Regulation of Intracellular Cyclic GMP Concentration by Light and Calcium in Electropermeabilized Rod Photoreceptors

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ABSTRACT   This study examines the regulation of cGMP by illumination and by calcium during signal transduction in vertebrate retinal photoreceptor cells. We employed an electropermeabilized rod outer segment (EP-ROS) preparation which permits perfusion of low molecular weight compounds into the cytosol while retaining many of the features of physiologically competent, intact rod outer segments (ROS). When nucleotide-depleted EP-ROS were incubated with MgGTP, time- and dose-dependent increases in intracellular cGMP levels were observed. The steady state cGMP concentration in EP-ROS (0.007 mol cGMP per mol rhodopsin) approached the cGMP concentration in intact ROS. Flash illumination of EP-ROS in a 250-nM free calcium medium resulted in a transient decrease in cGMP levels; this occurred in the absence of changes in calcium concentration. The kinetics of the cGMP response to flash illumination of EP-ROS were similar to that of intact ROS. To further examine the effects of calcium on cGMP metabolism, dark-adapted EP-ROS were incubated with MgGTP containing various concentrations of calcium. We observed a twofold increase in cGMP steady state levels as the free calcium was lowered from 1 μM to 20 nM; this increase was comparable to the behavior of intact ROS. Measurements of guanylate cyclase activity in EP-ROS showed a 3.5-fold increase in activity over this range of calcium concentrations, indicating a retention of calcium regulation of guanylate cyclase in EP-ROS preparations. Flash illumination of EP-ROS in either a 50- or 250-nM free calcium medium revealed a slowing of the recovery time course at the lower calcium concentration. This observation conflicts with any hypothesis whereby a reduction in free calcium concentration hastens the recovery of cytoplasmic cGMP levels, either by stimulating guanylate cyclase activity or by inhibiting phosphodiesterase activity. We conclude that changes in the intracellular calcium concentration during visual transduction may have more complex effects on the recovery of the photoresponse than can be accounted for solely by guanylate cyclase activation.

INTRODUCTION

Visual excitation in vertebrate photoreceptors is initiated upon activation of rhodopsin by light in the outer segment portion of the photoreceptor. Rhodopsin in turns

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activates a photoreceptor-specific G-protein, transducin, which then activates its target enzyme, cGMP phosphodiesterase (PDE). As a result of PDE activation, cytosolic cGMP levels are believed to decrease upon illumination. The lowering of the steady state cGMP concentration then results in a net dissociation of bound cGMP from binding sites on the cGMP-gated ion channel, which causes closure of the ion channels through which sodium and, to a lesser extent, calcium flow. This event is directly responsible for generation of the receptor potential in photoreceptor cells (for recent reviews, see McNaughton, 1990; Kaupp and Koch, 1992; Lagnado and Baylor, 1992; Pugh and Lamb, 1993).

The recovery from visual excitation is less well understood than the excitation pathway itself. During the recovery from illumination, distinct biochemical reactions serve to interrupt the activation sequence at several points, including phosphorylation of rhodopsin, binding of arrestin to phosphorylated rhodopsin, and the intrinsic GTPase activity of transducin (see above reviews for details). In addition, the enzyme responsible for cGMP synthesis, guanylate cyclase (GC), is clearly required in order to restore the cytosolic cGMP concentration to its pre-illumination level (for reviews, see Lolley and Lee, 1990; Stryer, 1991; Kaupp and Koch, 1992; Koch, 1992).

Changes in the rate of turnover of cGMP have been reported in studies of intact retina exposed to illumination (Goldberg, Ames, Gander, and Walseth, 1983; Ames, Walseth, Heyman, Barad, Graeff, and Goldberg, 1986; Dawis, Graeff, Heyman, Walseth, and Goldberg, 1988; Ames and Barad, 1988). Dramatic increases in cGMP turnover in response to illumination are accompanied by little or no change in the measured total cGMP content. These results imply a coordinated regulation of PDE and GC, but the biochemical mechanism by which both PDE and GC activity are modulated remains obscure. This lack of knowledge results in part from the inherent difficulty of measuring both PDE and GC activities simultaneously and under conditions that prevail in the cytosol of ROS during visual transduction.

Decreases in cytosolic calcium concentration in ROS also occur as a result of photoreceptor stimulation (Yau and Nakatani, 1985; McNaughton, Cervetto, and Nunn, 1986; Ratto, Payne, Owen, and Tsien, 1988; Lagnado, Cervetto, and McNaughton, 1992; Younger, McCarthy and Owen, 1992). This decrease is attributed to closure of the cGMP-gated ion channel preventing calcium entry into the outer segment, while the plasma membrane Na-Ca-K exchanger continues to extrude intracellular calcium (for review, see McNaughton, 1990). Extensive physiological evidence implicates calcium as an important intracellular messenger for photoreceptor recovery and adaptation but its exact biochemical roles are not yet known.

One current model for the restoration of cytosolic cGMP levels after photoreceptor illumination hypothesizes that the light-induced decrease in calcium concentration stimulates cGMP recovery by activating GC (for reviews, see Stryer, 1991; Kaupp and Koch, 1992). GC activity can be allosterically regulated by physiologically relevant changes in calcium concentration (Koch and Stryer, 1988; Dizhoor, Ray, Kumar, Niemi, Spencer, Brolley, Walsh, Philipov, Hurley, and Stryer, 1991; Horio and Murad, 1991; Lambrecht and Koch, 1991a, 1991b), although the identity of the calcium regulatory protein(s) that modulates GC activity is unknown at present. There is also biochemical evidence that the lifetime of activated PDE may be regulated in a calcium-dependent manner (Kawamura and Murakami, 1991;

Kawamura, 1993). Calcium has little direct effect on PDE itself (Kawamura and Bownds, 1981; Barkdoll, Pugh, and Sitaramayya, 1989), but rather acts via a calcium regulatory protein that interacts with rhodopsin kinase (Kawamura, 1993). Other sites of negative feedback regulation by calcium to promote the recovery process have been suggested but not substantiated to date (Kaupp and Koch, 1992; Lagnado and Baylor, 1992).

To examine the involvement of calcium in the regulation of cGMP metabolism during visual transduction, we have employed an electropermeabilized rod outer segment (EP-ROS) preparation that retains the morphological integrity of intact ROS while permitting access of low molecular weight solutes to and from the cytosol. The utility of this EP-ROS preparation for studies of other aspects of visual transduction has already been demonstrated (Binder, Biernbaum, and Bownds, 1990; Gray-Keller, Biernbaum, and Bownds, 1990). We show that re-establishment of physiological levels of cGMP can be achieved with the addition of millimolar levels of MgGTP in an external medium containing a calcium buffer. This EP-ROS preparation undergoes light-induced changes in total cGMP concentration when exposed to flash illumination. Dark-adapted EP-ROS also retain the ability to regulate the cGMP steady state and GC activity in response to changes in the free calcium concentration. However, we find that the recovery process is slowed-rather than accelerated-by a fivefold reduction in the buffered free calcium concentration in the EP-ROS medium; this slowing occurs both in the presence or absence of millimolar levels of ATP. We conclude that decreases in free calcium concentration that normally occur in intact ROS during illumination may have effects other than to hasten the recovery of cGMP levels during visual transduction.

