Functional Divergence of Platelet Protein Kinase C (PKC) Isoforms in Thrombus Formation on Collagen

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Arterial thrombosis, a major cause of myocardial infarction and stroke, is initiated by activation of blood platelets by subendothelial collagen. The protein kinase C (PKC) family centrally regulates platelet activation, and it is becoming clear that the individual PKC isoforms play distinct roles, some of which oppose each other. Here, for the first time, we address all four of the major platelet-expressed PKC isoforms, determining their comparative roles in regulating platelet adhesion to collagen and their subsequent activation under physiological flow conditions. Using mouse gene knock-out and pharmacological approaches in human platelets, we show that collagen-dependent α-granule secretion and thrombus formation are mediated by the conventional PKC isoforms, PKCα and PKCβ, whereas the novel isoform, PKCδ, negatively regulates these events. PKCδ also negatively regulates thrombus formation but not α-granule secretion. In addition, we demonstrate for the first time that individual PKC isoforms differentially regulate platelet calcium signaling and exposure of phosphatidylserine under flow. Although platelet deficient in PKCα or PKCβ showed reduced calcium signaling and phosphatidylserine exposure, these responses were enhanced in the absence of PKCδ. In summary, therefore, this direct comparison between individual subtypes of PKC, by standardized methodology under flow conditions, reveals that the four major PKCs expressed in platelets play distinct non-redundant roles, where conventional PKCs promote and novel PKCs inhibit thrombus formation on collagen.

Blood vessel damage or rupture of an atherosclerotic plaque exposes subendothelial collagen. Platelets rapidly adhere to collagen and are activated, inducing granule secretion and integrin αIIbβ3 activation and leading to large platelet aggregates. In addition, sustained intracellular calcium signaling induces phosphatidylserine (PS) exposure, which accelerates thrombin generation and coagulation. Together, these processes can result in formation of occlusive thrombi, leading to myocardial infarction or stroke.

The protein kinase C (PKC) family is comprised of multiple isoforms, which are responsible for a substantial part of the serine/threonine phosphorylation events in many cell types including platelets. The PKC isoforms are grouped into three classes: conventional forms (α, βI, βII, γ) that are activated by Ca2+/diacylglycerol, novel forms (δ, ε, η, θ) activated by diacylglycerol alone, and atypical forms (ζ, ι/λ), which are diacylglycerol-independent (1). Human and mouse platelets highly express the conventional PKC isoforms α and β and the novel isoforms δ and θ (2–6), whereas mouse platelets in addition express PKCε (7, 8). Early pharmacological studies, which could not distinguish between different isoforms, showed that key platelet activation processes, such as secretion, integrin αIIbβ3 activation, and aggregation, are positively regulated by PKC activity (9–13). However, there is also good evidence that PKC has a negative role in platelets, in particular by suppressing Ca2+ signal generation, for example by promoting Ca2+ extrusion (14, 15) and desensitizing agonist receptors (16). This raised the intriguing question whether different conventional and novel PKC isoforms may have distinct or even opposing roles in the control of these platelet responses (17).

There is some evidence to support this hypothesis. In human and mouse platelets, PKCα has been proposed as a key kinase regulating α-granule and dense granule secretion (18, 19) and platelet aggregate formation (20) in response to collagen and other platelet agonists (21). In mouse platelets, PKCβ positively regulates outside-in αIIbβ3 signaling (22) but not inside-out integrin activation. However, the role of the novel PKC isoforms, PKCδ and PKCθ, is less straightforward because both positive and negative signaling functions have been reported (8, 23–25).

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4 The abbreviations used are: PS, phosphatidylserine; FITC, fluorescein isothiocyanate; GPVI, glycoprotein VI; PKC, protein kinase C; PMA, phorbol myristate acetate; PPACK, H-Phe-Pro-Arg chloromethyl ketone; mAb, monoclonal antibody.
In light of this lack of a clear role for all PKC isoforms, which may result partly from differences in platelet preparation conditions, anticoagulant, and other ex vivo handling of platelet samples by different laboratories, we sought to compare directly platelet responses in physiological flow settings in whole blood. We compared platelet function and thrombotic response to flow over collagen, using blood from mice deficient in each of the four PKC isoforms. The data point to markedly divergent and partly antagonistic roles of the conventional and novel PKC isoforms in collagen-induced platelet activation and thrombus formation. In addition, we show for the first time that individual PKC isoforms differentially regulate platelet calcium signaling and procoagulant activity.

