Experimental Neurobiology
Vol. 13, pages 39~46, June 2004

Calcium-Induced Expression of *Period1* in SK-N-SH Human Neuroblastoma Cells

Jehui Kim[1], Youngshick Choe[1], Gi Hoon Son[1], June-Hee Park[1], Sukwon Lee[1], Inkoo Khang[1], Chung Choo Lee[2], Kun Ho Lee[1] and Kyungjin Kim[1]*

[1]School of Biological Sciences, Seoul National University, Seoul 151-742, Korea, [2]Division of Biological Sciences, Gachon Medical School, Ganghwa 417-980, Korea

ABSTRACT

In mammals, the suprachiasmatic nuclei (SCN) of the hypothalamus control endogenous circadian rhythms and entrainment to the environment. Light stimuli transiently increase the gene expression of *Period 1* (*Per1*) that is important in resetting of the mammalian central clock in the SCN. Pervious studies demonstrated that glutamate and N-Methyl-D-Aspartate (NMDA) mediate the effect of light stimuli on the central clock *in vivo*. However, molecular mechanism underlying the acute induction of *Per1* by the extra stimuli remains unclear. The present study is designed to set up an *in vitro* model and to examine the effect of calcium ion on *Per1* gene expression in the SK-N-SH human neuroblastoma cells using ribonuclease protection assay. Calcium ionophore A23187 (A23187) dramatically increased *Per1* mRNA levels in a time- and dose-dependent manner although glutamate and NMDA were unable to alter *Per1* gene expression in the SK-N-SH human neuroblastoma cells. In addition, the activity of human *Per1* promoter (-3829 to +123) was significantly promoted by treatment with A23187. The transcriptional inhibitor actinomycin D completely blocked A23187-induced increases in *Per1* mRNA levels, whereas cycloheximide, a potent translational inhibitor, did not. These results suggest that calcium ion may play an important role in the activation of *Per1* gene expression at the transcriptional level probably through the calcium responsive cis-elements located in 4.0 kb promoter region of *Per1* gene.

Key words: *Period 1*(*Per1*), clock gene, calcium ion, A23187, circadian clock, SK-N-SH human neuroblastoma cells

INTRODUCTION

All living organisms on earth have unique biological rhythms at period of approximately 24 h. That is so called circadian rhythm. In mammals, the master circadian clock resides in the suprachiasmatic nucleus (SCN) located in the hypothalamus of the brain (Johnson et al., 1988; Weaver, 1998). Light stimuli synchronize circadian oscillations of the core clock genes in SCN. Interactions among these

*To whom correspondence should be addressed.
TEL: 82-2-880-6694; FAX: 82-2-884-6560
e-mail: kyungjin@snu.ac.kr

"clock cells" in the whole SCN serve to synchronize individual circadian clocks to generate coordinated circadian outputs. These outputs ultimately control a vast array of circadian rhythms in physiology and behavior.

A clear view for the mammalian molecular clock within SCN neurons is beginning to emerge (Ikeda and Nomura, 1997; Tei et al., 1997; Reppert, 1998). The central clockwork appears to involve the transcriptional/translational feedback loop, which is similar to that in *Drosophila melanogaster*. In the fly, the positive transcriptional regulation of *Period (Per)* and *timeless (tim)* is controlled by two basic helix-loop-helix (bHLH)/PAS proteins, dCLOCK and dBMAL1, which heterodimerize and bind to the E box (CACGTG) (Hao et al., 1997; Allada et al., 1998; Darlington et al., 1998; Rutila et al., 1998; Lee et al., 1999).

Prominent differences between mammals and the other lives so far are discovered in the anatomical and molecular pathways by which light entrains the clock. In *Drosophila*, light leads to a degradation of TIM that is independent of eyes. In mammals, light coming into the eyes is transsynaptically communicated to the SCN, where it leads to an induction of *Per* mRNA. Photic signals are communicated via the retinohypothalamic tract (RHT), a direct neural projection from the retina to the SCN (Van den Polan, 1991; Gannon and Rea, 1993; Johnson et al., 1988). The master clock restricts its own sensitivity to stimulation so that only nocturnal light adjusts its timing, causing phase delays in early night and phase advances in late night (DeCousery, 1960; Takahashi and Zatz, 1982; Summers et al., 1984). This ensures that daily behavioral rhythms are synchronized appropriately to phases of the environmental cycle of darkness and light.

