Phosphatidylinositol transfer protein α (PITPα) participates in the supply of phosphatidylinositol (PI) required for many cellular events including phospholipase C (PLC) β and γ signaling by G-protein-coupled receptors and receptor-tyrosine kinases, respectively. Protein kinase C has been known to modulate PLC signaling by G-protein-coupled receptors and receptor-tyrosine kinases, although the molecular target has not been identified in most instances. In each case phorbol myristate acetate pretreatment of HL60, HeLa, and COS-7 cells abrogated PLC stimulation by the agonists formyl-Met-Leu-Phe, ATP, and epidermal growth factor, respectively. Here we show that phosphorylation of PITPα at Ser166 resulted in inhibition of receptor-stimulated PLC activity. Ser166 is localized in a small pocket between the 165–172 loop and the rest of the protein and was not solvent-accessible in either the PI- or phosphatidylinositol-loaded structures of PITPα. To allow phosphorylation at Ser166, a distinct structural form is postulated, and mutation of Thr59 to alanine shifted the equilibrium to this form, which could be resolved on native PAGE. The elution profile observed by size exclusion chromatography of phosphorylated PITPα from rat brain or in vitro phosphorylated PITPα demonstrated that phosphorylated PITPα is structurally distinct from the non-phosphorylated form. Phosphorylated PITPα was unable to deliver its PI cargo, although it could deliver phosphatidylinositol. We conclude that the PITPα structure has to relax to allow access to the Ser166 site, and this may occur at the membrane surface where PI delivery is required for receptor-mediated PLC signaling.

Phosphatidylinositol transfer proteins (PITPs) are a family of lipid-binding proteins that transfer individual molecules of phosphatidylinositol (PI) or phosphatidylinositol (PC) between membrane compartments (1). Originally identified as soluble proteins of ~35 kDa, the family of PITP-related proteins has subsequently grown to include three subgroups of proteins all containing a PITP domain (2, 3); the classical PITPs α and β (35 kDa), the larger related proteins Rgdpβ1 and -II (160 kDa), and the soluble Rgdpβ protein (38 kDa). The yeast Sec14p and its related family members form a separate group of proteins that, although they share lipid binding properties and transfer function with the mammalian PITPs, have no sequence or structural similarity (4–7).

PITPα and PITPβ are 77% identical (and 94% similar) in amino acid sequence and have been implicated in both signaling and membrane traffic (8–14). Biochemical studies involving reconstitution of cytosol-depleted cell preparations with crude cytosol have consistently identified PITP (α and β) as a reconstitution factor in phospholipase C (PLC)-mediated PI 4,5-bisphosphate hydrolysis, the synthesis of 3-phosphorylated lipids by phosphoinositide 3-kinases, regulated exocytosis, and the biogenesis of vesicles at the Golgi (8–16). Although both PITP isoforms can be used interchangeably in the reconstitution assays, they are likely to have some distinct functions in vivo. Deletion of the PITPα or PITPβ genes give distinct phenotypes; deletion of the PITPα gene leads to neurodegeneration and early death, while deletion of PITPβ is embryonically lethal (17, 18). Studies using genetic manipulation have failed to clarify the role of PITPα in PLC signaling possibly because of the overlapping roles of these isoforms (19, 20).

The ability of PITPs and PITPβ to transfer PI makes these soluble proteins ideally suited for regulating the spatial and temporal provision of PI in specific membrane compartments or in the nucleus where it can be phosphorylated by specific lipid kinases (2, 21). Recently we have reported the plasma membrane association of both PITPα and β isoforms when cells are activated by EGF to stimulate phospholipase C activity (20). Phosphorylated forms of PI play essential roles in many cellular processes. The major requirement is for PI 4,5-bisphosphate.
at the plasma membrane where it is a substrate for both PLC and phosphoinositide 3-kinases, enzymes whose activity is regulated by cell surface receptors. Additional roles include regulation of ion channels, enzymatic activity of phospholipase D, maintenance of the cytoskeleton, and recruitment of target proteins by interaction with their phosphoinositide-binding domains including PH (pleckstrin homology), PX (pho × homology), FYVE, and ENTH (epsin/N-terminal homology) (21, 22). Proteins containing these domains appear to be involved in membrane traffic including endocytosis, exocytosis, and vesicle budding.

Many previous studies have reported that activation of protein kinase C (PKC) attenuates receptor-coupled PLC activity thus providing a negative feedback signal to limit the magnitude and duration of receptor signaling. Despite numerous examples of such regulation, the target for PKC-mediated inhibition has not been identified (Ref. 23; for reviews, see Refs. 24 and 25). These studies include both the G-protein-regulated PLCβ family and receptor-tyrosine kinase-regulated PLCγ family. Additionally no single lipid kinase has yet been identified that provides a dedicated pool of PI 4,5-bisphosphate for PLC-mediated hydrolysis. There are altogether four PI 4-kinases (Type II p55 isoforms (α and β) and Type III PI 4-kinase α and PI 4-kinase β) and at least three phosphatidylinositol 4-phosphate 5-kinases (plus splice variants) (26, 27). So far there has been no evidence for negative regulation by phosphorylation of any of the lipid kinases by PKA. The signal transducing system and the phospholipases are different depending on the receptor, and the only common molecule that participates in both G-protein-regulated systems and receptor-tyrosine kinase is PITPα (10, 19, 28).

In this study we examined the possibility that phosphorylation of PITPα by PKC may be responsible for the negative feedback inhibition. While this study was in progress, van Tiel et al. (29) reported that PITPα was phosphorylated on Ser166 in vitro by PKC. In the present study, we identified Thr59 (minor) and Ser166 (major) as two residues that when phosphorylated provided a dedicated pool of PI 4,5-bisphosphate for PLC-mediated hydrolysis. This exchange for exchange for PC at the plasma membrane.

**EXPERIMENTAL PROCEDURES**

**Materials—** All standard chemicals were obtained from Sigma. γ-Labeled [32P]ATP, [3H]inositol, and [3H]acetate were obtained from Amersham Biosciences. Rat brain PKC (catalog no. 539494) was purchased from Calbiochem. This preparation predominantly contains the conventional PKC isoforms α, β1, β2, and γ. Endoproteinase Glu-C (V8 protease) and trypsin (modified) were sequencing grade and were obtained from Roche Applied Science. The lipids used for exchange were dimyristoyl PC, egg yolk PC, and bovine brain PI all from Sigma.

**Phosphorylation of PITPα by PMA Treatment of HL60 Cells—** HL60 cells (2 × 10⁶ cells) were washed and resuspended in 2 ml of buffer (20 mM Tris, 137 mM NaCl, 3 mM KCl, 1 mM CaCl₂, and 1 mM MgCl₂, pH 7.4), and the cells were incubated for 1 h with 1 mM of [32P]-labeled sodium orthophosphate at 37 °C. The cells were washed, resuspended in 4 ml, and allowed to equilibrate for 15 min at 37 °C. 900 µl of the cells were then added to tubes containing 100 µl of PMA (100 nM final concentration), and the cells were incubated for 0, 30, 60, and 300 s at 37 °C. The reaction was stopped by addition of 500 µl of lysis buffer (1% Nonidet P-40, 50 mM PIPES, pH 6.8) supplemented with phosphatase inhibitors, and insoluble material was removed by centrifugation. PITPα was immunoprecipitated using 5F12 monoclonal antibody (30) coupled to Sepharose, and the immunocomplexes were washed five times in lysis buffer. The immunoprecipitates were resolved by SDS-PAGE, Western blotted, phosphorimaged, and probed using the monoclonal antibody 5F12.

