Phosphoinositide 3-Kinase Signaling in Retinal Rod Photoreceptors

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PURPOSE. Phosphoinositide 3-kinase (PI3K) consists of a p110 catalytic protein and a p85 α regulatory protein, required for the stabilization and localization of p110-PI3K activity. The biological significance of PI3K was investigated in vertebrate rod photoreceptors by deleting its regulatory p85 α protein and examining its role in photoreceptor structure, function, and protein trafficking.

METHODS. Mice that expressed Cre recombinase in rods were bred to mice with a floxed p85 α (*pik3r1*) regulatory subunit of PI3K to generate a conditional deletion of *pik3r1* in rods. Functional and structural changes were determined by ERG and morphometric analysis, respectively. PI3K activity was measured in retinal homogenates immunoprecipitated with an anti-PY antibody. Akt activation was determined by Western blot analysis with a pAkt antibody.

RESULTS. Light-induced stress increased PI3K activity in retinal immunoprecipitates and phosphorylation of Akt. There was no effect of *pik3r1* deletion on retinal structure. However, twin flash electroretinography revealed a slight delay in recovery kinetics in *pik3r1* knockout (KO) mice compared with wild-type controls. The movement of arrestin in the *pik3r1* KO mice was slower than that in the wild-type mouse retinas at 5 minutes of exposure to light. At 10 minutes of exposure, the ROS localization of arrestin was almost identical between the wild-type and *pik3r1* KO mice.

CONCLUSIONS. The results provide the first direct evidence that rods use PI3K-generated phosphoinositides for photoreceptor function. The lack of phenotype in *pik3r1* KO rod photoreceptors suggests a redundant role in controlling PIP₃ synthesis. (*Invest Ophthalmol Vis Sci.* 2011;52:6355–6362) DOI:10.1167/iov.10.7138

The expression and activity of phosphoinositide 3-kinase (PI3K) and the formation of PI3K-generated D3 phosphoinositides have been demonstrated in intact retinal rod outer segment (ROS) membranes.^{1–4} To date, studies have implicated D3 phosphoinositides in a variety of cellular activities

such as vesicular trafficking, cytoskeletal reorganization, cell growth, adhesion, and survival,^{4,5} as well as photoreceptor-specific functions such as modulation of phototransduction,⁶ disc biogenesis,⁷ protein translocation,⁸ synaptic ribbon formation, and glutamate release.⁹ These studies clearly demonstrate that phosphoinositides generated by PI3K could facilitate intracellular protein trafficking and modulate phototransduction. However, the functional role of PI3K in any of these activities in rod photoreceptors is not fully established. In adult retina, our previous *in vivo* studies have shown that moderate light exposure activates PI3K/Akt survival signaling pathway in rod photoreceptor outer segments through light-induced tyrosine phosphorylation of the retinal insulin receptor (IR).^{10,11} Also, in retina, deletion of the IR leads to photoreceptor degeneration due to increased susceptibility to damage from light-induced stress, in part because of the inability to activate the PI3K/Akt survival pathway.¹² Receptor activation of PI3K has been shown to protect 661W,^{13,14} R28,¹⁵ and retinal ganglion cells¹⁶ from stress-induced cell death. These findings demonstrate the biological significance of the PI3K signaling pathway in neuroprotection of retinal photoreceptor cells.

Systemic deletion of p85 α regulatory or p110 α catalytic subunits of PI3K results in embryonic or neonatal lethality.^{17,18} To circumvent the problems associated with the global PI3K KO and to investigate functional significance of PI3K in a particular tissue or subset of cells, several studies have used the Cre-lox technology. Conditional deletion of cardiac *pik3r1* resulted in attenuated Akt signaling, reduced heart size, and altered cardiac gene expression.¹⁹ Conditional deletion of *pik3r1* in skeletal muscle resulted in reduced muscle weight and size.²⁰ The functional role of PI3K in the retina or photoreceptor cells is not known, although earlier studies from our laboratory have suggested its involvement in neuroprotection through the IR/PI3K/Akt signaling pathway.^{21–23} In the present study, using Cre-lox technology, we conditionally deleted the p85 α (*pik3r1*) regulatory subunit of PI3K in rod photoreceptors to evaluate the functional significance of PI3K in these cells.

MATERIALS AND METHODS

Polyclonal anti-p85 α antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal anti-Akt and anti-pAkt (Ser473) antibodies and LY294002, a pan-PI3K inhibitor, was purchased from Cell Signaling (Beverly, MA). Polyclonal anti-Cre antibody suitable for Western blot analysis was purchased from Novogene (Darmstadt, Germany) and monoclonal anti-Cre antibody suitable for immunohistochemistry was purchased from Abcam (Cambridge, MA). Robert S. Molday (University of British Columbia, Vancouver, Canada) kindly provided monoclonal 1D4 anti-rhodopsin antibody. Monoclonal anti-PY99 (anti-phosphotyrosine), polyclonal anti-p85 β , and polyclonal anti-rod transducin α (T α) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-arrestin antibody was a gift from Paul Hargrave (University of Florida, Gainesville). Monoclonal anti- β -actin antibody was purchased from Affinity BioReagents

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(Golden, CO). DAPI used for nuclear staining was purchased from Invitrogen (Eugene, OR). [γ - 32 P] ATP was purchased from Perkin Elmer Life Sciences (Shelton, CT). Phosphatidylinositol-4,5-bisphosphate (PI-4,5-P₂) was purchased from Echelon Research Laboratories, Inc. (Salt Lake City, UT). All other reagents used were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO).

