Ceramide kinase regulates phospholipase C and phosphatidylinositol 4, 5, bisphosphate in phototransduction


Phosphoinositide-specific phospholipase C (PLC) is a central effector for many biological responses regulated by G-protein–coupled receptors including Drosophila phototransduction where light sensitive channels are activated downstream of NORPA, a PLCβ homolog. Here we show that the sphingolipid biosynthetic enzyme, ceramide kinase, is a novel regulator of PLC signaling and photoreceptor homeostasis. A mutation in ceramide kinase specifically leads to proteolysis of NORPA, consequent loss of PLC activity, and failure in light signal transduction. The mutant photoreceptors also undergo activity-dependent degeneration. Furthermore, we show that a significant increase in ceramide, resulting from lack of ceramide kinase, perturbs the membrane microenvironment of phosphatidylinositol 4, 5, bisphosphate (PIP2), altering its distribution. Fluorescence image correlation spectroscopic studies on model membranes suggest that an increase in ceramide decreases clustering of PIP2 and its partitioning into ordered membrane domains. Thus ceramide kinase–mediated maintenance of ceramide level is important for the local regulation of PIP2 and PLC during phototransduction.

Results

Identification and Characterization of Drosophila CERK (DCERK). A BLAST search of the Drosophila melanogaster genome identified CG16708 as the Drosophila homolog of the CERK gene and was named DCERK. DCERK is on the right arm of the third chromosome at 82F11–83A1. It encodes a protein of 687 aa and is 35% identical to human CERK (Supporting Information (SI) Fig. S1). Identification and characterization of the Drosophila homolog of CERK were performed. DCERK is expressed in many tissues during development, including the nervous system, and is a member of the family of enzymes that converts ceramide to sphingosine, ceramide 1–phosphate and sphingosine 1–phosphate (9).

Activation of PLC leads to gating of two transduction channels, transient receptor potential (TRP) and TRP-like. Although many of the proteins involved in phototransduction have been well characterized, we are only beginning to understand how lipids and enzymes involved in lipid metabolism regulate this cascade (4–7). Sphingolipids are integral components of all eukaryotic cell membranes and also act as second messengers in diverse signaling pathways (8). The sphingolipid biosynthetic pathway is an evolutionarily conserved route that generates and interconverts various sphingolipids such as ceramide, sphingosine, ceramide 1–phosphate and sphingosine 1–phosphate (9). We showed earlier that modulating this biosynthetic pathway by targeted overexpression of Drosophila neutral ceramidase (CDase), an enzyme that converts ceramide to sphingosine, rescues retinal degeneration in an arrestin mutant, and facilitates membrane turnover in a rhodopsin null mutant by modulating the endocytic machinery (10–12). Although these studies established that ceramide metabolism is important for survival of photoreceptors, they did not evaluate its role in signaling events during phototransduction.

Ceramide kinase (CERK), a recently cloned lipid kinase, phosphorylates ceramide to ceramide 1–phosphate (C-1-P), thereby decreasing ceramide levels (13, 14). Here we show that Drosophila ceramide kinase (DCERK) regulates PLC activity, function, and the local organization of PIP2 in GPCR signaling by controlling the ceramide level. Genetic, biochemical, and electrophysiological analyses of DCERK deficient flies reveal a severe down regulation of NORPA and failure in phototransduction. Increased ceramide levels in the mutant also alter the level and membrane microenvironment of PIP2 that correlates with a failure of NORPA to localize to the membranes. Using fluorescence image correlation spectroscopy in supported bilayers, we show that ceramide perturbs both the protein-dependent and -independent compartmentalization of PIP2, thus providing a biophysical basis for the effect of ceramide on PIP2. These findings show that sphingolipids and phospholipids cooperate in vivo to establish a suitable membrane microenvironment for signaling mediated by PLC.


The authors declare no conflict of interest.

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that DCERK is an integral membrane protein that is released from membranes by detergent treatment (Fig. S2B). Hydrophobicity analysis predicts two transmembrane helices (residues 258–282 and 515–533) in the protein. Immunofluorescence analyses of Schneider cells expressing the DCERK transgene in dcerk1 showed no significant degeneration. Bars, 2 μm.

dcerk1 Mutant Does Not Respond to Light, and Its PLC Level and Activity Are Severely Downregulated. To test whether photoreceptor function was also defective in dcerk1, we performed electoretinogram recordings (ERGs) from control, mutant, and rescued flies raised in the dark. Surprisingly, despite their intact morphology, mutants did not respond to the light stimulus (Fig. 2A). However dcerk1 rescued with a DCERK transgene showed normal ERGs (Fig. 2A and B). The lack of light response in dcerk1 retina suggested that CERK regulates a crucial step in phototransduction. Mutations in NORPA, the critical effector of phototransduction, led to defective light-induced electrical responses, with null mutants being blind (16–18). Because dcerk1 were blind, we checked the steady-state level of NORPA by immunoblotting using 1-day-old dcerk1 maintained in the dark. We observed that NORPA protein was absent in dcerk1 and that the expression of a DCERK transgene in the mutant rescued the NORPA level (Fig. 2C). In overexposed blots, a small amount of NORPA can be detected in 1-day-old flies (Fig. S5C). Real-time PCR analysis revealed that the amount of NORPA transcript was not different between w1118 and dcerk1, suggesting that DCERK primarily regulates NORPA posttranscriptionally (Fig. S5D). Although w1118 retained extracts showed high PLC activity, mutant extracts lacked this activity, and it was restored in the rescued flies (Fig. 2D). Our results thus far show that the lack of DCERK downregulates NORPA and that this results in loss of its activity and function in photoreceptor cells.

