

Light-induced Losses and Dark Recovery Rates of Guanosine 3',5'-Cyclic Monophosphate in Rod Outer Segments of Intact Amphibian Photoreceptors

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ABSTRACT We used an apparatus in which pieces of dark-adapted amphibian retinas (*Rana pipiens*, *Bufo marinus*) obtained under infrared illumination were exposed to precise intervals of 500-nm illuminations, and then frozen by contact of their outer segment surface with a liquid helium-cooled copper mirror. Sections of the frozen outer segment layer were obtained in a cryostat and then assayed for total extractable cyclic 3',5'-guanosine monophosphate (cGMP). Significant losses of cGMP with respect to the dark level were evident as early as 60 ms after light onset. With dim subsecond illuminations these losses were surprisingly large, which suggests a previously underestimated magnification in the cGMP cascade, or a transient substantial inhibition of guanylate cyclase activity in combination with increased cyclic GMP phosphodiesterase activity. Within the subsecond period, significant losses that were proportional to light intensity (2-log-unit range) and duration (60–550 ms) were generally not evident. However, losses significantly proportional to these factors became evident with durations of 1 s or longer. When pieces of retina were first illuminated (10 or 60 ms), then held in darkness for increasing periods before freezing, we observed a continuous loss of cGMP during the early postillumination dark period, followed by a recovery of the total cGMP level. The times for recovery to the preillumination level appear to be significantly longer than times reported for the recovery of the photoreceptor membrane potential after similar light exposures.

INTRODUCTION

The absorption of photons by rhodopsin molecules in rod outer segments (ROS) leads to the activation of guanosine 5'-triphosphate (GTP) binding protein (transducin), which then, in turn, activates molecules of 3',5'-cyclic guanosine monophosphate (cGMP) phosphodiesterase (PDE) (for review cf. Stryer, 1986). This results in the hydrolysis of cGMP, a molecule present in high concentration in photoreceptors (Orr et al. 1976). However, much of the cGMP is thought to be in a bound fraction

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(Yamazaki et al., 1980). Since exposure of the cytoplasmic face of the ROS plasma membrane to cGMP opens membrane conductances (Fesenko et al., 1985; Yau and Nakatani, 1985*b*; Zimmerman and Baylor, 1986; Matthews, 1987) it has been postulated that fewer such conductances are open after a light exposure that significantly reduces the concentration of free cGMP. For this reason the free concentration of cGMP is thought to be the major regulatory factor of the photomodulated current in ROS (Yau and Nakatani, 1985*b*; Pugh and Cobbs, 1986). Although some early studies suggested that light-induced declines in total cGMP were only evident at times after the electrical response of rods and thus could not be involved in phototransduction (Kilbride and Ebrey, 1979; Govardovskii and Berman, 1981), more recent studies have demonstrated cGMP losses on a subsecond time scale consistent with such a role (Kilbride, 1980; Cote et al., 1984; Blazynski and Cohen, 1986). However, two reports (Goldberg et al., 1983; Ames et al., 1986) appear to indicate that while light proportionally affected the rate of turnover of cGMP in the briefest intervals studied, this occurred with either no change in the concentration of cGMP or a modest increase in its level. Our laboratory, using an apparatus that first illuminates pieces of intact retina and then rapidly stops biochemical processes by pressing the ROS surface to a liquid helium-cooled copper mirror, has reported very early light-induced declines in total, extractable cGMP per unit protein in ROS shaved from the frozen retinas (Blazynski and Cohen, 1986; Cohen and Blazynski, 1987). In the current report we address three issues: a difficulty in observing intensity-related losses in cGMP shortly after illumination, the effects of prolonged illumination (seconds) on the cGMP level of ROS, and the time course of cGMP changes elicited by a brief flash followed by varying periods of darkness.

We found that there was a surprising extent of loss of cGMP with brief weak illuminations. However, with subsecond illuminations differing in intensity over two log units and in durations from 60–550 ms, while most values were significantly different from dark levels, they were usually not different from each other. This differed from findings with longer illuminations. We also present evidence that after a brief light stimulus, a loss of cGMP continues for a time in darkness and that the recovery of total extractable cGMP levels in darkness proceeds at a rate that is significantly slower than the reported rates for the recovery of the rod membrane potential after comparable illuminations.

METHODS

Frogs (*Rana pipiens pipiens*) or toads (*Bufo marinus*) were obtained periodically from commercial suppliers, kept in an animal room that had a lighting regimen of 12-h light, 12-h dark, and fed live crickets. We have previously published a detailed description of our methods (Blazynski and Cohen, 1986), which are only briefly summarized here except for certain procedures particularly pertinent to the current experiments. All experiments reported here were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication 85-23, 1985).

Retinas were isolated under infrared illumination from a frog or toad that had been dark adapted for at least 16 h. They were cut into a variable number of pieces and stored in the dark in oxygenated cold medium. This medium, based on that used by electrophysiologists studying amphibian retinas, contained 100 mM NaCl, 1 mM NaHCO₃, 1.5 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 10 mM HEPES, and 15 mM glucose. We have reported (Blazynski and

Cohen, 1986) that storing retinal pieces in the dark in this cold medium for as long as 40 min had no effect on the level of cGMP in ROS.

