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Rhodopsin Phosphorylation Sites and Their Role in Arrestin Binding*

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Rhodopsin, the rod cell photoreceptor, undergoes rapid desensitization upon exposure to light, resulting in uncoupling of the receptor from its G protein, transducin (G_t). Phosphorylation of serine and threonine residues located in the COOH terminus of rhodopsin is the first step in this process, followed by the binding of arrestin. In this study, a series of mutants was generated in which these COOH-terminal phosphorylation substrate sites were substituted with alanines. These mutants were expressed in HEK-293 cells and analyzed for their ability to be phosphorylated by rhodopsin kinase and to bind arrestin. The results demonstrate that rhodopsin kinase can efficiently phosphorylate other serine and threonine residues in the absence of the sites reported to be the preferred substrates for rhodopsin kinase. A correlation was observed between the level of rhodopsin phosphorylation and the amount of arrestin binding to these mutants. However, mutants T340A and S343A demonstrated a significant reduction in arrestin binding even though the level of phosphorylation was similar to that of wild-type rhodopsin. Substitution of Thr-340 and Ser-343 with glutamic acid residues (T340E and S343E, respectively) was not sufficient to promote the binding of arrestin in the absence of phosphorylation by rhodopsin kinase. When S343E was phosphorylated, its ability to bind arrestin was similar to that of wild-type rhodopsin. Surprisingly, arrestin binding to phosphorylated T340E did not increase to the level observed for wild-type rhodopsin. These results suggest that 2 amino acids, Thr-340 and Ser-343, play important but distinct roles in promoting the binding of arrestin to rhodopsin.

Receptor desensitization is a critical process in the regulation of G protein-coupled receptor signaling pathways. Serine and threonine residues located either in the COOH terminus or in the third cytoplasmic loop of these receptors serve as substrates for phosphorylation by members of the G protein-coupled receptor kinase family. The G protein-coupled receptor kinases are unique serine/threonine kinases that phosphorylate only the ligand-activated form of G protein-coupled receptors (1, 2). Phosphorylation is followed by the binding of arrestin to the receptor, resulting in rapid termination of G protein activation (3–5). The physiological importance of rapid receptor desensitization has been demonstrated directly in studies of the visual signal transduction system, in which expression of a truncated form of rhodopsin missing the phosphorylation sites and a selective reduction in the levels of arrestin both lead to extended rhodopsin activity (6, 7).

Rhodopsin, the photoreceptor of the rod cell, has been used extensively as a model for investigating the regulation of G protein-coupled receptor desensitization (4). As many as 7 serines and threonines located in the COOH terminus of rhodopsin are substrates *in vitro* for rhodopsin kinase, the rod cell G protein-coupled receptor kinase, when rhodopsin is activated by light (8). However, several studies have suggested that only 1–2 phosphates are incorporated into rhodopsin *in vivo*. The binding of rod cell-specific arrestin to light-activated phosphorylated rhodopsin and the reduction of all-*trans*-retinal to all-*trans*-retinol after light exposure appear to prevent higher levels of phosphorylation (9, 10). The preferred sites of phosphorylation have been reported to be Ser-334, Ser-338, and Ser-343 depending on whether experiments were performed *in vivo*, *in vitro*, or with synthetic peptides as substrates for rhodopsin kinase (9–16). Phosphorylated rhodopsin induces a conformational change in arrestin that promotes its binding to light-activated rhodopsin, interfering with the ability of rhodopsin to activate its G protein, G_t (17, 18). However, the requirement for specific phosphorylation sites for arrestin binding has not been addressed.

In this study, site-directed mutants containing alanine substitutions for the COOH-terminal serine and threonine residues of rhodopsin were expressed in HEK-293 cells and examined for their ability to be phosphorylated and to bind arrestin. The results indicate that rhodopsin kinase can efficiently phosphorylate other serine and threonine residues in the absence of its preferred substrate residues (Ser-334, Ser-338, and Ser-343). A correlation was observed between the levels of rhodopsin phosphorylation and the amount of arrestin binding to the mutants. Two amino acids, Thr-340 and Ser-343, were found to be particularly critical for efficient arrestin binding to rhodopsin, but they may play different roles in promoting this process.

EXPERIMENTAL PROCEDURES

Materials—The mammalian cell expression vector pcDNA1/Amp was purchased from Invitrogen. An expression vector for SV40 T antigen (pRSV-TAg) and the cDNA for bovine opsin were gifts from Dr. Jeremy Nathans. The cDNA for bovine arrestin was a gift from Dr. Toshimichi Shinohara. 11-*cis*-Retinal was a gift from Hoffmann-La Roche. The monoclonal antibody R2-15N, which recognizes the NH_2-terminal 15 amino acids of bovine rhodopsin (19), was kindly provided by Dr. Paul Hargrave. Frozen bovine retinas were obtained from J. A. Lawson Inc. (Lincoln, NB). HEK-293 cells were purchased from American Type Culture Collection. [^{32}P]ATP and [^{35}S]methionine were from Amersham Corp. The rabbit reticulocyte lysate system for *in vitro* synthesis of bovine arrestin was purchased from Promega. The Bio-Spin columns were from Bio-Rad.

Mutagenesis—The cDNA for bovine rhodopsin (20) was inserted into the *Hin*dIII site of the vector pSelect (Promega) to generate a single-

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stranded DNA template for mutagenesis (21). Mutations in the COOH terminus of rhodopsin were made using a Promega Altered Sites mutagenesis kit as described previously (21). The mutants were sequenced for verification using Sequenase (Amersham Corp.) according to the manufacturer's directions.

Expression of Rhodopsin Mutants—Mutants were transiently expressed in HEK-293 cells using DEAE-dextran-mediated transfection as described previously (21). HEK-293 cells were cotransfected with plasmids pRSV-TAg and pcDNA1/Amp containing the rhodopsin cDNA. Approximately 65–70 h after transfection, membranes were prepared by sucrose density gradient centrifugation (22, 21). Protein concentrations were determined as described by Bradford (23). The mutants were analyzed for their ability to activate G_t and to bind 11-*cis*-retinal using methods described previously (21, 24).

