J. Phycol. **24**, 17–22 (1988)

INTRACELLULAR LOCALIZATION OF SAXITOXINS IN THE DINOFLAGELLATE *GONYAULAX TAMARENSIS*[1]

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ABSTRACT

The potent neurotoxin saxitoxin, and possibly several of its derivatives, are localized in two types of sites within the marine dinoflagellate Gonyaulax tamarensis *Lebour. Immunocytochemical techniques using a polyclonal antibody and epifluorescence microscopy demonstrate toxin localization within the nucleus as well as on the periphery of small granules thought to be starch grains. In the nuclear region, the labelling occurred on or close to the permanently-condensed chromosomes as well as in an area within the two arms of the nucleus in the vicinity of the nucleolus. No binding was observed in a closely-related, non-toxic dinoflagellate. Different binding affinities were observed between the nucleus and the grains at high and low antibody dilutions. This may relate to the polyclonal nature of the antiserum and to the presence of multiple toxins within the* G. tamarensis *isolate studied. Mechanistic interpretations of these labelling patterns remain speculative, especially the localization of the antigen at the outer edge of starch grains, but the distinct labelling in the nuclear region suggests that saxitoxin, with its two positively charged guanidinium groups, may bind to nucleic acids or nuclear proteins in a manner analogous to the polyamines and other cations. The labelling patterns reported here suggest that the saxitoxins may not simply be secondary metabolites but instead could be important compounds involved in the structure and function of the* G. tamarensis *genome.*

Key index words: antibody; dinoflagellate; Gonyaulax tamarensis; *immunocytochemistry; immunofluorescence; saxitoxin*

Gonyaulax tamarensis Lebour (synonyms *Protogonyaulax tamarensis* and *Alexandrium tamarense* (Taylor 1979, Balech 1985) is one of several marine dinoflagellates that produce potent neurotoxins that can cause outbreaks of paralytic shellfish poisoning (PSP). One of the least understood aspects of this phenomenon is the physiological role of the toxin molecules in dinoflagellate metabolism. This is true despite considerable success in the isolation and chemical characterization of the 12 different toxins from *Gonyaulax* species. The toxins known from this group of dinoflagellates are the parent compound, saxitoxin, and its eleven derivatives (Schantz et al. 1975, Shimizu 1978, Hall 1982). These compounds have slightly different structures and widely varying toxicities (Hall and Reichardt 1984). A given *G. tamarensis* isolate may differ from others both in the relative amounts of the various toxins it contains (Hall 1982, Cembella et al. 1987) and in its overall toxicity (Alam et al. 1979, Maranda et al. 1985). It is also known that the toxicity of a given isolate can vary significantly with different growth stages or culture conditions (Hall 1982, Boyer et al. 1985). The biosynthetic pathway for saxitoxin remains unresolved, although it has recently been shown that the amino acids arginine and ornithine are precursors to the toxin (Shimizu et al. 1984).

These results all have a bearing on the general issue of toxin biosynthesis, but they do not address toxin function directly. One popular speculation has been that the toxins might be a nitrogen storage product (Loeblich 1984), yet no significant decrease in toxicity has been observed in nitrogen-limited cultures compared to controls in the same stage of growth but with an excess of nitrogen (Hall 1982). A hint of toxin functionality is found in the report of Mickelson and Yentsch (1979) who describe the co-fractionation of toxins with nucleic acids and speculate that saxitoxin might be bound to DNA or RNA and possibly act as a recognition signal during transcription (Abbott and White 1979).

In other cell types, the function of a particular metabolite is often inferred through comparisons between strains or mutants that do or do not produce the compound. In this context, non-toxic *G. tamarensis* isolates do exist, but they have no obvious growth deficiencies and have not been examined in detail or compared with toxic strains at the biochemical level other than through isozyme electrophoresis (Cembella and Taylor 1986, B. Hayhome, unpubl. data). In this paper, we address the question of toxin functionality using an immunocytochemical approach. We report the localization of saxitoxin in

