A Role for GCAP2 in Regulating the Photoresponse

GUANYLYL CYCLASE ACTIVATION AND ROD ELECTROPHYSIOLOGY IN GUCA1B KNOCK-OUT MICE

Accepted for publication, June 10, 2008. Published, JBC Papers in Press, August 22, 2008, DOI 10.1074/jbc.M804452000

Clint L. Makino,† Igor V. Peshenko,† Xia-Hong Wen,§ Elena V. Olshevskaya,‡ Ronald Barrett,¶ and Alexander M. Dizhoor

1 From the Department of Ophthalmology, Massachusetts Eye and Ear Infirmary and Harvard Medical School, Boston, Massachusetts 02114. 2 Basic Sciences and Haftor Research Laboratories, Pennsylvania College of Optometry, Salus University, Elkins Park, Pennsylvania 19027, and 3 Department of Anatomy and Cell Biology, Wayne State University, Detroit, Michigan 48201.

Cyclic GMP serves as the second messenger in visual transduction, linking photon absorption by rhodopsin to the activity of ion channels. Synthesis of cGMP in photoreceptors is supported by a pair of retina-specific guanylyl cyclases, retGC1 and -2. Two neuronal calcium sensors, GCAP1 and GCAP2, confer Ca2+ sensitivity to guanylyl cyclase activity, but the importance and the contribution of each GCAP is controversial. To explore this issue, the gene GUCA1B, coding for GCAP2, was disrupted in mice, and the capacity for knock-out rods to regulate retGC and generate photoresponses was tested. The knock-out did not compromise rod viability or alter outer segment ultrastructure. Levels of retGC1, retGC2, and GCAP-1 expression did not compromise rod viability or alter outer segment ultrastructure. Levels of retGC1, retGC2, and GCAP-1 expression did not undergo compensatory changes, but the absence of GCAP2 affected guanylyl cyclase activity in two ways; (a) the maximal rate of cGMP synthesis at low [Ca2+] dropped 2-fold and (b) the half-maximal rate of cGMP synthesis was attained at a higher than normal [Ca2+]. The addition of an antibody raised against mouse GCAP2 produced similar effects on the guanylyl cyclase activity in wild type retinas. Flash responses of GCAP2 knock-out rods recovered more slowly than normal. Knock-out rods became more sensitive to flashes and to steps of illumination but tended to saturate at lower intensities, as compared with wild type rods. Therefore, GCAP2 regulation of guanylyl cyclase activity quickens the recovery of flash and step responses and adjusts the operating range of rods to higher intensities of ambient illumination.

In visual transduction, photon absorption by rhodopsin leads to the hydrolysis of cGMP, closure of the cGMP-gated cation channels in the plasma membrane, and membrane hyperpolarization (for review, see Ref. 1). To reset the sensitivity of rods, cGMP levels are restored by retina-specific guanylyl cyclases (retGCs)3 (2–4), transmembrane enzymes present in rods and cones (5). Although other membrane guanylyl cyclases are regulated by extracellular ligands, such as peptide hormones (for review, see Ref. 6), the rod versions have an extracellular segment that apparently lacks the peptide binding capacity. Instead, retGCs fall subject to regulation by Ca2+ through their intracellular domains (7, 8), a feature conferred by guanylyl cyclase activating proteins (GCAPs) (1, 9–11), which are members of the EF-hand superfamily of Ca2+-binding proteins. GCAPs inhibit cGMP production when Ca2+ is high in the dark-adapted state of photoreceptor (12–13) and stimulate it when Ca2+ is lowered by illumination and becomes replaced in GCAPs by Mg2+ (1, 9, 14–16). Rods of all vertebrate species express two guanylyl cyclases, retGC1 and retGC2 (17) or GC-E and GC-F, respectively (18), as well as two types of GCAPs, GCAP1 and GCAP2 (1, 10–11, 19). Cones in some vertebrates even express a third isoform, GCAP3 (20). GCAPs 1 and 2 are widely expressed in the Vertebrata subphylum, from fish to the mammals. Such preservation over 400 million years of evolution argues in favor of distinct roles for the two proteins in timing the photon response recovery and/or in light adaptation of photoreceptors; however, the functional basis for this complexity is not yet understood. Knock-out of the entire tail-to-tail oriented tandem of genes coding for GCAPs 1 and 2 increased the amplitude of the single photon response in mouse rods and lengthened its duration (3–4). Re-introduction of either GCAP1 or -2 accelerated recovery in the double GCAP1/2 knock-out mice, although GCAP1 expression alone restored dim flash response kinetics and sensitivity more efficiently than expression of GCAP2 (3, 21). Therefore, the individual roles for GCAP1 and GCAP2 in phototransduction remain unclear, and an individual gene knock-out approach would be beneficial to a proper understanding. In this paper we investigate the physiological significance of GCAP2 in rods by disrupting the GUCA1B gene coding for GCAP2 and describing the effects on retGC regulation and rod photoresponse characteristics. We
A Role for GCAP2 in Regulating the Rod Photoreceptor