**EXPERIMENTAL PROCEDURES**

**Animals**—Animal studies were approved by the local animal care and use committees. All mice were generated as described earlier for PKCo (26), PKCB (27), PKC8 (28), or PKC (29). In all mouse strains, platelet and erythrocyte counts in blood were in the normal range. Wild-type mice were used of the same background and same breeding program as the corresponding knock-out mice.

**Materials**—H-Phe-Pro-Arg chloromethyl ketone (PPACK) was obtained from Calbiochem, as were Ro-318425, G6976, PKCB inhibitor (3-(1-(3-imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrole-2,5-dione), and rottlerin. PKC inhibitor (30) was a kind gift from Boehringer Ingelheim Pharmaceuticals. Annexin A5 labeled with fluorescein isothiocyanate (FITC) was from Nexins Research. Fura-2 and Flu−4 acetoxyethyl esters, pluronic F-127, and Alexa Fluor 467-labeled annexin A5 came from Molecular Probes. Apyrase (grade V), bovine serum albumin, dichlorofluorescein, and phosphor myristate acetate (PMA) were from Sigma. Fibrillar type I collagen (Horn) was from Nycomed. Convulxin was purified as described (31). FITC-labeled PAC1 mAb against activated human CD62, came from BD Biosciences. FITC-labeled anti-human-CD62 (P-selectin) mAb from Sanquin. FITC-labeled anti-mouse CD62 mAb was from Emfret Analytics, as was FITC-labeled JON/A mAb. Other materials were obtained from sources indicated before (32, 33).

**Blood Collection and Platelet Preparation**—Blood was taken from aspirin−free healthy volunteers, who gave full informed consent. Human platelet-rich plasma and washed platelets were prepared by centrifugation (34). Platelets were resuspended in Hepes buffer, pH 7.45 (136 mM NaCl, 10 mM Hepes, 2.7 mM KCl, 2 mM MgCl₂, 1 mg/ml glucose, 1 mg/ml bovine serum albumin, 0.2 units/ml apyrase) at a concentration of 2 × 10⁵/ml.

Mouse blood was obtained by cardiac puncture under terminal anesthesia. For flow studies, blood was collected into 40 μM PPACK, 5 units/ml heparin, and 40 units/ml fragmin. For washed platelets, mouse blood was collected into 129 mM citrate, 1 units/ml heparin, and 5 mM glucose (32). Washed cells were suspended in modified Tyrode’s Heps buffer, pH 7.45 (134 mM NaCl, 20 mM Heps, 12 mM NaHCO₃, 2.9 mM KCl, 1 mM MgCl₂, 0.34 mM Na₂HPO₄, 5 mM glucose). Platelets were counted with a Coulter counter and adjusted to the appropriate density.

**Measurement of Single Cell Ca²⁺ Responses under Flow**—Washed murine platelets were incubated with 8 μM Flu−4 acetoxyethyl ester and 0.2 mg/ml pluronic F-127 for 45 min at ambient temperature under gentle rotation (37). Dye-loaded platelets were added to PPACK-anticoagulated blood from the same mouse strain to give 10% labeled platelets. During the first min of high shear blood flow, images were captured from the collagen surface at 5 Hz. Pseudo-ratio F/F₀ values were converted into nanomolar concentrations of [Ca²⁺]i, using predefined calibration parameters (38).

**Measurement of Single Platelet Adhesion to Collagen**—Flu−4-loaded platelets were added to whole blood as described above. Even in unactivated Flu−4-loaded platelets, there is basal fluorescence, which can be used to track the platelets under flow conditions, as described previously (37). Stably adhered platelets were defined as labeled cells that remained in one position on a collagen fiber for >30 s.