**Analysis of Receptor-stimulated Phospholipase C in Intact Cells—** For HL60 cells, confluent suspension cultures (10⁶ cells/ml) were incubated with 300 µM dibutyryl cAMP to differentiate the cells for 48 h in Medium 199 in the presence of 1 µCi/ml [3H]inositol. HeLa cells and COS-7 cells were labeled overnight with 2 µCi/ml [3H]inositol in Medium 199 supplemented with 1.5% dialyzed fetal calf serum and 5 µg/ml insulin and transferrin. In all cases, the cells were washed with HEPES buffer (20 mM HEPES, 137 mM NaCl, 3 mM KCl, 5.6 mM glucose, 1 mg/ml bovine serum albumin, 10 mM LiCl, 1 mM CaCl₂, and 1 mM MgCl₂, pH 7.2) and incubated with agonist. For HL60 cells, the cells were stimulated with FMLP (1 µM) for the indicated times. COS-7 cells were stimulated with EGF (100 ng/ml), and HeLa cells were stimulated with ATP (1 mM) for 20 min. Cells were pretreated with PMA (100 nM) for 5 min prior to stimulation with the agonist where indicated. Inositol phosphates were measured as described previously (8).

**Production of Polyclonal Antiserum to Phosphorylated Ser166—** Peptide synthesis and immunization were performed by Eurogentec (Seraing, Belgium). The peptide sequences CEDPALKSFKSITKGRGP and CEDPA-KFSGSKITKGRGP (where pS is phosphoserine) were synthesized, and two rabbits were immunized with the latter phosphorylated peptide. (Cysteine was added at the N terminus to aid coupling for the immunization process.) The serum was first purified using a Protein G-agarose column (10 ml). Serum was filtered through a 0.45-µm filter, applied to the column at 1 ml/min, and then washed extensively with TBS (25 mM Tris, pH 7.4, 137 mM NaCl, 5 mM KCl), 0.02% NaN₃. Bound antibody was eluted using 100 mM glycine, pH 2.5, and then neutralized with 20 mM Tris, pH 7.4. The Protein G column was then washed with 6 M urea before reuse). The eluted antibody was concentrated and desalted to TBS, 0.02% NaN₃, (8 ml).

Peptide (10 mg) was coupled to 1 ml of N-hydroxysuccinimide-agarose columns (Amersham Biosciences) using the manufacturer’s suggested coupling conditions. Two columns were made, a column containing the phosphorylated peptide and a second with the non-phosphorylated peptide. Protein G-purified immunoglobulins were purified using the peptide columns. Initially the Protein G-purified immunoglobulins were applied to the phoshopeptide column at 0.2 ml/min, and the flow-through was then reapplied to recover losses. The column was then washed extensively with TBS, 0.02% NaN₃ and the bound immunoglobulins were eluted with 100 mM glycine, pH 2.5 (3 ml) and immediately desalted to TBS/Nan₃ (4 ml). The resultant immunoglobulins were tested on dot blots containing dilutions of the synthetic phospho- and non-phosphopeptides. The antibody was further purified by applying to the non-phosphopeptide column. Briefly 1 ml was applied to the column and allowed to stand at room temperature for 30 min. The non-bound immunoglobulin (containing phosphopeptide antibodies) was washed through with 1 ml of TBS and then reapplyed to the column in 2 × 1 ml lots (leaving to stand for 30 min in between). The bound immunoglobulins were then washed with 1 ml of TBS resulting in 3 ml of non-bound immunoglobulin. This preparation, depleted of antibodies against the non-phosphorylated peptide, was then used for Western blots. The specificity of the phosphoantibody was validated using the following controls. The antibody was tested using PITPα mutants where the phosphorylation site was mutated to alanine (Fig. 4B) and by colorimetric antibody binding with prior incubation with phosphopeptide and A-phosphatase (New England Biolabs) treated PITPα (data not known).

**Production of PITPα-specific Polyclonal Antiserum—** Antibodies were raised in two rabbits against a specific PITPα peptide C-terminal sequence (CMRQKDVPVKGMADD). Peptide synthesis and immunization was performed by Eurogentec. Cysteine was added at the N terminus to aid coupling for the immunization process. The antisera was checked against recombinant PITPα to confirm that they were specific for PITPα.

**Phosphorylation of PITPα in Acutely Permeabilized Cells—** HL60 cells were acutely permeabilized with 0.6 IU/ml streptolysin O in PIPES buffer (20 mM PIPES, 137 mM NaCl, 3 mM KCl, 1 mM bovine serum albumin, 1 mM glucose, pH 6.8) in the presence of 1 mM MgATP, 2 mM MgCl₂, and calcium buffered with EGTA at pCa 7 or 5 (31). His-tagged wild-type and 759A PITPα proteins were included as controls. Phospholipase C was stimulated with 10 µM GTPγS (31). PMA (100 nM) was added to the cells for 10 min prior to permeabilization. Following incubation at 37 °C for 20 min, cells were removed by centrifugation, and the proteins were recaptured using nitroltriacetic acid (NTA)-agarose (Qiagen). Bound proteins were washed with PIPES buffer (containing no bovine serum albumin or glucose). Proteins were eluted with 500 mM imidazole, desalted to 20 mM Tris-HCl, pH 7.6, and concentrated. Quantities of each protein were estimated, and equal amounts were examined for phosphorylation by Western blotting.

**Preparation of Rat Brain Cytosol Enriched in PITPα—** Rat brains (9.5 g) were homogenized in 19 ml of buffer (20 mM PIPES, pH 6.8, 137 mM
Phosphorylation of PITPα at Ser166

NaCl, 3 mM KCl, 5 mM EGTA, 5 mM EDTA) supplemented with 1 ml of protease inhibitors (Sigma catalog no. P-2714). The homogenate was centrifuged to pellet membranes and insoluble material at 100,000 × g for 4 h at 4 °C. Cytosol (20 ml) was filtered through a 0.45-μm membrane and concentrated in an Amicon pressure filtration device with a 10-kDa membrane to 10 ml (16 mg of protein/ml). PITP was purified from 9 ml of this cytosol (total protein, 144 mg) by gel filtration using a Superdex-75 HR 26/60 column (Amersham Biosciences). 5-ml fractions were collected, and PITP-containing fractions were located by Western blotting with PITPα and with phospho-Ser166-specific antibodies. Fractions enriched in PITPα were pooled and concentrated for use in the in vitro phosphorylation assay.

