Physiological Evidence that Light-mediated Decrease in Cyclic GMP Is an Intermediary Process in Retinal Rod Transduction

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ABSTRACT Brief, intracellularly injected pulses of cyclic GMP transiently depolarize toad retinal rod outer segments (ROS). The depolarization is antagonized by light, perhaps by the activation of phosphodiesterase (PDE), as shown in the biochemical studies of others. As measured by the antagonism of cyclic GMP pulses by light, PDE activity peaks after the peak of the receptor potential and has approximately the same recovery time as the membrane voltage after weak illumination, but recovers more slowly than the membrane potential after strong illumination, as sensitivity does in other preparations. A cyclic GMP pulse delivered just after the hyperpolarizing phase of the receptor potential tends to turn off the light response. The kinetics of recovery from this turnoff are similar to those of the initial phase of the receptor potential. This similarity suggests that the initial phase of the receptor potential is controlled by light-activated PDE. Both EGTA and saturating doses of cyclic GMP block the light response, but only cyclic GMP increases response latency, which suggests that if calcium is involved in transduction, it is controlled by the hydrolysis of cyclic GMP. After brief pulses of cyclic GMP, a new steady state of increased depolarization occasionally develops. The effects described above also occur under these conditions. The results are consistent with the hypothesis that light-activated hydrolysis of cGMP is an intermediary process in transduction.

INTRODUCTION

The purpose of the experiments reported here is to test the hypothesis that the hydrolysis of cyclic GMP caused by light-activated PDE is an intermediary process controlling ROS transduction. The origins of this theory are the observations that the ROS contains light-activated enzymes in the cyclic nucleotide enzymatic cascade (Bitensky et al., 1971); that light both increases the hydrolysis of cyclic GMP and decreases the permeability of isolated ROS (Woodruff et al., 1977); that the speed and power of this light-activated PDE hydrolysis of cyclic GMP is sufficient to mediate transduction (Yee and Liebman, 1978; Woodruff and Bownds, 1979); that the first amplification step in the activation of PDE has each photolyzed rhodopsin molecule catalyzing the exchange of 500 GTP's for GDP's to make the guanyl nucleotide-binding site inactive.
protein, transducin, capable of activating PDE (Fung and Stryer, 1980; Stryer et al., 1981); and that the injection of cyclic GMP into the ROS of intact rods increases the receptor potential latency as if the excess must be hydrolyzed to produce the receptor potential (Nicol and Miller, 1978).

In the study reported here, it is suggested that the amplitude and kinetics of ROS membrane voltage responses to pulse injections of cyclic GMP reflect net ROS cyclic GMP hydrolysis rates and hence PDE activity. The recovery from a cyclic GMP injection given just after the initial phase of the receptor potential has kinetics similar to the initial phase, which suggests that the hydrolysis of cyclic GMP by light-activated PDE is an intermediary process in transduction.

MATERIALS AND METHODS

Patches of the retina are removed from decapitated and double-pithed *Bufo marinus* that had been dark adapted more than 2 h. Such retinas are mounted receptor side up and superfused with amphibian Ringer's: 108 mM NaCl, 0.6 mM NaSO₄, 0.13 mM NaHCO₃, 2.5 mM KCl, 1.2 mM MgSO₄, 1.6 mM CaCl₂, 5.6 mM glucose, and 3 mM HEPES. The Ringer's is adjusted to pH 7.6 with NaOH. Intracellular recordings and injections are made with glass micropipettes filled with 2 M KCl or 25 mM cyclic GMP (potassium salt; P-L Biochemicals, Inc., Milwaukee, WI), the latter having a resistance of ∼1,000 MΩ. Pipettes filled with higher concentrations of cyclic GMP tended to leak, as judged by the growth of microscopic crystals at the pipette tip. 1-nA current pulses are delivered through the recording pipette using a constant current generator triggered 1-20 ms so as to deliver pulses of 1-20 pC. When negative current is passed, the upper bound, assuming a transferance number of 1, is 6 × 10⁶ ions of cyclic GMP⁻/pC. The frog ROS contains 4 × 10⁷ cyclic GMP's (Woodruff and Bownds, 1979), whereas the *Bufo* rods used here (of 6.5 μM diameter and 80 μM length) have twice the volume so that each picocoulomb should increase the dark concentration of cyclic GMP by 7.5% at the most. The pipette is lowered onto the preparation and either spontaneously enters an ROS when the mechanical artifact indicating contact appears, or is oscillated in using negative capacitance feedback, applied as close to the contact point as possible to ensure entry into the outer segments. The whole retina is stimulated with flashes of white light: 0 log units = 80 μW cm⁻², or 2.0 × 10⁶ effective photons per second per rod.

