G protein-coupled receptor kinases (GRKs) initiate pathways leading to the desensitization of agonist-occupied G-protein-coupled receptors (GPCRs). Here we report that the cytoskeletal protein actin binds and inhibits GRK5. Actin inhibits the kinase activity directly, reducing GRK5-mediated phosphorylation of both membrane-bound GPCRs and soluble substrates. GRK5 binds actin monomers with a $K_d$ of 0.6 μM and actin filaments with a $K_d$ of 0.2 μM. Mutation of 6 amino acids near the amino terminus of GRK5 eliminates actin-mediated inhibition of GRK5. Calmodulin has previously been shown to bind to the amino terminus of GRK5 (Pronin, A. N., and Benovic, J. L. (1997) J. Biol. Chem. 272, 3806–3812) and here we show calmodulin displaces GRK5 from actin. Calmodulin inhibits GRK5-mediated phosphorylation of GPCRs, but not soluble substrates such as casein. Thus in the presence of actin, calmodulin determines the substrate specificity of GRK5 by preferentially allowing phosphorylation of soluble substrates over membrane-bound substrates.

G protein-coupled receptor kinases (GRKs) comprise a family of serine-threonine kinases that desensitize G protein-coupled receptors (GPCRs) by phosphorylating agonist occupied receptors (1). The GRKs contain a highly conserved catalytic domain flanked by more divergent amino (NH$_2$-) and carboxyl (COOH)-terminal regulatory regions. Based on sequence homology outside the catalytic domain, the GRKs are divided into three subfamilies consisting of GRK1 (Rhodopsin kinase); GRK2 (β-adrenergic receptor kinase 1) and GRK3 (β-adrenergic receptor kinase 2); and GRK4, -5, and -6. GRK1 and -4 are expressed exclusively in the retina (2) and testis (3, 4), respectively, while the other GRKs are expressed in most cell types and provide a ubiquitous mechanism for the desensitization of GPCRs. Since GPCR desensitization ultimately results in the termination of many important signaling cascades, it is not surprising that phosphorylation of GPCRs by GRKs is highly regulated.

Initially regulation of GPCR phosphorylation focused on the agonist-induced conformational changes receptors undergo to become GRK substrates. However, recent evidence demonstrates GRK activity is regulated differentially by protein kinase C phosphorylation, calcium-binding proteins, and lipid second messengers (1). Membrane GPCRs and soluble substrates GRK activity is regulated differentially by protein kinase C phosphorylation, calcium-binding proteins, and lipid second messengers (1). Membrane GPCRs and soluble substrates GRK activity is regulated differentially by protein kinase C phosphorylation, calcium-binding proteins, and lipid second messengers (1).

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine milk β-casein was purchased from Sigma. Highly purified bovine brain calmodulin was purchased from Calbiochem (San Diego, CA). GRK2 and GRK5 were purified from baculovirus-infected Sf9 cells (9, 10). NH$_2$-terminal and COOH-terminal polybasic (NTPB and CTPB) mutants were created by polymerase chain reaction mutagenesis of lysine and arginine residues between amino acids 22–29 and 547–557, respectively, to alamines (11). Preparations enriched for these mutant enzymes were made from baculovirus-infected Sf9 cells homogenized in 20 mM Tris, pH 7.5, 2 mM EDTA, 1 mM DTT, 100 μM phenylmethylsulfonyl fluoride, 4 μg/ml leupeptin, 1 μg/ml pepstatin, and 5 μg/ml aprotinin and clarified by centrifugation at 100,000 × g for 45 min. Rod outer segment membranes containing rhodopsin were prepared (12) and β$_2$-adrenergic receptor was purified from Sf9 cells and reconstituted in crude lipids (13, 14). Actin was purified from rabbit skeletal back and leg muscle (15) and gel filtered and sepharose C-300 (16) equilibrated in Buffer A (0.2 mM ATP, 0.5 mM DTT, 0.1 mM CaCl$_2$, 1 mM Na$_2$SO$_4$, 2 mM Tris, pH 8.0) at 4 °C. The actin concentration was determined by absorbance at 280 nm using a molar extinction coefficient of 2.66 × 10$^4$ M$^{-1}$ cm$^{-1}$ (17). Actin was labeled on C374 with pyrene/iodoacetamide (18), gel filtered, and used at 5% of the total actin concentration. Mg$^{2+}$-actin was made from Ca$^{2+}$-actin in Buffer A by adding 60–80 μg MgCl$_2$ and 200 μg EGTA. Mg$^{2+}$-actin was polymerized with 0.1 volume of 10 × MKEI (500 mM KC$_2$, 10 mM MgCl$_2$, 10 mM EGTA, 100 mM imidazole, pH 7.0) to generate a stock solution of filaments in 1 × polymerizing buffer.

