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Dynamics of Cyclic GMP Synthesis in Retinal Rods

Marie E. Burns,[1,3,4] Ana Mendez,[2] Jeannie Chen,[2] and Denis A. Baylor[1]
[1]Department of Neurobiology
Stanford University Medical Center
Stanford, California 94305
[2]The Mary D. Allen Laboratory for Vision Research
Doheny Eye Institute and
Departments of Ophthalmology and Cell and Neurobiology
Keck School of Medicine of the
University of Southern California
Los Angeles, California 90033
[3]Center for Neuroscience and
Department of Psychiatry
University of California, Davis
Davis, California 95616

Summary

In retinal rods, Ca^{2+} exerts negative feedback control on cGMP synthesis by guanylate cyclase (GC). This feedback loop was disrupted in mouse rods lacking guanylate cyclase activating proteins GCAP1 and GCAP2 (*GCAPs*$^{-/-}$). Comparison of the behavior of wild-type and *GCAPs*$^{-/-}$ rods allowed us to investigate the role of the feedback loop in normal rod function. We have found that regulation of GC is apparently the only Ca^{2+} feedback loop operating during the single photon response. Analysis of the rods' light responses and cellular dark noise suggests that GC normally responds to light-driven changes in [Ca^{2+}] rapidly and highly cooperatively. Rapid feedback to GC speeds the rod's temporal responsiveness and improves its signal-to-noise ratio by minimizing fluctuations in cGMP.

Introduction

Retinal rod photoreceptors use cyclic GMP (cGMP) signaling to transduce incident photons into changes in membrane potential. In rods, cGMP is synthesized by the membrane-bound forms of guanylate cyclase (GC), GC-1 and GC-2. In the dark, cGMP holds open cGMP-sensitive cation channels in the plasma membrane; these channels provide an inward current that partially depolarizes the cell. Upon absorbing a photon, rhodopsin activates many copies of the G protein transducin, which in turn activate cGMP phosphodiesterase (PDE). Hydrolysis of cGMP by PDE reduces the cGMP concentration ([cGMP]) in the outer segment, allowing cGMP-gated channels to close. This reduces the inward current and allows the rod to hyperpolarize until GC restores the original cGMP concentration.

During the response to light, the influx of Ca^{2+} into the outer segment declines, while Ca^{2+} continues to be extruded from the outer segment by a Na^+/Ca^{2+}, K^+ exchanger (Yau and Nakatani, 1984; Cervetto et al., 1989). As a result, the intracellular [Ca^{2+}] falls. This drop in [Ca^{2+}] causes coordinated changes that antagonize the response to light. The Ca^{2+} signal is thought to decrease rhodopsin activity (Kawamura and Murakami, 1991), increase the sensitivity of the channel for cGMP (Hsu and Molday, 1993), and increase the activity of GC (Lolley and Racz, 1982). To date, the specific quantitative contribution of each Ca^{2+}-dependent process to rod function has only been inferred (see Koutalos et al., 1995b; Nikonov et al., 2000). We therefore sought to directly determine how rapidly and powerfully Ca^{2+} feedback to GC acts in intact rods.

Figure 1 is a diagram of the negative feedback loop that couples the rate of cGMP synthesis to the size of the inward membrane current. A change in the intracellular [cGMP] rapidly changes the inward current, and thus, the intracellular free [Ca^{2+}]. The change in [Ca^{2+}] is detected by GCAPs, which alter the rate of synthesis of cGMP to oppose the initial change in [cGMP]. The steady-state gain of the feedback loop, g_L, is not known. This parameter is defined as the ratio of the relative change in the rate of synthesis of cGMP to the relative change in the [cGMP], both changes being small. The loop gain is perhaps the single most important property of the loop, for it determines how powerfully small changes in [cGMP] are nulled in the steady state. Since the cooperativity of channel activation (n_{ch}) by cGMP is 3 (Haynes et al., 1986; Zimmerman and Baylor, 1986; Ruiz et al., 1999), a small relative change in [cGMP] will produce a 3-fold larger change in the relative Ca^{2+} influx in the steady state (see Experimental Procedures). Likewise, if the activation of GCs by GCAPs is linear for small changes in [Ca^{2+}], the relative change in the rate of synthesis will be n_{GC}-fold greater than the relative change in the [Ca^{2+}], where n_{GC} is the cooperativity of Ca^{2+} regulation of GCs. Therefore, the ratio of the relative change in the rate of synthesis to the relative change in the [cGMP] will be the product of the cooperativities, $n_{ch} \times n_{GC}$ (see Experimental Procedures). The dynamics of the loop will fix the speed with which the steady-state attenuation is achieved. Lags in the rate of change of the intracellular [Ca^{2+}] as well as lags in the operation of the GCAPs themselves should contribute to the loop kinetics and define the contributions of the loop to rod function.

To abolish Ca^{2+} feedback to GC, we disrupted GCAP1 and GCAP2 expression in mice using standard transgenic techniques described in Mendez et al. (2001). Using suction electrodes, we then recorded from single intact rods from wild-type (+/+) or *GCAPs*$^{-/-}$ rods. Comparison of responses in which the phototransduction cascade operated with the feedback loop closed (+/+) or opened (−/−) allowed us to estimate the loop gain (g_L) and kinetics, as well as the maximal light-induced increase in GC activity. The *GCAPs*$^{-/-}$ rods also allowed us to measure the rate of thermal isomerization of mouse rhodopsin and to assess the contributions of other Ca^{2+}-dependent mechanisms to the form of the single photon response and to light adaptation.

[4]Correspondence: meburns@ucdavis.edu

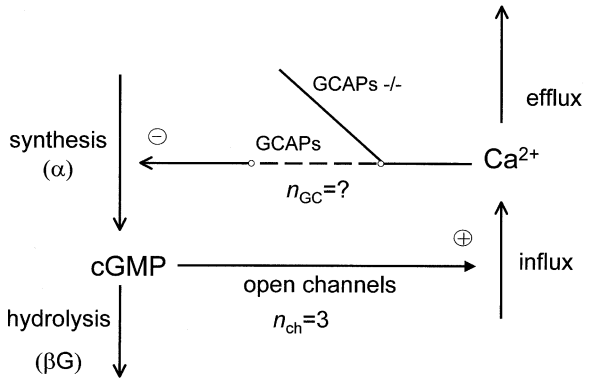

Figure 1. The Negative Feedback Loop that Regulates [cGMP] in Rod Cells

Evidence for this scheme comes from previous work in many labs. A change in the [cGMP] changes the number of open channels and the size of the inward current, part of which consists of Ca^{2+} influx. The alteration in Ca^{2+} influx changes the internal [Ca^{2+}], which in turn changes the rate of cGMP synthesis in the direction that opposes the initial change. For a given steady-state change in the cGMP concentration, there is a g_L-fold larger change in the relative rate of cGMP synthesis. The loop gain, g_L, is given by $g_L = n_{ch} \times n_{GC}$, where n_{ch} is the Hill coefficient for channel activation by cGMP and n_{GC} is the Hill coefficient for the dependence of guanylate cyclase activity on [Ca^{2+}]. Deletion of GCAPs expression opens the feedback loop (*GCAPs*$^{-/-}$). The strategy in our experiments was to compare the behavior of normal cells with that of cells in which the loop was opened.

Results

Ca^{2+} Regulation of GC Activity in Darkness and during the Single Photon Response

To study Ca^{2+} feedback to GC, we recorded the membrane current of single mouse rods in which the feedback loop was operating normally (+/+) or was opened by disruption of the expression of GCAP1 and GCAP2 using gene-targeting techniques (*GCAPs*$^{-/-}$). In a previous study, we found that loss of GCAPs expression abolished Ca^{2+} regulation of GC activity but had little effect on the expression of other genes or retinal morphology (Mendez et al., 2001). The mean dark currents of wild-type and *GCAPs*$^{-/-}$ rods were also not significantly different (as reported in Mendez et al., 2001; 12.3 ± 0.5, n = 28 wild-type cells; 13.3 ± 0.8 pA, n = 42 *GCAPs*$^{-/-}$ cells). Assuming that cGMP-gated channels are present at similar density in +/+ and −/− rods, the latter result suggests that the Ca^{2+} feedback loop to GC does not set the dark [cGMP]. Thus, the *GCAPs*$^{-/-}$ rods provide a useful system for examining the function of the feedback loop in intact rods under physiological conditions.

As demonstrated previously (Mendez et al., 2001), the single photon response of *GCAPs*$^{-/-}$ rods rose for a longer time after the flash, reached a 4-fold larger amplitude, and recovered more slowly (Figure 2A). This difference could not be attributed to decreased Ca^{2+} buffering in *GCAPs*$^{-/-}$ rods since the rate of decline of free intracellular [Ca^{2+}] was little affected by the loss of GCAPs expression (Table 1) and since BAPTA loading had no effect on the kinetics of the dim flash responses in *GCAPs*$^{-/-}$ rods (see below). We tested whether the difference in the single photon responses might have arisen from an unexpected increase in light-triggered PDE activity in the *GCAPs*$^{-/-}$ rods. Using the rising phase analysis of Pugh and Lamb (1993), we calculated the time course of the light-triggered PDE activity in *GCAPs*$^{-/-}$ and wild-type rods (see Experimental Procedures). At all flash strengths examined, the rate of rise of the light-evoked PDE activity at early times was the same in *GCAPs*$^{-/-}$ and control rods (Figure 2B). Thus, there was no evidence for changes in the expression levels or catalytic activities of the proteins that generate the response. We infer that the differences between *GCAPs*$^{-/-}$ and wild-type responses arise solely from opening the feedback loop that normally controls GC activity.

