Amplitude, Kinetics, and Reversibility of a Light-Induced Decrease in Guanosine 3′,5′-Cyclic Monophosphate in Frog Photoreceptor Membranes

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ABSTRACT The concentration of guanosine 3′,5′-cyclic monophosphate (cyclic GMP) has been examined in suspensions of freshly isolated frog rod outer segments using conditions which previously have been shown to maintain the ability of outer segments to perform a light-induced permeability change (presence of calf serum, anti-oxidant, and low calcium concentration). Illumination causes a rapid decrease in cyclic GMP levels which has a half-time ~125 ms. With light exposures that bleach less than 100 rhodopsin molecules in each rod outer segment, at least 10^4–10^5 molecules of cyclic GMP are hydrolyzed for each rhodopsin molecule bleached. Half of the total cyclic GMP in each outer segment, $\sim 2 \times 10^7$ molecules, is contained in the light-sensitive pool. If outer segments are exposed to continuous illumination, using intensities which bleach between 5.0×10^1 and 5.0×10^4 rhodopsin molecules/outer segment per second, cyclic GMP levels fall to a value characteristic for the intensity used. This suggests that a balance between synthesis and degradation of cyclic GMP is established. This constant level appears to be regulated by the rate of bleaching rhodopsin molecules (by the intensity of illumination), not the absolute number of rhodopsin molecules bleached. After brief exposure to light of varying intensities, cyclic GMP concentration returns to near the value observed in unilluminated outer segments within 30–60 s. The recovery is most rapid after dim illumination. Decreases in cyclic GMP levels induced by light superimposed on background illumination are reduced by an amount proportional to the intensity of the background light. Background light appears to have no effect on the sensitivity of the light-dependent cyclic GMP decrease. Calcium ions lower the level of cyclic GMP without influencing either the final level to which cyclic GMP is reduced by illumination or the light sensitivity of the reduction. These data, together with those of a previous paper presenting several correlations between cyclic GMP levels and light suppressible ionic permeability of isolated frog rod outer segments (Woodruff et al., 1977. *J. Gen. Physiol.* **69:** 667–679), suggest that cyclic GMP is involved in visual transduction, perhaps mediating between photon absorption and the permeability decrease.

INTRODUCTION

In vertebrate rod photoreceptors biochemical machinery for excitation and adaptation is contained within the outer segment, a cylindrical organelle which

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in the amphibian is 6–8 μm in diameter and 50–80 μm in length. The rod outer segment is a modified cilium composed of a plasma membrane which encloses a stacked series of 1,500–2,000 disc membranes. When light strikes one of the $\sim 3 \times 10^9$ rhodopsin molecules in the disc membranes, a transient suppression of the permeability of the plasma membrane occurs. This permeability decrease causes a hyperpolarization of the plasma membrane. The disc membranes are physically separate from the plasma membrane, and for this reason as well as others (see Hagins, 1972; Tomita, 1970), it is commonly assumed that an internal transmitter substance must transfer information from the site of photon absorption in the disc membranes to the permeability mechanism of the plasma membrane. The concentration of the presumed transmitter might increase or decrease in response to illumination, and be regulated by trans-disc membrane fluxes or by enzymic synthesis and breakdown. Ions, small organic molecules, and proteins are all potential transmitters. Considerable interest and effort has focused on the specific suggestion that light absorption by rhodopsin causes the release of calcium ions stored within the disc membrane. This calcium is assumed to bind to and inhibit the conductance mechanism of the plasma membrane. Definitive evidence proving or disproving this hypothesis has not been obtained (see Hagins and Yoshikami, 1977; Szuts and Cone, 1977).

In the past few years evidence has been accumulating that cyclic GMP may be an important molecule in photoreceptor physiology. Illumination causes a decrease in the level of cyclic GMP in photoreceptor cells (Orr et al., 1976; Cohen et al., 1978) and in isolated outer segments (Fletcher and Chader, 1976; Woodruff et al., 1977), apparently by activating the degradative enzyme, cyclic GMP-phosphodiesterase (Miki et al., 1973, 1975; Keirns et al., 1975; Krishna et al., 1976; Goridis and Weller, 1976; and Wheeler and Bitensky, 1977). The level to which cyclic GMP is reduced reflects the amount of incident radiation (Woodruff et al., 1977). Drugs which inhibit phosphodiesterase and increase endogenous cyclic GMP levels also increase the ionic permeability of isolated outer segments assayed in vitro (Brodie and Bownds, 1976), and cause depolarization of toad rods (Lipton et al., 1977 *b*). Woodruff et al. (1977) have recently shown that cyclic GMP levels and the ionic permeability of frog rod outer segments are suppressed over the same range of light intensity, and that pharmacological agents affect both similarly. The cyclic GMP decrease triggered by illumination was found to occur within 1 s, with several thousand cyclic GMP molecules disappearing for each rhodopsin molecule bleached.

This paper reports further studies of the rapid cyclic GMP decrease in isolated outer segments using conditions which maintain their transduction mechanism (Bownds and Brodie, 1975; Brodie and Bownds, 1976). The questions asked here are: (*a*) how rapidly is cyclic GMP decreased upon illumination? (*b*) is the decrease reversed when illumination stops? (*c*) Is the amplitude of the cyclic GMP decrease regulated by the total number of rhodopsin molecules bleached or the rate of their bleaching (by total illumination or light intensity)? (*d*) What is the sensitivity of the decrease (i.e., how many cyclic GMP molecules are hydrolyzed for each rhodopsin molecule bleached)? (*e*) Is the cyclic GMP response desensitized ("light-adapted") by a prior exposure to illumination? and (*f*) Do calcium ions influence the cyclic GMP decrease?

METHODS

Bullfrogs (Rana catesbeiana), 10–15 cm in length, were used in these experiments. The frogs were kept in light-tight holding tanks for 2–6 wk before use. Each animal was force-fed ~5 g of Purina Dog Chow (Ralston Purina Co., St. Louis, Mo.), vitamin supplemented, three times a week. During this holding period the frogs received a 12-h light-dark cycle, with intermittent illumination during the light part of the photoperiod: 5 min of dim white light, 5 min of light flashes at a frequency of one per second, and 5 min of darkness. This regimen of illumination was used to provide more retinal stimulation (alteration of light and dark adaptation) than would be provided by continuous illumination. Frogs were sacrificed for experiments 1–6 h before the end of the 12-h dark period. All experimental manipulations were carried out in infrared illumination, a head-mounted IR converter (FJW. Industries, Mount Prospect, Ill.) being used for visualization. Retinas were removed as described previously (Woodruff et al., 1977) and gently rinsed in a modified frog Ringer's solution (115 mM sodium chloride, 2.5 mM potassium chloride, 10 mM Hepes buffer [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 10% vol/vol undialyzed calf serum, 1 mM dithiothreitol and 3 mM EGTA [ethylene glycol-bis-(β-aminoethyl ether) *N,N'*-tetraacetic acid], pH 7.5). This modified Ringer's solution was used because it has been found to be optimal for maintaining rod outer segment structure and function in vitro (Bownds and Brodie, 1975). The presence of serum prevents extensive breakage of outer segments which occurs in ordinary Ringer's solution, and Woodruff et al. (1977) have shown that rapid hydrolysis of endogenous cyclic GMP occurs when outer segments are disrupted. The calf serum contained negligible amounts of cyclic GMP. (Calibration curves for the radioimmunoassay [see below], constructed with known amounts of cyclic GMP were similar with and without added serum.) Each retina was then placed in 500 μl of modified Ringer's solution and slowly agitated for 2 min to detach rod outer segments. The resulting suspension of outer segments and outer segment fragments (containing >1,000 outer segments/microliter) was used without further purification (see Woodruff et al., 1977 for discussion of purity). Approximately 80% of the outer segments were osmotically intact as determined by the fluorescence assay of Yoshikami et al. (1974). In experiments where more than one retina was needed, dissections were performed by more than one person so that the time between frog sacrifice and detachment of outer segments from the retina was fairly constant between experiments. The time between sacrifice and beginning of the 2-min retinal agitation period was between 3 min and 5 min in all experiments.

In a typical experiment 50-μl portions of the rod outer segment suspension were withdrawn, exposed to differing conditions of illumination, or incubated for different periods of time, and then mixed with 90 μl of 9% perchloric acid (PCA) to quench endogenous reactions. Cyclic GMP content was assayed as described below. Details for each experiment are given in the figure legends.

