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Protein kinase Cι/λ is dispensable for platelet function in thrombosis and hemostasis in mice

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ABSTRACT

Platelet activation at sites of vascular injury is crucial for hemostasis, but it may also cause myocardial infarction or ischemic stroke. Upon platelet activation, cytoskeletal reorganization is essential for platelet secretion and thrombus formation. Members of the protein kinase C family, which includes 12 isoforms, are involved in most platelet responses required for thrombus formation. The atypical protein kinase Cι/λ (PKCι/λ) has been implicated as an important mediator of cell polarity, carcinogenesis and immune cell responses. PKCι/λ is known to be associated with the small GTPase Cdc42, an important mediator of multiple platelet functions; however, its exact function in platelets is not known. To study the role of PKCι/λ, we generated platelet- and megakaryocyte-specific PKCι/λ knockout mice (*Prkci*$^{fl/fl,\ Pf4\text{-}Cre}$) and used them to investigate the function of PKCι/λ in platelet activation and aggregation *in vitro* and *in vivo*. Surprisingly, lack of PKCι/λ had no detectable effect on platelet spreading and function *in vitro* and *in vivo* under all tested conditions. These results indicate that PKCι/λ is dispensable for Cdc42-triggered processes and for thrombosis and hemostasis in mice.

1. Introduction

Blood vessel wall injury results in the exposure of the subendothelial extracellular matrix (ECM), which initiates stable platelet adhesion and aggregation [1,2]. These processes are crucial for normal hemostasis, but in diseased blood vessels they may lead to pathological thrombus formation and infarction of vital organs [3]. Platelet activation by multiple signaling pathways leads to their shape change, release of intracellularly stored granules, and spreading on immobilized ligands. Members of the serine/threonine protein kinase C (PKC) family are important mediators of these processes [4,5]. In particular, conventional and novel PKC isoforms were previously identified as important mediators of central platelet signaling pathways with distinct functions in platelet granule release (PKCα, ε, δ, θ) [4,6,7], thromboxane synthesis (PKCδ, θ, η) [8–10], spreading [4,6,11] and thrombus formation [4].

In contrast to conventional and novel PKCs, the role of the atypical PKCs (aPCKs), PKCι/λ and PKCζ, in platelet physiology has remained elusive. APKCs depend on phospholipids and are, among others, allosterically activated in a DAG- and Ca^{2+}-independent manner by interacting with the small Rho GTPases Cdc42 and Rac1 [12–14]. This interaction relieves the allosteric inhibition on aPKCs by interacting with Par6 [13,15]. Previously, Horikoshi et al. reported that aPKCs mediate cell polarity as part of the Par3/Par6 complex *via* their association with cell junctions and are necessary for apical membrane development [16], immune cell responses as well as cell migration *in vivo* in *C. elegans* [17]. PKCι/λ forms a complex with the small GTPase Rab2a and subsequently phosphorylates glyceraldehyde-3-phosphate dehydrogenase (GAPDH), thereby modulating microtubule dynamics in the early secretory pathway [18]. Moreover, PKCι/λ has been suggested to function downstream of phosphatidylinositol 3-kinase (PI(3)K) and PDPK1 and promoting cell survival in glioblastoma cells [19]. Likewise, PKCι/λ prevents amyloid beta protein-induced apoptosis in cultured neurons [20] and has been shown to mediate BCL-ABL-mediated drug resistance, rendering leukemia cells resistant for drug-induced apoptosis [21,22].

Lack of functional PKCι/λ results in embryonic lethality [23,24]. Deficiency of aPKCζ specifically in immune cells leads to a defective immune response and compromised NF-κB signaling concomitant with a reduced number of mature B cells as well as impaired T cell differentiation [25,26]. Studies with PKCι/λ-deficient T cells showed similar results [27].

