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9. TH was assayed as described by A. L. DeVries [*Methods Enzymol.* **127**, 293 (1986)]. Purified AFP was adjusted to a protein concentration of 1 mg/ml; homogenate of leaves from transgenic tobacco line 35-9-CA was assayed at a total protein concentration of 14.2 mg/ml, and that from line 35-9-CB was assayed at a total protein concentration of 7.5 mg/ml.
10. Monoclonal antibody YZ1/2.23 [M. T. McManus *et al.*, *Planta* **175**, 56 (1988)] was used for immunodetection of *N*-glycans. Enzymic deglycosylation: Purified carrot AFP (2.5 mg/ml) was incubated with α-mannosidase (50 U/ml), β-hexosaminidase (10 U/ml), and β-galactosidase (1 U/ml) from jack bean; β-mannosidase (4 U/ml) from *Helix pomatia* (all from Oxford Glycosciences, Abingdon, UK); β-xylosidase (0.1 U/ml) from tobacco (F. Khan and D. Ashford, unpublished data); and α-fucosidase (2 U/ml) from bovine kidney (Boehringer-Mannheim) in 0.1 M citrate-phosphate buffer (pH 5.0) containing 0.02% sodium azide and bovine serum albumin (1.1 mg/ml) for 16 hours at 37°C together with appropriate controls.
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13. First-strand cDNA was obtained by reverse transcription of total RNA from cold-acclimated carrot storage root with the oligonucleotide primer OG1 (5′-GAGAGAGGATCCTCGAGTTTTTTTTTTTTTT-3′) and the enzyme Superscript (Gibco BRL), according to the manufacturer's instructions. One percent of the cDNA was used as a template for polymerase chain reaction with the primers OG1 and cp3 (5′- GGICCIGTICCIYTITTYTTYCC-3′, where I is inosine and Y is T or C). The reaction was carried out in a thermal cycler with Taq DNA polymerase (Gibco BRL), for 30 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 74°C, according to manufacturer's instructions, with 2 mM $MgCl_2$ and primer concentrations of 1 μM. The ~800–base pair amplified fragment was cloned and used to screen a cold-acclimated carrot tap root cDNA library (ZAP cDNA kit; Stratagene) to obtain the full-length AFP clone. Seventeen hybridizing plaques were identified, seven were partially sequenced, and two were sequenced to completion, both of which contained identical coding regions representing the carrot AFP.
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18. Ten microliters of PG enzyme [*A. niger* PG (1 U/ml); Sigma] was added to 40 μl of 300 mM sodium acetate buffer with purified carrot AFP (±0.2 mg/ml). After 15 min of preincubation at 37°C, 100 μl of polygalacturonan substrate [0.5% (w/v) deesterified citrus polygalacturonic acid] was added and incubated at 37°C for 20 min. Reducing end groups released by PG were assayed [K. C. Gross, *Hortic. Sci.* **17**, 933 (1982)]. PG activity was eluted from a cell wall fraction of ripe tomato fruit [L. S. Zheng, R. C. Heupel, D. Dellapenna, *Plant Cell* **4**, 1147 (1992)] and assayed as above. When testing for inhibition of the tomato enzyme, AFP was also preincubated with substrate present at limiting concentrations (>0.05% polygalacturonan) before assay.
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Role for the Target Enzyme in Deactivation of Photoreceptor G Protein in Vivo

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Heterotrimeric guanosine 5′-triphosphate (GTP)–binding proteins (G proteins) are deactivated by hydrolysis of the GTP that they bind when activated by transmembrane receptors. Transducin, the G protein that relays visual excitation from rhodopsin to the cyclic guanosine 3′,5′-monophosphate phosphodiesterase (PDE) in retinal photoreceptors, must be deactivated for the light response to recover. A point mutation in the γ subunit of PDE impaired transducin-PDE interactions and slowed the recovery rate of the flash response in transgenic mouse rods. These results indicate that the normal deactivation of transducin in vivo requires the G protein to interact with its target enzyme.

In numerous signaling cascades, G proteins relay signals from seven-helix receptors to target effector molecules. G proteins become activated upon receptor-catalyzed binding of GTP to the α subunit and then modulate the activity of an effector until the bound GTP is hydrolyzed to guanosine diphosphate (GDP) (*1*). Two different mechanisms control the rate of GTP hydrolysis. First, the guanosine triphosphatase (GTPase) activities of at least two G proteins (transducin and G_q) can be increased by their target enzymes in vitro (*2, 3*). Second, the rate of GTP hydrolysis can be accelerated by members of the regulators of G protein signaling (RGS) protein family. RGS proteins are strong GTPase activators for a large spectrum of G proteins (*4*). The individual roles of the effector and the RGS protein in controlling G protein deactivation in vivo are not known.

