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Evidence that potassium channels mediate the effects of serotonin on the ocular circadian pacemaker of *Aplysia*

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Summary. The eye of the marine mollusk *Aplysia californica* contains a photo-entrainable circadian pacemaker that drives an overt circadian rhythm of spontaneous compound action potentials in the optic nerve. Serotonin is known to influence the phase of this ocular rhythm. The aim of the present study was to evaluate whether potassium channels are involved in effects on the ocular circadian rhythm. Our experimental approach was to study the effect of the potassium channel antagonist barium on serotonin-induced phase shifts of this rhythm. The application of barium was found to block serotonin-induced phase shifts whereas barium alone did not cause significant phase shifts. The effects of barium were found to be dose dependent. In addition, barium blocked forskolin-induced phase advances but did not interfere with serotonin-induced increases in cAMP content. Finally, barium antagonized serotonin-induced suppression of compound action potential activity. These results are consistent with a model in which the application of serotonin phase shifts the ocular pacemaker by causing a membrane hyperpolarization which is mediated by a cAMP-dependent potassium conductance.

Key words: Aplysia – barium – circadian – potassium channels – serotonin

Introduction

The eye of the marine mollusk *Aplysia californica* contains a circadian oscillator which drives an overt rhythm in the frequency of spontaneous compound action potentials (CAPs) recorded from the optic nerve (Jacklet 1969). Serotonergic fibers innervate the eye via projections from the cerebral ganglion (Goldstein et al. 1984; Olsen and Jacklet 1985; Takahashi et al. 1989) and a variety of studies indicate that 5-HT treatments can influence the phase of this oscillator (Corrent et al. 1978; Corrent and Eskin 1982; Colwell 1990). The mechanisms by which 5-HT produces phase shifts have been extensively studied and appear to involve a cAMP-dependent process. Forskolin, which increases cAMP through activation of adenylate cyclase, and membrane permeable cAMP analogs mimic the phase shifting effects of 5-HT (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). 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.

Barium (Ba^{++}) is a broad spectrum potassium channel antagonist whose mechanism of action involves the physical blockade of the ion channel (Armstrong and Taylor 1980; Cook and Quast 1990). Previous studies have shown that bath-applied Ba^{++} can be an effective antagonist of potassium channels in *Aplysia*. For example, in the spontaneously bursting neuron R15, 5-HT activates an inwardly rectifying potassium current which results in membrane hyperpolarization. This 5-HT-induced response is mediated by cAMP and blocked by the bath application of Ba^{++} (Benson and Levitan 1983; Benson and Adams 1987). Tetraethylammonium (TEA), another general antagonist of potassium channels, did not block this 5-HT-induced hyperpolarization. In general, bath applied Ba^{++} has been shown to block a variety of both ligand and voltage regulated potassium channels in *Aplysia* (e.g. Kauer et al. 1987; Brenzina et al. 1987; Halliwell 1990).

Abbreviations: ASW, artificial seawater; Ba^{++}, barium; CAP, compound action potential; CT, circadian time; 5-HT, serotonin; TEA, tetraethylammonium

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The purpose of the present study was to further investigate the role of potassium channels in mediating 5-HT-induced phase shifts of the circadian system of the marine mollusk *Aplysia*. We first sought to determine whether bath application of the potassium channel blocker Ba^{++} would prevent 5-HT-induced phase shifts of the circadian rhythm of CAP frequency recorded from the isolated eye of *Aplysia*. We also examined the effect of Ba^{++} on 5-HT-induced increases in cAMP and on forskolin-induced phase shifts. Finally, we examined the effect of Ba^{++} on 5-HT's acute suppression of CAP frequency.

Methods

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 h before the onset of darkness, animals were immobilized with an injection of isotonic $MgCl_2$ and then dissected.

