



Differential dephosphorylation of the Protein Kinase C-zeta (PKC ζ) in an integrin α IIb β 3-dependent manner in platelets

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ABSTRACT

Protein kinase C-zeta (PKC ζ), an atypical isoform of the PKC family of protein serine/threonine kinases, is expressed in human platelets. However, the mechanisms of its activation and the regulation of its activity in platelets are not known. We have found that under basal resting conditions, PKC ζ has a high phosphorylation status at the activation loop threonine 410 (T410) and the turn motif (autophosphorylation site) threonine 560 (T560), both of which have been shown to be important for its catalytic activity. After stimulation with agonist under stirring conditions, the T410 residue was dephosphorylated in a time- and concentration-dependent manner, while the T560 phosphorylation remained unaffected. The T410 dephosphorylation could be significantly prevented by blocking the binding of fibrinogen to integrin α IIb β 3 with an antagonist, SC-57101; or by okadaic acid used at concentrations that inhibits protein serine/threonine phosphatases PP1 and PP2A *in vitro*. The dephosphorylation of T410 residue on PKC ζ was also observed in PP1c γ null murine platelets after agonist stimulation, suggesting that other isoforms of PP1c or another phosphatase could be responsible for this dephosphorylation event. We conclude that human platelets express PKC ζ , and it may be constitutively phosphorylated at the activation loop threonine 410 and the turn motif threonine 560 under basal resting conditions, which are differentially dephosphorylated by outside-in signaling. This differential dephosphorylation of PKC ζ might be an important regulatory mechanism for platelet functional responses.

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1. Introduction

In most cell types, extracellular signals are relayed from the surface receptors to intracellular signaling molecules, such as the protein kinase C (PKC) family of serine/threonine kinases. These PKCs phosphorylate key serine and threonine residues present in target proteins leading to conformational changes resulting in alteration of their functions, which will eventually dictate the cellular responses [1,2]. Thus, PKCs are an integral part of cellular signal transduction processes. Various members of the classical and novel classes of PKC have been shown to be important for platelet functional responses such as aggregation, granule secretion, and

thromboxane generation [3–7]. PKC-zeta (PKC ζ) is a member of the atypical class of the PKC family [8]. Unlike the conventional and the novel isoforms, the atypical PKCs do not respond to second messenger diacylglycerol (DAG) because its C1 domain is modified to contain only one zinc-finger motif [8]. They also do not bind calcium because they lack the calcium binding C2 domain [8]. They are, however, sensitive to phosphoinositide 3-kinase (PI3-K) lipid product phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) and are activated by 3-phosphoinositide dependent protein kinase-1 (PDK1) in a PIP₃-enhanced manner [9]. PKC ζ , in addition, can be activated by phospholipase D2 (PLD2) via protein–protein interaction [10]. Phosphorylation of the threonine 410 residue present in the activation loop and autophosphorylation of threonine 560 residue present in the turn motif, of the kinase domain of the enzyme, have been shown to be important for its kinase activity [10–12]. However, evidence also exists that it can be catalytically active in the absence of threonine 410 phosphorylation [13].

PKC ζ functions as a MEK1 kinase in human alveolar macrophages [14]. Atypical PKCs transduce signals from the receptors of TNF α and IL-1 to the activation sites of NF κ B *in vivo* [15]. It has recently been shown that PKC ζ phosphorylates its upstream target, insulin

Abbreviations: PKC, protein kinase C; DAG, diacylglycerol; PI3-K, phosphoinositide 3-kinase; PIP₃, phosphatidylinositol (3,4,5)-trisphosphate; PDK1, 3-phosphoinositide dependent protein kinase-1; PLD2, phospholipase D2; PP1, serine/threonine protein phosphatase 1; PP2A, serine/threonine protein phosphatase 2A.

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receptor substrate-1, -3 and -4, but not -2, and this represents a negative feedback mechanism that may contribute to signal specificity in insulin action [16]. Furthermore, the polarity complex PAR-3/PAR-6/PKC ζ plays an essential role in polarization in epithelial cells and migrating astrocytes [17]. An alternatively spliced variant PKM ζ is involved in long-term potentiation (LTP) maintenance in hippocampal pyramidal cells [18]. Thus, this enzyme is involved in many functional responses in various types of cells.

In mice deficient in PKC ζ , phenotypic alterations are seen in the secondary lymphoid organs such as spleen and lymph nodes [19]. The genetic deletion of PKC ζ results in an impairment of B-cell receptor signaling and defects in the transcription of NF κ B-dependent genes [20]. PKC ζ knock-outs also have a defective T-cell-dependent immune response [20]. However, these mice are grossly normal [19].

PKC ζ has been shown to be expressed in human platelets [21–23], although it is not known whether it gets phosphorylated and/or activated downstream of receptor activation by physiological agonists. The molecular mechanisms of its regulation as well as the physiological functions of this kinase, in platelets, are also unknown. Since this enzyme is expressed in both platelets and immune cells, it can be speculated that it might play a role in linking immune responses with thrombosis. In this study, we investigated the mechanism of regulation of PKC ζ activation in platelets. We have confirmed its expression in human platelets, and have also found it to be basally phosphorylated at the activation loop threonine 410 and the turn motif threonine 560 under resting conditions. Upon agonist stimulation, the threonine 410 residue gets dephosphorylated, possibly in an integrin α IIb β 3-dependent manner, whereas the phosphorylated threonine 560 remains unaffected. This differential dephosphorylation might be important for platelet functional responses.