Phosphorylation of PITPα in Vitro—Partially purified rat brain PITPα (16 μg of protein) and 0.34 μg of thrombin-cleaved recombinant PITPα were phosphorylated with PKC (0.1 unit) in a volume of 50 μl containing 20 mM Tris-Cl, 5 mM MgCl2, 200 μM CaCl2, 100 μM EGTA, 100 μM MnATP, 200 μCi/μl γ-labeled [32P]ATP (pH 7.5) for 30 min at 30 °C for 1 h. SDS-PAGE was performed using 4–12% bis-Tris gels (Invitrogen) containing 20 mM Tris, 150 mM NaCl, 2.5 mM CaCl2, pH 8.4) and incubated with 50% acetonitrile, 100 mM ammonium bicarbonate for 15 min. The resulting crushed gel pieces were incubated with 100% acetonitrile to PITP was phosphorylated with purified rat brain PKC (Calbiochem). PITPα was preincubated with PI vesicles (40-fold molar excess) for 5 min at 30 °C to exchange the bound ligand to PI from the bacterially derived phosphatidyglycerol (32). The mixture was phosphorylated with 70–350 ng (0.1–5 units) of PKC/5–10 μg of PITP in a volume of 50 μl containing 20 mM Tris-Cl, 5 mM MgCl2, 100 μM MnATP, 200 μCi/μl γ-labeled [32P]ATP (pH 7.5) at 30 °C for 5 min or 1 h as indicated. Phosphorylated proteins were analyzed by SDS-PAGE, and the gels were imaged using a phosphorimaging system (Fuji BAS1000).

HPLC Analysis of Phosphopeptides—For HPLC analysis of the phosphopeptides, the phosphorylated protein was initially digested with proteinase K (1:50 proteinase K to protein). The phosphorylated protein in vitromicrosomes to non-labeled liposomes as described previously (28). Proteins were resolved by size exclusion chromatography using a Leica DMRBE microscope including an SP2 confocal head with AOBs (Accousto optical beam splitter) with a 63 × oil immersion objective (numerical aperture, 1.2) and the Leica confocal software (LCS).

Size Exclusion Chromatography and Isoelectric Focusing (IEF)—Recombinant PITPα was exchanged with the PC form by the inclusion of a 40 μM excess of dimyristoyl PC during the thrombin cleavage reaction. The exchanged protein was phosphorylated on Thr59 and Ser166 with PKC and then further resolved using size exclusion chromatography to remove PKC and excess [γ-32P]ATP. The column was equilibrated with 1 mM Tris, pH 7.5. Fractions were analyzed by SDS-PAGE and phosphorimaged, and the fractions containing phosphorylated PITPα were combined and concentrated. Phosphorylated PITPα was then analyzed by IEF, and its ability to exchange lipid was examined. A 40 μM excess of PI was incubated with the phosphorylated PITPα at 30 °C for 5 min. The mixture was resolved on Immobiline IEF gels (Amersham Biosciences). IEF gels consisted of a linear pH 4–7 gradient precast by the manufacturer and utilized an immobilized pH gradient. The gels were reswollen in water and run on a Multiphor flat bed electrophoresis system at 200 V for 1 min, 0–3500 V for 90 min, and 3500 V for 3 h. The gel was stained and phosphorimaged.

Fluorescence Resonance Energy Transfer by Fluorescence Lifetime Imaging Microscopy between EGF-PITPα and BODIPY-labeled Lipids—COS-7 cells were transfected with wild-type PITPα, and the cells were incubated with BODIPY lipids. Measurements of GFP lifetime were done exactly as described previously (20).

RESULTS

PMA Pretreatment Causes Phosphorylation of PITPα and Inhibits Receptor-stimulated Phospholipase C Activity—PITPα has five consensus phosphorylation sites (Thr59, Ser166, Thr169, Thr251, and Thr296) for protein kinase C. To examine PITPα phosphorylation, HL60 cells, metabolically labeled with [32P]Pi, were treated with PMA. PITPα was phosphorylated within 30 s, and the degree of phosphorylation was increased at 300 s (Fig. 1A). The G-protein receptor agonist fMLP was also found to inhibit the phosphorylation of PITPα in COS-7 cells transfected with EGFP-PITPα co-transfected with BODIPY-labeled phosphatidylserine.
Phosphorylation of PITPα at Ser\textsuperscript{166} to stimulate PITPα phosphorylation weakly (data not shown). Under conditions in which PITPα phosphorylation occurred by PMA pretreatment, fMLP-stimulated inositol phosphate formalization was also inhibited (Fig. 1B). The inhibition by PMA was not restricted to fMLP-stimulated HL60 cells where the phospholipase C responsible for inositol phosphate production is PLC\textsubscript{β}2 and is activated by βγ subunits. Fig. 1C shows that COS-7 cells stimulated with EGF and HeLa cells stimulated with ATP (1 mM) for 20 min. Inositol phosphates were measured as an indicator of phospholipase C activation. A, COS-7 cells stimulated with EGF (35 ng/ml), and HeLa cells were stimulated with ATP (1 mM) for 20 min. Inositol phosphates were measured as an indicator of phospholipase C activation. B, acutely permeabilized HL60 cells were incubated with His-tagged PITPα wild type (a) or PITPα mutant T59A (b) in the presence of GTP\textsubscript{γ}S or PMA and Ca\textsuperscript{2+} as indicated for 20 min at 37 °C. Proteins were captured using nitrolotriacetic acid-agarose and blotted with both PITPα antibodies. It was noted that phosphorylation of T59A was greatly enhanced compared with wild-type protein (3.7 ± 0.2-fold, n = 8).

Identification of Ser\textsuperscript{166} Phosphorylated PITPα in Rat Brain—Attempts to identify phosphorylated PITPα with the phosphoantibody in HL60 cells were not possible because of the low concentration of PITPα in HL60 cells (2 ng/100 μg of cell lysate). In contrast, rat brain has the highest tissue concentration of PITPα at 40 ng/100 μg of cell lysate. We therefore examined whether Ser\textsuperscript{166} phosphorylated PITPα could be detected in crude rat brain cytosol. Phosphorylated PITPα could only be detected following fractionation of rat brain cytosol by gel filtration. Individual fractions expected to be enriched in PITPα were blotted with both a PITPα-specific antibody and the phospho-Ser\textsuperscript{166} antibody. It was consistently observed that phosphorylated PITPα eluted slightly earlier than the nonphosphorylated protein (see Fig. 2A). The fractions containing PITPα were pooled and used in an in vitro assay using PKC.

Recombinant PITPα was also analyzed in parallel. Both the partially purified PITPα from rat brain cytosol and recombinant PITPα could be phosphorylated by PKC at Ser\textsuperscript{166} (Fig. 2B).

PITPα Is Phosphorylated at Thr\textsuperscript{59} and Ser\textsuperscript{166} in Vitro—To assess the stoichiometry of phosphorylation, recombinant PITPα was phosphorylated with purified PKC using \textsuperscript{32}P\textsubscript{ATP} in vitro. We initially used the His-tagged proteins in the phosphorylation assay and found that the linker region highlighted in italics, MRGSHHHHHHGMASMTGGQQMRGDLYD-DDDKDPMVLLKE, despite not having a consensus PKC phosphorylation site, was phosphorylated at the two serine residues (underlined) by PKC (identified by Edman sequencing). The experiments were therefore repeated with the His tag...
we therefore tried digestion with Glu-C, which cleaves C-terminal to trypsin cleavage sites, and the resultant HPLC profile had unresolved peptides likely resulting from incomplete digestion.

