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RGS Expression Rate-Limits Recovery of Rod Photoresponses

Report

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Summary

Signaling through G protein-coupled receptors (GPCRs) underlies many cellular processes, yet it is not known which molecules determine the duration of signaling in intact cells. Two candidates are G protein-coupled receptor kinases (GRKs) and Regulators of G protein signaling (RGSs), deactivation enzymes for GPCRs and G proteins, respectively. Here we investigate whether GRK or RGS governs the overall rate of recovery of the light response in mammalian rod photoreceptors, a model system for studying GPCR signaling. We show that overexpression of rhodopsin kinase (GRK1) increases phosphorylation of the GPCR rhodopsin but has no effect on photoresponse recovery. In contrast, overexpression of the photoreceptor RGS complex (RGS9-1·Gβ5L·R9AP) dramatically accelerates response recovery. Our results show that G protein deactivation is normally at least 2.5 times slower than rhodopsin deactivation, resolving a long-standing controversy concerning the mechanism underlying the recovery of rod visual transduction.

Introduction

Signaling through heterotrimeric G proteins is a ubiquitous mechanism in cell biology. Despite the intense study of processes that regulate G protein pathways (e.g., G protein receptor kinases, arrestins, regulators of G protein signaling [RGSs]), it is unknown which regulatory steps determine the overall duration of the resulting intracellular responses. Even in the well-studied G protein cascade of vertebrate rod photoreceptors, where many molecular perturbations have been found to slow photoresponses (reviewed in Makino et al., 2003), the identity of the biochemical step that dictates the time course of the response under normal conditions has been unclear. In rods, timely recovery of the photoresponse requires efficient deactivation of both rhodopsin and the G protein transducin, with the slower of these two steps determining the overall rate of response recovery (Nikonov et al., 1998). While the results of some experiments have been interpreted to imply that rhodopsin lifetime is the slowest, or rate-limiting, step in recovery (Pepperberg et al., 1992; Rieke and Baylor, 1998), results of other studies have been interpreted to support GTP hydrolysis by transducin as the rate-limiting step (Sagoo and Lagnado, 1997; Nikonov et al., 1998; Krispel et al., 2003). This controversy clouds the understanding of transduction, for example, by confounding the interpretation of experiments focused on other fundamental photoreceptor attributes, such as the molecular basis of reproducible single-photon responses (Hamer et al., 2003).

A major difficulty in resolving this controversy is that most experimental approaches that have investigated these mechanisms have abnormally prolonged deactivation and slow the recovery of physiological responses. Such manipulations include "knocking out" rhodopsin kinase (Chen et al., 1999) and RGS9-1 (Chen et al., 2000), as well as a number of other pharmacological and molecular biological manipulations (e.g., Sagoo and Lagnado, 1997). Manipulations that lengthen the lifetime of rhodopsin or transducin cannot resolve which of these two species' deactivation normally rate-limits recovery, since a process that is not rate-limiting can become so when its kinetics are slowed. Settlement of the controversy requires a specific manipulation that accelerates the rate-limiting step in intact cells. The theoretical rationale for this approach was developed several years ago by Nikonov et al. (1998), and the experimental feasibility addressed subsequently in the biochemical study by Kennedy et al. (2003). Here, we have addressed this question systematically by overexpressing (and thus increasing the total activity of) the two regulators that control rhodopsin and transducin deactivation in intact mouse rods and comparing the time courses of the flash responses.

Results and Discussion

RK Overexpression Does Not Accelerate Photoresponse Recovery

Deactivation of rhodopsin requires phosphorylation by rhodopsin kinase (RK, or GRK1) and the binding of arrestin, both of which normally occur on the timescale of the flash response (Xu et al., 1997; Chen et al., 1999). It is unlikely that arrestin binding rate-limits response recovery because lowered expression of arrestin has no effect on photoresponse duration (Xu et al., 1997; Burns et al., 2006). On the other hand,

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reduced expression of rhodopsin kinase does slow recovery of the light response (Chen et al., 1999). To determine whether the converse could also occur, i.e., whether overexpression of RK could accelerate response recovery, we generated RK transgenic mice that overexpressed bovine RK in rod photoreceptors. The expression level of RK in transgenic animals was determined by quantitative Western blotting (Figure 1), and four lines with various levels of RK overexpression were selected for analysis. No changes in the expression levels of other key phototransduction proteins were observed (see Figure S1 in the Supplemental Data), nor were there significant changes in photoreceptor number or structure (Figure S2 and Experimental Procedures). If the binding of RK rate-limits rhodopsin deactivation and if rhodopsin deactivation is rate-limiting for photoresponse recovery, overexpression of RK should accelerate response recovery. In contrast, if RK binding is normally not rate-limiting, increased RK levels should have little effect on response kinetics (Nikonov et al., 1998).