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 395 (in m*M*) NaCl, 10 KCl, 10 $CaCl_2$, 50 $MgCl_2$, 28 Na_2SO_4, 30 Hepes buffer, 100000 units/L penicillin and 100000 μg/L streptomycin. Ba^{++} was applied in an ASW which contained no antibiotic or Na_2SO_4 but otherwise had the same composition as listed above. These compounds were omitted when it was discovered that they caused the precipitation of Ba^{++}. This solution (Na_2SO_4-free and antibiotic-free ASW) by itself did not cause phase shifts nor did it interfere with 5-HT-induced phase shifts. 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. A polygraph was used to amplify and record the compound action potentials (CAPs). 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 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.

The effects of experimental treatments were calculated by measuring the phase difference between the experimental and control rhythms on the 4th cycle (the 2nd cycle after treatment), less the phase difference from the 1st (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. Experimental solutions were made by adding compounds directly to ASW within 2 h prior to use. Forskolin was administered in ASW containing 0.1% DMSO. Both forskolin and 5-HT were purchased from Sigma Chemicals, St. Louis, MO. Solution changes were made without illumination of the eyes.

Changes in the level of cAMP were obtained by comparing the amount of cAMP in an experimental and matched control group. For experiments in which 5-HT was applied for 15 min each group contained 2 eyes; for experiments in which 5-HT was applied for 6 h each group contained 6 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).

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.

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. Differences between treatment groups were evaluated using a 1-way ANOVA, followed by Tukey's multiple comparison procedure where appropriate. Values are shown as means ± 95% confidence interval (unless otherwise noted) and were considered significantly different if $P<0.05$.

Results

The application of Ba^{++} blocked 5-HT induced phase shifts in *Aplysia* (Fig. 1). At CT 18, a 6 h treatment of 5-HT (10 μ*M*) caused a phase delay of 98.6 min (±41.5, $N=7$). An example of a 5-HT-induced phase delay in the CAP activity rhythm is shown in Fig. 2. Ba^{++} (0.1 m*M*) alone at this phase did not cause a significant phase shift of the rhythm (4.0 min ±29.5, $N=6$). The simultaneous application of Ba^{++} (0.1 m*M*) prevented 5-HT-induced phase shifts of the ocular rhythm (−20.5 min, ±46.2, $N=6$). This value was significantly different than that of the group treated with 5-HT alone ($P<0.01$). Figure 3 shows an example of Ba^{++} preventing a 5-HT-induced phase shift in the CAP activity rhythm. Barium's inhibition of 5-HT-induced phase delays was dose dependent (Fig. 4). The co-treatment with TEA (100 m*M*) did not inhibit 5-HT-induced phase shifts (−131.8 min ±60.2, $N=5$).

The application of Ba^{++} also prevented 5-HT-induced phase advances (Fig. 1). A 6 h treatment of 5-HT (10 μ*M*), starting at CT 6, caused a phase advance of 100.2 min (±38.4, $N=6$). Treatment with Ba^{++} (10 m*M*) at the same phase did not produce a significant phase shift (−11.4 min ±68.7, $N=5$). When 5-HT (10 μ*M*) and Ba^{++} (10 m*M*) were applied together at this phase, no significant phase shift was observed (3.8 min ±38.1, $N=5$). This value was significantly different than that of the group treated with 5-HT alone ($P<0.01$). The inhibitory effect of Ba^{++} on 5-HT-induced phase advances was dose dependent (Fig. 4). The dose-response curve for barium's inhibitory effect on 5-HT-induced phase advances is markedly different from that of phase delays.

