# Intracellular calcium mobilization induces *period* genes via MAP kinase pathways in NIH3T3 cells

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**Abstract Mammalian *period* genes have a pivotal role in generating circadian rhythms and are rapidly induced by several stimuli in mammalian cells. In the present study, we revealed that treatment with thapsigargin significantly induced transcripts of mouse *period 1* and *2* (*mPer1* and *mPer2*) but not *mPer3* among circadian related genes in NIH3T3 cells. Thapsigargin-induced *mPer1* and *mPer2* mRNA expressions took distinct signaling pathways from protein kinase C and cAMP, but were partially inhibited by inhibitors of MEK1 and p38 mitogen-activated protein kinase, respectively. Thus, the present study suggested that intracellular calcium is one of multiple signaling stimuli triggering *mPer* gene expression in NIH3T3 cells.** 

*Key words:* Thapsigargin; $Ca^{2+}$; *mPer1*; *mPer2*; Mitogen-activated protein kinase; NIH3T3 cell

## 1. Introduction

Almost all organisms have endogenous biological rhythms called circadian rhythms, that have an intrinsic period of approximately 24 h [1]. In mammals, the hypothalamic suprachiasmatic nucleus (SCN) is the center of circadian rhythms such as sleep–wakefulness, glucose utilization and hormonal rhythms [2]. The cycle time of the intrinsic circadian rhythm is not precisely 24 h, and animals adjust their internal clocks to local environmental day and night by periodic resetting. Light is known as a strong regulator that causes a phase shift of the clock, and this information is conveyed to the SCN neurons directly via the retinohypothalamic tract (RHT) [3–5]. SCN neurons show an intracellular $Ca^{2+}$ increase in response to glutamate, a neurotransmitter in the RHT [6–8]. This influx of $Ca^{2+}$ through ionotropic glutamate receptors is required for clock resetting [9].

Three homologs of a *Drosophila period* gene (*Per*) and several genes concerned with rhythm generation have been identified in mouse [10–13]. The molecular mechanisms of the generating rhythms endogenously consist of positive and negative transcriptional feedback loops involving *mPer*s, two cryptochrome (*Cry*) and *Clock* genes [14,15]. Among them mouse *Per1* (*mPer1*) and *Per2* (*mPer2*) were shown to be induced in the SCN by light stimulation during night, and were also considered essential for resetting the circadian clock and shifting the firing rhythm in the SCN [16–19].

Recently, several studies have reported that circadian genes are expressed in both SCN and peripheral tissue [20,21]. Using peripheral cells and cell lines, several stimuli such as high concentrations of serum, forskolin, or phorbol 12-myristate 13-acetate (PMA) were reported to induce *period* genes [21–23]. However, little is known about the precise effects of the intracellular calcium on the expression of these genes.

Here we show that the treatment of NIH3T3 cells with thapsigargin, which elevates the intracellular calcium levels by inhibiting endoplasmic reticulum $Ca^{2+}$-ATPase, induced *mPer1* and *mPer2* genes among several circadian genes. Moreover, we suggest that this *mPer1* mRNA induction by thapsigargin used a signaling pathway distinct from that by PMA or forskolin induction.

## 2. Materials and methods

### 2.1. Materials

A specific inhibitor of p38 mitogen-activated protein (MAP) kinase (SB202190), a MAP kinase kinase (MEK1) inhibitor (U0126) and 1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra-(acetoxymethyl)-ester (BAPTA-AM) were purchased from Calbiochem (La Jolla, CA, USA). Forskolin and thapsigargin were obtained from Seikagaku Co. Ltd. (Tokyo, Japan).

### 2.2. Cell culture and treatment

NIH3T3 cells were maintained with Dulbecco's modified Eagle medium (DMEM) (Gibco BRL) containing 10% fetal calf serum, 100 mg/ml penicillin and 100 U/ml streptomycin. Before all experiments, cells ($3\times10^5$ cells/ml) were re-suspended in serum-free DMEM medium, seeded to the plates and then used for the experiments. To induce circadian genes, cells were treated with thapsigargin (0.5 μM), forskolin (20 μM), or PMA (1 or 0.5 μM), respectively. The concentrations of other drugs used were BAPTA-AM at 10 μM, U0126 at 40 or 10 μM and SB202190 at 10 or 2.5 μM.

