Differential Phosphorylation of the Rhodopsin Cytoplasmic Tail Mediates the Binding of Arrestin and Its Splice Variant, p44[†]

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ABSTRACT: Deactivation of the vertebrate photopigment rhodopsin is achieved through a two-step process. Rhodopsin is first phosphorylated by rhodopsin kinase and subsequently deactivated by the binding of the regulatory protein arrestin or its splice variant, p44. Although much is known about the overall differences between arrestin and p44 binding to different rhodopsin species (photolyzed versus unphotolyzed and/or phosphorylated versus unphosphorylated), the exact role of p44 during phototransduction remains to be fully elucidated. Our current study addresses this question by identifying structural differences between arrestin and p44 and characterizing the interaction between the negatively charged rhodopsin tail and either p44 or arrestin. Our results demonstrate that arrestin and p44 bind differently to different phosphorylated rhodopsin species and that this may be due to a structural difference between p44's and arrestin's basal states. This difference offers a potential regulatory mechanism that could regulate p44 and arrestin binding and, as a result, regulate the kinetics of the rod's light response.

G protein-coupled receptors (GPCR) are responsible for cellular signaling as a response to a wide spectrum of agonists including hormones, chemical odorants, neurotransmitters, and photons of light. The visual photopigment rhodopsin, a member of the largest subfamily of GPCRs (Family A), has become a paradigm for GPCR structure/function studies (*1*−*3*). In vertebrate rods, rhodopsin activation occurs when the covalently bound chromophore (retinal) absorbs a photon of light. The absorption leads to an isomerization event, converting 11-*cis* retinal into an all-trans conformation. Isomerization of the chromophore activates rhodopsin by forcing structural rearrangements in the transmembrane region of the protein (opsin). In its active form, rhodopsin can activate the heterotrimeric G protein, transducin. However, once activated, rhodopsin is immediately targeted for deactivation. This occurs through a two-step process. First, rhodopsin is phosphorylated at serine and threonine residues on its cytoplasmic tail by a specific kinase, rhodopsin kinase (RK) (*4*, *5*). Phosphorylated rhodopsin is subsequently targeted by a second regulatory protein, arrestin, which actively competes with transducin for binding sites on the rhodopsin cytoplasmic loops (*6*, *7*). As a result, arrestin is able to quench the rhodopsin signal.

Although the role of arrestin in phototransduction has been well-characterized, the role of its splice variant, p44, is not as clear. As with other protein in the arrestin family (*8*−*10*), a variant of arrestin is generated through an alternative splicing event, yielding a truncated form of the arrestin protein. p44 differs from arrestin only in that it lacks the last 35 C-terminal amino acids. p44 is found in the retina of mice, cows, and humans (*11*, *12*), and like arrestin, it has selectivity for both photolyzed and phosphorylated rhodopsin (*11*−*13*).

Several lines of evidence suggest that p44 can play an important role in shaping the rod's light response. One group of studies shows how p44 can bind with higher affinity than arrestin to different rhodopsin species (*11*, *14*). Another line of evidence that suggests the importance of p44 is its permanent presence in the rod outer segment (ROS) (*12*). Immunohistochemical studies have shown that arrestin undergoes a massive translocation from the rod inner segment to the ROS upon illumination of the rod. p44, however, is permanently localized to the ROS. Therefore, although p44 is present in the rod at $^1/_{10}$ the concentration of arrestin (*12*), it is ideally localized during the initial stages of phototransduction.

In this current study, we attempt to elucidate the role of p44 in phototransduction by characterizing its interaction with rhodopsin. Previous studies with arrestin have found that the identity and position of the phospho-residues on the rhodopsin cytoplasmic tail[1] can affect the binding of arrestin to rhodopsin (*15*−*17*), demonstrating the need for the proper alignment between rhodopsin and arrestin residues. This alignment is likely to be necessary for arrestin activation, which requires the displacement of the arrestin carboxyl tail

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[1] Abbreviations: Rho*, photolyzed rhodopsin; P-Rho*, photolyzed/phosphorylated rhodopsin; D-tail and E-tail Rhodopsin, rhodopsin with all seven serine and threonine residues substituted with aspartic acids (D-tail) or glutamic acids (E-tail); arrestin carboxyl tail, last 35 C-terminal amino acids of the arrestin sequence (370−404); rhodopsin cytoplasmic tail, last 20 C-terminal amino acids of the bovine sequence (329−348); polar core, acidic and basic amino acids at the fulcrum of the arrestin molecule (Asp^{30}, Arg^{175}, Asp^{296}, Asp^{303}, and Asp^{383}).

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and disruption of the salt-bridges with the polar core (*18*). Because p44 lacks the C-terminal carboxyl tail, the residues required for p44 activation may differ from those required by arrestin. If this is indeed the case, differential phosphorylation of rhodopsin could present a possible regulatory mechanism by which p44 and arrestin binding to rhodopsin is controlled. Using a transducin GTPγ^{35}S-binding assay, we indirectly evaluated p44 and arrestin binding to rhodopsin and the effect that negative charges on the rhodopsin cytoplasmic tail had on binding. We find that the position of the phospho-residues required for the association of p44 with rhodopsin differs from the position required for arrestin−rhodopsin interaction and that this differential binding may be due to structural differences between the basal structures of arrestin and p44.

MATERIALS AND METHODS

Measure of Arrestin-Mediated and p44-Mediated Deactivation of Rhodopsin through Transducin Inhibition Assays. The ability of arrestin and p44 to functionally bind and inhibit rhodopsin was determined by measuring transducin activation. GTPγ^{35}S-binding filter-binding assays were performed, and arrestin- and p44-mediated deactivations were determined as previously described (*15*).

Purification of Transducin from Bovine Rod Outer Segments. Bovine retinas were obtained from Preston Van Hooser and Shenk, Inc. (Seattle, WA). Transducin was purified as previously described (*19*). Purified transducin was stored at −20 °C in 50% glycerol in 10 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, and 1 mM DTT.

Expression and Purification of Rhodopsin Kinase and Rhodopsin Mutants. Recombinant rhodopsin kinase and rhodopsin mutants were expressed and purified as previously described (*15*).

