Constitutive Activity of the Light-Sensitive Channels TRP and TRPL in the *Drosophila* Diacylglycerol Kinase Mutant, *rdgA*

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Summary

Mutations in the *Drosophila* retinal degeneration A (*rdgA*) gene, which encodes diacylglycerol kinase (DGK), result in early onset retinal degeneration and blindness. Whole-cell recordings revealed that light-sensitive Ca^{2+} channels encoded by the *trp* gene were constitutively active in *rdgA* photoreceptors. Early degeneration was rescued in *rdgA;trp* double mutants, lacking TRP channels; however, the less Ca^{2+}-permeable light-sensitive channels (TRPL) were constitutively active instead. No constitutive activity was seen in *rdgA;trpl;trp* mutants lacking both classes of channel, although, like *rdgA;trp*, these still showed a residual slow degeneration. Responses to light were restored in *rdgA;trp* but deactivated abnormally slowly, indicating that DGK is required for response termination. The findings suggest that early degeneration in *rdgA* is caused by uncontrolled Ca^{2+} influx and support the proposal that diacylglycerol or its metabolites are messengers of excitation in *Drosophila* photoreceptors.

Introduction

Phototransduction in *Drosophila* is mediated by a phosphoinositide (PI) cascade, whereby absorption of light by rhodopsin activates sequentially a heterotrimeric G_q protein and phospholipase C (PLC) (reviewed by Hardie and Minke, 1995; Minke and Selinger, 1996; Scott and Zuker, 1998a). This cascade results in the opening of two classes of light-sensitive channels: a highly Ca^{2+}-permeable channel (TRP) encoded at least in part by the transient receptor potential (*trp*) gene (Montell and Rubin, 1989; Hardie and Minke, 1992) and a nonselective cation channel (TRPL) encoded by the *trp*-like (*trpl*) gene (Phillips et al., 1992; Niemeyer et al., 1996; Reuss et

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al., 1997). As in vertebrate photoreceptors, mutations in many of the genes encoding elements of the transduction cascade result not only in defects in phototransduction, but also in retinal degeneration (reviewed by Pak, 1995; O'Tousa, 1997), though the reasons are at best only incompletely known. Despite differences in the transduction cascades of vertebrates and invertebrates, mutations in homologous genes, including rhodopsin, arrestin, and PLC, result in degeneration in both cases (Stark et al., 1989; Dryja et al., 1990; Leonard et al., 1992; Dolph et al., 1993; Fuchs et al., 1995; Rao et al., 1995).

Probably the most severe *Drosophila* retinal degeneration mutant is the retinal degeneration A (*rdgA*) mutant. Unlike most other such mutants, degeneration in *rdgA* is light independent and, in severe alleles, already advanced at the time of eclosion (Hotta and Benzer, 1970; Harris and Stark, 1977; Johnson et al., 1982; Matsumoto et al., 1988). There is also virtually no response to light. The *rdgA* gene encodes an eye-specific diacylglycerol kinase (DGK) (Masai et al., 1993), which controls cellular levels of diacylglycerol (DAG) and its metabolic fate by phosphorylating DAG to phosphatidic acid (PA) (reviewed by Topham and Prescott, 1999). A recently discovered novel DGK isoform (DGK_ι) expressed in human brain and retina has 49% identity to *rdgA* and maps to a known locus of inherited retinitis pigmentosa (Ding et al., 1998).

Until recently, the major role of DAG in *Drosophila* phototransduction was presumed to be in the activation of a protein kinase C (PKC) isoform encoded by the *inaC* gene, which is required for response inactivation and light adaptation (Smith et al., 1991; Hardie et al., 1993b). In principle, therefore, genetic elimination of DGK might lead to excess DAG and overstimulation of PKC. However, DAG levels are reportedly normal in *rdgA* mutants (Inoue et al., 1989), and degeneration was not rescued in the double mutant *rdgA;inaC* (Masai et al., 1997). DGK is also important in PI recycling (Figure 1); other mutations in this pathway (CDP diacylglycerol synthase [*cds*] and *rdgB*; see Figure 1) also lead to degeneration; however, this is later in onset and light dependent, suggesting a different pathogenesis (Rubenstein et al., 1989; Sahly et al., 1992; Vihtelic et al., 1993; Wu et al., 1995). A new and potentially more important role for DAG was suggested by the recent finding that the light-sensitive channels TRP and TRPL could be activated by polyunsaturated fatty acids (PUFAs) (Chyb et al., 1999), which themselves could, in principle, be released from DAG. If this is the case, DGK might be a strategic enzyme in controlling the availability of DAG for excitation.

In the present study, we have analyzed the *rdgA* mutation using a combination of genetics, electrophysiology, and light and electron microscopy. Our key findings were that the light-sensitive channels were constitutively active and that degeneration was largely rescued in *rdgA;trp* double mutants also lacking the Ca^{2+}-permeable, light-sensitive TRP channels. In the genetically rescued photoreceptors, the response to light was restored but showed a severe defect in deactivation. The results are consistent with the recent proposal that excitation
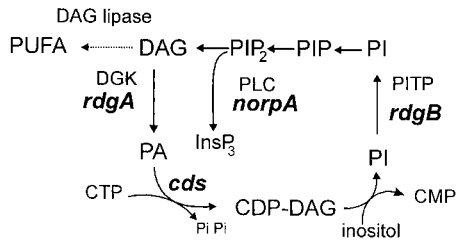
Figure 1. Lipid Signaling Pathways in *Drosophila* Photoreceptors

PLC encoded by the *norpA* gene hydrolyzes phosphatidyl inositol 4,5 bisphosphate (PIP_2) to generate $InsP_3$ and DAG. DGK phosphorylates DAG to PA, and *cds* adds cytidine diphosphate (CDP) to form CDP-DAG, which is the activated donor for inositol, forming PI via PI synthase. PI is transported from the endoplasmic reticulum to the plasma membrane via a PI transport protein (PITP) encoded by the *rdgB* gene and then serially phosphorylated to regenerate PIP_2. In many cells, PUFAs can be released from DAG via the action of DAG lipase. It is not known whether this pathway exists in *Drosophila* photoreceptors; however, PUFAs are potent activators of the light-sensitive channels (Chyb et al., 1999).

in *Drosophila* photoreceptors may be mediated by the DAG branch of the PI pathway and suggest that degeneration in *rdgA* is caused by uncontrolled Ca^{2+} influx via the TRP channels during a critical time window of pupal development.

Results

Electrophysiological Phenotype of *rdgA*

Severe loss-of-function *rdgA* alleles have been reported to be virtually unresponsive to light when measured using the electroretinogram (ERG) (Harris and Stark, 1977) but have not previously been investigated at the single cell level. In the present study, we investigated the most severe allele reported, *rdgA^BS12*, a null or near null mutation with a nonsense mutation in the C-terminal and less than 5% of normal DGK activity (Inoue et al., 1989; Masai et al., 1993). We also examined another severe allele (*rdgA^P35*) that has not, however, been characterized at the molecular level; we found it to be similar in all respects, but data are only presented for *rdgA^BS12*. In neither allele was any response to light detected in whole-cell recordings, even with the brightest flashes, which would normally saturate the wild-type (wt) response.