It is of notice that glutamate is the primary neurotransmitter transmitting light signals to the SCN (Johnson et al., 1988; Castel et al., 1993; Ding et al., 1994; Hannibal et al., 2000). Throughout night, a phase-resetting light stimulus evokes release of glutamate from the RHT and activation of multiple glutamate receptor types, of which NMDA receptors are critical (Colwell and Menaker, 1992; Ding et al., 1994; Mintz et al., 1999). The consequent influx of Ca^{2+} activates nitric oxide synthase to produce nitric oxide (Ding et al., 1994; Amir et al., 1995). After liberation of nitric oxide, the light signaling pathways diverge (Gillette, 1997). Light stimulus induce phase delay of the master clock through activation of neuronal ryanodine receptors in early night (Ding et al., 1998), whereas it promote the phase advance through activation of cGMP/PKG-dependent signal transduction cascade in late night (Weber et al., 1995; Ding et al., 1998). In both case, light-induced signaling cascades eventually induce phosphorylation of Ca^{2+}/cAMP response element binding protein (CREB) and CRE-mediated transcriptional activation (Ginty et al., 1993; Ding et al., 1997; Obrietan et al., 1998, 1999) and expression of a number of immediate-early genes including *c-fos* and *junB* in the retinorecipient zones of the SCN. These data strongly support the possibility that transcriptional regulation may play a critical role on resetting of the mater clock in the SCN.

Rapid and transient induction of *Period 1* expression is a prominent result of light-induced signaling cascades in both early and late night (Albrecht et al., 1997; Shearman et al., 1997; Shygeyoshi et al., 1997; Takumi et al., 1998; Zylka et al., 1998) and this increase is most critical for the entrainment of circadian behavior. However, the molecular mechanism underlying resetting of the master clock is largely unknown manly because of the lack of *in vitro* model. The present study is designed to set up an *in vitro* model and to examine the direct effect of calcium ion on the expression of *Per1* gene was examined in SK-N-SH human neuroblastoma cells.

MATERIALS AND METHODS

Materials

The human neuroblastoma cell line SK-N-SH (HTB-11) was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and cell culture reagents were from Gibco (NY, USA). A23187 were from Calbiochem (La Jolla, CA, USA). pGL3-luciferase vectors were from Promega (Madison, W1, USA) and pCMV-βgal that consists of cytomegaloviral promoter fused to the β-galactosidase gene was obtained from Stratagene (La Jolla, CA, USA). Other chemicals were purchased from Sigma.

Cell culture and drug treatment

SK-N-SH cells were maintained in Dulbeco's modified Eagle's medium (DMEM) with 4 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100μg/ml streptomycin, and 10% fetal bovine serum under a humidifying atmosphere containing 5% CO_2 at 37°C. Medium was refreshed every 2～3 d. For drug treatment, medium was changed to serum-free DMEM and incubated for 24 h. After incubation, medium was changed to 0.5μM A23187 containing medium. Actinomycin D (5μg/ml) and cycloheximide (10μM) were treated 3 h and 2 h before A23187 treatment, respectively.

Ribonuclease protection assay

Total RNAs were isolated by an acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Total RNA from SK-N-SH cells was reverse-transcribed into cDNA using random hexamer. A 220 bp *hPer1* (position: 599-818) fragment was generated by reverse transcription-polymerase chain reaction (RT-PCR) with primers of 5'-ctcatgacagcacttcgaga- 3' and 5'-cagacgtgatgtgctc cagc-3' using cDNA as a template. This *hPer1* cDNA fragment was first cloned into pGEM-T easy vector (Promega) to generate *hPer1*-T and the sequence was confirmed by sequencing. Antisense strand riboprobe was generated by *in vitro* transcription using T7 polymerase (Promega) in the presence of ^{32}P-UTP using Sal-linearized *hPer1*-T as a template.

A 248 bp cyclophillin (position: 160～407) fragment was generated by RT-PCR with primers of 5'-agcactggggagaaaggatt-3' and 5'-agccactcagtcttggcagt-3' using cDNA as a template. This cyclophillin cDNA fragment was first cloned into pGEM-7G vector (Promega) to generate hcyclophillin-7G using SmaI and the sequence was confirmed by sequencing. Antisense strand riboprobe was generated by *in vitro* transcription using T7 polymerase in the presence of ^{32}P-UTP using SpeI linearized cyclophillin-4G as template.

