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Light Adaptation through Phosphoinositide-Regulated Translocation of *Drosophila* Visual Arrestin

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Summary

Photoreceptor cells adapt to bright or continuous light, although the molecular mechanisms underlying this phenomenon are incompletely understood. Here, we report a mechanism of light adaptation in *Drosophila*, which is regulated by phosphoinositides (PIs). We found that light-dependent translocation of arrestin was defective in mutants that disrupt PI metabolism or trafficking. Arrestin bound to PIP_3 in vitro, and mutation of this site delayed arrestin shuttling and resulted in defects in the termination of the light response, which is normally accelerated by prior exposure to light. Disruption of the arrestin/PI interaction also suppressed retinal degeneration caused by excessive endocytosis of rhodopsin/arrestin complexes. These findings indicate that light-dependent trafficking of arrestin is regulated by direct interaction with PIs and is required for light adaptation. Since phospholipase C activity is required for activation of *Drosophila* phototransduction, these data point to a dual role of PIs in phototransduction.

Introduction

Photoreceptor cells are remarkable in their capacity to respond to their agonist, light, with great rapidity and over an intensity range that spans >10 orders of magnitude (Rodieck, 1998). These features of the photoresponse are similar between vertebrates and invertebrates. While there are some notable parallels in the phototransduction cascades in rods, cones, and invertebrate photoreceptors, the signaling cascades also exhibit major differences. Each of these cascades begins with the photoactivation of a G protein-coupled receptor (GPCR), rhodopsin, followed by activation of a heterotrimeric G protein. In rods and cones, the G protein, referred to as transducin, activates a cGMP-phosphodiesterase leading to closure of the cGMP-gated channels (reviewed in Arshavsky et al., 2002). In *Drosophila* photoreceptors, the trimeric G protein (Gq) stimulates a phospholipase C (PLC) (Bloomquist et al., 1988), resulting in activation of the cation channels TRP and TRPL (Montell and Rubin, 1989; Hardie and Minke, 1992; Phillips et al., 1992; Niemeyer et al., 1996).

Despite these differences in the mammalian and *Drosophila* phototransduction cascades, many of the same proteins that regulate PI metabolism are expressed in rods, cones, and *Drosophila* photoreceptor cells, and the activities of some of these proteins have been shown to change in a light-dependent manner (reviewed in Giusto et al., 2000). Nevertheless, the roles of PIs in mammalian phototransduction are enigmatic. One possible phenomenon that could be regulated by PIs is adaptation, though this possibility has not been explored.

The ability of mammalian photoreceptor cells to adjust to different intensities of light appears to involve multiple mechanisms, which are mediated through signaling proteins such as the rhodopsin kinase, phosphodiesterase, guanylate cyclase, cGMP-gated channels, and recoverin (reviewed in Fain et al., 2001). Adaptation is necessary for preventing photoreceptors from saturating their response, as the light intensity increases, and for improving temporal resolution in the visual response. This is accomplished in part by adjusting the magnitude of the initial light response and altering the kinetics of response termination.

A poorly understood phenomenon, which has been proposed to contribute to adaptation, is light-regulated translocation of signaling molecules to and from the outer segments and rhabdomeres, which are the sites of phototransduction in mammalian and *Drosophila* photoreceptor cells, respectively. Light-dependent movement of signaling proteins is well documented in mammals and fruitflies and includes the $G\alpha$ and $G\beta\gamma$ subunits (Sokolov et al., 2002; Kosloff et al., 2003) and the TRPL cation channel (Bahner et al., 2002). Shuttling of transducin and TRPL from the outer segments and rhabdomeres, respectively, participates in reducing the sensitivities of the photoreceptor cells to increasing intensities of light (Bahner et al., 2002; Sokolov et al., 2002).

Both vertebrate and invertebrate visual arrestins also undergo light-dependent translocations (Broekhuyse et al., 1985; Philp et al., 1987; Mangini and Pepperberg, 1988; Whelan and McGinnis, 1988; Kiselev et al., 2000; Peterson et al., 2003); however, the function of the dynamic reorganization of arrestin has not been addressed. It has been proposed that the intracellular movement of visual arrestin contributes to adaptation (McGinnis et al., 2002), although this suggestion has not been tested experimentally. Of equal importance, the mechanisms underlying the light-dependent shuttling of signaling proteins, including visual arrestin, are not understood.

During the last few years, arrestins have received additional scrutiny due to the demonstration that they are multifunctional proteins (reviewed in Hall and Lefkowitz, 2002). While arrestins have long been known to bind to rhodopsin and other GPCRs and participate in response termination, more recent studies show that they also function as molecular scaffolds. Among the many arrestin binding proteins is clathrin, and this interaction mediates endocytosis of GPCRs. Moreover, excessive endocytosis of GPCR/arrestin complexes has been shown to contribute to apoptosis (reviewed in Dolph, 2002).

PIs might be involved in the light-regulated movement of visual arrestins in photoreceptor cells, since they

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Table 1. Protein Products and Functions of Loci Described or Characterized in the Current Manuscript

A. Proteins Immunolocalized in Figure 1 and Supplemental Figure S1

Locus	Protein	Function
arr2	arrestin	inactivation of rhodopsin
inaC	protein kinase C	serine/threonine kinase
inaD	PDZ-containing protein	scaffold protein
ninaC	myosin III	protein kinase/myosin
ninaE	rhodopsin	light receptor
norpA	phospholipase C-β	production of second messengers DAG & IP_3
trp	cation channel	light-responsive channel

B. Other Loci Described in the Current Manuscript

Locus	Protein	Function
cds	CDP-DAG synthase	production of intermediate in PI cycle
dAkt	protein kinase B	serine/threonine kinase
Dp110	PI3-kinase	phosphorylation of 3-phosphoinositides
dPTEN	PI phosphatase	dephosphorylation of 3-phosphoinositides
Gβ76C	Gqβ	β subunit of trimeric G protein
rdgA	DAG kinase	production of phosphatidic acid in PI cycle
rdgB	PI transfer protein	transfer of PI in photoreceptor cells
rdgC	protein phosphatase	dephosphorylation of rhodopsin
trpl	cation channel	light-responsive channel

function as spatially restricted membrane second messengers and bind to and affect endocytosis of β arrestin in vitro (Gaidarov et al., 1999; Naga Prasad et al., 2002). Many proteins regulating the biosynthesis of PIs are photoreceptor cell enriched, and *Drosophila* phototransduction depends on the expression of a PLCβ encoded by the *norpA* locus (reviewed in Montell, 1999; Hardie and Raghu, 2001). However, the direct physiological targets and functions of PIs in visual transduction are not known.

In the current work, we found that the light-induced shuttling of the major arrestin (Arr2) in *Drosophila* photoreceptor cells functioned in adaptation. Localization of Arr2 was impaired in three mutants, *rdgB*, *cds*, and *PTEN*, which affected PI trafficking or biosynthesis. Arr2 bound to PIP_3 in vitro, and mutation of this site resulted in defects in the normal translocation of Arr2 and light adaptation. Mutations in Arr2 that decreased the interaction with PIP_3 in vitro also suppressed light-dependent retinal degeneration caused by rapid endocytosis of Arr2. These data raise the possibility that translocation of mammalian visual arrestin may also be regulated by PIs, providing a potential explanation for the presence of many rod and cone proteins that function in PI biosynthesis and which are activated in a light-dependent manner.

Results

Arr2 Trafficking Is Impaired in *rdgB* and *cds*

To test whether the retention or trafficking of one or more signaling proteins is regulated by PIs, we set out to compare the spatial distributions of a variety of signaling proteins (Table 1A) in wild-type flies and mutants that disrupt PI biosynthesis or trafficking. We performed immunolocalizations using two mutants, *rdgB* and *cds*, which disrupt a phosphatidylinositol transfer protein and CDP-DAG synthase, respectively (Figure 1A and Table 1B) (reviewed in Montell, 1999).

We determined the immunolocalizations of each of the signaling proteins after exposing dark-adapted flies to a pulse of bright blue light, which maximally converts Rh1 to the light-activated form metarhodopsin. In wild-type flies, each of the proteins was detected almost exclusively in the rhabdomeres. INAD, TRP, PKC, and NINAC (myosin III) displayed indistinguishable rhabdomere-enriched staining patterns in *rdgB* and *cds* photoreceptor cells (Figures 1B–1G; also see Figures S1A–S1F in the Supplemental Data available at http://www.neuron.org/cgi/content/full/39/1/121/DC1). Rh1 was also concentrated in *cds* rhabdomeres (Figure 1J); however, in *rdgB* flies, the Rh1 protein level was significantly decreased, as previously reported (Figure 1I and also see Supplemental Figure S1J in the Supplemental Data) (Milligan et al., 1997).

