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# Calcium channel blockers inhibit retinal degeneration in the retinal-degeneration-B mutant of *Drosophila*

(neuronal cell death/light-induced degeneration/$Ca^{2+}$ spikes/excessive phosphorylation)

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ABSTRACT **Light accelerates degeneration of photoreceptor cells of the retinal degeneration B (rdgB) mutant of *Drosophila*. During early stages of degeneration, light stimuli evoke spikes from photoreceptors of the mutant fly; no spikes can be recorded from photoreceptors of the wild-type fly. Production of spike potentials from mutant photoreceptors was blocked by diltiazem, verapamil hydrochloride, and cadmium. Little, if any, effect of the (−)-cis isomer or (+)-cis isomer of diltiazem on the light response was seen. Further, the (+)-cis isomer was ≈50 times more effective than the (−)-cis isomer in blocking the $Ca^{2+}$ spikes, indicating that diltiazem action on the rdgB eye is mediated by means of blocking voltage-sensitive $Ca^{2+}$ channels, rather than by blocking the light-sensitive channels. Application of the $Ca^{2+}$-channel blockers (+)-*cis*-diltiazem and verapamil hydrochloride to the eyes of rdgB flies over a 7-day period largely inhibited light-dependent degeneration of the photoreceptor cells. Pulse labeling with [$^{32}$P]phosphate showed much greater incorporation into eye proteins of [$^{32}$P]phosphate in rdgB flies than in wild-type flies. Retarding the light-induced photoreceptor degeneration in the mutant by $Ca^{2+}$-channel blockers, thus, suggests that toxic increase in intracellular $Ca^{2+}$ by means of voltage-gated $Ca^{2+}$ channels, possibly secondary to excessive phosphorylation, leads to photoreceptor degeneration in the rdgB mutant.**

The mechanism of hereditary retinal degeneration is largely unknown in spite of identification of the defective gene products causing retinal degeneration in mammals (1, 2) and extensive studies in invertebrates (3–5). In the retinal degeneration B (rdgB) mutant of *Drosophila* (precisely, *rdgB*$^{KS222}$ mutation), the major class of photoreceptor cells degenerates when mutant flies are exposed to a 12 hr/12 hr light/dark cycle regime for several days (24°C); raising the flies in the dark largely reduces this degeneration (3–5). Recent experiments have revealed that application of phorbol ester to eyes of rdgB flies raised in the dark leads to a degeneration of the photoreceptor cells that is indistinguishable from the degeneration caused by light (5). In the crab, the daily cyclical changes in phototransductive membranes is modified by okadaic acid, a phosphoprotein phosphatase inhibitor, to a sequence of events that exactly parallels that seen during light-induced degeneration of the rdgB fly photoreceptors (6). These results suggest that excessive phosphorylation of the rdgB mutant proteins by protein kinase C, activated by the light-induced inositol phospholipid phototransduction cascade, may be important in accelerating photoreceptor degeneration (5). A specific characteristic of the rdgB phenotype is the appearance of regenerative EGTA-sensitive spikes at early stages of the degeneration after light exposure (7) or treatment with phorbol ester in the dark (5). No spikes can be recorded from photoreceptors of wild-type fly (7). To test whether malfunction of voltage-dependent $Ca^{2+}$ channels leads to a toxic increase in intracellular $Ca^{2+}$ and thereby to photoreceptor degeneration, we examined the ability of blockers of voltage-dependent $Ca^{2+}$ channel to block the spikes and to inhibit the light-induced degeneration in rdgB fly photoreceptors.

