

Rapid and Reproducible Deactivation of Rhodopsin Requires Multiple Phosphorylation Sites

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Summary

Efficient single-photon detection by retinal rod photoreceptors requires timely and reproducible deactivation of rhodopsin. Like other G protein-coupled receptors, rhodopsin contains multiple sites for phosphorylation at its COOH-terminal domain. Transgenic and electrophysiological methods were used to functionally dissect the role of the multiple phosphorylation sites during deactivation of rhodopsin in intact mouse rods. Mutant rhodopsins bearing zero, one (S338), or two (S334/S338) phosphorylation sites generated single-photon responses with greatly prolonged, exponentially distributed durations. Responses from rods expressing mutant rhodopsins bearing more than two phosphorylation sites declined along smooth, reproducible time courses; the rate of recovery increased with increasing numbers of phosphorylation sites. We conclude that multiple phosphorylation of rhodopsin is necessary for rapid and reproducible deactivation.

Introduction

Retinal rod cells use a G protein cascade to amplify the effects of single absorbed photons into macroscopic electrical responses. Absorption of a photon excites the seven-transmembrane protein rhodopsin, which catalyzes the exchange of GTP for GDP on the G protein

transducin. Transducin-GTP in turn activates cGMP phosphodiesterase (PDE), which hydrolyzes cGMP and reduces the intracellular cGMP concentration, allowing cGMP-gated channels to close and hyperpolarize the cell. Because this amplified response persists as long as a rhodopsin remains catalytically active (Nakatani and Yau, 1988; Chen et al., 1995; Sagoo and Lagnado, 1997; Rieke and Baylor, 1998), timely deactivation of rhodopsin is required for unambiguous detection of subsequent photon absorptions.

At the carboxyl terminus of rhodopsin and other G protein-coupled receptors, there is a cluster of serine and threonine residues that can undergo stimulus-dependent phosphorylation (Wilden and Kühn, 1982; Thompson and Findlay, 1984; Sibley et al., 1986; Palczewski and Benovic, 1991). Two critical events, phosphorylation and arrestin binding, participate in the deactivation of these G protein-coupled receptors. In rod cells, the phosphorylation of light-activated rhodopsin (R*) by rhodopsin kinase (RK) initiates deactivation and limits the response amplitude (Chen et al., 1995; Sagoo and Lagnado, 1997; Chen et al., 1999). In the absence of arrestin, phosphorylation alone can decrease the catalytic activity of R* by at least 50% (Xu et al., 1997). Arrestin binding following phosphorylation is necessary to complete the quench (Wilden et al., 1986; Xu et al., 1997).

Each photoexcited rhodopsin molecule must be deactivated along a stereotypical time course (Rieke and Baylor, 1998) in order to elicit reproducible single-photon responses, which enable photon absorption to be accurately encoded (Baylor et al., 1979, 1984). The mechanism that confers reproducibility to rhodopsin deactivation is not known. One way to explain it is to assume that multiple independent steps, perhaps 10–20, are required to quench the activity of one photoexcited rhodopsin (Rieke and Baylor, 1998). Alternatively, a one- or two-step deactivation process (e.g., monophosphorylation and arrestin binding) that is regulated by a negative feedback mechanism might confer sufficient reproducibility (Whitlock and Lamb, 1999). As a first step in understanding reproducibility, it is important to determine how many sites on photoexcited rhodopsin become phosphorylated during deactivation, since multiple phosphorylations by RK might themselves contribute to reproducibility.

Biochemical studies of light-dependent phosphate incorporation into rhodopsin molecules have yielded widely varying stoichiometries. Many *in vitro* studies have reported multiple phosphorylation of rhodopsin (Wilden and Kühn, 1982; Miller and Dratz, 1984; Wilden, 1995). Analysis by liquid chromatography–mass spectrometry (LC-MS) has suggested that 3 serine residues—S334, S338, and S343—are the preferred sites for phosphorylation by RK (McDowell et al., 1993; Papac et al., 1993; Ohguro et al., 1993, 1994, 1996). In contrast, *in vivo* studies have reported that monophosphorylated rhodopsin predominates (Ohguro et al., 1995; Hurley et al., 1998), with S338 being the primary site phosphory-

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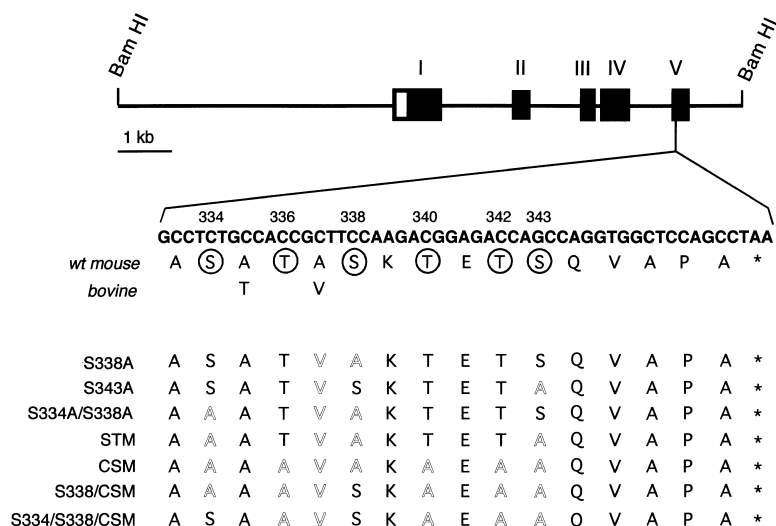


Figure 1. Rhodopsin Phosphorylation Site Mutants

Top, the 11 kb BamHI fragment of the mouse opsin gene. Solid boxes indicate the protein coding region. Open box is the 5' untranslated region. Middle, enlarged map of exon 5 sequence coding for the last carboxy-terminal 16 amino acids of mouse opsin. The circled amino acids represent the serine and threonine residues that are substrates for phosphorylation by rhodopsin kinase. Sequence differences between mouse and bovine opsins at codons 335 and 337 are indicated. Bottom, last 16 amino acid sequence for seven rhodopsin mutants. Amino acid substitutions are outlined. A conserved change that confers to murine opsin a linear epitope for mAb 3A6, A337V, was introduced in all mutants (Hodges et al., 1988).

lated after a bright flash and S334 being the primary site phosphorylated under constant illumination (Ohguro et al., 1995). Although chemical analyses can measure phosphorylation directly, it has not been possible to achieve the sensitivity and time resolution necessary to measure the phosphorylation that accompanies deactivation of rhodopsin under lighting conditions dimmer than those producing rod saturation.

To investigate the stoichiometry of phosphorylation, as well as its contribution to the time course and reproducibility of rhodopsin deactivation, we undertook a functional analysis in intact mouse rods expressing mutant rhodopsin molecules in which selected phosphorylation sites were mutated to Ala. We then recorded single-photon responses from individual rods to determine the effects of the mutations on the amplitude and time course of the responses.

Results

Expression of Rhodopsin Mutants in Transgenic Mouse Rods

To investigate the role of individual C-terminal phosphorylation sites in rhodopsin deactivation, we used a series of mutant transgenes in which one or more of the sites were replaced by Ala (Figure 1). To confirm that rhodopsin phosphorylation is restricted to the carboxy-terminal Ser and Thr residues, we generated a mutant in which all of these sites were substituted to Ala, the completely substituted mutant (CSM). Other mutants lacked one or more of the three Ser residues that are reportedly the preferred sites for phosphorylation by rhodopsin kinase: S338A, S343A, serine triple mutant (STM; S334A/S338A/S343A). Finally, one site (S338) or two sites (S334 and S338) were restored in the CSM (S338/CSM; S334/S338/CSM) to assess whether phosphorylation of these residues is sufficient to support normal rhodopsin deactivation, as has been suggested (Ohguro et al., 1995).