To assess how rapidly the feedback loop responds to changes in [cGMP], we examined the mean single photon responses on an expanded time scale (Figure 2C). The *GCAPs*$^{-/-}$ response diverged from the wild-type response on the rising phase, indicating that the feedback loop responds rapidly to changes in [cGMP]. To estimate the entire time course of GC activation, we calculated the cyclase activity (α) as a function of time after the flash from the mean single photon responses (see Experimental Procedures). This calculation converts inward current to [cGMP](t), assuming a value of 3 for the Hill coefficient for the channel activation by cGMP, and assuming that the dark GC activity is the same in wild-type and *GCAPs*$^{-/-}$ rods. The derived time course of α after a flash is superimposed upon the mean wild-type single photon response in Figure 2D. After a lag of about 40 ms, GC activity rose at a rate similar to that of the flash response, reaching a maximum 150 ms after the flash. The peak of the GC activity was followed by a rapid restoration of the membrane current, presumably the result of cGMP injected by the sudden rise in GC activity. Following the initial restoration of the inward current, the cyclase activity underwent a rapid decline followed by an undershoot during which the GC activity was less than the dark value. Presumably the decline and undershoot reflect rapid inhibition of GC activity by the sudden increase in [Ca^{2+}] that accompanied the restoration of the inward current. The derived time course of GC activity suggests that Ca^{2+} initially activates and then inhibits GC during the flash response.

To quantitatively assess the steady-state gain of the feedback loop, g_L, we compared the time integrals of the mean single photon responses of wild-type and *GCAPs*$^{-/-}$ rods. The time integral of the linear flash response is proportional to the amplitude of the steady-state response to a step of light (Baylor and Hodgkin, 1973), and the ratio of the time integrals of the mean single photon responses in the −/− and wild-type rods thus should equal the ratio γ_L of the steady-state responses to a dim step of light with the loop open and closed. For small responses that cause linear perturbations in the loop, it can be shown that $\gamma_L = 1 + g_L$. The mean time integral (in pA s) of the *GCAPs*$^{-/-}$ responses was 12-fold larger than that of wild-type responses (0.12 ± 0.01, n = 25 wild-type cells; 1.4 ± 0.2, n = 41 *GCAPs*$^{-/-}$ cells). This gives a value for γ_L of 12, and a value for g_L of 11. Thus, in the steady state, the small drop in [cGMP] resulting from the response to dim light would cause an 11-fold increase in the rate of synthesis of cGMP. Since $g_L = 11$, $g_L = n_{ch} \times n_{GC}$, and $n_{ch} = 3$, the

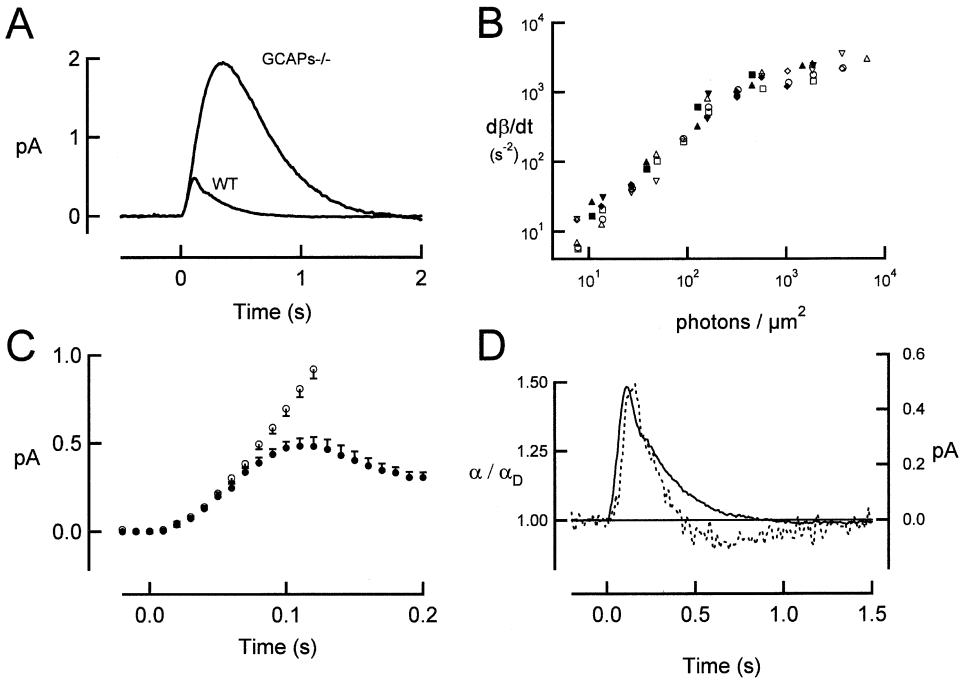

Figure 2. Effect of GCAPs Deletion on Light-Evoked Responses

(A) Population mean single photon responses from wild-type rods (WT, n = 20) and *GCAPs*$^{-/-}$ rods (n = 31). Mean dark currents (in pA) were 12.1 (wild-type) and 12.8 (*GCAPs*$^{-/-}$).

(B) Rate of change of light-activated PDE activity as a function of flash strength for five control rods (filled symbols) and five *GCAPs*$^{-/-}$ rods (open symbols).

(C) Mean single photon responses from (A), shown on an expanded time scale. Mean wild-type response is shown in filled symbols and mean *GCAPs*$^{-/-}$ response in open symbols. Error bars indicate SEM.

(D) Comparison of the calculated time course of normalized GC activity (α/α_D, dashed line) and the wild-type single photon response of (A) (solid line).

apparent Hill coefficient for the Ca^{2+} dependence of GC's regulation (n_{GC}) is 3.7.

As a second measure of g_L, we compared the steady-state responses of −/− and wild-type rods to steps of light. The intensity at which the *GCAPs*$^{-/-}$ step response was half-maximal was 13-fold dimmer than the corresponding intensity for wild-type rods (see Figure 7B), corresponding to a loop gain (g_L) of 12, and thus a value of 4 for n_{GC}. This value, as well as that of 3.7 obtained above, is larger than that expected from biochemical studies of the Ca^{2+} dependence of guanylate cyclase activity (see Discussion).

The values of g_L derived above would only hold if there are small, linear perturbations in [cGMP] and [Ca^{2+}]. Thus, the larger than expected Hill coefficient might have arisen if the fall in [cGMP] induced by the single photon response is not in fact a small relative change. To explore this possibility, we examined the effect of the feedback loop on the much smaller, spontaneous changes in cGMP concentration that occur in the dark (see below).

Effect of GCAPs Deletion on Rod Dark Noise

Since the feedback loop that controls GC activity acts to oppose changes in [cGMP], one would expect it to reduce the rod's dark noise, the low-frequency components of which arise from fluctuations in the concentration of cGMP (Rieke and Baylor, 1996). These fluctuations are generated by two principal sources: (a) thermal activation of rhodopsin (Baylor et al., 1980) and (b) thermal activation of PDE (Rieke and Baylor, 1996). To as-

Table 1. Kinetic Parameters of Wild-Type and *GCAPs*$^{-/-}$ Rods with and without BAPTA

	τ_{exch}[a] (ms)	Dim Flash Time to Peak (ms)	Dim Flash Integration Time (ms)	n
Wild-type	85 ± 10	101 ± 4	216 ± 20	14
Wild-type + BAPTA	374 ± 68	193 ± 16	ND	9
GCAPs$^{-/-}$	79 ± 12	309 ± 15	590 ± 32	16
GCAPs$^{-/-}$ + BAPTA	239 ± 37	340 ± 17	580 ± 25	15

[a] Time constant of the decline in the exchange current, measured following a bright flash that held the cell in saturation for at least 1 s. Mean ± SEM.