**Measurement of PKC Activity**—PKC activity was determined by Ser phosphorylation of modified PKC pseudo-substrate RFARKGLRQKNV (39), using a biotinylated mAb recognizing the phosphorylated form (Calbiochem). Washed
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platelets (1 × 10⁸/ml), pretreated with indicated inhibitors (10 min), were pelleted and immediately sonicated on ice and then further processed as indicated by the manufacturer. Platelet Aggregation and Flow Cytometry—Washed platelets were pretreated for 10 min with Me₂SO vehicle or indicated inhibitor. Platelet aggregation was measured under stirring by light transmission aggregometry at 37 °C. Using flow cytometry, α-granule secretion was assessed as P-selectin exposure with FITC-labeled anti-human or anti-mouse CD62 mAb. Similarly, α₁β₃ activation was assessed with FITC-labeled mAb against activation epitopes of this integrin (36).

Results

Deficiency in Conventional Isoforms PKCa or PKCB Impairs Collagen-induced Thrombus Formation and Platelet Activation under Flow—To investigate the functional consequences of specific PKC isoform deficiency, we used an in vitro flow model of platelet-vessel wall interaction in which whole blood is perfused over a collagen-coated surface at defined, arterial shear rate (32, 37, 40, 41). Previous human studies demonstrated that general inhibition of PKC almost fully abrogates the formation of platelet aggregates, whereas adhered platelets show increased procoagulant activity, as assessed by labeled annexin A5, which detects exposed PS (17). During flow over collagen at 1000 s⁻¹, wild-type mouse platelets rapidly assembled into large aggregates, and by contrast, after 4 min (Fig. 1A) or longer perfusion times, PKCa⁻/⁻ platelets were markedly impaired in aggregate formation, in agreement with our previous report (18). Formation of pseudopods and lamellipods of the adhered PKCa⁻/⁻ platelets was, however, normal. Quantitative analysis showed a reduction in platelet deposition by 70% (Fig. 1A), whereas morphometric analysis of the PKCa⁻/⁻ thrombi showed that mostly single cells or small sized aggregates were present (Fig. 1C). The latter observation was confirmed by measurement of thrombus volume after perfusion, using platelets prelabeled with dichlorofluorescein. Mean volumes of 323 ± 76 and 89 ± 18 μm³ for wild-type and PKCa⁻/⁻ platelets, respectively, were achieved (mean ± S.E., n ≥ 34, p < 0.01).

Interestingly, deficiency in PKCB also resulted in diminished platelet deposition and formation of smaller sized aggregates on collagen (Fig. 1), and thrombus volume was reduced to 95 ± 24 μm³. Platelet deposition during thrombus formation involves both primary adhesion of platelets to collagen and platelet-platelet aggregation. Analysis of primary platelet adhesion to collagen, however, showed no effect of loss of either PKCa or PKCB because fractions of stably adhered platelets were similar for wild-type and PKCa⁻/⁻ blood (80 ± 4% and 77 ± 3%), as well as for wild-type and PKCB⁻/⁻ blood (80 ± 5% and 81 ± 4%; n = 4 experiments, p > 0.5). Hence, the impaired thrombus formation is not a consequence of defective adhesion to collagen but rather a result of reduced platelet-platelet cohesion.

To investigate whether the reduced thrombus formation was accompanied by altered platelet activation, thrombi formed on the collagen surface were labeled for the α-granule secretion marker, P-selectin, and for procoagulant activity, with PS-binding annexin A5. Fig. 2A shows images obtained after dual staining (staining with each probe individually gave similar results). For both PKCa⁻/⁻ and PKCB⁻/⁻ thrombi, measurement of integrated fluorescence intensity demonstrated a dramatic reduction in surface expression of P-selectin and PS when compared with wild types (Fig. 2B). Although this reduction was partly explained by the diminished platelet deposition, the P-selectin mean fluorescence intensity per pixel was also reduced, suggesting a lower degree of α-granule secretion per platelet. Interestingly, in contrast to general PKC inhibition studies (17), where greater numbers of platelets show PS exposure, fewer platelets exposed PS in the absence of PKCa or PKCB when compared with wild-type controls (Fig. 2A).

