Regulation of G Protein-coupled Receptor Kinases by Calmodulin and Localization of the Calmodulin Binding Domain*

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G protein-coupled receptor kinases (GRKs) specifically phosphorylate and regulate the activated form of multiple G protein-coupled receptors. Recent studies have revealed that GRKs are also subject to regulation. In this regard, GRK2 and GRK5 can be phosphorylated and either activated or inhibited, respectively, by protein kinase C. Here we demonstrate that calmodulin, another mediator of calcium signaling, is a potent inhibitor of GRK activity with a selectivity for GRK5 (IC$_50$ ~ 50 nM) > GRK6 >> GRK2 (IC$_50$ ~ 2 μM) >> GRK1. Calmodulin inhibition of GRK5 is mediated via a reduced ability of the kinase to bind to both receptor and phospholipid. Interestingly, calmodulin also activates autophosphorylation of GRK5 at sites distinct from the two major autophosphorylation sites on GRK5. Moreover, calmodulin-stimulated autophosphorylation directly inhibits GRK5 interaction with receptor even in the absence of calmodulin. Using glutathione S-transferase-GRK5 fusion proteins either to inhibit calmodulin-stimulated autophosphorylation or to bind directly to calmodulin, we determined that an amino-terminal domain of GRK5 (amino acids 20–39) is sufficient for calmodulin binding. This domain is abundant in basic and hydrophobic residues, characteristics typical of calmodulin binding sites, and is highly conserved in GRK4, GRK5, and GRK6. These studies suggest that calmodulin may serve a general role in mediating calcium-dependent regulation of GRK activity.

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The abbreviations used are: GRKs, G protein-coupled receptor kinase(s); PKC, protein kinase C; ROS, rod outer segments; GST, glutathione S-transferase; SPR, surface plasmon resonance; CaM, calmodulin; MARCKS, myristoylated alanine-rich protein kinase C substrate.

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suggest that calmodulin may play an important role in regulating GRK function in a subtype-specific manner.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, Vent DNA polymerase, and other molecular biology reagents were purchased from New England Biolabs or Boehringer Mannheim. SP Sepharose was obtained from Pharmacia Biotech Inc., Calmodulin (bovine brain, >98% pure), calmodulin-agarose, and phosphatidylcholine (soybean type II-S) were from Avanti Polar Lipids, Inc. Rat PKC-α and bovine GRK1, overexpressed and purified from SF9 cells, were generous gifts from Dr. C. Stubbs and Drs. R. J. Lefkowitz and J. A. Pitcher, respectively. All other materials were from sources previously described (17).

Transfection of COS-1 Cells and Assessment of GRK Activity—Expression plasmids for GRKs were constructed by cloning the coding sequences of bovine GRK2 (20) and human GRK5 and GRK6 in the expression plasmids for GRKs were constructed by cloning the coding sequences of bovine GRK2 (20) and human GRK5 and GRK6 in the vector pBC12Bl (21). COS-1 cells were grown to ~80–90% confluence in 60-mm dishes at 37 °C in a humidified atmosphere containing 5% CO2, 95% air in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected with 4 μg of DNA using LipofectAMINE following the manufacturer’s instructions. Forty-eight hours after transfection, cells were harvested and lysed by scraping into 1 ml of ice-cold 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 200 mM NaCl, 1% Triton X-100 with protease inhibitors (5 μM aprotinin, 5 mM benzamidine, 20 mM leupeptin, 2 mM pepstatin A, 1 mM phenylmethylsulfonyl fluoride) and supernatants were prepared by centrifugation for 7 min at 100,000 × g (4°C). GRKs were then partially purified by chromatography on SP Sepharose as described (17).

