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Rhodopsin-regulated Insulin Receptor Signaling Pathway in Rod Photoreceptor Neurons

Raju V. S. Rajala · Robert E. Anderson

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Abstract The retina is an integral part of the central nervous system and retinal cells are known to express insulin receptors (IR), although their function is not known. This article describes recent studies that link the photoactivation of rhodopsin to tyrosine phosphorylation of the IR and subsequent activation of phosphoinositide 3-kinase, a neuron survival factor. Our studies suggest that the physiological role of this process is to provide neuroprotection of the retina against light damage by activating proteins that protect against stress-induced apoptosis. We focus mainly on our recently identified regulation of the IR pathway through the G-protein-coupled receptor rhodopsin. Various mutant and knockout proteins of phototransduction cascade have been used to study the light-induced activation of the retinal IR. Our studies suggest that rhodopsin may have additional previously uncharacterized signaling functions in photoreceptors.

Keywords Insulin receptor · Photobleaching · Rhodopsin · Phosphoinositdie 3-kinase · Retina · Rod outer segments

R. V. S. Rajala · R. E. Anderson
Department of Ophthalmology,
University of Oklahoma Health Sciences Center,
Oklahoma, OK 73104, USA

R. V. S. Rajala · R. E. Anderson
Department of Cell Biology,
University of Oklahoma Health Sciences Center,
Oklahoma, OK 73104, USA

R. V. S. Rajala · R. E. Anderson
Dean A. McGee Eye Institute,
Oklahoma, OK 73104, USA

R. V. S. Rajala (✉)
608 Stanton L Young Blvd Rm 409,
Oklahoma, OK 73104, USA
e-mail: raju-rajala@ouhsc.edu

Introduction

Insulin receptors (IR) and insulin signaling proteins are widely distributed throughout the central nervous system (CNS) [1]. Previous studies have suggested a role for insulin signaling in the regulation of food intake [2, 3] and neuronal growth and differentiation [4, 5]. Dysregulation of insulin signaling in the CNS has been linked to the pathogenesis of neurodegenerative disorders such as Alzheimer's and Parkinson's disease [6, 7]. Insulin and insulin growth factor-1 receptors are expressed at high levels in many brain areas and different cell types, including glial and neuronal cells [1]. Because neurons metabolize glucose in an insulin-independent manner and ablation of the IR in brain results in increased food intake, moderate diet-dependent obesity, and hypergonadotropic hypogonadism; the latter is associated with impaired maturation of ovarian follicles in females and reduced spermatogenesis in males and leads to reduced fertility [8]. These studies indicate that IRs play a role in the control of appetite suppression and reproduction.

Cells of bovine and rat retina contain high affinity receptors for insulin [1]. IR signaling provides a trophic signal for transformed retinal neurons in culture [9], but the role of the IR in vivo is unknown. We have used a targeted deletion strategy to specifically inactivate the IR gene in rod photoreceptors [10]. Reduced IR expression in rod photoreceptors significantly decreased retinal function and caused the loss of photoreceptors in mice exposed to bright light stress [10].

IR activation has been shown to rescue retinal neurons from apoptosis through the phosphoinositide 3-kinase (PI3K) cascade [9]. We previously reported that light induces tyrosine phosphorylation of the retinal IR and that this activation leads to the binding of PI3K to rod outer segment (ROS) membranes [11]. More recently, we

demonstrated that light-dependent IR activation is mediated through the G-protein-coupled receptor rhodopsin [12], the major protein in ROS. IR signaling is also involved in 17ß-estradiol-mediated neuroprotection in the retina [13]. Recent evidence suggests a down-regulation of IR kinase activity in diabetic retinopathy that is associated with the deregulation of downstream signaling molecules [14]. Our laboratory has shown that light-induced activation of the IR leads to the activation of downstream effectors, PI3K and Akt [12, 15]. Activated Akt phosphorylates and inactivates components of the apoptotic machinery [16–19]. There are three isoforms of Akt [20–25] and we found that all three isoforms are expressed in rod photoreceptor cells [26]. We also found that physiological light-activated IR results in the activation of Akt1 and Akt3 but not in the activation of the Akt2 isoform [15]. Deletion of several downstream effector molecules of the IR signaling pathway, such as IRS-2 [27], Akt2 [26], and bcl-xl [28], in the retina resulted in photoreceptor degeneration. These studies clearly indicate the importance of the IR signaling pathway in the retina.

