saturation. After resuspension and dialysis [20 mM tris-HCl (pH 7.5)], the proteins were separated by IEF in the pH range 4 to 6.5 with the RF3 Protein Fractionator (Texas Instruments). Active fractions were pooled, desalted, and exchanged into 20 mM sodium phosphate buffer (pH 7.0) with 0.2 M sodium chloride with the use of Centricon C-30 spin columns (Amicom, Beverly, MA). Gel filtration was done on a 7.8 × 250 mm TSK G3000 SW XL HPLC column (Tosoh Corporation, Japan) in the same buffer. Active fractions were pooled and then fractionated by SDS–polyacrylamide gel electrophoresis. The band corresponding to avenacinase (*10*) was electroeluted and used to immunize Wistar rats. The resulting polyclonal antiserum was used for protein immunoblot analysis and cDNA library screening at a dilution of 1:2000. Control experiments with preimmune serum (diluted 1:100) gave no signal. Rabbit antibody to rat immunoglobulin G alkaline phosphatase conjugate was used as the secondary antibody. A *Gga* cDNA expression library was constructed in lambda gt11 by standard methods [J. M. Chirgwin, A. E. Przybyla, R. J. MacDonald, W. Rutter, *Biochemistry* **18**, 5294 (1979); H. Aviv and P. Leder, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1408 (1972)] and plaques were screened with antiserum [R. Young and R. Davies, *ibid.* **80**, 1194 (1983)]. Immunoreacting plaques were purified and the insert DNA was amplified. All cDNAs isolated in this way cross-hybridized on a Southern blot and were used as probes to isolate further clones from the original cDNA library and also from a *Gga* genomic library constructed in pGM32. A 1.1-kb cDNA clone (pA312) and a 10.6-kb genomic clone (pA3G1) were selected for use in the subsequent experiment. The full DNA sequence of an additional cDNA clone (pA3171), which encompasses pA312, was determined completely for both strands (GenBank accession number U17568). This was achieved by sequencing restriction fragments of pA3171 that had been subcloned into pBluescript II SK (Stratagene, La Jolla, CA) and by use of specific primers to sequence over gaps and restriction sites. DyeDeoxy terminator cycle sequencing and the Model 373 DNA sequencing system (Applied Biosystems, La Jolla, CA) were used.

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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 guanosine 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 shutoff. 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, photoexcitation 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 and the subsequent binding of arrestin (*2*). It has not been demonstrated, however, that this mechanism causes shutoff in vivo. Furthermore, the kinetics of shutoff 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 shutoff (*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 five lines expressed high amounts of

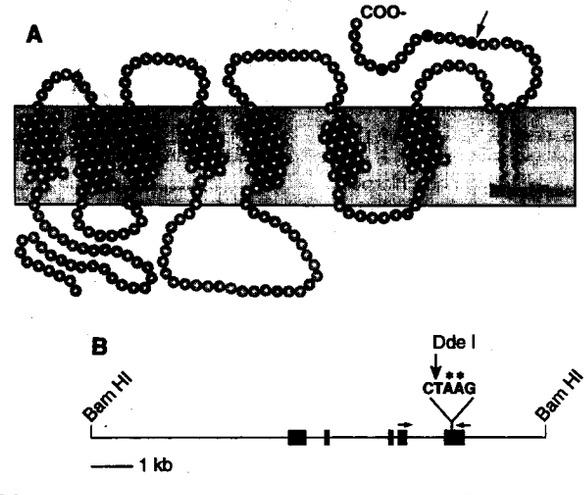

Fig. 1. Truncation mutant of rhodopsin. (**A**) A model for rhodopsin in the disc membrane (cross-sectional view, each circle representing an amino acid). Sites of rhodopsin kinase phosphorylation in normal rhodopsin are Ser^{334}, Ser^{338}, and Ser^{343} (*23*), shown by solid, filled circles. The 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 of two residues (TCC → 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′-GAGCTCTTCCATCTATAACCCGG-3′ and 5′-GGCTGGAGCCACCTGGCTG-3′).

