

# Electrical and Adaptive Properties of Rod Photoreceptors in *Bufo marinus*

## *II. Effects of Cyclic Nucleotides and Prostaglandins*

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**ABSTRACT** Substances known to alter cyclic nucleotide levels in cells were applied to the isolated toad retina and effects on rod electrical and adaptive behavior were studied. The retina was continually superfused in control Ringer's or Ringer's containing one or a combination of drugs, and rod activity was recorded intracellularly. Superfusion with cGMP, Bu<sub>2</sub>cGMP, isobutylmethylxanthine (IBMX; a phosphodiesterase inhibitor), or PGF<sub>2α</sub> (a prostaglandin) caused effects in rods that closely match those observed when extracellular Ca<sup>2+</sup> levels were lowered. For example, short exposures (up to 6 min) of the retina to these substances caused depolarization of the membrane potential, increase in response amplitudes, and some changes in waveform; but under dark-adapted or partially light-adapted conditions receptor sensitivity was virtually unaffected. That is, the position of the V-log I curve on the intensity axis was determined by the prevailing light level, not by drug level. These drugs, like lowered extracellular Ca<sup>2+</sup>, also decreased the period of receptor saturation after a bright-adapting flash, resulting in an acceleration of the onset of membrane and sensitivity recovery during dark adaptation.

Long-term (6–15 min) exposure of a dark-adapted retina to 5 mM IBMX or a combination of IBMX and cGMP caused a loss of response amplitude and a desensitization of the rods that was similar to that observed in rods after a long-term low Ca<sup>2+</sup> (10<sup>-9</sup> M) treatment. Application of high (3.2 mM) Ca<sup>2+</sup> to the retina blocked the effects of applied Bu<sub>2</sub>cGMP. PGE<sub>1</sub> superfusion mimicked the effects of increasing extracellular Ca<sup>2+</sup>. The results show that increased cGMP and lowered Ca<sup>2+</sup> produce similar alterations in the electrical activity of rods. These findings suggest that Ca<sup>2+</sup> and cGMP are interrelated messengers. We speculate that low Ca<sup>2+</sup> may lead to increased intracellular cGMP, and/or that applied cGMP may lower cytosol Ca<sup>2+</sup>, perhaps by stimulating Ca<sup>2+</sup>-ATPase pumps in the outer segment.

## INTRODUCTION

There is increasing evidence that cyclic nucleotides and calcium ions serve as interrelated second messengers in cell activation and that they may regulate each other's intracellular levels (Rasmussen, 1970; Rasmussen et al., 1975; Berridge, 1975). This evidence has been obtained largely from the study of hormonally responsive cells (Sutherland, 1972). For example,  $\text{Ca}^{2+}$  concentration may influence cyclic nucleotide levels by stimulating or inhibiting cyclase or phosphodiesterase activity (Rasmussen and Goodman, 1975; Rasmussen et al., 1975). On the other hand, cyclic nucleotides may regulate intracellular  $\text{Ca}^{2+}$  levels by enhancing or depressing  $\text{Ca}^{2+}$  pumps (Malaisse, 1973; Sulakhe et al., 1973; Shlutz and Marinetti, 1972; McCollum et al., 1972; Borle, 1974; Lehninger, 1970a; Howell and Montague, 1975). The exact manner in which cyclic nucleotides and  $\text{Ca}^{2+}$  interact appears to vary from tissue to tissue, and a number of systems have been described (Rasmussen and Goodman, 1975; Berridge, 1975).

The widespread occurrence of cyclic nucleotides and of calcium ions suggests that they may be interrelated in other systems. For example, in the retinal rod there is evidence that calcium serves as an intracellular messenger (see preceding paper). In addition, a number of experiments suggest a role for cyclic nucleotides in outer segment function (Bitensky et al., 1971). For instance, there are high concentrations of cyclic nucleotides and their anabolic (cyclase) and catabolic (phosphodiesterase) enzymes in rod outer segments (representative references include Bitensky et al., 1972; Pannbacker, 1973; Miki et al., 1973; Goridis et al., 1974; Manthorpe and McConnell, 1974). Of particular interest was the observation that phosphodiesterase (PhDE) activity is greatly enhanced by light, resulting in a fall in cyclic nucleotide levels in stimulated outer segments (Miki et al., 1973; Chader et al., 1973, 1974a, b; Goridis et al., 1974; Pannbacker, 1974).

The rod PhDE has been localized to the disk membrane (Robb, 1974), and its activation spectrum matches the absorption spectrum of rhodopsin (Keirns et al., 1975). The enzyme has a greater affinity for guanosine 3',5'-monophosphate (cGMP) than for adenosine 3',5'-monophosphate (cAMP). Consistent with this observation is the fact that light activation of rod outer segments causes a much greater *decrease* in cGMP than in cAMP content (Goridis et al., 1974, 1976; Pannbacker, 1974). Conversely, application of PhDE inhibitors causes a much greater *rise* in cGMP than cAMP in rod outer segments (Miki et al., 1973; Lipton, Rasmussen, and Liebman, unpublished observations). These data indicate that the cyclic nucleotide of importance for rod outer segment function is cGMP.

The only report on the effects of cyclic nucleotides themselves on the electrical activity of photoreceptors is that by Hood and Ebrey (1974) who applied adenosine 3',5'-cyclic monophosphate (cAMP) to a frog retina while recording the receptor potential extracellularly. They found no effects of the drug on either the receptor potential itself or on the time course of dark adaptation of the receptor potential. On the other hand, they observed earlier (Ebrey and Hood, 1973) that inhibitors of the phosphodiesterase enzyme that degrades cyclic nucleotides in rods generally decreased extracellularly recorded

receptor potential amplitudes. In contrast, Zaret (1972) found that the aspartate-isolated PIII of the frog increased in amplitude and broadened in shape with the addition of caffeine or aminophylline (PhDE inhibitors) to the retina.

The present experiments were undertaken in an effort to define the relationship, if any, between cGMP and calcium in vertebrate photoreceptor function. By intracellular recordings in the perfused toad retina, we have examined the effects of a variety of substances on receptor adaptive and response properties. The results showed that application of exogenous cGMP caused effects similar to that produced by lowering external calcium and that several substances known to enhance cGMP concentrations exhibited similar actions. Models to explain the interrelationship of cGMP and  $\text{Ca}^{2+}$  are discussed.

#### MATERIALS AND METHODS

The techniques involved in superfusing, recording, and stimulating the rods of the toad *Bufo marinus* were described in the previous paper. The same type of protocol was followed. Responses to brief flashes in the dark-adapted retina and during light and dark adaptation were elicited and evaluated as described previously. Two periods of drug superfusion were commonly used: short-term additions lasting 10 s to 5 min; and long-term additions, of 6–15 min. For most drugs, less than millimolar concentrations added for less than 2 min were needed to see an effect; i.e. about 1  $\mu\text{mol}$  of drug on the retina caused effects. For some test agents long-term additions generated a series of effects different from the short-term applications. Although responses to brief flashes were monitored continuously, results are generally reported in the figures at 2 min after addition of agent when some effect was seen, at 4 min when effects were often maximal, and at 10–15 min when the effects were reversed or long-term effects were evident.

#### *Drug Strategy*

Both cGMP and cAMP can be directly applied to cells and effects noted. However, cAMP and cGMP do not readily cross lipid membranes because of their hydrophilic characteristics. Therefore, the more permeable dibutyl derivatives ( $\text{Bu}_2\text{cAMP}$  and  $\text{Bu}_2\text{cGMP}$ ) are more effective when used in exogenous applications. Micromolar concentrations of these agents within the cell bring about cell activation (Sutherland, 1972), but when these drugs are applied extracellularly, millimolar concentrations are generally necessary (Sutherland, 1972).

