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Research Article

Hematology

Platelets are central players in atherothrombosis development in coronary artery disease. The PKC family provides important intracellular mechanisms for regulating platelet activity, and platelets express several members of this family, including the classical isoforms PKC $\alpha$  and PKC $\beta$  and novel isoforms PKC $\delta$  and PKC $\theta$ . Here, we used a genetic approach to definitively demonstrate the role played by PKC $\alpha$  in regulating thrombus formation and platelet function. Thrombus formation in vivo was attenuated in *Prkca*<sup>-/-</sup> mice, and PKC $\alpha$  was required for thrombus formation in vitro, although this PKC isoform did not regulate platelet adhesion to collagen. The ablation of in vitro thrombus formation in *Prkca*<sup>-/-</sup> platelets was rescued by the addition of ADP, consistent with the key mechanistic finding that dense-granule biogenesis and secretion depend upon PKC $\alpha$  expression. Furthermore, defective platelet aggregation in response to either collagen-related peptide or thrombin could be overcome by an increase in agonist concentration. Evidence of overt bleeding, including gastrointestinal and tail bleeding, was not seen in *Prkca*<sup>-/-</sup> mice. In summary, the effects of PKC $\alpha$  ablation on thrombus formation and granule secretion may implicate PKC $\alpha$  as a drug target for antithrombotic therapy.

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# PKC $\alpha$ regulates platelet granule secretion and thrombus formation in mice

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**Platelets are central players in atherothrombosis development in coronary artery disease. The PKC family provides important intracellular mechanisms for regulating platelet activity, and platelets express several members of this family, including the classical isoforms PKC $\alpha$  and PKC $\beta$  and novel isoforms PKC $\delta$  and PKC $\theta$ . Here, we used a genetic approach to definitively demonstrate the role played by PKC $\alpha$  in regulating thrombus formation and platelet function. Thrombus formation in vivo was attenuated in *Prkca*<sup>-/-</sup> mice, and PKC $\alpha$  was required for thrombus formation in vitro, although this PKC isoform did not regulate platelet adhesion to collagen. The ablation of in vitro thrombus formation in *Prkca*<sup>-/-</sup> platelets was rescued by the addition of ADP, consistent with the key mechanistic finding that dense-granule biogenesis and secretion depend upon PKC $\alpha$  expression. Furthermore, defective platelet aggregation in response to either collagen-related peptide or thrombin could be overcome by an increase in agonist concentration. Evidence of overt bleeding, including gastrointestinal and tail bleeding, was not seen in *Prkca*<sup>-/-</sup> mice. In summary, the effects of PKC $\alpha$  ablation on thrombus formation and granule secretion may implicate PKC $\alpha$  as a drug target for antithrombotic therapy.**

## Introduction

The PKC family comprises 10 isozymes grouped into 3 classes: conventional ( $\alpha$ ,  $\beta$ I,  $\beta$ II,  $\gamma$ ), novel ( $\delta$ ,  $\epsilon$ ,  $\eta$ /L,  $\theta$ ), and atypical ( $\zeta$ ,  $\iota$ / $\lambda$ ) (1). PKCs have long been known to play major roles in a number of platelet processes (2), most importantly aggregation and secretion, in which stimulation of platelets with diacylglycerol (DAG) or phorbol ester can induce aggregation and agonist-induced secretion can be prevented by broad spectrum pharmacological inhibition of PKC isoforms (3–6). Recently, it has been shown pharmacologically that PKC isoforms may exert a dual-control role in thrombus formation by mediating secretion and integrin activation under flow while also suppressing phosphatidylserine exposure and subsequent thrombin generation and coagulation (7).

At least 4 PKC isoforms ( $\alpha$ ,  $\beta$ ,  $\delta$ ,  $\theta$ ) are expressed in human platelets (8–14), and it is becoming clear that each isoform may play a different role in platelet function. Using genetic and pharmacological approaches, we have shown PKC $\delta$  to play a negative role in regulating filopodia formation and platelet aggregation in response to collagen through a functional interaction with the actin regulatory protein VASP (15, 16). Using biochemical approaches, PKC $\alpha$  has been identified as an essential factor in positively regulating  $\alpha$ -granule and dense-granule secretion in platelets (17) as well as platelet aggregation (18). We have recently shown, using biochemical and pharmacological approaches, that 2 tyrosine kinases, Syk and Src, physically and functionally interact with PKC $\alpha$  to regu-

late each other and cellular activities in platelets (19). It is now important that the function of PKC $\alpha$  in platelets be addressed definitively by a genetic approach.

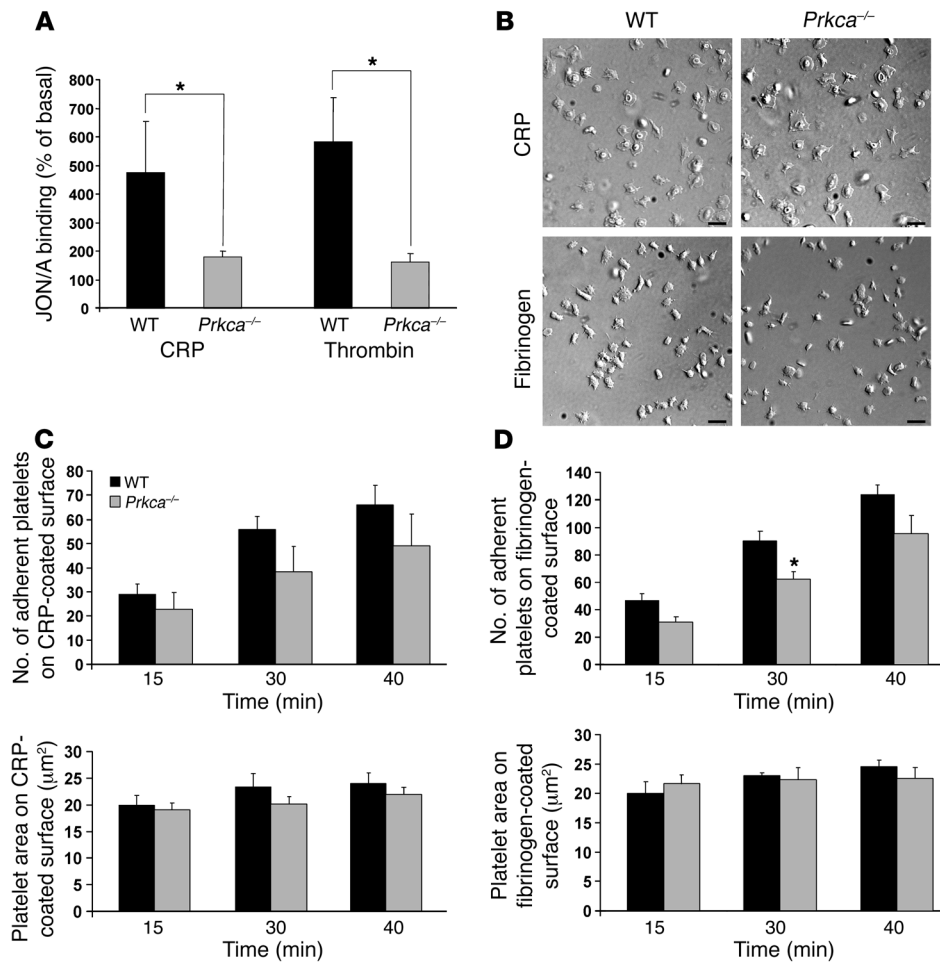
Genetic and molecular approaches have revealed a wide range of roles for PKC $\alpha$  in other cell types. Cell proliferation, differentiation, apoptosis, motility, and adhesion are all regulated by PKC (1, 20), with consequent roles for PKC $\alpha$  in regulation of tumor progression. Although there is evidence that in some tumors, PKC $\alpha$  may have a suppressor role (21, 22), in the majority of cases, PKC $\alpha$  expression and activity are higher in tumors than in normal tissues (23, 24) and PKC $\alpha$  activity promotes a more aggressive phenotype in breast cancer cells, with an increased metastatic potential (25–27). PKC $\alpha$  has therefore become a major target for therapeutic intervention in various cancers (28). In immune regulation, PKC $\alpha$  mediates T cell–dependent interferon generation (29) and is also critical for T cell receptor downregulation (30). Insulin signaling to PI3K and subsequently to glucose transport is enhanced in mice lacking PKC $\alpha$  (31), suggesting a negative feedback role for PKC $\alpha$  in metabolic processes. Finally, in the heart, PKC $\alpha$  regulates cardiac contractility and Ca<sup>2+</sup> handling in myocytes, and deficiency of PKC $\alpha$  protects against heart failure induced by pressure overload and against dilated cardiomyopathy (32).