The IR is highly conserved with a high degree of IR signaling homology between *Caenorhabditis elegans*, *Drosophila* and humans suggests functional conservation in the mammalian retina. The IR regulates neuronal survival in *C. elegans* [29]. In *Drosophila*, the IR serves an important function to guide retinal photoreceptor axons from the retina to the brain during development [30] and the IR influences the size and number of photoreceptors [31]. Mutation in either IR autophosphorylation sites [30] or its binding partner Dock [32] in *Drosophila* results in a severe photoreceptor axonal misguidance phenotype. In humans, defects in IR signaling in the central nervous system are associated with Alzheimer's disease [33–35]. The lack of IR activation leads to neurodegeneration in brain/neuron-specific IR knock-out mice [36]. These studies clearly suggest that the IR pathway is important for neuronal survival and maintenance.

This article focuses on our recently identified regulation of the IR pathway through the G-protein-coupled receptor rhodopsin. Our studies suggest that rhodopsin photoexcitation may trigger signaling events alternative to the classical transducin activation.

Increased IR Phosphorylation and PI3K Enzyme Activity Associated with IRs of Light-Adapted Rat Retinas

Ghalayini et al. [37] previously reported that light stimulates tyrosine phosphorylation of multiple proteins in ROS in vivo. To determine whether light has an effect on PI3K activity and phosphorylation of the IR, rats were dark adapted overnight, and one half were subjected to normal room light for 30 min [11]. Retinal lysates were immunoprecipitated with anti-PY-99 and anti-IRβ antibodies. The PI3K activity was higher in retinas from light-adapted rats, compared to those from dark-adapted animals (Fig. 1). These experiments suggested the light-induced activation of PI3K through light-induced tyrosine phosphorylation of IR [11].

The catalytic loops within the tyrosine kinase domain of the IR contain three (Y1158, Y1162, and Y1163) tyrosine motifs [38, 39]. It is generally believed that autophosphorylation within the activation loop proceeds in a processive manner initiated at the second tyrosine (1162), followed by phosphorylation at the first tyrosine (1158), and finally the last (1163), upon which the IR becomes fully active [38, 39]. To determine whether light activates the IR in the same manner as insulin, we immunoprecipitated the IR (Fig. 2b) from retinal lysates that were prepared from light- and dark-adapted mice followed by Western blot analysis with phosphospecific anti-IR ($pYpYpY^{1158/1162/1163}$) antibody. The results indicated increased phosphorylation in light-adapted compared to dark-adapted retinas (Fig. 2a). Light-adapted rod outer segments (LROS) and dark-adapted ROS were solubilized with 1% NP-40 and the IR was immunoprecipitated followed by Western blot analysis with anti-IR ($pYpYpY^{1158/1162/1163}$) antibody. The results indicated increased tyrosine phosphorylation of the IR in LROS (Fig. 2c). The blot was

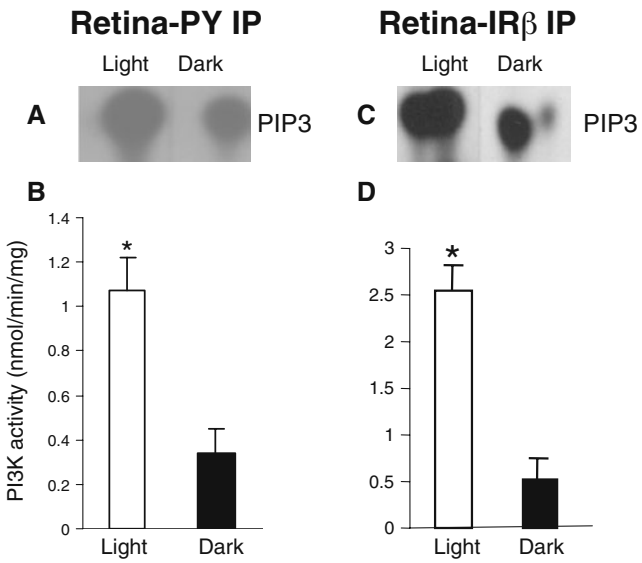

Fig. 1 PI3K enzyme activity in anti-PY-99 and anti-IRβ immunoprecipitates from dark- and light-adapted rat retina homogenates. PI3K activity was measured from anti-IRβ immunoprecipitates of lysates from light- and dark-adapted retinas. PI3K activity was measured using PI-4,5-P_2 and [γ^{32}P]ATP as substrate. The radioactive spots of PI-3,4,5-P_3 (**a**, **c**) were scraped from TLC plates and counted (**b**, **d**). Data are mean ± SD, n=6, *p<0.05. Reprinted with permission from [11]

