Rhodopsin kinase and recoverin modulate phosphodiesterase during mouse photoreceptor light adaptation

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Light stimulates rhodopsin in a retinal rod to activate the G protein transducin, which binds to phosphodiesterase (PDE), relieving PDE inhibition and decreasing guanosine 3′,5′-cyclic monophosphate (cGMP) concentration. The decrease in cGMP closes outer segment channels, producing the rod electrical response. Prolonged exposure to light decreases sensitivity and accelerates response kinetics in a process known as light adaptation, mediated at least in part by a decrease in outer segment Ca^{2+}. Recent evidence indicates that one of the mechanisms of adaptation in mammalian rods is down-regulation of PDE. To investigate the effect of light and a possible role of rhodopsin kinase (G protein–coupled receptor kinase 1 [GRK1]) and the GRK1-regulating protein recoverin on PDE modulation, we used transgenic mice with decreased expression of GTPase-accelerating proteins (GAPs) and, consequently, a less rapid decay of the light response. This slowed decay made the effects of genetic manipulation of GRK1 and recoverin easier to observe and interpret. We monitored the decay of the light response and of light-activated PDE by measuring the exponential response decay time (τ_{REC}) and the limiting time constant (τ_D), the latter of which directly reflects light-activated PDE decay under the conditions of our experiments. We found that, in GAP-underexpressing rods, steady background light decreased both τ_{REC} and τ_D, and the decrease in τ_D was nearly linear with the decrease in amplitude of the outer segment current. Background light had little effect on τ_{REC} or τ_D if the gene for recoverin was deleted. Moreover, in GAP-underexpressing rods, increased GRK1 expression or deletion of recoverin produced large and highly significant accelerations of τ_{REC} and τ_D. The simplest explanation of our results is that Ca^{2+}-dependent regulation of GRK1 by recoverin modulates the decay of light-activated PDE, and that this modulation is responsible for acceleration of response decay and the increase in temporal resolution of rods in background light.

INTRODUCTION

Light-stimulated rhodopsin (Rh*) activates the rod heterotrimeric G protein transducin by facilitating exchange of GTP for GDP on the transducin guanine–nucleotide-binding site (see Fain, 2014). Transducin-GTP then binds to an inhibitory γ subunit of phosphodiesterase (PDE), releasing inhibition and activating PDE to hydrolyze cGMP, the second messenger controlling the photoreceptor light-dependent channels. Transducin turns itself off by hydrolyzing bound GTP to GDP with a rate that is greatly accelerated by a GTPase-accelerating protein (GAP) complex consisting of three components: RGS9-1, Gβ5-L, and R9AP (see Arshavsky and Wensel, 2013). Transducin-GDP is then released from the PDE γ subunit, extinguishing PDE activation.

Sensory receptors adapt in the presence of maintained stimulation, but the mechanism of adaptation remains unresolved. In mammalian rods, adaptation seems to be produced by modulation of the synthesis and hydrolysis of cGMP. Considerable evidence indicates a role for Ca^{2+}-binding guanylyl cyclase–activating proteins (GCAPs; see Arshavsky and Burns, 2012; Morshedian and Fain, 2014), in the following way. Light activates PDE, which decreases cGMP, reduces channel conductance, and decreases outer segment Ca^{2+}. The decrease in Ca^{2+} reduces Ca^{2+} binding to the GCAPs, stimulating guanylyl cyclase to increase cGMP synthesis and oppose the decrease in cGMP produced by light.

Although the GCAPs clearly contribute, rods still show considerable adaptation in constant light or after bleaches in rods for which the GCAPs have been deleted (Mendez et al., 2001; Burns et al., 2002; J. Chen et al., 2010; Nymark et al., 2012). We (Woodruff et al., 2008; J. Chen et al., 2010) and others (Soo et al., 2008) have proposed that the decrease in cGMP produced by light is also countered by negative regulation of PDE activity, producing an important additional component

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Abbreviations used in this paper: GAP, GTPase-accelerating protein; GCAP, guanylyl cyclase–activating protein; GRK1, G protein–coupled receptor kinase 1; PDE, phosphodiesterase; Rh*, light-stimulated rhodopsin.

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of adaptation (see Fain, 2011; Morshedian and Fain, 2014). Background light can decrease the limiting time constant (τ_D) of response decay (Woodruff et al., 2008), which under the conditions of our experiments directly reflects light-dependent acceleration of the decay of PDE (Krispel et al., 2006; Tsang et al., 2006; C.K. Chen et al., 2010). Rods lacking GCAP proteins show large current overshoots after steady light exposure (Burns et al., 2002; J. Chen et al., 2010), which are most likely caused by a transient increase in cGMP concentration. We believe that this increase in cGMP is produced by a decrease in the rate of spontaneous and light-activated PDE, either through direct modulation of PDE itself or one of the other proteins controlling PDE activity such as transducin or the GAP proteins. A detailed model of adaptation including both cyclase and PDE regulation can account for all of the changes in sensitivity and waveform of rods in background light (J. Chen et al., 2010).

How is PDE activity controlled? Our experiments suggest that rhodopsin kinase (G protein receptor kinase 1 [GRK1]) and the Ca^{2+}-binding protein recoverin, in addition to their well-known roles in phosphorylating and turning off light-activated rhodopsin, may also alter the rate of PDE decay by phosphorylating some component of the PDE–transducin–GAP complex. Overexpression of GRK1 or deletion of recoverin can shorten τ_D, and recoverin deletion eliminates the acceleration of response decay by background light (Chen et al., 2012). Our results differ from those of Krispel et al. (2006), Sakurai et al. (2011), and Gross et al. (2012), who also recorded from mouse rods with varying degrees of increased GRK1 expression but did not observe a significant effect on the limiting time constant and decay of light-activated PDE. To resolve this discrepancy, we reasoned that effects on response kinetics might be easier to observe if the decay of the rod response was further slowed by underexpressing the GAPs.

In this paper, we show in GAP-underexpressing rods that background light produces a systematic decrease in τ_D that is linear with the decrease in circulating current, but there is little change in τ_D if recoverin has been deleted. Moreover, in GAP-underexpressing rods, overexpression of GRK1 and recoverin deletion both produce large and highly significant reductions of the limiting time constant τ_D. Because under the conditions of our experiments the limiting time constant is a direct reflection of the decay of PDE (Krispel et al., 2006; Tsang et al., 2006; C.K. Chen et al., 2010) and is not affected by the decay of Rh*, which is much too rapid to limit the decay of the rod light response (Burns and Pugh, 2010), our results strongly suggest that GRK1 may also act at targets in addition to Rh*, and that Ca^{2+}-dependent regulation of rhodopsin kinase by recoverin is largely responsible for acceleration of the decay of the rod light response in background light.

