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Involvement of a specific protein in the regulation of a circadian rhythm in *Aplysia* eye

(serotonin/cAMP/34-kDa protein/gel electrophoresis)

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ABSTRACT **Our previous results indicated that protein synthesis was necessary for serotonin (5-hydroxytryptamine, 5-HT) to regulate the phase of the biological clock in the *Aplysia* eye. Also, we showed that 5-HT appeared to increase the synthesis of a 34-kDa protein with an isoelectric point of 7.2. Subsequent studies were carried out to quantitate the effect of 5-HT on the 34-kDa protein and to examine whether the 34-kDa protein was involved in the circadian timing system. The regional specificity of the effect of 5-HT on the 34-kDa protein was investigated. The proximal portion of the eye appeared to synthesize much more of the 34-kDa protein than the distal portion. Also, 5-HT had a much larger effect on the synthesis of the 34-kDa protein in the proximal portion than in the distal portion. The proximal location of synthesis and the 5-HT effect on the synthesis of the 34-kDa protein correlate with the proximal location of cells and processes that are necessary for the expression of the circadian rhythm. The relationship between the effect of 5-HT on the circadian rhythm and the effect of 5-HT on the 34-kDa protein was also examined. As 5-HT causes phase shifts in the rhythm by activating adenylate cyclase to increase cAMP, forskolin and 8-benzylthioadenosine 3′,5′-cyclic monophosphate mimicked the effect of 5-HT on the 34-kDa protein. We also found that 5-HT significantly increased the synthesis of the 34-kDa protein at phases when 5-HT delays or advances the phase of the rhythm but did not increase the synthesis of the 34-kDa protein at a phase when 5-HT did not phase shift. This phase-dependent effect of 5-HT on the 34-kDa protein qualitatively accounts for the phase dependence of the effect of 5-HT on the circadian rhythm. These results, when considered together with our earlier data, suggest that the synthesis of the 34-kDa protein is directly involved in the phase shift produced by 5-HT. The 34-kDa protein is worthy of future investigation as a candidate for a component of the circadian oscillator.**

Serotonin (5-hydroxytryptamine, 5-HT) regulates the phase of a biological clock in the eye of *Aplysia californica* (1–3). An objective of our research is to identify steps involved in the pathway through which 5-HT regulates the phase of this biological clock. Tracing this regulatory pathway should help us identify components of the oscillating mechanism because the pathway must end upon some component of the biological clock.

Our previous results indicated that 5-HT produced phase shifts by activating adenylate cyclase and increasing cAMP (4, 5). Anisomycin, an inhibitor of protein synthesis, blocked phase shifts produced by 5-HT and 8-benzylthioadenosine 3′,5′-cyclic monophosphate (8-benzylthio-cAMP) (6). These results and others suggested that protein synthesis was necessary for the production of phase shifts by 5-HT, and the step in the phase-shifting pathway that was sensitive to anisomycin was after the increase in cAMP produced by 5-HT. Moreover, 5-HT appeared to increase the synthesis of a 34-kDa protein* (6). Taken together, our previous results suggested that protein synthesis might be an integral step in the production of phase shifts by 5-HT.

In the research reported here, we investigated the possible involvement of the 34-kDa protein in the phase shift produced by 5-HT. We examined the correlation between the effect of 5-HT on the circadian rhythm and the effect of 5-HT on the synthesis of the 34-kDa protein by asking the following questions: Does elevation of cAMP increase the incorporation of labeled amino acid into the 34-kDa protein? Is the effect of 5-HT on the incorporation of labeled amino acid into the 34-kDa protein phase-dependent? Is the effect of 5-HT on the incorporation of labeled amino acid into the 34-kDa protein localized in a particular region of the *Aplysia* eye? The results of the experiments presented in this paper are consistent with a direct role of the 34-kDa protein in phase-shifting by 5-HT. A preliminary report of some of these studies has been published (7).

