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Discrimination of muscle and neuronal Na-channel subtypes by binding competition between [^{3}H]saxitoxin and μ-conotoxins

(brain/electric organ/heart/tetrodotoxin)

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ABSTRACT **The effect of two μ-conotoxin peptides on the specific binding of [^{3}H]saxitoxin was examined in isolated plasma membranes of various excitable tissues. μ-Conotoxins GIIIA and GIIIB inhibit [^{3}H]saxitoxin binding in *Electrophorus* electric organ membranes with similar K_ds of $\approx 50 \times 10^{-9}$ M in a manner consistent with direct competition for a common binding site. GIIIA and GIIIB similarly compete with the majority (80–95%) of [^{3}H]saxitoxin binding sites in rat skeletal muscle with K_ds of ≈ 25 and $\approx 140 \times 10^{-9}$ M, respectively. However, the high-affinity saxitoxin sites in lobster axons, rat brain, and rat heart are virtually insensitive to GIIIA concentrations up to 10 μM. These results and previously published data suggest that three Na-channel subtypes can be distinguished on the basis of toxin pharmacology: Na channels of skeletal muscle and *Electrophorus* electroplax have high affinity for μ-conotoxins and tetrodotoxin, neuronal Na channels have low affinity for μ-conotoxins and high affinity for tetrodotoxin, while heart Na channels and a similar subtype also found in denervated muscle have low affinity for both μ-conotoxin and tetrodotoxin.**

Excitable tissues such as nerve, skeletal muscle, and heart contain voltage-dependent Na channels that mediate the rapidly activating and inactivating inward Na^+ current of action potentials in these cells. Purified preparations of Na channels from electric organ, muscle, brain, and heart contain a similar large glycoprotein, while muscle and brain preparations also contain smaller peptides (1–4). Since the large glycoprotein from *Electrophorus* electric organ was first purified as a toxin receptor and has now been shown to produce functional Na channels (5), it appears that this protein contains both the channel and the external receptor site for heterocyclic guanidinium toxins such as tetrodotoxin (TTX) and saxitoxin (STX). In recent years, much effort has been devoted to the identification of Na-channel subtypes based on the binding affinity of TTX and STX (6–15). These studies have identified a subtype with low affinity for TTX/STX that is present in cardiac muscle and noninnervated skeletal muscle cells of mammals. However, previous comparison studies of Na channels in normal adult skeletal muscle and nerve concluded that Na channels in these two tissue types may be the same molecular species because of their very similar high affinity for TTX/STX and similar electrophysiological properties (9, 16).

Recently, a new class of peptide toxins with blocking activity against Na channels has been identified in the venom of *Conus geographus*, an Indo-Pacific mollusc capable of paralyzing fish by injecting them with small venomous barbs (17, 18). Seven homologs of the μ-conotoxin class have been purified, sequenced, and shown to consist of a single chain of 22 amino acids with amidated carboxyl termini (18). One of these toxins, GIIIA, has recently been shown to block muscle action potentials (18) and macroscopic Na current in a voltage-clamped frog muscle fiber (19). At the single channel level, the kinetics of GIIIA block have been shown to conform to a single-site binding model (K_d, 110×10^{-9} M at 0 mV), from analysis of the statistics of discrete blocking events induced in batrachotoxin-activated Na channels from rat skeletal muscle (18). These studies revealed close similarities between the blocking action of GIIIA and the classical guanidinium toxins TTX and STX, including similar voltage-dependent binding to batrachotoxin-activated Na channels. These results suggested that μ-conotoxin peptides might share the same receptor site for TTX and STX.