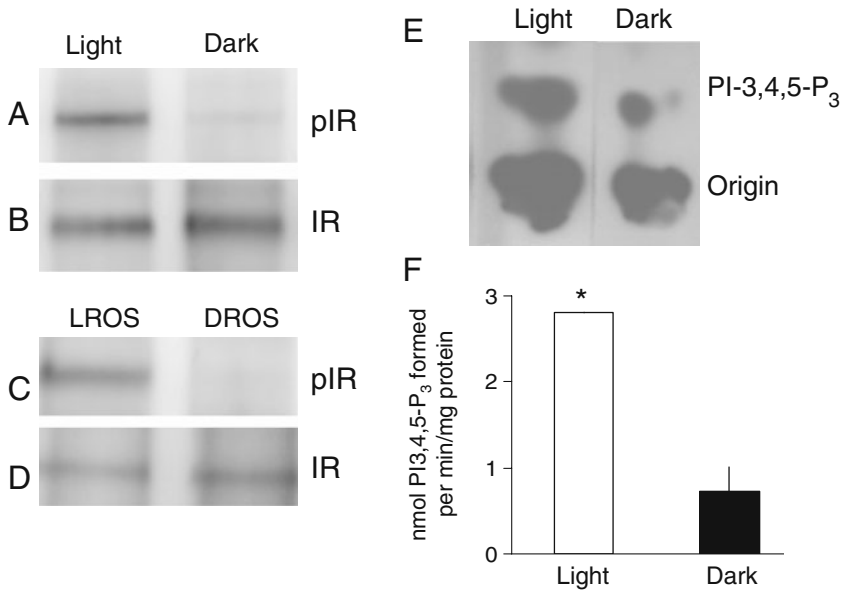

Fig. 2 Light activation of tyrosine phosphorylation in the catalytic loop of the IR. Two hundred micrograms of protein were immunoprecipitated with anti-IRβ antibody from either mouse retina lysates (**a**) or ROS (**c**) prepared from light- and dark-adapted rats. The immunoprecipitates were subjected to Western blot analysis with anti-IR ($pYpYpY^{1158/1162/1163}$) antibody. The blots were stripped and reprobed with anti-IRβ antibody to ensure equal amount of protein in each lane (**b** and **d**). PI3K activity was measured from anti-IRβ immunoprecipitates of lysates from light- and dark-adapted retinas (**e**). PI3K activity was measured using PI-4,5-P_2 and [γ^{32}P]ATP as substrate. The radioactive spots of PI-3,4,5-P_3 were scraped from TLC plates and counted (**f**). Data are mean ± SD, $n=6$, $^*p<0.05$. Reprinted with permission from [12]

stripped and reprobed with anti-IRß antibody to ensure equal amount of protein in each line (Fig. 2d). These results suggest that light activates IR phosphorylation in the catalytic loop within the tyrosine kinase domain. When retinal homogenates were immunoprecipitated with anti-IRβ antibodies, PI3K activity was more than threefold higher in IPs from light-adapted rats compared to those from dark-adapted animals (Fig. 2e, f). These results

