RESEARCH ARTICLE

Circadian and pharmacological regulation of casein kinase I in the hamster suprachiasmatic nucleus

PATRICIA V. AGOSTINO, SANTIAGO A. PLANO and DIEGO A. GOLOMBEK*

Laboratorio de Cronobiología, Departamento de Ciencia y Tecnologia, Universidad Nacional de Quilmes / CONICET, Roque Sáenz Peña 352, Bernal (B1876BXD), Buenos Aires, Argentina

Abstract

In mammals, the mechanism for the generation of circadian rhythms and entrainment by light–dark (LD) cycles resides in the hypothalamic suprachiasmatic nuclei (SCN), and the principal signal that adjusts this biological clock with environmental timing is the light:dark cycle. Within the SCN, rhythms are generated by a complex of molecular feedback loops that regulate the transcription of clock genes, including *per* and *cry*. Posttranslational modification plays an essential role in the regulation of biological rhythms; in particular, clock gene phosphorylation by casein kinase I , both epsilon (CKIε) and delta (CKIδ), regulates key molecular mechanisms in the circadian clock. In this paper, we report for the first time that CKI activity undergoes a significant circadian rhythm in the SCN (peaking at circadian time 12, the start of the subjective night), and its pharmacological inhibition alters photic entrainment of the clock, indicating that CKI may be a key element in this pathway.

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Introduction

In mammals, the mechanism for the generation and entrainment of circadian rhythms resides in the hypothalamic suprachiasmatic nuclei (SCN), and the principal signal that adjusts this biological clock with environmental timing is the light:dark cycle. Within the SCN, rhythms are generated by a complex of molecular feedback loops that regulate the transcription of clock genes, including *per* and *cry* (Ko and Takahashi 2006). Posttranslational modification plays an essential role in the regulation of biological rhythms (Gallego and Virshup 2007); in particular, clock gene phosphorylation by casein kinase I, both epsilon (CKIε) and delta (CKIδ), regulates key molecular mechanisms in the circadian clock (Virshup *et al*. 2007). In this paper we report, for the first time, that CKIε activity undergoes a significant circadian rhythm in the SCN, peaking at circadian time 12 (CT12), the start of the subjective night, and its pharmacological inhibition alters photic entrainment of the clock, indicating that CKIε may be a key element in this pathway.

Light reaches the SCN through the retinohypothalamic tract (RHT) and causes the release of glutamate, which initiates a signal transduction cascade in SCN neurons that ultimately results in a phase shift of the circadian system (Morin and Allen 2006). Nocturnal light pulses entrain the clock, delaying its phase in the early night and causing phase advances during the late night. Both responses involve the activation of calcium-related signals, including Ca^{2+}/calmodulin-dependent kinase II and neuronal NOS (nitric oxide synthase) (Agostino *et al*. 2004; Golombek *et al*. 2004). Transductional signals entrain the activity of the molecular clock by inducing the expression of clock genes and changing the dynamics of molecular feedback loops that ultimately establish the timing of the oscillator.

The protein kinases, casein kinase I epsilon (CKIε) and delta (CKIδ) mediate posttranscriptional regulation of levels and activity in components of the molecular clock. Mutations altering activity of *CKIε* and *CKIδ* can produce profound changes in circadian period in mammals (Ralph and Menaker 1988; Lowrey *et al*. 2000; Xu *et al*. 2005; Meng *et al*. 2008) and can alter both the accumulation of PER in the cytoplasm as well as nuclear translocation of PER-CRY

*For correspondence. E-mail: dgolombek@unq.edu.ar.

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dimers (Lee *et al*. 2001; Akashi *et al*. 2002; Eide *et al*. 2002, 2005). In humans, advanced sleep phase syndrome can result from either a mutation in *CKIδ* or loss of a phosphorylation target of CKIε, δ in the *hper2* gene (S662G) (Toh *et al*. 2001; Xu *et al*. 2005). This study was conducted to investigate the possibility of rhythmic control of CKI activity and the role of CKIε in sensitivity to light during the late night.

Materials and methods

Animals

Syrian hamsters (*Mesocricetus auratus*) were raised in our colony and were maintained in a 14:10 h light:dark cycle (14:10 LD, lights on at 0600 h) with food and water provided ad libitum, and room temperature set at 20 ± 2°C. Male adult animals (3–4 months old) were used in all the experiments. Locomotor activity in 17 cm-diameter wheels was recorded every 5 min. CT12 was defined as the time of activity onset in constant dark conditions. All animal procedures were performed in strict accordance with NIH rules for animal care and maintenance.