[1] *Accepted: 30 September 1987.*
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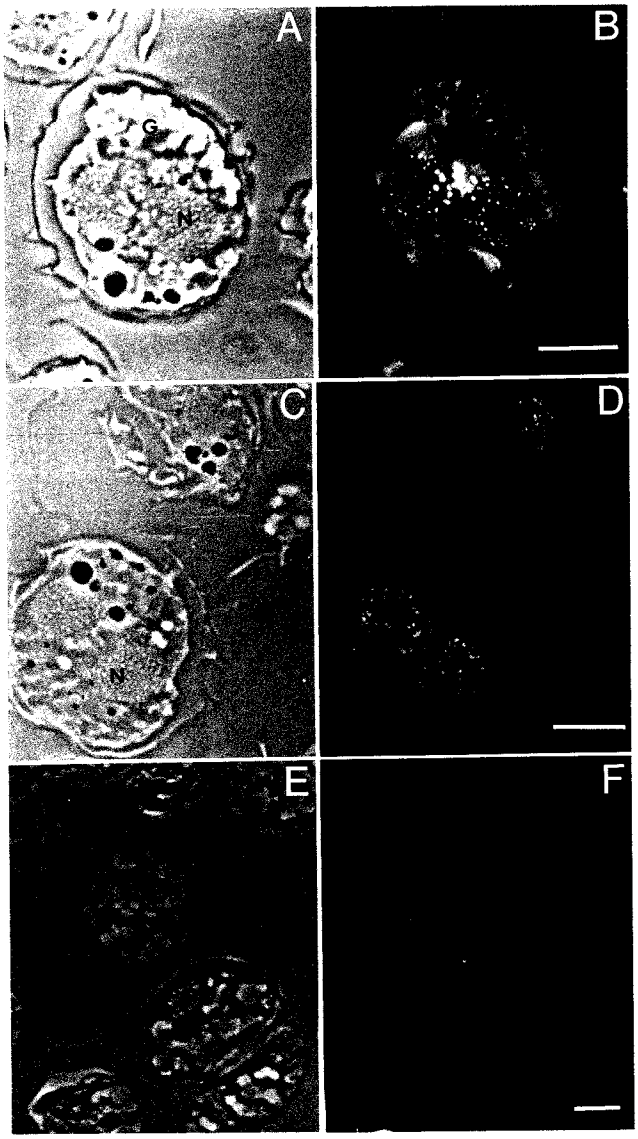

FIG. 1A–F. Phase and fluorescent images of *G. tamarensis*. Scale bar = 10 μm. A. Phase contrast image of a toxic *G. tamarensis* strain after fixation, embedding, and sectioning. Note the osmium-stained lipid bodies, starch grains (G) and the nucleus (N) with condensed chromosomes. B. Epifluorescent image of the same field as Fig. 1A, 1:3200 antibody dilution. Note the apparent labelling of the chromosomes, appearing as spots or rods depending on whether they were oriented perpendicular or parallel to the plane of the section. Some of the larger and brighter objects appear to lie between the two arms of the nucleus. The grains are not labelled. C. Phase contrast image, toxic strain, 1:3200 antibody dilution. The nucleus (N) appears as two circles. D. Epifluorescence image of the same field as Fig. 1C. E. Phase contrast image of non-toxic species, 1:100 antibody dilution. F. Epifluorescence image of the same field as Fig. 1E showing the absence of antigen labelling.

two types of cellular sites—one within the dinoflagellate nucleus and the other on the periphery of small granules thought to be starch grains. The binding of toxins to specific cellular sites not only provides new insights on function and biosynthesis but also has important implications with respect to the retention and accumulation of these toxins in the food chain.

MATERIALS AND METHODS

Two dinoflagellate isolates were used in this study, both members of the "*tamarensis* complex" (Taylor 1975), but one being toxic and the other non-toxic (unpubl. data). The toxic *Gonyaulax tamarensis* strain (GTCA29) was isolated from the Gulf of Maine near Cape Ann, MA. The non-toxic strain (GTM242) originates from Town Cove, Orleans, MA. That isolate is smaller than GTCA29 and has a characteristic 6th precingular plate on its theca that may justify its description as a new species (E. Balech, pers. comm.). Cultures were maintained in k medium (Keller and Guillard 1985) at 8° C on a 14:10 h LD cycle at approximately 150 $\mu E \cdot m^{-2} \cdot s^{-1}$ cool white fluorescent illumination.

During early exponential growth, cultures were harvested by centrifugation. Ten mL of a dense cell suspension were fixed for 10 min in buffered artificial seawater containing 4% paraformaldehyde and 0.5% glutaraldehyde and postfixed for 1 h in 2% osmium tetroxide in buffered artificial seawater. The pellets were dehydrated with an ethanol series and embedded in plastic for microtomy. Thin (0.5 μm) sections of the dinoflagellate cells were etched with sodium ethoxide and sodium meta-periodate (Maxwell 1978, Bendayen and Zollinger 1983) prior to 1 h incubations with various dilutions of antisera raised against saxitoxinol, an hydroxy-methyl derivative of saxitoxin. Preparation of the antiserum is described in Carlson et al. (1984). Sections were then treated with fluorescein (FITC) labelled goat antibody to rabbit IgG (1 h) and viewed and photographed with epifluorescence microscopy (Zeiss filter set 487710).

