There is a high degree of cross-talk between tyrosine phosphorylation and the serine/threonine phosphorylation signaling pathways. Here we show a physical and functional interaction between the classical protein kinase C isoform (cPKC), PKCα, and two major nonreceptor tyrosine kinases in platelets, Syk and Src. In the presence of a cPKC-selective inhibitor Gö6976, platelet 5-hydroxytryptamine release was abolished in response to co-activation of glycoproteins VI and Ib-IX-V by the snake venom alboaggregin A, whereas platelet aggregation was substantially inhibited. Of the two platelet cPKCs, PKCα but not PKCβ was activated, occurring in an Syk- and phospholipase C-dependent manner. Syk and PKCα associate in a stimulation-dependent manner, requiring Syk but not PKC activity. PKCα and Syk also co-translocate from the cytosol to the plasma membrane upon platelet activation, in a manner dependent upon the activities of both kinases. Although PKC is phosphorylated on tyrosine downstream of Syk, we provide evidence against phosphorylation of Syk by PKCα, consistent with a lack of effect of PKCα inhibition on Syk activity. PKCα also associates with Src; although in contrast to interaction with Syk, PKCα activity is required for the association of these kinases but not the stimulation-induced translocation of Src to the cell membrane. Finally, the activity of Src is negatively regulated by PKCα, as shown by potentiation of Src activity in the presence of the PKC inhibitors GF109203X or Gö6976. Therefore, there is a complex interplay between PKCα, Syk, and Src involving physical interaction, phosphorylation, translocation within the cell, and functional activity regulation.

The protein kinase C (PKC) family comprises 10 isozymes grouped into the following three classes: conventional (α, γ, βI, and βII), novel (δ, ε, η, λ, and θ), and atypical (ζ and ηA). In addition, PKCµ is considered as a fourth class, now generally referred to as a separate family termed protein kinase D (1). 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 diacylglycerol (DAG) or phorbol ester can induce aggregation, and agonist-induced secretion can be prevented by pharmacological inhibition of a broad range of PKC isoforms (2–5).

At least seven PKC isoforms (α, β, δ, θ, ε, η, and ζ) are expressed in platelets (6–12), and it is becoming clear that each isoform may play different roles in platelet function and may have different modes of activation and downstream targets. PKCα has been identified recently as an essential factor in positively regulating α- and dense granule secretion in platelets (13) as well as platelet aggregation (14). We were therefore interested to determine how PKCα activity may be modulated and its signaling role in platelets.

There are now known to be a variety of different mechanisms by which PKC activity and localization may be regulated, important among which is the phosphorylation of serine, threonine, and more recently tyrosine residues (15). Regulation of novel PKC isoforms by tyrosine phosphorylation has been well characterized in a variety of cell types and in response to a variety of stimuli (7, 16–30). We have shown recently (6, 31) that the novel isoforms PKCθ and PKCδ may be phosphorylated on tyrosine through physical and functional associations with the nonreceptor tyrosine kinases Btk and Fyn, respectively. Some reports have also documented tyrosine phosphorylation of PKCα in several other cell types and in response to a variety of stimuli including insulin (32), β1 integrin ligation (33), and oxidative stress induced by H2O2 in COS-7 cells (34). This last report concludes that tyrosine phosphorylation of PKCα recovered from these cells was catalytically active independent of DAG binding, indicating a possible pathway for early activation of PKC not dependent on PLC hydrolysis of inositol phospholipid. It has also been shown that PKCα may be positively regulated by Src (35), and therefore there is a precedent in the literature for regulation of this classical isoform by tyrosine phosphorylation.

Here we were interested to investigate the mutual regulation of PKCα, Syk, and Src kinases in human platelets. We show that the two tyrosine kinases Syk and Src physically interact with PKCα leading to distinct functional consequences. Syk-PKCα interaction does not depend upon PKCα activity in contrast to Src-PKCα interaction, of which does depend upon the activity of PKCα. Although PKCα activity was dependent upon Syk, as may be expected because of the proximal role played by Syk in GP VI-dependent signaling (36), there was no reciprocation because Syk activity was not regulated by PKCα. However, Src activity was negatively regulated by PKCα. The results suggest that PKCα, Syk, and Src interact to regulate each other and cellular activities in platelets.

