Genetic Analysis of the Role of Protein Kinase Cα in Platelet Function and Thrombus Formation

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Abstract

Background: PKCα is a novel protein kinase C isozyme, predominately expressed in T cells and platelets. PKCα−/− T cells exhibit reduced activation and PKCα−/− mice are resistant to autoimmune disease, making PKCα an attractive therapeutic target for immune modulation. Collagen is a major agonist for platelets, operating through an immunoreceptor-like signalling pathway from its receptor GPVI. Although it has recently been shown that PKCα positively regulates outside-in signalling through integrin αIIbβ3 in platelets, the role of PKCα in GPVI-dependent signalling and functional activation of platelets has not been assessed.

Methodology/Principal Findings: In the present study we assessed static adhesion, cell spreading, granule secretion, integrin αIIbβ3 activation and platelet aggregation in washed mouse platelets lacking PKCα. Thrombus formation on a collagen-coated surface was assessed in vitro under flow. PKCα−/− platelets exhibited reduced static adhesion and filopodia generation on fibrinogen, suggesting that PKCα positively regulates outside-in signalling, in agreement with a previous report. In contrast, PKCα−/− platelets also exhibited markedly enhanced GPVI-dependent α-granule secretion, although dense granule secretion was unaffected, suggesting that PKCα differentially regulates these two granules. Inside-out regulation of αIIbβ3 activation was also enhanced downstream of GPVI stimulation. Although this did not result in increased aggregation, importantly thrombus formation on collagen under high shear (1000 s−1) was enhanced.

Conclusions/Significance: These data suggest that PKCα is an important negative regulator of thrombus formation on collagen, potentially mediated by α-granule secretion and αIIbβ3 activation. PKCα therefore may act to restrict thrombus growth, a finding that has important implications for the development and safe clinical use of PKCα inhibitors.
although none of those currently in development have yet become commercially available.

We have previously shown that PKCθ is physically associated with, and phosphorylated by, the tyrosine kinase, Btk [4]. However, lack of available PKCθ-selective inhibitors has curtailed research on the role of this isoform in human platelets. Shattil and co-workers have reported PKCθ-deficient platelets spread poorly on fibrinogen, suggesting that PKCθ positively regulates outside-in signalling. In addition, they demonstrated that PKCθ does not regulate platelet activation in response to a Gq/Gi coupled agonists PAR4 agonist or to ADP [24]. However, this study did not examine the role of PKCθ in collagen-induced platelet activation.

Given the primary role played by collagen in inducing platelet activation during the very early stages of thrombosis, and the parallels between signalling downstream of the collagen receptor GPVI and that downstream of immunoreceptors, it was now important to determine the role played by PKCθ in collagen-induced platelet activation and thrombus formation. We report that PKCθ negatively regulates GPVI-dependent α-granule secretion and integrin αIIbβ3 activation and thereby is the only PKC isoform yet described with this function. Furthermore, loss of this negative regulation in PKCθ−/− platelets leads to enhanced thrombus formation under flow in vitro. These results reveal a novel negative regulatory pathway in platelet activation, and have relevance to the current clinical and pharmaceutical interest in PKCθ inhibitors.

**Methods**

**Materials**

Unless stated, all reagents were from Sigma Aldrich (Poole, Dorset, U.K.). Cross-linked collagen-related peptide (CRP) was from Professor Richard Farndale (Biochemistry, University of Cambridge, U.K.). Heparin collagen was from Axis Shield (Biciton, Cambbs., U.K.). Phycocyanin (PE)-labelled JON/A and fluorescein isothiocyanate (FITC)-labelled Wug.E9 (anti-P-selectin) antibodies were from Emfret Analytics (Eibelstadt, Germany). Anti-PKCζ, -PKCβ, -PKCγ, -PKCε and anti-tubulin antibodies were from BD Transduction Laboratories (Oxford, U.K.). Anti-PKCθ antibody was from Cell Signaling Technology (New England BioLabs, Hitchin, U.K.). horseradish-peroxidase (HRP)-conjugated antimouse IgG and anti-rabbit IgG secondary antibodies, and enhanced chemiluminescent (ECL) reagents were from Amersham (Little Chalfont, Bucks., U.K.). Luciferin-luciferase reagent was from Chronolog (LabMedics, Manchester, U.K.).

