

Phorbol ester induces photoreceptor-specific degeneration in a *Drosophila* mutant

(*rdgB* mutant/protein kinase C/phosphoprotein phosphatase/invertebrate phototransduction)

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Communicated by Seymour Benzer, August 25, 1989

ABSTRACT In the retinal degeneration B (*rdgB*) mutant of *Drosophila*, the major class of photoreceptors degenerate when the fly is raised in the light for several days; raising the fly in the dark largely prevents the degeneration. Thus, the *rdgB* is a conditional mutant that requires the operation of some stages of the phototransduction cascade to express its characteristic phenotype. We report here experiments that examine the ability of chemical agents to mimic light by causing photoreceptor-specific degeneration in the dark. Application of a specific activator of protein kinase C, phorbol ester, to eyes of *rdgB* flies led to a degeneration of the photoreceptors that was indistinguishable from that caused by light: both light and phorbol ester-induced degeneration were characterized by (i) selective degeneration of one class of photoreceptors; (ii) a unique pattern of degeneration; and (iii) the appearance of light-induced regenerative spikes at early stages of degeneration. Application of phorbol ester to the eyes of wild-type flies had no effect. We suggest that light or phorbol ester activates a protein kinase C and results in a sustained or excessive phosphorylation of proteins in the *rdgB* mutant, leading to photoreceptor degeneration. Furthermore, the results are consistent with identification of the *rdgB* gene product as a phosphoprotein phosphatase that is nonfunctional or absent in the mutant.

The *Drosophila* compound eye contains 800 repeat units referred to as ommatidia. In each ommatidium, there are eight photoreceptor cells, six peripheral (R1–R6) and two central (R7 and R8). In the *rdgB*^{KS222} mutant, only the R1–R6 photoreceptors degenerate after illumination, while R7 and R8 are spared (1, 2). After an extensive study on the *rdgB* mutant, Harris and Stark (2) suggested that the normal *rdgB* gene product is required for inactivation of a stage in the phototransduction pathway. According to this view, a defective *rdgB* gene product in the mutant no longer inactivates this phototransduction stage, thereby giving rise to an unbalanced response to illumination, which leads to degeneration (2).

Visual transduction in invertebrate photoreceptors now appears to involve a phospholipase C enzyme (PLC) (4–14), which is activated by photoexcited rhodopsin via a guanine nucleotide binding regulatory protein (G protein) (6, 7, 9, 14). PLC hydrolyzes inositol phospholipids to liberate inositol trisphosphate (4, 7, 10–14), which mimics visual excitation by light (4, 5, 7), and diacylglycerol (DG) (15), whose function in photoreceptor cells is unknown.

Application of chemical agents that mimic the effect of light to the eye of the mutant should lead to retinal degeneration if these chemicals operate prior to or at the site of action of the *rdgB* gene product (4–7). The phorbol ester, applied daily for 8 days in the present experiments, can enter the photo-

receptors via diffusion or via endocytosis (16), which is very pronounced in fly photoreceptors (17).

Specificity of the phorbol ester-induced degeneration was demonstrated by the following criteria: selective degeneration of R1–R6 relative to R7 and R8 photoreceptors; close similarity in the spatial distribution of the degenerating cells in *rdgB* flies treated with light or phorbol ester; close similarity between the electrophysiological and ultrastructural modifications from light- and phorbol ester-induced degeneration; and absence of any degeneration in the photoreceptors of wild-type flies due to light or phorbol ester treatment. Nonspecific action of phorbol ester is not expected to fulfill the above criteria. The specificity in phorbol ester action (3) should help to verify the model proposed by Harris and Stark (2) and to identify the transduction step that interacts directly or indirectly with the *rdgB* gene product and thereby provide a clue regarding the molecular mechanism of the degeneration and its relationship to phototransduction.

