

Calcium Effects on Frog Retinal Cyclic Guanosine 3', 5'-Monophosphate Levels and Their Light-Initiated Rate of Decay

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ABSTRACT When retinal sections were isolated from dark-adapted bullfrogs and placed in normal Ringer's solution, they contained 40.7 ± 0.2 pmol cGMP/mg protein (mean \pm SEM, 30 samples). When isolated, dark-adapted retinal sections were removed from normal Ringer's solution and placed in calcium-deficient Ringer's solution with 3 mM EGTA, there was about a threefold rise in cyclic GMP (cGMP) levels by 1.5 min and about a 10-fold rise by 5 min. The cGMP level remained high with no detectable decrease for at least 40 min (the longest time measured). When isolated, dark-adapted retinal sections were removed from normal Ringer's solution and placed in Ringer's solution which contained high-calcium (20 mM CaCl_2), there was a slow but significant decrease in cGMP levels. After 20 min in high-calcium Ringer's solution the cGMP level was 0.58 ± 0.07 (mean \pm SEM, eight samples) of the cGMP level in normal Ringer's solution incubated for the same time. The rate at which 10-fold elevated cGMP levels in low calcium decreased upon illumination was examined using quick-freezing techniques on the retinal sections. The elevated cGMP level in retinal sections incubated in low-calcium Ringer's solution was found to decay about 15-fold faster than cGMP levels in retinal sections incubated in normal Ringer's solution. The cGMP level in low calcium was significantly different ($P = 0.005$) after 1 s illumination, whereas the cGMP level in normal calcium was not significantly different.

INTRODUCTION

There are several possible roles for a light-regulated cyclic guanosine 3',5'-monophosphate (cGMP) level in the vertebrate photoreceptor:

- (a) cGMP could be involved in light excitation of the photoreceptor (see review by Hubbell and Bownds, 1979), perhaps in some intermediate role between the bleaching of rhodopsin and the closure of sodium channels on the plasma membrane;
- (b) cGMP could be involved in the adaptation of the photoreceptor. Thus, cGMP could serve to regulate the photoreceptor's sensitivity to light;
- (c) cGMP could be involved in a light-regulated metabolism not directly

involved in the photoreceptor light response (Lolley et al., 1977). For example, cGMP could regulate the rod outer segment (ROS) renewal process in which new disk membranes are added at the bottom of a ROS and eliminated at the ROS apex. The rate of the renewal process has been shown to be light sensitive (Young, 1967; Young and Bok, 1969). Another possible metabolic role could be the maintenance of ionic gradients across the disk or plasma membrane. Cyclic nucleotides have been found to be involved with the regulation of calcium levels across membranes in other systems (see Berridge, 1975);

(d) cGMP could be involved in some yet unknown process. cGMP could well serve in more than one of the above four possible roles.

In deciding which of the above possibilities cGMP could be involved in, it is important to know how fast the cGMP levels are changing after photon absorption in the ROS. The involvement of cGMP in the light excitation process would demand a rapid change in cGMP at its site of action before the plasma membrane potential of the ROS is affected. In an earlier study (Kilbride and Ebrey, 1979), the cGMP levels in isolated frog retinas were found not changed significantly 1 s after the initiation of illumination even with the brightest light used (ca. 7×10^7 rhodopsins bleached/s per ROS). The first significant decrease in cGMP from dark-adapted levels was detected only 3–5 s after the initiation of illumination (Kilbride and Ebrey, 1979). Our result of no significant cGMP level decay after 1 s of illumination is in apparent disagreement with other work on somewhat different preparations (Woodruff et al., 1977; Yee and Liebman, 1978; Woodruff and Bownds, 1979; Liebman and Pugh, 1979). Woodruff and Bownds (1979) found in isolated frog ROS a 35% decrease in cGMP levels with a half-time of 125 ms with a saturating light. Yee and Liebman (1978) found, using rod disk preparations, a proton release accompanying cGMP hydrolysis could be detected within 100 ms after a bright light flash; these experiments were done with saturating cGMP concentrations and in the presence of high GTP concentrations. Interestingly, Cohen et al. (1978) found that incubation of mouse retinas in darkness (dim red light) with calcium-deficient Earle's medium with 3 mM EGTA caused a 10–20-fold increase in the cGMP level, peaking at 2–3 min, but no change occurred in cAMP levels. Congenic rodless (retinal dystrophy/retinal dystrophy) mouse retinas have < 5% of control cGMP and were found (Cohen et al., 1978) to have only traces of calcium sensitivity; thus, Cohen et al. (1978) concluded that the changes in cGMP are likely to largely be occurring in rods. In the following study the effects of varying the calcium concentration of the Ringer's solution bathing retinal sections on the retinal cGMP levels and their decay rate after photon absorption were examined. These results may help to clarify the different rates of cGMP level changes observed by different authors.

