Physical and functional interaction between PKCδ and Fyn tyrosine kinase in human platelets

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Abbreviations:
5-HT, 5-hydroxytryptamine; ACD, acid citrate dextrose; ATP, Adenosine trisphosphate; BIM, bisindoylmaeleimide 1; ECL, enhanced chemiluminescence; GP, glycoprotein; HEPES, N-[2-hydroxyethyl] piperazine-N-[2-ethanesulphonic acid]; ITAM, immunoreceptor tyrosine-based activation motif; NP40, Nonidet P 40; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; PLC, phospholipase C; PRP, platelet-rich plasma; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulphate; SH2, Src homology 2; TBS-T, tris-buffered saline with Tween; vWF, von Willebrand factor
SUMMARY

An increasing number of tyrosine kinases have been shown to associate with isoforms of the protein kinase C (PKC) family. Here, we show evidence for physical and functional interaction between PKC´ and the Src family kinase Fyn in human platelets activated by alboaggreggin-A, a snake venom capable of activating both GPIb-V-IX and GPVI adhesion receptors. This interaction involves phosphorylation of PKCδ on tyrosine, and is specific in that other isoforms of PKC, PKCµ and λ, which also become tyrosine-phosphorylated, do not interact with Fyn. In addition, PKC´ does not interact with other platelet-expressed tyrosine kinases Syk, Src or Btk. Stimulation also leads to activation of both Fyn and PKC´ and to serine phosphorylation of Fyn within a PKC consensus sequence. Alboaggreggin-A-dependent activation of Fyn is blocked by bisindolylmaleimide I, suggesting a role for PKC isoforms in regulating Fyn activity. Platelet activation with alboaggreggin-A induces translocation of the two kinases from cytoplasm to the plasma membrane of platelets, as observed by confocal immunofluorescence microscopy. Translocation of Fyn and PKCδ are blocked by PP1 and bisindolylmaleimide I, showing a dependence upon Src and PKC kinase activities. Although PKC activity is required for translocation, it is not required for association between the two kinases since this was not blocked by bisindolylmaleimide I. Rottlerin, which inhibited PKCδ activity, did not block translocation of either PKCδ or Fyn, but potentiated platelet aggregation, 5-hydroxytryptamine secretion and the calcium response induced by alboaggreggin-A, indicating that this kinase plays a negative role in the control of these processes.
INTRODUCTION

The protein kinase C (PKC) family comprises 10 isoymes grouped into 3 classes: conventional (α, γ, βI, βII), novel (δ, ε, η, θ) and atypical (ζ, ι/λ). In addition, PKCμ and ν are considered as a fourth class, although some authors classify them as a separate family termed protein kinase D (1). Protein kinases C are critical for the regulation of several functional events in cells including exocytosis and adhesion. In platelets, activation of PKC by diacylglycerol or phorbol esters can induce platelet degranulation (2), and inhibitors of PKC effectively inhibit receptor-mediated platelet secretion (3-6), although synergism between Ca2+- and PKC-mediated processes is required for a full secretory response of platelets to external stimuli (4,7-9). Stimulation of PKC also leads to activation of the integrin αIIbβ3 (10) although there is conflicting evidence concerning the sensitivity of agonist-induced aggregation to pharmacological inhibition of PKC (4,11-13). It is now becoming clear however that different isoforms of PKC may play differential roles in the regulation of these events. Leitges et al. (2002) (14) have recently shown that PKCδ is a negative regulator of degranulation in mast cells, demonstrating a more complex role for the PKC family in control of this functional event.

There are now known to be a variety of different mechanisms by which PKC activity and localisation may be regulated, important amongst which is phosphorylation of serine, threonine and more recently tyrosine residues (15). We have recently shown that the novel isoform, PKCθ, may be phosphorylated on tyrosine through a physical and functional association with the non-receptor tyrosine kinase Btk (16). The closely related kinase PKCδ has also been shown to be phosphorylated on tyrosine residues in a variety of cell types and in response to a variety of stimuli including phorbol ester, growth factors and hormones (17-31). We had shown that phosphorylation of PKCθ on tyrosine is associated with an inhibition of this isoform, but there is presently controversy concerning the functional role of tyrosine phosphorylation of PKCδ. In some reports activity of PKCδ is reduced by tyrosine phosphorylation (20,21,32) whereas in others the modification enhances its activity (15,17,22,23,31). Differential effects upon activity may be accounted for by different sites of tyrosine phosphorylation within the protein by different
upstream kinases. It has been shown that PKCδ may be phosphorylated by Fyn (18,20,21), Lyn (24,33), Src (18,20,26,33-35), Abl (36,37) and growth factor receptor kinases (20,23).

In addition to tyrosine phosphorylation of PKC isoforms, tyrosine kinases may in turn become reciprocally phosphorylated on serine and threonine residues. There is substantial evidence of this for members of the Src family kinases. Src itself is phosphorylated by PKC isoforms (38,39) and one isoform, PKCε, has been shown to lie upstream of activation of Src and Lyn in cardiac myocytes (40). On the other hand, PKCδ has been shown to associate with Src and Lyn in mast cells, leading to reciprocal phosphorylation and a decrease in functional activity of Src and Lyn (33). Fyn has also been shown to be phosphorylated on serine residues (41,42). Cabodi et al. (2000) (43) have recently shown that one isoform of PKC, PKCη, associates with Fyn and is necessary and sufficient for activation of Fyn in keratinocytes. There is therefore evidence that, in other cell types, Fyn may associate physically and functionally with PKC isoforms, leading to modulation of its activity.

In the present report we were interested to investigate the mutual regulation of PKCδ and Fyn in human platelets. We were able to show a physical interaction between the two kinases and phosphorylation of each kinase on both serine/threonine residues and tyrosines. Functionally Fyn is shown to lie upstream of PKCδ, and PKC isoforms other than PKCδ lie upstream of Fyn positively regulating its activity and translocation from cytosol to plasma membrane.
EXPERIMENTAL PROCEDURES

