PKCα regulates collagen-induced platelet aggregation through inhibition of VASP-filopodia formation

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Protein kinase Cα (PKCα) has been shown by pharmacologic approaches to negatively regulate collagen-induced platelet aggregation. Here we addressed the molecular and cellular mechanisms underlying this negative regulation. Using PKCα−/− platelets, we show that the mechanism did not involve altered inside-out signaling to integrin α5β3 and did not affect early signaling events downstream of GPVI, because there was no difference in tyrosine phosphorylation of PLCγ2 between wild-type and PKCα−/− platelets. There was also no increase in secretion of dense granule content, in contrast to studies using rottlerin where secretion was enhanced. Importantly, however, there was marked enhancement of filopodia generation in PKCα−/− platelets upon adhesion to collagen compared with wild-type platelets. Filopodia play an essential role regulating adhesive events leading to platelet aggregation by increasing platelet-platelet contact. We show that the critical effector for PKCα is vasodilator-stimulated phosphoprotein (VASP), a major regulator of actin cytoskeleton dynamics. PKCα physically interacts with VASP constitutively and regulates its phosphorylation on Ser157. In VASP−/− platelets, the enhancement of filopodia generation, actin polymerization, and platelet aggregation by rottlerin is ablated. PKCα is therefore a critical negative regulator of filopodia, and hence platelet aggregation, through a functional interaction with the actin organizer VASP. (Blood. 2006;108:4035-4044) © 2006 by The American Society of Hematology

Introduction

The protein kinase C (PKC) family is a family of kinases that is critically involved in the regulation of several functional events in cells, including exocytosis and cell adhesion. Human platelets express predominantly 4 isoforms of PKC, α, β, δ, and θ. Many functional responses of platelets are regulated by PKC, including aggregation, which has been shown to be positively regulated by PKC isoforms in general, as suggested by its potentiation by PKC activators (eg, PdBu)1 and its inhibition by broad-spectrum pharmacologic antagonists of PKC isoforms.2-5 In platelets, PKC is also involved in the regulation of the cytoskeleton.6,7 Recently, with newer isoform-selective pharmacologic and genetic tools becoming available, the study of the roles of individual PKC isoforms has been made possible. PKCα is critically involved in calcium-induced α5β3-mediated platelet aggregation,8 and calcium-induced secretory responses depend on PKCα.9 We have previously shown that both aggregation and dense granule secretion induced by stimulation of glycoproteins VI and Ib-IIα-V strictly depend on PKCα activity.1 PKCα has also been reported to be involved in actin reorganization and proplatelet formation in murine megakaryocytes.10 PKCβ has been less extensively studied, although it has recently been shown critically to regulate outside-in signaling through α5β3 integrin.11 PKCβ has also recently been shown to play a similar critical role in inside-in signaling12 and physically and functionally interacts with Btk, a tyrosine kinase that plays a role in controlling calcium entry.13 PKCβ is unique, however, in that it has been shown to play a negative signaling role as well as a positive one. It has been shown to suppress platelet aggregation activated through glycoproteins VI and Ib-IIα-V,14,15 although it positively regulates secretion in response to protease-activated receptor-activating peptides SFLLRN and AYPGKF.15 The mechanisms underlying the negative regulation of platelet aggregation by PKCβ are not presently known and are the focus of the present report.

Filopodia are dynamic membrane protrusions supported by bundles of linearly extending actin filaments, with their fast-growing barbed end at the tip.16 They function to sense environmental cues in many cell types for the control of cellular migration, adhesion, and growth.16,17 In platelets, upon adhesion to collagen, filopodia are transiently formed and then superceded by the sustained lamellipodia of the spread platelet. Filopodia, however, critically contribute to the platelet aggregation response,18 and their formation is therefore of primary importance in regulating platelet activity in hemostasis and thrombosis. Many regulators of filopodia have been identified, including the Arp2/3 complex, the Rho family GTPase cdc42, Rif, and vasodilator-stimulated phosphoprotein (VASP). VASP is a regulator of actin polymerization, and it is primarily the anticapping activity of VASP that promotes filopodia formation by allowing linear actin polymerization.19-22 VASP is a major substrate of protein kinase A (PKA) and protein kinase G (PKG), which phosphorylate it at 3 main sites: Ser,157 Ser239, and Thr278.23 Phosphorylation of VASP, particularly at Ser157, regulates its anticapping activity24 and hence the ability of VASP to regulate filopodia development.

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In this study we confirm by a genetic approach the observation that PKCδ has a negative signaling role in regulating platelet aggregation. This negative regulation is mediated through a physical and functional interaction of PKCδ with VASP, leading to inhibition of filopodia formation such that in the absence of PKCδ filopodia formation is no longer transient but sustained. VASP is essential for mediating signaling by PKCδ, because the effects of the PKCδ-selective inhibitor rottlerin on filopodia formation, actin polymerization, and platelet aggregation are ablated in VASP−/− platelets. The work therefore reveals a new pathway for regulation of actin and filopodia and, thereby, platelet aggregation and shows PKCδ to be a major negative regulator of these events.

