Title
GAP-independent termination of photoreceptor light response by excess gamma 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 (Tα,GTP) before the Tα,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 Tα,GTP. They also suggest that in the presence of Tα,GTP, the PDE6γ remains attached to the rest of the PDE6 molecule, but after conversion of Tα,GTP to Tα,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 (Tα,GTP). The Tα,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 Tα,GTP back to Tα,GDP. The intrinsic rate of transducin GTP hydrolysis is slow (Antony 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; Antony 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 Tα,GTP to Tα,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...
this enhancement by mutation of the PDE6\textgamma also slows photoreceptor response turnoff (Tsang et al., 1998).

The PDE6\textgamma may also have an additional role in the inactivation of the photoreceptor light response. Several studies have shown that added PDE6\textgamma can reduce the activity of PDE6 by a mechanism that is independent of the hydrolysis of PDE6/5 (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 PDE6\textgamma in mouse rods. Our experiments show that excess PDE6\textgamma 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 PDE6\textgamma to the activated catalytic PDE6\textalpha and \textbeta 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 PDE6\textgamma contained 4.4 kb of the mouse opsin promoter, the complete open reading frame of the PDE6\textgamma cDNA (Pde6g\textgamma), and the polyadenylation signal of the mouse protamine gene (Lem et al., 1991). The entire Pde6g\textgamma coding region in the transgenic construct was sequence verified. KpnI and XbaI 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 wt6G were crossed with the Rgs9\textalpha and \textbeta subunits. These results were presented previously subunits as well as with the cone PDE6\textgamma. To detect PDE6\textalpha and \textbeta 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 immobilized 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\textalpha and \textbeta 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\textalpha and \textbeta subunits as well as with the cone PDE6\textalpha 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 Sensitize\textregistered protocol (Amersham Biosciences). Signals were quantified by densitometric scanning.

Several 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\textalpha (CT317, 1:3000), G\Beta\textgamma (CT215, 1:3000), PDE6\textgamma (PAI–723, 1:1000; Affinity Bioreagents, Golden, CO), G\E\textgamma–E (K285, 1:5000; from D. Garbers, University of Texas Southwestern Medical Center at Dallas, Dallas, TX), G\E\textgamma–F (A670, 1:5000; from D. Garbers), PDE6\textalpha (PAI–720, 1:500; Affinity Bioreagents), RK (MA–712, 1:5000; Affinity Bioreagents), arrestin (Marr, 1:20,000), transducin \textalpha subunit (UUTA1, 1:5000), phosducin (Gerti, 1:5000; from R. Lee, Sepulveda Veterans Affairs Hospital, Los Angeles, CA), GCAP1(\alpha–GCAP1, 1:1000; from A. Dizhoo, Pennsylvania College of Optometry, Elkins Park, PA), GCAP2(\alpha–GCAP2, 1:1000; from A. Dizhoo), 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 \textsuperscript{-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 then fixed 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 \textmu m) 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 wt6G 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 \mu m 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: PDE6\textgamma (PAI–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 turn-off, we overexpressed PDE6γ in mouse retinas. Germ-line transmission was obtained from nine founders, which showed varying levels of PDE6γ expression compared with WT C57BL/6 controls (Fig. 1A). No PDE6γ was detected either in postnatal day 13 (P13) (Fig. 1A) or adult Pdeo6tm1/Pdeo6tm1 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. 1B), as well as the catalytic subunits of PDE6α and PDE6β (Fig. 1C). 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.

Immunohistochemical experiments showed that PDE6γ in both WT and wt6C animals was localized to the ROS 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 Tα⊥ and arrestin. As in previous studies (Brann and Cohen, 1987; Philip et al., 1987; Whelan and McGinnis, 1988; Sokolov et al., 2002; Zhang et al., 2003), the Tα⊥ 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 (Philip 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 3C. 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 4A–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 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 Pdeo6tm1/+ genetic background. Lane 1, Transgenic line wt6H; lane 2, transgenic line wt6I; lane 3, transgenic line wt6J; lane 4, transgenic line wt6K; lane 5, transgenic line wt6L; lane 6, B6+/+ WT C57BL/6 control; lane 7, P13 Pdeo6tm1/Pdeo6tm1; lane 8, transgenic line wt6A; lane 9, transgenic line wt6B; lane 10, transgenic line wt6C; lane 11, transgenic line wt6D; lane 12, transgenic line wt6E; lane 13, B6+/+ WT C57BL/6 control; lane 14, P13 Pdeo6tm1/Pdeo6tm1. B, Overexpression (OX; wt6C) of PDEγ in the wt6C transgenic retina, expressed in the Pdeo6tm1/Pdeo6tm1 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.
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. 4A, 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. 4B, inset), indicating that the difference in gain of transduction is virtually unaffected.

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. 

The results in Figure 4A–C indicate that the amount of PDE6γ in wt6C rods binding to Tα-GTP before it can bind to endogenous PDE6γ (Wensel and Stryer, 1990; Otto-Bruc et al., 1993), the results in Figure 4A–C indicate that the amount of PDE6γ is sufficient to alter the gain of transduction cascade but that at bright intensities the Tα-GTP is so abundant in proportion to PDE6γ that the gain of transduction is virtually unaffected.

