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Light-dependent translocation of arrestin in rod photoreceptors is signaled through a phospholipase C cascade and requires ATP

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ABSTRACT

Partitioning of cellular components is a critical mechanism by which cells can regulate their activity. In rod photoreceptors, light induces a large-scale translocation of arrestin from the inner segments to the outer segments. The purpose of this project is to elucidate the signaling pathway necessary to initiate arrestin translocation to the outer segments and the mechanism for arrestin translocation. Mouse retinal organotypic cultures and eyes from transgenic *Xenopus* tadpoles expressing a fusion of GFP and rod arrestin were treated with both activators and inhibitors of proteins in the phosphoinositide pathway. Confocal microscopy was used to image the effects of the pharmacological agents on arrestin translocation in rod photoreceptors. Retinas were also depleted of ATP using potassium cyanide to assess the requirement for ATP in arrestin translocation. In this study, we demonstrate that components of the G-protein-linked phospholipase C (PLC) pathway play a role in initiating arrestin translocation. Our results show that arrestin translocation can be stimulated by activators of PLC and protein kinase C (PKC), and by cholera toxin in the absence of light. Arrestin translocation to the outer segments is significantly reduced by inhibitors of PLC and PKC. Importantly, we find that treatment with potassium cyanide inhibits arrestin translocation in response to light. Collectively, our results suggest that arrestin translocation is initiated by a G-protein-coupled cascade through PLC and PKC signaling. Furthermore, our results demonstrate that at least the initiation of arrestin translocation requires energy input.

1. Introduction

Cellular function ultimately depends upon the interaction of the complement of components contained in a particular cell. For proteins, functional activity can be regulated by a variety of mechanisms, including selective partitioning. For example, G-protein-coupled receptors signal different pathways depending on their compartmentalization, such as the β-adrenergic receptors which can activate the MAPK pathway when the receptors are contained in clathrin-coated vesicles [1], or function as canonical receptors when on the cell surface membrane.

Rod photoreceptors are one of the most highly polarized cells in vertebrates. The outer segment is an extreme elaboration of the primary cilium, functioning to transduce light into a change in membrane potential. The inner segment houses the metabolic and synthetic machinery for maintaining the function of the cell. Considering this polarity, there is obviously a flow of components from the inner segment, where proteins are synthesized, to the outer segment where many of these proteins are utilized in phototransduction. A few proteins, however, undergo massive reversible changes in distribution between the outer and inner segments in a light-dependent manner. In dark-adapted eyes, arrestin localizes primarily in the rod inner segments. In response to light, arrestin almost completely translocates to the rod outer segments [e.g., 2–4].

Current evidence supports the idea that the initial signaling of arrestin translocation is through light activation of rhodopsin. In animals that lack functional rhodopsin due to a deficiency in 11-*cis* retinal, arrestin translocation is absent [5]. Further, Strissel et al. [6] show that arrestin translocation is initiated at a threshold where approximately 3% of the total molecules of rhodopsin are bleached. At this threshold, a 30-fold excess of arrestin moves into the outer segments compared to the number of activated rhodopsin molecules. This excess of arrestin moving at a threshold level is a hallmark of a signaling cascade. Since rhodopsin, but not the visual G-protein transducin [5,7], is required for arrestin translocation, we reasoned that the signaling for arrestin translocation likely occurs through rhodopsin activation of an alternative G-protein-coupled cascade.

Abbreviations: PKC, Protein kinase C; PLC, Phospholipase C.

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The phosphoinositide pathway is one logical choice for this signaling cascade since several studies have identified components of the phosphoinositide pathway in photoreceptor cells, including G_i and G_q guanine nucleotide-binding proteins, phospholipase C (PLC) isozymes, and protein kinase C (PKC) isozymes [e.g. 8–11]. Both PLC and PKC activities in rod photoreceptors are modulated by light exposure, but have not been linked into a specific signaling pathway [8,12].

In this study, we hypothesize that phosphoinositide signaling triggered by rhodopsin activation initiates arrestin translocation. We show that arrestin translocation is initiated by light-activated rhodopsin through an alternative G-protein-coupled cascade that utilizes PLC and PKC and is parallel to the visual transduction cascade. Consistent with this observation, we also show that arrestin translocation is an energy-dependent process, requiring ATP.

2. Materials and methods

2.1. Animals

Xenopus laevis were housed in a continuous flow water system and maintained on a cycle of 12 h of light (800 lx) and 12 h of darkness. C57BL/6J mice were maintained on a cycle of 12 h of light (200 lx) and 12 h of darkness, with food and water *ad libitum*. All animal procedures followed the rules and regulations set forth by the Institutional Animal Care and Use Committee at the University of Florida and the University of Mainz.

2.2. Treatment of tadpoles with agonists and antagonists

Arrestin (Arr)-GFP tadpoles were produced by mating a male frog expressing arrestin fused with GFP at the C-terminus driven by the rod opsin promoter prepared as previously described [13]. Arr-GFP tadpoles (4–6 weeks) were dark adapted overnight in 0.1× tadpole Ringer's solution followed by treatments with 1 μM phorbol 12, 13-diacetate (PDA, Sigma) for 10 min in the dark. The PDA was prepared at 30 mM in 33% dimethylsulfoxide (DMSO), and diluted in tadpole Ringers such that the final DMSO concentration was 3.3%. PDA is a diacylglycerol analog that has been shown to activate protein kinase C [14]. A set of control tadpoles was treated with 3.3% DMSO in parallel. Tadpoles were then fixed as described later. Dark-adapted Arr-GFP tadpole eyes were also treated with a phospholipase C activator, 2,4,6-trimethyl-N-(m-3-trifluoromethylphenyl) benzenesulfonamide (m-3M3FBS, Calbiochem). Arr-GFP tadpole eyes were placed in Niu-Twitty buffer, (310 μM Na_2HPO_4, 150 μM KH_2PO_4, 58 mM NaCl, 670 μM KCl, 340 μM $Ca(NO_3)_2$, 830 μM $MgSO_4$, 2.4 mM $NaHCO_3$, 340 μM $CaCl_2$, and pH 7.3) and then treated with 1 μM m-3M3FBS (3.3% DMSO final concentration) for 15 min in the dark. The eyes were transferred to a new tube and fixed immediately after incubation. Another set of eyes was treated with 3.3% DMSO as a control.

Arr-GFP tadpoles were also treated with a dopamine receptor agonist. Following overnight dark adaptation in 0.1× tadpole Ringers overnight, the tadpoles were treated with 10 μM (−)-quinpirole hydrochloride (Sigma), a D_2-dopamine receptor agonist, for 10 and 30 min in 3.3% DMSO. The control tadpoles and quinpirole-treated tadpoles were fixed immediately after each time point.

To investigate if arrestin is utilizing a G-protein-regulated pathway, we used cholera toxin as a G_i activator. In a separate experiment, sulpiride was used as a D_2-dopamine receptor antagonist. Arr-GFP and wild-type eyes were treated with freshly prepared 15 μg/mL cholera toxin (Sigma) in 3.3% DMSO for 0–30 min under dim red lights. Arr-GFP eyes were also treated with 10 μM (s) −(−) sulpiride (Sigma) for 4 h in the dark and then light adapted for 60 min. A set of eyes remained in the dark in Niu-Twitty buffer, treated and untreated, while another set of eyes were light adapted for 60 min.

To determine if sulpiride penetrated into the photoreceptors during this incubation, dark-adapted wild-type tadpole eyes were also treated with 1 μM ^{3}H-sulpiride (−) [methoxy-^{3}H] (Perkin Elmer) for 4 h. A set of control eyes and sulpiride-treated eyes remained in the dark and a set were light adapted for 60 min. The eyes were fixed, rehydrated, and sectioned. To detect the tritiated sulpiride, sections were dipped in diluted Kodak emulsion in total darkness. Sections of eyes were exposed to the emulsion at 4 °C for 3 months. After 3 months, the slides were warmed to room temperature and then developed (Kodak D19 developer). Subsequently, the slides were rinsed in water and then fixed (Kodak fixer). The slides were rinsed and visualized with an Olympus BH-2 epifluorescent microscope. This procedure was adapted from Pardue [15].