MATERIALS AND METHODS

Preparation of Rod Outer Segments

Frogs (*Rana catesbeiana*) were kept on a 12-h light, 12-h dark cycle for at least two weeks before use in experiments, and were fed a vitamin-supplemented, pureed dog food. Retinas were isolated during the dark portion of the daily light-dark cycle. All procedures were performed in darkness using infrared illumination and infrared imaging devices (Electrophysics Corp., Fairfield, NJ). Isolated retinas were briefly rinsed in a Na^+-Ringer's (105 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 10 mM HEPES, pH 7.5) containing 5% Percoll. ROS were obtained by gentle agitation of the retinas in several 0.5 ml portions of Ringer's-5% Percoll solution. The detached ROS were purified from retinal debris and from osmotically disrupted ROS on a discontinuous Percoll gradient containing layers of 5, 30, 44, and 70% Percoll, as described in Biernbaum and Bownds (1985a). Osmotically intact ROS were removed from the 44–70% interface of the Percoll gradient.

For experiments where the cGMP content of osmotically intact ROS was determined (Fig. 4 *A*), purified ROS were mixed with various concentrations of the calcium chelator 1,2-bis (2-aminophenoxy)ethane-*N*,*N*,*N'*,*N'* tetraacetic acid (BAPTA) in Ringer's solution, incubated for 7 min and portions quenched by addition of 25% HCl. The concentrations of calcium and calcium buffer required to obtain a given free calcium concentration were calculated using a computer program (BAD; Brooks and Storey, 1992) that also accounts for metal chelation by nucleotides. The predicted free calcium concentrations obtained by the computer program were verified by measurements of Fluo-3 fluorescence emission at 525 nm after excitation at 495 nm (Minta, Kao, and Tsien, 1989).

For experiments utilizing homogenized cells, purified intact ROS in Percoll were diluted in a pseudo-intracellular medium [77 mM KCl, 35 mM NaCl, 2 mM MgCl$_2$, 5 mM dextran (M$_r$ = 6,000), 10 mM HEPES, pH 7.5], gently pelleted, resuspended in intracellular medium, and homogenized with a Potter-Elvehjem homogenizer. No cellular structure was apparent at the light microscopic level after this treatment.

In each experiment, each experimental condition was assayed in triplicate, and data points represent the results of at least three separate experiments. The concentration of rhodopsin (Rho) was determined spectroscopically by the method of Bownds, Gordon-Walker, Gaide-Huguenin, and Robinson (1971).

Electroporation

Percoll-purified ROS (0.35 ml) were transferred to a pre-cooled, 0.2 cm gap-width electroper-meabilization cuvette. The cell suspension was exposed to 12 square-wave pulses (3750 V/cm, 99-μs duration; BTX Model T-800, BTX, San Diego, CA) to create permanent pores in the plasma membrane of ROS, as described in detail elsewhere (Cote, Hammett, Martin, Arm-strong, Coccia, and Forget, manuscript in preparation). The cells were removed from the cuvette, diluted with intracellular medium, and pelleted gently by centrifugation for 30 s at 1,000 g. (The dextran in the intracellular medium serves to maintain the colloid osmotic pressure across the EP-ROS plasma membrane and prevents swelling.) The pellet was resuspended in pseudo-intracellular medium, and the cell suspension incubated for 20 min at room temperature in total darkness to permit the loss of > 95% of the endogenous nucleotides. The efficacy of electropermeabilization was ascertained by epifluorescent light microscopy of EP-ROS cells incubated with the membrane-impermeant dye, didansylcysteine (Yoshikami, Robinson, and Hagins, 1974); in all experiments, > 80% of the total EP-ROS population were made permeable to the fluorescent dye by the electropermeabilization conditions cited above. The morphology of EP-ROS, as judged by phase contrast microscopy, was indistinguishable from cell suspensions which had not undergone the electropermeabilization procedure.

Exposure of EP-ROS and Homogenized ROS to Test Solutions and Illumination

Nucleotide-depleted, dark-adapted EP-ROS were gently mixed with an equal volume of the pseudo-intracellular medium containing 2× concentrated nucleotides, CaCl$_2$, and calcium buffer; both EGTA and BAPTA were used with no difference in results. In the experiments where EP-ROS were exposed to flash illumination, we added a GTP-regenerating system (2 mM phosphoenolpyruvate and 2 mM GDP) to the external medium to help maintain constant GTP levels in the cytosol of EP-ROS. This strategy relies on the observation that ROS pyruvate kinase (Lopez-Escalera, Li, Szerencsi, and Schnetkamp, 1991) is the major route of GTP synthesis in EP-ROS (Cote et al., manuscript in preparation). The presence of the GTP-regenerating system had no significant effect on the steady state cGMP levels of dark-adapted EP-ROS incubated with 5 mM MgGTP and 250 nM free calcium (0.006 ± 0.001 mol cGMP per mol Rho; n = 14).

Incandescent room lighting was employed for procedures requiring saturating continuous illumination of EP-ROS. Flash illumination (2-ms duration) was delivered by a photographic flash unit attached to a set of neutral density filters to attenuate the intensity of the flash. The EP-ROS suspensions were loaded onto the centrifugal separation tubes, and the flash unit positioned above a microcentrifuge. This permitted rapid centrifugation after the flash stimulus was presented to EP-ROS. The intensity of the light stimulus was determined by spectroscopi-cally measuring the extent of bleaching of Rho in ROS suspensions exposed to multiple flashes. The intensity is reported as the number of Rho isomerizations per ROS, assuming 3 × 10^9 Rho molecules per frog ROS (Liebman and Entine, 1968).

Centrifugal Separation Method

After exposure of EP-ROS to test solutions, the intracellular concentration of cGMP was determined by partitioning the EP-ROS from the external medium by centrifugation (12,000 g for 1 min) through silicone oil into acid (Forget, Martin, and Cote, 1993). The 0.4 ml polyethylene microcentrifuge tubes were prepared by layering 150 μl of silicone oil on top of 100 μl of 25% HCl. The silicone oil (ρ = 1.028 g/ml) was prepared by mixing 5 vol of Dow Corning DC550 (ρ = 1.07 g/ml) with 1 part of Dow Corning DC200 (ρ = 0.818 g/ml). At this density of oil, EP-ROS sedimented to the acid layer while the pseudo-intracellular medium was retained on top of the oil. Greater than 95% of the EP-ROS passes through the oil layer within 10 s after the onset of centrifugation, as judged by the recovery of Rho in the bottom of the tube.

Immediately after centrifugation, the tubes were placed in a dry ice-ethanol bath. The tubes were cut near the oil/acid interface, and the acid layer and membrane pellet removed. After centrifugation to pellet precipitated material, the acid supernatant was dried down under vacuum in preparation for radioimmunoassay.

