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CYCLIC GUANOSINE 3′:5′-MONOPHOSPHATE MIMICS THE EFFECTS OF LIGHT ON A CIRCADIAN PACEMAKER IN THE EYE OF *APLYSIA*[1]

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Abstract

Environmental light regulates the phase of a circadian oscillator in the eye of *Aplysia*. We are attempting to define the events involved in transmitting light information from the environment to the circadian pacemaking mechanism in the eye. In this paper, we present several lines of evidence that cyclic guanosine 3′:5′-monophosphate (cGMP) is involved in the photic entrainment pathway. Light increases the level of cGMP in eyes without having detectable effects on cyclic adenosine 3′:5′-monophosphate (cAMP). An analogue of cGMP, 8-bromoguanosine 3′:5′-cyclic monophosphate (cGMP), can shift the phase of the circadian rhythm from the eye; the phase response curves for light and for 8-bromo cGMP are indistinguishable. Neither 8-bromo cAMP nor 8-bromo 5′-GMP mimics the effect of light or of 8-bromo cGMP on the rhythm. Light and 8-bromo cGMP appear to use convergent mechanisms for entrainment since the effects of these two treatments are nonadditive. Also, low Na^+ solutions antagonize the effects of both treatments. Finally, the kinetics of phase shifting by 8-bromo cGMP are similar to the kinetics of phase shifting by light.

In addition to perturbing the circadian rhythm, 8-bromo cGMP increases the frequency of spontaneous optic nerve impulses. The pattern of nerve impulses during 8-bromo cGMP treatment is the same as the pattern of impulses produced by light. The excitatory effect of 8-bromo cGMP, the low Na^+ blockade of the effects of 8-bromo cGMP, and the involvement of membrane depolarization in phase shifting by light suggest that depolarization mediates the effect of 8-bromo cGMP on the rhythm. The cellular site at which 8-bromo cGMP acts remains to be determined. However, 8-bromo cGMP does not appear to affect the rhythm by acting on R-type photoreceptors in the eye since 8-bromo cGMP had no effect on the membrane potential of these photoreceptors. At least two roles for cGMP are possible to explain our results. cGMP may be involved in transduction in non-R-type photoreceptors in the eye. Alternatively, cGMP may not be involved in transduction but may be elevated in cells as a result of the photoreceptor potential.

Environmental light cycles regulate or entrain circadian rhythms by shifting the phase of the circadian oscillator underlying the rhythm. Although the formal properties of entrainment have been thoroughly studied, little information exists concerning the neurophysiological and biochemical mechanisms that regulate circadian oscillators (Takahashi and Zatz, 1982). The eye of the mollusk, *Aplysia*, contains a circadian pacemaker that can be isolated and studied *in vitro* (Jacklet, 1969). A photic entrainment pathway contained within the isolated eye, and a serotonergic pathway that presumably mediates efferent information from the nervous system, converge on the ocular circadian pacemaker to regulate its phase (Eskin, 1971; Corrent et al., 1978; Prichard and Lickey, 1981; Eskin and Maresh, 1982). The cellular and biochemical identification of the steps in these two pathways should aid not only in understanding the process of entrainment but also in the search for the molecular components of the oscillatory mechanism since entrainment pathways must terminate upon components of the oscillator to regulate the phase of the rhythm.

The photic entrainment pathway appears to be quite direct and does not seem to involve action potentials, chemical neurotransmission, or secretion (Eskin, 1977). A sodium-dependent depolarization appears to be required for photic entrainment, and agents that cause depolarization mimic the phase-shifting effects of light (Eskin, 1972, 1977, 1979; Jacklet and Lotshaw, 1981). These results and others suggest that the circadian pacemaker is contained within a cell that is photo-

[1] We dedicate this paper to Dr. C. S. Pittendrigh on the occasion of his sixty-fifth birthday for the conceptual guidance that he has provided to us.

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receptive or that is electrotonically coupled to a photoreceptor cell by gap junctions. In either case the pathway from photoreceptor to circadian oscillator includes a membrane depolarization event.

The serotonin pathway is distinct from the photic entrainment pathway (Corrent et al., 1982). Serotonin activates adenylate cyclase, leading to an increase in cAMP which shifts the phase of the circadian oscillator through a process that appears to require protein synthesis (Eskin, 1982; Eskin et al., 1982; Eskin and Takahashi, 1983). Thus, cAMP appears to act as an intracellular "second messenger" for the effects of serotonin on the circadian pacemaker. We investigated whether the effects of light upon the circadian pacemaker are also mediated by cyclic nucleotides. We report here that light increases the level of cGMP but not that of cAMP in the *Aplysia* eye and that the effects of light are precisely mimicked by cGMP.

