Shifting the Phase of the Circadian Rhythm in Bioluminescence in *Gonyaulax* with Vanillic Acid[1]

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ABSTRACT

Exposure for 4 hours to vanillic acid (4-hydroxy 3-methoxy benzoic acid) caused large delay phase shifts (5 to 6 hours) in the circadian rhythm of bioluminescence in *Gonyaulax polyedra*, when assayed at either 10 to 14 circadian time or 22 to 02 circadian time in constant light and temperature, provided that the pH of the medium was 7.1 or lower. Corresponding changes in the pH with acetic acid did not shift phase. Vanillic acid caused detectable depolarization of the membranes of *Gonyaulax*, as demonstrated with the cyanine dye fluorescence technique.

Circadian rhythms in a wide variety of physiological processes are now known to be of common occurrence in both plants and animals (2, 7, 11, 16). While it is generally accepted that these oscillations are generated intracellularly, the biochemical mechanism has so far proven elusive. It is clear, however, that the biochemistry associated with the physiological processes by which circadian rhythms can be detected, *e.g.* photosynthesis and bioluminescence, is not responsible for generating the information regarding either the phase or period of the rhythmicity. Many biochemical rhythms have been measured in various cells, but it has proven difficult to determine whether any of these is the driving oscillator or pacemaker. The basic oscillator can theoretically be distinguished from the processes that it controls by the observation that a short perturbation in its biochemistry will result in a phase change, while a long lasting alteration will change the period. Perturbations in driven rhythmic processes may alter the amplitude of the rhythm but do not change period or phase. The study of phase-shifting after short perturbations has several advantages over the detection of period changes. Large phase changes have been observed following short exposures to bright light or altered temperature, while period changes are usually small. Cells can tolerate short exposures to chemicals that are toxic if present over long times. Phase changes following short exposure to chemical agents of which the specific metabolic effects are known and can be verified in the experimental organism under study are thus perhaps the best available indicators of the nature of the circadian oscillator.

Recent evidence has accumulated suggesting that the oscillator generating circadian rhythms is a feedback loop setting up a limit cycle (12, 13, 14, 22), and that the components of this loop include membrane properties and ion gradients (11, 13, 17, 19, 21), although the exact nature of the membrane changes and the identification of the ions involved have not yet been established. In

the marine dinoflagellate, *Gonyaulax polyedra*, circadian changes in membrane potential (1) and phase changes following short exposures to the K^+ ionophore, valinomycin (18), point to the electrical properties of membranes as important components of the circadian oscillator. If this is true, then chemical agents that depolarize membranes would be predicted to alter phase. Pulses of potassium that depolarize the membranes of the optic nerve of *Aplysia* have been found to shift the phase of the circadian rhythm in spontaneous nerve activity (3).

We report here the results of phase-shifting experiments with *Gonyaulax* using the phenolic acid derivative, vanillic acid (4-hydroxy 3-methoxy benzoic acid). Phenolic acids have been observed to depolarize the membranes of barley root cells and alter ion uptake in these and other cells (4). We report evidence that vanillic acid also depolarizes the membranes in *Gonyaulax* and produces large and reproducible phase shifts in the circadian rhythm of bioluminescence in these cells. Preliminary experiments showed that of a number of benzoic acid derivatives tested, including salicylic, 4-hydroxy cinnamic, and 4-hydroxy 3-methoxy cinnamic acids, vanillic acid was the most effective, so this acid was examined in detail. Our results extend to a single-celled organism the observation that depolarization of the membrane results in phase changes and strengthens the membrane hypothesis for the generation of circadian oscillations.

MATERIALS AND METHODS

Cultures of *G. polyedra*, strain 70A, a clone isolated by one of us (B. S.) from a red tide off Santa Barbara in 1970, were grown in an enriched seawater medium (Guillard's f/2 with silicon omitted [5]), on a 12-hr light, 12-hr dark cycle at 22 C. Cell suspensions were transferred to continuous light (200 μw cm^{-2} at 21 C) at the beginning of a normal light cycle. At this time, the cells were in the stationary phase of growth, about 15,000 cells ml^{-1}, and showed a strong circadian rhythm in bioluminescence.

