The Potassium Content of *Gonyaulax polyedra* and Phase Changes in the Circadian Rhythm of Stimulated Bioluminescence by Short Exposures to Ethanol and Valinomycin

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ABSTRACT

A circadian rhythm in the intracellular level of K⁺ in *Gonyaulax polyedra* is reported. When axenic cultures of *Gonyaulax* in continuous light (60–75 foot candles) are exposed for 4 hours to 0.1 or 0.2% ethanol, the subsequent free-running rhythm in stimulated bioluminescence is phase-shifted, the amount and direction of the shift being dependent on the time in the circadian cycle when cells are treated. The phase-response curve for ethanol closely resembles that for light in similarly maintained cells. When valinomycin (0.1 or 0.2 µg ml⁻¹) is present in addition to ethanol, the phase of the bioluminescence rhythm is returned to that of an untreated cell suspension. Valinomycin thus negates the effect of ethanol on phase. The intracellular K⁺ level immediately after treatment of a cell suspension for 4 hours with ethanol (0.1%) is about half that of untreated cells. If valinomycin (0.1 µg ml⁻¹) is also present during the 4-hour treatment, the intracellular K⁺ is only slightly lower than in untreated cells. Increasing the external concentration of K⁺ or Na⁺ for 4 hours has no effect on the rhythm of stimulated bioluminescence. These results are interpreted as support for the hypothesis that the mechanism by which circadian oscillations are generated involves changes in membrane properties.

A number of models for the generation of circadian oscillations have been proposed, but none have proven very satisfactory. The most explicit recent hypothesis is the chronon theory (8), which postulates that oscillations arise from the repeated sequential transcription of a portion of the cellular DNA, presumably composed of genes controlling the various processes which show rhythmicity. This model suffers from the fact that it predicts a severe disruption of rhythmicity by inhibitors of transcription; although effects of such substances on rhythms can be observed (12, 21, 22), they are not always present (31, 36, 37). For example, actinomycin D inhibits the glow rhythm in *Gonyaulax* but not the photosynthetic rhythm (21). Such an effect could be due to uncoupling the glow from the clock or inhibiting the glow. Furthermore, it has not been possible to demonstrate cyclic synthesis and degradation of the enzymes concerned in the various rhythmic processes such as luciferase in *Gonyaulax* (25). Recently, it has been suggested that an os-

cillation of the physical properties or permeability of membranes may mediate the observed rhythms (6, 9, 11). Support for such a hypothesis cites the phase changes brought about in *Phaseolus* by short exposures to substances which can interact with membranes such as alcohol (3, D₂O (4), or valinomycin (6), or by wilting for short times (5). Exposure of *Phaseolus* plants to ethanol, methanol (23), and D₂O (4) increases the length of the free-running period of the leaf movement rhythm. Similar effects of ethanol and D₂O on the period of the tidal rhythm in activity in the isopod *Excirolana* have also been reported (9, 10). The rhythm firing of the optic nerve in the excised eye of *Aplysia* can be phase-shifted by short exposures to higher than normal concentrations of K⁺ (11). The rhythm opening and closing of the leaflets of *Albizia* are mediated by movement of K⁺ (30). All these effects could be understood as temporary cyclic alterations in membrane properties, although no direct measurements of such changes have been reported.

Because the unciallar marine dinoflagellate *Gonyaulax polyedra* is known to show four circadian rhythms, in stimulated bioluminescence, in bioluminescent glow, in photosynthetic capacity, and in cell division (33), and because these rhythms persist for many cycles in continuous light and constant temperature, it is a particularly favorable test object for examining the role of membrane changes in circadian rhythmicity. Since valinomycin at low concentrations is known to form a complex with K⁺, enhancing its transport through biological membranes (14, 27), its effects on rhythms are especially interesting. This paper reports experiments in which cell suspensions of *Gonyaulax* in continuous light were exposed for short times to ethanol and valinomycin, and the rhythm in stimulated bioluminescence was subsequently examined for phase shifts. Determinations of intracellular potassium levels at different times in the circadian cycle are also reported.

