lazaro Encodes a Lipid Phosphate Phosphohydrolase that Regulates Phosphatidylinositol Turnover during *Drosophila* Phototransduction

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Summary

An essential step in *Drosophila* phototransduction is the hydrolysis of phosphatidylinositol 4,5 bisphosphate PI(4,5)P_2 by phospholipase Cβ (PLCβ) to generate a second messenger that opens the light-activated channels TRP and TRPL. Although the identity of this messenger remains unknown, recent evidence has implicated diacylglycerol kinase (DGK), encoded by *rdgA*, as a key enzyme that regulates its levels, mediating both amplification and response termination. In this study, we demonstrate that *lazaro* (*laza*) encodes a lipid phosphate phosphohydrolase (LPP) that functions during phototransduction. We demonstrate that the synergistic activity of *laza a*nd *rdgA* regulates response termination during phototransduction. Analysis of retinal phospholipids revealed a reduction in phosphatidic acid (PA) levels and an associated reduction in phosphatidylinositol (PI) levels. Together our results demonstrate the contribution of PI depletion to the *rdgA* phenotype and provide evidence that depletion of PI and its metabolites might be a key signal for TRP channel activation in vivo.

Introduction

Calcium influx through plasma membrane channels regulates a range of functions in both adult and developing neurons, including the transduction of a number of sensory modalities, learning and memory, and the navigation of dendrites during neuronal development (Berridge, 1998; Gomez and Spitzer, 1999). A number of different ion channels mediate calcium influx that subserves these functions; one major family are the TRP proteins (Montell et al., 2002). Several classes of TRP channels have been described (Montell et al., 2002), and members of all classes mediate key physiological processes in neurons (Voets and Nilius, 2003; Wang and Poo, 2005). Despite the importance of TRP proteins in neuronal function, the signaling mechanisms that regulate channel activity in vivo remain controversial and poorly understood. A common theme underpinning the regulation of several classes of TRP channels is the role of PI(4,5)P_2 and/or its metabolites (Clapham, 2003). When heterologously expressed, TRPC (Estacion et al., 2001), TRPV (Voets and Nilius, 2003), and TRPM (Rohacs et al., 2005; Runnels et al., 2002) channels are all reported to be modulated by changes in PI(4,5)P_2 levels during signaling. However, the role of PI(4,5)P_2 depletion in the activation of endogenous TRP channels remains limited by the lack of suitable in vivo models.

Drosophila phototransduction is a well-established model system for the analysis of calcium influx triggered by G protein-coupled phosphoinositide hydrolysis (Hardie and Raghu, 2001). In photoreceptors, the absorption of a photon of light by rhodopsin triggers PLCβ activity. This signaling cascade triggers the opening of at least two classes of plasma membrane channels, TRP and TRPL, resulting in the influx of calcium into the cell (Reuss et al., 1997). Despite intense investigation, the mechanism by which PLCβ activity results in TRP and TRPL opening remains unclear. The essential role of PI(4,5)P_2 hydrolysis in phototransduction is well-established; null mutants in *norpA*, which lack eye-enriched PLCβ, show no response to light (Bloomquist et al., 1988). The hydrolysis of PI(4,5)P_2 generates the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Berridge, 1993). IP_3 is insufficient for activation of TRP and TRPL in vivo (Hardie and Raghu, 1998), and the IP_3 receptor does not appear to be essential for phototransduction (Acharya et al., 1997; Raghu et al., 2000a). However, recent studies have suggested that lipid second messengers generated by PLCβ activity might play a role in *Drosophila* TRP and TRPL activation. Application of both DAG and PI(4,5)P_2 has been reported to modulate heterologously overexpressed TRPL channels (Estacion et al., 2001), and polyunsaturated fatty acids, metabolites of DAG, can activate TRP and TRPL (Chyb et al., 1999).

In most eukaryotic cells, DAG is phosphorylated by DGK to generate PA (reviewed in Kanoh et al., 2002). In *Drosophila*, *rdgA* mutants that lack eye-enriched DGK (Masai et al., 1993) show severe retinal degeneration that can be rescued by *trp* mutants (Raghu et al., 2000b). The retinal degeneration phenotype of *rdgA* has recently been shown to be modulated by light, Gq, and PLCβ (Georgiev et al., 2005), all of which are key elements required for PI(4,5)P_2 hydrolysis during phototransduction. Electrophysiologically, *rdgA* photoreceptors show constitutive TRP channel activity (Raghu et al., 2000b) as well as defects in amplification and response termination (Hardie et al., 2002; Raghu et al.,

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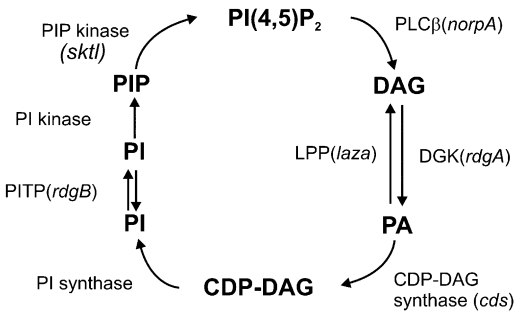

Figure 1. Key Lipid Intermediates in G Protein-Coupled $PI(4,5)P_2$ Turnover

The intermediates during this signaling cascade: [$PI(4,5)P_2$; DAG; PA; CDP-DAG, PI, PIP-phosphatidylinositol phosphate] are shown. Enzymes involved in the turnover of these lipid intermediates are shown; loss-of-function mutants affecting these molecules, where available, are shown in italics. PITP, phosphatidylinositol transfer protein.

2000b). Thus, the analysis of the *rdgA* mutant strongly suggests that in vivo the tight regulation of $PI(4,5)P_2$-derived lipid metabolites is essential for normal TRP channel activation. While the analysis of *rdgA* demonstrates the key role of DGK in this process, the biochemical and molecular basis for its requirement is unclear. In particular, it has thus far not been possible to identify the specific lipid messenger(s) responsible for the abnormal TRP channel activation in *rdgA*. One possibility is that reduced DGK activity results in an elevation of DAG and/or its metabolites in the face of ongoing $PI(4,5)P_2$ hydrolysis. Alternatively, it is possible that a reduction in levels of PA, the product of DGK, is a key determinant of the *rdgA* phenotype. In addition to being a potential second messenger in its own right, PA can also be recycled to resynthesize $PI(4,5)P_2$ (Figure 1). PA can bind to a number of proteins (Delon et al., 2004) and in some cases regulate their activity. These include two molecules involved in PI turnover: type I phosphatidylinositol 4 phosphate 5 kinase (type I PIPkin) in mammalian cells (Jenkins et al., 1994) and Opi1p, an ER resident transcription factor in yeast (Loewen et al., 2004). Opi1p, when bound to PA, translocates to the nucleus and stimulates the transcription of genes regulating phosphatidylinositol (PI) biosynthesis. Thus, it is likely that PA levels would be tightly regulated and linked to PI turnover in vivo. It also raises the possibility that a depletion of PI and consequently $PI(4,5)P_2$ might underlie the *rdgA* phenotype.

As part of a study to understand the biochemical basis of the *rdgA* phenotype, we looked for additional enzymes encoded in the *Drosophila* genome that might regulate lipid turnover during $PI(4,5)P_2$ hydrolysis. An immediate possibility is that lipid phosphate phosphohydrolases (LPPs) might act in synergy with DGK in controlling the balance of DAG and PA. LPPs were originally designated as type II PA phosphatases (PAP), since their activity was distinct from the type I PAP activity involved in glycerolipid biosynthesis (Jamal et al., 1991). However, to date it is unclear whether LPPs can metabolise PA, an intracellular bioactive lipid generated during $PI(4,5)P_2$ hydrolysis. In this study, we have tested this hypothesis and identified the endogenous LPP that functions during *Drosophila* phototransduction. We describe the biochemical consequence of the concerted action of DGK and LPP during phototransduction and its implications for the mechanism of TRP channel activation.

Results

A Number of Different LPPs Are Expressed in the Adult *Drosophila* Retina

To identify LPP that might function during phototransduction, we carried out a bioinformatic analysis of the completed *Drosophila* genome. A BLAST search using human LPP2 (Hooks et al., 1998) identified seven genes that showed homology to LPP. Two of these genes, *wunen* (*wun*) (Zhang et al., 1997) and *wunen-2* (*wun2*) (Starz-Gaiano et al., 2001), have previously been studied extensively for their role in *Drosophila* germ cell migration (Renault et al., 2004). When expressed in cell culture models, they show a catalytic activity profile similar to that of vertebrate LPP (Burnett and Howard, 2003). Five others, namely CG11425, CG11426, CG11437, CG11438, and CG11440, are clustered within an ~8 kb genomic region at 79E on chromosome 3. For reasons described later, we have named CG11440 as *lazaro* (*laza*). Analysis of the protein sequences of *Drosophila* LPP-like genes shows that they all contain six putative transmembrane domains as well as three domains previously identified as being characteristic of vertebrate LPP (Brindley and Waggoner, 1998). Indeed, 5 of the 7 residues shown to be essential for catalysis in human LPP were identical in all seven *Drosophila* genes and the three human orthologs (Figure 2A). To identify LPPs that are expressed in the retina, we performed RT-PCR on dissected retinae. This revealed that RNA for five of the seven *Drosophila* LPP genes, *wun*, *wun2*, *laza*, CG11425, and CG11426, was expressed in adult photoreceptors (Figure 2B). No expression could be detected for the other two genes, CG11437 and CG11438. These results show that LPPs are expressed in adult *Drosophila* photoreceptors and might play a signaling role in these cells. We also tested whether transcripts for any of these LPP genes were enriched in the retina, usually an indication of a role in photoreceptor function. We compared transcript levels for all five genes in total RNA from wild-type heads with those from so^D (a mutant that lacks eyes) using semiquantitative RT-PCR. This analysis revealed that only *laza* transcripts were enriched in the eye (Figure 2C).