2. Materials and methods

2.1. Materials

Apyrase (type VII), bovine serum albumin (fraction V), and acetylsalicylic acid were obtained from Sigma (St. Louis, MO, USA). PGE₁ was purchased from Enzo Life Sciences (Plymouth Meeting, PA, USA). AYPGKF, a PAR-4 agonist, was custom synthesized at Invitrogen (Carlsbad, CA, USA). SC-57101 was a gift from Searle and Co (Greenwich, CT, USA). Ethyl alcohol (Absolute, 200 proof) was purchased from Aaper Alcohol Co. (Shelbyville, KY, USA). The β -actin antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). The phospho-PKC ζ/λ (Thr410/403) (Cell Signaling Technology, Beverly, MA, USA) [24–26] and phospho-PKC ζ (pT560) (Epitomics, Burlingame, CA, USA) [27,28] antibodies have been validated by other investigators, as well as by the company (product datasheets). Furthermore, platelets do not express PKC λ isoform [29,30], so the phospho-PKC ζ/λ (Thr410/403) antibody will detect PKC ζ in platelets only when it is phosphorylated at Thr410. Both antibodies have also been shown to be reactive in both human and mouse tissues (company product datasheets). Total PKC ζ antibody, okadaic acid (a general serine/threonine inhibitor) and HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Chemiluminescent HRP-substrate was from Millipore (Billerica, MA, USA). Ketamine (Ketaset) was purchased from Fort Dodge Animal Health (Fort Dodge, IA, USA). All the other reagents were of reagent grade, and de-ionized water was used throughout.

2.2. Animals

CD-1 mice carrying PP1cy-null mutation and subsequently backcrossed for 10 generations onto Balb/C background were

generated in the laboratory of Susannah Varmuza (University of Toronto, Toronto, Canada) [31].

2.3. Isolation of human platelets

All experiments using human subjects were performed in accordance with the Declaration of Helsinki. Whole blood was drawn from healthy consenting human volunteers into tubes containing one-sixth volume of ACD (2.5 g of sodium citrate, 1.5 g of citric acid, 2 g of glucose in 100 ml of deionized water). Blood was centrifuged (Eppendorf 5810R centrifuge) at $230 \times g$ for 20 min at room temperature (25 °C) to obtain PRP (platelet-rich plasma). PRP was incubated (or not) with 1 mM aspirin for 30 min at 37 °C. The PRP was then centrifuged for 10 min at $980 \times g$ at room temperature to pellet the platelets. Platelets were resuspended in Tyrode's buffer pH 6.5 (138 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 3 mM NaH₂PO₄, 5 mM glucose, 10 mM PIPES, and pH 6.5) containing 0.1 U/mL apyrase and 1 μ M PGE1 (or not). The platelets were also prepared without aspirin and PGE1 treatment for some experiments. The platelet suspension was then centrifuged for 10 min at $980 \times g$ at room temperature to pellet the platelets. Platelets were then resuspended in Tyrode's buffer pH 7.4 (138 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 3 mM NaH₂PO₄, 5 mM glucose, 10 mM Hepes, and pH 7.4) containing 0.1 U/mL apyrase. Platelets were counted using the Hemavet (Drew Scientific Inc., Dallas, TX, USA) and concentration of cells was adjusted to 2×10^8 platelets/mL. The washed platelet suspension contained approximately 200,000 platelets/ μ L, less than 100 leukocytes/ μ L and RBCs were not detectable. All experiments using isolated platelets were performed within 4 h after drawing blood without or with (for some experiments) added extracellular calcium (1 mM).

2.4. Okadaic acid stock solutions preparation

A master stock solution (1 mM) of okadaic acid was first prepared by dissolving it in 200 proof absolute ethanol. This was serially diluted in absolute ethanol to get stock solutions of 250, 100, 50 and 5 μ M.

2.5. Isolation of murine platelets

Blood was collected from ketamine-anesthetized mice by cardiac puncture into syringes containing 3.8% sodium citrate as anticoagulant. The whole blood was centrifuged (IEC Micromax Centrifuge, International Equipment Components, CA, USA) at $100 \times g$ for 10 min to isolate the PRP. Prostaglandin E1 (1 μ M) was added to PRP. The platelets were centrifuged at $400 \times g$ for 10 min, and the pellet was resuspended in Tyrode's buffer pH 7.4 containing 0.1 U/mL apyrase.

2.6. Platelet aggregation

Platelet aggregation was measured using a lumi-aggregometer (Chrono-Log, Haverton, PA, USA) at 37 °C under stirring conditions (900 rpm). A 0.5 mL sample of aspirin-treated (or non-aspirin - treated) washed human platelets was stimulated with 500 μ M AYPGKF and the change in light transmission was measured. In okadaic acid studies, platelets were pre-incubated with 1 μ L okadaic acid stock solutions of 5, 50, 100, and 250 μ M (giving final concentrations of 10, 100, 200 and 500 nM, respectively) or 1 μ L ethanol for 5 min at 37 °C before agonist stimulation. The chart recorder (Kipp and Zonen, Bohemia, NY, USA) was set at 0.2 mm/s.

2.7. Western immunoblot analysis

Platelets were stimulated with AYPGKF and the reaction was stopped by the addition of SDS-Laemmli buffer (1 \times is 10% by

volume glycerol, 62.5 mM Tris–HCl, pH 6.8, 2% SDS, 0.01 mg/ml bromophenol blue, 1 mM dithiothreitol (DTT)). Proteins were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked by incubation with Tris (tris(hydroxymethyl)aminomethane)-buffered saline (TBS: 20 mM Tris, 140 mM NaCl) containing 3% (wt/vol) bovine serum albumin (BSA) for 1 h at room temperature. Membranes were incubated overnight at 4 °C with the primary antibody (1:1000 dilution) in loading buffer (TBS with 3% BSA) with gentle agitation. After three washes for 5 min each with Tris-Buffered Saline Tween-20 (TBS-T), the membranes were probed with an HRP-conjugated secondary antibodies (1:10,000 dilution) in TBS-T for 1 h at room temperature. After additional washing steps, membranes were then incubated with the chemiluminescent HRP-substrate and immunoreactivity was detected using a Fuji Film Luminescent Image Analyzer (LAS-3000 CH, Tokyo, Japan).

