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PKCθ is required for hemostasis and positive regulation of thrombin-induced platelet aggregation and α-granule secretion

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ABSTRACT

Platelet activation due to vascular injury is essential for hemostatic plug formation, and is mediated by agonists, such as thrombin, which trigger distinct receptor-coupled signaling pathways. Thrombin is a coagulation protease, which activates G protein-coupled protease-activated receptors (PARs) on the surface of platelets. We found that C57BL/6J and BALB/C mice that are deficient in protein kinase C θ (PKCθ), exhibit an impaired hemostasis, and prolonged bleeding following vascular injury. In addition, murine platelets deficient in PKCθ displayed an impaired thrombin-induced platelet activation and aggregation response. Lack of PKCθ also resulted in impaired α-granule secretion, as demonstrated by the low surface expression of CD62P, in thrombin-stimulated platelets. Since PAR4 is the only mouse PAR receptor that delivers thrombin-induced activation signals in platelets, our results suggest that PKCθ is a critical effector molecule in the PAR4-linked signaling pathways and in the regulation of normal hemostasis in mice.
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Introduction

Platelets play a fundamental role in the physiological response to vascular injury that control blood loss by the mechanisms of hemostasis. In addition to their contribution to hemostatic plug formation, they also secrete soluble factors that stimulate healing of damaged tissue. Their activation is mediated by different agonists, which are initially generated by the injured tissues, and later by the platelets themselves. These initial steps are characterized by a series of morphological and functional changes that promote platelet spreading and formation of pseudopods, resulting in adherence to the endothelium of the injured blood vessels, while concomitant expression of specific surface receptors promotes platelet aggregation. At this stage, platelets discharge the content of their granules, which further promote platelet activation and clot formation.

A major regulator of the activation cascade in platelets, is the thrombin serine protease (factor IIa), which triggers platelets by activating their surface protease-activated receptors (PARs) [1]. The thrombin cleaves the N-terminal extracellular domain of the PAR molecule and promotes the secretion of platelet granules, releasing their content into the surroundings. The PAR family includes four known isoforms of heterotrimeric G protein-coupled receptors [2], among which the PAR1 and PAR4 are expressed on human platelets [3], whereas mouse platelets express the PAR3 and PAR4 [4]. However, thrombin binding to murine PAR3 is insufficient for platelet activation, and studies have demonstrated that murine PAR3 serves as a cofactor for thrombin-induced activation of PAR4 [2,5]. Thrombin-mediated activation of PAR initiates a signaling cascade leading to activation of effector molecules, including the phospholipase C (PLC), which generates inositol 1,4,5-trisphosphtae (IP_3) and 1,2-diacylglycerol (DAG). The DAG directly activates most of the protein kinase C (PKC) isoforms, while IP_3 mediates Ca^{2+} release from intracellular stores, thereby supplying Ca^{2+} cofactors for the activation of the conventional PKC (cPKC) isoforms [6].

PKC enzymes play essential roles in platelet activation, and although distinct receptors are coupled to specific signaling pathways, different pathways may converge on the activation of PKC that regulate critical steps in platelet activation. The PKC family includes 10 structurally related isoforms, five of which, including PKCα, β, δ ζ, and θ, were found to be expressed in platelets at relatively high levels ([7] and Fig. 1).