To determine whether increased RK expression affected the response time course, we recorded from individual intact rods from each mouse line using suction electrodes. The average dim flash responses of wild-type and RK-overexpressing rods were very similar (Table 1; Figure 2). To quantify response recovery in each rod, we fitted the final falling phase of the average dim flash response with a single exponential function. On average, the recovery time constant (τ_{rec}) was not significantly different for responses of wild-type rods and rods that overexpressed RK ($p = 0.24$ for Line 1; $p = 0.12$ for Line 2; Table 1).

Because rods signal over a range of light intensities, we also studied the recovery kinetics of bright flash responses. Bright flash responses remain in saturation for characteristic times that depend on the flash strength, with the initial slope of the relation between the time in saturation and the natural log of the flash strength corresponding to the dominant time constant of recovery (τ_D; Pepperberg et al., 1992; Lyubarsky et al., 1996). The value of τ_D is similar to that of τ_{rec} in wild-type mouse rods, suggesting that the slowest step in response recovery occurs at the same rate under both dim- and bright-light conditions (Chen et al., 2000). Increasing the expression level of RK did not shorten τ_D (Table 1, Figure 2D; $p = 0.63$ for Line 2); in fact, 2-fold RK

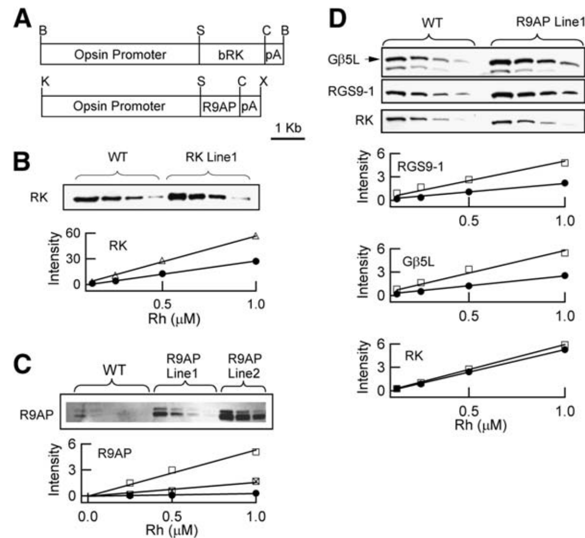

Figure 1. Incorporation of Transgenes Produces Protein Overexpression

(A) Transgenic constructs used to overexpress RK and R9AP in mouse photoreceptors. B: BssHII, C: ClaI, K: KpnI, S: SalI, X: XbaI, pA: polyadenylation signal.

(B) Representative Western blot quantifying the expression of RK in retinal homogenates of wild-type and RK transgenic mice (RK Line 1). For each line, 5, 2.5, 1.25, and 0.625 pmol rhodopsin was loaded. The relative expression of RK, calculated as the ratio of the slopes of the transgenic and wild-type relations (bottom), was 2.4-fold higher than that of wild-type (WT).

(C) Western blot showing the expression of R9AP in homogenates of wild-type and two R9AP-transgenic lines (R9AP Line 1 and R9AP Line 2), loaded as in (B).

(D) Quantification of RGS9-1 and Gβ5L protein levels in retinal homogenates. The slopes of the transgenic and wild-type relations (bottom) were 2.3 for both RGS9-1 and Gβ5L, and 1.1 for RK, indicating 2.3-fold higher expression for the RGS9 complex and normal expression of RK.

overexpression lengthened τ_D very slightly ($p = 0.051$; Table 1 and Figure 2E). Thus, overexpression of RK did not alter the rate-limiting step for recovery over a wide range of flash strengths.

