SHORT COMMUNICATION

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Cell-based inhibitor screening identifies multiple protein kinases important for circadian clock oscillations

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Molecular oscillation of the circadian clock is based on E-box-mediated transcriptional feedback loop formed with clock genes and their encoding products, clock proteins. The clock proteins are regulated by post-translational modifications such as phosphorylation. We investigated the effects of a series of kinase inhibitors on gene expression rhythms in Rat-1 fibroblasts. The period of the cellular circadian rhythm in culture was lengthened by treatment with SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor), IC261 (CKI inhibitor) and Roscovitine (CDK inhibitor). On the other hand, the period was shortened by SB216763 (GSK-3 inhibitor) or KN93 (CaMKII inhibitor) treatment. Application of 20 μM KN93 completely abolished the rhythmic gene expression. The activity of CaMKII exhibited circadian variation in a phase close to the E-box-mediated transcriptional rhythms. *In vitro* kinase assay revealed that CaMKII directly phosphorylates N-terminal and Ser/Pro-rich domains of CLOCK, an activator of E-box-mediated transcription. These results indicate a phosphorylation-dependent tuning of the period length by a regulatory network of multiple kinases and reveal an essential role of CaMKII in the cellular oscillation mechanism.

Introduction

Many physiological and behavioral processes are under the control of the circadian clock.[1] In mammals, the circadian clock system is organized in a hierarchy of multiple layers of oscillators. A master pacemaker in the suprachiasmatic nucleus (SCN) of hypothalamus orchestrates an array of slave oscillators existing in peripheral tissues via humoral factors and neuronal controls.[2] The cell-autonomous molecular oscillation is based on the transcriptional feedback loop driven by the clock genes and their products in both the SCN and peripheral tissues. Circadian locomotor output cycles kaput (CLOCK) and Brain and Muscle Arnt-like protein-1 (BMAL1) both encode bHLH-PAS containing transcriptional factors, which form a heterodimer to activate transcription of *Period (Per)* and *Cryptochrome (Cry)* genes through a CACGTG E-box *cis*-element. In turn, translated and nuclear accumulated PER and CRY proteins interfere with CLOCK-BMAL1-dependent transcriptional activation, thereby repressing expression of their own genes. Disruption of these circadian regulators abolishes cellular gene expression rhythms, indicating their essential roles in the oscillation mechanism.

The period length of the transcriptional feedback loop is finely tuned by post-translational modifications of clock proteins, such as phosphorylation. Inhibition of casein kinase (CK)Iε and δ or c-Jun N-terminal kinase (JNK) results in lengthening of the period of the gene expression rhythms in mammalian cells.[3-10] In chick pineal cells, SB203580, an inhibitor of p38 mitogen-activated protein kinase (p38 MAPK), lengthens the period of the circadian rhythm of the melatonin release.[11] SB202190, another inhibitor of p38 MAPK, lengthens the period of the gene expression rhythms in cultured human cells.[10] Inhibitors of cyclin-dependent kinase (CDK) such as Roscovitine lengthen ocular circadian rhythm in Bulla and Aplysia and the gene expression rhythms in cultured human cells.[10,12,13] Inhibition of glycogen synthase kinase (GSK)-3β or dual-specificity tyrosine phosphorylation-regulated kinase (DYRK)1A results in shortening of the period length of the gene expression rhythms in mammalian cells.[10,14]

Here we investigated the effects of a series of protein kinase inhibitors on bioluminescence rhythms from *Bmal1*-luciferase reporter in Rat-1 fibroblasts. The inhibitors examined in this study include those already reported in similar and/or other systems cited above.

Results

Screening of kinase inhibitors affecting molecular clock

We investigated the effects of a series of kinase inhibitors on the cellular circadian rhythm by using Rat-1 fibroblasts expressing a

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Bmal1-luciferase reporter.[15,16] No significant change in the period length was observed in the presence of U0126 [mitogen-activated protein kinase kinase (MEK) inhibitor], Gö6983 [protein kinase C (PKC) inhibitor], TBBt [casein kinase II (CKII) inhibitor], KT5720 [protein kinase A (PKA) inhibitor] or KT5823 [protein kinase G (PKG) inhibitor] (**Table 1**). Inhibitors of phosphoinositide 3-kinase (PI3K), wortmannin and LY294002, had no significant effect or only marginal effects on the period length of the cellular rhythms (**Table 1**). The period of the rhythm was lengthened by treatment with SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor), IC261 (CKI inhibitor) or Roscovitine (CDK2/7/9 inhibitor] (**Table 1 and Fig. 1**). On the other hand, the period length was shortened by SB216763 (GSK-3α/β inhibitor) or KN93 [Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) inhibitor]. These results demonstrate that the period length of the cellular clock is finely tuned by a network of the phosphorylation signaling mediated by multiple protein kinases. We found that the cellular oscillation was severely dampened in the presence of 20 μM KN93 (**Fig. 1**), and knock-down of CaMKIIγ and δ, 2 ubiquitous CaMKII isoforms, abolished the cellular rhythm in cultured cells.[16] These results demonstrate that CaMKII activity plays an essential role for robust oscillation of the cellular clock in addition to the regulatory role for the period length.

