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Mechanisms of Rhodopsin Inactivation in Vivo as Revealed by a COOH-Terminal Truncation Mutant

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Although biochemical experiments suggest that rhodopsin and other receptors coupled to heterotrimeric guanine nucleotide triphosphate—binding proteins (G proteins) are inactivated by phosphorylation near the carboxyl (COOH)-terminus and the subsequent binding of a capping protein, little is known about the quenching process in vivo. Flash responses were recorded from rods of transgenic mice in which a fraction of the rhodopsin molecules lacked the COOH-terminal phosphorylation sites. In the single photon regime, abnormally prolonged responses, attributed to activation of individual truncated rhodopsins, occurred interspersed with normal responses. The occurrence of the prolonged responses suggests that phosphorylation is required for normal shutdown. Comparison of normal and prolonged single photon responses indicated that rhodopsin begins to be quenched before the peak of the electrical response and that quenching limits the response amplitude.

In rod photoreceptors, photoreceptor inactivation of rhodopsin triggers activation of a G protein that leads to a decrease in the intracellular concentration of guanosine 3',5'-monophosphate (cGMP). Membrane channels gated by cGMP then close, and the rod photoreceptor hyperpolarizes (1). The processes mediating the recovery of the response to light are not well understood. In vitro, rhodopsin's activity can be quenched by the phosphorylation of the COOH-terminus by the retinal enzyme of arrestin (2). It has not been demonstrated, however, that this mechanism causes shutdown in vivo. Furthermore, the kinetics of shutdown in vivo are not known.

To address these questions, we generated transgenic mice that produced a form of rhodopsin in which the COOH-terminal sites that are phosphorylated by rhodopsin kinase (3) or protein kinase C (4) were deleted (Fig. 1A). Studies on the structurally related β-adrenergic receptor indicated that removal of 15 COOH-terminal amino acids from rhodopsin would probably yield a molecule capable of excitation but resistant to shutdown (5). The mouse genomic construct, which contained all coding sequences and introns as well as 5 kb of the 5' and 1.5 kb of the 3' flanking regions, was altered by site-directed mutagenesis to create a stop codon at residue 334 (S334ter) (Fig. 1B). Expression of this transgene was restricted to rods and occurred at the same time as the expression of normal opsin during development of the retina. Because overproduction of normal or modified rhodopsin invariably causes the retina to degenerate (6, 7), we screened several lines of transgenic mice for animals with low amounts of transgene expression. Four of these lines expressed high amounts of

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**Fig. 1. Truncation mutant of rhodopsin.** (A) A model for rhodopsin in the disc membrane (cross-sectional view, each circle representing an amino acid). Sites of rhodopsin kinase phosphorylation in normal rhodopsin are Ser358, Ser369, and Ser363 (23), shown by solid, filled circles. The polypeptide chain in the rhodopsin truncation mutant ended at the site marked by the arrow. (B) Transgene construct. The 11-kb Bam HI fragment of the mouse rhodopsin gene contained 5 kb of the 5' upstream sequence, all coding sequences (indicated by filled boxes) and introns, and 1.5 kb of the 3' downstream sequence. A stop codon was created at amino acid position 334 through site-specific mutagenesis. (TCG → TAA; indicated by the asterisks) in exon five. This mutation also created a Dde I restriction site in the transgene construct. The arrows indicate the positions of the PCR primers used in RT-PCR (5'-GAGGCTTTCATCTAAACC6GGC-3' and 5'-GCCTGAGCCACCTGGTTG-3').

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S334ter rhodopsin. In these animals, rods failed to make an outer segment and degenerated at an early age (8). One line produced relatively low amounts of S334ter and was used for our experiments.

The morphology of retinas in 2-month-old adult animals was indistinguishable from that of normal siblings. After 3 months, some differences became discernible. The thickness of the outer nuclear layer was reduced to ~80% of normal, and the rod outer segments were shorter. The loss of photoreceptors was progressive; by 1 year of age, the S334ter outer nuclear layer was further reduced to ~60% of its normal thickness.

