Temperature Compensation and Temperature Entrainment of the Chick Pineal Cell Circadian Clock

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We have used an *in vitro* model system of the circadian clock, dispersed chick pineal cells, to examine the effects of temperature on the circadian clock of a homeotherm. This preparation enabled us to isolate a circadian clock from *in vivo* homeostatic temperature regulation and expose cells to both constant temperatures and abrupt temperature changes. By manipulating the temperature of the pineal cells, we have demonstrated that (1) the circadian clock compensates its period for temperature changes over the range of 34–40°C; $Q_{10} = 0.83$, a value within the range of Q_{10} values measured for poikilothermic circadian clocks; (2) temperature pulses (42°C, 6 hr duration) shift the phase (advance and delay) of the circadian rhythm in a phase-dependent manner; and (3) a temperature cycle (18 hr at 37°C, 6 hr at 42°C) will entrain the circadian clock *in vitro*. This is the first demonstration of temperature entrainment of the circadian clock of a homeotherm *in vitro*. In addition we have found that temperature directly influences the synthesis and release of melatonin, the primary hormonal product of the pineal gland. The biosynthesis of melatonin is strongly temperature dependent with a $Q_{10} > 11$ when melatonin release is measured at ambient temperatures between 31°C and 40°C. In contrast, 6 hr 42°C temperature pulses acutely inhibit melatonin release in a manner similar to that seen previously with light pulses. These results demonstrate that a circadian clock from a homeothermic vertebrate is temperature compensated, yet temperature cycles can entrain the circadian melatonin rhythm. Thus, the chick pineal circadian oscillator has retained all the fundamental properties of circadian rhythms.

[Key words: circadian, temperature, pineal, chicken, temperature compensation, phase response curve, entrainment, melatonin, heat shock]

Organisms are exposed to 24 hr environmental cycles of both light and temperature. Because diurnal variations in light and temperature are inextricably linked in nature, each may have

affected the evolution of the circadian system so that circadian oscillators respond to light and temperature in similar ways. In general, light has two effects on the circadian system: cyclic light acts as a zeitgeber, entraining the circadian system and constant light alters the free-running period of the circadian clock. Temperature may have similar effects on the circadian system.

Most biological reaction rates are temperature-dependent, however a biological clock must be relatively independent of temperature changes. A temperature-dependent clock would run faster at higher temperatures and slower at lower temperatures so that it would be unreliable for time measurement. Pittendrigh (1954) first rigorously tested the hypothesis that the clock system must function relatively independently of temperature effects. He demonstrated that the eclosion rhythm of *Drosophila* persisted with a circadian period (~24 hr) within a range of ambient temperatures. Pittendrigh's seminal manuscript established temperature compensation as a criterion for defining a circadian clock, but 40 years later we still do not know the biochemical or molecular mechanism mediating this phenomenon.

Most temperature effects on the circadian clock have been observed in poikilothermic organisms, most of which entrain to temperature cycles but whose free-running period is temperature compensated (Zimmerman et al., 1968; Menaker and Wisner, 1983; Underwood, 1985; Chiba et al., 1993). Temperature compensation has also been demonstrated in heterothermic organisms (animals that lower their body temperature) (Menaker, 1959; Lee et al., 1990; Grahn et al., 1994). However, the effects of temperature on circadian rhythms have only recently been examined in obligate homeotherms (nonhibernating bird or mammal) (Barrett and Takahashi, 1993; Grahn et al., 1994; Zatz et al., 1994); it has been generally assumed that the ambient temperature would have little effect on the temperature of neural structures containing circadian pacemakers, such as the suprachiasmatic nucleus, pineal or retina. However brain temperature may increase in circumstances such as high levels of physical activity, exposure to extreme ambient heat, or fever. For example, the physiological brain temperature of the resting chicken is 39°C but it may reach 45°C under conditions of high activity (Prinzinger et al., 1991).

We tested the effects of temperature on a circadian oscillator from an homeotherm, the chicken, *Gallus domesticus*. The chicken pineal gland contains a circadian oscillator that drives the rhythmic synthesis and release of the hormone melatonin; there is no evidence that pineal cells store melatonin, and accordingly the melatonin released closely approximates the amount synthesized. Pineal melatonin synthesis persists *in vitro*

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in both organ culture and dispersed cell culture (Deguchi, 1979a,b; Robertson and Takahashi, 1988a,b). In addition, the melatonin rhythm is entrained by light and free runs in constant conditions *in vitro* (Robertson and Takahashi, 1988a,b; Zatz et al., 1988). We used this *in vitro* model system to ask whether the pineal circadian oscillator is temperature compensated and whether temperature pulses can phase shift the pineal circadian oscillator. We report here that the chick pineal clock is temperature compensated, is phase shifted by temperature pulses and can be entrained by temperature cycles. These results demonstrate that a cellular circadian oscillator from a homeotherm retains all the fundamental properties of circadian systems.