Materials

Trimeresurus albolabris venom was a kind gift from Professor R.G.D. Theakston (Liverpool, U.K.). Alboaggregin-A was prepared from venom by ion-exchange chromatography as previously described (Falati et al. 1999). Anti-phosphotyrosine monoclonal antibody 4G10 was from Upstate Biotechnology Inc (TCS Biologicals Ltd, Bucks, UK). All anti-PKC antibodies were from Transduction Laboratories (BD Biosciences, Oxford, UK). Anti-Btk, Fyn, Src and Syk antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-specific anti-Src (Tyr416) and phospho-specific anti-PKC substrate antibody were from Cell Signaling Technology (New England Biolabs, UK). Src family kinase inhibitor PP1 was from Alexis Corp (Nottingham, UK). PKC inhibitor Bisindoylmaleimide I was from Tocris (Bristol, UK). PKCδ↑ specific inhibitor rottrlerin was from BIOMOL Research Labs Inc. FITC-labelled anti-mouse, rabbit and goat antibodies, calcium indicator Fura 2-AM and myelin basic protein were from Sigma (Poole, Dorset, U.K.). LFM-A13 and Raytide™ peptide were from Calbiochem (La Jolla, CA, USA). [3H]-5-HT and [32P]γ-ATP were from Amersham (Amersham, 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. Acid Citrate Dextrose (ACD: 120 mM sodium citrate, 110 mM glucose, 80 mM citric acid, used at 1:7 vol/vol) was used as anticoagulant. Platelet rich plasma (PRP) was prepared by centrifugation at 200g, for 20 min and platelets were then isolated by centrifugation for 10 min at 1000g, 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 Tyrodes-HEPES buffer (145 mM NaCl, 2.9 mM KCl, 10 mM HEPES, 1 mM MgCl₂, 5 mM glucose, pH 7.3). To this platelet suspension, 10 µM indomethacin was added, and a 30 min resting period was allowed before stimulation. Stimulation of platelets was performed in an aggregometer at 37°C, with continuous stirring at 800 rpm, unless stated. Unless otherwise specified, all platelet stimulation occurred in the presence of EGTA (1 mM). Alboagreggin A was used at 3.5 µg/ml, unless otherwise stated, a concentration previously determined to be the EC₅₀ value for induction of 5-HT release (44).

**Immunoprecipitation of proteins**

Reactions were stopped by lysis of platelets with an equal volume of either 2X NP-40 extraction buffer (1% Nonidet P40, 300 mM NaCl, 20 mM Tris, 1 mM phenylmethylsulphonyl fluoride, 10 mM EDTA, 2 mM Na₃VO₄, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 µg/ml pepstatin, pH 7.3) or 2X RIPA buffer (2% Triton X-100, 2% Sodium deoxycholate, 0.2% SDS, 300 mM NaCl, 20 mM Tris, 1 mM phenylmethylsulphonyl fluoride, 10 mM EDTA, 2 mM Na₃VO₄, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 µg/ml pepstatin, pH 7.3). Lysates were incubated with protein-A sepharose plus 1-2 µg immunoprecipitating antibody for 2 hours or overnight at 4 °C. Beads were then washed before addition of 2X Laemmli sample solvent and boiling for 5 min.

**Immunoprecipitation with immobilised antibody**

For experiments involving assessment of phosphorylation of Fyn, in order to avoid contamination of immunoprecipitation samples with IgG heavy chain, it was necessary to covalently couple immunoprecipitating antibody to beads. This was achieved using Seize X protein G immunoprecipitation kit (Perbio Science UK, Cheshire, UK), following the described protocol.
Briefly, 100 µg of antibody was incubated with protein G agarose beads for 1 hour at room temperature to allow binding. Cross-linking reagent disuccinimidyl suberate (DSS) was added for 1 hour and beads were then washed extensively to remove all free antibody. Antibody-linked beads were then added to samples and were incubated overnight at 4 °C. Beads were then washed and trapped protein was eluted by addition of elution buffer (pH 2.8). To the eluate, 5X Laemmli sample solvent was added, and samples boiled for 5 min.

Electrophoresis of proteins and western blotting

Proteins were resolved by electrophoresis in 10-15% gradient SDS-PAGE gels. Samples were then transferred to PVDF membranes, using a Bio-Rad Trans-Blot SD semi-dry transfer cell, blocked with 10% bovine serum albumin and incubated for 1 hour at room temperature with appropriate primary antibody (1 µg/ml). Membranes were then washed before incubation with appropriate secondary antibody followed by thorough washing. Bound peroxidase activity was detected using enhanced chemiluminescence (ECL, Pharmacia-Amersham, UK).

In vitro kinase assays

Autophosphorylation - PKCδ was immunoprecipitated from platelets lysed into NP40 buffer, re-suspended in 20µl of kinase assay (KA) buffer (5 mM MgCl₂, 5 mM MnCl₂, 100 mM NaCl, 10 µM ATP, 2 mM Na₃VO₄, 20 mM HEPES pH 7.2) and the reaction started by addition of [γ-³²P]-ATP (250 µCi/ml). After incubation for 10 min at room temperature, the reaction was terminated by addition of 0.5 ml of ice-cold 100 mM EDTA. Immunoprecipitated proteins were then washed in RIPA buffer before separation by SDS-PAGE and detection of phosphorylated proteins by autoradiography.

Raytide Phosphorylation - Fyn activity was assayed using Raytide peptide as an exogenous
substrate. Immunoprecipitated kinase was resuspended in 20µl of KA buffer and 10 µg Raytide peptide added to each sample. The reaction was started by addition of 10µl of ATP buffer (0.15mM ATP, 30mM MgCl₂ and 200µCi/ml γ-[³²P]-ATP in KA buffer). After incubation at 30°C for 30 min the reaction was terminated by addition of 10% phosphoric acid. Samples were applied to 2x2 cm squares of P81 ion exchange chromatography paper, extensively washed in 0.5% phosphoric acid followed by a wash in acetone. Papers were then dried and labelled Raytide quantified by liquid scintillation counting.

**Measurement of cytosolic calcium**

Measurement of cytosolic calcium was performed as previously described (45). Briefly, 3µM Fura 2-AM was added to platelet rich plasma, and incubated at 30°C for 45 min in the presence of 10 µM indomethacin. Platelets were washed and re-suspended in modified Tyrodes and stimulated at room temperature in the absence of EGTA. Fluorescence excitation was made at 340 and 380 nm and emission at 510 nm was measured using a Perkin-Elmer LS5 spectrofluorimeter. Data are presented as the excitation fluorescence ratio (340:380 nm).

**Measurement of released 5-HT**

Platelets were loaded by incubation of PRP with 0.2 µCi/ml [³H]-5-HT for 1 hour at 37°C. Platelets were pre-incubated with 1 mM EGTA before stimulation, to prevent aggregation. Reactions were terminated by addition of an equal volume of 6% glutaraldehyde in Tyrodes, followed by brief microcentrifugation and [³H]-5-HT released into the supernatant was determined by liquid scintillation counting, and expressed as a percentage of the total tissue content, as described previously (46).
**Immunofluorescence confocal imaging of platelets**

Platelets were stimulated with alboaggregin-A and reactions terminated by addition of 4% paraformaldehyde in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄). Samples were left under agitation for 30 minutes at room temperature. Platelets were then pelleted by centrifugation at 4000 rpm for 2 minutes in a micro-centrifuge and washed twice in PBS. Platelets were immobilised on poly-Lysine coated coverslips overnight, permeabilised by incubation of coverslips with 0.05% Triton X/PBS at room temperature for 10 minutes, and were then incubated for 30 minutes at room temperature with PBS 1% Bovine Serum Albumin (BSA) to block non-specific antibody binding. Samples were then incubated overnight with primary antibody at a concentration of 1 µg/ml in PBS 1% BSA at 4°C. Coverslips were then washed 3 times in PBS 0.05% Triton X-100. Fluorescein isothiocyanate (FITC)-labelled secondary antibody was then added at a concentration of 2 ng/ml in PBS 1% BSA, for 1 hour at room temperature. Subsequent to this, coverslips were washed 3 times in PBS 0.05% Triton X-100 and then mounted onto slides using a 13.5% Mowiol solution containing 2.5% 1,4-diazobicyclo-[2,2,2]octane (DABCO) to prevent bleaching of fluorescence. Platelets were imaged using a Leica TCS-NT confocal laser scanning microscope equipped with Kr/Ar laser (488, 568, 647 nm lines) attached to a Leica DM IRBE inverted epifluorescence microscope with phase-contrast.
RESULTS

