

# Regulation of phototransduction responsiveness and retinal degeneration by a phospholipase D-generated signaling lipid

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**D***rosophila melanogaster* phototransduction proceeds via a phospholipase C (PLC)-triggered cascade of phosphatidylinositol (PI) lipid modifications, many steps of which remain undefined. We describe the involvement of the lipid phosphatidic acid and the enzyme that generates it, phospholipase D (Pld), in this process. *Pld*<sup>null</sup> flies exhibit decreased light sensitivity as well as a heightened susceptibility to retinal degeneration. Pld overexpression rescues flies lacking PLC from light-induced,

metarhodopsin-mediated degeneration and restores visual signaling in flies lacking the PI transfer protein, which is a key player in the replenishment of the PI 4,5-bisphosphate (PIP<sub>2</sub>) substrate used by PLC to transduce light stimuli into neurological signals. Altogether, these findings suggest that Pld facilitates phototransduction by maintaining adequate levels of PIP<sub>2</sub> and by protecting the visual system from metarhodopsin-induced, low light degeneration.

## Introduction

The phototransduction cycle of *Drosophila melanogaster* is one of the best understood examples of inositol lipid signaling. As in most invertebrates, phototransduction in *D. melanogaster* uses a G protein-coupled phosphoinositide pathway (Fig. 1; for reviews see Montell, 1999; Hardie, 2001; Hardie and Raghu, 2001) wherein photoisomerization of rhodopsin to metarhodopsin in the photoreceptor rhabdomeric microvilli activates heterotrimeric Gq. Gq- $\alpha$  then activates a phosphatidylinositol (PI)-specific PLC, which hydrolyzes PI 4,5-bisphosphate (PIP<sub>2</sub>), generating inositol 1,4,5-triphosphate (IP<sub>3</sub>) and DAG. Direct action of IP<sub>3</sub> on endoplasmic reticulum calcium stores and the stimulation of protein kinase C by DAG are not thought to underlie the subsequent Ca<sup>+</sup> surge that triggers depolarization because genetic inactivation of either the single IP<sub>3</sub> receptor or

the single protein kinase C isoform does not eliminate phototransduction (Raghu et al., 2000a). Instead, through some other mechanism (Raghu et al., 2000b), DAG mediates the opening of several classes of light-sensitive cation channels: transient receptor potential (TRP; Montell and Rubin, 1989; Hardie and Minke, 1992), TRP-like (Phillips et al., 1992; Niemeyer et al., 1996; Xu et al., 2000), and TRP- $\gamma$  (Xu et al., 2000). It has been proposed that conversion of DAG into polyunsaturated fatty acids mediates TRP activation (Chyb et al., 1999; Raghu et al., 2000b), but this remains controversial as DAG lipase activity has not yet been demonstrated in *D. melanogaster* (Agam et al., 2000, 2004; O'Tousa, 2002).

At the base of the rhabdomeric microvilli, a network of subrhabdomeral cisternae (SRC) plays a role in signal termination and in PIP<sub>2</sub> replenishment. DAG is eliminated through its conversion to phosphatidic acid (PA) via DAG kinase; PA, in turn, is converted to PI through additional steps, including the formation of CDP-DAG by CDP-DAG synthase (Wu et al., 1995). PI is then transported to the rhabdomeric microvillar membrane via one or more actions (Milligan et al., 1997) undertaken by the PI transfer protein retinal degeneration B (RdgB; Hotta and Benzer, 1970; Vihtelic et al., 1991, 1993) because flies lacking RdgB exhibit light-stimulated PIP<sub>2</sub> depletion (Hardie et al., 2001).

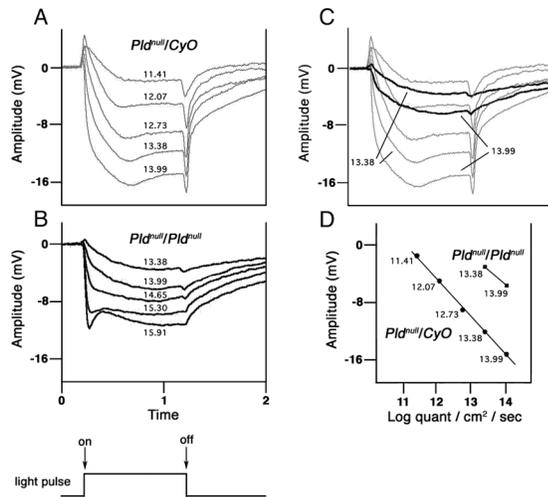
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M. LaLonde and H. Janssens contributed equally to this paper.

Abbreviations used in this paper: Arr2, arrestin2; ERG, electroretinogram; IP<sub>3</sub>, inositol 1,4,5-triphosphate; *norpA*, no receptor potential A; PA, phosphatidic acid; PAP, PA phosphatase; PI, phosphatidylinositol; PIP<sub>2</sub>, PI 4,5-bisphosphate; PI4P5K, PI 4-phosphate 5-kinase; Pld, phospholipase D; RdgB, retinal degeneration B; ROS, rod outer segments; SRC, subrhabdomeral cisternae; TRP, transient receptor potential.

The online version of this article contains supplemental material.