METHODS

Eyes were isolated from *A. californica* that were entrained to a 12-hr light:12-hr dark cycle. The connective tissue surrounding the isolated eyes was trimmed, and the optic nerves were cut away. Then, the eyes were pinned through the optic nerve stumps onto Sylgard (Dow Corning) in small polyethylene centrifuge tubes. Each experimental group consisted of six eyes (unless stated otherwise); the control group consisted of the contralateral eyes from the same animals. An eye with the optic nerve left attached was added to each group of trimmed eyes to monitor the circadian rhythm after exposure of the eyes to the experimental and control conditions. Both groups of eyes were kept in constant darkness at 15.5°C in 360 μl of buffered filtered seawater (BFSW) [artificial seawater (Instant Ocean, Aquarium Systems, Mentor, OH), pH 7.65 at 22°C, containing 30 mM Hepes, 100 units of penicillin G per ml, and 100 μg of streptomycin per ml].

The ocular circadian rhythm of compound action potentials is particularly sensitive to radiation (8), and the deleterious effect of ^{3}H-labeled or ^{35}S-labeled amino acids on the circadian rhythm has also been reported (6). To ensure that the events produced by the experimental treatments occurred in the presence of functioning circadian timing mechanisms, the experiments were done at radioactive concentrations (≈25 μCi of L-[4,5-^{3}H]leucine per ml; 1 Ci = 37 GBq) that allowed

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Abbreviations: 5-HT, serotonin (5-hydroxytryptamine); CT, circadian time; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; 8-benzylthio-cAMP, 8-benzylthioadenosine 3′,5′-cyclic monophosphate; BFSW, buffered, filtered seawater.

*Because of problems with molecular weight standards, the apparent molecular weight of this protein was incorrectly reported as 67,000 (6). Two independent methods of molecular weight calibration of two-dimensional polyacrylamide gels placed the apparent molecular weight of the protein at 34,000.

circadian rhythms in the frequency of optic nerve impulses to be observed in the eyes with optic nerves after the labeling treatments.

To investigate whether experimental treatments such as 5-HT or forskolin increased the incorporation of labeled amino acid, a group of eyes was exposed for 6 hr to the experimental treatment at a chosen time (phase). During the last 4 hr of the treatment, the experimental eyes were exposed to the treatment and L-[4,5-^{3}H]leucine (136 Ci/mmol; Amersham). The matched control group was treated for 4 hr only with L-[4,5-^{3}H]leucine. After exposure to the labeled amino acid, the eyes were rinsed with ice-cold BFSW. Then, the eyes without optic nerves were homogenized (as described below) for electrophoresis, and the eyes with optic nerves were set up for *in vitro* recording of spontaneous optic nerve impulses as described (9). The time of an experimental treatment was given as circadian time (CT), where CT 12 was the hour of offset of light in the light:dark cycle to which the intact animals had been entrained. Forskolin was first dissolved in dimethyl sulfoxide (Me_2SO). The final concentration of Me_2SO was <0.05% (vol/vol), and this concentration of Me_2SO has no effect on the *Aplysia* circadian rhythm (10).

Eyes, or portions of eyes, were homogenized on ice in 0.2-ml glass tissue grinders (Wheaton Scientific, Millville, NJ) containing 50 μl of grinding buffer (5 mM $MgCl_2$/10 mM Tris·HCl, pH 7.4, at 22°C), 5 μl of DNase (1 mg/ml), and 5 μl of RNase (1 mg/ml). When no pieces of tissue were visible, 50 μl of lysis buffer {9.1 M ultrapure urea, 2% (wt/vol) ampholytes, 5% (vol/vol) 2-mercaptoethanol, 3% (wt/vol) Nonidet P-40 (Sigma), or 3% (wt/vol) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Sigma)} was added, and grinding was continued for 5 more min. The homogenate was then transferred to a polyethylene centrifuge tube containing 28 mg of ultrapure urea. After the urea was dissolved, the sample was frozen and stored at −70°C. The sample was thawed and frozen at least twice before electrophoresis. In some experiments, the grinding buffer contained protease inhibitors (0.012% bacitracin, 0.012% trypsin inhibitor, 0.16% benzamidine, 0.03 trypsin inhibitor unit of aprotinin per ml, 0.018% phenylmethylsulfonyl fluoride) but no difference was noted between gels from samples with protease inhibitors and samples without inhibitors.