**Washed platelet preparation**

PKCθ−/− C57BL6/J mice have been described previously [17]. Wildtype C57BL6/J mice were used as control. Use of mouse platelets was approved by local research ethics committee at the University of Bristol, U.K. and mice were bred for this purpose under UK Home Office licence (PPL 30/2386) held by AWP. Washed platelets were prepared as previously described [13]. Of note, platelets were treated with indomethacin [10 μM]. Platelets were rested for 30 min after centrifugation.

**Electrophoresis and Western blotting**

Washed platelets (2×10^7/ml) were lysed in Laemmli sample buffer. Proteins were resolved by electrophoresis in 9% SDS-polyacrylamide gels. Samples were then transferred to polyvinylidene difluoride membranes, blocked with 10% bovine serum albumin, and subjected to immunoblotting with specific antibodies to various PKC isoforms, as described in the text. Primary antibody binding was detected by HRP-conjugated secondary antibodies are revealed using ECL reagents.

**Aggregation**

Washed platelets (2×10^7/ml) were stimulated by CRP or collagen in an aggregometer (Chrono-Log, Labmedics, Manchester, U.K.) at 37°C, under continuous stirring at 1000 rpm. Aggregation was monitored by optical turbidometry.

**Dense granule secretion**

ATP release from dense granules was monitored using Chrono-Log luciferin-luciferase reagent according to the manufacturer’s instructions.

**Analysis of αIIbβ3 activation and α-granule secretion by flow cytometry**

Washed platelets (4×10^7/ml) were aliquoted into tubes containing optimal concentrations of PE-JON/A or FITC-anti-C626P, which bind to active integrin αIIbβ3 and surface-exposed P-selectin (CD62P), respectively, and CRP at the final concentrations indicated, for 15 min. Analysis of 20,000 events was performed using a Becton Dickinson FACScan. The platelet population as identified by forward and side scatter profile. Data were analysed using WinMDI version 2.8.

**DIC imaging of platelet adhesion and spreading**

Measurement of static platelet adhesion and spreading was performed as previously described [13]. Glass coverslips were coated with fibrinogen, CRP or collagen and mounted in a live-cell chamber. Adhesion and spreading of washed platelets (2×10^7/ml) was followed by differential interference contrast (DIC) microscopy with a wide-field microscope DM IRB attached to an ORCA ER camera (63x/1.40 NA oil objective) (Leica Microsystems, Milton Keynes, UK). Images were processed with OpenLab 4.03 (Improvision). The surface area of adherent platelets was measured using Volocity software (Improvision), while the number of adherent platelets was counted manually.

**In vitro thrombus formation**

Flow-induced thrombus formation was assessed basically as described before [25]. A Leica wide-field microscope DM IRB (63x/1.40 NA oil objective), attached to an ORCA ER camera was used for image capture (Leica Microsystems, Milton Keynes, UK). Heparin/PPACK-anticoagulated mouse blood was flowed over immobilised collagen through a parallel plate perfusion chamber, at a fixed shear rate of 1000 s^{-1} for 4 minutes. For each experiment, at least 10 random phase-contrast images were captured, which were then averaged. Recorded images were analyzed with ImagePro software.

**Statistics**

Statistical analyses were performed using GraphPad Prism software, unless stated otherwise, using two-way ANOVA with Bonferroni post-test; p<0.05 was considered significant. Bar charts show mean data±SEM (where ‘n’ denotes the number of individual mice used).

**Results**

PKCθ−/− platelets exhibit normal expression of other PKC isoforms

In order to be confident that any differences seen between PKCθ−/− and wild-type (WT) platelets were due to loss of PKCθ,
Figure 1. PKC isoforms are not upregulated in PKCα−/− mice. Platelets lysates from wild-type (WT) or PKCα−/− (KO) mice were assessed for PKC isofrm expression by SDS-PAGE and western blotting using specific antibodies for PKCα, β, δ, θ and ε. Membranes were stripped and re-probed for α-tubulin as indicated to ensure equal loading of protein. Blots are representative of three independent experiments. doi:10.1371/journal.pone.0003277.g001

and not due to altered expression of other PKC isoforms, we assessed the expression of the major PKC isoforms in platelets by western blotting. In addition to PKCα, mouse platelets strongly express PKCα−, β, δ, and ε. No difference in expression of these isoforms was seen in PKCα−/− platelets relative to WT platelets (Fig. 1). The blotting membranes were stripped and re-probed for α-tubulin, to ensure equal loading of proteins between samples (Fig. 1, lower panels).

