J Comp Physiol A (1987) 161:335–346

Journal of Comparative Physiology A Sensory, Neural, and Behavioral Physiology

The *Bulla* ocular circadian pacemaker

I. Pacemaker neuron membrane potential controls phase through a calcium-dependent mechanism

Douglas G. McMahon* and Gene D. Block**
Department of Biology, Gilmer Hall, University of Virginia, Charlottesville, Virginia 22901, USA

Accepted April 15, 1987

Summary. In an effort to understand the cellular basis of entrainment of circadian oscillators we have studied the role of membrane potential changes in the neurons which comprise the ocular circadian pacemaker of *Bulla gouldiana* in mediating phase shifts of the ocular circadian rhythm. We report that:

1. Intracellular recording was used to measure directly the effects of the phase shifting agents light, serotonin, and 8-bromo-cAMP on the membrane potential of the basal retinal neurons. We found that light pulses evoke a transient depolarization followed by a smaller sustained depolarization. Application of serotonin produced a biphasic response; a transient depolarization followed by a sustained hyperpolarization. Application of a membrane permeable analog of the intracellular second messenger cAMP, 8-bromo-cAMP, elicited sustained hyperpolarization, and occasionally a weak phasic depolarization.

2. Changing the membrane potential of the basal retinal neurons directly and selectively with intracellularly injected current phase shifts the ocular circadian rhythm. Both depolarizing and hyperpolarizing current can shift the phase of the circadian oscillator. Depolarizing current mimics the phase shifting action of light, while hyperpolarizing current produces phase shifts which are transposed approximately 180° in circadian time to depolarization.

3. Altering BRN membrane potential with ionic treatments, depolarizing with elevated K^+ seawater or hyperpolarizing with lowered Na^+ seawater, produces phase shifts similar to current injection.

4. The light-induced depolarization of the basal retinal neurons is necessary for phase shifts by light. Suppressing the light-induced depolarization with injected current inhibits light-induced phase shifts.

5. The ability of membrane potential changes to shift oscillator phase is dependent on extracellular calcium. Reducing extracellular free Ca^{++} from 10 mM to 1.3×10^{-7} M inhibits light-induced phase shifts without blocking the photic response of the BRNs.

The results indicate that changes in the membrane potential of the pacemaker neurons play a critical role in phase shifting the circadian rhythm, and imply that a voltage-dependent and calcium-dependent process, possibly Ca^{++} influx, shifts oscillator phase in response to light.

Introduction

Endogenous biological oscillators, used to time daily rhythms in physiology and behavior, are a ubiquitous feature of eukaryotic organization. In order for an organism's internal pacemaker to be useful as a 'biological clock' it must be synchronized, or entrained, to local environmental time. The cellular events governing the entrainment of circadian pacemakers by external cues are of fundamental interest in the field of biological rhythms. Since the causal chain of events which mediates phase shifting must at some point terminate on an element of the circadian oscillator mechanism itself, tracing entrainment pathways at the cellular level may help identify oscillator components. In an effort to add to the current understanding of the cellular mechanisms of circadian pacemaker

Abbreviations: *BRN* basal retinal neuron; *CT* circadian time

* Present address: Department of Cellular and Developmental Biology, The Biological Laboratories, Harvard University, Cambridge, MA 02138, USA
** To whom offprint requests should be sent

entrainment, we have tested the proposition that changes in the transmembrane potential of putative circadian pacemaker neurons play an important role in resetting the phase of a neuronal circadian pacemaker. We have also examined the role of extracellular calcium in phase shifts produced by light pulses.

The preparation which has been used in these experiments is the retina of the marine mollusc *Bulla gouldiana*. The *Bulla* eye, like the eyes of several other opisthobranch molluscs (*Aplysia,* Jacklet 1969; *Bulla,* Block and Wallace 1982; *Bursatella,* Block and Roberts 1981; *Hamonea,* McMahon and Block 1982; *Navanax,* Eskin and Harcombe 1977) contains a circadian pacemaker which expresses a circadian rhythm in the frequency of spontaneously occurring optic nerve impulses in vitro. The *Bulla* ocular circadian pacemaker and its photic entrainment mechanism are contained within a group of approximately 100 electrically coupled neurons at the base of the *Bulla* retina (Block and McMahon 1984). Intracellular electrophysiological recordings from these basal retinal neurons (BRNs) reveal that they generate the optic nerve impulses which express the circadian rhythm, and exhibit circadian rhythms in membrane potential which appear to drive the impulse frequency rhythm (Block et al. 1984; McMahon et al. 1984). *Bulla* BRNs do not appear to have efferent connections within the retina because electrical activity in the BRNs is not reflected in other retinal cells (Block et al. 1984). While we are confident in our localization of the circadian oscillator to the BRNs as a group, we prefer to call them putative circadian pacemaker neurons because the test of whether individual neurons continue to exhibit circadian rhythmicity in isolation has not yet been performed.

Previous experiments in molluscs (Eskin 1972, 1977, 1982; Jacklet and Lotshaw 1981) and rodents (Rusak and Groos 1982) have suggested that membrane potential changes play a role in shifting the phase of neuronal circadian pacemakers. In these instances membrane potential changes were inferred, not measured directly, and the experimental treatments were provided to the entire pacemaker organ. Because of the indirect methods used, these experiments could not localize the critical site of action of the phase shifting agents to a particular cell-type within the pacemaker organ. In either case the phase shifting stimulus may have acted directly on circadian pacemaker cells, or indirectly by stimulation of cells or fibers connected to oscillator cells (Eskin 1977; Rusak and Groos 1982). In *Bulla* however, it is possible to test the involvement of membrane potential changes in phase shifting by monitoring directly the membrane potential effects of phase shifting agents and by selectively stimulating the putative circadian pacemaker neurons with intracellularly injected electrical current.

We now report that: 1) light, serotonin and 8-bromo-cAMP all produce sustained changes in basal retinal neuron membrane potential, 2) altering the membrane potential of the basal retinal neurons by intracellular current injection can phase shift the *Bulla* ocular circadian pacemaker, 3) altering membrane potential with ionic treatments phase shifts the ocular rhythm in a manner similar to current injection, 4) suppressing the depolarizing light response of the BRNs inhibits light-induced phase shifts, and 5) reduction of the extracellular calcium concentration inhibits light-induced phase shifts without suppressing the light-induced depolarization of the BRNs. Taken together the results suggest that altering calcium flux by changing the transmembrane potential is a critical cellular event in shifting the phase of the *Bulla* ocular circadian rhythm.

Methods

Adult *Bulla* were obtained from Alacrity Marine Biological Services (Redondo Beach, CA) and maintained on L:D 12:12 light cycles, in Instant Ocean artificial seawater at 15 °C. Dissections were performed in the late subjective day on animals injected with 10 ml of isotonic $MgCl_2$. Except as noted, eyes were maintained in artificial seawater consisting of (in m*M*) NaCl, 395; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 50; Na_2SO_4, 28; Hepes buffer, 30; and penicillin/streptomycin, 100000 units, 100000 mcg/l.

For phase shifting experiments eyes were maintained in light until Circadian Time 12 (time of lights off during the light cycle). One eye of the pair from each animal was randomly assigned as the experimental eye, while the other served as a matched control. Extracellularly recorded optic nerve compound action potential activity in both eyes was counted into half hour bins for analysis. Phase shifts were calculated by comparing the time of one half-maximum impulse frequency of experimental eyes to their matched controls on the rising phase of the second circadian cycle after stimulation. The time of half-maximum impulse frequency is a commonly used phase reference point for determining phase shifts of molluscan ocular rhythms and has been used exclusively for determining phase shifts of the *Bulla* ocular rhythm (Block and Wallace 1982; Block and McMahon 1984; Roberts and Block 1983, 1986). This reference point has been found to be stable even when the amplitude of the rhythm is diminished by preparation of the eye for intracellular recording (see below), or when the eye has been surgically reduced (Block and Wallace 1982; Block and McMahon 1984). Unless otherwise noted phase shifts are stated $\pm$the 95% confidence interval.