## MATERIALS AND METHODS

White-eyed *rdgB*$^{KS222}$ flies (Canton S strain) were raised at 19°C on instant *Drosophila* medium (formula 2-24 supplemented with vitamin A) and maintained continuously in the dark at all stages of development. In such conditions, there is no gross morphological manifestation of degeneration up to the age of 8 days (7). *In vitro* measurements of light-dependent photopigment phosphorylation and GTPase activity in cell-free membrane preparation of wild-type and dark-raised rdgB flies showed very similar light-dependent enzymatic activity in eyes of 1- to 4-day-old flies (24°C, data not shown). We used white-eyed flies only in our study. Before initiation of the experiments, vials were cleared, and 1-day-old flies were collected and kept at 25–26°C in the dark. Test compounds in Ringer's solution (2 mM KCl/130 mM NaCl/2 mM $CaCl_2$/5 mM $MgCl_2$/10 mM Hepes buffer, pH 7.0) were applied for 7 days to the eyes of flies (anesthetized with $CO_2$) in the following manner. Fine forceps with curved tips were used to put a drop of solution on each eye; the procedure was done either under dim red light (Schott RG-630 filter) in dark-raised flies or during light in light-raised flies and lasted <3 min.

The cornea of *Drosophila* has many intraommatidial bristles. The hydrophilic solutions most likely enter the eye through the bristle sockets (8). Chemicals that must be dissolved in organic solvents could not be used in this application method. The need to counteract the continuous (12-hr) action of light by application of $Ca^{2+}$-channel blockers with reversible action required a long-acting drug. We, therefore, applied high concentration of diltiazem (20 mM; Sigma) twice a day or 20 mM verapamil hydrochloride, dissolved in 1% cyclodextran, once a day. Previous studies using the single application of a chemical to *Drosophila* eye showed that dilution from the eye to the body took ≈90 min (8). Application of diltiazem twice a day and the slow release of verapamil hydrochloride from the cyclodextran during 7 days presumably led to the build-up of a sufficient eye concentration. The need to use a high concentration of hydrophilic drugs during 7 days limited the number of $Ca^{2+}$-channel blockers that could be used to diltiazem and verapamil

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Abbreviations: rdgB, retinal degeneration B; ERG, electroretinogram.

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hydrochloride. Cadmium, which blocked the spikes very efficiently, was toxic when applied in high concentration (see below).

The experiments were done on four groups of rdgB flies: (*i*) flies treated with Ringer's solution and raised in the light/dark cycle regime; (*ii*) flies treated with chemicals and raised in the light/dark cycle regime; (*iii*) flies treated with Ringer's solution and raised in the dark; and (*iv*) flies treated with chemicals and raised in the dark. In separate experimental runs the effect of the chemicals on wild-type flies was also examined, and no effect was found. The four groups of flies were raised, treated, and processed for histological examination together to ensure similar conditions. In part of the experiments, groups 3 and 4 were raised together, and in some experiments, groups 1–3 were raised together.

The procedure for both light and transmission electron microscopy has been described (5, 7, 8).

An electroretinogram (ERG) was prepared by placing the recording electrode on the cornea and the reference electrode filled with Ringer's solution on the thorax. Maximal-intensity blue light pulses (originated from a 12-V 100-W halogen lamp in conjunction with a Schott BG-28 blue filter or from a 150-W xenon light source with the same filter) were used. The unattenuated intensity of the blue light was $2.5 \times 10^{16}$ photons·cm$^{-2}$·s$^{-1}$ and $1.2 \times 10^{17}$ photons·cm$^{-2}$·s$^{-1}$ at the level of the eye for the halogen and xenon light sources, respectively.

