Evidence against a direct role for inositol phosphate metabolism in the circadian oscillator and the blue-light signal transduction pathway in *Neurospora crassa*

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The inositol-depletion hypothesis proposes that the effects of Li^+ on cellular functions are the result of inhibition by Li^+ of the inositol monophosphate phosphatase and subsequent depletion of inositol lipids. This mechanism has been proposed to account for the effects of Li^+ on the period of the circadian oscillator. Inositol phosphate metabolism has also been proposed as part of the blue-light signal-transduction pathway through which the phase of the circadian oscillator can be reset by light pulses. Four

predictions of these two hypotheses have been tested in the fungus *Neurospora crassa* and all have been found to fail: (1) inositol supplementation does not reverse the effects of Li^+ on the period of the circadian rhythm; (2) inositol depletion of an inositol-requiring mutant does not mimic the effects of Li^+ ; (3) depletion of inositol lipids does not inhibit the response to light; and (4) a phase-resetting pulse of light does not increase the levels of inositol phosphates, including $\text{Ins}(1,4,5)\text{P}_3$.

INTRODUCTION

Circadian (daily) rhythms are found in all eukaryotes and are driven by an endogenous, self-sustaining, oscillator; in the absence of external time-cues, the rhythm will free-run with a period close to 24 h. The oscillator can receive information from the environment, which allows it to synchronize with the environmental day/night cycle; the phase of the oscillator can be reset by light, and in many plants and micro-organisms this involves the blue-light photoreceptor. The biochemical basis for circadian rhythmicity has not yet been described for any organism, nor has the mechanism of blue-light signal transduction through which the oscillator receives input from the environment. Inositol phosphate metabolism has been suggested as a potential component of both the circadian oscillator and blue-light phototransduction.

One line of evidence in favour of the participation of inositol phosphate metabolism in the mechanism of the circadian oscillator comes from the well-established effects of lithium (Li^+) on the period of circadian rhythms. Chronic Li^+ treatment will change the period of the rhythm in many organisms, from micro-organisms to mammals (reviewed by Engelmann, 1987). In most organisms, the period is lengthened, but in some it is shortened. Li^+ has several other effects in whole organisms: it is used in the treatment of manic depression, and may act by inhibiting signal transduction in the central nervous system; and it is teratogenic in developing embryos. The 'inositol-depletion hypothesis' developed by Berridge et al. (1989) proposes that these effects can be explained by a single biochemical mechanism: the inhibition by Li^+ of inositol phosphate metabolism. This inhibition can result in a depletion of cellular pools of inositol and inositol lipids, and the subsequent inhibition of the production of second messengers may explain Li^+ 's effects in manic depression and teratogenesis. Berridge et al. (1989) also propose that the effects of Li^+ on circadian oscillators could be explained if the period of the oscillator was dependent on the rate of phosphoinositide turnover.

There is as yet little evidence in favour of a role for

phosphoinositide turnover in blue-light signal transduction. Blue light has been shown to activate G-proteins in plants (Warpeha et al., 1991) and fungi (Kozak and Ross, 1991) and to activate a plasma-membrane protein kinase in plants (Reymond et al., 1992). White-light-induced phase-resetting of the circadian oscillator in *Samanea* may involve phosphoinositide turnover (Morse et al., 1990). In the filamentous fungus *Neurospora crassa*, blue-light-induced resetting of the oscillator may involve protein kinases, Ca^{2+} and ion fluxes across the plasma membrane (reviewed by Lakin-Thomas et al., 1990). I have been testing the hypothesis that blue light resets the phase of the *Neurospora* oscillator through a phosphoinositide signal-transduction pathway, and I have found that an inositol-requiring mutant grown on low-inositol medium is several orders of magnitude more sensitive to light than on high inositol medium (Lakin-Thomas, 1992).

Several testable predictions follow from the two hypotheses described above. If inositol depletion accounts for the effects of Li^+ on the period of circadian oscillators, then: (1) inositol supplementation should reverse the effects of lithium; and (2) inositol depletion of an inositol-requiring mutant should mimic the effects of Li^+ . If blue light resets the phase through a phosphoinositide signal transduction system, then (3) depletion of inositol lipids should inhibit the response to light; and (4) a phase-resetting pulse of light should increase the levels of inositol phosphates. As I report in the present paper, all four predictions fail when tested in *Neurospora*.

