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ARRESTIN TRANSLOCATION IN ROD PHOTORECEPTORS

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1. INTRODUCTION

The vertebrate photoreceptor is the epitome of polarized neurons, containing two specialized compartments—the outer segment and the inner segment, connected by a narrow non-motile cilium. The outer segment of rod and cone photoreceptors is principally dedicated to capturing light and converting the energy of a photon into a change in membrane potential. The primary function of the inner segment is to provide the metabolic and synthetic demands of the photoreceptors. In order to maintain this high degree of specialization, molecules are routinely targeted to their appropriate compartment during protein synthesis. However, in addition to this relatively slow transport process, photoreceptors have a much more rapid process whereby some molecules are rapidly moved between the inner segment and outer segment through the connecting cilium in response to the light adaptational state of the eye. This translocation process has been conclusively demonstrated for two molecules involved in the phototransduction cascade—transducin and arrestin (Broekhuysse *et al.* 1985; Mangini and Pepperberg 1988; Whelan and McGinnis 1988; Sokolov *et al.* 2002; Peterson *et al.* 2003).

Recent studies have shown that the light-driven movement of arrestin is independent of activation of the phototransduction cascade since translocation is normal in transgenic mice that are deficient for transducin (Mendez *et al.* 2003; Zhang *et al.* 2003). Various ideas have been proposed regarding the potential function of protein translocation. These hypotheses include a role in the regulation of photoreceptor sensitivity (McGinnis *et al.* 1991; Sokolov *et al.* 2002), and a protective function in preventing light damage (Elias *et al.* 2004).

In this study, we address some of the mechanistic questions regarding arrestin translocation. Specifically we ask whether the light-driven redistribution of arrestin is a consequence of arrestin movement, or whether *de novo* protein synthesis coupled with arrestin degradation plays a significant role in the apparent redistribution of arrestin. In addition, we utilize a variant of arrestin that is unable to bind to rhodopsin to investigate the role of arrestin binding to light-activated phosphorhodopsin in the translocation of arrestin. Finally, we address whether vertebrate arrestins rely on an affinity for phosphoinositol lipids for translocation.

2. METHODS AND MATERIALS

2.1 Cycloheximide Treatment of Transgenic Tadpoles

Transgenic *Xenopus* expressing a fusion of GFP at the C-terminus of *Xenopus* arrestin (xAr-GFP) were obtained from breeding pairs of adult transgenic *Xenopus* as previously described (Peterson *et al.* 2003) and dark-adapted overnight. Tadpoles were placed in 0.1x tadpole Ringers with or without 100 μ M cycloheximide (CHI) for 1 h. Tadpoles then either remained in the dark, or were exposed to laboratory lighting for 45 min or for 240 min. At this point,

tadpoles were euthanized in 0.025% benzocaine, one eye removed, the cornea punctured with a scalpel, and placed in 100 μ L 1x tadpole Ringers with 20 mM glucose and 70 μ Ci 35 S-labeled methionine and cysteine (Amersham) for 2 h at room temperature. After rinsing, the eye was disrupted by vigorous pipetting and vortexing in 50 μ L 1x Laemmli sample buffer (Laemmli 1970), and viscosity reduced by the addition of 25 units of benzonase (Novagen). An aliquot of the extract was separated on 12% SDS-PAGE, the gel stained with Coomassie Brilliant blue, and the dried gel exposed to x-ray film to detect incorporated radiolabeled proteins.

The contralateral eye was fixed in methanolic formaldehyde as described (Peterson *et al.* 2003), and processed for confocal microscopy to detect the localization of the arrestin/GFP fusion using the endogenous fluorescence of GFP.

2.2 Myc-Tagged Arrestin

The ten amino acid myc tag (EQKLISEEDL) was incorporated into *Xenopus* arrestin using overlapping PCR products that introduced the cDNA for the myc-tag between the codons for Leu-76 and Thr-77. The overlapping products were combined and the complete cDNA amplified using primers against the 5' and 3' ends of the arrestin cDNA, incorporating *Xho*I and *Not*I sites, respectively. This product was cloned into the *Xho*I and *Not*I sites of the XOPS1.3 vector under the control of the 1.3 kb *Xenopus* rod opsin promoter (Tam *et al.* 2000). The cDNA for GFP was inserted immediately prior to the arrestin stop codon at an introduced *Nhe*I site. Transgenic animals expressing this myc-tagged xAr-GFP (xAr-myc-GFP) were prepared by nuclear transplantation (Kroll and Amaya 1996).

