Guanosine 3′,5′-Cyclic Monophosphate and the in Vitro Physiology of Frog Photoreceptor Membranes

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ABSTRACT Frog rod outer segments freshly detached from dark-adapted retinas contain approximately 1–2 molecules of guanosine 3′,5′-cyclic monophosphate (cyclic GMP) for every 100 molecules of visual pigment present. This cyclic GMP decays to 5′-GMP, and the conversion is accelerated upon illumination of the outer segments. Bleaching one rhodopsin molecule can lead to the hydrolysis of 1,000–2,000 molecules of cyclic GMP within 100–300 ms. The decline in cyclic GMP concentration becomes larger as illumination increases, and varies with the logarithm of light intensity at levels which bleach between 5×10^2 and 5×10^5 rhodopsin molecules per outer segment-second. Light suppression of plasma membrane permeability, assayed in vitro as light suppression of outer segment swelling in a modified Ringer's solution, occurs over this same range of light intensity. The correlation between cyclic GMP and permeability or swelling is maintained in the presence of two pharmacological perturbations: papaverine, a phosphodiesterase inhibitor, increases both cyclic GMP levels and the dark permeability of the plasma membrane; and β,γ-methylene ATP increases the effectiveness of light in suppressing both permeability and cyclic GMP levels.

INTRODUCTION

This paper presents data which correlate light-dependent changes in cyclic GMP with the in vitro physiology of frog retinal rod outer segments. Two papers previously presented in this journal provide background for the experiments presented here. The first described the development of an assay for observing outer segment function in vitro (1); the second demonstrated that this physiology can be correlated with light-sensitive enzyme activities (2). The in vitro assay makes use of the observation that light suppresses the swelling of isolated outer segments. This swelling is most probably driven by sodium chloride entry (1); light suppression of permeability slows the influx of ions and swelling.

The isolated outer segment contains a system that controls light sensitivity. Suppression of permeability, or swelling, is observed over 4 log units of light intensity and approximately follows Weber's law at light levels which bleach between 5×10^2 and 5×10^4 rhodopsin molecules per outer segment-second (2). The system which regulates light sensitivity may be related to light-dependent

rhodopsin phosphorylation (3, 4), for inhibitors of rhodopsin phosphorylation increase light sensitivity (5).

We have found also that the magnitude of the dark permeability available for suppression by light is increased by papaverine and other drugs which slow cyclic GMP breakdown in outer segments (2). These compounds act, at least in part, by inhibiting a light-activated cyclic GMP phosphodiesterase (6–10). Ebrey and Hood (11) have also demonstrated a physiological effect of these inhibitors. Goridis et al. (12) have demonstrated in whole retinas that light causes a decrease in cyclic GMP levels, and both this laboratory (2) and Fletcher and Chader (13) have made the same observation in isolated outer segments. In this paper we report more detailed studies on light-dependent changes in cyclic GMP in rod outer segments.

MATERIALS AND METHODS

Retinas were removed from dark-adapted bullfrogs (*Rana catesbeiana*) under infrared illumination, by use of an infrared image converter (F.J.W. Industries, Mt. Prospect, Ill.) to visualize the preparation. Each eye was opened with a shallow circumferential incision so that the retina remained attached to the front of the eye as the rest of the eye cup containing pigment epithelium was removed. Removing the retina this way minimized contamination from the pigment epithelium. The retina was then cut from the front half of the eye and gently rinsed in a modified Ringer's solution to remove any remaining contamination from the vitreous humor and pigment epithelium. The Ringer's solution consisted of 115 mM sodium chloride, 2.5 mM potassium chloride, 10 mM HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid), 10% vol/vol calf serum, 1 mM dithiothreitol, and 3 mM EGTA, pH 7.5.

Each retina was then placed in 1 ml of modified Ringer's solution and slowly agitated for 1–2 min to break loose rod outer segments. The resulting suspension contained approximately 10^6 outer segments and outer segment fragments which were used directly for cyclic GMP analysis or determination of in vitro physiology. Outer segments were exposed only to gentle treatment and to the modified Ringer's solution, since ordinary Ringer's solution or fragmentation destroys the ability of these outer segments to transduce light into a permeability decrease. More than 90% of the outer segments in this suspension were judged intact by the fluorescent staining procedure of Yoshikami et al. (14). In experiments using several retinas, dissections were simultaneously performed by several people so that all outer segments could be used soon, and at the same time after their detachment from the retina.

