Saxitoxin Binding in Nerves from Walking Legs of the Lobster *Homarus americanus*

Two Classes of Receptors

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**Abstract** The binding of exchange-labeled saxitoxin (STX) to sodium channels has been investigated in the nonmyelinated fibers of the walking leg nerves of the lobster. The properties of the STX binding site differed systematically among the nerves from different walking legs. The equilibrium dissociation constant for STX binding ($K_{STX}$) to the front legs is approximately twice that for the binding to the rear legs; the average ratio of $K_{STX}$ (front):$K_{STX}$ (rear) from five separate experiments was 1.80 ± 0.12 (mean ± SE). The actual $K_{STX}$ values ranged from 14.0 to 22.7 nM for the front leg nerves and from 8.6 to 12.7 nM for the rear leg nerves. $K_{STX}$ values for the middle two walking leg nerves fell between those for the front and rear legs. The inhibitory dissociation constant for tetrodotoxin ($K_{TTX}$), calculated from tetrodotoxin's inhibition of labeled STX binding, was 3.02 ± 0.27 nM for the front legs and 2.20 ± 0.33 nM for the rear legs. The ratio $K_{STX}:K_{TTX}$ was different in the front and rear leg nerves, being 5.5 and 4.2, respectively. The apparent $pK_a$ of the STX receptor also differed between the two legs, being 4.6 ± 0.3 for the front legs and 5.1 ± 0.1 for the rear legs. These results demonstrate that one tissue type in one organism can contain different toxin binding sites. The difference in the receptors can be qualitatively accounted for by the location of an additional negative charge near the receptor site of the rear walking leg.

**Introduction**

Saxitoxin (STX) and tetrodotoxin (TTX) are small organic molecules that specifically block sodium channels in many excitable membranes. The physical chemical properties of the receptor for STX and TTX have been studied in both electrophysiological experiments and in binding experiments with radiolabeled toxins. Earlier results revealed strong similarities between the properties of the toxin receptor and of the conductance of open sodium channels; for example, both toxin binding and sodium conductance were blocked by protons at pH 5.2–5.4, by calcium ions, and by thallous ions (Colquhoun et al., 1972; Henderson et al., 1973 and 1974; Hille, 1968 b, Woodhull, 1973). The blocking action of TTX was also reduced by acidic
Ringer's solutions, in good agreement with the results from toxin binding experiments (Ulbricht and Wagner, 1975 a and 1975 b). The binding activities of STX and TTX to sodium channels are mutually exclusive (Henderson et al., 1973; Wagner and Ulbricht, 1975); thus, the competition between protons and cations implied that there was a single toxin receptor site located at the outer opening of the sodium channel that was also an acidic binding site for cations (Henderson et al., 1974). Very specific models suggested that the toxin receptor was identical to the sodium channel's ion "selectivity filter," the structure that discriminates among permeant and impermeant cations (Hille, 1975). However, more recent results show that chemical modifications of channels resulting in an insensitivity to TTX and STX do not alter their ion selectivity properties (Spalding, 1980), so the exact location of the receptor for TTX and STX is still undetermined.

One method for testing the locus and structure of the toxin receptor is to compare the action of structurally complex inhibitory agents on toxin binding and sodium currents (Hansen Bay and Strichartz, 1978). However, a valid comparison is only possible when the same sodium channels are investigated in each type of measurement. In this paper, we report two different classes of toxin receptors in the walking leg nerves of the lobster. In a subsequent paper we shall describe the detailed characteristics of ion and drug binding to these receptors. Our findings show that apparently different classes of sites occur in the same organism and caution against any quick comparison of toxin binding and electrophysiological data from different tissues.

METHODS

Labeled saxitoxin (*STX), prepared by the method of H exchange (Ritchie et al., 1976), was purified by high-voltage paper electrophoresis, eluted into 10 mM morpholinoethane sulfonate (MES) buffer at pH 6.5, and stored at -80°C. The results reported here are the combination of results from experiments using two separate *STX preparations, one of ~70% purity, in which all fractions under the fastest moving radioactive peak during electrophoresis were pooled, and one of >90% purity, in which only fractions from the leading edge of the fastest moving radioactive peak were used. Binding results from both *STX preparations were comparable and in close agreement with previously published values.