Four-μl aliquots of the partially purified GRKs were then assayed by incubating with rod outer segment (ROS) membranes (100 pmol of rhodopsin) in 20 μl of 20 mM Tris-HCl, pH 8.0, 4 mM MgCl2, 0.1 mM CaCl2, 0.1 mM [γ-32P]ATP (1,000 cpm/pmol) in the presence of the indicated concentration of calmodulin for 6 min at 30 °C in room light. The reactions were stopped with 200 μl of ice-cold buffer (20 mM Tris-HCl, pH 8.0, 4 mM EDTA, 100 mM NaCl) and centrifugation for 10 min at 100,000 rpm (4°C). Pellets containing phosphorylated rhodopsin were dissolved in SDS loading buffer, and the samples were then electrophoresed on a 10% SDS-polyacrylamide gel (22). Gels were stained with Coomassie Blue, dried, and autoradiographed, and the 32P-labeled proteins were excised and counted to determine the pmol of phosphate transferred. Urea-treated ROS membranes containing rhodopsin or phosvitin (10 μg) in 20 μl of 20 mM Tris-HCl, pH 8.0, 4 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 0.1 mM [γ-32P]ATP (1,000 cpm/pmol) for 6 min at 30 °C in room light. When the effect of calmodulin was tested the reactions also included the indicated concentration of calmodulin and 0.2 mM CaCl2 (with or without EGTA) and were incubated for 2 min at 30 °C. 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 was phosphorylated with GRK5 autophosphorylated in the presence or absence of calmodulin in 20 mM Tris-HCl, pH 8.0, 4 mM MgCl2, 1 mM EDTA, 0.1 mM [γ-32P]ATP (6,000 cpm/pmol). Kin and V max values were derived from double-reciprocal plots of the data.

Effect of GRK5 Phosphorylation on Kinase Activity—Sixty pmol of GRK5 was autophosphorylated in a 40-μl reaction at 30 °C for 15 min in the presence or absence of either 3 μM calmodulin or 0.07 μM PKC, 1 μM phorbol 12-myristate 13-acetate, and 0.85 mg/ml phospholipid vesicles as described above and then purified by batchwise chromatography on SP Sepharose. Briefly, phosphorylation reactions were stopped on ice mixed with 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 2 mM EGTA, 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, 1 mM EGTA, 50 mM NaCl, 0.02% Triton X-100). The resin was pelleted, washed two or three times with 1 ml of buffer A, and the bound kinase was eluted with two 75-μl aliquots of 20 mM Tris-HCl, 0.1 mM EDTA, 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. Aliquots of the phosphorylated kinase 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. Typically, 70–80% of the GRK5 was recovered by this procedure, whereas PKC and calmodulin did not bind to SP Sepharose. Aliquots (~20 ng) of the SP Sepharose-purified GRK5 were also electrophoresed and subjected to Western blot analysis using antibodies raised against either amino acids 556–571 or 489–590 of human GRK5 as described (17).

Four-μl aliquots (~0.6 pmol) of the SP Sepharose-purified GRK5 were incubated at 30 °C with either ROS membranes (100 pmol of rhodopsin) or phosvitin (10 μg) in 20 μl of 20 mM Tris-HCl, pH 8.0, 4 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 0.1 mM [γ-32P]ATP (1,000 cpm/pmol) for 6 min at 30 °C in room light. When the effect of calmodulin was tested the reactions also included the indicated concentration of calmodulin and 0.2 mM CaCl2 (with or without EGTA) and were incubated for 2 min at 30 °C. 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 was phosphorylated with GRK5 autophosphorylated in the presence or absence of calmodulin in 20 mM Tris-HCl, pH 8.0, 4 mM MgCl2, 1 mM EDTA, 0.1 mM [γ-32P]ATP (6,000 cpm/pmol). Kin and V max values were derived from double-reciprocal plots of the data.

GRK5 Binding to Phospholipid Vesicles and ROS Membranes—The ability of GRK5 to associate with either receptor or phospholipid was analyzed by incubating 8-μl aliquots (~1.2 pmol) of SP Sepharose-purified 32P-labeled autophosphorylated GRK5 in the presence or absence of the indicated concentration of phospholipid vesicles or ROS membranes (250 pmol of rhodopsin) in 60 μl of 20 mM Tris-HCl, pH 8.0, 2 mM MgCl2, 0.1 mM CaCl2, 80 mM NaCl, 0.1 mg/ml ovalbumin, and the indicated concentration of calmodulin at 30 °C for 5 min in room light. The samples were centrifuged at 100,000 rpm 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. Pelleted GRK5 was expressed as a percentage of the total after subtracting the amount of GRK5 pelleted in the absence of phospholipids or ROS (~10–15%).

