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cGMP induces phase shifts of a mammalian circadian pacemaker at night, in antiphase to cAMP effects

(suprachiasmatic nuclei/circadian rhythms/brain slice/circadian clock/rat)

REBECCA A. PROSSER*, ANGELA J. MCARTHUR†, AND MARTHA U. GILLETTE*†‡

University of Illinois at Urbana–Champaign, *Neural and Behavioral Biology Program, and †Department of Physiology and Biophysics, 524 Burrill Hall, 407 South Goodwin, Urbana, IL 61801

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ABSTRACT **The suprachiasmatic nuclei (SCN) of mammals contain a circadian clock that synchronizes behavioral and physiological rhythms to the daily cycle of light and darkness. We have been probing the biochemical substrates of this endogenous pacemaker by examining the ability of treatments affecting cyclic nucleotide-dependent pathways to induce changes in the phase of oscillation in electrical activity of rat SCN isolated in brain slices. Our previous work has shown that daytime treatments that stimulate cAMP-dependent pathways induce phase shifts of the SCN pacemaker *in vitro* but treatments during the subjective night are without effect. In this study we report that the phase of SCN oscillation is reset by treatments that stimulate cGMP-dependent pathways, but only during the subjective night. Thus, the nocturnal period of SCN sensitivity to cGMP is in antiphase to the diurnal period of sensitivity to cAMP. These results suggest that cAMP and cGMP affect the SCN pacemaker through separate biochemical pathways intrinsic to the SCN. These studies provide evidence that changing biochemical substrates within the SCN circadian clock may underlie some aspects of differential temporal sensitivity of mammals to resetting stimuli.**

Mammals, like other eukaryotic organisms, possess an endogenous pacemaker that keeps near 24-hr time (1). This circadian clock, which can be entrained by the environmental lighting cycle, organizes an animal's behavior and physiology into daily rhythms. The primary circadian clock in mammals is located in the suprachiasmatic nuclei (SCN) (2, 3). The endogenous nature of this oscillator is apparent from experiments demonstrating that the SCN generate a stable circadian rhythm of neuronal activity for at least three cycles when isolated in a hypothalamic brain slice (4, 5). Furthermore, the pacemaker appears unperturbed by the process of brain-slice preparation during the animal's day (6) since this *in vitro* rhythm matches the rhythm of electrical activity produced by the SCN *in vivo* (7).

Circadian rhythms in intact animals are reset by step changes in constant lighting conditions at different phases of their entrained lighting cycle. Organisms undergo circadian changes in sensitivity to resetting stimuli so that in constant darkness their rhythms are reset by 15-min pulses of light during the night, but not the day (8, 9). Conversely, in constant light their rhythms are advanced by 2- to 3-hr pulses of darkness primarily when they occur during the day, although longer (6 hr) pulses of darkness can induce delays during the night (10, 11). Specific neurotransmitters injected into the SCN region can also induce phase resetting of behavioral rhythms (12–17). Like photic stimuli, their efficacy is usually limited to a specific portion of the circadian cycle, although the results of the *in vivo* pharmacological studies are sometimes contradictory (13, 15–17). The cellular basis of circadian change in sensitivity to resetting stimuli is unknown and is the subject of our investigations.

We have been studying the resetting characteristics of the SCN circadian pacemaker *in vitro*. This approach should reveal which properties predicted from *in vivo* studies are endogenous to this biological clock and should allow identification of their biochemical substrates. In earlier experiments we showed (4, 5) that *in vitro* treatments that increase endogenous cAMP levels reset the phase of the SCN clock when applied during subjective day (the time of lights-on in the donor colony) but not during subjective night. These results suggested that changing sensitivity to cAMP, within either an entrainment pathway or the clock mechanism, may underlie the sensitivity of the organism to some daytime phase-shifting stimuli. To further probe biochemical mechanisms associated with the SCN clock, we have investigated the ability of treatments that stimulate cGMP-dependent pathways to reset the SCN clock *in vitro*. cAMP and cGMP are involved in a wide variety of cell functions (18, 19), sometimes in an antagonistic relationship (20), and so it was of interest to determine whether the SCN clock shows a different sensitivity to cGMP as compared with its sensitivity to cAMP.