The phototransduction cascade of retinal rods is a particularly accessible G protein signaling system (*5*). Upon absorption of a photon,

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photoexcited rhodopsin activates transducin, which in turn activates its effector, cyclic guanosine 3′,5′-monophosphate (cGMP) PDE. Transducin activates PDE binding to its γ subunits (PDEγ), which removes their inhibitory constraint on the α and β catalytic subunits of PDE (PDEαβ). Hydrolysis of cGMP by PDE lowers the cytoplasmic cGMP concentration and closes cGMP-gated cationic channels in the rod plasma membrane, which reduces the inward current. During the recovery of the photoresponse, transducin deactivates hydrolyzing its bound GTP, thus permitting PDEγ to rapidly reinhibit PDEαβ. However, GTP hydrolysis by transducin in vitro is much slower than the recovery of the photoresponse, suggesting that one or more GTPase activators speed transducin's deactivation in vivo. The addition of PDEγ to reconstituted photoreceptor membranes accelerates transducin's GTPase activity (*2*). However, this effect of PDEγ requires the presence of another membrane-associated photoreceptor protein, RGS9 (*6, 7*). A fragment of RGS9 that contains the RGS homology domain, as well as other RGS proteins, accelerates transducin GTPase activity in the absence of PDEγ (*7, 8*), however, PDEγ can further enhance the catalytic activity of RGS9 (*7*).

The Trp^{70} of PDEγ is crucial for its interaction with transducin. Studies in reconstituted systems indicated that the substitution of Ala for Trp^{70} (W70A) has two effects: It reduces the affinity of PDEγ for transducin (*9, 10*) and it abolishes the acceleration of GTP hydrolysis in the transducin-PDEγ complex by photoreceptor membranes that contain RGS9 (*9*). We used the W70A mutation to determine whether transducin-PDEγ interactions are also required for the normal deactivation of transducin in the intact rod. We analyzed two mouse lines in which the PDEγ gene was replaced by the W70A transgene and one mouse line in which the PDEγ gene was replaced by the wild-type

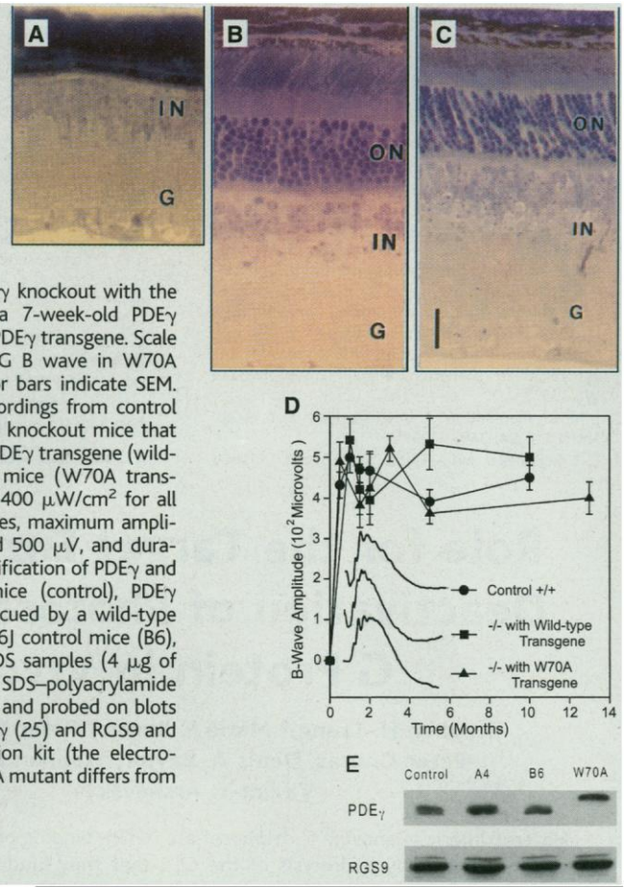

Fig. 1. Rescue of retinal degeneration in PDEγ knockout mice by expression of the W70A transgene. Photomicrographs of cross sections of mouse retinas that were stained with toluidine blue (ON, outer nuclear layer; IN, inner nuclear layer; G, ganglion cell layer) showing (**A**) a 7-week-old PDEγ knockout; (**B**) a 13-month-old PDEγ knockout with the W70A transgene, and (**C**) a 7-week-old PDEγ knockout with the wild-type PDEγ transgene. Scale bar, 40 μm. (**D**) Normal ERG B wave in W70A retinas over 13 months. Error bars indicate SEM. Inset shows corneal ERG recordings from control outbred mice (control), PDEγ knockout mice that were rescued by a wild-type PDEγ transgene (wild-type transgene), and W70A mice (W70A transgene). Flash intensities were 400 μW/cm² for all responses. For inset ERG traces, maximum amplitudes were between 400 and 500 μV, and durations were 300 ms. (**E**) Quantification of PDEγ and RGS9 in control outbred mice (control), PDEγ knockout mice that were rescued by a wild-type PDEγ transgene (A4), C57BL/6J control mice (B6), and W70A mice (W70A). ROS samples (4 μg of rhodopsin) were subjected to SDS–polyacrylamide gel electrophoresis (12% gel) and probed on blots with rabbit antibodies to PDEγ (*25*) and RGS9 and a chemiluminescence detection kit (the electrophoretic mobility of the W70A mutant differs from that of the wild-type PDEγ).