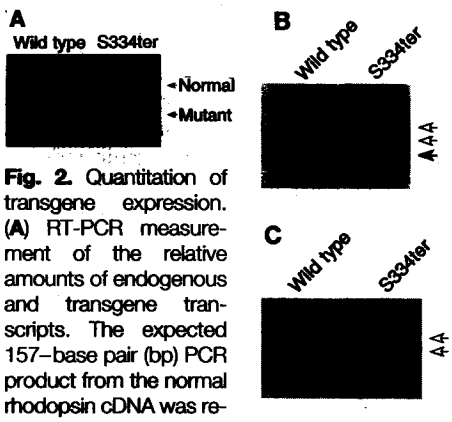

Fig. 2. Quantitation of transgene expression. **(A)** RT-PCR measurement of the relative amounts of endogenous and transgene transcripts. The expected 157–base pair (bp) PCR product from the normal rhodopsin cDNA was resistant to Dde I digestion, whereas the mutant rhodopsin PCR product was cleaved into 112- and 45-bp fragments. Only the 112-bp fragment was visible because the small quantity of the smaller fragment was below the detection limit of ethidium bromide. **(B)** Protein immunoblot analysis of rhodopsin in outer segments. When probed with an antibody to the NH_2-terminus, two bands for rhodopsin appeared in the sample from wild-type animals (white arrows), which may reflect different degrees of posttranslational modification. In addition to these two bands, samples from S334ter animals contained a band (filled arrow) that migrated at the position expected for the smaller S334ter species. **(C)** The additional band did not appear when the blot was probed with an antibody to the COOH-terminal amino acid residues 340 to 348. Thus, the smaller species was due to a COOH-terminal truncated form of rhodopsin. Densitometric scanning of the blot in (B) indicated that S334ter composed ~10% of the total rhodopsin in the transgenic animals.

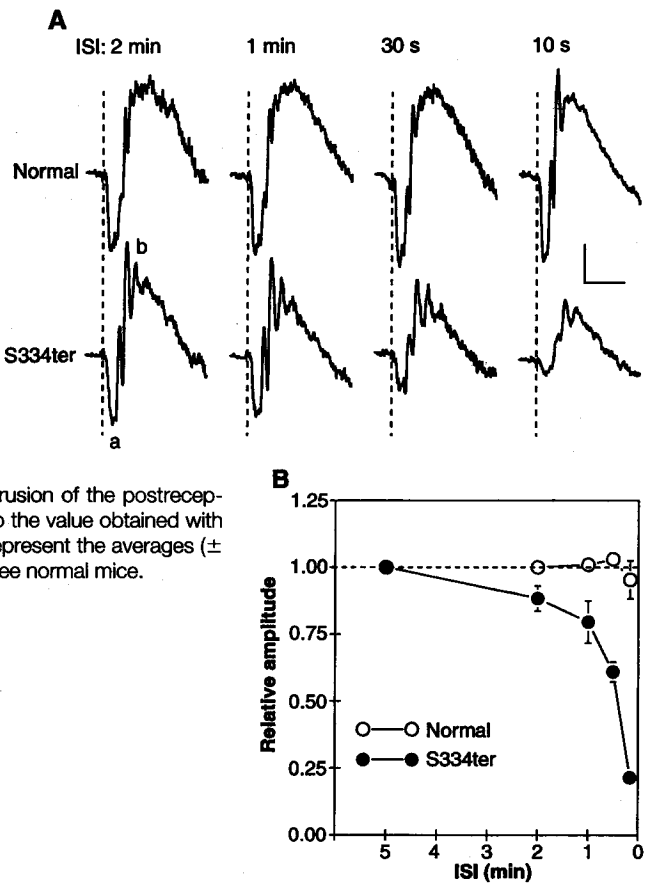

Fig. 3. ERGs of normal and S334ter mice. **(A)** ERGs recorded from a normal mouse and a transgenic one to a flash of 0.85 log cd s m^{-2} presented in darkness with different intervals elapsing between flash presentations. Vertical dashed lines indicate times of stimulus presentations. ERGs are averages of two or three responses. Scale bars are 250 μV vertically and 50 ms horizontally. **(B)** Amplitude of the a-wave as a function of ISI. The amplitude of the a-wave was measured at 8 ms after presentation of the stimulus, a point before the intrusion of the postreceptoral b-wave, and normalized to the value obtained with an interval of 5 min. Symbols represent the averages (± SEM) of four transgenic and three normal mice.

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 wild-type and mutant complementary DNAs (cDNAs) were identical, so PCR should have amplified both types equally. Because the site-directed mutagenesis created a restriction site in the transgene construct (Fig. 1B), the product of the transgene could be distinguished from that of the wild type by digestion of the PCR products with Dde I (Fig. 2A). On the basis of the relative intensities of the amplified product, we estimated the amount of S334ter mRNA to be about 0.12 ± 0.06 (mean ± SD; $n = 5$) relative to the normal. Protein immunoblot analysis of enriched rod outer segment preparations from transgenic mice showed two bands corresponding to normal rhodopsin and a third, faster migrating band corresponding to truncated rhodopsin when an antibody to the NH_2-terminus was used (Fig. 2B) (*10*). Densitometric scanning of the gel showed that the truncated product was present at an amount ~10% that of native rhodopsin. Consistent with its identity as the COOH-truncated form, the low molecular weight band was not labeled by an antibody to the COOH-terminus (Fig. 2C).