In a recent elegant study reconstructing the signaling pathway regulating platelet integrin  $\alpha$ <sub>IIb</sub> $\beta$ <sub>3</sub> in a nucleated cell system, it was shown that expression levels of PKC $\alpha$  equivalent to those found in platelets are required for activation of the integrin through the Rap1 pathway (33). This is in agreement with previous biochemical evidence suggesting an essential role for PKC $\alpha$  in regulating integrin (18). Importantly also, genome-wide association analysis of coronary artery disease revealed a cluster of SNPs in the *PRKCA*

**Conflict of interest:** The authors have declared that no conflict of interest exists.

**Nonstandard abbreviations used:** CRP, collagen-related peptide; DIC, differential interference contrast; GPVI, glycoprotein VI; PB, phosphate buffer.

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**Figure 1**

PKC $\alpha$  plays a major role in regulating inside-out signaling to integrin  $\alpha_{\text{IIb}}\beta_3$  but no significant role in outside-in signaling or in adhesion to fibrinogen or collagen. (A) Washed platelets from WT or *Prkca*<sup>-/-</sup> mice were labeled with PE-anti- $\alpha_{\text{IIb}}\beta_3$  antibody and stimulated with CRP (5  $\mu\text{g}/\text{ml}$ ) or thrombin (1 U/ml) for 15 minutes; immunofluorescence intensity was measured by flow cytometry. The data presented are geometric means as percentages of basal non-stimulated levels. Error bars represent SEM.  $n = 3$ . \* $P < 0.05$ . (B–D) Washed platelets from WT or *Prkca*<sup>-/-</sup> mice were added to CRP- or fibrinogen-coated coverslips, and the levels of spreading and adherence were analyzed. (B) Representative images of murine platelets 40 minutes after addition to CRP- or fibrinogen-coated surfaces as indicated. Images were taken under oil immersion. Original magnification,  $\times 63$ . Scale bars: 5  $\mu\text{m}$ . (C and D) Time course of WT and *Prkca*<sup>-/-</sup> platelet adherence (number of platelets estimated per field of view; upper panel) and spreading (as assessed by measurement of cell surface area; lower panel) on CRP-coated (C) and fibrinogen-coated (D) surfaces. Data shown are mean  $\pm$  SEM.  $n = 4$ .

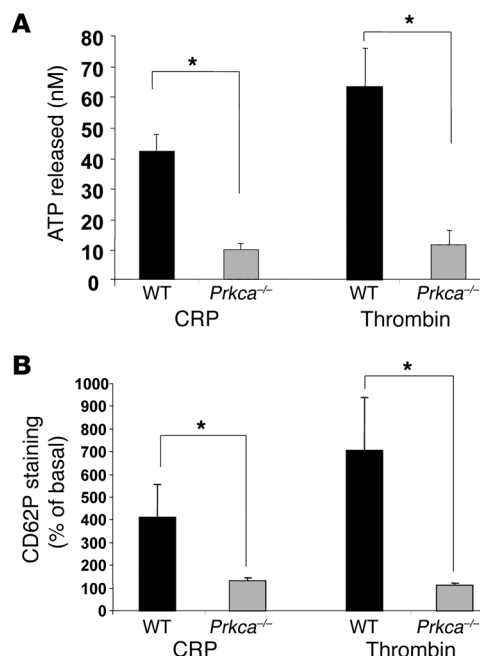
gene, with a maximal  $P$  value association of just over  $10^{-3}$  (SNPs rs12600582) (34, 35). This analysis marks the gene as one requiring further phenotypic analysis. It was essential to identify definitively by a genetic approach the role of PKC $\alpha$  in regulating platelet function and thrombus formation. Here, we report that genetic ablation of PKC $\alpha$  in platelets reveals nonredundant roles in regulating platelet dense-granule biogenesis and exocytosis and in the control of thrombus formation in vitro and in vivo.

**Results**

*Major role for PKC $\alpha$  in inside-out regulation of  $\alpha_{\text{IIb}}\beta_3$  but no significant role in outside-in signaling through this integrin.* The PKC family of kinases has been implicated in agonist-induced  $\alpha_{\text{IIb}}\beta_3$  integrin activation (36), and although an elegant reconstruction study showed a critical role for PKC $\alpha$  in regulating the integrin (33), an involvement of PKC $\alpha$  in this event in platelets has not yet been addressed directly and definitively. Here, we report for what we believe is the first time in platelet functional and thrombosis analysis, data from a mouse gene knockout lacking PKC $\alpha$ . Supplemental Figure 1 (supplemental material available online with this article; doi:10.1172/JCI34665DS1) shows baseline data demonstrating lack of expression of PKC $\alpha$  but normal expression of other platelet PKCs ( $\beta$ ,  $\delta$ , and  $\theta$ ) in *Prkca*<sup>-/-</sup> platelets. Figure 1A shows that both collagen-related peptide (CRP) (5  $\mu\text{g}/\text{ml}$ ) and thrombin (1 U/ml) produced significantly greater  $\alpha_{\text{IIb}}\beta_3$  activation in WT platelets compared

with *Prkca*<sup>-/-</sup>. This suggests that PKC $\alpha$  is a major regulator of inside-out signaling to  $\alpha_{\text{IIb}}\beta_3$ , although the residual response in the absence of PKC $\alpha$  suggests a component independent of this kinase. By way of control, Supplemental Figure 2 shows no significant difference in surface expression of  $\alpha_{\text{IIb}}$  (CD41) in *Prkca*<sup>-/-</sup> versus WT irrespective of activation state of the platelets and irrespective of agonist (CRP or thrombin).