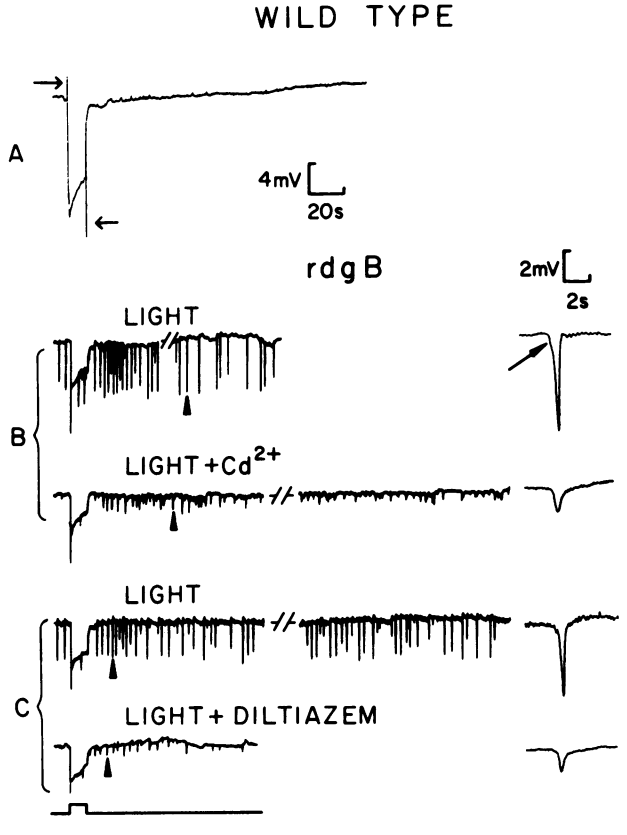


FIG. 1. Inhibition of $Ca^{2+}$ spikes of degenerating rdgB photoreceptors by $Ca^{2+}$-channel blockers. ERG recorded from normal (white-eyed) (*A*) and 8-day-old *Drosophila* rdgB mutants raised under 12 hr/12 hr light/dark cycle (*B* and *C*). No spikes were seen after the light stimulus in the normal fly (*A*), even when intensity of test light was increased 1000-fold (data not shown). Upper and lower traces in *B* and *C* were recorded from same eyes before and 10 min after application of a drop of Ringer's solution containing 20 mM $CdCl_2$ (*B*) and 20 mM diltiazem (*C*). Horizontal arrows in *A* indicate "on" and "off" transients arising from lamina neurons. Arrow in *B* (right) indicates the transition phase in generation of the spike from a slow wave to regenerative potential. Break in traces indicates 2-min pause. At right are magnified single spikes, each indicated by an arrowhead at left. Test stimulus was a blue light (12-V 100-W halogen lamp combined with a Schott BG-28 filter) in *B* and *C*. The light was attenuated 1000-fold in *A*.

## RESULTS

Fig. 1*B* shows that in rdgB flies raised for 8 days in a light/dark cycle regime, application of prolonged intense illumination produced multiple regenerative spikes in the retina. These spikes usually disappear within a few minutes in the dark as described (7). This activity contrasts with the retina of wild-type *Drosophila*, which did not produce spikes (Fig. 1*A*). To further characterize the spike potentials, we tested whether they are affected by $Ca^{2+}$-channel blockers. Blockers that are fully soluble in aqueous solution were used because the fly photoreceptors are very sensitive to organic solvents (5). The chemicals are diluted by the hemolymph flow in the eye (8, 9). Comparison of the ERG response to light before and after application of a drop of solution containing either cadmium or diltiazem showed that both agents severely decreased amplitude of the spike potentials (Fig. 1*B*) or eliminated them, while having little effect on receptor potential (Fig. 1 *B* and *C*). Application of verapamil hydrochloride (5 mM in Ringer's solution) also blocked the

