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Marie E. Burns and Edward N. Pugh, Jr.
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Lessons from Photoreceptors: Turning Off G-Protein Signaling in Living Cells

Phototransduction in retinal rods is one of the most extensively studied G-protein signaling systems. In recent years, our understanding of the biochemical steps that regulate the deactivation of the rod’s response to light has greatly improved. Here, we summarize recent advances and highlight some of the remaining puzzles in this model signaling system.

Major Features of Rod Phototransduction

The absorption of photons by the G-protein-coupled receptor (GPCR), rhodopsin, in the outer segments of retinal rod photoreceptors activates a series of biochemical reactions, called the phototransduction cascade, which generates the electrical response to light and thus mediates the first steps in vision. The understanding of the molecular basis of phototransduction and its regulation has greatly expanded with the application of mouse gene-targeting techniques, which made it possible to knock out or otherwise perturb the phototransduction machinery of intact photoreceptors. With the adaptation of suction electrode recording to mouse rods in the mid-1990s in Denis Baylor’s laboratory, it became feasible to assess how the photoreceptor is affected by deletions, mutations, and transgenic overexpression of proteins thought to regulate the cascade. Many lines of mice in which phototransduction proteins have been knocked out or mutated have been characterized physiologically; some of these are given in Table 1. The goal of this review is to summarize recent findings in the deactivation of this prototypical G-protein cascade and to articulate several remaining questions. We begin by a brief introduction to the activation and deactivation steps in phototransduction and then focus on the quantitative physiological measures that provide kinetic insights into the biochemical events underlying response recovery in living photoreceptors, as exemplified by mouse rods.

Activation reactions are highly amplifying

In rods, the molecular machinery of the cascade is concentrated in the outer segment, a subcellular reaction chamber containing several thousand square microns of highly structured, protein-laden membranes, the disc stack (FIGURE 1A). The biochemical events that initiate signaling are known in detail (FIGURE 1B): the absorption of a photon isomerizes the 11-cis retinal chromophore of rhodopsin (120), triggering a conformational change to a catalytically active state [metarhodopsin II (R*)] within milliseconds. R* catalyze GDP/GTP exchange on multiple copies of the heterotrimeric G-protein transducin. Each activated transducin alpha subunit (Gα-GTP; hereafter G*), binds to the gamma subunit of the cGMP phosphodiesterase (PDEγ), relieving its inhibition of the PDE6 catalytic subunits (50, 122); the activated transducin-PDE6 complex (G*E*) hydrolyzes cGMP. The resulting fall in the cGMP concentration causes the cGMP-gated channels in the plasma membrane to close (21, 51), leading to membrane hyperpolarization that reduces the rate of glutamate release onto second-order retinal neurons. For a more detailed, historically developed perspective on the activation reactions, see other reviews (e.g., Refs. 1, 99).

The ability of rods to signal the absorption of single photons arises from amplification contributed by three primary molecular or biophysical mechanisms: 1) G-protein activation by R*; 2) hydrolysis of cGMP by G*-E*; 3) cooperative gating of the cGMP-gated channels. After photon capture, G-protein activation proceeds at a rate of ~150 s⁻¹ per R* in amphibian rods and more than twice that in mammalian rods (1, 42). PDE activation per R* is thought to proceed at very nearly the same rate (63). These high rates of G-protein and effector activation result in part from the rate constants of the reactions but also from the high density of G-proteins (~3,000 μm⁻²) and PDE molecules (~80 μm⁻²) in the disc membrane. At the next stage of amplification, each G*-E* hydrolyzes cGMP at a rate near the diffusion limit (kcat/Km ~ 10⁸ M⁻¹·s⁻¹) (63), causing the local cGMP concentration to fall quickly. An important biophysical contribution to amplification arises from the cytoplasmic reaction volume: the same number of G*-E* in a smaller outer segment more rapidly changes the cGMP concentration than it would in a larger volume. This reaction-volume effect provides a basis for the >100-fold higher amplification constant of slender mammalian vs. hefty amphibian rods (99). Finally, the cooperative activation of the cGMP-gated channels (Hill coefficient of ~3; Ref. 105) causes a given fractional change in cGMP...
concentration to produce a threefold larger fractional change in the inward current. In summary, the amplification needed for high sensitivity is determined primarily by the two enzymatic amplifiers, the activated GPCR, R*, and the activated phosphodiesterase complex, G*-E*, with a further boost from the cGMP channel cooperativity. Many deactivation reactions are required for normal recovery of the photoresponse

Because of the high amplification of phototransduction, relatively modest light intensities could easily drive rods into saturation, i.e., close all of the cGMP-gated channels were it not for timely deactivation. If the active lifetimes of R* and G*-E* are prolonged, this saturation occurs at lower light intensities, and when not in saturation a rod with a prolonged response is less able to signal rapid changes in illumination. Therefore, timely recovery of the photoreceptor is critical for maintaining sensitivity in steady light and for signaling increments and decrements of light intensity. In rods, the timing of response recovery is determined by regulatory proteins that act to 1) speed deactivation of R*, 2) speed deactivation of G*-E*, and 3) accelerate the rate of cGMP synthesis. In humans, mutations that cause defects in each of these three categories of biochemical reactions cause 1) nightblindness (2, 20, 30, 40, 130), 2) bradyopia (39, 77, 86), and 3) early onset Leber's congenital amaurosis and cone-rod dystrophy (48), respectively.