ExperimenTal Procedures

GCAP2 Gene Knock-out—The targeting construct was assembled in a pPNT vector originating from Mulligan and co-workers (22). Long and short arms were amplified from a mouse genomic BAC clone RP23-164J5 (CHORI BACPAC Resources, Berkeley, CA) using a high fidelity thermophilic EXL DNA polymerase mix (Stratagene). The 1.9-kb short arm was amplified using 5′-GAACCTCTGCTGCTCTCAATCATGA-3′ and 5′-AAAAAGAATTCACCCCTCTTTCAATTACT-3′ primers and inserted into the BamHI/EcoRI sites of the vector. The 4.5-kb-long arm was then amplified using 5′-CTCTCTCTGAGCTGTGCTACATGCTTCCAGTCAAAGGGTTCATGCACGAGTTCAA-3′ and 5′-AGTGAGACGTGCTACTTCCATTGTCA-3′ primers and inserted into the single Xhol site of the vector. The resultant construct replaced a 1.2-kb fragment of GCAP2 that included the entire first exon, a 620-bp portion upstream from the start codon, and a 0.3-kb portion of the first intron with the PGKNeo:ttts cassette of the pPNT vector in a direct orientation to take advantage of the PGK transcription termination signal (ttts) of the cassette (Fig. 1A). The targeting construct was verified by restriction digestion and DNA sequencing, purified, linearized with NotI digestion, and electroporated into mouse B6/129 hybrid ES cells (inGenious Targeting Laboratory, Stony Brook, NY). DNA from neomycin-resistant ES clones was screened for homologous recombination of the long arm using primers 5′-ATGCCTGAATGAGTACAAATGA-3′ (from 0.4 kb upstream of the 5′-end of the long arm, marked as f1 in Fig. 1) and 5′-AGTGAAGCCGTCACTCAGTCAATGGCCTGG-3′ (from inside the PGKNeo cassette, r1 in Fig. 1) and that of the short arm using primers 5′-AGATCAGAGCTCTCTTTCCACAT-3′ (from inside the PGKNeo cassette, r2 in Fig. 1) and 5′-CGATGACACCACTCCTGAGTCA-3′ (0.2 kb downstream from the 3′-end of the short arm, r2 in Fig. 1). The characteristic fragments of 5 and 2.2 kb, respectively, resulting from the homologous recombination of the targeting construct, were detected in three ES clones. Two of them were injected into B6/129 mouse blastocysts (inGenious Targeting Laboratory) and developed into chimera mice of which one gave germline transmission. Integration of the targeting allele via homologous recombination as in Fig. 1B was also verified in mouse tail DNA.

Recombinant Myristoylated Mouse GCAP2—The GCAP2 coding region was PCR-amplified from mouse retina cDNA using Pfu polymerase (Stratagene), inserted in the pET11d vector (Novagen), verified by sequencing, and expressed in the BLR(DE3) Escherichia coli strain harboring yeast N-myristoyl transferase using the procedures described in Dizhoor et al. (11–13) and Hwang and Koch (23) and purified using the procedures of Peshenko and Dizhoor (24).

Antibodies against full-size recombinant mouse GCAP2 and GCAP1 were raised in rabbits and purified on the corresponding immobilized GCAPs. Antibodies against human retGC1 and retGC2 were raised in rabbits and conjugated with either horseradish peroxidase for immunoblotting (Pierce) or fluorescein isothiocyanate (Cappel/ICN).

Protein levels were determined by immunoblots of whole retinal homogenates as described in Olshhevskaya et al. (25). Blots were developed using a Pierce Femto Supersignal luminescent substrate kit (Thermo Scientific), exposed on x-ray film, and scanned for optical density of the signal as described in Woodruff et al. (26).

GC activity was assayed using [α-32P]GTP as a substrate (24, 26–27) except that creatine phosphate and creatine phosphokinase were added as described in Woodruff et al. (28). The resultant [32P]cGMP was analyzed using polyethyleneimine cellulose TLC, as described previously (24, 29).

Histology—Mice were killed by lethal injection of ketamine/xylazine and perfused through the heart, first with phosphate-buffered saline and then with 2% glutaraldehyde. The eyes were enucleated and fixed overnight in 2.5% glutaraldehyde, 2.5% paraformaldehyde solution in 0.1M cacodylate buffer, pH 7.4 (Electron Microscopy Sciences, Ft. Washington, PA), on ice. The eyes were then dehydrated by a series of ethanol solutions and mounted in Embed resin. Semi-thin sections were taken from plastic blocks and stained with methylene blue/azur mix. In some cases the dehydrated fixed eyes were embedded in paraffin, sectioned at 3-μm intervals and stained with hematoxylin/eosin. For electron microscopy, the eyes were additionally fixed with 1.5% glutaraldehyde, 2% OsO4 solution in 0.1 M Sorensen phosphate buffer (all reagents were from Electron Microscopy Sciences, Ft. Washington, PA). Sections were taken and photographed as described in Woodruff et al. (26).