RESULTS

Figure 1A, C shows the phase contrast images and Figure 1B, D the corresponding fluorescent images of cells treated with a 1:3200 dilution of antibody. The FITC label is seen predominantly within the nucleus where the fluorescence has a constellation-type pattern of numerous individual spots. It is clear that many of these spots are within the nucleus, which has a distinctive horseshoe shape in *G. tamarensis*, but labelling also occurs between the two arms or lobes of the nucleus (Figs. 1B, 2B). The latter sites are elongate and significantly brighter than the smaller, circular spots within the nucleus. Using the light microscope, it is not possible to determine whether the elongate shapes are located within the nuclear membrane or outside of it but in close proximity.

The second general area of antibody labelling is seen at higher antibody concentrations. A 1:100 dilution results in labelling in two general types of sites—one in the nuclear region as described above, and the other around the edges of numerous small (2 μm) grains throughout the cell (Fig. 2). These granules stained purple when the sections were bathed in an iodine solution (Fig. 3). No label was seen in the chloroplasts, cell wall, general cell membranes, or osmium-stained lipid bodies at the light microscope level of magnification (Fig. 2).

A non-toxic species exposed to a 1:100 antibody dilution showed no label within the cells (Fig. 1E, F). Similar negative results were obtained when the toxic strain of *G. tamarensis* was treated with normal

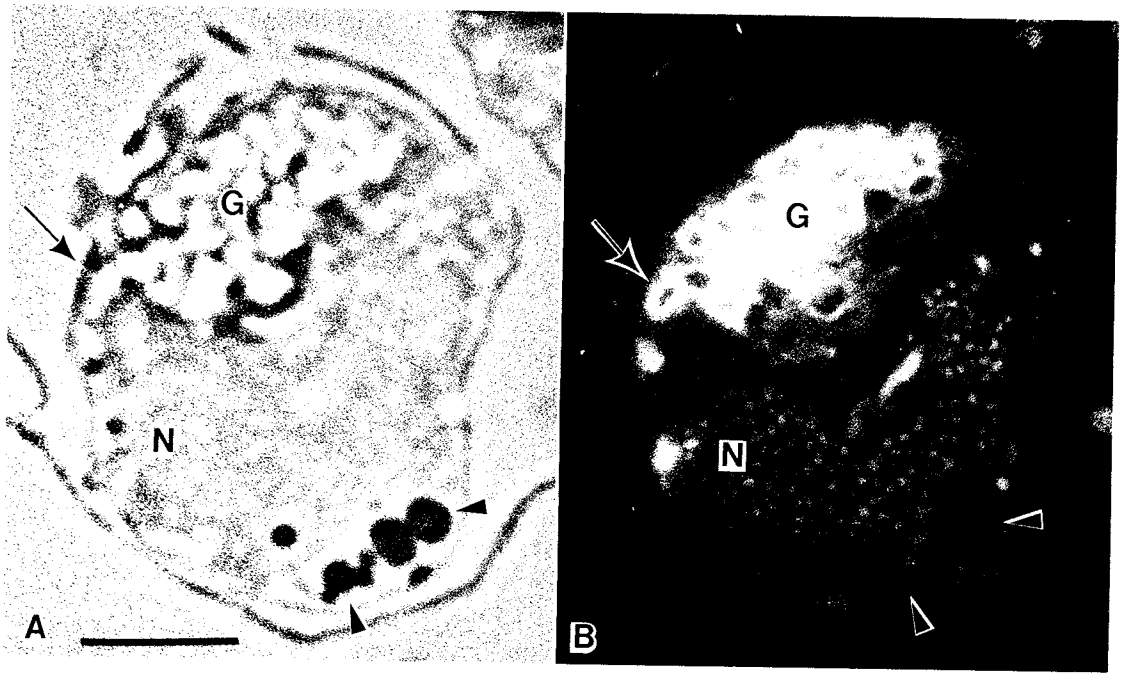

FIG. 2A, B. High magnification image of toxic cell treated with 1:100 antibody dilution. Scale bar = 10 μm. A. Phase contrast image highlighting the nucleus (N), darkly stained lipid bodies (arrowhead), and starch grains (G, arrow). B. Epifluorescence image of the same field as Fig. 2A. Note the labelling of the grains on the periphery only (arrow) as well as the chromosomes within the nucleus and a bright, elongate object between the two arms of the nucleus. The lipid bodies (arrowheads) and other organelles are not labelled.

rabbit serum or with a mixture of antibody and purified saxitoxin.