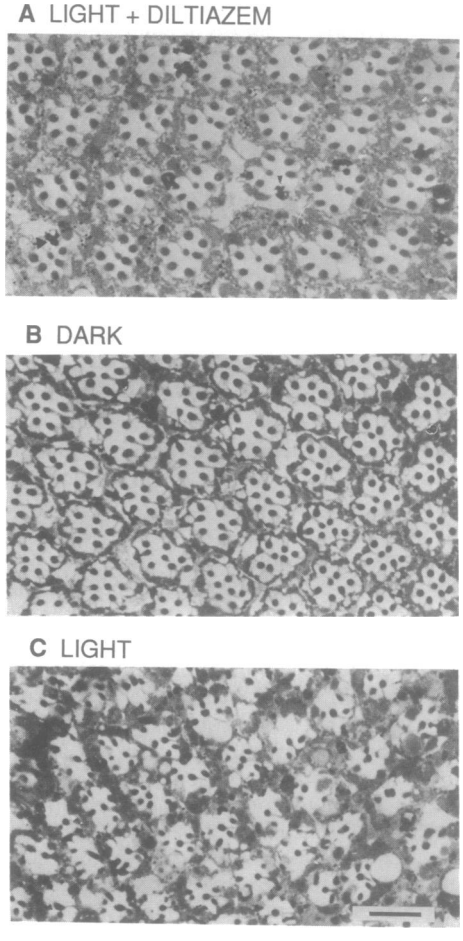


FIG. 2. Tangential light-micrograph sections of rdgB mutant eyes showing the effect of diltiazem in preventing retinal degeneration. Mutant rdgB flies were raised in the dark (*B*) or in 12 hr/12 hr dark/light for 8 days at 25°C (*A* and *C*). Twice a day one drop of Ringer's solution (*B* and *C*) or Ringer's solution/20 mM diltiazem (*A*) was applied to the cornea of the light-raised fly eyes. Arrow and arrowhead, partially degenerated rhabdomeres. Sections were chosen to illustrate maximal morphological differences; quantitative analysis is given in Fig. 3. (Bar = 10 μm.)

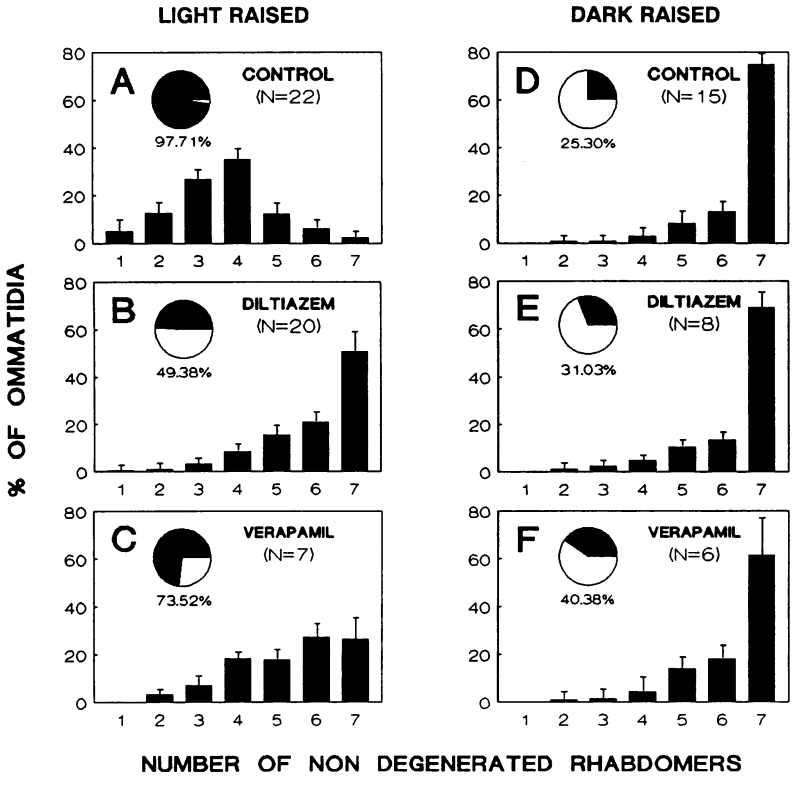


FIG. 3. Histograms and pie charts summarizing extent of degeneration induced by light and inhibition of light-induced degeneration when $Ca^{2+}$-channel blockers were applied to rdgB eyes. (*A*) Light-induced degeneration. (*B*) Inhibition of light-induced degeneration after application of diltiazem (20 mM). (*C*) Reduction in light-induced degeneration after application of verapamil hydrochloride (20 mM in 1% cyclodextran). (*D*) Dark-raised rdgB flies. (*E*) Dark-raised rdgB flies treated with diltiazem (20 mM). (*F*) Dark-raised rdgB flies treated with verapamil hydrochloride (20 mM in 1% cyclodextran). Degeneration was monitored morphologically from data similar to Fig. 2. Partially degenerated rhabdomeres (arrow and arrowhead, Fig. 2) were scored as visible rhabdomeres. Total number of ommatidia scored was 1305, 1549, 305, 997, 714, and 350 for groups in *A–F* respectively. Similar experiment with 10 mM diltiazem (five flies) gave results similar to that of *B* except that degree of degeneration was higher (64%, data not shown). Pie charts summarize fraction of defective ommatidia (i.e., ommatidia with one or more missing rhabdomeres, dark slices). The Ringer's solution applied to the control of the verapamil hydrochloride experiments contained 1% cyclodextran, and results of these control experiments are included in Fig. 3*D*. Number of rdgB flies used for each histogram (*A–F*) is indicated by N.

