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Saxitoxin Binding to Synaptosomes, Membranes, and Solubilized Binding Sites from Rat Brain

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Summary. Binding of ^{3}H-saxitoxin to Na^+ channels was studied in subcellular fractions prepared from rat brain homogenates. Saxitoxin binding to synaptosomes was saturable with an apparent dissociation constant of about 1 nM; about 1 pmol/mg protein was bound at saturating saxitoxin concentrations. A linear, nonsaturable component of saxitoxin binding accounted for less than 3% of the total binding at 30 nM. Saxitoxin binding to synaptosomes was unaffected by depolarization with elevated K^+ concentrations, or by activation of the Na^+ channels with batrachotoxin plus a purified polypeptide toxin from the scorpion *Leiurus quinquestriatus*. A procedure is described for preparing a membrane fraction that contains 70–80% of the total saxitoxin binding activity of the crude homogenate. The specific activity of this fraction was about 4 to 6 pmol/mg protein. About 60–70% of the saxitoxin binding sites were solubilized by incubating these membranes with the nonionic detergent Triton X-100; the detergent-solubilized binding sites eluted at a position corresponding to a mol wt of about 700,000 on gel filtration chromatography. Both membrane-bound and solubilized saxitoxin binding were assayed by a new cation exchange column method. The binding of saxitoxin to both membrane-bound and detergent-solubilized binding sites was saturable with an apparent dissociation constant of about 2 nM. Dissociation of the saxitoxin-receptor complex followed a single exponential decay with a rate constant at 0° of 0.1 min^{-1} for membrane bound and 0.2 min^{-1} for detergent-solubilized binding sites. The measured association rate constant was 6×10^8 M^{-1} min^{-1} at 0° for membrane-bound saxitoxin binding sites.

The rising phase of the action potentials in many excitable cells is due primarily to an influx of Na^+ ions through specific voltage-sensitive "Na^+ channels" (Hille, 1977). Tetrodotoxin (TTX) and saxitoxin (STX) bind with high affinity ($K_d = 1 - 10$ nM) to the outside of the Na^+ channels, thereby blocking entry of Na^+ ions (Kao, 1966; Narahashi, 1974;

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Hille, 1975*a*). Kao and Nishiyama (1965) and Hille (1975*b*) have proposed that the cationic guanidinium group of TTX and STX enters and blocks an acidic site in the Na^+ channel.

The interaction of TTX and STX with Na^+ channels in excitable membranes has been studied in two ways. First, in electrophysiological experiments, the blockade of Na^+ currents has been used as a measure of the toxin-receptor interaction. Second, TTX and STX have been labeled with tritium and the tritiated toxins have been used to measure directly the binding of TTX and STX to excitable membranes. The labeled toxins have been used to estimate the number (density) of Na^+ channels (generally 25–500 per μm^2). It has been concluded from these two types of experiments that TTX and STX both bind to the same, high-affinity site on the Na^+ channel and that toxin binding is directly associated with block of the channel (Ritchie & Rogart, 1977).

Labeled TTX and STX also provide a useful assay for following the purification of the STX binding component of the Na^+ channel. Using ^{3}H-TTX, Henderson and Wang (1972) and Benzer and Raftery (1973) demonstrated that TTX binding sites in garfish olfactory nerve membranes can be solubilized by incubation with the non-ionic detergent Triton X-100. Recently, Agnew *et al.* (1978) purified by 100-fold a TTX binding protein from a detergent extract of electric eel electroplax membranes.

We report here the results of studies on the binding of ^{3}H-STX to Na^+ channels in three different preparations from rat brain. First, the binding of ^{3}H-STX has been studied in synaptosomes under conditions where the membrane potential can be varied. Second, a membrane preparation is described that provides both high yield and high specific activity of ^{3}H-STX binding sites. Finally, STX binding sites have been solubilized from rat brain membranes and characterized in the soluble form. Both membrane-bound and detergent-solubilized STX binding sites were found to have similar kinetic properties.

A preliminary report of some of these findings has been published (Krueger, Ratzlaff & Blaustein, 1978).

Materials and Methods

Preparation of Synaptosomes

Rats (150 g female albino) were stunned and killed by decapitation. Forebrains from four rats were rapidly removed and homogenized in 20 ml of ice-cold sucrose solution (0.32 M sucrose-5 mM Na HEPES, pH 7.4). All subsequent procedures were performed at 0°. The brains were homogenized at 900 rpm (12 strokes) in a glass-Teflon homogenzer (0.15 mm

clearance). The homogenate was diluted to 60 ml and was centrifuged at $1000 \times g$ for 10 min, and the supernatant was retained. The pellet was washed by resuspending in 20 ml sucrose solution, homogenizing with 4 strokes, and centrifuging again at $1000 \times g$ for 10 min. The pooled supernatants were centrifuged 10 min at $1000 \times g$, and the pellet was discarded. The supernatant was then centrifuged at $12{,}000 \times g$ for 20 min, and the supernatant was discarded. The pellet was removed, taking care to exclude the small amount of dark, densely-packed material (mitochondria) at the bottom; the pellet was suspended in 40 ml of sucrose solution and centrifuged at $12{,}000 \times g$ for 20 min. The supernatant was discarded, and the pellet (P_2) was resuspended in sucrose solution (30 ml). 10 ml of the P_2 suspension were layered on 20 ml of 0.8 M sucrose-5 mM Na HEPES, pH 7.4, in each of three centrifuge tubes. The gradients were centrifuged at $9{,}000 \times g$ for 30 min in a Beckman SW 25.1 swinging bucket rotor (Hajos, 1975). The 0.32-M sucrose layer and the white material at the 0.32- to 0.8-M sucrose interface were removed by aspiration and discarded. The 0.8-M sucrose (about 50 ml), which contained synaptosomes, was decanted; the small pellet was discarded. Synaptosomes were equilibrated by the addition, with stirring, of ice-cold *Na+5K* (145 mM NaCl, 5 mM KCl, 1.4 mM $MgCl_2$, 1.0 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose, and 20 mM Tris-HEPES, pH 7.4) in small (1–5 ml) aliquots over a period of 20–30 min. The synaptosomes were pelleted by centrifugation at $20{,}000 \times g$ for 6 min and resuspended in *Na+5K* at about 5 mg protein/ml.

Preparation of Membrane Fractions

Forebrains from nine rats were minced with scissors in 50 ml of ice-cold sucrose solution and disrupted using a Polytron homogenizer (Brinkman) at setting 6 for 15 sec followed by a 15 sec cooling period and then another 15 sec of homogenization. The crude homogenate was diluted to 150 ml and was subjected to differential centrifugation as described by De Robertis *et al.*, (1967). The $1{,}000 \times g$ pellet (P_1) and the $12{,}000 \times g$ pellet (P_2) were suspended in sucrose solution for analysis. The $12{,}000 \times g$ supernatant was centrifuged at $120{,}000 \times g$ for 30 min. The pellet was resuspended in 25 ml sucrose solution and again centrifuged at $120{,}000 \times g$ for 30 min. This pellet (P_3) was suspended in sucrose solution and could be stored at $-70°$ for at least 2 months without loss of ^{3}H-STX-binding activity.