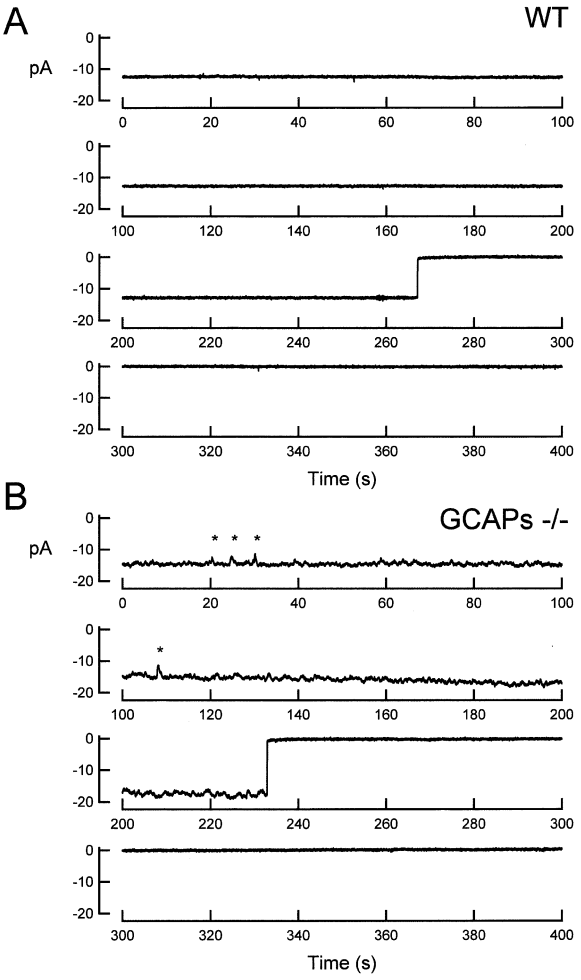

Figure 3. Increased Cellular Dark Noise in *GCAPs*$^{-/-}$ Rods

Representative currents collected in darkness and in saturating light from a (A) wild-type (WT) rod and (B) *GCAPs*$^{-/-}$ rod. The seal resistances were 11.6 and 16.1 MΩ and the dark currents were 12.9 and 17.8 pA, respectively. Constant bright light (500 nm) was presented at t = 267.1 s (A) and t = 232.9 (B). Asterisks denote discrete noise events (see text).

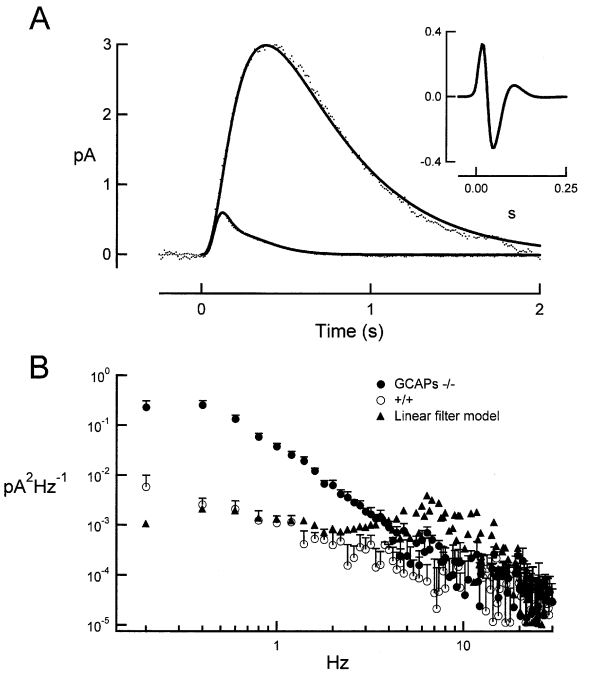

Figure 4. Test for Linearity of the Ca^{2+} Regulation of GC

(A) Theoretical functions (solid lines) were fitted to the population mean single photon responses (dots) from four *GCAPs*$^{-/-}$ rods (mean dark current, 14.0 pA) and five control rods (mean dark current, 12.9 pA) from which dark noise was also collected. Using Fourier deconvolution, the fitted functions were used to generate the impulse response of the theoretical linear filter (inset).

(B) Mean power spectra for the four *GCAPs*$^{-/-}$ rods (filled circles) and five control rods (open circles) from (A). In the frequency domain, the mean *GCAPs*$^{-/-}$ spectrum was multiplied by the spectrum of the impulse response of the theoretical filter to predict the wild-type spectrum. The predicted spectrum (triangles) was comparable to the mean +/+ spectrum, suggesting that the Ca^{2+} regulation of GC operates linearly on the single photon responses in normal mouse rods.

sess the loop's effect on the dark noise, we analyzed the membrane current of wild-type and *GCAPs*$^{-/-}$ rods in darkness and in the presence of saturating light (Figure 3). When all of the channels were closed by saturating light, the remaining current noise was instrumental. The recordings from the control rod (Figure 3A) revealed only marginally larger fluctuations in darkness than in saturating light. The recordings from the *GCAPs*$^{-/-}$ rod (Figure 3B) displayed larger fluctuations in darkness than in saturating light, indicating a considerable amount of cellular dark noise.

To quantify the loop's effect on the dark noise, we calculated the power spectrum of the noise and the noise variance. For each cell analyzed, spectra were determined in darkness and in saturating light, and on the assumption that the cell noise and instrumental noise are independent and additive, we found the spectrum of the cell noise as the difference spectrum (dark-light). The spectra in saturating light were flat (white) except for a small 1/f elevation at very low frequency (data not shown). In each case the amplitude of the flat portion agreed well with the prediction of the Nyquist equation (see Experimental Procedures) for the Johnson noise in the measured leakage resistance between the outer segment and the tip of the suction electrode. In both types of cells, most of the cellular dark noise was present at low frequencies (Figure 4B). The variance of the dark noise, calculated as the variance in darkness minus that in saturating light within the band 0.2–20 Hz was 0.166 ± 0.025 pA^2 (n = 6 cells) in *GCAPs*$^{-/-}$ rods and 0.0042 ± 0.0012 pA^2 (n = 5 cells) in wild-type rods. The 40-fold greater dark noise variance in *GCAPs*$^{-/-}$ rods and the form of the spectra indicate that the feedback loop in wild-type rods strongly reduced spontaneous fluctuations in [cGMP] that occurred in darkness.

The local cGMP fluctuations occurring in the dark are effectively very small because most of the dark noise results from spontaneous PDE activation (Rieke and Baylor, 1996; see later results). Therefore, the feedback loop should operate on the dark noise fluctuations linearly. The local changes in cGMP during the single photon response will be larger, however, and it is not clear

a priori whether the loop will operate on them linearly or not. To test this point, we attempted to use the loop's effect on the single photon response to predict its effect on the dark noise, assuming that both the single photon response and the shot effect of PDE noise perturb the loop linearly. After fitting the mean *GCAPs*$^{-/-}$ and +/+ single photon responses with theoretical functions (Figure 4A), we calculated the impulse response of the theoretical filter which, convolved with the *GCAPs*$^{-/-}$ response, would generate the wild-type response (see Experimental Procedures). This filter is an electrical representation of the dynamics of the loop; it partially differentiates the *GCAPs*$^{-/-}$ response into the smaller, briefer wild-type response. The impulse response of the filter is shown in the inset to Figure 4A. The modeling makes no assumption about the internal mechanism of the filter, but only that the filter operates linearly on changes in membrane current produced by either spontaneous activation of the cascade or the absorption of a single photon.

To calculate the filter's effect on the dark noise, we determined the power spectrum of the filter's impulse response and multiplied this by the mean power spectrum of the *GCAPs*$^{-/-}$ cellular dark noise. Because of the limited resolution and the presence of noise, we expected that the theory could give only general agreement for the overall magnitude of the noise, rather than its temporal characteristics. Indeed, the predicted spectrum of the filtered noise (triangles, Figure 4B) was similar to the observed dark noise spectrum of normal rods in the band 0.2–5 Hz. This analysis suggests that the feedback loop operates approximately linearly on the single photon response.

Thermal Activation of Rhodopsin in *GCAPs*$^{-/-}$ Rods

In a normal mouse rod, the discrete noise events that arise from thermal activation of rhodopsin (Baylor et al., 1980) are difficult to discern because of their small size. For this reason, the rate of occurrence of thermal activation of rhodopsin has not previously been measured in mouse rods. In *GCAPs*$^{-/-}$ rods, however, the single photon response amplitude was nearly five times larger, so that the discrete noise events were easier to resolve (Figure 3B, asterisks). In *GCAPs*$^{-/-}$ rods, the rate of occurrence of discrete noise events was $0.012 \pm 0.003\ s^{-1}$ (mean $\pm$ SD) at 36°C, based on a total of 16 events counted in 6 rods. In monkey rods, the mean rate of thermal activation is also about $0.01\ s^{-1}$ (Baylor et al., 1984). Because a mouse rod contains about 6-fold fewer pigment molecules (see Experimental Procedures), however, one would expect a 6-fold lower rate of occurrence if the two rhodopsins have the same stability. The results suggest that mouse rhodopsin may be less stable than macaque rhodopsin.