The inability of RK overexpression to accelerate response recovery was not due to a defective transgenic RK, as evidenced by two control experiments. First, we assessed the ability of the transgene to rescue the

Table 1. Kinetic and Sensitivity Parameters of Transgenic Lines

	Dim flash τ_{rec} (ms)	τ_D (ms)	Time to peak (ms)	I_d (pA)	Io (Φ μm^{-2})	Flash sensitivity (pA/(Φ μm^{-2}))	Elementary amplitude (pA)
Wild-type	190 ± 9 (33)	246 ± 13 (29)	126 ± 9 (33)	12.8 ± 0.4 (34)	49 ± 3 (33)	0.2 ± 0.02 (33)	0.53 ± 0.05 (32)
RGS9-ox Line 2 (4-fold)	74 ± 8 (31)	80 ± 5 (27)	111 ± 5 (31)	14.9 ± 0.6 (33)	54 ± 3 (31)	0.19 ± 0.02 (31)	0.48 ± 0.05 (29)
RGS9-ox Line 1 (2-fold)	117 ± 13 (20)	108 ± 10 (20)	110 ± 7 (20)	14.0 ± 0.6 (24)	60 ± 5 (21)	0.18 ± 0.02 (20)	0.47 ± 0.08 (19)
RK-ox Line 1 (2-fold)	170 ± 12 (30)	284 ± 15 (27)	127 ± 6 (30)	12.9 ± 0.4 (36)	39 ± 3 (28)	0.24 ± 0.03 (28)	0.66 ± 0.10 (28)
RK-ox Line 2 (4-fold)	159 ± 21 (13)	256 ± 20 (9)	119 ± 18 (13)	13.5 ± 0.8 (14)	52 ± 6 (12)	0.27 ± 0.07 (13)	0.61 ± 0.18 (12)
RK transgene in *RK*$^{-/-}$	258 ± 14 (19)	270 ± 17 (17)	134 ± 22 (19)	14.2 ± 0.8 (21)	48 ± 5 (19)	0.24 ± 0.04 (19)	0.60 ± 0.05 (17)
RK$^{-/-}$	5979 ± 1030 (28)	ND	337 ± 30 (19)*	10.8 ± 0.2 (19)*	ND	ND	0.91 ± 0.10 (19)*

(Φ) Photons.
(I_d) Maximal response amplitude.
(Io) Flash strength that elicited a half-maximal response.
(*) Values taken from Chen et al. (1999).