METHODS

The isolation of the retinal sections and the quick-freezing techniques were similar to those described before (Kilbride and Ebrey, 1979). In the present experiments, bullfrogs (*Rana catesbeiana*) were dark-adapted for at least 12 h before use. To avoid

the bleaching of rhodopsin which may trigger the extremely light-sensitive phosphodiesterase (PDE) activity, all operations up until the retina is frozen were carried out with the use of an infrared image converter (FJW Industries, Mt. Prospect, Ill.). To obtain an isolated retina, a frog was pithed, and 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 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 separate petri dishes. The Ringer's solution was continuously oxygenated in all experiments except during the time the retinal sections were being teased away from the pigment epithelium (~6 min).

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 ethanesulfonic acid), and 5.6 mM glucose adjusted to pH 7.5 with NaOH (Kilbride and Ebrey, 1979). In some of the Ringer's solutions the calcium content was varied. For high calcium solutions, additional CaCl₂ was added to bring the calcium concentration to 20 mM; the remaining chemicals were unchanged. For low calcium solutions, 3 mM EGTA (ethyleneglycol-bis- β -aminoethyl ether) *N,N'*-tetraacetic acid) was added to "calcium-free" Ringer's solution; the remaining chemicals were unchanged. This low calcium solution should contain approximately 10⁻⁹ M Ca²⁺ (Lipton et al., 1977).

In the present experiments, to examine the effects of varying the calcium concentration in the Ringer's solution bathing the retinal sections, the retinal sections were first isolated in normal Ringer's solution (containing 1.6 mM CaCl₂). Then when all the retinal sections had been isolated, they were transferred by small spoons (ca. 0.4 ml) first to a large petri dish containing at least 60 ml of Ringer's solution having a modified calcium concentration. In every case except for the first two points in Fig. 2 (where the sections were in the high calcium Ringer's solution for 1 min or less), they were then transferred to a second smaller storage petri dish (ca. 25 ml) of the same oxygenated Ringer's solution and incubated with continuous oxygenation in a light-tight chamber. At appropriate times, the retinal sections were transferred by a spoon to a large test tube (30-ml Corex, Corning Glass Works, Corning, N.Y.) and quick-frozen with liquid nitrogen cooled metal hammers (Kilbride and Ebrey, 1979). In the following experiments, to account for animal to animal variation, comparisons were made between cGMP levels (picomole cGMP/milligram protein) of retinal sections from the same frog with the cGMP level of the control retinal section being normalized to one. The retinal sections from one frog were randomly chosen with respect to left vs. right eye and with respect to the line of hemisection of the retina.

After a retinal section was quick-frozen with a "cold" hammer, the tube containing the frozen retinal section with hammer was placed in an ethanol-dry ice bath (195°K). After all sample tubes were in the 195°K bath, the room lights were turned on. The tube-hammer assembly was allowed to warm up slowly (several hours) in the presence of perchloric acid (PCA) to denature any protein present. The protein and cGMP were assayed as described before (Kilbride and Ebrey, 1979).

RESULTS

When isolated, dark-adapted retinal sections were removed from normal Ringer's solution and placed in modified Ringer's solution in which calcium was not added and which also contained 3 mM EGTA, there was about a 10-fold rise in cGMP levels (Fig. 1). The cGMP levels rose noticeably higher (ca. threefold) at 1.5 min and by about 5 min the maximum was reached. The

cGMP remained high with no detectable decrease for at least 40 min (the longest time measured). As a control for EGTA effects in the presence of normal free calcium concentrations (1.6 mM Ca^{2+}), a modified Ringer's solution containing 3 mM EGTA with 4.6 mM CaCl_2 was used. In this case, there was no significant change in the cGMP levels compared to retinal sections in normal Ringer's solution.

