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Reduction of translation rate stabilizes circadian rhythm and reduces the magnitude of phase shift

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ABSTRACT

In the intracellular environment, the circadian oscillator is exposed to molecular noise. Nevertheless, cellular rhythms are robust and show almost constant period length for several weeks. To find which molecular processes modulate the stability, we examined the effects of a sublethal dose of inhibitors for processes in the molecular clock. Inhibition of PER1/2 phosphorylation by CKIε/δ led to reduced amplitude and enhancement of damping, suggesting that inhibition of this process destabilized oscillation. In contrast, moderate inhibition of translation led to stabilization of the circadian oscillation. Moreover, inhibition of translation also reduced magnitude of phase shift. These results suggest that some specific molecular processes are crucial for stabilizing the circadian rhythm, and that the molecular clock may be stabilized by optimizing parameters of some crucial processes in the primary negative feedback loop. Moreover, our findings also suggested that rhythm stability is closely associated with phase stability against stimuli.

1. Introduction

A wide variety of physiological and behavioral activities of most organisms oscillate with the daily environmental cycle, primarily the light/dark cycle. Autonomous daily cycles, known as circadian rhythm, are controlled by the circadian clock. In mammals, transcription-translation feedback loops of clock genes have been shown to be the basis of the oscillation generating circadian rhythm [1].

Most cells in the body are thought to possess a circadian clock [2,3]. Cellular clocks of the whole body are under control of the primary integrator of circadian rhythm located in the suprachiasmatic nucleus (SCN), via neuronal and/or hormonal signaling [4]. External environmental signals, notably photic signals, are transmitted to the SCN, and the circadian phase of the SCN is entrained to adapt to the external light cycles. However, it is likely that peripheral clocks are not entirely dependent on the SCN, but to some extent function as independent oscillators. Other than signals from the SCN, peripheral tissues can also receive local signals from physical and physiological stimuli [5]. In some cases, local signals are sufficiently strong to desynchronize and shift the rhythm phase from the SCN in peripheral tissues, particularly in the liver and kidneys [6,7].

In addition to being flexible in terms of circadian phase, circadian rhythm is also highly robust and accurate. The robustness of behavioral rhythm is attributed to the robust oscillation of the primary circadian integrator in the SCN. The neuronal cells within the SCN are tightly connected by a neuronal network of neurotransmitters and electrical signaling. Synchronized molecular oscillators generate robust circadian output signals which define behavioral rhythms [8,9].

Inside the body, there are a wide variety of tissues and cells, with a wide variety of differences in physiological activities. Even within histologically identical cells, physiological conditions, such as the number of molecules, vary from cell to cell [10,11]. Despite inherent noise due to the fluctuations of physical and physiological conditions, cellular clocks can sustain robust oscillation. Single-cell monitoring revealed that mouse embryonic fibroblasts (MEF) from *Per2*::*dLuc* knock-in mice, sustained circadian rhythm for at least 6 weeks [12]. It is likely that robust molecular oscillation is attributed to the complicated network structure of the molecular clock. Comprehensive genetic perturbation analysis using RNAi

Abbreviations: CHX, cycloheximide; SCN, suprachiasmatic nucleus.

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demonstrated that network properties, such as proportional response, signal propagation through activator and repressor modules, and paralog compensation, contributed to the buffering influence of genetic perturbations on circadian oscillation [13].

A distinct example of stability is temperature compensation, i.e. period length is almost invariant within the physiological range of temperature. The most accepted model for temperature compensation is that negative and positive contributions of increasing reactions rates on period length are mutually canceled out [14]. However, in mammals, phosphorylation of PER1/2 by casein kinase Iε and δ (CKIε/δ) was revealed to be a temperature-insensitive reaction, and it was suggested that this process may contribute to determining temperature compensation, as well as period length [15]. These findings suggest that, in addition to network structure, few processes in the molecular clock have important roles for sustaining robust circadian rhythm.