The 2',3'-cyclic monophosphates occur naturally *in vivo* but are not metabolized by the PhDE and are inactive as second messengers (Lehninger, 1970*b*). The 2',3'-cyclic monophosphates were used as control test substances for the 3',5'-cyclic monophosphate studies. Also, PhDE inhibitors can be used to prevent destruction of cyclic nucleotides, thereby increasing the intracellular concentration of cyclic nucleotides (Miki et al., 1973). In this regard, isobutylmethylxanthine (IBMX) is of special value because it rather selectively enhances cGMP levels in rods. IBMX can be added alone or in conjunction with cGMP to increase greatly the intracellular level of this cyclic nucleotide. For example, a 3-min incubation of toad outer segments in 5 mM IBMX increased the cGMP concentration (by radioimmunoassay) about 2.5-fold over the control dark-adapted value (Lipton, Rasmussen, and Liebman, unpublished observations). Such an increase in cGMP levels is probably substantially greater than an exogenous application of cGMP or  $\text{Bu}_2\text{cGMP}$  could produce. It should be noted that some PhDE inhibitors also directly decrease intracellular  $\text{Ca}^{2+}$  concentrations in other tissues (Friedman et al., 1974). No report has yet appeared concerning IBMX effects on  $\text{Ca}^{2+}$  levels in cells.

Other experiments examined the effects of the prostaglandins PGE<sub>1</sub> and PGF<sub>2α</sub> on the electrical activity of rods. The intracellular levels of cyclic nucleotides may be affected by the exposure of cells to certain prostaglandins which have been shown to stimulate specific cyclase enzymes in many tissue types. Addition of PGE<sub>1</sub>, for example, is associated with an increase in cAMP concentration in many cells, whereas PGF<sub>2α</sub> is generally associated with an increase in cGMP levels in cells, but these correlations may vary because of dose or tissue type. Prostaglandins may also have a direct influence on intracellular Ca<sup>2+</sup> levels by some other mechanism (Clegg et al., 1966; Berti et al., 1967; Strong and Bohr, 1967; Ramwell and Shaw, 1970, 1971; Shaw et al., 1971; Ramwell, 1973; Samuelsson et al., 1975). Prostaglandins have not yet been reported in the retina, but their precursors (arachidonic and linoleic acids) exist in extremely high concentrations in the pigment epithelium compared to other tissues (E. R. Berman, personal communication).

Finally, another set of experiments was performed to study possible interrelationships between Ca<sup>2+</sup> and cGMP in rods. To answer this question, combinations of test agents (calcium, Bu<sub>2</sub>cGMP, and/or Bu<sub>2</sub>cAMP) were added to the retina.

cAMP and cGMP were obtained from Sigma Chemical Co. (St. Louis, Mo.). Bu<sub>2</sub>cAMP and Bu<sub>2</sub>cGMP were purchased from Boehringer/Mannheim (New York). PGE<sub>1</sub> and PGF<sub>2α</sub> (THAM) were gifts from Dr. John Pike, Upjohn Co. (Kalamazoo, Mich.) and Prof. E. J. Corey, Chemistry Department, Harvard University (Cambridge, Mass.), respectively. PGE<sub>1</sub> was solubilized in toad Ringer's by sonication. IBMX was obtained from Aldrich Chemical Co. (Milwaukee, Wis.) and was solubilized in Ringer's by gentle heating.

## RESULTS

The substances tested segregated into two groups: agents which elicited effects similar to a reduction in extracellular Ca<sup>2+</sup> concentration and those which produced effects similar to a rise in extracellular Ca<sup>2+</sup>. These two groups of substances will be discussed separately.

### *Drug Effects on Rod Electrical Activity*

Fig. 1 shows effects typical of three test agents that produced actions similar to reducing extracellular Ca<sup>2+</sup> concentrations. Two of these agents, Bu<sub>2</sub>cGMP and IBMX, are known to increase intracellular levels of cGMP in rods. In Fig. 1 A the rods were exposed to the test agents for short periods of time (10–45 s), and records from the same cell are illustrated during superfusion in control Ringer's and after addition of the agent. Although the magnitude of the effects varied between test agents, all showed the same basic changes. About 2 min after exposure to the drugs, the rods began to depolarize and the response amplitudes to increase. These changes were maximal at about 4 min (Fig. 1 A), but by 10–15 min the responses had returned to control levels. In this set of experiments IBMX caused the greatest degree of depolarization, and with this depolarization there was a loss of the initial transient of the response at about 4 min. Note that after 10–15 min the waveform of the response had also returned to normal. We also found that 1 mM cGMP added alone for 2 min exerted a small but statistically significant effect (in eight of eight recordings). On the other hand, the application of guanosine 2',3'-cyclic monophosphate to the retina produced no effects. Thus, it appears that the changes we observed