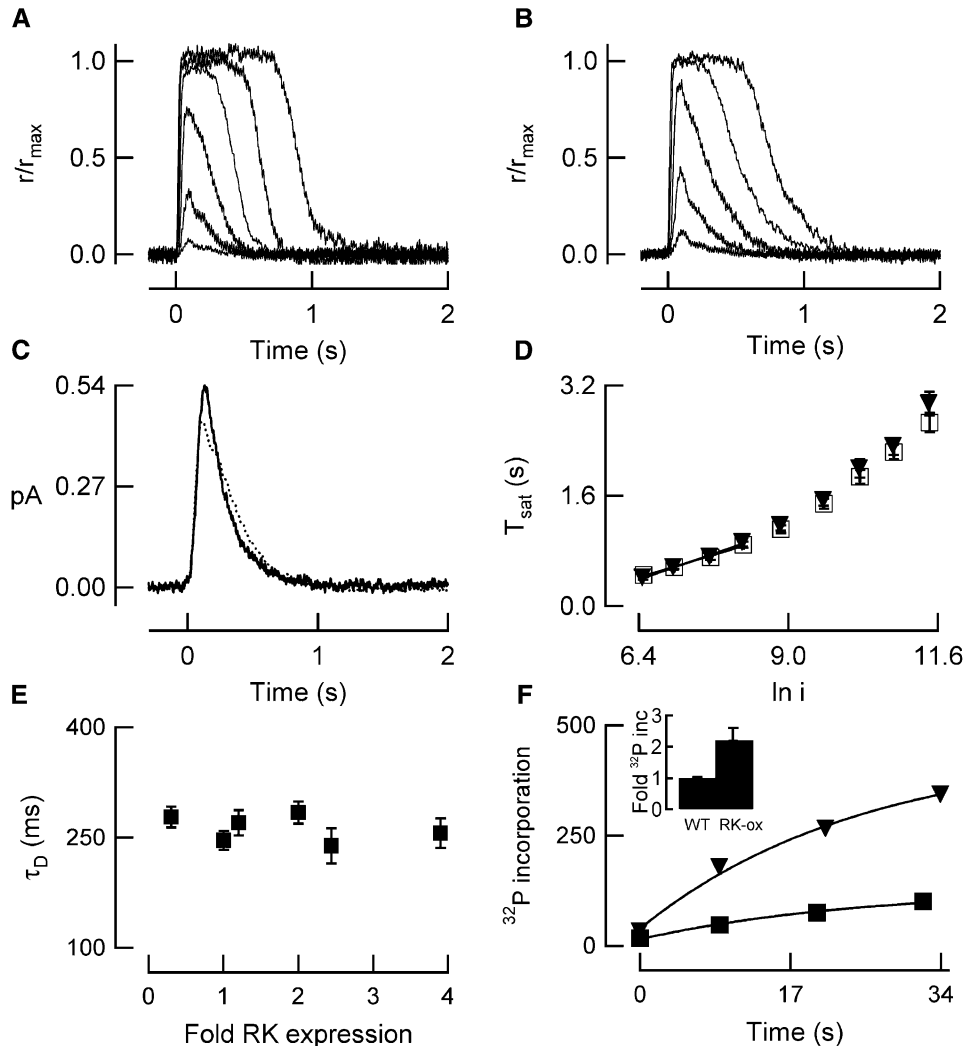

Figure 2. Overexpression of Bovine RK Transgene Has No Effect on Response Recovery

(A) Representative responses of a wild-type rod to flashes (500 nm, 10 ms, delivered at t = 0) that varied from 8 to 4000 photons/μm^2 by factors of 4. Dark current was 12.1 pA.

(B) Representative responses from an RK-overexpressing rod (RK Line 1). Dark current was 13.5 pA.

(C) Population mean single-photon responses for wild-type (dashed line; n = 31) and RK-overexpressing (RK Line 1, solid line; n = 27) rods.

(D) The time that a bright flash response remained saturated (T_{sat}) increased with the natural log of the flash strength (i, in photons/μm^2) for 30 wild-type (squares; τ_D = 250 ms) and 28 RK-overexpressing (RK Line 1, triangles; τ_D = 288 ms) rods. τ_D is given by the initial slope of this relation (see text).

(E) RK expression has no effect on τ_D. Each point represents a population of rods from a different transgenic line. Average parameters for the two highest-expressing lines (RK Line 1 and RK Line 2) are given in Table 1.

(F) Representative time course of the light-dependent rhodopsin phosphorylation in wild-type (squares) and RK-overexpressing (RK Line 1, triangles) rod outer segments. ^{32}P incorporation is shown in arbitrary units. The amount of ^{32}P incorporation is averaged from four independent wild-type and six independent RK-overexpressing (RK-ox) experiments, and shown in the inset.

Error bars = SEM.

phenotype of rods of RK knockout ($RK^{-/-}$) mice. The lack of rhodopsin phosphorylation in $RK^{-/-}$ rods lengthens rhodopsin's active lifetime and greatly prolongs flash responses (Chen et al., 1999). Transgenic expression of RK at levels comparable to that of endogenous RK in wild-type rods resulted in responses that recovered ten times faster than those of $RK^{-/-}$ rods, and had an average τ_D that was not significantly different from that of wild-type responses (Table 1, p = 0.25). Thus, the transgenic RK effectively reversed the physiological $RK^{-/-}$ phenotype. Second, we performed phosphorylation assays on purified rod outer segments from wild-type and RK-overexpressing retinas, and found that the rate of rhodopsin phosphorylation following a full bleach was significantly increased by RK overexpression (Figure 2F). On average, a 2-fold increase in RK expression was accompanied by an ~2-fold increase in the rate of rhodopsin phosphorylation in vitro (Figure 2F, inset). Thus, the transgenically-expressed RK was able to phosphorylate and deactivate rhodopsin, but was not able to speed response recovery. We therefore conclude that RK binding to photoexcited rhodopsin does not rate-limit flash response recovery in normal, dark-adapted rods.

Photoresponse Recovery Is Rate-Limited by RGS9

Normal recovery of the light response also requires timely hydrolysis of the terminal phosphate of GTP from the activated α subunit of transducin (Chen et al., 2000), which is catalyzed by the GTPase-activating complex consisting of RGS9-1 (He et al., 1998), the obligate subunit Gβ5L (Makino et al., 1999), and the membrane-anchoring protein R9AP (Hu and Wensel, 2002). The hydrolysis of GTP to GDP on transducin has also been hypothesized to be the slowest step in recovery of the light response (Sagoo and Lagnado, 1997; Nikonov et al., 1998; Krispel et al., 2003). To directly test whether transducin deactivation is normally rate-limiting for response recovery, we needed to overexpress all three members of this RGS9 complex. Although RGS9-1 and Gβ5L transgenes were unable to drive complex expression (Chen et al., 2003; and data not shown), we were able to increase the expression of all three members of the complex by introducing a transgene for R9AP (Figures 1A, 1C, and 1D). In retinas of different transgenic lines, the expression of R9AP ranged from 3- to 14-fold higher than that of wild-type and was accompanied by an increase in RGS9-1 and Gβ5L protein levels (1.3- to 4-fold in different lines). Within a given line, the extent of RGS9-1 and Gβ5L overexpression was similar (Figure 1D). These results suggest that R9AP is essential for stable complex formation and that R9AP expression determines the cellular concentration of the entire RGS9 complex. This requirement for R9AP is consistent with the loss of RGS9-1 protein in *R9AP*$^{-/-}$ rods and substantially lowered expression in *R9AP*$^{+/-}$ rods (Keresztes et al., 2004), and likewise consistent with the largely unaltered RGS9-1 levels in *RGS9*$^{+/-}$ and *Gβ5*$^{+/-}$ rods, which have normal levels of R9AP (Chen et al., 2000, 2003). Overexpression of the RGS9 complex did not alter the expression level of any of the other major phototransduction proteins (Figure S1) and was not associated with changes in photoreceptor number or structure (Figure S2 and Experimental Procedures).