**EXPERIMENTAL PROCEDURES**

Materials—Trimeresurus albolabris venom was a kind gift from Professor R. G. D. Theunke (Liverpool, UK). Alboaggregin A was pre-
obtained from four independent experiments are expressed as means ± S.E. from four independent experiments. B, concentration-response curve for Go6976-mediated inhibition of 5-HT release. [3H]5-HT-labeled platelets were pretreated for 10 min with various concentrations of Go6976 and stimulated with alboaggregin A (1 μM; 3 min). [3H]5-HT release was measured by liquid scintillation counting, and values of % inhibition of 5-HT release obtained from four independent experiments are expressed as means ± S.E. (IC50 = 180 nM). C, Go6976 inhibits platelet aggregation. Platelets were pretreated for 10 min with 100 μM or 1 μM (IC50) or vehicle solution (0.1% Me2SO final concentration). Platelets were stimulated with alboaggregin A (1 μM) and aggregation was assessed by turbidimetric aggregometry over a period of 3 min. Data shown are from one experiment representative of at least three independent experiments. D, concentration-response curve for Go6976-mediated inhibition of platelet aggregation. Platelets were pretreated for 10 min with various concentrations of Go6976 and stimulated with alboaggregin A (1 μM; 3 min). Inhibition of platelet aggregation by Go6976 from four independent experiments is expressed as mean ± S.E. (IC50 = 90 nM).

FIG. 1. Role of classical PKCs in 5-HT release and platelet aggregation. A, Go6976 and GF109203X abolish 5-HT release induced by platelet activation. [3H]5-HT-labeled platelets were pretreated for 10 min with GF109203X (20 μM) or Go6976 (1 μM) or vehicle solution (0.1% Me2SO final concentration). Platelets were stimulated with alboaggregin A (1 μg/ml; 3 min), and the [3H]5-HT released was measured by liquid scintillation counting. Results shown are mean ± S.E. from four independent experiments. B, concentration-response curve for Go6976-mediated inhibition of 5-HT release. [3H]5-HT-labeled platelets were pretreated for 10 min with various concentrations of Go6976 and stimulated with alboaggregin A (1 μg/ml; 3 min). [3H]5-HT released was measured by liquid scintillation counting, and values of % inhibition of 5-HT release obtained from four independent experiments are expressed as means ± S.E. (IC50 = 180 nM). C, Go6976 inhibits platelet aggregation. Platelets were pretreated for 10 min with 100 μM or 1 μM (IC50) or vehicle solution (0.1% Me2SO final concentration). Platelets were stimulated with alboaggregin A (1 μg/ml) and aggregation was assessed by turbidimetric aggregometry over a period of 3 min. Data shown are from one experiment representative of at least three independent experiments. D, concentration-response curve for Go6976-mediated inhibition of platelet aggregation. Platelets were pretreated for 10 min with various concentrations of Go6976 and stimulated with alboaggregin A (1 μg/ml; 3 min). Inhibition of platelet aggregation by Go6976 from four independent experiments is expressed as mean ± S.E. (IC50 = 90 nM).
PKCα, and immunoblotted for either PKCα or PKCβ. Lysates and resuspended in kinase assay (KA) buffer (5 mM MgCl₂, 5 mM MnCl₂, 100 mM NaCl, 1 mM ATP, 2 mM Na₃VO₄, 20 mM HEPES, pH 7.2). After incubation for 10 min at 37 °C, the reaction was terminated by addition of 0.5 ml of ice-cold EDTA (100 mM). Immunoprecipitated proteins were then washed twice with SDS-PAGE and immunoblot with anti-phosphoserine PKC substrate or anti-phosphothreonine antibodies (1:1000). In this way the assay is a direct, nonradioactive in vitro kinase assay for PKC.

**In Vitro Kinase Assay for Protein Kinase C**—PKC isoforms were immunoprecipitated from platelet lysates, and activation by alboaggregin A was assayed by in vitro kinase assay buffer before separation by SDS-PAGE and immunoblot with anti-phosphoserine PKC substrate or anti-phosphothreonine antibodies (1:1000). In this way the assay is a direct, nonradioactive in vitro kinase assay for PKC.

**Inositol Phosphate Accumulation Assay**—Platelets were loaded by incubation of platelet-rich plasma with 50 μCi/ml myo-[³H]inositol for 3 h at 30 °C. After addition of LiCl to a final concentration of 20 mM, platelets were separated from the supernatant by centrifugation and resuspended in Tyrode's-HEPES buffer containing 20 mM LiCl. After incubation with different inhibitors or vehicle solution (0.1% Me₂SO final concentration) for 10 min at 37 °C, platelets were activated and reactions stopped by addition of an equal volume of 0.8M perchloric acid. 350 μl of 0.72 N KOH, 0.6 M KHCO₃ solution was added to neutralize the sample solution, and samples were centrifuged for 2 min at 20,000 × g and inositol phosphates were separated on AG 1-X8 resin columns. Total labeled inositol phosphates were then determined by liquid scintillation counting, and data are presented as counts/min.

