Photoreceptor Deactivation and Retinal Degeneration Mediated by a Photoreceptor-Specific Protein Kinase C

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The protein kinase C (PKC) family of serine-threonine kinases has been implicated in the regulation of a variety of signaling cascades. One member of this family, eye-PKC, is expressed exclusively in the *Drosophila* visual system. The *inaC* (*inactivation-no-afterpotential C*) locus was shown to be the structural gene for eye-PKC. Analysis of the light response from *inaC* mutants showed that this kinase is required for the deactivation and rapid desensitization of the visual cascade. Light adaptation was also defective in *inaC* mutant flies. In flies carrying the retinal degeneration mutation *rdgB*, absence of eye-PKC suppressed photoreceptor cell degeneration. These results indicate that eye-PKC functions in the light-dependent regulation of the phototransduction cascade in *Drosophila*.

THE PROTEIN KINASE C (PKC) FAMILY OF SERINE-THREONINE protein kinases are activated by calcium, diacylglycerol, and phorbol esters (*1–3*). Although PKC's have been implicated as modulators of many signaling cascades, ranging from growth control and tumorigenesis to ion channel modulation, little is known about the in vivo functions of these kinases in their normal cellular environment.

Members of the PKC family have diverse expression profiles in vertebrates; some are expressed in many tissues, while others have more restricted expression patterns (*4*). Three PKC genes have been identified in *Drosophila melanogaster* (*5, 6*). One of these, eye-PKC, which is also known as PKC2 (*7*), appears to be expressed exclusively in the visual system, suggesting a function in visual transduction (*6*).

Phototransduction in *Drosophila*, as in vertebrates, begins with light activation of the G protein (guanosine triphosphate binding protein)–coupled receptor rhodopsin (*8*). The effector molecule that mediates the vertebrate visual cascade is a G protein–activated cyclic GMP (guanosine monophosphate)–phosphodiesterase, which lowers cyclic GMP concentrations in response to light (*9, 10*). In *Drosophila*, a G protein–coupled phospholipase C (PLC) functions as an effector. The central role of PLC in *Drosophila* phototransduction was demonstrated with the isolation of *norpA* (*no-receptor-potential A*) mutants. Strong *norpA* alleles completely eliminate the light response (*11*) and show reduced amounts of PLC activity in the head (*12*). The *norpA* gene encodes a PLC that is abundantly expressed in the retina (*13*). Thus, light activation of rhodopsin is thought to activate a PLC that catalyzes hydrolysis of phosphatidylinositol bisphosphate (PIP_2) into the second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG). While IP_3 appears to be a mediator of photoreceptor cell activation (*14, 15*), DAG may function in feedback regulation, perhaps through activation of eye-PKC.

Two interesting aspects of photoreceptor biology are the sensitivity and adaptability these cells display in response to light stimuli. Photoreceptors are capable of responding to single photons of light in the dark-adapted state (*16*). This remarkable sensitivity is achieved by amplification of the signal when this biochemical cascade is activated. Much is known about the biochemical mechanisms responsible for activation of the visual cascade (*9*), and, indeed, phototransduction has served as the prototype model for the study of G protein–coupled transduction cascades. However, the molecular mechanisms effecting the regulation of these processes remains largely unknown.

The visual systems of both vertebrates and invertebrates are able to modulate their sensitivity and respond to light stimuli that vary more than six orders of magnitude in intensity without response saturation, a process known as light adaptation. Light adaptation in both vertebrates and invertebrates appears to be mediated through changes in the concentration of calcium within the photoreceptor cells (*17–22*). During excitation of the phototransduction cascade, extracellular calcium ions flow into *Drosophila* photoreceptors through the light-activated conductance, and this calcium is required for triggering deactivation (defined as the recovery of the photoresponse after termination of the stimulus) and rapid desensitization (defined as the attenuation of the photoresponse during a sustained stimulus) (*23*). Moreover, *inaC* mutant photoreceptors are specifically defective in these processes (*23*). We now show that the *inaC* locus encodes the structural gene for eye-PKC and that eye-PKC is required for normal calcium-dependent photoreceptor cell deactivation and rapid desensitization.

Because eye-PKC is an important regulator of the phototransduction cascade, it should be possible to identify interacting components by studying the genetic interactions between eye-PKC mutants and other visual mutants. Indeed, we now demonstrate that retinal degeneration in the *rdgB* (*retinal degeneration B*) mutant is dependent on eye-PKC activity.

Localization of eye-PKC to the transduction organelle of photoreceptors. The RNA expression pattern of eye-PKC is restricted to the visual system of adult flies (*6*). In order to study the subcellular distribution of eye-PKC, we generated polyclonal anti-

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bodies to eye-PKC (anti–eye-PKC) using a synthetic peptide to the V3 variable domain (residues 333 to 347) and used these antibodies to examine eye-PKC distribution in frozen tissue sections of wild-type adult animals. The V3 or "hinge" region separates the regulatory and catalytic domains in the PKC family and varies in sequence among the known *Drosophila* PKC's (*6*). The antibodies revealed that eye-PKC was localized in the photoreceptor cells, both in the compound eyes and ocelli (Fig. 1A). In order to determine whether eye-PKC is expressed in all or a subset of photoreceptor cells, and whether it is also expressed in other cells that may escape detection by the antibodies, we generated transgenic flies that expressed a chimeric gene consisting of the promoter and first ten amino acids of eye-PKC fused to a lacZ reporter containing a nuclear localization signal. The targeting of the β-galactosidase reporter to the nucleus of the cells in which it is expressed facilitates cell identification (*24*). The results (Fig. 1C) show that eye-PKC is expressed in all types of photoreceptors found in the retina and ocelli. No other sites of expression were detected.