Materials and methods

Materials

Collagen was obtained from Nycomed (Linz, Austria), and collagen-related peptide (CRP) was a kind gift from Dr Richard Farndale (University of Cambridge, United Kingdom). The P2Y12-selective antagonist AR-C69931MX was a gift from AstraZeneca (Macclesfield, United Kingdom). The cd42 inhibitor secretase A was a kind gift from Prof Tom Kirchhausen and Prof Matthew Shair, Harvard University, Cambridge, MA. Rottlerin was supplied by Merck Biosciences (Beeston, United Kingdom), and NSC23766 was from Tocris (Avonmouth, United Kingdom). Anti–PKCδ antibody for immunoprecipitation and immunofluorescence and anti–PKCa, anti–PKCb, anti–PKCc, and anti–PKCd antibodies were from BD Biosciences (Oxford, United Kingdom), and anti–VASP antibody was from Alexis Biochemicals (Bingham, United Kingdom). Anti–PKCδ antibody for immunoblotting, secondary antibodies for immunofluorescence, and anti–PKCa antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). The cd42 activation assay kit and Rac activation assay kit were from Cytoskeleton (Denver, CO). Anti–phospho-VASP, anti–phospho-Ser/Thr PKA substrate, and anti–phospho–Ser/Thr PKC substrate antibodies were from Cell Signaling Technology (New England Biolabs, Hitchin, United Kingdom). R-phycocerythrin–conjugated anti–αIIbβ3, antibody JON/A was obtained from Emfret (Würzburg, Germany), while PhosphoDetect anti-PKG substrate antibody, forskolin, and 8-pCPT-cGMP were from Calbiochem (Merck Biosciences, Darmstadt, Germany). Anti-phosphotyrosine 4G10 antibody was from Upstate Biotechnology (Lake Placid, NY). Apyrase, adenosine-3′-phosphate-5′-phosphate (AP35P), prostaglandin E1, bovine serum albumin (BSA), Protein Phosphatase Cocktails I and II, bicinechonic acid (BCA), poly-l-lysine, the anti–β-tubulin antibody, TRITC-phalloidin, 1,4-diazobicyclo-[2,2,2]octane (DABCO), and Mowiol were purchased from Sigma (Poole, United Kingdom). The P2Y12-selective antagonist AR-C69931MX was a gift from AstraZeneca (Macclesfield, United Kingdom). Anti-phosphotyrosine 4G10 antibody was from Upstate Biotechnology (Lake Placid, NY). Apyrase, adenosine-3′-phosphate-5′-phosphate (AP35P), prostaglandin E1, bovine serum albumin (BSA), Protein Phosphatase Cocktails I and II, bicinechonic acid (BCA), poly-l-lysine, the anti–β-tubulin antibody, TRITC-phalloidin, 1,4-diazobicyclo-[2,2,2]octane (DABCO), and Mowiol were purchased from Sigma (Poole, United Kingdom). Enhanced chemiluminescence (ECL) system, [3H]5-hydroxytryptamine ([3H]5-HT), and horseradish peroxidase (HRP)–conjugated secondary antibodies were from Amersham (Little Chalfont, United Kingdom).

Preparation of platelets

Human blood was drawn from healthy, drug-free volunteers on the day of experiment, and platelets were prepared as described previously. Informed consent was provided according to the Declaration of Helsinki. For mouse experiment, and platelets were prepared as described previously. Human blood was drawn from healthy, drug-free volunteers on the day of preparation of platelets.

Platelet stimulation

Stimulation of platelets in suspension was achieved with either 30 μg/mL collagen or 5 μg/mL CRP. Alternatively, platelets were stimulated by adhesion to coated coverslips. Coverslips were coated with either 100 μg/mL collagen or 50 μg/mL CRP for 2 hours, followed by nonspecific binding saturation with 2% BSA for 1 hour.

SDS-PAGE and immunoblotting

Proteins were resolved by electrophoresis in 8% to 10% sodium dodecyl sulfate–polyacrylamide gels electrophoresis (SDS-PAGE). Samples were then transferred to PVDF membranes using a Bio-Rad (Hercules, CA) Trans-Blot semidy transfer cell, blocked with 5% to 10% BSA, and incubated for 1 hour or overnight at room temperature with appropriate primary antibody. Following incubation with anti–rabbit IgG HRP-conjugated antibodies, bound peroxidase activity was detected using enhanced chemiluminescence (ECL).

Protein immunoprecipitation

Twenty micrograms of either anti–PKCδ or anti–VASP antibody was immobilized onto Aminolink Plus Coupling Agarose Gel using Seize Primary Mammalian Immunoprecipitation Kit (Perbio, Rockford, IL). Four hundred micromolars of protein from platelet lysates was incubated at 4°C on immunocoupled gel-loaded columns. Beads were washed with TBS (25 mM Tris, 150 mM NaCl, pH 7.2), and protein was eluted and analyzed by immunoblotting.

Immunofluorescence confocal imaging

Platelets were stimulated by adhesion to either collagen- or CRP-coated surfaces. Reactions were terminated by addition of 4% paraformaldehyde. Platelet permeabilization and immunolabeling were performed as previously described. Primary antibodies were used at 1 μg/mL (anti–PKCδ) or 1:200 (anti–VASP). FITC- or TRITC-conjugated secondary antibodies were used at 4 μg/mL. For F-actin detection, 2 μg/mL TRITC-phalloidin was used. Platelets were imaged using a Leica TCS-NT confocal laser scanning microscope equipped with a krypton/argon laser attached to a DM IRBE inverted epifluorescence microscope with phase contrast (Leica, Wetzlar, Germany). Images were captured with a 63×/1.32 NA objective and were processed with OpenLab 4.03 (Improvision, Lexington, MA) and Adobe Photoshop (Adobe Systems, San Jose, CA).

Analysis of platelet adhesion and spreading by DIC microscopy

Glass coverslips were prepared as described under “Platelet stimulation” and mounted into a live-cell microscopy chamber. Platelets were resuspended to 2 × 10^7/mL in modified Tyrodes-HEPES buffer and dispensed onto the coverslip. Adhesion and spreading of platelets was followed by differential interference contrast (DIC) microscopy with a wide-field microscope DM IRB attached to an ORCA ER camera (63×/1.40 NA objective) (Leica). Images were processed with OpenLab 4.03 (Improvision) and Adobe Photoshop. The perimeter of adherent platelets was measured using Velocity software (Improvision), while the number of adhered platelets was manually counted.

