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Structure of the Saxitoxin Binding Site at Sodium Channels in Nerve Membranes

Exchange of Tritium from Bound Toxin Molecules

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SUMMARY

The exchange of tritium into water from saxitoxin molecules that were radiolabeled at the C-11 methylene position was measured at 37° in solution and in suspensions of brain membranes. High concentrations of membrane receptors were used to assure that more than 80% of the total saxitoxin (STX) present was specifically bound. The amount of back-exchanged tritium was determined either by measuring the radioactivity remaining in the STX, using a second binding assay, or by measuring the tritium in water using ion-exchange chromatography. The results show that the back-exchange is accelerated in the presence of the membranes, and that this is attributable solely to the nonspecific toxin binding. Little change in the back-exchange rate over that in solution occurs in specifically bound toxin molecules. These results place certain restrictions on the possible bonds and configurations of receptor-toxin complexes.

INTRODUCTION

The sodium ion channels in most excitable cells are specifically blocked by the natural agents TTX[1] and STX. These two toxins bind reversibly to a common receptor at the outer surface of the membrane in a one-to-one stoichiometry (1–5). The toxins reduce the current through the membrane without changing either the kinetics or the voltage dependence of the remaining permeability, and it appears that the configurational changes associated with the opening of sodium channels continue to occur normally in membranes completely blocked by TTX (6). The simplest interpretation of all of these results is that TTX and STX prevent ion fluxes through otherwise normally functioning channels by occluding the channel pore somewhere near its external opening.

Interest in the structure of the sodium channel has led to extensive study of the molecular properties of the binding site for TTX/STX. Both toxins are positively charged at pH 7, by virtue of guanidinium groups which are intrinsic parts of the molecules (7, 8), and both molecules contain many hydroxyl and amino groups on their surfaces which are potential hydrogen bond donors. Several hypotheses for a mechanism of toxin action have been proposed in which a guanidinium group on the toxins is positioned in the outer opening of the channel, as a permeant guanidinium ion would be in its passage through the channel pore, while the rest of the molecule prevents its further passage, forming reversible bonds with the channel structure (9, 10). In these models the toxin receptor is envisioned as part of the essential functional structure of the channel.

However, this intimate identity between receptor and channel pore has been disproven by more recent experiments. For one, chemical modification of sodium channels can render them insensitive to the toxins while causing only minor changes in their ion permeation properties (11, 12); and sodium channel permeabilities that are catalyzed by drugs and have a different ion selectivity than those activated by membrane potential still have the same sensitivity to TTX (13, 14). Biological modifications of sodium channels also occur. In mammalian skeletal muscle, denervation is followed in 6–7 days by the appearance of toxin-resistant channels which are otherwise almost identical with normal, toxin-sensitive channels in their ion selectivity and voltage-dependent functions (15). These results imply that the toxin receptor requires specific groups which are only superficially involved in determining the permeability of normal channels.

Despite the existence of several molecular hypotheses and the detailed results of toxin binding and electrophys-

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[1] The abbreviations used are: TTX, tetrodotoxin; STX, saxitoxin; MOPS, morpholinopropanesulfonate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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iological experiments, there is little direct evidence about the bonds which are formed between toxin molecules and receptors. Structure-potency studies have been reported for TTX (16) and for STX (17, 18), but assignments of essential groups from these experiments are complicated by the interactions among different parts of the toxin molecules. In some specific hypotheses the receptor structure was deduced by comparing analogous portions of the TTX and STX molecules, which were made to form the same bonds with their common receptor. However, recent results show that the physical-chemical properties of the TTX-receptor complex differ markedly from those of the STX-receptor complex. For example, the temperature dependence of the TTX affinity for mammalian skeletal muscle is about 3 times that of the STX affinity (19), and the blocking action of STX on sodium channels in frog nerve is selectivity enhanced in ^3H_2O solvents which have no effect on the action of TTX (20). Thus, it is unlikely that the bonds from the receptor to TTX are identical with those to STX, and arguments based on analogous bonding patterns must be tentative.

This paper is a study of the configuration of STX bound to its receptor. The experiments measure the accessibility to the bathing solution of identified, exchangeable hydrogens on the bound toxin molecule. Saxitoxin exists in solution in an equilibrium mixture of gem-diol and keto forms at the C-12 position (Fig. 1). The carbonyl group at C-12 participates in a keto-enol tautomerism with carbon atom 11, thus permitting a slow exchange of hydrogen atoms from solution into the methylene group at C-11 (21). Tritium is incorporated into STX by this reaction, during incubation in tritiated water (22). Likewise, tritium will exchange off the C-11 group under conditions which favor the keto form of the toxin in a configuration where this group is accessible to the water of the solution. In the experiments reported here the rates of back-exchange of tritium from [^{3}H]STX are compared in solution and when [^{3}H]STX is bound specifically and nonspecifically to nerve membranes. The results show that the rate of back-exchange is accelerated by nonspecific binding but is not significantly affected by specific toxin binding.