MATERIALS AND METHODS

Cultures of a strain of *Gonyaulax polyedra* Stein isolated at Scripps Institution of Oceanography in 1960 were used in these experiments. In all cases where cells were exposed to ethanol, axenic cultures of this strain purified by Dr. R. R. L. Guillard and designated "*Gonyaulax 60P*" were employed, because, if bacteria were present, they used ethanol as a carbon source, multiplied excessively, and killed the *Gonyaulax*. The medium for cultures with bacteria was that employed previously (35), in which sea water was diluted to 75% salinity and enriched with nitrate, phosphate, iron, EDTA, and soil extract. For axenic cultures, "f" medium (15) with half-strength nutrients and the addition of 2% v/v soil extract was used. Cultures were grown to stationary phase (cell density of 5,000–10,000 cells ml⁻¹) in alternating light (300 ft-c) and darkness, each of

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12 hr duration at 22 C. All light sources were cool white fluorescent lamps.

For experiments, cells were transferred to continuous light (60–75 ft-c, 22 C) at the end of a light period. In one experiment, a large sample of “red tide,” composed of more than 99.5% Gonyaulax polyedra, was collected at night from Santa Barbara Harbor, divided into liter aliquots, and transferred to continuous light at 0700 the next morning. Liter samples were harvested at intervals over 2 days for the determination of intracellular potassium.

In experiments where the effects of valinomycin were to be observed, 100-ml samples were withdrawn from a culture of Gonyaulax in continuous light, and valinomycin dissolved in 95% ethanol was added. The concentration of ethanol in the cell suspension was 0.1 or 0.2% v/v. Four hours later, cells were sedimented by centrifugation at 30g for 30 sec in an International clinical centrifuge, the medium containing valinomycin was decanted, and the cells were resuspended in medium without additive. Two-milliliter aliquots were then pipetted into sterile shell vials and replaced in continuous light for the measurement of stimulated luminescence. Ethanol at the same concentration as that present in the valinomycin was added to another sample of the culture and removed in the same manner as valinomycin. A third sample of the cell suspension without the addition of either ethanol or valinomycin was centrifuged and resuspended as a further control. Experiments were repeated at least twice.

Experiments were carried out at different circadian times after the cell suspensions were transferred to continuous light at 1200 circadian time. The time of the 4-hr exposure is given according to the convention of a circadian time scale of 24 hr, in which 0 hr is taken as biological dawn (25). In one experiment, the effects of continuous exposure to ethanol and valinomycin were also examined.

The bioluminescence of aliquots was measured by stimulating a fresh sample of the cell suspension in a photomultiplier photometer previously described (35). Mechanical stimulation was provided by a small motor-driven stirrer like that employed by Hamman and Seliger (16) and was continued for 1 min. The total light emitted from each of two or three samples was recorded at 3-hr intervals over three or four cycles of the rhythm in stimulated luminescence. Valinomycin (mol wt 1111.3) was obtained from Cal Biochemical Co.

Experiments in which the sodium or potassium concentration in the cell suspension was increased by 100 mM by the addition of NaCl or KCl, and the medium replaced after 4 hr, were carried out as described for ethanol and valinomycin.

For the determination of the potassium content of Gonyaulax, cells were harvested by filtration on a No. 1 filter, 25 cm in diameter, which had been prewashed three times in double-distilled water. Cells were washed from the filter with double-distilled water and were extracted by freezing and thawing and grinding in a glass homogenizer. Cell debris was removed by centrifugation, and the K+ and Na+ in the supernatant were determined with an Eppendorf flame photometer. The extent of contamination by the ions from the medium was estimated by adding 14C-inulin (0.5 μCi) to the cell suspension just before harvesting, and determining the radioactivity of both the original cell suspension and the supernatant after extraction, in a Packard scintillation counter in 5 ml of POPPOP in toluene. The protein content of the extract was determined by the Lowry procedure (24), after hydrolyzing the extract and the bovine serum albumin used as a standard in 0.5 N NaOH overnight at room temperature. In some experiments, the packed cell volume was estimated by centrifugation in calibrated tubes. The standard error for the determinations of umoles K+ per mg protein by this procedure was ± 3.5%.