All reagent used in the ribonuclease protection assay (RPA) are from the Ambion RPA II (Ambion), unless otherwise specified. Total RNA (10μg) was hybridized with *hPer1* (100000 cpm) and cyclophillin (10000 cpm) riboprobes at 42°C overnight. The hybridized mixture was then treated with RNase A/T1 mix (1 : 200) at 37°C for 1 h. The ribonuclease digestion was terminated by addition of 300μl of RNase inactivation and precipitation solution and protected fragment was precipitated at 20°C for 30 min. The pellets were dissolved in loading buffer and subjected to electrophoresis in the denaturing gel (8 M urea, 5% polyacrylamide) at 250 V for 2 h. The gel was dried and exposed to x-ray film for 24～48 h using intensifying screens.

Transfection

p*hPer1*-Luc vector containing 4 kb *hPer1* promoter (-3829 to +123) was generated by RT-PCR using primers (5'-aagctagcgggatcatgcaaatacgtga-3' and 5'-aa ctcgaggtagcccatcagattggaag-3'). For the transfection experiments, cells were plated in 60 mm dishes and grown to 30～40% confluence for 2～3 d. Cells were washed twice with 1X Dulbeco's phosphate-buffered saline (D- PBS) and medium was changed to DMEM with 10% fetal bovine serum. After two days, cells were washed twice with 1X D-PBS, received 5 ml of fresh medium, and incubated at 37°C for 2 h before transfection. Reporter plasmids for transfection experiments were purified by Qiagen columns (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions and dissolved in 0.1X TE buffer (1 mM Tris (pH 8.0), 0.1 mM EDTA) at a concentration of 0.5～1 mg/ml. DNA-calcium phosphate mixture was prepared by mixing one part of 2X BES-buffered saline (50 mM BES (pH 6.96), 280 mM NaCl, and 1.5 mM Na_2HPO_4) with one part of DNA-calcium chloride solution (60μg/ml DNA, 250 mM $CaCl_2$) and incubated at room temperature for 15 min before transfection. Each dish received 0.5 ml of resulting DNA-calcium phosphate mixture containing 5μg β-galactosidase reporter vector (pCMV-βgal) drop wise and was incubated at 37°C under 5% CO_2 tension for 24 h. Excess DNA-calcium phosphate precipitates were washed out by rinsing twice with calcium and magnesium-free D-PBS. Then cells were cultivated in an appropriate drug-added medium and further incubated for 24～48 h before making cell extracts. Cell extracts were prepared by incubating cells in 0.4 ml of 1X reporter lysis buffer (Promega) for 15 min at room temperature. After centrifugation, supernatants were kept at

-70°C till assay. Protein contents were measured by commercial protein assay kit (Bio-Rad laboratories, Hercules, CA, USA). Beta-galactosidase assay and luciferase assay were performed using β-galactosidase assay kit and luciferase assay kit (Promega), respectively. p*hPer1*-Luc exhibited high luciferase activity (3×10^4 to 1×10^5 light unit per 30 seconds depending on experimental conditions). Untransfected SK-N-SH cell extracts showed no background luciferase activity and endogenous β-galactosidase activity was negligible under the assay condition used. Cytomegaloviral promoter-driven β-galactosidase activity was used to compensate transfection efficiency.

Statistical analysis

Data for *hPer1* mRNA induction by ionophore and promoter activity were statistically evaluated using Student's *t*-test. Statistical significance was set at $p < 0.05$.

RESULTS

Glutamate and NMDA failed to regulate hPer1 mRNA levels in SK-N-SH cells

SK-N-SH cells are derived from human neuroblastoma, and their neuronal origin has been extensively characterized (Biedler et al., 1973). To investigate whether gluatmatergic neurotransmitters influence *Per1* gene expression, 0.5μM A23187, 1 mM glutamate and 100μM NMDA were treated for 2 h (Fig. 1). A23187 significantly increased *hPer1* mRNA levels in the SK-N-SH cells. At this dose and time window, glutamate and NMDA did not increase *Per1* mRNA levels. Similarly, excess amount of NMDA (1 mM and 10 mM) also had no alteration in *hPer1* mRNA levels (data not shown).