Surgery and intracerebroventricular injections

Hamsters were deeply anesthetized with 75 mg/kg ketamine and 10 mg/kg xylazine. Stainless steel guide cannulae (22 gauge, Plastics One, Roanoke, USA) for intracerebroventricular (ICV) injection were implanted into the third ventricle (0.6 mm anterior to bregma and 8.2 mm ventral to the skull surface, directly on the midline, with the tooth bar set at −2.0 mm), and dummy cannulae were inserted to prevent blockade of the tubing. Animals were maintained for recovery for at least two weeks under LD conditions before drug administration. Injections of CKI-7 (100 μM, 2 μl) were made after 10 days in DD with the aid of a dim red safe light. Phase shifts were measured as described by Agostino *et al*. (2008).

In vivo inhibition of CKIε

Wild-type hamsters received an ICV injection of 2 μl vehicle (sterile saline) or 100 μM CKI-7 (US Biological, Swampscott, USA), either 30 min before or 15 min following a light pulse (15 min, 50 lux) at CT18. Dark controls were handled in the same way but without the light.

Determination of CKIε activity

CKIε activity was assessed by a phosphorylation assay in wild-type hamsters at CT 4, 8, 12, 16, 20 and 24. Animals were kept under constant dark conditions for 2–3 days and were sacrificed at the corresponding circadian hours. CT12 was determined as the previous time of lights off, since no significant changes in circadian phase would have occurred after two days in DD. Hamsters were sacrificed by decapitation and their brains were quickly excised and placed in an ice-cold environment. An 800 μm hypothalamic slice was cut and tissue containing the SCN was punched out with a micropuncher (1 mm diameter). SCN-containing tissue was mechanically disrupted with a glass rod in 25 mM ice-cold Tris-HCl pH 7.4 containing 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 200 mM sodium vanadate and a mammalian protease inhibitor cocktail (Sigma, Cat. No. P8340; dilution 1:100). CKIε activity was determined following the method of Lowrey *et al*. (2000), slightly modified. Briefly, samples (~125 μg of homogenized SCN tissue) were incubated 15 min at 37°C in 100 μl of reaction mix (25 mM Tris-HCl pH 7.5, 7 mM $MgCl_2$, 0.5 mM DTT, 200 μM ATP, 50 μg/ml BSA, 0.5 mg/ml substrate, 5 μCi of γ^{32}P-labelled ATP, 3000 Ci/mmol (NEN Research Products, Boston, USA). Dephosphorylated casein (Sigma, Cat. No. C8032) was used as substrate. Initiation of the reaction occurred upon addition of the γ^{32}P-labelled ATP. Reactions were terminated by the addition of 2× sample buffer (100 mM DTT, 2% SDS, 80 mM Tris-HCl pH 6.8, 0.0006% bromophenol blue, 15% glycerol), followed by boiling for 2 min.

Because this was not an immunoprecipitated kinase assay, the phosphorylation reaction was performed in the presence of other kinase inhibitors, such as PKA/PKC (Ro 31-8220 1 μM), PKG (KT-5823, 100 μM) and CaMK (KN-93 100 μM) inhibitors. We also used the inhibitor CKI-7 (which is a potent inhibitor for CKI compared to CK2 and other kinases) as a control, and phosphorylation was significantly reduced in control samples (data not shown).

Products were resolved on 12% SDS-polyacrylamide gels. After electrophoresis, gels were fixed in 12% acetic acid, 50% methanol and 0.05% formaldehyde for 2 h and then exposed to a film (AGFA) for two weeks at −80°C. Densitometry was carried out using Sigma Gel software (Jandel Scientific Software, San Rafael, USA). Protein levels were measured by staining the gels with silver staining method. Data are expressed as mean ± S.E. (n = 3/time point).

