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THE EFFECTS OF ALTERING EXTRACELLULAR POTASSIUM ION CONCENTRATION ON THE MEMBRANE POTENTIAL AND CIRCADIAN CLOCK OF *PARAMECIUM BURSARIA*

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Summary

In some neural models of circadian rhythmicity, membrane potential and transmembrane flux of potassium and calcium ions appear to play important roles in the entrainment and central mechanisms of the biological clock. We wondered whether these cellular variables might be generally involved in circadian clocks, even non-neural clocks. Therefore, we tested the impact of changing extracellular potassium level on the circadian rhythm of photoaccumulation of *Paramecium* cells, whose membrane potential responds to changes of extracellular potassium in a manner similar to that of neurones. We found that pulse or step changes of extracellular potassium concentration did not phase-shift the circadian clock of *P. bursaria* cells in a phase-specific manner. Furthermore, modifying the extracellular concentration of calcium did not affect the magnitude of light-induced phase resetting. Therefore, while membrane potential and calcium fluxes may be crucial components of the circadian clock system in some organisms, especially in neural systems that involve intercellular communication, the *P. bursaria* data indicate that membrane potential changes are not *necessarily* an intrinsic component of circadian organization at the cellular level.

Introduction

Circadian rhythms are expressed by organisms at all levels of organization, both eukaryotic and prokaryotic (Johnson and Hastings, 1986; Kondo *et al.* 1993). Despite considerable study of their molecular basis, however, almost nothing definitive is known about the underlying clockwork mechanism. One class of models postulates ionic fluxes across membranes as central components of the clockwork (Njus *et al.* 1974; Sweeney, 1987).

Key words: *Paramecium bursaria*, circadian rhythms, membrane potential, photoaccumulation, potassium.

In invertebrate neural models of circadian rhythmicity, several lines of evidence point to a crucial role of membrane potential and transmembrane calcium fluxes in the rhythm generator and entrainment pathway. For example, changing the extracellular concentration of potassium ions alters the expression of the circadian rhythm of spontaneous neural activity in the retina of the molluscs *Aplysia californica* and *Bulla gouldiana* (Block *et al.* 1993). This treatment is known to depolarize the plasma membrane of molluscan neurones (Hodgkin and Keynes, 1955; Eskin, 1972). One of the ways that an increased extracellular concentration of potassium ions affects the circadian clock of these retinae is by lengthening the free-running period (McMahon and Block, 1987*b*). The second major way that extracellular potassium affects these clocks is by phase-dependent resetting of the rhythm (Eskin, 1972; Jacklet and Lotshaw, 1981). Apart from the well-known effects of extracellular potassium on the membrane potential of neurones in general, the likelihood that increased extracellular potassium concentration is affecting molluscan retinal clocks *via* membrane potential was demonstrated in *Bulla*, where it was shown that directly depolarizing or hyperpolarizing the pacemaker cells within the retina causes phase-dependent resetting of the rhythm which coincides with that caused by changes of extracellular potassium concentration (McMahon and Block, 1987*a*,*b*). Because the resting membrane potential of these pacemaker cells also oscillates in a circadian fashion (McMahon *et al.* 1984), the resetting of the circadian rhythm by an alteration in the potassium concentration in the medium is consistent with a crucial role for membrane potential in the oscillator's mechanism.

In *Bulla* eyes, the well-established phenomenon of entrainment of the circadian clock by light appears to be mediated by membrane potential and calcium ions. Experiments using direct current injections demonstrated that membrane depolarization is not only sufficient to phase-shift the *Bulla* ocular pacemaker, but that the depolarization is a required step in the light-induced phase-resetting pathway (McMahon and Block, 1987*a*). In addition, it appears that light itself causes a membrane depolarization in which the incoming ions are calcium. Reduction of extracellular [Ca^{2+}] blocks phase-shifting by light or by depolarizing potassium pulses in *Bulla* eyes (McMahon and Block, 1987*a*; Khalsa and Block, 1988; Block *et al.* 1993). These and other data have been invoked to build a model for the circadian clockwork in molluscan retinae which proposes that membrane potential, transmembrane Ca^{2+} fluxes and intracellular free Ca^{2+} levels are key components of the light entrainment pathway and/or of the generator of the free-running rhythm (Block *et al.* 1993).

Does membrane potential play a key role in the circadian mechanism in other types of cells? We decided to answer this question in a unicellular system by testing the response of a protozoan, *Paramecium bursaria*, to treatments which change its membrane potential. *P. bursaria* is an excellent cell for this investigation. It expresses a circadian rhythm of photoaccumulation which can be monitored automatically for many cycles to assay the phase and period of its circadian oscillator (Johnson *et al.* 1989; Nakajima and Nakaoka, 1989). *Paramecium* cells have long been a favourite subject for electrophysiological investigations, with the result that the response of these cells to ionic changes in the medium is well documented (Naitoh and Eckert, 1968; Machemer, 1989). Two characteristics that *P. bursaria* cells share with cells of the *Bulla* retina are (1) that

the resting membrane potential oscillates during the daily cycle (in the case of *P. bursaria* cells, the membrane potential undergoes an oscillation of approximately 5 mV in a light/dark cycle; Nakajima and Nakaoka, 1989), and (2) that light depolarizes the membrane of *P. bursaria* cells, the magnitude of the light-induced depolarization being dependent upon the extracellular concentration of calcium (Nakaoka *et al.* 1987).