METHODS

The strategy for the experiments reported here is diagrammed in Fig. 2. Tritiated saxitoxin ([^{3}H]STX) was incubated for up to 20 hr at 37° or 44° in solution alone,

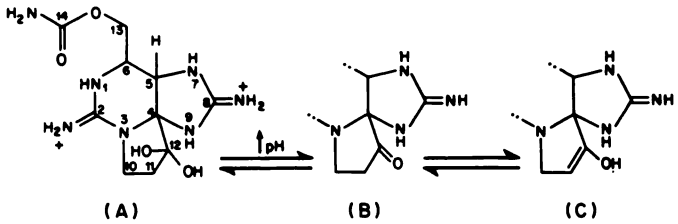

FIG. 1. *Structure of saxitoxin*

The hydrated ketone, or gemdiol, (A) is the predominant form at neutral pH. At alkaline pH, more of the unhydrated ketone (B) occurs which participates in a rapid keto-enol tautomerism ($B \rightleftharpoons C$) catalyzing the exchange of hydrogens into the C-11 nucleus. The portion of the molecule to the *left* of N-3 and C-5 remains essentially unchanged for pH values below 10.

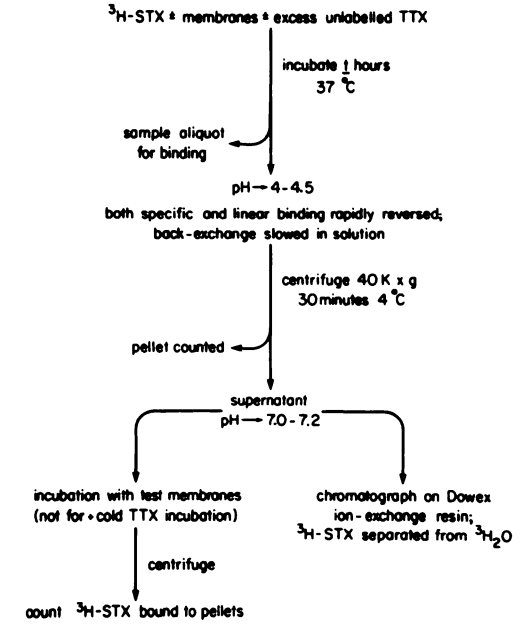

FIG. 2. *Flow diagram outlining the steps followed to measure the back-exchange reaction (see Methods for details)*

and with or without brain membranes. A high membrane content (1.4–1.9 mg) of protein was used to ensure that at equilibrium about 80% of the total [^{3}H]STX was bound in a saturable manner to sodium channel receptors. There was always an additional, small, nonsaturable component of [^{3}H]STX binding; when high concentrations of unlabeled STX or TTX were also present, the saturable binding of [^{3}H]STX was abolished and only the nonsaturable binding remained (see Fig. 3). Thus, the concentrations of labeled and unlabeled toxins and of receptors could be manipulated to produce either no binding, a high degree of saturable binding with little nonsaturable binding, or only nonsaturable binding.

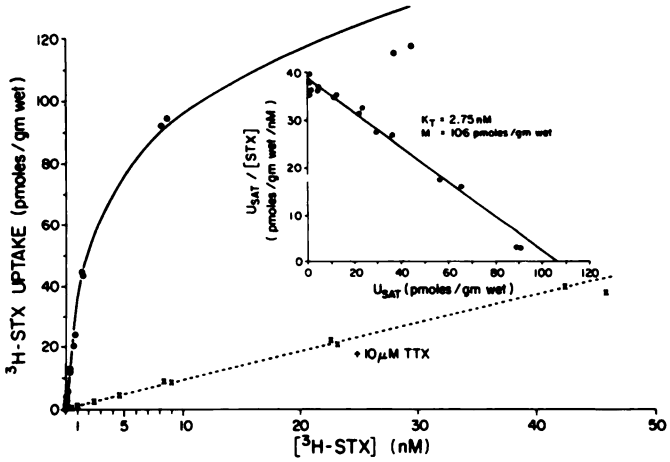

FIG. 3. *Uptake of [^{3}H]STX by brain membranes*

Equilibrium binding was measured in the absence (●) or presence (×) of unlabeled TTX to give respectively, the total and the linear uptake of [^{3}H]STX, divided by the equivalent mass of unhomogenized wet brain tissue. The difference between these two curves represents the saturable STX binding (U_{SAT}) and is graphed as a Scatchard plot in the *inset*. The *line* in the *inset* is a least-squares fit to the data points and is described by the equation $U_{SAT}/[STX] = (M - U_{SAT})/K_T$, where M is the STX receptor density and K_T is the equilibrium dissociation constant for STX binding.