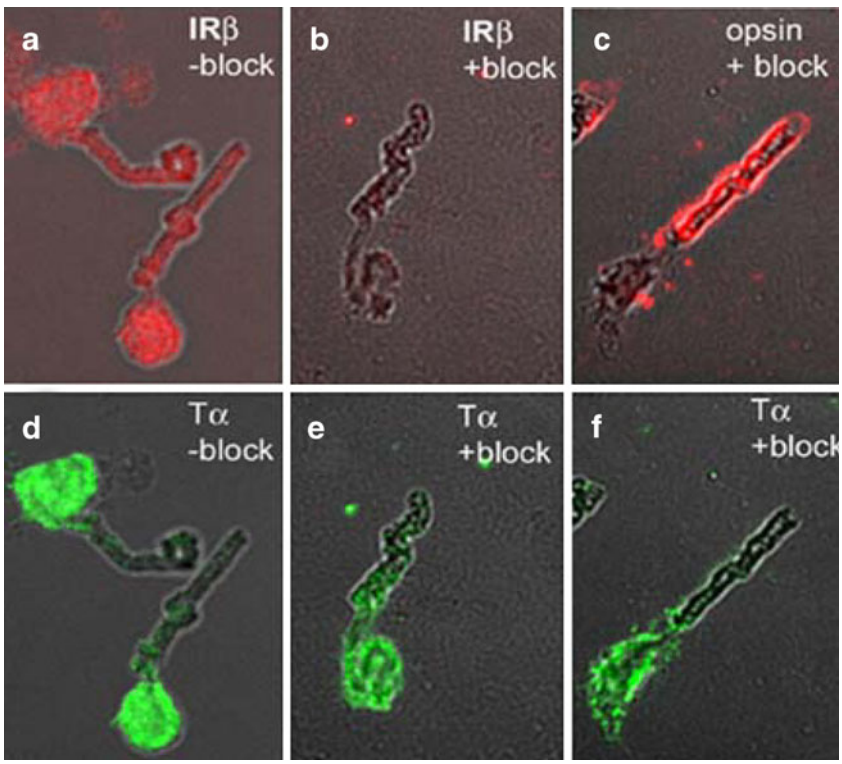

Fig. 3 Immunolocalization of IRβ in methanol fixed ROS. Bovine ROS were prepared on glass slides as described [62]. Slides were co-immunolabeled with opsin (**c**) and transducin alpha (**d**), or with IRβ (**a**), and transducin alpha (**e**). The immuno-staining of IRβ was significantly blocked using the peptide from which the antibody was generated (**b**). The same peptide did not block opsin (**c**) or transducin alpha staining (**e** and **f**). Reprinted with permission from [11]

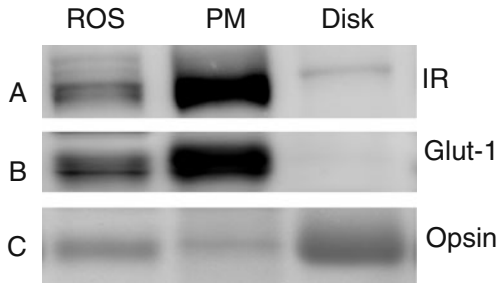

Fig. 4 IR localization to the plasma membrane. Bovine ROS, plasma membrane and disk proteins were subjected to Western blot analysis with anti-IRβ (**a**), anti-Glut1 (**b**), and anti-opsin antibodies (**c**). Reprinted with permission from [12]

demonstrate light-induced phosphorylation of the IRβ, as well as light-induced binding of p85 to the insulin receptor.

Location of Insulin Receptors in the Rod Photoreceptors

Rodrigues et al. [40] found the insulin receptor was localized in photoreceptor and neuronal cell bodies, with lower immunoreactivity in ROS. To further demonstrate that IRs are present in photoreceptor outer segments, we immunolabeled bovine ROS (containing some attached inner segment) with anti-IRβ antibody. Immunostaining was found in both outer and inner segments (Fig. 3a). Inclusion of the IRβ-blocking peptide inhibited the immunoreactivity of IRβ (Fig. 3b). For positive controls, anti-opsin antibody showed the localization of opsin only in the ROS (Fig. 3c), and transducin immunoreactivity was observed primarily in the inner segment of this light-adapted preparation (Fig. 3d, e). The IRβ blocking peptide did not block the binding of the anti-opsin (Fig. 3c) or transducin alpha antibodies (Fig. 3f). These results provide strong evidence that the IR is present in ROS membranes.

Insulin Receptors are Localized to Plasma Membrane

Crude bovine rod outer segments were subjected to FICOLL gradient centrifugation to isolate ROS disks [41]. ROS, plasma membrane, and isolated disks were subjected to Western blot analysis with anti-IRβ, anti-Glut1, and anti-opsin antibodies. The results indicate that IR, Glut1, and opsin immunoreactivity was present in ROS (Fig. 4). IR and the plasma membrane marker Glut1 were enriched in the plasma membrane fraction of the ROS (Fig. 4a, b). Opsin blots show the enrichment of opsin in the disk membranes (Fig. 4c). These results suggest that IRs is localized to the plasma membrane.