We expressed each rhodopsin transgene in the rhodopsin null homozygous background ($\rho^{-/-}$; Lem et al., 1999) so that observed photoresponses were known to be generated by the mutant rhodopsin. Since rod

photoreceptors degenerate when they express too little (Lem et al., 1999) or too much (Li et al., 1996; Mendez et al., 2000) rhodopsin, we studied lines in which the transgene expression level approximated that of endogenous rhodopsin. Retinas from transgenic mice had largely normal retinal morphology at 1 month of age, with approximately the same number of photoreceptor cells (as judged by outer nuclear layer thickness) and rod outer segment length as a wild-type control retina (Figure 2A). Normal retinal morphology was maintained for at least 2 months in S338A, S343A, STM, S338/CSM, and S334/S338/CSM lines (data not shown). CSM retinas showed a progressive degeneration that halved the number of photoreceptor nuclei at 12 postnatal weeks. This degeneration correlated with a higher expression of the transgene in this line and was not prevented by raising the mice in constant darkness (data not shown).

Transgenic mutant rhodopsin expression in $\rho^{-/-}$ rods was restricted to the outer segments (Figures 2B and 2C), indicating that the Ser and Thr residues are not part of the carboxy-terminal recognition signal for rhodopsin transport (Tai et al., 1999). Western blot analysis indicated that the expression levels of rhodopsin kinase, recoverin, arrestin, and transducin were indistinguishable in transgenic mutant and wild-type retinas (data not shown).

STM, S338/CSM, and S334/S338/CSM Rhodopsins Are Phosphorylated by RK In Situ

To assess phosphorylation of mutant rhodopsins, we examined light-dependent ^{32}P incorporation in intact retinas. Light exposure of the isolated retinas from control, STM, and S338/CSM mice resulted in phosphorylation of rhodopsin (Figure 3A), whereas retinas that were not exposed to light did not show appreciable levels of rhodopsin phosphorylation. No rhodopsin phosphorylation was detected in CSM retinas, confirming previous findings that light-dependent rhodopsin phosphorylation is restricted to the carboxy-terminal Ser and Thr residues (Thompson and Findlay, 1984; Palczewski et al., 1991; Brannock et al., 1999). Because $\rho^{-/-}$ retinas contain fully functional cones at 4–5 weeks of age (Toda et al., 1999); the absence of signal in the $\rho^{-/-}$ sample verifies

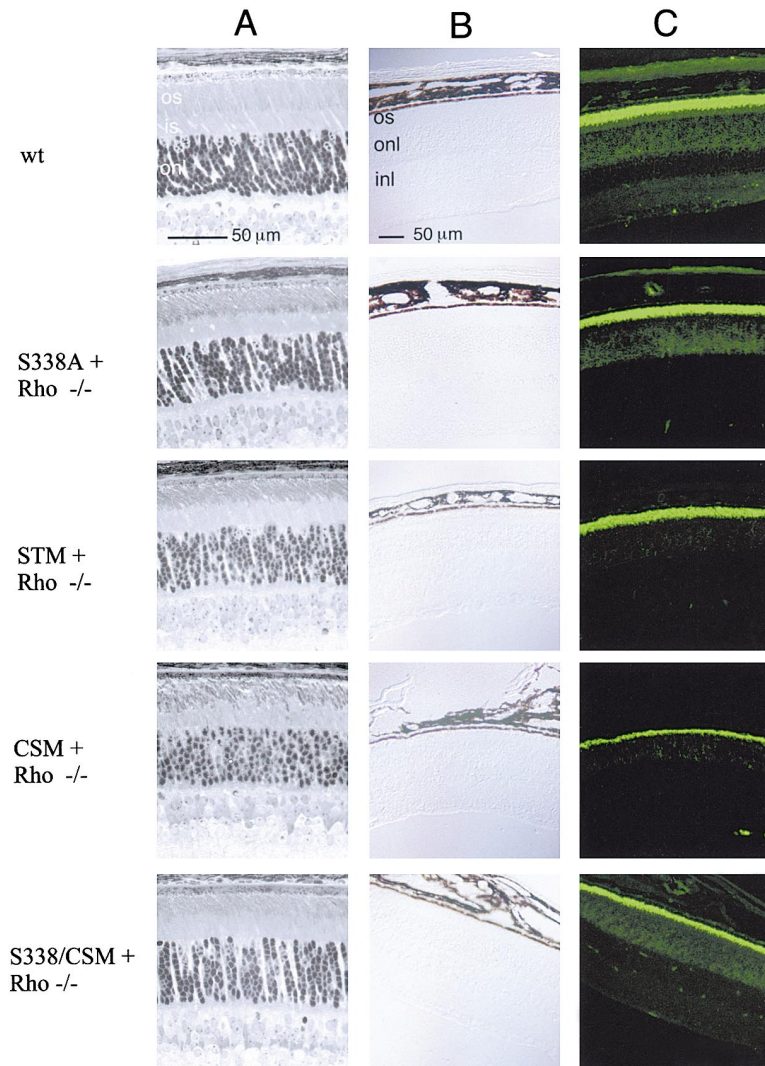


Figure 2. Retinal Morphology of Age-Matched Wild-Type Mice and Rhodopsin Null Homozygous Mice Expressing Mutant Rhodopsins

(A) shows light micrographs of 1 μm sections of central retina from 1-month-old mice. The number of rod photoreceptor nuclei in the transgenic lines at this age is very similar to that of the wild-type retina. Shown in (B) and (C) are cryosections of the indicated lines at 1 month of age (for wt, S338A, STM, and S338/CSM) and at 2 months of age (for CSM) immunostained with mAb R2-12N, which recognizes residues 2–12 from the amino terminus of rhodopsin at bright field (B) or dark field (C). Mutant rhodopsin, expression was restricted to rod outer segments. os, outer segment; is, inner segment; onl, outer nuclear layer; inl, inner nuclear layer.

that the labeled band in the other samples did not result from phosphorylation of cone opsin. The same samples were probed with an antibody against the amino terminus of rhodopsin to assess the amount of rhodopsin present (Figure 3B). The presence of phosphorylated rhodopsin was also verified in the membrane fraction from light-exposed isolated retinas using isoelectric focusing (IEF; Figure 3C). Western blot of IEF gels of solubilized membrane fraction from S338/CSM, S334/S338/CSM, and STM retinas exposed to light revealed one, two, or three species of phosphorylated rhodopsin respectively (Figure 3C, lanes 2, 3, and 5). Light-exposed wild-type rhodopsin showed six different phosphorylated species (lanes 4 and 7), whereas CSM was not phosphorylated (lane 6).

Thus, we conclude that all available carboxyl terminus phosphorylation sites in mutant and wild-type rhodopsins can be phosphorylated in a light-dependent manner in intact, living rods.

Effect of Phosphorylation Site Mutants on the Kinetics of the Single-Photon Response

To assess the contribution of particular phosphorylation sites to the time course of rhodopsin deactivation, we

recorded flash responses from $\text{rho}^{-/-}$ rods expressing each mutated rhodopsin (Figures 4 and 5). The responses of the mutant rods took on one of two general phenotypes (Figure 4). Rhodopsin mutants with fewer than three potential phosphorylation sites gave rise to responses that were greatly prolonged and maintained a steady amplitude until abruptly turning off at widely varying times (e.g., Figures 4A and 6). Rhodopsin mutants with three or more potential phosphorylation sites produced responses that more closely resembled those of normal rods, being briefer and more reproducible from trial to trial (Figures 4B, 5B, and 7).

