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Calcium channels mediate phase shifts of the *Bulla* circadian pacemaker

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Summary. 1. Light-induced phase advances of the activity rhythm of the *Bulla* ocular circadian pacemaker are blocked when the extracellular calcium concentration is reduced with EGTA to 0.13 μ*M*. Phase advances are also blocked in low calcium solutions without EGTA ([Ca] < 50 μ*M*).

2. The dependence of light-induced phase delays on extracellular calcium concentration in EGTA-free seawater was determined. Phase delays are blocked at calcium concentrations below 400 μ*M*, and reduced at concentrations of 1 m*M* and 3.5 m*M* (relative to shifts in normal ASW, [Ca] = 10 m*M*). Phase delays are also reduced and blocked at calcium concentrations higher than normal (60 m*M* and 110 m*M*, respectively).

3. Low calcium EGTA also blocked both phase delays and phase advances induced by pulses of depolarizing high K^+ seawater. Low calcium EGTA pulses presented alone at the same times did not generate significant phase shifts.

4. The organic calcium channel antagonists verapamil, diltiazem and nitrendipine as well as the inorganic calcium channel antagonists La^{3+}, Co^{2+}, Cd^{2+}, and Mn^{2+} were applied along with light pulses, however, the treated eyes were either phase shifted by these substances, or these substances were found to be toxic.

5. The inorganic calcium channel antagonist Ni^{2+} blocked both light-induced phase delays and advances at a concentration of 5 m*M*. Ni^{2+} applied alone did not generate significant phase shifts. Phase delays induced by high K^+ seawater were blocked in the presence of 50 m*M* Ni^{2+} but not in 5 m*M* Ni^{2+}. The light-induced CAP activity of the putative pacemaker cells was not inhibited by Ni^{2+}, suggesting that its blocking action was probably via its known role as a calcium channel antagonist.

Introduction

The role of biological clocks in timing behavior and physiology is well established; however, efforts to understand the cellular basis of these oscillators are just beginning to provide information about the mechanisms underlying rhythm generation and its entrainment by environmental cycles (Jacklet 1984).

The eye of the mollusc *Bulla gouldiana* is proving to be an exceptional preparation for studying the basis of circadian rhythmicity, since a circadian pacemaker resides within the retina, specifically within a group of electrically coupled cells located at the base of the retina (Block and Wallace 1982; Block and McMahon 1984; Jacklet and Colquhoun 1983). These basal retinal neurons (BRN's) are effectively independent from the 'photoreceptor layer' (save for an inhibitory input) and are themselves apparently photoreceptive (Block and McMahon 1983, 1984; Block et al. 1984). They express a circadian rhythm in compound action potentials (CAP's) which can be recorded from the optic nerve in vivo (Block and Davenport 1982) or in vitro (Block and Wallace 1982) and can be phase shifted with light pulses. In addition, intracellular recordings from BRN's in isolated eyes reveal a circadian modulation in membrane potential which appears responsible for the observed rhythm in CAP frequency (McMahon et al. 1984).

In order to elucidate the mechanisms underlying rhythm generation and its entrainment by light cycles, we have adopted the strategy of identifying

Abbreviations: *CAP* compound action potential; *BRN* basal retinal neuron

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in serial fashion the elements in the pathway by which light entrains the *Bulla* ocular pacemaker. This strategy has been successful in identifying components of entrainment pathways in the similar system of *Aplysia* (Eskin 1979) and this approach should ultimately lead to the identification of processes involved in the generation of the circadian oscillation.

It is now known that depolarization of the basal retinal neurons, whether achieved through intracellular current injection into BRN's, elevated extracellular K^+ concentration, or by illumination, can generate phase shifts of the circadian rhythm (McMahon and Block 1987). Furthermore, light-induced phase shifts are blocked by preventing the change in membrane potential associated with illumination, suggesting that membrane depolarization is a critical process in the light entrainment pathway.

Attempts to identify additional processes in the phase shifting pathway reveal that light-induced phase delays are blocked in EGTA buffered artificial seawater (ASW) solutions with low calcium concentration ([Ca]) = 0.13 μM), suggesting that extracellular calcium is necessary for light-induced phase shifts of the *Bulla* ocular rhythm (McMahon and Block 1987). In this study we have further quantified the requirement for extracellular calcium in depolarization and light-induced phase shifts and provide the first evidence that calcium channels are involved in mediating phase shifts of the *Bulla* ocular pacemaker.

Methods

Bulla gouldiana were obtained from Alacrity Marine Supply, Redondo Beach, California, and maintained in a temperature controlled seawater tank at 15 °C. Animals were exposed to a light cycle of 12 h of light and 12 h of darkness (LD 12:12) for at least one week prior to experimental set-up. Two hours before onset of darkness, animals were immobilized with an injection of 10 ml of isotonic $MgCl_2$, and then placed on ice for dissection.

For extracellular recordings, both eyes including the optic nerves were removed from each animal and placed in separate dishes of artificial seawater (ASW, 20 ml per dish), with one eye from each animal serving as the control for the contralateral one. The composition of ASW was 395 mM NaCl, 10 KCl, 10 $CaCl_2$, 50 $MgCl_2$, 28 Na_2SO_4, 30 Hepes buffer, 100000 units penicillin/l and 100000 µg streptomycin/l. The optic nerve from each eye was pulled up into a seawater-filled micropipette suction electrode mounted on a recording dish which was then placed in a light-tight recording chamber and maintained at 15 °C for the duration of the experiment.

For phase shifting treatments, one peak of activity of the experimental and control eyes was recorded to assess any phase difference between them and to ensure that the rhythms were properly entrained. The experimental pulses were applied prior to the second peak of activity, either in the early subjective night as the CAP activity is decreasing, or in the late subjective night just before and as CAP activity begins. For these experiments clock time was used as an approximation of circadian time to determine the phase of experimental pulse treatments. Therefore, pulses delivered at CT 13–16 and CT 21–24, for early and late subjective night respectively, are in fact occurring at a slightly later real circadian time since the period of the in vitro *Bulla* ocular rhythm is on the average slightly less than 24 h.

In order to quantitatively evaluate phase shifts, the phase difference between activity rhythms for experimental and control eyes for a given pulse treatment was taken as the phase difference on the third cycle (the second cycle after the treatment), less the phase difference from the first (pretreatment) cycle. Since eyes from the same animal have very similar rhythms, the phase difference of the first cycle was typically small. Recording was continued for at least 6 days before termination of an experiment. Light pulses were delivered by two green light emitting diodes (LED's) fixed at a distance of 8 cm from the eyes. The measured light intensity was approximately 22 lux.

For each solution exchange, at least 95% of the volume of the dish was first removed and 20 ml of the exchanging solution (maintained at 15 °C) infused. At the beginning of the pulse, one exchange of the experimental treatment solution was used for phase shifting agents and three exchanges were used for solutions with different calcium concentrations. At the end of the pulse 3 exchanges of ASW with 0.1% DMSO were followed by 2 exchanges of ASW for treatments with agents dissolved in 0.1% DMSO, and 5 exchanges of ASW were used for all other treatments. For treatments where agents were applied in order to block phase shifting pulses, the blocking treatment was applied 10 to 15 min prior to the start of the phase shifting pulse, and removed about 10 min after the phase shifting treatment was terminated.