DISCUSSION

The immunofluorescent visualization of the localized nature of saxitoxin (and possibly some of its derivatives) in *G. tamarensis* cells provides valuable insights into the presently unknown role of those toxins within the dinoflagellate. Although it may be that some of the toxin was lost during the fixation and dehydration steps of our procedure and thus that our data only represent a partial picture of toxin distribution, the remaining toxin is clearly not dispersed throughout the cytoplasm but instead is localized in two general types of sites—one within or near the nucleus, and the other at the periphery of numerous small grains scattered throughout the cell. No labelling was observed with a non-toxic isolate, with a toxic isolate treated with an antibody/saxitoxin mixture, or with toxic cells treated with normal rabbit serum. We thus know that the binding observed within the cells is specific for antigens produced by toxic *G. tamarensis* and that in all likelihood, these antigens are the saxitoxins.

When the sections were exposed to different concentrations of antibody, the labelling was variable; at high concentrations, both the nuclear region and the grains were fluorescent. At low concentrations, only the nucleus was visible. Variation in the intensity of labelling at the two types of sites following treatment with different dilutions may reflect the polyclonal nature of the antibody preparation and different affinities of the antigenic sites for the antibody. This latter possibility is consistent with the fact that the toxic strain of *G. tamarensis* used in these experiments contains six of the twelve saxitoxins (saxitoxin, gonyautoxins 2, 3, and 4, toxin C2, and neosaxitoxin; unpubl. data). The saxitoxin antibody cross-reacts with the first three of these and possibly with C2 as well (Carlson et al. 1984). Small structural differences give these toxins different intrinsic potencies as a result of their varying affinities for the sodium channel (Hall and Reichardt 1984). Their binding affinities for an antibody raised against saxitoxin should differ as well , resulting in a variation in labelling intensity at different antibody dilutions. We are unable at this time to specify which of these toxins, alone or in combination with others, is responsible for the observed antibody labelling.

The identity of the localization sites will remain uncertain until ultrastructural analysis using the transmission electron microscope is complete, but we believe that the small grains are starch grains. These are commonly observed within *G. tamarensis*, and as seen in Figure 3, they stain purple with iodine. The pattern of the fluorescent labelling, which is visible only around the periphery of the grains (Fig. 2B), is similar in appearance to starch grains following iodine staining (Fig. 3) or fixation with osmium for transmission electron microscopy, both of which leave a clear region at the center of the granules and a dark ring around the perimeter. To our knowledge, the material being stained by the osmium in starch grains has not been identified; os-

FIG. 3. Bright field image, toxic strain, of cell section after treatment with iodine solution. Scale bar = 10 μm. The same grains (G) that label with antibody are purple following this treatment. Note that the iodine also stains only the periphery of these grains. Nucleus (N) is not stained.

mium does not bind to simple starch. We thus have no explanation at this time for the apparent localization of toxin at the surface of these grains.

The size, number, and orientation of labelled sites within the nucleus is consistent with binding on or near the chromosomes. Dinoflagellates have several unique nuclear features (Spector 1984) including: i) a large number of chromosomes (143 in *G. tamarensis* (Dodge 1963)) that remain condensed throughout all stages of vegetative growth; and ii) a lack of histones and a high level of divalent metal cations within those chromosomes (Ris 1962, Dodge 1963, Kearns and Sigee 1979). Given the immunocytochemical observations reported here, we can hypothesize a role for saxitoxin within the nucleus, based in part on the recent report that arginine and ornithine are precursors for the toxin (Shimizu et al. 1984). These amino acids are also precursors for polyamines, which, by virtue of their positively charged amino groups, are known to bind to anionic sites on DNA, RNA, and cell membranes (Slocum et al. 1984, Tabor and Tabor 1985). The function of this binding is not fully understood, but it does provide a degree of structural rigidity (Liquori et al. 1967), is linked to chromosome condensation in interphase mammalian cells (Rao and Johnson 1971) and is thought to increase the fidelity of transcription and translation (Tabor and Tabor 1985). We believe that the two positively charged guanidinium groups on the saxitoxins might allow these compounds to bind to sections of DNA, RNA, or perhaps nuclear proteins in a manner analagous to the polyamines. A close association with the dinoflagellate chromosomes might then be expected, with antibody labelling visible at the light microscope level due to the permanently condensed nature of those chromosomes. A similar role has been suggested for divalent metal cations in the dinoflagellate nucleus since they could also bind to the negatively charged sites on the DNA molecule (Kearns and Sigee 1980).

