Light-Initiated Changes of Cyclic Guanosine Monophosphate Levels in the Frog Retina Measured with Quick-Freezing Techniques

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ABSTRACT Although there is good agreement that light reduces the amount of cyclic GMP (cGMP) in the retina, the exact time-course of this decrease is not well established. Bullfrog retinal sections were isolated under infrared light and quick-frozen with liquid nitrogen-cooled, metal hammers after exposure to various intensities of continuous illumination. This quick-freezing should stop the degradation of cGMP within 50–100 ms. The frozen retinal sections were then slowly warmed up in the presence of perchloric acid to denature enzymes involved in cGMP metabolism. cGMP was determined by radioimmunoassay and comparison was made between light- and dark-adapted retinal sections from the same animal. The average cGMP concentration was $44.3 \pm 0.7$ pmol cGMP/mg protein or $170.9 \pm 3.2$ pmol cGMP/retina. After 1 s of illumination no significant change in cGMP concentration was found even with the brightest light used ($\sim 7 \times 10^7$ rhodopsins bleached/second per rod. At this intensity the first significant decrease in cGMP from dark-adapted levels was detected 3–5 s after the initiation of illumination; cGMP decayed to 70–75% of the dark-adapted value after $\sim 30$ s. With lower intensity illumination the cGMP levels recovered to dark-adapted levels after the initial decrease even though the bleaching light remained on.

INTRODUCTION

Several observations implicate cyclic guanosine 3', 5' monophosphate (cGMP) as an important factor in the response of a vertebrate photoreceptor cell to light. The highest concentration of cGMP in any mammalian tissue has been found in the retina (Farber and Lolley, 1974; Gordis et al., 1974, 1977; Ferrendelli and Cohen, 1976; Krishna et al., 1976). Most of this cGMP is found in rod outer segment preparations (ROS) (Fletcher and Chader, 1976; Woodruff et al., 1977). Moreover, microdissection of retinal layers has located as much as 90% of retinal cGMP in the photoreceptor layers (Farber and
Lolley, 1974; Orr et al., 1976). In addition, mice that have retinas deficient in photoreceptors have much lower amounts of cGMP than normal mice (Farber and Lolley, 1976; Ferrendelli and Cohen, 1976). Light can lower these high cGMP concentrations. A light-adapted retina has about one-half the amount of cGMP as a dark-adapted one (Goridis et al., 1974, 1977; Ferrendelli and Cohen, 1976; Cohen et al., 1978; Mitzel et al., 1978). This decrease is believed to be due to a light-activated, cGMP-specific and GTP-dependent phosphodiesterase (PDE) (Krishna et al., 1976; Wheeler and Bitensky, 1977; Bitensky et al., 1978). Interestingly, inhibitors of PDE activity can dramatically affect the amplitude of the rod receptor potential (Ebrey and Hood, 1973; Lipton et al., 1977).

Although there is good agreement that light reduces the amount of cGMP in the retina, the exact time-course of this decrease is not well established. Goridis et al. (1974) found that a light exposure of 5 and 15 s caused 30 and 70% drops, respectively, of the cGMP levels of bovine retinas; the cGMP-consuming reaction was stopped by dropping the retina in perchloric acid (PCA). Goridis et al. (1977), using frog retinas, found that 10 s after a light flash that bleached 60% of the rhodopsin, there was no significant change in cGMP levels, but a large decrease was attained by 1 min. They stopped the cGMP-consuming reaction by immersion of the retina in PCA. With an attenuated light flash they found a significant drop of cGMP levels in only 3 s and a near maximal decrease of the cGMP level after 30 s. Mitzel et al. (1978), using mouse retinas, found a 70% decrease in cGMP levels within 30-60 s after turning on a continuous bleaching light (250 ft-c); they stopped the reaction by freezing the retinas on dry ice.

Light is also known to cause a decrease in cGMP levels in isolated ROS (Brodie and Bownds, 1976; Fletcher and Chader, 1976; Krishna et al., 1976; Woodruff et al., 1977). Woodruff et al. (1977) found with continuous saturating illumination of frog ROS that cGMP levels decreased by 40-50% within 6 s; approximately half of this decrease occurred within 200-400 ms. At appropriate times they stopped the cGMP-consuming reactions by rapidly mixing the ROS with PCA.

Recently, Yee and Liebman (1978), using rod disc membrane preparations, found that increased PDE activity in the presence of high GTP concentrations could be detected within 100 ms by a bright flash, while very low intensities of light or a low GTP concentration could cause delays up to seconds for full activation.

