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COINCIDENT REGULATION OF PKC δ IN HUMAN PLATELETS BY PHOSPHORYLATION OF TYROSINES 311 AND 565 AND PHOSPHOLIPASE C SIGNALING

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Abbreviations: GPCR, G protein-coupled receptor; PKC, protein kinase C; ADP, adenosine 5'-diphosphate; PLC, phospholipase C; DMEM, Dulbecco's modified Eagle's medium; DNM, dominant negative mutant; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; ECL, enhanced chemiluminescence; AM, acetoxymethyl; HEPES, N-[2-hydroxyethyl] piperazine-N-[2-ethanesulphonic acid]; ACD, Acid citrate dextrose; PRP, Platelet rich plasma; PGE₁, prostaglandin E₁; IBMX, isobutyl methylxanthine; TE, Tris-EDTA; TCA, trichloroacetic acid; A3P5P, adenosine-3', 5'-diphosphate; 2MeSADP, 2-methylthioadenosine 5'-diphosphate.

Word count: 3,846 (Introduction, Experimental, Results & Discussion)

SYNOPSIS

PKC δ plays a complex role in platelets, having both positive and negative signaling functions. It is phosphorylated on tyrosine in response to thrombin and collagen, and it has recently been shown that residue Y311 is phosphorylated in response to PAR1 and PAR4 receptor activation. Here we show Y311 and Y565 to be phosphorylated in response to thrombin, and have addressed the interplay between phosphorylation and the classical lipid-mediated activation of PKC δ . Phosphorylation of both Y311 and Y565 is dependent upon Src kinase and phospholipase C activity in response to thrombin. Importantly however, direct allosteric activation of PKC δ with phorbol myristate (PMA) also induced phosphorylation of Y311 and Y565, dependent upon Src kinases, but not phospholipase C. Membrane recruitment of PKC δ is essential for phosphorylation of the kinase on tyrosine, but tyrosine phosphorylation is not required for membrane recruitment of PKC δ . Both thrombin and PMA induce recruitment of PKC δ to the membrane, which for thrombin is by a PLC-dependent process. In order to address the functional role of tyrosine phosphorylation of PKC δ , we show phosphorylation to potentiate the activity of the kinase, although it does not play a role in membrane recruitment of the kinase. PKC δ is therefore regulated in a coincident fashion by PLC-dependent signals, recruiting it to the plasma membrane, and phosphorylation on tyrosine potentiating its activity.

INTRODUCTION

The protein kinase C (PKC) family comprises 10 isozymes grouped into 3 classes: conventional (α , γ , β I, β II), novel (δ , ϵ , η /L, θ) and atypical (ζ , ι / λ) [1], of which human platelets express predominantly 4 isoforms, α , β , δ and θ [2-8]. The PKC family has long been known to be involved in a number of platelet processes, most importantly aggregation and secretion, where stimulation of platelets with diacyl glycerol (DAG) 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]. Individual PKC isoforms however are likely to play distinct roles, and we have recently shown PKC δ to play a critical role negatively regulating collagen-induced platelet aggregation through inhibition of VASP-mediated filopodia formation [13].

Activation mechanisms for protein kinase C (PKC) family members involve the summation or synergy between multiple input signals, including classically cytosolic free calcium and diacylglycerol. These signals have in turn been thought to regulate recruitment of PKCs to the plasma membrane, where the kinase is able to be fully activated and where it encounters many of its substrates. Very recently it has been elegantly shown for example that PKC α is capable of very rapidly moving to and from the plasma membrane [14], controlled by rapid changes in cell calcium, demonstrating PKC to be a key readout sensor for the wide variety of transient calcium responses seen in many cell types.

Additionally however PKC isoforms undergo a series of phosphorylation events on serine/threonine residues, and for PKC δ in particular also on tyrosines [3, 15-29]. Human PKC δ contains 20 tyrosine residues, several of which have been shown to be phosphorylated including Tyr52, Tyr155, Tyr187, Tyr311, Tyr332, Tyr525 and Tyr565 [30]. These residues lie primarily either within the catalytic domain or the hinge region of PKC δ , and are generally not conserved between other PKC family members, making regulation by tyrosine phosphorylation relatively specific for PKC δ . PKC δ has been shown to be phosphorylated on tyrosine in human platelets in responses to agonists [31-33], including thrombin, and it has recently been shown that Tyr311 is phosphorylated in response to PAR1 and PAR4 activatory peptides [33]. Tyr311 lies within the hinge region of PKC δ , 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 directly addressed.

It was now important to assess the causal relationship between tyrosine phosphorylation of PKC δ and other mechanisms of activation of the kinase, in particular membrane recruitment and activation of phospholipase C. We show that PKC δ is phosphorylated on Tyr311 and Tyr565 in response to thrombin, and that this event potentiates the activity of the kinase. Phosphorylation on tyrosine appears not to regulate recruitment of the kinase to the membrane, but membrane localisation is critical for phosphorylation of PKC δ on Tyr311 and Tyr565. These data address the functional interplay between the various input signals to PKC δ in platelets, and reveal PKC δ as a coincidence effector for PLC-dependent signalling and tyrosine phosphorylation in these cells.

EXPERIMENTAL

Materials

Anti-phosphotyrosine monoclonal antibody 4G10, anti-phospho-Src (Tyr-416) clone9A6, anti-Src antibody and recombinant protein tyrosine phosphatase 1B (PTP-1B) were from Upstate Biotechnology Inc. (Lake Placid, NY). Phospho-specific antibodies against specific tyrosine sites on PKC δ and anti-GPIb (anti-CD42b antibody) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-PKC δ antibody was from BD Biosciences (Oxford, UK). Anti-actin antibody was purchased from Lab Vision Corp (Suffolk, UK). Horseradish peroxidase-conjugated secondary antibodies, enhanced chemiluminescence (ECL) kits and [γ - 32 P]ATP were purchased from Amersham Biosciences. Collagen was purchased from Nycomed (Munich, Germany) 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 from Sigma-Aldrich. The PAR-1 specific agonist SLLRN and PAR-4 specific agonist AYPGKF were from Bachem (Merseyside, UK). The PKC inhibitor GF109203X, Phorbol 12-myristate 13-acetate (PMA), U73122 and PP2 was from Tocris (Avonmouth, UK). Piceatannol and PP1 were from Alexis Corp (Nottingham, UK). Protein kinase C δ peptide substrate, PP3 and LFM-A13 were purchased from Calbiochem (LaJolla, CA). Anti-phosphothreonine (Thr-505) PKC δ antibody was from Cell Signalling Technology (New England Biolabs, Hitchin, UK). 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 into the anticoagulant sodium citrate (1:20 (v/v) dilution of a 4% solution). 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. Platelet-rich plasma (PRP) was prepared by centrifugation at 180 x g for 20 min, and platelets were then isolated by centrifugation for 10 min at 550 x g in the presence of prostaglandin E1 (40 ng/ml). The 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 MgCl₂, 5 mM glucose, pH 7.3). Indomethacin (10 μ M) was added to this platelet suspension, and a 30-min rest period was allowed before stimulation. All platelet stimulation occurred in the presence of EGTA (1 mM). Platelets were preincubated with

different inhibitors or vehicle solution (DMSO) for 10 min at 37 °C, and stimulated in an aggregometer (Chrono-Log Corp., Labmedics Ltd., Manchester, UK) at 37 °C, with continuous stirring at 800 rpm. Reactions were halted by either addition of 5xSDS sample buffer (24 mM Tris, pH 6.8, 10% (v/v) glycerol, 0.8% (v/v) SDS, 6 mM 2-mercaptoethanol, and 0.04% bromophenol blue) to produce whole cell lysates (WCL) or by addition of 2xNonidet P-40 lysis buffer for immunoprecipitation (as described below).