The severe nature of the *rdgA* mutation was immediately obvious from the appearance of dissociated ommatidia prepared for recording. wt adult ommatidia have a robust and rigid appearance, probably due to a well-developed cytoskeleton. By contrast, *rdgA^BS12* ommatidia are smaller and less robust in appearance, resembling, at least superficially, ommatidia prepared from pupae. Under whole-cell voltage-clamp conditions, further similarities with pupal photoreceptors were apparent. First, cell capacitance was only 8–15 pF compared with ~50 pF in wt adults (Figure 2) and similar to values recorded in pupae at 70%–75% of pupal development (Hardie et al., 1993a; see also Figure 6). This is indicative of greatly reduced membrane surface area, due to the near absence of microvilli. Second, adult *Drosophila* photoreceptors express at least two classes of K channels at high density: delayed rectifier channels and rapidly inactivating A channels encoded by the *Shaker*
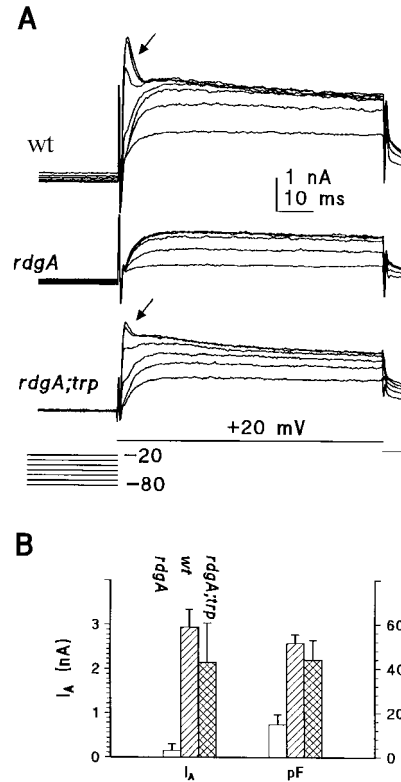

Figure 2. Whole-cell Voltage-Gated K^+ Current Profiles in Adult wt, *rdgA*, and *rdgA;trp^343* Photoreceptors

A voltage step to +20 mV was preceded by a series of hyperpolarizing prepulses in 10 mV steps. In wt (−20 to −80 mV), these progressively removed inactivation of first delayed rectifier (I_{Ks}) and then rapidly inactivating *Shaker* (I_A, arrow) channels. In *rdgA* (−20 to −60 mV), I_{Ks} appeared normal, but there was virtually no indication of I_A. I_A was restored to near wt values in *rdgA;trp^343* (−30 to −80 mV). The somewhat different appearance of the currents is due to a shift in voltage dependence to more negative values, which is also found in wt cells after rundown (Hardie, 1991a).

(B) Peak amplitudes of I_A, determined by subtracting a trace in which inactivation of I_A had not been removed (e.g., −30 mV prepulse in wt profile) from one in which inactivation had been fully removed (prepulse to −80 mV), and cell capacitance in wt, *rdgA*, and *rdgA;trp^343* adult photoreceptors.

gene. *Shaker* channels are normally first expressed late in pupal development, generating currents in excess of 2 nA by the time of eclosion (Hardie, 1991a). In adult *rdgA* photoreceptors, although delayed rectifier currents were normal, there was virtually no sign of *Shaker* channels in whole-cell currents (Figure 2).

The most significant phenotype detected under whole-cell voltage clamp was the presence of a small (~50 pA), constitutively active inward current, characterized by high-frequency noise, which was detected immediately on establishing the whole-cell configuration in all *rdgA* photoreceptors recorded from (n = 45). This constitutive activity appeared to be a specific feature of the *rdgA* mutation and has not been observed in a wide range of other mutants. Among others, these include two other retinal degeneration mutants (*rdgB* and *rdgC*); null mutants of rhodopsin (*ninaE*), which have similar severe defects in rhabdomere morphogenesis;

and *InaD¹* (B. Minke, personal communication), which is a null mutant of the INAD "scaffolding" protein that binds elements of the phototransduction cascade into a supramolecular complex (reviewed by Scott and Zuker, 1998a).

Several lines of evidence indicated that the constitutive current was mediated by the light-sensitive TRP conductance. First, it was blocked by micromolar concentrations of La³⁺, which blocks the *trp*-dependent light-sensitive current (Hochstrate, 1989; Hardie and Minke, 1992; Niemeyer et al., 1996). The current also showed a marked voltage-dependent inhibition by both Ca²⁺ and Mg²⁺ (data not shown) similar to that reported for TRP channels (Hardie and Minke, 1994b; Hardie and Mojet, 1995). Second, the voltage-dependent properties closely resembled those of the TRP conductance determined in the *trpl* mutant, displaying similar rectification (Figure 3B) and time-dependent voltage relaxations (data not shown). Third, the current showed high-frequency noise characteristics indistinguishable from those of the TRP channels isolated in the *trpl* mutant, power spectra being fitted by single Lorentzians with time constants of 0.4–0.6 ms (Figure 3C). No other constitutive or leak currents were detected in these cells; as shown in Figure 3, the current–voltage (I–V) relationship and power spectrum of the component blocked by La³⁺ appeared indistinguishable from the *trp*-dependent conductance in *trpl* photoreceptors, and after La³⁺ block, the input resistance (typically >2 GΩ) was at least as high as in dark-adapted wt cells.

