Regulation of the Circadian Oscillator in *Xenopus* Retinal Photoreceptors by Protein Kinases Sensitive to the Stress-activated Protein Kinase Inhibitor, SB 203580*

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Circadian rhythms are generated by transcriptional and translational feedback loops. Stress-activated protein kinases (SAPKs) are known to regulate transcription factors in response to a variety of extracellular stimuli. In the present study, we examined whether the SAPKs play a role in the circadian system in cultured *Xenopus* retinal photoreceptor layers. A 6-h pulse of SB 203580, an inhibitor of SAPKs, reset the circadian rhythm of melatonin in a phase-dependent manner similar to dark pulses. This phase-shifting effect was dose-dependent over the range of 1–100 μM. Treatment with SB 203580 also affected light-induced phase shifts, and light had no effect on the circadian oscillator in the presence of 100 μM SB 203580. In-gel kinase assays showed that SB 203580 selectively inhibited a small group of protein kinases in the photoreceptor cells. These SB 203580-sensitive kinases correspond to two isoforms of phosphorylated p38 MAPK and three isoforms of c-Jun N-terminal kinase (JNK). Further in vitro study demonstrated that SB 203580 also inhibited casein kinase Iε (CKIε), which has been shown to regulate circadian rhythms in several organisms. However, a pharmacological inhibition of CKIε reset the circadian oscillator in a phase-dependent manner distinct from that of SB 203580. This argues against a primary role of CKIε in the phase-shifting effects of SB 203580. These results suggest that SB 203580 affects the circadian system by inhibiting p38 MAPKs or JNKs and that these protein kinases are candidate cellular signals in the regulation of the circadian oscillator in the *Xenopus* retinal photoreceptors.

The central mechanisms of circadian oscillators are transcriptional, and translational autoregulatory feedback loops consisting of several clock gene products (1–3). Many of the clock proteins are phosphorylated, and some of the relevant protein kinases have been identified. It has been shown in hamster and *Drosophila* that mutations of casein kinase Iε (CKIε), and double-time, a *Drosophila* homologue gene of CKIε, changed the periods of the circadian oscillators (4–6). Phosphorylation of FRQ protein, a central component of the *Neurospora* circadian system, has also been shown to affect the period length of the circadian oscillator (7). These results indicate that protein kinases play important roles in regulation of circadian feedback loops.

The stress-activated protein kinases (SAPKs) are candidates for involvement in circadian system regulation. The SAPKs are members of the mitogen-activated protein kinase (MAPK) family and are activated by a variety of extracellular stimuli such as UV light, heat shock, osmotic stress, or inflammatory cytokines. The activated SAPKs regulate several transcription factors to control gene expression (reviewed in Refs. 8 and 9). The SAPKs are classified into at least two groups based on the amino acid sequences of their dual-phosphorylation sites. One group is c-Jun N-terminal kinases (JNKs, also known as SAPK1), and the other group is p38 MAPKs (also known as SAPK2, -3, and -4). Each SAPK is activated by a different set of upstream kinase and has a different substrate preference (8–10). Because some of the stimuli that activate SAPKs are known to reset the circadian oscillator (11–13), the SAPKs may play a role in regulation of the circadian oscillator feedback loops.

In the present study, we investigated whether the SAPKs are involved in the regulation of the circadian oscillator in *Xenopus* retinal photoreceptors. We used SB 203580, an inhibitor of SAPKs, to pharmacologically manipulate SAPK pathways. SB 203580 was originally described as an exceptionally specific inhibitor of some of the p38 MAPKs (9, 14–17). More recently it has also been shown to inhibit JNKs at higher concentrations (18, 19). We found that SB 203580 reset the photoreceptor circadian oscillator and prevented light-induced phase shifts. We also showed that the inhibitory effect of SB 203580 is relatively specific to a small group of protein kinases in the photoreceptor cells and that these SB 203580-sensitive kinases include at least JNKs and p38 MAPKs. We also found that SB 203580 suppresses not only SAPKs but also CKIε in an *in vitro* kinase assay.