The amount of labeled amino acid incorporated into protein was estimated by measuring the radioactivity in material precipitable by trichloroacetic acid. Ten microliters of the sample homogenate was first mixed with 250 μl of distilled water and 50 μl of bovine serum albumin (1 mg/ml). The mixture was cooled down on ice, and then 100 μl of trichloroacetic acid [50% (wt/vol)] was added. After 20 min, the precipitate was collected by suction filtration on a glass fiber filter (Whatman GF/A), which was then rinsed with trichloroacetic acid [5% (wt/vol)] and dried with ethanol [95% (vol/vol)]. The dried filter was put into 5 ml of Liquiscint (National Diagnostics, Manville, NJ). The protein concentration in a sample was measured by using the microassay method of a Bio-Rad dye-reagent protein assay kit.

The proteins in the homogenates were separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Approximately equal amounts of trichloroacetic acid-precipitable radioactivity were loaded onto the experimental and control gels. Normally 20–25 μl of sample (roughly equivalent to the material in one eye) was loaded onto each tube gel. Experimental samples and their matched control samples were run on the same batch of gels, and the gels were run and processed at the same time under identical conditions. The gel pattern of label incorporation was visualized by fluorography. At least two internal replica gels were run with each experimental and control sample.

O'Farrell's technique for 2D-PAGE (11) was employed with a few changes. The size of the gels was reduced by using Minigels (Idea Scientific, Corvallis, OR) in the second dimension. A pH gradient of about 4–9 was established with a mixture of ampholytes—1 part of Pharmalyte (Pharmacia) (pH 3–10), 2 parts of Pharmalyte (pH 4–6.5), and 2 parts of Pharmalyte (pH 6.5–9)—such that the final concentration of ampholytes in the isoelectric focusing gel solution was 2% (wt/vol). The isoelectric focusing gels were pre-run for 30 min by setting the voltage limit to 300 V and the current limit such that the starting voltage was 200 V. Then proteins were focused by running the gels at a constant voltage of 300 V for at least 14 hr.

After fixation of slab gels in trichloroacetic acid [50% (wt/vol)] for 1 hr and then in acetic acid [7% (vol/vol)] for at least 1 hr, the slab gels were rinsed with distilled water for 10 min and equilibrated with Autofluor (National Diagnostics) for 1 hr. After equilibration, the experimental and matched control gels were dried onto the same piece of filter paper. A single sheet of x-ray film (X-Omatic AR, Kodak) was placed over the dried gels and exposed for 2 weeks at −70°C. The resulting developed film had the fluorographs of the experimental and control gels.

A microscope (Leitz Dialux 20) equipped with a photometer (Leitz MPV compact microscope photometer) was used for quantitating the density of the 34-kDa spot on the fluorographs. The instrument was calibrated for transmittance measurement by focusing the microscope on a region of the film outside the boundaries of the gels and setting the meter reading to 100% transmittance. The window passing the transmitted light from the fluorograph to the phototube was just big enough to enclose the largest 34-kDa spot among all of the gels. To compare the transmittance of the spot for the 34-kDa protein between experimental and control gels, a ratio of the transmittance of the 34-kDa spot in the experimental gel to that in the control gel was calculated. To correct any bias due to a difference in total radioactivity between experimental and matching control gels, three matched reference spots of similar sizes to the 34-kDa spot were chosen on each gel. The ratio of the transmittance of each reference spot in the experimental gel to that in the control was then calculated. The transmittance ratio of the 34-kDa spot for each pair of experimental and control gels was then corrected for differences in sample radioactivity by dividing the ratio of the transmittance of the 34-kDa spot by the mean ratio of the transmittance of the three reference spots. Since at least two pairs of gels were run from the samples of each experiment, a representative relative transmittance was obtained for each experiment by averaging the relative transmittance of the 34-kDa spot over all pairs of gels for that particular experiment. Statistics were then calculated using this mean relative transmittance for each experiment with n being the number of completely separate experiments.