MATERIALS AND METHODS

Strains and culture methods

The strains used were *bd csp* and *bd csp inl*, as described in Lakin-Thomas (1992). Stocks were maintained on solid-agar slant cultures, as described in the preceding paper (Lakin-Thomas, 1993). For all experiments reported here, cultures were incubated at $22 \pm 1^\circ\text{C}$.

Cultures were grown on solid-agar medium (Vogel's N, with 0.5% maltose and 0.01% arginine) in glass growth tubes ('race tubes'), either 32 cm or 23 cm long, as described by Lakin-

Thomas (1992). For Li^+ experiments (Figure 1 below), a stock of minimal medium was prepared with trilitium citrate in place of trisodium citrate, and the final medium was made by mixing appropriate proportions of Na^+ - and Li^+ -containing stocks. The Na^+ concentration in minimal medium is normally 26.8 mM, and therefore the 20 mM Li^+ medium contained 6.8 mM Na^+ .

The liquid-culture system was as described in the preceding paper (Lakin-Thomas, 1993). Mycelial discs were grown in microtitre wells in 1 ml of Vogel's N medium with 2% glucose.

Assay of period of phase

The period and phase of the circadian oscillator were determined from the rhythmic pattern of bands of conidiation produced by race tube cultures, as described previously (Lakin-Thomas, 1992). Phase is reported in 'circadian time' (CT): one circadian cycle is divided into 24 'circadian hours' and CT 12 is defined as the phase at the time of transfer from constant light to constant darkness. Because liquid cultures do not display any visible rhythm, the phase of liquid-culture discs can only be determined by transferring discs to solid medium, allowing the cultures to grow for several days to produce bands of conidiation and extrapolating the phase of the conidiation rhythm back to the time of transfer. The phase of each experimental culture was compared with the pooled phase of a set of control discs transferred to race tubes at the light-to-dark transition.

For phase-resetting experiments (Figure 3 below), liquid cultures were incubated in constant white light for 48 h and transferred to constant darkness. At appropriate times, discs were removed from the microtitre wells and transferred to plastic Petri dishes for exposure to blue-light pulses, then immediately transferred to race tubes containing solid-agar medium (Vogel's N with 0.5% maltose and 0.01% arginine) and the same inositol concentration as the liquid medium at the time of inoculation. Race tubes were incubated in constant darkness for the remainder of the growth period. All manipulations after the initial transfer to darkness were carried out under a red safelight.

Blue-light pulses were given at 77 h after inoculation (29 h after the transfer to constant darkness). The light source was an incandescent slide projector lamp filtered through blue acetate (peak transmission at 440 nm with a half-maximal bandwidth of 110 nm) and 10 cm of water as an i.r. filter. The photon flux density was measured with a PAR quantum sensor (Skye Instruments, Llandrindod Wells, Powys, Wales, U.K.). For light doses above $10 \mu\text{mol} \cdot \text{m}^{-2}$, the intensity was held constant at either 3.6 or $18 \mu\text{mol} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$ and the pulse length was varied between 5 and 60 s; for low doses, pulse length was held constant at 5 s and intensity was varied with neutral-density filters.

Assay of $\text{Ins}(1,4,5)\text{P}_3$ levels in liquid culture

Liquid cultures were grown as described in the preceding paper (Lakin-Thomas, 1993) and were labelled during growth with $\text{myo}-[^3\text{H}]\text{inositol}$ at either 10 or 20 $\mu\text{Ci}/\text{ml}$. After 29 h in darkness, discs were transferred to Petri dishes and given pulses of blue light as described above. The photon flux density of the blue light source was held constant at $3.6 \mu\text{mol} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$ and pulses were of various durations. At the end of the light pulse the discs were immediately freeze-clamped under an aluminium block pre-cooled in liquid N_2 and were stored at -70°C . Extraction with HClO_4 and analysis of inositol phosphates by h.p.l.c. were as described in the preceding paper (Lakin-Thomas, 1993). The total radioactivity in each peak was normalized to the level of the phytic acid (InsP_6) peak to correct for potential differences in specific activity of the label, total mass or extraction efficiency,

on the assumption that the size of the relatively large pool of InsP_6 would not change significantly in response to brief pulses of light.