After dissecting the retinas, pieces from one animal were successively used. A piece was transferred under infrared to oxygenated medium at 22°C (the experimental temperature) and mounted ROS-up over a thin slice of fixed, thoroughly washed lung that was required for cushioning. This, in turn, rested on a piece of black filter paper glued to a numbered metal tab. The tab was affixed to a specimen holder which was then inverted and attached to the lower end of a long rod so that the retinal ROS surface then faced the surface of a copper mirror. The latter was covered by a retractable shield. The mirror was then brought to the liquid-helium temperature, and the specimen was illuminated for a known duration and intensity, at the end of which the shield covering the copper mirror retracted and the specimen was rapidly propelled to a collision of its ROS surface with the mirror surface. All these actions were electronically controlled. It takes 46–47 ms for a specimen to fall to a collision with the copper mirror. When the illumination sequence is programmed so that the light beams remain on while the specimen falls to freeze, although the retinas fall out of the beam path, they receive the equivalent of an extra 10 ms of light (as determined by mounting a photodiode in place of the retina) during the early part of the fall; the remaining dark-fall interval was 36 ms. If the fall occurs after the light beams are turned off, the 46-ms fall occurs in darkness. During the cooling of the copper mirror by liquid helium, the cold helium gas that forms is displaced from the area by the action of a fan. A baffle in front of the mounted piece of retina protects it from this air current and, by a thermistor test, the temperature at the tab remains constant (22°C). In the experiments involving the recovery of cGMP levels in the dark after a light flash, we initiated the light flash either before, during, or after the period required to cool the copper mirror to liquid-helium temperatures in an attempt to standardize the total time the specimen was suspended above the freezing block. In most cases, the time required to cool the copper block varied between 50–70 s. Thus, for those samples that required long dark durations (50 s or longer), light onset was initiated simultaneously with the cooling of the copper mirror, while for samples that required intermediate dark durations the light sequence was initiated during cooling, and for the briefest light-dark sequence or unilluminated specimens, the sequence was initiated after cooling.

This protocol could be used as long as the time interval between freezing successive specimens approximated 5 min. When all of the retinal pieces from a given animal were processed, retinas from another animal had to be isolated and this permitted warming of the liquid-helium transfer line, which necessitated a longer cooling period for the copper mirror for the first sample that was frozen. However, since the number of retinal pieces obtained per animal varies, and the experimental treatment varies in sequence for successive pieces of retina (i.e., dark, 10-ms flash, flash plus a certain dark recovery interval, etc.) the particular treatment of these initial pieces was randomized.

After freezing the specimens, each tab with a frozen specimen was rapidly transferred to liquid nitrogen and the accumulated tabs were subsequently stored at –80°C. For sectioning, the samples were placed on dry ice and then transferred to a cryostat (–35°C) where three 6- μ m tangential sections of the flattened ROS layer were obtained from a specimen. The tissue was collected in a tube surrounded by dry ice. Samples were homogenized in trichloroacetic acid and cGMP was measured by radioimmunoassay (Steiner et al., 1972; Harper and Brooker, 1975) and proteins by the method of Lowry et al. (1951). The term cGMP levels' as used in this report refers to total extractable cGMP per milligram of OS protein.

Light Calibration

To standardize our light source, two methods were employed. (a) Radiometric: The specimen was illuminated by two arms of a split fiber-optic bundle. The energy in each light beam was

separately determined using a radiometer (J16 with a J6512 probe, Tektronix, Inc., Beaverton, OR) placed at the position of the mounted piece of retina, with the photocell window placed normal to the incident beam. This illumination totaled 580 mw/m^2 , from which we calculated a flux of $\sim 1.4 \times 10^6 \text{ quanta}/\mu\text{m}^2 \text{ s}$ at the ROS surface of the retina.

(b) Rhodopsin bleaching: In this procedure, which was undertaken as a check on the photometry, $500 \mu\text{l}$ of an octylglucoside extract of rhodopsin ($5\text{--}10 \mu\text{M}$) was placed in a test tube (o.d., 1.2 cm; i.d., 0.99 cm) with the bulk of the rhodopsin solution positioned at the usual level of a piece of retina. Heat-filtered, unattenuated, 500-nm (narrow band width) illumination was applied for 15 or 30 s. The absolute absorbance loss at 500 nm was obtained after exhaustive bleaching (10 min) in the presence of hydroxylamine and was used to calculate the concentration of rhodopsin. Full spectra indicated little or no scattering contribution to the absorbance at 500 nm. The percent loss at 500 nm after the initial bleaching was used to calculate the rhodopsin molecules bleached, assuming that the outer segment contained 3×10^9 rhodopsin molecules. The radiometric measurement of light flux, when corrected for quantum efficiency of bleaching, closely predicted the measured bleaching of rhodopsin.

