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Light-Induced Tyrosine Phosphorylation of Rod Outer Segment Membrane Proteins Regulate the Translocation, Membrane Binding and Activation of Type II α phosphatidylinositol-5-phosphate 4-kinase

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Abstract

Type II phosphatidylinositol 5-phosphate 4-kinase (PIPKII α) catalyzes the synthesis of phosphatidylinositol-4,5-bisphosphate (PI-4,5-P₂), an essential lipid second messenger that may be involved in the regulation of phototransduction, neuroprotection, and morphogenesis in the vertebrate retina. Here we report that in rodent and transgenic frogs, the light-mediated activity and membrane binding of PIPKII α in rod outer segments (ROS) is dependent on tyrosine phosphorylation of ROS proteins. The greater type II α PIP kinase activity in the light-adapted ROS membrane results from light-driven translocation of PIPKII α from the rod inner segment to ROS, and subsequent binding to the ROS membrane, thus improving access of the kinase to its lipid substrates. These results indicate a novel mechanism of light regulation of the PIPKII α activity in photoreceptors, and suggest that the greater PIPKII α activity in light-adapted animals and the resultant accumulation of PI-4,5-P₂ within the ROS membrane may be important for the function of photoreceptor cells.

Keywords

Type II α Phosphatidylinositol 5-phosphate 4-kinase; Phosphatidylinositol-4,5-bisphosphate; Rod outer segment; Tyrosine phosphorylation; Membrane binding; Lipid second messengers; Retina

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Introduction

The membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PI-4,5-P₂) is a key molecule in the phosphatidylinositol signaling pathway and plays a critical role in diverse cellular processes [1]. PI-4,5-P₂ is the substrate for phospholipase C (PLC) [2] and class I phosphoinositide 3-kinases (PI 3-kinases) [3]. In addition, PI-4,5-P₂ directly affects the localization and activity of many cellular proteins via specific interactions with unique phosphoinositide-binding domains, thereby regulating actin reorganization [4], vesicle trafficking [5], endocytosis [6], exocytosis [7], ion channel activity [8], gene expression [9], and cell migration [10]. The multiple functions of PI-4,5-P₂ imply that the level of this molecule must be spatially and temporally regulated to maintain the integrity of the signaling pathways and cellular functions in which it participates.

Vertebrate retinal photoreceptor cells undergo active phosphoinositide metabolism. A complete phosphoinositide metabolic cycle is found in the vertebrate rod outer segments (ROS) [11–13]. Light activation of phospholipase C in retinas leads to hydrolysis of PI-4,5-P₂ [14]. This reaction produces two important intracellular second messengers, 1,2-diacylglycerol and inositol-1,4,5-trisphosphate, which activate protein kinase C (PKC) [15,16]. Activated PKC can phosphorylate several proteins involved in the phototransduction cascade in the vertebrate retina. Phosphorylation of rhodopsin prevents further activation of transducin and thus attenuates visual excitation [17,18]. Phosphorylation of the inhibitory subunit of phosphodiesterase (PDE_γ) can increase its binding to PDE_{αβ}, which then deactivates the enzyme and reduces cyclic GMP hydrolysis [19]. Cyclic GMP levels can be elevated by the PKC phosphorylation of guanylyl cyclase (GC) [20]. The net result of activation of PI-4,5-P₂-dependent PKC regulates visual transduction by mediating the light adaptation of photoreceptors and helping to restore the dark state of the ROS. PI-4,5-P₂ can activate PDE, which results in the inhibition of current flow through cyclic nucleotide-gated channels in ROS [21]. Modulation of phototransduction gain by changes in phosphoinositide levels has been reported [22]. Further, the light-dependent translocation of phototransduction proteins influences the enzymatic activities of phospholipase D, lipid phosphate phosphatase, diacylglyceride lipase and diacylglyceride kinase, all of which are responsible for the generation of the second messenger molecules [23–25].