2.3. Potassium cyanide treatment of tadpole eyes

Arr-GFP tadpoles were dark adapted for at least 12 h and then treated with 0.05% benzocaine until euthanized, at which point the eyes were removed with a scalpel. The eyes were then placed in MOPS-Twitty buffer (50 mM MOPS, 58 mM NaCl, 670 μM KCl, 340 μM $Ca(NO_3)_2$, 830 μM $MgSO_4$, 2.4 mM $NaHCO_3$, and 340 μM $CaCl_2$), pH 7.5 with or without 5 mM KCN under dim red lights. Eyes were incubated in 5 mM KCN for 1 h in the dark and then light adapted for 1 h under laboratory lighting. In eyes where the cyanide was removed, after treatment with 5 mM KCN for 1 h in the dark, the eyes were washed three times with MOPS-Twitty buffer, and then incubated in either MOPS-Twitty or MOPS-Twitty containing 5 mM ATP for 2 h in the dark. Eyes either remained in the dark or were light adapted for 1 h under laboratory lighting. Both dark-adapted eyes and light-adapted eyes were then fixed in methanolic formaldehyde and processed for confocal microscopy. A set of unfixed eyes were also collected for measurement of ATP levels using the Cell TiterGloMax (Promega) system. For this measurement, eyes were processed in the same way as above for microscopy except that instead of fixing in formaldehyde, the eyes were sonicated in 100 μL MOPS-Twitty buffer, allowed to sit for 30 min (25 °C) for the luciferase activity to stabilize and then luminescence measured, following the manufacturer's protocol to determine ATP levels.

2.4. Treatment of organotypic retina cultures

Mouse organotypic retina cultures were prepared under dim red light as previously described [16,17]. Briefly, eyes of C57/BL6J mice in the age of post-natal day 14–20 were treated with 1.2 mg/mL proteinase K for 15 min at 37 °C and quenched by adding Dulbecco's Modified Eagle's Medium (DMEM F-12) containing 10% fetal calf serum. After dissecting accessory tissue from the eye, retinas were cultured in supplemented DMEM F-12.

Organotypic retina cultures obtained from mice dark adapted for 12 h were treated with 100 nM PMA (phorbol 12-myristate 13-acetate, Sigma) or 100 nM m-3M3FBS (Calbiochem) for 10 min. Other retina cultures were treated with 10 μM chelerythrine chloride (Calbiochem) and 10 μM U73122 (Calbiochem) for 4 h in the dark. All agonists and antagonists were prepared according to the manufacturer's instructions and diluted for the incubation in DMEM F-12. Untreated and treated eyes either remained in the dark or were light adapted for 60 min. After fixation in 4% buffered paraformaldehyde, specimens were embedded, cryosectioned and further processed for immunohistochemistry.

2.5. ATP depletion of mouse retina cultures

Wild-type C57/BL6J mice were dark adapted for at least 12 h and then placed in isoflurane for euthanizing. The eyes were promptly removed following cervical dislocation. Organotypic retina cultures were incubated in 5 mM KCN or DMEM (D-Glucose-free, sodium

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pyruvate-free) supplemented with 2 mM 2-deoxy-D-glucose for 1 h in the dark and then light adapted for 1 h. After fixation, cultures were processed for immunohistochemistry.

2.6. ATP depletion of mouse retina cultures and eye cups

Wild-type C57/BL6J mice were dark adapted overnight, and then euthanized in isoflurane. Following cervical dislocation, the eyes were removed and placed in DMEM or glucose-free DMEM with 5 mM KCN for 1 h at 37 °C. A set of eyes were exposed to light (1400 lx) for 1 h. The eyes were then immediately fixed in 4% paraformaldehyde and processed for immunohistochemistry.

2.7. Immunohistochemistry of Xenopus retina

Wild-type *X. laevis* tadpoles (stage 50–54, either lab reared or obtained from *Xenopus* Express) were dark adapted overnight in the dark or under dim red lights. For dark- and light-adaptation studies, the tadpoles were either left in the dark or were light adapted for 60 min under laboratory lighting (approximately 850 lx). After dark adaptation or light adaptation, the tadpoles were fixed in 3.7% formaldehyde and 73% methanol in deionized water overnight at 4 °C. The tadpoles were rehydrated through serial dilutions of methanol, incubating for 30 min on ice in 60% methanol in phosphate buffered saline (PBS), 40% methanol in PBS, 20% methanol in PBS, and 100% PBS. After rehydration, the tadpoles were cryoprotected in 30% sucrose in PBS overnight at 4 °C. The eyes were dissected from the tadpoles and embedded in Optimal Cutting Temperature media and sectioned at 12 µm.

For immunohistochemistry, the sections were rinsed with PBS for 30 min to remove residual OCT and processed through the following series of treatments to optimize antibody penetration. The sections were incubated in freshly prepared 0.1% $NaBH_4$ for 30 min at room temperature, followed by 1% TritonX-100 in PBS for 30 min. The sections were then denatured with 6 M guanidinium hydrochloride in 50 mM Na_2PO_4, pH 7.0 for 20 min. The sections were rinsed with several changes of deionized water, and then blocked with 1% reduced gamma globulin fetal bovine serum or reduced gamma globulin horse serum with 0.2% TritonX-100 in PBS for 2 h. All antibodies used in this study were diluted in 1% reduced gamma globulin horse or fetal bovine serum with 0.2% TritonX-100 in PBS. The following antibodies at 1:50 or 1:100 dilutions were used: anti-*Xenopus* visual arrestin1 (xAr1-6; [13]), anti-arrestin (SCT-128; gift from Paul Hargrave), anti-$G_{i\text{-}1}$ G-protein (R4; Santa Cruz), anti-$G_{i\text{-}2}$ G-protein (L5; Santa Cruz), anti-$G_{i\text{-}3}$ G-protein (C-10; Santa Cruz), anti-$G_{i\text{-}o}$ G-protein (A2; Santa Cruz), anti-$G_{\alpha 11}$ G-protein (D-17; Santa Cruz), anti-transducin alpha G-protein (K-20; Santa Cruz), anti-phospholipase $C_{\gamma 1}$ (1249; Santa Cruz), anti-PLCβ4 (C-18; Santa Cruz), anti-protein kinase Cα (A9; Santa Cruz), and D2-dopamine receptor (H-50; Santa Cruz). The sections were incubated with the primary antibody for 18–24 h at room temperature in a hydrated chamber with gentle agitation. After incubation, the sections were washed with three changes of PBS (30 min each). The secondary antibody was added to the sections and incubated for 18–24 h. In this study, we used both Texas Red®-X goat anti-mouse (Invitrogen) and Texas Red®-X goat anti-rabbit (Invitrogen) at 1:200 dilution (these are shown in the images using the red channel). We also used Alexa Fluor® 647 goat anti-rabbit (Invitrogen) secondary antibody, which also fluoresces red, but at a longer wavelength. To make the distinction between the two fluorophores, we show Alexa Fluor 647 fluorescence using the blue channel. Two nuclear stains were used in this study and were incubated along with the secondary antibody: 0.2 µM SYTOX® Green (Invitrogen, shown in green) and 4′, 6-diamidino-2-phenylindole (DAPI) (Invitrogen, shown in blue). After the secondary antibody incubation, the sections were washed with PBS with three changes at 30 min intervals. They were then covered with Mowiol [13] to reduce autofluorescence and sealed with a cover slip.

2.8. Immunohistochemistry of organotypic mouse cultured retinas

Mouse monoclonals directed against arrestin (MAb 3D1.2, MAb 5C6), were applied on mouse retina cryosections as previously described [16,17]. Secondary antibodies were purchased as conjugates to Alexa 488 or Alexa 568 (Molecular Probes). Sections were mounted in Mowiol 4.88, containing 2% n-propyl-gallate. No reactions were observed in control sections.