MATERIALS AND METHODS

The preparation, maintenance, and recording procedures used in these experiments have been reported in detail (5). Briefly, 500-μm coronal brain slices were prepared during lights-on from adult Long–Evans rats born in our inbred colony and housed under 12 hr of light/12 hr of dark conditions. Slices were maintained in a brain-slice chamber (21) continuously perifused with Earle's balanced salt solution (GIBCO) supplemented with 24.6 mM glucose and 26.2 mM sodium bicarbonate (pH 7.4) at 37°C and saturated with an atmosphere of 95% O_2/5% CO_2. In the 3-day experiment, 0.5% gentamicin was added to the perifusion medium that was sterilized by filtration. At various times after slice preparation, perifusion was stopped and the bath medium was replaced for 1 hr with freshly made medium containing either 0.5 mM 8-bromoguanosine 3′,5′-cyclic monophosphate (Br-cGMP) or 0.5 mM N^2,$O^{2'}$-dibutyrylguanosine 3′,5′-cyclic monophosphate (Bt_2-cGMP) at pH 7.4 that had been oxygenated and warmed for 5 min. These membrane-soluble analogs of cGMP activate cGMP-dependent protein kinases and resist degradation by phosphodiesterase (22, 23). After 1 hr this medium was replaced completely with normal medium, and perifusion was resumed. Previous experiments

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Abbreviations: BzA-cAMP, 8-benzylaminoadenosine 3′,5′-cyclic monophosphate; Br-cGMP, 8-bromoguanosine 3′,5′-cyclic monophosphate; CT, circadian time; Bt_2-cGMP, $N^2O^{2'}$-dibutyrylguanosine 3′,5′-cyclic monophosphate; SCN, suprachiasmatic nuclei.

‡To whom reprint requests should be addressed.

have shown that the procedure of changing the perifusion medium by itself does not phase-shift the SCN clock (4).

In each experiment the effect of this treatment upon the circadian pacemaker was assessed by monitoring the subsequent cycle(s) of neuronal activity in one SCN. The spontaneous activities of single SCN cells encountered at random throughout the nucleus were monitored extracellularly by using glass microelectrodes filled with 5 M NaCl. Only clearly isolated single units with a signal/noise ratio of ≥2 were studied. After assessing the stability of its firing rate, each cell was monitored for two 120-sec periods using 10-sec bins and then the mean firing rate was determined. On average, four cells were monitored per hour over the course of 7–18 hr during each experiment. The firing rates of individual cells were averaged over 2-hr intervals using 1-hr lags to obtain a measure of the SCN neuronal population's circadian rhythm of firing rate, and from this we determined the time of peak firing rate in the circadian cycle(s) after treatment.

Our previous work showed that the time of peak neuronal activity recorded from SCN in untreated slices varies little across animals from our inbred line (6). Furthermore, the rhythm remains stable for at least 3 days *in vitro*: the mean time of peak electrical activity in unperturbed slices occurs at circadian time (CT) 7.1 ± 0.1 hr (n = 3), 6.9 ± 0.2 hr (n = 8), and 6.6 ± 0.4 hr (n = 3) on days 1–3 *in vitro*, respectively (where CT divides the circadian cycle into 24 hr and CT 0 = lights-on in the donor colony) (5). Thus the time of peak activity is a reliable measure of the phase of the underlying circadian pacemaker, and we could, therefore, determine phase shifts by comparing the time of peak activity in treated slices with that of untreated slices (5).