The subcellular distribution of eye-PKC was determined by indirect immunofluorescence examination of cross sections (1 μm) of wild-type retinas. Most of the signal is confined to the rhabdomeres of the photoreceptor cells (Fig. 1B). Rhabdomeres are the specialized microvillar organelles that contain the visual pigment rhodopsin and most of the proteins necessary for phototransduction (*8*). The localization of eye-PKC to the rhabdomeres is consistent with a function for PKC in the phototransduction cascade.

Assignment of the structural gene for eye-PKC to the *inaC* locus. Eye-PKC maps to position 53E6-10 on the right arm of the second chromosome. For molecular characterization, genomic DNA (90 kb) from the 53E region was isolated in a chromosomal walk. A second *Drosophila* PKC gene, PKC1 (*5, 7*), which is closely linked to eye-PKC (*6*), is expressed throughout the nervous system, including the brain and retina (*6*). The chromosomal walk showed that eye-PKC and PKC1 consist of two transcriptional units separated by 25 kb (Fig. 2).

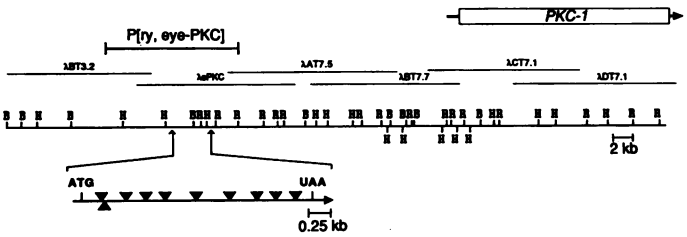


Fig. 2. Genomic structure of 53E genomic region that contains eye-PKC. A bidirectional chromosome walk was initiated from the eye-PKC gene. Clones were isolated from a lambda FIX II genomic library (Stratagene) and a restriction map was generated as described by the supplier. The bars above the map refer to the individual lambda clones isolated in the chromosomal walk. Restriction endonuclease sites are B, Bam H1; E, Eco RI; H, Hind III. The expanded diagram below the map displays the structure of the eye-PKC gene. Eye-PKC contains ten introns (filled triangles) ranging in size from 55 to 162 nt. DNA used to rescue the *inaC* mutant phenotype is shown above the walk (P[ry, eye-PKC]). *PKC-1* refers to the location of the *PKC-1* (protein kinase C) gene (*5*). This gene was identified by detailed restriction mapping and by hybridization with radiolabeled probes specific to the 5′ and 3′ ends.

If eye-PKC regulates the phototransduction cascade, then flies defective in eye-PKC function would be expected to have abnormal visual physiology. To identify potential eye-PKC mutants, we used anti–eye-PKC to examine known phototransduction mutants that map to the second chromosome for the presence of the eye-PKC polypeptide. Three mutants, US^{3741}, $inaC^{209}$, and $inaC^{207}$, all falling into a single complementation group, were defective in eye-PKC (Fig. 3). US^{3741} and $inaC^{209}$ showed a complete loss of the protein, while $inaC^{207}$ expressed ~5 percent of the wild-type amounts of the protein. Examination by indirect immunofluorescence of eye-PKC in tissue sections from these mutant flies showed a reduced signal in