Robustness and stability is an important feature of circadian rhythms. Weak rhythms are commonly observed in old people and patients with some health problem [16,17]. Thus, it is hypothesized that regeneration of robust rhythm may improve therapeutic efficiency and health conditions. However, there are few studies on the control of robustness and stability of the circadian rhythm. As shown in some reports, reduced amplitude due to a mutation in a clock gene, or desynchrony of cellular clocks, affected the speed of entrainment [18,19]. These results also suggest that better understanding of the mechanism of stability and robustness is required for the regulation of circadian rhythms.

To find which molecular processes in the transcription-translation feedback loop of the circadian clock modulate the stability of the mammalian peripheral clock, we focused on the effect of perturbations of cellular reactions that consist of the molecular clock. There were characteristic responses of the molecular clocks to sublethal doses of each inhibitor. Interestingly, moderate inhibition of translation led to stabilizing oscillation and reducing the magnitude of phase shift induced by forskolin. These results suggested that some specific molecular processes are crucial to stabilize the circadian rhythm, and the circadian clock may be stabilized by optimization of some processes in the primary negative feedback loop of the molecular clock.

2. Materials and methods

2.1. Construction and transfection of mPer::dLuc in Rat-1 cells

A 3410 bp region upstream of the mouse *Per2* sequence [20] was cloned into the KpnI/BglII sites of the pGL4.19 vector (Promega) to generate the *mPer2*::*dLuc* construct. Rat-1 cells was transfected with the *mPer2*::*dLuc* vector, and selected with 400 μg/ml G418 as previously described [21].

2.2. Real-time monitoring of circadian bioluminescence

The transfected cells in 35 mm dishes were treated with 100 nM dexamethasone, and following a further 1 h incubation period, the medium was replaced with 2 ml DMEM with 25 mM Hepes (GIBCO), supplemented with 10% FBS and 0.2 μM Luciferin (Nacalai Tesque). Bioluminescence was measured using photomultiplier tube (PMT) detector assemblies (Kronos, ATTO and C8801–01, Hamamatsu Photonics).

2.3. [^{35}S]Methionine incorporation assay

To assess protein synthesis activity, incorporation of L-[^{35}S] Methionine in Rat-1 cells was measured as described in a previous report with some modifications [22]. 35 mm dishes of Rat-1 cells in the stationary phase were incubated with 2 mCi/ml of L-[^{35}S] Methionine, sp act-1000 Ci/mmol, (New England Nuclear, Boston, Mass.). After 2 to 3 h, the medium containing [^{35}S]Methionine was removed, and the cell monolayer was washed twice with 500 μl of ice-cold PBS. After washing, 1 ml ice-cold 10% TCA was added, and the dishes were incubated for 1.5 h on ice. The precipitate was harvested with a cell scraper. After precipitation by centrifugation, the precipitate was washed twice with 500 μl of ice cold 10% TCA and solubilized in 150 μl 9 M Urea and 2% Triton X-100. Portions of the solubilized material were dissolved in 5 ml Atomlight liquid scintillation cocktail (PerkinElmer) and counted in a liquid scintillation counter (LSC-6100, Aloka). Protein concentration was determined by BCA Protein Assay Kit (Thermo Scientific).

2.4. Bioluminescence data analysis

First, data sets were detrended by subtracting the 24 h running average from the raw data. Amplitude was estimated as the height of the first peak after 24 h from assay start after subtraction of the count of the subsequence trough. Detrended data of the first 36 h were excluded from the estimation of period length and damping rate. Period and damping rate were estimated by fitting a model equation for damping oscillation to detrended data:

$$y(t) = a + be^{-kt}\cos\left(\frac{2\pi(t+\phi)}{\tau}\right)$$

in which each symbol represents: a, baseline; b, amplitude; k, damping rate; ϕ, phase and τ, period. For calculating the phase shift, phase differences between the peaks after stimulation by 0.001% DMSO and by 0.1 μM forskolin were calculated.

3. Results

3.1. SP600125 destabilizes the circadian oscillation, as well as prolongs period length

In a previous study, it was reported that circadian rhythm was resilient to reduction of transcription activity with sublethal dose of transcription inhibitors, α-amanitin and actinomycin D [23]. Moreover, the rhythm of Per1-knockout cells became more robust by actinomycin D. To further evaluate response of the molecular clock to perturbations on primary processes of the molecular clock network, we measured the bioluminescence rhythm of the mPer2:dLuc reporter in Rat-1 cells in the presence of sublethal doses of protein phosphorylation and translation inhibitors.