Lobster walking legs were removed from live lobsters, usually weighing 0.7-1.0 kg, and the nerves were dissected by the method of Furasawa (1929). Nerves were soaked in artificial seawater (NaSW) of the following composition; NaCl, 0.44 M; MgCl₂ 0.05 M; CaCl₂, 0.01 M; KCl, 0.01 M; HEPES¹ buffer, 0.005 M, pH 7.4. Experiments to test the pH dependence of *STX binding usually used NaSW containing the following buffers: 2 mM HEPES, 2 mM propionate (Na), 2 mM MES, with the final pH adjusted as noted by the addition of HCl or NaOH. In one pH experiment, 10 mM of each of the three buffers was used, and the results were indistinguishable from those using lower buffer concentrations.

Dissected nerves were sectioned lengthwise into four to six pieces and soaked in 5- or 10-ml volumes of test solutions containing *STX for a minimum of 8 h at 4°C. Longer soaking periods produced no changes in toxin uptake. Nerve pieces were removed, blotted gently on filter paper, weighed on a torsion balance (0-100 mg;

¹ N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid.
Bethlehem Apparatus Co., Hellertown, Pa.) and digested in glass vials by 0.5 ml of tissue solubilizer (Protosol, New England Nuclear, Boston, Mass.) and 0.1 ml H$_2$O for 6-10 h at 60°C. 5 or 10 ml of scintillant (Aquasol-2, New England Nuclear) was added to the digestate after it had cooled to room temperature, and the tritium content was measured in a liquid scintillation counter (Nuclear-Chicago Mark II or Beckman LS-8100 [Beckman Instruments, Inc., Fullerton, Calif.]).

Toxin uptake was composed of saturable binding and a nonsaturating linear component. The saturable component of *STX uptake was resolved from the total uptake data by subtracting the linear component of toxin uptake, that which remained in the presence of unlabeled TTX at concentrations of 1-10 μM. The linear uptake is the sum of free toxin distributed both in the interaxonal space and inside the axons, having entered from the cut ends, and of toxin that associates with and binds to the nerves with very low affinity at sites other than the sodium channels. The interaxonal and intracellular compartments can be estimated from the uptake of [14C]mannitol by the nerve, so that the actual nonsaturable binding of *STX to the nerves can also be measured. This nonsaturable component when measured with the 70° pure *STX was quite similar to that measured with the >90% pure toxin, showing that nonsaturable binding is largely due to radioactive toxin and not to impurities.

Data are presented as mean values ± standard errors. Statistically significant differences are expressed as P values calculated using a two-tailed Student's t test.

The drugs used were prepared as a stock solution, usually 10$^{-2}$-10$^{-4}$ mg/ml in 10 mM HEPES, pH 7.2 (anesthetics, maculotoxin, scorpion venoms). The pharmacological activities of these drugs were tested by monitoring their effects on extracellularly recorded compound action potentials of the lobster walking leg nerves at 20°C in NaSW.

Leiurus quinquestriatus scorpion venom was obtained from Sigma Chemical Co., St. Louis, Mo., and HEPES and MES buffers and TTX from Calbiochem, San Diego, Calif. Lidocaine and its quaternary derivatives were kindly provided by Dr. Bertil Takman, Astra Pharmaceuticals, Framingham, Mass., Centruroides sculpturatus scorpion venom was generously provided by Dr. William Culp, Dartmouth Medical School, Hanover, N. H., and crude preparation of maculotoxin venom was the generous gift of Dr. Peter Gage, University of New South Wales, Kensington, Australia. A standard for saxitoxin was obtained as paralytic shellfish poison from the U. S. Food and Drug Administration, Cincinnati, Ohio.

