Protein Kinase C Isoform ε Negatively Regulates
ADP-Induced Calcium Mobilization and Thromboxane
Generation in Platelets

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Objective—Members of the protein kinase C (PKC) family are shown to positively and negatively regulate platelet activation. Although positive regulatory roles are extensively studied, negative regulatory roles of PKCs are poorly understood. We investigated the mechanism and specific isoforms involved in PKC-mediated negative regulation of ADP-induced functional responses.

Methods and Results—A pan-PKC inhibitor, GF109203X, potentiated ADP-induced cPLA₂ phosphorylation and thromboxane generation as well as ERK activation and intracellular calcium (Ca²⁺) mobilization, 2 signaling molecules, upstream of cPLA₂ activation. Thus, PKCs inhibit cPLA₂ activation by inhibiting ERK and Ca²⁺ mobilization. Because the inhibitor of classic PKC isoforms, GO-6976, did not affect ADP-mediated thromboxane generation, we investigated the role of novel class of PKC isoforms. ADP-induced thromboxane generation, calcium mobilization, and ERK phosphorylation were potentiated in PKCe null murine platelets compared with platelets from wild-type littermates. Interestingly, when thromboxane release is blocked, ADP-induced aggregation in PKCe knockout and wild-type was similar, suggesting that PKCe does not affect ADP-induced aggregation directly. PKCe knockout mice exhibited shorter times to occlusion in an FeCl₃-induced arterial injury model and shorter bleeding times in tail-bleeding experiments.

Conclusion—We conclude that PKCe negatively regulates ADP-induced thromboxane generation in platelets and offers protection against thrombosis. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: calcium  G proteins  hemostasis  platelets  thrombosis

Platelets play a crucial role in thrombosis and hemostasis. On vascular injury, subendothelial collagen activates platelets via α₂β₁ and GPVI receptors to change shape, secrete granule contents, generate thromboxane and thrombin, and finally aggregate to form the platelet plug. ADP (secreted from dense granules), thrombin, and thromboxane thus generated are important positive feedback activators of platelets that amplify thrombus growth and increase thrombus stability.¹

ADP activates 2 distinct G-protein–coupled receptors on platelets, P2Y₁ and P2Y₁₂, which couple to Gq and Gi, respectively.¹ Gq activates phospholipase C β (PLC β), which leads to diacylglycerol (DAG) generation and intracellular calcium increases.¹ On the other hand, Gi is involved in inhibition of cAMP levels and PI-3 kinase activation.¹ Synergistic activation of Gq and Gi proteins leads to the activation of the fibrinogen receptor integrin α₁bβ₃ and thromboxane generation.¹ Thrombin activates platelets via PAR1 (PAR3 in mice) and PAR4 receptors. AYPGKIF is a peptide agonist that activates platelets via the PAR4 receptor, which couples to Gq.¹ Convulxin activates platelets via GPVI and GPIb receptors, which signals through Immune Tyrosine Activated Motifs (ITAMs) to activate PLCγ.²

Thromboxane (TXA₂) synthesis is a multistep process that begins at the membrane of the platelet or dense tubular system. Cytosolic phospholipase A₂ (cPLA₂) is the primary enzyme that cleaves membrane phospholipids such as phosphatidyl choline at the Sn-2 ester position, leading to formation of arachidonic acid (AA).³–⁵ cPLA₂ consists of an N-terminal regulatory domain and C-terminal catalytic domain connected by a hinge region in the middle. Regulatory domain consists of the C2 domain, which binds calcium and regulates cPLA₂ mobility to the membrane and thereby its activity.⁶ Furthermore, cPLA₂ activity is also shown to be regulated by phosphorylation at Ser 505 residue mediated by ERK 1/2 or p38 MAP kinase.⁸ Some studies also suggest that S505 phosphorylation is required for cPLA₂ enzyme activity.⁹,¹⁰ AA is then metabolized to thromboxane by...
enzymes such as prostaglandin G/H synthase (PGHS) (also called as cyclooxygenases [COX]) and thromboxane synthase to generate TXA₂.¹¹ TXA₂ thus generated activates platelets via TPₐᵣ receptors in human platelets that couple to Gq and G₁₂/₁₃ pathways.

