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GAP-Independent Termination of Photoreceptor Light Response by Excess γ Subunit of the cGMP-Phosphodiesterase

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We have generated a mouse with rod photoreceptors overexpressing the γ inhibitory subunit (PDE6γ) of the photoreceptor G-protein effector cGMP phosphodiesterase (PDE6). PDE6γ overexpression decreases the rate of rise of the rod response at dim intensities, indicating a reduction in the gain of transduction that may be the result of cytoplasmic PDE6γ binding to activated transducin α GTP (T_α-GTP) before the T_α-GTP binds to endogenous PDE6γ. Excess PDE6γ also produces a marked acceleration in the falling phase of the light response and more rapid recovery of sensitivity and circulating current after prolonged light exposure. These effects are not mediated by accelerating GTP hydrolysis through the GAP (GTPase activating protein) complex, because the decay of the light response is also accelerated in rods that overexpress PDE6γ but lack RGS9. Our results show that the PDE6γ binding sites of PDE6 α and β are accessible to excess (presumably cytoplasmic) PDE6γ in the light, once endogenous PDE6γ has been displaced from its binding site by T_α-GTP. They also suggest that in the presence of T_α-GTP, the PDE6γ remains attached to the rest of the PDE6 molecule, but after conversion of T_α-GTP to T_α-GDP, the PDE6γ may dissociate from the PDE6 and exchange with a cytoplasmic pool. This pool may exist even in wild-type rods and may explain the decay of rod photoresponses in the presence of nonhydrolyzable analogs of GTP.

Key words: **rod; phototransduction; retina; phosphodiesterase; G-protein; RGS protein**

Introduction

Photoexcited rhodopsin in a vertebrate rod binds to and activates the G-protein transducin, facilitating the exchange of GTP for GDP on the transducin α subunit (T_α or GNAT1). The T_α-GTP then binds to the inhibitory γ subunit (PDE6γ) of the phosphodiesterase effector enzyme (PDE6), relieving the inhibition of the PDE6 α and β catalytic subunits. Activated PDE6 hydrolyzes cGMP, leading to the closing of the cGMP-gated channels in the outer segment. This produces the hyperpolarizing light response that signals the detection of the light to the rest of the nervous system (Fain, 2003).

The turnoff of the photoreceptor response and reopening of the channels requires the inactivation of rhodopsin by phosphorylation and subsequent binding of arrestin, as well as the return of the PDE6 to its dark resting level by hydrolysis of T_α-GTP back to T_α-GDP. The intrinsic rate of transducin GTP hydrolysis is slow (Antonny et al., 1993) but is facilitated by interaction of transducin with other proteins (Arshavsky et al., 2002). The first of these to be identified was PDE6γ, which was initially thought to act by itself to accelerate GTP hydrolysis (Arshavsky and Bownds, 1992) but was later shown to have no effect on the rate of hydrolysis in isolation (Angleson and Wensel, 1993; Antonny et al., 1993) and to require additional components, subsequently identified as RGS9–1 (He et al., 1998), Gβ5 (Makino et al., 1999), and a membrane anchor protein, R9AP (Hu and Wensel, 2002). These together form a GTPase activating protein (GAP) complex that is essential for the rapid conversion of T_α-GTP to T_α-GDP. The GAP complex proteins function in concert, because elimination of any one greatly reduces the rate of GTP hydrolysis and slows the rate of turnoff of the rod response (Chen et al., 2000; Krispel et al., 2003; Keresztes et al., 2004). The PDE6γ enhances the activity of the GAP complex probably by increasing the affinity of transducin for RGS9/Gβ5 (Skiba et al., 2000), and disruption of

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this enhancement by mutation of the PDE6γ also slows photoreceptor response turnoff (Tsang et al., 1998).

The PDE6γ may also have an additional role in the inactivation of the photoreceptor light response. Several studies have shown that added PDE6γ can reduce the activity of PDE6 by a mechanism that is independent of the hydrolysis of T_α-GTP (Wensel and Stryer, 1990; Erickson et al., 1992; Angleson and Wensel, 1993, 1994; Otto-Bruc et al., 1993; Yamazaki, 1992; Yamazaki et al., 2002). We have re-examined this phenomenon by using genetic techniques to overexpress PDE6γ in mouse rods. Our experiments show that excess PDE6γ slows the rate of rise and decreases both the gain and sensitivity of the response; it also accelerates turnoff even in the absence of the GAP complex, probably by direct binding of the PDE6γ to the activated catalytic PDE6 α and β subunits. These results were presented previously at a meeting of the Association for Research in Vision and Ophthalmology (Tsang et al., 2004).

Materials and Methods

Generation of mutant mouse lines. Mice were raised in normal room lighting (12 h on/off) and used in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research of the Association for Research in Vision and Ophthalmology, as well as the Policy for the Use of Animals in Neuroscience Research of the Society for Neuroscience. The ages of the animals ranged from 6 weeks to 1 year with no apparent difference in the results. DNA constructs for the expression of PDE6γ contained 4.4 kb of the mouse opsin promoter, the complete open reading frame of the PDE6γ cDNA (*Pde6g*), and the polyadenylation signal of the mouse protamine gene (Lem et al., 1991). The entire *Pde6g* coding region in the transgenic construct was sequence verified. *Kpn*I and *Xba*I were used to excise vector sequences from the construct.

Oocytes were obtained from super-ovulated F1(DBA × C57BL/6) females mated with F1(DBA × C57BL/6). The construct was injected into the male pronuclei of oocytes under a depression slide chamber. These microinjected oocytes were cultured overnight in M16 and transferred into the oviducts of 0.5 d *post coitum* pseudopregnant F1 females. To limit the potential variation caused by differences in genetic background, all founders were backcrossed with the C57BL/6 strain. All mice were tested for the absence of the *rd1* mutation (Pittler and Baehr, 1991). To generate mice without the GAP complex, transgenic lines expressing the wt6C were crossed with the $Rgs9^{tm1}/Rgs9^{tm1}$ to place the transgene into RGS9–1 knock-out ($Rgs9^{-/-}$) background.

Identification of transgenic mice. DNA was isolated from tail tips or liver samples by homogenizing the tissue, digesting extensively with proteinase K, and extracting with phenol. Genotyping was determined by analyzing the 400 bp PCR products generated by forward primer CTGGAATGGAAGGCCTGG and reverse primer ATGGTGTATGAGCGGCG. The DNAs were also analyzed by Southern blot hybridization with a *Pde6g* probe. Additional restriction digests were performed to analyze the structure of the integrated sequences and to insure that the DNA flanking the transgene was intact.