**Figure 2. *Pld*<sup>null</sup> mutant flies display decreased light sensitivity.** ERGs were recorded from heterozygous *Pld*<sup>null</sup>/*CyO* (A) and homozygous *Pld*<sup>null</sup> (B) adult flies 7 d after eclosion. An intensity–response series to 570-nm stimuli is shown for each fly with intensities of 13.99, 13.38, 12.73, 12.07, and 11.41 log quanta/cm<sup>2</sup>/s for the control (A) and 15.91, 15.3, 14.65, 13.99, and 13.38 log quanta/cm<sup>2</sup>/s for the *Pld*<sup>null</sup> mutant (B). A significant response was not observed for the *Pld*<sup>null</sup> mutant at the three lower intensities at which a response was obtained for control flies. Stimulation at 570 nm was chosen because the effects of differences in eye color pigmentation are lower, and on- and off-transients are larger at longer wavelengths (Stark and Wasserman, 1972). Potential was recorded in millivolts as indicated. Three flies were examined for each strain, with similar results observed. (C) The wild-type response was superimposed on the two *Pld*<sup>null</sup> mutant ERGs that were conducted within the range examined for the control flies. Light-induced plateau amplitudes were estimated and plotted (D) as a function of the log of the light stimulation intensities to enable a comparison of the wild-type with the *Pld*<sup>null</sup> mutant responses. Similar results were obtained when *Canton-S* was used as the wild-type control.

### ***Pld* localizes to the cell body and to the base of the rhabdomere, but is not required for retinal ultrastructure**

The findings in Fig. 2 suggested that *Pld* might function by regulating the phototransduction lipid cycle. As one possibility, *Pld* could act in the SRC to add PA into the phototransduction lipid cycle in order to generate more PI and ultimately replenish the PLC-depleted PIP<sub>2</sub>. As a second, nonexclusive possibility, *Pld* might function in the rhabdomere to directly stimulate the PI4P5K-catalyzed phosphorylation of PIP to generate PIP<sub>2</sub>. Protein localization was examined using an affinity-purified polyclonal rabbit antiserum that was directed against the *Pld* NH<sub>2</sub> terminus. *Pld* staining was observed in the photoreceptor

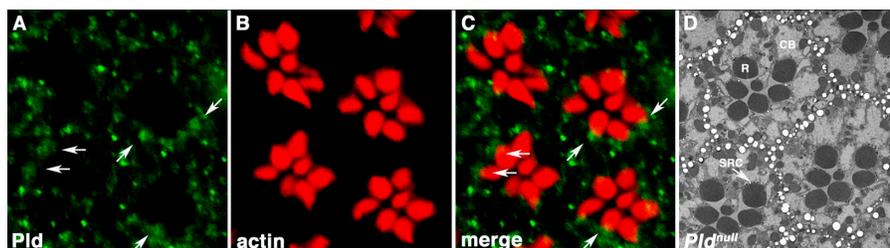
cell body, in some cases most strongly at the base of the rhabdomere (Fig. 3, A–C), which would be consistent with partial localization to the SRC. *Pld* was not observed in the remainder of the rhabdomere where light transduction occurs.

The adult retina in *Pld*<sup>null</sup> mutant flies was examined by using both light and electron microscopy. No apparent changes in morphology were observed (Fig. 3 D), suggesting that *Pld* is not intrinsically required for photoreceptor and ommatidial structural integrity.

### ***Pld* is not activated by light, but does rescue retinal degeneration in the *norpA*<sup>7</sup> mutant**

As demonstrated in Fig. 2, the phototransduction response is diminished in the absence of *Pld*-generated PA. A priori, PA could feed forward as a substrate or could act as a regulator (Fig. 1) to increase the PIP<sub>2</sub> available for PLC hydrolysis during light stimulation. Alternately, mammalian *Pld* is well known as a signaling-regulated enzyme that is activated by a wide variety of agonists through G protein-coupled receptors, including ones in the rhodopsin family (Mitchell et al., 1998). Accordingly, the activation of *Pld* as a downstream effector of photoactivated rhodopsin could generate PA that, in turn, could be dephosphorylated by PA phosphohydrolase (Starz-Gaiano et al., 2001) to form DAG and to trigger a TRP-mediated Ca<sup>2+</sup> influx.

To maximize potential light-stimulated, *Pld* phototransduction events, we used the UAS–GAL4 system (Brand and Perrimon, 1993) with the *ninaE-GAL4* (*Rh1*) driver to ectopically overexpress wild-type *Pld* cDNA in photoreceptor cells R1–6. To eliminate PLC-mediated phototransduction events in parallel and, thus, permit the isolated detection of any potential *Pld*-generated signal, the experiment was performed in flies lacking the eye-enriched isoform of PLC (*no receptor potential A* [*norpA*]), which eliminates PIP<sub>2</sub> hydrolysis and overt light-stimulated signals (Fig. 4 B). Light-stimulated transduction was not observed in *norpA*<sup>7</sup> flies that overexpressed *Pld* (Fig. 4 C), indicating that the contribution of *Pld* to phototransduction responses is not mediated by the rhodopsin-triggered activation of *Pld* and conversion of the ensuing PA to DAG. This finding only addresses whether light stimulation elicits a *Pld*-mediated change in membrane potential. It does not indicate whether *Pld*-generated PA is converted to DAG and subsequently activates TRP at a significant steady-state level, because ERGs only report changes in electrical activity; i.e., they do not



**Figure 3. *Pld* is expressed in the retina and localizes to the photoreceptor cell body.** (A–C) Immunostaining of whole-mounted retinas with an affinity-purified, anti-*Pld* antiserum (A) and with fluorescently labeled phalloidin to visualize the actin-rich rhabdomeres (B). *Pld* was detected in the photoreceptor cell body and at the base of the rhabdomere (arrows), where a small region of overlap with actin was observed in many cells (C). (D) Retinal tissue sections from *Pld*<sup>null</sup> mutant flies raised

for 6 wk under a 12-h light/12-h dark cycle were prepared and imaged using electron microscopy. Indistinguishable images were obtained from *Canton-S* flies raised under identical conditions (not depicted). CB, cell body; R, rhabdomere; SRC, subrhabdomeric cisterna.

