

Partial Characterization of a Tetrodotoxin-Binding Component from Nerve Membrane

(garfish olfactory nerve/membrane-bound protein/excitable ion channels)

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ABSTRACT Tetrodotoxin from Japanese puffer fish has been labeled with tritium and purified from the crude mixture obtained. The interaction between the purified [³H]tetrodotoxin and membrane suspensions from the olfactory nerve of long-nosed garfish has been investigated by equilibrium dialysis. Tetrodotoxin binds to membrane suspensions with a dissociation constant $K_D = 8.3$ nM. The nerve preparation binds 42 pmol of [³H]tetrodotoxin/g of wet tissue at saturating toxin concentrations. With various hydrolytic enzymes, the binding component is shown to be a protein embedded in a phospholipid environment. The binding is inhibited below pH 4.0 and is not stable towards heat. Tetrodotoxin binding is not inhibited by the local anesthetic, procaine.

Electrical excitability of nerve axons generally depends on a transient influx of sodium ions followed by an efflux of potassium ions (1). These ionic fluxes are pharmacologically distinct processes, and it is generally agreed that they are carried by distinct ion-specific channels. Sodium flux is reversibly and specifically inhibited by nanomolar concentrations of tetrodotoxin (TTX), while potassium efflux is unaffected at 100-times the concentration of TTX necessary to block the sodium influx (2). TTX has no effect on the post-synaptic potential at the neuromuscular junction (3), and it does not inhibit either active transport of sodium ions or the ATPase activated by sodium-potassium in toad bladder (4).

Recent studies on myelinated fibers from frog sciatic nerve have determined that several organic cations are able to substitute for sodium in the transient influx of ions (5). From these data Hille proposes a model for the ion-selectivity filter of the sodium channel as a small pore, 3×5 Å, lined with oxygens capable of forming hydrogen bonds and having one anionic site capable of interacting with cations. The structure of TTX (6, 7), with its positively charged guanidinium group and hydroxyl functions capable of forming hydrogen bonds, is certainly consistent with the hypothesis that blockage of the sodium channel is a result of a specific and tight binding of the toxin to the channel. Some preliminary experiments have been performed in an attempt to measure the binding of TTX to nerves (8-10). The experiments indicate that TTX does bind to nerves and that very low concentrations of binding sites occur on the nerve membrane.

The specificity of TTX action does not guarantee a specific association of the toxin with the relevant components of the excitable membrane, even if we accept the quite plausible mechanism of binding proposed by Hille (5). To insure such interaction in tissue homogenates, the toxin must be used at concentrations close to those that are active *in vivo* and under conditions that minimize nonspecific interactions of the cationic TTX molecule with anionic components in the tissue.

Abbreviations: TTX, tetrodotoxin.

Two major problems must be overcome before a biochemical study of the molecules involved in electrical excitability can be begun. Tetrodotoxin must be labeled to a high enough specific activity to be detectable in nanomolar concentrations. This has been accomplished by a modified Wilzbach exchange method (11). Secondly, a preparation of excitable membranes must be found that is suitable for biochemical studies. The olfactory nerve of the long-nosed garfish (*Leptostosteus osseus*) was chosen as an easily available preparation of unmyelinated fibers having a large surface area of excitable membrane (12). The preliminary studies reported here are directed toward characterization of the TTX binding component in excitable tissue; equilibrium dialysis was used to measure binding; susceptibility to enzymatic degradation was used to determine the nature of the membrane components involved.

MATERIALS AND METHODS

Tetrodotoxin (free of citrate) was obtained from Sankyo Co. Ltd. Tokyo. Tritium labeling was performed by International Chemical and Nuclear Corp. *Leptostosteus osseus* was obtained live from Minnesota Fish and Game or frozen from Gulf Specimen Co. (Panacea, Fla.). Phospholipases, nucleases, hyaluronidase, neuraminidase, and procaine were obtained from Sigma, α -chymotrypsin from Armour and Co., trypsin from Worthington, and Pronase from California Corp. for Biochemical Research.