MATERIALS AND METHODS

White-eyed *rdgB*^{KS222} and *rdgB*^{P36} flies (Canton S and Oregon R strains, respectively) were raised at 19°C on instant *Drosophila* medium (formula 2-24 supplemented with vitamin A) and maintained continuously in the dark. In such conditions, there is essentially no degeneration up to the age of 8 days (18). Measurements of light-induced GTPase activity in cell-free membrane preparation of wild-type and dark-raised *rdgB* flies showed very similar light-dependent activity in eyes of 5-day-old flies (24°C) (data not shown). We used white-eyed flies in our study since these flies are more accessible than the red-eyed flies for physiological and biochemical assays. For example, the prolonged depolarizing afterpotential can be easily induced in white-eyed but not in red-eyed flies (19). No differences were found between *w rdgB* and *rdgB* regarding degeneration or phototransduction (2). Before initiation of the experiments, vials were cleared and 1-day-old flies were collected and kept at 24°C in the dark. Every day for 8 days a test compound in Ringer's solution (2 mM KCl/140 mM NaCl/2 mM CaCl₂/5 mM MgCl₂/10 mM Hepes buffer, pH 7.0) was applied to the eye of flies anesthetized with CO₂ in the following manner. Fine forceps with curved tips were used to put a drop of solution on each eye. The procedure was carried out under dim red (RG 630 Schott) light and lasted <3 min. The flies were subsequently returned to the vials and kept in the dark. The water-soluble phorbol 12,13-diacetate ester (20) was used instead of the more common hydrophobic analogs because small amounts of the organic solvents used to dissolve the hydrophobic analogs affected fly photoreceptors (data not

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Abbreviations: PLC, phospholipase C; PKC, protein kinase C; DG, diacylglycerol; G protein, guanine nucleotide binding regulatory protein; ERG, electroretinogram.

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shown). The organic solvents mimicked dim-light excitation in the fly by producing photoreceptor noise in the dark in a manner similar to the effect found in the locust (21). The cornea of *Drosophila* has many intraommatidial bristles, which are missing in the cornea of the larger flies (e.g., *Musca*). The hydrophilic solutions most likely enter the eye through the bristle sockets. This conclusion was derived from the fast (<1 min) penetration of a drop of solution into the eye of *Drosophila* (22), while no penetration was observed into the eye of *Musca* flies. Control and treated flies were always raised, treated, and processed for histological examination together to ensure similar conditions.

For both light and transmission electron microscopy, the fixation and embedding stages were identical. Heads were cut off and sliced midsagittally. The eyes were fixed for 1 hr in 2.5% glutaraldehyde in 0.1 M cacodylate buffer with 5% sucrose. Eyes were then rinsed three times (10 min each) in 0.1 M cacodylate buffer (pH 7.2). For 1 hr postfixation, the eyes were placed in 2% osmium tetroxide/0.1 M cacodylate buffer, dehydrated in an ethanol series (in the range of 30–100%), and embedded in Spurr's epoxy resin. For light microscopy, 3- μ m-thick sections were stained with Richardson's methylene blue. For electron microscopy, thin sections were stained with 2% each uranyl acetate and lead citrate in ethanol/water (1:1). Electron microscopy was carried out with a Philips (EM300) electron microscope at 60 kV.

An electroretinogram (ERG) was prepared by putting a recording electrode on the cornea and a reference electrode filled with Ringer's solution on the thorax. Maximal-intensity blue light pulses (originated from 12-V, 100-W halogen lamp in conjunction with Schott BG-28 blue filter) were used. The unattenuated intensity of the blue light was 2.5×10^{16} photons \cdot cm $^{-2}$ \cdot s $^{-1}$ at the level of the eye.

To examine whether light- or phorbol ester-induced degeneration of a given photoreceptor in an ommatidium is a spatially independent event, we used the binomial distribution according to Feller (23) and found that p^6 , $6p^5q$, $15p^4q^2$, $20p^3q^3$, $15p^2q^4$, $6pq^5$, q^6 are the probabilities of all six rhabdomeres, five, four, three, two, one, or none to degenerate, respectively. The probability of a rhabdomere to degenerate is p and to remain intact is q ($q = 1 - p$), assuming that the degeneration of one rhabdomere is independent of the degeneration of other rhabdomeres and a homogeneous value of p for all ommatidia under given experimental conditions.