The results so far suggested that deficiency in PKCa or PKCB impairs platelet activation by collagen. To investigate this further and mechanistically, we monitored Ca²⁺ signaling in collagen-adhered platelets during whole blood perfusion (36, 37).
Deficiency of PKCa or PKCb suppresses collagen-induced activation of platelets in thrombi. Whole blood from indicated mice was flowed over collagen (Fig. 1), and platelet thrombi were double-stained with FITC-anti-mouse CD62P mAb (green) and Alexa Fluor 647-annexin A5 (red). A, representative confocal images (180 × 180 μm) after staining. B, integrated fluorescence (IF) intensity presenting activated platelets in arbitrary units to quantify cumulative expression of P-selectin and PS. Means ± S.E. (n = 3–5); **, p < 0.01 versus corresponding wild types. Numbers above each bar indicate the mean integrated pixel density of P-selectin staining, an indication of staining per platelet.

Analysis of the Ca\(^{2+}\) responses showed that although platelets from PKCa\(^{-/-}\) and PKCb\(^{-/-}\) mice were still capable of prolonged rises in [Ca\(^{2+}\)], after adhesion, these rises were lower in amplitude by comparison with those of corresponding wild-type platelets (Fig. 3, A and B). Together, these results indicate that deficiency in either PKCa or PKCb does not change primary platelet adhesion to collagen under flow but results in impaired secretion and aggregate formation and in lower GPVI-induced Ca\(^{2+}\) rises and PS exposure. Furthermore, PKCa and PKCb isoforms appear to play non-redundant roles.

Deficiency in Novel Isoforms PKCb and PKCd Enhances GPVI-induced Murine Thrombus Formation and Platelet Activation under Flow—To investigate the contribution of the major novel PKC isoforms in thrombus formation, flow experiments on collagen were carried out with blood from mice lacking PKCb or PKCd. Platelets from PKCb\(^{-/-}\) or PKCd\(^{-/-}\) mice readily assembled into aggregates (Fig. 4A). In comparison with wild-type controls, these thrombi covered more of the collagen surface and more frequently consisted of larger sized aggregates (Fig. 4, B and C). Thrombus volumes from wild-type, PKCb\(^{-/-}\), and PKCd\(^{-/-}\) thrombi were 490 ± 136, 1923 ± 456, and 2304 ± 261 μm\(^3\), respectively (n ≥ 16, p < 0.05). Thrombi on collagen were then stained for P-selectin and PS (Fig. 5A). The increased deposition of PKCb\(^{-/-}\) platelets was accompanied by higher integrated fluorescence intensities of both stains (Fig. 5B) and a higher fluorescence per pixel for P-selectin staining, suggesting an overall higher activation state of the platelets. In contrast, this was not observed for PKCd\(^{-/-}\) platelets. As for PKCa\(^{-/-}\) and PKCb\(^{-/-}\), similar fractions of labeled wild-type (87 ± 5%), PKCb\(^{-/-}\) (80 ± 2%), and PKCd\(^{-/-}\) (84 ± 4%) platelets were found stably adherent to collagen under flow (n = 4–5 experiments, p > 0.2), suggesting that enhanced thrombus formation does not result from increased adhesion to collagen but rather enhanced ability to form large aggregates.

Using Fluo-4-loaded platelets, collagen-adhered PKCb\(^{-/-}\) platelets, but not PKCd\(^{-/-}\) platelets, exhibited higher amplitude rises in [Ca\(^{2+}\)], in comparison with the corresponding wild-type cells (Fig. 6A). The increased Ca\(^{2+}\) signal in PKCb\(^{-/-}\) platelets was detectable at both earlier (30 s) and later (60 s) time points (Fig. 6B). Together, these results suggest that the absence of PKCb augments collagen-dependent thrombus formation under flow by increasing GPVLI-induced Ca\(^{2+}\) signaling and downstream responses such as α-granule secretion and procoagulant activity.