Rod outer-segment isolation. Rod outer segments (ROS) from dark-adapted mice were isolated under dim red light in a HEPES/phosphate-buffered balanced salt solution containing the following (in mM): 4.09 NaH_2PO_4, 148.4 NaCl, 4.91 KCl, 2.45 $CaCl_2$, 1.23 $MgSO_4$, and 14.7 HEPES, pH 7.2 (Zimmerman and Godchaux, 1982). Rhodopsin content was determined by the difference in absorbance at 500 nm before and after bleaching.

Immunoblot analyses. To facilitate the quantification of transgenic PDE6γ, transgenic mice were backcrossed to $Pde6g^{tm1}/Pde6g^{tm1}$ to obtain mice that expressed only the transgenic form of PDE6γ (Tsang et al., 1996). Mouse retinas were homogenized in PBS. Protein concentration was determined against BSA using the BCA kit (Pierce, Rockford, IL). The indicated amounts of retinal extracts were resolved by SDS-PAGE and transblotted onto nitrocellulose membrane. The relative levels of retinal proteins were determined by immunoblotting and enhanced chemiluminescence (ECL) with primary antibodies against RGS9–1 (CT317, 1:3000), Gβ5 (CT215, 1:3000), and PDE6γ (PA1–723, 1:1000), followed by peroxidase-conjugated secondary goat anti-rabbit IgG (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA). ECL signals were captured by a Kodak (Rochester, NY) IS440 imaging station and quantified with Kodak 1D4.5 software.

To detect PDE6 α and β subunits, protein containing 185 pmol of rhodopsin/lane from murine ROS was separated by electrophoresis on a 6.5–9.5% acrylamide/1.5% crosslinker inverted gradient gel, as described previously (Tsang et al., 1996). Proteins were then transferred to 0.2 mm immunoblot polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) and left overnight at 4 V/cm by the method of Towbin et al. (1979). Membranes were blocked in 3% BSA in 500 mM NaCl, 20 mM Tris, pH 7.6, and 0.1% Tween 20. The PDE6 α and β subunits were detected by incubation with a polyclonal antiserum raised against a 17-mer peptide (Piriev et al., 1993), 100% homologous with the rod PDE6 α and β subunits as well as with the cone PDE α subunit. Western blots were visualized with the DuoLux Chemiluminescence substrate kit (Vector Laboratories, Burlingame, CA) and a goat anti-rabbit IgG-alkaline phosphatase conjugate. Blots were exposed to Hyperfilm-MP (Amersham Biosciences, Piscataway, NJ) preflashed to increase sensitivity and linearity according to the SensitizeJ protocol (Amersham Biosciences). Signals were quantified by densitometric scanning.

Similar methods were used to assess relative levels of other retinal proteins (supplemental Fig. 1, www.jneurosci.org as supplemental material), by immunoblotting and ECL with primary antibodies against RGS9–1 (CT317, 1:3000), Gβ5 (CT215, 1:3000), PDE6γ (PA1–723, 1:1000; Affinity Bioreagents, Golden, CO), GC-E (K285, 1:5000; from D. Garbers, University of Texas Southwestern Medical Center at Dallas, Dallas, TX), GC-F (A670, 1:5000; from D. Garbers), PDE6α (PA1–720, 1:500; Affinity Bioreagents), RK (MA1–721, 1:5000; Affinity Bioreagents), arrestin (Marr, 1:20,000), transducin α subunit (UUTA1, 1:5000), phosducin (Gerti, 1:5000; from R. Lee, Sepulveda Veterans Affairs Hospital, Los Angeles, CA), GCAP1 (α-GCAP1, 1:1000; from A. Dizhoor, Pennsylvania College of Optometry, Elkins Park, PA), GCAP2 (α-GCAP2, 1:1000; from A. Dizhoor), followed by peroxidase-conjugated secondary goat anti-rabbit or goat anti-mouse IgG (1:5000; Santa Cruz Biotechnology).

Histology and immunocytochemistry. Mice were deeply anesthetized with pentobarbital (100 mg kg^{-1}) and fixed by vascular perfusion for 5 min with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3. Eyes were enucleated and a section of cornea removed. The eyes were again immersed in fixative for 2 h. The lens was removed, and the eyes were left in fixative overnight. The eyeballs were postfixed in osmium tetroxide, dehydrated through a graded series of ethanol, and bisected through the optic nerve. Each half was then embedded in Epon (Eponate 812; Ted Pella, Redding, CA). For light microscopy (supplemental Fig. 2, available at www.jneurosci.org as supplemental material), sections (1 μm) were cut, mounted on glass slides, and stained with toluidine blue. Similar areas were examined from each eye, as determined by measurement from the optic nerve head. For analysis of the outer segment diameters, silver–gold sections (60–90 nm) were cut on an ultramicrotome, picked up on grids, and stained with uranyl acetate and lead citrate. Sections were photographed, the negatives were scanned, and the average outer segment diameter was determined from each of 18 outer segments from both wild-type (WT) and wt6C retinas. The means for each eye were then averaged.

For immunohistochemistry, mouse eyeballs were fixed in 4% paraformaldehyde in PBS at 4°C overnight and cryoprotected in 30% sucrose in PBS. The eyeballs were sectioned at 12 μm thickness, washed twice with PBS, and blocked with 10% goat serum in 0.3% Triton X-100 in PBS for 30 min. They were then incubated with primary antibodies overnight at the following concentrations: PDE6γ (PA1–723), 1:100; GNAT1 (UUTA1), 1:100; arrestin (Marr), 1:200. Sections were washed three times for 5 min with PBS and treated with FITC-conjugated goat anti-rabbit IgG (1:100; Southern Biotechnology, Birmingham, AL) for 1 h at room temperature. After washing with PBS three times for 10 min, they were coverslipped with Vectashield and examined under a Leitz (Wetzlar, Germany) fluorescent microscope. Fluorescent images were cap-

tured with a SpotMosaic digital camera and exported to and edited with Microsoft (Redmond, WA) PowerPoint software.

Suction electrode measurements. Methods for recording responses of mouse rods have been given previously (Woodruff et al., 2002, 2003). In brief, rods were perfused with physiological solution containing amino acids and nutrients kept at 37°C. Stimuli at 500 nm were attenuated with calibrated absorptive neutral-density filters. Responses were amplified by a Warner Instruments (Hamden, CT) patch-clamp amplifier (PC-501A) and recorded with pClamp hardware and software (Molecular Devices, Foster City, CA). The single-photon response was calculated from the squared mean and variance (Chen et al., 2000). All errors given here (including in the table and figures) are SE.