Immunoprecipitation of PKC δ

Reactions were stopped by lysis of platelets with an equal volume of 2x Nonidet P-40 lysis buffer (NP-40) (100 mM Tris, 300 mM NaCl, 20mM EDTA, 1mM Na₃VO₄ and 2% (v/v) Nonidet P-40 substitute, pH 7.5), plus complete protease inhibitors. Lysates were precleared with protein-A-Sepharose beads for 1 hour. Antibody-protein-A complexes were formed by incubation of protein-A-Sepharose with 1 μ g of antibody for 1 hour at room temperature. Precleared lysates were added to the antibody-protein-A complexes and incubated at 4 °C with constant rotation overnight. Immunoprecipitates were washed 3 times with 1x NP-40 before addition of 5 x SDS sample buffer and boiling for 5 minutes.

Electrophoresis of Proteins and Western Blotting

Proteins were resolved by electrophoresis in 9–12% SDS-PAGE gels. Samples were then transferred to polyvinylidene difluoride membranes, blocked with 5–10% bovine serum albumin, and incubated for 1 h or overnight at room temperature with the appropriate primary antibody. Membranes were then washed before incubation with the appropriate secondary antibody followed by thorough washing. Bound peroxidase activity was detected using enhanced chemiluminescence (ECL).

***In Vitro* Kinase Assays**

PKC δ was immunoprecipitated from Nonidet P-40 lysates as described and washed 3 times with 1x Nonidet P-40 wash buffer containing 0.5mM Na₃VO₄. Some thrombin treated samples were dephosphorylated by exposure to recombinant PTP-1B (1 μ g, specific activity 13nmol \cdot min⁻¹ \cdot μ g⁻¹ using *p*-nitrophenylphosphate) for 10 mins at 37°C. All immunoprecipitates were then washed 3 times with 1x Nonidet P-40 wash buffer containing 1mM Na₃VO₄, and once in Kinase Assay Buffer (100mM NaCl, 20mM HEPES, 5mM MgCl₂, 5mM MnCl₂, pH 7.2). Immunoprecipitates were resuspended in 20 μ l of kinase assay buffer with 10 μ g of PKC δ peptide substrate. Reactions were started by addition of 10 μ l of ATP Buffer (0.15mM ATP,

30mM MgCl₂, 200 μ Ci/ml [γ -³²P]-ATP). After incubation at 30°C for 30 mins, the reaction was terminated by addition of 10% phosphoric acid and samples were added to 3cm squares of P81 ion exchange chromatography paper. The P81 papers were washed 5 times in 0.5% phosphoric acid, followed by a wash in acetone. Papers were then dried and labelled PKC δ peptide substrate was quantified by liquid scintillation counting.

Subcellular fractionation of human platelets

Basal and stimulated platelets were resuspended in ice-cold 2x sonication buffer (final concentration 320mM Sucrose, 4mM HEPES, 0.5mM Na₃VO₄, pH 7.4) containing complete protease inhibitors, and sonicated for 4 x 20s pulses. Intact platelets were removed by centrifugation at 1500 x g for 10mins before centrifugation at 100,000 x g for 60min. The supernatant was removed (cytosolic fraction) and the pellet (particulate fraction) was resuspended in Tris-HCl buffer containing 10mM Tris, 158mM NaCl, 1mM EGTA, 0.5mM Na₃VO₄, 0.1% SDS, 1% sodium deoxycholate, 1% Triton-X100, pH7.2, with complete protease inhibitors. Protein concentrations were quantified using the BCA-assay (Sigma-Aldrich). Equal protein concentrations of the fractions were either resolved by SDS-PAGE and western blotted for tubulin or GPIb to confirm fractionation had occurred, or PKC δ was immunoprecipitated from each fraction as described above, resolved by SDS-PAGE and western blotted using anti-PKC δ or phospho-specific antibodies.

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 PKC δ on Y311 and Y565 in human platelets

PKC δ has been shown to be phosphorylated on tyrosine in multiple cell types including platelets [3, 31-33]. A recent report had shown that PAR1 and PAR4 peptides were able to induce phosphorylation of Y311 [33], a residue shown by others to be important in regulating proteolytic degradation of PKC δ [34]. It was now important to understand the interplay between phospholipase C signalling and tyrosine phosphorylation of PKC δ , and in particular whether these two events were interdependent or unrelated, and how they contributed to the activity of PKC δ . In the first instance it was important to determine whether thrombin could induce phosphorylation of PKC δ on Y311 and other putative tyrosine phosphorylation sites. Both thrombin and collagen were able to induce phosphorylation of PKC δ on tyrosine (Fig. 1A) and thrombin induced a transient phosphorylation of both Y311 and Y565 (Fig. 1B), which was apparent at an early time point (20 s) but had substantially disappeared by 3 min. This was paralleled by a time course of phosphorylation on these residues in response to peptide activation of PAR1 and PAR4 receptors (Fig. 1C), with PAR1 activation profile being highly transient, and PAR4 being more sustained. A time course of phosphorylation on Y311 and Y565 showed that for collagen these events occurred more slowly and achieved maximal phosphorylation by 3 min (Fig. 1C(ii)). It has been demonstrated that Y52, Y155, Y332 and Y525 may also be phosphorylated in other cell types [15, 22, 27, 35-37]. Figure 2 shows that, in response to thrombin, collagen or PAR1/4 peptides, these tyrosines are not phosphorylated, although pervanadate used as a positive control is able to induce phosphorylation of these sites.

Phospholipase C signaling is necessary and sufficient to induce phosphorylation of PKC δ on Y311 and Y565 downstream of Src family kinases

Platelets express a variety of non-receptor tyrosine kinases that may be responsible for phosphorylation of PKC δ on Y311 and Y565. Figure 3A shows that the Src family kinase (SFK) inhibitors PP1 and PP2, but not the inactive analogue PP3 nor the Syk inhibitor piceatannol nor the Btk inhibitor LFM-A13, are able to block phosphorylation on these two sites induced by thrombin. Phosphorylation of PKC δ on Y311 and Y565 is therefore downstream of SFKs, but also requires phospholipase C (PLC) activity, since it is blocked by the PLC inhibitor U73122.