RESULTS

The ommatidial ultrastructures from three 8-day-old *rdgB* flies subjected to different treatments are shown in Fig. 1. The morphological studies focused on changes occurring in the rhabdomeres. Rhabdomeres are composed of tightly packed microvilli (Fig. 1, large arrow) containing the visual pigment. Several stages of degeneration were apparent in the eyes of both light-raised (Fig. 1B) and phorbol ester-treated (Fig. 1C) *rdgB* flies. Early stages of degeneration were characterized by the appearance of vesicles (V arrowheads) between the microvilli, phagocytotic vacuoles (P) in the cytoplasm, and partial internalization of the whole rhabdomere into the cytoplasm (twin arrowheads). Another characteristic of degeneration is an increase in electron density throughout some of the photoreceptors (24) (twin arrowheads in Fig. 1B and C). Finally, shedding of the rhabdomeres was observed, as indicated by strands detaching from the microvillar membrane. Some of these strands form multilamellar bodies (24). An electron-dense network was observed beneath the degenerated rhabdomere, giving the appearance of a tightly packed rough endoplasmic reticulum (Fig. 1, D, arrow). There was a reduction in the number and length of the microvilli resulting in smaller R1–R6 rhabdomeres (relative to the R7 and R8). Although there are other *Drosophila* mutants

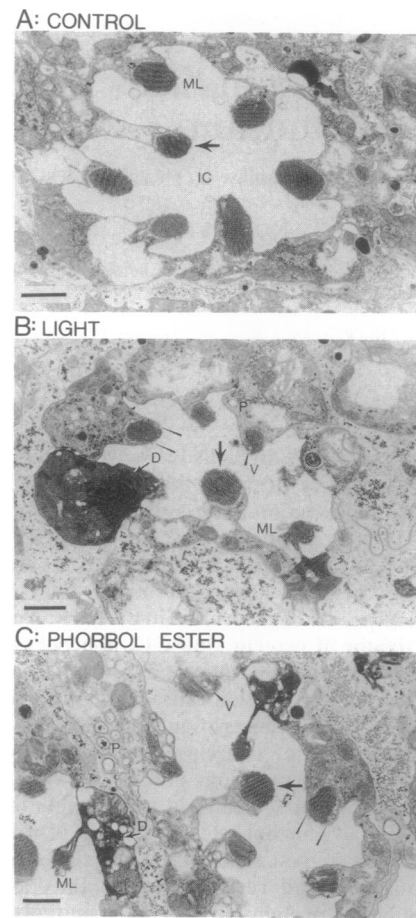


FIG. 1. Ultrastructure of a largely nondegenerated ommatidium compared to a partially degenerated ommatidium at early stages of degeneration of the *rdgB*^{K8222} mutant flies exposed to a light/dark cycle or phorbol ester treatment for 8 days at 24°C. (A) Cross-section of an ommatidium of the mutant raised in the dark. One drop of Ringer's solution ($\approx 1/10$ th the vol of the eye) was applied daily to the cornea under a dim red light (RG 630 edge filter, Schott). The typical structure of a fly ommatidium with seven rhabdomeres is shown. Initial stages of degeneration are observed as multilamellar bodies (ML) in the intraommatidial cavity (IC). The central rhabdomere, R7, is indicated by an arrow. The diameters of the rhabdomeres vary at different depths along the photoreceptors. This is the reason for the smaller diameter of R7 in A relative to B and C. (B) Cross-section of an ommatidium of a fly raised in a light/dark cycle. Illumination source was a 40-W fluorescent neon lamp. The various stages of degeneration were evident by the appearance of vesicles (V), phagocytotic vacuoles (P), multilamellar bodies (ML), electron densification of the cell and partial internalization of the whole rhabdomere into the cytoplasm (two arrowheads), a reduction in the number of microvilli and rhabdomeres, and appearance of electron-dense rough endoplasmic reticulum (D). In some cells, the rhabdomere is connected to the cell body via an electron-dense narrow bridge. Two rhabdomeres are missing. (C) Cross-section of an ommatidium from a fly raised in the dark and treated daily for 8 days with a drop of Ringer's solution containing 1.5 mM phorbol 12,13-diacetate, a hydrophilic form of phorbol ester. The effective concentration of phorbol 12,13-diacetate in modifying several electrical properties of hippocampal pyramidal neurons *in vitro* is around 0.5–1 μ M (i.e., 100- to 1000-fold higher than the hydrophobic phorbol esters). This concentration parallels the affinity of this compound for PKC (20). The apparent small differences between B and C are within the variability observed in the ultrastructure of a single eye. All flies used for the study were 8 days old (24°C). (Bars = 1.0 μ m.)

