Coincident regulation of PKCδ in human platelets by phosphorylation of Tyr^{311} and Tyr^{565} and phospholipase C signalling

Kellie J. HALL, Matthew L. JONES and Alastair W. POOLE[1]

Department of Pharmacology, School of Medical Sciences, University of Bristol, University Walk, Bristol BS8 1TD, U.K.

PKC (protein kinase C)δ plays a complex role in platelets, having effects on both positive and negative signalling functions. It is phosphorylated on tyrosine residues in response to thrombin and collagen, and it has recently been shown that Tyr^{311} is phosphorylated in response to PAR (protease-activated receptor) 1 and PAR4 receptor activation. In the present study, we show that Tyr^{311} and Tyr^{565} are phosphorylated in response to thrombin, and have examined the interplay between phosphorylation and the classical lipid-mediated activation of PKCδ. Phosphorylation of both Tyr^{311} and Tyr^{565} is dependent on Src kinase and PLC (phospholipase C) activity in response to thrombin. Importantly, direct allosteric activation of PKCδ with PMA also induced phosphorylation of Tyr^{311} and Tyr^{565}, and this was dependent on the activity of Src kinases, but not PLC. Membrane recruitment of PKCδ is essential for phosphorylation of this tyrosine residue, but tyrosine phosphorylation is not required for membrane recruitment of PKCδ. Both thrombin and PMA induce recruitment of PKCδ to the membrane, and for thrombin, this recruitment is a PLC-dependent process. In order to address the functional role of tyrosine residue phosphorylation of PKCδ, we demonstrate that phosphorylation can potentiate the activity of the kinase, although phosphorylation does not play a role in membrane recruitment of the kinase. PKCδ is therefore regulated in a coincident fashion, PLC-dependent signals recruiting it to the plasma membrane and by phosphorylation on tyrosine residues, potentiating its activity.

Key words: phospholipase C (PLC) phosphorylation, protease-activated receptor (PAR), protein kinase C (PKC), Src family kinase (SFK), thrombin.

INTRODUCTION

The PKC (protein kinase C) family is composed of ten isoenzymes, which are grouped into three classes: conventional (α, γ, βI, βII), novel (δ, ε, η/L and θ) and atypical (ζ and ι/λ) [1]. Of these PKC members, human platelets predominantly express four isoforms: α, β, δ and θ [2–8]. The PKC family has long been known to be involved in a number of platelet processes, with the most important of these processes being aggregation and secretion, where stimulation of platelets with DAG (diacylglycerol) or phorbol ester can induce aggregation, and agonist-induced secretion can be prevented by pharmacological inhibition of a broad range of PKC isoforms [9–12]. However, individual PKC isoforms are likely to perform distinct roles, and we have recently shown that PKCδ plays a critical role in the negative regulation of collagen-induced platelet aggregation, through the inhibition of VASP (vasodilator-stimulated phosphoprotein)-mediated filopodia formation [13].

Activation mechanisms for PKC family members involve the summation or synergy between multiple input signals, including classically cytosolic free elements, such as calcium and DAG. These signals have, in turn, been thought to regulate the recruitment of PKCs to the plasma membrane, where the kinase is able to be fully activated, and where it encounters many of its substrates. Recently, it has been shown that PKCα is capable of very rapid movement to and from the plasma membrane [14], which is controlled by rapid changes in cellular calcium levels, demonstrating that PKC is a key readout sensor for the wide variety of transient calcium responses seen in many cell types.

Additionally, PKC isoforms undergo a series of phosphorylation events on serine/threonine residues, and, for PKCδ in particular, tyrosine residues are also phosphorylated [3,15–29]. Human PKCδ contains 20 tyrosine residues, several of which have been shown to be phosphorylated, including Tyr^{52}, Tyr^{155}, Tyr^{187}, Tyr^{311}, Tyr^{332}, Tyr^{525} and Tyr^{565} [30]. These residues lie primarily within either the catalytic domain or the hinge region of PKCδ, and are generally not conserved between other PKC family members, making regulation by tyrosine phosphorylation relatively specific to PKCδ. PKCδ has been shown to be phosphorylated on tyrosine residues in human platelets in response to agonists [31–33], including thrombin, and it has also recently been demonstrated that Tyr^{311} is phosphorylated in response to PAR (protease-activated receptor) 1 and PAR4 activatory peptides [33]. Tyr^{311} lies within the hinge region of PKCδ, between the C1 and catalytic domains, and sits within an optimal Src substrate consensus sequence. Phosphorylation of this residue in other cell types has been linked to increased kinase activity and altered down-regulation or degradation of the kinase [34], although the role of this phosphorylation in platelets has not been addressed directly.

It was decided to assess the causal relationship between tyrosine phosphorylation of PKCδ and other mechanisms of activation of the kinase, in particular, activation by membrane recruitment and activation of PLC (phospholipase C). We show that PKCδ is phosphorylated on Tyr^{311} and Tyr^{565} in response to thrombin, and that this event potentiates the activity of the kinase. Phosphorylation of tyrosine residues does not appear to regulate the recruitment of PKCδ to the membrane, but membrane localization is critical for the phosphorylation of PKCδ on Tyr^{311} and Tyr^{565}.

Abbreviations used: DAG, diacylglycerol; ECL, enhanced chemiluminescence; GPIb, glycoprotein Ib; NP40, Nonidet P40; PAR, protease-activated receptor; PKC, protein kinase C; PLC, phospholipase C; PTP, protein tyrosine phosphatase; SFK, Src family kinase.

[1] To whom correspondence should be addressed (email a.poole@bris.ac.uk).

© The Authors Journal compilation © 2007 Biochemical Society

These data address the functional interplay between the various input signals for PKCδ in platelets, and reveal PKCδ as a coincidence effector for PLC-dependent signalling and tyrosine phosphorylation in these cells.

EXPERIMENTAL

Materials

The anti-phosphotyrosine monoclonal antibody, 4G10, anti-[Src (pTyr^{416})] clone9A6, anti-Src antibody and recombinant PTP (protein tyrosine phosphatase)-1B were bought from Upstate Biotechnology. Phospho-specific antibodies against specific tyrosine sites on PKCδ and anti-GPIb (glycoprotein Ib) (anti-CD42b antibody) were purchased from Santa Cruz Biotechnology. Monoclonal anti-PKCδ antibody was purchased from BD Biosciences. Anti-actin antibody was purchased from Lab Vision Corporation. Horseradish-peroxidase-conjugated secondary antibodies, ECL® (enhanced chemiluminescence) kits and [γ-^{32}P]ATP were purchased from Amersham Biosciences. Collagen was bought from Nycomed and Complete™ mini protease inhibitor tablets were from Roche Applied Science. Monoclonal anti-β-tubulin antibody, monoclonal anti-α-tubulin antibody, protein phosphatase inhibitor cocktail mixture I, bovine thrombin, ADP and Protein A–Sepharose were all purchased from Sigma–Aldrich. The PAR-1 specific agonist Ser-Phe-Leu-Leu-Arg-Asn and PAR-4 specific agonist Ala-Tyr-Pro-Gly-Lys-Phe were from Bachem. GF109203X (an inhibitor of PKC), PMA, U73122 and PP2 were all bought from Tocris. Piceatannol and PP1 were purchased from Alexis Corporation. PKCδ peptide substrate, PP3 and LFM-A13 were purchased from Calbiochem. Anti-[PKCδ (pThr^{505})] antibody was from Cell Signalling Technology. All other reagents were of analytical grade.