In these experiments manipulations in the dark used infrared illumination instead of the "dim red" light which many laboratories have used to approximate dark conditions during biochemical or physiological studies. We have found that red light from the General Electric BCJ bulb used at the intensity required for observing a dissection lowers cyclic GMP levels as much as a calibrated light bleaching 5×10^3 rhodopsin molecules per outer segment-second.

Obtaining the data in this report also requires that the outer segments be prepared and used as soon as possible after the frog is sacrificed. In two determinations where rod outer segments were obtained from eyes 30 min after sacrifice, the cyclic GMP changes were not as rapid as those shown in Fig. 3. Cyclic GMP levels were lower than normal in these and several other instances in which the preparation of outer segments was delayed for 20–40 min after sacrifice.

Cyclic GMP levels were assayed by using the radioimmunoassay technique described by Steiner et al. (15) as modified by Weinryb (16). Components of the assay were purchased

from Collaborative Research Incorporated, Waltham, Mass. At appropriate intervals 50-μl portions of the outer segment suspension were withdrawn and added to 60 μl of 9% perchloric acid (PCA). In the experiment of Fig. 3, where rapid changes in cyclic GMP levels were monitored, a series of 12 silicon-treated Pasteur pipettes, each containing 80 μl of 9% PCA, was mounted above a series of 50-μl samples from the outer segment suspension. In all experiments, modified Ringer's solution containing known amounts of cyclic GMP was extracted in the same way and processed in parallel with experimental samples to generate a standard curve. (The calf serum used in the modified Ringer's solution did not contain cyclic GMP.) All the above manipulations were carried out at room temperature, 22°C.

Samples containing PCA were centrifuged for 10 min at 1,500 *g*, 100 μl of supernate were withdrawn, and excess EGTA was complexed by making the solution 4 mM in calcium chloride. Samples were neutralized with potassium hydroxide and centrifuged for 10 min at 1,500 *g* to remove potassium perchlorate, and 100 μl of the supernate were used for assay. In some experiments this supernate was passed over a BioRad AG1-X8 column (Bio-Rad Laboratories, Richmond, Calif.) for further purification of the cyclic GMP (17). This procedure had no significant effect on the measured cyclic GMP levels; thus we observe no interference from contaminating compounds. Each incubation contained 100 μl of experimental sample, 100 μl of 0.1 M imidazole buffer (pH 7 at 4°C), 100 μl ^{125}I-succinyl cyclic GMP tyrosinemethylester, and 100 μl of cyclic GMP antiserum, the latter two components in the concentrations specified by the supplier of the assay. After 14–18 h of incubation, 2 ml of 0.1 M imidazole buffer were added, and after a further 20–30 min the solution was passed through a Millipore filter (type HA, 0.5-μm pore size, Millipore Corp., Bedford, Mass.) to which the antibody adsorbed. The filter was dissolved in 1 ml of 2-ethoxyethanol, 5 ml of scintillation fluid were added (Aquasol, New England Nuclear, Boston, Mass.), and radioactivity was determined with a Searle Mark II liquid scintillation counter (Searle Analytic, Inc., Des Plaines, Ill.). All procedures for the cyclic GMP assay were carried out at 4–5°C.

Outer segment suspensions were illuminated with the calibrated light source previously described (2). Continuous illumination was used in all experiments. Light suppression of swelling, or permeability, was measured by using the assay described by Bownds and Brodie (1). Reagents were obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS

The following experiments have been carried out in conditions which are optimal for observing in vitro physiology of rod outer segments (1). We have observed that these same conditions are most appropriate for finding and characterizing light-dependent chemical changes (2). Such an approach requires that we use crude suspensions of gently treated outer segments which may be contaminated with other membrane fractions. Several observations suggest that cyclic GMP is associated mainly with outer segments, rather than with possible contaminants. (*a*) We have determined that cyclic GMP levels in vitreous humor and pigment epithelium are much lower than in outer segments. (*b*) Cyclic GMP remains with outer segments and does not pass to the supernate after gentle sedimentation. (*c*) Approximately 80% of the cyclic GMP present in these suspensions is rapidly hydrolyzed if the outer segments are lysed by gentle homogenization. (*d*) In different outer segment preparations in which mitochondrial contamination varies between 2% and 20%, cyclic GMP levels remain proportional to the rhodopsin content.

