Rhythmicity of the cGMP-related signal transduction pathway in the mammalian circadian system

GABRIELA A. FERREYRA AND DIEGO A. GOLOMBEK
Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, 1876 Buenos Aires, Argentina
Received 18 August 2000; accepted in final form 3 January 2001

Rhythmicity of the cGMP-related signal transduction pathway in the mammalian circadian system. Am J Physiol Regulatory Integrative Comp Physiol 280: R1348–R1355, 2001.—Entrainment of mammalian circadian rhythms requires the activation of specific signal transduction pathways in the suprachiasmatic nuclei (SCN). Pharmacological inhibition of kinases such as cGMP-dependent kinase (PKG) or Ca\(^{2+}\)/calmodulin-dependent kinase, but not cAMP-dependent kinase, blocks the circadian responses to light in vivo. Here we show a diurnal and circadian rhythm of cGMP levels and PKG activity in the hamster SCN, with maximal values during the day or subjective day. This rhythm depends on phosphodiesterase but not on guanylyl cyclase activity. Five-minute light pulses increased cGMP levels at the end of the subjective night (circadian time 18 (CT18)), but not at CT13.5. Western blot analysis indicated that the PKG II isoform is the one present in the SCN. Inhibition of PKG or guanylyl cyclase in vivo significantly attenuated light-induced phase shifts at CT18 (after 5-min light pulses) but did not affect c-Fos expression in the SCN. These results suggest that cGMP and PKG are related to SCN responses to light and undergo diurnal and circadian changes.

MAMMALIAN CIRCADIAN RHYTHMS are generated by a hypothalamic biological clock in the suprachiasmatic nuclei (SCN) (27, 41). Light affects the clock through a direct projection of the retinohypothalamic tract (RHT) (35), releasing glutamate upon different kinds of receptors (2, 9). Entrainment can be achieved by stimulating the animal with short photic pulses delivered at different times of day, correlated with the acute light-induced expression of the mammalian period genes 1 and 2 (mper1 and 2) (4, 47, 48), which appear to be core components of the circadian clock (8). Moreover, antisense inhibition of mper1 blocks light and glutamate-induced phase shifts (3). Phase-shifting light stimulation induces the SCN expression of several immediate early genes (IEGs), including c-fos, junB, NGFI-A, and others (1, 30, 43, 46), as well as of the phosphorylation of the transcription factor cAMP response element binding protein (CREB) in the SCN (16).

Inhibition of the Ca\(^{2+}\)/calmodulin-dependent protein kinase blocks behavioral responses to light (18) and CREB phosphorylation in the SCN (19). As for the phosphorylation pattern of clock genes, it is known that the Drosophila per gene undergoes time-dependent phosphorylations that could be related to the regulation of the circadian clock feedback loop (10). In hamsters, Per appears to be phosphorylated by the casein kinase I\(\alpha\) (32), whose hamster mutation \(\tau\) (42) is analog to the fly’s double-time (29).

The cGMP-dependent pathway appears to play an important role in circadian entrainment. In the rat hypothalamic slice preparation, cGMP induces phase advances of the circadian rhythm of firing rate when applied during the subjective night (40, 49). The effects of exogenous application of cGMP on circadian phase might be dependent on an internal rhythm of the cyclic nucleotide in the rat hypothalamus (24, 25, 50) or SCN (55). Inhibition of the cGMP-dependent protein kinase (PKG) but not the cAMP-dependent kinase (PKA) blocks light-induced phase advances of hamster circadian rhythms (33). In addition, activation of nitric oxide synthase (NOS), which could be considered as a previous step in the cGMP-related pathway (6), might also be involved in the response to light, because circadian responses to light or glutamate are blocked by NOS inhibitors and enhanced by NO donors (7, 34, 54), and NOS activity itself showed a diurnal variation in the SCN (12).