Protein kinase C (PKC) isoforms are a family of kinases that phosphorylate their substrates at serine/threonine residues. PKCs are classified into 3 groups, based on their structure and cofactor requirements.¹² (1) Classic isoforms (cPKCs: α, β₁, γ, δ, ε, η, ζ, ι) contain both C₁ and C₂ domains and thus require both DAG and Ca²⁺ for activation. (2) Novel isoforms (nPKCs: δ₁, θ, ι, κ) contain the C₁ domain and lack the calcium-binding C₂ domain. Thus, nPKCs require only DAG for activation. (3) Atypical PKC isoforms (ζ, η, λ) lack a part of the C₁ domain and C₂ domain and thus do not require Ca²⁺ and DAG for activation.¹³ Instead, aPKCs contain a PBI domain that binds PS and also is involved in several protein-protein interactions.¹⁴ There are also reports that indicate the presence of a pleckstrin homology (PH) domain that is regulated by phosphoinositide-3,4,5-trisphosphate (PIP₃).¹⁵,¹⁶ PKCs are established positive regulators of various platelet functional responses.¹⁷ PKCs are shown to cause platelet aggregation independent of calcium and play an important role in granule secretion.¹⁸,¹⁹ Interestingly, we have previously shown that PKCs are shown to negatively regulate ADP-induced thromboxane generation.²⁰ Thus, we investigated the mechanism by which PKC isoforms negatively regulate ADP-induced thromboxane generation and PKC isoforms involved in this process.

We show that PKCs negatively regulate ERK activation and intracellular calcium mobilization, which in turn inhibits cPLA₂ activation and thromboxane generation. We also demonstrate that PKCe is the isoform involved in negative regulation of ADP-induced calcium mobilization, ERK phosphorylation, and thromboxane generation. We observe that AYPGKF-mediated (low concentration) but not Convulxin-mediated functional responses are potentiated in the absence of PKCe. Furthermore, we show that PKCe only inhibits thromboxane generation and does not affect ADP-induced aggregation or dense granule secretion directly. Finally, for the first time, we show that deficiency of the PKCe isoform results in protection against thrombosis, using an FeCl₃- induced arterial injury model.

**Methods**

Approval for this study was obtained from the Institutional Review Board and from the Institutional Animal Care and Use Committee of Temple University (Philadelphia, PA).

**Materials**

Apyrase (type VII), bovine serum albumin (fraction V), thrombin, 2MeSADP, MRS-2179 (N²-methyl-2-¹-deoxyadenosine-3’, 5’-bis-phosphate) (teta sodium salt), fibrinogen (type I), and acetylsalicylic acid were obtained from Sigma (St Louis, MO). Phospho-ERK antibodies against threonine 202 and tyrosine 204 residues, phospho-cPLA₂ antibody against Ser-505 residue, β₃-integrin antibodies, and β₂-actin antibodies were obtained from Cell Signaling Technologies (Beverly, MA). Anti-pPKCe Ser 729 antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Alkaline phosphatase–labeled secondary antibody was from Kirkegaard & Perry Laboratories (Gaithersburg, MD). AR-C69931MX (N²-(2-methyl-tioethyl)-2-(3,3,3-trifluoropropylthio)-β, γ-dichloromethylene ATP) (teta sodium salt) was a kind gift from the Medicines Company, Parsippany NJ. Pan-PKC inhibitors GF109203X, Go-6976, and Ro31-8220 were obtained from Biomol (now ENZO Life Sciences Inc, Plymouth Meeting, PA). PKCe knockoút (KO) mice and anti-PKCe antibody were a kind gift from Dr Robert Messing (Gallo Centre, San Francisco, CA).

**Isolation of Human Platelets**

All experiments using human subjects were performed in accordance with the Declaration of Helsinki. Whole blood was drawn from healthy, consenting human volunteers into tubes containing one-sixth volume of ACD (2.5 g sodium citrate, 1.5 g citric acid, and 2 g glucose in 100 mL deionized water). Blood was centrifuged (Eppendorf_5810R centrifuge, Hamburg, Germany) at 230g for 20 minutes at room temperature to obtain platelet-rich plasma (PRP). If indicated, PRP was incubated with 1 mmol/L acetylsalicylic acid (aspirin) for 30 minutes at 37°C. The PRP was then centrifuged for 10 minutes at 980g at room temperature to pellet the platelets. Platelets were resuspended in Tyrode buffer (138 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L MgCl₂, 3 mmol/L Na₂HPO₄, 5 mmol/L glucose, 10 mmol/L HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], pH 7.4, 0.2% bovine serum albumin) containing 0.1 U/mL aprotinin. Cells were counted using the Coulter Z1 Particle Counter (Miami, FL), and concentration of cells was adjusted to 2×10⁶ platelets/mL. All experiments using washed platelets were performed in the absence of extracellular calcium unless otherwise mentioned.

**Platelet Cell Lysate Preparation**

Platelets were stimulated with agonists for the appropriate time under nonstirring or stirring conditions at 37°C. The reaction was stopped by the addition of 3× SDS-Laemmli buffer. Platelet lysates were boiled for 10 minutes and stored for Western blotting analysis.

