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Multiple Saxitoxin-Binding Sites in Bullfrog Muscle: Tetrodotoxin-Sensitive Sodium Channels and Tetrodotoxin-Insensitive Sites of Unknown Function

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SUMMARY

The possible presence of multiple sodium channel subtypes in bullfrog skeletal muscle was investigated in binding experiments with [^{3}H]saxitoxin and in single-channel studies using planar lipid bilayers. Two classes of [^{3}H]saxitoxin-binding sites were identified in membrane preparations. One class displayed a toxin specificity characteristic of voltage-dependent sodium channels: high affinity for saxitoxin ($K_D \simeq 0.5$ nM), neosaxitoxin ($K_D \simeq 0.1$ nM), and tetrodotoxin ($K_D \simeq 1.3$ nM). A second class of membrane-associated binding sites exhibited high affinity for saxitoxin ($K_D \simeq 0.1$ nM), lower affinity for neosaxitoxin ($K_D \simeq 25$ nM), and complete insensitivity to tetrodotoxin at concentrations up to 32 μM. The first class corresponded to functional tetrodotoxin-sensitive sodium channels that could be incorporated and observed in planar bilayers in the presence of batrachotoxin. Similar attempts to incorporate tetrodotoxin-insensitive sodium channels from bullfrog muscle and heart membranes were unsuccessful. The unusual, tetrodotoxin-insensitive binding activity for [^{3}H]saxitoxin was also found at nM levels in the high speed supernatant of homogenized skeletal muscle without the addition of detergents. This soluble class of sites exhibited low affinity for neosaxitoxin ($K_D \simeq 60$ nM) and a very slow dissociation rate of [^{3}H]saxitoxin ($t_{0.5} \simeq 90$ min), properties nearly identical to those of the tetrodotoxin-insensitive sites in membranes. The soluble saxitoxin-binding activity is also characterized by a more basic pH dependence and a complete lack of binding competition between saxitoxin and alkali cations. Bullfrog muscle appears to be a good tissue source for the purification of this soluble saxitoxin-binding protein.

Voltage-dependent sodium channels have long been recognized as the only specific target of TTX and STX, two potent and widely distributed neurotoxins that bind at a common receptor site (for reviews see Refs. 1–3). These toxins have been invaluable as specific ligands for the assay, purification, and subtype characterization of sodium channel proteins. Our laboratory has recently investigated functional differences between sodium channel subtypes in mammals by comparing the properties of single sodium channels from rat brain, rat skeletal muscle, and canine heart incorporated into planar lipid bilayers in the presence of batrachotoxin (4). Comparison of the kinetics of block and binding of TTX, STX, and μ-conotoxin allowed us to distinguish three sodium channel subtypes in mammals (4–6). The m-type found in skeletal muscle exhibits high affinity for all three of these toxins, whereas the n-type of neuronal tissue is insensitive to μ-conotoxin but has high affinity for TTX/STX, and the h-type found in heart and denervated skeletal muscle is particularly insensitive to both TTX and μ-conotoxin. This subtype classificiation in mammals is also supported by a diverse body of evidence from many laboratories that includes radioligand binding studies, sodium flux, and voltage clamp experiments (7–9). However, comparative studies suggest that such a subtype classification scheme for mammals is not directly applicable to other vertebrates. For example, sodium channels of avian nerve and heart exhibit much smaller differences in their affinity for STX and TTX than the corresponding mammalian tissues (9).

In amphibians such as frogs, the current literature on STX and TTX pharmacology presents several confusing issues. Electrophysiological studies of frog heart have shown that cardiac sodium currents are completely blocked by TTX concentrations less than 300 nM, with a reported $K_{0.5}$ of 5 nM (10, 11). However, [^{3}H]STX binding experiments with frog heart ventricular tissue (12) have revealed two types of high affinity binding sites, one that has high affinity for TTX and one that is virtually insensitive to TTX (tested up to 1 μM). Thus, in frog heart, the high

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ABBREVIATIONS: TTX, tetrodotoxin; STX, saxitoxin; NEO, neosaxitoxin; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

affinity site for TTX appears to correspond to functional sodium currents, whereas a TTX-insensitive sodium current has not been reported. Another laboratory that also investigated [^{3}H]STX binding in frog heart found high affinity sites in both plasma membranes and the supernatant fraction of soluble protein (11, 13). In these studies, [^{3}H]STX binding in the intact ventricle exhibited competition by TTX, whereas that in the supernatant was insensitive to high concentrations of TTX.

[^{3}H]STX binding studies with membranes from bullfrog brain revealed only binding sites with high affinity for STX and TTX (12). However, recent voltage clamp experiments with bullfrog sympathetic neurons have distinguished two types of sodium currents (14). One type of rapidly inactivating current was blocked by both STX and TTX at concentrations that produce 50% inhibition at 10 nM or less for both toxins. A second type of sodium current that inactivated about 3-fold more slowly was blocked by 0.1–1 μM STX but not by 1–10 μM TTX. Thus, in certain frog neurons, there seems to be a functional sodium current that is insensitive to TTX but not to STX, a pharmacology that corresponds to the unusual TTX-insensitive binding site observed in frog heart (11, 12).

Frog skeletal muscle also exhibits a different behavior than mammalian skeletal muscle with respect to the toxin sensitivity of sodium currents after denervation. In mammals, sodium currents of normal skeletal muscle are very sensitive to TTX, with 50% inhibition occurring at about 5 nM (15). Within 2–5 days after denervation, mammalian skeletal muscle fibers show a TTX-insensitive component of sodium current (15, 16) that has been shown to correspond to the appearance of a TTX-insensitive subtype at the single-channel level (4, 17). In contrast, denervation of frog skeletal muscle fibers does not result in any change in the TTX sensitivity of action potentials (18, 19).

Previous binding studies on amphibian muscle have revealed high affinity binding of both [^{3}H]TTX and [^{3}H]STX that appears to follow single-site binding isotherms for both toxins (20–22). However, electrophysiological experiments and binding studies with CssII toxin from *Centruroides suffusus suffusus* (23) and γ-toxin from *Tityus serrulatus* (24) have suggested that these scorpion toxins have high affinity for sodium channels on the surface membrane but do not bind to sodium channels in the transverse tubular membrane system of frog skeletal muscle. Other binding studies with two different ethylenediamine derivatives of TTX (25) suggested that these two derivatives bind with different affinities to transverse tubules and surface sarcolemma of frog skeletal muscle. Evidence for different sodium channel subtypes within different regions of muscle fibers has also recently been obtained in mammals using monoclonal antibodies to purified rat muscle sodium channels (26).

The possibility that normal skeletal muscle contains different subtypes of sodium channels in sarcolemma and transverse tubules was of interest to our laboratory, since planar bilayer methods are a possible approach to functional distinction of these subtypes at the single-channel level. For this reason, we began to study [^{3}H]STX binding in homogenized bullfrog skeletal muscle with the aim of using a pharmacological approach to the identification of sodium channels from sarcolemma and transverse tubule membranes. The present report shows that we did indeed find two types of high affinity STX-binding sites; however, we could not directly correlate these types with different membrane locations. One of the types of [^{3}H]STX-binding sites had high affinity for TTX and corresponded pharmacologically to functional sodium channels that could be incorporated into planar bilayers from bullfrog muscle and heart. The second type of high affinity site for [^{3}H]STX was completely insensitive to TTX and was also present in the high speed supernatant of soluble protein from skeletal muscle. These results lead to questions regarding the physiological significance of this soluble saxitoxin binding activity and its relationship to functional sodium channels.

Experimental Procedures

Materials. [^{3}H]STX prepared by exchange with ^3H_2O was obtained from Amersham-Searle (Arlington Heights, IL). Two different lots of this product were found to have radiochemical purities of 55 and 73%. About 10% of the radioactivity in these samples did not bind to cation exchange resin, indicating substantial amounts of ^3H_2O or uncharged radiolysis products. These samples were repurified by a method suggested by Dr. S. Hall (U. S. Food and Drug Administration). A sample of the labeled STX was applied to a 20-ml column of Bio-Gel P6 (Bio-Rad Laboratories, Richmond, CA) that was previously equilibrated with 10 mM acetic acid, pH 5.0 at 4°. The column was eluted with 20 ml of equilibration buffer followed by 50 ml of 100 mM acetic acid, pH 3.0. This method routinely resolved two peaks of radioactivity. The first peak eluting at pH 5.0 did not bind specifically to brain membranes and was discarded. The second peak eluting at pH 3.0 was pooled and adjusted to pH 7.2 with Tris. Aliquots of this preparation were stored at −80°. Radiochemical purity of the toxin was determined by binding titration of fixed amounts of toxin with increasing concentrations of rat brain membranes. The concentration of [^{3}H]STX in the sample was determined by isotope dilution with standard STX solutions using specific binding to rat brain membranes as a biological assay. Such assays of two preparations of purified [^{3}H]STX gave radiochemical purities of 78–86% and specific activities of 24–26 Ci/mmol. Also, greater than 99% of the radioactivity in samples purified in this way was retained on cation exchange resin. Such preparations of [^{3}H]STX stored at dilutions of 10 μCi/ml or less were stable for at least 12 months.