Although Na-channel block by GIIIA can be readily demonstrated in frog and rat skeletal muscle, significant Na-channel blocking effects with this toxin have not been demonstrated in nerve or brain Na channels from these same species (18). This discrimination between neuronal and muscle Na channels suggested that Na channels in these two tissues might represent different channel subtypes. These implications are pursued in this paper by examining the effect of two purified μ-conotoxins on the specific binding of [^{3}H]STX to Na channels in various tissues. The μ-conotoxins studied are GIIIA and GIIIB, which differ by only 2 amino acids out of 22. GIIIB has substitutions of arginine for glutamine at residue 14 and methionine for glutamine at residue 18 of GIIIA (18). We find that GIIIA and GIIIB competitively inhibit the binding of [^{3}H]STX to sites in rat muscle and *Electrophorus* electric organ membranes but not in rat brain, rat heart, or lobster axon membranes. Some of these results have been presented in preliminary form (19, 20).

MATERIALS AND METHODS

Native membranes from various sources were prepared at 0°C–4°C as follows. The final fraction of each preparation was resuspended at $\approx$10 mg of protein per ml in 0.3 M sucrose buffer (10 mM Mops-NaOH, pH 7.4/0.2 mM EDTA, 3 mM NaN_3) and stored frozen at −80°C.

Crude microsomal fractions from rat skeletal muscle and *Electrophorus electricus* electric organ (World Wide Scientific Animals, Apoka, FL) were prepared according to a published method (21) except that *Electrophorus* membranes were not extracted with KCl. Five milliliters of crude microsomes (10–20 mg of protein per ml in 0.3 M sucrose buffer) was layered over 30 ml of 0.95 M sucrose buffer and centrifuged at 85,000 × *g* for 15 hr. The resulting low-density band was diluted in buffer, pelleted at 100,000 × *g*, and used in [^{3}H]STX binding studies.

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Abbreviations: STX, saxitoxin; NEO, neosaxitoxin; TTX, tetrodotoxin.

Whole rat brains (20 g) plus 0.3 M sucrose buffer (150 ml) were homogenized in a Teflon/glass homogenizer and centrifuged at 2000 × *g* for 15 min. This supernatant was saved and the pellets were rehomogenized in 150 ml of 0.3 M sucrose buffer and centrifuged as described above. The combined supernatant was pelleted at 100,000 × *g* for 40 min. The resulting pellets were rehomogenized in 150 ml of buffer without sucrose and centrifuged at 8000 × *g* for 10 min. The supernatant was pelleted at 100,000 × *g* and used in [^{3}H]STX binding sites.

A membrane preparation from rat heart ventricular muscle was prepared according to procedure II of Jones *et al.* (22). The Ca^{2+} loading step was not used and a fraction sedimenting at the interface of a 0.25 M/0.6 M sucrose gradient was saved at the final step.

A plasma membrane fraction from nerve axons of lobster walking legs was prepared by the method of Balerna *et al.* (23).

The standard assay for [^{3}H]STX binding contained 0.2 M choline Cl, 10 mM Mops-NaOH (pH 7.4), 0.5 mg of bovine serum albumin per ml, 4.5 nM [^{3}H]STX, and various membrane preparations at 0.3–2 mg of protein per ml in a final vol of 125 or 250 μl. [^{3}H]STX binding was allowed to equilibrate for 30–60 min at 0°C before separating the bound ligand at 4°C on 1-ml columns of Dowex 50X-200 (Tris$^+$ form) cation exchange resin in Pasteur pipettes. The assay was performed by rapidly layering 50 or 100 μl of the assay mixture on the column, briefly injecting the sample into the bed with a tight-fitting syringe, adding 0.5 ml of 20 mM Tris·HCl (pH 7.2), and rapidly forcing this eluant into a scintillation vial for counting. The processing time of each sample was 10–15 sec. Specific [^{3}H]STX binding was determined from the difference between a control for nonspecific binding that included 20 μM TTX (Calbiochem). Data points are the means of duplicate measurements. [^{3}H]STX used in these experiments was prepared according to Ritchie *et al.* (24). μ-Conotoxins GIIIA and GIIIB were purified from crude venom of *C. geographus* as described (18).