A more difficult problem is relating the above radiometric and bleaching data to the bleaching actually achieved in the dense rhodopsin layer represented by the stratum of rod outer segments in the mounted piece of retina. Because we use 500-nm (narrow band) illumination most of the incident photons will be captured by photopigment. Each light beam makes an angle of about 25° with the plane on which the piece of retina is mounted, but the freezing procedure required the pieces of retina to be draped over a cushioning slice of lung. Thus the surface of the retina was convex, and there was no fixed relationship between the axes of the ROS and the slightly divergent beams of incident light. As is well appreciated (cf. Dartnall, 1972), due to the molecular arrangement of rhodopsin in the planar rod disk membranes, the better the alignment of an axis of a ROS with the direction of light, the greater the bleaching. If the retinal pieces were truly planar and all ROS axes were normal to this plane, they would constitute a layer $\sim 60 \mu\text{m}$ deep. An impinging light beam at 25° to this plane would be refracted as it entered the denser medium of the retina and penetrate the retina at a 45° angle to the ROS axes. This permits calculating the length of the light path through this layer. The rhodopsin concentration in this volume (ROS plus interspaces) is $\sim 2.5 \text{ mM}$. The molar extinction coefficient for light (500 nm) striking the disks at 45° is 45,000. This gives an optical density of 0.955. The quantum efficiency of bleaching for amphibian rhodopsin is in the range of 0.5–0.67 (Kropf, 1967; Dartnall, 1968; Baylor et al., 1979). The above considerations, taken together, suggest that the actual number of molecules bleached would be 3–5 times that predicted for a dilute solution of rhodopsin subjected to the radiant energy we used. We have used a $3\times$ correction in modeling the hydrolysis expected for a given stimulus but the nonplanarity of the retina pieces could make a value above $3\times$ possible. Because of these uncertainties, the radiometric value is reported as the standard illumination at the surface of the retinal pieces, and for each experiment the log units of attenuation at 500 nm for the calibrated neutral density filters is stated. Since in our earlier report (Blazynski and Cohen, 1986) we referred changes in cGMP levels to values for bleached rhodopsins that did not take into account the high degree of photon capture by the dense aggregate of rhodopsin in the ROS layer, the degree of bleaching reported in those experiments should be increased by three- to fivefold.

In the initial paper using this methodology (Blazynski and Cohen, 1986) we also called attention to another possible source of error. Sections of the frozen ROS layer were obtained with a microtome in a cryostat under white light and it is probable that pressure at the knife edge produced a highly transient ice-to-water phase change that might have been accompanied by a transient but minimal temperature elevation since the thick steel microtome knife is a substantial and conductive heat sink. A temperature rise might either transiently activate the cGMP cascade or produce further hydrolysis by experimentally activated PDE, perhaps in

TABLE I
cGMP Concentration at Three Light Intensities*

Duration	N.D. 5.0	N.D. 4.0	N.D. 3.0	Dark
<i>ms</i>	<i>cGMP concentration</i>			
20	126.3 ± 4.3 (n = 19)	122.1 ± 5.8 (n = 17)	121.3 ± 3.7 (n = 16)	134.9 ± 4.6 (n = 21)
110	118.6 ± 4.8 (n = 17)	116.8 ± 3.5 (n = 14)	119.3 ± 4.4 (n = 15)	
510	105.4 ± 3.9 (n = 16)	102.3 ± 5.4 (n = 17)	94.4 ± 4.8 (n = 18)	
	<i>Percentage of dark cGMP</i>			
20	93.6 (NS)	90.5	89.9	
110	87.9	86.5	88.4	
510	78.1	75.8	70.0	

(NS) Not significantly different from dark levels.

*Picomoles of total extractable cGMP per milligram of protein (±SEM).

proportion to the extent of such activation. While the latter effect should not influence the times at which losses appear to be initiated or when recoveries appear to occur, they could influence the absolute levels of cGMP. Arguing against this are the smooth curves we reported (Blazynski and Cohen, 1986) from averaging randomly processed pieces of retina for different durations of light exposure at a particular intensity.

RESULTS

In our previous report, we had shown that differences in the extent of light-induced cGMP losses in proportion to intensity could be demonstrated for light durations of 1–2 s, but not for shorter durations. However, in all light-exposed samples the cGMP levels were significantly reduced from dark levels. This conclusion was based on a comparison of results from several separate experiments. To further investigate this observation, in a single experiment we exposed a large number of retinal pieces to very brief flashes (20, 110, 510 ms) of one of three dim illuminations followed by their fall to freeze (36 ms; see Methods). As is evident from Table I, with the exception of the 20-ms exposure to the dimmest intensity, there was a significant decrease in the levels of cGMP for all conditions. However, there were typically no significant differences in cGMP levels among ROS exposed to the three intensities at a given duration. The exception to this was observed for the longest duration when the levels were compared at 5.0 N.D. (log units of neutral density) and 3.0 N.D. ($P < 0.05$). With longer durations of illumination, significant differences in cGMP content could be discerned (Table II).