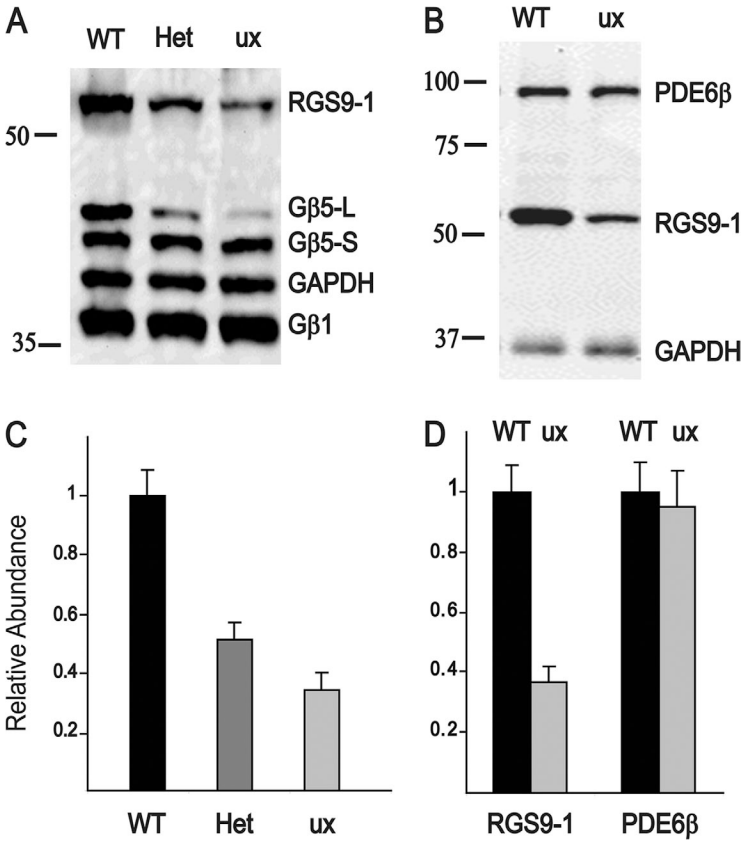

Figure 1. Reduction of transducin GAP level in *GAPux* mouse retinas. As shown previously by Keresztes et al. (2004), inactivating one copy of the R9AP gene leads to a noticeable reduction of transducin GAP level. (A) Representative immunoblot simultaneously probed for RGS9-1, Gβ5-L, Gβ5-S, Gβ1, and GAPDH in 10 µg of retinal extracts derived from WT, R9AP heterozygous knockout (Het), and compound R9AP and RGS9-1 heterozygous (ux) mice. (B) Representative immunoblot simultaneously probed for RGS9-1, PDE6β, and GAPDH in WT and ux retinal extracts. (C) Quantification of RGS9-1 level for experiments described in A, showing in Het (middle bar) and ux (right bar) mouse retinas a decrease to 51 ± 3 and 34 ± 3 (mean ± SEM) percent of WT level (left bar). GAPDH level was used for normalization. Similar degree of reduction was seen in Gβ5-L level but not in Gβ5-S or Gβ1 level (not depicted). (D) Quantification of PDE6β expression relative to GAPDH level in experiments of B showed a comparable level in ux retinal extracts to that of WT at 95 ± 7%, while RGS9-1 level dropped to 37 ± 3% (n=3). Error bars are SEMs.

MATERIALS AND METHODS

Transgenic mice

WT mice were C57BL/6 from The Jackson Laboratory. Homozygous R9AP knockout mice (Keresztes et al., 2004) were provided by V. Arshavsky (Duke University, Durham, NC). They were mated with WT C57BL/6J mice to produce heterozygous *R9AP+/−* mice with about half the transducin-GAP level in the retina (see Results). To reduce the transducin-GAP level further to below 50%, we generated compound heterozygous mice that were *R9AP+/−* and *RGS9-1+/−* by mating individual heterozygous knockouts, genotyping, and comparing GAP expression levels in resulting offspring. In compound *R9AP+/−* and *RGS9+/−* heterozygous knockouts, the GAP level could be reliably reduced to ∼34% (see Fig. 1). Genotypes of these various lines were determined by PCR before electrical recoding with procedures described previously (see, for example, Krispel et al., 2006). All experiments were performed on pigmented mice of either sex in accordance with the rules and regulations of the National Institutes of Health guidelines for research animals, as approved by the institutional animal care and use committees of the Virginia Commonwealth University and the University of California, Los Angeles. Animals were kept in cyclic 12/12 h on/off lighting in approved cages and supplied with ample food and water. Animals in all experiments were killed before tissue extraction by approved procedures, usually CO_2 inhalation or decerebration.

Antibodies

Rabbit anti-Gβ5 (CT-215), anti–RGS9-1 (CT318), and anti-Gβ1 (BN-1) antibodies were provided by M. Simon (California Institute of Technology, Pasadena, CA). Rabbit anti-GAPDH antibody was obtained from Cell Signaling Technologies. Mouse anti-PDE6B antibody and horseradish peroxidase–conjugated secondary antibodies were from Santa Cruz Biotechnology, Inc.

Immunoblotting

Retinal extracts (10 µg) were resolved by 12% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 10% dry milk in TBST buffer containing 25 mM Tris, pH 7.5, 137 mM NaCl, and 0.05% Tween-20. For the detection of both forms of Gβ5, CT215 was used at a 1:4,000 dilution. To detect RGS9-1, CT318 was used at a 1:4,000 dilution. For Gβ1, BN-1 was used at a 1:50,000 dilution. Anti-R9AP antibody was used at a 1:500 dilution. For PDE6β, the mouse antibody was used at a 1:500 dilution. Anti-GAPDH antibody was used at a 1:100,000 dilution, and the GAPDH signal was used as a loading control. Species-specific secondary antibodies were used at a 1:25,000 dilution. The signal was detected by enhanced chemiluminescence with the SuperSignal West Dura substrate kit (Thermo Fisher Scientific). Gel images were captured and quantified in an imaging station (IS440; Kodak) with an accompanying 1-D image analysis program (Kodak).

Electrophysiology

Methods for making suction-electrode recordings from mouse rods have been given previously (C.K. Chen et al., 2010; Chen et al., 2012). Rods were perfused at 37°C with Dulbecco's modified Eagle's medium (D-2902; Sigma-Aldrich), supplemented with 15 mM $NaHCO_3$, 2 mM Na succinate, 0.5 mM Na glutamate, 2 mM Na gluconate, and 5 mM NaCl, bubbled with 95% O_2/5% CO_2, pH 7.4. Unless otherwise indicated, data were filtered at 35 Hz (eight-pole Bessel) and sampled at 100 Hz. Flashes of 500-nm light at 20 ms in duration were attenuated to different light levels by absorptive neutral density filters. A 500-nm light was also used for background illumination. Other information about the details of response presentation are given in the figure legends. The values of τ_D were measured as in Woodruff et al. (2008) by giving a series of five flashes at each of between four to seven intensities chosen for each rod to fall within one and a half log units above the flash intensity that just produced saturation of that rod's response amplitude. The time in saturation (Tsat) was measured as the time from the beginning of the flash to the time at which the mean circulating current recovered to 25% of its dark-adapted value. Single-photon responses were calculated from the squared mean and variance as described previously (Chen et al., 2000; Tsang et al., 2006). Unless otherwise stated, errors are given as SEM, and significance was tested either with ANOVA or Student's *t*. Curve fitting, statistical tests, and plotting of data were done with the program Origin (OriginLab).