Results and discussion

We have recently reported that sensitivity to light is significantly increased in homozygous *CKIε*$^{tau/tau}$ hamsters (Agostino *et al*. 2008), indicating a role for *CKIε* not only in the rhythm generation mechanism but also in signal transduction mechanisms that participate in entrainment. That suggestion is now supported by results from direct pharmacological inhibition of CKI in wild-type hamsters. We administered CKI-7 (N-(2-aminoethyl)-5-chloroisoquinoline-8-sulphonamide) that has been characterized as a selective CKI inhibitor, without affecting CK2 activity (Chijiwa*et al*. 1989; Cheng and Louis 2001), although it does not discriminate between the epsilon and delta forms. This inhibitor has been widely used not only in circadian studies (e.g., Miyazaki *et al*. 2004; Preuss *et al*. 2004) but in other experimental models as well (e.g., Alappat *et al*. 2005). CKI-7, administered 30 min before a light pulse, significantly increased the size of phase shifts induced by light at CT18

while having no effect on phase when given in the dark (figure 1). The simplest explanation for this is that CKI activity is responsible for inhibitory regulation of RHT input via NMDAR transmission. Thus, inhibition of CKI activity results in increased input and larger phase shifts. CKIε has been reported to downregulate signalling via glutamate NMDA receptors (Chergui *et al*. 2005) which carry the photic signal from the retinohypothalamic tract into the SCN (Morin and Allen 2006). In this context, the circadian rhythm of CKI activity suggests that control of glutamatergic neurotransmission via modulation of NMDAR is a mechanism by which the clock is able to restrict its photic responsiveness to the subjective night. This may be accomplished even though intensity and duration appear to set the light pulse above saturating levels. Increases in phase-shift magnitude can be explained if PER and CRY proteins accumulated more rapidly in the hours following the light pulse in the presence of CKIε inhibition, consistent with the current negative feedback model. Moreover, light-induced shifts were unaltered when CKI-7 was administered 15 min after the light pulse (figure 1) suggesting that the transduction of the photic signal by the retinorecipient cells occurs rapidly, and is essentially complete in 15 min.

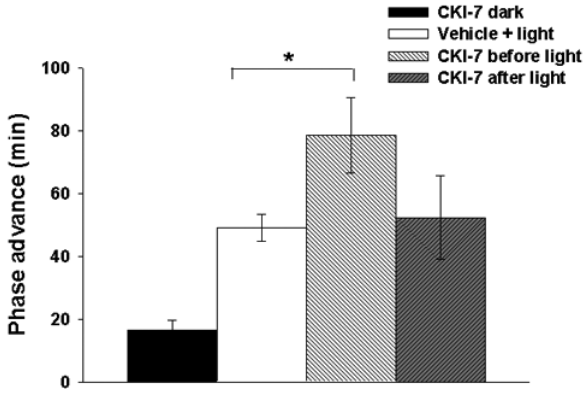

Figure 1. *In vivo* inhibition of CKI in wild-type hamsters. Animals received an ICV injection of vehicle (sterile saline) or CKI-7 (100 μM, 2 μl) either 30 min before or 15 min following a light pulse (15 min, 50 lux) at CT18. Dark controls were handled in the same way but without the light. Vehicle administration in the dark or after the light pulse had no effect on circadian phase (data not shown). Data are expressed as mean ± S.E. (n = 6 animals per group). $^{*}P < 0.05$; ANOVA followed by Tukey's test.

We analysed whether CKIε was indeed active at this time point. Although it has been reported that mRNA and protein levels for CKIε do not vary through the circadian cycle (Ishida *et al*. 2001; Lee *et al*. 2001), we found that the activity of the enzyme exhibits a robust circadian rhythm (figure 2) with a peak at CT12. This timing coincides with the peak activity of another circadian regulatory kinase, GSK3β, as well as the peak expression of mPER2 and mCRY2 proteins.