Results

To understand in greater detail the role of PDE6γ in photoreceptor response turnoff, we overexpressed PDE6γ in mouse rods. Germ-line transmission was obtained from nine founders, which showed varying levels of PDE6γ expression compared with WT C57BL/6 controls (Fig. 1*A*). No PDE6γ was detected either in postnatal day 13 (P13) (Fig. 1*A*) or adult *Pdeg*^{tm1}/*Pdeg*^{tm1} mice (Tsang et al., 1996), in which the *PDE6γ* gene had been knocked out. The line wt6C, which from densitometric scanning we determined to express a level of PDE6γ protein twofold higher than in WT animals, was selected for additional experimentation. Immunoblot analyses showed that the levels of other components of the RGS complex, RGS9–1 and Gβ5, were normal in these animals (Fig. 1*B*), as well as the catalytic subunits of PDE6α and PDE6β (Fig. 1*C*). This was also true of other proteins involved in phototransduction cascade (supplemental Fig. 1, available at www.jneurosci.org as supplemental material), including rhodopsin kinase, arrestin, the GCAPs, and guanylyl cyclase (both E and F). Thus, the PDE6γ overexpression appeared to have no effect on the expression of other photoreceptor proteins important in the transduction cascade. The effect on transduction (see below) can thus be attributed solely to the increase in PDE6γ level.

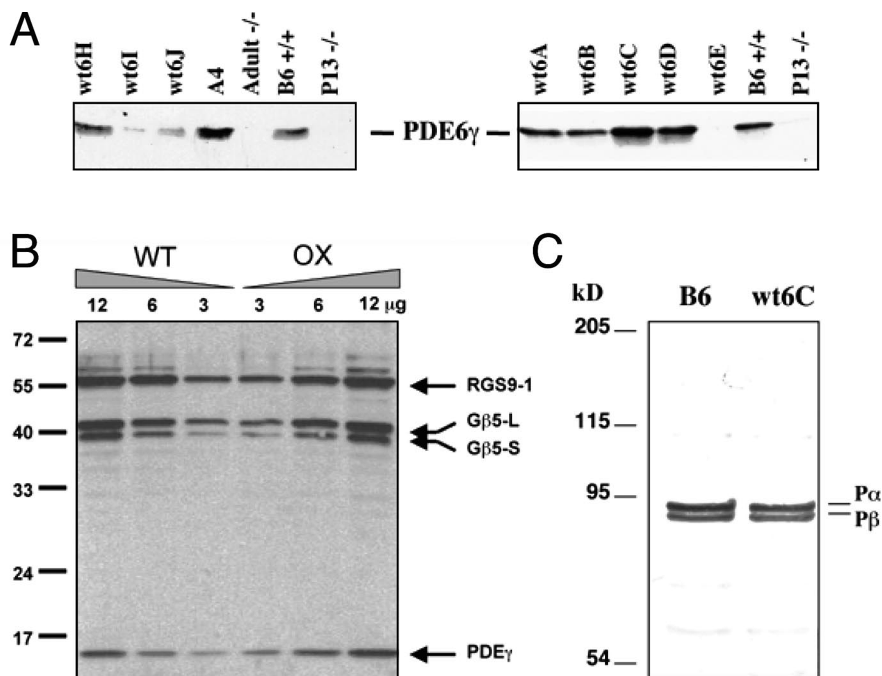

Figure 1. Transgenic overexpression of the 11 kDa PDE6γ subunit (PDEγ). ***A***, Immunoblot screening of PDEγ in ROSs of C57BL/6 (control) and indicated transgenic mouse lines normalized to 150 pmol rhodopsin content, with a polyclonal antibody recognizing the N-terminal part of the PDE6γ subunit. Transgenic PDE6γ is expressed in the *Pdeg6*^{tm1}/+ genetic background. Lane 1, Transgenic line wt6H; lane 2, transgenic line wt6I; lane 3, transgenic line wt6J; lane 4, transgenic line A4 (+/+); lane 5, adult *Pdeg6*^{tm1}/*Pdeg6*^{tm1}; lane 6, B6+/+ WT C57BL/6 control; lane 7, P13 *Pdeg6*^{tm1}/*Pdeg6*^{tm1}; lane 8, transgenic line wt6A; lane 9, transgenic line wt6B; lane 10, transgenic line wtC6; lane 11, transgenic line wt6D; lane 12, transgenic line wt6E; lane 13, B6+/+ WT C57BL/6 control; lane 14, P13 *Pdeg6*^{tm1}/*Pdeg6*^{tm1}. ***B***, Overexpression (OX; wt6C) of PDE6γ in the wt6C transgenic line, expressed in the *Pdeg6*^{tm1}/*Pdeg6*^{tm1} genetic background. The indicated amount of protein extracts from age-matched wt6C and WT mouse retinas were analyzed by immunoblot for RGS9–1, Gβ5, and PDE6γ simultaneously. Size markers are indicated at left. The PDE6γ level is approximately twofold higher in wt6C retina, whereas RGS9–1 and Gβ5 levels are similar to those of the control. ***C***, Immunoblot analysis of the PDE6α (Pα) and PDE6β (Pβ) catalytic subunits in control and transgenic ROSs normalized for rhodopsin content as in ***A***. Immunoblot incubated with the MOE polyclonal antibody recognizing all of the subunits of the PDE6 in B6 control and wt6C transgenic retinas is shown.

Immunohistochemical experiments showed that PDE6γ in both WT and wt6C animals was localized to the ROSs and that the distribution of PDE6γ was the same in the light and in the dark (Fig. 2). This is in contrast to the localization of T_α and arrestin. As in previous studies (Brann and Cohen, 1987; Philp et al., 1987; Whelan and McGinnis, 1988; Sokolov et al., 2002; Zhang et al., 2003), the T_α in the dark in both WT (data not shown) and wt6C retinas is mostly concentrated in the outer segments of the rods and moves in the light to the inner segments. Arrestin, on the other hand, is abundant in the inner segments in the dark but is found in both WT (data not shown) and wt6C retinas almost exclusively in the outer segments in the light (Philp et al., 1987; Whelan and McGinnis, 1988; Mendez et al., 2003; Zhang et al., 2003; Nair et al., 2005). These experiments provide no indication that the PDE6γ of wt6C animals and the other components of the transduction cascade behave in any way different from those in WT mice. Furthermore, as Figure 2 shows (see also supplemental Fig. 2, available at www.jneurosci.org as supplemental material), the morphology of wt6C mouse retina was normal, and the amplitude of the a-wave of the electroretinogram of wt6C mice was also normal up to 9 months of age (data not shown). The diameter of the ROSs was also the same in wt6C mice as in wild type (~1.3 μm).