We found that treatments which change the membrane potential of *P. bursaria* cells do not affect the circadian rhythm in these cells. Specifically, pulsatile or step changes of extracellular potassium levels did not elicit reproducibly significant phase shifts. Changes in the extracellular calcium level did not block or potentiate phase-shifting by light pulses. We conclude that changes in membrane potential are not of central importance to the circadian mechanism in *P. bursaria* and, therefore, that this parameter is not part of the circadian clockwork of all organisms.

Materials and methods

Strains, media

Paramecium bursaria with intracellular symbiotic *Chlorella* were used for all experiments. A variety of cultured strains (named Mit-B, Kz-1 and T316) derived from different isolates from nature were used in the phase-shifting experiments of Figs 3–7, as noted. A nitrosoguanidine-induced mutant of the Mit-B strain, named BND-1, was used for measurements of membrane potential, because it does not discharge its trichocysts in response to being impaled with microelectrodes. The cells were grown in 1.25 % (w/v) fresh lettuce juice medium (Hiwatashi, 1968) which had been inoculated with *Klebsiella pneumoniae*. Cultures were grown at 22 °C in light/dark (LD) cycles of 12 h of light (at about 25 μmol m^{-2} s^{-1}, cool-white fluorescence light) followed by 12 h of darkness. 1–3 days prior to photoaccumulation experiments or to measurement of membrane potential, the cells were washed and resuspended in ‘KCMT’ medium (0.25 mmol l^{-1} $CaCl_2$, 0.5 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} Tris–HCl, pH 7.2–7.4, with various concentrations of KCl as indicated) to allow the cells to adjust to the experimental medium. All experiments were performed at 22–25 °C.

Assay and analysis of circadian photoaccumulation rhythms

Photoaccumulation rhythms were monitored as described previously (Johnson *et al.* 1989) using cells in KCMT medium at densities of 3000–4000 cells ml^{-1}. For experiments in which the medium was replaced (i.e. pulses of high-K^+ medium), the cells were spun in a clinical centrifuge for 10 s in 10 ml ‘oil-test’ tubes, the supernatant was aspirated, and they were gently resuspended in fresh medium. Control experiments indicated that this centrifugation treatment did not cause reproducible phase shifts. In experiments which tested whether altering the potassium content of the medium had any effect on the phase or period of the photoaccumulation, the ciliary response duration (see below) to high-K^+ medium of the cells to be used was tested to confirm their sensitivity to high K^+ levels.

For phase-response experiments, cells were entrained by a light/dark cycle, then released into continuous conditions (continuous light, LL, or continuous darkness, DD).

'Step' or 'pulse' stimuli were presented to the cells at various circadian phases during the free run in LL or DD. For calculation of the period and phase shifts of the circadian clock, the trough or peak of the photoaccumulation rhythm was used as the phase reference point. The data were analysed by least-squares regressions (Johnson and Hastings, 1989; Johnson *et al.* 1989). Circadian phase was defined by 'circadian time' (=CT) as described in Johnson and Hastings (1989) and Johnson *et al.* (1989).

Measurement of membrane potential

Methods for intracellular recording of membrane potential were similar to those described previously (Matsuoka and Nakaoka, 1988). The microelectrodes were filled with 0.1 $mol\,l^{-1}$ KCl and their resistance was about 100–150 MΩ. To immobilize the cells for microelectrode recording, BND-1 cells that had been adapted to the experimental solution were deciliated by washing in 5 % ethanol (Nakaoka *et al.* 1987) and then resuspended in the experimental solution. Membrane potential measurements were made within an hour after deciliation.

Measurement of the duration of ciliary reversal

The duration of ciliary reversal was measured by the method of Naitoh (1968). Cells were transferred in a minimal amount of KCMT medium to 0.5 ml of stimulation medium (32 $mmol\,l^{-1}$ KCl, 1 $mmol\,l^{-1}$ $CaCl_2$, 1 $mmol\,l^{-1}$ Tris–HCl, pH 7.2). The cells were observed with a dissecting microscope and the interval between the initiation of backward swimming and the resumption of forward swimming was timed with a stopwatch. At room temperature, the duration of ciliary reversal ranged from about 3 s for cells from high-K^+ medium to 120 s for cells from low-K^+ medium.

For measurements of the ciliary reversal duration at different circadian or diurnal phases, the continued functioning of the circadian clock was confirmed by testing the mating reactivity rhythm concurrently (Miwa *et al.* 1987).

