Binding of Arrestin to Cytoplasmic Loop Mutants of Bovine Rhodopsin†

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ABSTRACT: The binding of arrestin to rhodopsin is a multistep process that begins when arrestin interacts with the phosphorylated C terminus of rhodopsin. This interaction appears to induce a conformational change in arrestin that exposes a high-affinity binding site for rhodopsin. Several studies in which synthetic peptides were used have suggested that sites on the rhodopsin cytoplasmic loops are involved in this interaction. However, the precise amino acids on rhodopsin that participate in this interaction are unknown. This study addresses the role of specific amino acids in the cytoplasmic loops of rhodopsin in binding arrestin through the use of site-directed mutagenesis and direct binding assays. A series of alanine mutants within the three cytoplasmic loops of rhodopsin were expressed in HEK-293 cells, reconstituted with 11-*cis*-retinal, prephosphorylated with rhodopsin kinase, and examined for their ability to bind in vitro-translated, ^{35}S-labeled arrestin. Mutations at Asn-73 in loop I as well as at Pro-142 and Met-143 in loop II resulted in dramatic decreases in the level of arrestin binding, whereas the level of phosphorylation by rhodopsin kinase was similar to that of wild-type rhodopsin. The results indicate that these amino acids play a significant role in arrestin binding.

G protein-coupled receptors (GPCRs)[1] transduce extracellular signals through their specific interaction with heterotrimeric G proteins. The activation of G proteins initiates a series of cellular responses that are critical for the control of growth, differentiation, and a number of metabolic processes (*1*, *2*). Attenuation of G protein signaling is mediated by a process known as desensitization, which can be defined as reduced responsiveness to continual stimulation by agonist. The desensitization of GPCRs is initiated when the agonist-activated receptor is phosphorylated by specific serine-threonine kinases known as G protein-coupled receptor kinases (GRKs), followed by the binding of a member of the arrestin family of proteins (*3*). The arrestin family presently includes two visual arrestins, rod arrestin (also known as S-antigen or 48K protein) and cone arrestin, and two β-arrestins, β-arrestin 1 and β-arrestin 2 (*4*, *5*). The binding of arrestin results in the uncoupling of receptors from their G proteins (*5*). A number of studies have described a new role for β-arrestin family members in the internalization of receptor−arrestin complexes through clathrin-coated pits (*6*−*11*), although such a role has not been identified for the visual arrestins.

Rhodopsin, the photoreceptor of the rod cell, has been used widely as a model for studying the regulation of GPCR desensitization through phosphorylation and arrestin binding. Rhodopsin kinase (GRK1), the rod cell member of the GRK family, has been shown in vitro to phosphorylate a series of seven serine and threonine residues located in the C terminus of rhodopsin (*12*). However, only one or two sites are thought to be phosphorylated in vivo (*13*). This appears to be sufficient for the binding of rod arrestin, a process that blocks the activation of G_t (transducin, the rod cell G protein) by sterically hindering its interaction with rhodopsin (*14*−*16*). Several studies have indicated that the binding of arrestin to rhodopsin is a multistep process (*17*−*20*). The first step is a conformational change in arrestin mediated by its interaction with the phosphorylated C terminus of rhodopsin. The second is the binding of this activated arrestin to a site on rhodopsin predicted to be within the cytoplasmic loops. Despite reports from peptide competition studies that implicate cytoplasmic loops I and III in the binding of arrestin (*18*, *21*), the key residues that mediate this process have not been identified.

This report describes the use of site-directed mutagenesis in defining sites within the cytoplasmic loops of rhodopsin that are critical for its interaction with rod arrestin. A series of mutants within the cytoplasmic loops of rhodopsin, previously analyzed for their ability to activate G_t and to be phosphorylated by rhodopsin kinase (*22*), were examined for their ability to bind arrestin in vitro. Our results suggest, for the first time, the participation of specific residues in cytoplasmic loops I and II in the formation of a contact site for arrestin.