Animals

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the NIH Guide for the Care and Use of Laboratory Animals. Protocols used were approved by the IACUC at Oklahoma University Health Sciences Center (OUHSC) and Dean A. McGee Eye Institute (Oklahoma City, OK). Animals were born and raised in our vivarium and kept under dim cyclic light (40–60 lux, 12-hour light/dark cycle for mice and 5 to 10 lux, 12-hour light/dark cycle for rats).

Generation of Rod Photoreceptor-Specific p85 α Knockout Mice

Mice expressing Cre recombinase under the control of the mouse rod opsin gene promoter²⁴ were cross-bred with the p85 α floxed mice (kindly provided by Lewis Cantley, Harvard Medical School, Boston, MA). The obtained mice were heterozygous for the p85 α (*pik3r1*) floxed allele. To create photoreceptor-specific *pik3r1* KO mice, we bred floxed p85 α mice carrying the *cre* transgene with p85 α floxed homozygous mice (backcross). The 2.6-kb fragment of the mouse *pik3r1* gene containing exon 7 was flanked with *loxP* sites, enabling deletion of all three p85 α isoforms (p85, p55, and p50) as previously described.¹⁹ Generated transgenic mice were genotyped for the mouse rod opsin promoter, Cre and the floxed p85 α regulatory subunit of PI3K, using PCR screening of tail DNA. For Cre genotype screening, a forward primer AGG TGT AGA GAA GGC ACT TAG designed within the promoter DNA sequence and reverse primer CTA ATC GCC ATC TTC CAG CAG G designed within the Cre recombinase DNA sequence were used to amplify a PCR product of 411 bp. To distinguish the p85 α floxed allele from the wild-type p85 α allele, we used the primer pairs CAC CGA GCA CTG GAG CAC TG and CCA GTT ACT TTC AAA TCA GCA CAG to amplify a 252-bp fragment from the wild-type p85 α allele and a 301-bp fragment from the floxed p85 α allele.

Rhodopsin Photobleaching

The photobleaching of rhodopsin was measured according to a published method.²⁵ The mice were dark-adapted overnight and killed in the morning, and the eyes were removed under dim red light. The eyes of each mouse were homogenized in 200 μ L of buffer (10 mM Tris [pH 7.4], 100 mM NaCl, and 1 mM EDTA) containing 2% *N*-octyl- β -D-glucopyranoside (Calbiochem, La Jolla, CA) and 50 mM of freshly prepared hydroxylamine. The suspension was centrifuged at 17,000g for 5 minutes and the supernatant was collected. The volume was measured for each sample, and the clear supernatant was scanned from 200 to 800 nm (Ultrospec 3000; Amersham Biosciences, Cambridge, UK), after which the samples were bleached by exposure to desk lamp light for 5 minutes and scanned again. The difference in absorption at 500 nm was used to determine the concentration of rhodopsin using a molar extinction coefficient of 42,000. The amount of rhodopsin was expressed as picomoles per eye.

Twin-Flash Electrophoretography

Twin-flash ERG was used to examine recovery kinetics in rods of our p85 α KO mice. Briefly, dark-adapted animals were analyzed by ERG consisting of five steps with two components, an initial flash and a second flash. Interstimulus intervals (ISIs), the time between the initial and second flash, were increased from step 1 to step 5 (1, 2, 4, 16, and 150 seconds). Between each step, the animals were dark adapted for 3 minutes, to allow for maximum recovery, as estimated in comparison to wild-type controls. Intensities of the flashes were kept constant at 2.3 log cd $\cdot$ s/m², which is

sufficient to bleach less than 0.3% of the rhodopsin (reviewed in Ref. 26). The amplitude of the a-wave was measured from baseline to the a-wave trough. The amplitude of the b-wave was measured from the trough of the a-wave to the peak of the b-wave.

Statistical Analysis

All values are presented as $\pm$ SEM. One-way ANOVA and post hoc statistical analysis using the Bonferroni pair-wise comparison were used to determine the significance of the difference. Only differences with $P < 0.05$ were considered statistically significant.

Other Methods

Preparation of whole-mounted bovine rod outer segments and immunolabeling was performed according to a published method.¹⁰ Photoreceptor outer segments were prepared by using a discontinuous sucrose gradient. PI3K activity was assayed on anti-PY99 immunoprecipitates,¹⁰ and the structure of *pik3r1* KO and control retinas was examined after tissue fixation and sectioning.²⁷

RESULTS

Expression of p85 α Regulatory Subunit of PI3K in Rod Photoreceptor Outer Segments

Commercially available anti-p85 α antibodies are not suitable for immunohistochemical analysis of cellular p85 α expression and localization in fixed tissues. To circumvent this technical problem, we examined the expression of p85 α in isolated, whole-mounted bovine rod photoreceptors by immunofluorescence microscopy. To identify rod photoreceptors, we co-labeled flat mounts with anti-rhodopsin and anti-p85 α antibodies (Figs. 1A–C). The localization of p85 α (Fig. 1A, green) appeared to be present predominantly in the ellipsoid regions of the rod inner segments, but was also present in the outer segments and in the region containing the connecting cilium. Rod photoreceptor outer segment membranes were labeled by the anti-rhodopsin antibody (Fig. 1B, red). Merged images are shown in Figure 1C. We verified the presence of the p85 α in the ROS by Western blot analysis of isolated ROS membranes. ROS and inner segment enriched band II retinal membranes were prepared from rat retinas by discontinuous sucrose gradient centrifugation. Western blot analysis of the two preparations were probed with antibodies to the $\alpha 3$ isoform of Na⁺K⁺-ATPase, p85 α , and opsin (Fig. 1D). The $\alpha 3$ isoform, found in the inner (but not the outer) segments,¹⁰ was absent from rat ROS, but was present in band II. Similar blots probed with the anti-p85 α and anti-opsin antibodies indicated the presence of p85 α and opsin in ROS and band II (Fig. 1D). It is interesting to note that the expression of p85 α is more in ROS than in band II, and the result of p85 α immunolabeling in isolated rods shows the opposite pattern (Figs. 1A–C). These observations suggest that p85 α could be more soluble in inner segments than in ROS, as the method we used is a discontinuous sucrose gradient centrifugation that yields membranes. These results provide strong evidence that p85 α is present in rod photoreceptors.

