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

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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, PKCθ, 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 PKCθ. 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 nonredundant 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 $\alpha_{IIb}\beta_3$ activation and leading to large platelet aggregates. In addition, sustained intracellular calcium signaling induces phosphatidylserine (PS)[4] 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 Ca^{2+}/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 $\alpha_{IIb}\beta_3$ 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 Ca^{2+} signal generation, for example by promoting Ca^{2+} 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 $\alpha_{IIb}\beta_3$ 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 PKCα (26), PKCβ (27), PKCδ (28), or PKCθ (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, Gö6976, PKCβ inhibitor (3-(1-(3-imidazol-1-ylpropyl)-1*H*-indol-3-yl)-4-anilino-1*H*-pyrrole-2,5-dione), and rottlerin. PKCθ inhibitor (30) was a kind gift from Boehringer Ingelheim Pharmaceuticals. Annexin A5 labeled with fluorescein isothiocyanate (FITC) was from Nexins Research. Fura-2 and Fluo-4 acetoxymethyl esters, pluronic F-127, and Alexa Fluor 647-labeled annexin A5 came from Molecular Probes. Apyrase (grade V), bovine serum albumin, dichlorofluorescein, and phorbol myristate acetate (PMA) were from Sigma. Fibrillar type I collagen (Horm) was from Nycomed. Convulxin was purified as described (31). FITC-labeled PAC1 mAb against activated human $\alpha_{IIb}\beta_3$ 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 $MgCl_2$, 1 mg/ml glucose, 1 mg/ml bovine serum albumin, 0.2 units/ml apyrase) at a concentration of 2×10^8/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 Hepes buffer, pH 7.45 (134 mM NaCl, 20 mM Hepes, 12 mM $NaHCO_3$, 2.9 mM KCl, 1 mM $MgCl_2$, 0.34 mM Na_2HPO_4, 5 mM glucose). Platelets were counted with a Coulter counter and adjusted to the appropriate density.

Thrombus Formation—Thrombus formation on collagen under flow was determined as described (32). Briefly, PPACK-anticoagulated mouse blood was flowed through a transparent, parallel plate flow chamber (50 μm deep, 3 mm wide), sealed with a collagen-coated coverslip, at a shear rate of 1000 s^{-1}. To measure thrombus volumes, platelets in blood were prelabeled with dichlorofluorescein (35). Activated platelets in thrombi were post-stained with FITC-annexin A5 (0.5 μg/ml) or with Alexa Fluor 647-annexin A5 (1:400) and FITC-labeled anti-P-selectin mAb (1:40).

Phase-contrast and fluorescence images were recorded with a DM-IRB fluorescence microscope and an Orca ER camera (Leica) or with a Nikon Diaphot 200 microscope and an EM-CCD camera (Hamamatsu). Confocal images were captured in real time using a Zeiss/Bio-Rad E600FN multiphoton system (36). Phase-contrast and (confocal) fluorescence images were taken from at least 10 randomly chosen microscopic fields. Images were analyzed for area coverage, integrated fluorescence intensity, or integrated fluorescence pixel density using ImagePro software (Media Cybernetics), as described before (34). For P-selectin staining, the extent of integrated fluorescence intensity depends on the number of platelets (*i.e.* the thrombus size) and the fluorescence per platelet. In contrast, integrated pixel density gives a measure of the amount of fluorescence per platelet. In control experiments, treatment of a preformed thrombus with thrombin resulted in a further increase (~2.5-fold) in mean pixel density of P-selectin fluorescence (data not shown). Both integrated fluorescence intensity and pixel density are given in Figs. 2 and 4. In contrast, individual platelets strain strongly with annexin A5 or remain unstained because annexin A5 polymerizes when binding to PS-exposing surfaces. The pixel density (*i.e.* staining per platelet) was therefore the same for all mouse strains, but the integrated fluorescence intensity is indicative of the number of PS-exposing platelets. Thrombus volume of dichlorofluorescein-labeled platelets was measured from confocal stacks of fluorescence images (35).

Measurement of Single Cell Ca^{2+} Responses under Flow—Washed murine platelets were incubated with 8 μM Fluo-4 acetoxymethyl 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/F_o values were converted into nanomolar concentrations of $[Ca^{2+}]_i$ using predefined calibration parameters (38).

Measurement of Single Platelet Adhesion to Collagen—Fluo-4-loaded platelets were added to whole blood as described above. Even in unactivated Fluo-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 RFARKGSLRQKNV (39), using a biotinylated mAb recognizing the phosphorylated form (Calbiochem). Washed

platelets (1×10^8/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 Me_2SO 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, $\alpha_{IIb}\beta_3$ activation was assessed with FITC-labeled mAb against activation epitopes of this integrin (36).

Calcium Responses in Suspension—Fura-2-loaded platelets (1×10^8/ml), prepared as described (33), were used to measure changes in cytosolic $[Ca^{2+}]_i$ by calibrated ratio fluorimetry. Time integrals of $[Ca^{2+}]_i$ (in nM) were determined as before.

Statistical Analysis—Differences between experimental groups were tested for significance with the non-parametric Mann-Whitney *U* test, using SPSS. Differences in frequency distributions of features with mean values per field were determined by χ^2 test (34).

