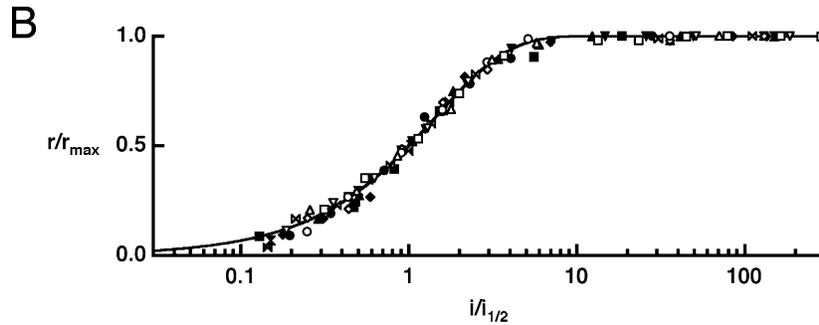


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Fig. 2 B appeared incorrectly in the original version of this article. The labeling was missing from the x axis. The corrected Fig. 2 B is below.



Recoverin Regulates Light-dependent Phosphodiesterase Activity in Retinal Rods

CLINT L. MAKINO,¹ R.L. DODD,² J. CHEN,^{3,4} M.E. BURNS,⁵ A. ROCA,³ M.I. SIMON,⁶ and D.A. BAYLOR²

¹Department of Ophthalmology, Harvard Medical School and the Massachusetts Eye and Ear Infirmary, Boston, MA 02114

²Department of Neurobiology, Stanford University School of Medicine, Stanford, CA 94305

³Zilkha Neurogenetic Institute, Beckman Macular Research Center, Doheny Eye Institute, ⁴Departments of Ophthalmology and Cell and Neurobiology, Keck School of Medicine of the University of Southern California, Los Angeles, CA 90089

⁵Center for Neuroscience and Department of Psychiatry and Behavioral Sciences, University of California, Davis, CA 95616

⁶Division of Biology, California Institute of Technology, Pasadena, CA 91125

ABSTRACT The Ca²⁺-binding protein recoverin may regulate visual transduction in retinal rods and cones, but its functional role and mechanism of action remain controversial. We compared the photoresponses of rods from control mice and from mice in which the recoverin gene was knocked out. Our analysis indicates that Ca²⁺-recoverin prolongs the dark-adapted flash response and increases the rod's sensitivity to dim steady light. Knockout rods had faster Ca²⁺ dynamics, indicating that recoverin is a significant Ca²⁺ buffer in the outer segment, but incorporation of exogenous buffer did not restore wild-type behavior. We infer that Ca²⁺-recoverin potentiates light-triggered phosphodiesterase activity, probably by effectively prolonging the catalytic activity of photoexcited rhodopsin.

KEY WORDS: calcium-binding proteins • knockout mice • light adaptation • photoreceptors • phototransduction

INTRODUCTION

In retinal rods, calcium orchestrates several mechanisms that counteract the effect of light on the phototransduction cascade, thus shortening the light response and preventing signal saturation (for reviews see Pugh et al., 1999; Pugh and Lamb, 2000; Fain et al., 2001). In darkness, Ca²⁺ enters the outer segment through cGMP-gated channels and is extruded by an electrogenic exchanger which couples the movement of 4 Na⁺ and 1 K⁺ down their electrochemical gradients to the removal of 1 Ca²⁺ (Cervetto et al., 1989). In the presence of light, fewer cGMP-gated channels are open, reducing Ca²⁺ influx in the face of continued Ca²⁺ extrusion; this results in a fall in the intracellular Ca²⁺ concentration. The lowered [Ca²⁺]_i is thought to affect three aspects of the transduction machinery: (a) it decreases light-dependent cGMP phosphodiesterase (PDE) activity, (b) it increases the channel's sensitivity for cGMP, and (c) it increases the activity of guanylate cyclases. Since the cell's responses to light reflect the contributions of all of these control mechanisms, a full understanding of Ca²⁺'s actions requires experimental designs that allow quantitative assessment of the indi-

vidual contributions. The purpose of this work is to investigate the contribution of recoverin, which has been proposed to mediate calcium control of PDE activity (Kawamura, 1993; Chen et al., 1995a; Klenchin et al., 1995).

Recoverin is a 23-kD Ca²⁺-binding protein present in certain retinal neurons: rods, cones, a subset of bipolar cells, and a minor subpopulation of cells in the ganglion cell layer (Dizhoor et al., 1991; Milam et al., 1993; Wiechmann and Hammarback, 1993). Recoverin is also expressed by pinealocytes (Korf et al., 1992), ocular ciliary epithelial cells (Bertazzoli-Filho et al., 2001), olfactory receptor neurons (Bastianelli et al., 1995), and some types of carcinomas (Polans et al., 1995; Maeda et al., 2000). Related proteins are found at numerous loci in the CNS. Biochemical experiments on rod outer segment preparations in vitro demonstrated that Ca²⁺-recoverin inhibits phosphorylation of rhodopsin (Kawamura, 1993) by binding to rhodopsin kinase (Chen et al., 1995a; Klenchin et al., 1995). Consistent with such an effect, dialysis of exogenous Ca²⁺-recoverin into isolated, intact rod outer segments delayed the recovery of the flash response (Gray-Keller et al., 1993). In truncated rods, addition of exogenous recoverin was shown to lengthen the response duration by prolonging the lifetime of catalytically active, photoexcited rhodopsin (Erickson et al., 1998). These results argue that recoverin imparts Ca²⁺ dependence to the shutoff of rhodopsin by rhodopsin kinase. Yet, experiments on

Address correspondence to Clint L. Makino, Department of Ophthalmology, Harvard Medical School and the Massachusetts Eye and Ear Infirmary, 243 Charles Street, Boston, MA 02114. Fax: (617) 573-4290; email: cmakino@meei.harvard.edu

outer segments permeabilized with staphylococcal α -toxin produced no evidence that the level of Ca^{2+} controlled the light-dependent phosphorylation of rhodopsin (Otto-Bruc et al., 1998). Recoverin's specific role in phototransduction in vivo, as well as its mechanism of action, therefore remain unclear. We approached these questions by observing the effects of recoverin deletion on visual transduction in intact mouse rods.

MATERIALS AND METHODS

Gene Targeting

A targeting vector was constructed by inserting the gene conferring neomycin resistance, MC1Neo (Stratagene), into the Not I restriction site of the first exon of the recoverin gene (Fig. 1 A). The vector was transfected into CJ7 embryonic stem (ES) cells by electroporation and 960 individual neomycin-resistant clones were isolated and expanded in 96-well plates. Proper targeting of the vector was confirmed by Southern analysis in seven of these isolated clones using the strategy illustrated in Fig. 1 A. Two of the recombinant ES clones were selected, expanded, and injected into 3.5 d blastocysts of C57/B6 mice. Chimeric mice were then bred to obtain mice in which one or both copies of the recoverin gene were disrupted. For some experiments, recoverin knockout ($\text{rec}^{-/-}$) mice were crossed with mice in which guanylate cyclase activating proteins 1 and 2 were knocked out ($\text{GCAPs}^{-/-}$; Mendez et al., 2001) to obtain rods in which both sets of proteins were absent ($\text{GCAPs}^{-/-}$, $\text{rec}^{-/-}$).

Immunocytochemistry

Eye cups from 2-mo-old mice were fixed in 4% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M phosphate buffer solution for 1 h on ice, rinsed in cold buffer, and embedded in acrylamide (Johnson and Blanks, 1984). Frozen sections, 10 μm in thickness, were collected and incubated with antirecoverin antibody (dilution, 1:200; Dizhoor et al., 1991). Bound antibody was visualized using a FITC-conjugated secondary antibody (dilution, 1:100; Vector Laboratories).

Analysis of Gene Transcription

RNA samples were prepared as recommended by Affymetrix, Inc. Briefly, a sample of 20–30 μg total RNA was isolated from four retinas using Trizol (Life Technologies). Duplicate samples were prepared from four wild-type and four recoverin knockout mice. All mice were killed at the same time of day, after being dark-adapted for 2–3 d. 10 μg of total RNA from each sample was reverse transcribed to generate cDNA, which was then used to synthesize biotin-labeled cRNA by in vitro transcription (Enzo Diagnostics, Inc.). Labeled cRNA was cleaned up using Rneasy Mini kits (QIAGEN) and fragmented to sizes from 35 to 200 bases by incubating at 94°C for 35 min.