Preparation and Purification of [³H]Tetrodotoxin. 10 mg of TTX was labeled with tritium by a catalytic exchange method in the presence of 30 Ci of tritium. The easily exchanged tritium was removed by solution in dilute acetic acid, followed by evaporation. The resulting solid was dissolved in 0.1 M acetic acid and stored at -20° . Purification of [³H]TTX was performed on small aliquots of this solution.

About 10 μ Ci of the above solution was diluted to 0.4 ml with distilled water titrated to pH 5.0 with acetic acid. The crude material was chromatographed on a 1.5×55 -cm Biogel P-2 (100-200 mesh) column, equilibrated in distilled water titrated to pH 5.0 with acetic acid. 1.0-ml fractions were collected and the radioactivity of 10- μ l aliquots was determined. Fractions that contained nerve blocking activity, as assayed with frog sciatic nerve, were pooled and lyophilized. The material was chromatographed again on a P-2 column, and the active fractions were lyophilized.

This material was dissolved in 10 mM ammonium acetate (pH 7.0) and applied to a 1.0 ml Bio-Rex-70 (200-400 mesh) (NH_4^+ form) column, equilibrated in 10 mM ammonium acetate (pH 7.0). [³H]TTX was eluted with a 100-ml linear salt gradient from 10-200 mM ammonium acetate (pH 7.0). 1.0-ml fractions were collected and the radioactivity of

10- μ l aliquots was determined. The physiologically active fractions were pooled and lyophilized.

Thin-Layer Chromatography of [3 H]TTX. Typically, 2–4 μ l of solution were spotted on prepared cellulose or silica-gel sheets and developed about 9.0 cm in the solvent tank, cut into 0.5-cm strips, and placed in scintillation vials with 50 μ l of water and 1 ml of NCS solubilizer. The radioactivity was determined in a toluene-based scintillator.

Assay of Nerve Blocking Activity. Frog sciatic nerves, dissected and desheathed, were placed in a Bionix nerve chamber. The diphasic compound action potential was amplified and displayed on a cathode-ray oscilloscope. A 5-mm length of the nerve, between the stimulating and recording electrodes, was immersed in 200 μ l of frog Ringer's solution containing the sample of toxin to be assayed. The percentage decrease in action-potential amplitude after 10 min was taken as a measure of TTX nerve-blocking activity. Appropriate controls were performed by use of Ringer's solution with no toxin added. The concentration of active TTX in a given sample was standardized against known concentrations of unlabeled TTX with the same nerve preparation. The action of TTX was fully reversible upon 0.5 hr of washing with Ringer's solution under these assay conditions.

Preparation of Membranes. Garfish olfactory nerve was dissected according to Easton (12), minced, diluted 1 to 5 with physiological buffer (12), and homogenized in a Virtis "60" homogenizer for 4 min (0°) at 40,000 rpm. The suspension was either used at this stage (initial binding curve) or centrifuged for 0.5 hr at 45,000 $\times g$. The pellet was suspended in the appropriate buffer for the enzyme treatments.

