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TETRODOTOXIN RECEPTORS IN PLASMA MEMBRANES ISOLATED FROM LOBSTER NERVE FIBERS

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SUMMARY

Tetrodotoxin binds to the axolemma and specifically blocks its Na^+ channels. The present work deals with the demonstration of tetrodotoxin receptors in an axolemma-enriched preparation. Walking-leg nerve fibers of the lobster *Panulirus argus* were utilized. The binding of tritiated tetrodotoxin to different fractions obtained at different stages of the isolation procedure was studied. The binding, in 10^{-12} mole of tetrodotoxin per mg of protein, is: 2.1 for the $4.2 \cdot 10^5 \times g \cdot$min pellets from nerve homogenates; 5.1 for the total nerve plasma membranes; 9.5 for plasma membrane Fraction I; and 3.4 for Fraction II. Fraction I is considered to be an axolemma preparation because of its morphological appearance, yield and high transport ATPase and acetylcholinesterase activities. Saxitoxin, which like tetrodotoxin specifically blocks the Na^+ channels, displaces tetrodotoxin from its binding sites. Tetrodonic acid, an inactive derivative of tetrodotoxin, does not displace tetrodotoxin from its receptors. The apparent dissociation constant of the tetrodotoxin–receptor complex is $4.0 \cdot 10^{-9}$ M, similar to that found in living axons of the squid and rabbit. The number of tetrodotoxin receptors in the axolemma preparation, calculated from binding data, membrane composition, density and thickness, is 28 sites/μm^2. This value is in good agreement with the number of Na^+ channels determined in living lobster axons.

INTRODUCTION

The most direct approach for obtaining information about the molecular phenomena associated with the functioning of the axon excitable membrane (axolemma) is to characterize, in a preparation of this membrane, the structures involved in the regulation of the ionic passage. One type of these structures are the so-called Na^+ channels, pathways normally used by this ion to move through the axolemma during the nervous impulse and during the early phase of conductance increase in voltage-clamped axons (see refs. 1 and 2).

The elucidation of the nature of the ionic channels has been hampered by difficulties in obtaining a homogeneous axolemma preparation containing these pathways. In our laboratory a procedure was developed for the isolation of plasma membranes from squid nerve fibers[3]. This procedure permits the isolation of a

fraction which seems to correspond to the axolemma, based on yield, electron microscopy appearance, and content of total, Na^+-, K^+-dependent, and cardiac glycoside-sensitive ATPases[3]. This method, slightly modified, has been applied in the present work to lobster nerves. Other procedures for the isolation of axolemma from squid optic nerves[4] and lobster walking-leg nerves[5], using similar identification criteria, have been recently described. In addition, acetylcholinesterase activity, which has been proposed and utilized as an enzymic marker of the axolemma in lobster nerve fibers[5–7], was also used as a marker in the present work.

The presence and integrity of ionic channels in isolated axolemma has not been demonstrated. Tetrodotoxin, which binds to this membrane and specifically blocks its Na^+ pathways (see refs 8–13) appears to be an appropriate marker for the detection of structures related to the Na^+ channels.

The present work deals with the demonstration of tetrodotoxin receptors in an axolemma-enriched preparation (Fraction I) obtained from lobster walking-leg nerves. The number of receptors per unit of membrane surface area, and the apparent dissociation constant (equilibrium constant) of tetrodotoxin with the receptors are about the same as those found in living axons[11,13]. This membrane preparation (Fraction I) appears to be a useful material for the characterization of the structures controlling the ionic movements across the axon excitable membrane.