CaMKII as an essential component of oscillation system

CaMKII activity in the SCN exhibits a circadian variation in a phase close to that of the E-box-dependent transcription rhythm.[1,17] The circadian variation of the CaMKII activity was also observed in the peripheral tissue, such as the lung (**Fig. 2**). Recently, we have shown that CaMKII stimulates expression of E-box-regulated genes by enhancing the heterodimerization of CLOCK and BMAL1 and that overexpression of CaMKII in HEK293 cells increases the phosphorylation level of CLOCK.[16] Here we used 2 partial domains of CLOCK protein termed as N-terminal (NT) and Ser/Pro-rich (SP) domains for *in vitro* CaMKII phosphorylation assay (**Fig. 3A**). A constitutive active catalytic domain of CaMKII, 30K-CaMKII, phosphorylated GST-SP, a fusion protein of SP domain with glutathione S-transferase (GST) and MBP-NT, a fusion protein of NT domain with maltose-binding protein (MBP) (**Fig. 3B**). On the other hand, no significant phosphorylation was detected with GST or MBP alone. These results indicate that CaMKII directly phosphorylates the SP and NT domains of CLOCK. It is possible that CaMKII-mediated phosphorylation of these domains is important for the heterodimerization of CLOCK with BMAL1 and for activation of the E-box-dependent gene expression.

Table 1. Effects of kinase inhibitors on period length of Rat-1 cellular rhythm in culture

Inhibitor	Target kinase	Effect on period (concentration)
U0126	MEK	n.s.* (10 μM), n.s. (40 μM)
SB203580	p38-MAPK	+5.9 hr (10 μM), +17.0 hr (40 μM)
SP600125	JNK	+5.1 hr (10 μM)
Wortmannin	PI3K	n.s. (4 μM), n.s. (20 μM)
LY294002	PI3K	+1.8 hr (10 μM)
Roscovitine	CDK2/7/9	+0.5 hr (5 μM), +3.6 hr (10 μM)
IC261	CKIδ/ε	+0.5 hr (10 μM), +2.4 hr (40 μM)
Gö6983	PKC	n.s. (2 μM), n.s. (4 μM)
KT5720	PKA	n.s. (2 μM), n.s. (4 μM)
KT5823	PKG	n.s. (2 μM)
TBBt	CKII	n.s. (20 μM)
SB216763	GSK-3α/β	−1.8 hr (10 μM), −7.3 hr (40 μM)
KN93	CaMKII	−0.2 hr (2 μM), −2.3 hr (5 μM), −5 hr (10 μM)

*no significant effect was observed on the period length.