The present experiments were undertaken to determine a more precise rate of decrease of cGMP in the retina. By comparison to similar techniques on muscle (see Brooker, 1975) the use of rapid-freezing techniques with the retina should stop the PDE activity within 50-100 ms. If, as indicated above, most of the cGMP is in the photoreceptors, then this change in retinal cGMP levels should fairly accurately reflect changes in photoreceptor cGMP levels.

METHODS

Bullfrogs (Rana catesbeiana) were dark-adapted for at least 12 h before use. All the following operations were performed under infrared illumination, using an infrared
After pithing, an eye was excised and hemisected with a razor blade. The posterior half of the eye was cut in two and the sections were placed into freshly oxygenated frog Ringer's solution. The Ringer's solution contained 108 mM NaCl, 2.5 mM KCl, 0.6 mM Na₂SO₄, 0.13 mM NaHCO₃, 1.6 mM CaCl₂, 1.2 mM MgSO₄, 3.0 mM HEPES (N-2 hydroxyethylpiperazine N'-2 ethanesulphonic acid), and 5.6 mM glucose adjusted to pH 7.5 with NaOH (modified from Brown and Pinto, 1974). The retina was gently teased away from the pigment epithelium and transferred to a petri dish containing about 25 ml of freshly oxygenated Ringer's solution, keeping the retinal sections for each frog in separated petri dishes. These sections were stored approximately 20-25 min in a light-tight chamber before being used.

Pairs of retinal sections from the same frog were removed from the light-tight chamber, and by using a small glass spoon, placed along with a volume (~0.4 ml) of Ringer's solution into pairs of large test tubes (30 ml Corex, Corning Glass Works, Corning, N.Y.). Hammers made of aluminum were designed to just fit inside the large tubes; the hammer's bottom was curved to match the tube's bottom curve. The hammers were kept in liquid nitrogen (77°C) until just prior to use. One retinal section of the pair was placed in the test tube and had a "cold" hammer dropped on it; this tube was then placed in an ethanol-dry ice bath (~75°C) and served as a dark-adapted control. The other tube with retinal section was placed in the automated "cold" hammer dropping apparatus. A "cold" hammer was suspended by an electromagnet above the tube, and air was blown between the hammer and the top of the tube to prevent cold air drafts from precooling the retinal section. The temperature in the bottom of the tube was within 0.5°C of the ambient temperature. A triggering system both opened a shutter to expose the retinal section to a continuous light source and at the appropriate time dropped the "cold" hammer on the retinal section. The tube containing the frozen retinal section and the hammer was put into the ethanol-dry ice bath (~75°C).

At the conclusion of a set of experiments, after all samples tubes were in the ~75°C bath, the room lights were turned on. To the tube-hammer assembly was added 1.5 ml of a PCA (10%): methanol, 1:1 mixture prechilled to ~20°C. The tube-hammer assembly was allowed to warm up slowly (several hours) until the PCA-methanol had melted. Then the hammer was removed and rinsed with 1 ml PCA-methanol (~20°C), the frozen retinal section was homogenized with a syringe, and finally the syringe was washed with 0.5 ml PCA-methanol. We assumed that after this treatment all the enzymes in the preparation had been denatured and that samples could be worked with at room temperature. Although this assumption seems reasonable, partial controls were performed by adding radioactive cGMP to frozen test tubes with and without retinas and then adding PCA-methanol as outlined above. At the the end of the pH neutralization process described below the samples contained identical (~±5%) amounts of cGMP measured by a modification of the method of Thompson and Appleman (1971) indicating no metabolism of cGMP after the retinas were frozen. Even this control did not rule out the somewhat unlikely possibility that when the samples were slowly warmed up from ~75°C in the presence of PCA, some cGMP metabolism might occur which the added exogenous cGMP did not monitor.

The homogenized retina was centrifuged at 36,000 g (4°C) for 1 h. The pellet was saved for protein determination after adding 0.5 N NaOH and heating in a 40°C water bath for 12 h (Hess and Lewin, 1965) along with standard protein solutions. Protein was measured by the method of Lowry et al. (1951). The supernate was neutralized with 6 N KOH using a pH indicator dye to mark the end point. This mixture was centrifuged at 25,000 g for 20 min to remove the PCA precipitate. 1 ml
of this supernate was added to 1 ml of Tris-EDTA buffer (0.2 M Tris and 16 mM EDTA) at pH 7.5. cGMP in the Tris-EDTA-buffered supernate was determined by a cGMP-specific radioimmunoassay (RIA) (Amersham/Seale Corp., Arlington Heights, Ill.). Standard samples of cGMP (Sigma Chemical Co., St. Louis Mo.) were added to PCA-methanol and carried through the procedure to obtain a standard curve. cGMP concentrations are expressed as picomoles cGMP/milligram of total retinal protein.