RESULTS

Effects of Li^+ on the period of the circadian oscillator

The *bd csp* strain was grown in race tubes on solid-agar medium in which the sodium citrate was partially replaced with various concentrations of lithium citrate, and the period of the conidiation rhythm was determined. Cultures were inoculated with conidia from stocks, exposed to white light for 24 h, and transferred to continuous darkness for the remainder of the growth period. The effects of various Li^+ concentrations are shown in Figure 1. All four concentrations of Li^+ tested increased

Figure 1 Effects of Li^+ and inositol on the period of the circadian oscillator in the *bd csp* strain

Cultures were grown on solid-agar medium in race tubes with various concentrations of Li^+ and inositol. The period and growth rate were determined as described in the Materials and methods section. Each point represents the mean $\pm$ S.E.M. for between four and ten individual race tubes. (a) Period (h); (b) growth rate (mm/h). —, Without inositol. ---, 277.5 μM inositol; 2 mM inositol.

Figure 2 Effect of inositol depletion on the period of the circadian oscillator in the *bd csp inl* strain

Cultures were grown on solid-agar medium in race tubes with various concentrations of inositol. The period and growth rate were determined as described in the Materials and methods section. Each point represents the mean $\pm$ S.E.M. for five individual race tubes. —, Period (h); growth rate (mm/h).

the period: compared with the zero- Li^+ controls, 5 mM Li^+ was significant at the $P = 0.05$ level of significance, and 10, 15 and 20 mM were significant at the $P = 0.01$ level (in one-tailed unpaired t tests). Engelmann (1987) reported that 10 mM Li^+ increased the period of the *bd* strain by 1.5 h, a larger increase than that reported here. However, the *bd* strain has a longer period on minimal medium than *bd csp*, owing to the period-shortening effect of the *csp* mutation (see Table 1 in Lakin-Thomas, 1992), and the period-lengthening effect of Li^+ may therefore be greater in *bd*.

The effect of Li^+ on the period was not reversed by added *myo*-inositol, as shown in Figure 1(a). Inositol was added to the growth medium in race tubes, and two concentrations were tested with various concentrations of Li^+ . Inositol alone had no significant effect on the period, and in most cases did not decrease the period in the presence of Li^+ . The only significant decrease in period due to inositol was seen in the 2 mM inositol cultures with 20 mM Li^+ ($P < 0.01$ in one-tailed unpaired t tests). Added inositol did not substantially reverse the inhibition of growth rate due to Li^+ (Figure 1b). Note that 277.5 μM inositol is sufficient to fully supplement the growth rate of the inositol-requiring *bd csp inl* strain (Figure 2), and would therefore be expected to reverse any effects of Li^+ which were the result of inositol depletion.

Effects of inositol depletion on the period of the circadian oscillator

The *bd csp inl* strain, which has an absolute requirement for inositol, was grown in race tubes on solid-agar medium at various concentrations of *myo*-inositol to assay the effects on the period. Cultures were inoculated with conidia from stocks, exposed to white light for 24 h, and transferred to constant darkness for the remainder of the growth period. As shown in Figure 2, growth rate declined rapidly below about 25 μM inositol. The cultures failed to conidiate below 20 μM , and therefore the period could not be assayed. At 20 μM , when growth rate was 84% of the rate at 2 mM inositol, there was a

slight but significant decrease in period in comparison with 40 μM inositol ($P < 0.05$ in a one-tailed t test).

When compared with cultures of the inositol-independent strain *bd csp* exposed to chronic Li^+ (Figure 1), *bd csp inl* cultures depleted of inositol (Figure 2) had a very different appearance. The rhythmic pattern of bands of conidiation was very clear in *bd csp* exposed to Li^+ , even at high Li^+ concentrations where the growth rate was severely inhibited. By contrast, the *bd csp inl* strain showed signs of 'inositol-less death' below 20 μM inositol, ceasing conidiation and producing a dark pigment typical of inositol starvation (Rana and Munkres, 1978).