RESULTS

STX and TTX Binding Parameters

The saturable binding of *STX to front and rear walking leg nerves is shown in Fig. 1 A. The walking leg nerves from three lobsters were separated into front and rear nerve “pools”; the average *STX uptakes by the different pooled nerves in the presence of 10 μM unlabeled TTX were subtracted from the total toxin uptake to yield the saturable uptakes. The lobster has four pairs of walking legs, but we usually used only the most anterior and posterior pairs to emphasize the difference in binding properties (see below). The curve for saturable toxin uptake ($U_{sat}$) is described by the binding equation

$$U_{sat} = \frac{U_{max} [STX]}{[STX] + K_T},$$

(1)

Where $U_{max}$ is the total number of binding sites and $K_T$ the dissociation
Figure 1. (A) Saturable binding of [3H]STX to separated front and rear walking leg nerves from three lobsters. Each point is the average uptake of 3–4 pieces of nerve. The curve is drawn from Eq. 1 using the binding parameters from the line fit to the Scatchard plot of B. $T = 4^\circ C$ 8-h incubation. (B) Scatchard plot of the data in A. The standard errors of the average values are shown as vertical lines when they exceeded the dimension of the symbols. Lines are drawn from a least-squares linear regression fit to the data from the separate legs.

constant for the toxin:receptor complex. Eq. 1 can be rewritten as

$$\frac{U_{\text{sat}}}{[\text{STX}]} = \frac{U_{\text{max}} - U_{\text{sat}}}{K_T}.$$  (2)

A graph of Eq. 2, $U_{\text{sat}}/[\text{STX}]$ vs. $U_{\text{sat}}$, shown in Fig. 1 B, is a Scatchard plot of the data of Fig. 1 A. From Eq. 2, the negative inverse of the slope of this
plot is the equilibrium dissociation constant for the toxin binding reaction, $K_T$, and the x-axis intercept is the toxin binding capacity, $U_{max}$. In this particular experiment the toxin binding capacities of front and rear walking leg nerves were equal when expressed on a wet weight basis, but the dissociation constants differ by a factor of 2.4.

The parameters for STX binding to walking leg nerves are collected in Table I. The binding capacities are equal in front and rear walking leg nerves and are close to the published value for pooled lobster walking leg nerve, $94 \pm 5$ fmol · mg wet wt⁻¹ (Ritchie et al., 1976). In contrast, the affinity ($K_T^{-1}$) for STX of the rear legs is consistently about twice that of the front legs. The $K_T$ values for the second and third pair of walking leg nerves lie between those for the front and rear pair; i.e. the toxin affinity increases in nerves taken from progressively posterior walking legs (Fig. 2). Previous studies did not detect multiple classes of binding sites in preparations of lobster walking leg nerves when the nerves were indiscriminately pooled. Nerves from the front two pairs of legs account for >75% of the total nerve weight, so the higher affinity sites of the rear leg nerves were probably obscured in the total uptake. In experiments for which we intentionally pooled nerves from all walking legs it was impossible to resolve significantly different binding constants.

The inhibition of *STX binding by a low concentration of unlabeled TTX is illustrated in Fig. 3 A. In Fig. 3 B the data are graphed as a Scatchard plot; the slope ($-1/K_T$) of the plot is reduced by TTX, but the X-intercept, $U_{max}$, is unchanged, showing that TTX acts as a competitive inhibitor of *STX binding. The inhibitory dissociation constant for TTX binding, $K_I$, is

$$K_T = K_T(1 + [I]/K_I)$$

where $K_T$ is the measured dissociation constant for *STX binding in the presence of TTX (at concentration [I]), and $K_T$ the dissociation constant with no inhibitor present.