Materials and Methods

Aplysia californica were obtained from Alacrity Marine Biological Services and kept in Instant Ocean sea water at 15°C. The animals were entrained to a light-dark cycle consisting of 12 hr light/12 hr dark before being used in experiments. Isolated eyes were maintained at 15.5°C in a medium (BFSW) consisting of Instant Ocean sea water with 30 mM HEPES (pH 7.7), penicillin at 100 units/ml, and streptomycin at 100 μg/ml. Eyes were maintained in constant darkness throughout experiments, unless light was used as an experimental treatment. The circadian rhythm of spontaneous nerve impulses from isolated eyes was measured by extracellular recording from optic nerves as described previously (Eskin, 1977).

Most chemical treatments were administered by two complete exchanges of the solution surrounding the eyes. Low sodium solutions (LoNa) were introduced by four complete solution changes with LoNa. Treatments were terminated by six rinses with BFSW. The following drugs were obtained from Sigma Chemical Co.: 8-bromoguanosine 3′:5′-cyclic monophosphate (8-bromo cGMP), 8-bromoadenosine 3′:5′ cyclic monophosphate (8-bromo cAMP), 8-bromoguanosine 5′-monophosphate (8-bromo 5′-GMP), and *N*-methyl glucamine. The osmolality of the LoNa solution was adjusted to equal BFSW using *N*-methyl glucamine. The components of the LoNa solution were as follows: NaCl (12 mM), KCl (10 mM), $CaCl_2$ (10 mM), $MgCl_2$ (50 mM), *N*-methyl glucamine (438 mM); 422 mM Cl^- was added by adjusting the pH to 7.7 with HCl. In the phase-shifting experiments with light, the light source was a Bausch and Lomb 6V microscope illuminator providing an intensity of about 1200 lux at the surface of the eye.

In the phase-shifting experiments, eyes were exposed to treatments during the first day of isolation and constant dark conditions. The time of treatments is given as circadian time (CT) where CT 12 is the time at the end of the light portion of the light-dark cycle and the time when the isolated eyes were placed into constant darkness. The rhythms of control eyes peaked on the average at CT 2.5 (2.5 hr after the projected onset of light) during the first day of constant darkness. The effects of treatments on the rhythm are given as phase shift values. Individual phase shift values are the differences in peak times of the rhythms during the fourth day of constant darkness from an experimental and control eye both from the same animal. Phase shifts calculated over the fourth or fifth cycle yielded the same values.

In the experiments in which the levels of cGMP and cAMP were measured, the optic nerves were removed from the eyes, and the eyes were pinned in a Petri dish containing Sylgard (Dow-Corning). The isolated eyes were maintained in darkness in an incubator at 15.5°C. Experimental and control groups consisted of four eyes each and the two groups were matched by distributing one eye of an animal into the experimental group and the other eye into the control group. After isolation *in vitro* for 3 to 15 hr in darkness, experimental groups of eyes were exposed to fluorescent light of 1500 lux. Before the light was turned on, control groups of eyes were placed in homogenizers using a Kodak no. 2 safelight. All but one of the experiments in which cyclic nucleotides were measured were performed early in the projected nighttime around CT 15. Cyclic nucleotide levels at this time after exposure of eyes to light were not different from cyclic nucleotide levels in the one other experiment performed at CT 4. Although we observed no differences in light-stimulated cyclic nucleotides at different times, a more systematic study is required to establish the importance of the circadian time of measurement. Control and experimental eyes were homogenized in 200 μl of ice-cold 1.5 M perchloric acid. The homogenates were centrifuged at 10,000 × *g* for 5 min, and measured aliquots of supernatant were neutralized with 2 M $KHCO_3$. The neutralized extract was centrifuged at 1500 × *g* for 10 min, and the supernatant was assayed for cGMP or cAMP by the radioimmunoassay described by Steiner et al. (1972) using the acetylation procedure of Harper and Brooker (1975). Assays were performed using New England Nuclear radioimmunoassay kits. Samples were assayed in duplicate for each nucleotide. Protein was measured using the method of Lowry et al. (1951).