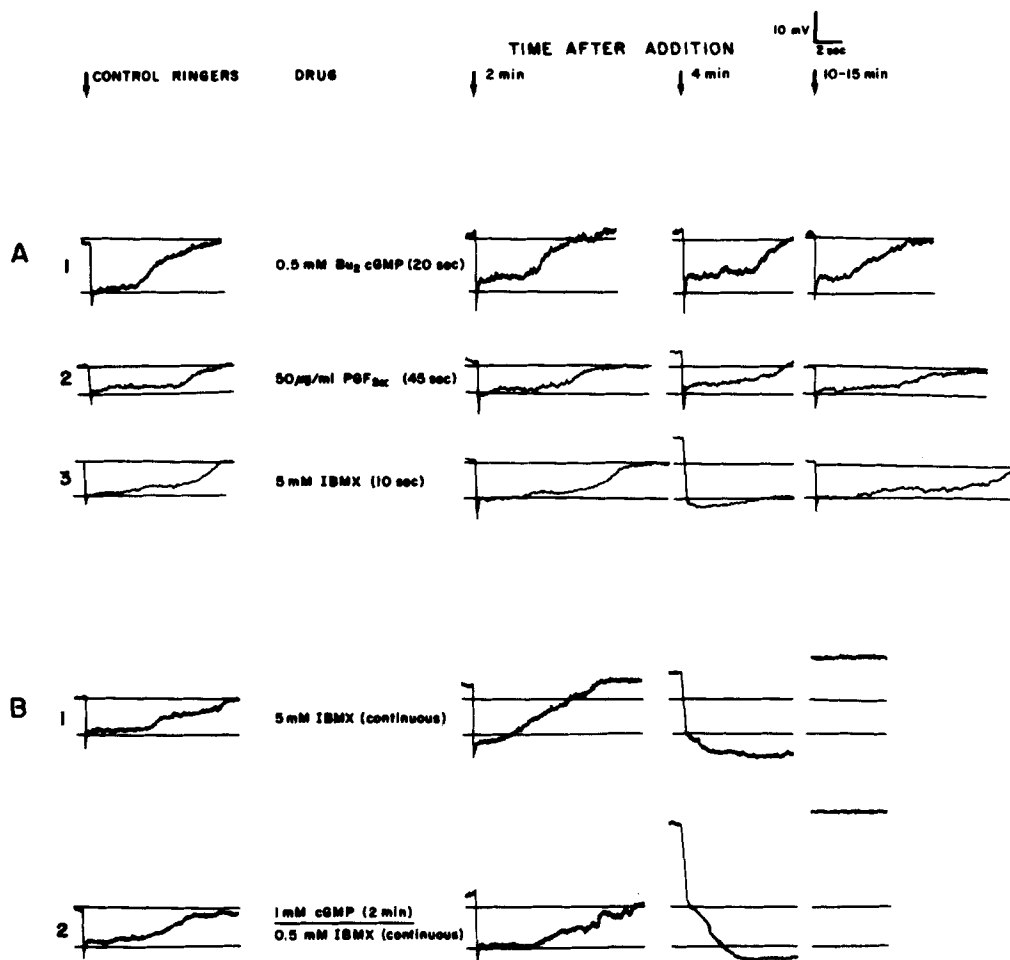


FIGURE 1. Effects of test agents that mimic a decrease in extracellular  $\text{Ca}^{2+}$  concentration on responses of dark-adapted toad rods to diffuse 200-ms flashes of 500-nm light. Each horizontal row represents responses from the same cell. The horizontal lines superimposed on each response indicate the original resting potential and plateau potential. The responses illustrated were elicited from each rod with a constant flash intensity, but the flash intensity varied between rods (3,000–30,000 photons absorbed per rod flash at 500 nm). Concentration of agent in reservoir is indicated in the drug column along with the length of addition. In A, the concentration and length of addition were approximately the minimum values necessary to give consistent effects. In both A and B, qualitatively similar effects were observed at 2 min. That is, all the cells had depolarized and their response amplitudes had increased. At 4 min the effect exerted by  $\text{Bu}_2\text{cGMP}$  and  $\text{PGF}_{2\alpha}$  were greater but qualitatively similar to the 2-min effects. However, IBMX alone or in combination with cGMP led to changes in waveform; e.g., the initial transient disappeared. By 10–15 min the effects of short-term additions of  $\text{Bu}_2\text{cGMP}$ ,  $\text{PGF}_{2\alpha}$ , and IBMX had all reversed (A). On the other hand, continuous addition of 5 mM IBMX or IBMX plus cGMP led to a loss of all light-evoked activity by 10–15 min (B). See text and Table I for further details.

with cGMP or Bu<sub>2</sub>cGMP can be attributed specifically to the 3',5'-cyclic monophosphate.

In Fig. 1 B are shown the effects of continuous exposure of rods to 5 mM IBMX and to a combination of 5 mM IBMX plus a short exposure to 1 mM cGMP. The effects seen at 2 and 4 min are qualitatively similar but of considerably greater magnitude than those seen in Fig. 1 A. After 2 min of drug exposure, both receptors were significantly depolarized, and the responses to the test flashes were correspondingly larger. By 4 min, further depolarization of the membrane was seen, light-evoked responses were still larger, and the initial transient had disappeared. Superfusion of IBMX closely mimicked the effects of low Ca<sup>2+</sup> Ringer's described in the previous paper, but the PhDE inhibitor tended to produce slightly more prolongation in response time course for comparable increases in amplitude. Between 4 min and 10 min the cells depolarized somewhat further, but they also lost responsiveness in a manner very similar to that observed after long exposure to low (10<sup>-9</sup> M) Ca<sup>2+</sup> Ringers (preceding paper, Fig. 9). That is, the cell lost sensitivity since the amplitude of the light-evoked responses gradually became smaller and, at the same time, the intensity of light necessary to evoke a half-maximum response ( $\sigma$ ) increased significantly. By 10–15 min no light-evoked activity whatsoever could be elicited from these rods. No recovery in such cells was observed even after return of the retina to control Ringer's. As was the case for retinas exposed to low (10<sup>-9</sup> M) Ca<sup>2+</sup> Ringers for long periods, other rods in the same retina were responsive when impaled after a 15-min control Ringer's washout. Thus, in combination with a 15-min continuous superfusion of certain drugs, impalement of the rods appeared to cause some permanent alteration in cells.

In Fig. 2, the degree of depolarization induced by various drugs and by low Ca<sup>2+</sup> Ringer's is compared with the increase in response amplitude. These data were taken at 2 and 4 min after addition of the test solutions. At these early times after drug addition the increase in response amplitude ( $\Delta V$ ) was, within experimental error, equal in absolute magnitude to the depolarization of the plasma membrane. At later times ( $\geq 6$ –7 min) with continuous superfusions of 5 mM IBMX or 10<sup>-9</sup> M Ca<sup>2+</sup>, the extent of depolarization was often greater than the increase in response amplitude. Note in Fig. 2 that the greatest extent of depolarization and increase of light response amplitude was induced by the combined addition of cGMP and IBMX, and this was a constant finding. In the experiment shown in Fig. 1 B in which these drugs were combined, one response recorded at about 4 min after drug addition was 42 mV in amplitude. This is larger than any receptor response we have ever recorded in the vertebrate retina. A summary of the effects of various test solutions on the response amplitudes of toad rods is presented in Table I.

In Fig. 3 are shown the effects on a rod of a short exposure to PGE<sub>1</sub>. The effects observed were similar to those seen with superfusion in high (3.2 mM) Ca<sup>2+</sup> or normal (1.6 mM) Ca<sup>2+</sup> plus the ionophore A23187 (see Fig. 4, preceding paper). Within 2 min after exposure of the retina to these substances, the rods began to hyperpolarize and the response amplitudes to decrease. At 4 min, these effects were maximal (see Table I), but by 10–15 min, the rods had returned almost to control levels. When either cAMP or Bu<sub>2</sub>cAMP was added

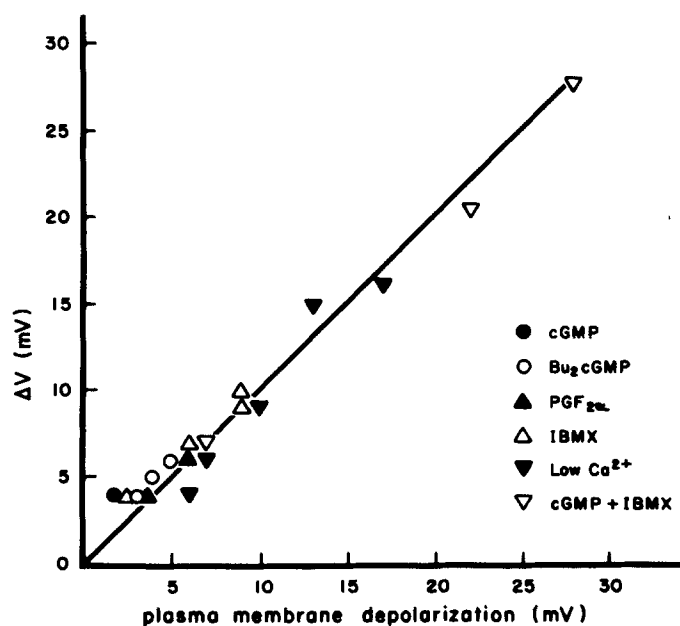


FIGURE 2. A comparison of the extent of depolarization and the increase of peak response amplitude induced in toad rods by various test agents. The increase in response amplitude ( $\Delta V$ ) was determined by subtracting response amplitudes evoked by brief flashes in control Ringer's from response amplitudes evoked 2 min and 4 min after test agent additions. Similarly, the plasma membrane depolarization was determined by measuring the difference between the resting membrane potential before and at 2 min and 4 min after addition of the test agents. The test agent concentration and lengths of addition are the same as in Table I. The straight line plots the values expected if the absolute magnitudes of  $\Delta V$  equal the depolarization of the plasma membrane potential.

TABLE I  
EFFECTS OF TEST SOLUTIONS ON ROD RESPONSE AMPLITUDES

Drug	Mean ratio of amplitude	Range	No. of recordings
1 mM cGMP (2 min)	1.4	1.2-2.5	8/8
0.5 mM Bu <sub>2</sub> cGMP (20 s)	1.6	1.2-2.7	14/15
50 $\mu$ g/ml PGF <sub>2<math>\alpha</math></sub> (45 s)	1.6	1.4-1.7	8/9
5 mM IBMX (10 s)	1.5	1.3-2.5	17/18
5 mM IBMX (continuous)	1.6	1.3-2.6	9/9
10 <sup>-9</sup> M Ca <sup>2+</sup> (3.5 min)	1.6	1.2-2.3	12/13
10 <sup>-9</sup> M Ca <sup>2+</sup> (continuous)	2.4	1.3-7.5	15/15
1 mM cGMP (2 min)	4.7	3.1-10.9	8/8
5 mM IBMX (continuous)			
50 $\mu$ g/ml PGE <sub>1</sub> (30 s)	0.6	0.4-0.7	8/10
3.2 mM Ca <sup>2+</sup> (3 min)	0.6	0.5-0.8	9/9
10 <sup>-6</sup> M A23187 (1 min)	0.6	0.5-0.7	7/8

Analysis of the effects of test solutions on the response amplitude of rods recorded 4 min after the start of test solution superfusion. The first column indicates the concentration of the drug along with the length of superfusion. Column 2 represents the ratio of responses during drug addition normalized to the control. The range of amplitude change is shown in columns 2 and 3. The number of recordings in which a significant change took place (at  $P < 0.01$ ) is indicated in the last column along with the total number of experiments carried out with each test solution.

to the retina for a short period, effects similar to those seen in Fig. 3 were usually observed. However, these effects were only occasionally reversible. That is, the cells usually remained hyperpolarized and response amplitudes were decreased long after the drugs had been washed out of the chamber. Why this is so is not clear, but one might speculate that direct cAMP effects are very long lasting because the PhDE in rods is much more effective in degrading cGMP as compared with cAMP (Miki et al., 1973). In any case, since the concentration of cAMP in vivo is so much smaller than cGMP, cAMP may be physiologically less important (Goridis et al., 1976; Miki et al., 1973).