Abbreviations

The following abbreviations are used: cyclic GMP: cyclic guanosine 3',5'-monophosphate; 8-bromo cyclic GMP: 8-bromo cyclic guanosine 3',5'-monophosphate; cyclic AMP: cyclic adenosine 3',5'-monophosphate; PDE: phosphodiesterase; GDP: guanosine 5'-diphosphate; GTP: guanosine 5'-triphosphate; IBMX: isobutylmethylxanthine; ROS: rod outer segment; HEPES: N-2 hydroxyethylpiperazine-N' -2-ethanesulfonic acid; Kᵢ: concentration of inhibitor for 50% enzyme inhibition; Kₐ: concentration of substrate for 50% enzyme activation; EGTA: ethyleneglycol-bis (beta-amino-ethyl ether) N,N' -tetraacetic acid.

RESULTS

Small Pulses of Cyclic GMP Transiently Depolarize

When small pulses of cyclic GMP are iontophoresed into an ROS in darkness, each pulse causes a transient depolarization and successive pulses have pro-
gressively slower decay rates (Miller and Nicol, 1981) as shown in Fig. 1. Passage of negative current is indicated on the lower (signal) trace by a downward deflection, positive current by an upward deflection. The last two upward deflections signal light, not current. The upper trace shows the membrane voltage response. The spike-like negative deflection (a) is caused by the current pulse; the slow transient depolarization (b) is the membrane voltage response to the injection of cyclic GMP⁻. Two controls demonstrate that the response to cyclic GMP is not artifactual. The spike-like positive deflections (c) are the artifacts of the passage of the same amount of positive current (16 pC of K⁺), which have a negligible lasting effect on membrane voltage. Similarly, when the electrode is removed from the ROS, the only response to negative current is a negative spike (Miller and Nicol, 1981). The first -4 log unit, 0.1-s light flash (d) shown in Fig. 1 (200 photons/ROS) is given shortly after the injection of cyclic GMP and antagonizes the transient depolarization (e), as evidenced by its more rapid decay, but more convincing evidence for this antagonism is presented in Figs. 7B and C. The main purpose of the record in Fig. 1 is to show the depolarizing response to cyclic GMP, its increasingly gradual decay in the absence of light, as well as the effect of a control K⁺ injection. The depolarization caused by cyclic GMP is associated with an increased conductance, as would be expected from increased Na⁺ permeability (Woodruff and Bownds, 1979; Miller and Nicol, 1981). The molecular mechanism of the depolarization caused by cyclic GMP is unknown. The hyperpolarizing recovery phase or gradual decay of the depolarization we previously hypothesized to be the result of PDE activity, whereas the continual slowing of the recovery would be the result of decreasing PDE activity with dark adaptation.
Threshold Response Is $6 \times 10^6$ Ions of Cyclic GMP$^-$ at Most

Progressively larger pulses of cyclic GMP$^-$ (Fig. 2) cause graded transient depolarizations of progressively larger amplitudes (Miller and Nicol, 1981) until the response saturates. The threshold membrane depolarizing response is $\sim 1 \text{ pC}$ of cyclic GMP$^-$, which should increase the dark concentration of ROS cyclic GMP by 7.5% at most (Fig. 2). Initially, the local concentration would be much higher near the pipette tip. The time until the first detectable membrane depolarization to the injection can be resolved at 70 ms (Miller, 1981). The leading edge of the cloud of injected cyclic GMP would have traveled 8 $\mu$m in that time, assuming radial symmetry, an aqueous medium, and a diffusion coefficient equal to that of cyclic AMP (Dworkin and Keller, 1977).

![Figure 2](image)

**Figure 2.** ROS membrane voltage response to injections of 1, 2, 3, 4, 5, 6, 7, 8, 7, 6, 5, 4, 3, 2, 1, 1 pC of cyclic GMP$^-$, respectively, left to right. Titration of cyclic GMP response as a function of injection current.