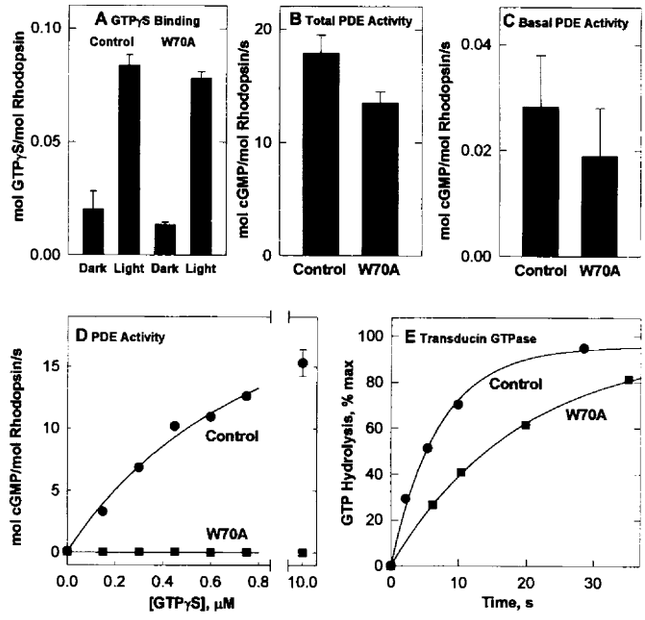

Fig. 2. Functional expression of phototransduction proteins in W70A rods. (**A**) Light-dependent binding of GTP-γ-S in ROS from control and W70A mice. Dark-adapted ROS (16 μl) containing 15 μM rhodopsin were mixed with 8 μl of 9 μM [^{35}S]GTP-γ-S under infrared illumination. After a 10-s incubation, the amount of bound GTP-γ-S was determined in a 7-μl portion by the nitrocellulose filter binding assay (*26*). The rest of the sample was bleached, and the GTP-γ-S binding was determined in another portion. Error bars indicate SD. (**B**) Trypsin-activated and (**C**) basal PDE activity. The reaction was started by mixing ROS containing 7.5 μM rhodopsin (14 μl) with 7 μl of 1 mM [^{3}H]cGMP, proceeded for 2 to 6 s (B) or 2 to 10 min (C), and was stopped by the addition of 100 μl 0.1 M HCl; the amount of hydrolyzed cGMP was then determined (*16*). Before the reaction was initiated, the ROS were incubated with either trypsin (10 μg/ml) (B) for 30 min to achieve complete PDE activation or 3 μM cGMP (C) for 2 min to stabilize basal PDE activity (*25*). The results are averages from two (B) or three (C) separate experiments; error bars indicate the range of determined values. (**D**) PDE activation by transducin. Bleached ROS (14 μl) containing 15 μM rhodopsin were incubated with 3 μM cGMP for 2 min to stabilize PDE basal activity, and the reaction was started by adding 7 μl of 2 mM [^{3}H]cGMP, which was supplemented by the indicated amounts of GTP-γ-S. The reaction was stopped either at 2 s (control) or 20 s (W70A) by the addition of 100 μl of 0.1 M HCl, and the amount of hydrolyzed cGMP was determined (*16*). The curve is a hyperbolic fit to the results from one of three similar experiments. The addition of GTP-γ-S in amounts less than the amount of transducin resulted in complete binding that was light dependent. The results are averaged from two independent determinations. Error bar indicates the range of determined values. (**E**) Transducin GTPase activity. The single-turnover GTPase reaction was started by mixing bleached ROS (14 μl) containing 30 μM rhodopsin with 7 μl of 0.3 μM [γ-^{32}P]GTP, and the time course of $^{32}P_i$ (P_i, inorganic phosphate) formation was determined by the activated charcoal assay (*27*) after the samples were quenched with perchloric acid. The rate constants of transducin GTPase activity that were determined by exponential fits of the results were 0.141 ± 0.021 s^{-1} (mean ± SD) for control ROS and 0.052 ± 0.004 s^{-1} for W70A ROS. The results shown are from one of two similar experiments.

transgene. Mutant or wild-type PDEγ cDNAs under the control of the opsin promoter were constructed and used to generate transgenic mice by conventional means (*11*). The PDEγ-deficient parental mice displayed rapid and severe retinal degeneration (*12*) (Fig. 1A). However, expression of either W70A or wild-type PDEγ transgenes restored normal retinal morphology (Fig. 1, B and C) and stable electroretinogram (ERG) recordings for up to 13 months (*13*) (Fig. 1D). Protein immunoblot analysis suggested that the expression level of both PDEγ (or W70A) and RGS9 in rod outer segments (ROS) of the transgenic mice was similar to that in control rods (*14*) (Fig. 1E). Likewise, the rhodopsin content in the retinas of transgenic mice was indistinguishable from that in controls (*15*).

