G protein-coupled receptor kinases (GRKs) specifically recognize and phosphorylate the hormone-occupied form of numerous G protein-coupled receptors, ultimately resulting in termination of receptor signaling. While little is presently known about the regulation of GRK function, recent studies suggest a role for protein kinase C (PKC) phosphorylation of the β-adrenergic receptor kinase in membrane association and activation of the kinase. To assess a potential general role for PKC in regulating GRK function, we characterized the ability of PKC to phosphorylate GRK5, a recently identified member of the GRK family. We demonstrate that GRK5 can be rapidly and stoichiometrically phosphorylated by PKC in vitro. Intact cell studies reveal that GRK5 is also phosphorylated when transiently expressed in COS-1 cells following treatment with the PKC activator, phorbol 12-myristate 13-acetate. In vitro analysis reveals two major sites of PKC phosphorylation within the C-terminal 26 amino acids of GRK5. GRK5 phosphorylation by PKC dramatically reduces its ability to phosphorylate both receptor (light-activated rhodopsin) and non-receptor (casein and phosvitin) substrates. Kinetic analysis reveals an ~5-fold increased \( K_m \) and ~3-fold decreased \( V_{max} \) for rhodopsin, with no change in the \( K_m \) for ATP. The reduced affinity of PKC-phosphorylated GRK5 for rhodopsin was also evident in a decreased ability to bind to rhodopsin-containing membranes, while direct binding of GRK5 to phospholipids appeared unaltered. These results suggest that PKC might play an important role in modulating the ability of GRK5 to regulate receptor signaling and that GRK phosphorylation by PKC may serve as a disparate mechanism for regulating GRK activity.

A basic feature of most cells is their ability to regulate their responsiveness to extracellular stimuli. This phenomenon, often termed desensitization, has been extensively studied for the β2-adrenergic receptor (β2AR), which mediates catecholamine stimulation of cAMP production (1, 2). Desensitization appears to involve a multistep process that is initiated by receptor activation and culminates in receptor phosphorylation and functional uncoupling of receptor signaling. Phosphorylation of the β2AR can be mediated by multiple protein kinases, including second messenger activated kinases such as protein kinase A and protein kinase C (PKC), as well as a specific class of kinases termed G protein-coupled receptor kinases (GRKs) (3, 4). GRKs are unique in that they specifically recognize and phosphorylate the agonist occupied or activated form of a receptor. To date, six members of the GRK family have been identified.

While GRKs are involved in regulating G protein-coupled receptor function, the activity of GRKs themselves also appears to be regulated. Examples of such regulation include the activation of rhodopsin kinase (5, 6), β-adrenergic receptor kinase (βARK) (7), and GRK5 upon binding to an activated receptor. This activation is manifested as an increased ability of the kinase to phosphorylate exogenous peptide substrates and will likely be a property shared by all of the GRKs. Another mechanism for regulating GRK function appears to be via phospholipid interaction. All of the GRKs can directly interact with phospholipids either via covalent modifications such as farne-sylation (rhodopsin kinase) (8) or palmitoylation (GRK4 and 6) (9, 10), or via lipid binding domains such as the pleckstrin homology domain in βARK1 and 2 (11) or a poorly defined polybasic domain in GRK5 (12, 13). In addition, βARK1 and 2 have the ability to interact directly with G protein βγ subunits, an interaction that may be involved in kinase localization (14–16). At least two of the GRKs also undergo rapid autophosphorylation (12, 13, 17). Autophosphorylation of rhodopsin kinase reduces its affinity for phosphorylated rhodopsin, suggesting a potential role in dissociation of the kinase from the receptor (18). In contrast, autophosphorylation of GRK5 appears to significantly activate the kinase (12).

More recent studies have implicated calcium in the regulation of GRK activity. In the visual system, rhodopsin kinase has been shown to be inhibited by the Ca\(^{2+}\)-binding protein recoverin (19). Calcium binding to recoverin promotes its association with rhodopsin kinase, inactivating the kinase and thereby reducing its ability to phosphorylate rhodopsin. Since calcium levels are decreased upon light activation of rod cells (20), recoverin binding to rhodopsin kinase might provide a mechanism for adaptation of the system to ambient light. Recent studies have also demonstrated that βARK can be phosphorylated by PKC, a Ca\(^{2+}\) and phospholipid-dependent kinase, leading to an ~2–3-fold activation of the kinase possibly via an increased ability of the kinase to bind to membranes (21, 22).