that display retinal degeneration, the specific features of the *rdgB* retinal degeneration described above are unique and are not observed in other mutants (24–27). The fact that both phorbol ester and light induced such a specific pattern of

degeneration satisfies one criterion for the specificity of the phorbol ester effect. Control mutant flies raised in the dark, to which Ringer's solution was applied daily under dim red light, showed only a few morphological signs of degeneration, such as vesicles and multilamellar bodies in the intraommatidial cavity (Fig. 1A). However, the degree of degeneration was much greater in the mutant raised under a light/dark cycle (Fig. 1B) or treated with phorbol ester in the dark (Fig. 1C).

The retinae of 8-day-old *rdgB* flies treated with either light or phorbol ester had ommatidia showing various degrees of degeneration (see Fig. 4). Fig. 1 shows a case of partial degeneration, and Fig. 2 A and B shows *rdgB* ommatidia at the final stages of degeneration induced by light (Fig. 2A) or by phorbol ester (Fig. 2B). The central photoreceptors R7 (arrow) and R8 (residing below R7 and not shown in this section), remain morphologically intact in both cases. No sign of degeneration was apparent in wild-type ommatidia treated with phorbol ester (Fig. 2C), thus satisfying an additional criterion for the specificity of the phorbol ester action. The relatively long R7 cell process connecting the rhabdomere to the cell body (Fig. 2 B and C) is within the variability observed in wild-type control (27).

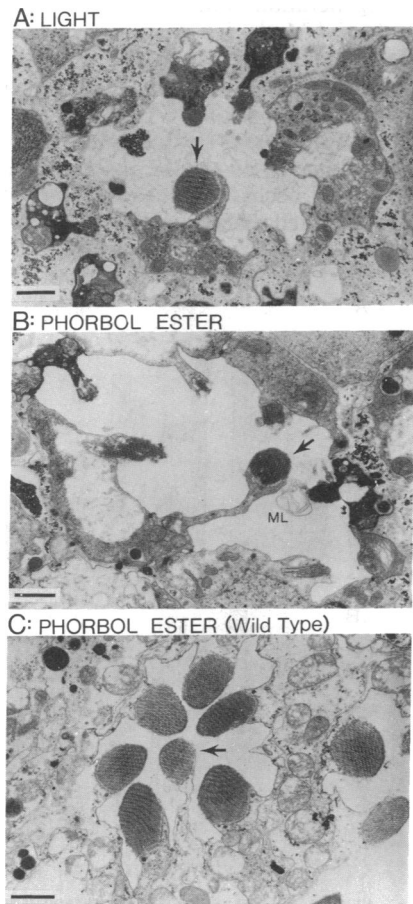


FIG. 2. The ultrastructure of highly degenerated ommatidium induced by light (A) compared to a highly degenerated ommatidium of the *rdgB* mutant exposed to phorbol ester (B). Phorbol ester was applied for 8 days as described in Fig. 1. The intact appearance of wild-type ommatidium treated with phorbol ester is included as a control (C). R1–R6 cells are almost completely degenerated in A and B but the central cell remained intact in mutants exposed to light (A) or phorbol ester (B). ML, multilamellar bodies. No sign of degeneration was observed in any of the ommatidia of 11 (1330 ommatidia scored) wild-type (white-eyed) flies treated with phorbol ester in the same manner as the mutant (C), which were used in the present study. (Bars = 1.0 μm .)