Hydrophobic compounds (nitrendipine, diltiazem, verapamil) were first dissolved in DMSO which was then dissolved in ASW to a final DMSO concentration of 0.1% (unless otherwise indicated). The composition of ASW solutions with alterations in ion concentrations is summarized in Table 1; these were prepared with the concentrations of Na_2SO_4, Hepes buffer, penicillin and streptomycin normally used in ASW. All other solutions were prepared using ASW with the added compound of interest. The high K^+ treatment used in the EGTA experiments was 50 mM KCl with osmolarity adjusted by lowering NaCl concentration (Table 1); phase shifts to high K^+ (50 mM KCl) were often variable with some eyes failing to shift and the majority exhibiting larger shifts than those generated by light pulses in this study, furthermore, during high K^+ (50 mM) treatment there was no CAP activity. For the Ni^{2+} experiment a preferred solution of high K^+ was used which was composed of 40 mM KCl in a hyperosmolar solution; phase shifts to high K^+ (40 mM) exhibited more regularity, and similar to that observed with light application, continuous CAP activity was observed during the pulse. Although the 40 mM high K^+ solution was hyperosmolar, the average phase shift to this treatment was still identical to that for the 50 mM high K^+ treatment (compare Fig. 5 and Fig. 8).

Extracellulary recorded CAP's were recorded on a Grass polygraph and fed to an analog to digital converter where a computer program counted them into 15 min bins. The phase relationship between the daily peaks of activity for each pair of eyes was determined by comparing the time of occurrence of the half maximum spike frequency on the rising phase of each daily cycle of activity. In order for data to contribute to the study, both eyes were required to complete four cycles of activity, where an acceptable cycle was defined as one which

had at least one bin of 8 events (a minimum frequency of 16 impulses per 0.5 h). All statistics in the text for average phase shifts are given in 95% confidence intervals, whereas average phase shifts in the figures are presented with standard errors.

Results

Blockage of light-induced phase shifts

A 3 h light pulse is capable of generating a phase delay or phase advance of the CAP rhythm depending on the time of application. In ASW with reduced extracellular calcium light-induced phase shifts were blocked.

A light pulse applied at CT 21–24, in the late subjective night, generated a phase advance of the circadian rhythm which remained stable throughout the subsequent cycles of activity (Fig. 1A). Enhanced CAP activity during the 3 h light pulse is evident in the record from the experimental eye (solid trace) at the beginning of the second peak of activity.

Unlike the response observed in normal ASW, when a light pulse is delivered to an eye in EGTA

Table 1. Composition of ASW solutions with concentrations of components in m*M*. All solutions also included 28 m*M* Na_2SO_4, 30 m*M* Hepes buffer, 100000 units penicillin/l, 100000 µg streptomycin/l and were adjusted to a pH 7.8 unless otherwise indicated in the text

Solution	NaCl	MgCl	KCl	CaCl	Other
ASW	395	50	10	10	–
EGTA ASW	419	50	10	5	10 EGTA
High K	383	50	50	10	–
High K + EGTA	379	50	50	5	10 EGTA
Low Ca	395	50	10	0	–
0.1 m*M* Ca	395	50	10	0.1	–
0.4 m*M* Ca	395	50	10	0.4	–
1.0 m*M* Ca	395	50	10	1.0	–
3.5 m*M* Ca	395	50	10	3.5	–
60 m*M* Ca	395	50	10	60	–
110 m*M* Ca	395	50	10	10	–
100 m*M* $MgCl_2$	395	100	10	10	–
150 m*M* $MgCl_2$	395	150	10	10	–
5 m*M* $NiCl_2$	395	50	10	10	5 $NiCl_2$
Hi K (40 m*M*)	395	50	40	10	–
HiK + Ni − Mg	395	0	40	10	50 $NiCl_2$
Ni − Mg	395	0	10	10	50 $NiCl_2$
HiK + Ca − Mg	395	0	40	60	–
Ca − Mg	395	0	10	60	–