The biotinylated, fragmented cRNA (15 μg) was hybridized to Affymetrix Murine genome U74Av2 arrays for 16 h at 40°C in the GeneChip Fluidics Station 400 (Affymetrix, Inc.). These arrays represented all 6,000 genes in the Mouse UniGene database that have been functionally characterized as well as $\sim 6,000$ expressed sequence tag clusters. After hybridization, the arrays were washed, stained with streptavidin-conjugated phycoerythrin, and scanned with a Hewlett-Packard Scanner. To compare results obtained with different arrays, GeneChip algorithms (Affymetrix,

Inc.) were used to normalize the hybridization intensity for each probe by the average hybridization intensity of all the chips. Then pairwise comparisons in the gene expression levels were made between retinas from wild-type and recoverin knockout mice to search for genes that were consistently up- or down-regulated by more than twofold.

Western Analysis of Retinal Proteins

Retinas from C57/B6 \times 129svev control and $\text{rec}^{-/-}$ mice, 6-wk to 3-mo-old, were homogenized in buffer (80 mM Tris-HCl, pH 7.4; 10 mM EDTA; 4 mM MgCl_2 ; 2 mM CaCl_2 ; 0.5 mg/ml complete mini protease inhibitors; Boehringer). Equal amounts of retinal homogenates from control and $\text{rec}^{-/-}$ mice were fractionated on 12% SDS-PAGE and transferred to nitrocellulose membranes (Protran, Schleicher and Schuell). The membranes were blocked with 5% nonfat dry milk in TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.05% Tween 20), incubated with primary antibodies diluted in TBST and 1% bovine serum albumin, followed by secondary antibodies conjugated to horseradish peroxidase (Sigma-Aldrich), and visualized using ECL (Amersham Biosciences). The primary antibodies consisted of antirhodopsin R2-12N (Hargrave et al., 1986), antirhodopsin kinase 8585 (Inglese et al., 1992) and G8 (Zhao et al., 1998), antirecoverin (Dizhoor et al., 1991), antiarrestin C10C10 (Knospe et al., 1988), antirod transducin α -subunit $\text{T}\alpha 1\text{A}$ (compare Calvert et al., 2000), antirod transducin β -subunit βN_1 (Amatruda et al., 1988) and $\text{G}\beta 1$ (Santa Cruz Biotechnology), anti-PDE α -, β - (CytoSignal), and γ -subunits (Arshavsky et al., 1992), anti-RGS-9 (Chen et al., 2000), and antiguanylate cyclase-E (Yang and Garbers, 1997). Each protein was analyzed one to three times.

Single Cell Physiology

Photoresponses were recorded from single rods of $\text{rec}^{-/-}$ and control mice as described in Sung et al. (1994). Control mice were obtained from Jackson ImmunoResearch Laboratories, as well as from the California Institute of Technology. Briefly, mice were dark adapted overnight, anesthetized with CO_2 , and then killed by cervical dislocation. The retinas were isolated into Leibovitz's L-15 medium under infrared light and stored on ice. Small samples were removed, chopped finely in L-15 containing $\sim 1.5 \mu\text{g/ml}$ DNase I (Type IV, Sigma-Aldrich), and transferred to an experimental chamber perfused continuously with an enriched Locke's solution heated to 35–38°C. Locke's contained (mM): Na^+ 144, K^+ 3.6, Ca^{2+} 1.2, Mg^{2+} 2.4, Cl^- 123.3, HEPES 10, HCO_3^- 20, EDTA 0.02, glucose 10, glutamate 0.5, succinate 3, BME vitamins, BME amino acid supplement, pH 7.4. A rod outer segment was drawn into a silanized glass pipette filled with a similar solution except that vitamins and amino acids were omitted and HCO_3^- was replaced with an equimolar amount of Cl^- . Cells were stimulated with an electronically shuttered tungsten halogen lamp. Monochromatic light (10-nm bandwidth) at 500 nm (flashes or steps of light) or at 520 nm (steps) was obtained with interference filters. Photoresponses were recorded with a current-to-voltage converter (Axopatch 200; Axon Instruments, Inc.) and digitized online with a Macintosh computer. Records were low-pass filtered at 30 Hz (-3 dB, 8-pole Bessel, AI2040; Axon Instruments, Inc.), except where noted otherwise. No corrections were made for the delay introduced by filtering. In addition, the traces in Figs. 2, 3, 4 and 7 were digitally filtered at 7 Hz (Igor version 3.15; Wavemetrics).

For the purposes of estimating the intracellular concentrations of ions and proteins, we calculated a rod outer segment aqueous volume of 9.2 to 15.4 fl. The calculation was based on a rod diameter of 1.4 μm (Carter-Dawson and LaVail, 1979) and a

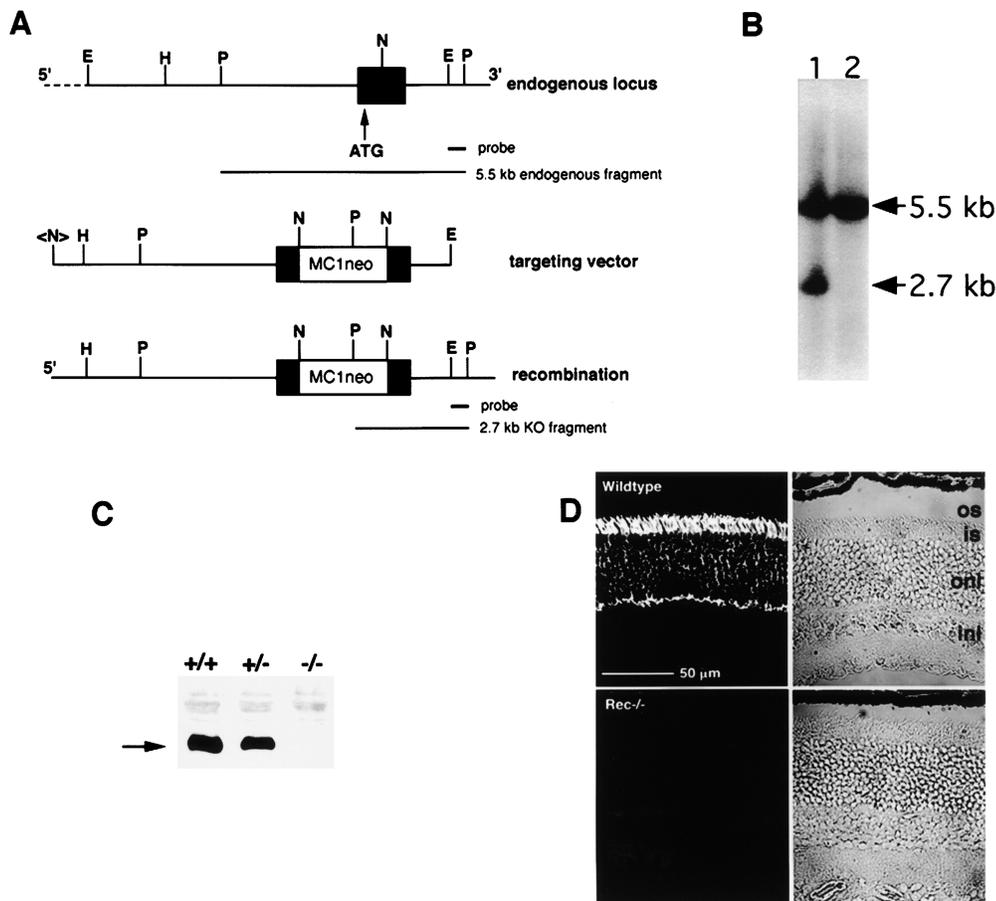


FIGURE 1. Targeted disruption of recoverin. (A) Partial restriction map of the endogenous locus, targeting vector and predicted genomic structure after homologous recombination. The targeting vector contained 7 kb of 5' and 1.5 kb of 3' homologous sequence. An MC1neo polyA cassette was inserted into the NotI site of exon 1, disrupting the gene. The probes used for Southern blots, representing wild-type and disrupted alleles are illustrated below their corresponding genomic structures. E, EcoRI; H, HindIII; N, NotI; P, PstI; <N>, NotI site from the polylinker of the lambda phage genomic clone. (B) Representative Southern blot of ES clones. DNA prepared from the G418-resistant ES clones was digested with PstI, size fractionated, blotted, and probed for the EcoRI-PstI fragment, located on the 3' side of the targeting vector. The 5.5-kb fragment derived from the wild-type allele, whereas the 2.7 kb band in lane 1 confirmed that the expected recombination event had occurred. (C) Recoverin immunoblot of retinal extracts prepared from wild-type (+/+), hemizygous (+/-), and homozygous knockout (-/-) mice probed with a polyclonal antibody raised against purified recoverin (Dizhoor et al., 1991). Recoverin (arrow) was reduced in the +/- lane as compared with the wild-type lane and undetectable in the -/- lane. (D) Localization of recoverin in the outer retina of normal mice and its disappearance in knockout mice. Sections from wild-type and recoverin knockout mice were immunostained for recoverin. os, outer segments; is, inner segments; onl, outer nuclear layer; inl, inner nuclear layer.