Localization and Levels of Other Phototransduction Components Are Not Affected in dcerk1 Mutant. In Drosophila photoreceptors, INAD, a scaffolding protein, organizes signaling components including NORPA into a supramolecular complex to maximize the efficiency of phototransduction (19, 20). We tested whether DCERK specifically influenced NORPA or if it could affect the concentration or localization of other signaling components by western and immunofluorescence analyses. As seen in Fig. S6 A and B, no significant difference was observed between control and dcerk1 for any of the
proteins tested in 1-day-old flies. Immunostaining with a NORPA antibody revealed that its level was too low to be detected (Fig. S6B). By day 14, dark-raised dcerk1 showed loss of TRP (Fig. S6C). Fig. S6D shows immunostaining of eyes with antibodies to DCERK. Dicerk 

![Fig. 2. Severe downregulation of NORPA expression in dcerk1 flies renders them unresponsive to light. (A) ERG recordings from dark-raised w1118, dcerk1, and rescued flies. The dcerk1 do not respond to light, whereas rescued flies exhibit robust response. Cn bw flies were also used as control, as the rescued flies were in cn bw background. (B) Quantitative analysis of ERG recordings shows that the amplitude in the rescued flies is comparable to that in control flies. (C) Western blot of head extracts probed with NORPA antibody shows that its expression is severely reduced in dcerk1 and rescued in dcerk1 expressing DCERK transgene. (D) Retinal extracts of dcerk1 do not show PLC activity, whereas activity of rescued flies is comparable to that of w1118. Error bars denote standard deviation (n = 3).

![Fig. 3. dcerk mutant accumulates ceramide, and reducing the ceramide level restores NORPA expression and function. (A) Total lipids extracted from dcerk1 and w1118 flies are subjected to UPLC-MS/MS. Individual ceramide and ceramide 1-phosphate species (with tetracaphosphinamine backbone to which 18-, 20-, 22-, or 24–carbon chain fatty acids are attached) are measured and values calculated for dcerk1 relative to w1118. (B) Expression of ceramidase (CDase) transgene rescues NORPA expression in dcerk1. (C) Quantification of NORPA shows that the level is comparable in the CDase dcerk1 rescued flies compared with the control. (D) ERGs showing CDase transgene rescues light response in dcerk1. (E) CDase overexpression rescues retinal degeneration in dcerk1.]

and Rh1 in w1118 and dcerk1. In these sections, CERK is present in rhabdoreres and other membranous compartments of photoreceptors, overlying cone, and lens cells, whereas Rh1 shows specific rhabdomere staining of R1–R6 cells. That CERK can localize to rhabdoreres is also seen in thin sections of photoreceptors contained with antibodies to DCERK and INAD (Fig. S7A).

**DCERK Regulates NORPA and Photoreceptor Function by Modulating the Ceramide Level.** To test whether the substrate, ceramide, or the product C-1-P was responsible for regulating NORPA, we measured their levels in lipid extracts from control and dcerk1 by UPLC-MS/MS. The mutant flies showed a 250–300% increase in ceramide levels in all of the species measured and a modest decrease (40%) in C-1-P levels when compared with w1118 (Fig. S4A). The levels of other sphingolipids such as ceramide phosphoethanolamine and glucosyl ceramide did not change significantly (<30%) between w1118 and dcerk1 extracts. It is likely that other pathways or proteins could contribute to the synthesis of C-1-P, as it is decreased by only 40% in the mutant. To determine whether the significant increase in ceramide level in dcerk1 was crucial for downregulating NORPA, we overexpressed CDase in dcerk1. CDase overexpression decreases the ceramide level by catalyzing the hydrolysis of ceramide to sphingosine and a fatty acid (10). Targeted overexpression of CDase in dcerk1 photoreceptors rescued NORPA, suggesting that an increase in ceramide contributed to a decrease in NORPA level (Fig. 3 B and C). Lipid extracts from dcerk1 flies expressing CDase indeed showed a decrease in ceramide in all species measured except C-20 (Fig. S7B). ERGs carried out on dcerk1 expressing CDase showed that overexpression of CDase rescues the lack of light response observed in dcerk1 (Fig. 3D). CDase expression also rescued the retinal degeneration in dcerk1 (Fig. 3E).