Materials and Methods

Animals

Fertilized chicken eggs (*Gallus domesticus*, White Leghorn) were obtained from Northern Hatchery, Inc. (Beaver Dam, WI). The eggs were incubated and hatched in the Northwestern University animal care facilities. Newly hatched male chicks were raised under a light cycle of 12 hr light:12 hr dark (LD12:12) with lights on at 0700 CST for 2–4 weeks prior to sacrifice. Food (Wayne Pellet Growers) and water were available *ad libitum*.

Cell culture

Chicks ($N = 10$ – 15 per primary culture) were killed by decapitation and the pineal glands were removed and chilled in sterile complete culture medium. Complete medium had the following composition: Medium M-199 with Hanks' salts and L-glutamine (GIBCO, 400-1200EB) supplemented with 10 mM HEPES buffer (GIBCO), 10% bovine calf serum (Hyclone), 2.5 μ g/ml amphotericin B (GIBCO), 100 U/ml penicillin G, 100 U/ml streptomycin (Sigma), L-glutamine (2 mM, Sigma), and NaHCO_3 . The tissue was washed 3 times in Hanks' salts solution containing 500 μ g/ml gentamicin, 1000 U/ml penicillin G, 1000 U/ml streptomycin, and 25 μ g/ml amphotericin B. The glands were transferred to 2 ml of Hanks' salts solution and minced. The minced tissue was transferred to a 15 ml tube and incubated at 37°C for 30 min in Hanks' solution containing 1 mg/ml collagenase (Worthington Life, CLSS-1). The tissue was triturated with a siliconized, fire-polished Pasteur pipette and then centrifuged at $50 \times g$ for 2 min (25°C) to pellet the partially dispersed fragments. The supernatant was collected in a 15 ml tube and set aside in an incubator (37°C, 5% CO_2). The pellet was resuspended in 2 U/ml papain (Worthington Life) and incubated at 37°C for 15 min. The fragments were triturated and complete culture medium was added to stop the digestion. The tissue was centrifuged at $50 \times g$ for 2 min and the supernatant was collected and set aside at 37°C. The pellet was resuspended in culture medium, triturated, and centrifuged at $50 \times g$, and then the supernatant was collected. All the collected supernatants were centrifuged at $500 \times g$ to pellet the dispersed cells. The supernatant was aspirated and the pelleted cells were resuspended in culture medium (2 ml/tube). The cells were counted with a hemacytometer. The typical dispersal resulted in a yield of 4×10^5 cells per pineal gland (1 pineal gland has about 10^6 cells). The cells were plated onto poly-L-ornithine-coated (Sigma) 96-well tissue culture dishes (Costar) at a density of 25–50,000 cells/well. The cells were immediately transferred to an incubator (37°C, 5% CO_2) and maintained under a 12 hr:12 hr light:dark cycle.

Flow-through cell culture technique

The plated cells were housed in four light-tight chambers with individual light and temperature regulation. Air-equilibrated culture medium with the following composition was used for the flow-through experiments: Medium M-199 with Hanks' salts and L-glutamine (GIBCO, 400-1200EB) supplemented with 10 mM HEPES buffer (GIBCO), N1 components (Bottenstein, 1984), 2% bovine calf serum (Hyclone), 100 U/ml penicillin G, 100 U/ml streptomycin (Sigma), L-glutamine (2 mM, Sigma), and sodium pyruvate (1.3 mM, Sigma). Culture medium was supplied by 16 parallel channels composed of 60 cc syringes on an automatic pump (Harvard, 2265) and microbore Teflon tubing (Biolab Products, 0.028 inches i.d., 0.046 inches o.d.). The syringes and tubing were sterilized by autoclaving (20 min). The culture medium flowed at a rate of 0.25 ml/hr and perfusate was collected every 2 hr. The per-

fusate was collected by an automatic fraction collector (ISCO, 328) in a humidified atmosphere then removed from the fraction collector at 8–12 hr intervals and frozen at -20°C until termination of the experiment. Light was supplied to the chambers by microscope illuminators (Dolan-Jenner, 180) fitted with fiber optic light guides (Dolan-Jenner). The light intensity at the level of the plated cells was 100 $\mu\text{W}/\text{cm}^2$ measured with a United Detector Technology S350 photometer with a radiometric filter. The entire flow-through system was contained in a temperature-controlled chamber at 37°C. Precise temperature regulation was controlled by Plexiglas water jackets connected to heating water circulators (Lauda, M3). Temperature of each tissue culture plate was continuously monitored by a multi-probe thermometer (Pace Scientific, XR230) which uploaded the temperature data to a personal computer.