For identifying additional sites of phosphorylation, the phosphoprotein was digested with trypsin, but chromatography of the resulting peptides by HPLC revealed no distinct peaks labeled with 32P (Fig. 2A, see inset). There are 39 potential trypsin cleavage sites, and the resultant HPLC profile had unresolved peptides likely resulting from incomplete digestion. We therefore tried digestion with Glu-C, which cleaves C-terminal to glutamate, since PITPα has 27 potential cleavage sites. The peptides were analyzed by HPLC, and five peaks with radiolabel were observed (Fig. 3A). Phosphopeptides in these peaks were quantified by Cerenkov counting according to the specific activity of 32P used for phosphorylation (Fig. 3C). The major peptide fragment (peak 5) was subjected to mass spectrometric analysis by MALDI-TOF mass spectrometry and solid phase Edman degradation (35). No peptide mass that matched a PITPα truncated mutant was identical to wild-type protein indicating that this change in conformation does not influence the availability of Ser166 for PKC-mediated phosphorylation (Fig. 4, A and B). The greater degree of phosphorylation observed in the permeabilized cell assay could be due to a structural change induced by the proximity of the protein to the membrane.

Since T59A is more highly phosphorylated at Ser166 in vitro, it must contain a larger population of a structurally distinct form of PITPα protein in which Ser166 is accessible to PKC. Fig. 4D shows native PAGE analysis of both wild-type and T59A PITPα. In the T59A sample, the PITPα resolved into two forms (Fig. 4D, lane 4, indicated by the arrows). One form (lower arrow) corresponded to the wild-type protein, which resolved predominantly as a single species (Fig. 4D, lane 3). Using SDS-PAGE each of the proteins, wild-type and T59A, resolved as a single band (Fig. 4E, lanes 3 and 4, respectively).

Identification of Ser166 and Thr251 on PITPα as Important Residues for PLC Signaling—To analyze the effects of phosphorylation on PLC signaling and lipid transfer, each of the five putative PKC phosphorylation sites (Ser166, Thr251, Thr198, Thr169, and Thr198) were mutated to glutamic acid as a phosphomimetic. Their locations in the PITPα structure are shown in Fig. 5A (5, 6). Thr198 and Thr251 are located on β-strands with Thr251 very close to the lipid binding site, and Thr169 and Ser166 are located in the regulatory loop, while Thr198 is located in the C-terminal α-helix (G-helix). The phosphomimetic mutant proteins and the wild-type protein were expressed in E. coli and examined for their ability to support the production of inositol phosphates following activation of PLCβ2 by βγ subunits in HL60 cells. The purified proteins were analyzed by SDS-PAGE (Fig. 5B). While the purity and expression levels of the wild-type PITPα and the mutants T198E and T251E were comparable, T59E, S166E, and T169E were less pure and had reduced expression levels compared with wild type. Protein concentrations were therefore estimated from the SDS-poly-
acrylamide gel relative to wild-type PITPα protein. Only the T59E and S166E PITPα mutants were unable to restore G protein-stimulated PLCβ-mediated inositol phosphate production, and these mutants were also unable to transfer PI in an in vitro assay (Fig. 5, C and D).

A more extensive analysis of the phosphomimetic mutants S166E and T59E was undertaken, and we included the phosphorylation-defective mutants (S166A and T59A) for comparison. To examine the localization of the mutants, PITPα proteins were expressed as GFP fusion proteins and transfected into COS-7 cells (Fig. 6A). GFP-PITPα S166E and S166A localized to the cytosol and nucleus similarly to wild-type PITPα (20, 37). In contrast, GFP-PITPα T59E and T59A were excluded from the nucleus. By confocal microscopy, it was observed that the number of cells expressing S166A and S166E was low in comparison to cells expressing wild type, T59A, or T59E. Expression of the GFP fusion proteins was also examined by Western blot. The antibody for PITPα identified two bands in the GFP fusion-expressing cells, one band representing the endogenous PITPα and the upper band representing the fusion protein. While expression of wild-type PITPα and the mutants T59A and T59E was clearly observed, we were unable to observe the expression of the GFP fusions for S166A and S166E. This result is not surprising since the number of GFP fusion-expressing cells represented 1–2% of the total cell population. It was observed that overexpression of S166A-GFP consistently led to increased levels of endogenous PITPα, and this was not explored further (Fig. 6A).

Thr59 and Ser166 mutants (Ala and Glu) were examined for PI and PC binding and transfer using a cell-based assay (33). In this assay, the PITPs were incubated with cytosol-depleted HL60 cells, which act as a source of radiolabeled lipids. Lipid binding is monitored after repurification of the PITPs, and lipid transfer is measured by monitoring transfer to exogenously added liposomes. T59E was unable to transfer PI, while PC transfer was similar to wild type (compare Fig. 6, B and C). This inability to transfer PI was entirely due to loss of PI transfer (see Fig. 6, D and G). In comparison, T59A showed reduced PI and PC transfer. A detailed analysis of several independent preparations showed that both PI and PC transfer were reduced to 60 ± 7 and 65 ± 9% (n = 4), respectively, when analyzed at a fixed concentration of 100 μg/ml (6). Interestingly PI binding was reduced to 36 ± 11 (n = 4), while PC binding was unaffected. We also examined the PITPα mutant T59A for its ability to reconstitute PLC activity, and it showed a modest decrease compared with wild type (data not shown).

Analysis of the Ser166 mutants revealed that both the phosphomimetic and phosphodefective mutants were severely impaired for both PI and PC transfer (Fig. 6E). Since the expression of the Ser166 mutants in E. coli was greatly reduced, they were only tested at a single concentration for transfer. S166A was also examined in the microsome-liposome assay, which requires less protein, and it was clear that this protein retained some activity. When examined for its ability to restore PLC signaling, the protein also retained some activity (data not shown) unlike S166E (Fig. 5C). Examination of binding revealed that S166A and S166E still bound PI and PC although at a reduced level (Fig. 6, F and G). S166E was more impaired than S166A, and this was reflected in their ability to transfer lipids.

**Fig. 3. Identification of Ser166 as the major phosphorylation site for protein kinase C. A, 2.7 nmol (100 μg) of PITPα was phosphorylated with PKC in vitro (phosphorimage) and enzymatically digested with Glu-C, and the peptide fragments were separated by HPLC. Inset, 0.27nmol (10 μg) of PITPα was phosphorylated with PKC in vitro and enzymatically digested with trypsin, and peptide fragments were separated by HPLC. B, Western blot analysis of peak fractions from Glu-C-digested PITPα. Fractions were dried under vacuum and solubilized in 20 μl of N,N-dimethylformamide. 2 μl of each was spotted onto polyvinylidine difluoride. Synthetic phosphorylated (PHOS) and non-phosphorylated (NON) peptides (0.5 μg) were spotted onto polyvinylidine difluoride to illustrate specificity of the phosphopeptide antibody. The membrane was air-dried, wetted with MeOH, and washed with PBS/Tween extensively. The membrane was probed with the phosphopeptide antibody (purified only on a phosphopeptide column and preincubated with 250 μg of synthetic peptide for 1 h at room temperature) for 1 h at room temperature. Bound antibody was detected with enhanced chemiluminescence. C, pmol of phosphopeptide in each Glu-C peak. Peak 1, fractions 116–118; peak 2, fractions 137–140; peak 3, fractions 147–149; peak 4, fractions 162–165; peak 5, fractions 167–169.**
FIG. 4. Phosphorylation of PITPa mutants demonstrates that Ser\textsuperscript{166} is a major phosphorylation site and solvent accessibility is conformation-dependent. A, mutant PITPa proteins (2 μg) were phosphorylated with PKC. 1 μg of each phosphorylated protein was subjected to SDS-PAGE, stained with Coomassie (upper panel), and phosphorimaged (lower panel). PITPa S166A (S166A) phosphorylation was dramatically reduced, while PITPa T59A (T59A) phosphorylation was enhanced compared with wild-type protein (1.7 ± 0.1-fold, n = 13). B, two further SDS-polyacrylamide gels were run with 100 ng of the phosphorylated proteins analyzed in A. Proteins were transferred to polyvinylidene difluoride and probed with a polyclonal antibody to PITPa (upper panel) and the PITPa Ser\textsuperscript{166} phosphoantibody (Phos166) (lower panel). Phosphorylated PITPa S166A was not detected by the phosphopeptide antibody. C, stereodogram of the pocket where Ser\textsuperscript{166} resides. In PITPa the side chain of Ser\textsuperscript{166} is in a small pocket between the 165–172 loop and the rest of the protein. Several other conserved residues in or adjacent to the loop apparently stabilize its conformation. Main chain hydrogen bonds are found at N-166–O-171 and N-170–O-166. Side chain to main chain hydrogen bonds are found at Asp\textsuperscript{234}–N-168, Asp\textsuperscript{234}–N-167, Lys\textsuperscript{165}–O-170, Lys\textsuperscript{165}–O-172, and Arg\textsuperscript{171}–O-12; and side chain to side chain hydrogen bonds; main chain atoms have carbon atoms in blue, nitrogen atoms in light blue, and oxygen atoms in red. This loop is identical in the PI and PC form of PITPa and the aepstructures. D, wild-type PITPa and T59A (10 μg) were resolved using native PAGE. Ovalbumin and albumin (10 μg) were included for reference, and gels were stained with Coomassie Blue. Lane 1, ovalbumin; lane 2, albumin; lane 3, wild-type PITPa; lane 4, T59A PITPa; arrows indicate position of the two forms of T59A. E, proteins (1 μg of each) resolved by 14% SDS-PAGE. Lane 1, ovalbumin; lane 2, albumin; lane 3, wild-type PITPa; lane 4, T59A PITPa. All gels shown are representative of experiments performed three to five times with separate PITPa preparations. WT, wild type.