Unlabeled STX and TTX were purchased from Calbiochem (La Jolla, CA). NEO was generously supplied by Dr. S. Hall. Batrachotoxin was obtained from Dr. J. W. Daly (Laboratory of Bioorganic Chemistry, National Institutes of Health). All other chemicals were of the purest grade available from commercial sources.

Bullfrog muscle preparations. Four types of skeletal muscle preparations are referred to in this study: "KCl membranes," "LiBr membranes," S1 supernatant, and S2 supernatant.

For membrane preparations, the total hind leg muscle was dissected from six to eight large specimens of *Rana catesbiana* after pithing. The muscle was divided into batches of 100 g and placed on ice; subsequent procedures were performed at 0–4°. Tissue batches of 100 g were minced in a food processor, combined with 300 ml of sucrose buffer (10 mM MOPS-NaOH, pH 7.4, 0.3 M sucrose, 3 mM NaN_3, 0.2 mM EDTA), and homogenized twice for 30 sec at high speed in a blender. Immediately before blending, 0.2 mM phenylmethylsulfonyl fluoride was added from a 200 mM stock solution in dry acetone. The homogenate was centrifuged at 2500 × g for 5 min and the supernatant was passed through cheesecloth. The pellets were combined with an original volume of buffer and homogenized and centrifuged as before. The pellets from this step were saved on ice for preparation of LiBr membranes, and the combined supernatant was used to prepare KCl membranes.

Solid KCl was added to the above supernatant to a final concentration of 0.6 M and stirred for 30 min. This mixture was centrifuged at 110,000 × g for 40 min and the clear supernatant was discarded. The pellets were resuspended in about 80 ml of sucrose buffer and centrifuged twice at 5,000 × g for 10 min to remove a pellet containing

mitochondria. The supernatant was centrifuged for 40 min at 110,000 × *g* and the pellets were resuspended in a final volume of 20–30 ml of sucrose buffer. This membrane suspension (10–15 mg/ml) was stored as 1-ml aliquots at −80° and is referred to in the text as KCl membranes.

Pellets previously saved from the low speed centrifugation step were rehomogenized in an original volume of sucrose buffer containing 0.5 M LiBr and stirred overnight at 0–4°. On the following day, the viscous mixture was rehomogenized with additional LiBr to a final concentration of 1.0 M. After this step, the viscosity of the mixture was greatly reduced and the suspension was centrifuged at 2,500 × *g* for 10 min. The supernatant was passed through cheesecloth and centrifuged at 110,000 × *g* for 40 min. The pellets were resuspended in 400 ml of sucrose buffer containing 0.6 M KCl and stirred for 30 min. This suspension was centrifuged at 110,000 × *g* for 40 min and the pellets were resuspended in 20–30 ml of sucrose buffer. The mixture was centrifuged at 5,000 × *g* for 10 min to remove a pellet containing mitochondria. The remaining supernatant (10–15 mg of protein/ml) was stored at −80° in 1-ml aliquots and is referred to in the text as LiBr membranes.

For the S1 and S2 supernatant preparations, skeletal muscle was dissected from one bullfrog and minced in a food processor. Sucrose buffer was added to the minced tissue (1 ml/g of muscle) and the mixture was processed with a Tissumizer (Tekmar, Cincinnati, OH) three times for 15 sec each at 8,000 rpm followed by three times for 15 sec at 20,500 rpm with 15-sec rest periods between bursts. The paste was centrifuged at 2,500 × *g* for 10 min, and the supernatant is referred to as S1 supernatant. S2 supernatant was prepared by centrifuging S1 supernatant for 1 hr at 110,000 × *g*.

A preparation of frog heart ventricular membranes was prepared from eight bullfrog hearts using a scaled-down version of a method previously described for canine heart (4).

Protein concentrations were determined according to the method in Ref. 27 after precipitation in the presence of deoxycholate and trichloroacetic acid as described (28). Bovine serum albumin was used as the protein standard.

Equilibrium binding assay. The standard assay for [^{3}H]STX binding contained 5 nM [^{3}H]STX, 10 mM MOPS-NaOH, pH 7.4, 200 mM choline chloride, 0.1 mM EDTA, and 1.5–3 mg of protein/ml of bullfrog membranes or supernatant in a final volume of 0.25 ml. In displacement experiments with unlabeled toxins or for estimates of total binding activity, the concentrations of binding sites was adjusted so that about 20% or 1 nM of the total [^{3}H]STX was bound initially in the absence of competitor. Binding was allowed to equilibrate at 0° for 30–60 min before separating the bound ligand at 4° on 1-ml columns of AG 50W-X2 cation exchange resin (100–200 mesh, Bio-Rad) as described previously (6). Tritium was counted at 40% efficiency using 6 ml of Ecoscint (National Diagnostics, Highland Park, NJ) as a scintillation fluid. Specific [^{3}H]STX binding was determined by subtracting nonspecific binding measured in the presence of 10 or 20 μM STX. In titrations involving labeled or unlabeled toxins, data points are the means of duplicate determinations that did not differ by more than 10%. Binding parameters were obtained by fitting the data to one- or two-site models using the non-linear least squares programs, EBDA, LIGAND, and KINETIC, written by G. A. McPherson (Elsevier-Biosoft, Cambridge, UK).

Planar bilayers and single channel recording. Single sodium channels from bullfrog skeletal muscle and heart membranes were incorporated into planar lipid bilayers in the presence of batrachotoxin and were recorded and analyzed as described previously for various rat muscle and canine heart preparations (4).

Results

Identification of TTX-sensitive and TTX-insensitive binding sites for [^{3}H]STX in skeletal muscle membranes. In most excitable tissues previously studied, binding of STX and TTX in strictly competitive, and the level of nonspecific binding measured with one of these radiolabeled toxins can be evaluated by an excess of either of the unlabeled toxins. We first observed that [^{3}H]STX binding is more complex in bullfrog skeletal muscle when we found that the level of nonspecific binding was always higher when 20 μM TTX was used as a control for nonspecific binding instead of 20 μM STX. This behavior is illustrated in Fig. 1 for two different preparations of KCl membranes. In this experiment [^{3}H]STX binding is plotted as a function of increasing concentrations of free STX or TTX in the assay, with zero binding defined as that measured in the presence of 20 μM STX. These results show that concentrations of TTX up to 32 μM fail to inhibit a certain fraction of the total [^{3}H]STX sites as indicated by saturation of the TTX titration at levels well above the STX blank. We also found that the TTX-insensitive fraction of specific [^{3}H]STX binding varied somewhat in different preparations (Fig. 1, A and B). For three different preparations of KCl membranes, the fraction of TTX-insensitive sites ranged from 32 to 63% of the total sites.