Intracellular recording. Eyes with attached optic nerves were dissected from animals and prepared for intracellular recording as previously described (Block et al. 1984). BRNs were pene-

trated using glass capillary microelectrodes filled with 3 *M* KCl or 4 *M* KAc (80–120 MΩ). For experiments measuring intracellular responses to phase shifting agents the recording chamber was maintained at 15 °C using a Lauda K/2R recirculating cooler, or at room temperature (23 °C). No consistent differences in results were observed at the two temperatures. Light pulses (5000 lux) from a projector bulb (EKE 150W) were delivered to the eyes through fiber optic light guides. Pharmacological agents were dissolved in a small volume of ASW and added as stock solutions to the recording chamber in order to establish the indicated final concentrations. For the Ca^{++} experiments BRN light responses to 5 min light pulses were recorded in both normal and low Ca seawater allowing 15 min dark adaptation between tests. Solution changes were accomplished by exchange of 10 volumes of the recording dish. Eyes were allowed 15 min to equilabrate to a new solution before testing.

Current injection experiments. One eye of the pair from each animal was selected as the experimental eye and was prepared for recording as previously described (Block et al. 1984). The surgical procedure did not produce phase shifts (mean phase difference of surgically treated eyes vs. pristine controls is +0.1 h, ±0.3 h, $N=4$). Intracellular penetrations of BRNs were made with glass capillary microelectrodes as described above. Eyes were maintained at 15 °C in darkness for the duration of the experiment except for exposure of both eyes to brief light pulses during positioning of the intracellular microelectrode (total exposure less than 5 min). Eyes were placed side by side in the recording chamber and illuminated by a relatively distant fiber optic to ensure that both eyes received approximately the same light intensity. Reimpalement was performed if the penetration was lost during current injection. Two of the 26 data points reported involved reimpalement.

Ionic experiments. Control eyes were maintained in artificial seawater at 15 °C in darkness for the duration of the experiment. Experimental eyes were also maintained in artificial seawater in constant conditions except for receiving an experimental treatment on the first or second circadian cycle following dissection. In high K^+ experiments eyes were exposed to pulses of seawater which consisted of (in m*M*): NaCl, 383; KCl, 50; $CaCl_2$, 10; $MgCl_2$, 50; Na_2SO_4, 28. In low Na^+ experiments eyes were exposed to pulses of seawater which consisted of (in m*M*): NaCl, 12; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 50; N-methyl-d-glucamine, 438 to substitute for Na^+ (Eskin et al. 1984). In calcium experiments eyes were exposed to either pulses of light (5000 lux), low Ca seawater (m*M*: NaCl, 419; KCl, 10; $CaCl_2$, 5; $MgCl_2$, 50; Na_2SO_4, 28 ; EGTA, 10; final free Ca^{++} concentration $=1.3\times10^{-7}$ *M*, calculated after Eskin (1977) by the method of Portzehl et al. 1964), or light and low Ca seawater. In the latter case low Ca seawater was added 15 min prior to exposure to light. All solutions contained 30 m*M* HEPES and 100000 units/100000 mcg per liter penicillin/streptomycin. Solution changes were accomplished by exchange of 10 volumes of the recording dish. The effects of these treatments on the phase of the ocular circadian rhythm were assayed as described above.

Results

Basal retinal neuron responses to light, 5-HT, and 8-bromo-cAMP

The effects on BRN membrane potential of the phase shifting agents light, 5-HT and 8-bromo-cAMP were measured directly using intracellular recording. In agreement with the findings of previous studies (Block et al. 1984) it was found that light depolarized *Bulla* BRNs. A typical BRN light response is shown in Fig. 1A. Light of 5000 lux produces an initial transient depolarization. This phasic response decays within 30 s to 1 min to a tonic depolarization of lower amplitude. The mean phasic depolarization produced by light of this intensity was 24 mV, while the tonic response averaged 7 mV (both $N=18$). The tonic response persisted as long as the eye was exposed to light. Our longest recording was approximately 2.0 h in duration and demonstrated the persistence of the light-induced depolarization during the entire recording period. Persistence of the depolarization is also consistent with the observation from extracellular

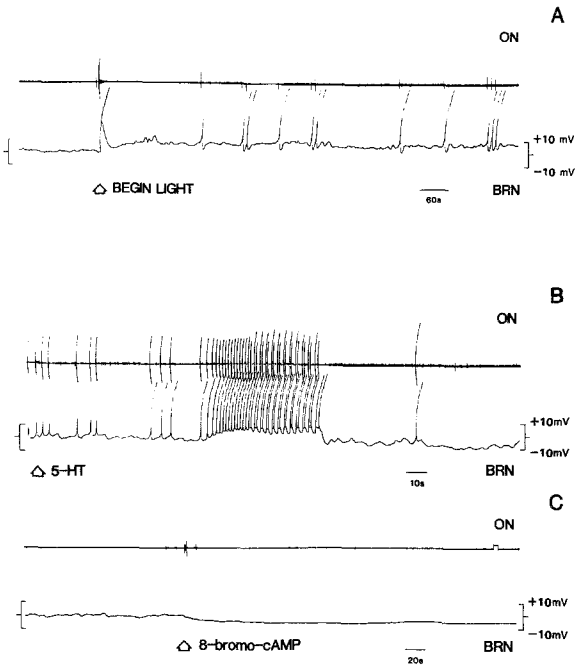

Fig. 1A–C. BRN responses to light, 5-HT and 8-bromo-cAMP. Simultaneous intracellular recording from a BRN and extracellular recording from the optic nerve (ON). The height of intracellularly recorded action potentials is attentuated due to clipping of the recorder pen. **A** Light pulse (5000 lux) begins at arrow and continues for the remainder of the trace. At onset, BRN shows a phasic depolarization of 24 mV which decays within 30 s to a tonic depolarization of 7 mV and which then persists for the duration of the recording. Light also evokes action potentials in the previously quiescent BRN which are recorded as compound action potentials in the optic nerve. **B** Addition of 5-HT to the recording chamber (arrow) causes, after some delay, a transient depolarization of 7 mV measured at the midpoints between action potentials. This is followed by a persistent hyperpolarization of 6 mV, and suppression of impulse activity in the BRN. **C** Addition of 2×10^{-3} *M* 8-bromo-cAMP to the recording chamber causes a persistent hyperpolarization of 4 mV

records that the increase in CAP frequency is sustained for the duration of light exposure.

Serotonin (5-HT) and its intracellular second messenger cAMP have been inferred to hyperpolarize retinal cells in *Aplysia* (Eskin 1982). In *Bulla*, it was found that 5-HT (10^{-5} *M*) produced a biphasic membrane potential response (Fig. 1B). Application of 5-HT produced an initial transient depolarization followed within approximately 1 min by a long lasting hyperpolarization. The mean amplitude of the phasic depolarization to 5-HT was 8 mV, and the mean tonic hyperpolarization was also 8 mV (both $N=6$). As with the tonic response to light, the tonic hyperpolarizing response to 5-HT persisted as long as the agent was present. The longest intracellular record obtained showed a hyperpolarization to 5-HT which was sustained for 4 h. This also agrees with extracellular observations in *Bulla* demonstrating a sustained reduction in the CAP frequency in response to 5-HT.