spikes in a manner similar to Fig. 1 *B* and *C* (data not shown). The diltiazem that we used contains the (+)-cis isomer. The (+)-cis isomer is a known $Ca^{2+}$-channel blocker, whereas the (−)-cis isomer blocks cGMP-sensitive channels (10). To examine whether diltiazem action on the rdgB eye is mediated by means of blocking voltage-dependent $Ca^{2+}$ channels or the light-sensitive channels (11), we applied 20 mM (−)-*cis*-diltiazem to wild-type *Drosophila* and measured the ERG response to light; no effect was found (six flies), and responses very similar to that of Fig. 1*A* were obtained. Application of (−)-*cis*-diltiazem to the spiking retina of rdgB eye blocked the $Ca^{2+}$ spikes in a manner similar to the result shown in Fig. 1*C*. To compare the effective concentration of (−)-*cis*-diltiazem and (+)-*cis*-diltiazem in blocking the spikes, we applied each compound at various concentrations to a light-induced spiking rdgB eyes. The minimal concentration of the (+)-cis isomer that blocked the spikes was 0.1 mM (six flies), whereas 5 mM of (−)-*cis*-diltiazem was the minimal concentration needed to block the spikes (eight flies). Both isomers had no effect on the light response of wild-type or rdgB flies. The above result is consistent with previous reports showing that both isomers of diltiazem block $Ca^{2+}$ channels, but the (+)-cis isomer is effective at a lower concentration relative to the (−)-cis isomer (12). Previous data (5, 7) and Fig. 1 suggest that early in the degeneration process large $Ca^{2+}$ fluxes occur through voltage-dependent $Ca^{2+}$ channels located at the axon terminals, leading to photoreceptor cell degeneration. To verify this pathophysiological mechanism, we tested whether application of $Ca^{2+}$-channel blockers to the intact rdgB eyes can retard the light-induced retinal degeneration and inhibit spike appearance.

Fig. 2*A* shows in histological cross sections of rdgB retina that daily application of diltiazem to the eyes of rdgB flies, which were raised in light/dark cycle for 8 days, inhibited photoreceptor degeneration relative to light-raised rdgB flies in the control (Fig. 2*C*). Normal appearance of most ommatidia of rdgB flies raised in the dark is demonstrated in Fig. 2*B* for comparison.

To quantitate extent of degeneration in rdgB eyes, the visible rhabdomeres per ommatidium were counted from light micrographs of histological cross sections of fly eyes (Fig. 2). Electron microscopy of the same eyes showed that our analysis represents a reliable and easily detectable estimate of the degree of degeneration (data not shown). The histograms in Fig. 3 summarize the distribution of ommatidia under the various conditions, according to degree of degeneration. Bars 7 show the fraction of ommatidia in which all seven rhabdomeres in each ommatidium were present [only one of the two central rhabdomeres, which do not degenerate in light (3, 4) is visible in a given section], whereas columns 1–6 show the fraction of ommatidia in which one to six rhabdomeres were still present. The shaded area in the pie chart summarizes the percentage of defective ommatidia (i.e., ommatidia in which one or more rhabdomeres were missing). Fig. 3*B* shows the histogram obtained from rdgB flies raised in light/dark cycle and treated with diltiazem; about half of the ommatidia in this group of flies did not show even a single degenerated photoreceptor, whereas minimal degeneration of one or two cells per ommatidium was seen in most of those affected. This degeneration contrasts to that in the ommatidia of control rdgB flies raised in a light/dark cycle without diltiazem, which showed several degenerated photoreceptors in most ommatidia (Fig. 3*A*). Mutant flies raised in the dark and treated with Ringer's solution showed a distribution of degenerated ommatidia similar to that of the diltiazem-treated rdgB flies but with a reduced level of degeneration (Fig. 3*D*). Histological cross sections of dark-raised rdgB flies treated with 20 mM diltiazem showed a minimal degree of degeneration similar to dark-raised and Ringer's-treated rdgB flies of the same experimental run (Fig. 3*E*). Diltiazem (20 mM) applied for 7 days to light- or dark-raised wild-type *Drosophila* had no effect on either the morphology of the retina, when inspected with light microscopy, or on the ERG of the flies (five flies).