After a measured incubation time, a small aliquot of the incubation suspension was sampled to determine the extent of toxin binding, and the toxin was then freed from the receptor at 4°. This was accomplished by transiently reducing the pH to 4.0–4.5 by the addition of 5 N HCl, centrifuging the membranes at 40,000 × *g* for 30 min, removing the supernatant, and returning its pH to 7.0–7.2 by addition of 3 N NaOH. Acidification leads to reversal of greater than 95% of [^{3}H]STX binding within 20 min (see Fig. 4) and actually slows the rate of back-exchange of tritium relative to that at pH 7.2. [Slowing results because exchange probably occurs at measurable rates only in the keto form of STX, and essentially none of the keto form exists at acid pH (7, 8).] All samples were acidified, even those without membranes, and the pH was monitored with a glass electrode during additions of acid and base.

The extent of back-exchange from the recovered STX was measured in two ways. In the first, the recovered toxin solution was equilibrated with a suspension of fresh brain membranes and the uptake of radioactivity was compared with that from an identical aliquot of [^{3}H]STX which either had been incubated at 0° or had been freshly diluted from frozen stock solutions. No significant difference between these latter two preparations could be detected; they produced uptakes which agreed within 5%. The back-exchange reaction is quite dependent on temperature (23); the half-time of exchange at 4° is >100 hr, and that is why the experiments reported here were conducted at or above 37°.

In the second procedure, the amount of tritium which had exchanged into water was directly assayed by column chromatography. The supernatant was loaded onto a cation exchange column (Dowex 50), which bound all of the toxin molecules and permitted rapid elution of the labeled water. This second method was essential for the samples in which unlabeled TTX or STX was used, since these would interfere with any binding of [^{3}H]STX to specific receptors of test membranes; thus, most of the experiments were conducted using the ion-exchange resin.

The recovery of toxin, as opposed to radioactivity, from the membrane incubation suspension was determined by a bioassay method. The degree of block of the compound action potential in a frog (*Rana pipiens*) desheathed sciatic nerve was compared for toxin solutions before the addition of membranes and after an 8-hr incubation with membranes followed by an acid wash, centrifugation, and neutralization (see Fig. 2 and preceding Methods). The inhibitions were identical within the reproducibility of the measurement, ±8%; the nominal STX concentration of 5 nM reduced the height of the action potential by 25–41% (the sensitivity of nerves varies among preparations), and the unincubated toxin was as active as the solution recovered from the membranes after incubation.

Membrane preparation. Membranes were prepared from fresh or frozen rabbit (2–3.5 kg New Zealand White) brain; toxin binding properties were unaffected by freezing. A whole brain or some fraction thereof, 2–7 gm in weight, was minced with scissors and then homogenized in 30 ml of ice-cold 0.25 M sucrose, 50 mM MOPS (pH 7.2) in a Virtis homogenizer for 10 sec at 4,000 rpm. The fragmented membranes were centrifuged at 40,000 × *g* for 30 minutes at 4°, the supernatant was decanted, and the membranes were resuspended in 20–30 ml of Locke's solution. Uniform resuspension was achieved by homogenizing the membranes in a glass-Teflon Potter-Elvehjem homogenizer with six to eight strokes at 1,000 rpm. The final membrane homogenate contained 1.6–2.4 mg of protein per milliliter.

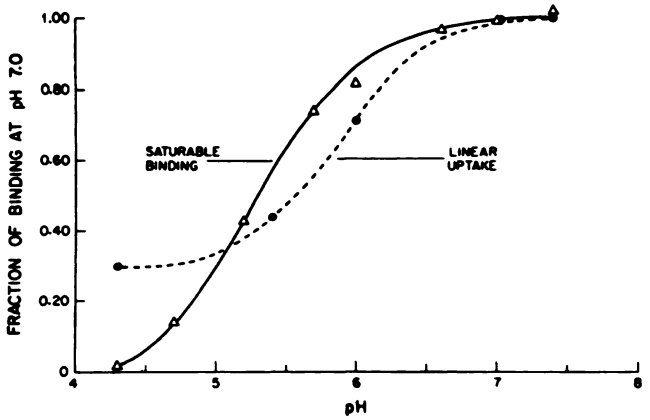

FIG. 4. *Fractional decrease of saturable and linear [^{3}H]STX uptake as the pH is lowered from 7.4*

The points are the mean of two measurements for each condition with the nominal [^{3}H]STX concentration at 3 and 4 nM.

Binding measurements. Brain membranes were incubated for 90 min at 4–6° in a solution containing differing concentrations of [^{3}H]STX, with or without unlabeled TTX. The latter conditions abolished saturable STX binding, leaving only a component of uptake proportional to [^{3}H]STX, i.e., linear binding. At the end of 90 min the membranes were pelleted by a 1-hr centrifugation at 40,000 *g*. The supernatant was decanted and its radioactive content sampled to determine the free [^{3}H]STX. Pellets were dislodged from the tubes following the addition of a 5% sodium lauryl sulfate solution and placed in 7-ml scintillation vials to which was added 0.2 ml of tissue solubilizer (Protosol, New England Nuclear Corporation, Boston, Mass.). The membranes were then digested in tightly capped vials by heating to 60° for 8–12 hr. After cooling to room temperature, 5 ml of scintillant (Aquasol-2, New England Nuclear Corporation, or Dimiscint, National Diagnostics, Somerville, N. J.) were added and the radioactivity was measured in a liquid scintillation counter. Counting efficiencies were determined using samples containing no [^{3}H]STX, by the addition of a calibrated 100 μl of ^3H_2O. Supernatants (100 μl) were also counted in 5 ml of scintillant and their efficiencies likewise measured.

