Ionic Channels and Nerve Membrane Lipids

Cholesterol-tetrodotoxin interaction

RAIMUNDO VILLEGAS, FLOR V. BARNOLA, and GERMAN CAMEJO

From the Centro de Biofisica y Bioquímica, Instituto Venezolano de Investigaciones Científicas (IVIC), Apartado 1827, Caracas, Venezuela

ABSTRACT Experiments were carried out to investigate possible interactions of tetrodotoxin (TTX) with lipid molecules isolated from nerve fiber plasma membranes of the squid Dosidicus gigas. TTX has a highly selective ability to block the channel normally used by Na+ to cross the axolemma during nervous impulse conduction. In order to investigate the interaction each lipid sample was spread on 5 × 10^-7 M TTX and TTX-free 0.15 M NaCl solutions adjusted to pH 7.4 with 7 × 10^-3 M phosphate buffer. The surface pressure–area diagrams of the lipid monolayers revealed that TTX interacts only with cholesterol. The expansion of the cholesterol monolayers at 5 × 10^-7 M TTX was 2 Å²/molecule at zero pressure for the experiments at 20°C and 2.5 Å²/molecule for those at 25°C. Similar results were obtained in KCl subphases. The apparent dissociation constant of the cholesterol-TTX complex calculated from dose-response experiments is 2.6 × 10^-7 M. Experiments at pH 10.1 revealed that the zwitter ionic form of TTX is less active. Experiments with cholesterol derivatives (cholesteryl acetate, cholesterol methyl ether, cholestanol, and cholesteryl acetate) indicate that for the interaction with TTX a partial negatively charged group at C-3 and a double bond between C-5 and C-6 on the steroid nucleus are required. Tetrodonic acid, a biologically inactive derivative of TTX, does not interact with cholesterol. The results lead us to propose that cholesterol is part of the Na+ channel.

A significant proportion of nerve membranes consists of lipids (1). However little is known about their precise function. Some experimental results indicate that polar lipids may be related to the excitation and conduction phenomena (2–6). Furthermore, recent experiments on the nonelectrolyte permeability of the axolemma revealed that during conduction of the nervous impulse a transient widening of the hydrophilic pores takes place, as well as a transient opening of hydrophobic pathways through the nonpolar regions of membrane lipids (7, 8). It has been suggested that at least part of these hydrophilic and hydrophobic routes may correspond to the channels used by Na+ and K+ to cross the axolemma during activity (7, 8).
Tetrodotoxin (TTX) has a highly selective ability to block the pathways normally used by Na⁺ to move through the axolemma during the nervous impulse and during the transient early phase of conductance increase in voltage-clamped axons (9-14). Therefore, TTX appears at present to be the finest tool available to deal with the problem of identifying the molecule or molecules of the axolemma associated with the Na⁺ channel. It thus follows that a detailed investigation of the interaction between TTX and lipids isolated from nerve membranes may provide useful information about the chemical nature of the Na⁺ channel.

The present work deals with the interaction between lipid molecules isolated from squid nerve fiber plasma membranes and TTX, as measured by the effect of TTX on the surface pressure-area (π-A) diagrams of lipid monolayers. The results revealed a highly specific interaction between TTX and cholesterol. Dose-response experiments suggested that the expansion of the π-A diagram of cholesterol caused by TTX is due to the interaction of single TTX molecules with only one type of site at the monolayers. The site might be some repeating structure formed by cholesterol. Experiments with cholesterol derivatives revealed that for the interaction with TTX, a partial negatively charged group at C-3 and a double bond between C-5 and C-6 on the steroid nucleus are required. Experiments at high pH indicated that the zwitter ionic form of TTX is less active than its cationic form. No interaction was found between cholesterol and tetrodonic acid (TTA), a biologically inactive derivative of TTX (15). These findings suggest that cholesterol is part of the Na⁺ channel in the axolemma.