2.8. Statistical analysis

Western blot data were compiled from at least three independent experimental results. Densitometric analyses of the phospho-PKC ζ immunoreactive bands were performed using Fuji Film Science Lab 2003 Image Gauge Version 4.22 software and were normalized to

their corresponding β -actin bands, and then the percent change (of the normalized values) was calculated taking the unstimulated samples as 100%. The results were expressed as mean \pm SEM. Statistical significance was tested by one-way ANOVA followed by Bonferroni post hoc analysis. *P* values < 0.05 was considered statistically significant, which are indicated by *, ** or *** for *P* < 0.05, <0.01 or <0.001, respectively; and non-significance is indicated by N.S.

3. Results

3.1. PKC ζ is expressed in human platelets and is phosphorylated under basal resting conditions

PKC ζ has been shown to be expressed in human platelets [21–23]. In this study, we have confirmed the presence of this enzyme in human platelets by immunoblotting for total protein. Different concentrations of unstimulated platelet samples (100, 200 and 500 million per ml) were first prepared and whole cell lysates were analyzed by Western blotting for the total protein. As shown in Fig. 1A, the signal strength of the protein of \sim 75 kDa increased with increasing concentrations and we conclude that PKC ζ is expressed in human platelets.

The T410 residue present in the activation loop has been shown to be important for its kinase activity [10–12]. We probed the unstimulated platelet lysates with a phospho-specific antibody and our results show that PKC ζ is phosphorylated at T410 under basal resting conditions in platelets (Fig. 1B). We did not observe any phosphorylation of other enzymes, such as Erk, in these resting platelet samples (data not shown), which verifies that the high basal phosphorylation of PKC ζ was not a consequence of inadvertent platelet activation during blood collection and platelet isolation.

Since phosphorylation of this residue has been shown to be important for its activity, we speculate that PKC ζ may be constitutively active under resting conditions in platelets. One potential mechanism for this is via protein–protein interaction. It was shown that the phox homology (PX) domain of phospholipase D2 (PLD2) directly interacts with the kinase domain of PKC ζ , and this association results in phosphorylation and activation of PKC ζ , which is independent of PLD2's lipase activity [10]. Of note, PLD2 has been shown to be present in platelets [32]. In co-immunoprecipitation experiments, we did not observe an association between these two proteins (data not shown). A plausible explanation could be that these two proteins dissociate after activation and hence we did not detect their association.

Additionally, there are reports that cleavage of PKC ζ by caspases could result in generation of a catalytically active subunit (the kinase domain) of this enzyme [12]. We have used a pan-caspase inhibitor in our experiments but did not observe any effect on the dephosphorylation event (data not shown), suggesting that caspase-mediated cleavage of PKC ζ probably does not occur in platelets.

3.2. Dephosphorylation of T410 residue upon agonist stimulation of platelets

We observed that PKC ζ has a high basal phosphorylation. For most other enzymes, agonist stimulation leads to an increase in the levels of phosphorylation and/or activity from a low basal state. A high basal phosphorylation could mean a constitutively phosphorylated state, which could decrease with agonist treatment. To test this hypothesis, we activated platelets with a PAR4 agonist AYPGKF (500 μ M) under both non-stirring and stirring conditions, and have found that the T410 residue gets dephosphorylated upon agonist stimulation only under stirring conditions (Fig. 2A). T560, another residue present in the turn motif of the kinase domain, also has been shown to be important for its activity [8]. Surprisingly,

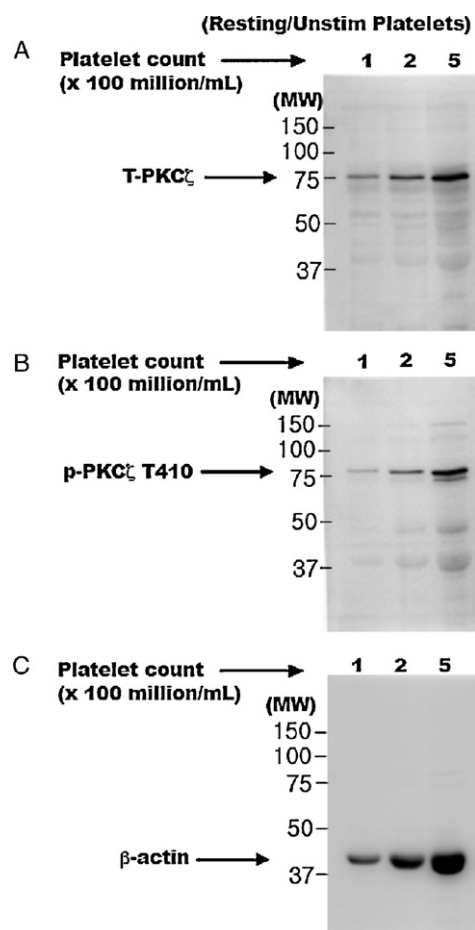


Fig. 1. Expression of PKC ζ in human platelets. Whole cell lysates of various concentrations of unstimulated human platelet samples (100, 200 and 500 million platelets per mL of Tyrode's buffer) were prepared and run on an SDS–PAGE. Western blots, representative of three independent experimental results, for total PKC ζ (Panel A) and phospho-PKC ζ T410 (Panel B). Corresponding lane loading controls with β -actin are also shown (Panel C). Unstim: unstimulated; MW: molecular weight in kDa; T-PKC ζ : total-PKC ζ ; p-PKC ζ T410: phosphorylated PKC ζ at threonine 410.