Importantly, allosteric activation of PKC δ by the phorbol ester PMA, which bypasses receptor activation, is also able to induce phosphorylation of Y311 and Y565 in a Src kinase-dependent manner (Fig. 3B). Because receptor activation is avoided, phosphorylation induced by PMA is not dependent upon PLC activity, and is not blocked by U73122. It was possible therefore either that SFK activity was dependent upon PMA, and in the case of thrombin was dependent upon diacyl glycerol, or that these lipid signals recruit PKC δ to the membrane where a basally active SFK may phosphorylate it, or a combination of both. We therefore assessed whether PMA or thrombin were able to activate SFK by using a phosphospecific antibody to pY416(Src), which recognises the autophosphorylation site in SFKs and is therefore a marker of activity in the cell. Figure 4 shows a basal SFK activity, which is ablated by PP1, and which is increased by approximately 2 fold by stimulation with thrombin. There is still substantial thrombin-dependent SFK activation in platelets pre-treated with U73122, the PLC inhibitor, 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 PKC δ on Y311 and Y565, and therefore that cellular localisation of PKC δ is a more critical regulatory step than regulation of Src kinase activity *per se*.

Phosphorylation of PKC δ on Y311 and Y565 is not required for membrane recruitment, whereas membrane recruitment is required for phosphorylation on these residues

The data from Figs 3&4 were consistent with an activation process where PLC signaling products lead to recruitment of PKC δ to the membrane, whereupon SFKs are able to phosphorylate it. If this were the case then SFKs should not be required for membrane recruitment. Figure 5A shows that PKC δ translocates rapidly but transiently from cytosol to membrane compartments, in a manner that parallels its tyrosine phosphorylation (Fig. 1). Importantly, phosphorylation of Y311 and Y565 occur only in membrane-associated fractions, and not in PKC δ localised to the cytosol, consistent with recruitment to the membrane being required for phosphorylation. Phosphorylation on tyrosine was however not required for translocation, since blockade of phosphorylation by PP1 had no effect upon membrane recruitment of the kinase (Fig. 5B). Conversely, membrane recruitment of PKC δ did require PLC activity, since blockade of PLC by U73122 substantially reduced its translocation.

PMA, which bypasses receptor-mediated PLC activation, also induced membrane recruitment of PKC δ (Fig. 6), and also induced tyrosine phosphorylation of Y311 and Y565 only when

the kinase was localised to the membrane. The time courses of membrane recruitment and phosphorylation on Y311 or Y565 show that membrane recruitment is rapid, being maximal by 30 s and sustained over 5 min, yet phosphorylation does not take place until later time points (Fig. 6A). Again, PP1 abolished tyrosine phosphorylation of PKC δ , but had no effect upon membrane translocation (Fig. 6B). In summary therefore these data are consistent with a model whereby PKC δ translocates to the membrane and is subsequently phosphorylated on Y311 and Y565 in a SFK-dependent manner.

Tyrosine phosphorylation of PKC δ potentiates its kinase activity

From Figs 5 & 6 it can be concluded that tyrosine phosphorylation of PKC δ has no role to play in translocating the kinase from cytosol to membrane, and in fact membrane recruitment is a requirement for tyrosine phosphorylation. It was therefore important to ascertain whether tyrosine phosphorylation of Y311 and Y565 played a role in regulating the kinase activity of PKC δ . Figure 7A(i) shows an *in vitro* kinase assay, demonstrating that thrombin stimulation of platelets leads to an increased activity of PKC δ . This increased activity is partially but non-significantly inhibited when platelets were pretreated with PP1, which abolished tyrosine phosphorylation of Y311 and Y565 (Fig. 7A(ii)). Importantly, because of other potential effects of SFK inhibition in the cell, we were able to dephosphorylate PKC δ *in vitro* using the tyrosine phosphatase PTP1b (Fig. 7A(ii)). Dephosphorylation *in vitro* also partially but non-significantly reduced the activity of PKC δ (Fig. 7A(i)), to the same extent as PP1 when applied to platelets. This suggested that phosphorylation on Y311 and Y565 may partially contribute to and potentiate kinase activation of PKC δ downstream of thrombin.

In order to demonstrate that the activity of PKC δ in platelets, rather than *in vitro*, was similarly partially dependent upon tyrosine phosphorylation by SFKs, Fig. 7B shows data demonstrating phosphorylation of PKC δ on Thr505. Autophosphorylation on this site provides a read-out of kinase activity in the cell, and Fig. 7B shows that thrombin induces a marked increase in phosphorylation of this residue, thereby confirming activation of the kinase by this agonist in platelets. Pre-treatment of platelets with PP1, the SFK inhibitor, partially inhibits activity of the kinase, paralleling data from *in vitro* kinase assay shown in Fig. 7A. As positive controls for the assay, PKC δ activity is also shown to be dependent upon PLC activity, as shown by an ablation of Thr505 phosphorylation in the presence of the U73122. The phorbol ester PMA is also able to directly induce activation of PKC δ and phosphorylation on Thr505 (Fig. 7B).

DISCUSSION

The protein kinase C family plays essential roles in regulating functional activities in most mammalian cell types. Regulation of the activity of the family members is complex and involves multiple convergent mechanisms, and for PKC δ phosphorylation on tyrosines is a prominent feature, although its regulation and function in primary cells is not yet clear. Here we have demonstrated that, in human platelets, Y311 and Y565 become phosphorylated in response to activation of PAR1 and PAR4 receptors by thrombin. Phosphorylation is downstream of Src family kinases, and importantly requires activity of phospholipase C to allow targeting of PKC δ to a membrane localisation which permits phosphorylation by Src family kinases present in that compartment of the cell. Phosphorylation on tyrosine therefore does not regulate localisation of PKC δ in the cell, but was shown to potentiate the activity of the kinase in the cell and *in vitro*. It is possible therefore that the role of phosphorylation of Y311 (hinge region) and Y565 (kinase domain) is to stabilise the active conformation of the kinase, at least *in vitro*.

Although it had previously been shown that PKC δ is phosphorylated on tyrosine in human platelets in response to thrombin [3, 32], only one report has so far identified any sites of phosphorylation in these cells [33]. Murugappan et al. (2005) had shown that Tyr311 to be phosphorylated in response to activation by PAR1- and PAR4-selective peptide agonists, although the authors do not demonstrate phosphorylation on any other tyrosine residues (33). Here we have extended this initial observation by demonstrating that the physiological agonist thrombin is able to induce phosphorylation on Tyr311, but additionally that phosphorylation also occurs at Tyr565, although not Tyr52, Tyr155, Tyr332 or Tyr525. The reason for the discrepancy between our study and that of Murugappan et al. (2005) (33), who did not observe phosphorylation of Tyr565, is not known at present. Throughout this study the phosphorylation status of Tyr565 paralleled phosphorylation of Tyr311, and we were not able to distinguish any circumstances in which phosphorylation of one residue would occur in the absence of the other. Blake et al. (1998) had shown that although PKC δ could be phosphorylated on multiple Tyr residues, Tyr311 phosphorylation was a 'master' site, in that mutation of this residue to a non-phosphorylatable Phe ablated phosphorylation of PKC δ on all other tyrosines. This may be the case in the platelet system also, where Tyr311 may be required for phosphorylation of Tyr565, but we were not able to determine this within the present study. The time courses of phosphorylation of each residue are very rapid and do not

substantially differ from each other, although at least for PAR4 peptide activation (Fig. 1C) Tyr311 phosphorylation is maximal by 20s, whereas for Tyr565 phosphorylation is not maximal until 1 min. This may suggest that phosphorylation of Tyr311 may precede that of Tyr565, least in response to activation of PAR4, and therefore that Tyr311 phosphorylation may be a pre-requisite for phosphorylation of Tyr565.