Western Blotting and Electrophoresis—Rhodopsin expressed in HEK-293 cells was analyzed by Western blotting using monoclonal antibody R2-15N. Details for immunoblotting and electrophoresis have been described previously (25). The level of rhodopsin expression was quantified using a Molecular Dynamics PhosphorImager. Because of the heterogeneity observed in the mobility of the expressed rhodopsin, due to multiple forms of glycosylation, the entire lane was measured for each sample (24). After subtraction of a background estimated from a lane of nontransfected cell membranes, the amount of rhodopsin was calculated using rod outer segment rhodopsin as a standard. A 62-kDa band was observed in Western blots of wild-type rhodopsin expressed in HEK-293 cells (21, 24, 26) and in mutants T340E/S343E, T340E, and S343E, but was reduced in the alanine-containing mutants (data not shown). This band, which accounts for ~10% of the total rhodopsin, was not phosphorylated (data not shown) and was considered to be inactive. Therefore, the amount of rhodopsin represented by this band was subtracted from the total estimated by immunoblot analysis.

Purification of Rod Outer Segment Proteins—Urea-stripped rod outer segment membranes were purified from frozen, dark-adapted bovine retinas as described previously (27). Rhodopsin kinase was prepared as a crude extract from light-exposed rod outer segment membranes (28, 29).

Phosphorylation of Rhodopsin by Rhodopsin Kinase—Phosphorylation of bovine rhodopsin expressed in HEK-293 cells was performed using methods similar to those described by Shi *et al.* (24). HEK-293 cell membranes expressing bovine rhodopsin were reconstituted with 14 μM 11-*cis*-retinal for 1 h at room temperature in the dark. The phosphorylation reaction was initiated by the addition of rod outer segment extract containing rhodopsin kinase (~20 μl) to a 70-μl reaction mixture consisting of the HEK-293 cell membranes, 10 mM Tris-HCl, pH 7.4, 260 mM NaCl, 5 mM $MgCl_2$, 0.125 mM EDTA, 0.125 mM EGTA, 2 mM dithiothreitol, 500 nM okadaic acid, and 150 μM [γ-^{32}P]ATP (300 μCi/ml). The amount of rhodopsin in each reaction was 0.33 μg unless otherwise specified. The amount of total membrane protein in each sample was equalized by the addition of nontransfected HEK-293 cell membranes. The reaction mixture was incubated in the dark under Eastman Kodak No. 2 safelights or under fluorescent room light for 8 min unless otherwise indicated in the figure legends. The time of 8 min was chosen because the progress of the reaction is close to linear while still giving a detectable signal (24). The reaction was terminated by placing the samples on ice for 2 min, followed by the addition of 1 ml of ice-cold buffer containing 0.1 mM Tris-HCl, pH 7.5, 50 mM NaF, and 10 mM ATP (buffered to pH 7.5 with Tris-HCl). After centrifugation at 12,000 × *g* for 15 min at room temperature, the pellets were dissolved in 10 mM Tris-HCl, pH 7.4, and 150 mM NaCl (TBS) containing 1.5% octyl glucoside, and the reaction mixture was centrifuged to remove insoluble material. The rhodopsin in the supernatant was immunoprecipitated by incubation with the R2-15N monoclonal antibody and protein A-Sepharose beads for 1 h each at room temperature. After three washes in TBS containing 0.1% sodium deoxycholate and 50 mM NaF, the protein was extracted from the beads with Laemmli SDS sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (30). The level of phosphorylation was quantified by PhosphorImager analysis of the dried gels. After subtraction of the amount of phosphorylation in samples incubated in the dark from the amount obtained in samples exposed to light, the data were normalized to the level of phosphorylation of wild-type rhodopsin. To determine the stoichiometry of the phosphate incorporated into wild-type rhodopsin, the lanes of the gels were cut, and the radioactivity was measured by liquid scintillation spectroscopy. During 8–10-min reactions, ~0.4 ± 0.2 mol (S.E.) of phosphate/mol of wild-type rhodopsin is incorporated.

Arrestin Binding—For the arrestin binding studies, HEK-293 cell membranes containing bovine rhodopsin were prephosphorylated by rhodopsin kinase in a buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM ATP, and 6 mM $MgCl_2$ at 30 °C for 1 h under fluorescent light or in the dark, as described for each experiment in the figure legends (31, 32). The level of phosphorylation ranged from 0.4 to 0.6 mol/mol of wild-type rhodopsin. Equal amounts of rhodopsin were used for each mutant. To correct for differences in the expression levels of the various mutants, nontransfected cell membranes were added so that the amount of total protein was also the same for each sample. After a 1-h incubation with rhodopsin kinase to phosphorylate the rhodopsin, the reaction was diluted with 1 ml of ice-cold buffer containing 20 mM Tris-HCl, pH 7.5, and 2 mM EDTA (Buffer A). The membranes phosphorylated by rhodopsin kinase were washed twice with Buffer A by centrifugation at 12,000 × *g* for 15 min. The rhodopsin was regenerated with 14 μM 11-*cis*-retinal as described above.

The cDNA for bovine arrestin (33) was inserted into pSP73 (Promega) and transcribed *in vitro* using T7 RNA polymerase. The synthesized arrestin RNA was translated *in vitro* using rabbit reticulocyte lysate at 30 °C for 1 h in the presence of 2 μl of [^{35}S]methionine (1200 μCi/ml) in a final volume of 25 μl according to the manufacturer's instructions. The synthesized product was centrifuged through a Bio-Spin 6 chromatography column to remove unincorporated [^{35}S]methionine and to exchange the buffer for one containing 30 mM HEPES, 2 mM $MgCl_2$, and 150 mM potassium acetate, pH 7.5 (Buffer B). The amount of synthesized arrestin was estimated from the amount of radioactive methionine incorporated into a hot trichloroacetic acid-insoluble fraction measured by liquid scintillation spectroscopy (34). Approximately 10 fmol of arrestin was used in the arrestin binding assay unless otherwise noted.