Application of the membrane permeable cAMP analog, 8-bromo-cAMP, also produced sustained hyperpolarization of the BRNs (Fig. 1C). However, in most cases the BRN response to 8-bromo-cAMP (2×10^{-3} *M*) lacked the initial phasic depolarization observed for 5-HT. The phasic depolarization was present in only one out of the five experiments, and then it was a weak effect (+2 mV). In contrast with the weak phasic response, BRNs consistently responded to 8-bromo-cAMP with a tonic hyperpolarization averaging 10 mV ($N=5$). The longest intracellular record of cAMP's action was one hour in duration. It is also apparent from extracellular records that its suppression of CAP activity in the BRNs is sustained for a number of hours.

Phase shifting with current injection

In order to perform these experiments, pairs of *Bulla* eyes were dissected from anesthetized animals and placed in a petri dish filled with artificial seawater. At appropriate times during the circadian cycle after dissection, a BRN in the experimental eye was penetrated and current was injected into the electrically coupled BRN population. Only those preparations in which a satisfactory impalement was obtained in less than one half-hour of manipulation, and in which current injection was effective for longer than one hour were assayed. Of approximately 180 attempted experiments 26 met these criteria and are reported here. The average resting membrane potential of the BRNs was −58 mV, and the average duration of current injection was 1.7 h.

A comparison of the short term effects of depolarizing current injected into the BRNs and the short term effects of light is illustrated in Fig. 2A. Both light and depolarizing current result in depolarization of the BRNs and an increase in the frequency of optic nerve compound action potentials produced by the electrically coupled BRN population. The continued synchronous firing of the BRNs in response to current injected into one cell indicates that the current spreads throughout the population. Similarly, injection of hyperpolarizing current into a single BRN suppresses impulse activity in the entire population (not shown), again indicating that injected current spreads within the BRNs.

An example of a phase shift obtained by current injection into the BRNs is shown in Fig. 2B. Depolarizing current (0.4 nA) was delivered in the early subjective night, from circadian time 14–16. This caused an increase in optic nerve impulse activity in the treated eye during stimulation and resulted in a phase delay of −1.2 h, measured on the second circadian cycle following stimulation. We were also successful in shifting the phase of the ocular circadian pacemaker by injecting hyperpolarizing current into the BRNs. An example of a phase shift obtained with hyperpolarization is shown in Fig. 2C. In this example hyperpolarizing current was injected into the BRNs during the late subjective day resulting in a phase advance of the ocular rhythm.

Summarized data from all of our current passing experiments are shown in Fig. 3. Plotting the phase shifts engendered by depolarizing current relative to the phase in the circadian cycle when current was delivered, yields a coarse phase response curve which is similar in shape and slightly lower in amplitude than the phase response curve previously obtained for 6 h light pulses (Fig. 3A). Depolarizing the BRNs with injected current produced phase delays in the early subjective night (mean phase shift = −1.6 h, ±0.6 h, $N=4$), phase advances in the late subjective night (+1.5 h, ±0.7 h, $N=4$), and caused no phase shift in the subjective day (time of light during previous light cycle, +0.1 h, ±0.5 h, $N=4$). The average injected current was +0.2 nA, resulting in a mean depolarization of the BRNs of +9 mV measured through the current passing electrode.

Summary data for hyperpolarizing current pulses is shown in Fig. 3B. Hyperpolarizing the BRNs produced phase advances in the late subjective day (+1.4 h, ±0.9 h, $N=3$) and no phase shift

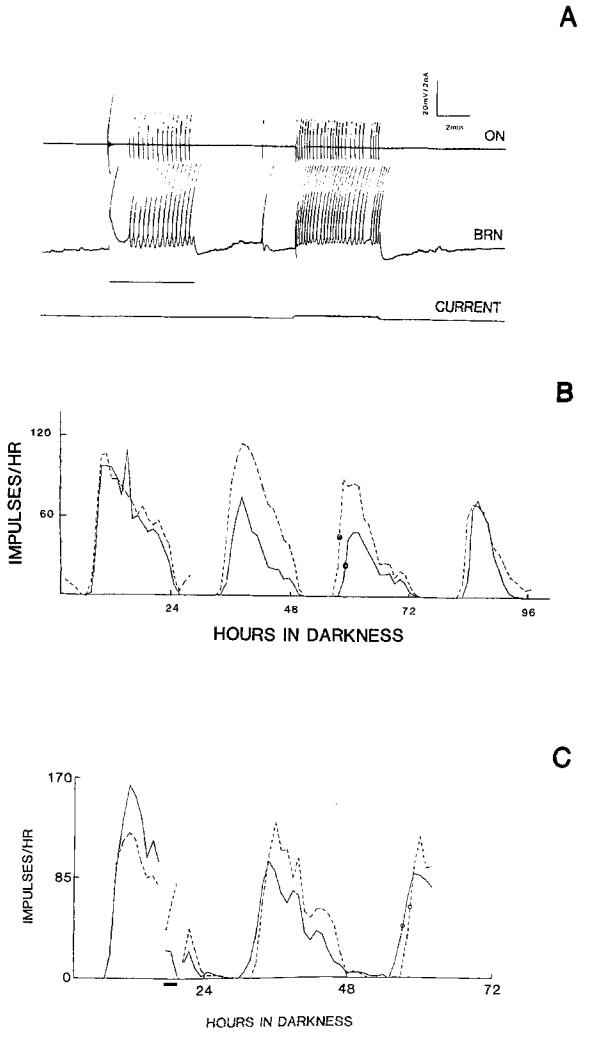

Fig. 2. A BRN responses to light and depolarizing current. Five min pulses of light (5000 lux, indicated by bar) and depolarizing current (0.15 nA). Height of intracellularly recorded action potentials is attentuated due to clipping. **B** Phase shift of the ocular circadian rhythm by depolarizing current. Solid line: experimental eye; dashed line: control. Discontinuities indicate times of transfer from incubator to recording apparatus. At CT 14 on the first circadian cycle following dissection depolarizing current of 0.4 nA was injected into the BRNs of the experimental eye for 2 h (bar). This caused an increase in the optic nerve impulse activity in the treated eye, and resulted in a phase delay of −1.2 h, measured on the second circadian cycle following stimulation. **C** Phase shift of the ocular circadian rhythm by hyperpolarizing current. At CT 6 on the first circadian cycle following dissection hyperpolarizing current of 0.4 nA was injected into the BRNs of the experimental eye for 2 h (bar). This caused a decrease in the optic nerve impulse frequency of the treated eye, and resulted in a phase advance of +1.0 h, measured on the second circadian cycle following stimulation