Saturating Pulses of Cyclic GMP$^-$ Increase Latency

In the absence of light, the response to the cyclic GMP injection decays back to the baseline (X’s, upper and middle records, Fig. 3) and, in contrast to the light response, never hyperpolarizes below the baseline. Responses to brief injections of cyclic GMP decay more slowly with dark adaptation and sometimes with aging of the preparation independent of dark adaptation, as illustrated in Fig. 3, where in spite of frequent light flashes, the response to cyclic GMP-labeled $X_5$ is more prolonged than $X_4$. When the depolarization caused by cyclic GMP saturates, the response to a light flash delivered during the plateau shows an increase in latency that is inversely proportional to the light intensity, which suggests that the excess cyclic GMP must be hydrolyzed by light-activated PDE to produce the response (Nicol and Miller, 1978). The latency, measured from the start of the flash to the initial response as shown by the arrows, bottom record, Fig. 3, is increased by a factor of 2.5 over the control in this case. After large saturating injections of cyclic GMP$^-$, the latency of the light response to dim flashes is increased more than the response.
to bright flashes, as shown by Fig. 4 taken from the data of the record on Fig. 6 of Miller and Nicol (1981). The latencies to the −5 and −4 log unit flashes are ~13 times that of the controls, whereas the −3 log unit (saturating flash) response latency is only six times the control latency. However, the rate of rise of the responses (millivolts per second) to the flashes is about the same after cyclic GMP− when compared with the controls (Fig. 5), as would be expected if the excess cyclic GMP− had to be hydrolyzed to produce the membrane voltage response to light and if the rate of rise of the response were controlled by light-activated PDE. Note that the PDE activity, as judged by the antagonism to cyclic GMP pulses, is maintained for a sufficiently long period both to hydrolyze the excess and to produce the response. The rate of rise is about the same for both the control and after the 12-pC saturating injection of cyclic GMP (Fig. 3, bottom record). The similarity between the kinetics of
the receptor potential of the control and of the delayed response after a saturating injection of cyclic GMP is evidence that both hyperpolarizations have the same underlying mechanism: the hydrolysis of cyclic GMP mediated by light-activated PDE.

This effect of excess cyclic GMP on latency appears to be unique, but cyclic GMP is not unique in causing transient depolarizations. Cyclic AMP, 5'-GMP (Miller and Nicol, 1979, 1981), and Cl⁻ (record A, Fig. 6) do also. The chloride injections shown in record A, Fig. 6, cause a transient depolarization sometimes followed by a plateau of depolarization or oscillations, and this type of voltage response to chloride is not substantially altered by the −2 log unit light flash.

![Graph showing latency as a function of flash intensity](image)

**Figure 4.** Latencies of light responses as a function of flash intensity after saturating doses of cyclic GMP and in the absence of injections. Latency is measured as time from beginning of flash to beginning of response to light as shown on bottom record, Fig. 3. Data are from experiment of Fig. 6, Miller and Nicol (1981). Increase in latency after saturating dose of cyclic GMP is ascribed to time for light-activated PDE to hydrolyze excess injected cyclic GMP.

Responses to light flashes delivered during the plateau of depolarization caused by Cl⁻ are smaller in amplitude and of the same latency as controls (not shown). The same is true for responses to illuminations during depolarizations caused by cyclic AMP, 5'-GMP (Miller and Nicol, 1979), and EGTA (Miller, 1981, and Discussion below). In contrast to all of these substances, in the presence of saturating doses of cyclic GMP, both the latency and amplitude of the receptor potential are increased.

*Response to Cyclic GMP Pulses May Reflect Underlying ROS PDE Activity*

If illumination activates PDE, as biochemical experiments suggest (Polans et al., 1981; Yee and Liebman, 1978), light flashes would be expected to
antagonize the effects of pulses of injected cyclic GMP. If, as suggested by the results of Fig. 2, the amplitude of the response to cyclic GMP is dose dependent, the increased rate of cyclic GMP hydrolysis would be reflected in a more rapid recovery from the depolarization caused by cyclic GMP or by the suppression of the depolarization. In contrast to light’s lack of effect on

![Graph](image)

**Figure 5.** Rate of rise of light response hyperpolarization before and after saturating doses of cyclic GMP as a function of light intensity is similar, which adds weight to the argument that the hyperpolarizing phase of the receptor potential is controlled by light-activated PDE. Rate of rise is measured on lines tangent to response as shown on bottom record, Fig. 3, and slopes are compared at about the same absolute membrane potential. From experiment of Fig. 6, Miller and Nicol (1981).

