FREEZE-FRACTURE STUDIES OF THE THECAL MEMBRANES
OF GONYAULAX POLYEDRA: CIRCADIAN
CHANGES IN THE PARTICLES OF ONE MEMBRANE FACE

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ABSTRACT

Intramembrane faces were visualized in the marine dinoflagellate Gonyaulax polyedra by the freeze-fracture technique, in order to test a prediction of a membrane model for circadian oscillations—i.e., that membrane particle distribution and size change with time in the circadian cycle. Cells from each of four cell suspensions in continuous light (500 lx, 20-21°C) were frozen, without fixation or cryoprotection, at four circadian times in a cycle. This paper reports findings concerning the membranes associated with the theca, particularly the cytoplasmic membrane and the membrane of the large peripheral vesicle. While the number and size distribution of the particles of the PF face of the cytoplasmic membrane were constant with time, those of the EF face of the peripheral vesicle doubled in number at 18 h circadian time as compared with 06 h. Particles of the 120-Å size class, in particular, were more numerous at 12 and 18 h circadian time than at 00 and 06 h. While the finding does not provide definitive confirmation of the membrane hypothesis for circadian rhythms, it is consistent with this model. It is suggested that the peripheral vesicle may be the site of bioluminescence in Gonyaulax.

The circadian rhythms of the dinoflagellate Gonyaulax polyedra are probably the most thoroughly investigated of any rhythms in unicellular organisms (12, 28, 31, 32). One circadian system controls both stimulated and spontaneous bioluminescence, photosynthetic capacity, and cell division in this organism (20). The manner by which such diverse processes are coordinated within a circadian framework presents a formidable problem. Recently it has been suggested that the common denominator may be membrane localization of key biochemical steps in these processes (4, 8, 25, 30). The pigments and enzymes of photosynthesis are associated with the thylakoid membranes of the chloroplast, and evidence has accumulated that the enzyme and substrate which produce bioluminescence are membrane bound (9). The control of cell division is less well understood, but this process may depend on the electrical properties of the cell membrane (19). These considerations have given rise to a membrane model for the generation of circadian oscillations (21, 29).

In view of the importance of membranes to the overt rhythmic processes, and possibly to circadian timing itself, an investigation of the ultrastructure of the various membranes of Gonyaulax seemed appropriate. Furthermore, the membrane model for the feedback generation of rhythms predicts that membrane particle number and distribution alter over circadian time. A direct examination of these particles at different circadian times might thus provide a test of this hypothesis.
The technique of freeze-fracture allows visualization of membrane cleavage planes and the associated particles. The frozen membranes are now known to cleave through the center of the bimolecular lipid layer and thus expose the protein molecules within (2). To date, the membranes of dinoflagellates have scarcely been examined by this method. One short paper on the distribution of membrane cleavage planes and the amphiesma (17) in

The study of dinoflagellate structure. The membranes associated with the theca or the amphiesma (17) in Gonyaulax have not previously been examined by freeze-fracture. This study was initiated to fill this deficiency, particularly to examine cells fractured at different times in a circadian cycle under conditions where rhythms are free running in continuous light. Light-dark cycles in the environment were avoided, since light is known to affect membrane function directly (10) and the object of this study was to test the prediction of the membrane model that spontaneous changes may occur during a circadian cycle. This is the first report of these studies and is limited to a discussion of the membranes associated with the theca or the amphiesma (17) in Gonyaulax.

MATERIALS AND METHODS

Cells

A clonal strain of G. polyedra Stein (strain 70A), isolated by the author in 1970 from a red tide, was used in most of these studies. The culture medium was filtered seawater enriched with NaN_O, NaH_2PO_4, trace metals, and vitamins (F/2 of Guillard and Ryther, 11) to which soil extract (2%) was added. Cultures were grown at 22°C with illumination for 12 h each day (3,500 lx from cool white fluorescent lamps). Three sequences of samples were prepared from cultures and one from a Gonyaulax red tide. On the day before the cells were to be frozen, the culture or red tide cell suspension was transferred to continuous light (500 lx, 20–21°C) at the beginning of a light period. Samples (2 ml) were pipetted for the determination of the circadian rhythm in bioluminescence and also placed in continuous light.

Freeze-Fracture Procedure

For freeze-fracture, 200-ml portions were removed from a cell suspension in continuous light and harvested by centrifugation at 30 g for 0.5 min. Small samples (about 0.05 ml) were pipetted onto cardboard disks and frozen without fixation or cryoprotection in liquid Freon 22 held close to its freezing point in a bath of liquid N_2. Cells were stored on these disks in liquid N_2 until cleaved. Samples were taken from a cell suspension at 00, 06, 12, and 18 h and the following 00 circadian time (c.t.). Bioluminescence was measured concurrently, using the photomultiplier photometer previously described (31) and stimulating luminescence by injecting 0.2 ml of 0.05 M acetic acid into each tube. Circadian time was calculated from the curve for the rhythm in stimulated bioluminescence, according to the convention of Pittendrigh (24), where 00 c.t. is equivalent to the phase at dawn in a 12:12 h light-dark cycle. The circadian cycle, which may not be exactly 24 h long in continuous light, is divided into 24 parts or circadian hours; 06 c.t. then corresponds to the middle of the day phase, 12 c.t., to dusk, and 18 c.t. to the middle of the night phase.