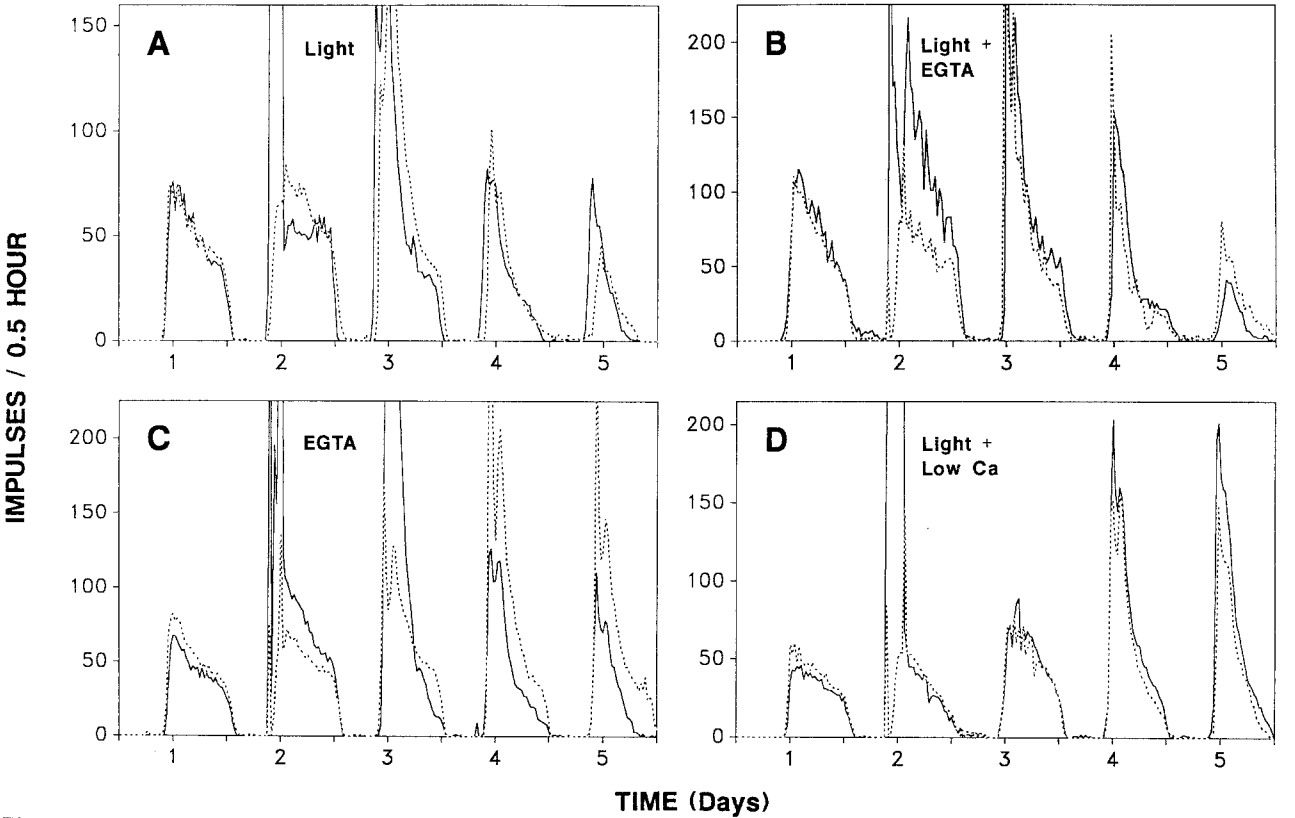

Fig. 1A–D. Records of CAP activity rhythms. In each plot the vertical axis is impulse frequency in impulses per half hour, and the horizontal axis is time in days where the numbered tick marks represent projected dawns. The solid trace is the experimental eye and the broken trace the control eye from the same animal. All panels are records where pulses were delivered in the late subjective night (CT 21–24); solution exchange artifacts at the beginning of the second peak indicate the time of the pulse. **A** A pulse of light alone where a phase advance is evident; **B** light in the presence of EGTA ASW; **C** EGTA ASW alone; **D** light in the presence of low calcium ASW without EGTA. No significant phase shifts appear in **B, C** and **D**

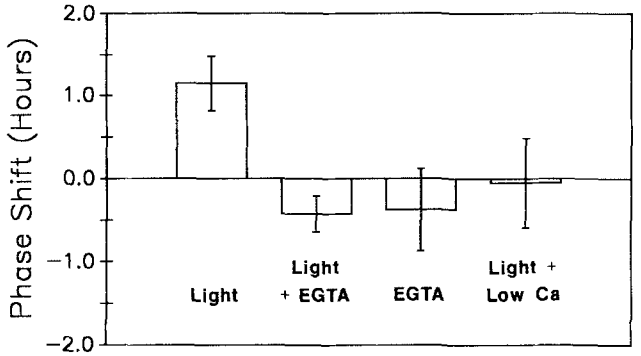

Fig. 2. Average phase shifts plotted with standard errors to pulses in the late subjective night (CT 21–24) where positive phase shifts are advances and negative phase shifts are delays. From left to right are values for treatments to light alone, light in the presence of EGTA ASW, EGTA ASW alone, and light in the presence of low calcium ASW without EGTA

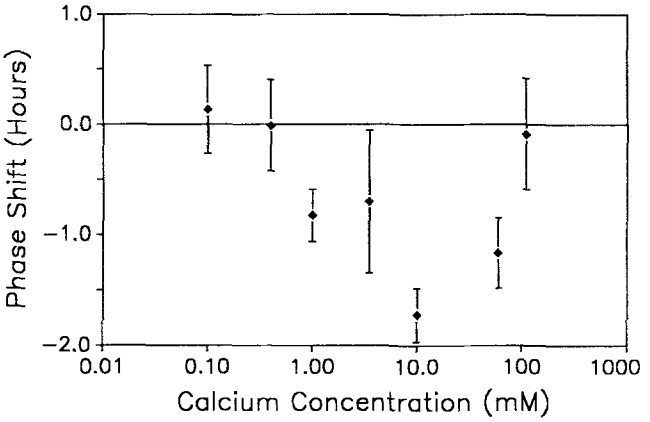

Fig. 3. Average light-induced phase shifts (in hours, delay shifts are negative) plotted with standard errors as a function of extracellular calcium concentration in m*M*

ASW ([Ca]=0.13 μ*M*), no phase advance was apparent (Fig. 1 B); in fact, a small phase delay was observed. The eyes were still responsive to light in EGTA ASW since the CAP activity increased during the pulse (in treatments with solution exchanges the pulses are bounded on either side with electrical artifacts due to the exchange process). In order to control for the possible effects of the EGTA ASW itself on the pacemaker, a pulse of EGTA solution alone was applied at the same time (Fig. 1 C). No phase difference was observed between experimental and control eyes. Finally, to control for any unwanted intracellular effects of EGTA, a light pulse was delivered in ASW which was prepared without the addition of calcium or EGTA (Low Ca^{2+}, Table 1); Figure 1 D displays a record of such an experiment where it is clear that there is no resultant phase shift of the light pulse whereas the sensory response of the eye to light is still apparent.

Statistical summary data for all the above treatments is represented with standard errors in Fig. 2. Light phase advances averaged approximately 1 h (+1.1 h, ±0.3 h, 95% C.I., $n=9$), whereas EGTA ASW with or without light generated small insignificant phase delays (−0.4 h, ±0.2, $n=5$ and −0.4 h, ±1.5, $n=5$, respectively). No phase advances were apparent following light pulses in the presence of seawater prepared without calcium or EGTA (−0.1 h, ±0.5, $n=4$).

[Ca]-dependence of light-induced phase shifts

In order to determine the relationship between extracellular calcium concentration and phase shifts, light pulses were delivered in the early subjective night (CT 13–16) to eyes in the presence of ASW containing various concentrations of $CaCl_2$ ranging from 100 μ*M* to 110 m*M* (normal calcium concentration 10 m*M*). The calcium concentrations of the solutions were verified following the treatments by atomic absorption spectroscopy.

The summary phase shifting data is plotted with standard errors in Fig. 3. At low calcium concentrations of 100 μ*M* and 400 μ*M* phase shifts were entirely blocked (+0.1 h, ±0.9, $n=6$ and 0.0 h, ±1.0, $n=6$, respectively). At concentrations of 1.0 and 3.5 m*M* calcium phase shifts were still submaximal (−0.8 h, ±0.5, $n=8$ and 0.7 h, ±1.5, $n=7$, respectively), relative to the normal shift of −1.7 h in normal 10 m*M* calcium ASW (±0.5, $n=9$). As calcium concentration was increased to 60 m*M*, phase shifts were again reduced in size, and at 110 m*M* calcium phase shifts were completely blocked (−1.2 h, ±0.8, $n=5$ and −0.1 h, ±1.2, $n=5$, respectively).

At lower than normal concentrations of calcium, the light-induced CAP activity was not inhibited, and in fact was increased and appeared with a distinctive bursting pattern. At 60 m*M* calcium the light-induced CAP activity was greatly reduced, a trend which was continued in 110 m*M* calcium where no CAP's were observed during the light pulse.

Blockage of depolarization-induced phase shifts

In addition to light-induced phase shifts, we found that depolarization-induced phase shifts to pulses of high K^+ (50 m*M* KCl) were similarly blocked in the presence of EGTA.