We recorded electroretinograms (ERGs) from S334ter mice and littermate control mice to test for abnormal photoreceptor function (*11*). Measurable at the corneal surface, the ERG is a light-evoked extracellular field potential that is generated by the 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 photoceptors (*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

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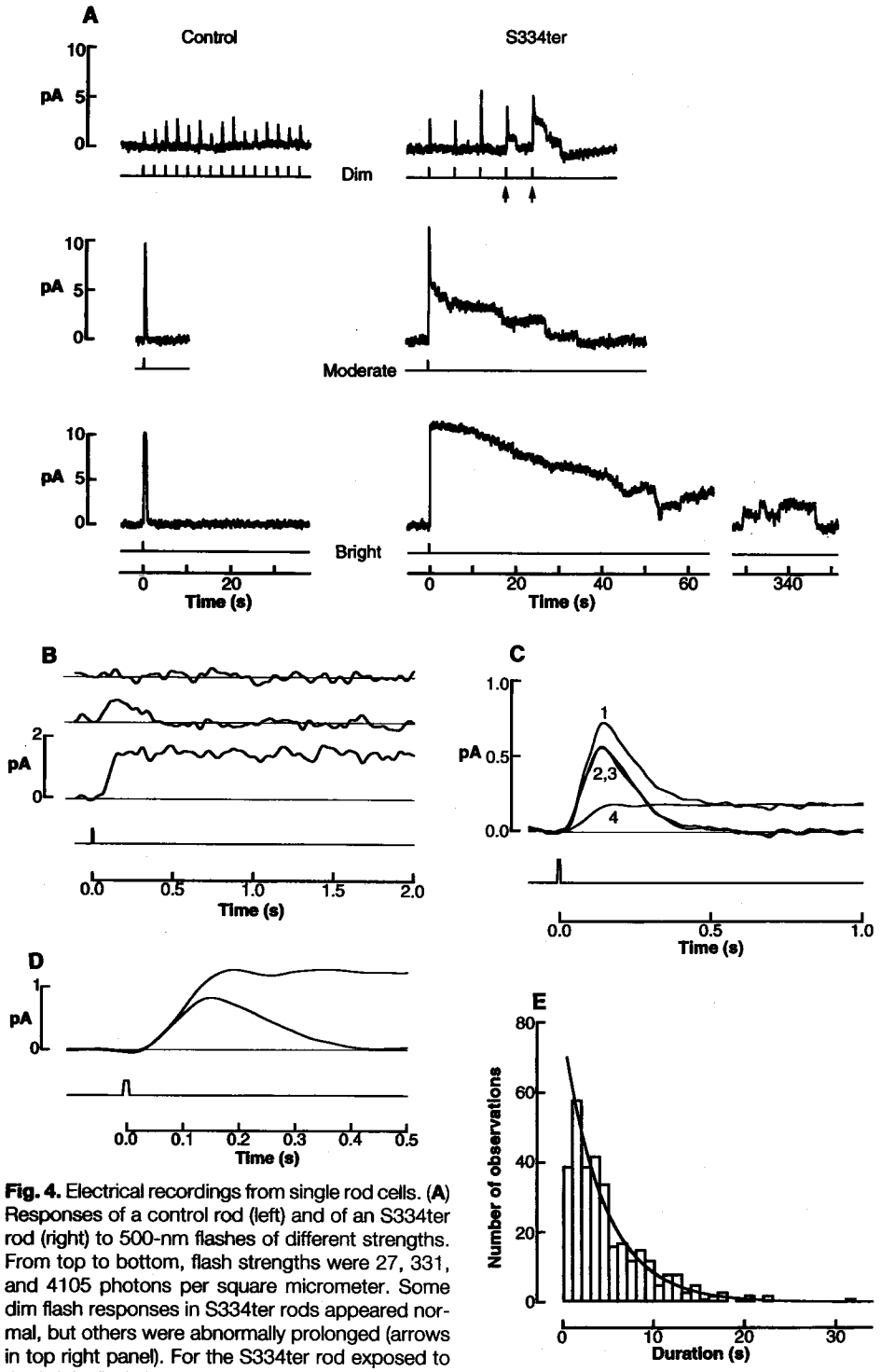