Fig. 1*a* demonstrates the effects of illumination on cyclic GMP levels. The concentration of cyclic GMP is highest in outer segment preparations freshly separated from the retina in the dark. The initial concentration of 0.015 mol

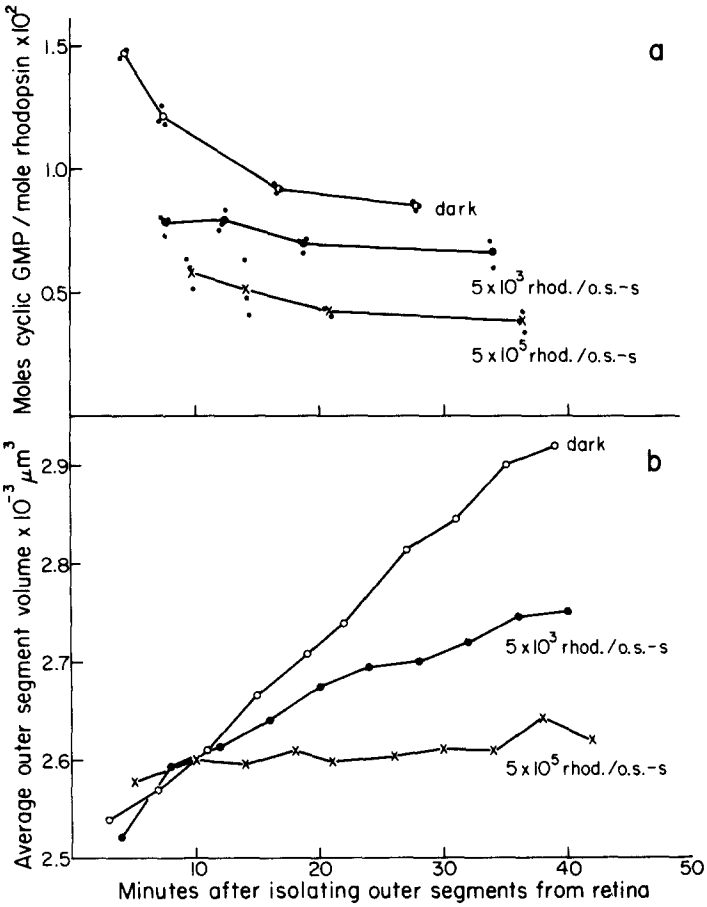

FIGURE 1. (*a*) Light-dependent decrease in cyclic GMP levels. Outer segments from four retinas were combined and then divided into three portions. After a sample of each was removed for determination of rhodopsin content, one was left dark (○), the second illuminated with continuous light bleaching 5×10^3 rhodopsin molecules per outer segment-second (●), and the third illuminated with light bleaching 5×10^5 rhodopsin molecules per outer segment-second (×). At the indicated intervals, three 50-μl samples were withdrawn from each suspension for cyclic GMP assay. (*b*) Light suppression of permeability under similar conditions of illumination. Outer segments were divided into three beakers containing the modified Ringer's solution with 10^{-4} M papaverine. Light suppression of swelling, or permeability, was monitored by use of the particle-sizing apparatus described previously (1).

cyclic GMP/mol rhodopsin decays rapidly during the first 10 min and then more slowly over the next 40 min. It is during the slower phase of cyclic GMP decay that we find it technically most practical to measure the light effects demonstrated in this paper. The dark decay of cyclic GMP may have its origin in the

decay of its precursor GTP, for we have determined that the GTP level falls rapidly after outer segments are detached from the retina.[1]