To determine what fraction of the total dark noise variance arises from discrete events, we compared the total cellular dark noise variance (0.2–10 Hz) with that attributable to the shot effects identical to the mean *GCAPs*$^{-/-}$ single photon response. We obtained the variance attributable to photon-like dark events by integrating the power spectrum of the single photon response over frequency and multiplying it by 0.06, the mean number of discrete events that would have occurred in each of the 5 s sweeps of record used to determine the spectrum. The total variance of the discrete events across this bandwidth was only 17% ($0.0280\ pA^2$) of the total cellular dark noise variance ($0.135\ pA^2$); therefore, about 80% of the cellular dark noise variance in mouse rods can be attributed to continuous noise, most of which results from the spontaneous activation of PDE (Rieke and Baylor, 1996). Thus, in mouse rods, like macaque rods (Baylor, Nunn and Schnapf, 1984), the total noise variance is dominated by the continuous component.

cGMP Resynthesis Limits the Recovery of Dim Flash Responses in *GCAPs*$^{-/-}$ Rods

In wild-type rods, the final falling phase of the dim flash response recovers along an approximately exponential time course, with a time constant (τ_{dim}) of about 0.2 s (Chen et al., 2000). This is similar to the time constant ($\tau_{sat} = 0.2$) (Lyubarsky and Pugh, 1996; Chen et al., 2000) determined by the slope of the relation between the time that bright flash responses remain in saturation and the log of the flash strengths (Pepperberg et al., 1992). This slope reflects the time constant of the "dominant" or rate-limiting step in turning off the saturated responses, assuming that the responses remain in saturation for sufficient time to allow $[Ca^{2+}]$ to fall to a minimum and that responses recover along a common, translatable time course (Lyubarsky et al., 1996; Nikonov et al., 1998). The similarity of the time constants τ_{dim} and τ_{sat} in wild-type mouse rods has suggested that the same process rate-limits recovery from both dim and bright flashes (Chen et al., 2000; this study). It is interesting to note that in *GCAPs*$^{-/-}$ rods, the τ_{dim} of dim flash responses (0.313 ± 0.018 s, n = 40 *GCAPs*$^{-/-}$ cells) was about 60% longer than those of wild-type rods (0.188 ± 0.013 s, n = 26 cells), yet the τ_{sat} values were not significantly different (0.226 ± 0.014 s, n = 24 wild-type cells; 0.243 ± 0.020 s; n = 19 *GCAPs*$^{-/-}$ cells; two-tailed t test, p = 0.46; Figure 5A, straight lines). This confirms that Ca^{2+} feedback to GC does not affect the rate-limiting step in deactivation per se (Lyubarsky et al., 1996; Nikonov et al., 1998; Calvert et al., 2002). It also suggests that in wild-type mouse rods, the feedback loop speeds the rate of resynthesis of cGMP enough that the final time constant of recovery of the dim flash response is mainly determined by the rate-limiting step of deactivation.

Estimation of the Maximal Stimulation of Cyclase Activity by GCAPs in Normal Mouse Rods

During a saturating flash response in a wild-type rod, the rate of synthesis of cGMP rises to its maximal value (α_{max}) because Ca^{2+} falls to a minimum. The time at which a bright flash response comes out of saturation is determined by the time required to restore the [cGMP], which is governed by the light-activated PDE activity and α_{max}. In a *GCAPs*$^{-/-}$ rod, however, the rate of synthesis of cGMP is fixed at the wild-type rod's dark rate (α_{dark}), and the time required for the saturated response to begin to recover is thus longer. Given that the light-activated PDE activity was the same in *GCAPs*$^{-/-}$ and wild-type rods (Figure 2B), and assuming wild-type and *GCAPs*$^{-/-}$ rods have channels with comparable sensitivities for

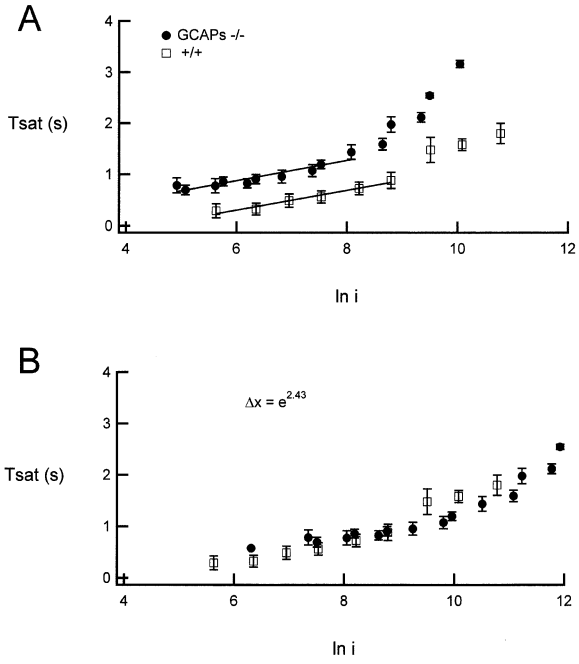

Figure 5. Loss of GCAPs Expression Has No Effect on the Dominant Time Constant of Recovery

(A) Saturation times for responses of wild-type and GCAPs-deficient rods as a function of the natural log of the flash strength in photons/μm^2. Data are the pooled results from 8 wild-type and 12 *GCAPs*$^{-/-}$ rods. The slopes of the lines fitted to the cumulative data, which reflect the mean dominant time constant of recovery, are 0.192 s (wild-type) and 0.197 s (*GCAPs*$^{-/-}$).

(B). Same plot as in (A), but with the *GCAPs*$^{-/-}$ results shifted 2.43 ln units to the right, reflecting an 11-fold reduction in GC activity (see text). Error bars indicate SD.

cGMP, the different saturation times for *GCAPs*$^{-/-}$ and wild-type bright flash responses can be used to estimate the maximal stimulation of GC activity in wild-type rods. Translating the *GCAPs*$^{-/-}$ relation in Figure 5A to the right by 2.43 ln units brought the *GCAPs*$^{-/-}$ and wild-type relations into coincidence, indicating that the saturation time of a *GCAPs*$^{-/-}$ response was equivalent to that of a wild-type rod's response to an $e^{2.43}$ or 11-fold brighter flash (Figure 5B). Since the light-activated PDE activity for a given flash strength was the same in wild-type and *GCAPs*$^{-/-}$ rods (Figure 2B), the 11-fold difference in the equivalent flash strengths reflects an 11-fold reduction in the rate of synthesis of cGMP in *GCAPs*$^{-/-}$ rods. This gives the maximal activation of GC in wild-type rods ($\alpha_{max}/\alpha_{dark}$) as 11-fold.

We also estimated the maximal activation of GC by comparing the absolute times that responses from wild-type and *GCAPs*$^{-/-}$ rods remained in saturation at a particular flash strength. Assuming the light-evoked PDE activity (β) undergoes first-order exponential decay, the PDE activity at late times after the flash can be approximated by

$$\beta(t) = \beta_o e^{-t/\tau_{sat}},$$

where β_o is the maximal PDE activity activated by the flash. In the instant just before the response begins to recover, the rate of synthesis of cGMP will equal the rate of hydrolysis by PDE ($\alpha = \beta G$, where G is the [cGMP]). In a wild-type rod that comes out of saturation at time t_1,

$$\alpha_{max} = \beta_o e^{-t_1/\tau_{sat}} G,$$

and in a *GCAPs*$^{-/-}$ rod that comes out of saturation at time t_2,

$$\alpha_{dark} = \beta_o' e^{-t_2/\tau_{sat}'} G',$$

Assuming that $G = G'$, $\tau_{sat} = \tau_{sat}'$, and $\beta_o = \beta_o'$,

$$\frac{\alpha_{max}}{\alpha_{dark}} = \frac{e^{-t_1/\tau_{sat}}}{e^{-t_2/\tau_{sat}}}$$

$$= e^{t_2 - t_1/\tau_{sat}}$$

$$= e^{\Delta t/\tau_{sat}}.$$

For the range of flash strengths over which the PDE decay time constant was invariant (50–3000 photons/μm^2), the average value of Δt was 0.5 s (Figure 5A). This corresponds to a 12-fold relative maximal activation of GC by GCAPs in mouse rods, very similar to the 11-fold value obtained above.

Do Other Ca^{2+}-Dependent Mechanisms Shape the Dark-Adapted Single Photon Response?

Ca^{2+} is thought to regulate not only GC, but also rhodopsin deactivation and the channel's sensitivity to cGMP. It has been proposed that Ca^{2+} feedback to rhodopsin deactivation might help to confer reproducibility on an otherwise noisy, stochastic deactivation (Whitlock and Lamb, 1999). However, it has not been shown whether Ca^{2+} dynamically regulates rhodopsin activity during the dark-adapted single photon response.

To test whether other Ca^{2+}-dependent feedback mechanisms shape the dark-adapted flash response, we loaded *GCAPs*$^{-/-}$ rods with the cell-permeant Ca^{2+} buffer BAPTA-AM. BAPTA increases the buffering capacity of the rod and therefore slows the rate at which the free [Ca^{2+}] concentration changes during the light response. Loading wild-type mouse rods with BAPTA-AM slowed the time course and increased the amplitude of the dim flash response (Figure 6A), as has been observed in many other studies (e.g., Torre et al., 1986). In contrast, BAPTA-AM had no effect on the amplitude or time course of the dim flash response in *GCAPs*$^{-/-}$ rods (Figure 6B; Table 1). To confirm that BAPTA had indeed been incorporated, we measured the time constant of the Na^+/Ca^{2+}, K^+ exchanger in wild-type and *GCAPs*$^{-/-}$ rods. The Na^+/Ca^{2+}, K^+ exchanger is electrogenic and generates a small inward current as it extrudes Ca^{2+} from the cytoplasm. The rate of decline of the inward current is directly proportional to the rate of decline of the free intracellular [Ca^{2+}]. When the intracellular Ca^{2+} is strongly buffered, the exchange current and the free intracellular [Ca^{2+}] decline more slowly. Indeed, BAPTA slowed the rate of Ca^{2+} extrusion in *GCAPs*$^{-/-}$ rods about 3-fold on average but failed to produce a change in the dim flash responses of the same cells (Table 1). There was thus no evidence that other Ca^{2+}-dependent mechanisms shape the dim flash response in *GCAPs*$^{-/-}$ rods. The other presumed targets for regulation by Ca^{2+} (modulation of rhodopsin activity and