[^{3}H]STX. Saxitoxin was tritiated by the method of Ritchie *et al.* (22). The STX was exposed to 25 Ci of ^3H_2O by New England Nuclear Corporation for 3 hr at 50°. Following removal of the ^3H_2O, the rapidly exchangeable tritium nuclei were washed out by water at 10°. The resulting labeled STX was further purified by high voltage paper electrophoresis and its concentration determined by the bioassay procedure noted above. Paralytic shellfish poison (Food and Drug Administration, Cincinnati, Ohio) was used as the standard in this bioassay. The purity of the toxin was determined by the method of Levinson (23) and ranged from 76% to 89% for the three

[^{3}H]STX preparations used. The true specific radioactivity of the toxins ranged from 8.2 to 11.8 Ci/mmole.

Preparation and operation of ion-exchange columns. Dowex resin (20 g of 50X2-100 beads) was washed three times in distilled water to remove fine particles, washed in 0.5 liter of 1 M NaOH, and washed again in distilled water, four times. Beads were then mixed in 200 ml of "Na buffer" containing 150 mM NaCl, 20 mM Hepes (pH 7.4), and bovine serum albumin (0.1 mg/ml). A small (8 mm × 4 mm) column was packed in a disposable plastic syringe and the column was pre-equilibrated with 20 ml of a low ionic strength solution containing 0.5 M glucose and 10 mM MES buffer (pH 6.0). The substance to be chromatographed, be it ^3H_2O or [^{3}H]STX, was diluted in this glucose-MES solution, loaded onto the column, and eluted first with approximately 30 ml of glucose-MES, followed by a high ionic strength solution of 0.5 M KCl or NaCl and 10 mM MES (pH 6.0). Fractions were collected by drop counting using a Gilson Microfractionator. A small degree of ^{3}H back-exchange from STX was produced by the column itself at room temperature, so all [^{3}H]STX chromatography was conducted at 4°. Duplicate and sometimes triplicate measurements were always made, and these agreed with each other within 10%.

Solutions and chemicals. Incubations were performed in a mammalian Locke's solution of the following composition: NaCl, 0.154 M; KCl, 0.0056 M; $CaCl_2$, 0.0022 M; Hepes buffer, 0.010 M, pH 7.2 at 37°. The buffer used was sometimes MOPS, at the same concentration and pH. Bioassays were conducted in frog Ringer's solution: NaCl, 0.110 M, KCl, 0.0025 M; $CaCl_2$, 0.002 M; MOPS, 0.005 M, pH 7.2 or 7.0.

Saxitoxin was obtained from the National Institutes of Health for labeling or from the Food and Drug Administration for use as a bioassay standard. TTX, MOPS, and Hepes were purchased from Calibiochem-Behring (La Jolla, Calif.). Dowex resin was purchased from Sigma Chemical Company (St. Louis, Mo.). All other salts and chemicals were of reagent grade.

RESULTS

Membrane fragments from rabbit brain have a high density of high-affinity STX receptors. The saturation of these receptors is shown by the curves in Fig. 3, which represent the equilibrium binding to membranes after a 90-min incubation period, since STX uptake is at 95% of its steady-state value after 1 hr of incubation. The *solid line* plots the total uptake of [^{3}H]STX against free toxin concentration; the *broken line* plots the uptake in the presence of unlabeled TTX at 200–1000 times the concentration of [^{3}H]STX which abolishes all saturable binding, leaving only the linear uptake. The difference between the two uptakes equals the saturable binding, which is graphed as a Scatchard plot in the *inset* of Fig. 3. Saturable binding is attributable to a class of high-affinity receptors with equilibrium dissociation constant (K_T) of 2.75 nM at 37° in Locke's solution.

The linear uptake of [^{3}H]STX results from a weak association of toxin with membranes and from trapping of toxin in the interstitial spaces of the membrane pellet. The size of the linear component was the same, regardless of the purity of the [^{3}H]STX, proving that linear uptake does not result from radiolabeled impurities. The weak binding to brain membranes probably involves some ionic interactions, since elevation of the divalent cation concentration of the incubation medium reduces this component of the linear uptake (24). An ionic interaction also is inferred from the results of Fig. 4 (described below), in which linear uptake is titrated by acidification of the incubation medium.