Expression of the W70A transgene had no effect on other proteins of the phototransduction cascade. The total amount of transducin in W70A ROS, which was determined by measuring the maximal light-dependent binding of a nonhydrolyzable GTP analog, guanosine 5′-*O*-(3′-thiotriphosphate) (GTP-γ-S), was 0.084 ± 0.004 mol per mol of rhodopsin (mean ± SD; $n = 7$) in control and 0.078 ± 0.003 mol per mol of rhodopsin ($n = 6$) in W70A ROS (Fig. 2A). Total PDE activity, determined by removing the inhibition conferred by PDEγ or its W70A mutant by trypsinolysis (*16*), was also very similar between W70A and control ROS (Fig. 2B). Basal PDE activity, which is restrained by PDEγ in the dark, was similar in dark-adapted W70A and control ROS (Fig. 2C), indicating that the W70A mutation does not grossly alter the ability of the γ subunit to inhibit the α and β subunits of PDE. We conclude that W70A expression caused minimal alterations in the expression of functionally active rhodopsin, transducin, and PDE.

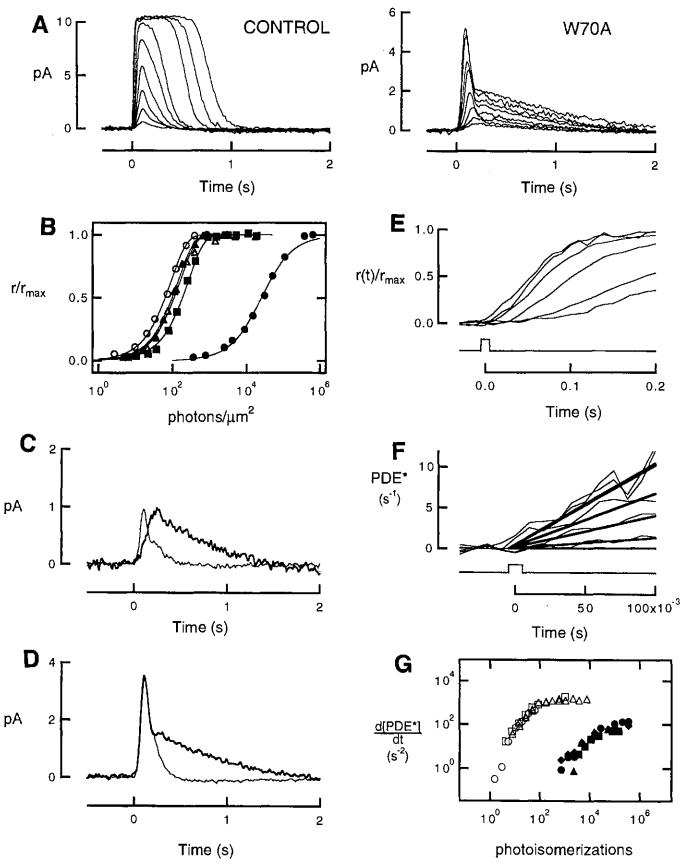

Fig. 3. Reduced flash sensitivity and slow response kinetics in W70A rods. (**A**) Suction electrode recordings of responses to flashes of increasing strength from a control (129/SvJ) and a W70A mouse rod. The control traces are the average of 3 to 66 responses to flashes that varied in strength from 9.83 to 2520 photons per square micrometer by factors of 2. The W70A traces are the averages of 5 to 29 responses to flashes that varied from 3460 to 283,000 photons per square micrometer by factors of 2. Flashes were delivered at $t = 0$. (**B**) Dependence of normalized response amplitude on flash strength for W70A rods (solid circles) and various control rods, including hemizygous rods expressing the W70A transgene (solid triangles), PDEγ knockout rods expressing the wild-type PDEγ transgene (squares), 129/SvJ rods (open circles), and C57BL/6J rods (open triangles). The control relations were fitted with saturating exponential functions, and the W70A relation was fitted by the Michaelis relation (*19*). (**C** and **D**) Superimposed average flash responses from a control rod (thin lines) and a W70A rod (thick lines). Flashes that elicited ~2 (control) or 950 (W70A) photoisomerizations in (C) or 7.6 (control) or 18,216 (W70A) photoisomerizations in (D) were delivered at $t = 0$. The maximal response amplitudes in (C) were 8.0 pA (control) and 6.1 pA (W70A). The maximal response amplitudes in (D) were 10.6 pA (control) and 5.2 pA (W70A). (**E**) Normalized responses $[r(t)/r_{max}]$ of a W70A rod to flashes of increasing strength (734 to 361,000 photons per square micrometer). (**F**) PDE activity (PDE*) as determined from the normalized flash responses in (E) (*20*). Straight lines are fitted to the first 100 ms of the response, beginning at the onset of the flash (lower trace). (**G**) The rate of PDE activation [that is, the slope of the lines fitted in (F)] was determined for four control rods [two 129/SvJ and two C57BL/6J (open symbols)] and four W70A rods (solid symbols). The maximal rate of PDE activation in W70A rods occurred at ~100,000 photoisomerizations, or ~100 photoisomerizations per disc face.