RESULTS

Fig. 1 compares the displacement of specific [^{3}H]STX binding to rat muscle and rat brain by increasing concentrations of three guanidinium toxins: TTX, STX, and neosaxitoxin (NEO). The ordinate axis of Fig. 1 is expressed as a fraction, f, of the specifically bound [^{3}H]STX that was measured initially in the absence of unlabeled toxin. The concentration, $K_{0.5}$, of unlabeled toxin that displaced one-half (f = 0.5) of the [^{3}H]STX bound initially was obtained from linear regression fits of Hill plots of $\log[f/(1-f)]$ vs. $\log[\text{toxin}]_{\text{free}}$. The slopes of such plots gave Hill coefficients ranging from n = 0.8 to 1.2. The solid line fits in Fig. 1 were drawn by using a Hill coefficient of 1.0 according to

$$f = K_{0.5}/(K_{0.5} + [\text{toxin}]_{\text{free}}). \quad [1]$$

These fits suggest that a single-site competition model is a reasonable description of these results in muscle and brain membranes as concluded by other groups (25–27). With this model, a simple relation exists between the free competitor concentration at half-displacement, $K_{0.5}$, and the K_d of the competitor:

$$K_d = K_{0.5}/\{1 + ([^3\text{H-STX}]_{0.5}/K_{d,\text{STX}})\}, \quad [2]$$

where $K_{d,\text{STX}}$ is the K_d for [^{3}H]STX binding, as determined directly by Scatchard plot analysis and [^{3}H-STX]$_{0.5}$ is the free concentration of [^{3}H]STX at half-maximal displacement, which is ≈3.5×10^{-9} M in these experiments.

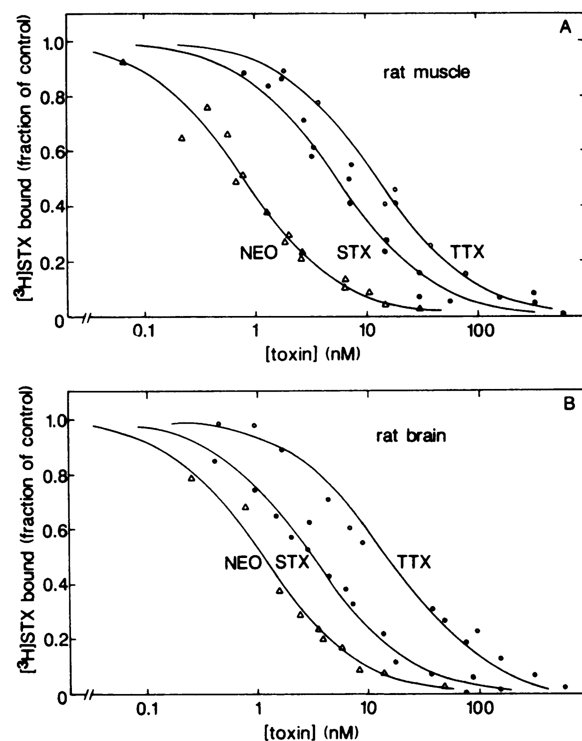

FIG. 1. Displacement of specific [^{3}H]STX binding to rat skeletal muscle (*A*) and rat brain (*B*) membranes by NEO, STX, and TTX. The ordinate is defined as the fraction of specifically bound [^{3}H]STX that remains in the presence of increasing concentrations of free toxins, plotted along the abscissa. The initial specific binding of [^{3}H]STX in the absence of other toxins is defined as f = 1, with f = 0 equivalent to the level of nonspecific binding measured in the presence of 20 μM TTX. The initial levels of specific [^{3}H]STX binding in the absence of other toxins were 0.9–1.2 pmol/mg in various experiments with the muscle preparation and 6.5–8.8 pmol/mg with the brain preparation. Solid lines are fits to Eq. 1 with the following values for $K_{0.5}$ ($\times 10^{-9}$ M): (*A*) NEO, 0.80; STX, 5.4; TTX, 13; (*B*) NEO, 1.2; STX, 3.1; TTX, 15.