The stoichiometry of the inhibition by any competitive inhibitor may be determined by application of the Hill equation (Hill, 1910). If the receptor, R, can combine with n molecules of inhibitor, I, to produce a blocked state

$$R + nI \rightleftharpoons RI_n$$

as well as combining with toxin to form a bound state

$$R + T \rightleftharpoons RT,$$

then the saturable uptake in the presence of inhibitor is given by

$$U_{sat} = \frac{U_{max} [STX]}{[STX] + K_T(1 + [I]^n/K_I)}$$

Dividing $U_{sat}$ by $U_{sat}$ (Eq. 1) gives the relative uptake, $\alpha$, which leads directly to

$$\frac{1}{\alpha} - 1 = \frac{[I]^n/K_I}{([STX]/K_T + 1)}.$$
<table>
<thead>
<tr>
<th></th>
<th>$K_{\text{STX}}$</th>
<th>$K_{\text{TX}}$</th>
<th>$K_{\text{STX}}$</th>
<th>$U_{\text{max}}$</th>
<th>$pK_a$</th>
<th>e.c.s.</th>
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<td></td>
<td>$\text{nM}$</td>
<td>$\text{nM}$</td>
<td>$\text{fmol} \cdot \text{mg wet wt}^{-1}$</td>
<td>$\mu l \cdot \text{mg wet wt}^{-1}$</td>
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<tr>
<td>Front leg nerve</td>
<td>$18.7 \pm 1.2(6)$</td>
<td>$3.02 \pm 0.27(4)$</td>
<td>$2.18 \pm 0.29(3)$</td>
<td>$128 \pm 13.2(6)$</td>
<td>$4.63 \pm 0.29(3)$</td>
<td>$1.90 \pm 0.12(23)$</td>
</tr>
<tr>
<td>Rear leg nerve</td>
<td>$9.2 \pm 1.4(4)$</td>
<td>$2.20 \pm 0.33(4)$</td>
<td>$2.18 \pm 0.29(3)$</td>
<td>$120 \pm 21(4)$</td>
<td>$5.11 \pm 0.09(3)$</td>
<td>$1.22 \pm 0.12(19)$</td>
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* For front leg vs. rear leg, binding parameters are significantly different at $0.0005 < P < 0.005(*)$, $0.05 < P < 0.10(\ddagger)$, and $0.10 < P < 0.25(\dagger)$.

§ Averaged ratios of STX dissociation constants between paired front and rear walking legs from three separate experiments, in each of which three animals were used. The ratio of the separately averaged uptakes is $1.80 \pm 0.12$. 
Taking the logarithm of both sides gives an equation which is linear in $n$,

$$\log\left(\frac{l - 1}{\alpha - 1}\right) = n \log I - \log[K_1(1 + [\text{STX}]/K_T)]$$

(5)

A plot of $\log\left(\frac{l - 1}{\alpha - 1}\right)$ against $\log I$, a Hill plot, will have a slope equal to the molecularity of the inhibition reaction, $n$ (Hill, 1910).

The results of one TTX competition experiment are graphed as a Hill plot in Fig. 4. The TTX concentration is varied while *STX is kept constant.

Linear regression analysis of the data yields a best-fitting line with slope of 1.22; the $K_1$ for TTX calculated from this experiment is 3.69 nM. Slopes of the Hill plots for all experiments measuring TTX inhibition of STX binding ranged from $n = 0.96$ to $n = 1.22$, demonstrating that only one bound TTX molecule is required to displace an *STX molecule. The averaged inhibitory binding constants for TTX are listed in Table I. As with STX, the affinity of TTX for the receptors of rear walking leg nerves is greater than that for front walking leg nerves ($0.05 < P < 0.10$). However, the ratio of $K_{\text{STX}}$ to $K_{\text{TTX}}$ is slightly larger in front than in the rear leg nerves.
Figure 3. (A) [3H]STX uptake by walking leg nerves in the presence of nonradioactive TTX. The total uptake in 0 TTX is shown by the filled circles through which no line is drawn. The uptake in the presence of 10 μM TTX (x), fit by the dashed line, shows the linear, nonsaturable uptake. The difference between these two is the saturable uptake, shown by the open symbols. The solid lines are described by Eqs. 1 and 3 using the binding parameters from the Scatchard plot analysis of B. (B) Scatchard plots of the saturable uptake data from A. For the front leg nerves, $K_{STX} = 14.0$ nM and $K_{TTX} = 3.66$ nM (5 nM TTX). For the rear leg nerves, $K_{STX} = 6.1$ nM and $K_{TTX} = 2.83$ nM (5 nM TTX).