**Overlay Method for Detection of GRK5-binding Proteins—** GRK5-binding proteins were identified using a modification of a procedure initially of Leiser et al. (19). Proteins in samples to be probed were separated by SDS-PAGE (20) and electrophoretically transferred to nitrocellulose membranes. The nitrocellulose filters were blocked in 5% non-fat dry milk, and 0.02% Na$_2$SO$_4$ for 1 h at 4 °C and subsequently washed three times with binding buffer (100 mM Tris, pH 7.4, and 50 mM NaCl). GRK5-binding proteins were detected by incubating the nitrocellulose filters with purified autophosphorylated GRK5. GRK5 (3 μM) was au-
ttophosphorylated by incubation in 20 mM Tris, pH 7.5, 10 mM MgCl₂, 2.0 mM EDTA, 1 mM DTT containing 60 μM [γ-³²P]ATP (~6000 cpm/μmol) at 30 °C for 30 min. Prior to incubation with the nitrocellulose filters the GRK5 was desalted over G-25 columns (1 ml) to remove excess [γ-³²P]ATP. The [γ-³²P]-labeled GRK5 (0.2 μM) was incubated with the nitrocellulose filters in binding buffer for 1 h at 4 °C. Blots were washed five times with 500 ml of binding buffer to reduce nonspecific binding and subsequently exposed to x-ray film.

Binding to Actin Monomers—Binding to actin monomers was determined from a change in the fluorescence intensity of pyrenyl-actin (λₐ = 365 nm, λₑm = 407 nm). GRK5 induces the polymerization of actin when [GRK] >> [Actin] (Fig. 3) so fluorescence intensities were measured 5 s after mixing kinase and actin. Data was collected over a range of kinase concentrations and was fitted to Equation 1,

\[ F_I = F_{I{o}} + \frac{(K_a + [A] + [GRK]) - (K_d + [A] + [GRK])^2 - 4[A][GRK])}{2[A]} \]  

where \( F_I \) is the fluorescence in the absence of GRK, \( [A] \) is the actin monomer concentration (0.4 μM), [GRK] is the GRK5 concentration, and \( K_a \) is the dissociation equilibrium constant of the complex. Parameters determined from the fits were the amplitudes (\( F_{I{o}} - F_{I} \)) and the \( K_d \).

Actin Filament Polymerization and Depolymerization—Assembly of actin filaments was monitored by fluorescence of pyrene-actin (λₐ = 365 nm, λₑm = 407 nm). Depolymerization of Mg²⁺-actin filaments was initiated by a 30-fold dilution of a 0.5 μM stock solution of filaments (5% pyrene-labeled) in 1 × KMEI buffer containing 60 mM CaCl₂ in the presence and absence of GRK. GRK induced assembly of actin monomers was measured by pyrene fluorescence after mixing actin monomers with GRK in Buffer A.

GRK5 Kinase Activity Determination—GRK5 and the indicated concentration of actin were incubated with either rod outer segments containing rhodopsin, β₂-adrenergic receptor (120 nM), or casein (23 μM) at 30 °C for 5, 10, or 15 min, respectively, in 20 mM Tris, pH 7.5, 10 mM MgCl₂, 2 mM EDTA, 1 mM DTT, and 60 μM [γ-³²P]ATP (~0.25 μCi/μl). For reactions examining the effect of calmodulin, the reaction also contained 2 mM CaCl₂, which alone had no effect on GRK5 activity. An equal volume of sample loading buffer (100 mM Tris, pH 7.2, 4% SDS, 200 mM DTT, 0.5% pyronin Y, 20% glycerol) was added to terminate the 25-μl reaction and the samples were subjected to SDS-PAGE. The gels were dried and the amount of phosphorylation was quantified using Image Quant (Molecular Dynamics).