In the present study we evaluated whether GRK5 can be regulated by PKC. We show that GRK5 is rapidly phosphorylated by PKC in vitro, suggesting a role for PKC in modulating the ability of GRK5 to regulate receptor signaling and that GRK phosphorylation by PKC may serve as a disparate mechanism for regulating GRK activity.
Regulation of GRK5 by Protein Kinase C

For metabolic labeling, cells were incubated for 1 h at 37 °C either in RPMI 1640 (lacking methionine) with 10% dialyzed fetal bovine serum for the [35S]Met labeling or in DMEM (lacking phosphate) with 1% fetal bovine serum for [32P]labeled GRK5. Cells were then incubated with either [35S]Met (0.1–0.2 mCi/ml) or [32P]orthophosphate (2000 cpm/200 μl) in 1 ml of DMEM for 2.5 h. PMA (0.2 μM final concentration) or vehicle were added to the wells, and the cells were incubated for 30 min at 37 °C, washed with phosphate-buffered saline, harvested, and analyzed by immunoprecipitation.

Immunoblotting and Immunoprecipitation—GRK5 rabbit polyclonal antibodies were raised against glutathione S-transferase (GST) fusion proteins containing either amino acids 98–136 or 489–590 (12) of human GRK5. GST and the GST-GRK5 fusion proteins were expressed in *Escherichia coli* and purified on a glutathione-Sepharose column using standard procedures (28). A rabbit polyclonal antibody was also generated against a peptide corresponding to amino acids 556–571 of GRK5 that had been covalently coupled to hemagglutinin. The resulting GRK5-specific peptide antibodies were affinity-purified on a Sepharose column containing the covalently coupled peptide. The GRK5 rabbit polyclonal antibody SC-565, generated against amino acids 571–590 of GRK5, was from Santa Cruz Biotechnology, Inc.

Transfected COS-1 cells were harvested by scraping into 0.4 ml of ice-cold 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 200 mM NaCl, 50 mM NaF, 0.8% Triton X-100 with protease inhibitors (5 mM aprotinin, 5 mM benzamidine, 20 μg/ml leupeptin, 2 μM pepstatin A, 1 mM phenylmethylsulfonyl fluoride). Cells were lysed by freeze/thaw and supernatants prepared by centrifugation for 7 min at 100,000 × g. Fifteen μg of total protein were then electrophoresed and subjected to Western blot analysis with the indicated antibodies. Proteins were visualized by ECL (Amersham), while protein concentrations were determined by dye binding assay (Bio-Rad) using bovine serum albumin as a standard.

Metabolically labeled cells were harvested by scraping into 0.2 ml of ice cold 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 200 mM NaCl, 50 mM NaF, 0.1% Triton X-100 with protease inhibitors. Cells were lysed by freeze/thaw and supernatants prepared by centrifugation for 7 min at 100,000 × g. Supernatants were diluted 5-fold with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 20 mM NaF, 0.02% Triton X-100 with protease inhibitors, and enriched in GRK5 by batchwise SP-Sepharose chromatography. SP-Sepharose eluates (150 μl) were added to 300 μl of Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.05% Tween 20) and 30 μl of GST-GRK5 (489–590) antiserum. Where indicated, 100 μg of GST-GRK5 (489–590) was added to block antibody binding to GRK5. Samples were incubated on ice for 15 min, and then 50 μl of a 50% protein A-agarose suspension were added and incubated an additional 30 min. The resin was washed twice with 1 ml of Tris-buffered saline, and labeled proteins were eluted with two 70-μl aliquots of SDS sample buffer heated to 70 °C. Supernatants were combined, and 25-μl aliquots of each sample were electrophoresed on a 10% SDS-polyacrylamide gel. The gel was fixed, soaked in 20% (v/v) PPO in acetic acid, washed with water, dried, and autoradiographed, and the 32P-labeled proteins were excised and counted to determine the picomoles of phosphate transferred.

