

## Thematic Review Series: Lipids and Lipid Metabolism in the Eye

## Phosphoinositide 3-kinase signaling in the vertebrate retina

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**Abstract** The phosphoinositide (PI) cycle, discovered over 50 years ago by Mabel and Lowell Hokin, describes a series of biochemical reactions that occur on the inner leaflet of the plasma membrane of cells in response to receptor activation by extracellular stimuli. Studies from our laboratory have shown that the retina and rod outer segments (ROs) have active PI metabolism. Biochemical studies revealed that the ROs contain the enzymes necessary for phosphorylation of phosphoinositides. We showed that light stimulates various components of the PI cycle in the vertebrate ROs, including diacylglycerol kinase, PI synthetase, phosphatidylinositol phosphate kinase, phospholipase C, and phosphoinositide 3-kinase (PI3K). This article describes recent studies on the PI3K-generated PI lipid second messengers in the control and regulation of PI-binding proteins in the vertebrate retina.—Rajala, R. V. S. Phosphoinositide 3-kinase signaling in the vertebrate retina. *J. Lipid. Res.* 2010. 51: 4–22.

## THE PI CYCLE

In the early 1950s, Hokin and Hokin (1, 2) discovered that addition of acetylcholine to brain slices stimulated the incorporation of phosphate and inositol but not glycerol into lipids; the major products of this incorporation were phosphatidylinositol (PI) and phosphatidic acid. Subsequent studies defined the reactions of the PI cycle and showed that the initial event was receptor-mediated activation of a phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PI-4,5-P<sub>2</sub>) to 1,2-diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP<sub>3</sub>). This increase in lipid synthesis reported by the Hokin was a recovery reaction that rapidly replenished PI separate from de novo PI synthesis. The role of 1,4,5-IP<sub>3</sub>

was established by Streb et al. (3) in their classic paper that showed elevations in IP<sub>3</sub> caused intracellular release of bound calcium. Subsequently, 1,2-DG was found to stimulate protein kinase C (PKC), a serine/threonine kinase that phosphorylates a number of cellular proteins (4). Activation of the PLC/PKC cascade affects a variety of cellular events, including secretion, phagocytosis, smooth muscle contraction, proliferation, neurotransmission, and metabolism [see reviews by Rhee (5), Rhee and Choi (6), and Berridge (7)]. In 1989, Auger et al. (8) discovered the receptor-mediated conversion of PI-4,5-P<sub>2</sub> to phosphatidylinositol 3,4,5-trisphosphate (PI-3,4,5-P<sub>3</sub>) in platelet-derived growth factor (PDGF)-stimulated smooth muscle cells and PI to phosphatidylinositol 3-phosphate (PI-3-P) in yeast. Subsequent studies showed that phosphorylation of the D3-position of the inositol ring by phosphoinositide 3-kinase (PI3K) can be stimulated by several extracellular molecules, including PDGF, insulin, insulin-like growth factor-1 (IGF-1), and nerve growth factor [see reviews by

Abbreviations: AMD, age-related macular degeneration; BDNF, brain derived neurotrophic factor; bFGF, basic fibroblast growth factor; CNTF, ciliary neurotrophic factor; DG, diacylglycerol; DHA, docosahexaenoic acid; 4E-BP, eukaryotic translation initiation factor 4E binding protein; eIF, eukaryotic initiation factor; EPO, erythropoietin; GPCR, G-protein coupled receptor; Grb14, growth factor receptor-bound protein 14; GSK3, glycogen synthase kinase 3; IGF-1, insulin like growth factor 1; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; IR, insulin receptor; IRS, insulin receptor substrate; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; PDGF, platelet-derived growth factor; PDK1, phosphoinositide-dependent protein kinase-1; PH, pleckstrin homology; PHR1, pleckstrin homology domain retinal protein; PI, phosphatidylinositol; PI-3,4-P<sub>2</sub>, phosphatidylinositol 3,4-bisphosphate; PI-3,4,5-P<sub>3</sub>, phosphatidylinositol 3,4,5-trisphosphate; PI3K, phosphoinositide 3-kinase; PI-3-P, phosphatidylinositol 3-phosphate; PI-4,5-P<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; p70S6K, ribosomal protein S6 kinase; PTEN, phosphatase and tensin homolog; PTP, protein tyrosine phosphatase; RGC, retinal ganglion cell; ROS, rod outer segment; RPE, retinal pigmented epithelium; SH, Src homology; VSP34, vacuole sorting protein 34; 4E-BP, eukaryotic initiation factor 4E binding protein.

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Vanhaesebroeck and Waterfield (9), and Datta et al. (10)]. The formation of all of these phosphoinositides has been demonstrated in mammalian cells [reviewed by Rameh and Cantley(11)] and we have shown their formation (except for PI-3-P) in intact rod outer segment membranes (ROSs) prepared from fresh bovine retinas (12–15).

## ACTIVATION OF PHOSPHOINOSITIDE SIGNALING PATHWAYS

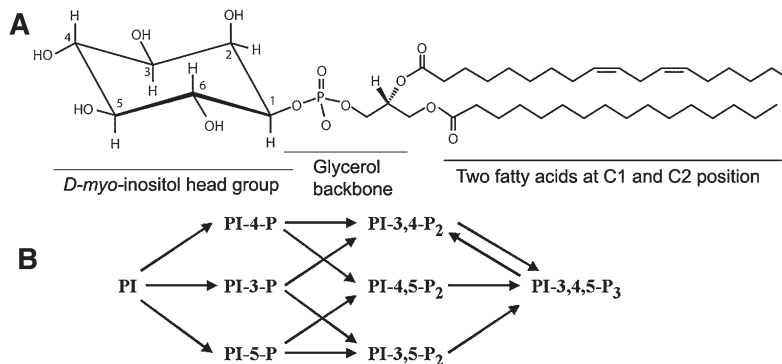
PIs, as components of phospholipids in the cell membrane, contain a *D-myo*-inositol head group, a glycerol backbone, and two fatty acids at the C1 and C2 acyl positions of glycerol (Fig. 1A). Phosphorylation of multiple free hydroxyls in the inositol head group generates several phosphorylated PI derivatives. Differential phosphorylation at the 3, 4, and 5 positions allows for the generation of seven distinct phosphoinositides (Fig. 1B). The intracellular levels of the phosphoinositides are controlled by PI-specific kinases and phosphatases that can rapidly convert one phosphoinositide into another (16). PI signals regulate signal transduction, cytoskeletal assembly, membrane binding, and fusion that are spatially restricted to specific membrane domains (16). The classic work of Grado and Ballou (17) characterized phosphatidylinositol 4-phosphate (PI-4-P) and PI-4,5-P<sub>2</sub> as D4 phosphoinositides. The discovery that phosphorylation at the D3 hydroxyl of the inositol head group (18) led to the generation of PI-3-P, phosphatidylinositol 3,4-bisphosphate (PI-3,4-P<sub>2</sub>), and PI-3,4,5-P<sub>3</sub>, which are referred to as D3 phosphoinositides, increased this diversity. The formation of all of these phosphoinositides has been demonstrated in mammalian cells (11).

### PI3K

The PI3K pathway is highly conserved among different species, including *Drosophila melanogaster*, *Caenorhabditis*

*elegans*, and mammals (9, 19). Studies in *Drosophila* have established the involvement of this pathway in the regulation of cell size and number (20–22). Genetic studies in *C. elegans* have linked this pathway to regulation of dauer formation. The dauer phenotype is a larval state characterized by developmental arrest and reduced metabolic rate triggered by adverse environmental conditions, including nutrient deprivation and overcrowding. Genetic dissection of the genes involved in this pathway led to the identification of the *daf* (*d*auer *a*ffected) genes (23, 24), some of which are homologs of the mammalian components of the insulin-PI3K signaling pathway.