To assess the ability of transducin to activate PDE, we added various amounts of GTP-γ-S to light-activated ROS (Fig. 2D). In control ROS, GTP-γ-S at saturating concentrations activated PDE to the same maximal level as that observed when PDE was trypsin-activated. In W70A ROS, however, activation of the entire transducin pool with GTP-γ-S was not sufficient to produce an increase in PDE activity above basal levels. In addition, the rate of GTP hydrolysis by transducin in ROS purified from W70A mice was slower than that in ROS from the control animals (*17*) (Fig. 2E). Because RGS9 expression in W70A rods was normal (Fig. 1E), we conclude that the prolongation of transducin's lifetime was caused by the uncoupling of activated transducin and W70A PDE.

To assess the effects of the W70A mutation on visual transduction in vivo, we recorded the photoresponses of single intact rods from W70A and control mice (*18*) (Fig. 3). All W70A rods examined ($n = 48$) were extremely insensitive to light (Fig. 3, A and B) and exhibited photoresponses that recovered more slowly than those of controls (Fig. 3, C and D). The flash strength required to elicit a half-maximal response was 21,600 ± 640 photons/μm² for W70A (mean ± SEM; $n = 20$) and 74.8 ± 1.9 photons/μm² ($n = 14$; strain 129/SvJ) and 112 ± 11 photons/μm² ($n = 14$; strain C57BL/6J) for control rods (*19*) (Fig. 3B). To determine whether the inability of transducin to increase PDE activity results from reduced binding affinity between transducin and W70A or from lower hydrolytic activity of the transducin-PDE holoenzyme complex, we calculated the light-evoked PDE activity as a function of time (*20*) (Fig. 3, E to G). PDE activity in W70A rods rose linearly for 100 ms after a flash that activated ~100 rhodopsins per disc face. Because such a bright flash would have normally activated all of the PDE within a few milliseconds (*21*), the rate of PDE activation in W70A rods proceeded about three orders of magnitude more slowly than normal. This impairment, which is consistent with the biochemical measurements in Fig. 2D, indicates that PDEαβ reduced the affinity of transducin for W70A in our experiments or that the W70A mutation slows the disinhibition of PDEαβ after transducin binds.

The impaired transducin-PDE interactions in W70A rods were associated with slow photoresponse kinetics (Fig. 3, C and D). The time to the peak of the dim flash response was 309 ± 14 ms (mean ± SEM; $n = 17$) in W70A rods and 100 ± 1 ms ($n = 17$) in controls. The time course of recovery was assessed by fitting a single exponential function to the final decline of the response. Responses of W70A rods declined seven times more slowly (exponential time constant $\tau =$ 1047 ± 22 ms; $n = 24$) than the responses of control rods ($\tau = 139.7 \pm 1.7$ ms; $n = 28$).