### RESULTS

If the circadian rhythms that are observed in Gonyaulax have a common origin in cyclic differences in membrane properties, then a rhythm in intracellular ion levels might be expected. A preliminary examination of the potassium concentration in Gonyaulax cells showed changes over the course of the environmental light-dark cycle, cells containing about twice as much K+ at the end of the light period as at its beginning (Table I). Since such a change could be the result of ion accumulation in photosynthesis during the light period, measurements of the ion content in cells in continuous light were made. An intensity was chosen where the circadian rhythm of photosynthesis is not expressed, so that the rate of photosynthesis was constant over time (32). These measurements also showed a maximum in intracellular potassium at the end of the biological day phase, at 12 hr circadian time (Table II). Two cycles in potassium content were observed in the “red tide” sample in continuous light (Table III). Thus, differences in K+ persist in continuous light and represent another circadian rhythm in Gonyaulax. While a rhythm in intracellular K+ is consistent with the hypothesis that the overt rhythms in Gonyaulax have a common origin in cyclic membrane permeability, it does not constitute proof of such an hypothesis.

#### Table I. Intracellular Cation Concentration of Gonyaulax polyedra at Different Times in a Light-Dark Cycle

<table>
<thead>
<tr>
<th>Ion</th>
<th>Medium</th>
<th>Circadian Time Cells Sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 hr</td>
</tr>
<tr>
<td>K+</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td>Na+</td>
<td>315</td>
<td>54</td>
</tr>
<tr>
<td>Mg2+</td>
<td>51</td>
<td>24</td>
</tr>
</tbody>
</table>

#### Table II. Intracellular Potassium Ion Concentration of Gonyaulax polyedra at Different Times in Continuous Light

A culture was divided and half (No. 2) was rephased by 180°, so that all sample preparation could be carried out during a 12-hr time space. The concentration of K+ in the medium was 12 mM. The light intensity was 75 ft-c, and the temperature was 22 C.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Circadian Time Cells Sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr</td>
</tr>
<tr>
<td>Na+</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>

#### Table III. Intracellular Potassium Ion Concentration of a Gonyaulax "Red Tide" at Different Times after Transfer to Continuous Light at Circadian Time 0

The concentration of K+ in the sea water was 10 mM. The temperature was 22 C, and the light intensity was 75 ft-c.

<table>
<thead>
<tr>
<th>Time Cells Sampled after Transfer to Continuous Light (hr)</th>
<th>8</th>
<th>11</th>
<th>14</th>
<th>17</th>
<th>26</th>
<th>31</th>
<th>36</th>
<th>39</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration K+ (nm)</td>
<td>23</td>
<td>23</td>
<td>21</td>
<td>21</td>
<td>17</td>
<td>19</td>
<td>20</td>
<td>14</td>
</tr>
<tr>
<td>umoles/mg protein</td>
<td>4.4</td>
<td>5.0</td>
<td>4.3</td>
<td>3.9</td>
<td>3.2</td>
<td>4.9</td>
<td>5.2</td>
<td>3.7</td>
</tr>
</tbody>
</table>
Gonyaulax does not contain a large central vacuole, and cells examined by electron microscopy (34) are seen to consist largely of membrane-bound organelles including the large nucleus, chloroplasts, mitochondria, trichocysts, and vesicles of unknown nature. The total intracellular potassium is only slightly higher than that in the medium, but the concentration within small vacuoles could be much higher, and could change by a greater fraction over a cycle than the total potassium. Thus nothing can be said at present concerning the accumulation of K⁺ in Gonyaulax. Although measurements of Na⁺ and Mg²⁺ in extracts were made, the contamination of the extracts with the medium, which contain high concentrations of these ions, prevented accurate determinations of intracellular levels.

Increasing the potassium or the sodium content of the medium by 100 mM for 4 hr had no effect on the phase of the rhythm in stimulated bioluminescence in Gonyaulax, irrespective of the time in the cycle when the high ion pulse occurred. However, Gonyaulax can tolerate rather large changes in the salinity of the medium, and did not lose motility on the addition of either ion, so it is likely that this cell is able to regulate the internal ion concentration irrespective of the external medium.