EXPERIMENTAL METHODS

Isolation of nerve fiber membranes

Membranes were isolated following a modification of the method previously developed in our laboratory[3]. Nerves dissected from walking-legs of living *Panulirus argus* lobsters, were placed in cold lobster physiological solution[14] and processed within 4 h. 40 g of nerves were transferred from physiological solution to 200 ml of 0.33 M sucrose buffered with 5 mM Tris–HCl, pH 7.4, and containing 0.2 mM $MgSO_4$ (sucrose A). The tissue was minced with a stainless steel blender for 2 min. The minced tissue was subsequently homogenized by 10 up and down strokes with a teflon-pestle homogenizer rotating at 500 rev./min. The homogenate was centrifuged at $70000 \times g$ for 60 min ($4.2 \cdot 10^5 \times g \cdot$min) at 2 °C. The supernatant was discarded and the pellet suspended in 90 ml sucrose A and homogenized again with a teflon-pestle homogenizer. 30-ml portions of homogenate were layered on top of 30 ml of 1.195 M sucrose buffered as sucrose A solution. This discontinuous gradient was centrifuged for 60 min at $74000 \times g$ at 2 °C in a Beckman SW 25.2 rotor. The band at the interface was collected and diluted to 90 ml with 5 mM Tris–HCl buffer at pH 7.4, containing 0.2 mM $MgSO_4$. 30-ml portions were relayered on 30 ml of 1.195 M sucrose. This gradient was centrifuged at $74000 \times g$ for 60 min at 2 °C. The sharp white band at the interface was collected and diluted 3-fold with 5 mM Tris–HCl buffer. The suspension was centrifuged at $70000 \times g$ for 30 min at 2 °C and the pellet identified as total nerve plasma membranes was collected in 6 ml of 2 mM Tris buffer at pH 7.4. The suspension was homogenized with a teflon-pestle homogenizer and 2-ml portions were placed on top of SW 25.1 rotor tubes containing a sucrose linear gradient from 0.66 to 1.195 M, buffered with 5 mM Tris–HCl at pH 7.4. The gradients were centrifuged at $74000 \times g$ during 3 h at 2 °C. After centrifugation two well defined cloudy regions banding at densities of 1.072 (Fractions I) and

1.124 g/ml (Fraction II) were collected, diluted 3–4-fold with 5 mM Tris–HCl buffer at pH 7.4, and centrifuged for 30 min at $70000 \times g$ at 2 °C. The pellets were collected, resuspended in 5 mM Tris–HCl buffer and centrifuged again. For the experiments on tetrodotoxin binding the pellets used were suspended in lobster physiological solution. It has been found that the yield and homogeneity of the axolemma preparation (Fraction I) is improved by the hypotonic shocks produced by homogenization of the nerve fibers in 0.33 M sucrose and the homogenization of the total nerve plasma membranes in 5 mM Tris–HCl.

Three subcellular fractions were used to study tetrodotoxin binding: (a) the first pellet containing the material present in the nerve fiber homogenate which sediments by centrifugation at $70000 \times g$ for 60 min ($4.2 \cdot 10^5 \times g \cdot \text{min}$); (b) the total nerve plasma membrane preparation containing axolemma and plasma membrane of periaxonal cells; and (c) the two membrane fractions obtained from the total plasma membrane preparation which are identified as membrane Fraction I and membrane Fraction II. As seen below, membrane Fraction I is identified as an axolemma preparation.

Analytical procedures

The method of Lowry *et al.*[15] was used to measure the protein content of the preparations. The lipid content was established by dry weight determinations[3]. ATPase activities were assayed in 50 mM Tris–HCl at pH 7.4, containing 3 mM $MgSO_4$, 1 mM Tris–ATP and with or without 150 mM NaCl, 25 mM KCl, and 0.2 mM ouabain. Incubations were carried out for 1 h at 20 °C and the phosphate liberated by the enzyme was evaluated, according to Baginski *et al.*[16]. Acetylcholinesterase activities were measured by the method of Hestrin[17] as utilized by Welsch and Dettbarn[7] for lobster walking-leg nerves. The substrate was 5 mM acetylcholine in lobster physiological solution, adjusted with 0.1 M Tris–HCl to pH 7.6. Incubations were at 20 °C during 5–10 min and were terminated by the addition of 0.2 ml of 1 M HCl to lower the pH to 2.5.

Preparation of [^3H]tetrodotoxin

Tetrodotoxin free of citric acid (Sankyo Ltd., Tokyo) was labelled by the Wilzbach method[18] (ICN, Tracerlab, Irvine, Calif.). This material was purified according to the procedure described by Hafemann[19] and the concentration of [^{3}H]tetrodotoxin in the purified preparation was evaluated by a mouse survival assay[19]. The specific activity of the toxin was determined by measuring the radioactivity corresponding to the biologically active component separated by thin-layer chromatography. Two solvent systems[20] were used for the chromatographic analysis: butanol–acetic acid–water (50:3:10, by vol.), and ethanol–acetic acid (96:4, v/v). These measurements indicated that the material has a radiochemical purity of 72%. From this value a specific activity of 37.4 Ci/mole was calculated for [^{3}H]tetrodotoxin. The experimental method to be described eliminates the contribution of radioactive impurities to the [^{3}H]tetrodotoxin binding measurements.