### 2.3. Reverse transcriptional-polymerase chain reaction (RT-PCR)

To estimate the mRNA level of each circadian related gene by RT-PCR, total RNA in the cells was extracted from lysed cells with Trizol (Gibco BRL), and converted to cDNA by reverse transcriptase (RT) using random hexamers to prime superscript II RNase-RT (Gibco BRL) according to the manufacturer's instructions. The reaction buffer contained 2.5 mM $MgCl_2$, 0.5 mM dNTP mixture and 1 mM dithiothreitol. Samples were incubated at 42°C for 50 min and the reaction was performed by heating at 70°C for 15 min. Specific DNAs were mixed and amplified with PCR reaction mixture (Taq PCR core

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*Abbreviations:* BAPTA-AM, 1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra-(acetoxymethyl)-ester; CREB, cAMP responsive element binding protein; Cry, cryptochrome; Erk, extracellular signal-regulated kinase; MAP kinase, mitogen-activated protein kinase; Per, period; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SCN, suprachiasmatic nucleus

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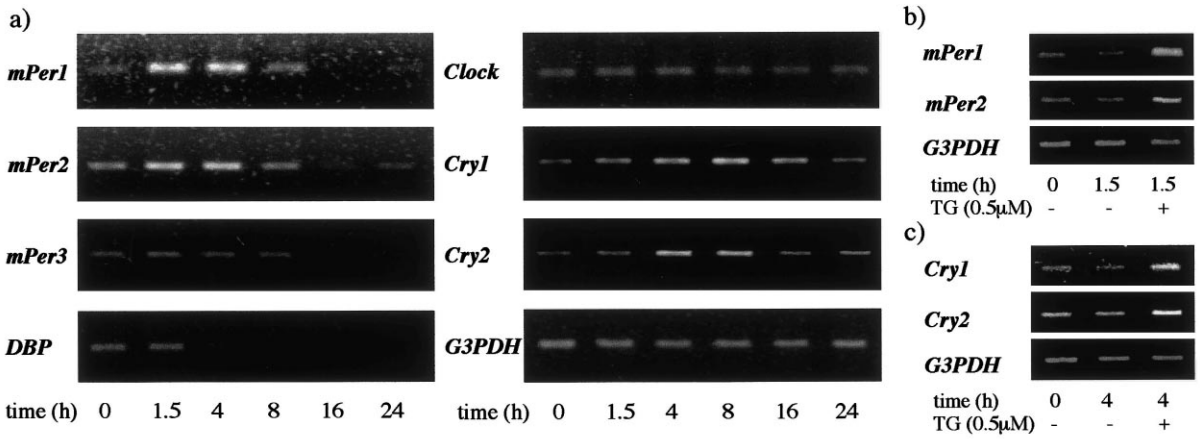


Fig. 1. Thapsigargin affects circadian related gene expression in NIH3T3 cells. NIH3T3 cells were exposed to thapsigargin at 0.5 μM for the indicated time (a). Cells were treated with vehicle (Con) or thapsigargin (TG, 0.5 μM) to detect *mPer1* and *mPer2* mRNA for 0 or 1.5 h (b), or *Cry1* and *Cry2* mRNA for 0 or 4 h (c). Total RNA was isolated from cells and each mRNA was analyzed by RT-PCR as described in Section 2.