TABLE II
Intensity-related Differences in cGMP Levels After 5-s Illuminations

Log N.D.	pmol cGMP/mg protein (± SEM)	n
Dark	155 ± 5.2	19
5.0	117.6 ± 8.8	17
3.0	116.6 ± 7.7	18
1.0	100.3 ± 5.4	22
0	95.6 ± 7.7	18

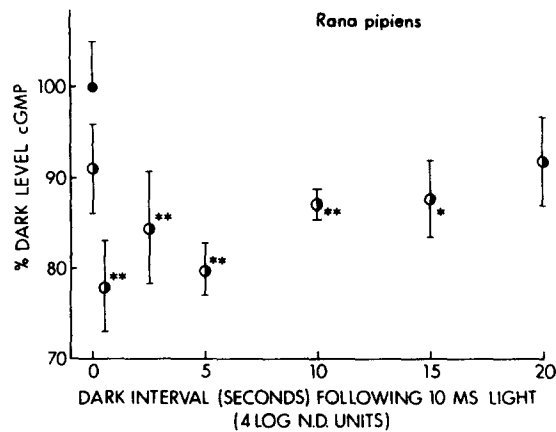
Light-sensitive cGMP Hydrolysis Is Extremely Rapid and Sensitive

It has been reported that the light-induced decrease in cGMP levels is extremely rapid (Woodruff and Bownds, 1979; Cote et al., 1984; Blazynski and Cohen, 1986). The data in Table I emphasize just how rapid this process is. For example, with a 20-ms light exposure, followed by a dark fall to a freeze of 36 ms (total elapsed time 56 ms) significant losses of cGMP were observed for the brightest of the three intensities. The losses averaged ~10% of the dark levels, which corresponds to ~3 million molecules of cGMP hydrolyzed in 56 ms. If this is entirely attributable to PDE activity, rather than to some combination of an inhibition of guanylate cyclase and a stimulation of PDE, these results appear to require extremely high turnover numbers for PDE (see Discussion).

cGMP Levels Recover In Darkness after a Flash

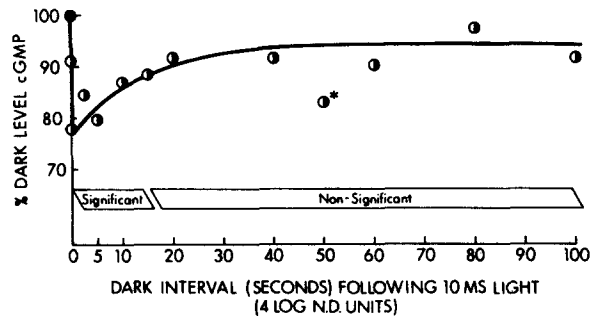
It is now widely accepted that free cGMP, acting at plasma membrane channels of ROS, opens the conductance to sodium and some other ions. The light-induced

FIGURE 1. Time course of the recovery of cGMP levels after a brief weak 500-nm illumination. The radiant flux at the ROS surface of the retina was 1.4×10^6 quanta/ μm^2 per s attenuated by a log neutral density of 4.2. Retinal pieces were either never exposed to light before freezing (filled circle) or frozen after a light stimulus plus a known dark interval (half-filled circles). For all samples a 46-ms fall in the dark to contact the freezing block followed the experimental period. Each datum point represents the mean (\pm SEM) of ~20 ROS samples. The means were significantly different from darkness at either the $P < 0.05$ (*) or the $P < 0.01$ (**) level between 250 ms and 15 s after light onset.



decrease in cGMP is thought to result in the closing of these conductances. It follows that the restoration of the dark level of free cGMP following a flash should return ROS conductances to their dark condition. We have determined that after brief dim flashes, the level of total cGMP declines and then recovers to the level observed before illumination in amphibian ROS. The results of experiments using pieces of intact frog retinas are presented in Figs. 1 and 2. In these experiments we used a 10-ms flash as the stimulus and all samples were frozen in the dark. Except for the dark control, after the flash we varied the time the specimen was held in the dark before the dark fall to freeze. In this experiment, in order to reduce the anticipated recovery time we used a very dim 10-ms flash. While this reduced the cGMP level at the earliest postillumination time point sampled, the first cGMP level that

FIGURE 2. The extended time course of recovery of cGMP levels after brief weak illumination at the same intensity as Fig. 1. The data represent the means of several experiments where retinas were illuminated for 10 ms and freezing was delayed for increasing durations of darkness (filled circle, dark only; half-filled circles, light followed by dark). The rapid decrease in the level of cGMP was followed by a much slower recovery to the preillumination level. For dark durations of 20 s or greater, the cGMP concentration was not significantly different from dark levels. In one experiment, at 50 s after the light exposure, the mean was different from the dark level.



was significantly different from the dark level was seen when freezing was at 510 ms after the onset of the 10-ms flash. In order to combine experiments, all values in each experiment were expressed as percent of the dark level for that experiment, and we averaged the normalized values representing the same time points in the combined experiments. In several experiments, no light response whatsoever was observed and these were not included in the pooled data (see Discussion). Fig. 1 presents data from the earlier part of the experiment, and in Fig. 2, data from the entire experiment is presented. We describe in Methods a procedure we used to have retinas suspended over the freezing block for similar times despite differing total periods of light plus dark, when the total period was 50 s or less. cGMP levels continued to fall after the light offset and remained significantly different from the dark condition for ~20 s after the flash. After this period the cGMP gradually increased toward the preillumination level. Once the cGMP measured reached a level that was statistically indistinguishable from the dark condition it remained stable for the remainder of the 100-s period studied.