Measurements of [^3H]tetrodotoxin binding to nerve membranes

The suspension of nerve membranes, incubation, and washing procedures were carried out in lobster physiological solution[14] at 4 °C. The composition of

this solution was: 465 mM NaCl, 10 mM KCl, 8 mM $MgCl_2$, 25 mM $CaCl_2$, and 10 mM Tris–HCl, pH 7.5.

The nerve membranes were suspended in physiological solution for 30 min. Afterward, 1-ml aliquots of the suspensions containing between 0.5 and 3.0 mg of protein were incubated during 30 min in 25 ml of either 5 μM non-radioactive tetrodotoxin or tetrodotoxin-free solutions. These solutions were 0.24 mM in acetic acid, required to dissolve the toxin. Their final pH was maintained at 7.5 due to the presence of 10 mM Tris–HCl. [^{3}H]tetrodotoxin was added to all vessels up to a final concentration of 100 nM [^{3}H]tetrodotoxin, and the incubation was prolonged during 30 additional min. At the end of this period the membrane suspensions were centrifuged at $65000 \times g$ during 30 min. The supernatant was drained, and the fluid adhered to the walls of the centrifuge tube was removed by suction. The membrane pellets were suspended in 2 ml of 5 ml Tris–HCl buffer, pH. 7.4, and transferred to counting vials to which were added 4 ml of Instagel liquid scintillation counting solution (Packard Instruments Co., Ill.). The samples were counted in a liquid scintillation spectrometer (Tricarb, Model 3320, Packard Instruments Co., Ill.). The S.D. in net counting was less than 2.5%.

Binding of [^{3}H]tetrodotoxin was calculated by subtracting the radioactivity (cpm/mg protein) present in pellets of membranes preincubated and incubated with an excess of non-radioactive tetrodotoxin from the radioactivity of paired samples preincubated in tetrodotoxin-free solution. The nonradioactive tetrodotoxin is assumed to displace only [^{3}H]tetrodotoxin. The subtractive procedure allows the calculation of the net amount of [^{3}H]tetrodotoxin bound to the subcellular fractions.

RESULTS

Characteristics of the nerve membranes used to measure tetrodotoxin binding

Table I shows the protein and lipid composition of the plasma membrane preparations. In the same table are presented the total Na^+-, K^+-dependent, and cardiac glycoside-sensitive ATPases and acetylcholinesterase activities of the pellet obtained from the nerve fiber homogenate, the total nerve plasma membrane preparation and the two plasma membrane fractions. As shown in Fig. 1, membrane Fractions I and II banded in the linear sucrose gradient at densities of 1.072 and 1.124 g/ml, respectively. These densities are similar to those found for equivalent fractions obtained from squid stellar nerve fibers[3].

Evidence has been presented for isolated plasma membranes of squid stellar nerve fibers which indicates that membrane Fraction I corresponds to the axolemma, and Fraction II corresponds mainly to plasma membranes of periaxonal cells (see ref. 3). Similar evidence has been obtained for the lobster walking-leg nerve fractions in the present work. These indications are: (1) the morphological appearance at high resolution electron microscopy of the isolated membrane fractions, similar to that observed in the intact nerve fibers (Villegas, G. M., unpublished); (2) the ratio of the amount of membrane Fraction I to the amount of membrane Fraction II obtained from the nerve fibers, which is 0.47; and (3) the higher content of total, Na^+-, K^+-dependent, and cardiac glycoside-sensitive ATPase in Fraction I than in Fraction II (Table I). A higher ATPase activity has been observed in the axolemma than in the periaxonal cell plasma membranes of the squid[21].