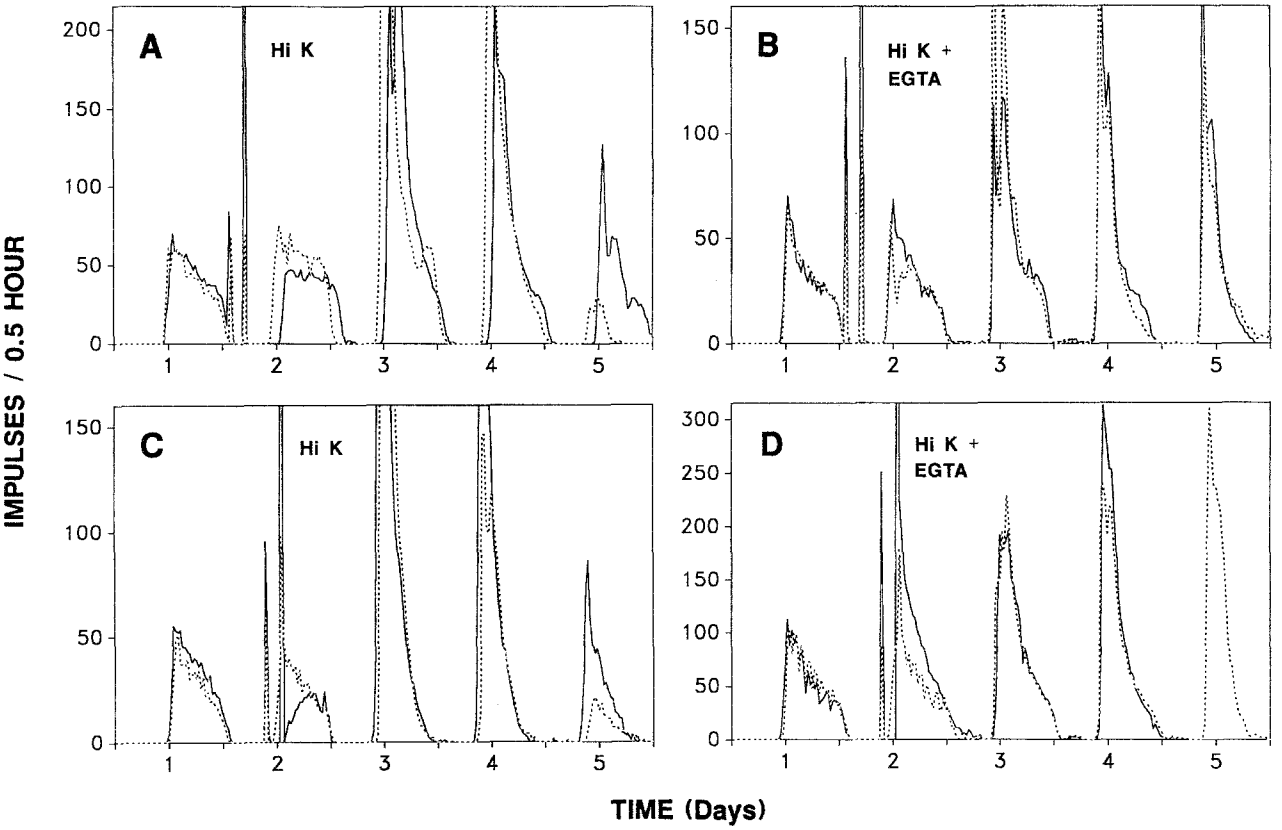

Fig. 4A–D. Plots of CAP activity records for high K^+ (50 m*M*) pulses displayed as in Fig. 1 with solid traces as the experimental eyes and broken traces the controls. **A** high K^+ alone at CT 13–16 resulting in a phase delay; **B** high K^+ in EGTA ASW at CT 13–16 resulting in no phase shift; **C** high K^+ alone at CT 21–24 resulting in a phase advance; **D** high K^+ in EGTA ASW at CT 21–24 resulting in no phase shift. Only solution exchange artifacts appear at the time of the pulses in all records

Figure 4A shows an activity rhythm of an eye which received a high K^+ pulse at CT 13–16. The phase delay following the treatment is readily apparent. When the calcium concentration of the high K^+ ASW was reduced with EGTA, the above treatment no longer generated a phase shift (Fig. 4B). Similar results were observed for high K^+-induced phase advances. As displayed in Fig. 4C, a high K^+ pulse at CT 21–24 generated a phase advance whereas a high K^+ pulse with EGTA did not (Fig. 4D). During all high K^+ treatments the eyes did not generate CAP activity regardless of whether EGTA was present.

Average phase shifts plotted with standard errors for the above treatments are represented in Fig. 5. The phase delay to a high K^+ pulse applied in the early subjective night (-1.3 h ± 0.5, $n=8$) was blocked in the presence of EGTA (-0.1 h, ± 0.2, $n=6$). (EGTA alone at this phase did not phase shift; ± 0.2 h, ± 0.5, $n=3$.) Similarly, the phase advance to a high K^+ pulse applied in the late subjective night ($+1.0$ h, ± 0.3, $n=8$) was also blocked in the presence of EGTA (-0.3 h, ± 0.4, $n=8$).

Phase shifts and calcium channel antagonists

To assess the involvement of calcium channels in mediating phase shifting, a variety of organic and inorganic calcium channel antagonists were applied concurrently with phase-shifting light pulses in an attempt to block phase shifts.

The organic calcium channel antagonist verapamil when applied with light at a concentration of 100 μ*M* did not block light-induced phase delays ($n=5$). At 200 μ*M*, verapamil treatment resulted in mostly irreversible inhibition of CAP activity; of 4 eyes tested only one survived the treatment and a light phase delay was still evident. Attempts to block light-induced phase advances with 100 μ*M* verapamil also lead to irreversible inhibition of CAP activity.

The trivalent inorganic calcium channel blocker La^{3+} resulted in irreversible inhibition of CAP

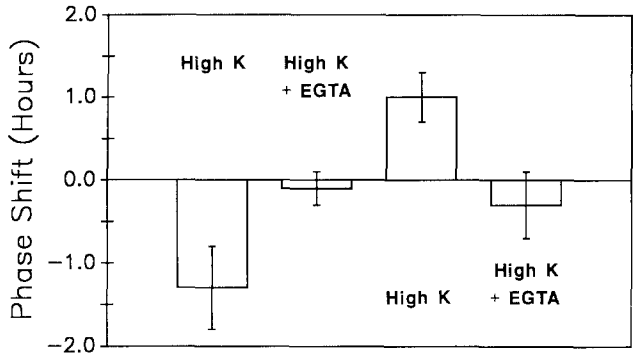

Fig. 5. Average phase shifts for high K^+ (50 m*M*) pulses plotted with standard errors. From left to right are a phase delay to high K^+ alone at CT 13–16, no phase shift to high K^+ in EGTA ASW at CT 13–16, a phase advance to high K^+ alone at CT 21–24, and small but insignificant phase delay to high K^+ in EGTA ASW at CT 21–24

activity or severe degradation of the subsequent activity peaks when applied in combination with light in ASW at pH 7 at 1 m*M* ($n=6$) or 500 μ*M* ($n=2$), or when applied alone during the subjective day when CAP activity is high (1 m*M*, $n=2$). When applied alone in the early subjective night when CAP activity is low, the activity following 1 m*M* treatment in pH 7 ASW was normal with no resultant phase shifting (1 m*M*, $n=2$); in normal pH 7.8 ASW La^{3+} alone at the same time was irreversible. Both spontaneous and light-induced CAP activity was severely reduced in the presence of La^{3+}.