Phospholipid vesicles were prepared by sonicating 76 mg of phosphatidylcholine and 9 mg of phosphatidylserine in 5 ml of 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1 mM EDTA on ice four times for 20 s.

Expression and Purification of Glutathione S-Transferase (GST) Fusion Proteins—DNA sequences coding for various regions of GRK5 were generated using the polymerase chain reaction and then used to replace a BamHI/SalI fragment in the vector pGEX-4T-2 (Pharmacia). The polymerase chain reaction-derived portions of the constructs were sequenced in their entirety using the dyeodeoxy chain termination method. The GST-GRK5 fusion proteins were expressed in Escherichia coli and purified over glutathione-agarose using standard procedures (25). The purity of the proteins was >95% as determined by Coomassie Blue staining. Protein concentrations were determined by dye binding assay (Bio-Rad) using bovine serum albumin as a standard.

Interaction of GST Fusion Proteins with Calmodulin—To assess the ability of GST fusion proteins to block calmodulin-mediated activation of GRK5, 4 pmol of GRK5-DD was autophosphorylated in the presence of 0.07 μM calmodulin and the indicated fusion protein in the absence or presence of 0.1 μM calmodulin. Reactions were processed by gel electrophoresis as described above, and the level of autophosphorylation was determined by excising and counting the 32P-labeled bands. None of the GST fusion proteins significantly affected the basal autophosphorylation of GRK5-DD.

The binding of GRK5 and GST-GRK5 fusion proteins to calmodulin-
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Effect of Calmodulin on GRK Activity—In an effort to elucidate further the potential role of calcium in regulating GRK function, we tested whether calmodulin could modulate the activity of various GRKs. Our initial studies compared the effect of calmodulin on COS-1 cell overexpressed preparations of GRK2, GRK5, and GRK6 to phosphorylate light-activated rhodopsin. Protein extracts from control COS-1 cells displayed very low rhodopsin phosphorylation activity, whereas cells transfected with GRK2, GRK5, or GRK6 expression constructs had a much higher level of phosphorylation (Fig. 1A). In the presence of calmodulin the phosphorylation of rhodopsin by GRK5 and GRK6 was significantly inhibited with IC_{50} values of $-0.25 \mu M$ for GRK5 and $-0.7 \mu M$ for GRK6 (Fig. 1B). In contrast, GRK2 was inhibited only at the highest concentration of calmodulin tested (IC_{50} > 3 \mu M). Thus, although all three GRKs tested were inhibited by calmodulin, GRK2 was much less sensitive than GRK5 and GRK6.

Because the COS-1 cell extracts contain many other proteins that could potentially influence the assay, we also studied the effect of calmodulin on purified GRKs. Calmodulin effectively inhibited the ability of GRK5 to phosphorylate rhodopsin with an IC_{50} = 50 \mu M (Fig. 2). Calmodulin also inhibited the activity of GRK2, although much less effectively (IC_{50} = 2 \mu M) than GRK5, whereas the activity of GRK1 was only modestly inhibited (IC_{50} = 10 \mu M calmodulin). The effect of calmodulin on GRK5 was also completely dependent on the presence of Ca^{2+} (data not shown). The higher IC_{50} values observed for the COS-expressed GRKs versus the purified GRKs might be because of the presence of additional calmodulin-binding proteins in the crude preparations which could bind calmodulin and reduce its effective concentration.

The high sensitivity of GRK5 to inhibition by both Ca^{2+} and calmodulin and PKC (17) strongly suggests that GRK5 will not be involved in regulating receptors coupled to G_{q/11} and phospholipase C since these receptors promote increased free calcium levels when activated, presumably leading to inhibition of GRK5. Thus, even if GRK5 can phosphorylate such receptors in vitro, it is unlikely that this would occur in intact cells. This may explain why coexpression of \alpha_{1p}-adrenergic receptors with GRK5 results in enhanced basal phosphorylation but no significant agonist-induced phosphorylation of the receptor (26). Similarly, recent studies have demonstrated that although GRK5 can phosphorylate myocardial type 1A angiotensin II receptors in vitro (27), desensitization of this receptor in transgenic mice overexpressing GRK5 was not affected (28). In contrast, the lower affinity of calmodulin for GRK2 suggests that it would not be regulated by calmodulin in most cells, although calmodulin levels in brain are high (1–10 \mu M) (18).