PI3K belongs to the large family of PI3K-related kinases or PIKK. Other members of the family include mammalian target of rapamycin (mTOR), ataxia-telangiectasia mutated, ataxia-telangiectasia mutated and RAD3 related, and DNA-dependent protein kinase. All possess the characteristic PI3K-homologous kinase domain and a highly conserved carboxy-terminal tail (25). However, only PI3K is known to have an endogenous lipid substrate. Mammalian cells carry at least eight different genes with significant homology and yeast contains only one PI3K gene (26). The PI3K enzymes are broadly divided into classes I, II, and III, depending upon their substrate specificity (27, 28) (Table 1). The class I PI3K phosphorylates PI-4,5-P<sub>2</sub> to produce PI-3,4,5-P<sub>3</sub> and class III enzymes produce PI-3-P from PI (16, 29). The activity of class II PI3K is debatable and probably involved in the production of both PI-3,4-P<sub>2</sub> and PI-3-P (26). Existing data suggest that class II and III PI3K may be involved in vesicular trafficking (30, 31). The class I PI3K is the most characterized and best understood enzyme (29). Class I PI3K enzymes are heterodimers composed of a catalytic subunit and an adaptor regulatory subunit (32). Class I catalytic subunits share significant homology and have an apparent molecular weight of p110 kDa and thus are referred to as p110 subunits (32). There are four class I *PI3Kp110* genes known in mammals; these are named *Pik3ca*, *Pik3cb*, *Pik3cg*, and *Pik3cd* and are referred



**Fig. 1.** A: Phosphatidylinositol (PI). B: Naturally occurring PIs. Phosphatidylinositol contain a *D-myo*-inositol head group, a glycerol backbone, and two fatty acids at the C1 and C2 acyl positions of glycerol (A). Phosphorylation of multiple free hydroxyls in the inositol head group generates several phosphorylated PI derivatives. Differential phosphorylation at the 3, 4, and 5 positions allows for the generation of seven distinct phosphoinositides (B). PI-4-P, phosphatidylinositol 4-phosphate; PI-3-P, phosphatidylinositol 3-phosphate; PI-5-P, phosphatidylinositol 5-phosphate; PI-3,4-P<sub>2</sub>, phosphatidylinositol 3,4-bisphosphate; PI-4,5-P<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PI-3,5-P<sub>2</sub>, phosphatidylinositol 3,5-bisphosphate; PI-3,4,5-P<sub>3</sub>, phosphatidylinositol 3,4,5-trisphosphate.

TABLE 1. PI3K family members

Class	Catalytic Subunit	Regulatory Subunit	Activator	Phosphoinositide Products
1a	p110 $\alpha$ p110 $\beta$ p110 $\delta$	p85	RTK, RAS	PI-3-P PI-3, 4-P <sub>2</sub> PI-3,4,5-P <sub>3</sub>
1b	p110 $\gamma$	p101		PI-3-P PI-3, 4-P <sub>2</sub> PI-3,4,5-P <sub>3</sub>
II	PI3KC2 $\alpha$ PI3KC2 $\beta$ PI3KC2 $\gamma$		RTK, integrins	PI-3-P PI-3, 4-P <sub>2</sub>
III	VSP34p			PI-3-P

to as PI3K $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  (33). *Pik3ca* and *Pik3cb* genes are ubiquitously expressed; *Pik3cg* and *Pik3cd* genes are specifically found in leukocytes with the exception of *Pik3cg*, which was recently detected in the cardiovascular system (34). The protein products of these genes have the highest homology at the N-terminal end, and all have a GTPase Ras domain, a C2-lipid binding domain, the phosphatidylinositol kinase domain, and a catalytic domain (35).

In mammals, the PI3K catalytic subunits (p110 $\alpha$ , p110 $\beta$ , and p110 $\delta$ ) are bound to any of five distinct regulatory subunits (p85 $\alpha$ , p85 $\beta$ , p55 $\gamma$ , p55 $\alpha$ , and p50 $\alpha$ , collectively referred to as “p85s”) (36). These p85 adapters result from three genes: *Pik3r1*, *Pik3r2*, and *Pik3r3* (35). The *Pik3r1* can be expressed in splice variants that encode p85 $\alpha$ , p55 $\alpha$ , and p50 $\alpha$ . The adapters p85 $\alpha$  and p85 $\beta$  are ubiquitously expressed (35), whereas p50 $\alpha$  and p55 $\alpha$  are present in fat, muscle, liver, and brain (37, 38), and p55 $\gamma$  is mainly expressed in the brain (39). 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 IA p110 catalytic domains (40).

Phosphorylation of cell surface receptors can be stimulated by several extracellular molecules, including PDGF, insulin, IGF-1, and nerve growth factor (9, 10). The adaptor subunit of these enzymes contains an Src homology (SH)2-domain that mediates recruitment to phosphotyrosine residues on the cytoplasmic domain of receptors, which results in the activation of the PI3K (29). Class II enzymes preferentially phosphorylate PI and PI-4-P, but not PI-4,5-P<sub>2</sub>, in vitro and contain a C2 domain that can mediate membrane interactions (11, 29). Evidence also indicates that class IA PI3K $\beta$  is activated by both G-protein-coupled receptor (GPCR) and tyrosine kinase receptors (41). The N-terminal p85-binding motif is absent in class IB PI3K and PI3K $\gamma$ , but interacts with the p101 (42) and p84/87 adaptor for regulation (43, 44). The GPCR can activate PI3K $\gamma$  and is regulated by free G $_{\beta\gamma}$  subunits of heterotrimeric G proteins, mainly the subtype G $_i$  (34). Class II comprises three members, PI3KC2 $\alpha$ ,  $\beta$ , and  $\gamma$ , which are characterized by a carboxy-terminal phospholipid-binding domain. Although no regulatory subunit has been identified, class II enzymes are predominantly membrane-bound and activated by membrane receptors including receptor tyrosine kinases and integrins (35). Class III enzymes, which phosphorylate only PI, are heterodimers of a catalytic subunit associated with the serine/threonine protein

kinase adaptor subunit that is required for membrane recruitment (16, 29). The class III kinase, VSP34p, is responsible for producing the majority of the cellular PI-3-P and is involved in protein trafficking through the lysosome.

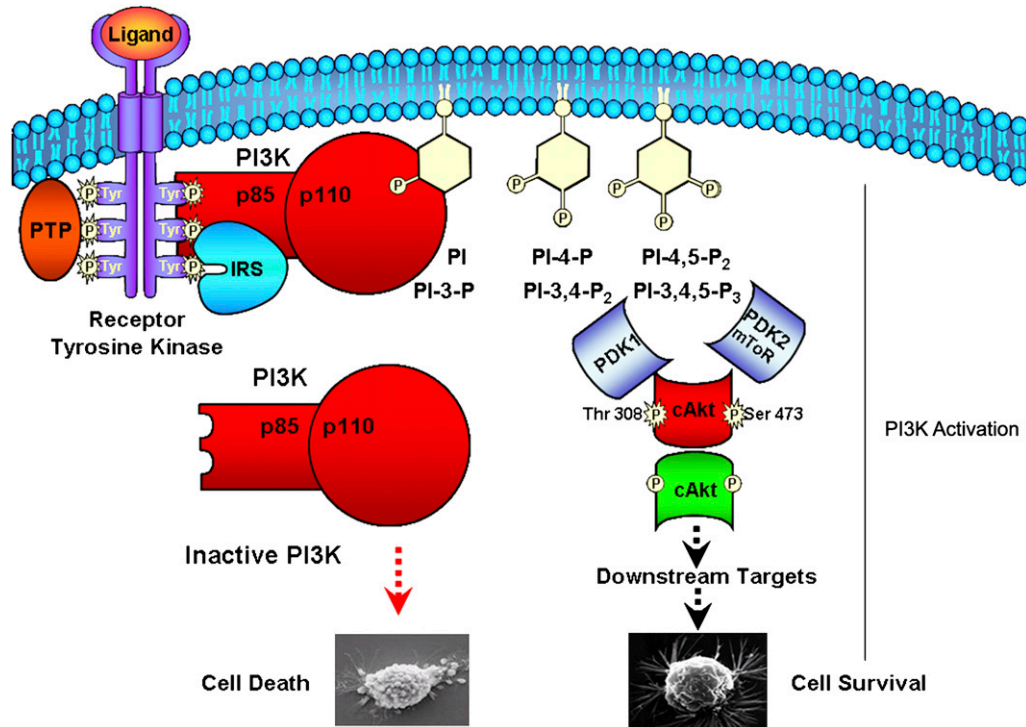
The regulatory p85 subunit contains an SH3 domain capable of binding to proline-rich sequences, a region of homology to the breakpoint cluster region gene product, a p110 binding domain, and two SH2 domains (N and C terminus). The regulatory subunit maintains the p110 catalytic subunit in a low-activity state in quiescent cells and mediates activation by direct interaction with phosphotyrosine residues of activated growth-factor receptors or adaptor proteins (29). The activated PI3K converts the plasma membrane lipid PI-4,5-P<sub>2</sub> to PI-3,4,5-P<sub>3</sub>. The termination of PI3K signaling by the degradation of PI-3,4,5-P<sub>3</sub> can be mediated by at least two different types of phosphatases. The SH2-containing phosphatases, SHIP1 and SHIP2, dephosphorylate the 5-position of the inositol ring to produce PI-3,4-P<sub>2</sub> (45). Although this dephosphorylation impairs some signaling downstream of PI3K, PI-3,4-P<sub>2</sub> can also mediate PI3K-dependent responses, and may mediate events that are independent of those stimulated by PI-3,4,5-P<sub>3</sub>. Loss of SHIP2 causes a dramatic increase in insulin sensitivity, suggesting that this phosphatase critically regulates PI3K signaling downstream of insulin (45). In contrast, the phosphatase PTEN (phosphatase and tensin homolog) dephosphorylates the 3-position of PI-3,4,5-P<sub>3</sub> to produce PI-4,5-P<sub>2</sub> (46). The loss of PTEN protein function has been found in a large fraction of advanced cancers, suggesting that uncontrolled signaling through PI3K may contribute to metastatic cancers (47).