A saturated solution of vanillic acid, obtained from the Aldrich Chemical Company, Milwaukee, Wis., was made up in double-distilled H_2O. Aliquots from this stock solution were added to the cell suspension to give a final concentration of 0.5 to 3 mM vanillic acid at either 10 or 22 hr after the cells had been transferred to continuous light. An equivalent amount of water was added to control cell suspensions. After the cells had been exposed to vanillic acid for 4 hr, they were sedimented by centrifugation at low speed in an International Clinical centrifuge at room temperature and resuspended in fresh medium without added vanillic acid. Two-ml aliquots were pipetted to glass shell vials for the measurement of bioluminescence and returned to constant light for 2 to 3 days to recover. Measurements of bioluminescence were then made at 3-hr intervals for 2 additional days. Bioluminescence was stimulated by the addition of acetic acid (final concentration 5 mM) and the resulting bioluminescence was recorded with a photomultiplier photometer as described previously (20). The

TABLE I

Phase shifts of the circadian rhythm in bioluminescence in Gonyaulax polyedra following a four-hour exposure to vanillic acid at the end of the day phase (10-14 c.t.), at the beginning (12-16 c.t.), and the end (22-02 c.t.) of the night phase in continuous light (200 W cm^{-2}) at 21°C. The standard error follows the phase shift in hours. The number of determinations for each point is in parentheses. * indicates that the pH was estimated from the addition of vanillic acid to a cell suspension at a later date. Delays in phase are negative.

Vanillic Acid Concentration (mM)	pH (Final)	Time of Treatment (c.t.)		
		10-14 Hours	12-16 Hours	22-02 Hours
0.5	7.2*	-	0(2)	0(2)
1.0	6.3	-1.8 ± 0.5(2)	-	-
	6.8*	-2(1)	-2.8 ± 0.3(2)	-0.5 ± 0(2)
2.0	6.3	-5.8 ± 1.3(2)	-	-4.8 ± 0.7(6)
	7.1	-	-	-6.0 ± 1(2)
	7.5	-1.8 ± 0.8(2)	-	-
	7.9	0(2)	-	0(2)
3.0	5.6*	-10(1)	-	-3.8 ± 1.1(4)

output of the photomultiplier was calibrated in quanta by the use of a ^{14}C standard solution in scintillation fluid (8).

Phase shifts were calculated from the time of the maximum in bioluminescence of the treated as compared to the control cell suspensions. Maxima that appeared *later* in the treated than in the control cell suspensions were interpreted as delays (negative phase shifts), according to the usual practice. Circadian time was calculated from the beginning of the day phase (00 c.t.)[2] according to Pittendrigh (15).

For the detection of changes in membrane potential, *Gonyaulax* cells were sedimented by gentle centrifugation and resuspended in 100 mM NaCl at pH 7.8. A 2-ml aliquot of the concentrated cell suspension (100,000 cells ml^{-1}) was placed in a cuvette and the cyanine dye diS C$_3$ (5) (final concentration 2.4 × 10^{-6} M) was added. Fluorescence was excited at 622 nm and measured at 670 nm, using an Aminco-Bowman spectrofluorimeter with a xenon light source and a Beckman X-Y recorder, model 814A. A few min were allowed for the fluorescence to reach a steady value, then vanillic acid was added (final concentration 2 mM). The pH was adjusted to the desired value by the simultaneous addition of a predetermined amount of NaOH. The cyanine dye was not toxic to *Gonyaulax*, since the cells recovered motility after dilution with fresh medium, as do untreated cells following centrifugation. Membrane depolarization has been shown to be accompanied by an increase in the intensity of cyanine dye fluorescence (9). The magnitude of the depolarization could not be calculated in absolute units in *Gonyaulax* because of its low internal K$^+$ concentration (15-30 mM, [18]), which precludes detection of K$^+$ efflux as an increase in fluorescence, so a zero value for membrane potential cannot be assigned (1).