RESULTS

Deficiency in Conventional Isoforms PKCα or PKCβ 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. 1*A*) or longer perfusion times, PKC$\alpha^{-/-}$ platelets were markedly impaired in aggregate formation, in agreement with our previous report (18). Formation of pseudopods and lamellipods of the adhered PKC$\alpha^{-/-}$ platelets was, however, normal. Quantitative analysis showed a reduction in platelet deposition by 70% (Fig. 1*B*), whereas morphometric analysis of the PKC$\alpha^{-/-}$ thrombi showed that mostly single cells or small sized aggregates were present (Fig. 1*C*). 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 μm^3 for wild-type and PKC$\alpha^{-/-}$ platelets, respectively, were achieved (mean ± S.E., $n \geq 34$, $p < 0.01$).

Interestingly, deficiency in PKCβ also resulted in diminished platelet deposition and formation of smaller sized aggregates on collagen (Fig. 1), and thrombus volume was reduced to 95 ± 24 μm^3. 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 PKCα or PKCβ because fractions of stably adhered platelets were similar for wild-type and PKC$\alpha^{-/-}$ blood (80 ± 4% and 77 ± 3%), as well as for wild-type and PKC$\beta^{-/-}$ 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.

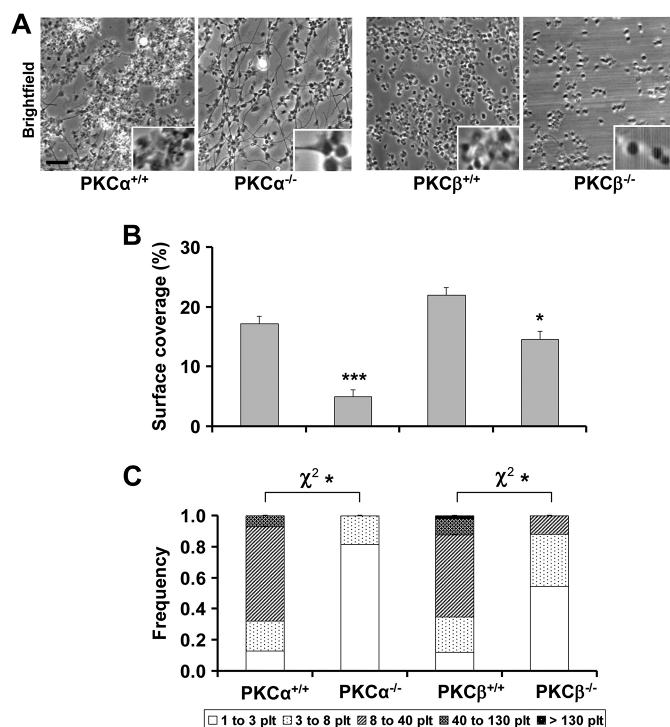

FIGURE 1. **Deficiency of PKCα or PKCβ suppresses thrombus formation on collagen under flow.** Whole blood from PKC$\alpha^{-/-}$, PKC$\beta^{-/-}$, or matched wild-type mice was flowed over a collagen surface at a shear rate of 1000 s^{-1}. *A*, representative phase-contrast images after 4 min of perfusion (*bar*, 20 μm; *insets*, 5× magnified). *B*, quantification of platelet deposition on collagen surface. *C*, morphometric analysis of aggregates on collagen surface with indicated numbers of platelets (*plt*) per feature. Means ± S.E. ($n = 3$–6), *, $p < 0.05$, ***, $p < 0.001$ *versus* corresponding wild types; χ^2 test, *, $p < 0.05$.

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. 2*A* shows images obtained after dual staining (staining with each probe individually gave similar results). For both PKC$\alpha^{-/-}$ and PKC$\beta^{-/-}$ thrombi, measurement of integrated fluorescence intensity demonstrated a dramatic reduction in surface expression of P-selectin and PS when compared with wild types (Fig. 2*B*). 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 PKCα or PKCβ when compared with wild-type controls (Fig. 2*A*).

The results so far suggested that deficiency in PKCα or PKCβ impairs platelet activation by collagen. To investigate this further and mechanistically, we monitored Ca^{2+} signaling in collagen-adhered platelets during whole blood perfusion (36, 37).