SP-Sepharose Purification of Phosphorylated GRK5 and Determination of Phosphorylation Sites—Following phosphorylation by PKC, GRK5 was purified by batchwise chromatography on SP-Sepharose. Briefly, phosphorylation reactions were stopped on ice, mixed with an equal volume of 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 100 mM NaCl, 0.4% Triton X-100, and then incubated for 10 min with 50 μl of a 50% suspension of SP-Sepharose in buffer A (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 200 mM NaCl, 0.02% Triton X-100). The resin was pelleted, washed two to three times with 1 ml of buffer A, and the bound kinase was eluted with two 75-μl aliquots of 20 mM Tris-HCl, 1 mM EDTA, 600 mM NaCl, 0.02% Triton X-100. The supernatants were combined, diluted with 150 μl of 20 mM Tris-HCl, pH 8.0, 4 mM MgCl2, 1 mM EDTA, and then used for further analysis. Typically, 70–80% of the GRK5 was recovered by this procedure, while PKC did not bind to SP-Sepharose.

For phosphoamino acid analysis, 300 μl of GRK5-RDD was phosphorylated with 7 μl of PKC in a 120-μl reaction for 15 min and then purified on SP-Sepharose as described above. The stoichiometry of phosphorylation was 2.1 mol of phosphoserine/mol of GRK5-RDD. Fifty μl of the SP-Sepharose eluate was hydrolyzed, dried down in a 200-μl 5% TCI/TCA, and subjected to acid hydrolysis for 2 h at 105 °C. Phosphoamino acids were separated on cellulose plates by two-dimensional TLC using isobutryic acid, 0.5 M ammonium hydroxide (5.3 v/v) in the first dimension, and 2-propanol, HCl, water (7.1:5.1:5 v/v) in the second dimension (29).

To determine the sites of phosphorylation, 3.6 nmol of purified GRK5-(98–136) was phosphorylated with PKC and then purified on SP-Sepharose. The phosphorylated protein was subjected to phosphoamino acid analysis, and 0.05% SDS sample buffer was used instead of the original buffer. The sample was electrophoresed on a 10% SDS-polyacrylamide gel, autoradiographed, and the 32P-labeled amino acids were excised and counted. The molecular weight of the phosphorylated protein was determined by SDS-PAGE on a 10% gel. The phosphorylated protein was excised from the gel, and the amino acid sequence was determined by Edman degradation.

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GRK5-R was incubated with 60 pmol of rat PKC in a 1.2-mL reaction for 15 min and then purified on SP-Sepharose. The stoichiometry of phosphorylation was ~2.7 mol of phosphate/mol of GRK5-R. The eluate from SP-Sepharose was mixed with 400 pmol of 10 mM Tris-HCl, pH 8.0, containing 6 μg of trypsin and then digested at 25 °C for 16 h. The resulting peptides were separated by reverse-phase HPLC on a C18 Vydac column eluted with a 15-mL linear gradient of 4–33% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.5 mL/min. Peptides were detected by absorbance at 220 nm, while radioactivity was determined by Cerenkov counting. This yielded one major peak of radioactivity (90%) eluting at ~10% acetonitrile and one minor peak (10%) at ~23% acetonitrile. The fraction containing the major radioactive peak was diluted to ~5% acetonitrile, 0.1% trifluoroacetic acid, reapplied to the column, and eluted with a 15-mL linear gradient of 7.8–10.6% acetonitrile, 0.1% trifluoroacetic acid. This resulted in the resolution of two peaks of radioactivity, which were collected and then sequenced using a gas-phase amino acid sequencer (Applied Biosystems model 477A).

**Effect of PKC Phosphorylation on GRK Activity**—Sixty pmol of GRK5, GRK5-DD, or βARK were phosphorylated with 5 pmol of bovine PKC in a 40-μL reaction for 10 min and then purified on SP-Sepharose. Aliquots of the phosphorylated kinases before and after SP-Sepharose purification were electrophoresed on an SDS-polyacrylamide gel to enable assessment of autophosphorylation, phosphorylation by PKC, and recovery from SP-Sepharose.