Light Activation of IR is Localized to Photoreceptor Neurons

Light absorption by rhodopsin activates transducin, a G-protein, which in turn promotes cGMP hydrolysis by cGMP-phosphodiesterase, leading to hyperpolarization of rod photoreceptor cells [42, 43]. FVB/N mice are homozygous for the Pdeb^{rd1} mutation (formally known as rd1) in the cGMP-phosphodiesterase β-subunit. As a result, these mice undergo rapid photoreceptor degeneration beginning at postnatal day 9 [44–47]. The retinas from adult FVB/N mice completely lack photoreceptors. To determine whether the light-induced tyrosine phosphorylation is mediated through photoreceptor neurons, we conducted experiments

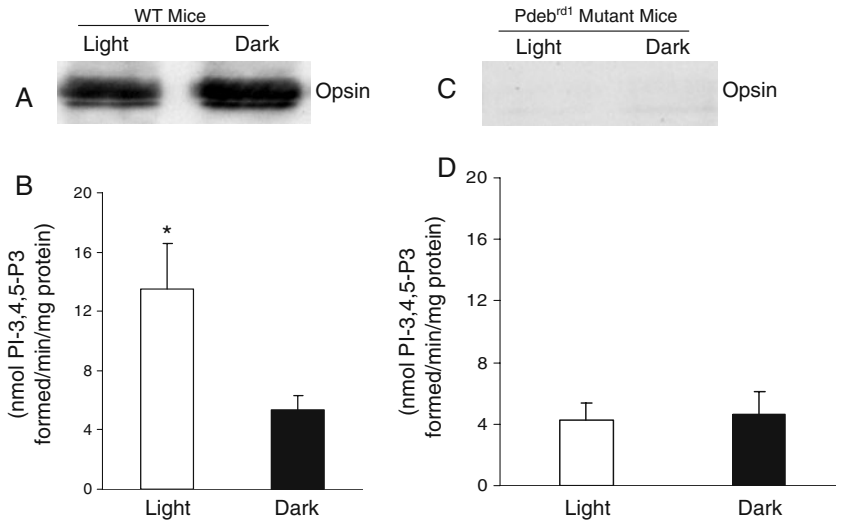

Fig. 5 PI3K activity and opsin in retinas of wild-type and FVB mice. PI3K activity was measured in the immunoprecipitates of IRβ from (**b**) wild-type (n=9) and [4] FVB mouse retinas. Data are mean ± SE. *P<0.05. Opsin (**a**, **c**) expression was examined with 10 μg of protein from light- and dark-adapted wild-type and FVB mice. Reprinted with permission from [11]

on 2-month-old wild-type and mutant FVB/N mice to investigate the involvement of photobleachable visual pigments in the regulation of PI3K activity. Also, as shown in Fig. 1, PI3K activity was significantly higher in anti-IRβ IPs of light-adapted wild-type mouse retinas compared to dark-adapted mouse retinas (Fig. 5b). However, there was no difference in PI3K activity in light- and dark-adapted $Pdeb^{rd1}$ mutant mice, which lack photoreceptors (Fig. 5d). Lack of photoreceptors was confirmed by probing the retina lysates with anti-opsin antibody, where no detectable opsin was observed in FVB/N mutant (Fig. 5c) compared to wild-type retinas (Fig. 5a). The results suggest that the observed light/dark differences in IRβ phosphorylation and subsequent binding of PI3K are photoreceptor-specific phenomena that are mediated by photon capture in the ROS. These studies also suggest that the phototransduction cascade is coupled to the activation of IR phosphorylation.

The Visual Transduction Cascade and IR Phosphorylation

To determine if phototransduction events are required for IR phosphorylation, we examined IR phosphorylation in mice lacking transducin ($Tr\alpha^{-/-}$). These mice contain normal amounts of rhodopsin, but its photoexcitation does not initiate phototransduction due to the lack of transducin. $Tr\alpha^{-/-}$ mice were dark-adapted overnight and half were exposed to normal room light for 30 min. Retinal lysates from light- and dark-adapted $Tr\alpha^{-/-}$ mice were subjected to glutathione S-transferase (GST) pull-down assays using the p85(N-SH2) domain (which specifically binds to the phosphorylated form of the IR) followed by Western blot analysis with anti-IRß antibody. The blot shows that $Tr\alpha^{-/-}$ mice still exhibit a light-dependent phosphorylation of IR (Fig. 6a). These results suggest that the visual transduction cascade, downstream of rhodopsin, is not necessary for light-dependent IR phosphorylation.