Binding to Actin Filaments—GRK5 was clarified in a Beckman TLA 100.2 rotor (70,000 rpm; 200,000 × g) for 20 min. The supernatant was mixed with a range of actin filament concentrations and equilibrated at 22 °C for 45 min. The final conditions were 50 mM KCl, 1 mM MgCl₂, 10 mM imidazole, pH 7.0, 200 μM ATP, 0.5 mM DTT, 2 mM CaCl₂, 600 nM GRK, and actin filament concentrations from 0 to 20 μM in the presence or absence of 1.2 μM calmodulin. The mixture was spun at 200,000 × g for 30 min to pellet actin filaments and bound GRK5. Free GRK5 in the supernatants was precipitated with 100% methanol. An aliquot of myoglobin was included in each sample to correct for any differences in recovery from the precipitation. Extracted proteins were separated by SDS-PAGE and analyzed by twodimensional scanning densitometry of wet, Coomassie-stained gels. Data from scanned gels were analyzed with NIH Image. Binding constants were determined from a least squares fit to a modified form of Equation 1.

RESULTS

GRKs Interact with Actin Monomers and Filaments—To identify proteins that interact with GRK5, cellular extracts were probed with radiolabeled GRK5 using a gel-overlay technique. An abundant protein of approximately 42 kDa in the particulate (P) fraction of heart tissue interacts with GRK5 (Fig. 1). Its abundance and electrophoretic mobility suggested that it might be actin, so we confirmed that purified actin from rabbit skeletal muscle interacts with GRK5 under these denaturing conditions (Fig. 1).

Both GRK5 and GRK2 bind actin monomers in solution. GRKs enhance the fluorescence of the pyrene-labeled actin monomers suggesting that they bind near the COOH terminus of actin near the site of fluorophore attachment (C374). GRK5 binds with a \( K_d \) of 0.62 (± 0.07) μM (Fig. 2A), while the affinity of GRK2 is approximately 10-fold lower, \( K_d = 5.6 \) μM (data not shown).

Both GRK2 and GRK5 cosediment with actin filaments. The equilibrium binding affinity of GRK5 for actin filaments in physiological salt is 0.2 ± 0.1 μM (Fig. 2B). As with actin
monomers, GRK2 binds with a 10-fold lower affinity ($K_\text{d} = 2.9 \mu M$). GRK5 binding to pyrene-labeled actin filaments quenches the fluorescence by $>50\%$ (data not shown). The affinity determined from pyrene fluorescence was similar to the sedimentation assay ($K_\text{d} = 0.18 \pm 0.13 \mu M$). These results indicate that GRK5 binds to actin filaments as well as actin monomers, with an affinity that is similar to other actin-binding proteins (21), whereas the affinity of GRK2 is lower. For the remainder of these studies we focused on the higher affinity interaction between actin and GRK5. At substoichiometric concentrations, GRK5 does not affect the critical concentration of actin filament assembly (data not shown), demonstrating that GRKs do not sequester monomers and prevent their incorporation into filaments.

Saturating concentrations of GRK5 inhibit the depolymerization of actin filaments upon dilution (Fig. 3A), demonstrating that GRK5 binding does not sever filaments, but stabilizes their structure. In addition, GRK5 induces the assembly of monomers under non-polymerizing conditions, presumably by stabilizing the formation of oligomers; in contrast, GRK2 has no effect on actin assembly (Fig. 3B). Taken together, these results suggest that GRK5 binds at least two actin monomers, thereby stabilizing the intersubunit contacts and inhibiting depolymerization from filament ends. Similar effects result from tropomyosin and myosin-binding actin (22, 23).

**Actin Inhibits the Catalytic Activity of GRK5**—GRK5 phosphorylates agonist-occupied GPCRs, the only physiological substrates identified for these kinases. Although no physiological soluble substrates are known for GRK5, casein is an *in vitro* model substrate for GRKs.

Actin binding to GRK5 inhibits GRK5-mediated phosphorylation of membrane bound (rhodopsin and $\beta_2$-adrenergic receptor) and soluble (casein) substrates with similar IC$_{50}$ values, between 1.5 and $3 \mu M$ (Fig. 4). These IC$_{50}$ values are consistent, within experimental error, with the binding affinities reported above (Fig. 2). The inhibition by actin differs from the action of calmodulin, another known inhibitor. Calmodulin physically inhibits GRK5 phosphorylation of membrane-bound substrates by preventing association with phospholipids and receptor substrates (24) but does not inhibit casein phosphorylation.