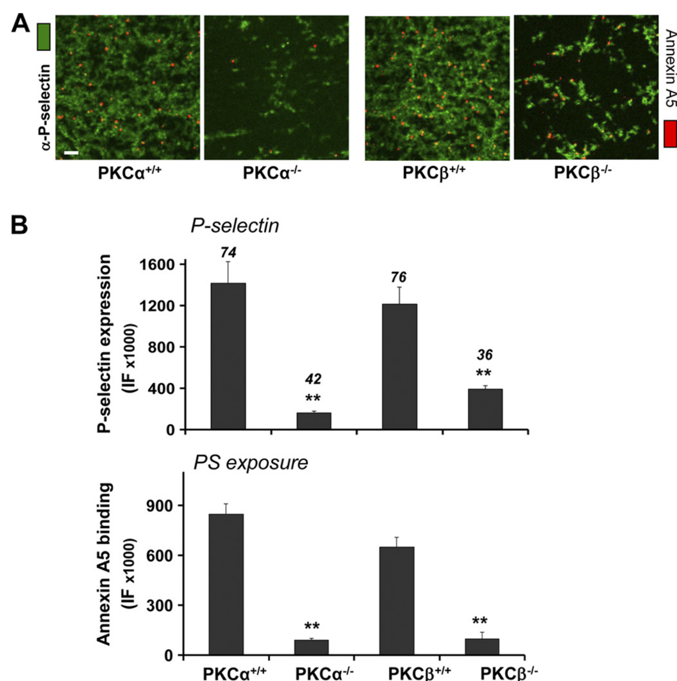

FIGURE 2. **Deficiency of PKCα or PKCβ suppresses collagen-induced activation of platelets in thrombi.** Whole blood from indicated mice was flowed over collagen (Fig. 1), and platelet thrombi were double-stained with FITC-anti-mouse CD62P mAb (*green*) and Alexa Fluor 647-annexin A5 (*red*). *A*, representative 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 PKC$\alpha^{-/-}$ and PKC$\beta^{-/-}$ mice were still capable of prolonged rises in $[Ca^{2+}]_i$ 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 PKCα or PKCβ 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, PKCα and PKCβ isoforms appear to play non-redundant roles.

Deficiency in Novel Isoforms PKCθ and PKCδ 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 PKCθ or PKCδ. Platelets from PKC$\theta^{-/-}$ or PKC$\delta^{-/-}$ mice readily assembled into aggregates (Fig. 4*A*). 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, PKC$\theta^{-/-}$, and PKC$\delta^{-/-}$ thrombi were 490 ± 136, 1923 ± 456, and 2304 ± 261 μm^3, respectively ($n \geq 16$, $p < 0.05$). Thrombi on collagen were then stained for P-selectin and PS (Fig. 5*A*). The increased deposition of PKC$\theta^{-/-}$ platelets was accompanied by higher integrated fluorescence intensities of both stains (Fig. 5*B*) 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 PKC$\delta^{-/-}$ platelets. As for PKC$\alpha^{-/-}$ and PKC$\beta^{-/-}$, similar fractions of labeled wild-type (87 ± 5%), PKC$\theta^{-/-}$ (80 ± 2%), and PKC$\delta^{-/-}$ (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 PKC$\theta^{-/-}$ platelets, but not PKC$\delta^{-/-}$ platelets, exhibited higher amplitude rises in $[Ca^{2+}]_i$ in comparison with the corresponding wild-type cells (Fig. 6*A*). The increased Ca^{2+} signal in PKC$\theta^{-/-}$ platelets was detectable at both earlier (30 s) and later (60 s) time points (Fig. 6*B*). Together, these results suggest that the absence of PKCθ augments collagen-dependent thrombus formation under flow by increasing GPVI-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 PKC$\alpha/\beta>\epsilon$) and PKCβ inhibitor (potency PKC$\beta>\alpha>\epsilon$); the phloroglucinol derivative, rottlerin (potency PKC$\delta>\alpha/\beta$); and PKCθ inhibitor (compound A, potency PKC$\theta>\delta>\alpha/\beta$). 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 PKCβ inhibitor abolished this response, although at higher concentrations (supplemental Fig. 1*A*). In contrast, rottlerin and PKCθ inhibitor were much less inhibitory and, in the case of rottlerin, potentiated platelet aggregation to PMA. Similarly, Ro-318425, Gö6976, and PKCβ inhibitor each dose dependently suppressed PMA-induced $\alpha_{IIb}\beta_3$ activation as well as α-granule secretion (supplemental Fig. 1, *B* and *C*). In contrast, PKCθ inhibitor potentiated $\alpha_{IIb}\beta_3$ 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 × 10^8 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 PKCβ inhibitor (2.5 μM) reduced phosphorylation to 54 ± 4 and 38 ± 6%, respectively. The inhibitors of novel PKC isoforms, rottlerin (10 μM) and PKCθ 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

