

Influence of Calcium on Guanosine 3',5'-Cyclic Monophosphate Levels in Frog Rod Outer Segments

ARTHUR S. POLANS, SATORU KAWAMURA, and
M. DERIC BOWNDS

From the Laboratory of Molecular Biology, the Department of Zoology, and the Neurosciences Training Program, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT In the presence of 10^{-9} M calcium, rod outer segments freshly detached from dark-adapted frog retinas contain between 0.01 and 0.02 moles of guanosine 3',5'-cyclic monophosphate (cyclic GMP) per mole of rhodopsin. The dark level of cyclic GMP is reduced ~50% by illumination that bleaches 5×10^5 rhodopsin molecules/outer segment-s. The dark levels of cyclic GMP also can be suppressed to ~0.007 mol/mol of rhodopsin by increasing the concentration of calcium from 10^{-9} M to 2×10^{-9} M, and they remain at this level as calcium concentration is raised to 10^{-3} M. The final level to which illumination reduces cyclic GMP is unaffected by the calcium concentration between 10^{-9} and 10^{-3} M. The maximal light-induced decrease in cyclic GMP occurs within 1 s from the onset of illumination at all calcium concentrations. The magnitude and time-course of the light-induced decrease in cyclic GMP measured in these experiments are comparable to values obtained previously (Woodruff et al. 1977. *J. Gen. Physiol.* **69**:677-679; Woodruff and Bownds. 1979. *J. Gen. Physiol.* **73**:629-653). The data are consistent with a role for cyclic GMP in visual transduction irrespective of the calcium concentration.

INTRODUCTION

Studies of vertebrate rod photoreceptors have suggested that guanosine 3',5'-cyclic monophosphate (cyclic GMP) may function as an internal transmitter mediating between photon absorption by rhodopsin and the permeability decrease of the outer segment plasma membrane (Woodruff and Bownds, 1979; Yee and Liebman, 1979; Miller and Nicol, 1979; for a review of earlier work see Hubbell and Bownds [1979]). Interest has recently focused on the kinetics of the light-induced cyclic GMP decrease measured in rod outer segments, because a slow rate might argue against a direct role for cyclic GMP in the transduction process.

In two recent studies (Kilbride and Ebrey, 1979; Kilbride, 1980), cyclic GMP levels were measured in retinal sections with a quick-freezing technique. This work raised the possibility that the rapid, light-sensitive decrease in cyclic GMP observed in isolated rod outer segments (Woodruff et al., 1977; Woodruff

and Bownds, 1979) might occur only at low calcium concentrations and be much slower at millimolar levels. This suggestion arose from the observation that a cyclic GMP decrease measured in retinal sections could not be detected 1 s after illumination in Ringer's solution containing 1.6 mM calcium but was observed when the calcium concentration was lowered by the addition 3 mM ethyleneglycol-*bis*(β -aminoethyl ether)-*N,N*-tetraacetic acid (EGTA). At either calcium level, the cyclic GMP decrease measured in retinal sections is slower than the corresponding change measured in isolated outer segments.

The experiments presented here demonstrate that in isolated outer segments the dark level of cyclic GMP decreases as the calcium concentration is increased from 10^{-9} to 10^{-3} M, but the final level to which cyclic GMP is suppressed by saturating illumination does not change. Furthermore, the maximum suppression of cyclic GMP levels occurs within 1 s from the onset of the light stimulus, regardless of the calcium concentration. Therefore, the different time-courses of the cyclic GMP decreases measured in isolated outer segments and retinal sections cannot be attributed to calcium concentration.