2.9. Microscopy and quantification

The slides prepared for this study were visualized by confocal microscopy (1024ES, BioRad; Zeiss LSM Multiphoton Laser Scanning Confocal Microscopy; Olympus Spinning Disc Confocal Microscope; or Leica SP5). The images were collected through a series of z-sections at 0.5 µm intervals and projected to a two-dimensional image. The images were then imported and processed with Adobe Photoshop. To quantify fluorescence in the images, the images were gray scaled and inverted. The inverted images were then imported to Scion Image version 4.0.2. The fluorescence in the outer and inner segments was measured by multiplying the encircled area and the density of that same area. The relative fluorescence in the rod outer segment and the rod inner segment of each photoreceptor was then calculated. The calculated fraction of fluorescence in the outer segments was measured from a minimum of two images collected from each eye from at least three animals. Average values were statistically compared using Student's two-tailed *t*-test for unpaired values.

3. Results

3.1. Arrestin translocates in response to activators of PKC and PLC

Since phospholipase C (PLC) and protein kinase C (PKC) biochemical activity have been previously demonstrated in rod outer segments [9,18], we hypothesized that perhaps these enzymes may also function to signal arrestin translocation. If so, then PLC and PKC should be localized to rods and in a location that could report information to initiate arrestin translocation. Using antibodies against PLC and PKC in indirect immunofluorescence experiments, we show that PLC and PKC immunoreactivities localize to the ellipsoid region of the inner segments (Fig. 1A–C). In comparison, arrestin is distributed uniformly throughout the inner segments and along the axoneme (Fig. 1D). This localization of PKC and PLC at the interface between the inner segment and outer segment is a reasonable location for regulating movement of arrestin between the two compartments of the rod.

Since the components of the phosphoinositide pathway are expressed in rod photoreceptors, we next wanted to determine if activating PLC and PKC could initiate arrestin translocation to the outer segments. Arrestin-GFP tadpoles were treated with phorbol 12, 13-diacetate (PDA), an activator of PKC, while maintaining the tadpoles in dark conditions (DA). In response to this activator of PKC, more than 70% of the arrestin translocated to the outer segments within 10 min of exposure (Fig. 1F) which was statistically different than the untreated animal ($\rho < 0.05$). Arrestin returned back to the inner segments after 30 min or longer exposure (Fig. 1F, inset). To our knowledge, this is the first time arrestin translocation has been initiated in the absence of light. Similarly, we were able to stimulate translocation of nearly 60% of the arrestin to the outer segments in explanted Arr-GFP eyes using an activator of PLC, m-3M3FBS, applied for 15 min (Fig. 1G). Again, arrestin did not remain in the outer segments during longer exposure to m-3M3FBS (Fig. 1G, inset).

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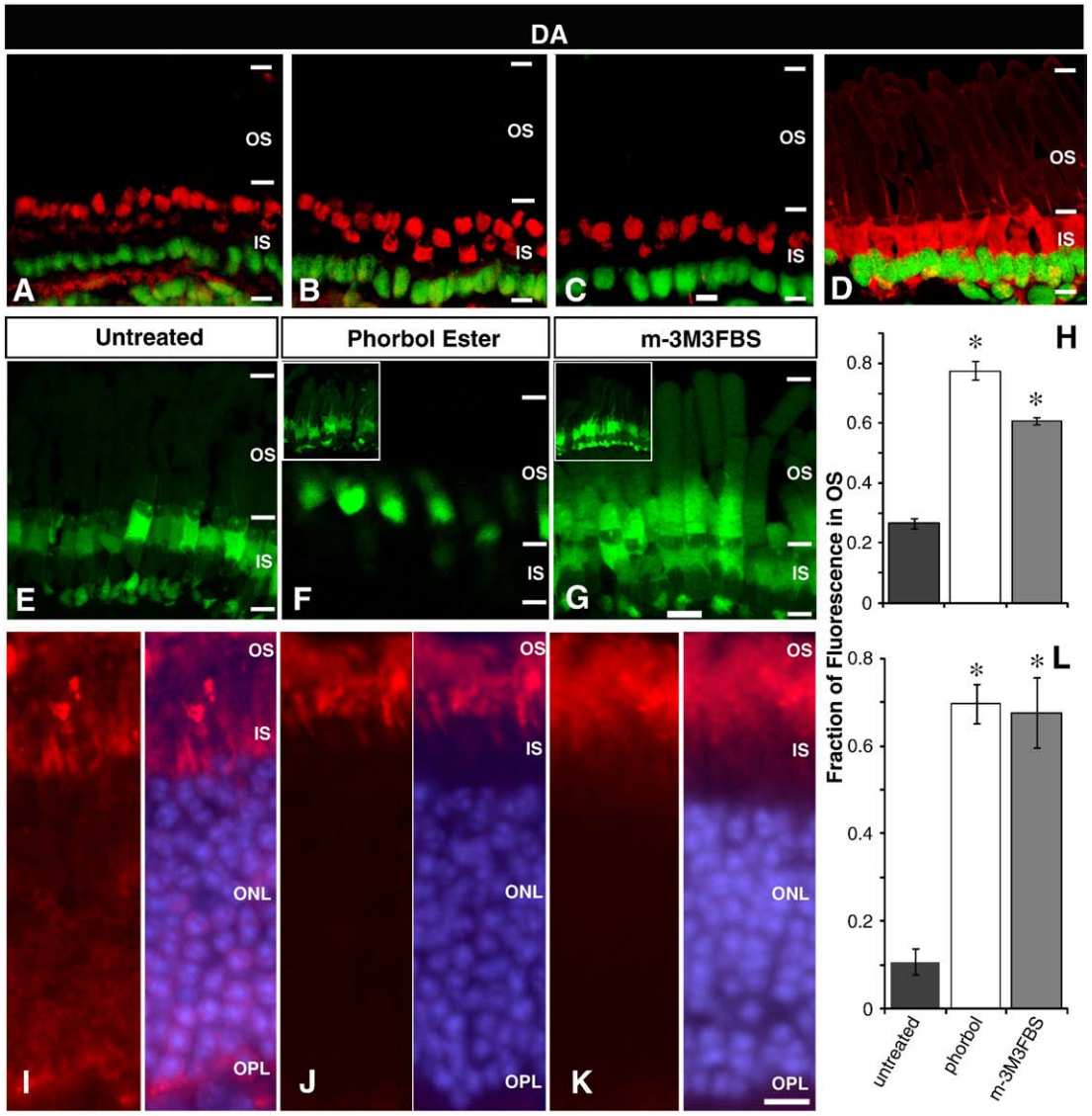

Fig. 1. A role for phospholipase C and protein kinase C in arrestin translocation in rod photoreceptors. PLC and PKC were immunolocalized in *Xenopus* retinal sections (A–D). Cryosections of wild-type tadpole eyes were immunostained using antibodies against (A) phospholipase C γ, (B) phospholipase C β, (C) protein kinase C α, or (D) arrestin (red). Nuclear DNA was stained with Sytox Green (green). PLC and PKC localized to the ellipsoid region of the inner segments (IS); outer segments (OS). Agonists of PKC and PLC induce arrestin translocation to the rod outer segments (E–L). Dark-adapted arrestin-GFP transgenic tadpoles were treated with the DMSO carrier (E, untreated control), 1 μM phorbol 12, 13-diacetate for 10 min (F), or explanted eyes from Arr-GFP transgenic tadpoles were treated with 1 μM m-3M3FBS for 15 min (G). *Insets* in F and G show the distribution of Arr-GFP in tadpole retinas after 30 min exposure to the activator. Endogenous fluorescence of GFP was imaged in cryosections, and the fraction of fluorescence in the outer segments quantified (H) (bars represent means ± SEM; $n = 7$). Mouse organotypic retina cultures were also treated with the DMSO carrier (I, untreated control), the PKC activator phorbol 12-myristate 13-acetate (J), or the PLC activator m-3M3FBS (K). Cryosections through the retinas were stained with antibodies to arrestin (red), and nuclear DNA stained with DAPI (blue). Fluorescence in the outer segments of the mouse organotypic cultures was quantified (L) (bars represent means ± SEM; $n = 3$). Activation of either PKC or PLC induced translocation of arrestin from inner segments (IS) to the outer segments (OS). Asterisks indicate a significant difference ($\rho < 0.05$) from the untreated controls. Outer nuclear layer (ONL); outer plexiform layer (OPL). Scale bars: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

To ensure this phenomenon was not specific to *X. laevis* photoreceptors, dark-adapted mouse retinal organotypic cultures were treated with a similar activator of PKC, phorbol 12-myristate 13-acetate (PMA). Like *Xenopus*, a significant amount of arrestin translocated to the outer segments in response to PMA treatment in the absence of light (Fig. 1J). Quantitation of the fluorescence in the outer segments (Fig. 1L) showed that amounts of arrestin similar to that observed in *Xenopus* were induced to translocate in mouse photoreceptors. Arrestin also translocated to the outer segments in response to m-3M3FBS in mouse retinas within 20 min (Fig. 1K). Note the absence of arrestin immunoreactivity in the inner segments, outer nuclear and outer plexiform layers. The confluence of these results from both *Xenopus* and mouse photoreceptors suggests a role for PKC and PLC in initiating arrestin translocation to the outer segments.

