Effects of Membrane ATPase Inhibitors on Light-Induced Phase Shifting of the Circadian Clock in *Neurospora crassa*

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ABSTRACT

Effects of several membrane ATPase inhibitors on light-induced phase shifting of the circadian conidiation rhythm in *Neurospora crassa* were examined using mycelial discs in liquid culture. Suppression of phase shifting by the inhibitors was strongly dependent on the pH of the liquid medium in which the discs were cultured during the time from light-dark transition (beginning of free-run) to light irradiation. When discs were cultured in pH 6.7 medium, azide, the inhibitors of plasma membrane ATPase (diethyldithiobestrol and *N*,*N*-dicyclohexylcarbodiimide), and ethanol completely suppressed the effect of light on the clock. In contrast, mycelial discs cultured in pH 5.7 medium were fully phase-shifted by light in the presence of the same and even higher concentrations of the chemicals. However, sensitivity to light of the discs cultured in relatively acidic medium was eight times higher than that of the discs cultured at neutral pH. Oligomycin and venturicidin, inhibitors of mitochondrial ATPase, did not suppress phase shifting by light at either pH.

Circadian rhythms are daily biological oscillations which persist in constant external conditions. One characteristic of circadian rhythms is that the phase of the rhythm is shifted by light pulses that interrupt constant darkness. The amount of the phase shift is dependent on the phase in which the light pulse is given (19). It has been suggested that membrane structure and function are components of the mechanism of the clock that generates circadian rhythms (6, 17, 27). In these models, light is thought to shift the phase by changing permeability of membranes to ions. In fact, some chemicals that alter membrane permeability, such as ionophores and alcohols, were reported to shift the phase of the clock and to mimic the effect of light (5, 27). Furthermore, daily oscillations of ion concentration in cells were reported in *Albizia* (22), *Gonyaulax* (26), and *Trifolium* (24).

However, we know little about the reactions connecting photoreceptor and the clock. Eskin (11) found that phase shifting by light in *Aplysia* could be nullified by certain changes in the ionic composition of the medium and suggested that membrane potential or conductance is altered by light and that the clock is phase-shifted as a result. Comparing phase response curves for light at different temperatures, Nakashima and Feldman (15) suggested that, in *Neurospora*, a heat-labile reaction couples the photoreceptor and the clock.

If light affects the clock through membrane functions, e.g. permeation of ions, one candidate for the site affected by light is membrane bound ATPases, because such enzymes are usually involved in active transport of ions. Changes in the activities of these ATPases should result in changes of ionic concentrations in cells. In addition, the blue-light photoreceptor of *Neurospora* was reported by Brain *et al.* (4) to exist in the plasma membrane.

Bowman *et al.* (2) recently characterized several inhibitors specific for the plasma membrane ATPase and for the mitochondrial ATPase in *Neurospora*. These inhibitors should be useful in determining whether membrane ATPases are involved in reactions connecting the photoreceptor system and the clock in *Neurospora*. In this report, we examined the effects of inhibitors specific for both plasma membrane and mitochondrial ATPases on phase shifting of the clock by light using a liquid culture system which was recently developed by Perlman *et al.* (18) and modified by Nakashima (16).

MATERIALS AND METHODS

The bd (band) strain of *Neurospora crassa* was a gift from J. F. Feldman. Procedures for maintenance of stock cultures and for liquid culture are the same as those reported previously (16). Conidia from 7-d-old slant cultures were suspended in distilled H2O. Conidia (13 × 10⁷) were added to a disposable Petri dish (11-cm diameter) with 25 ml of liquid medium containing Fries' salts (12), 0.3% glucose, and 0.5% arginine (21) and cultured for 33 h in continuous light at 26°C. Small discs were cut from the mycelial mats with a cork borer (11-mm diameter). Six discs were transferred into a 125-ml Erlenmeyer flask with 25 ml of liquid medium containing Fries' salts (12), 0.03% glucose, and 0.05% arginine and cultured on a reciprocal shaker (120 cycles per min) in continuous darkness at 26°C. The pH of the medium was adjusted using 1 N NaOH or 1 N HCl. Phosphate was replaced by the same concentration of chloride when the effect of vanadate was examined. Chemicals were added to the medium 48 h after transfer from light to dark, and mycelial discs were irradiated with white light for 5 min at 1,600 lux 1 h later. They were washed once with fresh liquid medium containing the usual concentration of glucose (0.3%) and arginine (0.5%) 2 h after irradiation. Then they were transferred individually to race tubes with 8 ml solid agar medium containing Fries' salts (12), 0.15% glucose, 0.25% arginine, and 1.5% agar and were cultured in continuous darkness at 26°C. The light pulse at the 49th h produces the largest phase shift of any time in the cycle (16); the phase is advanced about 10 h in control cultures.

The phase of the rhythm, i.e. the position of the first conidial band after the initial growth front mark (at the 64th h), was calculated using the method of Dharmananda and Feldman (9). All data points are the averaged values from six race tubes. All manipulations after the transition from light to dark were done under red safelight from 30-w white fluorescent lamps with red acrylic plate filters (Acrylite, Mitsubishi).