Preparation of ^{3}H-STX

Saxitoxin was exchange-labeled with tritium as described by Ritchie, Rogart and Strichartz (1976). The concentration of saxitoxin was determined by comparison with standard shellfish toxin (FDA, Cincinnati, Ohio) by bioassay. The radiochemical purity was estimated as follows: ^{3}H-STX, 1 nM, was incubated in 0.4 ml of *Na+5K* with increasing amounts of rat brain membranes (fraction P_3) from 25 to 2000 μg. After 10 min on ice, the membranes were pelleted by centrifugation at $100{,}000 \times g$ for 30 min. The amount of ^{3}H remaining in the supernatant was determined by liquid scintillation counting and was found to decrease asymptotically, with increasing protein, to about 25% of the total (mean of four experiments, range: 33–22%). At 1 nM STX, virtually all of the membrane-bound ^{3}H can be displaced by a large excess of unlabeled TTX (10 μM); thus we conclude that the bound ^{3}H represents genuine STX while the ^{3}H that cannot be bound, even at high concentrations of protein, is due to a labeled component that is not STX. All calculations have been based on a radiochemical purity of 75% and a sp act of 20 Ci/mmol.

Filter Assay of STX Binding

Binding of STX to intact synaptosomes and to membrane fractions was determined by filtration with Whatman GF/C glass fiber filters to separate bound from free STX. Aliquots of membrane fractions (0.01–1.0 mg protein) were incubated with 5 or 30 nM ^{3}H-STX in a total volume of 0.25 ml. Some aliquots at each condition were incubated with 10 μM unlabeled TTX in order to displace specifically bound ^{3}H-STX. Following incubation on ice for varying periods of time (10–30 min for dissociation constant determinations), 5 ml of ice-cold Na-buffer (150 mM NaCl in 20 mM Tris-HEPES, pH 7.4) were added and the mixture was filtered under suction (200 mm Hg). The filters were washed twice with 5 ml aliquots of Na-buffer (the total filtration time including washes was about 5 sec) and then placed in 10 ml of Omnifluor-toluene-Triton X-100 scintillation cocktail (Blaustein & Russell, 1975). The radioactivity trapped on the filters was counted by liquid scintillation spectroscopy.

Cation Exchange Column Assay of STX Binding

Binding of STX to both particulate and solubilized binding sites was assayed using Dowex columns to separate protein-bound from free toxin. The Dowex resin (50X2-100, Sigma) was prepared by suspending 20 g in 1 liter of distilled H_2O; after the beads settled, the supernatant was decanted and the wash process was repeated three times. The beads were then washed with 0.5 liter of 1-M NaOH followed by four one-liter distilled H_2O washes, and were suspended in Na-buffer containing 0.01 mg/ml bovine serum albumin. Columns of beads (0.3×2 cm), prepared in Pasteur pipettes with glass wool plugs, were washed with 4 ml Na-buffer before use.

For assay of STX binding, intact or detergent-solubilized membranes (P_3) were incubated with 5 nM ^{3}H-STX in a total volume of 0.25 ml of Na-buffer. After 10–30 min on ice (binding was maximal within 10 min and remained constant for at least 30 min), a 0.2-ml aliquot from each sample was layered on top of a cation exchange column; a separate column was used for each sample. Immediately after the sample had passed into the beads, the column was washed with 1 ml Na-buffer. The 1.2-ml volume, containing protein-bound STX, was eluted from the column in 15–20 sec. The effluent from each column was collected in a vial and was counted after the addition of 12 ml of scintillation cocktail. Sixty or more columns could be run within about 30 min. Columns were stored at 4° and could be used at least five times, provided that they were washed with 4 ml of Na-buffer prior to use.

Control experiments indicated that the columns retained more that 90% of the free STX; approximately 93% of the protein-bound ^{3}H-STX passed through in the 1.2-ml volume which was collected. All determinations using the column method were corrected for this 7% loss of protein. About 12% of the total ^{3}H was present in a form that was not retained on the columns in the absence of protein, and that contributed to the blank which was subtracted from all experimental determinations (*see* Fig. 4).

Solubilization of STX Binding Sites

Membranes were solubilized by diluting P_3 (12.5 mg/ml in sucrose solution) with 4 volumes of 0.625% (vol/vol) Triton X-100 (Sigma) in Na-buffer. After 1 hr on ice, the solution was centrifuged at $120{,}000 \times g$ for 30 min. The clear supernatant was removed and saved for analysis. The pellet was resuspended in 0.5% Triton X-100 in Na-buffer.

Gel Filtration Chromatography

Detergent-solubilized STX binding was analyzed by gel filtration on Sepharose CL6B. The column (0.9 × 53 cm) was equilibrated with a solution containing 150 mM NaCl, 20 mM Tris-HEPES, 1 mM $MgCl_2$, 1.2 nM ^{3}H-STX, 0.1% Triton X-100, 0.025% soybean phospholipids (Associated Concentrates), pH 7.5. An aliquot (1 ml) of the 120,000 × *g* supernatant of P_3 (containing 0.5% Triton X-100) was applied to the column and the column was eluted at 5.5 ml/hr. 0.5 ml fractions were collected and 50 µl aliquots of each fraction were analyzed by liquid scintillation counting as described above. In one experiment the 120,000 × *g* supernatant was heated to 50 °C prior to chromatography. The enzyme β-D-galactosidase (Sigma) was also (independently) chromatographed on the Sepharose column, in order to provide a molecular weight marker; the eluted fractions were assayed for enzymatic activity as described by the supplier.

Determination of STX Dissociation Rate Constant

The rate of dissociation of STX from its binding site was determined after incubating intact membranes or Triton X-100-solubilized binding sites with 5 nM ^{3}H-STX in 0.25 ml for 15 min; 10 µM TTX was added and 0.2 ml aliquots were applied to the cation exchange columns at various times and washed with Na-buffer as described above. The natural logarithm of the fraction of ^{3}H-STX remaining bound was plotted as a function of time in order to determine the dissociation rate constant (k_{-1}). In experiments where the STX dissociation rate was determined as a function of temperature, the cation exchange columns were kept in a cold room maintained at 5 °C and were washed with Na-buffer kept at 0 °C. In some experiments at 5 °C, protein equilibrated with ^{3}H-STX was applied directly to the columns, without addition of TTX, and the columns were washed with Na-buffer at various times. Under these conditions, free STX and any STX that dissociates from binding sites is immediately bound to the column. The same STX dissociation rate at 5 °C was obtained with both methods.

Other Methods

The polypeptide composition of P_3 and of the supernatant and pellet after Triton X-100 solubilization were analyzed by polyacrylamide slab gel electrophoresis, using the discontinuous system of Studier (1973) as described by Rudolph and Krueger (1979). Gels were stained with Coomassie brilliant blue as described by Fairbanks, Steck and Wallach (1971). Protein was determined by the method of Lowry *et al.* (1951). In experiments with Triton X-100, the detergent was removed prior to protein determination as described by Peterson (1977).

A polypeptide neurotoxin from the venom of the scorpion *Leiurus quinquestriatus* (Sigma), used in some STX-binding experiments, was purified as described by Catterall (1976).