In contrast to rods that overexpressed RK, rods that overexpressed the RGS9 complex 4-fold over the endogenous levels (R9AP Line 2) showed flash responses that recovered significantly faster than those of wild-type rods (Table 1, Figure 3; for τ_{rec}, $p = 7 \times 10^{-11}$ and for τ_D, $p = 6 \times 10^{-13}$). The rising phases and peak amplitudes of the dim flash responses were unaltered (Table 1, Figures 3C–3D; $p = 0.18$), consistent with the activation stages of transduction being unaffected by the overexpression. Comparison of the recovery kinetics in six different lines that expressed the GTPase-activating complex at various concentrations revealed a striking dependence of response recovery kinetics on the relative expression level. As the expression level of the RGS9 complex increased, both τ_{rec} for dim flash responses and τ_D for bright flash responses decreased. The relation between τ_D and expression level (Figure 3F) was well-fitted by a single exponential function that approached an asymptote of 77 ms. The curve for τ_{rec} was also well-fitted by an exponential function approaching an asymptote of 78 ms (Figure 3F, inset). The asymptotic nature of the relation indicates that, at the very highest levels of expression, the responses can be speeded no further by additional increases in RGS9 complex expression. Thus, either GTP hydrolysis has been sped maximally or the next slowest step in cascade deactivation has become rate-limiting. We conclude that, under normal conditions, deactivation of the G protein by the endogenous levels of the RGS9 complex is the slowest step in cascade recovery, occurring with a time constant of 0.2 s in normal rods. The second slowest step has a time constant of < 80 ms, which sets an upper limit for the average lifetime of rhodopsin.

Rate-Limiting of Recovery by RGS9 Is Consistent with Previous Findings

The identification of GTP hydrolysis catalyzed by the RGS9 complex as the rate-limiting step of the rod photoresponse is generally consistent with the findings of previous investigations in which manipulations targeting rhodopsin deactivation either did not affect, or resulted in minimal changes to, τ_D. Rhodopsin phosphorylation by RK is inhibited by recoverin in a calcium-dependent manner in vitro (Kawamura, 1993; Chen et al., 1995; Klenchin et al., 1995), and theoretical analysis has predicted that the decline in intracellular free calcium concentration that accompanies light adaptation should decrease the lifetime of rhodopsin in the living rod (Hamer, 2000; Nikonov et al., 2000). The finding that the mechanism underlying τ_D is *not* calcium-dependent (Nikonov et al., 1998), while other evidence shows that a faster deactivation mechanism *is* calcium dependent (Matthews, 1995, 1997; Murnick and Lamb, 1996; Nikonov et al., 2000), strongly supports the identification of rhodopsin deactivation as the faster of the two deactivation steps of the disc-associated reactions of the transduction cascade. In contrast, some perturbations of rhodopsin deactivation, including genetic deletion of recoverin (Makino et al., 2004) and mutation of rhodopsin's palmitoylation site (Wang et al., 2005), have slightly shortened τ_D. However, these studies have not demonstrated a dose-dependence of the hypothesized deactivation mechanism on τ_D (Figure 3F), nor have they been characterized in quantitative detail in a manner that excludes cell-to-cell variation or side-effects of the manipulation as explanations. Notably, these manipulations of rhodopsin deactivation did shorten the integration time of dim flash responses (Makino et al., 2004; Wang et al., 2005), which likely reflects the influence of calcium feedback mechanisms (Burns et al., 2002; Makino et al., 2004) and nondominant time constants on the overall time course of dim flash responses.