Oligomycin, venturicidin, DES,¹ and DCCD were dissolved in absolute ethanol. The final concentration of ethanol in the medium was less than 0.15%. Ethanol below this concentration does not shift the phase of the dark control and also does not affect

¹ Abbreviations: DCCD, *N*,*N*-dicyclohexylcarbodiimide; DES, diethyldithiobestrol.
phase shifting by light. The same concentration of ethanol was always added to flasks of the control series. Other chemicals were dissolved in water.

The respiration rate was determined polarographically using a Clark type O\textsubscript{2} electrode connected to a model 53 YSI oxygen monitor. The samples (12 discs) were immersed in the bathing medium containing Fries’ salts (12), and O\textsubscript{2} consumption was assayed at 26°C under red safelight.

RESULTS

Effects of Membrane ATP\textsubscript{ase} Inhibitors on Light-Induced Phase Shifting. Mycelial discs were cultured in media of various pHs, and 1 mM of azide, a mitochondrial ATP\textsubscript{ase} inhibitor, was added 1 h before the light treatment (Fig. 1). Azide, itself, shifted the phase when it was added to medium of initial pH lower than 5.4. Phase shifting by azide was very large in medium of initial pH 5. Azide did not inhibit phase shifting by light when the discs were cultured in the medium of about pH 6, but it completely inhibited light phase shifting at initial pH 6.7. The inhibition decreased again when the medium pH was higher than 6.7. However, azide was added into medium of different pHs, shown as final pH in Figure 1. Such differences in pH may cause differences of sensitivity to azide. In fact, sensitivity of respiration to azide is dependent on the pH of the assay medium (Fig. 2). Azide inhibited respiration more strongly at acidic pHs. For this reason, discs were cultured in pH 5.7 or in pH 6.7 medium, and the pH was adjusted by addition of NaOH or HCl at the same time as 2 mM azide was added (Fig. 2). Azide completely inhibited light-induced phase shifting of discs cultured in pH 6.7 medium at every pH examined but did not affect phase shifting of discs cultured in pH 5.7 medium. The different sensitivity of the discs to azide was, therefore, not due to difference of pH of the medium when azide was added.

The above results indicated that the initial pH of the culture medium is very important in examining effects of inhibitors on phase shifting by light. The following experiments were done using discs cultured in medium of two different pHs, 5.7 (acidic) and 6.7 (neutral). The former pH is that of usual (unadjusted) Fries’ medium (12). At high concentrations, most of the inhibitors changed the phase of dark controls. Therefore, we used concentrations below those that caused such phase shifts.

As shown in Figure 3, 1 mM azide completely suppressed phase shifting by light in discs cultured in the neutral medium but did not suppress it even at a concentration of 5 mM in discs cultured in acidic medium.

DES, a plasma membrane ATP\textsubscript{ase} inhibitor, at 10 μM completely suppressed the effect of light in mycelial discs cultured at neutral pH but did not affect it in cultures from acidic medium even at 35 μM (Fig. 4). This differential sensitivity to DES is not due to the difference in pH of the culture medium when discs were treated by DES, since light-induced phase shifting of discs from acidic medium was the same when the pH of the medium was varied at the same time that DES was added (Fig. 5). Light-induced phase shifting was largely suppressed in discs cultured at neutral pH, especially when DES was added to media of pH greater than 7 (Fig. 5). DES inhibited O\textsubscript{2} consumption by about 60% at a concentration of 60 μM (Fig. 5). However, inhibition of respiration was not affected by the pH of the assay medium.
DCCD inhibits both the mitochondrial and plasma membrane ATPases of *Neurospora* (1). DCCD inhibited the light effect at both pHs (Fig. 6). It was effective at 0.8 \( \mu \)M in the neutral medium and at 8 \( \mu \)M in the acidic medium; sensitivity to DCCD was, therefore, 10 times greater in discs cultured in the neutral medium than it was in those from the acidic medium. Respiration was inhibited only 20% by DCCD even at 40 \( \mu \)M, and the degree of inhibition was independent of external pH (data not shown).

Figure 7 shows the effect of vanadate on light-induced phase shifting. Vanadate, a plasma membrane ATPase inhibitor, resulted in a partial inhibition of phase shifting in discs cultured at both pHs.

Venturicidin (Fig. 8) and oligomycin (Fig. 9), mitochondrial ATPase inhibitors, did not affect light-induced phase shifting up to concentrations of 0.15 \( \mu \)g/ml. Growth stopped for about 1 d in discs treated with higher concentrations of these drugs, and the clock was completely reset.

**Effects of Ethanol and Light Intensity on Light-Induced Phase Shifting.** Preliminary experiments indicated that the final concentration of ethanol (used for solubilization of inhibitors) in the medium is important for obtaining significant phase differences between dark and light controls. Ethanol suppressed phase shifting by light in discs cultured in neutral medium but not in acidic medium (Fig. 10). Thus, sensitivity to ethanol is another difference caused by culture in media of the two pHs.