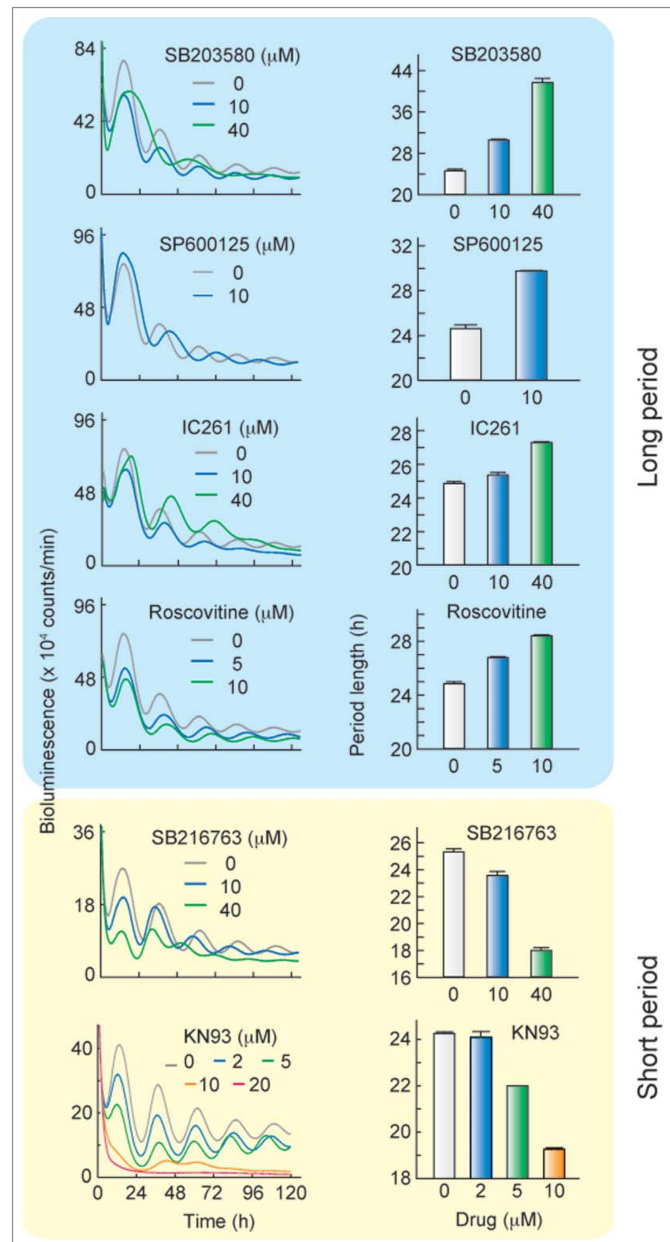

Figure 1. Period of the cellular clock is affected by treatment with SB203580, SP600125, IC261, Roscovitine, SB216763 or KN93. After treatment with 0.1 μM dexamethasone for 2 h, Rat-1-*Bmal1*-luc cells were transferred to the fresh medium containing 0.1 % DMSO with or without the kinase inhibitor. Left panels show representative raw results and right panels show mean period length with SEM from 4 independent experiments. The period length in the presence of 20 μM KN93 was not determined due to disruption of the bioluminescence rhythm.

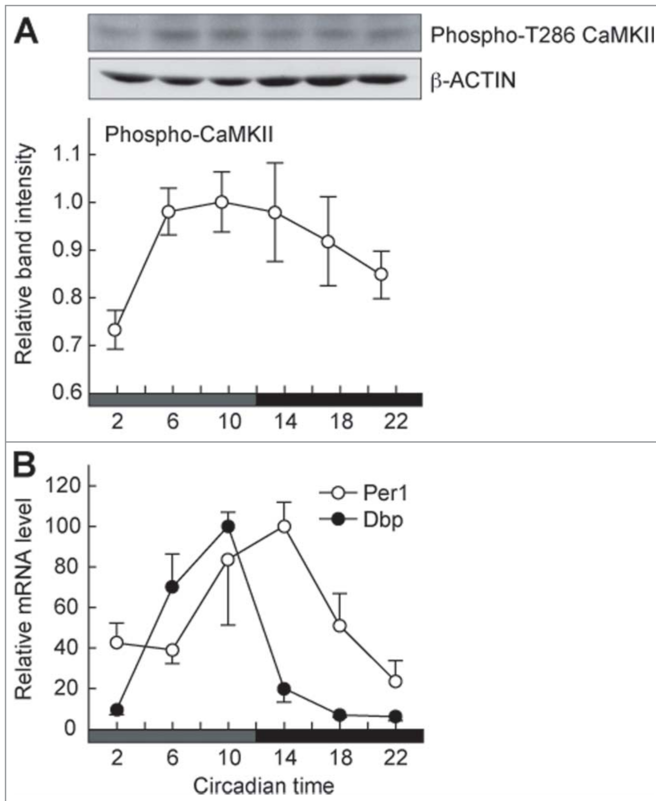

Figure 2. Circadian activation of CaMKII in phase with E-box-regulated gene expression rhythm. Mice were entrained to 12-h light/12-h dark cycles, and the lung was isolated from mice sacrificed every 4-hours on the first day under the constant dark condition. The samples were subjected to immunoblotting (**A**) or RT-PCR analysis (**B**). (**A**) Circadian profile of the phosphorylation (activation) levels of CaMKII. The activation levels of CaMKII were estimated by using an antibody recognizing phosphorylated T286 on CaMKII (Sigma-Aldrich), which represents its activated form. Top and middle panels show raw data for phospho-CaMKII and β-actin, respectively, and the band intensities of the former were quantified from 6 independent experiments (bottom panel). Data are mean with SEM, and the significant change is observed ($P < 0.05$, ANOVA). (**B**) Circadian changes in *Per1* and *Dbp* mRNA levels. The mRNA signals obtained by RT-PCR analysis were normalized to *Gapdh* mRNA. Data are mean with SEM from 4 independent experiments.

