Mechanism for shifting the phase of a circadian rhythm by serotonin: Involvement of cAMP

(*Aplysia*/isolated eye/phosphodiesterase inhibitors/cAMP analogues/levels of cAMP)

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ABSTRACT Serotonin (5-hydroxytryptamine, 5-HT) shifts the phase of the circadian rhythm in the eye of *Aplysia*. We have examined the role of cAMP in mediating the effects of 5-HT on the rhythm. The phase shifts produced by 5-HT are mimicked by treatments that should increase intracellular levels of cAMP. An analogue of cAMP, 8-benzylthio-cAMP, advanced and delayed the rhythm at phases in which 5-HT had similar effects on the rhythm. In addition, two phosphodiesterase inhibitors, Ro-20-1724 and papaverine, caused advance phase shifts where 5-HT advances the rhythm. The phosphodiesterase inhibitors Ro-20-1724 and 3-isobutyl-1-methylxanthine each potentiated the effect of subthreshold doses of 5-HT on the rhythm. The effects of 5-HT and 8-benzylthio-cAMP on the rhythm were nonadditive, indicating that 5-HT and 8-benzylthio-cAMP affect the rhythm through a common pathway. Finally, 5-HT produced large changes (13-fold) in the levels of cAMP in the eye. These results indicate that cAMP mediates the effect of 5-HT on the rhythm. There are two possible roles for cAMP in the circadian system. Either the cAMP system is an intracellular step in an entrainment pathway or it is part of the biological clock. Because 5-HT, 8-benzylthio-cAMP, and three phosphodiesterase inhibitors inhibit impulses from the eye, cAMP may also mediate the inhibition produced by 5-HT, or it might be involved in regulating the frequency of spontaneous impulses throughout the day.

Entrainment of circadian rhythms from animals requires that environmental information be processed through one or more neurons. Ultimately, the information must be translated into a form that perturbs the endogenous oscillating mechanism. Our research is focused upon the pathways by which entrainment information acts on circadian oscillators. Serotonin (5-hydroxytryptamine, 5-HT) shifts the phase of the circadian rhythm of the isolated eye of *Aplysia* (1). The characteristics of the phase shifts produced by 5-HT and light indicate that 5-HT is used in a different pathway from the one involved in entrainment of the isolated eye by light–dark cycles (2). We have hypothesized that 5-HT plays a role in transmitting circadian information from the central nervous system to the eye via efferent activity in the optic nerve (1–3). Because it is likely that circadian oscillators involve some part of the metabolic machinery of the cell, some of the final steps of entrainment pathways may be intracellular second messengers. We investigated whether cAMP mediates phase shifting by 5-HT because many different effects of 5-HT in *Aplysia* appear to involve cAMP (4–12). Preliminary results of some of these studies have appeared elsewhere (13).

METHODS

Eyes were removed from the sea hare *Aplysia californica* and maintained individually at 15.5°C in constant darkness through-

out the experiment. The recording chambers contained filtered sea water with 30 mM Hepes (pH 7.7), penicillin at 100 units/ml, and streptomycin at 100 µg/ml. The rhythm of spontaneous nerve impulses was assayed by recording impulses from the optic nerve, which was pulled into a polyethylene tube. A more detailed discussion of the apparatus has been published (14).