The physiological integrity of the central photoreceptors from the above-treated *rdgB* flies and from photoreceptors from similarly treated wild-type flies was confirmed by ERG recordings from dark-adapted flies (Fig. 3 Left) and light-adapted flies (Fig. 3 Right). The ERG measures changes in potential due to extracellular current flow in the eye in response to light. An ERG response was obtained from R7 and R8 but probably not R1–R6, in both light-raised (2) (Fig. 3C) and phorbol ester-treated (Fig. 3B) *rdgB* flies. This was suggested by the absence of the on and off transients (2) and the absence of a prolonged depolarizing afterpotential in response to maximal-intensity blue light (traces B–E). In trace A, the blue light intensity was attenuated 100-fold to prevent the appearance of the prolonged depolarizing afterpotential. Wild-type flies treated with phorbol ester gave normal ERG responses arising from all eight reticular cells, pigment cells (28), and lamina neurons (arrows) (Fig. 3A). An *ora* (outer rhabdomere absent) mutant fly, which does not have R1–R6 rhabdomeres, also does not display a light response from the R1–R6 cells (29) (Fig. 3D). After illumination with intense light, spike potentials appeared in the ERG of the degenerating *rdgB* flies (Fig. 3 E and F) but not in the ERG of wild-type flies (18). The appearance of the

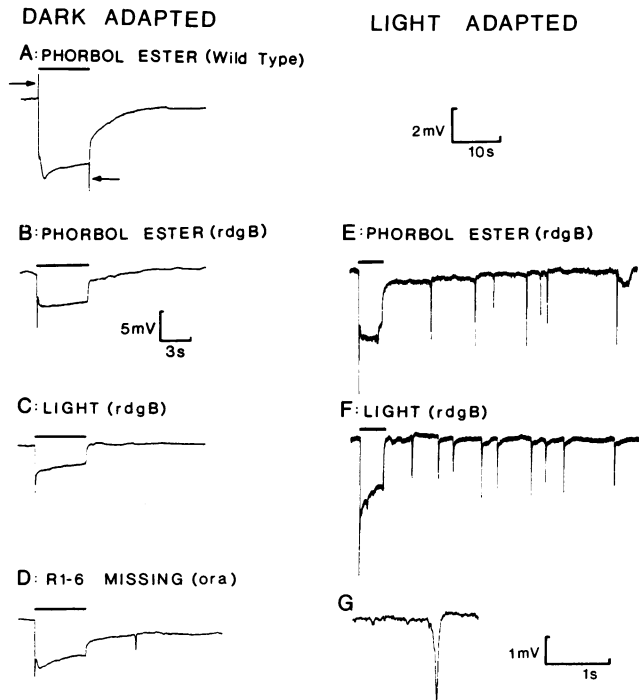


FIG. 3. ERG evoked from 8-day-old flies raised at 24°C. (Left) Recorded from dark-adapted flies (5 min). (Right) Recorded from light-adapted flies. (A) Wild-type fly treated with phorbol ester. (B) Phorbol ester-treated *rdgB* fly. (C) Light-raised *rdgB* fly. (D) Outer rhabdomere absent (*ora*) mutant, which does not have R1–R6 rhabdomeres and has only central (R7 and R8) functioning cells. Maximal intensity blue light pulses were used in B–F and were attenuated by 2 logarithmic units in A. The similar ERG light responses in B–F arose from R7 and R8 cells, indicating that they are functioning in both light- and phorbol ester-treated *rdgB* eyes. The remaining R1–R6 cells at this age showed deteriorated plasma membrane and were incapable of producing any response. Spike potentials, which usually appear in the ERG of degenerating *rdgB* flies (18), appear in E and F. (E and F) Recorded from the light-adapted eyes illuminated with the maximal intensity blue light 40 s before traces were recorded. (G) Single spike (from trace in E) in a faster time scale. The larger ERG in A arose from all eight photoreceptor cells (2). The “on” and “off” transients (arrows) arose from the second order lamina neurons (2), while the slow components during the rise and decay of the response arose from the pigment cells (28).