Early Onset Degeneration in *rdgA* Is Rescued by the *trp* Mutation

TRP channels are highly permeable for Ca²⁺ (Hardie and Minke, 1992; Reuss et al., 1997), suggesting that Ca²⁺ influx via the constitutively active channels might be responsible for degeneration. To test this, we asked whether degeneration in *rdgA* could be rescued by mutations in the *trp* gene. Double mutants (*rdgA;trp*) were generated using *rdgA^BS12* and three different *trp* alleles: *trp³⁰¹*, a severe mutant that expresses only ∼5% functional protein (Niemeyer et al., 1996); *trp^CM* (Cosens and Manning, 1969), a temperature-sensitive allele that appears functionally null when reared at 25°C but that generates substantial levels of functional protein at 19°C (Pollock et al., 1995); and *trp³⁴³*, which is a functional null (Scott et al., 1997). We also generated an *rdgA;trpl* double mutant and an *rdgA;trpl;trp³⁴³* treble mutant. Double mutants using any of the three *trp* alleles resulted in an obvious rescue of the retina of *rdgA* flies. On the day of eclosion, the appearance of the rhabdomeres, when monitored using optical neutralization of the cornea, was similar to that of wt or *trp* controls. Nevertheless, at the electron microscope (EM) level, *rdgA;trp* photoreceptors showed slight defects, with rhabdomeres having somewhat irregular borders and signs of vesiculation, even on the day of eclosion (Figure 4). Significantly, well-developed submicrovillar cisternae (specialized smooth endoplasmic reticulum at the base of the microvilli), which are reported to be absent in *rdgA* (Matsumoto et al., 1988), could be detected in favorable sections of *rdgA;trp* double mutants (data not shown). The residual defects were largely rescued in
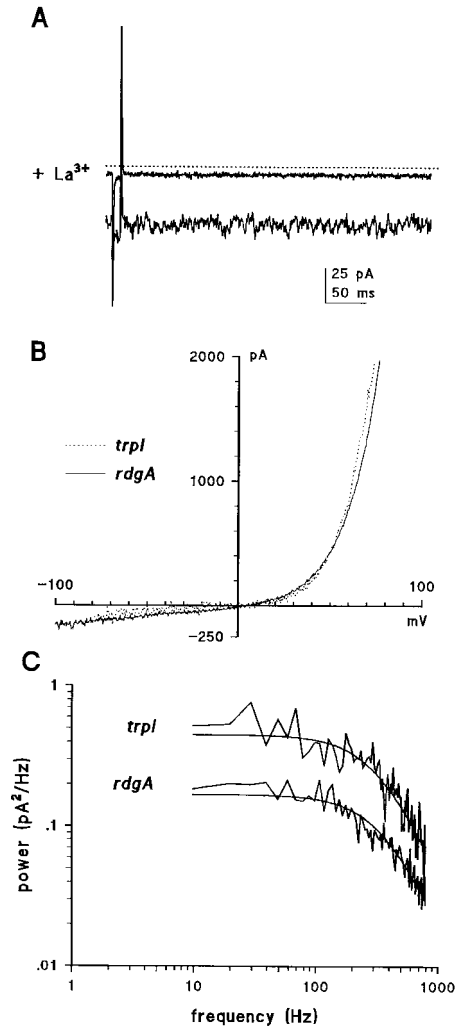

Figure 3. Constitutive Channel Activity in *rdgA*

(A) All *rdgA* photoreceptors exhibited an inward current with high-frequency noise immediately upon establishment of whole-cell recording configuration at a holding potential of −70 mV (n = 45). This current was blocked by 10 μM La³⁺. A 10 mV pulse at the start of each trace monitored resistance (∼1 GΩ before and 3 GΩ after block) and clamp time constant; dotted line, zero current level.

(B) The I–V relationship was determined from voltage ramps, subtracting a template recorded after block by La³⁺. This showed rectification and reversal potential that were similar to those of the light-induced current measured in *trpl* photoreceptors (scaled to overlap). Similar data were obtained from three other cells.

(C) Power spectra determined from steady-state noise of the constitutive channel activity in *rdgA* and from the "rundown current" in a *trpl* photoreceptor; spectra were fitted by single Lorentzians with time constants of 0.43 ms (*rdgA*) and 0.47 ms (*trpl*). Similar data were obtained from four other cells.

rdgA;trpl;trp³⁴³, which had an essentially wt appearance on the day of eclosion. In confirmation of earlier studies, photoreceptors in *rdgA^BS12* were largely degenerate at the time of eclosion (Figure 4B), as were photoreceptors in *rdgA^BS12;trp^CM* reared at the permissive temperature of 19°C (Figure 4C). The rescue was specific to the more Ca²⁺-permeable TRP channels, since no rescue was observed in *rdgA;trpl* double mutants lacking the second class of light-sensitive channel. A similar rescue
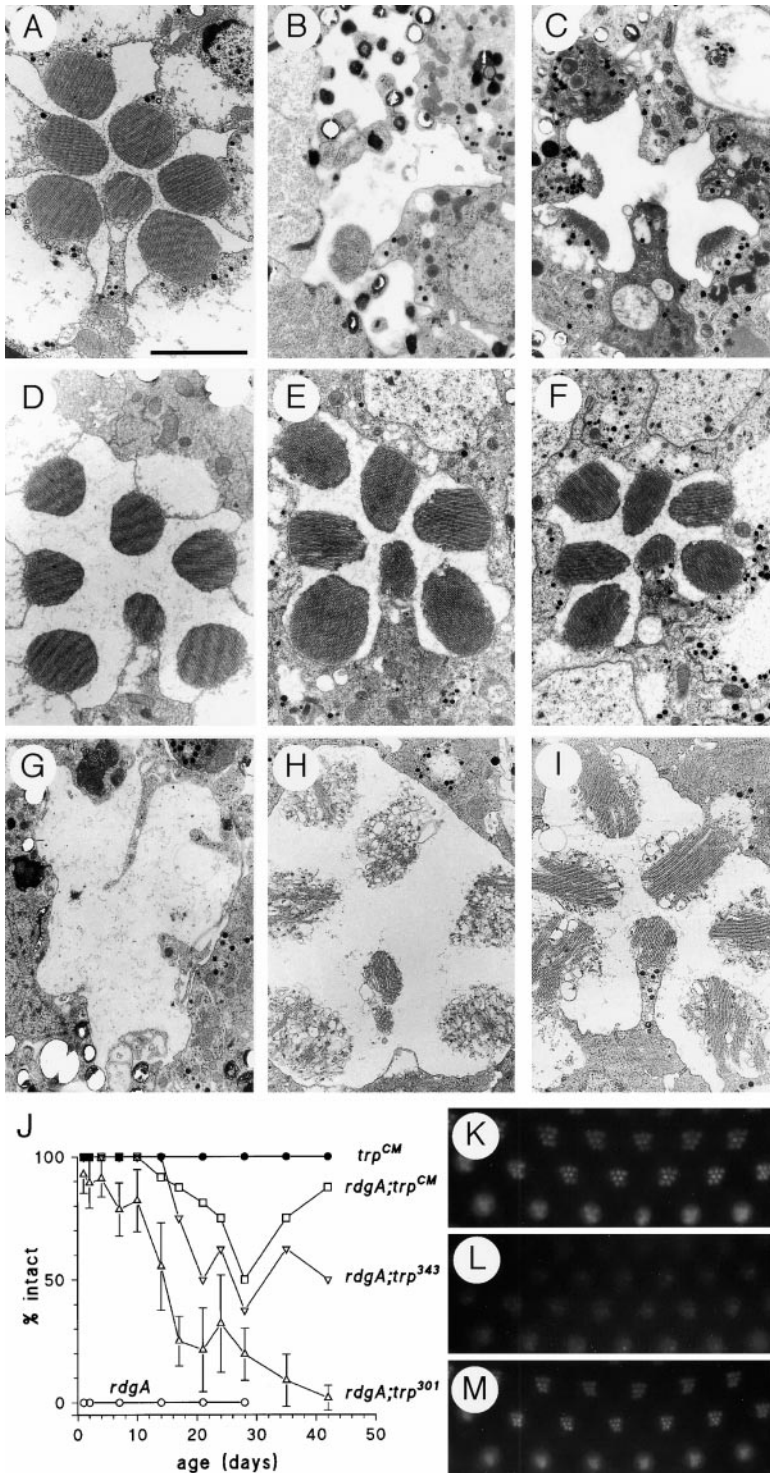
Figure 4. Structural Rescue of Degeneration in *rdgA;trp*

