Phosphorylation of GRK1 and GRK7 by cAMP-dependent Protein Kinase Attenuates Their Enzymatic Activities*

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Phosphorylation of G protein-coupled receptors is a critical step in the rapid termination of G protein signaling. In rod cells of the vertebrate retina, phosphorylation of rhodopsin is mediated by GRK1. In cone cells, either GRK1, GRK7, or both, depending on the species, are speculated to initiate signal termination by phosphorylating the cone opsins. To compare the biochemical properties of GRK1 and GRK7, we measured the $K_m$ and $V_{max}$ of these kinases for ATP and rhodopsin, a model substrate. The results demonstrated that these kinases share similar kinetic properties. We also determined that cAMP-dependent protein kinase (PKA) phosphorylates GRK1 at Ser21 and GRK7 at Ser28 and Ser29 in vitro. These sites are also phosphorylated when FLAG-tagged GRK1 and GRK7 are expressed in HEK-293 cells treated with forskolin to stimulate the endogenous production of cAMP and activation of PKA. Rod outer segments isolated from bovine retina phosphorylase the FLAG-tagged GRKs in the presence of dibutyryl-cAMP, suggesting that GRK1 and GRK7 are physiologically relevant substrates. Although both GRKs also contain putative phosphorylation sites for PKC and Ca$^{2+}$/calmodulin-dependent protein kinase II, neither kinase phosphorylated GRK1 or GRK7. Phosphorylation of GRK1 and GRK7 by PKA reduces the ability of GRK1 and GRK7 to phosphorylate rhodopsin in vitro. Since exposure to light causes a decrease in cAMP levels in rod cells, we propose that phosphorylation of GRK1 and GRK7 by PKA occurs in the dark, when cAMP levels in photoreceptor cells are elevated, and represents a novel mechanism for regulating the activities of these kinases.

G protein-coupled receptors (GPCRs) belong to the largest family of signal-transducing proteins in eukaryotes. They mediate cellular responses to a variety of environment stimuli, including light, taste, odors, ions, nucleotides, peptides, and lipids, through the activation of heterotrimeric G proteins (1–4). Phosphorylation of ligand-activated GPCRs by G protein-coupled receptor kinases (GRKs) is the initial step in the rapid termination or desensitization of GPCR signal transduction. For example, in rod cells of the vertebrate retina, desensitization occurs when light-activated rhodopsin is phosphorylated by GRK1, followed by the binding of visual arrestin to the phosphorylated rhodopsin, which blocks its interaction with transducin, the rod cell G protein (5). Although desensitization also occurs in cone cells, less is known about the proteins involved and their regulation. GRK1 and a cone-specific GRK, GRK7, are coexpressed in human and monkey cone cells (6–8) where both may contribute to the deactivation of cone opsins (7–9). In contrast, only GRK7 is expressed in the cones of pigs and dogs. However, it is absent from the mouse genome altogether and mouse cones express only GRK1 (7). Therefore, differences in cone visual transduction between species may result in part from variations in the expression pattern of GRK1 and GRK7. We recently implicated GRK7 in the phosphorylation of cone opsins in retinas from the pig and the 13-lined ground squirrel, rod-dominant and cone-dominant mammals, respectively (10). These results suggest that the role of GRK7 in cones might be similar to the role of GRK1 in rods. Very little is known regarding the regulation of GRKs in the retina apart from the interaction of GRK1 with recoverin, a retina-specific Ca$^{2+}$ sensor protein that inhibits the binding of GRK1 to rhodopsin (11). Since both GRK1 and GRK7 may phosphorylate cone opsins in primates, a comparison of their enzyme activities and their regulation by other signaling molecules is essential for understanding their potential contributions to cone opsin deactivation.

In this report, we compare the activities of human GRK1 and GRK7 in vitro. GRK1 and GRK7 share a similar $K_m$ and $V_{max}$ for bovine rhodopsin, a model GPCR substrate. We also determined that PKA phosphorylates the amino termini of GRK1 and GRK7, resulting in reduced ability of these two kinases to phosphorylate rhodopsin in vitro. Because cAMP levels are regulated by light in photoreceptor cells (12–15), our results provide the basis for a novel mechanism whereby GRK1 and GRK7 activities may be regulated by PKA in a light-dependent manner. Phosphorylation by second messenger-regulated kinases has been reported to regulate the activities of GRK2 and GRK5 (16–19) and now appears to be a common posttranslational modification that alters the activities of the retin-specific GRK family members.

**EXPERIMENTAL PROCEDURES**

Expression and Purification of Human FLAG-GRK1 and FLAG-GRK7—The FLAG amino acid sequence (Sigma), DYKDDDDK, was placed at the amino terminus of GRK1 and GRK7, resulting in a fusion protein of 37 amino acids.FLAG-GRK1 and FLAG-GRK7 activities were determined by light activation of PKA. Point mutations in GRK1 and GRK7 were generated by site-directed mutagenesis using the QuikChange® multi site-directed mutagen-
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In Vitro Phosphorylation of GRK1 and GRK7—Phosphorylation by PKA was measured in vitro by incubating the catalytic subunit of PKA (100 units/μl reaction mixture, New England Biolabs Inc.) at 30 °C with wild-type or mutant FLAG-tagged GRK1 and GRK7 (500 ng) and 150 μM [γ-32P]ATP (500–1000 cpm/pmol) in a 50-μl reaction volume for 10 min or 30 min as described in the legends to Figs. 3 and 7. Proteins were separated by SDS-PAGE or spotted onto P81 Whatman® phosphocellulose discs, washed as described above, and quantified using the Cerenkov method to determine the amount of phosphorylation. Phosphorylation by PKCα (Invitrogen) was assessed using a mixture of phosphatidylserine (Sigma) and diacylglycerol (Avanti Polar Lipids, Alabaster, AL) as described by the manufacturer. Histone H1 Type III (Sigma) was used as a control substrate to monitor PKCα activity. Phosphorylation of GRK1 and GRK7 by Ca2+/calmodulin-dependent protein kinase II (CaMKII) (Calbiochem) was measured in the presence of calmodulin (Calbiochem). Syntide 2 (Sigma) was used as a control substrate to monitor CaMKII activity.

