinogen ■ secretion

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proteins and platelet-specific proteins. One of the components of α-granules is P-selectin, which is expressed on the surface of activated platelets and aids in platelet-leukocyte interaction. After platelet secretion, TXA2 is generated which diffuses across the platelet membrane and recruits more platelets to the site of injury. Activation of platelets by physiological agonists such as thrombin and collagen result in the generation of thromboxane. At low doses of agonist, secretion is dependent on TXA2 generation and thus further amplification of platelet functional responses ensue. These events result in the formation of platelet plug. Inside-out signaling results in the activation of fibrinogen receptor αIIbβ3. There are 2 pathways by which the fibrinogen receptor can be activated, a Ca2+-dependent pathway and a PKC-dependent pathway. However, the specific PKC isoforms that can cause activation of αIIbβ3 in the absence of Ca2+ has not been investigated yet.

Protein Kinase Cs have been implicated in platelet secretion. Of the novel isoforms, PKCδ is implicated in inhibition of cell growth, cell differentiation, apoptosis, and tumor suppression in immune cells. PKCδ is activated by strong agonists such as thrombin and collagen, but not by ADP. Activated PKCδ positively regulates PAR-mediated dense granule secretion and negatively regulates GPVI-mediated dense granule secretion. However, recent reports have raised concerns regarding the specificity of rottlerin.

In this study, by combining the pharmacological approaches with the use of platelets from PKCδ knockout mice, we investigated the role of PKCδ in platelet functional responses. We show that PKCδ differentially regulates PAR and GPVI-mediated dense granule secretion and TXA2 generation. These effects translate into faster time to occlusion leading to thrombus formation in vivo.

Materials and Methods

Approval for this study was obtained from the Institutional Review Board of Temple University (Philadelphia, Pa), and mice were used for physiological measurements using the protocol approved by the Institutional Animal Care and Use Committee (IACUC).

Materials

Convulxin, fibrinogen (Fraction I, type I), apyrase grade VII, thrombin, and acetylsalicylic acid were obtained from Sigma. Luciferin-luciferase reagent was purchased from Chrono-Log. Antiphospho and anti-PKCδ antibodies were obtained Santa Cruz Biotechnology Inc. The acetyloxyethyl ester of the calcium chelator 5,5′-dimethyl-bis-(o-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid (dimethyl BAPTA) was purchased from NEN. All the other reagents were of reagent grade, and deionized water was used throughout.

Animals

PKCδ−/− (C57BL/6 background) mice and their age-matched wild-type littermates were used. These mice were obtained from Dr Keiko Nakayama (Tohoku Graduate School of Medicine, Japan). All the mice obtained from the source were transferred to the Central Animal Facility (CAF) under the head and director of the CAF in Temple University School of Medicine.

Preparation of Washed Human and Murine Platelets

The platelets were isolated from humans and mice according to previously established protocol.

Measurement of Platelet Dense Granule Secretion in Human and Mouse Platelets

ATP secretion from platelet dense granules was determined in aspirin-treated human platelets or indomethacin-treated mouse platelets by using the Luciferin-Luciferase assay. The platelets were treated with aspirin (1 mmol/L) or indomethacin (10 μmol/L) for 1 minute and then stimulated with agonists in a lumi-aggregometer at 37°C with stirring at 900 rpm, and the corresponding luminescence was measured.

Measurement of Platelet α-Granule Secretion in Murine Platelets

P-selectin (CD62) expression on platelets from PKCδ−/− murine platelets and those from wild-type littermates was measured according to a established protocol using FITC-labeled anti-CD62 antibody (Becton Dickinson).

Measurement of thromboxane A2 Generation

Washed murine platelets without indomethacin treatment were stimulated in a platelet aggregometer, and levels of TXB2 were determined according to established protocol.

In Vivo Thrombosis Model

Adult mice (6 to 8 weeks old, weight ~25 gms) were anesthetized by intraperitoneal injection of pentobarbital (40 mg/kg). Experimental groups consisted of PKCδ−/− and PKCδ+/+ mice (n=11). The mice were subjected to thrombosis according to a established protocol. The operator was blinded to mouse genotype while performing all experiments.

Statistical Analysis

The results were quantitated, expressed as mean±SD. The data were statistically analyzed using Student t test and P<0.05 was considered significant.