RESULTS

Effects of Forskolin and 8-benzylthio-cAMP. The effect of 5-HT on the circadian rhythm is mediated by cAMP, and anisomycin blocks the effect of 5-HT and 8-benzylthio-cAMP on the rhythm (4–6). If the 34-kDa protein is involved in mediating the effect of 5-HT on the rhythm, then treatments capable of elevating cAMP should change the expression of the 34-kDa protein as it is changed by 5-HT. Since forskolin, an activator of adenylate cyclase (12), and 8-benzylthio-cAMP have been shown to mimic the effect of 5-HT on the rhythm (4, 5), we investigated whether forskolin and 8-benzylthio-cAMP would mimic the effect of 5-HT on the 34-kDa protein.

Six-hour treatments with forskolin (1–10 μM) from CT 06 to 12 significantly increased the incorporation of labeled

amino acid into the 34-kDa protein (Fig. 1). The mean relative transmittance of the 34-kDa spot on the fluorographs (experimental transmittance divided by control transmittance; see *Methods* for definition) was 0.50 ± 0.22 (±95% confidence interval, see Table 1). Also, 6-hr 8-benzylthio-cAMP (1 mM; ICN) treatments starting at CT 06 increased the amount of label associated with the 34-kDa protein (Table 1). The effects of forskolin and 8-benzylthio-cAMP on the 34-kDa protein were very similar to the effect of 5-HT on the 34-kDa protein at this same phase of the circadian cycle (Table 1).

Phase Dependence of the Effects of 5-HT. As illustrated by the phase–response curve of the effect of 5-HT on the circadian rhythm (2), the effect of 5-HT on the phase of the circadian rhythm was clearly phase-dependent. 5-HT produced advance, delay, or zero phase shifts, depending on the phase when the treatment was applied. To investigate whether or not the effect of 5-HT on the 34-kDa protein was also phase-dependent, 5-HT treatments (5 μM) for 6 hr were applied at three phases of the circadian rhythm (Fig. 2). 5-HT had significant effects on the 34-kDa protein when eyes were treated at phases when 5-HT produced phase shifts and had no effect at a phase when 5-HT produced no phase shift. When 5-HT advanced the circadian rhythm (CT 06–12), 5-HT significantly increased the synthesis of the 34-kDa protein (mean relative transmittance = 0.55 ± 0.06, Table 1). When 5-HT delayed the circadian rhythm (CT 21:30–03:30), 5-HT also significantly increased the synthesis of the 34-kDa protein (mean relative transmittance = 0.76 ± 0.20). However, when 5-HT did not produce phase shifts (CT 15:30–21:30), 5-HT produced no difference between the spots for the 34-kDa protein on experimental and control fluorographs (mean relative transmittance = 1.02 ± 0.16). The values of the relative transmittance of the 34-kDa spot at each of the three phases were all significantly different from one another (Student's t test, $P < 0.02$), and a one-way analysis of variance showed that the groups were significantly different (F ratio = 33.08, $P < 0.0001$).

Regional Variation of Label Incorporation. We decided to compare the incorporation of labeled amino acid into the 34-kDa protein in different portions of the eye for two reasons. First, we wondered if the effect of 5-HT on the synthesis of the 34-kDa protein was uniformly distributed over the eye or localized to some particular portion of the eye. If the effect were localized, this would indicate that the effect might be specific for a certain localized type of cell rather than nonspecific for all cells or a type of cell such as glia, connective tissue, or smooth muscle that is spread evenly over the eye. Second, the proximal portion of the eye contains cells or processes that are necessary for the expression of the circadian rhythm (8, 13, 14). If a protein were involved with the biological clock in the *Aplysia* eye, it might be located mainly in the proximal portion of the eye.