**Aggregation**

Aggregation of 0.5 mL washed platelets was analyzed using a P.L.C.A. lumi-aggregometer (Chrono-log Corp, Haverton, PA). Aggregation was measured using light transmission under stirring conditions (900 rpm) at 37°C. Each sample was allowed to aggregate for at least 3 minutes. The chart recorder (Kipp and Zonen, Bohemia, NY) was set for 0.2 mm/s.

**Measurement of TXA₂ Generation**

Washed human platelets without aspirin treatment were prepared as noted and brought to a concentration of 4×10⁹ platelets/mL. Stimulations were performed in a platelet aggregometer under stirring conditions (900 rpm) at 37°C. When mentioned, pharmacological inhibitors GF109203X and Go-6976 were added for 10 minutes before addition of the agonist. Stimulations were performed for 3.5 minutes, and the reaction was stopped by snap freezing. Samples were stored at −80°C until TXB₂ analysis was performed. Levels of TXB₂ were determined in duplicates using a Couer-EIA Thromboxane B₂ Enzyme Immunoassay Kit (Assay Designs, Ann Arbor, MI), according to the manufacturer’s instructions. The mean±SEM was derived from experiments performed in triplicate using platelets obtained from 3 independent donors. Same protocol was followed during measurement of thromboxane generation in murine platelets.

**Western Blotting Analysis**

Lysates prepared from platelets were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane. Nonspecific binding sites were blocked by incubation in Tris-buffered saline and Tween (TBST; 20 mmol/L Tris, 140 mmol/L NaCl, 0.1% (vol/vol) Tween 20) containing 0.5% (wt/vol) milk protein and 3% (wt/vol) bovine serum albumin for 30 minutes 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 3 washes for 5 minutes each with TBST, the membranes were probed with an
HRP-labeled secondary antibody (1:5000 dilutions in TBST with 2% bovine serum albumin) for 1 hour at room temperature. After additional washing steps, membranes were then incubated with HRP substrate (Sigma Chemical Co, St Louis, MO) for 10 minutes at room temperature, and immunoreactivity was detected using a Fuji Film Luminescent Image Analyzer (LAS-1000 CH, Japan).

Intracellular Calcium Measurements
PRP was incubated with 5 μmol/L Fura AM and acetylsalicylic acid for 30 minutes. Platelets were then washed and isolated as described above. Platelets were preincubated with GF109203X or Ro-318220 or DMSO at 37°C for 10 minutes. Changes in fluorescence were measured using an Aminco-Bowman Series 2 luminescence spectrometer with a water-jacketed cuvette holder thermostatted at 37°C and set at constant stirring. Calcium measurements were done using methods described in Quinter et al.21

Isolation of Murine Platelets
Blood was collected from vena cava of anesthetized mice into syringes containing one-tenth blood volume of 3.8% sodium citrate as anticoagulant. Red blood cells were removed by centrifugation at 100g for 10 minutes. Platelet-rich plasma (PRP) was removed, and platelets were pelleted at 400g for 10 minutes. The platelet-poor plasma (PPP) was removed and platelet pellet was resuspended in Tyrode buffer (pH 7.4) containing 0.1 U/mL apyrase. The washed platelets were subsequently used for experiments.

FeCl₃-Induced Arterial Injury Model of Thrombosis
Male and female mice (4–5 month old) were anesthetized by intraperitoneal injection of pentobarbital (40 mg/kg). Right carotid artery of mice was exposed to 2×1 mm (LXB) filter paper dipped in 5% FeCl₃ for 90 seconds. Blood flow was measured using Doppler flow probe, and time taken for total cessation of blood flow in carotid artery due to thrombus formation was recorded and is plotted as time to occlusion.

Bleeding Time
Male and female mice (20–30 g) were anesthetized by exposing mice to isofluorine vapor. A cut was made longitudinally in the mouse tail using a Simplate device (Organon Teknika BV, Boxtel, The Netherlands). The tail was immediately immersed in 0.9% isotonic saline at 37°C. The bleeding time was defined as the time required for blockade of blood flow.

Statistical Analysis
We analyzed statistical significance of our data by using the Paired Student t test or ANOVA. Statistically significant data bearing a probability value *P<0.05 are annotated by an asterisk. Data are expressed as mean±SEM.

Results
Role of PKC Isoforms in ADP-Induced cPLA₂ Activation
Pan PKC inhibitors are known to enhance thromboxane generation in platelets. cPLA₂-mediated AA liberation from membrane phospholipids is the rate-limiting step to thromboxane generation in platelets. We investigated whether PKC isoforms negatively regulate thromboxane generation by regulating cPLA₂ activation. cPLA₂ activation is regulated by phosphorylations at various serine residues. Among these, phosphorylation of S505 is shown to be required for cPLA₂ activity.5,10 We pretreated platelets with GF109203X or DMSO, activated with 2MeSADP, an ADP analog in a time-dependent manner, and measured cPLA₂ activation by Western blot analysis with anti-phospho S505 cPLA₂ antibody. Aspirin-treated platelets were used in the above experiments to avoid feedback activation of cPLA₂ by thromboxane. As shown in Figure 1A and 1B, GF109203X potentiates 2MeSADP-induced cPLA₂ phosphorylation. These data sug-
gest that PKCs negatively regulate thromboxane generation by inhibiting cPLA₂ activation.