Flow cytometric analysis of activation of the αIIbβ3 integrin

The activation of mouse platelets was assessed with R-phycocerythrin–conjugated JON/A antibody that preferentially binds to the active form of αIIbβ3 integrin. Washed platelets (5 × 10^7/mL in Tyrodes-HEPES buffer modified with 1 mM CaCl2 and 0.35% BSA) were stimulated with 5 μg/mL CRP in the presence of PE-JON/A (5 μg/mL) for 15 minutes at 37°C. Flow cytometry was performed using a FACS Calibur flow cytometer (Becton Dickinson, Oxford, United Kingdom), and a total of 50,000 events per sample were collected. Fluorescent labeling was measured, and analysis of results was performed using WinMDI 2.8 (The Scripps Research Institute, La Jolla, CA).

Measurement of platelet aggregation

Platelets were prepared as described under “Preparation of platelets,” preincubated with different inhibitors or vehicle solution (0.1% Me2SO final concentration) for 10 minutes at 37°C, and stimulated in an aggregometer (Chrono-Log, Labmedics, Manchester, United Kingdom) at 37°C, with
Platelet aggregation was induced with collagen (30 μg/mL) and pretreated either with rottlerin (5 μM) for 15 minutes or DMSO vehicle as control. Platelet aggregation was induced with collagen (30 μg/mL) and monitored by turbidimetric aggregometry over a 10-minute period. Traces shown are representative of 3 independent experiments. (B) Whole cell lysates from PKCζ−/− and wild-type mouse platelets were analyzed for the expression of PKCα, PKCζ, PKCδ, and PKCβ by immunoblotting (top panels). Uniformity of protein loading was confirmed by immunoblotting with anti-β-tubulin antibody (bottom panels). The immunoblots are representative of 3 independent experiments.

Measurement of 5-HT release

Platelets were loaded by incubation of platelet-rich plasma with 0.5 μCi/mL (1.85 × 10⁶ Bq/mL) [3H]5-HT for 1 hour at 37°C. Platelets were then pelleted and resuspended in Tyrodes-HEPES buffer. After incubation with different inhibitors or vehicle solution (0.1% Me2SO final concentration) for 10 minutes at 37°C, platelets were stimulated with continuous stirring at 800 rpm. Following rapid centrifugation of the platelets, the [3H]5-HT released into the supernatant was determined by scintillation counting and expressed as a percentage of the total cell content as described previously.5

Rac and cdc42 activation assays

Activation assays were performed as advised by the supplier (Cytoskeleton). Briefly, 500 μL washed human platelets (4 × 10⁹/mL) were stimulated as indicated and lysed into 1% NP-40 buffer for 45 minutes at 4°C. Subsequently, 10 μg p21-activated kinase-protein binding domain (PAK-PBD) beads were added to the lysates and incubated for 45 minutes at 4°C. As a positive control, platelet lysates were incubated with GTPγS (200 μM), and platelet lysates incubated with GDP (1 μM) were used as negative controls. Beads were precipitated by centrifugation, washed, resuspended in Laemmli buffer, and boiled for 5 minutes. Supernatants were analyzed by immunoblot for the presence of either Rac1 or cdc42 using specific antibodies (250 ng/mL and 1 μg/mL, respectively).

Statistical analysis

Significance of difference between data was analyzed by 2-way or 1-way analysis of variance (ANOVA) with the Bonferroni posttest.

Results

PKCζ negatively regulates platelet aggregation by mechanisms independent of inside-out signaling to αIIbβ3, dense granule secretion, and early GPVI signaling

We and others had already shown that inhibition of PKCζ with rottlerin was able to potentiate the aggregation response of human platelets to collagen.14,15 A similar effect was observed as a consequence of PKCζ ablation in mouse platelets. As shown in Figure 1Ai, PKCζ−/− platelets were characterized by more extensive aggregation (maximal reduction in turbidity, 64.6% ± 7.1%) compared with wild-type platelets (46.8% ± 4.0%) (mean ± SEM, n = 5). Importantly, treatment with rottlerin (5 μM) potentiated the aggregation of wild-type platelets (Figure 1Aii), increasing the maximal decrease of turbidity from 46.2% ± 4.3% to 65.2% ± 2.9% (mean ± SEM, n = 5). On the other hand, no significant effect of rottlerin was observed on PKCy−/− platelets (Figure 1Aiii). Figure 1B shows that expression levels of other PKC isoforms (α, β, ε, and θ) in PKCζ−/− platelets were all equivalent to those in wild-type platelets, and therefore no functional compensation or up-regulation of related PKC isoforms was apparent.