Intracellular recordings were obtained from photoreceptor cells of eyes from adult *Aplysia*. The eye was removed and positioned lens upward in a U-shaped suction electrode. The lens was removed, and impalement of the distal segments of photoreceptors was accomplished with 40- to 60-megohm glass microelectrodes. All experiments were conducted during the subjective day. Once a successful impalement was obtained, the retina was left undisturbed in constant darkness for 1 to 2 hr to obtain a base line membrane potential. Membrane potentials were usually followed for at least 2 hr after addition of 8-bromo cGMP to the bath. The exposure time of eyes to 8-bromo cGMP ranged from CT 4 to CT 8 in these experiments. This exposure time included some phases at which 8-bromo cGMP caused phase shifts in the rhythm.

Results

Effects of light on cGMP. In 12 separate experiments, groups of isolated eyes were exposed to either 10 or 50 min of light. Light increased the level of cGMP in each of these experiments (Fig. 1). The mean increase in cGMP after 10 min of light was 50 ± 15% ($\bar{X}$ ± SE, seven experiments) and 40 ± 6% (five experiments) after 50 min of light. In these same experiments, light had no detectable effect on the amount of cAMP in eyes. The change in cAMP after 10 min of light was 4 ± 20% and after 50 min of light was −1 ± 20%.

Effects of cGMP on the rhythm. The increase in cGMP produced by light raised the possibility that an increase in cGMP could mediate the phase-shifting effect of light on the rhythm from the eye. This possibility was examined by comparing the effects of light and 8-bromo cGMP on the rhythm. Advance phase shifts in the rhythm were produced by 6-hr treatments of 8-bromo cGMP ranging in concentration from 1.5×10^{-4} M to 2×10^{-3} M when administered at phase CT 18

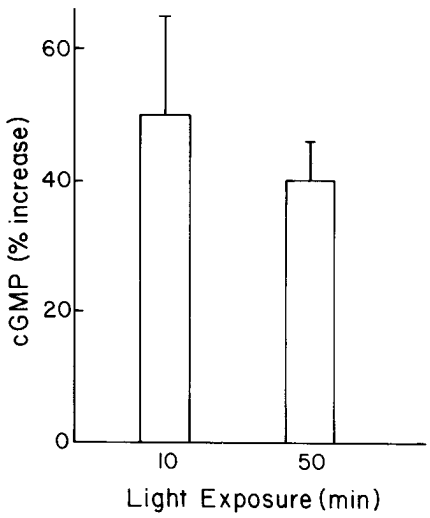

Figure 1. Increase in intracellular cGMP produced by exposure of isolated eyes to light. The change in cGMP was measured by comparing cGMP in a group of four experimental eyes with that in a group of four control eyes from the same animals. Seven separate experiments were performed at 10 min, and five experiments were performed at 50 min. *Error bars* are standard errors calculated with *N* as the number of experiments. The basal level of cGMP averaged 2.30 and 2.67 pmol/mg of protein in the 10- and 50-min light experiments, respectively.

to 24 (Fig. 2, Table I). At other phases of the rhythm, 8-bromo cGMP produced delay phase shifts (Fig. 2). A phase response curve for 8-bromo cGMP was obtained by exposing eyes to the analogue at various phases of the rhythm. The phase response curve for 8-bromo cGMP is indistinguishable from the phase response curve for 6-hr light pulses previously measured from the *Aplysia* eye (Fig. 3). The similarity in phase response curves for 8-bromo cGMP and light is significant because some other treatments on the eye produce response curves that are very different from the one produced by light (Corrent et al., 1982; Eskin and Takahashi, 1983). The superimposable phase response curves for 8-bromo cGMP and for light suggest that the two treatments perturb the circadian oscillator through a common process.

The possible role of cGMP in mediating phase shifting by light was investigated further by testing whether the effects of light and 8-bromo cGMP on the rhythm were additive. The mean phase shift produced by exposing eyes to treatments of light plus 8-bromo cGMP was not different from the phase shifts produced by either light or 8-bromo cGMP alone (Table II). Thus, the effects of 8-bromo cGMP and light on the rhythm were nonadditive. Nonadditivity in this experiment was not the result of a ceiling or limit on the amount that the phase of the rhythm can be shifted, since other treatments, such as increased extracellular K^+, have produced 6-hr advance phase shifts in the ocular rhythm (Eskin, 1972). The nonadditive effects of 8-bromo cGMP and light support the suggestion that 8-bromo cGMP and light are regulating the circadian oscillator through some common mechanism.