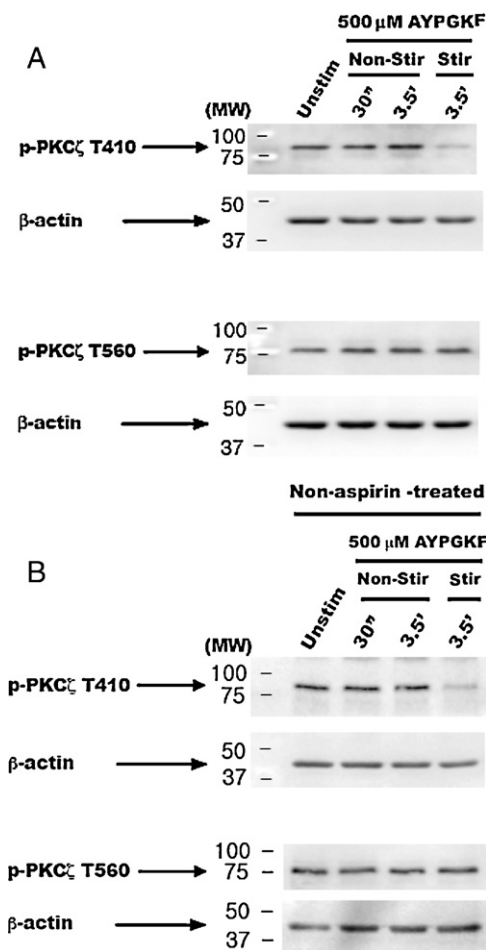


Fig. 2. Dephosphorylation of T410 residue of PKC ζ with agonist stimulation under stirring conditions. Washed human platelets were stimulated with PAR4 agonist AYPGKF (500 μ M) for various time-points under non-stirring (non-stir) and stirring (stir) conditions, and lysates prepared. Western blots, representative of three independent experimental results, for phospho-PKC ζ T410 and phospho-PKC ζ T560. Aspirin-treated (Panel A); and non-aspirin, non-PGE1-treated (Panel B) samples. Corresponding lane loading controls with β -actin are also shown. Unstim: unstimulated.

T560 phosphorylation was not affected by agonist stimulation, either under non-stirring or stirring conditions (Fig. 2A). We have also performed the same experiments using non-aspirin, non-PGE1-treated platelets (Fig. 2B), as well as in the presence of 1 mM extracellular calcium (data not shown), and observed the similar differential dephosphorylation responses.

3.3. T410 becomes dephosphorylated in an aggregation- and integrin α IIb β 3-dependent manner

Next, we performed a full panel of time-course and dose-response of agonist stimulation under stirring conditions (Fig. 3A) to further evaluate the dephosphorylation event. Platelets were stimulated with 500 μ M of AYPGKF for various time points (0–4 min) and lysates were analyzed by Western blotting (Fig. 3B). We observed that T410 gets dramatically dephosphorylated in a time-dependent manner (\sim 80%, at 4 min) (Fig. 3B and C). Platelets were also stimulated with varying concentrations of AYPGKF (100–500 μ M) for 4 min. Again, the T410 residue was found to get dephosphorylated in a concentration-dependent manner (Fig. 3B and D). Therefore, the T410 dephosphorylation is dependent on aggregation. The T560 phosphorylation, on the other hand, remained unaffected by aggregation (Fig. 3B, E and F).

From the previous observation, it was evident that the T410 dephosphorylation could be mediated via fibrinogen binding to activated integrin α IIb β 3 (outside-in signaling). To confirm this hypothesis, we blocked the integrin with an antagonist, SC-57101 (10 μ M) and stimulated the platelets with the agonist for 4 min (Fig. 3A). The T410 dephosphorylation was significantly prevented by blocking the interaction of integrin with fibrinogen and the subsequent outside-in signaling (Fig. 3B–D). This result strongly suggests that α IIb β 3 activation-mediated signaling leads to dephosphorylation of T410 on PKC ζ . Alternatively, other potential signaling (besides α IIb β 3 activation-mediated signaling) that could arise from cell–cell contact could contribute to the dephosphorylation. It is worth mentioning here that basal T560 phosphorylation is not affected either by agonist stimulation or by integrin blockade (Fig. 3B, E and F). The differential dephosphorylation of PKC ζ was also observed with other agonists, such as SFLLRN (acting on PAR1) and convulxin (acting on GPVI) (data not shown).