the depolarizations caused by other ions (e.g., Cl⁻, record A, Fig. 6), light strongly antagonizes the depolarizations caused by picocoulomb injections of cyclic GMP. The peak of this antagonism occurs later than the peak of the voltage response to light. The 10 injections of 5 pC each shown on Fig. 6, record B, are numbered consecutively. Number 1 is in the dark and shows a
Figure 6. Down on signal trace indicates iontophoresis of negative current: upper record 60 pC Cl\(^-\), all other records 5 pC cyclic GMP\(^-\). Up on signal trace indicates light flashes: record A, -2 log units, 0.1 s; records B, C, and D, -4 log units, 0.1 s. The responses to large pulses of Cl\(^-\) (A) are little affected by light in comparison with those of cyclic GMP (B), which are suppressed. Recovery from pulse number 2 (B) is shown in the expanded trace (C) and is superimposed on the initial phase of the receptor potential in (D). Similarity in kinetics of recovery from injection and initial phase of the receptor potential strengthens the argument that light-activated PDE underlies both processes.
spike-like depolarization. Number 2, given shortly after the delivery of the 0.1-s \(-4\) log unit flash, is smaller in amplitude, and the recovery from the depolarization is faster than for injection 1, as if light-activated PDE were antagonizing the depolarizing response to cyclic GMP\(^{-}\). At injection 3, which occurs after the peak of the hyperpolarization to light, the depolarization that would have occurred if the injection had been given in the dark-adapted preparation is completely suppressed, as if strong PDE activity hydrolyzed the injected cyclic GMP\(^{-}\) before it could exert a physiological effect. By injection 10, the state reflected at number 1 is reached.

One can argue that the electrical response to pulses of cyclic GMP reflect PDE activity. If PDE activity in turn determines membrane potential and/or sensitivity, one would expect a degree of correlation between the antagonism of depolarizations caused by cyclic GMP and the membrane potential and/or sensitivity. Fig. 7, record C, illustrates that for weak flashes, the return of PDE activity to the level before the flash corresponds with the time for the return of the membrane potential. For stronger illumination, the return of the membrane potential precedes the decrease in PDE activity to pre-flash levels (Figs. 7B and 6B). This point is illustrated by the dashed lines on Figs. 6B and 7B and C, which indicate the resting potential. Although sensitivity has not been measured on this preparation, it is known that membrane potential returns before the full recovery of sensitivity after strong illumination (Kleinschmidt and Dowling, 1975). It is therefore suggested that the decline in PDE activity, as measured by this physiological technique, will be found to correlate with the return of sensitivity. The PDE activity that is reflected in the membrane voltage response to cyclic GMP would actually be expected to be the result of cyclase and PDE activities. It is concluded that the antagonism of the depolarization to cyclic GMP pulses shown in Fig. 6B reflects increased PDE activity caused by the light flash, that the activity peaks after the membrane voltage response, and decreases as the membrane potential recovers after weak illumination, but is restored more slowly than the membrane potential following strong illumination.

Record B, Fig. 6, is reproduced on a slower time scale at Fig. 7A. After the 0.1-s flash is a 14.4-s flash at the same intensity. Just before the 14.4-s flash, the recovery following a cyclic GMP pulse is seen to be very slow. The response to the 14.4-s flash is not strictly comparable with the previous flash because of the different kinetics that would be expected for a response to a longer flash. When the results of Figs. 7A–C are analyzed so as to derive PDE recovery time as a function of flash energy, it appears that the larger the flash energy, the longer is the time for PDE recovery to the lower activity preceding the light flash (Fig. 8). Like the latency effect, the antagonism of the cyclic GMP depolarization caused by light appears to be a unique property of the system that may principally reflect underlying PDE activity. This reasoning suggests that the response to light starts to recover before the PDE activation begins to decrease. To know the role of voltage- and time-dependent conductances in this recovery, the outer segment light-dependent current rather than voltage should be compared with the pulse responses.
Kinetics of Recovery from Cyclic GMP Pulses Compared with Initial Phase of Receptor Potential

If the PDE activity increases more slowly than the response, it could be asked whether PDE activity is responsible for the initial phase of the receptor potential. If the light-activated PDE activity determines the hyperpolarizing phase of the receptor potential, the recovery from a cyclic GMP pulse placed as near as possible after that hyperpolarization should show similar kinetics.