MATERIALS AND METHODS

Mutagenesis of Bovine Rhodopsin

The cDNA for bovine rhodopsin (*23*) was inserted into the vector pSelect (Promega). Single-stranded DNA was generated and subjected to site-directed mutagenesis using

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[1] Abbreviations: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; DTT, dithiothreitol; HEK-293, human embryonic kidney-293; ROS, rod outer segment; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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the Altered Sites mutagenesis kit (Promega) as described previously (*24*). All mutants were sequenced for verification using Sequenase (Amersham Corp.). The entire sequence of mutants that demonstrated reduced arrestin binding was determined at The University of North Carolina at Chapel Hill Automated DNA Sequencing Facility. For expression in HEK-293 cells, the wild-type and mutant cDNAs were inserted into the vector pcDNAI/Amp (Invitrogen) at a *Hin*dIII site within the polylinker region.

Expression of Mutant Rhodopsin in HEK-293 Cells

The vector pcDNAI/Amp, containing either wild-type or mutant rhodopsin cDNA inserts (*22*), was cotransfected with pRSV-TAg (*25*), a vector containing the gene for simian virus 40 T antigen, into HEK-293 cells using a DEAE-dextran protocol (*22*, *24*, *26*). Cells were harvested approximately 70 h after transfection, and plasma membranes were prepared by sucrose density gradient centrifugation (*24*). Protein assays were performed as described by Bradford (*27*). The level of rhodopsin expression in these membranes was determined by Western blot analysis using an anti-rhodopsin monoclonal antibody, R2-15N, that recognizes an epitope at the N terminus of rhodopsin (provided by P. Hargrave, University of Florida, Gainesville, FL) (*28*), followed by incubation with [^{125}I]protein A. The level of expression was quantified using a Molecular Dynamics PhosphorImager. Urea-stripped rod outer segment (ROS) membranes, in which the level of rhodopsin has been determined spectrophotometrically using a molar extinction coefficient of 42 700 M^{-1} cm^{-1} at 498 nm (*29*), were used as a standard. Because of the heterogeneous pattern observed for the glycosylation of rhodopsin expressed in HEK-293 cells (*24*), the entire lane was measured for each sample.

Phosphorylation of Rhodopsin by Rhodopsin Kinase

Rhodopsin kinase was extracted from the bovine ROS membranes as described (*30*) and used to phosphorylate bovine rhodopsin expressed in HEK-293 cells as described previously (*20*, *22*). HEK-293 cell membranes containing 0.4 μg of rhodopsin were diluted into buffer A (described below) and preincubated in the dark with 14 μM 11-*cis*-retinal for 1 h at room temperature followed by centrifugation at 12000*g* for 10 min. The level of total protein was adjusted through the addition of nontransfected cell membranes so that all samples contained equal amounts of total protein. The phosphorylation reaction was carried out in a 40 μL volume of 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 6 mM $MgCl_2$, 1 mM DTT, 10 mM NaF, aprotinin (2 μg/mL), and leupeptin (1 μg/mL) (buffer A) containing 150 μM [γ-^{32}P]-ATP (125 μCi/mL) at 30 °C under fluorescent room lights for 8 min. The reaction was terminated by placing the samples on ice and adding 0.5 mL of ice-cold buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM NaF, and 10 mM ATP. After centrifugation at 38500*g* for 15 min, the pellet was resuspended in 20 mM Tris-buffered saline (TBS, pH 7.5) containing 2 mM EDTA, 1 mM DTT, 10 mM NaF, aprotinin (2 μg/mL), and leupeptin (1 μg/mL). After solubilization of the membranes in the same buffer containing octyl glucoside (1 mg of octyl glucoside/21 μg of membrane protein; 1.5−1.7% w/v), the mixture was incubated at room temperature for 1 h, and then centrifuged to remove the insoluble material. The phosphorylated rhodopsin in the supernatant was immunoprecipitated by incubation with the R2-15N anti-rhodopsin antibody for 1 h and then with protein A−Sepharose beads for 30 min at room temperature. The beads were washed three times in TBS containing 0.1% deoxycholate and 10 mM NaF and once with 10 mM Tris-HCl (pH 6.8). Rhodopsin was extracted from the beads with Laemmli sample buffer and analyzed by SDS−PAGE (*31*). The level of phosphorylation was quantified by phosphorimage analysis of the dried gels.

