Differential Role of Protein Kinase Cδ Isoform in Agonist-induced Dense Granule Secretion in Human Platelets*

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Swaminathan Murugappan‡§, Florin Tuluc‡, Robert T. Dorsam‡¶, Haripriya Shankar‡, and Satya P. Kunapuli‡§**

From the Departments of ‡Physiology and ¶Pharmacology and the §§Sol Sherry Thrombosis Research Center, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Several platelet agonists, including thrombin, collagen, and thromboxane A₂, cause dense granule release independently of thromboxane generation. Because protein kinase C (PKC) isoforms are implicated in platelet secretion, we investigated the role of individual PKC isoforms in platelet dense granule release. PKCδ was phosphorylated in a time-dependent manner that coincided with dense granule release in response to protease-activated receptor-activating peptides SFLLRN and AYPGKF in human platelets. Only agonists that caused platelet dense granule secretion activated PKCδ. SFLLRN- or AYPGKF-induced dense granule release and PKCδ phosphorylation occurred at the same respective agonist concentration. Furthermore, AYPGKF and SFLLRN-induced dense granule release was blocked by rottlerin, a PKCδ selective inhibitor. In contrast, convulxin-induced dense granule secretion was potentiated by rottlerin but was abolished by Go6976, a classical PKC isoform inhibitor. However, SFLLRN-induced dense granule release was unaffected in the presence of Go6976. Finally, rottlerin did not affect SFLLRN-induced platelet aggregation, even in the presence of dimethyl-BAPTA, indicating that PKCδ has no role in platelet fibrinogen receptor activation. We conclude that PKCδ and the classical PKC isoforms play a differential role in platelet dense granule release mediated by protease-activated receptors and glycoprotein VI. Furthermore, PKCδ plays a positive role in protease-activated receptor-mediated dense granule secretion, whereas it functions as a negative regulator downstream of glycoprotein VI signaling.

Platelets are an important part of the hemostatic mechanism that is activated following vascular injury (1). Upon activation, platelets secrete their granule contents that help amplify platelet responses to many of the physiological agonists. Human platelets contain two types of morphologically distinguishable storage granules: α-granules and dense granules. Substances released from the α-granules supplement the coagulation cascade at the site of vascular injury and hence contribute to the procoagulant function of platelets. The dense granules contain, among other components, ADP that is essential for recruiting platelets to the site of vascular injury.

Downstream of G protein-coupled receptor (GPCR) stimulation, G₁₃ is important for platelet secretion (2). In platelets from patients deficient in G₁₃, or phospholipase Cγ₂ (PLCγ₂), thrombin and thromboxane A₂ receptor stimulation results in markedly decreased platelet secretion (3, 4). Also, G₁₃-deficient mouse platelets do not secrete in response to thromboxane A₂ and thrombin (2). Similarly, collagen fails to cause dense granule secretion in PLCγ₂-deficient mouse platelets (5). Activation of PLC leads to generation of inositol 1,4,5-triphosphate and diacylglycerol (3, 6). Downstream of PLC activation, platelet dense granule secretion is dependent on increases in intracellular calcium and protein kinase C (PKC) activation, because blocking calcium or PKC significantly inhibits dense granule secretion (7–14).

PKC enzymes are members of the extended AGC (protein kinase A, G, and C) family, comprising phospholipid-dependent serine/threonine kinases that are involved in a wide spectrum of signal transduction pathways in response to a variety of extracellular stimuli (15–17). Following activation, these kinases migrate to different subcellular locations including the plasma membrane (18) and cytoskeletal elements (19) where they regulate different physiological functions (15–17). PKC isoforms are subdivided into three groups based on their lipid and cofactor requirements (20, 21): the diacylglycerol- and calcium-sensitive conventional isoforms (α, β₁, β₁I, and γ), the diacylglycerol-sensitive and calcium-insensitive novel isoforms (δ, η, θ, and ε), and the phosphatidylinositol triphosphate-sensitive atypical isoforms (ζ, ι, μ, and λ). Like the other members of the AGC family of kinases, PKC has three distinct phosphorylation sites: the activation loop, the turn motif, and the hydrophobic residues in the carboxyl terminus (22–25). The activation loop phosphorylation site sequence is highly conserved among the PKC isoforms, and this phosphorylation event is required to align the residues properly at the active site and for the catalytic activity of the enzyme (22–25).