recorded outer segment length of 12 to 20 μm. Half of the space was assumed to be occupied by the disks and was excluded.

RESULTS

Rec-/- Retinal Morphology and Protein Content

The targeting vector disrupted the first exon of the recoverin gene and prevented its expression (Fig. 1). The effect of recoverin knockout on gene expression in the retina was analyzed using Affymetrix Murine genome U74Av2 arrays, which contain all known phototransduction genes in addition to ~12,000 functionally characterized genes and expressed sequence tags. Comparisons of expression profiles between dark-adapted *rec-/-* and control retinas revealed only two significant changes. There was a 10-fold decrease in the signal for recoverin. The persistence of a low signal for recoverin in knockouts could result from the presence of a partial mRNA sequence for recoverin or from weak crosshybridization with transcript for another protein.

In addition, two probe sets for transducin β-subunit mRNA revealed 6- and 16-fold decreases, respectively. This large difference in expression levels for transducin β was verified using semiquantitative, real time RT-PCR. The lack of immunohistochemical staining of photoreceptor and bipolar cells in retinal sections and the absence of recoverin in Western blots of retinal protein confirmed that recoverin was indeed absent from knockout mice. Western analyses, however, did not show large changes in the amount of transducin β-subunit. Levels of rhodopsin, rhodopsin kinase, arrestin, transducin α-subunit, the α-, β-, and γ-subunits of PDE, RGS9-1, and guanylate cyclase E were also similar in knockout and control retinas. Disruption of recoverin expression was therefore considered to be functionally selective in that it did not give rise to compensatory changes in the levels of phototransduction proteins or to changes in the levels of mRNA for proteins not directly involved in phototransduction.

Knockout of recoverin did not appear to adversely af-

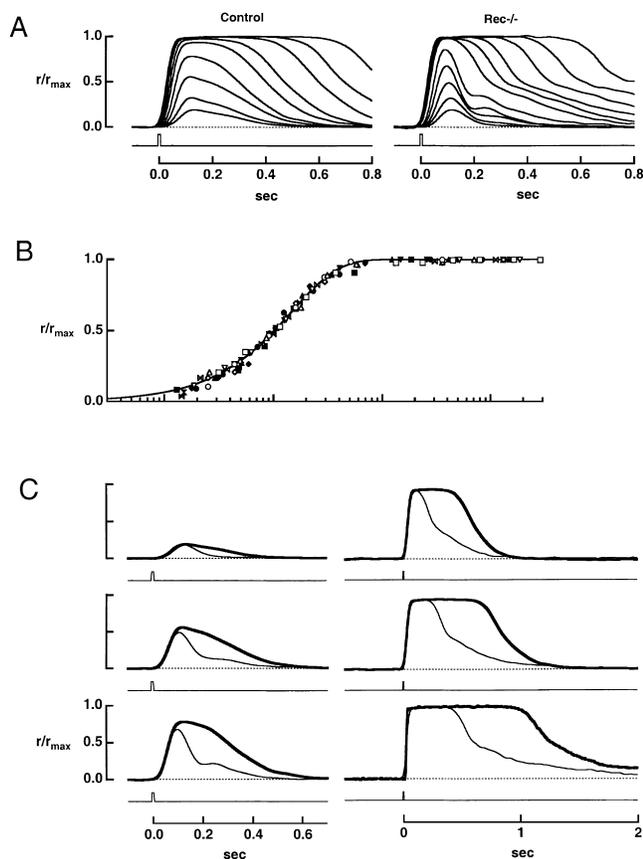


FIGURE 2. The effect of recoverin deletion on flash responses. (A) Averaged responses of a control rod and a *rec-/-* rod to flashes of increasing strength. The maximal response amplitudes were 16.4 and 12.9 pA for control and *rec-/-* rods, respectively. Flash strengths for the control rod were: 11.8, 20.6, 42.8, 74.9, 139, 243, 504, 882, and 1,690 photons μm^{-2} , while for the *rec-/-* rod, they were 12.8, 22.4, 46.5, 81.4, 151, 548, 960, 1,840, 3,230, 6,700, and 11,700 photons μm^{-2} . (B) Stimulus-response relation for seven control (filled symbols) and seven *rec-/-* rods (open symbols). The continuous line is a saturating exponential, $r/r_{max} = 1 - \exp(-ki/i_{1/2})$, where r_{max} is the saturating response amplitude, $k = \ln(2)$, i is the flash strength, and $i_{1/2}$ is the flash strength producing a half-maximal response for each cell. The cells in A are shown by the (\blacktriangledown , control), and by the (\square , knockout). (C) Faster flash response recovery in a *rec-/-* rod. Each pair of traces shows the averaged responses elicited by flashes of similar strength from the control (thick trace) and *rec-/-* (thin trace) rods in A. The flash strengths were (top to bottom, left to right): 11.8, 42.8, 74.9, 504, 1,690, and 6,160 photons μm^{-2} for the control rod and 12.8, 46.5, 81.4, 548, 1,840, and 6,700 photons μm^{-2} for the *rec-/-* rod.

fect the development or long-term viability of the retina. Rods elaborated outer segments of normal size and the outer nuclear layer, consisting of photoreceptor nuclei, attained normal thickness. The thickness of the inner nuclear layer, which contains the somas of bipolar cells, was also normal. There was no sign of a retinal degeneration in *rec-/-* mice up to 1 yr of age, the oldest examined.

Faster Flash Response Recovery in Dark-adapted *rec-/-* Rods

Fig. 2 A shows flash response families from a representative *rec-/-* and control rod. Although the rising phases of the responses were similar, *rec-/-* responses recovered more rapidly—a characteristic difference that is analyzed more fully below. After flashes of intermediate strength, the recovery phase of the knockout rod response often displayed a prominent “kink” usually not present in responses of control rods. The kink in the flash response of *rec-/-* rods may explain the anomalous electroretinograms of *rec-/-* mice, which exhibit two corneal positive b-waves rather than one in response to a single midrange scotopic flash (Ron Bush, personal communication; Hurley and Chen, 2001).

Collected results on the relation between normalized peak response amplitude and normalized flash strength are shown in Fig. 2 B. The smooth curve, a saturating exponential (Lamb et al., 1981), provided a good fit to the results from both populations. Although the half-saturating flash strength was lower for *rec-/-* rods, the difference was small, as shown in Table I. The table also summarizes other parameters of the flash responses from control and *rec-/-* rods. It can be noted that the dark current and the time-to-peak of the dim flash response were very similar for the two populations, as was the estimated amplitude of the single photon response.

Fig. 2 C compares the responses of the rods in Fig. 2 A to flashes whose strength varied over a wide range. For subsaturating flashes, the responses of the *rec-/-* rod recovered more rapidly than those of the control rod. The integration time of the dim flash response, defined as the area under the response divided by its peak height, was on average 37% lower in *rec-/-* rods than in control rods (Table I). The difference in effective response duration became more pronounced at higher flash strengths that saturated the response (Fig. 3, see also Hurley and Chen, 2001).

