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Calcium plays a central role in phase shifting the ocular circadian pacemaker of *Aplysia*

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Abstract The eye of the marine mollusk *Aplysia californica* contains an oscillator that drives a circadian rhythm of spontaneous compound action potentials in the optic nerve. Both light and serotonin are known to influence the phase of this ocular rhythm. The aim of the present study was to evaluate the role of extracellular calcium in both light and serotonin-mediated phase shifts. Low calcium treatments were found to cause phase shifts which resembled those produced by the transmitter serotonin. However, unlike serotonin, low calcium neither increased ocular cAMP levels nor could these phase shifts be prevented by increasing extracellular potassium concentration. Low calcium-induced phase shifts were prevented by the simultaneous application of the translational inhibitor anisomycin and low calcium treatment resulted in changes in [^{35}S]methionine incorporation into several proteins as measured by a two-dimensional electrophoresis gel analysis. Finally, light treatments failed to produce phase shifts in the presence of low calcium or the calcium channel antagonist nickel chloride. These results are consistent with a model in which serotonin phase shifts the ocular pacemaker by decreasing a transmembrane calcium flux through membrane hyperpolarization while light-induced phase shifts are mediated by an increase in calcium flux.

Key words Aplysia · Calcium · Circadian · Light Serotonin

Abbreviations ASW artificial seawater
EGTA ethylene glycol-bis(β-amino-ethyl ester) N,N,N´ N´-tetraacetic acid
CAP compound action potential · CT circadian time
5-HT serotonin · Ni^{++} nickel

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Introduction

Circadian rhythms are endogenously generated oscillations with periods of approximately 24 h. In order to function adaptively, the circadian system must be synchronized (or entrained) to the 24-h period of the environment. Entrainment is thought to occur through daily phase advances and delays of the endogenous oscillator. By determining the elements in the signal transduction cascades by which environmental signals cause phase shifts, it should be possible to identify processes responsible for the generation of circadian oscillations. Thus, a major goal of circadian rhythm research is to understand the cellular/molecular mechanisms which mediate phase shifts of the circadian system.

When the eyes of the marine mollusk *Aplysia californica* are removed and placed in constant conditions, the frequency of spontaneous compound action potentials (CAPs) recorded from the optic nerve exhibits a circadian rhythm (Jacklet 1969). Serotonergic fibers innervate the eye of this animal (e.g. Takahashi et al. 1989) and the pulsatile application of both serotonin (5-HT) and light cause phase shifts of the ocular pacemaker in vitro (Eskin 1971; Corrent et al. 1978). Previous work suggests that 5-HT causes phase shifts through a cAMP-dependent membrane hyperpolarization while light acts through a cGMP-dependent depolarization (Eskin et al. 1982; Eskin and Takahashi 1983; Eskin et al. 1984a). The steps which occur after membrane potential changes are unknown although ultimately changes in protein synthesis may be involved (Eskin et al. 1984b; Yeung and Eskin 1987; Raju et al. 1990; Koumenis and Eskin 1992). In the related mollusk *Bulla gouldiana*, evidence suggests that a transmembrane calcium flux is a part of the phase shifting mechanism which occurs after changes in membrane potential (McMahon and Block 1987; Khalsa and Block 1988, 1990; Khalsa et al. 1993). The present study was designed to investigate the role of extracellular calcium in mediating both 5-HT and light-induced phase shifts of the circadian ocular rhythm of the marine mollusk *Aplysia*. The results demonstrate that

calcium plays an important role in phase shifting this ocular pacemaker.

The objectives of this study were to determine: (1) whether bath application of a low calcium/ethylene glycol-bis(β-amino-ethyl ester) N,N,N´,N´-tetraacetic acid (EGTA) buffered artificial seawater (ASW) would cause phase shifts of the circadian rhythm of CAP frequency recorded from the isolated eye of *Aplysia*; (2) if this treatment caused an increase in ocular cAMP content; (3) whether low calcium-induced phase shifts could be prevented by the application of a high potassium ASW or the protein synthesis inhibitor anisomycin; (4) if this treatment led to changes in the rate of [^{35}S]methionine incorporation into proteins; (5) whether application of low calcium ASW or the calcium channel blocker nickel (Ni^{++}) would prevent light-induced phase shifts.

The present study's central hypothesis is that 5-HT causes phase shifts by decreasing a calcium-flux following membrane hyperpolarization while light causes phase shifts by increasing a calcium-flux. If this hypothesis is correct, low calcium treatments should mimic 5-HT-induced phase shifts but should not increase cAMP levels, hyperpolarize the membrane, or cause phase shifts sensitive to high potassium ASW. In addition, low calcium ASW should alter amino acid incorporation and cause phase shifts which are inhibited by anisomycin. Finally, low calcium treatments would be expected to prevent light-induced phase shifts.

