Delayed Phosphorylation of Classical Protein Kinase C (PKC) Substrates Requires PKC Internalization and Formation of the Pericentrion in a Phospholipase D (PLD)-dependent Manner*3

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It was previously demonstrated that sustained activation (30–60 min) of protein kinase C (PKC) results in translocation of PKC α and βII to the pericentrion, a dynamic subset of the recycling compartment whose formation is dependent on PKC and phospholipase D (PLD). Here we investigated whether the formation of the pericentrion modulates the ability of PKC to phosphorylate substrates, especially if it reduces substrate phosphorylation by sequestering PKC. Surprisingly, using an antibody that detects phosphosubstrates of classical PKCs, the results showed that the majority of PKC phosphosubstrates are phosphorylated with delayed kinetics, correlating with the time frame of PKC translocation to the pericentrion. Substrate phosphorylation was blocked by PLD inhibitors and was not observed in response to activation of a PKC B11 mutant (F663D) that is defective in interaction with PLD and in internalization. Phosphorylation was also inhibited by blocking clathrin-dependent endocytosis, demonstrating a requirement for endocytosis for the PKC-dependent major phosphorylation effects. Serotonin receptor activation by serotonin showed a similar response to phorbol 12-myristate 13-acetate, implicating a potential role of delayed kinetics in G protein-coupled receptor signaling. Evaluation of candidate substrates revealed that the phosphorylation of the PKC substrate p70S6K kinase behaved in a similar manner. Gradient-based fractionation revealed that the majority of these PKC substrates reside within the pericentrion-enriched fractions and not in the plasma membrane. Finally, proteomic analysis of the pericentrion-enriched fractions revealed several proteins as known PKC substrates and/or proteins involved in endocytic trafficking. These results reveal an important role for PKC internalization and for the pericentrion as key determinants/amplifiers of PKC action.

Protein kinase C (PKC) isoenzymes constitute a family of serine threonine kinases involved in cell signaling in response to the generation of lipid second messengers. PKC isoenzymes are divided into three families (classical, novel, and atypical) that have in common a regulatory amino-terminal domain and a carboxyl-terminal kinase domain containing motifs required for catalysis (1–3). The three major PKC isoforms (α, βI and II, and γ) constitute the classical PKC (cPKC)2 class and are activated by diacylglycerol (DAG) and calcium, which bind the C1 and C2 domain, respectively, in the amino terminus. Novel PKC (nPKC) isoforms (θ, δ, ε, and η) are calcium-independent isoforms because of a truncated non-functional C2 domain, whereas the atypical PKCs (ξ and λ/ι) are both calcium- and DAG-independent because of a truncation in both their C1 and C2 domains (1, 4). In the carboxyl terminus, the C3 domain is a typical ATP-binding domain, whereas the C4 domain contains a substrate-binding site (2). The plant-derived tumor promoter, phorbol 12-myristate 13-acetate (PMA), functions as a direct and potentiator activator of cPKCs and nPKCs (5).

According to current understanding, PKC activation occurs upon occupancy of the G protein-coupled receptors (GPCR) or tyrosine kinase receptors by ligands, leading to activation of phospholipase C and the resulting hydrolysis of phosphatidyl-inositol 4,5-bisphosphate in the inner leaflet of the plasma membrane to produce membrane-bound DAG and inositol 1,4,5-trisphosphate, which mediates the release of intracellular calcium. This concerted action leads to translocation of PKC to the plasma membrane within seconds of receptor activation (6, 7), upon which PKC is able to phosphorylate local substrates and regulate consequent events. Notably, this process is short lived because PKC returns to the cytosol within 2–5 min, which correlates with the metabolism and loss of DAG from the membrane in a process that is regulated by autophosphorylation of PKC (8).

In contrast to the classic acute pathway of PKC activation, sustained activation of the cPKCs α and βII for 15–60 min by either phorbol esters (9) or activation of GPCRs (10) results in translocation of cPKCs to a juxtanuclear region associated with the MTOC/centrosome and co-localization of PKC with Rab11, a marker of the perinuclear recycling compartment. Indeed, sustained activation of PKC was shown to induce co-localization and sequestration of several recycling membrane proteins and lipids to this novel compartment (10) termed the pericentrion, and these were defined as the PKC-

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2The abbreviations used are: cPKC, classical PKC; nPKC, novel PKC; DAG, diacylglycerol; GPCR, G protein-coupled receptor; PLD, phospholipase D; Bis, bisindolylmaleimide; PMA, phorbol 12-myristate 13-acetate; ESI, electrospray ionization; RFP, red fluorescent protein; 5-HT, serotonin; MARCKS, myristoylated alanine-rich C kinase substrate; FICI, 5-fluoro-2-indolyl deschlorohalopamilde.
dependent sequestered recycling endosomes. Very recently, it was shown that one important function of the pericentrion is to redirect the fate of some membrane receptors, such as the thrombin receptor, from the degradative endolysosomal pathway to the recycling pathway (10). The formation of the pericentrion (or similar appearing structures) has been observed following sustained stimulation of PKC with various ligands and in various cell lines (9, 11, 12), leading to sequestration of respective receptors or channels (13–15).

Mechanistically, the formation of the pericentrion was found to require the activity of PKC and that of phospholipase D (PLD) (16). Thus, PKC translocation and retention of sequestered proteins in the pericentrion were inhibited by the specific PKC inhibitor Gö 6976 (9). Moreover, kinase-inactive mutants of PKC α were unable to translocate to the pericentrion. This should be contrasted with acute translocation to the plasma membrane, which does not require PKC activity (12). Interestingly, Gö 6976 was also able to reverse translocation to the pericentrion even after it was formed, indicating a need for sustained activity of PKC for the persistence of the pericentrion (11). Studies from our laboratory (12) and the Exton laboratory (17) also demonstrated that the translocation of PKC to the pericentrion is PLD-dependent because inhibition of PLD by 1-butanol or by interfering RNA or the use of dominant negative PLD1 (10) blocked PKC translocation. PKC translocation to the pericentrion also depends on the clathrin-dependent pathway of endocytosis (11).