TABLE I

PERCENTAGE COMPOSITION AND SPECIFIC ACTIVITY OF ATPase AND ACETYLCHOLINESTERASE OF MEMBRANE FRACTIONS ISOLATED FROM LOBSTER NERVE FIBERS

Fraction	*Percentage composition*		*ATPase activity (μmoles P_i/mg protein per h)*			*Acetylcholinesterase (μmoles acetylcholine/mg protein per h)*
	Protein	*Total lipids*	*Total ATPase*	*Na^+-, K^+ dependent*	*Ouabain sensitive*	
Nerve homogenate pellet	—	—	2.8	2.5	2.5	45.9
Total nerve plasma membranes	32.0	68.0	18.3	10.6	6.1	192.8
Membrane Fraction I	24.0	76.0	64.2	44.0	23.0	300.0
Membrane Fraction II	39.4	60.6	46.4	13.9	6.5	184.0

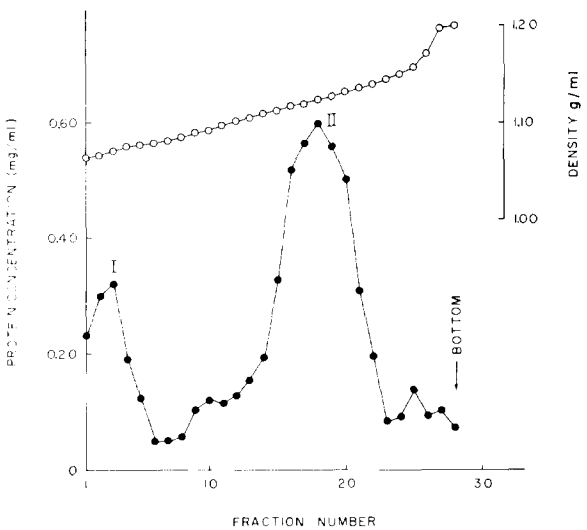

Fig. 1. Separation of membrane Fractions I and II obtained after 3 h centrifugation of nerve plasma membranes at 74000 × *g* in a linear sucrose-density gradient. A SW 25-1 rotor was used and 0.6-ml fractions were collected from the top of the tubes.

The acetylcholinesterase activity found to be higher in lobster membrane Fraction I than in Fraction II, as shown in Table I, gives further support to the identification of fraction I as the axolemma, according to the previously described distribution of this enzyme in lobster nerve[5–7].

The homogenity of the plasma membrane fractions has been explored by again subjecting each fraction to hypotonic shock and recentrifugation in a sucrose gradient. Membrane Fraction I was once again found as a single band located at its density of 1.072 g/ml. On the other hand, from Fraction II, a small amount of Fraction I was obtained, in addition to the main band corresponding to this fraction.

These results indicate that the axolemma-enriched preparation (Fraction I) is homogeneous, but that Fraction II, when obtained from the first linear gradient, contains up to a 20% contamination with Fraction I. Since Fraction I, as obtained from the first linear gradient, appears to be a homogeneous preparation and the lability of the tetrodotoxin receptors in the isolated membranes is unknown, all experiments were carried out with the fractions obtained from the first linear gradients. The presence of residual axolemma in Fraction II obtained from the first linear gradient, should contribute to the results obtained with this fraction.

Binding of [^3H]tetrodotoxin to nerve membranes

Fig. 2 shows the amount of [^{3}H]tetrodotoxin bound from 100 nM [^{3}H]tetrodotoxin solution by the first pellet obtained from the nerve fiber homogenate, the total nerve plasma membranes, and the plasma membrane Fractions I and II. The results are expressed in pmoles (10^{-12} moles) of [^{3}H]tetrodotoxin bound per mg of protein in each subcellular fraction. The values are: 2.1 for the nerve fiber homogenate pellet; 5.1 for the total plasma membrane preparation; 9.5 for membrane Fraction I (axolemma); and 3.4 for membrane Fraction II (plasma membranes of periaxonal cells *plus* residual Fraction I).

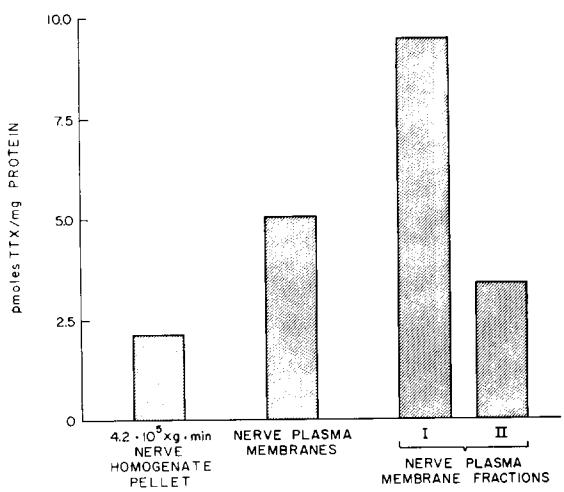

Fig. 2. Binding of [^{3}H]tetrodotoxin (TTX) in lobster physiological solution to the $4.2 \cdot 10^5 \times g \cdot$min pellet obtained from a total nerve fiber homogenate, the nerve plasma membrane preparation, and the two membrane fractions obtained from it after 3 h centrifugation at $74000 \times g$ in a linear sucrose gradient. The pH of the incubation media was 7.5.