Several PKC isoforms become tyrosine phosphorylated upon platelet activation by alboaggregin-A

Tyrosine phosphorylation of various isoforms of PKC has been demonstrated in a number of cell types (15,32,47), and we have recently reported tyrosine phosphorylation of PKCθ induced by alboaggregin A in platelets (16). Alboaggregin A is a purified lectin-type snake venom capable of binding to and activating both GP Ib and GP VI adhesion receptors on platelets for von Willebrand factor and collagen respectively. Here we show that several other PKC isoforms are also phosphorylated on tyrosine upon alboaggregin-A stimulation of platelets (Fig. 1). These include the classical isoform β, the novel isoforms ε & δ and PKCλ, all of which were not phosphorylated under basal conditions, but became so upon platelet activation. PKCη was also investigated, but showed no tyrosine phosphorylation under these conditions.

Association of PKC isoforms with platelet non-receptor tyrosine kinases

We had previously shown that PKCθ physically and functionally associates with the non-receptor tyrosine kinase Btk upon platelet activation (16). It was important therefore to investigate whether other isoforms of PKC that become tyrosine phosphorylated also associate with non-receptor tyrosine kinases. Syk, Src, Fyn and Btk are four major platelet tyrosine kinases known to be involved in the alboaggregin-A activated signalling pathway through activation of both GPIb-V-IX receptor complex and GPVI (16,44,48,49). Each of these kinases was immunoprecipitated from alboaggregin-A activated platelets, and Fig. 2A shows that there is specific association between PKCδ and Fyn, whereas there is no co-association between PKCs ε or λ with any of the tyrosine kinases investigated. The association between PKCδ and Fyn was
dependent upon activation (Fig. 2B) and was inhibited by PP1, a selective inhibitor of Src-family kinases. Bisindolylmaleimide I (BIM), the broad-spectrum PKC inhibitor, had no effect upon the association between PKCδ and Fyn (Fig. 2C).

**Tyrosine phosphorylation and activity of PKCδ is dependent upon Src kinase activity and is upregulated by pre-treatment of platelets with PKC inhibitors**

Fig. 3A shows that under basal conditions, PKCδ is not tyrosine phosphorylated. By 30 s of alboaggregin A stimulation, PKCδ had become tyrosine phosphorylated, reaching a maximal by 60 seconds, and then subsequently decreasing by 120 seconds, although not back to basal levels. As PKCδ and Fyn associate, it was decided to investigate whether inhibition of either of these kinase activities would affect the level of tyrosine phosphorylation of PKCδ. Figure 3B shows that pre-incubation of platelets with the Src family kinase inhibitor PP1 ablates the alboaggregin-A induced tyrosine phosphorylation of PKCδ. However, pre-treatment of platelets with non-isozyme specific inhibitor BIM (20 µM), or specific inhibition of PKCδ using rottlerin (also called mallotoxin, 10 µM), caused a marked increase in the tyrosine phosphorylation of PKCδ. Immunoprecipitated PKCδ was subjected to in vitro kinase assay where activity is measured by autophosphorylation, and incorporation of 32P-labelled phosphate is visualised by autoradiography. Fig. 3C shows that PKCδ from resting platelets had a low level of activity, but that subsequent to alboaggregin A stimulation, its activity increased. On addition of BIM or rottlerin to the kinase assay buffer, activity of PKCδ was ablated. When PKCδ was immunoprecipitated from platelets pre-incubated with either BIM or rottlerin prior to stimulation however, the in vitro activity of PKCδ was enhanced. PKCδ activity in contrast was fully inhibited when platelets were pre-incubated with the Src family kinase inhibitor PP1.
PKCδ translocates to the plasma membrane upon platelet activation in a manner dependent upon Src kinase activity

Translocation of PKC isoforms from cytosol to plasma membrane is characteristic of PKC activation. It was therefore decided to examine the subcellular localisation of PKCδ upon activation of platelets by alboaggregin-A, and to investigate the effect of inhibition of Src and PKC upon this translocation. Fig. 4A shows that upon stimulation of platelets with alboaggregin-A, PKCδ, along with the other tyrosine-phosphorylated isoforms PKCλ and PKCe, translocates from the cytosol to the plasma membrane. This translocation is prevented by pre-treatment of platelets with PP1, the inhibitor of Src family kinases, as shown in Fig. 4B. This effect is specific to inhibition of Src kinases, as inhibition of Btk with LFM-A13 had no effect on translocation of PKCδ. Figure 4B shows that the activity of PKC is essential for translocation of PKCδ to take place, since it is blocked by BIM, although not the activity of PKCδ itself, as inhibition of PKCδ with rottlerin did not affect its redistribution to the cell surface.

Fyn is phosphorylated on Tyr 419 upon activation in a manner dependent upon PKC and Src kinase activity

For full activity of Src kinases, a tyrosine residue within the activation loop of the catalytic domain (Tyr 419 for Fyn) must undergo autophosphorylation (reviewed in (50,51)). Using a phosphorylation site-specific antibody, it is possible to assess the phosphorylation state of this residue. Fig. 5A shows that under basal conditions there is some minimal phosphorylation of this residue (Tyr 419), but that upon stimulation of platelets with alboaggregin-A, tyrosine phosphorylation is enhanced. Addition of the Src family kinase inhibitor PP1 to the platelets (20 µM) ablated the observed phosphorylation. Addition of the non-selective PKC inhibitor BIM (20
µM) to the platelets resulted in a decrease in the stimulated level of phosphorylation back to, but not below, the basal level of phosphorylation, whereas addition of rottlerin (10 µM) had no effect.

**Fyn is phosphorylated on serine within a PKC consensus sequence**

It has long been known that in various cell types, Fyn may be phosphorylated on serine residues (41,42). Fig. 5B shows that upon platelet activation by alboaggregin-A, Fyn becomes phosphorylated on serine residues that are located within a PKC consensus sequence. This was shown by western blotting using an antibody that recognises phosphoserine within a PKC phosphorylation consensus sequence, specifically phosphoserine with arginine or lysine residues at the −2 and +2 positions with a hydrophobic residue at the +1 position. Furthermore, this stimulation-dependent phosphorylation was shown to be dependent on the activity of PKC, as inhibition with BIM (20 µM) ablated the phosphorylation.