MATERIALS AND METHODS

Bullfrogs, *Rana catesbeiana*, were kept in holding tanks and conditioned to the photoperiod described by Woodruff and Bownds (1979) for ~1–3 wk before use. Animals were removed from the holding tanks during the dark cycle and further dark-adapted at least 1 h before they were killed. All darkroom manipulations were performed at room temperature, under infrared illumination, with an image converter (F.J.W. Industries, Mt. Prospect, Ill.). Eyes were removed and dissected as described by Woodruff et al. (1977). Retinas were rinsed in a Ringer's solution (115 mM NaCl, 2 mM KCl, 2 mM MgCl₂, 0.1 mM CaCl₂, 1 mM dithiothreitol, and 10 mM *N*-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid [HEPES], pH 7.5) and then gently agitated for 2 min in ~500 μ l Ringer's solution to detach rod outer segments. Portions of the outer segment suspension were adjusted to the correct calcium concentration by mixing with Ringer's solution containing appropriate amounts of EGTA according to the calculations given by Caldwell (1970). The apparent affinity constants used were $K'_{Ca} = 4.70 \times 10^7$ and $k'_{Mg} = 1.76 \times 10^2$. EGTA was added to the Ringer's solution as follows: for 10^{-9} M Ca⁺², 2.78 mM EGTA; for 2×10^{-9} M Ca⁺², 1.49 mM EGTA; for 10^{-8} M Ca⁺², 0.386 mM EGTA; for 10^{-7} M Ca⁺², 0.129 mM EGTA; for 10^{-6} M Ca⁺², 0.102 mM EGTA; for 10^{-5} M Ca⁺², 0.090 mM EGTA. The EGTA concentration was doubled in experiments in which outer segments in Ringer's solution were diluted 1:1 with Ringer's solution containing EGTA. All solutions were maintained at pH 7.5. The calcium concentration of some solutions was verified with a calcium ion electrode (model F2112Ca, Radiometer A/S, Copenhagen). To test the possibility that EGTA might exert an influence on the system independent of its calcium buffering function, starting concentrations of 0.1, 0.2, and 1.0 mM calcium were used, and appropriate amounts of EGTA were added to obtain the free calcium concentrations given in Figs. 1 and 2. Cyclic GMP levels at a given calcium concentration were not significantly influenced by the differing EGTA levels. The calcium-induced changes in the concentration of cyclic GMP given in Results were rapid and complete by the time the first points were quenched (in some experiments only 1–2 min after the calcium concentration was adjusted). Cyclic GMP was measured during a period when levels were stable, as noted in the legend of Fig. 1. Aliquots (50 μ l) from each outer segment suspension were either kept dark or exposed

to a light bleaching a known amount of rhodopsin. Light intensity was calibrated as described by Brodie and Bownds (1976). Portions (50 μ l) of the outer segment suspension were used to determine rhodopsin concentration by difference spectroscopy after the outer segments had been dissolved in 200 μ l of 0.04 M hexadecyltrimethyl ammonium chloride (Bownds et al., 1971). Experimental samples were quenched by the addition of 100 μ l of 9% perchloric acid (PCA). In the experiments of Fig. 2, samples were quenched rapidly and simultaneously using disposable pipets containing PCA as described by Woodruff and Bownds (1979). Cyclic GMP levels were measured as described by Woodruff et al. (1977) with the radioimmunoassay technique of Steiner et al. (1972) as modified by Weinryb et al. (1972).

RESULTS

In the dark, the amount of cyclic GMP contained in a suspension of isolated outer segments varies with the calcium concentration. Fig. 1 demonstrates that in low calcium (10^{-9} M) outer segments contain between 0.01 and 0.02 mol of cyclic GMP/mol rhodopsin. This range of cyclic GMP content is the