Melatonin radioimmunoassay

Sample volumes of 3–60 μl (depending on the experiment) were assayed for melatonin content using a melatonin radioimmunoassay (Rollag and Niswender, 1976) modified by Takahashi et al. (1980). Melatonin was purchased from Sigma. Iodinated melatonin analog was supplied by Hazleton Washington (Vienna, VA). Rabbit anti-melatonin antibody (R1055 pool) was supplied by Dr. Gordon Niswender (Colorado State University). The assay has been validated for pineal culture medium without extraction of the samples (Takahashi et al., 1980). The culture medium did not interfere with measurements of the assayed sample volumes. The upper and lower limits of the assay are 500 and 1 pg/tube, respectively.

Data analysis

Phase reference point. The phase of the melatonin rhythm was determined by designating the midpoint of the cycle as the phase reference point. This phase reference point was calculated for each channel of the flow-through system. The midpoint was defined as the time midway between the half-rise and the half-fall values of each melatonin peak. The half-rise point was calculated by interpolating the time points that corresponded to the mean of the lowest melatonin concentration preceding the peak of the cycle and the peak melatonin concentration of the cycle. The half-fall was calculated by interpolating the time points that corresponded to the mean of the peak melatonin concentration and the lowest melatonin concentration following the peak of the cycle.

Normalization. Because we are primarily interested in phase and period measurements some of the data are presented in normalized form which reduces the variance among individual cell chambers. The normalization procedure enhances visualization of phase and period when comparing records. The normalization was performed by dividing the melatonin concentrations of each channel by the mean of the time series of that channel and then multiplying by 100 (the mean value of the channel = 100%). This calculation eliminates variation in the amplitude of the rhythm due to procedures which change the absolute amount of melatonin (e.g., number of cells plated per well).

Design of temperature compensation experiment. Four treatment groups of primary pineal cell cultures (four replicates per group) were entrained to a LD12:12 light cycle for 3 d in an incubator at 37°C, then transferred to the flow-through system and exposed to LD12:12 and 31°C, 34°C, 37°C, and 40°C. The cells remained in LD12:12 for 3 days to ensure synchronization of the melatonin oscillation after the temperature change and then placed in constant darkness (DD) until termination of the experiment. The melatonin oscillation in constant darkness was measured to determine the phase and period. The temperature coefficient was calculated by using the equation $Q_{10} = (R_2/R_1)^{10/(T_2-T_1)}$, where R is rate and T is temperature.

Calculation of period of the rhythm. The period length of a cycle was defined as the difference of two consecutive phase reference points. For every individual channel, three different period measurements were calculated from the first four cycles in DD and averaged to determine the mean period for that channel. The period lengths of the four replicate channels of a treatment group were averaged to determine the mean period of the treatment group.

Design of perturbation experiments. Determination of the most effective temperature pulse for phase shifting was made by exposing cells to temperature pulses of varying magnitudes and duration. The temperature pulses ranged from 39.5°C to 43°C and the duration of the pulse was either 3 or 6 hr. All the pulses were presented to the experimental group at circadian time (CT)12 of the first cycle in DD after 5 d of entrainment to LD12:12; the control group was held at constant environmental chamber temperature (36.5–37°C).

Measurement of the phase response curve required three sets of experiments. Each experiment contained one control group held at constant environmental chamber temperature (36.5–37°C) and three experimental groups (four replicates per group) each of which received a single 42°C temperature pulse of 6 hr duration. The cells were entrained to a LD12:12 light cycle for 5 d then exposed to DD until termination of the experiment. Temperature pulses were administered during either the first or second cycle in DD.

Calculation of phase shifts. The phases of each culture within a treatment group were averaged for the second cycle following the pulse. The mean of the experimental group was subtracted from the mean of the control group to determine the amount of phase shift; 95% confidence limits were calculated using the standard deviations (SD) of the two groups to calculate a weighted SD for the difference of the mean values.

Design of temperature entrainment experiment. Two groups of primary pineal cell cultures (five replicates per group) were entrained to a 12 hr:12 hr light:dark cycle for 3 d in an incubator at 37°C then were transferred to the flow-through system and exposed to 12 hr:12 hr light:dark at 37°C. The cells remained in 12 hr:12 hr light:dark for 2 d to ensure synchronization of the melatonin oscillation. One group was put in DD and exposed to a temperature cycle advanced 6 hr relative to the light cycle. The second group was put in DD and exposed to a temperature cycle delayed 6 hr relative to the light cycle. The temperature cycle was 6 hr at 42°C and 18 hr at 37°C because 6 hr temperature pulses induce phase shifts but 12 hr pulses abolish the ability of the cells to release melatonin. After three cycles of temperature the cells were exposed to constant conditions (DD and 37°C) for 4 d. The melatonin oscillation was measured to determine phase.