**Ceramide Levels Regulate the Localization and Level of PIP2.** Because PLC acts on PIP2, its localization and function is modulated by the lipid bilayer (21). We evaluated whether an increase in ceramide could alter global membrane properties such as bilayer fluidity. We measured fluorescence polarization as a measure of membrane fluidity, and these experiments showed that membrane fluidity did not change significantly in dcerk1 (22, Fig. S7C). As bulk membrane properties like fluidity were not affected, we decided to test whether the membrane microenvironment was perturbed in dcerk1 due to an increase in ceramide concentration. Because conventional procedures that involve 3Hinositol labeling followed by HPLC separation of radiolabeled phosphoinositides could not be adapted to flies, we measured the PIP2 level in control and dcerk1 by standardizing a coupled nonradioactive TLC separation of phosphoinositides followed by an enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody that recognizes PIP2 with high specificity (23). dcerk1 showed a 60% increase in the PIP2 level compared with the control (Fig. 4A). NORPA null mutants lacking the major PLC used as a control showed an accumulation of PIP2. We then analyzed whether increased ceramide could affect the distribution of PIP2, using the PIP2 antibody in thin sections of control and dcerk1 mutant photoreceptor cells. As seen in Fig. 4B (labeled PIP2), PIP2 appeared to be enriched in doughnut-shaped clusters and spots at the rhabdoreres and their base. We then confirmed that these clusters recruit PLC by expressing PLC6PH-GFP in photoreceptors and staining thin sections of photoreceptors with a GFP antibody. In this widely used tool to visualize the distribution of PIP2, the PH domain of PLC6, which specifically binds to PIP2 at the plasma membrane, is fused to GFP (24). As seen in Fig. 4B (labeled GFP), in addition to rhabdomere staining, clusters were visible at the membrane. The plasma membrane was stained with cadherin in these sections (labeled Cadherin). Fig. 4B (labeled PIP2 + GFP) shows the overlay of PIP2 and GFP, suggesting that PLC6 can co-localize to PIP2 enriched clusters at the membrane. To test whether clustering is unique to PIP2, we also expressed the PH domain of general receptor for phosphoinositides-1.
Ceramide level disrupts the organization of PIP2-enriched areas. Taken together, a likely explanation for our results is that NORPA organization is affected in null mutant ommatidium, whereas there were two to three in dcerk	extsuperscript{+}. Whereas DTS5 flies showed a light response, DTS5 flies in dcerk	extsuperscript{−} were light insensitive, whereas most of the NORPA in dcerk	extsuperscript{+} were in the soluble fraction and hence cytosolic. To test whether this NORPA was functional, ERGs were carried out on expressing DTS5. Lanes 1: w$^{1118}$; lane 2: dcerk	extsuperscript{−}; lane 3: UAS-DTS5; dcerk	extsuperscript{−}; lane 4: GMR-Gal4; dcerk	extsuperscript{−}; and lane 5: GMR-Gal4/UAS-DTS5; dcerk	extsuperscript{−}. We used a dominant temperature-sensitive proteosomal subunit (25). If NORPA is targeted for degradation in vivo would allow us to detect NORPA in dcerk	extsuperscript{−}. We used a dominant temperature-sensitive (DTS) mutation, DTS5, that affects the B6 proteosomal subunit (25). If NORPA is targetted for degradation in dcerk	extsuperscript{−}, then expressing DTS5 in dcerk	extsuperscript{−} photoreceptors should result in restoration of NORPA. As seen in Fig. 5A, this was indeed the case; quantitative analysis of NORPA levels is shown in Fig. S8A. We fractionated head extracts from control and dcerk	extsuperscript{−} expressing the DTS5 subunit into pellet and supernatant fractions and looked for NORPA by western analysis of dcerk	extsuperscript{−} expressing DTS5. Lanes 1: w$^{1118}$; lane 2: dcerk	extsuperscript{−} showed no response (Fig. 5C). Photoreceptor degeneration in dcerk	extsuperscript{−} could also not be rescued in these flies (Fig. 5D). Taken together, a likely explanation for our results is that NORPA that is not membrane associated because of the disorganization of PIP2 is unstable and is targeted for degradation.

Ceramide Affects PIP2 Clustering and Its Partitioning into Liquid Ordered Membrane Domains. Cellular membranes are not homogeneous but compartmentalized into liquid ordered (L$\text{O}$), liquid disordered (L$\text{d}$), and gel (L$\text{g}$) phases. In the L$\text{d}$ phase, lipids diffuse freely, while in the L$\text{g}$ phase they are immobile and the L$\text{g}$ phase is an intermediate stage. The existence of PIP2 compartmentalization in vivo has been rationalized by two hypotheses: (i) PIP2 molecules can cluster independent of proteins or other lipids, for example, through hydrogen bonding between their head groups; or (ii) PIP2 can be laterally sequestered by certain proteins such as MARCKS, GAP43 into cholesterol-rich membrane domains (26). We explored each of these scenarios in model membranes with defined components to understand how ceramide accumulation could influence PIP2. To address protein-independent clustering, we prepared supported bilayers made of dioleoylphosphatidylcholine (DOPC), 3% molar brain PIP2, and a trace amount of fluorescent PIP2 (BodTMRPIP2) for visualization. Under these conditions, BodT-MRPIP2 forms clusters that can be visualized using fluorescence.
Fig. 6. Ceramide affects protein-independent PIP<sub>2</sub> clustering and GAP-43P-induced PIP<sub>2</sub> partition in ordered domains. Fluorescence images of BodTMRPIP<sub>2</sub> (0.005% mol) in supported bilayers containing (A) DOPC, (B) DOPC + 3% BrP<sub>2</sub>, (C) B + 5% Cer-1-P, (D) B + 5% cholesterol, (E) B + 5% C<sub>6</sub> ceramide, and (F) B + 5% brain ceramide at room temperature. The presence of ceramide affects clustering of PIP<sub>2</sub>. (G) Degree of clustering of PIP<sub>2</sub> for all samples described above. This quantity is defined as inverse of the cluster density, as calculated via ICS on an average of eight 23 x 23-μm<sup>2</sup> images for each sample. **E and F** are statistically distinguishable from B with P < 0.01. **B** is statistically distinguishable from B with P < 0.1. (H) Fluorescence image of a supported bilayer showing Lo domains in Ld phase. Red color refers to BodTMRPIP<sub>2</sub> and green to BodChol (0.01% mol). The Lo domains appear dark in both channels. (I) When the bilayer is treated with palmitoylated GAP-43P, the Lo domains are fragmented and show a significant degree of fluorescence in the red channel. (J and K) Analogous imaging of bilayers with ceramide-rich domains in Ld phase, without and with GAP-43P respectively. Ceramide-rich domains are dark in both channels. (L) Partition of BodTMRPIP<sub>2</sub> in Lo or ceramide-rich domains. This is calculated as the ratio between the average red signal in Lo (or ceramide) domains—dark in the green channel and the signal in the Ld phase—bright in the green channel. *I is statistically distinguishable from H with P < 0.01 and **K is statistically indistinguishable from sample J with P = 0.3. (Scale bars, 2 μm.)