Because the pericentrion is emerging as a dynamic and PKC-dependent recycling compartment, one compelling question for our study was to determine the role of formation of this compartment in regulating phosphorylation of PKC substrates; in particular, we were interested to determine if formation of the pericentrion served to attenuate phosphorylation of PKC substrates. For these studies, we initially employed an antibody specific for PKC phosphosubstrates. Unexpectedly, we found that the majority of CPKC phosphosubstrates are phosphorylated with delayed kinetics and require activation of PLD and formation of the pericentrion. Direct data from fractionation assays demonstrated that the majority of these PKC substrates reside mostly within the pericentrion. Finally, using mass spectrometry, we report on potential PKC substrates in the pericentrion. The significance of these studies for PKC signaling and function are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—HEK293 cells were purchased from the American Tissue Culture Collection (Manassas, VA). Eagle’s minimal essential medium, Optiprep, 10% SDS solution, Lipofectamine 2000, fluorescent stains (Sypro Ruby protein gel stains), and Geneticin solution (G418) were purchased from Invitrogen. PMA, Gö 6976, and bisindolylmaleimide (Bis) were purchased from Calbiochem. 1-Butanol and Trizma were purchased from Fluka. Protease and phosphatase inhibitor mixtures, glycine, urea, CHAPS, iodoacetamide, ammonium carbonate, and DTT (electrophoresis grade; catalog no. D9163) were obtained from Sigma. For electrophoresis, SDS-polyacrylamide gels were purchased from Bio–Rad. Other electrophoresis ingredients were purchased from Bio–Rad (30% acrylamide/bisacrylamide and Tris-HCl buffer). M2 anti-FLAG antibody and agarose bead-conjugated M2 anti-FLAG antibody used for immunoprecipitation were purchased from Sigma. Anti-Rab11 antibody was purchased from BD Biosciences. Anti-phospho(Ser)-PKC α antibodies, anti-phospho-p44/42 MAPK (Thr202/Tyr204), anti-phospho-MAPKs (Ser185/187), anti-phospho-erzb (Thr676)/radixin (Thr566)/moesin (Thr558), and anti-phospho-p70S6K (Thr389) were purchased from Cell Signaling Technology (Danvers, MA). Anti-phospho-p38 (Thr180/Tyr182) was purchased from Promega (Madison, WI). For two-dimensional electrophoresis, Immobiline DryStrip IPG strips and ampholytes were purchased from GE Healthcare. Other ingredients for in-gel tryptic digestions, including Trypsin Gold (porcine modified sequencing grade), were obtained from Promega (Madison, WI). For PKC kinase activity, [32P]ATP was obtained from PerkinElmer Life Sciences. Histone, ATP, and phosphoric acid were obtained from Sigma, and DAG was obtained from Avanti.

**Plasmid Construction**—PKC βII-GFP-FLAG WT was made using the plasmid pBK-CMV-GFP-human PKC βII as template. The PCR product was amplified by a polymerase chain reaction using 5′-oligonucleotide primer (5′-AAACCCGGAT-CCACCATGGTGACCGGAGGAG-3′) and 3′-primer (5′-CGGGGTACCTACTTTATCGTGCCTGTTGATGTGGCCTC-CTGACCCGGGTTTAAAAATGTC-3′). The polymerase chain reaction fragment containing the FLAG cassette was gel-purified, digested with BamHI and KpnI, and directly inserted between the BamHI and KpnI sites of plasmid pBK-CMV-GFP-human PKC βII. An out of frame mock PKC βII-GFP-FLAG was made using the plasmid pBK-CMV-GFP-human PKC βII K371R as template. The PCR product was amplified using 5′-oligonucleotide primer (5′-GGCGGATCC AAAATGGAC-CTACGAGAGGAGGATTTATC originating from the transfer initiation codon (TIT)) and 3′-primer (5′-CAGGCGGGATCCTACGAGGAGGATTTATC originating from the transfer initiation codon (TIT)) but with a frameshift at amino acid 373 so that everything downstream is out of frame. PBK-GFP-PKC βII F663D was made using the plasmid pBK-CMV-GFP-human PKC βII F663D as template. The PCR product was amplified by polymerase chain reaction using 5′-oligonucleotide primer (5′-ACACCTAACTCTGGA-GATTAAAAACCCGAGTCT-3′) and 3′-primer (5′-GAC-TTCGGGTTTTAATCTTCAGAGTTAGGTGTT3′), which introduces a point mutation at amino acid 663. The phenylalanine (TIT) was changed to aspartic acid (GAT or ATC reverse complement). RFP-PKD1 was generously provided by Dr. Osvaldo Rey (Geffen School of Medicine, UCLA).

**Stable Cell Line Expression**—HEK 293 cells were grown in Eagle’s minimal essential medium containing 10% (v/v) FBS at 37 °C in an incubator providing 5% CO2. Transient transfections were carried on as follows. A medium containing 200 μl of Opti-MEM, 3 μl of Lipofectamine 2000, and 1 μg cDNA was added to 10-cm dishes containing 4 × 106 cells and kept overnight. The next day, the medium was changed, and G418 antibiotic was added for mammalian cell selection. From a titration
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curve, a concentration of 1 mg/ml was found to be optimal for selection. 7–10 days later, GFP-expressing cells selectively remained in culture and were later sorted (by flow cytometry) to collect the upper 20% bright population. GFP-positive cells in culture achieved a level above 90% and were maintained with the addition of G418 at a concentration of 0.4 mg/ml.

Confocal Microscopy—35-mm glass-bottomed culture dishes (MatTek) were used to plate cells expressing GFP-PKC BII at a density of 1 × 10^5 cells/dish and incubated overnight to allow cell attachment to glass. Medium was removed, and cells were promptly fixed for 10 min in 3.7% formaldehyde. The cells were observed under confocal microscopy using a laser-scanning confocal microscope (LSM 510 Meta, Carl Zeiss, Thornwood, NY).