Methods similar to those used to prepare KCl membranes have been used previously to prepare a starting fraction for a preparation rich in transverse tubular membranes (29). A different method, involving prolonged extraction of a low speed

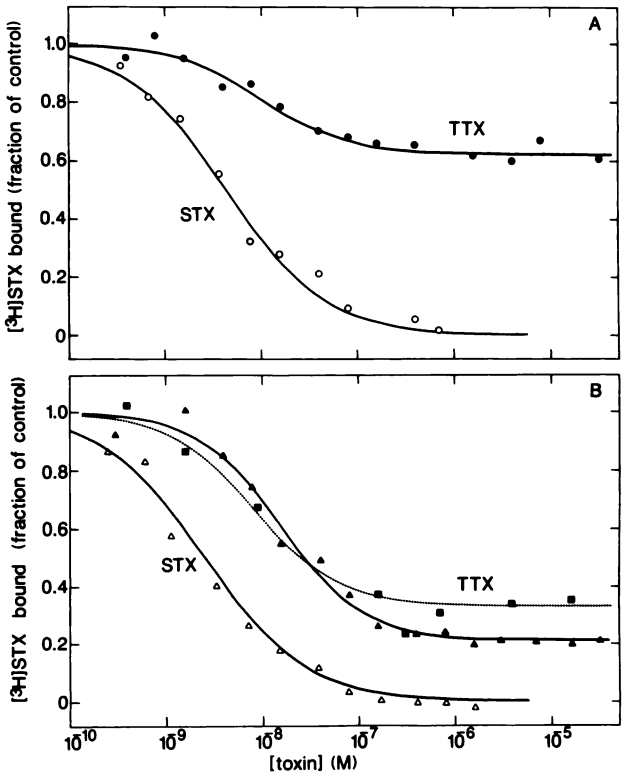

Fig. 1. Effect of STX and TTX on [^{3}H]STX binding in two preparations of skeletal muscle membranes. Specific binding of [^{3}H]STX to KCl membranes prepared from bullfrog muscle was assayed under standard conditions as described under Experimental Procedures. The data are plotted as the fraction of specifically bound [^{3}H]STX at various concentrations of free STX (○, △) or TTX (●, ▲, ■) relative to that measured in the absence of unlabeled toxin. Specific binding is the difference between total binding and nonspecific binding measured in the presence of 20 μM STX. A. Preparation of 7/8/86: ○, ●, no saponin. B. Preparation of 1/21/87: △, ▲, 0.4% saponin; ■, no saponin. Initial levels of specific binding in the absence of unlabeled toxins ranged from 0.42 to 1.1 nM bound or 0.16–0.42 pmol/mg of protein. Non-linear least squares fits of the data to binding models are described in the text.

pellet with LiBr, has been cited to yield fractions enriched in surface sarcolemmal membranes (29, 30). To investigate whether TTX-insensitive sites for [^{3}H]STX are also present in this fraction, we performed experiments similar to those in Fig. 1 using LiBr membranes instead of KCl membranes. Results quite similar to those in Fig. 1 were obtained with LiBr membranes (not shown); however, the fraction of TTX-insensitive sites was slightly lower (range of 17–41% in two preparations) than that observed in KCl membranes.

It has been shown previously that [^{3}H]STX and [^{3}H]TTX binding in isolated skeletal muscle membranes is increased in the presence of low concentrations of mild detergents (31, 32), presumably by increasing the permeability of sealed, inside-out membrane vesicles originating from transverse tubules to these positively charged toxins. To investigate whether the fraction of TTX-insensitive sites was affected by the presence of detergent, we also performed similar STX and TTX displacement experiments in the presence of saponin. We found that 0.5% saponin increased specific [^{3}H]STX binding by about 2-fold for both KCl and LiBr membranes at membrane protein concentrations of 2 mg/ml. Under these conditions, the TTX-insensitive fraction of [^{3}H]STX binding was still present but was reduced by up to one-half (35–51% reduction, range of four preparations) of that measured in the absence of saponin (Fig. 1B). This observation suggests that more of the TTX-sensitive sites than TTX-insensitive sites are inside sealed vesicles, implying that the TTX-insensitive sites may be preferentially located in unsealed or right side-out vesicles rather than in the sealed transverse tubule fraction. The fact that TTX-insensitive sites are still observed in the presence of detergent argues that this differential toxin sensitivity is not due to differential accessibility of the toxins to binding sites in vesicles.

Since the observation of "TTX-sensitive" and "TTX-insensitive" sites suggested that the behavior in Fig. 1 could be due to two distinct populations of sites, we performed binding analyses and further experiments to test this possibility. STX displacement titrations such as those in Fig. 1 were fit to the following empirical logistic function:

$$f = K_{0.5}^n/(K_{0.5}^n + [\text{toxin}]^n) \quad (1)$$

where f is the fraction of specifically bound [^{3}H]STX measured in the absence of unlabeled toxin, $K_{0.5}$ is the concentration of free unlabeled toxin at which $f = 0.5$, and n is an empirical Hill coefficient. In several different STX titrations, including experiments with both KCl and LiBr membranes in the absence and presence of saponin, we found that the Hill coefficient was always less than 1.0 with a mean value of 0.79 ± 0.04 (SD) for four experiments. This result indicates that the STX titration occurs over a wider concentration range than is predicted by a one-site system; however, these shallow, but smooth-shaped, STX titration curves did not allow accurate resolution of binding parameters for a two-site model. In fact, good fits were obtained using a one-site model with a K_D of 0.5 nM for [^{3}H]STX and K_D values in the range of 0.3–0.5 nM for STX. Thus, if two distinct sites are present in the membrane preparation, they apparently have very similar K_D values for STX.

TTX displacement titrations such as those in Fig. 1 were analyzed by a function similar to Eq. 1, except that the level of zero binding was defined by the saturation level observed at high TTX concentrations. The mean Hill coefficient of six experiments with different preparations was 0.92 ± 0.04 (SD). The closeness of this value to 1.0 indicates that the TTX displacement curves analyzed in this fashion are better described by a one-site model than are the STX titrations, suggesting that there is a single class of TTX-sensitive sites. To estimate the K_D of TTX for this class of sites, the TTX titrations were fit to a one-site model with the K_D for [^{3}H]STX fixed at 0.5 nM.[1] From these fits a mean K_D for TTX of 1.3 ± 0.3 (SD) nM was obtained from six experiments. This analysis of the TTX displacement data assumes that one class of binding sites for [^{3}H]STX exhibits simple competition between STX and TTX, whereas the other class of [^{3}H]STX sites does not recognize or interact with TTX. Further support for this interpretation is shown in the experiments of Figs. 2 and 3.

Fig. 2 shows a displacement titration of [^{3}H]STX binding that is similar to that of the same KCl preparation of Fig. 1B, except that NEO was used as the displacing toxin. NEO is a naturally occurring derivative of STX that differs only by the replacement of a hydroxyl group for a hydrogen atom at the N-1 nitrogen of STX (33). This experiment exhibited a biphasic displacement curve that could be fit by a binding model for two populations of receptors, with each receptor exhibiting a competitive interaction between [^{3}H]STX and NEO. By using fixed K_D values of 0.5 nM[2] for [^{3}H]STX at site 1 and 0.1 nM[3] for [^{3}H]STX at site 2, the use of the LIGAND fitting program resulted in best fit K_D values for NEO of 0.095 ± 0.022 nM at site 1 and 25 ± 11 nM at site 2, with 19 ± 2% of the total sites belonging to the site 2 population with low affinity for NEO. This percentage of sites with low affinity for NEO corresponds to the same percentage of TTX-insensitive sites in Fig. 1B and implies that the TTX-insensitive sites have about 250-fold lower affinity for NEO than the TTX-sensitive sites.

Fig. 3 shows the results of an investigation of the rate of [^{3}H]STX dissociation from frog muscle membranes as measured by the addition of an excess of unlabeled STX or TTX. In the presence of excess STX, a biphasic dissociation reaction was observed that could be fit by a sum of two exponential components. A nonlinear least squares fit resulted in [^{3}H]STX dissociation rate constants of 2.3 ± 0.5 × 10^{-3} sec^{-1} ($t_{0.5}$ ≃ 5 min)

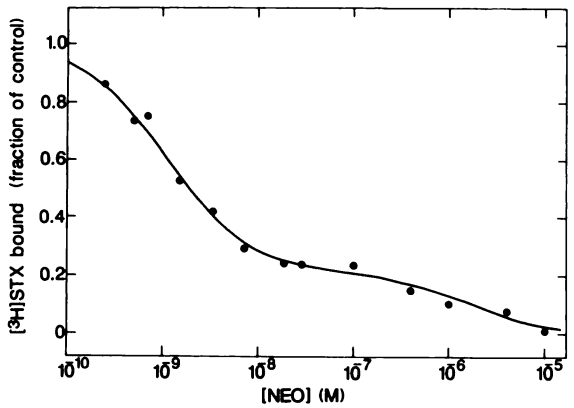

Fig. 2. Effect of NEO on specific binding of [^{3}H]STX to skeletal muscle membranes. An experiment similar to that of Fig. 1 was performed using NEO as the competing toxin. Membranes were from the same KCl preparation used in Fig. 1, B and 0.4% saponin was included in the assay. The initial level of specific binding in the absence of unlabeled toxin was 0.42 pmol/mg or 1.1 nM bound.

