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Cyclic changes in cAMP concentration and phosphodiesterase activity in a mammalian circadian clock studied in vitro

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The mammalian suprachiasmatic nuclei (SCN) contain a circadian pacemaker that continues to keep 24-h time when isolated in vitro. We are investigating the role of cAMP in the cellular mechanisms underlying SCN function. We have previously shown that increasing intracellular cAMP during the subjective day resets the SCN pacemaker in the in vitro rat brain slice preparation[8,20]. We now report that the level of cAMP fluctuates within the rat SCN under constant conditions in vitro. The level of endogenous cAMP is high during late day and late night, and low during early night. These changes in cAMP concentration are accompanied by opposite changes in phosphodiesterase activity; we detected no significant change in adenylate cyclase activity. These results provide further support for the hypothesis that cAMP is involved in circadian function in the SCN.

INTRODUCTION

Mammals display a wide variety of behavioral and physiological rhythms that are organized around the daily solar cycle. The timing for many of these circadian rhythms is generated by a pacemaker located within the suprachiasmatic nuclei (SCN). Although this clock is normally synchronized to the environment by information it receives through the retina[15,19], it does not depend upon environmental timing cues to continue running with a circadian period. Under constant environmental conditions these rhythms continue with a near 24-h period provided that the SCN and their efferent pathways are intact.

The endogenous nature of the time-keeping mechanism is apparent when the SCN are isolated in a brain slice and maintained in vitro. Pacemaker characteristics attributed to the SCN from in vivo studies are preserved in the suprachiasmatic brain slice. Under these conditions the SCN produce 24-h oscillations of spontaneous electrical activity[8,20] and vasopressin secretion[4,10] for several days. The rhythm of electrical activity, and thus the underlying pacemaker, is stable across inbred animals, such that the time of peak activity shows little variability[8,20]. The clock appears unperturbed by the process of tissue preparation during the daytime[6,7], since the electrical activity rhythm in vitro matches the electrical activity rhythm seen in vivo[13].

Circadian systems are composed of cellular processes that can participate in circadian timing in 3 ways: (1) within an entrainment pathway afferent to the pacemaker; (2) as a part of the time-keeping mechanism of the pacemaker; or (3) as an output of this clock. Those processes that function as inputs to, or as elements of, the pacemaker can be identified through the ability of specific treatments to perturb the phase of circadian rhythms of SCN in vitro. One such cellular process may involve the intracellular second messenger cAMP. In vitro treatments that stimulate cAMP-dependent processes (e.g. application of cAMP analogs or agents that increase endogenous cAMP levels) advance the SCN clock by 4–5 h[8,20]. In contrast to the nocturnal phase-shifting effects of cGMP analogs[21], these cAMP-stimulating treatments are effective only during the subjective daytime, the hours corresponding to the period that lights are on in the donor colony. The largest advances are induced by treatments between circadian time 3 and 7 (where circadian time, CT, measured in hours, begins at CT 0 when the lights come on in the colony, and continues for 24 h before starting over).

These results suggest that cAMP could act within an entrainment pathway afferent to the pacemaker. If

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cAMP is solely part of an input pathway, then the concentration of cAMP within the SCN should remain constant unless perturbed by an external signal. Additionally, or alternatively, cAMP could be controlled by the pacemaker, either as part of the time-keeping mechanism or as an output from it. If either of the latter cases applies, then the concentration of cAMP should oscillate within the SCN in the absence of external timing input.

To determine whether cAMP levels oscillate with the progression of the pacemaker cycle, we measured the concentration of cAMP within SCN at 4 time-points from the first circadian cycle in vitro. Measurements were made on SCN from brain slices maintained under conditions where they have been shown to generate a circadian rhythm of neuronal firing rate in the absence of afferent input[7,10,20]. We further measured the levels of cAMP in the nearby anterior hypothalamus of the same brain slices to compare with the levels seen in the SCN.

Changes in level could result from changes in the rate of synthesis and/or degradation of cAMP. These processes are controlled by the enzymes adenylate cyclase (AC) and phosphodiesterase (PDE), respectively. In the second part of this study, the activities of AC and PDE were measured in the SCN and hypothalamus maintained in vitro in order to assess their contributions to cAMP regulation.