Class IA PI3K $\alpha$  has been extensively studied in the retina and photoreceptors (14, 15, 48–52). The retinal transducin  $\beta\gamma$  subunits can stimulate G-protein-regulated PI3K, presumably PI3K $\gamma$  or class IA PI3K $\beta$  (53). Class III PI3K-generated PI-3-P was found to be involved in the vesicular membrane targeting of rhodopsin (54). Biochemical and functional studies related to other class of PI3K or different isoforms have yet to be performed on the retina and photoreceptors.

## ACTIVATION OF CLASS IA PI3K

The growth factor-stimulated PI3K pathway is described in **Fig. 2**. PI3K activity increases in response to PDGF binding to its receptor, in a large part because the class IA p85/p110 complex is translocated from the cytosol to the plasma membrane by the direct binding of the p85 SH2 domain to tyrosine-phosphorylated sites on the receptor (55, 56). Insulin receptor (IR) activation stimulates intrinsic tyrosine kinase activity that leads to autophosphorylation. Unlike the epidermal growth factor and PDGF receptors, the phosphorylated IR does not usually directly associate with SH2 proteins (57). Rather, the activated IR usually phosphorylates insulin receptor substrate (IRS)-1, a principal substrate of the IR, on multiple tyrosine residues, which, in turn, recognizes and binds to the SH2 domain of PI3K (58). With few exceptions (59), PI3K is activated when





**Fig. 2.** Signaling pathway downstream of PI3K affect cell death and cell survival. Activation of growth factor receptor protein tyrosine kinases results in autophosphorylation on tyrosine residues and transphosphorylation of adaptor proteins such as IRS. PI3K can also be stimulated by integrin-dependent cell adhesion and by G-protein coupled receptors (not shown). PI3K is brought to the membrane and activated by directly binding to phosphotyrosine residues of growth factor receptors or adapters. The lipid products of PI3K, PI-3,4-P<sub>2</sub>, or PI-3,4,5-P<sub>3</sub>, recruit a subset of signaling proteins with pleckstrin homology (PH) domains to the membrane where they are activated. The proteins include protein serine-threonine kinases, protein tyrosine kinases, exchange factors, and adaptor proteins (downstream effectors). Ultimately these proteins initiate complex sets of events that control protein synthesis, actin polymerization, cell survival, and cell entry. PI3K, phosphoinositide 3-kinase; IRS, insulin receptor substrate; PH, pleckstrin homology.

phosphorylated IRS-1 binds to the SH2 domain in its p85 $\alpha$  regulatory subunit, which establishes a direct molecular connection between circulating insulin and cellular PI3K (60). Insulin, IGF-1, IGF-2, brain-derived neurotrophic factor (BDNF), basic fibroblast growth factor (bFGF), ciliary neurotrophic factor (CNTF), erythropoietin (EPO), and PDGF can activate the PI3K pathway in the retina. Insulin and IGF-1 can stimulate PI3K in the photoreceptors (52, 61). PDGF can stimulate PI3K in ganglion (62, 63) and retinal pigmented epithelium (RPE) cells (63), whereas bFGF activates PI3K in Muller glial cells (64). EPO and BDNF can activate PI3K in ganglion cells (65, 66). CNTF protects the murine retina from constant light via activation of PI3K in vivo (67). We (68) and others (52) have shown that IR activation does not induce the activation of IRS-1 but does induce IRS-2 activation in the retina. We have also found that the IR can directly interact with the p85 subunit of PI3K (50). It should be mentioned that IRS-2 knockout mice lose up to 50% of their photoreceptors by 2 weeks of age, resulting from increased apoptosis (69).

#### PI3K REGULATED DOWNSTREAM EFFECTORS

The serine/threonine protein kinase B/Akt is a key mediator of signal transduction processes downstream of

PI3K. Upon growth factor stimulation, PI3K is the key enzyme catalyzing the transfer of phosphate from ATP to the D-3 position of the inositol ring of membrane-localized phosphoinositides, thereby generating 3'-phosphorylated phosphoinositides such as PI-3-P, PI-3,4-P<sub>2</sub> and PI-3,4,5-P<sub>3</sub>. This lipid second messenger recruits Akt to the membrane by engaging its pleckstrin homology (PH) domain (70–75). Once localized in the membrane, Akt is phosphorylated on two sites of the protein: the activation loop (Thr 308) and the hydrophobic motif (Ser 473) (76, 77). Phosphorylation at the activation domain is mediated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) (78) and the kinase that mediates phosphorylation at the hydrophobic motif is catalyzed by mTOR (79). Akt is fully activated when it is phosphorylated on both sites, after which it dissociates from the membrane and phosphorylates many substrates in the cytoplasm and nucleus (80–83) and thereby plays an important role in the regulation of metabolism, apoptosis, cell cycle, and transcription of various genes (84, 85).

The PI3K-generated phosphoinositides are able to recruit phospholipid binding proteins such as PDK1 and Akt. PDK1 phosphorylates Akt, and the activated Akt phosphorylates multiple proteins on serine and threonine residues (86). These substrates include glycogen synthase

kinase 3 (GSK3), ribosomal protein S6 kinase (p70S6K), Bcl-2<sub>XL</sub>-antagonist causing cell death, IκB kinase, endothelial nitric oxide synthase, mTOR, eukaryotic translation initiation factor 4E binding protein (4E-BP), forkhead transcriptional factor, and caspase 9. Through phosphorylation of these targets, Akt carries out its role as a key regulator of a variety of critical cell functions including glucose metabolism, cell proliferation, and survival.

Insulin stimulates protein synthesis via two major effects: the rapid activation of existing components of the translational apparatus and the longer term increase in the capacity of the cell or tissue for protein synthesis, which includes an increase in ribosome number (87). The rapid activation of protein synthesis by insulin is mediated primarily through the GSK3β inactivation mediated by activated Akt. Insulin, by inhibiting GSK3β, stimulates the dephosphorylation and activation of eukaryotic initiation factor (eIF)2B, a protein required for recycling of eIF2, itself necessary for all cytoplasmic translation initiation events. Insulin-activated Akt also phosphorylates the tuberous sclerosis TSC1–TSC2 complex to relieve its inhibitory action on mTOR, a key controller of translation initiation and elongation. The cap-binding factor eIF4E can be sequestered in inactive complexes by 4E-BP1. By activating mTOR, insulin induces phosphorylation of 4E-BP1 and its release from eIF4E, allowing eIF4E to form initiation factor complexes. It also induces dephosphorylation and activation of eukaryotic translation elongation factor 2 to accelerate elongation. Insulin inactivates eukaryotic translation elongation factor 2 kinase by increasing its phosphorylation at several mTOR-regulated sites. Insulin also stimulates synthesis of ribosomal proteins by promoting recruitment of their mRNAs into polyribosomes.

