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Calcium Inhibits Phase Shifting of the Circadian Conidiation Rhythm of *Neurospora crassa* by the Calcium Ionophore A23187[1]

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ABSTRACT

Effects of the calcium ionophore, A23187, and antimycin A on the circadian conidiation rhythm of *Neurospora crassa* were examined. A23187 at a concentration of 1 μM in medium not containing divalent cations delayed the phase by 10 hours at CT 10 and advanced it by 5 hours at CT 14 (CT 12 corresponds to the time that discs are transferred from light to dark). This phase shifting was completely inhibited by addition of 0.1 millimolar $CaCl_2$ but not by $MgCl_2$ at any concentrations examined.

Antimycin A inhibited respiration by 90% at a concentration of 0.2 micrograms per milliliter and lowered the ATP content by 85%. Antimycin A alone caused small phase advances but in combination with A23187 resulted in a large phase delay at CT 10. This phase shifting was not reversed by addition of $CaCl_2$ lower than 10 millimolar.

Neurospora crassa is one of the most useful organisms for studying molecular mechanisms of circadian rhythmicity, which is exhibited in many physiological activities of eukaryotes ranging from lower plants to higher animals (6). To evaluate chemical reactions involved in the mechanism of the circadian clock in *Neurospora*, many kinds of metabolic inhibitors have been examined. They include cycloheximide (8), quinidine and similar compounds that alter cyclic AMP metabolism (12), respiratory inhibitors (6), and diethylstilbestrol and related compounds (10). These compounds give phase response curves that differ from one another.

Calcium is known to work as a "second messenger" that coordinates many kinds of intracellular reactions (2). Eskin and Corrent (5) examined effects of the calcium ionophore A23187 on the circadian rhythm of nerve cell activity in the *Aplysia* eye and suggested that A23187 alters the rhythm by affecting intracellular gradients of the divalent cations Ca and Mg. On the other hand, Sweeney (17) reported A23187 to be ineffective on the rhythm of stimulated bioluminescence in *Gonyaulax*. Since Ca plays a major role in metabolic regulation, it was desirable to test whether Ca is involved in the mechanism of the circadian clock in *Neurospora crassa*. In this report, the effects of A23187 were examined. At the same time, antimycin A was used for inhibiting activity of the Ca-transporting system.

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MATERIALS AND METHODS

Culture conditions and Determination of Conidiation Rhythm. The bd (band) strain of *Neurospora crassa* was used throughout. Maintenance of stocks and methods for liquid culture were the same as reported previously (9). Conidia (13×10^5) were added to 25 ml of liquid medium containing Fries' salts (7), 0.3% glucose, and 0.5% arginine and cultured for 35 h in continuous light at 26 °C. Discs were cut from mycelial mats with a cork borer 11 mm in diameter. Six discs were transferred into 125-ml Erlenmeyer flasks with 25 ml of liquid medium. The medium contained Fries' salts, 0.03% glucose, and 0.05% arginine (pH adjusted to 7.0 by addition of NaOH before autoclaving). The discs were cultured on a reciprocal shaker (100 cycles/min) in continuous darkness at 26°C. A23187 and antimycin A were solubilized in ethanol and added to the medium. Except as noted, the medium of controls contained the same concentration of ethanol, which does not affect the rhythm. After treatment with chemicals, discs were transferred individually to race tubes with 8 ml of solid agar medium containing Fries' salts, 0.15% glucose, 0.25% arginine, and 1.5% agar. They were cultured in continuous darkness at 26°C.

The phase, or time of the first band, of the conidiation rhythm in race tubes after culturing in the liquid medium was determined by the linear regression analysis described by Dharmananda and Feldman (3). The phase of the liquid cultures was calculated from the first band in the race tube with the assumptions that periodicity in liquid medium is the same as that in the race tubes and that the rhythm is unaffected by transfer from liquid to solid medium (9, 13). Divalent cation-free medium contained (g/l): NH_4Cl, 7.2; NH_4NO_3, 5; KH_2PO_4, 5; NaCl, 0.5. Medium pH was adjusted to 7.2 before autoclaving.