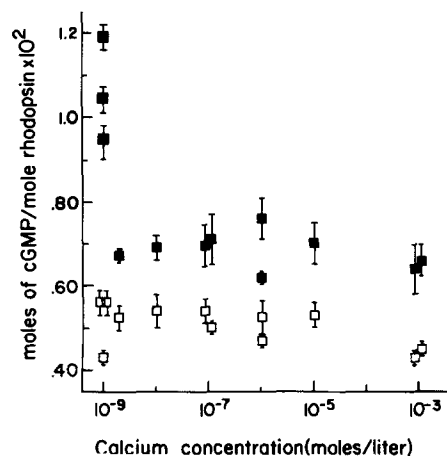


FIGURE 1. Dark and light levels of cyclic GMP as a function of calcium concentration. Portions of a dark-adapted outer segment suspension are adjusted to the indicated calcium concentrations by mixing with a Ringer's solution containing appropriate amounts of EGTA (see Methods). Triplicate samples (50 μ l) from each condition were either kept dark or exposed to 30 s of light bleaching 5.0×10^5 rhodopsin molecules/outer segment-s. Dark points were quenched before and after light points to ensure that no appreciable decay of cyclic GMP levels occurred during the measurements. Aliquots (50 μ l) from each condition were also taken to determine the rhodopsin concentration as described in Methods. Samples were quenched with 100 μ l of 9% perchloric acid and assayed for cyclic GMP content by a radioimmunoassay technique. Presented in this figure are the accumulative results from five separate experiments, dark (■) and light (□) points, respectively. Each point in the figure represents three determinations from a single experiment, mean \pm SEM. In both this figure and Fig. 2, outer segment samples were quenched with acid between 10 and 20 min after detachment of outer segments from the retina, when cyclic GMP levels are most stable (Woodruff et al., 1977).

same as that obtained by Woodruff et al. (1977), although it should be noted that in most experiments reported here the concentration of cyclic GMP resided toward the lower end of this range (0.0118 ± 0.0005 mol of cyclic GMP/mol rhodopsin, mean \pm SEM, $n = 8$). This probably is caused by the absence of 10% (vol/vol) calf serum in the Ringer's solution used in these experiments. Serum minimizes the breakage of outer segments and the subsequent hydrolysis of cyclic GMP that occurs in disrupted outer segments (Woodruff et al., 1977; Woodruff and Bownds, 1979). Serum was omitted from these experiments to accurately adjust the calcium concentration. In 11 experiments performed in the presence of serum and 10^{-9} M calcium, outer segments contained 0.0173 ± 0.0007 mol of cyclic GMP/mol rhodopsin in the dark (mean \pm SEM).

The dark level of cyclic GMP is reduced $\sim 30\text{--}40\%$ by increasing the external calcium concentration from 10^{-9} to 2×10^{-9} M, and it remains at that level up to millimolar calcium concentration. Fig. 1 shows that calcium in the concentration range of 2×10^{-9} to 10^{-3} M lowers the dark level of cyclic GMP to ~ 0.0068 mol of cyclic GMP/mol rhodopsin. At all concentrations of calcium examined, saturating illumination suppressed the level of cyclic GMP to ~ 0.0055 mol of cyclic GMP/mol rhodopsin.

The light-induced decrease in cyclic GMP occurs within 1 s from the onset of illumination, regardless of the calcium concentration. In the experiments of Fig. 2, samples containing 10^{-9} and 10^{-6} M calcium were simultaneously quenched in the dark and at the times indicated after the onset of illumination. In the presence of 10^{-9} M calcium, the average dark level of cyclic GMP was 0.0093 ± 0.0014 mol of cyclic GMP/mol rhodopsin (mean \pm SEM, $n = 9$). In these experiments, the level of cyclic GMP was reduced $\sim 25\%$ in the presence of 10^{-6} M calcium. Illumination completely suppressed the level of cyclic GMP at each calcium concentration within 1 s. There was no further decrease in the levels of cyclic GMP at either calcium concentration measured between 1 and 10 s after the onset of illumination. The same results were obtained at 10^{-3} M calcium with the protocol given in the legend of Fig. 2. The dark level of cyclic GMP in the presence of 10^{-3} M calcium was 0.0074 ± 0.0002 mol of cyclic GMP/mol rhodopsin (mean \pm SEM, $n = 3$). Within 1 s of illumination the concentration of cyclic GMP was suppressed to a level of 0.0056 ± 0.0007 mol of cyclic GMP/mol rhodopsin (mean \pm SEM, $n = 3$) and did not decrease further over the next 10 s (0.0061 mol of cyclic GMP/mol rhodopsin, duplicate points).

The magnitude and time-course of the light-induced decrease in cyclic GMP in these experiments matches closely the values obtained previously by Woodruff et al. (1977) and Woodruff and Bownds (1979).