The Rho GTPases RhoA, Cdc42 and Rac1, are essential for cytoskeletal rearrangements [28] and Cdc42 and Rac1 are crucial for platelet

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shape change, adhesion and spreading on the exposed components of the ECM, by inducing the formation of filopodia and lamellipodia [29–33]. Moreover, Cdc42 and RhoA act as a regulatory circuit in megakaryocytes (MKs), the platelet progenitors, by modulating cell polarization and transendothelial platelet biogenesis [34]. The MK and platelet-specific mechanoreceptor glycoprotein Ib (GPIb) triggers cell polarization *via* PI(3)K and Cdc42, while RhoA counteracts this process. Of note, loss of PKCι/λ mimicked the absence of Cdc42, demonstrating that PKCι/λ is downstream of this GTPase in GPIb-signaling [34]. Likewise, PKCι/λ has been identified as a downstream mediator of Cdc42-triggered processes in endothelial cells [14,35]. Thus, we aimed to assess whether PKCι/λ is a mediator of GTPase signaling pathways in platelets. Therefore, we generated platelet- and megakaryocyte specific $Prkci^{fl/fl,\ Pf4\text{-}Cre}$ mice (PKCι/λ KO) and found that PKCι/λ is dispensable for platelet function *in vitro* and *in vivo*.

2. Material and methods

2.1. Animals

Animal studies were approved by the district government of Lower Franconia (Bezirksregierung Unterfranken). $Prkci^{fl/fl}$ mice [24] were intercrossed with PF4-Cre [36] mice to generate platelet- and megakaryocyte-specific PKCι/λ-knockout mice. Male and female $Prkci^{fl/fl}$ mice (mixed SV129/C57BL/6J background) were analyzed between 8 and 14 weeks of age.

Mice were genotyped by PCR, with 5′ TTGTGAAAGCGACTGGATTG 3′ and 5′ AATTGTTCATGTTCAACACTGCT 3′ for the wild-type, and 5′ TTGTGAAAGCGACTGGATTG 3′ and 5′ CTTGGGTGGAGAGGCTATTC 3′ for the PKCι/λ knockout allele.

2.2. Chemicals and antibodies

Midazolam (Roche Pharma AG), dorbene (Pfizer), fentanyl (Janssen-Cilag GmbH) and their antagonists atipamezol (Pfizer), flumazenil and naloxone (both Delta Select GmbH) were used according to regulations of the local authorities. Human fibrinogen was from Sigma-Aldrich (Schnelldorf, Germany). Iron(III)chloride ($FeCl_3$) was from Roth (Karlsruhe, Germany). Polyclonal rabbit anti-actin antibody (Antibody ID: A2066) was from Sigma-Aldrich (Schnelldorf, Germany). Fibrillar type I collagen (Horm) was from Nycomed (Munich, Germany). Integrilin was from GlaxoSmithKline (Munich, Germany). U46619 was from Enzo Life Sciences GmbH (Lörrach, Germany), thrombin was from Roche (Mannheim, Germany), apyrase type III was from GE Healthcare (Chalfont St. Giles, UK), low-molecular-weight heparin was from ratiopharm GmbH (Ulm, Germany) and botrocetin from Loxo GmbH (Dossenheim, Germany). Polyclonal anti-vWF antibody (Antibody_ID: AB_2315604) was from Dako Cytomation (Hamburg, Germany). Monoclonal rabbit anti-PKCι/λ antibody (clone C83H11, antibody ID: #2998S, Lot: #2), monoclonal rabbit anti-PKCζ (clone: C24E6, antibody ID: #9368, Lot: #2) and anti-rabbit IgG-HRP (Antibody_ID: AB_2099233) were from Cell Signaling (Frankfurt, Germany). All monoclonal antiplatelet glycoprotein antibodies (host: rat) (antibody: p0p/6, antigen GPIX [37]; antibody: JON/A, antigen αIIbβ3 [38]; antibody: WUG1.9, antigen: P-selectin [39]), unconjugated or conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), DyLight 488 or HRP were obtained from EMFRET Analytics (Eibelstadt, Germany). Collagen-related peptide (CRP) was generated as previously described [40].