Overexpression of LPP Enhances Degeneration in *rdgA*

To test the ability of LPP to dephosphorylate PA generated during $PI(4,5)P_2$ hydrolysis, we exploited the *rdgA* mutant (Masai et al., 1993, 1997). In $rdgA^3$, a hypomorphic allele, photoreceptors look ultrastructurally normal on eclosion (Figure 3B) and degenerate with time (Figure 3D) when grown in laboratory incubator conditions (see Experimental Procedures). We generated transgenic flies and tested whether overexpression of one of the *Drosophila* LPPs, CG11426, affected the phenotype of *rdgA*. When CG11426 was overexpressed in $rdgA^3$ using Rh1-GAL4, there was obvious degeneration of the

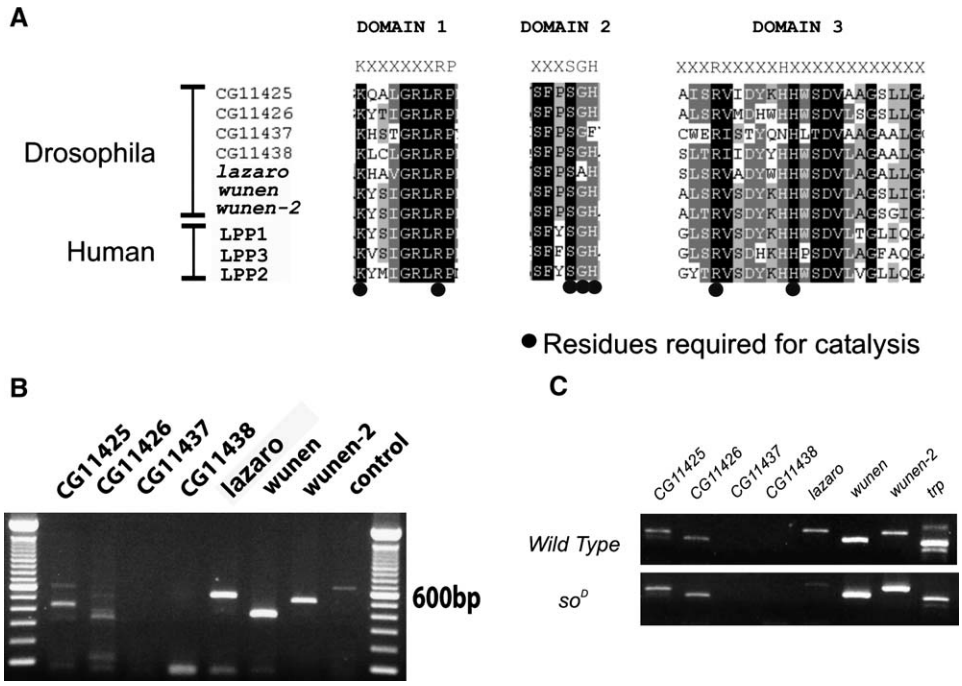

Figure 2. LPP Genes in the *Drosophila* Genome

(A) Multiple alignment of protein sequences from the seven potential LPP from the *Drosophila* genome and the three human genes are shown. Three conserved domains defined by Brindley and Waggoner (Brindley and Waggoner, 1998) are identified, and residues experimentally shown in the human genes to be required for catalysis are marked with filled circles.

(B) RT-PCR analysis of products obtained from retinal RNA for the seven *Drosophila* LPPs. No expression was detected for CG11437 and CG11438. The control gene is the transcript for the inositol 1,4,5-trisphosphate receptor (*itpr*).

(C) Comparative RT-PCR analysis for seven LPP genes from wild-type and so^D head RNA. Enrichment of *trp*, a known eye-enriched transcript in wild-type heads, is shown as a positive control. Enrichment of *laza* RNA can be seen in wild-type heads compared to that in so^D heads.

rhabdomeres in newly eclosed flies (Figure 3C). Over a period of 72 hr post-eclosion, $rdgA^3$+ LPP photoreceptors degenerated more rapidly than $rdgA^3$ alone (Figure 3D). By contrast, when CG11426 was overexpressed in otherwise wild-type photoreceptors, there was no detectable effect on photoreceptor ultrastructure (Figure 3D). Similar results were obtained with three other *Drosophila* LPPs, *wun*, *wun2*, and *laza* (see Figure S1 in the Supplemental Data available online). These results provide strong genetic evidence that LPP can functionally antagonise the DGK activity encoded by *rdgA*.

The enhancement of $rdgA^3$ by LPPs suggests that these enzymes can dephosphorylate PA in vivo. We tested this by measuring retinal PA levels using liquid chromatography followed by mass spectrometry, comparing $rdgA^3$ with $rdgA^3$ + CG11426. We found that levels of PA in $rdgA^3$ retinae were ~60% of wild-type. Importantly, this reduction was dramatically enhanced in $rdgA^3$+ LPP: these retinae had ~20% of total wild-type PA levels (Figure 3E). These results demonstrate that PA levels in $rdgA^3$ are reduced and that when overexpressed, LPP enhances this reduction in vivo.

Retinal degeneration in $rdgA^3$ can be completely blocked by $norpA^{P24}$ (Georgiev et al., 2005), a strong hypomorph in PLCβ essential for phototransduction, suggesting that the enhancement of *rdgA* by LPP was most likely mediated by PA derived from $PI(4,5)P_2$. To confirm this idea, CG11426 was overexpressed in $norpA^{P24}$, $rdgA^3$. This analysis revealed normal rhabdomeres in $norpA^{P24}$, $rdgA^3$ + LPP (Figure 3D) and shows that the enhancement of $rdgA^3$ by LPP requires ongoing light-induced $PI(4,5)P_2$ hydrolysis. Our results demonstrate that LPP can antagonize DGK function, most likely by dephosphorylating PA generated by $PI(4,5)P_2$ hydrolysis.

Isolation of Mutants in *laza*, an Eye-Enriched LPP

Several LPPs are expressed in the adult retina, and overexpression of each enhanced $rdgA^3$, raising the question of which endogenous LPP functions during phototransduction. To test this, we analyzed loss-of-function mutants in *wun*, *wun2*, and *laza*. We generated loss-of-function mutants in *laza* by transposon mutagenesis (Figure 4A). A number of mutant alleles were isolated; two of these, $laza^{15}$ and $laza^{22}$, are described (Figure 4A). $laza^{22}$ is a 703 bp deletion that removes the presumed start codon and the first 28 amino acids of the encoded protein. $laza^{15}$ is a larger deletion of 2.996 kb that removes the entire open reading frame. Both $laza^{15}$ and $laza^{22}$ leave adjacent genes physically intact and give homozygous viable adults. RT-PCR analysis revealed that the transcript encoding *laza* was completely absent in $laza^{15}$ and $laza^{22}$ (Figure 4B), and transcript levels of the other four LPPs were unaffected.

laza Mutants Show Light-Dependent Degeneration

To test the potential role of *laza* in phototransduction, we examined retinal ultrastructure in $laza^{15}$ and $laza^{22}$ using electron microscopy. Essentially similar results were obtained with both alleles and are henceforth