Results

Response of membrane potential to potassium ions

Although the membrane-potential response of *Paramecium* cells to changes in extracellular potassium level is well documented, most of these studies have been in the species *P. caudatum* (Naitoh and Eckert, 1968; Oka *et al.* 1986; Machemer, 1989). We wanted to be certain that the species we used, *P. bursaria,* responded to potassium as expected. Fig. 1 shows that the resting membrane potential of *P. bursaria* cells is a function of extracellular potassium concentration. Cells from both day and night phases depolarize in high-K^+ medium. The membrane potential in 16 $mmol\,l^{-1}$ K^+ medium is approximately 50 % of that in 2 $mmol\,l^{-1}$ K^+ medium for both day and night cells.

To test whether altered membrane potential affects circadian clock properties, we exposed the cells either to pulses or to step changes of extracellular potassium level (see below). To assess the impact of these treatments, it was necessary to know how long the potassium treatment altered the resting membrane potential or, to put it another way, we needed to know how rapidly the cells adapted to the potassium treatment. Were the

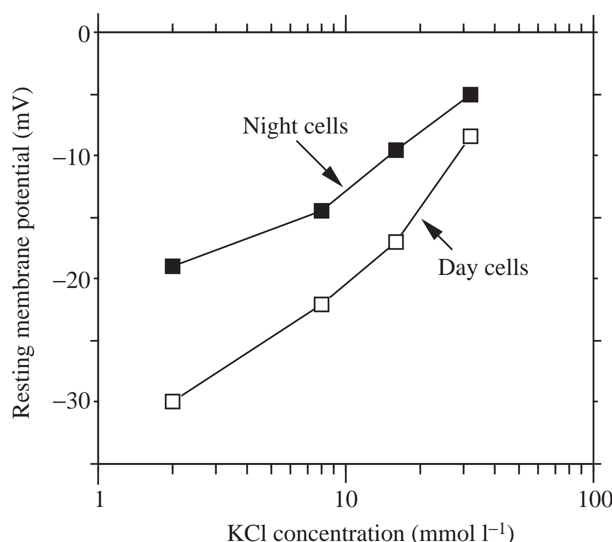

Fig. 1. Resting membrane potential of *Paramecium bursaria* cells (strain BND-1) in medium containing various concentrations of potassium (25 °C). Membrane potential was measured with microelectrodes filled with 0.1 mol l^{-1} KCl (Matsuoka and Nakaoka, 1988). Cells were tested at two different phases of a light/dark cycle: day (between 0 and 6 h after lights-on) and night (between 0 and 6 h after lights-off). Each point is the mean of measurements on 2–3 cells. In addition to potassium as indicated, the medium contained 0.25 mmol l^{-1} $CaCl_2$, 0.5 mmol l^{-1} $MgCl_2$ and 2 mmol l^{-1} Tris–HCl (pH 7.2).

potassium-induced changes in membrane potential only transient or were they long-lived? *P. caudatum* cells are known to adapt to changes in extracellular potassium concentration within about 2 h (Oka *et al.* 1986). As shown in Fig. 2, the response of *P. bursaria* cells to an increase of extracellular potassium level (from 2 to 16 mmol l^{-1}) is initially to depolarize, followed by a rapid adaptation to a new steady-state membrane potential. This new membrane potential (about −13 mV) is depolarized relative to the original potential (about −30 mV). Fortunately for our subsequent analyses, the new steady-state depolarized membrane potential is stable for at least 7 h. This means that the altered membrane potential is probably relatively constant for the duration of the 4–6 h pulses used to perturb the circadian rhythm of photoaccumulation. In the case of step increases of extracellular potassium level, the data of Fig. 2 indicate that the altered potential change may persist for at least 7–8 h.

Corroborative evidence that the membrane potential of our cells was relatively depolarized for a long time after the transfer from low-K^+ medium to high-K^+ medium was obtained using the duration of ciliary reversal as an assay of membrane potential. Consistent with the above observation, measurements of ciliary reversal duration after transfer of cells to 16 mmol l^{-1} potassium showed a short reversal duration (3–8 s) for at least 3–4 h after transfer of the cells to high-K^+ medium (data not shown). The correspondence of these results supports the idea that the duration of ciliary reversal after transfer to solutions containing very high potassium levels (32 mmol l^{-1}) is a reliable, albeit indirect, measure of the initial membrane potential (Naitoh, 1968).