Identical membrane hyperpolarizing responses should occur irrespective of whether the PDE substrate is natural cyclic GMP or that introduced by the pulse. In theory, the cyclic GMP pulse could always be made large enough to overcome the most active PDE to give a transient depolarization shutting off the light response. This has not proved possible after strong flashes but is usually feasible after a $-4 \log$ unit flash as in Fig. 6B. Pulse 2, Fig. 6B, which closely follows after the initial phase of the receptor potential, is expanded in...
Fig. 6C and superimposed on the receptor hyperpolarization in Fig. 6D. The kinetics of the hyperpolarizing recovery from this cyclic GMP pulse are similar to the kinetics of the initial phase of the receptor potential. The pulse of cyclic GMP briefly restores the membrane toward the resting level and as the light-activated PDE activity hydrolyzes the injected cyclic GMP, the turnoff of the light response (pulse of darkness, as it were) is reversed with kinetics similar to the light response. This similarity can be demonstrated under widely varying conditions, as in, for example, Fig. 11, top trace. Both in this figure and in Fig. 6D, the discrepancy between the absolute membrane potentials of the superimposed responses is small. When the pulse just coincides with the onset of the receptor potential as in Figs. 1 and 7C, the recovery from the effects of the pulse of cyclic GMP and the initial phase of the receptor potential are identical, as seen in the figure. Note also that the initial phase of the receptor potential in this case is steeper than the recovery from the cyclic GMP pulse.

**Figure 8.** Results of Fig. 7 plotted as equivalent flash intensity (flash duration times intensity) as a function of PDE recovery time estimated from the responses to pulses of cyclic GMP injected into the ROS. The PDE recovery time is longer with higher flash energies, regardless of how the energy is distributed over time.
in the dark that just precedes it, which indicates an increase in PDE activity. And as mentioned previously, saturating pulses of cyclic GMP delay the light response. Therefore, the data indicate the following sequence of events: light-activated PDE turns on at a rate that controls the latency and the initial phase of the hyperpolarizing receptor potential. Regardless of time- and voltage-sensitive conductances, PDE activity continues to build to a peak and to decrease gradually to the pre-flash level.

**Picocoulomb Cyclic GMP**-Pulses Sometimes Initiate a Slowly Developing Maintained Depolarization and an Increased Amplitude and Duration Receptor Potential

Most preparations age, as shown in the top record of Fig. 3, giving identical responses to light flashes and with saturating cyclic GMP injections causing equal amplitude depolarizations that become more prolonged. Occasionally, a slowly developing depolarization follows the iontophoresis of small amounts of cyclic GMP (record A, Fig. 7, and lower record, Fig. 9) that resembles the phenomenon first reported by Waloga and Brown (1979) and Brown and Waloga (1981). The depolarization develops slowly, on a time scale of minutes, and the responses to light flashes given during this period appear to resemble responses to brighter flashes. For example, all of the responses on the lower record, Fig. 9, are to flashes of $-4 \log$ units intensity, but the last two responses (A and B) seem closer in shape to the response to the $-1 \log$ unit control (Fig. 9, upper record) than to the $-4 \log$ unit controls on both records, Fig. 9. The resemblance, however, is superficial. Both the latency and rise time of all the responses on the bottom record, Fig. 9, are about the same as each other compared with the much shorter latencies and faster rise times of the control
responses to brighter lights. The initial transient on the latter responses, A and B, bottom record, Fig. 9, is also much slower than that of the brighter controls, e.g., C. The maintained depolarization, when it occurs, is apparently a response to events that might be triggered by injections but then proceed in the absence of further pulses, as Figs. 7 and 9 show. It is impossible to rule out a slow leak of cyclic GMP$^-$ from the pipette as the initial cause of this drift because cells that maintain a steady baseline (Fig. 3) are more common. Note that the responses below the line representing the original resting potential are nearly identical. Thus the responses from the depolarized state (e.g., A and B) can be interpreted as consisting of three phases: (a) a rapid hyperpolarization to the original baseline caused by initial PDE activity; (b) the original light response; and (c) the slow depolarization caused by accumulation of cyclic GMP presumably leaking from the pipette tip.