In the time-course experiments of Figs. 2, and 5–7, a series of silicone-treated Pasteur pipettes, each containing PCA, was mounted above a series of rod outer segment samples. Acid was sequentially ejected onto the samples by squeezing a bulb attached to the top of each pipet. In the rapid time-course experiments of Figs. 3 and 4, acid quenching of the series of outer segment samples was automated to increase time resolution. Each Pasteur pipet contained 100 μl of PCA mounted above a 20-μl portion of rod outer segment suspension so that enzymic reactions could be quenched more quickly. Acid was ejected from each pipet as a motor driven wheel was rolled across rubber tubing attached to the pipet. The motor (115 V, DC Bodine Typeframe, 1/8 hp, 1,725 rpm, Bodine Electric Co., Chicago, Ill.) was connected to the wheel by a wafer magnetic clutch (PIC Design Div. of Benrus Corp., Ridgefield, Conn.). 12 individual

outer segment samples could be quenched within 750–1,500 ms. Microswitches were used to signal the actual time between quencing the first and twelfth samples and to activate a shutter which exposed the samples to illumination between the quenching of the fifth and sixth samples. A high speed movie camera (Lo Cam, Redlake Corp., Photo Instrument Div., Campbell, Calif.) adjusted to 100 frames/s, was used to photograph 12 calibration runs; three runs each at four different motor speeds. The ejection of PCA from the Pasteur pipets onto the samples was linear with time in all experiments, and the error in estimating the time at which each sample was physically contacted by the acid was <5 ms. The time required for mixing acid and sample was also determined during the photographic calibration by monitoring a pH color change (bromophenol blue, pH range = 3.0–3.6; deep blue → clear). Mixing was complete within 52 ± 18 ms (mean ± SD) of initial exposure to the acid. Occasionally (3 cases in 128 determinations) the acid inverted with the sample and mixing was very slow—longer than 120 ms. These samples were not used in calculating the mixing time.

To estimate the additional time required for acid to penetrate outer segment membranes after mixing, a stopped flow spectrophotometer was used (Durrum Model D-110, modified to reduce temperature artifacts, Durrum Instrument Corp., Sunnyvale, Calif.). The device mixed outer segments and acid in 1 ms and then monitored an optical density increase at 515 nm caused by an increase in light scattering. (No such increase was observed when outer segments were mixed with Ringer's solution instead of perchloric acid.) The increase in density had a half-time of 60 ± 13 ms (mean ± SD). The time for mixing and acid penetration of the rod outer segments was also estimated by visually monitoring the acid-induced color change accompanying the denaturation of rhodopsin (red to orange-yellow). The time for the color change was compared with flashes of light of various durations. The color change was completed within 125–250 ms.

Cyclic GMP levels were assayed as described previously (Woodruff et al., 1977) using the radioimmunoassay technique of Steiner et al. (1972) as modified by Weinryb et al. (1972). Components of the assay were purchased from Collaborative Research Inc., Waltham, Mass. Phosphodiesterase activity was assayed using the two-step procedure outlined by Thompson et al., (1974) and Kemp and Huang (1974). [^{3}H]Cyclic GMP (10^3 dpm/mmol, New England Nuclear, Boston, Mass.) was added to each 50-μl sample of rod outer segment suspension. After an appropriate incubation time and light exposure, samples were placed in a hot sand bath (~100°C) for 1 min to stop enzymic reactions. Samples were brought to room temperature, and 200 μl *Crotalus atrox* venom (2 mg/ml, 50 mM Trizma base buffer, pH 7.5) was added. Samples were incubated with venom for 30 min at 37°C to convert 5'GMP to guanosine. This reaction was also stopped by placing the samples in hot sand for 1 min. Samples were then passed through an anion exchange column (DEAE-Sephadex, A-25, 7.5 × 0.5 cm, Pharmacia Fine Chemicals, Piscataway, N.J.) equilibrated with 50 mM Trizma base, pH 7.5. The radioactivity from the first 5 ml of eluant ([^{3}H]guanosine) was determined by liquid scintillation counting (Mark II, Searle Radiographics Inc., Des Plaines, Ill.). Unreacted cyclic GMP and 5'GMP do not elute from the column until the ninth ml. Recovery from exchange columns was better than 90%. All chemicals were obtained from Sigma Chemical Co., St. Louis, Mo.

Techniques for illumination of outer segments and for measuring rhodopsin content are described in an earlier paper (Brodie and Bownds, 1976).

RESULTS

The Amplitude of the Light-Dependent Cyclic GMP Decrease Is a Function of Time after Isolating Rod Outer Segments from the Retina

The cyclic GMP content of dark outer segments slowly decreases after they are isolated from the retina (Fig. 1 *a*), and the maximum obtainable light-induced

decrease becomes proportionally less (Fig. 1 *b*). In the experiment of Fig. 1 *a* the concentration of cyclic GMP decayed in the dark from 0.0125 mol cGMP/mol rhodopsin to 0.0078 mol cGMP/mol rhodopsin between 6 and 30 min after isolating the outer segments. This slow decrease in cyclic GMP is a common feature of these preparations and was noted previously (Woodruff et al., 1977). The concentration of cyclic GMP after 5–10 min of isolation varies from

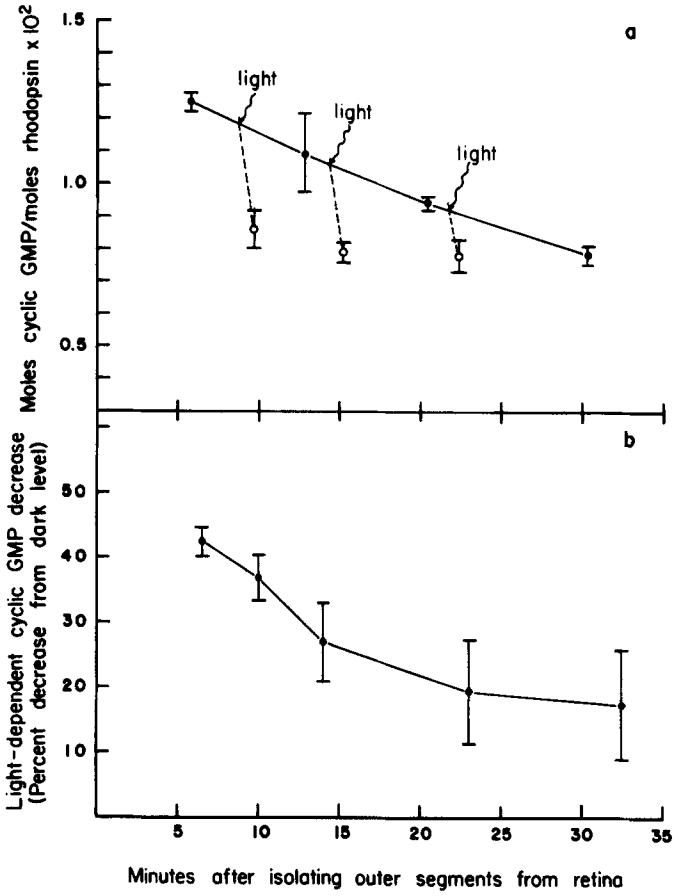

FIGURE 1. The light-induced decrease in cyclic GMP becomes smaller with time after detaching rod outer segments from the retina. (*a*) Outer segments from two retinas were combined in a total of 2.0 ml modified Ringer's solution (see Methods). At 6, 13, 20, and 30 min three 50-μl portions from the dark outer segment suspension were quenched by addition of 90 μl of 9% perchloric acid (●). At 9, 14, and 22 min, a 200-μl portion of the outer segment suspension was exposed to saturating illumination that bleached 5.0×10^5 rhodopsin molecules/outer segment per second for 30 s and then three 50-μl portions from the illuminated suspension were quenched as above (○). (A maximum decrease in cyclic GMP is obtained in 30 s; see Fig. 2.) Each data point represents a mean cyclic GMP level ± SE of the triplicate samples. (*b*) Averages of the percent decreases taken from 14 further experiments in which maximal cyclic GMP responses to saturating illumination were determined during this time period.

preparation to preparation but is generally between 0.010 and 0.020 mol cGMP/mol rhodopsin. If one assumes that there are 3×10^9 rhodopsin molecules in each outer segment (Liebman and Entine, 1968) this corresponds to $3–6 \times 10^7$ cyclic GMP molecules per outer segment.

Illuminating the outer segments with light of saturating intensity for the cyclic GMP decrease (5.0×10^5 rhodopsin molecules bleached/outer segment per second [Woodruff et al., 1977]; and see Fig. 7) at 9, 14, and 22 min of isolation (Fig. 1 *a*) decreased cyclic GMP levels 27, 25, and 15%, respectively. In Fig. 1 *b* the percent decrease in cyclic GMP averaged from many experiments is represented as a function of the time after isolating the outer segments from the retina. The average light-dependent decrease is approximately half as large at 22 min (20% decrease) as it is at 7 min (42% decrease). No light-dependent decrease in cyclic GMP was obtained 90 min after the outer segments were removed from the retina (two determinations, data not shown).

It was considered desirable to determine the effects of illumination as soon as possible after outer segments were isolated from the retina so that large, easily measurable responses could be obtained, and any effects of outer segment deterioration that might occur with time would be minimized. However, experimental manipulations made it difficult to routinely examine light effects earlier than 9 min after isolation. In the following experiments (with noted exceptions) the effects of illumination on rod outer segment cyclic GMP levels were determined between 9 and 15 min after isolating the outer segments from the retina.