**EXPERIMENTAL PROCEDURES**

**Animals and Photoreceptor Layer Preparation—**Adult, male *Xenopus laevis* (length, 5–6.5 cm) were purchased from Nasco (Fort Atkinson, WI) and exposed to 12-h light:12-h dark (LD 12:12) cycles for at least 3 weeks before use. Photoreceptor layers were prepared as described previously (20). Briefly, eyecups were prepared, and the inside of each eyecup was sequentially washed with 0.3% Triton X-100, distilled water, and culture medium to lyse the inner nuclear layer. Then the damaged inner retina was peeled out. The photoreceptor layers and attached pigment epithelium were isolated and incubated overnight in modified Wolf and Quinby amphibian tissue culture medium (Invitro-activated protein kinase; SCN, suprachiasmatic nucleus; ZT, Zeitgeber time; LD, light/dark cycle.)
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A. Culture medium was delivered by a syringe pump at a rate of 0.4 ml/h. Experimental photoreceptor layers were exposed to drug pulses by changing syringes and/or to pulses of light at times specified relative to the animals’ previous LD cycles (Zeitgeber time (ZT), dark onset = ZT 12). White light was delivered by fiber optic cables from an illuminator (Cole Parmer, Vernon Hills, IL) with a 150-watt quartz-halogen lamp. Control photoreceptor layers were exposed to the control medium containing Me2SO and kept in constant darkness throughout the experiments with syringe changes at the times of drug pulses. Melatonin in superfuse samples was measured by radioimmunoassay, using an 125I-melatonin analogue (Covance, Vienna, VA) and the antiserum produced by Rollag and Niswender (22).

Quantitative measurement of phase shifts of melatonin rhythms was performed as described previously (25). First, the melatonin release record from each photoreceptor layer was smoothed by a three-point moving average, and long-term trends were removed by subtracting the average of values 12 h before and after each point (using the Chrono program, written by Till Roenneberg, Universität München). The times of half-rise and half-fall of the third, fourth, and sometimes fifth circadian peaks were measured as phase reference points for each recording from each photoreceptor layer, the time of each phase reference point was compared with the corresponding mean time in the control group, and the magnitude of the overall phase shift for each photoreceptor layer was taken as the mean of the phase differences measured at those phase reference points.

Tissue Collection for Protein Kinase Assays—For each sample, four photoreceptor layers were cultured together in a 24-well tissue culture dish, starting at the dark onset time on the day after dissection. Culture medium was the same as that used for the superfusion culture. The culture dishes were placed in the CO2 incubator and kept in constant darkness. Tissue collections were performed at ZT 6 as follows. First, the receptor layer was taken as the mean of the phase differences measured at those phase reference points.

Immunoprecipitation—The protein samples were incubated overnight with phospho-JNK antibody (1: 50), phospho-p38 MAPK antibody (1:50) (New England Biolabs, Beverly, MA), or CK1 antibody (1:19; BD Transduction Laboratories, Lexington, KY) at 4 °C. On the next day, the samples were incubated for 2–3 h with either 10 μl of protein A-Sepharose beads (Amersham Biosciences, Piscataway, NJ) or mixture of protein A-Sepharose beads and protein G-agarose beads (Invitrogen) at 4 °C. For in-gel kinase assay, the samples were centrifuged (16,000 × g) at 4 °C. The supernatant fractions were kept at –80 °C until immunoprecipitation or in-gel kinase assay.

In-gel Kinase Assay—In-gel kinase assays were performed as described (24) with slight modifications. Briefly, equal amounts of total protein samples or immunoprecipitated samples solubilized in 2× SDS gel loading buffer were resolved on 10% SDS-polyacrylamide gel containing 0.5 mg/ml myelin basic protein (MBP). After electrophoresis, the gel was washed with 20% 3-propanol in 50 mM Tri-citrate (pH 8.0) for 1 h and then gelled in buffer A (50 mM Tri-citrate (pH 8.0), 5 mM 2-mercaptoethanol) for 1 h to remove SDS. The proteins on the gel were denatured by 6 Å guanidine-HCl in buffer A for 1 h, then renatured by 0.04% Tween 40 in buffer A for 19 h at 4 °C. After renaturation, the gel was preincubated in in-gel kinase assay buffer (40 mM Hepes-NaOH (pH 8.0), 2 mM DTT, 0.1 mM EGTA, 5 mM MgCl2) for 30 min, then the kinase reaction was carried out in in-gel kinase assay buffer containing [γ32P]ATP (25 μCi/ml buffer) for 1 h at room temperature, in the absence or presence of SB 203580 (1, 10, or 100 μM). The in-gel kinase assay buffer for the control group contained 0.13% Me2SO. After the kinase reaction, the gel was washed with 5% trichloroacetic acid con-
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FIG. 2. Phase-response curve (PRC) and phase-transition curve (PTC) for SB 203580-induced phase shifts of the circadian oscillator in retinal photoreceptor layers, and lack of effect of PD 98059. A (PRC): each experimental photoreceptor layer was exposed to a 6-h pulse of SB 203580 (30 μM) or PD 98059 (30 μM) starting at ZTs indicated on the horizontal axis. Phase shifts were determined as described in the text, and magnitude of the phase shifts (mean ± S.E.; n = 5 in each group) were plotted. SB 203580 (○) produced the PRC similar to dark, and PD 98059 (□) given at either midday or midnight did not reset the circadian oscillator. Phase advances and phase delays are represented as positive and negative values, respectively. *, p < 0.05, significant phase shift compared with control groups (ANOVA, Dunnett two-tailed test). Note that the values of S.E. of several points are too small to be shown. B (PTC): vertical axis represents new phases (ZTs) to which the circadian oscillator was moved by a 6-h pulse of SB 203580 (30 μM) started at ZTs shown on the horizontal axis. Data are derived from A. SB 203580 always moved the oscillator phase to the next time regardless of the timing of the pulse.