The aim of this study was to analyze rhythmicity of cGMP and PKG in the hamster SCN under different environmental conditions, as well as to assess the effect of light pulses on these parameters and the role of PKG and guanylyl cyclase (GC) in behavioral and cellular responses to light.

METHODS

Animals and experimental procedures. Syrian hamsters (Mesocricetus auratus) were raised in our colony and housed under a 14:10-h light-dark cycle (LD, lights on at 0600), 4–6 per cage, with food and water ad libitum. Male adult (3–4 months, roughly 100 g) were used in all experiments. Animals were obtained from animal facility of our institution and were maintained under these conditions for at least 2 weeks before experimentation.

Animals were kept in an environmentally controlled room (LD 14:10). For each experiment, animals were weaned from their dams at about 20 days of age and housed in a group of three to five animals per cage, with food and water ad libitum. Male adult (3–4 months, roughly 100 g) were used in all experiments. Animals were obtained from animal facility of our institution and were maintained under these conditions for at least 2 weeks before experimentation.

METHODS

Animals and experimental procedures. Syrian hamsters (Mesocricetus auratus) were raised in our colony and housed under a 14:10-h light-dark cycle (LD, lights on at 0600), 4–6 per cage, with food and water ad libitum. Male adult (3–4 months, roughly 100 g) were used in all experiments. Animals were obtained from animal facility of our institution and were maintained under these conditions for at least 2 weeks before experimentation.

METHODS

Animals and experimental procedures. Syrian hamsters (Mesocricetus auratus) were raised in our colony and housed under a 14:10-h light-dark cycle (LD, lights on at 0600), 4–6 per cage, with food and water ad libitum. Male adult (3–4 months, roughly 100 g) were used in all experiments. Animals were obtained from animal facility of our institution and were maintained under these conditions for at least 2 weeks before experimentation.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
mo) animals were used throughout the experiments. When animals had to be killed in the dark, we used a dim red light source (<1 lx). Under LD conditions, zeitgeber time 12 (ZT12) was defined as the time of lights off (i.e., 2000). Under constant dark conditions (DD), circadian time 12 (CT12) was defined as the onset of wheel-running activity. For neurochemical determinations, animals were kept under DD for 48 h and killed at 4-h intervals on the third day of DD. The average circadian period for hamsters in our colony is 24.1 h, so we estimated that the CTs would be approximately equal to their previous ZTs and killed the animals assuming that CT12 = ZT12.

Unless stated, all drugs came from Sigma (St. Louis, MO). Data were analyzed by Student’s t-test or one-way ANOVAs followed by Tukey tests. Rhythmic patterns were determined by least-squares adjustments to a 24-h period cosine waveform (cosinor method). The percentage of the signal that is explained by the adjustment is shown as R% (rhythmic percentage); the acrophase (h ± SE) indicates the time when the maximal value of the adjusted curve occurs.

Determination of cGMP levels. Animals were killed by decapitation, and the brains were quickly excised and placed in an ice-cold environment. The tissue was frozen and stored at −20°C until assayed to prevent postmortem changes in cGMP (26). A 500- to 800-μm slice was cut with a sharp razor, and tissue containing the SCN was punched out with a micropuncher. SCN-containing tissue was homogenized in 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) with a glass rod and centrifuged for 5 min at 13,200 rpm; cGMP content was determined by radioimmunoassay in the supernatant fraction. Samples and standards were acetylated (acetic anhydride: triethyamine, 1:2) and mixed with 1214-labeled cGMP (15,000–20,000 dpm; specific activity, 140 mCi/mmol) and rabbit antiserum against cGMP (Chemicon International, Temecula, CA; 1:150) and incubated overnight at 4°C. The antibody complex was precipitated with ethanol at 4°C using 2% bovine serum albumin (BSA) as a carrier and centrifuged at 2,000 g for 30 min, and the supernatant was separated by aspiration. The range of the standard curve was 5–5,000 femtol of cGMP, and the radioactivity was measured in a gamma counter.