When isolated, dark-adapted retinal sections were removed from normal Ringer's solution and placed in Ringer's solution which contained high calcium (20 mM CaCl_2), there was a slow but significant decrease in cGMP levels (Fig. 2). In these high calcium experiments, the values for the cGMP concentration were found to be more erratic (Fig. 2). The only statistically

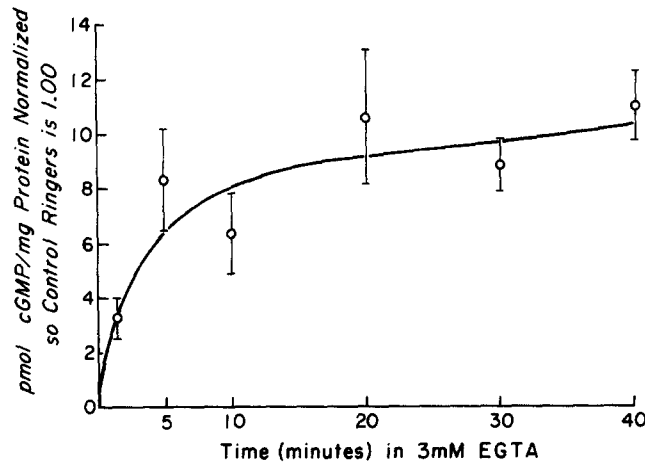


FIGURE 1. Concentrations of cGMP (picomole cGMP/milligram protein) as a function of dark-adapted retinal section's incubation time (minutes) in low calcium concentration with 3 mM EGTA Ringer's solution normalized so the cGMP values of dark-adapted retinal sections incubated in normal Ringer's solution are one. The points represent the mean \pm SEM with an average of four EGTA-treated retinal sections assayed per point.

significant difference ($P < 0.001$) was found after 20 min in high calcium Ringer's solution with a value of 0.58 ± 0.07 (mean \pm SEM, eight samples) of the cGMP level in normal calcium incubated for the same time. The cGMP may be decreasing sooner, as shown by the line drawn in Fig. 2, but, because of variation in this data, this is uncertain.

Since the cGMP levels of dark-adapted retinal sections were greatly (10-fold) increased in low calcium solutions, I was interested in how fast the elevated cGMP levels would decay upon illumination of the retinal sections. For these experiments, to minimize as much as possible the time between when the dark- and light-adapted retinal sections were frozen, a light-tight box was built which contained an automated "cold" hammer-dropping apparatus to allow a dark-adapted retinal section to be frozen simultaneously

with a light-exposed but otherwise similar retinal section. The measurements of cGMP levels in normal calcium and EGTA-treated retinal sections exposed to light and then quick-frozen to stop enzyme activity are shown in Fig. 3. The initial cGMP levels were taken to be the average of the dark-adapted values of retinal sections in either low or normal calcium Ringer's solution; the cGMP levels from the illuminated retinal sections were normalized to these average values. The cGMP levels for the normal calcium- and low calcium- (3 mM EGTA) treated retinal sections were found to be 40.7 ± 0.2 (mean \pm SEM, 30 samples) and 492.2 ± 18.4 (mean \pm SEM, 43 samples) pmol cGMP/mg protein, respectively. Although there is a large cGMP level increase in low calcium, Fig. 3 gives some perspective on how much faster the

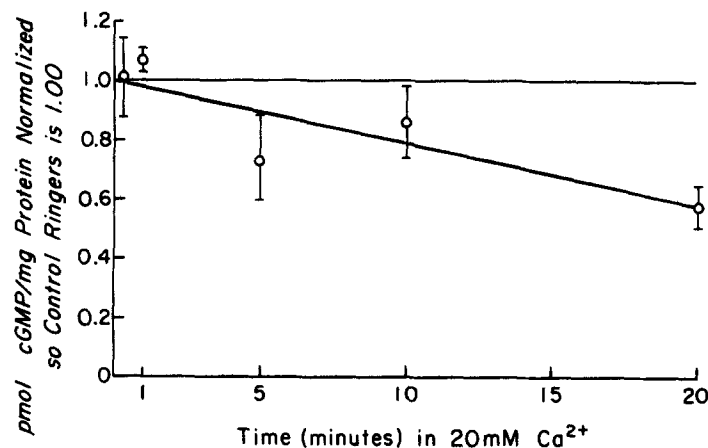


FIGURE 2. Concentrations of cGMP (picomole cGMP/milligram protein) as a function of dark-adapted retinal section's incubation time (minutes) in high calcium concentration (20 mM Ca²⁺) Ringer's solution normalized so that the cGMP values of dark-adapted retinal sections incubated in normal Ringer's solution are one. The points represent the mean \pm SEM with an average of six high calcium concentration-treated retinal sections assayed per point.