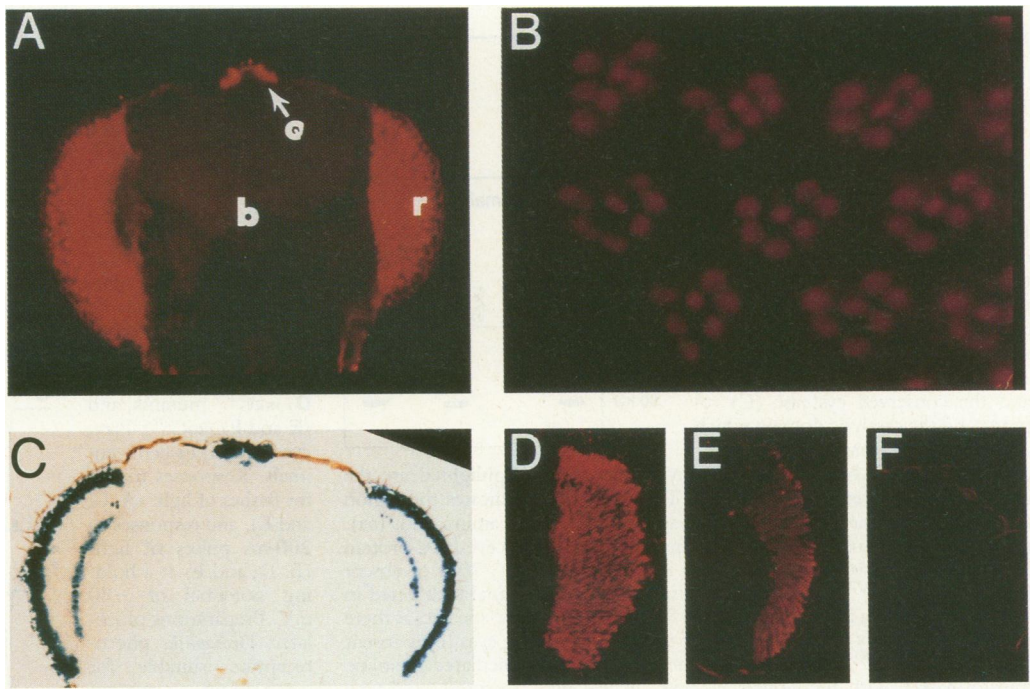


Fig. 1. Tissue-specific expression of eye-PKC. Indirect immunofluorescence staining of eye-PKC on (**A**) frozen tissue section through the adult head of wild-type animals, demonstrating specific expression in the visual system. R, retina; Q, ocelli; B, brain. (**B**) Cross section 1 μm thick through an adult retina. R1 to R6 rhabdomeres surround a central R7 rhabdomere in each ommatidial cluster. Expression of eye-PKC is restricted to the photoreceptor rhabdomeres. Tissue sections were prepared and processed as described (*53*), except that saponin (0.1 percent) was added to all washing solutions. Antibodies were affinity-purified on an Affi-Gel column (Bio-Rad) that contained the immunizing peptide ARLRDEVRHDRRPNE. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. Rhodamine-conjugated goat antibodies to rabbit immunoglobulin G were obtained from Jackson Immunoresearch (West Grove, Pennsylvania). (**C**) Expression of β-galactosidase in transgenic animals that contained a lacZ structural gene under the control of the eye-PKC promoter. The eye-PKC–LacZ fusion gene was generated by ligating a 6.8-kb promoter fragment of eye-PKC, including the initiator methionine and first ten amino acids, to a lacZ gene containing the SV40 T-antigen nuclear localization signal (*24*). Transgenic flies were generated by P element–mediated germline transformation as described (*54*). All photoreceptor cell classes in the retina and ocelli express β-galactosidase. No other sites of expression were detected. (**D** to **F**) Indirect immunofluorescence of anti–eye-PKC on (D) wild-type controls, and (E) $inaC^{207}$ and (F) $inaC^{209}$ mutants. The sections shown in (D), (E), and (F) were processed on the same slide to minimize any variability during staining.

the retina of $inaC^{207}$ flies (Fig. 1E) and no signal in the retinas of $inaC^{209}$ (Fig. 1F) and US^{3741} flies (*25*).

The US^{3741}, $inaC^{209}$, and $inaC^{207}$ mutants were originally identified in screens of mutagenized flies for defects in their electroretinograms (ERG). An ERG is an extracellular recording of light-induced electrical activity in the eye; it represents the summation of the entire electrical output of all photoreceptors, pigment cells, and neurons in the first optic lobe (*8*). The US^{3741} mutant was identified as defective in repolarization kinetics after termination of a light stimulus (*26*), whereas $inaC^{207}$ and $inaC^{209}$ were identified in screens for defects in the prolonged depolarizing afterpotential (PDA) (*27*). *Drosophila* photoreceptors, like those of many invertebrates, undergo a prolonged depolarizing afterpotential that persists after cessation of the light stimulus whenever a substantial amount of rhodopsin (R) has been converted to the dark-stable metarhodopsin (M) (*28, 29*). The physiological basis of the PDA is not known, but may be the result of the continued activity of an internal transmitter in the transduction cascade (*30*). During a PDA, photoreceptor cells become refractory to subsequent PDA-inducing stimuli and are said to be "inactivated" or nonresponsive. A PDA can be terminated at any time by photoconversion of metarhodopsin back to rhodopsin. The *inaC* mutants lacked the PDA characteristic of wild-type flies, but appeared to inactivate normally (thus the name *inactivation-no-afterpotential*) (*27*).

Using the polymerase chain reaction (PCR) we isolated the eye-PKC gene from US^{3741}, $inaC^{209}$, and inaC^{207} mutants and determined their nucleotide sequences. All three alleles have single-base changes that result in either nonsense or missense mutations (Table 1). Both US^{3741} and $inaC^{209}$ have nucleotide changes that introduce premature termination codons at residues 138 and 92, respectively. The $inaC^{207}$ gene has a nucleotide alteration that results in the substitution of an aspartic acid for a valine residue in the regulatory domain of the protein. This residue is not conserved among members of the PKC protein family, although an acidic amino acid residue is not normally found at this position (*6*).

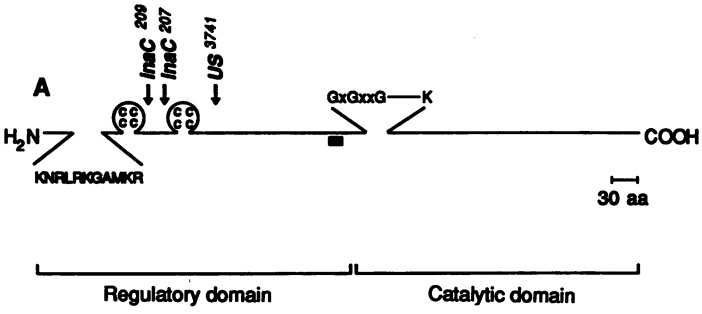
Table 1. Mutations of eye-PKC in *inaC* mutants. Sequencing of mutant alleles is described in (*52*).

Gene	Nucleotide*	Amino acid*
US^{3741}	$G^{417} \rightarrow A$	$Trp^{139} \rightarrow$ STOP
$inaC^{209}$	$G^{279} \rightarrow A$	$Trp^{93} \rightarrow$ STOP
$inaC^{207}$	$T^{602} \rightarrow A$	$Val^{201} \rightarrow$ Asp

*Numbered according to (*6*).