Since GRK5 was more sensitive to inhibition by calmodulin than the other GRKs, we focused the remainder of the study on the GRK5/calmodulin interaction. To assess further the effect of calmodulin on the activity of GRK5, we utilized soluble substrates such as casein and phosvitin. Although phosphorylation of casein by GRK5 was not altered by calmodulin (data not shown), GRK5 phosphorylation of phosvitin was inhibited with an IC_{50} = 0.6 \mu M (Fig. 2C). The inhibition of phosvitin phosphorylation suggests that calmodulin interacts with regions of GRK5 which are likely involved in substrate binding. However, the 10-fold reduced sensitivity of inhibition of phosvitin phosphorylation by calmodulin relative to rhodopsin phos-
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Figure 2. Effect of calmodulin on purified GRK activity. Panel A, 0.8 pmol of purified Sf9 cell-expressed GRK1, GRK2, or GRK5 was used to phosphorylate ROS membranes (4 μM rhodopsin) in the presence of the indicated concentrations of calmodulin. Proteins were separated on a 10% SDS-polyacrylamide gel and visualized by autoradiography. Panel B, 32P incorporation into proteins was determined by excising and counting the radioactive bands. The activity of GRK1 (●), GRK2 (○), or GRK5 (●) in the presence of calmodulin is expressed as a percentage of the rhodopsin phosphorylation in the absence of calmodulin. Panel C, 10 μg of phosvitin was phosphorylated with 0.8 pmol of GRK5 in the presence of the indicated concentrations of calmodulin. Proteins were separated on a 10% SDS-polyacrylamide gel and visualized by autoradiography.

Effect of Calmodulin on GRK5 Binding to Membranes—Unlike the other GRKs that utilize either covalent lipid modifications (GRK1, 4, and 6) or interaction with G protein subunits (GRK2 and 3) to enhance binding to phospholipid membranes, GRK5 appears to interact directly with phospholipids via regions rich in basic amino acids. GRK5 displays significant association with either phospholipid vesicles or with rhodopsin-containing ROS membranes (11, 17, 29). When tested in a direct binding assay, calmodulin was found to inhibit GRK5 binding to ROS membranes significantly with an IC50 of 0.3–0.4 μM (Fig. 3 A and B). However, this IC50 was some 6–8-fold higher than the IC50 for inhibition of rhodopsin phosphorylation. Indeed, at the highest calmodulin concentration tested, ~20% of the kinase remained bound to the ROS membranes even though rhodopsin phosphorylation was reduced >99% (compare Figs. 2B and 3B). These results suggest that calmodulin can directly inhibit GRK5 interaction with receptor. The binding of GRK5 to phospholipid vesicles was also inhibited by calmodulin (Fig. 3 C and D). However, although this inhibition was substantial at relatively low lipid concentrations (0.017 mg/ml), it could be largely overcome at higher phospholipid (0.85 mg/ml). These results imply a competitive type of inhibition and taken together with the rhodopsin studies suggest that calmodulin can directly compete for both the lipid and receptor binding sites of GRK5.

Calmodulin Activates GRK5 Autophosphorylation—GRK5 appears to be activated via a rapid phospholipid-stimulated autophosphorylation at residues Ser484 and Thr485 (11, 29). To our surprise calmodulin significantly enhanced the autophosphorylation of GRK5 (Fig. 4A). In an attempt to further characterize this finding we studied the effect of calmodulin on the autophosphorylation-defective mutant GRK5-DD, which has both Ser484 and Thr485 mutated to aspartate (17). Although autophosphorylation of GRK5-DD was not enhanced by phospholipids, calmodulin still significantly enhanced the autophosphorylation with an overall increase comparable to that seen for wild type GRK5. These data indicate that interaction with calmodulin results in increased autophosphorylation of GRK5 at sites distinct from Ser and Thr. Interestingly, calmodulin also significantly activates autophosphorylation of GRK6, but it has no effect on the autophosphorylation of GRK1 and GRK2 (data not shown).