When  $\text{Bu}_2\text{cAMP}$  or high  $\text{Ca}^{2+}$  was added to a retina that was also exposed to  $\text{Bu}_2\text{cGMP}$ , the effects usually observed after treatment with this agent were either much reduced or absent. In the experiment illustrated in Fig. 4, for

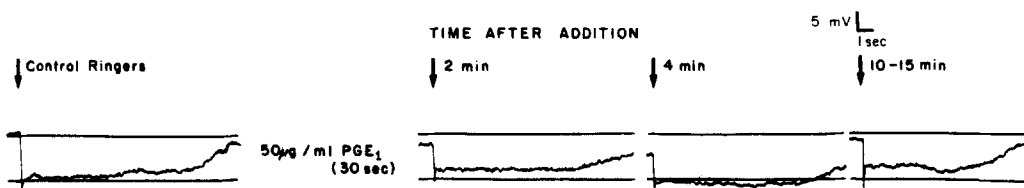


FIGURE 3. Effects of  $\text{PGE}_1$  on responses of dark-adapted toad rods to diffuse 200-ms flashes of 500-nm light. The arrangement of the figure is the same as Fig. 1. The addition of  $\text{PGE}_1$  resulted in effects similar to those observed with high (3.2 mM)  $\text{Ca}^{2+}$  and A23187 (see Fig. 4, preceding paper); i.e., the cell hyperpolarized, the response amplitudes decreased, and the time to peak increased. These effects reversed by 10–15 min after drug addition. See Table I and text.

example, a rod was exposed to 0.5 mM  $\text{Bu}_2\text{cGMP}$  for 30 s while being continuously bathed in Ringer's containing 3.2 mM  $\text{Ca}^{2+}$ . The depolarization and increase of response amplitude usually seen at 2 and 4 min after  $\text{Bu}_2\text{cGMP}$  exposure was not observed. Rather, at these times the cell was slightly hyperpolarized and its response amplitude correspondingly decreased, effects associated with the increase of  $\text{Ca}^{2+}$ . By 10–15 min, after the effects of  $\text{Bu}_2\text{cGMP}$  had worn off, the full extent of the membrane hyperpolarization and decrease of response amplitude induced by the continuously superfused high  $\text{Ca}^{2+}$  Ringer's was evident. How these antagonistic effects are being mediated is not clear; they may or may not involve the same locus.

#### *Drug Effects on Sensitivity of Dark- and Light-Adapted Rods*

Figs. 5–7 show voltage-intensity curves recorded from dark-adapted rods after a short-term addition of three different test agents. Two of the agents,  $\text{Bu}_2\text{cGMP}$  and IBMX, increase cGMP activity in the cells, while  $\text{PGE}_1$  may increase cAMP levels or affect  $\text{Ca}^{2+}$  movements in some other way. As can be seen from the figures, in all three experiments significant changes in response amplitudes occurred at all flash intensities as a result of drug addition;  $\text{Bu}_2\text{cGMP}$  and IBMX increased response amplitudes while  $\text{PGE}_1$  decreased response amplitudes. In no case, however, was the position of the voltage-



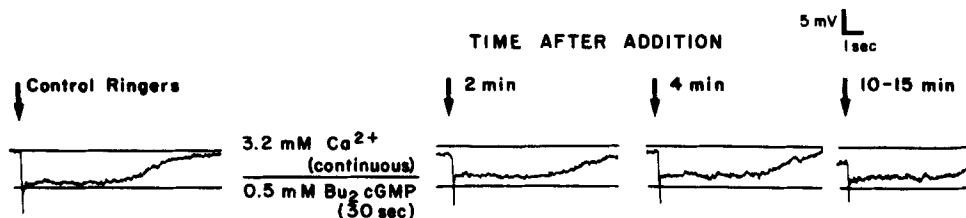


FIGURE 4. Effects of combinations of test agents on responses of dark-adapted toad rods to diffuse 200-ms flashes of 500-nm light. The arrangement of the figure is the same as Figs. 1 and 3. 30 s of 0.5 mM Bu<sub>2</sub>cGMP was added, followed immediately by continuous superfusion in high (3.2 mM) Ca<sup>2+</sup>. No effects of the Bu<sub>2</sub>cGMP were observed. Rather, at 2 min and 4 min the cell was slightly hyperpolarized, and its response amplitude had decreased. By 10-15 min the full effects of the continuously superfused high Ca<sup>2+</sup> Ringer's were evident. Similar results were observed in eight of eight experiments.

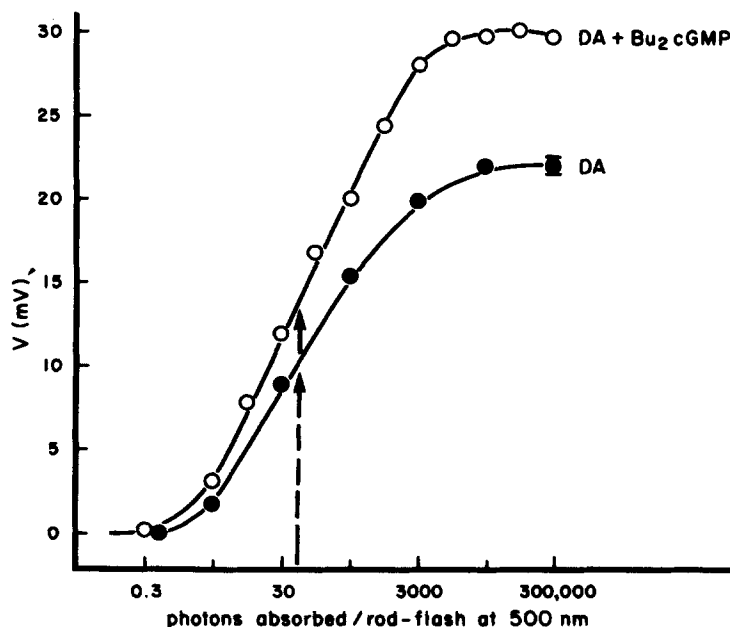


FIGURE 5. Effects of Bu<sub>2</sub>cGMP on the intensity-response relationship of a dark-adapted toad rod. The duration of each flash was 200 ms, and its intensity is expressed in photons absorbed per rod-flash. The lower curve was compiled during superfusion in control Ringer's and the upper curve 4 min after a 20-s addition of 0.5 mM Bu<sub>2</sub>cGMP. Note that Bu<sub>2</sub>cGMP affected the amplitude of response compared to control, but it virtually did not shift the curve along the intensity axis. Thus, there was no significant change in  $\sigma$  (indicated by arrows) in 14 of 15 intracellular recordings. The error bar represents  $\pm$ SE of the mean response amplitude.