pH Effects
Protons compete with STX binding at receptor sites on both front and rear walking leg nerves (Fig. 5). The pH dependence of saturable binding is different for the different nerves; the $pK_a$ for the receptor of the front legs is...
about 0.5 pH unit lower than that for the rear legs (0.1 < P < 0.25). Hill plots of this inhibition reaction yield both molecularity and $K_I$ values ($pK_a$). For the front walking leg nerves, the $pK_a = 4.63 \pm 0.29$ and $n = 1.05 \pm 0.20$ (three determinations), and for the rear leg nerves the $pK_a = 5.11 \pm 0.09$ and $n = 1.28 \pm 0.45$ (three determinations). In two experiments we preincubated walking leg nerves and STX separately at pH 4.5 for 6 h at 4°C and then measured the binding of this STX by these lobster leg nerves after a 6-h incubation at pH 7.4. The saturable uptake was 89-94% of the control uptake by nerves preincubated at pH 7.4, indicating that irreversible acid denaturation accounts for little of the observed inhibition by protons.

**Surface Potential at the Toxin Receptors**

One possible explanation for the differences of both toxin affinities and $pK_a$ values between the receptors in front and rear walking legs is a difference in
the local electrostatic potential at the toxin receptor. These “surface” potentials would arise from charges fixed on the membrane near the binding site. Such fixed charges would result in a diffuse layer of non-specific ions adjacent to the membrane, (McLaughlin et al., 1971), thereby modulating the local concentration of any charged ligand, including the toxins, and this affecting both the apparent toxin affinity and the pKₐ.

The magnitude of the surface potential at the STX/TTX receptor in several different membranes has been estimated using toxin binding (Henderson et al., 1974) and by voltage-clamp methods (Hille et al., 1975a). These surface potentials were calculated from equations derived in the following way. The concentration at the receptor of a toxin with charge \( z_t \), \([T_t]_R\), is related to its concentration in the bulk solution, \([T_t]_s\), by the Boltzmann equation:

\[
[T_t]_R = [T_t]_s \exp(-z_t \psi_R F/RT).
\] (6)

where \( \psi_R \) is the difference in electric potential between the receptor and the bulk solution, \( R \) is the gas constant, and \( T \) is the temperature in °K. The apparent dissociation constant, \( K_{t,app} \), thus differs from \( K_t \), the intrinsic dissociation constant of the receptor, with \( \psi_R = 0 \):

\[
K_{t,app} = K_t \exp(z_t \psi_R F/RT).
\] (7)

If any one toxin binds to two populations of receptors that differ only in their local surface potential, the potential difference can be determined using

![Figure 4. Hill plot of TTX inhibition of STX binding to front walking leg nerves. The points are mean values of four measurements; the error bars show standard deviations. The line is a least-square fit to all the data points and is described by EQ. 5 where \( n = 1.22 \) and \( K_t = 3.69 \) nM TTX. [STX] = 14 nM.](image-url)
the ratio of apparent dissociation constants. From Eq. 7,
\[
\frac{K_{a\text{pp}}(R_1)}{K_{a\text{pp}}(R_2)} = \exp \left( \psi_{R_1} - \psi_{R_2} \right) \frac{z_1 F}{RT}.
\]  
(8)
Eq. 8 can be solved for \( \psi_{R_1} - \psi_{R_2} \) using the measured toxin binding parameters from Table I. If diffuse surface potentials alone account for the observed differences in receptor properties, then the ratios between front and rear leg nerves of \( K_{STX} \), \( K_{TTX} \) and \( pK_a \) should yield the same difference in surface potential. Substituting the measured values from Table I in Eq. 8, the calculated difference in \( \psi_R \) between front and rear legs is 8.9, 7.9, and 27.6 mV, from the ratios of \( K_{STX} \), \( K_{TTX} \), and \( pK_a \), respectively.