Fluorescence correlation spectroscopy was used to show that membrane fluidity is not affected by the presence of the clusters (Fig. S8C). It was recently shown that in free-standing membranes, PIP<sub>2</sub> forms similar clusters that depend on the presence of divalent cations such as Ca<sup>2+</sup> (27). Next, we evaluated the effects of incorporating ceramides in these preparations on the clustering and distribution of PIP<sub>2</sub> and image correlation spectroscopic (ICS) analysis was performed to quantify the degree of PIP<sub>2</sub> clustering (Fig. 6G). The spatial segregation of BodTMRPIP<sub>2</sub> depends on the presence of brain PIP<sub>2</sub> in the lipid mixture, as simple DOPC samples show no significant heterogeneities (Fig. 6A). The incorporation of either brain ceramide or short-chain C6 ceramide significantly decreases cluster formation (Fig. 6E and F). C-1-P does not prevent PIP<sub>2</sub> clustering, whereas cholesterol seems to promote it (Fig. 6C and D). From these experiments, it appears that the presence of ceramide has a direct effect on the clustering of PIP<sub>2</sub> in model membranes. A possible explanation for this phenomenon lies in the peculiar biophysical properties of the ceramide molecule. Ceramide has a small polar head (like cholesterol), and it is both donor and acceptor for hydrogen bonding. The former feature might explain why ceramide and cholesterol interact with PIP<sub>2</sub>, the energetically unfavorable contact between water and their hydrophobic moieties are avoided by the large PIP<sub>2</sub> polar head (“umbrella effect”) (29). Nevertheless, the distinctive structural properties of ceramide (e.g., the amicid group that is absent in cholesterol and phospholipids) could influence the hydrogen bond network of surrounding molecules. Ceramide might act as a local chaperone that perturbs the PIP<sub>2</sub>—water—hydrogen bond network, thus allowing the electrostatic repulsion between PIP<sub>2</sub> molecules to disperse the clusters (30).

Protein/lipid dependent PIP<sub>2</sub> clusters (scenario 2): A second line of thought regarding PIP<sub>2</sub> compartmentalization is that certain proteins, for example, the diacylated protein GAP-43, can bind PIP<sub>2</sub> and partition into small raft domains, thus offering a molecular basis for PIP<sub>2</sub> segregation (31). In this context, ceramide might play an important role inducing structural alterations in the lateral organization of the membrane bilayer. Nonphosphorylated long-chain ceramides can form specific membrane microdomains that can exist in gel phase (L<sub>α</sub>) within raft domains of Lo phase (32). Such ceramide-rich domains are characterized by very tight lipid packing. To test whether such lateral segregation properties of ceramide within membrane bilayers could explain the loss of PIP<sub>2</sub> segregation seen in vivo in CERK mutants, we examined the effect of increasing ceramide on PIP<sub>2</sub> organization in supported bilayer showing raft-like phase separation. BodTMRPIP<sub>2</sub> was added to the lipid mixture in trace amounts to study the organization of PIP<sub>2</sub> whereas a green fluorescent lipid analog (BodChol) was used as a marker for the Ld phase. In these preparations, PIP<sub>2</sub> localizes to the Ld phase of the membranes (Fig. 6H) because of the multiple unsaturation in the sn-2 hydrocarbon chain that would make PIP<sub>2</sub> partitioning into tightly packed domains energetically unfavorable. In this set of experiments, we monitored PIP<sub>2</sub> partition into ordered domains, either raft-like or ceramide-rich, as this could be a more relevant mechanism for PIP<sub>2</sub> segregation in vivo, and we did not focus on the Ca<sup>2+</sup>-dependent PIP<sub>2</sub> clustering only partially observable in the Ld phase because of washing with 15 mM ethylenediaminetetraacetic acid (EDTA). Following a published protocol (31), we verified that the addition of doubly palmitoylated peptide GAP-43P (i.e., the N terminus plus the basic effector domains of GAP-43) relocates a portion of the PIP<sub>2</sub> to the Lo (raft-like) phase of the membrane (Fig. 6J). Substitution of ~50% of sphingomyelin with ceramide results in bilayers containing ceramide-rich domains in a matrix of Lo phase. Here again, PIP<sub>2</sub> is localized to the Lo phase (Fig. 6J). Interestingly, the addition of the acylated GAP-43P showed no significant change in the dimension of the ordered domains (Fig. 6K). Furthermore, very little of the PIP<sub>2</sub> relocates to these ceramide rich domains. Figure 6L shows quantification of the
fluorescent intensity originating from PIP2 in the ordered domains compared with that from PIP2 in the disordered phase. These experiments suggest where PIP2 clustering in vivo is determined by the partition of this lipid into small protein/lipid domains, the accumulation of ceramide, and the formation of highly ordered, ceramide-rich domains that effectively decrease PIP2 segregation. The conclusions drawn from fluorescence experiments described above correlate well with the observed effects of increased ceramide in DCERK mutants on PIP2 organization in photoreceptor cells.

Discussion

In this study, we show that DCERK regulates the ceramide level to maintain PLC and membrane organization of PIP2 during phosphoinositide-mediated GPCR signaling in Drosophila. Our results are summarized in a model depicted in Fig. S9. Morphometric analysis of PIP2 clusters suggests that there are still some clusters in dcerk; thus, the loss of NORPA in dcerk mutant may not be due only to the loss of PIP2 clustering. Although the effect of ceramide on PIP2 is central, increased ceramide could affect other membrane properties that can downregulate NORPA or other proteins. Also, INAD is required for localization of NORPA and TRP is required for localization of INAD (20). Because TRP protein is affected over time in dcerk, additional interactions mediated by INAD and TRP could also contribute to NORPA stability. Photoreceptor degeneration seen in cerk mutants is also not simply caused by loss of NORPA protein, as it is more severe and sets in earlier than in norpA null mutants. There could be other effects of ceramide contributing to degeneration.

Recent clinical studies have identified mutations in the human ceramide kinase like (CERKL) gene in patients with autosomal recessive retinitis pigmentosa (33). No CERKL homolog has been identified in the Drosophila genome. Interestingly, DCERK shares 31% identity with human CERKL (Fig. S1), and possibly DCERK could perform some CERKL functions also. It would be worthwhile to test whether CERK regulates PLCβ4, the closest homolog of NORPA among mammalian PLCs, thereby participating in the mammalian visual process. Recent analyses of cerk null mice revealed that CERK could function in cerebellar Purkinje cells (which are also enriched in PLCβ4) and in neutrophil homeostasis (34, 35). Because the mechanism by which mammalian CERK regulates these processes is not known, it would be interesting to test whether these functions are also mediated through PLC. Although our current understanding limits a direct co-relationship between mammalian CERK and PLCs, it is likely that similar ceramide-regulated microenvironments could operate in other phosphoinositide-dependent signaling such as insulin signaling in adipocytes.