MATERIALS AND METHODS

Brain slice preparation

Slices were prepared from 7.5–9-week-old Long–Evans rats of both sexes obtained from our colony, which has been inbred for more than 15 generations to improve genetic homogeneity and thus ensure little variability in the circadian clock[20]. All animals were housed in a 12-h light, 12-h dark cycle. The process of slice preparation and maintenance has been described previously[20]. Briefly, coronal slices containing the SCN and surrounding tissue were prepared during lights-on and placed in the center of a Hatton-style brain slice dish[12]. One 500 μm slice containing the middle section of the SCN was prepared from each rat. The outer chamber of the dish was filled with dH_2O warmed to 37°C and continuously aerated with 95% O_2/5% CO_2. The inner chamber was perfused at 30 ml/h with warmed, oxygenated Earle's balanced salt solution (GIBCO) supplemented with 24.6 mM glucose and 26.3 mM sodium bicarbonate, pH 7.4.

SCN were studied at 4 time points separated by 6-h intervals. The necessity of preparing slices during the daylight hours[6,7] caused tissue to be maintained in vitro for differing lengths of time. Slices to be assayed for cAMP concentration and/or adenylate cyclase activity at CT 4, 10, 16 and 22 were maintained in the slice dish for 3.00–17.75 h, 3.75–7.50 h, 5.25–15.00 h, and 14.75–18.75 h, respectively, prior to tissue collection. Slices to be assayed for PDE activity at CT 4, 10, 16 and 22 were maintained 3.25–3.75 h, 4.25–9.25 h, 6–14 5 h, and 12.25–15.5 h, respectively Possible effects of these varying incubation times were examined in slices initiated at different times but incubated for similar periods.

Tissue collection

Discrete brain regions were obtained by micropunch of frozen brain slices (method modified from Palkovits and Brownstein[18]) At the designated time ±0 5 h, a slice was removed from the slice chamber and rapidly frozen on a slide cooled with dry ice. Once frozen, the two SCN and two nearby areas of the anterior hypothalamus were removed using a frozen 26-gauge syringe needle with the tip filed to a round, bevelled opening. Punches from 2–3 slices (tissue processing method 1), 5–6 slices (tissue processing method 2), or 4 slices (tissue processing method 3) were collected in Eppendorf tubes kept frozen in dry ice; samples were subsequently stored for 1.5 h to 10 days at –70°C (mean storage time = 2.5 days)

Tissue processing

Method 1. Punches were disrupted with a probe sonicator (Vibra Cell by Sonics Materials) for 10 s in 500 μl cold (4°C) dH_2O containing 6% trichloroacetic acid and 0.05 mM RO 20-1724 (a specific inhibitor of cAMP phosphodiesterase[3]). Samples were then centrifuged at 2500 *g* for 15 min at 4°C. The supernatant of each sample was removed and saved. The pellet was resuspended in 150 μl 0.1 *N* NaOH and stored frozen for protein assay. The supernatant from each sample was extracted 4 times with 2 ml cold H_2O-saturated ethyl ether, discarding the upper ether phase each time. The samples were then dried overnight in a 70°C oven. Dried samples were resuspended in 150 μl of 50 mM sodium acetate (pH 6.2), acetylated with 3 μl triethylamine and 1.5 μl acetic anhydride, and then assayed for cAMP[24].

Method 2. Punches were sonicated for 10 s in 200 μl cold dH_2O containing 1 mM dithiothreitol, 1 mM EGTA, 0.05 mM RO 20-1724, and 24 mM sodium HEPES (pH 7.4) (after ref. 14). A 50 μl aliquot of this solution was removed and stored frozen for protein assay. A 70-μl aliquot of the solution was removed and frozen for cAMP assay. The remainder of the sample was used to measure AC activity. The 70-μl aliquot was later centrifuged at 4°C for 15 min at 2500 *g*; 60 μl of the supernatant was saved, and the rest discarded This 60-μl aliquot was diluted 1 1 with 50 mM sodium acetate (pH 6.2), acetylated with 2.4 μl triethylamine and 1.2 μl acetic anhydride, and assayed for cAMP

Method 3. Punches were sonicated for 10 s in 80 μl cold 100 mM Tris-HCl, pH 8 0, containing 3.75 mM mercaptoethanol[27]. Of the 80 μl, 20 μl were used to assay for PDE activity, and the rest of the sample was stored frozen for protein assay.

Protein assay

Protein concentrations were measured by the protein dye method of Bradford[2]. Samples were assayed in duplicate, using 50-μl aliquots (for samples processed by Method 1) or 20 μl aliquots (Methods 2 and 3), with bovine serum albumin (Sigma, fraction V) as the protein standard. Protein levels averaged 2.82 ± 0.15 μg per tissue punch.

cAMP assay

All samples were assayed for cAMP using the method of Steiner, with acetylation[24]. Briefly, 50 μl of either acetylated sample or acetylated cAMP standard was added to 50 μl sodium acetate buffer (50 mM, pH 6.2), 50 μl cAMP antiserum (a gift of Dr. V D. Ramirez; diluted to obtain 30–60% binding), and 50 μl ^{125}I-labeled cAMP (10,000 cpm). The mixture was then incubated for 18–24 h at 4°C. After incubation, 100 μl gamma-globulin (4 mg/ml) and 2 ml 95% ethanol were added to precipitate the bound cAMP. The mixture was centrifuged at 2500 *g* for 20 min at 4°C, the supernatant discarded, and the pellet counted on a gamma counter. The sensitivity of the assay was 1 fmol. The inter- and intra-assay coefficients of variation were 24% and 7.4% at 100 fmol/tube, respectively.