Illumination accelerates the decay of cyclic GMP, and we have found that a maximum decrease occurs with continuous light that bleaches approximately 5×10^5 rhodopsin molecules per outer segment-second (see also Fig. 2). Intermediate suppression of cyclic GMP levels is caused by continuous light bleaching 5×10^3 rhodopsin molecules per outer segment-second. The suppression of plasma membrane permeability caused by similar light levels is shown in Fig. 1*b*. Dark outer segments have relatively high permeability and swell rapidly. Maximal suppression of swelling, or permeability, is caused by light bleaching 5×10^5 rhodopsin molecules per outer segment-second and half suppression of swelling by light bleaching 5×10^3 rhodopsin molecules per outer segment-second. (The experiments in this paper show swelling during the 1st h after detachment of outer segments from the retina. The divergence between light and dark swelling (1) and the difference between cyclic GMP levels in the light and dark can persist for at least 2 h in these preparations. However, light suppression of dark cyclic GMP levels is no longer observed by 90 min, and we have found that light suppression of dark swelling is not always observed. Because of this variability, we no longer routinely work (as in reference 1) with outer segments more than 60 min after their detachment from the retina.)

The correlation between light-dependent decreases in cyclic GMP levels and permeability is presented in Fig. 2. The percent decrease in cyclic GMP and percent suppression of dark swelling are plotted as a function of the continuous light intensity used. The data on permeability changes have been taken from an earlier paper (2). The decreases in cyclic GMP and plasma membrane permeability occur over the same range of illumination and saturate with illumination bleaching approximately 5×10^5 rhodopsin molecules per outer segment-second. It should be noted that the permeability suppression was measured, for technical reasons (2), in the presence of the phosphodiesterase inhibitor papaverine, while cyclic GMP decay was not. A comparison is valid, however, for the inhibitor does not affect light sensitivity (reference 2, and see below).

Fig. 3 demonstrates that the cyclic GMP decrease in response to continuous illumination is very rapid. On exposure to light of saturating intensity (Fig. 3*a*) cyclic GMP levels decrease by 40–50% within 6 s. Approximately half of this decrease occurs within 200–400 ms. Fig. 3*b* shows that a decrease is also registered within 100–300 ms of the onset of illumination bleaching 1.3×10^4 rhodopsin molecules per outer segment-second. From the data of Fig. 3*b* we can obtain an estimate of a lower limit for the number of cyclic GMP molecules hydrolyzed per rhodopsin molecule bleached within 100–300 ms after the onset of illumination. In the three separate experiments shown in Fig. 3*b*, the averaged dark cyclic GMP levels were 1.00×10^{-2} (■), 0.90×10^{-2} (▲), and 1.03×10^{-2} (●) mol cyclic GMP/mol rhodopsin. The first data point after onset of illumination shows a 14% drop within 300 ms (■), a 13% drop within 100 ms (▲), and a 15% drop within 200 ms (●). Given the rate of bleaching and the number of rhodopsin molecules (3×10^9) per outer segment (reference 18),[2] we calculate 1.08×10^3

[1] M. Biernbaum, A. Shedlovsky, and D. Bownds. Manuscript in preparation.

[2] D. Bownds. Unpublished observations.

(■), 2.70 × 10^3 (▲), or 1.78 × 10^3 (●) mol of cyclic GMP hydrolyzed per mol of rhodopsin bleached within the indicated times. It seems appropriate to conclude that at least 1,000–2,000 molecules of cyclic GMP can be hydrolyzed per rhodopsin molecule bleached within 100–300 ms of the onset of illumination. A similar calculation for Fig. 3*a* yields a lower number, 50–65 molecules of cyclic GMP hydrolyzed per rhodopsin molecule bleached within 200–400 ms. (We should point out that this simple linear calculation is approximate, for continuous