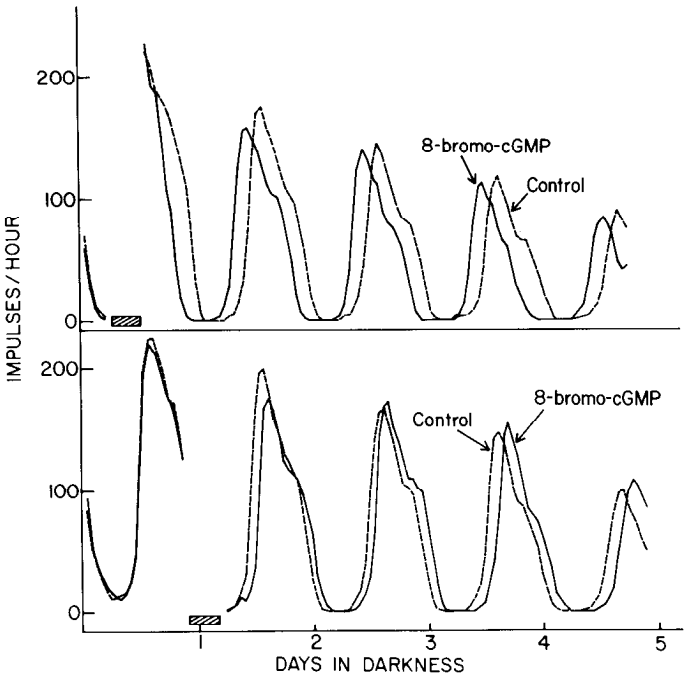

Figure 2. Advance (*top*) and delay (*bottom*) phase shifts produced by a 6-hr treatment with 8-bromo cGMP (2×10^{-3} M). The frequency of spontaneous optic nerve impulses is plotted as a function of the time isolated eyes were in constant darkness. The two rhythms shown in each *graph* are from eyes of the same animal. The time of exposure to 8-bromo cGMP is shown by the *hatched bars* at the *bottom* of each *graph*.

TABLE I

Specificity of 8-bromo cGMP-induced phase shift

Treatment[a]	Concentration	Phase Shift[b]	N
	M	*hr*	
8-Bromo cGMP	2×10^{-3}	+3.56 ± 0.99	9
	1×10^{-3}	+2.30 ± 1.36	5
	1.5×10^{-4}	+1.75 ± 1.37	4
8-Bromo 5′-GMP	2×10^{-3}	0.00 ± 0.65	4
8-Bromo cAMP	2×10^{-3}	−2.25 ± 2.29	4

[a] Treatments were 6 hr in duration and were given from CT 18 to 24.

[b] Phase shift is mean ± 95% confidence interval. Advance phase shifts are positive, and delay phase shifts are negative.

Specificity. The specificity of the phase-shifting ability of 8-bromo cGMP was examined by exposing eyes to other cyclic nucleotide analogues. The 5′-monophosphate analogue, 8-bromo 5′-GMP, did not phase shift the rhythm (Table I). The cAMP analogue, 8-bromo cAMP, did produce phase shifts; however, it did not mimic the effect of 8-bromo cGMP on the rhythm. 8-Bromo cAMP produced delay phase shifts at the phase (CT 18 to 24) where 8-bromo cGMP produced advance phase shifts (Table I). The delay phase shift produced by 8-bromo cAMP is consistent with our earlier results showing that a different analogue of cAMP, 8-benzylthio cAMP, produced delay phase shifts at CT 22 to CT 4 (Eskin et al., 1982). The lack of an effect of 8-bromo 5′-GMP and the inability of 8-bromo cAMP to mimic 8-bromo cGMP establish that the phase-shifting effect of 8-bromo cGMP is due to the cGMP moiety.