In Situ Phosphorylation of GRK1 and GRK7—Phosphorylation of wild-type and mutant GRK1 and GRK7 was assessed after transient expression of the proteins in HEK-293 cells using the Lipofectamine Plus transfection method (Invitrogen). Three days after transfection, cells were washed in phosphate-free DMEM and preincubated with phosphate-free DMEM containing 0.1% serum for 30 min. Cells were washed in phosphate-free DMEM and preincubated with the buffer containing PKA catalytic subunit for 10 min at 30 °C. Cells were incubated with 25 μM forskolin (Sigma) in Me2SO or Me2SO alone for only 15 min, then lysed for 30 min at 4 °C in TBS containing 1% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 50 mM NaF, 1 mM EDTA, and 10 mM sodium pyrophosphate. The lysates were centrifuged at 40,000 × g and incubated with anti-FLAG M2 affinity resin for 3 h at 4 °C. The resin was washed five times with TBS and incubated with L-aminoethyl dextran method as described previously (20). After 72 h in acetone, phosphorylation of PKA was measured after chemical digestion with formic acid or iodosobenzoic acid (2 mg/ml in acetic acid, final concentration). The supernant was diluted with 2 volumes of Buffer A and centrifuged at 14,000 rpm in a Beckman JA-14 rotor for 1 h. The supernatant was collected for the Western blot analysis using the anti-FLAG M2 monoclonal antibody (Sigma) and the Western-Star™ protein detection kit (Applied Biosystems, Foster City, CA) for chemiluminescence detection.

Phosphorylation of FLAG-tagged PKA by PKA from Rod Outer Segment Membranes—Rod outer segment (ROS) membranes were isolated from bovine retinas (J. A. Lawson, Inc.) using a stepwise sucrose density gradient as described (23, 24). Phosphorylation of FLAG-tagged GRK1 and GRK7 was performed under dim red light using methods described previously (25) with the following modifications. ROS containing 20 μl rhodopsin were resuspended in 20 μl Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM MgCl2, and 0.1% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 50 mM NaF, 1 mM EDTA, and 10 mM sodium pyrophosphate. The lysates were centrifuged at 40,000 × g and incubated with anti-FLAG M2 affinity resin for 3 h at 4 °C. The resin was washed with lysis buffer, incubated with L-aminoethyl dextran, and the extracted proteins were separated by SDS-PAGE. The gels were transferred to nitrocellulose membrane and phosphorylation was observed by phosphorimaging analysis. Western blot analysis was performed with the same nitrocellulose membrane using the anti-FLAG M2 monoclonal antibody and a chemiluminescent detection system as described above.

Chemical Cleavage of GRK1—FLAG-tagged GRK1 (10 μg) was incubated with or without PKA catalytic subunit and 10 μM [γ-32P]ATP (10 μCi/reaction) for 30 min at 30 °C. Chemical digestion of GRK1 with 70% formic acid was performed at 37 °C for 48 h while shaking. Cleavage of GRK1 with iodosobenzoic acid was performed after heating GRK1 at 70 °C for 10 min with 0.1% SDS (final concentration). The heated samples were diluted with water (1:1, v/v) prior to the addition of iodosobenzoic acid (2 mg/ml in acetic acid, final concentration). The cleavage reaction was incubated under N2 in the dark for 48 h at room temperature. After chemical cleavage with formic acid or iodosobenzoic acid, the reaction mixtures were subjected to five wash cycles with water by SpeedVac lyophilization, resuspended in 500 μl Tris-HCl, pH 8.0, 2 mM EDTA, and incubated with the GRK glutathione S-transferase pull-down kit (Amersham Biosciences). Two-dimensional Chromatography of GRK1 and GRK7—Peptide maps were generated using proteins radiolabeled in vitro or in situ as described by Boyle et al. (26) and Schaller and Parsons (27). For in vitro experiments, 1–2 μg of GRK1 or GRK7 were incubated with PKA catalytic subunit (100 units/μl reaction mixture, New England Biolabs Inc.) and 10 μM [γ-32P]ATP (10 μCi/reaction) at 30 °C for 30 min. Phosphorylated proteins were separated by SDS-PAGE and transferred...
to nitrocellulose. The radioactive bands were excised and incubated with 0.5% polyvinylpyrrolidone in 100 mM acetic acid for 30 min at 37 °C. After washing with distilled water, the nitrocellulose was incubated with either 20 μg trypsin (GRK1) or chymotrypsin (GRK7) in 50 mM ammonium bicarbonate for 16 h at 37 °C. Following proteolysis, the reaction mixture was lyophilized and washed with distilled water by centrifugation in a SpeedVac. The GRK1 peptides were separated by thin layer electrophoresis (TLE) for 9–12 min at 1000 V in 0.126M ammonium bicarbonate buffer, pH 8.9, using a Hunter thin layer peptide mapping system (CBS Scientific, Del Mar, CA). The GRK7 peptides were separated by TLE in pH 1.9 buffer (88% formic acid:acetic acid:H2O, 25:78:897, v/v/v). The plates were dried and subjected to ascending TLC in chromatography buffer (butanol:acetic acid:pyridine:H2O:isobutyric acid, 2:3:5:29:65, v/v/v/v/v). The chromatography plates were dried and exposed to x-ray film to visualize the phosphopeptides.