Four-μL aliquots (~0.6 pmol) of the SP-Sepharose-purified GRKs were then assayed by incubating with either ROS membranes (60 pmol of rhodopsin), casein (10 μg), or phosphitin (10 μg) in 20 μL of 20 mM Tris-HCl, pH 8.0, 4 mM MgCl₂, 1 mM EDTA, 0.1 mM [γ-32P]ATP (1000 cpm/pmol) for 6 min at 30 °C in room light. The reactions were stopped with 5 μL of SDS sample buffer, the samples were electrophoresed on a 10% SDS-polyacrylamide gel, gels were stained with Coomassie Blue, dried, and autoradiographed, and the 32P-labeled proteins were excised and counted. To assess the kinetics of receptor phosphorylation, 25–660 pmol of rhodopsin were phosphorylated with control or PKC-phosphorylated GRK5 in the presence or absence of phospholipid vesicles (90%) eluting at ~23% acetonitrile, 0.1% trifluoroacetic acid, reapplied to the column, and eluted with a 15-mL linear gradient of 7.8–10.6% acetonitrile, 0.1% trifluoroacetic acid. This resulted in the resolution of two peaks of radioactivity, which were collected and then sequenced using a gas-phase amino acid sequencer (Applied Biosystems model 477A).

**SRK Binding to Phospholipid Liposomes and ROS Membranes**—The ability of GRK5 to associate with phospholipids and rhodopsin was analyzed by incubating 8-μL aliquots (~1.2 pmol) of SP-Sepharose-purified 32P-labeled GRK5 in the presence or absence of phospholipid liposomes (30% RO membranes (250 pmol of rhodopsin) in 60 μL of 20 mM Tris-HCl, pH 8.0, 2 mM MgCl₂, 80 mM NaCl at 30 °C for 5 min in room light. The incubations were then pelleted at 100,000 × g for 6 min, the pellets were resuspended in 60 μL of reaction buffer, and equal aliquots of the supernatant and pellet fractions were electrophoresed on a 10% SDS-polyacrylamide gel. The gels were dried, autoradiographed, and the 32P-labeled proteins were excised and counted.

**RESULTS AND DISCUSSION**

**PKC Phosphorylates GRK5 in Vitro**—Previous studies have demonstrated that GRK5 is rapidly autophosphorylated in a phospholipid-dependent manner (12). GRK5 autophosphorylation results in the appearance of two slower moving forms of GRK5 when analyzed by SDS-PAGE (Fig. 1A, wt, 10 and 30 min). This transition of GRK5 into slower moving forms was not observed in the absence of ATP and paralleled the increased incorporation of radioactivity into the kinase when incubated with [γ-32P]ATP (Fig. 1B). Since the major sites of GRK5 autophosphorylation were previously identified as Ser484 and Thr485 (12, 13), phosphorylation of one of these residues likely causes a shift in the electrophoretic mobility of GRK5 while incorporation of a second phosphate creates an even slower moving form of the kinase.

Since GRK5 autophosphorylation might make it difficult to assess the ability of GRK5 to serve as a substrate for other protein kinases, we generated several mutants, which would have a reduced ability to autophosphorylate. These mutants were expressed in Sf9 cells using the baculovirus system and purified to >95% homogeneity (Fig. 1A). One of these mutants targeted a lysine residue (Lys215 in GRK5) that is conserved in all protein kinases and appears to be involved in the phosphate transfer reaction (31). Mutation of this conserved lysine into arginine generally results in a protein kinase with significantly reduced catalytic activity. The corresponding K215R mutation in GRK5 yielded a protein (GRK5-R) that had ~100–200-fold reduced ability to phosphorylate activated rhodopsin and β2-adrenergic receptor compared to wild type GRK5 (data not shown). Similarly, GRK5-R was reduced ~100–200-fold in autophosphorylation (Fig. 2).

Replacement of Ser484 and Thr485 in the autophosphorylation site with alanine leads to a significant reduction in the ability of the protein to phosphorylate rhodopsin and β2-adrenergic receptor (12). Here we generated a mutant with Ser484D and Thr485D replaced by negatively charged aspartate residues, which we expected would mimic autophosphorylation of GRK5. Indeed, the corresponding mutant protein (GRK5-DD) showed a significant reduction in autophosphorylation (Fig. 1B) with no loss in its ability to phosphorylate rhodopsin compared to wild type GRK5. We also generated a mutant with both the catalytic site (K215R) and the autophosphorylation site (S484D and T485D) mutated. Similar to GRK5-R, this mutant (GRK5-DD) had no significant catalytic activity (data not shown) and was not autophosphorylated (Fig. 2A). Interestingly, the introduction of negative charges into the autophosphorylation site also resulted in a reduced electrophoretic mobility of both GRK5-DD and GRK5-RDD compared to wild type GRK5 (Fig. 1A).