The density of high-affinity STX receptors in the incubation conditions of Fig. 3 is sufficient to bind most of the toxin molecules, markedly reducing the concentration of free [^{3}H]STX. For example, if the free [^{3}H]STX concentration would be "nominally" 1.0 nM in the absence of any binding, its measured value is 0.96 nM when only linear binding occurs (in the presence of TTX), but is reduced to 0.13 nM by the extensive saturable binding (in the absence of TTX). Similarly, saturable binding reduces a nominal 5.0 nM [^{3}H]STX concentration to 0.71 nM. The back-exchanged experiments described below were conducted mostly at nominal [^{3}H]STX concentrations of 5.0 nM, for which the measured free [^{3}H]STX was less than 1.0 nM, requiring that >80% of [^{3}H]STX was saturably bound.

Acid conditions reverse both the saturable and linear uptake of STX (Fig. 4). Saturable binding of nominal 3–4 nM STX is reduced to 50% at pH 5.3; the *solid line* of Fig. 4 is not a titration curve because as the pH is lowered the free toxin concentration rises, exaggerating the relative binding at lower pH values. Nevertheless, the inhibition of STX binding at low pH is completely consistent with other reports of the competition between protons and STX or TTX at the sodium channel (25, 26). In several preliminary experiments the reversal rate of bound [^{3}H]STX from brain membranes at pH 5 was measured using rapid filtration techniques (26, 27). The toxin dissociated with a half-time of 10 min. Therefore, over the 30-min period during which the membranes were centrifuged at pH 4.3, more than 90% of the [^{3}H]STX would have dissociated from the receptors. Linear binding is also inhibited by acid conditions, although some linear uptake remains at pH values below 4.2. Referring to Figs. 3 and 4, the total uptake of 5 nM STX by a membrane suspension at pH 7.2 can be reversed by 90–95% when the pH is lowered to 4.3. Acidification does not accelerate the back-exchange of tritiated STX, and has negligible effects on the structure of the STX and TTX molecules, below pH 7.0 (8). Therefore, mild acidification is a valid method for rapidly removing bound STX from brain membranes without changing toxin properties.

The first experiments to test the effect of binding on tritium back-exchange rates employed a subsequent binding assay to measure the radioactivity remaining in STX. A nominal concentration of 4.0 nM [^{3}H]STX was incubated with or without membranes at 44.5°. Supernatant samples from these incubations were then used in a second binding study to determine the new specific radioactivity of the [^{3}H]STX. In the absence of membranes, the tritium exchanged from [^{3}H]STX exponentially with a half-time of 18.6–19 hr, measured over a 20-hr period. In the presence of membranes, the back-exchange was exponential over the first 10 hr, but then

slowed dramatically. (Measurement of the pH of the incubation suspension after 20 hr showed that it had spontaneously fallen to 4.6, from an initial value of 7.0. The membrane suspension also took on a "curdled" appearance. Evidently the membranes are degraded at elevated temperatures after about 10 hr; the pH then falls, displacing the bound STX and, by itself, slowing back-exchange. Therefore, exchange reactions in membrane suspensions were only followed for the first 10 hr.) The decline of the tritium content of [^{3}H]STX over the first 10 hr in the presence of membranes was characterized by a reaction with a half-time of 2.5–2.8 hr. The rate of back-exchange was thus accelerated by a factor of about 7 in the presence of membranes.

Saxitoxin is bound to all membranes in both a saturable and a linear mode, either of which could catalyze the tritium back-exchange. Although linear binding alone remains in the presence of high concentrations of unlabeled, competitively binding TTX or STX, these unlabeled toxins would prevent, through this very competition, any subsequent assay of [^{3}H]STX by membrane binding. A different method, using column chromatography, was therefore employed to assay back-exchange in the presence and absence of unlabeled toxins.

The elution profile of [^{3}H]STX from a cation-exchange column is shown in Fig. 5. The first 32 fractions, eluted with a low ionic-strength solution, contained the tritiated water on the column. The STX was bound tightly to the column and was eluted only by concentrated (2–4 M) salt solutions, which remove no additional ^3H_2O (*broken line*). Figure 5 shows that an aliquot of [^{3}H]STX contains about 4% of tritium as ^3H_2O, even before incubation at elevated temperatures begins. This is an expected contamination from back-exchange which occurs during purification and subsequent thawing and freezing. After 24 hr at 37° the radioactive content of the water peak has grown, whereas that of the STX has declined by about 65%. This result agrees with the 19-hr half-time for back-exchange determined by the membrane-binding assay just described. The biological activity of several of the toxin fractions was determined on the action potential of the frog sciatic nerve, and is shown by the *asterisks* in Fig. 5. No biological activity is eluted at low ionic strength, whereas for the high ionic strength elution the biological activity is collected in parallel with the eluted tritium, showing that this radioactivity is indeed associated with saxitoxin.