RESULTS

Comparison of the Kinetic Parameters of GRK1 and GRK7—A comparison of the enzymatic activities of GRK1 and GRK7 may help distinguish their potential roles in cone opsin desensitization. FLAG-tagged human GRK1 and GRK7 were purified from transiently transfected HEK-293 cells by affinity chromatography as described under “Experimental Procedures.” The positions of the FLAG-tagged GRKs were confirmed by immunoblot analysis of the same sample using an anti-FLAG antibody (data not shown). GRK1 exhibited a slightly higher mobility than GRK7, although its predicted molecular size is slightly larger (64.8 kDa for FLAG-GRK1 and 63.5 kDa for FLAG-GRK7). Both kinases were composed of 2–3 bands, possibly due to heterogeneous isoprenylation or phosphorylation. The expressed proteins were ~70–80% pure (Fig. 1B). To measure the activities of these kinases in vitro, UROS membranes containing bovine rhodopsin were used as a model substrate. Rhodopsin was selected as a substrate because of its ease of isolation in large quantities from bovine retina. In contrast, the cone opsins cannot be isolated in sufficient amounts for these studies. The cone opsins possess serine/threonine-rich carboxyl termini that serve as substrates for phosphorylation (28, 29). Similarly, rhodopsin possesses seven serines and threonines in the carboxyl terminus that serve as substrates for GRK1 (5). To define the appropriate conditions for analyzing the activities of these kinases, a time course of rhodopsin phosphorylation by GRK1 and GRK7 was performed (Fig. 1C). The results demonstrated that phosphorylation reaches a plateau in ~60 min and the reaction is linear for at least 2 min (Fig. 1C, inset). Phosphorylation of rhodopsin in the dark was negligible over the 60-min time period (data not shown). Therefore, a 2-min assay was used for all subsequent experiments. The kinetic parameters of GRK1 and GRK7 were determined in vitro for ATP (Fig. 2A) and rhodopsin (Fig. 2B) and are summarized in Table I. GRK7 has a $K_m$ for ATP (21.4 μM) that is twice that of GRK1 (10.6 μM). The $K_m$ values for rhodopsin...
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were 1.9 $\mu M$ and 3.5 $\mu M$ for GRK7 and GRK1, respectively. GRK7 and GRK1 also display similar catalytic activities and phosphorylate rhodopsin with a $V_{\text{max}}$ of 916 and 1132 nmol/min/mg kinase, respectively. The $K_m$ values for ATP and rhodopsin reported here are similar to those reported by Pelczewski and colleagues using bovine GRK1 to phosphorylate bovine rhodopsin in membranes (30, 31). The affinity of GRK1 for detergent-solubilized rhodopsin reported by Thurmond et al. (32) is 10-fold higher (0.45 $\mu M$) than the affinity of GRK1 for rhodopsin measured in our experiments. Their studies also reported a lower $V_{\text{max}}$ for detergent-extracted rhodopsin. Although the use of rhodopsin in membranes represents a more physiological substrate than detergent-extracted proteins, the interpretation of such measurements is limited, due to several factors. For example, varying the amount of rhodopsin in the assay also varies the amount of membrane. Since these GRKs may partition into the membrane, the resulting $K_m$ values also may reflect changes that occur with increasing amounts of membrane. In addition, since all seven serines and threonines in bovine rhodopsin (six in human rhodopsin) have been shown to be phosphorylated in vivo and in vitro as well as in phosphorylation substrates (5, 33–34), it is likely that the rhodopsin in our assays represents a heterogeneously phosphorylated population. Nevertheless, our results indicate that GRK1 and GRK7 share similar enzymatic activities toward rhodopsin and ATP.

**GRK1 and GRK7 Are Substrates for PKA in Vitro**—Amino acid sequence analysis suggests that GRK1 and GRK7 have a number of potential PKA phosphorylation sites. In the absence of PKA, GRK1 and GRK7 are autophosphorylated, as reported previously (6, 36). When incubated with the catalytic subunit of PKA, wild-type GRK1 and GRK7 demonstrated increased levels of phosphorylation (Fig. 3A). Kinase-inactive GRK1 (K219R) and GRK7 (K220R) mutants were generated for comparison with the wild-type proteins to distinguish between autophosphorylation and phosphorylation by PKA. The corresponding kinase-inactive GRK2 mutant acts as a dominant negative and cannot phosphorylate the $\beta_2$-adrenergic receptor (37, 38). Similarly, the kinase-inactive mutants, K219R-GRK1 and K220R-GRK7, are unable to phosphorylate rhodopsin in vitro (data not shown). These mutants do not undergo auto-phosphorylation but can be phosphorylated by PKA (Fig. 3A). A comparison of the amino acid sequences of human GRK7 and GRK1 suggests that GRK7 has a conserved autophosphorylation site at Ser$^{490}$ (7). The mutants, S490A-GRK1 and S490E-GRK7, do not undergo autophosphorylation, indicating that Ser$^{490}$ is the only autophosphorylation site in GRK7 (Fig. 3B). Mutation of this serine to alanine or glutamic acid does not reduce phosphorylation by PKA (Fig. 3B). Therefore, the site(s) in GRK7 phosphorylated by PKA is distinct from the GRK7 autophosphorylation site.

There are also potential phosphorylation sites for PKCa and CaMKII in the sequences for GRK1 and GRK7. However, PKCa, a calcium and lipid-dependent Ser/Thr kinase present in rod outer segments (39–42), does not phosphorylate GRK1 or GRK7 in vitro (data not shown). Likewise, CaMKII, which is abundant in neural tissues (43) and fish photoreceptor cells (44), phosphorylates phosducin (45) but does not phosphorylate GRK1 or GRK7 in vitro (data not shown). PKCa and CaMKII phosphorylated the control substrates histone H1 and syntide 2, respectively, indicating that both kinases were active in our assay (data not shown).