**Fyn is activated by alboaggregin and is positively regulated by PKC activity**

It has previously been shown that Fyn is activated in platelets downstream of collagen and thrombin receptor activation (52,53). In Fig. 5C, the activity of Fyn immunoprecipitated from platelets is assayed in vitro by examining its ability to phosphorylate the exogenous substrate peptide Raytide™. Inhibitors were added either to the platelets, prior to stimulation with alboaggregin A, or to the kinase assay buffer (KAB) during the in vitro assay stage, to observe the role of PKC and Src family kinases in regulating the activity of Fyn. There is considerable Fyn activity even in basal platelets, since addition of the Src family kinase inhibitor PP1 (20 µM) to the KAB ablated this measured activity. Upon stimulation of platelets with alboaggregin A however, the activity increases. Pre-incubation of platelets with PP1 prior to stimulation caused
ablation of Fyn activity. Pre-incubation of platelets with the non-selective PKC inhibitor BIM (20 µM) prevented the stimulation-dependent increase in Fyn activity, but did not decrease activity below basal, unlike PP1. Pre-incubation of platelets with rottlerin (10 µM) had no effect on Fyn activity.

Fyn translocates to the plasma membrane in a Src kinase- and PKC kinase-dependent manner

There are various contrasting reports concerning the subcellular localisation of Fyn in basal and activated platelets. While some suggest that Fyn translocates to the cytoskeleton in an aggregation dependant manner (53), others have reported a constitutive association of Fyn with the GPVI-Fc receptor γ chain complex (52,54), indicating that some Fyn would be located at the plasma membrane regardless of stimulation. Figure 6 shows that in resting platelets most Fyn is diffusely distributed throughout the platelet, as indicated by the uniform distribution of staining, whereas upon stimulation of platelets with alboaggregin A, Fyn becomes localised to the plasma membrane, thus demonstrating a stimulation-dependent translocation. Pre-incubation of platelets with the non-selective PKC inhibitor BIM (20 µM) prevented the alboaggregin-A-induced translocation, however when platelets were pre-incubated with rottlerin (10 µM), Fyn still translocated as normal upon alboaggregin-A stimulation. Pre-incubation of platelets with the Src family kinase inhibitor PP1 (20 mM) however prevented translocation of Fyn to the plasma membrane.

Rottlerin potentiates alboaggregin-A-induced platelet aggregation, dense granule secretion and calcium response

Figure 7A shows that rottlerin, at both 5 mM and 20 mM, markedly potentiated alboaggregin-A-induced platelet aggregation, when the agonist was used at concentrations of 1.75 or 0.875 µg/ml,
which are submaximal for induction of aggregation. In addition, when alboaggregin-A-induced dense granule secretion was examined, by means of measurement of release of $^3$H-5-HT from platelets loaded with this compound, it was observed that inhibition of PKCδ caused a marked increase in $^3$H-5-HT released (Fig. 7B). This potentiatory effect was also seen when the effects of rottlerin on calcium mobilisation were examined. Figure 7C shows that pre-incubation of platelets with 5 and 20 µM rottlerin caused concentration-dependent increases in the maximum intracellular calcium concentration achieved by stimulation of platelets with alboaggregin A.
DISCUSSION

It is well established that the PKC and Src kinase families play key roles in signalling events in most cell types. In platelets it has been known for many years that PKC is essential for full activation of secretion and aggregation responses, although not for the shape change response, induced by a wide variety of platelet agonists from adhesion receptors such as GP VI to GPCRs such as the thrombin and thromboxane receptors. Activation of PKC is not an immediate early event, but is generally subsequent to activation of phospholipase C and release of DAG. The Src family of kinases has been shown to play a vital early signalling role in GP VI signaling, being responsible for phosphorylation of FcR γ-chain (52,55). We and others have also shown them to be involved in a similar early event in signaling downstream of GP Ib-V-IX (44,48), and in this report we use the snake venom alboaggregin A to activate both these adhesion receptors to induce early phosphorylation of the γ-chain by Src kinases.

It has become clear however that there is a greater level of complexity involved in these signalling events. In particular, the multiple of isoforms of PKC and multiple members of the Src kinase family are likely each to play different signalling and functional roles. We have recently shown for example that one novel PKC isoform, PKCθ, physically and functionally associates with the tyrosine kinase Btk. This is a selective association, since other members of the PKC family do not associate with Btk. In this paper we have chosen to extend this study of cross-talk to investigate further selective interactions between PKC family members and tyrosine kinases. In so doing we have uncovered a selective physical and functional association between PKCδ and Fyn, which is summarised in the diagram shown in Fig. 8.

PKCδ: tyrosine phosphorylation, activation and translocation to the plasma membrane

Since we had already shown that PKCθ becomes phosphorylated on tyrosine upon activation of
platelets with alboaggregin A (16), it was important to assess whether other members of the PKC family would also become tyrosine phosphorylated upon activation. The classical isoform β, the novel isoforms ε and δ, and the atypical isoform λ were all tyrosine phosphorylated in a stimulation-dependent manner. The novel isoform PKCη was not phosphorylated on tyrosine. Several of these isoforms have previously been shown to be phosphorylated on tyrosine in other cell types in response to a variety of stimuli (15,27,47,56), but their phosphorylation is clearly a cell- and stimulus-specific event. Many different agonists have been shown to cause tyrosine phosphorylation of the novel PKC isoform PKCδ, including growth factors (19,20,25,47), H2O2 (57) and GPCRs (25,58-60). Indeed one of these reports shows phosphorylation of PKCδ in platelets in response to thrombin (60), showing the phosphorylation to be biphasic, possibly corresponding to the phases of activation of Src kinases in platelets in response to this agonist (61). In the present study, in response to alboaggregin A, the time course peaked at 60 s and then dropped to a sustained plateau over the 5 min time course studied. This pattern is similar to, although more rapid to peak than, that seen in other cell types such as pancreatic acinar cells in response to CCK (27) and skeletal muscle cells in response to insulin (47).

It was then important to address the mechanism and role of tyrosine phosphorylation of PKCδ, which has previously been shown to be a calcium-independent, DAG-dependent kinase (1,19). First, PP1 was used as a selective Src family kinase inhibitor and was shown to abolish tyrosine phosphorylation of PKCδ in response to alboaggregin A. Src family kinases are therefore likely to lie upstream of this event, as expected since we and others have shown Src kinases to lie very proximal in the GP VI and GP Ib-V-IX signalling pathways (44,52,55), and therefore blockade of these kinases would lead to inhibition of most signalling and functional events. Src kinases may also be directly responsible for the phosphorylation of PKCδ, and this is in agreement with
other reports in other cell types showing that Src kinases are capable of phosphorylating this PKC isoform \((18,20,24,26,33-35)\). Src kinases clearly lie functionally upstream in this signalling pathway since PP1 also lead to inhibition of PKC\(\delta\) activity (Fig. 3C). Addition of the non-selective inhibitor bisindolylmaleimide (BIM) and the selective inhibitor rottlerin to the kinase assay buffer caused full inhibition of PKC\(\delta\) activity (Fig. 3C). This is in contrast to Davies et al. (2000) (62) who showed in a purified enzyme in vitro kinase assay system that rottlerin did not inhibit PKC\(\delta\), but did inhibit PRAK and MAPKAP-K2. In addition, others have shown poor activity and specificity of rottlerin \textit{in vivo} (63) and \textit{in vitro} (64). Our assay system differed from Davies et al. (62) and involved the use of native PKC\(\delta\) isolated from activated platelets by immunoprecipitation, which may explain the difference between our data and theirs, although it is clear that the specificity of rottlerin for PKC\(\delta\) may be poor. This limits us from drawing definitive conclusions regarding the role of PKC\(\delta\) in signalling pathways, but since we were able to show clear and complete inhibition of PKC\(\delta\) by rottlerin in platelets, this allows us to rule out a role for PKC\(\delta\) if rottlerin is shown to have no effect upon specific cellular responses. When inhibitors were added to the platelet suspension, both BIM and rottlerin were shown to enhance tyrosine phosphorylation of PKC\(\delta\) (Fig. 3B), and in the case of rottlerin, this was associated with an increase in the activity of PKC\(\delta\). This may indicate a negative feedback inhibition of PKC\(\delta\) by itself or other kinases inhibited by rottlerin, leading to enhanced apparent activity when isolated from platelets pre-treated with the PKC inhibitor. These data are in agreement with a recent report showing enhanced tyrosine phosphorylation of PKC\(\delta\) upon activation of pancreatic acinar cells by phorbol ester in the presence of BIM-1 at concentrations less than 30 \(\mu M\) (27).