Even the small dimension columns used were never saturated by STX, and at least 10 aliquots of [^{3}H]STX:^3H_2O could be run reproducibly through one column. Since the total tritium placed on the column could be accounted for within 5% by the sum of material eluted at low and high ionic strength, there was little permanent retention on the column. The procedure normally followed was to load the column with one aliquot of [^{3}H]STX:^3H_2O from an incubation, elute the ^3H_2O only, at low ionic strength, and, with the [^{3}H]STX still on the column, load and elute a second, identical aliquot. Thus, at least two measurements of ^3H_2O were obtained for each time of incubation, which agreed within 10% of each other. There was evidence that tritium slowly exchanged from [^{3}H]STX bound to the ion exchange resin at room temperature. For this reason all chromatography was conducted at 4°.

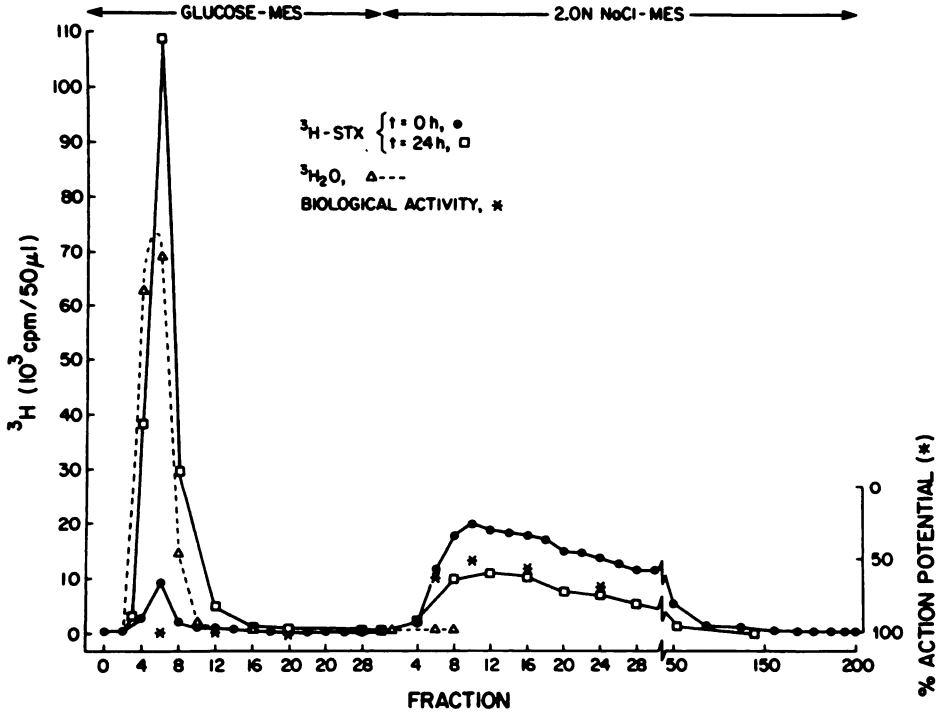

FIG. 5. *Chromatography on Dowex-50 column*

The *solid lines* show the elution profile for [^{3}H]STX before (●) and after (□) incubation in solution at 37° for 24 hr. The *asterisks* graph the blocking potency of a 1:10 dilution of the [^{3}H]STX fractions on a frog sciatic nerve. The degree of block is a nonlinear yet monotonic function of toxin concentration. The *broken line* (△) describes the elution of ^3H_2O only.