Immunocytochemistry—The mice were killed and perfused as above except that freshly prepared 4% paraformaldehyde in Tris-buffered saline was used for perfusion instead of glutaraldehyde, and the eyes were fixed in the same solution for 8–12 h. Cryosections were stained with antibodies (25) and viewed using an Olympus IBX 81 microscope integrated with an FV1000 Spectral confocal system. Images were captured using the Olympus FluoView FV10-ASW software supplied with the confocal microscope. Where indicated, nuclei were counterstained with TO-PRO-3 iodide (Invitrogen), and fluorescence...
A Role for GCAP2 in Regulating the Rod Photoresponse

Results

Expression of GCAPs and Other Phototransduction Proteins in GCAP2 Knock-out Mice—Expression of GCAP2 was blocked in mice by replacing the entire first exon of the GUCA1B gene together with its putative upstream promoter region with the PGK:Neo cassette (22) containing a transcription termination site (tts) in the direct orientation of the cassette (Fig. 1). Homologous recombination was confirmed by a series of PCR reactions on genomic DNA from mouse ES cell clones and from the mouse tails (Fig. 1, A and B). GCAP2, present in outer segments and synaptic termini of wild type rods, was not detectable by immunofluorescent staining of GCAP2−/− retinal sections using anti-GCAP2 antibody (Fig. 2C). There were no compensatory changes in retGC1, retGC2, or GCAP1 levels in immunoblots nor in the pattern of immunofluorescence of GCAP1 between the outer and inner segments in the GCAP2−/− retina (Fig. 2C). Because photoreceptor amplitude and kinetics depend upon the concentrations of key proteins (for review, see Refs. 31 and 32), we also measured the levels of other phototransduction proteins by immunoblotting. Transducin, PDE6, and RGS9.1 were unchanged; however, we did detect a decrease in the recoverin content by e.g. Fig. 3, even at 7 months. Outer nuclear layer thickness was preserved, indicating a full complement of photoreceptors. Average outer segment length (~22−23 μm) and diameter were unaffected by GCAP2 knock-out. At an ultrastructural level, the orderly arrangement and spacing of the disk membranes within the outer segment in GCAP2−/− were also normal (Fig. 3).
A Role for GCAP2 in Regulating the Rod Photoresponse

Alteration in Calcium-sensitive Guanylyl Cyclase Activity—We used a retGC assay on homogenates of dark-adapted retina (24) that measured retGC activity originating from photoreceptors, without a significant contribution from other potential guanylyl cyclase activities in the inner retina (25). In wild type retinal extracts retGC activity varied with Ca$^{2+}$ according to the Hill function with a $K_{0.5}$ for Ca$^{2+}$ of 69 nM and a Hill coefficient $n_H$ of 1.7. More than half of the total activity at low Ca$^{2+}$ was lost as a result of GCAP2 knock-out. The remainder followed a Hill function with a $K_{1/2}$ for Ca$^{2+}$ of 133 nm and $n_H = 2.1$ (Fig. 4). The “missing” activity (i.e. that driven by GCAP2), given by the difference between WT and knock-out activities, conformed to a Hill function with $K_1/2 = 47$ nM and $n_h = 1.9$. These experiments were conducted at the physiological level of free Mg$^{2+}$ of 1 mm (33), but three additional experiments at 0.5, 2, and 10 mm Mg$^{2+}$ (not shown) all yielded comparable differences between the knock-outs and the wild type (the values of $K_{1/2}$ in each case varied as function of free Mg$^{2+}$ (24)). High maximal retGC activity was restored to the GCAP2$^{−/−}$ homologene by the addition of either recombinant bovine (not shown) or recombinant mouse GCAP2 protein (Fig. 4B, inset).

Preincubation of wild type mouse retinal homogenate with anti-GCAP2 antibody produced a similar effect on total retGC activity as GCAP2 knock-out (Fig. 5). Maximal retGC activity at low Ca$^{2+}$ was suppressed 2-fold, and Ca$^{2+}$-sensitivity was shifted toward higher free [Ca$^{2+}$].

Alterations in Rod Responses to Flashes and Steps of Light—Sensitivity to flashes was increased in GCAP2$^{−/−}$ mice, normalized to the respective maximal activities (A), and normalized to the WT maximal activity (B). There were four determinations for knock-outs (C) and three determinations for WT (D) preparations except for the two highest [Ca$^{2+}$] in WT for which single determinations were made. Error bars mark S.E. Continuous lines show the fits to the Hill equation: retGC activity = (maximal activity − minimal activity)/(1 + ([Ca$^{2+}$]/$K_{1/2}$)) + minimal activity. Because of the unequal number of measurements at some [Ca$^{2+}$], fits were made to collected results but plotted against mean values for clarity. For WT (○), $K_{1/2} = 69$ nM, $n_H = 1.7$. For GCAP2$^{−/−}$ (□), $K_{1/2} = 133$ nm, $n_H = 2.1$. The dashed line shows the Hill fit to the difference (△); $K_{1/2} = 47$ nm, $n_H = 1.9$. The GC activity was assayed as described under “Experimental Procedures.” Inset, retGC activity in GCAP2$^{−/−}$ retinas stimulated by recombinant myristolated mouse GCAP2. In each of three experiments, 4 mouse retinas originally collected in 50 μl of Tris-buffered saline with 5 mm MgCl$_2$ were thawed, added to 200 μl of 2× GC buffer containing 5 mm MgCl$_2$ and 20 mm creatine phosphate, and homogenized. Then 225 μl of the suspension were mixed with 45 μl of 20 mm EGTA and 2 μl of 5 units/μl creatine phosphokinase and were assayed at 1 mm [Mg$^{2+}$], $I_{max}$, for retGC activity without (left) or with (right) GCAP2 at a final concentration of 4 μM. At $2.1$, the retGC activity was lost as a result of GCAP2 knock-out. The remainder followed a Hill function with a $K_{1/2}$ for Ca$^{2+}$ of 133 nm and $n_H = 2.1$ (Fig. 4). The “missing” activity (i.e. that driven by GCAP2), given by the difference between WT and knock-out activities, conformed to a Hill function with $K_{1/2} = 47$ nM and $n_H = 1.9$. These experiments were conducted at the physiological level of free Mg$^{2+}$ of 1 mm (33), but three additional experiments at 0.5, 2, and 10 mm Mg$^{2+}$ (not shown) all yielded comparable differences between the knock-outs and the wild type (the values of $K_{1/2}$ in each case varied as function of free Mg$^{2+}$ (24)). High maximal retGC activity was restored to the GCAP2$^{−/−}$ homologene by the addition of either recombinant bovine (not shown) or recombinant mouse GCAP2 protein (Fig. 4B, inset).