Arrestin Binding Assay

Prephosphorylation of Rhodopsin by Rhodopsin Kinase. Rhodopsin kinase extracted from bovine ROS membranes was used to phosphorylate bovine rhodopsin expressed in HEK-293 cells as described previously (*20*). HEK-293 cell membranes containing 0.4 μg of rhodopsin were preincubated in the dark with 14 μM 11-*cis*-retinal for 1 h at room temperature as described in the previous section. The level of total protein was also adjusted for these experiments through the addition of nontransfected cell membranes. The phosphorylation reaction was carried out in buffer A containing 2 mM ATP at 30 °C under fluorescent room lights. After 1 h, the reaction mixture was diluted with 1 mL of ice-cold buffer A and washed twice with the same buffer by centrifugation at 38500*g* for 15 min. Following phosphorylation, the rhodopsin was again regenerated with 14 μM 11-*cis*-retinal for 1 h at room temperature in buffer B, which consists of 30 mM HEPES (pH 7.5), 2 mM $MgCl_2$, 150 mM potassium acetate, 1 mM DTT, 10 mM NaF, aprotinin (2 μg/mL), and leupeptin (1 μg/mL). The membranes were pelleted by centrifugation at 12000*g* for 10 min and resuspended in buffer B for the arrestin binding assay.

In Vitro Translation of Arrestin. The plasmid (*32*) containing the cDNA for bovine arrestin (*33*) was a gift from V. V. Gurevich (Sun Health Research Institute, Sun City, AZ). The arrestin constructs were linearized, transcribed with SP6 RNA polymerase, and translated in the presence of [^{35}S]methionine using rabbit reticulocyte lysate (Promega) (*20*). The amount of protein synthesized was determined by measuring the level of the [^{35}S]methionine incorporated into a hot trichloroacetic acid-insoluble fraction using liquid scintillation spectroscopy (*34*).

Arrestin Binding Assay. The arrestin binding assay was performed essentially as described previously (*20*) with minor modifications. Briefly, phosphorylated and 11-*cis*-retinal-regenerated bovine rhodopsin (0.4 μg) was mixed in the dark with radiolabeled arrestin (30 000 cpm or approximately 10 fmol) in buffer B in a volume of 35 μL, and then exposed to fluorescent room lights for 5 min at 4 °C, followed by incubation at 37 °C for an additional 5 min. The binding reaction was terminated by 6.7-fold dilution with ice-cold buffer B. The diluted reaction mixture was layered over a cushion of ice-cold 0.2 M sucrose in buffer B and centrifuged at 100000*g* for 30 min at 2 °C. For some experiments, the centrifugation time was varied between 3 and 30 min (3, 5, 10, and 30 min), as described in the Results. The pelleted rhodopsin containing bound arrestin was washed with buffer

Table 1: Site-Directed Mutants of Bovine Rhodopsin[a]

	62												73
Loop I	T	V	Q	H	K	K	L	R	T	P	L	N	
T62A/V63A/Q64A	A	A	A	-	-	-	-	-	-	-	-	-	
H65Y	-	-	-	Y	-	-	-	-	-	-	-	-	
K66A/K67A	-	-	-	-	A	A	-	-	-	-	-	-	
L68A/R69A/T70A	-	-	-	-	-	-	A	A	A	-	-	-	
P71A	-	-	-	-	-	-	-	-	-	A	-	-	
L72A/N73A	-	-	-	-	-	-	-	-	-	-	A	A	
L72A	-	-	-	-	-	-	-	-	-	-	A	-	
N73A	-	-	-	-	-	-	-	-	-	-	-	A	

	141												152
Loop II	K	P	M	S	N	F	R	F	G	E	N	H	
K141A/P142A/M143A	A	A	A	-	-	-	-	-	-	-	-	-	
K141A	A	-	-	-	-	-	-	-	-	-	-	-	
P142A	-	A	-	-	-	-	-	-	-	-	-	-	
M143A	-	-	A	-	-	-	-	-	-	-	-	-	
S144A/N145A/F146A	-	-	-	A	A	A	-	-	-	-	-	-	
R147A/F148A/G149A	-	-	-	-	-	-	A	A	A	-	-	-	
E150A/N151A/H152A	-	-	-	-	-	-	-	-	-	A	A	A	