Two subfamilies of phosphatidylinositol 5-phosphate 4-kinase kinases (PIPK) produce PI-4,5-P₂ from independent pools of substrates [26]. In addition, type I and II subfamilies of PIPK can localize in different subcellular compartments and are involved in the synthesis of PI-4,5-P₂ in distinct sites within cell [27–30]. These findings suggest that the regulation of specific PIPK accumulation in specific regions of the cell is one of the major mechanisms for mediating the level of PI-4,5-P₂ within specific compartments of the cell. Previously, we reported that PIPKII α is present in vertebrate rod outer segments (ROS), and its activity is mediated by tyrosine phosphorylation *in vitro* [13]. Here, we provide evidence that light induces the tyrosine phosphorylation of unidentified ROS proteins, which facilitates the PIPK binding to ROS membranes *in vivo*. Here, we provide evidence that light induces the tyrosine phosphorylation of unidentified ROS proteins, which promotes translocation of PIPKII α from the cell body and rod inner segments (RIS) to the ROS, and in turn facilitates

the PIPK binding to ROS membrane *in vivo*. This resulting increase in light-stimulated PI-4,5-P₂ synthesis specifically in ROS may play a role in light adaptation.

Experimental Procedure

Materials

Rabbit polyclonal anti- PIPKII α (C-terminal) and anti-type II β PIP kinase (C-terminal) antibodies were obtained from Abgent, (San Diego, CA). Mouse monoclonal anti-phosphotyrosine antibody, anti-PY99, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-flag antibody was purchased from Sigma (St. Louis, MO). The secondary Alexa Fluor 594 goat anti-mouse IgG (catalog no. A-1105) was obtained from Molecular Probes (Eugene, OR). [γ -³²P]ATP was from Dupont NEN (Boston, MA). Monoclonal anti-arrestin antibody was a kind gift from Dr. Paul Hargrave (University of Florida, Gainesville, FL). Echelon Research Laboratories Inc. (Salt Lake City, UT) provided synthetic dipalmitoyl-PI-4-P and -PI-5-P, which had >99% isomeric purity according to the manufacturer's analysis. All other chemicals were obtained from Sigma (St. Louis, MO).

Animals

Albino mice were from The Jackson Laboratory (Bar Harbor, ME) and Sprague Dawley albino rats were from Harlan Sprague Dawley (Indianapolis, IN). Both were maintained in cyclic light (12 h off and 12 h on; 15 lux intensity for rats; 80 lux for mice) and had access to food and water *ad libitum*. All animal work was performed in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals, and the protocols were approved by the IACUC of the University of Oklahoma Health Science Center and the Dean A. McGee Eye Institute.

Plasmids and Retroviral Constructs

Full-length type I γ and type II α PIP kinase cDNA were cloned by PCR amplification by using mouse brain or retina cDNA, respectively, as templates with the following oligos as primers: for type II α PIP kinase, 5' primer GCTCGAGATGGCGACCCCT GGCAACCTG and 3' primer GTCTAGATTACAA GATGTG CCCAATAAAGTCCAAAAAGCG and for type I γ PIP kinase, 5' primer GCTCGAGATGGAGCTAGAGGTGCCGGACG and 3' primer GTCTAGATTATGTGTCGCTCTCGCCGTCG. The PCR products were first cloned into pGEM-T easy vector (Promega, Madison, WI.). The constructs of PIP kinase-pGEMT-easy were sequenced to verify that the PCR products did not contain any mutations. The inserts were excised with XhoI and XbaI restriction endonucleases and then cloned into the respective sites within pcDNA3 (Invitrogen, Carlsbad, CA.). Flag-tagged PIPK-pcDNA3 was generated by inserting the oligonucleotide containing the Flag tag sequence into the XhoI site at the 5' end of PIPK in PIPK-pcDNA3 constructs. Retroviral constructs for PIPKII α were generated by PCR using flag-PIPKII α -pcDNA3 as a template with the following oligonucleotides: sense GTTCGAAGCCACCATGGACTACAAGGATGACGATGACAAA and anti-sense GCTCGAGATG TCG TCCAAC TGCACCAGCACC. Retrovirus productions were carried out as described previously [31].

Cell Lines and Transfection

The 661W cell line (a gift of Dr. Muayyad Al-Ubaidi, OUHSC, Oklahoma City, OK) was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The packed Flag-PIPKII α -Migr-1 retrovirus construct was used to build up the stable expression of PIPKII α in 661W cells. The cells were grown on cover slips, treated with or without pervanadate, and then collected for immunocytochemistry.