Results

Binding of ^{3}H-STX to Synaptosomes

The binding of ^{3}H-STX to rat brain synaptosomes was studied using the filter method. Binding was saturable (Fig. 1) with an apparent disso-

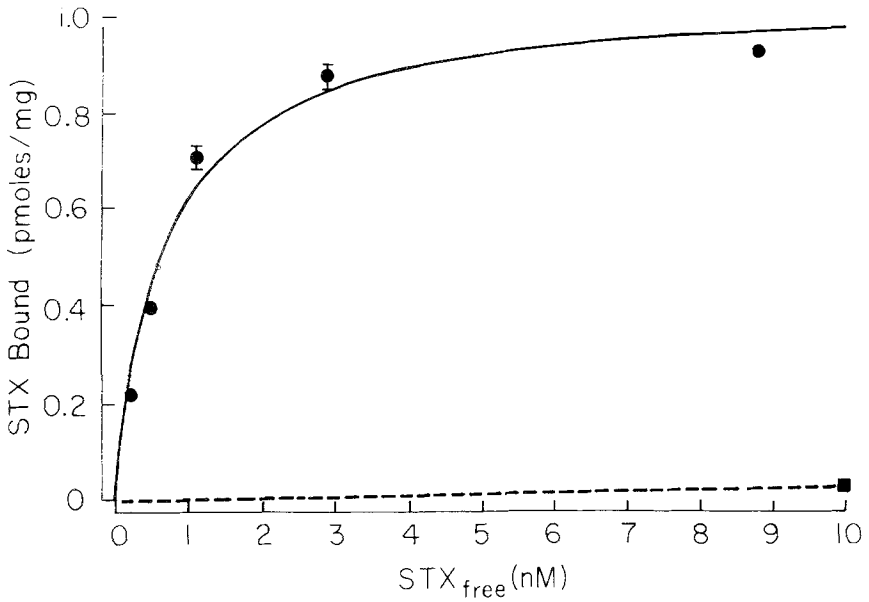


Fig. 1. Binding of ^{3}H-STX to intact synaptosomes. Synaptosomes were prepared as described under Methods and were suspended in *Na+5K* (about 5 mg protein/ml) at 0°. Aliquots (.25 ml) were then incubated with increasing concentrations of ^{3}H-STX (1–10 nM) for 30 min at 0°. Bound ^{3}H-STX was determined by filtration with glass fiber filters with three 5 ml washes of ice cold *Na+5K*, followed by liquid scintillation counting of the filters. The STX concentrations shown on the abscissa were calculated by subtracting the amount of STX bound from the total STX added. The data shown are means of triplicate determinations. Standard errors are shown when larger than the dimensions of the symbols. (●): "Specific" binding, i.e., (total binding) minus (binding + 10 μM TTX). (■): "Nonspecific" STX binding in the presence of a large excess of unlabeled TTX (10 μM). The curve shown is a least-squares fit to the equation:

$$\text{STX Bound} = B_m/(1 + K_d/[\text{STX}])$$

where $B_m = 1.05$ pmol/mg and $K_d = 0.7$ nM

ciation constant of 0.7 nM (0.6–0.7 nM in three experiments) at 0°. At saturating concentrations of STX, about 1 pmol/mg protein was bound; however, only about 10% of the total STX binding sites in the crude homogenate was recovered in the synaptosome fraction (data not shown). If there are 300 cm^2 of plasma membrane surface area per mg of synaptosome protein (Blaustein, 1975), the STX binding (1 pmol/mg) corresponds to a density of about 20 sites per μm^2. This value is of the same order as those reported for other vertebrate nerve preparations (*cf.* Ritchie & Rogart, 1977).

The affinity of TTX or STX for Na^+ channels in cardiac muscle has been postulated to be membrane potential-dependent (Baer, Best

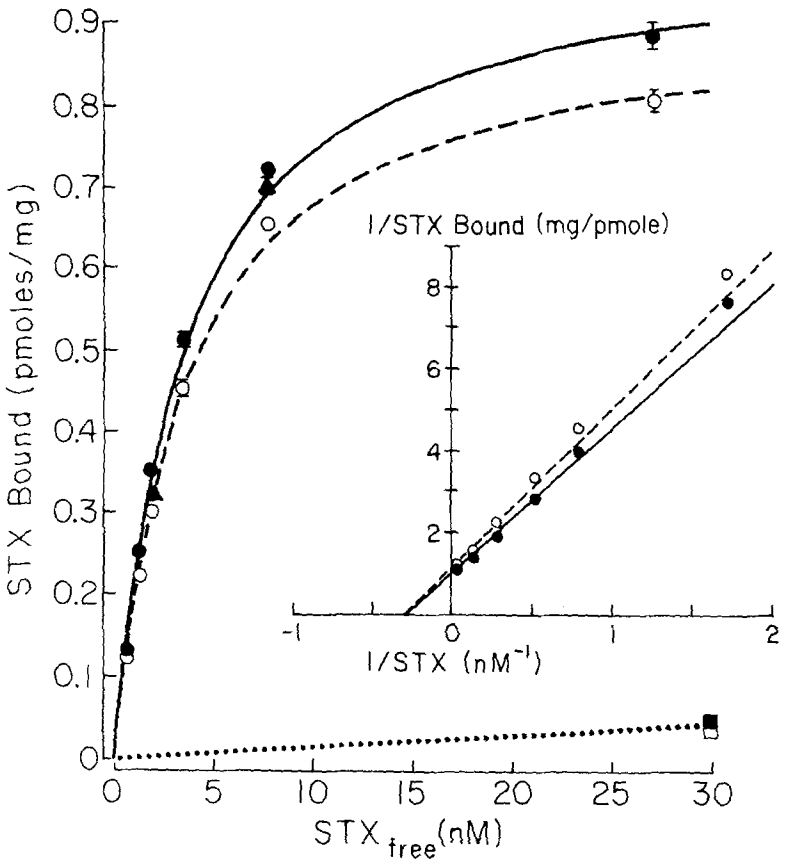


Fig. 2. Binding of ^{3}H-STX to depolarized intact synaptosomes. Synaptosomes were assayed for STX-binding as described in the legend to Fig. 1, except that the experiment was conducted at 30°. Prior to the addition of varying concentrations of ^{3}H-STX, the synaptosomes were incubated for 10 min in either *Na+5K* (●), *Na+5K*+1 μM bactrachotoxin+0.1 μM scorpion toxin (○), or 53 mM K^+, 97 mM Na^+ (▲). Three minutes after the addition of ^{3}H-STX, the synaptosomes were filtered as described in the legend to Fig. 1. (■, □): Binding in the presence of 10 μM TTX and either in the absence (■) or presence (□) of batrachotoxin plus scorpion toxin. The data shown are means of triplicate determinations; the error bars indicate the SEM's where they exceed the dimensions of the symbols. The curves shown are least-squares fits to the equation:

$$\text{STX bound} = B_m/(1+K_d/[\text{STX}])$$

where $B_m = 1.0$ pmol/mg and 0.9 pmol/mg for the solid and dashed curves, respectively. $K_d = 3.5$ nM for both curves. *Inset:* Same data shown as a double reciprocal plot

& Reuter, 1976). However, TTX binding to frog skeletal muscle was found to be independent of membrane potential (Almers & Levinson, 1975). Since the membrane potential of synaptosomes can be varied by changing the external K^+ concentration (Blaustein & Goldring, 1975), we were able to test directly the effect of membrane potential on STX

binding in this preparation. Binding of ^{3}H-STX to synaptosomes at 30° was determined using the filter method (Fig. 2). In the control condition (●) synaptosomes have membrane potentials of about −50 to −60 mV (inside negative: Blaustein & Goldring, 1975). In the presence of 53 mM K^+, the membrane potentials are about −10 to −15 mV (*c.f.* Blaustein & Goldring, 1975). This depolarization did not affect STX binding (▲). Synaptosomal Na^+ channels can be activated by several pharmacological agents including veratridine and batrachotoxin; moreover, the effects of these agents are potentiated by a polypeptide neurotoxin from the scorpion *L. quinquestriatus* (Catterall, 1976; Krueger & Blaustein, 1978; Blaustein, Ratzlaff, & Krueger, 1979). The combination of 1 μM batrachotoxin and 0.1 μM scorpion toxin maximally activates Na^+ channels and, in the presence of normal (145 mM) Na^+, depolarizes the synaptosomes (Krueger & Blaustein, 1978). In electrophysiological studies, both of these toxins also inhibit Na^+ channel inactivation (Koppenhöfer & Schmidt, 1968; Khodorov *et al.*, 1975; Okamoto, Takahashi & Yamashita, 1977). As shown in Fig. 2, STX binding was not significantly affected by the prior addition of batrachotoxin and scorpion toxin (○). This is consistent with the observation that neither batrachotoxin (2 μM) nor scorpion toxin (2 μM) affect STX binding to neuroblastoma cells in culture (Catterall & Morrow, 1978). Thus, STX binding to synaptosomes is independent of the membrane potential. Moreover, since excitable Na^+ channels in nerve are normally inactivated at membrane potentials less negative than −30 mV, STX binding would appear to be the same to Na^+ channels that are either closed (at normal "resting" potential), activated and depolarized (in the presence of batrachotoxin and scorpion toxin), or inactivated and depolarized (with K^+-rich media).