The arrestin binding assay was performed using methods similar to those described by Gurevich and Benovic (31, 32). The radiolabeled arrestin and the membranes containing phosphorylated bovine rhodopsin regenerated with 11-*cis*-retinal were incubated together in Buffer B at 37 °C for 5 min under fluorescent room light. The samples contained 0.1–1.0 μg of rhodopsin as noted in the figure legends. The reaction was terminated by dilution with 200 μl of ice-cold Buffer B. The reaction mixture was layered over a 200-μl cushion of 0.2 M sucrose in Buffer B and centrifuged at 100,000 × *g* for 30 min at 2 °C. After washing with Buffer B, the membrane pellets containing bound arrestin were dissolved in Laemmli SDS sample buffer and chromatographed on 10% SDS-polyacrylamide gels. The amount of arrestin bound to rhodopsin was quantified by PhosphorImager analysis. The amount of arrestin bound to nonphosphorylated rhodopsin was subtracted from the amount bound to phosphorylated rhodopsin. The results were normalized to the amount of arrestin bound to wild-type rhodopsin.

Statistical and Kinetic Analyses—Statistical tests were performed using the Macintosh computer program Statview (Abacus Concepts, Inc.). Apparent K_d and apparent B_{max} values (referred to below as K_d and B_{max}) were determined by nonlinear regression analysis of the arrestin binding data using the computer program Prism (GraphPad Software, Inc.).

RESULTS

Phosphorylation of and Arrestin Binding to Rhodopsin Mutants Expressed in HEK-293 Cells—The COOH-terminal 7 serine and threonine residues within the rhodopsin sequence Ser-334–Ser-343 have been proposed to be substrates for rhodopsin kinase *in vitro* (Fig. 1) (8). To investigate the ability of these serine and threonine residues to be phosphorylated by rhodopsin kinase and to evaluate their importance for arrestin binding, we compared the phosphorylation of wild-type rhodopsin with that of phosphorylation site mutants expressed in HEK-293 cells and examined the ability of these mutants to bind arrestin.

An assay was developed to measure the binding of *in vitro* translated arrestin to rhodopsin expressed in HEK-293 cells. Fig. 2*A* demonstrates that the binding of arrestin to HEK-293 cell membranes containing light-exposed wild-type rhodopsin is phosphorylation-dependent; the binding of arrestin to phosphorylated rhodopsin is typically ~10 times the level of binding to nonphosphorylated rhodopsin. Fig. 2*A* also shows that arrestin does not bind significantly to membranes from nontransfected cells. Previously, our laboratory developed an assay to measure the light-dependent phosphorylation of rhodopsin mutants by rhodopsin kinase (24). This assay was used to determine whether the sites phosphorylated by rhodopsin kinase

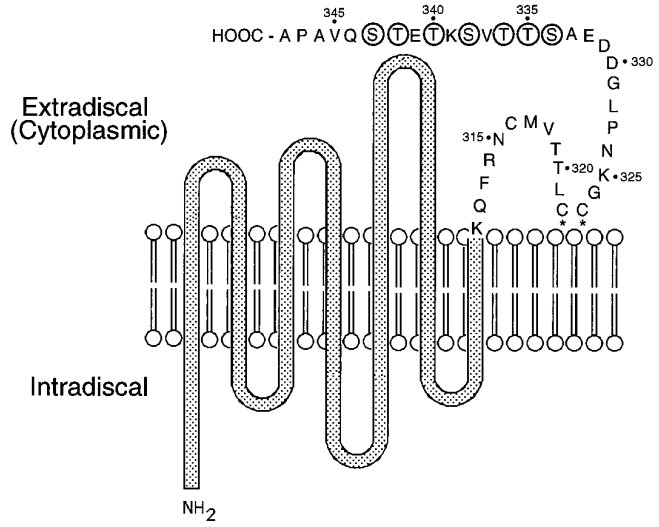

FIG. 1. **COOH terminus of bovine rhodopsin.** The *circled* amino acids represent the serine and threonine residues that are substrates for phosphorylation by rhodopsin kinase (3). Cys-322 and Cys-323 are marked with *asterisks* to indicate the sites of palmitoylation.

that promote arrestin binding are restricted to the COOH terminus. The rhodopsin mutant K325stop, in which Lys-325 is replaced by a stop codon causing a deletion of the COOH-terminal 24 amino acids (21), was expressed in HEK-293 cells and analyzed for its ability to be phosphorylated and to bind arrestin. The light-dependent phosphorylation of K325stop is <2% of the level observed for wild-type rhodopsin (Fig. 2*B*). This result is consistent with previous studies in which the removal of the COOH-terminal 21 amino acids by proteolysis abolished the phosphorylation of rhodopsin by rhodopsin kinase (35, 36). Our experiments also demonstrate that K325stop does not bind arrestin (Fig. 2*C*). Therefore, the phosphorylation sites that promote arrestin binding to rhodopsin are restricted to these COOH-terminal 7 serine and threonine residues.

To determine whether these phosphorylation sites differ in their ability to promote arrestin binding, a series of mutants was generated in which the 7 serine and threonine residues were substituted with alanines. Alanine was chosen because it is a neutral amino acid and is less likely to disrupt protein secondary structure (37–39). Initially, S334A/T335A/T336A, S338A/T340A, and T342A/S343A were generated to divide the 7 serine and threonine residues into three mutants (Fig. 3). The mutants were assayed for their ability to be phosphorylated by rhodopsin kinase and to bind arrestin. The triple mutant S334A/T335A/T336A demonstrated only an 18% decrease in phosphorylation, whereas phosphorylation was reduced by 40 and 33% for mutants S338A/T340A and T342A/S343A, respectively (Fig. 4*A*). S334A/T335A/T336A showed an 18% reduction in arrestin binding compared with wild-type rhodopsin (Fig. 4*B*). In contrast, S338A/T340A and T342A/S343A exhibited an 87% and a 68% reduction in arrestin binding, respectively, suggesting that the phosphorylation sites critical for promoting arrestin binding to rhodopsin are located within the sequence containing Ser-338, Thr-340, Thr-342, and Ser-343.