In this study, we show that PKCδ plays an important role in regulating agonist-induced platelet dense granule release and that it plays a differential role downstream of protease-activated receptors (PARs) and glycoprotein VI (GPVI).

EXPERIMENTAL PROCEDURES

Materials—Apyrase (type VII), fibrinogen (type I), bovine serum albumin (fraction V), thrombin, phosphor 12-myristate 13-acetate, and acetylsalicylic acid were obtained from Sigma. ADP, epinephrine, and CHRONO-LUME reagent were purchased from Chrono-log Corp. (Havertown, PA). 2,5-Dimethyl-5′,5′-dimethyl-bis-(o-aminophenoxycetone)-2,5′-tetraacetic acid; GPVI, glycoprotein VI; PAR, protease-activated receptor; PKC, protein kinase C; PLC, phospholipase C.

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** To whom correspondence should be addressed: Dept. of Physiology, Temple University, Rm. 224, OMS, 3420 N. Broad St., Philadelphia, PA 19140. Tel.: 215-707-4615; Fax: 215-707-4003; E-mail: spk@temple.edu.

1 The abbreviations used are: GPCR, G protein-coupled receptor; diethyl-BAPTA, 5,5′-dimethyl-bis-(o-aminophenoxycetone)-2,5′-tetraacetic acid; GPVI, glycoprotein VI; PAR, protease-activated receptor; PKC, protein kinase C; PLC, phospholipase C.
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**Isolation of Human Platelets**—Whole blood was drawn from healthy, consenting human volunteers into tubes containing one-sixth volume of ACD (2.5 g of sodium citrate, 1.5 g of citric acid, and 2 g of glucose in 100 ml of deionized water). Blood was centrifuged (Eppendorf 5810R centrifuge, Hamburg, Germany) at 230 × g for 20 min at room temperature to obtain platelet-rich plasma. Platelet-rich plasma was incubated with 1 mM acetylsalicylic acid for 30 min at 37°C. The platelet-rich plasma was then centrifuged for 10 min at 980 × g at room temperature to pellet the platelets. Platelets were resuspended in Tyrode’s buffer (138 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 3 mM NaH₂PO₄, 5 mM glucose, 10 mM Hepes, pH 7.4, 0.2% bovine serum albumin) containing 0.01 unit/ml aprotinin. Cells were counted using a Coulter Z1 particle counter, and the concentration of the cells was adjusted to 2 × 10⁹/ml. All experiments using washed platelets were performed in the absence of extracellular calcium unless otherwise mentioned.

**Measurement of Platelet Secretion**—Platelet secretion was determined by measuring the release of ATP using the CHRONO-LUME reagent. The activation of platelets was performed in a lumiggregometer at 37°C with stirring at 900 rpm, and the secretion was measured and expressed in nmol of ATP released/10⁹ platelets. The data were normalized to the maximum secretion as indicated in the figure legends. In experiments in which inhibitors were used, the platelet sample was incubated with the inhibitors for 15 min at 37°C prior to the addition of agonists. The secretion was subsequently measured as described above.

**Aggregation**—Aggregation of 0.5 ml of washed platelets was analyzed using a PICA lumiggregometer (Chrono-log Corp.). Aggregation was measured using light transmission under stirring conditions (900 rpm) at 37°C. Agonists were added simultaneously for platelet stimulation; however, each inhibitor was preincubated as follows: 10 μM dimethyl-BAPTA, for 10 min at 37°C; 10 μM Ro 31-8220, 3 min at 37°C; 5 μM rrotterlin and 100 nM Go6976, for 15 min at 37°C. Each sample was allowed to aggregate for at least 3 min. The chart recorder (Kipp and Zonen, Bohemia, NY) was set for 0.2 mm/s.