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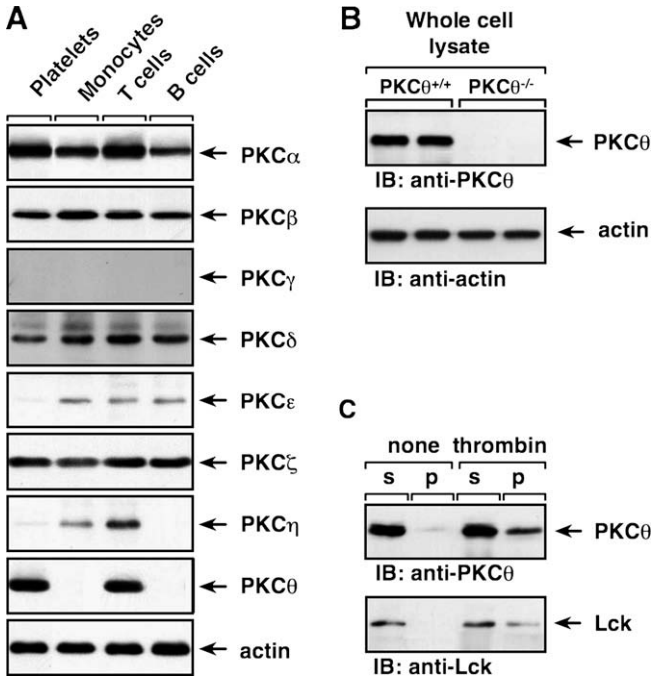

Fig. 1. PKCθ is expressed in platelets and respond to thrombin stimulation by translocation to the membrane. (A) Analysis of expression of distinct PKC isoforms in human peripheral blood platelets, monocytes, and B and T lymphocytes. The cells were isolated from human peripheral blood, and lysates containing 20 μg of proteins were loaded per lane. Proteins were resolved by SDS–PAGE and immunoblotted with the indicated Abs. Arrows indicate the position of the respective PKC protein band. (B) Analysis of PKCθ expression in platelets from wild type and $PKC\theta^{-/-}$ mice. Duplicate samples of platelet lysates were resolved by SDS–PAGE and immunoblotted with the indicated Abs. (C) Analysis of the effect of thrombin stimulation on the subcellular location of PKCθ in platelets. Platelets from C57BL/6J mice were stimulated with thrombin (0.1 U/ml) for 1 min at RT. Lysates were subsequently centrifuged and the soluble (s) versus particulate (p) fractions were collected and analyzed by SDS–PAGE and sequential immunoblotting with the indicated Abs.

Our studies demonstrated that the PKCθ isoform exhibits a relatively selective expression pattern in hematopoietic cells. For example, PKCθ is highly expressed in T lymphocytes [7], where it participates in TCR-coupled signaling pathways [8], while its expression in B lymphocytes is undetectable [7]. We also found that PKCθ is highly expressed in platelets [7], and since platelets express at least four additional PKC isoforms, we aimed at analyzing the role of PKCθ in platelet functions, in general, and in thrombin-mediated PAR-induced signaling cascades, in particular.

Analysis of PKCθ-deficient mice revealed that PKCθ is required for normal hemostasis; lack of PKCθ resulted in impaired blood coagulation and prolonged bleeding. More specifically, PKCθ was found to be essential for the regulation of thrombin-induced PAR4-coupled signaling pathways and induction of platelet activation.

Materials and methods

Reagents. Human thrombin, β-mercaptoethanol, aprotinin, leupeptin, prostaglandin E1 (PGE_1) and Triton X-100 were from Sigma Co. AEBSF was from ICN Biomedicals Inc. (Aurora, OH). Nitrocellulose membranes were from Schleicher & Schuell Inc. (Keene, NH). ECL was from Amersham Pharmacia Biotech Inc. (Uppsala, Sweden). Bradford protein assay reagent was from Bio-Rad Laboratories (Hercules, CA), and the MiniMACS magnetic separation system was from Miltenyi Biotec Gmbh (Bergisch Gladbach, Germany).

Antibodies. Abs directed against PKCα, β, γ, δ, ϵ, ζ, η or θ, and anti-Lck Abs were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-actin mAb (clone C4) was from ICN Biochemicals Ltd. (Bucks, UK). Anti-phosphotyrosine (pY) (4G10) mAb was from Upstate Biotechnology Inc. (Lake Placid, NY). Horseradish peroxidase (HRP)-conjugated sheep anti-mouse, and HRP-conjugated donkey anti-rabbit Ig Abs were from Amersham Pharmacia Biotech Inc. PE-conjugated rat anti-mouse CD41 mAb was from eBioscience (San Diego, CA), and Alexa fluor 647-conjugated mouse anti-human CD62P mAb was from Serotec (Oxford, UK).