In summary, our data show that modulation of the ceramide level by CERK regulates PIP2 and PLCβ function in Drosophila. Because PIP2 and PLC are fundamental components of GPCR signaling, uncovering their regulation by ceramide through CERK should lead to a better understanding of lipid regulation in signaling.

Methods

Genetic Screen and Isolation of dcerk Mutants. The genetic scheme and method to generate jump-out lines from DCERK-P element are outlined in SI Materials and Methods. Five hypomorphic mutants including dcerk1 were obtained from the screen.

Methods for electron microscopy and immunohistochemistry, assay for ceramide kinase activity, estimation of sphingolipids by mass spectrometry and FCS by ELISA, and model membrane experiments are described in detail in SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

Fly Stocks and Husbandry. Drosophila stocks were raised on standard corn meal agar and maintained at 25 °C unless otherwise mentioned. The DCERK P elements, Δ 2–3 transposase and norp A null mutants were obtained from the Bloomington Stock Center; DT55 flies were from Dr. Eric Baerarheke, University of Massachusetts Medical School; PLCδPH GFP flies were from Dr. Andy Zellhof, Indiana University. DCERK cDNA (EST AT18965) was cloned into the pUAST vector as a NotI-XhoI fragment. This construct was injected into w1118 embryos using standard techniques to generate transgenic flies. UAS DCERK transgenic flies were driven by actinGAL4 driver to rescue dcker1 phenotypes. PLCδPH GFP, Gmr-Gal4cn bw/cn bw; + flies were generated to check for PLCδPH expression and staining with GFP antibody. For Pip3 expression, Gmr-Gal4cn bw/cn bw; GRP1PH GFP+/+ flies were generated and photoreceptor sections from these flies were stained with GFP antibody.

Genetic Screen and Isolation of dcerk Mutants. DCERK P-element (w1118; P[GT1]CG16708BG01100, Bloomington Stock Center, stock #12454) females were crossed to Δ 2–3 transposase males. Single dysgenic males were crossed to balancer females, and potential jump-out males and females were collected and homozygous stocks generated. One fly head from each viable line was processed for western analysis and probed with a monoclonal antibody to DCERK to check for loss or reduction in DCERK protein. Five hypomorphic mutants including dcerk1 were isolated from the screen.

Immunohistochemistry

Antibodies. Mouse monoclonal antibodies to CERK was raised against bacterially expressed and gel purified DCERK. A 1.2-kb N-terminal fragment and 1.2-kb C-terminal fragment of DCERK were cloned as SalI-HindIII fragments into pMalc2x. N-terminal fragment and 1.2-kb C-terminal fragment of DCERK were cloned as SalI-HindIII fragments into pMalc2x. The purified N- and C- fragments were injected into mice. DCERK antibody was used at (1:50), Scramblase (1:250), and staining of third instar larval imaginal discs and S2 cells were carried out as described earlier (12). For imaginal discs and S2 cells, DCERK antibody was used at (1:50), Scramblase (1:250), and fluorescein-conjugated streptavidin was at 1:100 dilution. Biotinylated affinity purified anti-mouse IgG was used at 1:500, and fluorescein-conjugated streptavidin was at 1:100 dilution.

Immunofluorescence of Photoreceptor Cells. Thin eye sections (0.5 μm) were blocked in PBT (phosphate-buffered saline [PBS] + 0.1% Tween-20) + 5% bovine serum albumin (BSA) and 2% normal goat serum for 1 h, stained with primary antibody for 2 h, washed with PBT, and stained with secondary antibody for 1 h, washed, and mounted. The sections were stained with mouse anti-DCERK (1:5), rabbit anti-Rh1 (1:100), rabbit anti-NORPA (1:50), rabbit anti-INAD (1:50), rabbit anti-TRP (1:20, preadsorbed on w1118 head extract for 2 h), mouse anti-Pip3 (1:200), Cadherin (1:20), GFP antibody (1:300), and secondary antibodies (1:1000). Stained sections were visualized using Zeiss Axioplan imaging system using Hamamatsu-ORCA-ER camera and Axiosview 4.5 software. Quantum dot 565 goat anti-mouse Fab2 was used at 1:500 dilution, and the sections were viewed using Q dot filter sets on a spinning disc microscope (Solamere Technology Group modified Yokogawa CSU10 Spinning Disk Confocal).

Morphometric analysis was performed on confocal images of photoreceptors using Metamorph program 7.1 (Universal Imaging, Downingtown, PA). Pixel to micron ratio was assigned and the program measured area of Pip3 stained structures. In each condition, several images were taken to cover the whole eye, and then the area of Pip3 stained structures was averaged for the whole eye. The results denote a final average of three whole eyes from three independent experiments.

Purification and Enzyme Assay for CERK Activity. DCERK protein was isolated from stable Drosophila S2 cell line expressing His-V5 tagged DCERK. Cell pellet from 80 ml of induced S2 cells expressing DCERK was resuspended in lysis buffer (10 mM Mops pH 7.2, 150 mM KCl, 1 mM EDTA, 5 mM DTT, Protease Inhibitor Mixture (Sigma) and dounced in a glass homogenizer. The cell lysate was centrifuged at 100,000 g for 1 h. The pellet was re-suspended in 10 mM Mops pH 7.2, 150 mM KCl, 10% glycerol, 0.5% Triton X-100, Protease Inhibitor Mixture, and incubated at 4 °C for 1 h with gentle swirling. The re-suspended membrane fraction was centrifuged at 30,000 g for 30 min and the supernatant passed through His-Spin Trap column (GE Healthcare). DCERK protein was eluted with 300 mM imidazole buffer containing 0.5% Triton X-100 and used for assays. The assay for CERK activity was carried out according to the procedure described before with minor modifications (15). Ceramide (0.25 mM) with a sphingoid backbone of d18 and fatty acyl chain of C16 and sphingosine were dried under nitrogen and resuspended in solution containing 5 mM cardiolipin, 20 mM Mops, 1 mM diethylenetriaminepentaacetic acid pH6.6, 7.5% β-octyl glucoside, 50 mM NaCl, 1 mM DTT, and 10 mM CaCl2. The mixture was vortexed and sonicated in a bath sonicator for 15 min, after which ATP was added. This mixture was then added to a 96-well assay plate (Microlite+, Thermo Scientific); the final assay wells contained 250 μM ceramide, 12.5 μM ATP, and 5 μg of purified DCERK protein. Reactions were performed in duplicate and incubated for 1 h at 28°C. After incubation, 50 μl of Kinase glo (Kinase-Glo, Promega Corporation) was added to each reaction, mixed well and read on a Counter using Wallac 1420 software (Victor3 1420 Multilabel, Perkin-Elmer Life