**Western Blot Analysis**—Platelets were stimulated with agonists for the appropriate time, and the reaction was stopped by the addition of 3 × SDS-Laemmli buffer. Platelet lysate were boiled for 10 min, and proteins were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membrane. Non-specific binding sites were blocked by incubation in Tris-buffered saline and Tween (TBST; 20 mM Tris, 140 mM NaCl, 0.1% (v/v) Tween 20) containing 0.5% (w/v) milk protein and 3% (w/v) bovine serum albumin for 30 min at room temperature, and membranes were incubated overnight at 4°C with the primary antibody (1:10,000 dilution in TBST with 2% bovine serum albumin) with gentle agitation. After three washes for 5 min each with TBST, the membranes were probed with an alkaline phosphatase-labeled secondary antibody (1:5,000 dilution in TBST with 2% bovine serum albumin) for 1 h at room temperature. After additional washing steps, membranes were then incubated with CDP-Star chemiluminescent substrate (Roche, Indianapolis, Bedfont, MA) for 10 min at room temperature, and immunoreactivity was detected using a Fuji Film Luminescent Image Analyzer (LAS-1000 CH, Japan).

**RESULTS**

**Presence of Different PKC Isoforms in Human Platelets**—We first investigated the presence of various PKC isoforms in human platelets. This was achieved by Western blot analysis of platelet protein lysate using commercially available antibodies directed against specific PKC isoforms. As shown in Fig. 1, human platelets express PKC isoforms α, β, δ, η, ε, and ζ. Despite loading the wells with excess protein, we could not detect the isoforms γ, λ, and η.

**Time-dependent Dense Granule Release and Activation of PKC8 in Human Platelets by PAR-activating Peptides SFLLRN and AYPGKF**—PKC8 has been implicated in secretion in other cell systems (26–28), hence we investigated the role of this isoform in platelet dense granule secretion. We evaluated the activation of this isoform by analyzing the activation-dependent phosphorylation of the threonine 505 residue in the activation loop following agonist stimulation (23, 29–34). When aspirin-treated, washed human platelets were stimulated for various time periods with 50 μM PAR1-activating peptide SFLLRN and 500 μM PAR4-activating peptide AYPGKF, dense granule secretion started as early as 15 s (Fig. 2, A and C). PKC8 activation, as measured by phosphorylation at threonine 505, occurred in a time-dependent manner starting at 15 s and was sustained for 2 min (Fig. 2, B and D). We also checked the phosphorylation states for extended time period as late as 10 min. The phosphorylation was still present following agonist stimulation until 10 min (data not shown). Whereas SFLLRN-induced dense granule release was maximal at 15 s, AYPGKF-induced dense granule release reached maximum at 30 s. The difference in the time to maximum dense granule secretion between SFLLRN and AYPGKF could be the result of the differential kinetics of receptor activation of the two peptides (35). Thus, the time required for PAR agonist-induced PKC8 phosphorylation correlated with dense granule secretion.

Note that PKC8 phosphorylation is maximal at 2 min, whereas the dense granule release occurred maximally at 30 s (AYPGKF). The probable reason for this finding could be that the contents of the α granules might cause further activation of PKC8. Previous studies have shown that dense granule secretion occurs much earlier than α granule secretion following stimulation with agonists such as thrombin (36, 37). Hence, the initial secretion and PKC8 phosphorylation might be mediated downstream from the agonist receptor, and the subsequent phosphorylation might be potentiated by the secreted contents from the α granules. The secretion does not increase further because this process could have been saturated during those time periods even in the presence of increased PKC8 phosphorylation. By this mechanism, the PKC8 phosphorylation could be amplified over an extended time period even though the primary signaling event ends much earlier.