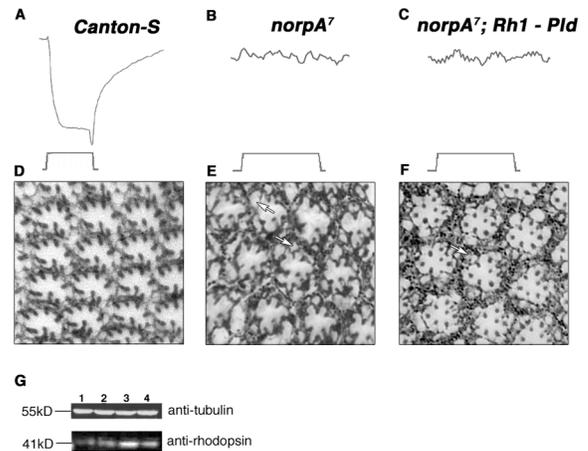
vide a means to assess differences in baseline current between different individuals.

In addition to the absence of a phototransduction response, the *norpA*<sup>7</sup> fly is also characterized by progressive, light-dependent retinal degeneration (Meyertholen et al., 1987). The degeneration ensues from a combination of generating metarhodopsin in a normal, light-dependent manner but failing to subsequently hydrolyze PIP<sub>2</sub>, generate DAG, activate TRP channels, and trigger a light-induced calcium influx (Steele and O'Tousa, 1990). In wild-type flies, metarhodopsin transiently complexes with arrestin2 (Arr2). This interaction ceases once Arr2 is phosphorylated by Ca<sup>2+</sup>/CaM-dependent kinase II (Fig. 1), which is activated by the TRP-triggered influx of Ca<sup>2+</sup> during phototransduction (Kahn and Matsumoto, 1997). In *norpA*<sup>7</sup> flies, Arr2 does not become phosphorylated and, thus, does not dissociate from activated rhodopsin (Alloway and Dolph, 1999; Alloway et al., 2000). The stable metarhodopsin–Arr2 complexes then undergo endocytosis and trigger light-dependent retinal degeneration through an apoptotic (Kiselev et al., 2000) or apoptotic-like (Hsu et al., 2004) pathway.

Consistent with previous papers (Meyertholen et al., 1987; Zinkl et al., 1990), *norpA*<sup>7</sup> flies displayed light-dependent retinal degeneration by 21 d after eclosion (Fig. 4 E). The degeneration was characterized by irregularly shaped ommatidia with intracellular vacuoles and by the loss of multiple photoreceptor cells (Fig. 4, compare E with the wild-type control in D, where a regular pattern of seven photoreceptor cells is observed in all ommatidia). In *norpA*<sup>7</sup> flies overexpressing Pld, however, there was a partial morphological rescue of the *norpA*<sup>7</sup> phenotype (Fig. 4 F). *norpA*<sup>7</sup> retinal degeneration is also inhibited when rhodopsin protein levels are decreased (to <3% of wild-type levels; Alloway et al., 2000); however, decreased rhodopsin was not observed in *Pld*-overexpressing flies (Fig. 4 G). Moreover, as PIP<sub>2</sub> hydrolysis and PLC activity in *norpA*<sup>7</sup> are only 2–3% of the wild type (Inoue et al., 1985, 1988), the substrate (PIP<sub>2</sub>) should not be limiting. Accordingly, altogether, the rescue of retinal degeneration in the *norpA*<sup>7</sup> mutant by the targeted expression of Pld suggests that the inability to generate DAG via PLC was compensated for by converting Pld-generated PA to DAG via PAP to activate the TRP ion channels and trigger dissolution of the metarhodopsin–Arr2 complexes in a constitutive, nonlight regulated manner.

#### Pld overexpression results in TRP channel-mediated retinal degeneration

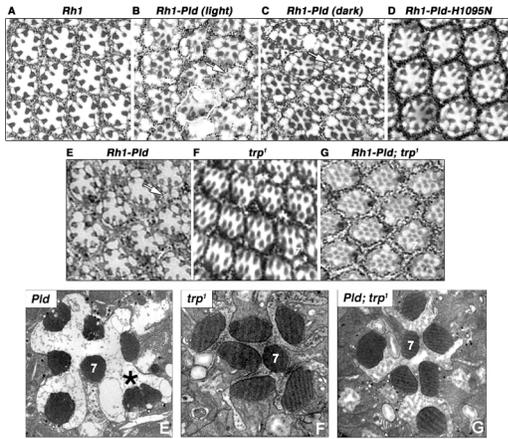
The maintenance of appropriate levels of DAG in the phototransduction system is critical. As discussed above, the failure to generate DAG and to subsequently activate the TRP channels leads to apoptotic or apoptotic-like death via metarhodopsin–Arr2 complex persistence (Alloway and Dolph, 1999; Alloway et al., 2000; Kiselev et al., 2000). Conversely, the rapid conversion of DAG to PA via DAG kinase is fundamental to response termination, and elevated levels of DAG, such as those that occur in the *rdgA* (DAG kinase) mutant, cause necrotic cell death that is secondary to the sustained opening of the TRP Ca<sup>2+</sup> channels (Raghu et al., 2000b).