Results

Effects of constant temperature on the chick pineal

With *in vitro* cell culture preparations of the chick pineal, we have the unique ability to manipulate the ambient temperature and study the effects of temperature on a circadian clock from a homeotherm. In preliminary experiments, we found that exposure of pineal cells in culture to elevated temperatures (42–43°C) for 12 hr or to temperatures below 27°C abolished the cells' ability to release melatonin (unpublished observations). To study the effects of constant temperature on the pineal circadian clock and melatonin release, four groups of cell cultures were continuously exposed to 31°C, 34°C, 37°C, and 40°C in both a light cycle (12 hr light:12 hr dark) and in constant darkness (DD) (Fig. 1A). Temperature affected two physiological functions of the pineal cells in very different ways: higher temperature *only slightly* increased the period of the circadian clock, whereas higher temperature dramatically increased melatonin output.

Because the pineal melatonin rhythms of cells exposed to temperatures between 34°C and 40°C continued with circadian periods in DD, the chick pineal circadian clock was temperature compensated in the range of 34–40°C (Fig. 1A). Actual average period values are shown in Figure 2: the periods were 22.7 ± 0.31 hr at 34°C, 23.6 ± 0.22 hr at 37°C, and 25.4 ± 0.24 hr at 40°C (mean $\pm$ SEM). The temperature coefficient, Q_{10} , over the range 34–40°C was 0.83; the Q_{10} for the range of 37–40°C was 0.79. We observed no persistent rhythm in DD at 31°C. Although the clock was synchronized to the light cycle at each of the temperatures, the phase angle between the light cycle and the melatonin rhythm was different at 31°C. The peak of melatonin release had a positive phase angle to the onset of light in the cells exposed to 34°C ($+4.5 \pm 2.1$ hr), 37°C ($+5.2 \pm 2.5$ hr), and 40°C ($+4.5 \pm 1.9$ hr), but the phase angle between the peak of melatonin release and light onset at 31°C was $+0.17 \pm 0.6$ hr (Fig. 1B). Differences in the phase angle of entrainment and expression of the rhythm in DD at 31°C could be due to any of several factors including uncoupling of the clock and melatonin synthesis or abolition of the circadian rhythm.

The synthesis and release of melatonin was temperature-dependent in chick pineal cells. Peak melatonin release from pineal cells was highest at 40°C and lowest at 31°C (Fig. 1A). Average peak melatonin release in LD was 2.0 ng/2 hr at 31°C, 9.4 ng/2 hr at 34°C, 20.8 ng/2 hr at 37°C, and 22.9 ng/2 hr at 40°C. However, there was no difference in the relative amplitude of melatonin release, after normalization, from pineal cells exposed to temperatures between 31°C and 40°C (Fig. 1B). The peak amount of melatonin release, 250% to 300% of the mean, occurred during the dark phase of the light–dark cycle at each of the temperatures. In addition to releasing more melatonin at higher temperatures in LD, pineal cells also released more melatonin at higher temperatures in DD. Quantitative analysis of total melatonin release in LD and in DD is shown in Figure 3. The average values for total melatonin production were calculated for three cycles of LD and five cycles of DD conditions. There was a significant difference between treatment groups ($p < 0.0001$; two-way ANOVA) with the cells exposed to 40°C producing nine times more melatonin than those exposed to 31°C. There was no significant difference between LD and DD conditions within the treatment groups exposed to 31°C, 37°C, and 40°C; the difference in mean amount of melatonin released was significantly different at 34°C ($p < 0.05$; post hoc Student-Newman-Keuls multiple comparison test following the two-way ANOVA). The temperature coefficient, Q_{10} , for melatonin production over the temperature range of 31–40°C was 11.1. So although the circadian clock of the chick pineal is temperature compensated, at least one other metabolic process within the same cells, the biosynthesis of melatonin, is not compensated for changes in temperature.

Phase-shifting effects of temperature

Light and temperature are the strongest environmental determinants of phase of circadian oscillators. Light pulses shift the melatonin rhythm of dissociated chick pineal cells in a phase-dependent manner (Robertson and Takahashi, 1988b). We examined the effects of temperature pulses presented to cultured chick pineal cells to determine whether high temperature pulses would also phase shift the melatonin rhythm. Figure 4 shows the phase-shifting effects of 6 hr temperature pulses (42°C) presented at CT12. Two groups of cultures were used to assay the phase shift: a control group was maintained at constant temperature in DD to measure the steady-state phase of the oscillation and an experimental group was exposed to a 6 hr, 42°C pulse in DD. The difference in phase between the two groups, measured from the second cycle after the pulse, was the net phase shift generated by the temperature pulse. Figure 4A shows the individual records of the four cultures of the experimental group which were exposed to the temperature pulse at CT12. Figure 4B shows the individual records of the control group which were maintained in constant conditions. The ensemble averages of both groups are plotted in Figure 4C to demonstrate the phase shift generated by the temperature pulse. The temperature pulse presented at CT12 generated a phase delay of 5.6 ± 0.9 (mean $\pm$ 95% C.L.) hr relative to the unperturbed control. Table 1 shows that the magnitude and duration of temperature pulses are critical determinants of the amount of phase shift of the circadian melatonin rhythm at CT12. Six hour temperature pulses of 39.5°C produced small phase shifts; whereas 6 hr pulses of 41°C or 42°C produced large phase shifts. Three hour pulses of 41°C were ineffective; while 3 hr pulses of 42°C produced moderate phase shifts. Surprisingly, 3 hr and 6 hr pulses at 43°C caused