Light-Induced Stress Activation of PI3K and Akt In Vivo

To determine whether the stress of exposure to light induces the activation of PI3K, we exposed the rats to light for 3 hours at 5000 lux²⁷ and immediately thereafter prepared retinal lysates from dark control or light-stressed rats, immunoprecipitated the tyrosine-phosphorylated proteins, and measured the PI3K activity. The PI3K activity associated with the anti-PY-99 immunoprecipitates in light-stressed retinas was greater than that from the dark-adapted controls (Figs. 1E, 1F), suggesting

FIGURE 1. Expression p85 α in bovine rod photoreceptor cells. (A) Immunolocalization of p85 α (green) in intact ROS (faint and punctuate staining), connecting cilium, and ROS. (B) Anti-rhodopsin antibody (red) was localized to outer segment membranes. Merged images are shown in (C). (D) Expression of p85 α regulatory subunit of PI3K in rod photoreceptor outer segments. ROS and band II were immunoblotted with anti-p85 α , anti- α 3-Na⁺/K⁺-ATPase, and anti-rhodopsin antibodies. Light stress (LS) stimulated phosphotyrosine protein-associated PI3K activity (E). Dark and light-stressed (5000 lux for 3 hours) retinas were lysed, immunoprecipitated with anti-PY99 antibody, and PI3K activity measured using PI-4,5-P₂ and [γ -³²P]ATP as substrates. The radioactive spots of PI-3,4,5-P₃ shown in (E) were scraped from the thin layer chromatography plate and counted (F). Data are the mean $\pm$ SD ($n = 3$), * $P < 0.05$. Representative Western blots show the presence of p85 α (G), pAkt (Ser473) (H), Akt (I), and β -actin (J) in the retinal lysates. Quantitative histogram of three independent experiments where pAkt (Ser473) levels on the Western blot analysis were normalized to Akt (K). Data are the mean $\pm$ SD, $n = 3$, * $P < 0.05$. OS, outer segment; CC, connecting cilium; IS, inner segment.

that exposure to light induces the activation of PI3K by binding to the activated growth factor receptors.

To determine whether exposure to light induces the activation of Akt, proteins from control and light-stressed rats were immunoblotted with anti-pAkt (Ser473), anti-Akt, anti-p85 α , and anti- β -actin antibodies. Light-induced stress resulted in the activation of Akt in the retina (Fig. 1H), as evidenced by the increased immunoreaction compared with the dark-adapted controls, quantified in Figure 1K. Total Akt and p85 α were not affected by light-induced stress (Figs. 1G, 1I). The blot was reprobed for β -actin to ensure equal amounts of protein in each line (Fig. 1J). These experiments suggest that light-induced stress activates Akt in vivo.

Generation of Rod-Specific p85 α KO Mice

Mice lacking the p85 α regulatory subunit die a few days after birth and disruption of the PI3K catalytic subunit (p110 α) is lethal in utero.^{17,18} Therefore, it is not possible to analyze retinal phenotypes in adult global KO mice. To circumvent these problems and to investigate the functional significance of PI3K in rods, we generated rod photoreceptor-specific *pik3r1* KO mice using Cre-lox technology (Fig. 2A). We assessed Cre recombinase expression and cellular localization in the retinas of wild-type and KO littermates by immunoblot analysis and immunofluorescence microscopy using an anti-Cre antibody. Cre expression was localized to rod photoreceptor nuclei in the conditional *pik3r1* KO retinas, whereas Cre recombinase staining was not detected in wild-type

controls (Fig. 2B). We also assessed Cre expression in the retinas of wild-type and KO littermates by immunoblot analysis. As expected, Cre expression was not observed in retinal lysates from wild-type controls (Fig. 2C). β -Actin expression was used as the loading control (Fig. 2C). These experiments suggest that Cre expression was present only in the rod photoreceptors. To evaluate the efficiency of Cre-mediated deletion of the p85 α in rod photoreceptors, RO and band II membranes were prepared from the conditional *pik3r1* KO mice and wild-type controls and subjected to Western blot analysis. ROS from conditional *pik3r1* KO mice had approximately 3% to 4% of p85 α protein content compared with that from wild-type controls (Figs. 2D, 2E). The expression of p85 β remained constant between conditional *pik3r1* KO and wild-type mice (Fig. 2D). Rhodopsin expression was used as a loading control in this experiment. These results indicate that mouse opsin-driven Cre successfully deleted most of the *pik3r1* gene expression in rod photoreceptor cells.