Preparation and stimulation of human platelets

Human blood was drawn from healthy, drug-free volunteers on the day of the experiment and diluted 1:20 into 4 % sodium citrate, which acts as an anticoagulant. ACD (acid citrate dextrose) [120 mM sodium citrate, 110 mM glucose, 80 mM citric acid, used at 1:7 (v/v)] was used as a further anticoagulant. PRP (platelet-rich plasma) was prepared by centrifugation at 180 ***g*** for 20 min at 30 °C, and platelets were then isolated by centrifugation at 550 ***g*** for 10 min at 30 °C in the presence of 40 ng/ml PGE_1 (prostaglandin E_1). The resultant pellet was resuspended to a density of 4×10^8 platelets/ml in a modified Tyrode's-Hepes buffer (145 mM NaCl, 2.9 mM KCl, 10 mM Hepes, 1 mM $MgCl_2$ and 5 mM glucose, pH 7.3). Indomethacin (10 μM) was added to this platelet suspension which was then incubated for 30 min before stimulation. All platelet stimulation experiments were performed in the presence of 1 mM EGTA. Platelets were pre-incubated with different inhibitors or the vehicle solution (DMSO) for 10 min at 37 °C, and stimulated in an aggregometer (Chrono-Log Corporation) at 37 °C, with continuous stirring at 800 rev./min. The stimulation reactions were halted by either the addition of 5 × SDS sample buffer [24 mM Tris/HCl, pH 6.8, 10 % (v/v) glycerol, 0.8 % (v/v) SDS, 6 mM 2-mercaptoethanol and 0.04 % (w/v) Bromophenol Blue] to produce whole-cell lysate preparations or by the addition of 2 % NP40 (Nonidet P40) lysis buffer [100 mM Tris/HCl, pH 7.5, 300 mM NaCl, 20 mM EDTA, 1 mM Na_3VO_4 and 2 % (v/v) NP40 substitute] for immunoprecipitation.

Immunoprecipitation of PKCδ

Reactions were stopped by lysis of platelets with an equal volume of 2 % NP40 lysis buffer, plus Complete™ protease inhibitors. Lysates were pre-cleared with Protein A–Sepharose beads for 1 h. Antibody–Protein A complexes allowed to form by incubation of Protein A–Sepharose with 1 μg of antibody for 1 h at room temperature (20 °C). Pre-cleared lysates were added to the antibody–Protein A complexes and incubated at 4 °C with constant rotation overnight. Immunoprecipitates were washed three times with 1 % NP40 lysis buffer before addition of 5 × SDS sample buffer, boiling for 5 min and resolution by SDS/PAGE.

SDS/PAGE and Western blotting

Proteins were resolved by SDS/PAGE (9–12 % gels). Samples were then transferred on to PVDF membranes (Millipore), blocked with 5–10 % (w/v) BSA in TBS (Tris-buffered saline: 25 mM Tris and 1.4 M NaCl) and 0.1 % (v/v) Tween 20, and incubated for 1 h or overnight at room temperature with the appropriate primary antibody. Membranes were then washed before incubation with the appropriate horseradish-peroxidase-conjugated secondary antibody, followed by thorough washing. Bound peroxidase activity was detected using ECL®.

In vitro kinase assays

PKCδ was immunoprecipitated from NP40 lysates as described above and washed three times with 1 % NP40 lysis buffer containing 0.5 mM Na_3VO_4. Some of the thrombin-treated samples were dephosphorylated by exposure to 1 μg of recombinant PTP-1B (specific activity 13 nmol/min per μg as determined using *p*-nitrophenyl phosphate) for 10 min at 37 °C. All immunoprecipitates were then washed three times with 1 % NP40 lysis buffer containing 1 mM Na_3VO_4 and once in kinase assay buffer (100 mM NaCl, 20 mM Hepes, 5 mM $MgCl_2$ and 5 mM $MnCl_2$, pH 7.2). Immunoprecipitates were resuspended in 20 μl of the kinase assay buffer, along with 10 μg of PKCδ peptide substrate. The reactions were started by the addition of 10 μl of ATP buffer (0.15 mM ATP, 30 mM $MgCl_2$ and 200 μCi of [γ-^{32}P]ATP/ml). After incubation at 30 °C for 30 min, the reaction was terminated by the addition of 10 % phosphoric acid, and samples were subsequently blotted on to 3 cm^2 squares of P81 ion-exchange chromatography paper. The P81 papers were washed five times in 0.5 % phosphoric acid, followed by a wash in acetone. The papers were then dried, and labelled PKCδ peptide substrate was quantified by liquid-scintillation counting.

Subcellular fractionation of human platelets

Basal and stimulated platelets were resuspended in ice-cold 2 × sonication buffer (final concentration of 320 mM sucrose, 4 mM Hepes and 0.5 mM Na_3VO_4, pH 7.4) containing Complete™ protease inhibitors, and sonicated for four 20 s pulses. Intact platelets were removed by centrifugation at 1500 ***g*** for 10 min at 4 °C before centrifugation at 100 000 ***g*** for 60 min at 4 °C. The supernatant was removed (cytosolic fraction) and the pellet (particulate fraction) was resuspended in Tris/HCl buffer [10 mM Tris/HCl, pH 7.2, 158 mM NaCl, 1 mM EGTA, 0.5 mM Na_3VO_4, 0.1 % (v/v) SDS, 1 % sodium deoxycholate and 1 % (v/v) Triton X-100] with Complete™ protease inhibitors. The protein concentrations were quantified using the BCA (bicinchoninic acid) assay (Sigma). Either equal protein concentrations of the fractions were resolved by SDS/PAGE and Western-blotted for tubulin or GPIb to confirm that fractionation had occurred, or each fraction was immunoprecipitated for PKCδ, resolved by SDS/PAGE and Western-blotted using anti-PKCδ or phospho-specific antibodies.

© The Authors Journal compilation © 2007 Biochemical Society

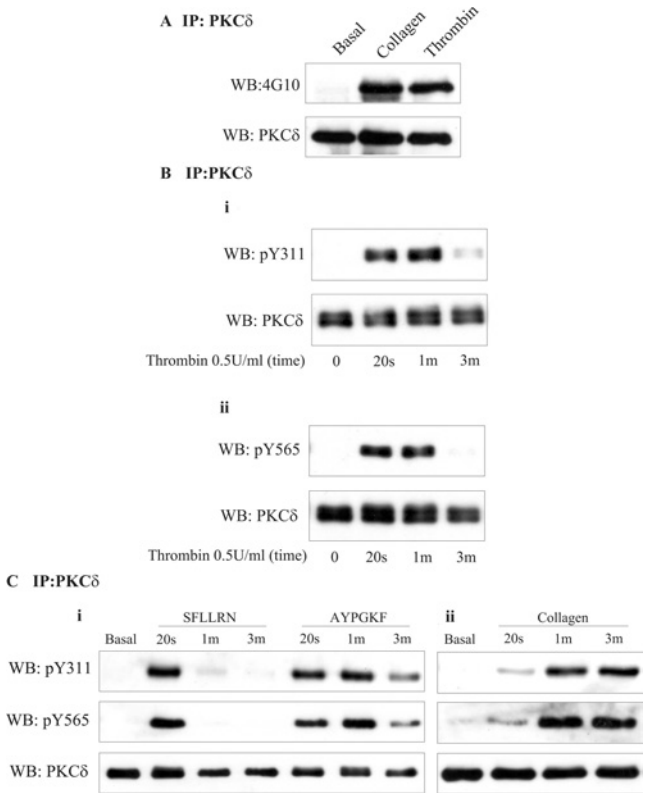

Figure 1 PKCδ is phosphorylated on Tyr^{311} and Tyr^{565} downstream of PAR1 and PAR4 activation

(**A**) Immunoprecipitation (IP) of PKCδ was performed from 1% NP40 lysates of platelets stimulated with 0.1 unit/ml thrombin for 1 min or 30 μg/ml collagen for 3 min. Proteins were separated by SDS/PAGE, transferred on to PVDF membranes and Western-blotted (WB) with the monoclonal anti-phosphotyrosine antibody, 4G10. Blots were reprobed with the anti-PKCδ antibody as indicated. (**B**) PKCδ was immunoprecipitated from 1% NP40 lysates of basal platelets or from platelets stimulated with 0.5 unit/ml thrombin for the time periods indicated. Samples were Western-blotted using phospho-specific antibodies directed against the PKCδ residues (i) Tyr^{311} (pY311) or (ii) Tyr^{565} (pY565). Samples were reprobed for PKCδ as indicated. (**C**) PKCδ was immunoprecipitated from 1% NP40 lysates from basal platelets or platelets stimulated for the indicated time periods with either (i) PAR1 or PAR4 agonist peptides SFLLRN (50 μM) or AYPGKF (500 μM) respectively, or (ii) collagen (30 μg/ml). Samples were Western-blotted for Tyr^{311} or Tyr^{565} as indicated. Membranes were stripped and reprobed for PKCδ to ensure equal loading. Blots are representative of three independent experiments.