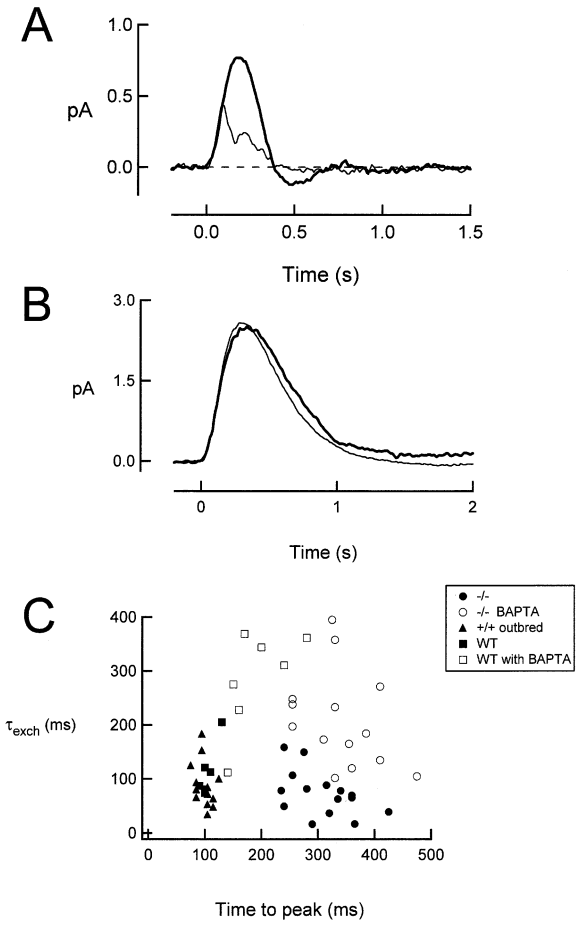

Figure 6. BAPTA Had No Effect on Dim Flash Responses of *GCAPs*$^{-/-}$ Rods

Form of the single photon response from representative wild-type (WT) rods (A) and *GCAPs*$^{-/-}$ rods (B) with (bold) and without (thin) BAPTA. (C) Scatterplot of the time to peak of the linear flash response and the time constant of the Na^+/Ca^{2+}, K^+ exchange current in *GCAPs*$^{-/-}$ and control rods, with and without BAPTA.

channel sensitivity) may require larger or longer-lasting changes in $[Ca^{2+}]$, such as occur during light adaptation.

Light Adaptation in *GCAPs*$^{-/-}$ Rods

In normal mouse rods, the response to a bright step of light displays a slow decline that develops over tens of seconds before reaching steady state (Figure 7A). This change causes the relation between response amplitude and light intensity to progressively shift to the right (Lamb et al., 1981). A shift of similar magnitude was observed in wild-type and *GCAPs*$^{-/-}$ rods (Figure 7B), suggesting that the mechanisms responsible for the slow component of adaptation operated normally in *GCAPs*$^{-/-}$ rods. Although our experiments do not identify the mechanisms that underlie this slow component of adaptation, the results demonstrate that it does not involve modulation of GCs by GCAP1 or GCAP2. As the membrane current typically shows a marked undershoot when the background light is extinguished (e.g., Figure 7A), modulation of the channel's sensitivity for cGMP by lowered Ca^{2+} may play a role. Other slower mechanisms of adaptation recently identified in bullfrog rods (Calvert et al., 2002) may also be involved.

The time for which a bright flash response remains in saturation decreases in the presence of strong background light as a result of the accompanying fall in intracellular $[Ca^{2+}]$ (Fain et al., 1989). Because this shortening cannot be attributed to Ca^{2+}-dependent feedback to GC, it has been attributed to a Ca^{2+}-dependent reduction in the time course of light-triggered PDE activity (Matthews, 1995) via the action of recoverin (Dodd, 1998). Indeed, the time in saturation did shorten in *GCAPs*$^{-/-}$ rods (Figure 7C), confirming that this adaptation mechanism does not involve Ca^{2+} feedback to GC. A background light that turned off at least half of the dark current decreased the time that *GCAPs*$^{-/-}$ rods remained in saturation following a bright flash (11 of 11 cells) as well as in wild-type rods (5 of 5 cells).

Discussion

Static and Dynamic Actions of the Feedback Loop to GC

We have used *GCAPs*$^{-/-}$ rods to quantitatively evaluate the static and dynamic actions of Ca^{2+} regulation of GCs in intact photoreceptors. Our results suggest that GC is both activated and inhibited by GCAPs depending on the free $[Ca^{2+}]$. However, in darkness, the resting $[Ca^{2+}]$ is such that there is neither a net activation nor inhibition of GC, for the dark currents of wild-type rods, in which the loop is closed, and *GCAPs*$^{-/-}$ rods, in which the loop is open, were very similar (Mendez et al., 2001).

The loop does however exert a strong steady-state effect in bright background light. We have found that the maximal light-induced change in cyclase activity ($\alpha_{max}/\alpha_{dark}$) in wild-type mouse rods is 11–12. Previous estimates for the maximal light-induced change in cyclase activity from other preparations, such as intact salamander rods (Cornwall and Fain, 1994; Hodgkin and Nunn, 1988) and truncated salamander rods (Koutalos et al., 1995a), as well as in vitro biochemical measurements in bovine (Koch and Stryer, 1988; Dizhoor et al., 1994; Gorczyca et al., 1995), frog (Calvert et al., 1998), and mouse (Tsang et al., 1998; Mendez et al., 2001) rod outer segment membranes have given widely varying figures (3- to 40-fold). The large magnitude of the Ca^{2+}-dependent cyclase activation that we observed helps to explain the significant GC-dependent extension of the light intensities over which a rod can respond (Koutalos et al., 1995b; Mendez et al., 2001) This, in conjunction with the mean PDE activity, sets the mean cGMP concentration and inward current upon which incremental responses are generated. Without the loop's static action on GC activity, the steady PDE activity generated by modest background light would reduce cGMP levels to near zero, saturating the electrical response and defeating signaling of incremental changes in light intensity (Pugh et al., 1999).

In response to small incremental changes in [cGMP], such as those that occur during the single photon response or during spontaneous noise events in the dark, the loop dynamically opposes changes in [cGMP]. Comparison of the responses to dim light with the loop open and closed gave an apparent loop gain, g_L of 11 (single

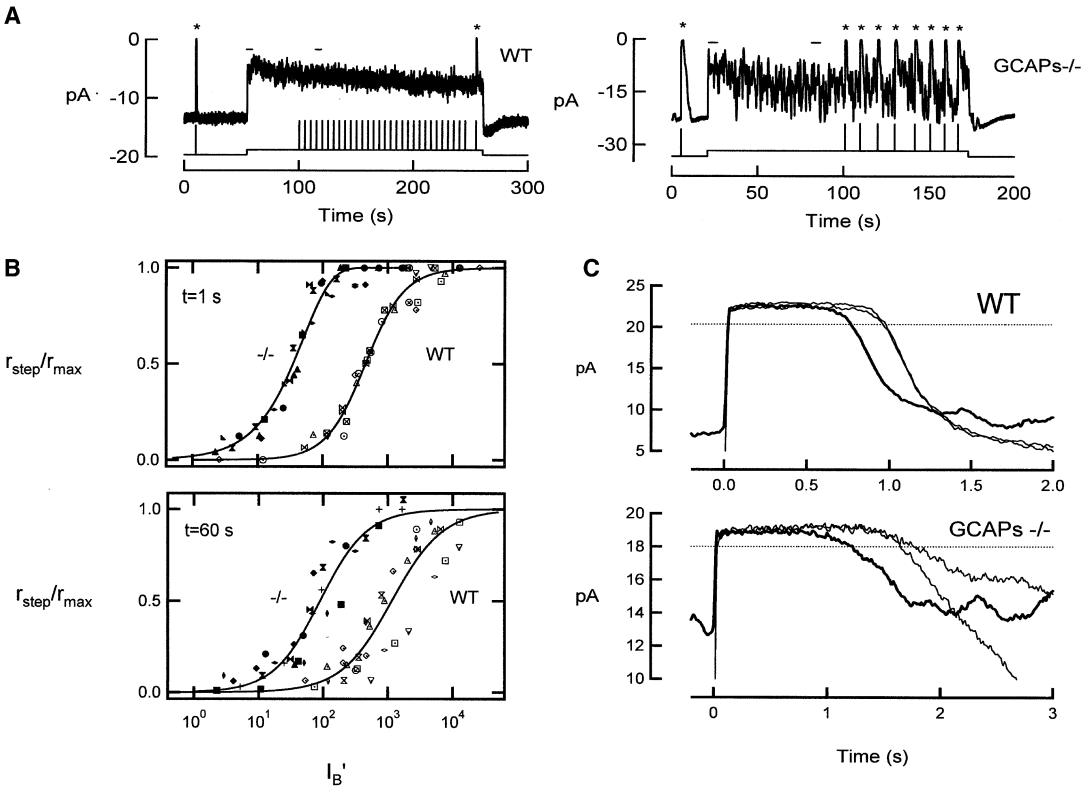

Figure 7. Two Manifestations of Light Adaptation in Wild-Type and *GCAPs*$^{-/-}$ Rods

(A) Representative current recordings from a wild-type (WT) and a *GCAPs*$^{-/-}$ rod during an adaptation experiment. Asterisks denote saturating flashes used to determine the amplitude of the dark current in darkness and in the presence of the steady illumination. In these examples, incremental flashes were delivered on top of the steady illumination, as indicated by the light stimulus monitor (bottom trace). Horizontal bars indicate the times at which the steady-state response amplitudes were measured.