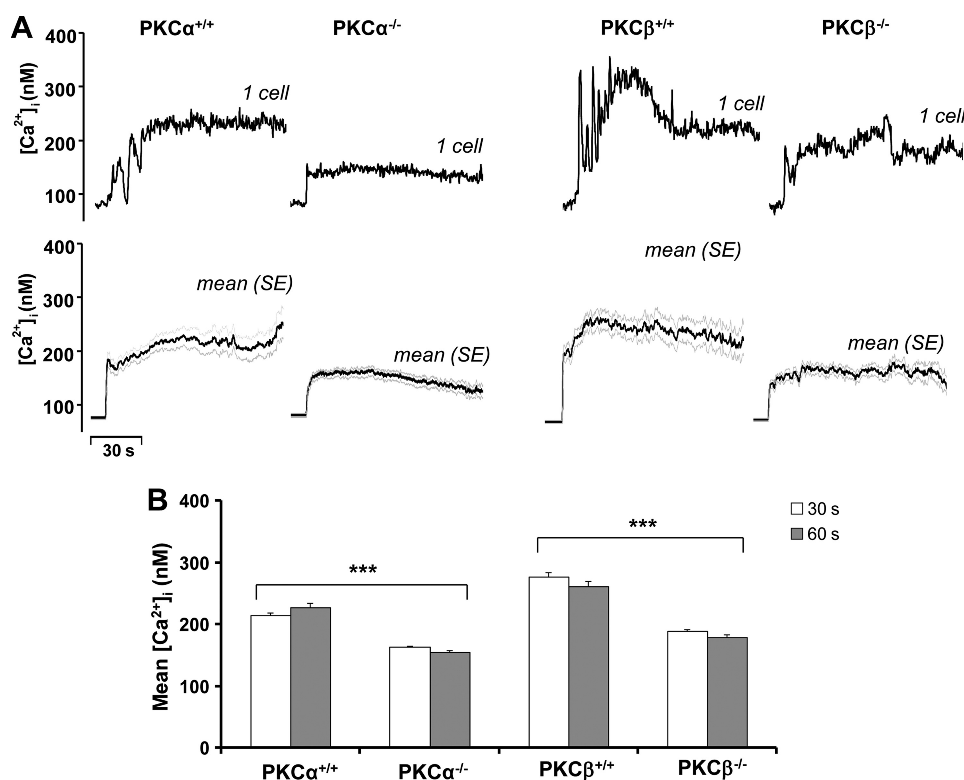

FIGURE 3. **Deficiency of PKCα or PKCβ suppresses collagen-induced Ca^{2+} signaling under flow but not adhesion.** Whole blood containing autologous Fluo-4-loaded platelets was flowed over collagen (Fig. 1), whereas fluorescence images of adherent platelets were captured in real time (5 Hz). *A*, traces of pseudo-ratioed changes in $[Ca^{2+}]_i$ from representative, single platelets as well as mean traces (S.E.) from overlays of 30–40 platelets. *B*, quantitative analysis of increased $[Ca^{2+}]_i$ at 30 s (*white*) and 60 s (*gray*) after initial response. No quantitative differences in response between wild-type mice were seen. After 30 s, averaged rises in $[Ca^{2+}]_i$ were 225 ± 26.3 nM for PKC$\alpha^{+/+}$ platelets, and 270 ± 33.9 nM for PKC$\beta^{+/+}$ platelets ($n = 3$). Data are means ± S.E. (n = 30–40, 3–4 experiments); ***, $p < 0.001$ *versus* corresponding wild types.

isoforms 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 Ca^{2+} signaling is not responsible for the inhibition of aggregation (Fig. 7*B*). Inhibition of novel PKC isoforms with rottlerin or PKCθ inhibitor enhanced convulxin-induced platelet aggregation. In general, measurements of convulxin-induced $\alpha_{IIb}\beta_3$ 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 Ca^{2+} Signaling—Finally, we measured GPVI-induced Ca^{2+} responses in washed human platelets. Gö6976 and PKCβ inhibitor suppressed the $[Ca^{2+}]_i$ peak and $[Ca^{2+}]_i$ time integral (Fig. 7, *E* and *F*), whereas rottlerin was without effect, and pretreatment with PKCθ inhibitor markedly increased the platelet Ca^{2+} signal. This resembled the potentiatory effect seen with Ro-318425 and also noted in earlier studies with human platelets (17). Hence, the enhancement of Ca^{2+} 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 Ca^{2+} signaling were not caused by regulated shedding of GPVI.

Relative Contribution of Conventional and Novel PKC Isoforms to Secretion and Ca^{2+} Signaling—Because PKCα and PKCθ regulate α-granule secretion in opposite directions, we investigated which isoform was dominant during platelet activation by treating PKC$\alpha^{+/+}$ and PKC$\alpha^{-/-}$ platelets with PKCθ inhibitor and measured P-selectin expression by flow cytometry. Consistent with the above data, P-selectin expression was significantly inhibited in PKC$\alpha^{-/-}$ platelets. Interestingly, although PKCθ inhibitor slightly increased the P-selectin expression in PKC$\alpha^{+/+}$ platelets, there was little effect of PKCθ inhibitor on PKC$\alpha^{-/-}$ platelets (Fig. 8*A*). These data suggest that the conventional isoforms are essential for secretion, whereas PKCθ regulates the extent of secretion.

PKCα and PKCθ also regulate Ca^{2+} signaling under flow conditions in opposite directions. Treatment with PKCθ inhibitor enhanced the Ca^{2+} signal in platelets flowed over collagen in PKC$\alpha^{+/+}$ platelets, consistent with the data obtained from PKC$\theta^{-/-}$ platelets. Moreover, PKCθ inhibitor also enhanced the Ca^{2+} signal in PKC$\alpha^{-/-}$ platelets (Fig. 8*B*). These data suggest that PKCθ is a more important regulator of Ca^{2+} 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,