It has long been recognised that translocation of PKC isoforms from cytosol to plasma membrane is characteristically associated with activation of the kinase (1). Here we have used a confocal immunofluorescence approach to examine movement of PKC isoforms from cytosol to
membrane upon cell activation. PKCs δ, ε and λ, all of which become phosphorylated on tyrosine, translocate from cytosol to a peripheral position in the platelet very rapidly upon activation by alboaggregin A. It was important to ascertain the role of PKC and Src kinase activities upon this translocation event for PKCδ. In correlation with the phosphorylation and activation of PKCδ, addition of PP1 blocked translocation to the membrane. Addition of BIM however also blocked translocation to the membrane, although rottlerin did not do so. This suggests that the activity of PKC isoforms other than PKCδ are important in controlling the localisation of PKCδ. One intriguing possible explanation for this may be that Fyn, which clearly associates with PKCδ, may act as a scaffold, or novel RACK protein (receptor for activated C-kinase) for PKCδ, mediating its transport to the plasma membrane, since BIM-1 was also shown to inhibit Fyn activity whereas rottlerin did not (Fig. 5C). Interestingly however, BIM did not inhibit the interaction between PKCδ and Fyn (Fig. 2C). Therefore PKC activity is not required for interaction between the two kinases, but is required for translocation to the membrane, as has been shown previously for novel isoforms of PKC, which require phosphorylation on serines within their C2 domain for full translocation to the plasma membrane (65).

Fyn: activation and phosphorylation by PKC and translocation to the plasma membrane

Interestingly Fyn is phosphorylated by a PKC isoform in a BIM-sensitive manner. Phosphorylation of Fyn by PKC correlates with activity of Fyn as assessed either by phosphorylation of tyrosine 419 (Fig. 5A), the autophosphorylation site within the catalytic domain of the kinase required for full activity of Fyn, or phosphorylation of an exogenous peptide Raytide (Fig. 5C). Alboaggregin A induces an activation of Fyn from a high resting basal level, and inhibition of PKC activity by BIM leads to inhibition of Fyn activity to the resting level. The high basal activity of Fyn is in contrast to the low basal activity of Src (Crosby & Poole,
unpublished observations) and is in agreement with data showing a constitutive association of active Fyn and Lyn with GP VI (54). Interestingly PP1, the Src kinase inhibitor, reduces the activity and phosphorylation of Fyn on Y419 apparently to zero, to a level well below that of the basal resting state. From this we infer that there are therefore two phases of Fyn activity; a basal activity which does not require PKC activity, and a stimulated activity which is dependent upon PKC activity. This stimulated activity may however not be dependent upon PKCδ activity, since rottlerin is not able to inhibit either the PKC-dependent phosphorylation of Fyn or its activity. We interpret this as meaning that Fyn activity is positively regulated by a PKC kinase activity which does not include PKCδ.

The activity of Src kinases correlates with their translocation from cytosol to plasma membrane in platelets and other cells (66). Fig. 6 showed that Fyn translocates upon alboaggregin A stimulation, and that this translocation depends upon a PKC activity, since it is inhibited by BIM. This PKC activity does not however include PKCδ since rottlerin was not able to ablate this response, and therefore we conclude that, as for Fyn activity, Fyn translocation requires a PKC isoform other that PKCδ. In parallel with its activity, Fyn translocation also depends upon its own activity, since PP1 fully inhibits it.

We had already shown that BIM and PP1 markedly inhibit the secretion of 5-HT induced by alboagreggin A (16,44), demonstrating the dependence of this response upon PKC and Src kinase activities. Here we showed however that PKCδ may exert a negative effect upon dense granule secretion, since rottlerin potentiated the response to alboagreggin A. This is in agreement with a recent finding by Leitges et al (2002) (14) who show a similar negative regulation of mast cell degranulation by PKCδ. This demonstrates a complex role for PKC isotypes in the regulation of secretion. The potentiation of secretion by rottlerin is paralleled by a potentiation of platelet aggregation and the cytosolic calcium response, the latter of which may therefore underlie the
enhanced secretion and aggregation responses in the presence of this inhibitor. Finally, the platelet shape change response is inhibited only by PP1, but not by inhibition of PKC isoforms by BIM or rottlerin (data not shown). This is in agreement with other reports showing that Src kinases play an early signalling role downstream of GP VI and GP Ib-V-IX receptors (44,52,55), but that PKC isoforms play no significant role in the regulation of shape change (67).

In conclusion, it is clear that many PKC isoforms become phosphorylated on tyrosine upon cell activation, and that PKCδ at least associates with a member of the Src kinases, Fyn (see Fig. 8 for summary diagram). Activity of PKCδ and its translocation to the plasma membrane are dependent upon Src kinase activation, as would be predicted from the early signalling role played by Src kinases in adhesion signalling in platelets. In addition however, translocation of PKCδ is regulated by a PKC activity which is not PKCδ itself. Fyn kinase however is phosphorylated not only on tyrosine residues but also on serine in a PKC-dependent manner. Fyn has a high basal activity which is not dependent upon phosphorylation by PKC, but its activity can be increased upon stimulation of platelets in a manner dependent upon PKC activity, although this activity is not likely to involve the associating PKCδ. It may be concluded that another member or members of the PKC family therefore regulate the localisation of both PKCδ and Fyn to the plasma membrane, and this activity is also required for agonist-dependent activation of Fyn. It is possible that either Fyn or PKCδ acts as a scaffold protein to permit translocation of the other kinase to the plasma membrane as part of its activation process. Interestingly, PKC activity is required for translocation of the PKCδ-Fyn complex to the plasma membrane, but not for the activation-dependent association of the two kinases. Finally, the role of PKCδ in functional control of platelet activity may primarily be as a negative regulator of platelet aggregation and secretion, since rottlerin markedly potentiates these functional responses.
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REFERENCES