kit, Qiagen), which contained 100 μM dNTP mixture, 1.5 mM $MgCl_2$, 1.25 U Taq polymerase together with 0.2 μM of each primer and cDNA. Each RT-PCR primer used in this study was as follows: *mPer1* sense primer, 5′-TGCTCTAGAAAGCTTATGAGTGGTCC-CCTAGAAGG-3′; *mPer1* antisense primer, 5′-GCTCTAGATGG-GCTCTGTGAGTTTGTAC-3′; *mPer2* sense primer, 5′-AATGGAT-ACGTGGACTTCTC-3′; *mPer2* antisense primer, 5′-TGGCTCTCA-CTGGACATTAG-3′; *mPer3* sense primer, 5′-TGCTGAGGAGAA-AAGTGCCC-3′; *mPer3* antisense primer, 5′-AGACATTCTGTTTC-GGTCTTCG-3′; *DBP* sense primer, 5′-CTCATCTCTTCGACTCT-CCA-3′; *DBP* antisense primer, 5′-TCCAGGTCCACGTATTCCAC-3′; *Clock* sense primer, 5′-CTTCCTGGTAACGCGAGAAA-3′; *Clock* antisense primer, 5′-TTTCCAGCAGATGAGTAGAG-3′; *Cry1* sense primer, 5′-GAGAGAATGTCCCGAGTTGT-3′; *Cry1* antisense primer, 5′-TTACTGCTCTGCCGCTGGAC-3′; *Cry2* sense primer, 5′-GAGGTGGTGACTGAGAACT-3′; *Cry2* antisense primer, 5′-CAAGTCCCACAGGCGGTAGT-3′; *G3PDH* sense primer, 5′-TCCACCACCCTGTTGCTGTA-3′; and *G3PDH* antisense primer, 5′-ACCACAGTCCATGCCATCAC-3′. The typical reaction conditions were 0.5 min at 95°C, 0.5 min at 60°C, and 1 min at 72°C. The results represent 18–30 cycles of amplification, after which cDNAs were separated by electrophoresis on 2.0% agarose gels and visualized using ethidium bromide. Experiments were repeated at least twice and reproducibility was confirmed.

### 2.4. Immunoblot analysis

Phosphorylated extracellular signal-regulated kinase (Erk), p38 MAP kinase and cAMP responsive element binding protein (CREB) were analyzed by immunoblotting. Cells treated with thapsigargin or forskolin were lysed with 20 mM Tris buffer, pH 8.0, containing 137 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium vanadate and 1% (v/v) Triton X-100. The protein concentration was determined by the method of Bradford [24]. Cell lysate containing 40 μg protein was separated on 8.0% SDS–polyacrylamide electrophoresis gels, immunoblotted onto polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) and identified by enhanced chemiluminescence using antibodies against phosphorylated p38, Erk and CREB. After immunoblotting, electrophoresis gels were stained with Coomassie blue stain to confirm the protein level of each lane.

## 3. Results

### 3.1. Thapsigargin induced mPer1 and mPer2 mRNA in NIH3T3 cells

We investigated by RT-PCR whether intracellular calcium level affects circadian gene expression. Stimulation of the NIH3T3 cells with thapsigargin strongly induced *mPer1* and *mPer2* mRNA expression among circadian genes (Fig. 1a,b). The level of thapsigargin-induced *mPer1* and *mPer2* mRNA reached a peak within 1–4 h and declined to basal levels before 8 h, but *mPer3* was less responsive. In other circadian related genes, the levels of *DBP* mRNA were constitutively low and decreased further in response to thapsigargin. *Clock* mRNA, a positive transcriptional regulator of *mPer1*, was hardly affected by thapsigargin. On the other hand, *Cry1* and *Cry2* mRNA, known as negative regulators of *mPer1*, were induced 4–8 h after thapsigargin treatment (Fig. 1a,c). Expressions of these genes were weak and did not fluctuate without thapsigargin treatment.

Compared with other stimuli, which have been reported to affect circadian genes such as PMA and forskolin, thapsigargin induced *mPer1* mRNA in NIH3T3 cells to the same extent (data not shown). To test whether intracellular calcium affects *mPer1* expression by PMA or forskolin, we measured the expression of *mPer1* in NIH3T3 cells in the presence of BAPTA-AM, a cell permeable intracellular calcium chelator. Although forskolin or PMA alone induced *mPer1* mRNA and co-stimulation markedly augmented its expression, treatment with BAPTA-AM reduced *mPer1* mRNA expression to basal levels (Fig. 2).