The time of experimental treatments is given as circadian time (CT). CT 12 is the time of projected dusk. The effects of experimental treatments are phase shifts ($\Delta\phi$) measured during the second cycle after treatment as the difference in peak times of the rhythms of matched (same animal) experimental and control eyes. One eye from each of six pairs of untreated eyes was randomly defined as an “experimental” eye. The mean difference between the peak of the rhythms of six pairs of “experimental” and control eyes during the cycle in which our results are normally tabulated was 0.25 hr (standard deviation 0.41 hr). Thus, by comparing matched experimental and control eyes, one can detect phase shifts as small as 1 hr. Data were obtained during the second cycle of the rhythm after treatments because the phase shifts during this cycle were essentially the same as those during the third cycle after treatments. For instance, the mean phase shifts during the second and third cycle after treatments for those eyes that were run at least three cycles after treatment were, respectively: +3.2, +3.5 hr [2 mM 8-benzylthio-cAMP (8-BzLS-cAMP), $n = 3$, CT 7–13]; +1.9, +1.9 hr (60 µM Ro-20-1724, $n = 4$, CT 8–14); +2.5, +2.5 hr (10 µM 5-HT, $n = 7$, CT 7–13); –2.3, –2.2 hr (10 µM 5-HT, $n = 5$, CT 22–4). The peak of the rhythm was chosen as an assay of the phase of the underlying oscillator instead of other parameters because the peak is usually sharp and easy to define. In addition, the variance appears to be smaller when the peak rather than the maximal rate of rise of the rhythm is used. The phase shifts between experimental and control eyes reported in this study are not affected by different choices of the parameter used to measure phase. The average difference in phase shifts determined by using the peak of the rhythm and the time of maximal rate of rise of the rhythm for all treatments used in this study was 0.10 hr. No single phase shift that was insignificant became significant or vice versa when maximal rate of rise was used to calculate the results instead of peak time. Ninety-five percent confidence intervals are given after most results.

Changes in the level of cAMP were obtained by comparing the amount of cAMP in an experimental and matched control group, each containing three eyes. The eyes were maintained at 17°C in very dim light. The experiments were performed during CT 12–15, which was 3 hr after isolation of the eyes. After experimental treatments, the eyes were rinsed with ice-cold sea water, separated from their optic nerves, and placed in a ho-

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Abbreviations: 5-HT, serotonin (5-hydroxytryptamine); CT, circadian time; iBuMeXan, 3-isobutyl-1-methylxanthine; 8-BzLS-cAMP, 8-benzylthio-cAMP.

mogenizer. cAMP levels were determined by using a New England Nuclear radioimmunoassay kit and by the acetylation procedure. The amount of protein was determined by the method of Lowry (15).

RESULTS

cAMP Analogues and Phosphodiesterase Inhibitors. One way to determine whether intracellular changes in cAMP are involved in mediating the effect of 5-HT is to test if an increase in the intracellular level of cAMP can mimic the response of the eye to 5-HT. 5-HT causes advance, delay, or no phase shift in the rhythm, depending on the subjective time or phase of the rhythm at which it is applied (1, 2). Advance phase shifts were produced by 6-hr 5-HT treatments (10 μ M) that began between CT 5 and CT 12 (Fig. 1A) and delay phase shifts were produced by similar 5-HT treatments that began between CT 19 and CT 23 (Fig. 2B). A treatment that mimics the effect of 5-HT on the rhythm should produce advance phase shifts at phases in which 5-HT causes advances and delays at phases in which 5-HT causes delays.

An increase in the intracellular level of cAMP may be mimicked by using the cAMP analogue 8-BzIS-cAMP. Six-hour treatments of 8-BzIS-cAMP (2 mM) produced advance phase shifts at CT 7–13 ($\Delta\phi = +3.25 \pm 0.80$ hr, $n = 4$) (Fig. 1B) and delay phase shifts at CT 22–04 ($\Delta\phi = -3.75 \pm 2.38$ hr, $n = 4$) (Fig. 2A). Thus, treatments of 8-BzIS-cAMP successfully mimicked the effects of 5-HT. A smaller concentration of 8-BzIS-cAMP (1 mM) also produced phase shifts at CT 7–13 ($\Delta\phi = +2.2$ hr $\pm$ 0.88). Another analogue of cAMP, 8-parachlorophenylthio-cAMP, may also cause phase shifts, because the rhythm from one eye was reported to have been delayed 2 hr by a 12-hr treatment with this compound (16).