3.2. Inhibitors of PKC and PLC modify arrestin translocation

Arrestin translocation to the outer segments in response to PKC and PLC activation strongly supports our hypothesis that arrestin translocation is associated with the phosphoinositide pathway. If this is an essential pathway to initiate arrestin translocation, then inhibition of PKC and PLC should affect arrestin translocation during light adaptation. Accordingly, mouse retinal organotypic cultures were treated with 10 μM chelerythrine or 10 μM U73122, which are PKC and PLC inhibitors, respectively. In the presence of these inhibitors, the light-induced translocation of arrestin was altered, with approximately 60% of the arrestin remaining in the inner segments and outer nuclear layer in mouse retinas treated with the inhibitors (Fig. 2C, D), unlike the untreated eye where much more of

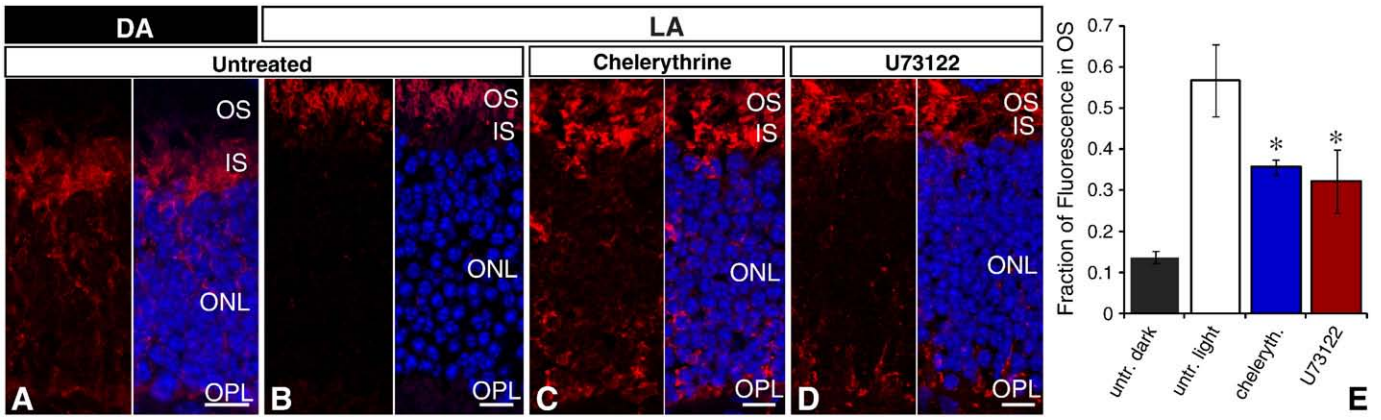

Fig. 2. Arrestin translocation to the outer segments is reduced in mouse photoreceptors treated with PKC and PLC inhibitors. Dark-adapted mouse organotypic retina cultures were treated with DMSO carrier (A, B untreated controls), 10 μM chelerythrine (C) or 10 μM U73122 (D) for 4 h and then light adapted for 1 h. Cryosections through the retina were stained with antibodies for arrestin (red) and nuclear DNA stained with DAPI (blue). In light-adapted cultures treated with either chelerythrine (C) or U73122 (D), significant amounts of arrestin remained in the inner segments (IS), outer nuclear layer (ONL), and outer plexiform layer (OPL), compared to the untreated control (B) where nearly all of the arrestin translocated to the outer segments (OS). The fraction of fluorescence in the outer segment layer was quantified (E; mean ± SEM). Asterisks indicate where the differences between the treated and untreated light-adapted eyes are statistically significant ($\rho < 0.05$). Scale bar: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the arrestin (57%) was in the outer segments under these conditions (Fig. 2B). This difference in localization in the presence of the PKC and PLC inhibitors during light adaptation is statistically different ($\rho < 0.05$).

3.3. A G-protein mediates arrestin translocation to outer segments

There are several G-proteins that could potentially activate PLC. To distinguish between some of these G-proteins as candidates for signaling arrestin translocation, we treated eyes from transgenic Arr-GFP tadpoles with cholera toxin. Cholera toxin targets stimulatory G-proteins (G_s, G_{t1}, G_{t2}, and G_{olf}) and a few of the inhibitory G-proteins [e.g., 19,20], activating the G-protein following ADP-ribosylation of the α-subunit. In transgenic tadpole eyes treated with cholera toxin, arrestin translocated to the base of the outer segments with a sustained response for 30 min (Fig. 3). Approximately 50% of the arrestin translocated to the outer segments, which is significantly different than the untreated eye ($\rho < 0.05$). These results provide evidence that arrestin translocation to the outer segments may utilize a G-protein, and that this G-protein is cholera toxin sensitive.

To further investigate if arrestin translocation to the outer segments is G-protein regulated, we attempted to activate the G-protein by co-opting a G-protein-coupled receptor other than rhodopsin that is expressed in the retina. The dopamine receptor has been extensively studied and has been shown to play a role in light adaptation [21–24]. Because its expression has been detected in photoreceptor cells [24], the D_2-dopamine receptor seemed to be a good target for activating arrestin translocation. Our own immunolocalization studies with a D_2 receptor-specific antibody show localization of this dopamine receptor in the inner segments in *Xenopus* (Fig. 4A, B), similar to previously published studies [24,25], in both light- and dark-adapted retinas.

To activate the D_2-dopamine receptor, we treated dark-adapted Arr-GFP tadpoles with 10 μM quinpirole, a D_2-dopamine receptor agonist, and then assessed arrestin localization in the absence of light (Fig. 4D–F). In these treated animals, arrestin-GFP translocated to the outer segments in response to the dopamine agonist and its localization in the outer segments was sustained for at least 30 min. The amount of arrestin translocation initiated by quinpirole (Fig. 4C) was significantly different ($\rho < 0.05$) than in the untreated tadpoles.