To compare the kinetics of the flash responses from various mutants, we estimated the single-photon responses of rods expressing mutant or wild-type rhodopsin (Figure 5). The mean single-photon response of control rods rose to a peak amplitude of 0.5 pA in 110 ms. A single exponential function fitted to the final falling phase of the response gave a recovery time constant (τ) of 150 ms (see Table 1). The mean single-photon response of rods expressing CSM rhodopsin, which lacked all phosphorylation sites, rose along the time course of the control response at early times but became significantly larger by about 100 ms after the flash (Fig-

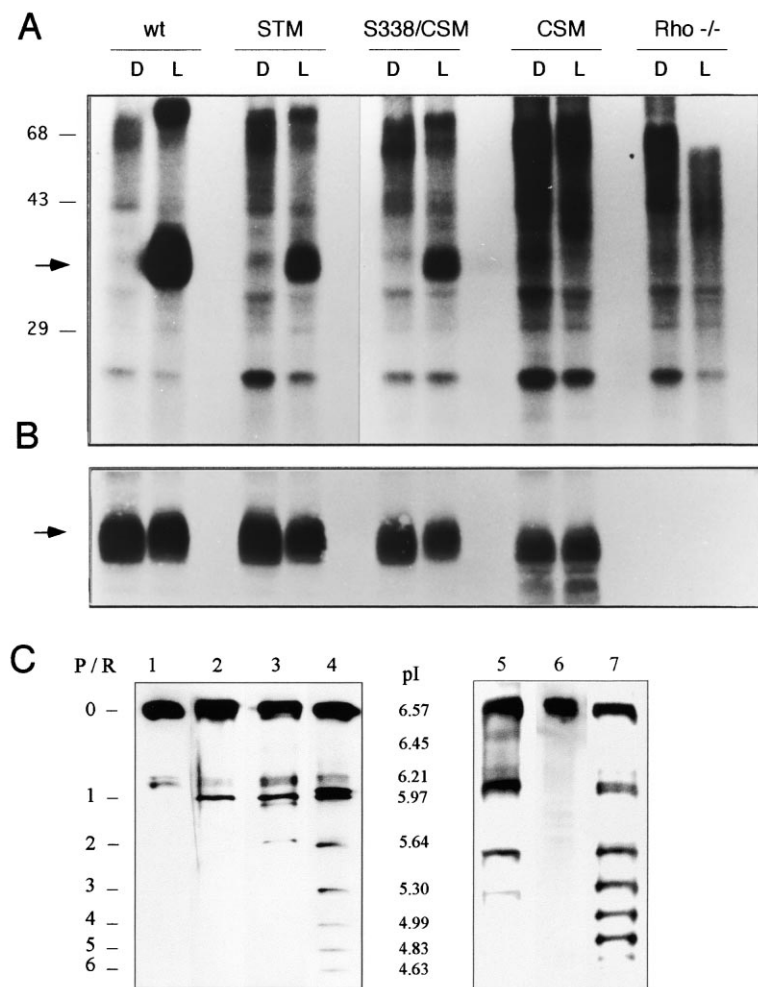


Figure 3. Light-Dependent Phosphorylation of Mutant Rhodopsins Ex Vivo

(A) In situ phosphorylation assay. Retinas were dissected under infrared light, incubated in phosphate-free Krebs buffer containing ^{32}P i, and exposed to white fluorescent light for 5 min (L) or maintained in darkness (D). Samples were quenched by homogenization in 6 M urea buffer, size-fractionated in a 12% SDS-PAGE gel, and blotted onto a nitrocellulose membrane. Phosphorylated rhodopsin (arrow) is visualized by autoradiography.

(B) Western blot of samples in (A) using a monoclonal antibody recognizing residues 2–12 of rhodopsin (R2–12N, generously provided by P. Hargrave, University of Florida).

(C) Separation of rhodopsin and its phosphorylated forms by isoelectrofocusing. Left and right panels correspond to two different experiments. Retinas from wild type (lanes 1, 4, and 7), S338/CSM (lane 2), S334/S338/CSM (lane 3), STM (lane 5), and CSM (lane 6) were dissected under infrared light, exposed to bright fluorescent light for 10 min (lanes 2–7), or kept in darkness (lane 1). Samples were processed as described in Experimental Procedures, and a fraction corresponding to 1/5 of a retina was loaded per lane. The isoelectric point (pI) of observed bands is indicated. The band at pI 6.57 corresponds to unphosphorylated rhodopsin, and bands at pIs 6.45 and 6.21 to unphosphorylated opsin. Phosphorylated rhodopsin species with increasing number of phosphates per rhodopsin (P/R) yielded bands at increasingly acidic pH: pI 5.97 (1 P/R), pI 5.64 (2 P/R), pI 5.30 (3 P/R), pI 4.99 (4 P/R), pI 4.83 (5 P/R), and pI 4.63 (6 P/R).

ure 5A). This indicates that phosphorylation normally begins to inhibit rhodopsin's catalytic activity by this time, consistent with previous descriptions of responses from rods expressing mutant rhodopsins that lack the entire C-terminal domain (Chen et al., 1995) and rods that do not express rhodopsin kinase (Chen et al., 1999). The average amplitude of single-photon responses from CSM was about 3-fold larger than normal, and this amplitude was maintained for widely varying times before the responses abruptly turned off (see below).

The specific roles of S334 and S338 in rhodopsin deactivation were investigated by restoring one or both of them in the CSM rhodopsin. S334 and S338 were reported to be the predominant sites that are phosphorylated in living mice, with S338 being the site phosphorylated after a light flash and S334 after continuous illumination (Ohguro et al., 1995). Like the responses of CSM rods, the S338/CSM responses were greatly prolonged and turned off abruptly at widely variable times. The amplitude of the single-photon response in S338/CSM rods was less than that in CSM rods, as if phosphorylation of S338 reduced rhodopsin's catalytic activity. This interpretation is strengthened by the observation that the initial rise of the response in the S338/CSM rods was identical to that in CSM rods, indicating

that the initial rates of PDE activation were identical (Figure 5A). The divergence of the responses at roughly 100 ms after the flash suggests that phosphorylation of S338 occurs around this time in S338/CSM rods but that S338 alone is not sufficient to allow normal rhodopsin deactivation.

Restoring both S334 and S338 in the CSM rhodopsin (S334/S338/CSM) also failed to restore normal single-photon response kinetics. Like elementary responses from CSM and S338/CSM rods, the S334/S338/CSM responses took the form of prolonged plateaus with exponentially distributed durations (see below). The mean amplitude and duration of S334/S338/CSM responses were not significantly different from those of S338/CSM rods (Table 1; Figure 5A), suggesting that the phosphorylation of S334 had little additional effect on the catalytic activity of rhodopsin. The severe prolongation of the S334/S338/CSM responses indicates that even the presence of two phosphorylation sites (S334 and S338) is not sufficient for normal rhodopsin deactivation.