PKCα has a small positive effect on platelet spreading on fibrinogen

Others have reported that platelet spreading on fibrinogen was partially defective in PKCα−/− platelets [24]. We were able to confirm and extend this result, demonstrating that both adhesion of platelets and specifically the degree of filopodia generation, rather than lamellipodia, 45 minutes after static deposition on fibrinogen-coated coverslips, were reduced in PKCα−/− platelets (Table 1). We analysed the kinetics of the spreading process to determine any further qualitative differences in spreading. Platelets during/after spreading were scored for number of filopodia and not due to altered expression of other PKC isoforms, we assessed the expression of the major PKC isoforms in platelets by western blotting. In addition to PKCα, mouse platelets strongly express PKCα−, β, δ, and ε. No difference in expression of these isoforms was seen in PKCα−/− platelets relative to WT platelets (Fig. 1). The blotting membranes were stripped and re-probed for α-tubulin, to ensure equal loading of proteins between samples (Fig. 1, lower panels).

Table 1. PKCα does not regulate adhesion or spreading on CRP or collagen.

| Adhesion | Surface area (μm²) |
|----------|--------------------|
| WT       | KO                 |
| WT       | KO                 |

Platelets were deposited on fibrinogen, CRP or collagen-coated coverslips in a live-cell chamber for 45 min and visualized by DIC microscopy. Five fields of view were selected at random and the number of adherent platelets was counted (adhesion) and spread surface area measured. Adhesion is total number of platelets adherent to the surface within a single 1000 μm² field of view. Shown are combined data from three independent experiments (mean ± SEM; * indicates p<0.05; ns = not significant).

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PKCα does not regulate adhesion or spreading on CRP or collagen

Since PKCα had a role in platelet adhesion and spreading on fibrinogen, its role in adhesion and spreading on CRP and collagen was also assessed. CRP is a selective GPVI agonist, whereas collagen activates both GPVI and integrin αIIbβ3. In contrast to fibrinogen, no significant effect was seen on adhesion or total platelet surface area on either of these substrates (Table 1). Platelet interaction with collagen is therefore not affected by absence of PKCα.

PKCα negatively regulates CRP-induced platelet activation

We further investigated whether PKCα regulates platelet activation following GPVI stimulation. Activation of GPVI leads to secretion of α-granules and dense granules, and activation of integrin αIIbβ3. The latter is known as inside-out signalling and is necessary for platelet aggregation.

CRP-induced surface expression of P-selectin, a marker of α-granule release was enhanced in the absence of PKCα. In WT platelets, 1 μg/ml CRP induced a 3.1 ± 0.5 -fold increase over basal in FITC-P-selectin fluorescence, which was increased to 10.2 ± 3.0 -fold in PKCα−/− platelets (n = 8; p<0.05; Fig. 3A), suggesting that PKCα negatively regulates the release of these granules. Interestingly, however, no difference in ATP secretion was seen between PKCα−/− and WT platelets in response to CRP (Fig. 3B) or collagen (Fig. 3C).

αIIbβ3 activation was determined by flow cytometry using JON/A, an antibody that recognises the active conformation of this integrin. Importantly, JON/A binding was almost double in platelets activated by 1 μg/ml CRP, from 3.8 ± 0.7 -fold over basal in WT to 7.5 ± 1.8 -fold in PKCα−/− platelets (n = 8; p<0.05; Fig. 4A). In contrast, a higher concentration of CRP (5 μg/ml) was not significantly affected (7.6 ± 1.2 -fold in WT compared to...
10.0±1.5–fold in PKC0−/−; n = 8; p = 0.81; Fig. 4A). These data suggest that PKC0 negatively regulates GPVI-dependent αIIbβ3 activation, but that at high concentrations this inhibition can be overcome. Interestingly however, platelet aggregation was not affected at either of these concentrations of CRP (Fig. 4B), nor was collagen-induced aggregation affected (Fig. 4C).