The differences in \( \psi_R \) calculated from the toxin affinities are almost equal, supporting the hypothesis that the observed differences in \( K_T \) values are due to simple electrostatic potential differences. (The range of \( K_T \) values within
the standard errors would give calculated differences of $\psi_R$ of 6.3–11.7 mV for $K_{STX}$ and 6.4–14.1 mV for $K_{TTX}$.) However, the $\Delta\psi_R$ probed by proton titrations is much larger. The discrepancy between the calculated values of $\psi_R$ can be accounted for by the presence of one additional acid group near the receptor in rear leg nerves. This hypothetical negatively charged acid group would raise both the apparent toxin affinity and the proton affinity in rear legs and would provide an additional site through which protons can “titrate” toxin binding, resulting in Hill coefficients that exceed unity and in apparently greater surface potential differences when probed by acid titration (see Discussion).

**Effects of Pharmacological Agents**

Several agents that affect sodium channels were tested for their interaction with the STX receptor. A crude extract of venom from the blue-ringed octopus, *Haplochleane maculosa* (maculotoxin; Dulhunty and Gage, 1971; Gage et al., 1976), inhibited saturable binding of STX in pooled nerves from all the walking legs. STX binding was halved at a venom concentration of 1–2 $\mu$g $\cdot$ ml$^{-1}$. Maculotoxin is reported to contain TTX (Crone et al., 1976), which is consistent with its competition for the STX receptor. Because this crude extract of the octopus salivary gland contained many impurities, we did not test it on the separate walking legs.

Other pharmacologically active agents tested had no effect on *STX binding. Scorpion venom from *Leiurus quinquestriatus* and *Centruroides sculpturatus* were without effect; relative *STX uptakes in the presence of these toxins at 1–5 $\mu$g $\cdot$ ml$^{-1}$ concentrations were 0.97 ± 0.75 (n = 6) and 1.04 ± 0.087 (n = 6), respectively. These results agree with the observed lack of effect of *Leiurus* toxin on STX binding to neuroblastoma cells (Catterall and Morrow, 1978). Also in agreement with previous results (Colquhoun et al., 1972; Henderson et al., 1973), we found that neither the local anesthetic lidocaine, nor its permeant (QX-572) or impermeant (QX-314) quaternary derivatives at 1 mM concentrations affected STX binding.

**Discussion**

The experiments reported here show that the receptors of the different pairs of lobster walking leg nerves differ significantly in STX and TTX affinity. This is the first report of direct measurements revealing a difference in toxin receptors in the same animal, although several electrophysiological studies have previously noted apparent differences in binding affinities. The most detailed of these is an analysis of voltage clamp currents in squid giant axons (Sevcik, 1976), which noted that the concentration dependence of the inhibitory activity of TTX could not be described by a single class of receptors, but rather by two classes whose TTX affinity differed by a factor of 45 (4.9 and 0.11 nM). The high-affinity sites accounted for 20% of the sodium channels, the low-affinity sites for the remaining 80%. (These percents are probably not correct, because the time of exposure of TTX (15 min) was too brief to allow equilibrium binding to be reached [Keynes et al., 1975]). In another kind of experiment, Schwarz et al. (1973) reported that action potentials of sensory
nerve fibers of the frog were more sensitive to TTX than were motor fibers. The different toxin affinities that we find in lobster nerves might arise from differences in the proportion of motor and sensory fibers in the walking leg nerves; the front walking legs terminate in a functional claw, the rear have no grasping function, although the total number of segments is the same in both appendages. However, we are unaware of any direct determination of fiber type in these different leg nerves and so can only speculate on the morphological basis for the observed difference.