spikes was found to be a sensitive monitor of early stages of the degeneration in *rdgB* flies (18). The results of Fig. 3 are additional support for the specificity of phorbol ester action. Nonspecific action such as down-regulation of protein kinase C (PKC) would be expected to affect wild-type photoreceptors as well as all photoreceptors in the *rdgB* eye in a similar manner.

To quantitate the amount of degeneration in *rdgB* flies, the number of visible rhabdomeres per ommatidium were counted from high-power ($\times 630$) light micrographs of 8-day-old flies. At this age, rhabdomeres with partial degeneration are still visible (Fig. 1). Therefore, our analysis represents a minimum estimate of the degree of degeneration. Flies were raised in the dark and treated with a chemical or in Ringer's solution without any chemicals. The histograms in Fig. 4 show the distribution of ommatidia under the various conditions, according to the degree of degeneration. Column 7 shows (in each histogram) the percentage of ommatidia in which all seven rhabdomeres were present (only one of the two central rhabdomeres is visible in a given section), while columns 1–6 show the percentage of ommatidia in which one to six rhabdomeres were still present. The shaded area in the

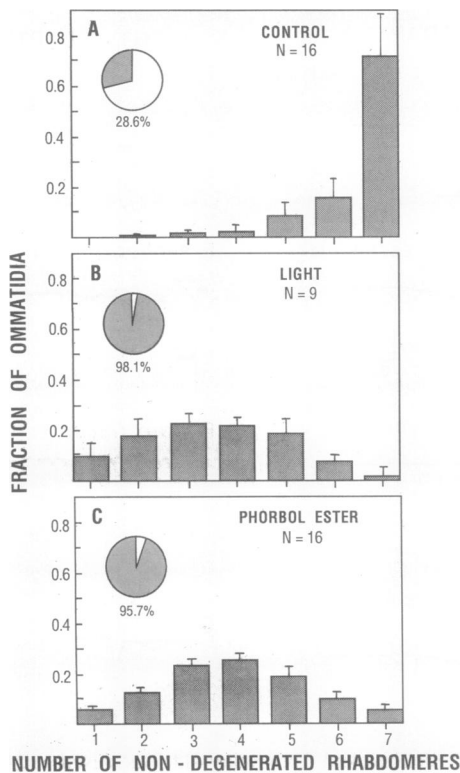


FIG. 4. Histograms and pie charts summarizing the extent of degeneration induced by light and phorbol ester applied in the dark. Degeneration was monitored morphologically. The flies were raised in total darkness for 8 days. The flies were treated by application of Ringer's solution containing the chemical agent (as indicated) during this period. Ringer's solution was applied to the control flies as well as to the light-treated flies (A and B). After 8 days, the eyes were fixed, stained, and viewed with a light microscope ($\times 630$). The missing rhabdomeres were scored in a double-blind manner. Partially degenerated rhabdomeres were scored as visible rhabdomeres. The histograms show the distribution of ommatidia under the various treatments according to the degree of degeneration. For example, bars 1 and 7 represent the percentage of ommatidia with 1 and 7 visible rhabdomeres, respectively. Pie charts present the percentage of degenerated ommatidia with one or more missing rhabdomeres (shaded area). The histograms were obtained from measurement of 16, 9, and 16 flies for A, B, and C, respectively, as indicated. The total number of ommatidia scored was 4460, 920, and 3320 for A, B, and C, respectively.

pie chart summarizes the percentage of defective ommatidia (i.e., ommatidia in which one or more rhabdomeres were missing). Fig. 4A shows the histogram obtained from *rdgB* flies raised in the dark and treated with Ringer's solution (control). A minor (28%) fraction of these ommatidia showed degeneration. In contrast, most (98%) ommatidia of *rdgB* flies raised under a light/dark cycle (Fig. 1B) showed some degeneration, with a majority having only three or four visible (two or three missing) rhabdomeres. Phorbol ester-treated mutants raised in the dark showed a distribution of degenerated ommatidia very similar to that found in illuminated flies (Fig. 2C).

