Kinetics of the cycloheximide-induced phase changes in the biological clock in *Gonyaulax*

**(Circadian rhythms/phase response curve/singularity/bioluminescence)**

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**ABSTRACT** Cytochalasin D, an inhibitor of protein synthesis in cytoplasmic ribosomes in eukaryotes, is shown to shift the phase of the circadian rhythm in stimulated bioluminescence in the marine dinoflagellate *Gonyaulax polyedra*. Kinetic analysis of the phase changes indicates that this effect may be subdivided into two distinctly different and well-separated parts. The first (early) phase change occurs with 15-min exposure to cycloheximide and is saturated at low concentrations (≈ 10 nM). The second (late) phase change requires about 150 min of exposure to cycloheximide and is saturated at 0.36 μM cycloheximide. Twenty-times-higher concentrations cause no further phase changes. The magnitudes of both early and late phase changes depend on the time of day when the cells are exposed to cycloheximide. Early phase changes vary from a 5-hr advance at circadian time (CT) 20 to 1 hr delay at CT 12; late phase changes are larger, the maximal advance being 12 hr at CT 16 and the greatest delay, 10 hr at CT 14. It is proposed that the early phase changes are caused by alterations in the ion distribution across membranes as a consequence of the permeation of cycloheximide. Late phase changes may be the result of inhibition of protein synthesis.

The phase response curve for the late phase change is identical to that obtained with saturating light pulses in otherwise constant darkness in *Gonyaulax*. Maximal phase changes arise in the part of the circadian cycle without effect on phase. Incubation of *Gonyaulax* with cycloheximide for a critical duration at a critical time induces arrhythmicity, but longer exposures to the inhibitor at the same time do not. This observation suggests the existence of a singularity in the circadian clock of *Gonyaulax*.

Circadian rhythms are biological oscillations with a period of about 24 hr. They persist in the absence of environmental cycles but may be entrained with an oscillatory parameter such as a light or temperature cycle. The phase of all circadian rhythms can be altered by a short exposure to light (1, 2). The amount and the direction of the changes depend on the time in the cycle when the light is given. The phase changes thus obtained may be expressed as a function of the time of exposure to light, usually given in circadian time (CT) in which CT 0 corresponds to daybreak and CT 12, to dusk in the entraining light cycle (3). The resulting curve is called a “phase response curve” (PRC).

Many attempts have been made to duplicate the PRC obtained with light by exposing organisms to various metabolic inhibitors, in the hope of identifying the cellular location of the clock. The rational for this approach is that a change in phase can be interpreted as evidence for a direct interaction of the substance with the circadian clock (1). Most inhibitors, however, are inactive in shifting the phase of a circadian oscillator (4, 5).

There are two prominent classes of active substances: inhibitors of protein synthesis on 80S ribosomes (6, 7) and substances that interfere with the distribution of ions across membranes such as ions, ionophores, uncouplers, and local anesthetics (8–10).

Years ago, Feldman (6) found that constant exposure to cycloheximide (CHX), an inhibitor of protein synthesis on cytoplasmic 80S ribosomes, lengthens the free-running period of the phototactic rhythm in *Euglena*. Both CHX and puromycin, an inhibitor of translation on both 70S and 80S ribosomes, cause phase changes in the circadian rhythm of photosynthesis in *Acetabularia* (7) and of neuronal activity in the isolated eye of *Aplysia* (11). Inhibitors of organellar transcription and translation show no effect on phase or period in all systems tested (12, 13). Therefore, protein synthesis in the organelles is not considered to have a role in the generation of circadian rhythmicity.

By contrast, most chemicals that are thought to affect the membrane systems of the cells in some way—example, ionophores (valinomycin)—yield small but definite changes in the *Gonyaulax* clock. PRs for these membrane-active compounds do not resemble the PRCs obtained with saturating pulses of light, darkness, or cycloheximide (this paper). Both the shape and the extent of the maximal phase changes are different. The PRC for saturating pulses of CHX in *Gonyaulax* is shown here to be identical to the light-induced PRC. The PRC for low concentrations of CHX is very similar to that obtained with the membrane-active compounds.

A kinetic analysis of the phase changes induced by CHX in the stimulated rhythm of bioluminescence in the marine dinoflagellate, *Gonyaulax polyedra*, is presented.