In the experiment presented in Fig. 3, retinal pieces were exposed to durations of

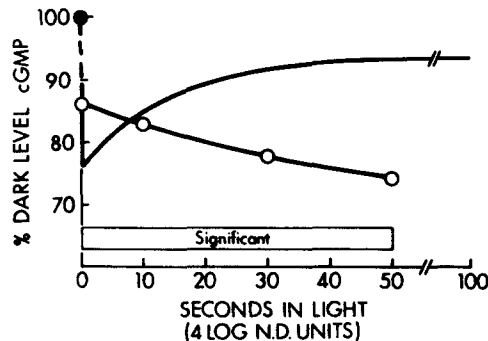


FIGURE 3. Comparison of the time course of cGMP concentration between samples that were continuously illuminated and those that were illuminated for 10 ms before the dark condition. The solid curve represents the data presented in Fig. 2; continuously illuminated samples demonstrate no recovery of cGMP levels. Symbols represent dark only (filled circle) and continuous light (empty circles) starting from a dark condition. For both sets of data, samples were illuminated with the same intensity of light.

continuous illumination of the same intensity as that used in the experiment illustrated in Figs. 1 and 2 (N.D. = 4.0). The continuous illumination resulted in a progressive slow decline in cGMP, in contrast to the gradual recovery in darkness seen in the previous experiment (replotted in Fig. 3 for comparison). The progressive decline during continuous illumination differs from a report by Kilbride and Ebrey (1979), who detected an apparent recovery of cGMP during prolonged illumination of whole frog retinas.

An interesting observation from these experiments is the fact that the decreases in total cGMP that occur in the dark interval immediately after flash offset are quantitatively similar to those reported for the same duration of continuous illumination

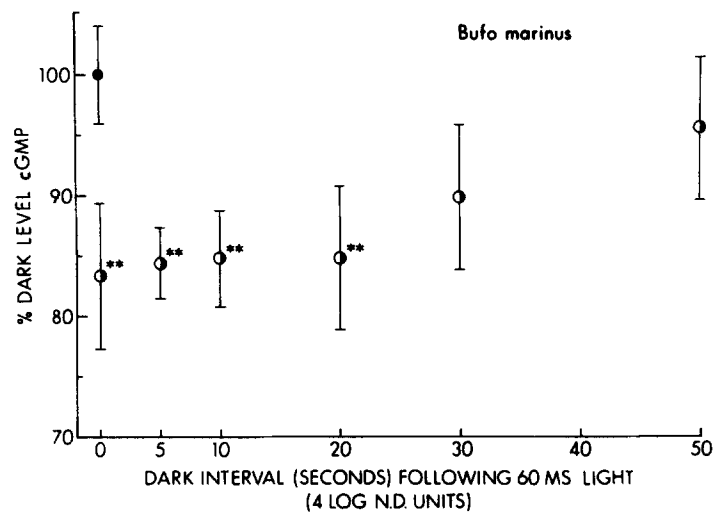


FIGURE 4. Time course of recovery of cGMP levels after a brief duration of illumination in toad retinas. The radiant flux (500 nm, narrow band) at the ROS surface of the retina was 1.4×10^6 quanta/ μm^2 per s, attenuated by neutral density filters as indicated. Retinas were exposed to either darkness (filled circle) or a 60-ms flash attenuated by 4.2 N.D. followed by increasing periods of darkness (half-filled circles). After the flash, for dark intervals of 20 s or less, the means (\pm SEM) of ~ 20 samples were significantly different from darkness (** $P < 0.01$).

at the same intensity. From Table I it is seen that at 510 ms, levels were 76% of the dark value, while in Fig. 1, at 500 ms after the 10-ms flash, levels were 78% of the cGMP measured in dark ROS. The initial velocities of cGMP loss appear to be the same despite the different durations of illumination.

The recovery studies were repeated using retinas isolated from the toad (*Bufo marinus*). This animal has a larger eye and is used by many electrophysiologists studying transduction in rods. In these studies retinas were illuminated for 60 ms at one of two intensities (N.D. = 4.0 or 3.0) to yield a larger effective bleach than that elicited in the frog experiments. For both illumination intensities, there was a significant decrease in cGMP levels after the flash. The decrease continued in darkness and then slowly recovered to the preillumination level (Figs. 4 and 5; note that in Fig. 4