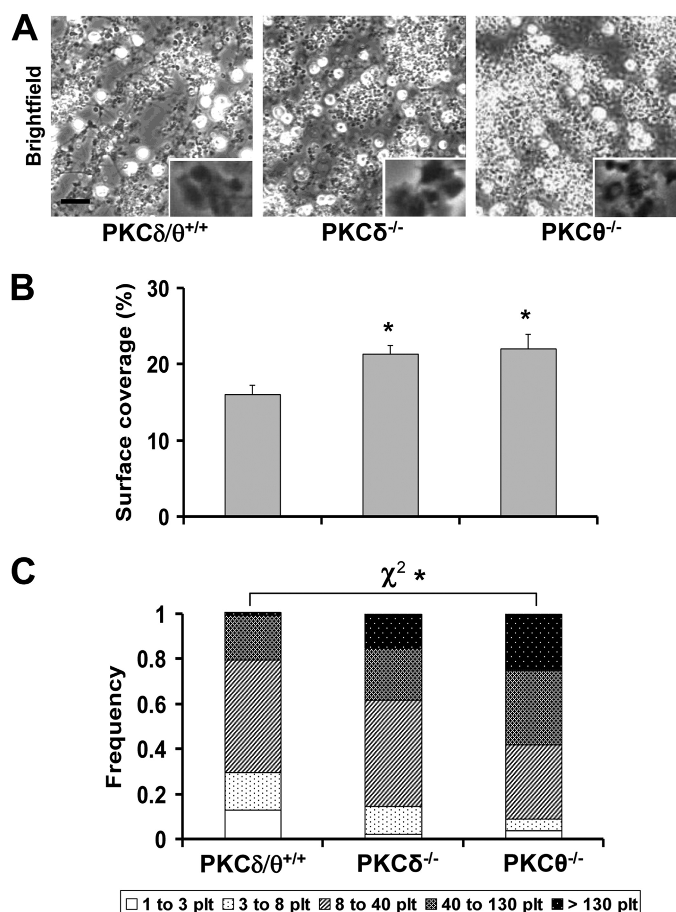

FIGURE 4. **Deficiency of PKCδ or PKCθ increases thrombus formation on collagen under flow.** Blood from PKC$\delta^{-/-}$, PKC$\theta^{-/-}$, or matched wild-type mice was flowed over collagen (Fig. 1). *A*, representative contrast images after 4 min of perfusion (*bars*, 20 μm; *insets*, 5× magnified). *B*, quantification of platelet deposition on collagen surface. *C*, morphometric analysis of aggregates on collagen surface with indicated numbers of platelets (*plt*) per feature. Means ± S.E. (n = 3–6); *, $p < 0.01$ *versus* corresponding wild types; χ^2 test, *, $p < 0.05$.

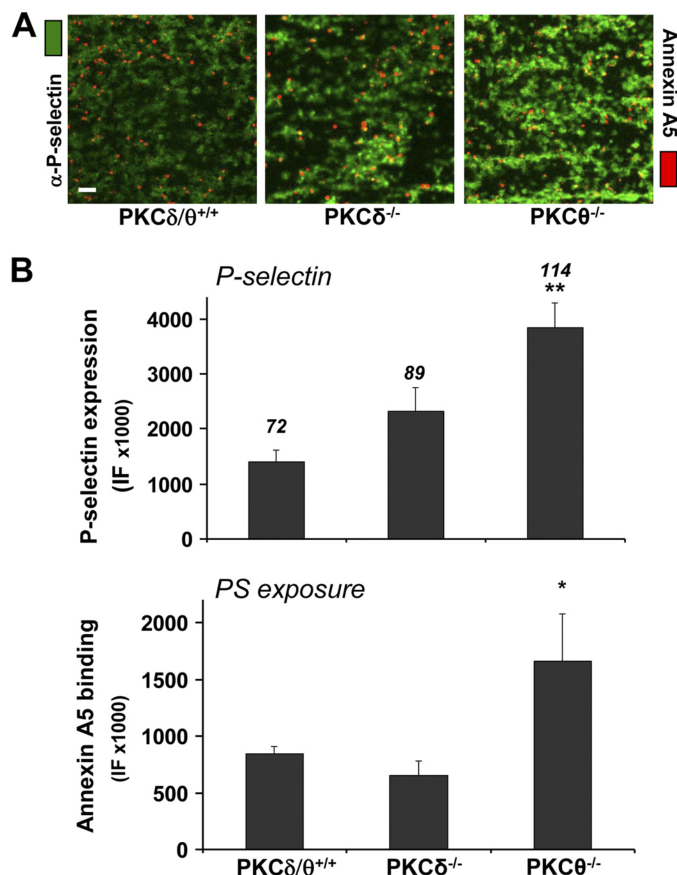

FIGURE 5. **Deficiency of PKCθ but not PKCδ increases collagen-induced activation of platelets in thrombi.** Blood from PKC$\delta^{-/-}$, PKC$\theta^{-/-}$, or matched wild-type mice was flowed over collagen, and platelet thrombi were double-stained with FITC-anti-mouse CD62P mAb (*green*) and Alexa Fluor 647-annexin A5 (*red*). *A*, representative confocal images (180 × 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); **, $p < 0.01$ and *, $p < 0.05$ *versus* corresponding wild types. *Numbers* above each bar indicate the mean integrated pixel density of P-selectin staining, an indication of staining per platelet.

in mediating thrombus formation. The absence of either isoform leads to marked suppression of secretion of α-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 PKCθ and PKCδ, however, leads to enhanced thrombus formation on collagen. The mechanisms for these isoforms are also distinct and non-redundant because although the absence of PKCθ also leads to enhanced secretion, calcium signaling, and phosphatidylserine exposure, the absence of PKCδ 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. 8*C*).