In experiments not utilizing the centrifugal separation technique, cell suspension aliquots were quenched by addition of 25% HCl in water. As in the centrifugal separation case, precipitated cell debris was pelleted by centrifugation before analysis of the supernatant by radioimmunoassay.

GC Assay

Nucleotide-depleted EP-ROS or homogenized ROS were pre-incubated with 250 μM zaprinast (M&B 22948, a specific inhibitor of the retinal cGMP PDE [Gillespie and Beavo, 1989]) for 5 min. To initiate the reaction, the suspension was mixed with a mixture of nucleotides and BAPTA (final concentration: 500 μM MgGTP, 100 μM ATP, and 1 mM BAPTA) along with various amounts of calcium to yield free calcium concentrations of 20, 100, 500, or 1000 μM. A lag period was typically observed (< 15 s) before the onset of cGMP formation. Aliquots of the final suspension were quenched in 25% HCl at 20, 25, and 30 s, and cGMP levels determined by radioimmunoassay. Initial rates were determined by linear regression. Experiments where [^{3}H]cGMP was added in order to quantitate PDE activity showed negligible hydrolysis (< 5%) of [^{3}H]cGMP over the time course of the reaction.

cGMP Radioimmunoassay

Radioimmunoassay reagents were added to the dried down samples in the following order: 100 μl 50 mM citrate buffer (pH 6.2), 100 μl of 2'-O-succinyl [^{125}I]iodotyrosine methyl ester cGMP (10^4 DPM), 100 μl of rabbit anti-cGMP antiserum (10^4 dilution), and 10 μl of normal rabbit serum (13 mg/ml). After overnight incubation at 4°C, bound cGMP was separated by isopropanol precipitation of the antibody followed by centrifugation at 4,900 g for 25 min at 4°C. The precipitate was counted in a gamma counter, and unknowns compared to a cGMP standard curve to determine the cGMP content of the sample.

HPLC Analysis of Guanine Nucleotides

EP-ROS samples were quenched by centrifugal separation into 5% perchloric acid. The acid layer was removed from the oil and cellular debris, and neutralized by treatment with Alamine 336© (a mixture of tri-n-octylamine and tri-n-decylamine) in chloroform (Khym, 1975). The aqueous layer was removed and injected on a Perkin Elmer C-18 column. The separation was performed under isocratic conditions with a mobile phase of 150 mM KH$_2$PO$_4$, 10 mM tetrabutyl ammonium hydrogen sulfate (an ion-pairing reagent), and 4% methanol.

Materials

Frogs were obtained by Niles Biologicals (Sacramento, CA). Electropermeabilization cuvettes were from BTX Corp. (San Diego, CA). All reagents were of the highest purity available and purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Silicone oils were obtained from William F. Nye Co. (New Bedford, MA). Alamine was from Henkel Corporation (Kankakee, IL). cGMP antiserum was purchased from Chemicon (El Segundo, CA). Radio-chemicals were from Dupont-New England Nuclear (Boston, MA).

R E S U L T S

To examine the regulation of steady state cGMP levels during visual transduction, we have developed an electropermeabilized ROS (EP-ROS) preparation that sustains physiological levels of cGMP when exogenous MgGTP is provided (Fig. 1). In order to control the intracellular milieu, EP-ROS were routinely incubated at room temperature in the dark for 15–20 min to allow endogenous nucleotides to either diffuse from the cytosol or be degraded by hydrolytic activities. After this incubation, cGMP levels inside EP-ROS were reduced by >90% (Cote and Brunnock, 1993). GTP and ATP levels inside EP-ROS also decreased by >95% during this incubation (data not shown).

Fig. 1 shows the time course of the net increase in total intracellular cGMP levels in nucleotide-depleted EP-ROS that were incubated with MgGTP. When EP-ROS are maintained in a dark-adapted state, 5 mM MgGTP addition restores the intracellular cGMP concentration to a level characteristic of purified, intact ROS (Table I) within several minutes. Recent work indicates that ~90% of this newly synthesized cGMP is likely to become bound to both high and moderate affinity cGMP binding sites present in the ROS (Cote and Brunnock, 1993).

Illumination of dark-adapted EP-ROS after a 10 min exposure to MgGTP results in a rapid, twofold decrease in total cGMP levels (Fig. 1, *dotted line*). The fact that the total cGMP content of EP-ROS drops only 50% may reflect cGMP compartmentation by high affinity cGMP binding sites in the outer segment (Cote and Brunnock, 1993) that could prevent hydrolysis of this bound pool of cGMP. This result also may be indicative of a change in the steady state PDE and GC activities in light-adapted EP-ROS, leading to a new steady state concentration of cGMP.

Exposure of nucleotide-depleted EP-ROS to saturating illumination before addition of MgGTP reduces the rate of the GTP-induced cGMP increase 10-fold (Fig. 1, *dashed line*). This time-dependent increase in cGMP levels observed under light-adapted conditions demonstrates that GC activity is capable of net synthesis of cGMP in the presence of light-activated PDE.

Fig. 2 shows that the total cGMP concentration attained by nucleotide-depleted EP-ROS incubated in a 100 nM free calcium medium increases as the MgGTP concentration is increased. At MgGTP concentrations less than 1 mM, the cGMP content is approximately constant at 0.004–0.005 mol cGMP per mol Rho. The constancy of the cGMP concentration over this range of MgGTP concentrations probably reflects cGMP bound to high affinity cGMP binding sites within the EP-ROS (binding site density, 0.005 mol cGMP per mol Rho; Cote and Brunnock, 1993). These high affinity cGMP binding sites dissociate bound cGMP slowly and are likely to protect newly synthesized cGMP from hydrolysis. At MgGTP concentrations >1 mM, the steady state cGMP concentration of EP-ROS increases to a maximum of
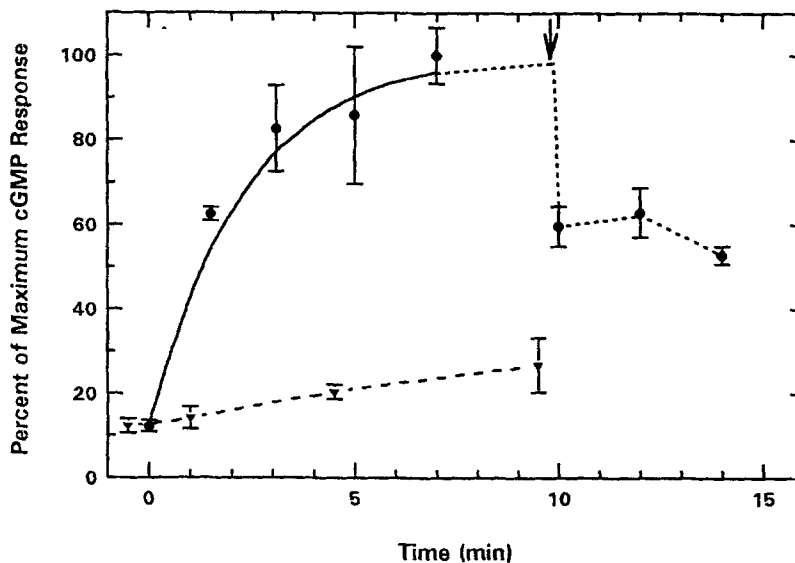
FIGURE 1.   Time course of stimulation of cGMP levels by MgGTP in dark-adapted and illuminated EP-ROS. Purified ROS were electropermeabilized as described in *Materials and Methods*, and depleted of endogenous nucleotides (0.0007 mol cGMP per mol Rho). At time zero, 5 mM MgGTP with 100 nM free calcium (final concentration) was added to either dark-adapted EP-ROS (*circles*) or to EP-ROS previously exposed to saturating, continuous illumination (*triangles*). At the indicated times, triplicate samples were quenched by the centrifugal separation method (see *Materials and Methods*), and the cGMP concentration inside EP-ROS determined by radioimmunoassay. The arrow represents the time at which dark-adapted EP-ROS were exposed to saturating continuous illumination. Data points represent the mean ± SD for an individual experiment ([Rho] = 4 μM), where the maximum cGMP response was 0.006 mol cGMP per mol Rho. Similar results were observed in two additional experiments, and the average dark time course for the 3 experiments is shown as the continuous line. Regression analysis of the light-exposed time course (*triangles*) shows a statistically significant increase in cGMP at the $P < 0.05$ level.