	231																				252
Loop III	K	E	A	A	A	Q	Q	Q	E	S	A	T	T	Q	K	A	E	K	E	V	T	R
K231A/E232A	A	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A233G/A234G/A235G	-	-	G	G	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A233N/A234N/A235N	-	-	N	N	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A233L/A234L/A235L	-	-	L	L	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Q236A/Q237A/Q238A	-	-	-	-	-	A	A	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E239A/S240A	-	-	-	-	-	-	-	-	A	A	-	-	-	-	-	-	-	-	-	-	-	-
T242A/T243A	-	-	-	-	-	-	-	-	-	-	A	A	-	-	-	-	-	-	-	-	-	-
Q244A/K245A	-	-	-	-	-	-	-	-	-	-	-	-	A	A	-	-	-	-	-	-	-	-
E247A/K248A/E249A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	A	A	-	-	-	-
V250A/T251A/R252A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	A	A	A

[a] Mutants in nonitalicized type have been characterized previously for their ability to be phosphorylated by rhodopsin kinase and to activate G_t (*22*).

B and dissolved in Laemmli sample buffer. The proteins were resolved by 10% SDS−PAGE. The amount of arrestin bound to wild-type or mutant rhodopsin was quantified by phosphorimage analysis of the dried gels. The amount of arrestin bound to nontransfected cell membranes was used as a measure of the level of nonspecific binding and was subtracted from the amount of arrestin bound to rhodopsin-containing membranes. This value was approximately 2−4% of the level of arrestin bound to phosphorylated, light-activated wild-type rhodopsin. The results of the binding experiments were normalized to the amount of arrestin bound to wild-type, phosphorylated rhodopsin. Arrestin binding to nonphosphorylated rhodopsin was also assessed for some mutants as indicated in the Results and figure legends.

RESULTS

Previously, our laboratory analyzed a series of clustered alanine mutants for their ability to be phosphorylated by rhodopsin kinase and to activate G_t (*22*). In this study, these mutants (Table 1) were examined for their ability to bind arrestin. Mutants demonstrating a specific and substantial influence on arrestin binding were separated into single amino acid mutants and also tested for their ability to bind arrestin to determine the individual residues that participate in this interaction.

Loop I. This is a relatively short, highly basic loop that is well-conserved between mammalian rod and cone opsins. All of the amino acids were changed to alanines except for

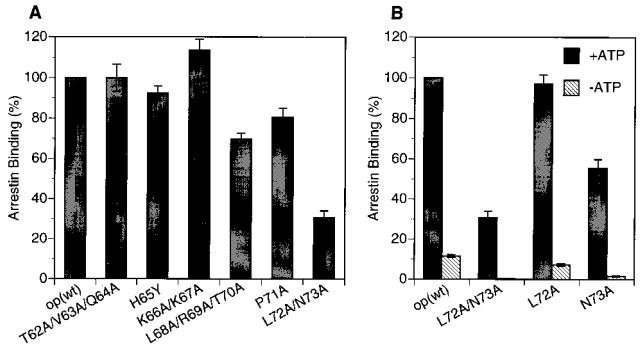

FIGURE 1: Arrestin binding to mutants in loop I of bovine rhodopsin. (A) Membranes prepared from HEK-293 cells expressing wild-type or mutant rhodopsin were reconstituted with 11-*cis*-retinal, phosphorylated by rhodopsin kinase, and assayed for their ability to bind ^{35}S-labeled arrestin prepared by in vitro translation as described in Materials and Methods. The value for arrestin binding to nontransfected cell membranes was subtracted from all values as a measure of nonspecific binding, and the results were normalized to the level of arrestin binding for wild-type [op(wt)] rhodopsin. Error bars represent the standard error. The results are representative of several experiments from at least two transfections. (B) Membranes prepared from HEK-293 cells expressing wild-type or mutant rhodopsin were reconstituted with 11-*cis*-retinal, phosphorylated by rhodopsin kinase (+ATP) or left untreated (−ATP), and assayed for arrestin binding as described for panel A. Error bars represent the standard error. The results are representative of three experiments from at least two transfections.