The studies here with mice lacking PKCα or PKCβ 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 α-granule secretion. A key role for murine PKCα in exocytosis of platelet α-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 PKCα or PKCβ 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 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 PKCα and PKCβ regulate 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 PKCα and PKCβ may each play essential, but distinct, roles.

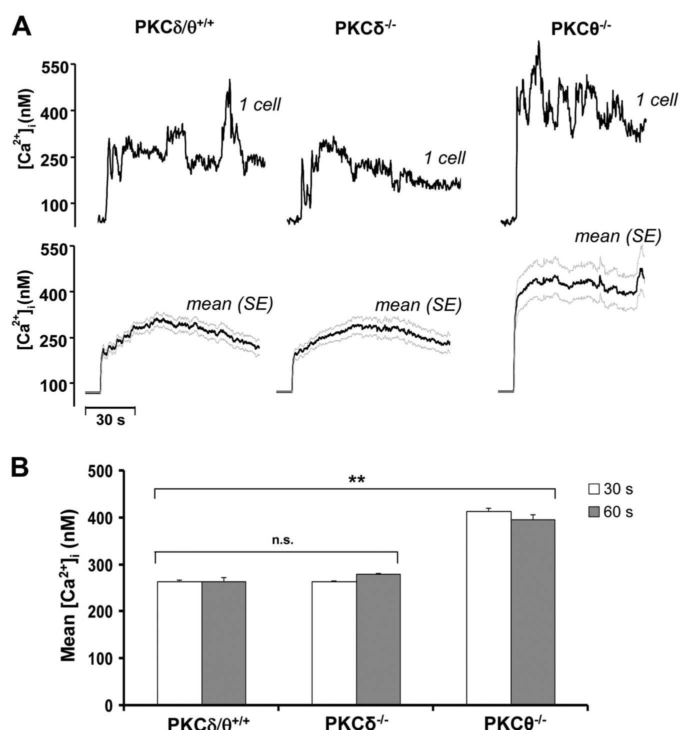

FIGURE 6. **Deficiency of PKCθ but not PKCδ increases collagen-induced Ca^{2+} signaling under flow.** Whole blood containing autologous Fluo-4-loaded platelets was flowed over collagen (Fig. 1), whereas fluorescence images of adhered platelets were captured in real time (5 Hz). *A*, traces of pseudo-ratioed changes in $[Ca^{2+}]_i$ from representative single platelets as well as mean traces (S.E.) from overlays of >38 platelets. *B*, graph of quantitative analysis of increased $[Ca^{2+}]_i$ at 30 s (*white*) and 60 s (*gray*) after initial response. Data are means ± S.E. (n = 38–40, 4 experiments); **, $p < 0.01$ *versus* corresponding wild types. *n.s.*, not significant.

It was important to extend the studies in mouse platelets by studies with human platelets, using the compounds Gö6976 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 PKC$\alpha^{-/-}$ 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 Gö6976 and PKCβ inhibitor point to a substantial reduction in platelet responses. The PKCβ inhibitor is, however, much less effective than Gö6976 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 positively 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 $\alpha_{IIb}\beta_3$ 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 PKC$\theta^{-/-}$ 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 $\alpha_{IIb}\beta_3$ 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 PKC 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 PKC$\theta^{-/-}$ blood is in apparent contrast to a previous report (25), in which PKC$\theta^{-/-}$ 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 PKCδ. 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 PKC$\delta^{-/-}$ mice in the present study. Although some reports suggest that GPVI-induced granule secretion is increased in PKC$\delta^{-/-}$ 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-