Mice. C57BL/6 J and BALB/C mice were from Harlan Laboratories, Rehovot. PKCθ-deficient ($PKC\theta^{-/-}$) mice were a gift from Dr. D. Littman (New York University, School of Medicine, New York, NY) [9]. The mice were originally on a 129- C57BL/6J mixed background, and were backcrossed onto the C57BL/6J or BALB/C background for more than ten generations. Wild type (wt; $PKC\theta^{+/+}$), heterozygous ($PKC\theta^{+/-}$), and PKCθ-deficient mice ($PKC\theta^{-/-}$) littermates were obtained by breeding of $PKC\theta^{+/-}$ heterozygote mice in each strain. All studies conform to the principles outlined by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research.

Preparation of enriched populations of cells from human peripheral blood. Human peripheral blood from healthy volunteers was fractionated by Ficoll–Hypaque gradient centrifugation, and polymorphonuclear cells were separated by dextran sedimentation and hypotonic lysis of erythrocytes. Monocytes and lymphocytes were obtained by successive separation of the mononuclear cell fraction on Percoll gradient. $CD3^+$ T cells and $CD19^+$ B cells were isolated using the MiniMACS magnetic separation system and the extent of purity of T and B lymphocyte was verified by FACS analysis and found to be at the range of 95–98%. Blood platelets were obtained by centrifugation of peripheral blood for 10-min at 120*g*, collection of the platelet-rich plasma, and precipitation of the platelets by centrifugation for 10-min at 1500*g*. All cells were resuspended in buffer A, and protein samples were prepared for SDS–PAGE analyses.

Isolation of mouse platelets. Mice were anesthetized by inhalation of isoflurane. Half ml of blood was collected from the inferior vena cava of anesthetized mice using a 1 ml syringes (with a 23 gauge needle), and transferred to polypropylene Eppendorf tube containing 50 μl of 3.8% acid citrate dextrose (ACD), as anticoagulant. Where required, red blood cells were removed by centrifugation at 200*g* for 15 min. Platelet-rich plasma (PRP) was diluted with an equal volume of EHS buffer (EDTA-HEPES saline)-containing 150 mM NaCl, 10 mM HEPES, pH 7.6, and 1 mM EDTA, that was supplemented with PGE_1 (1 μg/ml). PRP was recovered by centrifugation at 1000*g* for 10 min. Cell density was adjusted by adding platelet poor plasma (PPP) or modified Tyrode's solution-buffer (137 mM NaCl, 11.9 mM $NaHCO_3$, 0.4 mM Na_2HPO_4, 2.7 mM KCl, 1.1 mM $MgCl_2$, and 5.6 mM glucose, pH 7.4).

Stimulation of murine platelets. Washed platelets were obtained as described above. The pellet was resuspended in Tyrode's-HEPES buffer, pH 7.4 (10 mM HEPES-containing Tyrode's buffer) and concentration was adjusted to 2×10^9 platelets/ml. Samples were incubated with human thrombin (0.1 U/ml) at room temperature for 1 min or the indicated time interval. Reactions were terminated by the addition of 5× SDS sample buffer or 200 μl of ice-cold lysis buffer to 50 μl of PRP.

Aggregation assay. Platelets aggregation in whole blood samples diluted in lactated ringer's solution was initiated by the addition of thrombin (0.1 U/ml) and aggregation recording for 8 min using a Chrono-log Whole Blood Platelet Lumi-Aggregometer (Chrono-Log, Havertown, PA).

Subcellular fractionation of platelets. Platelet stimulation with thrombin was terminated by the addition of 1 ml ice-cold Triton X-100 lysis buffer (2% Triton X-100, 10 mM EGTA, 2 mM phenyl-

methylsulfonyl fluoride, 20 μg/ml leupeptin, 20 μg/ml benzamidine, and 100 mM Tris–HCl, pH 7.4), and samples were allowed to stand on ice for 30 min. The platelet lysates were subsequently centrifuged at 100,000*g* for 2 h at 4 °C and the soluble membrane fractions were collected, mixed with 2× SDS sample buffer and boiled for 5 min. The pellets (Triton X-100 insoluble fractions) were solubilized in 2× SDS sample buffer, boiled for 5 min, and both fractions were analyzed by SDS–PAGE and immunoblotting, as described.