The rising phases of responses from the S334/S338/CSM rods (and STM responses, see below) were considerably slower than those of responses from the other lines (Figures 5A and 5B), even at times too early to

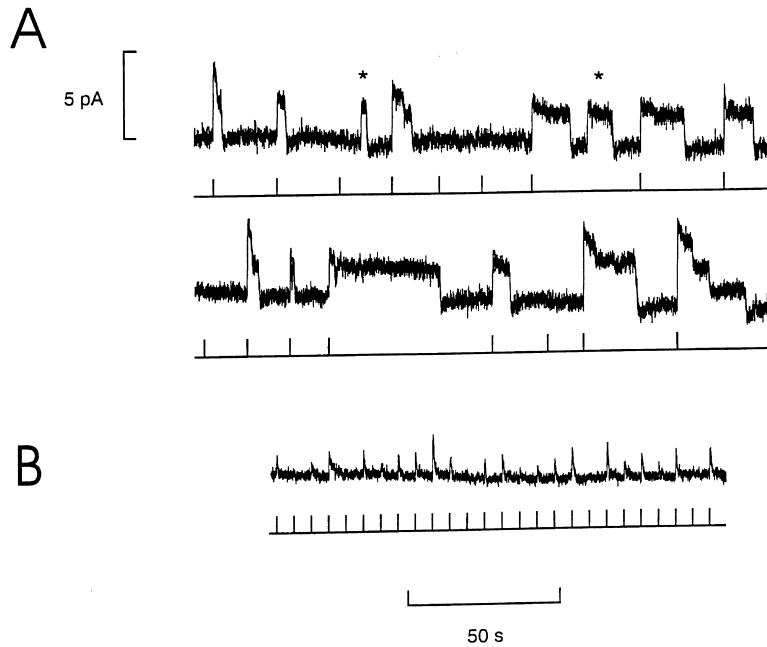


Figure 4. Flash Responses from Rods Expressing Mutant Rhodopsins

Responses from rods expressing CSM rhodopsin (A) or STM rhodopsin (B) to a series of dim flashes (3.9 photons/ μm^2) that activated an average of 1.2 (CSM) and 2.5 (STM) rhodopsin molecules per trial. Dark currents were 8.7 (CSM) and 8.5 (STM) pA. Trace beneath records is a flash monitor. Asterisks denote responses resulting from spontaneous isomerization of rhodopsin (Baylor et al., 1980).

reflect RK binding (100 ms; Chen et al., 1999). The slower rising phase at these early times reflects a reduced rate of PDE activation. This could arise from a decrease in the bimolecular rate constant of transducin activation by rhodopsin, a decrease in transducin or PDE concentration, or a small increase in the outer segment volume (Pugh and Lamb, 1993). Regardless of the mechanism, the early time of onset of this effect indicates that it is not related to phosphorylation or arrestin binding, and therefore, the effect was not pursued further.

The experiments described above indicated that S334 and S338 were not sufficient for normal rhodopsin deactivation. In order to determine whether the three Ser residues were *required* for normal deactivation, we recorded from rods expressing rhodopsin that lacked all 3 Ser residues but retained the 3 Thr residues (Ser triple mutant or STM). Single-photon responses from these rods had a smooth time course that generally resembled that of responses from wild-type rods. However, STM responses rose for longer times and recovered considerably more slowly than normal ($\tau \approx 350$ ms; Table 1; Figure 5B). These results indicate that phosphorylation of the Thr residues alone can support rhodopsin deactivation, although not at the normal rate.

When only one phosphorylation site was removed (S338A or S343A), the single-photon response was al-

most like that of control rods but recovery was slightly slowed ($\tau \approx 200$ ms; Table 1; Figure 5B). Consistent with this slower recovery, the mean integration times (see Experimental Procedures) of S338A and S343A responses were about 1.5 times longer than those of control responses. The results from STM, S338A, and S343A imply that the rate of rhodopsin deactivation depends not on the identity of the available sites, but on their total number. Taken together, these results are consistent with the notion that normal kinetics of rhodopsin deactivation require the presence of all six phosphorylation sites.

Effect of Phosphorylation Site Mutants on the Reproducibility of the Single-Photon Response

In rods expressing rhodopsins with fewer than three phosphorylation sites, the single-photon responses typically maintained a steady amplitude for several seconds before abruptly turning off at widely variable times. The distribution of the durations of 790 single-photon responses from 11 CSM rods was fitted by a single exponential function with a time constant of 4.6 s (Figure 6A). Likewise, the distributions of durations from S338/CSM and S334/S338/CSM rods were also exponential, with time constants of 2.9 s and 3.1 s, respectively (Figures 6B and 6C). This behavior is reminiscent of responses

Table 1. Characteristics of Dim Flash Responses and Dark Currents from Control and Rhodopsin Mutant Rods

Rhodopsin	Dark Current (pA)	Time to Peak (ms)	Integration Time (ms)	τ_{rec}^a (ms)	Single ϕ r Amplitude (pA)
Wild type (+/+)	10.4 \pm 0.6 (20)	108 \pm 3 (18)	207 \pm 15 (17)	150 \pm 15 (16)	0.56 \pm 0.08 (19)
S338A	10.5 \pm 0.8 (18)	122 \pm 12 (17)	291 \pm 24 (17)	191 \pm 14 (16)	0.57 \pm 0.08 (16)
S343A	11.0 \pm 0.9 (11)	100 \pm 4 (11)	278 \pm 20 (10)	184 \pm 41 (10)	0.40 \pm 0.05 (9)
STM	11.1 \pm 1.3 (14)	188 \pm 20 (12)	521 \pm 34 (12)	378 \pm 43 (12)	0.73 \pm 0.23 (8)
CSM	11.1 \pm 1.4 (11)	346 \pm 78 (11)	ND	5294 \pm 656 (7)	1.53 \pm 0.17 (10)
S338/CSM	10.4 \pm 0.9 (17)	120 \pm 8 (16)	2270 \pm 310 (13)	4402 \pm 997 (13)	0.68 \pm 0.11 (13)
S334/S338/CSM	10.3 \pm 0.6 (16)	199 \pm 30 (16)	2010 \pm 230 (15)	2931 \pm 277 (16)	0.62 \pm 0.10 (14)

^a τ_{rec} was determined by fitting a single exponential to the final falling phase of the mean dim flash response. Error bars indicate SEM.

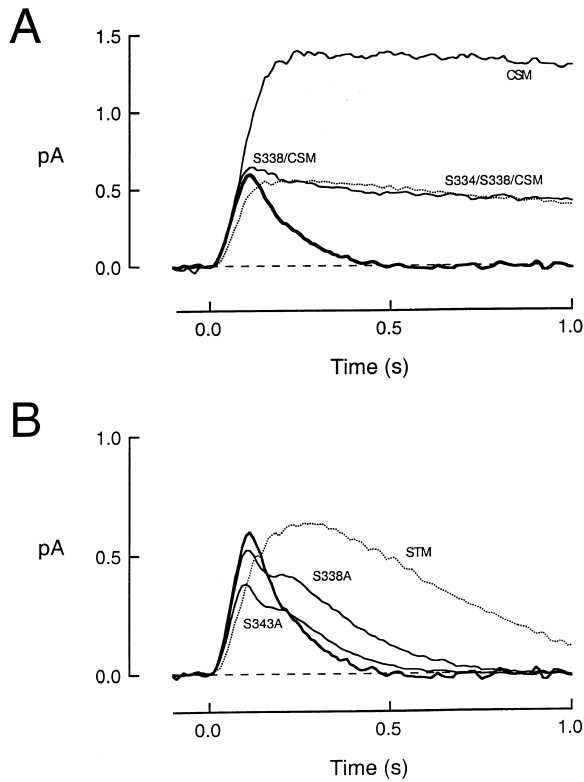


Figure 5. Population Mean Single-Photon Responses from Rods Expressing Wild-Type or Mutant Rhodopsins
(A) Mean responses from 10 wild-type (bold), 10 CSM, 13 S338/CSM, and 14 S334/S338/CSM (dotted trace) rods.
(B) Mean responses from 10 wild-type (bold, same trace as in [A]), 16 S338A, 9 S343A, and 8 STM (dotted trace) rods.

generated by rhodopsin lacking the entire C-terminal domain (S334ter; Chen et al., 1995) and rhodopsin that could not be phosphorylated because of a lack of rhodopsin kinase (Chen et al., 1999).