For *in vitro* studies, a His₆ tag was incorporated by PCR at the 5' end of the cDNA immediately after the initiating ATG for xAr-GFP and xAr-myc-GFP, and then cloned into the shuttle vector pPIC-ZA for heterologous expression in *Pichia pastoris* (Dinculescu *et al.* 2002). Expressed proteins were purified over nickel-agarose (Ni-NTA, Qiagen), followed by heparin agarose. *In vitro* binding assays with rhodopsin were performed using unphosphorylated and phosphorylated rod disc membranes prepared from *Rana catesbeiana* retinas as described for bovine disc membranes (McDowell 1993).

2.3 Four Lysine Mutants of Arrestin

Multiple alignment of *Drosophila*, bovine, and *Xenopus* arrestins was performed using ClustalX (Thompson *et al.* 1997). Two of the three lysines (Lys-228 and Lys-231) identified as important for promoting an association with inositol phospholipids in *Drosophila* (Lee *et al.* 2003) are conserved in bovine arrestin and were changed to alanines by site-directed mutagenesis. Because sequence conservation is not perfect, two additional lysines were substituted with alanines to insure disruption of the potential inositol phospholipid binding site, creating the four lysine mutant K232A/K235A/K236A/K238A (4K $\rightarrow$ A) in bovine visual arrestin. The 4K $\rightarrow$ A bovine arrestin mutant was expressed in *Pichia*, purified to homogeneity, and tested for binding to inositol phospholipids immobilized on nitrocellulose strips (PIP Strips, Echelon, Inc.). PIP-Strips were blocked for 60 min with 1% gamma globulin-free horse serum in phosphate-buffered saline (PBS). Wild-type arrestin, 4K $\rightarrow$ A arrestin, and the PH domain of phospholipase C δ 1 fused to glutathione-S-transferase (GST-GRIP; Echelon, Inc) (0.5 μ g/mL) were then incubated with the PIP strips for 4 h. After washing with 0.05% Tween-20 in PBS, the blots were immunoprobed to detect binding using anti-arrestin monoclonal SCT-128 for bovine arrestin and anti-GST monoclonal for the GRIP positive control. Binding of the primary antibody was detected using an anti-mouse antibody conjugated to alkaline phosphatase with nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate as the substrate.

Relative affinity of bovine arrestin and 4K→A arrestin for phytic acid was assessed by competitive elution from heparin agarose. Arrestin and 4K→A (1 mg) was immobilized on a 1 mL heparin sepharose column (Amersham) in 10 mM HEPES/30 mM NaCl, pH 7.0. Aliquots of phytic acid (1 μ M – 10 mM) in the same mobile phase were added to the column and the amount of arrestin eluted in each aliquot measured by absorbance at 278 nm.

Homologous mutations were also created in *Xenopus* arrestin by PCR site-directed mutagenesis, substituting lysines 232, 235, 236, and 267 with glutamine (4K→Q). This substituted arrestin was fused with GFP as previously described and used to create transgenic *Xenopus* tadpoles. Translocation of the Ar(4K→Q)-GFP was assessed by confocal microscopy using tadpoles that were dark-adapted for 3 days, then exposed to laboratory lighting for 50 min, or for 240 min.

3. RESULTS AND DISCUSSION

3.1 Does arrestin translocate from the outer segments?

Our previously published work clearly demonstrates that arrestin translocates from the inner segments (RIS) of dark-adapted rod photoreceptors, moving to the rod outer segments (ROS) upon exposure to light (Peterson *et al.* 2003). In these studies, it also appears that arrestin leaves the rod outer segments and returns to the inner segments in response to either dark adaptation or extended light adaptation. However, another equally tenable explanation is that the arrestin that translocates to the outer segments is proteolyzed and that the arrestin subsequently seen in the inner segment is a result of newly synthesized arrestin (Azarian *et al.* 1995).