Preparation of p44 Mutants. N-terminal His-tagged bovine p44 cDNA was cloned into pPIC-Zb (Invitrogen). Site-directed mutagenesis was performed using the QuikChange Mutagenesis protocol (Stratagene) with Pfu Ultra polymerase (Stratagene). GS115 clones with p44 gene insertions were confirmed with a PCR screen using 5′ and 3′ AOX1 primers. Positive clones were grown according to the Manual for Expression of Proteins in *Pichia pastoris* (Invitrogen). Cells were lysed using a French Press (20 000 psi) in 10 mM $NaPO_4$ (pH 6.4) with 300 mM NaCl and 10 mM imidazole. Lysate was centrifuged at >30 000*g* for 30 min. The supernatant was loaded onto a 10 mL column volume of NTA resin (Qiagen) and washed with 20 mM imidazole. p44 was eluted from the NTA column using 250 mM imidazole and loaded onto a 1 mL Hi-Trap Heparin column (Amersham). p44 was eluted from the Hi-Trap column using 400 mM NaCl in 10 mM HEPES (pH 7.5).

RESULTS

Effect of Glutamic and Aspartic Acid Substitutions on p44-Mediated Rhodopsin Deactivation. Previous studies have demonstrated that the presence of aspartic acid and glutamic acid residues in the rhodopsin cytoplasmic tail can increase arrestin binding to Rho* (*20*). However, these acidic residues were clearly found to be unable to substitute for phospho-residues for two reasons. (1) Aspartic and glutamic acids cannot increase arrestin-mediated deactivation to the same extent as P-Rho* and (2) Whereas a peptide mimicking the phosphorylated rhodopsin tail can induce a conformational change in arrestin, a peptide with aspartic and glutamic acid substitutions of the phosphorylatable residues cannot induce the same conformational changes (*20*). Therefore, although the negative charges can stabilize the interaction between arrestin and rhodopsin, they cannot induce the conformational changes required for high-affinity rhodopsin−arrestin interaction.

Since p44's ability to bind rhodopsin increases drastically upon rhodopsin phosphorylation, we examined whether aspartic and glutamic acid residues could substitute for phospho-residues during the rhodopsin−p44 interaction. Using a transducin GTPγ^{35}S-binding assay, we tested the effect of p44 on the activity of D-tail and E-tail rhodopsin (see Table 1 for rhodopsin mutant sequences). Our results show that, like arrestin, acidic residues on the rhodopsin tail can increase p44-mediated deactivation of Rho*, but they cannot completely substitute for phosphorylated residues (Figure 1). This implies that, like arrestin, the phosphorylated residues must provide certain structural changes within p44 that allow for high-affinity binding and that these structural changes cannot be achieved with the presence of acidic residues on the rhodopsin tail. However, there must be some stabilizing interactions that are provided by the acidic residues, given that the apparent affinity of p44 for rhodopsin increases in the presence of the mutations.

Position of Phospho-Residues on the Rhodopsin Tail Affect p44-Mediated Deactivation. The position of the phospho-residues on the rhodopsin tail can greatly affect the ability of arrestin to bind and inhibit rhodopsin (*16*). Previous studies have demonstrated that bovine rhodopsin mutants with only the three serines (Ser Only rhodopsin) or the four threonines (Thr Only rhodopsin) cannot be deactivated by arrestin to the same extent as wild-type (Wt) rhodopsin (*16*) (see Table 1 for C-terminal sequence of rhodopsin mutants). However, a rhodopsin with all three serines in addition to a threonine at position 340 (Serines-T340) can undergo Wt-level arrestin-mediated deactivation. The fact that Serines-T340, which has four phosphorylatable sites, is deactivated to a greater extent than Thr Only rhodopsin, which also has four phosphorylatable sites, demonstrates that the position, as well as the number, of phospho-residues on rhodopsin can modulate arrestin binding. To determine whether the position of the phospho-residues also affects p44-mediated rhodopsin deactivation, we tested the effect of p44 on rhodopsin mutants with altered phosphorylation sites.

As in previous arrestin studies, we tested the ability of p44 to deactivate Thr Only and Ser Only rhodopsin mutants. To avoid generating an artificial secondary structure in the rhodopsin tail, we decided to use glutamine, which aside from its inability to be phosphorylated is similar in size to a serine or threonine residue and unlike alanine does not induce helical structures. As previously seen with arrestin, the loss of either serines or threonines drastically reduces p44-mediated rhodopsin deactivation (Figure 2). p44-mediated inhibition of Ser Only and Thr Only rhodopsin mutants drops to nearly half the inhibition levels of Wt rhodopsin. Since the literature emphasizes the importance of serine residues during rhodopsin phosphorylation in vivo, we examined whether we could increase rhodopsin deactivation by p44 by adding threonine residues to Ser Only rhodopsin. Single

Table 1: Table of Rhodopsin Mutants[a]

	residue											
	333	334[b]	335[b]	336[b]	337	338[b]	339	340[b]	341	342[b]	343[b]	344
Wt	A	S	T	T	V	S	K	T	E	T	S	Q
E-tail	-	E	E	E	-	E	-	E	-	E	E	-
D-tail	-	D	D	D	-	D	-	D	-	D	D	-
S338/S343/T340	-	Q	Q	Q	-	-	-	-	-	Q	-	-
S334/S343/T340	-	-	Q	Q	-	Q	-	-	-	Q	-	-
S334/S338/T340	-	-	Q	Q	-	-	-	-	-	Q	Q	-
S334/T340	-	-	Q	Q	-	Q	-	-	-	Q	Q	-
S338/T340	-	Q	Q	Q	-	-	-	-	-	Q	Q	-
S343/T340	-	Q	Q	Q	-	Q	-	-	-	Q	-	-
D334/D340	-	D	-	-	-	-	-	D	-	-	-	-
D335/D343	-	-	D	-	-	-	-	-	-	-	D	-
D334	-	D	-	-	-	-	-	-	-	-	-	-
D340	-	-	-	-	-	-	-	D	-	-	-	-
D335	-	-	D	-	-	-	-	-	-	-	-	-
D343	-	-	-	-	-	-	-	-	-	-	D	-
Thr Only	-	Q	-	-	-	Q	-	-	-	-	Q	-
Ser Only	-	-	Q	Q	-	-	-	Q	-	Q	-	-
Serines-T335	-	-	-	Q	-	-	-	Q	-	Q	-	-
Serines-T336	-	-	Q	-	-	-	-	Q	-	Q	-	-
Serines-T340	-	-	Q	Q	-	-	-	-	-	Q	-	-
Serines-T342	-	-	Q	Q	-	-	-	Q	-	-	-	-
S343-Rho	-	Q	Q	Q	-	Q	-	Q	-	Q	-	-
S338/S343-Rho	-	Q	Q	Q	-	-	-	Q	-	Q	-	-
S334/S338-Rho	-	-	Q	Q	-	-	-	Q	-	Q	Q	-
S334-Rho	-	-	Q	Q	-	Q	-	Q	-	Q	Q	-

[a] Dashes (-) represent Wt residues. [b] These columns represent positions of Wt phosphorylatable residues.

