# Binding of Tetrodotoxin to Squid Nerve Fibers

## *Two Kinds of Receptors?*

CARLOS SEVCIK

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

ABSTRACT The effect of tetrodotoxin on the sodium currents of the squid (*Doryteuthis plei* and *Sepioteuthis sepiodea*) giant axons was studied under potential control conditions. The axons were immersed in artificial seawater at 21°C and pH 7.5. When the effect of the toxin is studied in concentrations ranging from 0.1 to 50 nM the Eadie-Haldane plot is not a straight line and indicates that there are two populations of sodium channels open during activity. 19.0 ± 4.7% of the channels are associated to receptors with an apparent dissociation constant of 0.11 ± 0.05 nM and 84.0 ± 4.1% of the channels are related to receptors having an affinity constant of 4.90 ± 0.49 nM (nine nerves).

### INTRODUCTION

Tetrodotoxin (TTX) is a fish poison well known for its ability to block the sodium current during activity in squid nerve and other preparations (1–3). The poison possesses a high affinity for the nerve membrane. Cuervo and Adelman (4) studied the effects of toxin concentrations ranging from 1 to 25 nM and found that in the squid *Loligo pealei*, 27% of the sodium current is suppressed irreversibly by tetrodotoxin and the remaining effect is fully reversible with an apparent dissociation constant of 3.31 nM. Similar results were reported by Schwarz et al. (12) which determined the rates of association and dissociation of TTX in nodal membrane from *Xenopus laevis* and *Rana esculenta*. Concentrations of the drug ranging from 0.31 to 31 nM were employed for *Xenopus*, and from 3.1 to 31 for *Rana*. The authors estimated a value of 3.6 nM as the dissociation constant in *Rana* and 4.08 in *Xenopus*. Other estimates in different nerve fibers employing parameters which are not linearly related to the sodium current, such as action potential amplitude, action potential rate of rise, or conduction velocity, have also been published by Keynes et al. (13) and Colquhoun and Ritchie (14, 15).

We have analyzed the effect of tetrodotoxin on the giant nerve fibers of the squids *Doryteuthis plei* and *Sepioteuthis sepiodea*. The suppression of the sodium current was studied under potential control conditions starting from toxin concentrations one order of magnitude lower than Cuervo and Adelman, ranging from 0.1 to 50 nM and at 21°C. Under such conditions we have found that the Eadie-Haldane (5, 6) transformation of the TTX dose-response curve is not a straight line; the experimental points may be fitted remarkably well with a line

assuming the existence of two kinds of tetrodotoxin receptors with apparent dissociation constant at least one order of magnitude different.

## METHODS

Giant axons of the tropical squid *D. plei* or *S. sepiodea* were dissected out of the mantle and cleaned of all surrounding fibers and most of the loose connective tissue. Artificial seawater (ASW) was prepared to the following compositions: $Na^+$, 445 mM; $K^+$, 10 mM; $Ca^{++}$, 11 mM; $Mg^{++}$, 53 mM; $Cl^-$, 580 mM; HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2 ethanesulfonic acid), 6 mM; and adjusted to pH 7.5 ± 0.01 with NaOH, at 21°C. The TTX was a crystalline preparation obtained from either Calbiochem (La Jolla, Calif.) or Sigma Chemical Co. (St. Louis, Mo.) in 1-mg vials with citrate buffer. 101 μM stock solutions in distilled water stored at 5°C were kept and used for several months. All experiments described in this communication were carried out at a temperature controlled at 21 ± 0.5°C. The concentrations of the toxin were 0.1, 0.2, 0.3, 0.5, 0.7, 1.0, 5.0, 10.0, and 50.0 nM. The TTX solutions in seawater were prepared all at the same time and never stored for more than 36 h to avoid toxin decomposition. The addition of 990 μl of TTX stock solution to 200 ml of artificial seawater to make a 500 nM working solution changes the osmolarity of the seawater only in 2 mosmol/kg, and the pH in about 0.002 U. The changes were considered negligible. The 50 nM TTX-ASW was always prepared from the toxin stock solution. The other toxin concentrations were prepared diluting aliquots of fresh 50 nM TTX-ASW with additional artificial seawater. The procedure minimized pipetting errors when small volumes of stock solution were handled and ensured that the same ASW was utilized in all the solutions employed in one experiment. To minimize evaporation, the solution flasks were tightly capped with Parafilm "M" (American Can Co., Greenwich, Conn.) with pinsize hole punctures to allow the drainage of the solution. Once the solutions were prepared, the pH remained constant due to the insensitivity of HEPES buffer to environmental $CO_2$ and temperature changes (16). HEPES is an excellent buffer in our pH range ($pK_a$ = 7.55).