Results

PKCδ Differentially Regulates PARs and GPVI-Mediated Dense Granule Secretion in Human Platelets

Previous studies from our laboratory and others have shown that PKCδ is important for PAR-mediated dense granule release, but it negatively regulates GPVI-mediated secretion. However, as these studies used a nonselective inhibitor rottlerin, we investigated the role of PKCδ using more selective tools to block this isoform. First, we used (δ V1-1)TAT (1 μmol/L), an anti-PKCδ RACK peptide, which specifically blocks translocation of PKCδ to its target substrate. When human platelets were preincubated for 15 minutes with (δ V1-1)TAT (1 μmol/L), 200 μmol/L AYPGKF-induced dense granule release in aspirin-treated human platelets was inhibited (Figure 1A). However, when the platelets were treated with a control peptide, there was no effect on AYPGKF-induced secretion (Figure 1A) and these were statistically significant (P<0.05). Similar experiments were performed by stimulating platelets with 60 ng/mL convulxin, a GPVI agonist. Contrarily, convulxin-induced dense granule release was potentiated by the pretreatment of...
platelets for 15 minutes with PKCδ specific antagonistic RACK peptide (Figure 1B). However, the control peptide did not affect the GPVI-mediated secretion (Figure 1B). The potentiation of convulxin-induced ATP secretion by (Δ V1-1)TAT was also statistically significant (Figure 1B). These results indicate that PKCδ positively regulates PAR-mediated dense granule secretion, whereas it negatively regulates GPVI-mediated dense granule secretion.

Regulation of Dense Granule Secretion in PKCδ Null Murine Platelets
As the specificity of the pharmacological agents might be argued, we used platelets from mice lacking PKCδ isoform. PKCδ knock out mice were generated by 2 groups.14,27–29 Although this PKC is ubiquitously expressed, the mice that do not express PKCδ show a clear phenotype only in immune cells.14 Indomethacin-treated platelets from PKCδ−/− mice and their age-matched wild-type littermate controls were stimulated with submaximal doses of AYPGKF (200 μmol/L), or GPVI agonists- convulxin (60 ng/mL), collagen-related peptide (CRP, 5 μg/mL), or collagen (20 μg/mL). Platelet aggregations and dense granule secretions were measured in these platelets on agonist stimulation. As shown in Figure 2A, aggregations and dense granule secretions were inhibited on stimulation with AYPGKF in PKCδ−/− murine platelets compared to wild-type littermates. On the other hand, secretion induced by GPVI agonists were potentiated. These results are consistent with the findings

Figure 1. PKCδ differentially regulates PARs and GPVI-mediated dense granule secretion in human platelets. Washed aspirin-treated human platelets were preincubated with 1 μmol/L (Δ V1-1)TAT, an anti- PKCδ RACK peptide or control peptide for 15 minutes, stimulated with 200 μmol/L AYPGKF or 60 ng/mL CVX, and dense granule secretions were measured. PAR-mediated dense granule secretions and GPVI-mediated dense granule secretions in the presence of indomethacin are shown along with the actual values of ATP secretion represented next to each secretion tracing (A). The data are representative of mean±SD from 3 independent experiments (n=3). The data were analyzed for statistical significance by Student t test, and P≤0.05 was considered significant. *P<0.05.

Figure 2. PAR and GPVI-mediated dense granule secretions are differentially regulated by PKCδ in murine platelets. Washed indomethacin-treated murine platelets were stimulated with 200 μmol/L AYPGKF, 60 ng/mL CVX, 5 μg/mL CRP, and 20 μg/mL collagen for 3 minutes at 37°C under stirring conditions. Agonist-induced aggregations and dense granule secretions were recorded. Secretions were measured in 4 independent experiments (n=4) and quantitated as ATP released in nmoles (A). Dense granule secretions were also measured in the absence of indomethacin (n=3) and quantitated as ATP released in nmoles and represented as bar graphs (B). The data were analyzed by Student t test, and P≤0.05 was considered significant. *P<0.05, **P<0.01.
using (δ V1-1) TAT in human platelets. Dense granule secretions were also measured in the absence of indomethacin and inhibition or potentiation was found to be statistically significant (Figure 2B). The actual ATP released in nmoles was quantitated and the inhibition or potentiation were statistically significant (Figure 2A and 2B). These data suggest that PKCδ differentially regulates PARs and GPVI-mediated dense granule secretions in murine platelets.

**PKCδ Regulates PAR and GPVI-Mediated α-Grainule Secretion**

The exposure of P-selectin from α-granules aids in different cell–cell interaction between platelets, leukocytes, and endothelial cells.\(^{11,30}\) As PKCδ differentially regulates dense granule secretion, we investigated the role of this isoform in α-granule secretion. Washed indomethacin-treated murine platelets from PKCδ\(^{-/-}\) and wild-type littersmates were stimulated with submaximal doses of AYPGKF or convulxin and P-selectin exposure was measured by flow cytometry. Our results showed that PAR-mediated α-granule secretion, expressed as Mean Fluorescence Intensity (MFI), was inhibited and GPVI-mediated α-granule secretion was potentiated marginally in PKCδ\(^{-/-}\) compared to wild-type littersmates (Figure 3). These results indicate that PKCδ plays a differential role in regulating α-granule secretion downstream of PARs and GPVI receptors.