Phase delays were evident when light pulses were applied (CT 13–16) in the presence of 1, 5 and 15 m*M* $CoCl_2$ and $MnCl_2$, respectively ($n=2$ each); at the higher concentrations the phase delays appeared progressively larger than those of light alone. Both Co^{2+} and Mn^{2+} reduced spontaneous CAP activity but had less effect on light-induced activity.

In contrast to the effects on CAP activity observed with La^{3+}, Co^{2+} and Mn^{2+}, the metals Cd^{2+} and Ni^{2+} applied alone induced CAP activity with increasing concentrations, and when applied with light modified the light-induced activity to a persistent bursting pattern similar to that observed with low calcium.

Irreversible inhibition of CAP activity or severe reduction and distortion of CAP frequency waveforms followed early subjective night treatments of Cd^{2+} under the following conditions: (a) with light in normal ASW at concentrations of 1 μ*M* ($n=4$) and 5 m*M* ($n=2$), (b) with light in normal ASW followed by a wash with pH 7.0 ASW at a concentration of 1 m*M* ($n=2$), (c) with light in pH 7.0 ASW at concentrations of 2 m*M* ($n=2$) and 5 m*M* ($n=2$), and (d) alone in pH 7.0 ASW at a concentration of 5 m*M* ($n=2$). Unlike Cd^{2+} and other treatments, Ni^{2+} treatments did not affect the survivability of subsequent cycles of activity and generated only minimal distortions in peak waveforms. Ni^{2+} was effective at blocking light-induced phase shifting. At a concentration of 10 m*M* it blocked phase shifts to light pulses applied in the early ($n=2$) and late ($n=1$) subjective night, and did not generate phase shifts of its own at the same phases ($n=2$ for each). Ni^{2+} was also effective at 5 m*M*. Figure 6A illustrates a phase delay to a light pulse in the early subjective night (CT 13–16). The phase delay was blocked in another preparation in Fig. 6B in the presence of 5 m*M* Ni^{2+}. No phase shift was apparent when Ni^{2+} was applied alone at the same phase (Fig. 6C).

Summary data is presented in Fig. 7 (with standard errors) for 5m*M* Ni^{2+} treatments. Light-induced phase delays (−1.7 h, ±0.2, $n=9$) were reduced to insignificant delays in the presence of Ni^{2+} (−0.3 h, ±0.2, $n=8$), and Ni^{2+} alone at the same phase generated no significant phase shift (−0.1 h, ±0.4, $n=5$). Light-induced phase advances (±1.1 h, ±0.3, $n=9$) were substantially reduced by Ni^{2+}, however the average phase shift for Ni^{2+} with light had a large standard error and the 95% confidence interval overlapped the average phase shift of light alone (+0.2 h, ±1.2, $n=7$). The average phase shift for treatments of Ni^{2+} alone at this phase also had a large standard error, and furthermore, there was an apparent small phase delay (−0.6 h, ±1.2, $n=7$).

To control for the variability in phase shifting with Ni^{2+} during the late subjective night, control eyes were also treated with Ni^{2+}. The average phase shift from experiments where both eyes were given 5 m*M* Ni^{2+} and the experimental eye a light pulse in the late subjective night was an insignificant −0.2 h (±0.3 h 95% C.I., $n=6$), indicating that 5 m*M* Ni^{2+} is indeed effective in blocking light-induced phase advances.

Unlike light-induced phase shifts, phase delays induced by treatments with high K^+ (40 m*M* KCl, CT 13–16) were not blocked in the presence of 5 m*M* Ni^{2+} ($n=2$). High K^+ phase delays were also not blocked at 10 m*M* or 25 m*M* Ni^{2+} ($n=2$ each) and it was necessary to increase the concentration to 50 m*M* Ni^{2+} before high K^+ phase shifts were substantially reduced ($n=2$). At this concentration it was necessary to control for the possibility that the high divalent cation concentration effect of Ni^{2+} or the hyperosmolarity was blocking phase

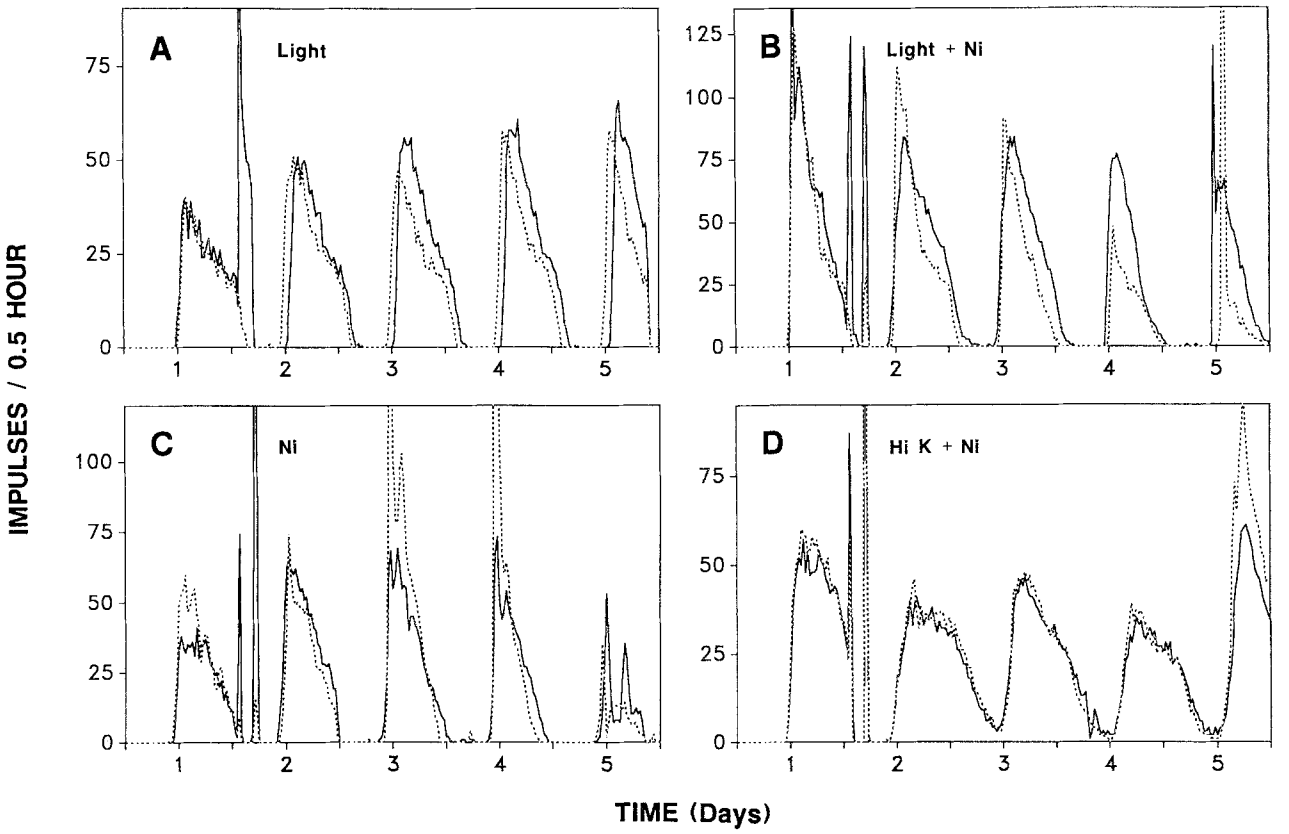

Fig. 6A–D. Plots of CAP activity records to phase-shifting and/or Ni^{2+} treatments at CT 13–16. **A** a pulse of light alone results in a phase delay; **B** in the presence of 5 m*M* $NiCl_2$ a light pulse results in no phase delay; **C** a pulse of 5 m*M* $NiCl_2$ alone yields no phase shift; **D** a pulse of high K^+ (40 m*M*) to the experimental eye where both eyes are in 50 m*M* $NiCl_2$, Mg-free ASW yields no phase shift