Methods

Animals

Aplysia californica were obtained from Alacrity Marine Supply (Redondo Beach, CA) and maintained in artificial seawater (ASW) at 15°C. Animals were entrained to a light-dark cycle (LD 12:12) for at least one week prior to experimental set-up. Two hours before the onset of darkness, animals were immobilized with an injection of isotonic $MgCl_2$ and then dissected.

Phase shifting experiments

For extracellular recordings, both eyes with their optic nerves were removed from each animal and placed in separate dishes of filtered (0.22 µm, Gelman) ASW. The composition of ASW was (in m*M*): 395 NaCl, 10 KCl, 10 $CaCl_2$, 50 $MgCl_2$, 28 Na_2S0_4, 30 Hepes buffer, 100,000 units/L penicillin and 100,000 µg/l streptomycin. The composition of low calcium ASW was the same as that of normal ASW except for a reduction of $CaCl_2$ to 5 m*M* and an addition of 10 m*M* EGTA. In this solution, the free calcium concentration was calculated to be 1.7×10^{-7} *M* ("Max Chelator" software, C. Patten, Hopkins Marine Station, CA). The optic nerve was sucked into a polyethylene tube embedded in Sylgard (Dow Corning) and a silver wire was inserted into the tube containing the nerve. The recording dish was placed in a light-tight recording chamber and maintained in darkness at 15°C for the duration of the experiment. Signals were amplified and recorded on a polygraph and the compound action potentials (CAPs) were counted by a computer. In these experiments, one eye from each animal served as the control for the contralateral (experimental) eye. 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 electrical activity.

The first cycle of CAP activity from experimental and control eyes was recorded prior to treatment to assess any phase difference between them and to ensure that the rhythms were properly entrained. The experimental treatments were applied prior to the second peak of activity, either in the late subjective day as the CAP activity was decreasing, or in the late subjective night just before and during the rising phase of CAP activity. Because the period of the ocular rhythm is close to 24.0 h, civil time was used as an approximation of circadian time to determine the phase of experimental pulse treatments.

Light treatments were delivered by a LED (green) with an intensity of 10 µW. Experimental solutions were made by adding compounds directly to ASW within 2 h prior to use. 5-HT, nickel chloride, and EGTA were purchased from Sigma Chemicals, St. Louis, MO. Solution changes were made without illumination of the eyes.

The effects of experimental treatments on the phase of the oscillation were calculated by measuring the phase difference between the experimental and control rhythms on the fourth cycle (the second cycle after treatment), less the phase difference from the first (pretreatment) cycle. By convention, positive values represent phase advances while negative values represent phase delays. Rhythms were recorded for at least 5 days before termination of an experiment to ensure that the phase shifts were stable. Phase shifts due to a treatment were considered to be significant if the 95% confidence interval of the group's mean did not overlap zero.

CAP frequency

Changes in CAP frequency were obtained by comparing the average number of CAPs/0.5 h during an experimental treatment with that of the untreated control eye from the same animal. The percent change was calculated with positive values representing increases in frequency and negative values decreases. The percent change per animal was then averaged to obtain the population responses shown.

Measurement of cAMP

Changes in the level of cAMP were obtained by comparing the amount of cAMP in an experimental and matched control group. Each group contained two eyes. The experimental protocol used in these experiments was the same as in the phase shifting experiments described above. After the experimental treatment, the eyes were placed into 0.1 *M* HCL for 30 min at which time they were sonicated for 30 s. This solution was then centrifuged to pellet the proteins and the supernatant was assayed. Levels of cAMP were determined by radioimmunoassay performed by the Diabetes-Endocrinology Research Center at University of Virginia (see Brooker et al. 1976 for description of method used).

Measurement of protein synthesis

In order to look for proteins in which the rate of amino acid incorporation was altered by experimental treatments, the technique of two-dimensional (2-D) gel electrophoresis was employed. The protocol was the same as described above with groups of three eyes exposed to the experimental treatment while contralateral eyes from the same animals served as controls. At the start of the treatment, 24 µCuries of radio-isotope [a mix of ^{35}S-methionine (75%) and ^{35}S-cysteine (15%) supplied by ICN,Irvine, CA] was used. At the end of the 6 h treatments, the eyes were homogenized in a Tris-HCl buffer (pH 7.4). Anderson's lysis buffer (2% each of SDS, glycerol, ampholine, CHAPS, and dithiothreitol) was added and the sample was heated at 95°C for 5 min. The sample was then frozen and stored at −80°C. These procedures were per-

formed under dim red light and the time required to prepare the samples never exceeded 15 min.

The amount of label incorporated into protein was determined using a standard trichloroacetic acid (TCA) precipitation protocol (Hames 1981). Neither low calcium nor 5-HT treatment had a significant effect on total protein synthesis as measured by TCA precipitation. By this same measure, anisomycin is known to cause a significant decrease (approximately 85%) in protein synthesis in the eye of *Aplysia* (Raju et al. 1990). For each experiment, a known amount of labelled protein (300,000 dpms) was then run on separate 2-D gels. The samples were run using pH 4–8 ampholines for the isoelectric focusing and then separated in the second dimension on a 12.3% polyacrylamide gel (modified procedure of O'Farrell 1975; Bollag and Edelstein 1991).