Our ability to stimulate a non-rhodopsin G-protein-coupled receptor to initiate arrestin translocation fits our hypothesis, but it also raises the possibility that dopamine receptors could be involved in arrestin translocation. To determine if arrestin actually uses the

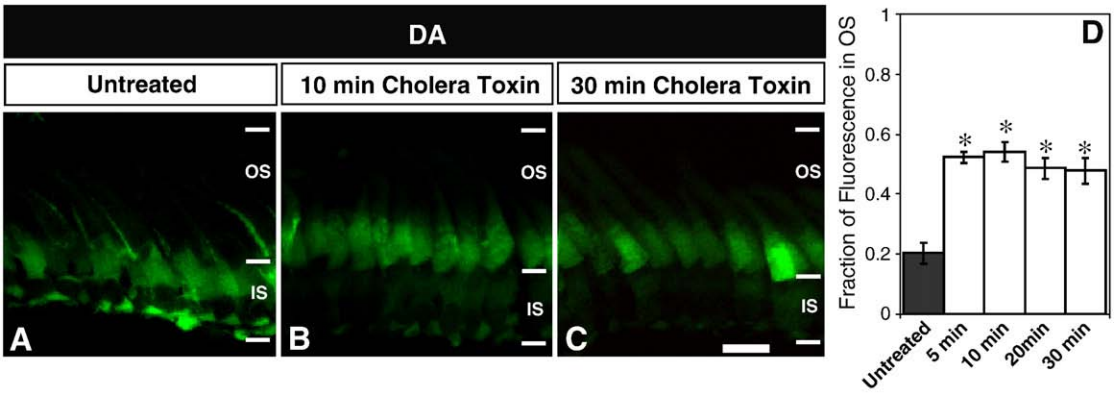

Fig. 3. Arrestin translocation is induced by cholera toxin. Dark-adapted arrestin-GFP transgenic tadpole eyes were treated with DMSO carrier (A, untreated control), or with cholera toxin (15 μg/mL) for 10–30 min in Niu-Twitty buffer in the dark (B–C). Cryosections of the photoreceptors were imaged for endogenous GFP fluorescence. Exposure to cholera toxin initiated translocation of arrestin to the base of the photoreceptor outer segments (OS) whereas arrestin remained in the inner segments (IS) of the untreated controls. For quantitation, the average fluorescence from the inner segments and outer segments was measured from a minimum of two images from each eye from three different tadpoles ($n \geq 12$), and the fraction of fluorescence in the outer segments plotted (D). Asterisks indicate where the differences in arrestin localization between the untreated tadpoles eyes and eyes treated with cholera toxin are statistically significant ($\rho < 0.05$). Scale bar: 20 μm.

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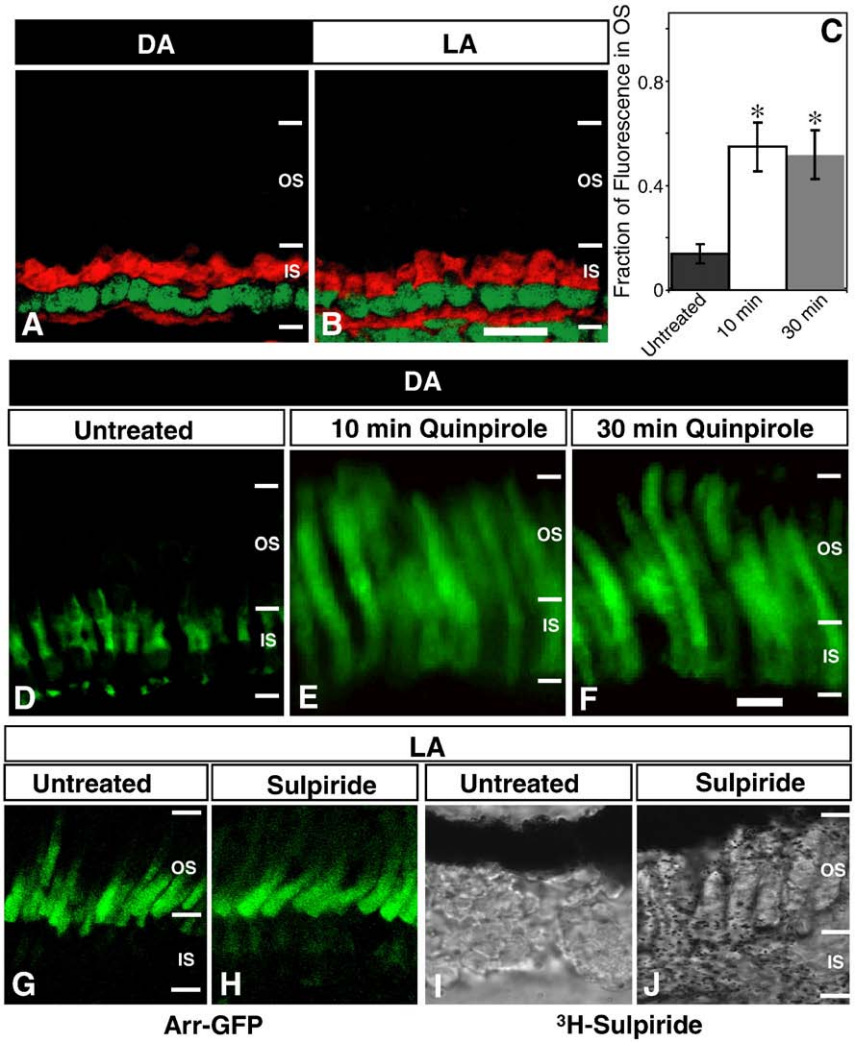

Fig. 4. D_2-dopamine receptor localization, activation, and inhibition in rod photoreceptors. Cryosections of dark-adapted (A) and light-adapted (B) wild-type tadpole eyes were immunostained with D_2-dopamine receptor antibody (red), and nuclear DNA stained with Sytox Green (green). The D_2-dopamine receptor localized primarily to the inner segments (IS) and axonemes of the rod photoreceptors. Dark-adapted arrestin-GFP tadpoles were treated with DMSO carrier (D, untreated control), or with 10 μM quinpirole, a D_2-dopamine receptor agonist, for 10–30 min (E, F) under dim red lights. Treatment with quinpirole changed the localization of the arrestin to both the outer segments (OS) and inner segments (IS), compared to only inner segments in the untreated control. The fraction of fluorescence in the outer segments of the photoreceptors following quinpirole treatment was measured (C), and averaged ($n = 9$). The differences between untreated tadpoles and quinpirole-treated eyes were statistically significant ($\rho < 0.05$). Arrestin localization was not affected by treatment with sulpiride, a D_2-dopamine receptor antagonist (G–J). Dark-adapted arrestin-GFP transgenic tadpole eyes were treated with DMSO carrier (G, untreated control) or with 10 μM sulpiride (H), a D_2-dopamine receptor antagonist, for 4 h and then light adapted for 1 h. To assess penetration of sulpiride into the eyes, tadpole eyes were incubated with DMSO carrier (I) or with 1 μM ^{3}H-sulpiride (J) for 4 h and then light adapted for 1 h. The eyes were fixed, cryosectioned, and dipped in photoemulsion to detect the radiolabel; developed photoemulsion was detected by epi-illumination throughout the entire photoreceptor layer. Scale bars: 25 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

dopamine receptor to initiate translocation to the outer segment in response to light, we treated Arr-GFP tadpole eyes with sulpiride, a D_2-dopamine antagonist, and then assayed for any effect on light-driven arrestin translocation (Fig. 4G, H). Arrestin translocation in light-adapted photoreceptors was indistinguishable between sulpiride-treated (92.2% ± 0.4) and untreated eyes (93.4% ± 0.2), which are not statistically different ($\rho > 0.05$). Because of these negative results, we assessed penetration of the sulpiride reagent using tritiated sulpiride (Fig. 4I, J). The presence of the ^{3}H-labeled sulpiride throughout the retina demonstrates clear penetration of sulpiride into the photoreceptors and suggests that arrestin translocation does not rely on the D_2-dopamine receptor to initiate translocation, but rather that we were able to co-opt the D_2-dopamine receptor with quinpirole treatment to initiate arrestin translocation.

Since the D_2-dopamine receptor couples primarily to G_i G-proteins, we also immunostained for several G-proteins in rod photoreceptors and found them to localize differentially in the inner segments. $G_{\alpha i\text{-}1}$ and $G_{\alpha i\text{-}2}$ were primarily localized in the inner segments (Fig. 5A, B) whereas $G_{\alpha i\text{-}3}$ was mostly localized in the ellipsoidal region of the inner segments and along the axoneme of the outer segments (Fig. 5C). For a comparison, immunoreactivity for $G_{\alpha\text{-}o}$ and $G_{\alpha 11}$ was also detected throughout the inner segments and also along the axoneme (Fig. 5D, E). These localizations were in contrast to $G_{\alpha t}$ (transducin) which localized to the outer segments in dark-adapted photoreceptors (Fig. 5F). Our results confirm previous studies indicating G_i-like activity in rod photoreceptors [26–28].