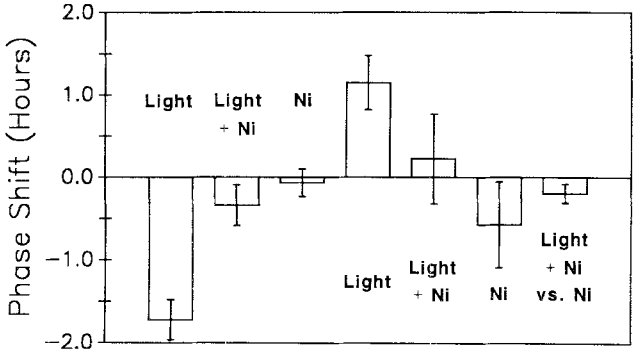

Fig. 7. Average phase shifts plotted with standard errors to light and/or 5 m*M* $NiCl_2$ treatments. From left to right are light alone (CT 13–16), light with $NiCl_2$ (CT 13–16), $NiCl_2$ alone (CT 13–16), light alone (CT 21–24), light with $NiCl_2$ (CT 21–24), $NiCl_2$ alone (CT 21–24), and light with $NiCl_2$ where the control eye also received $NiCl_2$ (CT 21–24)

shifting. Therefore, all subsequent 50 m*M* Ni^{2+} treatments were applied in ASW that was prepared without the usual 50 m*M* $MgCl_2$. This keeps the total divalent cation concentration constant, and the osmolarity normal for the control eyes and slightly higher for the high K^+ solutions (see Table 1). Also, due to the distortion of subsequent peaks of activity, Ni^{2+} in Mg^{2+}-free ASW was applied to the control eyes as well.

It is evident that the high K^+ treatment applied in the early subjective night (CT 13–16) does not phase shift the rhythm in the presence of 50 m*M* Ni^{2+} (Fig. 6D). The Ni^{2+} treatment resulted in significant distortions of subsequent peaks in both experimental and control eyes. Relative to typical peak waveforms, Ni^{2+} treatments tended to broaden and 'smooth' the peaks towards a more sinusoidal type waveform where spontaneous CAP activity did not drop to zero during the subjective night as it usually does (compare Fig. 6D to Fig. 6A).

The phase shift induced by high K^+ alone in normal ASW (40 m*M* KCl, CT 13–16) averaged −1.2 h (±0.6, $n=8$) and is illustrated in Fig. 8. In comparison, the high K^+ phase shift is substantially reduced in ASW where the $MgCl_2$ has been replaced with $NiCl_2$ for both the experimental and control eyes (−0.3 h, ±1.2, $n=6$). Although we

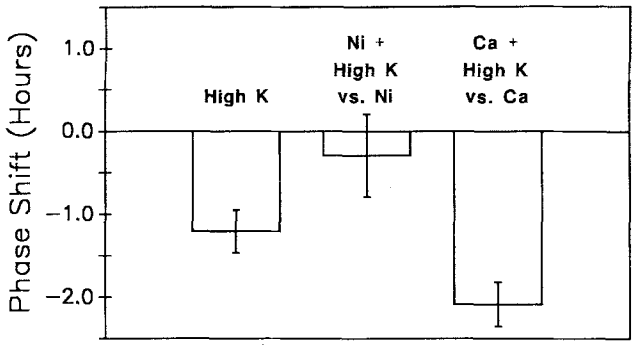

Fig. 8. Average phase shifts plotted with standard errors to high K^+ (40 m*M*) treatments delivered at CT 13–16. From left to right are high K^+ alone, high K^+ with 50 m*M* Ni^{2+} in Mg^{2+}-free ASW where the control eye also received 50 m*M* Ni^{2+} in Mg^{2+}-free ASW, and high K^+ with 60 m*M* Ca^{2+} in Mg^{2+}-free ASW where the control eye also received 60 m*M* Ca^{2+} in Mg^{2+}-free ASW

are confident that the high K^+ phase shifts are indeed blocked with 50 m*M* Ni^{2+}, the phase shifts with 50 m*M* Ni^{2+} and high K^+ treatments showed high variability and the 95% confidence interval overlapped the average phase shift to high K^+ alone.

To control for the consequences of using ASW with the $MgCl_2$ replaced with another divalent cation a pulse of high K^+ ASW with the $MgCl_2$ replaced with 50 m*M* calcium (total [Ca] = 60 m*M*) was applied to the experimental eye, while a pulse of $MgCl_2$-free high calcium ASW was applied to the control eye (CT 13–16). In this case the phase delay to high K^+ (−2.1 h, ±0.6, $n=6$) was in fact larger than that for high K^+ in normal ASW (Fig. 8), and certainly significantly different from the high K^+ treatment with 50 m*M* Ni^{2+}.

The effectiveness of Mg^{2+} as a blocker of light-induced phase shifts was also examined. The concentration of $MgCl_2$ in ASW was increased from 50 m*M* to 100 m*M* and 150 m*M*. Spontaneous CAP activity was inhibited with both concentrations and light-induced CAP activity was reduced in 100 m*M* $MgCl_2$ ASW and completely inhibited in 150 m*M* $MgCl_2$ ASW. Phase shifts to light pulses applied in the early subjective night (CT 13–16) were blocked with both concentrations of $MgCl_2$ (−0.3 h, ±0.9, $n=5$ for 100 m*M* and −0.1 h, ±0.7, $n=7$ for 150 m*M*), and no significant phase shift to 150 m*M* $MgCl_2$ ASW alone was observed (−0.2 h, ±0.7, $n=6$).

Discussion

Extracellular calcium and phase shifting

McMahon and Block (1987) demonstrated that phase delays induced by a 2 h light pulse in the early subjective night (CT 14–16) are blocked when low calcium EGTA ASW ([Ca] = 0.13 μM) is present during the pulse, and that a pulse of low calcium EGTA ASW alone at the same time does not generate a phase shift. Furthermore, they noted that the blocking action of EGTA is not simply due to hyerpolarization sufficient to counteract the depolarizing effect of light, since the basal retinal neurons continue to spike in response to light in the presence of EGTA ASW (suggesting depolarization), and intracellular recordings from BRN's indicated that light-induced depolarization is actually enhanced.