Preliminary reports of some of the data have been published (16, 17).

**EXPERIMENTAL METHODS**

*Isolation of Nerve Fiber Membranes*

The first stellar nerves of the squid, *D. gigas*, were isolated from the living animal. The nerves were immediately packed in dry ice and mailed from Lima to Caracas where they were processed. Since the method used for isolation of the nerve fiber membranes has been described in detail (see reference 1), only an outline of the procedure is given in Fig. 1. At the end two membrane fractions were obtained. The pellets containing membrane fractions I and II were suspended separately in 5 × 10⁻⁴ M Tris-HCl buffer at pH 7.4.

*Lipid Extraction and Fractionation*

The lipids of the two membrane fractions were extracted according to the method of Folch, Lees, and Sloane-Stanley (18). The total lipids from each membrane fraction were then fractionated by preparative thin layer chromatography into a polar and a nonpolar fraction. The plates were prepared with silica gel H (Merck-Darmstadt, Germany) and developed with petroleum ether:ethyl ether:acetic acid (85:15:1 v/v). Appropriate standards were run in each plate to identify the lipids, which were
Homogenate, 4 vol 0.33 M sucrose/volume tissue

Supernatant, discarded
Pellet suspended in 90 ml 0.33 M sucrose

Layered on:
Diluted 3x with buffer
Layered on:
30 ml 1.195 M sucrose

74000 x g, 60 min

Supernatant, discarded
Pellet diluted 3x with buffer

70000 x g, 15 min

Diluted 3x with buffer
Pellet diluted 3x with buffer

buffer = 5 x 10^{-3} M Tris-HCl buffer at pH 7.4

Figure 1. Outline of the procedure utilized to isolate the plasma membrane fractions I and II. Membrane fraction I (M-I) bands at a density of 1.090 g/ml and membrane fraction II (M-II) at 1.140 g/ml. For a detailed description of the procedure see reference 1.

visualized by spraying the plates with water. The polar lipids remaining at the origin and the nonpolar lipids running as cholesterol, fatty acids, and hydrocarbons, were scraped off the plate. The silica gel containing each lipid sample was then placed in a small glass column and the lipid eluted from the gel with chloroform-methanol (1:1 v/v). The eluates were centrifuged at 2000 rpm for 5 min to eliminate residual
silica gel. Then, 0.2 volume of H2O was added to the eluates, which were washed according to the procedure of Folch et al. (18). The lower chloroform phase was evaporated to dryness under N2 and the dry lipids dissolved in chloroform-methanol (85:15 v/v). The lipid solutions were kept in amber glass bottles with Teflon-lined screw caps at approximately 4°C until used.

The concentration of the solutions of total lipids, polar lipids, mixtures of nonpolar lipids, and of the individual nonpolar lipids of each membrane fraction was established by dry weight determinations. Appropriate aliquots were placed in tared aluminum pans, the solvent was evaporated by placing the pans on a hot plate at 60°C and then weighed in a Cahn RG electric recording balance (Cahn Instrument Co., Calif.) with a sensitivity of 4 µg.

Analytical Methods

The quantitative composition of membrane fractions I and II was established by gravimetric and colorimetric methods (19). Tentative identification of the lipid components was obtained by one and two dimensional thin layer chromatography in at least four solvent systems (20, 21). The fatty acids and hydrocarbon were tentatively identified by thin layer and gas liquid chromatography against appropriate standards.

Surface Pressure Measurements

The surface pressure–area (σ-A) diagrams of the membrane lipids were obtained using the Wilhelmy method (22). The surface pressure was continuously monitored by means of a sand-blasted platinum plate with a perimeter of 2.02 cm suspended from a Cahn RG automatic recording balance. The Kel-F coated trough of 100 ml capacity of the Cahn surface tension attachment was used. As liquid subphase a 0.15 mM NaCl solution adjusted to pH 7.4 with 7 × 10⁻⁴ M phosphate buffer was utilized. In some experiments the subphase was adjusted to pH 10.1 with 7 × 10⁻⁴ M bicarbonate buffer. In other experiments NaCl was replaced by KCl. The area of the liquid-air interface was 71.08 cm². The temperature of the solution was kept constant either at 20° or 25°C by circulating water around the trough.