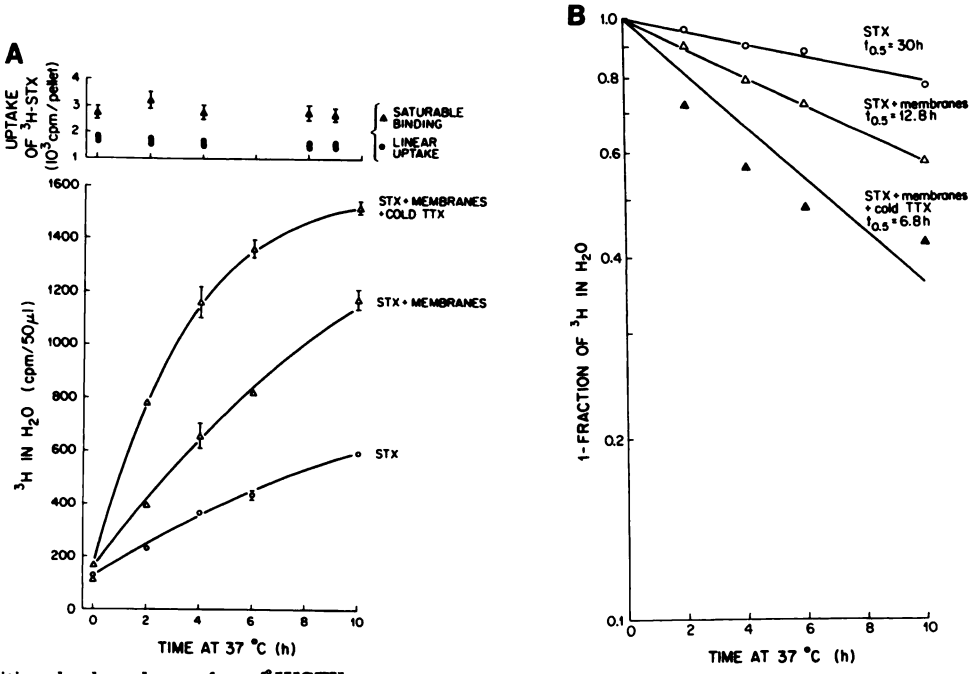

FIG. 6. *Kinetics of tritium back-exchange from [^{3}H]STX*

A. The total ^{3}H eluted by glucose-MES from a Dowex-50 column is plotted as a function of the incubation time at 37°. Each point is the average of three elution measurements of the incubation supernatant; *error bars* indicate standard deviations when they exceed the dimensions of the symbol. The *upper panel* shows the relative constancy of the linear and saturable binding capabilities of brain membranes preincubated at 37° in the absence of toxin and then bound with [^{3}H]STX at 4°.

B. Semilogarithmic plot of the data from the *lower panel* of A. Half-times are calculated from lines drawn by eye to fit the data.

The kinetics of back-exchange from [^{3}H]STX measured by column chromatography is shown in Fig. 6. The *upper panel* of Fig. 6A illustrates the stability of binding activity of a membrane preparation which was incubated in the absence of any toxin at 37° for times up to 10 hr and then added to a [^{3}H]STX solution at 4° to test for binding activity. Both linear and saturable binding activities are stable over this period, indicating that the degree of binding during the incubation of membranes with [^{3}H]STX is constant.

As with the membrane-binding assay, the tritium exchange rate is accelerated in the presence of membranes over the rate in solution only. One notable result appears in Fig. 6: when saturable binding of STX is abolished, the exchange rate increases. The semilogarithmic graph of Fig. 6B shows that the exchange rate is more than doubled by membranes alone, and about doubled again by the presence of 10 μM unlabeled TTX, which abolishes all saturable binding. This suggests that the acceleration of back-exchange is due, at least in part, to the linear binding. Linear binding is, by definition, proportional to the free [^{3}H]STX concentration. When unlabeled TTX saturates the specific STX receptors, the free [^{3}H]STX concentration rises and the extent of linear binding increases.

The relationship between linear binding and the catalysis of back-exchange is demonstrated in Fig. 7. As before, [^{3}H]STX is incubated alone in solution, or with membranes with or without excess TTX; an additional condition is also represented, in which one-half the membrane content is incubated with [^{3}H]STX and excess TTX to provide one-half of the linear binding. The rates of back-exchange are expressed as the rate constants of

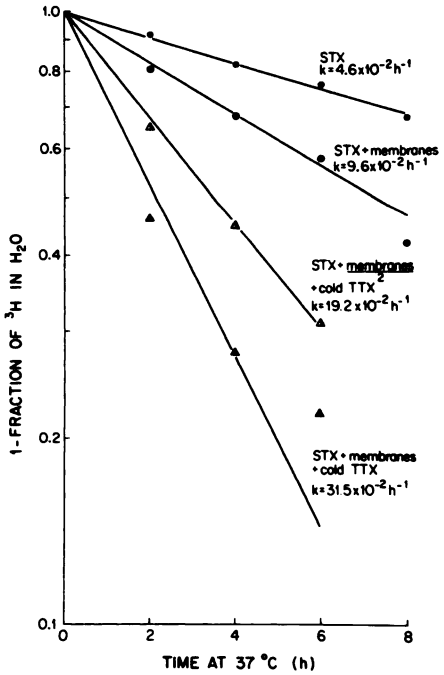

FIG. 7. *Semilogarithmic plot of tritium back-exchange kinetics*

The rate constants (k) are for the straight lines which fit the exponential appearance of ^{3}H in water: $1 - f = \exp(-kt)$, where f is the fraction of the ^{3}H which has back-exchanged into water at time t.

the exponential processes which are the best linear fits to the semilogarithmic plots. Again, membranes accelerate back-exchange, and membranes plus TTX accelerate it even more. Reducing the membrane content by one-half in excess TTX reduces the back-exchange rate.

TABLE 1

Relationship between back-exchange rate and linear binding[a]

Condition	Exchange rate k (10^{-2} hr^{-1})	Fraction of [^{3}H]STX which is free (α)[b]	Additional exchange rate due to membrane ($k - \alpha k_0$) (10^{-2} hr^{-1})	Linear STX uptake (β) (f moles/pellet)[b]	$(k - \alpha k_0)/\beta$ (10^{-4} hr^{-1} fmole^{-1} pellet)
STX in solution	4.6 ($=k_0$)	1.0	0	0	0
STX + membranes	9.6	0.33	8.08	101	8.0
STX + membranes + TTX	33.5	0.99	26.9	299	9.0
STX + (membranes/ 2 + TTX)	19.2	0.99	15.0	158	9.5

[a] Data from Fig. 7.