Figure 1. Effects of constant temperature on the chick pineal circadian clock and melatonin release. Points and bars represent mean $\pm$ SEM. Dark bars indicate the light–dark cycle. *A*, Average concentrations of melatonin release from chick pineal cells at 31–40°C. *B*, Normalized melatonin release from chick pineal cells exposed to 31–40°C.

arrhythmicity (Fig. 5). These data indicate that 6 hr pulses between 41°C and 42°C were most effective and so they were used in the subsequent perturbation experiments.

To determine whether the phase-shifting effects of elevated temperature were phase-dependent, 42°C temperature pulses were presented at other phases of the circadian cycle (Fig. 6). Six hour temperature pulses were presented at 3 hr intervals to

eight experimental groups; two additional experimental groups received temperature pulses at CT14 and CT16 to increase the resolution around the breakpoint. The response to single temperature pulses was dependent on the phase at which the pulse was administered. Pulses presented during the subjective day (CT6, 9) and early subjective night (CT12, 14) produced delay phase shifts in the melatonin rhythm (Fig. 6*E–H*). Temperature

Figure 1. Continued.

pulses during the mid-to-late subjective night (CT16, 18) produced phase-advances of the melatonin rhythm (Fig. 6*J,A*). Pulses at CT21, 24, 3 did not cause phase shifts (Fig. 6*B–D*). The phase response curve to the 6 hr, 42°C pulses shows that both the direction and amplitude of the phase shift is dependent on the phase at which the pulse is presented (Fig. 7). Maximum phase-delays generated by temperature pulses occurred at CT14 (11.2 hr) and maximum advances at CT18 (4.2 hr). Interestingly, the temperature pulse at CT15 caused a disruption of the circadian melatonin rhythm. This disruption of the rhythm could be due either to desynchronization of cellular pineal oscillators by the pulse occurring at the breakpoint, or to amplitude reduction by a “critical pulse” driving the system toward the singularity (Winfree, 1980).

The phase response curve for 6 hr temperature pulses is qualitatively very similar to the phase response curve for 6 hr light pulses (Fig. 8). In both phase response curves phase-delays occur in the subjective day and early subjective night, phase-advances occur in the mid-to-late subjective night, and the break-

point is the same phase in each curve occurring between CT15 and CT16. There are no differences between the two curves between phases CT0 and CT12. However, the advance portions of the curves differ in amplitude with the 6 hr light pulses generating larger phase-advances than 6 hr 42°C temperature pulses. This remarkable likeness of the light and temperature phase response curves suggests that light and temperature ultimately have similar effects on the chick pineal circadian clock.

Acute effect of temperature pulses on melatonin release

A rapid rise in pineal cell culture temperature has a dramatic effect on melatonin release. Figure 9 shows that increasing the temperature from 37°C to 42°C results in an inhibition of melatonin release. Temperature pulses presented at the representative phases CT14, CT18, and CT24 acutely inhibited melatonin synthesis; this effect is similar to the acute inhibition of melatonin synthesis caused by light pulses. Light presented at any phase of the circadian cycle inhibits melatonin synthesis (Robertson and Takahashi, 1988b). Likewise temperature pulses pre-

Figure 2. Period and frequency estimates of the melatonin rhythm of cultured chick pineal cells. The mean period and frequency of the melatonin rhythm were calculated for each treatment group (solid circles). The average of each channel in the treatment group is plotted as an open symbol.

sented at various phases reduce melatonin release (Fig. 6). Unlike the phase-shifting effect of temperature, the acute inhibitory effect of temperature on melatonin synthesis is not phase-dependent because melatonin synthesis is inhibited at all phases tested.

Entrainment by temperature

Because temperature pulses phase shift the pineal clock, we determined whether a temperature cycle could entrain the clock

Figure 3. Comparison of average melatonin release from chick pineal cells in a light cycle and in constant dark. Average concentrations for melatonin production by pineal cells during three cycles of a light cycle (open circles) and five cycles of constant dark (solid circles) were calculated for each treatment group. Points and bars represent mean $\pm$ SEM. The amount of melatonin produced by cells in different treatment groups was significantly different ($p < 0.0001$; two-way ANOVA). The cells within a treatment group produced similar amounts of melatonin in LD and in DD (* denotes statistical significance $p < 0.05$; post hoc Student-Newman-Keuls multiple comparison test following the two-way ANOVA). Linear regressions through the data are shown (dotted line, LD group; solid line, DD group).