cGMP levels decays from this initial high value. A very rough estimate indicates the cGMP in retinal sections is decaying about 15-fold faster, from its initial high value, in low calcium than in normal calcium. The decrease of cGMP in EGTA with 0.3 s of illumination (neutral density [ND] = 0) may be significantly different ($P = 0.08$) with a cGMP level of 0.95 ± 0.09 (mean \pm SEM, 16 samples) of the normalized control (dark-adapted, EGTA-treated retinal sections). The decrease in cGMP levels in EGTA with ND = 0, at 1 s after initiation of illumination, is definitely significantly different ($P = 0.005$) with a cGMP level of 0.84 ± 0.04 (mean \pm SEM, 10 samples) of the normalized control. The cGMP level in EGTA with ND = 0 has dropped to a value of about 25% of the normalized control after 60 s of illumination. With a less intense light (ND = 4) in EGTA, there may be a significant (P

= 0.19) decrease in cGMP level with 1 s of illumination with a cGMP level of 0.87 ± 0.08 (mean \pm SEM, five samples) of the normalized control. The (ND = 4) curve as a whole indicates a definite difference between matched pairs of light- and dark-adapted, EGTA-treated retinal sections. The cGMP levels with this dimmer light (ND = 4) decays only to a value (ca. 75% of the

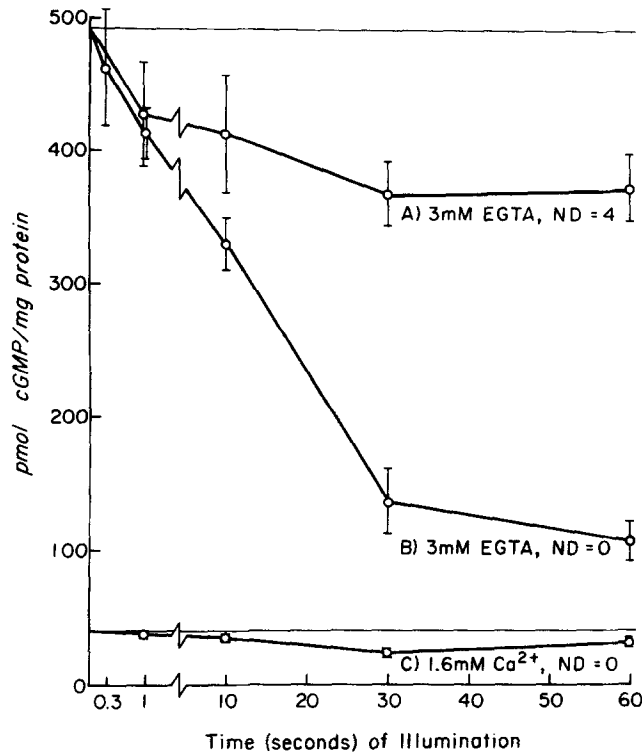


FIGURE 3. Concentration of cGMP (picomole cGMP/milligram protein) as a function of light exposure(s) in retinal sections normalized to the average cGMP value of dark-adapted, similarly calcium-treated retinal sections. The retinal sections were incubated in two different calcium concentration Ringer's solutions and the light intensity was attenuated by two different neutral density filters (A) 3 mM EGTA, ND = 4, (B) 3 mM EGTA, ND = 0, and (C) 1.6 mM Ca²⁺, ND = 0. The points represent the mean \pm SEM with an average in each case of (A) four, (B) eight, and (C) seven light-exposed retinal sections assayed per point. ND = 0 corresponds to $\sim 7 \times 10^7$ rhodopsins bleached/second/ROS.

control) about threefold higher than the brighter light (ND = 0) at 60 s. The result of the cGMP level changes in normal calcium Ringer's solution is similar to earlier work (Kilbride and Ebrey, 1979). There was no significant change after 1 s of illumination (ND = 0) in the normal calcium Ringer's solution (Fig. 3).