If a change in the permeability to potassium is a component of the process by which oscillations are generated, then artificially altering the intracellular potassium would be expected to alter the phase of the observable rhythms. Valinomycin is known to change the permeability of many biological membranes to potassium (27). Therefore, cells were exposed for short times to valinomycin and the rhythm of stimulated bioluminescence was measured over several cycles in continuous light. Since valinomycin is relatively insoluble in water, stock solutions were made up in ethanol and ethanol controls were included in all experiments. Phase shifts were observed in cells treated with valinomycin, as compared with ethanol controls, and the amount and direction of phase shift depended on the circadian time of exposure, confirming the findings of Bünning and Moser (6) in Phaseolus. However, considerable phase shifts occurred in the ethanol controls, as compared with the luminescence rhythm in cells which had merely been centrifuged and resuspended in fresh medium. Changing the medium had no effect on phase, confirming previous experience (18). Phase shifts with ethanol also depended on the time when the cells were treated. For example, exposing a Gonyaulax cell suspension to 0.1% ethanol for 4 hr between 12 and 16 hr circadian time resulted in a 3-hr phase delay in the bioluminescent rhythm (Fig. 1). The presence of valinomycin (0.1 µg ml⁻¹) in addition to ethanol (0.1%) returned the phase to that of the control, in which the medium was replaced but no additions were made. Thus, as compared with the ethanol control, valinomycin-treated cells were phase-advanced by 3 hr. Given later in the circadian cycle, ethanol alone advanced rather than delayed phase (Fig. 2), and again valinomycin reversed this effect, bringing the phase back to that of the untreated control. Neither valinomycin nor ethanol had any effect on the phase of the rhythm in bioluminescence between 5 and 9 hr circadian time, the biological day phase. The time-dependent phase changes caused by 4-hr exposures to ethanol are summarized in Figure 3A, a phase-response curve for ethanol. A similar phase-response curve for the effect of pulses of valinomycin as compared to those of ethanol alone is given in Figure 3B. A comparison of these curves shows that they are mirror images of each other and documents the opinion that valinomycin reverses the phase changes expected from the ethanol in which it is dissolved.

The effects of 0.2% ethanol were indistinguishable from those of 0.1% ethanol and were reversed by 0.2 µg ml⁻¹ valinomycin. A range of valinomycin concentrations from 0.02 to 10 µg ml⁻¹ was assayed for phase changes. Valinomycin at 0.25 µg ml⁻¹ or more damaged cells so extensively that a rhythm in bioluminescence could not be measured. The effects on phase were concentration-dependent in the concentration range 0.05 to 0.2 µg ml⁻¹ (Table IV).

The intracellular K⁺ of cells extracted at the end of a 4-hr exposure to ethanol from 12 to 16 hr circadian time was about half that of the untreated control cells, while the K⁺ content of cells exposed to valinomycin in ethanol was only slightly lower than that of the untreated control (Table V). Thus valinomycin partially reverses the effect of ethanol on intracellular K⁺. It is interesting to note that the greatest phase delay after ethanol treatment occurs at about 12 hr circadian time, at the peak of the rhythm in intracellular K⁺ in untreated cells.

Gonyaulax cell suspensions continuously exposed to valinomycin (0.2 µg ml⁻¹) in 0.2% ethanol showed no rhythm in bioluminescence over 3 days in continuous light. The level of luminescence was high and the cells were motile during this time. This observation requires repetition, however.