In contrast to the results described above, Arr2 was not rhabdomere specific but was diffusely distributed between the cell bodies and rhabdomeres in both *rdgB* and *cds* flies (Figures 1K–1M). The mislocalization of Arr2 was not simply due to an altered distribution or instability of Rh1, since Rh1 was normally localized in *cds* mutant flies (Figure 1J). NORPA (PLC) was also mislocalized in both *rdgB* and *cds*, though there was a more pronounced defect in *rdgB* (Supplemental Figures S1G–S1I available online). These data raise the possibility that PIs are required for proper localization of PLC and Arr2. PLC has a PI binding PH domain, which may be needed for rhabdomere targeting. However, Arr2 does not have such a domain. Nevertheless, mammalian visual arrestin has been reported to bind to inositol phosphates (IPs), though an interaction with PIs was not tested (Palczewski et al., 1991). Therefore, the question arises as to whether PIs regulate the trafficking of Arr2 directly.

C-Terminal Domain of Arr2 Bound to Phosphoinositides

To determine whether the visual arrestin bound directly to PIs, we probed a membrane containing various PIs (PIP-Strips) with the N- and C-terminal domains of Arr2

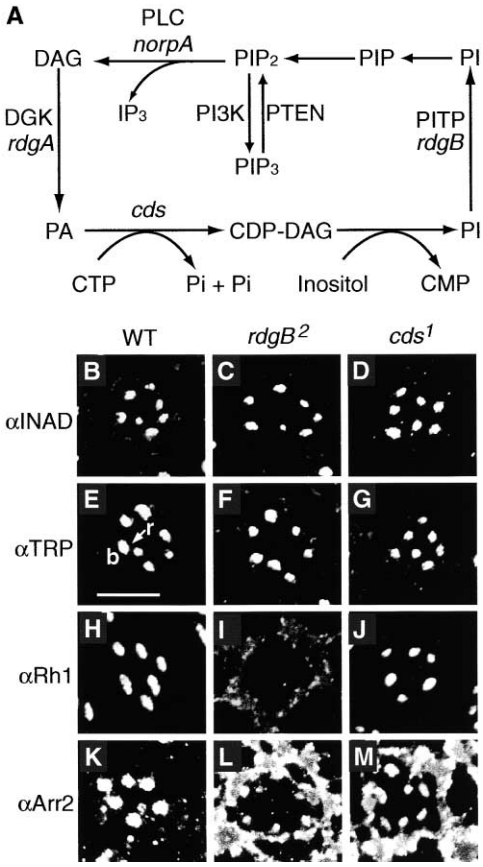

Figure 1. Phosphoinositide Signaling Pathway and Spatial Distribution of INAD, TRP, Rh1, and Arr2 in Wild-Type, *rdgB*, and *cds* Flies

(A) Schematic of PI cycle. See Table 1 for definition of the loci and proteins.

(B–M) Spatial distributions of signaling proteins in wild-type (wt), *rdgB*^{2}, and *cds*^{1} ommatidia. Shown are tangential sections through the distal region of white compound eyes (includes the six outer R1-6 cells and the smaller central R7 cell). Each panel includes one entire ommatidium from young adults (<2 days posteclosion). Prior to preparing the sections, the flies were dark adapted for ≥4 hr and exposed to blue (480 nm) light for 5 min. The sections were stained with antibodies against INAD, TRP, Rh1, and Arr2 as indicated. Rh1 stains only six rhabdomeres, as it is expressed only in R1-6 cells. Staining was detected using FITC-coupled secondary antibodies. The six or seven ovals seen in most panels correspond to the rhabdomeres (r). The cell bodies (b) are located near the periphery of the ommatidia. Scale bar, 10 μm.

fused to GST (GST-Arr2N and GST-Arr2C, respectively). We found that the GST-Arr2C bound to several PIs, while GST-Arr2N did not (Figure 2A). The binding pattern of GST-Arr2C was similar to that obtained with GST-CRAC-PH (GST-CRAC), which had been shown to bind PIP_3 preferentially (Parent et al., 1998) (Figure 2A).

To confirm that PIs or IPs bound to Arr2, we employed solution binding assays. We used IP_6 rather than a PI for these assays, since IPs are soluble in aqueous solutions and are available as labeled derivatives. ^{3}H-labeled IP_6 and GST-Arr2N or GST-Arr2C were combined in solution, precipitated, and the level of bound [^{3}H]IP_6 was measured. Consistent with the nitrocellulose phospholipid binding assay, GST-Arr2C but not GST-Arr2N bound to IP_6 (Figure 2B). Thus, it appeared that Arr2 bound to PIs or IPs and did so through the C-terminal domain.

Arr2 Preferentially Bound to PIP_3 In Vitro

We tested which PI preferentially bound to Arr2 by performing competition assays. [^{3}H]IP_6 and various concentrations (0.1–100 μM) of unlabeled PIs were mixed in solution with GST-Arr2C and the level of bound [^{3}H]IP_6 was determined. We found that IP_6 and PIP_3 displayed the lowest IC_{50} values (0.634 and 0.628 μM, respectively) (Figure 2C). However, using this assay system, we could not distinguish whether IP_6 or PIP_3 was the strongest binding partner.

To address whether there is specificity between IP_6 and PIP_3 for Arr2 binding, we performed a binding assay using PIP_3 beads. ^{35}S-labeled Arr2 bound to PIP_3 beads but not to control beads (Figure 2D). The C-terminal domain of RDGC, which did not bind to PIP-Strips (data not shown), did not interact with the PIP_3 beads (Figure 2D). To compare the binding specificity of IP_6 and PIP_3, we used a competition assay. Soluble PIP_3 competed for Arr2 binding to the PIP_3 beads at a lower concentration than IP_6 (IC_{50} = 2.0 μM versus 10.7 μM) (Figure 2E). Therefore, Arr2 appeared to bind preferentially to PIP_3 in vitro.

Lysine Residues Critical for Phosphoinositide Binding

To address the function of the PI association with Arr2 in vivo, we identified candidate amino acid residues critical for the Arr2/PIP_3 interaction. Since the residues that contributed to PI binding in vitro to β arrestin (Gaidarov et al., 1999) were not conserved in Arr2, we employed structural modeling, focusing on a corresponding region in Arr2 encompassing the PI binding site in β arrestin. The structures of several PI binding domains were determined in complex with PIs. These regions, such as the PH and the clathrin assembly lymphoid myeloid (CALM) domains, recognize PIs via hydrogen bond interactions with locally abundant positively charged residues rather than through a conserved structural motif. We found that lysines 228, 231, and 257 appeared to form a basic amino acid pocket, which resembled the IP_6 binding site of CALM (Ford et al., 2001). The Arr2/PIP_3 interaction model proposes that lysines 228, 231, and 257 form hydrogen bonds with the three phosphates in PIP_3 at distances ranging from 2.5 to 3.0 Å (Figures 3A and 3B).

We found that glutamine substitutions of all three lysine residues (3K/Q) identified in the structural modeling significantly impaired the interaction between Arr2 and IP_6 in a solution binding assay (Figure 3C; wt; 503 ± 26 c.p.m. versus 3K/Q; 171 ± 35 c.p.m.). Glutamine substitutions of unrelated lysine residues (391 and 393) did not decrease IP_6 binding (606 ± 63 c.p.m.; Figure 3C). Since lysine residues are subject to ubiquitination, and such a modification has been reported to affect trafficking of β arrestin (Shenoy et al., 2001), we also generated lysine to arginine substitutions (3K/R). The 3K/R mutations did not disrupt Arr2/IP_6 binding (Figure 3C; 545 ± 17 c.p.m.).

