MECHANISM OF INHIBITORY ACTION OF TETRODOTOXIN IN EXCITABLE MEMBRANES*

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Tetrodotoxin(Tx), the toxin from the Japanese Fugu or puffer fish, is a potent neurotoxin (1), but the mechanism of its toxic action has not yet been elucidated.

Several workers studying the blockage of action potential by Tx in various excitable tissues concluded that Tx might be a selective inhibitor on the Na-channel in the excitable membranes (2,3,4), while other workers proposed that Tx binds to the membrane macromolecules and hinders the exchange of univalent cations and divalent cations, thus causing the inhibition of action potential (5).

From experiments on the rat's neurohypophysis in vitro, it was found that Tx not only inhibits the release of vasopressin, posterior hormone, but also suppresses the movement of calcium ions (Ca) (6).

In the present communication, evidence will be presented that Tx chelates Ca strongly, and that Tx is able to alter the characteristics of the Ca-K exchange in a model membrane of phosphatidyl serine. These data present the possibility of elucidating the action of Tx on excitable membranes from a molecular point of view.

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MATERIALS AND METHODS

Binding between Tx and Ca ion was measured by titrating a solution of Ca by Tx in the presence of Murexide pigment, a Ca-indicator, and the resulting small absorption change was measured by a double-beam spectrophotometer (7,8,9).

Phosphatidyl serine (PLS) was isolated from beef erythrocytes and purified by repeated column chromatography (10). Benzene solution of PLS (1 μg) was spread on the air-water interface to form a monolayer (11). A polyethylene trough with an inner size of 27 mm (diameter) x 4 mm (depth) was used. Binding of ^{45}Ca in the water phase to the monolayer of phospholipid was measured by the increase of counts in a GM counter, which was set 5 mm above the monolayer (12). Using a ratemeter and a recorder, we can automatically measure the binding of ^{45}Ca to the membrane by an increase of the recording (13).

RESULTS

A titration curve of Ca by Tx is shown in Fig. 1. By extrapolating the linear portion of the curve to its intersection with the abscissa,

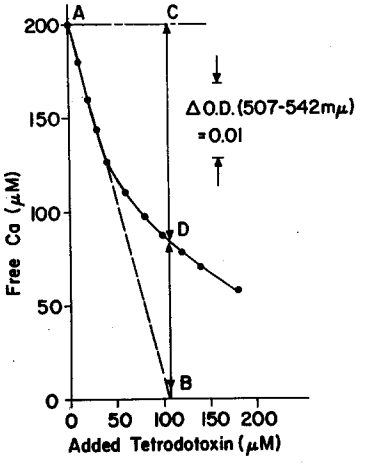

Fig. 1. Titration of a Ca solution by tetrodotoxin. Tris-HCl (pH 8), 20 mM; Murexide, 300 μM, 25°C.

we find that a Tx molecule binds approximately two Ca ions at pH 8.

If we assume a chemical equilibrium

$$Tx + 2\ Ca \rightleftharpoons TxCa_2 \quad , \tag{1}$$

the binding constant between Tx and Ca is expressed by

$$K_{TC} = \frac{(TxCa_2)}{(Tx)\ (Ca)^2} \quad M^{-2}$$

$$(Tx)_o = (Tx) + (TxCa_2) \tag{2}$$

$$(Ca)_o = (Ca) + 2\ (TxCa_2) \ ,$$

where $(Tx)_o$, $(Ca)_o$, (Tx), (Ca), and $(TxCa_2)$ are molar concentrations of total Tx, total Ca, free Tx, free Ca, and the complex, respectively. When we choose $(Ca)_o$ = OA and $(Tx)_o$ = OB in Fig. 1, we can easily see that Eq. 2 becomes

$$K_{TC} = \frac{1/2\ (CD)}{1/2(BD)\ (BD)^2} = 1.8 \times 10^8\ M^{-2} .$$

An example of absorption of ^{45}Ca in the water phase to the PLS monolayer is shown in Fig. 2. The increase of counts due to the monolayer formation corresponds to the amount of ^{45}Ca absorbed to the monolayer.

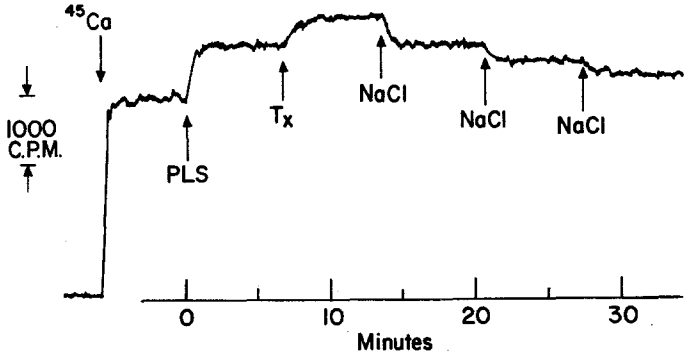

Fig. 2. Increase and decrease of counts recorded by using a GM counter and a ratemeter. All reagents except PLS were added into the water phase. Amount of ^{45}Ca was so chosen that the counts from just the water phase becomes approximately 3,000 cpm. Concentration of added reagents are 1 μg of PLS, 5 x 10^{-7}g/ml of Tx, each 5 mM of NaCl. Experimental conditions are 20 μM (total) of $CaCl_2$ and 1 mM of Tris-HCl (pH 8). 25°C.

When a small amount of Tx is added to the water phase, absorption of Ca is increased. When Na or K ion is added, a part of the Ca bound to the monolayer is replaced by these ions in accordance with both the amount of added univalent cations and the binding strength between PLS monolayer and univalent cations. Figure 3 shows the percent replacement of bound Ca by univalent cations and the effect of Tx on it. It is to be noted that K replaces Ca more than Na does, and that this difference almost disappears in the presence of Tx.