Extent of PITPa Phosphorylation Is Independent of the Bound Lipid Species—A previous study has reported that the PKC-dependent phosphorylation is controlled by the phospholipid species bound to PITPa (29). The PC-bound form of PITPa was found to be a better substrate than the PI-bound form. In light of the observation that the structures of PITPa bound to PI and PC are nearly identical (5, 6), we have re-examined whether the phospholipid species influences phosphorylation by PKC. PITPa proteins were exchanged with either egg yolk PC or bovine brain PI and purified by gel filtration to remove the vesicles. It was noted that some of the PITPa eluted in the void volume with the vesicles, leading to loss of protein. PI- and PC-loaded PITPa were phosphorylated by PKC to a similar extent (Fig. 7A).

Phosphorylated PITPa Can Exchange Bound PC for PI but Not Vice Versa—To examine whether phosphorylated PITPa could exchange its lipid-bound cargo, we used recombinant PITPa loaded with dimyristoyl PC for phosphorylation by PKC. As observed before with rat brain PITPa, the phosphorylated PITPa eluted slightly earlier compared with the bulk of the protein (Fig. 7B). Fractions enriched with the PC-loaded phosphorylated PITPa were pooled (Fig. 7B, i), half the sample was incubated with PI vesicles for lipid exchange, and the samples were analyzed by IEF. The proteins were silver-stained (Fig. 7C, panel a) and also phosphorimaged (Fig. 7C, panel b) to identify the phosphorylated proteins. In the silver-stained gel (Fig. 7C, panel a), the unphosphorylated PC form of PITPa focuses at pH 6, and when exchanged with PI, the majority of protein focuses at pH 5.7. This change in behavior is due to PITPa acquiring one net negative charge when PC is replaced with PI. In the phosphorimage, it is seen that the phosphorylated PC-ligated PITPa is shifted to a lower pH compared with the non-phosphorylated protein (Fig. 7C, compare lanes marked PC in panels a and b). This shift is due the acquisition of an additional negative charge, this time due to phosphorylation. The phosphorylated PITPa (PC form) shows a further
shift toward the positive side after it is exchanged with PI (panel b). Thus, phosphorylation of PITPα does not prevent exchange of PC for PI.

To examine whether phosphorylation of PITPα could affect the exchange of PI for PC, the same protocol was followed as above except that bacterially derived phosphatidylglycerol was exchanged with bovine brain PI. The PI-loaded protein was phosphorylated with PKC and subsequently incubated with dimyristoyl PC vesicles for lipid exchange as described above followed by analysis by IEF. On IEF, the proteins would not enter the gel, suggesting that the highly charged proteins were binding to the vesicles avidly and were not freely mobile. From this behavior we conclude that PI-loaded phosphorylated PITPα must not have exchanged its cargo for PC since the phosphorylated PITPα (PC form) had no difficulty in entering the gel as seen from Fig. 7C.

Phosphorylation of PITPα Perturbs Binding to PC in Intact Cells—Although the phosphoprotein (PC form) was still capable of exchange (PC for PI), the data described above suggested that the PI form of the phospho-PITPα was not able to exchange PI for PC. To analyze this behavior by a non-invasive technique we took advantage of a recent method for analyzing lipid-protein interactions using fluorescence lifetime imaging (20). Previous studies have already shown that EGF-stimulated inositol phosphate production is dependent on PITPα (10). We established that PMA pretreatment of COS-7 cells abolished the EGF-stimulated inositol phosphate production (Fig. 1C). COS-7 cells were transfected with GFP-PITPα and labeled with BODIPY PI and PC. As reported previously, upon EGF stimulation a significant reduction in GFP lifetime is observed indicating close proximity between PITPα and the lipid cargo (20). In PMA-pretreated cells, only the interaction between PITPα and membrane PC is inhibited but not that between PITPα and membrane PI (Table I). These results complement the in vitro analysis by IEF that PI-loaded PITPα, when phosphorylated, is unable to interact with PC, while the phosphorylated PC-loaded PITPα is still able to exchange its cargo for PI.

DISCUSSION

In this study, we found that PITPα can be phosphorylated in cells pretreated with PMA, and from an in vitro analysis identified Ser166 (major) and Thr59 (minor) as the residues that are phosphorylated. We also showed that PMA pretreatment disrupts both G-protein-stimulated phospholipase Cβ and EGF-stimulated phospholipase Cγ activation. Such negative feedback regulation by PMA pretreatment has been observed in many other cell types including FceR1 on mast cells (38), va-
soppressin-stimulated WRK-1 cells (39), angiotensin-stimulated adrenal glomerulosa cells, and carbachol-stimulated astrocytoma cells (40). The literature on the feedback regulation of phosphoinositide turnover has been reviewed, and in many cases the molecular target for PKC could not be identified (24, 25). Previous studies have reported that in FMLP-stimulated HL60 cells (a neutrophil-like cell line), inositol phosphate production due to PLC activation was inhibited by PMA pretreatment (23, 41). HL60 cells contain PLCβ2 and PLCβ3, and their activation occurs via Gβγ subunits exclusively when stimulated with FMLP (42). Knock-out of the PLCβ2 gene reduces inositol phosphate production and Ca2+ signaling by 70%, and while knock-out of both PLCβ2 and PLCβ3 genes completely abrogates the responses, indicating that PLCβ2 is the major phospholipase for inositol phosphate production in neutrophils (42, 43). In a recent study, the inhibition of FMLP-stimulated inositol phosphate production by PMA was examined, and it was concluded that phosphorylation and consequent inactivation of PLCβ2 or PLCβ3 isoforms was unlikely (23). Although PLCβ3 can be phosphorylated by PKC at Ser1103, this phosphorylation only inhibits Goα-stimulated PLCβ3 but not Gβγ-stimulated PLCβ3 (23). This serine residue is not conserved in PLCβ2. PITPα have not been examined previously, and in this study we demonstrated that phosphorylation of PITPα could be responsible for this PMA-induced inhibition. Although the extent of phosphorylation of PITPα in vitro was low, this should not disqualify PITPα. The low stoichiometry of PITPα phosphorylation observed in vitro is due to inacessibility of the Ser1166 residue to PKC (Fig. 4C). Since Ser1166 is in an identical environment in the three solved structures of PITPα (PC-PITP, PI-PITP, and apoPITP), this suggests this is the predominant form in solution. We suggest that it is in equilibrium with a small fraction of PITPα that is structurally distinct such that Ser1166 is accessible. This equilibrium is shifted in the mutant T59A since it is more highly phosphorylated in vitro. Although Thr59 is a long distance from Ser1166, perturbations in this region appear to be sufficient to shift the equilibrium to a different structural form.