Table 1 summarizes the K_d values for muscle and brain as derived by application of Eq. 2 to the data of Fig. 1. These results confirm previous reports that the relative affinity of these toxins in mammalian nerve and muscle is NEO > STX > TTX, as measured by binding, action potential, and single-channel measurements (28, 29). The K_d values for STX, NEO, and TTX show only a small variation between muscle and brain, indicating the similar pharmacological

Table 1. Equilibrium dissociation constants for toxin binding as estimated by various methods

Toxin	Rat brain, K_d × 10^{-9}M	Rat skeletal muscle, $K_d \times 10^{-9}$ M	*Electrophorus* electric organ, $K_d \times 10^{-9}$M
STX	0.22*	0.59*	1.1*
	0.19†	0.81†	
NEO	0.067†	0.11†	—
TTX	0.86†	1.8†	—
GIIIA	11,000?†	25†	56†
		30‡	36‡
GIIIB	—	140†	46†

*Determined directly by Scatchard plot analysis of [^{3}H]STX binding.
†Determined by analysis of the displacement of bound [^{3}H]STX by increasing concentrations of unlabeled toxin according to Eqs. 1 and 2.
‡Determined from the apparent K_d of [^{3}H]STX binding in the presence of several concentrations of unlabeled toxin according to Eq. 3.

specificity of the receptor sites for these classical toxins in these two tissues.

In the same assay, however, μ-conotoxin peptides are able to discriminate between muscle and brain Na channels. A comparison of Figs. 2*A* and 3*A* shows that GIIIA displaces [^{3}H]STX binding in rat muscle with a $K_{0.5}$ of $\approx 160 \times 10^{-9}$ M and has no effect in rat brain at concentrations up to $\approx 10 \times 10^{-6}$ M. There is, however, a consistently observed 20% inhibition of [^{3}H]STX binding in rat brain at 40×10^{-6} M GIIIA. We have not tried to examine the effect of higher GIIIA concentrations in brain because of the limited availability of this toxin. Figs. 2*B* and 3*B* also show that μ-conotoxin displaces [^{3}H]STX binding to *Electrophorus* electroplax membranes in the 10–1000 nM range but has no effect in lobster axon or in similar experiments with a rat heart preparation at concentrations up to 10 μM.

The data of Fig. 2*B* in electroplax are well-described by a single-site competition model with $K_{0.5}$ of 210×10^{-9} M for GIIIA and 170×10^{-9} M for GIIIB. By the relationship of Eq. 2 for the electroplax data, GIIIA and GIIIB have very similar K_ds of 56 and 46×10^{-9} M, respectively. However, for rat muscle, Fig. 2*A* shows that GIIIA has about a 5-fold higher affinity than GIIIB.

Fig. 2*A* also shows that high concentrations of GIIIA and GIIIB fail to inhibit 10–20% of specific [^{3}H]STX binding in this experiment with rat muscle. This consistently observed phenomenon suggested that a small proportion of the binding sites in the muscle preparation was insensitive to μ-conotoxin. To check whether a permeability problem such as sealed vesicles could explain this behavior, we performed some experiments in the presence of 0.24% saponin. In preliminary experiments, this detergent was found to increase [^{3}H]STX binding by a factor of ≈ 1.6 by presumably making sealed inside-out transverse tubule vesicles permeable to [^{3}H]STX (21). However, saponin treatment did not appear to significantly affect the proportion of sites that was sensitive to GIIIA, as also shown in Fig. 2*A*. To calculate an apparent K_d for GIIIA and GIIIB in the muscle experiments, we assumed that 16% of the sites in Fig. 2*A* were insensitive to these toxins and we fitted a normalized single-site binding isotherm to the remaining sites. This method gave K_d values of 25×10^{-9} M for GIIIA and 140×10^{-9} M for GIIIB in rat muscle using the relationship of Eq. 2.