**Figure 4. Pld does not directly transduce light signals, but does morphologically rescue retinal degeneration in the *norpA*<sup>7</sup> mutant.** ERGs were obtained from flies raised under continuous light conditions for 1 d: (A) *Canton-S*, (B) *norpA*<sup>7</sup>, and (C) *norpA*<sup>7</sup>; *P{UAS-Pld}/Rh1*. Retinal tissue sections were prepared from flies raised under a 12-h light/12-h dark cycle for 21 d: (D) *Canton-S*, (E) *norpA*<sup>7</sup>, and (F) *norpA*<sup>7</sup>; *P{UAS-Pld}/Rh1*. (D) All 7 photoreceptor cells in all 12 of the complete ommatidia are present in the wild-type *Canton-S* section, whereas the *norpA*<sup>7</sup> flies (E) display an irregular ommatidial array that is characterized by intracellular vacuolation (arrows) and missing photoreceptor cells. Only five of the nine complete ommatidia within this section contained seven intact photoreceptor cells. However, a more normal ommatidial structure was observed in *norpA*<sup>7</sup> flies that overexpressed Pld (F). Although some vacuolation was observed (arrow), all eight complete ommatidia in this section contained seven photoreceptor cells. The experiment was performed three times, and two eyes were sectioned per experimental condition with equivalent results. The entire set of sections was examined, and representative sections were selected for the figures. (G) Western blot analysis of rhodopsin protein levels using a mouse monoclonal antirhodopsin antibody. Tubulin was used as the loading control. Protein was extracted from the following adult heads: *Canton-S* (lane 1), *norpA*<sup>7</sup> (lane 2), *P{UAS-Pld}/Rh1* (lane 3), *norpA*<sup>7</sup>; *P{UAS-Pld}/Rh1* (lane 4). Rhodopsin levels were not decreased by Pld overexpression. Slightly elevated levels of rhodopsin in the *P{UAS-Pld}/Rh1* sample were observed in this experiment, but this was not a consistent finding based on the other experiments (not depicted).

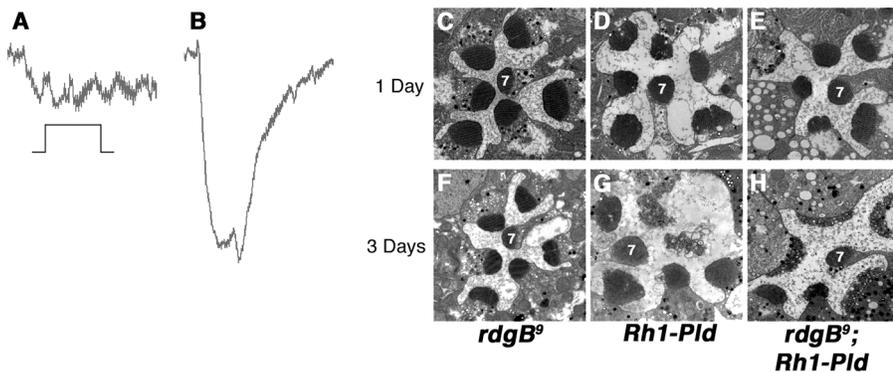
To explore further the possibility that Pld-generated PA can be converted to DAG and activate the TRP ion channels, we used several approaches. First, because Pld overexpression rescued *norpA*<sup>7</sup> metarhodopsin-mediated retinal degeneration, the levels of DAG that were generated by the Pld overexpression in the *norpA* mutant background were clearly not sufficient to trigger TRP-dependent cell death. Accordingly, Pld overexpression was examined in wild-type flies in which the physiologically normal DAG contribution from the light stimulation of PLC and subsequent PIP<sub>2</sub> hydrolysis would be present. Overexpression of Pld in wild-type flies resulted in the progressive retinal degeneration with clear loss of photoreceptor cells and increased intracellular vacuoles (Fig. 5 B) relative to controls (Fig. 5 A). This degeneration was light (PLC) dependent, as dark-reared, Pld-overexpressing flies displayed substantially less disruption of ommatidial morphology (Fig. 5 C).

Second, to demonstrate that the degenerative phenotype results from the production of PA as opposed to a non-enzymatic consequence of Pld overexpression, we generated UAS-Pld lines encoding a catalytically inactive allele of Pld, Pld-H1095N. These lines, when overexpressed by the *ninaE*-



**Figure 5. Pld overexpression causes activity and light-dependent retinal degeneration.** Retinal tissue sections were prepared from flies raised with the following conditions: under a 12-h light/12-h dark cycle for 21 d, (A) *Rh1*; (B) *P{UAS-Pld}/Rh1*; and (D) *P{UAS-Pld-H1095N}/Rh1*; in the dark for 21 d, (C) *P{UAS-Pld}/Rh1*; or under continuous light for 1 d, (E) *P{UAS-Pld}/Rh1*; (F) *trp<sup>1</sup>*; (G) *P{UAS-Pld}/Rh1, trp<sup>1</sup>*. The bottom panel consists of electron micrographs corresponding to E–G, with R7 labeled. (B) The overexpression of Pld resulted in changes in photoreceptor cell integrity with disarrayed architecture (white circle, outlining a single ommatidium) and widespread intracellular vacuolation (arrow). Only four of the nine complete ommatidia in this section had seven intact photoreceptor cells. The Rh1 promoter drives Pld expression only in R1–6 cells. R7/8 photoreceptors were largely spared, as can be observed in the circled ommatidia or pointed at by the arrow. (C) Maintaining the same flies in the dark substantially decreased the phenotype, as all 10 complete ommatidia in this section contained 7 intact photoreceptor cells, and only limited vacuolation was observed (arrow). (D) No degenerative changes were observed when a catalytically inactive point mutant allele of Pld (H1095N) was overexpressed. (E and F) Retinal disorganization and degeneration was observed in Pld-overexpressing flies after 1 d of continuous light stimulation (only three out of six complete ommatidia retained seven photoreceptor cells; asterisk in EM image in bottom panel shows an example of a degenerating cell; arrow shows that vacuolization and disorganization is also apparent) but not in *trp<sup>1</sup>* mutant flies. (G) Pld-induced degeneration was suppressed when Pld was overexpressed in the *trp<sup>1</sup>* null background, as all seven complete ommatidia maintained seven photoreceptor cells, and vacuolation was not observed (EM image, bottom). Sections are representative of three experiments performed.