**Immunofluorescence Confocal Imaging**—Platelets were prepared as described, before being pretreated with antagonists and stimulated. Reactions were terminated by addition of 4% paraformaldehyde in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄). Platelets were pelleted by centrifugation at 4000 rpm for 2 min in a microcentrifuge and washed twice in PBS. Platelets were immobilized on poly-L-lysine-coated coverslips overnight, permeabilized by incubation of coverslips with 0.05% Triton X/PBS at room temperature for 10 min, and incubated for 30 min at room temperature with 1% bovine serum albumin (BSA) in PBS to block nonspecific antibody binding. Samples were then incubated for 3 h in 1% BSA/PBS at room temperature with primary antibodies; coverslips were washed in PBS, 0.05% Triton X-100 and incubated for 30 min at room temperature with 1% BSA in PBS. Fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG secondary antibody and tetramethylrhodamine isothiocyanate (TRITC)-labeled anti-mouse IgG secondary antibody were added, and the reaction stopped by lysis into ice-cold 1% Nonidet P-40 buffer. PKCα or PKCβ phosphorylation of MBP was assessed by immunoblotting with anti-phospho-Ser PKC substrate antibody. Data shown are representative of three independent experiments.
FIG. 3. PKCα activity is regulated by Syk and PLC. A and B, PKCα activation is abolished by Syk inhibition and reduced by PLC inhibition. Platelets were then stimulated with alboaggregin A (Albo. A) (1 µg/ml) for 1 min, or control platelets were treated with vehicle solution (water). Reactions were stopped by lysis into ice-cold 1% Nonidet P-40 buffer, and immunoprecipitated (IP) PKCα was resuspended in kinase buffer containing 5 µg of dephosphorylated MBP and incubated for 10 min at 37 °C. Samples were separated by SDS-PAGE and immunoblotted with 1:1000 anti-phospho-Ser PKC substrate antibody. Data shown in A are representative of four independent experiments, quantified by densitometry, and represented in B, Western blot. B, shows data as mean ± S.E. (⁎ = p < 0.01 and ** = p < 0.001, compared with platelets pretreated with vehicle solution and stimulated with alboaggregin A). C, effect of piceatannol and U73122 on inositol phosphate (IP) accumulation. Platelets were labeled with myo-[3H]inositol, pretreated for 10 min with piceatannol (10 µg/ml), U73122 (10 µM), or vehicle solution (0.1% Me2SO final concentration) and incubated for 10 min at 37 °C. Samples were separated by SDS-PAGE and immunoblotted with 1:1000 anti-phospho-Ser PKC substrate antibody. Data shown are representative of three independent experiments. D, platelets were stimulated with alboaggregin A (1 µg/ml) for 1 min, or control platelets were treated with vehicle solution (water). Reactions were stopped by lysis into ice-cold 1% Nonidet P-40 buffer, and immunoprecipitated PKCα was resuspended in kinase buffer containing 5 µg of dephosphorylated MBP in the presence of either piceatannol (10 µg/ml), GF109203X (10 µM), or vehicle solution (0.1% Me2SO final concentration) and incubated for 10 min at 37 °C. Samples were separated by SDS-PAGE and immunoblotted with 1:1000 anti-phospho-Ser PKC substrate antibody. Data shown are representative of three independent experiments. E, effect of piceatannol and U73122 on Syk activity. Platelets were pretreated for 10 min with piceatannol (10 µg/ml), U73122 (10 µM), or vehicle solution (0.1% Me2SO final concentration) and stimulated with alboaggregin A (1 µg/ml) for 1 min, or control platelets were treated with vehicle solution (water). Immunoprecipitated Syk was immunoblotted with anti-Syk Tyr(P)-525/526 antibody and reprobed with anti-Syk antibody as shown. Data shown are representative of three separate experiments. F and G, effect of piceatannol on Fyn and Lyn activity. Platelets were pretreated for 10 min with piceatannol (10 µg/ml), PP1 (20 µM), or vehicle solution (0.1% Me2SO final concentration) and stimulated with alboaggregin A (1 µg/ml) for 1 min, or control platelets were treated with vehicle solution (water). Fyn (F) or Lyn (G) was immunoprecipitated, and their autophosphorylation was analyzed by immunoblotting with anti-phospho-Src family Tyr(P)-416 antibody. Data shown are representative of three independent experiments.
Anti-phospho-Thr antibody (Fig. 2) using anti-phospho-Ser PKC substrate antibody (Fig. 2). PKC-dependent phosphorylation of MBP was detected in vitro. Induced phosphorylation of MBP by PKC was mediated by classical isoforms, and GF109203X, which nonselectively inhibits PKC, only partially inhibited this response (Fig. 1A). The concentration-effect curve with variable slope using GraphPAD Prism was shown to be 390 nM. As shown in Fig. 1A, preincubation with maximally effective concentrations of either Go6976 (1 μM) or GF109203X (20 μM) abolished 5-HT release induced by platelet activation. The concentration dependence was mediated solely through activation of PLC. As signaling downstream of GP VI is mediated by activation of Src activity was substantially diminished, but a PLC-independent, Syk-dependent component remained. This suggested that Syk kinase, it was important to determine the activation dependence of PKCα by Syk in human platelets was analyzed by immunofluorescence. Platelets were pretreated for 10 min with piceatannol (10 μg/ml), and there is evidence that classical isoforms may be involved in these processes (13, 14). We chose to verify this involvement by using the PKC inhibitors Go6976, which has selectivity for the classical isoforms, and GF109203X, which nonselectively inhibits PKC isoforms. Platelets were stimulated with 1 μg/ml alboaggregin A, a purified lectin-type snake venom capable of binding and activating both GP Ib-IX-V and GP VI adhesion receptors for von Willebrand factor and collagen, respectively (39). As shown in Fig. 1A, preincubation with maximally effective concentrations of either Go6976 (1 μM) or GF109203X (20 μM) abolished 5-HT release induced by platelet activation. The concentration dependence of Go6976 for inhibition of 5-HT release shows an IC₅₀ of 180 nM (Fig. 1B). Although platelet aggregation was abolished by 20 μM GF109203X, 1 μM Go6976 only partially inhibited this response (Fig. 1C). Again, the concentration dependence of Go6976 for inhibition of this response was determined, and the IC₅₀ was shown to be 390 nM (Fig. 1D).