Rod responses in retinas with overexpressed PDE6γ

Representative responses of a WT rod and a wt6C rod to flashes of light of increasing intensity are given in Figure 3, *A* and *B*. Although rods in both animals have approximately the same average dark current (Table 1) and respond over a wide range of light levels, the responses of wt6C rods were desensitized, as can be seen from the mean response-intensity curves in Figure 3*C*. They also rise more slowly, especially at low intensities, and decay with an accelerated time course. A similar effect on sensitivity (although apparently not on response waveform) was seen after introduction of a much smaller concentration of excess PDE6γ into toad rods (Rieke and Baylor, 1996).

We examine the differences in waveform in greater detail in Figure 4*A*–*C*. Here, we have superimposed averaged responses from many rods of WT (black traces) and wt6C (red traces) mice at three different light intensities. The responses for each cell have been normalized before averaging to the maximum value of the

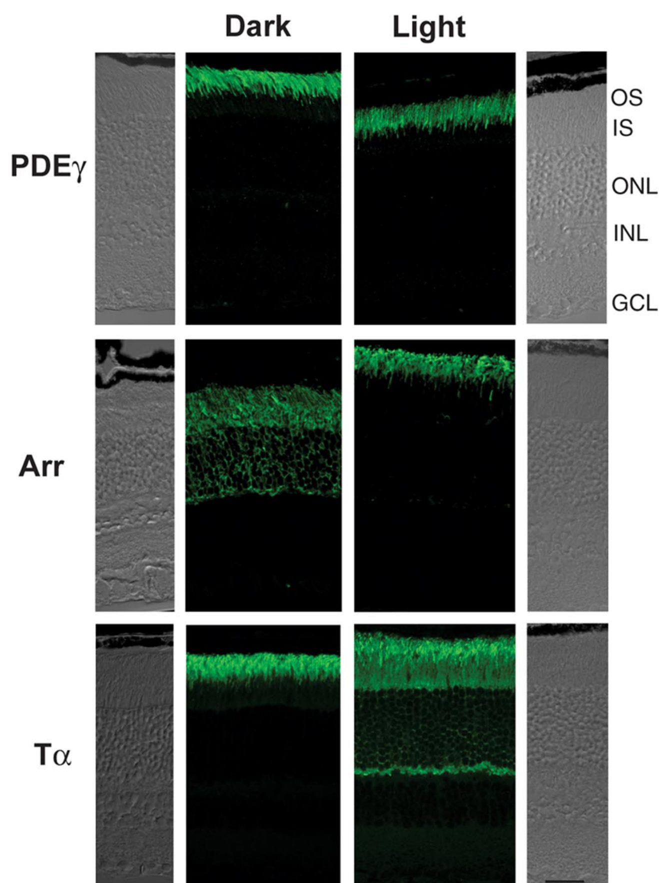

Figure 2. Absence of light-dependent movement of PDE6γ in wt6C transgenic mice. Fluorescent and bright-field images of retinal localization of PDE6γ (PDEγ; top panels), arrestin (Arr; middle panels), and transducin α subunit (Tα; bottom panels) in dark-adapted and light-adapted conditions were examined by immunohistochemistry (see Materials and Methods). For the light-adapted condition, mice were exposed to continuous room light (60 lux) for 6 h; for the dark-adapted condition, mice were killed after 12 h in darkness under infrared illumination. Although Tα and arrestin redistribute in opposite directions in and out of the outer segment (OS) layer, PDE6γ is found exclusively in the OS regardless of light. IS, Inner segment; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar, 25 μm.

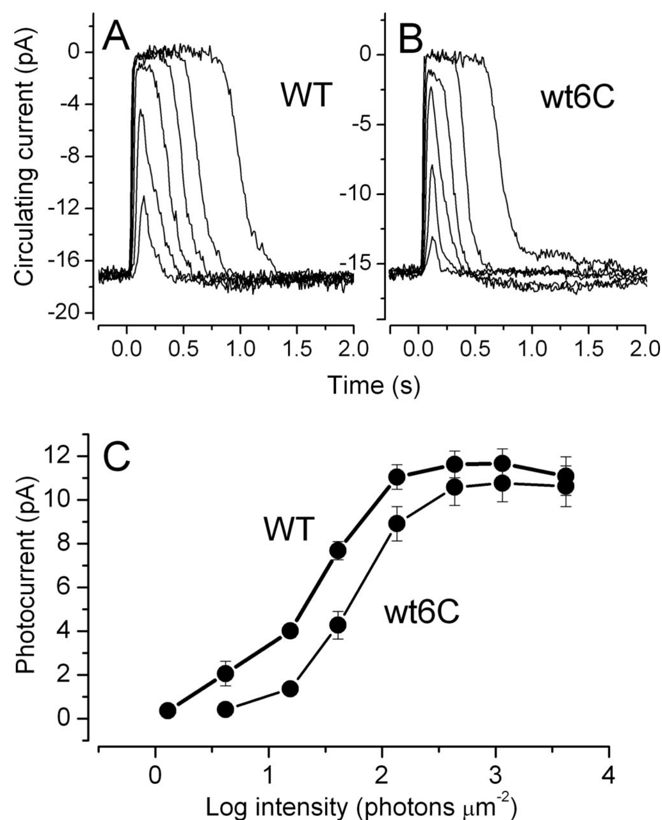

Figure 3. Responses of wt6C and WT rods to light. ***A***, WT rod, 20 ms flashes of 500 nm light at flash intensities of 17, 43, 160, 450, 1120, and 4230 photons μm $^{-2}$. The traces are averages of two to four flashes at each intensity. ***B***, Typical responses from a wt6C rod to the same flash intensities. Each trace was averaged from three to eight flashes. ***C***, Response amplitude versus flash intensity averaged from 34 WT and 20 wt6C rods. Flash intensity required to elicit a response of half-maximal amplitude was shifted to higher intensities by ~0.3 log units in the wt6C rods (see Table 1).

photo current for that cell. The insets compare the initial time course of the response at a higher temporal resolution.

At the dimmest intensity (Fig. 4*A*, inset), there is a clear difference in the initial rising phase of the light response that is manifest even at early times. This difference represents a reduction in the gain of the transduction cascade in the wt6C rods. It is also present at a moderate light intensity (Fig. 4*B*, inset), although in bright light (Fig. 4*C*, inset), the difference in rise time nearly disappears. If this difference is the result of excess PDE6γ in wt6C rods binding to T_{α}-GTP before it can bind to endogenous PDE6γ (Wensel and Stryer, 1990; Otto-Bruc et al., 1993), the results in Figure 4*A–C* indicate that the amount of PDE6γ is sufficient to alter the gain at dim to moderate intensities but that at bright intensities the T_{α}-GTP is so abundant in proportion to PDE6γ that the gain of transduction is virtually unaffected.