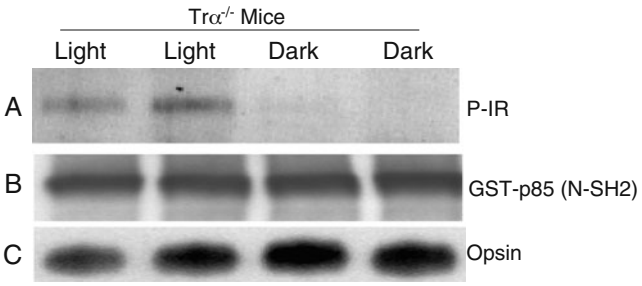

Fig. 6 Light-dependent phosphorylation of IR in $Tr\alpha^{-/-}$ mice. Lysates of retina from dark- and light-adapted $Tr\alpha^{-/-}$ mice were subjected to GST pull-down assays with GST-p85 (N-SH2) domain (**b**). Bound proteins were probed with anti-IRβ antibody (**a**). To ensure equal protein in both light and dark conditions, the original lysates were probed with anti-opsin (**c**) antibody. P-IR, phosphorylated IR. Reprinted with permission from [12]

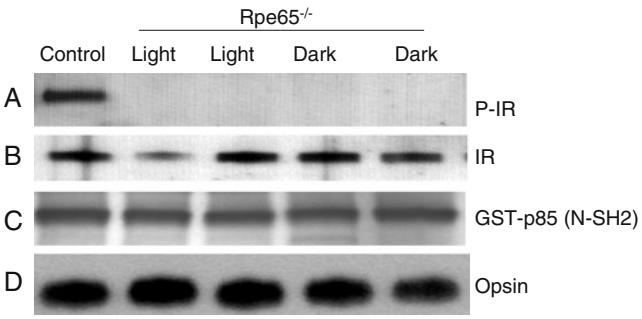

Fig. 7 Absence of IR phosphorylation in $Rpe65^{-/-}$ mice. Dark- and light-adapted $Rpe65^{-/-}$ mouse retinas were lysed and subjected to GST pull-down assays with GST-p85 (N-SH2) domain. The bound proteins were subjected to Western blot analysis with anti-IRβ antibody (**a**). The blot was probed with anti-IRβ (**b**), anti-GST (**c**) and anti-opsin (**d**) antibodies to demonstrated equal protein in each lysate. Control, wild-type retina stimulated with insulin was used as positive control (**a**). P-IR phosphorylated IR. Reprinted with permission from [12]

Photobleaching of Rhodopsin and IR Phosphorylation

To confirm that light-induced IR phosphorylation is signaled through rhodopsin, we examined the phosphorylation of the IR in retinas from retinal pigment epithelium protein (Rpe65) knockout mice that are deficient in 11-*cis*-retinal, the chromophore for rhodopsin [48]. These animals have the opsin apoprotein in their ROS, but do not have a bleachable rhodopsin due to the absence of the chromophore. $Rpe65^{-/-}$ mice were dark-adapted overnight and half were exposed to normal room light for 30 min. Retinal lysates from light- and dark-adapted $Rpe65^{-/-}$ mice were subjected to GST pull-down assays using the p85(N-SH2) domain, followed by Western blot analysis with anti-IRß antibody. The blot shows the absence of light-induced IR phosphorylation in $Rpe65^{-/-}$ mice (Fig. 7a). These results suggest that photobleaching of rhodopsin is necessary for IR phosphorylation.

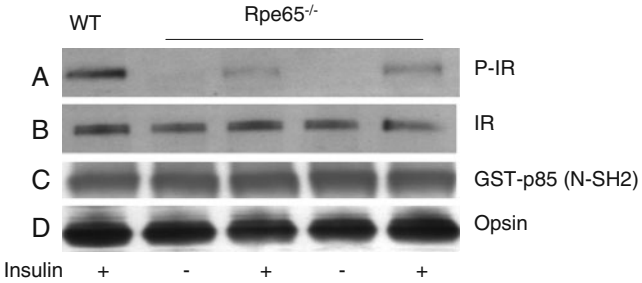

Fig. 8 Insulin-induced activation of the IR in $Rpe65^{-/-}$ mouse retinas. $Rpe65^{-/-}$ mouse retinas were stimulated with 1 μM insulin in organ cultures, lysed and subjected to GST pull-down assay with GST-p85 (N-SH2) domain. The bound proteins were subjected to Western blot analysis with anti-IRβ antibody (**a**). The blot was probed with anti-IRβ (**b**), anti-GST (**c**), and anti-opsin (**d**) antibodies to demonstrated equal protein in each lysate. Wild-type (WT) retinas stimulated with insulin were used as a positive control (**a**). *P-IR* phosphorylated IR. Reprinted with permission from [12]