Adenylate cyclase assay

Adenylate cyclase activity was determined as pmol cAMP produced/mg protein/min incubation at 37°C. Samples processed by Method 2 were assayed for cAMP production using a method described previously[1,22]. A 20 μl aliquot of a tissue sample was added to an assay tube that contained 10 μl dH_2O, 50 μl incubation me-

dium (dH_2O containing 2 mg/ml BSA, 0.8 mM EGTA, 2 mM dithiothreitol, 0.1 mM RO 20-1724, 24 mM creatine phosphate, 5 U/ml creatine phosphokinase, 100 mM HEPES at pH 7.4, and 16 mM $MgCl_2$), and 10 μl GTP (10 μM). To this, 10 μl ATP (10 mM) was added to start the reaction, and samples (assayed in duplicate) were incubated 15 min at 37°C. At the end of the incubation period, 400 μl of 50 mM sodium acetate, pH 4.75, was added to each tube and the tubes were placed in boiling water for 2 min. Samples were then centrifuged (15 min at 2500 *g*), and the supernatant assayed for cAMP, with acetylation, as described above. All measurements were corrected for basal cAMP levels and cross-reaction to reagents in the medium by subtracting measurements obtained from samples where the ATP was added after the incubation period.

Phosphodiesterase assay

Samples processed by Method 3 were assayed in duplicate for PDE activity by the method of Thompson[26,27]. Briefly 10 μl tissue sample and 10 μl snake venom (1 mg/ml *Ophiophagus hannah;* Sigma) were added to an assay tube containing, in final concentration, 0.57 μM cAMP, 4 mM Tris-HCl (pH 8.0), 1.11 mM mercaptoethanol, 1.46 μM 5′-AMP, 1.7 mM $MgCl_2$, and 2 μCi[^{3}H]cAMP (1 mCi/ml; New England Nuclear) to produce a final volume of 70 μl. Each tube was then incubated for 15 min at 30°C. (The remainder of each sample was stored frozen for protein assay). The reaction was stopped by adding 1 6 ml resin slurry (BioRad AG 1-X2, 200–400 mesh, mixed 1:2 with dH_2O) to each tube. Assay tubes were centrifuged 10 min at 2500 *g*, and the supernatant was removed to a clean tube. Another 1.5 ml resin slurry was added to the supernatant, the tubes were recentrifuged, and the supernatant from each removed to a scintillation vial. Twenty milliliters of scintillation fluid (Scintiverse; Fisher Scientific) was added to each vial, and the samples were counted for 10 min each on a scintillation counter (total counting efficiency = 36%). PDE activity was first expressed as pmol adenosine produced/mg protein. Because of variability between assays, PDE activity for each sample within an assay (which contained one sample from each circadian time) was normalized to the highest activity. Percent activity in SCN and hypothalamic samples was calculated separately.

Histology

To confirm placement of the tissue punches, a slice was saved that had one SCN punched and the contralateral hypothalamus punched. The slice was then immersed for 10 min in warm 0.01% Methylene blue, rinsed twice with normal perfusion medium, and stored in cold (4°C) medium overnight. This procedure stains the SCN differently from the surrounding hypothalamus, and thus allows for histological analysis[25].

Statistical analysis

One- or two-way ANOVA was used to analyze differences between multiple groups, with $P < 0.05$ required for statistical significance. Because of the degree of variation in the samples, the data were scrutinized for extreme outliers, identified as lying beyond 3 interquartile ranges from outer quartile boundaries[17]. For analytical classes with significant ANOVA levels, comparisons between individual groups were analyzed with reference to the mean squared variance of the entire group using Fisher's protected least significant difference procedure[17]. Pairwise comparisons were analyzed further using the more stringent Tukey–Kramer test[17].