Ba^{++} (0.1 m*M*) did not prevent 5-HT-induced increases in cAMP. In 5 separate experiments, groups of isolated eyes were exposed to 15 min of 5-HT starting at CT 18. In each of these cases, 5-HT increased the

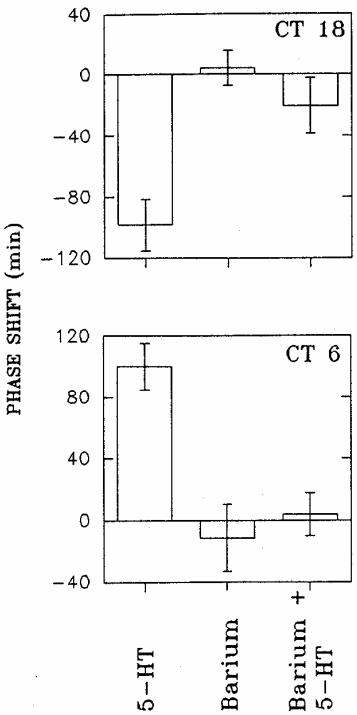

Fig. 1. Ba^{++} prevented 5-HT-induced phases. Values shown are mean phase shifts (± S.E.M.) in the CAP activity rhythm of *Aplysia* that resulted from a 6 h treatment of either 5-HT (10 μ*M*), Ba^{++}, or 5-HT and Ba^{++} applied simultaneously. *Top*: Treatments were given from CT 18–24; Ba^{++} (0.1 m*M*). *Bottom*: Treatments were given from CT 6–12; Ba^{++} (10 m*M*). $N=6$ per group

levels of cAMP compared to untreated controls from the same animals (Fig. 5A). This stimulation can be sustained for at least 6 h. When 5-HT was applied from CT 18–24, significant increases in cAMP levels could still be measured at CT 24 (174.5% of control values ±91.0, $N=5$). Furthermore, 5-HT was found to induce cAMP at all phases of the circadian cycle which were examined (Table 1). The acute induction of cAMP by 5-HT was not inhibited by the simultaneous treatment with Ba^{++}. In 6 separate experiments, groups of isolated eyes were exposed to 15 min of Ba^{++} and 5-HT and compared to controls which were treated with 5-HT alone (Fig. 5B). Ba^{++} did not have a significant effect on 5-HT stimulated levels of cAMP.

Ba^{++} did inhibit forskolin-induced phase delays of the ocular rhythm (Fig. 6). At CT 18–24, forskolin (1 μ*M*) caused a phase delay of −74.2 min (±17.5, $N=4$) while the treatment of Ba^{++} (1 m*M*) during this same interval did not cause a significant phase shift (−18.0 min ±22.6, $N=6$). When forskolin (1 μ*M*) and Ba^{++} (1 m*M*) were applied simultaneously, no significant phase shift was observed (5.0 min ±26.2, $N=6$). The phase shift which resulted from the simultaneous application of forskolin and Ba^{++} was significantly different than that produced by forskolin alone ($P<0.01$). Interestingly, lower doses of Ba^{++} (0.1 and 0.5 m*M*) did not block forskolin-induced phase shifts.

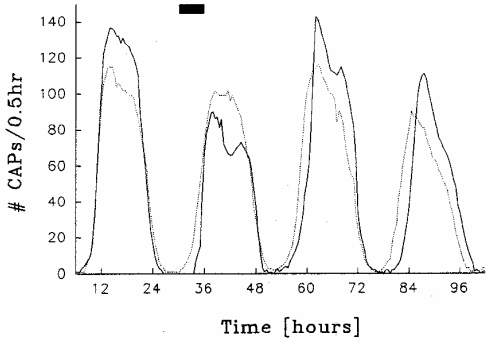

Fig. 2. A plot of CAP activity as a function of time showing the phase delay which resulted when 5-HT (10 μ*M*) was applied from CT 18–24. The *solid line* is the experimental eye and the *dashed line* is the control eye from the same animal. The *black bar* represents the time of treatment