p70S6K is one of the downstream effectors of PI3K (88–92). To date, PI3K (93, 94), PDK1, Akt (95–98), PKC (99, 100), the Rho family of small G proteins, and mTOR (101–106) are thought to be the upstream effectors of S6K1 phosphorylation. Many growth factors, including insulin, activate p70S6K in a PI3K-dependent manner. In addition, amino acids can activate p70S6K in a PI3K-independent manner. For example, branched-chain amino acids such as leucine are sufficient to activate mTOR, resulting in an increase in p70S6K phosphorylation and thereby activating it. mTOR is also in a pathway downstream of Akt. Akt is typically activated upon growth factor (such as insulin, IGF-1) stimulation of a cell. Akt then activates mTOR (by inhibiting the TSC complex), leading to p70S6K activation. Insulin promotes rat retinal neuronal cell survival in a p70S6K-dependent manner and this pathway is completely inhibited by the PI3K inhibitor LY294002. The mechanism by which p70S6K supports insulin-stimulated retinal cell survival remains uncertain. It has been suggested that internal ribosomal initiation of mRNA translation, a step that is affected by rapamycin and p70S6K, is critical for survival of cells under transient apoptotic stress (107). Postmitotic neurons require protein synthesis for survival (108) so it is possible that insulin stimulates protein synthesis via a rapamycin-dependent

mechanism. In support of this hypothesis are the data that show stimulation of the insulin/mTOR pathway delays cone death in a mouse model of retinitis pigmentosa (109). Cone cell death in retinitis pigmentosa could, at least in part, be a result of the starvation of cones. We recently observed that cone photoreceptor-specific deletion of p85 subunit of PI3K resulted in age-related cone degeneration (unpublished observations). These studies highlight the importance of protein synthesis in cone cell survival.

#### PROTEIN TYROSINE PHOSPHATASE-1B AND PI3K PATHWAY

The extent of tyrosyl phosphorylation on a given protein is controlled by the reciprocal action of protein tyrosine kinase and protein tyrosine phosphatase (PTP) activities. Specific PTPs, including Leukocyte Antigen-Related (LAR), SHP-2, and PTP1B, have been implicated in the regulation of normal IR signaling (110–123). Of these PTPs, PTP1B has received significant attention because it is an abundant enzyme expressed in all insulin-sensitive tissues (124, 125). PTP1B is an abundant and widely expressed nonreceptor tyrosine phosphatase, which is thought to be a key negative regulator of insulin signaling (126, 127). It was previously shown that PTP1B overexpression results in the inhibition of IR and IRS-1 signaling (117, 122, 128). Furthermore, introduction of anti-PTP1B antibodies into cells enhances IR signaling (129). Global deletion of PTP1B in mice results in increased systemic insulin sensitivity, enhanced glucose uptake into skeletal muscle, and improved glucose tolerance (130, 131). Increased and prolonged tyrosine phosphorylation of the IR was also found in mice lacking PTP1B (130, 131). The increased insulin sensitivity is due to the absence of PTP1B and results from failure to dephosphorylate the IR (130, 131). The IR, PI3K, and Akt pathway was found to be downregulated in diabetic retinopathy (132). Recently, we reported that diabetes downregulates IR activation and increases the activity of PTP1B (133).

Akt phosphorylates substrates at the consensus motif RXXRXS/T. PTP1B contains this motif (RYRDVS<sup>50</sup>), which can be phosphorylated by Akt on Ser<sup>50</sup> (134). Insulin stimulation can cause a significant increase in phosphorylation of PTP1B that can be blocked by pretreatment of cells with wortmannin or cotransfection of a dominant inhibitory Akt mutant. Phosphorylated PTP1B has an impaired ability to dephosphorylate the IR (134). These studies suggested that PTP1B is a novel substrate for Akt and that phosphorylation of PTP1B by Akt at Ser<sup>50</sup> may negatively modulate its phosphatase activity, creating a positive feedback mechanism for IR/PI3K signaling. Currently, we do not know whether such a mechanism exists in the retina; however, Akt2 knockout mice have increased susceptibility to light-induced photoreceptor degeneration (135). It is possible that failure to deactivate PTP1B by Akt2 may result in the degeneration phenotype.

## PI3K SIGNALING IN DIABETIC RETINOPATHY

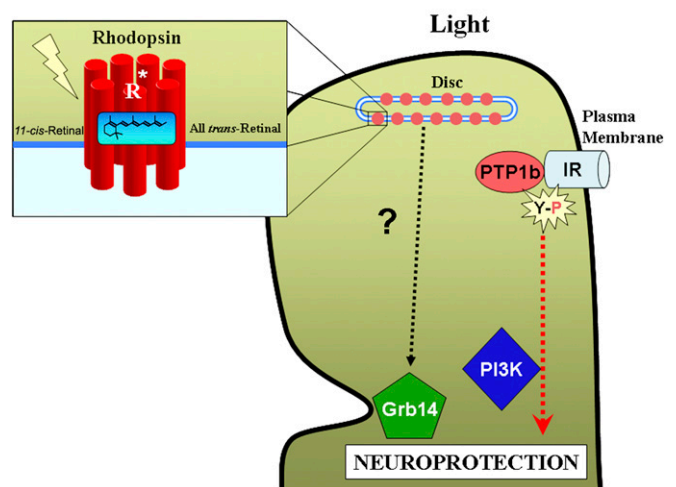
PI3K and Akt are downstream effectors of insulin signaling (136) as well as important signaling molecules in the regulation of glycogen metabolism in myocytes, lipocytes, and hepatocytes (137, 138). Uncoupling of insulin signaling at PI3K-Akt in these cell types in response to high glucose concentrations has been implicated in the pathogenesis of insulin resistance and Type II diabetes (139). However, PI3K-Akt also plays an important role in endothelial cells by regulating angiogenesis (140), proliferation (141), microvascular permeability (142), survival (86), cellular transformation, and embryonic differentiation (143). It has been shown previously that compromised muscle PI3K signaling contributes to symptoms of impaired insulin response and hyperlipidemia associated with human type 2 diabetes (144). The PI3K/Akt pathway plays an important role in diabetic retinopathy. The diabetic rat retina has a loss of PI3K, Akt-1 and Akt-3, mTOR, and p70S6K activities, but has greater GSK3 $\beta$  activity than in the wild-type retina (145). These studies clearly highlight the importance of this pathway in diabetic retinopathy.

We recently reported that in insulin-lacking cultures, photoreceptors from wild-type rat retinas exhibited an abnormal morphology with a wide axon cone and disorganization of the actin and tubulin cytoskeleton (146). Photoreceptors from IR knockout mouse retinas also exhibited a similar abnormal morphology (146). A novel finding in this study was that addition of docosahexaenoic acid (DHA), a photoreceptor trophic factor, restored normal axonal outgrowth in insulin-lacking cultures (146). These data suggest that IR-signaling pathways regulate actin and tubulin cytoskeletal organization in photoreceptors; they also imply that insulin and DHA activate at least partially overlapping signaling pathways that are essential for the development of normal photoreceptors. We further reported that high glucose and high insulin levels affected the cytoskeletal organization of the developing photoreceptors, and DHA prevented the abnormal development of these photoreceptors in culture (146). Further, the combination of insulin and DHA protects the Cytochalasin D mediated disruption of actin cytoskeleton of the developing photoreceptors (146). Phosphoinositides are the key molecules for regulation of actin cytoskeletal organization and membrane traffic from the plasma membrane (147). In photoreceptors, we previously reported that the IR regulates PI3K and Akt activation (49, 50, 148). Further, we recently reported that 3'-phosphoinositides generated through light-induced tyrosine phosphorylation of IR regulates the reorganization of actin cytoskeleton (61). The results from our current study also suggest that IR activation may regulate the actin cytoskeletal organization. Dysfunction of the actin cytoskeleton is a key event in the pathogenesis of diabetic nephropathy (149), diabetic neuropathy (150, 151), and diabetic cardiomyopathy (152, 153). Our findings that high glucose and high insulin concentrations induced severe morphological changes in actin cytoskeleton, altering growth cone struc-

ture, are consistent with reports showing impaired axonal growth and aberrant dystrophic structures in dorsal root ganglion neurons from diabetic animals and in neurons cultured under high glucose conditions (154, 155).