Based on these results, additional double amino acid mutants, S338A/S343A and T340A/S343A; the single amino acid mutants S338A, T340A, T342A, and S343A; and two mutants with five alanine substitutions, STTST and STTTT (Fig. 3), were constructed. A comparison of all of the mutants revealed that the level of phosphorylation decreased approximately in proportion to the number of remaining phosphorylation sites (Fig. 4*A*). The double amino acid mutants showed a greater decrease in phosphorylation than the single amino acid mutants. STTST, which is missing all of the phosphorylation sites except for Thr-340 and Ser-343, and STTTT, which is missing all of the COOH-terminal serines and threonines except for Ser-338 and Ser-343, showed the greatest reduction in phosphorylation, 60 and 64%, respectively. The single exception was the triple amino acid mutant S334A/T335A/T336A, which demonstrated a level of phosphorylation that was within the range of the single amino acid mutants.

These mutants were also tested for their ability to bind arrestin (Fig. 4*B*). All four mutants containing two alanine substitutions showed reduced arrestin binding compared with wild-type rhodopsin. For the single amino acid mutants, T340A and S343A exhibited a 55% and a 30% reduction, respectively, whereas S338A and T342A showed no significant decrease in arrestin binding. These data indicate that Thr-340 and Ser-343 are important for the binding of arrestin to rhodopsin. STTTT showed the greatest decrease (94%) in arrestin binding. The level of arrestin binding was plotted against the level of phosphorylation for each mutant to determine whether a correlation between these two properties could be observed (Fig. 5). Although there is variability within groups missing the same number of phosphorylation sites, the results demonstrate that arrestin binding increases as the level of phosphorylation increases. A Pearson correlation coefficient of 0.83 was calculated from these data, indicating that 69% (0.83^2) of the variation in arrestin binding among the different mutants is due to differences in phosphorylation (40). These data suggest that the level of phosphorylation has a significant influence on the level of arrestin binding.

The reduced binding of arrestin to T340A and S343A could be due either to a reduced affinity for rhodopsin or to a reduction in the number of binding sites. To distinguish between these possibilities, the amount of arrestin bound to T340A and S343A was measured as a function of arrestin concentration (Fig. 6). The K_d values (means ± S.E. of five to six experiments performed in duplicate) for T340A and S343A were 0.91 ± 0.12 and 1.0 ± 0.3 nM, respectively, similar to the value of 0.74 ± 0.11 nM for wild-type rhodopsin. Therefore, these rhodopsin mutants display only small changes in affinity for arrestin. In contrast, the number of binding sites (B_{max}) for the mutants was dramatically reduced for the binding of arrestin to the mutants compared with binding to wild-type rhodopsin. For the experiment shown in Fig. 6, the B_{max} values were 7.8 and 5.9 fmol for T340A and S343A, respectively, compared with 11.9 fmol for wild-type rhodopsin. Although the absolute values for B_{max} varied considerably between experiments, the values for the mutants were always significantly lower than those for wild-type rhodopsin; for T340A and S343A, the B_{max} values (±S.E.) were 50.1 ± 0.1 and 48.8 ± 0.1%, respectively, of the values for wild-type rhodopsin. These data indicate that mutation of Thr-340 and Ser-343 to alanine results in a change in B_{max} rather than a change in affinity for arrestin.

Phosphorylation of and Arrestin Binding to Glutamic Acid-substituted Rhodopsin Mutants—The negatively charged amino acids glutamic acid and aspartic acid have been shown to successfully mimic phosphorylated serine and threonine residues (41–44). To determine whether a negative charge at Thr-340 or Ser-343 is sufficient to promote arrestin binding, mutants T340E, S343E, and T340E/S343E were constructed (Fig. 3). Compared with T340A/S343A, which showed a 43% reduction in phosphorylation, T340E/S343E exhibited a level of phosphorylation only 19% lower than that of wild-type rhodopsin (Fig. 4*A*). This appeared to be due to an increased rate of phosphorylation (Fig. 7, *A* and *B*), suggesting that negative charges at these positions enhance the ability of rhodopsin kinase to phosphorylate the remaining serine and threonine