To confirm the effects of the 3K/Q and 3K/R substitutions on the Arr2/PI interaction, we performed PIP_3 bead binding assays using [^{35}S]Arr2. Consistent with the results of the IP_6 binding assay, Arr2$^{3K/Q}$ showed decreased

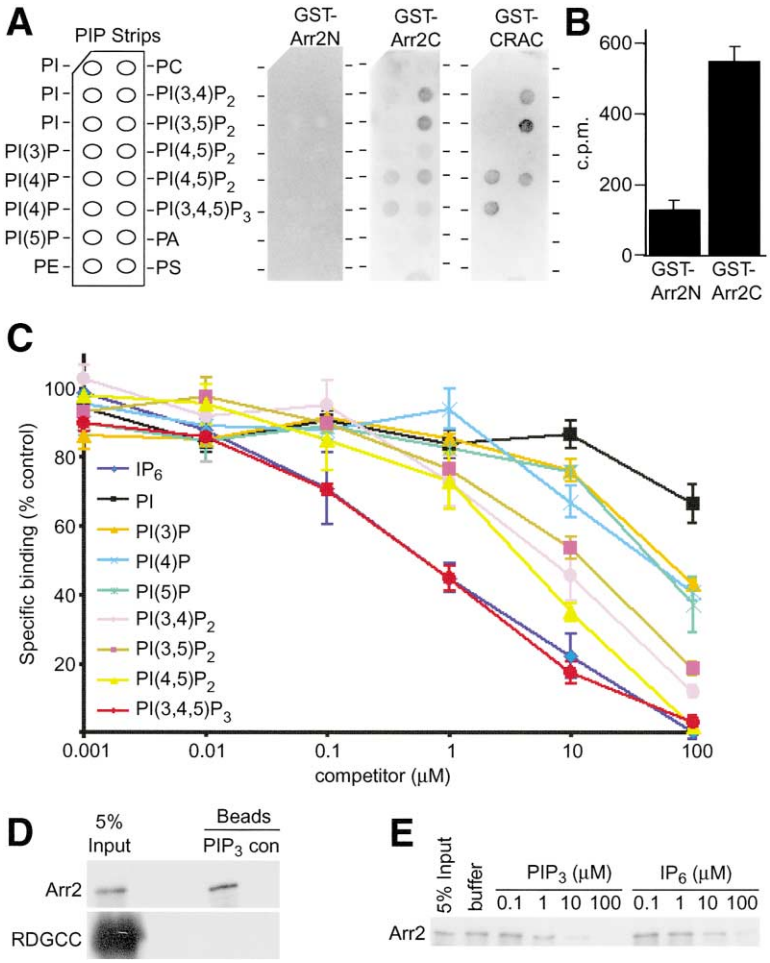

Figure 2. Direct Interaction of Arr2 with PIs In Vitro

(A) Nitrocellulose phospholipid binding assay. Purified GST-Arr2N (residues 1–210), GST-Arr2C (residues 204–401), or GST-CRAC (PH domain, residues 1–122) were incubated with nitrocellulose strips spotted with the indicated phospholipids (PIP-Strips; Echelon Research Laboratories). Bound protein was visualized by immunoblotting.

(B) Binding of $[^3H]IP_6$ to GST-Arr2N and GST-Arr2C. The levels of bound $[^3H]IP_6$ were measured using a scintillation counter. The SEMs were based on three sets of experiments.

(C) $[^3H]IP_6$ competition assay with various PIs. Binding of $[^3H]IP_6$ to recombinant GST-Arr2C was measured in the presence of the following cold competitors: IP_6 (IC_{50} = 0.634 μM), di-C8-PI ($IC_{50} \geq 100$ μM), di-C8-PI(3)P (IC_{50} = 62.4 μM), di-C8-PI(4)P (IC_{50} = 44.2 μM), di-C8-PI(5)P (IC_{50} = 46.3 μM), di-C8-$PI(3,4)P_2$ (IC_{50} = 6.8 μM), di-C8-$PI(3,5)P_2$ (IC_{50} = 12.6 μM), di-C8-$PI(4,5)P_2$ (IC_{50} = 4.3 μM), di-C8-$PI(3,4,5)P_3$ (IC_{50} = 0.628 μM). SEMs were based on at least three sets of experiments.

(D) PIP_3 bead binding assay. Full-length Arr2 or the C-terminal region of RDGC (RDGCC; residues 402–661), labeled with $[^{35}S]$methionine by coupled transcription/translation were incubated with PIP_3 beads or control beads (agarose beads without bound PIP_3). The bound probes were eluted, resolved by SDS-PAGE, and exposed to film.

(E) Competition of Arr2 binding to the PIP_3 resin with various concentrations of PIP_3 and IP_6 as indicated.

PIP_3 bead binding, while $Arr2^{3K/R}$ displayed a similar binding affinity to $Arr2^{WT}$ (3K/Q: 27.8% ± 8.6% versus 3K/R: 105.7% ± 14.7%; Figures 3D and 3E). We observed the same effects of the 3K/Q and 3K/R substitutions using the PIP-Strips binding assay. The 3K/Q but not the 3K/R derivative bound less effectively to the PIP-Strips than wild-type Arr2 (data not shown). These data indicated that lysine residues 228, 231, and 257 were necessary for the Arr2/PI interaction. Moreover, the $Arr2^{3K/R}$ derivative provided a control to address specifically the role of PI binding on Arr2 function in vivo, independent of an effect of the mutations on ubiquitination.

Arr2 Mutants Retained Rhodopsin Binding Ability

To determine the physiological role of PI binding to Arr2, we generated transgenic flies that expressed full-length $Arr2^{3K/Q}$, $Arr2^{3K/R}$, or transgenic wild-type Arr2 in a null *arr2* (*arr2*^{5}) background (Alloway and Dolph, 1999). Each of the three transgenic proteins were expressed at levels similar to that in wild-type (*y w*) (Supplemental Figure S2A at http://www.neuron.org/cgi/content/full/39/1/121/DC1). Since one of the roles of arrestin is to associate directly with rhodopsin and participate in deactivation of metarhodopsin, we evaluated whether rhodopsin/Arr2 binding was affected in the transgenic flies by employing an arrestin pellet assay (Kiselev and Subramaniam, 1997; Alloway et al., 2000). We found that neither $Arr2^{3K/Q}$ nor $Arr2^{3K/R}$ showed defects in association or dissociation from rhodopsin (Supplemental Figures S2B and S2C available online).

Defects in Light-Dependent Trafficking of $Arr2^{3K/Q}$ to and from the Rhabdomeres

We tested whether PI binding to Arr2 affected trafficking by examining the spatial distribution of Arr2 in the wild-type and the transgenic flies at several time points after light stimulation. Arr2 undergoes light-dependent shuttling from the cell body to the rhabdomere (Kiselev et al., 2000). In dark-adapted wild-type flies, Arr2 was dispersed throughout the rhabdomeres and the cell bodies (Figures 4A and 4P). In similarly treated *arr2*$^{3K/Q}$ flies, Arr2 displayed a 37% lower rhabdomeral concentration than in wild-type (25.6% ± 1.7% versus 35.0% ± 2.3%, respectively; Student's unpaired t test indicates a statistically significant difference, $p < 0.005$; Figures 4F and 4P). After exposing wild-type flies to a 5 min pulse of blue light, Arr2 was concentrated in the rhabdomeres (Figures 4B and 4Q). A similar light-dependent translocation was observed in *arr2*$^{3K/R}$ flies (Figures 4K, 4L, 4P, and 4Q). However, in *arr2*$^{3K/Q}$ flies, Arr2 was present diffusely throughout the cell bodies and rhabdomeres (Figures 4G and 4Q), similar to the wild-type Arr2 pattern in *rdgB* and *cds* (Figures 1L and 1M). These data indicated that

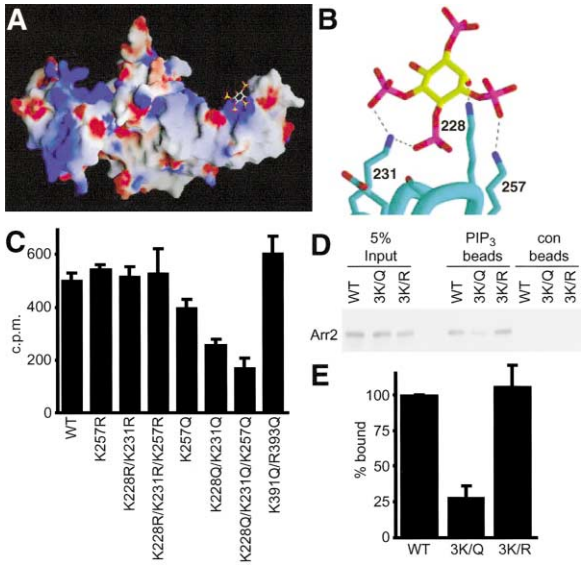

Figure 3. Residues in Arr2 Critical for PI Binding

(A) Surface representation of Arr2 modeled with IP_4, which is the IP derivative of PIP_3. Positively and negatively charged regions are indicated in blue and red, respectively.