The EC50 for calmodulin activation of GRK5-DD autophosphorylation was ~40 nM (Fig. 4B), very similar to the IC50 for calmodulin inhibition of rhodopsin phosphorylation. Kinetic studies reveal that calmodulin increases the Vmax of autophosphorylation (by ~8-fold), and the affinity for ATP (Km) is reduced from ~17 to ~5 μM (Fig. 4C). To characterize initially...
the potential role of calmodulin-stimulated autophosphorylation in inhibition of GRK5 we performed time course experiments. These studies reveal that after 1 min, rhodopsin phosphorylation was inhibited 95–96% (−20-fold), whereas the stoichiometry of GRK5 autophosphorylation was only 0.15 mol/mol (Fig. 4D). Moreover, as GRK5 autophosphorylation increased from 0.15 to 0.72 mol/mol, the fold inhibition of rhodopsin phosphorylation remained unchanged. These results suggest that in the presence of calmodulin, autophosphorylation does not appear to play a major role in inhibition of GRK5 activity.

To characterize directly the effect of calmodulin-stimulated autophosphorylation on GRK5 activity, GRK5 was incubated with ATP in the presence or absence of calmodulin or PKC, purified by SP Sepharose chromatography, and then assayed. Whereas phosphorylation of phosvitin by PKC-phosphorylated GRK5 was inhibited 3–4-fold with wild type GRK5, phosvitin phosphorylation by GRK5 autophosphorylated in the presence of calmodulin was slightly increased (27 ± 5%) (Fig. 5A). In contrast, GRK5 phosphorylation of rhodopsin was inhibited −6–7-fold by either PKC phosphorylation or calmodulin-stimulated autophosphorylation. To determine whether the reduced receptor phosphorylation by autophosphorylated GRK5 was due to a loss in either phospholipid or receptor binding, direct binding to phospholipid vesicles and ROS was tested. GRK5 association with phospholipids was not affected by calmodulin-stimulated autophosphorylation (57% bound with wild type GRK5 versus 59% with autophosphorylated GRK5; compare Figs. 3D and 5B). In contrast, calmodulin-stimulated autophosphorylation dramatically reduced GRK5 binding to ROS (from 57 to 12%, compare Figs. 3A and 5B). In addition, kinetic analysis revealed that autophosphorylated GRK5 had a −6-fold increased K_\text{m} and a ∼2-fold reduced V_\text{max} for rhodopsin phosphorylation compared with wild type GRK5 (data not shown). Taken together, these data suggest that calmodulin-stimulated autophosphorylation dramatically affects GRK5 interaction with receptor without disrupting the catalytic activity or association of GRK5 with phospholipids.

Our studies have revealed some similarities in the effects of calmodulin-stimulated autophosphorylation and PKC phosphorylation of GRK5 (17). In both cases phosphorylation inhib-

**FIG. 4.** Calmodulin activates autophosphorylation of wild type and mutant GRK5. Panel A, 4 pmol each of purified Sf9 cell-expressed wild type GRK5 or GRK5-DD was autophosphorylated at 30 °C for 10 min in the absence or presence of either 0.85 mg/ml phospholipid vesicles (lipo) or 0.2 μM calmodulin. The samples were then electrophoresed on a 10% SDS-polyacrylamide gel, visualized by autoradiography, and the 32P-labeled proteins were excised and counted. Histograms show the fold activation of GRK5 or GRK5-DD autophosphorylation in the presence of activator. Panel B, effect of calmodulin concentration on autophosphorylation of GRK5-DD. Four pmol (200 nM) of purified GRK5-DD was autophosphorylated in the presence of the indicated calmodulin concentrations for 10 min at 30 °C and then processed as described above. The data are presented as fold activation of the kinase autophosphorylation compared with control. Panel C, effect of ATP concentration on GRK5-DD autophosphorylation. Eight pmol of GRK5-DD was autophosphorylated in the absence (○) or presence (●) of 0.8 μM calmodulin with the indicated concentrations of ATP. The stoichiometry of GRK5-DD autophosphorylation was determined after excising and counting the radioactive bands. Panel D, time course of calmodulin-stimulated autophosphorylation of GRK5 and its effect on inhibition of rhodopsin phosphorylation. One hundred pmol of rhodopsin was phosphorylated with 2 pmol (0.1 μM) of GRK5 in the presence or absence of 0.5 μM calmodulin. At the indicated times, reactions were stopped with SDS sample buffer, proteins were separated on a 10% SDS-polyacrylamide gel, visualized by autoradiography, and the 32P-labeled proteins were excised and counted. Autoradiography, and the 32P-labeled proteins were excised and counted.
its GRK5 binding to ROS and phosphorylation of rhodopsin. However, several lines of evidence suggest that the site(s) phosphorylated by PKC and autophosphorylated in the presence of calmodulin are distinct. First, PKC phosphorylation inhibits phosvitin phosphorylation by GRK5, whereas calmodulin-stimulated autophosphorylation slightly activates phosphorylation of phosvitin. In addition, although PKC phosphorylation of GRK5 blocks binding of an antibody generated against residues 556–571 of GRK5 (17), calmodulin-stimulated autophosphorylation had no effect on antibody binding to GRK5 (data not shown). Identification of the calmodulin-stimulated autophosphorylation site is currently under investigation.