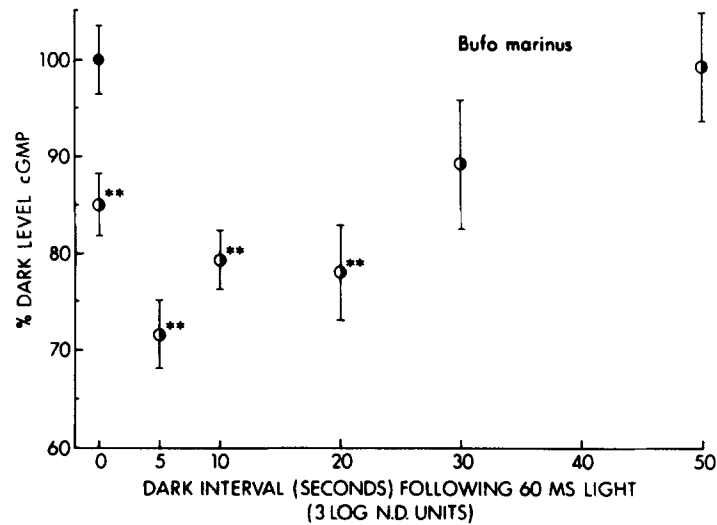


FIGURE 5. Retinas were exposed to darkness (filled circle) or to a 60-ms flash attenuated by 3.1 N.D. followed by darkness (half-filled circles). The illumination significantly (** $P < 0.01$) decreased cGMP levels compared with darkness until dark durations were 30 s or longer. Note that the ordinate scale differs from 4.

the ordinate scale is enlarged compared with that in Fig. 5). For the earliest time point (60-ms light), the percent fall in cGMP was virtually identical for both experiments despite a 10-fold difference in intensity. However, with a delay in darkness, intensity-related differences became obvious. For both experiments, after the light flash, the diminished levels of cGMP gradually recovered to become statistically

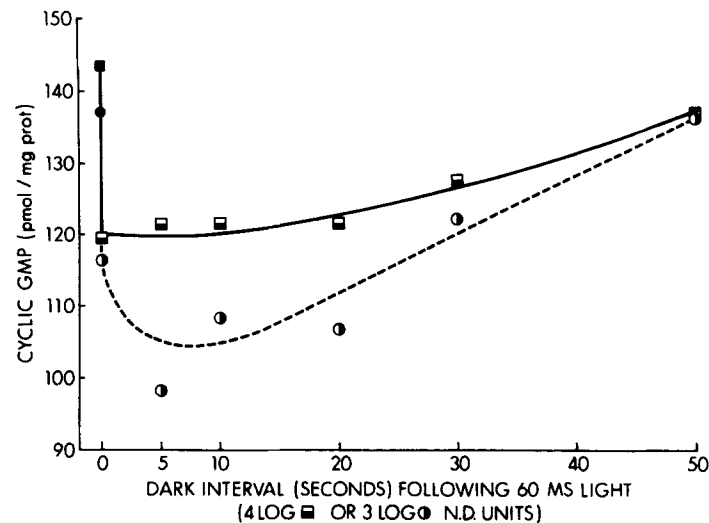


FIGURE 6. Comparison of recovery experiments illustrated in Figs. 4 and 5 without the normalization of data. Filled symbols represent retinas exposed only to darkness and half-filled symbols represent those exposed to light followed by dark.

indistinguishable from the preillumination level. The time course for the recovery was also similar despite the difference in intensity. The two experiments are compared in Fig. 6 where the data are *not* normalized.

DISCUSSION

Other investigators have reported dark levels of cGMP in amphibian ROS. In one case (Meyertholen et al., 1980) the assay was carried out on ROS shaken from toad retinas that had been incubated in a medium containing 24 mM bicarbonate, and the cGMP level was ~50% of that we report. The other reported values are based on ROS layers cut from freeze-dried sections from quick-frozen isolated retinas (Govardovskii and Berman, 1981), or from freeze-dried tangential sections of retinas within quick-frozen whole eyes (deAzeredo et al., 1978; Barbehenn et al., 1986). As levels were referred to dry weight, assuming protein to be 50% of dry weight, they range from 86–122 pmol cGMP/mg protein. The mean value we observe, based on many frog experiments, is 145 pmol cGMP/mg OS protein.

We had noted in an earlier report that when several experiments using widely varying illumination intensities were compared, intensity-related differences in the loss of cGMP were not evident until durations of over a second (Blazynski and Cohen, 1986; see Fig. 4). However, it was possible that subtle differences were lost due to variance in experimental noise in these individual experiments. This does not appear to be the case since we demonstrate in this report that within a single large experiment, for illumination periods of 550 ms or less, intensity-related differences in cGMP were not evident with stimuli ranging over 100-fold. It is likely that for these minimal duration and intensity variations, analytic noise within the experiment obscures small differences in cGMP levels between conditions, although when compared with dark levels in all combinations but one, significant differences were observed.