It has been shown recently that PKC $\beta$  and PKC $\theta$  play critical roles in regulating outside-in signaling from  $\alpha_{\text{IIb}}\beta_3$  (37, 38). Hence, in mouse platelets lacking either PKC $\beta$  or PKC $\theta$ , cell spreading on fibrinogen-coated surfaces was ablated. Additionally, we had recently shown that absence of PKC $\delta$  caused platelets to spread on CRP- or collagen-coated surfaces with markedly more sustained generation of filopodia than WT control platelets. It was important therefore to assess the role of PKC $\alpha$  in adhesion and spreading on either fibrinogen or CRP, and so we measured the number and surface area of adherent platelets by differential interference contrast (DIC) microscopy over a 40-minute period. Figure 1, B–D, shows that platelets lacking PKC $\alpha$  had no significant defect in their ability to spread on either fibrinogen-coated or CRP-coated surfaces, and importantly in the case of CRP, there was no evident sustained development of filopodia. In addition, although Figure 1, C and D, shows a trend toward decreased adhesion of platelets to both surfaces in *Prkca*<sup>-/-</sup>, significance was achieved only at a single time point (30 minutes;  $90.31 \pm 6.9$  platelets/field of view in WT



compared with  $62.18 \pm 5.6$  platelets/field of view in *Prkca*<sup>-/-</sup> mice; mean  $\pm$  SEM,  $P < 0.05$ ) in platelets adherent to fibrinogen.

*PKC $\alpha$  is a major regulator of dense- and  $\alpha$ -granule secretion.* Figure 2A shows that *Prkca*<sup>-/-</sup> platelets also exhibit a marked reduction in CRP- and thrombin-induced ATP secretion relative to WT controls ( $40.9 \pm 7.0$  nM ATP in WT vs.  $8.9 \pm 3.4$  nM ATP in *Prkca*<sup>-/-</sup> for CRP,  $P < 0.05$ ; and  $61.1 \pm 14.7$  nM in WT vs.  $10.8 \pm 5.7$  in *Prkca*<sup>-/-</sup> for thrombin,  $P < 0.05$ ). The defect in granule secretion was not restricted to dense granules, however, but also involved regulation of  $\alpha$ -granules. Stimulation with CRP induced P selectin surface expression in WT platelets, which was significantly greater than that in *Prkca*<sup>-/-</sup> platelets ( $411.9\% \pm 145.3\%$  over basal in WT vs.  $130.7\% \pm 14.6\%$  over basal in *Prkca*<sup>-/-</sup>). A similar marked knockdown of P selectin exposure was observed after thrombin activation (Figure 2B) ( $705\% \pm 235.7\%$  in WT vs.  $113.9\% \pm 5.5\%$  in KO;  $P < 0.05$ ), suggesting that PKC $\alpha$  plays a major role in platelet  $\alpha$ -granule and dense-granule release.

It has been demonstrated that SNAP23, a member of SNARE traffic and fusion protein complex, undergoes PKC-dependent phosphorylation during agonist-stimulated platelet degranulation, and in vitro PKC $\alpha$  was one of the isoforms able to phosphorylate recombinant SNAP23 (39). We therefore assessed the contribution of PKC $\alpha$  to phosphorylation of SNAP23 on Ser95, the site reported to be phosphorylated following thrombin stimulation

### Figure 3

PKC $\alpha$  regulates dense-granule but not  $\alpha$ -granule biogenesis. Washed platelets from WT or *Prkca*<sup>-/-</sup> mice were examined by transmission electron microscopy, and the number of dense granules (black arrows) and  $\alpha$ -granules (white arrows) was quantified as described in Methods. (A) Images of WT (left panel) or *Prkca*<sup>-/-</sup> (right panel) platelets are representative of 3 independent experiments. Scale bar: 1  $\mu$ m. Original magnification,  $\times 19,000$ . (B) Dense granules (left panel) and  $\alpha$ -granules (right panel) were counted per field of view (25–30 fields of view per preparation) and shown as mean  $\pm$  SEM for number of granules per  $\mu$ m<sup>2</sup>.  $n = 3$ . \* $P < 0.05$ .

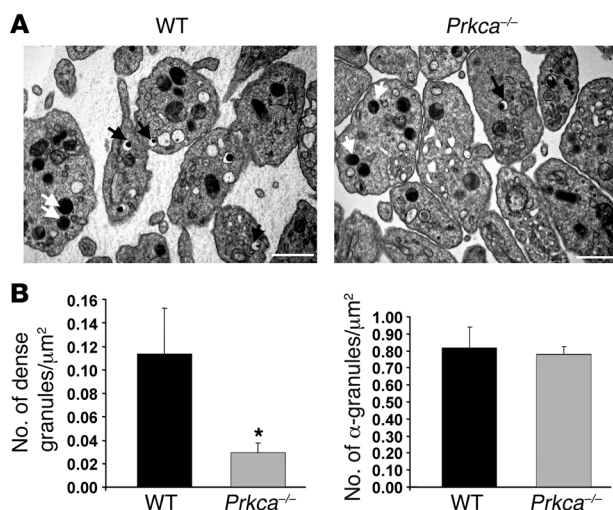
### Figure 2

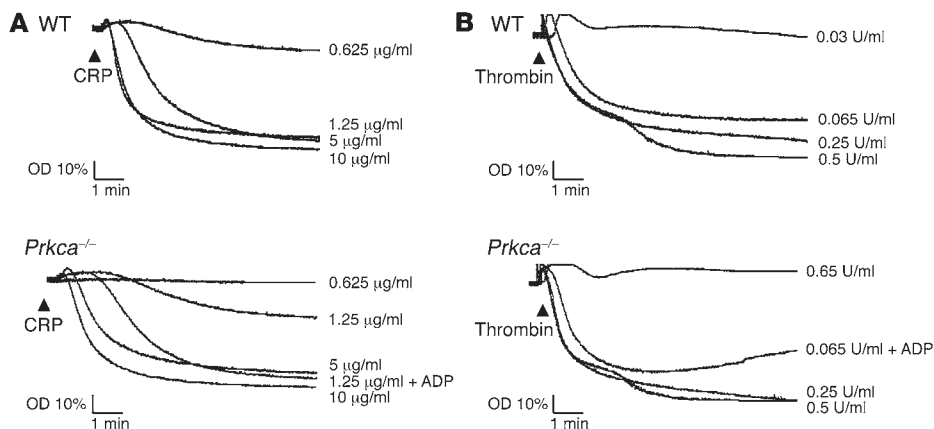
Key role for PKC $\alpha$  in secretion of dense granules and  $\alpha$ -granules. (A) Washed platelets from WT or *Prkca*<sup>-/-</sup> mice were stimulated with CRP (5  $\mu$ g/ml) or thrombin (0.25 U/ml) and secretion of ATP assessed by luminometry. Data shown represent maximal increase in ATP concentration and represent mean  $\pm$  SEM.  $n = 3$ . \* $P < 0.05$ . (B) Platelets from WT or *Prkca*<sup>-/-</sup> mice were labeled with FITC-C62P antibody and stimulated with CRP (5  $\mu$ g/ml) or thrombin (1 U/ml) for 15 minutes. Fluorescence intensity was measured by flow cytometry. Data presented represent geometric means as percentages of basal nonstimulated levels. Error bars represent SEM.  $n = 3$ .

(40). Supplemental Figure 3A shows a time course of phosphorylation of SNAP23 from WT mouse platelets. This time course correlated well with release of ATP from those platelets stimulated with thrombin. We then showed that both CRP and thrombin activation led to a potent increase in SNAP23 phosphorylation at Ser95, which was considerably although not completely inhibited by absence of PKC $\alpha$  (Supplemental Figure 3B). PKC $\alpha$ -mediated phosphorylation of SNAP23 may therefore play a role in regulating secretion or SNARE recycling.