Role of PKC Isoforms in ERK Activation

We have previously demonstrated that p42/44 MAPK or ERK regulates ADP-induced thromboxane generation. Furthermore, MAPKs are also shown to regulate S505 cPLA₂ phosphorylation or its activation. Hence, we investigated whether PKCs inhibit cPLA₂ activation by regulating ERK activation. Dual Thr and Tyr phosphorylations on ERK (Thr202/Tyr204 on ERK1 and Thr185/Tyr187 on ERK2) are well established and widely used activation markers of ERK. We pretreated aspirin-treated platelets with GF109203X or DMSO, activated with 2MeSADP for various time periods and subjected lysates to Western blot analysis with pThr/Tyr ERK1/2 antibody. As shown in Figure 1C and 1D, 2MeSADP-induced ERK phosphorylation is transient in DMSO-treated platelets, whereas it is hyperphosphorylated and sustained till 5 minutes in platelets pretreated with GF109203X. These results suggest that PKCs negatively regulate ERK activation.

Role of PKCs in 2MeSADP-Induced Intracellular Calcium Mobilization

In platelets, dense tubular system (DTS) has several endoperoxidases required for processing arachidonic acid to thromboxane. Therefore, cPLA₂ is mobilized from intracellular space to membranes of dense tubular system to render its enzymatic activity. Intracellular calcium increase is known to be required for such cPLA₂ translocation. Hence, we evaluated whether PKCs regulate intracellular calcium mobilization. We pretreated Fura-2 AM–labeled, aspirin-treated platelets with pan PKC inhibitors GF109203X or Ro 31 to 8220 and measured 2MeSADP-induced intracellular calcium mobilization. As shown in Figure 2A, pretreatment with GF109203X or vehicle control DMSO and activated them with 2MeSADP in the presence of AR-C69931MX, a P2Y₁₂ antagonist. As expected, pretreatment with AR-C69931MX decreased 2MeSADP-induced calcium mobilization. Interestingly, even in the presence of AR-C69931MX, GF109203X potentiated 2MeSADP-induced calcium mobilization in platelets (Figure 2B). These data suggest that PKCs negatively regulate ADP-induced calcium mobilization independent of P2Y₁₂ receptor.

Role of Classic and Nonclassic PKC Isoforms in ADP-Mediated Thromboxane Generation

We further investigated the specific isoforms that negatively regulate ADP-induced thromboxane generation by using pharmacological and gene KO approaches. We first investigated the role of classic PKC isoforms in ADP-induced thromboxane generation, using Go-6976, which inhibits the classic group of PKC isoforms at concentration below 10 nmol/L. As shown in Figure 3A, we pretreated Fura-2 AM–loaded platelets with GF109203X or Ro 31 to 8220 and measured 2MeSADP-induced intracellular calcium mobilization, compared with platelets pretreated with the vehicle DMSO. These data suggest that PKCs negatively regulate intracellular calcium mobilization, which may in turn inhibit cPLA₂ translocation. P2Y₁₂ receptor is shown to potentiate P2Y₁ receptor–mediated calcium mobilization. Furthermore, PKCs are shown to desensitize P2Y₁₂ receptors and thus inhibit P2Y₁₂-mediated responses. Thus, we evaluated whether PKCs regulate intracellular calcium mobilization by regulating P2Y₁₂ receptor desensitization. We pretreated Fura-2 AM–labeled platelets with GF109203X or vehicle control DMSO and activated them with 2MeSADP in the presence of AR-C69931MX, a P2Y₁₂ antagonist. As expected, pretreatment with AR-C69931MX decreased 2MeSADP-induced calcium mobilization. Interestingly, even in the presence of AR-C69931MX, GF109203X potentiated 2MeSADP-induced calcium mobilization in platelets (Figure 3B). These data suggest that PKCs negatively regulate ADP-induced calcium mobilization independent of P2Y₁₂ receptor.