3.4. Arrestin translocation in ATP-depleted photoreceptors

The mechanism of arrestin translocation has been studied by various groups that have reached somewhat disparate conclusions. Based on studies using ATP-depleted retinas, Nair et al. [29] suggested that arrestin translocation was entirely dependent on arrestin's affinity for two binding partners. During light adaptation, arrestin is

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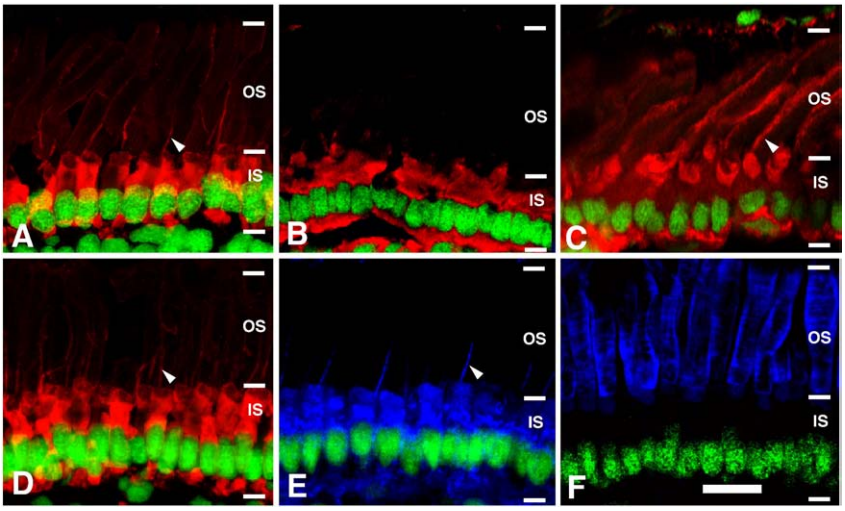

Fig. 5. Localization of G-proteins in rod photoreceptors. Dark-adapted wild-type tadpole eyes were immunostained for various G-proteins to determine their localization in the rod photoreceptors. Cryosections were immunostained for inhibitory G-proteins $G_{i\text{-}1}$ (A), $G_{i\text{-}2}$ (B), $G_{i\text{-}3}$ (C), and $G_{i\text{-}o}$ (D) (red). Sections were also stained for $G_{11\alpha}$ (E), and $G_{t\alpha}$ (F) (blue); nuclear DNA was stained with Sytox Green (green). Most of the G-proteins localize to the inner segments (IS) and along the axonemes (white arrowheads), with the exception of G_t which localizes to the outer segments (OS). Scale bar: 25 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

proposed to bind to light-activated rhodopsin in the outer segments, and during dark adaptation arrestin binds to microtubules present in the inner segment. Studies of arrestin's diffusion in rods show that arrestin is sufficiently mobile that passive diffusion could account for the changes in arrestin distribution [30,31]. In contrast, two other studies have shown that reagents that disrupt cytoskeletal elements have a potent effect on arrestin translocation [17,32]. The dependency of arrestin's movement on the cytoskeleton indicated the need for an energy supply. Our above observations demonstrate that arrestin translocation utilizes PKC and PLC, and argues that the machinery driving translocation might be more complex than the two-partner binding hypothesis previously proposed [29,33]. Furthermore, our implication of PKC in translocation argues for an ATP requirement which is in direct contrast to Nair et al. [29]. To help provide clarity, we recapitulated Nair et al.'s [29] ATP depletion study using explanted *Xenopus* eyes expressing arrestin-GFP. Analysis of the ATP levels in eyes treated for 60 min with 5 mM KCN revealed that ATP levels were depleted by three orders of magnitude compared to the untreated controls (Fig. 6H). In these dark-adapted ATP-depleted eyes, arrestin partitioned to the inner segments (Fig. 6C). Upon light exposure, however, arrestin did not move to the outer segments in cyanide-treated eyes (Fig. 6D), which was in contrast to the untreated controls where the arrestin localized to the outer segments (Fig. 6B).

One possible explanation for this observation is that the photoreceptor cells have been damaged in some non-specific manner by the cyanide treatment that would make them permanently refractory to light stimulation. To test this idea, eyes were washed with several changes of buffer without cyanide, and then supplemented with 5 mM ATP. In these animals, light adaptation led to translocation of arrestin to the outer segments to an extent that was statistically indistinguishable from untreated controls (Fig. 6F, G) ($\rho > 0.05$). Note that in eyes where the KCN was washed out, ATP levels recovered to about 6% of the level in untreated animals (Fig. 6H).

Our ATP-depletion results indicate that arrestin translocation does require ATP, which is in striking contrast to Nair et al. [29] where they concluded that arrestin translocation was not dependent on ATP. In order to determine whether this was due to a species difference, we reinvestigated the ATP dependence of arrestin translocation in mouse photoreceptors. Mouse organotypic retinal cultures were placed in high glucose media supplemented with 10% fetal calf serum, or glucose-free media supplemented with 2 mM deoxyglucose for 60–90 min, or in glucose-free media with 5 mM potassium cyanide. There was no difference in arrestin localization between the dark-adapted photoreceptors of high glucose or glucose-depleted retinas (data not shown). Arrestin translocation to the outer segment in response to light adaptation, however, was significantly ($\rho < 0.05$) reduced in retinas that were incubated in glucose-free media (Fig. 7C) or in 5 mM KCN (Fig. 7D) compared to the controls in which most of the arrestin was localized to the outer segments (Fig. 7B) with only about 20% of the arrestin translocating to the outer segments, compared to nearly 60% for the untreated control (Fig. 7E). In the treated retinas, most of the arrestin immunoreactivity remained in the inner segments, outer nuclear layer, and outer plexiform layer. The results obtained in organotypic retinal cultures were also confirmed using explanted mouse eyes (Supplement Fig. 1). These results indicate that in both frog and mouse rod photoreceptors, an ATP-dependent process plays an important role in the proper translocation of arrestin in response to light.

4. Discussion

4.1. The role of G-proteins in light-dependent movements of arrestin in rod photoreceptors

Our studies show that the initial step in signaling arrestin translocation appears to utilize a G-protein-regulated pathway. The evidence supporting this conclusion is several fold. First, we demonstrate that arrestin translocation is sensitive to cholera toxin, showing a highly significant increase in arrestin translocation after treatment with cholera toxin. Cholera toxin is known to affect only certain classes of G-proteins (G_s, G_{t1}, G_{t2}, G_{olf}, and a few G_i) [19,20], suggesting that one of these G-proteins is part of the translocation cascade. Obviously, the visual G-protein transducin (G_{t1}) is abundantly present in photoreceptors, but the dependence of arrestin translocation on this G-protein, transducin, can be excluded based on the experimental observation that arrestin translocates in mice in which $G_{t\alpha}$ expression is knocked out [5,7].

Additionally, our immunohistochemical investigations show that several types of G-proteins, particularly in the G_i class, are found in photoreceptors. For these G-proteins the localization is strongest in the inner segments and along the axoneme at the interface between the inner and outer segments. G_{11} expression in the inner segment, colocalizing with PKC and PLC, is intriguing since G_{11} can activate PLC. The presence of multiple G-proteins in this region of the rod