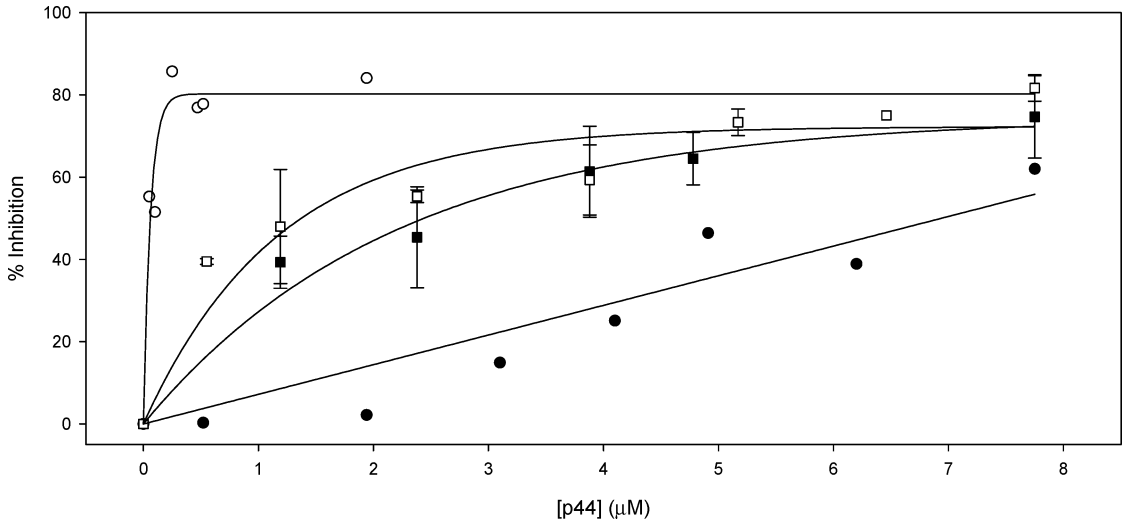

FIGURE 1: p44-mediated deactivation of D-tail and E-tail rhodopsin. GTPγ^{35}S-binding assays were performed as described in Materials and Methods. Rhodopsins were expressed in Cos-1 cells, and membranes were purified as described in Materials and Methods. D-tail rhodopsin mutant (solid squares) has all phosphorylatable sites on the rhodopsin cytoplasmic tail substituted with aspartic acid residues, and E-tail rhodopsin mutant (open squares) has all phosphorylatable sites substituted with glutamic acid residues. Unphosphorylated (solid cicles) and phosphorylated rhodopsins (open circles) have all endogenous phosphorylatable sites (Ser^{334}, Thr^{335}, Thr^{336}, Ser^{338}, Thr^{340}, Thr^{342}, and Thr^{343}). Traces are regression lines fitted to exponential functions using SigmaPlot equation. Error bars represent SEM ($n = 3-6$).

threonine residues were added to the Ser Only rhodopsin mutant at positions 335 (Serines-T335 rhodopsin), 336 (Serines-T336 rhodopsin), 340 (Serines-T340 rhodopsin), and 342 (Serines-T342 rhodopsin). The ability of p44 to deactivate these rhodopsin mutants was then tested using the transducin GTPγ^{35}S-binding assay. While Serines-T340 had been previously found to have Wt-level arrestin-mediated deactivation (*16*), we find that the addition of Thr^{335} (Serines-T335 rhodopsin) shows the greatest increase in p44 when compared with the Ser Only mutant. The addition of threonine residues at other positions actually attenuates p44 deactivation of Ser Only rhodopsin.

To determine the minimal number of phospho-residues required for Wt-level arrestin-mediated deactivation, we systematically altered the serine complement in the Serines-T340 mutant, generating rhodopsin mutants with only one or two serines present in the presence of Thr^{340}. As can be seen in Figure 3A, a rhodopsin mutant with all phosphorylatable sites replaced with glutamine except for Thr^{340} (T340 rhodopsin) is not deactivated by arrestin to the same extent as Wt rhodopsin. We added back individual serines to T340 rhodopsin to examine the effect that a single serine with Thr^{340} had on arrestin-mediated deactivation. We find that the position of the added serine impacts the level of

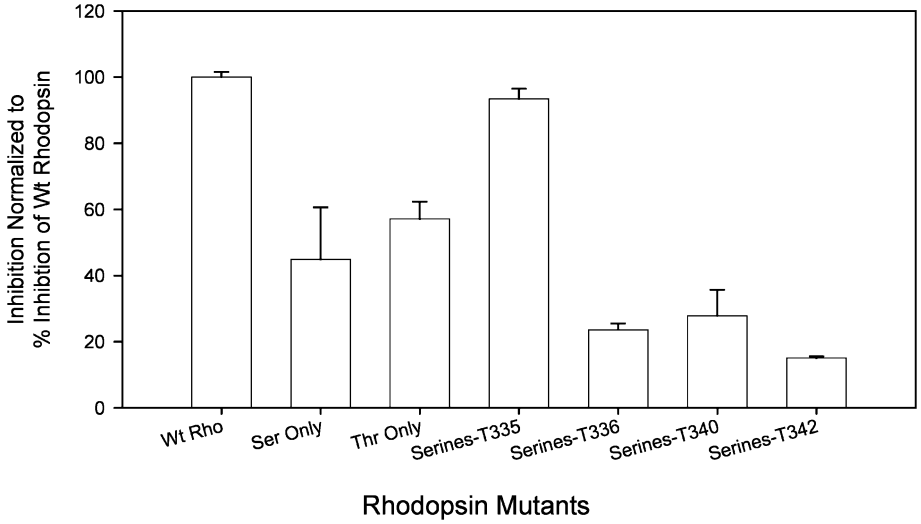

FIGURE 2: Effect of p44 on serine and threonine rhodopsin mutants. GTPγ^{35}S-binding assays were performed as described in Materials and Methods. Rhodopsin mutants were expressed in Cos-1 cells, and membranes were purified as described in Materials and Methods. The rhodopsin mutants were all phosphorylated by rhodopsin kinase prior to addition of p44. Table 1 outlines the amino acid sequence for the last 15 C-terminal residues for each of the rhodopsin mutants. The *y*-axis represents percent (%) inhibition of each rhodopsin mutant normalized to the % inhibition of phosphorylated Wt rhodopsin. The final concentration of p44 was 2 μM. (Error bars are SEM; $n = 3$.)