[1] The choice of these values is later justified in the text.
[2] The choice of these values is later justified in the text.
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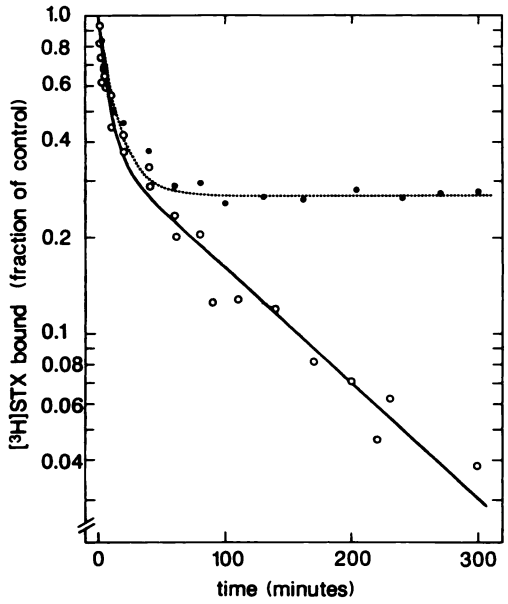

Fig. 3. Dissociation kinetics of specifically bound [^{3}H]STX from skeletal muscle membranes. [^{3}H]STX was bound to the same KCl membranes as those of Fig. 1B, in the presence of 0.5% saponin under conditions similar to those of Figs. 1 and 2. At time = 0, 20 μM unlabeled STX (○) or TTX (●) was added and [^{3}H]STX binding was assayed at various times. The data are plotted as the fraction of specifically bound [^{3}H]STX measured before addition of unlabeled toxins. The level of specific binding at time = 0 was 1.2 nM bound or 0.43 pmol/mg of protein.

for 63% of the population and $1.4 \pm 0.1 \times 10^{-4}$ sec^{-1} ($t_{0.5} \simeq 83$ min) for 37% of the population. In the presence of TTX, only the fast dissociation rate was observed, with no dissociation of [^{3}H]STX for up to 5 hr from the proportion of sites corresponding to the slowly dissociating fraction in the presence of STX. These results indicate that the rate of [^{3}H]STX dissociation is about 16-fold slower for the TTX-insensitive population than for the TTX-sensitive population.

Despite the compelling evidence for two types of STX-binding sites presented above, we were unable to resolve two sites by direct titrations of [^{3}H]STX binding to frog membranes by Scatchard plot analysis. Scatchard plots of [^{3}H]STX binding appeared to be linear within the scatter of the data, indicating that the two types of sites have very similar K_D values. By using a one-site analysis, we obtained K_D values of 0.54 ± 0.07 nM and 0.51 ± 0.05 nM for [^{3}H]STX in two different experiments (data not shown). We also attempted to estimate the K_D of [^{3}H]STX to the TTX-insensitive sites by analyzing Scatchard plots taken in the presence of TTX to inhibit binding to the TTX-sensitive fraction. Two-site analysis of these latter experiments resulted in K_D values of 0.19 ± 0.06 nM and 0.14 ± 0.04 nM (two experiments) for [^{3}H]STX binding to the TTX-insensitive sites. Taken together, these results suggest that the K_D of STX for binding to the TTX-sensitive site is about 0.5 nM, and that for binding to the TTX-insensitive site is about 0.1–0.2 nM.

Correspondence of the TTX-sensitive site to functional sodium channels from bullfrog muscle and heart membranes observed in planar lipid bilayers. Previous work in our laboratory has shown that it is possible to incorporate TTX-sensitive sodium channels from membrane preparations of rat brain and skeletal muscle and TTX-insensitive Na channels from canine heart and denervated rat skeletal muscle into planar lipid bilayers for pharmacological investigations at the single-channel level (4). We attempted to apply similar techniques to membrane preparations of bullfrog tissues to seek pharmacological correspondence of the two types of toxin sites with functional sodium channels.

For planar bilayer studies involving native membrane vesicles, it is necessary to use a purified preparation of membranes as a source of channels to incorporate into the bilayer. In the course of developing such preparations from bullfrog muscle, we subjected our crude preparations of KCl and LiBr membranes to equilibrium sucrose density gradient centrifugation and examined the gradient distribution of total protein, total [^{3}H]STX-binding sites, and TTX-insensitive sites. For KCl membranes we observed (Fig. 4A) a major peak of protein and [^{3}H]STX-binding activity at 34% sucrose and also a smaller peak of binding activity in the range of 20–25% sucrose, as reported previously (31). For the LiBr membranes we observed an additional high density peak at 40% sucrose (Fig. 4B). Previous studies of the purification of muscle plasma membranes have supported the suggestion that the low density membranes originate from surface and transverse tubule membranes, whereas the high density membranes consist of sarcoplasmic reticulum and triad junctions (34, 35). For planar bilayer experiments, we isolated the low density fraction (20–

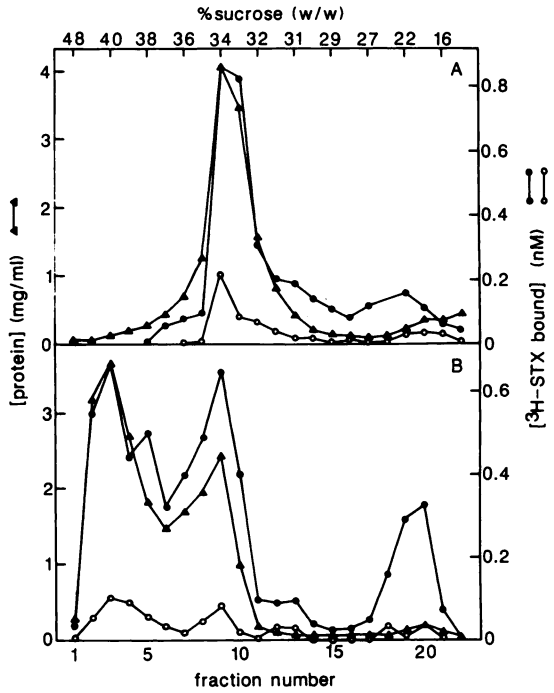

Fig. 4. Sucrose density gradient profiles of protein and [^{3}H]STX binding after equilibrium centrifugation of muscle membranes. Sucrose gradients were prepared from 16 ml of 25% (w/v) sucrose and 16 ml of 50% (w/v) sucrose in a linear gradient-mixing apparatus. Gradient solutions also contained 10 mM MOPS-NaOH, pH 7.4, and 3 mM NaN_3. Gradients were underlayed with 2 ml of 60% (w/v) sucrose and overlayed with 40 mg of protein of KCl membranes (A) or LiBr membranes (B). Samples were centrifuged in a Beckman SW28 rotor at 85,000 × g for 18 hr and fractionated into 22 equal volume fractions using a peristaltic pump. Each fraction was assayed for total protein (▲), total specific [^{3}H]STX binding (●), and TTX-insensitive [^{3}H]STX binding (○). Total specific [^{3}H]STX binding is defined as the difference between total binding and a blank containing 10 μM STX. TTX-insensitive [^{3}H]STX binding is defined as the difference between binding observed in the presence of 10 μM TTX and 10 μM STX. Binding assays included 0.5% saponin.

30% sucrose) of membranes as a source of membrane vesicles for bilayer fusion, since this fraction has been successfully used to incorporate plasma membrane channels from other species such as rabbit (36) and rat (4). However, we consistently observed that greater than 80% of the TTX-insensitive binding sites for [^{3}H]STX were distributed in the high density membrane peaks (Fig. 4), suggesting that these sites may be associated with sarcoplasmic or endoplasmic reticulum rather than sacrolemma or transverse tubule.

Bullfrog sodium channels from low density fractions of KCl and LiBr membranes were easily incorporated into planar lipid bilayers in the presence of 0.2 μM batrachotoxin using methods previously described for rat muscle membranes (4, 37). In the absence of TTX and STX, the batrachotoxin-activated sodium channels from bullfrog muscle exhibited behavior similar to that described for rat muscle sodium channels studied under the same conditions (37). The bullfrog muscle sodium channels exhibited a single channel conductance of 20.2 ± 0.8 (SD, n = 12) pS in the presence of symmetrical 0.2 M NaCl and an opening probability near 1.0 in the voltage range of −60 to +60 mV. The sensitivity of these Na channels to TTX is illustrated by the experiment of Fig. 5A, where 20 nM TTX added to the chamber corresponding to the extracellular side of the channel induced the appearance of long-lived blocked events in the single-channel records. As previously shown for other sodium channels, such blocking events correspond to the binding and unbinding events of individual toxin molecules and can be analyzed by described methods (4, 37) to obtain the blocking rate constants for TTX dissociation and association. The results of such an analysis are summarized in Table 1, which lists the measured mean blocked and mean unblocked dwell times at +50 and −50 mV in the presence of 20 nM TTX. Satisfactory agreement of the dwell time measurements with a single-site binding model is indicated by the close agreement of the sample mean with the standard deviation for each population of events, as expected for an exponential distribution.