Extraction and Assay of ATP. ATP was extracted and assayed by the method described by Slayman (14) with small modifications. Eighteen discs were put into 4 ml of ice-cold 6% $HClO_4$ and homogenized in a glass homogenizer. The homogenate was placed in an ice bath for 2 h and centrifuged. The precipitate was suspended in 2 ml of 6% $HClO_4$ and centrifuged. The supernatants were combined and neutralized with 1 N KOH after addition of glycylglycine to a final concentration of 10 mM. For neutralization, a double junction reference electrode was used with an outer solution of 1 N $(NH_4)_2SO_4$ for separating the electrode solution containing KCl from $HClO_4$. The neutralized solution was filtered with a Millipore filter and used for assay of ATP. ATP was determined using a CHEM-GLOW photometer (Aminco). The reaction cuvette contained 0.25 ml of a mixture (pH 7.4) containing 16 mM glycylglycine, 20 mM $MgCl_2$, 4 mM KH_2PO_4, and 50 μl of fire-fly extract prepared from desiccated fire-fly lanterns (Sigma) by the method of McElroy (15). A 0.1-ml aliquot was injected from the injection port and the amount

of light emitted in the first 5 s after injection was used for determination of ATP content. The results shown in the figures are averages ± SD from four different experiments.

Determination of O_2 Consumption. O_2 uptake by six mycelial discs was determined polarographically using a Clark-type O_2 electrode connected to a model 53 YSI O_2 monitor. The reaction medium was that in which the discs were cultured.

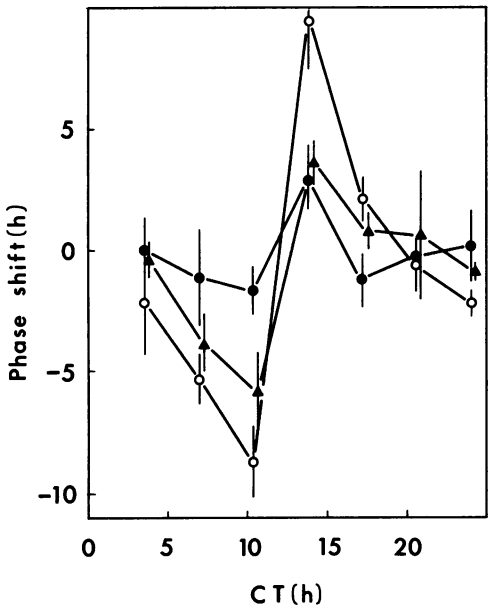

FIG. 1. Phase response curves for treatment with divalent cation-free medium containing A23187. Mycelial discs were treated for 3 h with: medium lacking divalent cations (▲), the same medium but containing 1 μM A23187 (O), and the same medium but containing 1 μM A23187 plus 0.5 mM $CaCl_2$ (●). They were then transferred to race tubes. All series were treated with the same concentration of ethanol (0.4%). Each point is the average phase of six discs ± SD.

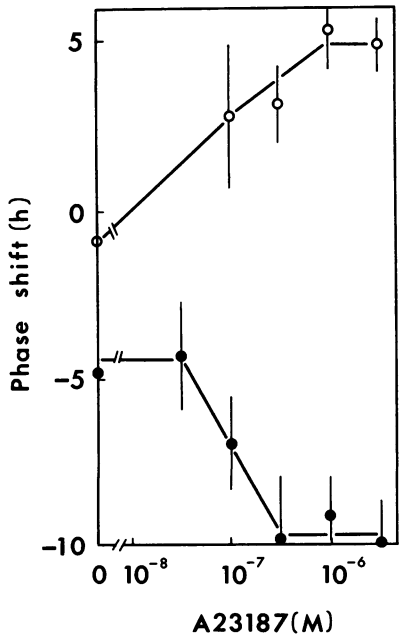

FIG. 2. Phase shifts caused by different concentrations of A23187. At CT 10 (●) and CT 14 (O), mycelial discs were treated for 3 h with different concentrations of A23187 in divalent cation-free medium.