RESULTS AND DISCUSSION

It is typical of circadian rhythms that they are reset by different amounts by the same stimulus given at different times in the circadian cycle. When visible or UV light is the stimulus for phase-shifting, large changes in phase are found during the night phase but none during the day phase of the circadian cycle. In *Gonyaulax*, short exposures to valinomycin at low concentration bring about the maximum phase advance at the end of the day phase and the greatest delay at the end of the night (18). Since vanillic acid was expected to change phase via depolarization of membranes, possibly in a manner similar to the K$^+$ flux caused by valinomycin, the end of the day phase and the end of the night phase, the times when valinomycin is most effective, were investigated. Four-hr pulses were also chosen by analogy with the experiments using valinomycin. The highest concentration of van-

illic acid that could be tested, 3 mM, was determined by the solubility of this compound in water. Large delay phase shifts (5-10 hr) were observed when cells were exposed to either 2 or 3 mM vanillic acid (Table I). At 0.5 mM, no reset was obtained, while the delay at 1 mM vanillic acid was intermediate.

Unexpectedly, delay phase shifts were observed following exposure to vanillic acid, irrespective of the time in the cycle when the chemical was present (three different times tested, Table I). The magnitude of the phase shift at a given concentration was about the same at all circadian times. This behavior contrasts sharply with that in response to valinomycin and ethanol, which varies markedly with the time of administration.

The magnitude of the phase shift proved to be highly dependent on the final pH of the cell suspension after the addition of vanillic acid. In experiments where the pH was adjusted to 7.1 or lower, large phase shifts were observed. If the pH was 7.5, the delay was smaller, while no phase change was seen at pH 7.9 (Table I). The addition of acetic acid to give pH 6.3 caused no phase change (Fig. 1). The increase in the effectiveness of vanillic acid at low pH is interpreted to indicate that the cell membranes of *Gonyaulax* are more permeable to the undissociated acid than to the ionized form, since the pK$_a$ of vanillic acid is 5.5. This is a commonly observed phenomenon, well known with respect to the uptake of IAA by plant cells, for example.

To confirm that vanillic acid does alter membrane potential in *Gonyaulax* as reported for barley roots (4), a necessary step for interpretation, changes in the fluorescence of the cyanine dye, diS C$_3$ (5), on the addition of vanillic acid (with and without *Gonyaulax*) were examined (Fig. 2). This method has been used successfully with this dinoflagellate to demonstrate depolarization of membranes on the addition of valinomycin and K$^+$ (1). This cyanine dye fluoresces strongly at 670 nm when excited at 622 nm. The addition of vanillic acid (final concentration 2 mM) causes a short term reduction in fluorescence in the absence of cells (Fig. 2, curve 1). In the presence of *Gonyaulax* (10^5 cells ml^{-1}), the same concentration of vanillic acid at pH 6.3 is followed by a persistent *increase* in fluorescence, an indication of membrane depolarization (Fig. 2, curve 2). This effect is less pronounced at pH 7.6 and 7.9 (Fig. 2, curves 3 and 4). No change in fluorescence is seen when the pH in the presence of vanillic acid is 8.2 (Fig. 2, curve 5). The changes in fluorescence on the addition of vanillic acid are pH-dependent, paralleling the phase shifts in the circadian rhythm of bioluminescence in response to this acid. The rapid increase of fluorescence on the addition of vanillic acid at low pH suggests depolarization of external membranes in *Gonyaulax*, but further work is needed to establish the site of the action of vanillic acid more precisely.

Large phase changes following short exposure to any biologically active molecule are unusual in circadian rhythms in general