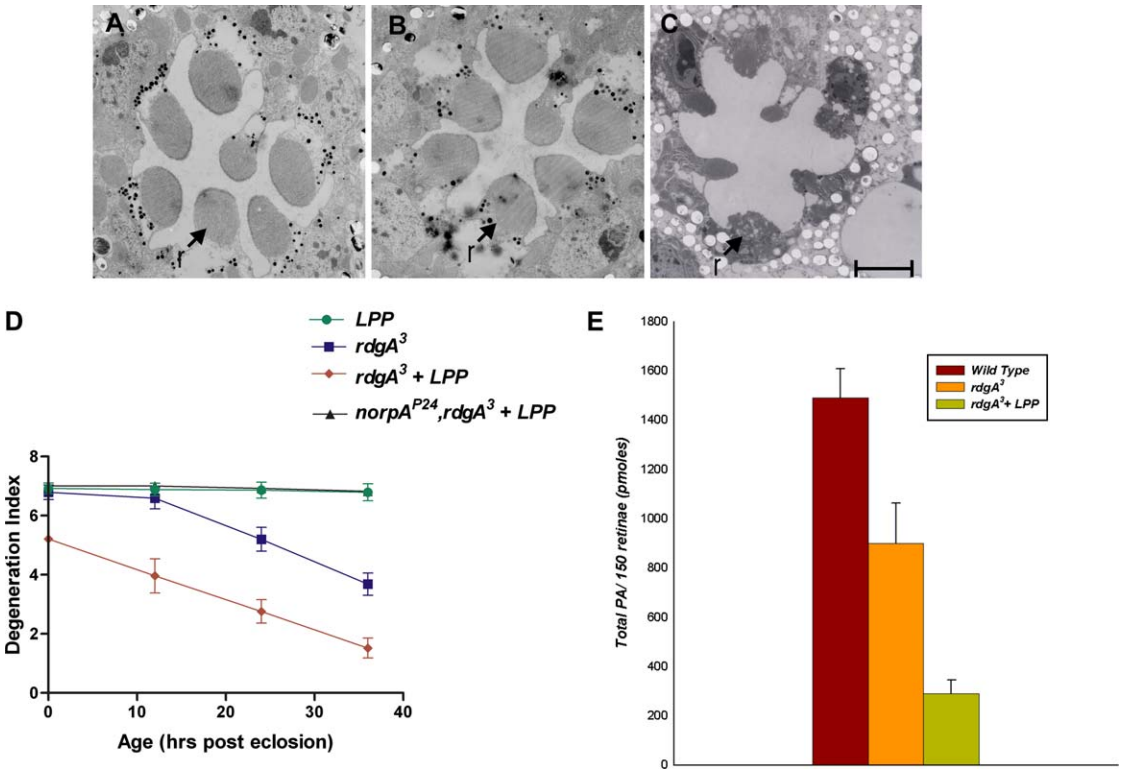

Figure 3. Effects of LPP Overexpression in Photoreceptors

EM images showing a single ommatidium from wild-type (A), *rdgA*^{3} (B), and *rdgA*^{3} + LPP (C) photoreceptors that are 0–12 hr after eclosion. Rhabdomeres are marked with r. Scale bar, 2 μm. The LPP used in these experiments was CG11426. (D) Time course of retinal degeneration compared between *rdgA*^{3} and CG11426 overexpression in *rdgA*^{3}. Overexpression in an otherwise wild-type background (*LPP*) as well as in photoreceptors lacking both PLCβ and DGK are shown (*norpA*^{P24}*, rdgA*^{3} + *LPP*). Each time point is mean ± SD of 50 ommatidia from at least five separate flies. (E). Total levels of PA measured in lipid extracts from retinae. Values shown are the means ± SD of three separate extractions and analysis.

presented for only *laza*^{22}, the smaller deletion. On eclosion, *laza*^{22} photoreceptors show normal ultrastructure. However, when grown in bright light, they undergo progressive vesiculation and degeneration of the rhabdomeres that is quite severe by 5 days post-eclosion (Figure 4D). Such degeneration was not seen when *laza*^{22} flies were grown in dim light (Figure 4C). The observation that *laza* mutants show light-dependent retinal degeneration strongly suggests that this gene product functions during phototransduction.

We analyzed levels of retinal phospholipids from *laza*^{22}, comparing samples from flies grown in dark with those grown in bright light. This analysis revealed an ~2.5-fold increase in PA levels when *laza*^{22} were grown in light compared to those grown in dark as well as wild-type flies grown in light (Figure 4E). By contrast, there were no significant changes in the levels of PC, PE, PI, and DAG (data not shown). These results demonstrate that light-induced PA levels increase in flies lacking the LPP activity encoded by *laza*.

To test the role of endogenous *wun* and *wun2* in phototransduction, we analyzed the phenotype of loss-of-function mutants in these genes (Figures S2A and S2B). No signs of rhabdomeral vesiculation and degeneration were seen in the case of either *wun* (Figure S2-CvD) or *wun2* (Figure S2-EvF), suggesting that the LPP encoded by these genes do not participate directly in phototransduction.

laza Functions Synergistically with *rdgA* during Phototransduction

If endogenous *laza* is a biochemical antagonist of DGK, an immediate prediction of our findings on overexpression of LPP in *rdgA* is that loss of the endogenous enzyme will have the opposite effect, i.e., suppress degeneration. To test this hypothesis, we generated *rdgA*^{3}*; laza*^{22} double mutants and studied their phenotype. When grown in dim light, *rdgA*^{3} undergoes degeneration that is enhanced by growing flies in bright light (Georgiev et al., 2005). In dim light, *rdgA*^{3}*; laza*^{22} degenerated at almost the same rate as *rdgA*^{3} (data not shown), suggesting that *laza* is probably not active in antagonizing DGK during low rates of $PI(4,5)P_2$ hydrolysis. However, when grown in bright light, *rdgA*^{3}*; laza*^{22} showed a dramatic reduction in the rate of degeneration compared to *rdgA*^{3} under the same conditions (Figures 5A and 5B); *laza*^{22} heterozygotes slowed the degeneration to a rate intermediate between *rdgA*^{3} and *rdgA*^{3}*; laza*^{22}. These results clearly demonstrate that the endogenous LPP encoded by *laza* functions during phototransduction and works in synergy with the DGK encoded by *rdgA*.

Owing to its ability to rescue degeneration in the blind mutant *rdgA* and work synergistically with it, we have named this gene (CG11440) *laza.* The name derives from the Spanish novel *Lazarillo de Tormes*, in which Lazaro is the boy servant of a blind man and helps him to see.

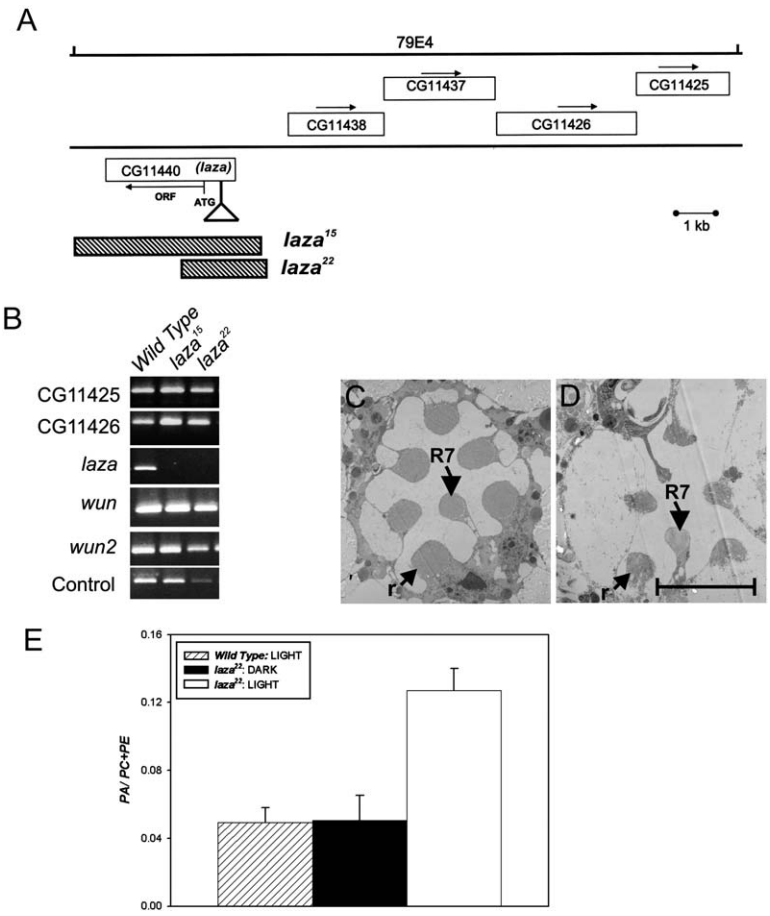

Figure 4. Photoreceptor Phenotypes of *laza* Loss-of-Function Mutants

(A) Genomic map of the 79E region on chromosome 3, showing genes for five LPPs described in the text. The site of insertion of EY04417 in *laza* is shown. Arrows indicate the strand of the open reading frame. The extent of deletions in $laza^{15}$ and $laza^{22}$ are shown. (B) RT-PCR analysis of RNA from heads of $laza^{15}$ and $laza^{22}$ compared to wild-type heads. RNA for *laza* is absent in the two *laza* mutant alleles. Levels of four other LPPs detected in wild-type heads are unaffected in the mutant alleles. EM images showing a single ommatidium from 5-day-old $laza^{22}$ flies grown in darkness (C) and bright light (D). Vesiculation and degeneration of rhabdomeres (r) is seen when this mutant is grown in bright light. The R7 rhabdomere is relatively normal as in other phototransduction mutants such as *rdgA* and *rdgB*. (E) Analysis of retinal PA levels in $laza^{22}$ mutants. Levels of PA in mutant retinae grown in dark and light are compared with wild-type retinae grown under similar illumination. Levels are show as pmol (PA)/pmol (PC + PE). Data are the means ± SD of three separate analyses.