In sum, our experiments are broadly consistent with a substantial body of evidence supporting the hypothesis that rhodopsin deactivation by RK is a "nondominant" calcium-dependent deactivation mechanism. Our experiments contribute to this evidence, showing that rhodopsin's active lifetime in mammalian rods must be brief (80 ms or less). Determination of rhodopsin's lifetime in the mammalian rod and identification of the molecular mechanisms underlying it (e.g., RK binding, multiple phosphoryl transfers, or arrestin binding) remain important topics for future investigations.

Implications for Other Aspects of Rod Signaling

Rapid rhodopsin deactivation has several significant implications for rod signaling. First, a relatively short

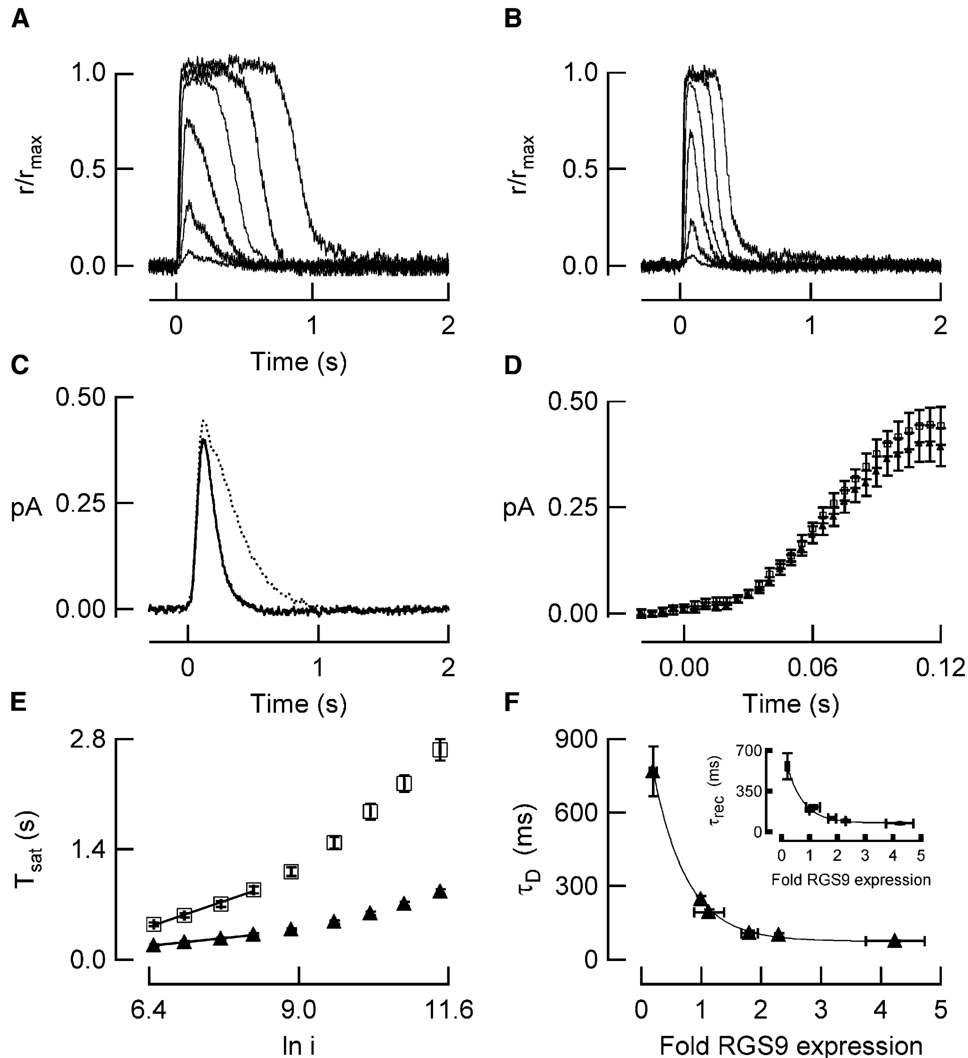

Figure 3. Expression Level of the RGS9 Complex Determines Time Course of Photoresponse Recovery

(A) Family of responses from a representative wild-type rod (same as Figure 2A).

(B) Family of responses from a representative RGS9-overexpressing rod (R9AP Line 2). Dark current was 15.9 pA.

(C) Population mean single-photon responses for wild-type (dashed line; n = 31) and RGS9-overexpressing (R9AP Line 2, solid line; n = 29) rods.

(D) (C) on an expanded time scale.

(E) Measurement of τ_D from 30 wild-type (squares, as in Figure 2D; τ_D = 250 ms) and 26 RGS9-overexpressing rods (R9AP Line 2, triangles; τ_D = 75 ms).