Figure 4. Temperature-induced phase shift of the circadian oscillation of melatonin release from dispersed chick pineal cells. Dark bars indicate the light-dark cycle. **A**, Superimposed individual records from four replicate cells chambers that received a 6 hr, 42°C temperature pulse beginning at CT12. **B**, Superimposed individual records from four replicate chambers that remained in DD at constant temperature. **C**, Superimposed ensemble averages from records presented in **A** and **B**. Solid circles represent the 6 hr, 42°C temperature pulse group and open circles represent the constant conditions group.

Table 1. Effects of different magnitude and duration temperature pulses on the amount of phase shift of the circadian melatonin rhythm in chick pineal cells

Temperature of pulse	Magnitude of phase shift	
	3 hr pulse	6 hr pulse
39.5°C	ND	-3.7 ± 2.3 hr (3, 4) -2.3 ± 2.0 hr (4, 3)
41°C	-1.7 ± 2.0 hr (3, 4)	-6.4 ± 1.9 hr (3, 4)
42°C	-4.6 ± 1.6 hr (4, 4)	-5.9 ± 1.7 hr (4, 4) -5.6 ± 0.9 hr (4, 4) -7.0 ± 2.4 hr (3, 4)
43°C	Arrhythmic (4, 4) Arrhythmic (3, 3)	Arrhythmic (5, 5) Arrhythmic (5, 5) Arrhythmic (3, 3)

All pulses began at CT12. Values are the mean ± 95% confidence limits. Values in parentheses are numbers of control and experimental replicates in the experiment, respectively. ND, not determined.

(Fig. 10). Cells were entrained to a light cycle then a temperature cycle was introduced in constant darkness. The temperature cycle was 6 hr at 42°C and 18 hr at 37°C. In one group the temperature cycle was advanced 6 hr relative to the previous light cycle (Fig. 10A) and in the other group the temperature cycle was delayed by 6 hr (Fig. 10B). After three temperature cycles, the cells were exposed to constant 37°C. The advanced temperature cycle phase-advanced the melatonin rhythm (Fig. 10A), and the delayed temperature cycle phase-delayed the melatonin rhythm (Fig. 10B). The two rhythms continued in constant conditions and remained 180° out of phase with each other until termination of the experiment. Because each group's rhythm continues in constant conditions in phase with the temperature cycle to which it was exposed and additionally out of phase with the original light cycle, the melatonin rhythm is entrained by the temperature cycle.

Discussion

Temperature compensation of the circadian clock has been demonstrated in several species of plants and animals, many of which have become classical models for the study of circadian biology (e.g., *Neurospora*, *Drosophila*, and *Aplysia*) (Pittendrigh, 1954; Zimmerman et al., 1968; Benson and Jacklet, 1977; Gardner and Feldman, 1981). Most studies investigating temperature compensation of the vertebrate circadian clock have examined the whole organism (Menaker, 1959; Rawson, 1960; Enright, 1966; Gibbs, 1981, 1983; Tokura and Aschoff, 1983; Underwood, 1985; Lee et al., 1990; Grahn et al., 1994). These *in vivo* preparations are adequate for investigating the long-term effects of temperature on the circadian clock in poikilothermic and heterothermic organisms because their body temperature fluctuates with the ambient temperature. Investigators using this approach have demonstrated temperature compensation of the clock in the poikilothermic lizard (Underwood, 1985), a heterothermic bat (Menaker, 1959), and a heterothermic squirrel (Lee et al., 1990; Grahn et al., 1994). However, the body temperature of an homeothermic organism cannot be altered for an extended period to assess temperature compensation of the clock. However, mice, hamsters, and rats have been exposed acutely (3–24 hr) to various ambient temperatures in attempts to determine if the circadian clocks of these organisms are temperature compensated (Rawson, 1961; Gibbs, 1981, 1983). With the advent

Figure 5. Three hour and 6 hr 43°C pulses cause arrhythmicity. Dark bars indicate the light–dark cycle. The vertical bar represents the temperature pulse presented at CT12. *A*, Phase shift generated by a 42°C, 6 hr pulse. *B*, Arrhythmicity due to a 43°C, 3 hr pulse. *C*, Arrhythmicity due to a 43°C, 6 hr pulse. Because the melatonin concentrations were low after the temperature pulse in *B* and *C*, the first 28 data points were normalized separately from the remaining time series; otherwise, these plotted values would be artificially high.

of *in vitro* systems for vertebrate circadian pacemakers, temperature compensation can be studied directly in homeotherms.