(A–I) EMs showing distal cross-sections of ommatidia at the level of the nuclei. wt flies (A) are characterized by well-formed and regular rhabdomeres. Scale bar, 2 μm. On day of eclosion (B–F), the rhabdomeres are completely absent in *rdgA*[BS12] (B); in *rdgA;trp*[CM], degeneration was almost as advanced when reared at the permissive temperature of 19°C (C), but structure was largely restored when reared at 25°C (F). Rhabdomeric structure on day 1 was also restored by other *trp* alleles, i.e., in *rdgA;trp*[343] (E) and *rdgA;trp*[301] (data not shown), but incipient signs of degeneration, including rough borders and/or vesiculation of the rhabdomeres, were observed. These signs were not seen in day 1 *rdgA;trpl;trp*[343] flies (D). In 28-day-old double mutants (G–I), degeneration had progressed further, being most severe using the weakest *trp* allele (*rdgA;trp*[301] (G), while rhabdomeres, albeit strongly vesiculated, were still visible in *rdgA;trp*[343] (H), *rdgA;trp*[CM] (I), and *rdgA;trpl;trp*[343] (data not shown).

(J–M) The time course of degeneration was followed using optical neutralization of the cornea (e.g., *trp*[CM] control [K], *rdgA*[BS12] [L], and *rdgA;trp*[CM] [M] reared at 25°C, all viewed on day 1). (J) shows the proportion of R1–R6 rhabdomeres still intact as a function of age in control (*trp*[CM]), *rdgA*[BS12], and the three double mutants. Data for *rdgA;trp*[301] were obtained by counting the proportion of R1–R6 rhabdomeres visible in each fly (expressed as mean ± SD, n = 8 flies for each age point). In *rdgA;trp*[CM] and *rdgA;trp*[343], the proportion of flies (n = 8–20 for each age point) still showing visible patterns is plotted (see Experimental Procedures). It should be noted, however, that the clarity of the patterns also deteriorated with age, correlating with the increased vesiculation seen in the EM.

was also obtained using an alternative *rdgA* allele (*rdgA*[P35];*trp*[301]; data not shown).

In older *rdgA;trp* double mutants, there was a further, allele-dependent deterioration of photoreceptor structure over a time period of 2–6 weeks. The most severe degeneration was observed using the weakest *trp* allele tested, i.e., in *rdgA;trp*[301]. Rhabdomere patterns viewed under optical neutralization disappeared within 14–28 days, and loss of virtually all microvillar structure by this time was confirmed by EM (Figure 4G). By contrast, most *rdgA;trp*[CM] and *rdgA;trp*[343] eyes still had discernible rhabdomeres after 4 weeks, although they became more difficult to resolve with optical neutralization and, at the EM level, showed increasing signs of vesiculation (Figures 4H and 4I). Unlike the minor defects on the day of eclosion, this slow degeneration was not prevented in

the *rdgA;trpl;trp³⁴³* treble mutant, the appearance of which at 4 weeks was now similar to that of *rgdA;trp³⁴³* and *rdgA;trp^CM* (data not shown).

We also examined genetically rescued flies using whole-cell recording techniques. The appearance of dissociated ommatidia from newly eclosed *rdgA;trp* flies (all alleles) now closely resembled wt. Furthermore, the "juvenile" electrophysiological features characteristic of *rdgA* photoreceptors, namely low capacitance and absence of *Shaker* channels, were restored to near wt levels (Figure 2). However, despite the nearly complete rescue of acute early degeneration, all photoreceptors still had constitutively activated channels, with currents at least as large as in *rdgA* alone. In *rdgA;trp³⁰¹*, the I–V relationship and block by La³⁺ indicated that these represented residual functional TRP channels (data not shown). In *rdgA;trp^CM* and *rdgA;trp³⁴³*, the channels appeared to be TRPL channels. They were not blocked by La³⁺, their I–V relationship closely resembled that of the light-induced current in *trp* mutants, and the noise power spectra were concentrated at somewhat lower frequencies and could be fitted by the sum of two Lorentzians with time constants of ∼2 and 0.2 ms, as previously reported for TRPL channels (Figure 5). Finally, the current was eliminated by the *trpl* mutation, i.e., in *rdgA;trpl;trp³⁴³* treble mutants, which no longer showed any constitutive activity (Figure 5).

Development of Constitutive Activity and Degeneration

The finding that *trp* mutations rescued early degeneration in *rdgA* suggested that Ca²⁺ influx via these channels might trigger degeneration. However, the constitutive activity of TRPL in *rdgA;trp* questions this interpretation, since they also mediate substantial Ca²⁺ fluxes (Peretz et al., 1994; Hardie, 1996). Since photoreceptors in *rdgA* show severe signs of degeneration on the day of eclosion, we wondered whether the crucial trigger for degeneration might occur at an earlier pupal stage and, if so, whether at this stage only *rdgA*—and not *rdgA;trp*—had begun to exhibit constitutive channel activity. To test this, we recorded from pupal photoreceptors starting at 70 hr postpuparium formation, which is about 10 hr before photoreceptors first develop any sensitivity to light (Hardie et al., 1993a), up to the time of eclosion (100 hr), monitoring constitutive channel activity and independent indicators of the course of degeneration, namely cell capacitance and *Shaker* channel activity (Figure 6).

In *rdgA* flies, constitutive activity was first noticed in photoreceptors from ∼75-hr-old pupae; in such cells, the activity consisted of random channel openings that could be blocked by La³⁺. By 80 hr, all cells showed significant maintained levels of spontaneous channel activity, increasing gradually to ∼50 pA by 85 hr, this level being maintained until eclosion. By contrast, in *rdgA;trp³⁴³*, the first consistent signs of constitutive channel activity were seen considerably later, at 85–90 hr (Figure 6A). No constitutive activity was detected at any pupal stage in wt or *trp* controls (n > 50).