**Role of PKCδ in PAR and GPVI-Mediated Thromboxane A\(_2\) Generation**

We have previously shown, using rottlerin, that PKCδ regulates PAR-mediated thromboxane A\(_2\) (TXA\(_2\)) generation.\(^{31}\) Therefore, we next investigated the contribution of PKCδ to TXA\(_2\) generation downstream of PARs and GPVI receptors using platelets from PKCδ-null mice. PAR-mediated TXA\(_2\) generated was inhibited in PKCδ\(^{-/-}\) murine platelets compared to the wild-type littersmates (Figure 4A). On the other hand, GPVI-mediated TXA\(_2\) generated was potentiated in PKCδ\(^{-/-}\) murine platelets compared to the wild-type littersmates (Figure 4B). These statistically significant results (\(P<0.05\)) suggest that PKCδ positively regulates PAR-mediated TXA\(_2\) generation.
tion, whereas it negatively regulates GPVI-mediated TXA₂ generation.

**PKCδ Plays a Role in Regulating PKC-Dependent Fibrinogen Receptor Activation**

Calcium and PKC pathways can independently cause PAR and GPVI-mediated fibrinogen receptor activation. To investigate the role of PKCδ in PKC-dependent fibrinogen receptor activation, washed aspirin-treated human platelets were preincubated with dimethyl BAPTA (10 μmol/L) for 5 minutes, which completely blocks the calcium-dependent pathways. We confirmed the effectiveness of BAPTA by measuring secretion on stimulation with AYPGKF, which was abolished. These BAPTA-treated platelets were then preincubated with (δ V1-1)TAT or control peptide for 10 minutes, stimulated with submaximal doses of PAR4 agonist peptide, AYPGKF (200 μmol/L) in the presence of fibrinogen (1 mg/mL). This resulted in the inhibition of AYPGKF-induced aggregation, whereas the control peptide had no effect (Figure 5A). Similarly, when indomethacin-treated PKCδ⁻/⁻ and wild-type platelets were preincubated with BAPTA for 5 minutes, AYPGKF-induced (200 μmol/L), aggregation was inhibited (Figure 5B). These results indicate that PKCδ is one of the isoforms contributing to PKC-dependent platelet aggregation. However, as the aggregation was not abolished in the case of pan-PKC inhibitors, suggesting that other PKC isoforms might also be contributing to this event.

**Regulation of Thrombus Formation by PKCδ In Vivo**

We investigated whether the results using ex vivo platelets translate into an in vivo aberration in thrombus formation in mice. Hence, we evaluated the role of PKCδ in thrombus formation in vivo using FeCl₃-induced carotid artery injury model. As shown in Figure 6A and 6B, the time to occlusion (TTO) in a representative wild-type and PKCδ⁻/⁻ mice were 6.5 minutes and 4 minutes, respectively. However, the time to occlusion of wild-type (12.85±11.13 minutes) and PKCδ⁻/⁻ (7.94±7.56 minutes) shown in Figure 6C (n=11) indicate that the differences in times to occlusion are not statistically significant (P>0.1) as judged by Student t test. These results indicate that PKCδ deficiency does not affect the thrombus formation in vivo.

**Discussion**

Previously, differential regulation of dense granule secretion by PKCδ isoform downstream of PAR and GPVI receptors was studied using rottlerin. However, recent reports have indicated problems regarding the specificity of rottlerin as a PKCδ inhibitor. It inhibits other protein kinases such as MAPK-activated protein kinase 2 and p38 regulated/activated kinase, and other enzymes such as β-lactamase, chymotrypsin, and malate dehydrogenase. To substantiate the previous findings with rottlerin, we used 2 complementary approaches in the current study. The first approach is the pharmacological approach, using (δ V1-1)TAT, an anti-PKCδ RACK peptide, and the second approach, extending the pharmacological findings, is the use of mice lacking PKCδ.

For the pharmacological inhibition of PKCδ, (δ V1-1)TAT was used. The specificity of the (δ V1-1)TAT peptide has been well established by previous studies, wherein the anti-PKCδ RACK peptide was shown to inhibit the translocation of PKCδ but not PKCα, β, etc. Furthermore, in platelets, 2MeSADP does not activate PKCδ. 2MeSADP-induced aggregations were not affected in human platelets that were pretreated with anti-PKCδ RACK peptide suggesting that this peptide is specific for PKCδ isoform (data not shown). Our results using (δ V1-1)TAT in human platelets and platelets from mice lacking PKCδ showed inhibition of PAR-mediated and potentiation of GPVI-mediated platelet dense granule secretions and TXA₂ generation. The effects of PKCδ were statistically significant only at submaximal doses of the agonist and not at higher doses (data not shown). Thus, PKCδ differentially regulates dense granule secretion and thromboxane generation in platelets downstream of PARs and GPVI.

Previous findings from Pula et al. reported that PKCδ does not play any significant role in GPVI-mediated dense granule secretion and fibrinogen binding. Our results are in contrast with these findings, because we observed a negative regulatory role for this PKC isoform downstream of GPVI in this study. There are plausible explanations for these differ-
In conclusion, PKCδ differentially contributes to the regulation of PAR- and GPVI-mediated secretion and TXA2 generation. However, the altered platelet functional responses in PKCδ null mouse platelets do not affect thrombus formation in the injured artery in these mice.
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Disclosures
None.

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