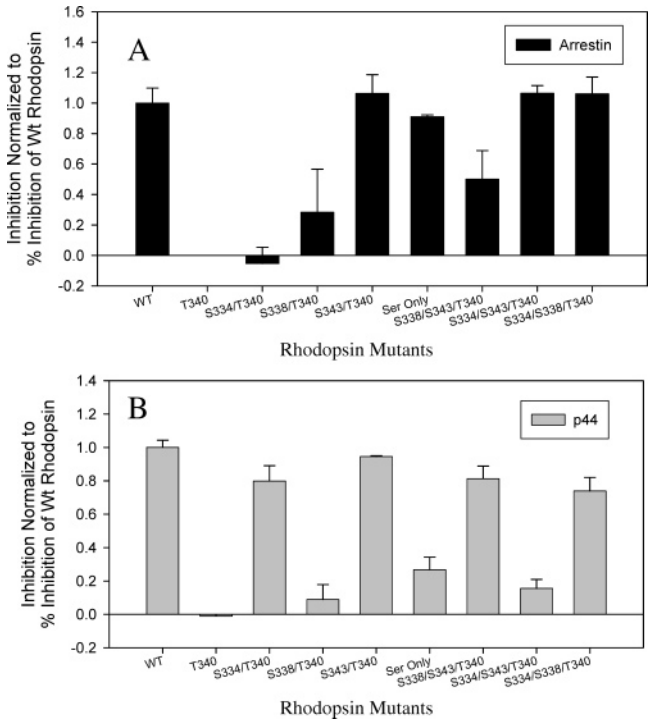

FIGURE 3: Effect of arrestin and p44 on Serine-T340 rhodopsin mutants. GTPγ^{35}S-binding assays were performed as described in Materials and Methods. Rhodopsin mutants were expressed in Cos-1 cells, and membranes were purified as described in Materials and Methods. Table 1 outlines the amino acid sequence for the last 15 C-terminal residues for each of the rhodopsin mutants. The *y*-axis represents % inhibition of each rhodopsin mutant normalized to the % inhibition of phosphorylated Wt rhodopsin. The final concentration of arrestin (panel A) and p44 (panel B) was 5 μM and 2 μM, respectively. (Error bars are SEM; $n = 2-4$.)

arrestin-mediated deactivation. Addition of Ser^{334} (S334/T340) does not increase rhodopsin inhibition relative to T340. However, addition of either Ser^{338} (S338/T340) or Ser^{343} (S343/T340) increases the arrestin-mediated inhibition. In fact, S343/T340 shows Wt-level inhibition by arrestin.

We also tested the effect that the addition of two serine residues to T340 had on arrestin-mediated deactivation. We find that addition of Ser^{334} and either Ser^{338} (S334/S338/T340) or Ser^{343} (S334/S343/T340) increases deactivation to the same extent as Wt rhodopsin. However, it was surprising to find that addition of serine residues at positions 338 and 343 (S338/S343/T340) attenuates rhodopsin deactivation compared to S338/T340 or S343/T340. These results suggest not only is the position of the serine residues added important for arrestin-mediated deactivation but also the context in which the serines are added.

We also tested the effect of p44 on the Serines-T340 rhodopsin mutants (Figure 3B). We find that there are similarities and differences in the way p44 and arrestin respond to the rhodopsin mutants. Both arrestin and p44 appeared to bind and inhibit S343/T340 to near-Wt levels. However, S334/T340 shows opposing levels of arrestin and p44 inhibition. Whereas S334/T340 rhodopsin shows no inhibition by arrestin, this mutant is deactivated by p44 to the same extent as Wt rhodopsin. In contrast, arrestin deactivates S334/S343/T340 to nearly 80% the level of Wt rhodopsin, yet p44 deactivates the same mutant to less than 20% Wt levels. Both S334/S338/T340 and S338/S343/T340 show increase inhibition as compared to T340. Although we see Wt-level deactivation of Serines-T335 rhodopsin by p44, we also see Wt-level deactivation with S343/T340, further suggesting the importance of the context in which particular phospho-residues are present.

Comparison of p44 to Arrestin Using a Computational Model. The positional requirements imposed on the phospho-residues during arrestin- and p44-mediated rhodopsin deactivation suggest a proper alignment of the rhodopsin and arrestin/p44 residues must be satisfied. In a previous study, we developed a computational model to simulate the interaction between arrestin and the rhodopsin cytoplasmic tail and probed for interactions formed between the two proteins (*15*). Using a combination of computer simulations and in vitro biochemical assays, we identified in that study certain interactions between residues on arrestin and two negative charges on the rhodopsin tail. Specifically, negative charges at positions 334 and 340 on rhodopsin were able to interact

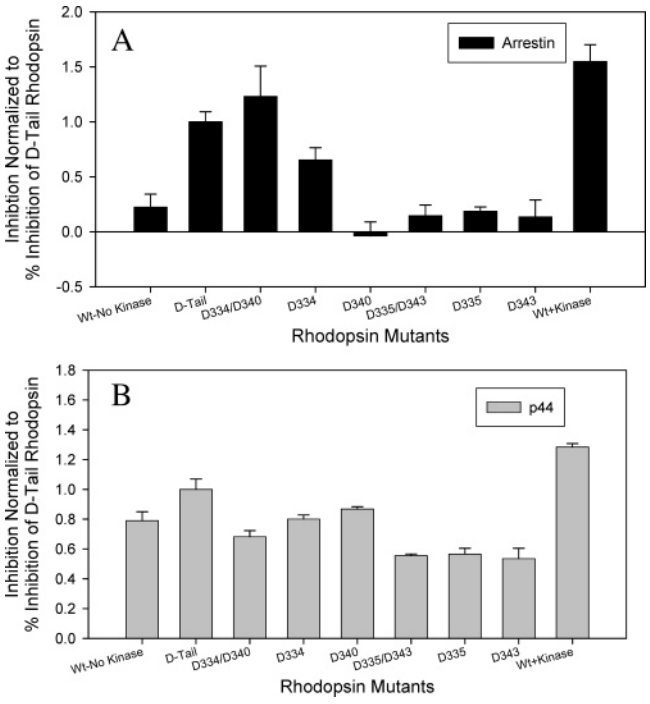

FIGURE 4: Effect of arrestin and p44 on rhodopsin D-tail mutants. GTPγ^{35}S-binding assays were performed as described in Materials and Methods. Rhodopsin mutants were expressed in Cos-1 cells, and membranes were purified as described in Materials and Methods. Table 1 outlines the amino acid sequence of the last 15 amino acids of the rhodopsin mutants. The *y*-axis represents % inhibition of each rhodopsin mutant normalized to the average % inhibition of D-tail rhodopsin for either arrestin or p44. Arrestin reactions (panel A) and p44 reactions (panel B) had final concentrations of 5 μM and 2 μM of arrestin and p44, respectively. Error bars are SEM ($n = 2-4$).