Preparation of rat rod outer segments

Three independent sets of ROS were prepared from rat retinas using a discontinuous sucrose gradient as previously described [13]. Each set had 24 rats (12 animals for light- and 12 animals for dark-adaptation). Retinas were homogenized in 4.0 ml of ice-cold 47% sucrose solution containing 100 mM NaCl, 1 mM EDTA, 1mM NaVO₃, 1 mM PMSF, and 10 mM Tris-HCl (pH 7.4) (buffer A). Retinal homogenates were transferred to 15-ml centrifuge tubes and sequentially overlaid with 3.0 ml of 42%, 3.0 ml of 37%, and 4.0 ml of 32% sucrose dissolved in buffer A. The gradients were spun at 82,000 $\times$ g for 1 h at 4 °C. The 32%/37% interfacial sucrose band containing ROS membranes was harvested and diluted with 10 mM Tris-HCl (pH 7.4) containing 100 mM NaCl and 1 mM EDTA, and centrifuged at 27,000 $\times$ g for 30 min. The ROS pellets were resuspended in 10 mM Tris-HCl (pH 7.4) containing 100 mM NaCl and 1 mM EDTA, and stored at -20 °C. All protein concentrations were determined by the BCA reagent following the manufacturer's instructions.

Single-Cell PCR

Single-cell rod photoreceptor cDNA was used as a template (a gift from Dr. Ruben Adler, Johns Hopkins University, School of Medicine, Baltimore, MD). Each type II isoform of PIPK was amplified with the specific primer pairs: for type II α , sense-CGGAAATACACTCAACAGCTCACCA, antisense-CAAGATGTGCCCAATAAAGTCCAAAAAG; for type II β , sense-GCAACCTCCTGAGCTTCCCC and antisense-CGCAGGATGTTGGACA GAACT G; and for type II γ , sense-CCTGAGGGTATCGGAGGCTAC and antisense-GGCAAAGATGTTGGCAATAAAGTCAG. Single-cell cDNA amplification and PCR analysis of PIPKII isoforms were carried out according to the methods described [32]. The β -subunit of cGMP-phosphodiesterase was used as a positive control with the primers: sense-GCCCTGGAGGAAGAAAAGAA and antisense-AGGCAGAGTCCGTATGCAGT to amplify the phosphodiesterase.

Real-Time PCR

The specific primers used for each PIPKII were as follow: for type II α , sense-TGGCTCCTGGAGAGTTTGATCCAAACATT, antisense-TTGCATCGTAATGAGTAAGGATGTCAATAATCGC; for type II β , sense-CCTCTGTGGACGTCTATGCCATGAA, antisense-GCGGCGTGTGCAGCTTTCTTCTTA; and for type II γ , sense-GCCCAGGAGAGTTTGAGTCCTTCAT, antisense-TCTTGGCATCATACTGTGTGCAAGTCAAT. The SYB green PCR master mix was

added to the PCR mixtures containing a pair of specific primers and rat retina cDNA. PCR was carried out with an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) following manufacturer's instructions. Data analysis was performed using the ABI PRISM 7000 sequence detection system software (v1.0; Applied Biosystems, Foster City, CA).

PIPK Assays

Either ROS membranes or PY-99 immunoprecipitates from light- and dark-adapted rat retinas were assayed for PIPKII activity by the method of Bazenot et al. [33] with slight modifications. Briefly, the enzyme assay reaction was performed for 15 min at room temperature in 50 μ L of kinase assay buffer plus 0.2 mM Na_3VO_4 , 50 μ M ATP, 5 μ Ci of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, and with or without 80 μ M synthetic dipalmitoyl-PI-5-P. The reactions were terminated by the addition of 80 μ L of 1 N HCl. Total lipids were extracted with 200 μ L of chloroform/methanol (1/1 v/v), separated using thin-layer chromatography (TLC) in a chloroform/methanol/ammonium hydroxide/water (70/100/25/15 by vol) solvent system, and subjected to autoradiography overnight at -80°C . PIP_2 was identified by comigration with a PI-4,5- P_2 standard, scraped from the TLC plates, and counted for radioactivity.

Type II PIPK-hrGFP Transgenic *Xenopus Laevis*

The expression vector FANSE-xop1.3-hrGFP-N1 was kindly provided by Dr. Papermaster (University of Connecticut Health Center, Farmington, CT). Full-length PIPKII α cDNA that was cloned from mouse retinal cDNA was digested from pGEMT-easy (Promega, WI) with XhoI and BamHI and inserted into the FANSE-xop1.3-hrGFP-N1 plasmid downstream of the opsin promoter and the upstream of hrGFP sequence. An open reading frame between the PIPKII α sequence and the hrGFP sequence remained. The transgenic construct was amplified in *E. coli* DH-5 α (Invitrogen, Carlsbad, CA), purified with Qiagen QIA prepTM spin miniprep kit (Valencia, CA), and linearized by digesting with Not I. The resulting construct was purified with QIAEX II Gel extraction kit (Valencia, CA), incubated with sperm nuclei and then injected into oocytes to generate transgenic *Xenopus laevis* that express PIPKII α -hrGFP according to the methods described by Wiechmann et al. [34].