TABLE I

Summary of ROS Intracellular Cyclic GMP Concentrations under Various Conditions

ROS Preparation	Intracellular [MgGTP]	Intracellular [Ca^{2+}]$_{free}$	Total [cyclic GMP]
	mM	*nM*	*mol cGMP per mol Rho*
EP-ROS	0.5	100	0.005 ± 0.001
		250	0.004 ± 0.001
	5.0	100	0.007 ± 0.001
		250	0.007 ± 0.001
Intact ROS	0.5*	100–200‡	0.007 ± 0.001
Intact ROS-IS	1.8*	100–220‡	0.010 ± 0.001§

*From Biernbaum and Bownds (1985a).

‡The two values are estimates based on the work of Ratto et al. (1988) and Younger et al. (1992).

§Data for cyclic GMP concentration in ROS-IS (ROS attached to the ellipsoid portion of the inner segment) taken from Cote et al. (1984).

0.007 mol cGMP per mol Rho, a value equal to that of intact ROS and only 30% less than that obtained for preparations of metabolically active ROS still attached to the inner segment (Table I). The predicted MgGTP concentration giving one-half of the maximal cGMP response is approximately 200 μM. These data agree with the K_M values reported for purified photoreceptor GC (Hakki and Sitaramayya, 1990; Hayashi and Yamazaki, 1991; Koch, 1991), as well as with the reported dose dependence of the membrane current on GTP concentration in truncated ROS (Kawamura and Murakami, 1989).

Fig. 3 demonstrates that flash illumination of EP-ROS pre-incubated with 5 mM MgGTP (and a GTP-regenerating system; see Materials and Methods) resulted in a
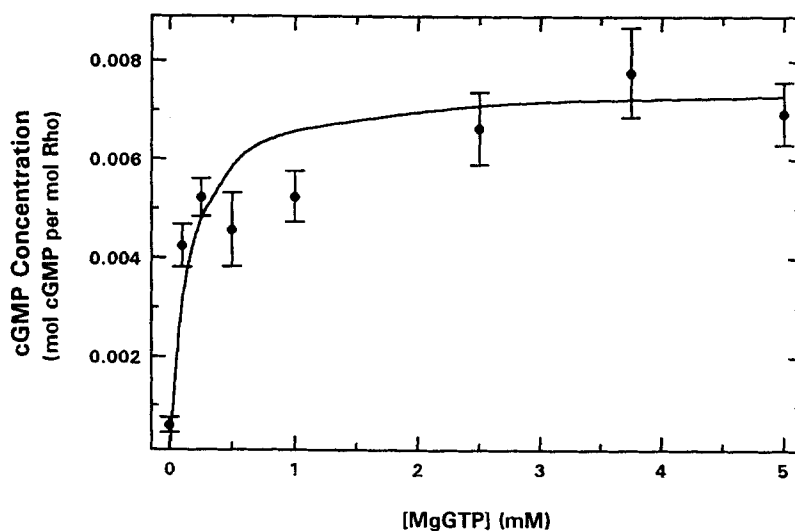

FIGURE 2. Dose dependence of MgGTP on the steady state levels of cGMP in EP-ROS. Dark-adapted, nucleotide-depleted EP-ROS were mixed with solutions containing various MgGTP concentrations and incubated for 8 min. Samples were quenched in triplicate and analyzed as described in *Materials and Methods*. The data points represent the mean ± SEM from three experiments where the free calcium concentration was 100 nM and where the Rho concentration in individual experiments ranged from 11 to 15 μM.

transient decrease in cGMP levels that was restored within 20 s. The EP-ROS were suspended in a medium containing 250 nM free calcium, a value similar to that measured for the dark adapted frog retina (Ratto et al. 1988). A flash isomerizing 1,170 Rho per ROS caused a 17 ± 4% (mean ± SEM, n = 5) decrease within 5 s after the stimulus. The cGMP response is restored to 97% of its pre-illumination steady state value within 20 s after flash illumination; this value is not statistically different from the cGMP concentration of dark-adapted EP-ROS. This kinetic behavior is similar to the flash response of purified ROS still attached to the mitochondria-rich portion of the inner segment and incubated in a 20 nM external calcium medium (Fig. 3, *dotted line;* taken from Cote, Nicol, Burke, and Bownds, 1989, Fig. 1). Unlike

the cGMP response in intact ROS, the response of EP-ROS shown in Fig. 3 occurred in the absence of changes in free calcium concentration that normally accompany visual excitation in intact ROS. This result suggests that both the initiation and termination of the cGMP response in EP-ROS do not require changes in calcium concentration.

To rule out changes in cytosolic GTP concentration as an explanation for the time course of the cGMP changes shown in Fig. 3, we monitored the intracellular GTP concentration in EP-ROS during the light response using the centrifugal separation
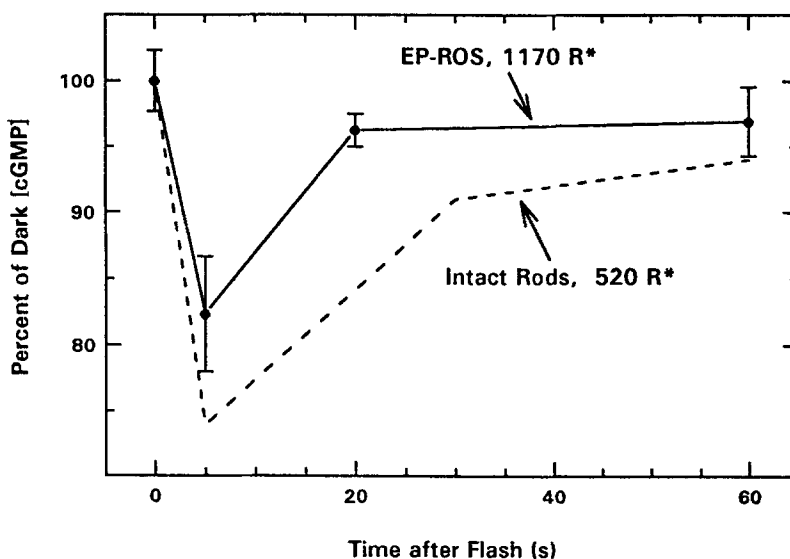