In permeabilized cells, PITPα phosphorylation was observed under conditions in which endogenous PKC is stimulated by PMA and Ca2+ or by GTPγS, which activates PLC (Fig. 1D). In cells, phosphorylation of PITPα will occur at the membrane, which may facilitate structural changes (see Fig. 8). There are precedents for such changes in proteins that are active at the membrane (44). We have identified two tryptophan residues that are essential for PITPα to mediate lipid transfer (6). Bulky aromatic tryptophan side chains are known to be able to penetrate the interfacial region, which is composed of the head groups, water, and portions of acyl chain methylene groups that extend toward the interface from the hydrocarbon core (45). They do not penetrate into the hydrophobic core of the membrane. Docking to the membrane, which is mediated by the interaction of these tryptophan side chains, may induce a conformational change. For the phosphorylation sites to become accessible, this may go further than the changes seen in the apostructure, converting PITPα into a “molten globule” that allows partial unfolding of the loop and so permitting PKC to gain access. The steroidogenic acute regulatory protein, STAR, which is also a transfer protein like PITP but for cholesterol, is reported to undergo such a transition when it binds to membranes (46–48). Thus, we anticipate that when PITPα docks to the membrane, the protein undergoes extensive changes including relaxation of the 165–172 loop such that the Ser1166 is available for phosphorylation. We cannot discount the possibility that ancillary proteins may aid this process.

Additional evidence that PITPα, phosphorylated at Ser1166, has a different structural conformation comes from analysis of its behavior on gel filtration. The phosphorylated fraction of both the native protein from rat brain and recombinant protein phosphorylated in vitro eluted earlier on a size exclusion column. The order of elution of proteins from a size exclusion column is inversely related to size and shape so that earlier
In PMA-pretreated cells, the EGF-stimulated interaction of GFP with BODIPY-PC but not BODIPY-PI is inhibited. Approximately 50 pixels was used to obtain a localized average lifetime value. The calculated GFP lifetimes of the individual pixels were averaged. The localized GFP lifetimes at the plasma membrane were quantified from three independent experiments. A region of interest containing approximately 200 µl of each fraction was combined and concentrated. Approximately 20 µg of protein was resolved by IEF. Panel a, silver-stained IEF gel; panel b, phosphorimage of the IEF gel. Lane PC, PITPα loaded with PC and phosphorylated, and then incubated with 20 µg of PI at 30 °C for 5 min. Following phosphorylation PC-loaded PITPα has an increased negative charge, and exchange to PI increases the net negative charge further shifting the protein toward the anode. Gels shown are representative of experiments performed three to five times with separate PITP preparations.

**Table I**

|           | Control | Me₂SO control | 10 nM PMA |
|-----------|---------|----------------|-----------|
| GFP lifetime at plasma membrane |          |                |           |
| GFP-PITPα | 2.12 ± 0.14 | 2.14 ± 0.1 | 2.00 ± 0.1 |
| BPC + PITPα | 2.15 ± 0.12 | 2.16 ± 0.1 | 1.95 ± 0.1 |
| BPI + PITPα | 2.20 ± 0.08 | 2.04 ± 0.15 | 2.10 ± 0.10 |
| BPC + PITPα + EGFR | 1.65 ± 0.10 | 1.70 ± 0.15 | 2.05 ± 0.10 |
| BPI + PITPα + EGFR | 1.70 ± 0.10 | 1.72 ± 0.07 | 1.75 ± 0.10 |

* Significant reduction in GFP lifetimes after EGF stimulation.

Phosphorylation of PITPα at Ser166

**Fig. 7.** Phosphorylated PC-loaded PITPα can still exchange its bound lipid for PI. A, 100 ng of PITPα exchanged with PI (brain) or PC (egg yolk) was phosphorylated at 30 °C with PKC for the indicated times. Blots were performed with 20 ng of protein/lane and probed with polyclonal antibody to PITPα (upper panel) and the PITPα Ser166 phosphoantibody (Phos166) (lower panel). Lane PC, PC-exchanged PITPα was phosphorylated with PKC in a volume of 200 µl (1 mg of protein) and resolved using a Superose-12 size exclusion column, collecting 200-µl fractions. 20 µl of each fraction was subjected to SDS-PAGE. Protein was stained with Coomassie R-250 (upper panel) and phosphorimaged (lower panel). Phosphorylated protein elutes slightly earlier (underlined and labeled i) than the bulk of the protein (overlined and labeled ii). C, the peak fractions containing the phosphorylated proteins (i) were combined and concentrated. Approximately 20 µg of protein was resolved by IEF. Panel a, silver-stained IEF gel; panel b, phosphorimage of the IEF gel. Lane PC, PITPα loaded with PC and phosphorylated, and lane PI, PITPα loaded with PC and phosphorylated, and then incubated with 20 µg of PI at 30 °C for 5 min. Following phosphorylation PC-loaded PITPα has an increased negative charge, and exchange to PI increases the net negative charge further shifting the protein toward the anode. Gels shown are representative of experiments performed three to five times with separate PITP preparations.

Alignment of 39 PITPα-related sequences identified in mammals, fish, amphibians, flies, soil amoebae, red photosynthetic algae, and parasites indicate that Ser166 is conserved in 36 of 39 sequences (Table II). In 31 sequences, a consensus sequence, EDX(SX)_4RG, can be identified. Ser166 is not conserved in one of the Caenorhabditis elegans and Caenorhabditis briggsae PITPs or in PITP from Encephalitozoon cuniculi. This strong conservation would indicate that the regulatory loop is impor-
Thr59 phosphorylation in cells may be greater. In addition, 4,5-bisphosphate (PIP2) of Ser166 for phosphorylation. Thr59 is conserved in all the docking onto the plasma membrane PITP lipase C.

Following stimulation of phospholipase C by cell surface receptors, production of diacylglycerol (DG) leads to activation of PKC. By docking onto the plasma membrane PITPs undergoes a structural change such that the Ser166 residue is accessible to protein kinase C. Phosphorylated PITPs is unable to deliver its PI cargo but not its PC cargo. The disruption in the delivery of PI leads to local depletion of PI 4,5-bisphosphate (PIP2) and cessation of the PLC response. Pretreatment of cells with PMA mimics this process by enhancing phosphorylation of PITPa at Ser166.