Materials—Culture medium salts were obtained from Sigma (St. Louis, MO), and all other medium components were from Invitrogen. SB 203580, PD 98059, and JNK inhibitor I were purchased from Calbiochem (La Jolla, CA). SP600125 was a gift from Signal Pharmaceuticals Inc. (San Diego, CA). For the melatonin radioimmunoassay, the 125I-melatonin analogue was from Covance (Vienna, VA), and the antiserum was donated by Dr. M. D. Rollag (Uniformed Services University). The kinase assay buffer for the control group contained 0.13% Me2SO. The kinase reaction was terminated by addition of 2% SDS gel loading buffer. The samples were heated at 95–100 °C for 5 min and then centrifuged. The supernatant fractions were resolved on 12% SDS-polyacrylamide gel. The phosphorylated substrates were detected by autoradiography and analyzed using MacBass as described above.

RESULTS

SB 203580 Resets the Photoreceptor Circadian Oscillator—A 6-h pulse of SB 203580 (30 μM) reset the phase of the melatonin release rhythm. The magnitude and direction of the phase shift depended on the timing of the pulse (Fig. 1). A pulse of SB 203580 beginning at ZT 3 shifted the phase of the melatonin rhythm −12 h (Fig. 1A), a pulse beginning at ZT 12 caused a 7-h phase advance (Fig. 1B), and a pulse at ZT 15 had no effect on the phase of the melatonin rhythm (Fig. 1C). The new phase was attained rapidly and persisted through the end of the experiment.

In contrast to its strong effect on the phase of the circadian oscillator, SB 203580 did not cause an apparent acute change in melatonin levels (Fig. 1). This is an interesting observation, because all of the entrainment stimuli reported previously such as light, dopamine, and cyclic AMP acutely affect melatonin level in the retinal photoreceptors (20, 23), indicating that those signals act on both the entrainment pathway and the melatonin output pathway. On the other hand, the effect of SB 203580 occurs distinctively on the entrainment pathway (or on the oscillator), and therefore, the SB 203580-sensitive cellular signal does not play a significant role in oscillator output for melatonin release.

We produced a phase-response curve (PRC) for SB 203580 by starting pulses at eight different phases throughout the circadian cycle (Fig. 2A). Pulses beginning in the late subjective night to the early subjective day caused phase delays, and pulses beginning in the late subjective day to the early subjective night caused phase advances, whereas a pulse centered at
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**Effects of SB 203580 on Light-induced Phase Shifts**

Cultures and sampling were started at dark onset. Experimental groups (C) were exposed to a 6-h pulse of light (indicated by the open bar at the top of each graph) with or without drug (indicated by the hatched bar) at midnight (ZT 15–21). Control groups (●) were kept in control medium without drug and in constant darkness with syringe changes at the time of drug pulse. Group means ± S.E. (n = 5 in each group) of normalized melatonin values are represented. A and C, light (~600 lux) suppressed melatonin levels and caused a phase delay of melatonin rhythm. B, treatment with light and SB 203580 (30 μM) together caused a small phase advance. D, treatment with light and PD 98059 (30 μM) together caused a phase delay indistinguishable from that caused by the light alone (compare with C).