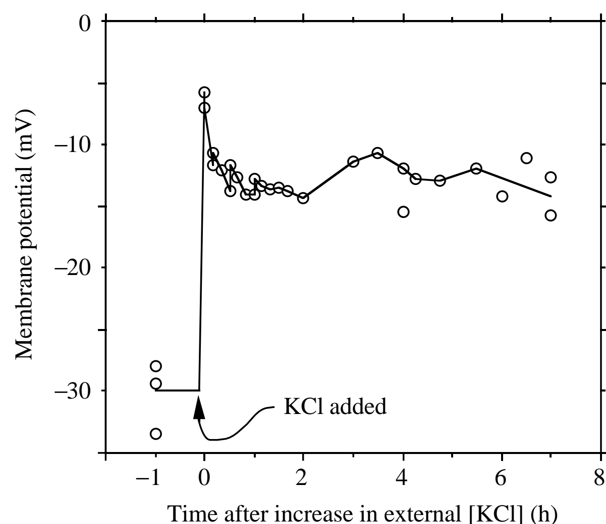

Fig. 2. Response of resting potential of *P. bursaria* cells (BND-1) to extended exposure to 16 mmol l^{-1} potassium medium. Cells which had been adapted to low-K^+ medium (2 mmol l^{-1} KCl, 0.25 mmol l^{-1} $CaCl_2$, 0.5 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} Tris–HCl at pH 7.2) were transferred at time zero to high-K^+ medium (16 mmol l^{-1} KCl, 0.25 mmol l^{-1} $CaCl_2$, 0.5 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} Tris–HCl at pH 7.2). Membrane potential was measured with microelectrodes filled with 0.1 mol l^{-1} KCl as in Fig. 1. The data in this figure are derived from measurements on 16 cells in LD at phases from 0 to 8 h after lights-on (25 °C).

Response of the photoaccumulation rhythm to potassium ions

Fig. 3 shows representative rhythms of cells in LL that have been exposed to ‘step’ changes of extracellular potassium concentration at different circadian phases. No large phase shifts are obvious in these raw data. Indeed, when the data are analysed and phase shifts provoked by the K^+ step are plotted as a function of the circadian time of the step (a phase response curve, or PRC, see Johnson and Hastings, 1989), it is clear that the phase shifts for this experiment are all less than 1.8 h (see PT111 data in Fig. 4A). Fig. 4A depicts the results from five independent K^+ step-up experiments in LL. Despite the scatter of the points, the data do not demonstrate any phase-specific response to K^+ step-ups, although there may be a slight tendency for small phase *advances*, which are independent of the circadian phase of treatment. One experiment in LL with K^+ step-downs is illustrated in Fig. 4B. Again, phase shifts are small and do not depend on the stimulus phase. There may be a tendency towards phase delays, but the data are not extensive enough to be confident of that trend. The change in K^+ concentration (1 or 2 mmol l^{-1} to 16 mmol l^{-1} KCl) should have changed the membrane potential by 10–14 mV, depending upon the time of day (Fig. 1). Preliminary experiments using a smaller change in K^+ concentration (transitions between 2 and 5–10 mmol l^{-1} KCl) also failed to obtain significant phase resetting (data not shown).

Similar experiments performed in DD are summarized in Fig. 5. Seven independent experiments in DD using K^+ step-ups are depicted in Fig. 5A. The scatter of the data points in DD is larger than in LL. This increased scatter is probably because the rhythm

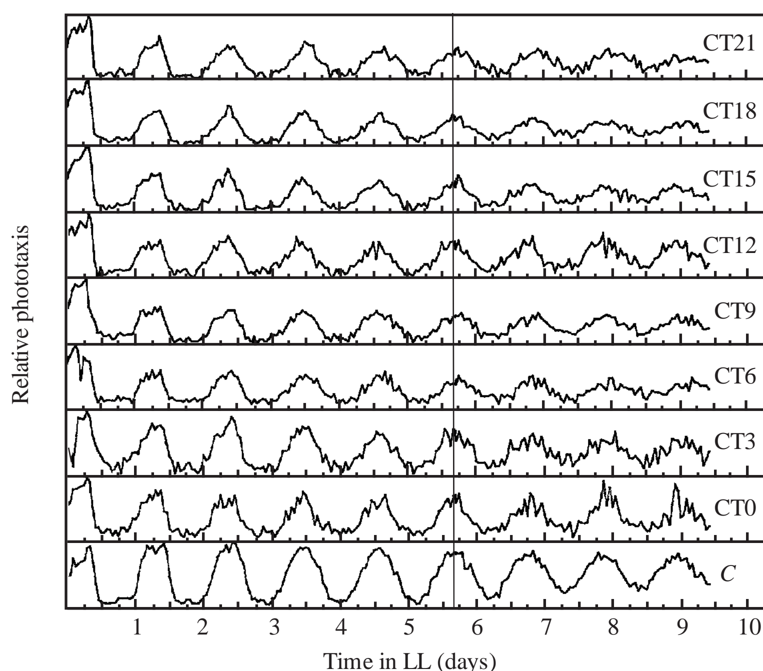

Fig. 3. Photoaccumulation rhythms of *P. bursaria* cells (strain Mit-B) exposed to 'step-up' increases in extracellular potassium concentration ($2\,\mathrm{mmol\,l^{-1}}$ KCl to $16\,\mathrm{mmol\,l^{-1}}$ KCl) at various circadian phases during the first day. The treatments are as follows, starting from the bottom: control (*C*, no KCl step), K^+ steps at CT0, CT3, CT6, CT9, CT12, CT15, CT18 and CT21, where CT is circadian time in hours. These raw data come from experiment PT111.