RESULTS

Tissue punch location

Examination of a Methylene blue stained slice from which punches had been obtained revealed that discrete, clean cores of tissue had been removed (Fig. 1). In this slice, the right SCN and part of the left hypothalamus were punched out, leaving the contralateral areas intact for comparison. The SCN punch does not extend outside the boundaries of the SCN, and is representative of the SCN punches obtained for this study. The hypothalamic punch is located dorsal and lateral to the SCN, and is within the anterior hypothalamus. Although the exact location of the hypothalamic punches varied slightly from slice to slice, they included primarily anterior hypothalamic tissue, with possibly a small amount of the paraventricular nucleus in some samples. In all cases the hypothalamic punches were obtained from the same slice as the SCN punches.

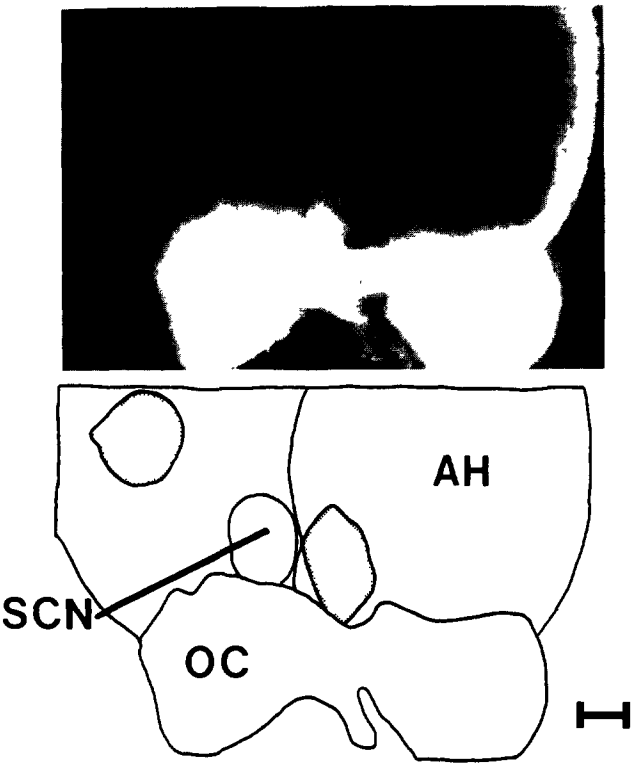

Fig. 1. Placement of SCN and hypothalamic tissue punches. Top: stained whole mount of the hypothalamic slice preparation. For the purpose of illustration, tissue punches were made of the right SCN and part of the left anterior hypothalamus in this slice. The left SCN, left intact for comparison, is stained darker than the surrounding hypothalamus. In experiments, punches were taken of the paired SCN and bilateral hypothalamic areas. Bottom: line drawing of the stained slice, indicating the location of the intact SCN, the optic chiasm (OC), and the anterior hypothalamus (AH). Shaded areas indicate the location of the tissue punches. Comparison with the intact half indicates that the SCN punch is located within the boundaries of the SCN; this placement is typical of the SCN punches. The hypothalamic punch is located within the anterior hypothalamus, in the general area of all the hypothalamic punches. However, the exact location of these punches varied. Calibration bar = 100 μm.

Basal cAMP levels

Basal cAMP levels were determined by two methods, the standard radioimmunoassay protocol of Steiner et

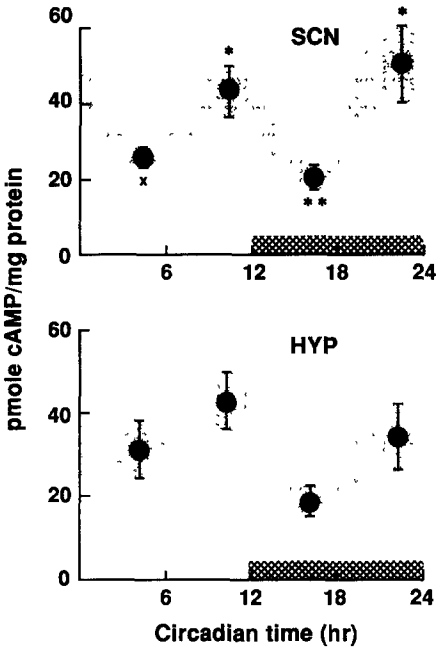

Fig. 2. Changes in cAMP levels in the SCN (top) and anterior hypothalamus (HYP, bottom) measured at 4 points over the circadian cycle in vitro. Plotted are the mean levels of cAMP ± S.E.M. from SCN (top) and HYP (bottom) samples obtained at the 4 circadian times. The area within the ranges of the S.E.M.s has been shaded to help visualize the pattern of changes. Only in the SCN are the fluctuations in cAMP levels significant (ANOVA, $P < 0.02$). Horizontal bar indicates the time of lights-off in the donor colony Fisher LSD analysis of pairwise comparisons of SCN time points indicate the following probability levels: *, the level is significantly different from CTs immediately succeeding ($P < 0.05$); **, the level is significantly different from that at the succeeding CT ($P < 0.020$); x, different at $P < 0.10$.