It was possible that the secretory defect seen in *Prkca*<sup>-/-</sup> platelets may be a result of a granule developmental problem. Transmission electron microscopy analysis of platelet ultrastructure showed an abnormality in the number of dense granules in *Prkca*<sup>-/-</sup> platelets, characterized by an approximately 3.7-fold reduction compared with WT platelets (Figure 3). Dense-granule biogenesis in megakaryocytes or peripheral distribution during proplatelet development could be the key points of regulation by PKC $\alpha$ , and this may additionally explain the major defect in dense-granule secretion seen in *Prkca*<sup>-/-</sup> platelets. Transmission electron microscopy quantification of  $\alpha$ -granule numbers, however, did not detect any substantial difference between WT and *Prkca*<sup>-/-</sup> platelets, indicating that the defect in  $\alpha$ -granule secretion is due to an essential role played by PKC $\alpha$  in regulation of secretion of these granules rather than their biogenesis.

*Platelet aggregation deficit in *Prkca*<sup>-/-</sup> platelets is rescued by exogenous ADP.* It was now important to determine whether the deficits in secretion and activation of integrin  $\alpha$ <sub>IIb</sub> $\beta$ <sub>3</sub> would translate into functional deficits in platelet aggregation and thrombus formation. Figure 4 shows concentration-response relationships for





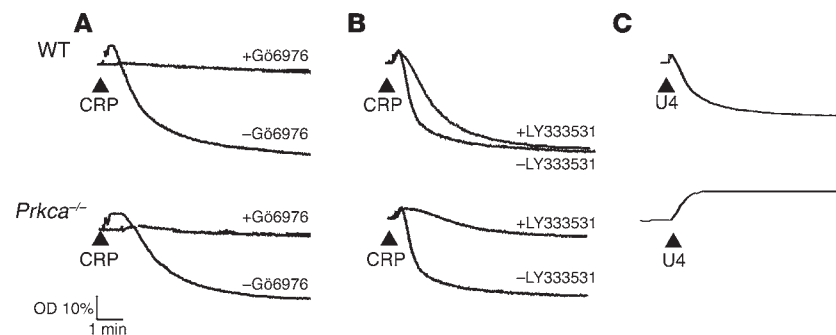
**Figure 4**  
ADP rescues deficient aggregation responses to CRP or thrombin in *Prkca*<sup>-/-</sup> platelets. Washed platelets from WT or *Prkca*<sup>-/-</sup> mice were stimulated with various concentrations of CRP (A) or thrombin (B) with or without simultaneous addition of ADP (10 µM) and aggregation assessed turbidimetrically. Aggregation traces shown are representative of 4 independent experiments.

platelet aggregation activated by CRP (Figure 4A) or thrombin (Figure 4B). At submaximal concentrations of either agonist, absence of PKC $\alpha$  had a marked effect upon the aggregation response, such that the aggregation responses to 1.25 µg/ml CRP or 0.065 U/ml thrombin were almost ablated in *Prkca*<sup>-/-</sup> platelets. Importantly, however, responses may be effectively rescued by addition of exogenous ADP (10 µM) simultaneously with CRP or thrombin. This indicates that the important functional deficit in *Prkca*<sup>-/-</sup> platelets is the lack of dense-granule secretion. The effect upon aggregation was also relative, since higher concentrations of either CRP or thrombin were able to restore aggregations to wild-type values.

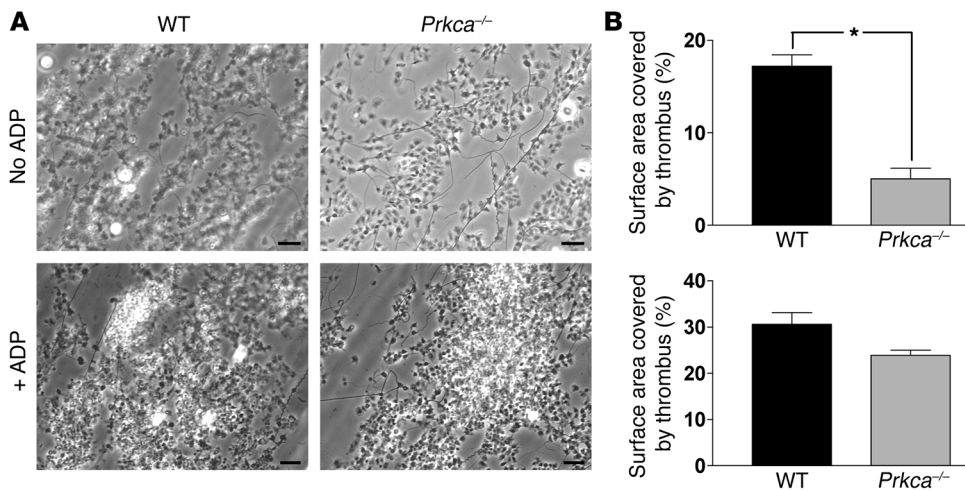
*Pharmacological inhibition of PKC $\beta$  reveals redundancy with PKC $\alpha$  for regulation of platelet aggregation.* Given that higher concentrations of agonists are capable of overcoming the aggregation deficits seen in the absence of PKC $\alpha$ , it was important to address whether there may be functional redundancy between PKC $\alpha$  and its closely related family member PKC $\beta$ . To assess this possibility, we preincubated platelets with the inhibitor of classical PKC isoforms Gö6976 at its maximally effective concentration (1 µM), which completely abrogated CRP-induced aggregation both in WT and *Prkca*<sup>-/-</sup> mouse platelets (Figure 5A). This confirmed an indispensable role for classical PKC isoforms in glycoprotein VI-mediated (GPVI-mediated) aggregation and suggested a potential redundancy between PKC $\alpha$  and PKC $\beta$  in regulating aggregation. This redundancy was demonstrated by using the PKC $\beta$ -selective inhibitor LY333531 (ruboxistaurin), which at a maximally effective concentration (10 µM) had no significant effect upon aggregation of WT platelets to CRP but was markedly inhibitory to this response in platelets lacking PKC $\alpha$  (Figure 5B). This was in parallel with thrombin (0.5 U/ml), for which addition of 10 µM LY333531 had no effect upon WT platelet aggregation response but markedly inhibited the response in *Prkca*<sup>-/-</sup> platelets (data not shown). Interestingly, for weaker agonists such as the thromboxane analogue U46619, aggregation was ablated in platelets lacking PKC $\alpha$ , although shape change in these platelets was unaffected (Figure 5C). This is consistent with pharmacological data that show a critical role for PKC in platelet aggregation induced

through the thromboxane receptor system (41). These data therefore suggest that for weak agonists, PKC $\alpha$  plays an essential role, whereas redundancy between PKC $\alpha$  and PKC $\beta$  exists for strong agonists in regulating the platelet aggregation response.