We show that Tyr311 phosphorylation is downstream of Src family kinases (Fig. 3A) in response to thrombin. This is consistent with Murugappan et al. (2005) who showed Src kinase-dependent phosphorylation of this residue in response to PAR1 and PAR4 activation [33], and is consistent with the fact that this residue sits within an excellent Src phosphorylation consensus sequence. We had shown previously that PKC δ selectively complexes with the tyrosine kinase Fyn in activated conditions [31], and this may therefore be the Src family kinase responsible for phosphorylating PKC δ in response to thrombin. Phospholipase C activity was also required for phosphorylation on both Tyr311 and Tyr565, since phosphorylation was blocked by the PLC inhibitor U73122. This suggested either that the Src kinase responsible for phosphorylating PKC δ was activated downstream of PLC, or that PKC δ recruitment to the plasma membrane and allosteric regulation was required to allow phosphorylation. We showed in Fig. 4A however that although thrombin was able to induce activation of Src family kinases, this was largely independent of PLC activity, since U73122 did not significantly decrease Src activity in response to thrombin. This suggests that the ablation of phosphorylation of PKC δ on Tyr311 and Tyr565 in the presence of U73122 may be because Src family kinases are able to phosphorylate PKC δ when allosterically modulated by diacylglycerol. It needs also to be mentioned that U73122 has been shown also to have actions other than inhibition of PLC, including inhibition of PLD [38] and stimulation of calcium release [39], and therefore it is possible that inhibition of translocation of PKC δ and its phosphorylation on tyrosine by this compound may be through an off-target inhibitory mechanism. The observation that the phorbol ester PMA also induced phosphorylation of both tyrosine residues on PKC δ indicated that membrane localisation and allosteric regulation were the important signals required to allow tyrosine phosphorylation. Consistent with this model, PMA had no effect upon Src kinase activity *per se*. Also consistent with this model is that basal activity of Src is sufficient to allow full phosphorylation of PKC δ on Y311 and Y565. This suggests therefore that cellular localisation of PKC δ is a more critical regulatory step for controlling its tyrosine phosphorylation than regulation of Src kinase activity *per se*.

We would therefore propose from the data that phosphorylation of PKC δ on Tyr311 and Tyr565 occurs in a Src kinase-dependent manner in response to thrombin, only when PKC δ is allosterically activated. This would suggest that tyrosine phosphorylation may only occur when PKC δ has translocated to the particulate fraction. This was indeed shown to be the case in Fig. 5A, where we show that thrombin induces a transient membrane translocation of the kinase, and that PKC δ is phosphorylated on Tyr311 and Tyr565 only when translocated to the membrane. Indeed, at late time points (3 min) when PKC δ had re-entered the cytosolic fraction, the kinase was also no longer phosphorylated on tyrosine. These data suggested therefore that the early translocation to the membrane would occur independent of tyrosine phosphorylation, and that phosphorylation occurred subsequent to the translocation event. Using PP1, which blocks tyrosine phosphorylation, Figs 5B & 6 showed that indeed tyrosine phosphorylation was not required for translocation to the membrane, whether induced by thrombin or PMA. These data would suggest that, in the case of PMA which does not significantly increase activity of Src above basal levels, translocation to the plasma membrane for sustained periods is sufficient to allow slow phosphorylation of Src on Y311 and Y565 (Fig. 6A). For the thrombin, which induces a doubling in Src activity (Fig. 4), the kinetics of phosphorylation of PKC δ on tyrosine are markedly more rapid (Fig. 5A). However, although U73122 substantially reduces translocation of PKC δ to the membrane, it does not abolish it (Fig. 5). Despite some translocation, phosphorylation of Y311 and Y565 is completely ablated (Fig. 5), and this may therefore suggest that translocation alone may not be sufficient to allow phosphorylation, at least over a 1 min time period in response to thrombin. It is still not clear also whether tyrosine dephosphorylation is required in order to allow PKC δ to detach from the membrane at late time points of activation, or whether PKC δ is dephosphorylated rapidly after membrane detachment. Similarly, the nature of the tyrosine phosphatase responsible for this dephosphorylation event is not presently known.

Finally, since tyrosine phosphorylation of PKC δ did not regulate its translocation to the membrane, it was important to address whether it may regulate its activity. By *in vitro* kinase assay and assay of autophosphorylation on Thr505 of PKC δ , we were able to show that blockade of its tyrosine phosphorylation or removal of phosphates from tyrosines *in vitro* was able to diminish partially the activity of the kinase (Fig. 7). This reduction in activity was non-significant, compared to thrombin-stimulated levels of activity, however there was a clear trend towards reduction in activity which was consistent between experiments. These data were paralleled by data for phosphorylation of PKC δ on Thr505, where a non-significant

reduction in the presence of PP1 was shown (Fig. 5B), although a consistent trend to inhibition was seen in experiments. It should be noted that, unlike other isoforms of PKC, phosphorylation on Thr505 in the activation loop of the kinase has been shown not to be essential for PKC δ activation [40, 41]. Phosphorylation on this site is also not necessarily as a result of autophosphorylation, as PKD1 has been shown also to phosphorylate this site [42]. Phosphorylation of Thr505 may therefore not reflect activity of PKC δ , but it is clear from a comparison of data shown in Fig 7A and 7B that the responses to thrombin in the presence and absence of PP1 are parallel, and therefore likely to be supportive of each other. They suggest that phosphorylation of PKC δ on Tyr311 and/or Tyr565 may potentiate activation of the kinase, consistent with a previous report for Y311 phosphorylation in cardiac myocytes [43], possibly by stabilising the kinase in the active conformation achieved after allosteric regulation by diacylglycerol binding to the C1 domain. In this way, PKC δ takes input signals from multiple sources, where translocation to the membrane is permissive for phosphorylation of two tyrosine residues which synergise with PLC-dependent signals to activate fully the kinase, and in this way this convergence of signals on PKC δ makes it a coincidence detector for these signalling events in the platelet.

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FIGURE LEGENDS**Figure 1. PKC δ is phosphorylated on tyrosines 311 and 565 downstream of PAR1 and PAR4 activation.**

A, PKC δ was immunoprecipitated (IP) from 1% Nonidet P-40 lysates of platelets stimulated with 0.1U/ml thrombin (1 min) or 30 μ g/ml collagen (3 mins). Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane and western blotted (WB) with monoclonal antiphosphotyrosine antibody 4G10. Blots were reprobbed with anti-PKC δ antibody as indicated. B, PKC δ was immunoprecipitated from 1% Nonidet P-40 lysates of basal platelets or platelets stimulated with 0.5U/ml Thrombin for the time periods indicated. Samples were western blotted using phospho-specific antibodies directed against the residues *i*, Y311 (pY311) or *ii*, Y565 (pY565) of PKC δ . Samples were reprobbed for PKC δ as indicated. C, PKC δ was immunoprecipitated from 1% Nonidet P-40 lysates of basal platelets or platelets stimulated with either (i) PAR1 or PAR4 agonist peptides SFLLRN (50 μ M) or AYPGKF (500 μ M) respectively, or (ii) collagen (30 μ g/ml), for the indicated time periods. Samples were western blotted for pY311 or pY565 as indicated. Membranes were stripped and reprobbed for PKC δ to ensure equal loading. Data shown are representative of three independent experiments.