The onset of constitutive activity in *rdgA* coincided with the first signs of degeneration. Up to ∼73 hr, microvillar growth in *rdgA*, as monitored by cell capacitance,
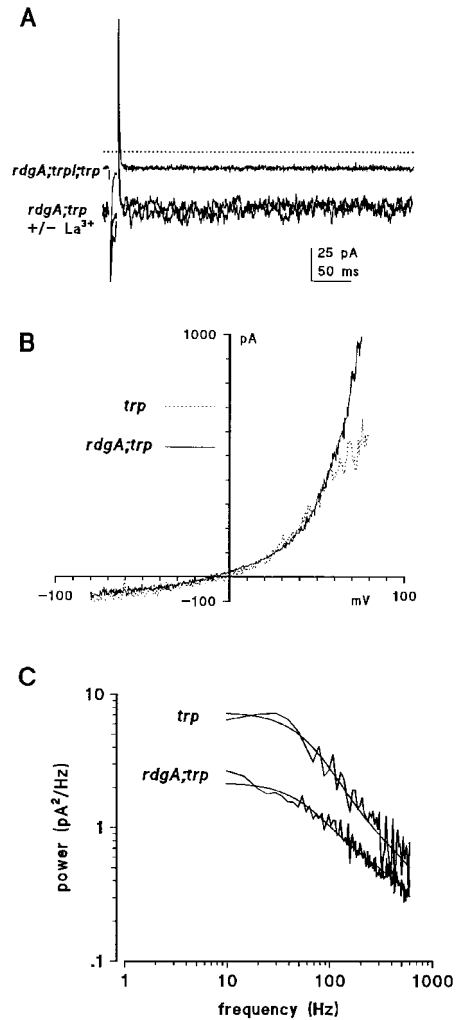

Figure 5. Constitutive TRPL Activity

All adult *rdgA;trp³⁴³* photoreceptors still exhibited a noisy inward current upon establishing the whole-cell configuration (n = 8). This current was not blocked by 10 μM La³⁺ but was eliminated by the *trpl* mutation (i.e., in the *rdgA;trpl;trp³⁴³* treble mutant, upper trace). (B) The I–V relationship, determined as in Figure 3, closely resembled that of the light-induced current in *trp* photoreceptors, which is mediated by TRPL channels (Niemeyer et al., 1996; Hardie et al., 1997).

(C) Power spectra of steady-state noise in *rdgA;trp³⁴³* compared with the rundown current in a *trp* photoreceptor (data from Hardie and Minke, 1994a). Both spectra have been fitted with the sum of two Lorentzians (Equation 1) with time constants of 2.3 ms and 0.2 ms (*trp*), and 2.3 ms and 0.23 (*rdgA;trp*).

had advanced to values indistinguishable from those of wt or *rdgA;trp³⁴³*; however, after 75 hr, capacitance failed to show significant further increases in *rdgA*, even appearing to decrease slightly between 75 and 85 hr. By contrast, in *rdgA;trp³⁴³*, as in wt or *trp*, cell capacitance continued to increase, reaching ∼35 pF by 85 hr and approaching adult wt values (40–60 pF) at the time of eclosion. At no stage during *rdgA* development was there significant functional expression of photoreceptor *Shaker* channels, while *rdgA;trp³⁴³* followed the wt pattern, with first expression at ∼80 hr, increasing rapidly to
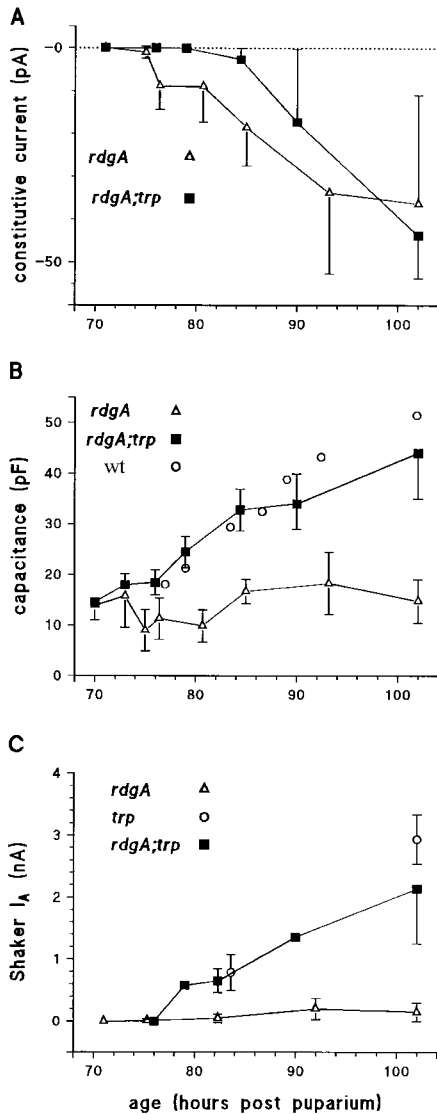
Figure 6. Time Course of Degeneration and Constitutive Activation during Pupal Development

(A) In *rdgA*, constitutive TRP channel activity was first observed at ~75 hr; however, in *rdgA;trp^343^*, spontaneous TRPL channel activity was first observed 10 hr later.

(B) Capacitance increase during development represents growth of the microvilli. In *rdgA*, growth appeared normal up to 70–75 hr (confirmed by EM; data not shown) but failed to show significant further increase. In *rdgA;trp^343^*, capacitance increased normally, in line with wt values (open circles; data from Hardie et al., 1993a).

(C) In controls (*trp*), *Shaker* channel currents (I_A, peak current at +20 mV; see Figure 3) first became detectable between 75 and 80 hr and increased to ~2–3 nA by the time of eclosion. In *rdgA* photoreceptors, I_A currents reached ~300 pA at most and often could not be detected at all. Normal developmental expression of I_A was restored in *rdgA;trp^343^*. Data are expressed as mean ± SD of four to twelve cells for each point. Data without SD bars represent means from only two cells.

values in excess of 2 nA (Figure 6). By contrast, delayed rectifier channels, which appear much earlier than *Shaker*, were expressed at approximately normal levels in *rdgA* (see Figure 2).

Taken together, these results suggest that Ca^{2+} influx

via constitutively active TRP channels may trigger degeneration at ~75 hr of pupal development in *rdgA*, presumably corresponding either to the first insertion of functional TRP channels into plasma membrane or to the expression of an enzyme or other protein essential for their activation. The *trp* mutation may prevent this by eliminating the more Ca^{2+}-permeable of the two light-sensitive channels and by delaying the onset of any constitutive channel activity beyond a critical period of development.

DAG Kinase Is Required for Response Termination

The onset of degeneration in *rdgA* at ~75 hr of pupal development precedes the development of the first responsiveness to light, which occurs at 80–85 hr (Hardie et al., 1993a). Possibly as a consequence of this, *rdgA* photoreceptors never become responsive to light, precluding analysis of a possible role of DGK in phototransduction. It was therefore particularly interesting to discover that *rdgA;trp* photoreceptors not only were structurally rescued, but also had recovered sensitivity to light. Responses, however, showed specific defects, suggesting a direct role for DGK in phototransduction.