intensity curve on the intensity axis significantly altered. In these dark-adapted rods, the intensity needed to elicit a half maximum response ( $\sigma$ ) was approximately 30 photons absorbed per rod-flash, and this value virtually did not vary regardless of the applied drug. Therefore, the sensitivity or gain ( $V_{\max}/\sigma$ ) of these rods varied only slightly with these test agents. It should be noted, however, that dark-adapted rods which were less sensitive, e.g.,  $\sigma = 100$ -200 photons absorbed per rod-flash, were often affected somewhat differently by

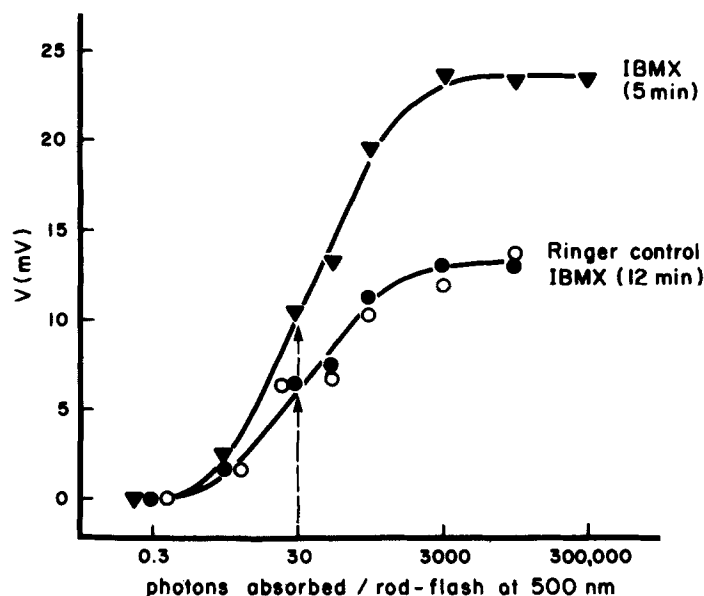


FIGURE 6. Voltage-intensity relationships before (filled circles), during (filled triangles), and after (open circles) a 10-s application of 5 mM IBMX to a dark-adapted rod. After IBMX addition the amplitude of responses increased at all stimulus intensities, but the curve did not significantly shift laterally on the intensity scale;  $\sigma$  (indicated by arrow) remained virtually the same in 17 of 18 experiments. Response amplitudes had returned to control levels by 12 min after drug addition (open circles).

$\text{Bu}_2\text{cGMP}$ ,  $\text{PGF}_{2\alpha}$ , or  $10^{-9}$  M  $\text{Ca}^{2+}$ . That is, in addition to an increase in  $V_{\max}$ ,  $\sigma$  would shift back toward 30 photons absorbed per rod-flash after application of test agent and thus sensitivity would increase.

In Fig. 6, the transient nature of the drug effects after short term applications is also illustrated. For example, the  $V$ -log  $I$  curve recorded 12 min after drug (IBMX) addition and washout perfectly matched the control curve. The maximal effect in this experiment was noted at 5-6 min, at which time the light-evoked responses were about twice those of control levels.

Fig. 8 shows  $V$ -log  $I$  curves obtained under both dark- and light-adapted conditions from a single rod bathed in control Ringer's and in Ringer's containing 5 mM IBMX for up to 6 min. As shown previously in Fig. 6, IBMX added under dark-adapted conditions increased the amplitude of responses,

but it did not significantly affect the position of the  $V$ -log  $I$  curve on the intensity scale. Under the light-adapted conditions tested ( $I_B = 2,500$  photons absorbed per rod-second at 500 nm), IBMX also increased the response amplitudes relative to those recorded from the rod in control Ringer's, but again it virtually did not shift the curve on the intensity axis. That is,  $\sigma$  was nearly identical in the partially light-adapted rod (3,000 photons absorbed per rod-flash at 500 nm) whether the rod was in control Ringer's or in Ringer's containing 5 mM IBMX.

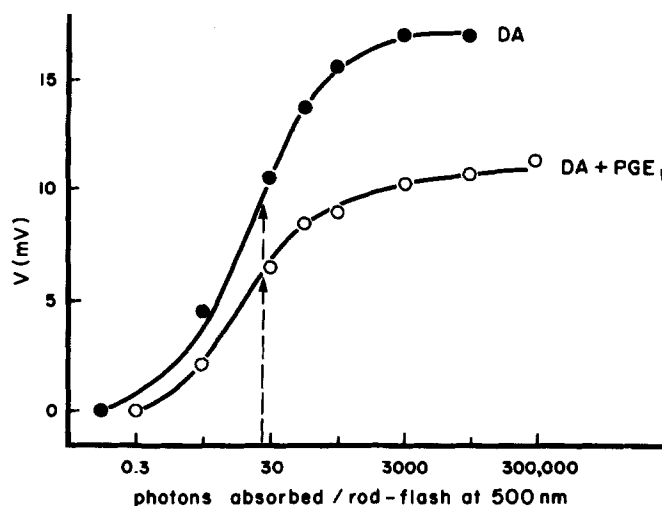


FIGURE 7. Effect of  $PGE_1$  on the  $V$ -log  $I$  curve of a dark-adapted toad rod. The experiment was exactly the same as in Fig. 5 except that 50  $\mu\text{g/ml}$   $PGE_1$  instead of  $Bu_2cGMP$  was applied for 45 s. Note that  $PGE_1$  decreased response amplitudes compared to control levels, but there was no significant lateral shift of the curve; i.e.,  $\sigma$  remained virtually constant (indicated by arrows) in 8 of 10 recordings. Difficulty in solubilizing  $PGE_1$  Ringer's may account for the two failures (see Materials and Methods).

Similar results were obtained with additions of  $cGMP$ ,  $Bu_2GMP$ , and  $PGF_{2\alpha}$ . That is, all of these drugs affected both membrane potential and response amplitude but not the major determinant of receptor sensitivity ( $\sigma$ ) whether the retina was dark or light adapted. These results are very similar to those observed when  $Ca^{2+}$  levels were altered extracellularly around toad photoreceptors for short periods ( $\leq 6$  min). On the other hand, prolonged exposure ( $>6$ –7 min) of the retina to 5 mM IBMX or a combination of IBMX and  $cGMP$  caused effects similar to those observed when the retina was exposed to  $10^{-9}$  M  $Ca^{2+}$  Ringer's for more than 6–7 min. Under these conditions, response amplitudes gradually declined, and  $\sigma$  increased tremendously; i.e. receptor sensitivity decreased just as in light adaptation.

Fig. 9 demonstrates the effect of IBMX on the intracellular response of a rod to an adapting background field. 3 min after the start of a continuous IBMX

infusion, the dark resting potential had depolarized, the response to the onset of the adapting light was fractionally larger than that observed when the rod was in control Ringer's, and the hyperpolarization maintained for the duration of the adapting light was also slightly larger. The incremental response elicited approximately 1 min after the background light was turned on was, however, significantly larger than the incremental response elicited under identical

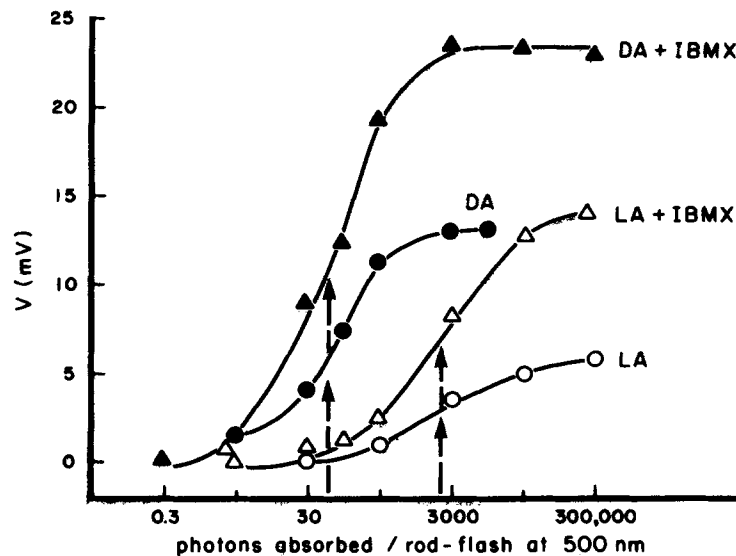


FIGURE 8. Voltage-intensity curves elicited from a single rod under both dark-adapted (DA) and light-adapted (LA) conditions during superfusion in control Ringer's or 5 mM IBMX. The intensity of the background illumination was 2,500 photons absorbed per rod-second at 500 nm. Data shown in this figure were collected first in control Ringer's and then 4-6 min after a 10-s addition of 5 mM IBMX. Under these conditions, IBMX affected response amplitudes in both the light- and dark-adapted states. However,  $\sigma$  (dashed lines) was virtually unaffected by drug addition in 17 of 18 recordings in the DA state and eight of eight rods in the LA state. The position of  $\sigma$  on the intensity axis was always determined by light level, not by drug level, for IBMX additions that lasted up to 6 min. See text.