3.4. Dephosphorylation of T410 is possibly mediated by α IIb β 3-associated ser/thr phosphatases

Our results showed that the integrin α IIb β 3 outside-in signaling possibly mediates the dephosphorylation of T410 residue. Although, α IIb β 3 does not have any intrinsic phosphatase activity, the catalytic subunits of protein phosphatase 1 (PP1c) [33] and protein phosphatase 2A (PP2Ac) [34] associate constitutively with the cytoplasmic tail of integrin α IIb subunit. Upon integrin engagement, PP1c dissociates from the integrin complex and gets activated [33]. In contrast, fibrinogen binding to the integrin does not alter PP2Ac– α IIb interaction and moderately reduces PP2Ac activity [34]. In order to determine if these phosphatases participated in the dephosphorylation of T410 residue on PKC ζ , we employed okadaic acid, a generic Ser/Thr phosphatase inhibitor [35]. Platelets were pre-incubated with various concentrations (1–500 nM) of okadaic acid for 5 min at 37 $^{\circ}$ C and then stimulated with the agonist for 4 min (Fig. 4A). Lysates were analyzed by Western blotting. Okadaic acid by itself did not affect the basal phosphorylation levels of T410 and T560 (Fig. 4B). We observed that the agonist-dependent dephosphorylation of T410 could be significantly prevented by okadaic acid concentrations of 200 nM and above (Fig. 4B and C), a concentration range that inhibits PP1 and PP2A in cell-free systems *in vitro* [35]. This suggested that the agonist-mediated T410 dephosphorylation is mediated by serine/threonine protein phosphatases. Alternatively, since okadaic acid inhibited aggregation, it is equally possible that the prevention of T410 dephosphorylation was a consequence of inhibition of integrin–fibrinogen interaction, in addition to its ability to inhibit the phosphatases. However, the basal T560 phosphorylation remained unaffected in platelets treated with okadaic acid and stimulated with the agonist (Fig. 4B and D).

Since the catalytic subunit of protein phosphatase 1 gamma isoform (PP1 γ) is required for thrombin-induced platelet integrin activation [33], we considered whether PP1 γ may participate in dephosphorylating PKC ζ T410 downstream of integrin engagement. We used a genetic approach wherein, the dephosphorylation of T410 in PKC ζ was analyzed using platelets from PP1 γ null mice [31]. In our experimental conditions, the agonist-mediated dephosphorylation of T410 on PKC ζ still occurred in PP1 γ null platelets (Fig. 5A and B), suggesting that other isoforms of PP1c or another phosphatase could be mediating this event. The basally phosphorylated state of T560 residue remained unaffected after agonist stimulation, even in PP1 γ null platelets (Fig. 5A and C).

We have tried to measure the enzymatic activity of PKC ζ by performing an *in vitro* kinase assay on the immunoprecipitated protein, but were unsuccessful (data not shown). Unfortunately,