The results in Figure 4*A–C* also show that the responses of wt6C rods decay more rapidly than those of WT rods, and this is apparent even at the dimmest intensity (Fig. 4*A*). At bright intensities (Fig. 4*C*), the waveforms of decay become similar in appearance but are shifted along the time axis. We constructed a Pepperberg plot (Pepperberg et al., 1992) for the WT and wt6C responses to bright flashes, by plotting the time for decay (T_{sat}) of the response to a criterion level of 25% of the dark current (or 75% of the maximum response), as a function of the natural log of the light intensity (Fig. 4*D*). We found that the curves for WT and wt6C rods had a similar shape but were shifted along the time axis. The curves also seem slightly different in slope, indicating a different value for the dominant time constant in response decay T_d. We investigated this possibility in greater detail by calculating the value of T_d for each rod separately and then averaging. This gave 173 ± 8 ms for WT ($n = 44$) and 147 ± 10 ms for wt6C ($n = 27$). A Student's *t* test gave a *p* value for this difference of 0.052, indicating that the difference in T_d is just above the 0.05 criterion for significance.

Figure 5 gives the mean single-photon responses of rods from the WT and wt6C animal strains, calculated as previously (see Materials and Methods) (Woodruff et al., 2003). Once again, the smaller and more rapidly decaying response is the one from the wt6C animals. Like the dim-intensity responses in Figure 4*A*, the rise time of the wt6C single-photon response is slower than that of WT rods (Fig. 5, inset), indicating a difference in the gain of transduction. There is also a marked acceleration in the decay of the single-photon response, demonstrating that the effect of excess PDE6γ on response turnoff occurs even at the limit of the smallest response.

We compare some parameters of the responses of rods from WT and wt6C mice in Table 1. The time to peak of wt6C rods was smaller than that of WT rods, and this difference was significant (*t* test; $p < 0.05$). The sensitivity of wt6C rods was smaller by a

Table 1. Kinetic and sensitivity parameters of WT and wt6C rods

	Dark current (pA)	Time to peak (ms)[a]	Flash sensitivity (pA photon^{-1} μm^{-2})	Flash intensity at half-saturation (photons μm^{-2})	Integration time (ms)[a]	τ for response recovery (ms)[b]
WT	11.9 ± 0.5 (44)	143 ± 4 (46)	0.26 ± 0.02 (46)	31 ± 2 (43)	199 ± 20 (47)	205 ± 19 (45)
wt6C	11.1 ± 0.7 (29)	126 ± 6 (29)	0.10 ± 0.01 (29)	71 ± 6 (29)	103 ± 9 (29)	91 ± 11 (29)
Rgs9$^{-/-}$	8.2 ± 0.7 (15)	214 ± 18 (19)	0.15 ± 0.02 (20)	31 ± 3 (15)	1820 ± 450 (21)	8420 ± 1000 (17)
Rgs9$^{-/-}$/wt6C	11.6 ± 0.7 (10)	127 ± 15 (9)	0.16 ± 0.05 (9)	78 ± 13 (9)	236 ± 68 (9)	326 ± 60 (9)

Values are means ± SE with the number of cells in parentheses.

[a]Values are for light responses at intensity of 15.5 photons μM^{-2}.

[b]Single exponential fit of just-saturating response.

factor of 2–3, and the flash intensity required to produce a half-saturation response was larger by approximately a factor of 2 (or 0.3 $\log_{10}$ units) (Fig. 3*C*); these differences were again significant (*t* test; $p < 0.05$). The integration time and time constant for response recovery are both significantly shorter for wt6C rods (*t* test; $p < 0.01$), reflecting the more rapid time course of response decay. Thus, the more extensive data in Table 1 support the conclusions of Figures 3–5: that responses of rods in wt6C mice are desensitized and decay more rapidly than those of WT rods.

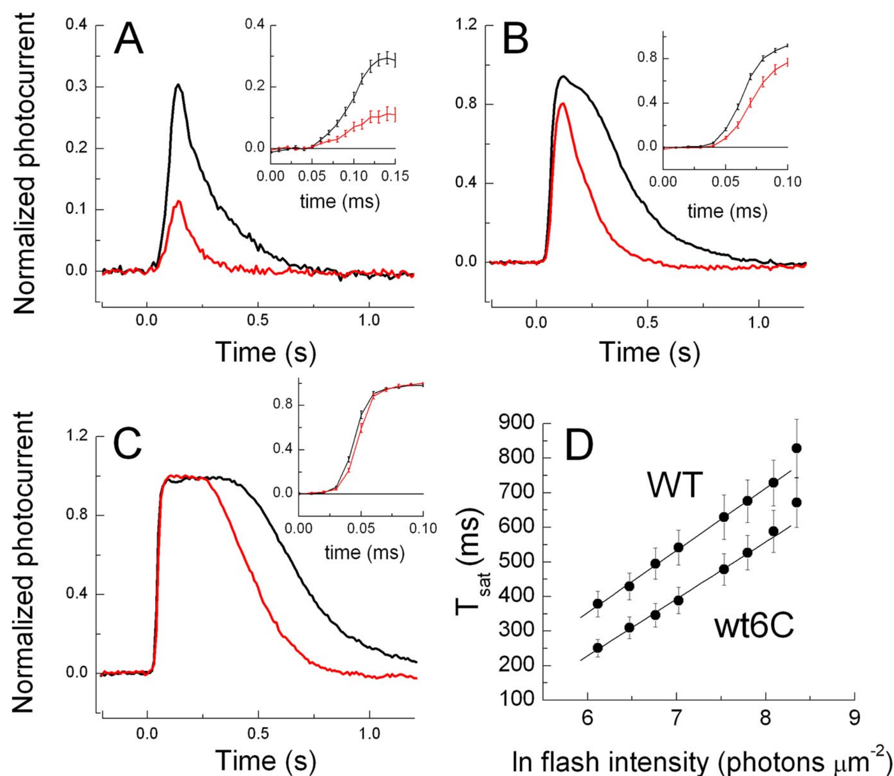

Figure 4. Response rise time and recovery in WT and wt6C rods. ***A***, Superimposed averaged responses of WT (black traces) and wt6C (red traces) rods at a flash intensity of 17 photons μm^{-2}. ***B***, As in ***A*** but at 160 photons μm^{-2}. ***C***, As in ***A*** but at 1120 photons μm^{-2}. Data were filtered at 30 Hz (eight-pole Bessel) and sampled at 100 Hz. The data in ***A–C*** were averaged from 31–34 WT and 18–20 wt6C rods after normalizing to the maximum photo current for each rod. The inset in each panel shows the rising phases of the responses on an expanded time scale with SE at 10 ms intervals. ***D***, Pepperberg plots of WT and wt6C rods. Time in saturation, T_{sat}, was estimated from the time for recovery to 25% of a dark circulating current after presentation of a bright flash. The dominant time constant for response turnoff was estimated from the best-fitting slope for intensities from 450 to 3250 photons μm^{-2}.