**Phosphorylation of PKC8 by Agonists That Cause Platelet Dense Granule Release**—We evaluated the ability of both GPCR and glycoprotein signaling to activate PKC8 and correlated it with their ability to cause dense granule secretion. Aspirin-treated and washed human platelets were stimulated with 1.0 unit/ml thrombin, 50 μM SFLLRN, 500 μM AYPGKF, 10 μM U46619, 10 μM ADP, 10 μM serotonin, 10 μM epinephrine, and 100 ng/ml convulxin, and both dense granule release and PKC8 phosphorylation were measured. As shown in Fig. 3, thrombin, SFLLRN, AYPGKF, U46619, or convulxin caused dense granule release (A), and phosphorylation of PKC8 (B), whereas ADP, serotonin, and epinephrine did not cause either. Phorbol 12-myristate 13-acetate, a known PKC activator, was used as a positive control for phosphorylation for PKC8. These results suggest that signaling downstream of both GPCR and glycoprotein receptors is capable of activating PKC8. In partic-
ular, agonists that cause dense granule secretion also activated PKC\(\delta\). We further investigated whether ADP transiently causes PKC\(\delta\) activation by measuring phosphorylation at various time points. As shown in Fig. 3C, ADP failed to cause PKC\(\delta\) phosphorylation until 2 min. These data show that agonists that cause granule secretion also cause activation of PKC\(\delta\), suggesting the involvement of this PKC isoform in the process of dense granule secretion.

**Correlation of PAR-mediated Dense Granule Secretion and PKC\(\delta\) Phosphorylation**—We investigated whether the concentration of agonist required for secretion of dense granules correlated with the concentration needed for activation of PKC\(\delta\). Human platelets were stimulated with increasing concentrations of the PAR1-activating peptide, SFLLRN, and the amount of secreted ATP was measured. As shown in Fig. 4A, SFLLRN began to cause dense granule secretion at a concentration of 5 \(\mu\)M, which corresponded to the same concentration at which phosphorylation and activation of PKC\(\delta\) began to occur (Fig. 4B).

We used the PAR4-activating peptide AYPGKF to correlate further PAR-mediated dense granule release and PKC\(\delta\) activation. As shown in Fig. 5A, AYPGKF began to cause dense granule secretion at a concentration of 100 \(\mu\)M, which corresponded to the same concentration at which phosphorylation of PKC\(\delta\) began to occur (Fig. 5B). These data suggest a correlation between dense granule release and activation of PKC\(\delta\) phosphorylation in human platelets.

**Effect of PKC\(\delta\) Isoform-selective Inhibitor on PAR-activating Peptides and Convulxin-induced Dense Granule Secretion**—PKC\(\delta\) is known to play an important regulatory role in exocytosis in other cell systems (26–28). We used a PKC\(\delta\) specific inhibitor, rottlerin (5 \(\mu\)M final concentration) (20, 21, 40–46) to investigate the role of this isoform in platelet dense granule secretion. Platelets were stimulated with different concentrations of SFLLRN, AYPGKF, or convulxin after preincubation for 15 min with either rottlerin or dimethyl sulfoxide (vehicle) at 37 °C, and the secreted ATP was measured. Whereas rottlerin inhibited (about 50%) both SFLLRN- and AYPGKF-induced ATP secretion (Fig. 6A, A and B), it potentiated convulxin-induced dense granule secretion (Fig. 6C). These results confirm the role of PKC\(\delta\) in regulating dense granule secretion in human platelets.