To check whether a degeneration of one photoreceptor is independent of the degeneration of its neighboring cells we used the data from Fig. 4B and C to determine the probability for photoreceptors to degenerate. Fig. 5 compares the degeneration induced by phorbol ester (A) and light (B) (solid bars) with a predicted random distribution of degenerated rhabdomeres across the retina, assuming that each degeneration is an independent event (hatched bars). Solid bars were replotted from Fig. 4B and C; hatched bars were calculated by using the binomial distribution with $p = 0.58$ and $q = 0.42$ for the light-induced and $p = 0.52$ and $q = 0.48$ for the phorbol ester-induced degeneration. p and q were derived from Fig. 4 (see *Materials and Methods*). A χ^2 test revealed that the observed and expected histograms are significantly different. A nonsignificant difference between an observed and expected distribution would indicate that the distribution of degenerated photoreceptors is random. However, the significant difference obtained between the observed and expected distributions does not necessarily mean a nonrandom distribution of the degenerated rhabdomeres. This result may arise

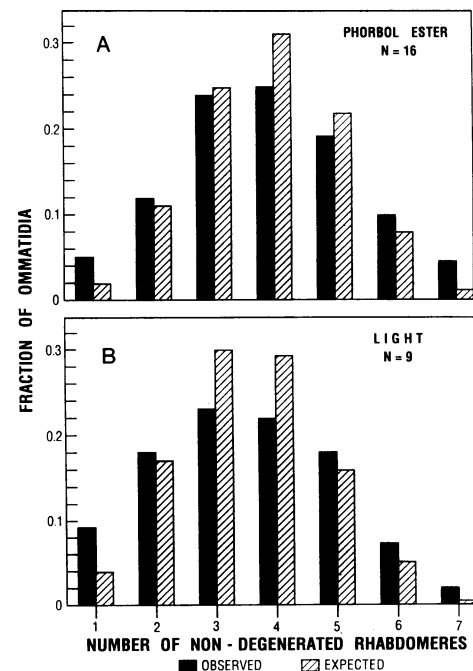


FIG. 5. A comparison between the observed and calculated fractions of nondegenerated rhabdomeres in the ommatidia of 16 and 9 *rdgB* flies treated with phorbol ester and light, respectively. The histograms were plotted similar to those in Fig. 4. Solid bars were replotted from Fig. 4B and C. Shaded bars were calculated by using the binomial distribution with $p = 0.52$ and $q = 0.48$ for the phorbol ester and $p = 0.58$ and $q = 0.42$ for the light-induced degeneration (which were derived from the data of Fig. 4), assuming that degeneration of a given cell is independent of degeneration of other cells. A χ^2 test indicated that there was a significant difference between the observed and expected distributions for both histograms A and B.

from a nonvalid assumption such as a homogeneous value of p for all ommatidia.

DISCUSSION

In the present study, we demonstrated that phorbol ester is able to mimic light by causing degeneration in a specific class of *Drosophila* mutant photoreceptors. It is unlikely that the selective degeneration of R1–R6 cells is due to much slower penetration of phorbol ester into R7 and R8 cells than R1–R6 cells. This is because of the close similarity between light and phorbol ester action on the morphology and electrophysiology of the photoreceptor cells. It is also unlikely that the chemically induced retinal degeneration was mediated by effects on energy metabolism. Application of the metabolic inhibitors cyanide (CN⁻) and 2-deoxy-D-glucose did not increase the extent of retinal degeneration over that observed in control flies treated with Ringer's solution (22), thus providing additional evidence that the phorbol ester-induced degeneration was a specific effect.