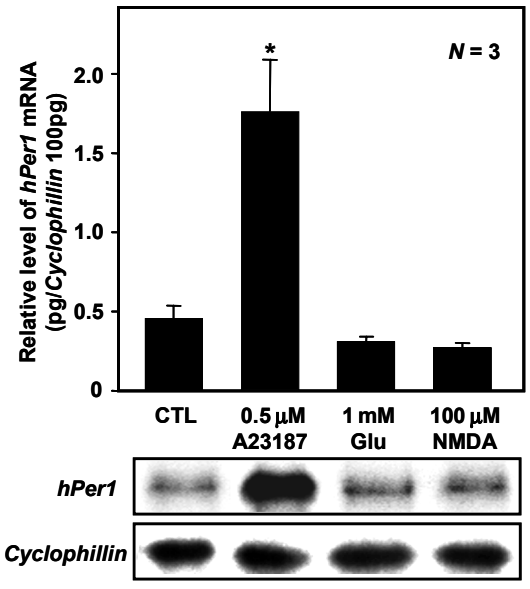

Fig. 1. Effects of calcium ionophore A23187, glutamate and NMDA on Per1 mRNA levels. SK-N-SH cells cultured for 24 h in the growth medium were incubated for 24 h in the serum-free medium. After incubation, SK-N-SH cells were treated with 0.5μM A23187, 1 mM glutamate and 100μM NMDA, respectively. Two hours after the treatment, total RNA was isolated and mRNA levels of Per1 were determined by RPA (*$p < 0.05$).

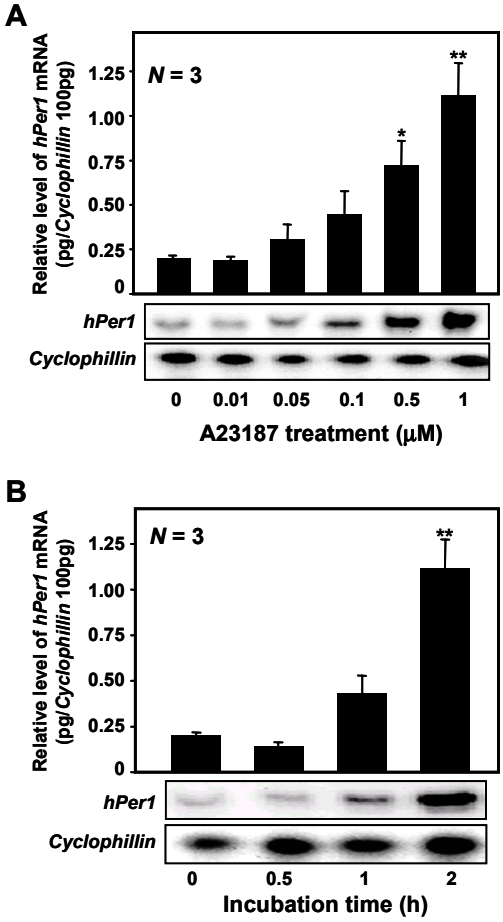

Fig. 2. Dose- and time-dependent increase in Per1 mRNA levels by A23187. SK-N-SH cells were treated with increasing amounts of A23187. Two hours after the treatment, total RNA was isolated and subjected to RPA (A). SK-N-SH cells were treated with 0.5μM A23187 and incubated for indicated time (B) (*$p < 0.5$, **$p < 0.01$).

A23187 increased Per1 mRNA levels in a time- and dose-dependent fashion

To examine effective time and dose-response, SK-N-SH cells were exposed to A23187. As shown in Fig 2A and B, the increases in *hPer1* mRNA levels by A23187 were time and dose dependent. In particular, increases in *hPer1* mRNA levels were peak after 2 h incubation with 0.5μM A23187. Also, these effects were inhibited by treatment with EGTA, a calcium chelator, indicating that calcium influx by A23187 appears to increase *Per1* mRNA levels (data not shown).