The light source used was from a tungsten lamp (Sylvania 500W DAK projector lamp, GTE Sylvania, Inc., Stamford, Conn.) which was passed through various neutral density (ND) filters and an electromagnetic shutter. The unattenuated light (ND = 0) was found to bleach a rhodopsin solution (2% LO, phosphate buffer) in the automated hammer-dropping apparatus at the rate of 2.4%/s. We estimate this is equivalent to bleaching \(7 \times 10^7\) rhodopsins/second per ROS.

In order to estimate how fast a retinal section was cooled by the hammer and tube assembly, a 36-gauge copper-constantan thermocouple was inserted through a small hole in the bottom of one of the 30-ml test tubes used in the experiments. A retinal section with Ringer's solution was placed over the thermocouple and a "cold" hammer dropped into the tube. The thermocouple registered \(-20^\circ\)C within 100 ms after the hammer hit the retina.

The condition and responsiveness of the retinal sections were monitored by recording their electroretinograms (ERGs) using an apparatus similar to one already described (Ebrey and Hood, 1973; Hood and Hock, 1973). In brief, the isolated retinal sections were placed in the above Ringer's solution in which 15 mM NaCl was replaced with 15 mM Na aspartate to isolate the receptor response. The retinal sections were placed in about 25 ml freshly oxygenated Ringer's solution in the same type petri dish as that used to store the retinal sections in the quick-freeze experiments. A pair of cotton-wick electrodes was placed above and below the retinal section while it was emersed in the Ringer's solution. The retinal sections were exposed to broad field illumination from a strobe flash after passing the light through a 500 nm interference filter and suitable neutral density filters. The ERGs appeared normal. After 25 min, the maximum time the isolated retinal sections were stored for the quick-freezing experiments, the amplitude of the ERGs of the retinal sections had decreased slightly, about 0.3 neutral density units from the maximum dark-adapted initial response. The latency of a response using light intensity comparable to a ND \(= 2\) light intensity in the cGMP experiment was \(\sim 50\) ms with the time to peak of \(\sim 300\) ms. Brighter lights gave shorter latencies. Although the ERG cannot be used to derive the exact kinetics of the rod outer segment's conductance changes, it should give a rough indication of how fast these changes must be.

**RESULTS**

Control experiments in which a retinal section was placed in a small amount of Ringer's solution in the bottom of a tube as was done in a quick-freezing experiment showed no significant decay in cGMP in the dark for 20 min (Table I). In our experiments the retinal sections were in a tube for less than 2 min. A comparison of dark-adapted values of picomole cGMP/milligram protein in retinal sections at the beginning and end of 12 sets of experiments showed a decrease of only 5% in those retinal sections that were stored longer. To account for animal-to-animal variation, the dark-adapted controls along with the retinal sections for each data point were done from the same frog; the control was frozen immediately prior to a light-exposed experiment. The
values of cGMP (picomole/milligram protein) were normalized to one so a comparison could be made among frogs. The actual dark value for the frog retinal cGMP concentrations in these experiments was found to be $44.3 \pm 0.7$ pmol cGMP/mg protein ($\pm$ SEM; 210 retinal samples) which corresponds to 170.9 $\pm 3.2$ pmol cGMP/retina.

The measurement of cGMP in retinal sections exposed to light and then quick-frozen to stop enzyme activity is shown in Fig. 1. Five different continuous light intensities were obtained by placing neutral density filters (ND --- 0, 2, 3, 4, and 5) in the light path. After a 1-s light exposure there is no significant change in cGMP levels at any light intensities used. Even at the highest intensity (ND --- 0), the first significant change in cGMP is not seen until 3–5 s. The time needed for one-half of the maximum decrease of cGMP is estimated to be 4 $\pm 1$ s for this light intensity (ND --- 0) and 14 $\pm 3$ s for the next most intense light (ND --- 2). These two intensities of light (ND --- 0 and 2) caused the cGMP to decay to about 70–75% of the dark-adapted level by 60 s.

Another striking feature of Fig. 1 is that at the lower light intensities (ND --- 3 or 4) which cause an initial decrease in cGMP, the cGMP level returned to its dark-adapted value even though the bleaching light remained on. With the ND --- 4 light intensity, the cGMP level recovered by 30 s while at the higher intensity (ND --- 3) it required about 60 s. At the time of minimal cGMP levels, about 20 s, the higher light intensity (ND --- 3) had about 75% of the dark-adapted level of cGMP while the lower intensity light dropped to only about 86%. With the lowest intensity light (ND --- 5) shown in Fig. 1, there is no significant change in cGMP levels from the dark levels with the exception of the point at 5 s. The value of the 5-s point is 0.86 $\pm 0.05$ (10 samples) of the normalized dark-adapted level, and probably indicates a significant decrease. If so, then the decrease in cGMP recovers by 10 s (see Fig. 1). These observations imply that the recovery of cGMP levels toward dark-adapted levels can occur in the light but is slower the greater the light intensity.