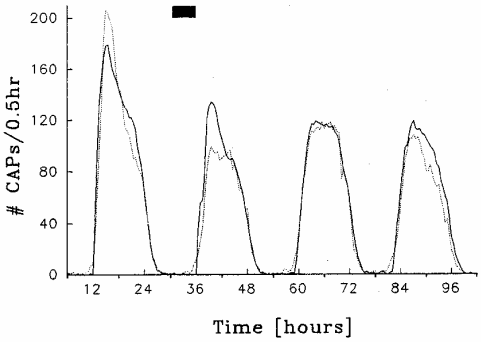

Fig. 3. A plot of CAP activity as a function of time showing that no phase shift resulted when Ba^{++} (1.0 m*M*) and 5-HT (10 μ*M*) were applied simultaneously from CT 18–24. The *solid line* is the experimental eye and the *dashed line* is the control eye from the same animal. The *black bar* represents the time of treatment

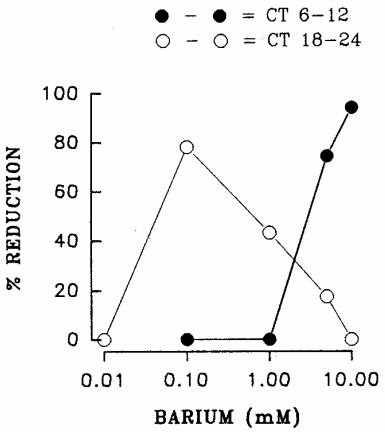

Fig. 4. Dose-response curve for Ba^{++}'s inhibition of 5-HT-induced phase shifts. Experimental eyes received 5-HT (10 μ*M*) and Ba^{++} (various doses) while the control eyes were untreated. Values shown are the calculated percent reduction in the magnitude of 5-HT-induced phase shifts caused by various doses of Ba^{++}. *Filled cycles* show results from treatments given from CT 6–12 while *hollow circles* show results of treatments from CT 18–24. $N=4$–7

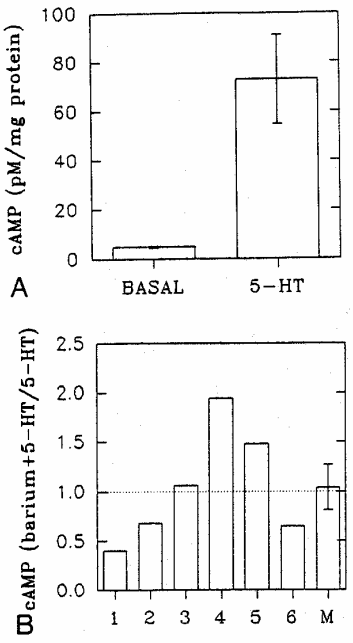

Fig. 5. A Exposure to 5-HT increases the levels of cAMP found in the isolated eyes. In 5 separate experiments, groups of isolated eyes ($N=2$/group) were exposed to 15 min of 5-HT starting at CT 18 and compared to controls from the same animals which were untreated. Values shown are means ± S.E.M. and are normalized for the total protein in the tissue. **B** The induction of cAMP by 5-HT was not inhibited by the simultaneous application of Ba^{++}. In 6 separate experiments, groups of isolated eyes ($N=2$/group) were exposed to 15 min of Ba^{++} and 5-HT and compared to controls from the same animals which were treated with 5-HT alone. Each column shows the effect of Ba^{++} on 5-HT-induced cAMP levels from a separate experiment while the mean difference (± S.E.M.) is shown in column labeled "M"

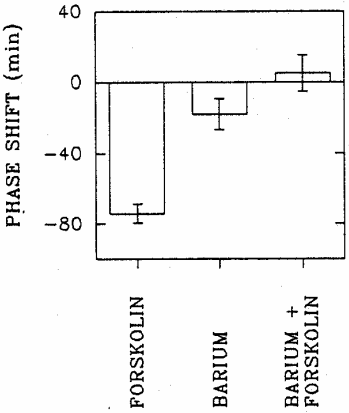

Fig. 6. Ba^{++} prevented forskolin-induced phase delays. Values shown are mean phase shift (+ S.E.M.) in the CAP activity rhythm of *Aplysia* that resulted from a 6 h treatment of either forskolin (1 μ*M*), Ba^{++} (1 m*M*) or forskolin and Ba^{++} simultaneously. Treatments were given from CT 18–24. $N=4$–6