Sensitivity in newly eclosed adult *rdgA;trp* photoreceptors, estimated from the intensity required to generate peak responses of similar amplitude, was reduced by ~3 log units compared with *trp* controls (Figure 7A). In addition, response termination was greatly slowed, responses decaying exponentially with a time constant of ~8 s (cf. ~20 ms in wt or 50 ms in *trp*). A possible explanation for the reduction in sensitivity is the background of constitutive channel activity and concomitant Ca^{2+} influx, which mediates the reduction in gain associated with adaptation. Ca^{2+} influx can be eliminated by recording in Ca^{2+}-free bath, and under these conditions, sensitivity in *rdgA;trp* was increased ~10-fold (data not shown). However, since the TRPL channels themselves are inhibited by Ca^{2+} (Reuss et al., 1997; Scott et al., 1997), removing Ca^{2+} also resulted in a large increase in constitutive channel activity, again precluding a meaningful comparison with *trp* controls.

As an alternative strategy, we identified a time window during pupal development (82–84 hr) when constitutive channel activity had yet to develop significantly in *rdgA;trp* but when the cells had already become competent to respond to light. Strikingly, peak responses of *rdgA;trp^343^* during this time window were now *increased* 2- to 3-fold with respect to *trp^343^* controls using the same intensity (Figure 7). This increase in sensitivity could be attributed to a specific defect in response termination, since there was no significant difference in the initial rate of rise of the responses; rather, the enhanced sensitivity was a consequence of responses in *rdgA;trp* continuing to rise longer. A severe defect could also be observed in the slow recovery of the responses to baseline, responses decaying with time constants of ~2 s in *rdgA;trp^343^*, almost 10-fold longer than those measured in *trp^343^* controls (Figure 7C).

Whole-cell recordings can only be readily made from adults within a few hours of eclosion; thus, sensitivity to light at later times was monitored using the ERG. Figures 7D and 7E summarize the results: in brief, *rdgA;trp* flies remained competent to respond as long
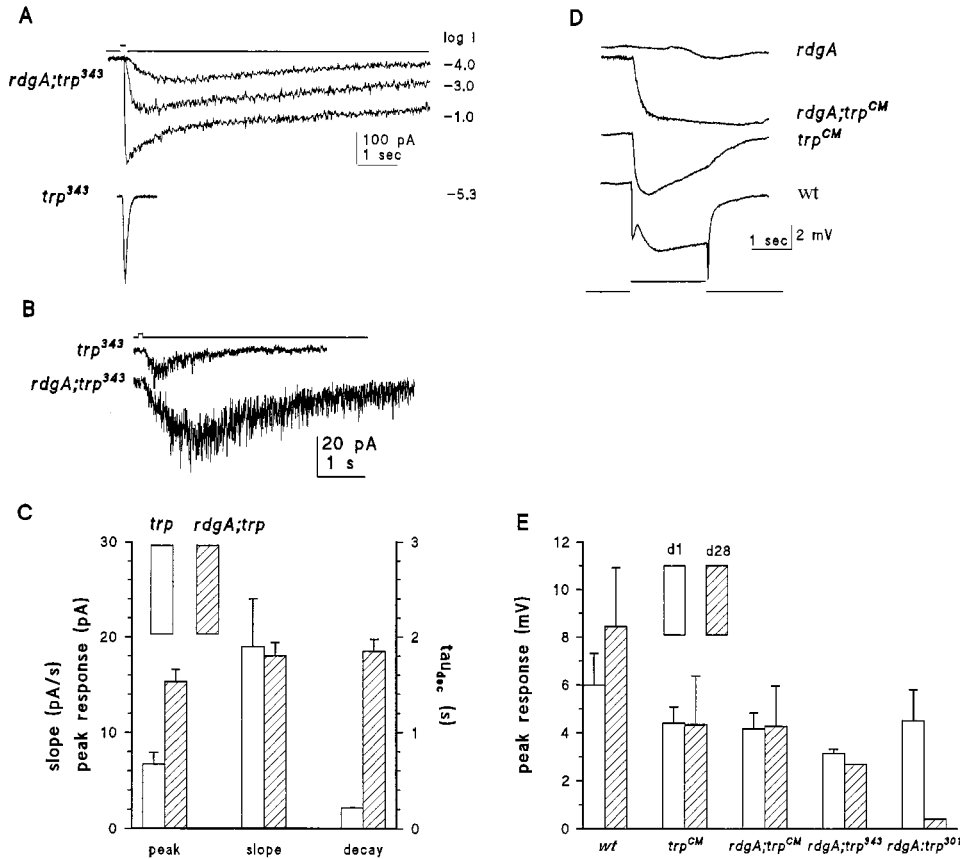
Figure 7. Light Responses in *rdgA;trp*

(A) Responses to 50 ms flashes of increasing intensity recorded in an adult (2 hr posteclosion) *rdgA;trp³⁴³* photoreceptor. At all intensities, there was an extremely slow recovery to baseline. A single response from a control *trp³⁴³* photoreceptor elicited with lower intensity is shown for comparison (log 0.0 corresponds to approximately 3×10^8 effectively absorbed photons per flash in a wt photoreceptor).

(B) Responses from 83-hr-old pupae. The same intensity now elicited larger responses in *rdgA;trp³⁴³* than in the *trp³⁴³* control at the same age. The response also peaked later and decayed more slowly in *rdgA;trp³⁴³*.

(C) Mean (±SD) data summarizing peak amplitudes, slope of initial rising phase, and decay time constant (first order exponential fitted to decaying phase of response) from six *trp³⁴³* and twelve *rdgA;trp³⁴³* photoreceptors from pupae between 82 and 84 hr, stimulated with the same intensity. There was no significant difference in the slope; however, peak amplitude was less than two times greater in *rdgA;trp³⁴³*, and the decay to baseline approximately ten times slower.

(D) Representative ERGs from wt, *trp^CM*, *rdgA;trp^CM*, and *rdgA* (1-day-old flies). Notice the marked deactivation defect in *rdgA;trp*.

(E) Peak ERG response (mV) to a 2 s flash of subsaturating intensity in 1-day-old (d1) flies and 28-day-old (d28) flies. Robust responses were maintained in *rdgA;trp^CM* and *rdgA;trp³⁴³*, but in *rdgA;trp³⁰¹*, there was virtually no response after 28 days. Data are expressed as mean ± SEM for n = 2–7 flies for each data point.

as their retinae were intact, i.e., *rdgA;trp³⁰¹* gave robust responses for up to 2 weeks, and *rdgA;trp³⁴³* for at least 4 weeks. The ERG results confirmed the whole-cell recordings, in that the most obvious phenotype was an extremely slow response termination. Peak responses in *rdgA;trp* to the same intensity (subsaturating) flashes in newly eclosed flies differed little from *trp* controls, although the slope of the rising phase of the ERG was reduced by approximately 2- to 3-fold (data not shown).