with arrestin residues Arg^{29}, Lys^{15}, His^{301}, and Lys^{300}. Given that p44 has the same amino acid sequence as arrestin between residues 1 and 369, we tested whether p44 made similar interactions as arrestin with the negative charges on the rhodopsin tail. Figure 4 outlines the results for both arrestin- (Figure 4A) and p44-mediated (Figure 4B) deactivation of different D-tail rhodopsin mutants. It is evident from these experiments that unlike arrestin, which has the same level of deactivation for D334/D340 rhodopsin as it has for D-tail rhodopsin, D334/D340 cannot be deactivated by p44 to the same level as D-tail rhodopsin. In fact, if p44's inhibition of D334/D340 is compared to its inhibition of unphosphorylated rhodopsin (Wt-No Kinase), it appears that the presence of Asp^{334} and Asp^{340} lowers p44's inhibitory activity. In addition, whereas the presence of a single negative charge at 340 (D340) on the rhodopsin tail does not increase arrestin-mediated deactivation of rhodopsin, p44-mediated deactivation of this rhodopsin mutant is actually higher than deactivation of D334/D340. In contrast, a single negative charge at 334 (D334) increases arrestin- and p44-mediated deactivation. We also tested whether negative charges at two other positions (335 and 343) could account for the binding of p44 to D-tail rhodopsin (D335/D343). These two positions do not show any interactions with arrestin residues during the computations simulations. Therefore, we used D335/D343 rhodopsin as a control to demonstrate that the interaction between arrestin and D334/D340 was specific to the negative charges at positions 334 and 340 and not due to the presence of two negative charges on the rhodopsin tail. Like arrestin, D335/D343 could not be deactivated by p44 to the same extent as D-tail rhodopsin. However, as is the case with D334/D340 rhodopsin, the presence of Asp^{335} or Asp^{343} actually lowers p44's inhibitory activity relative to unphosphorylated rhodopsin. Since the previous computational studies with arrestin and the D-tail rhodopsin mutants illustrate the need for the proper alignment between negative residues on rhodopsin and positive residue on arrestin, the inability of p44 to inhibit the same rhodopsin mutants suggests that this alignment is absent in the interaction between p44 and the tested D-tail mutants. Given that the computational modeling comprises simulations using the arrestin crystal structure, the fact that p44 responds to the D-tail rhodopsin mutants differently than arrestin suggests that there may be structural differences between arrestin and p44.

Timecourse of Arrestin and p44 Inhibition of Phosphorylated Rhodopsin. A previous study by Kennedy and colleagues characterized the sequential phosphorylation and dephosphorylation of rhodopsin upon illumination of a mouse rod to bright white light flashes (*21*). This study found Ser^{343} to be preferentially phosphorylated first, followed by Ser^{338}, and Ser^{334} last. The pattern of dephosphorylation follows the same order resulting in a series of phosphorylated rhodopsin species generated over time with phospho-Ser^{343} rhodopsin generated first, followed by phospho-Ser^{343}/phospho-Ser^{338} rhodopsin. The subsequent phosphorylation of Ser^{334} generates a triply phosphorylated rhodopsin modified at the three serine positions. The ordered dephosphorylation leads to the formation of phospho-Ser^{334}/phospho-Ser^{338} rhodopsin, followed by phospho-Ser^{334} rhodopsin. To determine whether this sequential phosphorylation/dephosphorylation could lead to differential binding of arrestin and p44, we generated rhodopsin mutants with the appropriate complement of phosphorylatable sites (Table 1) and tested arrestin- and p44-mediated deactivation of these rhodopsin mutants. Figure 5 outlines the results from these experiments.

We assayed the effect of p44 and arrestin on the different rhodopsin mutants over three concentrations of p44 and arrestin. The results reveal again that p44 and arrestin respond differently to the different phosphorylated rhodopsin species. Mostly, we find that p44 is a more powerful inhibitor of rhodopsin regardless of the complement of phosphorylatable sites present on the rhodopsin mutant. Of all the rhodopsin mutants, p44 seems to inhibit S334/S338/S343-Rho most effectively, reaching saturated inhibition levels at a p44 concentration of 2 μM. However, S334/S338-Rho is inhibited to a greater extent, approaching near 80% inhibition of rhodopsin activity at higher p44 concentrations. However, we do find that arrestin is more effective than p44 at inhibiting the activity of two rhodopsin mutants at low concentrations (S338/S343-Rho and S334-Rho). Under conditions where arrestin and p44 concentrations are at 2 μM, we see that arrestin is able to inhibit S338/S343-Rho and S334-Rho by 30% and 20%, respectively, more than p44. Although, at higher concentrations, p44 inhibits both rhodopsin mutants 15−20% more than arrestin. Interestingly, arrestin does not effectively inhibit S343-Rho, presumably the first phosphorylated rhodopsin species to appear after illumination of the rod, until higher arrestin concentrations. In contrast, p44 can inhibit S343-Rho activity 40% at 5 μM. Given that Kennedy and colleagues find a time-dependent