Figure 2. Protein kinase C (PKC) isoforms negatively regulate 2MeSADP-induced intracellular calcium mobilization. Fura-2 AM–labeled, aspirin-treated platelets were preincubated with 1% DMSO (vehicle), 5 μmol/L GF109203X, or 10 μmol/L Ro-318220 for 15 minutes at 37°C and stimulated with 100 nmol/L 2MeSADP under stirring conditions in the absence (A) or presence (B) of 1 μmol/L AR-C69931MX. Intracellular calcium increase was measured by spectrofluorimetry. Data from 3 different donors was pooled and statistical analysis was done using Student t test (*P<0.05, **P<0.01).
the role of nonclassic PKC isoforms. It was shown that GF109203X (also called GO-6850) inhibits various PKC isoforms in a concentration-dependent manner.29 At concentrations below 100 nmol/L, GF109203X was shown to inhibit classic PKC isoforms. Further increase of GF109203X concentration leads to inhibition of nonclassic PKC isoforms (novel and atypical PKC isoforms), as well.29 Hence, we pretreated platelets with various concentrations of GF109203X and measured 2MeSADP-induced thromboxane generation. As shown in Figure 3B, GF109203X did not affect 2MeSADP-induced thromboxane generation at concentrations below 100 nmol/L, but, at concentrations above 100 nmol/L, where GF109203X inhibits nonclassic PKC isoforms as well, it potentiated 2MeSADP-induced thromboxane generation. This result in addition to data with Go-6976 suggests that nonclassic PKC isoforms, that is, novel and/or atypical PKC isoforms, negatively regulate ADP-induced thromboxane generation.

**Activation of PKCε in Human Platelets**

Due to unavailability of specific tools to evaluate role of atypical PKC isoforms, we first investigated the role of novel class of PKC isoforms. Among the novel class of PKC isoforms δ, θ, η, and ε, we have previously shown that δ and η are activated in human platelets and positively regulate agonist-induced thromboxane generation.30–32 Hence, we first investigated whether PKCε is present and activated in human platelets. As shown in Figure 4A, we detected a band at 90 kDa in human platelet lysates and in wild-type (WT) murine platelet lysates. However, such a band was lighter (than WT) in HET and absent in KO platelet lysates. These data suggest that PKCε is expressed in human platelets. Activation of PKCε is shown to be regulated by phosphorylation at Ser729 residue in hydrophobic motif.33 Thus, we studied activation of PKCε by Western blot analysis, using anti-pSer729 PKCε antibody. As shown in Figure 4B, both 2MeSADP and AYPGKF phosphorylated PKCε at the Ser

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**Figure 3.** Nonclassic protein kinase C (PKC) isoforms negatively regulate ADP-induced thromboxane generation in platelets. Non–aspirin-treated platelets were preincubated at 37°C with various concentrations of A, Go-6976, and B, GF109203X or 1% DMSO for 15’ and activated with 100 nmol/L 2MeSADP for 3.5’ at stirring conditions. The reaction was stopped by freezing the samples on dry ice and TxB2, a stable analog of TxA2 was measured in these samples using ELISA. The data were pooled from 4 different donors and analyzed for statistical significance using ANOVA (*P<0.05, **P<0.01).

**Figure 4.** Activation of PKCε in human platelets. A, Human and mouse platelets were lysed using Lamelli-SDS buffer. Lysates were subjected to Western blot analysis using anti-PKCε antibody. The same blot was reprobed with anti-β3 integrin antibody to monitor protein loading in each lane. B, Aspirin-treated platelets were stimulated with 100 nmol/L 2MeSADP and 500 μmol/L AYPGKF at 37°C for 2 minutes. Reaction was stopped by adding Lamelli-SDS buffer. Samples were subjected to Western blot analysis using anti-pSer 729 PKCε antibody.
Role of PKCε in 2MeSADP-Induced Functional Responses

We studied the functional role of PKCε by using platelets from mice deficient in this isoform. As shown in Figure 5A, 2MeSADP-induced aggregation and secretion were increased in platelets from PKCε KO mice compared with WT littermates. Similarly, 2MeSADP-induced thromboxane generation was also enhanced in KO platelets compared with WT platelets (Figure 5B). These data suggest that PKCε negatively regulates ADP-induced functional responses such as aggregation, secretion, and thromboxane generation. Since thromboxane is a positive feedback activator of platelets, we investigated if PKCε/H9255α thromboxane is a positive feedback activator of platelets. We observed that average time taken for complete blockade of tail bleeding was 47.208 ± 3.90 seconds in KO, whereas it was 70.958 ± 5.38 seconds in WT mice. Furthermore, we observed that average bleeding time in HET is 51.298 ± 3.10 seconds (Figure 6A). These data suggest that PKCε negatively regulates hemostatic responses in vivo. Furthermore, we subjected KO and WT mice to FeCl3-induced arterial injury model to evaluate role PKCε in thrombosis. On injury, time to occlusion in KO mice was 10.583 ± 1.49 minutes, whereas it was 18.75 ± 1.84 minutes in WT mice. These data suggest that PKCε is a negative modulator of both hemostasis and thrombosis (Figure 6B).