**DISCUSSION**

The phase of all circadian rhythms that have been examined in this respect is altered by short exposures to light. The amount and direction of these phase changes is dependent on when in the circadian cycle the cells are exposed to light and may be
firing of the optic nerve is phase-shifted, and the amount and direction of the change in phase is dependent on the time of the high K⁺ pulse. It is also interesting in this connection that a circadian rhythm in intracellular K⁺ can be observed in Gonyaulax. Since the results of experiments with valinomycin in Phaseolus and increased K⁺ in Aplysia have been plotted on coordinates of time and phase shift different from each other and from those used in this paper, the data from these experiments have been replotted (Fig. 4), to allow a comparison between them, and with the data from Gonyaulax. Phase changes with increased potassium levels in Aplysia follow a pattern quite similar to the phase response curve for valinomycin represented by a phase response curve (28). Many attempts have been made to duplicate this phase-response curve by treating organisms with various biologically active substances for short times, with the hope of identifying components of the biochemical processes by which oscillations are generated. Until recently, these have been remarkably unsuccessful. Most metabolic inhibitors are ineffective in this respect (2, 18), although some appear to render biological timekeeping more erratic than usual. Specific inhibitors of RNA and protein synthesis likewise fail to duplicate the effects of light on phase (21, 22). Of a long list of substances assayed for phase-shifting in the Gonyaulax glow rhythm (18), only arsenite appeared to delay phase somewhat. More recently, however, some success in experiments of this type has been achieved. A high concentration of ethanol (25%) given to shoots of Phaseolus via the transpiration stream was reported to delay phase by 3 hr if given for 4 to 5 hr some time after the middle of the biological night phase (3). Temporary deuteration of Phaseolus is followed by transient cycles in leaf movement, but stable phase delays are also obtained (4). In the Euglena phototactic rhythm also, exposure for 24 hr or more to D₂O is followed by phase delays (1). Unfortunately, a complete phase-response curve for ethanol or deuterium pulses cannot be constructed for either Phaseolus or Euglena from the published data. Phaseolus plants allowed to wilt for 6 hr responded with phase changes in the leaf movement rhythm, the nature of the response depending on when wilting occurred (5).

In Phaseolus, valinomycin appears to be one of the most successful substances in imitating the phase changes caused by light (6). The effectiveness of this substance, both in Phaseolus and in Gonyaulax, suggests the implication of K⁺ in the generation of rhythmicity. This is supported by experiments with a circadian rhythm in a very different system, the isolated Aplysia eye (11). When the level of K⁺ in the medium surrounding this tissue is temporarily increased, the rhythm in the

**Fig. 2.** Effect of exposure of Gonyaulax polyedra strain 60P to ethanol and valinomycin for 4 hr at 20 to 24 hr circadian time on the phase of the rhythm in stimulated bioluminescence in continuous light (60 ft-c.) at 22 C. Data points are averages of total light emitted by two or three 2-ml samples of cell suspension. Luminescence of cells exposed to 0.1 μg ml⁻¹ valinomycin and 0.1% ethanol (---X--); luminescence of cells exposed to 0.1% ethanol (---Δ--); luminescence of cells resuspended in new medium without other treatment (---○--). The time of maximum luminescence is shown by arrow.

**Fig. 3.** Phase-response curves for the effects on the phase of the circadian rhythm in stimulated bioluminescence in Gonyaulax polyedra strain 60P brought about by 4-hr exposures to 0.1% ethanol (A) and to 0.1 μg ml⁻¹ valinomycin plus 0.1% ethanol calculated with reference to ethanol only (B). Note that phase advances are considered positive in sign and are plotted upward from zero on the ordinate. The abscissa is circadian time, using the convention that dawn equals 0 hr. The time of the dark period of the previous entraining light-dark cycle is shown as a shaded bar on the abscissa. Cells in all experiments were in 60 to 75 ft-c. continuous light at 22 C.
pulses in the leaf movement rhythm in Phaseolus. Although the change from delay to advance is more sudden in the *Aplysia* data, the maximum phase advance occurs in both systems at 24 hr circadian time. The phase-response curve for ethanol in *Gonyaulax* (Fig. 3A) is also similar in general form, and shows a maximum phase advance at 24 hr circadian time. This similarity suggests that all three effects share a common mechanism, possibly a transitory change in intracellular K+.