Even with saturating conditions of illumination, cyclic GMP levels were never observed to fall more than 50% (see also Woodruff et al., 1977), and thus there appears to be a "light-sensitive pool" of cyclic GMP containing approximately 2×10^7 molecules per outer segment. When outer segments are lysed by either freezing and thawing, homogenization, or hypoosmotic shock, at least 80% of the total cyclic GMP is degraded rapidly (Woodruff et al., 1977). This suggests that the cyclic GMP which is not degraded in light exposed outer segments is inaccessible to the phosphodiesterase. The location of cyclic GMP within the outer segment and its physical state (soluble or bound) is not known. Phosphodiesterase presumably is restricted to outer segment membranes (Miki et al., 1975).

The Light-Induced Decrease in Cyclic GMP Is Rapid

Fig. 2 illustrates a time-course for the light-induced decrease in cyclic GMP in response to illumination which bleaches 5.0×10^5 rhodopsin molecules/outer segment per second. In the experiment shown the level of cyclic GMP was reduced approximately 50% within 30 s. Half of this decrease had occurred by one second of illumination. The inset in Fig. 2 illustrates hydrolysis of exogenous [^{3}H]cyclic GMP by the rod outer segment phosphodiesterase which is activated by light. Activity of the enzyme is given by the slopes of the lines shown, and is enhanced at least 20-fold by light which bleaches 5.0×10^5 rhodopsin molecules/outer segment per second. This light activation of rod outer segment phosphodiesterase has been documented by several laboratories (Miki et al., 1973; Keirns, et al., 1975; Krishna et al., 1976; Goridis and Weller,

1976), and is probably the major cause of the cyclic GMP decrease shown in Fig. 2 (see below).

Several experiments similar to the ones shown in Fig. 2 indicated that the decrease in cyclic GMP and the activation of phosphodiesterase occur with little

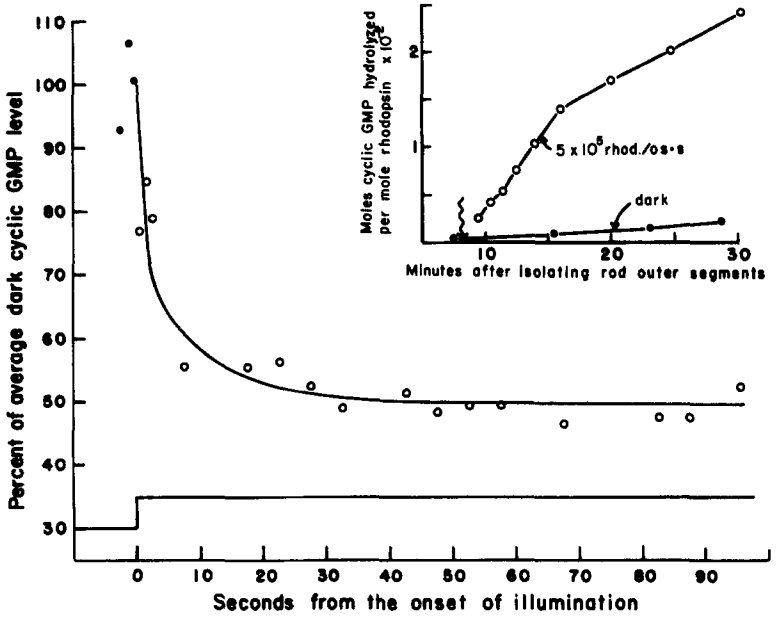

FIGURE 2. The light-induced decrease in cyclic GMP and light activation of rod outer segment phosphodiesterase. Rod outer segments from two retinas were divided into 50-μl portions. After the first three samples had been acid quenched (●), the remaining portions were sequentially acid quenched (see Methods) while exposed to continuous illumination that bleached 5.0×10^5 rhodopsin molecules/outer segment per second (○). The onset of illumination, denoted by a step at the bottom of the figure, occurred ~10 min after isolating the outer segments from the retinas. Four samples were masked from the light to serve as dark controls. The average of duplicate points for the controls taken at 37 and 75 s after the onset of illumination were 98.7% and 97.5% of the average dark cyclic GMP levels, respectively (data not shown). The line through the data points is drawn by hand. *Inset.* The rate of hydrolysis of exogenous cyclic GMP by phosphodiesterase is enhanced 20-fold by illumination bleaching 5.0×10^5 rhodopsin molecules/outer segment per second. Outer segments from two retinas were combined in modified Ringer's solution with 2 mM cyclic GMP ([^{3}H]cGMP; 100,000 cpm). No exogenous GTP or ATP was present. The radiotracer was added 5 min, and illumination was presented 8 min (arrow) after isolating the outer segments from the retinas. Reactions were stopped by exposing portions of the outer segment suspensions to a hot sand bath, and the amount of cyclic GMP hydrolyzed was determined as described in Methods.

delay on illumination. To determine how rapidly cyclic GMP levels decrease, the automated quenching device described in Methods was used. Fig. 3 shows a result obtained using this machine. When outer segments were illuminated with saturating light intensity (5.0×10^5 rhodopsin molecules bleached/outer segment

per second), the level of cyclic GMP was reduced 30% within 100 ms of illumination. Notice, however, that the concentration of cyclic GMP apparently started to decay before the onset of illumination. Evidently the effect of the acid on the sample was sufficiently delayed that endogenous reactions were not fully quenched by the time light was turned on. This means that the time between the onset of illumination and the initiation of the cyclic GMP decrease (latency) cannot be accurately determined with this acid-quenching technique. However, if one makes the reasonable assumption that the mixing and quenching time is constant for each sample, the data still yield accurately the rate at which cyclic GMP disappears. The half-time for this disappearance is 100–150 ms. The data

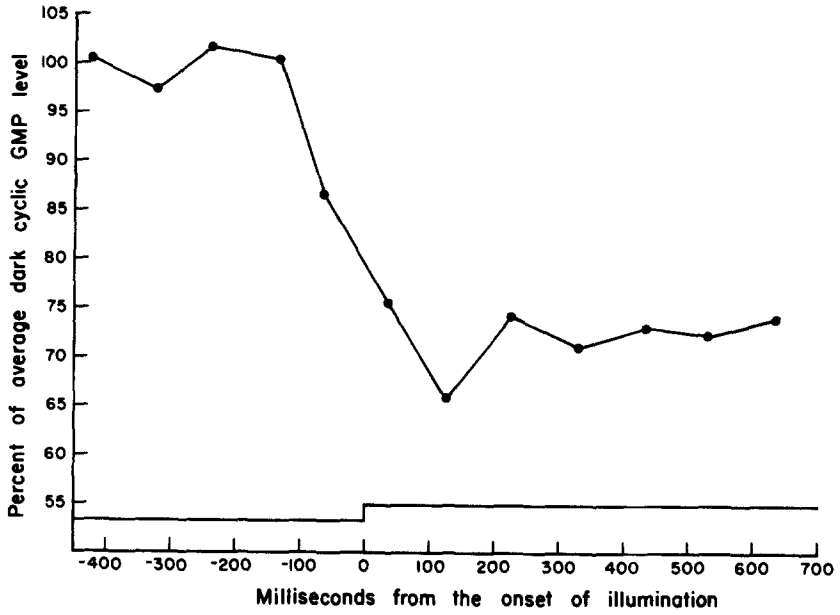

FIGURE 3. The light-dependent decrease in cyclic GMP measured in a rapid time-course experiment. 12 outer segment samples were acid-quenched in succession within 1.05 s (see Methods). Saturating illumination (bleaching 5.0×10^5 rhodopsin molecules/outer segment per second) was introduced after the fifth sample had been exposed to the perchloric acid. See text for details.

can be adjusted, as in Fig. 4, to reflect the fact that cyclic GMP levels actually fall after the onset of illumination. In 13 experiments similar to Fig. 3 the rate of decay was extrapolated, using linear regression analysis, to determine that the apparent beginning of the decrease occurred $\cong$145 ms before the onset of illumination. It was further determined for these experiments that the average time between the last point obviously not affected by illumination and the onset of illumination was 160 $\pm$ 20 ms. If one adds this time (160 ms) to the individual datum points, thereby shifting the response to the right relative to the onset of illumination, the decrease in cyclic GMP can be represented as occurring after, rather than before, illumination (Fig. 4).