Discussion

rdgA is probably the most severe retinal degeneration mutant in *Drosophila*, but the mechanism of degeneration is unknown. A key finding of the present study was that the light-sensitive TRP channels were constitutively active in *rdgA*. These channels are highly permeable to

Ca²⁺, and the resulting uncontrolled Ca²⁺ influx represents an obvious candidate for the primary cause of degeneration. This was supported by the rescue of the degeneration phenotype by *trp* mutations. Of particular interest was the finding that responsiveness to light was also restored in *rdgA;trp* double mutants and that the responses exhibited a specific defect in response termination. These results have implications, not only for understanding the mechanism of degeneration, but also for the mechanism of excitation, representing independent genetic evidence in support of the proposal that excitation may be mediated via the DAG branch of the PI pathway.

Implications for Degeneration

While it has long been recognized that degeneration in *rdgA* is light independent (Harris and Stark, 1977) and is

already advanced on the day of eclosion, the underlying mechanisms have remained obscure. The cytotoxicity of Ca^{2+} is well known and is implicated in both apoptosis and necrosis (reviewed by Trump and Berezesky, 1996; Lee et al., 1999); hence, the constitutive activity of Ca^{2+}-selective channels in *rdgA* provides a prime candidate for the initial trigger of degeneration. This is supported by two lines of evidence: first, the onset of constitutive activity at ~75 hr of pupal development coincided with the first detectable indicators of degeneration, namely the failure of significant further microvillar growth or insertion of functional *Shaker* channels into the plasma membrane. Second, genetic elimination of the Ca^{2+}-permeable TRP channel largely rescued this early onset degeneration. In addition, a similar severe form of light-independent early onset degeneration has very recently been reported in an allele of the *trp* gene itself (*trp*[P365]) in which the channels are also constitutively active (Yoon et al., 2000).

It seems unlikely that the lower Ca^{2+} permeability of the TRPL channels alone was a sufficient explanation for rescue from degeneration in *rdgA;trp* since the fractional Ca^{2+} current of TRPL channels is still ~30% of that of TRP channels (Hardie, 1996), and the constitutive currents in adult *rdgA;trp* photoreceptors were often larger than those in the *rdgA* mutants. We suggest instead that the delayed onset of the constitutive activity of the TRPL channels in *rdgA* may be the more significant factor. Adult photoreceptors tolerate massive and maintained light-induced Ca^{2+} influx, which would be toxic in most cell types. It seems plausible that the mechanisms that confer tolerance to Ca^{2+} influx may develop in parallel with the rest of the phototransduction machinery only during the last 20 hr of pupal development. This would explain why only Ca^{2+} influx prior to this period is capable of inducing the severe degeneration characteristic of the *rdgA* phenotype. We also note that when tested, the constitutive activity in *rdgA* appeared to be more or less completely blocked by La^{3+}, implying that there was no concomitant activity of TRPL channels. This suggests that the early onset of degeneration in *rdgA* may prevent incorporation of functional TRPL protein into the developing photoreceptor, much as appears to be the case for the *Shaker* channels.

Degeneration in *rdgA* was not completely rescued by the *trp*, and it remains unclear what underlies the residual degeneration in *rdgA;trp*. Constitutive activity of TRPL channels may be responsible for the minor defects seen on the day of eclosion, as these were largely rescued in *rdgA;trpl;trp*; however, this cannot account for the progressive slower degeneration, which was not rescued in the treble mutant. Possibilities might include hyperactivation of PKC or a disturbance in the phospholipid composition of the microvillar membrane.

Implications for Phototransduction
The mechanism of activation of the light-sensitive channels is the major unanswered question in *Drosophila* phototransduction and may have implications for the mechanism of activation of the large family of vertebrate TRP homologs (reviewed by Birnbaumer et al., 1996; Kiselyov and Muallem, 1999). Hence, the identification of a mutation that results in their spontaneous activation

is of unusual interest. Phototransduction in *Drosophila* is dependent upon PLC since severe mutations in the *norpA* gene, which encodes eye PLC, are blind (Pak et al., 1976; Bloomquist et al., 1988). Until recently, it was generally assumed that excitation was mediated by $InsP_3$, as is believed to be the case, e.g., in the Limulus ventral photoreceptor. However, in *Drosophila*, the only evidence in support of this, such as a recently reported correlation between light-induced Ca^{2+} release and sensitivity to light in the *trp* mutant (Cook and Minke, 1999), is indirect. By contrast, a mutation in the $InsP_3$ receptor was reported to have no detectable effect on phototransduction (Acharya et al., 1997), and $InsP_3$ appears not to be capable of mimicking excitation (Hardie and Raghu, 1998).

Recently, we demonstrated that arachidonic acid and other PUFAs could activate both TRP and TRPL channels in *Drosophila* photoreceptors, as well as recombinant TRPL channels expressed in cell lines (Chyb et al., 1999). This suggested that excitation may be mediated via the DAG branch of the PI pathway since PUFAs can be released from DAG via DAG lipase. Eukaryotic genes for DAG lipase have yet to be cloned; however, this enzyme is believed to play important signaling roles in a number of vertebrate cells by releasing arachidonic acid (reviewed by Topham and Prescott, 1999), which in some cases can also activate cation channels in the plasma membrane (Van der Zee et al., 1995; Shuttleworth, 1996; Broad et al., 1999). Assuming there is some basal production of DAG in the dark, the lack of excess DAG in *rdgA* suggests that DAG may be metabolized by alternative pathways. Diversion of DAG metabolism to generate PUFAs rather than PA (see Figure 1) would provide a working hypothesis for understanding how the *rdgA* mutation can lead to constitutive activity of the light-sensitive channels and subsequent degeneration.

While they cannot be excluded, other possible consequences of the *rdgA* mutation seem unlikely to account for the constitutive activity. First, by metabolizing DAG, DGK might be expected to lower the activity of PKC, which might thus be hyperactivated in *rdgA*. However, the eye PKC mutant *inaC* itself has a defect in response inactivation—exactly the opposite of what would be required to be consistent with the *rdgA* phenotype. DGK also generates PA, which could be hypothesized to play an inhibitory role in phototransduction; however, incorporation of up to 40 μM PA into photoreceptors via the patch pipette had no discernible effect on the wt light response or on the level of constitutive activity in *rdgA* mutants (data not shown). We also considered the possibility that constitutive activity might result from a nonspecific effect of defective rhabdomere morphogenesis and/or delocalization of the channel proteins from the microvilli. This seems unlikely, as constitutive activity (though now of TRPL channels) was also a characteristic feature of *rdgA;trp*[343] photoreceptors in which rhabdomere morphogenesis was restored to near normal. By contrast, null *ninaE* mutants, which show a degree of microvillar disorganization similar to that of *rdgA*, were never found to have constitutively active channels; neither was any such phenotype reported in another mutant with defective rhabdomere morphogenesis, *chaoptin* (Scott and Zuker, 1998b). Finally, no constitutive activity

has been detected in mutants of the INAD scaffolding protein, in which TRP protein is found throughout the plasma membrane (Chevesich et al., 1997; Tsunoda et al., 1997), demonstrating that localization of the channels to the microvilli and/or incorporation into the INAD signaling complex is not required to prevent constitutive activity.