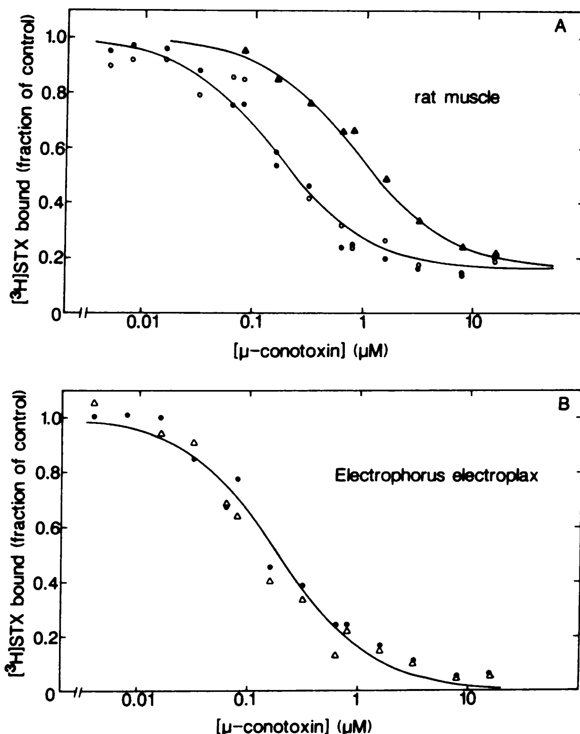

FIG. 2. Displacement of specific [^{3}H]STX binding to rat muscle (*A*) and *Electrophorus* electroplax (*B*) membranes by μ-conotoxins GIIIA and GIIIB. The initial levels of specific [^{3}H]STX binding were 1.1–1.8 pmol/mg for the muscle preparation and 3.3 pmol/mg for the eel preparation. (*A*) ▲, GIIIB; ○, GIIIA; ●, GIIIA in the presence of 0.24% saponin. (*B*) △, GIIIB; ●, GIIIA. The solid line in *B* is a fit to Eq. 1 with $K_{0.5} = 190 \times 10^{-9}$ M, and the solid lines in *A* are fits to Eq. 1 with f normalized by a 16% insensitive fraction and $K_{0.5} = 160 \times 10^{-9}$ M for GIIIA and $K_{0.5} = 850 \times 10^{-9}$ M for GIIIB.

