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Binding of Radioactively Labeled Saxitoxin to the Squid Giant Axon

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Summary. The binding of saxitoxin, a specific inhibitor of the sodium conductance in excitable membranes, has been measured in giant axons from the squid, *Loligo pealei*. Binding was studied by labeling saxitoxin with tritium, using a solvent-exchange technique, and measuring the toxin uptake by liquid scintillation counting. Total toxin binding is the sum of a saturable, hyperbolic binding component, with a dissociation constant at 2–4 °C of 4.3 ± 1.7 nM (mean SE), and a linear, nonsaturable component. The density of saturable binding sites is $166 \pm 20.4\,\mu m^{-2}$. From this density and published values of the maximum sodium conductance, the conductance per toxin site is estimated to be about 7 pS, assuming sequential activation and inactivation processes (F. Bezanilla & C.M. Armstrong, 1977, *J. Gen. Physiol.* **70**:549). This single site conductance value of 7 pS is in close agreement with estimates of the conductance of one open sodium channel from measurements of gating currents and of noise on squid giant axons, and is consistent with the hypothesis that one saxitoxin molecule binds to one sodium channel.

Saxitoxin (STX) and tetrodotoxin (TTX) specifically inhibit sodium currents in many excitable membranes (for reviews, *see* Kao, 1966; Evans, 1972). One toxin molecule reversibly binds to one membrane receptor (Hille, 1968; Cuervo & Adelman, 1970; Colquhoun & Ritchie, 1972) at a site probably located near the extracellular opening of the sodium channel (Henderson, Ritchie & Strichartz, 1974; Spalding, 1978). The potent and highly selective action of these toxins at nanomolar concentrations has led to their extensive use as probes of the sodium channel, and the density of sodium channels in a variety of tissues has been

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calculated from measurements of the saturable component of their binding to membranes.

Toxin binding has been determined either by bioassay or by the use of TTX or STX tritium-labeled by the Wilzbach, or modified Wilzbach, technique (for review, *see* Ritchie & Rogart, 1977*a*). Both methods, unfortunately, are relatively insensitive and require nerve preparations with a large membrane area per unit weight, nerve containing thousands of small nonmyelinated fibers, or muscles containing hundreds of fibers. We have recently developed a new method for labeling STX utilizing exchange of tritium from tritiated water to the toxin (Ritchie, Rogart & Strichartz, 1976). The final specific activity and the radiochemical purity of the STX is much higher than that of the previous Wilzbach labeled toxins, allowing us to determine the toxin binding of a single nerve process, the giant axon of the squid.

Materials and Methods

The ^{3}H-STX used in these experiments was the same as that used by Ritchie *et al.* (1976), but was re-electrophoresed to increase its purity. The radiochemical purity of the tritiated toxin, established by comparing the fraction of radioactivity taken up by lobster walking leg nerves from a bathing solution with the fraction of biological activity removed was about 0.87 (Levinson, 1975; Ritchie & Rogart, 1977*a*).

Lengths of 4–6 cm of the most medial giant axon, starting from the stellate ganglion, were removed from freshly killed squid (*Loligo pealei*) and freed of fine nerve fibers to restrict toxin uptake to the giant axon itself. The ends, and any damaged regions, were tied tightly with thread. The axonal areas were calculated by treating the axons as right cylinders, and using direct measurement of each axon's length and diameter; the latter which was measured at 3–6 points along each fiber, was 454 ± 11 μm ($n=62$).