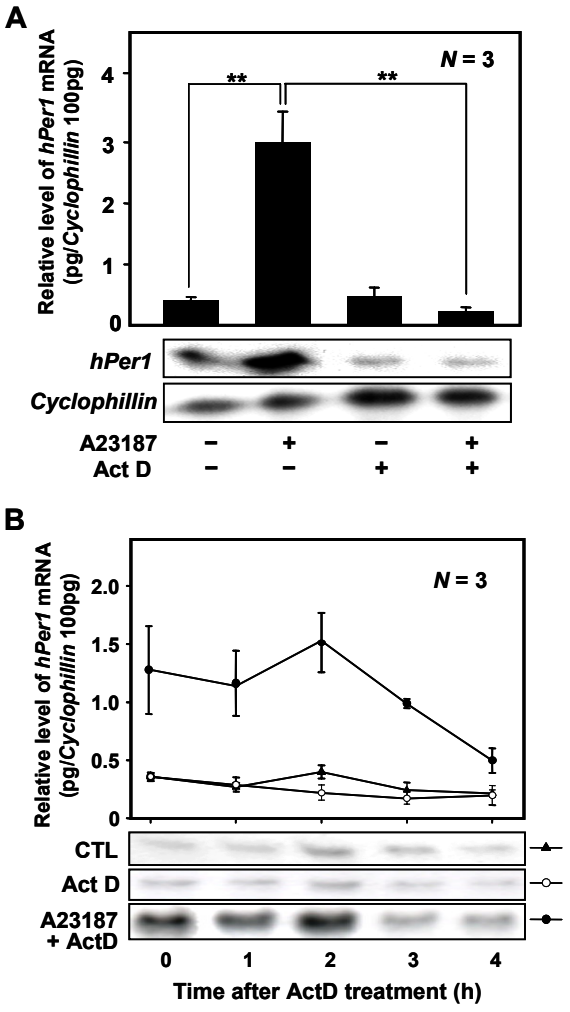

Fig. 3. Effect of actinomycin D on A23187-mediated Per1 induction. (A) SK-N-SH cells were incubated for 3 h in the presence (+) or absence (-) of 5μg/ml actinomycin D. After incubation, they were treated with 0.5μM A23187 and further incubated for 2 h. (B) the cells were pretreated with actinomycin D (5μg/ml) for indicated periods followed by co-treatment with 0.5μM A23187 for 2 h. Total RNA was isolated and subjected to RPA (**$p < 0.01$).

Actinomycin D, but not cycloheximide blocked A23187-mediated increase of hPer1 mRNA

To elucidate classical transcriptional aspect, SK-N-SH cells were preincubated with 5μg/ml of actinomycin D (Act D), a polymerase II inhibitor, 3 h before A23187 treatment and then 0.5μM A23187 was treated. After 2 h incubation, *Per1* mRNA levels were examined. Fiveμg/ml of Act D completely blocked A23187-mediated induction of *Per1* mRNA (Fig. 3A). Time course experiment showed that *Per1* mRNA levels remained until 2 h after Act D treatment, and then declined almost at the basal levels (Fig. 3B). These results indicate that A23187 regulates *Per1* mRNA level at transcription level.

CHX had no effect on A23187-mediated increase of hPer1 mRNA

To delineate whether protein synthesis is involved in A23187-mediated *Per1* induction, 10μM cyclohexamide, a protein synthesis inhibitor, was treated to SK-N-SH cells. While CHX did not alter A23187-induced increase in *hPer1* mRNA levels (Fig. 4), pretreatment of CHX alone for 2 h increased *hPer1* mRNA levels by 3 times. These results suggest

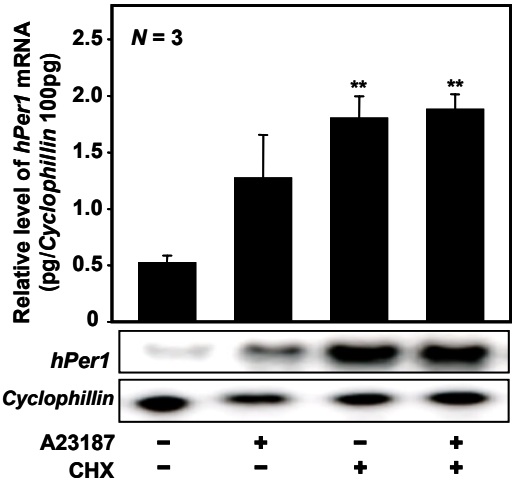

Fig. 4. Effect of cycloheximide on the A23187- mediated Per1 induction. The SK-N-SH cells were incubated for 3 h in the presence (+) or absence (-) of 10μM cyclohexamide (CHX). After incubation, they were treated with 0.5 μM A23187 and further incubated for 2 h. Total RNA was isolated and subjected to RPA (**$p < 0.01$).

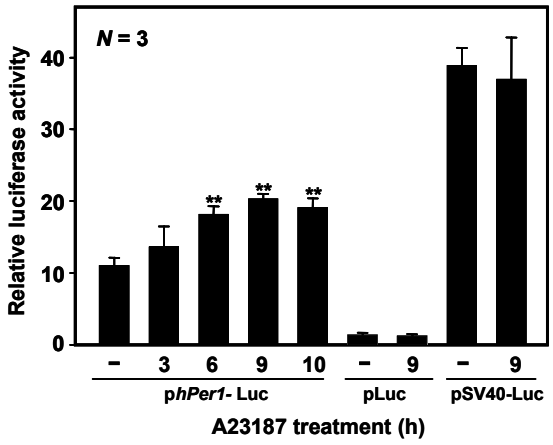

Fig. 5. Time course change of A23187-mediated activation of hPer1 promoter. SK-N-SH cells were transfected with phPer1-4.0 Luc or pGL3-basic by the calcium phosphate co-precipitation method. Twenty-four hours after the transfection, cells were incubated for 9 h in the presence (+) or absence (-) of 0.5μM A23187. Cells were then harvested and luciferase activity was determined (*$p < 0.05$).

that de novo protein synthesis is not required for *Per1* induction by A23187, rather is needed for rapid *Per1* degradation.