Toxin Affinity and Surface Potential

The dissociation constants measured for STX binding in the lobster walking leg nerves are very similar to those in other crustacean nerves (Ritchie et al., 1976; Baumgold, 1980) but are much larger than those measured in mammalian tissues or frog node, 1-5 nM and 1-2 nM, respectively (Weigele and Barchi, 1978a; Krueger et al., 1979; Wagner and Ulbricht, 1975; Hille, 1968a). A large contribution to this apparently weaker toxin affinity in the invertebrate axons arises from the much higher divalent cation content of the seawater solution, 60 mM compared with the 2 or 4 mM calcium concentration of frog Ringer or mammalian Locke solutions. In one experiment we measured a 50% reduction in the dissociation constants for STX of both front and rear walking legs when 50 mM Mg$^{2+}$ was absent from the NaSW solution. (Another factor is the relatively greater monovalent cation concentration of NaSW, 440 mM Na$^+$ compared with 110 or 154 mM Na$^+$ in frog Ringer or mammalian Locke solutions; see Weigele and Barchi [1978b]). As the ionic composition of the bathing solution approaches that of frog Ringer or mammalian Locke solutions, the toxin affinities approach those measured in frog or mammalian membranes. We believe that in most aspects the STX/TTX receptors in lobster walking legs nerves are identical to those in other tissues studied.

Elevated divalent cations reduce the apparent toxin affinity both through direct competition and by reducing the negative surface potential at the toxin binding site (Henderson, et al., 1974; Hille et al. 1975a). The magnitude of change in this surface potential was estimated at <7 mV per 20-fold increase in Ca$^{2+}$ concentration in rabbit vagus nerves (Henderson et al., 1974) and at ~7 mV per 10-fold increase in Ca$^{2+}$ for frog node of Ranvier (Hille et al., 1975a). Both these values are much smaller than the voltage shifts of sodium gating parameters in frog node, 15-22 mV per 10-fold increase in Ca$^{2+}$ (Hille et al., 1975b), so that gating parameters are affected differently than the STX/TTX receptors by changes in external cations.

In the experiments with lobster nerves reported here we made no attempt to measure the surface potential at one receptor directly but only calculated the differences between front and rear leg nerves. Because we measured a surface potential difference of only 8-9 mV when we compared the relative STX and TTX affinities, but of 28 mV when we compared the pKa values, we conclude that the differences between the receptors of front and rear leg nerves must be more complex than could be explained by diffuse surface potentials alone.
Still, a simple electrostatic difference between the microenvironment of the two receptors could account for the observed differences in $pK_a$s and toxin affinities. Consider, for example, a model wherein both front and rear leg receptors are identical, each containing a "primary" acid group that forms a primary ionic bond with both toxins (Sigworth and Spalding, 1980). The rear leg has in addition a "secondary" negative group located within several angstroms of the structural receptor, the membrane sites that make van der Waals contact with the toxins. As a consequence of the negative potential resulting from this second acid group, the apparent toxin affinities are increased, that of divalent STX more than that of monovalent TTX. Lowering the solution pH has several effects, the primary acid group becomes partially protonated, and the secondary acid group is also protonated, thereby reducing both the fraction of available structural receptors and the local toxin concentration. Thus, the pH dependence of rear leg receptors will show an apparently higher $pK_a$ and a larger Hill coefficient. All the observed differences between toxin binding to front and rear walking leg nerves are qualitatively accounted for by this model.

**Relationship between Toxin Receptor and Channel Conductance**

Is there a connection between the toxin affinities and other properties of the sodium channel? In lobster nerves, we have observed two different $pK_a$ values, 4.6 for front legs and 5.1 for rear legs. These proton dissociation constants for the STX/TTX receptor are affected by the high divalent and monovalent cation concentration of NaSW, just as the toxin $K_T$ values are. Indeed, if we assume that the front leg receptor is identical to that of most other toxin binding sites, and that proton binding is elevated in NaSW to the same degree as toxin binding, then the calculated equivalent $pK_a$ of receptors in this nerve in frog Ringer's solution would be 5.1. This value is close to that reported for toxin receptors in mammalian peripheral nerve (Henderson et al., 1973 and 1975), central nervous system membranes (Weigele and Barchi, 1978 a;) and rat skeletal muscle (Hansen Bay and Strichartz, 1980); all these $pK_a$ values are similar to the $pK_a$ for an acidic group that is essential for ion transport through open sodium channels in frog node of Ranvier (Hille, 1968 b and 1975, Woodhull, 1973). It was the strong similarity between the ion binding properties of these two functions that provided one of the major supports for the hypothesis that the receptor for TTX and STX is identical with the ion selectivity filter of the sodium channel (Hille, 1975).