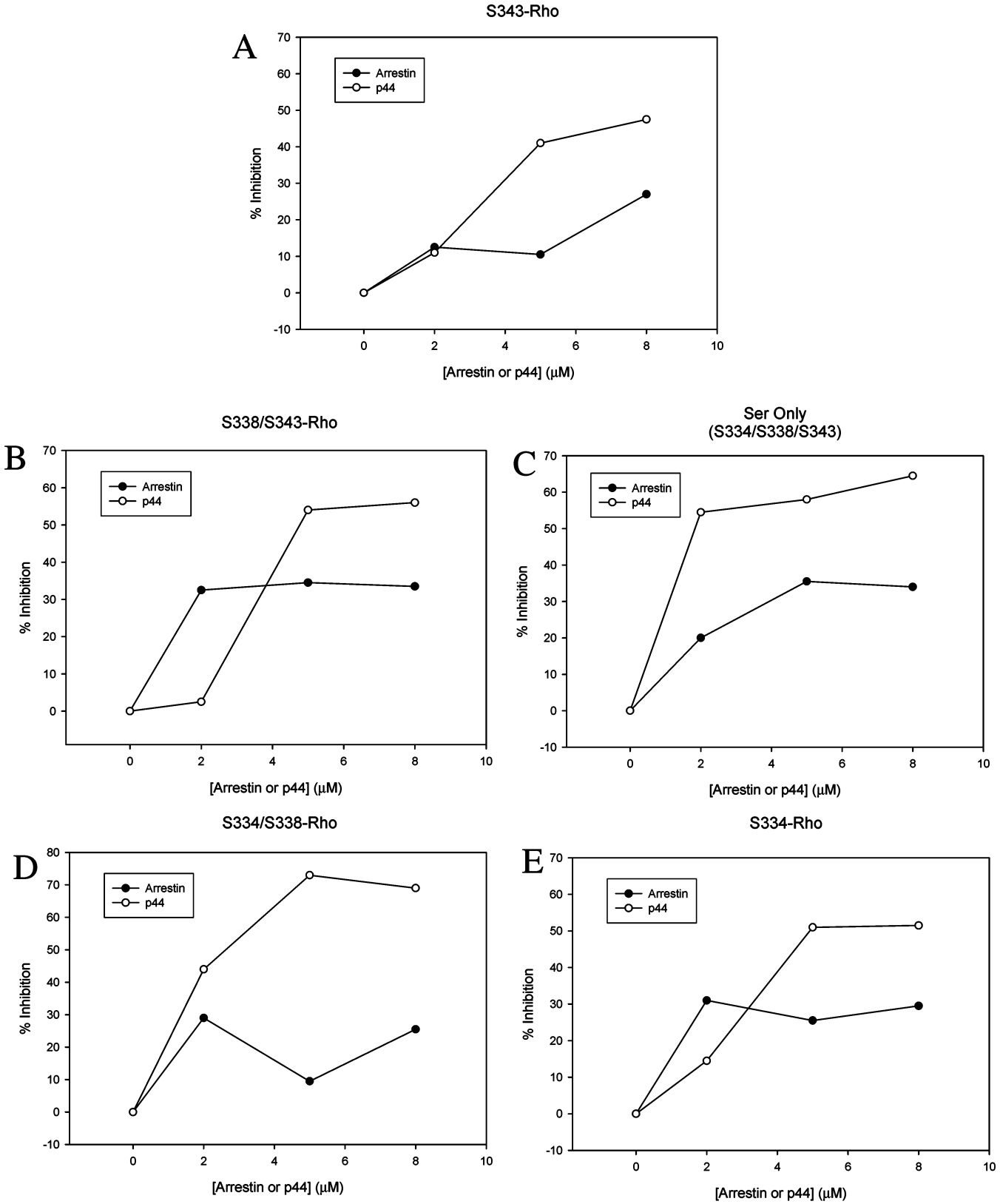

FIGURE 5: Effect of arrestin and p44 on singly, doubly, and triply phosphorylated rhodopsin mutants. GTPγ^{35}S-binding assays were performed as described in Materials and Methods. Rhodopsin mutants were expressed in Cos-1 cells, and membranes were purified as described in Materials and Methods. Table 1 outlines the amino acid sequence of the last 15 amino acids of the rhodopsin mutants. The effects of arrestin and p44 on S343-Rho (A), S338/S343-Rho (B), Ser Only (S334/S338/S343 Rhodopsin) (C), S334/S338-Rho (D), and S334-Rho (E) were analyzed. Values are the averages from two independent experiments.

change in the population of phosphorylated rhodopsin species found in the ROS and the current studies examining the translocation of arrestin from the rod inner segment to the outer segment, the differential binding of arrestin and p44 to phosphorylated rhodopsin may play a role in controlling kinetics of phototransduction deactivation in the rod.

Mutagenic Characterization of p44. To ascertain whether potential structural difference between arrestin and p44 could

Table 2: Table of p44 Mutants[a]

p44 mutant	substitution
D30A	$Asp^{30} \rightarrow Ala$
D296A	$Asp^{296} \rightarrow Ala$
D303A	$Asp^{303} \rightarrow Ala$
3DA	$Asp^{30} \rightarrow Ala$, $Asp^{296} \rightarrow Ala$, and $Asp^{303} \rightarrow Ala$
R175A	$Arg^{175} \rightarrow Ala$
R175E	$Arg^{175} \rightarrow Glu$
K15A	$Lys^{15} \rightarrow Ala$
2KA	$Lys^{14} \rightarrow Ala$ and $Lys^{15} \rightarrow Ala$

[a] The column labeled "substitution" describes the residues mutated in the specified p44 mutant.

explain their differential binding to different species of phosphorylated rhodopsin, we examined the role of certain residues that have been found to be involved in arrestin phosphate sensitivity. A great deal is known about the way arrestin responds to the phospho-residues on the rhodopsin tail (*18*). However, not much is know about the mechanism by which p44 responds to phosphorylated rhodopsin. Molecular dynamic computational simulations modeling the crystal structure of arrestin without the last 35 C-terminal amino acids suggest that the basal structures of both arrestin and p44 are very similar (Susan Gregurick, personal communication). For that reason, we employed a mutagenic approach to determine whether there are structural differences between the basal states of arrestin and p44.

Since several studies have identified phospho-residues-sensitive regions in arrestin that are involved in arrestin activation, we targeted the corresponding residues on p44 to establish whether they are also involved in p44's mechanism for selectivity toward phosphorylated rhodopsin. Some of the residues we targeted belong to a group of residues termed the "polar core" (*22*). These residues, which consist of arrestin residues Asp^{30}, Arg^{175}, Asp^{296}, Asp^{303}, and Arg^{382}, are a set of acidic and basic amino acids buried within the fulcrum of the two β-sheet sandwiches that comprise the arrestin molecule (*23*). They interact through a series of hydrogen-bonded salt-bridges, which maintain arrestin in an inactive conformation. Disruption of the charge equilibrium within the polar core either by the charge neutralization or the charge reversal of one of the polar core residues yields arrestin mutants that are constitutively active (*22*, *24*). This means that the phosphate selectivity of these arrestin mutants has been compromised and they are able to bind to Rho* with a higher affinity than Wt arrestin. For that reason, it has been suggested that the rhodopsin phospho-residues activate arrestin by disrupting the charge equilibrium within the polar core. We generated p44 mutants with single alanine substitutions for Asp^{30} (D30A-p44), Arg^{175} (R175A-p44), Asp^{296} (D296A-p44), and Asp^{303} (D303A-p44) (Table 2). The ability of these mutants to inhibit both phosphorylated and unphosphorylated Wt rhodopsin was then assayed (Figure 6). The corresponding Arg^{382} mutation cannot be generated in p44 since p44 lacks the carboxy-tail and therefore lacks this residue. We also generated a triple mutant where we substituted all three Asp residues (Asp^{30}, Asp^{296}, and Asp^{303}) with alanine residues (3DA).