In the current study we determined whether the blocking effect of EGTA is specific only to light-induced phase delays, or occurs at other phases of the circadian cycle as well. Light pulses were applied in the late subjective night (CT 21–24; a time when light pulses alone generate phase advances) in the presence of EGTA ASW. Phase advances were blocked (Fig. 2) suggesting that EGTA blocks all light-induced phase shifting. Furthermore, the fact that light-induced phase shifts were also blocked in low calcium ASW free of EGTA (Fig. 2) at calcium concentrations less than 400 μ*M* (Fig. 3) indicates that the effective blocking agent is the reduction of calcium and not the presence of EGTA.

In order to determine if the requirement for extracellular calcium is unique to light-induced phase shifting, phase shifting pulses of depolarizing high K^+ treatments were applied in the presence of EGTA low calcium ASW. Since phase shifts produced by pulses of high K^+ were blocked (Fig. 5), it suggests that the requirement for extracellular calcium in phase shifting is common to all depolarization-induced phase shifts.

Although, EGTA ASW alone in the late subjective night generated a small but statistically insignificant phase delay, it is not sufficient to account for the reduction in size of the light or high K^+-induced phase advances. Furthermore, light or high K^+ with EGTA ASW also generated a very similar small although insignificant phase delay.

The highest concentration of calcium that completely blocked phase delays in this study was 400 μM. In eyes of the closely related opisthobranch, *Aplysia*, phase shifts were blocked in EGTA ASW ([Ca] = 0.13 μ*M*) but were unchanged in EGTA ASW ([Ca] = 450 μ*M*) or in a high Mg^{2+} (125 m*M*)/low calcium (100 μ*M*) ASW solution (Eskin 1977). The calcium required for light-induced phase shifting in *Aplysia* in that study is lower than that observed here, but this difference may be due to the difference in animals, in light

intensities, and/or in the timing of the light pulses (3 h, CT 13–16 in this study versus 6 h, CT 18–24 in Eskin's study).

The increased CAP activity, in a multiple-CAP bursting pattern, with lowered calcium concentrations, and the decreased CAP activity in increased calcium concentrations has also been observed in the *Aplysia* eye (Eskin 1972, 1977; Jacklet 1973; Eskin and Corrent 1977). This effect of extracellular calcium concentration on neuronal excitability is well known and is likely due to the alteration of extracellular membrane surface charge due to the binding of divalent cations (Ca^{2+} and Mg^{2+}) to anionic sites on the membrane. An increase in extracellular divalent cation concentration causes a positive shift in the voltage dependence of ion channels and therefore an inhibition of spiking activity (Hagiwara and Takahashi 1967; Wilson et al. 1983).

Increasing the ASW calcium concentration to 60 m*M* reduced light-induced CAP activity and phase shifts, and a further increase to 110 m*M* eliminated both CAP activity and phase shifts (Fig. 3); the mechanism is likely via effects on surface charge blocking voltage dependent calcium channels. Increasing Mg^{2+} concentrations by identical amounts (from 50 m*M* to 100 m*M* and 150 m*M*) similarly inhibited light-induced CAP activity, as it does in the *Aplysia* eye (Jacklet 1973), but, unlike calcium, Mg^{2+} was effective at blocking phase shifting at both concentrations. The description of Mg^{2+} as a weak calcium channel blocker (Hagiwara and Byerly 1981) is consistent with this observation.

On the other hand, Wilson et al. (1983) have concluded that the blocking action of Mg^{2+} on calcium channels is through surface charge effects rather than through competitive binding at a channel site. If this were true, it would suggest that Mg^{2+} has a stronger surface charge effect in inhibiting calcium channel conductance than calcium does. However, high Ca^{2+} has been more effective than high Mg^{2+} at inhibiting bursting pacemaker potential activity, and therefore at least monovalent conductances, in *Aplysia* neurons (Barker and Gainer 1975). Clearly more information is necessary before any conclusions can be drawn concerning the mechanism of phase shift blockage by high Mg^{2+} in this study.

The solutions of high Ca^{2+} and high Mg^{2+} used were hyperosmolar (Table 1) with an increase of 300 mosm/l for the addition of 100 m*M* divalent salt. However, osmolarity itself is not sufficient to inhibit phase shifting since light-induced phase shifts were not blocked in the presence of ASW where the osmolarity was increased with 300 m*M* sucrose ($n=2$) or 200 m*M* NaCl ($n=2$) and high K^+-induced phase shifts were not blocked in the presence of ASW where the osmolarity was increased with 400 m*M* sucrose ($n=2$).

It should be noted that Eskin (1977) did not observe significant reductions in light-induced phase shifts in the *Aplysia* eye in solutions of high Mg^{2+}/low Ca^{2+} ([Mg]=125 m*M*, [Ca]=0.1 m*M*) and high calcium ([Mg]=50 m*M*, [Ca]=60 m*M*), although light-induced CAP activity was reduced. These discrepancies are possibly due to differences in the experimental preparations and treatments. In Eskin's study the NaCl concentration was reduced to maintain the osmolarity of normal ASW whereas in this study hyperosmolar solutions were used in the interest of maintaining normal NaCl concentration. Furthermore, if the observed reduction in phase shifting in solutions with high divalent cation concentrations is due to a decrease in the depolarizing effect of light, a higher light intensity may depolarize sufficiently to maintain phase shifting. This might account for the discrepancy with data in the *Aplysia* study since the light intensity in this study was lower.

Calcium channels and phase shifting

The organic calcium channel blockers did not appear to inhibit light-induced phase delays and were also toxic or irreversible; this was the case with verapamil and nifedipine (McMahon 1986). Diltiazem ($n=2$), as well as the dihydropyridine channel blocker nitrendipine ($n=2$) when applied with light resulted in atypically large phase delays suggesting that these compounds were generating their own effects on the pacemaker cells, possibily via their lipid solubility and consequent uptake into membranes or entry into the cells. It is also possible that the calcium channels in *Bulla* BRN's are relatively insensitive to organic blockers. Variable results have been reported as to their effectiveness in blocking invertebrate calcium channels (Hagiwara and Byerly 1981) and few neuronal calcium conductances appear to be affected by dihydropyridines (Godfraind et al. 1986).

Only one of the polyvalent cations tested, Ni^{2+}, proved to be effective at blocking light and depolarization-induced phase shifts. Although treatments of Ni^{2+} were relatively innocuous, the waveforms of subsequent peaks of CAP activity exhibited distortions following treatment with Ni^{2+} suggesting that it may either enter the cell or undergo some degree of irreversible binding. The concentration required to block light-induced phase shifting

(5 m*M*) is similar to that commonly required to block current through calcium channels. A higher concentration (50 m*M*) was required to block phase delays to high K^+ (40 m*M*), a concentration still within the expected range for calcium channel blockade, however, it is unclear why high K^+ phase shifts are more difficult to block. It should be noted that the preparation used in this study is the whole eye, and that a high K^+ treatment will depolarize not only BRN's, but all excitable membranes, possibly including terminals of efferent pathways to the pacemaker cells (Jacklet et al. 1987; Roberts and Block 1983, 1986).