Effects of inositol starvation on light sensitivity in liquid medium

In previous work (Lakin-Thomas, 1992), the *bd csp inl* strain was grown on solid-agar medium to assay the effects of low inositol on light-sensitivity. As described above, such cultures fail to produce conidial bands below about 20 μM inositol, and therefore light-sensitivity could not be assayed under more stringent starvation conditions. In addition, biochemical analysis is difficult in cultures growing on solid-agar medium. A liquid-culture system was therefore developed in which biochemical analysis and phase-resetting assays could be performed in parallel. Liquid-culture systems for *Neurospora* used in other laboratories rely on inhibition of growth by either carbon starvation (Nakashima, 1981) or pantothenate starvation (Perlman et al., 1981). In the liquid-culture system used here [and described in the preceding paper (Lakin-Thomas, 1993)], cultures are grown in microtitre wells to stationary phase, and inositol is growth-limiting at low (25 μM initial) concentrations. The disadvantage of this system is that cultures on high inositol, and inositol-independent cultures, undergo differentiation to produce conidia at the edges of the microtitre wells during the third day after inoculation.

To confirm that the circadian oscillator is functioning in these cultures, discs grown in 25 μM inositol were transferred to solid medium in race tubes with 25 μM inositol at various times after the transfer to constant darkness, and the phases of these cultures at the time of transfer were compared with race-tube cultures inoculated from conidia and transferred to darkness at the same time as the liquid cultures. The results (not shown) indicated that microtitre discs transferred to race tubes between 17 and 33 h after transfer to darkness remained in phase with the control tubes. The circadian oscillator must therefore be functional in the liquid cultures, and the period must not be very different from the period of the control cultures on solid medium.

Dose-response curves for the effect of light on phase were determined for liquid cultures of the *bd csp inl* strain inoculated at either 25 μM or 200 μM inositol (Figure 3). Discs were exposed to blue-light pulses at 29 h after transfer to darkness (approx. CT 22–24) and immediately transferred to race tubes to assay the phase. The phases of the discs given no light doses were approximately the same as the phase of the controls, indicating that there was no major observable effect produced by the transfer procedure.

The two dose-response curves in Figure 3, for low (25 μM) and high (200 μM) inositol, show similar features: at low light doses (below about 7 $\mu\text{mol} \cdot \text{m}^{-2}$) the phases of the light-exposed cultures were similar to the phase of the controls, and above 36 $\mu\text{mol} \cdot \text{m}^{-2}$ the light-exposed cultures were reset to a new phase. Variability between replicate tubes was very high at intermediate light doses (between 7 and 36 $\mu\text{mol} \cdot \text{m}^{-2}$ in these experiments). These are features which may be expected of a phase-resetting dose-response curve: intermediate doses can shift the oscillator into a state where phase is unstable or undefined (near the 'singularity' as described by Winfree, 1970),

Figure 3 Effect of inositol starvation on sensitivity to phase-resetting by blue light in the *bd csp inl* strain

Cultures were grown in liquid medium in microtitre plates as described in the Materials and methods section, and blue-light pulses were given after 29 h in darkness. Phase was determined by transferring discs to solid-agar medium in race tubes. Phase is reported as circadian time (CT) at the end of the light pulse (the time of transfer to race tubes). Because phase is a circular measurement, CT 0 = CT 24, and the top and bottom axes are the same, the plot should therefore be thought of as a horizontal cylinder. The phases of the zero light controls from all experiments at one inositol concentration have been pooled and are plotted (■) as mean phase $\pm$ mean angular deviation, calculated by the method of Zar (1984) for circular distributions. All other points (open symbols) represent the phase of a single race tube derived from a single disk. Different plot symbols represent different independent experiments. (a) 25 μ M inositol; (b) 200 μ M inositol.

while higher doses which are above a threshold for saturation of the phase-resetting response will shift the system to a new phase. Saturation of the response is defined as the lowest dose at which a stable new phase is attained. This new phase is 'stable' in two senses: (1) variability in phase between replicate tubes at a saturating dose is low (the same as the variability between replicate control tubes); and (2) doses above the saturation threshold shift the system to the same phase as the saturating dose. This saturating dose was found to be approximately the same (between 36 and 70 μ mol $\cdot$ m $^{-2}$) on both high and low inositol. A similar threshold for resetting was seen at 2 mM inositol (results not shown). Inositol starvation therefore does not appear to affect light-sensitivity under these conditions.