**GRK1 and GRK7 Are Phosphorylated in Forskolin-stimulated HEK-293 Cells**—Phosphorylation of the wild-type proteins and the kinase-inactive mutants was assessed in transfected HEK-293 cells to determine whether cAMP-dependent phosphorylation of GRK1 and GRK7 occurs in intact cells. After labeling with $^{32}$P-orthophosphate, cells were treated with forskolin to elevate cAMP levels and activate PKA. Wild-type GRK1 and GRK7 immunoprecipitated from forskolin-treated cells had increased levels of phosphorylation in comparison with cells treated with vehicle alone (Fig. 4A). The kinase-inactive mutants, K219R-GRK1 and K220R-GRK7, were also phosphorylated in a forskolin-dependent manner. The expression levels of wild-type and mutant FLAG-tagged GRK1 and GRK7 were assessed by Western blot analysis using anti-FLAG monoclonal antibodies (Fig. 4B). The expression of K219R-GRK1 and K220R-GRK7 is reduced in comparison to the wild-type proteins, leading to the lower levels of phosphorylation by PKA observed for these mutants in Fig. 4A.

**PKA Phosphorylates GRK1 on Ser$^{21}$**—Using the computer program, NetPhos (www.cbs.dtu.dk/services/NetPhos) (46), amino acid sequence analysis of GRK1 predicts the presence of five potential PKA phosphorylation sites. Chemical cleavage of PKA-phosphorylated GRK1 into large peptides with either formic acid or iodosobenzoic acid revealed that GRK1 is phosphorylated on the amino-terminal domain between amino acid residues 1–128 (data not shown). This region of GRK1 contains two potential PKA phosphorylation sites, Ser$^{21}$ and Ser$^{33}$. GRK1 mutants S21A and S33A were compared with wild-type and kinase-inactive GRK1 (K219R-GRK1) by phosphopeptide mapping. The major autophosphorylation sites in GRK1 are Ser$^{488}$ and Thr$^{489}$ (31, 36); the tryptic phosphopeptide maps of

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**Fig. 2. Kinetic parameters of human GRK1 and GRK7.** A, the $K_m$ and $V_{\text{max}}$ for ATP was determined using 50 nM GRK1 or GRK7, 10 $\mu M$ rhodopsin, and the indicated concentrations of [γ-$^{32}$P]ATP (500–1000 cpm/pmol) ($n = 5$). B, the $K_m$ and $V_{\text{max}}$ for rhodopsin were determined using 50 nM GRK1 or GRK7, 300 $\mu M$ [γ-$^{32}$P]ATP (500–1000 cpm/pmol), and the indicated concentrations of rhodopsin ($n = 4$). Phosphorylation reactions were performed in triplicate for 2 min at 30 °C as described under “Experimental Procedures” and graphed as the average of the indicated number of experiments. Error bars represent S.E.
wild-type GRK1 (Fig. 5A) likely represent a mixture of mono-
phosphorylated and diphosphorylated peptides (peptides 1–3).
Tryptic phosphopeptide maps were also prepared from wild-
type and mutant GRK1 phosphorylated by PKA in vitro. Phos-
phorylation of wild-type GRK1 by PKA, generates an addi-
tional phosphopeptide, peptide 4 (Fig. 5B). The map of K219R-
GRK1 is missing peptides 1–3, since this kinase-inactive
mutant does not undergo autophosphorylation but displays
peptide 4 because it is phosphorylated by PKA (Fig. 5C). Al-
though S21A-GRK1 undertakes autophosphorylation (Fig. 5D),
it is not phosphorylated by PKA, as evident from the absence of
peptide 4 in Fig. 5E. Phosphorylation of GRK1-S33A by PKA
was similar to wild-type GRK1, indicating that Ser23 is not
a substrate for PKA (data not shown). Therefore, Ser23 appears
to be the only residue in GRK1 phosphorylated by PKA in vitro.

To determine whether GRK1 can be phosphorylated by PKA
at Ser23 in cells, HEK-293 cells expressing wild-type or mutant
GRK1 were radiolabeled with [32P]orthophosphate and treated
with forskolin or vehicle (Fig. 6). GRK1 immunoprecipitated
from cell lysates was digested with trypsin for phosphopeptide
digestion. Similar to the results of the in vitro phosphorylation
experiments in Fig. 5, autophosphorylation of wild-type GRK1
and S21A-GRK1 results in a mixture of monophosphorylated
and diphosphorylated phosphopeptides (peptides 1–3; Fig. 6, A,
B, D, and E), whereas K219R-GRK1 is not autophosphorylated
(Fig. 6C). Forskolin treatment increased levels of peptide 4 in
cells expressing wild-type GRK1 and K219R-GRK1 (Figs. 6, B
and C). In contrast, S21A-GRK1 does not display forskolin-de-
pendent phosphorylation (Fig. 6E), implicating Ser23 as the site
of phosphorylation by PKA. In the absence of forskolin, peptide
4 is detectable at very low levels in wild-type GRK1 (Fig. 6C),
due either to weak autophosphorylation of Ser23, as described
previously (36), or to basal activity of PKA in HEK-293 cells.
Collectively, these results demonstrate that GRK1 is phos-
phorylated by PKA at Ser23 in vitro and in a forskolin-dependent
manner in HEK-293 cells.