## LIGHT-INDUCED ACTIVATION OF PI3K PATHWAY IN ROD PHOTORECEPTOR CELLS

We were the first to discover that light-induced tyrosine phosphorylation of the IR in rod photoreceptors leads to the activation of PI3K (Fig. 3). This novel pathway is independent of insulin secretion but dependent on photoreceptor neurons (50). We subsequently found that this pathway is mediated through the photobleaching of GPCR rhodopsin but is independent of transducin activation (49). Compared with the liver IR, retinal IR has a higher basal level of autophosphorylation (52), which is light-dependent (50). These observations led us to hypothesize that retinal IR phosphorylation could be modulated by a soluble factor(s) in the retina. To identify the regulators of IR, yeast two-hybrid screening of a bovine retinal cDNA library constructed with the cytoplasmic domain of retinal IR (68) identified growth factor receptor-bound protein 14 (Grb14) (156, 157), which binds to various tyrosine kinase receptors including IR (158–161). Grb14 is localized predominantly in the rod inner segment, nuclear layer, and synapse in dark-adapted rods, whereas Grb14 is redistributed throughout the entire cell, including the outer segment, in the light-adapted rods (162). The translocation of Grb14 requires photoactivation of rhodopsin, but



**Fig. 3.** Light-induced activation of PI3K. Grb14, an upstream regulator of IR, requires photobleaching of rhodopsin for membrane targeting. Grb14 protects light-dependent IR activation in rod photoreceptors against dephosphorylation by PTP1B. Our studies suggest that Grb14 translocates to photoreceptor outer segments after photobleaching of rhodopsin and protects IR phosphorylation in rod photoreceptor cells. Light-activated IR is subsequently associated with PI3K, a cell survival factor, and, thus, regulates the downstream survival pathway. R\*, photoactivated rhodopsin; Grb14, growth factor receptor-bound protein 14; IR, insulin receptor; PTP1B, protein tyrosine phosphatase; Y-P, phosphotyrosine.



not signaling through the phototransduction cascade, and is not based on direct Grb14-rhodopsin interactions (162). Previously, we hypothesized that Grb14 protects light-dependent IR activation in rod photoreceptors against dephosphorylation by PTP1B (49). We failed to observe light-dependent IR activation in Grb14 knockout mouse retinas, supporting our hypothesis (162). Our studies suggest that Grb14 translocates to ROSs after photobleaching of rhodopsin and protects IR phosphorylation in rod photoreceptor cells. These studies demonstrate that Grb14 can undergo subcellular redistribution upon illumination and suggest that rhodopsin photoexcitation may trigger signaling events alternative to the classic transducin activation.

The molecular mechanism behind the light-dependent translocation of Grb14 is not clearly understood. Identification of binding partner(s) of Grb14 may provide additional clues as to how this protein is translocated within rod photoreceptor cells. Grb14 has several functional domains that may potentially interact with a variety of proteins involved in intracellular signaling. It may also be important to investigate the nature of the interaction of Grb14 with motor proteins or other components of the polarized transport machinery. However, it should be recognized that the entire Grb14 translocation phenomenon could be potentially explained by a combination of the light-dependent changes in the affinity of Grb14 for binding sites outside ROS and intracellular diffusion.

Our results also suggest that a cross-talk exists between phototransduction and IR/PI3K signal transduction pathway. This cross-talk phenomenon has been shown for other GPCRs and many tyrosine kinase cascades are regulated by GPCRs (163, 164). Examples include mitogen-activated protein kinase (MAPK) cascade, such as extracellular-regulated kinases, and stress-activated protein kinases (164). The binding of PYK2, a nonreceptor protein tyrosine kinase, to N-terminal domain-interacting receptors is activated by GPCRs (165). The N-terminal domain-interacting receptor proteins are the human homologs of the *Drosophila* retinal degeneration B protein, a protein implicated in the *Drosophila* visual transduction pathway (165). These studies, along with our observations, suggest that photobleaching of rhodopsin may activate more than one signaling pathway. GPCR can activate the PI3K $\gamma$  and is regulated by the free G $_{\beta\gamma}$  subunits of heterotrimeric G proteins, mainly the subtype G $_i$  (35). Evidence also indicates that class 1A PI3K $\beta$  is activated both by GPCR and tyrosine kinase receptors (41); however, it has not been shown that class 1A PI3K $\alpha$  is regulated through GPCR, and our studies clearly suggest that rhodopsin regulates the activation of class 1A PI3K $\alpha$  in rod photoreceptors.

We found that Akt, the downstream effector of PI3K, is also activated by light adaptation (61). We further found that the membrane binding of Akt1 to ROS is IR/PI3K-dependent; reduced binding of Akt1 to ROS membranes was observed in the photoreceptor-specific IR knockout mice (61). Membrane binding of Akt1 is mediated through

its PH domain. Fluorescently-tagged protein domains (e.g., PH domains) as PI sensors have been used by a number of independent laboratories (166–170). We applied this approach to an in vivo model of transgenic frogs and found light-dependent generation of 3'-phosphoinositides specifically in photoreceptor cells using the Akt1-AH domain as the cellular probe (61). We expressed various green fluorescent protein/Akt1-PH domain fusion proteins in rod photoreceptors of transgenic *Xenopus laevis* under the control of the *Xenopus* opsin promoter and found light-induced trafficking and binding of Akt occurs (61). The light-dependent binding of this probe coupled with its in vitro binding affinity for 3'-phosphoinositides strongly suggests that PI-3,4-P $_2$  is generated in photoreceptor membranes in response to light exposure (61). Identification and quantitation of these PI species in response to light in vivo would further advance our understanding of the role of individual phosphoinositides in the regulation of specific phospholipid-binding proteins.

The functional consequence of light-dependent activation of PI3K in photoreceptor cells is not known. PI3K activity in rods is regulated by light, suggesting that it may function shedding of photoreceptor tips (171), biogenesis of new ROS membranes through addition of newly synthesized membranes at the base of the ROS (172), or light adaptation (173). Alternatively, it is possible that light-induced PI3K activity may mediate an innate self-protection mechanism. Receptor activation of PI3K has been shown to protect some neuronal cell types, such as cerebellar granular neurons (174) and PC-12 cells (175), from stress-induced neurodegeneration.

It has been well established that PI3K/Akt is crucial in mediating cell survival. Because bright light stimulation can cause the death of rod and cone photoreceptor cells (176, 177), activation of the PI3K/Akt pathway by relatively modest light levels may serve to prevent death of photoreceptors. Supporting this hypothesis are the data that show deletion of Akt2, one of the isoforms of Akt, results in stress-induced photoreceptor degeneration (135). Furthermore, rod-specific IR knockout mice have reduced PI3K and Akt recruitment to the membranes, suggesting that the IR regulates the downstream PI3K and Akt survival signal in rod photoreceptors (148). The IR knockout mice exhibited no detectable phenotype when they were raised in dim cyclic light. However, reduced IR expression in rod photoreceptors significantly decreased retinal function and caused the loss of photoreceptors in mice exposed to bright light stress (148).

The PI3K/Akt pathway is highly conserved throughout evolution and regulates cell size and survival and cell cycle progression. Very recently, PI3K/Akt was found to regulate purine nucleotide biosynthesis (178). Entry into S phase requires an abundant supply of purine nucleotides. PI3K/Akt can regulate the early steps of de novo synthesis by modulating phosphoribosylpyrophosphate production by the nonoxidative pentose phosphate pathway and the late steps by modulating activity of the bifunctional enzyme aminoimidazole-carboxamide ribonucleotide transformylase IMP cyclohydrolase (178). These studies defined

a new mechanism in which the PI3K/Akt cassette functions as a master regulator of cellular metabolism.