*Ablation of PKC $\alpha$  markedly attenuates thrombus formation in vitro and in vivo.* Finally, it was important to determine the role played by PKC $\alpha$  in thrombus formation both in vitro and in vivo. Figure 6A shows a major role for PKC $\alpha$  in vitro in mediating this function, where the images reveal no reduction in primary adhesion of platelets to the collagen-coated surface, consistent with the absence of effect upon static adhesion shown in Figure 1, but a marked reduction in aggregated platelet thrombus formation on top of the initially adherent cells. In order to confirm in a quantitative manner the lack of effect of PKC $\alpha$  ablation on primary adhesion, fluo-4-labeled platelets were added to the blood and used for measurements of platelet adhesion during flow over a collagen surface, as we previously described (42). Fluo-4-labeled cells flowing across the field of view were counted, and the percentage of these cells that adhered stably (adherent for >20 seconds) was estimated. At a shear rate of 1000 s<sup>-1</sup>, 81.7% ± 3.6% of WT and 78.1% ± 3.0% of *Prkca*<sup>-/-</sup> platelets became stably adherent



**Figure 5**  
Combined pharmacological and genetic approaches reveal redundancy between PKC $\alpha$  and PKC $\beta$  for regulation of platelet aggregation. (A and B) Washed platelets from WT or *Prkca*<sup>-/-</sup> mice were pretreated (10 minutes) with the classical PKC isoform inhibitor Gö6976 (1 µM) (A), the PKC $\beta$ -selective inhibitor LY333531 (10 µM) (B), or DMSO as vehicle control and stimulated with CRP (5 µg/ml). Aggregation was monitored by turbidimetric aggregometry. Traces shown are representative of 5 independent experiments. (C) Washed platelets from WT or *Prkca*<sup>-/-</sup> mice were stimulated with U46619 (U4) (10 µM) and aggregation assessed turbidimetrically. Traces shown are representative of 4 independent experiments.



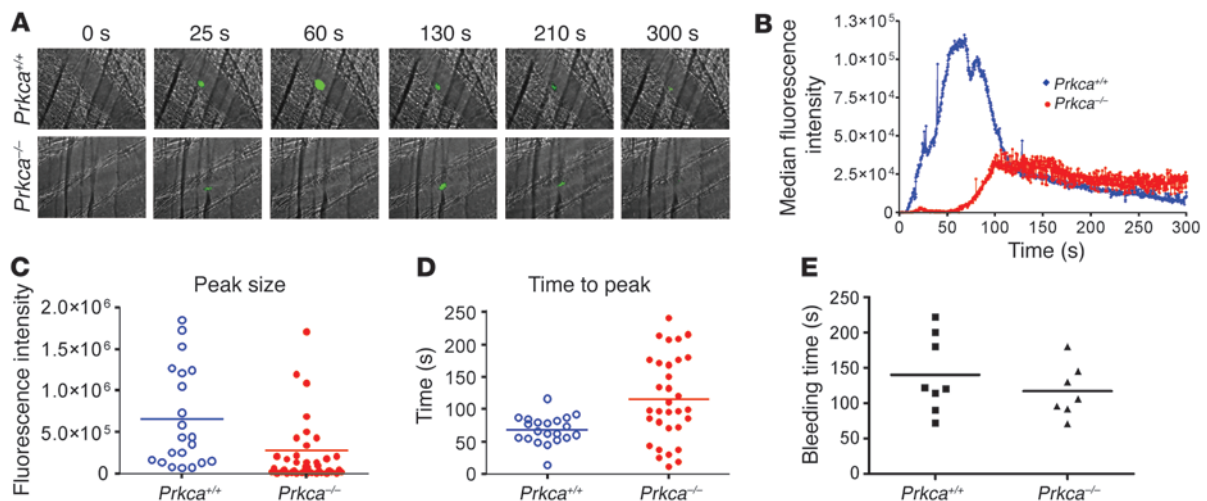
**Figure 6**

PKC $\alpha$  regulates thrombus formation in vitro. Heparin/D-phenylalanyl-prolyl-arginyl chloromethyl ketone-anticoagulated (heparin/PPACK-anticoagulated) blood from WT or *Prkca*<sup>-/-</sup> mice was passed over collagen (shear rate 1000 s<sup>-1</sup>). (A and B) ADP solution was confocused at a 10% flow rate (20  $\mu$ M ADP, final concentration) (lower panels). (A) Representative phase-contrast images after 4 minutes. Images were taken under oil immersion. Scale bars: 20  $\mu$ m. Original magnification,  $\times 63$ . (B) Surface area coverage with thrombi. Mean  $\pm$  SEM.  $n \geq 3$ . \* $P < 0.0001$ .

(mean  $\pm$  SEM,  $n = 5$ ;  $P > 0.05$ ), indicating no significant difference in initial stable adhesion to collagen. Also, in order to confirm that in the absence of PKC $\alpha$ , the 3D buildup of thrombus formation was markedly impaired, reconstructed images were generated from confocal Z-stacks (as described in Methods) and platelet aggregate (thrombus) volumes estimated. The mean volume of representative platelet aggregates on the coverslips after flow at 1000 s<sup>-1</sup> was 570  $\pm$  168  $\mu$ m<sup>3</sup> and 72.3  $\pm$  7.4  $\mu$ m<sup>3</sup> for WT and *Prkca*<sup>-/-</sup> blood, respectively (mean  $\pm$  SEM,  $n = 5$ ;  $P < 0.05$ ). The major secretory defect seen in *Prkca*<sup>-/-</sup> platelets was likely to be responsible for

the impairment of thrombus formation, since addition of ADP (20  $\mu$ M) to the blood in the flow chamber was capable of rescuing thrombus formation (Figure 6, A and B). This indicated that the thrombotic process under flow conditions markedly relies upon the contribution to secretion made by PKC $\alpha$ .

It was important finally to assess the role in vivo of PKC $\alpha$  in regulating thrombus formation. Figure 7 shows data reflecting application of a laser-induced endothelial injury model in cremaster muscle arterioles, described previously (43, 44). A marked difference is evident between WT controls and mice lacking PKC $\alpha$  (Figure 7),



**Figure 7**

In vivo thrombus formation is impaired in the absence of PKC $\alpha$ , but tail bleeding time is normal. (A–D) Mice were either *Prkca*<sup>-/-</sup> or littermate-matched wild-type controls. Platelets were labeled in vivo with Alexa Fluor 488, as described in Methods. (A) Platelets (green) composited with bright field images (black/white) of the cremaster arteriole were viewed, and images were acquired using a digital CCD camera (SensiCam II; Cooke Corp.) with a 640  $\times$  480 pixel array. Original magnification,  $\times 40$ . (B) Traces shown are median integrated platelet fluorescence of 15 thrombi induced in 3 or more mice for each group. Fluorescent intensity of platelets in arbitrary units is presented as a function of time. (C) Data are presented as a scatter diagram. Horizontal bar represents mean of 15 thrombi induced in at least 3 mice for each group. There is a significant reduction in thrombus intensity in *Prkca*<sup>-/-</sup> in comparison with WT controls (mean *Prkca*<sup>+/+</sup>, 652200; mean *Prkca*<sup>-/-</sup>, 279600;  $P < 0.05$ ). (D) Time to reach peak thrombus size also significantly differed from WT controls, with mean data shown by the horizontal bars (mean *Prkca*<sup>+/+</sup>, 68.1 seconds; mean *Prkca*<sup>-/-</sup>, 115.2 seconds;  $P < 0.01$ ). (E) Mice were anesthetized and a transverse incision made with a scalpel at a position where the diameter of the tail was 2.25 to 2.5 mm. The tail was immersed in normal saline (37°C) in a hand-held test tube. The time from incision to cessation of bleeding was recorded, and mean times are shown as horizontal bars. No significant difference was seen comparing WT mice (mean 140 seconds) with *Prkca*<sup>-/-</sup> (mean 117 seconds;  $P > 0.05$ ).



since in the absence of PKC $\alpha$ , thrombus formation is delayed and results in fewer accumulated platelets (see Supplemental Videos 1 and 2). Importantly, however, the ability of *Prkca*<sup>-/-</sup> to undergo normal hemostasis was demonstrated by the lack of alteration in tail-bleed time (Figure 7E) and by the lack of any fecal occult blood (data not shown). This may indicate that compensatory mechanisms are sufficient to allow normal hemostasis but are not sufficient to support normal thrombus formation and may reflect the observation that *Prkca*<sup>-/-</sup> platelets adhere normally to collagen under static and flow conditions (Figures 1 and 6) but have a marked reduction in subsequent platelet-platelet interaction (as evidenced by the lack of thrombus accumulation shown in Figure 6A).