As previously observed for other types of batrachotoxin-activated sodium channels in planar bilayers, the blocking kinetics of TTX are voltage dependent, with a longer mean blocked time and a shorter mean unblocked time observed at −50 mV compared to +50 mV. Assuming a single-site binding model, the mean dwell times can be used to estimate dissociation and association rate constants at +50 and −50 mV. A fit of these values to an exponential dependence of the respective rate constants on voltage can be used to estimate rate and equilibrium constants for TTX at 0 mV and effective valences (Table 1) for these reactions (4). The values obtained for these constants for bullfrog skeletal muscle (Table 1) may be compared to those previously determined for sodium channels from rat skeletal muscle (4). TTX binding to the frog muscle sodium channel has about the same voltage dependence as the rat muscle channel; however, the frog channel exhibits about a 4-fold higher affinity K_D (7.2 nM versus 29 nM) than the rat channel measured under the same conditions. This difference in K_D is due to a 4-fold slower dissociation rate constant for the frog muscle channel (0.029 sec^{-1} for frog versus 0.12 sec^{-1} for rat). The TTX association rate constants measured for these two channels are identical (4×10^6 sec^{-1} M^{-1}).

Taking into account the different conditions of the bilayer experiments with the binding experiments of Fig. 1, the single TTX-sensitive sodium channels observed in bilayers are likely to correspond to the sites with high affinity for TTX, observed in the experiment of Fig. 1. Despite our best efforts, we did not observe incorporation of any TTX-insensitive sodium channels corresponding to the TTX-insensitive site previously found in the binding experiments. In experiments involving 19 different bilayers containing one to three sodium channels (a total of 35 channels), all of the channels were TTX sensitive as demonstrated by channel block in the presence of 20 nM TTX.

Our failure to observe TTX-insensitive channels using the bilayer method suggested that this subtype of sodium channel was either nonfunctional or difficult to incorporate from bullfrog muscle. Since we previously found canine heart to be a good source of TTX-insensitive sodium channels, we also attempted to incorporate such channels from frog heart membranes. An example of an experiment with a single batrachotoxin-activated sodium channel from frog heart is shown in Fig. 5B. The frog heart sodium channels we observed were quite similar to the sodium channels observed from frog skeketal muscle except that the heart channels had a 10% larger conductance. The measured single-channel conductance of the frog heart channels was 22.7 ± 0.7 (SD, n = 9) pS, and these channels were also of the TTX-sensitive type as shown by the data in Fig. 5B and Table 1. In experiments involving 22 different bilayers containing one to three channels, we found that all of the 37 heart channels we observed were TTX sensitive as indicated by the potent blocking effect of 20 nM TTX. The kinetic parameters measured for TTX blocking of bullfrog heart channels were practically identical to those measured for the bullfrog skeletal muscle channels (Table 1), suggesting that functional sodium channels from both of these tissues have high affinity for TTX. However, bullfrog heart sodium channels have a 100-fold higher affinity for TTX than canine heart sodium channels previously studied in planar bilayers (Ref. 4 and Table 1).

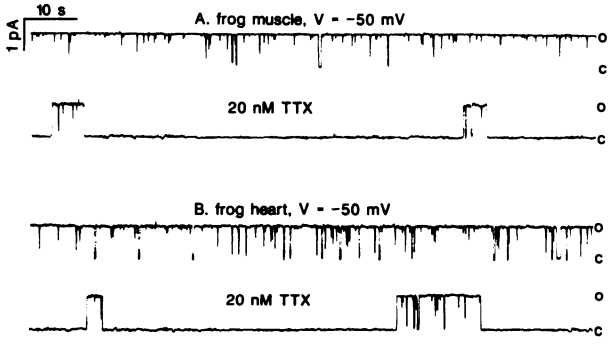

Fig. 5. Current records of single batrachotoxin-activated sodium channels from bullfrog muscle or heart. Batrachotoxin-activated sodium channels were incorporated into planar phospholipid bilayers, and current fluctuations were recorded as described previously (4). Records are filtered at 100 Hz. Closed (*c*) and open (*o*) current levels are indicated at the *right* of each *trace*. A. Record of a single channel incorporated from a low density fraction of a KCl preparation of bullfrog muscle was taken at −50 mV before and after the addition of 20 nM TTX to the extracellular side of the channel. B. Record of a similar experiment with a single channel incorporated from a membrane preparation of bullfrog heart ventricular muscle.

Pharmacological similarity of the TTX-insensitive, membrane-associated site to a water-soluble site in skeletal muscle. Our failure to incorporate functional sodium channels corresponding to the TTX-insensitive sites identified in binding experiments led us to consider the possibility that

TABLE 1

Kinetic parameters for TTX block of single Na channels from skeletal muscle and heart observed in planar bilayers

Single-channel experiments were performed as described in Fig. 5, and the measured mean blocked ($\bar{\tau}_b$) or mean unblocked ($\bar{\tau}_u$) dwell times at +50 and −50 mV were used to calculate dissociation (k_d) and association (k_a) rate constants at these voltages using methods and theory described previously (4, 37). Values are means ± standard deviations.

Tissue	Voltage	$\bar{\tau}_b$ (n)	$\bar{\tau}_u$ (n)	k_d	k_a	K_D[a]
	mV	*sec*	*sec*	*sec*$^{-1}$	10^6 *sec*$^{-1}$ *M*$^{-1}$	*(nM)*
Bullfrog muscle	+50	17 ± 18 (105)	19 ± 19 (104)	0.061 ± 0.006	2.8 ± 0.3	22
	−50	73 ± 58 (55)	8.8 ± 8.7 (57)	0.014 ± 0.002	5.8 ± 0.8	2.4
	0[b]			0.029 (0.38)	4.0 (0.19)	7.2 (0.56)
Rat muscle	0[c]			0.12 (0.40)	4.0 (0.19)	29 (0.60)
Bullfrog heart	+50	17 ± 19 (80)	27 ± 28 (80)	0.062 ± 0.007	2.0 ± 0.2	31
	−50	62 ± 55 (41)	11 ± 10 (44)	0.017 ± 0.003	4.5 ± 0.7	3.8
	0[b]			0.032 (0.34)	3.0 (0.21)	11 (0.55)
Canine heart	0[c]			0.52 (0.32)	0.46 (0.19)	1100 (0.51)

[a] K_D at +50 and −50 mV is the calculated ratio of k_d/k_a.

[b] The dependence of kinetic constants on voltage (V) was estimated by fitting the values measured at +50 and −50 mV to the following exponential functions of voltage: $k_d(V) = k_d(0)\exp(z_d'FV/RT)$, $k_a(V) = k_a(0)\exp(-z_a'FV/RT)$, and $K_D(V) = K_D(0)\exp(z'FV/RT)$ where $k_d(0)$, $k_a(0)$, and $K_D(0)$ refer to zero voltage rate and equilibrium constants, and z_d', z_a', and z' refer to the effective valences of these reactions as described previously (4). F is the Faraday constant, R is the gas constant, and T is the absolute temperature. The listed values are the derived zero voltage constants of the respective reactions with the effective valence given in parentheses.

[c] Similar zero voltage kinetic parameters described in Footnote *b* were taken from Ref. 4.

these binding sites might originate from a cytoplasmic pool. This notion was bolstered by the previous report that frog heart supernatant contained high affinity binding sites for [^{3}H]STX that were insensitive to displacement by TTX (13). To explore this possibility, we prepared supernatant fractions from bullfrog skeletal muscle as described under Experimental Procedures. The S1 supernatant preparation is a low speed supernatant of homogenized muscle which contains fragmented microsomes and is also used to prepare KCl membranes. Our S2 supernatant is a high speed supernatant of the S1 microsome fraction that is practically devoid of membranes as judged by its optical clarity. In preliminary experiments we indeed found that S2 supernatant contains high levels of [^{3}H]STX binding activity. The results of binding and protein assays on S1 and S2 supernatant preparations from five different bullfrogs are summarized in Table 2.

Table 2 shows that S2 supernatant contains [^{3}H]STX-binding sites at a concentration of about 5 nM and a specific activity of 0.3 pmol/mg of protein. This table also shows that about 80% of the total activity measured in the S1 microsome suspension remained in the supernatant after high speed centrifugation. Additional centrifugation of the S2 supernatant for 2 hr at 180,000 × g also failed to pellet this binding activity. By the usual criteria, these conditions of centrifugation would be expected to pellet all cellular membranes and organelles, raising the possibility that the S2 sites are associated with a water-soluble protein.