Effect of Deletion of p85 α in Rod Photoreceptor Cells

Light microscopic examination of the retinas from wild-type, heterozygous, and conditional KO mice at 1 month of age showed no difference in retinal structure among the three groups when each group was maintained in dim cyclic light (Figs. 3A–C). The retinas appeared normal and the ROS appeared to be well organized. There were 11 to 12 rows of

FIGURE 2. Generation of rod-specific p85 α KO mice. Rod photoreceptor-specific deletion of *pik3r1*, a pan-p85 α regulatory subunit of PI3K, was accomplished by cross-breeding floxed *pik3r1* mice to 0.2-kb mouse opsin promoter-controlled, rod-specific Cre mice (A). Primer pairs P1 and P2 were used to identify the wild-type and the floxed p85 α alleles. (B) Immunostaining of retinal sections from conditional *pik3r1* KO mice revealed that Cre was localized to rod nuclei (red) and was not present in wild-type control littermates. (C) Western blot analysis showed that Cre was expressed in the retinal protein extracts of conditional *pik3r1* KO mice, but not in the wild-type controls. β -Actin was used as a loading control. (D) Western blot analysis of p85 α and p85 β protein expression in ROS from conditional *pik3r1* KO mice and wild-type outer segment protein extracts. The p85 α levels were normalized to rhodopsin (E) and the results show that the p85 α levels in KO retinas were significantly reduced compared with wild-type littermates. OS, outer segment; IS, inner segment; ONL, outer nuclear layer.

photoreceptor nuclei in the outer nuclear layer (ONL), the number usually observed in rodents without retinal degeneration.²⁸ Quantitative analysis of the superior and inferior regions of the ONL showed no significant differences in the average ONL thickness measured at 0.25-mm intervals from the ONL to the inferior and superior ora serrata in the three groups (Fig. 3D), indicating that rod photoreceptor viability was not different among these mice at the ages examined.

Previous studies from our laboratory have shown that global deletion of Akt2²⁷ or conditional deletion of BCL-xL,²⁹ and the insulin receptor¹² in rod photoreceptors do not affect rod structure or function. However, in all three cases, the animals with deletions were more susceptible to light-induced damage than were the wild-type controls born and raised in identical conditions. Contrary to these findings, mice with a conditional deletion of p85 α were not more susceptible to light stress-induced retinal degeneration (results not shown).

ERG recordings were used to assess and measure light-driven rod photoreceptor functional responses in wild-type, heterozygous, and conditional *pik3r1* KO mice. $A_{\max}$ and $B_{\max}$ (mean $\pm$ SEM), the maximum values of the a- and b-wave amplitudes at saturating light intensities, respectively, were calculated for wild-type ($A_{\max}$, 594 $\pm$ 27.25; $B_{\max}$, 1243 $\pm$ 69.0, n = 6), *pik3r1* heterozygous ($A_{\max}$, 542 $\pm$ 24.5; $B_{\max}$, 1134 $\pm$ 50.1, n = 6) and *pik3r1* KO ($A_{\max}$, 516 $\pm$ 28.6; $B_{\max}$, 1167 $\pm$ 45.4, n = 9) groups, and no significant differences were found, indicating that the absence of *pik3r1* did not adversely affect the function of the retinas. Standard ERG recordings using increasing intensity of light stimulus showed no difference in light sensitivity among the three genotypes. We also found no difference in the rhodopsin content (mean $\pm$ SEM, n = 7, in picomoles per eye) between wild-type (353 $\pm$ 42.4) and conditional *pik3r1* KO (345 $\pm$ 49.1) mice. In *Dro-*

sophila, PI3K-generated phosphoinositides have been shown to affect phototransduction recovery kinetics.⁸ To determine whether loss of p85 α affects the phototransduction recovery kinetics in our mice, we used the previously established twin-flash method.²⁶ As shown above, there were no detectable differences between wild-type and conditional *pik3r1* KO littermates in the amplitude of the scotopic a-wave after the initial flash (Fig. 4A). However, twin flashes generated an ERG response, with a significant delay in recovery in conditional *pik3r1* KO mice compared with wild-type controls, in the first three steps, with interstimulus intervals of 1, 2, and 4 seconds (Figs. 4B, 4C). An interval of 16 seconds was sufficient for recovery in the KO mice, which was comparable to the response in wild-type controls. Our results in control mice are consistent with those of Weymouth and Vingrys,²⁶ who reported 95% recovery of the photoresponse in $\sim$ 13 seconds. Thus, conditional deletion of PI3K in mice causes a phototransduction phenotype, but does not affect retinal structure.

Effect of p85 α Deletion on Trafficking and Translocation of Proteins Involved in Phototransduction

It has been shown that PI3K-generated PI-3-P facilitates the trafficking of the rhodopsin-laden vesicles from ER and Golgi to outer segments and assembling into disc membranes.⁷ In the present study, we analyzed rhodopsin localization to rod photoreceptor outer segments in WT and conditional *pik3r1* KO animals by immunofluorescence microscopy using an anti-rhodopsin antibody. Results indicated proper rhodopsin localization to the rod photoreceptor outer segments in our conditional *pik3r1* KO animals (Fig. 5A). Furthermore, these results

FIGURE 3. Structural analysis of retinas from rod conditional *pik3r1* KO mice. (A–C) Representative retinal morphology from wild-type (WT), heterozygous for floxed allele (HET), and conditional *pik3r1* KO mice at 1 month of age. The sections were obtained along the vertical meridian. (D) Morphometric analysis showed no difference in ONL thickness in the mice at that age. ROS, rod outer segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

complemented a healthy appearance of ROS and normal rod function, as shown in our morphologic and ERG studies.