We also tested whether the concerted activity of *rdgA* and *laza* impacts on TRP and TRPL function during the light response. To do this, we analyzed responses to light in wild-type, $rdgA^3$, and $rdgA^3$; $laza^{22}$ double mutants. We compared the rate of deactivation of the light response following a 1 s stimulus of light (Figures 5C and 5D). Under these conditions, $rdgA^3$ mutants show a pronounced decrease in the rate of deactivation. This defect in deactivation was substantially rescued in the $rdgA^3$; $laza^{22}$ double mutant. In this study, we were unable to find a clear electrophysiological phenotype for the $laza^{22}$ single mutant using either ERG or whole-cell recording. We also studied the effect of overexpressing *laza* in $rdgA^3$ photoreceptors. Since $rdgA^3$ + *laza* photoreceptors show rhabdomeral degeneration which itself could affect deactivation kinetics, we used $rdgA^3$/+ photoreceptors for this analysis. $rdgA^3$/+ photoreceptors do not show retinal degeneration over the time periods used in this study; however, they do show a small but distinct defect in deactivation (Figures 5E and 5F). This defect was enhanced by overexpression of *laza* (Figures 5E and 5F). Overexpression of *laza* in wild-type photoreceptors did not have any effect on deactivation kinetics (data not shown). Together, our findings suggest that the combined activity of DGK (encoded by *rdgA*) and LPP (encoded by *laza*) can regulate the deactivation of TRP and TRPL during the light response.

To test the role of endogenous *wun* and *wun2* in phototransduction, we generated double mutants of these with $rdgA^3$ and studied their rates of degeneration under bright light illumination. We found that neither *wun* nor *wun2* were able to slow the rate of degeneration in $rdgA^3$ (Figure S2G), suggesting that the endogenous LPP encoded by these genes does not participate directly in phototransduction.

Changes in Retinal PA in *rdgA* Are Associated with Depletion of PI

While our results demonstrate the concerted action of *rdgA* and *laza* in regulating PA levels during phototransduction, they raise the question of how PA levels are linked to abnormal TRP channel activity seen in *rdgA*. One possibility is that PA directly regulates TRP channels, although it has previously been reported that supplementation of PA during whole-cell recording failed to suppress constitutive TRP activity in *rdgA* (Raghu et al., 2000b). To test the possibility that there might be changes in other phospholipid classes that could account for the *rdgA* phenotype, we performed a lipidomic analysis studying three other classes of phospholipids, including PI, phosphatidylcholine (PC), and phosphatidylethanolamine (PE). Since the reduction in absolute levels of phospholipids presumably partly reflects the ongoing degeneration process and associated loss of membranes, we expressed the levels of each phospholipid as a fraction of the level of PC + PE that are major building blocks of membranes. This analysis revealed a significant reduction in the levels of PI in $rdgA^3$ that was further enhanced on overexpression of LPP (Figure 6D). These findings demonstrate that the reductions

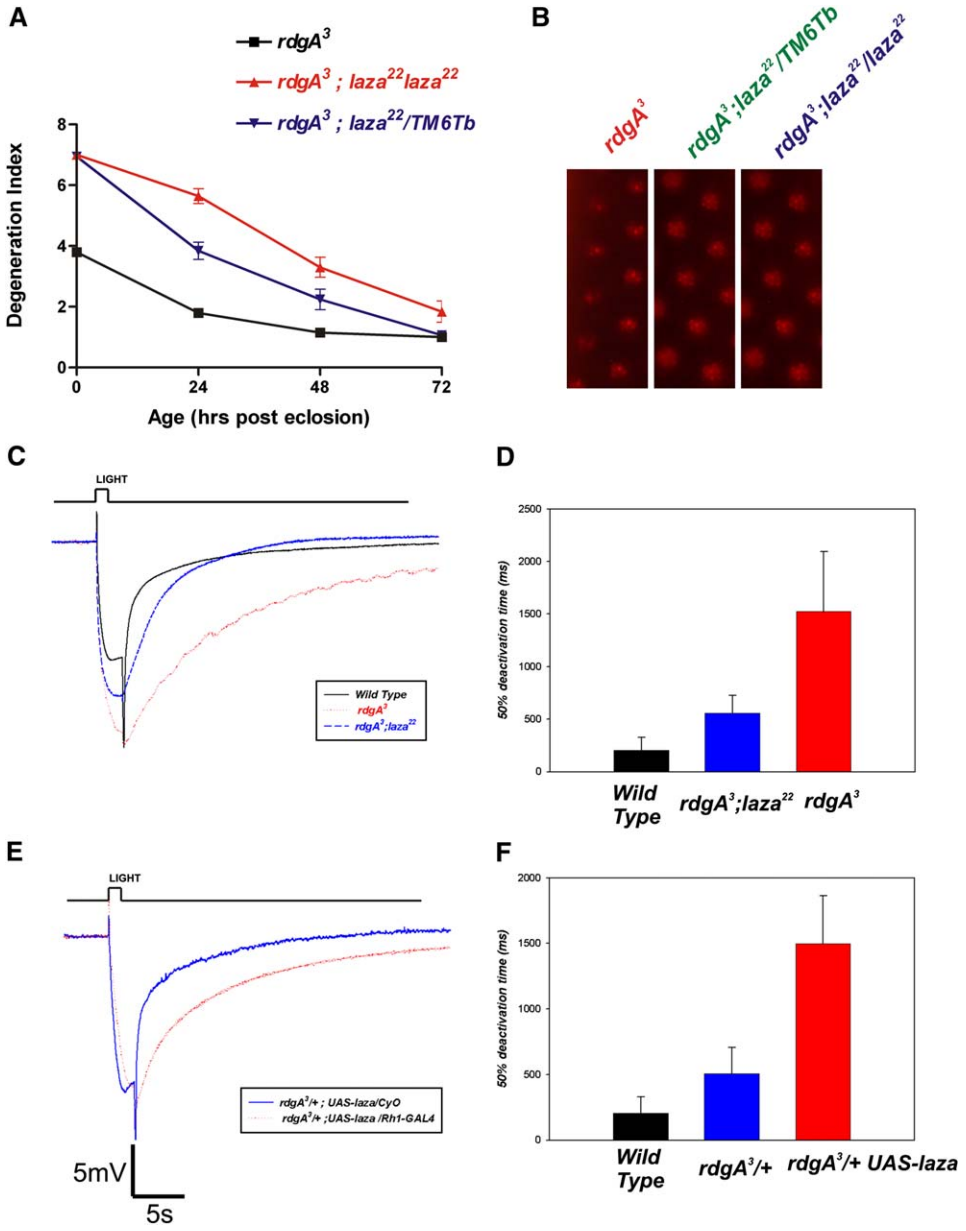

Figure 5. Concerted Action of *rdgA* and *laza* in Photoreceptors

(A) Rates of photoreceptor degeneration of $rdgA^3$ and $rdgA^3$; $laza^{22}$ mutants when grown in bright light. $rdgA^3$; $laza^{22}$ mutants degenerate slower that $rdgA^3$ after eclosion. The $laza^{22}$/*TM6Tb* heterozygote shows an intermediate phenotype. Each time point is mean ± SD of 50 ommatidia from at least five separate flies.

(B) Individual optical neutralization images showing ommatidia from $rdgA^3$, $rdgA^3$; $laza^{22}$, and $laza^{22}$/*TM6Tb*, showing the partial rescue of degeneration by the *laza* mutant.

(C) ERG responses to single 1 s flashes of light from wild-type, $rdgA^3$, and $rdgA^3$; $laza^{22}$. Traces show the slow rate of deactivation in $rdgA^3$ that is largely rescued in $rdgA^3$; $laza^{22}$.

(D) Rate of deactivation quantified as the time taken for the light response to decay to 50% of its peak amplitude. Data presented are the mean ± SD of approximately ten responses from each of five separate flies.

(E) ERG responses to single 1 s flashes of light from $rdgA^3$/+ retinae ($rdgA^3$/+; UAS-*laza*/CyO). These show a small but clear defect in deactivation. This deactivation is massively enhanced by overexpression of *laza* using Rh1-GAL4 ($rdgA^3$/+; UAS-*laza*/Rh1-GAL4).

(F) Rate of deactivation quantified as the time taken for the light response to decay to 50% of its peak amplitude. Data presented are the mean ± SD of approximately ten responses from each of five separate flies.

in PA levels during phototransduction are associated with a reduction in at least one other phospholipid class, namely PI, and raise the possibility that reduction in photoreceptor PI levels might contribute to the *rdgA* phenotype.