1. Newton, A. C. (2001) *Chem. Rev.* **101**, 2353-2364
2. Rink, T. J., Sanchez, A., and Hallam, T. J. (1983) *Nature* **305**, 317-319
3. Watson, S. P., McNally, J., Shipman, L. J., and Godfrey, P. P. (1988) *Biochem. J.* **249**, 345-350
4. Walker, T. R., and Watson, S. P. (1993) *Biochem. J.* **289**, 277 – 282
5. Yamada, K., Iwahashi, K., and Kase, H. (1987) *Biochem. Biophys. Res. Commun.* **144**, 35-40
6. Gerrard, J. M., Beattie, L. L., Park, J., Israels, S. J., McNicol, A., Lint, D., and Cragoe Jr., E. J. (1989) *Blood* **74**, 2405-2413
7. Kaibuchi, K., Takai, Y., Sawamura, M., Hoshijima, M., Fujikura, T., and Nishizuka, Y. (1983) *J. Biol. Chem.* **258**, 6701-6704
8. Knight, D. E., Niggli, V., and Scrutton, M. C. (1984) *Eur. J. Biochem.* **143**, 437-446
9. Siess, W., and Lapetina, E. G. (1988) *Biochem. J.* **255**, 309-318
10. Shattil, S. J., and Brass, L. F. (1987) *J. Biol. Chem.* **262**, 992-1000
11. Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F., and al., e. (1991) *J. Biol. Chem.* **266**, 15771-15781
12. Shattil, S. J., Cunningham, M., Wiedmer, T., Zhao, J., Sims, P. J., and Brass, L. F. (1992) *J. Biol. Chem.* **267**, 18424-18431
13. Pulcinelli, F. M., Ashby, B., Gazzaniga, P. P., and Daniel, J. L. (1995) *FEBS Lett.* **364**, 87 – 90
14. Leitges, M., Gimborn, K., Elis, W., Kalesnikoff, J., Hughes, M. R., Krystal, G., and Huber, M. (2002) *Molec. Cell Biol.* **22**, 3970-3980
15. Konishi, H., Tanaka, M., Takemura, Y., Matsuzaki, H., Ono, Y., Kikkawa, U., and Nishizuka, Y. (1997) *Proc. Natl. Acad. Sci. U S A* **94**, 11233-11237.

16. Crosby, D., and Poole, A. W. (2002) *J. Biol. Chem.* **277**, 9958-9965.

17. Konishi, H., Yamauchi, E., Taniguchi, H., Yamamoto, T., Matsuzaki, H., Takemura, Y., Ohmae, K., Kikkawa, U., and Nishizuka, Y. (2001) *Proc. Natl. Acad. Sci. U S A* **98**, 6587-6592.

18. Gschwendt, M., Kielbassa, K., Kittstein, W., and Marks, F. (1994) *FEBS Lett* **347**, 85-89.

19. Gschwendt, M. (1999) *Eur. J. Biochem.* **259**, 555-564.

20. Denning, M. F., Dlugosz, A. A., Threadgill, D. W., Magnuson, T., and Yuspa, S. H. (1996) *J. Biol. Chem.* **271**, 5325-5331.

21. Denning, M. F., Dlugosz, A. A., Howett, M. K., and Yuspa, S. H. (1993) *J. Biol. Chem.* **268**, 26079-26081.

22. Li, W., Mischak, H., Yu, J. C., Wang, L. M., Mushinski, J. F., Heidaran, M. A., and Pierce, J. H. (1994) *J. Biol. Chem.* **269**, 2349-2352.

23. Li, W., Yu, J. C., Michieli, P., Beeler, J. F., Ellmore, N., Heidaran, M. A., and Pierce, J. H. (1994) *Mol. Cell. Biol.* **14**, 6727-6735.

24. 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) *Biochem. Biophys. Res. Commun.* **214**, 888-894.

25. Soltoff, S. P., and Toker, A. (1995) *J. Biol. Chem.* **270**, 13490-13495.

26. Shanmugam, M., Krett, N. L., Peters, C. A., Maizels, E. T., Murad, F. M., Kawakatsu, H., Rosen, S. T., and Hunzicker-Dunn, M. (1998) *Oncogene* **16**, 1649-1654.

27. Tapia, J. A., Bragado, M. J., Garci, a, M., n, L. J., and Jensen, R. T. (2002) *Biochim. Biophys. Acta* **1593**, 99-113.
28. Brodie, C., Bogi, K., Acs, P., Lorenzo, P. S., Baskin, L., and Blumberg, P. M. (1998) *J. Biol. Chem.* **273**, 30713-30718

29. Kronfeld, I., Kazimirsky, G., Lorenzo, P. S., Garfield, S. H., Blumberg, P. M., and Brodie, C. (2000) *J. Biol. Chem.* **275**, 35491-35498

30. Wrenn, R. W. (2001) *Biochem. Biophys. Res. Commun.* **282**, 882-886

31. Blass, M., Kronfeld, I., Kazimirsky, G., Blumberg, P. M., and Brodie, C. (2002) *Mol. Cell. Biol.* **22**, 182-195

32. Joseloff, E., Cataisson, C., Aamodt, H., Ocheni, H., Blumberg, P., Kraker, A. J., and Yuspa, S. H. (2002) *J. Biol. Chem.* **277**, 12318-12323.

33. Song, J. S., Swann, P. G., Szallasi, Z., Blank, U., Blumberg, P. M., and Rivera, J. (1998) *Oncogene* **16**, 3357-3368

34. Zang, Q., Lu, Z., Curto, M., Barile, N., Shalloway, D., and Foster, D. A. (1997) *J. Biol. Chem.* **272**, 13275-13280

35. Blake, R. A., Garcia-Paramio, P., Parker, P. J., and Courtneidge, S. A. (1999) *Cell Growth Differ.* **10**, 231-241

36. Sun, X., Wu, F., Datta, R., Kharbanda, S., and Kufe, D. (2000) *J. Biol. Chem.* **275**, 7470-7473.

37. Yuan, Z. M., Utsugisawa, T., Ishiko, T., Nakada, S., Huang, Y., Kharbanda, S., Weichselbaum, R., and Kufe, D. (1998) *Oncogene* **16**, 1643-1648

38. Liebenhoff, U., Greinacher, A., and Presek, P. (1994) *Cell. Mol. Biol. (Noisy-le-grand)* **40**, 645-652.

39. Landgren, E., Blume-Jensen, P., Courtneidge, S. A., and Claesson-Welsh, L. (1995) *Oncogene* **10**, 2027-2035.

40. Ping, P., Zhang, J., Zheng, Y. T., Li, R. C., Dawn, B., Tang, X. L., Takano, H.,
Balafanova, Z., and Bolli, R. (1999) *Circ. Res.* **85**, 542-550

41. Kypta, R. M., Hemming, A., and Courtneidge, S. A. (1988) *EMBO J.* **7**, 3837-3844.

42. Cheng, S. H., Espino, P. C., Marshall, J., Harvey, R., Merrill, J., and Smith, A. E. (1991) *J. Virol.* **65**, 170-179.