_Excess Cyclic GMP Injected During Maintained Depolarization Slows Light Response Rate of Rise_

That the latency and rate of rise are changed very little by the slowly developing maintained depolarization after picocoulomb pulses of cyclic GMP is seen again in Fig. 10 where the control latencies and rates of rise (measured at the steep linear phase and at about the same membrane potential) are 184 and 172 ms, and 38 and 46 mV/s for the rapid phases of the responses following the flashes at A and B, Fig. 10, respectively. However, when the depolarization has approached the zero membrane potential level, although additional picocoulomb injections of cyclic GMP do not cause further depolarization (the driving force is apparently negligible, as if the membrane had reached the response's reversal potential), the response to light is greatly slowed. This can be seen by inspection of response C, Fig. 10, where even the fastest phase of the light response is only 20 mV/s. The system acts as if it were overloaded with cyclic GMP such that light-activated PDE is unable to reduce the concentration of cyclic GMP as rapidly as the control.

Characteristic of preparations that show the slowly developed, maintained depolarization, the membrane voltage responses to cyclic GMP$^-$ injections after illumination are increased in amplitude, as might be expected from additional cyclic GMP in an already overloaded system, but the pattern of increased rate of decay and suppression of the cyclic GMP response by light remains as previously described (Fig. 10, top record). This large amplitude of the voltage response to injected cyclic GMP provides another opportunity to compare the recovery rate of the depolarizing response to cyclic GMP with the hyperpolarization caused by light. The kinetics of the initial phase of the receptor potential and the recovery phase of the response to the 12-pC injection of cyclic GMP following it (Fig. 10C, bottom record) are similar; the recoveries from succeeding injections become progressively slower. This first injection after the flash at record C is labeled with an arrow. On the top record of Fig. 11 this injection is superimposed on the preceding hyperpolarization, the light response, to demonstrate how closely the result of hydrolysis of injected cyclic GMP resembles the actual light response.
Figure 10. Cyclic GMP injections show poorly on signal trace but are easily recognized by the sharp negative spikes on the records. Injections are 12 pC for the set shown on bottom record and 8 pC otherwise. The 12-pC set commences with the fourth injection preceding the flash at C and ends with the seventh injection after that light flash. Flash intensities on top record are from left to right: -4, -5, -5, -4, -4, -4, -4, -3.3 log units. The letters A, B, and C identify portions of the record that are expanded on the lower three traces. Time and amplitude calibrations on the middle of those three traces refer to all three; figures beside the initial phases of the receptor potentials on the bottom three traces are the rates of rise of the initial phases of the receptor potentials at the steepest segments measured approximately at the same absolute voltage. Figures below signal traces indicate response latencies. The slowly developing depolarization does not affect latency possibly because the system adjusts to slow leakage of cyclic GMP. When additional cyclic GMP is pulsed in at C, although the ROS cannot be further depolarized, the response shows signs discussed in text of saturation and overloading by cyclic GMP.
**Detailed Structure of Increased Latency Response**

When the ROS has been subjected to saturating injections of cyclic GMP, in spite of the fact that the receptor potential is delayed, there are sometimes signs of an initial process originating at the normal control latency. It would be expected that ROS PDE would be activated at the same time regardless of substrate concentration, but if the system is saturated there may be little or no physiological sign. As the ROS becomes unsaturated from cyclic GMP there is sometimes a slight hyperpolarization (arrowheads, Fig. 11) at the time when the photoresponse normally starts. This is most noticeable in the top record,

![Image](image_url)

**Figure 11.** Top record expanded response at C, Fig. 10. Middle and lower records from Fig. 3 can be identified from latencies under signal traces indicated in milliseconds. The recovery from the response to cyclic GMP injection on top trace is superimposed on the hyperpolarization caused by light. The similar kinetics of the two responses strengthens the argument that transduction is mediated by light-activated hydrolysis of cyclic GMP. Arrowheads on all records indicate possible electrical signals of responses in neighboring ROS transmitted by electrical coupling at level of inner segments.