Preincubation of wild type mouse retinal homogenate with anti-GCAP2 antibody produced a similar effect on total retGC activity as GCAP2 knock-out (Fig. 5). Maximal retGC activity at low Ca$^{2+}$ was suppressed 2-fold, and Ca$^{2+}$-sensitivity was shifted toward higher free [Ca$^{2+}$].

Alterations in Rod Responses to Flashes and Steps of Light—Sensitivity to flashes was increased in GCAP2$^{−/−}$ mice (Fig. 6, A–C, Table 1), but the difference was too small to resolve as a change in either rod outer segment dimensions (see above) or amplitude of the single photon response (Fig. 7, Table 1) as both parameters appeared to be normal in knock-out rods. Kinetics of the GCAP2$^{−/−}$ dim flash response began to deviate from normal after ~100 ms during the rising phase (Fig. 7).
A Role for GCAP2 in Regulating the Rod Photoresponse

Light triggers the hydrolysis of cGMP in photoreceptors through a rhodopsin-transducin-PDE6 pathway, which causes cGMP-gated channels to close. The photoreceptor hyperpolarizes as Na⁺ and a lesser amount of Ca²⁺ are prevented from entering the outer segment through the channels. Although Ca²⁺ makes only a minor contribution to the change in membrane potential, it signals a powerful feedback mechanism that stimulates rapid cGMP resynthesis (for review, see Ref. 1). Cyclic GMP synthesis in the outer segment is regulated by two Ca²⁺/Mg²⁺-sensing proteins, GCAPs 1 and 2, such that guanylyl cyclase activity increases when intracellular [Ca²⁺] is low (i.e. in the light when the cGMP gated channels are closed) and decreases when [Ca²⁺] is high (in darkness when the channels are open).

Without Ca²⁺ regulation over cyclase activity, the light-activated PDE6 reduces free cGMP within the outer segment to considerably lower levels and closes more channels. It also takes longer for the retGC to replenish the cGMP to re-open the channels. Hence, in GCAP1/2 double knock-out mouse rods the photocurrent response to a single photon response rises for a period 3 times longer to reach an amplitude nearly 5 times greater and then recovers ~2 times more slowly than normal (3–4).

To explore the significance of GCAP isoform type, each GCAP was earlier re-introduced separately back into the rods of the GCAP1/2 double knock-out background (3, 21). Overexpression of GCAP2 in the GCAP1/2−/− background limited the rise of the single photon response, although it was still larger than normal, and the response kinetics remained slow especially in the initial phase of the recovery (3). GCAP2 did greatly accelerate the recovery from a strong flash, making it as fast or even faster than in wild type (3). The results of these experiments suggested that GCAP2 contributes to the photoreponse only in situations where most or all of the channels are closed. A subsequent study reported that normal dim and bright flash responses were restored by overexpression of GCAP1 in the double knock-out background, at least in some rods (21). The

sloiner final ascent in knock-out rods delayed time to peak by ~10 ms. In addition, integration time increased 1.5-fold due to a brief shoulder in the recovery phase of the dim flash response just after the peak followed by a slower time constant, τᵣ, for the subsequent return to base line (Fig. 7, Table 1).

Na⁺/Ca²⁺, K⁺ exchange currents, measured after the sudden closure of all cGMP-gated channels in the outer segment by bright flashes, were normal in amplitude and kinetics in knock-out rods. Thus, the resting levels of Ca²⁺ and the rate of Ca²⁺ removal were undisturbed by GCAP2 knock-out (supplemental Fig. 1). Saturation time of the bright flash response increased linearly with the natural logarithm of flash strength (Fig. 6D), as described elsewhere (34–35). The slope of the relation, τᵣ, determined predominantly by the rate of transducin inacti-
A Role for GCAP2 in Regulating the Rod Photoresponse

TABLE 1
Photoreceptor parameters in wild type and GCAP2 knock-out mice

|                      | WT               | GCAP2−/−       |
|----------------------|------------------|----------------|
|                     | 47 ± 2 (n = 30)  | 41 ± 2 (n = 37, p = 0.03) |
| Single photon response |                 |                |
| Amplitude, pA        | 0.49 ± 0.04 (n = 14) | 0.43 ± 0.04 (n = 18) |
| Time to peak, ms     | 122 ± 3 (n = 21)  | 134 ± 2 (n = 22, p = 0.009) |
| Integration time, ms | 244 ± 16 (n = 21) | 366 ± 26 (n = 22, p = 0.0003) |
| Recovery time constant, τr, ms | 184 ± 15 (n = 21) | 271 ± 29 (n = 22, p = 0.02) |
| Saturating responses: τr, ms | 242 ± 19 (n = 24) | 261 ± 10 (n = 29) |

|                      |                 |                |
| I0.5, photons μm−2 |                |                |
| Measured at the response peak | 245 ± 26 (n = 14) | 147 ± 17 (n = 17, p = 0.003) |
| Measured 10 s after light onset | 432 ± 65 (n = 14) | 210 ± 30 (n = 16, p = 0.003) |