However, under physiological conditions we observe a toxin receptor with a $pK_a$ of 4.6 in the lobster leg nerve. Is this observation consistent with the hypothesis that the receptor resides at the ion selectivity filter? Yes, if the selectivity filter also has a lower $pK_a$ value in these channels. Although the ion selectivity properties of channels in the walking leg nerves cannot be studied directly, because they cannot be voltage clamped due to their extremely small diameter, other lobster nerves can be studied by this technique. In a voltage-clamp study of the large circumesophageal axon of *H. americanus*, Pooler and Valenzeno (1979) measured a $pK_a$ for the sodium conductance of...
4.8; the same pK_α was reported for Myxicola giant axons (Schauf and Davis, 1976). In both of these nerves, the dependence of the pK_α on the transmembrane potential was much weaker than the one measured in frog node. Woodhull (1973) originally interpreted her result from frog, which showed a relatively strong voltage dependence of the apparent pK_α for sodium conductance, to indicate that the acidic group with pK_α 5.2 was located ~25% of the way down the electrical gradient from the outside to the inside of the channel. In the lobster circumesophageal axon, Pooler and Valenzeno (1979) applied the same analysis and concluded that only 9% of the potential difference through the channel existed at the site of protonation with pK_α 4.8; the observations in Myxicola, while not made systematically, also revealed a comparatively small voltage dependence of the pK_α (see Discussion of Schauf and Davis [1976]). There appear to be two types of channels with regard to proton titration of the sodium conductance. We will call the channel with the more voltage-sensitive pK_α (5.2-5.4) the “inner blocking site” channel and the one with the less voltage-sensitive pK_α (4.8) the “outer blocking site” channel. In frog node of Ranvier the lipophilic drug aconitine converts normally occurring inner-site channels to the outer-site form (Naumov et al., 1979), so it is possible that these two physiological behaviors result from two different configurations of the same receptor protein and, perhaps, that the distribution between the configurations can be altered by endogenous membrane lipids as well as by drugs.

At this time we cannot equate the inner-site channels with those having an STX receptor with pK_α 5.2, nor the outer-site channel with a toxin receptor with pK_α 4.6-4.8, because both toxin binding and voltage-clamp experiments have not been performed on each type of channel. Even if these experiments were done and showed that the pK_α values for the channel conductance and toxin receptor were the same, this would not prove that one common site mediates toxin binding and ion transport. Two sites with equal pK_αs would yield the same result. In fact, Spalding (1980) has demonstrated that the abolition of TTX and STX sensitivity in frog node, after modification by covalent acylating reagents that react selectively with carboxyl groups, is accompanied by only partial reduction of the sodium conductance. The pK_α of the remaining TTX-insensitive conductance is lower than that of unmodified channels, and all the physiological results of acylation are consistent with the presence of two negatively charged groups at the sodium channel, one near the mouth and one deeper in the pore (Sigworth and Spalding, 1980).

The results of toxin-binding experiments and voltage-clamp studies show that several factors can modulate ion binding at the sodium channel. Some of these are exogenous, such as the composition of the bathing medium (Henderson et al., 1974; Hille et al., 1975 a; Weigele and Barchi, 1978 b) or the presence of certain drugs (Naumov et al., 1979), while others may be endogenous, structural components of the channel (this paper). Both diffuse surface potentials and specific local charges can affect toxin binding and sodium currents (Drouin and Neumcke, 1974; Hille et al., 1975 b). Therefore, attempts to correlate properties of toxin binding and sodium channel conductance must be constrained to comparison of the same tissue under the same conditions.
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