His-65, which was mutated to a tyrosine (Table 1). Three of these mutants, T62A/V63A/Q64A, H65Y, and K66A/K67A, showed no significant change in the level of arrestin binding compared to that of wild-type rhodopsin (Figure 1A). In contrast, two mutants, L68A/R69A/T70A and P71A, showed a moderate decrease (28 and 15%, respectively) and L72A/N73A demonstrated a substantial (70%) decrease in the level of arrestin binding. On the basis of these results, two single amino acid mutants, L72A and N73A (Table 1), were generated, expressed in HEK-293 cells, and examined for arrestin binding. L72A was similar to wild-type rhodopsin in its ability to bind arrestin (Figure 1B). However, N73A showed a 42% decrease in the level of arrestin binding. Since this mutant exhibited normal levels of phosphorylation (data not shown), the results suggest that Asn-73 is involved in arrestin binding. Previously, Gurevich and Benovic demonstrated that in vitro-translated arrestin binds to nonphosphorylated, light-activated rhodopsin, although at a much lower level than to phosphorylated light-activated rhodopsin (*17*). Similarly, the level of arrestin binding to nonphosphorylated wild-type rhodopsin in our experiments was approximately 10% of the level of binding to phosphorylated rhodopsin. In the absence of phosphorylation, mutants L72A/N73A and N73A displayed a reduced level of arrestin binding that was roughly proportional to the observed decrease in the level of binding to phosphorylated rhodopsin (Figure 1B). Although these effects are small, they are consistent with an influence on arrestin binding and independent of an influence on the phosphorylation of the mutants by rhodopsin kinase.

Loop II. Several studies have implicated the second cytoplasmic loop of rhodopsin in the activation of G_t and in phosphorylation by rhodopsin kinase (*22*, *35*, *36*). In the experiments described here, an approximately 98% decrease in the level of arrestin binding was observed for mutant K141A/P142A/M143A (Figure 2A), despite the fact that it

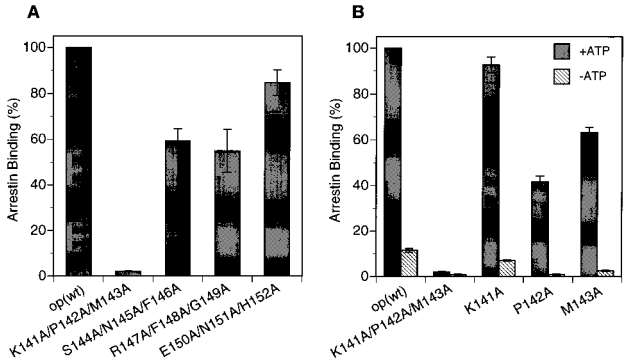

FIGURE 2: Arrestin binding to mutants in loop II of bovine rhodopsin. (A) Membranes prepared from HEK-293 cells expressing wild-type or mutant rhodopsin were reconstituted with 11-*cis*-retinal, phosphorylated by rhodopsin kinase, and assayed for their ability to bind ^{35}S-labeled arrestin as described in Materials and Methods. Error bars represent the standard error. The results are representative of several experiments from at least two transfections. (B) Membranes prepared from HEK-293 cells expressing wild-type or mutant rhodopsin and reconstituted with 11-*cis*-retinal were phosphorylated by rhodopsin kinase (+ATP) or left untreated (−ATP) and assayed for arrestin binding as described for panel A. Error bars represent the standard error. The results are representative of three experiments from at least two transfections.

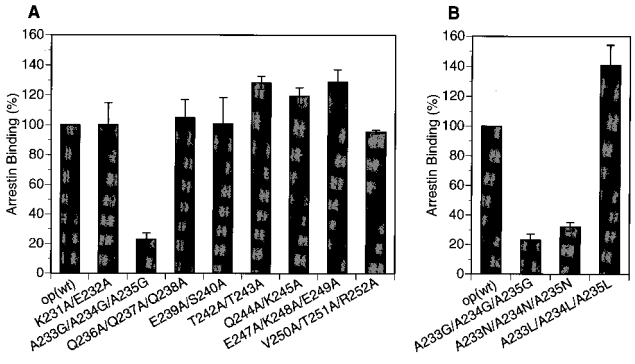

FIGURE 3: Arrestin binding to mutants in loop III of bovine rhodopsin. (A) HEK-293 cell membranes expressing wild-type or mutant rhodopsin were reconstituted with 11-*cis*-retinal, phosphorylated by rhodopsin kinase, and assayed for their ability to bind ^{35}S-labeled arrestin as described in Materials and Methods and in the legend of Figure 2. Error bars represent the standard error. The results are representative of several experiments from at least two transfections. (B) HEK-293 cell membranes expressing wild-type or mutant rhodopsin were reconstituted with 11-*cis*-retinal, phosphorylated by rhodopsin kinase, and assayed for arrestin binding as described for panel A. Error bars represent the standard error. The results are representative of four or five experiments from at least two transfections.