Role of depolarization in the effect of cGMP. The frequency

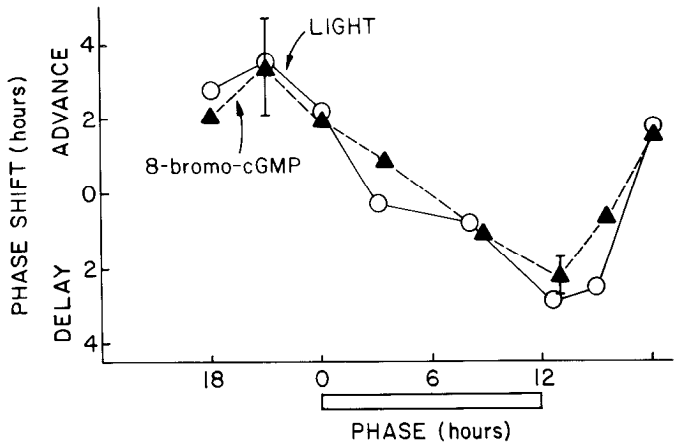

Figure 3. Comparison of phase response curves for 6-hr treatments with light and 8-bromo cGMP (2×10^{-3} M). The *graph* shows phase shifts produced in the rhythm as a function of the time (phase) of exposure of the eyes to the treatments. Treatments were given during the first day of isolation of the eyes as shown in Figure 2, and phase 0 is the time of projected dawn. Data are plotted with respect to the midpoint time of the treatment. The *error bars* are 95% confidence intervals with $N = 9$ for the maximum advance phase shift and $N = 7$ for the maximum delay phase shift. All other data points for the 8-bromo cGMP curve are averages of data from four pairs of eyes. Data for the light phase response curve are from Corrent et al. (1982).

TABLE II

Nonadditivity of light and cGMP and blockade by low sodium treatment

Treatment[a]	Phase Shift	N
	hr	
Light	+3.28 ± 0.45	36
8-Bromo cGMP	+3.56 ± 0.99	9
Light + 8-bromo cGMP	+2.83 ± 1.27	6
LoNa (12 mM)	−0.67 ± 1.18	6
Light + LoNa (12 mM)	+1.75 ± 0.57	12
8-Bromo cGMP + LoNa (12 mM)	+0.25 ± 0.80	6

[a] Treatments were 6 hr in duration and were given from CT 18 to 24.

of optic nerve impulses is elevated throughout exposure of the eye to light (Fig. 4). 8-Bromo cGMP also increases the frequency of optic nerve impulses, and the pattern of nerve firing produced by 8-bromo cGMP was similar to the pattern produced by light (Fig. 4). 8-Bromo 5′-GMP had no effect on the frequency of optic nerve activity.

The similarity in the increase in the frequency of optic nerve impulses produced by light and 8-bromo cGMP, together with the previously established involvement of membrane potential depolarization in mediating phase shifting by light, led us to ask whether 8-bromo cGMP phase shifted through depolarization. To address this question, we tested whether LoNa solutions could block the effect of 8-bromo cGMP on the rhythm. In these experiments, eyes were exposed to 8-bromo cGMP dissolved in a LoNa (12 mM) solution. The LoNa solution blocked the phase shift normally produced by 8-bromo cGMP (Fig. 5*B*, Table II). This LoNa solution did not phase shift the rhythm by itself (Fig. 5*A*, Table II). No optic nerve impulses were observed when eyes were exposed to either LoNa by itself or to LoNa plus 8-bromo cGMP. The phase shift produced by light was also significantly reduced by the LoNa solution used in these experiments (Table II).

Effect of 8-bromo cGMP on photoreceptor cell membrane potential. The results presented in the previous section suggest that cGMP mediates the phase-shifting effect of light by causing depolarization. These events may be occurring in the organized layer of microvillous photoreceptors (R cells, see Jacklet and Rolerson, 1982) beneath the lens in the eye. We examined this possibility by determining whether 8-bromo cGMP depolarized R-type photoreceptor cells. Membrane potentials of photoreceptor cells and optic nerve impulses were recorded simultaneously during exposure of the eye to 8-bromo cGMP. Long-term intracellular recordings were obtained from eight photoreceptor cells in eight different eyes. The membrane potentials of these cells were not affected by 8-bromo cGMP treatment, although the frequency of optic nerve impulses was increased in these same experiments (Fig. 6). Thus, if the phase-shifting effects of 8-bromo cGMP are a consequence of depolarization, this depolarization occurs in cells other than R-type photoreceptors.

Discussion

In order for photic information from the environment to shift the phase of the rhythm from the eye, light information must be transduced by a photoreceptor and propagated over some pathway to a molecular component of the circadian oscillator. Several lines of evidence presented in this paper argue that cGMP is involved in the photic entrainment pathway. Exposure of eyes to light increases intracellular cGMP levels. The phase response curves for light and for 8-bromo cGMP are identical, demonstrating that cGMP precisely mimics the phase-shifting effects of light. The action of 8-bromo cGMP is specific because neither 8-bromo 5′-cGMP nor 8-bromo cAMP mimics the effect of 8-bromo cGMP or light on the rhythm. Light and 8-bromo cGMP appear to act on the same pathway because the effects of these two treatments are nonadditive. The effects of both treatments are antagonized by LoNa solutions. Finally, the kinetics of phase shifting by the two treatments are similar. The advance phase shift produced by either light or 8-bromo cGMP can be detected shortly after termination of the treatment (see *top panel* of Fig. 2). The rapid time course of the light-induced phase shifts contrasts with the time courses seen with serotonin and cAMP treatments in which phase shifts develop 12 to 24 hr after the end of the treatment (Corrent et al., 1982). Taken together, these results strongly suggest that cGMP mediates the phase-shifting effect of light.