*GAL4 (Rh1)* driver, did not undergo retinal degeneration (Fig. 5 D), confirming that the degenerative mechanism requires active Pld.



**Figure 6. Pld overexpression restores light responses in the *rdgB<sup>0</sup>* mutant.** ERGs were performed on flies raised under continuous light conditions for 1 d: (A) *rdgB<sup>0</sup>* and (B) *rdgB<sup>0</sup>; P{UAS-Pld}/Rh1*. The abnormal ERG seen in the *rdgB<sup>0</sup>* mutant was rescued by the overexpression of Pld, resulting in normal response amplitude with both on- and off-transients. Retinal tissue sections from flies raised under continuous light for 1 or 3 d were prepared and imaged using electron microscopy: (C and F) *rdgB<sup>0</sup>*, (D and G) *P{UAS-Pld}/Rh1*, and (E and H) *rdgB<sup>0</sup>; P{UAS-Pld}/Rh1*. (C) *rdgB<sup>0</sup>* flies displayed some retinal degeneration 1 d after eclosion. (F) By 3 d after eclosion, more apparent retinal degeneration, with decreased rhabdomere size and increased vacuolation, had ensued. Flies overexpressing Pld had some degeneration 1 d after hatching (D) as described in Fig. 5; but, after 3 d, the flies had even more severe degeneration, with reduced rhabdomeres and degenerating photoreceptor cell bodies (G). In mutant flies overexpressing Pld (E and H), the degeneration caused by Pld expression was reduced. Note that photoreceptor cell R7 (7) appears relatively normal in G and H, which is consistent with the fact that the Rh1 promoter drives expression of Pld only in R1–6. Sections are representative of three experiments performed.

1 d after eclosion, *rdgB<sup>0</sup>* mutants did not respond appropriately to a light stimulus (Fig. 6 A), even though retinal degeneration was not obvious (Fig. 6 C). Pld expression in these mutants rescued the light response, as evidenced by the presence of the slow depolarization and repolarization waves with both on- and off-transients, which signified intact connections that were

Finally, although many mechanisms could be envisioned to account for the degeneration caused by elevated levels of Pld-generated PA, a cell death pathway that is mediated by persistently elevated levels of DAG would necessarily need to proceed through activation of the TRP  $Ca^{2+}$  channels. Thus, we examined whether Pld-mediated degeneration could be rescued by a null mutation in the *trp* gene, as degeneration in the absence of DAG kinase activity, which also leads to persistently elevated DAG levels, is suppressed in a *trp*-null background (Raghu et al., 2000b). Retinal tissue sections from *trp<sup>1</sup>* mutant flies that overexpressed Pld in R1–6 were analyzed for degeneration. The Pld overexpression phenotype (Fig. 5 E) was diminished (Fig. 5 G), suggesting that the increased Pld results in an increased activation of the TRP channels as a result of the increased levels of DAG.

Altogether, these findings suggest that Pld-generated PA can be converted to DAG in a light-independent manner. Because Pld is also required for normal light-dependent responses (Fig. 2), this implies that it should have at least two sites of action in the PI phototransduction pathway.

#### Pld expression rescues light responsiveness in the *rdgB<sup>0</sup>* mutant

RdgB is a PI transfer protein (Vihtelic et al., 1991) that has been proposed to mediate the transport of PI from the SRC back to the rhabdomere in order to regenerate the PIP<sub>2</sub> substrate that is required for hydrolysis in PLC-mediated phototransduction (Fig. 1). The *rdgB<sup>0</sup>* mutant is characterized by absent or rapidly extinguished ERGs because once the existing PIP<sub>2</sub> substrate is consumed, the photoreceptors are unable to readily regenerate it via PI recycling (Hardie et al., 2001). The ERG defects appear before any obvious morphological indication of the retinal degeneration that ensues through a complex mechanism involving, in part, metarhodopsin–Arr2 complex persistence (Alloway et al., 2000).

1 d after eclosion, *rdgB<sup>0</sup>* mutants did not respond appropriately to a light stimulus (Fig. 6 A), even though retinal degeneration was not obvious (Fig. 6 C). Pld expression in these mutants rescued the light response, as evidenced by the presence of the slow depolarization and repolarization waves with both on- and off-transients, which signified intact connections that were

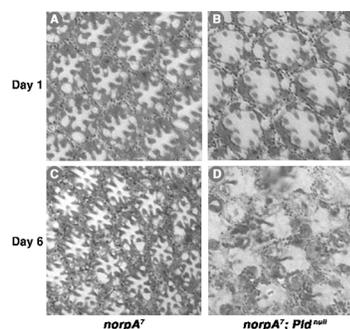
downstream of the photoreceptor (Fig. 6 B). Because the conversion of the Pld-generated PA to DAG showed no evidence of being light regulated (Fig. 4 C), the functional rescue of the *rdgB*<sup>9</sup> mutation by Pld overexpression suggests that Pld activity facilitates phototransduction via the maintenance of adequate PIP<sub>2</sub> substrate levels for PLC hydrolysis. These results are consistent with the findings described in Fig. 2, in which the loss of Pld activity resulted in blunted phototransduction.