Selective Activation of PKCα by Alboaggregin A in Platelets—The classical isoforms of PKC predominantly expressed in platelets are PKCα and -β (6, 7). By having determined the dependence of platelet responses upon classical isoforms of PKC, it was important to determine whether individual isoforms became activated upon platelet activation. Initially, it was important to confirm expression of both PKCα and PKCβ in human platelets and to demonstrate that the antibodies used were isoform-selective. In Fig. 2A, we immunoprecipitated either PKCα or PKCβ and blotted each immunoprecipitate with the antibodies for each isoform. The data show expression of each isoform in platelets and confirm that each antibody is only able to detect the single isoform it was raised against. PKCα or PKCβ were then immunoprecipitated using these isoform-specific antibodies, and the kinase activity was assessed in vitro using myelin basic protein (MBP) as a substrate. PKC-dependent phosphorylation of MBP was detected using anti-phospho-Ser PKC substrate antibody (Fig. 2B) or anti-phospho-Thr antibody (Fig. 2C). Stimulation of platelets induced phosphorylation of MBP by PKCα but not by PKCβ, as detected by anti-phospho-Ser PKC substrate antibody, suggesting that PKCα is selectively activated by alboaggregin A in platelets. The time dependence of PKCα activation by alboaggregin A was investigated by stimulating the platelets for different times, from 5 to 60 s. Kinase activity was measured as described by using anti-phospho-Ser PKC substrate antibody (Fig. 2D) and was shown to increase during the 60-s stimulation period. As a control, and in order to demonstrate that phosphorylation of MBP detected during in vitro kinase assay was mediated by classical PKCs, the assay was repeated in the absence or presence of Go6976 (1 μM). Fig. 2E shows that phosphorylation of MBP was markedly diminished in the presence of inhibitor, showing that it is mediated by classical PKC activity.

Regulation of PKCα Activity by PLC and Syk—The molecular mechanism underlying PKCα activation was next investigated. As showing downstream of GP VI is mediated by activation of Syk kinase, it was important to determine the activation dependence of PKCα by Syk and to determine whether this dependence was mediated solely through activation of PLC or by additional Syk-dependent signaling. PKCα activity, measured in vitro as described above, was shown to be completely dependent upon the activity of Syk kinase, because it is fully blocked in the presence of the Syk inhibitor piceatannol (Fig. 3, A and B). In the presence of the PLC inhibitor U73122, the activity was substantially diminished, but a PLC-independent, Syk-dependent component remained. This suggested that Syk-

RESULTS

Dependence of Platelet Responses upon Classical PKC Isoforms—It is clear that PKC isoforms play important roles in regulating both secretion and aggregation events in platelets, and there is evidence that classical isoforms may be involved in these processes (13, 14). We chose to verify this involvement by using the PKC inhibitors Go6976, which has selectivity for the classical isoforms, and GF109203X, which nonselectively inhibits PKC isoforms. Platelets were stimulated with 1 μg/ml alboaggregin A, a purified lectin-type snake venom capable of binding and activating both GP Ib-IX-V and GP VI adhesion receptors for von Willebrand factor and collagen, respectively (39). As shown in Fig. 1A, preincubation with maximally effective concentrations of either Go6976 (1 μM) or GF109203X (20 μM) abolished 5-HT release induced by platelet activation. The concentration dependence of Go6976 for inhibition of 5-HT release shows an IC₅₀ of 180 nM (Fig. 1B). Although platelet aggregation was abolished by 20 μM GF109203X, 1 μM Go6976 only partially inhibited this response (Fig. 1C). Again, the concentration dependence of Go6976 for inhibition of this response was determined, and the IC₅₀ was shown to be 390 nM (Fig. 1D).