Discussion

PKCs have been implicated in various platelet functional responses that are critical in hemostasis and thrombosis. The positive regulatory role of PKCs in platelet aggregation and secretion has been extensively studied.33 PKC α, δ, θ, and η have been implicated in this context.30–32 PKCs negatively regulate ADP-induced thromboxane generation.20 We investigated the PKC isoforms involved in negative regulation of ADP-induced thromboxane generation and mechanism by which PKCs regulate this process.

We show for the first time that PKCε negatively regulates ADP-induced thromboxane generation, by regulating calcium mobilization and ERK activation, without directly affecting ADP-induced aggregation. Such negative regulation of ADP-induced thromboxane generation was observed at both high and low agonist concentrations. We have also shown that PKCε negatively regulates PAR-mediated platelet functional responses induced by low concentrations of agonists, whereas it has no effect on responses induced by higher agonist concentrations. Furthermore, PKCε KO mice exhibit shorter tail-bleeding times and shorter time to occlusion in FeCl3-induced arterial injury model. These data collectively suggest that the negative regulatory role of PKCε in ADP-induced thromboxane generation affects the amplification and stability of thrombus in vivo.

We show that PKCs negatively regulate intracellular calcium mobilization and ERK activation. Consistent with our findings, PKCs were shown to negatively regulate calcium-mediated phosphatidylserine exposure.36 Unsworth et al have recently reported that PKCs negatively regulate ADP-induced aggregation and dense granule secretion.37 However, these studies were performed in platelets in which thromboxane generation was not blocked, and they did not evaluate thromboxane generation under these conditions. Thromboxane generated downstream of ADP reinforces aggregation and causes dense granule secretion in platelets.38 Such positive reinforcement of aggregation by generated thromboxane generation affects the amplification and stability of thrombus in vivo.

Role of PKCε in TP, PAR-Mediated, and GPVI-Mediated Platelet Functional Responses

We investigated the role of PKCε in PAR- and GPVI-induced platelet activation, using agonists AYPGKF and Convulxin, respectively. As shown in Figure 5E, aggregation induced by low concentrations of AYPGKF were potentiated in PKCε KO murine platelets compared with WT platelets, Convulxin-induced platelet aggregation was similar in PKCε KO and WT mice. Furthermore, aggregation induced by higher concentrations of AYPGKF in KO murine platelets was similar to WT platelets. These data suggest that PKCε negatively regulates only PAR-mediated platelet aggregation at low concentrations of agonist and does not regulate GPVI-mediated responses. We evaluated the role of PKCε in TxA2-induced platelet activation using U46619, a TP receptor agonist and PKCε KO murine platelets (Figure 5G). U46619-induced platelet responses were potentiated in PKCε KO murine platelets compared with WT littermates. Furthermore, unlike convulxin, collagen-induced platelet functional responses were also potentiated in PKCε KO murine platelets compared with WT controls (Figure 5G). These data suggest that thromboxane generation negatively regulated by PKCε plays a more important role in TxA2- and collagen-induced platelet activation.

Physiological Role of PKCε in Hemostasis and Thrombosis

Thrombin, ADP, and thromboxane are important agonists that contribute to thrombus formation, growth, and stability. Since we observed potentiation of platelet functional responses in KO mice, we further evaluated in vivo functional implications of this enhanced platelet function. We first measured tail-bleeding time in PKCε KO, Heterozygous (HET), and WT mice, which reflects primary hemostatic function. We observed that average time taken for complete blockade of tail bleeding was 47.208 ± 3.90 seconds in KO, whereas it was 70.958 ± 5.38 seconds in WT mice. Furthermore, we observed that average bleeding time in HET is 51.298 ± 3.10 seconds (Figure 6A). These data suggest that PKCε negatively regulates hemostatic responses in vivo. Furthermore, we subjected KO and WT mice to FeCl3-induced arterial injury model to evaluate role PKCε in thrombosis. On injury, time to occlusion in KO mice was 10.583 ± 1.49 minutes, whereas it was 18.75 ± 1.84 minutes in WT mice. These data suggest that PKCε is a negative modulator of both hemostasis and thrombosis (Figure 6B).