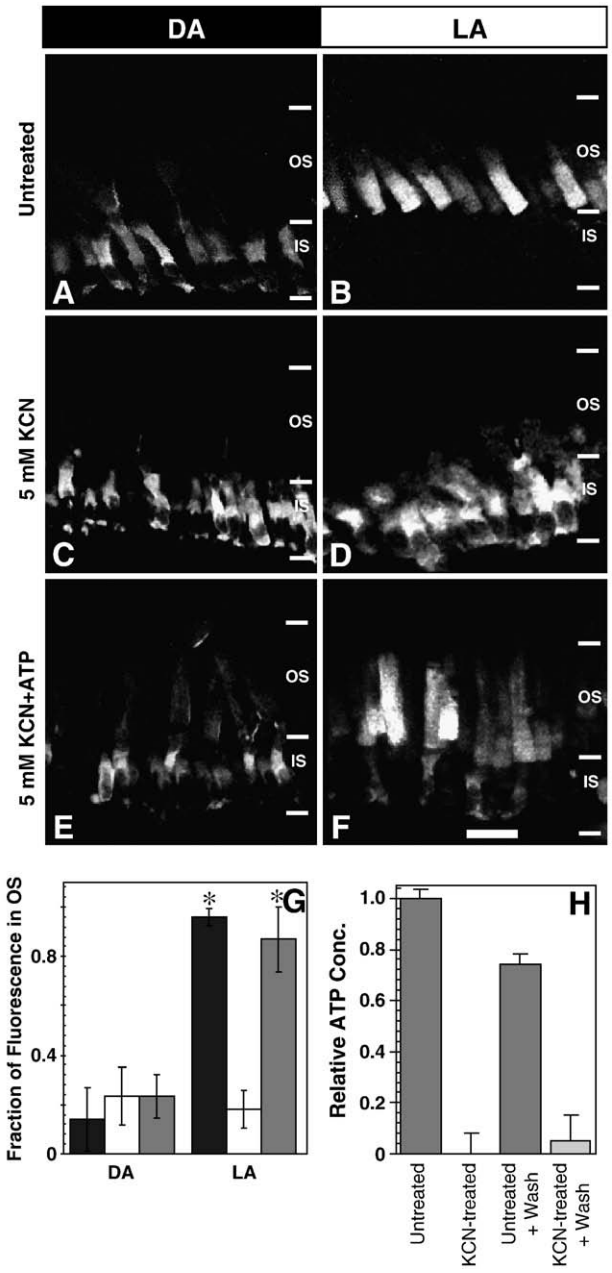

Fig. 6. Arrestin localization in response to ATP depletion in transgenic tadpoles. Dark-adapted arrestin-GFP transgenic tadpole eyes were incubated in MOPS-Twitty buffer in the dark for 2 h (A) or were light adapted for 1 h (B); eyes were depleted of ATP by treating with 5 mM KCN in MOPS-Twitty buffer for 1 h and then were kept in the dark for an additional 1 h (C), or were light adapted for 1 h (D). To remove KCN, after exposure to KCN for 2 h, the eyes were washed, supplemented with 5 mM ATP for an additional 2 h (E), and then were light adapted for 1 h (F). The relative amount of GFP fluorescence in the OS was quantified (G) for untreated controls (dark gray bars), KCN-treated eyes (white bars), and eyes supplemented with ATP after washing out KCN (light gray bars); bars represent the mean from at least eight eyes ±SEM. There was a statistically significant difference between light-adapted KCN-treated eyes and light-adapted control eyes ($\rho < 0.05$; asterisks), but not a statistically significant difference between control eyes and eyes in which the KCN was washed out and supplemented with exogenous ATP ($\rho > 0.05$). The relative amount of cellular ATP levels was measured using a luciferase assay showing a statistical difference between untreated eyes and KCN treated eyes (H). Scale bar: 25 μm.

photoreceptor highlights the complexity of the processes that are occurring at this location in this highly specialized cell. Our results demonstrating the presence of multiple G-proteins in rods confirm previous observations of G_i-like and G_s activity in the retina [26–28].

Because G_i G-proteins are able to couple to dopamine receptors [34,35], which are also expressed along the membranes of photoreceptor inner segments (Fig. 4), we reasoned that we might be able to co-opt these receptors to initiate arrestin translocation by activating the D_2-dopamine receptors. Using quinpirole, a D_2-dopamine receptor agonist, arrestin translocation to the outer segments was initiated in the absence of light. In contrast to the PLC and PKC agonists which caused only transient translocation of arrestin to the base of the outer segments, arrestin's localization to the outer segments in response to quinpirole was sustained for at least 30 min and was fully distributed within the outer segments. Although we are not sure of the reason behind these differences, arrestin's persistence in the outer segments may be attributed to the stability of the quinpirole compared to the PKC and PLC agonists. Alternatively, since the dopamine receptor is at the top of the cascade, it may take longer for the cascade to desensitize, therefore reducing the return of arrestin to the inner segments. It should be emphasized that we are not proposing that normal light-driven arrestin translocation is initiated by dopamine receptor activation. Instead, our studies only demonstrate that we are able to co-opt the D_2-receptors with a high dose of receptor agonist to initiate arrestin translocation. The lack of effect on arrestin translocation by sulpiride, a dopamine receptor antagonist, supports the view that dopamine receptors do not normally initiate arrestin translocation.

4.2. Role of PKC and PLC

In support of our hypothesis that arrestin translocation is signaled through a G-protein-regulated pathway, we also provide evidence implicating PKC and PLC in initiating arrestin translocation. We demonstrate that activators of both PKC and PLC initiate arrestin translocation to the outer segments in the absence of light. Apparently, the function of PKC and PLC in initiating this translocation is essential since antagonists of both PKC and PLC reduce the light-activated movement of arrestin to the outer segments. Although the results are consistent with a role for PKC in this pathway, it is important to note that the activators used in this study are not completely specific, and that they can also potentially activate chimaerins and diacylglycerol kinases [36,37].

Our immunohistochemical analysis also supports our hypothesis showing PKC and PLC co-localization in the inner segments. PLC activity has been shown to increase by 23% in light-adapted photoreceptors [8,18], suggesting a role for PLC during light adaptation. It is interesting that rhodopsin has been identified as the major substrate in the photoreceptor cell for PKC [9,11]. Whether this is relevant to arrestin translocation, or whether PKC activity couples to arrestin translocation through another substrate is not resolved in our present study.

4.3. Arrestin translocation is energy dependent

The first part of this study shows that arrestin translocation involves a G-protein-regulated pathway that would require both GTP and ATP. However, arrestin's translocation to the outer segments has been proposed as an energy-independent process, requiring only protein–protein interactions [29,33]. Contrary to Nair et al. [29], we demonstrate that arrestin translocation is an energy-dependent process that does not solely depend on protein–protein interaction. Our results show arrestin does not translocate to the outer segments in ATP-depleted tadpole photoreceptors, and is significantly reduced in ATP-depleted mouse photoreceptors. Importantly, translocation of arrestin can be recovered by the addition of ATP, demonstrating the viability of the ATP-depleted explanted eyes. The observed energy dependency is also consistent with an involvement of the cytoskeleton in the arrestin movement previously demonstrated [17,32]. Our results are strikingly different from Nair et al. [29], where they