The diminished time in saturation (T_{sat}) of *rec-/-* responses to bright flashes corresponds to an effective decrease in phototransduction gain. The magnitude of the decrease was assessed from the ratio of the flash strengths required to hold control ($n = 20$) and *rec-/-* ($n = 11$) rods in saturation for a given time (e.g., Fig. 3). Although the slope of the relation for the two types of rod differed slightly, a flash approximately ninefold brighter was required to hold knockout rods in saturation, indicating a ninefold lower gain (Fig. 3 B). The general conclusion from these experiments is that wild-type rods had a higher transduction gain during the recovery phase of the dark-adapted flash response.

TABLE I
Response Parameters of Control and *Rec*^{-/-} Rods

	Control	<i>Rec</i> ^{-/-}	p
Dark current (pA)	8.9 ± 0.3, 96	9.6 ± 0.5, 47	ns
Na ⁺ /Ca ²⁺ -K ⁺ exchange			
Time constant (ms)	113 ± 7, 73	86 ± 9, 51	2e-3
Initial amplitude (pA)	-0.53 ± 0.02, 73	-0.59 ± 0.05, 51	ns
Fractional amplitude	0.062 ± 0.003, 73	0.060 ± 0.04, 73	ns
Half-saturating flash strength, <i>i</i> _{1/2} (photons μm ⁻² at 500 nm)	47 ± 3, 59	37 ± 2, 39	2e-2
Single photon response characteristics			
Integration time, <i>T</i> _i (ms)	250 ± 11, 76	157 ± 9, 47	6e-8
Time to peak, <i>t</i> _p (ms)	138 ± 3, 76	135 ± 3, 47	ns
Amplitude (pA)	0.39 ± 0.04, 39	0.43 ± 0.04, 17	ns
Step sensitivity, <i>I</i> _{1/2} (photons μm ⁻² s ⁻¹ at 500 nm)	250 ± 31, 12	410 ± 71, 11	4e-2
Light adaptation, <i>I</i> ₀ (photons μm ⁻² s ⁻¹ at 500 nm)	178 ± 33, 4	192 ± 123, 2	ns

Values given as mean ± SEM, *n*. The kinetic parameters for the single photon response were taken from the responses to dim flashes, since their shapes are identical. Flashes eliciting a mean response less than a fifth of the maximum were considered dim. The amplitude of the single photon response was estimated by taking the ratio of the ensemble variance to the mean amplitude of the response to a dim flash. Sensitivity to steps of light was evaluated with either 500-nm or with 520-nm light. The intensity giving rise to a half maximal response, *I*_{1/2}, was given for 500-nm light, taking rod sensitivity to 520 nm relative to that at 500 nm as 0.833 (Calvert et al., 2000). *I*₀, found from light adaptation experiments of the type shown in Fig. 7 C, is the intensity of background light that halved flash sensitivity from the value observed in darkness. The value given for *I*₀ includes a conversion from 520- to 500-nm photons. Comparisons were made using a *t* test.

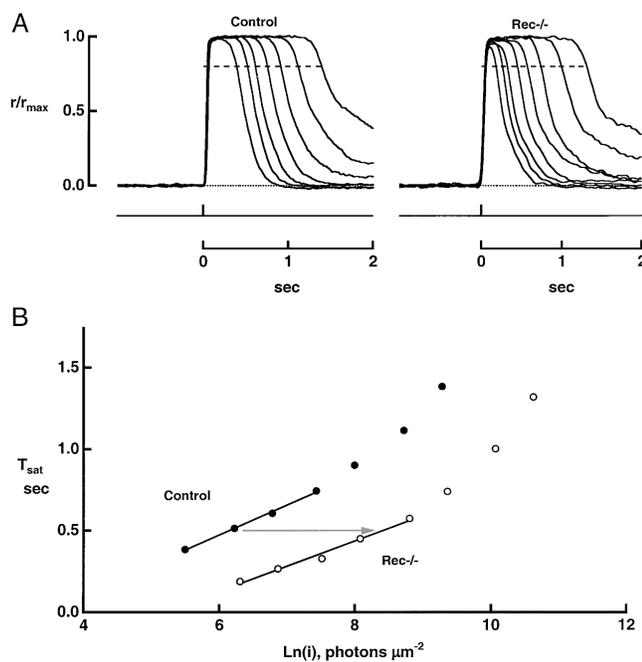


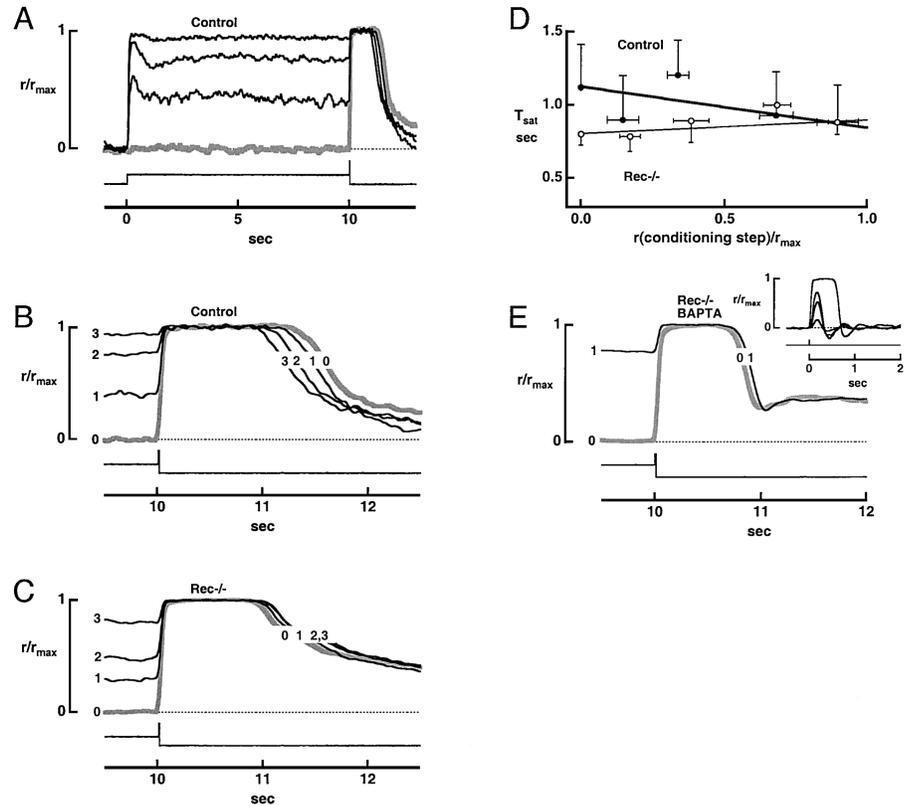
FIGURE 3. Faster recovery of *rec*^{-/-} rods from bright flashes. (A) Averaged responses of a control rod and a *rec*^{-/-} rod. *T*_{sat} was measured from midflash to 20% recovery of the circulating current, demarcated by the dashed line. Maximal response amplitudes were 16.4 and 10.4 pA for control and *rec*^{-/-} rods, respectively. (B) Pepperberg plots from the responses in A. *T*_{sat} was lower for the *rec*^{-/-} rod than for the wild-type rod at every flash strength. Linear regression over the indicated regions gave slopes of 184 ms for the control rod and 154 ms for the *rec*^{-/-} rod. The *rec*^{-/-} rod required 9.7-fold more light to produce a *T*_{sat} of 0.5 s (arrow).

Effect of Background Light on PDE Activity Evoked by a Bright Flash

Early biochemical studies suggested that recoverin exerts its effect in the Ca²⁺-bound form (Kawamura, 1993). If Ca²⁺-bound recoverin prolongs the response to a bright flash delivered in darkness, then at low Ca²⁺, control and *rec*^{-/-} rods should behave identically. This idea was tested using the step-flash protocol devised by Fain et al. (1989), as shown in Fig. 4 A. Steady background lights of varying intensities were used to lower the rod's [Ca²⁺]_i before the presentation of a saturating test flash. As expected (Fain et al., 1989), background light shortened the saturation time of the response to the flash in control rods (Fig. 4, A and B). Because the test flash response itself always lowered [Ca²⁺]_i to a minimal level and thus activated guanylate cyclase maximally, the reduction in *T*_{sat} should be caused by a reduction in the flash-evoked PDE activity (Matthews, 1996). The shortening of *T*_{sat} was a robust phenomenon, being present in each of 34 control cells.