Data analysis

Analysis of statistical significance was performed using one-way ANOVA with Bonferroni post-test if $P < 0.05$. In all cases, $P < 0.05$ was considered significant.

RESULTS

Thrombin induces phosphorylation of PKCδ on Tyr^{311} and Tyr^{565} in human platelets

PKCδ has been shown previously to be phosphorylated on tyrosine residues in multiple cell types, including platelets [3,31–33]. A recent report demonstrated that PAR1 and PAR4 peptides were able to induce phosphorylation of Tyr^{311} [33], a residue shown to be important in regulating the proteolytic degradation of PKCδ [34]. It was important to understand the interplay between PLC signalling and tyrosine phosphorylation of PKCδ, and whether these two events were interdependent or unrelated, and how they contributed to the activity of PKCδ. In the first instance, it was important to determine whether thrombin could induce phosphorylation of PKCδ on Tyr^{311} and other putative tyrosine phosphorylation sites. Both thrombin and collagen were able to induce phosphorylation of PKCδ on tyrosine residues (Figure 1A), and thrombin induced a transient phosphorylation of both Tyr^{311} and Tyr^{565} (Figure 1B), which was apparent at an early time point (20 s), but had disappeared by 3 min. This was paralleled by phosphorylation of these residues in response to peptide activation of the PAR1 and PAR4 receptors (Figure 1C), with the PAR1 activation profile being highly transient, and PAR4 activation being more sustained. A time course examining the phosphorylation states of Tyr^{311} and Tyr^{565} showed that, in the presence of collagen, phosphorylation occurred more slowly and achieved maximal phosphorylation by 3 min (Figure 1C, ii). It has been demonstrated that Tyr^{52}, Tyr^{155}, Tyr^{332} and Tyr^{525} may also be phosphorylated in other cell types [15,22,27,35–37]. Figure 2 shows that these tyrosine residues are not phosphorylated in response to thrombin, collagen or PAR1/4 peptides, although pervanadate (a PTP inhibitor, used as a positive control) was able to induce phosphorylation of these sites.

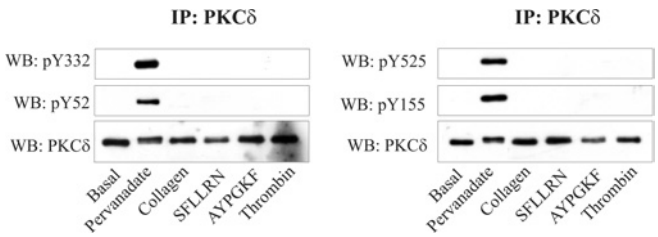

Figure 2 Collagen, SFLLRN, AYPGKF and thrombin do not induce phosphorylation of PKCδ on Tyr^{52}, Tyr^{155}, Tyr^{332} or Tyr^{525} in platelets

PKCδ was immunoprecipitated from: basal platelets, platelets stimulated with 50 μg/ml collagen for 3 min, 0.5 unit/ml thrombin for 1 min, 100 μM SFLLRN for 20 s or 500 μM AYPGKF for 1 min, or platelets treated with 200 μM pervanadate for 1 h at 37 °C. Immunoprecipitates (IP) were resolved by SDS/PAGE and phosphorylation of PKCδ was detected by Western blotting (WB) using phospho-specific antibodies directed against Tyr^{52} (pY52), Tyr^{155} (pY155), Tyr^{332} (pY332) or Tyr^{525} (pY525). Membranes were stripped and reprobed for PKCδ to ensure equal loading. Blots are representative of three independent experiments.

PLC signalling is necessary and sufficient to induce phosphorylation of PKCδ on Tyr^{311} and Tyr^{565} downstream of SFKs (Src family kinases)

Platelets express a variety of non-receptor tyrosine kinases that may be responsible for the phosphorylation of PKCδ at the residues Tyr^{311} and Tyr^{565}. Figure 3(A) shows that the SFK inhibitors, PP1 and PP2, can block thrombin-induced phosphorylation on these two sites, but the inactive analogue PP3, the Syk inhibitor piceatannol or the Btk inhibitor LFM-A13 could not block phosphorylation. Phosphorylation of PKCδ on Tyr^{311} and Tyr^{565} is therefore downstream of SFKs, but also requires PLC activity, since phosphorylation is blocked by the PLC inhibitor U73122.

Allosteric activation of PKCδ by the phorbol ester PMA, which bypasses receptor activation, is also able to induce phosphorylation of Tyr^{311} and Tyr^{565} in a Src kinase-dependent manner (Figure 3B). Because receptor activation is avoided, phosphorylation induced by PMA is not dependent on PLC activity, and is not blocked by U73122. It was possible that SFK activity was dependent on the presence of PMA, (and, in the case of thrombin, was dependent on DAG) or that these lipid signals recruit PKCδ to the membrane, where a basally-active SFK may phosphorylate it, or a combination of both. We therefore assessed whether PMA or

© The Authors Journal compilation © 2007 Biochemical Society

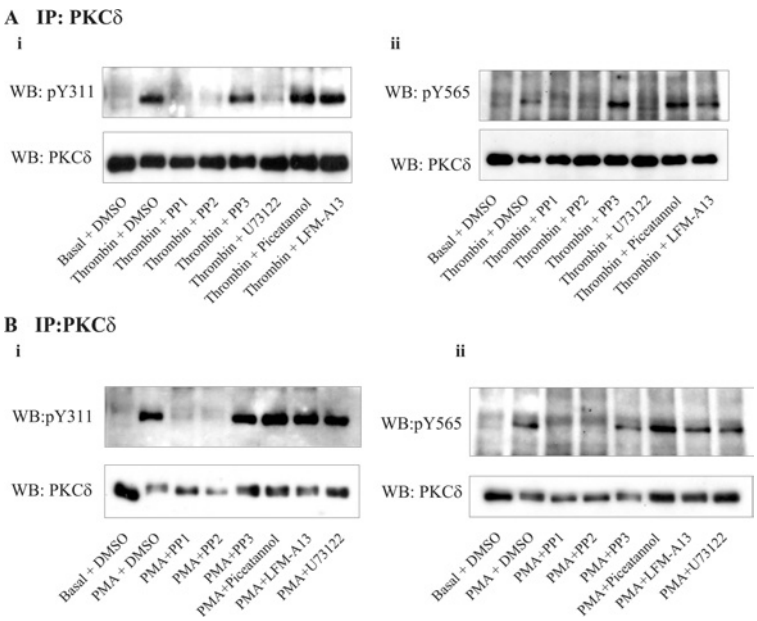

Figure 3 Tyrosine phosphorylation of PKCδ on Tyr^{311} and Tyr^{565} is inhibited by PP1, PP2 and U73122

Platelets were pre-incubated for 10 min with 0.1 % DMSO, 10 μM PP1, 10 μM PP2, 10 μM PP3, 10 μM U73122, 10 μg/ml piceatannol or 40 μM LFM-A13 as indicated before stimulation with (**A**) 0.5 unit/ml thrombin for 1 min or (**B**) 100 nM PMA for 5 min. (**A**, **B**) Platelets were lysed in 1 % NP40 lysis buffer and PKCδ was immunoprecipitated (IP) and Western-blotted (WB) for (i) pTyr^{311} (pY311) or (ii) pTyr^{565} (pY565). Membranes were stripped and reprobed for PKCδ to ensure equal loading. Blots are representative of three independent experiments.

thrombin was able to activate SFKs by using a phospho-specific antibody Src (pTyr^{416}), which recognizes the autophosphorylation site in SFKs, and is therefore a marker of activity in the cell. Figure 4 shows a basal level of SFK activity, which is ablated by PP1, and is increased approx. 2-fold on stimulation with thrombin. There is still substantial thrombin-dependent SFK activation in platelets which have been pre-treated with the PLC inhibitor U73122, and PMA alone is not able to induce SFK activation. These data suggest that basal activity of Src is sufficient to allow full phosphorylation of PKCδ on Tyr^{311} and Tyr^{565}, and that cellular localization of PKCδ is a more critical regulatory step than regulation of Src kinase activity alone.