729 residue, suggesting that PKCε is activated downstream of both P2Y and PAR4 receptors in human platelets.
Figure 5. Role of protein kinase C (PKCε) in 2MeSADP-induced platelet functional responses. Washed platelets isolated pooled from multiple WT and KO mice were stimulated with 100 nmol/L 2MeSADP for 3.5 minutes under stirring conditions in a lumi-aggregometer. A, Aggregation and secretion, or B, TxB₂ was measured. The data from 3 different days was pooled and was analyzed for statistical significance using Student t test (*P<0.05, **P<0.01). C, Washed platelets isolated pooled from multiple WT and KO mice were pre-treated with 10 μmol/L indomethacin and stimulated with 100 nmol/L or 30 nmol/L 2MeSADP for 3.5 minutes under stirring conditions in the lumi-aggregometer. Aggregation was measured. D, Fura-2 AM–labeled and washed murine platelets isolated from WT and KO mice were pretreated with 10 μmol/L indomethacin and activated with 100 nmol/L 2MeSADP under stirring conditions. Intracellular calcium flux was measured by fluorimetry. The data from 3 different days were pooled and analyzed for statistical significance, using the Student t test (*P<0.05, **P<0.01). E, Washed and indomethacin-treated murine platelets isolated from KO and WT mice were stimulated with 100 nmol/L 2MeSADP for various time periods under stirring conditions. Reaction was stopped by adding Lamelli-SDS buffer. Samples were subjected to Western blot analysis, using anti–phosphor-Thr/Tyr ERK1/2 antibody (p44/42 phospho-ERK) or anti-ERK1/2 antibody or anti-PKCε antibody. F and G, Washed platelets isolated pooled from multiple WT and KO mice were stimulated with various concentrations of AYPGKF or Convulxin (F) or U46619 or collagen (G) for 3.5 minutes under stirring conditions in a lumi-aggregometer. Tracings shown are representative of 3 separate experiments.
boxane also occurs during platelet activation by other agonists such as thrombin and collagen. Thus, to evaluate dependency of PKCε/H9255 to regulate platelet functional responses on generated thromboxane, we conducted our experiments in aspirin- or indomethacin-treated platelets, in which thromboxane generation was abolished. Under these conditions, loss of PKCε/H9255 in murine platelets neither affected ADP-induced aggregation nor induced dense granule secretion (Figure 5C). Similarly, in aspirin-treated human platelets pan-PKC inhibition with GF109203X did not cause dense granule secretion or potentiation of aggregation (data not shown). These data collectively suggest that PKCs (PKCε/H9255) negatively regulate intracellular calcium mobilization, which in turn regulates only thromboxane generation but not aggregation or dense granule secretion. There was some controversy regarding the role of generated thromboxane in U46619-induced platelet aggregation. Although it was previously shown that U46619-induced platelet aggregation is not affected by generated thromboxane, other groups have shown that abolishing thromboxane generation inhibits U46619-induced platelet aggregation.

In PKCε KO murine platelets, we evaluated role of generated thromboxane on U46619-induced platelet aggregation. Indomethacin inhibited U46619-induced platelet aggregation in PKCε KO murine platelets (data not shown). Similarly, in human platelets, aspirin inhibited U46619-induced platelet aggregation (data not shown). These data support the previous studies that generated thromboxane contributes to U46619-induced platelet aggregation.

We have previously shown that calcium positively regulates ADP-induced aggregation. However, in our studies, although PKC inhibition potentiates ADP-induced calcium mobilization (Figure 2A and Figure 5D), it does not result in potentiation of aggregation (Figure 5E). Although the reasons for this phenomenon need to be further investigated, it may be due to compartmentalization of calcium, which selectively increases activity of enzymes or molecules involved in thromboxane generation but not aggregation.

P2Y12 receptor-mediated signaling is shown to positively regulate P2Y1-mediated calcium mobilization in platelets. Furthermore, PKCs are shown to downregulate P2Y12 by facilitating P2Y12 receptor desensitization. Thus, it is intuitive to hypothesize that PKCs negatively regulate calcium mobilization by regulating P2Y12 receptor. However, we observed that PKCs regulate intracellular calcium mobilization independent of P2Y12 receptor signaling (Figure 2B).

The mechanism involved in PKC-mediated regulation of intracellular calcium concentration increases is yet to be studied. Intracellular calcium concentration is maintained by multiple mechanisms involving inositol 1,4,5 trisphosphate (IP3)-mediated calcium release from endoplasmic reticulum (ER) [dense tubular system in platelets] into cytosol, SERCA-mediated reuptake of cytosolic calcium into ER, calcium entry from the extracellular space into cytosol via store-operated calcium entry channels. Because we conduct all our ex vivo experiments in Tyrode buffer with minimal extracellular calcium, we could rule out the possibility that PKCs regulate cytosolic calcium concentration by regulating calcium entry from the extracellular space. Furthermore, on activation of P2Y1 receptor, it couples to Gq and activates PLCβ, which hydrolyzes phosphatidyl inositol 4,5-bisphosphate (PIP2) to DAG and IP3. DAG, thus generated, activates PKC and IP3 initiates calcium release from ER. Thus, PKC activation and calcium release would theoretically occur at similar time periods during platelet activation. Therefore, it is difficult to imagine that PKCs regulate intracellular calcium concentrations by regulating IP3-mediated calcium release from ER. Hence, it is quite possible...
that PKCs accelerate SERCA-mediated reuptake of cytosolic calcium in to ER and thereby negatively regulate cytosolic calcium concentration in platelets.