The membrane lipids dissolved at concentrations of 0.35–1.0 mg/ml in chloroform-methanol (85:15 v/v) were spread in successive 1 µl additions on the liquid surface by means of a 10 µl Hamilton microsyringe (Hamilton Co., Calif.) or a 50 µl microsyringe with a Hamilton PB600 dispenser attachment.

In order to investigate the effect of TTX or TTA on the σ-A diagrams, one of the substances was added to the subphase before spreading the lipids. TTX was obtained from Calbiochem (Los Angeles, Calif.) as a lyophilized powder containing citrate buffer. TTA, a kind gift of Dr. Toshio Narahashi from the Department of Physiology and Pharmacology, Duke University Medical Center, Durham, N.C., was dissolved in citrate buffer at pH 5. Only citrate buffer was added to the subphase in the TTX-free and TTA-free experiments.

The water used to prepare the solutions was tridistilled in an all-glass apparatus in the presence of KMnO₄. All solvents and salts were analytical reagent grade.
RESULTS

Composition of Membrane Fractions I and II

Membrane fraction I contains 29.5% proteins and 70.5% lipids; membrane fraction II is made up of 48.3% proteins and 51.7% lipids (see reference 1). The percentage weight composition of the lipids isolated from membrane fractions I and II is given in Table I.

Evidence has been presented which indicates that membrane fractions I and II should correspond to plasma membranes of different cells: membrane fraction I to the axolemma, and membrane fraction II to the plasma membrane of the periaxonal cells, mainly of the Schwann cells (see reference 1).

| Membrane fraction | Polar lipids | Nonpolar lipids |
|-------------------|--------------|-----------------|
|                   | Cholesterol  | Fatty acids     | Hydrocarbon |
| I                 | 58.5 ± 3.5   | 28.1 ± 2.3      | 6.2 ± 0.9   | 7.3 ± 1.2 |
| II                | 66.4 ± 2.1   | 25.2 ± 1.7      | 6.2 ± 1.0   | 2.1 ± 0.3 |

Values are the mean ± standard error of five membrane preparations analyzed. Membrane fraction I contains 29.5% proteins and 70.5% lipids; membrane fraction II contains 48.3% proteins and 51.7% lipids (see reference 1).

This evidence is: (a) the yield of membrane fractions I and II, which are 4 and 15 mg/g of nerve protein, respectively (1); (b) their morphological appearance and thickness at high resolution electron microscopy, which are similar to those described for the axolemma and Schwann cell membranes in the intact nerve fibers of *D. gigas* (23); and (c) their content of Mg++-, Na+-, K+-dependent cardiac glycoside-sensitive adenosine triphosphatase, higher in membrane fraction I than in membrane fraction II, in agreement with the observations that revealed a higher content of this ATPase in the axolemma than in the Schwann cell membrane (24). However, as shown in Table I, there is not a marked difference between the lipid compositions of the two membrane fractions. Experiments were carried out with the lipids isolated from the two membrane fractions.

**Interaction of TTX with Monolayers of Membrane Lipids**

Fig. 2 shows π-A diagrams of monolayers of total lipids isolated from membrane fractions I and II spread on 5 or 6 X 10⁻⁷ M TTX and TTX-free subphases at 25°C. They reveal that TTX produces expansion of the total lipid monolayers of both membrane fractions.
Figure 2. Surface pressure–area diagrams of monolayers of total lipids isolated from (a) membrane fraction I and (b) membrane fraction II, spread on TTX-free subphases (open circles), and subphases containing (a) $6 \times 10^{-7}$ M TTX and (b) $5 \times 10^{-7}$ M TTX (solid circles). Each value represents the mean ± standard error of 14 experimental measurements for (a), and 8 for (b). Subphase, 0.15 M NaCl solution; pH 7.4; temperature 25°C.