Time- and Light-Dependent Translocation of Arrestin and Transducin in *pik3r1* KO Mice

Previous analysis of arrestin and transducin translocation in mouse retina by single labeling have shown that the light-dependent translocation of transducin and arrestin was rapid and occurred over a period of minutes.³⁰ To simultaneously follow the time course of change in the relative concentrations of arrestin and transducin in ROS and RIS after the onset of light, dark-adapted wild-type and *pik3r1* KO mice were exposed to 300 lux light and killed at 0, 5, 10, and 30 minutes thereafter, and their eyes were fixed and processed for immunofluorescence microscopy. Figure 5B demonstrates the simultaneous progressive changes in the compartmentalization of arrestin (red) and transducin (green) at various times after exposure to light. After 5 minutes of exposure, almost all the transducin in the ROS of the dark-adapted retinas had moved to the inner segments in both wild-type and *pik3r1* KO mice (Fig. 5B). Likewise, in both groups, light induced the translocation of arrestin from the inner segment and outer plexiform layer (OPL) to the ROS. However, the movement of arrestin in the *pik3r1* KO mice was slower than in the wild-type mouse retina, evidenced by lower levels of immunoreactivity in the outer segments and residual immunoreactivity in the outer plexiform region at 5 minutes of exposure (Fig. 5B). After 10 minutes of exposure, the ROS localization of arrestin was almost identical between the wild-type and *pik3r1* KO mice (Fig. 5B).

DISCUSSION

The existence of the components of the IR/PI3K/Akt survival signaling pathways has been established in retinal photorecep-

tor cells.⁴ Our laboratory has shown that in mature retina, rod photoreceptor cells from IR,¹² Bcl-xl,²⁹ and Akt2²⁷ KO mice displayed an increased susceptibility to light-induced damage, mainly due to the inability to activate the PI3K/Akt survival pathway.^{12,27} These findings establish the biological significance of PI3K for maintaining cell viability and retinal neuroprotection. In the present study, we confirmed our earlier findings that light-induced stress leads to the activation of PI3K and subsequent phosphorylation of Akt. We recently reported that conditional deletion of *pik3r1* from cones results in an age-related cone degeneration.³¹ It is interesting to note that in this study, deletion of *pik3r1* from the rods did not lead to any photoreceptor degeneration phenotype. However, we did observe a subtle delay in phototransduction recovery kinetics and a delay in the trafficking of arrestin to outer segments in rod *pik3r1* KO mice. The lack of retinal degeneration phenotype in *pik3r1* KO mice suggests the complementation of PI3K functions in rods by a redundant pathway.

Class I_A PI3K is an obligatory heterodimeric complex comprising regulatory p85 and catalytic subunits.³² In mammals, the PI3K catalytic subunits (p110α, p110β, and p110δ) are bound to any one of five distinct regulatory subunits (p85α, p85β, p55γ, p55α, and p50α, collectively referred to as p85s).³³ These p85 adapters result from three genes, *pik3r1*, *pik3r2*, and *pik3r3*, respectively.³⁴ The *pik3r1* gene can be expressed in splice variants that encode p85α, p55α, and p50α. The adapters p85α and p85β are ubiquitously expressed,³⁴ whereas p50α and p55α are present in fat, muscle, liver, and brain^{35,36}; p55γ is expressed mainly in the brain.³⁷ All members of the p85 family contain a p110-binding region that interacts with a specific domain present at the N-terminal ends of the class I_A p110 catalytic domains.³⁸ The p110δ

FIGURE 4. Phototransduction recovery kinetics assay in conditional *pik3r1* KO mice. Twin-flash ERG recordings were used to examine recovery kinetics in WT and KO mice. (A) ERG recordings generated by the initial flash show no functional difference in rod photoreceptor response. (B) ERG recordings generated by the second flash show functional recovery delay in 1, 2, and 4 seconds recordings. However, 16 seconds between the initial and second flash was enough time for the full recovery of the photoresponse. (C) Histogram of response of the second flash relative to the initial flash ERG recording. Data are the mean $\pm$ SE, $n = 4$. * $P < 0.05$; ** $P < 0.01$.

isoform expression is specifically found in leukocytes.³⁹ The N-terminal p85-binding motif is absent in class I_B PI3K γ , but interacts with the p101⁴⁰ and p84/87 adapter for regulation.^{41,42} Retinal rod outer segment membranes express p110 α , p110 β , and p110 γ (data not shown) as well as both regulatory subunit of p85 α and p85 β . The absence of a phenotype in p85 α KO mice could be due to the complementation by regulatory p85 β , which may still signal p110 α and p110 β ,

or by p110 γ , which does not require a p85 subunit for regulation.

It is interesting to note that *pik3r1* deletion in liver exhibits a paradoxical improvement of hepatic and peripheral insulin sensitivity.⁴³ This study also showed decreased PI3K enzyme activity, but increased Akt activity correlated with increased PIP₃ levels, which was due to the diminished activity of PTEN,⁴³ a D3 phosphoinositide phosphatase. These and other studies clearly suggest that p85 α is a critical modulator of PI3K activation, but also is a positive regulator of PTEN activity.^{43,44} The lack of phenotype in rod *pik3r1* KO mice may also be due to steady state levels of PIP₃. Consistent with this idea, we failed to observe photoreceptor degeneration when we light-stressed *pik3r1* KO mice (data not shown).

Protein translocation has been documented in a broad range of animal species from flies to mammals.^{8,45,46} *Drosophila* arrestin has a phosphoinositide-binding domain and PI3K-generated PIP₃ controls the movement of arrestin.⁸ Furthermore, evidence shows that increase or decrease of expression of PTEN affects shuttling of arrestin; a decrease in PTEN expression (more PIP₃) affects movement of arrestin from microvilli to cell bodies; and an increase in PTEN expression (less PIP₃) affects movement of arrestin from cell body to the microvilli.⁴⁷ In our in vitro experiments, we were not able to demonstrate the binding of vertebrate visual arrestin to PI3K-generated phosphoinositides (data not shown) using an in vitro protein-lipid overlay assay.¹¹ Possible explanations would be an alteration of folding or conformation of arrestin and/or a need for scaffolding/docking protein to facilitate arrestin-phosphoinositide binding. Therefore, we cannot dismiss the possibility that these two molecules do not interact.