To estimate the actual latency of the cyclic GMP decrease one must know the total time required for mixing acid with the outer segments (≅50 ms, see Methods), and for quenching endogenous reactions. The quenching time is probably determined by the time required for acid to penetrate the outer

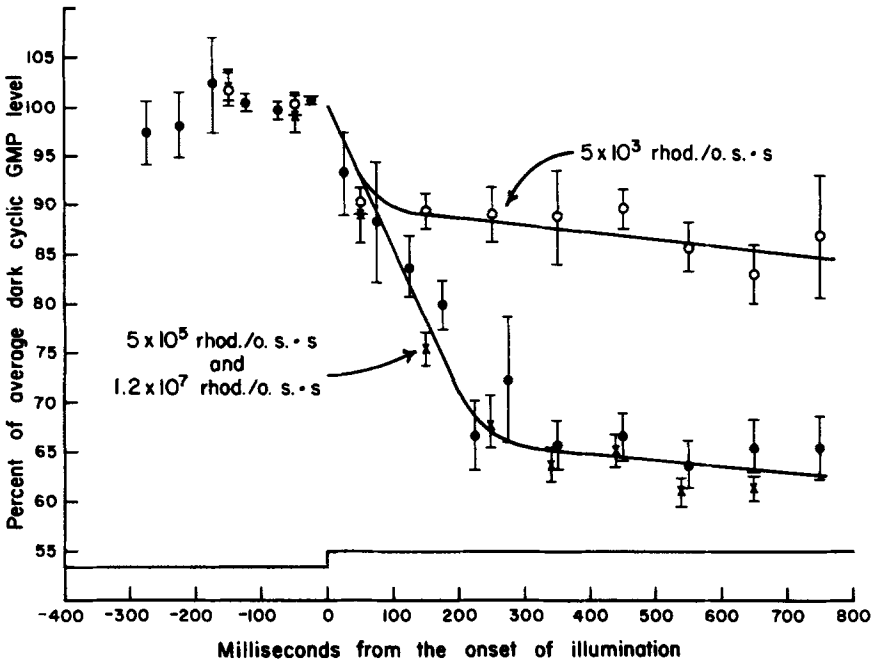

FIGURE 4. Rapid decrease in rod outer segment cyclic GMP levels measured at three different light intensities. 25 experiments were performed using the fast time-course device. 13 experiments used light intensity which was saturating for the cyclic GMP decrease (5.0×10^5 rhodopsins bleached/outer segment per second [●]), 6 experiments used light intensity two orders of magnitude below saturation (5.0×10^3 rhodopsins bleached/outer segment per second [○]), and another 6 used illumination two orders of magnitude above saturation (1.2×10^7 rhodopsins bleached/outer segment per second [X]). After adjusting each individual datum point by 160 ms, as described in the text, all of the data points were entered on a single graph and the points within discrete time intervals were averaged. The points shown in the figure represent the mean ± the standard error of the mean for all individual data points that fell within an interval. For the saturating intensity (●), enough data was available so that 50-ms intervals (between −300 ms and 300 ms) could be used for averaging. For the other intensities 100-ms intervals were used. Curves were drawn by hand. The latency and the rate of the cyclic GMP decrease were approximately similar at all intensities.

segment membranes ($t_{1/2} \cong 60$ ms, see Methods) and the nature of the reactions quenched. In soluble systems acid or alkali quenching requires <1–3 ms (Barman and Gutfreund, 1964). Liebman et al. (1978) have recently used a rapid kinetic assay to demonstrate that phosphodiesterase activity becomes maximal

within milliseconds after a bright flash. It seems likely then that cyclic GMP levels would begin to decrease within milliseconds after light absorption.

The results of 25 fast time-course experiments, in which the data were adjusted as just described, are presented in Fig. 4. The outer segments were exposed to three different light intensities. With a light that was just saturating for the response (5.0×10^5 rhodopsin molecules bleached/outer segment per second; ●) cyclic GMP was reduced by 35% from its dark-adapted concentration with a half-time of 125 ms. Light two orders of magnitude brighter (X) did not significantly increase the absolute latency, rate, or extent of cyclic GMP decay. Exposure to intermediate levels of illumination (○) caused a smaller decrease (10–15% compared to 30–35% for saturating intensity), but the latency of the decrease did not seem to be significantly longer. (This intermediate level of illumination causes a half-maximal permeability change in isolated outer segments [Brodie and Bownds, 1976; Woodruff et al., 1977]).

The total light induced decrease in cyclic GMP occurs in two phases. The rapid decrease shown in Figs. 3 and 4 is followed by a slower decay that occurs over the next few seconds (shown in Fig. 2; and in Fig. 3 of Woodruff et al., 1977). In two of the experiments in Fig. 4 (with light bleaching 5.0×10^5 rhodopsin molecules/outer segment per second) 10 additional data points were taken beyond 800 ms of illumination to illustrate this behavior. Within 1 s of illumination, cyclic GMP levels decreased by 30%, and within the next 20 s an additional fall of 10–15% completed the light-induced change (data not shown).

Given that there are between 3×10^7 and 6×10^7 cyclic GMP molecules/outer segment and 3×10^9 molecules of rhodopsin, one can calculate that the rate at which cyclic GMP molecules disappear during the rapid decrease shown in Fig. 4 is between 4×10^4 and 8×10^4 molecules/outer segment per millisecond. Assuming Michaelis-Menten kinetics, a cyclic GMP concentration of 10^{-5} M, a K_m of 0.07 mM (Miki et al., 1975), and a V_{max} of 20 mol cyclic GMP/mol rhodopsin per minute (initial velocity calculated from Fig. 2, *inset*), the fully light-activated phosphodiesterase might hydrolyze between 1.2×10^5 and 2.2×10^5 molecules of cyclic GMP/outer segment per millisecond. Thus, phosphodiesterase activation alone is probably sufficient to produce the rapid decrease shown in Fig. 4, if the rate of cyclic GMP synthesis is unaltered by illumination, and if PDE activation is as rapid as reported by Liebman et al. (1978).

The Light-Induced Decrease in Cyclic GMP Reverses When Illumination Ceases

The data of Figs. 5 and 6 demonstrate that cyclic GMP levels return to near their dark-adapted values after brief illumination at various light intensities. The results of these experiments, together with 16 others not shown, are summarized in Table I. If outer segments are exposed for 1 s or less to light bleaching between 5.0×10^2 and 5.0×10^5 rhodopsin molecules/outer segment per second, recovery of cyclic GMP levels is essentially complete (Figs. 5, 6, and Table I). After the most intense illumination used (1.2×10^7 rhodopsin molecules bleached/outer segment per second, delivered for 2 ms to 1 s) the rate of recovery was slowed, but cyclic GMP levels still recovered to within 6% of unilluminated control levels (Table I). (The extent of recovery was determined in each experiment after correcting for decay of cyclic GMP levels in an

unilluminated control. The average decay for this control was 8–9% during the 80-s period cyclic GMP levels were followed.) Recovery appeared to be compromised not by the intensity of the illumination but by its duration. For example, after 10 s of illumination bleaching 5.0×10^5 rhodopsin molecules/outer segment per second, cyclic GMP recovered to only 83% of the dark-incubated control, but after illumination which bleached 2.4 times more rhodopsin in 1 s, 96% recovery was obtained (Table I). With longer light exposures a longer delay

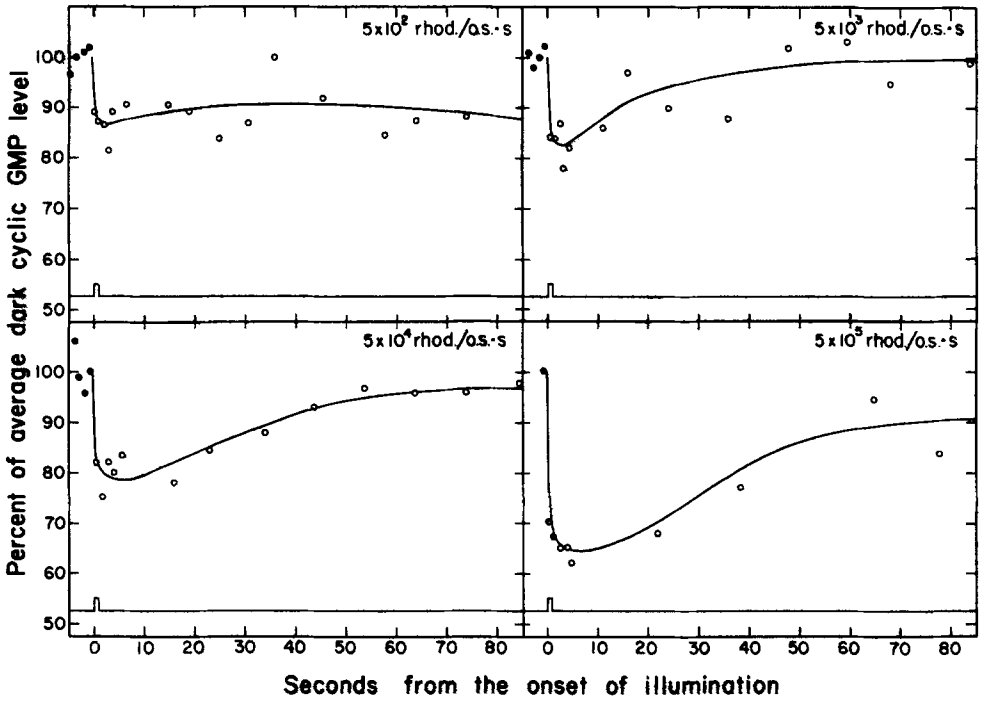

FIGURE 5. The light-dependent decrease in cyclic GMP as a function of light intensity. In four separate experiments rod outer segments were exposed for 1 s to light bleaching 5.0×10^2, 5.0×10^3, 5.0×10^4, or 5.0×10^5 rhodopsin molecules/outer segment per second. The average dark cyclic GMP level was determined for each individual experiment. As light intensity increased the amplitude of the cyclic GMP decrease increased (see also Fig. 7). Similar data for each intensity was obtained in three separate experiments.

between the cessation of illumination and the start of the cyclic GMP recovery was also observed. After exposure to 2 ms or 125 ms of illumination, cyclic GMP recovery was initiated in less than 10 s, but after a 1-s or 10-s exposure, 10–20 s were required (data not shown).