### PI3K KNOCKOUT PHENOTYPES

Mice lacking p85 $\alpha$  are viable but are hypoglycemic and exhibit increased insulin sensitivity (179, 180). This phenotype is due to the upregulation of the other *Pik3r1* splice variants, p50 $\alpha$  and p55 $\alpha$ , in fat and muscle (179, 180). Mice lacking the *Pik3r1* gene, which abolishes all splice variants (p85 $\alpha$ , p50 $\alpha$  and p55 $\alpha$ ), exhibit perinatal lethality, and loss of this deletion causes a significant reduction in the expression and activity of class IA PI3K catalytic subunits (181). Mice lacking p50 $\alpha$ /p55 $\alpha$  or p85 $\beta$  exhibit increased insulin signaling (182). Also, mice lacking p85 $\beta$  are viable and show hypoinsulinemia, hypoglycemia, and improved insulin sensitivity (183, 184). These studies suggest both positive and negative roles of the p85 regulatory subunits in insulin signaling. The p85 proteins are essential for p110 stabilization but conversely, free p85s exert negative effects on PI3K signaling (185). Under physiological conditions, p85 is more abundant than the catalytic p110 subunit, producing a competition between p85 monomers and p85-p110 dimers. PI-3,4,5-P<sub>3</sub> production is inhibited by monomeric p85, either by binding to phosphorylated IRS proteins (185) or by altering subcellular localization of p110/p85 dimers (186). These findings suggest that p85 family members have a complex role in regulating PI3K-mediated insulin signaling, which probably involves p110-independent activities.

The p85 regulatory subunit of PI3K downregulates IRS-1 signaling via the formation of a sequestration complex (187). In response to IGF-1, but not to PDGF signaling, p85 $\alpha$  localizes to discrete foci in the Chinese hamster ovary (CHO)-K1 cells, which contain the IRS-1 adaptor molecule, and the foci formation requires the binding of p85 to IRS-1 (187). Compared with the p85-p110 dimer, monomeric p85 is preferentially localized to these foci, which are not sites of PI-3,4,5-P<sub>3</sub> production (187). Ultrastructural studies reveal that p85-IRS-1 foci are cytosolic protein complexes devoid of a membrane (187). These studies suggest a mechanism of signal downregulation of IRS-1 that is mediated by monomeric p85 through the formation of a sequestration complex between p85 and IRS-1 (187). Modulation of PI3K-Akt signaling components in the membrane raft microdomains have been reported (188–190). Consistent with these reports, we have also demonstrated the localization of the IR and PI3K in detergent-resistant membrane rafts of rod photoreceptor outer segments (191). Currently, we do not know the specific signaling events mediated by IR and PI3K in rafts. Furthermore, heterozygous disruption of p85 improves insulin signaling and glucose homeostasis in normal mice and mice made insulin-resistant by heterozygous deletion of the IR and/or IRS-1 genes (192). We (68) and others (52) have shown that IR activation does not induce the activation of IRS-1 but does induce activation of IRS-2. It is possible that retinal monomeric p85 subunits may sequester

IRS-1 in the retina. Further studies are required to verify this hypothesis. However, the existence of free p85 monomeric subunits was recently challenged by Geering et al. (36), and their study found no free p85 or p110 PI3K subunits in murine WEHI-231 B lymphocyte cell line (36).

Deletion of p85 subunits ( $\alpha$  and  $\beta$ ) from the heart attenuated Akt signaling, reduced heart size, and altered cardiac gene expression (193). The p85 regulatory subunit of PI3K, a key mediator of insulin's metabolic actions, is also required for the activation of c-Jun N-terminal kinase (JNK) in states of insulin resistance including high-fat-diet-induced obesity and JNK1 overexpression (194). Skeletal muscle lacking both the p85 $\alpha$ /p55 $\alpha$ /p50 $\alpha$  and the p85 $\beta$  regulatory subunits of PI3K had reduced muscle weight and fiber size (144). We previously examined the retinal phenotype in p85 $\alpha$  subunit knockout mice (195) and these mice retain the ability to express p50 $\alpha$  and p55 $\alpha$  subunits (179). These mice do not exhibit any gross phenotype changes in the retinas, although a decreased IR-associated PI3K activity is found in p85 $\alpha$  subunit knockout mouse strains (195). The absence of lethal phenotype could be due to the complementation of p50 $\alpha$  and p55 $\alpha$  for the p85 $\alpha$  disruption. Disruption of the mouse gene encoding PI3K adaptor subunit p85 $\alpha$  and its splice variants p50 $\alpha$  and p55 $\alpha$  resulted in a lethal phenotype of impaired B cell development and proliferation (181). Tissue-specific deletion of p85 would give a precise functional role of PI3K in retinal functions. We recently observed that cone photoreceptor-specific deletion of p85 $\alpha$  subunit of PI3K resulted in age-related cone photoreceptor degeneration (unpublished observations).

Mice lacking either p110 $\alpha$  or p110 $\beta$  are embryonic lethal (196); however, heterozygous mice lacking PI3K $\alpha$  or  $\beta$  show normal responses to insulin and p110 $\alpha$ /p110 $\beta$  compound heterozygotes display decreased insulin sensitivity (196). These results suggest that both proteins may be involved in insulin signaling. Young heterozygous mice expressing a catalytically inactive PI3K $\alpha$  are normoglycemic; however, mice older than 6 months display glucose intolerance, hyperlipidemia, adiposity, as well as hyperglycemia, and deregulate hepatic gluconeogenesis, thus, assigning a fundamental role of PI3K $\alpha$  in insulin-dependent signaling (197). PI3K $\alpha$  is selectively recruited and activated by IRS proteins (197). PI3K inhibitors with significant isoform selectivity show that PI3K $\alpha$  is the major PI3K effector downstream of the IR and that PI3K $\beta$  maintains a marginal role (198). Inhibitor studies indicate that PI3K $\beta$  influences the PI3K $\alpha$  function, providing a basal threshold of PI-3,4,5-P<sub>3</sub> production that potentiates PI3K $\alpha$  activity, thus, suggesting that PI3K $\beta$  function may still be necessary for achieving full-scale signaling response. Further studies with mice expressing catalytically inactive mutants of PI3K $\beta$  may help to address this issue. There are no direct studies available on the disruption of the p110 subunit of PI3K in retinal cells. However, our previous work demonstrated that PI3K activity in photoreceptors is regulated through the IR (148). Rod-specific IR knockout mice have reduced PI3K and Akt association with the membranes, suggesting that the IR regulates the downstream



PI3K and Akt survival signal in rod photoreceptors. Reduced IR expression in rod photoreceptors significantly impairs the retinal function and loss of photoreceptors in mice exposed to bright light stress (148).

## PHOSPHOINOSITIDES IN CELLULAR REGULATION

The lipid products of PI3K serve as second messengers to recruit specific phospholipid-binding proteins to the plasma membrane and control the activity and subcellular localization of a diverse array of signal transduction molecules (199). The phospholipid-binding proteins bind to phosphoinositides through their PH domain. The PH domain, which was initially identified in the platelet PKC substrate pleckstrin, is a 120 amino acid colinear region identified by sequence comparison in more than 100 proteins (16). PH domains are present in cytoskeletal components (spectrin,  $\alpha$  actinin), guanine nucleotide exchange proteins, and GTPase-regulating proteins (RAS-GRF, Dbl, VAV, cdc25, SOS, RAS-GAP, Tiam-1, ARNO, GRP1/cytohesin-1), GTPases (dynammin), PI-regulated protein kinases (Akt/protein kinase B, PDK1), other protein kinases (BTK,  $\beta$  ARK), and PLC. All of these proteins bind to phosphoinositides with different affinities and specificities (16). These PH domains do not always bind phosphoinositides; sometimes, PH domains serve as protein-protein interaction domains (200, 201).

Several PH domain-containing proteins, which can be directly or indirectly regulated by phosphoinositides, have been identified in the retina. The best examples are Akt isoforms (52, 135), IRS-1, IRS-2 (52, 68, 69), Grb14 (157), Evictin-1 (202), PHR1 (pleckstrin homology domain retinal protein) (203, 204), and recently identified Akt dephosphorylating enzymes PHLPP (PH domain and leucine rich repeat protein phosphatases) and PHLPL (PH domain and leucine rich repeat protein phosphatase-like) (205). Deletion of two PI-binding proteins, Akt2 (135) and IRS-2 (69), in the retina results in photoreceptor degeneration.