conditions in control Ringer's. By 5 min of IBMX infusion, the dark resting potential had further depolarized, the response to background light was considerably larger, and the initial transient of the response had disappeared. In addition, the plateau hyperpolarization of the cell was not maintained with time. Within 1 min after the onset of the background light, the membrane potential had returned to dark membrane potential levels (note membrane potential before and after the break in the record). The incremental response elicited after the membrane potential had returned to the dark-adapted level was large in amplitude, and it displayed a prominent initial transient. The same intensity-flash without superimposed illumination would have elicited a response with no transient (shown in Fig. 1). Hence, with the addition of the

background light, the transient reappeared; i.e. the background illumination opposed the effect of the drug. Background illumination also antagonized the increased duration and response amplitudes seen after IBMX application. The behavior of the rod to background illumination when in the presence of IBMX for 5 min matched exactly that seen when the toad retina was bathed in low  $\text{Ca}^{2+}$  Ringer's for the same length of time (see Fig. 8, preceding paper).

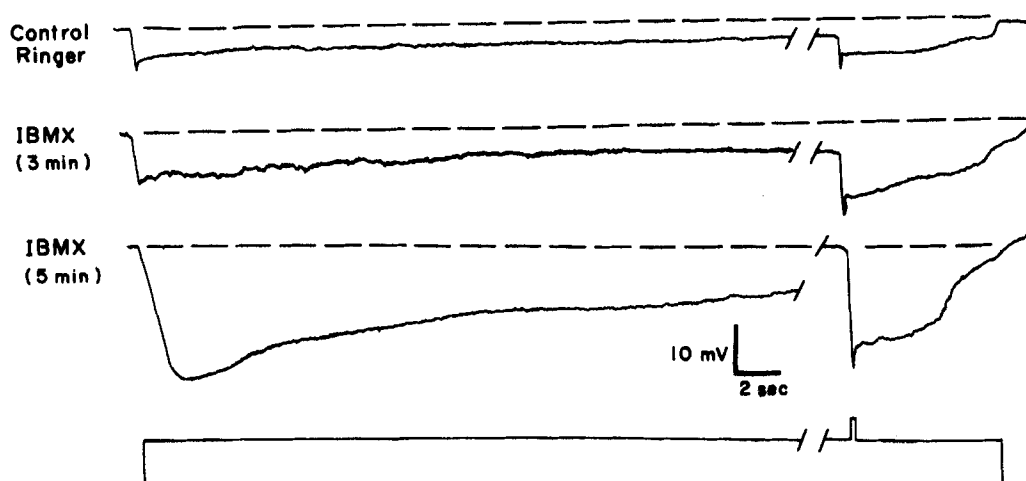


FIGURE 9. Effects of 5 mM IBMX upon intracellular responses of toad rods to background illumination and superimposed flashes. Lowest trace represents the light stimuli: long step is background ( $I_B = 2,500$  photons absorbed per rod-flash at 500 nm) and shorter pulse is superimposed 200 ms flash ( $I = 3,000$  photons absorbed per rod-flash). Hatch marks represent a break in the record of 34 s. A, The electrical response during superfusion in control Ringer's (1.6 mM  $\text{Ca}^{2+}$ ). Note the plateau potential was maintained throughout the background illumination. B, Response after 3 min of superfusion with 5 mM IBMX. The resting membrane potential had depolarized to a new level. Amplitude of responses to background and superimposed flashes were increased over control. Plateau potential to background was still maintained. C, Response after 5 min of superfusion with 5 mM IBMX. The resting membrane potential had further depolarized, and the amplitude of responses to background and superimposed flashes was further increased relative to the control. However, the plateau potential was no longer maintained throughout background illumination. Similar effects were obtained in nine of nine recordings. See text for further details.

#### *Drug Effects on Dark Adaptation*

In the previous paper it was shown that toad rods were saturated (i.e. unresponsive to any intensity of light flash) after a bright adapting flash that bleached a negligible amount of pigment ( $\leq 3\%$ ). The duration of this saturation period (17–100 s) matched closely the length of the plateau phase of the response. Thereafter the membrane potential returned relatively quickly to dark-adapted levels (5–20 s) during which time the rod rapidly increased in sensitivity. Although the length of the plateau component of the electrical

response and thus the period of saturation after a bright flash appeared to vary somewhat between rods, it was generally constant for any one rod from flash to flash when the retina was bathed in control Ringer's (see preceding paper). In the experiments to be described here, various drugs were applied to the retina, and the duration of the plateau phase after the adapting flash was measured. In all experiments, receptor sensitivity was also monitored and a close correspondence between plateau length and the onset of rapid sensitivity recovery was found.

Fig. 10 A-C shows the effects of test substances acting similarly to reduced extracellular  $\text{Ca}^{2+}$ . Plateau length of the response was clearly shortened by drug addition, whereas 10-15 min after the exposure to drug, this effect had reversed. Note in the case of IBMX, this was a rod in which the plateau was spontaneously lengthening with time (see previous paper), yet the effect of the drug is still clearly evident. Thus, these substances, like low  $\text{Ca}^{2+}$  Ringer's, accelerated the onset of membrane and sensitivity recovery during dark adaptation of single rods. Indeed, recovery to the dark-adapted state was often complete after  $\text{Bu}_2\text{cGMP}$ ,  $\text{PGF}_{2\alpha}$ , or IBMX treatment before recovery had even begun in the control. A summary of the effects of test solutions that decreased plateau length is shown in Table II.

Fig. 10 D shows that  $\text{PGE}_1$  induced effects opposite to those seen with the other agents. That is with  $\text{PGE}_1$  there was an increase in the duration of the plateau, and dark adaptation was delayed. Recovery was essentially complete in control Ringer's before it had ever begun after  $\text{PGE}_1$  treatment.

#### DISCUSSION

The changes in response characteristics produced by altering extracellular  $\text{Ca}^{2+}$  levels have been closely mimicked by applying to the retina substances that are known to alter intracellular cyclic nucleotide concentrations. Substances that are known or thought to increase cGMP levels (IBMX and  $\text{PGF}_{2\alpha}$ ) mimicked the effects of lowered extracellular  $\text{Ca}^{2+}$ , whereas  $\text{PGE}_1$  mimicked the effects of increased extracellular calcium concentration. Short-term ( $\leq 6$  min) additions of these drugs caused changes in membrane potential and light-evoked response amplitudes, but not changes in the major determinant of rod sensitivity ( $\sigma$ ) in either the dark- or the partially light-adapted toad. The position of the  $V\text{-log } I$  curve on the intensity scale of rods after short-term drug infusion was always determined by the prevailing light level and not by drug level. Thus, we conclude that intracellular cyclic nucleotide concentrations, like  $\text{Ca}^{2+}$  levels, affect both membrane potential and response amplitudes, but they influence receptor sensitivity only to a very minor degree in either the dark- or the partially light-adapted retina.