Fig. 11 (taken from Fig. 10C), possibly because the driving force for the hyperpolarization is greatest in such depolarized cells, but can sometimes be detected in the absence of a strong depolarization (Fig. 11, middle and bottom records, arrowheads). The main conclusion from these experiments is that whenever saturating amounts of cyclic GMP are injected into the dark-adapted ROS, and regardless of the state of membrane polarization, the receptor potential is delayed as if the excess cyclic GMP had to be hydrolyzed to produce the receptor potential.
DISCUSSION

Uniqueness of Increased Latency

Because many substances depolarize the ROS transiently and because the molecular mechanism by which cyclic GMP depolarizes rods is unknown (a kinase may be involved; Polans et al., 1979), the mere fact that excess cyclic GMP causes a transient ROS depolarization in the intact rod is a neutral finding with respect to the hypothesis that the receptor potential results from

![Graph of ROS responses to intracellular injections of EGTA. Numbers on bottom signal trace indicate response latencies. In contrast to cyclic GMP, EGTA blocks the light response without increasing latency. This is explained by the long-lasting PDE activation by light that is sufficient to hydrolyze the excess cyclic GMP and produce the membrane hyperpolarization response, whereas for EGTA blockage, no response is possible if calcium becomes available after the native cyclic GMP has been hydrolyzed. As calcium becomes available, hydrolysis of cyclic GMP initiated by light produces a smaller response of approximately normal kinetics. Thus if both cyclic GMP and calcium are intermediary processes in transduction, cyclic GMP controls calcium.](image)

PDE activation. The rapid decay and long-lasting suppression of these transients to picocoulomb injections by illumination is, however, consistent with the hypothesis. Nevertheless, the depolarization caused by the injection of EGTA into the ROS (Brown et al., 1977) is of particular importance because it has been shown that EGTA acts by chelating Ca^{++}, thereby causing an increased Na^+ permeability of the ROS plasma membrane (Oakley and Pinto, 1981). Therefore, EGTA would be expected to block the response to light, as shown by Brown et al. (1977, their Fig. 6). Fig. 12, top record, is a repetition of their experiment. EGTA alone causes a depolarization and increased
membrane voltage noise. The increased noise is also apparent from the data of Brown et al. (1977). After the second injection of EGTA, light flashes are delivered periodically. The responses to light are small at first and gradually recover to the control level along with a reduction in membrane noise. Light delays the recovery from depolarization, possibly because light causes a loss of Ca\(^{++}\) from the outer segment (Gold and Korenbrot, 1980; Yoshikami et al., 1980). However, although EGTA blocks the response to light, the response of essentially normal latency and kinetics gradually increases in amplitude as more Ca\(^{++}\) becomes available, as is apparent from the upper and lower records of Fig. 12. This is in marked contrast to the blockage of the light response by cyclic GMP in which the latency and amplitude are increased. The effects of EGTA again demonstrate the uniqueness of the latency effect of cyclic GMP, which suggest that the light response is controlled by the hydrolysis of cyclic GMP.

The essential difference between the cyclic GMP and EGTA responses is explained by the long-lasting light activation of PDE that not only hydrolyzes the excess cyclic GMP but also produces the delayed light response. In the case of the injection of EGTA that severely reduces the availability of calcium, the light response does not occur. Indeed, it cannot occur later because the cyclic GMP has already been hydrolyzed before calcium becomes available. Therefore, if both cyclic GMP and calcium are intermediaries in transduction, this difference between the effects of EGTA and cyclic GMP suggests that cyclic GMP controls calcium.

Because the rods are electrically coupled (reviewed in Gold, 1981) and most of the response to light normally comes from rods other than the one impaled with the microelectrode, the question arises as to what extent coupling influences the response of increased latency after the injection of cyclic GMP. This question is discussed in detail in Miller and Nicol (1981), but these points should be added: the physiological data leave little doubt that the response to light that hyperpolarizes to the same level as the control and with similar kinetics to the control is indeed a response to illumination in spite of its long latency. As discussed previously, there is not sufficient time for the cyclic GMP to diffuse into neighboring cells. One would like to know, therefore, why one does not observe the responses of neighbors at the normal latency. Several possible explanations that are not mutually exclusive can be advanced. First, if the injected cyclic GMP opens many additional sodium channels, neighboring responses may be reduced by shunting. Second, the very slight responses at normal latencies (Fig. 10, arrowheads) may derive from neighboring cells. Third, the cyclic GMP injection may act by unknown means to reduce coupling. Although it is difficult to devise an interpretation other than that the response of increased latency arises locally in the ROS in which the microelectrode is located, it would be desirable to reproduce these experiments in isolated rod cells.