displayed only a 20% decrease in the level of phosphorylation by rhodopsin kinase (*22*). S144A/N145A/F146A and R147A/F148A/G149A demonstrated 25 and 40% decreases in the level of arrestin binding, respectively. These mutants previously showed 26 and 40% decreases in the level of phosphorylation, respectively (*22*). Therefore, the observed decrease in the level of arrestin binding may be due to a reduced level of phosphorylation by rhodopsin kinase. In contrast to these mutants, E150A/N151A/H152A demonstrated only a 12% decrease in the level of arrestin binding.

Since K141A/P142A/M143A showed the greatest decrease in the level of arrestin binding of the loop II mutants, but exhibited only a small effect on phosphorylation, three individual point mutants, K141A, P142A, and M143A (Table 1), were generated, expressed, and examined for their ability to bind arrestin (Figure 2B). K141A bound arrestin to a similar extent as did wild-type rhodopsin. In contrast, P142A and M143A showed 58 and 37% decreases, respectively, in the level of arrestin binding. Similar to the results described for the loop I mutants, the level of binding of arrestin to nonphosphorylated mutant rhodopsin was proportionately lower. All three point mutants demonstrated normal levels of phosphorylation (data not shown), suggesting that Pro-142 and Met-143 play roles specifically in the binding of arrestin to rhodopsin. The residue corresponding to Met-143 in all human cone opsins is a phenylalanine (*37*). To determine whether phenylalanine might substitute for the methionine found in rhodopsin, mutant M143F was generated and expressed in HEK-293 cells. This mutant demonstrated a 26% decrease in the level of arrestin binding (data not shown). Although not a highly disruptive mutation, the result does suggest that the requirement for methionine at this position in rhodopsin is not simply due to its hydrophobicity.

Loop III. The third cytoplasmic loop has been implicated in both G protein activation and G protein selectivity for many GPCRs, including rhodopsin (*38*). A number of in vitro studies have also implicated this loop in the regulation of rhodopsin phosphorylation and arrestin binding (*18*, *21*, *22*, *36*, *39*). All of the mutants demonstrated arrestin binding levels similar to that of wild-type rhodopsin except for mutations at Ala-233-Ala-234-Ala-235 (Figure 3A). Our laboratory previously reported that both glycine (A233G/A234G/A235G) and asparagine (A233N/A234N/A235N) substitutions at these sites dramatically reduced the level of G_t activation and phosphorylation by rhodopsin kinase, although retinal binding is normal, suggesting that the overall tertiary structure of rhodopsin is preserved (*22*). In this study, A233G/A234G/A235G (Figure 3A,B) and A233N/A234N/A235N (Figure 3B) exhibited a substantial (70−80%) decrease in the level of arrestin binding, consistent with the 80% decrease in the level of phosphorylation reported previously for both mutants (*22*). In contrast, mutation of this sequence to leucines (A233L/A234L/A235L) caused a significant (40%) increase in the level of arrestin binding (Figure 3B). Phosphorylation of A233L/A234L/A235L by rhodopsin kinase demonstrated a similar increase (40%) compared to that of wild-type rhodopsin (Figure 4). Therefore, the changes in arrestin binding exhibited by these loop III mutants parallel changes in phosphorylation by rhodopsin kinase.

Combination Mutants: Loop I/Loop II and Loop II. The results from these experiments suggest that Asn-73 in loop I and Pro-142 and Met-143 in loop II of rhodopsin partially disrupt the interaction between rhodopsin and arrestin. To rule out the possibility that the observed decrease in the level of binding for the mutants is due to dissociation during centrifugation, the time of centrifugation during the arrestin binding assay was varied from 3 to 30 min. The level of arrestin binding to rhodopsin did not change for wild-type rhodopsin or these three mutants (data not shown), suggesting that the arrestin−rhodopsin complexes are stable during centrifugation and that the differences observed for arrestin binding to the mutants are not due to dissociation during this step. To determine whether mutation of a combination of these sites would enhance the level of disruption, mutants N73A/M143A, N73A/P142A, P142A/M143A, and N73A/