A vast literature has revealed rich details of the biochemical reactions that govern R* and G*-E* deactivation and cGMP synthesis, and parallel physiology experiments have sought to understand the relative rates of R* (kR) and G*-E* (kE) deactivation in living rods in this context (FIGURE 2). R* activity persists until it is quenched by two successive reactions: phosphorylation by rhodopsin kinase (GRK1) and the binding of the protein arrestin (Arr1; Refs. 61, 126). Likewise, G*-E* activity persists until the GTP bound to Gt/H9251 is hydrolyzed to GDP, a reaction that is speeded by RGS9-1, a retina-specific regulator of G-protein signaling (41), in a complex with Gt/H9252 and the membrane anchoring protein R9AP (47). Finally, guanylate cyclase activating proteins, or GCAPs (27, 36, 95), activate guanylate cyclases (GC-1 and GC-2) when intracellular calcium levels fall during the light-induced decline in inward current. Understanding the contributions of these biochemical steps to normal physiology requires manipulating the reactions in intact rods and quantitatively evaluating the kinetic consequences for the light response.

Table 1. Mouse genetic manipulations affecting rod phototransduction deactivation and response kinetics

<table>
<thead>
<tr>
<th>Gene</th>
<th>Refs.</th>
<th>Protein</th>
<th>Perturbation</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sag</td>
<td>129</td>
<td>Arrestin 1</td>
<td>Knockout</td>
<td>Delayed final recovery</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td>Transgenic</td>
<td>Restored knockout</td>
</tr>
<tr>
<td></td>
<td>110</td>
<td></td>
<td>Mutated transgenic</td>
<td>Restored knockout</td>
</tr>
<tr>
<td>Ar3/Arr4</td>
<td>14</td>
<td>Cone arrestin</td>
<td>Transgenic in Ar3 ko rods</td>
<td>Incomplete rescue</td>
</tr>
<tr>
<td>Grk1</td>
<td>10, 17</td>
<td>Rhodopsin kinase</td>
<td>Knockout</td>
<td>Long-lasting step-like responses</td>
</tr>
<tr>
<td></td>
<td>59</td>
<td>Transgenic overexpressor</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>Chimera</td>
<td>Slower recovery</td>
<td></td>
</tr>
<tr>
<td>Rho</td>
<td>13, 64</td>
<td>Rhodopsin</td>
<td>Underexpression</td>
<td>Faster kinetics - diffusion and volume effects</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>COOH-terminal deletion</td>
<td>Long-lasting step-like responses</td>
<td></td>
</tr>
<tr>
<td></td>
<td>121</td>
<td>Palmitoylation deletion</td>
<td>Shorter integration time</td>
<td></td>
</tr>
<tr>
<td>Rcvn</td>
<td>15, 68</td>
<td>Recoverin</td>
<td>Knockout</td>
<td>Shorter integration time</td>
</tr>
<tr>
<td>Rgs9</td>
<td>16</td>
<td>RGS9-1</td>
<td>Knockout</td>
<td>Slow, graded recovery</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>DEPless transgenic</td>
<td>Slow recovery like RGS9 ko</td>
<td></td>
</tr>
<tr>
<td></td>
<td>71</td>
<td>RGS9-2</td>
<td>Transgenic</td>
<td>Faster recovery to very bright flashes</td>
</tr>
<tr>
<td>Gnb5</td>
<td>58</td>
<td>Gβ5-L</td>
<td>Knockout</td>
<td>Slow recovery like RGS9 ko</td>
</tr>
<tr>
<td>Rgs9bp</td>
<td>54</td>
<td>R9AP</td>
<td>Knockout</td>
<td>Slow recovery like RGS9 ko</td>
</tr>
<tr>
<td></td>
<td>15, 59</td>
<td>Transgenic overexpressor</td>
<td>Much faster τrec and τD</td>
<td></td>
</tr>
<tr>
<td>Gucy2e</td>
<td>3, 131</td>
<td>GC1</td>
<td>Knockout</td>
<td>Increased sensitivity</td>
</tr>
<tr>
<td>Gucy2f</td>
<td>3</td>
<td>GC2</td>
<td>Knockout</td>
<td>Normal dark-adapted rod responses</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Double KO (GC1 and GC2)</td>
<td>No photoresponses; abnormal trafficking</td>
</tr>
<tr>
<td>Guca1a</td>
<td>11, 76</td>
<td>GCAP1</td>
<td>Double KO (GCAP1 and GCAP2)</td>
<td>Large, slow responses</td>
</tr>
<tr>
<td></td>
<td>46</td>
<td>Transgenic</td>
<td>Restores rod recovery in double ko</td>
<td></td>
</tr>
<tr>
<td>Guca1b</td>
<td>69</td>
<td>GCAP2</td>
<td>Knockout</td>
<td>Slow recovery; saturation in dim light</td>
</tr>
<tr>
<td></td>
<td>76</td>
<td>Transgenic in double GCAP knockout</td>
<td>Like GC1 ko</td>
<td></td>
</tr>
<tr>
<td>Pde6g</td>
<td>115</td>
<td>PDEγ</td>
<td>Knockout</td>
<td>Photoreceptor degeneration</td>
</tr>
<tr>
<td></td>
<td>116</td>
<td></td>
<td>Overexpression</td>
<td>Reduced gain; faster recovery in RGS9 ko background</td>
</tr>
<tr>
<td></td>
<td>117, 127</td>
<td></td>
<td>Phosphorylation site mutants</td>
<td>Modulated recovery by background light</td>
</tr>
<tr>
<td></td>
<td>114</td>
<td></td>
<td>W70A mutation</td>
<td>Reduced sensitivity and slower recovery</td>
</tr>
</tbody>
</table>
The Recovery of the Rod Photoresponse is Rate-Limited by a Single Process