Optimization of the Anti-phospho(Ser)-PKC α Substrate Antibody—Following immunodetection, a minimal but visible dark background with irregular patterns was observed in PMA-treated samples at the first time of antibody use. In an earlier study (18), to eliminate the possibility of nonspecific binding, the antibody was incubated with a mixture of phospho-Ser, -Thr, and -Tyr residues at a phosphoamino acid/antibody molar ratio of 10,000:1. Due to the antibody’s short shelf life (about 7 days after dilution) and to the rapid turnover of the antibody used in our study, we applied a different strategy by incubating the antibody aliquots with a blotted transfer membrane adsorbing a high concentration of proteins from PMA-treated HEK total cell lysates overnight (~16 h) with frequent changes before using the aliquot for immunodetection. The resulting immunodetection was significantly cleared from the background of both treated and untreated cells. However, due to the rapid decrement in signal intensity of the diluted antibody within a short period of time, the signal threshold showed some variation, depending on the number of times the aliquot was used, without any considerable effect on the overall pattern.

PKC Assay—PKC activity was quantitatively determined through the incorporation of a phosphate group from [32P]ATP onto histone in the presence of detergents (to allow formation of micelles), lipid cofactors (phosphatidylserine/DAG), and Ca^{2+}. The protocol is detailed elsewhere (19). Briefly, PKC was immunoprecipitated using anti-FLAG antibody, and the conjugated beads were washed and resuspended in lysis buffer. Two separate mixtures were prepared, one containing 100 μM ATP and 2 μCi of [32P]ATP and the other containing 10 mol % DiC18:1 phosphatidylserine and 5 mol % DiC18:1 DAG dried under a nitrogen gas stream. 10% Triton X-100, CaCl_2 (EGTA for basal measurement), Tris-HCl, and MgCl_2 were added to the second mixture before finally adding 200 μg/ml histone. A final solution was obtained by adding both mixtures to the beads, and the radioactivity (amount of phosphorylated histone) was measured by a scintillation counter at 30 min for the basal and activated states.

Cell Lysis and Immunoprecipitation—Cells were washed twice with cold PBS and lysed with detergent-based lysis buffer. Lysates were pulse-sonicated for 10 s and centrifuged at 12,000 rpm for 10 min. Protein concentration was determined by BCA, and the supernatant was used for immunoprecipitation and immunodetection. Agarose bead-conjugated M2 anti-FLAG antibody was incubated in the supernatant overnight at 4 °C and then boiled in Laemmli buffer.

Agonist Treatment—PMA, Gö 6976, and FIP1 were dissolved in 0.01% DMSO. PMA (100 nm) was used as a PKC activator for all time course assays. Gö 6976 (5 μM for 1 h prior to PMA) and Bis (3 μM for 1 h prior to PMA) were used as PKC inhibitors. 1-Butanol (0.5% for 30 min prior to PMA treatment) and FIP1 (20) were used as PLD inhibitors.

Inhibition of Clathrin-dependent Endocytosis—Cells were either incubated for 30 min with hypertonic sucrose solution (400 mm) or in K^+ -free buffer. For this protocol, a buffer containing 140 M NaCl, 20 M HEPES, 1 M CaCl_2, 1 M MgCl_2, 1 mg/ml glucose was used. Cells were rinsed twice with 1:1 H_2O-diluted buffer and incubated with the original buffer for 20 min at 37 °C before applying further treatment. KCl (10 mm) was added to this buffer as a positive control.

Subcellular Fractionation—All sample preparation procedures were carried out at 4 °C. The protocol was briefly modified from its previous application (11). In brief, cells were grown in batches of 150-mm culture dishes (3 dishes/treatment) and brought to confluence. Cells were lysed in the described hypotonic buffer containing 8.5% sucrose, briefly sonicated in pulses for 10 s, and centrifuged at 100 × g for 3 min to precipitate nuclear and unbroken particles. The lysates were ultracentrifuged at 120,000 × g for 1 h using a Beckman rotor type 70 Ti to collect the total membrane protein pellet. Gradient layers were generated in centrifugation tubes using different percentages of Optiprep (20, 15, 10, and 5%). In order to collect larger PKC-rich fractions, the volume of both 5 and 15% layers was changed from 3 ml each to 1 and 5 ml, respectively. Protein recovery was quantitated before pellet overlay on the Optiprep gradient. The collected pellets were next resuspended in lysis buffer, briefly sonicated, and overlaid on top of the gradients and then centrifuged for 18 h at 90,000 × g using SW-40 swing bucket rotor (Beckman Coulter). For different assays, 50 aliquots (250 μl each) were collected from the gradient and probed with either anti-FLAG, anti-phospho(Ser)-PKC substrates, or anti-Rab11 antibodies. Samples of 25 μl were taken from each fraction for immunodetection, and the Rab11-rich fractions were pooled for proteomic analysis. To remove the Optiprep polymer from solution, the PKC-rich fractions were mixed with a solution of hypotonic lysis buffer/H_2O at ratios of 1:2:1 (v/v/v) and centrifuged at 90,000 × g for 45 min, and the pellets were harvested for the two-dimensional electrophoresis procedures.

Two-dimensional Electrophoresis—Pellets, collected as above, were resuspended in ice-cold acetone for 30 min and centrifuged at 13,000 × g, and the resulting pellets were washed with proteomic grade water (Bio-Rad) twice without disturbing the pellets. IPG strips (13 cm) were rehydrated overnight (16–20 h) with 150 μl of rehydration buffer (8 M urea, 2 M thiourea, 0.5% CHAPS, 3% DTT, 0.5% amolyctes, and traces of bromphenol blue) and 1% amolycte. Pellets were dissolved in 100 μl of rehydration buffer, and protein content was determined by a non-interfering protein assay (G-Biosciences, Maryland Heights, MO) to ensure equal loading. A total of 75 μg of protein was used for the isoelectric focusing. The focusing protocol was carried out as follows: 1) step and hold 500/500 V-h; 2) gradient 1000/1000 V-h; 3) gradient 6000/12,000 V-h; 4)
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step and hold 6000/4000 V·h. After reduction/alkylation, strips were resolved in 12% SDS-PAGE and run for 5 h at 250 V.

**Gel Staining and Image Acquisition**—Gels were fixed in 50% methanol and 10% acetic acid for 3–5 h and stained with Sypro Ruby total protein stain for 6 h and destained by 10% methanol and 7% acetic acid for 6 h. Images were taken by a Typhoon laser scanner 9400 (GE Healthcare) using a fluorescent mode with an excitation/emission wavelength of 532/600 nm. The photomultiplier tube filter was set to 540 V, and a pixel size of 100 μm.

