Differential regulation of threonine and tyrosine phosphorylations on protein kinase Cδ by G-protein-mediated pathways in platelets

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Phosphorylation of activation loop threonine (Thr<sup>505</sup>) and regulatory domain tyrosine (Tyr<sup>311</sup>) residues are key regulators of PKC (protein kinase C) δ function in platelets. In the present study, we show that G<sub>q</sub> and G<sub>12/13</sub> pathways regulate the Thr<sup>505</sup> and Tyr<sup>311</sup> phosphorylation on PKCδ in an interdependent manner. DiC8 (1,2-dioctanoylglycerol), a synthetic analogue of DAG (diacylglycerol), caused Thr<sup>505</sup>, but not Tyr<sup>311</sup>, phosphorylation on PKCδ, whereas selective activation of G<sub>12/13</sub> pathways by the YFLLRNP peptide failed to cause phosphorylation of either residue. However, simultaneous activation by DiC8 and YFLLRNP resulted in Thr<sup>505</sup> and Tyr<sup>311</sup> phosphorylation on PKCδ. In addition, we found that the activation of SFKs (Src family tyrosine kinases) is essential for G<sub>12/13</sub>-mediated Thr<sup>505</sup> phosphorylation of PKCδ. These results were confirmed using G<sub>δ</sub>-deficient mouse platelets. Finally, we investigated whether Thr<sup>505</sup> phosphorylation is required for Tyr<sup>311</sup> phosphorylation. A T505A PKCδ mutant failed to be phosphorylated at Tyr<sup>311</sup>, even upon stimulation of both G<sub>δ</sub> and G<sub>12/13</sub> pathways. We conclude that (i) PKCδ binding to DAG, downstream of G<sub>q</sub> pathways, and its translocation results in Thr<sup>505</sup> phosphorylation, (ii) G<sub>12/13</sub> pathways activate SFKs required for the phosphorylation of Tyr<sup>311</sup> on Thr<sup>505</sup>-phosphorylated PKCδ, and (iii) Thr<sup>505</sup> phosphorylation is a prerequisite for Tyr<sup>311</sup> phosphorylation on PKCδ.

Key words: G<sub>12/13</sub>, G<sub>δ</sub>, phosphorylation, platelet, protein kinase Cδ (PKCδ), Src family tyrosine kinase.
the two events. Whereas Thr<sup>505</sup> phosphorylation requires only G<sub>δ</sub>-mediated DAG generation, Tyr<sup>311</sup> phosphorylation requires G<sub>12/13</sub>-mediated SFK activation, the presence of DAG, basal calcium levels and phosphorylated Thr<sup>505</sup>.

EXPERIMENTAL

Approval for this study was obtained from the Institutional Review Board of Temple University.

Materials

Apyrase (type VII), BSA (fraction V), thrombin and acetyl-salicylic acid (aspirin) were obtained from Sigma. The heptapeptide AYPGKF was custom-synthesized at Research Genetics. The heptapeptide YFLLRNP was custom-synthesized by New England Biolabs. Anti-phospho-PK<sub>C</sub>δ antibodies with specificity for threonine and tyrosine residues were obtained from Cell Signaling Technologies. Anti-GFP (green fluorescent protein) antibody and Protein-G Plus beads were obtained from Santa Cruz Biotechnology. YM-254890 was a gift from Yamanouchi England Biolabs. Anti-phospho-PKC<sub>δ</sub> antibodies with specificity for threonine and tyrosine residues were obtained from Cell Signaling Technologies. Anti-phospho-PKC<sub>δ</sub> antibodies with specificity for threonine and tyrosine residues were obtained from Cell Signaling Technologies. The heptapeptide YFLLRNP was custom-synthesized by New England Biolabs. Anti-phospho-PK<sub>C</sub>δ antibodies with specificity for threonine and tyrosine residues were obtained from Cell Signaling Technologies. Anti-phospho-PKC<sub>δ</sub> antibodies with specificity for threonine and tyrosine residues were obtained from Cell Signaling Technologies.