We applied, in addition, Ringer's solution containing 5 mM verapamil hydrochloride for 7 days to the rdgB eyes of flies raised in the light. No effect was found, possibly because of the insufficient concentration of verapamil used. To increase

the concentration of verapamil hydrochloride to 20 mM, we added 1% cyclodextran to the Ringer's solution. Application of 20 mM verapamil hydrochloride once a day retarded the light-induced degeneration relative to the control, but this concentration was less effective in comparison with diltiazem (Fig. 3*C*). Verapamil hydrochloride (20 mM) applied for 7 days to dark-raised rdgB flies (Fig. 3*F*) showed distribution of degenerated ommatidia similar to the dark-raised control rdgB flies treated with either Ringer's solution or Ringer's solution/1% cyclodextran. The apparent difference among the averaged fraction of "defective ommatidia" in Fig. 3 *D–F* is not statistically significant.

We also applied 5 mM and 10 mM cadmium to the eyes of rdgB flies in a manner similar to that of Figs. 2 and 3. However, the 5 mM cadmium did not block degeneration when applied for 7 days to intact rdgB flies, whereas the 10 mM concentration was toxic and killed all the other flies. Therefore, cadmium could not be used in these experiments.

To further examine the effect of diltiazem in retarding degeneration and to examine the correlation of spikes to degeneration, we measured the diltiazem effect on the appearance of spike potentials in the ERG of flies at various ages as a possible indicator of early stages of degeneration. Because even minor fractions of degenerated photoreceptors can produce spikes in the ERG (7), this experiment presumably constitutes a sensitive test for changes in the degree of early stages of degeneration. The ERG was measured 12 hr after the fly was last treated to minimize a direct effect of the treatment on the ERG. The histograms in Fig. 4 show the percentage of diltiazem-treated rdgB flies in which spikes could be elicited by bright light in their ERG as a function of age in light (Fig. 4*A*)- and dark (Fig. 4*B*)-raised flies. The frequency or amplitudes of the spikes were not considered in Fig. 4. Fig. 4 shows that up to 3 days essentially no spikes were seen in any group of flies; the percentage of flies with spikes increased with age. In all our experiments, light-raised and Ringer's solution-treated flies (light control) showed the highest percentage of flies with spikes; this percentage reached 100% at 7 days of age. Diltiazem (20 mM) significantly reduced the percentage of flies with spikes in both light- and dark-raised flies at early age.