The delay in phototransduction recovery kinetics observed in our rod-specific *pik3r1* KO mice could be an outcome of modification of multiple phototransduction steps, such as the retinoid cycle, rhodopsin bleaching, rhodopsin phosphorylation, arrestin/transducin translocation, or cyclic nucleotide-gated-channel opening/closing. In *Drosophila*, inactivation of phototransduction is achieved by myosin III-driven, PIP₃-mediated translocation of visual arrestin Arr2 from rhabdomeric cell body to the microvilli.^{8,48,49} The light-dependent translocation allows Arr2 to bind meta-rhodopsin, which prevents further interaction of GPCR with G-protein transducin and terminates phototransduction.⁵⁰ During prolonged exposure to light, the translocation of Arr2 is accompanied by the migration of T α to the cell body, thus lowering photoreceptor sensitivity to illumination and allowing sufficient time for inactivation and recovery of rhodopsin.⁵¹ Light-induced translocation of Arr2 to the microvilli, phototransduction termination, and recovery were impaired in invertebrate retina deficient in *cds* and *rdgB* (phosphoinositide biosynthesis and trafficking genes) or overexpression of *PTEN*.⁵²⁻⁵⁴ In our rod conditional *pik3r1* KO animals, twin-flash ERG recordings indicated a significant delay in phototransduction recovery kinetics. This subtle functional phenotype was not sufficient to affect overall rod photoreceptor cells viability and function. The delay in phototransduction recovery kinetics could also be due to an effect of PI3K-generated phosphoinositides on CNG channel activity. Consistent with this hypothesis, PI3K-generated PIP₃ has been shown to inhibit the action of olfactory⁵⁵ and cone CNG⁵⁶ channels. Such regulation may be true for the rod CNG channel as well. Further studies are needed to understand the role of PI3K-generated phosphoinositides in rod functions. This could be achieved by double knockout animal models. Such studies are under way in our laboratory.

The IR/PI3K/Akt pathway activation is tightly regulated and requires upstream photobleaching of rhodopsin, but does not require transducin signaling.⁵⁷ Our present study suggests that the light-induced activation of the IR/PI3K pathway that we

FIGURE 5. Rhodopsin localization and dark-to-light temporal translocation of arrestin and transducin in wild-type and conditional *pik3r1* KO mice. **(A)** Immunofluorescence analysis with anti-rhodopsin antibody in WT and KO retinal sections shows no abnormality in rhodopsin localization. Control, omission of primary antibody. **(B)** Translocation of arrestin and transducin ($T\alpha$) in retinal sections from WT and KO mice in response to exposure to 300 lux of light (0, 5, 10, and 30 minutes). Movement of arrestin, but not transducin, was slower in the KO mice at the 5-minute time point. The images shown are representative of five retinas examined from WT and KO mice at each of the four time points. The images for each time point are of the same section viewed with a filter to detect (green) transducin-, (red) arrestin-, and (blue) DAPI-stained nuclei. ROS, rod outer segment; RIS, rod inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer.

reported earlier^{10,57} is functionally important for phototransduction-induced protein trafficking. These studies strengthen our hypothesis regarding the existence of cross-talk between tyrosine kinase signaling and phototransduction and suggest that in addition to classic phototransduction, rhodopsin can initiate a second signaling pathway, the IR/PI3K/Akt defense mechanism, in retinal photoreceptors. Based on our present study, we speculate that in a timely manner, PI3K could synchronize the cross-communication between the modulation of phototransduction and the activation of mechanistic signaling pathways necessary to maintain photoreceptor cell viability.

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References

- Guo X, Ghalayini AJ, Chen H, Anderson RE. Phosphatidylinositol 3-kinase in bovine photoreceptor rod outer segments. *Invest Ophthalmol Vis Sci.* 1997;38:1873–1882.
- Guo XX, Huang Z, Bell MW, Chen H, Anderson RE. Tyrosine phosphorylation is involved in phosphatidylinositol 3-kinase activation in bovine rod outer segments. *Mol Vis.* 2000;6:216–221.
- Rajala RV, Anderson RE. Interaction of the insulin receptor beta-subunit with phosphatidylinositol 3-kinase in bovine ROS. *Invest Ophthalmol Vis Sci.* 2001;42:3110–3117.
- Rajala RV. Phosphoinositide 3-kinase signaling in the vertebrate retina. *J Lipid Res.* 2010;51:4–22.
- Cantley LC. The phosphoinositide 3-kinase pathway. *Science.* 2002;296:1655–1657.
- He F, Mao M, Wensel TG. Enhancement of phototransduction g protein-effector interactions by phosphoinositides. *J Biol Chem.* 2004;279:8986–8990.
- Chuang JZ, Zhao Y, Sung CH. SARA-regulated vesicular targeting underlies formation of the light-sensing organelle in mammalian rods. *Cell.* 2007;130:535–547.
- Lee SJ, Xu H, Kang LW, Amzel LM, Montell C. Light adaptation through phosphoinositide-regulated translocation of *Drosophila* visual arrestin. *Neuron.* 2003;39:121–132.
- Schmitz F, Drenckhahn D. Li(+)-induced structural changes of synaptic ribbons are related to the phosphoinositide metabolism in photoreceptor synapses. *Brain Res.* 1993;604:142–148.
- Rajala RV, McClellan ME, Ash JD, Anderson RE. In vivo regulation of phosphoinositide 3-kinase in retina through light-induced tyrosine phosphorylation of the insulin receptor beta-subunit. *J Biol Chem.* 2002;277:43319–43326.
- Li G, Rajala A, Wiechmann AF, Anderson RE, Rajala RV. Activation and membrane binding of retinal protein kinase BalphA/Akt1 is regulated through light-dependent generation of phosphoinositides. *J Neurochem.* 2008;107:1382–1397.
- Rajala A, Tanito M, Le YZ, Kahn CR, Rajala RV. Loss of neuroprotective survival signal in mice lacking insulin receptor gene in rod photoreceptor cells. *J Biol Chem.* 2008;283:19781–19792.