Animals

G<sub>δ</sub><sup>-</sup>-deficient mice were obtained from Dr T. Kent Gartner (Department of Microbiology and Molecular Cell Sciences, University of Memphis, Memphis, TN, U.S.A.) [39], with permission from Dr Stefan Offermanns (University of Heidelberg, Heidelberg, Germany).

Methods

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 and 2 g of glucose in 100 ml of deionized water). Blood was centrifuged (Eppendorf 5810R centrifuge) at 230 g for 20 min at room temperature (25 °C) to obtain PRP (platelet-rich plasma). PRP was incubated with 1 mM aspirin for 30 min at 37 °C. The PRP was then centrifuged for 10 min at 980 g at room temperature to pellet the platelets. Platelets were resuspended in Tyrode’s buffer (138 mM NaCl, 2.7 mM KCl, 1 mM MgCl<sub>2</sub>, 3 mM Na<sub>2</sub>HPO<sub>4</sub>, 5 mM glucose, 10 mM H<sub>2</sub>O<sub>2</sub>, pH 7.4, and 0.2% BSA) containing 0.01 unit/ml apyrase. The isolated platelets were subsequently used for experiments.

Platelet cell lysates preparation

Platelets were stimulated with agonists for the appropriate time under non-stirring conditions at 37 °C. The reaction was stopped by the addition of 3 × SDS/Laemmli’s buffer. Platelet lysates were boiled for 10 min and stored for Western blot analysis.

COS7 cell lysate preparation

COS7 cells were grown in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C with 5% CO<sub>2</sub>. COS7 cells were initially co-transfected transiently with PAR4 receptor for overexpression using Lipofectamine<sup>™</sup> 2000 (Invitrogen). PAR4-overexpressing COS7 cells were co-transfected further transiently with either GFP-tagged wild-type PK<sub>C</sub>δ or GFP-tagged T505A mutant PK<sub>C</sub>δ. The COS7 cells were activated with thrombin and the reaction was stopped by adding ice-cold PBS. The cells were lysed using cell lysis buffer (Cell Signaling Technologies), and PK<sub>C</sub>δ was immunoprecipitated using the anti-GFP antibody.

Western blot analysis

Lysates prepared from platelets or COS7 cells were separated by SDS/10% PAGE and transferred on to PVDF membrane. Non-specific binding sites were blocked by incubation in TBST (Tris-buffered saline/Tween 20: 20 mM Tris/HCl, pH 7.4, 140 mM NaCl and 0.1% Tween 20) containing 0.5% milk protein and 3% (w/v) BSA for 30 min at room temperature, and membranes were incubated overnight at 4 °C with the primary antibody (1:1000 dilution in TBST with 2% BSA) with gentle agitation. After three washes for 5 min each with TBST, the membranes were probed with an alkaline-phosphatase-labelled secondary antibody (1:5000 dilutions in TBST with 2% BSA) for 1 h at room temperature. After additional washing steps, membranes were then incubated with CDP-Star chemiluminescent substrate (Tropix) for 10 min at room temperature, and immunoreactivity was detected using a Fuji Film Luminescent Image Analyzer (LAS-1000 CH).

Statistical analysis

Western blots were analysed using ImageJ software (NIH). Results are expressed as fold increase over control. Significance was tested using Student’s t test. P < 0.05 was considered significant and is indicated by a single or double asterisk.

RESULTS

G<sub>δ</sub> and G<sub>12/13</sub> pathways mediate phosphorylation on PKC<sub>δ</sub>