Defective deactivation in the photoreceptor cells of eye-PKC mutants. As a means of demonstrating that the *inaC* gene product is eye-PKC we carried out P element–mediated germline transformation of the cloned eye-PKC gene into *inaC* mutant hosts. Using whole cell (*31*) and nystatin perforated (*32*) patch clamp recordings, we analyzed the electrophysiological responses of wild-type and *inaC* mutant photoreceptors to light stimuli (Fig. 4). The kinetics of the light response in wild-type cells showed rapid deactivation (Fig. 4A). In addition, sustained light stimuli (referred to as pulses) evoked a complex response with biphasic kinetics resulting from a rapid partial desensitization during the stimulus (Fig. 4B). Extracellular calcium entering the photoreceptor through the light-activated conductance is necessary for this rapid deactivation and desensitization of the light response (*23*). The *inaC* mutant photoreceptors were defective in their deactivation kinetics, thus resulting in an increase in the time integral of the response (Fig. 4, C and D). In addition, rapid desensitization is also defective, demonstrating that the fast kinetics of both of these processes share a requirement for eye-PKC activity. Introduction of a wild-type copy of the cloned eye-PKC gene into *inaC* mutant hosts was sufficient to completely rescue the mutant phenotype, thereby restoring normal visual function (Fig. 4, E and F). These results demonstrate that eye-PKC is a key effector molecule that regulates the kinetics of stimulus-dependent deactivation and desensitization of the photoreceptor cell.

Because influx of extracellular calcium through the light-activated



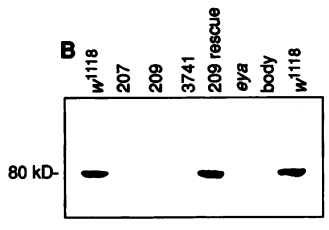


Fig. 3. Wild-type and mutant forms of eye-PKC. (**A**) The diagram shows the proposed structure of eye-PKC protein. Shown are the regulatory domain which contains the pseudosubstrate region (K, Lys; N, Asn; R, Arg; L, Leu; G, Gly; A, Ala; M, Met) and the conserved cysteine (C) repeats, and the catalytic domain which contains the conserved adenosine triphosphate (ATP) binding site (X, any amino acid). Highlighted are the location of the three *inaC* mutations. The shaded box indicates the region used to generate the eye-PKC–specific antibodies. Bar, 30 amino acids (aa). (**B**) Immunoblot analysis of eye-PKC mutants. The 80-kD eye-PKC protein is present in control flies (w^{1118}), but is reduced ($inaC^{207}$, 207) or absent ($inaC^{209}$, 209; US^{3741}, 3741) in *inaC* mutants. A weak signal is detected in $inaC^{207}$ mutants on long exposure. The 80-kD protein is eye-specific, as there is no signal in body extracts or in the heads of flies lacking eyes [eyes absent (*eya*) (*55*)]. $inaC^{209}$ mutants transformed by P element–mediated germline transformation with the cloned *eye-PKC* gene (Fig. 2) express wild-type amounts of eye-PKC (209 rescue, compare with w^{1118}). Protein extracts were prepared from five heads or three bodies by sonication in buffer (20 μl) consisting of 100 mM tris pH 6.8, 3 percent SDS, 0.7 M β-mercaptoethanol, 10 percent glycerol, and subjected to electrophoresis on an SDS polyacrylamide gel (10 percent) (*56*). Transfer and immunodetection were as described (*24*).

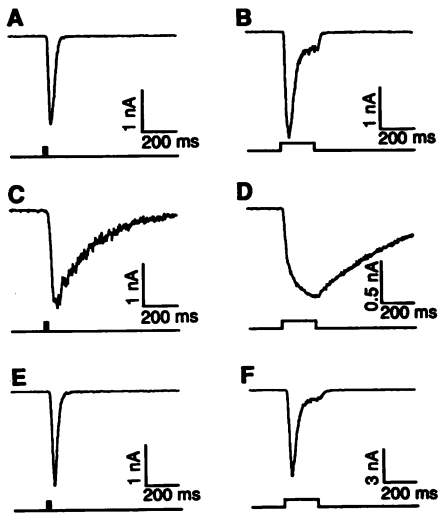


Fig. 4. Restoration of normal visual function to *inaC* mutants by the cloned eye-PKC gene. Whole cell voltage clamp recordings of light-activated currents from (**A** and **B**) wild-type, (**C** and **D**) $inaC^{209}$ mutants, and (**E** and **F**) $inaC^{209}$; P[ry, eye-PKC] transgenic animals. Responses to 10-ms flashes of light (A, C, and E), and responses to 200-ms pulses of light (B, D, and F) at a holding potential of −80 mV. Preparations of isolated *Drosophila* photoreceptors suitable for patch-clamp analysis were made as described (*23*), and details of patch-clamp methodology have been described (*57*). Recordings were made with the nystatin perforated patch technique (*32*) in symmetric cesium solutions with Ca^{2+} (1 mM) in the bath. Photoreceptors were stimulated with monochromatic light at λ = 520 ± 10 nm. Traces shown were obtained from different cells.

conductance is required for normal photoreceptor cell deactivation and rapid desensitization (*23*), we propose that a major role of the entry of extracellular calcium is to activate eye-PKC as a result of light excitation (see Fig. 5).