Mutants in *cds* Enhance Degeneration of $rdgA^3$

To test the role of PI depletion in the *rdgA* phenotype, we used mutants in CDP-DAG synthase (cds^1) (Wu et al., 1995), the enzyme that converts PA to CDP-DAG (Figure 1). cds^1 has previously been shown to affect the

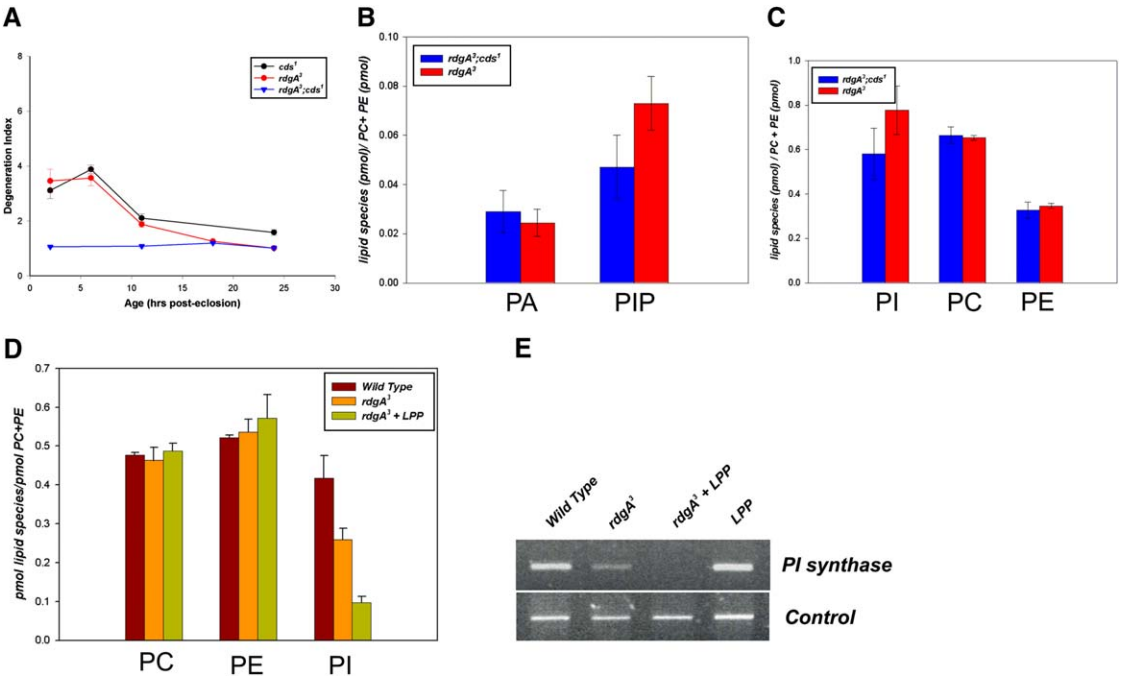

Figure 6. *cds* Enhances the *rdgA* Phenotype

(A) Comparison of the rate of degeneration of $rdgA^3$, cds^1, and $rdgA^3$; cds^1 when grown in bright light. The double mutant degenerates faster than each of the single mutants.

(B) Levels of PA and PIP in $rdgA^3$ and $rdgA^3$; cds^1. Levels of PA are not significantly changed, but PIP levels are decreased in $rdgA^3$; cds^1. Levels of each phospholipid are shown as pmol (lipid class)/pmol (PC + PE). Data shown are mean ± SD from three separate experiments. 150 retinae/genotype were used per experiment.

(C) Levels of PI, PC, and PE in $rdgA^3$ and $rdgA^3$; cds^1. Levels of PI show a marginal change. Levels of other lipids are not changed.

(D) Levels of PC, PE, and PI in wild-type, $rdgA^3$, and $rdgA^3$ + *LPP*. Levels of each phospholipid are shown as pmol (lipid class)/pmol (PC + PE). Data shown are mean ± SD from three separate experiments. 150 retinae/genotype were used per experiment. Significant changes are seen in the levels of PI.

(E) RT-PCR analysis of PI synthase transcripts in retinae. PCR analysis was done on an oligodT primed RT. As a control, levels for *itpr* RNA were measured using the same RT reaction. Reduced levels of transcript are seen in $rdgA^3$ and are almost completely lost in $rdgA^3$ + *LPP*.

rate of $PI(4,5)P_2$ resynthesis during the light response (Hardie et al., 2001). In addition, we have recently found that cds^1 mutants show reductions in the abundance of the two molecular species that constitute the major fraction of PI in wild-type photoreceptors (data not shown). We generated $rdgA^3$; cds^1 double mutants and compared the rate of degeneration to $rdgA^3$ and cds^1. We found that cds^1 enhanced the rate of degeneration in $rdgA^3$ (Figure 6A). To understand the biochemical basis of this degeneration, we analyzed phospholipid levels comparing $rdgA^3$ retinae with those from $rdgA^3$; cds^1. This study revealed that although PA and DAG levels were not different in the two genotypes, there were significant changes in the levels of PI ($0.05 < p < 0.1$) (Figure 6C) and PIP ($0.001 < p < 0.01$) (Figure 6C), suggesting that reduced levels of PI and PIP may contribute to the degeneration phenotype of $rdgA^3$.

PA Regulates Levels of PI Synthase Transcripts

An immediate metabolic fate of PA is conversion to PI by the sequential activity of CDP-DAG synthase and PI synthase (Figure 1). Thus, it is likely that the reduction of PI levels in $rdgA^3$ and $rdgA^3$ + LPP are partly explained by the reduced levels of PA available as substrate for CDP-DAG synthase. However, recently it has been shown that in yeast PA can transcriptionally regulate the levels of PI synthase (Loewen et al., 2004). To test whether this was also the case in *Drosophila* photoreceptors, we used RT-PCR to compare levels of PI synthase (CG9245-CDP-diacylglycerol-inositol 3-phosphatidyltransferase) transcript in wild-type retinae with those from $rdgA^3$ and $rdgA^3$ + *LPP*. Our analysis revealed that CG9245 transcript levels in $rdgA^3$ were reduced and were virtually obliterated in RNA from $rdgA^3$ + *LPP* retinae (Figure 6E). Thus, the levels of PI synthase transcript are directly correlated with the levels of retinal PA.

laza Modulates the Degeneration of *rdgB*

Since our biochemical analysis strongly indicated that PA levels generated during phototransduction are linked to PI resynthesis, we wondered whether this might also impact on the $PI(4,5)P_2$ resynthesis. To test this in vivo, we used the *rdgB* mutant that encodes the *Drosophila* homolog of PI transfer protein. Loss-of-function mutants in *rdgB* show (1) light-dependent retinal degeneration (Vihtelic et al., 1991, 1993) and (2) a reduced rate of $PI(4,5)P_2$ resynthesis during the light response (Hardie et al., 2001). We overexpressed LPP in *rdgB* mutants and compared $rdgB^{KS222}$ with $rdgB^{KS222}$ + LPP. This analysis showed that overexpression of LPP enhances degeneration in $rdgB^{KS222}$ (Figure 7A). Conversely,

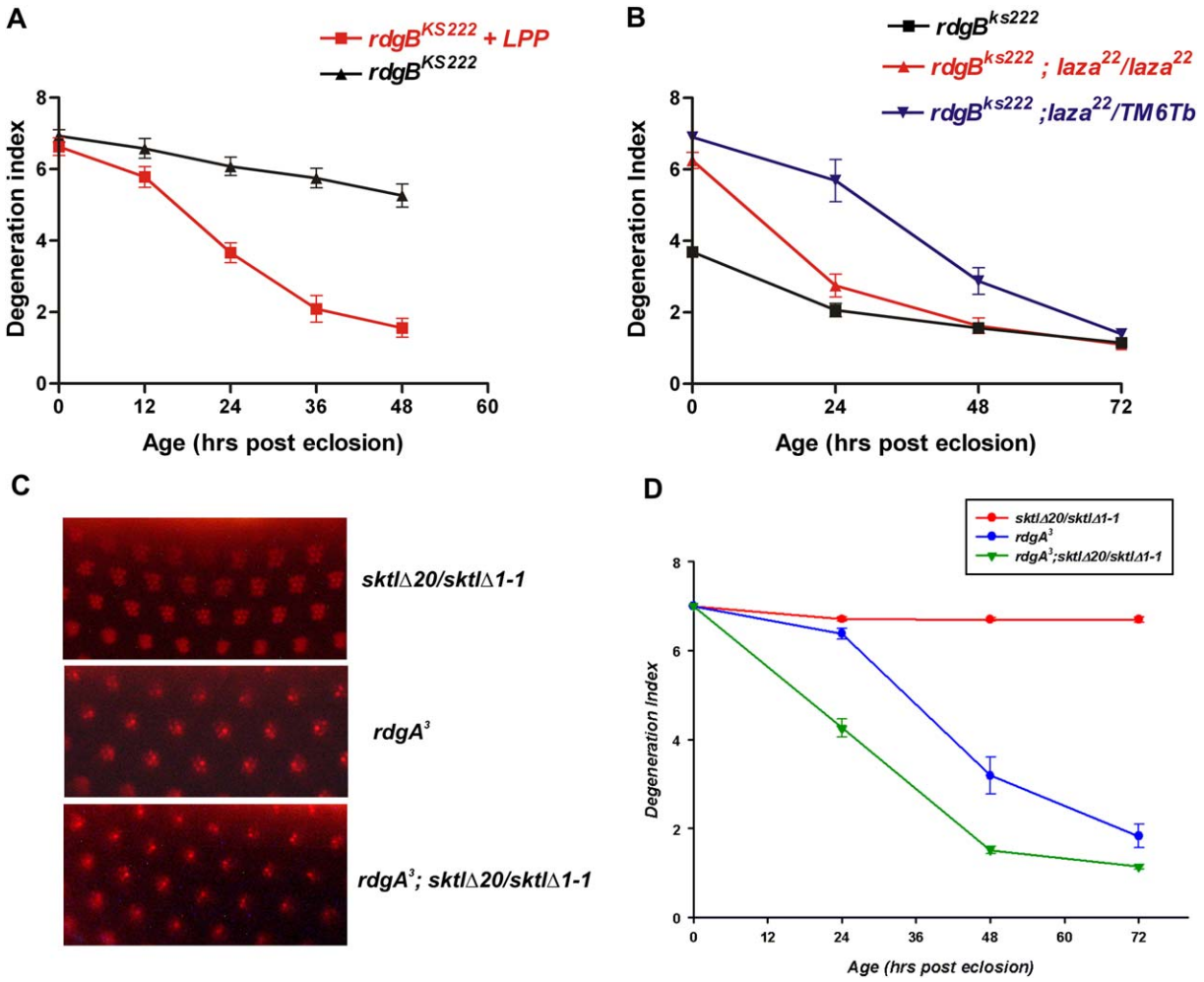

Figure 7. Effects of Diminished PI(4,5)P_2 Resynthesis in Photoreceptors

(A) Retinal degeneration in *rdgB*^{KS222} flies compared to that of the same strain overexpressing CG11426. A dramatic enhancement of degeneration is seen on overexpressing CG11426.