To test whether GRK5 serves as a substrate for PKC, we initially examined the ability of purified PKCs to phosphorylate wild type GRK5 or K215R (GRK5-R), K215R/S484D/T485D (GRK5-RDD), and S484D/T485D (GRK5-DD) mutant GRK5 were electrophoresed on a 10% SDS-polyacrylamide gel and visualized by Coomassie Blue staining. Wild type GRK5 and GRK5-DD were incubated with [γ-32P]ATP for 0, 10, or 30 min before electrophoresis as described under "Experimental Procedures." The positions of faster (f) and slower (s) moving forms of the proteins are indicated on the left, while the molecular sizes of protein standards are shown in kilodaltons on the right. B, autoradiogram of the same gel.

**FIG. 1.** Autophosphorylation of recombinant wild type and mutant GRK5 proteins. A, 2 μg each of purified Sf9 cell-expressed wild type GRK5 or K215R (GRK5-R), K215R/S484D/T485D (GRK5-RDD), and S484D/T485D (GRK5-DD) mutant GRK5 were electrophoresed on a 10% SDS-polyacrylamide gel and visualized by Coomassie Blue staining. Wild type GRK5 and GRK5-DD were incubated with [γ-32P]ATP for 0, 10, or 30 min before electrophoresis as described under "Experimental Procedures." The positions of faster (f) and slower (s) moving forms of the proteins are indicated on the left, while the molecular sizes of protein standards are shown in kilodaltons on the right. B, autoradiogram of the same gel.
Regulation of GRK5 by Protein Kinase C

FIG. 2. PKC phosphorylation of purified GRK5 proteins. A, 20 pmol of wild type or mutant GRK5 were phosphorylated in the absence (−) or presence (+) of 1 pmol of PKCα for 6 min at 30 °C as described under “Experimental Procedures.” The proteins were electrophoresed on a 10% SDS-polyacrylamide gel and visualized by autoradiography. The positions of GRK5 and PKC are indicated on the right. 32P incorporation into proteins was determined by excising and counting the radioactive bands. Phosphorylation stoichiometries of GRK5, GRK5-R, GRK5-RDD, and GRK5-DD were 1.1, 7.9 × 10−3, 2.5 × 10−3, and 0.08 mol/mol, in the absence, and 2.8, 2.2, 1.4, and 1.6 mol/mol in the presence of PKC, respectively. B, time course of GRK5-R (○) and GRK5-RDD (●) phosphorylation by PKC. 20 pmol of purified GRK5-R or GRK5-RDD were incubated with 1 pmol of PKCs for the indicated period of time at 30 °C, electrophoresed on a 10% SDS-polyacrylamide gel, and visualized by autoradiography. The stoichiometry of GRK5 phosphorylation was determined by excising and counting the radioactive bands. C, effect of PKC concentration on phosphorylation of GRK5-R (○) and GRK5-RDD (●). 20 pmol (1 μM) of purified GRK5-R or GRK5-RDD were phosphorylated with the indicated concentrations of PKCα for 3 min at 30 °C and then processed as described above.

FIG. 3. Expression of GRK5 in transiently transfected COS-1 cells. A, COS-1 cells were transiently transfected with the vector pBC12BI (vect) or the expression constructs pBC-GRK5 and pBC-GRK5-R using LipofectAMINE. The cells were harvested 48 h after transfection, lysed, and centrifuged for 7 min at 100,000 × g. 15 μg of supernatant protein were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and then immunoblotted using a rabbit polyclonal antiserum generated against the fusion protein GST-GRK5(489–590). 12 ng of purified recombinant GRK5 (std) was loaded in the left lane. Positions of faster (f) and slower (s) moving forms of the proteins are indicated on the right. B, COS-1 cells, transiently transfected with vector or pBC-GRK5, were metabolically labeled 48 h after transfection with either [35S]Met or [32P]P, for 2.5 h as described under “Experimental Procedures.” Cells were then treated for 30 min in the presence or absence of 0.2 μM PMA, washed with PBS, lysed, centrifuged, and immunoprecipitated with the polyclonal GRK5 antiserum. Where indicated immunoprecipitation was done in the presence of 100 μg of GST-GRK5(489–590) (FP). Immunoprecipitated radioactive proteins were separated on a 10% SDS-polyacrylamide gel, and the gel was then fixed, soaked in 20% PPO in acetic acid, dried, and autoradiographed.