With an *in vitro* culture system of chick pineal cells we were able to isolate the circadian clock from homeostatic temperature regulation and expose it to various constant temperatures. The melatonin rhythm persisted between 34°C and 40°C with a circadian period demonstrating temperature compensation of the chick pineal circadian clock. Because the frequency of the homeothermic circadian clock (like the poikilothermic circadian clock) is relatively invariant over a wide temperature range, temperature compensation can be considered a valid universal property of a circadian clock. Although the chick pineal clock is

Figure 6. Phase-dependent effects of 6 hr temperature pulses. Dark bars indicate the light–dark cycle. The vertical bar represents the 6 hr, 42°C temperature pulse. Solid circles represent the ensemble average of four replicate cell chambers that received a 6 hr, 42°C temperature pulse. Open circles represent the ensemble average of four replicate cell chambers maintained in DD and constant temperature.

Figure 7. Phase response curve to temperature pulses. The magnitude and direction of the phase shift is dependent upon the phase at which the temperature pulse was initiated. Points and bars represent mean phase difference $\pm$ 95% confidence limits. *, Significant phase shift, $p < 0.05$.

temperature compensated, the period lengthens as temperature increases. The temperature-dependent lengthening of period is expressed mathematically as a temperature coefficient, or Q_{10} , which is the ratio of rates of a reaction at two temperatures 10°C

Figure 8. Phase response curves to light and temperature. The magnitude and direction of the phase shift is dependent upon the phase at which the pulse was initiated. Points and bars represent mean $\pm$ 95% confidence limits. The light pulse data (open circles) is from Robertson and Takahashi (1988b). Temperature data (solid circles) is the same as in Figure 7.

Figure 9. Acute effect of temperature pulses on melatonin release. Three representative phases are shown: A, CT14 is in the delay portion of the phase response curve; B, CT18 is in the advance portion of the phase response curve; C, CT24 is where no significant phase shift occurs. Points and bars represent the mean melatonin concentrations $\pm$ SEM. The temperature pulse is represented by the stippled bar. Each point represents the concentration of melatonin release plotted at the onset of collection, for example, the point at hour 14 represents melatonin release from hour 14 to hour 16. The lower plot in each figure is the actual recorded temperature of the four replicate chambers exposed to the temperature pulse. Temperature readings were collected every 5 min.

apart. The range of Q_{10} values for circadian clocks measured from poikilothermic organisms is 0.8–1.2 (Takahashi et al., 1989). The calculated Q_{10} for the homeothermic chick pineal clock is within the same limits with a value of 0.83 for the range of $34\text{--}40^{\circ}\text{C}$. However, the period of the clock lengthened 1.8 hr over the temperature range of $37\text{--}40^{\circ}\text{C}$ ($Q_{10} = 0.79$), $39\text{--}41^{\circ}\text{C}$

Figure 10. *In vitro* entrainment of the circadian oscillation of the chick pineal melatonin rhythm by a temperature cycle. Points and bars represent the mean $\pm$ SEM. The upper dark bars of each panel represent the light-dark cycle and the lower dark bars of each panel represent the temperature cycle. The temperature cycle was 6 hr at 42°C (open bar) and 18 hr at 37°C (solid bar). A, Six hour advance of the temperature cycle (relative to the light cycle). B, Six hour delay of the temperature cycle (relative to the light cycle).

is physiological temperature. Whether this period difference between 37°C and 40°C could be significant for the organism remains an open question. However the increased amplitude of the rhythm at 40°C could be physiologically significant and could be similar to that seen in *Drosophila* (Pittendrigh et al., 1991), *Gonyaulax* (Broda et al., 1989), and *Neurospora* (Lakin-Thomas et al., 1991).

The melatonin rhythm did not persist at 31°C although the cells were still capable of synthesizing and releasing the hormone. The absence of a rhythm could be due to an uncoupling of the clock and melatonin synthesis or to an arrest of the motion of the circadian clock itself. If melatonin synthesis is uncoupled from the clock, then the possibility remains that the clock is also temperature compensated at 31°C and we simply cannot measure its output. Alternatively, it is possible that the pineal circadian oscillator does not run at 31°C. There is evidence from other species that the clock is stopped at a critical low temperature (Sweeney and Hastings, 1960; Roberts, 1962; Njus et al., 1977).

Although the chick pineal clock was relatively unresponsive to long-term exposure to different temperatures (i.e., only slight changes in period length), it was sensitive to acute temperature pulses. A 6 hr pulse of 42°C caused phase-dependent shifts in the melatonin rhythm. These shifts in the melatonin rhythm indicate that the phase of the underlying oscillator is altered by abrupt temperature changes (Pittendrigh, 1981a,b). Most temperature-pulse phase response curves have been measured in poikilothermic organisms but no rigorously measured phase response curves have been determined for obligate homeotherms (Johnson, 1990). The phase response curve to temperature pulses in chick pineal cells is qualitatively very similar to the phase response curve to saturating light pulses (Fig. 8). Because the light and temperature induced phase shifts are similar one