Phosphodiesterase inhibitors might also increase the intracellular level of cAMP if there is a reasonable basal level of ade-

nylate cyclase activity. Six-hour treatments of 500 μ M 3-isobutyl-1-methylxanthine (iBuMeXan) at CT 7–13 did not cause a phase shift ($\Delta\phi = +0.55 \pm 0.83$ hr, $n = 9$), nor did similar treatments of theophylline (5 mM) cause phase shifts ($\Delta\phi = -0.25$ hr, $n = 2$). However, one other phosphodiesterase inhibitor, papaverine (an isoquinoline), did cause phase shifts that mimicked the effects of 5-HT on the rhythm and another phosphodiesterase inhibitor, Ro-20-1724 (an imidazolidinone), partially mimicked the effects of 5-HT: 60 μ M Ro-20-1724, $\Delta\phi = +1.88 \pm 0.76$ hr, $n = 4$, CT 8–14 (Fig. 1D) and $\Delta\phi = -0.45 \pm 0.31$, $n = 21$, for a group of eyes whose treatments ranged between CT 18–24 and CT 2–8; 200 μ M papaverine, $\Delta\phi = +2.17 \pm 0.71$ hr, $n = 3$, CT 7–13 (Fig. 1C), and 300 μ M papaverine, $\Delta\phi = -1.83 \pm 1.43$ hr, $n = 3$, CT 18–24. High concentrations of dibutyryl-cAMP plus iBuMeXan may also shift the phase of the rhythm of the *Aplysia* eye (17).

Interactions Between 5-HT and Drugs Affecting Levels of cAMP. A more direct way to examine whether the 5-HT effect is mediated by changes in cAMP is to look for interactions between 5-HT and drugs that affect the levels of cAMP. Such interactions may be investigated by determining whether effects of 5-HT are potentiated by phosphodiesterase inhibitors and whether the effects of 5-HT and 8-BzIS-cAMP are additive. In the potentiation experiments, we asked if phase shifts would occur when phosphodiesterase inhibitors were added to subthreshold doses of 5-HT. No significant phase shifts were produced by either 10 nM 5-HT ($\Delta\phi = +0.17 \pm 0.33$ hr, $n = 9$) or 500 μ M iBuMeXan ($\Delta\phi = +0.55 \pm 0.83$ hr, $n = 9$) when the treatments were administered at CT 7–13. However, adding these two treatments together produced phase shifts ($\Delta\phi = +1.54 \pm 0.64$ hr, $n = 11$) that were significantly different from those with either treatment alone or the sum of the individual treatments (Fig. 3A). A similar experiment was performed with

FIG. 1. Advance phase shifts produced by 5-HT or treatments capable of increasing intracellular cAMP. The frequency of spontaneous optic nerve impulses is plotted as a function of the time that the isolated eyes were in constant darkness. Each graph contains rhythms from eyes of the same animal. Experimental eyes received 6-hr treatments during the time (CT 7–13) shown by the hatched bars on the bottom of the graphs. The open bars at the bottom of the graphs are 12 hr in length and represent the projected light portion of the light-dark cycle that was used to entrain the intact animals. The concentrations used in the treatments were: (A) 10 μ M, (B) 2 mM, (C) 200 μ M, (D) 60 μ M.

FIG. 2. Delay phase shifts in the rhythm produced by 6-hr treatments of 2 mM 8-BzIS-cAMP (A) or 10 μ M 5-HT (B).

Ro-20-1724 at CT 23–5. At this phase Ro-20-1724 causes only very small or no phase shifts. The effect of Ro-20-1724 by itself at this phase was -0.62 ± 0.48 hr, $n = 8$, and the effect of 5-HT (10 nM) at this phase was $+0.08 \pm 0.52$ hr, $n = 6$. Adding

Ro-20-1724 together with 5-HT produced a significant delay phase shift ($\Delta\phi = -1.43 \pm 0.76$ hr, $n = 7$), which was significantly different from that for either of the treatments by themselves or the sum of the two individual treatments (Fig. 3B). Thus, two phosphodiesterase inhibitors, Ro-20-1724 and iBuMeXan, appear to potentiate the effects of 5-HT on the rhythm.