Previous studies from our laboratory indicate that intracellular calcium release is required for ERK activation. Thus, it is possible that PKCs indirectly regulate ERK phosphorylation by regulating intracellular calcium mobilization. On the other hand, it is also possible that PKCs regulate ERK phosphorylation independent of calcium mobilization by regulating phosphatases that dephosphorylate ERK or upstream activators that phosphorylate ERK, such as Raf. In various cell systems, PP2A, a serine/threonine phosphatase, and MKP3, a dual-specificity phosphatase (that dephosphorylates the substrates at Thr and Tyr residues), are shown to dephosphorylate ERK.42–44

Using commercial antibodies, PKCe was previously reported in human platelets. However, Pears et al45 and Buenosuco et al47 failed to identify PKCe in human platelets. Thus, to resolve the issue, we subjected human and murine platelet lysates to Western blot analysis, using custom-made antibody against PKCe (gift from Dr Mesung, Gallo Centre, San Francisco). We identified PKCe mobilizing ~90 kDa in human and murine platelet lysates (Figure 4A). Such a band was absent in PKCe KO murine platelets, increasing the confidence in data (Figure 4A). We also observed a nonspecific band masking PKCe in all lanes. Furthermore, PKCe has been reported in human platelets by a group, which studied platelet protein repertoire using mass spectrometry.48 Thus, in this study, we show that PKCe is shown to be phosphorylated activation loop, turn motif and hydrophobic motif. Furthermore, phosphorylation at Ser729 residue in hydrophobic motif of PKCe is shown to be essential for activation.33 Thus, in this study, we show that PKCe is phosphorylated at Ser729 ×2MeADP and AYPGKF in human platelets. These data together suggest that PKCe is expressed and activated in human platelets. We also investigated the role of PKCe using pseudo-RACK inhibitors in human platelets (data not shown). However, the inhibitors elicited similar effect in KO mice, suggesting that it is nonspecific. Furthermore, extent of potentiation in ADP-induced thromboxane generation caused by GF109203X is much greater than potentiation in PKCe KO mice, suggesting that PKCe is only one isoform that negatively regulates ADP-induced thromboxane generation, and there could be other PKC isoforms that negatively regulate ADP-induced thromboxane generation. Furthermore, chemical inhibitors used at high enough concentrations are known to cause nonspecific effects. For example, we have recently demonstrated that Go-6976, when used at concentration above 50 nmol/L, also inhibits Syk kinase.49 Thus, we have used Go-6976 at concentrations below 50 nmol/L. Although some Pan-PKC inhibitors, which were claimed to specific such as Ro 31—8220, have been shown to inhibit other serine threonine kinases such as mitogen-activated protein kinases (MAPK) with IC50 similar to PKCs, there are no such reports on GF109203X. Thus we have performed our experiments using GF109203X.

Pears et al46 have reported that PKCe has positive regulatory role in GPVI-mediated platelet functional responses and has no effect on ADP-, and AYPGKF-mediated platelet functional responses. However, we observe potentiation in AYPGKF- and ADP-mediated functional responses and non-significant differences in GPVI-mediated functional responses in PKCe null murine platelets compared with WT littermates. Interestingly, in concurrence with our data, the same group47 has recently reported that PKCe indeed negatively regulates ADP-induced functional responses. Thus, it is difficult to comment on data published by this group previously. Unlike convulxin, collagen-induced platelet functional responses were negatively regulated by PKCe. This is due to the dependence of collagen, which activates platelets by adhesion on generated thromboxane and secreted ADP more than convulxin, a soluble agonist that activates platelets independent of these feedback regulators at higher concentrations.18,21 Since PKCe negatively regulates thromboxane generation, it is possible that PKCe effects are pronounced in platelet responses induced by collagen but not by convulxin.

In conclusion, we have shown for the first time that PKCe negatively regulates ADP-induced platelet functional responses, including calcium mobilization, ERK activation, cPLA2 activation, and thromboxane generation, and thereby inhibits hemostasis and thrombosis in vivo.

Acknowledgments

Y.B.S. designed and performed experiments, analyzed data, and wrote the report; P.L. performed experiments; J.J. performed experiments and analyzed data; K.B. and M.R. performed experiments; S.K. performed experiments and analyzed data; D.W. carried out overall direction, designed experiments, and analyzed data; P.K. carried out overall direction, designed experiments, and analyzed data.

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