Figure 2. Collagen, SFLLRN, AYPGKF and Thrombin do not induce phosphorylation of PKC δ on tyrosine residues 52, 155, 332 or 525 in platelets.

PKC δ was immunoprecipitated from either basal or stimulated platelets (collagen 50 μ g/ml 3 minutes, thrombin 0.5unit/ml 1 minute, SFLLRN 100 μ M 20 seconds or AYPGKF 500 μ M 1 minute), or platelets treated with 200 μ M pervanadate for 1 hour at 37°C. Immunoprecipitates were resolved by SDS-PAGE and phosphorylation of PKC δ was detected by Western Blotting using phospho-specific antibodies directed against residues Y52 (pY52), Y155 (pY155), Y332 (pY332) or Y525 (pY525). Membranes were stripped and reprobbed for PKC δ to ensure equal loading. Data shown are representative of three independent experiments.

Figure 3. Tyrosine phosphorylation of PKC δ on Y311 and Y565 is inhibited by PP1, PP2 and U73122.

Platelets were preincubated (10 min) with either DMSO (0.1%), PP1 (10 μ M), PP2 (10 μ M), PP3 (10 μ M), U73122 (10 μ M), piceatannol (10 μ g/ml) or LFM-A13 (40 μ M) as indicated before stimulation with thrombin (A, 0.5 U/ml) for 1 min or PMA (B, 100 nM) for 5 min.

Platelets were lysed in 1% Nonidet P-40 lysis buffer and PKC δ was immunoprecipitated. For *A* and *B*: samples were western blotted for *i*, pY311, *ii*, pY565. Membranes were stripped and reprobed for PKC δ to ensure equal loading. Data shown are representative of three independent experiments.

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

Platelets were preincubated (10 mins) with DMSO (0.1%), PP1 (10 μ M) or U73122 (10 μ M) before stimulation with thrombin (0.5 units/ml) for 1 min or PMA (100 nM) for 5 min. Platelets were lysed in Laemmli sample buffer, proteins were separated by SDS-PAGE and transferred to PVDF membrane. In *A* samples were western blotted using anti-pTyr⁴¹⁶ Src antibody and in *C* membranes were reprobed using anti-Src antibody to ensure equal loading. In *B*, bands from (*A*) were quantified by densitometry and represented in the histogram as a percentage of maximal Src phosphorylation in response to thrombin. Data is presented as mean \pm S.E.M (n=3) (*, p<0.01 compared to DMSO alone; **, p<0.001 compared to DMSO alone; ***, p<0.001 compared to thrombin pretreated with DMSO).

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

A, platelets were stimulated for the indicated time points with thrombin (0.5 units/ml), and *B*, platelets were preincubated (10 min) with DMSO (0.1%), PP1 (10 μ M) or U73122 (10 μ M) before stimulation with thrombin (0.5 units/ml) for 1 min. Cytosolic and particulate fractions were isolated as described under "Experimental Procedures". For *A* and *B*, *i* and *ii*, PKC δ was immunoprecipitated from cytosol and particulate fractions and samples were western blotted for *i*, pY311 or *ii*, pY565. Membranes were stripped and reprobed for PKC δ as indicated. For *A* and *B*, *iii*, equal protein concentrations of particulate and cytosolic fractions were lysed in laemmli sample buffer and separated by SDS-PAGE. Samples were western blotted, as indicated, for α -tubulin or β -tubulin as cytosolic markers and GPIb as a membrane marker to ensure complete fractionation of platelets. Data shown are representative of three independent experiments.

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

A, Platelets were stimulated for the indicated times with PMA (100 nM) and *B*, Platelets were preincubated (10 min) with DMSO (0.1%) or PP1 (10 μ M) before stimulation with PMA (100 nM) for 5 min at 37 °C. Cytosolic and particulate fractions were isolated as described under “Experimental Procedures”. For *A* and *B* PKC δ was immunoprecipitated from cytosol or particulate fractions, and samples were western blotted for (i), pY311 or (ii), pY565. Membranes were stripped and reprobed for PKC δ as indicated. In (iii), particulate and cytosolic fractions were lysed in Laemmli sample buffer and separated by SDS-PAGE. Samples were western blotted, as indicated, for α -tubulin as a cytosolic marker and GPIb as a membrane marker to ensure complete fractionation of platelets. Data shown are representative of three independent experiments.

Figure 7. Tyrosine phosphorylation potentiates the kinase activity of PKC δ .

A (i), PKC δ was immunoprecipitated from platelets preincubated with DMSO (0.1%) or PP1 (10 μ M) before stimulation with thrombin (0.5 units/ml) for 1 min. Immunoprecipitates from thrombin-stimulated platelets were dephosphorylated, where indicated, using PTP1b and an *in vitro* kinase assay performed as described under “Experimental Procedures”. Kinase activity is expressed as fold increase relative to unstimulated platelets. Data shown are mean \pm S.E.M (*, $p < 0.05$ compared to unstimulated platelets). *A* (ii), PKC δ immunoprecipitates from above were separated on SDS-PAGE and transferred to PVDF membrane. Samples were western blotted for pY311 (*left panel*) or pY565 (*right panel*) to ensure dephosphorylation occurred and reprobed for PKC δ as indicated to ensure equal loading. *B*, Platelets were preincubated (10 mins) with DMSO (0.1%), PP1 (10 μ M) or U73122 (10 μ M) before stimulation with thrombin (0.5 units/ml) for 1 min or PMA (100 nM) for 5 min. Platelets were lysed in Laemmli sample buffer, proteins were separated by SDS-PAGE and transferred to PVDF membrane. In *B* (i) samples were western blotted using anti-pThr⁵⁰⁵ PKC δ antibody (arrow corresponds to PKC δ) and reprobed in (iii) using anti-PKC δ and anti-actin antibodies to ensure equal loading. The apparent reduction in immunoreactivity of PKC δ in samples taken from PMA-stimulated platelets (far right lane) has been noted previously [44], and is likely not to reflect a true reduction in protein loading but an altered ability of PKC δ to bind antibody, possibly as a result of multiple phosphorylations. Anti-actin reprobe was therefore also included to demonstrate equal loading of the gel. In *B* (ii) bands from *B* (i) were

quantified by densitometry and represented as percentage of maximal PKC δ phosphorylation in response to thrombin. Data shown are mean \pm S.E.M (n=3: *, p<0.001 compared to DMSO alone; **, p<0.001 compared to thrombin alone; *, p<0.01 compared to DMSO alone).