GAP-independent acceleration of turnoff by overexpressed PDE6γ

Because PDE6γ is known to facilitate the interaction of RGS9 with T_α, the results in Figure 3 and Table 1 do not distinguish between a direct effect of PDE6γ on PDE6 turnoff and an indirect effect mediated through the GAP complex. We therefore intercrossed wt6C mice into an *Rgs9*$^{-/-}$ background that lacks both the RGS9–1 and Gβ5L proteins (Chen et al., 2000). *Rgs9*$^{-/-}$ rods have prolonged light responses (Fig. 6*A*), as reported previously (Chen et al., 2000). In *Rgs9*$^{-/-}$ rods expressing wt6C, however, the rate of decline of the rod response was greatly accelerated (Fig. 6*B*), although still much slower than in WT or wt6C rods. Note the differences in time scale between Figure 6*B* and Figures 3, *A* and *B*, and 4*A–C*. Single exponential fits to the decay phase of just-saturating responses gave a time constant of 91 ± 11 ms for wt6C rods and 205 ± 19 ms for WT rods but 8.42 ± 1.0 s for *Rgs9*$^{-/-}$ rods and 326 ± 60 ms for rods from progeny of the mating of *Rgs9*$^{-/-}$ and wt6C mice. The integration time was also longer for rods from these animals than for those from either WT or wt6C rods (Table 1).

In *Rgs9*$^{-/-}$ rods, recovery after a bright flash is delayed. This can be seen in the recording of Figure 7*A*, in which a rod was exposed to a saturating light for 4 min, ending at $t = 0$. Flashes were not given during the recovery of the photo current, so as not to prolong return of the current to the baseline even further. In rods from *Rgs9*$^{-/-}$ mice mated to wt6C (Fig. 7*B*), the recovery was greatly accelerated and flashes during recovery produced photo currents of normal amplitude. Recovery in these rods was still considerably slower than in WT (Fig. 7*C*) or wt6C (data not shown) rods, for which the current returned to the baseline only a few seconds after the turning off of the background light. These results complement those of Figure 6 and show that the overexpression of PDE6γ can at least, to some extent, reverse the effects of knock-out of the *Rgs9* gene (i.e., overexpression has an epistatic effect on rods lacking the GAP complex). We return to this observation in the Discussion.

Continuous component of dark noise

The experiments we have so far described indicate that excess PDE6γ can diffuse presumably from the cytoplasm to bind to the PDE6γ binding sites of the PDE6 α and β catalytic subunits. For this to occur, the binding sites must be accessible to the excess PDE6γ. We wondered whether these sites were also accessible in darkness. Rods in darkness exhibit two kinds of noise (Baylor et al., 1980, 1984), one consisting of discrete events resembling

J. Neurosci., April 26, 2006 • 26(17):4472–4480 • 4477

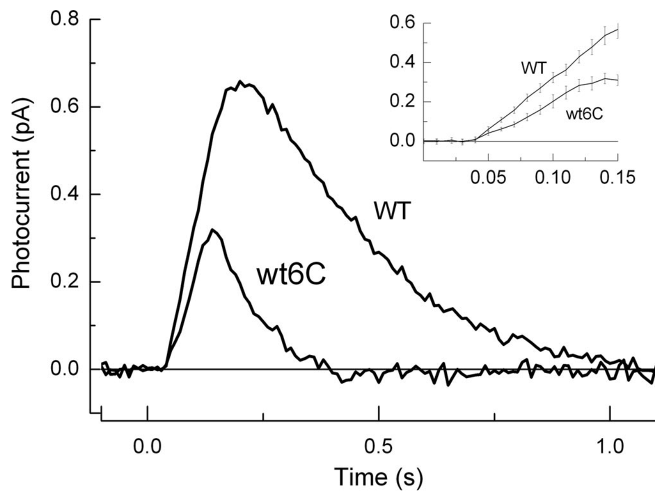

Figure 5. Averaged single-photon responses for WT and wt6C rods from 47 WT and 18 wt6C rods. Individual single-photon responses were calculated by dividing the mean response to 40–60 dim light-flash responses by the number of photoisomerizations per flash, which was estimated by the scaling factor needed to adjust the individual rising phases of the mean responses to the rising phases of the ensemble variances of the means. Inset, Rising phases of single-photon responses on an expanded time scale with the SE at 10 ms intervals.

single-photon responses and another continuous component, probably produced by spontaneous activity of PDE6 in the dark (Rieke and Baylor, 1996). We reasoned that if excess PDE6γ could bind to the PDE$\alpha\beta$ core in darkness, the wt6C rods might have a smaller component of continuous noise than WT rods.

We therefore recorded baseline noise in segments of 10 s duration; 20–30 such segments were recorded from each rod both in the dark and in the presence of saturating light. From these recordings, we determined the power spectrum of the dark fluctuations for each cell by subtracting the spectrum in the light from the spectrum in the dark. These difference spectra were then averaged for a total of 17 WT and 18 wt6C rods. We observed a clear light–dark difference in noise in both WT and wt6C rods, but we could detect no significant difference in the averaged spectra of the rods of the two kinds. We therefore conclude either that excess PDE6γ does not bind to the PDE6γ binding sites of the PDE6 α and β catalytic subunits in darkness (Rieke and Baylor, 1996) or that the excess PDE6γ does bind and have an effect on the continuous component, but this effect was too small for us to observe.

Discussion

Our experiments indicate that excess PDE6γ can decrease the rate of rise and gain of the rod response. Comparisons of waveforms of single-photon (Fig. 5) and dim-flash (Fig. 4*A*) responses show that the responses of wt6C and WT rods diverge at a time too early to reflect accelerated turnoff of PDE6 (Tsang et al., 1998). The simplest explanation is that excess PDE6γ can bind to T_α-GTP in the outer segments of wt6C rods (Wensel and Stryer, 1990; Otto-Bruc et al., 1993), so that fewer T_α-GTPs are available to bind to endogenous PDE6γ. The concentration of activatable transducin may also be reduced, if added PDE6γ were to bind to T_α-GDP and remove it from $T_{\beta\gamma}$ (Otto-Bruc et al., 1993). The effects on rise time and gain are marked at dim intensities (Fig. 4*A*) but become less easily observed for flashes of increasing intensity (Fig. 4*B*,*C*), perhaps because the number of PDE6γ molecules in the cytoplasm is not large enough to produce a significant effect when large amounts of T_α-GTP are generated. A comparison of the number of molecules is revealing. For Figure 4, *B* and *C*, the flash intensities were 160 and 1120 photons μm^{-2}, equivalent to ~80 and 560 Rh* per rod [assuming a collecting area of 0.5 μm^2 (Field and Rieke, 2002)]. If each bleached rhodopsin molecule produces 120 T_α-GTP molecules (Leskov et al., 2000), then, as a result of the two flashes, 9.6×10^3 and 6.7×10^4 T_α-GTP molecules will be formed. The number of excess γ molecules in our wt6C animals, on the other hand, is of the same order as the number of endogenous γ molecules, ~30 μM [twice the PDE concentration of ~15 μM (Hamm and Bownds, 1986)]. This is equivalent to ~2×10^5 molecules, substantially greater than our best estimates of the number of T_α-GTP molecules produced by the flashes. Because the brighter of the two flashes showed little effect of excess PDE6γ on the rising phase of the response, there are three possible conclusions. The effective con-