Axons were incubated for 1–2 hr at 2–4 °C in artificial seawater (ASW) containing ^{3}H-STX and ^{14}C-mannitol, or ^{3}H-STX, ^{14}C-mannitol, and 10 μM unlabeled TTX. Each axon was removed, gently blotted, freed of thread, and digested for 30 min at 60 °C in 0.5 ml of Protosol (New England Nuclear). Occasionally, axons were allowed to digest overnight at room temperatures. The uptake of ^{3}H and ^{14}C were measured by scintillation counting, using the external standard channels ratio to determine the ^{3}H and ^{14}C efficiencies as well as the ^{14}C spillover into the tritium window. The mannitol-accessible extracellular space, calculated individually for each nerve (1.01 ± 0.07 μl·cm^{-2}, $n=41$), was used to correct for the amount of ^{3}H in the extracellular space. A *Patternsearch* nonlinear least squares procedure (Colquhoun, Henderson & Ritchie, 1972), using all points, both in the presence and absence of a high concentration of unlabeled TTX, gave estimates of both the values of the binding parameters and of their standard errors. Standard errors are quoted throughout, where possible.

Results

Figure 1*A* shows the uptake of ^{3}H-STX from a single day's experiments (11 axons) at a variety of different STX concentrations, in the

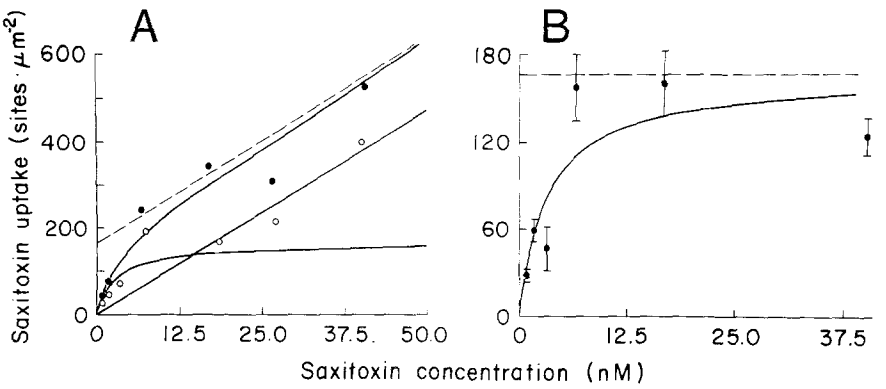

Fig. 1. The uptake of ^{3}H-STX by squid giant axon at different external concentrations of toxin. (*A*): Each point is the mean of 2–4 separate determinations in the absence (●) and presence (○) of 10 μM TTX. The interrupted line is the asymptote of the total binding curve. The linear and saturable components, calculated from the Patternsearch determinations, are drawn separately. (*B*): The linear component was determined in the presence of 10 μM unlabeled TTX (as in *A*) and subtracted from the points for binding in the absence of unlabeled TTX ($n=23$). The vertical bars represent ±1 SE. The interrupted line is the asympote (virtually horizontal) of the binding curve calculated by the *Patternsearch* method. The saturable uptake curve is defined by the parameters $K_t=4.3$ nM and $M=166$ sites μm^{-2}

absence and in the presence of an amount of TTX (10 μM) theoretically large enough to inhibit 99% of the saturable uptake. (Cuervo & Adelman, 1970). As in other preparations studied (*see* Ritchie & Rogart, 1977*a*), the total uptake curve can be fitted by the equation:

$$U_t = b\,[\mathrm{STX}] + M/(1 + K_t/[\mathrm{STX}])$$

where U_t is the total uptake, b is a constant, [STX] the external concentration of STX, M the maximum saturable binding capacity, and K_t the equilibrium dissociation constant. The linear component is relatively large, possibly because any tritium that has back-exchanged out of the labeled toxin into water has penetrated into compartments such as the intracellular space. There was some small variation in the size of the linear component from one day to the next, perhaps due to differences in the uptake of $[^3\mathrm{H}]\cdot\mathrm{H_2O}$. Therefore, to pool the results from different experiments done on different days, the linear component for each particular experiment (○) was substracted from the total uptake points (●) for each experiment to yield the saturable component alone. Figure 1*B* indicates the pooled, averaged results for the saturable component of ^{3}H-STX uptake.