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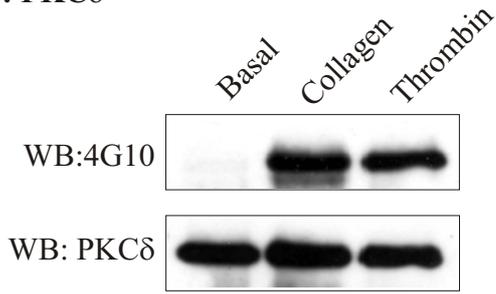
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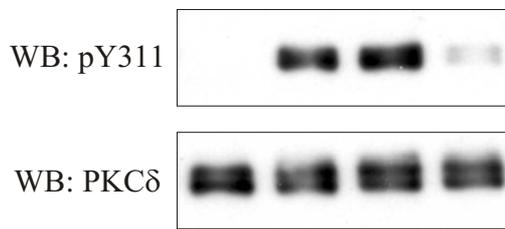
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A.) IP: PKC δ



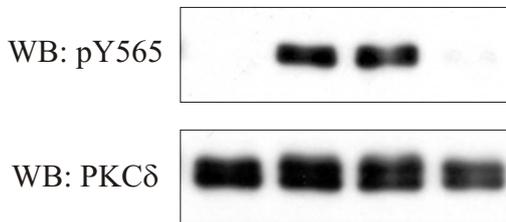
B.) IP:PKC δ

(i)



Thrombin 0.5U/ml (time) 0 20s 1m 3m

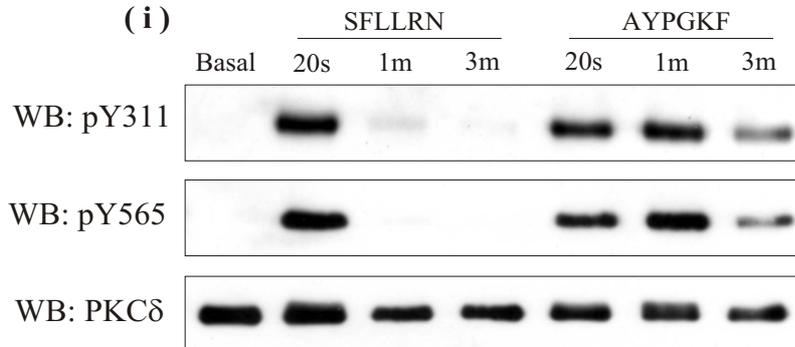
(ii)



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C.) IP:PKC δ

(i)



(ii)

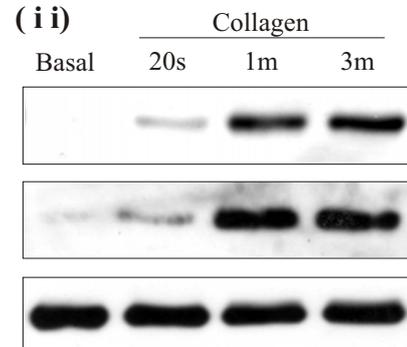


Fig. 1

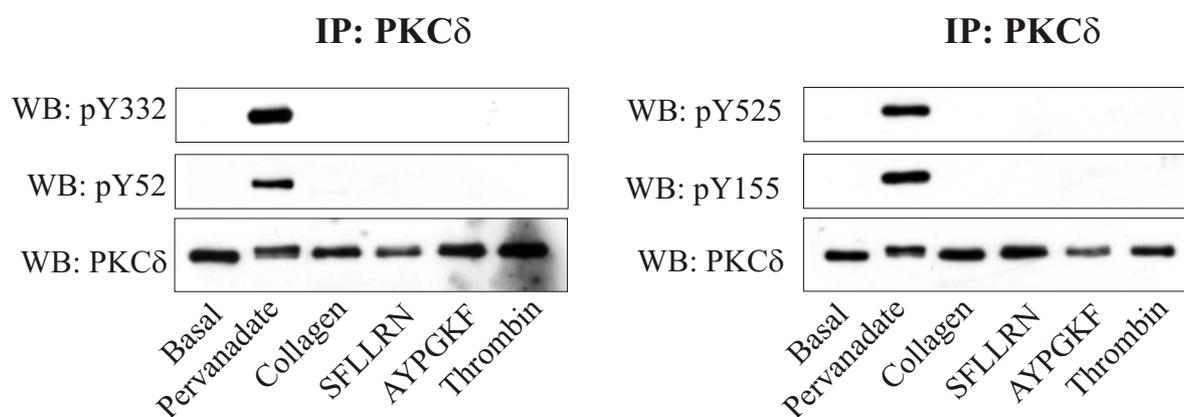
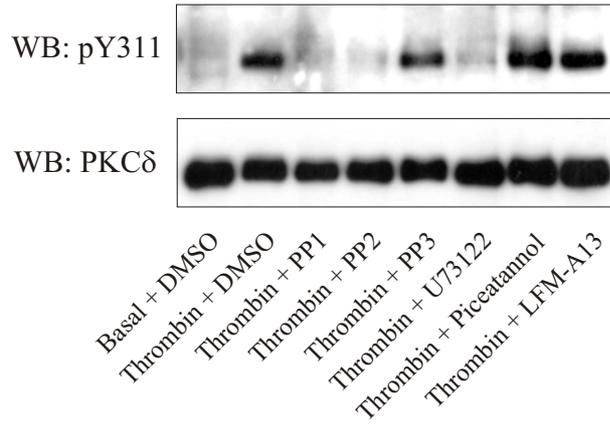


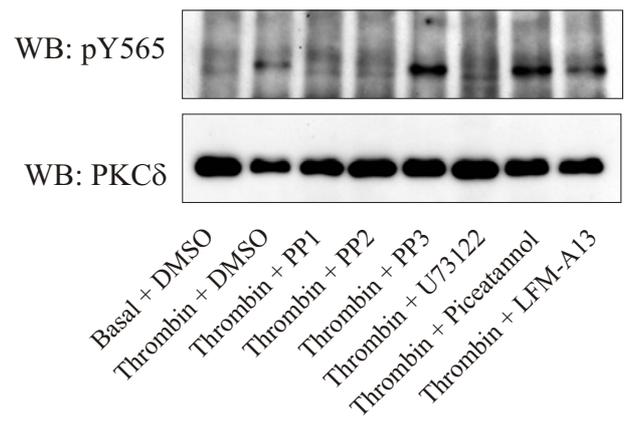
Fig. 2

A.) IP: PKC δ

(i)

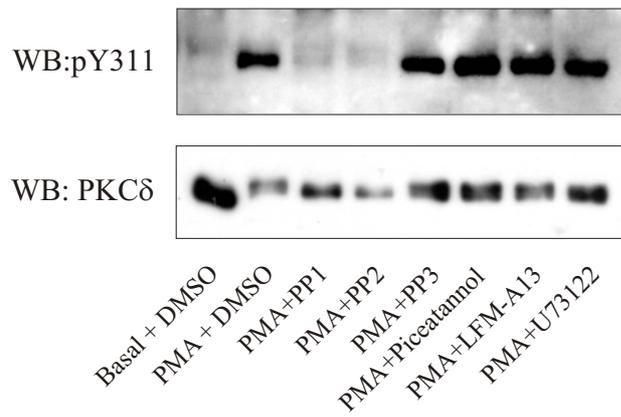


(ii)