DISCUSSION

These experiments demonstrate that raising the calcium concentration to above 10^{-9} M results in a decrease in the dark cyclic GMP level in isolated rod outer segments. The resultant dark level of cyclic GMP does not vary significantly between 2×10^{-9} and 10^{-3} M calcium. The dark activity of

phosphodiesterase is enhanced by raising the calcium concentration from 10^{-9} to 10^{-6} M, and this might be responsible for the observed decrease in the level of cyclic GMP (Robinson et al., 1980). (It should be noted that Woodruff and Bownds [1979] and we have observed a smaller effect of calcium concentration on cyclic GMP levels in isolated outer segments than reported for retina (Cohen et al., 1978; Kilbride, 1980). This may reflect differences in the metabolism of cyclic GMP in the two preparations. Another possibility is that elevated cyclic GMP levels in retina in low calcium are not confined to the outer segments and also may reflect changes in the inner segments.) Illumi-

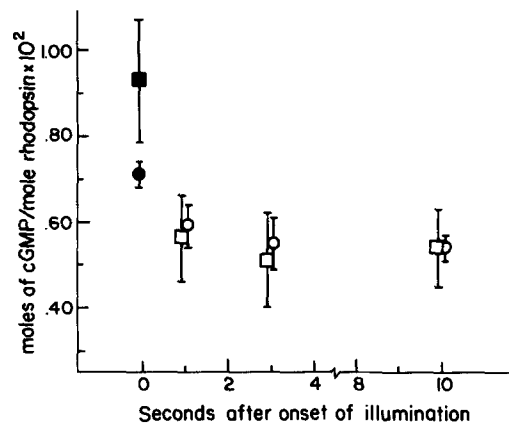


FIGURE 2. Time-course of the light-induced cyclic GMP decrease as a function of calcium concentration. Portions of a dark-adapted outer segment suspension were adjusted to 10^{-9} M calcium (squares) or 10^{-6} M calcium (circles) by mixing with a Ringer's solution containing appropriate amounts of EGTA (see Methods). Samples (50 μ l) from each condition were either kept dark (■, ●) or exposed to a light source (○, □) bleaching $\sim 3.8 \times 10^6$ rhodopsin molecules/outer segment-s. At 1, 3, and 10 s after the onset of illumination, samples from each condition were quenched with 100 μ l of 9% perchloric acid and subsequently assayed for their cyclic GMP content. Aliquots (50 μ l) from each condition also were taken to determine rhodopsin concentration. Presented in this figure are the results from three separate experiments. Each dark point represents triplicate determinations in each of three experiments (mean \pm SEM, $n = 9$). Each 1-s light point represents duplicate determinations in each of three experiments (mean \pm SEM, $n = 6$). All remaining points represent single determinations in each of three experiments (mean \pm SEM, $n = 3$).

nation further reduces the level of cyclic GMP, and maximal reduction occurs within 1 s from the onset of the light stimulus, regardless of the calcium concentration. It is possible that the failure of Kilbride and Ebrey (1979) and Kilbride (1980) to find a light-induced cyclic GMP decrease after 1 s of illumination in high calcium is explained by the action of the calcium on dark cyclic GMP levels, for no evidence is presented in that work that the percent decrease in cyclic GMP from dark levels is a different function of time in low and high calcium. Lowering calcium may have increased the detectability of the light-induced cyclic GMP decrease by elevating dark cyclic GMP levels.

