LETTER TO THE EDITOR

[Brief letters to the Editor that make specific scientific reference to papers published previously in THE JOURNAL OF GENERAL PHYSIOLOGY are invited. Receipt of such letters will be acknowledged, and those containing pertinent scientific comments and scientific criticisms will be published.]

On the Interaction of Tetrodoxin with Cholesterol Monolayers

Dear Sir:

In previous works published in this journal (1, 2), we described the interactions of tetrodotoxin (TTX) and saxitoxin (STX) with monolayers of cholesterol and some cholesterol derivatives. It has recently been suggested (3) that the expansion of the cholesterol monolayers at the liquid-air interface caused by TTX may be due to a surfactant impurity of the phosphate salts used in previous works to buffer the subphase, and/or to a time-dependent cholesterol modification at the interface. Although our experimental design allows discarding such causative agents, we study again the TTX-cholesterol monolayer interaction, taking these factors, specifically, into consideration.

A detailed description of the method is given in references 1 and 2. In the present experiments, either a 0.15 M NaCl solution (free of phosphate buffer) or tridistilled water was used as liquid subphase. Citrate-free TTX (Sankyo Co., Tokyo, Japan) dissolved in 0.01 M acetic acid, or the acid alone in the paired control experiments, was added to the subphase before spreading the cholesterol. The final TTX concentration was 1×10^{-6} M. The pH of the subphases was 5.0 and the temperature 25°C. Cholesterol (Applied Science Laboratories Inc., State College, Pa.), dissolved in chloroform-methanol (85:15 vol/vol) was spread on the liquid surface in successive 1.0-μl additions at constant time intervals, while recording the surface tension; the time required to spread the monolayers was 25 min (range 20–30 min).

The expansions of the monolayers caused by TTX on the 0.15 M NaCl solution (free of phosphate buffer) and on the tridistilled water subphases, are 2.2 and 1.6 A^2/molecule, respectively (Fig. 1), similar to those previously observed (1). These results allow excluding as cause of the expansions (*a*) a surfactant contaminant suggested to be present in the phosphate salts used in previous works, and (*b*) a cholesterol modification at the interface, since the maximum time-dependent expansion observed on a pure water subphase (free of TTX) is less than 0.3 A^2/molecule after 40 min (3).

It should be remarked that we have always calculated the expansion caused by TTX from paired experiments, one without and the other with TTX, the other experimental conditions being the same, and that the surface tension was recorded while

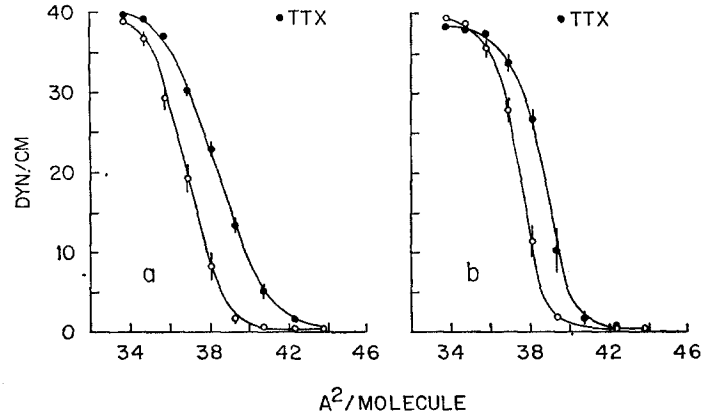

FIGURE 1. Surface pressure-area diagrams of cholesterol monolayers spread on 1 × 10^{-6} M TTX (solid circles) and TTX-free (open circles) subphases. The subphases were: (*a*) 0.15 M NaCl solution free of phosphate buffer, and (*b*) tridistilled water; pH 5.0; temperature 25°C. Each value is the mean ± standard error of six experiments.

spreading the cholesterol. It is possible that when the surface tension is obtained by compression of the film (3), the contribution of any surfactant contaminant is maximized.