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Molecular Techniques

RT-PCR Analysis. RNA was isolated from whole flies using TRIzol reagent (Invitrogen). A 200-ng quantity of DNase-treated RNA sample was amplified by SuperScript One-Step RT-PCR System using Dicer 5′ ATGAGCGAAGACACGAGCCAGCA 3′; 5′ AAAAATGACGGGGAGACCC 3′. These two primers, RT-PCR the full length ORF of CG16708, a length of 2186 nucleotides. The genomic fragment would be 2674 nucleotides due to inclusion of two introns. The primers for Actin 5′ ATGACGAGGAAGGCTGTGCT 3′; 5′ TTGCAAGCATTGGCGTGC AAATG 3′ include the ORF of actin 5C, a length of 1.13kb.

Real-Time PCR Analysis. RNA was isolated from fly retina using TRizol reagent. Approximately 1 µg RNA was reverse transcribed using SuperScript II reverse transcriptase with Oligo(dT)12–18 (Invitrogen). Real-time PCR was performed using SYBR Green PCR mastermix and the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The primers used were as follows: NORPA 5′ GGGCCAGGGATGGCATGTCAT3′ and 5′ TGGGCC, GTTGGCCGATCTGG3′; RP49 5′ AAAGCTGTGCGCAAAATGCG 3′ and 5′ GCACGTTGTCGACAGGAAC 3′. These two primers, the Ct values for NORPA were normalized using the values of RP49 to obtain the DCT. The DCT values for W1118 and deerk1 were divided with the DCT value of W1118 to obtain DDCt.

ERG Recordings. ERG recordings were performed as described before (1). Electrical signals were amplified using a DAM50 amplifier (World Precision Instruments), recorded using Powerlab 4/30, viewed and analyzed using Chart 5 software (AD Instruments, Colorado Springs, CO). Stimulating light was generated using the values of RP49 to obtain the DCt. The Ct values for Actin 5′ ATGACGAGGAAGGCTGTGCT 3′; 5′ TTGCAAGCATTGGCGTGC AAATG 3′ include the ORF of actin 5C, a length of 1.13kb.

Fluorescence Polarization Measurements. The isolation of plasma membrane and fluorescence polarization measurements were performed as described before (22). Plasma membrane was isolated by density gradient centrifugation. Flies were washed thoroughly in PBS containing 0.5% Triton X-100 to remove the residual food material sticking to the flies and were homogenized in homogenization buffer [100 mM TrisHCl (pH 7.4)/150 mM sodium chloride/0.2 mM EGTA with protease inhibitor mixture]. The homogenate was centrifuged at 1,000 × g for 10 min to remove cell debris. The resulting postnuclear supernatant was mixed 1:2 with Opti-Prep, resulting in 40% solution. Opti-prep solutions (30% and 50%) were overlaid on the top. The tubes were centrifuged for 3 h at 100,000 g. Plasma membrane appeared as a white dense band at 30–5% interface. The membrane was collected and used immediately for fluorescence polarization studies. TMA-DPH was added (500 pmol/mg of protein) to membranes and suspended in 1 ml of 50 mM potassium phosphate buffer (pH 7.4), and the mixture was incubated at 25 °C for 30 min with gentle shaking. Fluorescence polarization was measured in FluoroMax-2 spectrofluorimeter (ISA) with 360-nm excitation and 430-nm emission wavelengths. Fluorescence intensities in both parallel and perpendicular directions to the incident light were measured, and fluorescence
polarization was calculated. Membrane fluidity was expressed as the inverse function of polarization.

**Preparation of Supported Lipid Bilayers.** All unlabeled lipids were purchased from Avanti Polar Lipids (Alabaster) and used without further purification. Briefly, unlabeled lipids and fluorescent PIP2 (BodTMRPIP2, Echelon Bioscience, Salt Lake City, UT) were mixed in organic solutions in different proportions. For bilayers showing no phase separation, the lipid composition was either 100% molar dioleoylphosphatidylcholine (DOPC), 97% DOPC + 3% brain PI(4,5)P2 (BrPI(4,5)P2), 92% DOPC + 3% BrPI(4,5)P2 + 5% cholesterol, or 92% DOPC + 3% BrPI(4,5)P2 + 5% ceramide. Ceramides were either synthetic C6 or brain extract species. For bilayers showing phase separation, we used two different lipid mixtures. To obtain Lo/Ld phase separation, we mixed 37.5% molar DOPC + 37.5% C18:0 sphingomyelin (SM) + 25% cholesterol. To obtain ceramide/Ld phase separation, we mixed 37.5% DOPC + 21.5% SM + 16% C18:0 ceramide + 25% cholesterol. Cholesterol, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoate (BodChol, Invitrogen, Eugene, OR) was added in 0.01% molar proportion to visualize the Ld phase. The concentration of BodTMRPIP2 was 0.005 mol% in all cases. After solvent evaporation, the lipid film thus obtained was slowly rehydrated using PBS buffer without Ca2+ ions, slowly rehydrated using 15 mM EDTA SLB buffer and finally with 0.1 mM EDTA SLB buffer to remove the Ca2+ ions needed for vesicle fusion.