To discriminate between these two mechanisms, transgenic Ar-GFP tadpoles were treated with cycloheximide (CHI) to inhibit protein synthesis (Obrig *et al.* 1971), and translocation subsequently assessed. Figure 1 shows total homogenates prepared from one eye from each of these tadpoles, separated on 12% SDS-PAGE. Protein staining of total homogenates prepared from eyes treated with 100 μ M CHI revealed a similar profile as the untreated animals, although the total protein content extracted was slightly less in the treated animals (Figure 1A). Autoradiography of this same gel indicates that there was substantial incorporation of the radiolabeled cysteine and methionine in the untreated animals, but that new protein synthesis was largely blocked in the tadpoles treated with cycloheximide (Figure 1B).

The contralateral eyes from the same tadpoles used in Figure 1 were fixed in formaldehyde and processed for confocal microscopy to show the distribution of the Ar-GFP fusion protein, using the endogenous fluorescence of GFP (Figure 2). In both the untreated and CHI-treated tadpoles, the Ar-GFP localizes to the RIS and axonemes in dark-adapted animals. In response to 45 min of light exposure, the Ar-GFP almost completely translocates to the outer segments. If the cycloheximide-treated tadpoles are kept in light for 4 h or returned to the dark for 1 h (data not shown), the Ar-GFP again concentrates in the RIS and axonemes for both treated and untreated tadpoles. Because we have demonstrated that treatment of the tadpoles with CHI blocks new protein synthesis (Figure 1), we conclude that arrestin translocates both to and from outer segments, and that at least the vast majority of the redistribution of arrestin is a consequence of translocation and not proteolysis followed by replacement by newly synthesized protein.

These results are consistent with quantitative studies showing that the mass of arrestin-GFP is conserved during translocation from the RIS to the ROS in response to light (Peet *et al.* 2004). This study did not quantify the translocation from ROS to RIS during dark adaptation. A similar study using cycloheximide to inhibit protein synthesis in mouse retinas shows the light-driven redistribution of transducin is also largely a consequence of transducin translocation and not synthesis of new protein (Sokolov *et al.* 2002).

3.2 Is binding to light-activated rhodopsin required for arrestin translocation?

When the first observations were made showing that arrestin redistributes to the outer segments during light adaptation, it was assumed that this translocation was a consequence of arrestin binding to light-activated phosphorylated rhodopsin (Wilden *et al.* 1986; Mangini *et al.* 1994). However, in mice that are deficient for rhodopsin phosphorylation, either lacking rhodopsin kinase or in which the C-terminus of rhodopsin has been mutated to remove the phosphorylation sites, light-driven translocation of arrestin between the inner segment and outer segments occurs in a manner indistinguishable from that in wild-type mice (Mendez *et al.* 2003; Zhang *et al.* 2003). To corroborate and extend these findings, we analyzed the translocation of arrestin in transgenic *Xenopus* that expressed an arrestin that is unable to bind light-activated phosphorhodopsin (R*P). In previous studies, we demonstrated that binding of arrestin to R*P could be blocked by inserting a ten amino acid myc tag (EQKLISEEDL) in a loop of bovine arrestin between Leu-77 and Ser-78 (Dinculescu *et al.* 2002). Reasoning that this variant of arrestin could be used to address the necessity of arrestin binding to R*P for translocation, we created a homologous substitution in our *Xenopus* arrestin-GFP fusion, inserting the myc tag between Leu-76 and Thr-77 (xAr-myc-GFP).

To determine if this insertion in *Xenopus* arrestin has a similar effect as that documented for bovine arrestin, both xAr-GFP and xAr-myc-GFP were heterologously expressed, purified, and used in a centrifugation binding assay with rhodopsin in ROS disk membranes obtained from bullfrog retinas. As previously documented, xAr-GFP retains its selectivity for light activated phosphorhodopsin (Figure 3). In contrast, xAr-myc-GFP has essentially no binding to the rhodopsin-containing disc membranes for any of the four forms of rhodopsin.

In transgenic tadpoles expressing this same transgene, xAr-myc-GFP translocation is indistinguishable from xAr-GFP (Figure 4). In these animals, the xAr-myc-GFP clearly translocates to the ROS in response to light in a manner that is qualitatively and quantitatively identical to xAr-GFP (compare to untreated animals in Figure 2). Clearly the translocation of arrestin is independent of binding to rhodopsin.