Further evidence about the stability of cholesterol at the liquid-air interface, under our experimental conditions, was obtained from monolayers prepared with the same cholesterol solution to which 1 μCi of 4-[^{14}C]cholesterol/ml (New England Nuclear, Boston, Mass.) was added. The subphases were the same solution buffered with phosphate used in previous works (1, 2), or tridistilled water, both containing 1 × 10^{-6} M TTX. After spreading the monolayers in 30 min, 5 ml of hexane were added and then about 2.5 ml were withdrawn with glass syringes and evaporated under nitrogen. The residues dissolved in 0.3 ml of chloroform-methanol (2:1 vol/vol) and a control sample of the [^{14}C]cholesterol were applied to a silica gel plate which was developed with petroleum ether:diethyl ether (80:20 vol/vol). Each lane was divided into 17 segments, each segment suspended in 10 ml of Instagel counting solution (Packard Instrument Co., Downers Grove, Ill.), and the radioactivity determined in a liquid scintillation spectrometer (Tricarb, model 3320, Packard Instrument Co.).

The radioactivity distribution in all the thin layer chromatograms shows a single peak of radioactivity corresponding to cholesterol (Fig. 2). No other molecular species that could be separated by thin layer chromatography was observed. The oxidation products of cholesterol, if present, would have remained at the origin of the chromatograms (4).

Since the monolayer was found to reproduce some of the characteristics of the axolemma receptors (see references 1 and 2), two alternative interpretations of the toxin-monolayer interaction were proposed: (*a*) that cholesterol may be a component of the actual receptor, or (*b*) that the TTX and STX receptor may share some chemical features with the monolayer, the interaction being a property of the monolayer

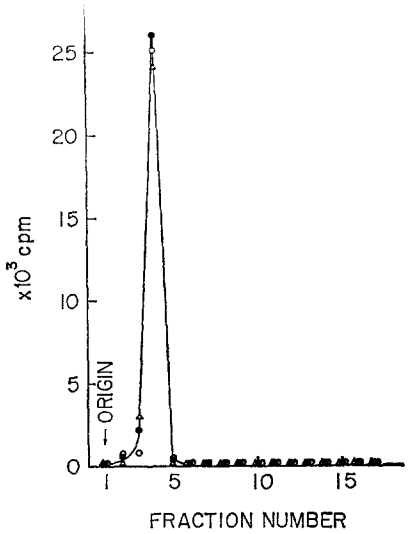

FIGURE 2. Distribution of radioactivity in thin-layer chromatograms of [^{14}C]cholesterol recovered from labeled cholesterol monolayers spread on 1×10^{-6} M TTX subphases. The subphases were 0.15 M NaCl solution buffered at pH 7.4 with 0.01 M phosphate (open circles), and tridistilled water at pH 5.0 (open triangles); temperature 25°C. The radiochromatogram of the original [^{14}C]cholesterol (solid circles) is also shown. The fractions correspond to segments of the chromatographic plate.

rather than of the individual cholesterol molecule. The differences found between the interactions of the toxins with the monolayer and with the receptor (e.g. disagreements of the dissociation constants and of the effects of pH), made the latter explanation the most likely. Should this be the case, the study of the film may help to gain knowledge about the true structure of the actual receptor.

Received for publication 1 August 1974.

RAIMUNDO VILLEGAS
FLOR V. BARNOLA
GERMÁN CAMEJO
Centro de Biofísica y Bioquímica
Instituto Venezolano de Investigaciones Científicas (IVIC)
Caracas 101, Venezuela

REFERENCES

1. VILLEGAS, R., F. V. BARNOLA, and G. CAMEJO. 1970. Ionic channels and nerve membrane lipids. Cholesterol-tetrodotoxin interaction. *J. Gen. Physiol.* 55:548.
2. VILLEGAS, R., and F. V. BARNOLA. 1972. Ionic channels and nerve membrane constituents.

Tetrodotoxin-like interaction of saxitoxin with cholesterol monolayers. *J. Gen. Physiol.* **59**:33.

3. GACESA, P., M. W. HILL, and A. D. BANGHAM. 1973. Tetrodotoxin-cholesterol interactions at the air-water interface. *Biochim. Biophys. Acta.* **307**:467.
4. KAMEL, A. M., N. D. WEINER, and A. FELMEISTER. 1971. Autoxidation of cholesterol monomolecular films. *J. Colloid. Interface Sci.* **35**:163.