Normal response kinetics need to be defined in native cells

The compartmentalization of phototransduction in the outer segment permits the light-induced changes in cGMP-gated current to be readily recorded by drawing an individual outer segment of an intact rod into a highly polished suction pipette electrode. The primary advantage of suction electrode recordings is that the integrity of the cell membrane and consequently the natural protein concentrations and their subcellular localizations are maintained. In contrast, in vitro assays that rely on cellular homogenates or reconstituted enzymes do not reproduce the more rapid kinetics observed in intact cells. The validity of using suction electrode recordings to determine deactivation kinetics from acutely isolated retinal slices is demonstrated by the remarkable agreement of the sensitivity and kinetics of rod responses recorded in Locke’s solution at 37°C to those recorded in vivo with electroretinographic methods (Figure 3).

The membrane currents recorded with suction electrodes provide a real-time readout of the cGMP concentration, owing to the rapid gating and voltage independence of the cGMP-gated channels at physiological membrane potentials. The time

FIGURE 1. The rod photoreceptor and the disc-associated proteins that mediate the response to light
A: rod photoreceptors are highly compartmentalized cells, with most organelles retained in the inner segment (IS) and synaptic ending (SE). The specialized outer segment (OS) is filled with a stack of intracellular membranous discs that house the transduction machinery. B: schematic of a portion of three disc membranes and plasma membrane shown in A, illustrating activation and deactivation reactions.
course of the cGMP concentration in turn can be further analyzed to derive the time course of PDE activity (44, 99). Conversely, theoretical modeling of the underlying biochemical reactions can be used to predict changes in cGMP concentration and thereby the time course of the photoresponse (e.g., Ref. 38).

Recovery time constant of dim and bright flash responses

Since the seminal investigation of Hodgkin and Nunn (44), it has been known that rod responses to both dim and moderately bright flashes recover to baseline along a time course that is exponential at adequately late times (83). For mouse rods, this exponential “tail phase” has a time constant of recovery (\(\tau_\text{D} \)) of \(\sim 200 \text{ ms} \).

The exponential recovery can also be revealed through the analysis of saturating responses (FIGURE 4). The time that a response to a bright flash remains in saturation (\(T_{\text{sat}} \), red circles in FIGURE 4A) is directly proportional to the logarithm of the flash strength, a graphical analysis colloquially termed a “Pepperberg plot” (FIGURE 4B; Ref. 83). The slope of the \(T_{\text{sat}} \) vs. flash strength relationship in this semilog plot measures the time constant \(\tau_\text{D} \) of the slowest kinetic step in photoresponse recovery, the so-called “dominant recovery time constant.” For the mouse, \(\tau_\text{D} \) is \(\sim 250 \text{ ms} \) for flashes producing up to \(\sim 1 \text{ R}^* \) per disc face (log-e isomerizations = 7.5 in FIGURE 4B). This value for \(\tau_\text{D} \) is close to \(\tau_\text{D} \) determined from the tail-phase recovery of dim flash responses and the time constant of recovery measured in vivo (FIGURE 3). The similarity of \(\tau_\text{D} \) and \(\tau_\text{D} \) in normal rods and in many genetic lines in which deactivation mechanisms have been perturbed suggests that the same biochemical step rate-limits recovery over an \(\sim 1,000\)-fold range, from a single photon up to \(\sim 1 \text{ R}^* \) per disc face.

Hypotheses regarding the mechanism underlying \(\tau_\text{D} \)

The molecular mechanism rate-limiting response recovery has been intensely investigated and debated since 1992. Theoretical analysis of the cascade reactions revealed that the exponential recovery could arise from either of two mechanisms, the deactivation of the G*-E* complex via GTP hydrolysis or the decay of R* activity (Ref. 83; FIGURE 2A). In the first case, R* would deactivate relatively rapidly, so that at the “long times” of the response tail phase the brief pulse of R* activity would have decayed completely, leaving only the slower deactivation of the G*-E* complexes to determine the recovery time course. In the second case, R* would continue to activate G* during the response tail phase, but the lifetimes of the resultant G*-E* complexes would be relatively short, so that the response recovery would track R* deactivation.