Samples treated with GAP43 were prepared by a procedure adapted from previously published protocols (5). Liposomes were prepared as described above for the phase separation mixtures. After brief sonication, liposomes in SLB buffer (1 mg/ml) were incubated for 1 h at 40 °C with 1 mM DTT, 15 μM palmitoyl CoA, and 3 μM GAP-43 derived peptide (GAP-43P, synthesized by Bachem, Torrance, CA). Subsequently, the liposomes were briefly sonicated again and transferred to the mica surface, as described above for the phase separating bilayers (i.e., in presence of EDTA).

**Fluorescence Imaging and Image Correlation Spectroscopy.** Fluorescence imaging was performed at room temperature (~25 °C) on a LSM 510 Meta (Zeiss, Jena, Germany). The excitation light of a Argon laser at 488 nm (or He-Ne 543 nm) was reflected by a HFT 488 (or HFT 700/543) dichroic mirror and focused onto the sample by a Zeiss C-Apochromat 40x. NA = 1.2 UV-VIS-IR water immersion objective. Fluorescence signal was then collected by the same objective and, after passing through a 525/50 bandpass (or 560-long pass) filter, measured by a photomultiplier (PMT). The confocal geometry was ensured by a 70 (80) μm pinhole in front of the PMT.

Image correlation spectroscopy (ICS) was performed as described before (6). For each sample, 16–24 × 23-μm2 images from two different preparations were acquired in the red channel. The width of a pixel was ~40 nm. A self-written Matlab program (MathWorks, Natick, MA) performed background and noise correction, and the calculation of the autocorrelation matrix, which was fitted using a 2-d Gaussian. The cluster density CD was calculated from the fit parameters. Provided that the amount of BodTMRPIP2 molecules is constant in the different samples and that the cluster dimensions are never larger than the optical resolution, the degree of clustering is defined as the inverse of CD (at high cluster density, the clusters are smaller with fewer fluorophores; at low cluster density, there are many fluorophores per cluster).

Measurement of BodTMRPIP2 partition into Lo domains was performed acquiring fluorescence images in both red and green channels. A self-written Matlab program performed the following: (i) background correction, (ii) precise spatial alignment of the two channels via a cross-correlation algorithm; (iii) phase assignment using the signal in the green channel; and (iv) calculation of the partition defined as \( \frac{I_{\text{Lo}}}{I_{\text{Lo} + I_{\text{Ld}}}} \) BodTMRPIP2/LoPoles, i.e., the ratio between the fluorescence signal from BodTMRPIP2 in the Lo phase (averaged over all of the pixels in the Lo phase) and the fluorescence signal from BodTMRPIP2 in the Ld phase (averaged over all of the pixels in the Ld phase). For each sample, we acquired 16–24 images from two different bilayer preparations. Image dimensions varied between ~15 × 15 and 23 × 23 μm2. Statistical significance of the variations between samples was assessed using the “ttest2” routine from Matlab (1% or 10% significance level).

Fig. S1. Comparison of the amino acid sequences of CERK homologs from Drosophila, human, mouse, C.elegans, and rice and human CERKL.