A23187 increased the human Per1 promoter activity

To test whether the changes in *hPer1* mRNA levels by A23197 are coupled with enhanced gene transcription, *hPer1* promoter activity was determined by transient transfection assay. SK-N-SH cells were transfected with p*hPer1*-4.0Luc containing 4kb (-3829 to +123) *hPer1* promoter-driven luciferase reporter. Treatment of SK-N-SH cells with 0.5μM A23187 increased the promoter activity by about 1.8 folds (Fig. 5). As expected, treatment of A23187 did not alter the basic reporter vector and SV40 promoter-driven luciferase activity as controls.

DISCUSSION

It is well known that light pulse or NMDA can elicit rapid induction of *Per1* mRNA in the SCN. For example, a single brief exposure to light induces m*Per1* RNA within 1 h and shifts phase of the oscillation of its own expression (Shigeyoshi et al 1997). In this regard, anti-sense *Per1* nucleotide blocks phase shifts induced by light, glutamate, and NMDA (Akiyama et al., 1999).

In the present study, we shows that Ca^{2+} influx induced by A23187, a calcium ionophore, increased *hPer1* mRNA levels significantly within 2 h in SK-N-SH human neuroblastoma cells. Such increase in *hPer1* mRNA levels was clearly time- and dose-dependent and attenuated by chelation of extracellular Ca^{2+} using EGTA. This observation is consistent with recent studies. For instance, light-stimulated m*Per1* induction in mice is a rapid, transient event that is initiated by 15～20 min in the retinorecipient SCN peaks at 60 min, and spread throughout the SCN (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997; Takumi et al., 1998; Zylka et al., 1998).

In the present study, we found that treatment with actinomycin D completely blocked the increase of *Per1* mRNA level induced by A23187. This result suggests that Ca^{2+} regulate the expression of *Per1* gene at the transcriptional level. Gene induction has been believed to be the basis on resetting the biological clock in the SCN. Similarly, light pulses that reset the clock also induce the expression of a number of immediate-early genes, including *c-fos* and *junB* in the retinorecipient zones of the SCN (Kornhauser et al., 1996; Best et al., 1999). Also, it has been reported that light-induced gene expression in the SCN is in the control of a well established signaling pathway, in which RHT synaptic glutamate acts through postsynaptic NMDA receptors to increase intracellular levels of Ca^{2+}, which in turn trigger a kinase cascade to activate transcription factors CREB and ERK (Kornhauser et al., 1996; Obrietan et al., 1998). Recently, it is demonstrated that there is a link between light signaling and CRE activation, and these effects are mediated by glutamate, nitric oxide synthase, MAPK, and CREB phosphorylation in the mouse (Obrietan et al., 1999). In addition to m*Per1* induction, light/glutamate stimuli promote phosphorylation of CREB and CRE-mediated transcription, either directly or indirectly (Ginty et al., 1993; Ding et al., 1997).

We observed that increased levels of *hPer1* mRNA induced by A23187 were quickly declined to control levels 4 h after treatment with actinomycin D. It is noticeable that degradation of *Per1* mRNA was faster in the A23187-treated group than in A23187- untreated group. This result suggests that stability of *hPer1* mRNA induced by A23187 is

shorter than 4 h and there may be an active degradation of *Per1* mRNA triggered by calcium ion. On the other hands, pretreatment with cycloheximide, a protein synthesis blocker did not block induction of *Per1* mRNA by A23187. This result suggests that protein synthesis is not required for A23187-mediated *Per1* induction. Treatment with cycloheximide alone also led to a 3.5 fold increase of *hPer1* mRNA, which may be resulted from prolonged half-lives (Balsalobre et al., 1998, 2000). Based on such results, we speculate that de novo protein synthesis is not required for induction of *Per1* mRNA mediated by A23187 but for rapid degradation of *Per1* mRNA.