Using very dim light (ND --- 7) the value for the cGMP level after 5 s of illumination was 0.95 $\pm 0.11$ (five samples) of the normalized dark-adapted level, indicating no significant decrease. This result and the small change for the ND --- 5 filter indicate that the threshold for a measurable decrease is
somewhere between these two intensities which we estimate to bleach $7 \times 10^2$ and 7 rhodopsins/second per ROS.

**DISCUSSION**

Orr et al. (1976) found over 90% of the total cGMP in the rabbit retina concentrated in the photoreceptor layer. We estimate from their data that about 50% of the cGMP in the retina is in the ROS. We find about 170 pmol cGMP/frog retina, so assuming that about 50% of this is in the ROS and that there are about $10^6$ ROS per retina (Gordon and Hood, 1976), then we find about $5 \times 10^7$ cGMPs/ROS. Woodruff et al. (1977) estimate that in isolated bullfrog ROS there are 1–2 cGMPs per 100 rhodopsins and $3 \times 10^9$ rhodopsins/ROS which gives about $3–6 \times 10^7$ cGMPs/ROS. Moreover, Goridis et
al. (1977) found 25.0 pmol cGMP/mg protein in *Rana esculenta*. We find a similar value, 44.3 ± 0.7 pmol cGMP/mg protein in our frogs, *Rana catesbeiana*. Recently, de Azeredo et al. (1978) report 43.50 ± 2.26 pmol cGMP/mg dry weight in the ROS layer upon microdissection of frog (*Rana pipiens*) retina. We estimate this value leads to about 11 µM cGMP in the ROS or about 1 × 10⁷ cGMPs/ROS. Thus, our levels of cGMP in the ROS are in fair agreement with these others despite different manipulations of the ROS.

How much of the cGMP decrease we see in the retina is due to decreases in the ROS's cGMP is unclear. Orr et al. (1976) found that cGMP in the ROS layer of rabbit retinas decreased about 30% after 1 h in the light; we estimate from their data that about 40% of the total decrease of cGMP in the retina is from the decrease in the ROS. de Azeredo et al. (1978), using frog (*Rana pipiens*) retinas, found a larger decrease of cGMP levels (70%) in the ROS layer. Although these results give some indication that a substantial percent of the total decrease of cGMP of the retina is due to decrease in the ROS's cGMP, they do not reflect any differences in the rate of decrease in cGMP in the various retinal layers. For example the ROS's cGMP could be a disproportionately large fraction of the retinal cGMP decrease that occurs during the initial second after illumination. Moreover, there may be two distinct pools of cGMP in the ROS, a small one which changes rapidly and a larger one which changes slowly. Our results must be viewed cautiously in light of these considerations. We find the retinal cGMP levels decrease about 25% after 15 s of illumination with a decay half-time of about 4 s with the brightest light (ND = 0). If all the initial decrease we see in retinal cGMP is entirely in the ROS, then the decay rate is about 6% of the ROS's cGMP per second, or about 3 × 10⁶ cGMPs/ROS per second. If only 50% of the initial decrease we see in retinal cGMP is from the ROS, then this decay would be reduced by half.

We detected a significant decrease from the dark-adapted level of cGMP only after several seconds. This result is surprising because it appears there should be enough light-stimulated PDE activity in the ROS to hydrolyze all the cGMP in the ROS much more rapidly than this. Miki et al. (1975) found a $V_{\text{max}}$ of 48,000 mol cGMP hydrolyzed/min per mol enzyme with a ratio of 930 rhodopsins per PDE and with a $K_{\text{m}}$ of 70 µM. From these values we estimate a $V_{\text{max}}$ of 2 × 10⁸ cGMPs/s per ROS and a $K_{\text{m}}$ of 3 × 10⁵ cGMPs/ROS. These numbers are probably conservative estimates of the maximum PDE activity.¹ Using them in the integrated Michaelis-Menten equation and assuming all the PDE is activated immediately, it can be shown that it should take <25 ms for the cGMP to be reduced by 50% from an initial concentration of 5 × 10⁷ cGMPs/ROS. We are unable to explain this apparent discrepancy between the calculated rate of cGMP hydrolysis and what we actually measure. Perhaps our result is due to there being only a small pool of cGMP accessible to the high activity PDE. Another possibility is that the activation of the PDE may not solely be due to the availability of bleached rhodopsins, but in vivo is controlled by some rate-limiting factor which is not rate-limiting in the biochemical experiments. For example, PDE activation in rod disk

preparations has been shown to be delayed for several seconds with low GTP concentrations (Yee and Liebman, 1978).