(B) Responses of control and *GCAPs*$^{-/-}$ rods to steady light. The amplitude of the response to continuous illumination of varying intensities was measured at 1 s or 60 s after the onset of illumination. The relations were fitted by a saturating exponential function (*GCAPs*$^{-/-}$, top) or a Michaelis function (*GCAPs*$^{-/-}$, bottom; wild-type top and bottom; exponent values 1.1–1.4). The mean light intensities that yielded half-maximal responses for the two populations varied by a factor of 13. Notice that both −/− and +/+ relations shift to the right by an equal amount over time, illustrating a slow component of light adaptation in both wild-type and *GCAPs*$^{-/-}$ rods.

(C) Background light shortened the time that a bright flash response remained in saturation. A bright flash (1590 photons/μm^2) was delivered in the absence (thin), presence (bold), and then in the absence (thin) of steady light that turned off at least half of the dark current. Bright flashes were delivered after the membrane current had stabilized following either the onset or offset of the background light.

photon response) or 12 (dim step response). These values are consistent with Hill coefficients of 3 for activation of the channel by cGMP (Zimmerman and Baylor, 1986; Haynes et al., 1986; Ruiz et al., 1999) and about 4 for the effect of Ca^{2+} on cyclase activity. Therefore, in spite of the lack of static regulation by the loop in darkness, the loop exerts a powerful action to oppose deviations from the dark set-point. With the loop closed, there was also a modest improvement in the signal-to-noise ratio of the single photon effect (the amplitude of the single photon response divided by the standard deviation of the cellular noise). On average, this improvement was about 40%. Apparently the loop's primary function in darkness is to shorten the amplitude and integration time of the single photon effect while at the same time reducing dark noise fluctuations. Our estimation of the time course of GC activation (Figure 2D) suggests that the feedback loop minimizes fluctuations in [cGMP] not only by dynamic activation of GC but also via dynamic inhibition of GC when the instantaneous free [Ca^{2+}] exceeds steady-state dark levels. Inhibition of GC activity at high [Ca^{2+}] has been previously observed in steady-state biochemical studies (e.g., Rudnicka-Nawrot et al., 1998; Dizhoor et al., 1998); our data suggest that such inhibition might be physiologically relevant.

Molecular Mechanisms of Ca^{2+} Regulation of GCs

The high functional cooperativity in intact rods indicates that GCs/GCAPs are more sensitive to changes in Ca^{2+} than previously thought. Most biochemical measurements of Ca^{2+} dependence of GC activity have yielded a Hill coefficient near 2 (reviewed in Pugh et al., 1997; but see Koch and Stryer, 1988). Dimerization of GCs and GCAPs (Yang and Garbers, 1997; Laura and Hurley, 1998; Olshevskaya et al., 1999; Tucker et al., 1999; Yu et al., 1999) might explain the higher apparent cooperativity in vivo, but this needs to be empirically tested.

Comparison of the rising phases of normal and *GCAPs*$^{-/-}$ single photon responses revealed that the activation of GC activity initially lags behind the flash response by about 40 ms. The speed with which the loop can respond to changes in [cGMP] is somewhat

surprising given that the observed time constant of the decline in Na/Ca^{2+}, K^+ exchange current, and thus the time constant of the decline of the free intracellular $[Ca^{2+}]$, is about 85 ms in normal mouse rods (see Table 1). This is likely aided by the high cooperativity of GC activation and suggests that the activation of GC apparently follows the dynamic change in $[Ca^{2+}]$ with little lag.

Other Ca^{2+}-Dependent Feedback Mechanisms

Our studies suggest that the only Ca^{2+}-dependent negative feedback during the single photon response is that which acts on GC activity. This finding strongly constrains models for the mechanisms underlying the reproducibility of the single photon response by failing to support the existence of a dynamic Ca^{2+} feedback that controls rhodopsin deactivation. Other Ca^{2+}-dependent processes undoubtedly contribute during brighter flashes and/or during adaptation to steady illumination. Indeed, *GCAPs*$^{-/-}$ rods showed several characteristic features of light adaptation, including modulation of flash sensitivity (Mendez et al., 2001), time-dependent decreases in the steady-state response to light (Figures 7A and 7B), and the speeding of response kinetics (data not shown). Clearly, Ca^{2+} regulation of GCs is not essential for such changes.

Experimental Procedures

Transgenic Techniques

The murine genes for GCAP-1 and GCAP-2 were disrupted using standard gene targeting techniques as described (Mendez et al., 2001). The molecular techniques and biochemical assays confirming that *GCAPs*$^{-/-}$ mice lacked GCAPs expression and Ca^{2+}-dependent GC activity have been presented elsewhere (Mendez et al., 2001).

Steady-State Gain of the Feedback Loop (g_L)

We assume that in mouse rods, as in rods of other species (Fesenko et al., 1985; Yau and Nakatani, 1985; Nakatani and Yau, 1988; Karpen and Brown, 1996), the channels' half-saturating [cGMP] is well above the dark [cGMP]. Then the membrane current I is related to the cGMP concentration G approximately by

$$I = \kappa G^{n_{ch}},$$

where κ is a constant and n_{ch} is the Hill coefficient for channel activation by cGMP. A small relative change in G will then produce an n_{ch}-fold larger relative change in I, as approximately given by

$$\frac{dI}{I} = n_{ch}\frac{dG}{G}.$$

We also assume that the rate of synthesis α of cGMP by GC varies with the concentration C of free internal Ca^{2+} as described by the Hill equation:

$$\alpha = \frac{\alpha_{max}}{1 + (C/K_{1/2})^{n_{GC}}},$$

where α_{max} is the maximum rate of synthesis, $K_{1/2}$ is the $[Ca^{2+}]$ that at which the activity is half-maximal, and n_{GC} is the Hill coefficient for GC activation by Ca^{2+}. Experimental estimates for $K_{1/2}$ have varied from 30 to 580 nM, depending on experimental conditions (reviewed in Pugh et al., 1997), with most reports clustering near ~200 nM. Experimental measurements for C in the dark have also varied, with most reports greater than 400 nM (Gray-Keller and Detwiler, 1994; McCarthy et al., 1994; Sampath et al., 1998; Detwiler and Gray-Keller, 2000; Matthews and Fain, 2000; but see Woodruff et al., 2002 for measurement in mouse rods).

For concentrations $C > K_{1/2}$, the dependence of α on C is approximately

$$\alpha = \frac{\alpha_{max}K_{1/2}^{n_{GC}}}{C^{n_{GC}}},$$

and the relative change in α for a small relative change in C is approximately

$$\frac{d\alpha}{\alpha} = -n_{GC}\frac{dC}{C}.$$

Since dI/I should be directly proportional to dC/C, this can also be expressed as

$$\frac{d\alpha}{\alpha} = -n_{GC}n_{ch}\frac{dG}{G}.$$

Thus, the gain of the feedback loop g_L becomes

$$g_L = \frac{d\alpha}{\alpha}\left(\frac{dG}{G}\right)^{-1} = -n_{GC}n_{ch}.$$

Suction Electrode Recording

Mice for electrophysiological study were housed in 12 hr cyclic light and dark-adapted overnight prior to an experiment. Retinas were removed under infrared light and stored in L-15 medium (GIBCO) supplemented with 10 mM glucose and 0.1 mg/ml bovine serum albumin (Sigma) on ice. A small piece of isolated retina was mechanically chopped in chilled L-15 and placed in a recording chamber. For BAPTA-AM incorporation, a piece of retina was first incubated in electrode-filling solution (see below) containing 100 μM BAPTA-AM (Molecular Probes, Eugene, OR) for 10 min at room temperature with gentle trituration. The BAPTA-loaded piece of retina was then placed in L-15, mechanically chopped, and placed in the recording chamber.

The tissue in the recording chamber was perfused with bicarbonate-buffered Locke's solution: 149 mM NaCl, 3.6 mM KCl, 2.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM HEPES (pH 7.4), 0.02 mM EDTA, 20 mM sodium bicarbonate, 3 mM disodium succinate, 0.5 mM sodium glutamate, 0.1% vitamins and amino acid supplement (GIBCO), and 10 mM glucose, bubbled with 95% 0_2/5% CO_2 and warmed to 34°C–37°C. The dimensions of the mouse rod outer segments selected for recording were approximately 12 μm long and 1.2 μm in diameter. Thus, the volume of a mouse rod outer segment is roughly 6-fold smaller than that of a monkey rod outer segment (Baylor et al., 1984) and thus should contain 6-fold fewer rhodopsin molecules.