Inhibition of Conventional or Novel PKC Isoforms Differently Influences Collagen-induced Human Platelet Aggregation and Activation—It was important to determine whether the roles defined for the major PKC isoforms in mouse platelets could be paralleled in human platelets. We therefore studied the relative effects of isoform non-selective and isoform-selective PKC inhibitors: the bis-indolyl maleimide, Ro-318425 (non-selective PKC inhibitor); the modified bis-indolyl maleimides, Gö6976 (potency PKCα/β/ε) and PKCb inhibitor (potency PKCβ/ε); the phloroglucinol derivative, rottlerin (potency PKCδ/ε/β); and PKCb inhibitor (compound A, potency PKCδ/ε/β). As a first approach to determine the effects of inhibitors on PKC-dependent platelet function, we assessed aggregation responses to PMA. Similar to Ro-318425, which completely abolished aggregation at 0.5 μM, both Gö6976 and PKCb inhibitor abolished this response, although at higher concentrations (supplemental Fig. 1A). In contrast, rottlerin and PKCb inhibitor were much less inhibitory and, in the case of rottlerin, potentiated platelet aggregation to PMA. Similarly, Ro-318425, Gö6976, and PKCb inhibitor each dose dependently suppressed PMA-induced α\(_{\text{IIb/IIIa}}\)β3 activation as well as α-granule secretion (supplemental Fig. 1, B and C). In contrast, PKCb inhibitor potentiated α\(_{\text{IIb/IIIa}}\)β3 activation, whereas rottlerin potentiated secretion. This indicates that inhibition of conventional PKC isoforms, but not novel isoforms, suppresses PMA-induced integrin activation and secretion.

To assess how the inhibitors suppressed platelet PKC kinase activity, their influence on PKC exogenous substrate peptide phosphorylation was determined under the same experimental conditions (1 × 10\(^6\) platelets/ml, no plasma). Although Ro-318425 (10 μM) abolished kinase activity to 2.1 ± 0.5% (n = 5) of control, Gö6976 (1 μM) and PKCb inhibitor (2.5 μM) reduced phosphorylation to 54 ± 4 and 38 ± 6%, respectively. The inhibitors of novel PKC isoforms, rottlerin (10 μM) and PKCb inhibitor (2.5 μM), reduced PKC activity to 63 ± 8 and 75 ± 5% of control, respectively. Combinations of inhibitors fully suppressed substrate peptide phosphorylation. These data are in agreement with previously published data (22) and demonstrate that the classical
isofoms of PKC represent the more major component of platelet PKC activity in human platelets.

Subsequently, we determined the influence of these compounds on human platelet activation via the collagen receptor, GPVI. Collagen-induced platelet aggregation was concentration dependently inhibited by Ro-318425 (Fig. 7, A), and near complete inhibition was achieved at 1 μM Gö6976, whereas PKCβ inhibitor was effective at higher concentrations. Ro-318425 was ineffective when platelets were co-stimulated with collagen and ADP, suggesting that the inhibition of aggregation is explained by loss of granule secretion. By contrast, the calcium ionophore, ionomycin, did not rescue aggregation, suggesting that reduced Ca2+ signaling is not responsible for the inhibition of aggregation (Fig. 7B). Inhibition of novel PKC isoforms with rottlerin or PKCθ inhibitor slightly enhanced convulxin-induced platelet aggregation. In general, measurements of convulxin-induced αIIbβ3 activation and P-selectin expression showed similar effects with these inhibitors (Fig. 7, C and D). However, strikingly, PKCθ inhibitor but not rottlerin markedly increased these responses, in agreement with previously published gene knock-out studies (8, 24).

Inhibition of Conventional or Novel PKC Isoforms Differently Influences GPVI-induced Ca2+ Signaling—Finally, we measured GPVI-induced Ca2+ responses in washed human platelets. Gö6976 and PKCβ inhibitor suppressed the [Ca2+]i, peak and [Ca2+]i time integral (Fig. 7, E and F), whereas rottlerin was without effect, and pretreatment with PKCθ inhibitor markedly increased the platelet Ca2+ signal. This resembled the potentiatory effect seen with Ro-318425 and also noted in earlier studies with human platelets (17). Hence, the enhancement of Ca2+ signaling by general PKC inhibitors is mimicked only by inhibition of the novel PKCθ isoform. Under these conditions, convulxin (70 ng/ml; 5 min) did not cause shedding of GPVI. Although treatment of platelets with a high concentration of convulxin (250 ng/ml) for 1 h induced ~50% GPVI shedding, this was not affected by treatment with Ro-318425 (supplemental Fig. 2), suggesting that differences in Ca2+ signaling were not caused by regulated shedding of GPVI.