Light-induced changes in intracellular calcium are important for light adaptation in vertebrate (*17, 18, 33–35*) and invertebrate photoreceptors (*21, 22*). Therefore, we examined the possibility that eye-PKC is a participant in the biochemical pathway that mediates light-adaptation. Adaptation mechanisms allow photoreceptors to modulate the gain of the transduction cascade so that the dynamic range of sensitivity is extended over many logarithmic orders of light intensity and saturation of the excitation mechanisms is prevented. As an assay for the stimulus-dependent changes in the gain (signal amplification) of the excitation mechanisms, we examined the ability of wild-type and *inaC* photoreceptors to modify their sensitivity to weak test flashes after an adapting light stimulus of variable intensity. The test flashes are of sufficiently weak intensity that they do not cause significant adaptation. Wild-type photoreceptors displayed a reduction in the sensitivity to the test flash, which is a function of the intensity of the adapting light (Fig. 6, A and B). Equivalent stimulus protocols in *inaC* photoreceptors caused significantly greater reductions in sensitivity to the test flash (Fig. 6, C and D), although the degree of reduction was still dependent on the intensity of the adapting flash. In addition, the time course of recovery from the light-adapted state was greatly protracted in the mutant photoreceptors (for example, compare the amplitude of the response to the test flashes before and 30 seconds after the adapting flash in Fig. 6, B and D). As a quantitative analysis of the data, the degree of adaptation is plotted as a function of the relative intensity of the adapting flash (I_a/I_t) (Fig. 6E). The results indicate that *inaC* mutant photoreceptors (dotted bars) display significant hyperadaptation relative to wild-type cells (black bars).

The simplest model for the mechanistic basis of this hyperadapting phenotype is that the excessive adaptation observed in *inaC* photoreceptors is the result of the defective deactivation of the light response in these mutant cells [that is, the larger impulse response (time integral) of *inaC* photoreceptors to a light flash may generate a greater driving force for adaptation mechanisms]. This model predicts that normalization for the difference in response kinetics

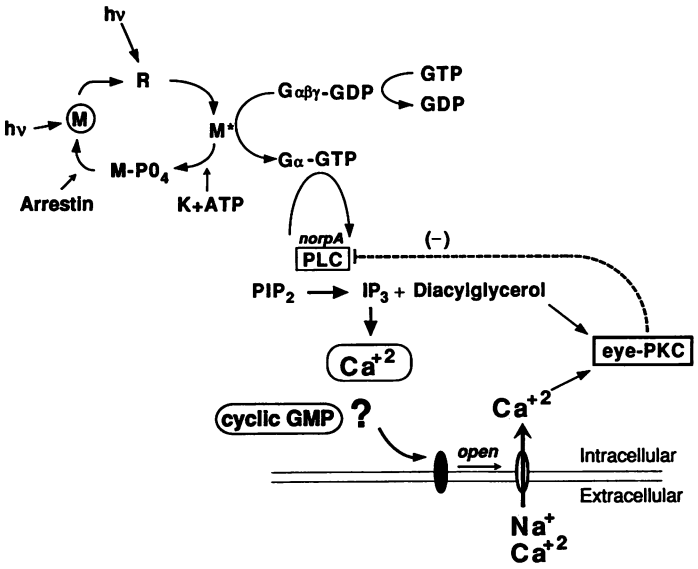


Fig. 5. Model of eye-PKC function. Absorption of a photon of light causes a conformational change in the rhodopsin molecule (R) and activates its catalytic properties. Active metarhodopsin (M*) catalyzes G protein activation. The G protein exchanges GDP for GTP and releases the inhibitory βγ subunits. Active G protein catalyzes the activation of the *norpA*-encoded PLC, and PLC hydrolyzes PIP_2 into IP_3 and DAG. The IP_3 is released from the membrane and diffuses to receptors located on the subrhabdomeric cisternae (SRC). This binding is thought to release intracellular calcium from SRC, which appears to be involved in excitation. Cyclic GMP has also been implicated as a possible intracellular messenger mediating excitation (*58, 59*). Extracellular sodium and calcium then enter the cell through the light-activated conductance. The large influx of extracellular calcium activates eye-PKC, which then negatively regulates the phototransduction cascade, perhaps through phosphorylation of PLC. The function of DAG in *Drosophila* phototransduction is not known, but it could modulate basal levels of eye-PKC activity or facilitate calcium activation of eye-PKC. K+ATP, rhodopsin kinase and ATP; $M-PO_4$, phosphorylated metarhodopsin; and filled and open ovals, closed and open light-activated cation channels, respectively. This signaling cascade has been described in detail (*8*).