Most of these drugs did, however, cause alterations in the duration of receptor saturation after a bright but nonbleaching light exposure; i.e. they affected the time required for both membrane potential and sensitivity to recovery during dark adaptation. Similar effects were also seen when extracellular  $\text{Ca}^{2+}$  levels were altered. It is interesting to note that background illumination antagonized the effects of both IBMX and low  $\text{Ca}^{2+}$  on responses of dark-adapted toad rods to brief flashes. Since light activates the PhDE in

outer segments and IBMX inhibits the enzyme, one interpretation of this antagonism of the amplitude and waveform changes is a competition between light and IBMX on the activity of the PhDE.

With prolonged ( $\geq 6$ –7 min) exposure of a dark-adapted retina to IBMX or

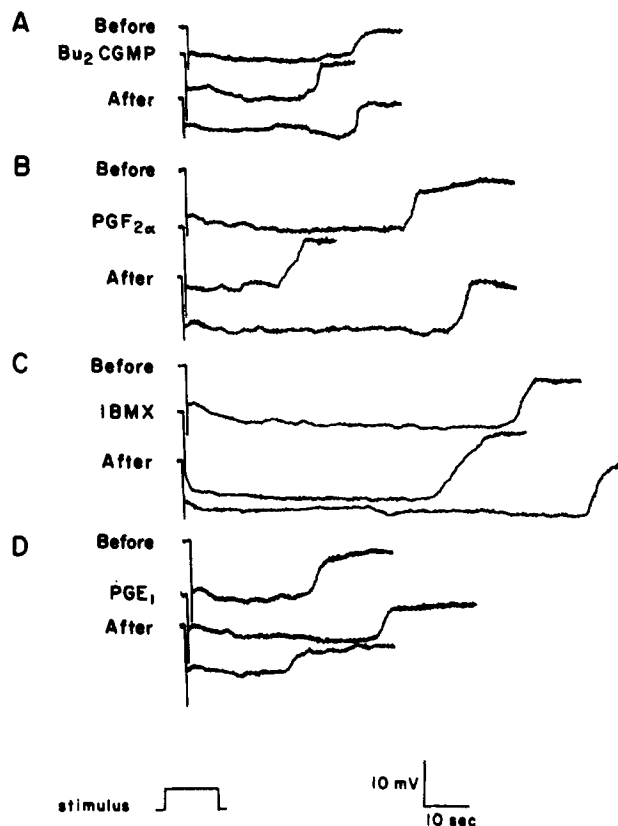


FIGURE 10. Effects of test agents on intracellular responses and membrane recovery during dark adaptation of toad rods after a bright-adapting flash. The adapting procedure consisted of a 12-s "yellow" (Corning cutoff filter 3484) unattenuated light with a retinal irradiance of 1.1 mW/cm<sup>2</sup> which bleached  $\leq 3\%$  of the pigment. Each group of three traces was from a single rod. Concentration and length of addition of test agent are the same as in Figs. 1 and 3 (only the 10-s addition of 5 mM IBMX is illustrated here). "Before" refers to control Ringer's response, and "After" refers to 10–15 min after drug addition. Drug effects were monitored at 4 min after addition. Bu<sub>2</sub>cGMP, PGF<sub>2α</sub>, and IBMX all accelerated and PGE<sub>1</sub> delayed the onset of membrane recovery after the adapting light step. See Table II and text for further details. In two rods the maximum effect of PGF<sub>2α</sub> was observed later than 4 min (e.g., about 9 min).

IBMX and cGMP, the rods lost responsiveness in a fashion identical to that observed after long-term exposure to low calcium Ringer's ( $\text{Ca}^{2+} = 10^{-9}$  M). Response amplitude diminished and sensitivity of the rod decreased, mimicking light adaptation. Thus every effect we observed as a result of altering extracel-

lular  $\text{Ca}^{2+}$  levels has also been seen by applying to the retina drugs that are known to alter cyclic nucleotide levels.

*The Interrelationship of  $\text{Ca}^{2+}$  and cGMP*

The experiments reported in this paper point toward a close relationship between  $\text{Ca}^{2+}$  and cGMP in the functioning of the vertebrate photoreceptor cell. To unravel the possible interrelationships between  $\text{Ca}^{2+}$  and cGMP, we have considered a number of alternatives: do the similar electrical effects of cGMP and low  $\text{Ca}^{2+}$  represent separate, "parallel" pathways, or might  $\text{Ca}^{2+}$  and

TABLE II  
EFFECTS OF TEST SOLUTIONS ON RECOVERY TIME ONSET

Drug	Mean ratio of recovery times	Range	No. of recordings
0.5 mM $\text{Bu}_2\text{cGMP}$ (20 s)	0.7	0.5-0.8	8/10
50 $\mu\text{g/ml}$ $\text{PGF}_{2\alpha}$ (45 s)	0.4	0.2-0.7	8/8
5 mM IBMX (10 s)	0.7	0.5-0.7	9/11
$10^{-9}$ M $\text{Ca}^{2+}$ (4 min)	0.6	0.4-0.7	7/8
50 $\mu\text{g/ml}$ $\text{PGE}_1$ (30 s)	1.5	1.2-1.7	8/10

Analysis of the effects of test agents on the acceleration or delay of membrane potential recovery during dark adaptation after a bright-adapting flash. The ratio of the times of onset of membrane recovery in various test and control Ringer's provides a normalized value of the acceleration or delay of membrane recovery. The mean and range of this ratio are shown in columns 2 and 3. The number of recordings that showed a significant change ( $P < 0.01$ ) is shown in the last column along with the total number of experiments tried. The mean ratio must be viewed only approximately since its value is dependent on the time of onset of membrane recovery in control Ringer's in each cell. For example, if a particular drug accelerated membrane recovery from a control of 60 s down to 30 s, the ratio of recovery would be 0.5 or 50% of the control value. However, if in another cell the same drug accelerated recovery from a control of 25 s down to 20 s, the change would not be significant (ratio 0.8). Yet it must be emphasized that in 152 rods a membrane recovery of about 20 s was the fastest observed and appeared to be a limiting value. In fact, in several cases the apparent failure of drug action may be explained by a membrane recovery in control Ringer's which was already very fast and near the limiting value before drug addition (e.g., hence only 9 of 11 rods were significantly affected by IBMX).

cGMP modulate the intracellular concentration of each other? For example, it is conceivable that the cyclic nucleotide regulates  $\text{Ca}^{2+}$  levels (Schultz et al., 1973; Berridge, 1975) which in turn produce the alterations in the electrical activity of the rod. If this is so, our data suggest that increasing cGMP levels in the rod reduces cytosol  $\text{Ca}^{2+}$  concentration. One way this could be accomplished is through the stimulation of  $\text{Ca}^{2+}$ -ATPase pumps by cGMP. It has been reported that  $\text{Ca}^{2+}$ -ATPase pumps are located in both the plasma and disk membranes (Bownds et al., 1971; Hagins and Yoshikami, 1974; Mason et al., 1974; Ostwald and Heller, 1972); thus, when cGMP levels in the rod outer segment are increased, the activity of the  $\text{Ca}^{2+}$  pumps is enhanced and cytosol  $\text{Ca}^{2+}$  levels are lowered. With lower intracellular  $\text{Ca}^{2+}$  levels, more  $\text{Na}^+$  channels are opened and available for blockade; the receptor depolarizes, and response amplitudes increase. Also, after a bright-adapting flash, cytosol  $\text{Ca}^{2+}$  levels are reduced faster, resulting in a shortened period of receptor saturation; hence, the onset of membrane recovery during dark adaptation is accelerated.