Phosphorylation of PKCδ on Tyr^{311} and Tyr^{565} is not required for membrane recruitment, whereas membrane recruitment is required for phosphorylation on these residues

The data from Figures 3 and 4 were consistent with an activation process where PLC signalling products lead to the recruitment of PKCδ to the membrane, where SFKs are able to phosphorylate it. If this were true, then SFKs should not be required for membrane recruitment. Figure 5(A) shows that PKCδ translocates rapidly but transiently from the cytosol to membrane compartments, in a manner that parallels its tyrosine phosphorylation (Figure 1). Importantly, phosphorylation of Tyr^{311} and Tyr^{565} is present only in membrane-associated fractions and is not detected in the cytosolic fractions when PKCδ is localized to the cytosol. This is consistent with recruitment of PKCδ to the membrane being required for tyrosine residue phosphorylation. However, phosphorylation on tyrosine residues was not required for translocation, since inhibition of phosphorylation by PP1 had no effect on recruitment of the kinase to the membrane (Figure 5B). Conversely, membrane recruitment of PKCδ did require PLC activity, since inhibition of PLC by U73122 substantially reduced its translocation.

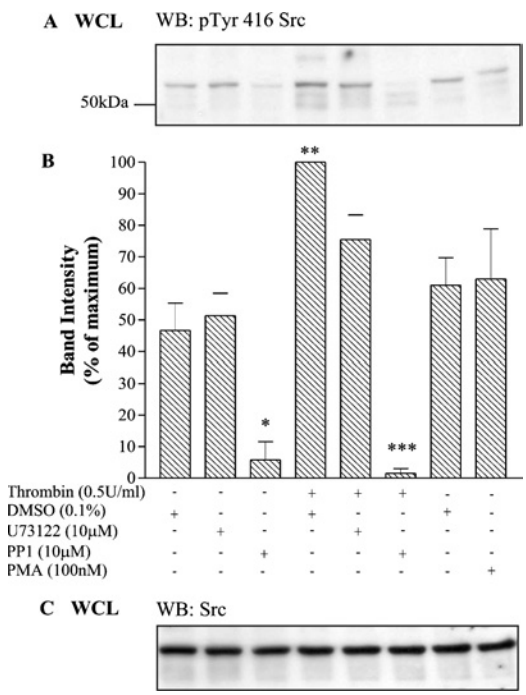

Figure 4 Thrombin, but not PMA, stimulates an increase in Src kinase activity which is inhibited by PP1, but not by U73122

Platelets were pre-incubated for 10 min with 0.1 % DMSO, 10 μM PP1 or 10 μM U73122 before stimulation with 0.5 unit/ml thrombin for 1 min or 100 nM PMA for 5 min. Platelets were lysed in Laemmli sample buffer and proteins were resolved by SDS/PAGE and transferred on to PVDF membranes. (**A**) Western blotting (WB) using [Src (pTyr^{416})] antibody. (**B**) Bands from (**A**) were quantified by densitometry and represented in the histogram as a percentage of maximal Src phosphorylation in response to thrombin. Results are means $\pm$ S.E.M ($n = 3$). * $P < 0.01$ compared with DMSO alone; ** $P < 0.001$ compared with DMSO alone; *** $P < 0.001$ compared with thrombin pre-treated with DMSO. (**C**) Membranes from (**A**) were reprobed using anti-Src antibody to ensure equal loading.

© The Authors Journal compilation © 2007 Biochemical Society

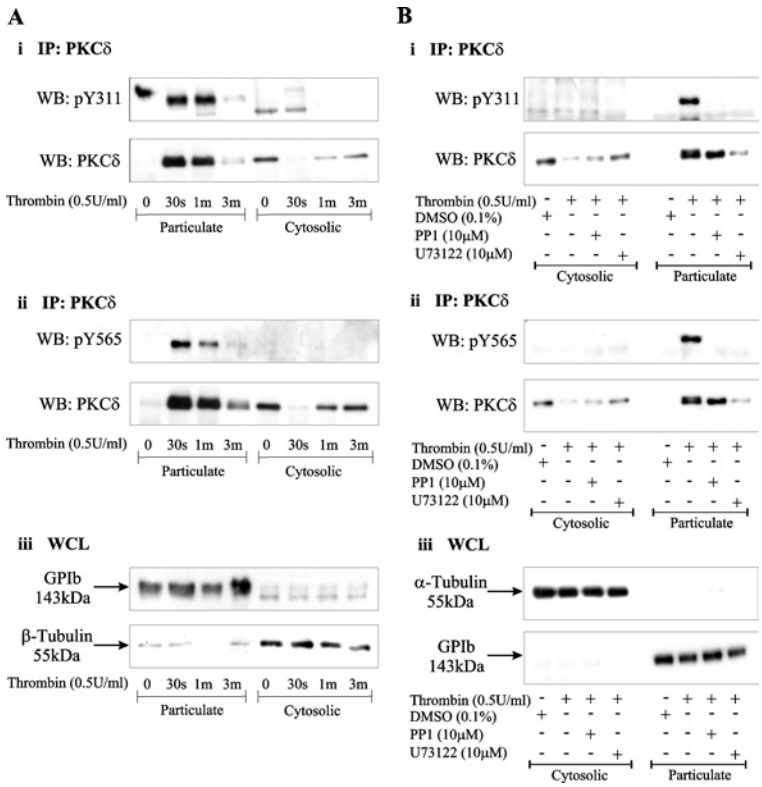

Figure 5 Thrombin stimulates transient translocation of PKCδ to the particulate fraction, which is downstream of PLC, but not Src kinase activation

(**A**) Platelets were stimulated for the indicated times with 0.5 unit/ml thrombin. (**B**) Platelets were pre-incubated for 10 min with 0.1 % DMSO, 10 μM PP1 or 10 μM U73122 before stimulation with 0.5 unit/ml thrombin for 1 min. Cytosolic and particulate fractions were isolated as described in the Experimental section. (**A**, **B**) PKCδ was immunoprecipitated (IP) from cytosol and particulate fractions and Western-blotted (WB) for (i) pTyr^{311} (pY311) or (ii) pTyr^{565} (pY565). Membranes were stripped and reprobed for PKCδ as indicated. (iii) Equal protein concentrations of particulate and cytosolic fractions were lysed in Laemmli sample buffer and resolved by SDS/PAGE. Samples were Western-blotted for α-tubulin or β-tubulin as cytosolic markers and GPIb as a membrane marker to ensure complete fractionation of platelets. Blots are representative of three independent experiments.

PMA, which bypasses receptor-mediated PLC activation, also induced membrane recruitment of PKCδ (Figure 6) and tyrosine phosphorylation at Tyr^{311} and Tyr^{565}, but only when the kinase was localized to the membrane. Examination of the time course of membrane recruitment and phosphorylation on Tyr^{311} or Tyr^{565} showed that membrane recruitment was rapid, reached a maximum level of recruitment by 30 s and this was sustained over 5 min. However, phosphorylation of Tyr^{311} and Tyr^{565} did not occur until after PMA stimulation for 5 min and 1 min respectively (Figure 6A). Again, PP1 abolished the tyrosine phosphorylation of PKCδ, but had no effect on membrane translocation (Figure 6B). In summary, these data are consistent with a model, in which PKCδ translocates to the membrane and is subsequently phosphorylated on Tyr^{311} and Tyr^{565} in a SFK-dependent manner.