Figure 3. Surface pressure–area diagrams of monolayers of polar lipids isolated from (a) membrane fraction I and (b) membrane fraction II, spread on TTX-free subphases (open circles), and subphases containing $5 \times 10^{-7}$ M TTX (solid circles). Each value represents the mean ± standard error of three experimental measurements for (a), and five for (b). Subphase, 0.15 M NaCl solution; pH 7.4; temperature 25°C.

Fig. 3 shows the π-A diagrams corresponding to the polar lipid monolayers of membrane fractions I and II spread on $5 \times 10^{-7}$ M TTX and TTX-free subphases at 25°C. It can be observed that TTX does not affect the monolayers of the polar lipids.

Fig. 4 shows the effect of TTX on monolayers of nonpolar lipids isolated
Figure 4. Surface pressure–area diagrams of monolayers of nonpolar lipids isolated from (a) membrane fraction I and (b) membrane fraction II, spread on TTX-free (open circles), and $5 \times 10^{-7} \text{M}$ TTX-containing subphases (solid circles). Each value is the mean ± standard error of seven experimental measurements for (a), and five for (b). Subphase, 0.15 M NaCl solution; pH 7.4; temperature 25°C.

Figure 5. Surface pressure–area diagrams corresponding to monolayers of (a) all nonpolar lipids of membrane fraction I, (b) the fatty acids–hydrocarbon mixture from the same fraction, and (c) cholesterol, spread on TTX-free subphases (open circles) and subphases containing $5 \times 10^{-7}$ M TTX (solid circles). Each value represents the mean ± standard error of seven experimental measurements for (a), three for (b), and 11 for (c). Subphase, 0.15 M NaCl solution; pH 7.4; temperature 25°C.

from membrane fractions I and II spread on $5 \times 10^{-7}$ M TTX and TTX-free subphases at 25°C. The diagrams reveal that TTX produces expansion of the nonpolar lipid monolayers of both membrane fractions.

In order to establish which molecule or molecules are responsible for the
TTX-caused expansion of the nonpolar lipid monolayers, experiments with the components of the nonpolar lipid mixtures were carried out. Since the largest expansion of the monolayers was observed with the nonpolar lipids obtained from membrane fraction I, their components were used.

![Figure 6](image)

**Figure 6.** Surface pressure–area diagrams of cholesterol monolayers spread on $5 \times 10^{-7}$ M TTX (solid circles) and TTX-free subphases (open circles). Experiments carried out at (a) 20°C, and (b) 25°C. Each value is the mean ± standard error of 8 experimental measurements for (a), and 11 for (b). Subphase, 0.15 M NaCl solution; pH 7.4.

![Figure 7](image)

**Figure 7.** Dosage-response of cholesterol monolayers to TTX. The expansion of cholesterol in $A^2$/molecule is plotted as a function of the molar concentration of TTX in the liquid subphase. The apparent dissociation constant used to calculate the curve is $2.6 \times 10^{-7}$ M. The values in parentheses are the number of experiments corresponding to each TTX concentration. Subphase, 0.15 M NaCl solution; pH 7.4; temperature 25°C. Experiments performed by Dr. V. Davila.

With the use of thin layer chromatography, as described in Experimental Methods, the nonpolar lipids of membrane fraction I were separated into a fatty acids–hydrocarbon mixture and cholesterol. Fig. 5 shows the $\pi$-$A$ diagrams corresponding to (a) all nonpolar lipids of membrane fraction I, (b) the fatty acids–hydrocarbon mixture, and (c) cholesterol, each of them spread on $5 \times 10^{-7}$ M TTX and TTX-free subphases at 25°C. The results show that TTX does not affect the monolayers of the fatty acids–hydro-
carbon mixture, but it does produce an expansion of the cholesterol monolayers similar to that found in the monolayers prepared with all the nonpolar lipids of membrane fraction I.