Having verified the negative regulatory role played by PKCζ in collagen-induced platelet aggregation, it was now important to

Figure 2. The activation of integrin αIIBβ3, secretion of ADP, and early signaling downstream of GPVI are not regulated by PKCζ. (A) Platelets from either wild-type (WT) or PKCζ−/− mice were loaded with JON/A antibody, stimulated with CRP (5 μg/mL) for 15 minutes, and surface labeling measured by flow cytometry. The bar graph shows the geometric mean of the intensity of PE-antibody labeling for WT or PKCζ−/− platelets nonstimulated or stimulated with CRP. The data represent mean ± SEM from 3 independent experiments, and the statistical significance of differences between PKCζ−/− and wild-type was analyzed by 1-way ANOVA (nonsignificant). The top inset shows the forward-scattering (FSC-Height) versus side-scattering dot plot for a representative sample of mouse platelets. The bottom inset shows the frequency distribution of the labeling values for the platelet population in the different experimental conditions. (B) Platelets from wild-type (WT) or PKCζ−/− mice were preincubated for 15 minutes with 0.2 μM L-AP4, 1 mM A3P5P5, and 1 μM ARC and stimulated with collagen (30 μg/mL). Platelet aggregation responses were monitored by turbidimetric aggregometry, and data shown are representative of 3 independent experiments. (C) Wild-type (WT) or PKCζ−/− mouse platelets were loaded with [3H]5-HT. Subsequently, the percentage of total [3H]5-HT content released by platelets stimulated for 5 minutes with 30 μg/mL or 3 μg/mL collagen in the presence or absence of rottlerin (5 μM) was determined by liquid scintillation counting. Results shown are mean ± SEM from 3 independent experiments. The difference between WT and PKCζ−/− platelets was analyzed by 1-way ANOVA, and differences were statistically nonsignificant (P = .42). The differences between rottlerin-treated and nontreated controls in both wild-type and PKCζ−/− platelets stimulated with 3 μg/mL collagen were significant (P < .01). (D) PLCγ2 was immunoprecipitated from PKCζ−/− and wild-type (WT) mouse platelets after activation by collagen (30 μg/mL) for the time periods shown. Samples were analyzed by immunoblotting using antiphosphotyrosine antibody 4G10 (top panels). The efficiency of immunoprecipitation was analyzed by reblotting with anti-PLCγ2 antibody (bottom panels). The arrow indicates the molecular weight of PLCγ2. The immunoblots are representative of 3 independent experiments.
Platelet aggregation in response to collagen (30 µg/mL) was monitored by turbidimetric aggregometry. Traces shown are representative of 3 independent experiments. (A) Washed human platelets were preincubated with secramine A at the concentrations indicated for 15 minutes. (i) Platelet aggregation in response to collagen (30 µg/mL) was monitored by turbidimetric aggregometry. Traces shown are representative of 3 independent experiments, either prior to adhesion (suspension) or at time points of activation in contact with collagen either at an early stage where filopodia are predominant or at a late stage where lamellipodia are predominant. (iii) The activity of secramine A was investigated by cdc42 pull-down using GST-tagged PAK-PBD protein beads. Washed human platelets were either not stimulated or stimulated with collagen (30 µg/mL) to the same levels. Taken together, these data suggest that PKCγ does not regulate dense granule release and that blockade of cdc42 by a specific inhibitor, secramine A, leads to a large degree subsequent lamellipodia, upon adhesion of human platelets to a collagen-coated surface (Figure 3Aii). Figure 3Aiii confirms that secramine is able to determine the underlying molecular mechanism. We initially ruled out several mechanisms (Figure 2). First we were able to rule out a regulation of αIIbβ3 integrin using Jon/A monoclonal antibody, which recognizes the integrin only when in the active conformation. Figure 2A shows a marked increase in Jon/A binding upon activation of platelets with the GPVI-selective agonist CRP in both wild-type and PKCδ−/− platelets. There was no significant difference in activation of the integrin between these 2 groups of platelets, suggesting that PKCδ does not regulate inside-out signaling to αIIbβ3.

Figure 3. PKCδ negatively regulates filopodia formation in human platelets. (A) Washed human platelets were preincubated with secramine A at the concentrations indicated for 15 minutes. (i) Platelet aggregation in response to collagen (30 µg/mL) was monitored by turbidimetric aggregometry. Traces shown are representative of 3 independent experiments. (ii) Platelets were added to collagen-coated coverslips and allowed to settle on the surface and adhere to collagen fibers under static conditions. Images shown are of single representative platelets from at least 3 independent experiments, either prior to adhesion (suspension) or at time points of activation in contact with collagen either at an early stage where filopodia are predominant or at a late stage where lamellipodia are predominant. (iii) The activity of secramine A was investigated by cdc42 pull-down using GST-tagged PAK-PBD protein beads. Washed human platelets were either not stimulated or stimulated with collagen (30 µg/mL) in the absence or presence of secramine A (3 or 30 µM, 15 minutes of preincubation). Cells were lysed and pull-downs performed as described in "Materials and methods," and the amount of GTP-loaded cdc42 pulled down was assessed by immunoblot. As a positive control, platelet extracts were treated with GTPγS (200 µM), while platelets treated with GDP (1 mM) were used as negative control. Images shown are representative of 3 independent experiments. (B) Washed human platelets were preincubated for 15 minutes with either rottlerin (5 µM) or DMPSO as control (vehicle) as indicated. In some experiments washed human platelets were also preincubated for 15 minutes with apyrase (0.2 U/mL), A3P5P (1 mM), and AR-C69931MX (1 µM) as indicated (ADP inhib.). Platelets were added to collagen-coated coverslips and allowed to settle on the surface and adhere to collagen fibers under static conditions. (i) Images shown are for platelets allowed to adhere to the collagen-coated surface for 40 minutes in the presence or absence of the various inhibitors as indicated. These images are representative of 5 independent experiments. The perimeter (ii) and the number (iii) of adherent platelets were measured 0, 10, 20, 30, or 40 minutes after being dispensed onto the collagen-coated surface. Rottlerin induced a statistically significant increase in the perimeter of adherent platelets (P < .01), while no statistically significant difference was detected (P = .66) for the number of adherent platelets. Incubation with ADP receptor antagonists did not influence the platelet perimeter (P > .01) but significantly reduced the number of adherent platelets (P < .01). Data shown are mean ± SEM from at least 3 independent experiments.