In the present study, using whole proximal promoter (-3829 to +123, 4 kb) of *hPer1* gene containing 3 E-boxes (Hida et al., 2000; Yamaguchi et al., 2000), we also found that *hPer1* promoter-driven luciferase activity was significantly increased 6 h after treatment with A23187 in SK-N-SH cells. Thus, it is conceivable that there resides the calcium responsive element in the 4.0 kb 5' flanking region of *hPer1* gene. These results provide evidence that calcium can play an important role in transcriptional activation of *hPer1* gene.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Research Laboratory (NRL) Program (2000-N-NL-01-C-149), Frontier Program in Neuroscience through the Brain Research Center and the Basic Research Program of the Korea Science and Engineering Foundation, and the Ministry of Science and Technology. S. Lee, J. Park, G.H. Son and K.H. Lee were supported by the Brain Korea 21 Research Fellowship from the Korea Ministry of Education and Human Resources Development.

REFERENCES

Akiyama M, Kouzu Y, Takahashi S, Wakamatsu H, Moriya T, Maetani M, Watanabe S, Tei H, Sakaki Y, and Shibata S (1999) Inhibition of light- or glutamate-induced m*Per1* expression represses the phase shifts into the mouse circadian locomotor and suprachiasmatic firing rhythms. *J Neurosci* 19: 1115-1121.

Albrecht U, Sun ZS, Eichele G, and Lee CC (1997) A differential response of two putative mammalian circadian regulators, m*Per1* and mper2, to light. *Cell* 91:1055-1064.

Allada R, White NE, So WV, Hall JC, and Rosbash M (1998) A mutant Drosophila homolog of mammalian Clock disrupts circadian rhythms and transcription of period and timeless. *Cell* 93:791-804.

Amir S, Robinson B, and Edelstein K (1995) Distribution of NADPH-diaphorase staining and light-induced Fos expression in the rat suprachiasmatic nucleus region supports a role for nitric oxide in the circadian system. *Neuroscience* 69: 545-555.

Balsalobre A, Damiola F, and Schibler U (1998) A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell* 93:929-937

Balsalobre A, Marcacci L, and Schibler U (2000) Multiple signaling pathways elicit circadian gene expression in cultured Rat-1 fibroblasts. *Curr Biol* 10:1291-1294.

Best JD, Maywood ES, Smith KL, and Hastings MH (1999) Rapid resetting of the mammalian circadian clock. *J Neurosci* 19:828-835.

Biedler JL, Helson L, and Spengler BA (1973) Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. *Cancer Res* 33:2643-2652.

Castel M, Belenky M, Cohen S, Ottersen OP, and Storm-Mathisen J (1993) Glutamate-like immunoreactivity in retinal terminals of the mouse suprachiasmatic nucleus. *Eur J Neurosci* 5:368-381.

Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156-159.

Colwell CS and Menaker M (1992) NMDA as well as non-NMDA receptor antagonists can prevent the phase-shifting effects of light on the circadian system of the golden hamster. *J Biol Rhythms* 7:125-136.

Darlington TK, Wager-Smith K, Ceriani MF, Staknis D, Gekakis N, Steeves TDL, Weitz CJ, Takahashi JS, and Kay SA (1998) Closing the circadian loop: CLOCK-induced transcription of its own inhibitors per and tim. *Science* 280:1599-1603.

Decoursey PJ (1960) Daily light sensitivity rhythm in a rodent *Science* 131:33-35.

Ding JM, Chen D, Weber ET, Faiman LE, Rea MA, and Gillette MU (1994) Resetting the biological clock: mediation of nocturnal circadian shifts by glutamate and NO. *Science* 266: 1713-1717.

Ding JM, Faiman LE, Hurst WJ, Kuriashkina LR, and Gillette MU (1997) Resetting the biological clock: mediation of nocturnal CREB phosphorylation via light, glutamate, and nitric oxide. *J Neurosci* 17:667-675.

Ding JM and Buchanan GF (1998) A neuronal ryanodine receptor mediates light-induced phase delays of the circadian clock. *Nature* 394:381-384.

Gannon RL and Rea MA (1993) Glutamate receptor immunoreactivity in the rat suprachiasmatic nucleus. *Brain Res* 622: 337-342.

Gillette MU (1997) Cellular and biochemical mechanisms underlying circadian rhythms in vertebrates. *Curr Opin Neurobiol* 7: 797-804.

Ginty DD, Kornhauser JM, Thompson MA, Bading H, Mayo KE, Takahashi JS, and Greenberg ME (1993) Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. *Science* 260:238-241.

Hannibal J, Moller M, Ottersen OP, and Fahrenkrug J (2000)

PACAP and glutamate are co-stored in the retinohypothalamic tract. *J Comp Neurol* 418:147-155.