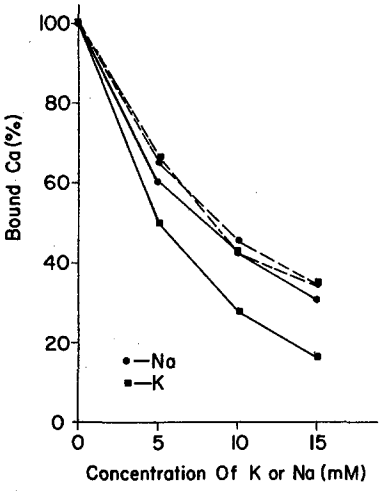

Fig. 3. Percent replacement of bound Ca by KCl or NaCl in the water phase. Solid lines: without Tx. Dotted lines: with 5×10^{-7}g/ml Tx. Experimental conditions are those of Fig. 2.

DISCUSSION

From these data we can depict the inhibitory action of Tx on excitable membranes as follows: (i) chelation of Ca ions, (ii) increase of the binding of Ca to the membrane, and (iii) suppression of Ca-K exchange which causes blockage of triggering of the action potential.

As to the first point, the structure of Tx helps us to understand the high binding constant between Tx and Ca. Since the proposed

structure (14,15,16) of the Tx molecule has six OH bases, it is possible that Ca ions are chelated by these OH bases. The fact that the binding ratio between Ca and Tx decreases with decreasing pH also supports this possibility (17). Under the same experimental conditions, the binding between EDTA and Ca becomes (17)

$$\text{EDTA} + \text{Ca} \rightleftharpoons \text{EDTACa} \tag{3}$$

$$K_{EC} = \frac{(\text{EDTACa})}{(\text{EDTA})\,(\text{Ca})} = 1.56 \times 10^{6}\ \text{M}^{-1}. \tag{4}$$

Concerning the second point, we have as yet no data showing whether Tx binds directly to the acidic phospholipid monolayer. However, even if Tx does not bind to the monolayer, it is possible that Ca is more tightly bound to the membrane due to the formation of a complex such as PLS-Ca-Tx-Ca-PLS. The fact that Ca-chelating reagents other than Tx, such as glycoletherdiaminetetraacetic acid (GEDTA), EDTA and ATP, also increase the binding of ^{45}Ca in a range of extremely dilute concentration (13) supports this possibility. In addition to this, we might expect that Tx binds to the biological membranes which should have a considerable amount of both positive and negative charged groups and traps calcium ions.

An outline of determining the binding constant of di- and univalent cations to PLS monolayer and the equilibrium constants in Ca-univalent cation exchange is presented (details to be published later) (13). When the concentration of chelating reagents in the water phase increases, it is natural that ^{45}Ca bound to the PLS monolayer begins to decrease in accordance with the relationship between the binding constants for the PLS-Ca and Ca-chelator complexes. From such experiments using EDTA as a chelator, for which the binding constants to Ca have been measured (Eq. 4), and assuming the following chemical equilibrium

$$2\ \text{PLS} + \text{Ca} \rightleftharpoons \text{PLS}_2\text{Ca} \tag{5}$$

$$K_{PC} = \frac{(PLS_2Ca)}{(PLS)^2\,(Ca)}\ M^{-2}\ , \qquad (6)$$

we can determine the value of K_{PC} as $1.03 \times 10^{12}\ M^{-2}$. This shows that the binding between PLS monolayer and Ca is very high, suggesting the importance of the binding of Ca to acidic phospholipids in the function of biological membranes. It is interesting to note that neutral phospholipids like phosphatidyl choline, phosphatidyl ethanolamine and sphingomyelin scarcely bind ^{45}Ca under the same experimental conditions (13). The chemical equilibrium constants in exchange reactions and the apparent binding constants of univalent cation M to PLS monolayer can be determined from the data of Fig. 3 using Eq. 5, 6 and assuming the following reactions

$$PLS_2Ca + 2\ M \rightleftharpoons 2\ PLSM + Ca \qquad (7)$$

$$K_{Ca \rightleftharpoons M} = \frac{(PLSM)^2(Ca)}{(PLS_2Ca)\,(M)^2}$$

$$PLS + M \rightleftharpoons PLSM \qquad (8)$$

$$K_{PM} = \frac{(PLSM)}{(PLS)\,(M)}\ M^{-1}\ .$$

The results are listed in Table I which offers quantitative bases for the third argument, i.e., in both the chemical equilibrium constant for reaction ($K_{Ca \rightleftharpoons M}$) and the apparent binding constant to PLS monolayer (K_{PM}), K is larger than Na, but Tx suppresses both of these values for K to near the corresponding values for Na.

TABLE I

Chemical Equilibrium Constants of Exchange Reactions and Apparent Binding Constants Between PLS and Monovalent Cations

	Chemical Equilibrium Constant		Apparent Binding Constant	
	$K_{Ca \rightleftharpoons K}$	$K_{Ca \rightleftharpoons Na}$	K_{PK}	K_{PNa}
Control	4.4×10^{-7}	1.6×10^{-7}	6.8×10^{2}	4.2×10^{2}
Tetrodotoxin (5×10^{-7} g/ml)	1.3×10^{-7}	1.3×10^{-7}	3.6×10^{2}	3.6×10^{2}

Quite similar results have been obtained by using a monolayer of polyelectrolyte (18), suggesting that the effect of Tx on membranes can be studied as a problem of interaction between charged groups of polyelectrolytes and hydrated cations.

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