13. Gomez-Vicente V, Doonan F, Donovan M, Cotter TG. Induction of BIM(EL) following growth factor withdrawal is a key event in caspase-dependent apoptosis of 661W photoreceptor cells. *Eur J Neurosci.* 2006;24:981-990.
14. Mackey AM, Sanvicens N, Groeger G, Doonan F, Wallace D, Cotter TG. Redox survival signalling in retina-derived 661W cells. *Cell Death Differ.* 2008;15:1291-1303.
15. Barber AJ, Nakamura M, Wolpert EB, et al. Insulin rescues retinal neurons from apoptosis by a phosphatidylinositol 3-kinase/Akt-mediated mechanism that reduces the activation of caspase-3. *J Biol Chem.* 2001;276:32814-32821.
16. Kermer P, Klocker N, Labes M, Bahr M. Insulin-like growth factor-I protects axotomized rat retinal ganglion cells from secondary death via PI3-K-dependent Akt phosphorylation and inhibition of caspase-3 *In vivo*. *J Neurosci.* 2000;20:2-8.
17. Bi L, Okabe I, Bernard DJ, Wynshaw-Boris A, Nussbaum RL. Proliferative defect and embryonic lethality in mice homozygous for a deletion in the p110alpha subunit of phosphoinositide 3-kinase. *J Biol Chem.* 1999;274:10963-10968.
18. Fruman DA, Mauvais-Jarvis F, Pollard DA, et al. Hypoglycaemia, liver necrosis and perinatal death in mice lacking all isoforms of phosphoinositide 3-kinase p85 alpha. *Nat Genet.* 2000;26:379-382.
19. Luo J, McMullen JR, Sobkiw CL, et al. Class IA phosphoinositide 3-kinase regulates heart size and physiological cardiac hypertrophy. *Mol Cell Biol.* 2005;25:9491-9502.
20. Luo J, Sobkiw CL, Hirshman MF, et al. Loss of class IA PI3K signaling in muscle leads to impaired muscle growth, insulin response, and hyperlipidemia. *Cell Metab.* 2006;3:355-366.
21. Rajala RV, Anderson RE. Rhodopsin-regulated insulin receptor signaling pathway in rod photoreceptor neurons. *Mol Neurobiol.* 2010;42:39-47.
22. Rajala RV, Anderson RE. Light regulation of the insulin receptor in the retina. *Mol Neurobiol.* 2003;28:123-138.
23. Yu X, Rajala RV, McGinnis JF, et al. Involvement of insulin/phosphoinositide 3-kinase/Akt signal pathway in 17 beta-estradiol-mediated neuroprotection. *J Biol Chem.* 2004;279:13086-13094.
24. Le YZ, Zheng L, Zheng W, et al. Mouse opsin promoter-directed Cre recombinase expression in transgenic mice. *Mol Vis.* 2006;12:389-398.
25. Ranchon I, LaVail MM, Kotake Y, Anderson RE. Free radical trap phenyl-N-tert-butyl nitrone protects against light damage but does not rescue P23H and S334ter rhodopsin transgenic rats from inherited retinal degeneration. *J Neurosci.* 2003;23:6050-6057.
26. Weymouth AE, Vingrys AJ. Rodent electroretinography: methods for extraction and interpretation of rod and cone responses. *Prog Retin Eye Res.* 2008;27:1-44.
27. Li G, Anderson RE, Tomita H, et al. Nonredundant role of Akt2 for neuroprotection of rod photoreceptor cells from light-induced cell death. *J Neurosci.* 2007;27:203-211.
28. Rapp LM, Smith SC. Morphologic comparisons between rhodopsin-mediated and short-wavelength classes of retinal light damage. *Invest Ophthalmol Vis Sci.* 1992;33:3367-3377.
29. Zheng L, Anderson RE, Agbaga MP, Rucker EB III, Le YZ. Loss of BCL-XL in rod photoreceptors: increased susceptibility to bright light stress. *Invest Ophthalmol Vis Sci.* 2006;47:5583-5589.
30. Elias RV, Sezate SS, Cao W, McGinnis JF. Temporal kinetics of the light/dark translocation and compartmentation of arrestin and alpha-transducin in mouse photoreceptor cells. *Mol Vis.* 2004;10:672-681.
31. Ivanovic I, Anderson RE, Le YZ, Fliesler SJ, Sherry DM, Rajala RV. Deletion of p85alpha regulatory subunit of phosphoinositide 3-kinase in cone photoreceptor cells results in cone photoreceptor degeneration. *Invest Ophthalmol Vis Sci.* 2011;52:3775-3783.
32. Carpenter CL, Duckworth BC, Auger KR, Cohen B, Schaffhausen BS, Cantley LC. Purification and characterization of phosphoinositide 3-kinase from rat liver. *J Biol Chem.* 1990;265:19704-19711.
33. Geering B, Cutillas PR, Nock G, Gharbi SI, Vanhaesebroeck B. Class IA phosphoinositide 3-kinases are obligate p85-p110 heterodimers. *Proc Natl Acad Sci U S A.* 2007;104:7809-7814.
34. Hirsch E, Costa C, Ciralo E. Phosphoinositide 3-kinases as a common platform for multi-hormone signaling. *J Endocrinol.* 2007;194:243-256.