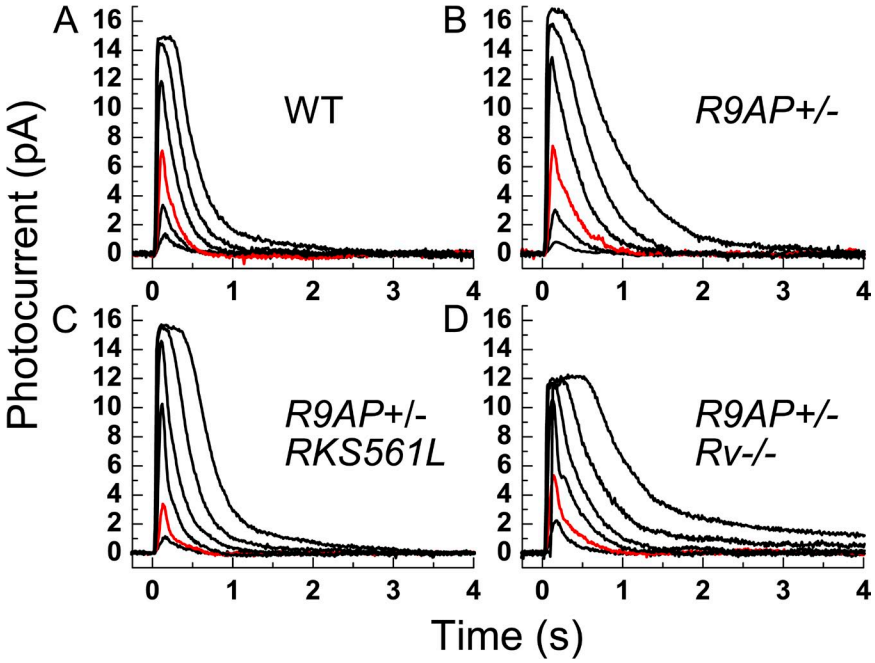

Figure 2. Comparison of mean response waveform of WT, *R9AP+/−*, *R9AP+/−;RKS561L*, and *R9AP+/−;Rv−/−* rods to 20-ms flashes given at t = 0 for each rod type at the following light intensities (in photons μm^{-2}): (A and B) 3, 9, 23, 75, 240, and 780; and (C and D) 9, 23, 75, 240, 780, and 2,800. (A) WT, mean of 12 rods. (B) *R9AP+/−*, mean of seven rods. (C) *R9AP+/−;RKS561L*, mean of nine rods. (D) *R9AP+/−;Rv−/−*, mean of nine rods. Red traces are responses for each rod type to flashes of 23 photons μm^{-2}. Note that averaging of rod responses tends to slur the decay phases of individual photoreceptors, which vary from rod to rod, with the result that the averaged response especially at bright intensities is not representative of any one individual cell. Mean decay times averaged cell by cell are given in Figs. 4 B and 5.

TABLE 1

Kinetic and sensitivity parameters of rods

Animal line (number of rods)	r_{max}	S_F^D	$I_{1/2}$	t_i	τ_D
	pA	*pA photon*$^{-1}$ *μm*^{2}	*photons μm*$^{-2}$	*ms*	*ms*
WT (22)	14.2 ± 0.7	0.34 ± 0.02	26 ± 2	262 ± 16	185 ± 11
R9AP+/− (24)	14.6 ± 0.7	0.29 ± 0.04	40 ± 4	319 ± 24	254 ± 18
R9AP+/−;RKS561L (28)	13.5 ± 0.7	0.12 ± 0.02	79 ± 8	254 ± 28	182 ± 10
R9AP+/−;Rv−/− (8)	12.0 ± 1.2	0.32 ± 0.09	28 ± 3	243 ± 45	187 ± 16
GAPux (28)	13.9 ± 0.8	0.32 ± 0.03	35 ± 4	418 ± 30	248 ± 12
GAPux;RKS561L (29)	13.5 ± 0.7	0.11 ± 0.01	89 ± 9	231 ± 17	179 ± 10
GAPux;Rv−/− (17)	9.9 ± 0.9	0.31 ± 0.03	36 ± 6	239 ± 18	204 ± 14

All values are means ± SEM. Numbers in parentheses in the first column give number of rods recorded. Values of r_{max} (maximum response amplitude) were determined cell by cell from responses to saturating flashes; S_F^D (dark-adapted flash sensitivity), by dividing the peak amplitude of the mean dim-flash response for each cell by the flash intensity; $I_{1/2}$ (the intensity required to produce a half-maximal response), from the fit of response-intensity data for each cell to a Boltzmann function in the program Origin; t_i (the integration time), from the time integral of the mean dim-flash response for each cell divided by the peak amplitude of the response; and τ_D (the Pepperberg constant) for dark-adapted rods as described in Materials and methods.

RESULTS

To produce rod responses with prolonged PDE activation and slowed decay, we used two lines of mice with reduced GAP expression (Fig. 1). The first was an R9AP heterozygous knockout mouse (*R9AP+/−*), with reduced expression of the RGS9-1 protein and also of Gβ5-L to a mean value of 51%. The second line of mice was doubly heterozygous for both *R9AP* and *RGS9*, which for convenience we call "*GAPux*" or simply "*ux*." Expression levels for RGS9-1 and Gβ5-L in *GAPux* rods were reduced to a mean value of 34%. The levels of other similar proteins not part of the rod GAP complex such as Gβ5-S and Gβ1 were unaffected in both mouse lines (Fig. 1 A). Moreover, the expression level of PDE6β was unaffected by underexpressing the GAP proteins (Fig. 1, B and D). Keresztes et al. (2004) showed previously that underexpression of GAP proteins is also without effect on the level of transducin.

R9AP+/− rods

In Fig. 2, we show that reduction of GAP expression in *R9AP+/−* rods resulted in responses to brief stimuli that decayed more slowly than those of WT rods for the same flash intensities (Fig. 2, A and B). To simplify comparison of waveforms, we show in red the responses to flashes of 23 photons μm^{-2}. The limiting time constant was greater in *R9AP+/−* rods than in WT rods (see Table 1), and this difference was highly significant (*t* test, P = 0.0026).

To test the effects of overexpression of rhodopsin kinase, we mated *R9AP−/−* mice with *RKS561L* mice, which our previous experiments have shown to express ~12 times more kinase than WT rods and which show