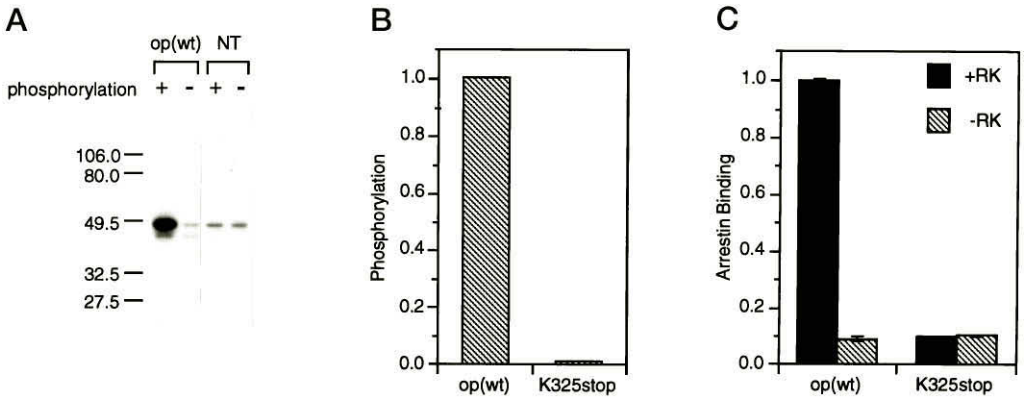

FIG. 2. **Phosphorylation of and arrestin binding to wild-type rhodopsin and the mutant K325stop.** *A*, autoradiogram of arrestin binding to HEK-293 membranes containing wild-type rhodopsin (1 μg; *op(wt)*) or to nontransfected cell membranes (*NT*). The binding of [^{35}S]methionine-labeled, *in vitro* translated arrestin to membranes phosphorylated by rhodopsin kinase (+) or to nonphosphorylated membranes (−) was followed by SDS-polyacrylamide gel electrophoresis and autoradiography as described under "Experimental Procedures." *B*, PhosphorImager analysis of the phosphorylation of wild-type rhodopsin and the truncation mutant K325stop by rhodopsin kinase. HEK-293 cell membranes containing 1 μg of wild-type rhodopsin or K325stop were reconstituted with 11-*cis*-retinal and incubated with 2 mM [^{32}P]ATP and rhodopsin kinase in the light or in the dark for 60 min, followed by SDS-polyacrylamide gel electrophoresis. The level of phosphorylation was quantified by PhosphorImager analysis of the dried gels. The amount of phosphorylation in the dark was subtracted from the amount of phosphorylation in the light, and the results for K325stop were normalized to the level of light-dependent phosphorylation for wild-type rhodopsin. The data represent the average of duplicates from three similar experiments from three different transfections. The stoichiometry of phosphorylation was ~0.6 mol of phosphate/mol for wild-type rhodopsin and 0.01 mol of phosphate/mol for the K325stop mutant. *C*, PhosphorImager analysis of the binding of arrestin to wild-type rhodopsin and the mutant K325stop phosphorylated as described for *B*. *+RK*, membranes phosphorylated by rhodopsin kinase; *−RK*, nonphosphorylated membranes. Arrestin binding was performed as described under "Experimental Procedures." After SDS-polyacrylamide gel electrophoresis, the level of binding was quantified by PhosphorImager analysis, and the results were normalized to the amount of arrestin bound to wild-type rhodopsin. The data are representative of three experiments. *Error bars* represent the range of duplicates from a single experiment.

	330				335					340				345					
op(wt)	D	D	E	A	S	T	T	V	S	K	T	E	T	S	Q	V	A	P	A
S334A/T335A/T336A	-	-	-	-	A	A	A	-	-	-	-	-	-	-	-	-	-	-	-
S338A/T340A	-	-	-	-	-	-	-	-	A	-	A	-	-	-	-	-	-	-	-
T342A/S343A	-	-	-	-	-	-	-	-	-	-	-	-	A	A	-	-	-	-	-
S338A/S343A	-	-	-	-	-	-	-	-	A	-	-	-	-	A	-	-	-	-	-
T340A/S343A	-	-	-	-	-	-	-	-	-	-	A	-	-	A	-	-	-	-	-
S338A	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-
T340A	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-
T342A	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-
S343A	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-
STTST	-	-	-	-	A	A	A	-	A	-	-	-	A	-	-	-	-	-	-
STTTT	-	-	-	-	A	A	A	-	-	-	A	-	A	-	-	-	-	-	-
T340E	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-
S343E	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-
T340E/S343E	-	-	-	-	-	-	-	-	-	-	E	-	-	E	-	-	-	-	-

FIG. 3. **Mutations in the COOH terminus of bovine rhodopsin.** *A*, alanine substitutions; *E*, glutamic acid substitutions. The *dashed lines* represent amino acid sequence identical to that of wild-type rhodopsin (*op(wt)*).

residues. The levels of phosphorylation for T340E and S343E were similar to those for the corresponding alanine mutants, T340A and S343A (Fig. 4*A*).

Studies of mutants T340E/S343E, S343E, and T340E demonstrated that negative charges at these positions alone are not sufficient to promote arrestin binding in the absence of phosphorylation by rhodopsin kinase (data not shown). However, after phosphorylation by rhodopsin kinase, T340E/S343E and S343E were able to bind arrestin as efficiently as wild-type rhodopsin (Fig. 4*B*). In contrast, arrestin binding to T340E, which was 47% lower than the binding to wild-type rhodopsin, was not significantly different from the binding to T340A. Therefore, Thr-340 may require a higher negative charge than Ser-343 to promote arrestin binding. Alternatively, the role of Thr-340 in arrestin binding may not be related to its ability to serve as a substrate for phosphorylation.

DISCUSSION

We have examined the phosphorylation of the 7 serine and threonine residues located in the COOH terminus of rhodopsin and analyzed their role in the binding of arrestin using the approach of *in vitro* mutagenesis. All of the mutants expressed in HEK-293 cells were able to activate G_t and displayed normal 11-*cis*-retinal binding (data not shown), indicating that the normal structure of rhodopsin is preserved in these mutants. Despite reports that all 7 residues can be phosphorylated *in vitro* (3), studies *in vivo* and in retinal homogenates have suggested that only 3 residues (Ser-334, Ser-338, and Ser-343) actually serve as phosphorylation sites (9, 11). *In vivo* and *in vitro* studies differ somewhat in their identification of the primary site of phosphorylation. *In vivo*, Ser-338 has been reported to be the primary site after a flash of light, whereas Ser-334 is the main phosphorylated residue in continuous light, possibly due to the slower rate of dephosphorylation of Ser-334 (10, 11). *In vitro* experiments using rod outer segment proteins also suggested that Ser-338 is the major substrate site, followed by Ser-343 (13–15). However, when synthetic peptides corresponding to the rhodopsin COOH terminus are used as phosphorylation substrates, rhodopsin kinase prefers Ser-343 (12, 16). Our results demonstrate that rhodopsin kinase can phosphorylate the COOH terminus almost as efficiently when any of these preferred residues are missing, suggesting that there is little selectivity for different substrate sites. As expected, reduced phosphorylation is observed as increasing numbers of these COOH-terminal sites are replaced with alanines. The single exception is the triple mutant S334A/T335A/T336A, which showed a smaller decrease in phosphorylation than would be expected from the substitution of 3 residues with alanines. These 3 amino acids in wild-type rhodopsin may not be phosphorylated efficiently in our assay system. Alternatively, they may be phosphorylated in a light-independent manner by a kinase such as protein kinase C during expression in HEK-293 cells. Protein kinase C has been reported to phosphorylate rhodopsin within the sequence Ser-334–Thr-335–Thr-336 (45).