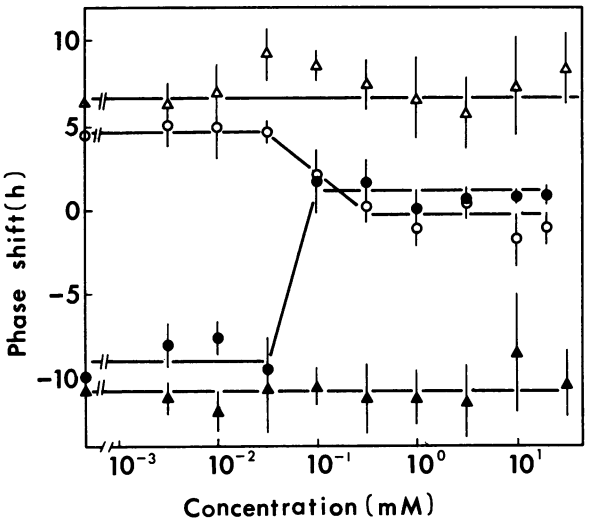

FIG. 3. Inhibition by $CaCl_2$ of phase shifting induced by A23187. At CT 10 (●, ▲) and CT 14 (O, △), mycelial discs were treated for 3 h with medium containing different concentrations of $CaCl_2$ (●, O) or $MgCl_2$ (▲, △) and 1 μM A23187.

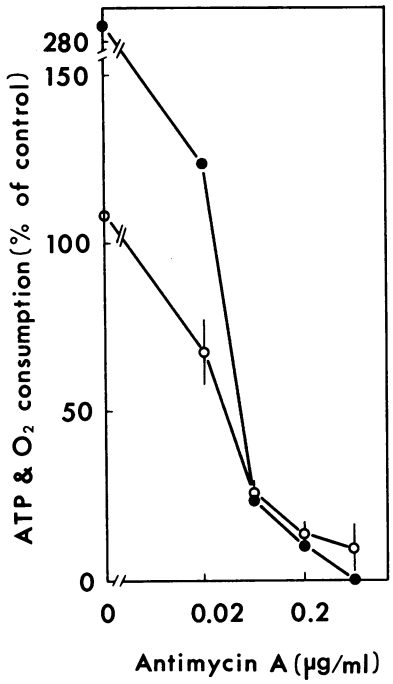

FIG. 4. Effects of various concentrations of antimycin A on ATP content in mycelial discs and on respiration. At CT 14, mycelial discs were treated for 3 h with various concentrations of antimycin A, and ATP content (O) and rate of O_2 consumption (●) were determined. Controls were cultured in medium not containing ethanol.

RESULTS

Phase Shifting by A23187. Mycelial discs were washed once with divalent cation-free medium and then transferred in the new divalent cation-free medium for every 3 h from 24 to 48 h after transfer from light to dark. The phase of the rhythm of the experimental series was compared with that of a control that was not transferred (Fig. 1). Treatment with divalent cation-free medium resulted in a small phase delay at CT 10. (The time that the discs are transferred from light to dark is designated CT 12.) However, the amount of phase shifting varied in different experiments, possibly due to contamination by divalent cations from