Determination of PKG activity. The SCN were obtained as mentioned and were mechanically disrupted with a glass rod in 20 mM Tris·HCl, 0.25 M sucrose, 200 μM EGTA, 200 μM EDTA, 50 μM phenylmethylsulfonyl (PMSF), and β-mercaptoethanol, pH 6.18. After a 5-min centrifugation at 13,200 rpm, a dilution of the supernatant was used in the assays to determine PKG activity, the reaction assay tubes contained 20 μl of the following mixture: tissue (3–7 μg protein), 20 mM Tris·HCl, 10 mM MgSO4, 50 μM EGTA, 50 μM EDTA, 50 μM 1,4-dithiothreitol, 100 μM ATP (γ-[32P]ATP, 10 Ci/ mmol), and a specific PKG substrate [5 μg histone H2B (Alexis)]. Tubes were stored on ice, and the reaction was initiated when γ-[32P]ATP was added. After 5 min at 37°C, the reaction was stopped by pouring 15 μl from each tube onto a phosphocellulose disc (Whatman P-81). Discs were washed twice for 10–15 min with 1% H3PO4 (vol/vol, 10 ml per sample) and twice for 5–10 min with water. 32P retained in the filters (that corresponds to [32P]incorporated in the substrate) was quantified in a liquid scintillation counter (1214 RackBeta, Wallac). All samples were assayed with and without histone 2B as control.

Determination of GC activity. SCN were obtained as described at ZT4 and 16. GC activity was determined as described previously (11) with minor modifications. Tissue was homogenized in (in mM) 40 Tris, 6 Cl2Mn, 1 PMSF, and 0.01 sodium orthovanadate, pH 7.6. The reaction assay tubes contained 100 μl of the following mixture: 40 mM Tris·HCl, 3 mM Cl2Mg, 0.5 mM PMSF, 0.005 mM sodium orthovanadate, 1 mM cGMP, 0.2 mM guanosine 5′-triphosphate (GTP), [32P]GTP (150,000 cpm/tube; specific activity, 15 Ci/mmol), 1 mM IBMX, 0.5 mg/ml BSA, 0.3 mg/ml creatine phosphokinase, 6.62 mg/ml phosphocreatine, and 50 μl tissue. Tubes were incubated for 15 min at 37°C, and the reaction was stopped by adding 0.5 ml 30 mM EDTA, [3H]cGMP (50,000 cpm/tube) (4°C) per tube and boiling for 1 min. After centrifugation for 4 min at 13,200 rpm, the supernatant fraction was used, and cGMP was separated by column chromatography (alumin, 100–200-μm mesh) with 6 ml 0.1 M Tris·HCl, pH 7.5. [32P]cGMP and [3H]cGMP were quantified in a liquid scintillation counter (1214 RackBeta, Wallac).

Phosphodiesterase activity. SCN were obtained as described at ZT4 and ZT16. cGMP-phosphodiesterase (PDE) activity was determined as described previously (11) with minor modifications. Tissue was homogenized in 80 mM Tris and 10 mM Cl2Mg, pH 8, and the supernatant of a 5-min centrifugation at 13,200 rpm was used in the assays. The reaction assay tubes contained 100 μl of the following mixture: 40 mM Tris·HCl, 5 mM Cl2Mg, 100 μM cGTP, 4 mM β-mercaptoethanol, 100 μM cGMP, [3H]GMP (150,000 cpm/tube; specific activity, 15 Ci/mmol), and 50 μl tissue. Tubes were incubated for 30 min at 37°C, and the reaction was stopped by boiling the tubes for 1 min. Samples were incubated for 10 min at 37°C with 50 μl 5′-nucleotidase (1 mg/ml). The unreaceted cyclic nucleotides were removed, and 1 ml of resin (Dowex X-8, 200–400 mesh) plus three parts of methanol were added. After centrifugation for 15 min at 13,200 rpm, the supernatant fraction was used to determine radioactivity in a liquid scintillation counter.