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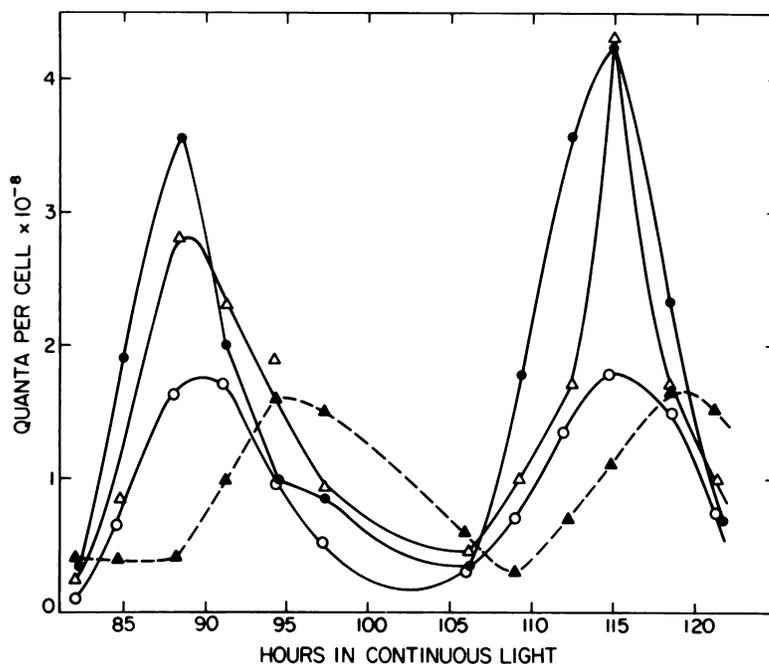[2] Abbreviation: c.t.: circadian time.

FIG. 1. Circadian rhythms in bioluminescence in *G. polyedra* in continuous light (200 μw cm^{-2} at 21 C). Cell suspensions were exposed for 4 hr from the 10th to the 14th hr in continuous light (10–14 c.t.) to (1) 2 mM vanillic acid in medium adjusted to pH 6.3 with 0.1 M NaOH (▲); (2) 2 mM vanillic acid in medium adjusted to pH 7.9 (△); (3) medium adjusted to pH 6.3 with acetic acid (●); and (4) medium only, pH 8.3 (○). Bioluminescence was stimulated by the addition of acetic acid to 2-ml aliquots of the cell suspension (final concentration 5 mM). Each point represents the average bioluminescence from three to five such aliquots.
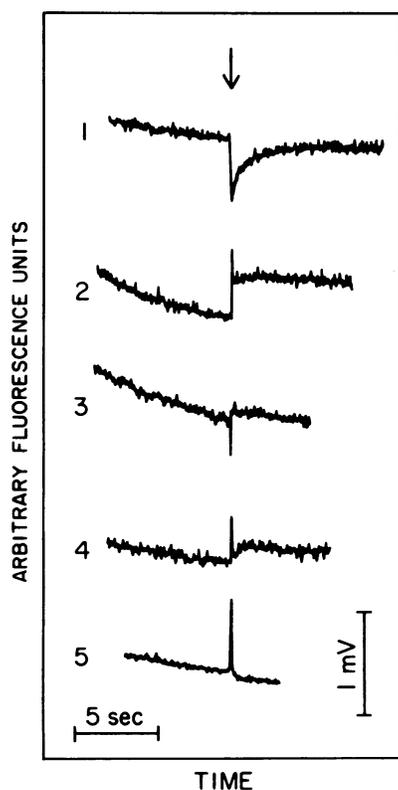

FIG. 2. Changes in the fluorescence of the cyanine dye diS C$_3$ (5), final concentration 2.4 × 10^{-6} M, in response to the addition of vanillic acid (2 mM final concentration), in the absence of cells (curve 1), in the presence of 100,000 cells ml^{-1} *G. polyedra* at pH 6.3 (curve 2), at pH 7.6 (curve 3), at pH 7.9 (curve 4), and at pH 8.2 (curve 5). All additions were made at the arrow. Curves traced directly from the recording. Fluorescence was excited at 622 nm and measured at 670 nm.

and in rhythms in *Gonyaulax* in particular. In experiments with alcohols and valinomycin (18) the phase changes were only 2 to 3 hr at most, rather than 5 to 6 hr as reported here following exposure to vanillic acid. No consistent phase changes were detected in a series of experiments assaying respiratory inhibitors, inhibitors of macromolecular synthesis, plant hormones, and other active substances for phase shifting the glow rhythm of *Gonyaulax* (6). A recent report of large phase changes following short exposures to cycloheximide (10) is an exception to this generalization. In this laboratory, the additional substances, ouabain, dibutyryl cyclic AMP (1 mM), and theophylline (1, 3, and 10 mM) have been found to be without phase-shifting effect in *Gonyaulax*. That vanillic acid, which our fluorescence experiments indicate depolarizes the membranes of *Gonyaulax*, causes such large phase delays adds support to the membrane hypothesis for the generation of circadian rhythms, at least in *Gonyaulax*.