If the effects of 5-HT and 8-BzIS-cAMP on the rhythm are related (i.e., nonindependent), then the effects of the treatments should be nonadditive if they are given simultaneously. The effect of 6-hr treatments of 5-HT (10 μ M) + 8-BzIS-cAMP ($\Delta\phi = +2.88 \pm 1.36$ hr, $n = 4$) at CT 7–13 does not equal the sum of the effects of the individual treatments (8-BzIS-cAMP = $+3.25 \pm 0.80$ hr, $n = 4$, and 5-HT = $+2.5 \pm 1.0$ hr, $n = 7$) (Fig. 3C). Nonadditivity in this experiment was not the result of a ceiling or limit on the amount that the phase of the rhythm can be shifted, because other treatments such as increased extracellular K^+ have produced 6-hr advance phase shifts in the rhythm from the eye (18). It is worth noting here that it is unnecessary to use saturating doses of treatments to establish nonadditivity, but saturating doses are necessary to establish additivity of effects.

Changes in the Level of cAMP. Another criterion for demonstrating that an effect is mediated by cAMP is to show that the effect is accompanied by increases in the level of cAMP. The results are expressed as a ratio of total cAMP in three experimental eyes to the cAMP in matched control eyes. Within 3 min of exposure to 5-HT (10 μ M) an increase in cAMP as large as 13-fold was observed (Fig. 4 *Inset*). The amount of cAMP after 3 min of exposure to 5-HT averaged over three experiments (nine experimental eyes) was 43 pmol/mg of total protein. Because the lens of the eye contains about 80% of the total protein, this corresponds to 210 pmol/mg of retinal protein. The level

FIG. 3. Potentiation of subthreshold doses of 5-HT by phosphodiesterase inhibitors (A, B) and nonadditivity of 8-BzIS-cAMP and 5-HT (C). (A) Experimental eyes received 6-hr treatments with 10 nM 5-HT, 500 μ M iBuMeXan, or 5-HT + iBuMeXan during CT 7–13. (B) Experimental eyes received 6-hr treatments with 10 nM 5-HT, 60 μ M Ro-20-1724 (Ro), or 5-HT + Ro-20-1724 during CT 23–5. (C) Experimental eyes received 6-hr treatments with 2 mM 8-BzIS-cAMP, 10 μ M 5-HT, or 8-BzIS-cAMP + 5-HT. Each error bar spans a 95% confidence interval.

FIG. 4. Time course of change in cAMP in eyes produced by 10 μ M 5-HT (Inset) and change in cAMP produced by different concentrations of 5-HT. The responses are ratios of cAMP measured in groups of three experimental and control eyes. For the concentration experiments, the 5-HT treatments were 6 min in duration.

of cAMP appears to decrease after 10 min of exposure to 5-HT, and by 1.5 hr of treatment there appears to be little or no stimulation of cAMP by 5-HT.

The effect of different concentrations of 5-HT on cAMP was determined by treating eyes with 5-HT for 6 min. In addition, both the experimental and control groups were exposed to Ro-20-1724 (60 μ M). Ro-20-1724 for 6 min does not appear to affect basal levels of cAMP at the phase of these experiments. The threshold for detecting a change in cAMP appears to be between 10⁻⁷ M and 10⁻⁶ M 5-HT, though there may be a low level of stimulation at 10⁻⁸ M and 10⁻⁷ M (Fig. 4). cAMP increased over the range 10⁻⁷ M to 10⁻⁴ M 5-HT. The time course and dose-response curve for the effect of 5-HT on levels of cAMP in the eye resemble similar curves obtained from other *Aplysia* tissues (5, 8, 10, 12, 19).