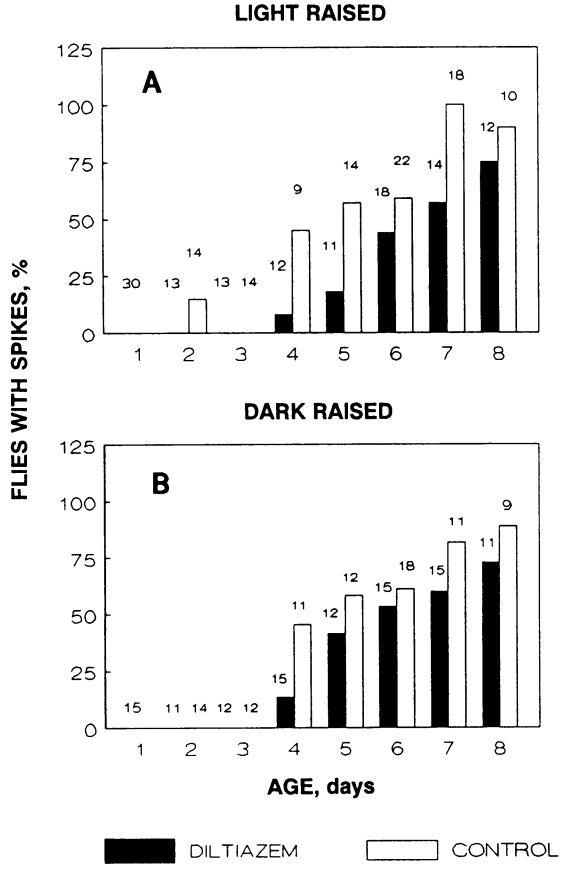


FIG. 4. Appearance of spike potentials in the ERG is inhibited by diltiazem in rdgB flies of various ages. Histograms show percentage of flies with spikes as a function of age in light-raised and Ringer's solution-treated versus diltiazem (20 mM)-treated and light-raised rdgB flies (*A*) and in dark-raised and Ringer's solution-treated versus diltiazem (20 mM)-treated and dark-raised rdgB flies (*B*). In each experiment (*A* and *B*) the flies were raised and treated together. Spikes were elicited by using several 10-s maximal-intensity blue light (Schott BG-28) pulses of the xenon light source. Flies without spikes were illuminated several times to be sure no spikes were elicited. Number above each column indicates number of flies used for experiment.

Figs. 2–4 thus demonstrate that blocking of voltage-dependent $Ca^{2+}$ channels inhibited photoreceptor degeneration in rdgB fly photoreceptor.

Phorbol ester applied to dark-raised rdgB flies was found to mimic light in causing photoreceptor degeneration (5), suggesting that excessive phosphorylation may initiate degeneration in rdgB fly photoreceptors. To test whether the rdgB mutant eye has excessively phosphorylated proteins we did pulse-labeling experiments in which [$^{32}$P]phosphate was applied under illumination to the eyes of rdgB and wild-type flies. After this treatment, flies were kept in the dark (19°C) to minimize photoreceptor cell degeneration. At various time intervals after application of the [$^{32}$P]phosphate, the flies were homogenized, and $^{32}$P-labeled proteins were revealed by SDS/PAGE and autoradiography. Fig. 5 shows that 12 hr after labeling phosphorylated proteins did not differ between the wild type and the mutant (lanes 1 and 2). However, 1, 2,

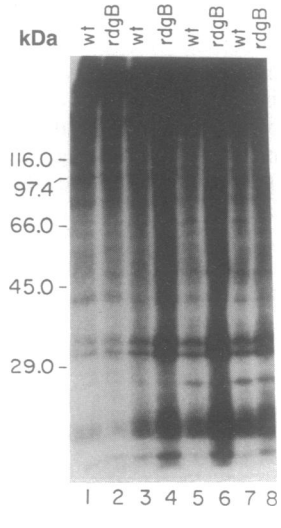


FIG. 5. Pulse labeling of proteins with [$^{32}$P]phosphate in rdgB and white-eyed normal *Drosophila* flies *in vivo*. The autoradiogram of eye membranes analyzed by SDS/PAGE 12 hr and 1, 2, or 3 days (lanes 1–8, respectively) after labeling with [$^{32}$P]phosphate shows that the rdgB eye proteins 1 and 2 days after labeling had incorporated much more $^{32}$P than normal flies. The rdgB and Canton S white-eyed flies were raised in the dark at 19°C. One-day-old flies were treated by application of a drop of [$^{32}$P]phosphate (20 mCi/ml, carrier-free; 1 Ci = 37 GBq) in 150 mM Tris·HCl, pH 7 buffer to each cornea. On the half, first, second, and third day after labeling, the eyes of 24 flies in each group were homogenized in 10 mM Mops buffer, pH 7.0/120 mM KCl/3 mM $MgCl_2$/1 mM dithiothreitol containing the protease inhibitors leupeptin at 60 $\mu$g/ml, EP475 at 10 $\mu$g/ml, and pepstatin A at 1 $\mu$g/ml. Membranes were precipitated (14,000 × *g* × 15 min), analyzed by SDS/PAGE (7.5–15% gradient), and autoradiographed. Analysis of six fly heads in each group 3 hr after labeling revealed that each fly received $\approx 10^6$ cpm of $^{32}$P, and the maximal difference between groups did not exceed 20%.