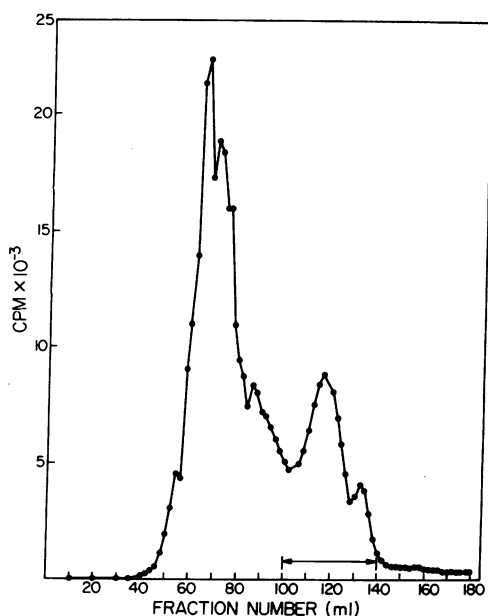


FIG. 1. Chromatography of TTX that had been tritiated by a modified Wilzbach process on a Biogel P-2 column (1.5 \times 55 cm) with water titrated to pH 5.0 with acetic acid, as the elution buffer. 1.0-ml fractions were collected. 10- μ l aliquots of appropriate fractions were removed for determination of radioactivity. Fractions 100–140 were pooled and contained physiological activity.

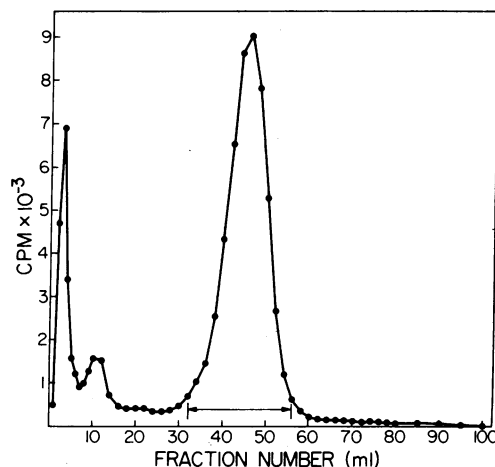


FIG. 2. Gradient elution chromatography on a Bio-Rex 70 column (1-ml bed volume) of the active fractions of [3 H]TTX twice chromatographed on Biogel P-2 columns. Elution was with a 100-ml ammonium acetate gradient from 10–200 mM at pH 7.0. 1.0-ml fractions were collected and 10- μ l aliquots were removed for determination of radioactivity. Fractions 32–56 were pooled and contained physiological activity.

Equilibrium Dialysis Binding Assays. The dialysis tubing was heated for 3 hr in several changes of water over a steam bath and then stored in physiological buffer at 4°. 0.35 ml of membrane suspension was placed in the tubing and dialyzed against 5.0 ml of physiological solution containing different amounts of [3 H]TTX for 12 hr at 4° with agitation. 0.25-ml aliquots were removed from inside and outside the tubing, dissolved in NCS solubilizer, and counted in a toluene-based scintillator in a Packard Tri-Carb Scintillation Spectrometer. It was necessary to determine the efficiency of counting for each sample since the membrane suspension quenched more than the outside solution. For each set of experiments, a blank was used to ensure that the [3 H]TTX had equilibrated across the dialysis tubing.

Enzyme Treatments. Nerve membranes were suspended in buffers appropriate for the various enzymes (13), which were used at a concentration of 1 mg/ml, except for neuraminidase, which was 2.5 mg/ml, and phospholipase A, which was 0.2 mg/ml. Incubation was for 1 hr at 25°. The enzyme-treated membranes were pelleted at 45,000 $\times g$ for 0.5 hr in a Sorvall centrifuge and resuspended in physiological solution for binding studies. Simultaneous controls were run with the various buffers, without addition of the enzymes.

RESULTS AND DISCUSSION

Preparation and purification of [3 H]TTX

The crude material (10 mg) labeled by International Chemical and Nuclear Corp. contained 30 mCi of tritium and about 3–4 mg of active tetrodotoxin, as measured by the frog sciatic-nerve assay. When extremely small quantities of this crude labeled TTX (about 0.4% of the total material) were chromatographed on a Biogel P-2 column with a buffer of dilute acetic acid (pH 5.0), the peak of radioactivity containing nerve-blocking activity was eluted after the bulk of the inactive radioactive material and with an elution volume greater than the total bed volume of the column (see Fig. 1), presumably due to the sorption of the TTX molecule to the resin at very low ionic strength (14). Repeated chromatography on Biogel P-2 columns further removed the faster moving, inactive, radioactive material.