Our result of no significant decay in cGMP after 1 s of illumination is in apparent disagreement with the experiments of Woodruff et al. (1977). In isolated frog ROS they found decreases of 20–25% in cGMP levels within 200–400 ms. They stopped the cGMP-consuming reactions by rapidly mixing the ROS with PCA; this procedure may not rapidly stop an enzyme reaction occurring inside the rod's plasma membrane. However, a more recent report by that group (Bownds et al., 1978) confirms a latency for cGMP decrease of 50 ms and a half-time of 125 ms. Another difference between our experiments and Woodruff et al. (1977) is that they used low-calcium (i.e., EGTA) Ringer's solution; Cohen et al. (1978) has shown this can affect the cGMP levels in the retina. Yet another reason for the difference in results could be the difference between the isolated frog ROS preparation of Woodruff et al., which may have alterations in its normal pools of metabolites and our retinal preparation which may more accurately reflect in vivo conditions.

Fig. 1 shows cGMP levels recover to dark-adapted levels within 30–60 s under continuous low intensity illumination (ND = 3, 4, and 5). Previous work has not found recovery of cGMP levels in the dark or in the light in the retina (Goridis et al., 1974; Goridis et al., 1977) perhaps because of higher light levels than those needed to observe recovery was used. The origin of the recovery we observed is unknown, but it might be due to the switching of the PDE from a high activity form to low activity form even though the light remains on. In such a case, the cGMP level in the ROS wouldn't go to zero if the hypothetical decay of PDE activity is rapid enough. Recently, Yee and Liebman (1978) and Liebman (1979) in their ROS disk experiments have presented some evidence for a lowering of PDE activity after initial activation.

In the rod photoreceptor, Yoshikami and Hagins (1973) have proposed an internal excitation transmitter which carries the light signal between the bleached rhodopsin and the permeability sites on the plasma membrane. Yoshikami and Hagins (1973) suggested calcium as a good candidate for the internal transmitter. Cyclic nucleotides have also been suggested as an internal transmitter in photoreceptors (see review by Hubbell and Bownds, 1979). One of the criteria a candidate for an internal transmitter should fulfill is that its concentration should undergo significant changes within several hundred milliseconds after light absorption, the approximate time range of the receptor potential (Baylor et al., 1974). As noted above, we found only significant cGMP level changes only after more than 1 s of illumination. Moreover, with our brightest light, which would saturate the electrical response, we estimate the maximum rate of decrease to be $1-3 \times 10^6$ cGMPs hydrolyzed/s per ROS, or about 0.6% of the cGMP in the ROS hydrolyzed during 100 ms. So the cGMP decrease we observed is probably too slow by more than an order of magnitude to be involved as an internal excitation transmitter.

Another property an internal transmitter must have is a high signal-to-noise ratio at low light intensities, since a photoreceptor can respond to single photon events (Hecht et al., 1942). When an attempt is made in the ROS to create a low concentration of cGMP due to local high PDE activity triggered
by a single photon, cGMP from the surrounding region will diffuse into this area of low concentration (see negative transmitter in Fig. 2 A). This diffusion will tend to wash out any large decrease in local transmitter concentration. Thus, it is somewhat difficult to see how one photon, even if it activated enzymes which lead to the hydrolysis of all the cGMP between two disks, could affect the sodium permeability sites associated with the ~20–60 disks that are shut off by one photon (see Cone, 1973; Yoshikami and Hagins, 1973). In the case of the release of a positive transmitter released against a low background (see Fig. 2 B) it is easier to obtain a high signal-to-noise ratio. However, the possibility of a negative transmitter cannot yet be dismissed.

In summary, there appears to be some serious problems with cGMP as a candidate for an internal excitation transmitter in vertebrate rods. However, cGMP as well as other transmitter candidates such as 5' GMP should be considered as possible adaptive signal transmitters. The time scale of cGMP recovery that we found in dim light is reminiscent of light adaptation in the cold-blooded vertebrate rod (gecko: Kleinschmidt and Dowling, 1975; toad:...
Moreover, a depletion signal like cGMP which is averaged over a large portion of the ROS could serve adaptative processes well.

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REFERENCES