PKCδ is a critical negative regulator of platelet filopodia formation and actin polymerization in response to collagen

Platelets rapidly and transiently generate filopodia upon activation by agonists in a manner dependent upon a number of key signaling components critically including the Rho GTPase cdc42. Filopodia play an important role regulating interaction between platelets, thereby regulating platelet aggregation. Figure 3Ai shows that blockade of cdc42 by a specific inhibitor, secramine A, leads to dose-dependent blockade of aggregation. Secramine A blocked formation of filopodia, and to a large degree subsequent lamellipodia, upon adhesion of human platelets to a collagen-coated surface (Figure 3Aii). Figure 3Aiii confirms that secramine is able to
inhibit cdc42 activity in platelets by assessment of GTP loading of cdc42 using a PAK-PBD pull-down assay. It was possible, therefore, that the potentiation of platelet aggregation to collagen seen in the absence of active PKCδ may be mediated by a potentiation of filopodia formation. Figure 3Bi shows human platelets adherent to a collagen-coated surface at a late adherent stage where the spread lamellipodial morphology is predominant. The time-lapse video in Video S1 (available at the Blood website; see the Supplemental Materials link at the top of the online article) shows that the spread lamellipodial phase is sustained but is preceded by a transient filopodial phase after initial adhesion of the platelet to the collagen fiber. In the presence of rotterlin, however, a marked morphologic difference is seen, where the transient filopodial generation becomes sustained (Video S2; Figure 3B). The responses seen are not dependent upon secreted ADP, because they are apparent in the presence of a cocktail of ADP receptor antagonists and apyrase. Quantitative data are displayed by assessing mean cell perimeter in Figure 3Bii, which shows a time-dependent increase in cell perimeter that is markedly potentiated in the presence of rotterlin. Figure 3Biii shows that the number of platelets adherent to collagen in the presence or absence of rotterlin is equivalent, although the presence of the ADP inhibitory cocktail potently reduced the number of adherent cells.

These data suggested a negative signaling role for PKCδ in regulating filopodia formation, and this was confirmed using PKCδ−/− mouse platelets. Figure 4A shows that platelets lacking PKCδ show a sustained filopodial development in contrast to wild-type platelets where the filopodial phase is transient. These data are illustrated in time-lapse live-cell videos shown in Videos S3-S4 (wild-type and PKCδ−/− platelets, respectively). Interestingly, rotterlin is also able to potentiate filopodial formation in mouse platelets, as in human platelets, but this potentiation is absent when applied to PKCδ−/− platelets. This indicates that the effect of rotterlin on this response is indeed mediated through inhibition of PKCδ.

Because filopodia are normally subsumed by developing lamellipodia, it was possible that the apparent potentiation in filopodia seen in the absence of active PKCδ was a result of regulation of lamellipodia by PKCδ rather than a genuine regulation of filopodia formation. To demonstrate definitively that PKCδ regulated filopodial formation, we used an inhibitor of GTPase Rac (NSC23766), which we show to inhibit Rac1 activity in platelets, by assessment of GTP loading of Rac1 using a PAK1-PBD pull-down assay (Figure 4B), as has been shown in Rac−/− platelets. In the presence of NSC23766, however, filopodia are formed normally, and the potentiation of filopodia seen in PKCδ−/− platelets is maintained upon treatment with the Rac inhibitor. These data demonstrate that PKCδ does negatively regulate filopodia.

Because filopodial structures are generated by coordinated actin polymerization, it was important to determine whether actin polymerization was also regulated by PKCδ. Figure 5A shows in human platelets that rotterlin induces a significant increase in actin polymerization in platelets adherent either to collagen- or CRP-coated surfaces. It is also evident from the images of single adherent platelets that, in spread platelets, the polymerized actin forms a “rosette” shape and that this configuration is markedly disrupted in platelets treated with rotterlin. The enhanced actin polymerization and the disrupted configuration are

Figure 4. PKCδ negatively regulates filopodia formation in mouse platelets. (A) Washed platelets from either wild-type (WT) or PKCδ−/− (δ-KO) mice were preincubated for 15 minutes with either rotterlin (5 μM) or DMSO as control (vehicle) as indicated. Platelets were added to collagen-coated coverslips and allowed to settle on the surface and adhere to collagen fibers under static conditions. (i) Images shown are for platelets allowed to adhere to the collagen-coated surface for 40 minutes, representative of 5 independent experiments. The perimeter (ii) and number (iii) of adherent platelets were measured 0, 10, 20, 30, or 40 minutes after being dispersed onto the collagen-coated surface. In the absence of rotterlin, the differences in perimeter (at 10, 20, 30, and 40 minutes) between WT and PKCδ−/− (δ-KO) platelets were statistically significant (P < .01), while no significant difference was detected (P > .05) for the number of adherent platelets. In the presence of rotterlin, the difference in perimeter between PKCδ−/− (δ-KO) and WT platelets was not significant (P > .05). Data shown are mean ± SEM from 3 independent experiments. (B) Washed platelets from either wild-type (WT) or PKCδ−/− (δ-KO) mice were preincubated for 15 minutes with either NSC23766 (200 μM) or DMSO as control (vehicle) as indicated. Platelets were added to collagen-coated coverslips and allowed to settle on the surface and adhere to collagen fibers under static conditions. (i) Images shown are for platelets allowed to adhere to the collagen-coated surface for 40 minutes, representative of 3 independent experiments. The perimeter (ii) and number (iii) of adherent platelets were measured 0 and 40 minutes after being dispersed onto the collagen-coated surface. The differences in perimeter between WT or PKCδ−/− (δ-KO) platelets at 40 minutes were statistically significant in the presence or absence of NSC23766 (P < .01). Values shown are mean ± SEM from 3 independent experiments, and the statistical differences were analyzed by 1-way ANOVA.
mirrored in mouse platelets lacking PKCδ upon adhesion to CRP (Figure 5B) or collagen (data not shown). These data demonstrate a negative signaling role for PKCδ in actin polymerization and a role for the kinase in organizing the actin cytoskeleton.