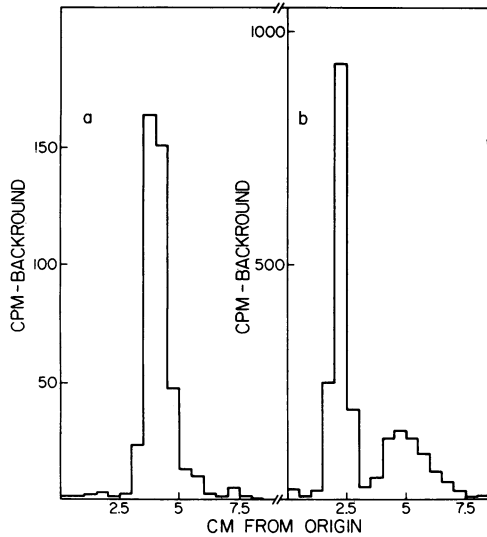


FIG. 3. (a) Thin-layer chromatography on silica gel of purified $[^3\text{H}]\text{TTX}$ with butanol:acetic acid:water 8:3:9 as a solvent.

(b) Thin-layer chromatography of crude labeled TTX on silica gel with butanol:acetic acid:water 8:3:9 as a solvent. Note the scale of the ordinate is about 1/10 that of a. Both chromatograms contained about equal amounts of active TTX as assayed with frog sciatic nerve.

Pooled active fractions from the P-2 column, when placed on a Bio-Rex 70 column and eluted with a linear salt gradient, gave three peaks (Fig. 2). The radioactive peak containing the physiological activity (fractions 32-56) was sharp and symmetrical, suggesting high purity of the labeled toxin.

Thin-layer chromatography on cellulose and silica gel with ethyl acetate:pyridine:water:acetic acid 5:5:3:1 and butanol:acetic acid:water 8:3:9 showed a single peak of radioactivity for the purified $[^3\text{H}]\text{TTX}$. Thin-layer chromatography of crude labeled material showed the majority of the radioactivity was not at the same position as the purified toxin peak (Fig. 3). The sharp symmetrical peak on the Bio-Rex 70 column and the single peaks on thin-layer chromatography with two different supports and two different solvent systems was taken as good evidence of the radiochemical purity of the $[^3\text{H}]\text{TTX}$ preparation. As a further check of the radiochemical purity of our $[^3\text{H}]\text{TTX}$, a sample of the puri-

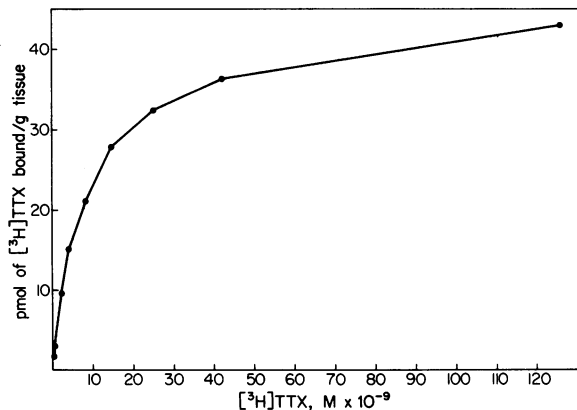


FIG. 4. Binding curve of $[^3\text{H}]\text{TTX}$ to olfactory nerve homogenate by equilibrium dialysis. Picomol of $[^3\text{H}]\text{TTX}$ bound per gram of tissue is plotted against the concentration of free $[^3\text{H}]\text{TTX}$.