We tested whether inhibition of extracellular signal regulated kinases (ERKs, other members of MAPK superfamily) reset the circadian rhythm of melatonin by using PD 98059, an inhibitor of ERK kinase. ERK has been suggested to regulate the circadian system in mouse suprachiasmatic nucleus (SCN) (25), chick pineal gland (26), but also see 27), and Bullfrog retinas (28) as well as several cell lines (29, 30). However, a 6-h pulse of PD 98059 (30 μM) given either during the midday or during the midnight had no effect on the phase of melatonin rhythms (Fig. 2A). This argues against a role for ERK signaling in regulation of the circadian oscillator in Xenopus retinal photoreceptors.

**A Dose-dependent Effect of SB 203580 on the Circadian Oscillator Phase**—We determined the dose-response relationship for SB 203580 effects on the circadian oscillator by starting pulses at ZT 6, where the oscillator is the most sensitive to the drug. A 6-h pulse of the drug induced phase shift of the circadian oscillator in a dose-dependent manner (Fig. 3). A pulse of 1 μM SB 203580 did not affect oscillator phase (Fig. 3A), whereas a pulse of 10 μM caused a significant phase shift (Fig. 3B). A larger phase shift was induced when the photoreceptor layers were given a pulse at a concentration of 100 μM (Fig. 3C). This result showed that the phase-shifting effects of SB 203580 are dose-dependent and that a threshold concentration for resetting the circadian oscillator is somewhere in the range of 1–10 μM in the photoreceptor cells (Fig. 3D).

**Effects of SB 203580 on Light-induced Phase Shifts**—We also investigated effects of SB 203580 on photic entrainment. When photoreceptor layers were exposed to a 6-h light pulse (~600 lux) beginning at ZT 15, the expected phase delays of the melatonin rhythms were induced (Figs. 4A and 5A). At 10 or 30 μM SB 203580, a 6-h pulse of the drug alone did not cause a significant phase shift at this time (Fig. 5A). When the light treatments were applied together with either 10 μM or 30 μM SB 203580, significant phase advances were induced (Figs. 4B and 5A). These phase advances were not caused by either light or the drug treatment alone, indicating a complex interaction in the effects of the two treatments on the oscillator. On the other hand, treatment with a higher concentration of SB 203580 (100 μM) together with light caused a phase delay that is indistinguishable from that caused by SB 203580 (100 μM) alone, but significantly different from that caused by the light alone (Fig. 5A). This indicates that light had no effect on oscillator phase in the presence of 100 μM SB 203580. We also performed similar experiments at ZT 21 to confirm the SB 203580 effects on the light-induced phase shift. When the photoreceptor layers were exposed to a 6-h light pulse beginning at ZT 21, the expected phase advances were induced (Fig. 5B). On the other hand, a 6-h pulse of SB 203580 caused phase delays of melatonin rhythms, and the effect was dose-dependent (Fig. 5B). When the light was applied together with either 10 or 30 μM SB 203580, the phase shift that is significantly different from that produced by either light or the drug treatment alone was induced (Fig. 5B). However, simultaneous treatment with light and 100 μM SB 203580 together caused a phase delay that is indistinguishable from that caused by SB 203580 (100 μM) alone. Thus, SB 203580 consistently affected both phase advances and phase delays induced by light, and light had no effect on the oscillator phase in the presence of 100 μM SB 203580.

In contrast, a 6-h pulse of PD 98059 (30 μM) did not affect the
phase shifts caused by a light pulse (~600 lux) at ZT 15 (A) or at ZT 21 (B). This result argues against a role for ERK signaling in regulation of the circadian oscillator in Xenopus retinal photoreceptors by light.

It has been reported that inhibition of protein synthesis resets the circadian oscillator in a phase-dependent manner similar to SB 203580 and blocks light-induced phase shifts (31–33). This raised the possibility that the effects of SB 203580 on the circadian system are due to nonspecific inhibition of protein synthesis. We therefore examined whether SB 203580 inhibited protein synthesis in our system. The photoreceptor layers were incubated under constant darkness in the culture medium in which leucine was replaced by [3H]leucine receptor layers were incubated under constant darkness in the presence or absence of SB 203580. The [3H]leucine incorporated into newly synthesized protein was measured by the trichloroacetic acid precipitation method (34). We found that a 6-h pulse of SB 203580 did not inhibit total protein synthesis (data not shown). It is unlikely, therefore, that the effects of SB 203580 are caused by nonspecific inhibition of protein synthesis.