Tissue Preparation and Immunocytochemistry

About three-week-old transgenic tadpoles were dark adapted overnight and either light adapted at 800 lux using Neodymium lamp (Bulbrite, Moonachie, NJ) for 40 min or kept in the dark. Each set had 20 transgenic tadpoles (10 tadpoles for light- and 10 tadpoles for dark-adaptation). The tadpoles were subsequently fixed in 4% paraformaldehyde in PBS buffer (pH 7.4) either under dim red light or light depending on adaptation status. The samples were dehydrated with 15% sucrose in PBS for 3–4 h, and then 30% sucrose for 16–20 h at 4°C . After dehydration, the tadpoles were embedded with O.C.T. and stored at -80°C overnight. A cryostat microtome was used to cut 15- μ m sagittal sections of the whole-head of the transgenic tadpoles. To verify adaptation status, arrestin was probed, and the antibody conjugated to Alexa Fluor 594 (red) was used as the secondary. To analyze the tissue distribution of PIPKII α -hrGFP, nuclei were labeled with 0.0005% 4',6-diamidino-2-phenylindole (DAPI) (Sigma,). Sections were rinsed in PBS (pH 7.4), and

cover slips were mounted onto the slides with mounting medium (Vector labs, Burlingame, CA). All sections were viewed using Nikon Eclipse 800 microscope. Images were captured with a digital camera and analyzed using Metamorph image analysis software (Version 6.0, Downingtown, PA).

Immunoblotting and Immunoprecipitation

Cells were collected and lysed in Triton X-100 lysis buffer containing 15 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 μ g of aprotinin/ml, 4 μ g of leupeptin/ml, and 2 μ g of pepstatin/ml. Supernatants were obtained after centrifugation at $16,000 \times g$ in microcentrifuge for 30 min at 4°C, and subjected to immunoprecipitation or Western blotting. The details of the methods for immunoblotting and immunoprecipitation of the rat ROS membranes were as previously described [13].

Data Analysis

PIPKII activity assays were done on at least three independent ROS preparations. The data were analyzed by ANOVA or Student's *t*-test, and statistical significance was defined as $P < 0.05$.

Results

PIPKII α is the major isoform in photoreceptor cells

PIPKII exists in three isoforms as α , β , and γ . To determine which PIPKII isoforms are expressed in rod photoreceptors, we used cDNA from five single mouse rod photoreceptor cells and subjected it to PCR using primers specific to mouse PIPKII α , β , and γ . We observed amplification of PIPKII α in 3 out of 5 individual single rod cell cDNAs (Fig. 1A). We could also amplify PIPKII β isoform in 1 out of 5 and none of the cDNAs were positive for PIPKII γ isoform (Fig. 1A). The results indicate that PIPKII α is the major isoform expressed in rod photoreceptors.

Real-time PCR was performed on the mouse retinal cDNA preparations to quantify the level of PIPKII isoform expression in the mouse retina. All three isoforms of PIPKII were expressed in the mouse retina; the mRNA level of type II γ was 2.9 fold and 6.3 fold higher than the mRNA levels of PIPKII α and PIPKII β , respectively (Fig. 1B). Real-time PCR of PIPKII was also performed on rat retina cDNA and the expression patterns of PIPKII isoforms were similar to the mouse retinal patterns (results not showed).

Light-dependent activation of PIPKII α in light-adapted rat ROS but not in light-adapted rat retina in vivo

We have previously reported that several phosphoinositide metabolizing enzymes are regulated through light [35–39]. To investigate the effect of light on PIPKII α activity, rats were dark-adapted overnight and half of the dark-adapted rats were exposed to normal room light for 30 min. Retinas were harvested and used for the preparation of either ROS or retinal lysates, to determine PIPK activity. There was no difference in PIPK activity in lysates from light- and dark-adapted retinas (Fig. 2A and B); however, there was greater

PIPK activity in light-adapted ROS than that in dark-adapted ROS (Fig. 3A and B). These experiments suggest that light does not activate the PIPKII α activity, but rather that it increases PIPKII α binding to ROS membranes.