Background light failed to shorten the *T*_{sat} of *rec*^{-/-} rods (19 of 20 rods) and in most cases increased it. Records from one such experiment are shown in Fig. 4 C. *T*_{sat} was extended slightly with conditioning step intensity in *rec*^{-/-} rods because recovery from the step alone was generally more prolonged at higher intensities. There was some evidence that the same effect partially masked the decline in *T*_{sat} with conditioning step intensity for control rods. In 7 out of 24 control rods where a wide range of conditioning step intensities

FIGURE 4. Absence of phosphodiesterase adaptation in *rec*^{-/-} rods. (A) Step-flash paradigm. A saturating flash of 57,800 photons μm^{-2} at 500 nm, presented to a control rod at time = 10 s, was preceded by darkness (response shown with thick gray line) or by a conditioning step of light of intensity: 2,080, 4,540, or 9,470 photons $\mu\text{m}^{-2} \text{s}^{-1}$ at 520 nm, which reduced $[\text{Ca}^{2+}]_i$. The maximal response amplitude was 7.3 pA. (B) Decrease in response saturation time for flashes given after a step of light. Responses are the same as those shown in A. (C) Lack of decrease in flash response saturation time after conditioning steps in a *rec*^{-/-} rod. The flash delivered 55,200 photons μm^{-2} at 500 nm while the step intensities were: 920, 1,920 and 4,190 photons $\mu\text{m}^{-2} \text{s}^{-1}$ at 520 nm. The maximal response amplitude was 11.4 pA. (D) Convergence of control and *rec*^{-/-} rod flash response saturation times after a conditioning step of light to diminish Ca^{2+} . Averaged responses to the conditioning step after 10 s of exposure relative to the maximal response were plotted on the abscissa. Error bars show SEM. Test flash strength ranged from 4,310 and 4,830 photons μm^{-2} at 500 nm. Continuous lines plot the mean slopes and intercepts for rods of each type, after linear regressions of the results from individual rods. For controls, the slope was -0.283 s while for *rec*^{-/-}, it was 0.092 s . *Rec*^{-/-} rods exhibited differences in both slope ($P < 0.0006$) and intercept ($P < 0.02$). (E) Failure of calcium buffer to restore PDE adaptation in a *rec*^{-/-} rod. Rods were incubated in 100 μM BAPTA-AM for a minimum of 10 min at room temperature, before being recorded. The flash strength was 36,300 photons μm^{-2} at 500 nm, dimmer than in A–C. The step intensity was 5,020 photons $\mu\text{m}^{-2} \text{s}^{-1}$ at 520 nm. The maximal response amplitude was 14.3 pA. (Inset) Averaged flash responses of a *rec*^{-/-} rod treated with BAPTA. The time to peak of the dim flash response was delayed to 170 ms but the integration time, measured to the first baseline crossing, was only 140 ms. The flash densities were: 7.9, 26.4, 46.2, and 3,800 photons μm^{-2} at 500 nm.



were tested, T_{sat} change little or even increased after dim to moderate conditioning steps, but eventually decreased at higher step intensities. Background light did shorten T_{sat} slightly in one *rec*^{-/-} rod. But the recovery phases of the responses to steps and steps plus flashes in this rod had undershoots that were not present in the responses to the flashes alone. Similar behavior was sometimes observed in control rods and hence appears to be unrelated to the deletion of recoverin. The fact that background light caused the saturation time of the response to a bright test flash in control rods and *rec*^{-/-} rods to become more similar (Fig. 4, B–D) is consistent with the notion that Ca^{2+} -recoverin boosts the latter portion of the bright flash response.

Altered Kinetics of $\text{Na}^+/\text{Ca}^{2+}\text{-K}^+$ Exchange in *rec*^{-/-} Rods

The light response is generated by changes in the concentration of cGMP, which in turn reflect not only activation of PDE by light but also Ca^{2+} mediated activa-

tion of guanylate cyclase by GCAPs (guanylate cyclase activating proteins). Recoverin's abundance in photoreceptors (17–40 μM ; Kawamura and Murakami, 1991; Klenchin et al., 1995), suggests that it could contribute significantly to a rod's Ca^{2+} buffering capacity. Thus, the deletion of recoverin could speed the light-induced decline in calcium and the resulting activation of guanylate cyclase. To assess the effect of recoverin deletion on Ca^{2+} dynamics, we recorded the $\text{Na}^+/\text{Ca}^{2+}\text{-K}^+$ exchange currents of control and knockout rods. A bright flash, which rapidly closed the light-sensitive channels, revealed the exchange current as a small, slowly decaying inward current (Fig. 5). The exchange transients are shown on an expanded, normalized ordinate scale in the lower panels (jagged traces) where they are fitted with single exponentials (smooth curves). In control rods the exponential time constant was $113 \pm 7 \text{ ms}$ (mean \pm SEM), while in *rec*^{-/-} rods, it was $86 \pm 9 \text{ ms}$ (Table I). In the same rods the initial amplitude of the exchange current, extrapolated to the time at which

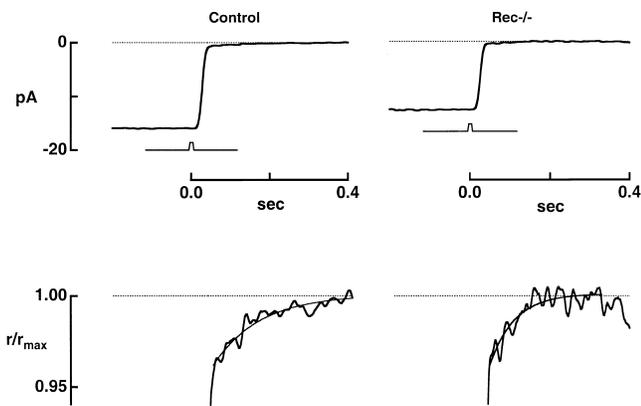


FIGURE 5. Accelerated Ca^{2+} dynamics in $\text{rec}^{-/-}$ rods. The top panel shows the averaged responses of a control and a knockout rod to bright, saturating flashes of 1,690 and 5,520 photons μm^{-2} , respectively. The response amplitude was 15.9 pA for the control rod and 12.6 pA for the knockout rod. The low amplitude, slowly declining inward current reflects the extrusion of Ca^{2+} by the $\text{Na}^{+}/\text{Ca}^{2+}\text{-K}^{+}$ exchanger. The exchange currents are expanded, normalized, and fitted with a single exponential in the bottom panel. For the control rod, the exponential time constant was 112 ms, while for the knockout, it was 61 ms. The initial amplitudes of the exchange currents were -0.7 and -0.6 pA for control and $\text{rec}^{-/-}$, respectively.

the bright flash response reached half its peak amplitude (Yau and Nakatani, 1985), was -0.53 ± 0.02 and -0.59 ± 0.05 pA in control and $\text{rec}^{-/-}$ rods, respectively. Because the stoichiometry of ionic exchange is fixed (Cervetto et al., 1989) the time integral of the exchange current is directly proportional to the amount of Ca^{2+} extruded. The initial amplitudes were the same for the two types of rods, consistent with their maintaining a similar free $[\text{Ca}^{2+}]_i$ in darkness. However, the longer time constant for control rods made the integrated area of the exchange current larger, indicating that more Ca^{2+} was extruded from control rods. The 9.1 fC of additional charge entering control rods during the exchange current corresponds to 6 to 10 μM Ca^{2+} in the outer segment, assuming a volume range of 9.2 and 15.4 fl (see MATERIALS AND METHODS). Since most of a rod's internal Ca^{2+} is bound (Lagnado et al., 1992), the extra Ca^{2+} content of controls presumably represents a fraction bound to recoverin. This fraction, which is apparently released and extruded rapidly after a bright flash, comprises $\sim 15\%$ of the total Ca^{2+} present in the outer segment.

Does the $\text{rec}^{-/-}$ phenotype result simply from a faster fall in intracellular Ca^{2+} concentration and earlier activation of guanylate cyclase? We examined this in two ways.