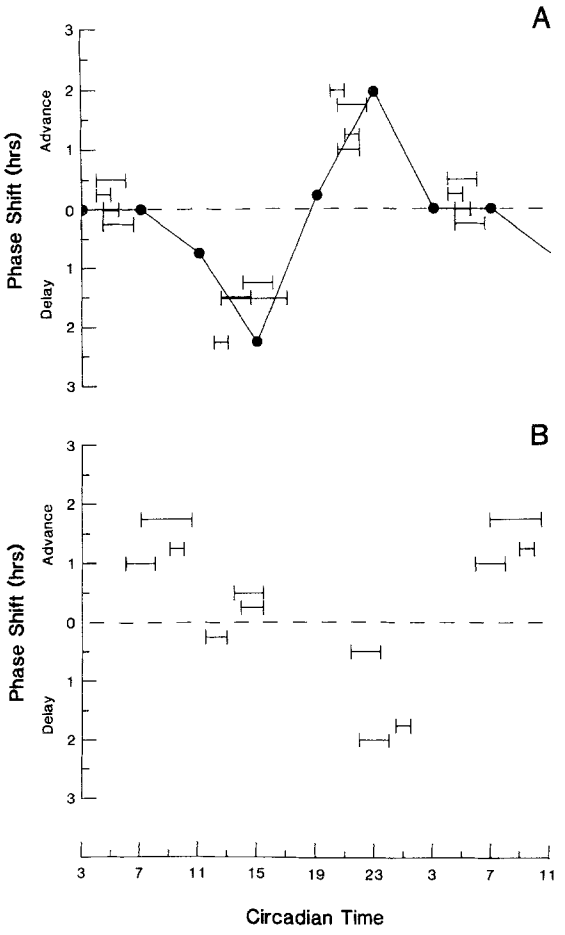

Fig. 3A, B. Phase shifts to intracellular current injection. Bars: circadian time and duration of injected current. Phase shifts plotted to the nearest 0.25 h. **A** Phase shifts by depolarizing current (bars) plotted with phase response curve to 6 h light pulses (solid line, redrawn from Block and McMahon 1984) shown for comparison. **B** Phase shifts by hyperpolarizing current. Data points during the subjective day have been replotted to facilitate visualization of the phase response curve

in the early subjective night (+0.2 h, ±1.3 h, $N=3$). Hyperpolarizing current delivered in the late subjective night and early subjective day produced phase delays on all three trials, however, the 95% confidence interval for these responses overlaps zero (−1.4 h, ±1.9 h, $N=3$) and thus these phase delays are not statistically significant. The average injected current was −0.3 nA which resulted in a mean hyperpolarization of −15 mV.

As mentioned previously, eyes which were surgically prepared for current injection experiments typically exhibited lower amplitude rhythms than the corresponding unoperated control eye (mean reduction in peak amplitude = 13%). This raises the issue of whether changes in the waveform of the circadian rhythm might lead to erroneous estimates in the magnitude of experimentally generated phase shifts. For example with reference to Fig. 2B, it could be the case that the lower amplitude of the experimental eye rhythm and not a phase shift is responsible for the difference in the times of half-maximum impulse frequency. While

Table 1. Phase reference points (1/2 maximum and peak frequency) for current injection experiments

	Depolarization			
	First cycle		Second cycle	
	1/2 max	peak	1/2 max	peak
Delay	−0.6	−2.4	−1.6	−2.2
(s.e.m)	(0.5)	(2.0)	(0.2)	(0.5)
Advance	+1.5	+2.0	+1.5	+1.1
(s.e.m)	(1.5)	(1.2)	(0.2)	(1.2)
No shift	+0.1	0.0	+0.1	−0.3
(s.e.m)	(0.2)	(0.0)	(0.2)	(0.6)
	Hyperpolarization			
	First cycle		Second cycle	
	1/2 max	peak	1/2 max	peak
Delay	−0.3	+0.3	−1.4	−1.3
(s.e.m)	(0.8)	(1.8)	(0.3)	(0.3)
Advance	+0.4	+0.7	+1.4	+0.2
(s.e.m)	(0.3)	(0.3)	(0.2)	(0.7)
No shift	+0.2	+1.0	+0.2	−0.2
(s.e.m)	(0.1)	(0.5)	(0.3)	(0.3)

Mean phase shifts (in hours) and standard errors of the mean (s.e.m) are tabulated into delay, advance and no shift phases as defined in the text

this possibility cannot be entirely excluded, several lines of evidence suggest that differences in the timing of half-maximum impulse frequency accurately reflected phase shifts in the experimental eye. First, eyes which were surgically prepared for recording but not impaled, exhibited rhythms that were in phase with their unoperated counterparts (see Methods). Second, experimental eyes phase delayed, advanced or revealed no shift, depending upon the time at which the current pulse was delivered (Fig. 3). The reduction in amplitude of the rhythm in the experimental eye cannot account for these phase dependent changes in the direction of the phase shift. Finally, as Table 1 illustrates, a phase response curve generated using peak impulse frequency on the second circadian cycle as the phase reference point, while more variable than the one generated using midrise point, still reveals the phase dependent nature of the responses to current injection (average standard error for peak comparisons is ±0.6 h compared with ±0.2 h for midrise points). Phase differences measured by midrise or peak on the first circadian cycle after treatment are even more variable, but are generally consistent with those measured on the second circadian cycle. Thus we strongly suspect that our measurements faithfully reflect the states of the underlying pacemakers. Although we did not as a matter of practice monitor current injection preparations beyond their measurement cycles, Fig. 2B does show persistence of a phase shift by current injection on the third circadian cycle after treatment. In addition, phase shifts to depolarizing and hyperpolarizing ionic treatments also persist on the circadian cycle following the measurement cycle (see Figs. 4 and 5 below) suggesting that membrane potential-induced phase shifts are stable.

Phase shifts by high K^+ and low Na^+ seawater

While the above results demonstrate that phase shifts of the ocular circadian rhythm can be initiated by current injection into the BRNs, we also sought to evaluate the phase shifting effects of changing membrane potential by ionic manipulations. We have assayed the effects of pulses of seawater in which the K^+ concentration was elevated from 10 to 50 m*M*, and pulses of seawater in which the Na^+ concentration was reduced from 451 to 12 m*M*. Although the precise magnitude of BRN membrane potential changes induced by these solutions was not measured, similar raised K^+ and lowered Na^+ solutions have been shown to respectively depolarize and hyperpolarize *Bulla* BRNs (McMahon and Block 1987).

High K^+ seawater was tested at two different phases in the circadian cycle. Pulses delivered in the early subjective night from CT 14-16 (Fig. 4A) produced phase delays of the ocular circadian rhythm (−1.1 h, ±0.8 h, $N=7$) while pulses delivered from CT 22-0 in the late subjective night resulted in phase advances (+0.7 h, ±0.4 h, $N=4$). As shown in Fig. 4B, these phase shifts are similar in direction and lower in magnitude than shifts produced by depolarizing current injection delivered at similar phases in the circadian cycle. This pattern of phase shifts is also similar to those obtained with light, which depolarizes BRNs.

Low Na^+ seawater was tested at three different phases of the circadian cycle. Pulses delivered in the mid to late subjective day at CT 8-11 produced phase advances of the ocular circadian rhythm (+0.7 h, ±0.5 h, $N=8$) while pulses delivered in the late subjective day and early subjective night at CT 10-13 produced no phase shift (+0.2 h, ±0.7 h, $N=3$) and pulses delivered in the late subjective night and early subjective day at CT 22-1 (Fig. 5A) produced phase delays (−1.0 h, ±0.6 h, $N=7$). As shown in Fig. 5B these phase shifts are similar in direction to phase shifts induced by hy-

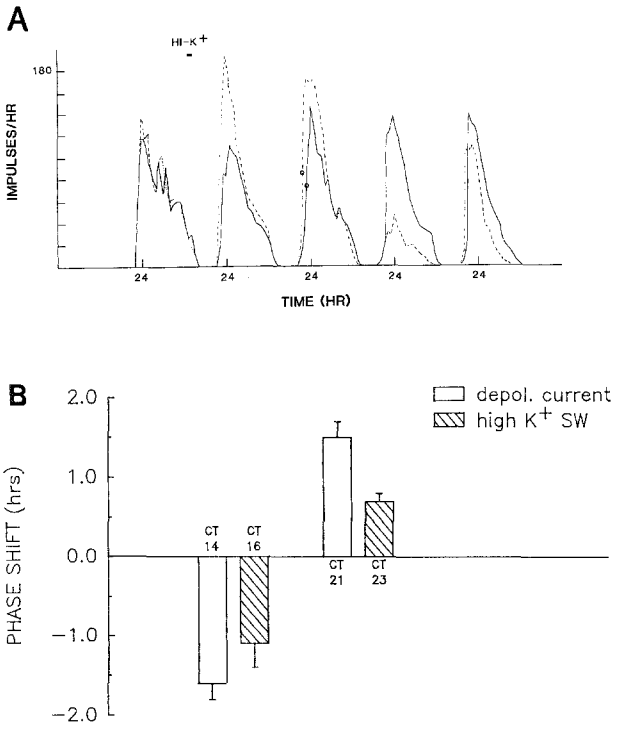

Fig. 4A, B. Phase shifts to high K^+ seawater. **A** Phase delay of experimental eye (solid line) by pulse delivered between CT 14–16. Circles on waveforms indicate phase reference point on the measurement cycle. **B** Mean phase shifts to depolarizing current and high K^+ seawater. Bars are standard errors. Circadian time indicated is midpoint of pulse