FIGURE 7. Slowed recovery of the GCAP2−/− single photon response. The mean, fractional single photon response from 18 knock-out rods (gray trace) was similar in size to that from 14 WT rods (black trace), but the recovery was slower. The divergence of the two traces late in the rising phase suggests that GCAP2 normally activates retGC at this time.

apparently normal physiological photoresponses conferred solely by GCAP1 regulation over retGC raised questions about the role of GCAP2. However, differing results with the two isotypes of GCAPs could have arisen, for example, from unavoidable cell-to-cell variations in levels of transgene expression. Furthermore, although these experiments confirmed that each of the two GCAPs could regulate the cyclase not only in vitro, but also in vivo, it was not possible for either study to answer the question of what each GCAP does in the normal rod. The proper approach requires individual gene knock-out to isolate the specific components of the recovery driven by each GCAP, uniformly missing from all rods. Our results from GCAP2 knock-out mice described here identify the conditions in which GCAP2 contributes to the normal physiological behavior of rod photoreceptors.

The properties of individual GCAPs assayed in vitro (13, 23–24) implied that regulation of retGC by GCAPs might be a two-step process in which GCAP1 mediates the first-response reaction that boosts cGMP production after illumination as soon as intracellular Ca2+ starts to fall, whereas GCAP2, which is more readily inhibited by Ca2+ (24), provides additional stimulation of cGMP synthesis only after Ca2+ drops substantially, i.e. in response to brighter light (3, 23) (Fig. 9). We confirmed the main elements of this hypothesis by selectively deleting GCAP2 function in mouse rods without perturbing the expression of retGC1, retGC2, or GCAP1.

Knock-out of GCAP2 did not eliminate the Ca2+-dependence of cyclase activity; however, maximal guanylyl cyclase activity at low Ca2+ was reduced by a factor of 2.5 (Fig. 4B). The decrease in the total cyclase activity was not a result of an irreversible change caused by the lack of GCAP2 because the cyclase activity rose nearly 2.5-fold when a recombinant myristoylated mouse GCAP2 was added back to the retinal homogenate of the knock-out mice (Fig. 4B, inset) at a concentration matching the physiological level of GCAP2 estimated for bovine rods (38). The K1/2 for Ca2+ measured at the physiological concentration of free [Mg2+]~1 = 1 μM (33) increased to 133 nM with a Hill coefficient of 2.1 in the knock-out. Subtraction of this GCAP1 activity in the knock-out from the wild type activity revealed the effect of GCAP2 that was missing from GCAP2−/− retinas. It conformed to a Hill function with K1/2 = 47 nM and a Hill coefficient of 1.9 (Fig. 4). Similar results were obtained by measuring cyclase activity after suppressing the GCAP2 component with an antibody (Fig. 5). These values are in good agreement with the K1/2 of 50 nM obtained for recombinant myristoylated bovine GCAP2 at 1 μM Mg2+ (24). The K1/2 of 133 nM Ca2+ for the remaining GCAP1 component directly measured in the GCAP2−/− knock-out retinas was noticeably lower than the corresponding value of ~300 nM measured for recombinant bovine GCAP1 under the same conditions (24). Murine GCAP1 may resemble recombinant human GCAP1 in that respect, because the latter is also more sensitive to Ca2+ than its bovine ortholog (39). Thus, GCAP1 and GCAP2 each command roughly half of the total (maximal) guanylyl cyclase activity to extend continuous regulation over a greater range of [Ca2+] than could be provided by either GCAP alone. Although it is impossible to rule out potential regulatory effects that we are missing as a result of disruption of the photoreceptor cells, we expect that the overall perturbation of retGC regulation measured in the conditions of our assay should be minimal compared with other in vitro approaches. This analysis assumes that cyclase regulation in retinal homogenates reflects its regulation in rods exclusively, based on observations that retGC activity is immeasurable in retinas lacking photoreceptors (25) and retGC activity is not Ca2+-sensitive in retinas with photo-
receptors that fail to express both GCAPs 1 and 2 (3) as well as the following argument.

Distributing the guanylyl cyclase activity of ~1 nmol of cGMP retina⁻¹ min⁻¹ (e.g. Fig. 4, inset) over the ~6.5 million rods in a mouse retina (40) would correspond to ~2.6 x 10⁻¹⁸ mol of cGMP rod⁻¹ s⁻¹. Rod outer segments have a diameter of 1.4 μm and a length of 23 μm (Fig. 3, C–F) giving them a volume of 0.035 pl. Because half the volume is occupied by disks, the maximal activity within a rod outer segment (ROS) would be 149 μM cGMP ROS⁻¹ s⁻¹. The concentration of PDE6 catalytic subunits in mouse rod outer segments is not known, but if it were the same as estimated for bovine outer segments, i.e. ~30 μM (41–42), and if each catalytic subunit were to maintain a basal activity of 0.3–0.8 s⁻¹ in vivo in darkness (2, 43), then guanylyl cyclase activity must be somewhere between 9 and 24 μM ROS⁻¹ s⁻¹ to match the PDE6 activity. According to physiological estimates, guanylyl cyclase activity increases 11–12-fold when intracellular Ca²⁺ falls to its minimum in intact mouse rods (4), reaching between 99 and 290 μM cGMP ROS⁻¹ s⁻¹, in reasonable agreement to the biochemically determined maximum of 149 μM cGMP ROS⁻¹ s⁻¹ in our experiments.