Electrophoresis and immunoblotting. Cell lysates were resolved by electrophoresis on 10% acrylamide gels as described [7], and immune reactive proteins were visualized using an ECL reagent and autoradiography.

Flow cytometry. Whole blood was diluted 1:1000 in Tyrode's buffer, and platelets in 50 μl of diluted blood were stained with 2 μl Alexa fluor 647-labelled anti-CD62P mAb and 2 μl phycoerythrin (PE)-anti-CD41 mAb, for 15 min at room temperature, and incubated with or without 0.1 U/ml of thrombin for additional 1 min. Reaction was stopped by addition of 1 ml of Tyrode's buffer. Cell staining was analyzed on a BD FACSCanto II flow cytometer (BD Biosciences, Mountain View, CA) equipped with DIVA 6.1.1 Software. Platelets were initially gated according to their typical light scatter characteristics followed by electronic gating of $CD41^+$ events (platelets). Expression of CD62P was analyzed on gated platelets from $PKC\theta^{+/+}$ and $PKC\theta^{-/-}$ C57BL/6J mice.

Bleeding time. Tail bleeding times were performed blind to genotype in 10–12 weeks old isoflurane anesthetized mice. Tails were amputated 5 mm from tail tips were saved for genotyping. The cut end was immediately immersed in saline at 37 °C in a 50 ml clear conical tube, and cessation of blood flow was recorded. Tubes were then centrifuged at 1500*g* for 10 min, cells were resuspended in 6 ml of lysis buffer (NH_4Cl 8.3 mg/ml, $KHCO_3$ 1.0 mg/ml, and EDTA 37 μg/ml), and hemoglobin and total protein content were determined by measuring sample absorbance at 575 and 280 nm, respectively.

Statistics. Statistical significance was analyzed by Student's *t*-test. Unless otherwise indicated, data represent the mean ± SD, with $p < 0.05$ considered statistically significant.

Results and discussion

Analysis of PKC isoform and PKCθ expression pattern in platelets

To test the expression of distinct PKC isoforms in platelets, and compare it with that of other hematopoietic cells, we used peripheral blood of healthy human volunteers and isolated platelets, monocytes, B lymphocytes and T lymphocytes. Proteins from the cell lysates were resolved by SDS–PAGE, electroblotted onto nitrocellulose membranes, and immunoblotted with Abs specific for different PKC isoforms. The results demonstrated that platelets express relatively high levels of PKCα, β, δ, ζ, and θ, and low or undetectable levels of PKCη, γ and ϵ (Fig. 1A). Some isoforms were found in all cell types tested, while PKCθ, which is expressed at high levels in platelets and T lymphocytes, was undetectable in monocytes and B lymphocytes. Sample immunoblotting with anti-actin Abs indicated the loading of a similar amount of proteins in each lane.

In order to test the requirement for PKCθ in hemostasis and platelet functions in mice, we obtained PKCθ-deficient ($PKC\theta^{-/-}$) mice from Dr. D. Littman (New York University, School of Medicine, New York, NY), which were originally on a 129- C57BL/6J mixed background [9], and backcrossed them onto the C57BL/6J and BALB/C background for more than ten generations. To verify the absence of PKCθ in the 'knock-out mice', we isolated platelets from wild type and $PKC\theta^{-/-}$ C57BL/6J mice and determined their PKCθ expression levels by Western blot. High expression levels of PKCθ were observed in platelets of wild type mice, while PKCθ was undetectable in platelets of $PKC\theta^{-/-}$ mice (Fig. 1B). Similar results were obtained when testing platelets from wild type versus $PKC\theta^{-/-}$ BALB/C mice (not shown). To test whether $PKC\theta^{-/-}$ is involved in platelet responses to thrombin, we utilized platelets from $PKC\theta^{+/+}$ C57BL/6J mice and compared the subcellular distribution of PKCθ before and after thrombin stimulation. The results indicated that the majority of the PKCθ proteins reside in the soluble fraction of resting platelets (Fig. 1C). In contrast, thrombin stimulation resulted in translocation of a fraction of the PKCθ proteins into the particulate fraction. Similar results were obtained when analyzing the subcellular distribution of the Lck protein tyrosine kinase (PTK), an essential signaling molecule in platelets, and a regulator of PKCθ [10].