and short incubation times, PKC showed little discrimination between GRK5-R and GRK5-RDD (Fig. 2C), suggesting that Ser484 and/or Thr485 are most likely not the primary phosphorylation targets of PKC. Kinetic studies reveal a $K_m = 0.6$ μM and $V_{max} = 60$ nmol phosphate/min/mg for both GRK5-R and GRK5-RDD in the absence of PKC activators, while a $K_m = 2$ μM and $V_{max} = 900$ nmol phosphate/min/mg were obtained in the presence of PKC activators. These kinetics are comparable to those of a number of well established PKC substrates (32) and demonstrate that GRK5 is a good substrate for PKC in vitro.

Phosphorylation of GRK5 in COS-1 Cells—Since GRK5 is a good substrate for PKC in vitro, we next wanted to assess whether GRK5 could also serve as a substrate for PKC in intact cells. COS-1 cells, either untransfected or transfected with the vector pBC12BI, contain undetectable levels of GRK5 as assessed by immunoblotting (Fig. 3A, lane 2). When transfected with the expression construct pBC-GRK5, GRK5 was expressed at a high level (~3 μg/mg) and exhibited a broad immunostaining pattern suggestive of a heterogeneous state of autophosphorylation (lane 3). In contrast, GRK5-R was expressed at a much lower level (~0.5 μg/mg) (lane 4), precluding us from further studying this construct in COS-1 cells.

To determine whether GRK5 is phosphorylated by PKC in COS-1 cells, cells transfected with vector alone or with wild type GRK5 were metabolically labeled with either [35S]methionine or [32P]P. The labeled cells were then treated with the PKC activator PMA for 30 min, and GRK5 was immunoprecipitated using an anti-GRK5 rabbit polyclonal antibody (12). No [35S]- or [32P]-labeled proteins were immunoprecipitated from COS-1 cells transfected with vector alone (Fig. 3B, lanes 1 and 5). However, in cells transfected with wild type GRK5, two or three [35S]-labeled proteins were immunoprecipitated (lane 2). Immunoprecipitation of these proteins was completely blocked by the GRK5 fusion protein used to generate the antibodies (lane 4). The pattern of [35S]-labeled bands observed in the absence of PMA treatment suggests that 60–70% of the GRK5 was autophosphorylated (slower moving form of the kinase), while the remainder has the same mobility as purified recombinant GRK5 and likely corresponds to GRK5 that is not autophosphorylated.
only the slower moving band was radiolabeled (Fig. 3B, lane 6). Since autophosphorylation appears to activate GRK5, the high basal autophosphorylation level implies that most of the GRK5 expressed in COS-1 cells is in an activated state.

When the cells were treated with PMA, a significant change in the electrophoretic mobility of GRK5 was apparent with a noticeable decrease in the amount of slower moving and corresponding increase in the faster moving form (Fig. 3B, compare lanes 2 and 3). Since autophosphorylation decreases the electrophoretic mobility of GRK5, this suggests that PMA treatment may reduce the amount of autophosphorylated GRK5. Direct evidence for PKC phosphorylation of GRK5 in COS-1 cells was assessed by immunoprecipitation of the 32P-labeled proteins following treatment with PMA. These studies show that the total amount of immunoprecipitated 32P-labeled GRK5 was increased significantly (180 ± 20% compared to untreated cells, n = 3). Since phosphorylation of both the slower and faster moving forms of GRK5 was observed following PMA treatment (Fig. 3B, lane 7), this suggests that PKC phosphorylation does not alter the electrophoretic mobility of GRK5 and that Ser484 and Thr485 are not the primary sites of PKC phosphorylation. Thus, under basal conditions 60–70% of the GRK5 appears to be stoichiometrically autophosphorylated, whereas following PMA treatment ~30% of the GRK5 is auto-phosphorylated while ~70% is stoichiometrically phosphorylated, most likely by PKC.