The stochastic termination of responses in CSM, S338/CSM, and S334/S338/CSM rods suggests that the rate-limiting step in quenching these mutant rhodopsins is a memoryless, first-order process. Although the mechanism of the single, rate-limiting step is not known, it might represent the binding of an inhibitory molecule, such as arrestin or p44 arrestin (Smith et al., 1994; Palczewski et al., 1994; Pulvermuller et al., 1997) to unphosphorylated rhodopsin. The stochastic behavior is highly abnormal, since the rod's single-photon response is typically very reproducible (Baylor et al., 1979, 1984; Rieke and Baylor, 1998). The dramatic loss of reproducibility in the CSM, S338/CSM, and S334/S338/CSM mutants suggests that three or more phosphorylation sites are required for reproducible rhodopsin deactivation.

To test whether reproducibility was more subtly affected in the other phosphorylation site mutants, we compared the form of the time-dependent ensemble variance and the mean dim flash response. If the elementary response has a reproducible amplitude and waveform, the form of the time-dependent ensemble variance should take the form of the mean dim flash response squared, as dictated by the Poisson statistics

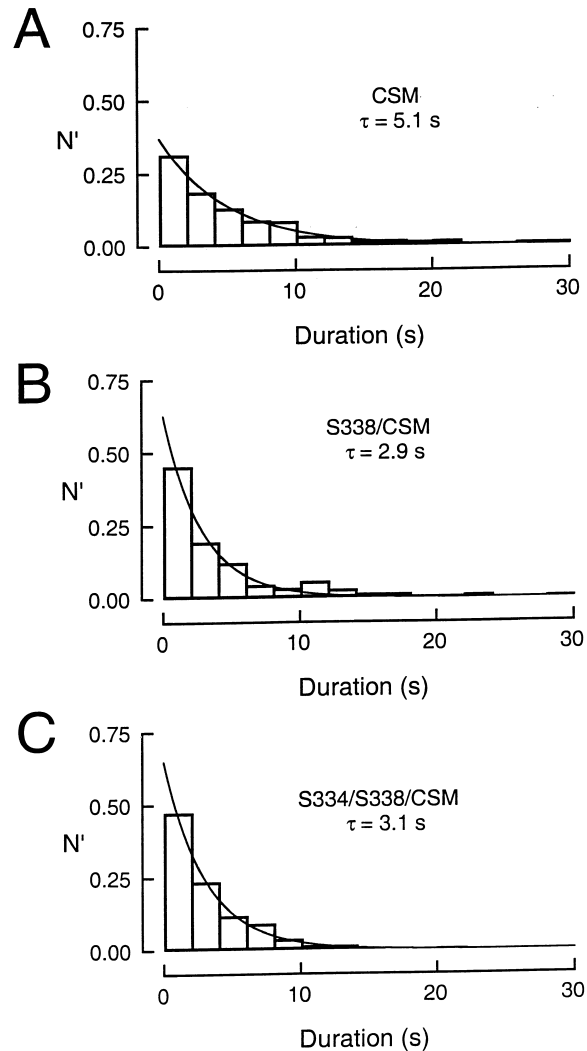


Figure 6. Stochastic First-Order Deactivation of Responses from Rods Expressing Rhodopsins with Fewer Than Three Phosphorylation Sites

Distributions of unitary event durations from CSM (A), S338/CSM (B), and S334/S338/CSM (C) rods were scaled by the total number of events observed. Single exponential functions with the time constants listed were fitted to the experimental observations over an interval of 0–30 s. The mean duration of each distribution was 6.3 s (CSM; 790 events from 11 cells), 4.2 s (S338/CSM; 348 events from 5 cells), and 3.1 s (S334/S338/CSM; 426 events from 4 cells). Two of the means are larger than the taus because of a few events with very long durations. For an exponential distribution, the ratio of the standard deviation to the mean is unity; for the experimental distributions the ratios were 1.2 (CSM), 1.2 (S338/CSM), and 1.1 (S334/S338/CSM).

of photon absorption (see Experimental Procedures). Indeed, in control rods, the form of the mean dim flash response squared was very similar to that of the ensemble variance (Figure 7A), consistent with there being little variability in the time course of the elementary response itself. In all of the rhodopsin mutants, however, the same analysis revealed an increase in the ensemble variance at late times (Figures 7B–7D; Table 2). The amount of the residual variance increased as the number of available phosphorylation sites decreased (Table 2). Because the

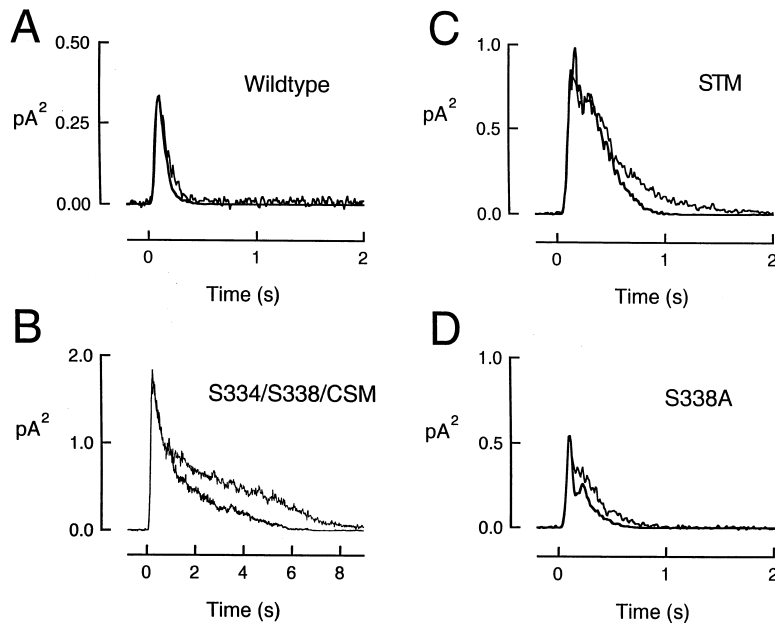


Figure 7. Increased Variability in Response Recovery in Transgenic Rods Expressing Mutant Rhodopsin

The mean dim flash response from a wild-type (A), S334/S338/CSM (B), STM (C), or S338A (D) rod was squared and scaled so that the rising phase coincided with that of the time-dependent ensemble variance (thin). The additional variance at late times indicates variability in the recovery of the elementary response. The time integral of the residual variance for each (in pA^2s) was 0.0107 (wild type), 1.64 (S3384/S338/CSM), 0.104 (STM), and 0.0501 (S338A). Dark currents (in pA) were 6.9 (wild type), 9.2 (S334/S338/CSM), 10.4 (STM), and 7.7 (S338A). Mean number of photoisomerizations per flash (inverse of the scaling factor, above) were 3.5 (wild type), 3.2 (S3384/S338/CSM), 2.2 (STM), and 3.8 (S338A).

absolute residual variance is also dependent on the mean number of photoisomerizations as well as the amplitude and mean duration of the single-photon response, we also examined the *relative* reproducibility of the elementary response. The relative variation of the single-photon response, represented by the variation index, V (see Experimental Procedures), was markedly increased in mutants that gave step-like responses but was not increased in the single, double, and triple serine mutants (Table 2). This result suggests that reproducibility requires at least three phosphorylation sites and that additional sites provide no additional improvement in the relative reproducibility of the response.