Hao H, Allen DL, and Hardin PE (1997) A circadian enhancer mediates PER-dependent mRNA cycling in Drosophila melanogaster. *Mol Cell Biol* 17:3687-3693.

Hida A, Koike N, Hirose M, Hattori M, Sakaki Y, and Tei H (2000) The human and mouse Period1 genes: five well-conserved E-boxes additively contribute to the enhancement of m*Per1* transcription. *Genomics* 65:224-233.

Ikeda M and Nomura M (1997) cDNA cloning and tissue-specific expression of a novel basic helix-loop-helix/PAS protein (BMAL1) and identification of alternatively spliced variants with alternative translation initiation site usage. *Biochem Biophys Res Commun* 233:258-264.

Johnson RF, Moore RY, and Morin LP (1988) Loss of entrainment and anatomical plasticity after lesions of the hamster retinohypothalamic tract. *Brain Res* 460:297-313.

Kornhauser JM, Mayo KE, and Takahashi JS (1996) Light, immediate-early genes, and circadian rhythms. *Behav Genet* 26:221-240.

Lee C, Bae K, and Edery I (1999) PER and TIM inhibit the DNA binding activity of a Drosophila CLOCK-CYC/dBMAL1 heterodimer without disrupting formation of the hetrodimer: a basis for circadian transcription. *Mol Cell Biol* 19:5316-5325.

Mintz EM, Marvel CL, Gillespie CF, Price KM, and Albers HE (1999) Activation of NMDA receptors in the suprachiasmatic nucleus produces light-like phase shifts of the circadian clock in vivo. *J Neurosci* 19:5124-5130

Obrietan K, Impey S, Smith D, Athos J, and Storm DR (1999) Circadian regulation of cAMP response element-mediated gene expression in the suprachiasmatic nuclei. *J Biol Chem* 274:17748-17756.

Obrietan K, Impey S, and Storm DR (1998) Light and circadian rhythmicity regulate MAP kinase activation in the suprachiasmatic nuclei. *Nat Neurosci* 1:693-700.

Reppert SM (1998) A clockwork explosion! *Neuron* 21:1-4.

Rutila JE, Suri V, Le M, So WV, Rosbash M, and Hall JC (1998) CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of Drosophila period and timeless. *Cell* 93:805-814.

Shearman LP, Zylka MJ, Weaver DR, Kolakowski LF Jr, and Reppert SM (1997) Two period homologs: circadian expression and photic regulation in the suprachiasmatic nuclei. *Neuron* 19:1261-1269.

Shigeyoshi Y, Taguchi K, Yamamoto S, Takekida S, Yan L, Tei H, Moriya T, Shibata S, Loros JJ, Dunlap JC, and Okamura H (1997) Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the m*Per1* transcript. *Cell* 91:1043-1053.

Summers TL, Ferraro JS, and McCormack CE (1984) Phase-response and Aschoff illuminace curves for locomotor activity rhythm of the rat. *Am J Physiol* 246:299-304.

Takahashi JS and Zatz M (1982) Regulation of circadian rhythmicity. *Science* 217:1104-1011.

Takumi T, Taguchi K, Miyake S, Sakakida Y, Takashima N, Matsubara C, Maebayashi Y, Okumura K, Takekida S, Yamamoto S, Yagita K, Yan L, Young MW, and Okamura H (1998) A light-independent oscillatory gene mPer3 in mouse SCN and OVLT. *EMBO J* 17:4753-4759.

Tei H, Okamura H, Shigeyoshi Y, Fukuhara C, Ozawa R, Hirose M, and Sakaki Y (1997) Circadian oscillation of a mammalian homologue of the Drosophila period gene. *Nature* 389:512-516

Van den Pol An (1991) Glutamate and aspartate immunoreactivity in hypothalamic presynaptic axons. *J Neurosci* 11: 2081-2101.

Weaver DR (1998) The suprachiasmatic nucleus: a 25-year retrospective. *J Biol Rhythms* 13:100-112.

Weber ET, Gannon RL, and Rea MA (1995) cGMP-dependent protein kinase inhibitor blocks light-induced phase advances of circadian rhythms in vivo. *Neurosci Lett* 197:227-230.

Yamaguchi S, Mitsui S, Miyake S, Yan L, Onishi H, Yagita K, Suzuki M, Shibata S, Kobayashi M, and Okamura H (2000) The 5' upstream region of m*Per1* gene contains two promoters and is responsible for circadian oscillation. *Curr Biol* 10:873-876.

Zylka MJ, Shearman LP, Weaver DR, and Reppert SM (1998) Three period homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. *Neuron* 20:1103-1110.