**Tryptic Digestion**—Protein spots were manually excised from gels and tryptically digested in-gel. Gel plugs were rinsed and treated with 100 mM ammonium carbonate in 50% acetonitrile for 45 min twice to remove SDS and then dehydrated for 5 min at room temperature in 100% acetonitrile. Complete drying was achieved in a SpeedVac for 10–15 min to remove the remaining acetonitrile traces. Acid-dissolved Trypsin Gold was diluted to 20 μg/ml and added to the plugs and incubated overnight at 37 °C. The next day, plugs were extracted with 50% acetonitrile/5% TFA to collect digestion solution and dried in a SpeedVac at room temperature for 2–4 h in preparation for MS analysis.

**Mass Spectrometry**—Tryptic-digested samples were analyzed by liquid chromatography (LC)-electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) on a linear ion trap mass spectrometer (LTQ, Thermo Finnigan) coupled to an LC Packings nano-LC system. A 15-cm × 75 μm C-18 reversed phase LC column (packed by the Proteomics Core Facility, Medical University of South Carolina) was utilized with a 60-min gradient from 2% acetonitrile, 0.1% formic acid to 60% acetonitrile, 0.1% formic acid at a flow rate of 200 nl/min. A blank was analyzed between samples to limit carry over. Data-dependent analysis was utilized on the mass spectrometer to perform MS/MS on all ions above an ion count of 1000. Dynamic exclusion was set to exclude ions from MS/MS selection for 3 min after being selected two times in a 30-s window.

The MS/MS data search was performed against the human database using Thermo Finnigan Bioworks 3.3 software. Post-translational modifications of methionine oxidation, carboxyamidomethylation of cysteines, and phosphorylation of serine, threonine, and tyrosine were considered in search settings.

**Phosphorylation of Known PKC Substrates**—HEK293 cells were grown on 12-well plates to about 80% confluence, starved primarily mediated by cPKC. Similar levels of transfection were evaluated by immunodetection using anti-GFP antibody.

Based on the above results, we sought to establish cell lines with stable overexpression of PKC βII and a PKC βII F663D mutant that was employed in subsequent studies because of its lack of translocation to the pericentriol. The expression level of the proteins was measured by Western blotting, and the results showed a similar signal for both WT and F663D displaying a protein with a molecular mass around 105 kDa, consistent with the anticipated molecular mass of the recombinant PKC (PKC βII + GFP + 2× FLAG) (Fig. 1C). On the other hand, as a control, the PKC expressed out of frame did not yield any detectable protein (Fig. 1C). To assess the activity of these enzymes, a PKC kinase assay was used, and the results showed that both recombinant enzymes (WT and F663D) demon-

**RESULTS**

**Anti-phospho(Ser)-PKC Substrate Antibody Detects Phosphorylation Specific to the Classical PKC Isoforms**—The subfamilies of PKC isoenzymes preferentially target distinct substrates distinguished by the amino acid residues in proximity to the phosphorylatable Ser/Thr. This has allowed the development of an antibody that detects preferentially phosphosubstrates of the cPKC family (21–23). We first sought to determine the basal phosphorylation profile that can be detected by the anti-phospho(Ser)-PKC substrate antibody. Control cells and cells overexpressing PKC βII were treated with either DMSO or 100 nM PMA for 1 h and probed with the antibody (Fig. 1A). The results showed that the antibody detected basal phosphorylation in vehicle-treated cells among both control cells and those overexpressing PKC βII. Treatment with PMA for 60 min resulted in significant enhancement of phosphorylation in both cell types. However, the signal exposure necessary to display the basal phosphorylation overwhelmed a clear detection of the phosphorylation pattern for samples treated with PMA. Thus, this antibody appears to detect both basal and PMA-stimulated phosphorylation, which was enhanced significantly by overexpression of PKC βII. This significant enhancement of phosphorylation by overexpression of PKC indicated the ability of the antibody to detect specific PKC-mediated phosphorylation.

This in turn allowed us to determine whether the observed phosphorylation profile occurred exclusively due to the activation of the cPKC subclass. In order to optimize the detection of substrates specific to PKCs, we employed conditions that allow higher sensitivity of the antibody (higher dilution and longer exposure). In turn, this allowed us to observe primarily the phosphorylation pattern resulting from overexpression of PKC and was not confounded by basal phosphorylation or phosphorylation induced by endogenous kinases. Therefore, HEK 293 cells were transiently transfected with six isoforms representing the different classes of the PKC, namely βII, α, βI, γ, θ, and δ. The results demonstrated that only isoforms that translocate to the pericentriol, namely α and βII, showed major phosphorylation activity at 60 min post-PMA treatment (Fig. 1B), thus suggesting that the antibody detects phosphorylation primarily mediated by cPKC. Similar levels of transfection were evaluated by immunodetection using anti-GFP antibody.

**Statistics**—All immunodetection assays are representative of a minimum of 20 fields over two experiments and were cropped and resized for publication.
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Stratified increased basal activity compared with the control. Lipid activation significantly increased the activity level of both proteins in a similar manner (Fig. 1D). These results indicate that both proteins show comparable in vitro activity. Next, the optimal PMA concentration that induces maximal phosphorylation was determined. As shown (supplemental Fig. 1A), substrate phosphorylation was partially detected in response to 3 nM PMA with a weak signal that reached its peak between 30 and 100 nM. Because our earlier studies employed 100 nM PMA as a preferred concentration in stimulating formation of the pericentriol (9, 11), this concentration was selected in this work for consistency. Next, we sought to determine if an inhibitor of cPKCs would specifically inhibit the PMA-induced phosphorylation. Cells were pretreated with different concentrations of Gö 6976 (0.1–5 μM) for 30 min, followed by a sustained stimulation (1 h) with 100 nM PMA. As shown (supplemental Fig. 1B), a significant inhibitory effect of Gö 6976 was observed at a concentration of 0.75 μM. Nearly total inhibition was obtained at 5 μM. Consequently, 5 μM was used as a standard concentration for further work. To confirm that the effects of Gö 6976 are specific to PKC inhibition, another PKC inhibitor, bisindoylmaleimide, was used for this pur-
pose. The latter also inhibited PMA-induced phosphorylation in a concentration-dependent manner, ranging from 0.3 to 3 μM (supplemental Fig. 1C). These results suggest that the observed PMA-stimulated phosphorylation is exclusively due to PKC activity and not to other PMA potential targets.