Whereas calmodulin-stimulated autophosphorylation directly inhibits rhodopsin phosphorylation by GRK5, the addition of calmodulin further inhibits GRK5 (Fig. 5, C and D). This demonstrates that calmodulin is still able to bind to autophosphorylated GRK5. However, the effect of calmodulin appeared to be neither additive nor synergistic with autophosphorylation since both forms of GRK5 (wild type and calmodulin-stimulated autophosphorylated) were inhibited comparably by 150 or 500 nM calmodulin.

Taken together, our data suggest that calmodulin binding to GRK5 can directly inhibit GRK5 activity and that this inhibition is not mediated primarily via kinase autophosphorylation. First, calmodulin inhibits GRK5 binding to phospholipid and ROS membranes even in the absence of ATP (Fig. 3), whereas the addition of ATP has no effect on binding. Second, calmodulin-stimulated autophosphorylation increases slightly the phosphorylation of phosvitin by GRK5 (Fig. 5A), whereas phosvitin phosphorylation is inhibited in the presence of calmodulin (Fig. 2C). Finally, whereas calmodulin-stimulated autophosphorylation inhibits GRK5 activity 6–7-fold (Fig. 5D), high concentrations of calmodulin (>1 μM) are able to inhibit the kinase activity >100-fold (Fig. 2B). Thus, although calmodulin-stimulated autophosphorylation may contribute to a higher sensitivity of GRK5 to calmodulin, the direct binding of calmodulin to GRK5 likely plays the major role in inhibiting GRK5 activity.

Based on our data we propose the following model for GRK5 regulation by calmodulin. At resting calcium concentrations GRK5 is active and able to phosphorylate agonist-occupied receptors. When a cell is stimulated and intracellular calcium levels rise, calmodulin binds to GRK5 and inhibits directly receptor phosphorylation. However, since calmodulin-stimulated autophosphorylation also inhibits GRK5 activity, the kinase should remain inhibited even when calcium levels go down and calmodulin dissociates from the enzyme. Presumably, GRK5 will eventually be dephosphorylated and return to its basal level of activity. Thus, calmodulin-stimulated autophosphorylation may prolong the inhibitory effect of a transient increase in intracellular calcium levels on GRK5. A similar regulatory cycle has been demonstrated for the calmodulin-dependent protein kinase, CaM-kinase II, where calmodulin binding and calmodulin-stimulated autophosphorylation activate rather than inhibit the kinase (18). GRK5 is the first example of an enzyme negatively regulated by calmodulin in this manner.