Labeled proteins were detected by autoradiography. To analyze the large number of detected proteins (>750), a computer assisted system (BioImage, Millipore, Ann Arbor, MI) was utilized. With this system, changes in the density of protein spots were measured by integrating optical density values of spot. In pilot studies, film was exposed for durations of one to three weeks and an exposure of 15 days was found to give the best range of optical density values for these gels. With this exposure time, none of the protein spots of interest appeared to saturate the optical density scale. Despite these efforts, it is quite possible that there is a non-linear relationship between the optical density measurements and the label associated with a protein spot in the gel. Thus, the changes in optical density values are most accurately viewed as approximations of experimental changes in incorporation of label. Any differences detected by the program were verified by direct visual inspection. To examine the relative abundance of individual proteins, additional 2-D gels were run and silver stained (procedure of Merril et al. 1984).

Analysis of data

Values are shown as means ±95% confidence interval (unless otherwise noted). For the phase shifting experiments, differences between treatment groups were evaluated using a 1-way ANOVA, followed by Newman-Keul's multiple comparison procedure. For the other studies, differences between experimental and control groups were determined using a paired *t*-test. Values were considered significantly different if $P<0.05$.

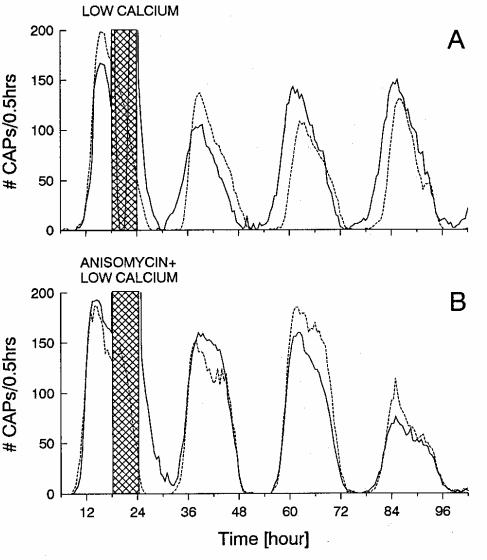

Fig. 1A,B Plots of CAP activity as a function of time. **A** A phase advance which resulted when a low calcium (5 m*M*)/EGTA (10 m*M*) solution was applied alone from CT 6–12. **B** This phase shift was prevented when low calcium ASW and anisomycin (1 μ*M*) were applied simultaneously from CT 6–12. The *solid line* is the experimental eye and the *dashed line* is the untreated control eye from the same animal. The *cross hatched* bar represents the time of treatment

Results

Effects of low calcium ASW on phase of ocular rhythm

The application of a low calcium (5 m*M*)/EGTA buffered (10 m*M*) ASW caused phase shifts of the ocular pacemaker. Application of this low calcium ASW from CT 18–24 caused a phase delay of −97.2±64.8 min (N=5) and when applied from CT 6–12 caused a phase advance of 88.2±51.9 min (N=11). Three h treatment with this solution did not cause significant phase shifts at any of the phases tested (data below). Low calcium ASW also increased the extracellularly recorded CAP frequency. For example, at CT 6–12, low calcium ASW increased CAP frequency during treatment by a factor of 10 (N=4) compared to untreated controls from the same animals. An example of a low calcium-induced phase advance is shown in Fig. 1A. These phase shifts were very similar to those produced by 5-HT. As previously shown, the application of 5-HT (10 μ*M*) caused a phase delay of −90±24 min (N=6) when applied from CT 18–24 and a phase advance of 102±36 min (N=6) when applied from CT 6–12 (Colwell 1990). Since low calcium ASW caused phase shifts which resemble those produced by 5-HT, we sought to determine whether these two treatments were acting through similar mechanisms.

Effects of low calcium ASW on ocular cAMP levels

There is strong evidence that 5-HT causes phase shifts through a cAMP-dependent mechanism (e.g. Eskin and Takahashi 1983), so the effect of the low calcium ASW on ocular cAMP content was determined. In 6 separate experiments, groups of isolated eyes were exposed to 15 min of the low calcium ASW starting at CT 6. The cAMP content per eye was 23.9±4.6 p*M*/mg protein in the experimental groups compared to 24.2±5.1 p*M*/mg protein in untreated control groups from the same animals. Longer (6 h) treatments of this low calcium ASW were also without effect on cAMP levels (20.4 p*M*/mg protein, N=2). In contrast, in two groups exposed to 5-HT (10 μM) for 15 min, the ocular cAMP content was 332.1 p*M*/mg protein. Thus, low calcium treatments do not appear to cause phase shifts through the activation of adenylate cyclase.