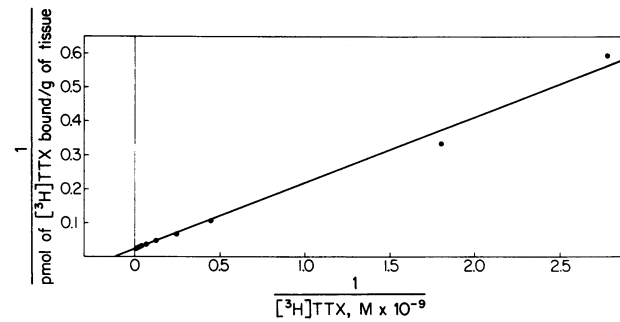


FIG. 5. Reciprocal plot of $[^3\text{H}]\text{TTX}$ binding to olfactory nerve homogenate. The ordinate shows the reciprocal of the amount of $[^3\text{H}]\text{TTX}$ bound in pmol/g of wet tissue. The abscissa is the reciprocal of the concentration of free $[^3\text{H}]\text{TTX}$.

fied $[^3\text{H}]\text{TTX}$ was chromatographed on a Beckman 120-B amino-acid analyzer. The column effluent was collected and the fractions were assayed for radioactivity. A single sharp peak of radioactive material was eluted slightly after the normal elution position of ammonia, as would be expected for the basic tetrodotoxin molecule. A sample of $[^3\text{H}]\text{TTX}$ was incubated at pH 9.0 for 2 hr at 25°, and then applied to the amino-acid analyzer. This alkaline treatment destroys about 50% of the nerve-blocking activity of tetrodotoxin (16). The elution profile of the degraded sample showed four major peaks of radioactivity, and the peak corresponding to the $[^3\text{H}]\text{TTX}$ was greatly reduced in size. The co-chromatography of biological activity and radioactivity in various systems plus the disappearance of the $[^3\text{H}]\text{TTX}$ peak on the analyzer simultaneously with the loss of nerve-blocking activity indicate that the radioactive material in our sample of $[^3\text{H}]\text{TTX}$ does have the properties of native tetrodotoxin. Hafemann (10) has reported difficulty in obtaining radio-

TABLE 1. Effects of enzyme treatment on tetrodotoxin binding

Treatment	(cpm bound treated material/cpm bound control)
Ribonuclease (1 hr)	0.91 ± 0.05*
Deoxyribonuclease (1 hr)	1.09 ± 0.05
Hyaluronidase (1 hr)	1.04 ± 0.05
Neuraminidase (1 hr)	1.62 ± 0.05
Phospholipase A (1 hr)	0.69† ± 0.10
Phospholipase A (2 hr)	0.62† ± 0.10
Phospholipase C (1 hr)	0.99 ± 0.05
Phospholipase D (1 hr)	1.28 ± 0.05
Phospholipase A (1 hr), then	
Trypsin (1 hr)	0.10† ± 0.10
α-Chymotrypsin (1 hr)	0.22† ± 0.10
Pronase (1 hr)	0.30† ± 0.10
Trypsin (1 hr)	1.10 ± 0.05
α-Chymotrypsin (1 hr)	0.80 ± 0.05
Pronase (1 hr)	0.72 ± 0.05
Heat 100° (15 min)	0.18 ± 0.05
pH 2	0.09 ± 0.05
pH 4	0.22 ± 0.05
Procaine 10 ⁻³ M	1.10 ± 0.05
10 ⁻² M	0.99 ± 0.05

* Standard error of the mean.

† Binding measured in supernatant as well as in pellet.

chemically pure [³H]TTX presumably because he has not taken advantage of the sorption properties of Biogel P-2 or the possibility of ionic strength gradient elution on Bio-Rex 70. The purified preparation of [³H]TTX obtained here was standardized against known concentrations of unlabeled TTX with the frog sciatic nerve as a bioassay. A specific activity of 0.33 Ci/mmol was determined for the purified active [³H]TTX.