### Effects of SB 203580 on Photoreceptor Protein Kinases

We assessed the overall specificity of SB 203580 for protein kinase activities in the photoreceptors by in-gel kinase assays of total protein extracts. This technique detects both substrate-dependent phosphorylation and autophosphorylation of kinases separated by molecular mass. Using MBP as a substrate, we detected various protein kinases in the photoreceptor cells (Fig. 6). The signals represent specific incorporation of radiolabeled phosphates into the substrate protein, because no signal was detected when [γ-32P]ATP was replaced by [α-32P]ATP (data not shown). The apparent molecular masses of the detected protein kinases were distributed approximately between 30 and 110 kDa. For quantitative analysis, those kinases were divided into 10 groups based on the differences in molecular mass, and the group numbers (peak numbers) are shown on the right side of the panels. A, the film was overexposed to show most of the detected bands in one film; B, the film was exposed to the same set of the gels for the shorter period of time to show the inhibition of the 43- to 45-kDa protein kinase group (peak 6) by SB 203580. Asterisks indicate the kinase groups significantly inhibited by SB 203580.

#### Table I

Control value was defined as 100% in each peak, and the experimental values are relative to the control. Mean ± S.E. from three-four independent experiments are represented.

| Peak (kDa) | 1 μM | 10 μM | 100 μM |
|-----------|------|-------|--------|
| Peak 1 (80–105) | 99.3 ± 7.5 | 102.0 ± 10.8 | 86.1 ± 6.6 |
| Peak 2 (74) | 102.9 ± 15.1 | 106.7 ± 10.0 | 129.4 ± 16.6 |
| Peak 3 (68) | 88.2 ± 16.6 | 87.8 ± 20.7 | 119.3 ± 17.4 |
| Peak 4 (61) | 85.4 ± 5.8 | 89.2 ± 6.4 | 70.3 ± 9.0 |
| Peak 5 (55) | 84.1 ± 7.0 | 52.7 ± 13.9 | 20.8 ± 1.8 |
| Peak 6 (43–45) | 90.9 ± 5.9 | 82.9 ± 5.9 | 61.0 ± 8.3 |
| Peak 7 (39) | 102.7 ± 2.9 | 114.3 ± 14.6 | 113.3 ± 4.6 |
| Peak 8 (38) | 97.7 ± 20.9 | 113.6 ± 6.1 | 98.1 ± 4.1 |
| Peak 9 (34) | 109.1 ± 14.6 | 99.4 ± 21.9 | 59.5 ± 2.9 |
| Peak 10 (33) | 109.4 ± 22.6 | 101.9 ± 12.1 | 108.4 ± 9.3 |

* p < 0.05, significantly different from the group treated with 1 μM SB 203580 (ANOVA, Dunnett one-tailed test).
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**Fig. 7.** Dose-response effects of SB 203580 on phospho-JNKs and phospho-p38 MAPKs measured by in-gel kinase assay. A, crude extract from photoreceptor cells (Ex) and samples immunoprecipitated with either phospho-p38 MAPK antibody (IP-p38) or phospho-JNK antibody (IP-JNK) were resolved on 10% SDS-polyacrylamide gel containing MBP. After renaturation treatment, the gels were incubated in the in-gel kinase assay buffer in the absence (Control) or presence of SB 203580 (1, 10, or 100 μM). A, retinal photoreceptor cells expressed at least two isoforms of p38 MAPKs (43 and 45 kDa) and three isoforms of JNK (45, 52, and 54 kDa), and the mobility of those SAPKs was comparable to that of SB 203580-sensitive protein kinases in the crude extract (peaks 5 and 6). The molecular weight markers are shown on the right side of the panel, and the peak numbers on the left side of the panel are the protein kinase groups in the crude extract samples. B, quantitative result of the inhibitory effects of SB 203580 on each p38 MAPK isoforms (a and b) and JNK isoforms (c–e). For each SAPK, the signal intensity on the control gel was defined as 100%. The kinase activities of all SAPK isoforms were inhibited by SB 203580, but the dose dependencies were different among the SAPKs. The data are representative results of two independent experiments with similar results.