Conclusion

A cell-based phenotype screening of small molecule compounds is a very useful approach to identify modifying enzymes involved in the cellular clockwork.[7,10,15,18,19] The present study revealed that the period of the cellular clock was lengthened by SB203580, SP600125, IC261 and Roscovitine, consistent with the previous studies.[3-13] On the other hand, the period was shortened by SB216763 or KN93. We recently reported the roles of CaMKII in regulation of the circadian clock at multiple levels.[16] In the cellular level, CaMKII mediates Ca^{2+}-dependent regulation of the transcriptional feedback loop by activating E-box-dependent gene expression. CaMKII directly phosphorylates CLOCK (**Fig. 3B**), and the NT or SP domain of CLOCK contains 5 or 4 CaMKII consensus sequences, R/KXXS/T,[20] respectively (**Fig. 3C**). In the SCN, CaMKII activity is essential for synchronization of individual neuronal rhythms and for the synchronized oscillation between left and right SCN nuclei.[16] In contrast to the effect of KN93 on the period length in the cultured cells (**Fig. 1**), mice carrying a kinase-dead mutation in CaMKIIα (K42R) showed prolonged period length in wheel running rhythms.[16] Because the previous study demonstrated that inhibition of neuronal coupling among the SCN neurons resulted in prolongation of the period in behavioral rhythms,[21,22] it is possible that disruption of the neuronal coupling by the CaMKII mutation might have affected strongly the period length of the behavioral rhythms. Further behavioral analysis of the CaMKIIα mutant mice revealed that the kinase activity is important not only for the robust wheel running rhythm but also for the coupling between the morning and evening activity rhythms. In this way, our cell-based kinase inhibitor screening revealed CaMKII as an important mediator in the communication between the morning and evening oscillators in the behavioral rhythms.[16] Such behavioral phenotypes were quite unique, and further analysis of the mutant mice will provide a novel insight into the circadian regulation of the behavioral activities.

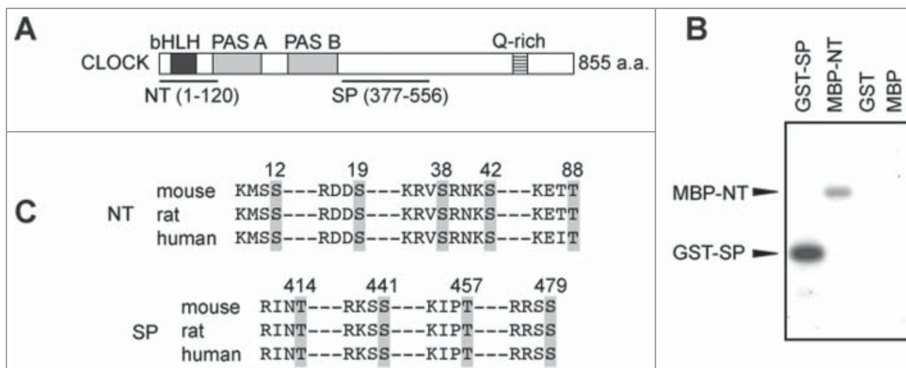

Figure 3. N-terminal region and Ser/Pro-rich region of CLOCK is phosphorylated by CaMKII. (**A**) Schematic drawing of the structure of mouse CLOCK protein. The N-terminal (NT) and Ser/Pro-rich (SP) region of CLOCK protein were subjected to the *in vitro* CaMKII phosphorylation assay. (**B**) *In vitro* CaMKII phosphorylation assay. GST-SP, MBP-NT, GST or MBP was used as a substrate protein for the *in vitro* CaMKII phosphorylation assay. GST-SP and MBP-NT were phosphorylated by 30K-CaMKII, whereas no significant phosphorylation was detected with GST or MBP. (**C**) Consensus CaMKII phosphorylation sequences (R/KXXS/T) in NT and SP region of CLOCK. The consensus sequences of mouse CLOCK were aligned with the corresponding regions of rat and human CLOCK. Gray areas indicate potential phosphorylation sites.

Materials & Methods

Real-time monitoring of circadian rhythms in cultured cells

Real-time monitoring of the circadian gene expression was performed using Rat-1 cells expressing a luciferase reporter under the control of the upstream region of *Bmal1* gene.[15,16] Rat-1 cells were treated with 0.1 μM dexamethasone for 2 h for the rhythm synchronization, and the bioluminescence signals from the cells were continually recorded at 37°C under air with dish-type bioluminescence detector, Kronos (ATTO, AB-2500) or LumiCycle (Actimetrics).

***In vitro* CaMKII phosphorylation assay**

The *in vitro* kinase assay was carried out at 30°C in a reaction mixture (10 μl) consisting of 1 mM dithiothreitol, 100 μM [γ-^{32}P]ATP, 10 ng 30K-CaMKII and 100 ng substrate protein.[23] The substrate proteins, GST-SP, MBP-NT, GST or MBP (each 100 ng) were prepared as described previously.[24] After incubation for 30 min, the reaction was stopped by the addition of 10 μl of 2 × SDS sample buffer. Phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography.

Animal Experiments

The animal experiments were conducted in accordance with the guidelines of the University of Tokyo. The mice (C57BL/6 background, male, 10-week-old) were housed individually at 23°C in cages with food and water available *ad libitum*. Immunoblotting and RT-PCR analyses were performed as described previously.[15,16,24]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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