Neither the data in Fig. 1 *A* nor those in *B* define a hyperbolic binding curve nearly as well as do data from studies of binding of this ^{3}H-STX to other nerves or to muscle (Ritchie *et al.*, 1976; Ritchie & Rogart, 1977*a*). But this is not surprising since the uptake is very much smaller than in preparations with more membrane surface area. Furthermore, there may be variations because we are dealing with individual axons in each experiment rather than large populations of fibers. Finally, the estimation of axon area is less accurate than is the corresponding measurement of wet weight for the other preparations studied. Nevertheless, from inspection of Fig. 1 *B* it is apparent that a saturable component of 120–180 sites μm^{-2} does exist in the squid giant axon. Furthermore, if one assumes that the binding is hyperbolic, as in all other tissues studied, there are sufficient points to allow statistical analysis and estimation of the parameters and their standard errors. The results indicate values for M of 166.2 ± 20.4 sites μm^{-2} and for K_t of 4.3 ± 1.7 mM. A measurement of the ability of exchange-labeled ^{3}H-STX to reversibly

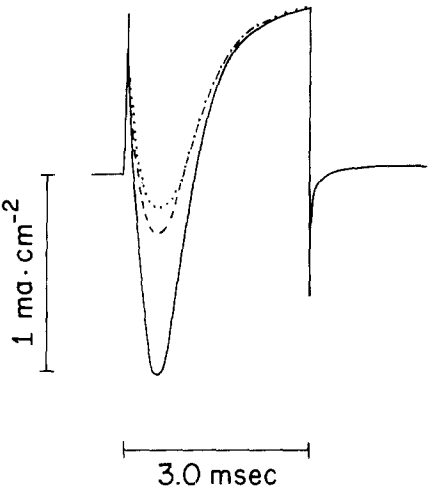

Fig. 2. Reduction of sodium currents by STX in voltage-clamped squid axon (Resting potential; 68 mV). The membrane was first hyperpolarized with a 50 mV prepulse for 13 msec, before the 3 msec long depolarization of 50 mV to activate the currents. Curves are pen recorder traces of the averages of 50 current measurements. Solid line, control seawater (1.05 mA·cm^{-2}); broken line, after 7 min in 18.4 nM ^{3}H-STX (0.35 mA·cm^{-2}); dotted line, after 11 min in 18.4 nM ^{3}H-STX (0.23 mA·cm^{-2}; steady-state block). The peak current returned to 89% of control 7 min after the axon was returned to seawater (not shown). $T = 13$ °C. A dissociation constant for STX was calculated from the modified binding equation: $K_t = [STX]\alpha/(1-\alpha)$ where $[STX] = 18.4$ nM and α is the ratio of the peak inward current in the presence of STX to the average peak inward current in seawater before and after STX application

block sodium currents in a voltage-clamped axon from *L. pealei* gave a dissociation constant of 4.8 nM at 13 °C (Fig. 2), in close agreement with the K_t value from the binding study. Earlier binding experiments, using ^{3}H-TTX on *Loligo forbesi* (Levinson & Meves, 1975) indicated a value for M of 553 sites μm^{-2}. The difference in density between our measurements on *L. pealei* and those of Levinson and Meves (1975) on *L. forbesi* may reflect a true species difference. This is not unreasonable, for Ritchie and Rogart (1977*b*) have demonstrated that, even among different varieties of the same species of frog, significant differences in toxin binding densities occur.