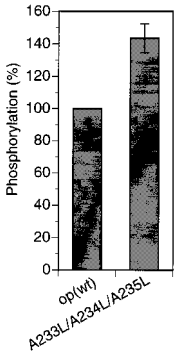

FIGURE 4: Phosphorylation of the loop III mutant, A233L/A234L/A236L, by rhodopsin kinase. Membranes from HEK-293 cells expressing either wild-type or A233L/A234L/A236L rhodopsin were incubated with 11-*cis*-retinal, phosphorylated by rhodopsin kinase, and immunoprecipitated as described in Materials and Methods. The results were normalized to the level of phosphorylation for wild-type rhodopsin. Error bars represent the standard error. The results are representative of six experiments from at least two transfections.

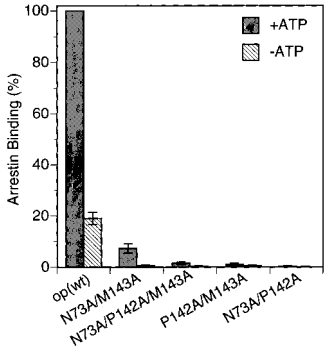

FIGURE 5: Arrestin binding to loopI/loop II combination mutants. HEK-293 cell membranes expressing wild-type or mutant rhodopsin were reconstituted with 11-*cis*-retinal, phosphorylated by rhodopsin kinase, and assayed for their ability to bind ^{35}S-labeled arrestin prepared by in vitro translation as described in Materials and Methods and in the legend of Figure 1. Error bars represent the standard error. The results are representative of three experiments from at least two transfections.

Table 2: Combination of Loop I and Loop II Mutants That Disrupt Arrestin Binding

loop II
P142A/M143A
loop I/loop II
N73A/P142A
N73A/M143A
N73A/P142A/M143A

P142A/M143A (Table 2) were constructed, expressed in HEK-293 cells, and examined for their ability to be phosphorylated by rhodopsin kinase and to bind arrestin (Figure 5). The levels of phosphorylation ranged from 73 to 90% of the levels for wild-type rhodopsin (data not shown). However, each combination mutant showed a greater than 95% decrease in the level of arrestin binding compared to that of wild-type rhodopsin. These results suggest that Asn-73, Pro-142, and Met-143 cooperate in the binding of arrestin to rhodopsin.

DISCUSSION

As described above, the results from a number of studies have led to a multistep model for arrestin binding to rhodopsin. The first step is an interaction between the phosphorylation recognition domain of arrestin and the phosphorylated C terminus of rhodopsin (*17*, *40*). This is thought to induce a conformational change in arrestin that can be detected as an increase in the sensitivity of arrestin to proteolytic digestion (*41*, *42*). A synthetic peptide corresponding to the phosphorylated C terminus of rhodopsin was shown to induce this conformational change in vitro (*19*). The second step is the stable binding of the activated arrestin to rhodopsin at a site presumed to be within the cytoplasmic loops. Incubation with the synthetic phosphopeptide induces arrestin to bind rhodopsin even when the carboxyl-terminal phosphorylation domain has been removed (*19*), suggesting that the stable binding of arrestin to rhodopsin does not require the C terminus. Peptide competition studies have implicated part of the first and third cytoplasmic loops of rhodopsin in arrestin binding (*18*). Consistent with these results, peptides representing the third cytoplasmic loops of the m_2- and m_3-muscarinic acetylcholine receptors and the $\alpha_{2A/D}$-adrenergic receptor were shown to bind directly to different members of the arrestin family (*21*).

Our own studies represent the first attempt to address the role of specific amino acids in the binding of arrestin to the cytoplasmic loops of rhodopsin. Initially, a series of clustered alanine substitution mutants of rhodopsin, used previously to identify sites that are involved in G_t activation and interaction with rhodopsin kinase (*22*), were examined for their ability to bind arrestin. The identification of several mutants that showed reduced levels of arrestin binding led to the construction and analysis of single amino acid mutants for defining specific residues within the cytoplasmic loops that are important for arrestin binding. Our results demonstrate that Asn-73 in loop I and two amino acids, Pro-142 and Met-143, in loop II are critical for this interaction. Mutations at these positions (N73A, P142A, and M143A) reduce the level of arrestin binding but do not affect the ability of rhodopsin to be phosphorylated by rhodopsin kinase. Any two of these mutations in combination almost entirely disrupt arrestin binding, also without significant effects on phosphorylation. These data imply that both cytoplasmic loops participate in the interaction of arrestin with rhodopsin. Although the level of binding of arrestin to nonphosphorylated rhodopsin mutants was low, the pattern was similar to that for phosphorylated rhodopsin, consistent with an influence on binding that is independent of phosphorylation by rhodopsin kinase (*17*).