(B) Schematic representation of the PIP_3 binding site. Broken black lines represent polar interactions between IP_4 and lysines 228, 231, and 257.

(C) Site-directed mutagenesis of the IP_6 binding domain of Arr2. [^{3}H]IP_6 binding to the wild-type (wt) GST-Arr2C fusion protein (residues 204–401) and to derivatives containing the indicated alterations were expressed and tested for binding. Error bars reflect standard errors from three replicates.

(D) PIP_3 bead binding assay. [^{35}S]-labeled full-length Arr2 (wt) or derivatives containing the 3K/Q or the 3K/R mutations were incubated with PIP_3 beads or control beads. The probes were resolved by SDS-PAGE and exposed to film.

(E) Quantification of the PIP_3 bead binding assay. The percent of wt, 3K/Q, and 3K/R bound to the PIP_3 beads from (D) was determined from the pixel intensities of the bands. The amount of wt bound to the beads is defined as 100%. SEMs were based on three sets of experiments.

the *arr2*$^{3K/Q}$ but not the *arr2*$^{3K/R}$ flies had defects in light-dependent movement of Arr2 into the rhabdomeres.

The lack of rhabdomere enrichment in *arr2*$^{3K/Q}$ could have reflected a defect in the kinetics in the light-dependent movement of the mutant Arr2 into the rhabdomeres. Alternatively, Arr2$^{3K/Q}$ might not become rhabdomere enriched even after prolonged exposure to light. To discriminate between these possibilities, we stimulated the flies for 1 hr with blue light and found that the Arr2$^{3K/Q}$ displayed a rhabdomere-enriched distribution (Figures 4H and 4R). Thus, the light-dependent movement to the rhabdomeres was delayed rather than eliminated in *arr2*$^{3K/Q}$ flies.

We also assessed whether PI binding is necessary for Arr2 movement from the rhabdomeres to the cell bodies. Rhodopsin/Arr2 complexes undergo clathrin-dependent endocytosis and movement to the cell bodies after exposing the flies to blue light, followed by incubation in the dark (Kiselev et al., 2000). Therefore, we exposed the transgenic flies to 1 hr of blue light and subsequently placed the flies in the dark for 3 hr. Both wild-type Arr2 and Arr2$^{3K/R}$ redistributed to the cell bodies following this paradigm (Figures 4D, 4N, and 4S). This was in contrast to the *arr2*$^{3K/Q}$ photoreceptor cells, which showed a rhabdomere-enriched Arr2 signal under these conditions (Figures 4I and 4S). After 72 hr in the dark, Arr2$^{3K/Q}$ redistributed between the rhabdomeres and cell bodies (Figures 4J and 4T). The Arr2 protein levels remained unchanged after exposure to all of these experimental conditions (Supplemental Figure S2D at http://www.neuron.org/cgi/content/full/39/1/121/DC1). The observations that both light-dependent rhabdomere localization and shuttling to the cell bodies were slowed in *arr2*$^{3K/Q}$ indicated that PI binding was necessary for normal trafficking of Arr2 in both directions, to and from the rhabdomeres.

arr2$^{3K/Q}$ Suppressed Light-Dependent Retinal Degeneration in the *norpA* Mutant

Null mutations in the gene encoding the eye-enriched PLC (*norpA*) eliminate light-induced Ca^{2+} influx. Due to the lack of Ca^{2+} influx, Arr2 is not phosphorylated by Ca^{2+}/calmodulin-dependent kinase II (Matsumoto et al., 1994; Kahn and Matsumoto, 1997) and does not dissociate from rhodopsin (Alloway and Dolph, 1999). As a consequence of the stable rhodopsin/arrestin complexes in *norpA*, there is excessive rhodopsin/Arr2 endocytosis, which in turn causes light-dependent retinal degeneration. This degeneration has been reported to be suppressed by removing Arr2 (Alloway et al., 2000). However, this suppression is quite incomplete, as by 2 weeks posteclosion some of the rhabdomeres in *norpA;;arr2*^{5} flies have completely degenerated (Supplemental Figure S3 at http://www.neuron.org/cgi/content/full/39/1/121/DC1). This partial suppression of the *norpA* degeneration could have been due to a lack of formation of rhodopsin/arrestin complexes or to the absence of arrestin-mediated endocytosis of rhodopsin. Furthermore, since arrestins are multifunctional proteins (reviewed in Hall and Lefkowitz, 2002), the suppression may have been due to some other arrestin function.

Since Arr2$^{3K/Q}$ does not appear to affect its interaction with rhodopsin but appears to be defective in endocytosis, we addressed whether the degeneration in *norpA* was suppressed in combination with *arr2*$^{3K/Q}$. We assayed retinal degeneration in live flies by examining for the presence of the deep pseudopupil (DPP; see figure legends). Those flies that have undergone retinal degeneration fail to display a DPP. As previously reported, *norpA* flies undergo light-dependent retinal degeneration, and all animals lose the DPP after 10 days (Figure 5A; *norpA;;arr2*$^{+}$). In the *norpA;;arr2*$^{3K/Q}$ double mutant, the DPP was retained (Figure 5A). In contrast, most of the *norpA;;arr2*$^{3K/R}$ double-mutant flies lost the DPP after 10 days (Figure 5A).

We confirmed that *arr2*$^{3K/Q}$ suppressed the *norpA*-induced retinal degeneration using transmission EM. While *norpA* flies showed severe retinal degeneration after 2 weeks of a light/dark cycle, consistent with previous reports (reviewed in Pak, 1994), and the DPP assay described above (Figures 5A and 5B and Supplemental Figure S3 at http://www.neuron.org/cgi/content/full/39/1/121/DC1; *norpA;;arr2*$^{+}$ and *norpA*), the rhabdomeres were retained in *norpA;;arr2*$^{3K/Q}$ (Figure 5B). Though the morphology of the rhabdomeres was not wild-type, there

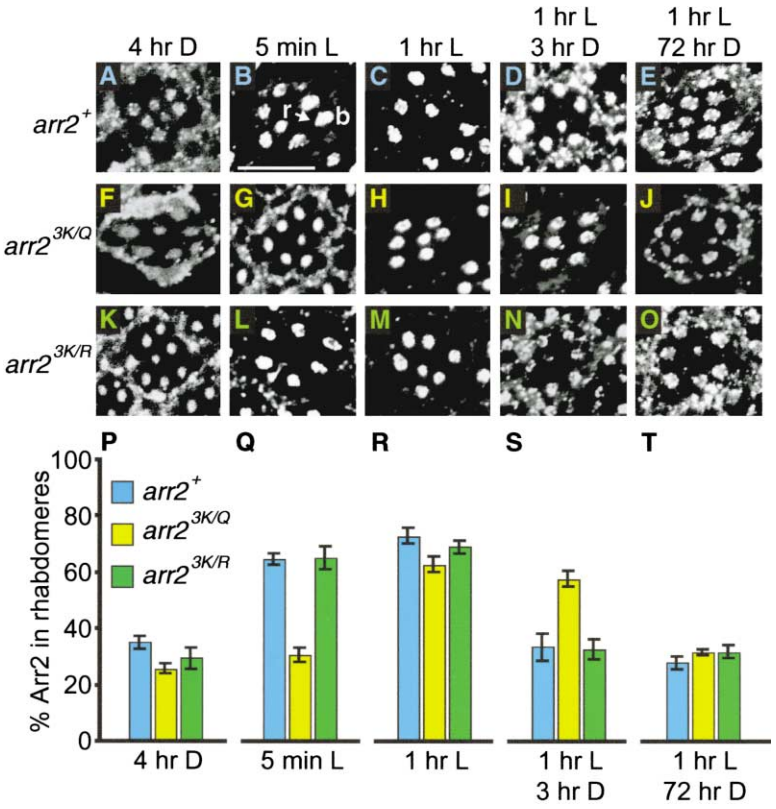

Figure 4. Light-Dependent Translocation of Arr2 in Transgenic Flies

Tangential sections of adult compound eyes shown were obtained from *arr2*$^{+}$, *arr2*$^{3K/Q}$, and *arr2*$^{3K/R}$ white-eyed flies ≤2 days old. The flies were reared under a normal light/dark cycle and then either dark-adapted for 4 hr (A, F, and K), exposed to 5 min blue light (B, G, and L), 1 hr blue light (C, H, and M), 1 hr blue light followed by 3 hr in the dark (D, I, and N), or 1 hr blue light followed by 72 hr in the dark (E, J, and O). Staining was performed with anti-Arr2 antibodies. Scale bar, 10 μm. (P–T) Quantification of Arr2 staining in the rhabdomeres from (A)–(O) (see Experimental Procedures). SEMs were based on examination of 10–12 ommatidia.

was much greater suppression of the *norpA* degeneration by *arr2*$^{3K/Q}$ than by the *arr2*^{5} null mutation (Figure 5B and Supplemental Figure S3 available online). Collectively, the results presented here and in previous sections suggest that Arr2$^{3K/Q}$ suppresses retinal degeneration in *norpA* due to a decrease in endocytosis of rhodopsin/Arr2 complexes.