al.[24] and as part of the adenylate cyclase assay[22]. The samples were assayed in 8 separate determinations, with 4–11 samples analyzed per assay. Two-way ANOVA indicated no significant differences in the results due to the type of tissue processing method used (see Table I). Therefore, the data for cAMP levels from the two methods were combined for further analysis. Outlier analysis identified 3 samples out of the 45 cAMP determinations as lying more than 3 interquartile ranges beyond the outer quartile boundaries. All were in the CT 4 group from SCN analyzed by method 2. These were excluded from further analysis. The combined data include the results of 7–14 samples obtained at each time point.

The mean levels of cAMP found in the SCN at the 4 times sampled are summarized in Fig. 2 (top) and Table II. There is a significant variation in cAMP levels in SCN obtained at the 4 circadian times ($P < 0.02$, one-way ANOVA). Pairwise differences examined relative to the mean square of the variance were significant at $P < 0.05$ for 3 pairs: CTs 10 vs 16 ($P < 0.05$), CTs 16 vs 22 ($P < 0.020$), and CTs 22 vs 4 ($P < 0.05$, Fisher's protected least significant difference test). The remaining comparison, mean cAMP levels at CTs 4 vs 10, differed only at $P < 0.10$. Thus, the significant points of variation in cAMP levels are the decrease from CT 10 to a low at CT 16, followed by a second high at CT 22, and the subsequent drop at CT 4.

The levels of cAMP found in the anterior hypothalamus at the 4 time points sampled from the same slices are summarized in Fig. 2 (bottom) and Table II. As with the SCN cAMP data, two-way ANOVA indicated no significant differences in the data due to tissue processing method so the data were combined for further analysis. While the general pattern of cAMP levels is similar to that seen in the SCN, the differences are not significant ($P = 0.09$, one-way ANOVA). Because these data did not demonstrate significant changes in cAMP levels in the anterior hypothalamus over the day, they were not analyzed further.

TABLE I

In vitro levels of cAMP in the SCN and hypothalamus obtained using two tissue processing methods

cAMP expressed as pmol/mg protein.

	Circadian time (h)			
	4	*10*	*16*	*22*
SCN				
Method 1	27.27 ± 0.62 ($n = 5$)	44.22 ± 12.56 ($n = 6$)	25 74 ± 4.52 ($n = 5$)	66.44 ± 16 3 ($n = 6$)
Method 2	32.76 ± 10.0 ($n = 5$)	42.97 ± 9.23 ($n = 8$)	13 74 ± 0.61 ($n = 4$)	35.43 ± 10.74 ($n = 6$)
Hypothalamus				
Method 1	36.05 ± 11.11 ($n = 5$)	52 17 ± 3.84 ($n = 6$)	26.19 ± 3.92 ($n = 5$)	41.63 ± 14.15 ($n = 6$)
Method 2	27.15 ± 10.72 ($n = 5$)	36.67 ± 11.69 ($n = 8$)	9 46 ± 1.28 ($n = 4$)	27.11 ± 9.9 ($n = 6$)

TABLE II

Levels of cAMP, adenylate cyclase activity, and phosphodiesterase activity in the SCN and hypothalamus in vitro

cAMP expressed as pmol/mg protein. AC expressed as pmol cAMP/mg protein/min. PDE expressed as % activity.

	Circadian time (h)			
	4	*10*	*16*	*22*
SCN				
cAMP[a]	26.52 ± 1.13[b] (n = 7)	43.5 ± 6.68[c] (n = 14)	20.41 ± 3.01[d] (n = 9)	50.97 ± 9.7[c] (n = 12)
AC	16.51 ± 5.25 (n = 7)	11.51 ± 3.88 (n = 7)	8.93 ± 2.76 (n = 7)	31.88 ± 19.27 (n = 7)
PDE[a]	64.98 ± 13.38[e] (n = 7)	27.18 ± 4.73[d] (n = 6)	68.71 ± 12.69[e] (n = 7)	36.4 ± 11.58[f] (n = 7)
Hypothalamus				
cAMP	31.6 ± 7.04 (n = 10)	43.31 ± 6.83 (n = 14)	18.75 ± 3.7 (n = 9)	34.37 ± 8.17 (n = 12)
AC	16.98 ± 7.32 (n = 7)	22.58 ± 7.05 (n = 7)	12.89 ± 4.65 (n = 7)	39.21 ± 18.5 (n = 7)
PDE	40.85 ± 10.91 (n = 7)	74.68 ± 13.55 (n = 7)	40.73 ± 16.87 (n = 6)	53.74 ± 22.18 (n = 6)

[a] ANOVA across circadian time is significant ($P < 0.05$).
[b] Different from the CT 10 at $P < 0.10$, Fisher's LSD procedure.
[c] Significantly different from the CT immediately succeeding ($P < 0.05$, Fisher's LSD).
[d] Significantly different from the CT immediately succeeding ($P < 0.025$, Fisher's LSD).
[e] Different from CT 22 at $P < 0.06$, Fisher's LSD.
[f] Different from CT 4 at $P < 0.09$, Fisher's LSD.