Retinal degeneration was apparent in *rdgB*<sup>9</sup> mutant flies by 3 d after eclosion (Fig. 6 F), and ommatidial architecture was completely destroyed by 7 d after eclosion (not depicted). Given that Pld overexpression ameliorated the retinal degeneration in *norpA*<sup>7</sup> mutant flies (Fig. 4) and that Pld overexpression restored the photoresponse in the *rdgB*<sup>9</sup> mutant, it might be anticipated that morphological improvement would be observed in *rdgB*<sup>9</sup> mutant flies overexpressing Pld. Surprisingly, however, Rh1-driven Pld expression in this mutant did not prevent or slow the rate of retinal degeneration (Fig. 6, C–H). However, the absence of RdgB did slow the degeneration caused by Pld overexpression (Fig. 6, compare G with H). This further suggests that Pld overexpression causes retinal degeneration by increasing the DAG-mediated TRP channel activation; this is achieved by increasing the levels of the PIP<sub>2</sub> substrate for PLC and by increasing the DAG production from PA. When one arm of the pathway is hindered (by decreasing PLC-mediated hydrolysis of PIP<sub>2</sub>), Pld overexpression results in less extensive degeneration.

#### Pld deficiency exacerbates the apoptotic-like degeneration observed in light-stimulated *norpA*<sup>7</sup> flies

Our findings thus far suggested that Pld may function to maintain the PIP<sub>2</sub> substrate pool that is required for PLC-mediated light responses, and that the PA generated via Pld overexpression may undergo conversion to DAG in opposition to the DAG kinase step that terminates phototransduction responses. To explore whether the potential conversion of Pld-generated PA to DAG occurs in a physiologically significant context through endogenous Pld action, we examined the consequences of removing the Pld function in the background of the *norpA*<sup>7</sup> mutant, which exhibits metarhodopsin–Arr2-mediated neurodegeneration that is secondary to the inability to activate TRP channels via PLC-generated DAG. If the endogenous Pld pathway functions to elevate DAG levels constitutively, then the *Pld*<sup>null</sup>; *norpA*<sup>7</sup> double mutant would be anticipated to have a more severe phenotype than either mutant alone.

1 d after eclosion, neither the *Pld*<sup>null</sup> (not depicted, but see Fig. 3 D) nor the *norpA*<sup>7</sup> (Fig. 7 A) null mutants individually displayed retinal degeneration, and, in fact, retinal degeneration in *norpA*<sup>7</sup> did not become evident until several days later (Fig. 7 C). However, at 1 d after eclosion, the *Pld*<sup>null</sup>; *norpA*<sup>7</sup> double mutant (Fig. 7 B) already exhibited initial signs of degeneration with small rhabdomeres and decreased cell body size. By 6 d after eclosion, retinal degeneration in the *Pld*<sup>null</sup>; *norpA*<sup>7</sup> double mutant was sufficiently advanced so that only R7/8 remained intact (Fig. 7 D). Together with the previous findings, these results demonstrate that Pld plays physiological roles both in supporting PIP<sub>2</sub> levels during phototransduction



**Figure 7. Endogenous levels of Pld action protect against retinal degeneration.** Retinal tissue sections were prepared from flies raised under continuous light for 1 or 6 d: (A and C) *norpA*<sup>7</sup> and (B and D) *norpA*<sup>7</sup>; *Pld*<sup>null</sup>. Although the *norpA*<sup>7</sup> (A) mutant flies had a fairly normal retinal morphology with intact photoreceptor cells and cell bodies soon after eclosion (and the *Pld*<sup>null</sup> was indistinguishable from wild type; not depicted), the *Pld*<sup>null</sup>; *norpA*<sup>7</sup> double mutant (B) already displayed advanced degeneration, with small rhabdomeres and severely reduced cell bodies. No cell bodies were evident except for photoreceptor R7/8. By 6 d after eclosion, degeneration in the *norpA*<sup>7</sup> (C) mutant was becoming evident (smaller rhabdomeres and some vacuolation); however, by this time, the *norpA*<sup>7</sup>; *Pld*<sup>null</sup> double mutant (D) had undergone extensive retinal degeneration, with only rhabdomeres R7/8 remaining apparent in some ommatidia.

and in maintaining photoreceptor viability in the absence of ongoing phototransduction events.

## Discussion

In this study, we used genetic approaches to explore a novel player that is active in the phototransduction pathway in *D. melanogaster*. We show that Pld, through its generation of PA, modulates both the photoreceptor responsiveness and the structural integrity of the retina.

#### Facilitation of phototransduction

The diminished, wild-type light responsiveness and the exacerbation of the *norpA* phenotype in the absence of Pld reveals a role for Pld in the phototransduction pathway in regulating light sensitivity and photoresponse amplitude. There are four potential steps in which Pld could be involved: provision of additional PA for the purpose of generating PI; movement of the PI from the SRC to the rhabdomere; stimulation of PI4P5K to spur conversion of PI to PIP<sub>2</sub>; and production of DAG via PAP dephosphorylation of PA.