In previous work (Lakin-Thomas, 1992) I determined dose-response curves for the *bd csp inl* strain on solid-agar medium using pulses of cool-white fluorescent light. To obtain

Figure 4 Effects of blue light on the level of Ins(1,4,5) P_3 in the *bd csp inl* strain

Cultures were grown in liquid medium labelled with [3 H]inositol at 200 μ M and blue-light pulses were given as described in Figure 3. Light intensity was 3.6 μ mol $\cdot$ s $^{-1}$ $\cdot$ m $^{-2}$. Extraction and analysis of inositol phosphates were as described in the Materials and methods section. Results are from five experiments with various pulse durations, giving a total of three independent determinations at each time-point. Within each experiment, the changes in Ins(1,4,5) P_3 level in the light-treated samples were calculated as a percentage of the zero-light control. Each point represents the mean $\pm$ S.E.M. for three determinations.

the data in Figure 3, I improved the method by switching to liquid cultures and using a defined blue-light source. To directly compare light-sensitivity in liquid and solid medium, disc cultures in liquid medium were given pulses of white light with the same blue photon flux density as the blue pulses used in Figure 3, and the same phase-resetting threshold was found (results not shown). This result indicates that the response of the blue-light photoreceptor is the same in white light as in blue light, and the thresholds in the previous work can be directly compared with the present results. As reported above, cultures in liquid medium inoculated at both 25 μ M and 200 μ M inositol required about 36–70 μ mol $\cdot$ m $^{-2}$ for saturation. On solid-agar medium, cultures at 25 μ M inositol required about 25 μ mol $\cdot$ m $^{-2}$ of blue light to saturate the phase-resetting response (very similar to the results reported here), while cultures at 250 μ M inositol required about 25000–50000 μ mol $\cdot$ m $^{-2}$ for saturation (Lakin-Thomas, 1992).

Effects of light on the levels of Ins(1,4,5) P_3

Liquid cultures of the *bd csp inl* strain were grown under conditions identical with those described in Figure 3, at 200 μ M inositol with the addition of *myo*-[3 H]inositol. Disc cultures were given pulses of blue light and were immediately freeze-clamped at the end of the pulse. Extracts were assayed for inositol phosphate levels by h.p.l.c. as described in the preceding paper (Lakin-Thomas, 1993), and the results are presented in Figure 4. The data were analysed by a two-tailed *t* test, and no significant changes were seen in the level of Ins(1,4,5) P_3 between 5 and 120 s after the beginning of the blue-light pulse. The levels of other inositol phosphates were also determined in these samples (results not shown), and no reproducible changes were seen in the levels of the Ins P_2 peak (two samples per time point), four peaks of Ins P_3 (three samples), two peaks of Ins P_4 (two samples) and two peaks of Ins P_5 (two samples). The levels of inositol monophosphates could not be assayed in these unwashed samples because of contamination with inositol phosphates from the

extracellular medium [the preceding paper (Lakin-Thomas, 1993)].

DISCUSSION

Does the inositol-depletion hypothesis account for the effects of Li^+ on the period of the oscillator?

There is a growing list of cases in which the effects of chronic Li^+ treatment can be reversed by adding *myo*-inositol, and there is good reason to suspect that the effects of Li^+ are the result of disruption of the operation of a phosphoinositide signalling system in these cases. For example, inositol reverses Li^+ -induced disruption of development in frog embryos (Busa and Gimlich, 1989) and sea-urchin embryos (Giudice et al., 1992) and reverses Li^+ effects on behaviour in rats (Kofman et al., 1991). The reversal by inositol of Li^+ -induced effects is also seen in various tissues such as isolated rat islets (Zawalich et al., 1989), rat hippocampal slices (Pontzer and Crews, 1990), frog muscle fibres (Zhu and Fu, 1990) and human lymphocytes (Jenkins et al., 1991).