The rate of the light-induced cyclic GMP decrease measured in isolated outer segments ($t_{1/2} = 125$ ms) is clearly different from values obtained with retinal sections. At light intensities similar to the ones employed in these and previous experiments (Woodruff and Bownds, 1979), the $t_{1/2}$ for the cyclic GMP reduction measured in frog retinal sections was 4 ± 1 s (Kilbride and Ebrey, 1979), and in a subsequent set of experiments (Kilbride, 1980) appeared to be even longer. Slower kinetic data, however, do not permit one to conclude that the cyclic GMP decrease is slower than the conductance change occurring in the outer segments of the retinal section used, because the relevant conductance change was not measured. This is an important point because extracellular current recordings from sections of toad retinas have revealed that the time to peak of the outer segment photoreponse can be much slower (0.6–4 s) than previous intracellular voltage recordings had indicated (Baylor et al., 1979). It is possible that the slower time-course of the cyclic GMP decrease in frog retinal sections correlates with a slower physiological response. Measurements with frog retinal sections further assume that the only light-sensitive changes in the levels of cyclic GMP occur in the rod outer segments. This assumption is based on work with other species (Orr et al., 1976; Farber and Lolley, 1974). If this were not the case in frog, the time-course of the cyclic GMP decrease in retina would represent a composite of changes rather than solely the outer segment response.

That the time-course of the cyclic GMP reaction in outer segments is different from that of retinal sections may reflect differences in the control of enzymes involved in cyclic GMP metabolism. In isolated outer segments and purified disks, phosphodiesterase inactivates after flashes of light (Liebman and Pugh, 1979)¹ and thus corresponds to a return of cyclic GMP to its dark level (Woodruff and Bownds, 1979). In continuous light, however, phosphodiesterase remains activated (Robinson et al., 1980) and there is no apparent return of cyclic GMP (Woodruff and Bownds, 1979). These results contrast with data obtained with retinal sections which indicate that cyclic GMP levels first decrease and then return in the presence of continuous dim light (Kilbride and Ebrey, 1979). It is possible that phosphodiesterase is inactivated rapidly in retina, which might account for the slower time-course of the cyclic GMP decrease. Although little is known about the synthetic enzyme, guanylate cyclase, some differences in its activity in the two preparations may contribute to the different time-courses.

In these and previous experiments (Woodruff and Bownds, 1979; Woodruff et al., 1977), the light-induced cyclic GMP decrease occurs rapidly enough to be involved in the transduction process in rod outer segments. The data presented here further indicate that increasing calcium concentration does not impede the cyclic GMP decrease to an extent that could discount cyclic GMP from a role in transduction. The calcium concentration, however, does affect one characteristic of the cyclic GMP decrease. In 10^{-9} M calcium, the light-induced cyclic GMP decrease measured in isolated outer segments is maximal

¹ Kawamura, S., and M. D. Bownds. Light adaptation of the cyclic GMP phosphodiesterase of frog photoreceptor membranes mediated by ATP and calcium ions. Submitted for publication.

at continuous light levels that bleach between 5×10^4 and 5×10^5 rhodopsin molecules/outer segment-s (Woodruff et al., 1977; Woodruff and Bownds, 1979). At 10^{-3} M calcium the cyclic GMP decrease is maximal between 5×10^3 and 5×10^4 rhodopsin molecules bleached/outer segment-s, because the initial dark level of cyclic GMP is reduced at high calcium concentration (Woodruff and Bownds, 1979). These light levels are still comparable to those that saturate the photoresponse in living rod photoreceptors (Normann and Werblin, 1974; Fain, 1976).

There are many ways in which cyclic GMP could contribute to the control of permeability. It may function as an internal transmitter, mediating between photon absorption and the permeability mechanism of the plasma membrane, or it may participate less directly in the transduction process, perhaps in visual adaptation. It will be necessary to determine more about the steps between cyclic GMP and the permeability mechanism before its possible role as an internal transmitter can thoroughly be evaluated.

This work was supported by grant EY00463 from the National Institutes of Health, an award from Fight for Sight, New York, and a Naito research grant.

Received for publication 26 June 1980.