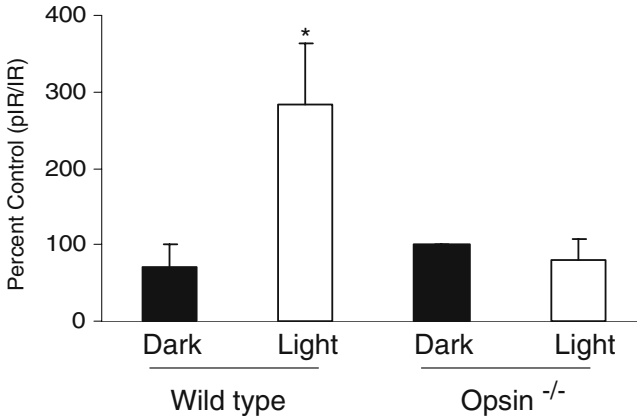

Fig. 9 Activation of the IR through light stimulation of rods. Two hundred micrograms of protein were immunoprecipitated with anti-IRβ antibody from retina lysates from light- and dark-adapted wild-type and homozygous opsin$^{-/-}$ mice. The immunoprecipitates were subjected to Western blot analysis with anti-IR (pYpYpY$^{1158/1162/1163}$) antibody. The blots were stripped and reprobed with anti-IRβ antibody to ensure equal amount of protein in each lane. Densities were calculated from the immunoblots and the results are expressed as phospho-IR/total IR. Data mean ± SD, n=6, *p<0.05. Reprinted with permission from [11]

Insulin Activation of IR Phosphorylation in Rpe65$^{-/-}$ Mice

Rpe65$^{-/-}$ mouse retinas were stimulated with 1 μM insulin in organ cultures, after which the retinas were lysed and subjected to GST pull-down assays using the p85 (N-SH2) domain followed by Western blot analysis with anti-IRß antibody. The results indicate that the IR can be phosphorylated in response to insulin stimulation in Rpe65$^{-/-}$ retinas (Fig. 8a) and suggest the existence of a light-mediated IR pathway in the retina that is different from the known insulin-mediated pathway in non-neuronal tissues. Collectively, these experiments show that photobleaching of rhodopsin is necessary for light-mediated phosphorylation of the IR.

Activation of IR Signaling Through Light Stimulation of Rods

To further explore whether the activation of the IR is signaled through light stimulation of rods, we examined the phosphorylation of the IR in retina lysates from opsin$^{-/-}$ mice. Although these mice have the full complement of rod photoreceptor cells, they lack rod outer segments and scotopic electroretinographic (ERG) signal [49]. However, retinas of opsin$^{-/-}$ mice exhibit normal photopic ERG responses, reflecting the existence of a functional cone transduction pathways [49, 50]. Therefore, these mice afforded the ideal system to test the activation of IR signaling through light stimulation of rods and to determine if any contributions are made by cones. To that end, C57Bl/6 control, and homozygous opsin$^{-/-}$ mice were dark-adapted overnight and half were exposed to normal room light for 30 min and the rest were kept in the dark. Retinal lysates from light- and dark-adapted mice from the two groups were subjected to immunoprecipita-