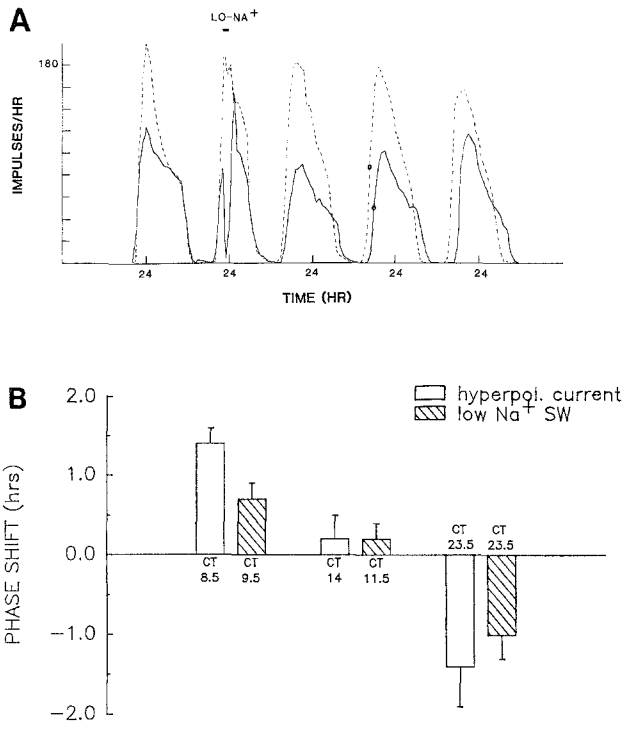

Fig. 5A, B. Phase shifts to low Na^+ seawater. **A** Phase delay of experimental eye (solid line) by pulse delivered between CT 22-1. Circles indicate phase reference points on measurement cycle. **B** Mean phase shifts to hyperpolarizing current and low Na^+ seawater. Bars standard errors. Circadian time indicated is midpoint of pulse

perpolarizing current injection delivered at similar phases in the circadian cycle. Thus both depolarizing and hyperpolarizing ionic treatments produce patterns of phase advances and delays much like depolarizing and hyperpolarizing current injection. Importantly, the significant phase delays observed in response to pulses of low Na^+ seawater in the late subjective night suggest that the delays observed in response to hyperpolarizing current at this phase, though not statistically significant, are a valid indication of the response of the oscillator to hyperpolarization.

Blocking phase shifts with injected current

In order to test whether depolarization of the BRNs is a necessary step in the light entrainment pathway we assayed the effect on light-induced phase shifts of suppressing the light-induced depolarization of the BRNs. Experiments were performed in the early subjective night. Eyes exposed to light at this phase exhibited average phase delays of 1.2 h (± 0.7, $N=7$) compared with unexposed controls. In blocking experiments, however, both eyes in the recording dish were exposed to light while the depolarizing light response of the BRNs in one eye was suppressed by intracellular injection of hyperpolarizing current. If the hyperpolarizing current had no effect on phase shifts then both eyes would be expected to be delayed equally by the light pulse, and there should be no phase difference between them. If, on the other hand, the hyperpolarizing current completely inhibited phase shifts in the treated eyes then only the untreated eyes would be delayed by the light pulse, and there should be an average 1.2 h phase difference between them, as if the eyes subjected to current injection had been kept in darkness.

In fact, it was found that suppressing the light induced depolarization of the BRNs inhibited light-induced phase delays. Eyes receiving light only were phase delayed with respect to those receiving both light and hyperpolarizing current by an average of 0.9 h (± 0.8 h, $N=5$) (Fig. 6). This phase delay was not statistically different from that observed in eyes exposed to light versus eyes kept in darkness (t-test of means, $P>0.20$). Our apparent failure to inhibit 100% of the expected delay phase shift in some preparations could be due to an inability to suppress the depolarizing light re-

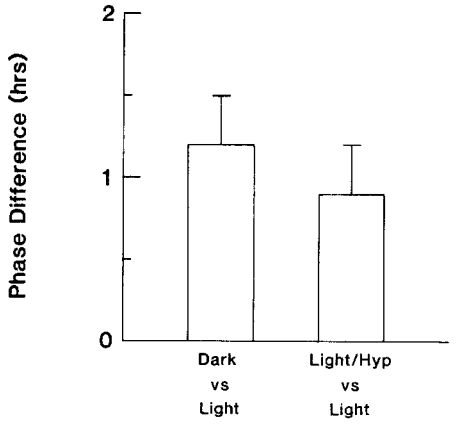

Fig. 6. Inhibition of light-induced phase shifts by hyperpolarizing current. Left bar shows mean phase difference of eyes exposed to a light pulse (1.5 h centered at CT 14, 5000 lux) compared with unexposed control eyes ($N=7$). Right bar: mean phase difference of eyes exposed to light and hyperpolarizing current injection, compared with control eyes simultaneously receiving light (average duration of 1.5 h centered at CT 14, $N=5$). Error bars standard errors of the mean

sponse in all cells of the electrically coupled BRN population by injecting current into a single neuron.

The average injected current used in the blocking experiments was -0.4 nA which resulted in a mean hyperpolarization of -11 mV. As reported above, hyperpolarizing current alone delivered during the early subjective night did not cause phase shifts ($+0.2$ h, ± 1.3, $N=3$). Thus the reduction in phase delay of the experimental eyes is due to actual inhibition of the light-induced phase shift, and cannot be attributed to cancellation of the light-induced delay by an offsetting phase advance due to BRN hyperpolarization.

Blocking phase shifts by reducing extracellular calcium

The results obtained with intracellular current injection into BRNs suggest that a voltage-dependent process, controlled by BRN membrane potential, is a critical element in resetting the phase of the *Bulla* ocular circadian rhythm. One possible mechanism by which transmembrane potential could affect pacemaker phase is by control of ion fluxes through voltage-dependent ion channels. While any permeable ion, such as Na^+, K^+, Cl^-, or Ca^{++}, could be involved, Ca^{++} would seem a particularly likely candidate for this role because of its well documented function as an intracellular second messenger in many biological systems. For this reason we have examined whether reducing Ca^{++} influx during exposure to light, by reducing extracellular Ca^{++}, inhibits light-induced phase shifts.

Experiments were performed during the early subjective night, between CT 14 and 16. Light pulses delivered during this interval produce phase delays of the ocular circadian rhythm in eyes maintained in normal artificial seawater. An example of a phase shift to a light pulse delivered at this time is shown in Fig. 7A. However, when eyes were exposed to light while in low Ca^{++} seawater (1.3×10^{-7} M free Ca^{++} in EGTA buffer), the light-induced phase delay was inhibited (Fig. 7B). Low Ca^{++} seawater alone at this phase did not produce phase shifts of the ocular circadian rhythm (Fig. 7C).