It is also puzzling that such large cGMP losses are detected for these short light durations. If one uses literature values for rates of transducin activation (Vuong et al., 1984; each frog R* activates 500 transducins in 500 ms at 23°C), and selects the full turnover capacity for amphibian PDE at 21°C as reported (1,250 molecules cGMP hydrolyzed per second; Liebman and Pugh, Jr., 1982) one finds that the rate of loss of cGMP that we observed with very short times exceeds the rates calculated. For example, applying the indicated 3× correction for estimating actual bleaching as in Methods, in one experiment we calculated that 480 R*/ROS were formed by a 20-ms flash. The tissue was frozen 56-ms after flash onset. We assume that rhodopsins were bleached at a rate of 24 per ms for 20 ms, and that each bleached rhodopsin activated one transducin per ms from the time of its bleaching to the end of the experimental period (56 ms). Also, we assume that each activated transducin activated a single PDE in 1 ms (Cobbs and Pugh, Jr., 1987), and that the latter hydrolyzed cGMP from the time of its activation to the end of the 56 ms period. Ignoring turnoff processes because of the short time involved, we calculate a loss of 0.6×10^6 molecules of cGMP as compared with the observed loss of 2.8×10^6 molecules of cGMP (assuming a dark content of 30 μ M cGMP and a mean ROS volume of 1.5×10^{-12} liters). The actual loss is 4.7 times that calculated. However, the above modeling used a PDE-turnover value based on the enzyme saturated with substrate.

Amphibian outer segments have been said to contain 30–60 μM cGMP (Yee and Liebman, 1978). If but 5–10% or 1.5–6 μM of cGMP is free, as the results of electrophysiological experiments seem to require if free cGMP is the predominant regulator of photoconductances in ROS (Yau and Nakatani, 1985*b*; Cobbs and Pugh, Jr., 1985) and as the K_m for amphibian PDE in the light is 500 μM (Robinson et al., 1980; Kawamura and Murakami, 1986), only a small percentage of cGMP could interact with PDE. Accordingly, the 4.7-fold disparity might actually be a disparity of 400–1,600-fold. However, Deterre et al. (1988) have recently reported that cGMP-PDE contains two inhibitory subunits and that the removal of both yields an enzyme with an activity comparable to that of trypsin-activated PDE. They suggest that, in possible contrast to the situation in vivo, the concentrations of active α -transducin obtained in assays of PDE in vitro may be lower by two orders of magnitude from that required to fully activate PDE. Therefore, the turnover capacity of amphibian PDE used in our modeling may be too low. It is also of interest that high-affinity binding sites for cGMP on inactive PDE have been demonstrated for amphibian PDE (Yamazaki et al., 1980) and mammalian photoreceptors (Gillespie and Beavo, 1988).

However, one is faced with the possible existence of an artifact that magnifies the light-induced loss. One such possibility is mentioned in the Methods section and relates to cryomicrotomy. Another might be based on ionic changes in the cold-stored pieces of retina, although we have shown that such storage did not affect the dark level of cGMP (Blazynski and Cohen, 1986) and retinal pieces were placed in 22°C medium just before mounting.

On the other hand, losses comparable to those we observed have been reported by Cote et al. (1984, 1986) when they used a preparation of isolated frog outer segments of which 60–80% have attached inner segments (OS-IS). In general, these studies were carried out in media with reduced calcium levels, but in the earlier of these two reports, two of the studies, like our own, were performed in a medium with a normal calcium level and yielded results similar to those reported here. For example, in one experiment they observed a 10% loss of cGMP with continuous illumination bleaching 8,000 rhodopsins/s for 50 ms (Cote et al., 1984; Fig. 5). In their more usual studies in which the OS-IS were in a medium with 20 nM calcium, with illumination bleaching 160 rhodopsins/rod per s, they observed a 10% loss of cGMP in 1 s (Cote et al., 1986; Fig. 2). In their earlier paper, Cote et al. (1984) state that a 1-nM calcium medium increases cGMP in OS-IS by 2.6-fold. Acid quenching was used to stop chemical reactions in all their experiments. Thus, quantitative results highly similar to those we report here were obtained by a very different method.

A loss of cGMP could be accomplished by any combination of the inhibition of guanylate cyclase and the stimulation of cGMP-PDE. If values taken from the literature are not seriously in error, and if there is not another multiplicative factor in the cascade, one must consider whether light elicited a large decrease in basal guanylate cyclase activity. The level of guanylate cyclase activity in dark ROS is unknown. Very low levels of calcium activity are known to markedly increase cGMP levels in retinas that possess photoreceptors (Cohen et al., 1978; Fleischman, 1981; Lolley and Racz, 1982). This is likely to be based on the relief of the known calcium inhibition of ROS guanylate cyclase (Krishnan et al., 1978; Troyer et al., 1978; Fleischman,

1981). Evidence has also been presented that demonstrates the inhibition of ROS-PDE activity by decreasing calcium activity (Bownds, 1980; Robinson et al., 1980). In any event, it now seems quite clear that light reduces calcium activity in outer segments (Yau and Nakatani, 1985a; Lamb et al., 1986; McNaughton et al., 1986). Thus, the indirect action of light on guanylate cyclase or PDE via its effects on calcium activity would seem to be in a direction that would tend to elevate cGMP levels. An additional, although small, contribution to free cGMP levels would also be made by the liberation of two molecules of cGMP from their noncatalytic binding sites on each amphibian PDE molecule whenever that PDE becomes activated (Yamazaki et al., 1980), unless the bound cGMP molecules become preferentially hydrolyzed by the activated PDE. However, the activation of guanylate cyclase by reduced calcium levels and the increased synthetic contribution to the cGMP pool probably lags behind hydrolysis of cGMP due to the light-induced activation of PDE, thus permitting the fall in the free cGMP concentration required for ROS photoconductances to close.