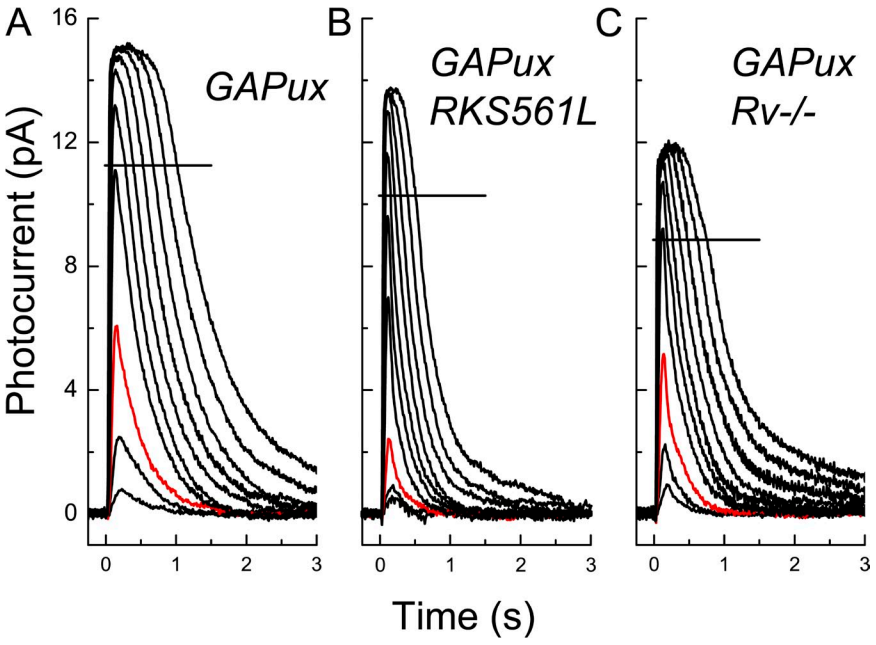

Figure 3. Comparison of mean response waveform of *GAPux*, *GAPux;RKS561L*, and *GAPux;Rv−/−* rods to 20-ms flashes given at t = 0 for each rod type at the same intensities (in photons μm^{-2}): 3, 9, 23, 75, 130, 240, 430, 780, 1,500, and 2,800. Horizontal lines show value of current at 25% of maximum used in estimating values of Tsat in Fig. 5. (A) *GAPux*, mean of 16 rods. (B) *GAPux;RKS561L*, mean of 20 rods. (C) *GAPux;Rv−/−*, mean of 10 rods. Not all of the rods in Table 1 were used for this figure because responses at every flash intensity were not recorded from every rod. Red traces are responses for each rod type to flashes of 23 photons μm^{-2}. Note that averaging of rod responses tends to slur the decay phases of individual photoreceptors, which vary from rod to rod, with the result that the averaged response especially at bright intensities is not representative of any one individual cell. Mean decay times averaged cell by cell are given in Figs. 4 B and 5.

more rapid phosphorylation of bleached rhodopsin (Chen et al., 2012). Responses of *R9AP+/−;RKS561L* rods decayed more rapidly than *R9AP+/−* rods (Fig. 2 C). Response decay was also accelerated when we deleted recoverin in the *R9AP+/−* background (Fig. 2 D). Deletion of recoverin should release rhodopsin kinase from inhibition by recoverin binding (Kawamura, 1993; Chen et al., 1995), effectively increasing the amount of rhodopsin kinase available to phosphorylate target proteins. Table 1 shows that the values of τ_D for *R9AP+/−;RKS561L* (182 ± 10 ms) and *R9AP+/−;Rv−/−* rods (187 ± 16 ms) were smaller than for *R9AP+/−* rods (254 ± 18 ms). A one-way ANOVA reported that the mean values for τ_D were significantly different among these three groups of animals, at least at the 0.001 level. Pairwise *t* tests revealed that the difference in the values of τ_D for *R9AP+/−* and *R9AP+/−;RKS561L* rods was highly significant (P = 0.0006), and the difference in the value of τ_D between *R9AP+/−* rods and *R9AP+/−;Rv−/−* rods was also statistically significant (P = 0.045).

GAPux rods

Because *GAPux* mice express the GAP-complex proteins at an even lower level than *R9AP+/−* mice, we subjected *GAPux* rods to more extensive analysis. *GAPux* responses (Fig. 3 A) again decayed more slowly than WT rods. The mean integration time increased from 262 ms in WT rods to 418 ms in *GAPux* rods (*t* test, P = 0.00016). Overexpression of GRK1 (Fig. 3 B) or deletion of recoverin (Fig. 3 C) both accelerated the decay of the response and reduced the integration time (see Table 1). The decreases in integration time were again highly significant (*GAPux* vs. *GAPux;RKS561L*, P = 0.00001; *GAPux* vs. *GAPux;Rv−/−*, P = 0.00011) and brought them nearly to the value of the integration time in WT animals. There were no significant differences in integration times between WT and *GAPux;RKS561L* (P = 0.21) or WT and *GAPux;Rv−/−* (P = 0.61), indicating that overexpression of rhodopsin kinase or the deletion of the recoverin gene can effectively compensate for the slowing of response kinetics produced by underexpressing the GAP proteins.

In Fig. 4, we examine the time course of response decay in more detail. In Fig. 4 A, we show mean responses to single photons calculated as in previous experiments from the squared mean and variance of a series of responses to dim-intensity flashes (see, for example, Chen et al., 2000; Tsang et al., 2006). Overexpression of rhodopsin kinase (red trace) produced about a twofold decrease in response amplitude and a marked acceleration of the single-exponential time constant of response decay. Deletion of recoverin (blue trace) had little effect on response amplitude but greatly quickened the rate of response decay. The decay time was quantitated by fitting a single-exponential decay function to the declining phases of the responses (smooth curves in Fig. 4 A). The value of the decay time constant (τ_{REC}) was considerably smaller for *GAPux;RKS561L* rods (193 ms) and *GAPux;Rv−/−* rods (174 ms) than for *GAPux* rods (331 ms). We also show for comparison the single-photon response of WT rods. The initial time courses of all of the responses are not detectably different, indicating that none of the genetic manipulations we have made had a significant effect on the time course of activation.

In Fig. 4 B, we show the effects of GRK1 overexpression and recoverin deletion on response decay in a different way. We fitted the waveform of responses rod by rod to exponential decay functions in the linear range of response amplitude to derive the mean value of the time constant τ_{REC} as a function of flash intensity. Fits were done from threshold to just-saturating flash intensities and did not include responses to flashes above saturation, which evoked slowly decaying "tails" (as in the responses to the brightest flashes in Fig. 3). The

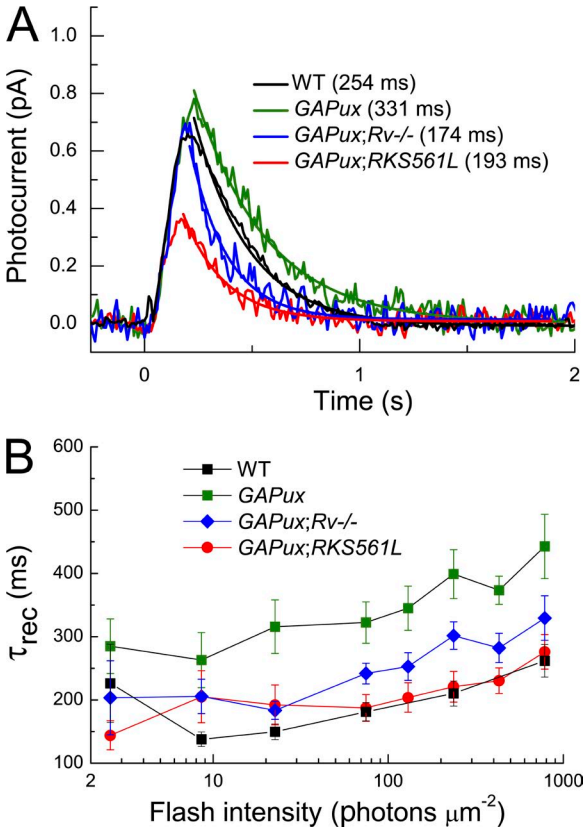

Figure 4. Exponential time course of flash decay. (A) Single-photon responses calculated from the squared mean and variance as in Chen et al. (2000) and Tsang et al. (2006). Traces give means of 41 WT rods (black), 21 *GAPux* rods (green), 18 *GAPux;RKS561L* rods (red), and 15 *GAPux;Rv−/−* rods (blue). Fits through data (solid curves) are exponential decay functions with values of the single time constant τ_{REC} of 254 ms (WT), 331 ms (*GAPux*), 193 ms (*GAPux;RKS561L*), and 174 ms (*GAPux;Rv−/−*). (B) Mean values of τ_{REC} as a function of flash intensity for 12 WT rods (black), 13 *GAPux* rods (green), 18 *GAPux;RKS561L* rods (red; only six rods were used for the lowest intensity data point), and 17 *GAPux;Rv−/−* rods (blue). Error bars are SEMs.

exponential fit was constrained only to the part of the current <0.5 of the peak current amplitude to avoid the nonlinearity produced by response saturation. No other constraints were placed on the fits. There was some variability in the value of τ_{REC} at different intensities for all three mouse lines, as well as a tendency for τ_{REC} to increase with increasing flash brightness. These results nevertheless document the systematic decrease in τ_{REC} and acceleration of flash decay for both the *GAPux*; *RKS561L* and *GAPux;Rv−/−* rods compared with the *GAPux* rods.