Requirement for Arr2/PI Interaction for Light Adaptation

A key question concerns the normal physiological role of the light-dependent trafficking of Arr2. To assay the light response, we used electroretinogram (ERG) recordings, which measure the summed light responses of all retinal cells. Using a conventional paradigm, the PDA, *arr2*$^{3K/Q}$ flies did not display a defect in activation or termination of the photoresponse (Supplemental Figure S4 at http://www.neuron.org/cgi/content/full/39/1/121/DC1).

To address whether there was a defect in some aspect of the photoresponse in *arr2*$^{3K/Q}$, we compared ERGs in wild-type and transgenic flies under white-light conditions similar to those that induce movement of Arr2 from the cell bodies to the rhabdomeres (Supplemental Figure S6 available online). We found that preexposure to light increased the speed of termination of the ERG response in wild-type flies. The termination of the light response, which was relatively slow in flies that had been dark adapted for 4 hr, was much faster after a 10 min preexposure to light and further accelerated after a 1 hr light treatment (Figures 6A and 6B; $t_{80\%}$ = the time for an 80% return to baseline after cessation of the light stimulus: dark, 2.01 ± 0.24 s; 10 min light, 0.83 ± 0.06 s; 1 hr light, 0.64 ± 0.06 s). This long-term adaptation phenomenon correlated with the time course of the light-dependent translocation of Arr2 to the rhabdomeres. Furthermore, preexposure to light had very little impact on the rate of termination of the ERG in *arr2*^{5} null mutant flies (Figures 6A and 6B).

If the termination of the light response is accelerated by an increase in concentration of rhabdomeral Arr2, then there should be a defect in termination in *arr2*$^{3K/Q}$, as these flies show a decrease in the rate of light-dependent shuttling to the rhabdomeres. Consistent with this proposal, we found that *arr2*$^{3K/Q}$ flies displayed slower termination of the ERG response than wild-type flies. This component of the light response was nearly as slow in *arr2*$^{3K/Q}$ flies maintained in the dark as similarly treated *arr2*^{5} null flies (Figures 6A and 6B; dark $t_{80\%}$: *arr2*$^{3K/Q}$, 3.19 ± 0.23 s; *arr2*^{5}, 3.90 ± 0.30 s). Following a 10 min preexposure to light, the rate of termination of the ERG increased in *arr2*$^{3K/Q}$ flies but was much slower than 10 min light-adapted *arr2*$^{+}$ or *arr2*$^{3K/R}$ (Figure 6C; 10 min light $t_{80\%}$: *arr2*$^{3K/Q}$, 1.84 ± 0.05 s; *arr2*$^{+}$, 0.83 ± 0.06 s; *arr2*$^{3K/R}$, 0.92 ± 0.12 s). Termination of the photoresponse was almost as slow in these *arr2*$^{3K/Q}$ flies as that observed in dark-adapted *arr2*$^{+}$ or *arr2*$^{3K/R}$ (Figures 6A and 6B; $t_{80\%}$: 10 min light *arr2*$^{3K/Q}$, 1.84 ± 0.05 s; dark *arr2*$^{+}$, 2.01 ± 0.24 s; dark *arr2*$^{3K/R}$, 2.33 ± 0.26 s). After 1 hr light stimulation, rapid termination was restored in *arr2*$^{3K/Q}$ (Figures 6A and 6B; 0.79 ± 0.03 s). Since Arr2$^{3K/Q}$ was concentrated in the rhabdomeres by similar light conditions, rapid response termination correlated with enrichment of Arr2 in the rhabdomeres. These results indicated that

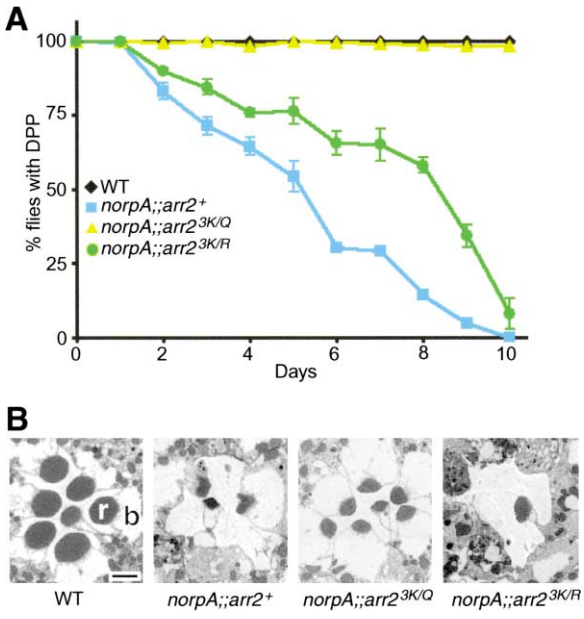

Figure 5. Suppression of Retinal Degeneration in *norpA* by $arr2^{3K/Q}$

(A) The time course of retinal degeneration assayed in live flies by examining the deep pseudopupil (DPP). The DPP is a pattern of dark spots on the surface of the fly eye which results from the optical superposition of rhabdomeres from neighboring ommatidia (Franceschini and Kirschfeld, 1971). The integrity of the DPP is directly correlated with proper structure of the ommatidia. Shown are the fraction of flies with DPPs as a function of age (days). The flies were exposed to a normal dark/light cycle. At least 40 flies were examined three times per day. The error bars represent the SEMs. (B) Transmission EMs of cross sections of compound eyes at a depth of 30 μm. The indicated flies in a white-eyed background were reared for 2 weeks under a 12 hr light/12 hr dark cycle, and samples were prepared as described (Lee and Montell, 2001). r, rhabdomere; b, cell body. Scale bar, 2 μm.

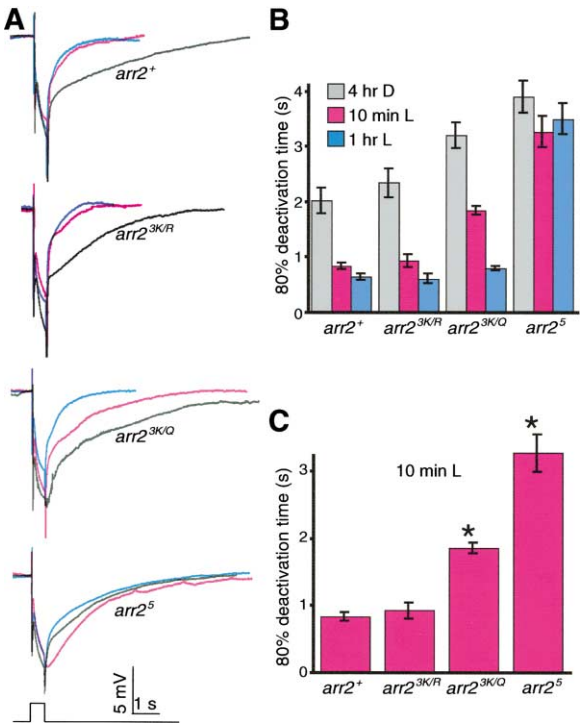

Figure 6. Requirement of PI/Arr2 Interaction for Long-Term Adaptation

(A) ERGs of transgenic flies. White-eyed $arr2^{+}$, $arr2^{3K/Q}$, $arr2^{3K/R}$, and *arr2* flies ≤2 day old were exposed to a 0.5 s pulse of orange light (indicated by the event marker below the ERGs). Before performing the ERGs, the flies were either maintained in the dark for 4 hr (4 hr D; black traces), or dark adapted ≥4 hr followed by an exposure to white light for 10 min (10 min L; red traces), or dark adapted followed by a 1 hr pulse of white light (1 hr L; blue traces). (B and C) Time required for an 80% recovery after termination of the light response in (A). Fifteen to twenty-five flies were examined, and the error bars represent the SEMs. (*$p < 0.0001$, Student's unpaired t test.)

Arr2 trafficking was necessary for the light-dependent increase in the rate of termination of the photoresponse.