There is some tentative evidence in support of such a scheme (Lipton, 1977). For instance, it was possible to negate the effect of a 30-s addition of 0.5 mM Bu<sub>2</sub>cGMP with a 15-s application of 0.5 mM Bu<sub>2</sub>cAMP, but the same level of Bu<sub>2</sub>cAMP did not block the actions of low (0.8 mM) Ca<sup>2+</sup>. On the other hand, high (3.2 mM) Ca<sup>2+</sup> opposed the effects of 0.5 mM Bu<sub>2</sub>cGMP. We would expect these results only if cGMP led to lowered Ca<sup>2+</sup> levels which then produced the observed electrical effects. If the pathway were the opposite, i.e. low Ca<sup>2+</sup> leading to increased cGMP which in turn brought about the effects, we would expect Bu<sub>2</sub>cAMP to be able to block not only the effects of Bu<sub>2</sub>cGMP but also the effects of low Ca<sup>2+</sup>. It is possible, however, that these preliminary results were due to fortuitous concentration effects; further experiments should settle this point.

A related hypothesis that could also explain the data proposes that cGMP and Ca<sup>2+</sup> are dual, sequential messengers in rod excitation. For example, it is known that light-activation of rhodopsin stimulates PhDE activity causing a precipitous fall in cGMP levels. Woodruff et al. (1977) have recently shown that photoactivation of one rhodopsin molecule results in the hydrolysis of 1,000–2,000 molecules of cGMP within 100–300 ms. This rapid decrease in cGMP could produce an inhibition of the Ca<sup>2+</sup> pumps, resulting in a rapid rise of cytosol Ca<sup>2+</sup>. Since increased Ca<sup>2+</sup> levels appear to decrease Na<sup>+</sup> conductance of outer segment plasma membrane, the rod would hyperpolarize. This scheme is summarized below:



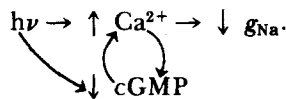
The kinetics of cGMP decrease after a light flash appear fast enough to be involved in visual excitation (Woodruff et al., 1977), but a definitive study of the time course of the whole set of reactions has not yet been carried out.

The alternative possibility to explain the interaction between Ca<sup>2+</sup> and cGMP is that Ca<sup>2+</sup> affects the cGMP concentration in the rod, and it is the cyclic nucleotide that produces the electrophysiological effects. In fact, by analogy to other tissues (Berridge, 1975), it is very possible that low Ca<sup>2+</sup> produces an increase in cGMP levels in rod outer segments and that high Ca<sup>2+</sup> produces a decrease. For example, it has been shown that Ca<sup>2+</sup> affects guanylate cyclase activity in smooth muscle, exocrine pancreas, and other cell types (Berridge, 1975). One model of excitation consistent with this scheme suggests that light-activation of rhodopsin leads to a rise in cytosol Ca<sup>2+</sup> which in turn lowers cGMP levels. The light-induced decrease in cyclic nucleotide might depress protein kinase activity responsible for phosphorylation (Sutherland, 1972) or, more rapidly, interact allosterically with a plasma membrane protein which normally maintains open Na<sup>+</sup> channels in the outer segment membrane in the dark. Hence, in the light the Na<sup>+</sup> permeability of the outer segment decreases, and the membrane hyperpolarizes. This scheme may be summarized as follows:



On the other hand, rather than high Ca<sup>2+</sup> depressing cGMP levels which in turn lead to hyperpolarization of the rod, the decrease in cGMP triggered by light and increased Ca<sup>2+</sup> could represent a positive feed-forward step (similar

to endochondral bone, [Rasmussen et al., 1972]). This fall in cGMP, with resulting relative inhibition of the  $\text{Ca}^{2+}$  pumps, would enhance the rise of cytosol calcium, ensure that it is not too rapidly removed, and result in the reaction cycle:



In conclusion, our results suggest that  $\text{Ca}^{2+}$  and cGMP are interrelated messengers in the rod cell and that their effects are inversely related, i.e. an increase in cGMP mimics low  $\text{Ca}^{2+}$  and vice versa. We speculate that  $\text{Ca}^{2+}$  levels in the rod may be modulated by cGMP, perhaps by stimulating  $\text{Ca}^{2+}$ -activated ATPase pumps on either the disk or the plasma membranes. However, there are testable alternatives, and it is difficult at present to fit the available data into a unique scheme that describes the relationship between these two intracellular control substances.

#### APPENDIX

#### Drug Effects on the Response Waveform

##### *Duration of Light Responses*

When dark-adapted rods were exposed to IBMX for more than 3–4 min, the light-evoked responses increased significantly in duration (Fig. 1 A). These findings, by themselves, suggested perhaps that IBMX, and hence increased cGMP levels, were prolonging the presence of the internal messenger (e.g.,  $\text{Ca}^{2+}$ ) on the plasma membrane by inhibiting rather than stimulating a  $\text{Ca}^{2+}$  pump. Other experiments, however, showed that this hypothesis could not be correct: (a) low extracellular  $\text{Ca}^{2+}$  (which in all probability decreased intracellular  $\text{Ca}^{2+}$ ) produced similar effects to cGMP. Hence, it did not appear likely that cGMP was prolonging the presence of cytosol  $\text{Ca}^{2+}$  by inhibiting a pump; (b) after a bright flash, cGMP or low  $\text{Ca}^{2+}$  accelerated the onset of membrane recovery during dark adaptation. That is, we observed that the response duration after a bright step was shortened with IBMX or low  $\text{Ca}^{2+}$  treatment (Fig. 10, and, in the preceding paper, Fig. 11). Thus, there is an apparent paradox because in the dark-adapted retina IBMX (increasing cGMP) or lowering extracellular  $\text{Ca}^{2+}$  prolonged response duration to brief flashes whereas after a bright step of light the onset of membrane recovery was accelerated by increased cGMP or low  $\text{Ca}^{2+}$ , i.e., duration of the response to the adapting flash was shortened.

The paradox can be explained in the following way. Suppose that the open  $\text{Na}^+$  channels and the pump sites compete for messenger (e.g.,  $\text{Ca}^{2+}$ ). After a bright step of light, all the  $\text{Na}^+$  channels in the rod outer segment are blocked. The onset of membrane potential recovery from the saturation level during dark adaptation will depend on how fast the blocking particles are removed from the plasma membrane. If  $\text{Ca}^{2+}$  is the blocking particle, and  $\text{Ca}^{2+}$  levels in the cytosol are lowered by pumps which can be stimulated by cGMP, then

increasing cGMP levels in the rod would accelerate the clearing of the cytosol of  $\text{Ca}^{2+}$ . Thus, the period of saturation would be shortened, as would the duration of the response.

In the dark-adapted retina, on the other hand, IBMX increases cGMP levels which stimulate pump activity and lower cytosol  $\text{Ca}^{2+}$ . This results in an increased number of open  $\text{Na}^+$  channels. Thus, the cell depolarizes and the amplitude of a response to a brief flash is increased. But also, because so many more  $\text{Na}^+$  channels are available for blocking, the number of  $\text{Na}^+$  channels statistically overwhelms the pumping sites in competition for released  $\text{Ca}^{2+}$ . Thus the duration of a response to a flash is prolonged, i.e. released  $\text{Ca}^{2+}$  is more likely to encounter an open  $\text{Na}^+$  channel than a pump site as compared to the control condition. If this notion is correct, we would also expect that low  $\text{Ca}^{2+}$  infusions (about 4 min) would increase response duration since additional  $\text{Na}^+$  channels are presumably opened under these conditions. This was observed (see Fig. 2 A, preceding paper).

Finally, the effects of cGMP and low  $\text{Ca}^{2+}$  on the kinetics of the time-to-peak of the receptor response are also consistent with the interpretation that cGMP is stimulating inactivation or pumping of the blocking particles, and that the blocking particles (or internal transmitter) are  $\text{Ca}^{2+}$  (Lipton, 1977).

We thank Patricia A. Sheppard for preparing the figures and Michael H. Zeldin for helpful discussions.

This research was supported in part by a National Eye Institute Grant EY-00824. Stuart A. Lipton received support from a National Institutes of Health Medical Scientist Training Program grant to the University of Pennsylvania School of Medicine. Many of the experiments reported here were described in a thesis presented by Stuart A. Lipton to the Department of Biochemistry and Biophysics in partial fulfillment for the degree of Ph.D.

Received for publication 17 March 1977.

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