**PKA Phosphorylates GRK7 on Ser23** and Ser36**.—**To deter-
mine the sites on GRK7 that are phosphorylated by PKA, seven
potential substrate sites (identified by NetPhos as described
above) were mutated to Ala or Gln and the effect on phos-
phorylation of GRK7 by PKA measured in vitro. Comparison
of Ser23 and Ser36 single point mutants, S23A and S36A, with
wild-type GRK7 revealed a partial reduction in phosphoryla-
tion of the mutants by PKA (Fig. 7). Ser23 and Ser36 appear
to be the only PKA phosphorylation sites, since the GRK7 double
mutants, S23A/S36A and S23E/S36E, were not phosphorylated
by PKA (Fig. 7). In contrast, mutation of the other five potential
PKA phosphorylation sites to alanine (S333A, T353A, S441A,
S445A, and T537A) did not reduce PKA phosphorylation (data
not shown). These results demonstrate that GRK1 and GRK7
are phosphorylated by PKA at Ser23 and Ser36 by a partial
mechanism.

**TABLE I**

|          | ATP        | Rhodopsin | V<sub>max</sub> | k<sub>cat</sub> | k<sub>cat</sub>/K<sub>m</sub> |
|----------|------------|-----------|----------------|---------------|-----------------|
|          | μM        | μM        | nmol/min/mg    | s<sup>-1</sup> | s<sup>-1</sup> μM<sup>1</sup> |
| GRK7     | 21.4 ± 5.6 | 1.9 ± 0.4 | 9157 ± 64.5     | 1.0 ± 0.2     | 5.5 ± 0.4 × 10<sup>5</sup> |
| GRK1     | 10.6 ± 4.0 | 3.5 ± 0.7 | 1132.0 ± 84.2   | 1.2 ± 0.2     | 3.6 ± 0.3 × 10<sup>5</sup> |
| GRK1 (31)| 7 ± 2      | 4 ± 2     | 450 ± 52        | N/A           | N/A             |
| GRK1 (32)| N/A        | 0.45 ± 0.04| 39 ± 2         | 0.04         | 0.9             |

**Fig. 3.** Phosphorylation of GRK1 and GRK7 by PKA in vitro. A, wild-type (WT) GRK1 and GRK7 and kinase inactive mutants of GRK1 (K219R) and GRK7 (K220R) were incubated without (−) or with (+) the catalytic subunit of PKA and 150 μM [γ<sup>32P</sup>]ATP (500–1000 cpm/pmol) for 30 min at 30 °C. B, GRK7 autophosphorylation site mutants (S490A and S490E) were incubated with 150 μM [γ<sup>32P</sup>]ATP for 60 min at 30 °C in the absence (−) or presence (+) of PKA. The phosphorylated proteins were separated by SDS-PAGE, and phosphorylation was visualized by phosphorimage analysis.

**Fig. 4.** Phosphorylation of GRK1 and GRK7 by PKA in HEK-293 cells. A, wild-type (WT) and kinase-inactive GRK1 (K219R) and GRK7 (K220R) were expressed in HEK-293 cells and labeled with [32P]orthophosphate. Nontransfected (NT) cells were used as a negative control. Cells were treated with 25 μM forskolin in MeSO (±) or MeSO alone (−) for 15 min and FLAG-tagged GRKs were immunoprecipitated from cell lysates using anti-FLAG affinity resin. The phosphorylated proteins were separated by SDS-PAGE and visualized using phosphorimage analysis. B, Western blot analysis using an anti-FLAG monoclonal antibody was performed to compare the expression levels of wild-type and mutant GRK1 and GRK7.
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and Ser\textsuperscript{36} did not affect the intrinsic kinase activity of GRK7. Wild-type and mutant GRK7 were phosphorylated in vitro, digested with chymotrypsin, and analyzed by two-dimensional chromatography to confirm the identity of the phosphorylation sites. Autophosphorylation of wild-type GRK7 results in one phosphopeptide (peptide 1; Fig. 8A), consistent with our earlier finding that GRK7 possesses only one autophosphorylation site, Ser\textsuperscript{490} (Fig. 3B). Phosphorylation by PKA results in two prominent phosphopeptides (peptides 2 and 3; Fig. 8B) that are distinct from the phosphopeptide generated by autophosphorylation. Compared with wild-type GRK7, S23A-GRK7 (Fig. 8C) and S36A-GRK7 (Fig. 8D) each lack one of the PKA-dependent phosphopeptides (peptides 3 and 2, respectively), while the double Ala mutant (S23A/S36A-GRK7), which can be autophosphorylated (Fig. 8E), lacks both PKA-dependent phosphopeptides (Fig. 8G). These experiments confirm that Ser\textsuperscript{23} and Ser\textsuperscript{36} are phosphorylated by PKA in vitro.

Chymotryptic phosphopeptide maps derived from radiolabeled HEK-293 cells expressing GRK7 (Fig. 9) are similar to those derived from GRK7 phosphorylated in vitro (Fig. 8). Consistent with Ser\textsuperscript{490} as the sole autophosphorylation site in GRK7, only one phosphopeptide (peptide 1) was observed when wild-type GRK7 was isolated from vehicle-treated cells and subjected to phosphopeptide mapping (Fig. 9A). In contrast, maps of wild-type GRK7 isolated from forskolin-treated cells contained two additional phosphopeptides (peptides 2 and 3; Fig. 9B). Chymotryptic maps of the kinase-inactive mutant (K220R-GRK7) isolated from forskolin-treated cells lacked pep-

**FIG. 5.** Two-dimensional chromatography of GRK1 phosphorylated in vitro. Wild-type (WT) and mutant GRKs were phosphorylated as described under “Experimental Procedures” and digested with trypsin for phosphopeptide mapping. The tryptic phosphopeptides were separated by TLE and TLC as described under “Experimental Procedures.” Phosphorylation of wild-type GRK1 was examined in the absence (A) or presence (B) of PKA. Phosphorylation of K219R-GRK1 was examined in the presence of PKA (C), while phosphorylation of S21A-GRK1 was examined in the absence (D) or presence (E) of PKA. The phosphorylated peptides were detected by autoradiography. The asterisk indicates the peptide origin. The Δ symbol indicates the absence of specific phosphopeptides compared with wild-type GRK1.