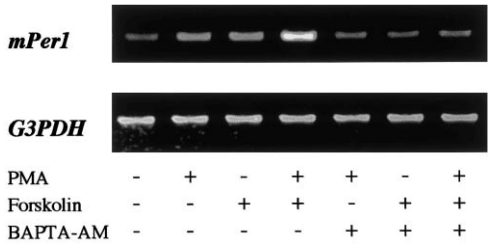


Fig. 2. BAPTA-AM suppresses PMA- and forskolin-induced *mPer1* mRNA expression in NIH3T3 cells. After 1 h pretreatment with BAPTA-AM (10 μM) or vehicle, cells were treated with PMA (0.5 μM) and/or forskolin (20 μM) for 60 min. Total RNA was isolated from cells and each mRNA was analyzed by RT-PCR as described in Section 2.

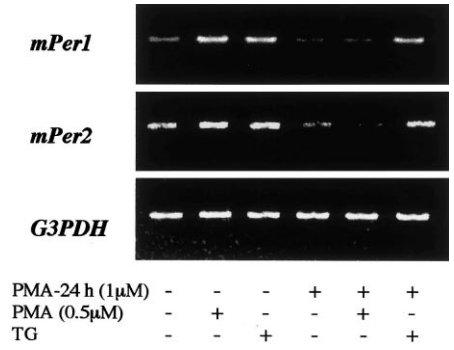


Fig. 3. Effect of PKC down-regulation on thapsigargin-induced *mPer1* and *mPer2* mRNA expression in NIH3T3 cells. After 24 h pretreatment with PMA (PMA-24 h, 1 μM) or vehicle, cells were treated with PMA (0.5 μM) or thapsigargin (TG, 0.5 μM) for 60 min. Total RNA was isolated from cells and each mRNA was analyzed by RT-PCR as described in Section 2.

### 3.2. Protein kinase C (PKC) and cAMP signaling did not contribute to thapsigargin-induced mPer1 and mPer2 mRNA in NIH3T3 cells

Next, we investigated whether PKC activation and/or increase in cAMP levels are downstream signaling of intracellular calcium mobilization by thapsigargin. Since prolonged exposure with PMA down-regulates PKC activities in several cells, we measured thapsigargin-induced *mPer1* and *mPer2* mRNA in NIH3T3 cells 24 h after treatment with PMA at 1 μM. The basal level of *mPer1* and *mPer2* mRNA was slightly reduced compared with unexposed cells. Thapsigargin significantly induced *mPer1* and *mPer2* mRNA within 1 h although retreatment with PMA at 0.5 μM did not induce *mPer1* and *mPer2* mRNA (Fig. 3). We measured the phosphorylation status of CREB, one of the downstreams of the cAMP pathway, to elucidate the role of cAMP on thapsigargin-induced *mPer1* mRNA. Treatment with forskolin immediately induced phosphorylated CREB and maintained its phosphorylation for more than 40 min. However, thapsigargin hardly augmented CREB phosphorylation in the present study conditions (Fig. 4).

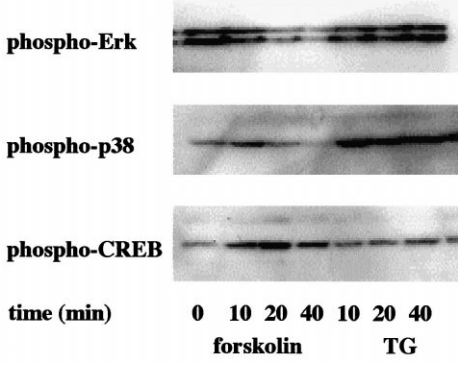


Fig. 4. Thapsigargin and forskolin affect the phosphorylation of Erk, p38 MAP kinase and CREB in NIH3T3 cells. The cells were incubated with forskolin (20 μM) or thapsigargin (TG) (0.5 μM) for the indicated time. Cell lysate was analyzed by immunoblotting with antibodies against phosphorylated Erk, p38 kinase and CREB as described in Section 2.