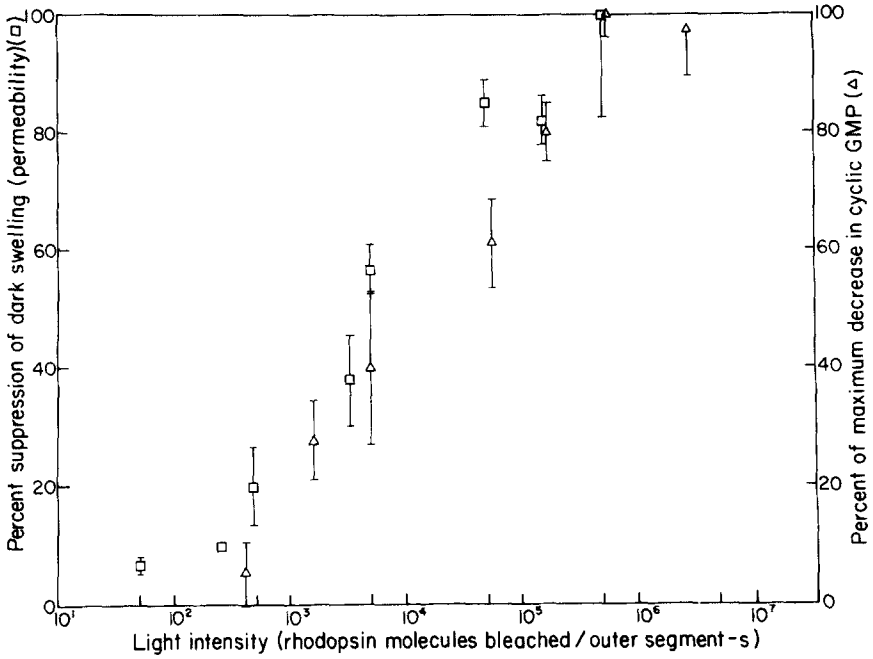

FIGURE 2. Light sensitivity of light-dependent cyclic GMP decrease (△) and dark swelling (□). Each data point for cyclic GMP decrease represents the mean (SE indicated) of eight determinations (eight different eyes) as follows. Rod outer segments isolated from each eye were divided into two suspensions; one was kept dark and the other was illuminated continuously at the appropriate light intensity for 10 min. Both dark and illuminated outer segments were kept in suspension during the incubation by gentle pipetting. Three 50-μl samples were then withdrawn from each suspension and assayed for cyclic GMP as in Fig. 1. The light sensitivity of swelling, or permeability, is taken from the data of Fig. 2 in reference 2.

illumination is used in Fig. 3, and thus rhodopsin bleaching is occurring continuously. A nonlinear calculation could increase the estimate of moles cyclic GMP hydrolyzed per mole rhodopsin bleached, but such a calculation requires kinetic assumptions which are not appropriate at this stage.)

The correlation between cyclic GMP levels and permeability changes is maintained when pharmacological agents are used to perturb this system. Fig. 4 demonstrates that papaverine increases the magnitude of the dark permeability and increases cyclic GMP levels approximately twofold. Other phosphodiester-

ase inhibitors have a similar effect (data not shown). An elevation of cyclic GMP levels by papaverine addition is shown in Fig. 4*a*. This suggests that isolated outer segments can synthesize as well as hydrolyze this compound. (A guanylate cyclase which converts GTP to cyclic GMP is present in outer segment [19–21].)