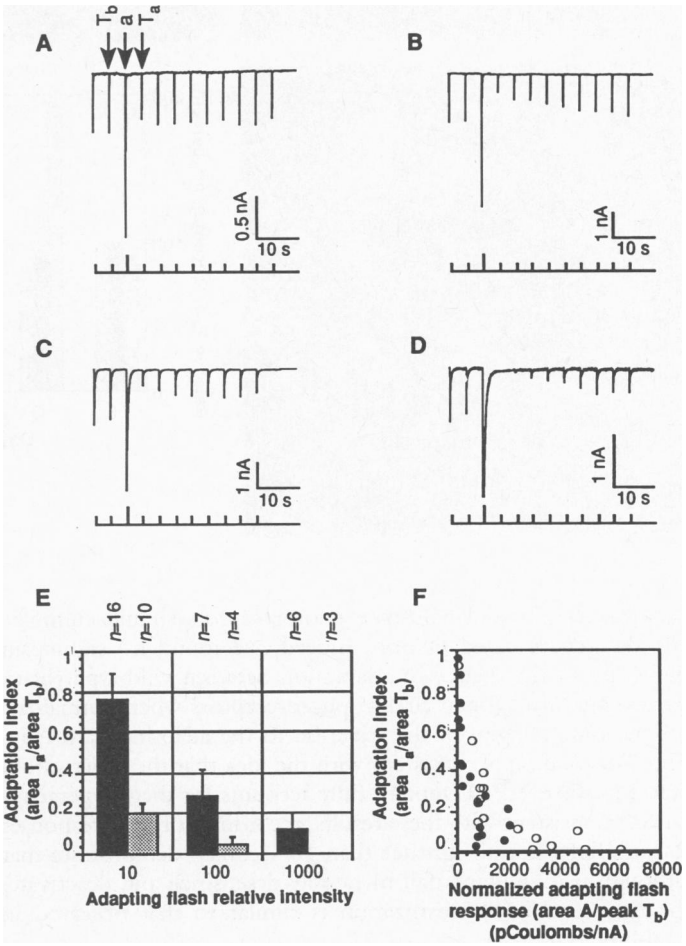


Fig. 6. Light adaptation of wild-type and eye-PKC mutant photoreceptors (*inaC*^{209}). The adaptation paradigm involved the stimulation of photoreceptors with weak test flashes (T_b) (small bars in the stimulation protocol trace) prior to a larger magnitude adapting flash (a) (large bar in the stimulation protocol trace), followed by test flashes (T_a). The duration of both test and adapting flashes was 10 ms. The test flashes were weak enough to avert significant adaptation (compare responses to the first and second flashes). (**A** and **B**) Responses of wild-type photoreceptors; (**C** and **D**) responses of *inaC* mutants. The adapting flash intensity was either ten times greater (A and C) or 100 times greater (B and D) than the test flash. (**E**) The degree of adaptation in wild-type (black bars) and *inaC* mutants (dotted bars) is plotted as a function of the relative intensity of the adapting flash (I_a/I_t). (**F**) A graph relating the degree of adaptation in wild-type (black dots) and *inaC* mutant (white dots) to the normalized time integral of the response to the adapting flash. The degree of adaptation was quantified as area T_a/area T_b. The responses to adapting flashes were normalized for variances in absolute sensitivity among cells with the use of the ratio of the time integral of the adapting flash response over the average peak response to prior test flashes (area A/avg [peak T_b]). Plots in (E) and (F) were made with the identical set of data. All data were acquired as in Fig. 4, except that standard whole cell patch clamp techniques were used.

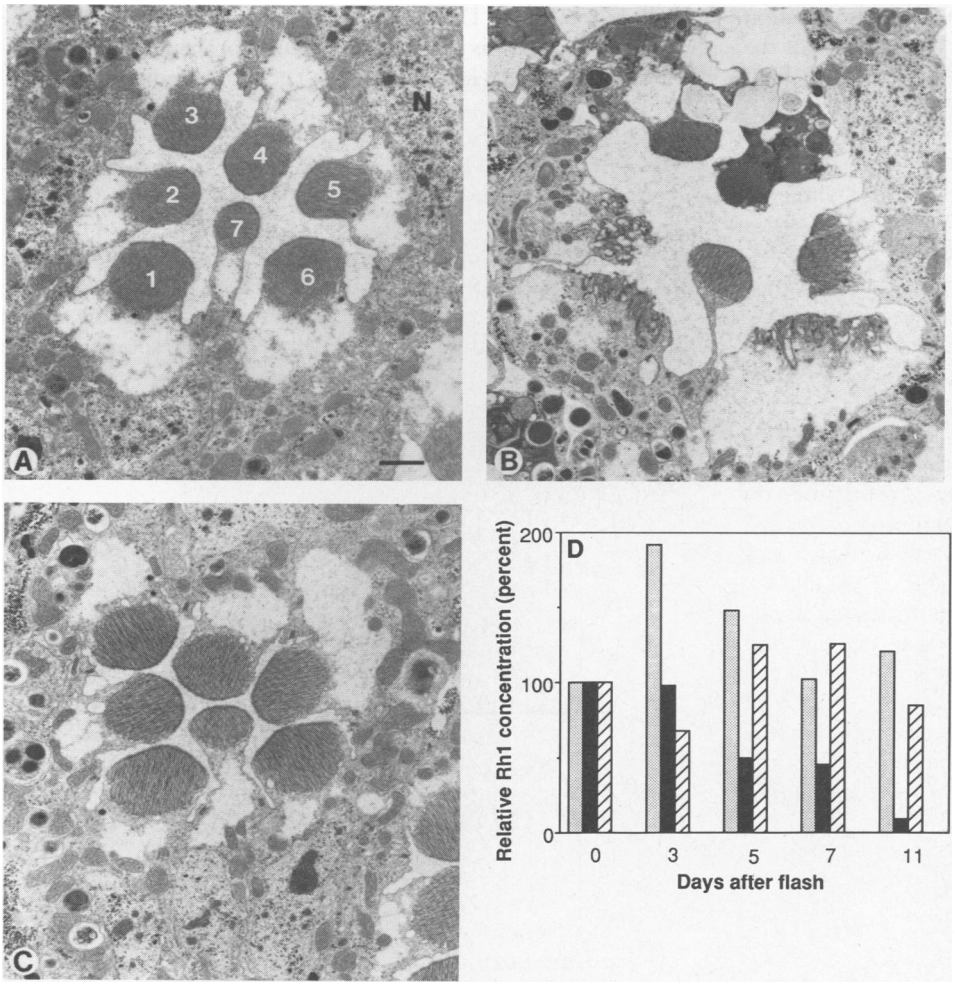


Fig. 7. eye-PKC mediates retinal degeneration in *rdgB* mutants. Flies were raised in the dark and given a 90-minute pulse of white light on the first day after eclosion. Animals were then kept in the dark and processed at the indicated times. Flies were fixed and sectioned for electron microscopy on day 12. (**A**) $inaC^{209}$ mutant photoreceptors. Numbers refer to rhabdomeres of the R1 through R7 photoreceptors cells. (**B**) $rdgB^{EE170}$ mutant photoreceptors, showing dramatic degeneration of the R1 through R6 photoreceptors. The R7 cell is more resistant to degeneration than the R1 through R6 cells. (**C**) The $rdgB^{EE170}$;$inaC^{209}$ double mutants, showing protection from light-dependent degeneration. Scale bar, 1 μm. (**D**) As an indicator of the overall extent of degeneration, opsin concentration was quantified in $inaC^{209}$ control (stippled bars), $rdgB^{EE170}$ (black bars), and $rdgB^{EE170}$;$inaC^{209}$ double mutants (hatched bars). Opsin concentrations were determined in extracts from five flies. Values were normalized to day zero amounts. The $rdgB^{EE170}$;$inaC^{207}$ double mutants gave essentially the same results as $rdgB^{EE170}$;$inaC^{209}$. Techniques for electron microscopy and opsin analyses have been described (*60*).