Sequence Alignment of CERKs and human CERKL

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Fig. S2. Characterization of *Drosophila melanogaster* CERK (DCERK). (A) Protein extracts prepared at different stages of *Drosophila* development are separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE), blotted and probed with a monoclonal antibody (3H7) to DCERK. The blot shows that DCERK is ubiquitously expressed. The blot is also probed for tubulin as a loading control. (B) Fractionation of head extracts shows that DCERK is an integral membrane protein. DCERK could be dissociated from the membrane by treatment with the detergent Nonidet P-40 but not by alkaline pH or high salt concentration. (C) *Upper panel* shows wing imaginal discs co-stained with an antibody to scramblase, a protein previously demonstrated to localize to the plasma membrane. (Scale bar, 20 μm.) *Lower panel* shows immunostaining with an antibody to cadherin. (Scale bar, 20 μm.) (D) DCERK does not co-localize with endosome, Golgi, or ER markers. Schneider cells were stained with a monoclonal antibody to DCERK and rabbit polyclonal antibodies to Rab5 (endosomal marker), Lava Lamp (Golgi marker), Calnexin (ER marker), and DAPI to stain the nucleus. Overlay images show there is no significant overlap between DCERK and these organelle markers.
Fig. S3. DCERK is a bona fide ceramide kinase. (A) The ability of DCERK to deplete ATP in the presence of ceramide or sphingosine is measured by a chemiluminescence assay. Membrane fractions from Schneider cells stably expressing either vector alone or DCERK were used in the assay. DCERK phosphorylates ceramide significantly \( n = 3 \). Error bar denotes standard deviation. (B) DCERK does not use DAG as a substrate. The ability of DCERK to deplete ATP is measured by the chemiluminescence assay using DAG as substrate. Purified DAG kinase was used as a positive control in this assay. (C) DCERK expression results in decreased ceramide and increased ceramide-1-phosphate levels. Lipid extracts were prepared from Schneider cells stably expressing either vector alone or DCERK and subjected to mass spectrometry. Cells expressing DCERK show 50% reduction in ceramide and \(-60\%\) increase in ceramide-1-phosphate when compared with cells expressing vector alone.
Fig. S4. Genetic scheme used to isolate dcerk mutants. (A) P element (w^{118}; P{GT1}CG16708BG01100, Bloomington Stock Center, stock# 12454) was mobilized and excision lines were established. Western analysis using 3H7 antibody to DCERK was carried out on one fly head sample from each viable line while lethal lines were analyzed by rescue experiments with a transgenic fly expressing DCERK. (B) PCR and sequence analysis of genomic DNA revealed that ~6 kb (shown by arrows) of the original 11.2kb P element was left behind in dcerk1. (C) Light induced progressive degeneration in dcerk1. Transmission electron micrographs show photoreceptors of newly eclosed, 1-, 5-, and 17-day-old dcerk1 mutants. Rhabdomeres can be seen in newly eclosed and 1-day-old photoreceptors, whereas those of 5- and 17-day-old flies show hardly any rhabdomeres and extensive vacuolation.
Fig. S5. NORPA level is down regulated in dcerk$^1$. (A) Western analysis of $w^{1118}$ and norpA null mutant head extracts probed for NORPA showing specificity of the NORPA antibody used in the study. The blot is probed with an antibody to IPP as loading control. (B) The level of ceramide kinase over-expression when UAS-CERK transgenic flies are driven using the actin GAL4 driver. Western blot of head extracts from 1-day-old dark-raised $w^{1118}$, CERK transgenic alone (UAS-CK), driver alone (actin GAL4), actin GAL4 driven CERK transgenic (actin Gal4.UASCK) and dcerk$^1$ flies. CERK is overexpressed significantly in the actin Gal4 driven flies. (C) A small amount of NORPA protein can be detected in 1-day-old dark-raised flies when the Western blot is significantly overexposed. (D) DCERK modulates NORPA posttranscriptionally. Real time PCR analysis of RNA isolated from $w^{1118}$ and dcerk$^1$ shows that DCERK primarily modulates NorpA posttranscriptionally. $n = 5$. Error bars denote standard deviation.
Fig. S6. Levels and localization of other phototransduction components are not significantly affected in dcerk<sup>1</sup> mutants. (A) Western analyses of various phototransduction components show that their levels are not changed in 1-day-old dcerk<sup>1</sup> compared with w<sup>1118</sup> extracts. (B) Thin sections of dark-grown w<sup>1118</sup> and dcerk<sup>1</sup> photoreceptors are stained with antibodies against different phototransduction components. Immunostaining reveals that localization of INAD, TRP, and rhodopsin 1 (Rh1) proteins remain unaffected in dcerk<sup>1</sup>. (C) Western analysis showing TRP protein is lost by day 14 in dcerk<sup>1</sup> compared with w<sup>1118</sup>. (D) Immunostaining of eyes with antibodies to DCERK and Rh1. Cryosections (10 μm) were co-stained for Rh1 (red, as a control marker for rhabdomere in R1-R6) and DCERK (green). Upper panel shows cryosection of w<sup>1118</sup> at ×10 magnification; lower panel shows dcerk<sup>1</sup>. These sections provide a view of the photoreceptors, underlying lamina, and the brain. In the control sections, CERK is present in the rhabdomeres and other membranous compartments of photoreceptors, overlying cone and lens cells, underlying lamina, and brain, whereas Rh1 shows specific rhabdomere staining of R1-R6 cells. The DCERK staining in the mutant is at the background level.
Fig. S7. CDase overexpression decreases ceramide level and rescues NORPA in dcerk^1. (A) Thin sections of w^{1118} and dcerk^1 photoreceptors are immunostained for DCERK and INAD proteins. DCERK co-localizes with INAD in wild-type photoreceptors. Error bars denote standard deviation. (B) Ceramide levels are reduced in flies overexpressing CDase in dcerk^1 mutant. Total lipids are extracted from fly heads of dcerk^1 and dcerk^1 overexpressing CDase and subjected to UPLC MS/MS. Individual ceramide species (with tetradecasphinganine backbone to which fatty acids of either 18, 20, 22, or 24 carbon chain are attached) are measured and values are calculated for dcerk^1 overexpressing CDase relative to dcerk^1. Ceramide species with fatty acids C-18, C-22, and C-24 show decrease in ceramide compared with mutant, whereas C-20 is not rescued. (C) Membrane fluidity is measured in plasma membrane preparations of w^{1118}, dcerk^1, and dcert^1 head tissue as the inverse function of fluorescence polarization. The dcerk^1 does not show altered membrane fluidity compared with w^{1118} and dcert^1, which show an increase in membrane fluidity serves as a positive control. (D) Immunostaining of thin sections of w^{1118}, dcerk^1, and norpA null mutant photoreceptors with an antibody to PIP2. Clusters of PIP2 can be seen in w^{1118} and norpA, whereas they are less in dcerk^1. Insets show PIP2 cluster in w^{1118}; this is not observed in dcerk^1. (E) Morphometric analyses of area occupied by PIP2 clusters in w^{1118}, dcerk^1 and norpA null mutant eyes. For all immunostaining experiments, nine sections from three different eyes for each condition were observed.
Fig. S8. Immunofluorescence of thin sections of w1118, dcerk1, and norpA null photoreceptors with an antibody to PIP2 and a quantum dot (565) conjugated secondary antibody. (A) Clusters of PIP2 can be seen in w1118 and norpA, whereas they are less in dcerk1. PIP2 also appears more diffuse in dcerk1 compared with w1118 and norpA. Bright field and overlay images for the three sections are also shown. (B) NORPA levels are comparable in dcerk1 expressing DTS5 subunit and w1118. (C) Diffusion coefficients of the fluorescent lipid analogue BodChol in the Ld and Lo phases of SM/DOPC/cholesterol supported bilayers, without and with 3% mol BrPIP2. The diffusion of the fluorescent probe is measured via line-scanning FCS and provides information about the local viscosity and the lipid packing in the membrane. Reported values (average and standard deviation) were obtained from three different positions in two independent samples.
Fig. S9. Model showing ceramide kinase regulates PLC activity, function and the local organization of PIP$_2$ in GPCR signaling by controlling the ceramide level. In normal photoreceptors, PIP$_2$ that is clustered recruits NORPA to the membrane, and this leads to further downstream signaling. In dcerk mutant flies, the increased ceramide level perturbs the membrane microenvironment of PIP$_2$ correlating with degradation of NORPA. There is loss of PLC function and a failure to transduce light signal.