35. Antonetti DA, Algenstaedt P, Kahn CR. Insulin receptor substrate 1 binds two novel splice variants of the regulatory subunit of phosphatidylinositol 3-kinase in muscle and brain. *Mol Cell Biol.* 1996;16:2195-2203.
36. Inukai K, Anai M, Van Breda E, et al. A novel 55-kDa regulatory subunit for phosphatidylinositol 3-kinase structurally similar to p55PIK is generated by alternative splicing of the p85alpha gene. *J Biol Chem.* 1996;271:5317-5320.
37. Pons S, Asano T, Glasheen E, et al. The structure and function of p55PIK reveal a new regulatory subunit for phosphatidylinositol 3-kinase. *Mol Cell Biol.* 1995;15:4453-4465.
38. Hiles ID, Otsu M, Volinia S, et al. Phosphatidylinositol 3-kinase: structure and expression of the 110 kd catalytic subunit. *Cell.* 1992;70:419-429.
39. Hirsch E, Lembo G, Montrucchio G, Rommel C, Costa C, Barberis L. Signaling through PI3Kgamma: a common platform for leukocyte, platelet and cardiovascular stress sensing. *Thromb Haemost.* 2006;95:29-35.
40. Stephens LR, Eguinoa A, Erdjument-Bromage H, et al. The G beta gamma sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. *Cell.* 1997;89:105-114.
41. Suire S, Coadwell J, Ferguson GJ, Davidson K, Hawkins P, Stephens L. p84, a new Gbetagamma-activated regulatory subunit of the type IB phosphoinositide 3-kinase p110gamma. *Curr Biol.* 2005;15:566-570.
42. Voigt P, Brock C, Nurnberg B, Schaefer M. Assigning functional domains within the p101 regulatory subunit of phosphoinositide 3-kinase gamma. *J Biol Chem.* 2005;280:5121-5127.
43. Taniguchi CM, Tran TT, Kondo T, et al. Phosphoinositide 3-kinase regulatory subunit p85alpha suppresses insulin action via positive regulation of PTEN. *Proc Natl Acad Sci U S A.* 2006;103:12093-12097.
44. Chaggar RB, Links PH, Pastor MC, et al. Direct positive regulation of PTEN by the p85 subunit of phosphatidylinositol 3-kinase. *Proc Natl Acad Sci U S A.* 2010;107:5471-5476.
45. Arshavsky VY. Protein translocation in photoreceptor light adaptation: a common theme in vertebrate and invertebrate vision. *Sci STKE.* 2003;2003:E43.
46. Hardie RC. Phototransduction: shedding light on translocation. *Curr Biol.* 2003;13:R775-R777.
47. Stocker H, Andjelkovic M, Oldham S, et al. Living with lethal PIP3 levels: viability of flies lacking PTEN restored by a PH domain mutation in Akt/PKB. *Science.* 2002;295:2088-2091.
48. Lee SJ, Montell C. Light-dependent translocation of visual arrestin regulated by the NINAC myosin III. *Neuron.* 2004;43:95-103.
49. Frechter S, Minke B. Light-regulated translocation of signaling proteins in Drosophila photoreceptors. *J Physiol Paris.* 2006;99:133-139.
50. Byk T, Bar-Yaacov M, Doza YN, Minke B, Selinger Z. Regulatory arrestin cycle secures the fidelity and maintenance of the fly photoreceptor cell. *Proc Natl Acad Sci U S A.* 1993;90:1907-1911.
51. Fain GL, Matthews HR, Cornwall MC, Koutalos Y. Adaptation in vertebrate photoreceptors. *Physiol Rev.* 2001;81:117-151.
52. Wu L, Niemeyer B, Colley N, Socolich M, Zuker CS. Regulation of PLC-mediated signalling *in vivo* by CDP-diacylglycerol synthase. *Nature.* 1995;373:216-222.
53. Vihtelic TS, Goebel M, Milligan S, O'Tousa JE, Hyde DR. Localization of Drosophila retinal degeneration B, a membrane-associated phosphatidylinositol transfer protein. *J Cell Biol.* 1993;122:1013-1022.
54. Huang H, Potter CJ, Tao W, et al. PTEN affects cell size, cell proliferation and apoptosis during Drosophila eye development. *Development.* 1999;126:5365-5372.
55. Brady JD, Rich ED, Martens JR, Karpen JW, Varnum MD, Brown RL. Interplay between PIP3 and calmodulin regulation of olfactory cyclic nucleotide-gated channels. *Proc Natl Acad Sci U S A.* 2006;103:15635-15640.
56. Bright SR, Rich ED, Varnum MD. Regulation of human cone cyclic nucleotide-gated channels by endogenous phospholipids and exogenously applied phosphatidylinositol 3,4,5-trisphosphate. *Mol Pharmacol.* 2007;71:176-183.
57. Rajala A, Anderson RE, Ma JX, Lem J, Al Ubaidi MR, Rajala RV. G-protein-coupled receptor rhodopsin regulates the phosphorylation of retinal insulin receptor. *J Biol Chem.* 2007;282:9865-9873.