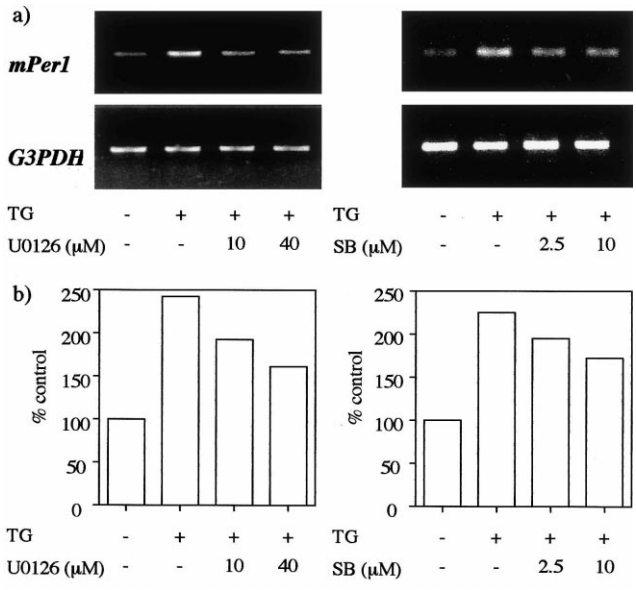


Fig. 5. Effect of U0126 and SB202190 on *mPer1* mRNA expression in NIH3T3 cells. After 1 h pretreatment with U0126 (40 or 10 μM), SB202190 (SB, 10 or 2.5 μM) or vehicle, cells were treated with thapsigargin (TG) at 0.5 μM for 60 min. Total RNA was isolated from cells and each mRNA was analyzed by RT-PCR as described in Section 2 (a). The relative mRNA level of *mPer1* was calculated by comparison of G3PDH-normalized values with the levels of untreated controls (b).

### 3.3. MAP kinase inhibitors down-regulated thapsigargin-induced mPer1 mRNA in NIH3T3 cells

We also studied the effects of MAP kinase on thapsigargin-induced *mPer1* and *mPer2* mRNA expression using U0126 and SB202190 (Fig. 5). As shown in Fig. 4, thapsigargin immediately induced p38 MAP kinase phosphorylation and maintained its status until 40 min. Forskolin induced p38 MAP kinase phosphorylation only transiently although CREB was continuously phosphorylated. The phosphorylation status of Erk was basically high in our study conditions compared with that of p38 MAP kinase, and its phosphorylation was maintained by the treatment with thapsigargin. On the other hand, forskolin continuously declined Erk phosphorylation in NIH3T3 cells. U0126 at 10 μM and SB202190 at 2.5 μM partially reduced thapsigargin-induced *mPer1* mRNA expression. Similar effects of both reagents on thapsigargin-induced *mPer2* mRNA expression were also detected (data not shown).

## 4. Discussion

Calcium-dependent gene expression regulates fundamental biological responses including cell survival, differentiation and proliferation. Thapsigargin, a non-phorbol ester-type tumor promoter, discharges intracellular $Ca^{2+}$ stores by specific inhibition of the endoplasmic reticulum $Ca^{2+}$-ATPase [25]. In the present study, we demonstrated that thapsigargin-dependent calcium mobilization transiently induced *mPer1* and *mPer2*, among circadian oscillatory genes, in NIH3T3 cells. Of the three *period* genes, *mPer1* mRNA tended to be the most sensitive to thapsigargin, but the induction of *mPer3* mRNA was only marginal. Recently, different functions, and the regulation, of the three *period* genes for the circadian

clock in mutant mice were reported [26,27]. Intracellular calcium may regulate each *period* gene by distinct mechanisms.