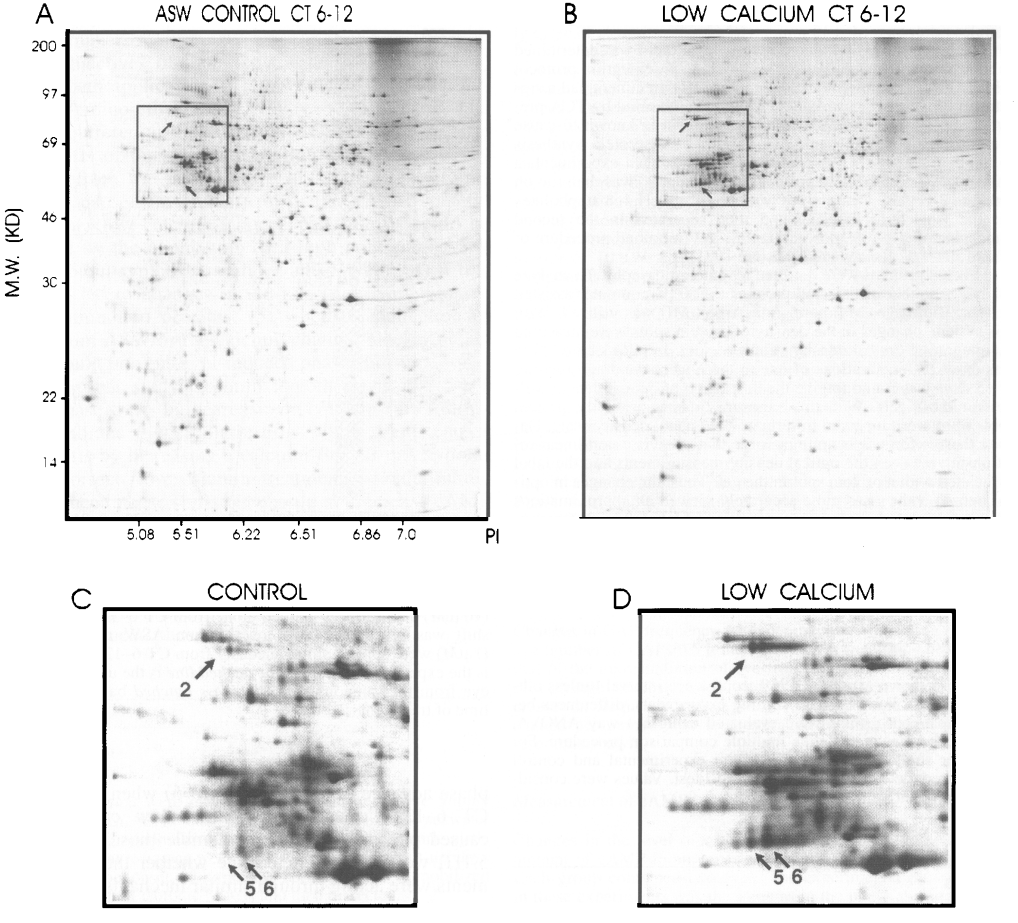

Fig. 2A–D Effect of low calcium ASW on the incorporation of [^{35}S]methionine into proteins detected by autoradiograms of two-dimensional gels. **A** Control gel obtained from eyes exposed to [^{35}S]methionine for 6 h starting at CT 6. **B** Experimental gel obtained from contralateral eyes from same animals exposed to low calcium ASW and [^{35}S]methionine for 6 h starting at CT 6. *Box* indicates area for enlargement in **C** and **D**. **C** and **D** Enlargements of portions of the control and experimental gels, respectively. *Arrows* indicate three proteins whose labeling increased as a result of the experimental treatment. The *numbers on the arrows* correspond to the numbered proteins in Table 1. The results shown are from a single experiment but are representative of 6 experiments

Effects of high potassium on low calcium ASW-induced phase shifts

Previous work has shown that high potassium ASW (30 m*M*), a treatment which should depolarize membrane potential, can prevent 5-HT-induced phase shifts (Eskin 1982). The present study evaluated the effect of this depolarizing treatment on low calcium-induced phase advances. The simultaneous application of the high potassium/low calcium ASW from CT 6–12 caused phase advances that were not significantly different from those produced by low calcium ASW alone (62.1±35.4 min, *N*=10; 51.2±31.9 min, *N*=6, respectively). By itself, high potassium ASW did not cause significant phase shifts when applied from CT 6–12 (31.3±55.1 min, *N*=6). Thus, treatment with high potassium ASW did not prevent low calcium-induced phase shifts. This result suggests that low calcium does not cause phase shifts through membrane hyperpolarization.