Discussion

Analysis of single-photon responses in transgenic mouse rods expressing mutated rhodopsin has enabled us to determine the requirements for timely and reproducible rhodopsin deactivation under physiological conditions. Our results indicate that multiple phosphorylation must occur for rapid and reproducible rhodopsin deactivation. The results also suggest that RK binding alone has no effect on the catalytic activity of R^* and

that at least three phosphorylations are required to induce high-affinity arrestin binding. With three or more available phosphorylation sites, the rate of recovery of the flash response increased with the number of available sites, indicating that all phosphorylation sites are needed for normal rhodopsin deactivation under normal physiological conditions.

Evidence for Multiple Phosphorylation of Rhodopsin

Rhodopsin kinase becomes activated when it binds to the third cytoplasmic loop of photoexcited rhodopsin (Palczewski et al., 1991). Once activated, it can phosphorylate a variety of substrates. Normally these are the clustered Ser and Thr sites on the carboxyl terminus of rhodopsin, but exogenous peptide fragments and the C-terminal residues of other G protein-coupled receptors (Palczewski et al., 1989) can also be phosphorylated by activated RK. Because of RK's promiscuity for substrates and the dynamic unstructured properties of rhodopsin's COOH-terminal domain (Langen et al., 1999), substitution of Ala for Ser and Thr residues at rhodopsin's COOH terminus is not expected to affect RK's ability to phosphorylate the remaining sites. Indeed, our experiments using IEF/Western blot showed that all available sites in various rhodopsin mutants could be phosphorylated in a light-dependent manner.

That any Ser and Thr site on the carboxyl terminus can be phosphorylated by RK has been demonstrated by numerous biochemical experiments (Wilden and Kühn, 1982; Wilden, 1995; Brannock et al., 1999). Using isolated frog or bovine rod outer segment (ROS) as a source of rhodopsin in the native membrane environment, Wilden and Kühn demonstrated incorporation of up to 7 phosphate/mol of rhodopsin (Wilden and Kühn, 1982), corresponding to the number of potential phosphorylation sites on the carboxyl terminus of bovine rhodopsin. Our IEF experiments similarly showed that all of the carboxy terminal Ser and Thr sites in the wild type as well as the mutant rhodopsins could be phos-

Table 2. Variations in the Time Course of Responses From Control and Rhodopsin Mutant Rods

Rhodopsin	Integrated variance (pA^2s)	Variation Index ^a V
Wild type (+/+)	0.025 ± 0.005 (19)	0.33 ± 0.08 (10)
S338A	0.046 ± 0.010 (10)	0.26 ± 0.06 (11)
S343A	0.047 ± 0.013 (8)	0.30 ± 0.08 (8)
STM	0.091 ± 0.012 (7)	0.23 ± 0.06 (7)
CSM	ND	ND
S338/CSM	ND	ND
S334/S338/CSM	0.73 ± 0.13 (13)	0.77 ± 0.16 (12)

^a Relative variation in the time course of the response (see Experimental Procedures). Error bars indicate SEM.

phorylated in intact retinas. Since the rate of arrestin-R* association depends on the number of phosphates on the carboxyl terminus of rhodopsin (Wilden, 1995), multiple phosphorylation events may be required for turning off rhodopsin's catalytic activity rapidly in vivo.

Indeed, our single-cell recordings from rods expressing the rhodopsin phosphorylation site mutations clearly demonstrate that multiple phosphorylation of rhodopsin's carboxy-terminal domain occurs under physiological conditions. This conclusion contrasts with the results of recent biochemical studies that found that monophosphorylated rhodopsin predominated in vivo (Ohguro et al., 1995; Hurley et al., 1998). These conclusions were based on mass spectrometry of proteolyzed peptide fragments from the carboxy-terminal domain of rhodopsin, obtained at long times (10s of seconds to 10s of minutes) after exposure to light that bleached 0.17%–100% of rhodopsin molecules. In addition to the inadequate time resolution, the timing and extent of rhodopsin phosphorylation are likely to be different at high bleach levels because the concentration of RK becomes limiting (Laitko and Hofmann, 1998).

Multiple Phosphorylation Confers Speed and Reproducibility on Rhodopsin Deactivation

When all the Ser and Thr residues at rhodopsin's C terminus were mutated to Ala (CSM), flash responses were greatly prolonged, consistent with the notion that phosphorylation is a necessary prerequisite for timely rhodopsin deactivation. Biochemical analysis of the carboxyl terminus of rhodopsin indicated that S338 and S334 are the preferred sites for phosphorylation in living mouse retinas (Ohguro et al., 1995). If these are the exclusive sites in normal rhodopsin deactivation, restoring these sites in the CSM should lead to normal deactivation. Although restoring one (S338) or two (S334/S338) phosphorylation sites did partially attenuate rhodopsin's ability to activate transducin, phosphorylation of these residues did not result in normal deactivation kinetics. Instead, the responses recovered after an exponentially distributed waiting time of mean 3 s. We conclude that while phosphorylation of S338 is sufficient to reduce the catalytic activity of rhodopsin at early times, phosphorylation at one (S338) or two (S334/S338) sites cannot trigger the rapid, high-affinity arrestin binding to R* that is required for normal deactivation. This is consistent with previous reports that phosphorylation of one site (S334 or S338; Brannock et al., 1999) or two sites (S338/S343 or T340/S343; Zhang et al., 1997) does not efficiently trigger arrestin-mediated quenching of rhodopsin in reconstitution assays.

When three or more of the phosphorylation sites were present, the flash responses were more similar to those of normal rods, but they had slightly longer durations. When the 3 Ser residues were mutated (STM), the flash responses were about 2.5 times longer than control responses. This indicates that phosphorylation of the Thr residues was sufficient to induce high-affinity binding of arrestin to R*. When only a single Ser was mutated (S338 or S343), the responses were slightly slower than normal. This suggests that all six sites contribute to normal, rapid deactivation of photoexcited rhodopsin in normal rods. The slowed recovery in these mutants

Table 3. Modified Sequence in Mutant Rhodopsin Transgenes and Oligonucleotides Used in the Genotyping Protocol

Line	Coding Region, Amino Acids 333–348 ^a	Primers ^b	PCR Conditions and Digestion
S338A	5'-GCC TCG GCC ACC GTG GCC AAG ACG GAG ACC AGC CAG GTG GCT CCA GCC TAA-3' (EaeI)	mRh7.2: 5'-GACGACGCCTCGGCCACCCGIG-3' (forward) mRh5: 5'-GGAGCCTGCATGACCTCATCC-3' (reverse)	94°C 5 m, (94°C 60 s; 63°C 90 s; 72°C 90 s) 30 cycles
S343A	5'-GCC TCT GCC ACG GTC TCC AAG ACG GAG ACC GCC CAG GTG GCT CCA GCC TAA-3' (BglI)	mRh7.1: 5'-GACGACGCCTCGGCCACCCGIG-3' (forward) mRh5: 5'-GGAGCCTGCATGACCTCATCC-3' (reverse)	94°C 5 m, (94°C 60 s; 63°C 90 s; 72°C 90 s) 30 cycles
STM	5'-GCC TCG GCC ACC GTT GCC AAG ACG GAG ACC GCC CAG GTG GCT CCA GCC TAA-3' (EagI)	mRh5: 5'-GTAGGGGACATGCTGAGGTGAGGC-3' (reverse) mRh5: 5'-GGAGCCTGCATGACCTCATCC-3' (reverse)	94°C 5 m, (94°C 30 s; 63°C 30 s; 72°C 60 s) 30 cycles, EagI digestion
CSM	5'-GCC GCG GCC GCC GTT GCC AAG GCG GAG GCC GCC CAG GTG GCT CCA GCC TAA-3' (EagI)	mRh5: 5'-GTAGGGGACATGCTGAGGTGAGGC-3' (reverse) mRh5: 5'-GGAGCCTGCATGACCTCATCC-3' (reverse)	94°C 5 m, (94°C 30 s; 63°C 30 s; 72°C 60 s) 30 cycles, EagI digestion
S338/CSM	5'-GCC GCG GCC GCC GTT TCC AAG GCG GAG GCC GCC CAG GTG GCT CCA GCC TAA-3' (EagI)	mRh5: 5'-GTAGGGGACATGCTGAGGTGAGGC-3' (reverse) mRh5: 5'-GGAGCCTGCATGACCTCATCC-3' (reverse)	94°C 5 m, (94°C 30 s; 63°C 30 s; 72°C 60 s) 30 cycles, EagI digestion
S334/S338/CSM	5'-GCC TCT GCG GCC GTT TCC AAG GCG GAG GCC GCC CAG GTG GCT CCA GCC TAA-3' (EagI)	mRh5: 5'-GTAGGGGACATGCTGAGGTGAGGC-3' (reverse) mRh5: 5'-GGAGCCTGCATGACCTCATCC-3' (reverse)	94°C 5 m, (94°C 30 s; 63°C 30 s; 72°C 60 s) 30 cycles, EagI digestion