## PHOSPHOINOSITIDE METABOLISM IN THE RETINA

We and others have established that PI metabolism is active in the vertebrate retina and ROS (13–15, 206–228). In ROS, light stimulates the enzymatic activities of PLC (219, 220, 224–226, 229), PKC (230, 231), PI synthetase (12), DG kinase (13), and PI3K (14, 49, 50). ROS PKC can phosphorylate a number of ROS proteins including rhodopsin (232–237) and phosphodiesterase (238, 239). The lipid second messengers generated from phosphatidylcholine, phosphatidic acid and diacylglycerol, are modulated by the different illumination states of the retina. 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.

Detailed studies are described by Giusto and her coworkers (to come in this series) in the thematic review on lipid second messengers and related enzymes in ROS.

## PHOSPHOINOSITIDES IN RETINA STRUCTURE AND FUNCTION

Emerging evidence from various laboratories shows the importance of phosphoinositides in photoreceptor functions. PI-4,5-P<sub>2</sub> can activate phosphodiesterase, which results in the inhibition of current flow through cyclic nucleotide-gated channels in ROSs (240). Modulation of phototransduction gain by changes in PI levels has been reported (241). PI3K-generated phosphoinositides are involved in the membrane targeting of rhodopsin as well as disc morphogenesis in mammalian photoreceptors (242). In the retina, deletion of the PI-binding proteins Akt2 (135) and IRS-2 (69) results in photoreceptor degeneration. *Drosophila* arrestin has a PI-binding domain that binds PI-3,4,5-P<sub>3</sub> and controls the movement of arrestin (243). Two other proteins with PH domains play potentially important roles in the retina. Evictin-1 can localize to rhodopsin-laden, postGolgi membranes in photoreceptor cells, suggesting that the protein may be involved in post-Golgi trafficking of membranes (202). PHR1 is present in outer segments and binds to transducin  $\beta\gamma$  subunits but does not bind to inositol phosphates; however, PI lipids were not tested (204). The PH domain of PHR1 is most similar to that of Akt, which we found in the ROS (135). We demonstrated that PH domain of Akt1 can bind to PI3K-generated phosphoinositides in ROS (61). Myosin II binds to the PH domain of Akt (244), which raises interesting questions, because myosin VIIA gene is mutated in Usher syndrome 1B patients (245). Usher syndrome is the most common condition that affects both hearing and vision. The major symptoms of Usher syndrome are hearing loss and an eye disorder called retinitis pigmentosa. Retinitis pigmentosa causes night-blindness and a loss of peripheral vision (side vision) through the progressive degeneration of the retina. However, the myosin-Akt interaction has not been demonstrated in the visual system. All of these studies clearly underline the importance of phosphoinositides in photoreceptor structure and function.

## NEUROPROTECTIVE ROLE OF PI3K IN THE VERTEBRATE RETINA

IR-activated PI3K activity can protect transformed retinal neurons in culture and can inhibit caspase-mediated retinal cell death (246). The neuroprotective activity of 17- $\beta$  estradiol in the retina is exhibited through PI3K activation (247). Deletion of PTEN, a negative regulator of mTOR pathway in adult retinal ganglion cells (RGCs), promotes robust axon regeneration after optic nerve injury (248). The PDGF-induced Akt activation has shown to protect from the death of retinal pericytes by advanced glycation end-products (249). This activation perhaps could be mediated through PI3K pathway in response to

PDGF (249). The PI3K-Akt signaling cascade plays a major role in mouse 661W cone photoreceptors and this pathway can be compromised in response to the activation of pro-apoptotic BCL-2 family members (250). PI3K-generated PI-3,4,5-P<sub>3</sub> accumulation is essential for apical membrane morphogenesis in *Drosophila* photoreceptor epithelial cells (251). During photoreceptor apoptosis in the retinal degeneration mouse model, PTEN, which counteracts the effects of PI3K by decreasing levels of 3'-phosphorylated phosphoinositides, is activated and the downstream effectors of PI3K, Akt, and forkhead in rhabdomyosarcoma are inactivated (252). Interestingly, inactivation of PTEN in RPE cells results in retinal degeneration (253). This phenotype is due to the loss of interaction of signaling proteins to the PDZ-binding domain of PTEN but not due to the enhanced Akt activation (253). These studies suggest that PTEN is essential for normal RPE cell function (253).

Impairment of RPE structural integrity is known to cause age-related macular degeneration (AMD). AMD is a leading cause of vision loss in Americans 60 years of age or older. Activation of PI3K-Akt pathway in RPE protects the RPE cells against the deleterious effects of oxidative stress. One of the major causes of AMD is oxidative stress, which is generated both endogenously during the course of phototransduction and also by a variety of exogenous insults (254–256). AMD-inducing oxidative stresses impact the PI3K-Akt neuron survival pathway (257–260). It has been proposed that this pathway protects RPE cells against the deleterious effects of oxidative stress (261–263).

The goldfish retina has been used extensively for the study of nerve regeneration and the PI3K-mediated process is critical for nerve regrowth (264). PI3K has a central role in mediating the survival of differentiated neurons in vivo and also plays an important role in retinal development (265). BDNF has a potential neuroprotective effect on axotomized RGCs and this protective effect is mediated through PI3K and MAPK signaling pathways (65). Furthermore, PI3K/Akt signaling pathway is activated intrinsically and has a neuroprotective effect on injured RGCs (266). bFGF may mediate activation of Muller glial cells in the ischemic-hypoxic retina and some of the protective effects of bFGF are mediated through PI3K pathway (64). CNTF can also protect the retinal cells in vitro and in vivo via PI3K activation (67). The cytokine EPO promotes regeneration of adult central nervous system neurons via Jak2/Stat3 and PI3K/Akt pathway (66).

Intraocular pressure elevation has often been used as an experimental model to study mechanisms underlying RGC death associated with ocular ischemic injury and glaucoma. PI3K/Akt pathway has differential roles in RGC survival in rats with or without acute ocular hypertension (267). Furthermore, PI3K/Akt pathway inhibition-dependent activation of macrophages is detrimental to RGCs (268). The PI3K pathway can protect the 4-hydroxy-2-nonenal-induced oxidative injury in RPE (269). Heme oxygenase 1 (HO-1) is a representative mediator of antioxidants and

cytoprotectants against various stress stimuli including oxidants in vascular cells (270). Insulin treatment can prevent H<sub>2</sub>O<sub>2</sub>-induced NF-kappaB and caspase-8 activation and apoptosis via IRS1/PI3K/Akt2/heme oxygenase 1 pathway in retinal pericytes, suggesting a potential explanation for how insulin is retarding the progression of microvascular complications induced by diabetes (270). PI3K-atypical protein kinase C signaling is also required for Wnt attraction and anterior-posterior axon guidance (271). PI3K-Akt mediated atypical protein kinase C signaling is also essential for apical membrane morphogenesis in *Drosophila* photoreceptor epithelial cells (251). PI3K/Akt signaling pathway serves as the circadian output in the retina (272). PDGF and insulin/IGF-1 can exhibit specific, distinct modes of class IA PI3K activation in normal rat retinas and RGC-5 retinal ganglion cells (62). These studies identify two distinct modes of retinal class IA PI3K activation that occur in response to PDGF receptor and insulin/IGF-1 receptor stimulation (62). Biswas et al. (62) propose the hypothesis that PDGF-induced PI3K/Akt pathway may provide novel therapeutic targets to ameliorate cell death in diabetic retinopathy and other retinal neurodegenerations. We recently reported that light stress activates tyrosine phosphorylation of ROS proteins, including IGF-1R, which promotes the binding of PI3K to ROS membranes (48). The activation of the IGF1-R/PI3K/Akt survival pathway may serve a protective role in the retina. In stress, insulin and p85 activates JNK via cdc42 and mitogen-activated protein kinase kinase 4; this cdc42/JNK pathway requires both an intact N-terminus and functional SH2 domains within the C terminus of the p85 $\alpha$  regulatory subunit (194). These studies suggest that p85 $\alpha$  plays dual roles in regulating insulin sensitivity and in mediating cross-talk between the PI3K and stress kinase pathways. No data on the cross-talk between PI3K and JNK stress kinase pathways in retina are available.