The observation that only one of many calcium channel antagonists applied was useful and effective in blocking phase shifting opens up the possibility that the action of Ni^{2+} in blocking phase shifting may not be via its action as a calcium channel blocker, and so conclusions drawn concerning the role of calcium channels based on this evidence must be viewed with some degree of caution. A direct measurement of intracellular calcium is needed to validate the effects of Ni^{2+} on transmembrane calcium flux.

If Ni^{2+} is acting to block calcium channels, the failure of other inorganic blockers should be addressed. Although the trivalent cation La^{3+} is the most potent inorganic calcium channel blocker (Hagiwara and Byerly 1981) its application resulted in irreversible inhibition of CAP activity. La^{3+} effects were reversible upon wash-out with pH 7 ASW (Okamoto et al. 1976) but only when the eyes were not undergoing spontaneous or light-induced spontaneous activity. It is possible that the presence of CAP activity renders La^{3+} binding irreversible, and/or that the CAP activity promoted the entry of La^{3+}. In *Aplysia* eyes La^{3+} is believed to act on intracellular Ca^{2+} transport to generate a lengthening of period when the eyes are continuously bathed in a low concentration (5 μ*M*) (Woolum and Strumwasser 1983). Since the La^{3+} pulses were 3 h in length at a much higher concentration in this study (1 m*M*), intracellular confounding effects of La^{3+} are likely. Similar effects on period in *Aplysia* have been observed with Mn^{2+} (Woolum and Strumwasser 1983), and phase delays to Mn^{2+} application have been observed in both *Bulla* (Roberts and Block 1983) and *Aplysia* (Eskin and Corrent 1977). Since Mn^{2+} is capable of crossing cell membranes its effects on the *Aplysia* pacemaker are believed to be intracellular (Woolum and Strumwasser 1983; Eskin and Corrent 1977).

The effect of Cd^{2+} on CAP activity was very similar to that of Ni^{2+} but the irreversible distortions of CAP activity waveforms precluded its usefulness. Since some reversibility was obtained by wash-out with ASW pH 7 it may be that Cd^{2+} binding is somewhat irreversible as La^{3+} is. However, Cd^{2+} (as well as Mn^{2+}) has also been observed to pass through Ca^{2+} channels (Fukuda and Kawa 1977) opening up the possibility that Cd^{2+} also exerts its confounding effects intracellularly.

It is possible that the failure of Mn^{2+}, Co^{2+}, and Cd^{2+} to block phase shifts is that light facilitates their entry into the cell where they exert their own delays, irrespective of whether they block the light-induced phase delaying mechanism by blocking the entry of calcium. The 3 h treatment is much longer than is used in studies of the calcium channel blocking effects of these divalent cations. It is unknown whether passage of these ions across the cell membrane occurs with long term exposure.

The success of Ni^{2+} in blocking phase shifts over other divalent cations may be due to several features: (a) it is possible that Ni^{2+} is substantially more effective than other divalent cations in blocking the calcium channels in BRN's, Akaike et al. (1978) have found Ni^{2+} to be substantially more effective than Cd^{2+} and Co^{2+} in reducing calcium currents in *Helix* neurons by 2 orders of magnitude in concentration, (b) the entry of Ni^{2+} into the cell, whether through calcium channels or otherwise, is less than for the other divalent cations, (c) the intracellular effects of Ni^{2+} are quantitatively or qualitatively different than the other ions, and/or (d) the binding of Ni^{2+} to the membrane or the calcium channels is not as irreversible as for the other divalent cations.

Calcium and the light phase shifting pathway

Assuming that calcium channels do mediate the entry of calcium ions and phase shifting, the mechanism for light and high K^+-induced phase shifting in the *Bulla* ocular pacemaker can be represented as the phase shifting pathway in Fig. 9. In this scheme, both light and high K^+ generate phase shifts by first depolarizing the BRN membrane. The membrane depolarization then activates voltage sensitive calcium channels and extracellular calcium enters the cell increasing the intracellular calcium concentration. Depolarization is necessary for the generation of light-induced phase shifts and is sufficient for the generation of light-type phase shifts, whereas calcium is necessary, but may or may not be sufficient. Current efforts in our laboratory are directed at evaluating whether calcium influx alone is sufficient to generate phase shifts.

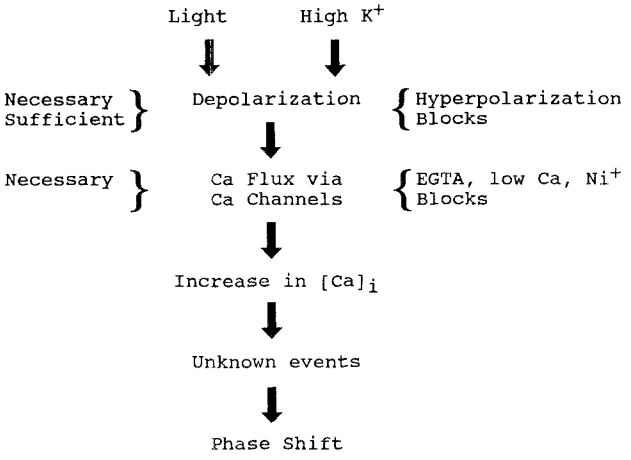

Fig. 9. Poposed sequence of events mediating light and high K^+-induced phase shifts of the *Bulla* ocular circadian rhythm

Changes in a transmembrane calcium flux may also be involved in phase shifts generated by hyperpolarization. Hyperpolarization of basal retinal neurons, either by direct current injection or by exposure of the retina to reduced extracellular sodium, generates phase shifts with phase delays in the early subjective day followed by advances in the late subjective day (McMahon and Block 1987). Recently, we have observed that similar phase shifts can be obtained by exposing the eye to pulses of EGTA low calcium ASW (Block and Khalsa 1987; Khalsa and Block, in preparation). In addition, removal of extracellular calcium for extended periods leads to a lengthening of the free-running period (Ralph and Block, in preparation). These findings raise the intriguing possibility that the circadian rhythm in membrane potential which has been reported in basal retinal neurons (McMahon and Block 1987) leads to a persistent calcium flux during the subjective day when the plasma membrane is relatively depolarized and the absence of a calcium flux during the subjective night. Phase shifts during the subjective night would be effected by depolarizing the membrane, activating voltage sensitive calcium channels and increasing the level of intracellular calcium. Alternately, phase shifts during the subjective day would occur by reducing the 'diurnal' flux and thereby reducing intracellular calcium levels. Such a scheme, if proven accurate, would have utility in explaining the action of a number of phase shifting agents by their effects on a single variable, the transmembrane calcium flux.

Clearly, a calcium second messenger system is a likely subsequent step in the light phase shifting pathway since it commonly occurs in other cells (Rasmussen and Barrett 1984), however it is unlikely that calmodulin is involved since calmodulin antagonists have proved ineffective at blocking light-induced phase delays (Khalsa and Block 1987; Khalsa and Block, in preparation).

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