Relative Contribution of Conventional and Novel PKC Isoforms to Secretion and Ca2+ Signaling—Because PKCα and PKCθ regulate α-granule secretion in opposite directions, we investigated which isoform was dominant during platelet activation by treating PKCα+/+ and PKCα−/− platelets with PKCθ inhibitor and measured P-selectin expression by flow cytometry. Consistent with the above data, P-selectin expression was significantly inhibited in PKCα−/− platelets. Interestingly, although PKCθ inhibitor slightly increased the P-selectin expression in PKCα+/+ platelets, there was little effect of PKCθ inhibitor on PKCα−/− platelets (Fig. 8A). These data suggest that the conventional isoforms are essential for secretion, whereas PKCθ regulates the extent of secretion.

PKCα and PKCθ also regulate Ca2+ signaling under flow conditions in opposite directions. Treatment with PKCθ inhibitor enhanced the Ca2+ signal in platelets flowed over collagen in PKCα+/+ platelets, consistent with the data obtained from PKCθ−/− platelets. Moreover, PKCθ inhibitor also enhanced the Ca2+ signal in PKCα−/− platelets (Fig. 8B). These data suggest that PKCθ is a more important regulator of Ca2+ signaling than of granule secretion.

DISCUSSION

Here, for the first time, we have addressed all four of the major platelet-expressed PKC isoforms, determining their comparative roles in regulating platelet activation by collagen under physiological flow conditions. The study is also the first to determine the role of individual PKC isoforms in regulating calcium responses, at the single cell level, in growing thrombi visualized in real time. Importantly, the study reveals major positive roles for the conventional isoforms, PKCα and PKCβ,
in mediating thrombus formation. The absence of either isoform leads to marked suppression of secretion of \( \alpha \)-granules, aggregate formation, calcium signaling, and PS exposure under flow. The data therefore suggest essential but non-redundant roles for these kinases in regulating these events. In contrast, the absence of the novel isoforms PKC\( \theta \) and PKC\( \delta \), however, leads to enhanced thrombus formation on collagen. The mechanisms for these isoforms are also distinct and non-redundant because although the absence of PKC\( \theta \) also leads to enhanced secretion, calcium signaling, and phosphatidylserine exposure, the absence of PKC\( \delta \) does not potentiate any of these functions.

We conclude that all four major expressed PKC isoforms play distinct non-redundant roles, where the conventional PKCs promote and the novel PKCs inhibit thrombus formation on collagen, by a variety of mechanisms (summarized in Fig. 8C).

The studies here with mice lacking PKC\( \alpha \) or PKC\( \beta \) demonstrated that platelet thrombi formed on collagen were significantly smaller in comparison with wild-type thrombi. This reduced thrombus formation was associated with impaired GPVI-induced \( \alpha \)-granule secretion. A key role for murine PKC\( \alpha \) in exocytosis of platelet \( \alpha \)-granules and dense granules has recently been demonstrated (18). Importantly, these effects were not caused by diminished primary adhesion to collagen because time lapse studies with Fluo-4-loaded platelets showed normal adhesion under shear in case of PKC\( \alpha \) or PKC\( \beta \) deficiency. Instead, our data suggest that the defect is in the ability of platelets to form aggregates on platelets that have already adhered.

In addition, in both PKC\( \alpha^{-/-} \) and PKC\( \beta^{-/-} \) mice, the procoagulant activity of collagen-adhered platelets was diminished, consistent with the reduced \( \text{Ca}^{2+} \) signal. Earlier, it had been shown that the platelet procoagulant response is a direct consequence of GPVI-induced activation of collagen-adhered platelets (42, 43). How PKC\( \alpha \) and PKC\( \beta \) regulate \( \text{Ca}^{2+} \) signaling is not yet understood, although a role for conventional PKCs in store-operated calcium entry has previously been proposed on the basis of pharmacological studies in human platelets (44). Together, these data support the concept that both conventional PKC isoforms positively regulate thrombus formation by enhancing GPVI-induced platelet activation, leading to secretion and procoagulant activity. Interestingly, there is a high degree of non-redundancy in this process, indicating that PKC\( \alpha \) and PKC\( \beta \) may each play essential, but distinct, roles.