Frozen samples were cleaved in a Balzers BA 360M (Balzers High Vacuum Corp., Santa Ana, Calif.). The surface thus exposed was shadowed with platinum and the replica was stabilized with carbon. Etching was avoided since intramembrane particles rather than surface features were of interest. Replicas were cleaned in 70% H_2SO_4 for 24 h, rinsed three times with water, cleaned with undiluted commercial bleach (Purex) for an additional hour, and again washed with water. Replicas were mounted on Formvar-coated copper grids (diameter 2 mm, 300 mesh) and examined and photographed in a Siemens Elmiskop 101 electron microscope. Particles on membranes were counted and measured on prints at x 120,000 with a measured area and a calibrated grid and magnifier (Edmund Scientific Co., Barrington, N.J.). The diameter of particles was measured at right angles to the direction of shadowing. Standard error of the means were calculated using an HP 45 calculator (Hewlett-Packard, Palo Alto, Calif.). The PF face of a membrane was considered to be that fracture face adjacent to the cytoplasm and the EF face, the opposing fracture face (3).

Transmission Electron Microscopy

For transmission electron microscopy, cells were harvested as described above and resuspended in 1% OsO_4 in 0.3 M NaCl made up in glass-redistilled water. Fixation was for 1 h at room temperature, followed by dehydration in a graded acetone series. The cells were embedded according to the method of Spurr (27), except that the plastic was changed three times during 24 h to ensure complete infiltration. Silver and gray sections were cut with a diamond knife and were double-stained with uranyl acetate and lead citrate. Cells were examined and photographed as above.

RESULTS

Transmission electron microscopy (1, 14, 26) has shown that the Gonyaulax cell is complex in structure and contains numerous membrane-bound organelles, including chloroplasts, mito-
FIGURE 1 A cross section through the outer portion of G. polyedra, showing the pellicle (P), the cytoplasmic membrane (PM), the distal end of a trichocyst organelle (T), a polyvesicular body (PVB), the microtubular layer (M), a chloroplast (C), and parts of the peripheral vesicle (PV). Cell fixed at 18 h c.t. in continuous light (LL). × 40,000.

FIGURE 2 A freeze-fracture replica of the outer portion of G. polyedra showing part of a cellulose plate (W), the pellicle (P), a chloroplast (C), two polyvesicular bodies (PVB) and a part of the peripheral vesicle (PV). Cell frozen at 06 h c.t. in LL. × 40,000.
chondria, trichocysts, polyvesicular bodies, Golgi-derived vesicles, a nucleus, and a number of unidentified vesicles enclosed by a single membrane (Figs. 1 and 2). The cell is an oblate spheroid, about 45 μm in length, and is bounded by an amphiesma (17), a series of structures associated with the wall. The most distal of these is a thin, fragile membrane beneath which lie the thecal plates. Both outer membrane and plates may be lost without disrupting the cell and are quickly replaced. In surface view, plates appear to be composed of interwoven strands, presumably cellulose (Figs. 2 and 3). Round pits are present in the plates, each of which contains one or several pores through which trichocysts can be discharged to the exterior (Figs. 1 and 3 and reference 1). These pits have not been seen previously in surface view.

The principal protective coat of the cell is the pellicle (Figs. 1 and 2). Its outer surface shows round, raised plaques and many small depressions (Fig. 4). No pores have been seen in the pellicle corresponding to those of the plates. The pellicle appears to be continuous over the distal tip of the trichocyst organelle (Fig. 1).

The cytoplasmic membrane lies directly against the inner surface of the pellicle (Fig. 1) and also appears to be uninterrupted. The PF face of this membrane contains a large number of particles, about 6,000/μm² (Figs. 5 and 6). The EF face is seldom seen in freeze-fractured cells, but in four replicas which showed this face, it carried fewer particles than the PF face, about 1,500/μm².

Inside the cytoplasmic membrane are what appear in cross sections to be a series of flattened vesicles bounded by single membranes (Figs. 1 and 2). Freeze-fracture reveals, however, that this is a single continuous peripheral vesicle encircling the entire cell, interrupted locally by polyvesicular bodies (Fig. 5) and by the distal ends of trichocyst organelles (Fig. 6). The EF face of the membrane of this vesicle shows characteristic small local elevations and depressions (Figs. 5 and 6) and circular craters which suggest pinocytosis. On the PF face of this membrane (Fig. 7) are many particles, about 4,000/μm². In the lumen of the peripheral vesicle are found the crystals formerly thought to be the source of bioluminescence in *Gonyaulax* (13) but now considered to be guanine (9) (Fig. 7).