Our results show that, unlike arrestin, the neutralization of the four major polar core residues does not confer constitutive activity to p44 (Figure 6). In fact, we find that all the p44 polar core mutants have decreased binding to

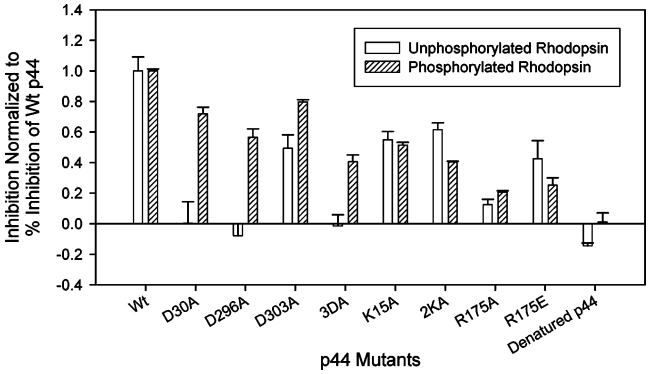

FIGURE 6: Effect of p44 mutants on phosphorylated and unphosphorylated rhodopsin. GTPγ^{35}S-binding assays were performed as described in Materials and Methods. Rhodopsin was expressed in Cos-1 cells, and membranes were purified as described in Materials and Methods. p44 mutants were expressed in *Pichia pastoris* and purified as described in Materials and Methods. Table 2 outlines the amino acid substitutions for each of the p44 mutants. The *y*-axis represents % inhibition normalized to % inhibition of each type of rhodopsin (Rho* or P-Rho*) by Wt p44. The final concentration of p44 in all the reactions with the p44 mutants (except the denatured p44 reactions) was 5 μM. The final p44 concentration for denatured p44 was 10 μM. To generate denatured p44, an aliquot of p44 was incubated at 95 °C for 5 min and cooled on ice for 5min prior to its addition to the reaction. (Error bars are SEM; $n = 2-4$.)

Rho* compared to Wt p44 (Figure 6, white bars). The p44 mutants D30A-p44, D296A-p44, 3DA-p44, and R175A-p44 have completely lost the ability to bind and inhibit Rho*, while D303A-p44 retains some inhibitory activity. We then tested the ability of these mutants to inhibit P-Rho* (Figure 6, hatched bars). D296A and 3DA show only background-level inhibitory activity, whereas D30A-p44, R175A-p44, and D303A-p44 show lowered inhibition levels relative to Wt p44. To demonstrate that the decrease in inhibition levels seen in some of the p44 mutants is not due to misfolding as a result of the alanine substitutions, we tested the inhibitory effect of 10 μM denatured p44. As can be seen in Figure 6, even at high concentrations, denatured p44 has no effect on rhodopsin activity, suggesting that the decrease in inhibition levels of p44 mutants D30A-p44, D303A-p44, and R175A-p44 is due to loss of stabilizing interactions between rhodopsin and p44 as opposed to the inability of these mutants to fold into a natured structure. Additionally, with the exception of D296A-p44, purification of all the p44 mutants entails passage through an affinity (Heparin) column. Although heparin does not induce the same conformational changes as a phosphorylated rhodopsin tail, only a properly folded p44 molecule would respond to heparin (*25*). Therefore, our purification methods would suggest that these proteins are not denatured. Mutagenic analysis of the presumed p44 polar core therefore reveals that these residues, which keep arrestin inactive, are not involved in maintaining p44 in basal inactive state, although they are clearly important for the overall interaction between p44 and Rho*. To determine whether phosphorylation can induce increased p44-mediated rhodopsin deactivation by the p44 mutants, we compared inhibition of both unphosphorylated and phosphorylated rhodopsin in the presence of 5 μM p44. We found that, although the p44 mutants exhibit a decreased ability to inhibit phospho-rhodopsin compared to Wt p44, phosphorylation does increase their ability to inhibit rhodop-

sin, suggesting that there is still a phospho-sensitive region in these p44 mutants.

Since the charge equilibrium within the arrestin polar core is important for maintaining arrestin in an inactive state, we tested whether a charge reversal of Arg^{175} (R175E-p44) could render p44 constitutively active. This mutation significantly affects arrestin's selectivity for phosphorylated rhodopsin (*24*). As a result, an $Arg^{175} \rightarrow$ Glu mutation in arrestin allows arrestin to bind Rho* in a manner that is indistinguishable from its binding to P-Rho*. However, it appears that the same mutation in p44 (R175E-p44) does not result in constitutive activity. Actually, R175E shows lowered inhibition of both Rho* and P-Rho* as compared to Wt p44. This further demonstrates that Arg^{175} is not a part of the putative p44 polar core, yet it is important for the overall binding of p44 to Rho*.

In addition to the polar core residues, two other residues play a significant role in arrestin's phosphate sensitivity. The two lysine residues, Lys^{14} and Lys^{15}, are found to be necessary for arrestin activation (*26*). When these two residues are absent, the arrestin carboxyl tail cannot be displaced in the presence of the phosphorylated rhodopsin tail, and therefore, arrestin cannot be activated (*27*). To determine whether these residues play a role in p44's phosphates sensitivity, we generated p44 mutants that lacked one (K15A) or both (2KA) of these lysine residues. We then tested their ability to bind and inhibit both P-Rho* and Rho*. As seen in Figure 6, we find that K15A and 2KA have lowered inhibition of Rho and P-Rho* compared to Wt p44. Neutralization of Lys^{14} and Lys^{15} affects p44's ability to inhibit P-Rho* more than p44's ability to inhibit Rho*, suggesting that the two lysines are important for the interaction between p44 and the phosphorylated rhodopsin tail.

DISCUSSION

Although a great deal is known about the biochemistry and binding kinetics of arrestin to rhodopsin, the function of p44 in the vertebrate rod has yet to be determined. In this study, we demonstrate that p44 has distinct requirements for rhodopsin binding and that these requirements are different from those we have identified for arrestin. We also demonstrate that structural differences between arrestin and p44 may explain why they differentially bind to phosphorylated rhodopsin. This, in turn, presents a regulatory mechanism for binding of both arrestin and p44.

The specific interactions between arrestin and the phosphorylated rhodopsin tail are well-characterized. One aspect of this interaction does not allow for the substitution of acidic residues for phospho-residues. This suggests that the mere presence of ionic partners on the rhodopsin tail is not sufficient to effectively inhibit transducin activation by rhodopsin. Our current studies with p44 suggest that a similar mechanism is in place for the interaction between p44 and rhodopsin. We find that the presence of acidic residues on the rhodopsin tail does not increase p44-mediated rhodopsin deactivation to the same extent as the presence of phospho-residues. Since we know that arrestin phospho-residues are needed to induce arrestin into an active conformation, these results suggest that p44 might also require a conformational change for its activation.