TABLE 2

[^{3}H]STX binding activity measured in S1 and S2 supernatant fractions

S1 and S2 supernatant fractions were separately prepared from total leg muscle of five different frogs. Protein concentration and [^{3}H]STX binding activity were assayed under standard conditions as described under Experimental Procedures. Values are expressed as the mean of five preparations from individual frogs ± standard deviations.

	Binding activity (n = 5)
Muscle weight (g)	42 ± 10
S1 volume (ml)	30 ± 4
S1 [protein] (mg/ml)	22 ± 9
S1 [STX sites] (pmol/ml)	5.1 ± 2.7
S2 [protein] (mg/ml)	12 ± 4
S2 [STX sites] (pmol/ml)	4.3 ± 2.8
% STX sites in S2/S1	82 ± 12
Total STX sites in S2 (pmol)	139 ± 109
Specific activity of S2 (pmol/mg)	0.33 ± 0.15
Yield of activity in S2 (pmol/g muscle)	3.1 ± 1.6

Figs. 6 and 7 show the results of a pharmacological characterization of the [^{3}H]STX binding activity in the S2 supernatant fraction. Titration of a fixed amount of S2 supernatant with increasing amounts of [^{3}H]STX resulted in a binding isotherm that was well described by a single site as shown by the Scatchard analysis of Fig. 6A. The equilibrium binding data of Fig. 6A were used to derive an equilibrium dissociation constant of 0.14 ± 0.02 nM. The association and dissociation kinetics of [^{3}H]STX binding to the receptor in the S2 supernatant are shown in Fig. 6B. The exponential time course of the association and dissociation reaction is also consistent with binding to a single class of sites. The derived association and dissociation rate constants for [^{3}H]STX from these experiments is 1.5×10^6 sec^{-1} M^{-1} and 1.2×10^{-4} sec^{-1}, respectively. The ratio of these rate constants gives an equilibrium K_D of 0.08 nM, which is in reasonable agreement with the value obtained by Scatchard analysis.

The results of displacement experiments designed to investigate competition of [^{3}H]STX binding in the S2 supernatant with STX, NEO, and TTX are shown in Fig. 7. Both STX and NEO exhibited single-site displacement curves (Hill n = 1.06, STX; 1.01, NEO), whereas TTX did not induce any significant displacement of [^{3}H]STX binding at concentrations up to 80 μM. The derived K_D values for STX and NEO from the data of Fig. 7 were 0.10 nM and 60 nM, respectively, assuming single-site competition and a K_D for [^{3}H]STX of 0.1 nM (Table 3). These derived K_D values for STX and NEO in the S2 supernatant fraction correspond closely to those estimated for the TTX-insensitive component of [^{3}H]STX binding in the KCl and LiBr membrane preparations (Table 3). Also, the slow dissociation rate of [^{3}H]STX from the S2 sites is similar to the slow component observed in KCl membranes (Fig. 2). The similar pharmacological properties of these two sites suggest that the TTX-insensitive activity exists in two physical forms

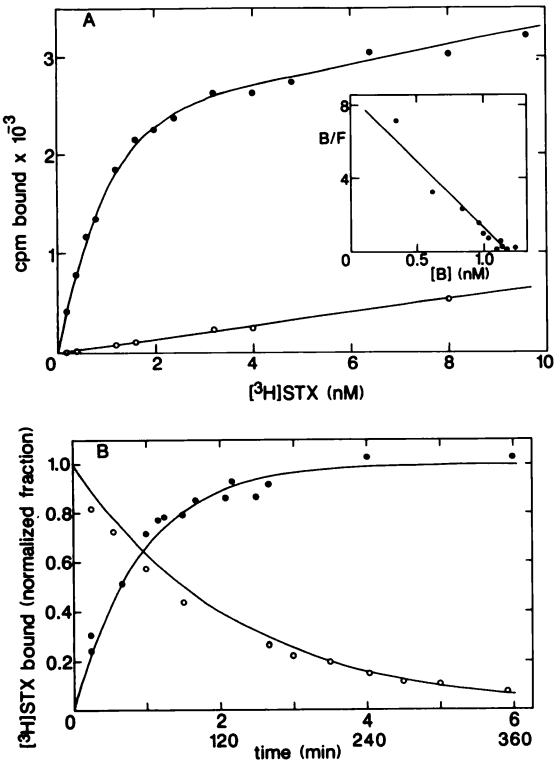

Fig. 6. Equilibrium and kinetic analysis of [^{3}H]STX binding to skeletal muscle supernatant. A. Titration of a fixed amount of S2 supernatant with increasing concentrations of [^{3}H]STX. The assay mixture contained 10 mM MOPS-NaOH, pH 7.4, 0.2 M choline chloride, 2.2 mg/ml S2 supernatant, and the indicated total concentrations of [^{3}H]STX plotted along the *abscissa*. Raw data are plotted on the *ordinate* as cpm of bound [^{3}H]STX measured in 0.1 ml of assay mixture in the absence (●) or presence (○) of 10 μM STX. The *inset* shows a Scatchard plot of bound/free (*B/F*) versus the concentration of bound, [*B*]. B. Dissociation and association kinetics of [^{3}H]STX binding to S2 supernatant. The upper time scale refers to the association experiment and the lower time scale refers to the dissociation experiment. In the dissociation experiment (○), the standard assay mixture for [^{3}H]STX binding containing 2.2 mg/ml S2 supernatant was incubated at 0° for 1 hr. At time = 0, 20 μM STX was added and 0.1-ml samples were assayed for binding at various times. The data are expressed as the fraction of specifically bound [^{3}H]STX measured before the addition of unlabeled STX (0.68 pmol/mg). In the association experiment (●), the standard assay mixture containing 2.2 mg/ml S2 supernatant was incubated at 0° and 13.8 nM [^{3}H]STX was added at time = 0 to initiate the binding reaction. Samples of 0.1 ml were assayed at various times. The level of binding was constant at times between 5 and 60 min (not shown), with a mean of 1.4 nM bound or 0.64 pmol/mg.

in frog muscle: a water-soluble form and a membrane-bound form.

Further discrimination of the two types of binding sites by pH dependence and competition by monovalent cations. The unusual properties of TTX insensitivity and apparent water solubility of the [^{3}H]STX-binding sites in the S2 supernatant led us to investigate other biochemical characteristics of relevance to sodium channels. One well known property of previously studied sodium channels is the presence of a carboxyl group in the TTX/STX-binding site that exhibits an apparent pK_a in the range of 5.3–6.0 as determined from studies of the inhibition of binding of these toxins by protons (38–41). Another previously documented property of TTX/STX binding is competition by monovalent alkali cations with the selectivity sequence $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$ (38, 39,

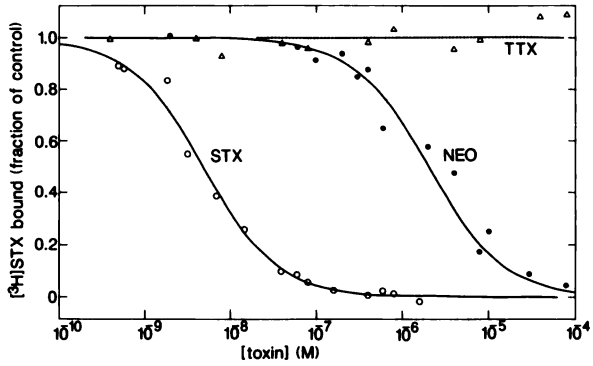

Fig. 7. Effect of STX, NEO, and TTX on specific binding of [^{3}H]STX to skeletal muscle supernatant. Specific binding of [^{3}H]STX to S2 supernatant was assayed under standard conditions as described under Experimental Procedures. Data are plotted in the same manner as in Fig. 1. ○, STX; ●, NEO; △, TTX. Initial levels of specific binding in the absence of unlabeled toxins ranged from 0.53 to 0.62 pmol/mg.