**Effect of Classical PKC Isoform Inhibitor on Convulxin-induced Dense Granule Release**—Agonist-mediated platelet dense granule secretion is inhibited by the PKC inhibitors Ro 31-8220 and GF109203X (47, 48). These nonselective inhibitors block both the conventional and novel isoforms (49). Previous studies have implicated PKC\(\delta\) in secretion, but it was also shown in the same study that this enzyme alone is not sufficient to cause dense granule release (50). Because rottlerin did not inhibit convulxin-induced dense granule release, we postulated that other PKC isoforms might be involved in convulxin-induced secretion. Hence we used a PKC\(\delta\)/\(\beta\)-specific inhibitor, Go6976 (100 \(n\)M final concentration) (51) to investigate the role of classical PKC isoforms in convulxin-induced dense granule release. As shown in Fig. 7A, Go6976 abolished convulxin-induced platelet secretion, suggesting that classical PKC isoforms play an important role in convulxin-induced dense granule release. However, Go6976 had minimal inhibitory effect on SFLLRN-induced dense granule secretion (Fig. 7B). When both rottlerin and Go6976 were used together, SFLLRN-induced dense granule secretion was inhibited to the same extent as that with rottlerin alone (data not shown), indicating that PKC\(\delta\)/\(\beta\) isoforms do not play a major role in SFLLRN-mediated dense granule secretion. In contrast, classical PKC isoforms seem to play an important role in GPVI-mediated dense gran-
ule secretion. Ro 31-8220, an inhibitor of both classical and novel PKC isoforms, completely abolished the dense granule secretion by PAR agonists or convulxin (data not shown) (47, 48).

**Effect of Rottlerin on SFLLRN-, AYPGKF-, and Convulxin-induced PKC/H9254 Phosphorylation**

As seen from the previous experiments, rottlerin inhibits the amount of dense granule secretion caused by the PAR-activating peptides while potentiating secretion caused by convulxin. This could be either because of rottlerin affecting the kinase activity following PKC/H9254 phosphorylation or that it could inhibit the phosphorylation event itself that is needed for kinase activity. We investigated the phosphorylation of PKC/H9254 following platelet stimulation with the PAR-activating peptides or convulxin in the presence of rottlerin. As shown in Fig. 8, rottlerin decreased the phosphorylation of PKCδ in response to all the three agonists. This indicated that inhibition of PKCδ activity by rottlerin could be the result of its ability to inhibit the phosphorylation of the activation domain that is essential for its activity.

**Effect of Rottlerin on SFLLRN-induced Platelet Aggregation**

PKC has been shown to play an important role in platelet fibrinogen receptor activation (48, 52). Hence, we investigated the role of the PKCδ isoform, which plays a positive regulatory role SFLLRN-induced dense granule release, in SFLLRN-induced platelet aggregation. As shown in Fig. 9, rottlerin had no effect on SFLLRN-induced platelet aggregation, whereas Ro 31-8220 caused inhibition of aggregation. We have shown recently that fibrinogen receptor can be activated independently
by calcium-dependent and -independent (PKC-dependent) pathways in human platelets (48). The calcium-dependent pathway can be blocked by chelating intracellular calcium with dimethyl-BAPTA without inhibiting the PKC-dependent pathway; under these conditions, the effect of rottlerin on PKC-mediated platelet aggregation was measured. As shown in Fig. 8, rottlerin failed to inhibit PKC-dependent platelet aggregation in the presence of dimethyl-BAPTA, whereas Ro 31-8220 completely blocked this aggregation. These results suggest that isoforms other than PKC play a role in SFLLRN-induced platelet aggregation.

DISCUSSION

Platelet secretion is an important component of the amplification of platelet activation. Several platelet agonists stimulate GPCRs and cause activation of the heterotrimeric G protein Gq, leading to activation of PLCβ. PLC activation leads to generation of IP3 and DAG that causes increase in calcium and PKC activation, respectively (3–6). Both of these signaling molecules, viz. calcium and PKC, are known to be important for dense granule release (7–14). Previously, several investigators have detected PKC isoforms α, β, δ, θ, ε, and ζ in human platelets but could not detect the isoforms γ, λ, and η (53–57). Consistent with the previous studies, we have also determined that human platelets express seven PKC isoforms: α, β, δ, θ, ε, η, and ζ (Fig. 1).

FIG. 5. Correlation between concentration-dependent dense granule release and PKC phosphorylation by AYPGKF. Aspirin-treated and washed human platelets were stimulated with increasing doses of AYPGKF, and dense granule release (A) and PKC phosphorylation (B) were determined as described previously. The secretion data were normalized to the maximum secretion (taken as 100%). The secretion data (mean ± S.E.) shown are from three independent experiments performed using platelets from different donors. The Western blot is from a single experiment representing three independent experiments performed using platelets from different donors.