between mutant and wild-type photoreceptors should eliminate any differences in adaptation. Indeed, we found no significant difference in the degree of adaptation between wild-type (black circles) and *inaC* (open circles) photoreceptors when normalized for the time integral of the response to the adapting flash (Fig. 6F). This finding is consistent with the idea that the deactivation defect in the eye-PKC mutant fully accounts for their hyperadaptation. Consistent with these results, recordings of light responses to long light stimuli (greater than 10 seconds) demonstrate that although *inaC* mutants fail to rapidly desensitize and deactivate, the final level of desensitization is similar to that observed in wild-type cells (*25*).

Protection of *rdgB* photoreceptors from retinal degeneration by eye-PKC mutants. Because eye-PKC is a key component of the visual transduction cascade, it is important to identify its upstream regulators and downstream targets. A powerful approach to address this issue is the study of epistatic interactions between *inaC* and other visual mutants. A number of mutants have been isolated in *Drosophila* in which photoreceptor cells degenerate in a light-dependent manner (*8*). One of the most interesting is *rdgB*. When *rdgB* mutants are grown at room temperature in the dark they have morphologically normal photoreceptor cells. However, as little as a single flash of light is sufficient to trigger an irreversible course of photoreceptor cell degeneration (*11*). The *rdgB-norpA* double mutants do not degenerate, indicating that the events responsible for degeneration occur downstream from PLC activation (*11*). Application of phorbol esters to the eyes of dark-reared *rdgB* flies result in photoreceptor cell degeneration, although wild-type flies are unaffected (*36*). These findings suggest that activation of PKC may mediate the photoreceptor degeneration in *rdgB* mutants. If eye-PKC is the PKC that mediates this effect, then *rdgB* flies deficient in eye-PKC should be protected from light-dependent degeneration. To test this hypothesis, we generated $rdgB^{EE170}$;$inaC^{209}$ double mutants and measured light-dependent photoreceptor cell degeneration by ultrastructural examination of the retina and immunoblot analysis of photoreceptor-specific proteins. The $rdgB^{EE170}$ photoreceptors displayed cellular degeneration and reduced concentrations of photoreceptor proteins 11 days after exposure to a light flash (Fig. 7, B and D). In contrast, $rdgB^{EE170}$;$inaC^{209}$ photoreceptors were morphologically indistinguishable from wild-type or $inaC^{209}$ controls (Fig. 7, C and D). These results demonstrate that *rdgB* photoreceptor degeneration is suppressed in eye-PKC mutants. Therefore, eye-PKC is a mediator of retinal degeneration in the *rdgB* mutant, and the light dependency of the *rdgB* phenotype probably reflects the activation of eye-PKC in the visual cascade (see below).

Function of eye-PKC in phototransduction. Although much is known about the biochemistry and diversity of the PKC protein family, little is known about the functions of these enzymes in vivo. Indeed, much of the work on the biology of PKC's has relied on the use of pharmacological activators and inhibitors of PKC (*3, 37*). The original identification of an eye-specific isoform of PKC in *Drosophila* suggested a function for this enzyme in phototransduction (*6*). Rescue of the *inaC* visual mutation by germline transformation with the cloned eye-PKC gene demonstrates that inaC is the structural gene for eye-PKC. The detailed phenotypic characterization of this mutant showed that eye-PKC is a key regulator of deactivation and desensitization of the phototransduction cascade.

Although light adaptation is affected in *inaC* mutants, our results suggest that the eye-PKC–dependent regulatory mechanisms are distinct from those involved in light adaptation. Thus, it may be that parallel molecular mechanisms are involved in feedback regulation of phototransduction and that the *inaC* mutation genetically separates these regulatory pathways. Furthermore, our demonstration of genetic interactions between *inaC* and *rdgB* sets the stage for a comprehensive definition of the eye-PKC–dependent biochemical pathway.

The existence of a cell-type specific isoform of this enzyme, expressed in tissues not required for viability, provides an ideal model for the study of PKC function in vivo. Specifically, the availability of PKC null mutants in *Drosophila* provides a genetic background suitable for structure-function studies of PKC and allows us to genetically identify and dissect PKC regulatory pathways. In this regard, it would be interesting to determine whether the other four *ina* complementation groups (*inaA, B, D, and E*) (*27*) have any relation to the eye-PKC biochemical pathway.

The *inaC* mutants were originally isolated based on the fact that they do not display a prolonged depolarizing afterpotential, but still were "normally" nonresponsive to subsequent PDA-inducing stimuli (*27*). The lack of the afterpotential in these flies is probably a result of the hyperadaptation that occurs after bright or prolonged stimuli. The inability of *inaC* photoreceptors to rapidly reverse this light-adapted state makes them nonresponsive to subsequent light stimuli, and they appear inactivated in electroretinogram recordings.