RESULTS

Treatment with Br-cGMP during the middle of subjective day had little effect upon the subsequent time of peak activity. For example, as shown in Fig. 1*A*, after Br-cGMP treatment between CT 7–8 on day 1 *in vitro* the electrical activity rhythm peaked at CT 6.25 of day 2, indicating a phase advance of 0.65 hr. The results of 1-hr Br-cGMP treatments during the subjective daytime are summarized in the phase–response curve in Fig. 2, where the response of the pacemaker (the change in the time of peak activity in each experiment) is plotted according to the circadian phase of the treatment. As can be seen, Br-cGMP treatments between CT 0 and 11 induced phase shifts ranging from −0.75 to 1.25 hr, with a mean phase advance of 0.24 ± 0.24 hr (n = 8).

In contrast to daytime treatments, application of Br-cGMP during subjective night induced large shifts in the phase of the electrical activity rhythm, with the magnitude of phase change dependent upon the time of treatment. For example, as shown in Fig. 1*B*, treatment at CT 18–19 induced a phase advance of 6.5 hr, such that the peak in activity occurred at CT 0.5. Overall, the mean phase advance induced by 1-hr Br-cGMP treatments between CT 12 and 22 was 4.38 ± 0.71 hr (n = 10). The phase shifts induced by these nocturnal Br-cGMP treatments are summarized in Fig. 2. The largest phase advances, 6–7 hr, occurred after 1-hr Br-cGMP treatments between CT 16 and 18, with smaller advances induced by treatments earlier and later during the subjective night.

The responses to nighttime Br-cGMP treatments were stable across two cycles, as shown in Fig. 3. The advanced time-of-peak (at CT 0.5) observed during the first cycle after treatment was seen again nearly 24 hr later (at CT 1.0) during the second cycle after treatment. This indicates that Br-cGMP treatment for 1 hr at CT 18 completely resets the underlying circadian pacemaker during the first 6 hr after treatment.

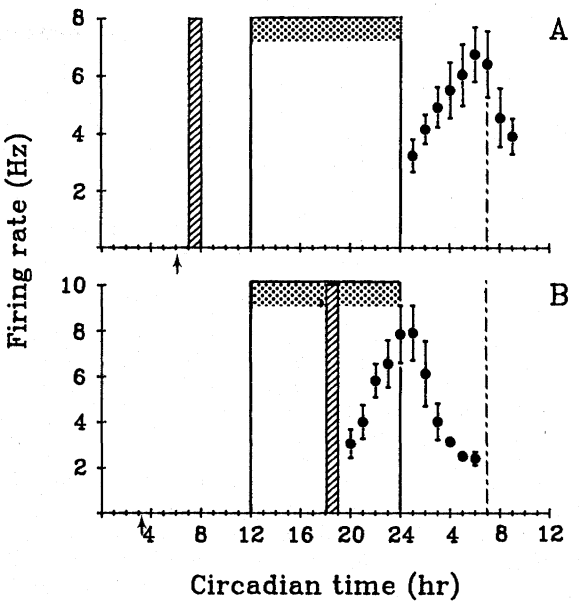

FIG. 1. Differential effects of Br-cGMP on the SCN's rhythm of electrical activity. Plotted are running 2-hr values (mean ± SEM), lagged by 1 hr, of all single units recorded in individual experiments according to the circadian time at which they were recorded. (*A*) Br-cGMP (0.5 mM) treatment at CT 7–8 had little effect on the phase of the electrical activity rhythm in the cycle after treatment, so that the subsequent time of peak activity occurred at CT 6.25. (*B*) Br-cGMP treatment at CT 18–19 phase-advanced the subsequent time of peak activity by 6.5 hr, to CT 0.5. Horizontal stippled bar, subjective night for the SCN, the time of lights-off in the donor colony; vertical hatched bar, time of treatment; dotted line, mean time of peak activity in untreated slices; arrow, time of tissue preparation.