The ability to rescue degeneration in *rdgA* by genetic elimination of the TRP Ca²⁺ influx channel provides evidence for the suggestion that this constitutive Ca²⁺ influx is causal for the severe degeneration seen in *rdgA*. In addition, the structural rescue was associated with the restoration of responsiveness to light, making it possible to assess the role of DGK in phototransduction. Unfortunately, adult *rdgA;trp* photoreceptors now had significant levels of constitutively active TRPL channels, complicating a direct comparison with *trp* controls. However, by recording from 80- to 85-hr-old pupae, it was possible to record responses in the absence of significant spontaneous activity, allowing direct comparison with *trp* controls under comparable conditions. At this developmental stage, the *rdgA* phenotype was found to be a specific and pronounced defect in response deactivation, implying a role for DGK in response termination.

In conclusion, the novel aspects of the *rdgA* phenotype revealed in the present study, namely constitutive activity of the light-sensitive channels and defect in response termination, are consistent with a role of DAG in excitation and provide independent genetic evidence in support of the recent proposal that DAG and its downstream metabolites may be messengers of excitation in *Drosophila* photoreceptors (Chyb et al., 1999).

Experimental Procedures

Flies

Experiments were performed on *Drosophila melanogaster* reared at 25°C in the dark. The wt strain was Oregon R. Mutants used included three alleles of *trp*: *trp³⁰¹*, *trpᶜᴹ* (Cosens and Manning, 1969), and *trp³⁴³* (Scott et al., 1997). The *rdgA* allele used, *rdgAᴮˢ¹²*, is the most severe reported allele (Hotta and Benzer, 1970; Masai et al., 1993). Double mutants (*rdgA;trp*) of all three *trp* alleles were generated using standard crosses with appropriate balancers. *rdgA;trpl* double and *rdgA;trpl;trp* treble mutants were generated using the *trpl³⁰²* null allele (Niemeyer et al., 1996). Pupal age was estimated by aging pupae selected at ~75% of pupal development, at which time there are a number of recognizable stages of short duration (Bainbridge and Bownes, 1981; Hardie et al., 1993a). We assumed the following ages for these short-lived stages: p11i (75 hr), p11ii (76 hr), and p12i (77 hr). For earlier stages, we assumed late p9 pupae (dark amber eyes) were 70 hr old, and p10 (red eyes, no bristles) 73 hr. Eclosion was assumed to have occurred at 100 hr postpuparium.

Electrophysiological Recordings and Stimulation

Dissociated ommatidia were prepared as previously described (Hardie, 1991b) and transferred to a recording chamber on an inverted Nikon Diaphot microscope. Cells were whole-cell voltage clamped at −70 mV, unless otherwise stated. Series resistance values were generally below 25 MΩ and were routinely compensated to 80% when measuring currents >200 pA. Data were collected and analyzed using an Axopatch 1-D amplifier and pCLAMP 6 or 8 software (Axon Instruments, Foster City, CA). As previously described (Hardie, 1996), cells were stimulated via a green LED, with a maximum effective intensity of ~10⁵ photons/photoreceptor/s. When higher intensities were required, monochromatic (560 nm) light from a 75 W Xe arc lamp was delivered via the microscope objective. Relative intensities

were calibrated using a photomultiplier and converted to absolute effective intensities by counting quantum bumps at the lowest intensities in wt photoreceptors.

Noise analysis was performed as previously described (Hardie and Minke, 1994a). Briefly, uncompensated steady-state noise was sampled at 2–5 kHz and filtered at 1–2 kHz, and fast Fourier transforms averaged from at least ten nonoverlapping 2048 point samples. Spectra were corrected for the clamp time constant and for recording noise. Power spectra were fitted by single or summed Lorentzian functions:

$$\text{power spectral density} = \qquad (1)$$
$$w_1 \{1/[1 + (2\pi \cdot f\tau_1)^2]\} + w_2 \{1/[1 + (2\pi \cdot f\tau_2)^2]\},$$

where f = frequency, τ_1 and τ_2 time constants (effective channel open times), and $w_1 + w_2$ relative weighting factors.

For ERGs, flies were immobilized in soft wax, and a platinum electrode was positioned in a drop of conducting gel on the surface of the eye, with an indifferent electrode contacting the abdomen again via conducting gel. Illumination was via a DC-regulated 50 W halogen lamp filtered with a 560 nm cutoff filter and Wratten neutral density filters covering 5 log units. Light was delivered to the eye via a liquid light guide positioned 5 mm from the eye.

Solutions

Standard extracellular solution was composed of (in mM): NaCl, 120; KCl, 5; TES, 10; MgCl₂, 4; CaCl₂, 1.5; proline, 25; and alanine, 5; intracellular solution was composed of K gluconate, 140; TES, 10; Mg ATP, 4; MgCl₂, 2; NAD, 1; and Na GTP, 0.4. To measure I–V relations, K channels were blocked by replacing the K gluconate with 115 mM CsCl and 15 mM TEACl. pH of all solutions was adjusted to 7.15.

Optical Neutralization

For observing rhabdomeres in the intact eye, heads were mounted on microscope slides using clear nail varnish and observed under a 40× or, for photography, 63× oil immersion objective using antidromic illumination (Franceschini and Kirschfeld, 1971). To obtain a semiquantitative index of degeneration, we used two approaches, depending on the nature of the degeneration. In *rdgA;trp³⁰¹*, degeneration proceeded such that the number of rhabdomeres visible decreased in an apparently random fashion, allowing a count of the proportion of rhabdomeres visible. In the other genetic backgrounds, the rhabdomeres became progressively and uniformly less well defined until they were no longer visible. For these genotypes, we scored the number of flies showing discernible patterns. By either measure, R1–R6 rhabdomeres were completely degenerated in *rdgA* flies on the day of eclosion (although occasional central, R7 rhabdomeres could be seen), while wt or *trp* controls showed no degeneration under the dark-rearing conditions used, up until at least 6 weeks.

Electron Microscopy

Eyes were prepared for histology by dissecting in ice-cold fixative (2% formaldehyde, 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer [pH 7.3]). After 4 hr fixation at 4°C, eyes were buffer washed, postfixed in 1% OsO₄ (1 hr), and stained en bloc in uranyl acetate (1 hr). Eyes were dehydrated in an alcohol series and embedded in Spurr's. Ultrathin sections (70 nm) were stained with uranyl acetate and lead citrate, and viewed on a Phillips CM100 transmission EM.

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