Membrane depolarization has been implicated in phase shifting by light in previous studies (Eskin, 1972, 1979; Jacklet and Lotshaw, 1981). Depolarizing treatments, such as elevated K^+_o

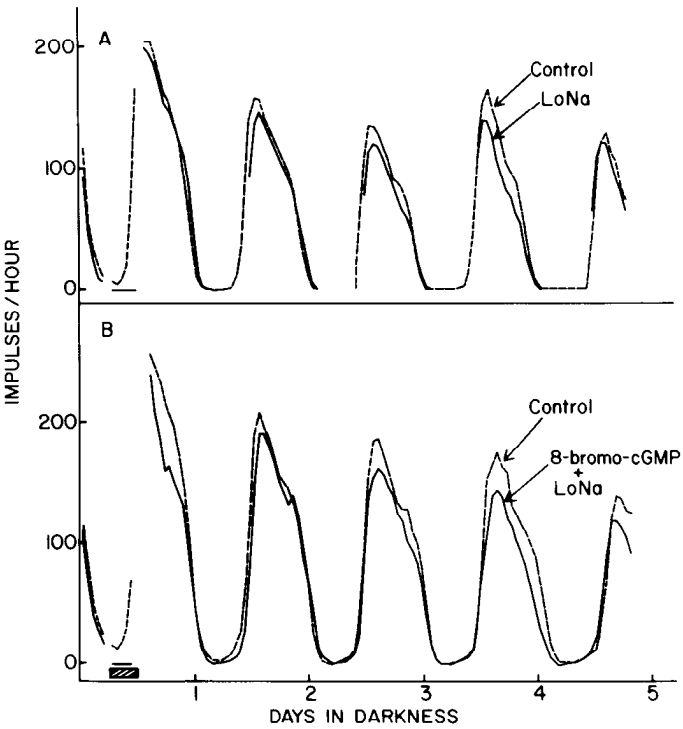

Figure 5. *A*, LoNa solution (Na^+ = 12 mM) does not phase shift the rhythm. *B*, The phase-shifting effect of 8-bromo cGMP is blocked when an eye is treated with 8-bromo cGMP plus LoNa solution.

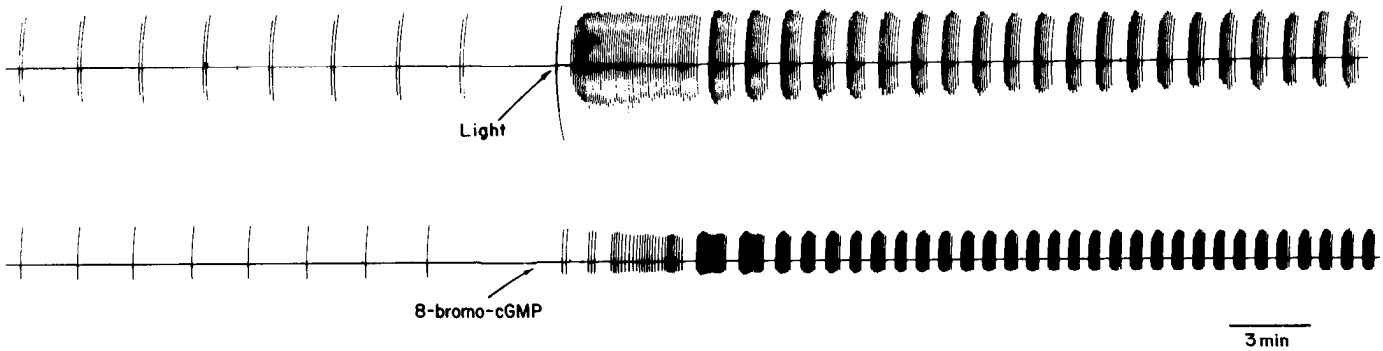

Figure 4. Similarity in the effects of light and 8-bromo cGMP on extracellularly recorded optic nerve impulses. At the beginning of both records, the isolated eyes were in darkness. At the times shown, the top eye was exposed to light and the bottom eye was exposed to 8-bromo cGMP (2×10^{-3} M).