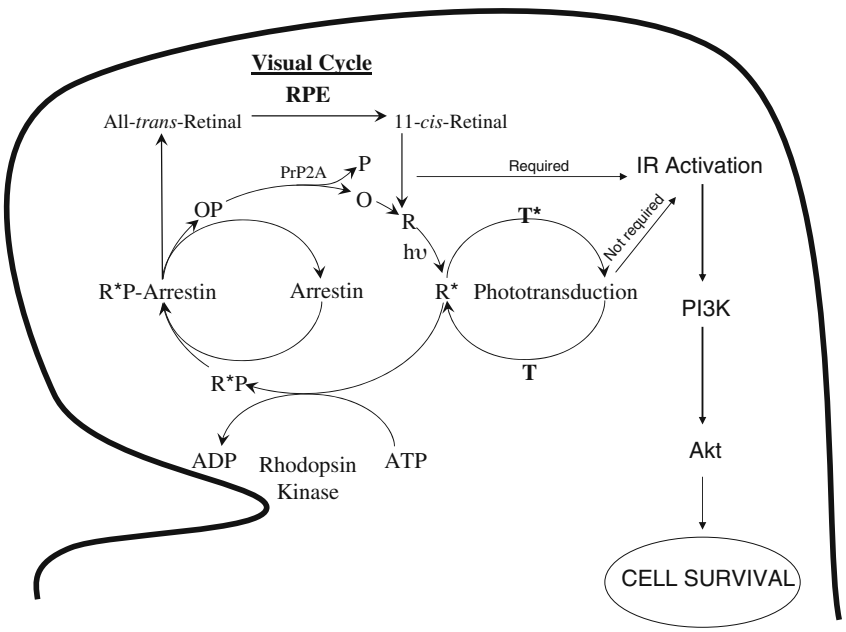

Fig. 10 Working model of IR activation in rod photoreceptor cells. The IR activation in photoreceptor cells requires the photobleaching of rhodopsin but not transducin signaling. Light-activated IR is subsequently associated with PI3K, a cell survival factor and thus regulates the downstream survival pathway. *R** photoactivated rhodopsin

tion with anti-IRß antibody followed by Western blot analysis with anti-IR ($pYpYpY^{1158/1162/1163}$) antibody. The blots were stripped and reprobed with anti-IRß antibody. Densities were calculated from the respective immunoblots and the results are expressed as phospho-IR/total IR. In control mice, we observed a significant increase in IR phosphorylation from light-adapted retinas compared to dark-adapted retinas (Fig. 9). In homozygous $opsin^{-/-}$ mice, the light-dependent activation of the IR was lost (Fig. 9). These results suggest that the light-dependent activation of the IR may be regulated through rod transduction pathway.

Our results also suggest that cross-talk exists between phototransduction and other signal transduction pathways. This cross-talk phenomenon has been shown for other G-protein-coupled receptors (GPCRs), and many tyrosine kinase cascades are regulated by GPCRs [51, 52]. Examples include mitogen-activated protein kinase cascade, extracellular-regulated kinases, and stress-activated protein kinases [52]. Further, the binding of PYK2, a non-receptor protein tyrosine kinase, to N-terminal domain-interacting receptors (Nir) is activated by GPCRs [53]. The Nir proteins are the human homologs of the *Drosophila* retinal degeneration B protein, a protein implicated in the visual transduction pathway in flies [53].

Several studies have shown that the retinal ROS contains intrinsic tyrosine kinase(s) that can be activated by light [37] to phosphorylate several ROS proteins [54, 55]. It has been shown that light exposure in vivo activates Src and promotes its association with a complex containing bleached rhodopsin and arrestin [56]. Retinal brain immunoglobulin-like molecules with a tyrosine-based activation motif protein has also been shown to be tyrosine phosphorylated in vitro in a light-dependent manner [57]. Evidence also indicates that the small G-protein Rac-1 may be regulated by rhodopsin in both *Drosophila* [58] and vertebrates [59]. The α-subunit of heterotrimeric G-protein, $G_{11}\alpha$, does not participate in visual transduction, but the opsin-G_{11}-mediated signaling pathway is important for photic entrainment of the chicken pineal circadian clock [60]. These earlier studies along with the present study clearly suggest that photobleaching of rhodopsin may activate more than one signaling pathway.

Conclusion

The retina expresses PI3K, which is regulated through the light-induced tyrosine phosphorylation of the IR in vivo [11, 61]. Light-induced activation of the retinal IR is independent of insulin secretion and the light effect is localized to photoreceptor neurons [11]. These studies suggest that there exists a cross-talk between phototransduction and other signal transduction pathways. Our studies also suggest that light-induced tyrosine phosphorylation of the IR requires the photobleaching of rhodopsin, but not transducin signaling (Fig. 10). Collectively, these studies suggest that rhodopsin can cross-talk with other signaling pathways in addition to the classical phototransduction cascade.

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