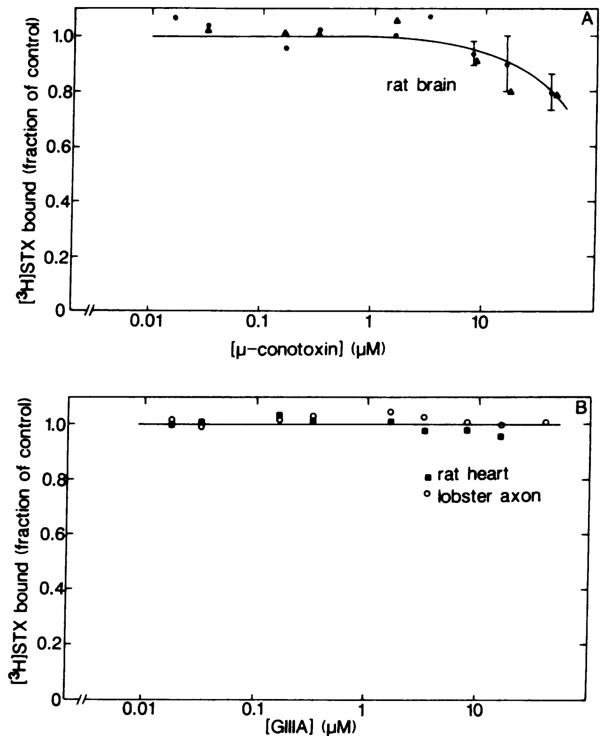

FIG. 3. Specific binding of [^{3}H]STX to rat brain (*A*), rat heart (*B*), and lobster axon (*B*) membranes in the presence of μ-conotoxins. Initial levels of specific [^{3}H]STX binding in the absence of μ-conotoxin were 5.7–8.0 pmol/mg for rat brain, 2.0 pmol/mg for rat heart, and 9.3 pmol/mg for lobster axon membranes. (*A*) ●, GIIIA; ▲, GIIIB. (*B*) ■, GIIIA, rat heart; ○, GIIIA, lobster axon. The error bars in *A* indicate the SEM of three different experiments. The solid line in *A* is drawn according to Eq. 1, assuming a $K_{0.5}$ of 160×10^{-6} M.

Although binding to [^{3}H]STX in rat brain is rather insensitive to GIIIA, the slight inhibition observed in Fig. 3*A* at high GIIIA concentration suggests that brain Na channels might recognize μ-conotoxins with low affinity. To estimate a K_d value for GIIIA in rat brain, we assumed that the 20% inhibition at 40 μM GIIIA was due to simple competition. Using the estimated $K_{0.5}$ displacement value of 160×10^{-6} M (corresponding to the fit of Fig. 3*A*) and Eq. 2, we obtain a K_d of 11×10^{-6} M for GIIIA in rat brain, as noted with a question mark in Table 1. Thus, the estimated relative affinity for GIIIA of Na channels in rat brain vs. rat muscle is $11{,}000 \times 10^{-9}$ M/25×10^{-9} M or a 440-fold lower affinity in brain.

To further pursue the mechanism of μ-conotoxin inhibition in electroplax and rat muscle, we performed a Scatchard plot analysis of [^{3}H]STX binding in the presence and absence of GIIIA. The results of Fig. 4 for the electroplax preparation show that 240×10^{-9} M GIIIA increases the apparent K_d of [^{3}H]STX without affecting the maximum number of binding sites. A replot of the apparent K_d for [^{3}H]STX vs. GIIIA concentration (Fig. 4 *Inset*) exhibits a reasonably linear relationship, as expected for single-site competition:

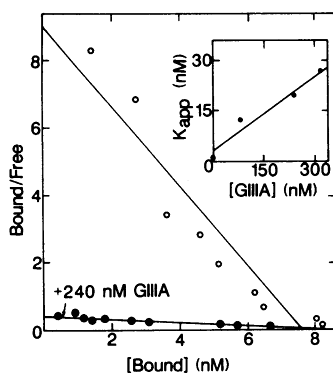

FIG. 4. Scatchard plot of [³H]STX binding to *Electrophorus* electroplax membranes in the absence and presence of GIIIA. The total concentration of eel membrane protein in the assay was 0.6 mg/ml. The parameters of linear regression fits to the Scatchard equation were $K_d = 0.85 \times 10^{-9}$ M and $n = 7.6 \times 10^{-9}$ M sites (12.7 pmol/mg) in the absence of GIIIA and $K_d = 20 \times 10^{-9}$ M and $n = 8.2 \times 10^{-9}$ M sites (13.7 pmol/mg) in the presence of 240 nM GIIIA. (*Inset*) Replot of the apparent K_d of [³H]STX vs. GIIIA concentration according to Eq. 3.

$$K_{d,\text{apparent}} = K_{d,\text{STX}}\,\{1 + ([\text{GIIIA}]/K_{d,\text{GIIIA}})\}. \quad [3]$$

Using Eq. 3, the results shown in Fig. 4 (*Inset*) yield a calculated K_d for GIIIA of 36×10^{-9} M, which is close to the value of 56×10^{-9} M obtained in the displacement experiment of Fig. 2*B*. This agreement supports the conclusion that the effect of GIIIA on [³H]STX binding in electroplax occurs by mutually exclusive competition for a common site.