^a Coding region corresponding to nucleotides 6082–6129 of mouse opsin gene, GenBank accession number M5171. Nucleotide changes are indicated in bold. Restriction sites engineered in the transgene are underlined in the sequence and indicated in parentheses.

^b Primers used in the genotyping PCR reaction. Primer regions that hybridize specifically with the transgene modified sequence are underlined. All other primers recognize the endogenous and the heterologous mouse opsin genes with the same efficiency.

could reflect a slower rate of phosphorylation by RK and/or a slower rate of arrestin association due to the decrease in the number of available phosphorylation sites. Future experiments will investigate whether it is phosphorylation or arrestin binding that is rate limiting for recovery in these slowed responses.

Mutant rhodopsins containing fewer than three phosphorylation sites yielded single-photon responses that turned off abruptly after exponentially distributed waiting times of several seconds. The stochastic termination of responses in CSM, S338/CSM, and S334/S338/CSM rods suggests that the rate-limiting step in quenching these mutant rhodopsins is a memoryless, first-order process. The dramatic loss of reproducibility in the CSM, S338/CSM, and S334/S338/CSM mutants suggests that phosphorylation of three or more sites is required for reproducible rhodopsin deactivation and single-photon responses. Surprisingly, the relative reproducibility of the single-photon response was the same in all of the mutants with three or more phosphorylation sites (Table 3), at least within the limits of our measurements. We conclude that three phosphorylations are required for normal reproducibility of the single-photon response and that additional phosphorylations increase the rate of deactivation, apparently without affecting reproducibility.

Rhodopsin Deactivation Does Not Begin Prior to Phosphorylation

Transducin binding to R* can inhibit rhodopsin phosphorylation *in vitro* (Pfister et al., 1983; Miller and Dratz, 1984), suggesting that transducin sterically hinders access of RK to R*. However, more recent light-scattering studies have shown that RK can effectively compete with transducin for binding to R* *in vitro*, suggesting that the binding of RK to R* might itself reduce rhodopsin's ability to activate the G protein (Pulvermuller et al., 1993). Since RK should bind to CSM rhodopsin (Palczewski et al., 1991; Shi et al., 1995; Thurmond et al., 1997), one would expect the elementary response in RK^{-/-} rods to rise longer and reach a larger amplitude than that in CSM rods. In RK^{-/-} rods, the amplitude of the single-photon response was about 1 pA and the time to reach this amplitude was about 340 ms (Chen et al., 1999). In CSM rods, the time to reach this plateau amplitude was 346 ± 78 ms (n = 11), and the mean amplitude was slightly larger (1.53 ± 0.17 pA, n = 10). These results suggest that RK binding alone has little effect on rhodopsin's catalytic activity in the intact cell.

Model of Rhodopsin Deactivation

In summary, our results indicate that rhodopsin normally becomes multiply phosphorylated rapidly ($\tau \leq 200$ ms in a wild-type rod) under physiological conditions. The first phosphorylation occurs within 100 ms of the flash, limiting the amplitude of the response by a factor of ~2.5. Accumulation of at least three phosphates on rhodopsin's carboxy-terminal domain appears to be required to trigger a conformational change in arrestin, allowing it to bind phosphorylated R* and complete the quench of rhodopsin's catalytic activity. The speed of rhodopsin deactivation seems to be further enhanced by the accumulation of additional phosphates. These

results support a simple model for rhodopsin deactivation in which multiple steps incrementally reduce the catalytic activity of R*, contributing to the reproducibility of the single-photon response (Rieke and Baylor, 1998). The results of our study do not preclude other mechanisms for reproducibility, such as a prescribed sequence of phosphorylation events or calcium-dependent feedback to rhodopsin kinase. Multiple phosphorylation of the G protein-coupled receptor may prove to be a general strategy for rapid and reliable deactivation of signals in systems that require strict temporal performance.

Experimental Procedures

Generation of Transgenic Mice

Polymerase chain reaction (PCR)-mediated mutagenesis (Ausubel et al., 1995) was used to introduce mutations into an 11 kb BamHI murine genomic clone that encompasses the entire opsin gene, with 5 kb of upstream and 1.5 kb of downstream flanking sequences (Chen et al., 1995). The whole PCR-generated DNA fragment for each mutant (0.7 kb) was sequenced for verification. The DNA coding region corresponding to amino acids 333–348 of rhodopsin is indicated for each rhodopsin construct in Table 3. Alanine was chosen as the phosphorylation site replacement amino acid because a neutral substitution is less likely to disrupt protein secondary structure (Chothia, 1976; Rose et al., 1985). A conservative change that confers to murine opsin a linear epitope for mAb 3A6, A337V, was introduced in all mutants. Additional silent mutations were introduced in the transgenes to generate unique restriction sites that were used for genotyping and determination of mutant-to-endogenous transcript ratio (Mendez et al., 2000).

Transgenic mice were generated by standard procedures (Hogan et al., 1994), according to approved NIH and USC animal guidelines. Microinjection was performed on F2 hybrid zygotes from C57Bl/6J and DBA/2J F1 strains. Transgene-positive mice were identified by selective amplification of the transgene by PCR or by selective digestion of transgene-derived PCR amplification product. PCR primers and conditions used in the genotyping protocol are detailed in Table 3. Transgene-positive mice were bred to rhodopsin null homozygous mice (Lem et al., 1999). Following two rounds of breeding, mice positive for the transgene and homozygous null for rhodopsin were identified by Southern blot as previously described (Mendez et al., 2000).

Histology

For analysis of the retinal morphology by light microscopy, eyecups were fixed overnight in 2.5% glutaraldehyde, 2% paraformaldehyde, 0.1 M cacodylate buffer, pH 7.2, and embedded in epoxy resin. Transverse 1 μ m sections from central retina were stained with Richardson's stain (0.5% methylene blue, 0.5% Borax, 0.5% Azure II).