Activation of PIPKII α activity through light-induced tyrosine phosphorylation of protein(s) in ROS

We showed that PIPKII α in bovine ROS is activated by tyrosine phosphorylation *in vitro* [13]. We hypothesize that light induces the tyrosine phosphorylation of specific protein(s) in the ROS, which leads to the binding of PIPK to the phosphorylated protein. To examine light-induced tyrosine phosphorylation, we immunoprecipitated tyrosine-phosphorylated protein(s) from light- and dark-adapted ROS with an anti-phosphotyrosine antibody (PY99) and measured the PIPK activity. We detected greater PIPK activity associated with the phosphotyrosine immunoprecipitates from light-adapted ROS than with the phosphotyrosine immunoprecipitates from dark-adapted ROS (Fig. 4). These experiments suggest that light causes the tyrosine phosphorylation of ROS protein(s), which provides a docking site for the binding of PIPK.

PIPKII α binding to photoreceptor membranes depends on tyrosine phosphorylation

To confirm PIPKII α binds to photoreceptor membranes and requires tyrosine phosphorylation, stable expression of PIPKII α in the photoreceptor cell line, 661W, was established. The expression of PIPKII α was stained with anti-Flag mouse monoclonal antibody and the Texas red conjugate as the secondary antibody. The expression of Flag-PIPKII α was visualized by using confocal microscopy. In absence of pervanadate in the cell culture, fluorescence associated with PIPKII α remained in the cytosol (5B, 5C, 5E, 5F). In the presence of pervanadate, fluorescence corresponding to PIPKII α was found in the plasma membrane of the cells (Fig. 5H and 5K). Under both tyrosine and non-tyrosine phosphorylation conditions, the expression of the PIPKII α could not be seen in the nuclei of the 661W cells (Fig. 5C, 5F, 5I, 5L). These results indicate that PIPKII α when tyrosine phosphorylated will translocate from the cytosol to the cell membrane in 661 W cells.

Light drives the translocation of PIPKII α from the inner to the outer segments of photoreceptor cells

To further understand the high PIPKII α activity present in light-adapted ROS membranes, a transgenic *Xenopus* model was developed. Full-length type II α PIP kinase cDNA was cloned into the FANSE-xop1.3-hrGFP-N1 expression vector, incubated with sperm nuclei and then injected into oocytes to create the transgenic frogs. The opsin promoter was selected to limit PIPKII α expression to photoreceptor cells. PIPKII α was fused with GFP for easy detection through green fluorescence. Distribution of the PIPKII α -GFP fusion protein in dark- and light-adapted animals was analyzed by immunocytochemistry. Images from the retinas of dark-adapted transgenic tadpoles showed the majority of PIPKII α -GFP fusion protein accumulated in the inner segments of photoreceptors (Fig. 6G), whereas light-adapted *Xenopus* had fusion proteins in the outer segments of the photoreceptor cells (Fig. 6C). Arrestin was observed in the IS of the dark-adapted *Xenopus* and in the ROS after 45 min of light exposure, which confirmed the light and dark adaptation status within *Xenopus*.

(Fig. 6B and F). DAPI staining of the retina indicated the location and orientation of the ROS (Fig. 6A and E). Merging the arrestin immunostaining images with GFP expression images further confirmed that PIPKII α localized to the inner segments of photoreceptors in the dark-adapted tadpoles, and translocated to the outer segments in response to light (Fig. 6D and H).

Discussion

PIPKII α is present in the vertebrate retina. With light stimulation, most of the immunoreactivity of the kinase is located in the ROS of rat photoreceptor cells, and its activity is mediated by tyrosine phosphorylation *in vitro* [13]. In the current study, we report three novel findings: 1) light stimulation is responsible for the activity of PIPKII α *in vivo*; 2) light-induced PIPKII α activity is mediated by tyrosine phosphorylation of an unidentified ROS protein(s); and 3) the higher PIPKII α activity in light adapted or tyrosine phosphorylated ROS is associated with greater amounts of PIPKII α in ROS membranes rather than direct activation of PIPKII α . In addition, real-time PCR and single-cell PCR of rod photoreceptors identified that PIPKII α is the major isoform of PIPK in the photoreceptor cells.