Finally, Ba^{++} was found to attenuate 5-HT's suppression of extracellularly recorded CAPs in darkness (Fig. 7). The average CAP frequency from CT 6–12 was 105.1 spikes/0.5 h (±23.7, $n=25$). Treatment with 5-HT

Table 1. A summary of the effects of a 15 min treatment of 5-HT (10 μ*M*) on cAMP levels measured from isolated eyes of *Aplysia*. Values are shown as means ± 95% confidence intervals. There were two eyes per group and the number of groups is shown in paranthesis. There are no significant differences between these values

Phase of Treatment	cAMP (p*M*/mg protein)
CT 0	37.3±13.2* (3)
CT 6	48.7±33.1* (3)
CT 12	51.8±23.4* (3)
CT 18	62.4±32.2* (15)

* indicates significant values

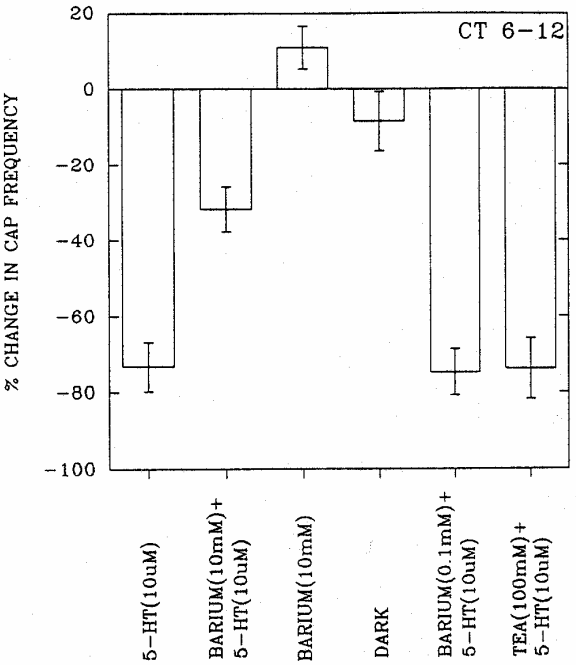

Fig. 7. Ba^{++} attenuated 5-HT-induced suppression of CAP frequency. The effect of various treatments on CAP frequency was determined by comparing the average number of CAPs/0.5 h during an experimental treatment with the untreated control eye from the same animal. The percent change was calculated with positive values representing increases in frequency. The column labeled "DARK" represents the difference in CAP frequency between two untreated eyes from the same animal. Values shown are means ± S.E.M.; $N=4$–30

at this phase caused a significant ($P<0.01$) decrease in the CAP frequency (−73.3%±16.6, $n=6$) compared to untreated controls from the same animal. On the other hand, Ba^{++} (10 m*M*) alone led to a small (non-significant) increase in the firing rate (10.9%±13.8, $n=7$). When the 5-HT and Ba^{++} (10 m*M*) treatments were given simultaneously from CT 6–12, the firing rate was still inhibited compared to untreated controls but only by −31.6% (±19.7, $N=11$). Thus, the application of Ba^{++} (10 m*M*) caused a significant attenuation of 5-HT's suppression of CAP frequency ($P<0.05$). A lower dose of Ba^{++} (0.1 m*M*) or TEA (100 m*M*) had no significant effect on either 5-HT-induced phase shifts (see

above) or 5-HT-induced inhibition of CAP frequency. 5-HT still inhibited CAP frequency in the presence of these lower doses Ba^{++} (0.1 m*M*) or TEA (100 m*M*) by an average of −74.8% (±19.3, $N=4$) and −73.8% (±25.7, $N=4$), respectively. At CT 18–24, 5-HT also decreased CAP frequency (−45.2% ±47.7, $N=7$); however, this inhibition was not significant.