**MATERIALS AND METHODS**

Cells and Culture Conditions. G. polyedra Stein, clone 70A, was grown in Fernbach flasks containing 1.5 liters of f/2 medium (14) in an alternating light/dark cycle (12:12), illuminated from cool white fluorescent lamps during the light period (1.8 mW/cm²). The temperature was 21 ± 1°C. The cells were transferred to constant light conditions (LL, 0.5 mW/cm²) in the early stationary phase of growth (1–1.4 × 10⁴ cells per ml). The cultures were divided into 50-ml aliquots and were allowed to adapt to the reduced light intensity for 3 days prior to the experiment.

Experimental Protocol. CHX pulses were administered as described in ref. 9. The inhibitor was removed by washing the cells and resuspending them in fresh f/2 medium. When the cells were continuously exposed to CHX, they died after 2–3 days. After a 3- to 8-hr exposure to CHX that was terminated as described above, however, the cells survived for at least 8 days.

The free-running phase of the cultures was estimated by monitoring the acid-stimulated bioluminescence, which was measured according to ref. 15, with a slight modification. (This

Abbreviations: CHX, cycloheximide; CT, circadian time; PRC, phase response curve.
modification greatly enhances the reproducibility of replicates—i.e., to within about 8%. Bioluminescence was measured immediately after the cells were pipetted into test vials with an automatic pipettor with a 2- to 3-mm orifice to minimize friction.

Three days later, bioluminescence was measured for 1.5 cycles or longer. In some instances, measurements were continued for another 1.5 cycles. No significant difference in the estimate of the phase could be seen in any of these cases. All experiments were repeated two or three times. To make comparison between experiments possible, all data were expressed in CT (3).

CHX was purchased from Sigma and Calbiochem.

RESULTS

Biphasic Nature of the CHX Effect. Phase shifts in the circadian rhythm of stimulated bioluminescence in Gonyaulax induced by 3-hr pulses of CHX were observed over a 10^4-fold concentration range (Fig. 1). The concentration required for the maximum phase change was 0.36 μM; concentrations 20 times higher had no further effect. Phase shifts were observed at much lower concentrations of CHX than those required for effective inhibition of translation in vitro in most plants and animals. The magnitude and the direction of the phase changes induced by the inhibitor were different at different phases of the circadian cycle.

The magnitude of the phase change did not increase in proportion to the concentration of CHX but showed two steps well separated from each other. The second step appeared at a concentration of CHX 10 times higher than that required for the first step. A concentration of 10 nM CHX was sufficient to induce the first or early phase shift, which varied from a 4-hr advance to a 1-hr delay, depending on the cycle time when the pulse of inhibitor was given (Fig. 1, data for delays not shown). The late phase change was larger and also varied with the CT when the pulse of CHX was given, from a 10-hr delay at CT 14 to a 12-hr advance at CT 16.

Similar biphasic kinetics are observed when the CHX concentration is kept constant but the duration of the incubation is varied (Fig. 2). The early phase shift was observed with 10- to 15-min exposure to 0.36 μM CHX and was between a 5-hr advance and a 3-hr delay, depending on the CT at which the pulse was given. The late phase change was seen when the duration of the exposure to CHX was 150 min or more. Small delays were observed after somewhat shorter pulses. We have assumed that the phase changes observed at CT 14 are delays because the effectiveness of CHX increased smoothly between pulse durations of 90 and 150 min. The magnitude of the phase change clearly depends on the dose of CHX, a short exposure to a high concentration being equivalent in effect to a long exposure to a low concentration (compare Figs. 1 and 2).

The late phase changes caused by CHX usually were larger than the early phase changes at any given time in the circadian cycle (Figs. 1 and 2). The relative contribution of the early and the late phase changes to the total, after 3-hr exposure to CHX, was different at different times in the circadian cycle. Even the direction of the two types of phase change was not always the same. At CT 14, for example, an early phase advance of 2 hr after 30 min was reversed after 3-hr exposure to give a total phase delay of 7 hr (Fig. 2).