**Fig. 8.** Effects of SP600125 on the circadian rhythms of melatonin from Xenopus retinal photoreceptor layers. A, experimental group (n = 4) (○) was exposed to a 6-h pulse of SP600125 (30 μM) beginning at ZT 6 (indicated by the open bar at the top of the graph), and control group (n = 3) (●) was exposed to control medium without drug, but with syringe changes at the time of the drug pulse. The melatonin values for each photoreceptor layer were normalized relative to the average melatonin release by that photoreceptor layer. Group means ± S.E. of normalized melatonin values are represented. B, quantitative summary of the magnitude of the phase shifts caused by the different concentrations of SP600125. Data represented are means ± S.E. (n = 3–5 in each group). *, p < 0.05, significant phase shift compared with control groups (ANOVA, Dunnett two-tailed test).

*Effects of SB 203580 on Photoreceptor SAPKs—*The apparent molecular masses of the SB 203580-sensitive protein kinases in Fig. 6 match the predicted sizes of the *Xenopus* SAPKs (35–38). We therefore examined whether active p38 MAPKs and JNKs are expressed in the *Xenopus* retinal photoreceptors. The homogenized photoreceptor samples were incubated with antibody against either phospho-p38 MAPKs or phospho-JNKs then the immunoprecipitated samples were analyzed by in-gel kinase assays. Fig. 7 shows that at least two isoforms of p38 MAPK and three isoforms of JNK are expressed in the retinal photoreceptor cells and that no other kinase is detected after the immunoprecipitation, indicating good specificity of these antibodies. The apparent molecular masses of those SAPKs were ~43 and 45 kDa for the p38 MAPKs and ~45, 52, and 54 kDa for the JNKs, consistent with the predicted molecular masses of SAPKs in *Xenopus* system (Fig. 5A) (35–38). Importantly, all of those SAPKs migrated at the positions comparable to both of the SB 203580-sensitive protein kinase groups observed in the total protein extracts (Fig. 7, peaks 5 and 6).

We also found that all of these SAPKs are inhibited by SB 203580 in the concentration range of 1–100 μM. The dose dependences, however, were somewhat different among the SAPKs (Fig. 7). Of the p38 MAPKs, the 43-kDa isoform was strongly inhibited by the 1 μM SB 203580, whereas the 45-kDa isoform was much less sensitive to the drug. Of the JNKs, the 54-kDa isoform was the most sensitive to the SB 203580, and the 45-kDa isoform was the least sensitive to the drug. This is consistent with the previous studies showing that the sensitivity to SB 203580 differed among p38 MAPKs and JNKs (9, 16, 19). These data indicate that the *Xenopus* retinal photoreceptors express active p38 MAPKs and JNKs and that these SAPKs are inhibited by SB 203580.

*Do JNKs Regulate the Photoreceptor Circadian System?—*We then tried to determine roles of JNKs in the photoreceptor circadian system by using SP600125, a new specific inhibitor of JNKs. SP600125 has been shown to inhibit JNKs with an IC50 of 5–10 μM, and the drug at a concentration of 25 μM does not affect ERKs or p38 MAPKs in Jurkat T cells (39). When photoreceptor layers were exposed to a 6-h pulse of SP600125 at a concentration of 30 μM beginning at ZT 6, a significant phase delay was observed (Fig. 8). This phase-shifting effect is consistent with the effects induced by the SB 203580 treatment at this time, suggesting that JNKs are involved in regulation of the circadian oscillator. However, this drug also caused contin-
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Fig. 9. Dose-response effects of SB 203580 on phosphorylation of phosvitin by CKIε. A, autoradiogram of phosphorylated phosvitin resolved on a 12% SDS-polyacrylamide gel. Samples immunoprecipitated with CKIε antibody were incubated with phosvitin as a substrate and [γ-32P]ATP in the kinase assay buffer in the absence (C: control) or presence of SB 203580 (1, 10, or 100 μM). The activity of CKIε is suppressed by SB 203580 in a dose-dependent manner. B, quantitative result of the inhibitory effects of SB 203580 on CKIε. The signal intensities of two forms of phosphorylated phosvitin were combined together in each lane and compared with that of control group. The value in the control lane (C) was defined as 100%.

Fig. 10. Dose-dependent effect of CKI-7 on the phase of the circadian rhythms of melatonin. Experimental groups (C) were exposed to a pulse of CKI-7 (A: 30 μM, B: 100 μM, C: 300 μM) at ZT 9–15 indicated by the open bar at the top of each graph. Control group (●) was exposed to control medium without drug with syringe changes at the time of drug pulse. Group means ± S.E. (n = 4–5 in each group) of normalized melatonin values are represented. D, magnitude of the phase shifts caused by the three different concentrations of the CKI-7 pulses. Data represented are means ± S.E. (n = 4–5). *, p < 0.05, significantly different from untreated controls (ANOVA, Dunnett two-tailed test).