Because the above results show cPKC-dependent phosphorylation and because the antibody was designed to detect phosphorylation of cPKC substrates, these results indicate that the observed phosphorylations are more likely due to direct effects of cPKC on these substrates and less likely due to the action of further downstream kinases because these kinases do not show similar substrate preferences. One kinase, PKD1, required specific evaluation, as a remote candidate because it can be activated by other PKCs (24, 25) and also because of its responsiveness to stimulation by PMA (26). First, to confirm the PKD phosphorylation activity, we conducted a time course assay of PKD kinase activity following 100 nM PMA treatment for 1, 5, 30, and 60 min. RFP-PKD was overexpressed, and its activity was measured by anti-phosphoserine antibody. As Fig. 2A shows, PKD is kinetically active, and its activity increases over the first 5 min and persists up to 1 h. Anti-RFP antibody was used to determine the level of expression. Next, cells overexpressing either PKC βII or RFP-PKD were incubated with 100 nM PMA for 5 or 60 min, and then the phosphosubstrates were evaluated as above. The results (Fig. 2B) demonstrated that PMA stimulation of cells overexpressing PKD did not show any major change in PKC substrate phosphorylation, confirming the specificity of PKC kinase activity in the substrate phosphorylation.

Requirement for Translocation to the Pericentriol for Major Phosphorylation of cPKC Substrates—Acute stimulation of cPKC by PMA results in initial translocation to the plasma membrane within 1–5 min, followed by translocation to the pericentriol in response to sustained stimulation (15–60 min). Therefore, it became important to determine the extent of substrate phosphorylation during early (5-min) versus late (30–60-min) stimulation. At 5 min of stimulation, there was minimal detection of phosphosubstrates in response to PMA (Fig. 3A). On the other hand, stimulation with PMA for 30–60 min showed robust substrate phosphorylation. These unexpected results suggested that the predominant majority of PKC βII substrates are detected in the time frame when this enzyme is already off the plasma membrane and when it has translocated to the pericentriol. Moreover, inhibition of PKC by Gö 6976 blocked substrate phosphorylation at all time points of PMA treatment (Fig. 3B). Thus, PKC βII displays robust activity, especially in later time points, raising the possibility that translocation to the pericentriol is required for this activity.
To determine if translocation to the pericentrion is indeed required for this phosphorylation, we employed approaches to inhibit the translocation of PKC and then determine if these affected phosphorylation of substrates. It was previously shown that PKC translocation to the pericentrion is PLD-dependent (12, 17). To determine if PLD is required for the observed phosphorylation, cells were treated with PMA (100 nM), and the effects of PLD inhibition on phosphorylation were determined. For these studies, 1-butanol was used to inhibit PLD, and the results showed that 1-butanol had a dominant inhibitory effect on PKC phosphorylation at 60 min (Fig. 4), whereas no significant change was observed at 5 min, suggesting that PKC-mediated phosphorylation is PLD-dependent. To confirm that the effects of 1-butanol are specific to PLD inhibition, FIP1, a potent and recently developed synthetic PLD inhibitor (20), was used at a concentration of 750 nM because this concentration was optimal to nearly block all PLD activity at both basal and PMA-stimulated levels in vivo as well as in vitro while still acting as a specific non-toxic PLD inhibitor without affecting the typical localization of PLD1 at the perinuclear membrane vesicles (18).

The results showed inhibition as significant as that shown by 1-butanol (Fig. 4). These data demonstrate a requirement for PLD in the induction of the major phosphorylation of PKC substrates.

To further define the role of PLD in substrate phosphorylation, we employed an active mutant of PKC, PKC F663D, which was shown by Hu and Exton (17) not to interact with PLD and not to translocate to the pericentrion. The results from Fig. 1D show that this mutant is active in vitro. Results shown in Fig. 5A demonstrate that this mutant translocates to the plasma membrane acutely in response to PMA, similar to WT PKC. However, unlike wild type PKC, this mutant failed to translocate to the pericentrion (Fig. 5A). Importantly, and although fully active in vitro, the F663D showed only modest substrate phosphorylation at 5 and 60 min (Fig. 5B). Interestingly, this phosphorylation was similar at 5 and 60 min, suggesting predominant phosphorylation at the plasma membrane and no further phosphorylation due to lack of translocation to the pericentrion. Taken together, these data demonstrate a requirement for PKC activation of PLD and a role for PLD in the major phosphorylation of PKC substrates.

Next, the role of endocytosis in substrate phosphorylation was evaluated. Previous results showed that formation of the pericentrion depends on clathrin-dependent endocytosis. Experimentally, inhibition of clathrin-dependent endocytosis with potassium-free buffer or through incubation in hypertonic sucrose solution blocked the juxtanuclear translocation of PKC and the formation of the pericentrion (11). To determine if phosphorylation of the detected substrates requires clathrin-dependent endocytosis, cells were either treated with hypertonic sucrose solution or incubated in a potassium-free buffer, and the results showed that inhibition of endocytosis by sucrose nearly blocked all PKC phosphorylation activity at 60 min of treatment (Fig. 6A). Likewise, endocytosis inhibition by potassium-free buffer showed significant inhibition of PKC phosphorylation (Fig. 6B). These results demonstrate a requirement for endocytosis for the PKC-dependent major phosphorylation.

The above results showed that the major phosphorylation of PKC substrates requires translocation of PKC to the pericentrion; however, due to the active nature of recycling from the
pericentriol, it could not be determined if PKC substrates reside in the pericentriol itself. To investigate this, we isolated subcellular fractions enriched in the pericentriol using gradient-based cellular fractionation as described under “Experimental Procedures.” Because previous studies showed that sustained stimulation of cPKC leads to translocation particularly to the Rab11-rich fractions (11), we focused on PKC substrate phosphorylation in these fractions (Fig. 7). Untreated cells showed some phosphorylation detected by the anti-phospho-(Ser)-PKC substrate antibody in the Rab11-enriched fractions, including a major band co-migrating with PKC H9252 II (Fig. 7A).