Tyrosine phosphorylation of PKCδ potentiates its kinase activity

From Figures 5 and 6, it can be concluded that tyrosine phosphorylation of PKCδ has no role in the translocation of the kinase from the cytosol to membranes and membrane recruitment is a requirement for tyrosine phosphorylation. It was therefore important to ascertain whether tyrosine phosphorylation of Tyr^{311} and Tyr^{565} played a role in regulating the kinase activity of PKCδ. Figure 7(A, i) demonstrates that thrombin stimulation of platelets leads to an increased activity of PKCδ. This increased activity was partially inhibited when platelets were pre-treated with PP1, which abolished tyrosine phosphorylation of Tyr^{311} and Tyr^{565} (Figure 7A, ii). However, this inhibition was determined to be statistically non-significant. Importantly, owing to the other potential effects of SFK inhibition in the cell, we were able to dephosphorylate PKCδ *in vitro* using PTP1b (Figure 7A, ii). Dephosphorylation *in vitro* also partially reduced PKCδ activity (Figure 7A, i) to the same extent as PP1 when applied to platelets, but this was determined to be non-significant. This suggested that phosphorylation of Tyr^{311} and Tyr^{565} may partially contribute to and potentiate the kinase activation of PKCδ, at a step downstream of thrombin.

The activity of PKCδ in platelets was examined to determine whether kinase activity was similarly partially dependent on tyrosine residue phosphorylation by SFKs, as demonstrated *in vitro*. Figure 7(B) shows data demonstrating phosphorylation of PKCδ on Thr^{505}. Autophosphorylation of this site provides an indication of kinase activity in the cell, and a marked increase in phosphorylation of this residue is observed in the presence of thrombin (Figure 7B), confirming the activation of the kinase by thrombin in platelets. Pre-treatment of platelets with PP1, an inhibitor of SFKs, partially inhibits the activity of the kinase, paralleling data from the *in vitro* kinase assay (Figure 7A). As positive controls for the assay, PKCδ activity was also shown to be dependent on PLC activity, as an ablation of Thr^{505} phosphorylation was detected in the presence of U73122. PMA is also able to directly induce activation of PKCδ and phosphorylation on Thr^{505} (Figure 7B).

© The Authors Journal compilation © 2007 Biochemical Society

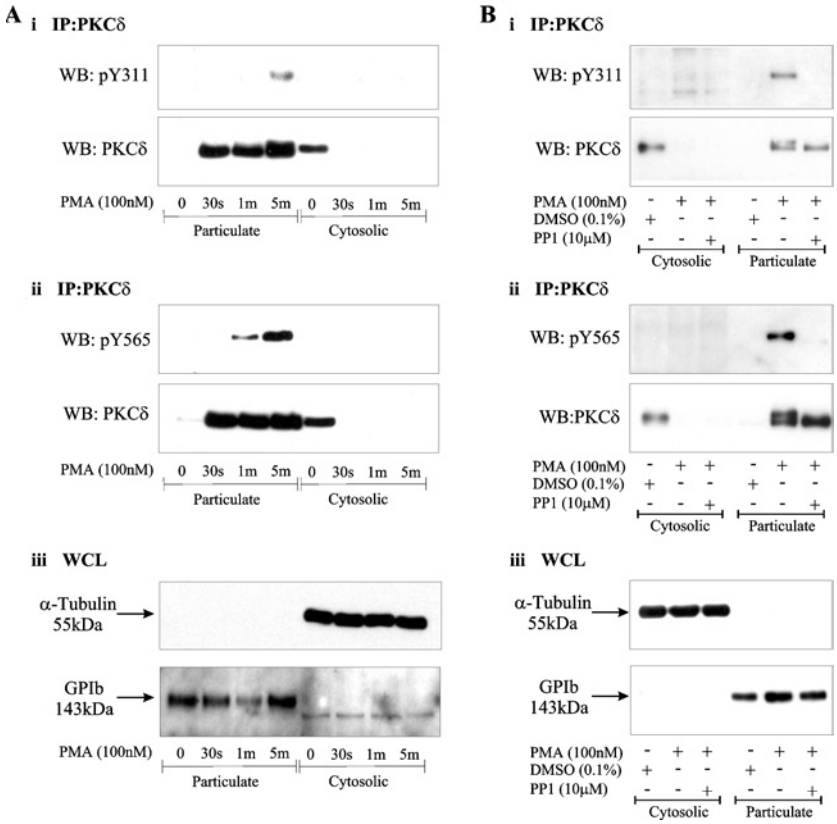

Figure 6 PMA stimulates translocation of PKCδ to the particulate fraction which is not inhibited by PP1

(**A**) Platelets were stimulated for the indicated times with 100 nM PMA. (**B**) Platelets were pre-incubated for 10 min with 0.1 % DMSO or 10 μM PP1 before stimulation with 100 nM PMA for 5 min at 37 °C. Cytosolic and particulate fractions were isolated as described in the Experimental section. (**A**, **B**) PKCδ was immunoprecipitated (IP) from cytosol or particulate fractions, and samples were Western-blotted (WB) for (i) pTyr^{311} (pY311) or (ii) pTyr^{565} (pY565). Membranes were stripped and reprobed for PKCδ as indicated. (iii) Particulate and cytosolic fractions were lysed in Laemmli sample buffer and resolved by SDS/PAGE. Samples were Western-blotted for α-tubulin as a cytosolic marker and GPIb as a membrane marker to ensure complete fractionation of platelets. Blots are representative of three independent experiments.

DISCUSSION

The PKC family plays essential roles in regulating functional activities in most mammalian cell types. Regulation of the kinase activity of the PKC family members is complex and involves multiple convergent mechanisms. For PKCδ, phosphorylation on tyrosine residues is a prominent feature, although its regulation and function in primary cells is not yet clear. In the present study we have demonstrated that, in human platelets, Tyr^{311} and Tyr^{565} become phosphorylated in response to activation of PAR1 and PAR4 receptors by thrombin. This phosphorylation was shown to be downstream of SFKs and required PLC activity, which allowed PKCδ to be targeted to a membrane localization, facilitating phosphorylation by SFKs present in that intracellular compartment. Therefore phosphorylation of tyrosine residues does not regulate the localization of PKCδ, but was shown to potentiate the activity of the kinase in the cell and also *in vitro*. It is possible that the role of phosphorylation of Tyr^{311} (hinge region) and Tyr^{565} (kinase domain) is to stabilize the active conformation of the kinase, at least *in vitro*.

Although it had been shown previously that PKCδ is phosphorylated on tyrosine residues in human platelets in response to thrombin [3,32], only one report had so far identified any phosphorylation sites in these cells: Murugappan et al. [33] have shown that Tyr^{311} was phosphorylated in response to activation by PAR1- and PAR4-selective peptide agonists, although phosphorylation was not demonstrated on any other tyrosine residues. In the present study, we have demonstrated that thrombin is able to induce phosphorylation on Tyr^{311}, but phosphorylation also occurs at Tyr^{565}, although not at Tyr^{52}, Tyr^{155}, Tyr^{332} or Tyr^{525}. The reason for the discrepancy between our study and that of Murugappan et al. [33] is not known at present. Throughout the present study, the phosphorylation status of Tyr^{565} has paralleled that of Tyr^{311}, and we were not able to deduce any circumstances in which phosphorylation of one residue would occur in the absence of the other. Blake et al. [34] have shown that, although PKCδ could be phosphorylated on multiple tyrosine residues, Tyr^{311} phosphorylation was a 'master' site, in that mutation of this residue to a non-phosphorylatable phenylalanine residue ablated phosphorylation on all other tyrosine residues in PKCδ. This may be the case in platelets as well, where Tyr^{311} may be required for the phosphorylation of Tyr^{565}, but we were not able to determine this. The time course of phosphorylation for each tyrosine residue was very rapid and did not differ substantially from each other, although for PAR4 peptide activation (Figure 1C), Tyr^{311} phosphorylation reached the maximal point by 20 s, whereas for Tyr^{565}, phosphorylation was not maximal until 1 min. This suggested that phosphorylation of Tyr^{311} may precede that of Tyr^{565}, at least in response to PAR4 activation, and therefore that Tyr^{311} phosphorylation may be a prerequisite for the phosphorylation of Tyr^{565}.