If the phase shifts induced by Br-cGMP treatment at night were the result of specifically stimulating cGMP-dependent pathways, then treatment with other active analogs of cGMP should induce similar shifts. To test this, we treated slices with Bt_2-cGMP, the only other cGMP analog we found commercially available, at two circadian times. Application of 0.5 mM Bt_2-cGMP during the subjective night at CT 18–19 phase-advanced the electrical activity rhythm by 4.71 ± 0.41 hr (n = 4), whereas application during the subjective day at

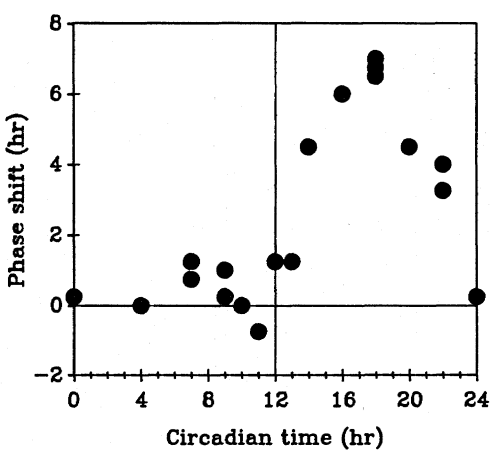

FIG. 2. Phase–response curve for 1-hr treatments with 0.5 mM Br-cGMP. Changes in the phase of the electrical activity rhythm induced by Br-cGMP treatments *in vitro* vs. unperturbed controls are plotted according to the circadian time of treatment. Each dot indicates the result of a single experiment. The largest phase advances occurred after treatments during mid-subjective night, whereas treatments during subjective day had little effect.

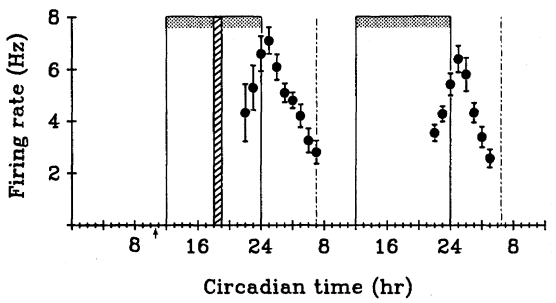

FIG. 3. Br-cGMP induces stable phase shifts *in vitro*. Slices were treated at CT 18–19 on day 1 *in vitro*. Peak activity on days 2 and 3 is at CT 0.5 and 1.0, respectively. Thus, the phase advances are complete within 6 hr and represent stable shifts of the underlying pacemaker. See Fig. 1 for details.

CT 7 had little effect on the time of peak electrical activity in the subsequent cycle (0.25-hr phase delay, $n = 2$).

The differential responsiveness of the SCN clock to analogs of cAMP and cGMP is illustrated in Fig. 4, where the phase–response curves for 8-benzylaminoadenosine 3′,5′-cyclic monophosphate (BzA-cAMP, results replotted from ref. 5) and Br-cGMP are shown together. This figure summarizes the results of 28 experiments with BzA-cAMP treatment at 16 CTs and 18 experiments with Br-cGMP treatment at 13 CTs. It can be seen that the sensitivities of the SCN clock to stimulation of cAMP- and cGMP-dependent pathways are 12 hr out-of-phase and virtually nonoverlapping: sensitivity to the cAMP analog appears in the subjective day between CT 3 and 12, whereas sensitivity to the cGMP analog occurs in the subjective night between CT 14 and 22.