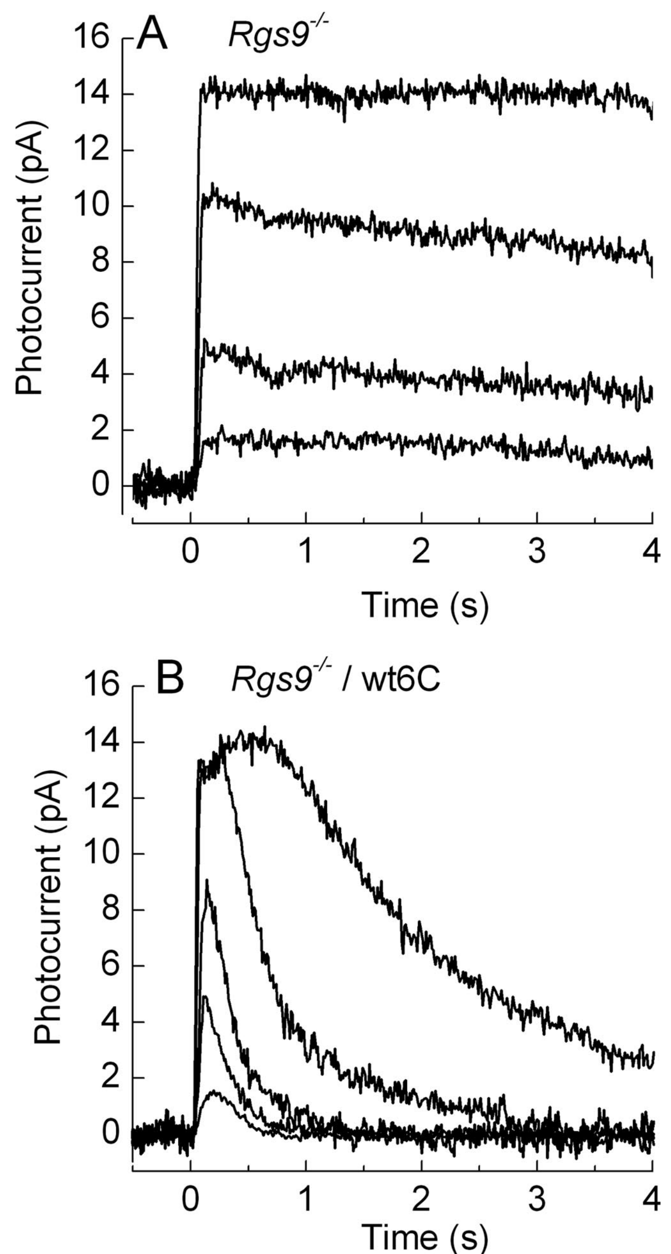

Figure 6. Responses of rods lacking the RGS9–1 complex are accelerated by excess PDE6γ. ***A***, Responses of a *Rgs9*$^{-/-}$ rod to flash intensities of 4, 17, 43, and 160 photons μm^{-2}. ***B***, Responses of rods from wt6C mice on an *Rgs9*$^{-/-}$ background. See Results. Flash intensities are 4, 17, 43, 160, and 450 photons μm^{-2}. The responses are averages of 5–20 flashes each.

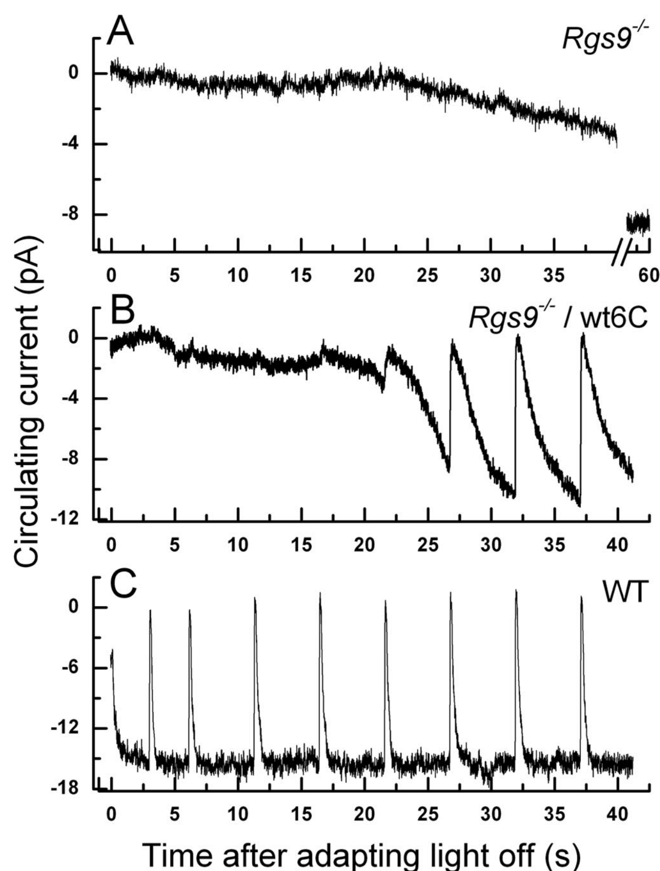

Figure 7. Recovery from bright-light exposure (4 min, 2830 photons $\mu m^{-2} s^{-1}$). The adapting light was turned off at $t = 0$. The pigment bleached was <0.4%. ***A***, *Rgs9*$^{-/-}$ rod. The break in the abscissa shows eventual recovery of *Rgs9*$^{-/-}$ rod response 50–60 s after the light was extinguished. ***B***, wt6C rod on *Rgs9*$^{-/-}$ background. Flashes of 20 ms at 437 photons μm^{-2} were presented every 5 s after turning off the adapting light. ***C***, WT rod with flashes as in ***B***.

centration of PDE6γ is much less than the total concentration, or estimates of the gain of production of T_α-GTP are too low by at least 1 order of magnitude, or T_α-GTP binds much more readily and tightly to PDE6γ on the membrane than to excess PDE6γ, presumably in the cytoplasm.