Early experiments aimed at identifying the biochemical step underlying \(\tau_\text{D} \) characterized its calcium dependence. Experiments of Lyubarsky et al. (67) showed that \(\tau_\text{D} \) was identical whether a rod’s internal calcium was clamped to its dark level or free to change during the light response. This result favored the identification of \(\tau_\text{D} \) with deactivation of G*-E*, which has no known calcium dependence. It also complemented earlier work by Matthews (73, 74), which had uncovered a short-lived calcium dependence of deactivation well before recovery of the current began; this latter dependence was hypothesized to be calcium-dependent regulation of R* deactivation and to be mediated by the calcium-binding protein recoverin (52). In contrast, other experiments led authors to suggest R* lifetime to be the rate-limiting step in recovery (98, 104), whereas still others favored the contrary conclusion that hydrolysis of GTP to GDP by G*-E* is rate limiting (57, 83, 106). These disagreements propagated through the analysis of many features of photoreceptors, including the molecular basis of reproducible single photon responses (38) and the molecular basis of the functional differences between rod and cone photoreceptors (24, 132). For many years, the principal difficulty in settling the
controversy was that most experimental manipulations abnormally prolonged deactivation, either by diluting endogenous regulators in biochemical experiments or by slowing the time course of physiological responses through pharmacological or genetic loss-of-function manipulations. What was needed was a targeted molecular manipulation that dramatically sped the rate-limiting step in deactivation and thus shortened the dominant time constant of recovery. Thus, if the targeted molecular mechanism were rate-limiting, speeding it up would shorten $\tau_{D}$, whereas were it not rate-limiting, further speeding it would not alter $\tau_{D}$.

**RGS9 Rate Limits the Recovery of the Normal Photoresponse**

The shortening of the dominant recovery time constant was finally achieved in experiments with rods that transgenically overexpressed R9AP, which in turn led to overexpression of the entire RGS9 complex (59). As mentioned above, the RGS9 complex (RGS9-1/Gβ5-L/R9AP) resides in the disc membrane (FIGURE 1), where it stimulates the GTPase activity of the G*-E* complex. Comparison of recovery kinetics for both dim and saturating responses (measurements of $\tau_{rec}$ and $\tau_{D}$, respectively) showed that overexpression of the RGS9 complex speeds response recovery in a dose-dependent manner (59). In rods in which RGS9-expression was approximately fourfold higher than wild-type levels, the recovery time constant for both dim and bright flash responses was sped roughly threefold, from 250 to 80 ms (59). In a more recent study, roughly sixfold overexpression of the RGS9 complex further reduced both time constants to 54 ms (15). These results establish that, for flashes activating from 1 to 4,000 R* in mouse rods, the rate-limiting step in recovery is RGS9-catalyzed GTP hydrolysis by the G*-E* complex.

To understand how the rate of G*-E* deactivation can be controlled by the RGS9 expression level, the RGS9-catalyzed GTP hydrolysis reaction needs to be considered in a framework of enzyme kinetics (FIGURE 2B). Generally, when an enzyme with Michaelis constant $K_m$ eliminates a substrate $S$, the terminal phase of substrate removal ($S < K_m$) is an exponential decay with time constant $1/(V_{max}/K_m)$, where $V_{max} = k_{cat}[\text{Enzyme}]$, i.e., the product of enzyme concentration and the turnover number of the enzyme. Remarkably, these simple ideas apply directly to RGS9-catalysis of G*-E* deactivation, with RGS9 identified as the enzyme and G*-E* as its substrate.

Incorporating a Michaelis scheme for RGS9 into a standard model of phototransduction (gray boxes in FIGURE 2) revealed that the dominant recovery time constant obeys $\tau_{D} = 1/(V_{max}/K_m) = 1/(k_{cat}[\text{RGS9}]/K_m)$ over most of the 20-fold range of RGS9 expression levels in the mice investigated by Krispel et al. (12). Thus the concentration dependence of $\tau_{D}$ on RGS9 expression level is a consequence of classic enzyme kinetics and reveals how the dominant time constant of the GPCR cascade can be dramatically tuned by changing the expression level of a single gene, in this case R9AP driving expression of the RGS complex.

**FIGURE 3.** Kinetics of responses recorded from mouse rods using suction electrodes closely match the kinetics of in vivo electroretinographic (ERG) recordings from mice

A: rod photoresponse extracted from paired-flash ERG experiments of Hetling and Pepperberg (symbols; Ref. 43; Figure 5B): flash produced a response of $\sim$55% the saturating a-wave amplitude corneal ERG a-waves (symbol). The black trace is a population average half-maximal response from 15 c57Bl/6 mouse rods recorded with suction electrodes; the flash strength was 48 photons/$\mu$m$^2$. Red curves are exponential functions, both with a 202-ms time constant. B: paired-flash mouse a-wave data of Lyubarsky and Pugh (1996) for a series of flashes of increasing strength that completely suppressed the a-wave (symbols) are compared with population average suction electrode recordings (smooth traces) from 15 mouse rods made in bicarbonate-buffered Locke’s solution at 37°C to flashes that ranged from 580 to 25,200 photons/$\mu$m$^2$ by factors of 2. The average maximal response amplitude for the suction electrode recordings in A and B was 16.5 pA; the average effective collecting area was 0.50 $\mu$m$^2$.
Mechanism of slowed recovery for flashes producing more than 1 R* per disc face
A mouse rod outer segment comprises ~800 membranous discs with 1,600 disc faces. Recovery slows dramatically when flashes produce in excess of 4,000 R* or ~2R* per disc face, as shown by a sharp break in the slope of the Pepperberg plot (FIGURE 4B). Recent work suggests that, for flashes producing 4,000–160,000 R*, the rate-limiting step is RGS9-catalyzed GTP hydrolysis of G* that is uncomplexed with E*, i.e., G* produced in excess of total PDE (71). Evidence for this came from experiments on mouse rods that expressed a brain-specific isoform of RGS9, RGS9-2, that does not require PDE (9253) for stimulating GTP hydrolysis of G*.