Table 1. Effect of treatments on the incorporation of [^{3}H]leucine into a 34-kDa protein

Treatment	Phase (effect on rhythm)	No. of experiments (gel pairs)	Experimental/control*
Forskolin	CT 06:00–12:00 (advance)	5 (9)	0.50 ± 0.22
8-benzylthio-cAMP	CT 06:00–12:00 (advance)	2 (4)	0.51
5-HT	CT 21:30–03:30 (delay)	3 (6)	0.76 ± 0.20
5-HT	CT 06:00–12:00 (advance)	13 (30)	0.55 ± 0.06
5-HT	CT 15:30–21:30 (no effect)	5 (9)	1.02 ± 0.16

*Mean relative transmittance of the 34-kDa spot ±95% confidence intervals. Any bias due to the difference in the total radioactivity between experimental and control gels has been compensated.

Ten eyes with optic nerves attached were treated with 5-HT (5 μM) for 6 hr starting at CT 06, and the eyes were labeled with [^{3}H]leucine during the latter 4 hr of the 5-HT pulse. After rinsing with ice-cold BFSW, each eye was dissected into a distal and a proximal portion in the following way. A cut was made near the cornea, and the lens was gently squeezed out. Then, a cut was made across the eye, dividing it into distal and proximal portions, and finally the optic nerve was removed (Fig. 3). The distal and proximal portions were homogenized separately for 2D-PAGE. Equal amounts of radioactivity from the distal portions and proximal portions were loaded onto separate gels.

Clearly, more label was associated with the 34-kDa protein in the proximal portions than in the distal portions of 5-HT-treated eyes (Fig. 3 *A* and *C*, mean relative transmittance = 0.24 ± 0.05, $n = 5$, nine gel pairs). Thus, the effect of 5-HT on the 34-kDa protein was not a general effect spread evenly over the eye but mainly localized in the proximal portion of the eye.

In addition to the 34-kDa protein, there were at least four other proteins with more label associated with them in the proximal portions than in the distal portions, and at least four other proteins had more label associated with them in the distal portions than in the proximal portions. The uneven distribution of label was to be expected since cell types and processes are not evenly distributed over the eye (15, 16).

The effect of 5-HT on the 34-kDa protein in proximal versus distal portions of *Aplysia* eyes might reflect only a difference in the relative amounts of synthesis (more synthesis in the proximal than in the distal portion) in the two portions of the eye. We investigated this possibility by exposing 10 eyes to 5-HT (CT 06–12) and [^{3}H]leucine and the other matched 10 eyes to [^{3}H]leucine only. The eyes were then dissected into proximal and distal portions as described above. Incorporation of label into the 34-kDa protein was observed to be greater in the proximal than in the distal portions (Fig. 3 *B* and *D*, mean relative transmittance = 0.67 ± 0.23, $n = 3$, four gel pairs). Also, 5-HT increased the incorporation of label into the 34-kDa

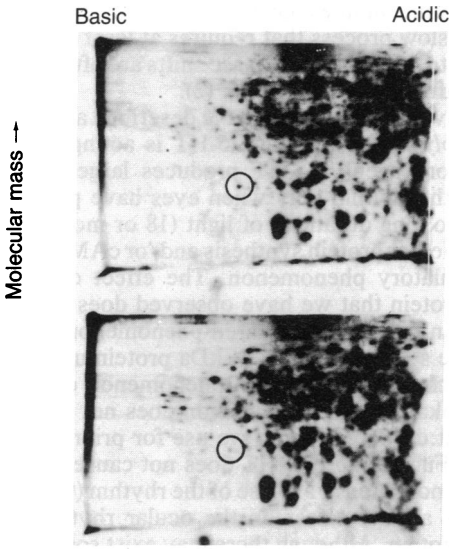

FIG. 1. Effect of forskolin on the incorporation of [^{3}H]leucine into proteins. (*Upper*) An experimental group of isolated eyes was treated with forskolin (1 μM) for 6 hr starting at CT 06 and with [^{3}H]leucine during the last 4 hr of the forskolin treatment. (*Lower*) A matched control group of isolated eyes was exposed only to [^{3}H]leucine. The amount of label associated with a protein (circled) was increased by the forskolin treatment. This protein has an apparent molecular mass of 34 kDa and an isoelectric point of 7.2.