An individual rod outer segment was drawn into a suction electrode containing 140 mM NaCl, 3.6 mM KCl, 2.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 3 mM HEPES, 0.02 mM EDTA, and 10 mM glucose. Single cell recordings were done as described (Mendez et al., 2000). Unless otherwise indicated, the membrane current was low-pass filtered at 20 Hz with an 8-pole Bessel filter and sampled at 100 Hz using an acquisition program written in IgorPro (Wavemetrics, Lake Oswego, OR) by Fred Rieke (University of Washington, Seattle, WA) or using NiDAQ for for Windows and IgorPro (National Instruments; Austin, TX). The light stimulus consisted of brief flashes (10 ms) of 500 nm light, or steady light at 520 nm. The light intensity was controlled by calibrated neutral density filters, and the lamp power at 500 and 520 nm was measured after each experiment using a silicon photodiode detector (Graseby Optronics, Orlando, FL). The form of each rod's single photon response was estimated by variance-to-mean squared analysis as described (Mendez et al., 2000). The integration time of the dim flash response was determined by dividing the time integral of the mean response by the peak amplitude (Baylor and Hodgkin, 1973). Wild-type rods were from mice of the same outbred genetic strain used in making the *GCAPs*$^{-/-}$, although in a handful of BAPTA experiments 129SvJ mice (Jackson Laboratories, Bar Harbor, ME) were also used and yielded very similar results. BAPTA had no effect on the dominant time constant of recovery (τ_{sat}) in either wild-type or *GCAPs*$^{-/-}$ rods (data not shown).

Current Noise Measurements

For measuring the cellular dark noise, membrane current was low-pass filtered at 20 Hz and digitized at 200 Hz. Sets of 23–25 sweeps, each 5 s long, were collected in complete darkness and then in saturating light. The one-sided power spectrum was calculated for

each sweep and used to obtain the average over the set; the mean spectrum in the light was then subtracted from the mean spectrum in the dark to obtain the spectrum of the cellular dark noise. Difference spectra were averaged across cells to determine the mean spectrum for the population (Figure 4B). The total cellular dark noise variance was obtained by integrating the difference spectrum over the bandwidth 0.2–20 Hz. The current variance σ_i^2 expected from Johnson noise in the electrode leakage resistance was calculated from the Nyquist relation

$$\sigma_i^2 = \frac{4KTB}{R},$$

where K is Boltzmann's constant (1.38×10^{-23} J/K), T is absolute temperature (308 K), B is bandwidth (0.2–10 Hz), and R is the leakage resistance (11.4–20.8 MΩ). The linear filter used to predict the dark noise of wild-type rods was derived by Fourier deconvolution of theoretical functions fitted to the mean single photon responses of wild-type and *GCAPs*$^{-/-}$ rods.

Time Course of the Light-Activated PDE Activity

The time course of the light-evoked PDE activity $P^*(t)$ was calculated using the method described by Pugh and Lamb (1993):

$$P^*(t) = -\frac{1}{n_{ch}} \frac{d[\ln(1 - r(t)/r_{max})]}{dt},$$

where $r(t)$ is the time course of the light-evoked response (i.e., the decrease in inward current, I, plotted upwards from zero), r_{max} is the maximal response amplitude, and n_{ch} is the Hill coefficient for channel activation by cGMP. Assuming that $n_{ch} = 3$, we measured the initial rate of rise of the light-evoked PDE activity for five wild-type and five *GCAPs*$^{-/-}$ rods across many flash strengths.

Time Course of the Light-Activated Change in GC Activity

Under all conditions, the rate of change of the cGMP concentration is determined by the rates of cGMP synthesis and hydrolysis, as described by

$$\frac{dG}{dt} = \alpha - \beta G,$$

where G is the cGMP concentration, α is the rate of synthesis, β is the hydrolytic activity of PDE and buffering of cGMP has been ignored. In *GCAPs*$^{-/-}$ rods (quantities denoted by $'$), α' is a constant which we assume to be α_D, and therefore the expression when solved for β' becomes

$$\beta' = \frac{1}{G'}\left(\alpha_D - \frac{dG'}{dt}\right).$$

Assuming $\beta = \beta'$ and solving for $\alpha(t)$ we obtain

$$\alpha(t) = \frac{dG}{dt} + \frac{G}{G'}\left(\alpha_D - \frac{dG'}{dt}\right).$$

Since the dark cGMP concentration in rods is well below the Michaelis constant for the cGMP-gated channels, the current is related to the cGMP concentration as

$$G = kI^{1/n_{ch}},$$

where n_{ch} is the cooperativity of the channels for cGMP and k is a constant describing the sensitivity of the channels. Assuming $n_{ch} = 3$, the rate of change in the cGMP concentration is given by

$$\frac{dG}{dt} = \frac{k}{3I^{2/3}} \frac{dI}{dt}.$$

Expressing $\alpha(t)$ in terms of I instead of G, we obtain

$$\alpha(t) = k\frac{d(I)^{1/3}}{dt} + \left(\frac{I}{I'}\right)^{1/3}\left(\alpha_D - k\frac{d(I')^{1/3}}{dt}\right).$$

The mean dark currents (in pA) were 12.1 (wild-type) and 12.8 (*GCAPs*$^{-/-}$) for the rods used to generate the mean currents used for this calculation. Furthermore, we assumed that a typical rod with 15 pA of dark current would have a dark [cGMP] of 3 μM (Nakatani and Yau, 1988; Pugh and Lamb, 1993), thus producing a value of 1.22 μM pA$^{-1/3}$ for the constant k. We also assumed a value of 10 μM s^{-1} for α_D (Tsang et al., 1998). The form of $\alpha(t)$ obtained by this method was essentially unchanged by the value of k and tolerated up to 2-fold differences in the dark currents of *GCAPs*$^{-/-}$ and wild-type rods and values of α_D up to 25 μM s^{-1}.

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References

Baylor, D.A., and Hodgkin, A.L. (1973). Detection and resolution of visual stimuli by turtle photoreceptors. J. Physiol. *234*, 163–198.

Baylor, D.A., Matthews, G., and Yau, K.W. (1980). Two components of electrical dark noise in toad retinal rod outer segments. J. Physiol. *309*, 591–621.

Baylor, D.A., Nunn, B.J., and Schnapf, J.L. (1984). The photocurrent, noise and spectral sensitivity of rods of the monkey *Macaca fascicularis*. J. Physiol. *357*, 575–607.

Calvert, P.D., Ho, T.W., LeFebvre, Y.M., and Arshavsky, V.Y. (1998). Onset of feedback reactions underlying vertebrate rod photoreceptor light adaptation. J. Gen. Physiol. *111*, 39–51.

Calvert, P.D., Govardovskii, V.I., Arshavsky, V.Y., and Makino, C.L. (2002). Two temporal phases of light adaptation in retinal rods. J. Gen. Physiol. *119*, 129–146.

Cervetto, L., Lagnado, L., Perry, R.J., Robinson, D.W., and McNaughton, P.A. (1989). Extrusion of calcium from rod outer segments is driven by both sodium and potassium gradients. Nature *337*, 740–743.

Chen, C.K., Burns, M.E., He, W., Wensel, T.G., Baylor, D.A., and Simon, M.I. (2000). Slowed recovery of rod photoresponse in mice lacking the GTPase accelerating protein RGS9–1. Nature *403*, 557–560.

Cornwall, M.C., and Fain, G.L. (1994). Bleached pigment activates transduction in isolated rods of the salamander retina. J. Physiol. *480*, 261–279.

Detwiler, P.B., and Gray-Keller, M.P. (2000). Measurement of light-evoked changes in cytoplasmic calcium in functionally intact isolated rod outer segments. Methods Enzymol. *316*, 133–146.

Dizhoor, A.M., Boikov, S.G., and Olshevskaya, E.V. (1998). Constitutive activation of photoreceptor guanylate cyclase by Y99C mutant of GCAP-1. Possible role in causing human autosomal dominant cone degeneration. J. Biol. Chem. *273*, 17311–17314.

Dizhoor, A.M., Lowe, D.G., Olshevskaya, E.V., Laura, R.P., and Hurley, J.B. (1994). The human photoreceptor membrane guanylyl cyclase, RetGC, is present in outer segments and is regulated by calcium and a soluble activator. Neuron *12*, 1345–1352.

Dodd, R.L. (1998). The role of arrestin and recoverin in signal transduction by retinal rod photoreceptors. PhD dissertation. Stanford University, Stanford, California.

Fain, G.L., Lamb, T.D., Matthews, H.R., and Murphy, R.L. (1989). Cytoplasmic calcium as the messenger for light adaptation in salamander rods. J. Physiol. *416*, 215–243.

Fesenko, E.E., Kolesnikov, S.S., and Lyubarsky, A.L. (1985). Induction by cyclic GMP of cationic conductance in plasma membrane of retinal rod outer segment. Nature *313*, 310–313.

Gray-Keller, M.P., and Detwiler, P.B. (1994). The calcium feedback signal in the phototransduction cascade of vertebrate rods. Neuron *13*, 849–861.