Limiting time constant τ_D

We also characterized rod response decay by estimating the value of the limiting time constant τ_D from measurements of the time in saturation (Tsat) as a function of flash intensity (Pepperberg et al., 1992). Values of Tsat for *GAPux*, *GAPux;RKS561L*, and *GAPux;Rv−/−* rods were determined as the duration between the beginning of the flash and the time at which responses decayed to 25% of the dark circulating current, as indicated by the horizontal lines in Fig. 3. We plotted Tsat as a function of the natural log of the flash intensity and took best-fitting straight lines as estimates of τ_D (Pepperberg et al., 1992).

In Fig. 5, we have plotted mean values (with SEMs) of Tsat for the three *GAPux* mouse lines. The mean values of the first three flash intensities in the figure were well fit by straight lines with slopes of 249 ms (*GAPux*), 176 ms (*GAPux;RKS561L*), and 209 ms (*GAPux;Rv−/−*). Because the *GAPux;RKS561L* rods were less sensitive than the other two (Table 1), we used a different range of intensities to ensure that the flash intensities for the measurements were uniformly a factor of ~30 (1.5 $\log_{10}$ units) above those just causing response saturation (see Materials and methods). We used only three data points in these fits, because the fourth brightest intensity for each of the mouse strains was slightly above the resulting straight line, indicating that by this fourth intensity Tsat was already beginning to depart from linearity (see Martemyanov et al., 2008). If four data points were included in our fits, the best-fitting values of τ_D uniformly increased to 276 ms (*GAPux*), 195 ms (*GAPux;RKS561L*), and 230 ms (*GAPux;Rv−/−*), but there was very little change in the difference between the value for *GAPux* rods on the one hand and *GAPux;RKS561L* rods or *GAPux;Rv−/−* rods on the other.

In Table 1, we show mean values of τ_D estimated as in Fig. 5 from Tsat values determined rod by rod from fits for each photoreceptor. The mean values of the fits in the table are in close agreement with the fit to the means in Fig. 5. The values of τ_D of both the *GAPux;RKS561L* rods (179 ± 10 ms) and the *GAPux;Rv−/−* rods (204 ± 14 ms) were smaller than *GAPux* rods (248 ± 12 ms). A one-way ANOVA reported differences in the mean values for τ_D at least at the 0.001 level. Pairwise *t* tests showed that the difference between *GAPux* and *GAPux;RKS561L* was highly significant (P = 0.00005), and the difference between *GAPux* and *GAPux;Rv−/−* was also significant (P = 0.022).

The effect of background light and circulating current on τ_D

We have shown previously that the limiting time constant in WT rods can be decreased by steady background light (Woodruff et al., 2008). Because the value of τ_D in darkness is larger for *R9AP+/−* rods and *GAPux* rods than for WT rods (Table 1), it seemed to us possible that rods underexpressing the GAP proteins would provide a larger range over which to investigate the effects of background light intensity on τ_D. We therefore measured τ_D by stimulating *GAPux* rods with bright,

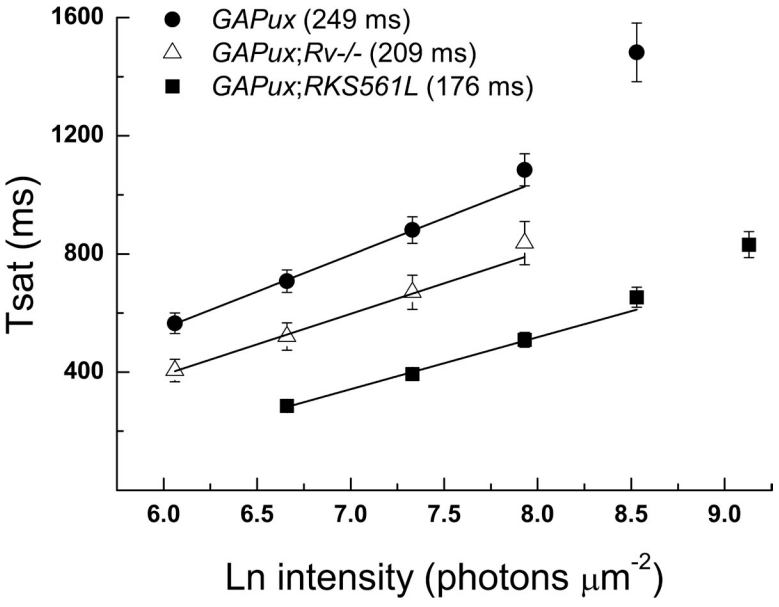

Figure 5. Tsat as a function of the natural log of the light intensity. Values of Tsat were determined rod by rod as the time from the beginning of the flash for the photocurrent to fall to 75% of its saturating value from the same rods used for Fig. 3. Data points give means and error bars give SEMs from 21 *GAPux* rods, 28 *GAPux;RKS561L* rods, and 17 *GAPux;Rv−/−* rods. Straight lines through data are for values of τ_D as follows: *GAPux*, 249 ms; *GAPux;RKS561L*, 176 ms; and *GAPux;Rv−/−*, 209 ms. See Results.

saturating flashes to measure Tsat as in the experiments of Fig. 5, but in the presence of a range of steady background intensities.