3.3 A role for inositol phospholipids in arrestin translocation?

Translocation of arrestin in *Drosophila* photoreceptors has been reported to be dependent upon an association with inositol phospholipids, particularly phosphoinositol trisphosphate (PIP₃) (Lee *et al.* 2003). Mutation of three lysines in *Drosophila* arrestin (Lys-228, Lys-231, and Lys-257) disrupts this association and significantly slows the translocation of arrestin from the cell body to the rhabdomere in response to light through a NINAC myosin III-dependent mechanism (Lee and Montell 2004). Accordingly, we investigated if a similar mechanism was involved in the translocation of vertebrate arrestin, particularly since bovine arrestin has a demonstrated affinity for inositol phosphates (Palczewski *et al.* 1991). Alignment of bovine and *Drosophila* arrestins shows that two of the three mutated lysines are conserved. From an earlier study we had prepared a mutant of arrestin that coincidentally substituted these two lysines with alanines along with the two nearby lysines (K232A/K235A/K236A/K238A, used herein as 4K→A). Although the sequences of *Drosophila* and bovine arrestins are not perfectly conserved, the placement of the amino acids in three-dimensional projections based on the crystal structure of bovine arrestin (Hirsch *et al.* 1999) allows us to assign conformational homology. In addition, there are no other lysine residues that project near this grouping of lysines, giving us confidence that we have identified any lysines that might potentially bind inositol phospholipids in a similar location in vertebrate arrestins as identified in *Drosophila* arrestin.

Utilizing the procedure used in *Drosophila* (Lee *et al.* 2003), we assessed the binding of wild-type arrestin and 4K→A arrestin to different types of phospholipids spotted on nitrocellulose

strips (Figure 5A). Unlike *Drosophila* arrestin, which showed significant binding to many forms of phosphoinositol bisphosphate and trisphosphate in this assay, we were unable to detect any binding of wild-type bovine arrestin or 4K→A arrestin to the phospholipid spots. Positive controls using the PH domain of phospholipase C- δ 1 (GST-GRIP) or arrestin from house flies (*Musca domestica*, not shown), show easily detectable binding. These results suggest that the affinity of bovine arrestin for inositol phospholipids is significantly lower than that of *Drosophila* arrestin. This conclusion is supported by previous studies, showing an IC_{50} of 10 μ M for phytic acid (inositol hexaphosphate) for bovine arrestin (Palczewski *et al.* 1991), compared to 0.6 μ M for *Drosophila* arrestin (Lee *et al.* 2003).

To assess whether the four lysine substitutions in bovine arrestin affected its affinity for phytic acid, WT and 4K→A arrestins were immobilized on heparin agarose, and competed off with increasing concentrations of phytic acid. Figure 5B shows that the 4K→A arrestin required a 50-fold lower concentration of phytic acid to elute the bound protein. This result indicates that the lysine substitutions had the predicted effect of reducing the affinity for inositol phospholipids and that we had correctly identified at least some of the lysines that participate in the association of arrestin with inositol hexaphosphate. These substitutions did not affect the specificity of 4K→A arrestin for R*P (data not shown).

Using these data, we prepared homologous substitutions of lysines in *Xenopus* arrestin to assess the impact of this reduced affinity for inositol phospholipids on arrestin translocation *in vivo*. Lysines 232, 235, 236, and 267 were substituted with glutamine (4K→Q), the same substitution used in *Drosophila* arrestin to disrupt translocation of *Drosophila* arrestin. In tadpoles expressing 4K→Q arrestin fused to the N-terminus of GFP, the translocation is indistinguishable from that of wild-type arrestin/GFP (compare to untreated tadpoles in Figure 2). In the dark-adapted state, 4K→Q arrestin localizes to the inner segments and axonemes of the rod photoreceptors. In response to 50 minutes of light adaptation, 4K→Q arrestin translocates to the outer segments qualitatively and quantitatively equal to wild-type arrestin. Like the native arrestin, 4K→Q arrestin also returns to the inner segments and axonemes during extended light adaptation, or if the tadpoles are subsequently dark adapted (data not shown). The similarity of these translocation results and the lower affinity of vertebrate arrestins for inositol phospholipids lead us to conclude that inositol phospholipids do not appear to play a role in the translocation of vertebrate arrestins, at least not through a direct interaction with arrestin as has been demonstrated for *Drosophila*.