In general, there appeared to be a correlation between the level of phosphorylation and the level of arrestin binding. Approximately 69% of the variability in arrestin binding was estimated by statistical analysis to be due to differences in

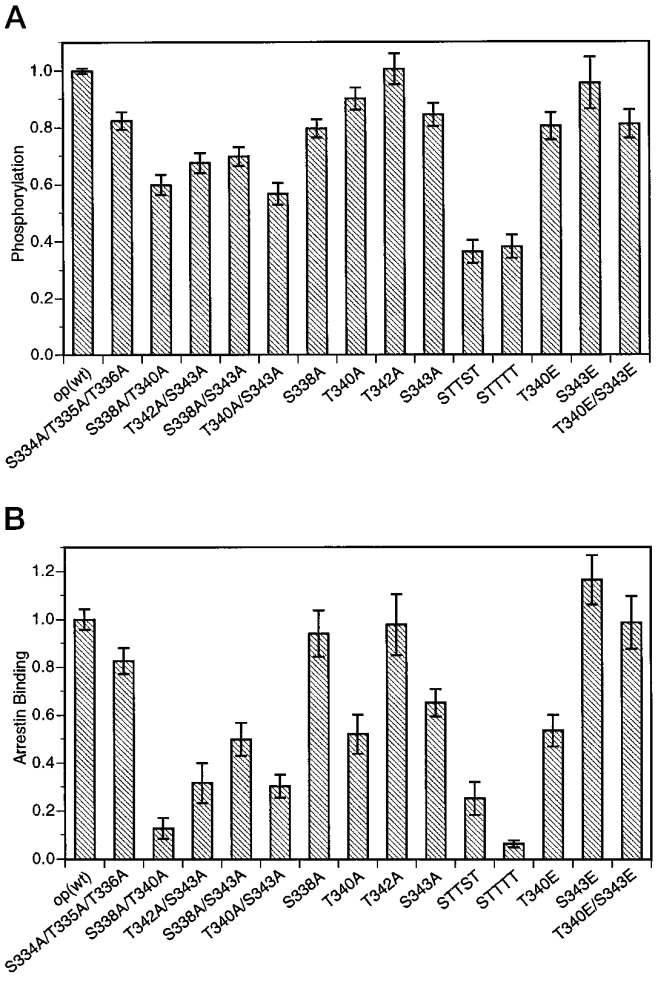

FIG. 4. **Phosphorylation of and arrestin binding to rhodopsin mutants.** *A*, phosphorylation. Membranes prepared from transfected HEK-293 cells were reconstituted with 11-*cis*-retinal and phosphorylated by rhodopsin kinase for 8 min. The results were quantified by PhosphorImager analysis as described under "Experimental Procedures" and normalized to the level of phosphorylation for wild-type rhodopsin (*op(wt)*). The data represent the averages of two to nine experiments, each performed in duplicate, from at least two transfections. *Error bars* represent S.E. *B*, arrestin binding. The membranes prepared from transfected HEK-293 cells were reconstituted with 11-*cis*-retinal and phosphorylated by rhodopsin kinase for 1 h as described under "Experimental Procedures." Arrestin binding was performed as described under "Experimental Procedures" using 1 μg (25 pmol) of rhodopsin and 10 fmol of arrestin per assay. The results were normalized to the level of arrestin binding for wild-type rhodopsin. The data represent the averages of two to five experiments performed in duplicate from at least two transfections. *Error bars* represent S.E.

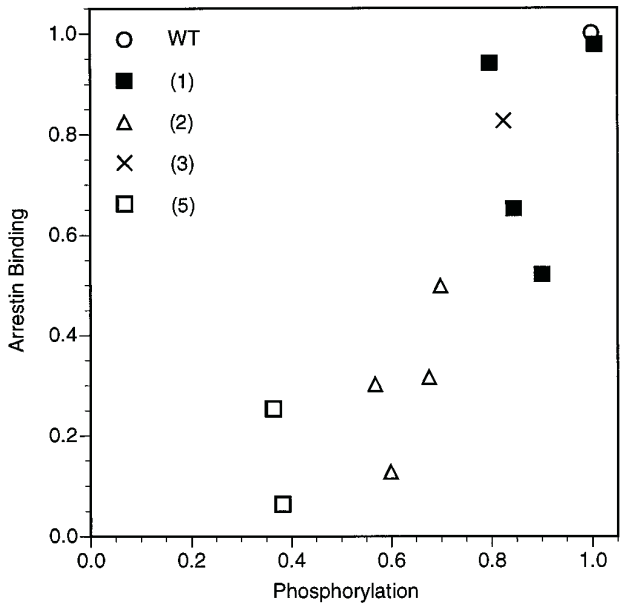

FIG. 5. **Correlation between phosphorylation by rhodopsin kinase and arrestin binding.** The data shown in Fig. 4 were replotted to analyze the relationship between the level of phosphorylation by rhodopsin kinase and the amount of arrestin binding. The *numbers* in *parentheses* for each symbol represent the number of mutated phosphorylation sites. *WT*, wild-type rhodopsin. A Pearson correlation test was performed, resulting in a correlation coefficient (r) of 0.833.

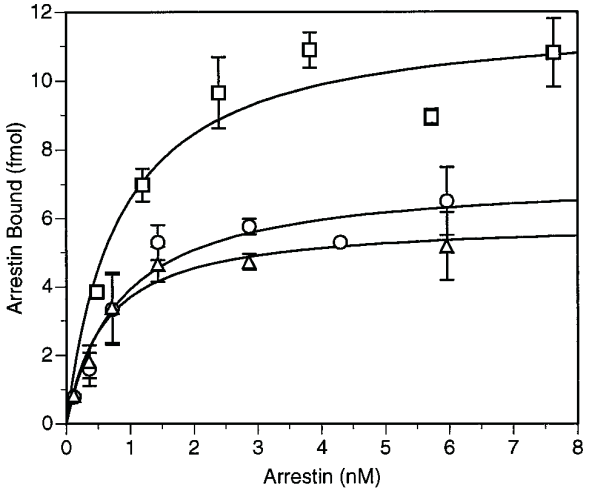

FIG. 6. **Arrestin binding to wild-type rhodopsin and the rhodopsin mutants T340A and S343A.** HEK-293 cell membranes containing 2.5 pmol (0.1 μg) of rhodopsin phosphorylated by rhodopsin kinase as described in the legend to Fig. 2*B* were incubated with varying amounts of [^{35}S]methionine-labeled arrestin (4.8–305.3 fmol) in a reaction volume of 40 μl under the conditions described under "Experimental Procedures." The results are representative of four to five independent experiments, each performed in duplicate. □, wild-type rhodopsin; ○, T340A; △, S343A. The K_d values for this experiment were 0.81, 0.95, and 0.60 nM for wild-type rhodopsin, T340A, and S343A, respectively. The B_{max} values were 11.9, 7.8, and 5.9 fmol, respectively.