Effect of anisomycin on low calcium-induced phase shifts

Since the protein synthesis inhibitor anisomycin can prevent 5-HT-induced phase shifts (Eskin et al. 1984b), the effect of anisomycin (1 μ*M*) on low calcium-induced phase advances was also evaluated. When low calcium

Table 1 Summary of the effects of low calcium and 5-HT on [^{35}S] incorporation into protein in isolated eyes of *Aplysia* (*Y* yes, *N* no)

Spot #	Mol. Wt. (KDa)	PI	Detected by silver stain	Effect of low Ca^{++} on optical density (% control)	Effect of 5-HT on optical density (% control)
1.	16.5	6.30	Y	59±9*	132±42
2.	19.3	6.91	Y	71±7*	63±7*
3.	23.4	5.32	N	10±3**	164±62
4.	23.4	5.14	N	43±12*	50±10***
5.	23.4	5.27	N	28±8*	128±31
6.	23.8	5.29	N	61±10**	97±7
7.	24.8	6.37	N	105±19	282±54*
8.	26.4	6.58	Y	83±5*	85±4**
9.	28.6	6.56	N	66±8*	56±6***
10.	31.6	5.43	N	93±24	908±242*
11.	43.9	7.01	N	266±22***	147±27
12.	49.9	5.31	Y	107±35	25±6***
13.	55.5	6.20	Y	86±10	73±10*
14.	59.5	5.53	N	413±81*	119±46
15.	59.5	5.58	N	455±76***	121±39
16.	59.5	5.67	N	314±32***	240±120
17.	59.5	5.73	N	310±39***	99±8
18.	60.5	6.71	N	173±16*	176±79
19.	66.1	6.87	N	72±10*	88±9
20.	92.3	5.57	Y	561±67***	84±5*
21.	92.3	5.62	N	734±110***	84±13
22.	92.9	5.51	Y	239±92*	87±18

* significantly different ($P<0.05$) compared to controls
** significantly different ($P<0.01$) compared to controls
*** significantly different ($P<0.005$) compared to controls

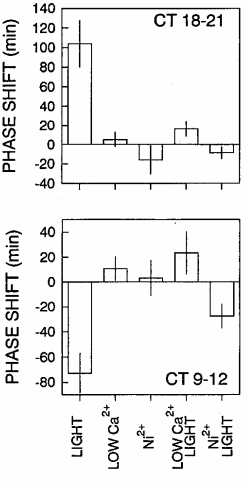

Fig. 3 Low calcium ASW as well as the bath application of the calcium channel antagonist Ni^{++} prevented light-induced phase shifts. Values shown are mean phase shifts (±S.E.M.) in the CAP activity rhythm of *Aplysia* that resulted from a 3 h treatment of either light (10 μW), low calcium ASW (5 m*M* $CaCl_2$, 10 m*M* EGTA), Ni^{++} (1 m*M*), light and low calcium ASW, or light and Ni^{++} applied simultaneously. *Top*: Treatments were given from CT 18–21. *Bottom*: Treatments were given from CT 9–12. *N*=5–7 per group

ASW and anisomycin were applied simultaneously from CT 6–12, anisomycin significantly ($P<0.01$) inhibited low calcium-induced phase shifts (29.8±40.1 min, *N*=7; 131.4±34.2 min, *N*=5, respectively). An example of anisomycin preventing a low calcium-induced phase advance is shown in Fig. 1B. By itself, anisomycin (1 μ*M*) did not cause significant phase shifts when applied from CT 6–12 (13.0±64.0 min, *N*=5). Thus, treatment with anisomycin prevented low calcium-induced phase advances. This finding suggests that low calcium may cause phase shifts through a process dependent on protein synthesis.

Effects of low calcium and 5-HT on [^{35}S] incorporation

We next sought to determine if low calcium and 5-HT could affect protein synthesis. In 6 separate experiments, groups of isolated eyes (*N*=3 per group) were exposed to low calcium or 5-HT in the presence of [^{35}S]methionine (Fig. 2). Following treatment, ocular proteins were extracted and separated by 2-D gel electrophoresis. Contralateral eyes, exposed to the labeled amino acid and treated with ASW, were used as controls. The autoradiograms were digitized and differences in labeling intensities between matched spots (over 750 matched spots were analyzed per experiment) from experimental and control autoradiograms were determined. A 6 h treatment of 5-HT applied from CT 6–12 was found to significantly and consistently affect the labeling of 9 proteins compared to untreated control eyes from the same animals in all 6 experiments: both increases and decreases in amino acid incorporation into specific proteins were observed. Similarly, a 6 h treatment of low calcium ASW applied from CT 6–12 was found to affect the labeling of 18 proteins in all 6 experiments. Interestingly, in 5 cases, the same proteins were altered by both 5-HT and low calcium treatments. A summary of the effects of the experimental treatments with the proteins characterized by apparent molecular weight and isoelectric points is found in Table 1. Only those changes which were significant and consistent i.e. occurred in 6 out of 6 experiments are included in this Table. In some cases, the total