Figure 8. Model depicting how phosphorylation of PITPa causes inhibition (negative feedback) following stimulation of phospholipase C. Following stimulation of phospholipase C by cell surface receptors, production of diacylglycerol (DG) leads to activation of PKC. By docking onto the plasma membrane PITPs undergoes a structural change such that the Ser166 residue is accessible to protein kinase C. Phosphorylated PITPs is unable to deliver its PI cargo but not its PC cargo. The disruption in the delivery of PI leads to local depletion of PI 4,5-bisphosphate (PIP2) and cessation of the PLC response. Pretreatment of cells with PMA mimics this process by enhancing phosphorylation of PITPa at Ser166.

In addition to the phosphorylation of Ser166, our analysis showed that Thr59 is also phosphorylated. Although it is a minor site in the in vitro studies reported here, the extent of Thr59 phosphorylation in cells may be greater. In addition, Thr59 clearly has an indirect role in regulating the accessibility of Ser166 for phosphorylation. Thr59 is conserved in all the currently known PITP sequences (6), and we show here that when mutated to a phosphomimetic, the protein effectively became a PC-binding and transfer protein. In the T59E mutant, PI binding was reduced by over 90%, and PI transfer was undetectable. Nonetheless PC binding and transfer were unaffected. A similar pattern has been observed previously where mutation of this residue to a variety of amino acids (T59V, T59E, T59D, T59S, T59N, and T59Q) all led to near complete loss of PI binding when Thr59 was mutated to glutamate. Since this residue is conserved in all the PITP sequences identified so far, it is anticipated that this residue plays a similar function in all of the PITP family. However, analysis of the PITP domain of Drosophila RDGBo protein indicates that the T59A mutation, not T59E, leads to a reduction in PI transfer, while PC transfer is unaffected (50). This result is surprising in the light of the PI-bound PITP structure and needs to be revisited.

In the mammalian RDGBo protein (also known as Nir2), the PITP domain of the wild-type protein localizes to the cytoplasm similar to PITPa, and mutation of Thr59 to glutamate causes the protein to translocate to lipid droplets (51). This striking relocalization is certainly not a feature of PITP, but may be due to a structural change that inhibits its nuclear targeting. As discussed above, T59A was a better substrate for PKC, and this is likely due to a structural change in the protein.

Recent reports from studies in cells derived from PITPa knock-out mice have been unable to show any effects on PLC signaling. In one study, embryonic stem cells were stimulated with serum and lysophosphatidic acid, and it was reported that inositol 1,4,5-trisphosphate production was not affected (18). In another study fibroblasts were stimulated with vasopressin, and again the authors found no effect on PLC signaling (52). These findings are hampered by the presence of other PI transfer proteins, in particular PITPβ present in the cells. We have observed the interaction of PITPβ with the plasma membrane following EGF stimulation (20), suggesting that PITPβ could fulfill the role of PITPa in these knock-out studies. We have previously shown that PITPβ, Sec14p, and Dictyostelium PITPs are able in vitro to substitute the PLC signaling role of PITPa (19, 34). We are currently using interference RNA to
Table II
Conservation of Ser166 in many PITP-related sequences

| Sequence       | Accession no. | PII PITP     | PII PITP     | E2 PT                             |
|----------------|---------------|--------------|--------------|-----------------------------------|
|                |               |              |              |                                   |

transiently deplete PITPα in a variety of cell types including neuronal cells with agonists that work through G-protein-coupled receptors. Using interference RNA we have been able to show that transiently decreased levels of PITPα do affect the production of inositol phosphates. Using different cell types we were able to show that disruption of PLC activity is both agonist- and cell type-dependent. We would suggest that these differing effects correlate with the levels of other PI transfer proteins such as PITPβ present in these cell types as well as the potency of the agonist used.

In conclusion, we identified two residues, Thr206 and Ser166, as phosphorylation sites on PITPα that had dramatic effects on PITPα-dependent phospholipase C signaling. The effect of phosphorylation was to specifically reduce the delivery of PI for PLC signaling and hence decrease inositol phosphate production. Retaining PC transfer is not sufficient for the protein to function in PLC signaling as exemplified by T98E. We anticipate that PITPβ, which also can participate in PLC signaling and is known to go to the plasma membrane during stimulation with EGF (19, 20), will be phosphorylated by PKC in a similar manner at both Thr206 and Ser166. Phosphorylation of Ser166 in PITPβ by PKC has been reported recently (53).

Acknowledgments—We thank Dr. Mark Crawford (University College London) for help in MALDI-TOF analysis and Dr. D. Alessi (University of Dundee) for allowing Clive Morgan to work in the laboratory. We thank Michelle Li for providing technical assistance.