The Amplitude of the Cyclic GMP Decrease Is a Function of the Intensity and Duration of Illumination

The data from experiments such as those shown in Figs. 5 and 6 yield information on the amplitude of the cyclic GMP decrease under varying

conditions of illumination, and can be used to confirm and extend the findings of Woodruff et al. (1977). In that paper it was shown that the amplitude of the cyclic GMP decrease increased with the logarithm of light intensity at levels which bleach between 5.0×10^2 and 5.0×10^5 rhodopsin molecules/outer segment per second. To examine this relationship further outer segments were exposed for 2 or 125 ms or 1, 10, and 60 s to illumination bleaching between 5.0×10^2 and 1.2×10^7 rhodopsin molecules/outer segment per second, and the

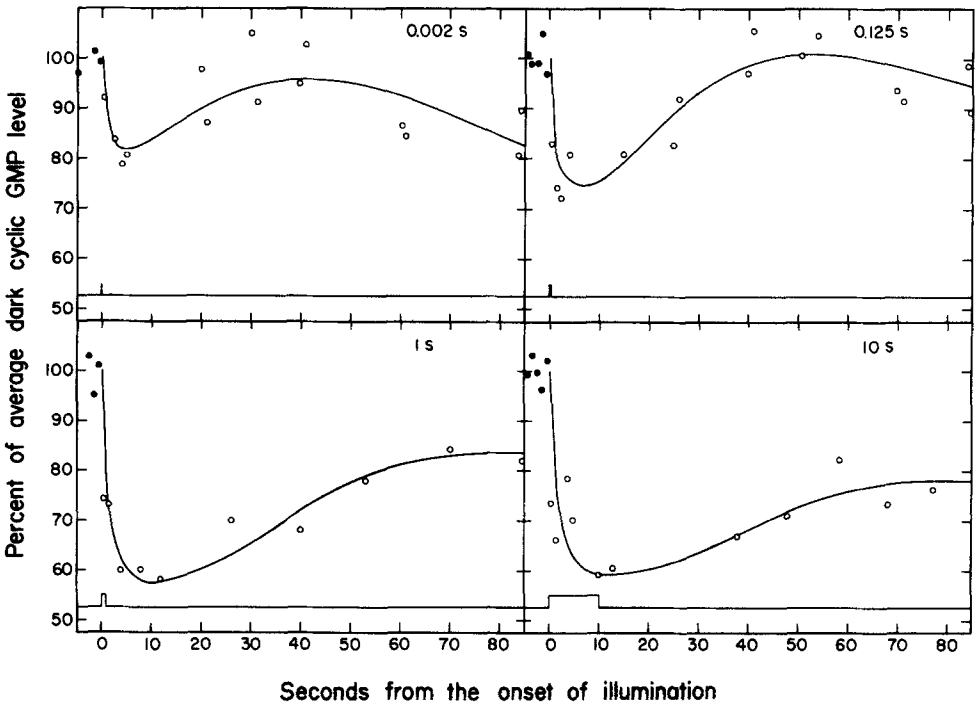

FIGURE 6. The light-induced decrease in outer segment cyclic GMP levels as a function of time of exposure to illumination. In four separate experiments rod outer segments were exposed to illumination bleaching 5.0×10^5 rhodopsin molecules/outer segment per second for 2 or 125 ms, or 1 or 10 s. The outer segments were prepared and extracted as in Fig. 2. Similar data for each condition was obtained in at least three separate experiments.

maximal cyclic GMP decreases were determined. The amplitudes of the responses for each condition are indicated in Fig. 7.

The amplitude of the cyclic GMP decrease is similar when measured after 1, 10, or 60 s of illumination and is graded with the logarithm of light intensity over the range between 5.0×10^2 and 5.0×10^4 rhodopsin molecules bleached/outer segment per second. This is consistent with the data and discussion in Figs. 2 and 4, and indicates that cyclic GMP concentration in outer segments falls to a value characteristic for a given level of illumination within several

seconds after the onset of illumination, and then remains at that level even though more rhodopsin is being bleached as illumination continues. For illumination lasting 1 s or longer, cyclic GMP levels must then be determined by the rate of rhodopsin bleaching, and not the absolute number of rhodopsin molecules bleached. Cyclic GMP is being continually synthesized and degraded in these isolated outer segments, and it would appear that light acts by shifting the balance between synthesis and degradation so that cyclic GMP level comes to a new steady value (see Discussion).

Illumination which lasts for 2 or 125 ms does not trigger the maximal cyclic GMP change possible for a given intensity at even the brightest intensity used (1.2×10^7 rhodopsin molecules bleached/outer segment per second). Even though 2 ms of this bright intensity bleaches approximately the same number of rhodopsin molecules as a 1-s exposure to an intensity of 5.0×10^4 rhodopsin

TABLE I

RECOVERY OF CYCLIC GMP LEVELS AFTER BRIEF ILLUMINATION

Time of light exposure	Light intensity	Extent of recovery	Rate of recovery
	Rhodopsin molecules bleached/ outer segment per second	*% of dark-adapted control*	*% increase/second*
2 ms	5.0×10^5	110±3	1.1
	1.2×10^7	98±1	0.5
125 ms	5.0×10^5	100±2	0.9
	1.2×10^7	94±3	0.4
1 s	5.0×10^3	100±4	0.7
	5.0×10^5	95±2	0.6
	1.2×10^7	96±1	0.2
10 s	5.0×10^3	92*	0.6
	5.0×10^5	83±1	0.4

In a number of experiments such as those shown in Figs. 5 and 6, the extent of recovery of cyclic GMP was determined by comparison with dark-adapted controls (see text). The rates of recovery are estimates and were determined by measuring the slopes of the curves after cyclic GMP started to return towards its dark level. Each value is the average from three determinations (±SE).

* Value is the average of two determinations.

molecules bleached/outer segment per second, the cyclic GMP decrease caused by the briefer illumination is significantly lower (see Fig. 7). For short light exposure the amplitude of the cyclic GMP decrease is a function of both the rate of rhodopsin molecule bleaching and the duration of exposure. This suggests that some component important in setting the maximum amplitude of the cyclic GMP decrease is expressed (or perhaps reaches a critical concentration) only if photoexcited rhodopsin molecules, or a short-lived rhodopsin intermediate, are present for a period of at least 1 s.

The first few photons captured in completely dark-adapted photoreceptors each cause 0.1–1.0% of the total possible permeability change (see Baylor et al., 1974; Schwartz, 1975; Fain, 1976). From the information in Fig. 7 one can

estimate how many cyclic GMP molecules disappear from the cyclic GMP pool in an outer segment for each rhodopsin molecule bleached. The largest value for this ratio is obtained for short exposure to dim light, the smallest for long exposure to bright light. Approximately 3×10^4 and 5×10^4 molecules of cyclic