FIGURE 3. Dim flash illumination causes a transient decrease in cGMP concentration in EP-ROS. Nucleotide-depleted EP-ROS ([Rho] = 5–15 μM) were incubated for 5 min in a medium containing (final concentrations): 5 mM MgGTP, 2 mM GDP, and 2 mM phosphoenolpyruvate. The calcium concentration of the solution was buffered at 250 nM. A portion of the EP-ROS suspension was exposed to a 2-ms flash isomerizing 1170 Rho per rod. Samples were quenched in triplicate at the indicated times. Data points represent the mean ± SEM for five individual experiments. The dashed line represents data for osmotically intact outer segments with attached inner segments that were incubated in a 20-nM calcium medium and exposed to a flash bleaching 520 Rho per rod (taken from Cote et al., 1989; Fig. 1).

technique and HPLC analysis of guanine nucleotides. We observed a <10% change in GTP concentration during the minute after illumination (data not shown). This result is consistent with the data of Biernbaum and Bownds (1985b) who reported no change in GTP concentration for intact photoreceptors exposed to a comparable intensity of illumination.

To examine the role of calcium in the maintenance of the steady state cGMP concentration in dark-adapted EP-ROS, we incubated EP-ROS in solutions containing 500 μM MgGTP and various free calcium concentrations. The data in Fig. 4 A
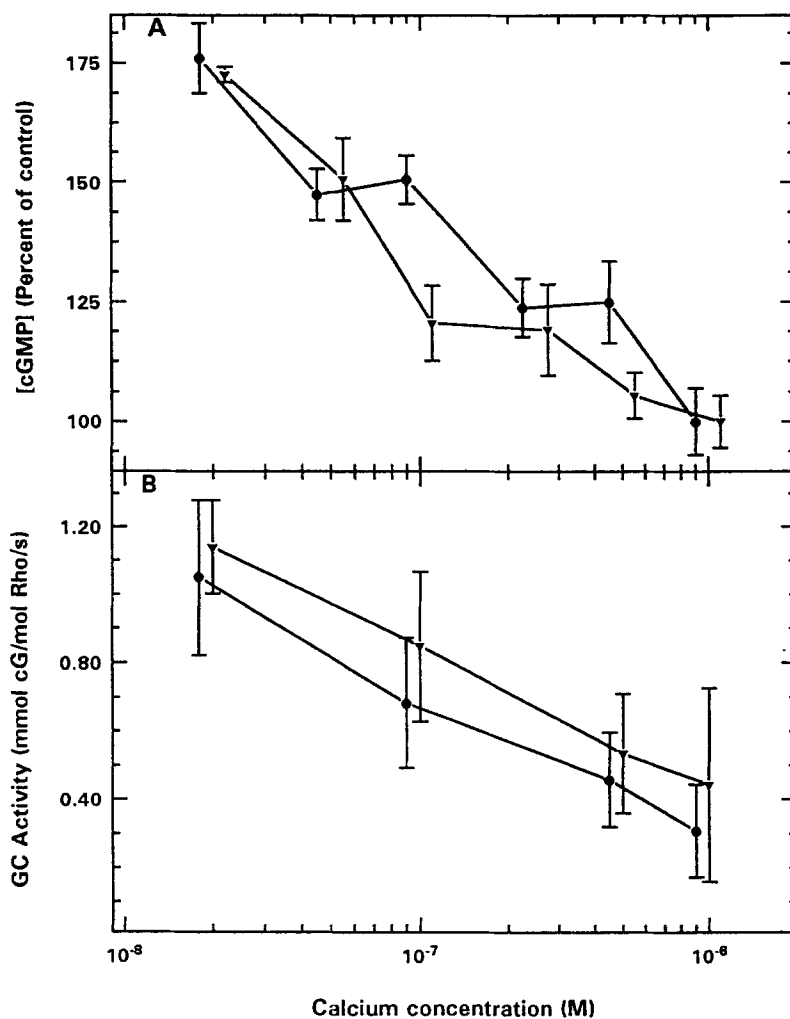
FIGURE 4. Changes in free calcium concentration modulate changes in both total cGMP content and in GC activity. (*A*) Nucleotide-depleted, dark-adapted EP-ROS (*circles*) were incubated with 500 μM MgGTP and various amounts of EGTA or BAPTA to yield free calcium concentrations between 20 nM and 1 μM. After a 5-min incubation, triplicate samples were centrifugally separated and cGMP levels determined. Osmotically intact ROS (*triangles*) were incubated for 7 min at the indicated calcium concentrations, and portions quenched by addition of acid. The cGMP concentration at 1 μM external calcium was defined as 100%, and was 0.007 ± .001 mol cGMP per mol Rho in intact ROS, and 0.0032 ± .0002 mol cGMP per mol Rho in EP-ROS. [This twofold difference in cGMP levels reflects the fact that a sub-saturating GTP concentration was used in the EP-ROS experiments; a GTP concentration near the K_M for GC was chosen to optimize detection of GC regulation by calcium.] The data

show a gradual 1.7-fold increase in the total cGMP concentration as the free calcium concentration is lowered from 1 μM to 20 nM. This same increase in cGMP content is also observed in intact ROS as the calcium concentration is lowered (Fig. 4 *A*, *triangles*). (Note that the cytosolic free calcium concentration is not known for intact ROS, and the abscissa in Fig. 4 *A* reflects in this instance the *external* calcium concentration.) These results with intact and EP-ROS are consistent with previous studies of isolated ROS which found a 1.5–3-fold increase in total cGMP levels upon lowering the external calcium concentration (Woodruff and Bownds, 1979; Polans, Kawamura, and Bownds, 1981; Cote, Biernbaum, Nicol, and Bownds, 1984). The excellent correlation between calcium-induced changes in total cGMP concentration in EP-ROS and in intact ROS supports the idea that EP-ROS retain calcium regulatory proteins believed to be involved in cGMP metabolism in intact rod photoreceptors.

To determine whether the calcium effect on total cGMP concentration could be ascribed to calcium-induced activation of GC, we directly determined the GC activity in EP-ROS under conditions where PDE activity was negligible. We incubated EP-ROS with the specific PDE inhibitor, zaprinast; ATP was also included because it has been reported that a calcium-sensitive activator of GC has a greater effect when phosphorylated (Lambrecht and Koch, 1991*b*). Fig. 4 *B* shows that GC activity in EP-ROS is stimulated 3.5-fold when the free calcium concentration is lowered from 1 μM to 20 nM. The lack of a plateau region at either the lowest or higher calcium concentrations in Fig. 4 *B* suggests that the observed extent of activation represents a minimum value.