We showed that Tyr^{311} phosphorylation in response to thrombin occurs downstream of SFKs (Figure 3A). This is consistent with

© The Authors Journal compilation © 2007 Biochemical Society

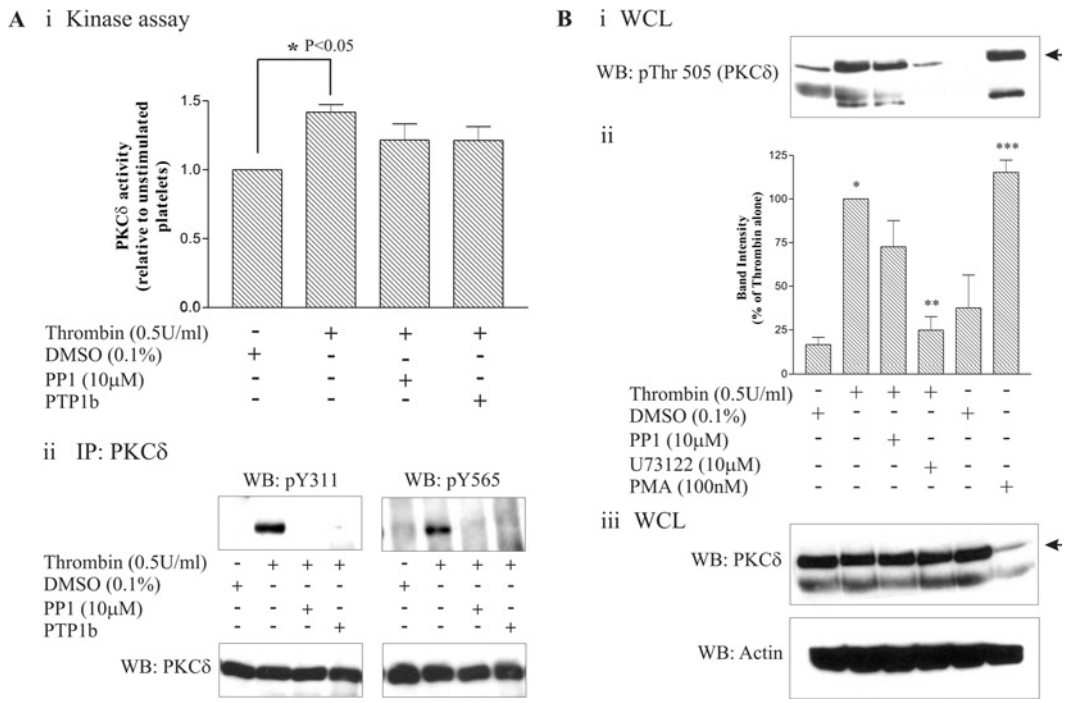

Figure 7 Tyrosine phosphorylation potentiates the kinase activity of PKCδ

(**A**) (i) PKCδ was immunoprecipitated (IP) from platelets pre-incubated with 0.1% DMSO or 10 μM PP1 before stimulation with 0.5 unit/ml thrombin for 1 min. Immunoprecipitates from thrombin-stimulated platelets were dephosphorylated using PTP1b, and an *in vitro* kinase assay was performed as described in the Experimental section. Results are means ± S.E.M. * $P < 0.05$ compared with unstimulated platelets. (ii) PKCδ immunoprecipitates were resolved by SDS/PAGE and transferred on to PVDF membranes. Samples were Western-blotted (WB) for pTyr^{311} (pY311) (left panel) or pTyr^{565} (pY565) (right panel) and reprobed for PKCδ as indicated to ensure equal loading. (**B**) Platelets were pre-incubated for 10 min with 0.1% DMSO, 10 μM PP1 or 10 μM U73122 before stimulation with either 0.5 unit/ml thrombin for 1 min or 100 nM PMA for 5 min. Platelets were lysed in Laemmli sample buffer, and proteins were resolved by SDS/PAGE and transferred on to PVDF membranes. (i) Samples were Western-blotted using anti-[PKCδ/(pThr^{505})] antibody (arrow corresponds to PKCδ). (ii) Bands from (i) were quantified by densitometry and expressed as a percentage of maximal PKCδ phosphorylation in response to thrombin. Results are means ± S.E.M ($n = 3$). * $P < 0.001$ compared with DMSO alone; ** $P < 0.001$ compared with thrombin alone; *, $P < 0.01$ compared with DMSO alone. (iii) The blots in (i) were reprobed using anti-PKCδ and anti-actin antibodies to ensure equal loading. The apparent reduction in immunoreactivity of PKCδ in samples taken from PMA-stimulated platelets (far right lane) has been noted previously [44] and is not likely to reflect a true reduction in protein loading, but rather an altered ability of PKCδ to bind to the antibody, possibly as a result of multiple phosphorylation. The reprobing of the lysates with the anti-actin antibody was also included to demonstrate equal loading of the protein.

Murugappan et al. [33], who have shown Src kinase-dependent phosphorylation of this residue in response to PAR1 and PAR4 activation. This is consistent with the fact that Tyr^{311} sits within a consensus sequence for Src phosphorylation. We have shown previously that PKCδ selectively complexes with the tyrosine kinase Fyn in activated conditions [31], and therefore this may be the SFK responsible for the phosphorylation of PKCδ in response to thrombin. PLC activity was also required for phosphorylation on both Tyr^{311} and Tyr^{565}, since phosphorylation was blocked by the PLC inhibitor U73122. This suggested either that the Src kinase responsible for the phosphorylation of PKCδ was activated downstream of PLC or that PKCδ recruitment to the plasma membrane and allosteric regulation was required to allow phosphorylation. However, we demonstrated that, although thrombin was able to induce activation of SFKs, this was largely independent of PLC activity, since U73122 did not significantly decrease Src activity in response to thrombin (Figure 4A). This suggests that the ablation of phosphorylation of PKCδ on Tyr^{311} and Tyr^{565} residues in the presence of U73122 may be because SFKs are able to phosphorylate PKCδ when it has undergone allosteric modulation by DAG. It should be noted that U73122 has been shown to have other actions as well as inhibition of PLC, including inhibition of PLD (phospholipase D) [38] and stimulation of calcium release [39]. It is therefore possible that inhibition of the translocation of PKCδ and its phosphorylation on tyrosine residues by this compound may be the result of an off-target inhibitory mechanism. The observation that PMA also induced phosphorylation of Tyr^{311} and Tyr^{565} indicated that membrane localization and allosteric regulation were important signals required for tyrosine phosphorylation. Consistent with this model, PMA had no effect on Src kinase activity alone. Basal activity of Src is sufficient to allow full phosphorylation of PKCδ on Tyr^{311} and Tyr^{565}, which is also consistent with our model. This suggests that cellular localization of PKCδ is a more critical regulatory step for controlling it tyrosine phosphorylation than regulation of Src kinase activity.

We therefore propose from the data that phosphorylation of PKCδ on Tyr^{311} and Tyr^{565} occurs in a Src kinase-dependent manner in response to thrombin, but only when PKCδ is allosterically activated. This suggests that tyrosine phosphorylation may only occur when PKCδ has translocated to the particulate fraction. It was demonstrated that thrombin induced a transient membrane translocation of the kinase, and that PKCδ is phosphorylated on Tyr^{311} and Tyr^{565} only when translocated to the membrane (Figure 5A). Indeed, at later time points (3 min), when PKCδ had re-entered the cytosolic fraction, the kinase was shown to no longer be phosphorylated on tyrosine residues. These data suggest that the early translocation to the membrane would occur independently from tyrosine phosphorylation and that phosphorylation occurred subsequently to the translocation event.