The IR and IGF-1R are structurally similar. Both are found at the cell surface as  $\alpha_2\beta_2$  heterotetramers with transmembrane ligand-binding  $\alpha$ -subunits and intracellular, tyrosine kinase-containing  $\beta$ -subunits. Furthermore, once activated, both receptors can phosphorylate and/or interact with the same intracellular protein substrates, including members of the IRS family and Src homologous and collagen domain protein. IR and IGF-1R also activate many of the same downstream signaling molecules such as PI3K and MAPK (273, 274). Insulin and IGF-1 in retinal endothelial cells respond differentially to oxygen (275). Insulin and IGF-1 signaling in endothelium plays a role in retinal neovascularization through the expression of vascular mediators, with the effect of insulin being more important in this process than IGF-1, as demonstrated with the advent of endothelial cell-specific insulin and IGF-1R knockout mice (275). Both IR (148) and IGF-1R (48) in photoreceptors activate neuroprotective PI3K and Akt. These studies clearly suggest redundant and nonredundant tissue-specific roles of IR and IGF-1R. Further studies are required to understand the specific receptor-activated PI3K in retina and photoreceptor functions.

## PHOSPHOINOSITIDES IN CYTOSKELETAL ORGANIZATION

Phosphoinositides are the key molecules for regulation of actin cytoskeletal organization and membrane traffic from the plasma membrane (147). Our studies suggest that light-dependent-generated phosphoinositides regulate the actin cytoskeleton (61). We also observed lower levels of ROS-associated actin (presumably F-actin) in IR knockout mouse retinas than in wild-type mouse retinas (unpublished observations). These observations, along with our current findings, suggest that light-induced activation of IR/PI3K may regulate the actin cytoskeletal organization. Dysfunction of the actin cytoskeleton is a key event in the pathogenesis of diabetic nephropathy (149), diabetic neuropathy (150, 151), and diabetic cardiomyopathy (152, 153). The light-induced activation of IR/PI3K-generated phosphoinositides in the retina may play an important role in the control of diabetic retinopathy. PI3K and Akt activations are downregulated in diabetic retinopathy, supporting this hypothesis (132).

## PHOSPHOINOSITIDE SIGNALING IN CONE PHOTORECEPTORS

Cone photoreceptors make up a very small percent (3–5%) of total retinal photoreceptors. In humans, cones are essential for an optimal acute, bright, and colored visual perception. Therefore, the quality of our daily lives relies highly on how healthy and functional our cones are. Specific mechanisms of cone cell death are very different depending on genetic predispositions as well as environmental factors. Many questions regarding these mechanisms are yet to be answered. A canonical Akt survival pathway has been shown to be constitutively active in cone photoreceptors (276). However, rod photoreceptors express the active form of Akt only transiently during the exposure to conditions such as physiological light (61), oxidative (269), hyperosmotic (277), or light stress (135). In mouse models of retinitis pigmentosa having genetic mutations in rods, the active form of Akt stays expressed until the photoreceptors die (109). A possible interpretation is that Akt survival pathway desperately attempts to rescue rods from a genetically predisposed death phenotype. Furthermore, recent findings from the model of retinitis pigmentosa showed that as rods die, cones are starved, primarily due to the downregulation of insulin/Akt/mTOR signaling pathway (109). mTOR plays a crucial role in a nutrient-sensitive signaling pathway that regulates cell growth, proliferation, and metabolic maintenance. Stimulation of the insulin/mTOR signaling pathway delays cone death (109). The classical link between the extracellular signals such as insulin/IR and intracellular survival pathways like Akt/mTOR is PI3K. In diabetic retinopathy, the leading cause of blindness in developed countries, cones rather than rods selectively die, primarily due to downregulation or inactivation of IR/PI3K/Akt survival pathway (132). Evidence showed that PI metabolism at the

synapse is important in synaptic ribbon formation and glutamate release, specifically, in cone photoreceptors (278). This PI metabolism is  $\text{Ca}^{+2}$ -dependent. Furthermore, in dark, when intracellular  $\text{Ca}^{+2}$  levels are high, PI metabolism increases at the synaptic termini compared with outer segments (278). Cones then release glutamate at the synapse, which in turn halts PI metabolism in adjacent horizontal cells. The opposite events take place in light, where the highest PI metabolism is in outer segments, perhaps to aid phototransduction processes (278). A drop in intracellular  $\text{Ca}^{+2}$  levels and a subsequent decrease in PI metabolism at the synapse causes cones to stop releasing glutamate, which in turn liberates PI metabolism in horizontal cells (278). At present, the biological significance of this cone phenomenon is not well studied. A study has suggested that in rod photoreceptors, proper synaptic ribbon formation and rod synapse pairing with the bipolar and horizontal cells into triads might be important in inner retinal phototransduction signal propagation (278).

Synaptic ribbons are specialized cytoskeletal components of the presynaptic exocytotic machinery in photoreceptors. In cone photoreceptors, these structures are highly dynamic, disappearing during darkness and reforming in the light phase. Synaptic ribbons in cone photoreceptors are very sensitive to both  $\text{Li}^+$  and  $\text{IP}_3$ , suggesting that inositol polyphosphates may play a physiological role in the disassembly of synaptic ribbons (278). The PI pathway can exist in isolated photoreceptor synapses, and PI metabolism in photoreceptor synapses is activated in the dark and correlated with the disappearance of synaptic ribbons during dark adaptation (278). Furthermore,  $\text{Li}^+$  and  $\text{IP}_3$  can influence synaptic ribbons in cones but not in rods; thus, the dark-activated PI metabolism is largely ascribed to cone synapses (278). We recently observed that cone photoreceptor-specific deletion of p85 subunit of PI3K resulted in age-related cone photoreceptor degeneration (unpublished observations). Whether the observed degeneration could be due to lack of PI3K generated phosphoinositides in the regulation of synaptic ribbons needs to be studied.


## POSSIBLE PHYSIOLOGICAL ROLES OF THE PI CYCLE IN PHOTORECEPTOR CELLS

Rod and cone photoreceptor cells are involved daily in several activities other than visual transduction. These include the rhythmic shedding of outer segments (279), the synthesis and transport of large amounts of membrane material destined for the outer segments (280), the movement of proteins between the inner and the outer segments in response to light/dark stimuli (281, 282), and the maintenance of a circadian clock (283). In addition, we showed that the photoreceptor cell is highly plastic and is capable of responding to stress by upregulating the synthesis of proteins that provide protection from damaging effects of constant illumination (284, 285). Each of these events requires the transfer of a signal from the outer segment to other regions of the photoreceptor cell. A mutation in several enzymes of the PI cycle can cause a retinal



degeneration in invertebrates (286–288). In humans, Lowe’s oculocerebrorenal syndrome is caused by a mutation in inositol polyphosphate-5-phosphatase (289, 290). Mutations in the PYK2-binding domain of phosphatidylinositol transfer membrane-associated protein (PIPNM3) caused autosomal dominant cone dystrophy in two Swedish families (291). PIPNM3, known as a human homolog of the *Drosophila rdgB*, lacks the N-terminal PIT domain needed for the transport of phospholipids, the renewal of photoreceptor membranes, and the electroretinogram response to light (291). A disruption in the orderly flow of metabolic events in the retina can lead to a retinal degeneration, as has been demonstrated repeatedly.

## CONCLUSIONS AND FUTURE DIRECTIONS

Studies from various laboratories, including ours, suggest that the PI3K pathway is neuroprotective. Our laboratory was the first to demonstrate the light-dependent regulation of PI3K pathway in rod photoreceptors. PI3K activation has been shown to be downregulated in diabetic retinopathy. Cell type specific activation of PI3K by various growth factors has also been reported in the retina, and the activated PI3K is shown to be neuroprotective. Neurodegeneration is an important component of diabetic retinopathy as demonstrated by an increased apoptosis in the neural retina during experimental and human diabetes. PI3K-Akt survival signals have been shown to be downregulated in diabetic retinopathy. Understanding the regulation of this pathway and identifying new regulator(s) would help to design therapeutic strategies to protect the dying retinal cells. One of the limitations in retina research is the involvement of different PI3K isoforms and their precise functional role in retinal structure and function. Currently, most of the studies are done with the use of chemical inhibitors, which tend to inhibit all isoforms, leading to difficulty in interpreting the isoform involvement. Availability of cell-specific conditional knockout mouse lines would address the individual role of specific isoforms in retina. Research on targeted therapies to activate the PI3K pathway in retina would be a promising avenue to pursue to protect the dying retinal cells in neurodegeneration. 

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