LITERATURE CITED

1. ADAMICH M, PC LARIS, BM SWEENEY 1976 *In vivo* evidence for a circadian rhythm in membranes of *Gonyaulax*. Nature 261: 583–585
2. DeCOURSEY PJ, ed 1976 Biological Rhythms in the Marine Environment. University of South Carolina Press, Columbia
3. ESKIN A 1972 Phase shifting a circadian rhythm in the eye of *Aplysia* by high potassium pulses. J Comp Physiol 80: 353–376
4. GLASS ADM, J DUNLOP 1974 Influence of phenolic acids on ion uptake. IV. Depolarization of membrane potentials. Plant Physiol 54: 855–858
5. GUILLARD RRL, JH RYTHER 1962 Studies of marine plankton diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* Cleve Gran. Can J Microbiol 8: 229–239
6. HASTINGS JW 1960 Biochemical aspects of rhythms: phase shifting by chemicals. Cold Spring Harbor Symp Quant Biol 25: 131–143
7. HASTINGS JW, H-G SCHWEIGER, eds 1976 The Molecular Basis of Circadian Rhythms. Dahlem Konferenzen, Berlin
8. HASTINGS JW, G WEBER 1973 Total quantum flux of isotopic sources. J Optical Soc Am 53: 1410–1415
9. HOFFMAN JF, PC LARIS 1974 Determination of membrane potentials in human and *Amphiuma* red blood cells by means of a fluorescent probe. J Physiol 239: 519–552
10. KARAKASHIAN MW, H-G SCHWEIGER 1976 Evidence for a cycloheximide-sensitive component

in the biological clock of *Acetabularia*. Exp Cell Res 98: 303–312

11. MENAKER M, ed 1971 Biochronometry. National Acad Sci, Washington DC

12. NJUS D, VD GOOCH, D MERGENHAGEN, F SULZMAN, JW HASTINGS 1976 Membranes and molecules in circadian systems. Fed Proc 35: 2353–2357

13. NJUS D, FM SULZMAN, JW HASTINGS 1974 Membrane model for the circadian clock. Nature 248: 116–120

14. PAVLIDIS T 1967 A model for circadian clocks. Bull Math Biophys 29: 781–791

15. PITTENDRIGH CS 1965 On the mechanism of entrainment of a circadian rhythm by light cycles. *In* J Aschoff, ed, Circadian Clocks. North Holland Publ Co, Amsterdam, pp 277–297

16. SWEENEY BM 1969 Rhythmic Phenomena in Plants. Academic Press, London

17. SWEENEY BM 1974 A physiological model for circadian rhythms derived from the *Acetabularia* rhythm paradoxes. Int J Chronobiol 2: 25–33

18. SWEENEY BM 1974 The potassium content of *Gonyaulax polyedra* and phase changes in the circadian rhythm of stimulated bioluminescence by short exposures to ethanol and valinomycin. Plant Physiol 53: 337–342

19. SWEENEY BM 1976 Evidence that membranes are components of circadian oscillators. *In* JW Hastings, H-G Schweiger, eds, The Molecular Basis of Circadian Rhythms. Dahlem Konferenzen, Berlin, pp 267–281

20. SWEENEY BM, JW HASTINGS 1957 Characteristics of the diurnal rhythm of luminescence in *Gonyaulax polyedra*. J Cell Comp Physiol 49: 115–128

21. SWEENEY BM, JM HERZ 1977 Evidence that membranes play an important role in circadian rhythms. Proc XII Int Conf. Int Soc Chronobiol, pp 751–761

22. WINFREE AT 1970 Integrated view of resetting a circadian clock. J Theor Biol 28: 327–373