First, we used BAPTA-AM incorporation to bolster the Ca^{2+} buffering capacity and slow the changes in intracellular free Ca^{2+} concentration in four $\text{rec}^{-/-}$

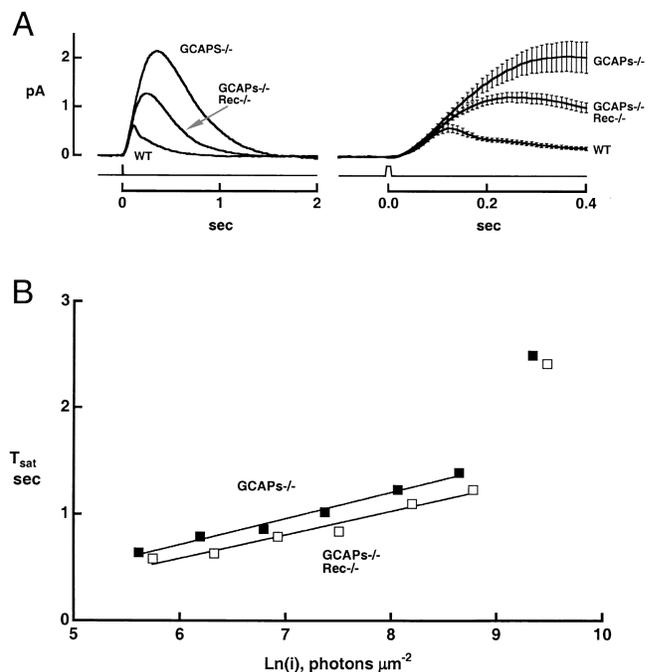


FIGURE 6. Changes in the flash responses of $\text{GCAPs}^{-/-}$ rods after deletion of recoverin. (A) Single photon responses of $\text{GCAPs}^{-/-}$ and $\text{GCAPs}^{-/-} \text{rec}^{-/-}$ rods. Traces show the averaged responses of 15 rods of each type on two time scales. Error bars demarcate the SEM. Low pass filtered at 20 Hz. The $\text{GCAPs}^{-/-}$ and wild type responses are reproduced from Mendez et al. (2001). (B) Saturation functions. The relation for the $\text{GCAPs}^{-/-} \text{rec}^{-/-}$ rod was shifted to flash strengths ~ 1.7 -fold greater than for the $\text{GCAPs}^{-/-}$ rod. Linear regression yielded slopes of 240 and 222 ms for $\text{GCAPs}^{-/-}$ and $\text{GCAPs}^{-/-} \text{rec}^{-/-}$ rods, respectively.

rods from one retina. Ca^{2+} buffering was significantly increased as demonstrated by the fact that the exchange current time constant was twofold longer than that in four untreated rods from the other retina of the same mouse (82 ± 13 ms, $\text{rec}^{-/-}$; 157 ± 29 ms, $\text{rec}^{-/-}$ with BAPTA). BAPTA did not restore a normal phenotype to $\text{rec}^{-/-}$ rods; instead it delayed the time to peak of the flash response and introduced an oscillation in the recovery phase (Fig. 4 E, inset). Furthermore, background light still failed to reduce the saturation time of a bright flash response (Fig. 4 E). These experiments suggest that recoverin does not act only as a Ca^{2+} buffer in the outer segment.

Second, we determined the effect of deleting recoverin in rods lacking calcium-regulated GC activity. We did so by comparing transduction in rods lacking guanylate cyclase-activating proteins ($\text{GCAPs}^{-/-}$; Mendez et al., 2001) with that in rods lacking both GCAPs and recoverin ($\text{GCAPs}^{-/-} \text{rec}^{-/-}$ double knockouts). If recoverin solely acts by controlling the time course of cyclase activation, deleting recoverin should have no effect in the $\text{GCAPs}^{-/-}$ background. Fig. 6 il-

TABLE 11
Flash Response Parameters of Rods after Deletion of GCAPs and Recoverin

	GCAPs ^{-/-}	GCAPs ^{-/-} Rec ^{-/-}	p
Dark current (pA)	13.3 ± 0.8, 42	12.3 ± 0.9, 19	ns
Half-saturating flash strength, $i_{1/2}$ (photons μm^{-2} at 500 nm)	11.3 ± 0.8, 33	19.0 ± 1.2, 18	1e-6
Single photon response characteristics			
Integration time, T_i (ms)	589 ± 19, 42	504 ± 27, 15	2e-2
Time to peak, t_p (ms)	315 ± 13, 42	255 ± 20, 17	2e-2
Amplitude (pA)	2.31 ± 0.20, 41	1.34 ± 0.10, 15	6e-3

Values are given as mean ± SEM, *n*. Results from GCAPs^{-/-} rods were reported previously by Mendez et al. (2001).

illustrates single photon responses averaged from a number of GCAPs^{-/-}, GCAPs^{-/-} rec^{-/-} double knockouts, and wild-type rods; Table II summarizes several parameters of the flash responses of the cell populations. As reported previously, GCAPs deletion increased the amplitude, time-to-peak, and integration time of the single photon response (Mendez et al., 2001). Deletion of recoverin in the GCAPs^{-/-} background gave a sizeable reduction in the single photon response amplitude (Fig. 6, Table II) and a corresponding increase in the half-saturating flash strength (Table II). Recoverin deletion also shortened the time-to-peak and integration time of the dim flash response in the GCAPs^{-/-} background. This indicates that in the absence of recoverin, light-stimulated PDE activity was effectively shorter. In addition, the “kinks” observed in subsaturating rec^{-/-} responses were absent in the GCAPs^{-/-} rec^{-/-} responses. Apparently, the kinks in the responses of the rec^{-/-} rods can be attributed to abnormally rapid cyclase activation. The characteristic differences in the flash responses of normal, rec^{-/-}, GCAPs^{-/-}, and GCAPs^{-/-} rec^{-/-} were satisfactorily predicted by a simple quantitative model of the response dynamics (Fig. 8 and APPENDIX).

Saturation times of bright flash responses in GCAPs^{-/-} rods were shifted to twofold higher flash strengths upon recoverin deletion (e.g., Fig. 6 B; *n* = 21 GCAPs^{-/-} and 11 GCAPs^{-/-} rec^{-/-} rods). In additional experiments (not depicted), exposure to a conditioning step shortened the saturation time of the response to a bright flash in each of 11 GCAPs^{-/-} rods, as expected from the presence of recoverin’s action, but this effect was absent in each of 7 GCAPs^{-/-} rec^{-/-} rods.

In summary, these results suggest that recoverin has a dual action on the flash response: (a) Ca²⁺-recoverin extends the effective duration of rhodopsin’s catalytic lifetime, and (b) by acting as a Ca²⁺ buffer, recoverin slightly delays the activation of guanylate cyclase.

Decreased Sensitivity of rec^{-/-} Rods to Steps of Light

The onset of a step of light elicited a response that rose to a peak and then partially recovered, as the rod

adapted (e.g., Fig. 4 A). The initial restoration of the current was faster in rec^{-/-} rods than in controls (Fig. 7 A, arrowheads), but was not faster in rods lacking both GCAPs and recoverin (GCAPs^{-/-} rec^{-/-}; unpublished data). This suggests that the initial, faster restoration of the current in rec^{-/-} rods arises from accelerated Ca²⁺ activation of guanylate cyclase. The absence of recoverin decreased step sensitivity, measured at the initial peak of the step response (Fig. 7 B and Table I). For control rods, an intensity of 250 photons $\mu\text{m}^{-2} \text{s}^{-1}$ elicited a half-maximal response while for rec^{-/-} rods, 410 photons $\mu\text{m}^{-2} \text{s}^{-1}$ was required (Table I). This 1.6-fold difference is attributable to the 1.6-fold lower integration time of the dim flash response in rec^{-/-} rods.