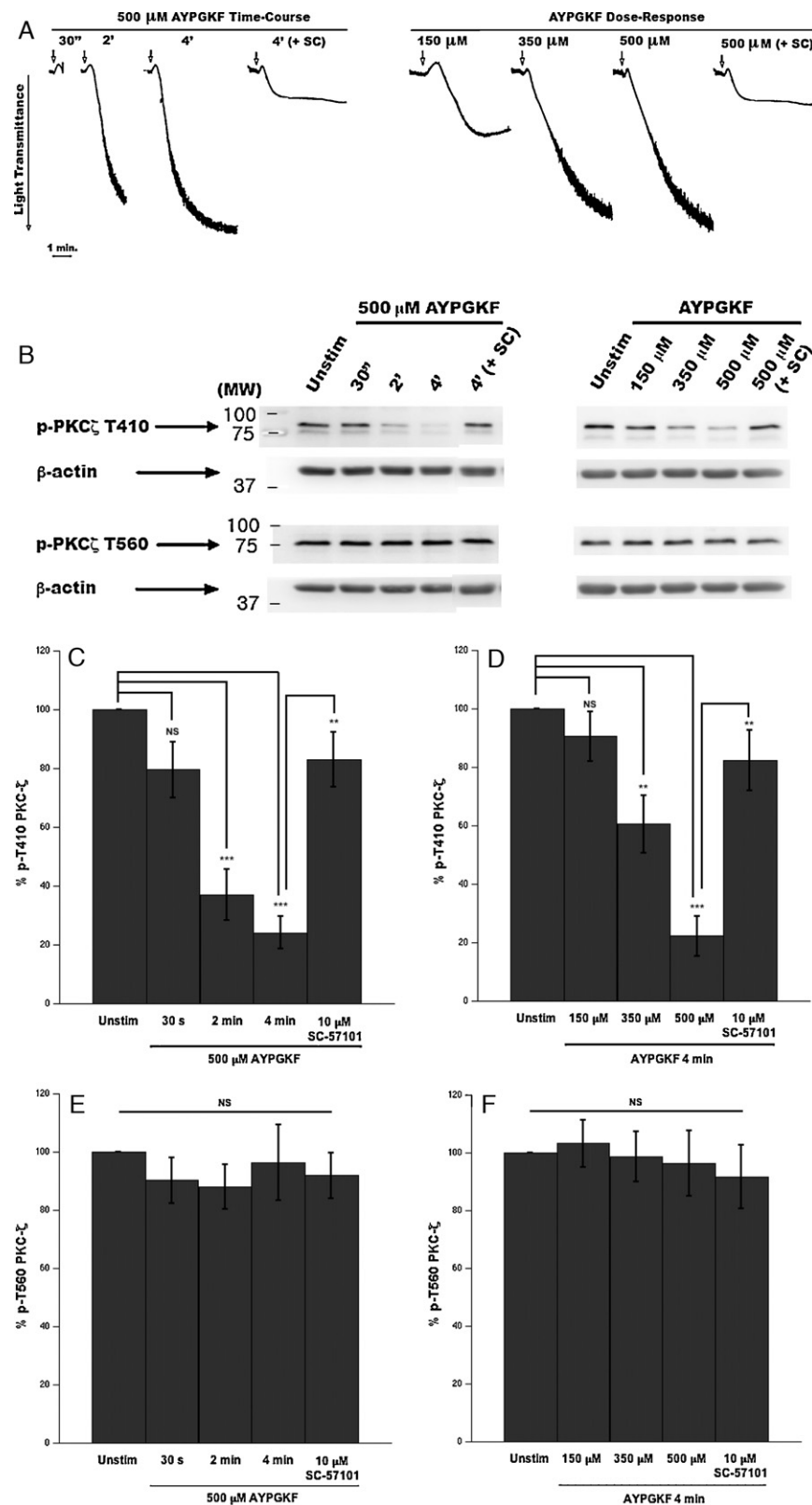


Fig. 3. Differential dephosphorylation of PKC ζ via agonist-stimulated platelet aggregation and prevention of T410 dephosphorylation by integrin α IIb β 3 blockade. Representative aggregation tracings of washed and aspirin-treated human platelets stimulated under stirring conditions, with 500 μ M AYPGKF for various time-points and for 4 min in the presence of 10 μ M of integrin α IIb β 3 antagonist SC-57101 (Panel A left); and with varying concentrations of AYPGKF for 4 min and with 500 μ M AYPGKF for 4 min in the presence of 10 μ M of integrin α IIb β 3 antagonist SC-57101 (Panel A right). Representative Western blots of the lysates probed with phospho-PKC ζ T410 and phospho-PKC ζ T560 antibodies (Panel B). Densitometric analysis of the phospho-PKC ζ T410 blots from the time-course (Panel C) and concentration-response (Panel D) experiments. Densitometric analysis of the phospho-PKC ζ T560 blots from the time-course (Panel E) and concentration-response (Panel F) experiments. Data compiled from three independent experimental results. Corresponding lane loading controls with β -actin are also shown. Unstim: unstimulated, SC: SC-57101, * p < 0.05, ** p < 0.01, *** p < 0.001, NS: non-significant.