The phosphorylation of the activation loop threonine residue (Thr<sup>505</sup>) on PKC<sub>δ</sub> is dependent on the G<sub>δ</sub>-mediated generation of DAG [40]. We investigated whether DiC<sub>8</sub>, a stable synthetic analogue of DAG, can cause tyrosine (Tyr<sup>311</sup>) phosphorylation on PKC<sub>δ</sub>. Isolated and aspirin-treated platelets were activated with various concentrations of DiC<sub>8</sub> and Thr<sup>505</sup> and Tyr<sup>311</sup> phosphorylation on PKC<sub>δ</sub> were measured by Western blotting. As shown in Figure 1A, DiC<sub>8</sub> resulted in robust phosphorylation on Thr<sup>505</sup> in a concentration-dependent manner. There was no detectable phosphorylation of Tyr<sup>311</sup> on PKC<sub>δ</sub> under these conditions. Similar results were also obtained with low concentrations of PMA (Figure 1B). However, very high concentrations of DiC<sub>8</sub> and 100 nM PMA resulted in both Thr<sup>505</sup> and Tyr<sup>311</sup> phosphorylation.
phosphorylation (results not shown). Since our goal is to identify the selective contribution of the Gαq and G12/13 pathways in PKCδ phosphorylation, we chose to use lower concentrations of DiC8 alone for the rest of our experiments. These results show that Gαq-mediated pathways are sufficient for Thr505 phosphorylation on PKCδ, but not for the Tyr311 phosphorylation.

Previous studies have shown that phosphorylation on Tyr311 of PKCδ occurs downstream of thrombin-activated PARs that couple to both Gαq and G12/13 pathways in platelets [28,31,36]. Given these findings, we investigated whether activation of the G12/13 pathways can induce phosphorylation of Tyr311 on PKCδ. Isolated and aspirin-treated platelets were activated with lower concentrations of YFLLRNP, which selectively activates the G12/13 pathways in platelets [37]. It is well established that low concentrations of YFLLRNP result in G12/13 activation without any increase in intracellular calcium levels. Thr505 and Tyr311 phosphorylation on PKCδ were measured by Western blotting. As shown in Figure 1(C), YFLLRNP failed to cause phosphorylation of Tyr311 on PKCδ. As expected, there was no Thr505 phosphorylation downstream of G12/13 pathways. These data showed that the G12/13 pathways by themselves are not sufficient for Thr505 or Tyr311 phosphorylation of PKCδ. Similar results were obtained when platelets were activated with PAR4-selective peptide, AYPGKF, in the presence of the Gαq inhibitor YM-254890 (see below).

Concomitant signalling by Gαq and G12/13 pathways is essential for Tyr311 phosphorylation on PKCδ

Previous studies have demonstrated that tyrosine phosphorylation of Tyr311 on PKCδ required the presence of PMA [30,38]. This suggested that binding of PKCδ to DAG or its analogues is essential for Tyr311 phosphorylation on PKCδ. However, DiC8 by itself failed to result in Tyr311 phosphorylation under lower concentrations (Figure 1A). Given these observations, it is evident that signalling mechanisms other than DAG binding might be essential for Tyr311 phosphorylation of PKCδ. We hypothesized that DAG binding to PKCδ along with signalling downstream of the G12/13 pathways are essential for Tyr311 phosphorylation on PKCδ. Isolated and aspirin-treated platelets were stimulated with DiC8, YFLLRNP or both, and the phosphorylation events on PKCδ were measured by Western blotting. As shown in Figure 2, phosphorylation on Tyr311 occurred only when both DiC8 and YFLLRNP were used together as agonists. Consistent with results shown in Figure 1, phosphorylation of Thr505 occurred only when both DiC8 and YFLLRNP were used together as agonists. Consistent with results shown in Figure 1, phosphorylation of Thr505 occurred even when DiC8 was present in a concentration of 4 μM. Phosphorylation of Tyr311, however, failed to occur when stimulated with DiC8 or YFLLRNP alone. Similar findings were obtained in experiments carried out with the PAR4 agonist peptide, AYPGKF, which was used to activate the G12/13 pathways in the presence of the Gαq inhibitor, YM-254890 (results not shown). These results suggested that both DAG binding to the C1 domain of PKCδ and G12/13-mediated signalling pathways are essential for phosphorylation of Tyr311 on PKCδ.