Disruption of PTEN Expression Caused Defects in Arr2 Trafficking and Light Adaptation

The analysis of $Arr2^{3K/Q}$ indicates that PIs have a role in regulating the light-dependent movements of Arr2, which participate in light adaptation. Based on the in vitro binding data, a candidate PI, which may regulate Arr2, is PIP_3. If so, then mutations that alter the levels of PIP_3 should affect Arr2 translocation and long-term adaptation. PIP_3 is generated by the phosphorylation of $PIP(4,5)P_2$ by PI3-kinase and is metabolized by PTEN, which is a PI3-phosphatase (Figure 1A). Therefore, mutations in both enzymes should alter the PIP_3 levels in vivo, as appears to be the case in *dPTEN* mutant flies (Stocker et al., 2002). However, overexpression or loss-of-function mutations in the major *Drosophila* PI3-kinase caused severe degeneration or developmental defects, respectively (see legend to Supplemental Figure S5 at http://www.neuron.org/cgi/content/full/39/1/121/DC1).

We then examined Arr2 trafficking in PTEN null mutant flies (Stocker et al., 2002) and PTEN overexpression flies (Huang et al., 1999) (*PTEN oe*: *hs-Gal4/UAS-PTEN*). Since the PTEN null mutation results in lethality and the morphology of the photoreceptor cells is abnormal in PTEN mosaic eyes, we took advantage of the finding that *dPTEN* null mutant flies are viable, in combination with a mutation affecting the protein kinase dAKT (Stocker et al., 2002). Moreover, *dPTEN;dAkt* photoreceptors display normal morphology and an ERG similar to wild-type, using a normal PDA paradigm (Figures 7E–7H and Supplemental Figure S4E at http://www.neuron.org/cgi/content/full/39/1/121/DC1).

We found that an increase or decrease in the expression of PTEN caused reciprocal defects in shuttling of Arr2 to and from the rhabdomeres, respectively. The *dPTEN;dAkt* flies showed normal rhabdomere localization of Arr2 after a 5 min stimulation with blue light (Figures 7F and 7N). However, Arr2 shuttling to the cell bodies seemed to be impaired, since the majority of Arr2 remained in the rhabdomeres after a 1 hr exposure to blue light, followed by incubating in the dark for either 3 or 72 hr (data not shown; Figures 7H and 7P). Under the same conditions in wild-type flies, Arr2 was detected throughout the cell bodies and rhabdomeres (Figures 4D, 4E, 4S, 4T, 7D, and 7P). In contrast to these results, *PTEN oe* flies showed diffuse Arr2 staining in the cell bodies and rhabdomeres after a 5 min blue light stimulus

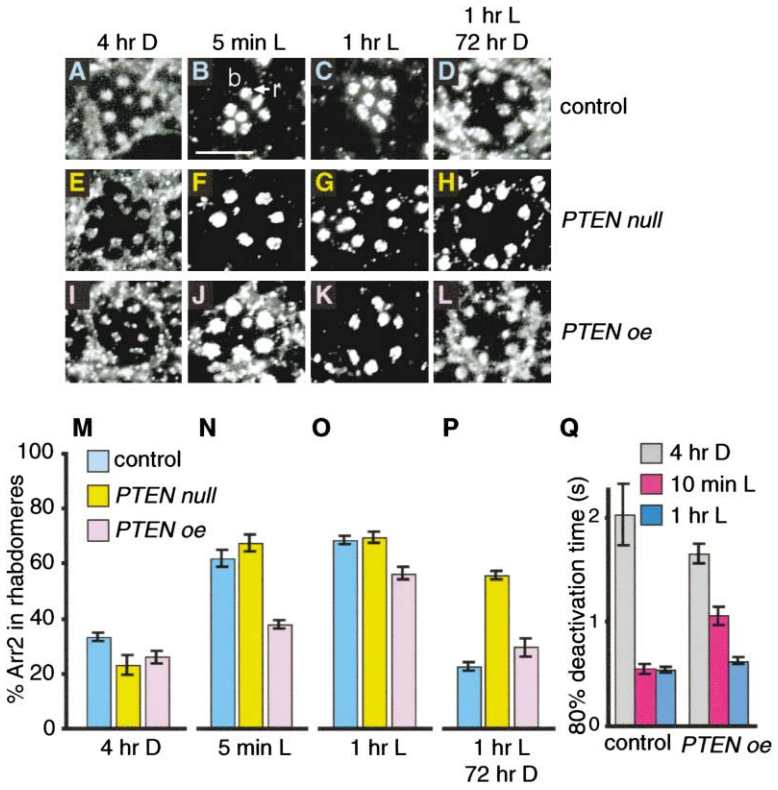

Figure 7. Intracellular Movement of Arr2 and Light Adaptation in Flies with Altered Expression of PTEN

All of the sections were obtained from white-eyed flies (either *w* or *cn bw*). (A–D) Tangential sections of compound eyes obtained from *hs-Gal4/TM3* flies (control). (E–H) Sections from *dPTEN;dAkt* flies (*PTEN null*). (I–L) Sections from *hs-Gal4/UAS-PTEN* flies overexpressing PTEN (*PTEN oe*). Sections were prepared from flies reared under a normal light/dark cycle and then either dark adapted for 4 hr (A, E, and I), exposed to blue light for 5 min (B, F, and J), 1 hr blue light (C, G, and K), or 1 hr blue light followed by 72 hr in the dark (D, H, and L). The sections were stained with anti-Arr2 antibodies. Scale bar, 10 μm. (M–P) Quantification of the Arr2 staining in the rhabdomeres from (A)–(L). SEMs were based on results from 10 to 12 ommatidia. (Q) Time required for an 80% recovery after termination of the light response. Wild-type control (*hs-Gal4/TM3*) and *PTEN oe* flies were reared under a light/dark cycle and dark adapted for ≥4 hr. Some of the flies were subsequently exposed to white light for either 10 min or 1 hr. ERGs were performed by exposing the flies to a 0.5 s pulse of orange light. Twelve to nineteen flies were examined, and the error bars represent the SEMs.

(Figures 7J and 7N). After stimulation with blue light for 1 hr, Arr2 was concentrated in the rhabdomeres, indicating that the kinetics of trafficking into the rhabdomeres was retarded rather than eliminated in *PTEN oe* (Figures 7K and 7O). Movement of Arr2 to the cell bodies seemed to be nearly normal, since the Arr2 was localized throughout the cell bodies and rhabdomeres after 1 hr in the light followed by either 3 or 72 hr in the dark (data not shown and Figure 7L). Thus, absence of PTEN resulted in impairment in shuttling Arr2 to the cell bodies, and higher PTEN levels caused defects in the light-dependent movement of Arr2 from the cell bodies to the rhabdomeres.

Since light-dependent rhabdomere localization of Arr2 is necessary for the light-induced acceleration in the termination of the ERG, we examined the light responses of *PTEN oe* flies, which showed slow trafficking of Arr2 to the rhabdomeres. We found that the *PTEN oe* displayed a defect in this component of the light response. After exposing dark-adapted flies to a 10 min pulse of light, the termination of the ERG response was much slower in *PTEN oe* than wild-type flies ($t_{80\%}$: 1.05 ± 0.09 s and 0.55 ± 0.05 s, respectively; Figure 7Q). Similar termination rates were observed in wild-type and *PTEN oe* flies after a 1 hr light stimulation (Figure 7Q). These data further support the conclusion that PIs play a crucial role in Arr2 translocation and in light adaptation.

Discussion

Translocation of Arrestin2 Functions in Light Adaptation

A curious but longstanding observation is that visual arrestin undergoes a dramatic light-dependent translocation from the inner segment to the outer segment of rods and cones (Broekhuyse et al., 1985; Philp et al., 1987; Mangini and Pepperberg, 1988; Whelan and McGinnis, 1988). However, the function of this dynamic movement has not been described. *Drosophila* visual arrestin, Arr2, also undergoes light-dependent shuttling between the cell bodies and the phototransducing portion of the photoreceptor cells, the rhabdomeres (Kiselev et al., 2000). This observation provided the potential for using a genetic approach to address two unresolved issues: (1) the mechanisms regulating this movement and (2) the function for this light-driven translocation. Due to the slow time course of the light-induced movements, which occur over a few to many minutes, an interesting possibility is that the trafficking of arrestin could contribute to long-term adaptation, as the concentration of arrestin has been proposed to limit response termination (Ranganathan and Stevens, 1995). One well-known illustration of long-term adaptation is experienced upon entering and leaving a darkened room, such as a movie theater. Interestingly, this delay occurs over a similar time course as the light-dependent translocation of visual arrestins.