This is consistent with basal activation of PKC and the partial induction of PKC-dependent formation of the juxtanuclear compartment. Interestingly and in further support of this, Gö6976, the inhibitor of cPKCs, resulted in near abrogation of this basal phosphorylation (Fig. 7B). Importantly, PMA treatment resulted in a robust increase in phosphorylation of substrates in the pericentriolar fractions resembled the one seen in total cell lysates, suggesting that the phosphorylation profile observed in total cells following PMA stimulation reflects the presence of the substrates within the pericentriol. Moreover, and as seen in total cells, Gö6976 inhibited the PMA-stimulated phosphorylation (Fig. 7D). Likewise, 1-butanol also inhibited phosphorylation detected in the pericentriol (Fig. 7, E and F). Taken together, the results show that the major phosphorylation of substrates seen upon sustained PKC stimulation reflects predominantly phosphosubstrates present in the pericentriol-enriched fractions.

Sustained Stimulation of Receptors Results in Induction of Substrate Phosphorylation—In a recent study (9), we showed that sustained stimulation of Gq-coupled GPCRs results in the PKC- and PLD-dependent formation of the pericentriol, and this may act to divert receptors from the degradative compartment to the Rab11-positive recycling compartment. To determine if receptor-mediated activation of PKC also results in similar delayed phosphorylation, cells were treated with 10 μM serotonin (5-HT) for either 5 min or 60 min (Fig. 8). The results
showed significantly more phosphorylation at 60 min post 5-HT treatment, and this activity was both PKC- and PLD-dependent because it was inhibited by Gö 6976 and by 1-butanol (Fig. 8). These results demonstrate that sustained 5-HT receptor stimulation results in induction of PKC-mediated phosphorylation in a PLD-dependent manner, revealing that this is not a peculiarity of PMA treatment.

Evaluation of the Role of the Pericentrion in Phosphorylation of Known PKC Substrates—In order to determine the kinetics of phosphorylation and the effect of the pericentrion on known PKC substrates, several proteins that are known to be phosphorylated upon PMA treatment were investigated. Cells were treated with PMA for 2, 5, 10, 30, and 60 min, and phosphorylation status of p44/42 MAPK, MARCKS, ezrin/radixin/moesin, p70S6K, and p38 was analyzed by Western blotting. As shown in Fig. 9, proteins were divided into two groups based on the time of PMA treatment required for their phosphorylation. One group displayed acute phosphorylation upon PMA (~2 min). This was observed for p44/42 MAPK, MARCKS, and ezrin/radixin/moesin (Fig. 9A). Another group comprised proteins that are phosphorylated later (starting at or after 10 min), in a time frame matching that of pericentrion formation. This group was represented by p70S6K and p38 MAPK (Fig. 9B). Although it is known that the phosphorylation of p44/42 MAPK (27), MARCKS (28), ezrin/radixin/moesin (29), p70S6K (30), and p38 (31) is PKC-dependent, it was of great interest to determine if cPKCs are selectively involved in this process. To address this, cells were pretreated with Bis, which inhibits all cPKC and nPKC isoforms, or with Gö 6976, which specifically inhibits cPKC isoforms. The results showed that although all proteins analyzed were phosphorylated in a PKC-dependent manner (inhibited by Bis), only phosphorylation of ezrin/radixin/moesin, p70S6K, and to a minor extent MARCKS and p38 was cPKC-dependent (inhibited by Bis and Gö 6976) (Fig. 9C). Thus, most of the acute phosphosubstrates appear to be substrates of non-classical PKCs.

Next, it became important to investigate if the phosphorylation of the cPKC-dependent substrates is dependent on formation of the pericentrion. Thus, the ability of PLD inhibitors to modulate phosphorylation of ezrin/radixin/moesin and p70S6K was evaluated. As shown in Fig. 9D, phosphorylation of p70S6K was PLD-dependent. Taken together, these results demonstrate that cPKC can phosphorylate substrates in both a delayed and pericentrion-dependent (p70S6K) manner or in an acute and pericentrion-independent manner (ezrin/radixin/moesin). Interestingly, phosphorylation of the remaining substrates appears to be mediated by non-cPKCs.

Identification of Candidate Phosphoproteins Present in the Pericentrion—The above results showed that many PKC substrates are more likely substrates of non-classical PKCs, and this limited the evaluation of the extent of cPKC substrates and which of those show delayed phosphorylation. Therefore, it became of significant interest to determine the major cohort of PKC substrates present in the pericentrion. To this end, LC/MS/MS approaches were employed to examine the pericentrion-enriched fraction. Cells were treated with PMA with or without Gö 6976, and the Rab11-positive fractions were resolved in two-dimensional electrophoresis using a linear pH range of 3–10 on 13-cm IPG strips. Gels were stained for total protein using Sypro Ruby. A total of 14 spots were readily visible from the gel of the PMA-treated sample and were not present in the control (Fig. 10A). These spots were then sequenced by LC/MS/MS.

The results led to the identification of PKC βII itself, consistent with the translocation of PKC βII to the pericentrion. Interestingly, most of the other identified proteins have been previously identified as PKC substrates and/or known to be associated with recycling components (Table 1). These included the transferrin receptor, which is considered a marker of recycling endosomes and has been previously found to relocalize from diffuse endosomes to the pericentrion in a PKC-dependent manner (11). Moreover, the transferrin receptor has been shown to be phosphorylated by PKC (32). The epidermal growth factor receptor pathway substrate 15-like 1 (EPS 15) is a tyrosine phosphosubstrate of EGFR that is also involved in clathrin-dependent endocytosis and has also been reported to colocalize with clathrin (33). The Na⁺/K⁺-ATPase β3 subunit is the non-catalytic component of the active transporter and is phosphorylated at conserved residues by PKC (34). Na⁺/K⁺-ATPase interacts with caveolin-1 (Cav1) which regulates its endocytosis, trafficking, and subcellular localization (35). Acid sphingomyelinase-like phosphodiesterase is related by...
sequence to acid sphingomyelinase; however, the function of this protein and its localization are not known. The isoform of the serine/threonine-protein phosphatase 2A catalytic subunit (PP2A) is a ubiquitous protein phosphatase that has been seen in the trans-Golgi network/early endosomal system, where it may regulate the phosphorylation-dephosphorylation cycle of furin (36). The H^+-ATPase has been also found to reside in the endosomes. The activities of vacuolar-H^+-ATPase and vacuolar H^+-pyrophosphatase are altered by GPCRs and tyrosine kinase receptors, confirming a synergetic role in membrane trafficking and in clathrin-mediated endocytosis (37). Their role in the endosomes is to facilitate the dissociation of ligand from receptors by maintaining a low pH in the endosomes (38).