Absence of PKCθ results in prolonged bleeding time from injured blood vessels

Platelets are an essential component of hemostasis, and several different PKC isoforms were found to regulate various platelet functions. To test the involvement of PKCθ in hemostasis, we compared bleeding times of wild type versus $PKC\theta^{-/-}$ mice following tail tip amputation.

The mice were anesthetized with isoflurane and their tail tips were amputated with a scalpel blade. The cut end was immersed in saline at 37°C in a conical tube, and bleeding time was inspected visually. In addition, blood loss during the bleeding time assay was quantified by measuring the absorbance of hemoglobin (A_{575}) and total proteins (A_{280}) of lysates of cells accumulated in the saline in which the tails were placed.

The results demonstrated that the bleeding period of $PKC\theta^{-/-}$ C57BL/6J mice (229.4 ± 14.4 s) was significantly longer than in $PKC\theta^{+/+}$ C57BL/6J mice (60.2 ± 2.6 s) ($P < 0.0004$, *t*-test) (Fig. 2A). A similar difference in bleeding time was also observed in BALB/C mice, where cessation of bleeding in $PKC\theta^{-/-}$ mice (78.7 ± 7.7 s) was significantly longer than in $PKC\theta^{+/+}$ mice (34.6 ± 4.2 s) ($P < 0.05$, *t*-test) (Fig. 2D).

The quantity of blood lost during the bleeding-time assays roughly paralleled the bleeding times, as demonstrated by measurement of the amount of hemoglobin (Fig. 2B and E) or total protein content (Fig. 2C and F) in the blood cells that accumulated in the saline in which the tails were placed. Bleeding time of heterozygote mice demonstrated an intermediate phenotype with greater variability between individuals. The results indicate that PKCθ plays an important role in platelet activation responses leading to blood coagulation.

Lack of PKCθ impairs thrombin-induced platelet aggregation

To analyze the potential involvement of PKCθ in thrombin-induced platelet aggregation, we used whole blood from $PKC\theta^{+/+}$ and $PKC\theta^{-/-}$ C57BL/6J mice. Blood samples were diluted in Ringer's solution, stimulated with thrombin, and aggregation was recorded using a Chrono-Log Whole Blood Aggregometer. Thrombin was found to induce a rapid platelet aggregation response in whole blood samples of $PKC\theta^{+/+}$ mice. In contrast, the rate of aggregation of platelets from $PKC\theta^{-/-}$ mice was markedly reduced and reached only 25% of that observed in platelets from $PKC\theta^{+/+}$ mice at 5 min post-stimulation (Fig. 3, left panel). Similar results were observed when the blood samples were inspected microscopically, where platelets from $PKC\theta^{-/-}$ mice produced fewer and smaller aggregates (Fig. 3, right panel).