**An NH$_2$-terminal Mutant of GRK5 Is Not Inhibited by Actin**—To determine if either of the polybasic regions at the extreme NH$_2$ terminus and COOH terminus of GRK5 interact with actin, we examined mutants with mutations of basic amino acids at the NTPB or CTPB of GRK5 (shown in Fig. 5A). In five independent experiments performed in duplicate, 2.2 $\mu M$ actin inhibited CTPB-mediated casein phosphorylation by 40% but NTPB-mediated casein phosphorylation by only 6% (Fig. 5B). Actin-mediated inhibition of CTPB is similar to that observed using purified wild-type GRK5 (Fig. 4). These results suggest that the amino terminus of GRK5, and specifically basic residues located in this region (Fig. 5A), bind actin. The polybasic region of NTPB that is required for inhibition by actin overlaps the calmodulin-binding site of GRK5 previously localized between residues 20 and 39 (24).

Calmodulin Reverses Actin Inhibition of the GRK5-mediated Phosphorylation of a Soluble GRK5 Substrate—To determine if calmodulin alters actin inhibition of GRK5-mediated phosphorylation, we assayed casein and rhodopsin phosphorylation in the presence of inhibiting concentrations of actin and increasing concentrations of calcium-calmodulin. CaCl$_2$ itself has no effect on GRK5-mediated phosphorylation (data not shown). Calmodulin specifically inhibits GRK5-mediated phosphorylation of membrane substrates (6), whereas actin inhibits GRK5-mediated phosphorylation of both rhodopsin and casein, membrane and soluble substrates (Fig. 4). Interestingly, calcium-calmodulin reverses the inhibitory effect of actin on casein phosphorylation (Fig. 6, filled bars), consistent with these two regulators binding to similar sites on GRK5. Calmodulin did not rescue receptor phosphorylation activity in the presence of actin (Fig. 6, open bars), suggesting that calmodulin dissociates GRK5 from actin to form a GRK5-calmodulin complex competent to phosphorylate soluble substrates, but incapable of phosphorylating receptor substrates.

Both the ability of calmodulin to reverse the actin-mediated inhibition of GRK5 and the apparently coincident binding sites of actin and calmodulin suggests that calmodulin disrupts the physical interaction between GRK5 and actin filaments. Indeed, in two experiments similar to Fig. 2B, 2 $\mu M$ actin filaments bound 570 ($\pm 9.5$) nM of 600 nM GRK5 in the absence of calmodulin and only 309 ($\pm 62$) nM GRK5 with 1.2 $\mu M$ calmodulin. In addition, calmodulin eliminates the effects of GRK5 on actin polymerization and depolymerization shown in Fig. 3, resulting in traces superimposable with the actin alone trace (data not shown).
DISCUSSION

We identify actin as a 42-kDa protein that interacts with GRK5. This high affinity interaction between GRK5 and actin manifests itself in the ability of actin to inhibit GRK5-mediated kinase activity. Actin inhibits the phosphorylation of soluble substrates as well as membrane-bound substrates. Interestingly, point mutations within the calmodulin-binding domain of GRK5 prevent inhibition by actin. These apparently overlapping binding sites allow calmodulin to displace GRK5 from actin filaments and to increase GRK5 activity toward soluble substrates in the presence of actin.

Actin is a very abundant cellular protein and is a major component of the microfilament network that is essential for many cellular processes including cell division, tension, movement, and signal transduction. Most known proteins associated with microfilaments assist in the organization and reorganization of the cytoskeleton. In vitro, GRK5 stabilizes filaments, but given the low cellular concentration of GRK5 we would not predict any effects on actin polymerization overall, although GRK5 could stabilize filaments near membranes where it is localized.

Few actin-associated proteins are reported to have enzymatic activities that are directly inhibited by actin. One enzyme known to associate with the cytoskeleton that is inhibited by actin is casein kinase II (25). Casein kinase II has some similarities to GRK5 including the ability to phosphorylate casein and the ability to be inhibited by heparin (26). The glycolytic enzyme aldolase is also regulated by actin binding. In this case, the substrate, fructose 1,6-bisphosphate, inhibits aldolase binding to actin (27, 28), suggesting that the actin-bound enzyme is in its resting state. These two examples support the suggestion that actin provides more than a means of statically localizing proteins, but plays a more dynamic role by specifically regulating enzymatic activity.