The results in Figure 4A–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. 4A). At bright intensities (Fig. 4C), 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 (Tsat), 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. 4D). 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 Td. We investigated this possibility in greater detail by calculating the value of Td 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 Td 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 4A, 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
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. 3C); 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.

GAP-independent acceleration of turnoff by overexpressed PDE6γ

Because PDE6γ is known to facilitate the interaction of RGS9 with Tø, 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<sup>−/−</sup> background that lacks both the RGS9–1 and Gβ5L proteins (Chen et al., 2000). Rgs9<sup>−/−</sup> rods have prolonged light responses (Fig. 6A), as reported previously (Chen et al., 2000). In Rgs9<sup>−/−</sup> rods expressing wt6C, however, the rate of decline of the rod response was greatly accelerated (Fig. 6B), 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 4A–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<sup>−/−</sup> rods and 326 ± 60 ms for rods from progeny of the mating of Rgs9<sup>−/−</sup> 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<sup>−/−</sup> rods, recovery after a bright flash is delayed. This can be seen in the recording of Figure 7A, in which a rod was exposed to a saturating light for 4 min, ending at t = 0. Flashlets 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<sup>−/−</sup> mice mated to wt6C (Fig. 7B), 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. 7C) 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 episodic 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

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

<table>
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<tr>
<th></th>
<th>Dark current (pA)</th>
<th>Time to peak (ms)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Flash sensitivity (pA photons&lt;sup&gt;−1&lt;/sup&gt; μm&lt;sup&gt;−2&lt;/sup&gt;)</th>
<th>Flash intensity at half-saturation (photons μm&lt;sup&gt;−2&lt;/sup&gt;)</th>
<th>Integration time (ms)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>τ for response recovery (ms)&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td>WT</td>
<td>11.9 ± 0.5 (44)</td>
<td>143 ± 4 (46)</td>
<td>0.26 ± 0.02 (46)</td>
<td>31 ± 2 (43)</td>
<td>199 ± 20 (47)</td>
<td>205 ± 19 (45)</td>
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<tr>
<td>wt6C</td>
<td>11.1 ± 0.7 (29)</td>
<td>126 ± 6 (29)</td>
<td>0.10 ± 0.01 (29)</td>
<td>71 ± 6 (29)</td>
<td>103 ± 9 (29)</td>
<td>91 ± 11 (29)</td>
</tr>
<tr>
<td>Rgs9&lt;sup&gt;−/−&lt;/sup&gt;/wt6C</td>
<td>8.2 ± 0.7 (15)</td>
<td>214 ± 18 (19)</td>
<td>0.15 ± 0.02 (20)</td>
<td>31 ± 3 (15)</td>
<td>1820 ± 450 (21)</td>
<td>8420 ± 1000 (17)</td>
</tr>
<tr>
<td>Rgs9&lt;sup&gt;−/−&lt;/sup&gt;/wt6C</td>
<td>11.6 ± 0.7 (10)</td>
<td>127 ± 15 (9)</td>
<td>0.16 ± 0.05 (9)</td>
<td>78 ± 13 (9)</td>
<td>236 ± 68 (9)</td>
<td>326 ± 60 (9)</td>
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Values are means ± SE with the number of cells in parentheses.

<sup>a</sup>Values are for light responses at intensity of 15.5 photons μm<sup>−2</sup>.

<sup>b</sup>Single exponential fit of just-saturating response.
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. 4A) 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 Tα-GTP in the outer segments of wt6C rods (Wensel and Stryer, 1990; Otto-Bruc et al., 1993), so that fewer Tα-GTPs are available to bind to endogenous PDE6γ. The concentration of activatable transducin may also be reduced, if added PDE6γ does bind and have an effect on the continuous component, but this effect was too small for us to observe.

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 Tα-GTP molecules (Leskov et al., 2000), then, as a result of the two flashes, 9.6 × 10\(^3\) and 6.7 × 10\(^4\) Tα-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 Tα-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-
centration of PDE6γ is much less than the total concentration, or estimates of the gain of production of Tγ-GTP are too low by at least 1 order of magnitude, or Tγ-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 more rapidly (Fig. 4C 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 PDE6 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, Tq (Fig. 4D). 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 Tγ-GTP hydrolysis. In animals overexpressing PDE6γ, the light response can be turned off in two ways, either by hydrolysis of Tγ-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 the inverses of the time constants of Tγ-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 Tγ-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 Tγ-GTP/PDE6γ complex must remain bound to the PDE6 heterotetramer until the terminal phosphate of the GTP has been hydrolyzed. If the Tγ-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 Tγ-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 Tγ-GTP 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 Tγ-GTP and endogenous PDE6γ remains attached to the PDE6 heterotetramer, but after hydrolysis of Tγ-GTP to Tγ-GDP, the PDE6γ/Tγ-GDP complex dissociates and is released to the cytosol, because PDE6γ binds much less tightly to Tγ-GDP than to Tγ-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 Tγ-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 Tγ-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...
that those of the rods of WT rods, are nevertheless much more similar to WT responses expressed, although slower to recover from light exposure than but debilitating blindness (Nishiguchi et al., 2004). The results in 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.

References