Interaction of TTX with Cholesterol

Fig. 6 shows the \( \pi - A \) diagrams of purified cholesterol spread on \( 5 \times 10^{-7} \text{ M} \) TTX and TTX-free subphases at 20°C and 25°C. The diagrams reveal that TTX causes, as described above, expansion of the cholesterol monolayers. The expansion caused by TTX at the concentration used was 2 A\(^2\)/molecule at zero pressure for the experiments performed at 20°C and 2.5 A\(^2\)/molecule for those at 25°C. Similar results were obtained with subphases containing NaCl or KCl.

Dose-Response Experiments

Further information on the binding of TTX to cholesterol may be obtained from a dose-response curve made by plotting the expansion of the cholesterol monolayers as a function of TTX concentration in the subphase. This approach is similar to that followed by Hille (14) to investigate the binding of TTX to the membrane of myelinated nerve fibers.

Fig. 7 shows the expansion of the cholesterol monolayers at various concentrations of TTX. The experimental data are well-fitted by a rectangular
hyperbola calculated by assuming that a small fraction of the total cholesterol expansion is due to the association of one TTX molecule with cholesterol. The sigmoid curve shown in Fig. 7 is a semilogarithmic plot of the rectangular hyperbolic function. Under the present experimental conditions the apparent dissociation constant of the cholesterol-TTX complex appears to be $2.6 \times 10^{-7}$ M. This value was used to calculate the curve shown in Fig. 7. The simplest interpretation of the dose-response curve is that one TTX molecule interacts with a single type of site at the cholesterol monolayers.

**Experiments with Cholesterol Derivatives**

In an effort to explore the possible structural features of cholesterol which may be responsible for its interaction with TTX, the effect of this toxin on monolayers of cholesteryl acetate, cholesterol methyl ether, cholestanol, and cholestanyl acetate was investigated.

Fig. 8 shows the $\pi$-A diagrams of cholesteryl acetate and cholesterol methyl ether monolayers spread on $5 \times 10^{-7}$ M TTX and TTX-free subphases at $25^\circ$C. The diagrams indicate that TTX produces an expansion of the cholesteryl acetate monolayers but not of those of the methyl ether derivative.

Fig. 9 shows the $\pi$-A diagrams of cholestanol and cholestanyl acetate monolayers spread on $5 \times 10^{-7}$ M TTX and TTX-free subphases at $25^\circ$C. The diagrams reveal that TTX does not interact with the monolayers of these molecules.
Experiments with TTX at High pH

Experiments with the liquid subphase buffered with bicarbonate to pH 10.1, instead of pH 7.4, were carried out. The results shown in Fig. 10 indicated that at this high pH the toxin has a small effect, if any, on the cholesterol monolayers.

Experiments with Tetrodonic Acid (TTA)

Experiments with TTA added to the liquid subphase instead of TTX were carried out. The results of these experiments, shown in Fig. 11, revealed that $5 \times 10^{-7}$ M TTA produces no expansion of the $\pi$-A diagrams of cholesterol at 25°C. Experiments performed at 20°C also revealed no effect of TTA.

Figure 10. Surface pressure–area diagrams of cholesterol monolayers spread on $5 \times 10^{-7}$ M TTX (solid circles) and TTX-free subphases (open circles) at pH 10.1. Each value is the mean ± standard error of six experimental measurements. Subphase, 0.15 M NaCl solution; temperature 25°C.

Figure 11. Surface pressure–area diagrams of cholesterol monolayers spread on subphases containing $5 \times 10^{-7}$ M TTA (tetrodonic acid, solid circles) and TTA-free subphases (open circles). Each value is the mean ± standard error of six experimental measurements. Subphase, 0.15 M NaCl solution, pH 7.4; temperature 25°C.
DISCUSSION

The experiments described in the preceding paragraphs revealed that TTX interacts with the total lipids and the nonpolar lipids isolated from nerve fiber plasma membranes of the squid, *D. gigas*. We have identified cholesterol as the molecule in the nonpolar lipids with which TTX interacts.