(F) τ_D (main panel) and τ_{rec} (inset) decreased with increasing expression of the RGS9 complex. Each point represents the population average from 10–38 rods from a different transgenic mouse line. The average parameters of the two highest-expressing lines (R9AP Line 1 and R9AP Line 2) are given in Table 1. The exponential fit approaches an asymptote of 77 ms and 78 ms for τ_D and τ_{rec}, respectively.

Error bars = SEM.

rhodopsin lifetime means that few G proteins become activated following photon absorption. Biochemical and electrophysiological experiments indicate that the transducin activation by a fully active R* proceeds at a rate of $\sim$120 s^{-1} in amphibian rods at room temperature (Leskov et al., 2000). Assuming that the rate is roughly 2-fold higher at mammalian body temperature (Heck and Hofmann, 2001), an 80 ms average lifetime for rhodopsin means that no more than $\sim$20 G proteins become activated on average following the absorption of a single photon. This estimate is 5-fold lower than the generally held value of over a hundred (c.f. Makino et al., 2003). Second, rapid rhodopsin deactivation highlights the remarkably small variation in the rising phases and peak amplitudes of single-photon responses (Rieke and Baylor, 1998; Whitlock and Lamb, 1999), and provides an important constraint for analysis of the molecular mechanisms that account for the overall reproducibility of the rod's single-photon response (e.g., Hamer et al., 2003).

RGS Proteins as Regulators of Precision Timing in GPCR Signaling

Finally, our results provide strong evidence that RGS proteins are not simply negative regulators of signaling pathways, but rather act as precision timers whose expression levels determine the duration of physiological responses. Because mammalian cones express

10-fold higher levels of RGS9-1 than rods (Cowan et al., 1998; Zhang et al., 2003), it seems likely that the faster response recovery in cones arises at least in part from the high expression level of the RGS9 complex (Zhang et al., 2003). The expression levels of many other neuronal RGS proteins are altered by neural activity, such as electroconvulsive seizures (Gold et al., 2002), administration of drugs of abuse (Traynor and Neubig, 2005), and by some forms of synaptic plasticity (Ingi et al., 1998). Additionally, abnormal RGS activity is associated with deficits in visual perception of low-contrast motion (Nishiguchi et al., 2004), and has been reported in a range of human diseases including schizophrenia (Mirnics et al., 2001) and Parkinson's disease (Tekumalla et al., 2001). Thus, regulation of RGS expression may dynamically adjust the duration of cascade signaling for a myriad of biological processes and suggests that the expression level in specific tissues may be an important target for therapeutic intervention in neural timing disorders.

Experimental Procedures

Generation of Transgenic Mice

Full-length bovine rhodopsin kinase, or R9AP cDNA, was engineered by PCR to be flanked by SalI and ClaI sites and cloned into the pRho4.4 vector (Li et al., 2005) between the 4 kb mouse opsin promoter and the 0.6 kb mouse protamine polyadenylation signal. Transgenic constructs were sequence verified, gel purified after restriction by BssHII (for RK) or KpnI/XbaI (for R9AP), and injected into C57BL/6 x SJL embryos. Transgenic founders were identified by PCR using a common forward primer RH1.1: 5′-TCAGTGCCTGGA GTTGCGCTGTGG and transgene specific primers RKb1: 5′- TCGTC GGCCGTGTCGTAGTCCTCG (for RK) and R9AP-230: 5′-CACCCA GAGCCGCTCAAACTC (for R9AP). The PCR conditions used were: 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min. The product sizes were 550 bp and 360 bp for RK and R9AP founders, respectively. All founders (13 for RK and 20 for R9AP) were backcrossed to a C57BL/6 background to remove the rd1 allele intrinsic to the SJL strain and are currently maintained in a mixed SJL and C57BL/6 background with wild-type alleles at the Pde6b locus. $R9AP^{+/-}$ mice were obtained by breeding R9AP knockout mice (Keresztes et al., 2004) with C57BL/6.

Physiology

Mice were reared in cyclic light, and dark adapted overnight prior to an experiment. Mice were euthanized with CO_2 and decapitated under infrared light. One retina was removed and placed in Ringer's solution (130 mM NaCl, 3.6 mM KCl, 2.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM HEPES, 0.2 mM EDTA) for Western blotting, while the second retina was placed in Leibowitz's medium (L-15; Invitrogen) on ice for single-cell recording. Methods and solutions for suction electrode recording from mouse rods are described elsewhere in detail (Keresztes et al., 2004).