Phosphorylation of Tyr311 on PKCδ in Gαq-deficient mouse platelets

Activation of platelets with PAR agonists in Gαq-deficient mice results in robust activation of the G12/13 pathways [36,41]. We tested the hypothesis that the G12/13 pathway is required for Tyr311 phosphorylation of DAG-bound PKCδ. Isolated platelets obtained from Gαq-deficient mice were treated with DiC8, AYPGKF or both, and Thr505 and Tyr311 phosphorylation on PKCδ were measured by Western blotting. As shown in Figure 3, Tyr311 phosphorylation on PKCδ occurred only when platelets were activated with both DiC8 and AYPGKF. AYPGKF alone failed to cause phosphorylation of Thr505 or Tyr311, and DiC8 alone resulted in phosphorylation of Thr505 only. These results show that Tyr311 phosphorylation on PKCδ requires the conformational change induced by DAG binding along with signalling mediators generated downstream of G12/13 pathways, consistent with results from human platelets.
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Figure 2 Concomitant signalling by Gq and G12/13 pathways are essential for Tyr311 phosphorylation on PKCδ

Isolated and aspirin-treated platelets were treated with DiC8, YFLLRNP or both at 37°C. The reaction was stopped after 1 min by adding Laemmli’s buffer. The cell lysates were analysed for Thr505 and Tyr311 phosphorylation by Western blotting using phospho-specific antibodies as indicated [pT505, phospho-Thr505; pY311, phospho-Tyr311]. Total PKCδ was used for lane loading control. The blot shown is representative of experiments performed using platelets from three different donors. Blots were analysed and quantified as described in the Experimental section. */**P < 0.05 compared with controls with similarly shaded bars.

Figure 3 Phosphorylation of Tyr311 on PKCδ in Gαq-deficient mouse platelets

Isolated platelets from Gαq-deficient mice were treated with DiC8, AYPGKF or both at 37°C for 1 min. The reaction was stopped after 1 min by adding Laemmli’s buffer. The cell lysates were analysed for Thr505 and Tyr311 phosphorylation by Western blotting using phospho-specific antibodies as indicated [pT505, phospho-Thr505; pY311, phospho-Tyr311]. Total PKCδ was used for lane loading control. The blot shown is representative of experiments performed using platelets from three separate sets of pooled blood from knockout animals. Blots were analysed and quantified as described in the Experimental section. */**P < 0.05 compared with controls with similarly shaded bars.

SFKs downstream of G12/13 pathways are essential for Tyr311 phosphorylation of PKCδ

It is well established that Tyr311 phosphorylation on PKCδ is mediated by SFKs in platelets [22,28]. On the basis of our results that G12/13 pathway activation is essential for Tyr311 phosphorylation, we hypothesize that SFKs, activated downstream of G12/13 pathways, mediate Tyr311 phosphorylation of DAG-bound PKCδ. Isolated and aspirin-treated human platelets were activated with DiC8 or AYPGKF/YM-254890 or both in the presence or absence of the SFK inhibitor PP2, and Tyr311 phosphorylation on PKCδ was measured by Western blot analysis. As shown in Figure 4(A), PP2 inhibited Tyr311 phosphorylation on PKCδ resulting from concomitant activation of platelets by DiC8 and AYPGKF/YM-254890. Consistent with results published previously [28], PP2 has no effect on the Thr505 phosphorylation under any conditions. PP3 was used as a negative control for PP2. There was no difference between using PP3 and DMSO in our experiments (results not shown). These results showed that the G12/13 pathway causes activation of SFKs and that this activation is essential for Tyr311 phosphorylation of DAG-bound PKCδ.

Results obtained from experiments carried out using human platelets were confirmed using platelets obtained from Gαq-deficient mice. As shown in Figure 4(B), inhibition of SFKs completely inhibited the Tyr311 phosphorylation resulting from simultaneous activation of platelets with DiC8 and AYPGKF. As shown with human platelets, PP2 had no effect on the Thr505 phosphorylation of PKCδ in Gαq-deficient mouse platelets.