Unresolved questions about the RGS9 reaction in rods and cones
The shortest recovery time constant observed thus far with RGS9 complex overexpression is ~50 ms, corresponding to an overall GTPase rate constant of 1/1 τD = 20 s⁻¹ (15). Could 1/ τD be reduced further in rods with still higher expression levels? Theoretical analysis of the data of Krispel et al. (59) suggests that indeed should be the case: RGS9-catalyzed hydrolysis of G*-E* likely has a kcat in situ exceeding 50 s⁻¹ (12), a turnover generally consistent with biochemical data (4, 65). Thus the 1/ τD of rods could in theory become as short as 1/50 s⁻¹ or 20 ms, assuming other processes, like R* deactivation, are sufficiently fast. Likewise, could the higher expression level of the RGS9 complex in cones underlie their more rapid deactivation (132)? To date, the rates of cone opsin deactivation and G*-E* deactivation in cones remain undetermined.

Questions remain about the molecular steps that immediately follow RGS9-catalyzed GTP hydrolysis and, in particular, about the role of PDEγ in the G*-E* complex. For example, what are the structural changes that drive the dissociation of the G*-E*-RGS9 complex on GTP hydrolysis and thus permit the high turnover number of the RGS9 reaction? Furthermore, how rapidly does PDEγ re-inhibit the PDEβ catalytic subunits? It is unknown whether the binding of G* to PDEγ causes PDEγ to completely dissociate during activation or whether it merely opens up the catalytic site while remaining bound. Experiments in truncated toad rods showed no change in cellular dark noise associated with dialysis of exogenous PDEγ into the rod, leading to the conclusion that PDEγ does not completely dissociate (103). However, in mice, overexpression of PDEγ can decrease the amplitude and speed recovery of rods lacking RGS9, suggesting that the exogenous PDEγ can indeed compete with the endogenous protein (116). The extent of PDEγ dissociation may also depend on the adaptational state of the cell: biochemical studies have suggested that cGMP-dependent changes in the affinity of the GAF domain of PDEγ may indeed cause complete dissociation under some conditions, such as during light adaptation (23). Additional evidence for this change in affinity with light adaptation stems from experiments showing steady light can alter the phosphorylation state of PDEγ, which in turn alters the kinetics of the flash response (117, 127). Thus many questions remain about the role of PDEγ in the regulation of G*-E* activity in both dark and light adapted conditions.

FIGURE 4. Dark-adapted responses of a wild-type mouse rod
A: population average response family from a Sv129 mouse rod. Brief (10 ms) flashes were delivered at t = 0 s and ranged in strength from 7.78 to 85,600 photons/μm² by factors of 2–4. B: Pepperberg plot of wild-type mouse rods. The time that a bright flash response remained in saturation (τsat) is plotted as a function of the natural logarithm of the flash strength in photoisomerizations per flash (R*). The slopes of the fitted lines, 0.26 and 0.80 s⁻¹, give the dominant time constants of recovery over the respective range of flash strengths.
Evidence for Rapid Deactivation of the GPCR Rhodopsin

Steps in R* deactivation necessary for normal recovery in intact rods

Following activation by light, R* becomes phosphorylated by rhodopsin kinase (GRK1; Refs. 17, 66, 87, 91, 108, 109, 112, 126). The sites of phosphorylation are clustered at the COOH-terminus of rhodopsin, and either truncating the COOH-terminus entirely (19) or mutating these COOH-terminal serine and threonine residues to alanines (28, 75) leads to abnormally slow response recovery. Indeed, responses of rods expressing either the COOH-terminal rhodopsin truncation mutant or those expressing a rhodopsin mutant lacking all six of the potential phosphorylation sites are indistinguishable from those of rods in which GRK1 has been knocked out (17). Furthermore, biochemical studies suggest that at least three phosphoryl groups on rhodopsin’s COOH-terminus are required to trigger high-affinity binding of arrestin (Arr1; Refs. 31, 33, 34, 118, 125, 129), the subsequent step that fully quenches R*’s activity and prevents further activation of G-protein subunits (129). Multiple phosphorylation and Arr1 binding are likewise required for normal recovery, since rhodopsin mutants with fewer than three phosphorylation sites give responses that are abnormally slowed (28, 75).

R* deactivation is normally rapid

One of the major consequences of the identification of G*-E* deactivation as the rate-limiting step of recovery is that it necessarily follows that R* deactivation is still faster. However, the question remains of exactly how much faster. The dominant time constant of recovery for both dim and saturating responses (τ_{rec} and τ_{D}) provide strong constraints on the lifetime of R*: in the highest RGS9 overexpression attained thus far (approximately sixfold), the recovery time constant, and thus the upper limit for R* lifetime in dark-adapted rods, is 54 ms (15). Such a short R* lifetime is consistent with manipulations of R* deactivation that cause little or no change in τ_{D} or τ_{rec}, including overexpression of GRK1 (59, 123), knocking out the calcium-dependent inhibitor of GRK1, recoverin (68), and underexpression of Arr1 (10, 32, 129). Nonetheless, it is puzzling that these perturbations have little effect on the dim flash response, because changes in R* lifetime should produce corresponding changes in the total number of G*-E* and thus would be expected to alter the response amplitude. One possible resolution to this puzzle is that calcium feedback to guanylate cyclase dampens amplitude variation that would otherwise arise from different R* lifetimes.