## Discussion

Platelets play a central role in mediating atherothrombosis and are therefore the target of numerous therapies aimed at reducing their activity, particularly in the prevention of coronary artery thrombosis in heart attacks (45). PKC is established, largely by pharmacological studies, as a major regulator of multiple platelet activities (2), and it is increasingly clear that the different isoforms of PKC expressed in platelets perform distinct functions. There is a difficulty of interpretation of data from some pharmacological studies, however, because of the lack of selectivity of the reagents available to target specific PKC isoforms. Here, we used a genetic approach to demonstrate definitively, for what we believe is the first time, the role played by PKC $\alpha$  in regulating platelet function and thrombus formation. Importantly, the study revealed a key role for PKC $\alpha$  in regulating granule biogenesis and exocytosis, which was essential for thrombus formation, since ablation of thrombus formation in *Prkca*<sup>-/-</sup> platelets could be rescued by addition of exogenous ADP. The findings reveal PKC $\alpha$  to be a potential drug target for antithrombotic therapy, since selective inhibitors would exert a major effect upon thrombus formation while sparing primary platelet adhesive functions.

It has recently been shown pharmacologically that PKC isoforms exert dual control of thrombus formation by mediating secretion and integrin activation under flow while suppressing phosphatidylserine exposure and subsequent thrombin generation and coagulation (7). There is incomplete and largely indirect evidence that, of the various isoforms of PKC expressed in platelets, PKC $\alpha$  plays a role in regulation of integrin  $\alpha_{IIb}\beta_3$  activation (36). The study by Han et al. (33) elegantly showed that PKC $\alpha$  was required for integrin  $\alpha_{IIb}\beta_3$  activation in a reconstituted cell system rather than in the platelet. The study by Tabuchi et al. (18) used an in vitro permeabilized platelet system to investigate receptor-independent calcium-mediated activation of platelet aggregation. Although this latter study demonstrated that added purified PKC $\alpha$  was able to support a calcium-induced platelet aggregation response, the approach has limitations in that receptor-mediated activation is lost. Nonetheless, these studies provided indications that PKC $\alpha$  may regulate platelet integrin activation and aggregation, and it was therefore essential to perform a genetic-based definitive study of the role played by PKC $\alpha$  in mediating these events.

The marked knockdown in activation of integrin  $\alpha_{IIb}\beta_3$  in response either to thrombin or CRP in platelets lacking PKC $\alpha$  paralleled a deficit in the ability of these platelets to undergo aggregation at submaximal concentrations of agonist. This was consistent with the impaired ability of *Prkca*<sup>-/-</sup> platelets to form a thrombus in blood flowing over a collagen-coated surface. The effect on aggregation could, however, be overcome by increasing the concen-

tration of agonist, and the reasons for this may derive from several factors. First, it is known that integrin  $\alpha_{IIb}\beta_3$  activation in response to strong agonists is in part regulated independently of PKC (3, 46). Further, studies with integrin blockers have shown that not all  $\alpha_{IIb}\beta_3$  receptors are required to be activated (e.g., by the PKC-independent pathway) for a full platelet aggregation response in the platelet aggregometer (47). Thus, it may be possible to achieve a maximal aggregation response with a markedly reduced expression of activated integrin. In contrast, in thrombus formation in flowing whole blood, stable platelet aggregation critically depends on limited levels of autocrine-produced ADP and hence limited and reversible integrin activation (48). Under these more physiological conditions, the number of activated integrin receptors may be low, and therefore the marked reduction in integrin activation seen in *Prkca*<sup>-/-</sup> platelets may cause a significant reduction in the ability of platelets to form thrombi. Additionally, we provide evidence for redundancy between PKC $\alpha$  and PKC $\beta$  in Figure 5, which shows that selective inhibition of PKC $\beta$  only significantly attenuated aggregation responses in *Prkca*<sup>-/-</sup> platelets, not WT platelets, indicating redundant functions of these 2 isoforms. It is also significant to note that in assays for occult blood in feces, no gastrointestinal bleeding was detected in either WT ( $n = 5$ ) or *Prkca*<sup>-/-</sup> ( $n = 6$ ) mice. Together with the data shown in Figure 7, where no difference in tail bleeding time is seen in *Prkca*<sup>-/-</sup> compared with WT controls, this also implies redundancy of signaling molecules for regulation of hemostasis in vivo.

There is significant contribution to the effects of PKC $\alpha$  ablation on aggregation and thrombus formation by regulation of granule secretion, since these processes greatly rely upon autocrine ADP release (49). Clearly, both dense- and  $\alpha$ -granule secretion are markedly disrupted in platelets lacking PKC $\alpha$ , and evidence that this is functionally critical is provided by the rescue experiments depicted in Figure 4 for aggregation and Figure 6 for in vitro thrombus formation, where addition of exogenous ADP recovers the deficits in these responses seen in *Prkca*<sup>-/-</sup> platelets.

The role of unspecified PKC isoforms as a family in the regulation of secretion has been shown pharmacologically by several groups (3–6). The role of the PKC $\alpha$  isoform in agonist-independent secretion has been demonstrated previously in an artificial permeabilized platelet system (17). Although valuable to indicate the role of PKC $\alpha$ , it was important to address the issue by a definitive genetic approach. Indeed, the only study to date to address specific PKC isoform function in secretion in platelets in a combined genetic and pharmacological approach had shown a major discrepancy in results obtained by the 2 approaches for PKC $\delta$ . Pula et al. (15) showed that genetic ablation of PKC $\delta$  had no significant effect upon dense-granule secretion, whereas in that study and previous studies (9, 16), the PKC $\delta$ -selective inhibitor rottlerin had been shown to enhance dense-granule secretion in response to collagen and alboaggregin, operating through the GPVI receptor. The present results provide direct evidence that PKC $\alpha$  is a major regulator of secretion of  $\alpha$ -granules, since P selectin expression is reduced almost to basal levels in *Prkca*<sup>-/-</sup> platelets in response either to CRP or thrombin. Importantly, we show that  $\alpha$ -granule numbers are equivalent between *Prkca*<sup>-/-</sup> and WT platelets and therefore the secretion defect for this granule type reflects a genuine deficiency in the secretory pathway. For dense granules, this study shows an additional knockdown in numbers of granules in *Prkca*<sup>-/-</sup> platelets. This suggests a role for PKC $\alpha$  in biogenesis of dense granules and is therefore an area that requires further analy-



sis. A number of different elements of the secretory machinery, including SNAP23, SNAP25, Munc18a and Munc18b, syntaxin 2, and Rab6, have been reported to be PKC substrates (40, 50–52), and therefore their phosphorylation may influence secretion. In this study then, phosphorylation of SNAP23 serves as a potential marker for these events and may contribute functionally to the process of secretion. However, although SNAP23 phosphorylation on Ser95 is markedly reduced in *Prkca*<sup>-/-</sup> platelets and this may play a role in regulating  $\alpha$ -granule secretion, its role in dense-granule secretion cannot be clarified from this study.