Discussion

In the present study, we examined the effect of the potassium channel blocker Ba^{++} on 5-HT-induced phase shifts of the circadian system of *Aplysia*. We found that the application of Ba^{++} blocked 5-HT-induced phase advances and delays but did not, by itself, cause phase shifts (see Fig. 1). This suggests that Ba^{++} interferes with part of the cascade of cellular events which underlies 5-HT-induced phase shifts. This signal transduction cascade has been extensively studied by Eskin and coworkers and appears to involve a cAMP mediated event (e.g. Eskin et al. 1982; Eskin and Takahashi 1983). Consequently, we examined the effect of Ba^{++} on both 5-HT-induced increases in ocular cAMP content and on forskolin-induced phase shifts.

Our results confirmed a previously published report which found that the application of 5-HT can acutely increase cAMP levels in the whole eye (Eskin et al. 1982; see Fig. 5). We found that this increase is maximal 15 min after the application of 5-HT; however, significantly elevated levels of cAMP could be detected as long as 6 h after the bath application of the transmitter. In addition, 5-HT was found to increase cAMP levels throughout the circadian cycle (Table 1). In each of 3 experiments in which we compared 5-HT induction of cAMP at different phases of the circadian cycle, we found the largest induction at CT 18. However, this phase dependence was not significant. Basal levels of cAMP were low at all phases tested and if an endogenous rhythm in cAMP content was present it occurred below the sensitivity of the methods of detection employed in this study. We then examined the effect of Ba^{++} on the induction of cAMP by a 15 min treatment of 5-HT. We found that Ba^{++} did not interfere with 5-HT's induction of cAMP. Some caution must be taken in the interpretation of these experiments as our measurements of cAMP were on intact eyes containing a heterogeneous population of cells of which the putative pacemaker neurons (the so-called "D-cells") would only be a small part (Jacklet 1989). Changes in cAMP in pacemaker cells could be masked by other cells in the retina.

Previous studies have shown that both forskolin, which acts to increase cAMP via a stimulation of adenylate cyclase, and cAMP analogs mimic the phase shifting effect of 5-HT (Eskin et al. 1982; Eskin and Takahashi 1983). These results provide strong evidence that cAMP is part of 5-HT's signal transduction cascade and also indicate that forskolin acts to cause phase shifts through its stimulatory action on cAMP levels. We found that Ba^{++} blocked forskolin-induced phase shifts although higher doses were required to prevent forskolin-induced phase shifts than were required to prevent 5-HT-induced phase shifts. Without knowing more about the effects of these agents on cAMP levels in the pacemaker cells themselves, it is difficult to explain this difference. Thus, Ba^{++} does not appear to block 5-HT's induction of cAMP but does prevent phase shifts presumably caused by increases in cAMP. The simplest interpretation of these data is that Ba^{++} acts to prevent phase shifts at some point in the signal transduction cascade after 5-HT's induction of cAMP.