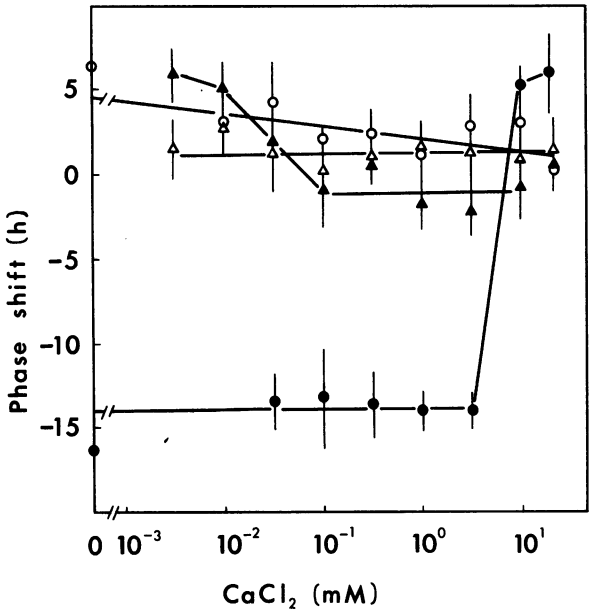

FIG. 5. Inhibition by $CaCl_2$ of phase shifting by antimycin A and by antimycin A plus A23187. At CT 10 (●, ▲) and CT 14 (○, △), mycelial discs were treated with divalent cation-free medium with various concentrations of $CaCl_2$ containing 0.2 μg/ml antimycin A (▲, △) and antimycin A plus 1 μM A23187 (●, ○). Controls were treated with the same concentration of ethanol.

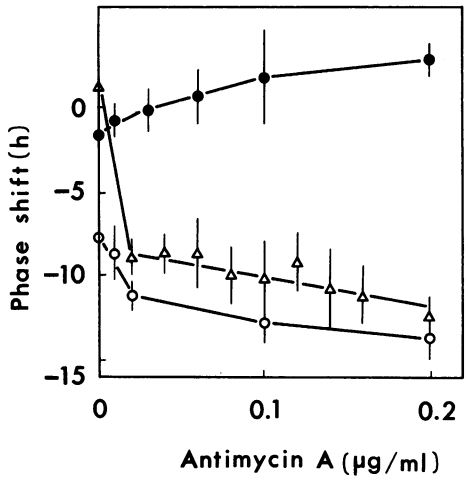

FIG. 6. Phase shifting by various concentrations of antimycin A and A23187 in medium containing various concentrations of $CaCl_2$. At CT 10, mycelial discs were treated for 3 h with divalent cation-free medium containing three different concentrations of $CaCl_2$ (0 mM, ○; 1 mM, △; and 10 mM, ●) with various concentrations of antimycin A (abscissa) plus 1 μM A23187.

the old culture medium. Addition of A23187 in divalent cation-free medium resulted in a large increase in phase shifting with 9-h phase delay at CT 10 and a 10-h phase advance at CT 14, even if treatment with divalent cation-free medium did not result any phase shifting. The amplitude of the phase advance was not reproducible; generally, phase advances of 5 or 6 h occurred at CT 14. However, phase shifting was completely blocked by addition of 0.5 mM $CaCl_2$ to the divalent cation-free medium containing A23187.

Figure 2 shows that the magnitude of phase shifting at CT 10 and CT 14 is dependent on the concentration of A23187; maximum phase shifts occurred at 3×10^{-7} M at both times.

As shown in Figure 1, 0.5 mM $CaCl_2$ inhibits phase shifting by 1 μM A23187. The minimum necessary concentration of Ca to block both phase delays at CT 10 and phase advances at CT 14 was 0.1 mM (Fig. 3). Treatment with concentrations higher than 0.1 mM $CaCl_2$ in the presence of A23187 did not change the phase in the reverse directions. $MgCl_2$ did not block phase shifting by 1 μM A23187 at any concentration examined.

Effects of Antimycin A on Phase Shifting by A23187. To examine the role of energy from respiration in the blocking action of Ca, antimycin A was used in the following experiments. As shown in Figure 4, antimycin A drastically inhibited respiratory activity. After treatment with 0.2 μg/ml antimycin A for 3 h, respiratory activity was about 10% of control levels. ATP content also decreased drastically to 15% of control levels.

When mycelial discs were treated with antimycin A in divalent cation-free medium containing different concentrations of $CaCl_2$ at CT 10, small phase advances occurred at $CaCl_2$ concentrations lower than 0.1 mM. Antimycin A did not cause any phase shifting when it was added to medium not containing divalent cations at CT 14 (Fig. 5). On the other hand, when antimycin A was added with 1 μM A23187, the magnitude of the phase delays (14 h) was larger than when mycelial discs were treated with only A23187 (8 h). Complete inhibition by Ca of these large phase delays occurred only at concentrations higher than 10 mM $CaCl_2$ which were actually accompanied by small phase advances. At CT 14, phase advances caused by antimycin A plus A23187 were more inhibited as $CaCl_2$ concentrations increased but the effect is not as striking since the phase advances were small compared to phase delays. Phase changes in the reverse direction were not observed at CT 14.