Summary data for all of our experiments examining the role of extracellular calcium are shown in Fig. 8. We found that light pulses delivered to eyes maintained in normal seawater generated a mean phase delay of -1.5 h (± 0.5 h, $N=11$), while the same light signal delivered to eyes in reduced calcium seawater resulted in a small phase advance ($+0.6$ h, ± 0.5, $N=11$). Pulses of low calcium seawater alone resulted in no significant phase shift ($+0.2$ h, ± 0.4, $N=10$). Phase shifts obtained in response to light, and to low Ca^{++} plus light are statistically different from one another while those of low Ca^{++}, and low Ca^{++} plus light are not (t-test of means, $P<0.01$, $P>0.20$, respectively). The fact that low Ca^{++} seawater does not generate a significant phase shift of its own when delivered at CT 14–16 indicates that the reduction in the light-induced phase delay observed in low Ca^{++} seawater is due to actual inhibition of the delay phase shift, and is not the result of addition of opposite sign phase shifts produced by light and low Ca^{++} seawater.

In order to exclude the possibility that low Ca^{++} seawater was inhibiting phase shifts by suppressing the light-induced depolarization of the BRNs, the effects of low Ca^{++} seawater on the BRN light response were measured by intracellular recording. All measurements were performed between CT 14 and 16. The light-induced depolarization of the BRNs is not suppressed in low Ca^{++} seawater (Fig. 9). Both the initial phasic depolarization and the tonic depolarization are greatly enhanced in low Ca^{++} seawater, possibly due to inactivation of Ca^{++} activated K^+ currents. The average light-induced phasic depolarization recorded in normal seawater was 31 mV, while in low Ca^{++} seawater the phasic depolarization averaged 60 mV ($N=6$ for both). The tonic light-induced depolarization increased from 12 mV in normal artificial seawater to 39 mV in low Ca^{++} seawater

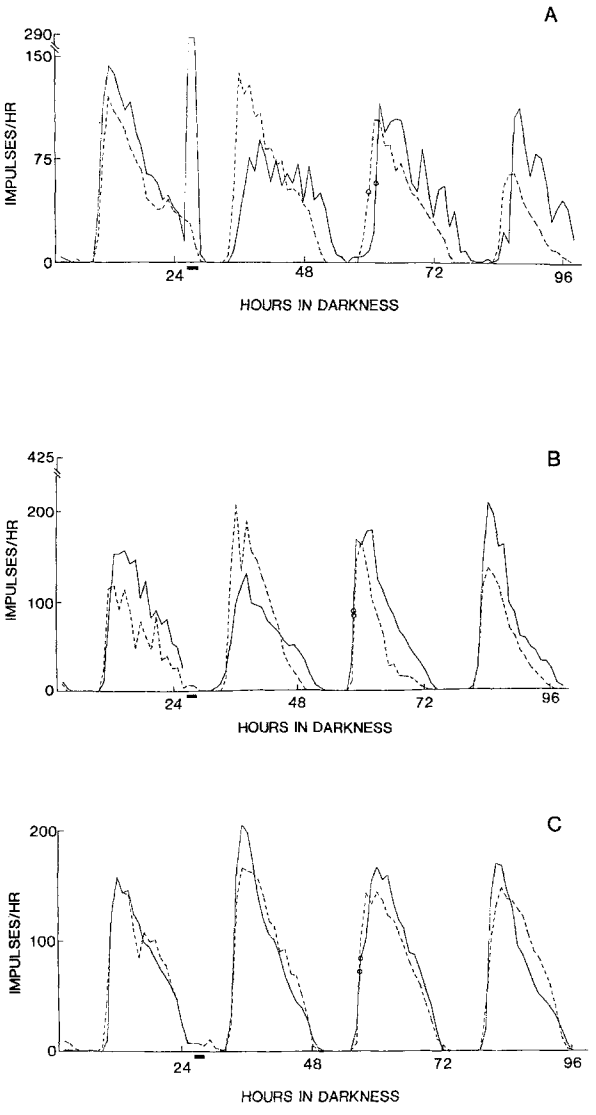

Fig. 7A–C. Effects of light, low Ca^{++} seawater, and light plus low Ca^{++} seawater delivered from CT 14 to 16 on the optic nerve impulse frequency rhythm. Solid line experimental eye, dashed line control. **A** Exposure to light (bar) increases impulse frequency, and phase delays the impulse frequency rhythm on subsequent circadian cycles. **B** Exposure to light and low Ca^{++} seawater (bar) results in a large increase in impulse activity, but does not phase shift the impulse frequency rhythm. **C** Exposure to low Ca^{++} seawater (bar) results in a modest increase in impulse frequency, but does not phase shift the impulse frequency rhythm

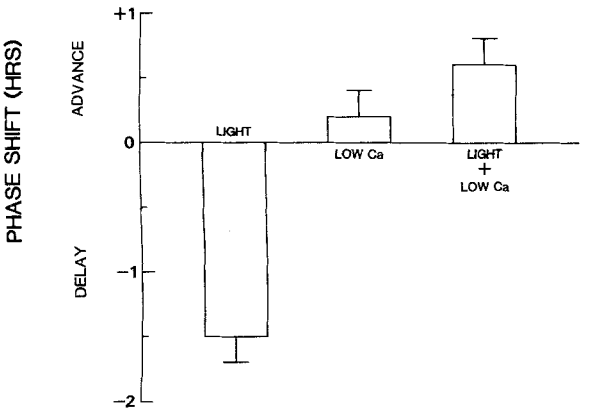

Fig. 8. Phase shifts by light and low Ca^{++} seawater. All experiments were performed from CT 14 to 16. Light, $N=11$; Low Ca, $N=10$; Light + Low Ca, $N=10$. Error bars standard errors of the mean

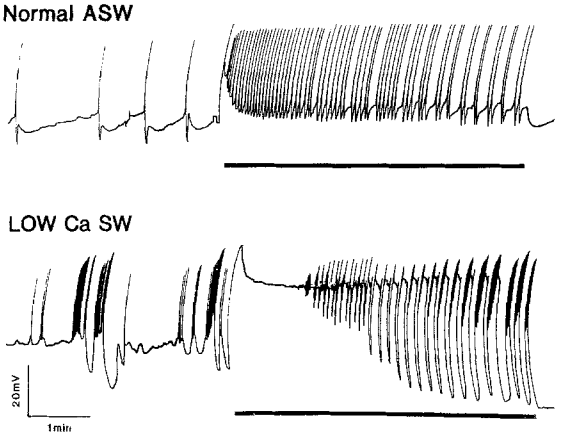

Fig. 9. BRN light response in normal and low Ca^{++} seawater recorded intracellularly. Upper panel shows response to a 5 min light pulse (5000 lux) in normal seawater (10 m*M* Ca^{++}). The initial phasic depolarization decays into a sustained tonic depolarization, and an increase in impulse frequency. Lower panel: response to a light pulse in low Ca^{++} seawater (1.3×10^{-7} *M* Ca^{++}). An initial phasic depolarization plateaus to a tonic depolarization. During final minute of the light pulse response consists of large depolarizing membrane potential oscillations, with bursts of action potentials superimposed on their peaks

(both $N=6$). The light-induced depolarization persists in low Ca^{++} seawater, as it does in normal artificial seawater. The large depolarizing waves (approximately 40 mV trough to peak) evident during the latter half of the low Ca^{++} light response in Fig. 9, were found to persist as long as exposure to light was maintained (up to 1 h). This accounts for the sustained increase in optic nerve impulse frequency observed during exposure to light while in low Ca^{++} seawater. Taken together these results demonstrate that low Ca^{++} seawater does not inhibit phase shifts by blocking photoresponses in the BRNs.