4. CONCLUSIONS

These studies on selected aspects of arrestin translocation reveal three important findings. First, the light-driven redistribution of arrestin is a consequence of arrestin translocation and not a result of arrestin proteolysis with subsequent replacement by newly synthesized protein. Second, our results further substantiate that the translocation of arrestin to the ROS following light is not a result of arrestin's affinity for photoactivated, phosphorylated rhodopsin. And finally, we show that unlike *Drosophila* arrestin, vertebrate visual arrestins do not appear to rely on an affinity for phospholipids to promote translocation. Future studies will focus on identifying the mechanism of arrestin translocation and identify how arrestin interacts with this translocation machinery.

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Figure 1.

Inhibition of protein synthesis in the eyes of tadpoles treated with 100 μ M cycloheximide (CHI). Treated (+) or untreated (–) tadpoles were dark adapted overnight (DA) or exposed to light for 45 min (LA45) or for 240 min (LA240). One eye was removed and new protein synthesis assessed as described in Methods. Proteins extracted from eye homogenates were separated by 12% SDS-PAGE and stained with Coomassie blue (A). Newly synthesized proteins that incorporated radiolabeled cysteine and methionine are revealed in the autoradiograph of the same gel (B).

Figure 2.

Arrestin-GFP translocation in rods from transgenic tadpoles treated or not treated with CHI. Contralateral eyes from the animals used in Figure 1 were cryosectioned and the endogenous fluorescence of the arrestin/GFP fusion protein visualized using confocal microscopy. Tadpoles were dark adapted overnight (DA), exposed to light for 45 min (LA 45 min), or to light for 240 min (LA 240 min).

Figure 3.

Binding of arrestin-GFP fusions to frog rhodopsin. *Xenopus* arrestin-GFP fusion protein (xAr-GFP) and arrestin-GFP fusion with a myc tag inserted between Leu-76 and Thr-77 (xAr-myc-GFP) were purified and bound to rhodopsin in disc membranes obtained from bull frog retinas in an *in vitro* centrifugation assay. The rhodopsin was either kept in the dark (R), light activated for 2 min (R*), phosphorylated but not activated (RP), or light activated and phosphorylated (R*P). Proteins bound to the disc membranes were analyzed by western blotting with an anti-GFP polyclonal antibody and quantified by scanning densitometry. Bound arrestin was normalized to the binding of xAr-GFP to R*P. Error bars represent SEM (n=3).

Figure 4.

Translocation of myc-tagged arrestin-GFP fusion in transgenic tadpoles. Confocal images were obtained from cryosections of xAr-myc-GFP transgenic tadpoles that were dark-adapted overnight (DA) or exposed to light for 45 min (LA 45 min). The endogenous fluorescence of the GFP protein was imaged.

Figure 5.

In vitro binding of arrestin to phospholipids. A. Native arrestin (WT), arrestin with four lysines substituted with alanine (4K→A), and GST-GRIP positive control were used to probe phospholipids immobilized on nitrocellulose. Binding was detected using anti-arrestin monoclonal antibody or anti-GST antibody for the GST-GRIP control with alkaline phosphatase-conjugated secondary antibody. Spotted lipids are lysophosphatidic acid (LPA), lysophosphocholine (LPC), phosphatidyl inositol (PI), phosphatidyl inositol 3-phosphate (PI3P), phosphatidyl inositol 4-phosphate (PI4P), phosphatidyl inositol 5-phosphate (PI5P), phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingosine-1-phosphate (S1P), phosphatidyl-3,4-bisphosphate (PI34P₂), phosphatidyl-3,5-bisphosphate (PI35P₂), phosphatidyl-4,5-bisphosphate (PI45P₂), phosphatidyl-3,4,5-trisphosphate (PI345P₃), phosphatidic acid (PA), and phosphatidylserine (PS). B. Relative affinity of WT arrestin (open circles) and 4K→A arrestin (closed squares) for inositol hexaphosphate was assessed by binding the arrestins to heparin agarose and competitively eluting with an increasing concentration of inositol hexaphosphate (phytic acid). Eluted protein was quantified by measuring absorbance at 278 nm.

Figure 6.

Confocal images of xAr-4K→Q-GFP translocation in rod photoreceptors. Transgenic tadpoles were dark-adapted overnight (DA), light adapted for 50 min (LA 50 min), or light adapted for 240 min (LA 240 min). Images show the endogenous fluorescence of the GFP in the fusion protein.