(B) Rates of photoreceptor degeneration of *rdgB*^{KS222} and *rdgB*^{KS222}*; laza*^{22} mutants when grown in bright light. *rdgB*^{KS222}*; laza*^{22} mutants degenerate slower that *rdgB*^{KS222} after eclosion. The *laza*^{22}*/TM6Tb* heterozygote shows a better suppression of degeneration.

(C) Representative images showing rhabdomere integrity in *rdgA*^{3}, *sktlΔ20/sktlΔ1-1*, and *rdgA*^{3}*; sktlΔ20/sktlΔ1-1*. *sktlΔ20/sktlΔ1-1* shows wild-type rhabdomeres. *rdgA*^{3}*; sktlΔ20/sktlΔ1-1* rhabdomeres are more degenerate than *rdgA*^{3} at the same age.

(D) Rate of degeneration in *rdgA*^{3}, *sktlΔ20/sktlΔ1-1*, and *rdgA*^{3}*; sktlΔ20/sktlΔ1-1*. *rdgA*^{3}*; sktlΔ20/sktlΔ1-1* degenerates faster than *rdgA*^{3} alone. In this time frame, *sktlΔ20/sktlΔ1-1* does not show significant degeneration.

loss-of-function mutants in *laza* were able to slow the rate of degeneration of *rdgB*^{KS222} (Figure 7B).

sktl Enhances Degeneration in *rdgA*^{3}

To test the role of reduced PI(4,5)P_2 synthesis consequent to the reduced PA and PI levels in *rdgA*, we analyzed the effect of reduced type 1 PIP kinase activity on the *rdgA* phenotype. For this we used mutants in *sktl* that encode one of the two type I PIP kinases in *Drosophila.* Since null mutants in *sktl* are cell-lethal in photoreceptors, we used a heteroallelic combination of *sktlΔ20* (a null allele) (Hassan etal., 1998) and *sktlΔ1-1* (a hypomorphic insertion in the upstream region of the *sktl* gene). *sktlΔ20/sktlΔ1-1* itself does not show any degeneration on the timescale of the experiments described (Figure 7E). We generated *rdgA*^{3}*; sktlΔ20/sktlΔ1-1* photoreceptors and compared the rate of degeneration to that of the *rdgA*^{3} single mutant. This analysis revealed that *sktlΔ20/sktlΔ1-1* enhanced the rate of degeneration in *rdgA*^{3}.

Subcellular Localization of LPP Overexpressed in Photoreceptors

The finding that LPP can regulate intracellular PA levels in *Drosophila* photoreceptors raises questions on where the enzyme is localized within these cells. We studied the subcellular localization of GFP-tagged *laza* in *Drosophila* photoreceptors between 0–12 hr after eclosion. Under these conditions, we could not detect expression of *laza*-GFP in the plasma membrane of either the rhabdomere or the cell body (Figures 8A and 4D). Rather, the protein appeared to be punctate and distributed throughout the cell body. The pattern of distribution showed considerable overlap to that of an antibody directed to the ER retention signal KDEL (Napier et al., 1992) (Figures 8B and 8D). These results suggest that

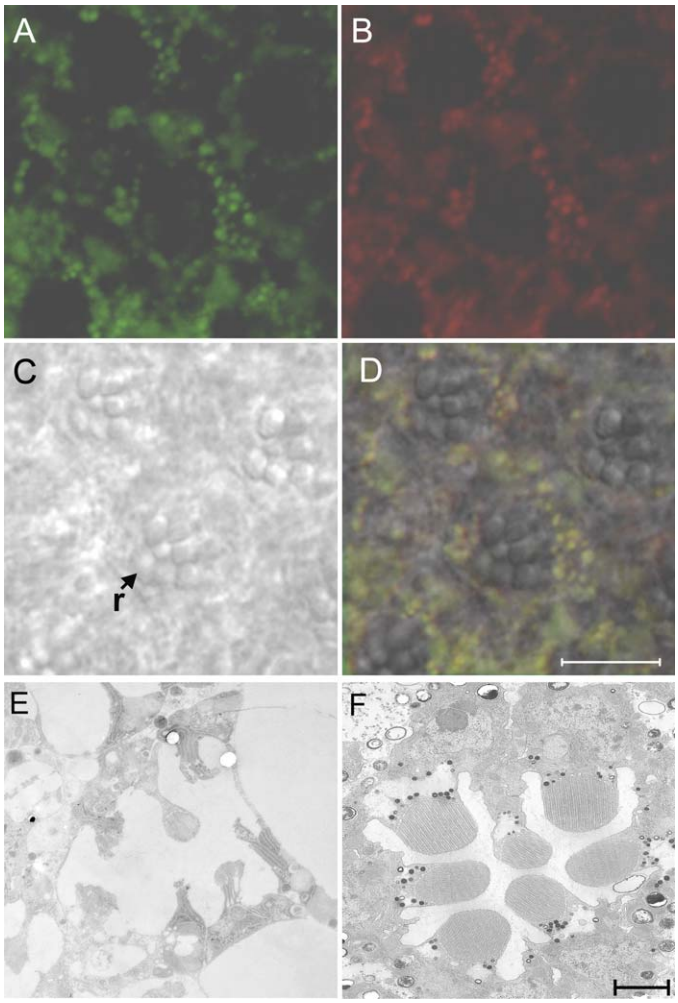

Figure 8. Subcellular Localization of *laza* in Photoreceptors

Confocal images of ommatida from retinae overexpressing GFP-tagged *laza* under conditions described in the manuscript. (A) GFP antibody used to detect the tagged *laza*. No signal can be seen from the plasma membrane of the rhabdomere. (B) Pattern of staining seen with a monoclonal antibody to KDEL, the ER retention signal. (C) DIC image of the same section, showing the rhabdomeres (r) that constitute the bulk of photoreceptor plasma membrane. (D) Overlay of the three previous images, showing substantial coincidence of the GFP and KDEL signal. Rescue of light-dependent retinal degeneration in *laza* mutants by the *laza*-GFp construct. Images shown are for $laza^{22}$ (E) and $laza^{22}$; UAS-*laza*-GFP/Rh1-GAL4 (F). Flies shown are both day five under constant illumination.

under these conditions the overexpressed enzyme is localized to the endoplasmic reticulum or a compartment thereof. Similar results were obtained with CG11426. Despite concerted efforts, we have not been able to raise an antibody that allows us to study the distribution of the endogenous protein. However, to test whether *laza*-GFP encodes a functional protein whose distribution might report that of the endogenous enzyme, we attempted to rescue the phenotype of $laza^{22}$ using the *laza*-GFP construct used in this study. This analysis revealed that *laza*-GFP was able to rescue the retinal degeneration phenotype of $laza^{22}$ restoring wild-type rhabdomere ultrastructure (Figure 8E versus 8F).

Discussion

During receptor-activated cell signaling cascades, it is important that the resynthesis of biochemical substrates is tightly coupled to their consumption by enzymatic reactions. This is particularly necessary in the context of neuronal signaling cascades, where at high rates of receptor stimulation, significant depletion of substrates might otherwise occur. For example, in bright daylight, *Drosophila* photoreceptors are able to successfully detect $\sim 10^6$ photons s^{-1} without significant loss of sensitivity (Hardie and Raghu, 2001). In the context of a G protein-coupled phosphoinositide signaling cascade, the hydrolysis of the substrate $PI(4,5)P_2$, a minor membrane phospholipid, needs to be coupled to its resynthesis by the sequential phosphorylation of a cellular pool of PI. Thus, an adequate rate of $PI(4,5)P_2$ resynthesis requires among other things the maintenance of this pool of PI. PI is synthesized in the ER by the condensation of inositol and CDP-DAG by PI synthase. Previous studies have suggested the importance of a constant supply of inositol generated from IP_3 to maintain ongoing G protein-coupled PLC signaling in neurons (Gani et al., 1993; Jope et al., 1992; Kendall and Nahorski, 1987; Kennedy et al., 1990). However, the role, if any, for the recycling of DAG to supply CDP-DAG required for PI synthesis has thus far not been addressed. In addition to being an allosteric activator of PKC (Nishizuka, 1992), PA can be recycled into PI resynthesis by the sequential action of CDP-DAG synthase and PI synthase (Figure 1). In the present study, we found that (1) in $rdgA^3$ photoreceptors, levels of total PA (Figure 3E) are reduced and that (2) this reduction is enhanced by overexpressing LPP. Together with previous findings that DAG levels are not elevated in the *rdgA* (Inoue et al., 1989), our observations demonstrate the critical role of PA levels generated

by DGK in the *rdgA* phenotype. However, the findings that elevation of PA levels via the patch pipette does not suppress constitutive TRP channel activity and that elevation of PA levels using the *cds*^{1} mutant did not rescue *rdgA*^{3} strongly suggest that PA does not directly influence TRP channel activity or contribute to the degeneration phenotype.