phosphorylation. The lowest levels of arrestin binding were exhibited by mutants such as STTST and STTTT, which displayed the lowest levels of phosphorylation. Despite this correlation, the data also imply that factors other than the degree of phosphorylation influence arrestin binding. Therefore, individual serine and threonine residues may differ in their ability to promote binding. The observation of only a small decrease in arrestin binding for mutant S334A/T335A/T336A suggests that the most critical sites are in the region of the COOH terminus containing Ser-338, Thr-340, Thr-342, and Ser-343. T340A and S343A demonstrated a 48% and a 35% decrease in arrestin binding, respectively, although phosphorylation levels were similar to that of wild-type rhodopsin. These data indicate that Thr-340 and Ser-343 play important roles in arrestin binding. In fact, all of the constructs in which Thr-340 was replaced with alanine (S338A/T340A, T340A/S343A, T340A, and STTTT) showed significantly reduced arrestin binding compared with other mutants with the same number of alanine substitutions.

The binding of arrestin to T340A and S343A was examined as a function of arrestin concentration to determine whether the observed differences are due to a change in K_d or B_{max}. Because the active conformation of rhodopsin, *meta*-rhodopsin II, decays progressively over time (3), it is difficult to define precise K_d and B_{max} values for the interaction of rhodopsin with other proteins. However, arrestin binding stabilizes *meta-*

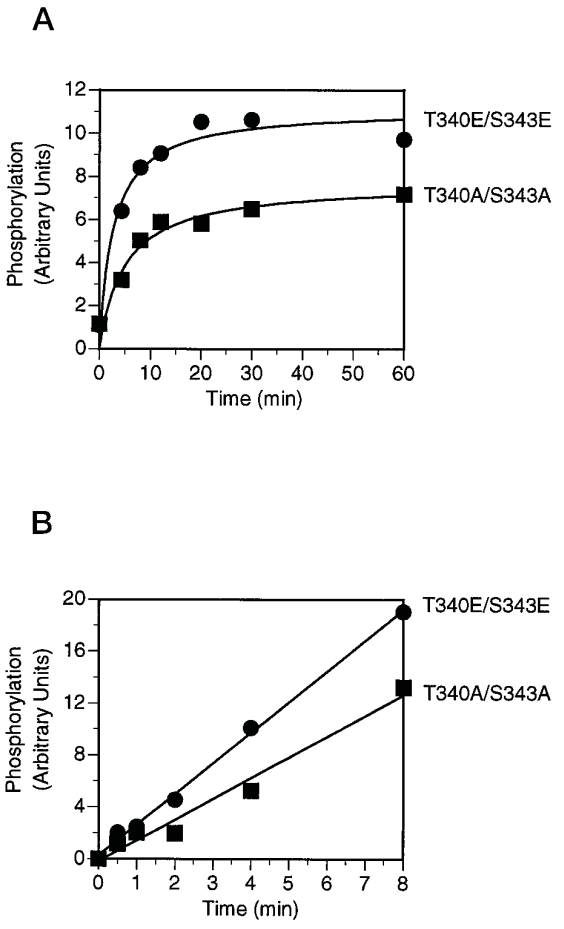

FIG. 7. **Time course of the light-dependent phosphorylation of T340E/S343E and T340A/S343A.** *A*, a 60-min time course; *B*, an 8-min time course. Transfected HEK-293 cell membranes containing 0.3 μg of rhodopsin were phosphorylated by rhodopsin kinase in the light or in the dark for the times indicated using the methods described under "Experimental Procedures." The amount of phosphorylation in the dark was subtracted from the amount of phosphorylation in the light at each time point. Each point represents the average of duplicate determinations from one experiment. ■, T340A/S343A; ●, T340E/S343E.

rhodopsin II (46). Therefore, direct binding studies can be used to obtain comparative values for the interaction of arrestin with wild-type and mutant rhodopsin. An average K_d of 0.74 ± 0.11 nM obtained in our studies for the binding of rod cell-specific arrestin to wild-type rhodopsin is similar to the K_d reported for the binding of *in vitro* translated β-arrestin to the m_2-muscarinic acetylcholine receptor (0.48 ± 0.04 nM) and is 11-fold lower than the K_d reported for the binding of rod cell-specific arrestin to the m_2-muscarinic receptor (7.2 ± 1.2 nM) (32, 47). Therefore, using similar methods, our data fall within a range of values reported by others for the binding of arrestins to G protein-coupled receptors. Previously, a K_d of ~50 nM for the binding of rod cell-specific arrestin to rhodopsin was reported, using light scattering techniques to measure the ability of the arrestin to stabilize *meta*-rhodopsin II (46). The reason for the lower affinity observed in these studies is unclear, but presumably is a result of differences in the methods employed in those experiments.