**FIG. 6.** Two-dimensional chromatography of GRK1 phosphorylated in situ. Wild-type (WT) GRK1, S21A-GRK1, and K219R-GRK1 were expressed in HEK-293 cells. Cells were labeled with \[^{32P}\]orthophosphate and treated with MeSO\(_4\) (A and D) or 25 μM forskolin in MeSO\(_4\) (B, C, and E) for 15 min as described under “Experimental Procedures.” GRKs were immunoprecipitated from cell lysates with anti-FLAG monoclonal antibodies and analyzed by TLE and TLC as described under “Experimental Procedures.” The asterisk indicates the peptide origin. The Δ symbol indicates the absence of specific phosphopeptides compared with wild-type GRK1.

**FIG. 7.** Phosphorylation of wild-type (WT) and mutant GRK7. Phosphorylation of wild-type GRK7 was compared with mutant proteins possessing single (S23A, S36A, S23E, and S36E) or double (S23A/S36A and S23E/S36E) Ala or Glu substitutions at Ser\textsuperscript{23} and Ser\textsuperscript{36}. Wild-type and mutant GRKs were incubated with 10 μM \[^{32P}\]ATP (10 Ci/reaction) in the presence (+) or absence (−) of PKA for 1 h at 30 °C. Phosphorylated proteins were separated by SDS-PAGE and visualized by phosphorimaging analysis.
rhodopsin, suggesting that substitution of the negatively charged residue, glutamic acid, for serine mimicked phosphorylation by PKA. Both S21A-GRK1 and S21E-GRK1 activities were insensitive to phosphorylation by PKA, consistent with our observation that Ser21 is the only PKA phosphorylation site on GRK1. Since the levels of autophosphorylation are similar for wild-type GRK1, S21A-GRK1, and S21E-GRK1 (Fig. 10B), their intrinsic activities appear to be unaffected by mutagenesis.

The effect of phosphorylation by PKA on the activity of wild-type and mutant GRK7 was also examined using rhodopsin as a substrate. Phosphorylation by PKA inhibited the ability of wild-type GRK7 to phosphorylate rhodopsin by 50% (Fig. 11), similar to the effect of phosphorylation by PKA observed for GRK1. In comparison, S23A and S36A mutants were unaffected by preincubation with PKA. The double mutant, S23A/S36A-GRK7, phosphorylated rhodopsin at higher levels than the wild-type protein, whereas S23A-GRK7 and S36A-GRK7 had activities similar to wild-type GRK7. In contrast, S23E/S36E-GRK7 had reduced activity compared with wild type GRK7 and was also unaffected by a preincubation with PKA. The mutants S23E-GRK7 and S36E-GRK7 also had reduced activities compared with wild-type GRK7. The activities of the single and double S23E and S36E mutants suggest that the Glu substitutions of GRK7 mimic phosphorylation by PKA. Autophosphorylation of the GRK7 Ser23 and Ser36 mutants was reduced by 11%, similar to the effect of phosphorylation by PKA observed for GRK1 (Fig. 10A).

Several other proteins localized to photoreceptor cell outer segments have been shown to be substrates for PKA, including phosducin, RGS9–1, PDEα, and PDEβ (25, 47–49). Therefore, we were interested to know whether PKA present in rod outer segments would phosphorylate GRK1 and GRK7. Rod outer segments were isolated from dark-adapted frozen bovine retina

FIG. 8. Two-dimensional chromatography of GRK7 phosphorylated in vitro. Wild-type (WT) and mutant GRKs were phosphorylated as described in the legend to Fig. 7 and digested with chymotrypsin. The chymotryptic peptides were separated by TLE and TLC as described under “Experimental Procedures.” Wild-type GRK7 was phosphorylated in the absence (A) and presence (B) of PKA. S23A-GRK7 (C) and S36A-GRK7 (D) were phosphorylated in the presence of PKA. S23A/S36A-GRK7 was phosphorylated in the absence (E) and presence (F) of PKA. Phosphopeptides were visualized by autoradiography. The asterisk indicates the peptide origin. The ∆ symbol indicates the absence of specific phosphopeptides compared with wild-type GRK1.

FIG. 9. Two-dimensional chromatography of GRK7 phosphorylated in situ. Wild-type (WT) GRK7 (A and B) and K220R-GRK7 (C) were expressed in HEK-293 cells and labeled with [32P]orthophosphate as described under “Experimental Procedures.” Cells were treated with Me2SO (A) or 25 μM forskolin in Me2SO (B and C) for 15 min. GRKs were immunoprecipitated from cell lysates with anti-FLAG monoclonal antibodies, and the phosphorylated proteins were digested with chymotrypsin for analysis by TLE and TLC as described under “Experimental Procedures.” The asterisk indicates the peptide origin. The ∆ symbol indicates the absence of specific phosphopeptides compared with wild-type GRK1.