Western blot. SCN were homogenized in 50 mM Tris·HCl (pH 7.4), 1 mM PMSF, 1 mM EDTA, 1 mM EGTA, and 0.32 M sucrose. SCN proteins (30 μg) were run on 9% acrylamide gels and electrophoretically transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) for 2.5 h at 400 mA, and the membranes were blocked in 1% BSA in TBS for 1 h. After a 24-h incubation with a specific antibody (antibody to rabbit, rabbit; 1:500, CalBiochem, La Jolla, CA), membranes were washed three times with Tween-Tris-buffered saline (TTBS), and immunoreactivity was assessed using a secondary antibody coupled to horseshad peroxidase (Bio-Rad; diluted 2.7 μl/10 ml TTBS), and visualized by means of a chemiluminescence reaction (ECL reaction kit, Amersham Life Science, Piscataway, NJ) on Agfa Curix RP1 film. Films were scanned, and relative protein content was estimated by performing an intensity densitometry of the immunoreactive bands. In separate assays we used specific antibodies raised in rabbits against isofrom I (PKG I, 1:1,000) or II (PKG II, 1:1,000) of the enzyme (a generous gift from Prof. F. Hofmann, Institut für Pharmakologie und Toxikologie, München, Germany), using smooth muscle or whole brain as positive controls, respectively (28, 36).

Modulation of light-induced phase shifts in vivo. Hamsters were anesthetized with pentobarbital sodium (80 mg/kg) and implanted with 22-gauge stainless steel guide cannulas (Plastics One, Roanoke, VA) aimed for the third ventricle. Cannulas were implanted 1.0 mm above the target site between the bilateral SCN nuclei (coordinates relative to bregma: anteroposterior +0.6, mediolateral 0.0, dorsoventral −8.0 to −8.2 depending on skull thickness; tooth bar was set at −2.0 mm). After recovery from anesthesia, the animals were housed in a 14:10-h LD cycle for 1 wk before being placed in constant dim red light for the duration of the experiment.
Wheel-running activity was monitored continuously and recorded using Dataquest III (Minimitter, Sunriver, OR). The onset of activity for each day or circadian cycle was used as a marker of the phase of the activity rhythm and was defined as CT12. Phase shifts in DD conditions were calculated using eye-fitted lines drawn through consecutive onsets of activity for the 7 days before each experimental manipulation (day 0) and on subsequent days 4 through 10. These were used to extrapolate CT12 on day 1 projected by the pre- and postpulse lines.

After stable rhythms under DD were monitored, animals received a 15-min light pulse (400 lx) at CT18 (the times at which maximum phase advances are obtained). Fifteen minutes before a light pulse, experimental animals received a 0.2-μl microinjection (over 2 min) of KT-5823, a selective PKG inhibitor (LC Laboratories; 20 μM in 50% DMSO-saline), 1H-[1,2,4]oxadiazolo[4,3-a]quinoline-1-one (ODQ), a selective GC inhibitor (Research Biochemicals, Natick, MA; 1 μM in 50% DMSO-saline), or vehicle. Control animals received drug injections without light pulses at CT18.

On completion of behavioral studies, animals were perfused intracardially with 100 ml saline, followed by 200 ml of 10% formalin (in 0.1 M phosphate buffer). Brains were then removed and postfixed in a formalin-sucrose solution (30%) for 24 h. Cresyl violet-stained sections (40 μm) were examined to verify injection sites.

Modulation of light-induced Fos expression in the SCN. For immunocytochemical localization of light-induced c-Fos in the SCN, animals received 0.2-μl icv injections of 50% DMSO-saline, 20 μM KT-5823, or 1 μM ODQ, that were followed 15 min later by a 15-min light pulse. One hour after this, hamsters were perfused with 4% paraformaldehyde (in 0.1 M phosphate buffer). The following day, sections were washed in PBS and incubated for 1 h with anti-rabbit biotinylated secondary antibody (Vector Labs; 1:1,000). Fos immunoreactivity was visualized through an avidin-biotin-peroxidase reaction (ABC kit, Vector Labs) with the addition of diaminobenzidine as a reagent.