Consistent with the proposal that the regulated movement of arrestin contributes to slow adaptation, we found that a reduction in the rate of Arr2 translocation had a major impact on a light-dependent component of the photoresponse. In wild-type, we found that the rate of termination of the photoresponse was significantly faster in flies that have had prior exposure to light; however, this adaptation feature was virtually eliminated in *arr2* null mutant flies. Of particular significance, termination of the ERG response was much slower in flies that had defects in the light-dependent movement of Arr2.

Based on these findings indicating that light-dependent movement of Arr2 contributes to long-term adaptation in *Drosophila*, we propose that dynamic movements of mammalian visual arrestin may contribute to long-term adaptation in humans, in addition to other established mechanisms, such as chromophore regeneration (McBee et al., 2001).

Transducin also undergoes a light-driven translocation between the rod outer and inner segments, and this movement out of the outer segment is correlated with a reduction in the amplitude of the photoresponse (Sokolov et al., 2002). Similar light-dependent movement of the Gqα occurs in fly photoreceptor cells, and this translocation is dependent on the presence of the G$\beta\gamma$ (Kosloff et al., 2003). However, the effect of this translocation on adaptation has not been addressed. Recently, light-regulated translocation of the TRPL channel has been suggested as a novel mechanism for reducing the sensitivity to increasing intensities of light (Bahner et al., 2002). Therefore, *Drosophila* appears to have at least two long-term light-adaptation mechanisms: light-dependent movement of Arr2 into the rhabdomeres for increasing the speed of termination of the photoresponse and light-dependent movement of TRPL into the cell body for enabling the photoreceptor cells to adjust the amplitude of their response to background light.

Regulation of Arrestin2 Movement by PIs

A second central question concerning the light-driven changes in the spatial distribution of Arr2 concerns the underlying mechanism. Several observations support the conclusion that the movements of Arr2, to and from the rhabdomeres, are regulated by PIs. The rate of Arr2 translocation was much slower as a result of alterations in the levels of expression of gene products, such as a PI-transfer protein (RDGB), CDS, and PTEN, that affect the metabolism or distribution of PIs. Furthermore, Arr2 bound to PIs in vitro, and mutations in this binding site caused translocation defects. In addition, endocytosis of β arrestin is mediated in vitro through interaction with PIs, though such a mode of regulation has not been demonstrated in vivo (Gaidarov et al., 1999). In the current report, we provide evidence that the movement of visual arrestin was regulated by PIs in photoreceptor cells in both directions, in and out of the rhabdomeres.

An issue raised by the current experiments is the identity of the PI or IP that interacts with and regulates the movement of Arr2 in vivo. It seems more likely that Arr2 binds to PIs than IPs, since the shuttling defects, which are observed in mutants affecting the PI-transfer protein or *cds*, are similar to those resulting from mutation of the PI/IP binding site in Arr2. PIP_3 is a prime candidate for regulating Arr2, since the IC_{50} is lowest for PIP_3. Furthermore, overexpression of the phosphatase that hydrolyzes PIP_3 (PTEN) results in impairment of Arr2 translocation to the rhabdomeres but not shuttling to the cell bodies. These results support the conclusion that PIP_3 facilitates shuttling of Arr2 to the rhabdomeres but also indicate that PIP_3 does not affect movement of Arr2 from the rhabdomeres to the cell bodies. Given that translocation of Arr2 is disrupted by mutation of the PI binding site in Arr2, we propose that another PI, which remains to be identified, is required for this latter movement. The defect in Arr2 shuttling to the cell bodies in the PTEN null might result from an increased level of PIP_3, which competes with another PI required for movement to the cell bodies.

Dual Role of Phosphoinositides in *Drosophila* Vision

The demonstration that translocation of Arr2 is regulated by PIs addresses a lingering question concerning potential roles of PIs in photoreceptor cells. The *Drosophila* visual transduction cascade is among the most intensively studied GPCR cascades. During the last 30 years, many proteins and mutations have been identified that perturb PI signaling (reviewed in Montell, 1999; Hardie and Raghu, 2001); however, the targets and mechanisms directly regulated by PIs have not been previously described.

The regulation of Arr2 shuttling by PIs occurs on the order of a few to many minutes. This is in contrast to the millisecond time scale, which operates in the activation of phototransduction. Although the specific activation mechanism involved in *Drosophila* phototransduction remains elusive, it is established that it depends on a PLCβ (NORPA) (Bloomquist et al., 1988). Thus, PLC-mediated hydrolysis of PIP_2 leads to rapid activation of the light-sensitive channels through the millisecond generation of PIP_2 metabolites or reduction in PIP_2 levels. Since adaptation occurs over a much slower timescale, regulation of this latter phenomenon exclusively by direct effects of second messengers on protein activities might be too rapid. Rather, regulation of adaptation by the translocation of signaling proteins provides a mechanism whereby changes in second messengers, such as PIP_3, result in delayed effects on the magnitude and the kinetics of signaling. Therefore, PIs appear to have the capacity to serve a dual role in activation and adaptation by modulating the activities and localization of signaling proteins.

Possible Roles of Phosphoinositides in Mammalian Vision

A major unresolved issue in mammalian vision is the function of PIs in rods and cones, since cGMP rather than lipid second messengers mediate activation of mammalian phototransduction. This question arises in part from the observation that several enzymes regulating PIs, including p110 PI3-kinase and DAG kinase, are activated in mammalian photoreceptor cells in a light-dependent manner (Huang et al., 2000; Rajala and Anderson, 2001). Furthermore, rods and cones express homologs of many eye-enriched proteins that function in *Drosophila* phototransduction. These include a PLCβ4 (Ferreira and Pak, 1994) and M-rdgB2 (Lu et al., 1999). However, the functions of these genes in rods and cones have not been identified, despite the generation of mouse knockouts (Jiang et al., 1996; Lu et al., 2001).

We propose that PIs are excellent candidates for regulating the intracellular translocation of mammalian photoreceptor proteins in general and visual arrestin in particular. Consistent with this proposal are the observations that mammalian visual arrestin undergoes a light-dependent translocation (Broekhuyse et al., 1985; Philp et al., 1987; Mangini and Pepperberg, 1988;

Whelan and McGinnis, 1988; Peterson et al., 2003), which appears to occur through an active mechanism rather than via passive diffusion (Mendez et al., 2003). Moreover, mammalian visual arrestin binds to IPs in vitro, although an interaction with PIs was not tested (Palczewski et al., 1991). If mammalian visual arrestin binds PIs, then disruption of PI metabolism may interfere with adaptation in rods and cones, similar to the defects in *arr2*$^{3K/Q}$, *rdgB*, *cds*, and *PTEN* mutant flies. Thus, it would be interesting to reevaluate the *PLC*β*4* and *m-rdgB2* knockout mice for effects on arrestin translocation and light adaptation.

Implications of Suppression of Retinal Degeneration by Disrupting PI/Arr2 Interaction

Previous reports showed that stable Arr2/rhodopsin complex formation leads to retinal degeneration in *norpA* or *rdgC* flies (Alloway et al., 2000; Kiselev et al., 2000). Removal of the *arr2* gene in a *norpA* or *rdgC* background partially suppresses the photoreceptor cell death. This partial suppression could be due to elimination of Arr2/rhodopsin complexes, reduction in endocytosis of rhodopsin, or disruption of some other Arr2 function. In this work, we uncoupled Arr2/rhodopsin binding and PI-regulated trafficking of Arr2 by expressing Arr2$^{3K/Q}$, which is defective in movement but not rhodopsin binding. Since the retinal degeneration in *norpA* was largely rescued in *arr2*$^{3K/Q}$ flies, our results suggest that apoptosis in *norpA* results from endocytosis of Arr2/rhodopsin complexes rather than a defect in Arr2/rhodopsin binding. This conclusion is further supported by the finding that there was even greater suppression of the *norpA* degeneration in an *arr2*$^{3K/Q}$ than in an *arr2*^{5} null background.

It is possible that endocytosis of stable arrestin/rhodopsin complexes may contribute to certain types of retinal degenerations in humans, as appears to be the case in *Drosophila*. If so, the findings in the current report that retinal degeneration is suppressed by interfering with the PI/Arr2 interaction raise the intriguing possibility that application of drugs that suppress PI production in the rods and cones or PI binding to visual arrestin may be an effective approach to suppress certain types of human retinal dystrophies.