In the present studies of rod physiology (Figs. 6–8), loss of GCAP2 alone affected the single photon response ~100 ms after the flash, during the rising phase, so one might have expected to find a change in response amplitude. The decrease in i_{0.5} (the number of photons per μm² delivered to the dark-adapted rods by a brief flash, producing a half-maximal response, Table 1) in GCAP2⁻/⁻/rods signified a modest increase in sensitivity and was consistent with an enlarged photoreceptor. Such a change was too small to resolve in determinations of single photon response amplitude. One consideration is that the effects of GCAP2 knock-out on a single-photon response amplitude may have been partially compensated by the decrease in recoverin expression. Recoverin restrains the shutoff of phototransduced rhodopsin (for review, see Refs. 44 and 45), so with less recoverin, rhodopsin shuts off sooner. Acceleration of this nondominant step in the photoreceptor response recovery (36) may decrease the amplitude of the response (46). Indeed, in the double GCAP1/2 knock-out rods, complete loss of recoverin lowers the amplitude of the single photon response and reduces recovery saturation time after bright flashes (47). But this effect of recoverin knock-out was not observed when both GCAPs were present (47), so retention of 80% recoverin and full GCAP1 activity in our GCAP2 knock-out rods described here seems unlikely to have substantially affected single-photon response amplitude. Instead, the loss of GCAP2 regulation over cytochrome slowed flash responses by affecting primarily the kinetics of the recovery phase and, to some extent, that of the rising phase. The difference in the GCAP2 knock-out and wild type dim flash responses was most apparent after the shoulder in the wild type recovery phase, ~300–800 ms after the flash, when the GCAP2⁻/⁻ response was more than 3 times higher than the WT response and returned to base line with a slower time constant (Fig. 6, Table 1). These differences argue that in normal rods, Ca²⁺ must fall low enough during the rising phase to where GCAP2-sensitive regulation of guanylyl cyclase activity becomes significant and that GCAP2 continues to be important throughout the response recovery, even for single photon responses.

Bright flashes drive the rod into saturation, during which time cGMP levels and [Ca²⁺]_{free} in the outer segment fall to very low levels (48). Cyclase ramps up to its maximal rate of cGMP production to reopen channels in a timely manner.
A Role for GCAP2 in Regulating the Rod Photoreponse

Knock-out of both GCAPs or a reduction to half the normal levels of both GCAPs greatly prolongs the saturation time (by factors of ~3–4 and 2–2.5, respectively, Ref. 3) because cyclase activity is unable to properly accelerate (3 and 4). Sufficiently high expression of either GCAP1 or GCAP2 in the double GCAP1/2 knock-out background nearly restored the normal recovery after the bright flash (3, 21) but did not answer the question about the specific contribution from each GCAP to the bright flash recovery. That question was addressed in the present study using the individual knock-out of GCAP2. By comparison with the wild type, the recovery after saturating flashes was delayed by ~120 ms in GCAP2−/− rods (Fig. 6D, Table 1). The corresponding lateral shift of the saturation function to lower flash strengths by 0.45 ln unit meant that GCAP2−/− rods were 1.6-fold more sensitive than WT rods. Even after emerging from saturation, it took ~70 ms longer for the GCAP2−/− response to return to base line (supplemental Fig. 2). This overall delay of ~200 ms in the recovery after bright flashes is the impairment caused specifically by the lack of GCAP2.

The initial photocurrent response to a step of light integrates the effects of photon absorptions, so it depends on the size and shape of the single photon response. The lengthened integration time of the GCAP2−/− dim flash response prolongs the rising phase of the step response and, together with a slightly increased i0.5 (Table 1), enhances the step response amplitude. The i0.5 (intensity of 500 nm constant light giving rise to a half-maximal response to prolonged illumination rather than after a brief burst of light; Table 1) determined at the peak of the response was 1.7-fold lower for mutant rods, as expected from the product of the 1.5-fold increase in integration time and the 1.15-fold drop in i0.5. After 10 s of continuous exposure, light lost its effectiveness, and the response began to wane, so that brighter light was required for a half-maximal response at that point (i0.5 increased, Table 1) in both wild type and GCAP2−/− rods. Although the mechanism(s) of this element of light adaptation (a gradual decline in sensitivity to the step light) is not fully understood, it appears to affect regulation of the lifetime of photoactivated rhodopsin rather than Ca2+-sensitive regulation of guanylyl cyclase (for review, see Ref. 49; see also Ref. 50). Consequently, this feature was characteristic of the response in both wild type and GCAP2−/− rods in our experiments (Fig. 8), and the light sensitivity of GCAP2−/− rods at 10 s of constant light exposure remained twice as high as in wild type (Table 1). After the light was extinguished, circulating current returned to GCAP2−/− rods more slowly (Fig. 8), because they were unable to recover as quickly from individual quanta as the wild type (Fig. 7).