For immunocytochemical analysis, eyecups were fixed in 4% paraformaldehyde, 0.5% glutaraldehyde, 0.1 M cacodylate buffer, pH 7.2, for 2 hr at room temperature, washed in 0.1 M cacodylate buffer, and cryoprotected for 12 hr in 30% sucrose at 4°C. Eyecups were embedded in OCT (Tissue-Tek, Sakura Finetech, Torrance, CA) and sectioned at 10 μ m thickness. Sections were incubated in 0.1 M phosphate buffer containing 0.5% normal goat serum, 1% BSA, and 0.1% Triton X-100 for 1 hr at room temperature. Sections were then incubated for 2 hr with mAb R2-12N (Adamus et al., 1991), diluted 1:50 in 0.1 M phosphate buffer containing 1% BSA and 0.1% Triton X-100, and for 1 hr with FITC-conjugated goat anti-mouse IgG Ab (Vector Laboratories, Burlingame, CA) at a dilution of 1:100 in 0.1 M PBS and 0.1% Triton X-100.

In Situ Phosphorylation Assays

All mice for *in situ* phosphorylation assays were 1 month old. Mice were dark adapted for a minimum of 12 hr prior to use. Retinas were isolated under infrared light and incubated for 1 hr in 100 μ l of phosphate-free Krebs solution (120 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM CaCl₂, 10 mM glucose, 100 mM HEPES [pH 7.4]) containing 1.25 mCi/ml [³²P]H₃PO₄ (10 mCi/ml, ICN Pharmaceuticals,

Irvine, CA) in the dark to allow incorporation of ^{32}P in the endogenous ATP pool. Following incubation, retinas were either washed with Krebs buffer and immediately homogenized in urea buffer in the dark (6 M urea, 20 mM Tris [pH 7.4], 5 mM EDTA) or exposed to 3500 lx white fluorescent light for 5 min prior to homogenization. Membrane fractions were collected by centrifugation at 13,000 g for 20 min, washed with 20 mM Tris (pH 7.4), resuspended in 50 μl of buffer (80 mM Tris-HCl [pH 8.0], 10 mM EDTA, 4 mM MgCl_2 , 2 mM CaCl_2 , 0.5 mg/ml complete mini protease inhibitors [Boehringer Mannheim, Indianapolis, IN]), and incubated for 20 min with 20 units of DNaseI (Boehringer Mannheim) at room temperature. A fraction of each sample corresponding to 1/8 of a retina was loaded in duplicate 12% SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes (Protran, Schleicher and Schuell, Keene, NH), one of which was directly exposed to an x-ray film for detection of ^{32}P -labeled proteins and the other processed for Western blot using rhodopsin mAb R2-12N (Adamus et al., 1991).

Separation of Phosphorylated Rhodopsin Species by Isoelectrofocusing

One-month-old mice were dark adapted overnight. Retinas were dissected under infrared light, exposed to saturating white fluorescent light for 10 min, and immediately frozen in ethanol/dry ice. A control wild-type retina was frozen in the dark immediately after dissection. Two retinas per sample were thawed and homogenized in 400 μl homogenization buffer (25 mM HEPES [pH 7.5], 100 mM EDTA, 50 mM NaF, 5 mM adenosine, 0.5 mg/ml complete mini protease inhibitors [Boehringer Mannheim]). Membrane fractions were collected by centrifugation at 13,000 g for 30 min and resuspended in 1 ml regeneration buffer (10 mM HEPES [pH 7.5], 1 mM MgCl_2 , 0.1 mM EDTA, 2% BSA, 50 mM NaF, 5 mM adenosine, 100 μM 11-cis-retinal). Regeneration of rhodopsin was allowed to proceed at 4°C for a minimum of 12 hr. After regeneration, membrane fractions were pelleted and solubilized for 3 hr at 4°C in solubilization buffer (10 mM HEPES [pH 7.5], 1 mM MgCl_2 , 10 mM NaCl, 0.1 mM EDTA, 1% dodecyl maltoside [Calbiochem, La Jolla, CA]). The samples were centrifuged at 13,000 g for 30 min, and a fraction of the supernatant corresponding to 1/5 of a retina was analyzed by isoelectric focusing. Isoelectric focusing of rhodopsin was conducted in the dark, under previously described conditions (Adamus et al., 1988). After the run, the resulting pH gradient of the gel was determined using a flat electrode (Beckman Instruments, Fullerton, CA) and proteins were transferred to a nitrocellulose membrane by capillary action. Rhodopsin was detected by Western blot with mAb 4D2 (a generous gift from Robert Molday, University of British Columbia, Canada), which recognizes an epitope on the amino terminus of rhodopsin (Molday and MacKenzie, 1983).

Electrophysiology

All mice for physiology experiments were adults (35–60 days old) and were dark adapted for a minimum of 15 hr prior to use. Mice were anesthetized and sacrificed by CO_2 narcosis and decapitation, according to approved NIH and Stanford animal guidelines. Retinas were isolated under infrared light and stored on ice in oxygenated L-15 medium supplemented with 10 mM glucose and 0.15 mg/ml bovine serum albumin. Small pieces of retina were mechanically chopped and placed into the recording chamber under infrared light. The chamber was perfused with bicarbonate-buffered Locke's solution containing 112.5 mM NaCl, 3.6 mM KCl, 2.4 mM MgCl_2 , 1.2 mM CaCl_2 , 10 mM HEPES, 0.02 mM EDTA, 20 mM NaHCO_3 , 10 mM glucose, 3 mM sodium succinate, 0.5 mM sodium glutamate, and 0.1% vitamin and amino acids supplement (Sigma, St Louis, MO). Prior to an experiment, this external solution was bubbled with 95% O_2 /5% CO_2 , warmed to between 35°C and 37°C, and the pH adjusted to 7.4 with NaOH. The solution in the suction electrode contained 140 mM NaCl, 3.6 mM KCl, 2.4 mM MgCl_2 , 1.2 mM CaCl_2 , 3 mM HEPES (pH 7.4), 0.02 mM EDTA, and 10 mM glucose. The responses were low-pass filtered at 20 Hz with an eight-pole Bessel filter and acquired at 100 Hz using an Igor-based program written by Dr. Fred Rieke (University of Washington). Light intensity was calibrated using a silicon detector (UDT350, Graseby Optronics, Orlando, FL) and controlled with calibrated neutral density filters. Brief flashes (10 ms) of 500 nm light were used for stimulation.

The amplitude of each flash response was measured relative to the mean current during a 750 ms window preceding the flash. The form of each rod's single-photon response was estimated by squaring the mean response to at least 24 dim flashes of fixed strength and comparing the squared mean to the time-dependent ensemble variance. The time-dependent ensemble variance was also set to zero over the 750 ms window preceding the flash. The scaling factor that brings the rising phase of the mean squared response into alignment with the rising phase of the time-dependent variance provides an estimate of $1/n$, where n is the mean number of photoisomerizations per flash (Rieke and Baylor, 1998). The observed mean response was divided by n to estimate the mean amplitude and form of the single-photon response. The time integral of the single-photon response was divided by the peak amplitude to obtain the integration time (Baylor and Hodgkin, 1973).

To assess the effect of rhodopsin mutations on the reproducibility of the time course of the single-photon response, the squared mean response was scaled (as above) and subtracted from the time-dependent ensemble variance. If the variance due to the Poisson statistics of light absorption and that due to variations in response deactivation are independent and additive, the residual variance is an estimate of the fluctuations in the time course of shutoff of the dim flash response. The time integral of the residual variance was measured from the time of the flash to the time at which the mean response had recovered back to baseline. The time integral of the residual variance reflects inherent differences in the response recovery from trial to trial, but it is also directly proportional to the mean number of photoisomerizations (n), the mean duration of the flash response (integration time, t_i), and the peak amplitude of the single-photon response squared (r_p^2). Therefore, to obtain an estimate of the relative variation in the time course itself, we calculated the dimensionless index V , defined as:

$$V = \frac{\int \sigma_{\text{res}}^2(t) dt}{t_i n r_p^2},$$

where σ_{res}^2 is the residual variance.

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