Our results show that, in addition to its effect on the rising phase, excess PDE6γ also accelerates the rate of turnoff of the photoreceptor response, confirming for intact rods earlier biochemical observations on isolated proteins or ROS preparations (Wensel and Stryer, 1990; Erickson et al., 1992; Angleson and Wensel, 1993, 1994; Antonny et al., 1993; Bondarenko et al., 1999;Yamazaki et al., 2002). The effect of excess PDE6γ on response decay is not simply attributable to a decrease in PDE activation, because even for WT and wt6C responses with similar rising phases and producing a similar suppression of circulating current, the wt6C response decays markedly more rapidly (Fig. 4*C* and supplemental Fig. 3). The acceleration of mouse rod response decline can occur even in the absence of RGS9–1 and the GAP complex, indicating that shutoff by excess PDE6γ is independent of any effect it may have on the hydrolysis of GTP.

The most likely interpretation of our experiments, in our view, is that the PDE6 is rate limiting for the decay of the rod photoreceptor light response (Sagoo and Lagnado,1997; Krispel et al., 2005) and that excess PDE6γ speeds up response decay by accelerating the rate of turnoff of PDE activity. One difficulty with this interpretation is that excess PDE6γ does not significantly alter the value of the rate-limiting time constant, T_d (Fig. 4*D*). We think the reason for this is that the effective concentration of the overexpressed PDE6γ is sufficient to turn off only a fraction of the PDE6 activated by bright light and that the majority is extinguished in the usual way by GTP hydrolysis, as in a WT rod. Because, however, the mechanisms of response turnoff are complex and still not completely understood, other explanations cannot at present be excluded.

Although PDE6γ binding and shut down of the PDE6 can occur in the absence of the GAP complex, these proteins do have an effect on the rate of binding. This can be seen from the following considerations. In WT mice, the response to a just-saturating light decays with a mean time constant of 205 ms (Table 1). It is likely from our own results and those of Krispel et al. (2005) that this represents the time constant of T_α-GTP hydrolysis. In animals overexpressing PDE6γ, the light response can be turned off in two ways, either by hydrolysis of T_α-GTP or by binding of free PDE6γ. If these two processes occur independently, then their rate constants should add and the time constant of turnoff in animals overexpressing PDE6γ (91 ms) should be the inverse of the sum of inverses of the time constants of T_α-GTP hydrolysis and of PDE6γ binding. The time constant for PDE6γ binding can therefore be calculated to be ~164 ms. If this same calculation is performed for rods lacking the GAP complex, the predicted time constant for PDE6γ binding is 339 ms, over twice as long. The simplest explanation is that the GAP complex facilitates excess PDE6γ binding, perhaps by removing T_α-GTP/PDE6γ further from the PDE6γ binding sites on the catalytic subunits and making these sites more easily accessible. One implication of these considerations is that the T_α-GTP/PDE6γ complex must remain bound to the rest of the PDE6 heterotetramer until the terminal phosphate of the GTP has been hydrolyzed. If the T_α-GTP/PDE6γ were to come entirely off the PDE6, as seems to occur in frog rods (Yamazaki, 1992), the rate of exogenous PDE6γ binding could not be affected by the presence or absence of RGS9–1 and the rest of the GAP proteins.

It is highly likely that excess PDE6γ turns off the cascade by binding directly to the PDE6γ binding sites on the PDE6 α and β catalytic subunits rather than by binding to T_α-GTP and facilitating the removal of the G-protein from the PDE6 heterotetramer. The experiments of Otto-Bruc et al. (1993) show that the W70F form of PDE6γ binds much more weakly to T_α-GTPγS than does WT PDE6γ, but both forms of PDE6γ inhibit activated PDE6 with similar efficiency. We propose that after the binding of exogenous PDE6γ to the catalytic site of activated PDE6, the complex of T_α-GTP and endogenous PDE6γ remains attached to the PDE6 heterotetramer, but after hydrolysis of T_α-GTP to T_α-GDP, the PDE6γ/T_α-GDP complex dissociates and is released to the cytosol, because PDE6γ binds much less tightly to T_α-GDP than to T_α-GTP (Otto-Bruc et al., 1993). This would recycle endogenous PDE6γ to the pool of free inhibitor.

We summarize our conclusions in the schema of supplemental Figure 4 (available at www.jneurosci.org as supplemental material). In wt6C mouse rods, the T_α-GTP produced by light stimulation (top panel) may bind either directly to excess PDE6γ (A), reducing the gain of the light response, or to endogenous PDE6γ, as in the WT rod (1), displacing the endogenous PDE6γ from its inhibitory binding sites and activating the PDE6. The T_α-GTP and endogenous PDE6γ then bind to RGS9–1 and its associated proteins (2), to form a complex that remains attached to the PDE6 heterotetramer.

The activity of the PDE6 can then be quenched in two ways

(bottom panel). The RGS9–1 complex can accelerate the rate of hydrolysis of T_α-GTP to T_α-GDP (3), causing the T_α-GDP and RGS9–1 to come off the PDE6 and the PDE6γ to return to its sites on the PDE6 catalytic α or β subunits. Alternatively, excess PDE6γ can bind directly to sites on the PDE6 catalytic α or β subunits (B). We then suppose that once the T_α-GTP is hydrolyzed to T_α-GDP, the T_α-GDP and RGS9–1 fall off the PDE6, and the associated PDE6γ is also released back into the cytoplasmic pool of PDE6γ. The postulated exchange of PDE6γ may also occur to some extent in WT mice, and this may explain the decay of rod photoresponses even in the presence of nonhydrolyzable analogs of GTP (Erickson et al., 1992).

Defects in RGS9–1 or R9AP in humans cause a condition that has been termed "bradyopsia," characterized by subnormal acuity, photophobia, and slow adaptation that produces a transient but debilitating blindness (Nishiguchi et al., 2004). The results in Figures 6 and 7 show that overexpression of PDE6γ has an epistatic effect on rods lacking the GAP complex, both for responses to flashes and for recovery after bright-light exposure. The responses of rods in *Rgs9*$^{-/-}$ mice in which PDE6γ has been overexpressed, although slower to recover from light exposure than WT rods, are nevertheless much more similar to WT responses that those of the rods of *Rgs9*$^{-/-}$ mice without PDE6γ overexpression. We predict that the bradyopsia of patients lacking the GAP complex would be substantially ameliorated by PDE6γ overexpression in rod and cone photoreceptors. Because RGS9–1 and the other components of the GAP complex are expressed in many parts of the nervous system but the PDE6γ protein is expressed only in the photoreceptors, future pharmacological upregulation of PDE6γ may provide a safer and more easily implemented cure for bradyopsia and related conditions.

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