damps more rapidly in DD (usually within three cycles), so the regression analyses are based on fewer points. Therefore, the phase determination of data obtained from experiments performed in DD is less certain. Nonetheless, the data in DD support the basic conclusion inferred from the data of Fig. 4: K^+ step-ups do not elicit phase-specific phase-shifting in DD, although there is again a slight tendency for phase-independent phase *advances* (Fig. 5A). The scatter of data for K^+ step-downs is even larger than for K^+ step-ups in DD; in this case, there is a tendency for phase advances to predominate in the subjective nighttime (=CT12–CT24, where CT is circadian time in hours; Fig. 5B). We do not consider this tendency to be significant because the scatter of data points is large and because there is no mirror-image delay region in the K^+ step-up phase response (Fig. 5A).

Phase responses are usually measured as responsiveness to pulse stimuli instead of to step stimuli. To be sure that the non-responsiveness of the photoaccumulation rhythm was not due to the type of stimuli we used, we also tested the impact of pulse stimuli. Fig. 6A illustrates the phase-shifting caused by K^+ step-up pulses in LL. As was observed with step stimuli, there is no significant phase-dependency of resetting with pulse stimuli (Fig. 6B shows the controls, cells that had been centrifuged and resuspended in $2\,\mathrm{mmol\,l^{-1}}$ K^+ medium during the test interval). Unlike the step stimuli, pulses did not

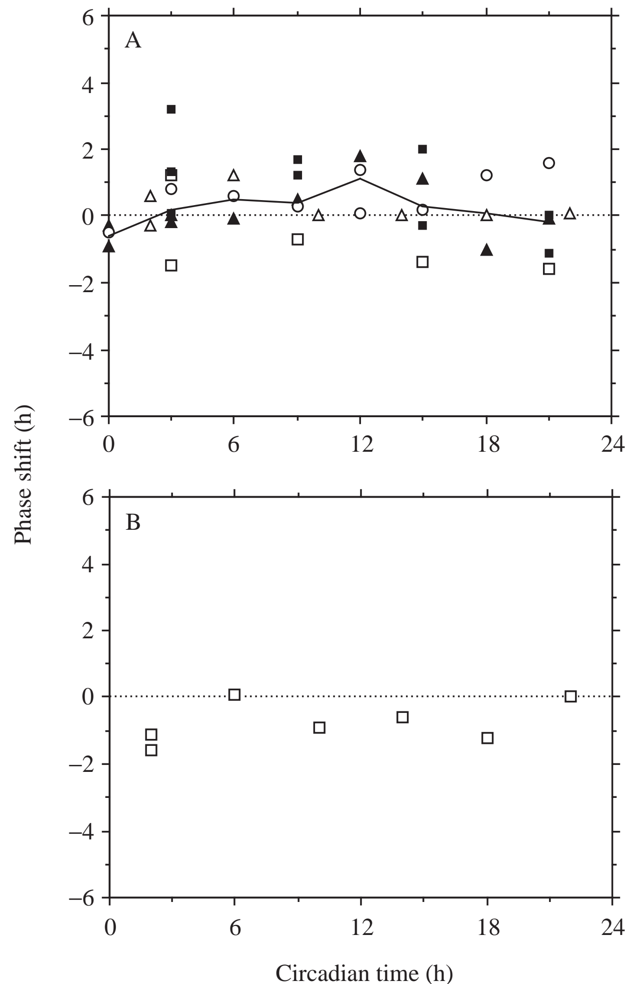

Fig. 4. Phase response summary of $[K^+]$ step data in continuous light (LL). Abscissae, circadian time (=phase) of the $[K^+]$ step; ordinates, phase shift in hours (positive values are advance shifts, negative values are delay shifts). (A) Step-ups of extracellular KCl concentration. The data come from five independent experiments: open squares (2 to 16 mmol l^{-1} steps, strain Kz-1, experiment IM137), filled squares (2 to 16 mmol l^{-1} steps, strain Kz-1, IM136), open triangles (1 to 16 mmol l^{-1} steps, strain Mit-B, PT130), filled triangles (2 to 16 mmol l^{-1} steps, strain Mit-B, PT111) and open circles (2 to 16 mmol l^{-1} steps, strain Mit-B, PT110). The solid line connects the averages of 3 h data pools. (B) Step-downs of extracellular KCl concentration. The data come from one experiment: PT130 (16 to 1 mmol l^{-1} steps, strain Mit-B).

appear to elicit phase-independent *advances* (or *delays*). On the basis of the data of Fig. 2, the membrane probably remained depolarized throughout the high-K^+ pulses.