43. Cabodi, S., Calautti, E., Talora, C., Kuroki, T., Stein, P. L., and Dotto, G. P. (2000) *Mol. Cell* **6**, 1121-1129.

44. Falati, S., Edmead, C. E., and Poole, A. W. (1999) *Blood* **94**, 1648-1656

45. Poole, A. W., and Watson, S. P. (1995) *Brit. J. Pharmacol.* **115**, 101-106

46. Poole, A., Gibbins, J. M., Turner, M., van Vugt, M. J., van de Winkel, J. G., Saito, T., Tybulewicz, V. L., and Watson, S. P. (1997) *EMBO J.* **16**, 2333-2341.

47. Braitman, L., Sheffi-Friedman, L., Bak, A., Tennenbaum, T., and Sampson, S. R. (1999) *Diabetes* **48**, 1922-1929.

48. Wu, Y., Suzuki-Inoue, K., Satoh, K., Asazuma, N., Yatomi, Y., Berndt, M. C., and Ozaki, Y. (2001) *Blood* **97**, 3836-3845

49. Dormann, D., Clemetson, J. M., Navdaev, A., Kehrel, B. E., and Clemetson, K. J. (2001) *Blood* **97**, 929-936

50. Morgan, D. O., and De Bondt, H. L. (1994) *Curr. Opin. Cell Biol.* **6**, 239-246.

51. Superti-Furga, G. (1995) *FEBS Lett.* **369**, 62-66.

52. Ezumi, Y., Shindoh, K., Tsuji, M., and Takayama, H. (1998) *J. Exp. Med.* **188**, 267-276

53. Wu, Y., Ozaki, Y., Inoue, K., Satoh, K., Ohmori, T., Yatomi, Y., and Owadab, K. (2000) *Biochim. Biophys. Acta* **1497**, 27-36.

54. Suzuki-Inoue, K., Tulasne, D., Shen, Y., Bori-Sanz, T., Inoue, O., Jung, S. M., Moroi, M., Andrews, R. K., Berndt, M. C., and Watson, S. P. (2002) *J. Biol. Chem.* **277**, 21561-21566.
55. Briddon, S. J., and Watson, S. P. (1999) Biochem. J. **338**, 203-209

56. Nakai, M., Hojo, K., Yagi, K., Saito, N., Taniguchi, T., Terashima, A., Kawamata, T., Hashimoto, T., Maeda, K., Gschwendt, M., Yamamoto, H., Miyamoto, E., and Tanaka, C. (1999) *J. Neurochem.* **72**, 1179-1186.

57. Yamamoto, T., Matsuzaki, H., Konishi, H., Ono, Y., and Kikkawa, U. (2000) *Biochem. Biophys. Res. Commun.* **273**, 960-966

58. Ho, A. K., Hashimoto, K., Matowe, W., and Chik, C. L. (1999) *Mol. Cell. Endocrinol.* **150**, 169-178

59. Soltoff, S. P., Avraham, H., Avraham, S., and Cantley, L. C. (1998) *J. Biol. Chem.* **273**, 2653-2660

60. Moussazadeh, M., and Haimovich, B. (1998) *FEBS Lett.* **438**, 225-230

61. Golden, A., and Brugge, J. S. (1989) *Proc. Natl. Acad. Sci. U S A* **86**, 901-905.

62. Davies, S. P., Reddy, H., Caivano, M., and Cohen, P. (2000) *Biochem. J.* **351**, 95-105

63. Leitges, M., Elis, W., Gimborn, K., and Huber, M. (2001) *Lab. Invest.* **81**, 1087-1095

64. Soltoff, S. P. (2001) *J. Biol. Chem.* **276**, 37986-37992

65. Pepio, A. M., and Sossin, W. S. (2001) *J. Biol. Chem.* **276**, 3846-3855

66. Horvath, A. R., Muszbek, L., and Kellie, S. (1992) *EMBO J.* **11**, 855-861.

67. Paul, B. Z., Daniel, J. L., and Kunapuli, S. P. (1999) *J. Biol. Chem.* **274**, 28293-28300
FIGURE LEGENDS

**Figure 1. Activation-dependent tyrosine phosphorylation of PKC isoforms.** i) & ii) PKCβ, η, ε and iii) & iv) PKCδ & λ were immunoprecipitated from RIPA lysates of basal platelets and platelets stimulated for 60 s with alboaggregin-A (3.5 µg/ml). i) & iii) Samples were western-blotted with monoclonal antiphosphotyrosine antibody 4G10, showing that PKCs β, ε & δ become tyrosine phosphorylated upon alboaggregin-A stimulation. ii) & iv) Blots were re-probed with appropriate anti-PKC antibody. Data shown here are representative of 3 experiments.

**Figure 2. PKCδ selectively associates with Fyn**

A) Tyrosine kinases Btk, Src, Fyn and Btk were immunoprecipitated from NP-40 lysates of platelets stimulated with alboaggregin-A (3.5 µg/ml) for 60 s. Samples were western-blotted with i) anti-PKCδ, ii) anti-PKCe or iii) anti-PKCλ. Whole cell lysate lane (WCL) was included in each for reference. B) NP-40 Lysates were prepared from basal platelets or platelets stimulated with alboaggregin A (3.5 µg/ml) for 60 s and PKCδ was immunoprecipitated. Samples, including a whole cell lysate (WCL), were western-blotted with i) anti-Fyn and ii) anti-PKCδ antibodies as shown. C) NP40 lysates were prepared from basal platelets and platelets stimulated with alboaggregin A for 60 s and Fyn immunoprecipitated. For some samples, platelets were pretreated with either PP1 (20 µM) or BIM (20 µM) prior to stimulation. Samples, including a WCL, were western-blotted with i) anti-PKCδ and ii) anti-Fyn antibodies. Results shown are representative of 3 experiments.

**Figure 3. Tyrosine phosphorylation and activation of PKCδ is inhibited by PP1**

PKCδ was immunoprecipitated from RIPA lysates of basal platelets or platelets activated by
alboaggregin-A (3.5 µg/ml). In (A), platelets were stimulated with alboaggregin-A for the time periods indicated. In (B), platelets were pre-treated (10 mins) with either rottlerin (10 µM), bisindolylmaleimide I (BIM, 20 µM) or PP1 (20 µM) as indicated before stimulation with alboaggregin-A (3.5 µg/ml) for 60 s. In (C), inhibitors (rottlerin, BIM or PP1) were added either to platelets or to the kinase assay buffer (KAB) 10 mins prior to either platelet stimulation or kinase assay respectively. For (A), (B) & (C), i) shows samples western-blotted for phosphotyrosine using mAb 4G10 and ii) shows a reprobe for PKCδ. Results shown are representative of 3 experiments.

Figure 4. Translocation of PKCδ to the platelet plasma membrane is blocked by PP1 and BIM

Fixed platelets were stained with antibodies to PKCδ, ε or λ as indicated, followed by FITC-conjugated anti-mouse secondary antibody as described in Methods. Basal resting platelets are shown in i), whereas platelets stimulated with alboaggregin-A (3.5 µg/ml) for 60 s are shown in ii) and iii). Platelets in iii) were pretreated with either PP1 (20 µM), LFM-A13 (40 µM), bisindolylmaleimide I (BIM, 20 µM) or rottlerin (10 µM) as indicated before stimulation. Images shown are from a single experiment but are representative of at least three repetitions.