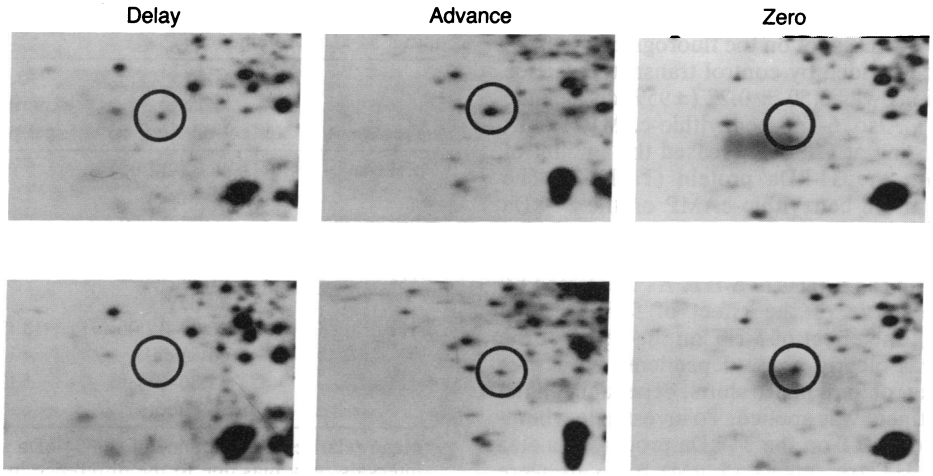

FIG. 2. Phase-dependent effect of 5-HT on the amount of [^{3}H]leucine incorporated into the 34-kDa protein. Experimental eyes (5 μM 5-HT and [^{3}H]leucine; *Upper*) and matched control eyes ([^{3}H]leucine only; *Lower*) were treated at three different phases of the circadian rhythm when 5-HT produces delay, advance, or no phase shifts (Zero). 5-HT increased the amount of label associated with the 34-kDa protein at phases when 5-HT produces phase shifts, but 5-HT had no effect on the 34-kDa protein at a phase when 5-HT does not produce a phase shift (see Table 1 for details). Also note that the spot representing the 34-kDa protein in the control gel at the phase when 5-HT does not phase shift was the darkest when compared with the control gels of the other phases.

protein in the proximal portions of the eyes (Fig. 3 *A* and *B*, mean relative transmittance = 0.36 ± 0.10, $n = 3$, four gel pairs) but had little or no effect on the incorporation of label into the 34-kDa protein in the distal portions (Fig. 3 *C* and *D*, mean relative transmittance = 0.88 ± 0.17, $n = 3$, four gel pairs). This last set of experiments confirmed that 5-HT had its major effect on the 34-kDa protein in the proximal portion of the eye. Also, this set of experiments yielded the additional finding that much more of the 34-kDa protein appeared to be synthesized in the proximal portions than in the distal portions of untreated eyes.

DISCUSSION

Our previous research showed that protein synthesis was necessary for 5-HT to regulate the phase of the *Aplysia* ocular rhythm and that 5-HT increased the synthesis of a 34-kDa protein (6). In addition to producing phase shifts in the circadian rhythm, 5-HT has at least two other effects on the *Aplysia* eye: 5-HT suppresses the generation of spontaneous optic nerve impulses and it increases the photosensitivity of the eye (1, 17). Which of these effects of 5-HT may involve the 34-kDa protein? 5-HT, 8-benzylthio-cAMP, forskolin, and phosphodiesterase inhibitors shift the phase of the circadian rhythm, inhibit spontaneous optic nerve activity, and increase photosensitivity of the eye (1, 4–6). However, anisomycin, an inhibitor of protein synthesis, does not interfere with the effects of 5-HT on the spontaneous optic nerve activity and photosensitivity (6). Thus, protein synthesis does not appear to be necessary for these two other effects of 5-HT. The time course of the effects produced by 5-HT also argues against an involvement of protein synthesis in the suppression of spontaneous optic nerve impulses and the increase in photosensitivity. The suppression of impulses and increase in photosensitivity are rapid effects that reach maxima within minutes. On the other hand, phase-shifting by 5-HT is a slow process that requires at least a 1-hr exposure of the eye to 5-HT, and the phase shifts are often not apparent for days after 5-HT treatments (2).