Endogenous cycles of membrane potential

As noted in the Introduction, the membrane potential of *P. bursaria* cycles in LD. The maximum potential of about −30 mV is achieved at LDT2–LDT4 (where LDT is light/dark

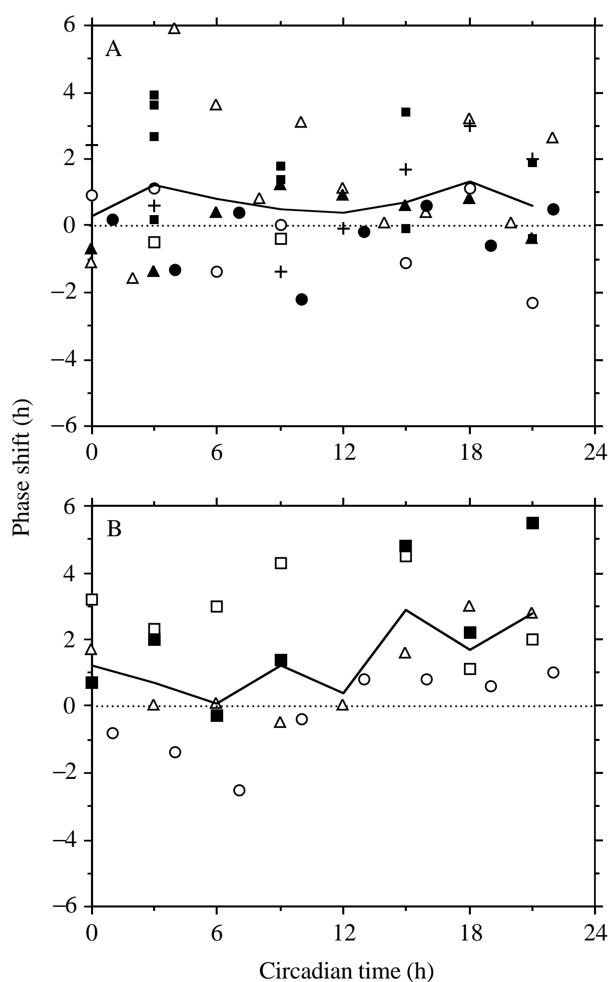

Fig. 5. Phase response summary of [K^+] step data in continuous darkness (DD). Data are plotted as in Fig. 4. (A) Step-ups of extracellular KCl concentration. The data come from seven separate experiments: crosses (1 to 10 $mmol\,l^{-1}$ steps, strain Mit-B, experiment PT68), filled circles (2 to 10 $mmol\,l^{-1}$ steps, strain Mit-B, PT72), open circles (1 to 16 $mmol\,l^{-1}$ steps, strain Mit-B, PT106), filled triangles (1 to 16 $mmol\,l^{-1}$ steps, strain Mit-B, PT108), open triangles (2 to 16 $mmol\,l^{-1}$ steps, strain Mit-B, PT109), filled squares (2 to 16 $mmol\,l^{-1}$ steps, strain T316, IM134) and open squares (2 to 16 $mmol\,l^{-1}$ steps, strain Kz-1, PT138). The solid line connects the averages of 3 h data pools. (B) Step-downs of extracellular KCl concentration. The data come from four independent experiments: open triangles (10 to 1 $mmol\,l^{-1}$ steps, strain Mit-B, PT68), open circles (10 to 2 $mmol\,l^{-1}$ steps, strain Mit-B, PT72), filled squares (16 to 1 $mmol\,l^{-1}$ steps, strain Mit-B, PT106) and open squares (16 to 1 $mmol\,l^{-1}$ steps, strain Mit-B, PT108). The solid line connects the averages of 3 h data pools.

time in hours), while the minimum potential is approximately −25 mV at LDT14–LDT16 (Nakajima and Nakaoka, 1989). Does membrane potential cycle in continuous conditions? We attempted to answer this question by using an indirect gauge of membrane potential, ciliary reversal duration (Naitoh, 1968). In LL, the ciliary reversal duration appeared to