At first, we examined the effect of SP600125, a JUN N-terminal kinase (JNK) inhibitor. Although knockdown of JNK had little to no effect on cellular circadian rhythm, administration of SP600125 to *mPer2*::*Luc*-NIH3T3 cells and *hPer2*-*Luc*-U2OS cells lengthened the period length more than 6 h [15]. The target molecule leading to period change was identified as CKIε/δ, of which its major phosphorylation substrates are PER1 and PER2 (PER1/2). In the present study, the period length of Rat-1 cells was also lengthened by the inhibitor (Fig. 1D). Moreover, inhibition of PER1/2 phosphorylation not only affected period length, but also overall bioluminescence level, amplitude, and damping rate. Overall bioluminescence level and the amplitude of the first peak were reduced (Fig. 1A and C), and damping rate was increased dose-dependently (Fig. 1D), suggesting that inhibition of this process destabilized oscillation. Thus, phosphorylation of PER proteins by CKIε/δ may be a crucial process for sustaining stable oscillation, as well as determining period length.

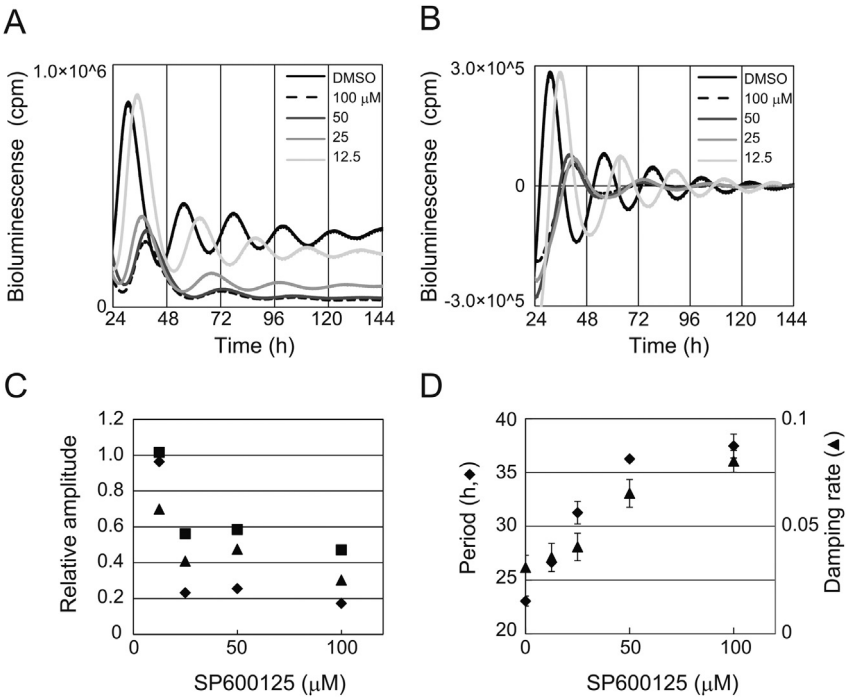

Fig. 1. Effect of phosphorylation inhibitor SP600125 on cellular rhythm. Representative raw (A) and detrended data (B) of mPer2:dLuc bioluminescence of Rat-1 cells in the presence of SP600125. C) Relative amplitude of rhythms in the presence of SP600125 to that of control (+0.001% DMSO). Amplitude was estimated as the height of the first peak after 24 h from assay start after subtracting the count of the subsequence trough. Data from three independent experiments are plotted individually. D) Dose-dependent effects of SP600125 on period length and damping rate. Each symbol represents the mean ± SD of three independent experiments.