[b] Measured directly during this experiment before acidification of incubation suspension at time 0.

A quantitative correlation between linear binding and accelerated back-exchange is established in Table 1. For each of the conditions of Fig. 7 the extent of linear binding was measured during the incubation. Table 1 compares the linear binding, the additional rate of exchange catalyzed by the membranes, and the ratio of these two measurements. When only linear binding occurs, in the presence of high concentrations of TTX, the additional exchange rate is proportional to the degree of linear binding, 9 to 9.5 $\times$ 10^{-4} hr^{-1} fmole^{-1} pellet. The added rate per linearly bound toxin when saturable binding also occurs is slightly reduced from this value, being 8 $\times$ 10^{-4} hr^{-1} fmole^{-1} pellet. In a second experiment of this design, the added rate per linearly bound STX was slightly greater for the incubation condition with both saturable and linear binding (no cold TTX) than for linear binding alone (cold TTX present). Thus, the results of both experiments show that linear binding alone accelerates the back-exchange of tritium from [^{3}H]STX; in fact, the additional exchange rate is proportional to the degree of linear binding. In contrast, it appears that the extensive saturable binding which occurs has no measurable effect on this back-exchange.

DISCUSSION

The back-exchange of tritium from the C-11 position of [^{3}H]STX into water is catalyzed by a weak binding of [^{3}H]STX to brain membranes. This binding is a linear function of the [^{3}H]STX concentration up to at least 10^{-5} M (4, 24) and is characteristic of STX uptake by membranes of both excitable and non-excitable tissues (3). Linear uptake has about the same magnitude regardless of the radiopurity of [^{3}H]STX, showing that it is not due to some tritiated impurity. Ritchie and Rogart (24) showed that the size of the linear uptake was markedly reduced in solutions containing elevated Ca^{2+}, and proposed that linear binding was due to concentration of the divalent STX cation in the diffuse double layer adjacent to the nerve membrane which is known to carry a net negative charge (28, 29). The results of this paper also suggest that ionic interactions contribute strongly to the linear uptake, which is inhibited at acid pH values, where some negatively charged groups on the membrane may be neutralized by protonation.

More specifically, the accelerated back-exchange which accompanies linear binding may be due to a weak ionic bonding of [^{3}H]STX to negatively charged membrane groups. In solution, the exchange of the C-11 methylene hydrogen atoms occurs through a keto-enol tautomerism between C-11 and the keto group at C-12 (Fig. 1). The keto form is present at less than 1% at pH 7 (8), but increases at alkaline pH as the guanidinium moiety centered at C-8 (pK_a 8.2) becomes unprotonated. The loss of this proton is paralleled by a shift in electron density back into C-4 with concomitant destabilization of the gem-diol relative to the unhydrated ketone and, therefore, an increase in the fractional population of the latter. If, in the linear binding reaction, the guanidinium group at C-8 formed an ionic bond with some negatively charged group, then the proton would also be drawn away from C-4, rendering it more electron-dense and shifting the equilibrium at C-12 toward the keto form. This seems a reasonable proposal for a mechanism of accelerated back-exchange associated with linear binding.

Specifically bound [^{3}H]STX has the same back-exchange rate as that of [^{3}H]STX in solution. This observation places some restrictions on the possible configurations of the STX-receptor complex at the sodium channel. Since a measurable back-exchange reaction within the times measured here (<10 hr) requires both the presence of the keto form of STX and accessibility of solvent hydrogen atoms to the C-11 methylene group, either (a) the keto form of the toxin does not occur during specific bonding or (b) such bonding does not catalyze solvent hydrogen exchange at C-11, either for energetic or steric reasons.

With regard to Option a, other experimental observations imply that the keto form is not required for the specific binding of STX. First, at neutral pH, less than 1% of the STX in solution is present as the ketone and the potency of STX is actually reduced relative to that at pH 7.0, by about 20% when the pH is elevated to 8.2 and by 60–75% at pH 9.0 (2),[2] conditions which increase the ketone form of STX by from severalfold to an order of magnitude, respectively (8). Second, a reduced form of STX, the alcohol 12α-OH STX (8), which cannot take on the keto form, is still 10% as potent as STX (18). Evidently, the ketone group is not essential for specific toxin binding.

But, whereas the ketone form may not be essential for binding, it could occur in the molecule as a consequence of ionic bonding of the C-8 guanidinium, as described above as a mechanism for the exchange acceleration by the linear uptake. However, if the portion of a specifically bound molecule around C-11 were buried in the receptor and inaccessible to solvent molecules, then exchange from an existing keto form would be sterically prevented,

[2] G. Strichartz, unpublished observations.

the circumstances of Option b. The results of these tritium exchange experiments do not permit the selection of either option, but they do exclude the possibility that a specifically bound saxitoxin molecule undergoes a keto-enol tautomerism at freely exposed C-11 and C-12 carbon atoms.

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