In *Gonyaulax*, valinomycin clearly negates the effect of ethanol alone, both in phase shifting the rhythm of stimulated bioluminescence and in changing the total intracellular K+. Both ethanol and valinomycin would be expected to exert effects on the structure and permeability of biological membranes. Ethanol is a lipid solvent and has been shown to stabilize red blood cell membranes against hemolysis (20, 26). Valinomycin is known to act as an ionophore for K+ and has recently been found to substitute for the K+ requirement of a membrane-bound K+ and Na+-dependent ATPase active in ion transport (19). The biochemical basis for the opposite effect of ethanol and valinomycin in *Gonyaulax* awaits further experimentation on isolated membrane systems. Spin-label and other such studies are in progress in this laboratory.

In *Gonyaulax*, the phase-response curve for the effect of short exposures to dilute ethanol mimics that to light reasonably closely. Phase changes are of about the same magnitude as those produced by bright light pulses in cells in continuous light (7). The effects of both light and ethanol pulses change from a phase delay to a phase advance in the early part of the biological night phase (7). Exposure to light or to ethanol in the middle of the biological day phase has no effect on the phase of the subsequent rhythm in stimulated bioluminescence. Light has been found to alter the electrical properties of *Acetabularia* (13, 17, 29). Thus it is possible that the phase-shifting effects of light may be membrane-mediated.

**Table IV. Phase Shifts after Treatment of Gonyaulax polyedra Strain 60P by Valinomycin**

The organism was treated for 4 hr with different concentrations of valinomycin from 8 to 12, or from 12 to 16 hr circadian time. Phase shifts are calculated relative to the ethanol control.

<table>
<thead>
<tr>
<th>Valinomycin Conc</th>
<th>Phase Shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg ml⁻¹</td>
<td>at 8-12 c.t.</td>
</tr>
<tr>
<td>0.02</td>
<td>0</td>
</tr>
<tr>
<td>0.05</td>
<td>3</td>
</tr>
<tr>
<td>0.1</td>
<td>3</td>
</tr>
<tr>
<td>0.2</td>
<td>3.8</td>
</tr>
<tr>
<td>0.25</td>
<td>3</td>
</tr>
</tbody>
</table>

1 Circadian time.

**Table V. Intracellular Potassium Concentration of Gonyaulax polyedra Strain 60P after a 4-hr Exposure to Ethanol or to Valinomycin and Ethanol from 12 to 16 hr Circadian Time**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>K⁺ in</th>
<th>Valinomycin + ethanol (0.1 µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmole/mg protein</td>
<td>12-16 c.t.</td>
</tr>
<tr>
<td>Untreated control</td>
<td>Ethanol (0.1%)</td>
<td>12-16 c.t.</td>
</tr>
<tr>
<td>I</td>
<td>3.0</td>
<td>1.6</td>
</tr>
<tr>
<td>II</td>
<td>3.3</td>
<td>1.8</td>
</tr>
</tbody>
</table>

**Fig. 4. Phase-response curves for the effect of 0.1 µg ml⁻¹ valinomycin in 0.1% ethanol on the phase of the leaf movement rhythm in *Phaseolus coccineus* calculated with reference to the ethanol control, from Bünning and Moser (6), (––––); and for the effects of high potassium ion (100 mM) pulses on the phase of the rhythm in firing rate of the optic nerve of isolated eyes of *Aplysia California*, from Eskin (11) (–X–). The data have been replotted so that circadian time 0 is dawn in both cases and phase advances are plotted upward. The time of the dark period of the previous entraining light-dark cycle is shown as a shaded bar on the abscissa.**

The phase and the period of a circadian rhythm are properties of the underlying oscillator, and are the only measurable features which can be unequivocally assigned to this oscillator at present, as distinct from the physiological processes which this oscillator controls. Substances which affect phase or period are thus indicators of the nature of this oscillator. The findings that the circadian rhythm in stimulated bioluminescence in *Gonyaulax* is phase-shifted by ethanol, that this phase shift is reversed by the presence of valinomycin in low concentrations, and that the intracellular K+ in untreated cells shows a circadian rhythm, strengthen the hypothesis that the mechanism by which circadian rhythms are generated involves an oscillation in the physical properties of biological membranes, or intracellular ion concentration.

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**LITERATURE CITED**