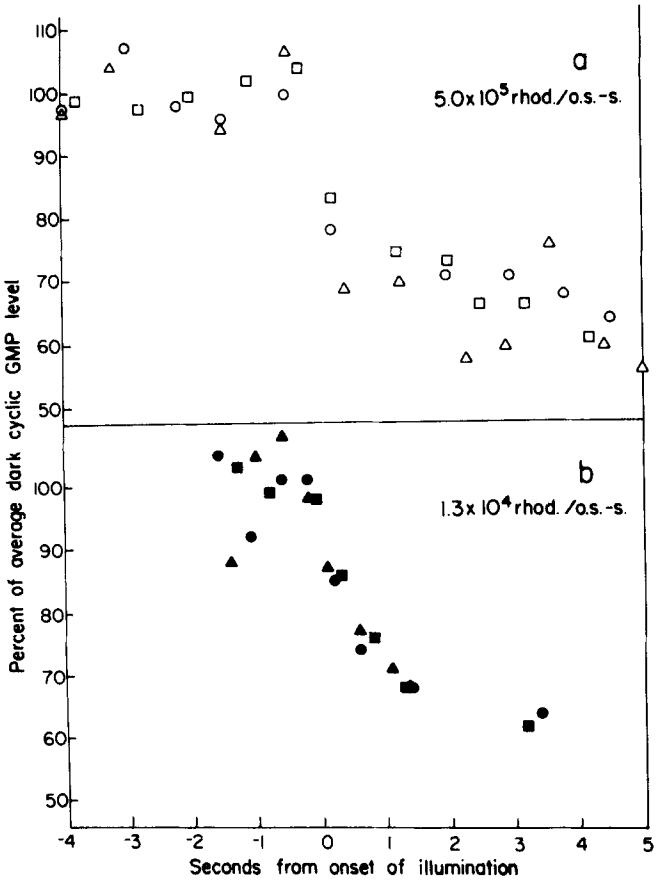

FIGURE 3. Time required for light-dependent decrease in cyclic GMP. In six separate experiments (designated △, ○, □, ▲ ●, ■), successive acid extractions were performed within approximately 11 s. In Fig. 3*a*, the rod outer segments were exposed to continuous light that bleached 5×10^5 rhodopsin molecules per outer segment-second, and in Fig. 3*b* to light bleaching 1.3×10^4 rhodopsin molecules per outer segment-second.

The corresponding physiological behavior is shown in Fig. 4*b*; the dark permeability of isolated outer segments is enhanced by papaverine addition.

In an earlier paper (2) we noted that although papaverine enhanced the magnitude of the dark permeability, it did not influence sensitivity, the relative amount of illumination required to suppress this permeability (Fig. 5, also reference 2). Similarly, a comparison of Figs. 1 and 2 (without papaverine) with Fig. 5*b* (with papaverine) suggests that papaverine does not influence the

amount of illumination required to decrease cyclic GMP levels. Bleaching approximately 5 × 10^3 rhodopsin molecules per outer segment-second results in

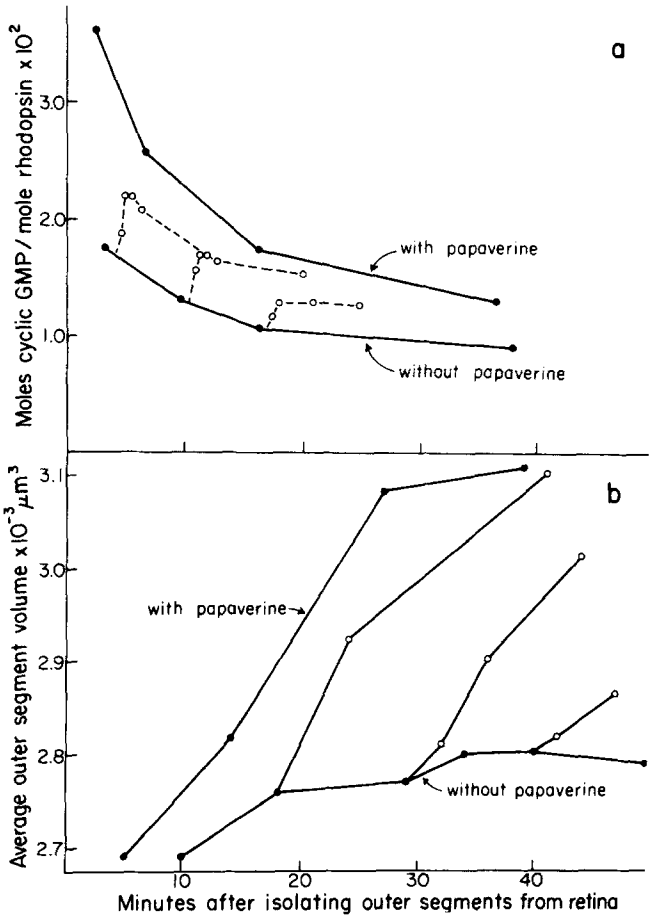

FIGURE 4. Effect of papaverine on dark-adapted rod outer segments. (*a*) Rod outer segments were shaken from the retina into modified Ringer's solution with 10^{-4} M papaverine and without papaverine as indicated. The points are the means of triplicate samples extracted and assayed as in Fig. 1. The open circles (○) demonstrate increases in cyclic GMP levels upon addition of papaverine to rod outer segments taken from the incubation without the inhibitor at 4, 10, and 17 min. (*b*) The effect of papaverine on dark permeability or swelling rate. Rod outer segments were shaken in modified Ringer's solution and 100-μl portions added to 15 ml of the same Ringer's solution with 10^{-4} M papaverine or without papaverine. The open circles (○) demonstrate the increased permeability observed when further 100-μl samples are added to Ringer's solution with papaverine at 18, 29, and 40 min of incubation.

half-maximal decrease with or without the inhibitor. Therefore, papaverine does not affect the light sensitivity of either the permeability decrease or the cyclic GMP decrease.