In summary, we have shown PKC $\alpha$  to play major roles in regulating platelet secretion of  $\alpha$ -granules, biogenesis and secretion of dense granules, regulation of integrin  $\alpha_{IIb}\beta_3$ , and thrombus formation in vitro and in vivo. PKC $\alpha$  also regulates platelet aggregation, although the defect in *Prkca*<sup>-/-</sup> platelets is relative, since high concentrations of agonists overcome the deficit and there is evident redundancy of the action of PKC $\alpha$  with the other expressed classical PKC isoform in platelets, PKC $\beta$ . PKC $\alpha$  plays no significant role in regulating adhesion of platelets to collagen-coated surfaces under static or flow conditions and does not regulate platelet spreading response or outside-in signaling through integrin  $\alpha_{IIb}\beta_3$ . It could be argued that, because PKC $\alpha$  is the most highly expressed of the PKC isoforms in platelets, its role in platelets may be wide ranging and effectively mask that of other PKC isoforms that are expressed at lower levels. The evidence presented here, however, suggests highly specific roles for PKC $\alpha$ , since, for instance, its absence has no significant effect upon platelet adhesion and spreading on collagen but markedly suppresses thrombosis. Additionally, if PKC $\alpha$  were so predominant functionally in platelets, knockout of the other PKC isoforms would be predicted not to have functional effects. This is clearly not the case, since we have shown absence of PKC $\delta$  to enhance platelet responses to collagen through enhanced filopodia formation (15) and we have more recently shown absence of PKC $\theta$  to enhance  $\alpha$ -granule secretion and integrin  $\alpha_{IIb}\beta_3$  activation in response to GPVI agonists (53). Shattil's group has also shown absence of PKCs  $\beta$  and  $\theta$  to ablate outside-in signaling through integrin  $\alpha_{IIb}\beta_3$  (37, 38). These data therefore demonstrate specific functional roles for the different PKC isoforms, including for PKC $\alpha$  in regulating secretion in particular, such that redundancy of activity is not apparent for specific functions.

Genome-wide association analyses of major human diseases have been conducted recently, based upon technical advances in high throughput microarray analyses of SNPs. These studies are introducing major new leads in genes that may be related to disease, and although PKC $\alpha$  is not in the top ranking of genes associated with coronary artery disease, a cluster of SNPs in this gene with a maximal *P* value of just over  $10^{-3}$  (SNP rs12600582, intronic, minor allele frequency of 0.233 in Europeans) (34, 35) may indicate some significance in this disease of polygenic cause. For these reasons, PKC $\alpha$  may represent a drug target for antithrombotic therapy, with inhibitors exerting an effect upon thrombus formation but sparing primary platelet adhesive functions. PKC $\alpha$  is already a target for the drug aprinocarsen, an antisense oligonucleotide therapy used in the treatment of specific neoplastic conditions (54, 55), and it will now be important to assess whether this or other small molecule-based approaches may represent opportunities in the development of platelet-based antithrombotic drugs in the management of coronary artery disease and other arterial thrombotic diseases.

## Methods

**Materials.** CRP was from Richard Farndale (University of Cambridge, Cambridge, United Kingdom). Thrombin, BSA, ADP, protein G-Sepharose, and fibrinogen were purchased from Sigma-Aldrich. Complete Mini Protease Inhibitor Tablets were from Roche Applied Science. Secondary HRP-conjugated anti-rabbit antibody was obtained from Santa Cruz Biotechnology Inc. SNAP23 (P-T95) antibody was a generous gift from Paul Roche (NIH). JON/A (anti- $\alpha_{IIb}\beta_3$ ) and WugE9 (anti-P selectin) antibodies were from Emfret Analytics. PE-conjugated anti-CD41 antibody and its isotype-matched control, PE-conjugated rat IgG1, were from AbD Serotec. Isoform-specific anti-PKC mouse monoclonal antibodies were from BD Biosciences. LY333531 was supplied by A.G. Scientific Inc. (Fluorochem Ltd.), U46619 was from ALEXIS Biochemicals, Gö6976 was from Calbiochem, and CHRONO-LUME reagent came from Chrono-log (Labmedics). The P2Y<sub>12</sub> antagonist AR-C69931MX was a gift from AstraZeneca. The P2Y<sub>1</sub> antagonist MRS2500 was from Tocris Bioscience.

**Platelet preparation.** Mice were bred and maintained in the University of Bristol animal facility in accordance with United Kingdom Home Office regulations. All procedures were undertaken with United Kingdom Home Office approval in accordance with the Animals (Scientific Procedures) Act of 1986 (project license numbers: 40/2212, 40/2749 and 30/2386). *Prkca*<sup>-/-</sup> mice were generated as previously described (32). Blood was drawn by cardiac puncture under terminal anesthesia into acid citrate dextrose (20 mM citric acid, 110 mM sodium citrate, 5 mM glucose), 1:7 ratio, v/v. Platelets were prepared as previously described (15). In brief, blood was diluted with 250  $\mu$ l of modified Tyrode's-HEPES buffer (134 mM NaCl, 0.34 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.9 mM KCl, 12 mM NaHCO<sub>3</sub>, 20 mM HEPES, 5 mM glucose, and 1 mM MgCl<sub>2</sub>, pH 7.3) and centrifuged at 180 *g* for 6 minutes at room temperature. Platelet-rich plasma was removed, and platelets were isolated by centrifugation at 550 *g* for 6 minutes in the presence of PGE<sub>1</sub> (140 nM) and indomethacin (10  $\mu$ M). Pelleted platelets were resuspended to the required density in modified Tyrode's-HEPES buffer and rested for 30 minutes at 37°C in the presence of 10  $\mu$ M indomethacin prior to stimulation.

**Platelet aggregation.** Platelets were resuspended in Tyrode's-HEPES buffer at a final concentration of  $2 \times 10^8$  per ml. Platelets were preincubated with different inhibitors or vehicle solution (0.1% Me<sub>2</sub>SO final concentration) for 10 minutes at 37°C, and aggregation of agonist-stimulated platelets was monitored in an optical aggregometer (Chrono-log; Labmedics) at 37°C, with continuous stirring at 800 rpm.

**Flow cytometry.** Two-color analysis of mouse platelet activation was conducted using PE-conjugated JON/A, an antibody that preferentially binds to the active form of  $\alpha_{IIb}\beta_3$  integrin, and with a FITC-conjugated antibody specific for CD62P (P selectin). 25  $\mu$ l of washed platelets ( $4 \times 10^7$ /ml in Tyrode's-HEPES buffer supplemented with 1 mM CaCl<sub>2</sub> and 0.35% BSA) was mixed with 10  $\mu$ l of antibody and subsequently stimulated either with 5  $\mu$ g/ml CRP or 1 U/ml thrombin for 15 minutes at room temperature. The reaction was stopped by addition of 400  $\mu$ l ice-cold PBS, and samples were analyzed within 30 minutes. For estimation of surface expression levels of total  $\alpha_{IIb}\beta_3$  integrin, CD41 was stained with PE-conjugated anti-CD41. Flow cytometry was performed on a FACSCalibur flow cytometer (BD Biosciences), using CellQuest version 3.1f software (BD Biosciences), and a total of 20,000 events per sample were collected.