Identification of the Sites of PKC Phosphorylation—In vitro and intact cell studies suggest that while PKC can phosphorylate Ser484 and/or Thr485, the primary sites of phosphorylation likely reside elsewhere in GRK5. Since GRK5-RDD can be phosphorylated by PKC to a stoichiometry of ~2 mol of phosphate/mol of GRK5-RDD (Fig. 2B), this suggests a minimum of two primary sites of PKC phosphorylation. Because the C-terminal tail of GRK5 is rich in serine, threonine, and basic residues, we tested whether a GST fusion protein containing the C-terminal 102 amino acids of GRK5 (GST-GRK5-(489–590)) could also serve as a substrate for PKC. Indeed, GST-GRK5-(489–590) was phosphorylated by PKC to a stoichiometry of ~1–1.5 mol of phosphate/mol of fusion protein, while GST itself was not a substrate for PKC (Fig. 4A). Thus, one or two potential PKC phosphorylation sites lie within the C-terminal 102 amino acids of GRK5.

We next tested the ability of control and PKC-phosphorylated GRK5-R to be immunoblotted by antibodies targeted to different regions of GRK5. Both control and PKC-phosphorylated GRK5-R were comparably recognized by antibodies generated against either residues 98–136 or residues 489–590 of GRK5 (Fig. 4B, lanes 1–4). However, an antibody generated against residues 556–571 of GRK5 clearly immunoblotted the nonphosphorylated form of GRK5-R, but it did not recognize PKC-phosphorylated GRK5-R (lanes 5 and 6). This suggests that PKC likely phosphorylates GRK5 within the 16 amino acid antibody epitope KRPQSNNKSSPSK. The binding of an antibody generated against residues 571–590 of GRK5 was also reduced after phosphorylation by PKC, although it was still detectable (lanes 7 and 8). This domain is also serine- and threonine-rich, although there is only one basic residue nearby (Lys387).

Phosphoamino acid analysis of PKC-phosphorylated GRK5-RDD revealed that most of the radioactivity was incorporated into phosphoserine (83 ± 2%) with a small amount in phosphothreonine (17 ± 2%) (data not shown). To further define the PKC-phosphorylation sites on GRK5, PKC-phosphorylated 32P-labeled GRK5-R was digested with trypsin and chromatographed by reverse phase HPLC. This yielded one major (~90%) and one minor (~10%) peak of radioactivity. When the major peak was rechromatographed and eluted with a shallower gradient, two separate peaks of radioactivity were observed. The peaks were collected and subjected to gas phase sequencing. The first peak yielded the sequence of the extreme C-terminus of GRK5 (Thr590-Ser506) (Fig. 4C). This peak contains 7 serine and 2 threonine residues and represents the same region that was used to generate the antibodies whose recognition of GRK5 was reduced by PKC phosphorylation (Fig. 4B, lanes 7 and 8). The second radioactive peak yielded the sequence SSPSSK (residues 565–570) (Fig. 4C). This region is part of the peptide (Lys556-Thr571) used to generate the antibodies whose binding was completely blocked by PKC phosphorylation (Fig. 4B, lanes 5 and 6). These results demonstrate that at least two PKC phosphorylation sites lie within the C-terminal 26 residues of GRK5. Since both of these peptides contain multiple serine and/or threonine residues, we cannot determine the exact residues phosphorylated by PKC. However, since there is a requirement for basic residues to serve as a PKC substrate (33) and phosphoserine is the predominant amino acid in GRK5 phosphorylated by PKC, we envision the most likely sites to be Ser572 and either Ser566 or Ser568. Of note is the fact that Ser572 and Ser566 are conserved in human (24), bovine (13), and rat (34) GRK5. However, it is also possible that PKC has little preference for specific residues within this C-terminal domain, and that the phosphorylation of any residue would have a similar consequence. For example, PKC can phosphorylate three different serine residues within pleckstrin, but phosphorylation of any two of the three has the same effect on pleckstrin’s ability to inhibit phosphoinositide hydrolysis (35).