A dim flash response is defined as a response whose amplitude does not exceed 20% of the maximal response amplitude, the upper limit of the linear range. The form of the single-photon response was estimated by squaring the mean response to a series of at least 30 dim flashes of fixed strength, and scaling it so that the initial rising phase coincided with that of the time-dependent ensemble variance. The scaling factor is an estimate of $1/n$, where n is the number of photoisomerizations per flash (Rieke and Baylor, 1998). The mean response was then divided by n to yield the estimated mean response to a single photoisomerization. The effective collecting area for each cell was also calculated empirically by dividing n by the flash strength used to elicit the dim flash responses (in photons μm^{-2}; Baylor et al., 1979). The effective collecting areas of wild-type (0.43 ± 0.04 μm^2, n = 32) and transgenic rods (RK Line 1: 0.41 ± 0.04 μm^2, n = 28, p = 0.73; R9AP Line 2: 0.46 ± 0.04 μm^2, n = 30, p = 0.65) were not different.

The time constant of the decline in the $Na^+/Ca^{2+},K^+$ exchange current was measured in cells with dark currents that ranged from 13.0–15.0 pA. A single exponential was fitted to the slow secondary rise of the mean response to bright flashes that held the cell in saturation for a minimum of 0.5 s. The time constant of the light-induced decline in exchange current was not significantly different between wild-type (188 ± 24 ms, n = 7) and transgenic rods (RK Line 1: 167 ± 28, n = 5, p = 0.53; R9AP Line 2: 181 ± 19, n = 6, p = 0.80).

Western Blotting

A retina in Ringer's solution was homogenized by sonication and kept on ice. An aliquot was solubilized in 70 mM n-octylglucoside containing 50 mM hydroxylamine (pH 7.4) and the rhodopsin content determined by difference spectroscopy at 500 nm before and after a full bleach, using 40,500 as the extinction coefficient for rhodopsin. The homogenate was diluted with sample buffer to a stock concentration of 1.0 pmol/μl rhodopsin. For comparison of protein expression levels, wild-type homogenates were processed side by side with transgenic homogenates; each were subjected to 2-fold serial dilutions, run on SDS-PAGE and Western blotted using the following antibodies: mouse anti-RK (MA1-720, Affinity Bioreagents); rabbit anti-RGS9 (CT-318; Chen et al., 2000), rabbit anti-Gβ5 (CT-215; Watson et al., 1994); sheep anti-R9AP (affinity purified sheep antibody against the CHAPRRPLVRTGVTG peptide of mouse R9AP). Signals were detected with the Li-Cor Odyssey system using secondary antibodies conjugated to IR dyes (Molecular Probes).

Phosphorylation Assays

Rod outer segments (ROS) were purified in darkness by vortexing retinas in 2% OptiPrep (Sigma) and centrifuging through a gradient of 8%, 12%, 16%, and 20% OptiPrep for 20 min. This centrifugation yielded three distinct bands. The middle and bottom band were collected, filtered, pelleted and washed with Ringer's solution. The resulting pellet was stored dry in the dark at −80°C. For phosphorylation assays, the ROS in the pellet were opened with 40 μl water in darkness and the rhodopsin concentration determined by difference spectroscopy described above. The final reaction buffer was composed of 10 mM rhodopsin, 11.9 mM phosphate buffers, 137 mM NaCl, 2.7 mM KCl, and 3 mM $MgCl_2$. A 20 μl ROS sample containing 10 μM rhodopsin was fully bleached with room light and stored on ice. Within 10 min of bleaching, the sample was brought to room temperature and phosphorylation was initiated by adding 10 μl of 200 μM [γ-^{32}P] ATP (20 μCi; Perkin Elmer). The reaction was terminated by adding 5 μl aliquots of the mixture to 1x SDS-PAGE sample buffer. Samples were subjected to SDS-PAGE and electrotransferred to a PVDF membrane (Biorad). The radioactivity of the blotted protein bands was analyzed on a STORM PhosphorImager (Amersham Biosciences). Phosphorylated ROS from wild-type and transgenic mice containing identical rhodopsin concentration were run side by side on the same gel for analysis. Total rhodopsin per lane was double-checked following ^{32}P detection using immunoblot analysis of the same PVDF membrane, which confirmed that equal rhodopsin levels were loaded per lane. Extent of phosphorylation of rhodopsin in darkness was comparable to the background and less than 10% of that for light-activated rhodopsin.

All p values were obtained from two-tailed t tests assuming equal variances. Throughout, error bars indicate SEM.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/51/4/409/DC1/.

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