The monophasic kinetics at CT 20 in Fig. 2 appear contradictory at first glance. However, the first phase change places

![Fig. 1](image-url)  
**Fig. 1.** Phase shifts in the circadian rhythm of acid-stimulated bioluminescence in G. polyedra in response to short exposures to different concentrations of CHX. Cultures of Gonyaulax, free-running in constant light, were exposed to 3-hr pulses of CHX at the concentrations indicated at different times in the circadian cycle. Three and 4 days later, the phase changes were measured by using a bioluminescence assay. Advances are plotted as positive; delays are plotted as negative. The cell suspensions were kept in constant light (0.5 mW/cm² at 21 ± 1°C) during the experiment. These experiments were repeated two or three times each. The variation in the estimation of the maximum bioluminescence is about ±0.7 hr. The numbers on the graph (CT 02 to CT 20) correspond to the phase in the circadian cycle at which the pulse was started. Exposures to CHX at phases intermediate to those shown gave intermediate phase shifts but are omitted for clarity. All dose–response curves were biphasic.
the clock into CT 4–9, where perturbations are ineffective. Thus, no late phase change is expected at this time.

Second pulses of CHX, given between 30 sec and 5 hr after a saturating pulse and lasting either 30 min or 3 hr, did not affect the phase. This shows that the saturation of the CHX effect is not caused by inactivation of the drug but is a function of the response of the clock mechanism.

**Evidence for a Singularity in the Circadian Clock in Gonyaulax.** The direction of the late phase change in all exposures that began between CT 14 and CT 17 was difficult to evaluate because the amplitude of the bioluminescence rhythm decreased dramatically with pulse duration in this part of the circadian cycle. Finally, rhythmicity was observed after 60–90 min of exposure to CHX. However, when the exposure to CHX was even longer, 150 min or more, a clear rhythm was again observed. Concurrently with the recovery of the amplitude of the rhythm, the maximal phase change was observed. Such behavior is characteristic of the singularity described in other rhythmic systems (16, 17). Once the cell suspensions became arrhythmic by this means, they did not recover rhythmicity even after 4 days. Second exposures to CHX of 30 min or 3 hr duration started between 30 sec and 5 hr after the singularity was reached were without additional effect on the amplitude. On the other hand, continuous exposure to CHX for 150 min or more over the same time span yielded normal rhythmicity and phase changes. When the singularity had been reached, it was remarkably stable in *Gonyaulax*. However, CHX had to be removed at a critical time or no singularity was observed.

**PRC for CHX.** It is possible to plot a PRC for saturating doses of CHX (0.36 μM for 3 hr) in the same way that a PRC is plotted for saturating light pulses. The PRC for CHX thus obtained (Fig. 3) was identical with that for 3-hr pulses with saturating light in otherwise constant darkness in *Gonyaulax*. In Fig. 3, the phase changes have been plotted as a function of the time in the cycle when the pulses began (lower abscissa). On the upper abscissa, 2 hr have been added to the beginning of the pulse because about 2 hr are required to bring about the saturating phase changes (Fig. 2). As with light, CHX given between CT 4 and CT 9 was without effect on the phase of the rhythm in bioluminescence. Delays were observed between CT 12 and CT 17 and advances in phase were observed between CT 18 and CT 2.

**Maximal Perturbation Drives the Oscillator into the Range of CT 4 to CT 9.** Fig. 4 is an "old phase versus new phase" curve (19) for 3-hr pulses of CHX at 0.36 μM. This curve is identical to the one obtained when the phase at which the pulse is given + phase shift induced (φ + Δφ) is expressed as a function of the phase at which the pulse is given (φ). The ordinate and the abscissa must then be marked from top to bottom and from right to left, respectively, for agreement with published curves. All new phases fell between CT 4 and CT 9. Saturating perturbations given during this interval appeared to be ineffective (Fig. 4, diagonal lines). However, submaximal pulses could give rise to small phase changes, which are then reversed by the late phase change (Fig. 2, CT 2 and CT 6). A similar range in the circadian cycle that appears to be immune for perturbations to light has been found in the pupal eclosion rhythm in *Drosophila* (20).

**DISCUSSION.** In *Gonyaulax*, a CHX pulse (0.36–1 μM) of about 2.5 hr is required to complete the phase-shifting process. About the same time is required for maximal phase changes with light. The phase change obtained at a given phase is the same whether the perturbing agent is light or CHX. To our knowledge, no other drug has been found that mimics the effects of light on the clock so closely in *Gonyaulax*. Unfortunately, we cannot decide if the clock is perturbed via the same pathway by light and CHX because the effects of light on the clock are so poorly understood.