Measuring R* lifetime. Given that R* deactivation is much more rapid than G*-E* deactivation, and the apparent insensitivity of the early phases of the response to changes in R* lifetime, how can one determine R* lifetime in the living rod? In a recent study, Gross and Burns (32) observed that a 50% decrease in Arr1 expression increased the absolute times that responses to strong flashes remained in saturation but did not change τ_{D}. This result is consistent with a lengthening of R* lifetime, without a change in τ_{D}, which remained determined by G*-E* deactivation. When Arr1 underexpression was accompanied by an increase in RGS9-overexpression (Arr1+/− RGS9-ox rods), an identical increase in the T_{sat} values was observed with no change in the 80-ms τ_{D}, indicating that even the slowed R* deactivation remained faster than 80 ms.

Gross and Burns also noted that the prolonged R* lifetime lengthened the apparent τ_{rec} in Arr1+/− RGS9-ox rods. As noted previously, normally τ_{rec} = τ_{D} indicating that one and the same process rate limits recovery from both dim and bright flashes. The reason τ_{rec} is longer than τ_{D} in Arr1+/− RGS9-ox rods appears to be straightforward: τ_{rec} only reflects the slower of τ_{R} and τ_{E} if the two time constants are sufficiently different from each other and τ_{rec} is extracted from the tail phase at sufficiently late times (31). When the two time constants become similar, as in Arr1+/− RGS9-ox rods, the “sufficiently late time” for dominance would only be reached well after the dim flash response has completely recovered. In contrast, τ_{D} in Arr1+/− RGS9-ox rods is extracted from responses at later times, after dominance has occurred (32).

Gross and Burns used the increase in the T_{sat} values for bright flash responses and the increase in τ_{rec} for dim flash responses to extract the rate of phosphorylation and Arr1 binding under normal conditions and conditions of reduced Arr1 expression. Using a scheme of photoresponse deactivation consistent with established biochemistry, they found that R* deactivation normally follows a single exponential decay with a time constant of ~40 ms, suggesting that phosphorylation and Arr1 binding proceed at effectively the same rate in wild-type rods. However, in rods expressing 50% of normal levels of Arr1, a single exponential time course for R* deactivation could not explain the experimental results. Rather, reduced Arr1 expression resulted in data best described by an R* decay with two time constants in which the first time constant was the same as for wild-type and represented phosphorylation by GRK1 (τ_{E} = 40 ms), whereas the second time constant was slower and represented slowed Arr1 binding (τ_{E}/H9270^{+}) = 67 ms). Given the association rate constant for Arr1 binding of ~1 μM^{-1}s^{-1} (101), the calculated binding rate for Arr1 in normal rods is consistent with
an effective Arr1 concentration of 28 μM, which is similar to previous biochemical estimates of Arr1 monomer in bovine rods (35).

R* deactivation is speeded during light adaptation

Rhodopsin phosphorylation is inhibited by the protein recoverin in a calcium-dependent manner in vitro (18, 52, 55), providing a mechanism by which rhodopsin lifetime can be regulated by intracellular calcium levels. As discussed above, calcium clamp experiments in salamander rods show that τD is not calcium sensitive (83), whereas another faster mechanism, presumably R* deactivation, is dependent on the calcium concentration at the time of the flash (73, 74, 81). Consistent with the idea that R* deactivation is normally very rapid, mouse rods lacking recoverin have the same τD as wild-type rods. However, the time that a bright flash response remains in saturation is dramatically reduced in rods lacking recoverin, consistent with a shorter R* lifetime. Recoverin knockout rods also fail to exhibit the “step-flash” effect, the shortening of response duration in the presence of steady light (68), supporting the notion that recoverin prolongs R* lifetime and that this prolongation is relieved during adaptation. Very strong evidence for the role of recoverin in directly controlling R* lifetime was provided by a recent study by Chen et al. (15). These authors made use of the transgenic overexpression of RGS9 to speed G*-E* deactivation and, in addition, slowed R* lifetime by expressing a GRK1 chimera with reduced enzymatic activity. Together, these manipulations made the R* lifetime rate-limiting for recovery. In these rods in which R* was rate limiting, background light shortened τD, but only in the presence of recoverin. The remarkable conclusion to be drawn from the cumulative results of all of these studies is that, in dark-adapted rods, recoverin prolongs R* lifetime by inhibiting GRK1, resulting in the “long” 40-ms R* lifetime. When calcium falls during light adaptation, this inhibition is relieved, and the lifetime of R* is shorter still. Precisely how much shorter remains to be determined.

Unresolved issues in R* deactivation

Given the importance of R* deactivation for maintaining the balance between signal amplification and rapid, reliable recovery, understanding the molecular details of R* deactivation in intact rods remains one of the most fundamental unresolved issues in phototransduction. How can R* deactivation, which requires phosphorylation by GRK1 and the binding of Arr1, occur within ~40 ms and be faster still when recoverin’s inhibition is relieved during light adaptation?