As described above, we observed that the K_d values for T340A and S343A were similar to that for wild-type rhodopsin, despite significant differences in the level of arrestin binding. In contrast, the B_{max} was reduced in all of our experiments by 35–50% for both mutants. Experiments using either full-length rhodopsin or COOH-terminal synthetic peptides have shown that the negatively charged phosphorylated residues of rhodopsin alter the conformation of arrestin, allowing it to bind to light-exposed nonphosphorylated rhodopsin (17, 18). The stable binding of this activated arrestin is thought to involve an interaction with rhodopsin's cytoplasmic loops (18, 48, 49). This implies that the interaction of arrestin with the rhodopsin COOH terminus converts arrestin from a low to a high affinity form. Since our centrifugation assay requires ~45 min to complete the separation of free from bound arrestin, we speculate that we are not able to measure the binding of the low affinity form. This assumption is based on calculations by Yamamura *et al.* (50) demonstrating that separation times would have to be <1.7 min to avoid a loss of >10% of a ligand-receptor complex if the K_d is $>10^{-9}$ M (see also Table III-2 in Ref. 51). Therefore, if the COOH-terminal mutations T340A and S343A weaken the binding of the low affinity form, the resulting change in K_d would probably not be detected in our assays. However, as a consequence of that weaker interaction, the conversion of arrestin to the high affinity form by the COOH terminus would be less efficient, resulting in a reduced amount of stable arrestin binding, therefore a reduced B_{max}. An alternative explanation might be that the mutations cause the improper folding, aggregation, or post-translational modification of rhodopsin, thus interfering with the binding of arrestin. We view these possibilities as unlikely since retinal binding, light-dependent G_t activation, and light-dependent phosphorylation by rhodopsin kinase are all normal. Nevertheless, we cannot rule out the possibility that the mutations modify the surface of rhodopsin in some way that partially alters its accessibility to arrestin, resulting in a reduced B_{max}. Experiments designed to resolve this question include the use of synthetic peptides corresponding to the phosphorylated COOH terminus of rhodopsin. We would predict that the phosphorylated wild-type peptide would be able to induce the binding of arrestin to the T340A and S343A mutant rhodopsin to levels similar to that of wild-type rhodopsin. In contrast, T340A and S343A mutant phosphorylated COOH-terminal peptides would be expected to induce arrestin binding to wild-type rhodopsin that demonstrates a reduced B_{max}.

The B_{max} values obtained in our studies are significantly lower than one would expect, based on the assumed 1:1 stoichiometry between phosphorylated rhodopsin and arrestin. For example, in the experiment shown in Fig. 6, an estimated 1.2 pmol of phosphorylated rhodopsin was used. However, the B_{max} for the binding of arrestin to wild-type rhodopsin was only 12.6 fmol. There are a number of reasons that may contribute to these low values. First, the stoichiometry of phosphorylation ranges from 0.4 to 0.6 mol/mol of rhodopsin in our experiments. Since our results suggest that the level of phosphorylation influences arrestin binding, it is possible that only a small fraction of rhodopsin has a sufficient number of phosphates to efficiently promote arrestin binding. A second possibility lies in the stability of light-activated rhodopsin. Gurevich *et al.* (47) observed that the binding of β-arrestin to the m_2-muscarinic acetylcholine receptor was ~30% and the binding of visual arrestin to the same receptor was only 14% of what would be expected based on a 1:1 stoichiometry. The authors suggested that this may be related to a lack of stability of the agonist-receptor-arrestin complex. Similarly, the instability of *meta*-rhodopsin II in our experiments could lead to lower values for B_{max}. Despite these drawbacks to determining B_{max}, our results clearly indicate that relative B_{max} values are reduced for mutants T340A and S343A compared with that for wild-type rhodopsin.

Negative charges were introduced through the substitution of Thr-340 and Ser-343 with glutamic acids, and the results of phosphorylation and arrestin binding were compared with

those of wild-type rhodopsin and the corresponding alanine mutants. None of the glutamic acid mutants was able to bind arrestin without first being phosphorylated by rhodopsin kinase. Mutant T340E/S343E showed enhanced phosphorylation compared with the equivalent alanine mutant (T340A/S343A). This result suggests that the introduction of two negative charges enhances the rate of phosphorylation of the remaining serine and threonine residues. A similar cooperativity has been observed by measuring the rate of phosphorylation of rhodopsin containing different numbers of phosphates or the phosphorylation of synthetic peptide substrates corresponding to the rhodopsin COOH terminus (8, 52–55). Glutamic acid substitution also enhanced the phosphorylation of the formyl-Met-Leu-Phe receptor by the G protein-coupled receptor kinase GRK2 (44), implying that cooperativity may be common to the phosphorylation of G protein-coupled receptors by other members of this kinase family. T340E/S343E also showed increased binding to arrestin compared with the equivalent alanine-substituted mutant, consistent with the role of phosphorylation in promoting a conformational change in arrestin that allows it to bind to other sites in rhodopsin (18, 48, 49). When Ser-343 was replaced with glutamic acid (S343E), an increase in arrestin binding to a level comparable to that of wild-type rhodopsin was observed, even though there was little change in the level of phosphorylation. In contrast, T340E showed no increase in arrestin binding compared with T340A. Interestingly, T340E/S343E binds arrestin better than the single amino acid mutant T340E. It may be that the enhanced phosphorylation observed for T340E/S343E increases the efficiency with which arrestin undergoes its conformational change and compensates for the partial loss of function at Thr-340.

It has been reported that Thr-340 is not phosphorylated *in vivo* under conditions where arrestin binds to rhodopsin. Therefore, the requirement for Thr-340 may not involve its ability to be phosphorylated; rather, substitution with either alanine or glutamic acid may disrupt its function. As described above, a phosphorylated synthetic peptide corresponding to the rhodopsin COOH terminus induces a conformational change in arrestin and promotes its binding to rhodopsin (18). In contrast, heparin, a negatively charged glycosaminoglycan, causes a conformational change in arrestin, but does not induce arrestin to bind to rhodopsin (17). Therefore, the COOH terminus of rhodopsin, or the corresponding synthetic peptide, appears to serve a function in addition to providing negative charges to promote the interaction of arrestin with rhodopsin. Perhaps Thr-340 plays a role in this function. Although we cannot rule out the possibility that the single negative charge of glutamic acid is not sufficient to mimic the electrostatic interactions of a phosphate in the case of T340E, a preliminary report of experiments in which all seven phosphorylation sites were mutated to glutamic acids indicated that the presence of multiple negative charges was not sufficient to promote arrestin-mediated inhibition of G_t activation (56). These results are consistent with the possibility that the COOH terminus serves a function apart from providing a negatively charged environment. The elucidation of that function will aid in the understanding of the interactions between rhodopsin and arrestin.

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