The hypothesized analogy between saxitoxin and polyamines is strengthened by our independent observations (unpubl. data) that variations in the rate and magnitude of toxin production in *G. tamarensis* closely parallel the dynamics of polyamine synthesis described for plants and other cell types (Slocum et al. 1984). This is seen in actively growing *G. tamarensis* cells whose toxin production is highest during exponential growth, as well as in stressed cells, where toxin accumulates to levels several times higher than is measured during growth under optimal conditions (Hall 1982; unpubl. data). Similar trends are observed in the patterns of polyamine synthesis in terrestrial plants during rapid growth or growth under temperature stress (Slocum et al. 1984). Although there is no *a priori* reason for saxitoxin localization in the nuclear region to be related to fluctuations in toxin production during growth, the well-documented connection between nucleic acid function and the size of polyamine pools in other cell types provides a useful analogy that is made more relevant by the amino acid precursors that these two systems have in common.

Localization of saxitoxin on or near the condensed *G. tamarensis* chromosomes is easily visible with the epifluorescence microscope, but the additional labelling observed near the nucleus is less distinct. As seen in Figures 1B and 2B, several larger objects fluoresce brightly in the region between the two arms of the horseshoe-shaped nucleus. Since we cannot tell where the plane of the microtome section cut the nucleus in these images, it is not possible to say whether this particular labelling is inside the nuclear membrane or outside. Some of the elongate objects are most likely the chromosomes cut in longitudinal section, since they are similar in width to the circular cross sections higher up in the arms of the nucleus. In some images however, the labelling between the arms of the nucleus is on objects significantly larger than the chromosomes (Figs. 1B, 2B). Until a companion study of toxin localization using the transmission electron microscope is complete, we can only offer the speculation that the observed labelling is within the nucleolus, an intranuclear organelle containing genes encoding ribosomal RNA (rRNA), rRNA precursors, rRNA-associated proteins, and enzymes required for synthesis of ribosomes. It is a site of intense RNA synthesis. In *G. tamarensis*, the nucleolus is located inside

the nuclear membrane and within the region between the two arms of the nucleus (Fritz 1986).

The binding between the antibody and nuclear material in our toxic strain of *G. tamarensis* is consistent with reports of the co-fractionation of toxicity with nucleic acids in extracts from this same species (Mickelson and Yentsch 1979) and with the release of saxitoxins from high molecular weight fractions of the digestive gland of toxic scallops following treatment with RNase (Kodama et al. 1982). In fact, based on their RNase data, Kodama et al. (1982) suggested that saxitoxin may be a constituent of *G. tamarensis* RNA.

More work is clearly needed to provide physiological explanations for these immunocytochemical observations. Not only do we need to examine toxin localization at the ultrastructural level for this species and for other toxin producers, but we also need to determine the distribution of each of the saxitoxins and the identity of the molecules to which the binding is occurring, be it DNA, RNA, or some other nuclear component. In this context, it is noteworthy that the change in labelling patterns observed with different antibody dilutions (Fig. 1B vs. 2B) suggests that the individual toxins may be spatially segregated within the cell. We also must examine non-toxic strains more closely, since it is possible that they produce compounds that are structurally and functionally similar to saxitoxin within the cell but that are not poisons due to small differences in conformation or charge.

Our results, combined with the recently proposed biosynthetic pathway for saxitoxin (Shimizu et al. 1984), provide a useful framework from which the metabolic role of toxins within the dinoflagellate and the mechanisms for the accumulation and movement of these toxins through the food chain can be addressed. The labelling patterns suggest that these toxins (and possibly their non-toxic analogues) are not simply secondary metabolites but instead may be important cellular constituents involved in the growth and replication of the dinoflagellates.

Research was supported in part by National Science Foundation, Grants OCE-8315262 and OCE-8614210, by the Office of Sea Grant, National Oceanic and Atmospheric Administration through grant NA86AA-D-SG090 (R/B 76) and by the National Institutes of Health. We thank Biometric Systems Inc., L. Provasoli, P. Presley, and S. Hall for assistance and discussions. Contribution No. 6395 from the Woods Hole Oceanographic Institution.

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