Experiments with saxitoxin

Experiments were carried out replacing with 5 μM saxitoxin, the 5 μM non-radioactive tetrodotoxin used to displace [^{3}H]tetrodotoxin. In two samples it was found that the ratio of the amount of [^{3}H]tetrodotoxin displaced by 5μM saxitoxin to the amount removed in one paired experiment by 5 μM non-radioactive tetrodotoxin, was 1.04. This result indicates that saxitoxin displaces [^{3}H]tetrodotoxin from nerve plasma membrane preparations as efficiently as tetrodotoxin.

Experiments with tetrodonic acid

The binding of [^{3}H]tetrodotoxin was studied in the presence and absence of tetrodonic acid. The tetrodonic acid experiments were carried out with nerve plasma membranes replacing the 5 μM nonradioactive tetrodotoxin used to displace [^{3}H]-tetrodotoxin by 5 μM tetrodonic acid. The ratio of radioactivity in two preparations with tetrodonic acid to that of paired samples incubated without tetrodonic acid was 1.02. This ratio indicates that tetrodonic acid does not displace tetrodotoxin from its binding sites.

[^{3}H]Tetrodotoxin dose–binding experiments

Further information on the association of tetrodotoxin to the receptor sites in isolated nerve membranes was obtained from experiments in which the binding of [^{3}H]tetrodotoxin was determined at toxin concentrations of 10^{-11}–10^{-7} M.

Fig. 3 shows a plot of the pmoles of [^{3}H]tetrodotoxin bound per mg of total membrane protein as a function of molar concentration of [^{3}H]tetrodotoxin in the incubation solution. The data are well fitted by a rectangular hyperbola. The sigmoid curve shown in Fig. 3 is a semilogarithmic plot of the rectangular hyperbolic function calculated by assuming an apparent dissociation constant (equilibrium constant) for the [^{3}H]tetrodotoxin–membrane receptor complex of $4.0 \cdot 10^{-9}$ M. The simplest interpretation of the curve is that there is a single type of binding site at the membrane and that each site reacts independently with one tetrodotoxin molecule.

Removal of [^{3}H]tetrodotoxin by successive washings

Experiments were carried out to explore the removal of radioactivity from plasma membrane preparations by successive washings with lobster physiological

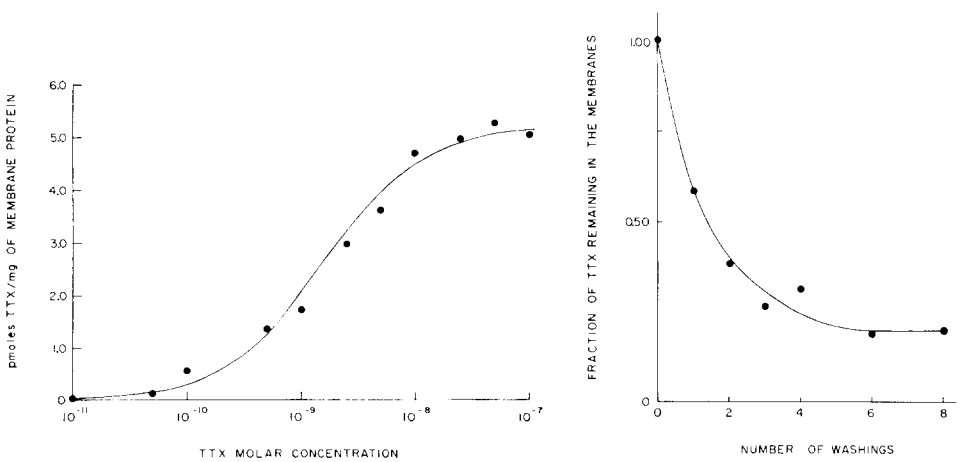

Fig. 3. Dose-binding of [^{3}H]tetrodotoxin to nerve plasma membranes. The binding of [^{3}H]-tetrodotoxin is plotted as a function of toxin concentration in the lobster physiological solution used to incubate the membranes. The pH of the incubation media was 7.5. The apparent dissociation constant used to calculate the best fitting curve was $4.0 \cdot 10^{-9}$ M.