Phosphorylation on Thr505 is a prerequisite for Tyr311 phosphorylation on PKCδ

In vitro studies have shown that SFKs are able to phosphorylate Tyr311 on PKCδ only in the presence of PMA in the reaction medium [30,38]. This finding implies that PMA binding to PKCδ induces conformational change/phosphorylations that are essential for SFKs to phosphorylate Tyr311. However, the relationship between Thr505 and Tyr311 phosphorylation is not clear. We have used PAR4-expressing COS7 cells that express GFP-tagged PKCδ and have Thr505 mutated to alanine in order to investigate the role of Thr505 phosphorylation on Tyr311 phosphorylation in vivo. As shown in Figure 5, thrombin-mediated Tyr311 phosphorylation is abolished completely in cells that express the T505A PKCδ mutant. Tyr311 phosphorylation is preserved in wild-type cells. As expected, there was no Thr505 phosphorylation in the mutant cells. This result shows that the DAG-mediated conformational change alone is not sufficient for SFK-mediated Tyr311 phosphorylation. Phosphorylation at Thr505 is essential for subsequent SFK-mediated Tyr311 phosphorylation.

DISCUSSION

PKCδ is a novel PKC isoform which is regulated through extensive phosphorylations on key threonine, serine and tyrosine...
residues. PKCδ has been implicated in platelet dense granule release and thromboxane generation [28–30]. In the present study, we have identified fundamental mechanisms by which two of the key residues (Thr505 and Tyr311) of PKCδ are phosphorylated downstream of G-protein-activated pathways in platelets. We find that Thr505 phosphorylation is dependent on Gq-mediated DAG generation, whereas Tyr311 phosphorylation requires DAG binding to PKCδ, Thr505 phosphorylation and activation of SFKs by G_{12/13} pathways. This is the first study to demonstrate a selective requirement of G-protein-activated pathways for key phosphorylation events on PKCδ, in addition to defining the unique temporal relationship between the highly conserved Thr505 phosphorylation and the non-conserved Tyr311 phosphorylation on PKCδ.

Translocation of PKCδ from the cytosol to the membranes requires the generation of DAG downstream of G_q-mediated phospholipase C activation [37,40]. A sustained presence of DAG in the membranes of activated cells engages more PKCδ molecules to the membranes, causing a net increase in the number of PKCδ molecules translocating to the membrane fraction of the cells. PKCδ that is translocated to the membrane fraction is subsequently phosphorylated at the Thr505 residues. As shown in Figure 1(A), the addition of DiC8 results in robust phosphorylation of Thr505 on PKCδ in a concentration-dependent manner, but not Tyr311 phosphorylation. The presence of strong Thr505 phosphorylation by DiC8 and PMA at lower concentrations confirms that robust translocation of PKCδ to the membranes occurs under these conditions. This suggests that translocation of PKCδ to the membranes is not sufficient for Tyr311 phosphorylation. These data are contradictory to the proposal by Hall et al. [30], who concluded that allosteric activation of PKCδ by PMA and membrane translocation of the kinase are sufficient for Tyr311 phosphorylation. They also concluded that basal level activation of SFK is enough to cause Tyr311 phosphorylation of translocated PKCδ. If this were true, lower concentrations of DiC8 used in the present study that cause Thr505 phosphorylation should also induce Tyr311 phosphorylation. Consistent with results shown by Hall et al. [30], higher concentrations of DiC8 (>20 μM) in the present study resulted in Tyr311 phosphorylation (results not shown). The use of lower concentrations of DiC8 eliminates non-specific activation of various kinases that could contribute to Tyr311 phosphorylation. We therefore measured Thr505 and Tyr311 phosphorylation at various doses of PMA. Lower doses of PMA (1 nM) caused only Thr505 phosphorylation, but no Tyr311 phosphorylation (Figure 1B). Higher doses of PMA caused both Thr505 and Tyr311 phosphorylation. Hall et al. [30] used a single concentration (100 nM) of PMA and observed both Thr505 and Tyr311 phosphorylation. The use of higher concentrations of PMA or DiC8 can cause secretion from the platelet granules in addition to non-specific activation of other C1-containing kinases and hence could complicate the interpretations. The low concentration of DiC8 and PMA used in the present study failed to cause
any granule secretion (results not shown). Alternatively, it is possible that Tyr311 phosphorylation on PKCδ is dependent on the extent of translocation of the kinase to the membranes. At lower concentrations, the extent of translocation is not enough for Src PTKs (protein tyrosine kinases) (that are active in unstimulated platelets) to tyrosine-phosphorylate PKCδ. But at very high DAG concentrations, the extent of PKCδ translocation that occurs is enough for the Src PTK to tyrosine-phosphorylate PKCδ.