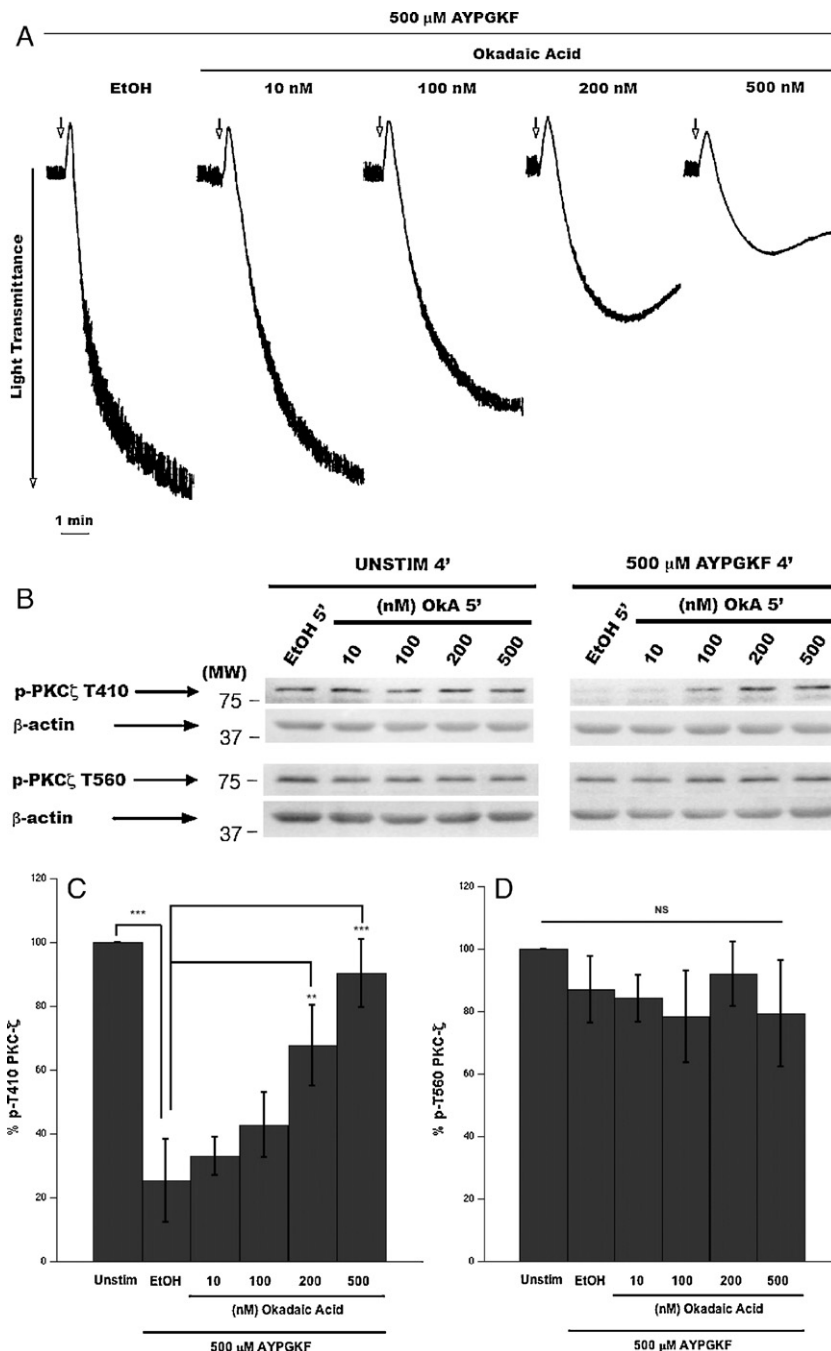


Fig. 4. Prevention of T410 dephosphorylation by okadaic acid. Washed and aspirin-treated human platelets were preincubated with 1 μ L absolute ethanol or various concentrations of okadaic acid (10–500 nM) at 37° C for 5 min under stirring conditions, and then stimulated with 500 μ M AYPGKF for 4 min under stirring conditions. Representative aggregation tracings (Panel A). Representative Western blots of the lysates probed with phospho-PKC ζ T410 and phospho-PKC ζ T560 antibodies (Panel B). Unstimulated (Panel B left) and stimulated with 500 μ M AYPGKF (Panel B right) samples. Densitometric analysis of the phospho-PKC ζ T410 blots, with each phospho-PKC ζ T410 signal in the AYPGKF-stimulated sample normalized to its corresponding phospho-PKC ζ signal in the unstimulated sample (Panel C). Densitometric analysis of the phospho-PKC ζ T560 blots, with each phospho-PKC ζ T560 signal in the AYPGKF-stimulated sample normalized to its corresponding phospho-PKC ζ signal in the unstimulated sample (Panel D). Corresponding lane loading controls with β -actin are also shown. Data compiled from three independent experimental results. Unstim: unstimulated, EtOH: ethyl alcohol (200 proof), Oka: okadaic acid, * p < 0.5, ** p < 0.01, *** p < 0.001, NS: non-significant.

the functional roles of PKC ζ in platelets could not be evaluated at this time. Addition of the myristoylated pseudosubstrate inhibitory peptide into the platelet suspension led to destruction/agglutination of platelets.

4. Discussion

The PKC family of serine/threonine kinases mediates a number of physiological functions in platelets such as aggregation,

secretion and thromboxane generation [3–7]. The novel and the conventional classes of PKC have been thoroughly studied in this regard [3–7]. However, nothing is known regarding the regulation and the physiological role of the atypical member PKC ζ in platelets [21–23]. The current study was undertaken to investigate the regulatory mechanism of this enzyme in platelets.

PKC ζ was detected by Western blot analysis of the lysates prepared from washed platelets, and is basally phosphorylated on threonine 410 (T410) and threonine 560 (T560) residues. At least in

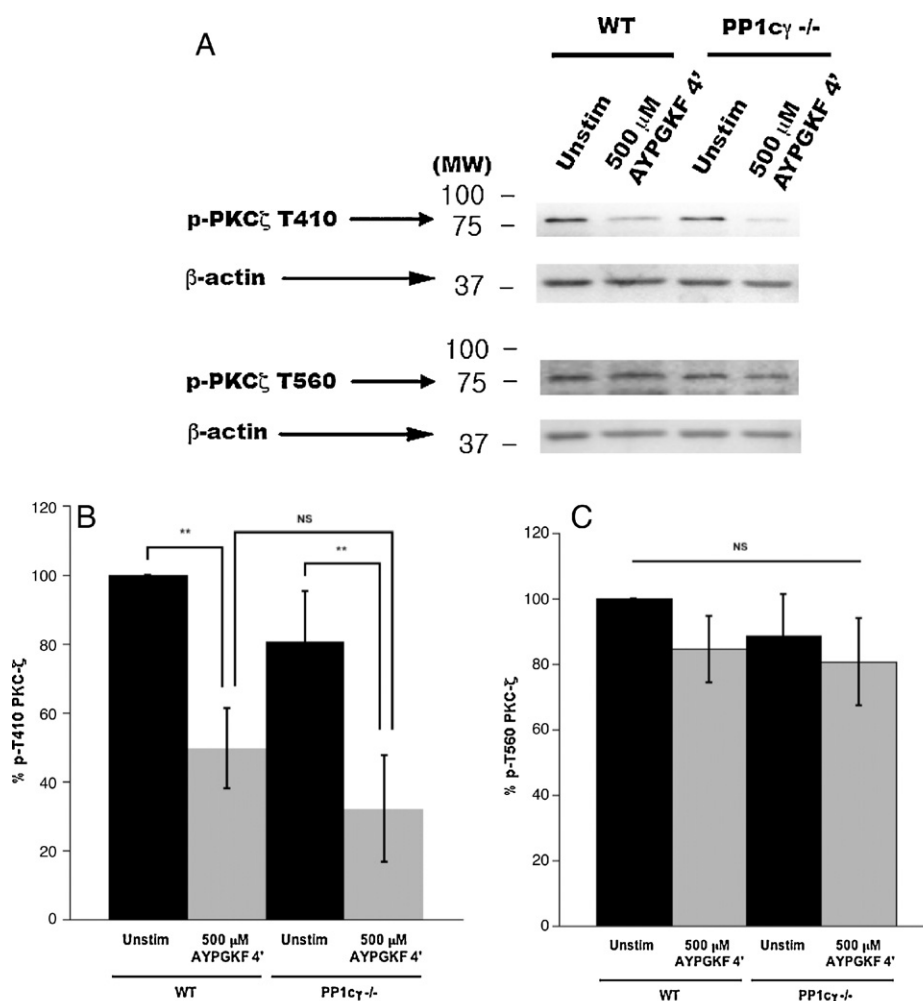


Fig. 5. Inability of PP1 γ deficient platelets to prevent the T410 dephosphorylation. Washed and aspirin-treated platelets from wild type or PP1 γ null mice were stimulated with 500 μ M AYPGKF for 4 min under stirring conditions, and lysates were prepared. Representative Western blots of the lysates probed with phospho-PKC ζ T410 and phospho-PKC ζ T560 antibodies (Panel A). Densitometric analyses of the phospho-PKC ζ T410 blots (Panel B) and the phospho-PKC ζ T560 blots (Panel C). Corresponding lane loading controls with β -actin are also shown. Data compiled from three independent experimental results. Unstim: unstimulated, WT: wild type, PP1 γ ^{-/-}: PP1 γ null mice, ** p < 0.01, NS: non-significant.

some cell types, PKC ζ is known to be constitutively phosphorylated and active under basal resting conditions [36]. Agonist-stimulation (only under stirring conditions) led to dephosphorylation of the T410 residue but not the T560 residue, suggesting that integrin activation and/or signaling might be necessary for T410 dephosphorylation. Consistently, T410 dephosphorylation could be significantly prevented by blocking fibrinogen binding to the integrin, even when the platelets were stimulated with the agonist.