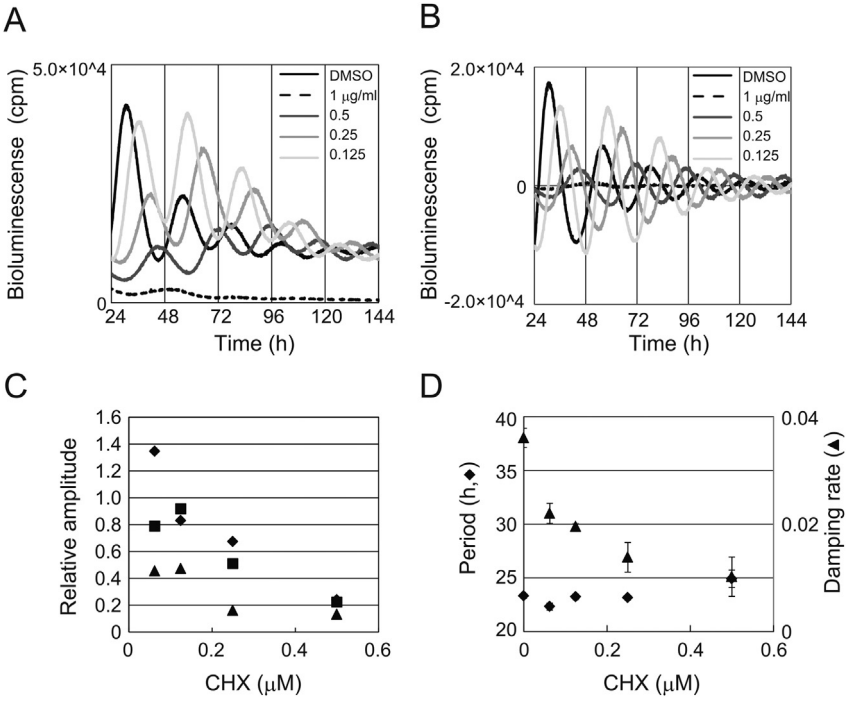

Fig. 2. Effect of translation inhibitor cycloheximide (CHX) on cellular rhythm. Representative raw (A) and detrended data (B) of mPer2:dLuc bioluminescence of Rat-1 cells in the presence of CHX. C) Relative amplitude of rhythms in the presence of CHX to that of control (+0.001% DMSO). Amplitude was estimated as the height of the first peak after 24 h from assay start after subtracting the count of the subsequence trough. Data from three independent experiments are plotted individually. D) Dose-dependent effects of CHX on period length and damping rate. Each symbol represents the mean ± SD of three independent experiments.

3.2. Cycloheximide stabilizes circadian oscillations

Next, we examined the effect of the translation inhibitor cycloheximide (CHX). Circadian oscillation was observed in the presence of CHX up to 0.5 μM, but was lost at 1.0 μM (Fig. 2A). The amplitude of the first peak was decreased along with the increase of inhibitor concentration (Fig. 2A and C), whereas period length was little affected (Fig. 2D). However, a larger amplitude could be observed in the middle of the measurement at concentrations from 0.125 to 0.25 μg/ml, compared to cells receiving vehicle (Fig. 2A and B). Consistent with the observations, the damping rate was reduced with increasing CHX concentration (Fig. 2D). Thus, initial amplitude appeared to be sustained for a longer period of the measurement in the presence of the inhibitor. These results suggested that moderate suppression of translation stabilized cellular rhythms. To test the hypothesis that the increase of amplitude was not due to recovery of oscillation by deactivation of CHX, we measured the protein synthesis rate by a methionine incorporation assay (Fig. 3A). At 0.25 μg/ml CHX, the incorporation rate was approximately 25% of that with vehicle at 0 h, and remained significantly reduced, of approximately 56%, at 48 h after administration of CHX (Fig. 3A), indicating that CHX suppressed protein translation during the measurement.