Intracellular localization is an important means of regulating GRK activity. GRK1, -2, and -3 are present in the cytosol in resting cells, and only when cells are exposed to agonist, translocate to membranes to phosphorylate their receptor substrates. GRK4, -5, and -6, on the other hand, are located in the particulate fraction which contains both GPCRs and actin. Actin inhibition of GRK5-mediated phosphorylation suggests a mechanism by which GRK5 is simultaneously localized to the

![Fig. 4. Inhibition of GRK5-mediated phosphorylation. A range of actin concentrations, 0.4 to 2.4 μM GRK5 in 60 μM γ-32P]ATP (6 μCi), 20 mM Tris, pH 7.5, 10 mM MgCl2, 2 mM EDTA, and 1 mM DTT were incubated with rod outer segment membranes containing rhodopsin (under visible light for 5 min), casein (15 min), or purified, lipid reconstituted β2-adrenergic receptor and 100 μM isoproterenol (10 min) at 30 °C. The data are expressed as the percentage of substrate phosphorylation in the absence of actin and are the mean of at least three independent experiments with the error bars representing the S.E. The IC50 values, calculated using Graphpad Prism, were 1.5 ± 1.9 μM for β2-adrenergic receptor phosphorylation, 2.6 ± 1.6 μM for rhodopsin phosphorylation, and 1.8 ± 1.5 μM for casein phosphorylation.

![Fig. 5. Calmodulin and actin-binding domains localize to the NH2 terminus of GRK5. A, the schematic of GRK5 highlights the NH2- and COOH-terminal polybasic domains. The sequence of the calmodulin-binding domain (CaMBD) is shown below the shaded box in bold text and the conserved catalytic domain is represented by the striped box. The amino acids mutated to alanines between amino acids 22–29 and 547–557 in the GRK5 NTPB and CTPB mutants, respectively (11), are underlined. B, soluble fractions enriched with GRK5 NH2- and COOH-terminal polybasic mutants were prepared from Sf9 cells and assayed as in Fig. 4. Actin-mediated inhibition of casein phosphorylation by NTPB and CTPB is measured and determined to be significantly different (p < 0.01). Data shown are the mean and S.E. from five independent experiments performed in duplicate.

![Fig. 6. Co-regulation of GRK5-mediated phosphorylation by calcium-calmodulin and actin. Calmodulin relieves actin inhibition of GRK5-mediated casein phosphorylation. GRK5 was assayed as in Fig. 4 plus 2 mM CaCl2 with rhodopsin or casein as substrates, the indicated concentration of calmodulin, and 3 μM rabbit skeletal actin. The data are expressed as the percent of substrate phosphorylation in the absence of actin. The mean and S.E. of three independent experiments are shown.](https://example.com/fig6.png)
cytoskeleton and prevented from desensitizing its receptor substrate. Given the overlapping binding sites of actin and calmodulin, we propose that these proteins act in concert to regulate GRK5 catalytic activity. Interestingly, MARCKS also binds calmodulin and actin. Hartwig et al. (29) demonstrate that calmodulin prevents the actin filament bundling ability of a synthetic peptide corresponding to residues 155–173 of MARCKS. In addition, the association of caldesmon (30) and dystrophin (31) with actin is reduced by calcium-calmodulin. Thus, GRK5 is similar to a number of other proteins that are displaced from actin by calmodulin.

The data reported here in an in vitro model system support the following model for GRK5 activation. A rise in the intracellular levels of calcium following agonist occupancy would activate calmodulin and displace the kinase-inhibited GRK5 from actin. This calmodulin bound GRK5 is capable of phosphorylating soluble substrates in vitro, suggesting the possibility that GRK5 could then phosphorylate non-receptor soluble substrates in vivo. Although there are, as yet, no known such endogenous soluble substrates for GRK5, Pitcher et al. (32) have recently identified tubulin as a soluble substrate for GRK2. Previous evidence demonstrating that agonist-occupied receptors increase GRK phosphorylation of peptide substrates (33–35) also support the possibility that endogenous substrates could be phosphorylated following agonist stimulation of GPCR activation of GRKs.

Previous work has focused on the inhibition of GRK5 by calmodulin, however, this inhibition extends only to membrane-bound receptor substrates. In contrast, actin is a direct inhibitor of GRK5, inhibiting both membrane-bound and soluble substrates. Since actin and calmodulin both interact with the NH₂ terminus of GRK5, we propose that the dynamic regulation of GRK5 by these two proteins could serve as a calcium-mediated mechanism to modulate the kinase specificity of GRK5 for either membrane or soluble substrates.

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