**Regulation of platelet function by PKCδ is mediated through a physical and functional interaction with VASP**

To elucidate the mechanism by which PKCδ regulated actin polymerization and filopodia formation, we screened for proteins that directly interacted with PKCδ by communoprecipitation. A previously unreported interaction was found with VASP (Figure 6A), a major regulator of actin polymerization. Importantly, through its anticapping activity, VASP promotes filopodia formation by allowing linear actin polymerization. The physical association between VASP and PKCδ is reflected in Figure 6B, which shows a high degree of colocalization of the 2 proteins throughout all stages of morphologic rearrangement, including the transient filopodial phase and the later sustained lamellipodial phase.

We and others have recently shown VASP to be a substrate not only of PKA and PKG but also the classical isoforms of PKC, which phosphorylate VASP on Ser157 but not Ser239 in platelets and vascular smooth muscle cells. Figure 6C shows that collagen and CRP induce phosphorylation on Ser157 but not Ser239 and that, paradoxically, phosphorylation of Ser157 was observed in an unusual role among PKC isoforms expressed in platelets, because broad-spectrum PKC inhibitors had shown this family of kinases to play largely a positive signaling role. It was therefore important to confirm the pharmacologic data from previous publications and to elucidate the mechanism underlying the negative regulation of platelet aggregation by PKCδ. In this study we confirmed by a genetic approach the negative signaling role of PKCδ in regulating platelet aggregation induced by collagen and revealed a novel and unexpected underlying mechanism. The negative regulation is mediated through a physical and functional interaction of PKCδ with VASP, leading to inhibition of filopodia formation such that in the absence of PKCδ filopodia formation is no longer transient but sustained and platelet aggregation is enhanced. VASP is essential for mediating signaling by PKCδ, because the effects of the PKCδ-selective inhibitor rottlerin on filopodia formation and actin polymerization are ablated in VASP−/− platelets. The work therefore reveals a new pathway for regulation of platelet aggregation mediated by PKCδ through a negative regulation of actin polymerization and filopodia formation.

We were first able to show that collagen-induced platelet aggregation is potentiated in the absence of active PKCδ either using a genetic approach (PKCδ−/− platelets) or a pharmacologic approach (rottlerin). These results are in agreement with previously

PKCδ isoform inhibitor, Go6976, is able to reduce phosphorylation to basal levels in response to collagen. There was minimal contribution from PKA or PKG, which we show to be not significantly activated by collagen (Figure S2), because inhibition of these kinases by H89 had no effect upon collagen-induced VASP Ser157 phosphorylation (Figure 6D). PKCδ therefore negatively regulates the ability of classical PKC isoforms to phosphorylate VASP on Ser157, although this is not through a direct regulation of the activity of these kinases, because their ability to phosphorylate platelet substrates is unaffected by rottlerin or the absence of PKCδ (Figure S3).

As an anticapping protein, VASP localizes to the tips of linearly growing actin filaments in the growth of actin-dependent membrane protrusions such as filopodia. Figure 6E shows that in platelets adherent to collagen- or CRP-coated surfaces, VASP forms a ringlike structure that surrounds the rosette-shaped F-actin arrangement. In human platelets pretreated with rottlerin (Figure 6Ei) or in mouse platelets lacking PKCδ (Figure 6Eii), this organization of both VASP and actin filaments is lost and the ringlike distribution of both proteins is entirely disrupted.

Finally, it was important to demonstrate definitively that VASP is the essential mediator of PKCδ function for regulation of platelet actin polymerization, filopodia generation, and platelet aggregation. Figure 7A shows that the potentiation of filopodia by rottlerin (shown quantitatively as an increase in cell perimeter) at a late time point (40 minutes) is ablated in mouse platelets lacking VASP. This is paralleled by data for both actin polymerization (Figure 7B) and platelet aggregation (Figure 7C) because in VASP−/− platelets the potentiatory effect of the PKCδ inhibitor rottlerin is also ablated. This indicates that PKCδ is a critical negative regulator of actin polymerization, filopodia formation, and platelet aggregation mediated through an essential association with VASP.

**Discussion**

PKCδ has been shown previously by us and others to play a role distinct from other PKC isoforms in negatively regulating platelet aggregation in response to GPVI activation. This placed PKCδ in an unusual role among PKC isoforms expressed in platelets, because broad-spectrum PKC inhibitors had shown this family of kinases to play largely a positive signaling role. It was therefore important to confirm the pharmacologic data from previous publications and to elucidate the mechanism underlying the negative regulation of platelet aggregation by PKCδ. In this study we confirmed by a genetic approach the negative signaling role of PKCδ in regulating platelet aggregation induced by collagen and revealed a novel and unexpected underlying mechanism. The negative regulation is mediated through a physical and functional interaction of PKCδ with VASP, leading to inhibition of filopodia formation such that in the absence of PKCδ filopodia formation is no longer transient but sustained and platelet aggregation is enhanced. VASP is essential for mediating signaling by PKCδ, because the effects of the PKCδ-selective inhibitor rottlerin on filopodia formation and actin polymerization are ablated in VASP−/− platelets. The work therefore reveals a new pathway for regulation of platelet aggregation mediated by PKCδ through a negative regulation of actin polymerization and filopodia formation.
published data showing a marked potentiation of aggregation of human platelets in response to low concentrations of alboaggregin A, a C-type lectinlike snake venom that activates glycoproteins VI and Ib-IX-V. Importantly, rottlerin potentiated the aggregation of wild-type but not PKCζ−/− platelets, confirming that at least for aggregation the effect of rottlerin was due specifically to inhibition of PKCζ. This was important because rottlerin has been reported to have effects other than inhibition of PKCζ.