Anisomycin also acts as an inhibitor of translation, which compete with the amino acid side chains of aminoacyl tRNAs for binding to the A-site cleft [24], whereas cycloheximide inhibits translation elongation through binding to the E-site of the 60S ribosomal unit [25]. Similar to the results with CHX, larger amplitude could be observed during the measurement in the presence of anisomycin, compared to cells receiving vehicle, and damping rate was decreased along with the increase of anisomycin concentration; however, period length was only slightly changed (Fig. S1). Methionine incorporation was also moderately suppressed at the minimum concentration (20 nM) at which the decrease of damping ratio was observed (Fig. S1). The effect of CHX was confirmed in a mouse C6 glioma cell expressing the Bmal1:dLuc reporter [26], in which the larger amplitude and the decreased damping ratio was also observed (Fig. S2). This result suggested that the moderate inhibition of translation stabilized circadian rhythm in various types of mammalian cells.

3.3. Moderate inhibition of translation reduces the magnitude of phase shift induced by forskolin

Circadian clocks are entrained to external environmental cycles by shifting the circadian phase. For cellular clocks, phase shift can be elicited by administration of chemical compounds, such as forskolin and dexamethasone. We examined whether translational inhibition had influence on the magnitude of the phase shift by forskolin. Circadian rhythm showed both advances and delays of circadian phase by approximately 12 h at most by administration of 0.1 μM forskolin, depending on the phase at time of administration (Fig. 3B and C). However, in the presence of 0.25 μM CHX, the magnitude of phase shift was suppressed (Fig. 3B and C), showing type 1 of the phase response curve. These results suggested that improved stability of the circadian rhythm affected the magnitude of phase shift.

4. Discussion

In the intracellular environment, the circadian oscillator is exposed to molecular noise. Nevertheless, cellular rhythms are robust and show almost constant period length for several weeks [12]. To evaluate the stability of cellular rhythms, we examined the response of molecular clocks to perturbations by sublethal doses of inhibitors for processes in the molecular clock. In addition to