We could also detect other circadian related genes including *Clock*, *DBP*, *Cry1* and *Cry2* by thapsigargin. The effects of thapsigargin on each gene expression were varied. We observed that *Cry1* and *Cry2* mRNA were induced in response to intracellular calcium mobilization for the first time, and they were induced later than *mPer1* or *mPer2*. Cry has been reported to negatively regulate *period* gene transcription [14,15]. Therefore, the induction of *Cry* mRNA may be a consequence of the immediate induction of *mPer1* and *mPer2* mRNA. In contrast to the enhancement of the expression of *mPer* and *mCry* genes, the expression of *DBP* mRNA was reduced in NIH3T3 cells by treatment with thapsigargin. The present results agree with results that endothelin-1-dependent *mPer* induction is accompanied with the reduction of *DBP* mRNA [28], but the precise mechanisms remain to be determined.

Recently, it was reported that cells in peripheral tissues also have circadian clock genes similar to the SCN, the central clock in mammals. Balsalobre et al. first reported that treatment with high concentrations of horse serum (serum shock) induced circadian genes such as *rPer1*, *rPer2* and *DBP* in addition to immediate early genes in rat-1 fibroblast [21]. In addition to serum shock, many stimuli such as forskolin, glucocorticoid, and PMA have been reported to induce circadian genes in cell line models [21–23,29]. We demonstrated that an intracellular calcium chelator, BAPTA-AM, reduced forskolin and/or PMA-induced *mPer1* mRNA expression to basal levels. Therefore, we next investigated the contribution of the cAMP- and PKC-mediated pathways to thapsigargin-induced *mPer1* and *mPer2* mRNA expression in NIH3T3 cells. The forskolin-dependent phosphorylation status of Erk, p38 MAP kinase and CREB was significantly different from thapsigargin-dependent ones. PMA is a strong activator of PKC but prolonged treatment with PMA down-regulates PKC signaling [30]. In the present study conditions, thapsigargin still induced *mPer1* and *mPer2* mRNA after prolonged treatment with PMA although they abolished PMA-dependent *mPer1* and *mPer2* mRNA expression. From these findings, protein kinase A and PKC appeared to have a minor role in thapsigargin-induced *mPer1* and *mPer2* mRNA in NIH3T3 cells.

MAP kinases are subdivided into Erk, p38 MAP kinase or c-JUN amino-terminal protein kinase. Specific pharmacological inhibitors such as U0126 for MEK1-Erk and SB202190 for p38 MAP kinase were utilized to demonstrate the involvement of each kinase in various cellular events [31–33]. In the study conditions, phosphorylation of Erk was still high under resting conditions and thapsigargin predominantly induced phosphorylation of p38 MAP kinase. Both inhibitors partially but significantly prevented thapsigargin-induced *mPer1* and *mPer2* mRNA expression in NIH3T3 cells. These results are consistent with the inhibitory effects of both reagents for thapsigargin-dependent histidine decarboxylase expression in RAW cells [34]. However, the limited effects of both inhibitors suggest that additional signaling pathways contributed to the thapsigargin-induced *mPer1* and *mPer2* mRNA expression in NIH3T3 cells. Very recently, a pharmacological inhibitor specific for $Ca^{2+}$/calmodulin-dependent kinase was reported to be involved in *Per1* induction in hamster SCN, although its contribution to the thapsigargin-mediated signaling pathway remains to be determined [35].

In conclusion, the findings here demonstrated that *mPer1* and *mPer2* mRNA, among several circadian related genes in NIH3T3 cells, are rapidly induced by intracellular calcium mobilization via the MAP kinase pathway. This $Ca^{2+}$ increase–MAP kinase pathway, distinct from pathways such as PMA–MAP kinase and forskolin-phosphorylated CREB, may also be involved in the resetting of the circadian clock rhythm. Further studies about the downstream signaling pathways of MAP kinase will give new insights into the molecular mechanisms of the mammalian circadian clock.

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