2.3. mRNA isolation, cDNA synthesis and reverse transcriptase PCR (RT-PCR)

Washed platelets were prepared as described previously [32]. Platelets from the same genotype were pooled and resuspended in TRIZOL. In parallel, 100 mg brain tissue was homogenized in TRIZOL. Chloroform was added to extract RNA and precipitated by incubation with isopropanol. mRNA was washed twice in 70% ethanol and resuspended in DEPC H_2O containing RNase inhibitor. cDNA synthesis from platelet and tissue mRNA was performed using super script reverse transcriptase III. RT-PCR primers were designed using Primer-Blast [41].

RT-PCR Primer for *Actb* (β-actin):
5′ TAGCTGCGTTTTACACCCT 3′
5′ TTTGGGGGATGTTTGCTCCA 3′
RT-PCR Primer for *Prkcz* (PKCζ):
5′ AGCAGGAGAGCCAACCTTCTA 3′
5′ TCTACTGGAGGCTCTTGGGA 3′.

2.4. Immunoblotting

Proteins of lysed platelets were separated by SDS-PAGE and blotted onto polyvinylidene difluoride membranes. After blocking, the membrane was incubated with antibody overnight at 4 °C. HRP-conjugated antibodies were incubated for 1 h at room temperature, and enhanced chemilumincescence was used for visualization.

2.5. Platelet life span

Mice were injected intravenously with 5 μg of an anti-GPIX IgG derivative coupled to DyLight 488. Each day for up to five days, 50 μL blood was withdrawn and the percentage of anti-GPIX IgG Dylight 488 labeled platelets was determined by flow cytometry [32].

2.6. Platelet preparation, aggregation and flow cytometry

Washed platelets were prepared as described previously [32]. For aggregometry, washed platelets (160 μL with 1.5×10^{8} platelets mL^{-1}) were analyzed in the presence (CRP) or absence (thrombin) of 70 μg mL^{-1} human fibrinogen. Light transmission was recorded on a four-channel aggregometer (Fibrintimer; APACT, Hamburg, Germany) for 10 min and expressed in arbitrary units, with buffer representing 100% light transmission. For flow cytometry, heparinized whole blood was diluted 1:20 in Tyrode's-HEPES buffer, incubated with saturating amounts of fluorophore-conjugated mAbs for 15 min at room temperature, and analyzed on a FACSCalibur (BD Biosciences, Heidelberg, Germany) [42].

2.7. ATP secretion

Washed platelets (80 μL with 5×10^{5} platelets/L^{-1}) were diluted into 160 μL Tyrode-HEPES buffer containing 2 mM Ca^{2+}. After addition of 25 μL luciferase reagent (CHRONO-LUME), agonists were added at indicated concentrations to the continuously stirred (1000 rpm) platelet suspension. Luminescence was recorded on a 700 Whole Blood/Optical Lumi-Aggregometer (Chrono-log) for 10 min. ATP release was calculated by the AggroLink 8 software using an ATP standard.

2.8. Adhesion under flow conditions to collagen or vWF

For adhesion to collagen, cover slips were coated with 200 μg mL^{-1} collagen I at 37 °C o/n [33]. For adhesion to von Willebrand Factor (vWF), cover slips were coated with rabbit anti-human vWF antibody at 4 °C o/n, washed with PBS, blocked for 1 h with 1% BSA in H_2O and incubated with 200 μL murine plasma obtained from control mice. Blood (700 μL) was collected into 300 μL heparin (20 U/mL in TBS, pH 7.3). Whole blood was diluted 2:1 in Tyrode's buffer containing Ca^{2+} and filled into a 1 mL syringe. For adhesion to collagen, before perfusion, anticoagulated blood was incubated with Dylight-488–conjugated anti-GPIX derivative (0.2 μg mL^{-1}) at 37 °C for 5 min. Transparent flow chambers with a slit depth of 50 μm, equipped with the coated cover slips, were connected to a syringe that was filled with