It is notable that Asn-73 in loop I and Pro-142 in loop II are conserved among the four opsins, rhodopsin and red, blue, and green opsin, suggesting that they play an important structural or functional role in these proteins. Both loops I and II are predicted to form β-turns, on the basis of NMR analysis of the equivalent synthetic peptides (*43*). Substitution with alanine at the position of Asn-73 might be expected to increase the propensity for α-helical conformation or, alternatively, disrupt the potential formation of hydrogen bonds with other sites in rhodopsin or with arrestin. However, insufficient information exists to propose a specific role for this residue at this time. Mutation of Pro-142 to alanine in loop II might be expected to have dramatic effects on the structure of that loop. Prolines are frequently found at bends or termination points in α-helices and can increase the

rigidity of the surrounding sequence (*44*). Therefore, replacement of this amino acid may result in increased flexibility as well as a change in secondary structure. However, no change in the level of phosphorylation by rhodopsin kinase was observed for mutations at this site, despite the influence of mutations in the neighboring residues (S144A/N145A/F146A and R147A/F148A/G149A) (*22*). This suggests that drastic changes in secondary structure do not occur, supporting the possibility that Pro-142 is one site that is necessary for arrestin binding.

Loop III is predicted to be an α-helix (*45*−*47*) with a turn at its center (*43*, *48*). In our experiments, only mutation of the sequence Ala-233-Ala-234-Ala-235 to glycines or asparagines (A233G/A234G/A235G and A233N/A234N/A235N, respectively), both of which are predicted to disrupt α-helices, reduced the level of arrestin binding. Since the same mutants previously demonstrated similar decreases in the level of phosphorylation by rhodopsin kinase (*22*), a direct influence on arrestin binding could not be elucidated. Interestingly, the introduction of leucines at Ala-233-Ala-234-Ala-235, which might be predicted to enhance α-helical structure, increased the levels of both phosphorylation and arrestin binding. The results of these experiments suggest that helical structure may be important for the interaction of rhodopsin with rhodopsin kinase. To determine whether this region directly influences arrestin binding, alternative strategies that bypass the requirement for rhodopsin to be phosphorylated to bind arrestin will be employed. For example, preliminary experiments demonstrate that a mutant arrestin, R175E, which binds rhodopsin in a phosphorylation-independent manner (*49*, *50*), shows a dramatically reduced level of binding to the loop III A233G/A234G/A235G and A233N/A234N/A235N mutants. These data support a role for this sequence in arrestin binding that is independent of phosphorylation by rhodopsin kinase. A second strategy will be to use a peptide corresponding to the phosphorylated C terminus of rhodopsin to induce the binding of arrestin to nonphosphorylated A233G/A234G/A235G and A233N/A234N/A235N mutants. A reduced level of binding under these conditions would also suggest a separate role for this sequence in arrestin binding.

The model for arrestin binding proposed by Gurevich and Benovic (*17*) implies that multiple regions of arrestin coordinate to promote its stable interaction with rhodopsin. Peptide competition studies have predicted the involvement of cytoplasmic loops I and III in arrestin binding (*18*). We have used a method developed previously (*20*) to screen a series of rhodopsin mutants for comparative changes in the level of arrestin binding. We have identified mutations in loops I and II that result in reduced levels of arrestin binding to both phosphorylated and nonphosphorylated rhodopsin. These mutations, Asn-73 in loop I and Pro-142 and Met-143 in loop II, dramatically influence the ability of arrestin to bind to rhodopsin without affecting either phosphorylation or G_t activation. These amino acids are strong candidates for direct interaction with arrestin for forming a stable complex as part of the desensitization process. Results from analysis of the crystal structure of arrestin predict that the N terminus of arrestin may be closely associated with the loops of rhodopsin (*51*). This work represents an important step forward in refining our understanding of this interaction.

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