Effects of 8-BzIS-cAMP and Phosphodiesterase Inhibitors on Optic Nerve Impulses. Exposure of eyes to 5-HT also decreases the frequency of spontaneous optic nerve impulses (1, 2) (see Figs. 1A and 2B during the treatment). 8-BzIS-cAMP and all the phosphodiesterase inhibitors used in this study except iBuMeXan also decreased (20–80%) the frequency of nerve impulses (Figs. 1C and D and 2A). iBuMeXan increased the frequency of impulses by about 200%. The fact that most of the drugs used in this study inhibited impulses indicates that the inhibitory effect of 5-HT on the eye may be mediated by cAMP. Interestingly, an inhibitory effect of 5-HT on a spontaneously active neuron (R15) in *Aplysia* is mediated by cAMP (6). Also, the endogenous cycling of the frequency of nerve impulses from the eye may be mediated by cycling of the intracellular level of cAMP (16).

The decrease in spontaneous nerve impulses produced by 5-HT, 8-BzIS-cAMP, and phosphodiesterase inhibitors raises the possibility that these treatments may shift the phase of the rhythm through their effects on the frequency of nerve impulses. However, the inhibitory effects of these compounds and their phase-shifting effects do not seem to be causally related, because there is no correlation between phase shifting and impulse inhibition in the data from this or other studies (3).

DISCUSSION

The maximal advance and delay phase shifts produced by 6-hr 5-HT treatments and other treatments used in these experiments are small. The small size of these phase shifts does not indicate that these are minor effects, because the maximal phase shifts produced by 6-hr pulses of bright light are equivalent to those produced by 5-HT and 8-BzIS-cAMP (2). Also, the magnitude of the effects reported here is well outside the limit for detecting phase shifts (≈ 1 hr, see *Methods*).

The 5-HT phase response curve is somewhat unusual with respect to the particular phases at which 5-HT produces advance and delay phase shifts. For instance, the 5-HT and light response curves are shifted about 12 hr relative to one another on the phase axis of the response curve, and most of the other response curves from *Aplysia* differ in significant ways from the 5-HT response curve (2). Thus, it is of some significance that 8-BzIS-cAMP and 5-HT advanced and delayed the rhythm at the same phases. In addition, two phosphodiesterase inhibitors, Ro-20-1724 and papaverine, produced advance phase shifts at the appropriate phase, but these agents were somewhat less effective in delaying the rhythm. It is not surprising that the effectiveness of phosphodiesterase inhibitors varied with the phase of treatment. A variation such as this would occur if the basal adenylate cyclase, phosphodiesterase activity, or both changed throughout the day. Diurnal rhythms of cAMP content and phosphodiesterase activity in a ganglion of *Helix* have been observed (20), and Strumwasser and Stephens have evidence for a rhythm of cAMP content in the eye of *Aplysia* (21).

Though Ro-20-1724 and papaverine had effects on the rhythm, the methylxanthines (theophylline and iBuMeXan) did not by themselves cause phase shifts. Others have found methylxanthines effective in triggering presumed cAMP-mediated responses in other parts of the *Aplysia* nervous system (e.g., refs. 7, 9, and 11). Variations in the effectiveness of phosphodiesterase inhibitors among different areas of the same tissue (e.g., mammalian brain) seem to be a common phenomenon (for review, see ref. 22) and have been related to the existence of different types of phosphodiesterases within the tissue. Thus, the types of phosphodiesterases present in some cells of the abdominal ganglion may be different from those in the eye. Though iBuMeXan did not shift the phase by itself, it potentiated the phase-shifting effect of 5-HT. A similar situation has been observed in the abdominal ganglion of *Aplysia*, in which theophylline alone did not increase cAMP but theophylline did increase the amount of cAMP produced by 5-HT (5).

The positive effects of the phosphodiesterase inhibitors in our studies appear to be of greater significance than their lack of effects in specific cases. Three structurally different phosphodiesterase inhibitors (Ro-20-1724, papaverine, and iBuMeXan) had effects either on the rhythm or on the phase-shifting effect of 5-HT. It seems unlikely that the effects of all three of these inhibitors could be explained by a mechanism that did not include an effect on phosphodiesterase, and thus on cAMP levels in the eye.