To examine whether some of the calcium-dependent GC activator protein may diffuse from the EP-ROS (and thus be unable to regulate GC activity in electropermeabilized cells), we compared the GC activity of EP-ROS with that of homogenized ROS. Fig. 4 *B* shows that homogenized ROS (*triangles*) exhibit a similar dependence of GC activity on calcium concentration, with no statistically significant difference in enzyme rates for the two ROS preparations. We conclude that EP-ROS retain the same extent of calcium regulation of GC activity as do homogenized ROS preparations in which calcium regulatory proteins presumably have complete access to the enzyme.

One proposed mechanism for the recovery of the photoresponse purports that the light-induced decrease in free calcium concentration stimulates GC activity and promotes more rapid recovery of the pre-illumination steady state cGMP levels in ROS (see *Introduction*). The EP-ROS preparation permitted us to add calcium buffer to the medium and thereby buffer the free calcium concentrations in the cytosol to test the effect of calcium on the flash response. If the above model of how calcium controls the rate of recovery is correct, we predicted that buffering the free calcium

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represent the mean ± SEM for six experiments for EP-ROS ([Rho] = 6–30 μM) or three experiments for intact ROS ([Rho] = 8–12 μM). (*B*) Nucleotide depleted, dark-adapted EP-ROS (*circles*) or homogenized ROS (*triangles*) were incubated with zaprinast to inhibit PDE activity, then mixed with nucleotides at various free calcium concentrations to initiate the GC reaction (see *Materials and Methods*). The data points represent the mean (±SEM) rate of cGMP synthesis (normalized for Rho content) where the [Rho] = 8–15 μM.

concentration in EP-ROS at a lower value would increase GC activity and thereby reduce the amplitude of the response and accelerate the recovery kinetics. Fig. 5 A demonstrates that reducing the free calcium concentration from 250 nM to 50 nM slows the response of cGMP to flash illumination, with only a minor effect on the response amplitude. In the dark, the steady state cGMP concentration of EP-ROS exposed to 5 mM MgGTP and 50 nM free calcium was 0.010 ± 0.002 mol cGMP per mol Rho. The light-induced decrease of EP-ROS in 50 nM free calcium was 24 ± 6% (n = 5), slightly greater than the response in 250 nM free calcium. The recovery of cGMP levels after flash illumination is significantly slower in 50 nM compared to 250 nM free calcium. This is the opposite of the predicted behavior if lowering free calcium were to accelerate the recovery phase of the response to flash illumination.

The experiments in Fig. 5 A were performed with EP-ROS that had been depleted of endogenous nucleotide (including ATP) and subsequently supplied only with MgGTP. It was possible that the lack of ATP could prevent calcium-sensitive processes involving GC or PDE from occurring (Lambrecht and Koch, 1991b; Kawamura, 1993). We therefore compared the cGMP response to flash illumination of EP-ROS incubated in the presence or absence of 1 mM ATP. Fig. 5 B shows that inclusion of ATP had no statistically significant effect on the cGMP response of EP-ROS maintained in a 50 nM calcium solution. One possibility is that ATP is not involved in the cGMP recovery kinetics at the intensity of flash illumination used in these experiments (1,170 isomerizations per ROS). Alternatively, residual adenine nucleotides remaining after depletion of >95% of endogenous adenine nucleotides in EP-ROS may be sufficient to carry out ATP-dependent events during the recovery process.

We conclude that the results obtained in Fig. 5 are inconsistent with the idea that lowering the calcium concentration in the cytosol accelerates the recovery of cGMP levels. The fact that lowering calcium accelerates cGMP synthesis by GC (Fig. 4) implies that calcium must be regulating some other aspect of cGMP metabolism and/or binding in order to result in a net slowing of cGMP recovery in EP-ROS after flash illumination.

DISCUSSION

In this paper we present the use of a novel EP-ROS preparation dialyzed of intracellular metabolites to study the effects of illumination and calcium on cGMP metabolism. This gently permeabilized cell preparation retains the ability to carry out visual excitation in response to dim flashes (i.e., less than 1 Rho molecule isomerized per disk membrane per ROS; Fig. 3). EP-ROS also retain the functional components to terminate the excitation pathway by restoring the light-induced cGMP decrease within 20–60 s (depending on the calcium concentration; Fig. 5 A). We have employed the EP-ROS preparation to introduce exogenous calcium buffers to control the intracellular calcium concentration, and find that cGMP metabolism (and GC in particular) responds to changes in free calcium concentration (Fig. 4). However, contrary to predictions of the currently prevalent model by which calcium is thought to regulate the recovery of cGMP levels after illumination, we find that lowering intracellular calcium causes a slowing of the recovery of cGMP concentration to its dark-adapted level (Fig. 5 A).
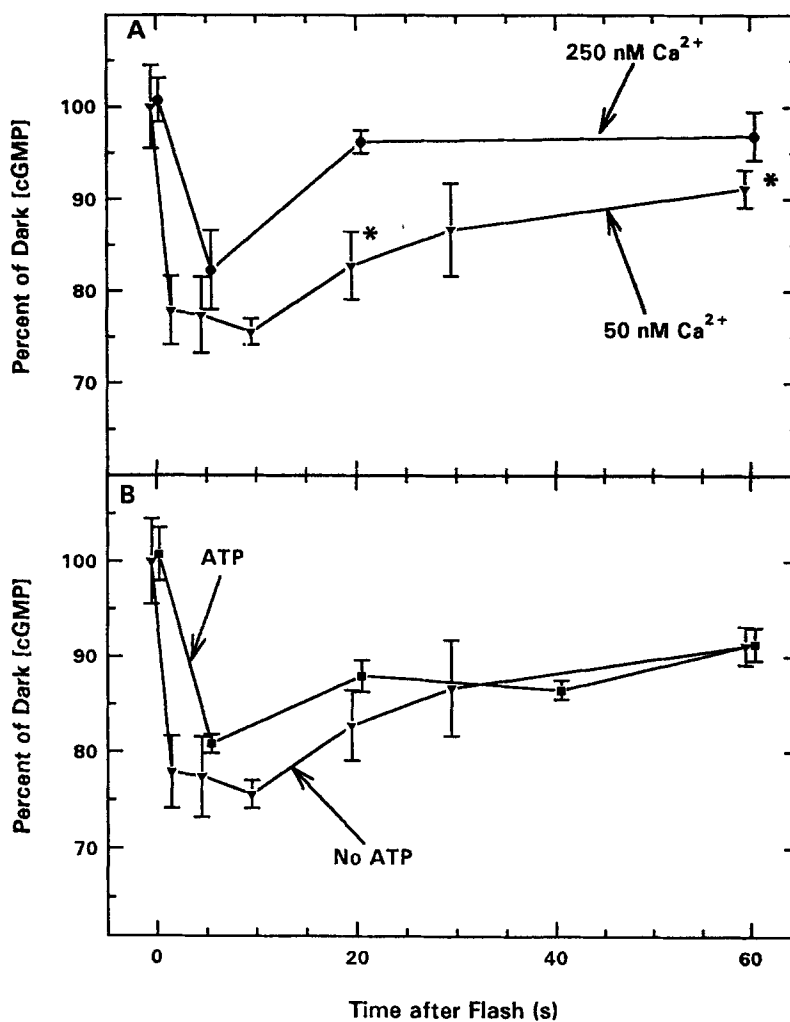
FIGURE 5.   Lowering the free calcium concentration to 50 nM slows the recovery of the cGMP response to flash illumination in EP-ROS. (A) EP-ROS were incubated with 5 mM MgGTP and either 250 nM (circles; from Fig. 3) or 50 nM (triangles) free calcium, as described in the legend to Fig. 3. The data points for the 50 nM calcium condition ([Rho = 4–11 μM) represent the mean (±SEM) for five experiments. Asterisks indicate a statistically significant difference in cGMP levels (compared to the corresponding value at 250 nM calcium) at the $P < 0.05$ level of confidence, as determined by a t test. (B) EP-ROS were incubated for 10 min in 50 nM free calcium and 5 mM MgGTP in the presence (squares) or absence (triangles; from Fig. 5 A) of 1 mM ATP before illumination. The results in the presence of ATP represent the mean of 4 experiments ([Rho] = 13–17 μM).