It was, however, clear from our data that although rottlerin was also able to enhance secretion of 5-HT induced by collagen, in line with previous reports for this inhibitor in platelets and in vitro—for example, inhibiting 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.

It was clear that filopodia play a critical role regulating platelet-platelet interaction, and it was therefore possible that enhanced filopodia formation could also underlie the enhanced platelet aggregation response seen in PKCζ−/− platelets. The study by Frojmovic and colleagues had been performed with ADP as agonist and, therefore, to verify the role played by filopodia in

Figure 6. Physical and functional association of PKCζ with VASP. (A) Washed human platelets were stimulated for 180 seconds with collagen (30 μg/mL) or unstimulated (vehicle). Reactions were stopped by addition of ice-cold NP-40 lysis buffer, and PKCζ (i) or VASP (ii) was immunoprecipitated and blotted each for PKCζ and VASP as indicated. The arrows indicate the molecular weight of VASP (i) or PKCζ (ii). The immunoblots are representative of 3 independent experiments. (B) Human platelets adherent to a CRP-coated surface were fixed, permeabilized, and immunostained with 1:250 anti–phospho-VASP (Ser157) and reprobed with 1:1000 anti-VASP antibody (top and bottom panels, respectively). The arrow indicates the molecular weight of VASP. All immunoblots shown are representative of 3 independent experiments. (C) Human platelets were either nonstimulated or stimulated with collagen (3 μg/mL) or collagen (30 μg/mL) or unstimulated (H9262) and their superimposition (third column) from 3 independent experiments. The phase-contrast image of the platelet is also shown (right column). The early stage of platelet attachment, the transient filopodial phase, and the late lamellipodial phase are represented on the top, center, and bottom rows, respectively. (C) Whole cell lysates (WCL) of (i) human platelets or (ii) platelets from either wild-type or PKCζ−/− mice were immunoblotted with 1:1000 anti–phospho-VASP (Ser157) and 1:1000 anti–phospho-VASP (Ser239) (top and middle panels, respectively). Bottom panels show the reblot with 1:1000 anti-VASP antibody. Platelets were either nonstimulated or stimulated with collagen (30 μg/mL) in the absence of presence of kinase inhibitors (H89, 3 μM; G66769, 1 μM; 15 minutes of preincubation) as indicated. The arrow indicates the molecular weight of VASP. All immunoblots shown are representative of 3 independent experiments. (D) Human platelets were either nonstimulated or stimulated with collagen (30 μg/mL) in the presence of CRP (5 μg/mL) or collagen (30 μg/mL) or unstimulated (H9262) (bottom panels) mice. The superimposition of VASP and F-actin labeling (third column) and the phase-contrast image (right) are also shown. Images shown are representative of 3 independent experiments.
adherent platelets in the different experimental conditions was measured 40 minutes after dispensing of platelets onto the surface. Data shown are mean ± SEM (n = 4), and the statistical significance was analyzed by 1-way ANOVA (*P < .01, comparison between data in the absence or presence of rottlerin). (B) Washed platelets derived from either wild-type (WT) or VASP (KO) mice were allowed to adhere to collagen-coated coverslips in the absence or presence of rottlerin (5 μM, 15 minutes of preincubation). Platelets were fixed 40 minutes after addition to the coated surfaces, permeabilized, and stained with TRITC-phalloidin. Fluorescence labeling was detected by confocal microscopy and quantified using Leica Confocal Software. Data represent mean ± SEM (n = 3), and the statistical significance of difference was analyzed by 1-way ANOVA (*P < .01, comparison between results in the absence or presence of rottlerin). Images below the graphs show representative examples of platelets obtained under the same conditions indicated in the bar graph and stained with TRITC-phalloidin (top row), with corresponding phase-contrast images (bottom row). (C) Washed platelets were prepared from (i) wild-type (WT) or (ii) VASP (KO) mice and pretreated either with rottlerin (5 μM) for 15 minutes or DMSO vehicle as control. Platelet aggregation was induced with collagen (30 μg/mL) and monitored by turbidimetric aggregometry over a 10-minute period. Traces shown are representative of 3 independent experiments.

collagen-induced platelet aggregation we used secramine A, a novel inhibitor of the Rho family GTPase cdc42 that plays a critical role in filopodia formation.38 Secramine inhibits activation of cdc42 by a mechanism dependent upon the guanine nucleotide dissociation inhibitor RhoGDI, mimicking the effects of dominant-negative cdc42 expression. Figure 3A showed that secramine markedly inhibited filopodia formation in platelets upon contact with collagen and dose-dependently inhibited collagen-induced platelet aggregation. Interestingly, secramine also substantially inhibited platelet adhesion to collagen and largely blocked lamellipodia formation in addition to filopodia. This is likely to be because filopodia are required for early firm adhesion of platelets to collagen and subsequent lamellipodia formation is a sequential event after filopodia formation. In the absence of filopodia formation, therefore, the development of lamellipodia is substantially inhibited.