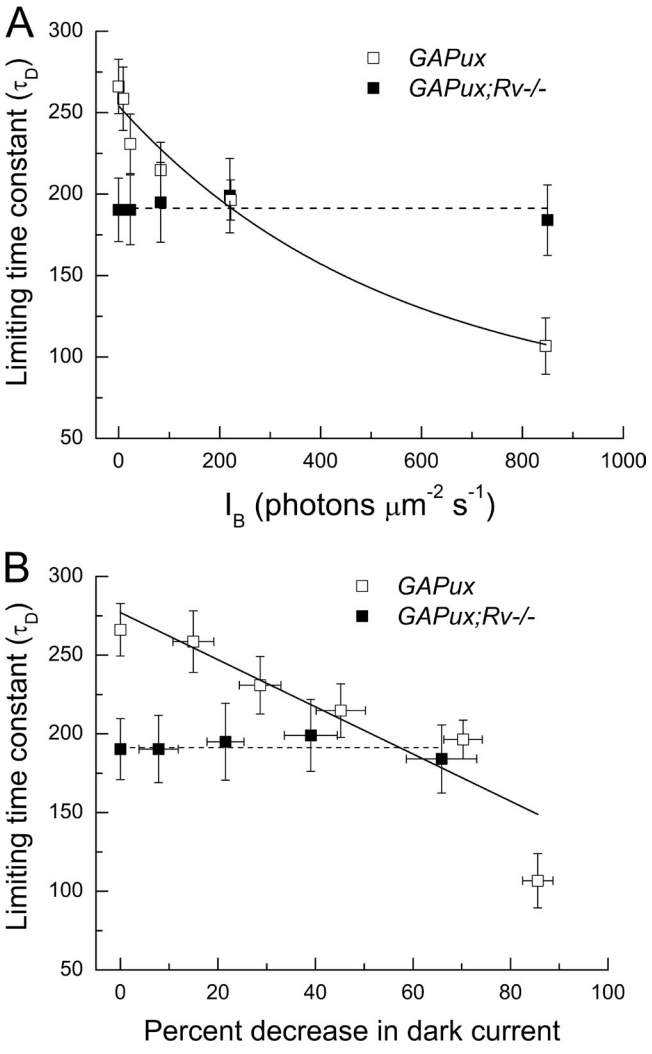

Figure 6. Limiting time constant (τ_D) as function of light intensity and circulating current. (A) Mean values of the τ_D as a function of the intensity of the background light (I_B) in photons μm^{-2} s^{-1} for 12 *GAPux* (□) and 6 *GAPux;Rv−/−* (■) rods (only 2 *GAPux;Rv−/−* rods at the brightest background intensity). Curve through *GAPux* data points is of the form $\tau_D = \tau_{D0} + A[\exp(-I_B/k)]$, with τ_{D0}, A, and k constants whose best-fitting values were τ_{D0} = 69 ms, A = 160 ms, and k = 538 photons μm^{-2} s^{-1}. Dashed line is linear regression for data from *GAPux;Rv−/−* rods with the slope constrained to be zero. The best-fitting value of τ_D was 191 ms. (B) Values of the τ_D for the same *GAPux* rods as in A (□) but plotted as a function of circulating current (r) normalized to its maximum value before presentation of the backgrounds (r_{max}). Circulating current was estimated rod by rod from the saturating value of the response to flashes in each of the backgrounds. Straight line through data are best fit of linear straight line with coefficient of determination r^2 = 0.76. Data for *GAPux;Rv−/−* rods (■) is also given as a function of circulating current, and the dashed line is again linear regression with the slope constrained to be zero and with a best-fitting value of τ_D of 191 ms. Error bars in A and B are SEMs.

The results of these experiments are given in Fig. 6 A. The open symbols show the mean value of τ_D for *GAPux* rods, plotted as a function of the background light intensity I_B (in photons μm^{-2} s^{-1}). The solid curve fitted to the data is the equation $\tau_D = \tau_{D0} + A[\exp(-I_B/k)]$, with τ_{D0}, A, and k constants whose best-fitting values were τ_{D0} = 69 ms, A = 160 ms, and k = 538 photons μm^{-2} s^{-1}. These data show that the value of the limiting time constant was systematically reduced as the intensity of the background light was increased and declined asymptotically for *GAPux* rods to a value of ∼70 ms in bright light, in approximate agreement with our previous measurement of τ_D in bright background light for WT rods of 75 ± 5 ms (Woodruff et al., 2008). The closed symbols give the data for *GAPux;Rv−/−* rods and indicate that there is a much smaller change (if any) in the value of τ_D with background light in rods lacking recoverin. The value of τ_D was smaller for *GAPux;Rv−/−* rods than for *GAPux* rods in darkness and was little changed by dim or even bright background light. The dashed curve was determined by linear regression with the slope constrained to be zero; the best-fitting value of τ_D was 191 ms.

Because photoreceptor response amplitude as a function of light intensity can be adequately fitted in some cases with exponential saturation functions (Lamb et al., 1981), we thought it possible that the exponential decrease in τ_D in Fig. 6 A might result from a linear dependence of τ_D on circulating current. We therefore estimated circulating current in background light for each of the rods in Fig. 6 A from the peak amplitude of saturating responses in the presence of each of the background intensities. The relationship of τ_D to circulating current is given in Fig. 6 B. For *GAPux* rods, the mean values of τ_D can be adequately fitted with a straight line, particularly at the dimmer background intensities. For the two brightest backgrounds, the means showed some departure from the best-fitting straight line; but for these bright backgrounds, the measurements of τ_D and of circulating current were more difficult to make accurately because responses were small. Even with all of the data points in Fig. 6 B used for the linear fit, the coefficient of determination r^2 had a value of 0.76. For *GAPux;Rv−/−* rods, there was again little change in τ_D. Collectively, the data in Fig. 6 (A and B) indicate that background light produces a decrease in τ_D nearly in proportion to the decrease in circulating current, and that this modulation of τ_D is dependent on the Ca^{2+}-binding protein recoverin.

Light adaptation in *GAPux* and *GAPux;Rv−/−* rods

Because background light reduces τ_D in *GAPux* rods but not in *GAPux;Rv−/−* rods, we were curious to know whether other aspects of light adaptation would also be affected if recoverin were deleted. Fig. 7 shows measurements of sensitivity (S_f) divided by sensitivity in darkness

(S_F^D) as a function of background light intensity. Sensitivity was calculated as the peak response amplitude for small-amplitude responses divided by the flash intensity in photons μm^{-2}. Means have been fitted with the Weber–Fechner equation, $S_F/S_F^D = I_0/(I_0 + I_B)$, where I_0 is a constant and I_B is the intensity of the background light. Both *GAPux* and *GAPux;Rv−/−* rods show decreases in sensitivity in approximate agreement with this equation, as has been shown previously for WT rods and *Rv−/−* rods on a WT background (Makino et al., 2004; J. Chen et al., 2010). The best-fitting value of I_0 is somewhat smaller for *GAPux* rods (20 photons μm^{-2} s^{-1}) than for WT rods (77 photons μm^{-2} s^{-1}), and I_0 is somewhat larger for *GAPux;Rv−/−* rods (154 photons μm^{-2} s^{-1}). Thus, *GAPux* rods are somewhat more sensitive and *GAPux;Rv−/−* rods somewhat less sensitive to background light than WT rods. The increase in sensitivity for *GAPux* rods may reflect in part the greater integration time of these photoreceptors (Table 1).

In the inset to Fig. 7, we show normalized responses to brief flashes of the same intensity in the presence of background light in *GAPux* rods (top) and *GAPux;Rv−/−* rods (bottom). The decay time of the *GAPux* rod response was progressively accelerated with increasing background light intensity, as we have shown previously for WT rods (Woodruff et al., 2008) and *GCAPs−/−* rods (J. Chen et al., 2010). There was, however, much less acceleration of the time course of decay in rods lacking recoverin (Chen et al., 2012). The results of Figs. 6 and 7 together show that background light produces a progressive decrease in τ_{REC} as well as in τ_D, and that both effects are largely ablated when recoverin is deleted from the genome. They also show that there is little effect produced by recoverin deletion on the decrease of sensitivity in background light, indicating that the control of sensitivity and response waveform during light adaptation may be produced by different mechanisms.