FIG. 10. Phosphorylation by PKA inhibits GRK1 activity. A, wild-type (WT) GRK1, S21A-GRK1, or S21E-GRK1 (50 nM, final concentration) were incubated with 300 μM [γ-32P]ATP (500–1000 cpm/pmnl in the absence (−) or presence (+) of PKA in vitro for 10 min at 30 °C prior to the addition of UROS (containing 2 μM rhodopsin, final concentration) in a 25 μl of reaction volume for 2 min. Levels of phosphorylation were quantified by Cerenkov counting. Phosphorylation of rhodopsin in the dark was subtracted from phosphorylation in the light and normalized to the levels of rhodopsin phosphorylation by wild-type GRK1 preincubated in the absence of ATP or PKA (−ATP/−PKA). The values for phosphorylation of wild-type and mutant GRK1 in the dark were ~101 ± 24 nmol/min/mg of kinase. In contrast, the values in the light range from 194 ± 29 to 548 ± 75. The results represent the average of three to four experiments. Error bars represent S.E. B, wild-type GRK1, S21A-GRK1, and S21E-GRK1 were incubated with [γ-32P]ATP for 60 min at 30 °C and examined by phosphorimaging analysis.
and incubated with [γ-32P]ATP and purified FLAG-tagged GRK1 or GRK7 in the presence or absence of Bt2cAMP (Fig. 12). Wild-type GRK1 and GRK7 demonstrated enhanced phosphorylation in the presence of Bt2cAMP. This phosphorylation was decreased in the presence of H-89, an inhibitor of PKA. The kinase-inactive mutants, K219R-GRK1 and K220R-GRK7, could only be phosphorylated in the presence of Bt2cAMP and phosphorylation was abolished in the presence of H-89. Western blot analysis demonstrated that equal amounts of FLAG-tagged GRK1 and GRK7 proteins were isolated from the reaction mixtures. These data indicate that GRK1 and GRK7 can be phosphorylated in rod outer segment extracts and provides evidence that this potential regulatory mechanism may be relevant in vivo.

**DISCUSSION**

In humans and monkeys, only GRK1 is expressed in rods, whereas cones express both GRK1 and GRK7 (7, 8, 50, 51). Deficiencies in their expression contribute to impaired vision observed in patients with Oguchi disease and enhanced S cone syndrome (9, 52). A biochemical comparison of GRK1 and GRK7 is critical to understanding their roles in desensitization in cones and may provide insight into the functional consequences of their disparate expression patterns in mammals (6–8). We have compared the $K_m$ and $V_{max}$ of GRK1 and GRK7 for rhodopsin and ATP and provide the first evidence that these two kinases are similar in their affinity and catalytic activity. Furthermore, our results for human GRK1 are similar to those previously reported for the phosphorylation of rhodopsin by bovine GRK1 (31). Although mammalian GRK1 is farnesylated in vivo (54), and GRK7 is predicted to be geranylgeranylated, based on the sequence of the carboxy-terminal CAXA motif (6, 7), our results suggest that differences in amino acid sequence (only 47% overall identity) and isoprenyl modification do not significantly affect their activities toward a model substrate. This conclusion is in agreement with that of Inglese and colleagues (55), who reported that exchange of the farnesyl for a geranylgeranyl CAXA motif on GRK1 alters membrane association but not the ability to phosphorylate rhodopsin.

We demonstrate for the first time that PKA phosphorylates serine residues on GRK1 and GRK7 in vitro, using either commercially supplied PKA catalytic subunit or isolated rod outer segments containing the PKA holoenzyme stimulated by Bt2cAMP. We have also shown that GRK1 and GRK7 expressed in HEK-293 cells are phosphorylated when the cells are treated with forskolin. PKCα and CaMKII, which phosphorylate the retinal-specific proteins RGS9–1 and phosducin, respectively (40, 45, 56), did not phosphorylate GRK1 or GRK7 in vitro (data not shown). Therefore, phosphorylation of GRK1 and GRK7 by PKA appears to be selective and suggests a novel mechanism for the regulation of these kinases by cAMP. Phosphorylation significantly decreases the ability of these kinases to phosphorylate rhodopsin. The sites phosphorylated by PKA, Ser21 in GRK1 and Ser23 and Ser36 in GRK7, are localized to similar positions in the amino termini (residues 1–192) (Fig 13). These domains share only 30% amino acid identity, whereas the catalytic and carboxy-terminal domains share 56 and 52% identity, respectively. Despite the low sequence identity in the amino termini, the similar positions of these PKA

![Figure 11](image1.jpg)

**Fig. 11.** Phosphorylation by PKA inhibits GRK7 activity. Wild-type (WT) GRK7 and Ser21 and Ser36 GRK7 mutants (50 nM, final concentration) in a 25-μl reaction volume for 2 min. Phosphorylation was evaluated as described in the legend for Fig. 10. The values for phosphorylation of wild-type and mutant GRK7 were $82 \pm 17 \text{ nmol/min/mg of kinase}$. In contrast, the values in the light ranged from $262 \pm 52 \text{ nmol/min/mg of kinase}$ to $591 \pm 64 \text{ nmol/min/mg of kinase}$ for wild-type and mutant GRK7. The results represent the average of 3–10 experiments. Error bars represent S.E.

![Figure 12](image2.jpg)