© The Authors Journal compilation © 2007 Biochemical Society

Using PP1, which inhibits tyrosine phosphorylation, we showed that tyrosine phosphorylation was not required for translocation to the membrane, whether induced by thrombin or by PMA (Figures 5B and 6). These data sugges that, in the case of PMA which does not significantly increase the activity of Src above basal levels, translocation to the plasma membrane for sustained periods is sufficient to allow slow phosphorylation by Src on Tyr^{311} and Tyr^{565} (Figure 6A). For thrombin, which induces a doubling in Src activity (Figure 4), the kinetics of phosphorylation of PKCδ on tyrosine residues is markedly more rapid (Figure 5A). However, although U73122 substantially reduces the translocation of PKCδ to the membrane, it does not abolish it (Figure 5), but phosphorylation of Tyr^{311} and Tyr^{565} was completely ablated (Figure 5). This implies that translocation alone may not be sufficient to allow phosphorylation, at least over a 1 min period when induced in response to thrombin. It is still not clear also whether tyrosine dephosphorylation is required to allow PKCδ to detach from the membrane at later time points after activation, or whether PKCδ is dephosphorylated rapidly after membrane detachment. Similarly, the nature of the tyrosine phosphatase responsible for this dephosphorylation event is not currently known.

Since tyrosine phosphorylation of PKCδ did not regulate its translocation to the membrane, it was important to address whether it may regulate the activity of PKCδ. By using an *in vitro* kinase assay and an assay of autophosphorylation on Thr^{505} of PKCδ, we were able to show that inhibition of tyrosine phosphorylation or removal of phosphates from tyrosine residues *in vitro* could diminish partially the activity of PKCδ (Figure 7). This reduction in activity was non-significant, compared with thrombin-stimulated levels of activity; however, there was a clear trend towards a reduction in activity which was comparable between experiments. These data were similar to the results on phosphorylation of PKCδ on Thr^{505}, where a non-significant reduction in phosphorylation in the presence of PP1 was shown (Figure 5B), although a consistent trend towards inhibition was observed in experiments. It should be noted that, unlike other isoforms of PKC, phosphorylation of Thr^{505} in the activation loop of the kinase is not essential for PKCδ activation [40,41]. Phosphorylation of Thr^{505} is not necessarily as a result of autophosphorylation, as PKD1 has been shown to also phosphorylate this site [42]. Phosphorylation of Thr^{505} may not reflect the activity of PKCδ, but it is clear that the response to thrombin in the presence and absence of PP1 is parallel (Figure 7). This suggests that phosphorylation of PKCδ on Tyr^{311} and/or Tyr^{565} may potentiate activation of the kinase, consistent with a previous report on the role of Tyr^{311} phosphorylation in cardiac myocytes [43]. This activation may possibly be achieved by stabilization of the kinase in the active conformation, which is achieved after allosteric regulation by DAG binding to the C1 domain. In this way, PKCδ can take input signals from multiple sources, where translocation to the membrane allows phosphorylation of two tyrosine residues, which synergize with PLC-dependent signals to fully activate the kinase, and it is this convergence of signals on PKCδ which makes it a coincidence detector for certain signalling events in platelets.

K.J.H. is supported by an MRC (Medical Research Cancil) studentship. The work was supported by programme and project grants from the British Heart Foundation (grant numbers PG/04/097/17620 and RG/05/015).

REFERENCES

1 Newton, A. C. (2001) Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. Chem. Rev. **101**, 2353–2364

2 Crosby, D. and Poole, A. W. (2002) Interaction of Bruton's tyrosine kinase and protein kinase Cθ in platelets. Cross-talk between tyrosine and serine/threonine kinases. J. Biol. Chem. **277**, 9958–9965

3 Murugappan, S., Tuluc, F., Dorsam, R. T., Shankar, H. and Kunapuli, S. P. (2004) Differential role of PKC-δ isoform in agonist-induced dense granule secretion in human platelets. J. Biol. Chem. **279**, 2360–2367

4 Grabarek, J., Raychowdhury, M., Ravid, K., Kent, K. C., Newman, P. J. and Ware, J. A. (1992) Identification and functional characterization of protein kinase C isozymes in platelets and HEL cells. J. Biol. Chem. **267**, 10011–10017

5 Crabos, M., Imber, R., Woodtli, T., Fabbro, D. and Erne, P. (1991) Different translocation of three distinct PKC isoforms with tumor-promoting phorbol ester in human platelets. Biochem. Biophys. Res. Commun. **178**, 878–883

6 Baldassare, J. J., Henderson, P. A., Burns, D., Loomis, C. and Fisher, G. J. (1992) Translocation of protein kinase C isozymes in thrombin-stimulated human platelets. Correlation with 1,2-diacylglycerol levels. J. Biol. Chem. **267**, 15585–15590

7 Wang, F., Naik, U. P., Ehrlich, Y. H., Freyberg, Z., Osada, S., Ohno, S., Kuroki, T., Suzuki, K. and Kornecki, E. (1993) A new protein kinase C, nPKCη', and nPKCθ are expressed in human platelets: involvement of nPKCη' and nPKCθ in signal transduction stimulated by PAF. Biochem. Biophys. Res. Commun. **191**, 240–246

8 Khan, W. A., Blobe, G., Halpern, A., Taylor, W., Wetsel, W. C., Burns, D., Loomis, C. and Hannun, Y. A. (1993) Selective regulation of protein kinase C isoenzymes by oleic acid in human platelets. J. Biol. Chem. **268**, 5063–5068

9 Shattil, S. J. and Brass, L. F. (1987) Induction of the fibrinogen receptor on human platelets by intracellular mediators. J. Biol. Chem. **262**, 992–1000

10 Walker, T. R. and Watson, S. P. (1993) Synergy between Ca^{2+} and protein kinase C is the major factor in determining the level of secretion from human platelets. Biochem. J. **289**, 277–282

11 Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F. et al. (1991) The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. J. Biol. Chem. **266**, 15771–15781

12 Shattil, S. J., Cunningham, M., Wiedmer, T., Zhao, J., Sims, P. J. and Brass, L. F. (1992) Regulation of glycoprotein IIb–IIIa receptor function studied with platelets permeabilized by the pore-forming complement proteins C5b–9. J. Biol. Chem. **267**, 18424–18431

13 Pula, G., Schuh, K., Nakayama, K., Nakayama, K. I., Walter, U. and Poole, A. W. (2006) PKCδ regulates collagen-induced platelet aggregation through inhibition of VASP-mediated filopodia formation. Blood **108**, 3959–3960

14 Reither, G., Schaefer, M. and Lipp, P. (2006) PKCα: a versatile key for decoding the cellular calcium toolkit. J. Cell Biol. **174**, 521–533

15 Konishi, H., Yamauchi, E., Taniguchi, H., Yamamoto, T., Matsuzaki, H., Takemura, Y., Ohmae, K., Kikkawa, U. and Nishizuka, Y. (2001) Phosphorylation sites of protein kinase Cδ in H_2O_2-treated cells and its activation by tyrosine kinase *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **98**, 6587–6592

16 Gschwendt, M., Kielbassa, K., Kittstein, W. and Marks, F. (1994) Tyrosine phosphorylation and stimulation of protein kinase Cδ from porcine spleen by src *in vitro*. Dependence on the activated state of protein kinase Cδ. FEBS Lett. **347**, 85–89