DISCUSSION

Our work has two principal conclusions. First, overexpression of GRK1 or deletion of recoverin can produce not only an acceleration in the time course of decay of the photoreceptor light response but also a significant decrease in the limiting time constant τ_D. This result is important, because Nikonov et al. (1998) showed that the limiting decay of light-activated PDE activity is given by a difference of exponential decay functions for Rh* and the Gα–PDE complex (PDE*), with the time constant of the slowest decay always dominating; previous experiments have shown that the decay of PDE* in WT mouse rods (∼200 ms) is much slower than the decay of Rh* (∼50 ms) and is directly responsible for the limiting time constant of response decay (Krispel et al., 2006; Tsang et al., 2006; Burns and Pugh, 2010; C.K. Chen et al., 2010). Although our experiments indicate that overexpression of GRK1 and recoverin deletion would be expected to accelerate the decay of both Rh* and of PDE*, we have shown previously for rods in a WT background that the decay of PDE* is slower than Rh* decay and continues to determine the time course of response decay even when GRK1 is overexpressed or recoverin deleted (Chen et al., 2012). Rods with decreased GAP expression have an even slower PDE* decay, and the difference in the decay time constants for Rh* and PDE* should be even greater. We are therefore confident that

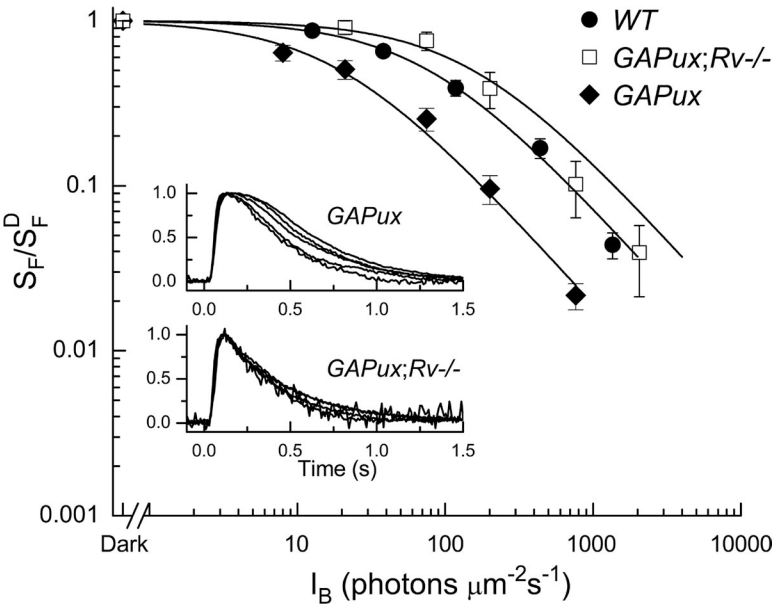

Figure 7. Adaptation to background lights. Ordinate plots sensitivity S_F in the presence of steady background light divided by sensitivity in the absence of background, S_F^D; abscissa gives intensity of background in photons μm^{-2} s^{-1}. Sensitivity was calculated as the peak response amplitude for small-amplitude responses divided by the flash intensity in photons μm^{-2}. Data points give means and error bars give SEMs for 20 WT rods (●), 6 *GAPux;Rv−/−* rods (□), and 16 *GAPux* rods (◆). Means have been fitted with the Weber–Fechner equation, $S_F/S_F^D = I_0/(I_0 + I_B)$, where I_0 is a constant and I_B is the intensity of the background light. The curve in the middle is the best-fitting curve for WT rods with I_0 = 77 photons μm^{-2} s^{-1}. The curve to the left is for *GAPux* rods with I_0 = 20 photons μm^{-2} s^{-1}, and the curve to the right is for *GAPux;Rv−/−* rods with I_0 = 154 photons μm^{-2} s^{-1}. (Inset) Superimposed normalized responses for *GAPux* and *GAPux;Rv−/−* rods to 20-ms flashes at 238 photons μm^{-2} in dark-adapted rods and in the presence of various background lights. Mean responses have been calculated from 11 *GAPux* rods at backgrounds of 8, 21, 75, and 204 photons μm^{-2} s^{-1}, and 6 *GAPux;Rv−/−* rods at background light intensities of 21, 75, 204, and 760 photons μm^{-2} s^{-1}.

the value of τ_D in our experiments reflects the decay of light-activated PDE in all of the rods we have examined.

We have now looked at GRK1 overexpression in three different backgrounds: WT mice (Chen et al., 2012) and, in this study, *R9AP*+/− and *GAPux* mice. The differences in the values of τ_D with and without GRK1 overexpression were highly significant for all three: P = 0.001 (WT), P = 0.0006 (*R9AP*+/−), and P = 0.00005 (*GAPux*). Moreover, the relative reduction in τ_D produced by GRK1 overexpression was approximately the same in all three mouse background strains, in WT and after reducing GAP expression. This is not the result we would expect if GRK1 were acting only on Rh* decay. Our results now firmly establish an effect of GRK1 on the rate of decay of light-activated PDE, and because deletion of recoverin also produces a significant reduction in τ_D (see also Makino et al., 2004; Bush and Makino, 2007), modulation of PDE decay by GRK1 seems to be produced at least in part through the action of the GRK1-binding protein recoverin. We conclude that, contrary to current thinking, GRK1 together with recoverin—in addition to phosphorylating rhodopsin—may alter the activities of one or more phototransduction proteins, either by directly phosphorylating them or indirectly through some unknown mechanism.

The second major conclusion of our work is that background light produces a progressive decrease in the value of the rod-limiting time constant (Fig. 6 A) in addition to a systematic acceleration of the rate of decay of the light response (Fig. 7 and Woodruff et al., 2008; Chen et al., 2012). The decrease in τ_D is nearly linear with the reduction in circulating current (Fig. 6 B). Little or no change was observed in either τ_D (Fig. 6 A) or τ_{REC} (Fig. 7 and Chen et al., 2012) after deletion of recoverin. Because a reduction in circulating current should produce a proportionate decrease in the rod outer segment Ca^{2+} concentration by decreasing the rate of Ca^{2+} influx, and because the $k_{1/2}$ for the binding of Ca^{2+} to recoverin is several micromolar (Chen et al., 1995) and much higher than the resting free-Ca^{2+} concentration in a mouse rod outer segment (Woodruff et al., 2002), the simplest explanation of our observations is that background light decreases Ca^{2+} and relieves inhibition of GRK1 by recoverin, which then increases the phosphorylation of some protein that accelerates the rate of light-activated PDE decay. This mechanism is apparently responsible for the acceleration of rod response decay in background light and the increase in the scotopic flicker-fusion frequency with increasing ambient light intensity. We discuss these two principal conclusions in more detail below.

GAP underexpression, GRK1 overexpression, and recoverin deletion

To record from rods with prolonged time constants of decay, we used animals heterozygous for the *R9AP* gene. Overexpression and underexpression of *R9AP* has been shown previously to produce commensurate changes in expression of the GAP proteins (Keresztes et al., 2004; Krispel et al., 2006), whereas inactivation of one copy of either *RGS9-1* (Chen et al., 2000) or *Gβ5* (Chen et al., 2003) has by itself little effect on GAP expression. The results in Fig. 1 show that *R9AP*+/− rods contain about half the normal amount of both R9AP and Gβ5-L; expression levels of other similar proteins and of PDE are unaffected. Our measurement of the level of GAP expression in *R9AP*+/− is similar to that of Keresztes et al. (2004) but considerably higher than the value of 20% of WT reported by Burns and Pugh (2009). The responses we recorded from *R9AP*+/− rods decayed more slowly than those of WT rods, but the difference in decay time was not as pronounced as that reported previously (see Fig. 1S in Supporting Material of Burns and Pugh, 2009). We are unable at present to offer an explanation for these differences, as the *R9AP*−/− animals used in both sets of experiments were taken from the same source (Keresztes et al., 2004).