**Fig. 12.** cAMP-dependent phosphorylation of GRK1 and GRK7 by rod outer segments. FLAG-tagged wild-type (WT) GRK1 and the kinase-inactive mutant, K219R-GRK1 (A), or wild-type GRK7 and the kinase-inactive mutant, K220R-GRK7 (B), were incubated with bovine ROS prepared as described under “Experimental Procedures” in the presence (○) or absence (■) of dibutyryl cAMP (db-cAMP) and H-89, an inhibitor of PKA. Phosphorylation was performed in the dark for 30 min at 30 °C in the presence of 5 μM [γ-32P]ATP (30 μCi). The FLAG-tagged GRKs were immunoprecipitated from the ROS, separated by SDS-PAGE, and transferred to nitrocellulose. The level of phosphorylation was assessed by phosphorimage analysis, and the levels of GRK1 and GRK7 were assessed by Western blot analysis using the anti-FLAG monoclonal antibody, M2, and a chemiluminescent secondary antibody detection system as described under “Experimental Procedures.” Arrowheads represent the positions of FLAG-tagged GRK1 and GRK7.
phosphorylation sites suggest a common function. Substitution of Ser\(^{21}\) in GRK1 and both Ser\(^{23}\) and Ser\(^{36}\) in GRK7 with glutamic acid appears to mimic phosphorylation by PKA, resulting in reduced ability of these mutants to phosphorylate rhodopsin. In contrast, the alanine mutants, S21A-GRK1 and S23A/S36A-GRK7, phosphorylate rhodopsin to similar or higher levels than the wild type proteins. None of the glutamic acid or alanine mutants is phosphorylated by PKA, and PKA does not affect the ability of these mutants to phosphorylate rhodopsin, indicating that we have correctly identified the major phosphorylation sites. Collectively, these data suggest that phosphorylation of GRK1 and GRK7 by PKA inhibits phosphorylation of rhodopsin, possibly by disrupting their interaction with substrates. This hypothesis is consistent with observations by others that the amino-terminal domain of GRK1 regulates rhodopsin binding (57, 58). An alternative hypothesis, that phosphorylation of GRK1 and GRK7 by PKA reduces their intrinsic activities, is unlikely, since mutation of the phosphorylation sites to alanine or glutamic acid does not affect auto-phosphorylation of either kinase. Phosphorylation by PKA may also be a step in a larger regulatory mechanism involving calcium binding proteins such as recoverin, since the amino terminus of GRK1 is the site of negative regulation by recoverin (5). Phosphorylation may also control the binding of other, as yet unidentified, regulatory proteins. We cannot entirely rule out the possibility that cGMP-dependent protein kinase (PKG) phosphorylates GRK1 and GRK7 in the retina, because the consensus sequences for substrate phosphorylation are so similar for PKG and PKA (59). However, PKA is generally expressed at much higher concentrations than PKG in most cell types (59) and colocalizes with other photoreceptor cell substrates in rod outer segments (see below) (25, 47–49). We observed that forskolin, a specific activator of adenylyl cyclase, induces phosphorylation of these GRKs in HEK-293 cells and that H-89, an inhibitor that is 10-fold more selective for PKA over PKG (60), blocks Bt2cAMP-stimulated phosphorylation of GRKs using rod outer segments. These data strongly suggest that PKA functions to phosphorylate these GRKs. Future work, perhaps using conditional knock-outs of these two kinases in mice, would be necessary to determine whether PKG can phosphorylate these GRKs in vivo.

Several other photoreceptor cell proteins have been identified as PKA substrates, including phosducin, RGS9–1, PDE\(_{\alpha}\), and PDE\(_{\beta}\) (25, 47–49). Therefore, these proteins may be regulated by light-dependent changes in cAMP levels, which are well documented in vertebrate photoreceptor cells (12, 13, 15, 61). Calmodulin (CaM), activated by higher Ca\(^{2+}\) concentrations in the dark, stimulates CAMP synthesis by adenylyl cyclase Type I (AC1) (12, 14, 62, 63). Both phosducin and RGS9–1 demonstrate increased phosphorylation in the dark, consistent with the higher levels of cAMP in photoreceptor cells observed under these conditions (25, 48), although recent work on phosducin suggests that higher cAMP in the dark may not always involve regulation by Ca\(^{2+}\) (35). Rods and cones also display a circadian regulation of cAMP levels through a complex mechanism that has yet to be fully elucidated. For example, transcription of AC1 has been shown to be circadian in rat rods, demonstrating greater activity at night (65). Therefore, cAMP levels are generally higher at night and may enhance the phosphorylation of photoreceptor cell substrates by PKA. Another mechanism for circadian regulation of PKA activity may be via dopamine, the major catecholamine in the retina. Dopamine released from amacrine and interplexiform cells (66, 67) as part of the circadian rhythm in the retina (68–70) acts on rod and cone cells through D2/D4 dopamine receptors (70) to inhibit adenylyl cyclase activity and reduce cAMP levels during the day (13, 71–74). We propose that PKA phosphorylates GRK1 and GRK7 when cAMP levels in photoreceptor cell are elevated, either in direct response to the absence of light in photoreceptor cells or through circadian regulation as described above. Under these conditions, GRK1 and GRK7 would have reduced ability to phosphorylate their substrates. When the retina is exposed to light, cAMP levels fall, allowing PKA substrates in photoreceptor cells, including GRK1 and GRK7, to be dephosphorylated. This would presumably increase the activities of these GRKs, enhancing phosphorylation of their substrates. Since cAMP does not appear to affect phototransduction in lizard rods after a flash stimulus (75), this signaling pathway may be more important for photoreceptor cells to adapt to persistent, higher light intensities.

Recent studies have suggested that the subcellular localization and enzymatic activities of several other GRKs are regulated by phosphorylation (16–19, 76, 77). For example, PKA phosphorylates the carboxy terminal of GRK2 at Ser\(^{695}\), causing enhanced binding of GRK2 to G\(_{\beta\gamma}\) subunits and translocation of GRK2 to the membrane (78). PKC, Src, and extracellular signal-regulated kinase 1/mitogen-activated protein kinase (ERK1/2MAPK) also regulate the activities of some GRK family members (16, 79–86). The data presented here provide new insights into a potential mechanism for regulating the activities of GRK1 and GRK7 in response to light-dependent or circadian changes in Ca\(^{2+}\) and cAMP levels. For GRK1, the most likely substrate in rods is rhodopsin (5), while in cones GRK1 and GRK7 phosphorylate cone opsins, based on recent work from our own (10, 64) and other laboratories (53). However, the possibility that other GPCRs and nonreceptor proteins important in photoreceptor cell metabolism, survival, and function are substrates for these kinases should not be overlooked. Ongoing studies seek to uncover the role of phosphorylation by PKA in GRK1 and GRK7 function in the vertebrate retina.

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