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Involvement of PKCα in Human Platelet Activation

FIG. 4. Syk and PKCα translocate to the cell membrane and co-localize upon platelet activation. The localization of Syk and PKCα in human platelets was analyzed by immunofluorescence. Platelets were pretreated for 10 min with piceatannol (10 μg/ml), Go6976 (1 μM), or vehicle solution (0.1% MeSO final concentration). Platelets were then stimulated with alboaggregin A (Albo. A) (1 μg/ml) for 1 min, or control platelets were treated with vehicle solution (water). Samples were fixed and stained with mouse anti-PKCα (1 μg/ml) and rabbit anti-Syk (1:500) antibodies, followed by anti-mouse IgG rhodamine-conjugated and anti-rabbit IgG fluorescein-conjugated secondary antibodies. The localization of Syk (FITC, left panels), PKCα (TRITC, center panels), and the superimposition of the two channels (right panels) are shown. Data shown are representative of four independent experiments.

In Vitro Raytide Phosphorylation Assay—Src activity was assayed using Raytide peptide as an exogenous substrate. Immunoprecipitated kinase was resuspended in 20 μl of KA buffer, and 10 μg of Raytide peptide was added to each sample. The reaction was started by the addition of 10 μl of ATP buffer (0.15 mM ATP, 30 mM MgCl₂, and 200 μCi/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 2 × 2 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 labeled Raytide was quantified by liquid scintillation counting.

Statistics and Data Presentation—Bar graphs were obtained by using GraphPAD Prism (GraphPAD software), and where appropriate, statistical significance was assessed using one-way analysis of variance with Bonferroni’s multiple comparison post-test. Log concentration-effect curves were fitted to logistic expressions for sigmoidal concentration-effect curves with variable slope using GraphPAD Prism.
Involvement of PKCα in Human Platelet Activation

![Image](108x367 to 514x737)

**Fig. 5.** PKCa and Syk associate in an activation-dependent and Syk kinase-dependent manner. A, inhibition of Syk interaction with PKCa by piceatannol. Platelets were pretreated for 10 min with U73122 (10 μM), piceatannol (10 μg/ml), or vehicle solution (0.1% MeSO final concentration) and stimulated with alboaggregin A (Albo. A) (1 μg/ml) for 1 min, or control platelets were treated with vehicle solution (water). IP, immunoprecipitation; WB, Western blot. B, interaction of Syk with PKCa is not dependent upon PKCa activity. Platelets were pretreated for 10 min with GF109203X (20 μM), G6976 (1 μM), piceatannol (10 μg/ml), or vehicle solution (0.1% MeSO final concentration) and stimulated with alboaggregin A (1 μg/ml) for 1 min or control platelets were treated with vehicle solution (water). C, Syk interacts with PKCa from early time points. Platelets were treated for 1 min with vehicle solution (water) or for 5, 15, 30, 60, and 180 s with alboaggregin A (1 μg/ml). A–C, reactions were stopped by lysis into ice-cold 1% Nonidet P-40 buffer, and PKCa was immunoprecipitated, separated by SDS-PAGE, and immunoblotted with 1:1000 anti-Syk antibody. The position of Syk on the blot is indicated. Both immunoprecipitating antibody (anti-PKCα) and blotting antibody (anti-Syk) were derived from mice, generating a prominent IgG heavy chain band as indicated (IgG HC). A and B, the presence of PKCa was assessed by reblotting with 1 μg/ml anti-PKCα antibody. C, Tyr phosphorylation of PKCa and associated Syk was assessed by reblotting with 1 μg/ml anti-phospho-Tyr 4G10 antibody (center panel). D, PKCa is phosphorylated on tyrosine in an Syk-dependent manner. Platelets were pretreated for 10 min with piceatannol (10 μg/ml) or vehicle solution (0.1% MeSO final concentration) and stimulated with alboaggregin A (1 μg/ml) for 1 min, or control platelets were treated with vehicle solution (water). Reactions were stopped by lysis into ice-cold 1% Nonidet P-40 buffer, and PKCa immunoprecipitates were immunoblotted with 1 μg/ml anti-phospho-Tyr 4G10 antibody. A–D, immunoprecipitation of PKCa was confirmed by reblotting membranes with 1 μg/ml anti-PKCα antibody. All data shown are representative of three independent experiments.

**VI**

where Syk lies essentially upstream of PLCγ2 (36). Fig. 3, F and G, shows that under our conditions piceatannol is also selective for Syk over members of the Src family kinases, because it does not inhibit either Fyn (Fig. 3F) or Lyn (Fig. 3G) activity, as assessed by autophosphorylation of each kinase detected by an antibody specific for phosphorylated tyrosine in the activation loop of the Src family kinase domain (anti-Src-phospho-Tyr-416) (41).