Fig. 4. Removal of [^{3}H]tetrodotoxin by successive washings of the membrane with lobster physiological solution (pH 7.5).

solution. Paired pellets of membrane preparations incubated as described above, one with 5 μM non-radioactive tetrodotoxin *plus* 100 nM [^{3}H]tetrodotoxin and the other with only 100 nM [^{3}H]tetrodotoxin, were resuspended in 25 ml of lobster solution and recentrifuged to obtain the pellets again; this procedure was repeated eight times. The radioactivity of the membranes before the first resuspension and after completion of one, two, three, four, six and eight washing cycles was measured.

As shown in Fig. 4, after eight washings 20% of [^{3}H]tetrodotoxin still remained bound to the membrane; this percentage could not be removed by dilution with tetrodotoxin-free nonradioactive solution. The contribution of radioactive contaminants to this percentage is discarded by the subtractive procedure used to calculate the net amount of [^{3}H]tetrodotoxin bound to the membranes. On the other hand, it should be noted that in the membranes which were preincubated with nonradioactive tetrodotoxin, 8.2% of the [^{3}H]tetrodotoxin bound before the first washing still remained after eight washings. This percentage may represent tightly bound contaminants and/or the amount of [^{3}H] tetrodotoxin that is not removed with the excess of nonradioactive tetrodotoxin in the present experiments.

DISCUSSION

The experimental results have shown the presence of tetrodotoxin receptors in the plasma membranes isolated from lobster walking-leg nerves by a slight modification of the procedure described for squid axons[3]. The binding of [^{3}H]tetrodotoxin per mg of protein increases, as shown in Fig. 1, from 2.1 pmoles for the first nerve fiber homogenate pellet to 9.5 pmoles for nerve membrane Fraction I, which corresponds to the axolemma.

It is important to notice that Fraction I contains 17.6 times more Na^+-, K^+-dependent ATPase than the nerve fiber homogenate pellet. This enzyme is in all probability an axolemma constituent, since the hypotonic condition used to prepare the membranes should not favor trapping of the enzyme into vesicles. The specific activity of the enzyme in Fraction I (44 μmoles P_i per mg protein per h) is 6.4 times higher than that found for an equivalent axolemma enriched preparation obtained from the lobster *Homarus americanus*[5], and 4.4 times lower than that for plasma membranes of retinal axons of the squid *Dosidicus gigas*[4]. The discrepancies in ATPase activity could be due to differences in the sources of the membranes and in the isolation procedures. As pointed out in previous paragraphs, the yield of membrane Fraction I, its morphological appearance, as well as its high ATPase and acetylcholinesterase activity, indicate that this nerve membrane fraction is an axolemma preparation. Additional support to this identification is the higher content of tetrodotoxin receptors in membrane Fraction I than in the other membrane preparations. Denburg[5] has recently isolated from lobster walking-leg nerves a fraction of low density with high ATPase and acetylcholinesterase activity. This membrane isolated in hypotonic conditions, as our Fraction I, is also considered an axolemma-enriched preparation. The membrane structure seems to be preserved since the electron microscopy appearance and thickness of membrane Fraction I is indistinguishable from axolemma in the intact nerve fibers (Villegas, G. M., unpublished). The apparent low density of the lobster axon plasma membrane suggests that in the fractionation

scheme of Welch and Detbarn[7] the axolemma should be in the floating pellet isolated by centrifugation in hyperosmotic or isosmotic sucrose.

The tetrodotoxin receptors present in the isolated membranes share similar properties with the tetrodotoxin receptors found in living nerve fibers: (a) tetrodonic acid does not bind to the tetrodotoxin sites; (b) saxitoxin and tetrodotoxin appear to compete for the same receptor; and (c) the apparent dissociation constant of the tetrodotoxin–receptor complexes in the isolated membranes and in living axons are about the same.