We have shown (5) that β,γ-methylene ATP increases the light sensitivity of outer segments as determined by permeability measurements. These results are confirmed and expanded here. Fig. 5*a* illustrates that β,γ-methylene ATP affects the in vitro physiology in two ways: first, it partially reverses the papaverine-induced dark permeability; second, it increases the effectiveness of light in

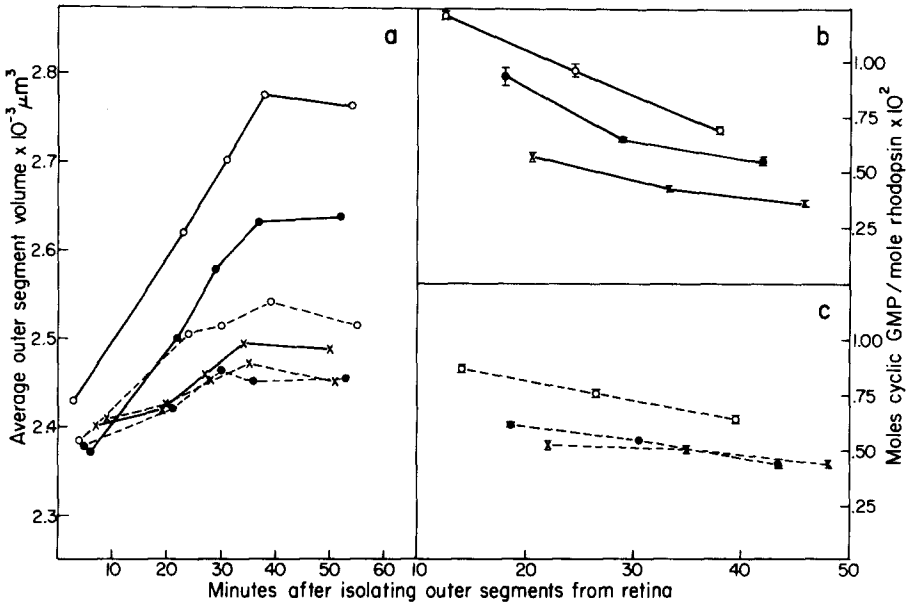

FIGURE 5. The effect of β,γ-methylene ATP on in vitro physiology and cyclic GMP. (*a*) Rod outer segments were shaken from the retina into modified Ringer's solution with 10^{-4} M papaverine and 100-μl portions added to 15 ml of the same Ringer's solution with 5 mM β,γ-methylene ATP (-----) and without, β,γ-methylene ATP (——). Outer segments were incubated in dark (○) or exposed to continuous light that bleached 5×10^3 (●) or 5×10^5 (×) rhodopsin molecules per outer segment-second. Similar results were obtained in six experiments. (*b*) Rod outer segments were shaken from the retina in modified Ringer's solution with 10^{-4} M papaverine. The outer segments were incubated in the dark (○) or exposed to light that bleached 5×10^3 (●) or 2.2×10^6 (×) rhodopsin molecules per outer segment-second. Each data point represents the mean of quadruplicate samples (with SE bars) extracted and assayed for cyclic GMP as in Fig. 1*a*. (*c*) To a portion of the same outer segment suspension 5 mM β,γ-methylene ATP was added. Illumination and assay were as in (*b*). Similar results were obtained in three experiments.

suppressing permeability. Analogous effects on cyclic GMP levels are observed. Fig. 5*b*, *c* shows that the high dark level of cyclic GMP induced by papaverine is decreased when β,γ-methylene ATP is added. Further, light that bleaches approximately 5×10^3 rhodopsin molecules per outer segment-second causes full rather than half-maximal suppression of cyclic GMP concentration.

DISCUSSION

These data demonstrate several correlations between cyclic GMP levels and the

swelling of isolated rod outer segments. Both are suppressed as a continuous function of light intensity over the same 4 log unit range of illumination and the effectiveness of light in decreasing both is enhanced if β,γ-methylene ATP is added. Also, cyclic GMP levels and swelling are increased by papaverine and decreased by β,γ-methylene ATP.