DISCUSSION

A large percentage of the cGMP level changes that I observed in the retina should be the cGMP in the photoreceptors, since Orr et al. (1976) found that over 90% of the total cGMP in the rabbit retina concentrated in the photoreceptor layer. Their data also indicates ~ 50% of the cGMP in the retina is in the ROS. Moreover, in low calcium ($\sim 10^{-9}$ M Ca^{2+}) Ringer's solution synaptic transmission is probably blocked (Douglas, 1968; Katz, 1969) which should mean light-induced changes in cGMP levels would only be in the photoreceptors.

The response to light of these retinal sections in Ringer's solution appears normal (Kilbride and Ebrey, 1979). However, Lipton et al. (1977) found prolonged exposure (7-15 min) of a *Bufo marinus* retina to low calcium (10^{-9} M) Ringer's solution caused the photoreceptors to become unresponsive. Lipton et al. (1977) found, that although light responsiveness was lost, the cells did not appear to die during this procedure since the membrane potential was maintained. Moreover, these workers routinely recorded normal rod responses elsewhere in the retina after 15 min of control Ringer's solution washout. Lipton et al. (1977) suggest the failure to recover after prolonged calcium exposure may be a result of the electrode penetration of the cell and not simply to low calcium treatment. These results indicate that in the present experiments, although the photoreceptors do not give a physiologically normal light response, the cells are probably viable. Indeed the change in cGMP levels in low calcium is one possible explanation for the change in the light responsiveness of the photoreceptor.

Woodruff and Bownds (1979) found a faster decay of cGMP levels in isolated frog ROS than we (Kilbride and Ebrey, 1979) found in retinal sections; this could be the result of their using a low calcium ($\sim 10^{-8}$ M Ca^{2+}) Ringer's solution in their quick-stopping experiments. Cohen et al. (1978) and my work (Fig. 1) show that low calcium ($\sim 10^{-9}$ to 10^{-8} M Ca^{2+}) solutions increase retinal cGMP levels about 10-fold. The increased cGMP level caused by low calcium is found to decay faster than the cGMP levels in normal Ringer's solution (Fig. 3). However, Woodruff and Bownds (1979) found only about a 25% increase in cGMP levels in ROS isolated in low calcium ($\sim 10^{-8}$ M Ca^{2+}) over ROS isolated in normal calcium (10^{-3} M Ca^{2+}). This may indicate that the cGMP metabolism has been altered within the isolated ROS in low calcium.

Yee and Liebman (1978) found a faster onset of phosphodiesterase (PDE) activity in rod disk preparations than we (Kilbride and Ebrey, 1979) found in retinal sections; this may also be the result of the difference in the preparations. They (Yee and Liebman, 1979) monitor the PDE activity by adding cGMP to their rod disks suspended in low calcium medium; whether this added cGMP is a valid monitor of intrinsic cGMP in the ROS is moot. Even if it is, the measurement of short lag times for increased PDE activity has been done with very high cGMP (1-2 mM) concentrations when compared to the physiological concentration of 60 μM (Kilbride and Ebrey, 1979), which is close to the PDE k_m of 70 μM (Miki et al., 1975; Yee and Liebman, 1978).

The estimate of 60 μM cGMP concentration assumes the cGMP is evenly distributed throughout the ROS. Possible compartmentalization of cGMP within the ROS could complicate estimates of the cGMP accessible to the PDE, which is especially important when the concentration of the cGMP is close to the k_m for PDE. Indeed because of complications due to compartmentalization of cGMP within the ROS, I believe the data does not yet warrant ruling out cGMP as an internal transmitter for phototransduction (see also Kilbride and Ebrey, 1979).

In summary, my finding in Fig. 3 that the 12-fold-elevated cGMP level in retinal sections incubated in low calcium Ringer's solution decays in the light \sim 15-fold faster than retinal sections in physiological Ringer's solution could be due to substrate concentration effects and/or altered metabolism caused by low calcium. Moreover, Yee and Liebman's (1978) finding of rapid (ca. 100 ms) onset of increased PDE activity with large amounts of externally added cGMP (1-2 mM) may also be due to substrate concentration and/or low calcium effects.

In this study, as in a previous work (Kilbride and Ebrey, 1979), cGMP levels in intact retinal sections were used to closely approximate the *in vivo* situation. Here, I have shown that very low calcium concentrations (10^{-9} M) cause a much more rapid decay of cGMP levels than in the presence of physiological calcium concentrations (10^{-3} M).

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