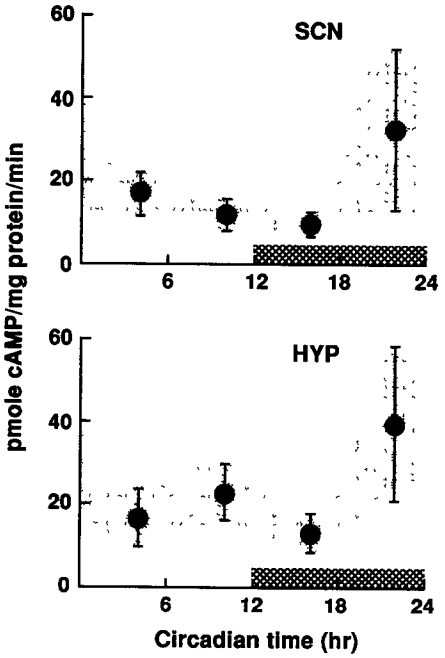

Fig. 3. AC activity in the SCN and anterior hypothalamus over the circadian cycle in vitro. Plotted are the mean amounts of cAMP produced by the SCN (top) and hypothalamus (HYP, bottom) ± S.E.M. for the 4 CTs samples. No significant changes in AC activity were observed in either brain region (ANOVA). See Fig. 2 legend for details.

Adenylate cyclase and phosphodiesterase activities.

The levels of AC activity in the SCN at the 4 circadian times sampled are summarized in Fig. 3 (top) and Table II. These data are the result of 7 samples at each time point, assayed in 7 separate determinations, with samples from either 2 or 4 circadian times assayed together. While the mean level of activity at CT 22 appears higher than those at the other times sampled, the changes in activity are not significant ($P < 0.3$, one-way ANOVA). Because the variance of the data for CT 22 is much greater than that at the other times, we transformed the data by taking their square roots. This equalized the variances (data not shown), but reanalysis still indicated no significant differences ($P < 0.5$). The levels of AC activity found in the hypothalamic samples (7 samples per time point except for CT 16, where one was lost; samples were assayed together with the SCN samples) are summarized in Fig. 3 (bottom) and Table II. The pattern of changes seen here is similar to that in the SCN, and again is not significant, either in its original form ($P < 0.3$) or after transformation ($P < 0.4$).

The levels of PDE activity found in the SCN are summarized in Fig. 4 (top) and Table II. These data consist of 7 samples at each time point except CT 10, where one sample was lost. Each assay contained one sample from each time point, and thus the data from each assay could be expressed as percent PDE activity. The range of PDE

activity is 4.59–145.46 pmol cAMP degraded/mg protein (mean = 29.81 ± 6.78). In contrast to the AC results, there are significant changes in PDE activity across the 24 h ($P < 0.05$; one-way ANOVA). These changes in PDE activity correlate with those seen in cAMP concentration, such that at intervals when cAMP levels increase (CTs 4 vs 10 and 16 vs 22), PDE activity decreases ($P < 0.05$ and 0.06, respectively, Fisher's LSD test). This trend appears again when cAMP levels decrease: PDE levels increase significantly between CT 10 and 16 ($P < 0.025$); between CT 22 and 4 the increase does not reach significance ($P < 0.09$). The most significant of these changes is the increase in PDE activity between CT 10 and 16 ($P < 0.05$, Tukey-Kramer).

The levels of PDE activity measured in the hypothalamus are summarized in Fig. 4 (bottom) and Table II. These data consist of 7 samples at CTs 4 and 10, and 6 samples at CTs 16 and 22 (two samples were lost). Hypothalamic PDE activity ranges from 0.1 to 237.21 pmol cAMP degraded/mg protein (mean = 48.27 ± 10.2). Unlike the results from the SCN, no significant changes in PDE activity are seen in the hypothalamus.