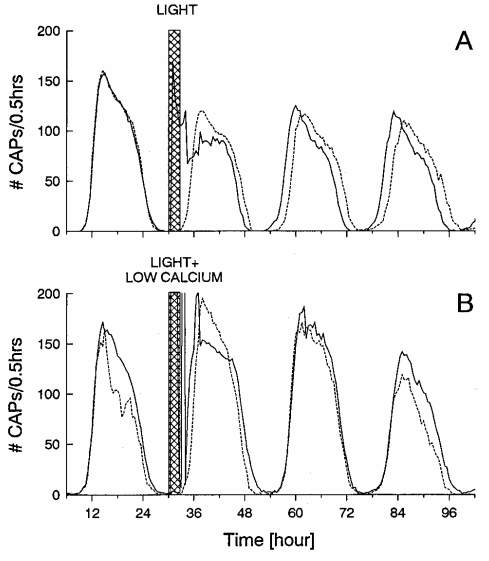

Fig. 4A,B An example of low calcium ASW preventing a light-induced phase advance. Plot of CAP activity as a function of time. **A** A phase advance resulted when light (10 μW) was applied alone from CT 18–21. **B** This phase shift was prevented when low calcium ASW (5 m*M* $CaCl_2$, 10 m*M* EGTA) and light (10 μW) were applied simultaneously during these same phases. The *solid line* is the experimental eye and the *dashed line* is the untreated control eye from the same animal. The *cross-hatched bar* represents the time of treatment

protein in the 2-D gels was visualized with a silver staining procedure. Since this is a relatively insensitive method to detect proteins, the results were included in Table 1 as a measure of the relative abundance of the identified protein. These results support the suggestion that low calcium treatments cause phase shifts through the regulation of protein synthesis.

Effects of low calcium and nickel on light-induced phase shifts

Previous work has suggested that a transmembrane calcium flux is required for light-induced phase shifts (Eskin 1977; McMahon and Block 1987; Khalsa and Block 1988). Thus, we also determined whether the application of the low calcium ASW or the calcium channel blocker Ni^{++} would prevent light-induced phase advances and delays (Fig. 3). At CT 18, a 3 h treatment of light caused a phase advance of 103.8±65.8 min (*N*=5). Three h treatments of low calcium ASW or Ni^{++} (1 m*M*) alone at this phase did not cause significant phase shifts of the rhythm (5.2±19.2 min, *N*=6 and −16.0±37.6 min, *N*=6, respectively). The simultaneous application of either low calcium ASW or Ni^{++} prevented light-induced phase shifts of the ocular rhythm (16.3±37.6 min, *N*=7; −8.7±15.6 min, *N*=6, respectively). These values are significantly different from that of the group treated with light alone ($P<0.01$). An example of low calcium ASW preventing a light-induced phase advance is shown in Fig. 4.

The application of these two treatments also prevented light-induced phase delays (Fig. 3B). At CT 9, a 3 h treatment of light caused a phase delay of −72.8±43.5 min (*N*=5). Low calcium ASW or Ni^{++} (1 m*M*) alone at this phase did not cause a significant phase shift of the rhythm (10.8±26.2 min, *N*=5; 3.2±36.2 min, *N*=6). The simultaneous application of either low calcium ASW or Ni^{++} prevented light-induced phase delays of the ocular rhythm (23.3±17.0 min, *N*=6; −27.7±24.8 min, *N*=6). These values are significantly different from that of the group treated with light alone ($P<0.01$; $P<0.05$). These results suggest that in *Aplysia* light-induced phase shifts are dependent on extracellular calcium and that the channels involved are sensitive to Ni^{++}.

Discussion

Work presented in this paper suggests that changes in a calcium flux is part of the signal transduction cascade by which both 5-HT and light cause phase shifts of the ocular rhythm in *Aplysia*. The 5-HT cascade has been extensively studied by Eskin and coworkers and is thought to involve a cAMP-dependent process. Forskolin, which increases cAMP through activation of adenylate cyclase, and membrane permeable cAMP analogs cause phase shifts which are similar to those caused by 5-HT (Benson 1980; Eskin et al. 1982; Eskin and Takahashi 1983). Furthermore, 5-HT can increase cAMP levels in the eye (Eskin et al. 1982; Eskin and Takahashi 1983). Also, 5-HT-induced phase shifts of the rhythm are mimicked by the bath application of solutions containing low potassium and blocked by the simultaneous application of solutions containing high concentrations of potassium (Eskin 1982). In addition, the broad spectrum potassium channel blocker barium was recently shown to prevent 5-HT and forskolin-induced phase shifts (Colwell et al. 1992b). These studies suggest that 5-HT is acting through a cAMP regulated change in membrane potential in order to cause phase shifts and that the channel involved is one permeable to potassium. In the current study, 6 h (but not 3 h) treatments of low calcium ASW were found to produce phase shifts. Although these low calcium-induced phase shifts were quite variable, they were clearly similar in phase dependence and average magnitude to those produced by 5-HT. This similarity raises the possibility that a decrease in calcium flux is a step in this signal transduction cascade which occurs after hyperpolarization.