Several protein kinases (45). If SB 203580 resets the circadian oscillator by inhibiting CKI, suppression of the CKI activity is predicted to mimic the phase-shifting effect of SB 203580. We, therefore, examined whether CKI-7 resets the circadian oscillator with similar phase dependence. When a 6-h pulse of CKI-7 was applied to the photoreceptor layers beginning at ZT 9, phase delays of the melatonin rhythms were induced in a dose-dependent manner (Fig. 10). We also observed a phase delay (−5.2 ± 0.3 h, n = 4) when photoreceptor layers were treated with CKI-7 (30 μM) for 6 h beginning at ZT 12. At both ZT 9 and ZT 12, SB 203580 causes strong phase advances, so the PRCs for the two inhibitors do not match. These data indicate that CKI-7 can reset the photoreceptor oscillator. This may be due to inhibition of CKI, although the dose dependence...
of the phase-shifting effect extends beyond the range expected to be specific. At any rate, these data argue against a primary role for CKI inhibition in the phase-shifting effects of SB 203580.

**DISCUSSION**

In the present study, we found that a pulse of SB 203580 given at any time of the circadian cycle strongly resets the circadian oscillator to night time in *Xenopus* retinal photoreceptor layers. The effects are strikingly specific in that these treatments had no acute effect on melatonin synthesis or long term effect on amplitude of the melatonin rhythm. Several other known resetting stimuli such as light, dopamine, and activation of cyclic AMP acutely suppress or stimulate melatonin synthesis (20, 21, 46–50). Several other manipulations have long term effects on the amplitude of the rhythm. These include high [K⁺] treatment (51) and several protein kinase inhibitors, including SP600125 (Fig. 8). The present data indicate that SB 203580 acts selectively on an input (entrainment) pathway or on the oscillator itself. The phase transition curve for the drug suggests that the protein kinase responsible for the drug effects regulate the circadian oscillator actively during the subjective day.

We also found that SB 203580 altered the light-induced phase shifts in a dose-dependent manner and that light had no effect on the circadian oscillator in the presence of a high concentration of SB 203580 (100 μM). If the drug does not have a phase-shifting effect at any time throughout the circadian cycle (not only the time when light causes a phase shift (23)), the results can be interpreted relatively simply. However, because SB 203580 itself resets the circadian oscillator, an interpretation of this result is problematic. Because the PRC of SB 203580 is dark-pulse type, the drug effect on the oscillator is opposite the effect of light (32, 52). There are at least two possible explanations for how the drug blocked the light-induced phase shifts; either 1) SB 203580 inhibits the cellular signals involved in the photic entrainment pathways, or 2) SB 203580 acts on the circadian oscillator via distinct pathway from the photic entrainment pathway, and the phase shift caused by light and SB 203580 together resulted from the counterbalancing effects of the two stimuli on the circadian oscillator. In the first case, light and the drug affect the same signaling pathway in an opposite fashion, and a high concentration of the drug is required to completely block the photic information. Therefore, one would predict that the drug causes a quantitative change in the light-induced phase shifts, because the effects of two stimuli are settled before reaching to the oscillator. In the latter case, on the other hand, one cannot easily predict the magnitude or direction of the phase shifts caused by the simultaneous treatment of light and SB 203580, because the drug could cause qualitative changes in the light-induced phase shifts by resetting the oscillator itself. When the light is applied together with a very high concentration of the drug, the light effect is overwhelmed by the drug effect that could move the oscillator state variables very far from the limit cycle. Intermediate responses to co-administration as we observed at ZT 21 (Fig. 5B) do not distinguish between these two explanations. However, the data at ZT 15 (Fig. 5A) fit in the latter explanation, because simultaneous treatment with light and either 10 or 30 μM SB 203580 at ZT 15 caused phase shifts that could not be explained by simple quantitative changes. It is likely, therefore, cellular signals responsible for the SB 203580 effects are distinct from the photic entrainment pathway.