Taken together, our experiments demonstrate that GCAP2 is necessary for normal recovery and light adaptation in rods. For retGC activation in response to light to be fast enough and not delay the recovery excessively (2, 4, 36, 51) GCAP1 alone is not sufficient, and GCAP2 is required. It needs to be pointed out that the contribution of each GCAP to the biochemical pathway behind the cyclase regulation via Ca2+ feedback cannot be described in terms such as “more” or “less” important no matter how tempting the use of such terms may be. By participating in the activation of retGC even at Ca2+ levels at which GCAP1 regulation is expected to predominate, GCAP2 quickens the recovery of the single-photon response as well as the responses to brighter flashes. By reducing the temporal overlap between the responses to individual quanta, GCAP2 also shifts the operating range of the rod to higher ambient illuminations. It is likely that GCAP1, which by itself is not fully sufficient to provide normal cyclase regulation in the absence of GCAP2, is responsible for most of the cyclase activation during recovery from a single-photon response. Direct verification by individual GCAP1 knock-out is needed, however, because the dual mode of operation for Ca2+ feedback on the cyclase activity depends on additional factors that cannot be resolved without comparison of two individual gene knock-outs, GCAP1 versus GCAP2. Our interpretation of the experimental results was based on the premise that knock-out of GCAP2 simply eliminated its function and isolated the function of GCAP1. However, there are suggestions that things may not be so simple. For example, GCAP1 and GCAP2 are each capable of binding both retGCs in vitro so GCAP2 knock-out might be partially compensated if a greater than normal share of cyclase activity becomes available for regulation by GCAP1. Alternatively, the presence of both GCAPs acting in accord might produce a different regulation of cyclase than each type alone. Because GCAP1 and 2 may bind to distinct domains on the cytoplasmic portion of retGC1 (52), their interaction on the cyclase remains a possibility. In the future it will be important to generate GCAP1−/− and GCAP1/2−/− rods to see if the GCAP1−/− behavior is complementary to that of GCAP2−/−.

Acknowledgments—We appreciate the gift of pPNT vector originating from Dr. Mulligan, and we thank N. Michaud for technical assistance.

REFERENCES

1. Fu, Y., and Yau, K.-W. (2007) Pfluegers Arch. Eur. J. Physiol. 454, 805–819
2. Hodgkin, A. L., and Nunn, B. J. (1988) J. Physiol. (Lond) 403, 439–471
3. Mendez, A., Burns, M. E., Sokal, I., Dizhoor, A. M., Baehr, W., Palczewski, K., Baylor, D. A., and Chen, J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 9948–9953
4. Burns, M. E., Mendez, A., Chen, J., and Baylor, D. A. (2002) Neuron 36, 81–91
5. Dizhoor, M. E., Lowe, D. G., Olshevskaya, E. V., Laura, R. P., and Hurley, J. B. (1994) Neuron 12, 1345–1352
6. Garbers, D. L. (1999) Methods 19, 477–484
7. Laura, R. P., Dizhoor, A. M., and Hurley, J. B. (1996) J. Biol. Chem. 271, 11646–11651
8. Laura, R. P., and Hurley, J. B. (1998) Biochemistry 37, 11264–11271
9. Gorczyca, W. A., Gray-Keller, M. P., Detwiler, P. B., and Palczewski, K. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4014–4018
10. Gorczyca, W. A., Polans, A. S., Surgucheva, I. G., Subbaraya, I., Baehr, W., and Palczewski, K. (1995) J. Biol. Chem. 270, 22029–22036
11. Dizhoor, A. M., Olshevskaya, E. V., Hensel, W. J., Wong, S. C., Stults, J. T., Ankoudinova, I., and Hurley, J. B. (1995) J. Biol. Chem. 270, 25200–25206
12. Dizhoor, A. M., and Hurley, J. B. (1996) J. Biol. Chem. 271, 19346–19350
13. Dizhoor, A. M., Boikov, S. G., and Olshevskaya, E. V. (1998) J. Biol. Chem. 273, 17311–17314
14. Koch, K.-W., and Stryer, L. (1988) Nature 334, 64–67
15. Peshenko, I. V., and Dizhoor, A. M. (2006) J. Biol. Chem. 281, 23830–23841
16. Peshenko, I. V., and Dizhoor, A. M. (2007) J. Biol. Chem. 282, 21645–21652
17. Lowe, D. G., Dizhoor, A. M., Liu, K., Gu, Q., Spencer, M., Laura, R., Lu, L.,...
A Role for GCAP2 in Regulating the Rod Photoresponse