Discussion

The results presented here indicate that membrane potential changes in the putative circadian pacemaker neurons of the *Bulla* eye play an important role in mediating phase shifts of the ocular circadian pacemaker. Three treatments previously

shown to phase shift molluscan circadian oscillators light, 5-HT and cAMP (Eskin 1982; Block et al. 1984) produce sustained changes in the membrane potential of the BRNs. In addition, altering BRN membrane potential selectively with intracellular current injection, or altering the membrane potential of all cells in the eye with ionic treatments phase shifts the pacemaker. Either depolarizing or hyperpolarizing the BRNs from rest can induce phase shifts. Since our measurements of changes in BRN membrane potential during current injection were monitored through the current passing electrode they must be considered only approximate. However, the current-induced membrane potential changes found to phase shift the ocular circadian rhythm are indeed similar in magnitude to the physiological responses of the BRNs to phase shifting agents.

Importantly, although we have tested at only a few phases, it appears as though direct depolarization and hyperpolarization mimic the phase shifting action of physiological stimuli. Injection of depolarizing current into the BRNs mimics the phase shifting action of light, producing delays in the early subjective night, advances in the late subjective night, and no shift during the subjective day. Pulses of high K^+ seawater also produce delays in the early subjective night and advances in the late subjective night. At the present time we do not have sufficient information to be certain if hyperpolarization alone mimics 5-HT and cAMP. The pattern of phase shifts produced by hyperpolarizing current and low Na^+ seawater, phase delays in the early subjective day, phase advances in the late subjective day, and no shift in the subjective night, is similar to the responses obtained from application of 5-HT and cAMP analogues to the *Aplysia* eye (Corrent et al. 1978; Eskin et al. 1982; Eskin and Takahashi 1983). These agents do indeed hyperpolarize *Bulla* BRNs, but we do not yet have phase response curves to them. The above results suggest the possibility that these phase shifting agents may also act to shift the phase of the ocular circadian rhythm by altering BRN membrane potential. This is clearly the case for light, because our results indicate that depolarization of the BRNs is an essential step in shifting the phase of the circadian rhythm by a light pulse.

Comparison of the phase shifts obtained with depolarization and those obtained with hyperpolarization shows that the hyperpolarization-induced phase shifts are shifted approximately 180° on the time axis from those produced by depolarization. For example, hyperpolarization elicits phase delays near CT 0, while depolarization elicits delays near CT 15; hyperpolarization elicits advances near CT 9, while depolarization elicits advances near CT 22. While we must be cautious in our interpretation of these results because of the relatively few phases evaluated, the similarity to results obtained in *Aplysia* is striking. Agents which are inferred to depolarize *Aplysia* retinal cells (light, high potassium, strophanthidin) and those which are inferred to hyperpolarize retinal cells (serotonin, 8-benzylthio-cAMP, zero potassium) exhibit phase response curves which are shifted approximately 12 h in CT (Eskin 1972, 1979, 1982; Jacklet and Lotshaw 1981). Our data, combined with the previously mentioned results from *Aplysia,* suggest that membrane polarization is involved as a critical step in generating both types of phase response curves (see also Eskin 1982). Interestingly, a similar situation may exist in mammals. Comparison of the phase response curve to dark pulses to the phase response curve to light pulses in hamsters (Boulos and Rusak 1982) reveals an alignment similar to that of phase response curves to hyperpolarization and depolarization in molluscs. Since depolarization has been suggested to be involved in light-induced phase shifts of the hamster suprachiasmatic nucleus (Rusak and Groos 1982) it is possible that there is some physiological similarity between dark pulses in the hamster and hyperpolarization in molluscan eyes.

In the instances where pulses of depolarizing current and hyperpolarizing current overlap in phase, they elicit different phase shifts (see Fig. 3). The overlap between the two treatments is most extensive during the early subjective night where depolarization induces phase delays, and hyperpolarization produces no phase shift. There is also overlap during the subjective day (CT 6) where depolarization produces no shift, and hyperpolarization produces phase advance, and during the late subjective night (CT 22) where depolarization induces phase advance, and hyperpolarization appears to produce phase delay. Thus the response of the oscillator at the phases of the circadian cycle tested depends on the polarity of the injected current. This gives assurance that the phase shifts are an effect of the injected current and are not an artifact of the experimental procedure.

While we have attributed the phase shifting action of both current injection and the ionic treatments to their effects on membrane potential, these manipulations may have other effects on the BRNs independent of their membrane potential effects. One possible concern is alteration of the intracellular concentration of ions by ionophoreses from the

current injection electrode or from the action of the ionically altered seawater solutions. Electrodes were filled with KCl or KAc. Injection of depolarizing current thus ionophoresed K^+ ions into the injected BRN, and elevated K^+ seawater may have also raised intracellular K^+ levels. We believe it is unlikely that K^+ accumulation and not membrane potential per se is responsible for phase shifts. If this were the case then depolarization by injected current or high K^+ seawater should not mimic light-induced phase shifts. It seems unlikely that BRNs accumulate K^+ during prolonged exposure to light since the sustained light-induced depolarization and the concomitant increase in impulse activity would activate outward potassium currents.

Injection of hyperpolarizing current iontophoresed Cl^- or Ac^- into the BRNs, while hyperpolarizing with low Na^+ seawater may have altered intracellular Na^+ or Cl^- levels. The similar phase shifts produced by low Na^+ and hyperpolarizing current are not easily explained in terms of their expected actions on Na^+ and Cl^-. The decreased concentration of these ions in low Na^+ seawater might lead to a reduction in their intracellular concentration, however, hyperpolarizing current would not be expected to lead to such decreases. Acetate is often not freely permeable and might be expected to accumulate during injection of hyperpolarizing current with a KAc-filled electrode. However, there were no consistent differences in the phase shifts produced by electrodes filled with KCl or KAc, and low Na^+ seawater contains no Ac^-. Thus, while we cannot rigorously exclude changes in Na^+, K^+, Cl^-, or Ac^- as sources of spurious phase shifts there are no consistent explanations suggesting that their effects, rather than changes in membrane potential, underly the phase shifts observed in response to current injection.

The presence of extracellular free Ca^{++} is essential for phase shifts of the *Bulla* ocular circadian pacemaker in response to light. Reducing the extracellular free Ca^{++} has also been shown to block light-induced phase advances in *Aplysia* (Eskin 1977). However, in this case, the blocking action was attributed to reduction in the photoresponse of the eye as a whole, as measured by a 90% reduction in the electroretinogram (ERG). Low Ca^{++} seawater does not block light-induced depolarization of *Bulla* BRNs, in fact the photoresponse is enhanced. The photic response of the compound action potential-producing cells in the *Aplysia* eye may also be facilitated in low Ca^{++} solutions because extracellular optic nerve recordings demonstrate an increased light response of compound action potential units in low Ca^{++} seawater, despite the reduction in overall ERG (Eskin 1977).