would expect both to entrain the circadian clock; entrainment of the chick pineal by light has been demonstrated previously (Deguchi, 1979b; Robertson and Takahashi, 1988a,b). Although temperature cycles are an effective zeitgeber in poikilotherms (Underwood, 1985), they have been ineffective in entrainment of most homeotherms (Bruce, 1960; DeCoursey, 1960; Erkert and Rothmund, 1981). However there are some data indicating that temperature may entrain some homeotherms; in these studies about 50% of the animals (macaques and squirrel monkeys) synchronized their locomotor activity patterns to a temperature cycle (12 hr at 17°C and 12 hr at 32°C) (Tokura and Aschoff, 1983; Aschoff and Tokura, 1986). Temperature entrainment of the chick pineal cells *in vitro* provides the strongest evidence that temperature can entrain the circadian clock of a homeotherm.

Temperature had two effects on melatonin synthesis independent of the effects on the clock: (1) elevated temperature increased the amplitude of melatonin release and (2) acute temperature pulses inhibited melatonin release. When the pineal cells were exposed to extended durations of constant temperature the cells at 40°C released nine times more melatonin than those at 31°C ($Q_{10} > 11$) (Fig. 3). This high reaction rate may be attributed to increased activity and/or induction of the enzymes (tryptophan hydroxylase, aromatic-L-amino acid decarboxylase, arylalkylamine-N-acetyltransferase, hydroxy-indole-O-methyltransferase) or to other temperature-dependent components (cAMP) of the melatonin synthesis pathway (Thibault et al., 1993; Zatz et al., 1994). This temperature-dependent difference in the amplitude of melatonin release from the pineal gland has been demonstrated in other systems as well (Menaker and Wisner, 1983; Zachmann et al., 1991; Max and Menaker, 1992; Bolliet et al., 1993; Zatz et al., 1994). Melatonin release

is also affected by acute temperature pulses; a 42°C pulse acutely inhibited melatonin release at every phase tested. This effect has also been observed with acute light pulses (Robertson and Takahashi, 1988b). However, others only observed acute inhibition by temperature when an extremely high temperature pulse (46.7°C) was presented (Zatz et al., 1994). This difference could be due to any of several technical procedures such as (1) the method of temperature measurement; Zatz et al. (1994) recorded incubator air temperature but we recorded the temperature (measurement resolution, $\pm 0.1^\circ\text{C}$) continuously in a well of the culture plate containing the pineal cells; (2) the method and frequency of collecting melatonin samples; Zatz et al. (1994) changed the cell culture medium of a static cell culture every 4 hr but we collected samples of a continuous cell perfusate every 2 hr; or (3) the culture conditions; the cells of Zatz et al. (1994) were maintained in static conditions interrupted by complete medium changes whereas our cells were continuously exposed to dynamic conditions in which the cells were never subjected to abrupt changes in the extracellular milieu. The differences in procedures 2 and 3 could affect the way the cells "condition" the medium and therefore affect their response to temperature changes.

How do cells detect temperature changes and respond appropriately to those changes? The heat shock response in which a specific set of proteins (heat shock proteins) is induced is the only well described cellular response to temperature changes. The magnitude of this response (level of heat shock protein induction) correlates with the stimulus strength, and the time course of heat shock protein induction is dependent upon the duration of the stimulus. For example, prolonged exposure of cells to 42°C results in a transient induction of the heat shock response but prolonged exposure to 43°C results in a sustained heat shock response (Morimoto, 1993). It is extremely interesting that in the chick pineal cells a 42°C pulse shifts the phase of the circadian clock but a 43°C pulse causes arrhythmicity (Fig. 5). Also, a higher amplitude melatonin rhythm is generated by continuous exposure to 40°C than at other lower temperatures but exposure to 43°C abolishes both the rhythm (3 and 6 hr exposure) and the ability of the cells to release melatonin (12 hr exposure). A possible explanation of the deleterious effects of 43°C on melatonin synthesis and circadian rhythmicity is that the sustained 43°C heat shock response is accompanied by an overall inhibition of protein synthesis. Both melatonin synthesis and circadian rhythmicity in chick pineal cells depend on ongoing protein synthesis (Takahashi et al., 1989). Because the heat shock protein system is a universal response in all cell types and the proteins are highly conserved, it is tempting to speculate that elements of the heat shock protein system may be involved in either temperature compensation or the transduction of temperature changes. If this is the case, then it should be possible to mimic the effects of temperature by manipulating the heat shock protein system.

The effects of temperature on the clock of an obligate homeotherm (chicken) are much like those observed in poikilothermic organisms. The circadian clock of an obligate homeotherm is temperature-compensated and temperature acts as a zeitgeber in that it shifts the phase of the clock. The effects of light and temperature on the circadian clock have been inseparable in nature; therefore convergent mechanisms may have been utilized to mediate the entraining effects of light and temperature on the chick pineal oscillator.

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