17 Gschwendt, M. (1999) Protein kinase Cδ. Eur. J. Biochem. **259**, 555–564

18 Denning, M. F., Dlugosz, A. A., Threadgill, D. W., Magnuson, T. and Yuspa, S. H. (1996) Activation of the epidermal growth factor receptor signal transduction pathway stimulates tyrosine phosphorylation of protein kinase Cδ. J. Biol. Chem. **271**, 5325–5331

19 Denning, M. F., Dlugosz, A. A., Howett, M. K. and Yuspa, S. H. (1993) Expression of an oncogenic *rasHa* gene in murine keratinocytes induces tyrosine phosphorylation and reduced activity of protein kinase Cδ. J. Biol. Chem. **268**, 26079–26081

20 Li, W., Mischak, H., Yu, J. C., Wang, L. M., Mushinski, J. F., Heidaran, M. A. and Pierce, J. H. (1994) Tyrosine phosphorylation of protein kinase Cδ in response to its activation. J. Biol. Chem. **269**, 2349–2352

21 Li, W., Yu, J. C., Michieli, P., Beeler, J. F., Ellmore, N., Heidaran, M. A. and Pierce, J. H. (1994) Stimulation of the platelet-derived growth factor β receptor signaling pathway activates protein kinase C-δ. Mol. Cell. Biol. **14**, 6727–6735

22 Szallasi, Z., Denning, M. F., Chang, E. Y., Rivera, J., Yuspa, S. H., Lehel, C., Olah, Z., Anderson, W. B. and Blumberg, P. M. (1995) Development of a rapid approach to identification of tyrosine phosphorylation sites: application to PKCδ phosphorylated upon activation of the high affinity receptor for IgE in rat basophilic leukemia cells. Biochem. Biophys. Res. Commun. **214**, 888–894

23 Soltoff, S. P. and Toker, A. (1995) Carbachol, substance P, and phorbol ester promote the tyrosine phosphorylation of protein kinase Cδ in salivary gland epithelial cells. J. Biol. Chem. **270**, 13490–13495

24 Shanmugam, M., Krett, N. L., Peters, C. A., Maizels, E. T., Murad, F. M., Kawakatsu, H., Rosen, S. T. and Hunzicker-Dunn, M. (1998) Association of PKCδ and active Src in PMA-treated MCF-7 human breast cancer cells. Oncogene **16**, 1649–1654

© The Authors Journal compilation © 2007 Biochemical Society

25 Tapia, J. A., Bragado, M. J., Garcia-Marin, L. J. and Jensen, R. T. (2002) Cholecystokinin-stimulated tyrosine phosphorylation of PKC-δ in pancreatic acinar cells is regulated bidirectionally by PKC activation. Biochim. Biophys. Acta **1593**, 99–113
26 Brodie, C., Bogi, K., Acs, P., Lorenzo, P. S., Baskin, L. and Blumberg, P. M. (1998) Protein kinase Cδ (PKCδ) inhibits the expression of glutamine synthetase in glial cells via the PKCδ regulatory domain and its tyrosine phosphorylation. J. Biol. Chem. **273**, 30713–30718
27 Kronfeld, I., Kazimirsky, G., Lorenzo, P. S., Garfield, S. H., Blumberg, P. M. and Brodie, C. (2000) Phosphorylation of protein kinase Cδ on distinct tyrosine residues regulates specific cellular functions. J. Biol. Chem. **275**, 35491–35498
28 Wrenn, R. W. (2001) Carbachol stimulates Tyr phosphorylation and association of PKCδ and PYK2 in pancreas. Biochem. Biophys. Res. Commun. **282**, 882–886
29 Blass, M., Kronfeld, I., Kazimirsky, G., Blumberg, P. M. and Brodie, C. (2002) Tyrosine phosphorylation of protein kinase Cδ is essential for its apoptotic effect in response to etoposide. Mol. Cell. Biol. **22**, 182–195
30 Steinberg, S. F. (2004) Distinctive activation mechanisms and functions for protein kinase Cδ. Biochem. J. **384**, 449–459
31 Crosby, D. and Poole, A. W. (2003) Physical and functional interaction between PKCδ and Fyn tyrosine kinase in human platelets. J. Biol. Chem. **278**, 24533–24541
32 Moussazadeh, M. and Haimovich, B. (1998) Protein kinase C-δ activation and tyrosine phosphorylation in platelets. FEBS Lett. **438**, 225–230
33 Murugappan, S., Shankar, H., Bhamidipati, S., Dorsam, R. T., Jin, J. and Kunapuli, S. P. (2005) Molecular mechanism and functional implications of thrombin-mediated tyrosine phosphorylation of PKCδ in platelets. Blood **106**, 550–557
34 Blake, R. A., Garcia-Paramio, P., Parker, P. J. and Courtneidge, S. A. (1999) Src promotes PKCδ degradation. Cell Growth Differ. **10**, 231–241
35 Song, J. S., Swann, P. G., Szallasi, Z., Blank, U., Blumberg, P. M. and Rivera, J. (1998) Tyrosine phosphorylation-dependent and -independent associations of protein kinase C-δ with Src family kinases in the RBL-2H3 mast cell line: regulation of Src family kinase activity by protein kinase C-δ. Oncogene **16**, 3357–3368

Received 15 February 2007/6 June 2007; accepted 15 June 2007
Published as BJ Immediate Publication 15 June 2007, doi:10.1042/BJ20070244

36 Okhrimenko, H., Lu, W., Xiang, C., Ju, D., Blumberg, P. M., Gomel, R., Kazimirsky, G. and Brodie, C. (2005) Roles of tyrosine phosphorylation and cleavage of protein kinase Cδ in its protective effect against tumor necrosis factor-related apoptosis inducing ligand-induced apoptosis. J. Biol. Chem. **280**, 23643–23652
37 Zrachia, A., Dobroslav, M., Blass, M., Kazimirsky, G., Kronfeld, I., Blumberg, P. M., Kobiler, D., Lustig, S. and Brodie, C. (2002) Infection of glioma cells with Sindbis virus induces selective activation and tyrosine phosphorylation of protein kinase Cδ. Implications for Sindbis virus-induced apoptosis. J. Biol. Chem. **277**, 23693–23701
38 Bosch, R. R., Patel, A. M., Van Emst-de Vries, S. E., Smeets, R. L., De Pont, J. J. and Willems, P. H. (1998) U73122 and U73343 inhibit receptor-mediated phospholipase D activation downstream of phospholipase C in CHO cells. Eur. J. Pharmacol. **346**, 345–351
39 Muto, Y., Nagao, T. and Urushidani, T. (1997) The putative phospholipase C inhibitor U73122 and its negative control, U73343, elicit unexpected effects on the rabbit parietal cell. J. Pharmacol. Exp. Ther. **282**, 1379–1388
40 Stempka, L., Schnölzer, M., Radke, S., Rincke, G., Marks, F. and Gschwendt, M. (1999) Requirements of protein kinase Cδ for catalytic function. Role of glutamic acid 500 and autophosphorylation on serine 643. J. Biol. Chem. **274**, 8886–8892
41 Stempka, L., Girod, A., Müller, H. J., Rincke, G., Marks, F., Gschwendt, M. and Bossemeyer, D. (1997) Phosphorylation of protein kinase Cδ (PKCδ) at threonine 505 is not a prerequisite for enzymatic activity. Expression of rat PKCδ and an alanine 505 mutant in bacteria in a functional form. J. Biol. Chem. **272**, 6805–6811
42 Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P. and Parker, P. J. (1998) Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. Science **281**, 2042–2045
43 Rybin, V. O., Guo, J., Sabri, A., Elouardighi, H., Schaefer, E. and Steinberg, S. F. (2004) Stimulus-specific differences in protein kinase Cδ localization and activation mechanisms in cardiomyocytes. J. Biol. Chem. **279**, 19350–19361
44 Rybin, V. O. and Steinberg, S. F. (2006) Immunoblotting PKC-δ: a cautionary note from the bench. Am. J. Physiol. Cell Physiol. **290**, C750–C756

© The Authors Journal compilation © 2007 Biochemical Society