Fly photoreceptors contain a large amount of G-protein-mediated PLC (7), which hydrolyzes phosphatidylinositol 4,5-bisphosphate into inositol trisphosphate and DG after illumination (4, 7, 12–14). Both light and guanosine 5'-[γ -thio]triphosphate GTP[γ S], which activate PLC via a G protein in *Drosophila* photoreceptors (7), also cause degeneration of R1–R6 but not R7 and R8 of the *rdgB* flies (22). This suggests that activation of a G protein precedes the step in the transduction cascade that leads to photoreceptor degeneration in *rdgB*. Activation of PLC also precedes the step that leads to degeneration, since mutation of the *norpA* gene, which encodes light-activated PLC (8, 30, 31) blocks light-induced degeneration in *rdgB* photoreceptors in the double mutant *norpA rdgB* (2). A direct interaction between the *norpA* gene product (PLC) and the *rdgB* gene product was suggested by Harris and Stark (2) based on the isolation of the *norpA*^{SUII} mutant, which blocks degeneration in *rdgB*^{K5222} in an allele-specific manner.

The present study suggests that both light and phorbol ester induce degeneration in *rdgB* flies by a similar mechanism. The simplest interpretation is that the light-induced degeneration in the *rdgB* mutant results from a DG-induced PKC activity, since phorbol ester has been shown previously to mimic DG in activating PKC (3). A photoreceptor-specific PKC was recently discovered in *Drosophila* (32). Application of a high (8 mM) dose of phorbol ester 12,13-diacetate into the photoreceptors of the fly *Musca* by extracellular pressure injection close to the photoreceptors during bright light (7, 17) did not excite the photoreceptors (data not shown) and thus excluded a role for DG in excitation but perhaps suggested a role for DG in light-activated turnover.

The degeneration of the mutant photoreceptors is best explained by the hypothesis that the *rdgB* gene product is a phosphoprotein phosphatase that is nonfunctional or missing in the mutant. Thus, in the presence of light or phorbol ester, sustained protein phosphorylation induced by activation of PKC, unbalanced by dephosphorylation, may lead to photoreceptor degeneration. This interpretation is consistent with the main hypothesis of Harris and Stark (2) that an unbalanced action of a phototransduction stage is the cause for degeneration in *rdgB* eyes. However, the current results neither support nor rule out a direct interaction between the *rdgB* and *norpA* gene product. Recent experiments (J. Kleiman, B.M., and Z.S.) have provided evidence that there is a defect in phosphatase activity in *rdgB* eyes. In these experiments, application of ³²P_i to eyes of *rdgB* and wild-type *Drosophila* followed by polyacrylamide gel electrophoresis and autoradiography showed a much greater and longer-lived incorporation of ³²P_i-labeled proteins in *rdgB* than in wild-type flies. Complementary biochemical assay of Ca²⁺ and

calmodulin-dependent phosphoprotein phosphatase using a synthetic peptide showed that this enzyme activity is greatly reduced in the *rdgB* relative to wild-type flies.

How a sustained protein phosphorylation leads to photoreceptor degeneration is an open question. A clue may be derived from examining the Ca²⁺ level in the mutant photoreceptors since phorbol ester was reported to recruit a previously covert class of Ca²⁺ channels in untreated cells in *Aplysia* bag cell neurons (33). High intracellular Ca²⁺ is known to be associated with degeneration in a large variety of cells (34). The appearance of Ca²⁺ spikes at an early stage of the degeneration in *rdgB* flies (18), but not in wild-type flies, may cause a toxic increase in Ca²⁺ in the *rdgB* photoreceptors.

We thank Drs. C. Montell, K.-W. Yau, and R. Payne for critical reading of the manuscript and for many valuable discussions and A. Rom, E. Suss, and S. Tsemach for help in the preparation of the figures. This work was supported by grants from the National Institutes of Health (EY-03529) and the United States–Israel Binational Science Foundation, Jerusalem.

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