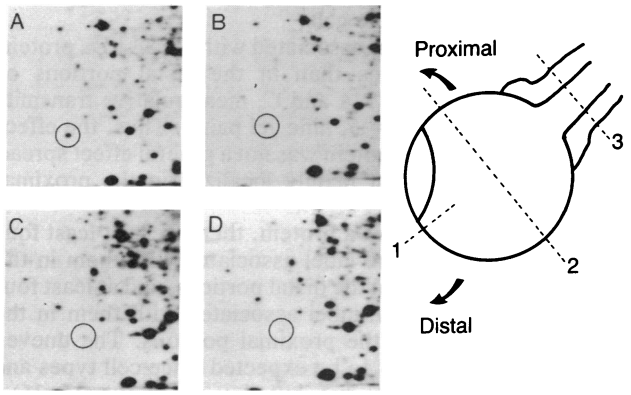

FIG. 3. Regional variation in the synthesis of the 34-kDa protein and the effect of 5-HT on the synthesis of the 34-kDa protein. A group of 10 eyes was treated with 5-HT (CT 06–12) and labeled with [^{3}H]leucine (*A* and *C*) while a matched control group was only exposed to [^{3}H]leucine (*B* and *D*). After the treatments, each eye was cut near the cornea (dashed line no. 1) to remove the lens. Then, the eye was cut into proximal and distal portions (dashed line no. 2), and the optic nerve was cut away (dashed line no. 3). The 34-kDa protein is circled. The 34-kDa protein was mainly synthesized in the proximal portions of the eyes (*B* vs. *D*). 5-HT increased the incorporation of label into the 34-kDa protein in the proximal portions (*A* vs. *B*) but had little or no effect in the distal portions of the eyes (*C* vs. *D*).

Nadakavukaren *et al.* (18) have described an effect of 5-HT on the *Aplysia* eye in which 5-HT is acting as a so-called "modulatory agent;" 5-HT produces large effects on the phase of the rhythm only when eyes have previously been exposed to long durations of light (18 or more hr). It is not known whether protein synthesis and/or cAMP is involved in this modulatory phenomenon. The effect of 5-HT on the 34-kDa protein that we have observed does not seem to be involved in the Nadakavukaren phenomenon because 5-HT affects the synthesis of the 34-kDa protein under conditions in which the Nadakavukaren phenomenon does not occur. The Nadakavukaren phenomenon does not occur following 12:12 light:dark cycles that we use for prior entrainment of animals. Furthermore, 5-HT does not cause the Nadakavukaren phenomenon at a phase of the rhythm (CT 06–12) when 5-HT has a large effect on the ocular rhythm and on the 34-kDa protein. Although there may exist some other effects of 5-HT that have not been observed, the phase-shifting effect of 5-HT that we have studied is so far the only known effect in which the 34-kDa protein may be involved.

A possible involvement of the 34-kDa protein in producing phase shifts by 5-HT was investigated by examining the specificity of the effect of 5-HT on the protein and by examining the correlation between the effect of 5-HT on the rhythm and the effect of 5-HT on the 34-kDa protein. The

synthesis of the 34-kDa protein and the effect of 5-HT on the synthesis of the protein exhibited spatial specificity. As shown by cutting eyes into proximal and distal portions, the synthesis and the effect of 5-HT on the synthesis of the 34-kDa protein occurred almost exclusively in the proximal portion of the eye. Therefore, the synthesis and the effect of 5-HT on the synthesis of the 34-kDa protein did not occur in all cells or in a type of cell distributed evenly throughout the eye. The distal and proximal portions of eyes should contain approximately equal amounts of smooth muscle and glial cells. The distal portions should contain all of the corneal cells and most of the cells and processes of photoreceptors and pigmented support cells, whereas the proximal portions should contain most of the neurosecretory cells [called secondary neurones by Jacklet *et al.* (19)] and neuropil (16). Hence, the neurosecretory cells or perhaps other not yet identified cells or processes located primarily in the proximal portion of the eye are candidates for the location of the stimulatory effect of 5-HT on the synthesis of the 34-kDa protein.