REFERENCES

1. Wirtz, K. W. A. (1997) Biochem. J. 323, 353–360
2. Hsuan, J., and Cockcroft, S. (2001) Genome Biol. http://genomebiology.com/1996–4906/2/REVIEWS011
3. Allen-Baume, V., Segui, B., and Cockcroft, S. (2002) FEBS Lett. 531, 74–80
4. Sha, B., Phillips, S. J., Bankaitis, V., and Luo, M. (1998) Nature 391, 506–510
5. Yoder, M. D., Thomas, L. M., Tremblay, J. M., Oliver, R. L., Yarbrough, L. H., and Helmkanp, G. M. Jr. (2001) J. Biol. Chem. 276, 9246–9252
6. Tillye, S. J., Skippin, A., Murray-Rust, J., Swigart, P., Stewart, A., Morgan, C. C., Cockcroft, S., and McDonald, N. Q. (2004) Structure (London) 12, 317–326
7. Schueten, A., Agianian, B., Westman, J., Kron, J., Wirtz, K. W. A., and Gros, T. (2002) EMBO J. 21, 2117–2121
8. Cunningham, E., Thomas, G. M., Ball, A., and Cockcroft, S. (1995) Curr. Biol. 5, 775–783
9. Fennesz, A., Cunningham, E., Prosser, S., Tan, K. S., Swigart, P., Thomas, J. H., and Cockcroft, S. (1999) Curr. Biol. 6, 730–738
10. Kauffmann-Zeh, A., Thomas, G. M., Ball, A., Prosser, S., Cunningham, E., and Cockcroft, S. (1997) Curr. Biol. 7, 1158–1161
11. Ohashi, M., Jan de Vries, K., Frank, R., Snoek, G., Bankaitis, V., and Wirtz, K. W. A. (1996) Nature 377, 544–547
12. Hay, J. C., and Martin, T. F. J. (1993) Nature 366, 572–575
13. Kolar, G., Loutzenhoven, M., Swigart, P., Whatmore, J., Ball, A., Cockcroft, S., and Wetzker, R. (1997) Biochem. J. 325, 299–301
14. Jones, S. M., Alb, J. G., Jr., Phillips, S. J., Bankaitis, V. A., and Howell, K. E. (1999) J. Biol. Chem. 274, 10349–10354
15. Hay, J. C., Fisette, P. L., Jenkins, G. H., Fukami, K., Takeawana, T., Anderson, R. M., and Telfer, R. P. F. (1995) Nature 374, 173–177
16. Panaretou, C., Demin, J., Cockcroft, S., and Waterfield, M. D. (1997) J. Biol. Chem. 272, 2477–2485
17. Hamilton, B. A., Smith, D. J., Mueller, R. T., van Berkel, V., Daly, M. J., Kroglyak, L., Reeve, M. P., Nernhauser, J. L., Hawkins, T. L., and Ruiter, M. (1997) Neuron 18, 711–722
18. Alb, J. G., Jr., Phillips, S. J., Stowens, K. E., Suidan, M., Ball, A., Swigart, P., Whatmore, J., and Reaves, J. W. (1999) J. Biol. Chem. 274, 10349–10354
19. Cunningham, E., and Tan, W. S. X., Swigart, P., Thomas, J. H., and Cockcroft, S. (1996) Proc. Natl. Acad. Sci. USA 93, 6589–6599
20. Larjian, B., Allen-Baume, V., Morgan, C. P., Li, M., and Cockcroft, S. (2003) Curr. Biol. 13, 788–793
21. Cockcroft, S., and De Matteis, M. A. (2001) J. Membr. Biol. 180, 187–194
22. Cullen, P. J., Cozier, G. E., Banting, G., and Mellor, H. (2001) Curr. Biol. 11, R882–R893
23. Yue, C., Ku, C. Y., Liu, M., Simon, M. I., and Sanborn, B. M. (2000) J. Biol. Chem. 275, 30202–30205
24. Rana, R. S., and Hokin, L. E. (1990) Physiol. Rev. 70, 115–184
25. Buse, S. G., and Wirtz, K. W. A. (1992) J. Biol. Chem. 267, 12393–12396
26. Gehrmann, T., and Heilmeyer, L. M., Jr. (1998) Eur. J. Biochem. 253, 375–370
27. Fruman, D. A., Meyers, R. E., and Cantley, L. C. (1998) J. Biol. Chem. 273, 4781–4787
28. Thomas, G. M., Cunningham, E., Fennesz, A., Ball, A., Totty, N. F., Truong, O., Hsuan, J., and Cockcroft, S. (1993) Cell 74, 919–928
29. van Tiel, C. M., Westernman, J., Fazan, A., Wirtz, K. W. A., and Snoek, G. T. (2000) J. Biol. Chem. 275, 21825–21838
30. Prosser, S., Sarra, R., Swigart, P., Ball, A., and Cockcroft, S. (1997) Biochem. J. 324, 19–23
31. Stutchfield, J., and Cockcroft, S. (1988) Biochem. J. 250, 375–382
32. Hara, S., Swigart, P., Jones, D., and Cockcroft, S. (1997) J. Biol. Chem. 272, 14099–14101
33. Segyi, D., Allen-Baume, V., and Cockcroft, S. (2002) Biochem. J. 366, 233–234
34. Swigart, P., Insall, R. H., Wilkins, A., and Cockcroft, S. (2000) Biochem. J. 347, 837–843
35. Casamayor, A., Morrice, N. A., and Alessi, D. R. (1999) Biochem. J. 342, 427–432
36. Vojnovic, J. P., Tremblay, J. M., Yarbrough, L. H., and Helmkanp, G. M. (1996) Biochemistry 35, 12526–12531
37. De Vries, K. J., Westernman, J., Bastiaens, P. I. H., Jovin, T. M., Wirtz, K. W. A., and Snoek, G. T. (1996) Exp. Cell Res. 227, 33–39
38. Cunha-Melo, J. R., Gonzaga, H. M., Ali, H., Huang, F. L., Huang, K.-P., and Beaven, M. A. (1989) J. Immunol. 143, 2617–2625
39. Monaco, M. E., and Mufson, R. A. (1986) Biochem. J. 236, 171–175
40. Orellana, S. A., Solski, P. A., and Brown, S. H. (1985) J. Biol. Chem. 260, 5236–5239
41. Cockcroft, S., and Stutchfield, J. (1989) Biochem. J. 263, 715–723

Downloaded from http://www.jbc.org/ at guest on July 23, 2018

2 A. Skippin, N. Carvou, and S. Cockcroft, unpublished data.
42. Jiang, H., Kuang, Y., Wu, Y., Xie, W., Simon, M. I., and Wu, D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7971–7975
43. Li, Z., Jiang, H., Xie, W., Zhang, Z., Smrcka, A. V., and Wu, D. (2000) Science 287, 1046–1049
44. Heitz, F., and Van Mau, N. (2002) Biochim. Biophys. Acta 1597, 1–11
45. Yau, W. M., Wimley, W. C., Gawrisch, K., and White, S. H. (1998) Biochemistry 37, 14713–14718
46. Bose, H. S., Baldwin, M. A., and Miller, W. L. (2000) Endocrinology 126, 629–637
47. Bose, H. S., Whittal, R. M., Baldwin, M. A., and Miller, W. L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7250–7255
48. Christensen, K., Bose, H. S., Harris, F. M., Miller, W. L., and Bell, J. D. (2001) J. Biol. Chem. 276, 17044–17051
49. Alb, J. G., Jr., Gedvilaite, A., Cartee, R. T., Skinner, H. B., and Bankaitis, V. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8826–8830
50. Milligan, S. C., Alb, J. G., Jr., Elagina, R. B., Bankaitis, V. A., and Hyde, D. R. (1997) J. Cell Biol. 139, 351–363
51. Litvak, V., Shaoul, Y. D., Shulewitz, M., Amarilio, R., Carmon, S., and Lev, S. (2002) Curr. Biol. 12, 1513–1518
52. Monaco, M. E., Kim, J., Ruan, W., Wieczorek, R., Kleinberg, D. L., and Walden, P. D. (2004) Biochem. Biophys. Res. Commun. 317, 444–450
53. van Tiel, C. M., Westerman, J., Paasman, M. A., Hoebers, M. M., Wirtz, K. W., and Snoek, G. T. (2002) J. Biol. Chem. 277, 22447–22452
54. Matsuzaki, M., Misumi, O., Shin-I, T., Maruyama, S., Takahara, M., Miyagishima, S. Y., Mori, T., Nishida, K., Yagisawa, F., Nishida, K., Yoshida, Y., Nishimura, Y., Nakao, S., Kobayashi, T., Momoyama, Y., Higashiyama, T., Minoda, A., Sano, M., Nomoto, H., Oishi, K., Hayashi, H., Ohta, F., Nishizaka, S., Haga, S., Miura, S., Morishita, T., Kabeya, Y., Terasawa, K., Suzuki, Y., Ishii, Y., Asakawa, S., Takano, H., Ohita, N., Kuroiwa, H., Tanaka, K., Shimizu, N., Sugano, S., Sato, N., Nozaki, H., Ogasawara, N., Kohara, Y., and Kuroiwa, T. (2004) Nature 428, 653–657
Phosphorylation of a Distinct Structural Form of Phosphatidylinositol Transfer Protein α at Ser\textsuperscript{166} by Protein Kinase C Disrupts Receptor-mediated Phospholipase C Signaling by Inhibiting Delivery of Phosphatidylinositol to Membranes

Clive P. Morgan, Alison Skippen, Bruno Segui, Andrew Ball, Victoria Allen-Baume, Banafshe Larijani, Judith Murray-Rust, Neil McDonald, Gopal Sapkota, Nick Morrice and Shamshad Cockcroft

J. Biol. Chem. 2004, 279:47159-47171.
doi: 10.1074/jbc.M405827200 originally published online August 18, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M405827200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 53 references, 27 of which can be accessed free at http://www.jbc.org/content/279/45/47159.full.html#ref-list-1