The experiments presented in this paper use a novel electropermeabilized photoreceptor preparation that is internally dialyzed of low molecular weight compounds. This allows the intracellular environment to be defined in terms of nucleotides and other solutes made available to the components of the signal transduction pathway in the ROS. When supplied with physiological levels of MgGTP, net cGMP synthesis occurs in the outer segment until a new steady state is reached that is characteristic of the cGMP content of osmotically intact, purified ROS (Table I). The use of a centrifugal separation procedure (see *Materials and Methods*) makes it possible to restrict our analysis to intracellular nucleotides in EP-ROS.

The relatively slow time course of restoration of cGMP concentration in EP-ROS after MgGTP addition (Fig. 1) is not completely understood at present. One partial explanation is that diffusion of MgGTP to the site of cGMP synthesis in the EP-ROS may be rate limiting. Restricted influx across the electropermeabilized plasma membrane, hindered longitudinal diffusion introduced by the disk membrane structure, or cytoplasmic buffering by nucleotide binding sites could all slow substrate access to the catalytic site (for discussion, see Pugh and Lamb, 1993). However, in our measurements of GC activity in EP-ROS reported in Fig. 4 B, we typically observed a lag time under 15 s before the onset of cGMP production. Because of the slow, gentle mixing needed to prevent breakage of EP-ROS, this lag period represents an upper estimate for the extent of hindered diffusion of GTP to GC in the EP-ROS preparation. This indicates a relatively rapid access of GTP to the site of cGMP synthesis in EP-ROS.

A more likely explanation for the slow attainment of the cGMP steady state in Fig. 1 is the following scenario: nucleotide-depleted, dark-adapted EP-ROS are likely to initially have a relatively high PDE activity due to partial dissociation of the inhibitory γ subunits resulting from loss of bound cGMP from high-affinity, noncatalytic binding sites on PDE (Arshavsky, Dumke, and Bownds, 1992). Addition of MgGTP to EP-ROS results in cGMP synthesis, some of which is immediately hydrolyzed by PDE, while some cGMP binds to the high affinity sites. cGMP binding to PDE promotes PDE γ subunit reassociation (Arshavsky et al., 1992), thus gradually reducing PDE activity to its basal level. The relatively slow attainment of the cGMP steady state in Fig. 1 might then reflect primarily the time course of reduction of PDE activation in the presence of a constant level of GC activity. Further evidence for significant PDE activity in nucleotide-depleted EP-ROS can be obtained by noting that the rate of increase in total cGMP concentration (~25 μM within the first min with [GTP] = 5 mM; Fig. 1) is an order of magnitude less than the observed GC rate of EP-ROS in a 100 nM calcium medium (300 μM cGMP per min; see below).

Compartmentation of cGMP by binding to specific binding sites may play a major role in understanding the regulation of cGMP metabolism by light and calcium. Continuous illumination of dark-adapted EP-ROS pre-incubated with MgGTP results in a rapid 50% decrease in cGMP concentration followed by a much slower continued decline (Fig. 1). This result is consistent with the presence of a class of high affinity cGMP binding sites (K_D = 60 nM, site density = 0.005 mol cGMP per mol Rho; Cote and Brunnock, 1993) which dissociates bound cGMP slowly, and thus protects this cGMP pool from PDE hydrolysis.

The dose-response data in Fig. 2 can also be interpreted from the perspective of

cGMP compartmentation in the ROS. Note that for MgGTP concentrations below 1 mM, the amount of intracellular cGMP in EP-ROS is essentially constant at a value of ~0.005 mol cGMP per mol Rho. This value is equal to the ROS site density of high affinity cGMP binding sites cited above. The lack of a concentration dependence at low GTP concentrations might result from newly synthesized cGMP titrating unoccupied cGMP binding sites and thus being protected from hydrolysis. The additional increase in cGMP concentration seen at GTP concentrations greater than 1 mM would then represent the sum of cGMP bound to moderate affinity sites (Cote and Brunnock, 1993) and free cytoplasmic cGMP within the ROS. It is presumably this latter pool of cGMP which is metabolically active on the time scale of visual excitation in ROS.

One of the most important aspects of the EP-ROS preparation is the ability to control the ionic composition of the cytosol. Due to the permanent pores in the plasma membrane resulting from electropermeabilization, it is possible to introduce millimolar levels of calcium buffer into the cytosol and to eliminate transmembrane ionic gradients that are responsible for the circulating membrane current of photoreceptor cells. Entry of the calcium buffer BAPTA or EGTA into the cytosol of EP-ROS is inferred to effectively control the free calcium concentration based on the following considerations: (*a*) molecules of similar size and charge (such as MgGTP [this paper], as well as didansylcysteine, sorbitol, and a variety of high energy phosphates [Cote et al., manuscript in preparation]) do freely enter EP-ROS. (*b*) The similar calcium dependence of GC activity for homogenized and EP-ROS (Fig. 4 *B*) indicate that the intracellular calcium concentration of EP-ROS is similar to that of the external medium. (*c*) The millimolar levels of calcium buffer used in our experiments exceeds by ~10-fold the endogenous calcium buffering capacity of ROS (see Kaupp and Koch, 1992, for review). The presence of pores in the plasma membrane of EP-ROS also will serve to equilibrate intracellular and extracellular ion concentrations. Without transmembrane ionic gradients there can be no net transport of calcium into or out of EP-ROS, since the driving force for operation of the Na-Ca-K exchanger is eliminated. Thus, the light-induced decrease in calcium concentration normally mediated by the Na-Ca-K exchanger in intact photoreceptors will be absent in EP-ROS.