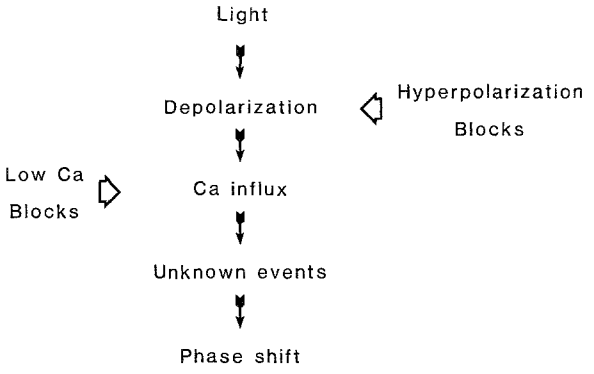

Fig. 10. Proposed sequence of events mediating light-induced phase shifts of the *Bulla* ocular circadian rhythm

The results of our current injection experiments indicate that depolarization of the BRNs is both necessary and sufficient to phase shift the ocular circadian rhythm. In light of this, our finding that reducing extracellular Ca^{++} inhibits phase shifts despite the persistence of light-induced depolarization in the BRNs, suggests that this treatment blocks the light entrainment pathway at a point downstream from the membrane potential step. While we cannot presently rule out the possibility that EGTA directly affected intracellular calcium levels, the simplest explanation of low Ca^{++} seawater's action is that it abolishes light-induced Ca^{++} influx into the BRNs which would normally occur through channels activated by the light-induced depolarization. This view is supported by recent experiments in which phase shifts to light pulses were blocked in lowered calcium seawater without EGTA added (Khalsa and Block, unpublished study). While we have not yet tested directly for light-induced Ca^{++} influx in the BRNs, calcium influx in response to light has been reported in other invertebrate photoreceptive cells (Brown and Blinks 1974; Brown 1983).

The results presented in this paper are summarized in our proposed light entrainment pathway shown in Fig. 10. Briefly, we believe that light induces phase shifts by depolarizing the BRNs thereby producing an influx of Ca^{++} through voltage-dependent channels. This scheme accounts for our findings that light-induced phase shifts are dependent on both voltage changes in the BRNs and extracellular Ca^{++}. It focuses attention on Ca^{++} flux as the probable transmembrane signal by which membrane potential changes in the putative pacemaker neurons shift the phase of the circadian oscillation. Future research will seek to determine

whether other treatments which reduce Ca^{++} flux, such as Ca^{++} channel blockers, inhibit light-induced phase shifts, and whether increasing intracellular Ca^{++} in the BRNs in the absence of depolarization mimics light-induced phase shifts. In addition it should be interesting to determine if hyperpolarizing treatments also regulate phase through a calcium-dependent mechanism.

It is now clear that the membrane potential of the BRNs, and the voltage-dependent processes it controls, are intimately associated with the workings of the *Bulla* ocular circadian pacemaker. The membrane potential of BRNs has been identified both as an output of the pacemaker (McMahon et al. 1984) and as a critical part of the entrainment mechanism. Experimental investigation of how voltage-dependent processes control circadian pacemaker phase, and how the oscillator mechanism in turn drives the BRN membrane potential rhythm, should reveal information about the fundamental mechanisms by which neuronal circadian pacemakers generate circadian rhythmicity, and are entrained by temporal cues.

Acknowledgments. We would like to thank A. Lusska for technical assistance and S.B.S. Khalsa for critical review of an earlier version of the manuscript. This study was supported by a Gwathmey Fellowship from the University of Virginia Society of Fellows to D.G.M. and NIH grant NS 15264 and RCDA NS 00714 to G.D.B. Experiments were undertaken in partial fulfillment of the requirements for a PhD degree.

References

Block GD, McMahon DG (1983) Localized illumination of the *Aplysia* and *Bulla* eye reveals new relationships between retinal layers. Brain Res 265:134–137

Block GD, McMahon DG (1984) Cellular analysis of the *Bulla* ocular circadian pacemaker system III: Localization of the circadian pacemaker. J Comp Physiol A 155:387–395

Block DG, Roberts MH (1981) Circadian pacemaker in the *Bursatella* eye: Properties of the rhythm and its effect on locomotor behavior. J Comp Physiol 142:403–410

Block GD, Wallace SF (1982) Localization of a circadian pacemaker in the eye of a mollusc, *Bulla*. Science 217:155–157

Block GD, McMahon DG, Wallace SF, Friesen WO (1984) Cellular analysis of the *Bulla* ocular circadian pacemaker system. I. A model for retinal organization. J Comp Physiol A 155:365–378

Boulos Z, Rusak B (1982) Circadian phase response curves for dark pulses in the hamster. J Comp Physiol 146:411–417

Brown HM (1983) The role of H^+ and Ca^{2+} in *Balanus* photoreceptor function. In: Grinnell AD, Moody WJ (eds) The physiology of excitable cells. Alan R. Liss, New York, pp 329–342

Brown JE, Blinks L (1974) Changes in intracellular free calcium concentration during illumination in invertebrate photoreceptors. Detection with aequorin. J Gen Physiol 64:643–665

Corrent G, McAdoo DG, Eskin A (1978) Serotonin shifts the phase of the circadian rhythm from the *Aplysia* eye. Science 202:155

Eskin A (1972) Phase shifting a circadian rhythm in the eye of *Aplysia* by high potassium pulses. J Comp Physiol 80:353–367

Eskin A (1977) Neurophysiological mechanisms involved in the photoentrainment of the circadian rhythm from the *Aplysia* eye. J Neurobiol 8:273–299

Eskin A (1979) Circadian system of the *Aplysia* eye: Properties of the pacemaker and mechanisms of its entrainment. Fed Proc 38:2573–2579

Eskin A (1982) Increasing external K^+ blocks phase shifts in a circadian rhythm produced by serotonin or 8-benzylthio-cAMP. J Neurobiol 13:241–249

Eskin A, Harcombe E (1977) Eye of *Navanax:* Optic activity, circadian rhythm and morphology. Comp Biochem Physiol 57A:443–449

Eskin A, Takahashi JS (1983) Adenylate cyclase activation shifts the phase of a circadian pacemaker. Science 220:82–84

Eskin A, Corrent G, Lin C-Y, McAdoo DJ (1982) Mechanism for shifting the phase of a circadian rhythm by serotonin: Involvement of cAMP. Proc Natl Acad Sci USA 79:660–664

Eskin A, Takahashi JS, Zatz M, Block GD (1984) Cyclic GMP mimics the effects of light on a circadian pacemaker in the eye of *Aplysia*. J Neurosci 4:2466–2471

Hagiwara SH, Byerly L (1981) Calcium channel. Annu Rev Neurosci 4:69–125

Jacklet JW (1969) Circadian rhythm of optic nerve impulses recorded in darkness from the isolated eye of *Aplysia*. Science 164:562–563

Jacklet J, Lotshaw DP (1981) Light and high potassium cause similar phase shifts of the *Aplysia* eye circadian rhythm. J Exp Biol 94:345–349

McMahon DG, Block GD (1982) Organized photoreceptor layer is not required for light responses in three opisthobranch eyes. Soc Neurosci Abstr 8:33

McMahon DG, Block GD (1984) Circadian pacemaker entrainment in *Bulla:* light and serotonin affect the membrane potential of putative circadian pacemaker neurons. Soc Neurosci Abstr 10:292

McMahon DG, Block GD (1987) The *Bulla* ocular circadian pacemaker. II. Chronic changes in membrane potential lengthen free running period. J Comp Physiol A 161:347–354

McMahon DG, Wallace SF, Block GD (1984) Cellular analysis of the *Bulla* ocular circadian pacemaker system: Neurophysiological basis of circadian rhythmicity. J Comp Physiol A 155:379–385

Portzehl H, Caldwell PC, Ruegg JC (1964) The dependence of contraction and relaxation of muscle fibers from the crab *Maia squinado* on the internal concentration of free calcium ions. Biochim Biophys Acta 79:581–591

Roberts MH, Block GD (1983) Mutual coupling between the ocular circadian pacemakers of *Bulla gouldiana*. Science 221:87–89

Roberts MH, Block GD (1986) Analysis of mutual circadian pacemaker coupling between the two eyes of *Bulla*. J Biol Rhythms 1:55–75

Rusak B, Groos G (1982) Suprachiasmatic stimulation phase shifts rodent circadian rhythms. Science 215:1407–1409