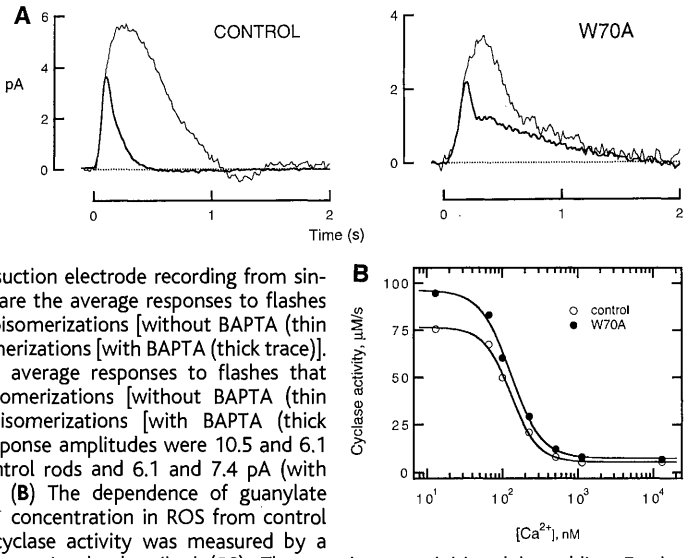

Fig. 4. Normal Ca^{2+}-dependent negative feedback in W70A rods. (**A**) BAPTA slowed or abolished the rapid recovery component of the control and the W70A responses. Small pieces of retina were incubated in 10 μM BAPTA-AM for 10 min with gentle agitation before suction electrode recording from single rods. Control traces are the average responses to flashes that elicited ~7.6 photoisomerizations [without BAPTA (thin trace)] or 10.6 photoisomerizations [with BAPTA (thick trace)]. W70A traces show the average responses to flashes that elicited ~3500 photoisomerizations [without BAPTA (thin trace)] or 2400 photoisomerizations [with BAPTA (thick trace)]. The maximal response amplitudes were 10.5 and 6.1 pA (with BAPTA) in control rods and 6.1 and 7.4 pA (with BAPTA) in W70A rods. (**B**) The dependence of guanylate cyclase activity on Ca^{2+} concentration in ROS from control and W70A mice. The cyclase activity was measured by a method similar to that previously described (*28*). The reaction was initiated by adding 5 μl of intracellular buffer (*14*) (containing 1 mM [α-^{32}P]GTP, 10 mM cGMP, 20 μM adenosine 5′-triphosphate, and Ca^{2+} buffered by BAPTA to the indicated concentration) to 10 μl of ROS suspension containing 15 μM rhodopsin, 100 μM zaprinast, and Ca^{2+} buffered to the same concentration. The reaction was stopped after 2 min by the addition of 50 mM EDTA (pH 7.0) and was immediately boiled for 1 min. The results are taken from one of two similar experiments. The dependence of the guanylate cyclase activity on Ca^{2+} concentration was fitted by the Hill equation $\alpha = \alpha_{max} - (\alpha_{max} - \alpha_{min})\ Ca^n/(Ca^n + K_{1/2}{}^n)$, where α is the rate of cGMP formation, α_{max} is the maximum rate at low Ca^{2+}, α_{min} is the minimum rate at high Ca^{2+}, $K_{1/2}$ is the half-saturating Ca^{2+} concentration, and n is the Hill coefficient. For control mice, the parameters are α_{max} = 76.5 μM/s, α_{min} = 5.5 μM/s, $K_{1/2}$ = 129 nM Ca^{2+}, and n = 2.45. For W70A mice, the parameters are α_{max} = 96.3 μM/s, α_{min} = 7.5 μM/s, $K_{1/2}$ = 129 nM Ca^{2+}, and n = 2.17.

The time constant of recovery in W70A rods was unchanged over a range of flash strengths that activated up to ~140,000 rhodopsin molecules (*22*), which indicated that the slow recovery kinetics were not the result of the depletion of other deactivation enzymes. Because the rate of association of PDEγ with PDEαβ was unaffected by the W70A mutation (*9*), the slow recovery of the W70A responses cannot be attributed to a reduced rate of reinhibition of the catalytic subunits. Instead, both the prolonged time to peak and the slow recovery of W70A responses are consistent with a lengthened lifetime of activated transducin. This indicates that transducin must bind to PDEγ for transducin to deactivate normally.

After bright flashes, when the amplitude of W70A responses exceeded ~1 pA, a rapid phase of recovery usually preceded the slow phase (in 44 of 48 cells) (Fig. 3, A and D). The Ca^{2+} buffer 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid–acetoxymethyl ester (BAPTA-AM), which slows changes in intracellular Ca^{2+} concentration, slowed or eliminated the rapid component of recovery in both W70A and control photoresponses, yet had no effect on the final slow recovery of the W70A responses (Fig. 4A). The Ca^{2+} regulation of guanylate cyclase, which is probably a primary calcium feedback mechanism in the W70A response (*23*), was very similar in control and W70A ROS (Fig. 4B). These experiments support the notion that the rapid component of recovery is due to normal calcium feedback to the cascade, whereas the slow component of recovery reflects slowed GTPase activity due to the W70A mutation.

Our results provide evidence that interaction with the effector enzyme influences the rate of G protein deactivation in vivo. Although RGS9 alone enhances the GTPase activity of transducin (*7*), transducin must interact with PDEγ to produce the rapid deactivation that is characteristic of the normal flash response. The dual requirement for an RGS protein and the effector may be a general strategy to ensure that the G protein relays excitation from the activated receptor to the effector with high efficiency.