Results of a similar experiment with a rat muscle membrane preparation are shown in Fig. 5. The results of Fig. 5 in the absence of GIIIA are fit to a single site with a K_d of 0.62×10^{-9} M and a site concentration of 1.8 nM (1.3 pmol per mg of protein). With two different preparations, we observed that Scatchard plots of [³H]STX binding in the presence of GIIIA were nonlinear. By using the same value for the total site capacity, the data in the presence of 480 nM GIIIA are fit to a sum of two independent populations with apparent K_ds of 0.20 and 25×10^{-9} M, using a graphical method (30). In this fit, the relative proportion of high-affinity [³H]STX sites to total sites was 16%, a value similar to the fraction of GIIIA-insensitive sites that was estimated in the displacement titrations of Fig. 2*A*. The effect of GIIIA on the apparent K_d of the low-affinity component in Fig. 5 was also analyzed according to the relationship of Eq. 3 at 240 and 480 nM GIIIA, in a similar manner as shown in the electroplax experiment in Fig. 4 (*Inset*). This analysis gave a value of 30×10^{-9} M for the K_d of GIIIA, which is close to that of 25×10^{-9} M obtained in the displacement experiment of Fig. 2*A*. The K_d of $25–30 \times 10^{-9}$ M for GIIIA in rat muscle measured in the present studies is ≈1/4th that (K_d, 110×10^{-9} M) measured previously at 0 mV using batrachotoxin-activated channels in planar bilayers (18). However, the higher temperature (22°C) and higher Na^+ concentration (200 mM) in the bilayer studies could explain this difference.

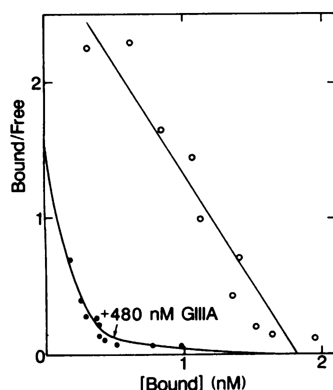

FIG. 5. Scatchard plot of [³H]STX binding to a rat muscle membrane preparation in the absence and presence of 480 nM GIIIA. The total concentration of membrane protein in the assay was 1.4 mg/ml. The parameters of the solid-line fits of the data are given in the text.

These results support the conclusion that the rat muscle preparation contains two populations of receptor sites, one sensitive to GIIIA and one insensitive to GIIIA. In the absence of GIIIA, these two populations are difficult to resolve with [³H]STX alone. The properties of the GIIIA-insensitive population appear similar to the high-affinity [³H]STX binding sites in rat brain (Table 1; Fig. 3*A*); however, it is difficult to make reliable K_d comparisons between brain and the GIIIA-insensitive sites in muscle from curved Scatchard plots such as that of Fig. 5. Nevertheless, it is possible that the GIIIA-insensitive population in the rat muscle preparation represents a small contamination by nerve terminals, which have μ-conotoxin insensitive Na channels. Alternatively, the two populations in muscle could represent two different Na-channel subtypes coexisting in the muscle plasma membrane. Other workers have proposed that certain TTX derivatives and scorpion toxins discriminate between Na channels in the muscle surface sarcolemma membrane and in the transverse tubule membrane (31). At present, we cannot distinguish between these alternative explanations.