Discussion

The value of the binding site density is important because it allows calculation of the conductance of a single channel from known values of the membrane conductance per unit area, based on the assumption that one toxin molecule binds to one sodium channel. This single channel conductance can then be compared with conductances estimated from electrophysiological measurements of gating currents and noise. The peak value for specific membrane sodium conductance observed during voltage-clamp in *Loligo pealei* at 13 °C is 111 ± 5 mS·cm^{-2} in ASW (calculated from 11 axons not internally perfused; in Table 1 of Yeh & Narahashi, 1974). The quantitative relationship between the measured peak sodium conductance and the total maximum sodium conductance, $\bar{g}_{Na}$, depends on the model for sodium channel kinetics that is assumed. In the classical Hodgkin and Huxley (1952) model, channel opening (activation) and channel inactivation are independent processes that occur simultaneously upon membrane depolarization. Because significant inactivation occurs before complete activation, the measured peak conductance will always be less than the maximum conductance. Allowance for these factors would yield a true value for $\bar{g}_{Na}$ of $111 \cdot D$ mS·cm^{-2}. The value of D is not known for *Loligo pealei* at 13 °C; however, using values from *L. forbesi* for the time constants of inactivation and activation, τ_h and τ_m of 0.67 and 0.16 msec., respectively (curve *B*, Table 2 of Hodgkin & Huxley, 1952) we calculated D to be $2.5/h_\infty$, where h_∞ is the steady-state value of inactivation at the holding potential (-70 mV) used by Yeh and Narahashi (1974). Taking h_∞ to be unity, (Taylor, 1959) and assuming the above time constants also apply to *Loligo pealei*, we thus obtained a value for D of 2–3. From this we calculated that

with a binding site density of 170 μm^{-2} the average conductance per single site would be about 14–20 pS. However, if a different kinetic scheme is assumed, in which the activation and inactivation processes are coupled and occur sequentially (Bezanilla & Armstrong, 1977), then the measured peak sodium conductance will closely approximate $\bar{g}_{Na}$. The calculated conductance per toxin binding site would then be about 7 pS. The coupled kinetic scheme describes the actual sodium conductance in *L. pealei* more faithfully than the Hodgkin-Huxley kinetics, so we believe that the single site conductance value of 7 pS is closer to the sodium channel conductance than the higher estimates of 14–20 pS.

Recent estimates of single sodium channel conductance from gating current measurements in squid axons range from 5–8 pS in *L. pealei* (Armstrong, 1975) to about 2.5 pS in *L. forbesi* (Keynes & Rojas, 1975); from noise measurements in *L. vulgaris* it is about 4 pS (Conti, de Felice & Wanke, 1975). Our estimate of about 7 pS for the single open channel conductance, based on a 1:1 stoichiometry of toxin receptors to sodium channels and a coupled kinetic scheme, is consistent with the estimated value from gating currents for the same species, *L. pealei.*

Comparison of these density calculations must be qualified by several factors: First, the electrophysiological measurements are made on lengths of axon only a few millimeters long, with the largest diameter from near the stellate ganglion, while binding studies involve the entire length of the axon. The toxin binding capacity averaged over the entire axon could differ from the density determined electrophysiologically on the largest portion. Secondly, the toxin binding is not affected by the physiological state of the axon. Thirdly, the axons are almost entirely free of small fibers. Finally, STX binds only to axonal membrane and not to the Schwann cells. The second of these assumptions is supported by the evidence that neither depolarization, homogenization, nor even membrane solubilization of small nonmyelinated fibers has a significant effect on TTX or STX binding capacity (Almers & Levinson, 1975; Henderson & Wang, 1972). Furthermore, axons examined carefully under 50× magnification had only three or four small fibers associated with them, usually at regions of small axon branchings. These latter fibers have a relatively small area of membrane and are thus unlikely to contribute significantly to the binding. We cannot rigorously exclude the possibility that STX binds to Schwann cell membranes of the squid. Villegas, Sevcik, Barnola and Villegas (1976) have reported a veratridine-induced depolarization of the Schwann cell membrane in the squid *Sepiotheuthis sepioidea* which is antagonized by TTX and is sodium dependent. How-

ever, in mudpuppy optic nerve tissue containing only glial cells, neither saturable toxin binding nor drug-activated TTX- or STX-sensitive sodium permeabilities could be detected (Strichartz, Tang & Orkand, 1979).

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