RESULTS

As shown in Fig. 1A, cGMP levels in the SCN exhibited a daily variation in animals kept under an LD cycle (ANOVA: F = 8.75, P < 0.0001), and this variation persisted under DD conditions (Fig. 1B, ANOVA: F = 5.87, P < 0.0015). Minimal values were measured...
around ZT/CT = 20 and maximal values around ZT/CT = 24–04. This daily modulation in the cGMP levels appears to be the result of a differential cGMP-PDE activity. As shown in Fig. 2A, PDE activity exhibited a significant variation at midphase points (t_{34} = 3.993, P < 0.001, Student’s t-test). GC activity did not exhibit a significant variation at the same time points (Fig. 2B, t_{22} = 0.6512, P > 0.5).

We observed a significant PKG activity in the SCN, which was dependent on the presence of the specific substrate histone 2B (basal activity, 2.39 ± 0.14 fmol P_1·µg protein −1·5 min −1; boiled tissue control, 0.20 ± 0.02 fmol P_1·µg protein −1·5 min −1; P < 0.01, Dunnett’s multiple comparisons test vs. basal values). Histone 2B-independent activity did not vary throughout the day (data not shown). The addition of 8-bromoadenosine-cGMP increased basal activity by 34% (P < 0.05, Dunnett’s multiple comparisons test vs. basal values).

As shown in Fig. 3A, PKG activity, measured in the presence of an exogenous substrate, presented a significant diurnal rhythm under LD that resembled the changes in cGMP levels (ANOVA: F = 28.16, P < 0.0001), with maximal values during the day. This rhythm persisted under constant dark conditions (ANOVA: F = 11.63, P < 0.0001) (Fig. 3B). This variation in PKG activity correlated with diurnal and circadian rhythms in the levels of the enzyme as determined by Western blots (Fig. 4, top). As shown by the Western blots of Fig. 4, bottom, the PKG II isoform is the one present in the SCN.

To understand the role of the cGMP-related system in circadian entrainment, we administered phase-shifting light pulses during the circadian night. cGMP levels were increased significantly after 5-min light pulses at CT18 (t = 3.019, P < 0.03, Student’s t-test) but were unaffected by the same photic stimulus at CT14 (t = 0.319, P > 0.7) (Fig. 5).

As we previously showed with KT-5823 (a specific PKG inhibitor, see Ref. 33), administration of the GC inhibitor ODQ at CT18 significantly blocked light-induced phase advances (vehicle + light: 2.06 ± 0.45 h; KT-5823 + light: 0.55 ± 0.16 h; ODQ + light: 0.69 ± 0.21 h) (Fig. 6, A-C) (ANOVA: F = 7.672, P < 0.025).

Drug or vehicle had no effect on circadian phase when administered without photic stimulus (data not shown).

Light pulses at CT18 induced c-Fos expression along ventrolateral portions of the SCN (Fig. 7A). Fos expression was not affected by administration of KT-5823 (Fig. 7B) or ODQ (Fig. 7C) before the light pulse.

DISCUSSION

The results in the present studies indicate that the cGMP-related signal transduction pathway presents a daily variation in the hamster SCN that persists in...
The variation in cGMP levels is related to temporal changes in specific PDE activity and not to GC activity, because PDE activity was minimal during the day in correlation with the increase of cGMP levels. It is possible that light pulses administered during the night modify the levels of cGMP and activity of PKG as one of the ways of phase-shifting the mechanism of the clock: as nocturnal values are low, a putative hypothesis is that light transiently increases the levels of these variables to entrain the oscillator. Indeed, light pulses during the late subjective night (CT18) resulted in a significant increase in cGMP levels, but were not effective at CT14, the time when light induces phase delays of hamster circadian rhythms. It has been suggested that signal transduction pathways might differ for delays and advances of the clock (17, 19); indeed, inhibition of PKG results in an impairment of light-induced phase advances but not delays (33, 55), suggesting that the cGMP-related system is effective in the transient acceleration of clock dynamics, but not for the slowing down that is apparently required for phase delays (37).