Reverse transcriptase and the polymerase chain reaction (RT-PCR) were used to estimate the relative amounts of mRNA encoding S334ter and normal rhodopsin in the rods (9). The primer sequences for the

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massed electrical activity of the retina. Particular attention was paid to the a-wave because this ERG component is most closely associated with the light-evoked electrical responses of the photoreceptors (12). The cone-mediated ERG was normal (13). The rod-mediated a-waves of transgenic mice were only slightly below the average amplitude of the normals, provided that the interstimulus interval (ISI) was sufficiently large. As the ISI was reduced, responses from normal mice changed little; whereas those of S334ter mice became progressively smaller (Fig. 3, A and B). The a-wave reduction seen in S334ter mice at short ISIs indicates that their rods recovered more slowly from a flash than those of normal mice.

We explored the apparent defect of rod function in S334ter by recording the light-evoked electrical responses of individual rods (14). Electrical responses of a control rod to flashes of several strengths were compared with those of an S334ter rod (Fig. 4A). At dim flash strengths, some S334ter rod responses were abnormally prolonged, whereas at greater flash strengths, all S334ter responses were prolonged. This prolonged recovery after bright flashes provides an explanation for the decrease in the ERG with shorter ISI in S334ter mice. The form of the recovery phase of the flash responses from S334ter rods consisted of a
sequence of step-like transitions. The number of steps, as well as the length of the treads, varied from trial to trial. At the highest flash strength, control rods only occasionally exhibited a single step (Fig. 4A), whereas 22 of 29 transgenic rods behaved similarly to the transgenic cell illustrated. With very bright flashes, recovery in S334ter rods sometimes was interrupted by a stepped secondary rise before the final decline, and late steps typically appeared after the current recovered to base line (Fig. 4A).

S334ter rods were stimulated by dim flashes that photomonomerized approximately one rhodopsin per trial. Many responses were indistinguishable from those of control rods, but some rose monophonically to a plateau with an amplitude nearly twice that of the normal single photon response and remained at that amplitude for up to 30 s (Fig. 4, B and E). This behavior suggests the absence of a key process that restrains and terminates the normal elementary flash response. In successive trials, normal and prolonged responses appeared in an unpredictable sequence, indicating that prolonged responses did not simply reflect a long-term change in the state of the cell. Thus, the staircase during recovery of the response to a moderately bright flash apparently resulted from the summation of multiple prolonged events, each of which shut off at a different time.

The probabilities that a single photon would trigger a prolonged or normal response were estimated. The average response to a large number of dim flashes was decomposed into normal and prolonged components (Fig. 4C). The amplitude of each component in the average, divided by the amplitude of the elementary event (for example, 0.9 pA for normal and 1.3 pA for prolonged, single photon responses in this cell), gave the relative frequencies of occurrence of each type of response. Prolonged responses occurred with a frequency of 0.15 ± 0.05 (mean ± SD); n = three rods from two mice) of that of normal responses. The agreement of this result with the relative amounts of S334ter and normal rhodopsin mRNA and with the estimated amounts of each opsin in the outer segments supports the notion that the prolonged responses arose from photostimulation of truncated rhodopsin.

The rising phase of the normal single photon response was compared with the rising phase of the prolonged response triggered by S334ter rhodopsin (Fig. 4D). The normal and prolonged responses began to rise identically, showing that the COOH-terminal amino acids were not required for rhodopsin to interact with transducin, the G protein found in photoreceptors (15). This is consistent with results
indicating that transducin activation maps to the second and third cytoplasmic loops of rhodopsin (16). The normal response began to diverge from the prolonged response by 100 ms after the flash, demonstrating that even before the normal response had reached its maximum, rhodopsin's catalytic activity was starting to be quenched. Thus, rhodopsin shutoff begins during the rising phase of the flash response.

Prolonged responses had variable durations (Fig. 4E). The experimental distribution was fitted by an exponential with a time constant of 4.5 s. In another rod, the time constant was 4.3 s. The exponential fit suggests that the prolonged responses are terminated by a stochastic, memory-less, first-order transition that quenches catalytic activity abruptly after a mean wait of several seconds. The nature of this reaction is not known. One possibility is inactivation due to phosphorylation at a site at the third cytoplasmic loop (17). Another is that a thermal transition inactivates the catalytically active form, metarhodopsin II (MII). This would require a faster transition than that previously described for formation of the inactive form MII, which occurs in 1 to 2 min at 37°C (18). The prolonged responses of late onset that occurred after bright flashes may represent spontaneous back reactions in a reversible equilibrium (Fig. 4A).