**Activation-dependent Association between PKCa and Syk**—As we had shown PKCa to be activated by Syk-dependent signals, both PLC-dependent and -independent, we were interested to determine whether the functional interaction of these two proteins also involves their association in an heteromeric complex. The cellular distribution of these two proteins was first analyzed by confocal microscopy. Fig. 4 shows that, in resting platelets, Syk and PKCa were broadly expressed in the platelet cytoplasm. Upon platelet activation,
both Syk and PKCα translocated from the cytoplasm to a sub-plasma membrane localization, where the two proteins were shown to co-localize. Pretreatment with either piceatannol or Gö6976 abolished the translocation of both Syk and PKCα, suggesting that the simultaneous activity of both proteins is necessary for translocation of either protein in response to platelet activation.

The association between Syk and PKCα was also investigated by co-immunoprecipitation. The association between the two kinases depends upon the activity of Syk but not upon the activity of PKCα, because piceatannol, but not U73122 or inhibitors of PKC, abolishes the interaction as assessed by co-immunoprecipitation (Fig. 5, A and B). As shown in Fig. 5C (top panel), Syk is abundantly co-precipitated with PKCα after 5 s of alboaggregin A stimulation. Longer stimulations (up to 180 s) do not further increase the amount of Syk co-precipitated with PKCα. Most interestingly, both PKCα and associating Syk are also rapidly tyrosine-phosphorylated in response to platelet activation (Fig. 5C (middle panel)), although phosphorylation of PKCα is weak by comparison with Syk. Fig. 5D shows that tyrosine phosphorylation of PKCα is dependent upon Syk activity, because piceatannol substantially inhibited tyrosine phosphorylation of PKCα.

By having shown that Syk may feed forward to induce phosphorylation of PKCα on tyrosine and positively regulate the activity of PKCα, it was also important to assess whether Syk was phosphorylated and/or reciprocally regulated by PKCα. In Fig. 6A, we have used the anti-phospho-Ser PKC substrate antibody, raised against a consensus sequence for phosphorylation by PKC, to show that although phosphorylation of Syk may be detected upon stimulation, this is not downstream of PKCα because there is no inhibition of phosphorylation by Gö6976. On the other hand, phosphorylation is abolished by the PLC inhibitor U73122 and partially inhibited by the broad spectrum PKC inhibitor GF109203X, suggesting that at least part of the phosphorylation event may be downstream of a nonclassical PKC isofrom. Phosphorylation data correlated with assay of Syk activity, because Fig. 6B shows autophosphorylation of Tyr-525/526 is induced upon activation and is not affected by inhibition of classical PKC isoforms using Gö6976. There was a small increase in Syk activity upon broad spectrum inhibition of PKCs using GF109203X, suggesting a possible negative feedback on Syk activity by nonclassical PKCs.

Platelet Activation-dependent Association of PKCα and c-Src—As it had been shown previously that PKCα may also be positively regulated by another nonreceptor tyrosine kinase, Src (35), we were interested to address whether PKCα would associate with Src in platelets. Fig. 7A shows that PKCα does not co-immunoprecipitate with Src in resting platelets, but upon activation with alboaggregin A, the two kinases associate. This association may be seen in both Src and PKCα immunoprecipitates (shown in Fig. 7A, i and ii). Unlike for the PKCα-Syk association, however, the interaction was dependent upon PKC activity, as pretreatment with GF109203X or Gö6976 abolished co-immunoprecipitation, possibly suggesting that PKCα needs to be in its active state to associate with Src. Consistent with this and with data from Fig. 3 showing that PKCα activity depends upon Syk activity, inhibition of Syk by piceatannol also substantially inhibited the association between PKCα and Src (Fig. 7B). In order to assess the selectivity of interaction between PKCα and Src, the presence of PKCβ in Src immunoprecipitates was investigated and shown not to be present in either basal or stimulated conditions (Fig. 7C). Additionally, PKCα was also shown not to associate with other members of the Src family kinases, Fyn and Lyn (Fig. 7, D and E), thereby demonstrating a degree of specificity in the association between PKCα and Src.

It was therefore important to determine whether associating Src was phosphorylated by PKCα. We reprobed Src immunoprecipitates in Fig. 7A with anti-phospho-Ser PKC substrate antibody or an anti-phosphothreonine antibody, but as shown in the figure we could not detect phosphorylation of Src on these residues. This suggests that Src is not phosphorylated by PKCα either in resting platelets or upon platelet activation. Nevertheless, we cannot completely exclude the possibility that Src is phosphorylated by PKCα on a site that is not recognized by the antibodies used.