Since the phase shifts caused by 5-HT appear to be mediated by increases in cAMP, it is possible that low calcium ASW also causes phase shifts through a cAMP

dependent mechanism. However, if this was the case, low calcium ASW should increase ocular cAMP content. Instead, this treatment had no consistent effect on cAMP content measured in the eye. Although it is possible that low calcium ASW produced changes too low to be detected or changes that are masked by the heterogeneous cell population in the retina, this treatment certainly did not mimic the effects of 5-HT on cAMP levels. These results are consistent with low calcium ASW causing phase shifts through a cAMP-independent mechanism.

Since 5-HT appears to generate phase shifts via membrane hyperpolarization, it is possible that low calcium ASW could also hyperpolarize the membrane and cause phase shifts through this mechanism. This does not appear to be the case. First, if the low calcium treatment hyperpolarized the membrane, the spontaneous CAP frequency should decrease. Instead, low calcium ASW significantly increased the extracellularly recorded CAP frequency compared to untreated controls from the same animals. Similar results were previously reported by Jacklet (1973). Second, high potassium ASW, a treatment which should depolarize retinal cells, did not prevent low calcium-induced phase shifts. These results support the view that low calcium ASW is causing phase shifts through a step on the signal transduction cascade after membrane potential.

Previous work suggests that 5-HT-induced phase shifts involves the synthesis of proteins. Simultaneous application of the protein synthesis inhibitor anisomycin blocked 5-HT-induced phase advances (Eskin et al. 1984b). In the present study, anisomycin was also found to prevent low calcium-induced phase advances. These results suggest that both low calcium and 5-HT cause phase shifts through a mechanism dependent upon protein synthesis. Interpretation of these experiments is simplified because anisomycin does not cause phase shifts when applied from CT 6–12. Unfortunately, at other phases (e.g. CT 18–24), protein synthesis inhibitors cause phase shifts in the ocular rhythm and protein synthesis may even be part of the basic oscillator mechanism (Rothman and Strumwasser 1976; Jacklet 1977, 1980; Lotshaw and Jacklet 1986; Yeung and Eskin 1988; Khalsa et al. 1992). Thus, it is not clear whether protein synthesis is generally required to phase shift the ocular rhythm.

Since both low calcium and 5-HT may cause phase shifts through the synthesis of proteins, we used 2-D gel electrophoresis to detect proteins whose amino acid incorporation was altered by these experimental treatments. The application of low calcium or 5-HT both lead to changes in labeling of proteins: both increases and decreases in the rate of amino acid incorporation were observed. The finding that protein synthesis inhibitors prevent low calcium and 5-HT-induced phase shifts suggests that those proteins whose amino acid incorporation was increased by these experimental treatments may be of particular interest for future study. Previous studies have also found that 5-HT treatment alters the amino acid incorporation of ocular proteins in *Aplysia* (Eskin et al. 1984b; Yeung and Eskin 1987; Koumenis and Eskin 1992). Unfortunately, the proteins identified by the present study do not directly correspond (at least in molecular weight or isoelectric point) to those previously described by Eskin and co-workers. The reason for these discrepancies are unclear; however, technical differences (e.g. in labeling isotope and 2-D gel electrophoresis protocols) are likely responsible. Regardless, the observation that both low calcium and 5-HT seem to alter the labeling of at least some of the same proteins supports the hypothesis that 5-HT causes phase shifts through a signal transduction cascade which involves a decrease in a transmembrane calcium flux.

In the present study, the effect of low calcium ASW and Ni^{++} on light-induced phase shifts of the circadian system of *Aplysia* was also examined. Three h treatments of either low calcium or Ni^{++} were found to block light-induced phase advances and delays but did not, by themselves, cause phase shifts. These results with low calcium confirm an earlier study by Eskin which also reported that treatment with a low calcium/EGTA solution could prevent light-induced phase advances (Eskin 1977). In addition, both reduction of extracellular Ca^{++} and the bath application of Ni^{++} have also been found to prevent light-induced phase shifts in *Bulla gouldiana* (McMahon and Block 1987; Khalsa and Block 1988). These results suggest that low calcium and Ni^{++} interfere with part of the cascade of cellular events which underlies light-induced phase shifts.