These results also suggest that two of the most ubiquitous second messenger systems, cAMP and cGMP, appear to play opposite roles in the regulation of circadian rhythmicity. Evidence from in vitro experiments suggests that cAMP and its related protein kinase PKA are more likely to mediate responses to nonphotic stimuli (38, 39). Accordingly, it has been reported that inhibition of PKA in the SCN in vivo does not affect light-induced phase shifts (33). PKA activity also shows a strong diurnal rhythm in the SCN that is constant darkness, suggesting an endogenous control. Although our experimental conditions do not allow us to determine the circadian period of these fluctuations, it appears that under DD the peak of the rhythm is phase-shifted with respect to entrained conditions.

Fig. 5. cGMP content in the hamster SCN after a 5-min light pulse applied at CT14 and 18. Hamsters were killed, and the cGMP content was assessed as described. Significant differences between light-treated and control animals were found at CT18 (t8 = 3.019, *P < 0.03, Student's t-test) but not at CT14 (t6 = 0.309, P > 0.7).

Fig. 6. Effects of KT-5823 and 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one (ODQ) on light-induced phase shifts at CT18. A-C show double-plotted actograms with successive days in the ordinate axis and CT (in h, ranging from 0 to 48) in the abscissa. Crosses, the time of manipulation; straight lines, eye-fitted regressions of activity onsets before and after treatments. A: representative actogram of an animal receiving intracerebroventricular ODQ administration followed by a light pulse at CT18. B: representative actogram of an animal receiving ODQ alone at CT18. C: representative actogram of an animal receiving vehicle intracerebroventricularly before a light pulse at CT18. Light pulses induced significant phase advances that were blocked by ODQ. D: summary of phase changes at CT18. The column labeled KT-5823 represents previously published data (33) showing that PKG inhibition blocks light-induced phase advances. (ANOVA, F = 7.672, P < 0.025; *P < 0.05 vs. vehicle, Tukey's test).
dependent on the LD cycle and disappears under DD conditions (13).

PKG inhibitors block photic phase advances to light in vivo, and here we show that ODQ, a specific inhibitor of NO-dependent GC (15, 45), is also effective in blocking light-induced phase shifts. As for NO itself, by immunocytochemistry and direct enzymatic reactions, both NOS and NADPH diaphorase were found throughout the SCN (5, 44, 54). Local administration of NOS inhibitors blocks light-induced phase shifts of circadian locomotor activity rhythms (7, 34, 53), whereas NO donors enhance the circadian response to light (34). We have determined that NOS activity exhibits a significant diurnal variation when assessed at midphase points in an LD cycle; however, no significant differences were observed when animals were kept in constant darkness and enzyme activity was assayed during the subjective night and day, suggesting that NOS activity is not directly controlled by the circadian clock. Indeed, light pulses augmented SCN NOS activity, but irrespective of the phase of stimulation (12).

One of the key targets of NOS in the central nervous system is activation of soluble GC, resulting in an increase of cGMP levels that in turn might activate a PKG, in particular, when NOS is activated by glutamate (6). It is possible that, at least during the late subjective night, light reaches the clock through a glutamate-Ca\(^{2+}\)-NOS-GC-cGMP pathway, inducing PKG activation and other downstream factors that could also be clock regulated. Indeed, this does not appear to be the case for diurnal variations of cGMP, because we found no changes in GC activity levels at different times of day. It is possible that there are different signal transduction pathways for cGMP rhythm generation and light-induced changes.