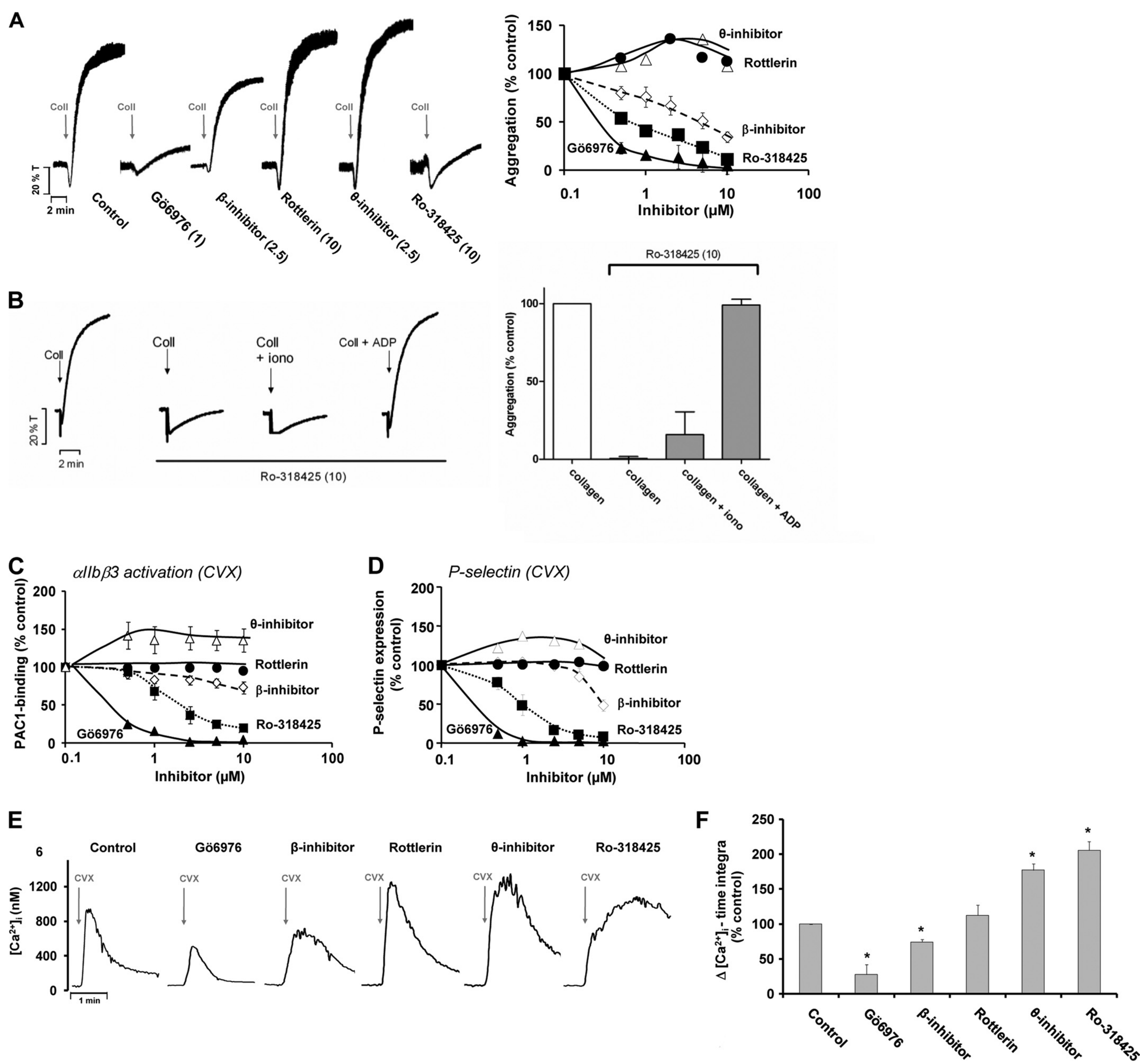

FIGURE 7. **Inhibition of conventional or novel PKC isoforms differentially affects collagen-induced platelet activation and calcium signaling.** *A–D*, washed human platelets were pretreated as described for supplemental Fig. 1 and stimulated with collagen (5 μg/ml) or convulxin (70 ng/ml). *A*, effect of inhibitors (μM) on collagen (*Coll*)-induced platelet aggregation. Shown are representative aggregation traces (optical light transmission) and dose-response curves. *β-inhibitor*, PKCβ inhibitor; *θ-inhibitor*, PKCθ inhibitor. *B*, rescue of collagen-induced aggregation by ADP (10 μM) but not ionomycin (*iono*; 100 nM). Shown are representative aggregation traces and mean ± S.E. ($n = 4$). *C* and *D*, inhibitor effects on platelet activation induced by convulxin (*cvx*) as measured by flow cytometric analysis of $\alpha_{IIb}\beta_3$ activation and P-selectin expression. Effects are expressed as percentages of control condition. Means ± S.E. ($n = 3$–5). *E* and *F*, washed human platelets (1×10^8/ml), loaded with Fura-2, were treated with Me_2SO vehicle (control), Gö6976 (1.0 μM), PKCβ inhibitor (2.5 μM), rottlerin (10 μM), or PKCθ inhibitor (1.0 μM) Ro-318425 (10 μM). *E*, traces of changes in $[Ca^{2+}]_i$ were measured in response to convulxin (70 ng/ml). *F*, quantitative effect on convulxin-induced $[Ca^{2+}]_i$ time integrals. Means ± S.E. ($n = 3$–4); *, $p < 0.05$ *versus* control condition.

tlerin had little effect on collagen-induced integrin $\alpha_{IIb}\beta_3$ activation and α-granule secretion but enhanced platelet aggregation. Therefore, although PKCθ and PKCδ 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 PKCα and PKCβ 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 PKCθ because the absence or pharmacological inhibition of PKCθ 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 PKCθ inhibitor on PKC$\alpha^{-/-}$ platelets. Importantly, combined loss of