In *Neurospora*, the existence of an inositol-requiring mutant has made it possible to test directly the relevance of the inositol-depletion hypothesis with respect to the circadian oscillator. If Li^+ affects the circadian oscillator in the inositol-independent *bd csp* strain by depleting inositol, then the period-lengthening effects of Li^+ should be reversed by added inositol; similarly, inositol depletion of the inositol-requiring *bd csp inl* strain should mimic the effects of Li^+ by lengthening the period. As shown by Figures 1 and 2, both of these predictions fail. Li^+ has been shown to inhibit the inositol monophosphate phosphatase in *Neurospora* (Hanson, 1991; Szabó et al., 1991), and the inhibition of growth rate seen in Figure 1 indicates that Li^+ was having some physiological effect, but neither the inhibition of growth rate nor the lengthening of the period appear to be due to inositol depletion. Li^+ may instead exert its effects through some other mechanism, such as perturbing Na^+ -dependent ion gradients (since Na^+ is replaced by Li^+ in the growth medium), or through effects on G-proteins (Avissar et al., 1988).

Does blue light reset the oscillator through an inositol phospholipid signalling system?

In my first attempt to answer this question (Lakin-Thomas, 1992) I used the inositol-requiring strain and assayed phase-resetting by white light in cultures grown on solid-agar medium in race tubes. Contrary to expectations, I found that low-inositol (25 μM) cultures were not less sensitive to light, but rather several orders of magnitude more sensitive than high-inositol (250 μM) cultures. I have subsequently assayed the levels of inositol metabolites in cultures under similar conditions [the preceding paper (Lakin-Thomas, 1993)] and have found an increase of about 70% in the level of PtdInsP_2 on low inositol medium.

If the increase in PtdInsP_2 was responsible for the increase in light-sensitivity, then depletion of PtdInsP_2 might be expected to decrease light-sensitivity. The use of the liquid-culture system allowed me to test light-sensitivity under conditions of severe inositol starvation which would have prevented expression of the conidiation rhythm on solid medium. Low-inositol liquid cultures inoculated at 25 μM inositol reach a final concentration of about 5 μM at the time of harvesting, and show signs of inositol-less death [the preceding paper (Lakin-Thomas, 1993)]. Compared with liquid cultures inoculated at 200 μM (final concentration about 73 μM), the level of PtdInsP_2 is depleted to about 25%

(Lakin-Thomas, 1993). In spite of this, there is no discernible change in light sensitivity (Figure 3). It appears, therefore, that there is not a simple correlation between the basal levels of the putative signalling precursor and the sensitivity to the light signal.

The most direct evidence for the operation of a phosphoinositide signalling system should be an increase in the level of $\text{Ins}(1,4,5)\text{P}_3$ in response to the signal. Between 5 and 10 s of light is required to saturate the phase-resetting response under the conditions of Figure 4, and therefore the intracellular signal would not be expected to peak before 5 s; and because the earliest-reported response to blue light in *Neurospora* is the induction of RNA synthesis at 2 min (Nawrath and Russo, 1990), the signal should be detectable before this time. The failure to find such an increase in $\text{Ins}(1,4,5)\text{P}_3$ within this time-scale (Figure 4) is consistent with the lack of correlation between inositol starvation and light-sensitivity. It is unlikely that a small localized increase in $\text{Ins}(1,4,5)\text{P}_3$ levels could be responsible for the physiological response, but would be undetectable above a high basal level, for two reasons: (1) the basal level of $\text{Ins}(1,4,5)\text{P}_3$ was calculated as 67 nM under the conditions of Figure 4 [the preceding paper (Lakin-Thomas, 1993)], well below the estimated K_m values for Ca^{2+} release from organellar stores, indicating that a 10–100-fold increase must occur for an effective stimulus, and (2) the entire mycelium of *Neurospora* is light-sensitive when grown on solid-agar medium (Dharmananda and Feldman, 1979) and there is no reason to expect cultures in liquid medium to behave differently.

These results make it unlikely that blue-light-induced phase-resetting is mediated by a phosphoinositide signalling system in *Neurospora*. The change in light-sensitivity on solid-agar medium in response to inositol depletion (Lakin-Thomas, 1992) still requires an explanation. Inositol depletion on solid medium may affect the synthesis of pigments associated with the photoreceptor or may alter the levels of other membrane lipids which may be components of signal-transduction pathways. One consequence of Li^+ -induced inositol depletion in animal cells is an accumulation of diacylglycerol, and this mechanism may have the potential to enhance protein kinase C activity (Berridge et al., 1989); it remains to be seen whether inositol depletion has the same effect in *Neurospora*.

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