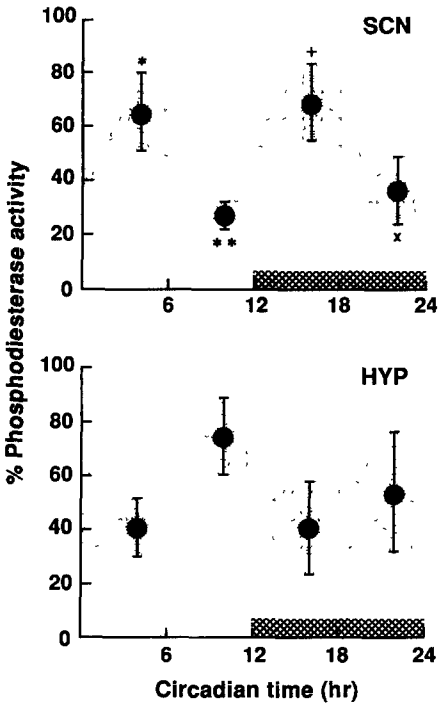

Fig. 4 PDE activity in the SCN and anterior hypothalamus over the circadian cycle in vitro. Plotted are the relative amount of cAMP degraded by SCN (top) and hypothalamic (HYP, bottom) samples ± S.E.M at the 4 CTs samples Only the variation in SCN PDE activity is significant (ANOVA, $P < 0.05$). *, PDE activity is significantly different from CT immediately succeeding ($P < 0.05$, Fisher's LSD test); **, PDE activity significantly different from CT 16 ($P < 0.025$, Fisher's LSD, $P < 0.05$, Tukey-Kramer); +, PDE activity different from that at CT immediately succeeding at $P < 0.06$; x, PDE activity different from CT 4 at $P < 0.09$ See Fig 2 legend for details

DISCUSSION

Basal cAMP levels

These results show that the level of cAMP in the SCN changes over the circadian cycle in vitro. The pattern of change suggests that cAMP levels are relatively high just prior to subjective dawn and dusk, and drop off significantly in the early night. Thus, the changes appear to have a circadian period, although more frequent sampling may confirm ultradian fluctuations. These changes in cAMP concentration are not likely to be the result of a general decrease or increase in cAMP levels beginning at the time of sacrifice, since some of the highest measurements of cAMP were obtained from tissue maintained in vitro for the shortest time (3.75–7.5 h for CT 10), as well as tissue maintained for the longest time (14.75–18.75 h for CT 22). Furthermore, a recent report has demonstrated that length of time in vitro does not significantly affect the level of a related purine cyclic nucleotide, cGMP, in SCN[29]. Rather, the differences in cAMP concentration appear to reflect regulated changes in the level of cAMP within the SCN in vitro. Whether the changes in cAMP concentration reflect a sustained oscillation in cyclic nucleotide levels associated with SCN timekeeping remains to be determined over longer incubation periods in vitro.

Our findings are similar in some aspects to those of a study by Murakami and Takahashi that examined cAMP levels from the SCN of freshly dissected slices of rat brain[16]. Both found a significant variation in cAMP levels in the SCN over the course of the day. The present study identified the source of change as endogenous to the SCN. The range of concentration changes in cAMP levels measured in the present study is similar to that seen ex vivo, with a difference between high and low mean levels of 30–35 pmol cAMP/mg protein in each case. However, the absolute levels of cAMP differ slightly between the two studies, with the previous study finding higher overall levels of cAMP. In both studies the changes in cAMP may be limited to a subset of cells in the SCN. By analyzing tissue punches, the studies would have averaged what may have been locally more robust fluctuation. In the freshly dissected tissue, a peak in cAMP concentration occurred at CT 9, which is close to the CT 10 high reported here. The difference between the two studies suggests that a true peak may occur earlier than CT 10, but well after CT 4. Murakami and Takahashi did not observe a second rise in cAMP concentration during subjective night; CT 22 was the nadir in each condition they tested. The most likely explanation for differences between the two studies must derive from procedural differences in preparation of the tissue.

If the changes seen in cAMP in the SCN were due

only to differences in the viability of the tissue in vitro or to inter-assay differences, then we would expect to find comparable changes in the hypothalamus. However, the changes in the hypothalamus were much smaller, suggesting that the SCN has some weak modulatory influence on hypothalamic cAMP levels. Neuroanatomical studies have shown that, in addition to the large output to the nearby paraventricular nuclei, some SCN efferents pass through the anterior hypothalamus[23,28]. Thus, some of the hypothalamic punches may have included tissue that normally receives SCN efferents. If these connections remained intact in the brain slice, then the SCN could have modulated the hypothalamic levels of cAMP to some extent. These results correlate with electrophysiological observations of regions of the slice peripheral to the SCN in which no circadian rhythm of firing rate was found[11] and with studies of surgically reduced slices in which clock properties could be localized to the SCN[9].