DISCUSSION

The competitive nature of the binding interaction between GIIIA and STX implies that the inhibition of STX binding by GIIIA is due to overlapping binding sites for these molecules instead of separate sites that interact allosterically. Our results thus identify μ-conotoxin peptides as a class of ligands for the TTX/STX receptor of voltage-dependent Na channels. The shared sensitivity of STX binding in *Electrophorus* electroplax and rat muscle to competition by μ-conotoxins suggests that the amino acid sequence or set of sequences that form the toxin binding site is highly conserved between these two tissues but not in other Na channels that we have tested. On the basis of toxin pharmacology at this site, we can now distinguish three distinct Na-channel subtypes in mammals. The two subtypes identified in this paper may be referred to as the muscle subtype or m-type, which has high affinity for both μ-conotoxins and TTX, and the n-type of neuronal tissues, which has low affinity for μ-conotoxin and high affinity for TTX. Since it is known that the electric organ of *Electrophorus* develops from embryonic skeletal muscle (32), the electroplax Na channel may be identical to the m-type in this species. In addition, other studies suggest the existence of a third subtype, the h-type in heart (7, 9, 12), which also appears to be expressed in denervated skeletal muscle (6, 8, 15) and in cultured muscle cells (10, 11, 14). This latter h-type Na channel can be identified by its low affinity for TTX (K_d, $\approx 1 \times 10^{-6}$ M). We have chosen tissue types as a convenient nomenclature for these subtypes because these pharmacologically different toxin receptors appear to correspond to the predominant functional channel types that are normally expressed in these three adult mammalian tissues. This tentative classification scheme does not answer the underlying question of the molecular basis for these pharmacological differences. We hypothesize that these multiple subtypes involve multiple Na-channel genes; however, post-translational modifications of a single precursor channel cannot be ruled out.

Na channels in lobster and crab axons have previously been shown to exhibit high affinity for TTX and STX with K_d values in the 2–20 × 10^{-9} M range (23, 24). The insensitivity of lobster axon Na channels to μ-conotoxin suggests that the channel modification that resulted in this sensitivity was a comparatively recent evolutionary development. Although crustaceans have skeletal muscles with clear morphological similarity to vertebrates, voltage-dependent excitation in crustacean muscle occurs by voltage-dependent Ca channels (33). On this basis, we would expect that Na channels of other invertebrate nerve tissues would also be insensitive to μ-conotoxins and that sensitivity probably only occurs in vertebrate muscles with sodium action potentials.

Previous work has identified the presence of two types of Na channels in rat heart homogenates and cultured heart cells. Binding studies have resolved both high- and low-affinity TTX/STX sites (12, 34), while functional electrophysiological or flux studies have found only low-affinity inhibition constants for these toxins (7, 9, 12). While there is general agreement that Na channels with low TTX/STX affinity represent the major functional subtype in mammalian heart, the interpretation of high-affinity toxin binding sites is controversial. One group has concluded that such high-affinity sites in homogenized heart preparations represent contamination by autonomic nerve endings (34), while another group has concluded that Na channels with high affinity for TTX/STX are intrinsically present in the rat cardiac plasma membrane (12). Under the conditions of our experiment with rat heart membranes in Fig. 3*B* we would be primarily measuring such high-affinity STX sites. The insensitivity of these sites to GIIIA places them in our n-type classification, since they cannot be distinguished from brain Na channels at present. Although we have not attempted to study the subtype with low TTX/STX affinity by direct binding studies, we have recently studied such channels by incorporation of batrachotoxin-activated Na channels from dog heart membranes into planar bilayers (19). By using this single-channel assay, we have identified channels that clearly have a low affinity for TTX (K_d, ≃1 × 10^{-6} M) and find that such channels are unaffected by 2 μM GIIIA. These channels thus comprise a class that exhibits low affinity for both μ-conotoxins and TTX/STX. Based on these results, we would expect the electrical activity of mammalian heart to be quite insensitive to μ-conotoxins, as also suggested by work with crude *C. geographus* venom (35). Recently, we have also identified a similar h-type Na channel from denervated rat muscle after incorporation in planar bilayers, and this channel is also insensitive to μ-conotoxin (36).

Note Added in Proof. During the course of publishing this paper, two additional reports of binding competition between conotoxins and [^{3}H]TTX/[^{3}H]STX appeared (37, 38).

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