The interaction of TTX with cholesterol monolayers is revealed by an expansion of their π-A diagrams. Dose-response experiments, similar to those carried out by Hille (14) to investigate the binding of TTX to the membrane of myelinated nerves, have shown that the increment in area per cholesterol molecule is a function of TTX concentration in the subphase. The data are well-fitted by a rectangular hyperbola calculated by assuming an apparent dissociation constant for the cholesterol-TTX complex of $2.6 \times 10^{-7}$ M. The sigmoid curve shown in Fig. 7 is a semilogarithmic plot of the rectangular hyperbolic function. The simplest interpretation of the dose-response curve is that one TTX molecule interacts with a single type of site at the cholesterol monolayers. This site might be some repeating structure formed by cholesterol.

The value obtained for the apparent dissociation constant of the cholesterol-TTX complex is about $10^2$ times higher than that calculated by Hille (14) for TTX with the frog nerve membrane. The difference between the dissociation constants might be due to the differences between the two systems. It should be noticed that the value of $2.6 \times 10^{-7}$ M calculated for the cholesterol-TTX complex is extremely low and thus cannot be accounted for by nonspecific interaction. The apparent dissociation constant is only valid for given experimental conditions and is somewhat dependent on the TTX batch used.

The interaction between cholesterol and TTX is rather specific. Two structural features of the steroid appear to be required: (a) a partial negatively charged group at C-3, and (b) a double bond between C-5 and C-6.

The importance of the functional group at C-3 in the steroid is revealed by the experiments with cholesteryl acetate and cholesterol methyl ether. The acetate ester favors the interaction and the methyl ether precludes the effect of TTX. The absence of interaction observed with the methyl ether derivative is probably due to a reduction of the polarity around C-3. That a partial negatively charged group at C-3 in the steroid molecules plays a key role suggests that besides steric considerations, a positive group in the TTX molecule is involved in its interaction with cholesterol. The experiments carried out at pH 10.1 support this latter proposal. Ionization of the hemilactal link of TTX, which has a pKₐ of about 8.8, converts the cationic to the zwitter ionic form at high pH. At pH 10.1, no interaction of TTX with cholesterol is observed. This result may be related to the observations which revealed that
only the cationic form of the toxin is biologically active and therefore capable of producing the blockage of the sodium channel in the axolemma (14, 25).

The requirement of a double bond between C-5 and C-6 for the interaction with TTX of the steroid molecules having a partial negatively charged group at C-3 is demonstrated by the experiments with cholestanol and cholestanyl acetate. It is possible that the unsaturation has an inductive effect favoring the localization of a partial negative charge at C-3 and/or that by locking the steroid ring, the double bond provides the specific steric conformation of the steroid required for the interaction with TTX.

The finding that TTA, obtained from TTX by opening its hemilactal link, does not affect cholesterol monolayers indicates that the intact structure of TTX is required for its interaction with cholesterol. This finding may be related to the absence of toxicity of TTA. Narahashi, Moore, and Poston (15) have suggested that the absence of biological activity of TTA is due to the presence of a negative charge close to the guanidinium group positive charge. This interpretation favors our postulate that in the interaction between TTX and cholesterol, the positively charged guanidinium group of TTX and the partial negatively charged group at C-3 in cholesterol are essential for the association of these molecules.

The most relevant result of this work is perhaps the demonstration of a specific interaction between TTX and cholesterol, an important lipid component of nerve fiber plasma membranes. Since TTX specifically blocks the Na\(^+\) channel in living axons, it is tempting to suggest that cholesterol is a constituent of the Na\(^+\) channel.

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