In considering correlations between chemistry and "in vitro physiology" two important limitations of the in vitro assay must be kept in mind. First, during the period of approximately 10 min after detachment of outer segments from the retina we observe "erratic" in vitro physiology. Divergence between dark and light swelling is not always observed (cf. Figs. 1, 4, and 5, and the figures of references 1 and 2). This initial variability may reflect a period of metabolic instability during which the outer segment is adapting to an in vitro "steady-state." In addition to the drop in cyclic GMP seen during this period (Fig. 1) we observe decreases in levels of GTP, ATP, GDP, and ADP.[1] Second, while the rate of swelling of isolated outer segments might be taken to reflect their relative permeability (1), it cannot be used to measure the kinetics of permeability changes. Permeability changes may be quite rapid, with the swelling assay only permitting us to see the consequences of those changes over many minutes (as the differences in volume between dark and illuminated outer segments become large enough for our apparatus to detect them). Apparently, we are permitted to see the correlations between cyclic GMP levels and permeability or swelling reported in this paper because both stay relatively constant, at each given level of illumination, for 10–40 min after detachment of outer segments from the retina.

A central point made by the data of this paper is that the light-dependent changes in cyclic GMP levels reflect the operation of a sensitivity-controlling mechanism similar to that which controls permeability. The effectiveness of light decreases as its intensity increases: the decrease in cyclic GMP and permeability both approximate a Weber relationship over 2–3 log units of light intensity. A link between cyclic GMP and a sensitivity control mechanism is also suggested by Fig. 5, which demonstrates that β,γ-methylene ATP enhances the effectiveness of light in suppressing both permeability and cyclic GMP levels.

In our previous paper we suggested a role of cyclic GMP not in sensitivity control but in regulating the maximum amplitude of the permeability change that can be effected by illumination (2). This suggestion was based on the finding that amplitude was increased by the addition of papaverine, a phosphodiesterase inhibitor which boosts cyclic GMP levels. This inhibitor, however, did not influence sensitivity. We now find that the light-dependent cyclic GMP decrease, like the permeability decrease, is desensitized at higher light intensities and that β,γ-methylene ATP increases the relative effectiveness of light. Thus, cyclic GMP may be part of the sensitivity- as well as amplitude-controlling mechanism.

It is important to point out that the conclusions we draw on the basis of adding pharmacological agents such as papaverine or β,γ-methylene ATP do not require us to specify their site(s) of action. These agents are used to produce perturbations and subsequently to determine whether the correlation between cyclic GMP levels and permeability still holds. It will be of interest to determine the molecular mechanism and specificity of papaverine and β,γ-methylene ATP

action on the cyclic GMP-controlling system. Such studies might clarify the connection between the pathways controlling cyclic GMP and permeability.

The experiments presented here raise the possibility that cyclic GMP is an internal transmitter which mediates between photon absorption in disk membranes and the permeability decrease of the outer segment plasma membrane (12, 13, 22). Cyclic GMP meets two of the criteria required of such a transmitter: its concentration changes rapidly (possibly more rapidly than 200 ms), and the gain of the system is high. Bleaching one rhodopsin molecule can trigger the hydrolysis of at least 1,000–2,000 molecules of cyclic GMP.

The light-induced decrease in cyclic GMP might be controlled in several different ways. We do not know all the rate-limiting steps in its pathway. An obvious candidate for primary rapid control is the light-sensitive phosphodiesterase whose activity can be enhanced at least 10-fold by illumination (6–10). A guanylate cyclase activity in outer segments may be inhibited by illumination (19, 23). The data of Fig. 4 suggest that cyclic GMP is being continuously synthesized and degraded in isolated outer segments, and thus the levels and light sensitivity of its precursor, GTP, must also be considered. Interaction with adenosine nucleotide pathways may also be important.

The light-dependent changes in cyclic GMP, whatever their origin, are likely to have physiological relevance, for cyclic GMP is thought to play a role in intracellular control mechanisms. Cyclic GMP might interact with calcium (24) or prostaglandin-related processes (25) in outer segments, for we have found significant effects of these agents on in vitro physiology (1).[3] A role for cyclic GMP as a general regulator of outer segment metabolism rather than as a specific trigger of permeability changes must also be considered.

It is too early to speculate on detailed pathways which regulate this system. Our preliminary results suggest that there are a number of small molecule transformations and covalent protein modifications proceeding at physiologically relevant light levels.

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