The molecule PI-4,5-P₂ is hydrolyzed by light-activated retinal PLC [14], which results in an activation of PKC, mediation of the light adaptation of photoreceptors, and restoration of the dark state of the ROS [16–20]. PI-4,5-P₂ is also a substrate for PI 3-kinase, an enzyme important for rhodopsin membrane targeting and disc morphogenesis [40]. In addition, PI-4,5-P₂ can stimulate cGMP PDE activity directly [22]. Therefore, it is likely that the increased production of PI-4,5-P₂ in ROS membranes induced by light is functionally significant in the phototransduction cascade, ROS disc morphogenesis, and photoreceptor cell survival.

Several enzymes involved in phosphoinositide turnover are stimulated by light. Those include PLC [41], PI synthetase [35], and PI 3-kinase [36,38]. In the case of PLC, a higher enzymatic activity is found in light-adapted rat ROS than in dark-adapted rat ROS [41]. Similar differences were also found in rat ROS following light exposure *in vivo*. The present study provides evidence to suggest that the activity of PIPKII α , another enzyme of the phosphoinositide cycle in photoreceptor cell, is also mediated by light in rat ROS membrane *in vivo*. Single-cell PCR indicated that PIPKII α is the major isoform of PIPKII in photoreceptors. Significant differences were observed in PIPKII activity from the light- and the dark-adapted ROS. Therefore, it is reasonable to assume that PIPKII α is the isoform responsible for the light-induced up-regulated activity of PIPKII in the ROS membrane. Our data showed that PIPK activity was higher in light-adapted retinal ROS than in dark-adapted ROS (Fig. 1). In general, an activated PIPK can increase the production of PI-4,5-P₂ in ROS. The low level of PI-4,5-P₂ present in light-adapted ROS may be the result of the initiation of PI-4,5-P₂ hydrolysis and/or phosphorylation during light stimulation, which occurred faster than its synthesis.

The increased activity of PIPKII α in light-adapted ROS may be either due to activation of the enzymatic activity or due to translocation of the kinase from the cytoplasm to the ROS

membranes in which the PIPKII α substrate is located. Light-dependent membrane association of phosphoinositide metabolic enzymes, such as PI 3-kinase, PLC γ 1 and diacylglycerol kinase have been reported by several investigators [35,36,38,41,42]. We found that the expression of GFP-fused PIPKII α in our transgenic frog model exhibited a differential localization in photoreceptors in response to light stimulation. The GFP-PIPK was in the RIS and cell body when the *Xenopus* were in the dark. After a 45-min exposure to light, PIPKII α translocated to the ROS. More importantly, the ROS was used for the immunoprecipitations that were prepared on a sucrose step gradient; no soluble proteins were present in these ROS membranes. These results indicate that light stimulates PIPKII α movement from cell body and RIS to ROS, which subsequently increases the association with the ROS membrane and results in the greater PIPKII α activity in the light-adapted ROS membranes than in the dark-adapted ROS membranes.

The mechanism of activation of PIPK is not yet well understood. Phosphorylation and dephosphorylation are reported to play important roles in the regulation of PIPK [43,44]. The cellular level of PI-4,5-P₂ in HEK-293 cells was elevated by use of a protein tyrosine phosphatase inhibitor [45]. Hinchliffe et al. [46] showed the phosphorylated PIPKII α on threonine 376 led to a loss of PIPKII α activity. Castellino and Chao [47] found that PIPKII co-immunoprecipitated with the activated EGF receptor in A431 and PC12-615 cells and with members of the ErbB tyrosine kinase family in activated MCF7 cells. In both instances, tyrosine phosphorylation of the EGF and ErbB receptors was necessary for these receptors to be immunoprecipitated by the anti-PIPKII antibody. We showed that the activity of PIPKII α in ROS was mediated by tyrosine phosphorylation *in vitro* [13]. In the current study, a higher activity of PIPKII α was found in light-adapted ROS than in the dark-adapted ROS. Thus, it seems reasonable to postulate that light may induce the tyrosine phosphorylation activation of PIPKII α activity. Our data showed that the higher kinase activity not only appeared in light-adapted, but also in tyrosine-phosphorylated ROS when compared with dark-adapted and non-tyrosine-phosphorylated ROS in anti-PY immunoprecipitates (Fig. 2). Therefore, the data suggest that the greater PIPKII α activity in light-adapted ROS is related to tyrosine phosphorylation of ROS. These results also suggest that some unidentified receptor tyrosine kinase or adapter protein in the ROS is responsible for the regulation of PIPKII α activity. Ghalayini et al. [37] reported that light stimulates tyrosine phosphorylation of several ROS proteins in rat ROS *in vivo*. Bell et al. [48] identified that at least 10 endogenous proteins in bovine ROS are tyrosine-phosphorylated *in vitro*. The identity of the tyrosine phosphorylated protein(s) responsible for PIPKII α translocation and binding to ROS membranes remains to be determined.