To better understand what role calcium plays in regulating cGMP metabolism during visual transduction, we examined the effects of altering the calcium concentration in EP-ROS on total cGMP levels, GC activity, and the cGMP response to flash illumination. The total cGMP content of EP-ROS shows a modest 1.7-fold increase over the range of 1 μM to 20 nM free calcium in the medium. This calcium effect is comparable to the effect of lowering the external calcium concentration on intact ROS (Fig. 4 *A*), suggesting that cGMP metabolism in EP-ROS and intact ROS was responding similarly to changes in calcium concentration.

The 3.5-fold increase in GC activity in lowering the calcium concentration from 1 μM to 20 nM (Fig. 4 *B*) must be accompanied by an increase in cGMP hydrolysis in order to account for the 1.7-fold increase in the steady state cGMP concentration of EP-ROS (Fig. 4 *A*). An increase in PDE activity at lower calcium concentrations is expected simply as a consequence of the increased cGMP concentration as the calcium is lowered. (PDE will hydrolyze cGMP with first-order kinetics because

[cGMP] < < K_M of PDE [reviewed by Pugh and Lamb, 1993]. Thus, an increase in cGMP concentration would cause a proportionate increase in the PDE hydrolytic rate.) Thus, the 1.7-fold increase in total cGMP concentration in EP-ROS is consistent with an equivalent increase in PDE activity accompanying the 3.5-fold increase in GC activity.

The rate of cGMP synthesis by GC in frog EP-ROS compares favorably to previous estimates of cGMP metabolic flux in intact amphibian photoreceptors. At 1 μM free calcium concentration, the rate of cGMP synthesis in EP-ROS or homogenized ROS is approximately 0.4 mmol cGMP per Rho per second at a MgGTP concentration of 500 μM (Fig. 4 B). Assuming Michaelis-Menten kinetics and a K_M for frog GC equal to the K_M for the major isoform of toad GC (160 μM; Hayashi and Yamazaki, 1991) we calculate a predicted maximum rate of 0.5 mmol cGMP synthesized per mol Rho per second. Referenced to the cytoplasmic concentration of Rho in ROS (6 mM; Liebman and Entine, 1968), the rate of cGMP synthesis in EP-ROS is predicted to be ~3 μM cGMP per second with millimolar levels of MgGTP and a free calcium concentration of 1 μM. At a free calcium concentration of 100–250 nM (thought to be the resting concentration of dark-adapted rod photoreceptors; Ratto et al., 1988; Younger et al., 1992), the rate of cGMP synthesis in EP-ROS is estimated to be 4–5 μM per second. These values are in general agreement with biochemical (2 μM cGMP per second; Dawis et al., 1988) or electrophysiological (3 μM cGMP per second; Hodgkin and Nunn, 1988) estimates of cGMP metabolic flux in dark-adapted amphibian photoreceptors (for discussion, see Pugh and Lamb, 1990). However, it is also possible that the somewhat higher rate of cGMP synthesis we observe in EP-ROS and disrupted ROS is significant, and reflects an artifactual elevation of the rate of cGMP synthesis compared to intact photoreceptors.

The value of the basal rate of GC activity in EP-ROS (0.5 mmol cGMP per Rho per second; defined at 1 μM free calcium concentration and saturating levels of MgGTP) is comparable to GC activity from disrupted toad ROS (Hayashi and Yamazaki, 1991) but 2–3-fold lower than basal GC rates in bovine ROS (Koch and Stryer, 1988; Dizhoor et al., 1991; Lambrecht and Koch, 1991a,b). The higher GC rates in mammalian rod photoreceptors may represent a biochemical correlate to physiological differences in the kinetics of the electrical response between mammalian and amphibian rod photoreceptors.

The 3.5-fold extent of activation of frog GC activity as the calcium concentration is lowered from 1 μM to 20 nM (Fig. 4 B) differs from previous estimates of the effects of calcium on GC activity. It should be first mentioned that the 3.5-fold extent of activation we observe for frog GC represents a minimum value; the lack of a plateau region at either end of the range of calcium concentrations we tested suggests that an additional stimulation might be observed at calcium concentrations lower than 20 nM and/or an additional decrease in GC activity might be seen at concentrations greater than 1 μM. Wide variations in the reported in vitro stimulation of bovine GC by calcium—from a low of twofold (Horio and Murad, 1991), to values of three and fivefold comparable to this study (Koch and Stryer, 1988, Fig. 1 A; Dizhoor et al., 1991; Lambrecht and Koch, 1991a, Fig. 4 C; Lambrecht and Koch, 1991b), to greater than 10-fold stimulation (Koch and Stryer, 1988, Figs. 2 and 3; Lambrecht and Koch, 1991a, Fig. 4 A)—make it difficult to assess the relative calcium sensitivity

of the amphibian and bovine enzymes. Physiological measurements of net cGMP synthesis in truncated frog photoreceptors demonstrate that lowering the calcium concentration from 1 µM to 20 nM elevates cGMP levels approximately fivefold (Kawamura and Murakami, 1989, Fig. 8), also in general agreement with the results of Fig. 4 B.

It is generally accepted that visual excitation results in a lowering of the cytosolic free calcium concentration in ROS. It has been hypothesized that cGMP synthesis is stimulated by the allosteric activation of GC resulting from this decrease in calcium concentration. Recent evidence also implicates calcium regulatory proteins in reducing the activation and lifetime of light-activated PDE upon a lowering of calcium (Kawamura and Murakami, 1991; Kawamura, 1993). Both the activation of GC and inactivation of PDE resulting from a decrease in intracellular calcium should thus cause a decrease in the amplitude of the light-induced cGMP decrease, as well as an acceleration of the recovery phase of cGMP response to light (Cohen and Blazynski, 1993).

The results shown in Fig. 5 A demonstrate that lowering the buffered calcium concentration in the EP-ROS medium from 250 to 50 nM slowed the recovery of cGMP, rather than accelerating it as predicted by the above-mentioned model. In addition, only a minor change in the response amplitude was noted. The result is not due to the lack of ATP in the incubation medium, since its inclusion (Fig. 5 B) does not alter the time course of the cGMP response. Our results are qualitatively consistent with data from and Rispoli and Detwiler (1992) who observed a faster recovery of the photoresponse under conditions where the internal calcium concentration of truncated or perfused ROS was elevated. These results suggest that the well-documented ability of calcium to alter the photoresponse of photoreceptors may be more complex than previously thought. It may be that *sustained* changes in calcium concentration (such as were carried out in this study and by Rispoli and Detwiler, 1992) may affect cGMP metabolism in a different manner than the *transient* change in calcium concentration that occurs during the response to flash illumination. If true, it may mean that calcium plays different roles as a second messenger during excitation and recovery (transient calcium change) versus light adaptation (sustained change in calcium).

Electropermeabilized photoreceptor outer segments represent a physiological cellular preparation for gaining access to the cytosol in order to examine the interrelationship of cGMP metabolism and calcium in the processes of visual excitation, recovery and light adaptation. This experimental system will be used in future studies to address the metabolic regulation of cGMP during light adaptation in vertebrate photoreceptors.

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