Ni^{++} is a widely used antagonist of voltage-sensitive calcium channels which apparently acts by binding to a site within the calcium channel and thus interferes with current flow (Akaike et al. 1978; Hagiwara and Byerly 1981; Winegar et al. 1991; Shibuya and Douglas 1992). Although in some cases Ni^{++} has been used to separate classes of calcium channels, it acts as a broad spectrum antagonist of calcium channels at the dose (1 m*M*) used in the present study (Bean 1989; Marcheno and Sage 1993). The observation that Ni^{++} prevents light-induced phase shifts suggests that a calcium flux through a voltage-sensitive calcium channel is part of the light signal transduction cascade. This possibility is also supported by the finding that the low calcium solution also prevents light-induced phase shifts. One consequence of reducing extracellular calcium would be to inhibit synaptic transmission. Thus, an alternative explanation for the data is that Ni^{++} and low calcium prevent light-induced phase shifts by interfering with the transmission of light information to the pacemaker. Although other studies have found that treatments which block synaptic transmission do not interfere with pacemaker function (Eskin 1972, 1977; Jacklet 1973), it is difficult to rule out a contribution from other cells in the retina. This is a general limitation of the whole retina preparation used in this study i.e. it is difficult to distinguish whether experimental treatments act directly on pacemaker cells or indirectly through other cells in the retina.

In *Aplysia*, light appears to cause phase shifts through a cGMP dependent membrane depolarization. Light

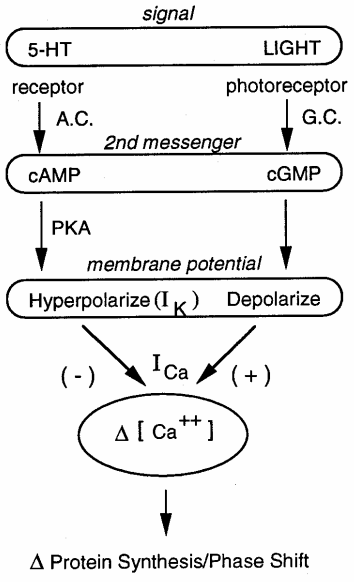

Fig. 5 Model for cellular mechanisms of entrainment in *Aplysia*. In this model, light acts via a cGMP dependent process to depolarize the membrane. 5-HT acts via a cAMP dependent process to open potassium channels and thus hyperpolarizes the membrane. Membrane depolarization leads to a persistent calcium flux through voltage-dependent calcium channels while membrane hyperpolarization leads to a decrease in calcium flux. The steps after changes in calcium flux are not known; however, protein synthesis is likely to be involved. *A.C.* adenylate cyclase; *G.C.* guanylate cyclase; *PKA* protein kinase A; I_{Ca} calcium currents; I_K potassium currents

acutely increases the cGMP content of isolated eyes and a cGMP analog can cause phase shifts which resemble those produced by light (Eskin et al. 1984a). The next step in the signal transduction cascade appears to be membrane depolarization. Solutions which presumably act to hyperpolarize the membrane prevent phase shifts induced by light as well as a cGMP analog (Eskin 1977; Eskin et al. 1984a). In addition, solutions which should depolarize the membrane mimic the phase shifting effects of light (Eskin 1972; Jacklet and Lotshaw 1988). Taken together these results suggest that in *Aplysia*, like *Bulla*, membrane depolarization leads to a persistent calcium flux through voltage-dependent calcium channels which are blocked by Ni^{++}.

The present results have led us to propose a model of the phase shifting pathway in the circadian system of the *Aplysia* eye (Fig. 5; Also see Block et al. 1993; Colwell et al. 1992a; Koumenis and Eskin 1992). In this model, 5-HT would cause phase shifts via cAMP regulation of a potassium conductance which results in membrane hyperpolarization. Light, on the other hand, causes phase shifts via cGMP regulation of ion channels which act to depolarize the membrane. The phase shifting pathways of light and 5-HT appear to converge on membrane potential. Based on the data presented in this paper, we propose that the step after membrane potential is a transmembrane calcium flux. Thus, in *Aplysia*, light acts to increase Ca^{++} flux and 5-HT acts to decrease Ca^{++} flux via their respective actions on membrane potential. These changes in calcium flux are responsible for the resulting phase shift and may also lead to changes in protein synthesis.

One of the most striking features of this model (which is currently untested) is that spontaneous, rhythmic changes in membrane potential drives a transmembrane calcium flux which would last for hours. There is some evidence in other systems for this type of long term calcium flux. For example, in pituitary cells, there is evidence for a voltage sensitive calcium conductance which is active at resting potentials (Scherubl and Hescheler 1991). In *Aplysia* neurons, a voltage regulated calcium flux is thought to underlie burst formation in spontaneously active neurons (e.g. Gorman et al. 1982; Kramer and Zucker 1985). Finally, *Bulla* retinal neurons will show a prolonged (>1 h) calcium influx in response to depolarization with elevated levels of extracellular potassium (Geusz et al. 1994). Recent advances in cell culturing retinal neurons in *Bulla* and *Aplysia* (e.g. Michel et al. 1993; Jacklet and Barnes 1993) should soon enable us to directly look for this type of voltage sensitive calcium conductance in putative pacemaker neurons.

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