The dose-response curve for 5-HT-induced changes in cAMP can be compared with the dose-response curve for the phase-shifting effect of 5-HT presented elsewhere (3). Some caution is warranted in doing this, because the amount of cAMP after 6 min of 5-HT (Fig. 4) is being compared with phase shifts produced by 6-hr pulses of 5-HT. In addition, our measurements of cAMP were on intact eyes containing a heterogeneous population of cells. Changes in cAMP related to phase shifting by 5-HT could be largely masked by changes in cAMP in cells that are not involved in producing phase shifts in the rhythm. The threshold dose for phase shifting by 5-HT was between 10⁻⁸ and 10⁻⁷ M, and a large change in cAMP was seen between 10⁻⁷

and 10^{-6} M 5-HT. Thus, the two thresholds are within at least one order of magnitude of each other and might even be closer together because small amounts of stimulation of cAMP were observed at 10^{-7} and 10^{-8} M 5-HT. The stimulation of cAMP by 5-HT increased over three orders of magnitude, between 10^{-7} and 10^{-4} M 5-HT, whereas the phase shift was constant in magnitude between 10^{-7} and 10^{-3} M 5-HT. This type of difference between the dose-response curve for cAMP and that for the physiological response has been reported in many systems utilizing cAMP (23–25). Apparently, the effective pools of cAMP involved in producing physiological effects are small, close to basal levels, and may be compartmentalized within cells (26, 27).

There is not enough information available yet for strict comparisons between the time course of change in cAMP and phase shifting by 5-HT. cAMP was increased a large amount after 10 min of exposure to 5-HT (Fig. 4 *Inset*), but there appeared to be little or no stimulation of cAMP after 1.5 hr of 5-HT. Small but significant phase shifts are caused by 1.5-hr treatments of 5-HT at a phase different from that at which cAMP was measured (2). We have not yet examined the effects of pulses shorter than 1.5 hr. However, it seems unlikely that pulses of 5-HT as short as 3 min, which significantly stimulated cAMP, will shift the phase of the rhythm. Such brief transient changes in cAMP, which may be caused by brief environmental events, may be of no significance for entrainment of biological clocks and, in fact, may need to be filtered out. It may be that cAMP has to be increased for some minimal amount of time (≈ 1 hr) to produce effects on the rhythm. If this is so, determining which intracellular process (e.g., activation of kinases or phosphorylation of proteins) requires a long-term increase in cAMP for activation may yield an important clue to other steps in the phase-shifting pathway.

Many of the criteria for demonstrating that a physiological effect is mediated by cAMP (28) have been met for the effect of 5-HT on the rhythm. The phase shifts produced by 5-HT can be mimicked by an analogue of cAMP, 8-BzIS-cAMP. Phosphodiesterase inhibitors cause phase shifts by themselves and potentiate the effect of 5-HT on the rhythm. The phase-shifting effects of 8-BzIS-cAMP and 5-HT are nonadditive. 5-HT is capable of changing the level of cAMP in the eye. Finally, some additional support for a role of cAMP comes from our recent finding that the same treatment, increasing extracellular K^+ to 30 or 40 mM, blocks the effect of both 5-HT and 8-BzIS-cAMP on the rhythm (29). It is unlikely that all of these results could be explained by some process other than a change in cAMP mediating the effect of 5-HT on the rhythm, but further work is necessary to establish this firmly.

Our results are consistent with two possible roles of cAMP in the circadian system of the *Aplysia* eye: the cAMP system is part of an entrainment pathway, or it is part of the biological clock. If a change in cAMP represents part of the entrainment pathway, the next steps might entail phosphorylation of a protein through activation of a kinase by cAMP. Other steps in the pathway might follow from this until the information is in a form that can perturb and shift the phase of the circadian oscillator. Alternatively, changes in the activity of adenylate cyclase might be the end of the entrainment pathway and the beginning of the

clock system if the cAMP generating and degrading system forms the basis of the oscillating system in the eye. A model of the biological clock involving such a cAMP system, which will oscillate under certain conditions, has been proposed (30). The significance of our results is that they focus attention on the cAMP system either as a step in an entrainment pathway or as a part of the biological clock and that experiments can be performed in an attempt to discriminate between these two possibilities.

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