Ba^{++} is an extensively studied potassium channel antagonist and this is likely to be the mechanism by which this ion prevents 5-HT-induced phase shifts. Previous studies have provided evidence that membrane hyperpolarization is a step in the signal transduction cascade by which 5-HT causes phase shifts of this circadian oscillator (e.g. Eskin 1982). Thus, agents which prevent 5-HT-induced hyperpolarization should also prevent phase shifts. In *Aplysia* neurons, Ba^{++} has been shown to block ligand regulated potassium channels (e.g. Benson and Levitan 1983; Ewald and Eckert 1983; Connor and Hockberger 1984; Lotshaw et al. 1986; Benson and Adams 1987). Thus, it is likely that Ba^{++} inhibits 5-HT-induced phase shifts by preventing the hyperpolarization normally induced by 5-HT. Although we did not intracellularly measure the membrane potential of *Aplysia* pacemaker neurons, the extracellularly recorded CAP frequency (which should be a reflection of the membrane potential of these neurons) supports this interpretation. By itself, 5-HT caused an inhibition of the spontaneous CAP frequency during CT 6–12. This inhibition was partially reversed by the administration of Ba^{++} (10 m*M*). TEA and low doses of Ba^{++}, which were ineffective at inhibiting 5-HT-induced phase shifts at this phase, were also ineffective at preventing 5-HT's inhibition of CAP activity. 5-HT also caused a suppression of CAP frequency during CT 18–24. However, spontaneous neural activity was already very low during these phases and 5-HT's suppression was not statisticly significant. These data provide additional evidence that 5-HT causes phase shifts through a membrane hyperpolarization which is caused by the opening of potassium channels.

Ba^{++} interfered with 5-HT-induced phase shifts in a dose dependent manner (see Fig. 4). The range of effective doses used (0.1 m*M*–10 m*M*) in this study is in the range of doses in which Ba^{++} has been found to act as a potassium channel antagonist in other studies (e.g. Armstrong and Taylor 1980; Benson and Levitan 1983; Brenzina et al. 1987). Interestingly, the dose-response relationship described for Ba^{++}'s inhibition of 5-HT-induced phase delays was very different from that found for phase advances. For example, a dose of 0.1 m*M* Ba^{++} which blocked phase delays had no inhibitory influence on phase advances. One explanation for this finding may lie in the observation that the Ba^{++} blockade of potassium channels can be voltage dependent. Previous studies have shown that membrane depolarization can raise the dose of Ba^{++} required to block a potassium conductance (e.g. Standen and Stanfield 1978; Armstrong and Taylor 1980). In the *Aplysia* retina, the pacemaker cells responsible for driving the rhythm in CAPs are thought to undergo a daily rhythm in membrane potential. During CT 6–12, spontaneous neural

activity is high and the pacemaker cells are presumable depolarized. Thus, higher doses of Ba^{++} may be required at this phase to block the potassium channel regulated by 5-HT.

The dose response curve for Ba^{++}'s inhibition of 5-HT-induced phase delays appeared inverted-"U" shaped, with doses higher than 0.1 m*M* proving less effective. Previous studies in *Aplysia* did not report the dose-response function of Ba^{++}'s action as a potassium channel blocker so we do not know if the inverted-"U" shaped dose-response function is unusual. We also do not know if the dose response curve for phase advances also had this shape for it was difficult to record the circadian rhythm after treatment with doses of Ba^{++} higher than 10 m*M*. It may be that at higher doses, Ba^{++} starts to affect processes other than potassium conductance. For example, at doses higher than those used in this study, Ba^{++} has been reported to act as a competitive antagonist of calcium mediated processes (e.g. see Connor 1979). However, we can not explain the inverted-"U" shaped dose response function.

This work contributes to the identification of the potassium channels involved in 5-HT-induced phase shifts. Ba^{++} is a fairly non-selective potassium channel antagonist and its antagonistic action is not restricted to a specific class of potassium channels. Nevertheless, this study does lay the groundwork for future biophysical analysis by demonstrating that the potassium channels of interest are antagonized by Ba^{++} but not by TEA (at least under the conditions found in this study). In addition, Ba^{++} can be used as a tool to help in the identification of components of the phase shifting pathway which are downstream from membrane potential. For example, Eskin and colleagues have identified proteins whose levels of synthesis change in response to a treatment with 5-HT (Eskin et al. 1984b; Yeung and Eskin 1987). The work presented in this paper suggests that only those changes which are blocked by Ba^{++} are likely to be part of the phase shifting mechanisms. In this way, the demonstration of Ba^{++} blockade could become an important criterion for the identification of components of the signal transduction cascade by which 5-HT causes phase shifts.

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