We also reduced GAP expression further by breeding animals to be heterozygous for both the *R9AP* and *RGS9-1* genes. Our results show that a reduction in the copy number of the *RGS9-1* gene can influence expression of GAP proteins in animals that are also *R9AP*+/−, because in *GAPux* animals we succeeded in reducing the expression of both RGS9-1 and Gβ5-L to about one third of WT levels. The response integration time was even greater in *GAPux* rods than in *R9AP*+/− rods (*t* test, P = 0.017), but we were surprised that we could not detect a significant difference in the limiting time constant between these two groups of animals. The difference might have been greater and more easily detected had we also deleted the GCAP proteins and prevented acceleration of response decay by the guanylyl cyclase (Gross et al., 2012).

We tested the effect of GRK1 expression on *R9AP*+/− and *GAPux* rods by interbreeding them with *RKS561L* mice, which our previous experiments have shown to overexpress GRK1 by a factor of ∼12 and to speed the rate of rhodopsin phosphorylation (Chen et al., 2012; see also Gross et al., 2012). Overexpression of GRK1 in both *R9AP*+/− and *GAPux* rods produced a reduction of integration time (Table 1), a decrease in the single-exponential decay time constant τ_{REC} (Fig. 4), and an acceleration of the limiting time constant τ_D (Table 1 and Fig. 5). The acceleration of τ_D in *R9AP*+/− and *GAPux* rods are in contrast to previous attempts to detect an effect of GRK1 overexpression on τ_D (Krispel et al., 2006; Sakurai et al., 2011; Gross et al., 2012), which all failed to show a statistically significant effect. We believe that these previous attempts were unsuccessful either because the amount of GRK1 expression was lower than in our experiments (Krispel et al., 2006; Sakurai et al., 2011) or because measurements were made on a WT background, in which a small decrease in τ_D could not be shown to be significant (Gross et al., 2012).

Decreases in integration time and τ_D were also produced in both *R9AP+/−* and *GAPux* rods by deleting the recoverin gene (Table 1). Similar effects of recoverin deletion have been observed previously on a WT background (Makino et al., 2004; Bush and Makino, 2007; Chen et al., 2012). Deleting recoverin would increase the effective activity of GRK1 by preventing recoverin-dependent inhibition. For *GAPux* rods, the effects of recoverin deletion were smaller than 12 times GRK1 overexpression, both on the limiting time constant (Table 1) and on the amplitude of the single-photon response (Fig. 4 A). One possible explanation for this difference is that the effective increase in GRK1 activity was smaller in *Rv−/−* mice than in *RKS561L* animals, but deletion of recoverin might also have other effects on rod responses, perhaps as the result of a change in outer segment Ca^{2+} buffering (Makino et al., 2004). These additional effects may explain why τ_D is smaller in the brightest background intensities than after recoverin deletion (Fig. 6 A).

Mechanism of modulation of light-activated PDE

Our experiments on *R9AP+/−* and *GAPux* rods, together with previous work on rods with normal GAP expression (Chen et al., 2012), indicate that the decay of light-activated PDE can be modulated by GRK1 in concert with recoverin. GRK1 overexpression and recoverin deletion accelerate τ_D, whose value under the conditions of our experiments reflects the rate of decay of light-activated PDE (Krispel et al., 2006; Tsang et al., 2006; C.K. Chen et al., 2010). These effects are unlikely to be caused by nonspecific protein interactions between GRK1 and the PDE, because modulation of the rod decay rate requires the GAP proteins; GRK1 overexpression has no effect on rod response decay if RGS9-1 and the other GAP proteins are completely deleted (Chen et al., 2012).

Because GRK1 overexpression and recoverin deletion have similar effects on light-activated PDE decay, we suggest that the two proteins act in concert. Recoverin may inhibit GRK1 at high levels of outer segment Ca^{2+} in darkness. During steady light exposure, the decrease in Ca^{2+} produced by the reduction in the probability of opening of the cGMP-gated channels would cause recoverin to be released from GRK1, freeing the kinase to phosphorylate target proteins. This mechanism would explain why τ_D decreases in proportion to the decrease in circulating current during background light exposure (Fig. 6 B) and would also clarify why deletion of recoverin largely prevents modulation of τ_{REC} (Fig. 7) and τ_D (Fig. 6). The linear relationship in Fig. 6 B could be the result of a linear dependence of outer segment free-Ca^{2+} concentration on circulating current (see, for example, Woodruff et al., 2007), together with the elevated $k_{1/2}$ for the binding of Ca^{2+} to recoverin (Chen et al., 1995). Our experiments do not indicate the nature of the phosphorylated protein, which we speculate to be PDE itself (see, for example, Tsang et al., 2007), one of the GAP proteins (Balasubramanian et al., 2001; Hu et al., 2001), or transducin. One possibility is that phosphorylation accelerates the binding of transducin to the GAP proteins so that transducin is shut off more rapidly. Rapid binding of transducin to the GAPs could cause transducin to be shut off even before it activates PDE, perhaps explaining why rods with six times overexpressed GAP proteins (C.K. Chen et al., 2010) or 12 times overexpressed GRK1 (Table 1 and Chen et al., 2012; Gross et al., 2012) show a two- to threefold decrease in sensitivity. Moreover, the value of the limiting time constant in rods with six times overexpressed GAP proteins is not further accelerated when GRK1 is also overexpressed (Chen et al., 2012), perhaps because the rate of binding is already so rapid that it cannot be made faster.

We propose that GRK1 and recoverin are together primarily responsible for the progressive acceleration of rod response decay (Fig. 7, inset) and the limiting time constant (Fig. 6) during adaptation to steady background light. Because the rate of photoreceptor response decay determines the sensitivity of the visual system to change and motion, the modulation of light-activated PDE provides an essential Ca^{2+}-dependent mechanism that permits the rods to respond more rapidly to changes in light intensity in the presence of brighter ambient illumination. We suggest that this mechanism is also responsible at least in part for the acceleration of the scotopic flicker-fusion frequency during light adaptation (Brindley, 1970).

Our experiments show, however, that the change in sensitivity in background light is nearly unaffected by recoverin deletion (Fig. 7). Although the fit of increment sensitivity to the Weber function for the *GAPux;Rv−/−* rods is not terribly good, the fit of these data to the exponential saturation function is even worse: the sensitivity of the *GAPux;Rv−/−* rods at a background intensity of 1,000 photons μm^{-2} s^{-1} is two orders of magnitude greater than the exponential saturation function would predict (Mendez et al., 2001). Moreover, both J. Chen et al. (2010) and Makino et al. (2004) have shown that deletion of the recoverin gene on a WT background has no effect on the change in increment sensitivity in steady light. These results indicate that other mechanisms must also be present in the rod outer segment in addition to GCAP modulation of cyclase and GRK1 regulation of PDE decay, which can also regulate the transduction cascade to produce adaptation during background illumination.

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