Gorczyca, W.A., Polans, A.S., Surgucheva, I.G., Subbaraya, I., Baehr, W., and Palczewski, K. (1995). Guanylyl cyclase activating protein.

A calcium-sensitive regulator of phototransduction. J. Biol. Chem. *270*, 22029–22036.

Haynes, L.W., Kay, A.R., and Yau, K.W. (1986). Single cyclic GMP-activated channel activity in excised patches of rod outer segment membrane. Nature *321*, 66–70.

Hodgkin, A.L., and Nunn, B.J. (1988). Control of light-sensitive current in salamander rods. J. Physiol. *403*, 439–471.

Hsu, Y.T., and Molday, R.S. (1993). Modulation of the cGMP-gated channel of rod photoreceptor cells by calmodulin. Nature *361*, 76–79.

Karpen, J.W., and Brown, R.L. (1996). Covalent activation of retinal rod cGMP-gated channels reveals a functional heterogeneity in the ligand binding sites. J. Gen. Physiol. *107*, 169–181.

Kawamura, S., and Murakami, M. (1991). Calcium-dependent regulation of cyclic GMP phosphodiesterase by a protein from frog retinal rods. Nature *349*, 420–423.

Koch, K.W., and Stryer, L. (1988). Highly cooperative feedback control of retinal rod guanylate cyclase by calcium ions. Nature *334*, 64–66.

Koutalos, Y., Nakatani, K., Tamura, T., and Yau, K.W. (1995a). Characterization of guanylate cyclase activity in single retinal rod outer segments. J. Gen. Physiol. *106*, 863–890.

Koutalos, Y., Nakatani, K., and Yau, K.W. (1995b). The cGMP-phosphodiesterase and its contribution to sensitivity regulation in retinal rods. J. Gen. Physiol. *106*, 891–921.

Lamb, T.D., McNaughton, P.A., and Yau, K.W. (1981). Spatial spread of activation and background desensitization in toad rod outer segments. J. Physiol. *319*, 463–496.

Laura, R.P., and Hurley, J.B. (1998). The kinase homology domain of retinal guanylyl cyclases 1 and 2 specifies the affinity and cooperativity of interaction with guanylyl cyclase activating protein-2. Biochemistry *37*, 11264–11271.

Lolley, R.N., and Racz, E. (1982). Calcium modulation of cyclic GMP synthesis in rat visual cells. Vision Res. *22*, 1481–1486.

Lyubarsky, A.L., and Pugh, E.N., Jr. (1996). Recovery phase of the murine rod photoresponse reconstructed from electroretinographic recordings. J. Neurosci. *16*, 563–571.

Lyubarsky, A., Nikonov, S., and Pugh, E.N., Jr. (1996). The kinetics of inactivation of the rod phototransduction cascade with constant Ca_i^{2+}. J. Gen. Physiol. *107*, 19–34.

Matthews, H.R. (1995). Effects of lowered cytoplasmic calcium concentration and light on the responses of salamander rod photoreceptors. J. Physiol. *484*, 267–286.

Matthews, H.R., and Fain, G.L. (2000). Laser spot confocal technique to measure cytoplasmic calcium concentration in photoreceptors. Methods Enzymol. *316*, 146–163.

McCarthy, S.T., Younger, J.P., and Owen, W.G. (1994). Free calcium concentrations in bullfrog rods determined in the presence of multiple forms of fura-2. Biophys. J. *67*, 2076–2089.

Mendez, A., Burns, M.E., Roca, A., Lem, J., Wu, L.W., Simon, M.I., Baylor, D.A., and Chen, J. (2000). Rapid and reproducible deactivation of rhodopsin requires multiple phosphorylation sites. Neuron *28*, 153–164.

Mendez, A., Burns, M.E., Sokal, I., Dizhoor, A.M., Baehr, W., Palczewski, K., Baylor, D.A., and Chen, J. (2001). Role of guanylate cyclase-activating proteins (GCAPs) in setting the flash sensitivity of rod photoreceptors. Proc. Natl. Acad. Sci. USA *98*, 9948–9953.

Nakatani, K., and Yau, K.-W. (1988). Guanosine 3′,5′-cyclic monophosphate-activated conductance studied in a truncated rod outer segment of the toad. J. Physiol. *395*, 731–753.

Nikonov, S., Engheta, N., and Pugh, E.N., Jr. (1998). Kinetics of recovery of the dark-adapted salamander rod photoresponse. J. Gen. Physiol. *111*, 7–37.

Nikonov, S., Lamb, T.D., and Pugh, E.N., Jr. (2000). The role of steady phosphodiesterase activity in the kinetics and sensitivity of the light-adapted salamander rod photoresponse. J. Gen. Physiol. *116*, 795–824.

Olshevskaya, E.V., Boikov, S., Ermilov, A., Krylov, D., Hurley, J.B., and Dizhoor, A.M. (1999). Mapping functional domains of the guanylate cyclase regulator protein, GCAP-2. J. Biol. Chem. *274*, 10823–10832.

Pepperberg, D.R., Cornwall, M.C., Kahlert, M., Hofmann, K.P., Jin, J., Jones, G.J., and Ripps, H. (1992). Light-dependent delay in the falling phase of the retinal rod photoresponse. Vis. Neurosci. *8*, 9–18.

Pugh, E.N., Jr., and Lamb, T.D. (1993). Amplification and kinetics of the activation steps in phototransduction. Biochim. Biophys. Acta *1141*, 111–149.

Pugh, E.N., Jr., Duda, T., Sitaramayya, A., and Sharma, R.K. (1997). Photoreceptor guanylate cyclases: a review. Biosci. Rep. *17*, 429–473.

Pugh, E.N., Jr., Nikonov, S., and Lamb, T.D. (1999). Molecular mechanisms of vertebrate photoreceptor light adaptation. Curr. Opin. Neurobiol. *9*, 410–418.

Rieke, F., and Baylor, D.A. (1996). Molecular origin of continuous dark noise in rod photoreceptors. Biophys. J. *71*, 2553–2572.

Rudnicka-Nawrot, M., Surgucheva, I., Hulmes, J.D., Haeseleer, F., Sokal, I., Crabb, J.W., Baehr, W., and Palczewski, K. (1998). Changes in biological activity and folding of guanylate cyclase-activating protein I as a function of calcium. Biochemistry *37*, 248–257.

Ruiz, M., Brown, R.L., He, Y., Haley, T.L., and Karpen, J.W. (1999). The single-channel dose-response relation is consistently steep for rod cyclic nucleotide-gated channels: implications for the interpretation of macroscopic dose-response relations. Biochemistry *38*, 10642–10648.

Sampath, A.P., Matthews, H.R., Cornwall, M.C., and Fain, G.L. (1998). Bleached pigment produces a maintained decrease in outer segment Ca^{2+} in salamander rods. J. Gen. Physiol. *111*, 53–64.

Torre, V., Matthews, H.R., and Lamb, T.D. (1986). Role of calcium in regulating the cyclic GMP cascade of phototransduction in retinal rods. Proc. Natl. Acad. Sci. USA *83*, 7109–7113.

Tsang, S.H., Burns, M.E., Calvert, P.D., Gouras, P., Baylor, D.A., Goff, S.P., and Arshavsky, V.Y. (1998). Role for the target enzyme in deactivation of photoreceptor G protein in vivo. Science *282*, 117–121.

Tucker, C.L., Woodcock, S.C., Kelsell, R.E., Ramamurthy, V., Hunt, D.M., and Hurley, J.B. (1999). Biochemical analysis of a dimerization domain mutation in RetGC-1 associated with dominant cone-rod dystrophy. Proc. Natl. Acad. Sci. USA *96*, 9039–9044.

Whitlock, G.G., and Lamb, T.D. (1999). Variability in the time course of single photon responses from toad rods: termination of rhodopsin's activity. Neuron *23*, 337–351.

Woodruff, M.L., Sampath, A.P., Matthews, H.R., Krasnoperova, N.V., Lem, J., and Fain, G.L. (2002). Measurement of cytoplasmic calcium concentration in the rods of wild-type and transducin knock-out mice. J. Physiol., *542*, 843–854.

Yang, R.B., and Garbers, D.L. (1997). Two eye guanylyl cyclases are expressed in the same photoreceptor cells and form homomers in preference to heteromers. J. Biol. Chem. *272*, 13738–13742.

Yau, K.W., and Nakatani, K. (1984). Electrogenic Na-Ca exchange in retinal rod outer segment. Nature *311*, 661–663.

Yau, K.-W., and Nakatani, K. (1985). Light-suppressible, cyclic GMP-sensitive conductance in the plasma membrane of a truncated rod outer segment. Nature *317*, 252–255.

Yu, H., Olshevskaya, E., Duda, T., Seno, K., Hayashi, F., Sharma, R.K., Dizhoor, A.M., and Yamazaki, A. (1999). Activation of retinal guanylyl cyclase-1 by Ca^{2+}-binding proteins involves its dimerization. J. Biol. Chem. *274*, 15547–15555.

Zimmerman, A.L., and Baylor, D.A. (1986). Cyclic GMP-sensitive conductance of retinal rods consists of aqueous pores. Nature *321*, 70–72.