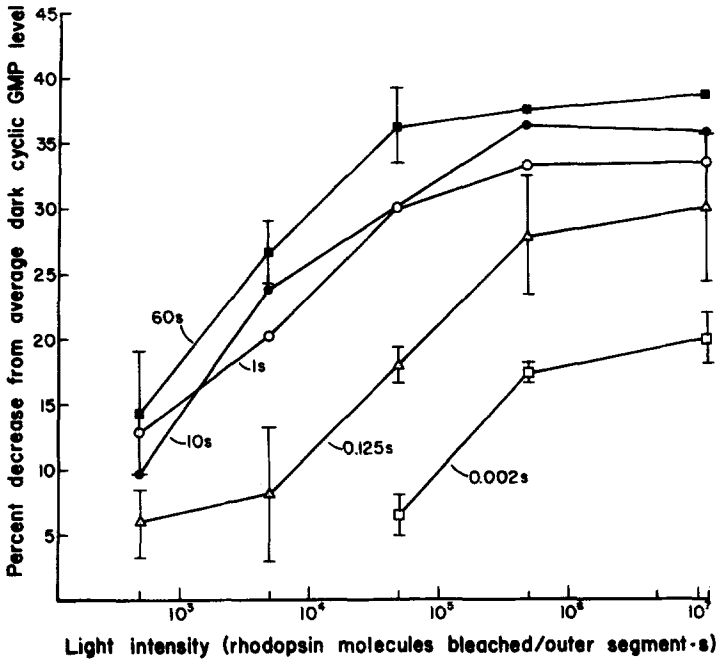

FIGURE 7. The amplitude of the cyclic GMP decrease as a function of light intensity and exposure duration. Outer segments were exposed for 2 ms (□), 125 ms (△), 1 s (○), 10 s (●), or 60 s (■) to illumination bleaching 5.0×10^2, 5.0×10^3, 5.0×10^4, 5.0×10^5, or 1.2×10^7 rhodopsin molecules/outer segment per second. The maximum decrease in each experiment (which occurred 2–20 s after the onset of illumination, depending on the duration of illumination) was determined using the three to five individual data points from the time-course (see Fig. 5 and 6) which gave the maximum difference between light and dark. The points shown are an average from at least three separate experiments (with the exception that points for the 60-s exposure at 5.0×10^5 and 1.2×10^7 rhodopsin molecules bleached/outer segment per second and the 10-s exposure at 5.0×10^5 rhodopsin molecules bleached/outer segment per second are the average of two determinations). Standard errors for 1-s and 10-s response curves are not indicated to permit the curves to be seen more clearly; they are similar to the errors shown for the 60-s curve. The response is larger at high light intensities, and is nearly complete after a 1-s exposure. Illumination for 125 or 2 ms at even the highest intensity does not give a maximal cyclic GMP decrease.

GMP disappear per rhodopsin molecule bleached after 2 ms of light bleaching 5.0×10^4 rhodopsin molecules/outer segment per second, and 125 ms of light bleaching 5.0×10^2 rhodopsin molecules/outer segment per second, respectively. The ratio rapidly decreases from this value as more rhodopsin molecules

are bleached. To obtain an estimate of the number of cyclic GMP molecules which disappear at even lower light intensities, one can extrapolate from the data of Fig. 7 graphed as shown in Fig. 8. Using logarithmic coordinates the decrease in the number of cyclic GMP molecules per rhodopsin molecule bleached is plotted as a function of the number of rhodopsin molecules bleached per outer segment. With light exposures that bleach > 10^5 rhodopsin molecules/outer segment, the slope of the curve is -1.0 ± 0.01 (mean ± SE; solid line) presumably because the cyclic GMP decrease has saturated and is not influenced

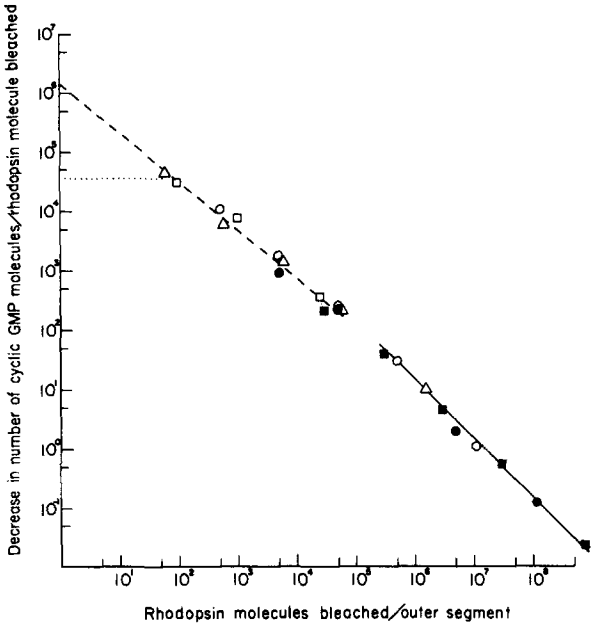

FIGURE 8. Decrease in the number of cyclic GMP molecules for each rhodopsin molecule bleached plotted as a function of the number of rhodopsin molecules bleached in each outer segment. Points were calculated assuming that dark-adapted outer segments contain 4.5×10^7 molecules of cyclic GMP. This value was multiplied by the percent decreases shown in Fig. 7 to obtain the actual number of cyclic GMP molecules disappearing after differing amounts of rhodopsin bleaching. The solid and dashed lines were determined by linear regression analysis for the data above and below 10^5 rhodopsin molecules bleached/outer segment, respectively. The symbols represent the different exposure durations and are defined in Fig. 7. (See text for details.)

by additional rhodopsin bleaching. With light exposures that bleach $<10^5$ rhodopsin molecules/outer segment, the slope is -1.22 ± 0.17 (mean ± SE; dashed line). If this is extrapolated to one rhodopsin molecule bleached per outer segment, $\sim 1.5 \times 10^6$ molecules of cyclic GMP, 3.3% of the total pool of outer segment cyclic GMP, would disappear after a single photon is captured. The actual decrease caused by a single photon absorption is probably between this extrapolated value and the experimentally derived value of $3\text{–}5 \times 10^4$ cyclic GMP molecules disappearing/rhodopsin molecule bleached, which represents approximately 0.1% of the total cyclic GMP.

The Decrease in Cyclic GMP Induced by Light Superimposed on Background Illumination Is Reduced by an Amount Proportional to the Intensity of the Background Light

In living photoreceptors the effect of dim background illumination is to make the receptor less responsive to light superimposed on that background (for example, see Fain, 1976). Thus, the effects of introducing steps of illumination were examined in the outer segment preparation. The data presented in Table II demonstrate that dim illumination (bleaching 5.0×10^1 rhodopsin molecules/outer segment per second) has no influence on the final level to which cyclic GMP is reduced by either an intermediate light intensity (bleaching 5.0×10^3 rhodopsin molecules/outer segment per second) or a saturating light intensity (bleaching 5.0×10^5 rhodopsin molecules/outer segment per second). The same result is obtained when the saturating illumination is superimposed on brighter background lights (Table II).

Because the first step of illumination (background light) partially decreased the level of cyclic GMP without affecting the final level to which cyclic GMP was decreased by the second step of illumination (test light), the cyclic GMP response to the test light was reduced compared to the response in outer segments not exposed to the background light. The magnitude of this reduction is proportional to the magnitude of the cyclic GMP decrease induced by the background light.

Removal of Calcium Enhances Cyclic GMP Levels in Dark-Incubated Outer Segments

In several electrophysiological studies it has been noted that procedures expected to lower calcium ion concentration in rod outer segments depolarize the membrane of the dark-adapted rod (presumably by increasing plasma membrane permeability) (Lipton et al., 1977 *a*; Brown et al., 1977). All of the experiments in this report and in our previous papers (Bownds and Brodie, 1975; Brodie and Bownds, 1976; Woodruff et al., 1977) have in fact been carried out in a modified Ringer's solution supplemented with 3 mM EGTA to bring the free calcium concentration to 10^{-8} M. These conditions increase the permeability of isolated outer segments as well as the magnitude of the light-induced permeability decrease (Bownds and Brodie, 1975) and have made the experiments which correlate enzyme activities or cyclic GMP levels with the permeability of isolated outer segments technically more feasible (Brodie and Bownds, 1976; Woodruff et al., 1977).

Lowering the calcium concentration increases not only the permeability of isolated outer segments (Brodie and Bownds, 1975) but also their cyclic GMP levels. Rod outer segments prepared in Ringer's solution containing 10^{-3} M Ca^{++} contained an average of $1.04 \pm 0.06 \times 10^{-2}$ mol cGMP/mol rhodopsin ($\pm$SE, $n = 3$), whereas those in Ringer's solution containing $\sim 10^{-8}$ M Ca^{++} (i.e., 3 mM EGTA) averaged $1.53 \pm 0.14 \times 10^{-2}$ mol cGMP/mol rhodopsin. Alternatively, if rod outer segments were prepared in a low calcium Ringer's solution (3 mM EGTA) and exposed to 4×10^{-3} M $CaCl_2$ (bringing free Ca^{++} to 10^{-3} M), cyclic GMP levels decreased by 27.5 $\pm$ 2.3% (mean $\pm$ SE, $n = 15$; for example,

see Fig. 9). It would appear that cyclic GMP concentration is influenced by changes in external calcium in the concentration range between 10^{-8} and 10^{-5} M, because the addition of 10^{-3} M $CaCl_2$ to outer segments prepared in Ringer's solution containing 10^{-4} M Ca^{++} did not influence cyclic GMP levels. (Calcium concentration in the serum-Ringer's solution containing no added EGTA was 10^{-4} M, and the cyclic GMP level was 0.95 ± 0.05 mol/mol rhodopsin.) Observations similar to these have been reported for living retinas by Cohen et al. (1977) who found that incubation in EGTA-containing solutions causes a large stimulation of cyclic GMP levels (10–20-fold), whereas increasing external calcium levels causes no depression of cyclic GMP levels.