The two sets of discs showed different sensitivities to light (Fig. 11). In discs cultured in the acidic medium, maximum phase shifting was caused by 1-min irradiation at 10 lux. Maximum phase shifting in the neutral medium required 8-min irradiation, indicating that the latter discs were 8 times less sensitive to light than were those cultured at pH 5.7.

**DISCUSSION**

Azide suppressed phase shifting by light in mycelial discs cultured in neutral (pH 6.7) medium and did not affect light phase shifting of discs cultured in acidic medium. However, it inhibited \( O_2 \) consumption, as measured with an \( O_2 \) electrode, immediately after addition to both sets of discs (data not shown). Azide is reported to be an inhibitor of flavin-mediated photoactivation processes (20, 23). In addition, Lang-Feulner and Rau (14) found that azide inhibits photoinduction of carotenoid synthesis of *Fusarium*. These results suggested that azide may suppress light-induced phase shifting through inhibition of the photoactivation process rather than through membrane ATPase activity.

For discs cultured in medium of neutral \( pH \), two inhibitors of plasma membrane ATPase, DES and DCCD, were very effective in suppressing light-induced phase shifting. Concentrations of these drugs, which result in inhibition of ATPase activity and
suppression of phase shifting by light, are very similar. Phase shifting by light was completely suppressed by 10 μM DES and by 0.8 μM DCCD. Bowman et al. (2) reported that concentrations of 9 μM and 3 μM of DES and DCCD, respectively, result in 50% inhibition of plasma membrane ATPase activity. The similarity of the effective concentration is consistent with the possibility that both inhibitors suppress light phase shifting through inhibition of plasma membrane ATPase activity. DES has been reported to inhibit plasma membrane ATPase activity (1) and also to affect membrane potential (13) in other plants. On the other hand, DES was reported to disrupt mitochondrial electron transport in animal cells (10). In Neurospora, DES inhibited O2 consumption by less than 10% at a concentration of 10 μM, which completely suppressed light-induced phase shifting. Furthermore, the amount of inhibition of respiration was not affected by medium pH in a range of pH 5.5 to pH 7.5 (Fig. 5). This insensitivity to external pH is in contrast to the observations presented here on light-induced phase shifting and indicates that DES does not suppress light-induced phase shifting by inhibiting respiratory metabolism. However, vanadate, also shown by Bowman et al. (2) to be a potent inhibitor of plasma membrane ATPase, inhibited phase shifting by light very little. The medium pH when vanadate was added probably did not influence its effectiveness; the plasma membrane ATPase in Neurospora is inhibited by the same amount between pH 6.5 and pH 7.7, as vanadate exists in solution predominantly as the monovalent anion in this pH range (3). To examine the effect of vanadate, phosphate was removed from the medium because phosphate is a strong competitor of vanadate (7, 25). However, it is possible that, in these studies, intracellular phosphate was competing with vanadate. Additionally, it has been reported in red blood cells that the effectiveness of vanadate is nearly three orders of magnitude lower in intact cells than in isolated membranes (8).

Oligomycin and venturicidin, mitochondrial ATPase inhibitors, did not affect phase shifting by light. In preliminary experiments, the drugs inhibited growth by 50 and 80%, respectively, when they were added at 0.05 μg/ml liquid medium. This indicates that both inhibitors are incorporated into the cells. Therefore, mitochondrial membrane ATPase activity apparently is not involved in the mechanism of phase shifting by light.

For discs cultured in neutral medium, a plausible explanation of the experimental results is that light is perceived by a flavin-associated photoreceptor which stimulates plasma membrane ATPase activity and shifts the phase of the clock. It is interesting that the inhibitors, at concentrations which suppress light-induced phase shifting, do not affect the phase of the clock in the dark control. This suggests that a decrease in plasma membrane ATPase activity does not affect clock function at this phase of the cycle, but stimulation of the activity by light does change the phase of the clock. However, we have little information about reactions that couple the photosystem to the clock in cultures from acidic medium, except that DCCD completely suppresses phase shifting by light. Discs cultured in acidic medium are 8 times more sensitive to light than are those from the neutral medium.

Mycelial discs had differential sensitivities to light and chemicals, including plasma membrane ATPase inhibitors and ethanol, depending on the pH at which they were cultured. In the liquid media used in these studies, the discs do not grow after the first 15 h in constant darkness (16). It is possible that turnover rates of
membrane components are affected by external pH and that membrane structure and function may change gradually. These changes may result in differential sensitivities to light and chemicals. Characterization of biochemical differences in membrane structure among discs cultured at various pHs may yield further information about reactions connecting the photoreceptor system to the clock.

Acknowledgments—I wish to express my thanks to Professor Y. Ota for his kind guidance throughout this study, to Dr. J. Perlman for her correction of the manuscript, and to Dr. J. F. Feldman for allowing me to start these experiments in his laboratory.

LITERATURE CITED