We also studied the translocation of PKCα and Src in the cell by confocal immunofluorescence microscopy. As shown in Fig. 8, these two kinases are sparsely distributed in the cytoplasm...
of resting platelets, but following platelet activation both PKCa and Src translocate to a sub-plasma membrane location where they appear co-localized. Pretreatment of platelets with PP1, an Src family kinase inhibitor, abolishes the translocation of both PKCa and Src. On the other hand, the classical PKC-selective inhibitor G6976 abolishes only PKCa translocation without inhibiting Src translocation. This is consistent with our finding that the association between PKCa and Src depends upon PKC activity (Fig. 7A) and suggests that although the two kinases physically associate, neither this association nor the activation of PKCa is necessary for translocation of Src.

**Functional Regulation of Src by PKC**—By having shown the association of Src and PKCa, it was important to determine whether PKC activity could regulate the activity of associating Src kinase. We chose to assay Src activity by the following two methods: analysis of autophosphorylation of Tyr-416, and in vitro phosphorylation of exogenous substrate peptide Raytide. Under basal conditions Src is not phosphorylated on Tyr-416 (Fig. 9, A and B), and upon activation this residue becomes phosphorylated. Pretreatment of platelets with PKC inhibitors, either G6976 or GF109203X, induced an increase in the stimulated phosphorylation of Tyr416, suggesting that PKCa may...
negatively regulate Src activity. Pretreatment of platelets with the Src inhibitor PP1 completely abolished activation-induced autophosphorylation of Src. When assayed in vitro by 32P-labelling of Raytide peptide by immunoprecipitated Src, inhibition of PKC isoforms by GF109203X also potenitized the activity of Src (Fig. 9C), confirming that PKCs negatively regulate the activity of Src in this system. Finally, we assessed the effect of the Syk inhibitor piceatannol on Src activity (Fig. 9D), and we show that the activity is not diminished in the presence of this inhibitor, demonstrating selectivity of piceatannol for Syk over Src in our assay. This is consistent with gene knock-out studies, where absence of Syk had no effect upon adhesion-mediated activation of Src (42). There is a small but reproducible increase in Src activity in fact, which is consistent with the working model we present in Fig. 10, as Syk positively regulates PKCα activity which in turn negatively regulates Src activity. It would be predicted that inhibition of Syk would therefore lead to an increase in Src activity.

**DISCUSSION**

It is widely accepted that tyrosine phosphorylation is critical for early activation of platelets by the adhesion receptors GP VI and GP Ib-IX-V. However, in addition to tyrosine kinases, the PKC family of serine/threonine kinases has been shown to be essential in aggregation and secretion (13, 14, 43–46). This study has defined a critical role for PKCα in complex with Syk and Src tyrosine kinases in human platelets activated downstream of GPVI and GP Ib-IX-V, and this study also demonstrates an essential role for this PKC isozyme in regulating secretion of 5-HT and a major contributory role in regulating platelet aggregation (illustrated diagrammatically in Fig. 10).

In this study we have analyzed the association, phosphorylation, activation, and translocation of PKCα, Syk, and Src in human platelets activated downstream of GP VI and GP Ib-IX-V. Here we have used the snake venom component alboaggrin A as an agonist to stimulate platelets through combined activation of GP Ib-V-IX and GP VI, receptors for von Willebrand factor and collagen, respectively (37, 39, 47). Co-activation of these receptors is likely to occur in vivo, and they may act synergistically with each other to induce substantial platelet activation (48). Murugappan et al. (7) have recently reported selective inhibition of platelet responses to collagen, rather than thrombin, by the classical PKC inhibitor G6976, suggesting a primary role for classical PKCs in signaling mediated by adhesion. Of the classical PKC family, platelets express PKCα and PKCβ isoforms predominantly, with little or no expression of PKCγ (6, 7). In our study, co-activation of GP VI and GP Ib-IX-V leads to selective activation of PKCα rather than the PKCβ isoforms, allowing us to infer that responses blocked by the classical isoform-selective inhibitor G6976 are likely to be primarily mediated by PKCα. We therefore conclude from data presented in Fig. 1 that platelet 5-HT secretion induced by alboaggrin A is absolutely dependent upon PKCα activity. Platelet aggregation is also completely dependent upon PKC activity, being blocked by GF109203X, but although a major component is PKCα-dependent, there is also a small component that is not blocked by G6976 and is therefore PKCα-independent, and may therefore be downstream of a nonclassical isoform of PKC. We have recently shown that PKCθ is activated by alboaggrin A (6), and this may be one of the nonclassical isoforms that play a role in mediating platelet aggregation. Indeed, deficiency of PKCθ has been shown recently to lead to signaling defects in the activation of integrin αIIβ3, in human platelets (49).