How many phosphoryl transfers occur during deactivation of R* in the intact rod?

Biochemical studies of light-dependent rhodopsin phosphorylation have reported widely varying stoichiometries. Although many in vitro studies have reported stoichiometries in excess of six phosphates per R* (e.g., Refs. 80, 124, 126), in vivo studies have reported that only one or a few phosphates get incorporated (e.g., Refs. 49, 88), at least on a time scale comparable to the time course of the flash response (53). The individual contributions of phosphorylation and Arr1 binding to R* deactivation in intact, normal rods have been difficult to determine because phosphorylation not only increases rhodopsin’s binding affinity for Arr1 (31, 33, 60, 118) but also itself reduces R* activity (94, 129).

Control of GRK1 activity.

Following photoactivation of rhodopsin to R*, GRK1 itself can become “activated” by R*, leading to the phosphorylation of non-photoexcited rhodopsins, a process referred to as “high-gain phosphorylation” (6, 8, 25, 26, 78, 79, 92, 109). In addition, autophosphorylation of GRK1 (62) lowers its affinity for phosphorylated R* (9, 102), and mutagenesis of GRK1’s autophosphorylation sites increases phosphorylation of rhodopsin in vitro (93), supporting the idea that autophosphorylation normally limits the extent or rate of rhodopsin phosphorylation by triggering dissociation. Thus it may be the dissociation of GRK1 from R* that determines the timing of arrestin binding. The activity of GRK1, as well as its counterpart in many mammalian cones, GRK7, is reduced by phosphorylation by cAMP-dependent kinase (45), and in cones dephosphorylation of GRK7 accompanies light exposure, suggesting that GRK7 activity is regulated during light adaptation (90). As yet, there have been no physiological investigations of GRK1 modulation by phosphorylation.

Despite the rapid rate of R* deactivation in rods, there are suggestions that the rate of deactivation may be higher still for cone opsins. GRK7 has higher specific activity for rhodopsin than GRK1 in vitro (119). Furthermore, the cone visual pigments have higher rates of spontaneous decay (107) and seem to rely on phosphorylation (85) and arrestin (Arr4; Ref. 84) binding to lesser extents than rhodopsin. Whether cone pigment deactivation is likewise subject to calcium-dependent modulation by recoverin during light adaptation remains unknown, but recoverin is expressed in cones and does bind to GRK7 (113).

Does Arr1 movement into the outer segment alter R* deactivation?

Prolonged steady illumination that cumulatively bleaches only a few percent of all of the rhodopsin molecules is sufficient to cause light-dependent movement of Arr1 into the outer segment, an intriguing phenomenon that has
been intensely studied in recent years (e.g., Refs. 82, 89, 96, 111). A drastic increase in total Arr1 concentration could reduce the rate at which R* activates the G-protein transducin by competition, thereby decreasing phototransduction gain and thus decreasing response amplitude. In addition, a higher level of Arr1 could shorten R* lifetime by decreasing the time needed for Arr1 to bind following phosphorylation. Theoretically, this could also affect response amplitude by decreasing the number of G-proteins that any given R* could activate during its (even briefer) lifetime. However, because high-affinity Arr1 binding to R* first requires multiple phosphorylation, increased Arr1 levels presumably could not shorten R* lifetime beyond that determined by the rate of phosphorylation.

**Role of Calcium Regulation of cGMP Synthesis in Shaping the Photoresponse**

**Molecular components of calcium-dependent guanylyl cyclase regulation**

The deactivation reactions discussed above are only part of the story: for the photoresponse to recover, cGMP concentration must be restored to its pre-flash levels. In rods, cGMP synthesis is maintained by two guanylyl cyclases, GC1 and GC2 (3), whose activities are regulated by calcium-sensitive, GC-activating proteins or GCAPs (in mouse rods, only GCAP1 and GCAP2). In normal rods, the light-induced decline of cGMP that leads to closure of some of the cGMP-sensitive channels causes a reduction in calcium influx. Because the Na\(^+\)/Ca\(^{2+}\)_exchanger continues to extrude calcium, the reduced calcium influx leads to a reduction in intracellular Ca\(^{2+}\). This decline in Ca\(^{2+}\) promotes dissociation of calcium from GCAPs, leading to activation of GC and an increased rate of cGMP synthesis. The increased rate of cGMP synthesis acts to more rapidly restore cGMP levels, opposing the light-induced decline triggered by the G*-E* activity. Loss of either GC1 or GC2 causes little change in rods, indicating functional redundancy at maintaining the physiological level of cGMP (3, 131). However, GCAP1 activates GC1 more efficiently than GC2, and GCAP1 and GCAP2 have slightly different calcium sensitivities, causing them to have different time courses and potencies in regulating cyclase activity in rods (69, 76). As a result, in normal mouse rods, the physiological consequences for calcium feedback activation of cGMP synthesis affect both the time course of the flash response (dynamic feedback) as well as the steady-state level of current in the presence of continuous illumination (static feedback).