The experimental cell is of the type used by Ehrenstein and Gilbert (7) slightly modified to decrease the dead space to 600 μl. Membrane potential was controlled with an electronic feedback circuit having a rise time of 0.8 μs connected to the axon via a "piggy-back" electrode (8). Membrane potential was measured through the internal glass capillary of the piggy-back assembly and a reference electrode placed in contact with the fiber to minimize series resistance (9). No compensation of the series resistance was attempted in the present work; the value of this resistance was estimated from the capacitive transient at the beginning of the pulse to be less than 3 Ω · $cm^2$. The axons were controlled at the resting level and depolarizing pulses lasting 1.6 ms, with the amplitude chosen to produce maximum inward sodium current, were employed to estimate the effect of tetrodotoxin on sodium conductance. The temperature of the seawater was monitored by means of a thermistor permanently placed in the vicinity of the nerve (Yellow Springs Instrument Co., Yellow Springs, Ohio).

## RESULTS

### *Reversibility of Tetrodotoxin Membrane Interaction*

The kinetic analysis, to be described later, of the interaction between tetrodotoxin and the nerve membrane is only possible if the reaction is reversible, so the reversibility was tested in four axons exposed for 15 min to 50 nM TTX, comparing the sodium current before the exposure to the toxin and after 30-min washing with toxin-free artificial seawater; 98.9 ± 2.7% of the initial current was

recovered after the wash period. Since this fraction is not significantly different from 100% as shown by Student's $t$ test ($P > 0.5$), we conclude that the TTX-receptor interaction in *D. plei* is fully reversible.

*Tetrodotoxin Dose-Response Curves*

The percentage of suppression of the sodium current was measured after 15 min of exposure to each toxin concentration (0.1, 0.2, 0.3, 0.5, 0.7, 1.0, 5.0, 10.0, and 50.0 nM) and Eadie-Haldane plots of effect vs. effect divided by the concentration of the toxin were drawn. The Eadie-Haldane plot of a dose-response curve is a straight line if the drug under study interacts reversibly with a receptor in the fashion described by the Michaelis-Menten equation and deviates very markedly from linearity otherwise (10).

Fig. 1 is a TTX dose-response curve drawn as an Eadie-Haldane plot. The

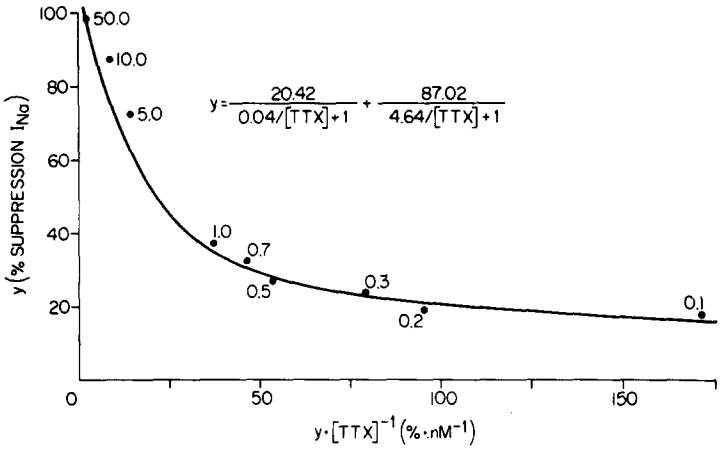


FIGURE 1. Tetrodotoxin dose-response curve from *D. plei*, drawn as Eadie-Haldane plot. The ordinate presents the effect of tetrodotoxin as percentage of suppression of the sodium current, the abscissa is effect times the concentration of the drug employed (nanomolar). Experimental points appear as dots; the nanomolar concentration of TTX used appears next to each dot. Theoretical line drawn according to the equation indicated in the figure.