The principal objective of this research was to test a prediction of the membrane model for the generation of circadian oscillation in *Gonyaulax* (21)—that membrane faces show changes in particle number or distribution with time over the 24-h cycle. This paper reports quantitative data concerning the number and size of particles found on the PF face of the cytoplasmic membrane (Fig. 8) and the EF face of the membrane of the peripheral vesicle (Fig. 9) at four times during a circadian cycle in cells showing a circadian rhythm in bioluminescence in continuous light. On the PF face of the cytoplasmic membrane there are about 6,000 particles/μm² at all circadian times examined (Fig. 8). The mean diameter of these particles is 70 Å and the distribution of particle size about this mean is symmetrical and approximately constant. On the EF face of the peripheral vesicle membrane, however, both the number and the size distribution of particles changes with time in the circadian cycle (Fig. 9). At 18 c.t. there are twice as many particles on this membrane face as at 06 c.t. Particle numbers at 00 and 12 c.t. are intermediate. This difference in particle number was confirmed by an enumeration of the particles from 64 different photographs representing 38 cells and 22 separate replicas from four different cell suspensions. Cells frozen at 00 h at the beginning and end of a circadian cycle were included in this enumeration. The difference in the number of particles on the EF face of the peripheral vesicle membrane in cells from the same cell suspension frozen at 06 c.t. and 18 c.t. the same day is clearly discernible (Figs. 10 a and b).

Although it is difficult to say with certainty, because of the small number of replicas showing the EF face of the cytoplasmic membrane and the PF face of the membrane of the peripheral vesicle, no marked differences in the particle number on these membrane faces were detected. The size distribution of the particles on the EF membrane face of the peripheral vesicle showed a most interesting change with time in the circadian cycle (Fig. 9). The greatest number of particles was always found in the 100-Å size class. At 00 c.t. the distribution of particle diameters was symmetrical. However, at 12 and 18 c.t., a second maximum appeared at 120 Å, representing a doubling in the number of particles in this size class in the interval between 00 and 12 c.t. Particles of other size classes also increased in number during the interval between 06 and 18 c.t., but the increase was not as dramatic.

The differences in both the total number of particles and the number in the 120 Å-size class at different circadian times appear to be significant, judging from the standard error values calculated
FIGURE 3 Replica of a cellulose plate of G. polyedra fractured along the surface, showing the fibrillar structure and a depression containing a trichocyst pore (TP). Cell frozen at 06 h c.t. in LL. x 60,000.

FIGURE 4 The pellicle of G. polyedra fractured along its outer surface, showing raised plaques and small depressions. A fragment of the cytoplasmic membrane (PM) and a section of the peripheral vesicle (PV) may be seen at the bottom of the figure. Cell frozen at 06 h c.t. in LL. x 60,000.
Figure 5. The EF face of the membrane of the peripheral vesicle perforated by a polyvesicular body (PVB) and the distal tips of two trichocyst organelles (T) and a small portion of the cytoplasmic membrane PF face (PM). Depressions, elevated areas, and craters can also be seen on the EF face of the peripheral vesicle membrane. Cell frozen at 06 h c.t. in LL. × 60,000.

Figure 6. Another view of the EF face of the membrane of the peripheral vesicle perforated by the distal tip of a trichocyst organelle (T) showing a crater (arrow), membrane particles, and a portion of the PF face of the cytoplasmic membrane with particles (PM). Cell frozen at 06 h c.t. in LL. × 120,000.
FIGURE 7 The PF face of the membrane of the peripheral vesicle of *G. polyedra* and a portion of the lumen of this vesicle in cross-fracture, showing two crystals (CR). Note the many particles on this membrane face. Cell frozen at 12 h c.t. in LL. × 120,000.

![Image of the PF face of the membrane of the peripheral vesicle of *G. polyedra*](image)

**Figure 8** Histograms showing the number and size distribution of the particles on the PF face of the cytoplasmic membrane in *G. polyedra* at four different times during a circadian cycle in LL. Lines at the top of each bar represent ± the SEM of the number of particles, for each determination (Figs. 8 and 9). For enumeration and measurement of particles, the same photographs were used for the data of Figs. 8 and 9 as far as possible. Additional photographs were used for Fig. 9, since the EF face of the membrane of the peripheral vesicle, but not of the cytoplasmic membrane, was shown in these photographs. Since no differences were noted in the cytoplasmic membrane, while the same photographs showed differences in the membrane of the peripheral vesicle, artifacts due to contamination of the surface before replication cannot be responsible for the differences observed. Fixation artifacts are ruled out since the cells were neither fixed nor glycerinated. Reproducibility was good between experiments using four different cell suspensions. Therefore, I consider that the differences in particle number and size distribution with time in the circadian cycle are real. Other membranes in

Three photographs were measured for each histogram. This figure includes measurements of seven cells from two cell suspensions and six different replicas. The total number of particles for each time is given in the upper right of each histogram followed by its standard error.