FIG. 6. Effect of rottlerin on SFLLRN-, AYPGKF-, and convulxin-induced platelet dense granule secretion. Aspirin-treated and washed human platelets were pretreated with vehicle (solid line) or 5 μM rottlerin (dashed line) for 15 min at 37 °C. These platelets were then stimulated with increasing concentrations of SFLLRN (A), AYPGKF (B), or convulxin (C), and the secreted ATP was measured using the CHRONO-LUME assay. The data were normalized to the maximum secretion (taken as 100%). The secretion data (mean ± S.E.) shown are from three independent experiments performed using platelets from different donors.
PKCα has been shown to be important in the secretion process in other cell systems and that it mediates positive stimulatory signals for secretion (26–28). Hence, we investigated whether PKCα plays an important role in regulating agonist-mediated dense granule secretion in human platelets. Based on the correlations of time- and concentration-dependent activation of PKCα and dense granule release by PAR-activating peptides and the inhibitory effect of the PKCα-selective inhibitor rottlerin on SFLLRN- and AYPGKF-induced dense granule secretion, we conclude that PKCα plays a positive regulatory role in PAR-mediated platelet dense granule secretion. The persistence of PKCα phosphorylation at time periods beyond the maximum dense granule secretion could be caused by the potentiating effects of substances that are released from the α granules. Our results show that in the presence of 5 μM rottlerin, PAR-activating peptides SFLLRN- and AYPGKF-induced platelet dense granule secretion was decreased but not completely abolished. This lack of complete inhibition suggests a possible role for other PKC isoforms that could also contribute to secretion. The phosphorylation state of PKCα was also reduced in the presence of rottlerin, suggesting that rottlerin inhibits the phosphorylation and activation of PKCα. Decreased phosphorylation and activation of PKCα would then result in decreased dense granule secretion. However, SFLLRN-induced dense granule release was not inhibited by the classical PKC isoform selective inhibitor Go6976, indicating that these PKC isoforms do not play any significant role in PAR-mediated dense granule release. All the above suggests that PKCα plays an important positive role in dense granule secretion downstream of PAR-mediated signaling.

Previous studies have shown that PKCα is essential but not sufficient for granule secretion from human platelets and that a yet unidentified cytosolic factor is needed for this secretory process (50). Our studies with convulxin are in agreement with this observation and indicate that classical PKC isoforms do not play an important role in GPVI-mediated platelet dense granule release. In contrast to the results with PAR-activating peptide, rottlerin potentiated convulxin-induced dense granule secretion, suggesting a negative role for PKCα in this signaling pathway. Convulxin-mediated PKCα phosphorylation was also inhibited in the presence of rottlerin and hence reduced the
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activity of PKCδ. Given that this isoform plays a negative role in dense granule secretion downstream of GPVI signaling (59), inhibiting the phosphorylation and, thereby activity of this isoform, would thus potentiate GPVI-mediated dense granule secretion. Our results with convulxin are consistent with the interpretations of Crosby and Poole (59). Whereas PLC-δ2 activation downstream of PAR signaling leads to PKC activation, Gq-mediated dense granule secretion but do not play any role in PAR-mediated dense granule secretion. Furthermore, the PKCδ isoform does not play any role in PAR-mediated platelet fibrinogen receptor activation.

In conclusion, we have shown that PKCδ plays an important role in PAR-mediated dense granule secretion in human platelets while negatively regulating secretion downstream of GPVI signaling. The failure of ADP to activate this isoform could account for its inability to cause dense granule secretion. In addition, PKCα/β isoforms play an important role in GPVI-mediated dense granule secretion but do not play any role in PAR-mediated dense granule secretion. Our results demonstrate that PKCδ does not play a role in this aggregation (Fig. 9).

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