ordinate is the percentage of suppression of the sodium current, the abscissa is the same percentage of suppression times the reciprocal of the concentration. The toxin concentrations employed (nanomolar) appear next to each point. Please note the marked derivation of the experimental points from a straight line and the very good fit between those points and the curve drawn. The curve was fitted by least squares assuming the existence of two kinds of receptors for tetrodotoxin, one kind (type 1) has a very high affinity for TTX, the other (type 2) has a lower affinity for the drug. The line obeys the equation:

$$Y = \frac{Y_{max_1}}{K_1/[TTX] + 1} + \frac{Y_{max_2}}{K_2/[TTX] + 1}$$

where $K_1$ and $K_2$ are apparent dissociation constants of each kind of receptor,

and $Y_{max_1}$ and $Y_{max_2}$ the suppression of the sodium current observed when the receptors of the type 1 or type 2, respectively, are all bound to the toxin. [TTX] is the nanomolar toxin concentration. The line in Fig. 1 was calculated with values: $K_1 = 0.04$ nM, $K_2 = 4.64$ nM, $Y_{max_1} = 20.42\%$, and $Y_{max_2} = 87.02\%$. The good agreement between the experimental points and the line fitted in Fig. 1 is a characteristic feature of all the experiments presented in Table I despite the variability that may be appreciated between different nerves.

Dose-response curves were determined in two kinds of experiments. In the first one, the toxin was applied starting from the lowest concentration (as in Fig. 1) and increased stepwise as indicated before. In the second group, the fibers were exposed to 50 nM TTX for 15 min followed by a 30-min washing period

TABLE I

DOSE RESPONSE RELATION OF TETRODOTOXIN IN *D. plei*

| Experiment number | $K_1$ | $Y_{max_1}$ | $K_2$ | $Y_{max_2}$ |
|---|---|---|---|---|
| | *nM* | % | *nM* | % |
| Pretreated with 50 nM TTX | | | | |
| 1 | 0.10 | 14.50 | 4.40 | 81.44 |
| 2 | 0.01 | 31.75 | 5.65 | 73.41 |
| 3 | 0.05 | 5.30 | 5.00 | 101.20 |
| 4 | 0.25 | 17.52 | 4.19 | 79.76 |
| 5 | 0.01 | 2.22 | 2.98 | 97.79 |
| Mean ± SEM | 0.08±0.04 | 14.19±5.20 | 4.44±0.45 | 86.72±5.41 |
| Not pretreated | | | | |
| 1 | 0.09 | 27.70 | 5.63 | 78.18 |
| 2 | 0.04 | 20.42 | 4.64 | 87.02 |
| 3 | 0.01 | 6.66 | 3.51 | 93.87 |
| 4 | 0.44 | 44.97 | 8.07 | 63.02 |
| Mean ± SEM | 0.15±0.10 | 24.94±7.98 | 5.46±0.97 | 80.52±6.66 |
| Mean ± SEM all nine experiments | 0.11±0.05 | 18.97±4.66 | 4.90±0.49 | 83.97±4.09 |

with toxin-free artificial seawater, after which a dose-response curve was determined as in the first batch. The results obtained in both situations are presented in the table as "pretreated with 50 nM TTX" and "not pretreated." The constants calculated for each nerve are presented in the table. At the bottom of each column, the means ± standard error of the mean are displayed. The absence of statistically significant differences between both groups is further evidence of the complete reversibility of the interaction between tetrodotoxin and the nerve membranes of *D. plei*. Due to the lack of significant differences among the two groups, general means for the parameters estimated for the nine nerves were calculated and appear at the very bottom of the table.

Even disregarding our kinetic interpretation of the findings, which may be subject to discussion, the evidence presented indicates that tetrodotoxin concentrations below 1 nM suppress sodium current to an extent which is incompatible with a single receptor that binds TTX with an apparent dissociation constant of

3–5 nM, and that exhibits no cooperativity (17). The results could be artefactually produced if deterioration during application of the lower TTX concentrations occurred to a significant extent. To rule out this possibility, a single toxin concentration was applied to squid axons for 15 min, the effect recorded, and the toxin then washed out with ASW for 20 min. Table II presents the results obtained by such procedure with six different toxin concentrations, each one tested in five different axons. Notice that over 96% of the initial sodium current was recovered after the 20-min wash period that followed toxin application. If we assume that the fraction of sodium current not recovered is all due to deterioration, and that axons run down linearly with time, we will estimate not more than 1.6% deterioration during the 15-min toxin application.

Although it is not a good practice to fit dose-response curves to the mean of the effects observed at each drug concentration since their analysis may lead to wrong kinetic interpretations (18), in Fig. 3 we present an Eadie-Haldane plot of

TABLE II

EFFECT AND REVERSIBILITY OF TETRODOTOXIN CONCENTRATIONS IN *D. plei*

| TTX concentration | Percentage of suppression of $I_{Na}$ | Percentage of recovery of initial $I_{Na}$ | Number of axons |
|---|---|---|---|
| *nM* | | | |
| 0.1 | 8.25±1.46 | 98.61±0.93 | 5 |
| 0.2 | 11.63±1.80 | 96.33±0.88 | 5 |
| 0.5 | 18.61±0.84 | 98.51±0.59 | 5 |
| 1.0 | 20.31±0.98 | 97.40±1.09 | 5 |
| 5.0 | 56.95±2.76 | 99.45±1.94 | 5 |
| 50.0 | 97.87±0.22 | 98.29±1.92 | 5 |

Values are mean ± standard error of mean.

the data given in Table II to further show the nonlinearity of the dose-response relation. The broken line was drawn by eye to help in the interpretation of the figure. Other details of Fig. 3 are similar to the ones presented in Figs. 1 and 2.