PKCθ is required for thrombin-induced expression of CD62P

CD62P (P-selectin, GMP-140, PADGEM) is a constitutively expressed platelet α-granule membrane protein [11] that is rapidly

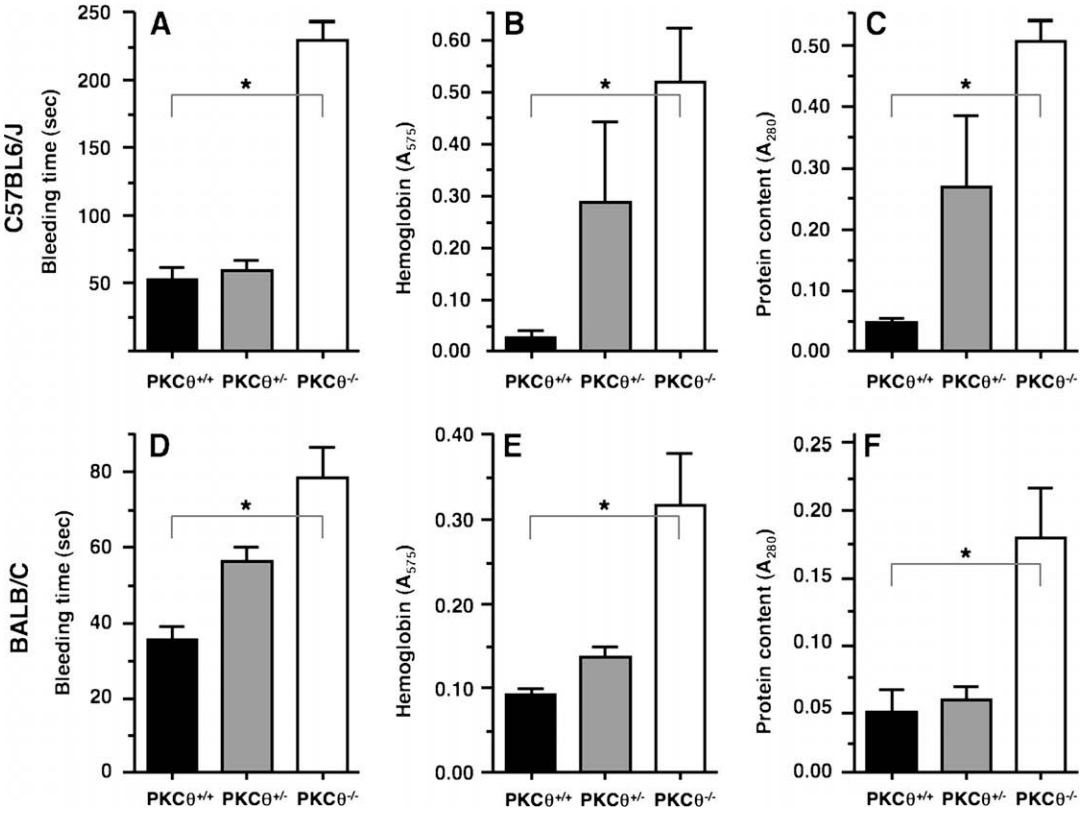

Fig. 2. Lack of PKCθ results in prolonged bleeding time. Bleeding time was determined in 10–12 weeks old PKCθ$^{+/+}$, PKCθ$^{+/-}$ and PKCθ$^{-/-}$ C57BL6/J and BALB/C male mice. The mice were anaesthetized with isoflurane and their tails were amputated 5 mm from the tip such that tail artery and veins were fully transected. The cut end was immediately immersed in a saline solution at 37°C in a 50 ml clear conical tube. (A,D) Bleeding time was inspected visually and measured using a stopwatch. Quantitation of bleeding was performed by measuring the amount of hemoglobin (B,E) or total proteins (C,F) in the blood sample collected in saline. Each column represents the mean ± SD of 12 mice. ($^{*}p < 0.05$; unpaired student t test).