The serine/threonine protein kinase MST4 isoform 2 is a member of the MST4 group. It is an STE20-like kinase functioning as an upstream kinase of the MAPK signaling pathway. It is regulated by different classes of membrane receptors, including G-protein-coupled receptors and tyrosine kinase receptors (39).

Motif-containing GAP-1 is a regulator of Rab GTPase activity. Interestingly, this specific GAP colocalizes with Rab11, which is important for the trafficking of Rab11 to the pericentriolar area. This GAP is targeted to vesicular compartments by activated Rab11 (40).

Tumor necrosis factor type 1 receptor-associated protein (TRAP1) is a member of the heat-shock family of proteins (HSP90) but has specific functions that differ from those of other members of the HSP90 family (41) because it regulates signal transductions from different receptors of the TNF receptor superfamily (42, 43).

The only protein that was found without any known significant relevance to the process of endocytosis and trafficking was lamin B. Lamin B is a component of the nuclear lamina on the nucleoplasmic side of the inner nuclear membrane.

Thus, among the identified proteins enriched in the pericentriol, several are already known as PKC substrates. These are Na^+ /K^+-ATPase β3 subunit (44), PP2A (45), H^+-ATPase (46), IQGAP1 (47), TRAF1 (48), and transferrin receptors (49). Most of the others are also known to be involved in endocytosis/trafficking.

Moreover, probing with the anti-phospho(Ser)-PKC substrate antibody of the pericentrion-rich fractions showed an interesting wide distribution of phosphorylated proteins across the entire pI/Mr scale (Fig. 10B).

DISCUSSION

In this study, we set out to identify whether internalization of cPKC and/or formation of the pericentriol influence cPKC-
mediated substrate phosphorylation. Specifically, we postulated that the translocation of cPKC to the pericentrion would dissociate it from plasma membrane substrates and perhaps expose it to a new subset of substrates, expecting that the major effect would be attenuation of substrate phosphorylation. The study employed three approaches, an antibody that specifically detects phosphosubstrates of PKC, evaluation of candidate substrates, and a proteomic approach.

Surprisingly, the results revealed that the predominant phosphorylation of cPKC substrates appears to require internalization of cPKC as well as the formation of the pericentrion. Indeed, sustained activation of cPKC resulted in concentration of several cPKC substrates in the pericentrion. These findings have significant implications for understanding PKC function and regulation, especially in defining the pericentrion as a candidate signaling compartment for cPKC.

The major conclusion of this study is that the predominant action of cPKCs on substrate phosphorylation does not correlate with the acute translocation of either PKC \( \alpha \) or PKC \( \beta II \) to the plasma membrane. Indeed, previous studies as well as confocal studies shown here demonstrate that acute translocation of PKC to the plasma membrane occurs within seconds, after which PKC "reverse translocates" from the plasma membrane as it becomes internalized (8). Importantly, the F663D mutant of PKC \( \beta II \), which is active and translocates to the plasma membrane but does not internalize, did not show the robust phosphorylation of substrates seen with the wild type enzyme. On the other hand, sustained activation of PKC (30–60 min) led to corresponding robust substrate phosphorylation, which peaked at 30–60 min. This time frame corresponded to internalization of PKC and formation of the pericentrion. Additionally, when phosphorylation of known PKC targets was analyzed upon PMA treatment, cPKC/PLD-dependent phosphorylation

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**FIGURE 8. Effect of 5-HT on phosphorylation of PKC substrates.** HEK cells overexpressing WT PKC \( \beta III \) were treated with 10 \( \mu M \) 5-HT for 5 or 60 min, and then PKC substrates were immunodetected.

**FIGURE 9. Phosphorylation of putative PKC substrates.**

A and B, HEK-293 cells were treated with 100 \( \mu M \) PMA for the indicated times. C, cells were treated with 100 \( \mu M \) PMA for 5 min (phospho-p44/42-MAPK, phospho-MARCKS, and phospho-ezrin) or 30 min (phospho-p70 S6K and phospho-p38) with or without a 1-h preincubation with 3 \( \mu M \) Go 6976 or 2 \( \mu M \) Bis. D, cells were treated with 100 \( \mu M \) PMA for 5 min (phospho-p44/42-MAPK) or 30 min (phospho-p70 S6K) with or without a 15-min preincubation with 0.4% 1-butanol. Levels of the indicated proteins were determined by Western blotting. \( \beta \)-Actin was used to normalize for loading. The results are representative of two independent experiments.
of p70S6K was evident starting at 15–30 min, correlating with the time of formation of the pericentrion. Interestingly, we could only observe a clear cut cPKC-induced phosphorylation that was not dependent on the pericentrion for ezrin/radixin/moesin. All other specific PKC substrates we evaluated showed modest to little inhibition by Go 6976 as compared with inhibition by Bis, suggesting that they are likely substrates of nPKCs.

Many lines of evidence support the utility of the anti-PKC phosphosubstrate antibody to detect specifically the activity of cPKCs. When used under conditions that depend on detection of phosphosubstrates only with overexpression of PKC, the antibody detected phosphorylation only with overexpression of cPKC α and βII and not other PKCs. Moreover, the detected phosphorylation was robustly inhibited by Go 6976, indicating the need for activation of cPKCs and not other PMA targets. Thus, the results clearly indicated that these phosphorylations are dependent on cPKC activity.

Although the above results strongly implicate cPKC in the induction of this phosphorylation, they do not directly distinguish between direct effects of cPKCs on these substrates or indirect effects. However, a number of considerations lead us to favor a direct effect. First, the antibody was developed against a sequence highly specific not only for PKC substrates (serine)
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but more specifically against cPKCs. Thus, the antibody did not detect nPKC-mediated phosphorylation. Second, there are no known kinases with high similarity for cPKC in substrate preference. The only relatively close kinase is PKD, and the results shown here negate a role for PKD. Third, the pattern of phosphorylation at 5 min when cPKC is still present at the plasma membrane resembles the pattern at 60 min, albeit demonstrating much less phosphorylation. Thus, if the early set is that of cPKC substrates, then the late set (which is very similar) is likely to be that of cPKC substrates. Finally, the roles of PLD and endocytosis (see below) also support the role of the cPKC-mediated pericentrion.