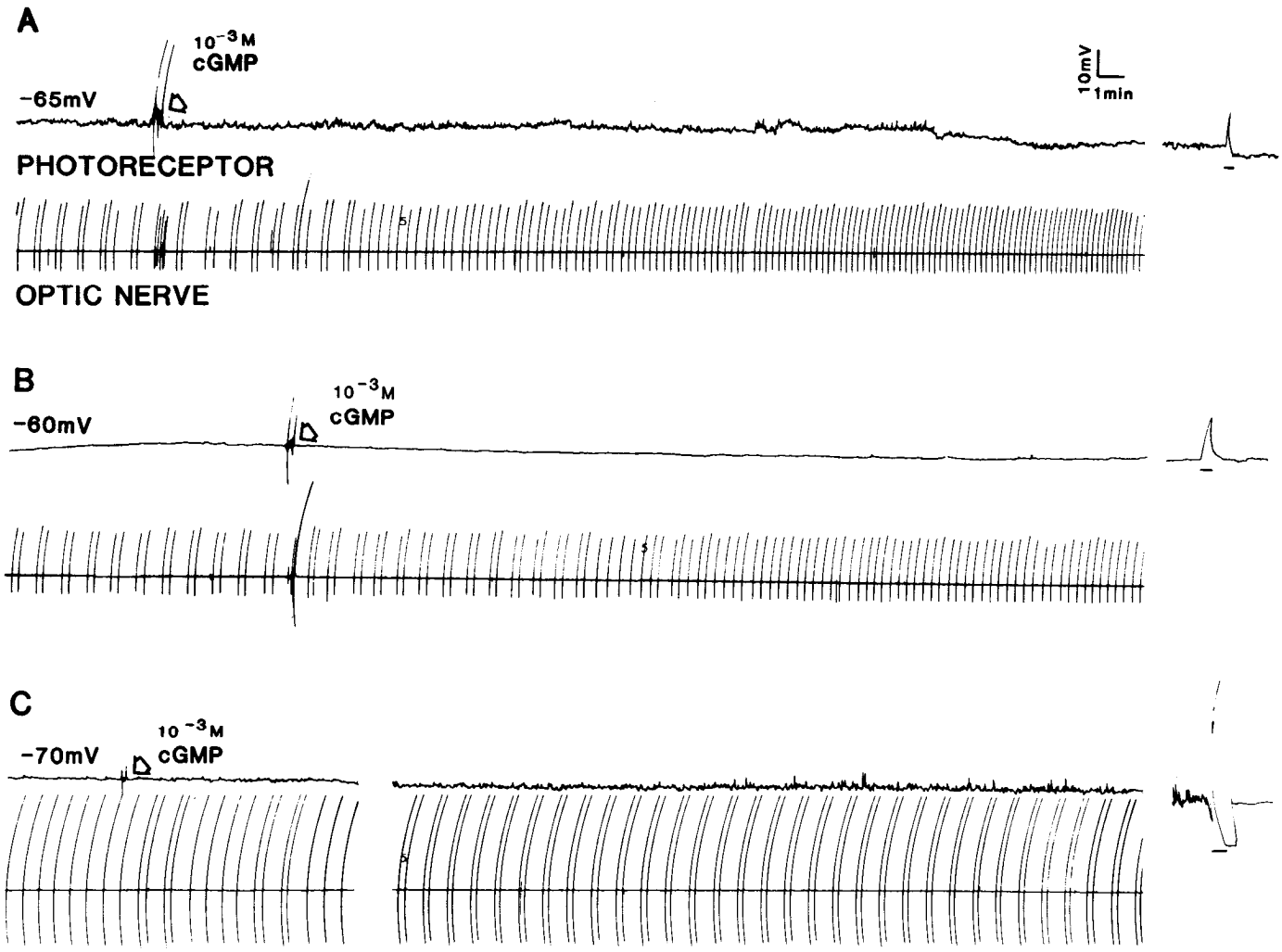

Figure 6. Effect of 8-bromo cGMP on simultaneous recordings of optic nerve impulses and the membrane potential of photoreceptor cells. *Records A* and *B* are continuous recordings, while *C* shows the initial effects of drug infusion (*left record*) and a sample of the record 4 hr after treatment (*right record*). Confirmation of photoreceptor impalement was accomplished by exposing the retina to a 20-sec light pulse. These receptor responses are shown to the *right* of each *record*.