41, 42). In order to characterize these properties in the S2 supernatant sites, we compared the dependence of [^{3}H]STX binding in frog KCl membranes and S2 supernatant on pH and alkali cations. The results shown in Fig. 8 indicate that both of these properties are altered in the TTX-insensitive, soluble sites as compared to the TTX-sensitive, membrane-bound sites. The data of Fig. 8A suggest that the pH dependence of [^{3}H]STX binding is shifted to more basic pH values by at least 0.4 pH unit. Also, a comparison of Fig. 8, B and C, shows that the soluble sites exhibit the same amount of [^{3}H]STX binding in the presence of 0.6 M choline chloride, 0.6 M LiCl, and 0.6 M NaCl, while the membrane-bound sites exhibit competition by the alkali cations. In other experiments, we also found that K^+, Rb^+, and Cs^+ do not affect binding of [^{3}H]STX to the soluble receptor at a concentration of 0.6 M compared to the same concentration of choline chloride (not shown). Thus, the soluble binding site for STX also exhibits a lack of competition by monovalent alkali cations.

Discussion

Our results discriminate two types of high affinity STX-binding sites in bullfrog skeletal muscle that differ in their toxin pharmacology and physiochemical properties. One type exhibits widely recognized properties of previously described sodium channels. These properties include competitive, high affinity binding of STX and TTX, competition of STX binding by alkali cations such as Li^+ and Na^+, and integral association with plasma membranes. This familiar type of STX site corresponds to the properties of functional sodium channels that have been thoroughly studied in frog muscle fibers by toxin binding and voltage clamp experiments (19–22, 43).

The second type of STX site that we found has several novel and peculiar properties such as an unusually slow dissociation rate of STX, relatively low affinity for NEO, practically complete insensitivity to TTX, existence in both membrane-associated and soluble forms, and a lack of competition of STX binding by alkali cations. The unusual properties of the soluble binding site provoke the question of whether this site is structurally or functionally related to genuine sodium channels that have been previously characterized. The altered binding properties do not provide a definitive answer, since it is possible that such changes could be due to a minor structural alteration

TABLE 3

Summary of toxin binding constants

Values were obtained in various [^{3}H]STX binding experiments carried out at 0–4° as described in the text.

	Membranes		S2 supernatant
	TTX-sensitive site	TTX-insensitive site	
STX			
K_D (nM)	0.51–0.54	0.14–0.19	0.14 ± 0.02
k_d (sec^{-1})	2.3 ± 0.5 $\times 10^{-3}$	1.4 ± 0.1 $\times 10^{-4}$	1.2 ± 0.1 $\times 10^{-4}$
k_a (sec^{-1} M^{-1})	ND[a]	ND	1.5 ± 0.1 $\times 10^{6}$
NEO K_D (nM)	0.095 ± 0.022	25 ± 11	60 ± 10
TTX K_D (nM)	1.3 ± 0.3	UND[b]	UND

[a] ND, not determined.
[b] UND, undetectable.

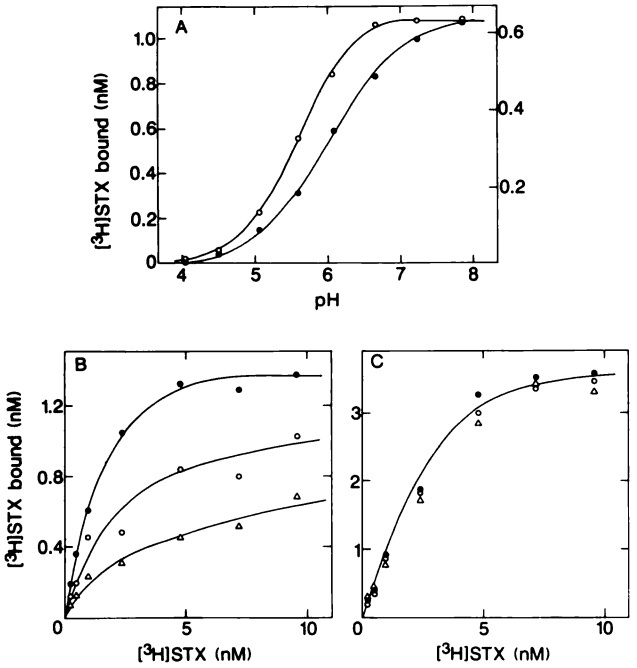

Fig. 8. Effect of pH and monovalent cations on specific binding of [^{3}H]STX to skeletal muscle membranes and supernatant. A. Specific binding at 0° was assayed in the presence of 2.4 nM [^{3}H]STX, 0.2 M choline chloride, 0.6 mM EDTA. 25 mM citric acid, 25 mM 2-(*N*-morpholino)ethanesulfonic acid, 25 mM MOPS, and 25 mM 3-[tris-(hydroxymethyl)methyl]aminopropanesulfonic acid adjusted to various pH levels with Tris. The *ordinate scale* on the *left* refers to the concentration of specifically bound [^{3}H]STX measured for S2 supernatant (●), and the *scale* on the *right* refers to that for KCl membranes (○) measured in the presence of 0.5% saponin. B and C. Specific [^{3}H]STX binding was assayed in the presence of the indicated total concentration of [^{3}H]STX, 10 mM MOPS-NaOH, pH 7.4, and 0.6 M choline chloride (●), 0.6 M NaCl (○) or 0.6 M LiCl (△). B. KCl membranes (3.2 mg/ml) including 0.5% saponin. C. S2 supernatant (5.4 mg/ml). *Solid lines* were drawn by eye and have no theoretical significance.

in the toxin-binding site. Also, the fact that we have not yet observed TTX-insensitive sodium channels from bullfrog muscle in planar bilayers is an ambiguous result, since this particular subtype might not be activated by batrachotoxin. As far as we are aware, no mention of a TTX-insensitive sodium current has been made in previous macroscopic voltage clamp studies of frog muscle fibers, aside from chemical modification experiments in which normal sodium channels were chemically rendered TTX insensitive (44). One explanation for this discrepancy may be that functional TTX-insensitive sodium channels in frog muscle reside deep within invaginations of the transverse tubular system, making this current inaccessible to macroscopic voltage clamp or patch clamp experiments on isolated fibers. It should be noted, however, that previous optical experiments using voltage-sensing dyes have shown that the action potential propagated in the transverse tubular system of frog muscle is blocked by μM concentrations of TTX (45). Although it seems unlikely that functional TTX-insensitive sodium channels exist in frog muscle, a reexamination of this question would be warranted in view of the reports on differential sensitivity of surface and transverse tubular sodium channels to scorpion toxins and TTX derivatives (23–25).

In the absence of direct structural information on the soluble STX binding component, one cannot conclude that it is a soluble form of a sodium channel protein. However, indirect evidence does leave this possibility open. Recently, two pharmacologically and kinetically distinct sodium currents have been described in bullfrog sympathetic neurons (14). Although both of these currents were sensitive to STX, one of them was not blocked by 10 μM TTX. Since this latter current exhibits a toxin specificity similar to that of the soluble binding sites observed here, it is possible that the protein components of these two activities are related despite their expression in different excitable tissues. With the identification of frog skeletal muscle as a good tissue source of this activity, purification of the soluble STX binding component may clarify its structural relationship to sodium channel proteins.

Acknowledgments

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References

1. Fuhrman, F. A. Tetrodotoxin, tarichatoxin and chiriquitoxin: historical perspectives. *Ann. N. Y. Acad. Sci.* **479**:1–14 (1986).
2. Schantz, E. J. Chemistry and biology of saxitoxin and related toxins. *Ann. N. Y. Acad. Sci.* **479**:15–23 (1986).
3. Ritchie, J. M., and R. B. Rogart. The binding of saxitoxin and tetrodotoxin to excitable tissue. *Rev. Physiol. Biochem. Pharmacol.* **79**:1–50 (1977).
4. Guo, X., A. Uehara, A. Ravindran, S. H. Bryant, S. Hall, and E. Moczydlowski. Kinetic basis for insensitivity to tetrodotoxin and saxitoxin in sodium channels of canine heart and denervated rat skeletal muscle. *Biochemistry* **26**:7546–7556 (1987).
5. Cruz, L. J., W. R. Gray, B. M. Olivera, R. D. Zeikus, L. Kerr, D. Yoshikami, and E. Moczydlowski. *Conus geographus* toxins that discriminate between neuronal and muscle sodium channels. *J. Biol. chem.* **260**:9280–9288 (1985).
6. Moczydlowski, E., B. M. Olivera, W. R. Gray, and G. R. Strichartz. Discrimination of muscle and neuronal Na-channel subtypes by binding competition between [^{3}H]saxitoxin and μ-conotoxins. *Proc. Natl. Acad. Sci. USA* **83**:5321–5325 (1986).
7. Frelin, C., P. Vigne, and M. Lazdunski. Na^+ channels with high and low affinity tetrodotoxin binding sites in the mammalian skeletal muscle cell. *J. Biol. Chem.* **258**:7256–7259 (1983).
8. Gonoi, T., Y. Ohizumi, H. Nakamura, J. Kobayashi, and W. A. Catterall. The *Conus* toxin geographutoxin II distinguishes two functional sodium channel