Binding of ^{3}H-STX to Rat Brain Membranes

An alternate membrane preparation procedure was developed in order to obtain a better yield of STX binding sites than was obtained with synaptosomes (10%). Rat brains were extensively disrupted using a Polytron homogenizer, and the homogenate was subjected to differential centrifugation (*see* Methods).

Representative data on the binding of ^{3}H-STX to the various subfractions of rat brain are given in Table 1. Binding was determined by the filter assay method at an STX concentration of 30 nM. More than 80% of the total STX binding sites in the crude homogenate were re-

Table 1. STX binding to subcellular fractions from rat brain

Fraction	STX binding (pmol/brain)	Recovery (%)	Sp act (pmol/mg protein)
Homogenate	201	100	1.5
P_1	7.2	4	0.4
P_2	28.9	14	1.1
P_3	138	69	4.7
Recovery in P_1–P_3	–	87	–

Rat forebrains (approximately 1.2 g each-wet wt) were homogenized using a Polytron tissue disrupter and were subjected to differential centrifugation as described under Methods. STX binding was determined by the glass fiber filter method. STX concentration was 30 nM. The values shown have been corrected for nonspecific binding (i.e., in the presence of 10 μM TTX) which was less than 5% of the total binding in each fraction.

covered in the three particulate fractions; 69% of this particulate binding activity was found in P_3. The $120{,}000 \times g$ supernatant was not assayed in the experiment of Table 1, but in other experiments was found to bind negligible amounts of STX (using the column assay). The specific activity of binding was enriched 3- to 6-fold in P_3 and ranged from 4 to 6 pmol/mg protein at 30 nM STX as compared to 1–2 pmol/mg in the crude homogenate and in synaptosomes (Fig. 1)

Binding of ^{3}H-STX to P_3 was saturable (Fig. 3*A*) with an apparent dissociation constant of 1–2 nM at 0°. Saxitoxin binding was also determined as a function of the STX concentration at pH 7.4 and at pH 5.5 (data not shown). Lowering the pH to 5.5 approximately doubled the apparent dissociation constant without significantly affecting the maximal binding. These results suggest that a titratable group with pK_a of 5–6 is associated with the STX binding site. Similar results have been reported by Weigele and Barchi (1978) in synaptosomes and by Henderson, Ritchie and Strichartz (1974) in garfish olfactory nerve and rabbit vagus nerve.

Column Assay of ^{3}H-STX Binding

In order to assay STX binding in detergent-solubilized preparations, it was necessary to utilize an assay method other than the filter method, since less than 20% of the STX binding activity in P_3 was retained on the glass fiber filters after the membranes were treated with Triton X-100 (data not shown). For this reason a cation exchange column assay procedure was developed that could be used to determine STX

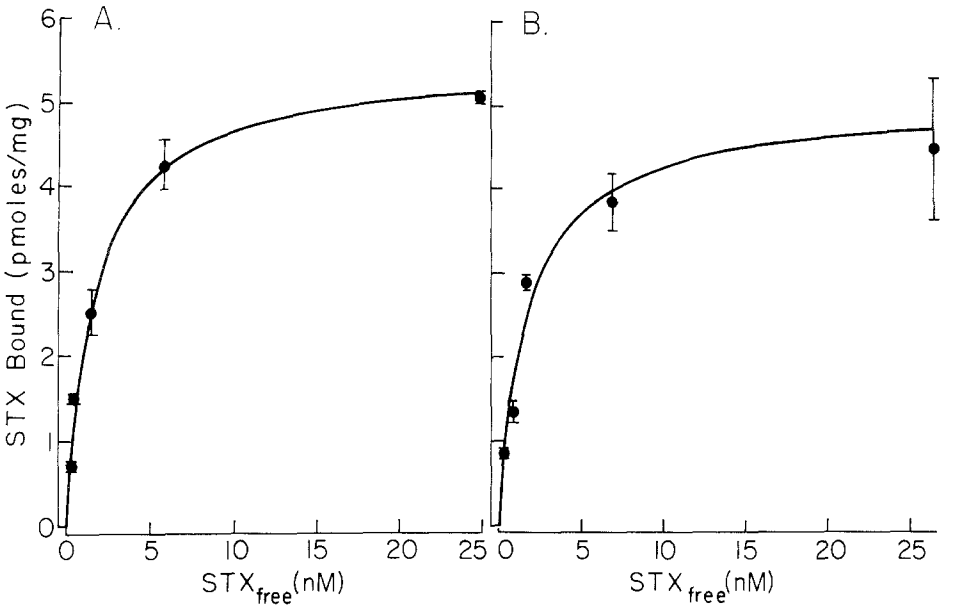


Fig. 3. Binding of ^{3}H-STX *vs.* STX concentration. (*A*): Binding to P_3 membranes (filter method). (*B*): Binding to Triton X-100 supernatant from P_3 (column method). Data were corrected for binding in the presence of 10 μM TTX. The error bars show ±SEM from triplicate determinations. The free concentration of STX was calculated as the total STX minus the bound STX. The curves were fit to the equation:

$$\text{STX bound} = B_m/(1 + K_d/[\text{STX}])$$

by the method of least squares. (*A*): $B_m = 5.5$ pmol/mg; $K_d = 1.8$ nM. (*B*): $B_m = 5.0$ pmol/mg; $K_d = 1.8$ nM

binding in both intact membranes and in solubilized preparations. Since the running time for each column is only about 15–20 sec, this method is useful for kinetic studies at temperatures up to 20° where the rate of dissociation of STX from its receptor is quite rapid.

Figure 4 shows data from an experiment in which STX binding (5 nM STX) was assayed by both filter and column methods. In the filter assay (Fig. 4*A*), more than 95% of the STX binding was "specific" (i.e., displaced by excess unlabeled TTX). In the column assay (Fig. 4*B*) there is a substantial blank (◐) because some ^{3}H not associated with STX passes through the columns, as does a small fraction of the free ^{3}H-STX. Nevertheless, when the counts in samples containing excess TTX (○) are subtracted from comparable samples without TTX (●), the net "specific" binding (▲) determined by the filter (Fig. 4*A*) and column (Fig. 4*B*) methods is similar. Addition of TTX did not affect the value of the blank in the absence of membranes. In the presence