TABLE II

EFFECT OF BACKGROUND ILLUMINATION ON THE AMPLITUDE OF CYCLIC GMP DECREASE

Light intensity			
Background light	Test light	*n*	Average dark cyclic GMP level
Rhodopsin molecules bleached/outer segment per second			%
–	–		100
5.0×10^{1}	–	6	91±3
–	5.0×10^{3}	4	70±6
5.0×10^{1}	5.0×10^{3}	4	68±5
–	5.0×10^{5}	2	51
5.0×10^{1}	5.0×10^{5}	2	57
5.0×10^{2}	–	2	76
–	5.0×10^{5}	2	47
5.0×10^{2}	5.0×10^{5}	2	47
5.0×10^{3}	–	2	64
–	5.0×10^{5}	2	57
5.0×10^{3}	5.0×10^{5}	2	54

Rod outer segments were exposed for 15 s to background light of indicated intensities and then for 6–8 s to a test light. The cyclic GMP decreases induced by the background light alone, the test light alone, and then the background followed by the test light are shown for various conditions of illumination. Background illumination does not significantly influence the level to which cyclic GMP is reduced by the test light. The number of experiments using each condition of illumination is indicated (*n*). In each experiment data points were averaged from triplicate samples.

Fig. 9 demonstrates further that although the level of cyclic GMP in dark-incubated outer segments is influenced by calcium concentration, the final level to which cyclic GMP is suppressed by saturating illumination is not. Between 10 and 30 min after isolating outer segments from the retina, the level of cyclic GMP in 10^{-3} M Ca^{++} was about 25% lower than the level in low-calcium Ringer's solution. When outer segments were illuminated with a saturating light intensity (bleaching 2.2×10^{6} rhodopsin molecules/outer segment per second) the level of cyclic GMP was reduced to approximately the same level (0.5×10^{-2} mol cGMP/mol rhodopsin) in both low and 10^{-3} M Ca^{++}. In six separate experiments like the one shown in Fig. 9, cyclic GMP concentration in the presence of 1 mM Ca^{++} was 96.0 ± 1.6% of that with ~10^{-8} Ca^{++} after exposure to saturating

illumination. Fig. 9 also shows that illumination bleaching 5.0×10^3 rhodopsin molecules/outer segment per second decreases cyclic GMP to an intermediate level in low calcium conditions (consistent with Fig. 7), but almost fully decreases cyclic GMP concentration in 1 mM Ca^{++} Ringer's solution. A conclusion drawn from a number of experiments at intermediate levels of illumination (bleaching 5.0×10^2 and 5.0×10^3 rhodopsin molecules/outer segment per second) is that the effectiveness of illumination (the number of cyclic GMP molecules which disappear for each rhodopsin molecule bleached) is not significantly altered by

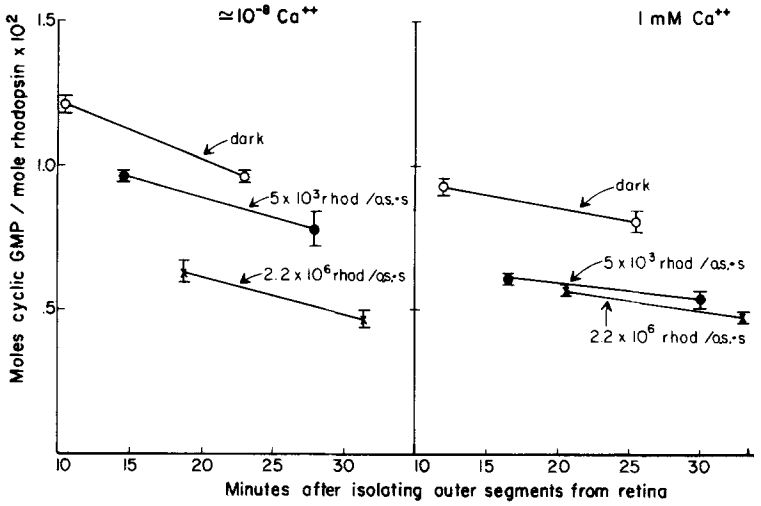

FIGURE 9. Calcium concentration and rod outer segment cyclic GMP levels. Outer segments from two retinas were combined in the modified Ringer's solution used in the experiments of Figs. 1–8 (containing 3 mM EGTA, $\sim 10^{-8}$ M Ca^{++}). A portion of this suspension was made 4 mM in $CaCl_2$, so that the free calcium concentration was 1 mM. The low calcium and 1 mM Ca^{++} outer segment suspensions were each divided into three portions. One was left dark (○), another was illuminated with light bleaching 5.0×10^3 rhodopsin molecules/outer segment per second (●), and the third was illuminated with light bleaching 2.2×10^6 rhodopsin molecules/outer segment per second (x). Each data point represents the average (± standard error) of triplicate samples (50 μl) taken at the times indicated. Calcium decreased the dark-adapted level (○) of cyclic GMP ~25%. Illumination bleaching 5.0×10^3 rhodopsin/outer segment per second (●) partially decreased cyclic GMP in the control Ringer's solution, and fully suppressed cyclic GMP in 1 mM Ca^{++}.

conditions of low or high calcium concentration. In high calcium the maximal light-induced decrease in cyclic GMP thus occurs at a lower light intensity (5.0×10^3 rhodopsin molecules bleached/outer segment per second), because in this condition there is less cyclic GMP in the dark available for hydrolysis on illumination.

DISCUSSION

The main finding of this work is that cyclic GMP levels in freshly isolated frog rod outer segments fall very rapidly upon illumination and within a few seconds

assume a steady value which is a function of the light intensity. It would appear that the light-dependent changes noted in this paper, at least among the nucleotides, may be unique to cyclic GMP. Orr et al. (1976) and Cohen et al. (1978), have shown that cyclic AMP levels are not light sensitive. Biernbaum and Bownds[1] have found that ATP levels are not changed by illumination, and that a light-induced decrease in GTP levels is slower than the cyclic GMP decrease.

The light-induced decrease in cyclic GMP occurs rapidly enough and with sufficiently high stoichiometry to be involved in the transduction process in rod outer segments. The cyclic GMP decrease probably occurs within milliseconds of light absorption, and the half-time for the initial rapid phase is 125 ms. The light-induced hyperpolarization of the amphibian rod has an absolute latency between 30 and 50 ms, and the time to peak at moderate to bright light intensities is between 100 and 200 ms (for example, see Fain, 1976). Adaptation processes may be initiated within 50–100 ms of the onset of illumination (Baylor and Hodgkin, 1974). The graph of Fig. 8 suggests that the fraction of total cyclic GMP which disappears for each of the first few rhodopsin molecules bleached is between 0.1%, measured after bleaching 60–100 rhodopsin molecules, and a value of 3.3%, obtained by extrapolation to one rhodopsin molecule bleached. (Experimental error prevented direct determination of the cyclic GMP decrease caused by <100 photon absorptions.) Because approximately half of the total cyclic GMP is light-sensitive, between 0.2 and 6.6% of the light-sensitive cyclic GMP disappears per rhodopsin molecule bleached for each of the first few photons captured. In fully dark-adapted photoreceptors, 0.1–1.0% of the total possible permeability suppression is caused by each photon absorption for the first 10–20 photons captured (see Baylor et al., 1974; Schwartz, 1975; Fain, 1976) and the absorption of only a few photons/rod per second triggers a light adaptation which lowers the sensitivity to about one-third of its dark-adapted value (Baylor and Hodgkin, 1974; Fain, 1976).

Continuous illumination at intermediate intensities (5×10^2 and 5×10^3 rhodopsin molecules bleached/outer segment per second) causes cyclic GMP concentration to fall to steady levels which are clearly intermediate between dark levels and the levels caused by saturating illumination (5×10^4 to 5×10^5 rhodopsin molecules bleached/outer segment per second). 90% of the change occurs over 3–4 log units of light intensity. The permeability of isolated outer segments (Brodie and Bownds, 1976) and the hyperpolarizing response of amphibian rod receptors to continuous illumination (Fain, 1976) are graded with light intensity over this same range. (Most vertebrate rod cells exposed to continuous illumination increase their response as the light intensity is raised to ~ 4 log units above threshold, with saturation of the response occurring when about 10^4 rhodopsin molecules are being bleached/outer segment per second. For example, see Normann and Werblin, 1974; Kleinschmidt and Dowling, 1975; Fain, 1976.)

In addition to similarities between the behavior of cyclic GMP and the electrical activity of photoreceptors, there are at least four significant differ-

[1] Biernbaum, M., and D. Bownds. Light-activated decrease of guanosine 5'-triphosphate in isolated frog rod outer segments. Manuscript in preparation.

ences. First, short flashes of bright light (10–100 ms in duration) can cause a saturating photoreceptor response, but illumination lasting for up to 1 s is required for a maximal cyclic GMP decrease to be registered (Fig. 7). Second, even though the photoreceptor potential change and the cyclic GMP response saturate at about the same light intensity, the half-maximal response for photoreceptor hyperpolarization occurs when only 10–20 photons have been absorbed, whereas the half-maximal response for cyclic GMP occurs when ~1,000 photons have been captured (or ~100 photons when calcium concentration has not been buffered to $\sim 10^{-8}$ M by EGTA addition). The cyclic GMP response is less sensitive than the potential response by 1–2 orders of magnitude. Third, dim background illumination desensitizes the rod receptors response to light but has no desensitizing effect on the cyclic GMP decrease (Table II). Finally, the receptor's membrane potential recovers rapidly after 10 s of illumination bleaching 5.0×10^5 rhodopsin molecules/outer segment per second, but cyclic GMP levels recover slowly and incompletely (Fig. 5, Table I).