Indeed, multiple lines of evidence, provided in this study, support a role for cPKC internalization and formation of the pericentrion for induction/enhancement of substrate phosphorylation. First, the time course noted above for substrate phosphorylation clearly correlated with internalization of cPKC and formation of the pericentrion. Second, the results revealed a requirement for endocytosis for optimal phosphorylation of PKC substrates because blocking clathrin-mediated endocytosis was able to inhibit the delayed PKC phosphorylation activity. Third, there was a clear requirement for PLD because both translocation of PKC and substrate phosphorylation were inhibited through the inhibition of PLD by 1-butanol or FIP1 or by blocking the PKC-PLD interaction using the active PKC mutant (F663D). Fourth, and most clearly, subcellular fractionation studies revealed that the majority of PKC phospho-substrates reside in the pericentrion-enriched fractions and not in the plasma membrane. Indeed, the complement of PKC substrates detected in the Rab11 rich fractions (corresponding to the pericentrion) was very similar to the set of substrates detected in total cell lysates. The phospho-substrates in the pericentrion fraction included PKC βII itself (Fig. 7C), consistent with previous results demonstrating the translocation of PKC to the Rab11-enriched fraction following sustained activation of PKC. Taken together, these results demonstrate that a significant portion of PKC phospho-substrates predominantly localize in the pericentrion-enriched fractions following sustained stimulation of PKC.

This work also implicates a potential role of delayed kinetics in GPCR-mediated signal transduction. It was previously demonstrated that 5-HT induces global sequestration of molecules to the pericentrion in a cPKC-dependent manner. In the current work, the results show that the sustained action of 5-HT, which induces sustained activation of cPKC, also results in sustained substrate phosphorylation.

The results from this study also identify a group of prominent proteins that localized to the pericentrion in a PKC-dependent manner using tandem mass spectrometry analysis. Indeed, several proteins were seen to accumulate in the pericentrion fraction upon PMA stimulation on two-dimensional gel analysis; however, the majority showed a very low abundance and could not be detected convincingly by MS. Nevertheless, several proteins were positively detected by these techniques. Interestingly, the identified proteins were mostly known PKC substrates and/or proteins known to recycle. Specifically, transferrin receptor, acid sphingomyelinase-like phosphodiesterase, and IQ-GAP1 (motif-containing GAP-activating protein 1) were defined as recycling proteins (50, 51). Two additional proteins are known to be involved in the clathrin-dependent endocytosis pathway (EPS 15 and H+ -ATPase).

The results from this study raise a tantalizing possibility of a role for the pericentrion as a recycling compartment with signaling activity. Indeed, the results from this study show that compared with the plasma membrane, the pericentrion is a major site for the presence of phospho-substrates for cPKC βII and cPKC α. cPKCs play diverse roles in the regulation of signaling events, including desensitization of membrane-bound signaling complexes. In the membrane, ligands bind to the extracellular domain of receptor tyrosine kinases or to GPCRs, leading to activation of phospholipase Cγ or β, respectively. This leads to acute translocation of PKC to the plasma membrane within seconds, where it presumably phosphorylates local substrates and regulates downstream signaling. The current data demonstrated modest cPKC-induced phosphorylation within this time frame. However, signal transduction is not limited to the plasma membranes. Receptors internalized into endosomes remain competent for signal transduction, with many emerging examples of activation of receptors in this compartment. For example, endosomes carry internalized TRK receptors to interact with endosomal signaling molecules of ERK1/2 and PI3K/AKT pathways (52, 53). Endosomes containing EGFR, Rab5, APPL1 (adaptor protein, phosphotyrosine interaction, pH domain, and leucine zipper-containing 1) and APPL2 (Rab5 effectors) are reported as signaling endosomes, transducing signaling pathways that mediate cell proliferation (54, 55). In addition, the ability of signaling complexes to form in endosomes has been demonstrated by the fact that downstream signaling components of phosphorylated EGFR, such as Shc, Grb2, and mSOS, were detected on the early endosomes of liver parenchyma cells (56). Another example, inhibition of endocytic vesicle formation by the dominant negative dynamin (K44A) abrogates MAPK activation in response to PMA-activated PKC (27). Also, EGF receptor endocytic trafficking controls membrane trafficking signaling pathways (57). Thus, formation of the pericentrion appears to be important in controlling the signaling functions of cPKCs. More studies are required to decipher the specific mechanisms operating on individual substrates.

Importantly, the majority of cellular cPKC phospho-substrates appear to locate to the pericentrion-enriched fraction and not in the plasma membrane. Thus, it can be concluded that the pericentrion is the subcellular site for location of many cPKC substrates. The results, however, do not allow us to distinguish whether the pericentrion is the site where phosphorylation occurs (i.e. if it is a signaling compartment per se). This is because the pericentrion is a dynamic structure whose formation requires sustained activation of PKC and PLD (11), and recycling appears to be ongoing, although recycling proteins become enriched in the pericentrion. Thus, another alternative is that PKC acts on these substrates at the plasma membrane (or in early endosomes), but the substrates become enriched in the pericentrion over the time course studied (up to 60 min). Therefore, more studies are required to distinguish these two possibilities.
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Irrespective of whether substrates are phosphorylated in the pericentrion itself or are simply concentrated there following phosphorylation in more proximal sites, the pericentrion is obviously involved in cPKC-mediated regulation of phospho-substrates. This mechanism could also modulate accessibility of phosphosubstrates to phosphatases, thus accounting for their enrichment. Functionally, it is possible that the pericentrion may control receptors and other plasma membrane proteins by either removing them from their normal signaling partners or, on the contrary, by bringing together previously unconnected components. In conclusion, this study reports on cPKC-mediated regulation of phospho-substrates by the pericentrion, and their enrichment. Functionally, it is possible that the pericentrion may play a critical role in regulating PKC function through the regulation of the degree of localization of the major PKC substrates.

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