**Identification of the Calmodulin Binding Site in GRK5—** Since calmodulin interaction with GRK5 reduces the binding of GRK5 to both phospholipid and receptor, we next focused on identifying the region of GRK5 which interacts with calmodulin. Analysis of the GRK5 amino acid sequence reveals that it contains several regions (in amino- and carboxyl-terminal domains) with features typical for a calmodulin binding site (i.e. mainly basic and hydrophobic residues that form an α-helix) (30). To identify the calmodulin binding domain in GRK5 we generated several GST-fusion proteins containing various regions of GRK5 and then assessed the ability of these proteins to inhibit calmodulin-stimulated autophosphorylation of GRK5-DD. Neither GST alone nor a GST fusion protein containing the carboxyl-terminal 102 amino acids of GRK5 had an effect on GRK5-DD autophosphorylation (Fig. 6a). However, a fusion protein containing residues 1–200 of GRK5 blocked almost completely the stimulation of autophosphorylation by calmodulin. Thus, GST-GRK5-(1–200) appears to bind calmodulin thereby effectively sequestering it from GRK5-DD and inhibiting calmodulin-stimulated autophosphorylation.

Since the amino-terminal domain of GRK5 contains four or five potential calmodulin binding domains, several additional constructs from this region were tested. A fusion protein containing the first 98 residues of GRK5 still effectively inhibited calmodulin-stimulated GRK5 autophosphorylation, whereas GST-GRK5-(50–200) had no effect. Constructs containing residues 20–49 or 20–39 of GRK5 also effectively blocked calmodulin activation of GRK5-DD autophosphorylation. As a further
Although calmodulin binding domains have no obvious consensus sequence, the calmodulin binding domain is within residues 20–39 of GRK5. Contingent on GST-GRK5 fusion proteins bound to calmodulin, the interaction, a value in good agreement with the EC50 for calmodulin, can interact directly with calmodulin (38), these regions containing calmodulin-dependent inhibition of rhodopsin phosphorylation. In addition, the interaction of GRK5 with calmodulin was specific since no GRK5 binding was observed when another calcium and hydrophobic residues to opposite sides of the helix. Hydrophobic residues are boxed, and positively charged residues are denoted with a +.

test of calmodulin binding, we measured direct binding of several GST-GRK5 fusion proteins to calmodulin-agarose. GST-GRK5-(1–200) and GST-GRK5-(20–49) bound to calmodulin-agarose in the presence of Ca2+ and could be eluted with EGTA, whereas GST-GRK5-(50–200) did not bind to calmodulin-agarose (data not shown).

The ability of GRK5 and several of the GST-GRK5 fusion proteins to bind calmodulin was also tested using SPR technology on a BIAcore instrument. This method detects directly the interaction among biological macromolecules because of an increase in the mass of the protein complex and a corresponding change in the refractive index of the solution close to the surface of the instrument’s sensor chip (31). GRK5 bound to immobilized calmodulin in a calcium-dependent manner (Fig. 6B). Additional studies suggested a Kd of ~10 nm for this interaction, a value in good agreement with the EC50 for calmodulin-dependent inhibition of rhodopsin phosphorylation. In addition, the interaction of GRK5 with calmodulin was specific since no GRK5 binding was observed when another calcium-binding protein, recoverin, was tested. In contrast, under similar conditions, GRK1 does display specific calcium-dependent binding to recoverin.2 As expected, the 1–200- and 20–39-containing GST-GRK5 fusion proteins bound to calmodulin, whereas the 98–136 and 50–200 fusions did not.

Taken together, these data clearly indicate that the major calmodulin binding domain is within residues 20–39 of GRK5. Although calmodulin binding domains have no obvious consensus sequence, most of them adopt a basic amphiphilic α-helical structure that contains a large number of positively charged residues as well as hydrophobic residues that repeat with a three to four period (30). The region of GRK5 which binds calmodulin has similar characteristics with a total of 9 basic and 5 hydrophobic residues (Fig. 7A). This portion of GRK5 also has significant homology with the myristoylated alanine-rich PKC substrate (MARCKS), a well-characterized calmodulin-binding protein (19). Helical wheel projection of this region of GRK5 shows the segregation of basic and hydrophobic residues to opposite sides of the helix, thereby making them available for interaction with acidic and hydrophobic patches of calmodulin (Fig. 7B).