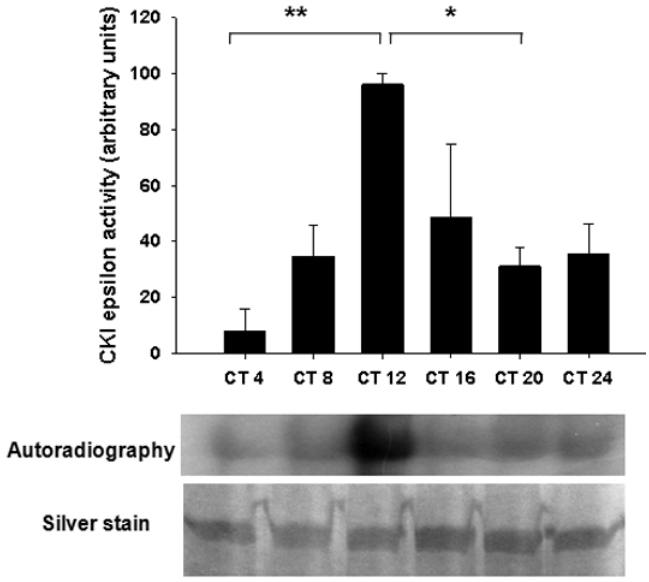

Figure 2. Circadian rhythm of CKI activity in wild-type hamsters. CKIε activity was assessed by phosphorylation assay in wild-type hamsters at CT 4, 8, 12, 16, 20 and 24. Plotted values are the mean values ± S.E. from three independent experiments. A clear circadian rhythm was found, peaking at CT12. $^{**}P < 0.01$: CT12 versus CT4; $^{*}P < 0.05$: CT12 versus CT 20; ANOVA followed by Tukey's test.

Our findings support the idea that CKIε activity is directly implied in circadian responses to light and that it represents a rhythmic target for molecular entrainment pathways. Photic entrainment exhibits two kind of gating controls. On one hand, light might change the phase of the clock (during the subjective night) or have no effect on circadian phase (during the subjective day). On the other hand, nocturnal light pulses can induce phase delays or advances depending on the timing of the stimulus. Two main hypotheses have been proposed for this kind of gating: either different signal transduction mechanisms are specifically responsible for light-induced phase delays or advances, or the same kind of mechanism undergoes cyclic changes that enable a differential responsivity to light at various times of the day. Our results suggest that the circadian rhythm in CKIε activity, together with its role in the strength of photic responses, makes it a likely candidate for the differential responses to light. Indeed, taking into account our finding that homozygous *CKIε$^{tau/tau}$* hamsters exhibit a higher sensitivity to light, we can conclude that CKIε is not only involved in the molecular clock dynamics but also in the regulation of circadian entrainment.

According to our experiments, a certain level of CKIε activity prior to or during a light pulse is necessary for normal phase shifting and entrainment, while the change of activity either by mutation (Agostino *et al*. 2008) or pharmacological interference results in larger shifts. CKIε therefore appears to be a suppressor of photic responses.

A different inhibitor, PF670462, was found to induce significant phase delays in rats at CT12, and a minimum effect on photic phase shifts at CT18 (Badura *et al*. 2007) This discrepancy with our results could be related to several factors, such as species (rats versus hamsters), dose (100 mg/kg versus 100 μM) and way of administration (SC versus ICV) It is interesting to note that the most robust delay seems to occur at CT12, coinciding with the time of peak in CKIε activity. Moreover, CKIε can phosphorylate multiple targets, including the clock proteins PER, CRY1 and BMAL1 (Lowrey *et al*. 2000; Eide *et al*. 2002), with diverse effects (Akashi *et al*. 2002). It is not clear whether CKI-7 is able to reduce all phosphorylation activity of CKIε or is rather specific for certain targets. In addition, it is important to consider that our enzymatic activity rhythm corresponds to casein phosphorylation, which might not necessarily be the same as that for clock proteins like PER.

Interestingly our results are in the same direction to those reported by (Shimomura *et al*. 1998) for the short period *tau* mutant hamster ($CKI\varepsilon^{tau/tau}$), raising the possibility that the mutation and the pharmacological inhibitor have some actions in common. The mutation, *tau*, has been shown to be a loss of kinase function (Lowrey *et al*. 2000; Eide *et al*. 2002) as well as a gain of function in nuclear translocation of the PER:CRY complexes (Gallego *et al*. 2006). *CKI*ε *tau* showed reduced activity for casein phosporylation *in vitro* (Lowrey *et al*. 2000; Eide *et al*. 2002; Vanselow *et al*. 2006) but *in vivo* studies suggest that CKIε *tau* increases phosphorylation of PER1 and PER2 (Gallego *et al*. 2006; Meng *et al*. 2008). It may be the case that while CKIε kinase activity is reduced by *tau*, the affinity for PER:CRY complexes is increased, resulting in greater net phosphorylation and potentiated nuclear transport.

In summary, our studies provide evidence in favour of a role for CKIε in the regulation of light-induced entrainment of circadian rhythms. Whereas the multiple substrates of this enzyme, even within the circadian system, make this a formidable challenge to study, its rhythmic activity in the SCN, tied closely to the day–night transition, attests to its importance in temporal regulation of physiology and a site for pharmacological intervention.

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