B.) IP:PKC δ

(i)



(ii)

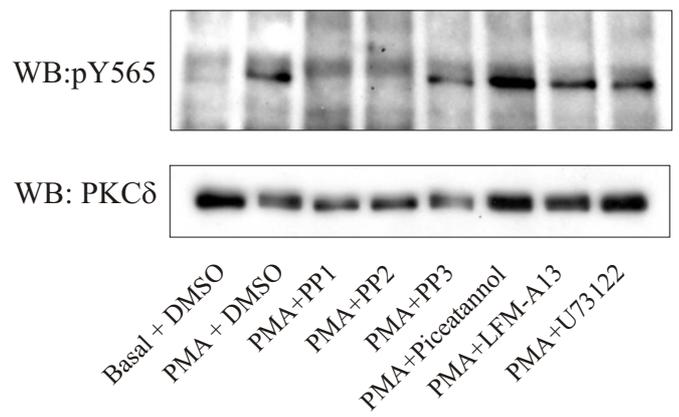


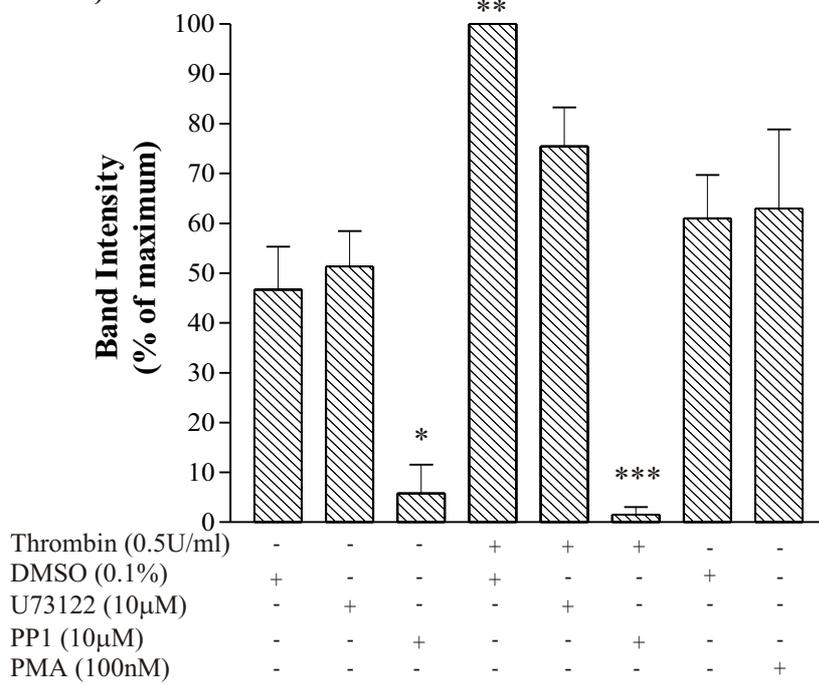
Fig. 3

A.) WCL

WB: pTyr 416 Src



B.)



C.) WCL

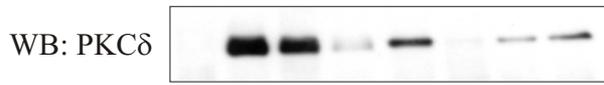
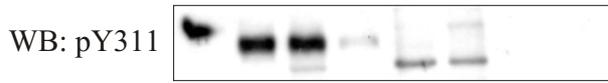
WB: Src



Fig. 4

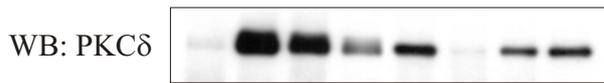
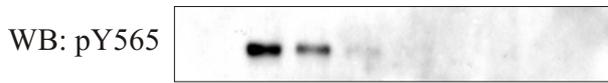
A.

(i) IP: PKC δ



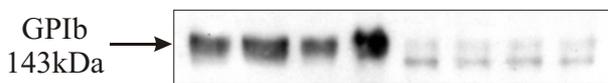
Thrombin (0.5U/ml) | 0 30s 1m 3m | 0 30s 1m 3m |
 Particulate | Cytosolic

(ii) IP: PKC δ



Thrombin (0.5U/ml) | 0 30s 1m 3m | 0 30s 1m 3m |
 Particulate | Cytosolic

(iii) WCL



Thrombin (0.5U/ml) | 0 30s 1m 3m | 0 30s 1m 3m |
 Particulate | Cytosolic

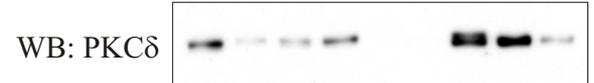
B.

(i) IP: PKC δ



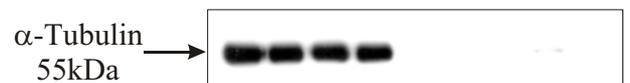
Thrombin (0.5U/ml)	-	+	+	+	-	+	+	+
DMSO (0.1%)	+	-	-	-	+	-	-	-
PP1 (10 μ M)	-	-	+	-	-	-	+	-
U73122 (10 μ M)	-	-	-	+	-	-	-	+
	Cytosolic				Particulate			

(ii) IP: PKC δ



Thrombin (0.5U/ml)	-	+	+	+	-	+	+	+
DMSO (0.1%)	+	-	-	-	+	-	-	-
PP1 (10 μ M)	-	-	+	-	-	-	+	-
U73122 (10 μ M)	-	-	-	+	-	-	-	+
	Cytosolic				Particulate			

(iii) WCL

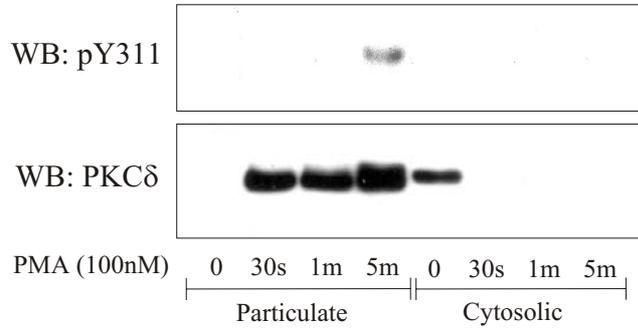


Thrombin (0.5U/ml)	-	+	+	+	-	+	+	+
DMSO (0.1%)	+	-	-	-	+	-	-	-
PP1 (10 μ M)	-	-	+	-	-	-	+	-
U73122 (10 μ M)	-	-	-	+	-	-	-	+
	Cytosolic				Particulate			

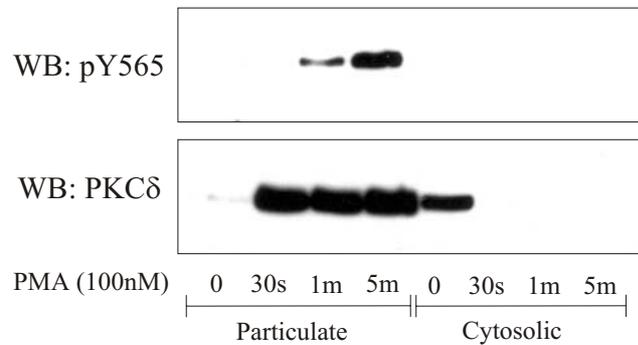
Fig. 5

A.)