After their rise, prolonged responses showed no diminution in amplitude before termination. This implies that a steady state of intense transducin activation and phosphodiesterase activity was maintained throughout the catalytic lifetime of the photoactivated S334ter molecule. Although the duration of the prolonged responses varied widely, final recovery required less than 0.5 s in each case. Therefore, transducin and phosphodiesterase inactivation must occur within this time, which is consistent with transducin's rate of guanosine triphosphate hydrolysis inferred from microcalorimetry (19).

Prolonged single photon responses were not observed in transgenic mouse rods that later degenerated because of the expression of a mutant rhodopsin lacking the last five amino acids from the COOH-terminus (7). Thus, prolonged responses do not appear to represent a nonspecific effect of rhodopsin truncation or eventual retinal degeneration. Prolonged single photon responses occur in normal monkey rods, but the probability of a prolonged response was estimated to be about 0.1% that of a normal single photon response (20). Our finding that S334ter rhodopsin produces prolonged responses suggests that the COOH-terminus of ~0.1% of the rhodopsin in normal mammalian rod is not available as a substrate for the quenching process. A possible explanation is that, in a small fraction of rhodopsin molecules, the polyepitope is truncated similarly to S334ter by endogenous protease activity or that protein synthesis fails to reach the COOH-terminus of the polyepitope chain. In conclusion, removal of 15 amino acids from the COOH-terminus of rhodopsin produces a molecule that is transported to the rod outer segment and activates transducin in rods that are not shut off properly. Phosphorylation of rhodopsin at the COOH-terminus is presumably essential for rapid termination of the photoreceptor in vivo. These results may be relevant to the function of other receptors that have seven transmembrane helical domains and are coupled to G proteins (21). Rhodopsin mutations that affect the COOH-terminal phosphorylation sites cause autosomal dominant retinitis pigmentosa (22). The S334ter mouse may be useful for studying this blinding human disease.

**REFERENCES AND NOTES**


9. RNA was extracted from both retinas of a normal mouse and a 3-month-old transgenic mouse and reverse-transcribed to cDNA on a hydroxylamine-activated herring sperm nuclei nucleotides. The cDNA was PCR amplified with the primers shown in Fig. 1B. The PCR products obtained were sequenced on an ABI 377 sequencer. The sequences were aligned with the cDNA clone (18) obtained from a dark-adapted overnight, anesthetized with 15 µl of solution per gram of body mass; the mouse was warmed to a heating pad at 30°C. ERGs were recorded with a stainless steel needle contacting the corneal surface through a layer of 1% methylcellulose. A reference electrode was placed in the mouth, and a needle electrode in the tail provided a ground. Flashes of white light, 1 ms in duration, were generated by a GS-2000 (Nicotex, Madison, WI) and presented as diffuse, full-field illumination. For each flash, the mean density function was generated; we delivered flashes at a rate of 2.3 Hz in the presence of a 1.6 log cd m−2 rod-desensitizing field of white light; exposure to the background light began 10 min before the flash presented. The light intensity was set at 4 to 14 log cd m−2, averaged, and stored with a Pathfinder II (Nicolet) signal-averaging system.


15. We found the amplitude and form of the rising phase of the prolonged event by averaging late prolonged events that had been aligned to base line. Before we averaged them, the prolonged events were aligned on the time axis so that the point at which each reached a criterion amplitude of 0.6 pm a coasted. Late events with a duration of less than 500 ms were excluded.

16. We estimated the amplitude of the normal single photon response by dividing the peak ensemble variance of 223 diom flash responses (less the variance introduced by prolonged responses) by the peak amplitude of the ensemble average diom flash response (less the amplitude introduced by the prolonged responses). The average of 13 normal flash responses was then scaled to this estimated amplitude of the single photon response amplitude. The flash delivered 2.5 photons per square micrometer at 500 µm.

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