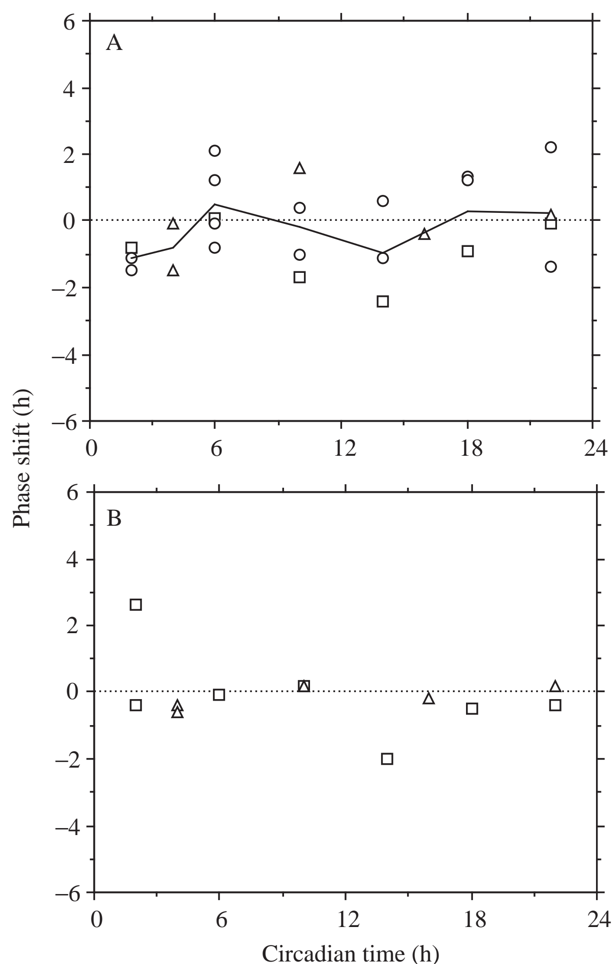

Fig. 6. Phase response summary of [K^+] pulse data in LL. Data are plotted as in Fig. 4; circadian time of the pulse *onset* is plotted on the abscissa. (A) The data come from three separate experiments: squares (6 h pulses of 16 mmol l^{-1} KCl, strain Mit-B, PT60), circles (4 h pulses of 16 mmol l^{-1} KCl, strain Mit-B, PT125) and triangles (6 h pulses of 20 mmol l^{-1} KCl, strain Mit-B, PT127). The cells were in 2 mmol l^{-1} KCl medium (KCMT) before and after the high-K^+ pulse. The solid line connects the averages of 3 h data pools. (B) Controls for pulse experiments. In these experiments, the cells were centrifuged and resuspended exactly as the experimental samples, except that the pulse medium contained 2 mmol l^{-1} KCl. The data come from two separate experiments: squares (6 h pulses, strain Mit-B, PT60) and triangles (6 h pulses, strain Mit-B, PT127).

have a low-amplitude oscillation, which was consistent with the potential measurements of LD cells. Previously published measurements of swimming velocity also suggested that membrane potential oscillates in LL (Nakajima and Nakaoka, 1989).

In DD, however, the ciliary reversal duration decreased monotonically, suggesting a progressive depolarization of the cells in prolonged darkness (data not shown), despite the fact that the circadian clock was operating in these cells (as assayed by the mating

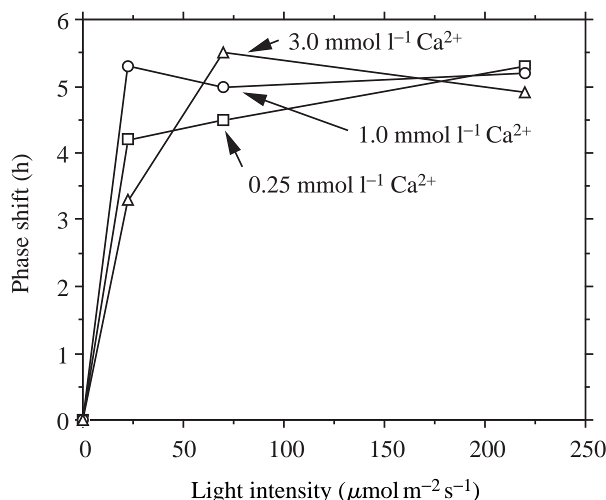

Fig. 7. Magnitude of phase-shifting by light as a function of extracellular calcium concentration. A 4 h light pulse was given at CT19–CT23 to *P. bursaria* cells in DD that had been adapted to KCMT medium (2 mmol l^{-1} KCl) with different concentrations of $CaCl_2$ (squares, 0.25 mmol l^{-1} $CaCl_2$; circles, 1.0 mmol l^{-1} $CaCl_2$; and triangles, 3.0 mmol l^{-1} $CaCl_2$). The light pulse was presented at a variety of intensities between 22 and 220 μmol m^{-2} s (abscissa), and the resulting phase advances of the photoaccumulation rhythm are plotted on the ordinate. The calcium concentrations are the total extracellular calcium concentrations. However, measurements with a Ca^{2+}-selective electrode (World Precision Instruments, New Haven, CT) confirmed that essentially all of the calcium in these solutions was in the free ionic form (experiment PT69; strain Mit-B).

reactivity rhythm). The data suggest that there is no strict linkage between the central mechanism of the circadian clock and rhythmic changes of membrane potential, but that the membrane potential may oscillate in LL as an output of the clock. This conclusion must be tempered, however, with the realization that the LL and DD data are based only on two indirect methods of membrane potential measurement (ciliary reversal duration and swimming velocity), which might be influenced by other factors.