The precise site of action of eye-PKC in the phototransduction cascade is not known, but probably involves phosphorylation of one or more substrates in the photoreceptor cells. For instance, phosphorylation of activated rhodopsin is thought to be important for its deactivation (*38*). The photoreceptor-specific arrestin-1 and arrestin-2 proteins of *Drosophila*, which are thought to be involved in opsin deactivation, are also phosphorylated in a light-dependent manner (*39, 40*). However, opsin deactivation does not appear to be the rate-limiting defect in *inaC*-dependent photoresponse deactivation. For instance, photoconversion of active metarhodopsin to rhodopsin during deactivation does not alter the kinetics of this process in *inaC* photoreceptors (*25*). This suggests that the site of action of eye-PKC is downstream of the opsin.

Other possible sites of action of eye-PKC include the G protein (see, for example, *41*), the light-activated ion channel (for examples, see *42*), and the PLC encoded by *norpA*. The mechanisms by which PLC enzymes are modulated are not known. The norpA protein is structurally related to the β class of phospholipase enzymes (*13, 43*), and PLC_β is phosphorylated in mammalian tissue culture cells in response to phorbol ester treatment (*44*). Treatment of various cell types with phorbol esters inhibits receptor-coupled PIP_2 hydrolysis, suggesting a negative feedback role for PKC in regulation of events at or upstream of PLC (*45–48*). Therefore, eye-PKC could be involved in modulating the *norpA* gene product or its regulators (Fig. 5). The persistent activity of PLC in eye-PKC mutants may explain the slow deactivation kinetics of the photoresponse in these flies.

It is not known whether vertebrate photoreceptors are also regulated by feedback mechanisms that involve PKC. However, calcium is a key regulator of vertebrate phototransduction (*17, 18, 33–35*). The availability of a PKC mutant in *Drosophila* provides the basis for genetic and biochemical studies to identify biologically relevant substrates and regulators of this enzyme. In this regard, we have identified eye-PKC as a genetic suppressor of *rdgB*, suggesting that the light-dependent activation of eye-PKC in the visual transduction cycle is responsible for triggering retinal degeneration in *rdgB* mutants. If the eye-PKC–*rdgB* interaction involves any other proteins, they should also be genetically identifiable as *rdgB* suppressors.

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52. The eye-PKC gene from US^{3741}, $inaC^{209}$, and $inaC^{207}$ mutants was amplified as two overlapping fragments in independent PCR reactions (*49*). Initial nucleotide concentrations were reduced to 40 μM to minimize PCR misincorporations. Four independently isolated subclones from different PCR reactions (representing two complete eye-PKC genomic regions) were sequenced for each mutant to eliminate errors occurring during PCR amplification.
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57. Photoreceptors were isolated and processed as described (*23*). Cells were visualized through Hoffmann interference contrast on a Leitz Fluovert inverted microscope. Recordings were made at room temperature (23° to 25°C) with patch pipettes with average resistances of 5 Mohm. Whole cell recordings were made with standard techniques (*31*), and nystatin-perforated patch recording techniques were adapted from (*32*). For perforated patch recording, a stock nystatin (Sigma) solution (50 mg/ml) in dimethyl sulfoxide was freshly diluted 1:500 into a filtered pipette solution for each pipette. Pipette tips were filled with nystatin-free solution to aid in seal formation. More than 80 percent series resistance compensation was used during recording. Signals were amplified with an Axopatch 1-D amplifier (Axon Instruments), filtered at 2 kHz, and digitized at 125 kHz for analysis. Photoreceptors were stimulated with light from a 75-W Xenon arc lamp, attenuated with neutral density filters (Oriel), regulated by an electronic shutter (Sutter Instruments), and focused through the microscope objective. Pipette solution, 124 mM CsCl, 10 mM Hepes, 11 mM EGTA, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 3 mM Mg^{2+}ATP, 0.5 mM Na^+GTP, pH 7.15. Bath solution, 124 mM CsCl, 10 mM

Hepes, 1 mM $CaCl_2$, 32 mM sucrose, pH 7.15.
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60. For electron microscopy, dissected heads were processed as described (*50*) except that the fixation solution contained formaldehyde (4 percent), glutaraldehyde (1 percent), and sucrose (2 percent). For immunoblots, the heads from three female and two male flies were removed for test at each time point and processed as in Fig. 3. Opsin was detected with a monoclonal antibody to Rh1 opsin (*51*). The I^{125}-labeled immunoblot was scanned quantitatively (Phosphorimager, Molecular Dynamics) and the results were analyzed with Imagequant software (Molecular Dynamics).
61. Fly stocks that carry the US^{3741} mutation were obtained from J. Merriam. Flies carrying the $inaC^{207}$ and $inaC^{209}$ mutations were obtained from W. Pak. We thank N. Colley for help with thin cryosections; M. Socolich for help with chromosome in situs; D. Cowan for embryo microinjections; W. Harris, T. Hunter, C. F. Stevens, and members of the Zuker laboratory for valuable comments on the manuscript; and D. Baylor, R. Aldrich, and J. Lisman for helpful discussions. Supported by the Howard Hughes Medical Institute; a training grant from the Medical Scientist Training Program (R.R.); the Pew Foundation, the McKnight Foundation, and the March of Dimes Basil O'Connor program (C.S.Z.). C.S.Z. is an investigator of the Howard Hughes Medical Institute.

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"I told you not to get emotionally involved. Now get back in there and sterilize that Petri dish."

Photoreceptor Deactivation and Retinal Degeneration Mediated by a Photoreceptor-Specific Protein Kinase C

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