Fig. 4. Electrical recordings from single rod cells. (**A**) Responses of a control rod (left) and of an S334ter rod (right) to 500-nm flashes of different strengths. From top to bottom, flash strengths were 27, 331, and 4105 photons per square micrometer. Some dim flash responses in S334ter rods appeared normal, but others were abnormally prolonged (arrows in top right panel). For the S334ter rod exposed to the bright flash (bottom right panel), the break in the record was 262 s. Lower trace of each pair is a flash monitor. (**B**) Representative responses of an S334ter rod to dim flashes, showing a failure to respond (top), a normal single photon response (middle), and a large, prolonged response (bottom). Flash strength was 3.2 photons per square micrometer at 500 nm. (**C**) Relative incidence of prolonged and normal responses. We scaled a replica of the averaged prolonged response (*24*) by a factor of 0.13 (curve 4) to match the amplitude of the average of 222 dim flash responses (curve 1) at 1 s after the flash. The difference (curve 2) resembled the normal single photon response of this cell (*25*) when the normal response was scaled by a factor of 0.67 (curve 3). The ratio of the scaling factors for the prolonged and normal components, 0.13/0.67, gave the fractional incidence of prolonged to normal responses, 0.20. (**D**) Kinetics of prolonged responses. We superimposed the average of 49 prolonged responses with the normal single photon response by aligning their initial rising phases. (**E**) Distribution of the durations of 325 prolonged responses measured at half maximal amplitude. The mean duration of the prolonged response in this rod was 5.2 s. We fitted the distribution with an exponential function of time constant 4.5 s, omitting the first bin because some short events were probably missed.

sequence of steplike 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 photoisomerized approximately one rhodopsin per trial. Many responses were indistinguishable from those of control rods, but some rose monophasically 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 photoexcitation 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 15 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 MIII, 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 a 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 polypeptide is truncated similarly to S334ter by endogenous protease activity or that protein synthesis fails to reach the COOH-terminus of the polypeptide 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 normally but fails to be shut off properly. Phosphorylation of rhodopsin at the COOH-terminus is presumably essential for rapid termination of the photoresponse 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.

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10. Retinas were collected from five normal or five S334ter mice, aged 2 to 3 months. Rod outer segments were isolated on a Percoll gradient [D. S. Williams, T. A. Shuster, M. R. Moldrawski, A. D. Blest, D. B. Farber, *Exp. Eye Res.* **49**, 439 (1989)]. Proteins were separated on a 12% acrylamide gel, blotted, and probed with R2-12N or K16-107, monoclonal antibodies specific for the NH_2-terminus (residues 3 through 8) and the COOH-terminus (residues 340 through 348) of rhodopsin, respectively [G. Adamus *et al.*, *Vision Res.* **31**, 17 (1991)].
11. Mice, aged 3 months, were dark-adapted overnight, anesthetized with 15 μl of solution per gram of body mass; the saline solution contained ketamine (1.0 mg ml^{-1}), xylazine (0.4 mg ml^{-1}), and urethane 40 mg ml^{-1}). The mouse was warmed on a heating pad at 38°C. ERGs were recorded with a stainless steel wire 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 (Nicolet, Madison, WI) and presented as diffuse, full-field illumination. For evaluating cone-mediated function, 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 flash presentation. Responses were filtered at 1 kHz, averaged, and stored with a Pathfinder II (Nicolet) signal-averaging system.
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14. Control mice aged 3 to 8 months and S334ter mice aged 4 to 6 months were dark-adapted for a minimum of 18 hours. Methods for isolating and chopping the retina, delivering light stimuli, and recording photocurrents with suction electrodes have been described (*7*). Responses were low-pass filtered at 30 Hz with an 8-pole Bessel filter (Axon Instruments, Foster City, CA) and digitized at 100 or 400 Hz. We also digitally low-pass filtered some records at 8 Hz by convolving with a Gaussian function. Temperature was 36° to 38°C. Responses were not corrected for the delay introduced by low-pass filtering.
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24. We found the amplitude and form of the rising phase of the prolonged event by averaging late prolonged events that rose after the responses to bright flashes had recovered 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 0.8 pA coincided. Late events with a duration of less than 500 ms were excluded.
25. We estimated the amplitude of the normal single photon response by dividing the peak ensemble variance of 223 dim flash responses (less the variance introduced by prolonged responses) by the peak amplitude of the ensemble average dim flash response (less the amplitude introduced by the prolonged responses). The average of 13 normal dim flash responses was then scaled to this estimate of the single photon response amplitude. The flash delivered 2.5 photons per square micrometer at 500 nm.

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