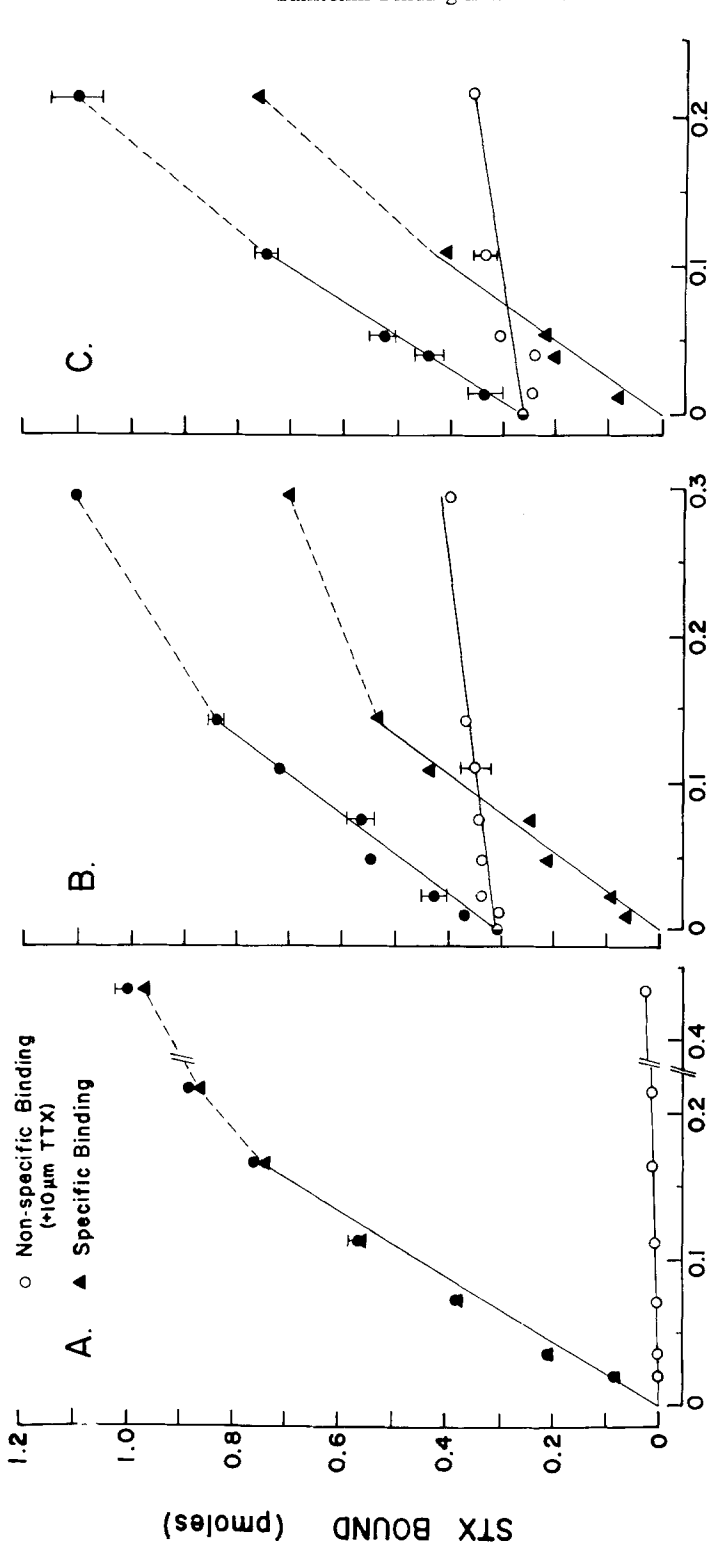


Fig. 4. Binding of ^{3}H-STX to P_3 from rat brain. (*A*): P_3 membranes assayed by glass fiber filter method. (*B*): P_3 membranes assayed by column method. (*C*): Triton X-100 supernatant of P_3 assayed by column method. (●) Total binding; (○) Binding in the presence of 10 μM TTX; (▲) "Specific binding", i.e., (total binding) minus (binding+TTX). The total STX concentration was 5 nM. The blank value (●) was unaffected by the presence of 10 μM TTX. Error bars show ±SEM from triplicate determinations when larger than the dimensions of the symbols. The data shown in *A*, *B*, and *C* are from the same membrane preparation. The lines were drawn by eye

of excess TTX, the addition of membranes caused only a small increase in the number of counts due to nonspecific binding to membranes that appeared in the column effluent.

The slopes of the difference curves (▲) correspond to specific activities of 4.4 pmol/mg (Fig. 4*A*) and 3.9 pmol/mg (Fig. 4*B*) for the filter and column assays, respectively. The curves appear to saturate at the higher protein concentrations because a significant fraction (30–50%) of the total ^{3}H-STX is bound to protein.

Assay of ^{3}H-STX Binding to Detergent-Solubilized Binding Sites

Figure 4C shows the binding of STX to Triton X-100-solubilized binding sites as a function of protein concentration, using the cation exchange column method. About 60–80% of the STX binding sites were solubilized when P_3 was incubated with 0.5% Triton X-100; the amount of STX bound was unaffected by the presence of 0.5% Triton X-100 (Table 2). About 80% of the protein was also solubilized under these conditions, so that there was no increase in specific activity. Increasing the Triton X-100 concentration from 0.5% to 4% did not increase significantly either the amount of protein or the number of binding sites solubilized. Effective solubilization of binding sites required that the ratio of Triton X-100 to protein (expressed as μl/mg) be 2 or greater. Thus, more than

Table 2. Solubilization of STX binding sites

Preparation	Assay method	Protein (mg)	STX binding (pmol ± SEM)	Sp act[a] (pmol/mg)
P_3	Filter	13.5	68.7 ± 2.0	5.1
P_3	Column	13.5	54.0 ± 2.1	4.3
P_3 + TX-100	Column	13.7	53.6 ± 1.3	4.2
TX-100 supernate	Column	9.8	33.4 ± 1.5	3.6
TX-100 pellet	Column	3.0	9.8 ± 0.8	3.5

STX binding was assayed by the filter and column methods as indicated. P_3 was solubilized with 0.5% Triton X-100 (TX-100) as described under Methods. Both the pellet and supernatant (120,000 × *g*; 30 min), as well as an aliquot of the membranes + Triton X-100 prior to centrifugation, were assayed. STX concentration was 5 nM. All data have been corrected for the amount of binding in the presence of 10 μM TTX. Binding assays were performed in duplicate (filter) or triplicate (column), and protein determinations were performed in duplicate. Temperature = 5 °C.

[a] These values represent the specific binding at 5 nM STX where only 60–70% of the available sites are occupied (*see* Fig. 3).

80% of the STX binding sites were solubilized in 0.25% Triton X-100, but only when the membrane protein concentration was reduced to 1.25 mg/ml (data not shown).

Solubilization of protein from P_3 by 0.5% Triton X-100 was relatively nonspecific, as indicated by analysis of the particulate and soluble fractions by SDS-polyacrylamide gel electrophoresis (data not shown). Most of the polypeptides in P_3 appeared quantitatively in the Triton X-100 supernatant. Three polypeptides in the 15,000 mol wt range were resistant to Triton X-100 and remained in the pellet.