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Figure 1. The level and the isoforms of PIPKII expression in mouse retina and photoreceptors
 Expression of PIPKII isoforms in rod photoreceptor cells. Lanes 1 to 5 were different sets of single rod photoreceptor cell cDNA templates; lane 6 is a negative control. β -PDE was as the positive control (A). mRNA levels of type II α , β and γ were determined by using real-time PCR (B). Means $\pm$ SEM of five independent mouse retina cDNA preparations are shown for each isoform. *Significance between γ and α or β * $P < 0.001$.

Figure 2. PIPKII activity in light- and dark-adapted retinas

Light- and dark-adapted rat retina lysates were assayed for PIPKII activity employing PI-5-P and [γ^{32} P]ATP as substrates. TLC autoradiogram of PIPKII products from light- and dark-adapted rat retinas (A). Quantification of light- and dark-adapted rat retina type II PIP kinase activity is shown by histogram (B). Data are means $\pm$ S.D. (n=3).

Figure 3. PIPKII activity in light- and dark-adapted ROS

Light- and dark-adapted rat ROS [LROS and DROS, respectively] were assayed for PIPKII activity employing PI-5-P and [$\gamma^{32}\text{P}$]ATP as substrates. A, TLC autoradiogram of PIPKII products from light- and dark-adapted rat ROS membranes. B, Quantification of light- and dark-adapted rat ROS membrane PIPKII activity is shown by histogram. Data are means $\pm$ S.D. (n=3). *P < 0.001.

Figure 4. Effect of tyrosine phosphorylation on PIPKII activity

Light- and dark-adapted rat ROS [LROS and DROS, respectively] membranes were immunoprecipitated with either mouse IgG (control) or anti-PY99 antibody followed by measurement of the PIPKII activity employing PI-5-P and [γ^{32} P]ATP as substrates. TLC autoradiogram of PIPKII products from light- and dark-adapted rat ROS membrane (A). Radioactive spots of PI-4,5-P₂ were scraped from the TLC plates and counted (B). Data are means $\pm$ S.D. (n=4). The PIPKII activities from light and dark were significant at *P < 0.01.

Figure 5. Localization of PIPKII α in photoreceptor cells

Flag-recombinant Flag-type II PIPK α is stably expressed in the 661W cell line. The cells were cultured for 20 h with DMEM plus 10% FBS and then starved for 4 h. The cells were treated with either PBS (A, B, C, D, E and F) or with 150 μ M pervanadate (G, H, I, J, K and L) for 20 min and then starved 6 hours. The resulting cells were fixed, probed with anti-flag as the primary antibody and Texas red conjugated as the secondary antibody. Confocal microscopy was conducted to view the subcellular localization of Flag-type II α PIP kinase. Panels A, B, C, G, H and I were obtained using lower magnification. Panel D, E,

F J, K, and L were obtained using higher magnification. Panel A, D, G and J show DAPI stained for nuclei. Panel B, E, H and K show cells probed the anti-flag primary antibody and Texas red conjugated as the secondary antibody for PIPKII α protein. Panel C, F, I and L were the merger of the images of the DAPI staining and immunostaining of PIPKII α . The nuclear staining and the merge of the images for cytosol and nuclei showed the position of the nucleus, cytosol and plasma membrane.

Figure 6. Light-dependent translocation of PIPK in transgenic frog photoreceptors

PIPK translocation was examined in dark- (E–H) and light-adapted (A–D) transgenic frogs. Nuclei were stained with DAPI in light- (A) and in dark (E)-adapted tadpoles. Endogenous arrestin was probed with anti-arrestin and the secondary antibody was conjugated with Texas red in light (B)- and dark (F)-adapted tadpoles. PIPKII α -hrGFP fluoresced green in light (C)-and dark (G)-adapted tadpoles. The yellow fluorescence represents colocalization of recombinant PIPKII α -hrGFP (green) and endogenous arrestin immunoreactivity (red) in

photoreceptor cells in light (D)- and dark (H)-adapted tadpoles. OS, outer segments; IS, inner segments.