External calcium does not potentiate light-induced phase-shifting

Light pulses reset the phase of circadian oscillators, and the clock of *Paramecium* is no exception (Ehret, 1960; Hasegawa and Tanakadate, 1987; Johnson *et al.* 1989). As noted in the Introduction, light depolarizes the membrane of *P. bursaria* cells and the magnitude of the light-induced depolarization is dependent upon the extracellular concentration of calcium (Nakaoka *et al.* 1987). If light-induced phase-shifting in *P. bursaria* cells were mediated by a similar mechanism to that proposed for *Bulla* (Block *et al.* 1993), then light would open calcium channels in the plasma membrane of *P. bursaria* cells, causing a membrane depolarization and calcium ion influx. If this scenario were correct, we would predict that the magnitude of light-induced phase-shifting in *P. bursaria* cells would be increased by increasing the extracellular calcium concentration.

This prediction is not supported by our data, as illustrated in Fig. 7. Light pulses of various intensities were given at CT19–CT23, a phase at which large phase shifts can be obtained (Johnson *et al.* 1989). As Fig. 7 shows, there is no significant trend in the data, which suggests that changing the extracellular calcium concentration does not modify the magnitude of light-induced phase resetting. The range of calcium concentrations tested in this experiment (0.25–3.0 $mmol\,l^{-1}$) is sufficient to double the membrane depolarization evoked by light (Nakaoka *et al.* 1987). Although it is difficult to eliminate a role for a transmembrane calcium flux in the absence of direct measurements of intracellular free calcium levels, the data indicate that the same criteria used in *Bulla* to implicate transmembrane calcium fluxes in the light-induced phase-shifting pathway do not support a role for calcium fluxes in *P. bursaria.*

Discussion

Paramecium bursaria is an ideal species in which to test whether the membrane potential of a unicellular organism is intrinsically involved in circadian clock function. The membrane potential can be directly measured and it responds to the ionic composition of the medium, so that it can be easily manipulated. The membrane potential of *P. bursaria* cells oscillates in a light/dark cycle and it also depolarizes in response to light pulses. This light-induced depolarization appears to be a result of the influx of calcium ions from the medium into the cells. All of these characteristics are reminiscent of the pacemaker cells of molluscan eyes (Block *et al.* 1993). It therefore seemed natural to test whether circadian clock function in *P. bursaria* cells responds to changes of membrane potential, as do the pacemaker cells of molluscan retinae.

Unfortunately, we found no evidence that the role of membrane potential in the circadian mechanism of molluscan eyes could be generalized to the clockwork of this unicellular organism. We regard the results of the experiments of Figs 4–6 to be negative; none of the phase shifts observed supports the hypothesis of phase-dependent resetting, which indicates that membrane potential does not play an important role in the circadian clockwork. The only possible exception to this conclusion are the data of Fig. 5B, where there appear to be phase-dependent advances in the subjective nighttime for the experiments in DD. This is probably not significant because there is no similar trend in the data from LL (Fig. 4B). Furthermore, we might expect that $[K^+]$ step-ups and $[K^+]$ step-downs should phase-shift the clock in opposite directions, and there is no evidence of delays in the subjective nighttime in Fig. 5A. The most important reason to believe that the inconsistent phase-shifting patterns depicted in Figs 4–6 are not physiologically relevant, however, is based on the realization that the membrane potential changes caused by our treatments are large compared with the natural daily changes of membrane potential in *P. bursaria*. In particular, changing the extracellular potassium levels between 2 and 16 $mmol\,l^{-1}$ will change the membrane potential by 10–20 mV, about 50 % of the total membrane potential (Fig. 1). But the diurnal change of membrane potential in *P. bursaria* cells in LD is only about 5 mV, less than 20 % of the total membrane potential (Nakajima and Nakaoka, 1989). Moreover, light pulses depolarize the cell membrane by up to 5 mV (Nakaoka *et al.* 1987), but can cause potent phase-

shifting (Johnson *et al.* 1989). If the clock of *P. bursaria* behaved like that of *Bulla* (Block *et al.* 1993), there should be a closer correlation between the magnitude of membrane potential changes and the magnitude of phase resetting. Clearly, there must be more to the story in *P. bursaria* than membrane potential.

It is possible that the tendency towards phase-independent resetting seen in Figs 4 and 5 is statistically significant. Nevertheless, because the responses are not a function of the phase of the step stimuli, it is likely that this is a rather non-specific effect which provides no information about the clock mechanism itself. Moreover, the insensitivity of light-induced phase-shifting of *P. bursaria* cells to the extracellular concentration of calcium is inconsistent with the results obtained with *Bulla* neurones (McMahon and Block, 1987*a*; Khalsa and Block, 1988). Therefore, we conclude that the clockwork model based on molluscan retinae (Block *et al.* 1993) cannot be extrapolated to the circadian mechanism of *P. bursaria* cells. Of course, the model based on molluscan retinae may be valid in other organisms, particularly for neural systems that involve intercellular communication. Nevertheless, the *P. bursaria* data argue that membrane potential changes are not a necessarily intrinsic component of circadian organization at the cellular level.

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