Effect of PKC Phosphorylation on GRK5 Activity—Since GRK5 is rapidly and stoichiometrically phosphorylated by PKC in vitro, we next tested the effect of PKC phosphorylation on GRK5 activity. Since βARK has been demonstrated to be phosphorylated and activated by PKC (21, 22), it was used as a control in this series of studies. βARK and GRK5 were phosphorylated by PKC, purified on SP-Sepharose, and then assayed for their ability to phosphorylate light activated rhodop-
ability of kinase. Thus, as might be expected, no significant change in the
by PKC to a stoichiometry of 5 mol of phosphate/mol of kinase, demonstrating that GRK5 is a
much better substrate for PKC than is GRK5-DD (phosphorylated to a stoichiometry of
2.4 mol of phosphate/mol of kinase), demonstrating again that the reduced catalytic activity is not simply due to reduced autophosphorylation of GRK5. In contrast, there was no change in the
$K_m$ for ATP for PKC-phosphorylated and control GRK5 (data not shown). Thus, these results demonstrate that PKC phosphorylation reduces the catalytic activity of GRK5 toward both receptor and non-receptor substrates. This is in contrast to βARK, where PKC phosphorylation results in an 1.6-fold reduction in the ability of βARK to phosphorylate a soluble peptide substrate (22) but a 2–3-fold increase in rhodopsin phosphorylation (21, 22).

Previous studies have demonstrated that GRK5 can bind directly to phospholipid membranes (12) as well as to ROS membranes in a light-independent fashion (13). Here we tested the effect of PKC phosphorylation on GRK5 binding to phospholipid vesicles and ROS membranes. A small amount of GRK5 was pelleted when centrifuged alone, while the addition of phospholipid vesicles significantly increased the amount of pelleted GRK5 (from 16% to 36%) (Fig. 6A). Incubation of GRK5 with ROS membranes resulted in an even larger increase in the amount of pelleted GRK5, probably due to direct binding to rhodopsin (to 62%). PKC phosphorylation of GRK5 did not change its binding to phospholipid vesicles (33% versus 36%); however, a significant decrease in binding to ROS membranes was observed (25% versus 62%) (Fig. 6B). Thus, PKC phosphorylation of GRK5 does not affect the ability of the kinase to bind to phospholipid vesicles, but it does significantly reduce the direct binding of the kinase to receptor. Again this is in contrast to βARK, where it has been suggested that the enhancement in rhodopsin phosphorylation was the result of increased membrane binding of βARK (22), although it is not clear whether this binding was to phospholipids or to the receptor itself.

Conclusions—In summary, our findings suggest that PKC phosphorylation may serve as a general mechanism for regulating GRK function. PKC rapidly and stoichiometrically phosphorylates GRK5 resulting in a significant reduction in GRK5 activity. This is in striking contrast with the ability of PKC to phosphorylate and activate βARK (20, 21). The differential regulation of βARK and GRK5 by PKC suggests that GRK diversity may play an important role in the differential desensitization of various receptors. GRK expression levels and sub-
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type may also prove critical in determining how rapidly receptor signaling is attenuated, in particular for those receptors coupled to phospholipase C stimulation. For example, myocardial β1ARK and GRK5 appear to play an important role in regulating β1AR function, which is coupled to stimulation of cAMP production (36, 37). In contrast, the myocardial type 1A angiotensin II receptor, which stimulates phospholipase C and consequently PKC, appears to be regulated by βARK but not by GRK5. Similarly, recent studies have demonstrated that coexpression of either the type 1A angiotensin II or GRK5. Similarly, recent studies have demonstrated that coexpression of either the type 1A angiotensin II or GRK5 results in enhanced basal phosphorylation but no significant agonist-induced phosphorylation of these receptors (38, 39). Perhaps PKC activation by either angiotensin II or βARK-adrenergic receptor stimulation may turn off GRK5 activity.

βARK appears to be the predominant GRK in many cells (40, 41). However, mRNA distribution reveals that GRK5 is particularly high in lung, heart, skeletal muscle, placenta, colon, and retina (13, 24, 34). Moreover, Nagayama et al. (34) recently showed that GRK5 is the predominant GRK in rat thyroid FRTL5 cells and is involved in desensitization of the thyrotropin receptor. Interestingly, overexpression of GRK6 in these cells has the same effect as GRK5, but FRTL5 expresses little if any GRK6. Thus, the expression of GRK5 in these cells is determined by reasons other than a simple requirement for thyrotropin receptor desensitization. We speculate that the expression of a particular GRK in a given cell is determined by a need to integrate regulation of receptor desensitization in the cell with other signals. In cells that express multiple GRKs, the relative contribution of each GRK in the desensitization of a particular receptor may well depend on the presence of other stimuli. Because PKC activates βARK and inhibits GRK5, stimulation of PKC would favor signals regulated by GRK5 in such cells. Thus, phosphorylation by PKC may play an important role in the heterologous regulation of GRK activity.

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