and 3 days after labeling much more incorporation of $^{32}P$ into eye proteins was found in rdgB flies than in wild-type flies. These findings are consistent with diminished protein phosphatase activity that results in a slower rate of, but greater and more persistent, protein phosphorylation after prolonged labeling periods.

## DISCUSSION

Several lines of evidence suggest that voltage-dependent $Ca^{2+}$ channels are critically affected by the *rdgB* mutation and that malfunction of these channels is involved in retinal degeneration. The temporal appearance of $Ca^{2+}$ spikes before overt morphological signs of degeneration (7) is consistent with recruitment and prolonged opening of voltage-dependent $Ca^{2+}$ channels as an essential step in the mechanism of retinal degeneration. We have previously determined that the sites at which these $Ca^{2+}$ spikes arise are the normally nonspiking axons and axon terminals of the photoreceptor cells (7). This normally nonspiking tissue presumably has a limited $Ca^{2+}$ buffering capacity. Consistent with this localization and its relevance to retinal degeneration is the finding that the axon terminals and axons of the rdgB photoreceptor degenerate before any other part of the photoreceptor cell (13). Voltage-dependent $Ca^{2+}$ channels are known to be regulated by phosphorylation and dephosphorylation reactions (14, 15) and are localized to the axon terminal as part of the mechanism of transmitter release (16). Toxic intracellular $Ca^{2+}$ concentration is a common mechanism of cell death (17).

Three lines of evidence suggest that inhibition of the light-dependent degeneration by the various chemicals could not be mediated by blocking the light-sensitive channels: (*i*) The chemicals had no effect on the response to light. (*ii*) There is no evidence in any tissue that verapamil blocks light-sensitive channels. (*iii*) The (+)-*cis*-diltiazem that is inefficient in blocking cGMP-sensitive channels (10) blocked the $Ca^{2+}$ spikes at ≈50-fold lower concentration relative to the (−)-cis isomer.

The degeneration induced by phorbol ester (5) and the excessive phosphorylation of eye proteins in rdgB mutant *in vivo* (Fig. 5) suggest that light absorption by the photopigment activates protein kinase C by means of G protein-mediated phospholipase C (18), leading to hyperphosphorylation of photoreceptor proteins in rdgB mutant due to some unknown defect.

The rdgB transcription unit has been cloned and characterized recently by Vihtelic *et al.* (19). The gene encompasses a complex transcriptional unit generating at least five different mRNAs. The 1054-amino acid protein inferred from the most abundant cDNA class contains 281 amino-terminal residues that have been shown to be >40% identical with the entire rat phosphatidylinositol transfer protein (D. R. Hyde, personal communication). How the impaired *rdgB* gene product leads to excessive phosphorylation at the rhabdomere region and to production of $Ca^{2+}$ spikes at the synaptic terminal is an open question. The results suggest, however, that malfunction of voltage-dependent $Ca^{2+}$ channels that can be blocked by diltiazem and verapamil leads to photoreceptor degeneration.

A pertinent question is whether the later stages of inherited retinal degeneration of mammals resemble the degeneration of photoreceptor cells in the rdgB mutant. A common feature of several of the mammalian retinal degeneration mutations is a striking increase in cGMP concentrations due to a decrease in cGMP phosphodiesterase activity (2). This finding is extensively documented for the *rd*-containing mouse (2, 20), the Irish setter (21), and in one case of human retinitis pigmentosa (22). The high level of cGMP may result in massive opening of the light-sensitive and $Ca^{2+}$-permeable channels (23), leading to toxic increase in cytosolic $Ca^{2+}$ and degeneration.

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