Experimental Procedures

Fly Stocks

All of the experiments were performed with white-eyed flies (*w* or *cn bw*). The following strains of *Drosophila melanogaster* were reared at 25°C under a 12 hr light/12 hr dark cycle: (1) *y w*^{67}, (2) *w*^{1118}, (3) *w f*^{1} *rdgB*^{2}, (4) *cn bw; cds*^{1}, (5) *y w;; arr2*^{5}, (6) *w norpA*^{EE5}*;; arr2*^{5}, (7) *y w;; FRT82B Dp110*^{A}*/TM6*, (8) *cn bw; UAS-Dp110*, (9) *y w; cn bw; UAS-Dp110 D954A/TM3*, (10) *y w; dPTEN*^{117}*; dAkt*^{1}*/dAkt*^{3}, (11) *cn bw; UAS-PTEN*^{12}, (12) *cn bw; hs-Gal4*, (13) *w;;G*β*76C*^{1}, and (14) *y w;; arr2* transgenic flies. Twelve to twenty-four hours prior to initiating the experiments, the flies (1–2 days posteclosion) were either maintained in the dark or under light. To overexpress PTEN, we generated *cn bw; hs-Gal4/UAS-PTEN*^{12} flies. The proteins were induced by exposing the flies to 1 hr heat shocks (37°C) at 24 hr intervals for 5 days. The control flies for these latter experiments (*cn bw; hs-Gal4/TM3*) were exposed to the same heat shock protocol.

Immunolocalizations and Quantitative Analysis

Unless otherwise noted, the immunolocalizations were performed on young (<2 day old) flies, which were dark adapted for ≥4 hr and subsequently treated with light or maintained in the dark. The immunostainings shown in Figures 1, 4, 7, and Supplemental Figures S1 and S5 (in the Supplemental Data available at http://www.neuron.org/cgi/content/full/39/1/121/DC1) were performed using blue light (~5 mW/cm^2) and the immunostainings presented in Supplemental Figure S6 were obtained using white light (~30 mW/cm^2). Fly heads were hemisected under a dim red photographic safety light, fixed with paraformaldehyde, and embedded in LR White resin as described (Porter and Montell, 1993). Cross sections (0.5 μM) of the compound eyes were obtained from the distal (R7) region of the retina and stained with primary antibodies (diluted 1:250 to 1:1000) and FITC-labeled secondary antibodies (diluted 1:50) as described (Porter and Montell, 1993). We had previously prepared antibodies to INAD (Wes et al., 1999) and TRP (Chevesich et al., 1997) in rabbits. Anti-Rh1 is a mouse monoclonal (4C5: the Developmental Studies Hybridoma Bank), and anti-Arr2 was a gift from Dr. S. Subramanium.

Quantification of the relative concentration of Arr2 in the rhabdomeres and cell bodies was determined using the "Segmentation and Measure Segment" function in the IP Lab Spectrum program. The area (*A*) and mean fluorescence intensity (*I*) in the rhabdomeres (*r*), whole ommatidia (*o*), and background (*b*) in the extracellular regions, such as the central matrix, were determined. The integrated optical density (*IOD*) in the rhabdomeres and whole ommatidia were calculated using the formula $IOD_r = (I_r - I_b) \times A$ and $IOD_o = (I_o - I_b) \times A$, respectively (Pelletier et al., 2000). The percentages of Arr2 in the rhabdomeres were calculated using the formula: $100 \times IOD_r/IOD_o$. Each data set was based on 10 to 12 ommatidia selected from at least three different sections.

Inositol Polyphosphate Binding Assay

Binding of [^{3}H]IP$_6$ to the recombinant proteins and the competition assays using unlabeled PIs was performed essentially as described (Gaidarov et al., 1996). In brief, purified GST-Arr2 fusion proteins (pGEX2TK; Pharmacia) were incubated at 4°C for 15–20 min in 100 μl of 25 mM Tris-HCl (pH 7.4), 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mg/ml γ-globulin (Sigma), 0.01 μCi of [^{3}H]IP$_6$ (NEN), and various concentrations of unlabeled competitors as indicated in the figure legends. The samples were then mixed with ice-cold 30% (w/v) PEG-8000 to yield a final 20% concentration and incubated on ice for 10 min. After centrifugation at 4°C for 10 min at 14,000 rpm, the supernatants were aspirated, the pellets were dissolved in 200 μl of 1% SDS, and the radioactivity was counted in 10 mls of a BCS scintillation mixture (Amersham Pharmacia). To determine the level of nonspecific binding of [^{3}H]IP$_6$ to the recombinant proteins, we performed assays in the presence of 100 μM unlabeled IP$_6$.

Nitrocellulose Phospholipid Binding Assays

PIP-Strips (Echelon Research Laboratories) spotted with 100 pmol of various lipids were blocked with 3% fatty acid-free BSA (Sigma A-6003) in TBS-T (50 mM Tris [pH 7.5], 150 mM NaCl, 0.5% Tween-20) and incubated with purified recombinant proteins (1 μg/ml) for 2 hr at 4°C in TBS-T (1 M NaCl). The membranes were washed three times in TBS-T (150 mM NaCl), incubated with anti-GST antibodies (1:1000 dilution; Amersham Pharmacia) in TBS-T (150 mM NaCl) for 2 hr at 4°C, and washed three times in TBS-T (150 mM NaCl) and incubated with horseradish peroxidase-coupled anti-goat IgG (1:1000 dilution; Boehringer Mannheim) in TBS-T (150 mM NaCl) for 2 hr at 4°C. After washing three times in TBS-T (150 mM NaCl) and once in TBS (50 mM Tris [pH 7.5]; 150 mM NaCl), the levels of proteins bound to membranes were determined using an enhanced chemiluminescence Western blotting detection reagent (Perkin-Elmer) and exposing the membranes to X-ray film.

PIP$_3$ Bead Binding Assay

Arr2^{WT}, Arr2$^{3K/Q}$, Arr2$^{3K/R}$, and the C-terminal region of RDGC (Lee and Montell, 2001) were labeled with [^{35}S]-methionine by coupled transcription/translation (TNT kit; Promega) after subcloning the corresponding cDNAs in pcDNA3. Five microliters of the probe alone (Figures 2D, 3D, and 3E) or together with the competitors (IP$_6$ or PIP$_3$; Figure 2E) were mixed with PIP$_3$ beads (40 μl slurry; Echelon Research Laboratories) or control beads in 200 μl of TBS-T buffer and 1× c[l]mplete protease inhibitor cocktail (Roche). After a 2 hr

incubation at 4°C, the beads were pelleted by centrifugation and washed three times with TBS-T. The washed beads were pelleted, and 40 μl of 2× SDS sample buffer was added. The eluted proteins were resolved by SDS-PAGE, and the gels were dried and exposed to BioMax MR film (Kodak).

Structural Modeling

The Arr2 structural model was prepared using the bovine visual arrestin structure as a template (Hirsch et al., 1999). The template PDB file was imported into program O (Jones et al., 1991), and residues of visual arrestin were replaced by those of the target (Arr2) based on an initial sequence alignment. The conformations of the side chains of residues in the binding site were adjusted to avoid steric clashes. The Quanta program (MSI Inc.) was used for docking IP_4 to Arr2 and for energy minimization. Figures 3A and 3B were prepared with VMD (Humphrey et al., 1996). Electrostatic surfaces were calculated with GRASP (Nicholls et al., 1991).

Generation of Transgenic Flies

Genomic DNA was prepared from *y w* flies, and a 5 kb fragment of Arr2 DNA was amplified by PCR and subcloned into pCRScript (Stratagene). The 3K/Q and 3K/R mutations were introduced by the QuikChange method (Stratagene). XhoI and NotI wild-type and the mutant genomic fragments were excised from pCRScript vectors and introduced into the *yellow* C4 transformation vector (gift of S. Patten and P. Geyer). The constructs were injected into the embryos of *y w;;arr2*^{5}, and transformants were identified on the basis of y^+ body color.

Electroretinogram Recordings

Electroretinogram (ERG) recordings were performed as described (Lee and Montell, 2001), using white-eyed flies fixed on microscope cover glasses under a dim red safety light.

Transmission Electron Microscopy

Transmission EM was performed as described (Lee and Montell, 2001), using hemisected fly heads, fixed in a buffered paraformaldehyde and glutaraldehyde solution and embedded in LR White resin. The tangential sections were cut at a depth of 35 μm from the surface of the eyes.

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