The possible substrates for PKG in the brain have not been completely identified (14, 51, 52), although in vitro studies have suggested that PKG might activate several sequence elements such as the serum response element, the AP-1 binding site and the cAMP response element (21). Our characterization experiments indicate that the PKG II isoform is the one present in the SCN. This kinase is mostly expressed in the brain and has been suggested to affect neurotransmitter release, as well as having other intestinal and renal effects (28, 31, 36).

Cellular markers of the circadian response to photic stimulation in the SCN include members of the Fos family (2, 17, 20, 22, 30, 43, 46). In addition to IEG expression, the transcription factor CREB is phosphorylated to its active form in SCN cells in response to light (16). This evidence has led to the suggestion that phosphoCREB formation and activation of one or more of these IEGs are required for normal light-induced phase shifting and entrainment. However, circadian rhythms and photic responses of mice with a targeted mutation of the c-fos gene appear to be quite normal (23). Here we show that inhibition of PKG or GC result in a significant impairment of light-induced phase advances of locomotor activity rhythms but do not affect Fos expression at the same circadian time, in a similar way to what has been reported with regards to NOS inhibition (55). These results suggest the existence of divergent pathways for light-induced phase shifting of rhythms and IEG expression, or, alternatively, both kinds of responses could have a differential sensitivity to light. However, because AP-1 transcriptional activation depends on both Fos and Jun DNA-binding, an alternative explanation is that Jun expression or phosphorylation has been blocked by the pharmacological treatment.

Fig. 7. Fos expression in the SCN following light pulses at CT18 after vehicle (A), KT-5823 (B), or ODQ (C) intracerebroventricular administration. The number of Fos-expressing cells per SCN in each 40-μm slice was not affected (vehicle + light: 112.5 ± 21.2; KT-5823 + light: 108.2 ± 22.5; ODQ + light: 117 ± 27). Drug administration did not induce Fos expression by itself in any case. Illv, third ventricle; OC, optic chiasm.
Collectively, these results suggest that photic entrainment, a fundamental requisite for adaptation of circadian rhythmicity to its natural environment, might rely upon a signal transduction pathway that includes cGMP and PKG-dependent steps (at least for phase advances of the clock), supported at least partly by endogenous circadian rhythmicity of the cGMP-related system. It is interesting that these mechanisms are primarily related to light-induced phase advances and not phase delays: it is possible that for nocturnal animals the ecophysiological requirements at different time points (day-night transition for delays, the opposite for advances) result in differential cellular mechanisms responsible at each phase.

Mechanisms downstream of PKG activation must lead to long-term changes in SCN neurons leading to stable synchronization. Currently, there is a gap between what is known about the input to the clock (via light, glutamate, and specific intracellular signals) and the core components of the circadian oscillator. Light-induced resetting of per and other genes (3, 4, 47, 48) are probably accompanied by concomitant posttranslational changes in clock proteins, of which phosphorylation is certain to play a key role. The first clock gene coding for an enzyme (casein kinase I) was recently reported (32), and its mutation has a profound effect of circadian rhythmicity. Identifying the kinases involved in the system will shed more light into how the clock tells time.

The help of Dr. H. de la Iglesia, Dr. R. Rosenstein, and M. I. Keller Sarmiento is gratefully acknowledged. Drs. P. Ruth and F. Hoffman (Institut für Pharmakologie und Toxikologie, München, Germany) kindly gave us the PKG I and II antibodies.

This work was supported by Consejo Nacional de Investigaciones Científicas y Tecnológicas, Agencia Nacional de Promoción Científicas y Tecnológicas, Universidad Nacional de Quilmes, Becas Carrillo-Oñativia (Ministerio de Salud), and Fundación Antorchas (Argentina). D. A. Golombek is the recipient of John Simon Guggenheim Memorial Foundation fellowship.

REFERENCES