How might reduced levels of photoreceptor PA contribute to the *rdgA* phenotype? In this study, we made two observations that provide an insight into this question. (1) The *cds*^{1} mutant that elevates photoreceptor PA levels while reducing PI and PIP levels enhanced rather than suppressed degeneration in *rdgA*^{3}. (2) The reductions in PA level were associated with a change in PI levels in *rdgA*^{3} retinae, and PI levels underwent a dramatic reduction when PA levels were further reduced by LPP overxpression (Figure 6D). Taken together, it is likely that our findings reflect the requirement for a constant supply of PA generated by *rdgA* so that CDP-DAG can be generated by CDP-DAG synthase to be used as a substrate for PI synthesis. Our finding that transcript levels of PI synthase in photoreceptors are directly correlated with PA levels suggests one mechanism by which PA might regulate PI resynthesis linked to the level of ongoing PI(4,5)P_2 hydrolysis. In our analysis, we found colocalization of the enzyme within the cell body in a pattern highly reminiscent of the ER within these cells (Figure 8D). This membrane compartment is the site at which the resynthesis of PI(4,5)P_2 is initiated by the enzyme CDP-DAG synthase using PA as the substrate (Lykidis et al., 1997). A previous study has concluded that in photoreceptors, DGK (encoded by *rdgA*) is also localized on the submicrovillar cisternae thought to be a specialization of the ER (Masai et al., 1997). Thus, three key enzymes that together contribute to the regulation of cellular PA and generation of PI appear to be localized to the ER, consistent with a role for PA in stimulating resynthesis of PI.

Since the sequential phosphorylation of PI is a major route of PI(4,5)P_2 generation, changes in PI levels could impact on photoreceptor PI(4,5)P_2 resynthesis. To test this possibility, we analyzed the effect of *laza* activity in *rdgB*, a mutant with reduced rates of PI(4,5)P_2 resynthesis at the rhabdomeral plasma membrane (Hardie et al., 2001). We tested the effect of altering PA levels by both overexpressing LPP as well as using *laza* mutants to remove the endogenous LPP that functions during phototransduction and found that the retinal degeneration phenotype of *rdgB* could be modulated by levels of LPP activity in the retina. Together with our biochemical data on the effects of altered PA levels in photoreceptors, these results suggest a role for PA levels in regulating the supply of PI to be transported to the plasma membrane for PI(4,5)P_2 resynthesis during phototransduction. How might PA levels modulate the *rdgB* phenotype? It has previously been shown that PA does not bind to and alter PITP function in vitro (Whatmore et al., 1999). One obvious mechanism is that the level of PI (a key substrate that is transported by PITP) is altered by changes in *laza* activity. An alternative mechanism is suggested by the finding that in vitro PA binds to and stimulates type I PIPkin activity (Jenkins et al., 1994). Our finding that reduced type 1 PIPkinase activity (*sktlΔ20/sktlΔ1-1)* enhances the rate of degeneration in *rdgA*^{3} (Figures 7C and 7D) supports this mechanism. Thus, there are at least three mechanisms by which PA derived from PI(4,5)P_2 can stimulate PI(4,5)P_2 resynthesis: (1) as a unique substrate for CDP-DAG synthesis, (2) as a transcriptional regulator of PI synthase, (3) as an allosteric regulator of type I PIPkin. In the present study, we provide evidence in support of these three mechanisms in photoreceptors. We therefore propose that the generation of PA by DGK during PLCβ-mediated signaling provides a key signal to regulate the resynthesis of PI(4,5)P_2.

LPP Functions Synergistically with DGK to Modulate PA Levels during Signaling

Given the critical role of PA during phototransduction, we identified LPP as an enzyme that might work in conjunction with DGK to regulate PA levels. LPP was originally designated as a type II PA phosphatase (PAP) (Jamal et al., 1991; Kai et al., 1996) and shows remarkable substrate promiscuity in vitro (reviewed in Brindley and Waggoner, 1998). When overexpressed in cell culture models, LPPs are able to dephosphorylate extracellular lipids presented to intact cells (Jasinska et al., 1999; Roberts et al., 1998) and localize mainly to the plasma membrane (Alderton et al., 2001; Burnett and Howard, 2003; Jasinska et al., 1999), with residues involved in catalysis facing externally (Zhang et al., 2000). These findings have led to the idea that LPPs are ectoenzymes catalyzing the hydrolysis of extracellular bioactive lipid, although, to date, the in vivo substrate of any LPP has not been identified. In particular, it is unclear whether LPPs can metabolise PA generated by hydrolysis of PI(4,5)P_2. During this study, we analyzed the enzymatic activity of a number of LPP gene products during *Drosophila* phototransduction. We found that overexpressing four different *Drosophila* LPPs individually can exacerbate the phenotype of hypomorphic mutants in DGK. Significantly, the effects of LPP overexpression were evident only in the sensitized background of DGK loss of function, providing strong evidence that LPP can function as antagonists of DGK. Our finding that the reduced levels of PA in *rdgA*^{3} retinae were further reduced on LPP overexpression provides biochemical support for the genetic data. Further, we also demonstrate that *laza*^{22} retinae show light-induced accumulation of PA. This finding supports the idea that *laza* regulates PA levels during phototransduction. Together, our findings provide compelling evidence that in vivo DGK and LPP operate as a kinase/phosphatase pair that controls the level of PA. Our finding that overexpression of LPP in flies lacking both DGK and PLCβ in their photoreceptors (*norpA*^{P24}, *rdgA*^{3}) did not result in retinal degeneration strongly supports the conclusion that in vivo LPP can dephosphorylate PA generated from PI(4,5)P_2 hydrolysis by the sequential enzymatic activity of PLCβ and DGK. Here we identify an in vivo substrate of LPP and also demonstrate a role for LPP in regulating the DAG/PA balance during G protein-coupled PI(4,5)P_2 signaling.

During our study, we found that when overexpressed LPPs encoded by a number of different gene products, including CG11426, *laza*, *wun*, and *wun2*, were all able to antagonize DGK function, reminiscent of the ability of all three isoforms of vertebrate LPPs (LPP1, LPP2,

Table 1. Sequences of Primers Used in This Study

Gene	EST	Forward Primer Sequence (5′-3′)	Reverse Primer Sequence (5′-3′)
CG11425	GH04282	CTTATTGTCCTGGTGGAG	AAAGATTTGGCCGACACTCG
CG11426	GH12758	AGCTAATTACCAGCAGGC	CACGATCAGGCCGAGC
CG11437	RE35159	AGCCTGAAGTATCCCTATCG	GTGTAGTATCCCGCGTAGC
CG11438	LP01491	ACTCGACGATTCACAGAGC	TCCATAAGGATAAGTCCTGC
lazaro	HL01743	CCGAAAGACATCGAAACG	GAGCAGGACCATCATGAGC
wunen	GH02203	GACCATAAAGAACTGGCCC	CACGATGAATATCACGCCG
wunen-2	RE26896	GAAAAGAACTAGACCAAGAACG	GAATGGCACTATTGCACC
itpr		CCGACACATCGATTTCCG	CACCTCAATCCAATATGG
CG9245		GCCTTCTGGTTCATGTCC	GTACAAGCACACGTAAAACAGC

and LPP3) to dephosphorylate PA (Roberts et al., 1998). In contrast, we found, using loss-of-function analysis, that the only LPP that was able to functionally antagonise DGK and hence suppress degeneration in *rdgA* was the eye-enriched LPP *laza*. It has recently been shown that when overexpressed, LPPs can form oligomers (Burnett et al., 2004). It is possible that high levels of expression result in the assembly of oligomers that lack substrate specificity.