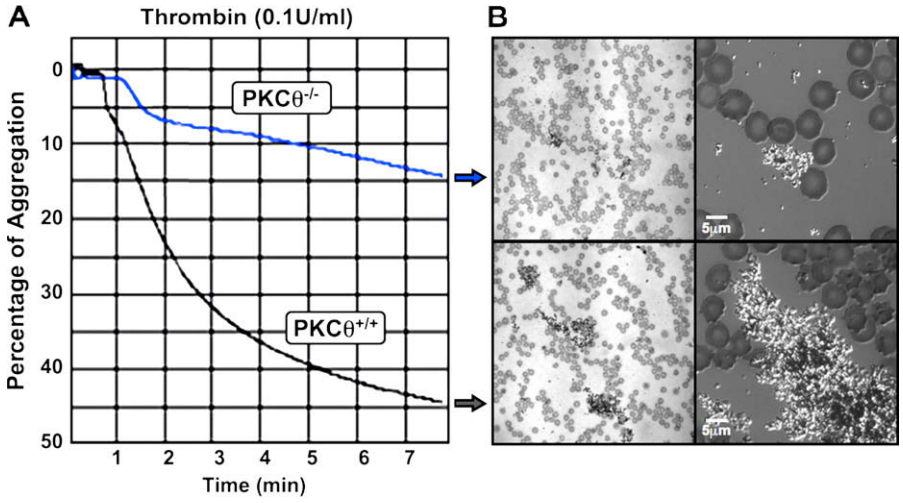

Fig. 3. Thrombin-induced platelet aggregation requires PKCθ. (A) Whole blood was collected from the inferior vena cava of anesthetized PKCθ$^{+/+}$ and PKCθ$^{-/-}$ C57BL6/J mice (450 μl per mouse) and transferred to polypropylene Eppendorf tubes containing 50 μl of 3.8% acid citrate dextrose (ACD) as anticoagulant. Whole blood samples were diluted in lactated Ringer's solution (1:10) and thrombin (0.1 U/ml)-induced aggregation was recorded for 8 min using a Chrono-Log Whole Blood Aggregometer. (B) Whole blood samples were stimulated with 0.1 U/ml thrombin for 1 min. A thick blood film was made, air-dried, stained with Giemsa and visualized using Differential Interference Contrast (DIC) microscopy (Nomarski). Four fields of view were selected at random and photographed, and one representative field is shown at lower (600×) and higher (3000×) magnification for blood samples from PKCθ$^{+/+}$ (lower) and PKCθ$^{-/-}$ (upper) mice.

redistributed to the plasma membrane following agonist stimulation [11]. The plasma membrane CD62P can interact with the leukocyte counter receptor P-selectin glycoprotein ligand-1 (PSGL-1) and promote platelet–leukocyte adhesion, and leukocyte activation, adhesion to endothelial cells, and transmigration to sites of inflammation [12].

The expression of CD62P on the surface of platelets is in direct correlation with platelet degranulation and secretion, and there-

