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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.

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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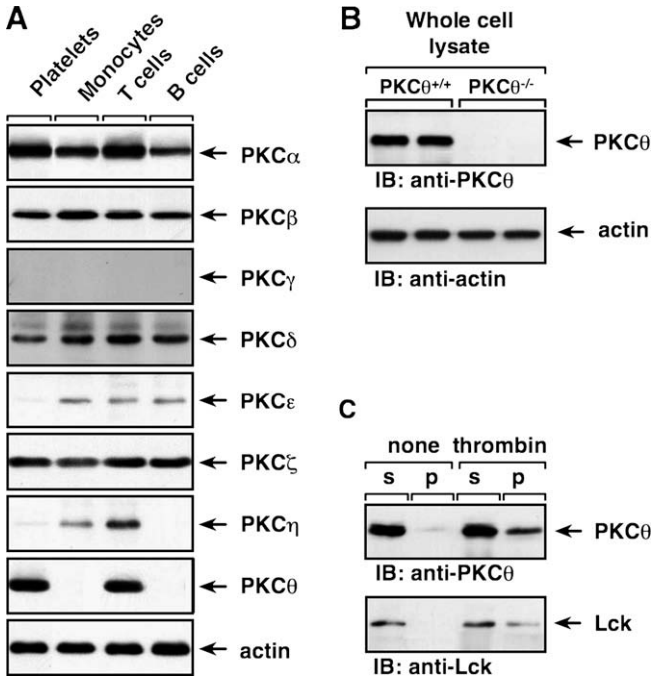

Fig. 1. PKCθ is expressed in platelets and respond to thrombin stimulation by translocation to the membrane. (A) Analysis of expression of distinct PKC isoforms in human peripheral blood platelets, monocytes, and B and T lymphocytes. The cells were isolated from human peripheral blood, and lysates containing 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-