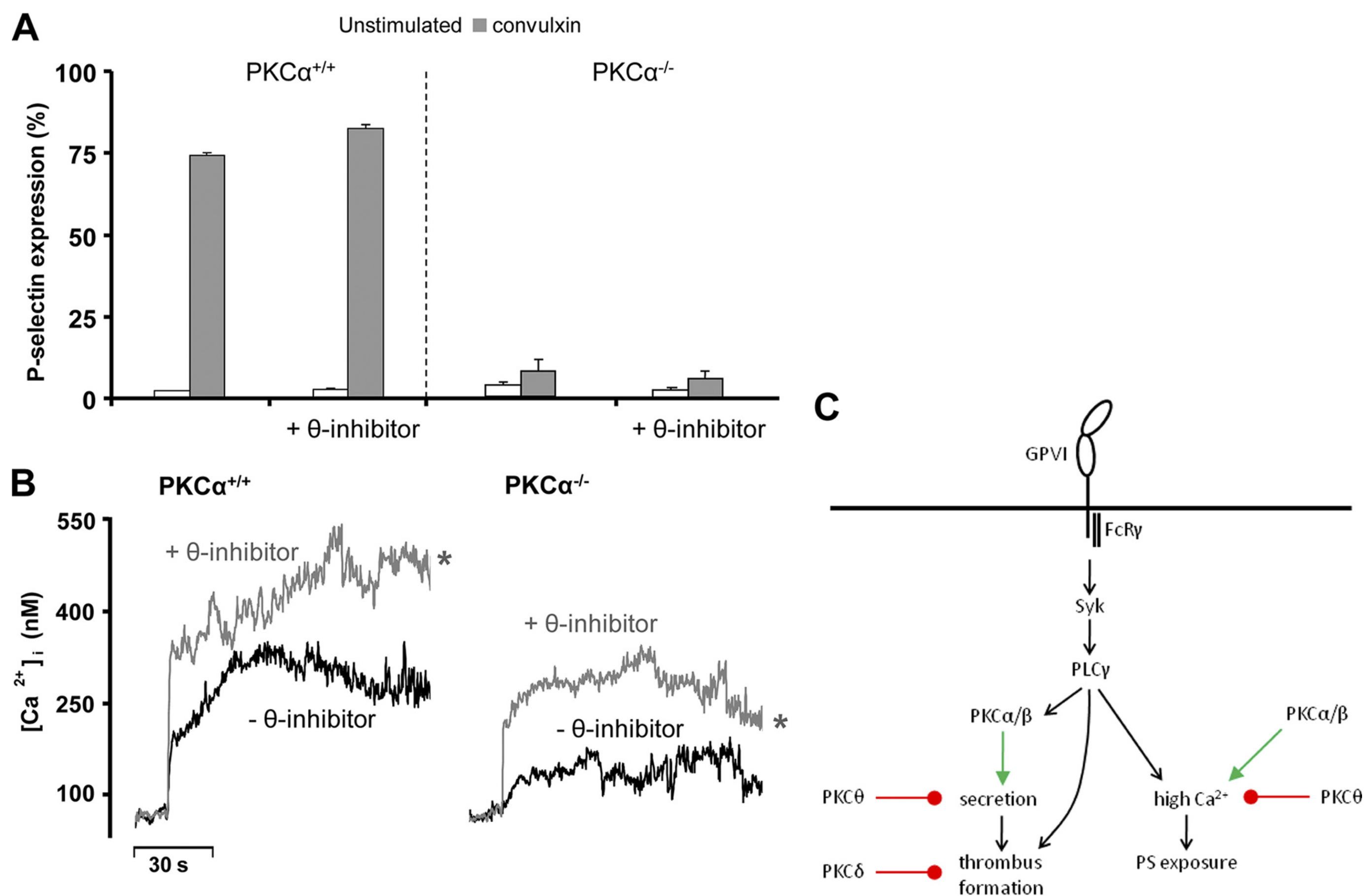

FIGURE 8. **Relative contributions of PKCα and PKCθ to secretion and Ca^{2+} signaling.** *A*, washed PKC$\alpha^{+/+}$ or PKC$\alpha^{-/-}$ platelets were pretreated with PKCθ inhibitor (*θ-inhibitor*) or vehicle control and were stimulated with convulxin (70 ng/ml). α-Granule secretion was assessed by flow cytometric analysis of P-selectin expression. Data are expressed as the percentage of cells expressing surface P-selectin ($n = 3$). *B*, blood from PKC$\alpha^{+/+}$ or $^{-/-}$ mice containing autologous Fluo-4-loaded platelets was treated with PKCθ inhibitor or vehicle control and then flowed over collagen, as described in the legend for Fig. 3. Representative traces are shown ($n = 3$). *, $p < 0.05$ PKCθ inhibitor *versus* untreated condition. *C*, summary diagram of the roles of different PKC isoforms in platelet secretion, thrombus formation, sustained Ca^{2+} signaling, and PS exposure. PKCα/β are necessary for collagen-induced granule secretion, which is negatively regulated by PKCθ. In the absence of PKCα/β, there is no granule secretion and so no effect of PKCθ. PKCα/β positively regulate sustained Ca^{2+} signaling and PS exposure, whereas it is negatively regulated by PKCθ even in the absence of PKCα. PKCδ negatively regulates thrombus formation independently of granule secretion and does not regulate Ca^{2+} signaling or PS exposure.

PKCα and PKCθ signaling produced a phenotype that resembled PKC$\alpha^{-/-}$ platelets for secretion and PKC$\theta^{-/-}$ platelets for Ca^{2+} 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 for PKCθ to regulate (Fig. 8*C*). In contrast, we suggest that collagen-induced sustained Ca^{2+} signals and subsequent PS exposure are positively regulated by PKCα and PKCβ and negatively regulated by PKCθ. Unlike secretion, however, the conventional isoforms are not essential for generation of the Ca^{2+} signal.

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, PKCδ, and PKCθ and reveal important distinctions in mechanism and function of these kinases. The data will provide a platform for future exploitation of these different family members in modulation of platelet function and thrombus formation.

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