The most striking observation of this study is illustrated in Figures 3B and 4 for human and mouse platelets, respectively, and supported by live-cell movies in Videos S1-S4. These data show the central observation of this study: that lack of PKCδ leads to markedly enhanced filopodia generation and, in particular, converts a transient filopodial phase into a sustained filopodial development upon adhesion of platelets to collagen. This study has therefore revealed a new function for PKCδ and a new mechanism for negative regulation of actin polymerization and filopodia formation, in turn regulating platelet-platelet interaction and aggregation.

PKC isoforms have previously been shown to play critical roles in regulating cell contractility and motility29,40 and in regulating the actin cytoskeleton through phosphorylation of multiple substrates, including adducin, fascin, MARCKS, and vinculin,41 although none so far has shown a functional interaction of any isoform with VASP. At the molecular level it is clear that a major determinant of PKC function is the immediate protein environment in which the kinase is found.42,43 and we therefore set out to identify novel interacting partners for PKCδ by a coimmunoprecipitation approach. VASP was identified as one of these proteins and interestingly was found to associate constitutively with PKCδ. It is not clear from our data at present whether the interaction is direct or mediated by an additional partner protein. VASP has multiple functions critically involved in remodeling the actin cytoskeleton, promoting profilin recruitment, actin nucleation, bundling and filament formation, and anticaepting and branching roles.39,20 The anticaepting role has been shown to regulate the branching and the polymerization of actin, in turn determining the development of filopodia.22

VASP is classically recognized to be phosphorylated on multiple sites by PKA and PKG.21,24 Phosphorylation has been shown to negatively regulate the ability of VASP to antagonize the capping of actin filament barbed ends by different capping proteins (such as CP, CapG, and gelsolin), resulting in the reduction of linear actin polymerization in vitro.24 Regulating the phosphorylation status of VASP therefore determines its ability to influence cell shape, including promoting filopodia formation.44,45 In addition, it has recently become clear that other kinases may regulate phosphorylation of VASP, importantly including PKC isoforms. Recent reports from our group and others have shown that phosphorylation of Ser157 but not Ser239 may be induced in other kinases may regulate phosphorylation of VASP, in turn determining the development of filopodia.
critical in regulating VASP activity, leading to potentiation of filopodia formation. It is not clear at this stage how inhibition of PKC8 may lead to hyper phosphorylation of Ser157. A recent report by Profirovic et al. of a cAMP-independent PKA-mediated phosphorylation of VASP in human umbilical vein endothelial cells (HUVECs) in response to thrombin suggested that the collagen-mediated activation of platelets might induce phosphorylation of VASP on Ser157 in a similar cAMP-independent fashion. Interestingly, Riondino et al. had shown that PKA-mediated pathways that lead to phosphorylation of VASP in platelets do not inhibit collagen-induced shape change in these cells. PKCδ could be a negative regulator of cAMP-independent PKA-mediated pathway, but data using the PKA inhibitor H89 (Figure 6D), which did not inhibit collagen-induced phosphorylation of VASP Ser157, makes an involvement of PKA unlikely. Figure S2 also provides evidence that rottlerin has no significant effect upon the phosphorylation status of PKA or PKG substrates, as detected by antibodies raised against consensus sequences for phosphorylation by these kinases. For PKG also, this would be consistent with a recent report showing that although collagen may induce NO generation, the effect leading to cGMP and PKG activation, this is highly dependent upon thromboxane generation, which would be absent from our experiments because they are conducted in the presence of indomethacin.

It is more likely that another member of the PKC family is responsible for the collagen-mediated Ser157 phosphorylation of VASP. We recently showed that, in platelets, thrombin induces phosphorylation of VASP Ser157 in a manner dependent upon classical isoforms of PKC. In this scenario, PKCδ would negatively regulate a classical PKC isoform responsible for VASP phosphorylation, consistent with data shown in Figure 6D where we show classical PKC isoforms are responsible for mediating VASP Ser157 phosphorylation. The precise mechanism underlying negative regulation of VASP phosphorylation is still not clear however, although the observation that rottlerin can inhibit the effect suggests that it is dependent upon PKCδ activity. Also, data shown in Figure S3 suggest that PKCδ does not directly regulate the activity of the classical PKC isoforms. Activity of PKCδ is therefore in some way may interfere with the ability of classical PKC isoforms to phosphorylate VASP on Ser157. Irrespective of the mechanism however, data from Figure 7 suggest that VASP is a critical downstream effector for PKCδ, mediating its negative effects upon actin polymerization, filopodia formation, and aggregation, because the potentiation of these activities by rottlerin is absent in platelets that lack VASP expression.

In summary, we have identified a new mechanism for negative regulation of actin polymerization and filopodia formation by PKCδ and, subsequently, platelet-platelet interaction and aggregation. This is mediated by a novel constitutive interaction between PKCδ and VASP in platelets, where activation by collagen leads to phosphorylation of VASP on Ser157. PKCδ negatively regulates phosphorylation on Ser157, functionally leading to suppression of actin polymerization and reduction of filopodia formation, because these two events are markedly enhanced in the absence of active PKCδ. In turn, filopodia formation is critically required for collagen-induced platelet aggregation. We therefore propose a novel mechanism for regulation of platelet aggregation induced by collagen whereby PKCδ is a critical negative regulator of filopodia formation through a functional interaction with the actin regulator VASP.

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Authorship

Contribution: G.P. designed and performed experiments and cowrote the manuscript; K.S. generated essential gene knockout mice and contributed to essential experiments; K.N. and K.I.N. generated essential gene knockout mice and contributed to experimental design; and A.W.P. designed experiments and cowrote the manuscript.

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PKCδ regulates collagen-induced platelet aggregation through inhibition of VASP-mediated filopodia formation

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