**Slowly Developing Depolarization**

Evidence is presented in the Results section that suggests that the depolarization depicted by Brown and Waloga (1981) may result from leakage of
cyclic GMP from the recording pipette. First, the depolarization develops slowly and in the absence of current pulses (Fig. 9). Second, the response to illumination during the depolarization is seen to resemble the response to the control flash when only the portion of the response below the original resting potential is considered. All of the response above that level (Fig. 9) would be directly attributable to the leakage. The explanation for the leakage may be found in the high sensitivity of the ROS to cyclic GMP and its hydrolysis. A 1-mV error in nulling the amplifier leakage current using an electrode of 10⁹ Ω resistance will result in 1 pA of leakage. If the leakage current is overcompensated, potassium will flow out of the pipette and Cl⁻ (the main cell anion) will flow into the pipette while cyclic GMP leakage is inhibited. If the leakage is undercompensated, cyclic GMP⁺ would be expected to flow continually from the pipette. And although such a degree of leakage would be acceptable if the pipette contained KCl or acetate, elevations of cyclic GMP levels will depolarize the cell.

Against the Hypothesis

Three lines of evidence have been presented that have cast doubt on the hypothesis that light-activated PDE hydrolysis of cyclic GMP controls hydrolysis. First, Meyertholen et al. (1980) have shown that removing bicarbonate/CO₂ from Ringer's reduces intracellular cyclic GMP and the amplitude of the light response to all intensities without changing sensitivity, as judged from the midpoint of the intensity response function. Because it is not clear how reducing bicarbonate/CO₂ reduces cyclic GMP or what other effects the loss of bicarbonate produces, and because there is nothing inconsistent with lower levels of cyclic GMP producing smaller amplitude receptor potentials, this result is inconclusive.

Second, experiments in which the concentration of cyclic GMP in the retina is measured at various times after illumination (e.g., Kilbride, 1980) question whether the light-induced decrease in cyclic GMP is fast enough to mediate transduction. Kilbride (1980) found that there was no detectable decrease in retinal cycle GMP until 1 s using intense illumination and 1.6 mM Ca++ in the Ringer's, whereas the time was reduced to 300 ms when Ca++ was reduced to a very low level with EGTA. However, Polans et al. (1981) confirmed that the half-life for cyclic GMP decrease is ~125 ms, independent of the Ca⁺⁺ level, as first found by Woodruff and Bownds (1977). Although the reasons for the discrepancy are not known (see Polans et al., 1981, for a full discussion of the problem), there can be no doubt that light-activated PDE has sufficient power and speed when measured in the isolated outer segment alone (Polans et al., 1981).

Third, based on effects of nonhydrolyzable analogues of cyclic GMP, it has been argued that it is unlikely that the hydrolysis of cyclic GMP controls transduction (Waloga and Bitensky, 1981). Because of the ROS cyclic GMP concentration is ~50 μM, analogues of cyclic GMP such as IBMX and 8-bromo cyclic GMP, which act as competitive inhibitors of PDE and have Kᵢ's in the 0.1-1 M range (Miller et al., 1973), can only achieve partial inhibition,
especially when present in micromolar concentrations. Because competitive inhibitors act to increase the normal substrate’s \( K_m \), the analogues should cause an accumulation of excess cyclic GMP and a slowing of the light-induced hydrolysis of cyclic GMP (Fletcher and Chader, 1976). Both IBMX (Lipton et al., 1977; Miller and Nicol, 1981) and 8-bromo cyclic GMP (Waloga and Bitensky, 1981) increase the amplitude and duration of the receptor potential, which sometimes appears slowed. Because neither IBMX nor 8-bromo cyclic GMP is easily hydrolyzed, the large receptor potential caused by these substances must be the result of the hydrolysis of the abnormal accumulation of cyclic GMP, because partial inhibition of PDE increases cyclic GMP levels without greatly affecting the light-induced hydrolysis, and because, based on the logic of Nicol and Miller (1978), the hydrolysis of cyclic GMP produces the receptor potential.

CONCLUSION

In summary, there is no conclusive evidence refuting the concept that the hydrolysis of cyclic GMP controls transduction even though the molecular mechanism by which hydrolysis controls the \( \text{Na}^+ \) channel is unknown. The similarity between the kinetics of the recovery from a cyclic GMP injection and the initial phase of the light response strengthens the argument that cyclic GMP hydrolysis is an intermediary process in transduction.

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