Some of these differences may derive from a "slowing down" of the endogenous chemistry of outer segments after their detachment from the retina. Even though isolated outer segments maintain their ability to synthesize and degrade cyclic GMP, it is clear that degenerative changes are taking place. Levels of cyclic GMP, ATP, and GTP decay in dark adapted outer segments (Fig. 1 *a*, and Woodruff et al., 1977; Biernbaum and Bownds[1]), presumably because crucial metabolites or cofactors can no longer be obtained from the inner segment portion of the photoreceptor. The response of cyclic GMP to illumination becomes less with time after isolating the outer segments from the retina (Fig. 1 *b*). The failure of cyclic GMP to recover after a lengthy exposure to illumination also may be symptomatic of degenerative changes. It is because of this deterioration that no attempt has been made to extend to aged preparations the correlation, noted by Woodruff et al. (1977), between light suppression of cyclic GMP and permeability. Although Bownds and Brodie (1975) observed light suppression of outer segment swelling (permeability) 2 h after detachment of outer segments from the retina, it has been found that in many preparations this is not observed. Light suppression of swelling often is lost in <1 h. Thus, the lability of both the permeability mechanism and the light-induced cyclic GMP decrease has made it impractical to pursue their correlation in outer segments detached from the retina for more than 60 min.

Further problems arise in comparing the effects of illumination on the intracellularly recorded rod response with the cyclic GMP measurements on isolated outer segments in suspension. The electrical responses recorded from a single rod derive from a pooling of the signals of many adjacent receptors and thus do not represent only the receptor which is impaled (Schwartz, 1975; Fain et al., 1975; Copenhagen and Owen, 1976); further, they might involve voltage-dependent conductances arising in the inner segment portion of the photoreceptor (Fain et al., 1978). It will be more relevant to compare the chemical changes which occur in individual outer segments with conductance changes which occur in individual outer segments. Yau et al. (1977) have recently introduced techniques for monitoring the membrane current of single rod

outer segments, and appropriate data on conductance changes in the outer segment plasma membrane should soon be available for comparison with the light-induced chemical changes. It is important also to emphasize that data in these experiments were obtained in low-calcium media; the presence of 3 mM EGTA brought the free calcium concentration to $\sim 10^{-8}$ M. Most of the electrophysiological data has been obtained by using Ringer's solutions containing millimolar levels of calcium.

In these outer segment preparations, cyclic GMP concentration probably is regulated by a balance between its synthesis and destruction. The ability of the outer segments to synthesize cyclic GMP is indicated by an increase in cyclic GMP levels when inhibitors of the degradative enzyme, phosphodiesterase, are added (Woodruff et al., 1977) and by the observation that cyclic GMP levels increase after termination of a brief light exposure (Figs. 5 and 6). Cyclic GMP concentration is clearly "buffered", for it remains fairly constant even when levels of its precursor GTP are changed (Biernbaum and Bownds[1]). The mechanism which permits cyclic GMP concentration to stabilize at intermediate levels of illumination then probably involves shifting the equilibrium between its synthesis and destruction. The controlling steps occur at least partially in the link between rhodopsin bleaching and phosphodiesterase activation, for Abramson et al.[2] have found that phosphodiesterase activation in minimally disrupted outer segments occurs over 3–4 log units of light intensity. Phosphodiesterase activity, like cyclic GMP levels, reaches and maintains a constant value at intermediate levels of continuous illumination, and is related to the rate of rhodopsin bleaching (to light intensity) rather than the absolute number of rhodopsin molecules bleached. Part of the light-induced lowering of cyclic GMP levels could also derive from inhibition of the guanylate cyclase activity. Krishna et al. (1976) have reported a two-fold inhibition of guanylate cyclase caused by illumination. Goridis and Weller (1976), however, failed to confirm this observation, and Abramson et al.[2] find no effect of illumination.

The return of cyclic GMP levels to their dark values after brief illumination probably is caused by deactivation of the phosphodiesterase. Abramson et al.[2] have observed an inactivation of phosphodiesterase after brief illumination, and Wheeler and Bitensky (1977) have isolated a GTPase activity which they suggest is involved in both activation and inactivation of phosphodiesterase. Also, it has been shown that after large rhodopsin bleaches the regeneration of rhodopsin by addition of 11-*cis*-retinal causes partial inactivation of phosphodiesterase (Keirns et al., 1975; S. Green and D. Bownds[3]). It is difficult to determine whether this mechanism of inactivation is used at the low light intensities employed in these experiments (which bleached <1% of the rhodopsin present), for the frog rod outer segments have an endogenous store of 11-*cis*-retinal sufficient to regenerate rhodopsin after a 1–3% bleach (Azuma et al., 1977). An

[2] Abramson, B. P., R. P. Robinson, M. L. Woodruff, and D. Bownds. Light sensitive enzymes regulating guanosine 3′,5′-cyclic monophosphate concentration in frog rod outer segments. Manuscript in preparation.

[3] Green, S., and D. Bownds. Unpublished observations.

attempt to enhance recovery of cyclic GMP levels after the cessation of illumination by adding exogenous 11-*cis*-retinal was not successful (Woodruff and Bownds[4]).

The kinetics and stoichiometry of the light-induced cyclic GMP decrease are consistent with a role for cyclic GMP in visual transduction. One possibility is that cyclic GMP functions as an internal transmitter mediating between photon absorption and the permeability decrease of the plasma membrane, a role previously suggested for calcium ions. If the presence of cyclic GMP were required to maintain high permeability (to "hold open channels"), its disappearance would lead to a suppression of permeability and photoreceptor hyperpolarization. Another possibility is that cyclic GMP plays an important role in visual adaptation, with changes in cyclic GMP modifying the effect of illumination on the permeability mechanism.

Following the suggestion of Hagins and Yoshikami that calcium might be an internal transmitter in outer segments, considerable interest has focused on measuring the physiological effects of low and high calcium conditions (see, for example, Hagins and Yoshikami, 1974; Brown et al., 1977; Lipton et al., 1977*a*; Wormington and Cone, 1978). There is general agreement that permeability of rod receptor cells is suppressed if calcium concentration is raised to 3–10 mM, and thus raising external calcium appears to mimic the effect of illumination. Conversely, dark permeability increases when calcium concentration is lowered by the addition of the chelating agent EGTA. Experiments using calcium ionophores or intracellular injections of calcium confirm that these permeability effects result from changes in intracellular calcium levels. Although there is agreement that both intracellular calcium and light can influence outer segment permeability, Bastian (1978) has provided evidence that calcium and light have very different effects on the sensitivity and waveform of the rod responses, and similar observations have been made for cone responses (Bertrand et al., 1978).

It is possible that the effect of exogenous calcium on permeability may derive from the calcium-induced changes in cyclic GMP concentration. Cyclic GMP levels, like permeability, are suppressed by calcium addition. The locus of this calcium effect may be the guanylate cyclase, for Troyer et al. (1978) and Krishnan et al. (1978) have demonstrated that calcium ions can inhibit this enzyme. Miki et al. (1973) have failed to note an effect of calcium on phosphodiesterase activity. Further work will be required to determine whether calcium effects other photoreceptor chemistry, and how calcium and cyclic GMP might interact in controlling rod outer segment permeability. Interaction between calcium and cyclic nucleotides as a physiological control mechanism has been suggested for other cell systems (for review, see Rasmussen and Goodman, 1977).

In other neural systems, it has been speculated that cyclic nucleotides control ion permeability by regulating the covalent modification of proteins, most notably through phosphorylation/dephosphorylation sequences (Greengard, 1978). If this were the case in rod outer segments, one might expect to find protein phosphorylation which is maximal in the dark, when cyclic GMP levels

[4] Woodruff, M. L., and D. Bownds. Unpublished observation.

are high, and dephosphorylation in the light as cyclic GMP levels decrease. This laboratory has identified two low molecular weight protein components of frog rod outer segments which undergo a dephosphorylation/phosphorylation sequence after brief illumination (Polans et al., 1978, Polans et al.[5]). Agents which enhance cyclic GMP levels and outer segment permeability increase the phosphorylation level of the proteins. Addition of calcium ions, which suppresses permeability and cyclic GMP levels in isolated outer segment, causes rapid dephosphorylation of the proteins. Establishing further correlations between permeability changes in outer segments and the phosphorylation-dephosphorylation of these proteins, as well as determining the locus of these proteins, are important steps in elucidating the possible function of cyclic GMP in visual transduction.

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