FIGURE 4. Deficiency of PKC\( \delta \) or PKC\( \theta \) increases thrombus formation on collagen under flow. Blood from PKC\( \delta^{-/-} \), PKC\( \theta^{-/-} \), or matched wild-type mice was flowed over collagen, and platelet thrombi were double-stained with FITC-anti-mouse CD62P mAb (green) and Alexa Fluor 647-annexin A5 (red). A, representative confocal images (180 \( \mu \text{m} \times 180 \mu \text{m} \) after staining. B, integrated fluorescence (IF) intensity presenting activated platelets in arbitrary units to quantify cumulative expression of P-selectin and PS. Means \( \pm \) S.E. (n = 3–5); *, \( p < 0.05 \) versus corresponding wild types; \( \chi^2 \) test, *, \( p < 0.05 \).
It was important to extend the studies in mouse platelets by studies with human platelets, using the compounds Go6976 and PKCβ inhibitor. Dose-response curves showed an inhibitory effect of both compounds on collagen-induced platelet aggregation as well as on GPVI-induced integrin activation and P-selectin expression. In addition, these compounds diminished GPVI-induced Ca^{2+} signal generation. The reduction in aggregation is likely to be mainly caused by loss of granule secretion as full aggregation could be restored by co-stimulation with ADP. This is consistent with our previous report that co-infusion with ADP could restore thrombus formation on collagen in PKCα−/− deficient platelets. By contrast, enhancing cytosolic Ca^{2+} by co-stimulation with ionomycin did not rescue collagen-inducing aggregation.

For mouse platelets, the present data indicate that both isoforms are required for full GPVI-dependent activation, suggesting a non-redundancy in function, particularly for regulation of P-selectin expression and annexin V binding, which are both markedly suppressed in the absence of either PKCα or PKCβ. For human platelets, the studies with Go6976 and PKCβ inhibitor point to a substantial reduction in platelet responses. The PKCβ inhibitor is, however, much less effective than Go6976 on convulxin-induced responses (Fig. 7, A–C). This may suggest that in human platelets, there is more redundancy of function between PKCα and PKCβ than in mouse platelets. It may, however, be a reflection of the selectivity profile of these inhibitors and may also reflect differences between the effects of gene deletion versus pharmacological inhibition of a kinase. In support of the concept that both PKCα and PKCβ contribute to human platelet activation are the findings in the literature that purified PKCα mediates granule secretion (19), that PKCα is phosphorylated upon GPVI stimulation (45), and that PKC in general and PKCβ in particular are implicated in α_{IIb}β_{3} signaling (22, 46).

In contrast, the current studies with mice deficient in PKCθ point to a negative role in collagen-dependent granule secretion and thrombus formation. In addition, increased GPVI-induced activation of PKCθ−/− platelets was apparent from the higher Ca^{2+} responses of adhered single platelets and the increased numbers of procoagulant, PS-exposing cells. Consistent with these data, the PKCθ inhibitor increased GPVI-induced human platelet aggregation as well as α_{IIb}β_{3} activation, Ca^{2+} mobilization, and α-granule secretion. There are discrepancies in the literature about negative or positive roles for PKCθ in regulating platelet function (24, 25, 47, 48), which may be a result of differences in platelet preparation conditions. We have, however, previously shown platelet responses to GPVI activation to be enhanced in the absence of PKCθ (24, 48), and taken together, these data would support the concept that PKCθ may down-regulate rather than up-regulate GPVI-mediated granule secretion and aggregate formation under physiological flow conditions. Mouse platelets express relatively high amounts of PKCθ when compared with the other novel PKC isoforms, PKCδ and PKCε (7, 24). Furthermore, all three isoforms are phosphorylated on tyrosine following GPVI stimulation (7, 45, 47, 49, 50).