REFERENCES

- BAYLOR, D. A., T. D. LAMB, and K.-W. YAU. 1979. The membrane current of single rod outer segments. *J. Physiol. (Lond.)*. **288**:589-611.
- BOWNDS, M. D., A. GORDON-WALKER, A. C. GAIDE-HUGUENIN, and W. ROBINSON. 1971. Characterization and analyses of frog photoreceptor membranes. *J. Gen. Physiol.* **58**:225-237.
- BRODIE, A. E., and M. D. BOWNDS. 1976. Biochemical correlates of adaptation processes in isolated frog photoreceptor membranes. *J. Gen. Physiol.* **68**:1-11.
- CALDWELL, P. C. 1970. Calcium chelation and buffers. In *Calcium and Cellular Function*. A. W. Cuthbert, editor. St. Martin's Press, New York. 11-16.
- COHEN, A. I., I. A. HALL, and J. A. FERRENDELLI. 1978. Calcium and cyclic nucleotide regulation in incubated mouse retinas. *J. Gen. Physiol.* **71**:595-612.
- FAIN, G. L. 1976. Sensitivity of toad rods: dependence on wavelength and background illumination. *J. Physiol.* **261**:71-101.
- FARBER, D. B., and R. N. LOLLEY. 1974. Cyclic guanosine monophosphate: elevation in degenerating photoreceptor cells of the C3H mouse retina. *Science (Wash. D. C.)*. **186**:449-451.
- HUBBELL, W. L., and M. D. BOWNDS. 1979. Visual transduction in vertebrate photoreceptors. *Annu. Rev. Neurosci.* **2**:17-34.
- KILBRIDE, P. 1980. Calcium effects on frog retinal cyclic guanosine 3',5'-monophosphate levels and their light-initiated rate of decay. *J. Gen. Physiol.* **75**:457-465.
- KILBRIDE, P., and T. G. EBREY. 1979. Light-initiated changes of cyclic guanosine monophosphate levels in frog retina measured with quick-freezing techniques. *J. Gen. Physiol.* **74**:415-426.
- LIEBMAN, P. A., and E. N. PUGH, JR. 1979. The control of phosphodiesterase in rod disk membranes: kinetics, possible mechanisms and significance for vision. *Vision Res.* **19**:375-380.
- MILLER, W. H., and G. D. NICOL. 1979. Evidence that cyclic GMP regulates membrane potential in rod photoreceptors. *Nature (Lond.)*. **280**:64-66.

- NORMANN, R. A., AND F. S. WERBLIN. 1974. Control of retinal sensitivity. I. Light and dark adaptation of vertebrate rods and cones. *J. Gen. Physiol.* **63**:37-61.
- ORR, H. T., O. H. LOWRY, A. I. COHEN, and J. A. FERRENDELLI. 1976. Distribution of 3':5'-cyclic AMP and 3':5'-cyclic GMP in rabbit retina in vivo: selective effects of dark and light adaptation and ischemia. *Proc. Natl. Acad. Sci. U. S. A.* **73**:4442-4445.
- ROBINSON, P. R., S. KAWAMURA, B. ABRAMSON, and M. D. BOWNS. 1980. Control of the cyclic GMP phosphodiesterase of frog photoreceptor membranes. *J. Gen. Physiol.* **76**:631-645.
- STEINER, A. L., C. W. PARKER, and D. M. KIPNIS. 1972. Radioimmunoassay for cyclic nucleotides. *J. Biol. Chem.* **247**:1114-1120.
- WEINRYB, I., I. M. MICHEL, and S. M. HESS. 1972. Radioimmunoassay of cyclic AMP without precipitating antibody. *Anal. Biochem.* **45**:651-663.
- WOODRUFF, M. L. and M. D. BOWNS. 1979. Amplitude, kinetics, and reversibility of a light-induced decrease in guanosine 3',5'-cyclic monophosphate in frog photoreceptor membranes. *J. Gen. Physiol.* **73**:629-653.
- WOODRUFF, M. L., M. D. BOWNS, S. H. GREEN, J. L. MORRISSEY, and A. SHEDLOVSKY. 1977. Guanosine 3',5'-cyclic monophosphate and the in vitro physiology of frog photoreceptor membranes. *J. Gen. Physiol.* **69**:667-679.
- YEE, R., and P. A. LIEBMAN. 1978. Light-activated phosphodiesterase of the rod outer segment: kinetics and parameters of activation and deactivation. *J. Biol. Chem.* **253**:8902-8909.