Integrins do not have any intrinsic phosphatase activity but a pool of Ser/Thr phosphatases interact with the integrin complex. We investigated a role for such phosphatases in the dephosphorylation of T410 residue. Okadaic acid (a ser/thr phosphatase inhibitor) by itself could alter the basal phosphorylation levels of many proteins because the inhibition of phosphatases could shift the equilibrium towards kinase mediated phosphorylation events. Therefore, control unstimulated platelet samples treated with the corresponding concentrations of okadaic acid were prepared, and the final PKC ζ phosphorylation levels were calculated by normalizing to the control unstimulated samples. Okadaic acid by itself did not affect the basal phosphorylation status of PKC ζ but prevented the agonist-mediated dephosphorylation of T410 on PKC ζ , strongly suggesting that PP1 and/or PP2A could be the phosphatases responsible for T410 dephosphorylation.

Protein phosphatase 1 (PP1) which is constitutively associated with the integrin and gets activated in an integrin-dependent manner [33] could be one of the possible candidates. The γ isoform of the catalytic subunit of PP1 has been shown to be one of the phosphatases responsible for the 2MeSADP-mediated dephosphorylation of PKC η [4]. However, no significant difference in the level of T410 dephosphorylation of PKC ζ was observed in the wild-type versus the PP1 γ knock-out mouse platelets, suggesting that this isoform may not be involved in the dephosphorylation process. Other isoforms of the catalytic subunit of PP1 (α , β / δ) or PP2A or another phosphatase, either alone or collectively, could be responsible for this phenomenon. However, such studies could not be conducted at the present time due to the lack of PP1c isoform-specific pharmacological inhibitors, embryonic lethality of PP2A α null mice [37] and the unavailability of PP1 α or PP1 β null mice. Of note, the basal phosphorylation of T560 remains unaffected under all the conditions used in this study.

The activation loop threonine 410 of PKC ζ is phosphorylated by PDK-1 (PIP2-dependent kinase-1) in many cell systems, and the turn motif threonine 560 is an autophosphorylation site. The phosphorylation of both these two residues have been shown to be required for its kinase activity. However, there is also evidence that this enzyme can still be fully active even in the absence of the T410 phosphorylation site [13]. Tyrosine phosphorylation was observed

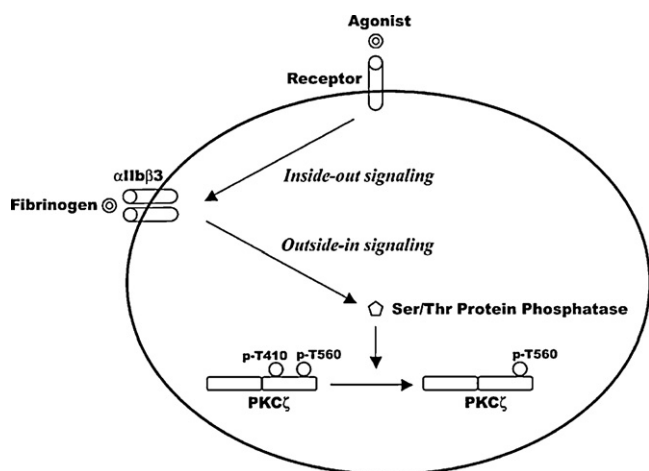


Fig. 6. Hypothetical model of regulation of PKC ζ in platelets. PKC ζ is expressed in human platelets and is basally phosphorylated on threonine 410 and 560 residues. Agonist stimulation, via aggregation- and activated integrin-mediated outside-in signaling that activates an unidentified serine/threonine phosphatase, leads to dephosphorylation of the T410 residue, but not the T560 residue.

in mutants where T410 was deleted, but which showed similar kinase activity as the wild-type protein. It was suggested that phosphorylation of tyrosine 428 present near the activation loop, could result in a similar active conformation as the threonine 410 phosphorylated protein [13].

The physiological significance of the basally phosphorylated state of PKC ζ and the subsequent dephosphorylation of T410 in an integrin-dependent manner, is not known at the current time. One plausible explanation could be that this enzyme is constitutively active and might act as a negative regulator of platelet functional responses, and integrin-mediated (outside-in) signaling leads to its deactivation. The T560 autophosphorylation signal alone might not be sufficient to maintain its full kinase activity. Studies in PKC ζ knock-out mice might hopefully shed some lights into the physiological role/s of this protein in platelets.

In summary, we have demonstrated that, in platelets, the atypical PKC isoform zeta (ζ) may be constitutively phosphorylated at the activation loop threonine 410 residue as well as the turn motif threonine 560 residue. Only the threonine 410 gets dephosphorylated, possibly in an integrin α IIb β 3-dependent manner (Fig. 6). Although serine/threonine phosphatases PP1c and PP2Ac could possibly regulate this dephosphorylation, PP1c isoform is not involved in this event.

Authors' contribution

Azad Mayanglambam designed the research, performed the experiments, analyzed the data and wrote the manuscript. Dheeraj Bhavanasi performed some of the experiments and analyzed data. K. Vinod Vijayan provided the PP1c γ null mice and edited the manuscript. Satya P. Kunapuli designed the research, analyzed the data, edited the manuscript and supervised the overall research.

Conflict of interest

The authors declare that there is no conflict of interest.

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