Our studies demonstrate that activation of SFKs downstream of G_{12/13} pathways and DAG-mediated PKCδ translocation is essential for Tyr311 phosphorylation (Figure 4). Stimulation of the G_{12/13} pathways is known to result in SFK activation in platelets [42]. The exact identity of the SFK family member(s) that is activated downstream of G_{12/13} is not known. Binding of DAG to PKCδ leads to conformational change that makes PKCδ a substrate for G_{12/13}-activated SFKs. However, it is not clear whether the association between PKCδ and the SFK member is mediated directly or indirectly through an adaptor protein.

Stimulation of the G_{12/13} pathways may lead to a complex formation between the adaptor protein and SFK, which may associate further with membrane-bound PKCδ. Further studies are required to delineate the exact mechanism underlying G_{12/13}-mediated Tyr311 phosphorylation of PKCδ. Although the present study shows the contribution of G_{12/13} pathways towards Tyr311 phosphorylation, we cannot rule out the contribution of signalling events downstream of G_{12/13} pathways, such as intracellular calcium increases and tyrosine kinases, regulating this phosphorylation. The DAG requirement for Tyr311 phosphorylation demonstrated in the present study is consistent with studies carried out in cardiac myocytes, which demonstrated that Tyr311 phosphorylation occurs only in the presence of phorbol esters [38].

Data from the present and previous studies demonstrate that SFK mediates Tyr311 phosphorylation of PKCδ only in the presence of DAG or PMA [30,38]. This implies that the conformational change on PKCδ induced by DAG binding makes the kinase a suitable target for SFK-mediated tyrosine phosphorylation. Phosphorylation of Thr505 is often used as a surrogate marker for translocation and subsequent activation of PKCδ [43]. Even though the role of DAG binding on Tyr311 phosphorylation is known, it is not clear whether Thr505 phosphorylation is required for the activated SFK to cause Tyr311 phosphorylation. In the present study, we demonstrated, using cell biological approaches, that Thr505 phosphorylation is a prerequisite for Tyr311 phosphorylation (Figure 5). This indicates that, in addition to the conformational change mediated by DAG, phosphorylation at Thr505 is essential for the SFK-mediated Tyr311 phosphorylation of PKCδ. The exact importance of such a temporal relationship between the two phosphorylation events is not clear. Hall et al. [30] have shown that the Tyr311 phosphorylation on PKCδ is a prerequisite for Tyr311 phosphorylation on PKCδ.
phosphorylation on PKCδ enhances its kinase activity in platelets. Other studies have shown that tyrosine phosphorylation of PKCδ accelerates proteolytic degradation in Src-transformed NIH 3T3 cells [25]. However, such a phenomenon has not been proved to occur in physiological systems until now. It is already known that Tyr311 phosphorylation is not required for Thr565 phosphorylation on PKCδ, since inhibition of SFKs using PP1 or PP2 does not have any effect on the Thr565 phosphorylation [28]. This is consistent with studies that have shown that tyrosine phosphorylation of PKCδ is not required for membrane recruitment [30].

Previous studies from our laboratory have shown that tyrosine phosphorylation of PKCδ is essential for PAR-mediated thromboxane A2 generation in platelets [28]. Given that Tyr311 phosphorylation is dependent on G12/13 pathways, it is possible that PAR-mediated thromboxane A2 generation requires concomitant G12 and G12/13 activation and this may occur by way of Tyr311 phosphorylation of PKCδ.

In conclusion, the present study demonstrates, for the very first time, the temporal relationship between the phosphorylation of two key residues on PKCδ, namely Thr565 and Tyr311. Whereas DAG binding alone is sufficient for Thr565 phosphorylation, Tyr311 phosphorylation has several requirements, including DAG binding, Thr565 phosphorylation and G12/13-mediated SFK activation. A schematic model summarizing the differential regulation of PKCδ phosphorylation in platelets is shown in Figure 6.

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