At CT 10, antimycin A caused phase delays in medium containing A23187 and low concentrations of $CaCl_2$. In the same medium with high concentrations of $CaCl_2$, antimycin A caused small phase advances (Fig. 6).

DISCUSSION

In divalent cation-free medium or medium containing very low concentrations of $CaCl_2$, Ca concentration in the cells should be lowered by the presence of A23187. Phase shifting by A23187 in such medium was completely blocked by addition of 0.1 mM $CaCl_2$, but not by $MgCl_2$. This indicates that decrease in the internal concentration of Ca results in slowing the clock at CT 10 and advancing it at CT 14. On the other hand, Ca concentrations in the cell should be increased in medium containing high concentrations of $CaCl_2$ and A23187. However, this presumed increase of Ca does not affect the clock. Two explanations are that Ca may only be needed above some threshold level for normal clock function, or extra Ca from the outside is extruded by a Ca transporting system in the plasma membrane. In normal cultures, Ca is extruded from *Neurospora* cells by an ATP-dependent, Ca-transporting system counteracting passive diffusion from the outside (16). If the second possibility is correct, ATP deprivation by respiratory inhibitors should cause a lowering of the external concentration of Ca necessary to block phase shifting by A23187. Tsien *et al.* (18) have reported that free cytoplasmic Ca in intact lymphocytes increases in the presence of mitochondrial poisons which cause severe ATP depletion. However, the opposite was found, *i.e.* much higher concentrations of Ca are necessary for inhibiting phase shifting by A23187 in the presence of antimycin A (10 mM $CaCl_2$) than in its absence (0.1 mM $CaCl_2$). This result suggests that phase shifting by A23187 may not be due to changes of cytoplasmic concentrations of Ca.

Calcium is also incorporated into mitochondria accompanying electron transport in animal cells (2). If a similar process occurs in *Neurospora* cells, it is easy to explain why antimycin A increased the magnitude of phase delays caused by A23187. Calcium accumulation in mitochondria was decreased by inhi-

bition of electron transport by antimycin A and at the same time the Ca concentration in mitochondria was lowered by increased passive diffusion by A23187. This suggests that the Ca concentration in mitochondria must be above some threshold level for normal clock function. Decreased Ca concentrations resulted in phase delays at CT 10 and phase advances at CT 14. An important question is whether the mitochondrial Ca concentration changes diurnally and whether this change is a component of the circadian clock. If so, and if mitochondrial Ca is reduced during the phase when it normally accumulates, it is reasonable to speculate that the clock should be delayed. According to this view, Ca depletion during the phase of normal decline should result in a phase advance. Such a role for Ca would be similar to that of K proposed by Njus *et al.* (11) and Burgoyne (1) to be the main component of the circadian clock. However, high external concentration of Ca with A23187 and antimycin A did not cause phase shifts toward reverse direction. The only exception was a small phase advance at CT 10 in medium containing concentrations of Ca greater than 10 mM. These results suggest that, even if it occurs, a circadian rhythm of Ca levels in the mitochondria is not a main component of the circadian clock. However, it is possible that the treatments described here were not effective in raising intramitochondrial Ca concentrations. Measurements of intracellular Ca would help to clarify this point.

A role for mitochondrial Ca transport in the *Neurospora* clock parallels suggestions by Eskin and Corrent (5) from studies on the *Aplysia* eye. An important role of mitochondria in the circadian clock has been postulated by Dieckman and Brody (4) from experiments using oligomycin-resistant mutants in *Neurospora.* Previous experiments using diethylstilbestrol also suggested that mitochondrial function is involved in the *Neurospora* clock (10). However, the phase response curve for diethylstilbestrol differs from that for A23187; the cross-over point from phase delay to advance is CT 5 in the former and CT 12 in the latter. Although both chemicals affect mitochondrial reactions, they must be affecting different reactions which are feeding into the clock. Further information on the relationship between the clock and mitochondrial function is needed for an understanding of molecular mechanisms of circadian rhythms.

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