**Dynamic and static feedback**

Calcium-dependent activation of cGMP synthesis during the dim flash response produces a sharply sculpted recovery phase shortly after the peak of the response. Evidence that this sharp initial downstroke just after the peak (the “chop”) in mouse rod responses arises from GC activation comes from the pronounced absence of this feature in rods loaded with the calcium buffer BAPTA (114) and in rods in which both GCAP1 and GCAP2 were knocked out (GCAPs\(^{-/-}\); Refs. 11, 76). The comparison of flash responses of wild-type and GCAPs\(^{-/-}\) rods clearly illustrates this sculpting effect: the single photon responses (SPRs) of GCAPs\(^{-/-}\) rods begin to diverge from those of wild-type by 40 ms after a flash, peak later, and recover more slowly (11). When only GCAP1 is expressed in rods, photoresponses do show the “chop” (46, 69), whereas rods that express only GCAP2 do not (76). These results indicate that rapid calcium feedback through GCAP1/GC1 sculpts the amplitude and time course of the dim flash response near the peak. However, GCAP2 also shapes the photoresponse dynamically, although at slightly later times, so that the integration time and recovery are affected in mouse rods lacking only GCAP2 (69). These results indicate that both GCAP1 and GCAP2 regulate GC activity, even during the presumably small changes in intracellular [Ca\(^{2+}\)] that occur during the SPR. Since the apparent K\(_{1/2}\) of calcium for GCAP2 is ~50 nM (69), the involvement of GCAP2 during the single photon response suggests that the local changes in calcium that are produced during the single photon response are larger and/or faster than what is predicted by calcium imaging of the bulk cytoplasm in response to a bright flash (128). Larger and more rapid changes in calcium might also arise due to the close proximity of the cGMP-sensitive channels and the NCKX exchanger (5). This remains an important unresolved issue in photoreceptor physiology, since the time course of calcium feedback is important for regulating sensitivity, light adaptation, and perhaps even reproducibility of the SPR.

It is precisely the dynamic, regulated feedback to cGMP synthesis that enables photoresponse recovery to track G*-E* deactivation. The power and speed of the GC-GCAP-calcium feedback is such that during the tail phase of the flash response the decaying G*-E* activity is effectively in equilibrium with GC activity so that the photoresponse recovery of normal rods closely tracks the decay of the light-evoked PDE activity (11, 44, 83). In addition, steady-state GC-GCAP feedback plays a crucial role in allowing the rod to operate in higher steady illumination than would be possible in the absence of such feedback (56, 100). In
mouse, this effect is ~13-fold; thus steady illumination suppresses a given fraction of the cGMP current of GCAPs’ rod s at 13-fold lower levels than in wild-type rods (11). The loss of GCAP2 causes rods to be more sensitive than normal to steady light and to lose roughly half of this static feedback control that normally extends their operating range in steady light (69). Thus both GCAP1/GC1 and GCAP2/GC2 serve distinct but overlapping roles resulting from their differences in Ca2+ sensitivity.

**Efficient Signal Integration: The Single Photon Response**

**Reproducibility in the framework of fast R* deactivation**

The SPR of vertebrate rods has been investigated for more than 30 years and generally found to be “unexpectedly” reproducible. The expectation of high variability arose in part from the realization that deactivation of a single R* would necessarily be stochastic. Historically, much of the research on the reproducibility of the rod SPR has taken place in a framework in which the deactivation of R* was considered rate limiting for recovery. In such a framework, accounting for the observed reproducibility has been argued to require many stochastic steps sequentially diminishing the activity of R*, which would include multiple phosphorylation events and subsequent Arr1 binding (28, 29, 38, 104).

SPR reproducibility remains poorly understood, especially in the context of a short R* lifetime. In an investigation by Bisgena and colleagues (7), it was found that longitudinal diffusion of cGMP contributes spatio-temporal filtering that materially improves predicted reproducibility. In addition, reproducibility similar to that observed experimentally could be achieved with three graded steps in R* deactivation, two phosphoryl transfers by GRK1, followed by Arr1 binding. Thus explaining SPR reproducibility does not require the postulation of a large number of R* deactivation steps, nor that the overall R* lifetime dominates recovery.

**Signaling is efficient when R* deactivation is fast**

Given that the active lifetime of R* in situ in mouse rods is ~40 ms (32) and that G*-E* deactivation in wild-type rods is normally ~250 ms, the ratio of the average lifetimes of the two enzymatic amplifiers, R* and G*-E*, stands as ~6:1. An interesting feature of this ratio is that it implies that the GPCR signaling is highly efficient (12): at the time to peak of the light response (125 ms), >70% of the E* that have been produced are simultaneously active. In contrast, in a situation in which the two lifetimes are reversed, the E* activity is <20% efficient—thus most of the E* produced would not contribute to signaling but would be “lost” by rapid hydrolysis before the response reaches its maximum.

**Overall Summary**

The identification of deactivation of the G-protein-effector complex as the slowest step in rod response recovery has resolved a long-standing controversy about the phototransduction cascade in living rods. This resolution has sharpened the focus on other unresolved problems in photoreceptor physiology, including the kinetics and molecular details of the mechanisms underlying the brief (<50 ms) lifetime of photoactivated rhodopsin R* and the molecular and biophysical processes that contribute to the reliability of the single photon response. The resolution has also revealed how the concentration of an RGS, acting through classic enzyme kinetics, can precisely control the principal time constant of a GPCR cascade.

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**References**