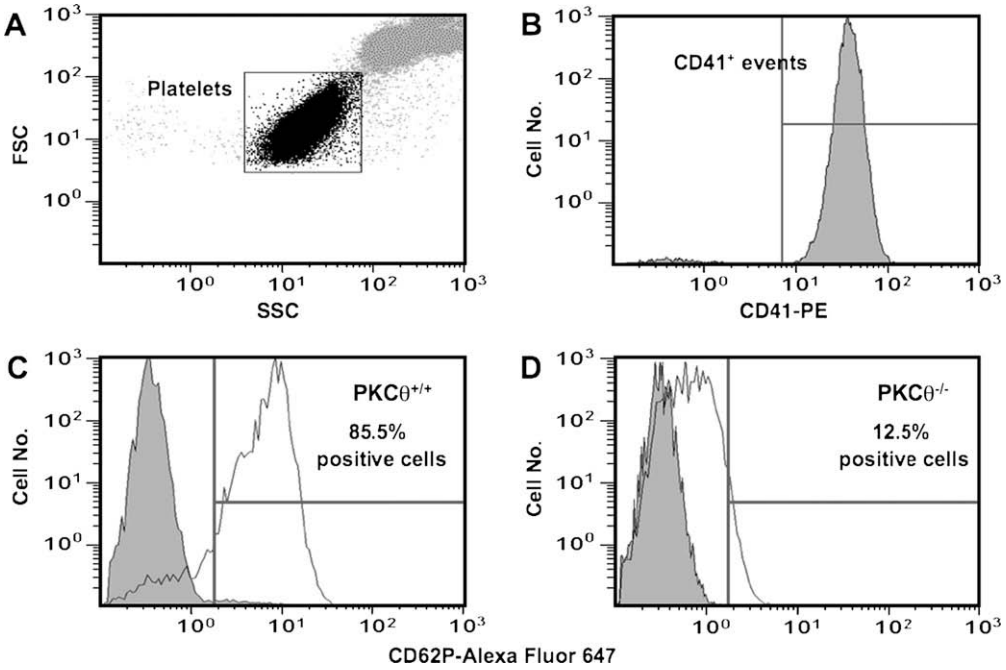

Fig. 4. Thrombin-induced P-selectin (CD62P) expression in platelets from wild type and PKCθ$^{-/-}$ mice. Whole blood was obtained from the inferior vena cava of anesthetized wild type and PKCθ$^{-/-}$ C57BL6/J mice. Platelets were stimulated with thrombin (0.1 U/ml) and stained with Alexa fluor 647-labelled anti-CD62P mAb and phycoerythrin (PE) anti-CD41 mAb, and analyzed by flow cytometry. Platelets were gated according to their typical SC and FSC characteristics (A) and CD41 expression analysis on the gated events indicated that <95% of them were CD41-positive (B). Expression of CD62P was then analyzed in the CD41^{+} gated platelets from PKCθ$^{+/+}$ (C) versus PKCθ$^{-/-}$ (D) mice. Grey and white histograms demonstrate the CD62P expression pattern in non-stimulated and thrombin-treated platelets, respectively.

fore serves as an indicator for the status of platelet activation [13]. To test whether PKCθ is involved in the regulation of thrombin-induced platelet degranulation, we utilized whole blood from PKCθ$^{+/+}$ and PKCθ$^{-/-}$ C57BL/6J mice. The blood samples were diluted in Tyrode's buffer, and the effect of thrombin on expression of CD62P in platelets from PKCθ$^{+/+}$ and PKCθ$^{-/-}$ C57BL/6J mice was studied by flow cytometry. Platelets were first identified as a population of events with a low forward and side scatter (Fig. 4A). More than 95% of these events were positively stained for CD41 (GPIIb/IIIa complex) (Fig. 4B), a known platelet specific surface marker [14]. Analysis of the expression of CD62P in gated CD41^{+} platelets demonstrated that 85% platelets from PKCθ$^{+/+}$ mice responded to thrombin by membrane expression of CD62P (Fig. 4C), compared to only 12% of the platelets of PKCθ$^{-/-}$ mice (Fig. 4D).

Although mouse platelets express both PAR3 and PAR4, thrombin signals mouse platelets predominantly via PAR4, with PAR3 serving as a facilitating cofactor for thrombin cleavage and activation of PAR4 [5]. This model was further substantiated by studies of platelets from PAR4$^{-/-}$ mice, which did not respond to thrombin stimulation [2,15].

The present results indicated that PKCθ is a major positive regulator of the PAR-coupled signaling pathways controlling platelet activation and aggregation, and is necessary for optimal thrombin-induced secretion of α-granules. Furthermore, PKCθ is required for blood clot formation following vascular injury. Since thrombin affect mouse platelets via the activation of PAR4, our results suggest that PKCθ is an essential component in the signaling cascade that delivers outside-in signals via PAR4. The observations that other PKC isoforms, such as PKCα, are also required for the induction of platelet aggregation or α-granules secretion [16] could indicate that different PKC isoforms possess some non-overlapping functions that are essential for platelet responses. For example, two or more PKC isoforms may operate downstream to a single receptor and phosphorylate two or more distinct PKC isoform-specific substrates required for a certain activity. Alternatively, the requirement for different PKC isoforms for a certain platelet function may be dependent on a cross talk between two or more receptor-coupled signaling pathways that involve distinct PKC isoforms. Further dissection of the contribution of distinct PKC isoforms to specific platelet functions will require the identification of PKC isoform-specific substrates in platelets in order to understand how their phosphorylation affects their activity and contributes to a specific platelet function.

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