Incremental Flash Responses in Background Light

Deleting recoverin had little effect on light adaptation, as assessed by measuring the dependence of flash sensitivity on background light intensity (Fig. 7 C). The plot shows the relative sensitivity to a dim test flash as a function of normalized background light intensity, with both scales logarithmic. The closed symbols show results from control rods, and open symbols show results from rec^{-/-} rods. The normalizing factor for the background light intensity is I_0 , the intensity that reduced the cell’s flash sensitivity to half the value in darkness. Values of I_0 were similar in control and rec^{-/-} rods: 178 and 192 photons $\mu\text{m}^{-2} \text{s}^{-1}$, respectively (Table I). The smooth curve is drawn according to the Weber-Fechner relation:

$$S_f/S_f^d = 1/(1 + I/I_0). \quad (1)$$

This expression provides an empirical description of the behavior observed in several types of mammalian rods (Tamura et al., 1989, 1991; Nakatani et al., 1991). For comparison, the dashed and dotted curves plot the relation expected for rods whose adaptation results strictly from response saturation:

$$S_f/S_f^d = \exp(-(\ln 2)(T_i I)/i_{1/2}). \quad (2)$$

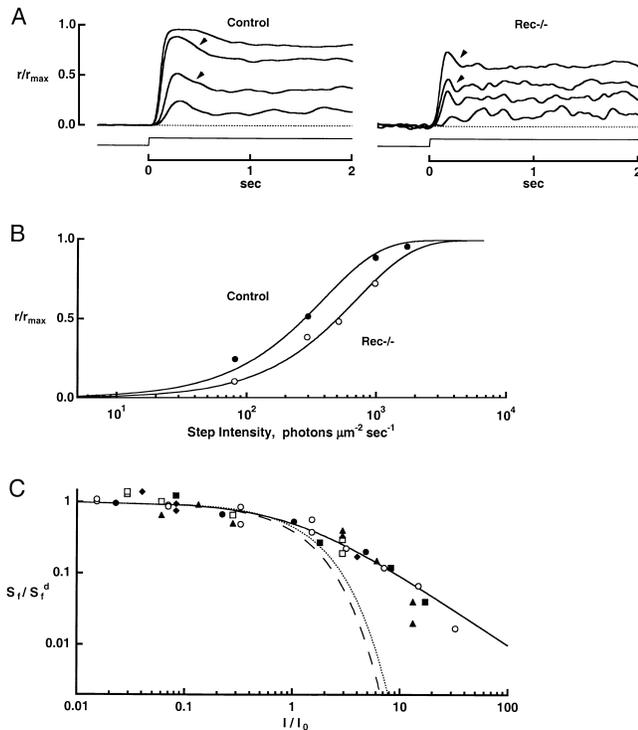


FIGURE 7. Responses to steps of light and to flashes superimposed on steps. (A) Averaged responses of a control rod and a $rec^{-/-}$ rod to steps of light. Arrowheads mark the partial recovery in current resulting from light adaptation. Maximal response amplitudes were 14.5 and 8.0 pA for control and $rec^{-/-}$ rods, respectively. (B) Diminished sensitivity of a $rec^{-/-}$ rod to steps of light. Results from A were fitted with a saturating exponential function (continuous lines). Half-maximal responses were elicited with intensities of 273 and 495 photons $\mu\text{m}^{-2} \text{s}^{-1}$ at 500 nm in the control and $rec^{-/-}$ rods, respectively. (C) Normal incremental flash sensitivity in $rec^{-/-}$ rods. Rods were adapted to a background light for a minimum of 40 s and then probed with dim test flashes. Dim flashes were also given in darkness before and after presentation of the background to monitor the condition of the cells. The continuous line plots the Weber-Fechner relation (Eq. 1). For comparison, the dashed and dotted lines plot the behaviors predicted by saturation alone (Eq. 2) for control and $rec^{-/-}$ rods, respectively. Collected results from four control (filled symbols) and two $rec^{-/-}$ rods (open symbols).

The behavior of both types of rod was very similar, and both populations truly adapted rather than simply saturating. We conclude that deletion of recoverin had little effect on the mechanisms that fix the dependence of incremental flash sensitivity on background light intensity.

DISCUSSION

Functional Role of Recoverin in Phototransduction

A photon absorbed in bright background light normally produces a smaller contribution to the steady-

state PDE activity than does a photon absorbed in darkness (Koutalos et al., 1995). This effect, referred to as "PDE adaptation", depends at least in part on the light-induced fall in steady-state free $[\text{Ca}^{2+}]_i$ within the outer segment and can amount to a seven-fold reduction in the incremental PDE activity (Matthews, 1996). By reducing the steady level of PDE activity that the background generates, PDE adaptation reduces the background's ability to elicit a saturating response, during which incremental stimuli cannot be effectively transduced. Our results identify recoverin as the Ca^{2+} sensor that triggers calcium-dependent PDE adaptation. Thus, the dark-adapted response to bright flashes was shorter in $rec^{-/-}$ rods than in wild-type rods, and this difference disappeared in background light, which lowered the free $[\text{Ca}^{2+}]_i$. There was no evidence that any other component of PDE adaptation persisted after the recoverin gene was knocked out. Evidence that the shorter incremental response of $rec^{-/-}$ rods arose from reduced PDE activity rather than an effect on the activity of guanylate cyclase is provided by the fact that recoverin's action was still present in GCAPs $^{-/-}$ rods, whose cyclase activity does not vary with background light level (Mendez et al., 2001; Burns et al., 2002). It should be emphasized that recoverin potentiates the dark-adapted response to light rather than having an effect on the response in background light. Thus, Ca^{2+} recoverin increases the duration of the light-triggered PDE activity. As a result, the integration time of the dark-adapted flash response is longer and the amplitude of the response to a dim step is larger.

Deletion of recoverin had little effect on the dependence of relative flash sensitivity on background light intensity (Fig. 7 C). This result, perhaps surprising at first, may be explained in the following way. At high background intensities, recoverin is in the Ca^{2+} -free state, where it exerts no effect, and thus knockout rods and control rods should behave identically. At low background intensities, Ca^{2+} -recoverin potentiates the late phase of the incremental flash response in control rods. However, flash sensitivity, measured at the peak response amplitude, is almost insensitive to recoverin's action, so that again little difference is expected between knockout and control rods. Had step sensitivity been plotted as a function of background intensity, rods containing recoverin would have exhibited a modest extension of response range, being nearly twofold more sensitive in darkness and converging with the $rec^{-/-}$ rods' behavior at high background intensity. We cannot explain the electroretinographic recordings that suggested a slightly elevated step sensitivity in $rec^{-/-}$ mice (Hurley and Chen, 2001).

Matthews (1997) showed that in salamander rod outer segments at room temperature there was very little PDE adaptation during the response to a bright

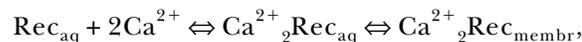
flash, yet a conditioning step of light preceding the bright flash gave a substantial effect. Negative feedback during the flash response itself was negligible because the target controlling PDE adaptation had mostly disappeared by the time the free $[Ca^{2+}]_i$ fell. Probably there is also little PDE adaptation (calcium-dependent modulation of rhodopsin activity) during the flash response of mouse rods because slowing Ca^{2+} dynamics in GCAPs $-/-$ rods has no effect on the kinetics of the flash response (Burns et al., 2002). The rapid disappearance of Ca^{2+} -recoverin's target suggests that recoverin mediates an effect in which the response to an incident photon can desensitize only the response to a subsequent photon. This is in contrast to the effect mediated by guanylate cyclase activation, which provides negative feedback during the single photon response.

Molecular Mechanism of Recoverin's Action

Our experiments show that Ca^{2+} -recoverin effectively prolongs light-triggered PDE activity, but they do not identify the target of its action. Because the initial rate of rise of the dark-adapted flash response was the same in *rec-/-* and control rods it is unlikely that recoverin affects the initial efficiency of transducin activation by photoexcited rhodopsin, the efficiency of PDE activation by activated transducin, or the catalytic activity of PDE itself. Therefore, Ca^{2+} -recoverin apparently acts on the effective catalytic lifetime of one of the species in the excitation chain. In truncated outer segments of salamander rods, Ca^{2+} -recoverin failed to affect the rate of deactivation of transducin (Erickson et al., 1998). Furthermore, Kawamura (1993) presented evidence that Ca^{2+} -recoverin inhibits the phosphorylation of rhodopsin by rhodopsin kinase, and subsequent experiments demonstrated that Ca^{2+} -recoverin binds rhodopsin kinase (Gorodovikova and Philippov, 1993; Chen et al., 1995a; Klenchin et al., 1995). Ca^{2+} -recoverin may impede the shutoff of rhodopsin simply by decreasing the effective concentration of free rhodopsin kinase. The feasibility of such a mechanism was demonstrated by models of rod photoresponses (compare Pugh et al., 1999; Hamer, 2000; Nikonov et al., 2000). Consistent with this notion, addition of exogenous recoverin into a rod outer segment prolonged the flash response (Gray-Keller et al., 1993; Erickson et al., 1998) and extended the catalytic lifetime of photoexcited rhodopsin (Erickson et al., 1998). Also consistent, reduction of the rhodopsin kinase content of mouse rods by knockout of one allele slowed the recovery phase of the flash response (Chen et al., 1999).