In addition, the present data suggest that PKCθ is the principal isoform mediating the earlier recognized effect of PKCθ in down-regulating GPVI-induced platelet Ca^{2+} signaling and procoagulant activity (17). The mechanism is currently unknown, although PKC may reduce phospholipase C activation (51) or increase Ca^{2+} extrusion via the plasma membrane Ca^{2+} ATPase (14, 15).

The enhanced thrombus formation that we report in PKCθ−/− blood is in apparent contrast to a previous report (25), in which PKCθ−/− mice showed reduced thrombus formation in vivo in a FeCl_{3}-induced carotid injury model. This is likely to reflect the additional contribution of thrombin generation in vivo. Importantly, although PKCθ negatively regulates collagen-dependent platelet activation, it appears to have a positive role in thrombin-induced signaling (25, 52). This agonist-dependent difference is similar to that proposed by Kunapuli and colleagues (23) for PKCθ. The relative importance of collagen and thrombin in vivo appears to depend on the injury model used and the extent of injury (53). We therefore suggest that the effect seen in vivo is a combination of enhanced collagen-dependent platelet activation and reduced thrombin-dependent signaling.

A tendency to increased thrombus formation was also seen with PKCδ−/− mice in the present study. Although some reports suggest that GPVI-induced granule secretion is increased in PKCδ−/− mice (23), we have not been able to show this (present study and Ref. 8) but rather demonstrate a potentiated aggregation response to collagen through enhanced filopodia formation in the absence of PKCδ. Consistent with this, we found with human platelets that rot-
Rottlerin had little effect on collagen-induced integrin \( \alpha_{IIb}\beta_3 \) activation and \( \alpha \)-granule secretion but enhanced platelet aggregation. Therefore, although PKC\( \beta \) and PKC\( \delta \) negatively regulate collagen-dependent thrombus formation, they act through distinct mechanisms.

Treatment of human platelets with broad spectrum PKC inhibitors blocks granule secretion and formation of large aggregates but also enhances platelet \(Ca^{2+}\) signaling and PS exposure (17). This suggests that the major regulators of granule secretion and thrombus formation are PKC\( \alpha \) and PKC\( \beta \) because inhibition of all PKC isoforms replicates the phenotype of PKC\( \alpha^{-/-} \) or PKC\( \beta^{-/-} \) platelets. In contrast, the major regulator of \(Ca^{2+}\) signaling and PS exposure appears to be PKC\( \theta \) because the absence or pharmacological inhibition of PKC\( \theta \) has a similar effect on \(Ca^{2+}\) signaling and PS exposure to broad spectrum PKC inhibition.

We tested this directly by examining the effect of PKC\( \theta \) inhibitor on PKC\( \alpha^{-/-} \) platelets. Importantly, combined loss of
PKCa and PKCθ signaling produced a phenotype that resembled PKCa+/− platelets for secretion and PKCθ−/− platelets for Ca2+ signaling. Moreover, the phenotype resembled that of platelets treated with a broad spectrum PKC inhibitor. We propose that the conventional PKC isoforms are essential for collagen-induced granule secretion, whereas PKCθ negatively regulates the extent of this secretion. This means that in the absence of conventional PKC signaling, there is no granule secretion and so no effect of PKCθ. PKCθ/β positively regulate sustained Ca2+ signaling and PS exposure, whereas it is negatively regulated by PKCθ even in the absence of PKCα. PKCθ/β negatively regulates thrombus formation independently of granule secretion and does not regulate Ca2+ signaling or PS exposure.

The data presented here are the first comparative analysis of platelet PKCs in thrombus formation on collagen and the first to determine calcium responses, secretion, and procoagulant activity at the single cell level in the growing thrombus in platelets lacking specific PKC isoforms. Together, the data form a comprehensive analysis of the roles played by PKCα, PKCβ, PKCθ, and PKCδ and reveal important distinctions in mechanism and function of these kinases. The data will provide a platform for future exploation of these different family members in modulation of platelet function and thrombus formation.

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