and strophanthidin, phase shift the rhythm and appear to mimic the effect of light. Also, there is a correlation between the effect of low Na^+ on the amplitude of the electroretinogram and on the magnitude of the phase shift produced by light (Eskin, 1977). The large increase in the frequency of spontaneous optic nerve impulses produced by 8-bromo cGMP and the similarity of this effect with that of light suggests that 8-bromo cGMP is either depolarizing or increasing the excitability of neurons in the eye. A similar effect of cGMP on the frequency of spontaneous activity was observed by Levitan and Norman (1980) on cell R15 in the abdominal ganglion of *Aplysia*. Our finding that LoNa solutions blocked the phase-shifting effect of 8-bromo cGMP supports the hypothesis that 8-bromo cGMP acts by causing membrane depolarization.

The cellular location of the light-induced cGMP increase in the *Aplysia* eye remains to be determined. In vertebrate rod photoreceptors, decreases in cyclic GMP are postulated to mediate the hyperpolarization produced by light (Hubbell and Bownds, 1979; Pober and Bitensky, 1979). Increases in intracellular cGMP can increase inward current and depolarize vertebrate photoreceptors (Lipton et al., 1977; Miller and Nicol, 1979; Lipton, 1983). To our knowledge, there are no similar results implicating cGMP in transduction in invertebrate photoreceptors. In *Limulus* ventral photoreceptors, light has no effect on intracellular levels of cGMP (Schmidt and Farber, 1980), and cGMP does not mimic the effect of light on the photoreceptor (Stern and Lisman, 1982). The largest, best defined, and probably the most abundant photoreceptor (R cells) in the *Aplysia* eye is located in a layer beneath the lens. These cells are depolarized by light, and this depolarization appears to be produced by a conductance increase to Na^+ (Eskin, 1977; Jacklet and Rolerson, 1982). If cGMP has a transduction role in R cells, then exposing them to 8-bromo cGMP should result in membrane depolarization. To the contrary, we observed no change in membrane potential when photoreceptors were exposed to concentrations of 8-bromo cGMP capable of phase shifting the rhythm. The lack of an effect of 8-bromo cGMP was not due to a permeability problem, because the frequency of spontaneous nerve impulses was increased by the drug in these same experiments. These results indicate that 8-bromo cGMP does not act upon R-type photoreceptors to phase shift the rhythm. There is evidence for other types of photoreceptors in the eye (Jacklet and Rolerson, 1982). For example, recent results of Block and McMahon (1983) have shown that the effect of light on spontaneous activity from the *Aplysia* eye may be mediated by photoreceptors near the base of the eye and not by photoreceptors located directly beneath the lens of the eye. Furthermore, in the *Bulla* eye, which is similar in several ways to the *Aplysia* eye, light can phase shift the rhythm in a reduced eye preparation which contains only

cells near the base of the eye (Block and Wallace, 1982). Thus, cGMP could act on or through these other photoreceptors to phase shift the rhythm.

There are at least two possible roles of cGMP in phase shifting by light. Cyclic GMP may be involved in transduction in non-R-type photoreceptor cells. The phase-shifting pathway for this possibility would be light → increase cGMP → photoreceptor potential → depolarization → phase shift of rhythm. Alternatively, cGMP may not be involved in transduction but is elevated in some cells as a result of the photoreceptor potential.

It is intriguing that the phase-shifting pathways for serotonin and for light both involve second messengers and cyclic nucleotides. cAMP mediates the effects of serotonin on the eye rhythm, while cGMP appears to mediate the effects of light. It is possible that these two pathways converge at some point on their way to the circadian oscillator or that they may converge upon some component of the oscillator itself. If such a convergence occurs, the two pathways must act in opposite ways to account for the reciprocal nature of the phase response curves for light and for serotonin (Corrent et al., 1982). Thus, for example, if serotonin or cAMP increases phosphorylation (Cromie et al., 1983) or the synthesis of some protein (Eskin, 1982), then light or cGMP might decrease phosphorylation or the synthesis of that same protein. The possibility that cAMP and cGMP might have opposite effects on steps of phase-shifting pathways or on a molecular component of the clock itself should provide us with a powerful new tool to identify such steps or components of the oscillator.

Note added in proof. A recent report has appeared indicating that light increases cGMP in squid photoreceptors (FEBS Lett. *168:* 213–216, 1984).

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