The finding that tetrodonic acid does not bind to the tetrodotoxin receptors agrees with the lack of neurotoxicity of tetrodonic acid[22]. It appears that the opening of the hemilactal ring in tetrodotoxin to produce tetrodonic acid abolishes the affinity of the molecule for the receptor site[22].

The observation that saxitoxin appears to bind at the same receptor as tetrodotoxin in the isolated membranes is in agreement with results obtained in intact nerve fibers. Both molecules have the same striking property of blocking the Na^+ channels in the axolemma (see refs 1, 2, 12 and 23). It should be noticed that tetrodotoxin and saxitoxin are produced by different organisms and have different chemical structures sharing only the presence of a guanidinium group (see refs 24–26).

The apparent dissociation constant (equilibrium constant) for the binding of [^{3}H]tetrodotoxin to the receptors in the isolated membranes is $4.0 \cdot 10^{-9}$ M. This value is in good agreement with those determined by electrophysiological experiments for the binding of tetrodotoxin to the excitable membrane of living axons, which are: $3.3 \cdot 10^{-9}$ M for the giant axons of the squid[27], and $3.5 \cdot 10^{-9}$ M for the non-myelinated fibers of the rabbit vagus nerve[28]. For saxitoxin with the frog nodes of Ranvier, the constant is $1.2 \cdot 10^{-9}$ M (see ref. 12).

The experiments on the removal of [^{3}H]tetrodotoxin bound to isolated membranes by successive washings with tetrodotoxin-free physiological solution reveals that with this procedure only a fraction (0.80) of the bound toxin can be washed out. It should be noted that the removal of tetrodotoxin by successive washings of the membranes with tetrodotoxin-free solution is a process different from that of exchange between [^{3}H]tetrodotoxin and non-radioactive tetrodotoxin. The failure to remove from the isolated membranes all the bound tetrodotoxin by washing with physiological solution may be related to results obtained in living nerve fibers[13,29]. In the lobster *Homarus vulgaris*, it has been found that even prolonged rinsing in tetrodotoxin-free solution failed to restore the action potential abolished by tetrodotoxin, except in one or two of the largest fibers[13]. A quantitative comparison between the results obtained with intact axons and isolated membranes can not be made at present, because the relationship among the time of exposure to tetrodotoxin, the extent of removal of tetrodotoxin by washing and the functional recovery after tetrodotoxin treatment of the axonal membranes in the three species of lobster used, has not yet been established.

From the data available, it is possible to calculate the number of receptors in nerve membrane Fraction I, identified as an axolemma preparation. Taking the density of this membrane fraction as equal to the mean density of the segment of the sucrose linear gradient at which it bands (1.072 g/ml), and taking its thickness from measurements of high resolution electron micrographs as 94 Å (Villegas, G. M., unpublished), it is possible to calculate that there are $9.9 \cdot 10^5$ cm^2 of surface area per

g of membrane. When the toxin concentration in the medium is 10^{-7} M, the binding sites in the isolated nerve plasma membranes appear to be saturated. At this concentration, membrane Fraction I has $9.5 \cdot 10^{-12}$ moles/mg protein. Since protein represent 24% of the membrane weight, the total membrane surface area per mg of protein is $4.1 \cdot 10^3$ cm^2. Tetrodotoxin has been demonstrated to act only at the external side of the axolemma[30]; therefore, assuming all the molecules of [^{3}H]tetrodotoxin to be bound at one membrane face only, it can be calculated that there are 28 molecules per μm^2. From electrophysiological measurements on the effect of tetrodotoxin in lobster living axons and assuming that each Na^+ channel is blocked by a single tetrodotoxin molecule (see refs 11, 13 and 27), it has been calculated that there are between 13 and 36 sodium channels in 1 μm^2 of axolemma external surface[11,13]. Therefore, the value of 28 tetrodotoxin receptors (or Na^+ channels) per μm^2 determined in membrane Fraction I, is in good agreement with those reported for the living lobster axons. It should be noted that this value could vary with the method used to isolate the plasma membranes and the extent of purification of the axolemma preparation.

The isolation from lobster nerve fibers of an axolemma preparation containing the expected number of tetrodotoxin receptors per unit area, could be considered an initial step toward the characterization of the structures associated with the ionic movements during the nervous impulse.

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