PKC0 negatively regulates thrombus formation in vitro
Since PKC0−/− aggregated normally, despite increased αIIbβ3 activation and α-granule secretion, we investigated whether the role of PKC0 might become more apparent during thrombus formation in the more physiological setting of flow conditions. Anticoagulated whole blood was passed over a collagen-coated
coverslip through a parallel-plate flow chamber at a shear rate of \(1000 \text{ s}^{-1}\), and thrombi observed under phase contrast after 4 min. Figure 5 shows that platelets from WT mice formed substantial thrombi on the collagen surface, however, platelets from PKC \(h/2\) formed significantly larger thrombi, suggesting that the negative role of PKC \(h\) is necessary to restrict thrombus size under flow conditions.

Discussion

PKC activation is generally considered to positively regulate platelet signalling, since platelet activation is inhibited by broad-spectrum PKC inhibitors, and PKC activators can enhance platelet activation. However, here we show that the role of PKC0 is more complicated than this, as it negatively regulates \(\alpha\)-granule secretion and inside-out signalling to integrin \(\alpha_{IIb}\beta_3\), yet positively regulates outside-in integrin signalling. In the absence of PKC0, thrombus formation was markedly enhanced, suggesting that the negative role of PKC0 is necessary to restrict thrombus size under flow conditions.

First, we observed a significant reduction in PKC0\(^{-/-}\) platelet adhesion and reduced spreading on fibrinogen compared to WT platelets, in agreement with Soriani et al. [24]. Interestingly, Soriani’s study showed an approximately 50% reduction in spread platelet surface area whereas our study only showed a 13% reduction. This apparent quantitative (though not qualitative) discrepancy could result from technical differences between our experiments. We used DIC microscopy to image platelet spreading, and the surface area of platelets was measured by manually outlining each cell (approximately 25 \(\mu\text{m}^2\)). Another study by McCarty et al [26] that used this approach saw a similar surface area. In both McCarty’s study and ours, mouse platelets rarely formed large lamellipodia when spreading on fibrinogen, in contrast to human platelets, which form full lamellipodia on fibrinogen) and filopodia were still apparent even after 45 minutes. In contrast, Soriani et al. [24] measured the surface area by confocal microscopy of rhodamine-phalloidin stained platelets, and reported a much lower surface area (approximately 8 \(\mu\text{m}^2\)). Rather than measure the surface area directly, this method measures F-actin coverage, perhaps suggesting that PKC0 regulates actin polymerization. WT platelets spread on fibrinogen and imaged using this method do not appear to exhibit the spiky morphology we and others [26,27] observe using DIC microscopy. Our analysis suggests that PKC0 positively regulates filopodia formation, since a smaller proportion of PKC0\(^{-/-}\) platelets showed many (>5) filopodia compared to WT. Regardless of these quantitative differences, both of our studies qualitatively agree that PKC0 is a positive regulator of outside-in signalling by integrin \(\alpha_{IIb}\beta_3\).

In contrast, PKC0 negatively regulates GPVI-induced \(\alpha_{IIb}\beta_3\) activation. The selective GPVI agonist, CRP, induced a concentration-dependent increase in binding of JON/A, an
PKCq Regulates Platelets

Figure 5. PKCq negatively regulates thrombus formation on collagen under flow in vitro. Whole blood was passed over a collagen-coated coverslip at 1000 s⁻¹ for four minutes then observed by phase contrast microscopy (A). Surface coverage was measured and is shown in B as mean±SEM for three independent experiments. Bar indicates 10 µm.

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activation state-specific α₁bβ₃ antibody. In PKC9⁻/⁻ platelets this was markedly enhanced compared to WT at an intermediate concentration of CRP, though not at a higher concentration, suggesting that PKC9 reduces expression of active α₁bβ₃ on the platelet surface, although inhibition can be overcome as agonist stimulation increases. It has been previously reported that PKC9 does not regulate α₁bβ₃ activation in response to ADP or AYPGKF [24], both of which act through G protein-coupled receptors, suggesting that the regulatory role of PKC9 may be specific to GPVI signalling.