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FIGURE 9 Histograms showing the number and size distribution of the particles on the EF face of the membrane of the peripheral vesicle of G. polyedra at four different times during a circadian cycle in LL. Lines at the top of each bar represent ± the standard error of the mean number of particles. For the 00 c.t. histogram, n = 3; for the 06 c.t. histogram, n = 6, and for both the 12 c.t. and the 18 c.t. histograms, n = 5. Six photographs were measured for each histogram. This figure includes measurements of 12 cells from three cell suspensions and nine different replicas. The total number of particles for each time is given in the upper right of each histogram followed by its standard error.

Gonyaulax will be discussed in future communications.

DISCUSSION

The fine structure of G. polyedra as revealed by the freeze-fracture technique conforms in general to that seen by transmission electron microscopy. However, the continuity of the peripheral vesicle would be very difficult to observe in sections examined by transmission electron microscopy because it is pierced by trichocysts and polyvesicular bodies in so many places. Sectioned material of many different dinoflagellates shows vesicles just beneath the cell surface (5-7, 16). These may well prove to be confluent as in Gonyaulax, when these species are examined by the freeze-fracture technique.

It is interesting to speculate regarding the function of the peripheral vesicle, especially since the particles on one fracture face of the membrane of this organelle change their frequency and size distribution with circadian time. Luminescence increases from 12 to 18 h circadian time, then decreases (31). The correspondence between the appearance of particles 120 Å in diameter and the increase in bioluminescence suggests that luciferase may be bound in this membrane during the time of high luminescence capacity. Specific markers for luciferase are required to confirm this possibility, and none is known at present. The localization in the lumen of the peripheral vesicle of crystals, once thought to be luminescent particles and associated with the particulate luminescence during gradient separation of homogenates of Gonyaulax (13), makes this possibility attractive. These crystals are birefringent and may act as a reflecting layer. Schmitter (26) suggested that the polyvesicular bodies may be luminescent organelles in Gonyaulax, but these are not present in the luminous Pyrodinium bahamense (unpublished observations of the author) and one would expect a uniformity among dinoflagellates in this respect.

Changes in particle number and size distribution on one membrane face could be brought about by incorporation of new protein molecules into the membrane or by a change in the orientation of particles from one membrane face to the other. Since the PF face of the membrane of the peripheral vesicle carries many particles (4,000/μm²) and few replicas showing this face could be found among all the replicas examined, it was not possible to detect whether or nor particles disappeared from the PF face when they appeared on the EF face of this membrane. Hence the two possibilities could not be distinguished. Changes in both the number and the arrangement of membrane particles have been reported in freeze-fracture studies. When dormancy is broken in the spring, the number of particles in the plasmalemma of the cells of the cambial zone of willow stems increases, while particles of the tonoplast decrease in number (23). A change in the particle number in the membranes of the frog rod outer segment on illumination has also been reported.
Figure 10 Particles on the EF face of the peripheral vesicle of G. polyedra. Local depressions and elevations are also shown. (a) Cells frozen at 06 c.t. in LL. (b) Cells from the same cell suspension frozen at 18 c.t., 12 h later than those of a. Both figures, x 240,000.
(18). On the other hand, stacking of the thylakoids in Chlamydomonas has been shown to be accompanied by lateral movement of large particles of the EF thylakoid face into the appressed region (22). Thus, it is clear that both number and arrangement of membrane particles can change over time in diverse membrane systems. Circadian changes of this type have not been reported previously, however.

The finding reported here, that particles of the EF face of the peripheral membrane change their number and size distribution with circadian time, is consistent with the membrane model which postulates that circadian oscillations are the result of a feedback mechanism, one component of which is the arrangement of the transport proteins within an unspecified membrane or membranes and the other the distribution of an ion or other molecule on either side of this membrane (21, 29). However, the changes observed in the particles of the membrane of the peripheral vesicle may be no more than another example of a process controlled by, rather than controlling circadian oscillations. If the particles are not themselves a part of the circadian clock, they are at least a molecular manifestation of its operation.

The particles of the PF face of the cytoplasmic membrane do not show circadian changes. It will be informative to determine whether other membrane faces, especially those of the chloroplast, do or do not change in a circadian time scale. These measurements are in progress and will be reported in the near future.

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