Syk is a major early tyrosine kinase component of the GP VI signaling pathway (36, 50) and is also activated by GP Ib-IX-V (37, 51). It has been shown recently to regulate classical PKC isoforms by direct tyrosine phosphorylation of PKCα (52, 53), in particular on Tyr-658 of PKCα, allowing us to infer that responses blocked by the classical isoform-selective inhibitor G6976 are likely to be primarily mediated by PKCα. We therefore interested to determine whether PKCα was activated in an Syk-dependent manner, whether it was associated with Syk, and whether it was phosphorylated by Syk in human platelets.

Our evidence, from Fig. 3, suggests that PKCα is activated in a manner absolutely dependent upon Syk activity, because inhibition of Syk using the inhibitor piceatannol abolishes PKCα activity. Although piceatannol has a narrow concentration range over which it is selective for Syk over members of the Src family (54, 55), our control experiments shown in Fig. 3 demonstrate that under our conditions piceatannol is at least selective for Syk inhibition relative to inhibition of the two major upstream Src kinases Fyn and Lyn. Also, piceatannol does not inhibit PKCα directly, as addition of this Syk inhibitor to the in vitro kinase assay for PKCα does not affect its kinase activity. It is important to state here that genetic approaches to selective modulation of Syk activity would also be valuable to support our findings, although the usefulness of the mouse Syk gene knock-out would be limited, because analysis of interaction of PKCα with Syk would not be possible. Future studies...
may however involve generation of a point mutant kinase-dead Syk gene knock-in, where full analysis of the role of Syk kinase activity would be made possible. PKCα is known to be regulated by products of phospholipase C, diacylglycerol, and inositol 1,4,5-trisphosphate-mediated calcium rise. Syk is established as lying upstream of PLCα2 in platelets, in both GP VI and GP Ib-IX-V-mediated pathways (36, 37), and it was therefore possible that the Syk dependence of PKCα activity was solely mediated by its activation of PLCα2. However, this was not the case, because Fig. 3 also shows that a maximally effective concentration of the PLC inhibitor is not able to abolish PKCα activity completely, in contrast to piceatannol that does abolish PKCα activity. We therefore conclude that PKCα is activated in an Syk-dependent manner through PLC-dependent and PLC-independent pathways. The latter may involve direct tyrosine phosphorylation of PKCα, and we show in Fig. 5 that although minor by comparison with tyrosine phosphorylation of Syk, there is a clearly discernible tyrosine phosphorylation of PKCα in platelets that occurs over a similar time course to that of PKCα activation. This phosphorylation is downstream of Syk kinase, because it is blocked by piceatannol, as shown in Fig. 5D. This would be consistent with other reports where tyrosine phosphorylation of PKCα has been shown in other cell types, for example in insulin-stimulated CHO cells expressing the insulin receptor (32) and in mast cells downstream of FceRI activation (52, 53). Indeed, Konishi et al. (34) showed that in response to H2O2 stimulation of COS-7 cells, PKCα becomes phosphorylated on tyrosine and is catalytically activated (34). Evidence is provided by these authors that phosphorylation of conserved tyrosine residues within the catalytic domain of PKC can activate the kinase in a manner unrelated to hydrolysis of inositol phospholipids and generation of diacylglycerol. In the present work, we show that PKCα is both tyrosine-phosphorylated and activated in an Syk-dependent manner. This is not a definitive proof that direct phosphorylation by Syk induces the activation of PKCα, rather that PKCα activity lies downstream of Syk activity. Most importantly, however, there is not likely to be a reciprocation by PKCα on Syk activity, because there was no change in activity seen in the presence of Go6976 (Fig. 6B). This was consistent with the lack of evidence for direct phosphorylation of Syk by PKCα, as shown in Fig. 5A using the antibody directed against PKC substrates in the consensus phosphorylation sequence.
Involvement of PKCα in Human Platelet Activation

PKCα is a key signaling molecule in platelets, which is activated by co-stimulation of adhesion receptors GP VI and GP Ib-IX-V. PKCα activation leads to the phosphorylation or regulation of other signaling molecules, such as Syk and Src. Syk and Src are involved in regulating both 5-HT secretion and aggregation, although there is some controversy regarding the mechanism of their activation in platelets.

The PLC-independent pathway to regulation of aggregation depends upon PKCα, which plays a critical role in regulating both 5-HT secretion and aggregation. Although PKCα is not feedback phosphorylated or regulated by PKCα, however, Syk is phosphorylated by PKCα, although this may not be direct phosphorylation.

In conclusion, PKCα plays a critical role in regulating both 5-HT secretion and aggregation, although the exact mechanism of their activation remains unclear. Further studies are needed to elucidate the role of PKCα in regulating these processes.
Spc. In addition to control of activities, PKCα physically associates with both Syk and Src in a stimulation-dependent manner. The complex interplay between these signaling partners now requires further detailed analysis to elucidate how the associations may take place, in order to develop tools to specifically disrupt interactions.

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