Figure 5. Fyn is phosphorylated on serine by PKC and is activated in a PKC-dependent manner

A) & B) Fyn was immunoprecipitated from RIPA lysates of basal platelets and platelets stimulated for 60 s with alboaggregin-A (3.5 µg/ml). Platelets were pretreated with either DMSO (0.1% vol as control), PP1 (20 µM), bisindolylmaleimide I (BIM, 20 µM) or rottlerin (10 µM) as indicated before stimulation. For A) i), samples were western-blotted with anti-phosphoserine PKC substrate antibody whereas for B) i) samples were western-blotted with an antibody recognising phosphorylated Y419 in Fyn. For A) & B) ii) blots were re-probed with
anti-Fyn antibody to show even loading. Data shown here are representative of 3 experiments.

C) Fyn was immunoprecipitated from 1% NP-40 lysates of basal platelets and platelets stimulated for 60 s with alboaggregin-A (3.5 µg/ml). Prior to stimulation some samples, as indicated, were pre-incubated for 10 mins with PP1 (20 µM), bisindolylmaleimide I (BIM, 20 µM) or rottlerin (10 µM) as indicated before stimulation. Immunoprecipitates were subjected to in vitro kinase assay, during which some samples were treated with PP1 (20 µM) in the kinase assay buffer (KAB) as indicated. Incorporation of $^{32}$P into Raytide was assayed by liquid scintillation counting. Data shown are mean +/- S.E.M. (n=3).

**Figure 6. Translocation of Fyn to the platelet plasma membrane is blocked by PP1 and BIM**

Fixed platelets were stained with antibodies to Fyn, followed by FITC-conjugated anti-rabbit secondary antibody as described in Methods. Samples were either from basal platelets or platelets stimulated with alboaggregin-A (3.5 µg/ml) for 60 s without stirring as indicated. Some samples were pretreated with either PP1 (20 µM), bisindolylmaleimide I (BIM, 20 µM) or rottlerin (10 µM) as indicated before stimulation. Images shown are from a single experiment but are representative of at least three repetitions.

**Figure 7. Rottlerin induces a marked potentiation of functional responses in activated platelets**

Platelets were pretreated for 10 min with rottlerin at the concentrations indicated, or with DMSO (0.1% vol.) as control. A) Platelet aggregation trace data are shown induced by submaximal concentrations of alboaggregin-A at (i) 0.875 µg/ml and (ii) 1.75 µg/ml, as assessed by standard turbidimetric aggregometry over a 3 min period. Data shown are the result of one experiment but are representative of at least three repetitions. B) Platelets were loaded by pre-incubation for 1 hr
with $[^3]H$-5-HT (0.2$\mu$Ci/ml). Released $[^3]H$-5-HT in response to alboaggregin A (3.5 $\mu$g/ml, 1 min) was assessed by liquid scintillation counting. Data shown are mean +/- S.E.M. (n=3). C) Platelets were loaded by pre-incubation for 45 mins with the calcium indicator Fura 2-AM (3 $\mu$M). Results show the rise in cytosolic calcium in response to alboaggregin A (3.5 $\mu$g/ml) in the presence or absence of rottlerin as indicated. Data shown are the result of one experiment but are representative of at least three repetitions.

**Figure 8. Model depicting the physical and functional interaction between PKC$\delta$ and Fyn**

Upon activation of platelets through the adhesion receptors GP Ib-V-IX and GP VI, Fyn and PKC$\delta$ physically associate, translocate to the plasma membrane and become activated and phosphorylated as shown. The physical interaction between the two kinases is independent of PKC activity, however the translocation of both kinases to the plasma membrane is dependent upon the activity of a PKC isoform other than PKC$\delta$ itself. Fyn becomes phosphorylated on a serine by a PKC isoform other than PKC$\delta$, and is activated in a PKC-dependent manner. PKC$\delta$ activity subsequently negatively modulates platelet activation processes as shown.
Fig. 1

| IP: | β | η | ε | Blot: 4G10 |
|-----|---|---|---|------------|
| i)  |   |   |   |            |

Alboaggregin: 
- + - + - +

| IP: | δ | λ | Blot: PKC |
|-----|---|---|------------|
| iii) |   |   |            |

Alboaggregin: 
- + - +

|   |   |   | Blot: PKC |
| iv) |   |   |            |

Blot: 4G10
Fig. 2

A)  WCL  IP:Syk  IP:Src  IP:Fyn  IP:Btk
   i)  ![Blot: PKCδ]
   ii) ![Blot: PKCε]
   iii) ![Blot: PKCλ]

B)  WCL  B  S
   i)  ![Blot: Fyn]
   ii) ![Blot: PKCδ]

C)  WCL  IP:Fyn
   i)  ![Blot: PKCδ]

| Treatment | PKCδ | Syk | Src | Fyn | Btk |
|-----------|------|-----|-----|-----|-----|
| Alboaggregin A | - | - | + | + | + |
| PP1 | - | - | - | + | - |
| BIM | - | - | - | - | + |

Blot: Fyn
Fig. 3

A) IP: PKCδ

i) Blot: 4G10

Time (s): 0 15 30 60 120 300

ii) Blot: PKCδ

B) IP: PKCδ

i) Blot: 4G10

Alboaggrelin A: - + + + + +
Rottlerin : - - - + -
BIM: - - - - +
PP1: - - + - -

ii) Blot: PKCδ

C) IP: PKCδ

i) Autoradiograph

Alboaggrelin A: - + + + + + +
Inhibitor in KAB: - - BIM Rott - - -
Inhibitor in platelets: - - - - BIM Rott PP1

ii) Blot: PKCδ
Fig. 5

PKCδ-Fyn cross-talk

A) i) IP:Fyn

Blot: αY419P

Alboaggregin A:
Rottlerin:
BIM:
PP1:

- + + + + +
- - - - + +
- - - + - -

ii) Blot: Fyn

B) i) IP:Fyn

Blot: αPKC substrate

Alboaggregin A:
BIM:

- + + +
- - + +
Fig. 6

Alboaggregin A: - + + + +
Inhibitor: - - BIM rottlerin PP1
**Fig. 7A**

PKCδ-Fyn cross-talk

i) Alboaggregin-A (0.875 μg/ml)

Rottlerin (μM)

Optical Density

0

5

20

3 min

---

ii) Alboaggregin-A (1.75 μg/ml)

Rottlerin (μM)

Optical Density

0

5

20

3 min
Fig. 7

B) % 5-HT secretion

C) Fluorescence ratio

PKCδ-Fyn cross-talk
Fig. 8

PKCδ-Fyn cross-talk

GPIb-V-IX

GPVI

Fyn

PKCδ

PKCδ

[Ca^{2+}]_i

5-HT secretion

aggregation
Physical and functional interaction between PKCδ and Fyn tyrosine kinase in human platelets
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