Adenylate cyclase and phosphodiesterase activities

No significant changes in AC activity were detected in either the SCN or the hypothalamus, although there was a trend toward higher AC activity at CT 22 in both areas. There are several possible explanations for the increased variability at CT 22. One is that AC activity increases around this phase in a pulsatile manner, so that the probability of sampling the tissue during one of the pulses of high activity is small. Another possibility is that AC activity peaks slightly earlier or later than CT 22, so that the samples in this study were taken when AC activity was either increasing or decreasing. More frequent sampling of AC activity at times surrounding CT 22 could help determine if this latter possibility is correct. Further research may determine whether this variability has functional importance in the SCN.

The changes in PDE activity seen in the SCN in vitro correlate with the changes observed in cAMP concentration. That is, the activity of this cAMP degradative enzyme decreases over the same time intervals that cAMP rises (CT 4–10 and CT 16–22) and increases between CT 10 and 16, when there is a significant decrease in cAMP levels. This correlation may be coincidental, reflecting separate processes in different cell types, or these changes may be the result of regulated changes in PDE activity within a single cell type. Until the changes in cAMP and PDE can be localized to the same cell, a causal relationship cannot be established.

No significant changes in PDE activity were seen in the hypothalamus. This does not mean that the level of PDE activity does not fluctuate in vitro in the hypothalamus. Rather, it means that we were not able to detect any changes using these methods and sampling at these circadian times. Together with the unchanging AC activity in the hypothalamic samples, these results are consistent with the finding that cAMP concentration does not change significantly in the hypothalamus.

The PDE changes in SCN do not appear to be related to the amount of time between tissue preparation and collection since both high and low levels of PDE activity were measured from tissue maintained in vitro short (CTs 4 and 10) and long (CTs 16 and 22) periods of time. Furthermore, the changes in PDE activity in the SCN were not seen in the hypothalamus, indicating that the differences were not due either to changes in the condition of the tissue in vitro or to interassay differences.

cAMP and circadian function of the SCN

Some definition of the relationship of cAMP to SCN function can be formed from our aggregate data. Previously we have shown that treatments that increase endogenous cAMP levels can reset the SCN circadian pacemaker in vitro, but only during the circadian day (CT 3–11)[8,20]. Whether this window of sensitivity represents a temporally limited set of receptors or effectors within an input pathway, or a temporally restricted effector element that is part of the pacemaker, cannot yet be determined. However, the finding that endogenous cAMP levels change significantly over the circadian cycle in SCN under constant conditions in vitro suggests that cAMP could function in a time-keeping or output role involving regulated changes in its concentration, even in the absence of cAMP changes mediating signal transduction from an input pathway during phase-shifting.

The temporal sequence of cAMP changes in this system suggests integrated circadian functions. The endogenously regulated changes between CT 10 and 16 follow the period of sensitivity to cAMP-induced resetting and thus may be hypothesized to be more related to time-keeping functions compared with the later increase at CT 22, which must be output related as cAMP stimulation at this time has no effect on phase. The results suggest that throughout the circadian cycle PDE activity may be an important clock-controlled point at which cAMP metabolism is regulated.

Perhaps not coincidently, the phase-shifts induced in the daytime by treatments that stimulate cAMP pathways advance the phase of the clock toward the time of the relative high in cAMP near dusk. This supports the notion that regulated changes in the level of cAMP late in the day may play a role in time-keeping. That is, the daytime treatments that stimulate cAMP pathways may induce phase advances by stimulating substrates already present in anticipation of a relative high in cAMP that occurs several hours later. Specific protein kinase A (PKA; cAMP-dependent protein kinase) substrates have recently been reported to be temporally restricted to this portion of the circadian cycle in SCN in vitro[5]. Perhaps the dephosphorylated state of one or several PKA -substrates is a substrate for time-keeping between mid- and late-day, and cAMP activation of PKA with attendant

phosphorylation events marks the end of the day by mediating the procession into a night-time state. Through early activation of PKA and specific phosphorylation events, input-stimulated synthesis of cAMP during the day could step-advance the clock to the point of night onset (dusk). In any case, the identity of the cells in which phase-shifts are induced, those in which cAMP levels fluctuate and those in which protein kinase A substrates appear must be determined in order to establish any functional relationship.

In summary, we have shown that the level of cAMP fluctuates in SCN in vitro with high levels during late day and late night, and low levels during early night. These changes in cAMP concentration within the SCN occur in antiphase to changes in PDE activity and in the apparent absence of changes in AC activity. These results suggest that regulation of PDE by the SCN pacemaker may produce an endogenous oscillation in cAMP. It remains to be determined whether this circadian change in cAMP comprises part of the clock mechanism or is strictly an output from it.

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