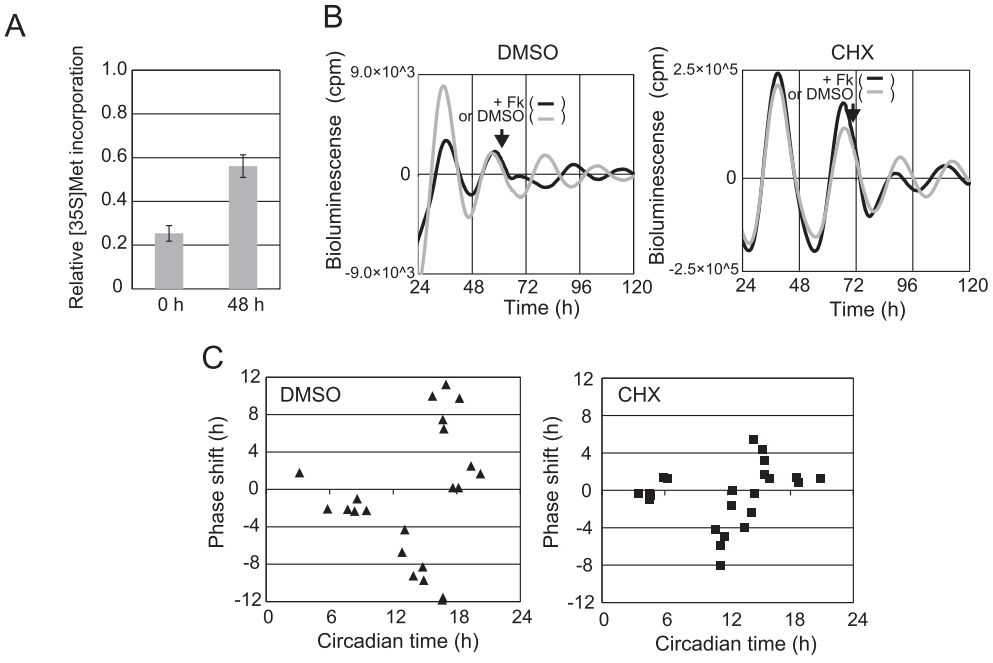

Fig. 3. Effect of cycloheximide (CHX) on phase shift induced by forskolin. A) [^{35}S]Methionine incorporation rate was measured in the presence of DMSO or 0.25 μg/ml CHX, and normalized to protein concentration. Each data represents the mean ± SEM of three independent experiments (n = 3). The relative translation rates to that of DMSO were 0.25 ± 0.036 at 0 h and 0.56 ± 0.052 at 48 h after addition of the inhibitor. B) Representative experimental data of phase shift induced by 0.1 μM forskolin in the presence of DMSO (left) or 0.25 μg/ml CHX (right). C) Phase response curve of mPer2:Luc Rat-1 cells induced by 0.1 μM forskolin in the presence of DMSO (left) or 0.25 mg/ml CHX (right). The horizontal axis represents time relative to PER2:Luc peak time (CT12) and the vertical axis represents magnitude of phase shift. Data are represented in circadian hours (1 circadian hour = period length (hour)/24).

prolonged period length [15], inhibition of PER1/2 phosphorylation by CKIε/δ led to reduced amplitude and enhancement of damping (Fig. 1). Interestingly, moderate inhibition of translation led to stabilization of the circadian oscillation (Fig. 2). Moreover, in the presence of the translation inhibitors, the magnitude of phase shift by forskolin was suppressed (Fig. 3). These results suggest that some specific molecular processes are crucial for stabilizing the circadian rhythm, and that the molecular clock may be stabilized by optimizing parameters of some crucial processes in the primary negative feedback loop. Moreover, our findings also suggested that rhythm stability is closely associated with phase stability against stimuli.

Many studies have reported that the modulation of CKIε/δ-dependent PER1/2 phosphorylation altered parameters of circadian rhythm [15,27–29]. In addition, our results suggested that phosphorylation of PER proteins is also a process ensuring the stability and the robustness of cellular rhythms. In many model organisms, phosphorylation of clock proteins have crucial roles for characterizing circadian rhythm [29]. Among others, it was demonstrated that the cycling of KaiC phosphorylation in cyanobacteria underlies the circadian oscillation generating temperature-compensated circadian rhythm [30,31]. Although the molecular components and network structures differ among organisms, the phosphorylation of clock proteins for characterizing circadian properties are likely to be a preserved molecular mechanism of circadian clocks.

The present study indicated that an appropriate dose of CHX stabilized circadian oscillation in Rat-1 cells. This was validated by further experiments using a different set of promoter of the luciferase reporter gene and C6 glioma cells. These findings suggest that the stabilization of the circadian oscillation by inhibition of translation is common to molecular clocks in various types of cultured cells and possibly, the relationship between the translation rate and the stability of cellular rhythms is common to all mammalian circadian oscillators. Although we could not discriminate whether the increased stabilization was due to amplification of single cell rhythm or improved synchrony (i.e. smaller distribution of period length) of cellular rhythms, it is significant that we can evaluate the stabilization of the circadian rhythm by luminescence from a population of cells. The circadian oscillation in cell lines is a model of peripheral circadian oscillators [32,33]; therefore it is useful to evaluate circadian rhythm at the population level, rather than at the single cell level, for considering circadian rhythms at the tissue level in vivo.

The magnitude of the phase shift of cellular rhythm was suppressed by inhibition of translation. This result suggested that rhythm stability is closely associated with phase stability. The magnitude of phase shift can be theoretically explained by the relative strength of the stimuli to the diameter of the limit cycle. The limit cycle with a larger diameter (i.e. large amplitude) may be less sensitive to stimuli than that with a small diameter [19]. Because stabilized oscillation by administration of CHX sustained a large amplitude for a longer duration, modest forskolin stimulation (0.1 μM) relative to the standard procedure (10 μM) could elicit only a smaller phase shift than in cells without the translation inhibitor. Another possibility is that the molecular clock is less sensitive to stimulation by forskolin, because moderate inhibition of translation may antagonize forskolin-induced acute and transient expression of PER1 protein [34].

It is widely acknowledged that weak rhythm is a common symptom observed in elderly people and patients with some specific health problem [16,17]. Our present study indicated that optimized regulation of translation and phosphorylation processes can improve robustness and tunability of the circadian rhythm, and also suggests that chemical compounds controlling translation and the phosphorylation state are candidate compounds of drugs for recovery of a healthy and robust rhythm.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.158.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.toxicon.2014.02.002.

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