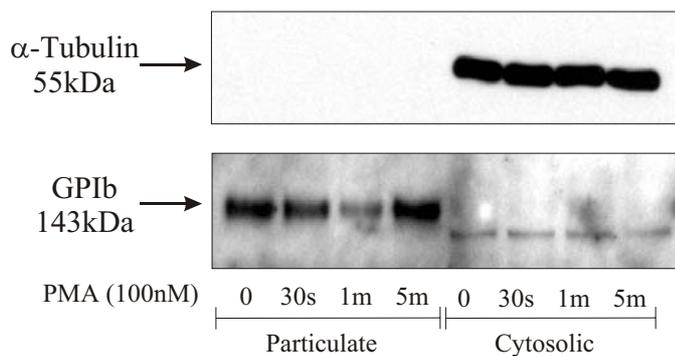
(i) IP:PKC δ



(ii) IP:PKC δ

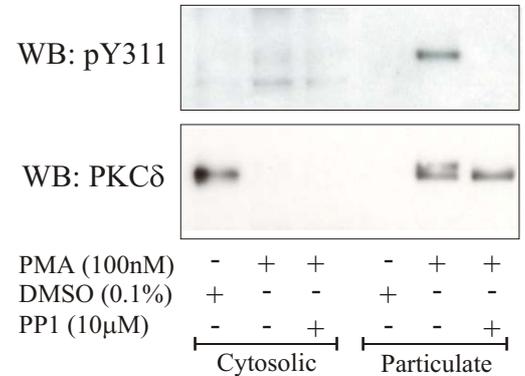


(iii) WCL

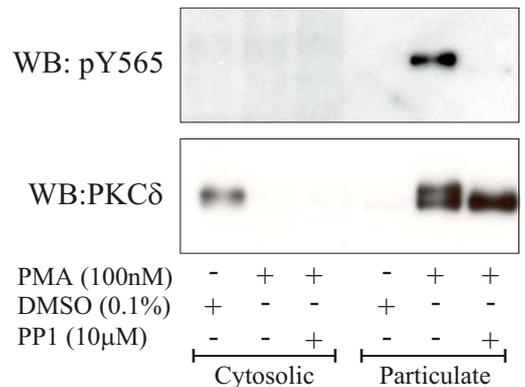


B.)

(i) IP:PKC δ



(ii) IP:PKC δ



(iii) WCL

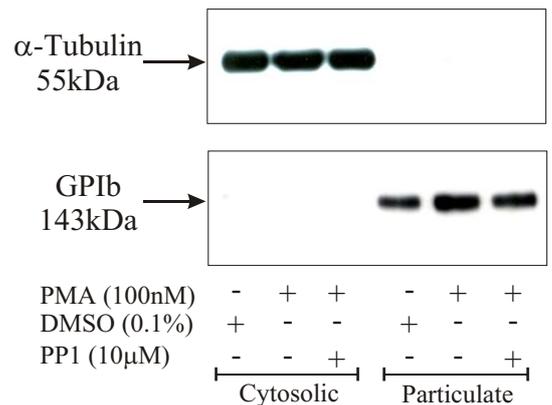


Fig. 6

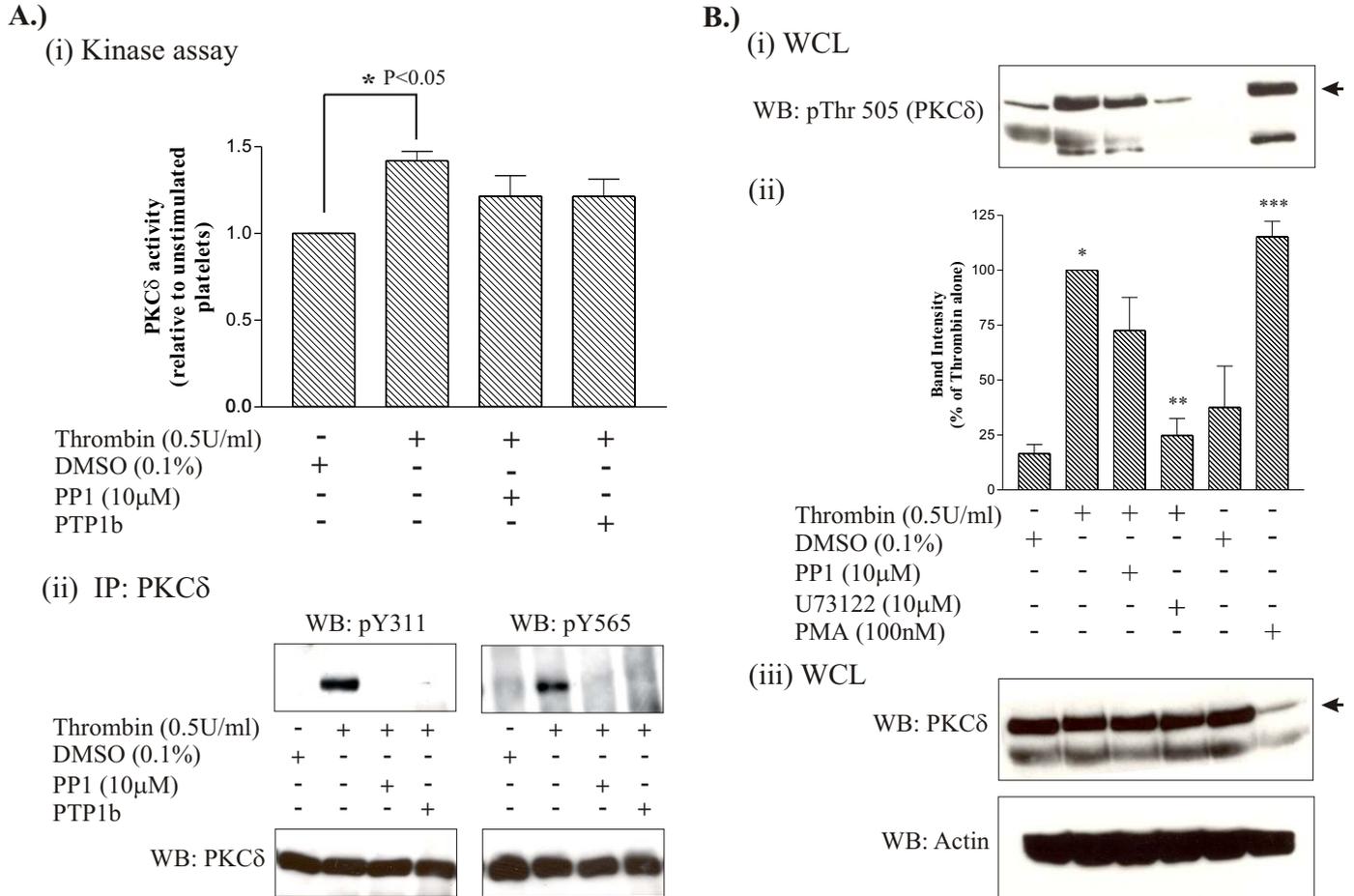


Fig. 7