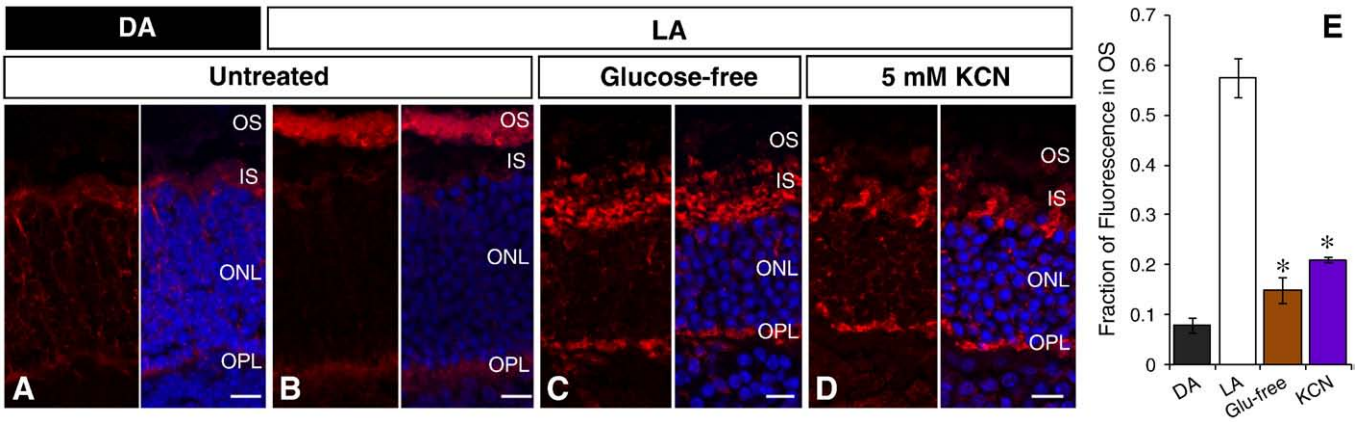

Fig. 7. Arrestin localization in response to ATP depletion in mouse retina. Mice were dark adapted for at least 4 h and subsequently retinas were prepared under dim red light. Organotypic retina cultures were then either placed in glucose-free medium (C) or glucose-free medium with 5 mM KCN (D). After 1 h incubation at 37 °C at 5% CO_2, the eyes were light adapted for 1 h. Untreated dark-adapted (A) and light-adapted (B) eyes are shown for comparison. Mouse sections were immunostained for arrestin (red) and the nuclear DNA stained with DAPI (blue). In photoreceptors that were either in glucose-free medium or in glucose-free medium with KCN, a large amount of the arrestin immunostaining is found over the inner segments (IS), the outer nuclear layer (ONL), and the outer plexiform layer (OPL) following light adaptation, compared to the untreated control where most of the arrestin was found in the outer segments (OS). Fluorescence fraction in the outer segments was quantified (E), showing the averages (±SEM; $n=4$). Asterisks indicated differences that are significantly different compared to the untreated light-adapted control ($\rho<0.05$). Scale bar: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

showed arrestin is able to translocate in ATP-depleted eyes. We are unsure as to the source of the discrepancy, although the differences in results may be attributed to more effective penetration of cyanide since we were able to deplete ATP levels by three orders of magnitude, whereas the previous study achieved only a 100-fold reduction of ATP. Regardless of the source of the difference, it is clear that in both frog and mouse photoreceptors, arrestin translocation is dependent on ATP. This ATP dependence further supports our hypothesis that arrestin translocation uses a G-protein-regulated pathway for the initial distribution to the outer segments. It is noteworthy that Nair et al. [29] saw an effect of ATP-depletion on the rate of arrestin translocation at 400 lx. In eye cups that were treated with cyanide, the half-time of arrestin translocation was nearly doubled. Although the authors attributed this slowed rate of translocation to a difference in the affinity of arrestin for light-activated rhodopsin compared to rhodopsin that was both light activated and phosphorylated, it is also possible that their ATP depletion affected the rate of arrestin release from the inner segment.

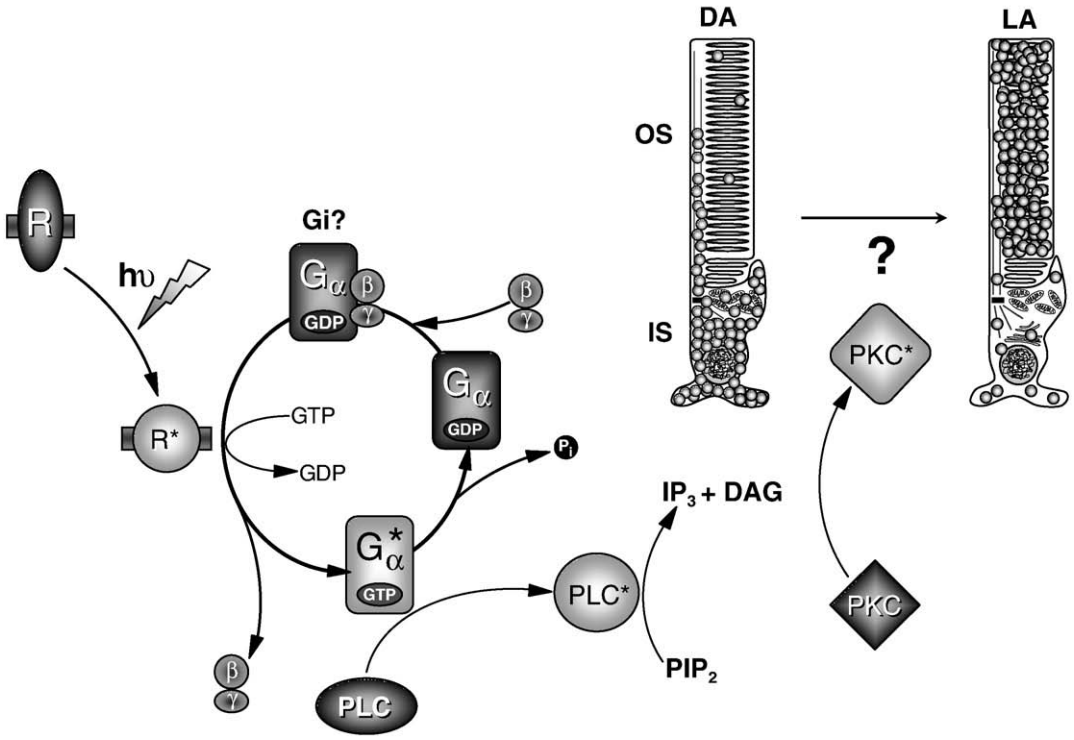

Fig. 8. Schematic illustration of the transduction cascade that initiates arrestin translocation in response to light ($h\nu$). Rhodopsin (R) is activated by light which subsequently activates a heterotrimeric G-protein, perhaps a Gi. G_α^* activates phospholipase C (PLC) to catalyze the hydrolysis of phosphatidyl bis-phosphate (PIP_2) to inositol trisphosphate (IP_3) and diacylglycerol (DAG). DAG activates protein kinase C (PKC) which in some undiscovered mechanism triggers the release and translocation of arrestin (small spheres) from the inner segment (IS) and axoneme to the outer segment (OS).

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4.4. Proposed pathway

Our ability to stimulate arrestin translocation to the outer segments in the absence of light demonstrates the complexity associated with arrestin translocation. In light of our findings, we are proposing that arrestin translocation to the outer segments requires two major steps. In our model, the initial distribution of arrestin in response to light is signaled by the phosphoinositide pathway with the final step in the phosphoinositide pathway serving as a "gatekeeper" to release arrestin to the outer segments (Fig. 8). Neither the mechanism nor the nature of this molecular gatekeeper has been identified, although it appears that ATP plays an important role. In the second step, arrestin can then rapidly disperse throughout the basal one-third of the outer segment, probably by diffusion. However, it appears that for arrestin to rapidly distribute throughout the length of the outer segments requires microtubule cytoskeletal elements since arrestin is slow to fully distribute throughout the outer segments when frog or mouse photoreceptors are treated with thiabendazole, a microtubule poison [17,32]. Under these conditions, arrestin strongly localizes to the base of the outer segments. In keeping with the two-partner binding hypothesis [29,33], it appears that binding to light-activated rhodopsin is important for retaining arrestin in the outer segment, since in our studies with the PLC and PKC agonists, arrestin moved to the outer segments but did not remain as occurs with light-activated translocation. We hypothesize that the PKC and PLC agonists have opened an arrestin "gate", but in the absence of activated rhodopsin to serve as an anchor, arrestin returns to the inner segments.

The observation that arrestin translocation to the basal portion of the outer segments appears to be different than its translocation throughout the remainder of the outer segment leads to the obvious question about what is unique about the basal one-third of the outer segments compared to the distal two-thirds? Structurally, there is no obvious difference, except that there is a larger cytosolic space associated with the axoneme at the base of the outer segments [38–40], perhaps creating fewer barriers for diffusion of arrestin. It is intriguing that a recent investigation of the kinesin motor proteins in rods revealed that a second kinesin, Kif17, is present along the singlet microtubules in the apical two-thirds of the outer segments, whereas kinesin II is present in the connecting cilium and along the doublet microtubules in the proximal one-third of the axoneme [38,41]. Perhaps this difference in kinesin distribution relates to the apparent difference in arrestin translocation along the outer segments.

In summary, our current studies show that light-dependent arrestin translocation in vertebrate rod photoreceptors is likely signaled by light-activated rhodopsin through a non-transducin G-protein pathway operating in parallel to phototransduction. This alternative, cholera toxin-sensitive G-protein signals through PLC and PKC to initiate arrestin translocation. Importantly, we show that arrestin translocation is an energy-dependent process.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cellsig.2009.10.016.

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