Although the 20–39 amino acid region of GRK5 is substantially conserved within the GRK4 subfamily (GRK4, 5, and 6), it differs significantly from the corresponding regions of GRK1, 2, and 3 (Fig. 7A). These differences may account for the much higher affinity of GRK5 for calmodulin compared with GRK1 and GRK2. Similarly, the apparent higher affinity of GRK5 for calmodulin compared with GRK6 may indicate that the three nonconserved amino acid differences among these kinases within this region are involved in calmodulin binding. Alternatively, other protein determinants may also play a role in calmodulin interaction. For example, since GRK6 is palmitoylated it might bind more tightly to phospholipid membranes than GRK5 and thus might be inhibited less effectively by calmodulin. Recently residues 22–29 of GRK5 were suggested to be involved in the binding of phosphatidylinositol 4,5-bisphosphate (12). Since this region overlaps with the calmodulin binding domain of GRK5 it is possible that calmodulin can compete with phosphatidylinositol 4,5-bisphosphate for binding to GRK5. This is supported by our observation that calmodulin inhibits directly the GRK5 binding to phospholipid vesicles. Indeed, it has been shown for a number of proteins, such as GAP-43 (32), MARCKS (33), and nitric oxide synthase (34), that the calmodulin binding site is involved directly in phospholipid interaction. However, since the carboxyl-terminal region of GRK5 has also been implicated in phospholipid binding (11), this may explain why calmodulin does not inhibit completely the membrane binding of GRK5 (Fig. 3B).

GRK4 is the only member of the GRK family demonstrated to undergo alternative splicing (9, 35). Exons in both the amino-terminal and carboxyl-terminal domains of GRK4 can be alternatively spliced, resulting in a total of four different protein variants of GRK4, each of which appears capable of promoting receptor phosphorylation and desensitization (9). Interestingly, the region homologous to the calmodulin binding domain of GRK5 (residues 20–39) lies entirely within the first alternatively spliced exon of GRK4 (residues 18–49). This raises the intriguing possibility that different splice variants of GRK4 could be differentially regulated by calmodulin (i.e. variants lacking this exon would not be inhibited by calmodulin). In addition, although there is no similarity in the exon-intron organization of the GRK2 and GRK4 genes (9, 36), organization of the GRK4, 5, and 6 genes appears to be highly conserved.3 This creates the possibility of calmodulin-insensitive splice variants of GRK5 which are expressed in certain cell types or during some stage of development.

While this manuscript was in preparation, Chuang et al. (37) also reported that calmodulin inhibits GRK-mediated phosphorylation of rhodopsin with IC50 values virtually identical to those observed in our experiments. However, one important difference between the two studies was that Chuang et al. included G protein βγ subunits in their assays to activate GRK2, whereas our studies were performed in the absence of Gβγ. Since Gβγ can interact directly with calmodulin (38), these authors speculated that calmodulin inhibition of GRK2 could be mediated via sequestering Gβγ from GRK2. However, since the IC50 values for calmodulin inhibition of GRK2 activity were identical in both studies, this suggests that the inhibition is the result of a direct interaction between calmodulin and GRK2.

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2 V. Z. Slepak, C.-K. Chen, M. I. Simon, and J. Hurley, manuscript in preparation.

3 J.-L. Parent and J. L. Benovic, unpublished observation.
Moreover, our preliminary studies also demonstrate that GRK2 binds directly to calmodulin as detected by SPR (data not shown).

In conclusion, our studies demonstrate that calmodulin is a potent inhibitor of GRK activity with a selectivity for GRK5 (IC\textsubscript{50} \sim 50 nM) > GRK6 >> GRK2 (IC\textsubscript{50} \sim 2 \mu M) >> GRK1. Calmodulin inhibition of GRK5-mediated receptor phosphorylation is caused by inhibition of kinase interaction with both receptor and phospholipid, and the major region of calmodulin interaction lies within amino acids 20–39 of GRK5, a region that is highly conserved in GRK4, 5, and 6. These studies also further establish the role of Ca\textsuperscript{2+} in the regulation of GRK activity. While GRK1 is inhibited by Ca\textsuperscript{2+/recoverin and GRK5 and GRK6 (and likely GRK4) are potently inhibited by Ca\textsuperscript{2+}/calmodulin, the effect of Ca\textsuperscript{2+} on GRK2 (and likely GRK3) will depend on the relative contribution of PKC-mediated activation and calmodulin-mediated inhibition of these kinases.

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