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11. The experimental animals used in these studies were derived from an outbred mouse line containing contributions from strains 129/SvJ (Jackson Laboratories, Bar Harbor, ME), C57BL/6J (Jackson Laboratories), DBAJ (Jackson Laboratories) and MF1 (Harlan Sprague Dawley, Indianapolis, IN). The constructs used for the normal and mutant PDEγ contained 4.4 kb of the mouse opsin promoter, the complete open reading frame of *Pdeg* [N. Tuteja and D. B. Farber, *FEBS Lett.* **232**, 182 (1988)], and the polyadenylation signal of the mouse protamine gene [J. Lem, M. L. Applebury, J. D. Falk, J. G. Flannery, M. I. Simon, *Neuron* **6**, 201 (1991); P. A. Johnson, J. J. Peschon, P. C. Yelick, R. D. Palmiter, N. B. Hecht, *Biochim. Biophys. Acta* **950**, 45 (1988)]. The point mutation was introduced by a standard polymerase chain reaction (PCR)–based site-specific mutagenesis strategy. The entire *Pdeg* coding region in the transgenic construct was sequenced. After excision and purification from the plasmid vector, the constructs were injected into the male pronuclei of oocytes from superovulated F_1 (CBA × C57/Bl6) females that were mated with homozygous $Pdeg^{tm1}/Pdeg^{tm1}$ males. Integration of the transgene was determined by Southern (DNA) blot and PCR analyses of tail DNA. Transgenic mice were backcrossed to $Pdeg^{tm1}/Pdeg^{tm1}$ homozygous offspring, which were screened for inheritance of the foreign gene. The sensitivity and kinetics of the photoresponses in two independent W70A transgenic lines were indistinguishable. The control biochemical experiments used retinas from littermates that did not carry the transgene, and the control physiological experiments used retinas from both 129/SvJ and C57BL/6J mice, whose rods behaved very similarly, except where indicated. All mice were genotyped for the absence of the *rdl* allele [S. J. Pittler and W. Baehr, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8322 (1991)] before use. The standard nomenclature of the W70A mouse is TgN(RhPdegW70A)1Goff $Pdeg^{tm1Goff}/Pdeg^{tm1Goff}$.
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13. ERGs were obtained from dark-adapted mice that were anesthetized with intraperitoneal urethane (40 mg/ml), ketamine (1 mg/ml), and xylazine (0.4 mg/ml) at 15 μl/g body weight. The corneal ERG was detected with a cotton wick electrode. Flashes were obtained from a modified strobe lamp with a 3-cm aperture that was positioned 9 cm from the dilated pupil of the mouse. The ERG response was measured with a Nicolet Instruments CA-1000 computer, which averaged 2 to 100 responses at a flash frequency of 0.05 to 1 Hz. The maximum flash intensity photoisomerized ~2000 rhodopsin molecules per rod [A. L. Lyubarsky and E. N. Pugh, *J. Neurosci.* **16**, 563 (1996)].
14. Mouse ROS were isolated on ice under infrared illumination in Ringer's solution, which contained 130 mM NaCl, 3.6 mM KCl, 2.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM Hepes with KOH (pH 7.4), and 0.02 mM EDTA, and was adjusted to 313 mosM. The retinas from five animals were removed from the eyecups, placed in 150 μl of 8% OptiPrep (Nycomed, Oslo, Norway) in Ringer's buffer, and vortexed at maximum speed for 30 to 60 s. The tubes were then centrifuged at 200g for 40 s, and the supernatant containing the ROS was gently removed. The vortexing and sedimentation sequence was repeated at least six times. The collected supernatant was loaded on a step gradient that was made with 10 and 18% OptiPrep in Ringer's solution in a 4-ml centrifuge tube. The tube was centrifuged for 15 min at 3300g,

and the ROS were collected from the interface between 10 and 18% OptiPrep. The supernatant was diluted three times with Ringer's solution and centrifuged at 30,000g for 20 min. The sedimented material containing the ROS was rinsed once with 200 μl of Ringer's solution. The ROS were osmotically intact. We disrupted the plasma membrane of the ROS by hypotonic shock; 90 μl of water was added directly to the sedimented material, and the ROS were resuspended by intense mixing for ~10 s. The osmolarity was adjusted by adding 10 μl of a 10× intracellular buffer containing 120 mM KCl, 5 mM $MgCl_2$, 10 mM Hepes (pH 7.5), 1 mM dithiothreitol, 10 μM leupeptin, and 100 kallikrein units per 1 ml of aprotinin (final concentrations).