Equilibrium dialysis binding studies

This technique has been used to measure the binding of [³H]-TTX to tissue homogenates of garfish olfactory nerve. A plot of the amount of [³H]TTX bound against free [³H]-TTX concentration (see Fig. 4) shows that at nanomolar concentrations, [³H]TTX binds to nerve homogenates and that the binding sites can be saturated. A double reciprocal plot (Fig. 5) yields a straight line and gives a dissociation constant of $K_D = 8.3$ nM TTX and a maximal binding of 42 pmol of [³H]TTX per g of wet tissue. The reciprocal plot also indicates only one type of high affinity binding site for the toxin. A Hill plot (15) of the data yields a Hill coefficient of 1, indicating that the binding of TTX is not a cooperative process. Subsequent experiments showed recovery of all the [³H]TTX binding in the $45,000 \times g$ pellet, confirming that the binding component is membrane bound.

TTX binding studies were performed on tissue homogenates of a branch of the myelinated trigeminal nerve, found adjacent to the olfactory nerve in the gar (12). No [³H]TTX binding was detectable over a range of TTX concentrations from 3.5–50 nM. The limits of sensitivity of the assay indicate a binding of less than 1.5 pmol/g of wet tissue. This low value is to be expected for myelinated fibers where the excitable membrane occupies only a small fraction of the membrane surface area.

In order to correlate the number of binding sites with physiological data, it is necessary to determine the number of sites per square micrometer. Calculations have been made for the olfactory nerve of cross-sectional area and surface area of axon per length of nerve (12). If we assume a density of 1 g/ml for the tissue, there are 6.5×10^{12} μm^2 of axonal surface area per gram of wet tissue. Under saturating concentrations of [³H]TTX there are 2.52×10^{13} binding sites per gram of tissue, and therefore 3.9 binding sites per square micrometer of surface area. This value is lower than values reported for other unmyelinated preparations (8, 9), but agrees well with a recent value obtained for gar olfactory nerve (16). The dissociation constant for TTX binding is also in agreement with physiological studies that determined the concentration of TTX at which the sodium influx was reduced to half its normal value (17). The agreement of the binding and dissociation data from experiments with whole nerves and the membrane suspension studies reported here encourages the investigation of the biochemical properties of the TTX binding component. Since the TTX binding component retains its binding properties when the axonal membranes are disrupted, the solubilization and isolation of a molecule or molecules involved in electrical excitability becomes possible.

Enzyme treatment

The only enzymes found capable of inhibiting toxin binding were phospholipase A and two proteases, chymotrypsin and Pronase (Table 1). Trypsin treatment had no effect upon binding. Prior treatment of the membranes with phospholipase A for 1 hr, followed by treatment with various proteolytic

enzymes, resulted in greater reduction of binding than that found without the prior treatment. This indicates that the protein part of the TTX binding component is embedded in a phospholipid environment, which partially protects against attack by proteolytic enzymes. Toxin binding is also inhibited below pH 4 and by heating membrane suspensions to 100° for 15 min. It is not known whether the inhibition below pH 4 is due to inactivation of the binding protein or merely to protonation of a residue in the active site, which must be in the deprotonated state for binding to occur. 10^{-2} M procaine had no effect upon the binding, which is in agreement with the notion that such local anesthetics block action potentials by some interaction with the lipid phase of the membrane and not at the specific TTX binding site.

Treatment of the membranes with neuraminidase caused a 62% increase in TTX binding. This same effect has been seen for [¹²⁵I] α -bungarotoxin binding to membranes of electroplax from electric fish (18). The increase in binding could be due to an "unmasking" of binding sites upon cleavage of sialic acid residues from the membrane, or it could simply be due to an increase in nonspecific binding sites on the membrane that could interact with the positive charge on the toxin molecule.

The results of the experiments presented here show that TTX binds to a small number of high affinity sites on the membranes of garfish olfactory nerve and that the membrane-binding component appears to be protein or phospholipoprotein in nature. Agreement with previous calculations of the number of sites and the binding affinity suggest that the TTX binding protein may be identical with or related to the molecule or molecules involved in the transient sodium influx of excitable tissue.

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