The results of several studies have shown that the normal functioning of the proximal portion of the *Aplysia* eye is both necessary and sufficient for the expression of the ocular circadian rhythm (8, 13, 14). Therefore, the location of both the synthesis and the effect of 5-HT on the synthesis of the 34-kDa protein correlate with the location of cells necessary for the expression of the circadian rhythm.

The effect of 5-HT on the 34-kDa protein also correlated with the effect of 5-HT on the circadian rhythm with respect to phase dependence. 5-HT significantly increased the synthesis of the protein at the phases when 5-HT produces phase shifts, and 5-HT did not affect the synthesis of the protein at the phase when 5-HT does not produce a phase shift. Why does 5-HT increase the synthesis of the 34-kDa protein when 5-HT advances and delays the circadian rhythm? Suppose the phase of the circadian rhythm is dependent on the amount of 34-kDa protein, and the amount of the 34-kDa protein oscillates throughout a cycle. An increase in synthesis of the 34-kDa protein produced by 5-HT during the rising phase of the oscillation would advance the phase, whereas an increase in synthesis of the 34-kDa protein during the declining phase of the oscillation would delay the phase. Therefore, the effect of 5-HT on the 34-kDa protein can at least qualitatively account for the effect of 5-HT on the circadian rhythm at various phases of the rhythm.

An important question is whether the synthesis or amount of the 34-kDa protein varies through a circadian cycle. The amount of labeled amino acid incorporated into the 34-kDa protein appeared to be highest at the phase when 5-HT does not shift the rhythm (Fig. 2), but the reliability of this observation is questionable because radioactivity was not standardized across experiments. Future work is necessary before we can know if there is a cyclic variation in labeled amino acid incorporation into the 34-kDa protein.

The results of our experiments are consistent with a direct involvement of the 34-kDa protein in phase-shifting by 5-HT. Protein synthesis is required for 5-HT to phase shift, and 5-HT appears to affect the synthesis of the 34-kDa protein (6). Forskolin and 8-benzylthio-cAMP mimicked the effect of 5-HT on the rhythm, and they mimicked the effect of 5-HT on the 34-kDa protein. The effect of 5-HT on the protein exhibited spatial specificity. Finally, the effect of 5-HT on the 34-kDa protein was phase-dependent.

Possible direct roles of the 34-kDa protein in phase-shifting may be as a component of the entrainment pathway, a component of the time-keeping mechanism, or a component of both. At some point along a pathway through which an entraining agent regulates a biological clock, the entrainment information must affect some component of the time-keeping mechanism. This point is the end of the entrainment pathway and an integral component of the time-keeping mechanism. Consequently, this component will have characteristics of the entrainment pathway and the time-keeping mechanism. An involvement of the 34-kDa protein in the time-keeping mechanism is consistent with previously described effects of protein synthesis inhibitors on the phase and period of the *Aplysia* ocular circadian rhythm (20–22).

Although all of our results point to a direct role of the 34-kDa protein in phase-shifting, we still cannot rule out the possibility that the 34-kDa protein plays a role in the output pathway of the circadian clock. For instance, the 34-kDa protein may be a neurosecretory product that varies with a circadian rhythm. The synthesis of the 34-kDa protein may be regulated by or tightly coupled to some other protein whose synthesis is an integral part of the oscillatory mechanism. Discrimination among various specific roles of the 34-kDa protein in the *Aplysia* ocular circadian system will require the ability to manipulate specific proteins within cells.

By using 5-HT as a probe of the circadian system in the *Aplysia* eye, we have found two specific molecules, cAMP and a 34-kDa protein, worthy of study as possible components of the circadian time-keeping mechanism. These results seem to justify our approach of tracing entrainment pathways and indicate that a similar approach using light, neurotransmitters, or hormones may be fruitful in other organisms. Many questions concerning the roles of cAMP and the 34-kDa protein remain unanswered. Because we have shown in this paper that cAMP appears to affect the expression of the 34-kDa protein, one of the most interesting and important questions to answer in the future is whether the 34-kDa protein controls the level of cAMP.

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