## DISCUSSION

The results indicate that the sodium channels in the squid membrane are associated to two types of TTX receptors with widely different apparent dissociation constants. One kind of receptor (type 1) has an affinity constant equal to 0.11 ± 0.05 nM and comprises 19.0 ± 4.7% of the receptors. The other kind (type 2) has an affinity constant of 4.90 ± 0.49 nM and constitutes 84.0 ± 4.1% of the total amount of receptors; the latter kind is the only one that may be evidenced easily when TTX is studied in a concentration range from 1 to 25 nM and in all likelihood corresponds to the one studied by Cuervo and Adelman (4) and Schwarz et al. (12), although it is possible that the high affinity fraction does not exist in *Xenopus* where the latter authors have found no "clear indication that tetrodotoxin bound irreversibly to a fraction of membrane sites as reported for the squid giant axon by Cuervo and Adelman (1970)."

The results just described are not unique for *D. plei* since we have confirmed

them also for another tropical squid, *S. sepiodea* (see Fig. 2). Very few experiments were carried out on *S. sepiodea* because the results obtained in this species are only confirmatory of the findings in *D. plei*, and also because the axons of the former squid are smaller in diameter, more branchy, and harder to dissect and clean, making the survival of a voltage clamped preparation shorter, and deterioration could severely impair the measurements. Thus, type 1 receptors could exist in the nerve membrane of all squids with perhaps an even higher affinity for tetrodotoxin. Then, small amounts of TTX present in the Frankenhaeuser-Hodgkin (11) space may saturate those receptors producing an apparent irreversible fraction due to incomplete washing. A word must be said about the tendency to overshoot 100% suppression of some of the regression lines fitted to the data. This awkward finding probably stems from two sources: first, the

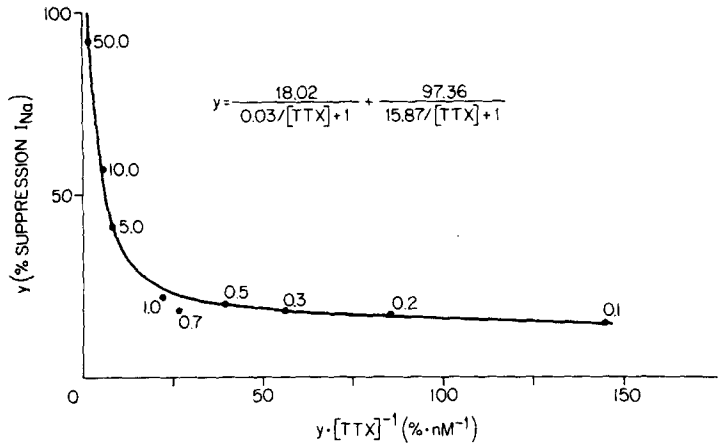


FIGURE 2. Tetrodotoxin dose-response curve from *S. sepiodea* as Eadie-Haldane plot. The figure presents the effect of TTX on one axon of *S. sepiodea* to illustrate the existence of type 1 receptors. Notice the similarities between this result and the one presented in Fig. 1 obtained in *D. plei*. For details on figure description see Fig. 1.

intercept of any regression line is affected by some random error which results in the line overshooting or undershooting the true values. This is certainly an important factor in our case since the sum of the mean values of $Y_{max_1}$ and $Y_{max_2}$ in Table I is not statistically different from 100%, indicating that the random error cancels, as expected, when a greater sample of data is averaged. Second, probably an important factor, determinative of overshooting of the expected 100% of suppression of sodium current, comes from an undue enhancement of the effect of the higher TTX concentrations (over 1 nM) from deterioration of the preparations after a rather lengthy experiment (1–2 h).

Since the nonlinearities of the Eadie-Haldane plot appear at very low TTX concentrations (under 1 nM) it is necessary to rule out several artifacts that may simulate two receptors where only one exists. In the first place: Is it possible that deterioration of the nerve, occurring during the application of low TTX concentrations, produces the observed results? We have two reasons for answering no.