15. Rhodopsin expression in control and W70A retinas was assayed by two different techniques. (i) The absorption of 500-nm light by rhodopsin was measured in retinal flatmount preparations. The fraction of absorbed light was calculated as $(I_2 - I_1)/I_2$, where I_1 and I_2 are the measured intensities of 500-nm light transmitted through the retina before and after bleaching the rhodopsin with bright white light for 10 min. The transmitted intensity of a spot of light (0.015 mm^2) was measured by a photomultiplier that was connected to a digital pulse counter. The mean percent of absorbed light (expressed as minimum, maximum, and *n*, number of determinations) was 27.6%, (23.6, 34.7, $n = 3$) in 129/SvJ retinas and 27.5% (21.3, 31.8, $n = 4$) in W70A retinas. (ii) The amount of rhodopsin in the retinas of four mice was determined through difference spectroscopy [M. D. Bownds, A. Gordon-Walker, A.-C. Gaide-Huguenin, W. Robinson, *J. Gen. Physiol.* **58**, 225 (1971)] after solubilization in 30 mM cetyltrimethylammonium chloride. The rhodopsin content of both control and W70A retinas was 0.3 nmol per retina.
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17. The absolute rates of GTP hydrolysis in control ROS in vitro were slower than the rate of recovery of the photoresponse. This is consistent with many previous reports that show that dilution of cellular components, most likely RGS9, slows the rate of GTP hydrolysis (*6*) [E. A. Dratz, J. W. Lewis, L. E. Schaechter, K. R. Parker, D. S. Kliger, *Biochem. Biophys. Res. Commun.* **146**, 379 (1987); V. Y. Arshavsky, M. P. Antoch, K. A. Lukjanov, P. P. Philippov, *FEBS Lett.* **250**, 353 (1989)]. Similarly, the 2.7-fold difference in GTPase rate between control and W70A ROS in Fig. 2E should be considered only as the lowest estimate for the difference in physiologically intact photoreceptors.
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20. At early times during the rising phase of the flash response, the light-evoked increase in PDE activity as a function of time, PDE*(*t*), is given by the expression (*24*) $PDE^*(t) = -(1/n)\ (d\{\ln[1 - r(t)/r_{max}]\}/dt)$, where $r(t)$ is the time course of the flash response and *n* is the cooperativity of the cGMP-gated channels. We calculated PDE*(*t*) with this formula, assuming $n = 3$ [A. L. Zimmerman and D. A. Baylor, *Nature* **321**, 70 (1986); L. W. Haynes, A. R. Kay, K.-W. Yau, *ibid.*, p. 66]. The cell in Fig. 3, E and F, was loaded with BAPTA to determine the saturating maximal amplitude (7.4 pA) and to delay the onset of calcium-dependent negative feedback to the cascade. BAPTA had no effect on the rate of PDE activation in any control or W70A rods examined. To determine PDE*(*t*) for W70A rods in which the flash response did not saturate, we assumed the dark current (r_{max}) to be 12 pA. For all cells, the mean number of photoisomerizations per flash was calculated by multiplying the flash strength (in photons per square micrometer) by the effective collecting area of the mouse rod (0.23 μm^2).
21. In a mouse rod that is stimulated by an instantaneous flash causing 100 photoisomerizations per disc face, the total complement of activatable PDE in a disc face will be depleted with a time course $f(t) = 1 - \exp(-t/\tau_{act})$, in which the time constant τ_{act} is given by the ratio of the total number of PDE subunits divided by the initial rate of activation (*24*). Assuming 1000 s^{-1} for the initial rate of PDE activation per photoactivated rhodopsin (*24*), one finds that the initial rate of PDE* production will be $1 \times 10^5\ s^{-1}$. With ~400 PDE subunits per disc face (based on ~1:100 ratio of PDE to rhodopsin) a τ_{act} value of ~4 ms is obtained. Allowing for the finite flash duration of 10 ms and assuming an effective delay of 3 ms (*24*), one would expect activation of the PDE in a normal mouse rod to be completed within a few milliseconds after the end of the flash.
22. M. E. Burns and D. A. Baylor, unpublished observation.
23. Because of the prolonged lifetime of PDE* in the W70A rods, any feedback reaction underlying the fast recovery component should be downstream of PDE*, as any upstream feedback should affect the response amplitude, not the recovery kinetics [for a detailed analysis, see S. Nikonov, N. Engheta, E. N. Pugh, *J. Gen. Physiol.* **111**, 7 (1998)]. Therefore, we propose that the acceleration of guanylate cyclase activity, which is caused by decreased intracellular Ca^{2+}, is mainly responsible for the rapid initial recovery phase of W70A responses to bright flashes.
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An Antimicrobial Activity of Cytolytic T Cells Mediated by Granulysin

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Cytolytic T lymphocytes (CTLs) kill intracellular pathogens by a granule-dependent mechanism. Granulysin, a protein found in granules of CTLs, reduced the viability of a broad spectrum of pathogenic bacteria, fungi, and parasites in vitro. Granulysin directly killed extracellular *Mycobacterium tuberculosis*, altering the membrane integrity of the bacillus, and, in combination with perforin, decreased the viability of intracellular *M. tuberculosis*. The ability of CTLs to kill intracellular *M. tuberculosis* was dependent on the presence of granulysin in cytotoxic granules, defining a mechanism by which T cells directly contribute to immunity against intracellular pathogens.

Cytolytic T lymphocytes are required for protective immunity against intracellular pathogens such as *Listeria monocytogenes* and *Trypanosoma cruzi*, pathogens known to escape from the phagocytic vacuoles into the cytoplasm of infected host cells. CTLs have