subtypes in rat muscle cells developing *in vitro*. *J. Neurosci.* **7**:1728–1731 (1987).
9. Rogart, R. B. High-STX-affinity vs. low-STX-affinity Na^+ channel subtypes in nerve, heart and skeletal muscle. *Ann. N. Y. Acad. Sci.* **479**:402–430 (1986).
10. Connor, J., L. Barr, and E. Jakobsson. Electrical characteristics of frog atrial trabeculae in the double sucrose gap. *Biophys. J.* **15**:1047–1067 (1975).
11. Tanaka, J. C., D. D. Doyle, and L. Barr. Sodium channels of vertebrate hearts: three types of saxitoxin binding sites in heart. *Biochim. Biophys. Acta* **775**:203–214 (1984).
12. Catterall, W. A., and J. Copperamith. High-affinity saxitoxin receptor sites in vertebrate heart: evidence for sites associated with autonomic nerve endings. *Mol. Pharmacol.* **20**:526–532 (1981).
13. Doyle, D. D., M. Wong, J. Tanaka, and L. Barr. Saxitoxin binding sites in frog myocardial cytosol. *Science (Wash. D. C.)*, **215**:1117–1119 (1982).
14. Jones, S. W. Sodium currents in bull-frog sympathetic neurones. *J. Physiol. (Lond.)* **389**:605–627 (1987).
15. Pappone, P. A. Voltage-clamp experiments in normal and denervated mammalian skeletal muscle fibers. *J. Physiol. (Lond.)* **306**:377–410 (1980).
16. Redfern, P., and S. Thesleff. Action potential generation in denervated rat skeletal muscle. II. The action of tetrodotoxin. *Acta Physiol. Scand.* **82**:70–78 (1971).
17. Weiss, R. E., and R. Horn. Functional differences between two classes of sodium channels in developing rat skeletal muscle. *Science (Wash. D. C.)* **233**:361–364 (1986).
18. Nasledov, G. A., and S. Thesleff. Denervation changes in frog skeletal muscle. *Acta Physiol. Scand.* **90**:370–380 (1974).
19. Colquhoun, D., H. P. Rang, and J. M. Ritchie. The binding of tetrodotoxin and α-bungarotoxin to normal and denervated mammalian muscle. *J. Physiol. (Lond.)* **240**:199–226 (1974).
20. Almers, W., and S. R. Levinson. Tetrodotoxin binding to normal and depolarized frog muscle and the conductance of a single sodium channel. *J. Physiol. (Lond.)* **247**:483–509 (1975).
21. Jaimovich, E., R. A. Venosa, P. Shrager, and P. Horowicz. Density and distribution of tetrodotoxin receptors in normal and detubulated frog sartorious muscle. *J. Gen. Physiol.* **67**:399–416 (1976).
22. Ritchie, J. M., and R. B. Rogart. The binding of labeled saxitoxin to the sodium channels in normal and denervated mammalian muscle and in amphibian muscle. *J. Physiol. (Lond.)* **269**:341–354 (1977).
23. Jaimovich, E., M. Ildefonse, J. Barhanin, O. Rougier, and M. Lazdunski. *Centruroides* toxin, a selective blocker of surface Na^+-channels in skeletal muscle: voltage-clamp analysis and biochemical characterization of the receptor. *Proc. Natl. Acad. Sci. USA* **79**:3896–3900 (1982).
24. Barhanin, J., M. Ildefonse, O. Rougier, S. V. Sampaio, J. R. Giglio, and M. Lazdunski. *Tityus* toxin, a high affinity effector of the Na^+ channel in muscle, with a selectivity for channels in the surface membrane *Pflügers Arch.* **400**:22–27 (1984).
25. Jaimovich, E., R. Chicheportiche, A. Lombet, M. Lazdunski, M., Ildefonse, and O. Rougier. Differences in the properties of Na^+ channels in muscle surface and T-tubular membranes revealed by tetrodotoxin derivatives. *Pflügers Arch.* **397**:1–5 (1983).
26. Haimovich, B., D. L. Schotland, W. E. Fieles, and R. L. Barchi. Localization of sodium channel subtypes in adult rat skeletal muscle using channel-specific monoclonal antibodies. *J. Neurosci.* **7**:2957–2966 (1987).
27. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
28. Peterson, G. L. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **83**:346–356 (1977).
29. Hidalgo, C., C. Parra, G. Riquelme, and E. Jaimovich. Transverse tubules from frog skeletal muscle: purification and properties of vesicles with the inside-out orientation. *Biochim. Biophys. Acta* **855**:79–88 (1986).
30. Barchi, R. L., J. B. Weigele, D. M. Chalikan, and L. E. Murphy. Muscle surface membranes: preparative methods affect apparent chemical properties and neurotoxin binding. *Biochim. Biophys. Acta* **550**:59–76 (1979).
31. Moczydlowski, E., and G. Latorre. Saxitoxin and ouabain binding activity of isolated skeletal muscle membranes as indicators of surface origin and purity. *Biochim. Biophys. Acta* **732**:412–420 (1983).
32. Jaimovich, E., P. Donoso, J. L. Liberona, and C. Hidalgo. Ion pathways in transverse tubules: quantification of receptors in membranes isolated from frog and rabbit skeletal muscle. *Biochim. Biophys. Acta* **855**:89–98 (1986).
33. Shimizu, Y., C. Hsu, W. E. Fallon, Y. Oshima, I. Miura, and K. Nakanishi. Structure of neosaxitoxin. *J. Am. Chem. Soc.* **100**:6791–6793 (1978).
34. Lau, Y. H., A. H. Caswell, and J. P. Brunschwig. Isolation of transverse tubules for fractionation of triad junctions of skeletal muscle. *J. Biol. Chem.* **252**:5565–5574 (1977).
35. Rosenblatt, M., C. Hidalgo, C. Vergara, and N. Ikemoto. Immunological and biochemical properties of transverse tubule membranes isolated from rabbit skeletal muscle. *J. Biol. Chem.* **256**:8140–8148 (1981).
36. Latorre, R., C. Vergara, and C. Hidalgo. Reconstitution in planar lipid bilayers of a Ca^{2+}-dependent K^+ channel from transverse tubule membranes isolated from rabbit skeletal muscle. *Proc. Natl. Acad. Sci. USA* **79**:805–809 (1982).
37. Moczydlowski, E., S. S. Garber, and C. Miller. Batrachotoxin-activated Na^+ channels in planar lipid bilayers: competition of tetrodotoxin block by Na^+. *J. Gen. Physiol.* **84**:665–686 (1984).
38. Henderson, R., J. M. Ritchie, and G. T. Strichartz. Evidence that tetrodotoxin and saxitoxin act at a metal cation binding site in the sodium channels of nerve membrane. *Proc. Natl. Acad. Sci. USA* **71**:3936–3940.
39. Reed, J. K., and M. A. Raftery. Properties of the tetrodotoxin binding component in plasma membranes isolated from *Electrophorus electricus*. *Biochemistry* **15**:944–953 (1976).
40. Weigele, J. B., and R. L. Barchi. Analysis of saxitoxin binding in isolated rat synaptosomes using a rapid filtration assay. *FEBS Lett.* **91**:310–314 (1978).
41. Barchi, R. K., and J. B. Weigele. Characteristics of saxitoxin binding to the sodium channel of sarcolemma isolated from rat skeletal muscle. *J. Physiol. (Lond.)* **295**:383–396 (1979).
42. Weigele, J., and R. Barchi. Saxitoxin binding to the mammalian sodium channel: competition by monovalent and divalent cations. *FEBS Lett.* **95**:49–53 (1978).
43. Campbell, D. T., and B. Hille, Kinetic and pharmacological properties of the sodium channel of frog skeletal muscle. *J. Gen. Physiol.* **67**:309–323 (1976).
44. Spalding, B. C. Properties of toxin-resistant sodium channels produced by chemical modification in frog skeletal muscle. *J. Physiol. (Lond.)* **305**:485–500 (1980).
45. Heiny, J. A., and J. Vergara. Optical signals from surface and T system membranes in skeletal muscle fibers. Experiments with potentiometric dye NK2367. *J. Gen. Physiol.* **80**:203–230 (1982).

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