and Hurley, I. B. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5532–5539
18. Yang, R.-B., Foster, D. C., Garbers, D. L., and Fulle, H.-J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 602–606
19. Palczewski, K., Subbaraya, I., Gorczyca, W. A., Helek, B. S., Ruiz, C. C., Ohguro, H., Huang, J., Zhao, X., Crabb, J. W., Johnson, R. S., Walsh, K. A., Gray-Keller, M. P., Detwiler, P. B., and Baehr, W. (1994) Neuron 13, 395–404
20. Imanishi, Y., Li, N., Sokal, I., Jackson, P. K., Bronson, R. T., and Howes, K. A., Pennesi, M. E., Sokal, I., Church-Kopish, J., Schmidt, B., Tybulewicz, V. L., Crawford, C. E., and Palczewski, K. (2002) EMBO J. 21, 1545–1554
21. Howes, K. A., Pennesi, M. E., Church-Kopish, J., Frederick, J. M., Rieke, F., Palczewski, K., Wu, S. M., Detwiler, P. B., and Baehr, W. (2002) EMBO J. 21, 1545–1554
22. Tybulewicz, V. L., Crawford, C. E., Jackson, P. K., Bronson, R. T., and Mulligan, R. C. (1991) Cell 65, 1153–1163
23. Hwang, J. Y., and Koch, K. W. (2002) Biochemistry 41, 13021–13028
24. Peshenko, I. V., and Dizhoor, A. M. (2004) J. Biol. Chem. 279, 16903–16906
25. Olshevskaya, E. V., Calvert, P. D., Woodruff, M. L., Peshenko, I. V., Savchenko, A. B., Makino, C. L., Ho, Y. S., Fain, G. L., and Dizhoor, A. M. (2004) J. Neurosci. 24, 6078–6085
26. Woodruff, M. L., Olshevskaya, E. V., Savchenko, A. B., Peshenko, I. V., Barrett, R., Bush, R. A., Sieving, P. A., Fain, G. L., and Dizhoor, A. M. (2007) J. Neurosci. 27, 8805–8815
27. Hurley, J. B., and Dizhoor, A. M. (2000) Methods Enzymol. 315, 708–717
28. Woodruff, M. L., Janisch, K. M., Peshenko, I. V., Dizhoor, A. M., Tsang, S. H., and Fain, G. L. (2008) J. Neurosci. 28, 2064–2074
29. Olshevskaya, E. V., Hughes, R. E., Hurley, J. B., and Dizhoor, A. M. (1997) J. Biol. Chem. 272, 14327–14333
30. Liu, X., Bulgakov, O. V., Wen, X.-H., Woodruff, M. L., Pawlyk, B., Yang, J., Fain, G. L., Sandberg, M. A., Makino, C. L., and Li, T. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 13903–13908
31. Detwiler, P. B., Ramanathan, S., Sengupta, A., and Shraiman, B. I. (2000) Biophys. J. 79, 2801–2817
32. Makino, C. L., Wen, X. H., and Lem, J. (2003) Curr. Opin. Neurobiol. 13, 404–412
33. Chen, C., Nakatani, K., and Koutalos, Y. (2003) J. Physiol. (Lond.) 533, 125–135
34. Pepperberg, D. R., Cornwall, M. C., Kahler, M., Hofmann, K. P., Jin, J., Jones, G. J., and Ripp, H. (1992) Visual Neurosci. 8, 9–18
35. Lyubarsky, A., Nikonov, S., and Pugh, E. N., Jr. (1996) J. Gen. Physiol. 107, 19–34
36. Krispel, C. M., Chen, D., Melling, N., Chen, Y.-J., Martemyanov, K. A., Quillinan, N., Arshavsky, V. Y., Wensel, T. G., Chen, C.-K., and Burns, M. E. (2006) Neuron 51, 409–416
37. Ma, J.-X., Znoiko, S., Othersen, K. L., Ryan, J. C., Das, J., Isayama, T., Kono, M., Oprian, D. D., Corson, D. W., Cornwall, M. C., Cameron, D. A., Harosi, F. I., Makino, C. L., and Crouch, R. K. (2001) Neuron 32, 451–461
38. Hwang, J. Y., Lange, C., Helten, A., Hoppper-Heitmann, D., Duda, T., Sharma, R. K., and Koch, K. W. (2003) Eur. J. Biochem. 270, 3814–3821
39. Tucker, C. L., Woodcock, S. C., Kelsell, R. E., Ramamurthy, V., Hunt, D. M., and Hurley, J. B. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9039–9044
40. Jeon, C. J., Sretto, E., and Masland, R. H. (1998) J. Neurosci. 18, 8936–8946
41. Sitaramayya, A., Harkness, J., Parkes, J. H., Gonzalez-Oliva, C., and Lieberman, P. A. (1986) Biochemistry 25, 651–656
42. Gillespie, P. G., and Beavo, J. A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 4311–4315
43. Koutalos, Y., Nakatani, K., and Yau, K.-W. (1995) J. Gen. Physiol. 106, 891–921
44. Philippov, P. P., Senin, I. I., and Koch, K.-W. (2006) Neuronal Calcium Sensors, pp. 139–151, Nova Science Publishers, New York
45. Bush, R. A., and Makino, C. L. (2006) Neuronal Calcium Sensors, pp. 153–180, Nova Science Publishers, New York
46. Sagoo, M., and Lagnado, L. (1997) Nature 389, 392–395
47. Makino, C. L., Dodd, R. L., Chen, J., Burns, M. E., Roca, A., Simon, M. L., and Baylor, D. A. (2004) J. Gen. Physiol. 123, 729–741
48. Woodruff, M. L., Sampath, A. P., Matthews, H. R., Krasnoperova, N. V., Lem, J., and Fain, G. L. (2002) J. Physiol. (Lond.) 542, 843–854
49. Calvert, P. D., and Makino, C. L. (2002) Adv. Exp. Med. Biol. 514, 37–60
50. Morinondo, A., and Rispoli, G. (2003) Photochem. Photobiol. Sci. 2, 1292–1298
51. Nikonov, S., Engheta, N., and Pugh, E. N., Jr. (1998) J. Gen. Physiol. 111, 7–17
52. Duda, T., Fik-Rymarkiewicz, E., Venkataaraman, V., Krishnan, R., Koch, K.-W., and Sharma, R. W. (2005) Biochemistry 44, 7336–7345