DISCUSSION

These results demonstrate that the phase of the SCN pacemaker can be reset *in vitro* by treatments that selectively stimulate cGMP-dependent pathways. Further, they indicate that the SCN clock is sensitive to cGMP analogs only during the subjective night of the circadian cycle, a time when light stimuli *in vivo* advance behavioral rhythms. The sensitivity of the SCN to cGMP analogs occurs in antiphase to the time of sensitivity to cAMP analogs. Thus, these results suggest that the changing sensitivity of the organism to resetting stimuli may have its origin, at least in part, in the differential

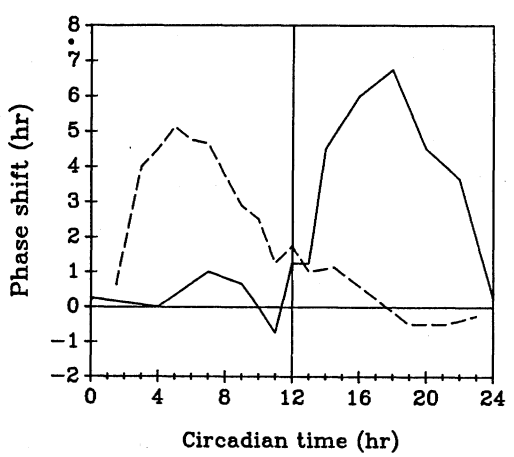

FIG. 4. Combined phase–response curves for 1-hr treatments of 0.5 mM BzA-cAMP (dashed line) and Br-cGMP (solid line). Changes in the phase of the SCN electrical activity rhythm induced by these treatments are plotted according to the CT of treatment. The phase–response curve for BzA-cAMP is based upon 28 experiments over 16 CTs (replotted from ref. 5). The data for Br-cGMP, replotted from Fig. 2, is based upon 18 experiments at 13 CTs.

sensitivity of the SCN to stimulation of cGMP- or cAMP-dependent pathways.

The phase advances induced by these 1-hr treatments with cGMP analogs are robust and appear to be complete within 6 hr of treatment. In these aspects the phase shifts induced by Br-cGMP and Bt_2-cGMP are similar to those induced by a series of three 10-min pulses of high (50 mM) K^+ *in vitro*, which also phase-advances the rhythm 6–7 hr when applied between CT 17 and 19 (24). The ability of cGMP analogs to phase-shift only at night is also similar to the effects of several *in vivo* treatments, including light pulses (9) and electrical stimulation (25). Although the mechanisms through which these treatments shift the SCN pacemaker have yet to be determined, our results suggest that at least one step in nighttime phase shifting may involve elevating endogenous cGMP within some SCN neurons.

One hypothesis that is consistent with these data is that in intact animals light pulses during mid-subjective night excite optic nerve afferents to release transmitter that stimulates guanylate cyclase. The increased level of cGMP could in turn stimulate phosphorylation of one or more proteins present only during the cGMP-sensitive period and thereby alter their activity. The consequence could be a cellular change in state that advances the pacemaker to a new phase, from which time-keeping proceeds normally. To begin testing this hypothesis one could investigate whether light or optic nerve stimulation can induce increases in cGMP levels in the SCN. Photic stimulation has been shown to increase cGMP levels in several other systems, including the ocular circadian pacemaker of mollusks (26).

The phase of the SCN clock's sensitivity to Br-cGMP differs by nearly 12 hr from its sensitivity to cAMP analogs, including 8-bromoadenosine 3′,5′-cyclic monophosphate, and treatments that increase endogenous cAMP levels (4, 5). These results are important for several reasons. First, they support the hypothesis that Br-cGMP phase-shifts the clock through stimulation of cGMP-dependent pathways, rather than through nonspecific effects. Since the molecular structures of the bromo analogs of cGMP and cAMP differ only slightly (22), they should have similar nonspecific effects. However, they act on the SCN clock during almost nonoverlapping times, and thus each analog appears to affect the SCN through its separate specific actions. This conclusion is further supported by the data showing that a second cGMP analog, Bt_2-cGMP, also phase-advances the SCN when applied during the subjective night but not during the subjective day.