Because the components of the cGMP cascade consist of integral and membrane-associated proteins exposed to the intracellular ionic milieu and compartmentation, some of their interaction kinetics may not correspond to those deduced from studying the properties of isolated components in a diluted solution, or they may not be faithfully represented by ROS membrane or broken ROS preparations. It is perhaps noteworthy that the highest rates for cGMP loss/ R^* have been obtained at subsecond times with preparations where ROS are intact (Cote et al., 1984; Blazynski and Cohen, 1986). As recently summarized by Liebman et al. (1987), reaction rates for cGMP hydrolysis in ROS appear to require rhodopsin, transducin (G-protein), and PDE interactions in or on the two-dimensional disk membrane, i.e., two-dimensional diffusion. Similar consideration has been given by others as to how coupled enzyme reactions involving concentrated enzymes and possible mechanisms involving the direct transfer of metabolites (minimizing diffusion) might affect the kinetics of sequential reactions (Sreva, 1987; Srivastava and Bernhard, 1987), but it is by no means clear how many of these considerations apply to ROS.

In the recovery experiments (Figs. 1, 2, 4, and 5) a very rapid loss of cGMP was again observed after a weak bleach. In a few initial recovery experiments that used frog retinas, no points significantly differed from the dark level of cGMP. The reason for this is unknown. Perhaps it was due to the nutritional state of the animals as supplied, or it merely reflected the fact that the intensity and duration of the stimulus approached the limits of detectability by our methods. When retinas from recently collected toads were used, the stimulus intensity was increased to generate more R^*/ROS for the period of illumination. For all of these experiments, the time for the return of cGMP to dark levels was 20–30 s, which is longer than the time for the return of membrane potential or current after similar bleaches (Lipton et al., 1977; Mueller and Pugh, Jr., 1983). A similar result was reported in a study using an OS-IS preparation in low Ca^{++} medium by Bownds et al. (1987). A likely minor contributory factor to the delay of recovery in our experiments is the fact that the rod tips were subjected to more bleaching than the remainder of the ROS; Baylor and Lamb (1982) have found slower electrophysiological recovery when bleaching is directed at ROS tips, as compared with their bases. The data also illustrate that the

decline in the total extractable cGMP level persists for a relatively long time after illumination. During the several hundred milliseconds immediately after bleaching, the decay of cGMP in the dark was highly similar to that seen with continuous illumination, which indicates a continued cGMP hydrolysis in excess of synthesis. Dark hydrolysis in excess of synthesis was suggested in several studies where cGMP was injected into amphibian ROS (Miller and Nicol, 1981; Miller, 1982; Kawamura and Murakami, 1986). These injections transiently depolarized the membrane and light delivered after the injection accelerated the recovery of polarity. For weak flashes the time course for the decay in the ability of light to antagonize the cGMP-induced depolarizations was similar to the time course of the return of the membrane potential after the test flash. For brighter flashes, the membrane potential returned faster than the duration of the light-dependent antagonism to effects of the injection of cGMP (Miller, 1982). More recently, gecko ROS were injected with large amounts of cGMP and the depolarizations induced were shown to decay on a time scale similar to the return of ROS sensitivity (Kawamura and Murakami, 1986), which suggests a role for PDE inactivation in adaptation. These electrophysiological experiments were assumed to measure PDE activity *in situ*. In the current report a net hydrolytic loss of cGMP over synthesis is directly demonstrated and persists for a relatively long time after a flash. This suggests that heightened cGMP-PDE activity persists for relatively long durations after a flash. The eventual recovery of cGMP in the dark reflects a rebalancing of these processes, in which it is highly likely that processes turning off the cGMP cascade are significant components together with a possibly delayed acceleration of cGMP synthesis.

That the time course for the recovery of the level of cGMP appears not to correlate with reported times for the return of membrane potential for similar bleaches, does not eliminate cGMP as the primary regulator of the light-sensitive channels. Yau and Nakatani (1985*b*) have suggested that a small fraction of the total channels are open in the dark and that a light-induced decrease of a "free" cGMP concentration, representing 5% of total cGMP, would suffice to close these channels. It is not clear whether restoration of the original size of the putative pool of "free" cGMP requires restoration of total cGMP. This depends, in part, on whether virtually all binding sites for cGMP are on PDE. If the free pool can recover faster than total cGMP, then either it is possible to replace free cGMP by synthesis, or the affinity of some of the noncatalytic binding sites for cGMP must change to permit replenishing of the free pool from the bound pool, followed by a metabolic restoration of the bound pool. The affinity of binding sites for cGMP might be regulated by the absolute or relative concentration of guanine and/or adenine nucleotides (Yamazaki et al., 1982; Stryer, 1986; Fesenko and Krapiviniskii, 1986). It is also possible that there exists a pool of low-affinity binding sites for cGMP (Volotovskii et al., 1984).

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