**Measurement of ATP secretion.** ATP secretion was measured using CHRONO-LUME reagent according to the manufacturer's protocol. 5  $\mu$ l luciferase-luciferin was added directly to the platelets, which were being continually stirred (1000 rpm), and 5  $\mu$ g/ml CRP or 1 U/ml thrombin was added to activate platelets for 1 minute. The luminescence intensity was measured at a setting of  $\times 0.05$ .

**Platelet spreading and adhesion assay.** Coverslips were coated with either 0.1 mg/ml fibrinogen or 50  $\mu$ g/ml CRP and left overnight, followed by





nonspecific blocking step with 2% BSA for 1 hour. Platelets were resuspended to  $2 \times 10^7$ /ml in modified Tyrode's-HEPES buffer, and 500  $\mu$ l was dispensed onto the coverslip mounted in a live-cell microscopy chamber. Adhesion and spreading of platelets was observed by differential interference contrast (DIC) microscopy, with a wide-field DM IRB microscope attached to an ORCA ER camera (63 $\times$ /1.40 NA oil objective) (Leica). Five images in different random parts of the coverslip area were taken at 15, 30, and 45 minutes and processed with OpenLab 4.03 (Improvision) and Adobe Photoshop software. The surface area and number of adherent platelets was estimated using ImageJ software (<http://rsbweb.nih.gov/ij/>).

**Immunoblotting.** For stimulated samples,  $2 \times 10^8$ /ml of washed platelets were activated with 5  $\mu$ g/ml CRP or 1 U/ml thrombin in the presence of 2 mM CaCl<sub>2</sub> for 3 minutes and solubilized in Laemmli sample buffer. Proteins were resolved by electrophoresis in 9.5% (for PKC isoform estimation) or 16% (for SNAP23 Ser95 phosphorylation estimation) SDS-PAGE. Samples were then transferred to PVDF membranes, blocked with 10% BSA, and subjected to immunoblotting with anti-PKC $\alpha$ , - $\beta$ , - $\theta$  or - $\delta$  antibodies, SNAP antibody, or phospho-SNAP23-Ser95 antibody.

**Thrombus formation under flow in vitro.** Flow-induced thrombus formation was assessed basically as described before (56). Heparin/D-phenylalanyl-prolyl-arginyl chloromethyl ketone-anticoagulated (Heparin/PPACK-anticoagulated) mouse blood was passed over immobilized collagen through a parallel plate perfusion chamber at a shear rate of 1000 s<sup>-1</sup> for 4 minutes. Where indicated, ADP (200  $\mu$ M in saline) was coinfused at a 10% rate immediately before the blood reached the flow chamber. For each perfusion surface, phase-contrast images from 10 random microscopic fields were collected. Surface coverage was analyzed using Image-Pro software, version 4.1 (Media Cybernetics). The average percentage area covered by adherent platelets was measured by automated setting of masks for the ranges of gray levels corresponding to the presence of platelets and thrombi. For each image, the (blinded) observer needed to approve the mask settings. Averaged pixel fractions of the masks from 10 images were considered as a best estimate of the surface area coverage with thrombi.

Aggregate volume was also estimated by confocal laser scanning microscopy, using a Bio-Rad 2100 multiphoton system (Bio-Rad Laboratories), as described before (57). Platelet aggregates that formed on collagen (4 minutes, shear rate 1000 s<sup>-1</sup>) were postlabeled with FITC-anti-mouse CD62 mAb (1:100). Confocal stacks were recorded for measurement of the volume of individual platelet aggregates. Analysis of confocal images (gray level bit maps) and 3D reconstruction of images were with LaserPix software (Media Cybernetics).

**Detection of occult fecal blood.** The presence of occult fecal blood was detected by Hemdetect (DIPRO, supplied by Autogen Bioclear) on freshly obtained stool samples.

**Electron microscopy.** Platelet-rich plasma was collected and spun at 590 g for 5 minutes. Supernatant was removed from the platelet pellet, and the pellet was fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) (pH 7.4). The pellet was washed in PB and then incubated in 1% osmium tetroxide in PB for 30 minutes. After washing in PB and deionized water, the pellet was incubated in 3% uranyl acetate in deionized water for 30 minutes. After washing with deionized water, the pellet was dehydrated in a graded series of increasing amounts of ethanol (70%, 80%, 90%, 96%, 100%, and 100%, with each step lasting for 10 minutes). After removal of the 100% ethanol, the pellet was incubated with pure Epon for 2 hours at room temperature. Thereafter, the Epon was replaced with fresh Epon, and this was hardened overnight in a 60°C oven. Ultrathin counterstained sections were imaged on a Philips CM100 equipped with a side-mount MegaView III camera (Olympus Soft Imaging Solutions).

To determine the dense-granule and  $\alpha$ -granule content, total numbers of granules in equivalent-sized fields of view were counted. For each genotype,

25–30 randomly chosen fields of view were examined. All microscopic images were taken at the same magnification ( $\times 19,000$ ), and the number of cells per field of view between WT and *Prkca*<sup>-/-</sup> preparations were equivalent.

**Analysis of bleeding time.** Experiments were conducted on 25–35 g male and female mice. Mice were anesthetized (75 mg/kg ketamine and 1 mg/kg medetomidine intraperitoneally), and a transverse incision was made with a scalpel at a position where the diameter of the tail was 2.25 to 2.5 mm. The tail was immersed in normal saline (37°C) in a hand-held test tube, and the time from incision to cessation of bleeding was recorded.

**Intravital microscopy of thrombus formation in vivo.** Intravital microscopy was performed essentially as described previously (44). Experiments were conducted on 25–30 g male *Prkca*<sup>-/-</sup> mice and their littermate-matched control WT mice. Anesthesia was induced by intraperitoneal ketamine (100 mg/kg Vetalar; Pharmacia & Upjohn Ltd.) and 2% xylazine (20 mg/kg; Millpledge Pharmaceuticals). The left cremaster muscle was exteriorized and spread flat over an optically clear coverslip and continuously superfused. High-speed intravital microscopy experiments were performed as previously described by Falati et al. (43).

Platelets were labeled fluorescently with Alexa Fluor 488-conjugated goat anti-rat antibody (Molecular Probes) and rat anti-murine CD41 antibody (BD Biosciences – Pharmingen) infused via a carotid cannula. Thrombi were induced in arterioles with a diameter of 25–35  $\mu$ m by a nitrogen ablation laser (MicroPoint; Photonic Instruments), which was introduced through the microscope objective. Bright field and fluorescent images were captured simultaneously for 4–5 minutes, and multiple thrombi were generated with a distance of at least 200  $\mu$ m between them and upstream of previous injuries. The integrated intensity value for the growing thrombus was plotted against time and peak size of thrombus, and time taken to reach peak was determined.

**Statistics.** Statistical analyses were carried out on raw data using unpaired, 2-tailed Student's *t* test, and *P* < 0.05 was considered statistically significant. Values are expressed as mean  $\pm$  SEM. For all data, *n* indicates number of mice tested.

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