The exacerbation of the *norpA* phenotype in the absence of Pld (Fig. 7) indicates that the PA generated by Pld functions physiologically in a context other than by simply increasing the pool of PI in the SRC, moving the PI to the rhabdomeres, or promoting PIP<sub>2</sub> synthesis from PIP through the stimulation of PI4P5K, because the *norpA* flies ultimately lack the ability to use PIP<sub>2</sub>. Although we do not provide biochemical evidence for the following, the most likely role for PA in this setting would be its dephosphorylation to DAG, which would then trigger transient opening of a limited number of TRP channels, the influx of a small amount of Ca<sup>2+</sup>, the activation of CaM kinase II, the dephosphorylation of Arr2, and the destabilization of metarhodopsin–Arr2 complexes that would otherwise internalize and initiate apoptotic-like pathways. Increasing DAG levels via the inhibition of DAG kinase

has previously been shown to rescue *norpA* degeneration (Hardie et al., 2003), and, most likely, overexpressed Pld rescues *norpA* degeneration (Fig. 4) by similarly raising DAG levels via production of PA, followed by the conversion of PA to DAG via PAP.

Could the elevation of DAG via the Pld production of PA affect phototransduction? The absence of a light-stimulated response when Pld is overexpressed in the *norpA* background indicated that Pld is not light responsive itself (Fig. 4). However, the provision of a low level of DAG could lower the threshold for PLC-mediated, light-stimulated depolarization, and the blunted phototransduction response that is seen in *Pld<sup>null</sup>* flies (Fig. 2) could reflect, in part, a loss of this “priming” level of DAG.

Even if Pld-generated PA does function to prime DAG levels, this cannot be the only step in the cycle at which it exerts an effect; DAG priming would not explain how Pld overexpression rescues the phototransduction response in *rdgB* (phosphatidylinositol transfer protein) mutant flies in which the transfer of PI from the SRC to the rhabdomeres is presumably diminished (Alloway et al., 2000). Although Pld-generated PA may help to maintain PI levels, this is unlikely to underlie the *rdgB* rescue because it is not PI production that is limiting in this mutant. Conversely, there would be challenges in proposing that the mechanism underlying the rescue involves the local production of PA to stimulate PI4P5K: Pld resides within the photoreceptor cell body (Fig. 3) rather than in the rhabdomere, where conversion of PI to PIP<sub>2</sub> presumably takes place; and rhabdomeric PI is already depleted in this mutant. We favor the hypothesis that Pld plays a role, similar to that undertaken in many mammalian systems, by promoting the exocytic vesicular trafficking outward from the Golgi and perinuclear storage vesicles toward the plasma membrane (Chen et al., 1997; Brown et al., 1998; Vitale et al., 2001; Choi et al., 2002; Du et al., 2003). Pld-promoted trafficking of exocytic vesicles could serve to transfer PI to the rhabdomeres and, if PA were also enriched in the vesicles, could additionally provide a means to stimulate PI4P5K in the absence of locally available Pld. The function of RdgB is complex, with the promotion of vesicular trafficking being one potential mechanism through which it helps to maintain rhabdomeric levels of PIP<sub>2</sub> (Lev, 2004). The loss of RdgB and the ensuing consequence for vesicular trafficking may be compensated for by the extensively studied mechanisms through which Pld promotes vesicular trafficking in yeast and in mammalian systems (for review see McDermott et al., 2004).

Ultimately, regardless of which multiple mechanism is most important, Pld-mediated generation of PA is a significant factor in phototransduction responses because flies lacking Pld manifest only ~2% of wild-type light sensitivity.

### Rescue of degeneration mutants

As described in Fig. 4, the overexpression of Pld opposed the apoptotic-like degeneration that is associated with the *norpA* mutant, in which rhodopsin is converted to metarhodopsin but signaling through PLC hydrolysis of PIP<sub>2</sub> is disrupted. The effect of Pld overexpression appears to be mediated through conversion of the Pld-generated PA to DAG because the degeneration that is observed with Pld overexpression in wild-type backgrounds is suppressed in the *trp<sup>1</sup>* mutant. This indicates

that the DAG-mediated Ca<sup>2+</sup> influx through the opening of the TRP channel is a requisite step in cell death progression.

Pld overexpression did not rescue all degenerative phenotypes. The identification of *rdgA* (Harris and Stark, 1977) as a DAG kinase mutant allele presented two possibilities: that the retinal degeneration observed in this mutant resulted either from excess DAG accumulation or from PA deficiency. Subsequent studies demonstrating that the necrotic degeneration could be rescued with the *trp* mutation suggested that the key element was excess DAG accumulation (Raghu et al., 2000b; Hardie et al., 2003). We examined this further by crossing the Pld-overexpressing line into the *rdgA* mutant background. No rescue was observed (unpublished data), indicating that the provision of PA to flies lacking DAG kinase does not address the basis of the degeneration. Accordingly, our findings support published proposals that DAG accumulation underlies the phenotype (Raghu et al., 2000b). Pld also did not rescue the degeneration associated with *rdgB*; however, the mechanistic pathways underlying degeneration in this setting are complex and are not fully understood because it is rescued in the mutant strains *norpA* (Harris and Stark, 1977), *trp*, and *inaC* (Smith et al., 1991).

Does the production of DAG from PA that is generated by endogenous levels of Pld play a role in retinal protection? Our observation (Fig. 7) that the light-elicited *norpA* phenotype is exacerbated in the absence of Pld suggests that the levels of DAG that are generated through Pld action are physiologically significant. The most likely scenario in which this would be relevant for wild-type flies is under conditions of low light, in which infrequent photoisomerization might lead to the gradual accumulation of metarhodopsin–Arr2 complexes in the absence of elevating Ca<sup>2+</sup> levels sufficient to trigger CaM kinase II dephosphorylation of Arr2. A low, but significant, trickle of DAG production via Pld action could serve to sufficiently stimulate CaM kinase II activity to prevent such accumulation and the ensuing apoptotic-like cell death.