Implications for TRP Channel Activation

A common but poorly resolved theme underlying the signaling mechanisms regulating TRPC channel activation is the role of $PI(4,5)P_2$ or its metabolites in activation. Recently, a number of studies have shown that a range of lipids generated by $PI(4,5)P_2$ hydrolysis can activate both vertebrate and invertebrate TRPC channels when applied exogenously to overexpressed channels (reviewed in Hardie, 2003). However, to date, the activation of endogenous TRPC channels has been studied in only two model systems: activation of TRP and TRPL during *Drosophila* phototransduction and the activation of TRPC2 channels in neurons of the mouse vomeronasal organ. In both of these systems, the inactivation of DGK (using the *rdgA* mutant in *Drosophila* and pharmacology in the mouse study; Lucas et al., 2003; Raghu et al., 2000b) results in a sustained activation of TRPC channels in the face of ongoing $PI(4,5)P_2$ hydrolysis. While these findings are consistent with a role for DGK in regulating response termination, they have thus far not allowed a definitive analysis of the messenger of activation. In the mouse vomeronasal organ, the finding that TRPC2-like channels can be activated by exogenous DAG (Lucas et al., 2003) implies that the build-up of this messenger is the biochemical basis of abnormal TRPC channel activation when DGK is inhibited. However, a build-up of DAG in the vomeronasal organ during DGK inhibition has yet to be demonstrated. A previous study showed that in *Drosophila* photoreceptors of the *rdgA* mutant, there was no elevation of DAG (Inoue et al., 1989), while demonstrating that light-induced PA levels were reduced. In the present study, we have demonstrated that in addition to a reduction in retinal PA levels, there is also a reduction in the level of PI, the precursor for $PI(4,5)P_2$ resynthesis, and an associated change in the levels of PI synthase, a key enzyme for PI resynthesis. These findings raise the possibility that the reduced level of PI might also result in a reduction in the rate of $PI(4,5)P_2$ resynthesis. Our finding that reduction in type I PIP kinase activity enhances degeneration in $rdgA^3$ supports this hypothesis. Previous studies have shown that both *rdgB* photoreceptors as well as wild-type photoreceptors (in zero extracellular calcium) show prolonged activation of TRP channels following a response to light (Hardie et al., 2001). In addition, TRPL channels expressed in S2 cells have been reported to be inhibited by $PI(4,5)P_2$ (Estacion et al., 2001). Taken together with the findings reported in this paper, we propose that an imbalance between the rate of $PI(4,5)P_2$ hydrolysis by PLCβ and its resynthesis from PA might underlie the excessive activation of endogenous TRP channels in photoreceptors of the *rdgA* mutant.

Experimental Procedures

Fly Culture and Stocks

Flies were reared on standard cornmeal, dextrose, yeast medium at 25°C and 50% relative humidity in a constant temperature incubator. There was no internal illumination within the incubator, and flies were only subject to brief pulses of light when the incubator door was opened. When required, flies were grown in a cooled incubator with constant illumination from a light source (fluorescent tube providing ~850 lux within the incubator). Rh1-GAL4 insertions were obtained from Dr. C. Desplan, Rockefeller University. $rdgA^3$ mutants were obtained from Dr. Seymour Benzer, Caltech; $rdgB^{KS222}$ mutants were from Dr. R.C.Hardie, Cambridge University. UAS-*wunen*, UAS-*wunen-2* were from Dr. K. Howard, University College, London; EY04417 were from the Bellen Lab/*Drosophila* Genome Project.; so^D, wun^{k16806} and wun^{k12382} were obtained from *Drosophila* Stock Center, Bloomington; and $EP2650^{ex34}$ were from Dr. Ruth Lehmann, NYU School of Medicine, New York.

Mutagenesis

In order to generate excisions of EY04417, the transposon was mobilized using a stable genomic source of transposase (Robertson et al., 1988). The progeny was screened for flies that had lost the P{white$^+$} marker. Such animals were crossed to w; TM3Sb/TM6Tb to balance the third chromosome, and several independent lines were set up. Each line was then analyzed as described below.

Molecular Analysis of Excisions

Initial analysis of excision lines was performed using a series of primers designed to the genomic region around the initial insertion in the 79E region. This allowed an initial estimate of the size of the deletions. Genomic DNA was prepared as described (Gloor et al., 1993), and genomic PCR was performed using the long-range high-fidelity EXL Polymerase (Stratagene) to generate a PCR product spanning the deletion. The PCR product was gel purified and cloned into pCR-XL-TOPO using the TOPO-XL TA cloning kit (Invitrogen). Recombinant colonies were identified, and three independent clones were sequenced. Sequences were assembled using STADEN, and alignment of sequences from mutant versus wild-type sequences was used to identify the breakpoints of the excision in each strain.

RT-PCR Analysis

Total RNA was extracted from freeze-dried dissected retinae as previously described (Raghu et al., 2000a). Reverse transcription was done using oligodT priming. The primers used for PCR analysis of LPP expression and PI synthase (CG9245) are shown in Table 1.

Generation of CG11426 and *lazaro* Overexpression Flies

The *LPP* cDNA used to generate UAS-CG11426 and UAS-*lazaro* transgenic flies in this study originated from the clone pOT2a-GH12578 and pOT2a-HL01743, respectively. Clones were completely sequenced to confirm the presence of a full intact open reading frame. Both LPPs were expressed as C-terminal GFP-tagged fusions generated by in vitro recombination (Horton, 1997) and subcloned into the EcoR1/Xho1 sites of the fly transformation vector pUAST (Brand and Perrimon,1993). Germline transformation was performed using established protocols (Rubin and Spradling, 1982). Single mapped insertions were used in the experiments described. Overexpression in the retina was performed using the modular GAL4/UAS system.

Optical Neutralization and Scoring Retinal Degeneration

Flies were immobilized by cooling on ice, decapitated using a sharp razor blade, and fixed on a glass slide using a drop of colorless nail varnish. Optical neutralization and quantitation of degeneration was done as previously described (Georgiev et al., 2005).

Antibodies

The following antibodies were used in this work: anti-rhodopsin mouse monoclonal 4C5 and the anti-syntaxin1 monoclonal 8C3 (Developmental Studies Hybridoma Bank, University of Iowa); anti-GFP monoclonal JL-8 (Clontech Laboratories, Palo Alto, CA) and anti-KDEL from Dr. Geoff Butcher, Babraham Institute. For immunofluorescence, anti-mouse Alexa Fluor 488 and anti-rat Alexa Fluor 633 (Molecular Probes) were used.

Immunohistochemistry

Whole-mount immunohistochemistry was performed on 0- to 12-hr-old flies using a modification of previously published methods (Karagiosis and Ready, 2004). Retinae were dissected in ice-cold PBS and fixed with 4% PFA in PBS for 1 hr on ice. Fixed eyes were given three 10 min washes in PBST (PBS with 0.25% Triton X-100). Blocking was performed using 10% FBS for 1 hr at room temperature. Incubations with primary antibodies (diluted in PBST with 5% FBS) were performed at 4ºC overnight. Following washes in PBST, samples were incubated in secondary antibodies diluted in PBST for 2 hr at room temperature. Samples were mounted in Citifluor (Agar Scientific, UK) and viewed using a Zeiss LSM 510 META confocal microscope using a Plan-Apochromat 63× N.A. 1.40 (oil) objective.

Electron Microscopy

Eyes were prepared for histology and viewed by EM as previously described (Raghu et al., 2000b).

Isolation of Pure Retinal Tissue

Pure preparations of retinal tissue were collected using previously described methods (Fujita et al., 1987; Matsumoto and Pak, 1982). Briefly, 0- to 12-hr-old flies were snap frozen in liquid nitrogen and dehydrated in acetone at −20ºC for 48 hr. The acetone was then drained off and the retinae dried at room temperature. They were cleanly separated from the head at the level of the basement membrane using a flattened insect pin.

Analysis of Retinal Lipids

150 freeze-dried retinae dissected as described above were homogenized in 0.5 ml methanol (containing 500 ng each of 12:0/12:0 species of DAG, PA, PtdCho, phosphatidylethanolamime, phosphatidylglycerol, and phosphatidylserine as internal standards) using a 1 ml glass homogeniser until completely disrupted (>100 strokes). The methanolic homogenate was transferred into a glass, screw-capped tube. Further methanol (0.5 ml) was used to wash the homogenizer and combined in the glass tube. Chloroform (2 ml) was added and left to stand for 10 min. 0.88% KCl (1 ml) was then added to split the phases. After removal of the upper phase and interfacial material, the lower organic phase containing the lipids was dried, resuspended in 15 μl chloroform, and finally transferred into a silanized autosampler vial ready for analysis.

Phospholipids (1 μl injection) were separated on a Luna silica column (3 μm, 1.0 × 150 mm; Phenomenex) using 100%-chloroform/methanol/water (90:9.5:0.5) containing 7.5 mM ethylamine changing to 100% acetonitrile/chloroform/methanol/ water (30:30:35:5) containing 10 mM ethylamine over 20 min at 100 μl/min. Detection was by electrospray ionization in both positive (PC) and negative modes (PA, PE, PG, PI, PS) on a Shimadzu QP8000α single quadrapole mass spectrometer (probe voltage, ±4 kV; nebulizer gas, 4 l/min N_2; desolvation line temperature, 300ºC).

Electroretinogram Recordings

Flies were anesthetised and immobilized at the end of a disposable pipette tip using a drop of molten wax. Recordings were done using glass microelectrodes filled with Ringer's solution, and improved electrical contact was achieved using a tiny drop of ultrasound gel. Voltage changes were recorded between the surface of the eye and an indifferent electrode placed elsewhere on the head capsule. Following fixing and positioning, flies were dark adapted for 5 min. Stimulating light from a 50 watt halogen lamp was delivered to within 5 mm of the fly's eye through a fluid-filled light guide and timed using a Uniblitz shutter. Voltage changes were amplified using a DAM50 amplifier (WPI) and recorded using pCLAMP. Analysis of traces was performed using Clampfit (Axon Laboratories).

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/49/4/533/DC1/.

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