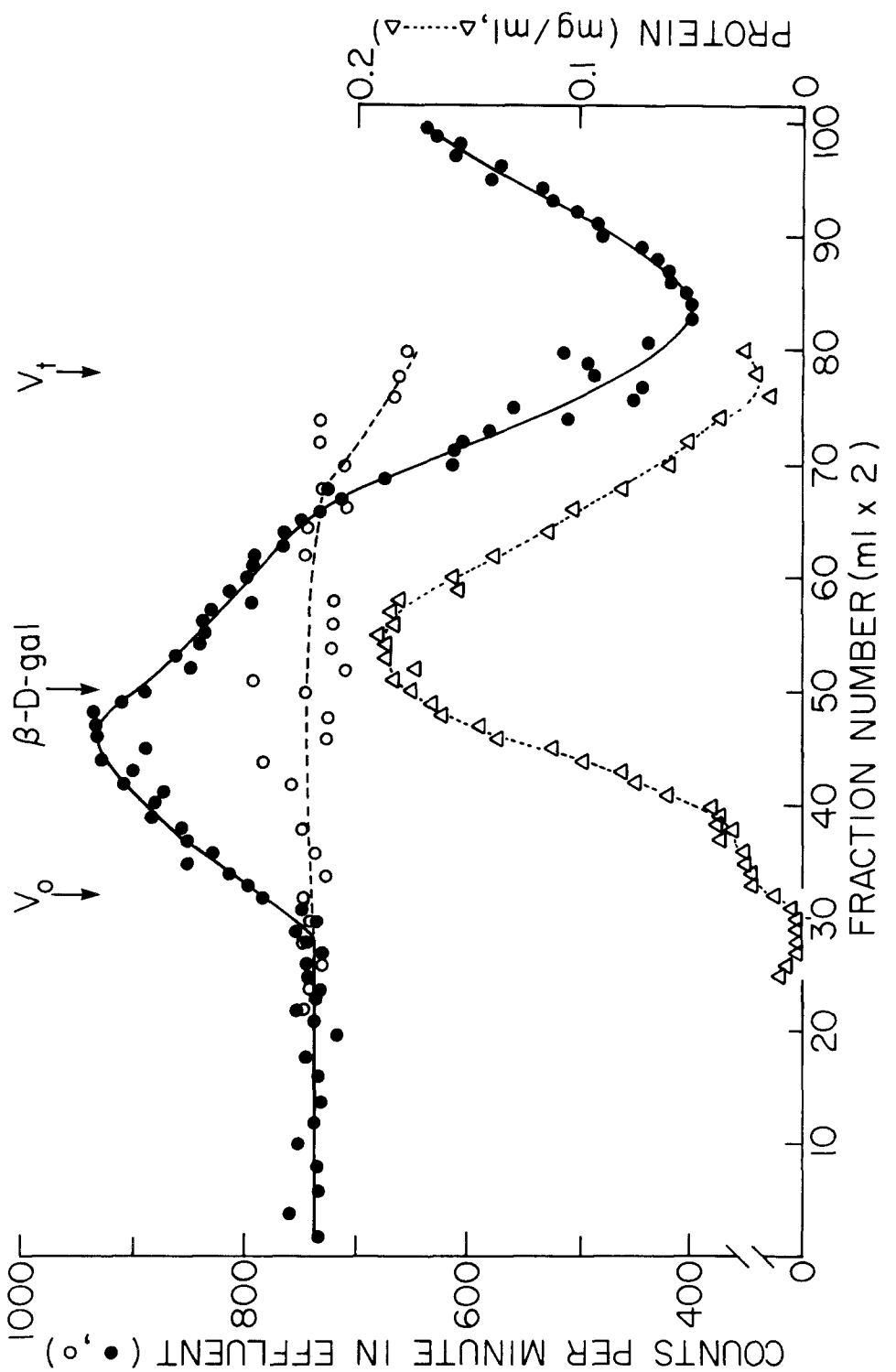
Gel Filtration of Detergent-Solubilized STX Binding Sites

Saxitoxin binding sites were recovered in the $120{,}000 \times g$ supernatant after incubation of P_3 with the non-ionic detergent Triton X-100 (Table 2). It is possible that the detergent treatment did not truly solubilize the binding sites but only disrupted the membranes into very small fragments that did not sediment at $120{,}000 \times g$. To examine this possibility, detergent-solubilized P_3 was applied to a gel filtration column (Sepharose CL 6B) that had been equilibrated with 1.2 nM ^{3}H-STX (Fig. 5). Binding activity (●) was retained on the column, eluting at a position slightly ahead of β-D-galactosidase (mol wt 540,000). The STX binding activity eluted earlier than the bulk of the solubilized protein (△). When the Triton X-100 supernatant was heated to 50 °C prior to gel filtration (○), no peak of STX binding was observed. Thus, the STX binding sites behave on the gel filtration column like a macromolecular species of mol wt 600,000 to 800,000.

Fractions containing binding activity from a similar column were assayed for STX binding by the cation exchange column method. Although a small amount of TTX-sensitive STX binding activity was observed in the eluted fractions corresponding to the peak of ^{3}H-STX binding (*see* Fig. 5), very little of the original solubilized binding activity was recovered from the column. This indicates that the activity was unstable under these conditions. When the soybean phospholipids were omitted from the elution buffer, no peak of STX binding activity was observed.

Determination of STX Equilibrium Dissociation Constant

The binding of ^{3}H-STX to both intact membranes (P_3) and solubilized binding sites was saturable (Fig. 3). STX binding to nonsolubilized P_3


PROTEIN (mg/ml, △-----△)
0.2
0.1
0
100
90
80
70
60
50
40
30
20
10
FRACTION NUMBER (ml x 2)
COUNTS PER MINUTE IN EFFLUENT (●, ○)
1000
800
600
400
0
V_t
β-D-gal
V_o

was determined by the filter method (Fig. 3*A*) and by the column method (data not shown). Both methods gave similar results. Binding to detergent-solubilized P_3 (using the same P_3 as in Fig. 3*A*) was assayed by the column method (Fig. 3*B*). Half-maximal binding occurred at about 1.8 nM in both preparations, indicating that the affinity of STX binding was unchanged by solubilization.

The apparent dissociation constants for STX binding to both intact P_3 membranes and solubilized membrane protein were determined as a function of temperature from 0 to 10°. In intact P_3 the dissociation constant was observed to be 1.2–1.8 nM at 0°, 2.2–2.6 nM at 5°, and 2.7–3.5 nM at 10°, suggesting a Q_{10} of about 2. In intact synaptosomes the apparent STX dissociation constant was 0.6–0.7 nM at 0° (Fig.1) and 3.5 nM at 30° (Fig. 2), indicating a Q_{10} of about 1.8; this is comparable to the Q_{10} of 1.5 reported by Weigele and Barchi (1978). The dissociation constant for STX binding to Triton X-100 solubilized binding sites ranged from 1.3 to 1.8 nM but was not observed to vary consistently with temperature from 0 to 10°.

Determination of Association and Dissociation Rate Constants

The rate of dissociation of the STX-receptor complex was determined in both intact membranes (P_3) and in Triton X-100 solubilized preparations (Fig. 6). The off-rate constant (k_{-1}) was found to be about 0.1 min^{-1} in intact membranes and about 0.2 min^{-1} in solubilized membranes at 0°. The STX dissociation rate constant was determined to be about 0.2 min^{-1} in intact synaptosomes at 0° using the filter method (data not shown).

The dissociation rate constant for P_3 membranes and solubilized STX binding sites was determined as a function of temperature from

Fig. 5. Gel filtration chromatography of STX binding sites. The Triton X-100 supernatant from P_3 was prepared as described under Methods. An aliquot of this supernatant (1 ml) was applied to a Sepharose CL 6B column (0.9 × 53 cm) that had been equilibrated with a solution containing 150 mM NaCl, 20 mM Tris-HEPES, 1 mM $MgCl_2$, 1.2 nM ^{3}H-STX, 0.1% Triton X-100, and 0.025% soybean phospholipids at 4°. The column was eluted at 5.5 ml/hr and 0.5 ml fractions were collected. The amount of ^{3}H in 50 μl of each fraction was determined by liquid scintillation counting (●, ○). (●), control Triton X-100 supernatant; (○), Triton X-100 supernatant that was heated to 50 °C for 5 min, prior to chromatography. (△), Protein, determined by the method of Peterson (1977). V_o is the void volume of the column determined with blue dextran; V_t is the total volume of the column; *β-D-gal* is the position at which the enzyme β-D-galactosidase eluted on the same column