CHX is thought to bring about its effects on various eukaryotic organisms by inhibiting protein synthesis on 80S ribosomes. Consequently, it is pertinent to know whether a similar inhibition of translation can be demonstrated in *Gonyaulax*. 

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**Graph Description:**

**Fig. 2.** Phase changes in the circadian rhythm of acid-stimulated bioluminescence in *Gonyaulax* in response to exposure to CHX at 0.36 μM for different lengths of time. The conditions were identical to those in Fig. 1. Exposures to CHX at phases intermediate to those shown were omitted for clarity, but all showed the same biphasic kinetics. The numbers in the graph (CT 06 to CT 20) correspond to the phase at which the pulse was started. The variation in the estimation of the maximal bioluminescence is about ±0.7 hr. Every experiment was repeated two to four times.
Preliminary work in our laboratory (unpublished data) has shown that the incorporation of [14C]leucine into acid-precipitable material can be inhibited by the presence of CHX. However, the inhibition is small (5%) at the CHX concentration (0.36 μM) and the exposure duration (3 hr) that brings about the maximal phase shift in Gonyaulax. In these experiments, chloramphenicol (100 μg/ml) was present to inhibit protein synthesis on organellar and bacterial ribosomes. No inhibition of the incorporation of [14C]leucine could be detected after 30-min incubation with CHX. In Acetabularia and Aplysia, much larger inhibition of translation on 80S ribosomes as a consequence of the presence of CHX has been reported (7, 11), but in these cases, the exposure to CHX and labeled amino acid precursors was much longer (8 and 12 hr, respectively). In Aplysia, much higher concentrations of CHX were used. The effect of low concentrations of CHX for short times on phase or on the incorporation of labeled amino acids was not reported in either Acetabularia or Aplysia. It is thus possible that a 5% inhibition of protein synthesis may be sufficient to perturb the circadian clock in these organisms as in Gonyaulax. If this is the case, there may be a protein essential to clock function as postulated by Schweiger and Schweiger (21). Such a protein must then be made on ribosomes that are unusually sensitive to CHX.

The interpretation of the biphase kinetics observed in these experiments when phase changes were plotted as a function of both concentration of CHX and duration of the exposure to the inhibitor is a matter of conjecture at present. The experiments were repeated a number of times and this biphase character of the response was always observed except in the case mentioned in Results (Fig. 2, CT 20).

There is some indication in the experimental data that the early and late phase changes arise from different mechanisms. Not only the amount but also the direction of the phase shift can be different at a given phase, as mentioned above. The PRC for the early effect has a different shape from that for the late effect. However, the PRC for weak light pulses is also different from that for bright light (19). The PRC for the early phase shift is similar to that obtained with certain agents that are known to interfere with ion distribution across membranes—for example, local anesthetics and decreased external pH (unpublished data). Thus, we suggest that the early phase shifts that are observed when Gonyaulax is exposed to low concentrations of CHX for short times may be mediated by membrane effects of CHX. It has been reported that CHX interacts with membrane potentials and transport systems in other organisms (22, 23). In the case of Euglena (22), the inhibition of transport could be mimicked by the ionophore nigericin which exchanges protons and sodium ions across membranes. The kinetics observed and the concentrations required in Euglena are similar to those that bring about the early phase shifts in Gonyaulax.

The late phase shifts brought about by CHX in Gonyaulax cannot be interpreted with certainty. Perhaps this effect may be the result of inhibition of translation on 80S ribosomes.

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FIG. 4. The effect of 5-hr exposures of G. polyedru to CHX at 0.36 µM, expressed as "old phase versus new phase" (19). This curve is identical to the one obtained when the phase at which the pulse was started (+2 hr for the delay time for the effect of CHX) + the phase shift induced (ϕ + Δϕ) is expressed as a function of the phase at which the pulse was started (+2 hr lag) (ϕ). The axes are labeled from top to bottom and from right to left, respectively, to conform to the conventional "old phase versus new phase" curves (19). Note that all the points fall between about CT4 and CT9. Again, every point is the mean (±SD) of two to four experiments.