Structural Differences between Arrestin and p44. The mutagenic studies on p44 presented in this study suggest that either p44 uses different residues for its phosphates sensitivity or the specific role of the phospho-sensitive residues has been altered in p44. It is known that arrestin is maintained in a basal inactive state through several ionic intramolecular interactions, mainly provided by the polar core and the anchored carboxyl tail. Disruption of the polar core interactions can be simulated by mutations that neutralize or reverse the charge of the pertinent residues. Arrestin mutants that have these altered charges show some level of constitutive activity (binding to unphosphorylated rhodopsin to a higher level than Wt arrestin). To determine whether p44 relies on the same complement of residues for phosphate sensitivity, we neutralized the corresponding "polar core" residues in p44 and assayed for constitutive activity in these p44 mutant. Not only did we not detect any constitutive activity in the p44 polar core mutants, but we also found that neutralizing the arrestin polar core residues leads to decreased inhibition of both Rho* and P-Rho*. This suggests that the role of these residues is altered in p44, such that their neutralization prevents proper binding of p44 to photolyzed rhodopsin.

The studies with the p44 polar core mutants illustrate the structural importance of the interaction between the arrestin carboxyl tail and the main arrestin body. Although one of the arrestin "polar core" residues lies on the carboxyl tail (Asp^{383}), most of the residues reside on the main arrestin body (Asp^{30}, Arg^{175}, Asp^{296}, and Asp^{303}). Therefore, one might suspect that the presence of the carboxyl tail provides only a steric barrier between the polar core residues and the rhodopsin phospho-residues, and it is only the disruption of the polar core interactions that aids in rearranging arrestin's structural state. However, this does not seem to be the case. Loss of the interactions between arrestin's main body and the carboxyl tail region, as is the case for p44, appears to alter the arrangement of resides within the polar core to the point were mutation of these polar core residues, which would normally convey constitutive activity in arrestin, actually decreases the ability of p44 to bind and inhibit Rho* and P-Rho*. Therefore, from our studies, it would appear that the residues that help maintain p44 in a basal, inactive state differ from the polar core residues that maintain arrestin in an inactive state.

Effect of Rhodopsin Charged Cytoplasmic Tail on p44-Mediated Rhodopsin Deactivation. We find that the potential structural differences between p44 and arrestin have interesting implications for the interaction of these molecules with the negative charges on the rhodopsin tail. As mentioned previously, the presence of negative residues on the rhodopsin tail can increase the binding of arrestin to rhodopsin. Computational and in vitro analyses of the arrestin−rhodopsin interaction suggest that negative residues at positions 334 and 340 on the rhodopsin tail are sufficient for stabilizing the interaction between arrestin and rhodopsin. Further analysis of the interaction between arrestin and D-tail rhodopsin reveals specific sites on arrestin that formed ionic interaction with the negative charges on the rhodopsin tail. When we analyze the effect of p44 on D-tail rhodopsin, we find that negative residues at positions 334 and 340 on the rhodopsin tail are not sufficient to account for the increased p44-mediated deactivation of D-tail rhodopsin.

Likewise, we found that p44 and arrestin prefer different complements of phospho-residues on the rhodopsin tail for optimal rhodopsin deactivation. In fact, three of the rhodopsin phosphorylatable mutants we tested show opposite arrestin and p44 effects. Rhodopsin mutants S334/S343/T340 and Serines-T340 show Wt-level arrestin-mediated deactivation while displaying minimal p44-mediated deactivation. In contrast, S334/T340 shows Wt-level p44-mediated deactivation while showing minimal arrestin-mediated deactivation. Although current molecular dynamic simulations with a p44-like molecule (arrestin structure lacking the C-terminal 35 amino acids) show no difference between the basal structures of arrestin and p44 (Susan Gregurick, personal communication), our mutagenic work strongly suggests conformational differences between arrestin and p44.

Since arrestin and p44 behave differently with the different rhodopsin mutants, differential phosphorylation of the rhodopsin tail may provide a mechanism for regulating the binding of the two regulatory proteins during rhodopsin deactivation. This seems an odd regulatory mechanism given that there is no evidence to suggest that rhodopsin is differentially phosphorylated under different illumination conditions. However, a study by Kennedy and colleagues shows that a progression of successive phosphorylation and dephosphorylation can generate a series of differentially phosphorylated rhodopsin molecules over time (*21*). Time-dependent mass spectrometry studies of an illuminated rod found that Ser^{343} is the first residue to be phosphorylated, followed by Ser^{338}, and then followed by the phosphorylation of Ser^{334} last. Subsequent dephosphorylation in the same order results in the generation of a series of phospho-rhodopsin species in order of P-Ser^{343}, P-Ser^{338}/P-Ser^{343}, P-Ser^{334}/P-Ser^{338}/P-Ser^{343}, P-Ser^{334}/P-Ser^{338}, and P-Ser^{334}. We show that arrestin and p44 are capable of inhibiting the activity of all these mutants by at least 30% (arrestin) and 60% (p44). In addition, we demonstrate that p44 is a more effective inhibitor of P-Ser^{343}, which is the first phospho-rhodopsin species to be generated. Meanwhile, we show that a single phospho-serine at position 334 (P-Ser^{334}), which is the last residue to be dephosphorylated, enhances arrestin-mediated rhodopsin deactivation better than a single phospho-serine at 343 (P-Ser^{343}).

Importance of Differential Arrestin/p44 Binding. Regulation of arrestin and p44 binding potentially affects the kinetics of a rod response. Recent in vivo studies of arrestin/p44 knock-out/knock-in mice suggest that p44 is important for recovery of the photic response under dim-light conditions (*28*). In contrast, arrestin appears to be involved in deactivation of phototransduction under bright, or constant, illumination. Recently, the translocation of photransduction machinery in the rod as a response to light has become of great interest. Nair and colleagues recently characterized the rate of arrestin translocation in live photoreceptors (*29*). They demonstrated that arrestin translocation into a bleached region of the rod has a half-time of less than 2 min. Together with the timeline of rhodopsin phosphorylation suggested by Kennedy and colleagues, the effective arrestin concentration of arrestin in the rod outer segment would be low relative to p44 when P−Ser^{343} rhodopsin appears. However, given that most of the p44 population is permanently in the outer segment, p44 would be predisposed to immediate binding to photolyzed, phosphorylated rhodopsin. After translocation of arrestin to the outer segment, and continued presence of P-Ser^{334} rhodopsin, deactivation of rhodopsin activity would most likely be dependent on arrestin binding to rhodopsin.

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