### Roles in mammalian phototransduction

Although phototransduction proceeds differently in mammals and flies, mammalian Pld is expressed in the retina (Lee et al., 2001) and in ROS (Salvador and Giusto, 1998). PAPs are also present and active in the retina (Pasquare de Garcia and Giusto, 1986) and appear to be regulated by phototransduction because PAP activity is inhibited by light stimulation (Pasquare et al., 2000). This would be consistent with the proposal that the conversion of DAG to PA, and accordingly, inhibiting conversion of PA to DAG, is important for signal termination. Conversely, the activation of PAP under dark conditions would lead to DAG production, downstream of and consistent with our proposal that Pld mediates protection from low-level, light-induced degeneration. It remains to be determined if Pld alleles associate with retinal dysmorphology or function.

## Materials and methods

### Fly strains

*D. melanogaster* strains were maintained on standard cornmeal, yeast, sugar, and agar media. Transgenic lines carrying *P{UAS-Pld}*, *P{UAS-Pld}*

H1095N], or  $P\{>w^{hs}, Pld^{*}>\}$  (termed  $Pld^{null}$ ) were generated by the embryo transformation of  $y^1w^{67c23}$  flies (Rubin and Spradling, 1982). In brief, targeting of the *Pld* locus was performed using the method of Rong and Golic (2000). The *P* (*ninaE-GAL4*) driver (also termed *Rh1-GAL4*) inserted on the second chromosome was a gift of C. Desplan (New York University, New York, NY). Phototransduction mutants included *norpA*<sup>7</sup> (also termed *norpA*<sup>P24</sup>; Pearn et al., 1996), *rdgA*<sup>3</sup> (also termed *rdgA*<sup>RC47</sup>; Harris and Stark, 1977), *rdgB*<sup>2</sup> (also termed *rdgB*<sup>ks222</sup>; Harris and Stark, 1977), and *trp*<sup>1</sup> (also termed *trp*<sup>CM</sup>; Montell and Rubin, 1989). All fly lines were in a *w*<sup>+</sup> (red eyed) background.

### Histological fixation and sectioning

Unless noted otherwise, light-reared flies for phototransduction experiments were maintained in continuous fluorescent light at 25°C upon eclosion for the desired period of time. Fly heads of the desired genotype were bisected and immersed in 2.5% PFA and 2% glutaraldehyde in 0.1 M sodium phosphate, pH 7.2, for 1 h on ice. A standard ethanol dehydration series was performed on ice, and the tissue was immersed in three 10-min washes of propylene oxide at room temperature. For embedding, Durcupan ACM (Fluka) was used as follows: 50% propylene oxide/50% Durcupan overnight and 100% Durcupan for at least 4 h. The tissue was then transferred to a Durcupan-containing mold and baked at 70°C for 24–48 h. Transverse thick (1–2 μm) sections were stained with toluidine blue (Sigma-Aldrich) for light microscopy. Each experiment was repeated at least twice, and at least two eyes (each from different heads) were sectioned per experiment. The entire set of sections was examined from the proximal to distal eye and from representative sections selected for the figures.

### Immunofluorescent staining of pupal retinas and confocal microscopy

Immunofluorescence on whole pupal retinas was performed following standard procedures. In brief, intact pupal heads were fixed in 4% PFA/0.1 M sodium phosphate, pH 7.2, for 15 min at room temperature. Retinas were then dissected in 1× PBS and were incubated with primary antibody overnight at 4°C. After washing, retinas were incubated with secondary antibody and fluorescently labeled phalloidin for 2 h at room temperature. Affinity-purified, rabbit polyclonal anti-*Pld* antibody was used at 1:1,000. Fluorescent secondary antibody and fluorescently labeled phalloidin were purchased from Molecular Probes. Images were captured using a confocal microscope (model TCS-2; Leica).

### Western blot analysis

Total adult head homogenates were prepared as previously described (Kiselev et al., 2000). In brief, five fly heads were manually disrupted in 2× sample buffer (60 mM Tris, pH 6.8, 2% SDS, 0.004% bromophenol blue, 10% β-mercaptoethanol, 20% glycerol, and 1× protease inhibitor cocktail) and were sonicated for 5 s. Western blot analysis was performed according to standard protocols. Primary antibodies were mouse monoclonal anti-rhodopsin1 antibody (1:1,000, 4C5; Developmental Studies Hybridoma Bank) and mouse monoclonal antitubulin antibody (1:20,000; Sigma-Aldrich).

### Electron microscopy

Adult heads were fixed and processed as previously described (Colley et al., 1995). The fixed tissue was dehydrated in serial changes of ethanol followed by propylene oxide and was embedded in Spur's medium (Polysciences, Inc.). Ultrathin sections were obtained on an ultramicrotome (model Ultratrac E; Reichert), stained with 2% uranyl acetate and lead citrate, and viewed at 80 kV on an electron microscope (model 410; Philips).

### Electrophysiology

ERGs were obtained as previously described (Chen and Stark, 1992). A NaCl micropipette was inserted through the cornea of one eye of each immobilized live fly, and an indifferent electrode was grounded on an agar-containing dish underlying the body of the fly. The fly was illuminated with 1-s pulses generated by a standard shutter of either 570-nm or 600-nm light from a 150-W Xenon arc lamp. Electrode output was transmitted through a differential preamplifier to a computer using the PowerLab 410 (AD Instruments) as the interface. Neutral density filters were used to regulate the intensity of the light stimulus.

### Online supplemental material

Fig. S1 provides evidence that dPld can be expressed as a full-length protein and can be recognized by an antisera prepared against it. Its activity in mammalian cells is detected under basal conditions and increases upon cellular stimulation by agonist signals. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200502122/DC1>.

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