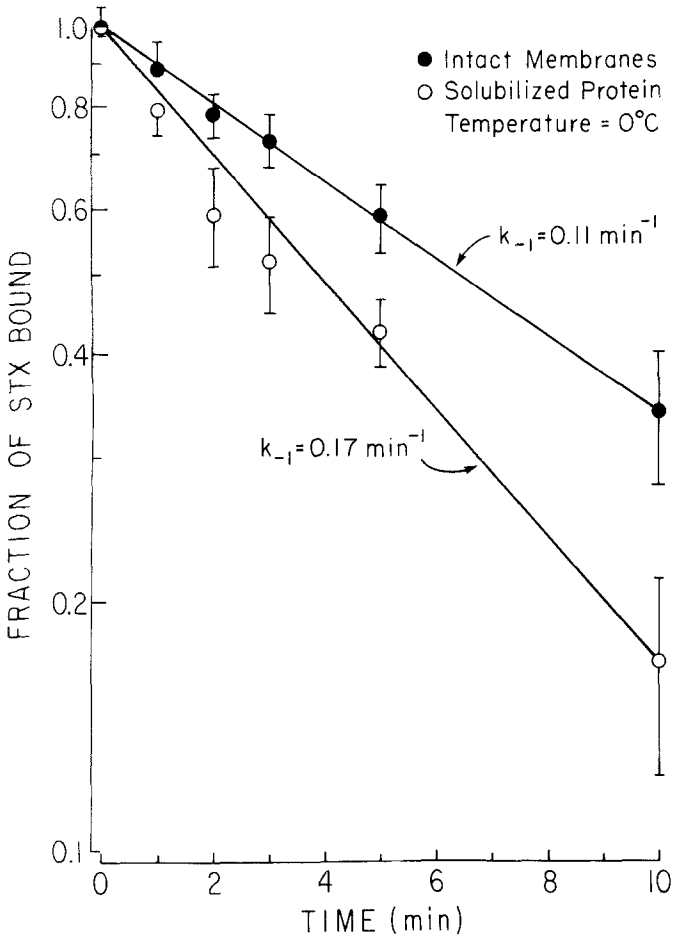


Fig. 6. Determination of STX-dissociation rate constant. Rat brain membranes (P_3, ●) or Triton X-100 solubilized membranes (○) (about 1.2 mg in 0.25 ml) were incubated with 1 nM ^{3}H-STX for 30 min at 0°. A large excess (10 μM) of unlabeled TTX was added, and the incubation was continued at 0°. At the indicated times, aliquots (0.2 ml) were assayed for bound STX by the column method. The data are plotted as the logarithm of the fraction of STX bound *vs.* time. The off-rate constants k_{-1} were determined as the slopes of the least-squares fit of the data to the equation:

$$\log_e (\text{fraction bound}) = k_{-1} \cdot t.$$

The data shown are the means (±SEM) of quadruplicate determinations

0–10° (Fig. 7). Higher temperatures were not studied because the dissociation rate became too fast to be determined accurately in solubilized preparations with the column method. The dissociation rate constant increased markedly with temperature in both particulate and soluble

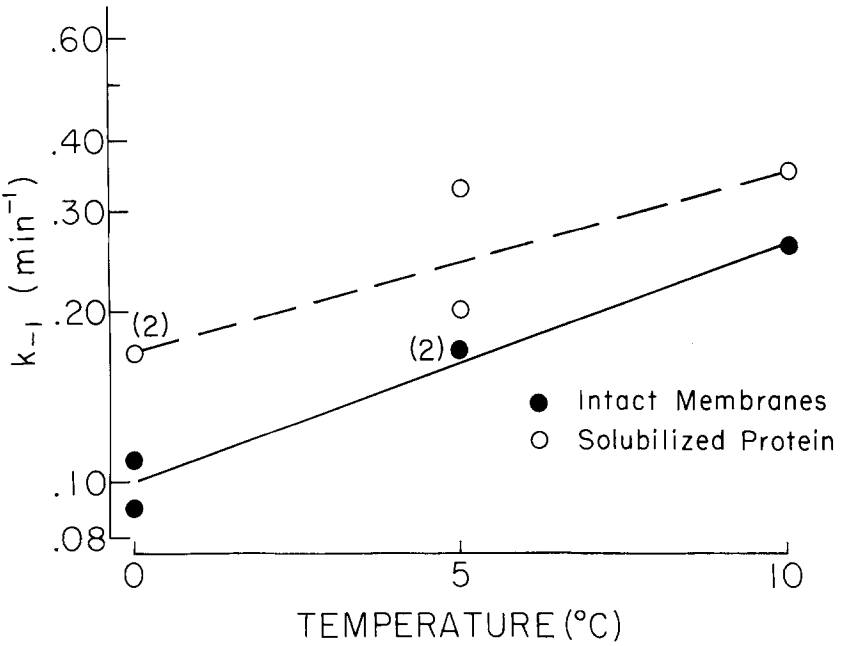


Fig. 7. Temperature dependence of STX-dissociation rate constants. The off-rate constants for rat brain membranes (P_3, ●) and Triton X-100-solubilized membranes (○) were determined as a function of temperature as described in the legend to Fig. 6. Each data point is the result of an individual experiment. *(2)* indicates that the same value was obtained in two separate experiments. The lines were drawn by eye

preparations; the estimated Q_{10}'s were about 2.5 and 2.0 for particulate and solubilized preparations, respectively.

The rate of STX binding was too fast to be resolved with the column method in solubilized preparations even at 0°. However, the association (on-rate) constant, k_{+1}, could be determined at 0° for the particulate fraction (P_3) using the filter method (Fig. 8). The binding of STX to P_3 followed an exponential time course with the apparent on-rate constant k'_{+1}, being proportional to the STX concentration from 1 to 10 nM. These apparent pseudo-first-order rate constants are related to the true association rate constant k_{+1} according to the relationship (Hill, 1909):

$$k'_{+1} = k_{+1} \cdot [\mathrm{STX}] + k_{-1}. \tag{1}$$

The true on-rate constant k_{+1} was determined to be about 6×10^8 M^{-1} min^{-1} (Fig. 8). The on-rate constant in intact synaptosomes at 0° was determined to be about 5×10^8 M^{-1} min^{-1} (data not shown).

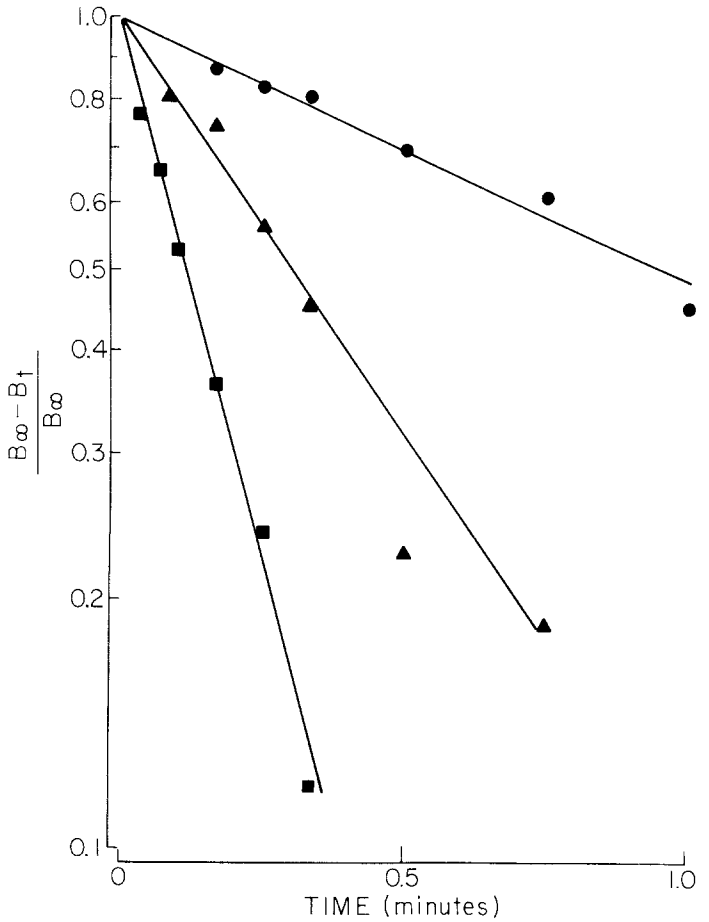


Fig. 8. Determination of STX-association rate constant. Rat brain membranes (P_3) were incubated at 0° with ^{3}H-STX (1.1 nM, ●; 3.3 nM, ▲; or 10 nM, ■) for the indicated times and were then assayed for ^{3}H-STX bound by the filter method. The value on the ordinate represents the fraction of unoccupied sites at time t. B_∞ at each concentration was the amount of ^{3}H-STX bound after 30 min. The data points are the means of triplicate determinations. The lines are least-squares fits of the data to the equation:

$$\log_e \frac{B_\infty - B_t}{B_\infty} = k'_{+1}\, t.$$

The on-rate constant k_{+1} was calculated from the equation:

$$k'_{+1} = k_{+1} \cdot [\mathrm{STX}] + k_{-1}$$

where k_{-1} was 0.11 min^{-1} as determined in Fig. 6. The following parameters were obtained:

[STX]	k'_{+1}	k_{+1}
1.1 nM	0.78 min^{-1}	6.1×10^8 M^{-1} min^{-1}
3.3 nM	2.4 min^{-1}	6.9×10^8 M^{-1} min^{-1}
10.0 nM	6.1 min^{-1}	6.0×10^8 M^{-1} min^{-1}

In this experiment the protein concentration was reduced to 0.05 mg/ml so that the free STX concentration would change by less than 10% during the course of the experiment.

Discussion

Kinetics of ^{3}H-STX Binding

The kinetic parameters for the interaction of STX and TTX with Na^+ channels in synaptosomes obtained in these experiments are in general agreement with those reported in other preparations. Representative values for excitable tissues other than muscle (but including electroplax) are given in Table 3. The apparent dissociation constant for both STX and TTX is in the range of 1–10 nM. If correction is made for a substantial temperature dependence of the dissociation rate constant k_{-1} ($Q_{10}=2$–3, Weigele & Barchi, 1978; Schwarz, Ulbricht & Wagner, 1973; this paper) the values for k_{-1} for both STX and TTX are similar in a variety of excitable membranes. Determination of the association rate constant k_{+1} was made less frequently and is probably of the order of 10^9 M^{-1} min^{-1} except in the squid axon (Cuervo & Adelman, 1970) where Schwann cells may present a significant diffusion barrier to TTX and STX (Keynes *et al.*, 1975).

Table 3. Kinetic constants for STX and TTX binding

Preparation	Temp.	K_d (nM)	k_{-1} (min^{-1})	k_{+1} (M^{-1} min^{-1})	Reference
Saxitoxin					
Synaptosomes	0°	0.5	0.25	–	Weigele & Barchi, 1978
Synaptosomes	20°	1.1	2.1	–	Weigele & Barchi, 1978
Synaptosomes	0°	0.7	0.2	5×10^8	This paper
Rat brain membranes	0°	1.8	0.1	6×10^8	This paper
Solubilized rat brain membranes	0°	1.8	0.2	–	This paper
Frog node [a]	16°	1.7	1.1	6×10^8	Wagner & Ulbricht, 1975
Tetrodotoxin					
Xenopus node [a]	20°	3.6	0.9	2×10^9	Schwarz *et al.*, 1973
Squid giant axon [a]	7°	3.3	0.12	2×10^7	Cuervo & Adelman, 1970
Electroplax	4°	6.0	0.7	–	Reed & Raftery, 1976
Crab nerve membranes	24°	2.9	0.1	–	Balerna *et al.*, 1975
Solubilized garfish nerve	20°	6.0	1.0	–	Henderson & Wang, 1972
Solubilized electroplax	0°	1.1	0.024	–	Agnew *et al.*, 1978

[a] Determined by blockade of Na-currents

An interesting and unexplained discrepancy arises in the present study when the values of K_d, k_{-1}, and k_{+1} in rat brain membranes (P_3) are compared. For the simple reaction scheme:

$$\mathrm{STX} + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} \mathrm{STX} \cdot R \tag{2}$$

where R is the STX binding site, the kinetic parameters should be related by the equation:

$$K_d = \frac{k_{-1}}{k_{+1}}. \tag{3}$$

The values that we obtained for k_{-1} and k_{+1} would predict that that K_d at 0° should be about 0.2 nM as compared to the observed value of 1–2 nM. This discrepancy was found in each of three experiments in which all three parameters were determined. It is possible that this discrepancy is the result of artifact in the filter assay procedure, although it would be expected that such an experimental artifact would result in a determined on-rate-constant (k_{+1}) that is *smaller* than the true constant. Our results, however, reveal an on-rate constant that is *larger* than would be expected from the equilibrium dissociation constant and the off-rate constant. Another possible explanation for this result is that the STX binding site undergoes a slow conformational change after the initial binding of the ligand, so that, in on-rate determination experiments, STX binding is to a different conformational form of the binding site than that from which dissociation is measured. Wagner and Ulbricht (1975) reported that the experimentally-determined equilibrium dissociation constant (K_d) was equal to the ratio of the off- and on-rate constants (k_{-1}/k_{+1}), suggesting a simple bimolecular reaction (Table 3, line 6). It should be mentioned, however, that these investigators were measuring the Na^+ conductance and not STX binding. A smaller (twofold) discrepancy between calculated and determined on-rate constants was observed in intact synaptosomes at 0° (*see* line 3, Table 3).

An advantage of the intact synaptosome preparation is that the synaptosomes are physiologically competent and exhibit a number of the functional properties of intact nerve terminals (*c.f.* Blaustein, 1975; Bradford, 1975; Blaustein, *et al.*, 1977). Thus, STX binding can be directly compared to the STX block of functional Na^+ channels in the same preparation under identical conditions. We have found (*manuscript in preparation*) that STX blocks the ^{22}Na influx through batrachotoxin-activated Na^+ channels with a K_i that is similar to the K_d observed for STX

binding to synaptosomes under the same conditions (about 4 nM at 30 °C, *see* Fig. 2 this paper).

We also examined the binding of ^{3}H-STX to intact synaptosomes that were depolarized by elevated external K^+ as well as to synaptosomes that were treated with batrachotoxin and scorpion toxin (Fig. 2). Neither of these treatments significantly altered STX binding, which implies that STX binds equally well to closed, open, and inactivated channels.

Solubilization of STX binding Sites

STX binding sites were solubilized from P_3 by the non-ionic detergent Triton X-100. In the presence of the detergent, the STX binding sites remained in the supernatant after centrifugation at $120{,}000 \times g$ for 30 min and the STX binding activity was retained on a gel filtration column (Sepharose CL6B, Figure 5), eluting slightly ahead of the enzyme β-D-galactosidase (mol wt 540,000). These results indicate that, in the presence of Triton X-100, the STX binding sites are associated with structures of molecular dimensions rather than with small membrane fragments.

An important consideration for the purification of STX binding sites, as well as for a kinetic analysis of STX binding to solubilized binding sites, is the availability of a convenient assay for STX binding that can be used once the membrane proteins have been solubilized. In this paper we describe a cation exchange column method that can be used to assay both particulate and detergent-solubilized STX binding sites (Fig. 4*B* and *C*). Kinetic studies using the column method indicate that the dissociation constant for STX binding is 1.8 nM at 0° for both particulate and soluble binding sites. The dissociation rate constant k_{-1} is only slightly larger in the soluble preparation than in the original membranes. This indicates that the STX binding sites from rat brain membranes appear to be largely unchanged following solubilization. Moreover, the observation that more than 50% of the STX binding sites in the whole rat brain homogenate are recovered in solubilized form, indicates that this preparation may be a very useful starting point for the purification and characterization of STX binding sites from the mammalian central nervous system.

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