CRP-induced aggregation was not affected by the absence of PKC9, however. Similarly, collagen-induced aggregation was also the same in WT and PKC9⁻/⁻ platelets. The lack of any effect on the rate or extent of aggregation was surprising, especially in response to 1 µg/ml CRP. At this concentration, the rate of aggregation was submaximal and yet the extent of integrin activation strongly enhanced. It might be expected, therefore, that the increased integrin activation would accelerate aggregation. However, since the extent of aggregation in response to 1 µg/ml CRP was almost maximal, further enhancement of α₁bβ₃ in PKC9⁻/⁻ platelets can have little further effect. The apparent disparity between absolute levels of integrin activation and extent of aggregation highlights the large level in integrin reserve believed to exist in platelets. β₃⁺⁻/⁻ platelets, with only 50% of the WT levels of β₃ on their surface, have almost identical bleeding times and aggregation responses to PMA, ADP, thrombin and arachidonic acid compared to WT platelets [28]. In like manner, although WT platelets show approximately 50% less integrin activation than PKC9⁻/⁻ platelets at 1 µg/ml CRP, we should not expect this necessarily to relate to a difference in the extent of aggregation.

PKC9 also negatively regulates α-granule secretion, although no difference in dense granule secretion was observed. This suggests that the release of different platelet granules is regulated by distinct mechanisms. The PKC family in general is a critical positive regulator of platelet granule secretion [2,10,11], although this positive function is likely to be mediated through conventional (Ca²⁺-dependent) isoforms [5,10,11]. Thus, it appears that the different PKC isoforms have contrasting roles in platelet α-granule secretion: PKCα is critically required for α-granule secretion, and PKC9 acts to counter this action. PKCγ is also critically important for dense granule secretion, which is not countered by PKC9. It has been suggested that PKCδ, closely related to PKC9, may negatively regulate GPVI-dependent dense granule secretion [5]. This interpretation was based on the use of rottlerin, a supposedly specific PKCδ inhibitor (though several PKCδ-independent targets have been reported [29–31]). However, we have previously reported that rottlerin enhances GPVI-dependent dense granule release even in PKCδ⁻/⁻ mice [13]. Thus, negative regulation of GPVI-dependent dense granule release does not appear to be mediated by either PKCδ or PKC9.

PKC9 negatively regulates thrombus formation under flow over a collagen-coated surface. Binding to collagen activates GPVI, leading to integrin α₁bβ₃ activation, which is enhanced in PKC9⁻/⁻ platelets. The increased number of adhesive contacts between platelets may accelerate the growth of the thrombus. Thus, negative regulation of inside-out signalling by PKC9 may be an important brake on thrombus growth at a site of injury. This effect is in contrast to the lack of effect seen in aggregation, highlighting the importance of physiological flow conditions [32]. In standard aggregometry, platelets exhibit a very large integrin reserve, whereas under flow, with higher shear force on any platelet-platelet interactions, integrin activation may be a limiting factor. Increased α₁bβ₃ activation would therefore enhance thrombus growth. This effect may be partially countered by the slightly reduced platelet adhesion to fibrinogen and reduced subsequent spreading, perhaps leading to fewer platelet-platelet contacts. Given the large effect on integrin activation compared to the smaller effect on spreading, however, the balance of these appears to favour increased thrombus size in PKC9⁻/⁻ platelets.

In summary, we have shown that PKC9 negatively regulates GPVI-dependent inside-out signalling, in contrast to the positive role generally ascribed to the PKC family in general. Although enhanced integrin α₁bβ₃ activation does not lead to increased aggregation in an aggregometer tube, PKC9⁻/⁻ platelets display enhanced thrombus formation on collagen under flow, suggesting that, under more physiological conditions, the regulatory role of PKC9 may restrict thrombus size. This may impact on the clinical safety of PKC9 inhibitors.
Acknowledgments

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Author Contributions

Perceived and conceived the experiments: KJH MTH KG JMC JH AWP. Performed the experiments: KJH MTH KG JMC JH. Analyzed the data: KJH MTH KG JMC JH AWP. Wrote the paper: KJH MTH JH AWP.

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