In the first place, the action of all TTX concentrations is quite reversible, so it is unlikely that any significant fraction of its effect comes from deterioration. Second, if deterioration occurred to a significant extent during the application of low tetrodotoxin concentrations an apparent "cooperativity" would be produced, but the line would consequently bend *away* from the origin of coordinates and not *towards* it. We, however, occasionally observe this behavior in fibers that depolarized markedly from their initial resting potential when released of the electronic potential control; the results obtained in those fibers were rejected. A different point could be raised about the sufficiency of the 15-min period the fibers were allowed to equilibrate in each toxin concentration. Fig. 4 presents sodium current of two fibers as a function of time elapsed after the onset of

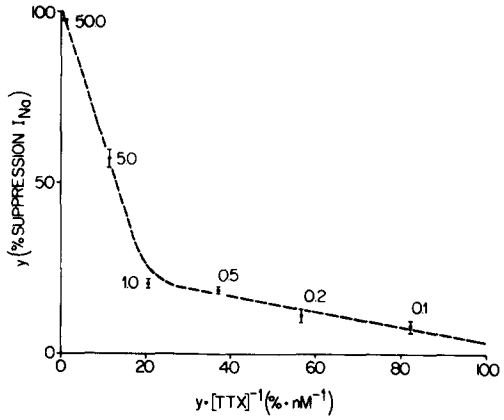


FIGURE 3. Eadie-Haldane plot of the effect of tetrodotoxin on *D. plei*. The points represent the mean (± SEM) the effect of six TTX concentrations. Each point was determined in five different nerves, each concentration was applied for 15 min and then washed out for 20 min with ASW. The percentage of sodium current suppression produced by the toxin and the fraction of the control sodium current obtained after the wash period appear in Table II. The broken line was fitted by eye to help interpretation. For additional details see Fig. 1 and the text of this communication.

perfusion with 50 and 0.1 nM TTX-ASW, respectively. It may be appreciated that steady state is reached in about 6-min.

Since some of the axons presented in this paper have type 1 receptors with affinity constants below 0.1 nM, or even lower than 0.03 nM, it might seem desirable to have utilized lower concentrations. However, if the parameters describing the curve in Fig. 1 are used, we can predict an effect equal to 4.27% suppression when 0.01 nM TTX is utilized. It seems unwise to work in such a range when the precision of the recording equipment is about 3%.

All the limitations of our technique (deterioration, concentrations higher than ideal, etc.) cannot, however, conceal the fact that the Eadie-Haldane plot of tetrodotoxin dose-response curves in *D. plei* and *S. sepiodea* at 21°C is markedly nonlinear and that, furthermore, under those conditions low concentrations of tetrodotoxin produce an effect significantly larger than the one predicted from a

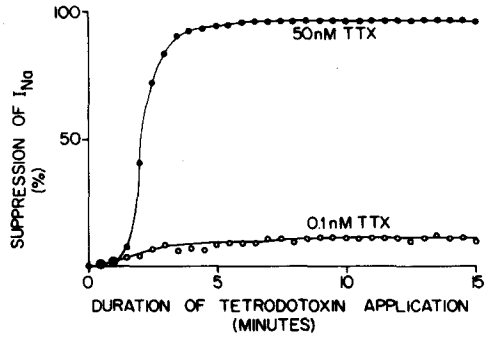


FIGURE 4. Suppression of sodium current versus duration of tetrodotoxin application. The figure shows the time course of sodium current suppression in two different axons, one of them exposed to 0.1 nM TTX and the other to 50 nM TTX. Ordinate is percentage of suppression of sodium current and abscissa, duration of tetrodotoxin perfusion in minutes. The effect of both concentrations is observed to reach a steady state in about 6 min. The sodium current at time zero was 7 mA/cm$^2$ for the axon exposed to 0.1 nM TTX and 13.7 mA/cm$^2$ for the other one. One hundred percent of the initial current was recovered after the wash period from the axon exposed to 0.1 nM TTX and 97% when the toxin was washed out from the axon exposed to 50 nM TTX.

single receptor that binds tetrodotoxin in a Michaelis-Menten fashion, with molecularity equal to 1, an affinity constant of about 3 nM, and no cooperativity. The limitations of our technique, at most, can decrease the accuracy of our estimates of $K_1$, $K_2$, $Y_{max_1}$, and $Y_{max_2}$.

The two types of receptors described may represent either two different structures in the membrane or a single structure that changes with time between two conformations, each one with different affinity for the drug.

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