To determine the protein kinases responsible for the SB 203580 effects we used two different approaches. First, we identified candidate kinases in photoreceptors and tested sensitivity of those kinases to SB 203580. Second, we asked whether any of the limited number of alternative inhibitors mimicked the effects of SB 203580. Our in-gel kinase assay data showed that the inhibitory effect of SB 203580 is relatively specific for a small group of protein kinases and that these SB 203580-sensitive protein kinases are members of SAPKs (p38 MAPKs and JNKs). We also revealed that at least five active SAPKs were expressed in *Xenopus* retinal photoreceptors and that all of those protein kinases were inhibited by SB 203580 in vitro over the concentration range that resets the circadian oscillator. SB 203580 was originally characterized as a specific inhibitor of SAPK2a (p38) and SAPK2b (p38β2) (14–16), but later studies showed that higher concentrations of the drug also inhibit JNKs (18, 19). Our results are consistent with those studies. Surprisingly, we also found that this drug suppressed the activity of CKI in vitro, suggesting CKI as an additional candidate for a drug target in our system. To our knowledge, this is the first report to show that SB 203580 inhibits CKIs. Thus, these data are consistent with SAPKs and CKIs as targets of SB 203580. However, because conditions are different between *in vivo* and *in vitro*, we cannot draw a strong conclusion based on the dose dependence. We then performed further pharmacological experiments. To determine roles of JNKs in regulation of circadian system, other available JNK inhibitors such as SP600125 and JNKI1 were tested. The results of those experiments, however, were inconclusive, because those two JNK inhibitors showed either similar effect to SB 203580 but problematically long-lasting (SP600125) or no phase-shifting effect that is inconsistent with the SB 203580 effects (JNKI1). If the long-lasting stimulatory effect of SP600125 on melatonin is caused by inhibition of JNKs, it would role out roles for JNKs in the phase-shifting effects by SB 203580, because such melatonin stimulation was not caused by the SB 203580. However, it is also possible that the stimulatory effect of melatonin by SP600125 is a nonspecific effect. We could not, therefore, determine whether JNKs are responsible for SB 203580 effects. To determine roles of CKI in regulation of the circadian system, we examined the effect of CKI-7 on the circadian rhythms of melatonin. We found that CKI-7 in the concentration range between 30 and 300 μM reset the circadian oscillator in the *Xenopus* retinal photoreceptors. However, the phase dependence of the CKI-7 effect did not match that of SB 203580. This argues against a CKI role in the phase-shifting effects of SB 203580. Although the simplest model of the SB 203580 action is inhibition of a single protein kinase, it is also possible that SB 203580 may reset the circadian oscillator by inhibiting multiple protein kinases whose functional balance determines the phase of the circadian oscillator. If this were true, any specific protein kinase inhibitor would not mimic the phase-dependent effects of SB 203580. Therefore we cannot completely rule out a role of CKI in the SB 203580 effects solely based on the different phase dependence.

As mentioned above, the phase transition curve for SB 203580 suggests that the protein kinase responsible for the drug effects regulate the circadian oscillator actively during the subjective day. We found, however, that the levels of active JNKs did not exhibit circadian rhythm in the photoreceptor cells under constant darkness,2 and others showed that the
levels of active p38 MAPK did not exhibit rhythmic change in chick pineal gland. There are several possible cellular mechanisms by which protein kinase pathways regulate the circadian oscillator in a phase-dependent manner, such as rhythmicity in the protein kinase activity, in substrate availability, or in its subcellular localization. Currently little is known whether those cellular mechanisms in p38 MAPKs, JNKs, or CKIs signaling pathways show circadian rhythms in vertebrate rhythms.

The ERKs have been suggested to be involved in vertebrate circadian systems. In mouse suprachiasmatic nucleus (SCN), a primary circadian oscillator in mammals, phosphorylation of the ERKs is rhythmic with high levels during the day and is increased by light pulse at night. Circadian and light regulation of ERKs has also been reported in chick pineal gland although in a manner that is opposite to that found in mouse SCN. In chick pineal gland, activity of the ERKs was reported to peak during the night and to be dephosphorylated in response to light. However, a more recent study by others failed to observe the daily and the light effect on the ERK activity in dispersed chick pineal cell culture. Circadian rhythms of phosphorylated ERKs have also been reported in amacrine cells of bullfrog retina with high levels during the night, and the inhibition of the ERKs signaling pathways by PD 98059 caused phase delays of the phosphorylated ERK rhythms. This suggests that the ERKs regulate circadian oscillators in bullfrog retina. However, in the present study, inhibition of the ERK signaling pathway by PD 98059 had no effect either on the phase of the circadian oscillator or on the light-induced phase shifts in our system. Although we cannot make a strong argument based on the negative results, our data does not support a role of ERKs for the regulation of the circadian oscillator in Xenopus retinal photoreceptors.

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