If recoverin is to effectively control the concentration of free rhodopsin kinase, its concentration must exceed that of rhodopsin kinase. Is this condition met? Recoverin has a relatively low affinity for Ca^{2+} (Ames et al., 1995) so that if the dark concentration of free Ca^{2+}

is 250 nM (Woodruff et al., 2002), most of the recoverin will be in the Ca^{2+} -free form. Assuming that recoverin binds two Ca^{2+} with a Hill coefficient of 1.4, that recoverin exists in a simple equilibrium between Ca^{2+} -free and Ca^{2+} -bound forms and that Ca^{2+} -bound recoverin partitions into the membrane, then



where $k_1 = [Rec]_{aq}[Ca^{2+}]_{free}^{1.4}/[Ca^{2+}_2Rec]_{aq} = (17 \mu M)^{1.4}$ and $k_2 = [Ca^{2+}_2Rec]_{aq}/[Ca^{2+}_2Rec]_{membr} = 0.1$ (Ames et al., 1995; Baldwin and Ames, 1998; Erickson et al., 1998). For $[Ca^{2+}]_{free} = 0.25 \mu M$ and $[Ca^{2+}_2Rec]_{aq} + [Ca^{2+}_2Rec]_{membr} = 3-5 \mu M$ (from the analysis of the exchanger current, above), the total recoverin concentration in a dark-adapted outer segment would be 106–176 μM . This range of values is larger than the biochemical determinations of 30–40 μM for recoverin (Kawamura and Murakami, 1991; Klenchin et al., 1995; but see Kawamura et al., 1993, 1996). At $>100 \mu M$, recoverin exceeds the estimated rhodopsin kinase concentration of $\sim 10 \mu M$ by tenfold (Shichi and Somers, 1978), making the model for kinase inhibition feasible. Therefore, the simplest interpretation of our data is that calcium-recoverin prolongs the lifetime of photoexcited rhodopsin, probably via its interaction with rhodopsin kinase. During light adaptation, intracellular Ca^{2+} falls and the effect of recoverin is removed.

A P P E N D I X

The effect of deletion of recoverin on the photocurrents generated by flashes was modeled using Rieke and Baylor's (1998) treatment. The model makes several simplifying assumptions: catalytically active rhodopsin in dark-adapted control rods decays exponentially, the ROS volume is well-stirred, and regulation of the cGMP-gated channel by Ca^{2+} is negligible. The model successfully described the responses of *rec-/-* rods if the absence of recoverin or low intracellular Ca^{2+} was assumed to cause an abrupt decline of the rhodopsin activity, the remaining activity then decaying with an exponential time course (Fig. 8 A).

The change in PDE activity induced by photoexcited rhodopsin is given by

$$\frac{dP(t)}{dt} = \sigma R(t) - k_{PDE}[P(t) - \beta_{PDE}], \quad (A1)$$

where R is rhodopsin activity, P is the total PDE activity, σ is the rate of PDE activation for unit R , β_{PDE} is the basal PDE activity, and k_{PDE} is the rate of decay of light-triggered PDE activity. The cGMP concentration, $G(t)$, depends on its rate of synthesis, γ , and its rate of hydrolysis:

$$\frac{dG(t)}{dt} = \gamma(t) - P(t)G(t). \quad (A2)$$

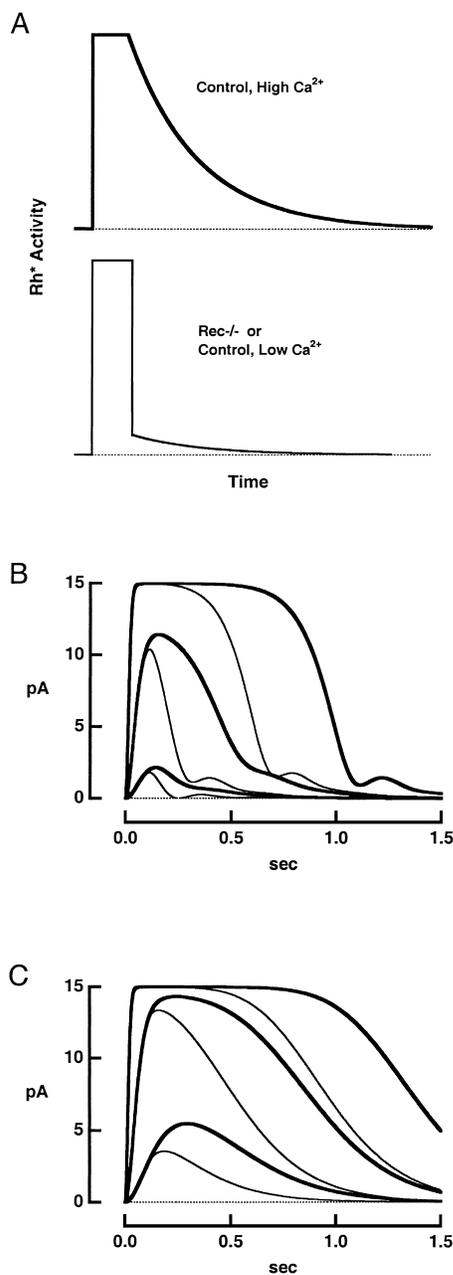


FIGURE 8. Calculated flash responses. (A) Assumed time courses of rhodopsin's catalytic activity. In the control rod (top), activity shut off exponentially after a delay of 100 ms (Chen et al., 1995b, 1999). The time constant of 205 ms was found from the mean slope of the relation between T_{sat} and the natural logarithm of the flash strength (compare Pepperberg et al., 1992). In the $\text{rec}^{-/-}$ rod (bottom), rhodopsin activity suddenly fell ninefold after 100 ms and then decayed exponentially with a time constant of 205 ms. (B) Photocurrents generated by the flash response model (APPENDIX) for control (thick traces) and $\text{rec}^{-/-}$ (thin traces) rods. Rhodopsin's catalytic activity was increased ten-fold for successive pairs of responses. (C) Responses from the model in the absence of Ca^{2+} feedback onto guanylate cyclase for rods with (thick traces) and without (thin traces) recoverin. The catalytic activity levels of rhodopsin are the same as those in B.

The synthesis of cGMP is Ca^{2+} dependent:

$$\gamma = \frac{\gamma_{\text{max}}}{1 + (Ca/K_{GC})^{3.7}}, \quad (\text{A3})$$

where Ca is the free $[\text{Ca}^{2+}]_i$ and K_{GC} is the Ca at which cGMP synthesis is half maximal. Changes in Ca follow

$$\frac{dCa(t)}{dt} = qhG^3(t) - \beta Ca(t), \quad (\text{A4})$$

where q is a constant relating membrane current to changes in Ca , β is the rate constant for Ca^{2+} extrusion, and h ($=0.551 \text{ pA } \mu\text{M}^{-3}$) is a constant relating the membrane current to the cube of the cGMP concentration.

In our simulations (Fig. 8 B), the maximal light-suppressible current was 15 pA, and Ca was set to 250 nM in darkness and assumed to fall to 20 nM in saturating light (Woodruff et al., 2002) along a single exponential time course. For control responses, the time constant β was set to 113 ms, while for $\text{rec}^{-/-}$, it was set to 86 ms. PDE decay was 7 s^{-1} , basal PDE activity set to 10 s^{-1} , and K_{GC} was $0.25 \mu\text{M}$ with a Hill coefficient of 3.7 (Burns et al., 2002). Feedback onto guanylate cyclase was removed in order to simulate the behavior of the rod after knockout of both recoverin and GCAPs by setting K_{GC} to 10,000 (Fig. 8 C).

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