Second, these results indicate that the isolated SCN clock retains the characteristic of differential day–night sensitivity to phase-shifting agents seen *in vivo*. In this regard, cGMP can be grouped with *in vivo* treatments, including light pulses (9), carbachol (refs. 16 and 17; however, see also ref. 15), and electrical stimulation (25), that phase-shift rodent circadian rhythms when applied during subjective night. Conversely, cAMP appears to act like a separate group of *in vivo* treatments that phase-advance only when applied during subjective day, including dark pulses (10, 11), neuropeptide Y (12), and glutamate (14). The retention by the isolated SCN of differential day–night sensitivity to resetting stimuli supports the idea that these separate entrainment pathways act on a clock that itself changes with the procession of time. It also reinforces the evidence that the SCN contain an autonomous circadian clock that continues to function normally when isolated in a brain-slice preparation.

Third, these results strongly support the conclusion that cAMP and cGMP affect the SCN circadian pacemaker through separate biochemical pathways. Their site of action could be within pathways afferent to the pacemaker or within the time-keeping mechanism itself. In either case, the indication that these two cyclic nucleotides access different,

temporally limited components may provide additional clues as to biochemical substrates within the SCN. For example, cAMP may affect the pacemaker through phosphorylating proteins available only during the day; conversely, cGMP may act through phosphorylating proteins that are available only during the night. Each phosphorylated substrate would have the ability to maximally advance the pacemaker by nearly a quarter of its cycle. Preliminary evidence suggests that indeed there are day–night differences in protein and phosphoprotein profiles in the SCN *in vivo* (27, 28). Thus, protein changes may underlie the differential sensitivity of the SCN clock to resetting by cAMP and cGMP.

The results of previous experiments provide further support for the hypothesis that cAMP and cGMP may play differential roles within the hypothalamus, and, more specifically, within the SCN. The levels of cAMP and cGMP have been shown to oscillate 180° out-of-phase with each other in freshly dissected hypothalami of rat, with cAMP levels high in the early day and cGMP levels high in the early night (29). Also, cGMP and cAMP analogs have been shown to increase and decrease the firing rates of some SCN cells *in vitro*, respectively (30). However, interpretation of these results with respect to the ability of cAMP and cGMP analogs to reset the SCN pacemaker is difficult, since it is not known at what CT these treatments were performed.

The differential sensitivity of the SCN clock to cAMP and cGMP analogs is in some ways similar to that seen in the circadian pacemaker located in the *Aplysia* eye. In the isolated eye preparation of this mollusk, 6-hr treatments with Br-cGMP induce phase advances when applied during subjective night and phase delays during subjective day; conversely, 6-hr treatments with BzA-cAMP induce phase advances when applied during the subjective day and phase delays during subjective night (26, 31). Several differences between these two systems should be noted, however. (*i*) Br-cGMP and BzA-cAMP appear to induce only phase advances in the SCN pacemaker, whereas they induce both advances and delays in *Aplysia*. (*ii*) While BzA-cAMP and Br-cGMP appear to phase-shift the SCN during nonoverlapping 12-hr periods, these analogs affect the *Aplysia* eye pacemaker during overlapping phases, although they produce opposite changes. (*iii*) The phase shifts seen in the SCN clock after 1-hr treatments of BzA-cAMP and Br-cGMP are much larger than those seen in *Aplysia* after 6-hr treatments with these analogs. Thus, although both pacemakers appear to be differentially sensitive to BzA-cAMP and Br-cGMP, their responses to these analogs differ both qualitatively and quantitatively.

In conclusion, the SCN pacemaker is reset *in vitro* by nighttime but not daytime treatments that stimulate cGMP-dependent pathways, suggesting that cGMP may underlie some aspects of light-induced phase shifts *in vivo*. The actions of cGMP must reside either within the SCN pacemaker or within a pathway accessing it. Nocturnal sensitivity to cGMP is distinct from the SCN's sensitivity to cAMP, which is restricted to the subjective daytime. This differential sensitivity to cAMP and cGMP parallels the differential day–night sensitivity of the SCN to a variety of *in vivo* treatments and suggests that these cyclic nucleotides normally may act on the SCN circadian clock through the stimulation of separate biochemical pathways.

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