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CHARACTERISTICS OF SAXITOXIN BINDING TO THE SODIUM CHANNEL OF SARCOLEMMA ISOLATED FROM RAT SKELETAL MUSCLE

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SUMMARY

1. The characteristics of saxitoxin (STX) binding to the mammalian Na channel have been studied in purified sarcolemma isolated from rat skeletal muscle.

2. STX binds specifically to isolated sarcolemma with a K_d of $1{\cdot}43 \times 10^{-9}$ M and B_{max} of 7–8 p-mole STX bound/mg membrane protein at 0°C in the presence of 140 mM-NaCl. In rat muscle homogenate under the same conditions the corresponding values are $K_d = 1{\cdot}53 \times 10^{-9}$ M and $B_{max} = 0{\cdot}15$–$0{\cdot}20$ p-mole/mg protein (18–20 p-mole/g wet wt.). Membrane purification produced a fortyfold increase in STX binding site concentration per milligram protein. Calculated binding site density in isolated sarcolemma was about 30 sites/μm^2 of membrane surface.

3. Denervation (10–14 days) results in a 43% reduction in the density of high-affinity STX binding sites in purified sarcolemma, but the K_d for this class of sites is not changed.

4. In sarcolemma, the apparent K_d for STX binding is dependent on temperature, pH and ionic strength. The Q_{10} for K_d between 0 and 40 °C is 1·3. Protonation of a group having a pK of 6·0 markedly raises K_d without affecting B_{max}. Apparent K_d increases eightfold when ionic strength is raised from 20 to 600 mM.

5. Dissociation and association rate constants for STX binding are temperature dependent with Q_{10} of 2·6 and 1·9 respectively between 0 and 20 °C.

6. STX binding is competitively inhibited by monovalent and divalent cations under conditions of constant total ionic strength. An affinity sequence of $Tl^+ > Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$ is seen for the monovalent cation-binding site.

7. The STX binding site is relatively stable to heat and to enzymic degradation. A specific modifier of carboxyl residues inactivates subsequent STX binding. This process can be prevented by the presence of STX during the reaction.

8. Characteristics of the STX binding site in isolated sarcolemma are compared to those reported for other isolated excitable membranes and for studies of whole muscle and muscle homogenate. Sarcolemma provides a potential source of enriched Na channels for further purification efforts in a mammalian system.

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INTRODUCTION

In most excitable membranes, the generation of an action potential requires rapid sequential changes in membrane conductance to Na and K ions (Hodgkin & Huxley, 1952). Although the membrane Na conductance system involved in this process has been studied most extensively using electrophysiologic techniques (see Armstrong, 1975 for a review), considerable indirect structural information has been obtained through the use of various neurotoxins which bind specifically to the channel (Narahashi, 1974; Ritchie, 1975; Catterall. 1977). Tetrodotoxin (TTX) and saxitoxin (STX) are the most thoroughly characterized of these neurotoxins (Ritchie & Rogart, 1977*b*). Recently published methods for the preparation and purification of both toxins in labelled form have greatly extended their application (Ritchie, Rogart & Strichartz, 1976; Hafeman, 1972).

Tritiated STX and TTX have been used to probe the Na channel in a number of invertebrate and vertebrate systems (see Ritchie & Rogart, 1977*b* for a review). Work in muscle, however, has been limited to studies employing whole muscle or crude homogenate and has been hampered by the low level of binding obtained and by problems of interpretation in regard to surface geometry (Almers & Levinson, 1975; Ritchie & Rogart, 1977*b*; Jaimovich, Venosa, Shrager & Horowitz, 1976). Methods for the preparation of isolated muscle surface membrane have been refined to the point where binding studies on purified sarcolemma can be undertaken (Barchi, Wong & Bonilla, 1977; Barchi, Weigele, Chalikian & Murphy, 1979).

We report here the characterization of STX binding to the Na channel of isolated and purified sarcolemma from rat skeletal muscle. Binding characteristics in this membrane system are dependent upon various physical factors including temperature, pH and total ionic strength of the incubation medium. We have determined cation-binding selectivity sequences based on equilibrium binding studies and find them qualitatively similar to those reported in other isolated excitable membranes (Reed & Raftery, 1976; Henderson, Ritchie & Strichartz, 1974; Weigele & Barchi, 1978*b*). Binding characteristics and site density in purified sarcolemma are compared to those previously reported in whole muscle.

METHODS

Purified saxitoxin (STX) was the generous gift of Dr E. J. Schantz of the University of Wisconsin. Tetrodotoxin (TTX) and the various enzymes were obtained from Sigma Chemical Corp. Saxitoxin was tritiated by the New England Nuclear Corp. after the method of Ritchie *et al.* (1976). The resultant labelled toxin was purified as previously described (Ritchie *et al.* 1976; Weigele & Barchi, 1978*a*). Bioactivity of the purified toxin was determined by comparison to 'standard shellfish toxin' as the hydrochloride salt obtained from the Food and Drug Administration. The desheathed sciatic nerve sucrose gap method of Strong, Smith & Keana (1973) was used for bio-assay. In addition, radiopurity was checked by displacement of bound [3H]STX from an excess of purified synaptosomes using unlabelled toxin as suggested by Ritchie & Rogart (1977*a*). The various saxitoxin preparations used for our studies had radiopurities of 60–70 % and specific activities between 15 and 20 c/m-mole.

Preparation of rat sarcolemma

Sarcolemma was isolated and purified using a modification of the LiBr extraction of Festoff & Engel (1974) as previously described and characterized by us (Barchi *et al.* 1979). Membrane was prepared from the mixed fibre type and fast muscles of the rat hind leg. Purity of isolated

membranes was monitored using marker enzyme assays and cholesterol determinations as previously reported (Barchi *et al.* 1977, 1979). Purified sarcolemma was suspended in Tris buffer (5 mM, pH 7.3) and stored frozen at −20 °C until needed.

Equilibrium STX binding determinations

Saxitoxin binding to isolated sarcolemma was determined using a rapid filtration technique recently reported from this laboratory (Weigele & Barchi, 1978*a*). Binding curves were constructed using duplicate samples at each of eight [^{3}H]STX concentrations between 0·125 and 10×10^{-9} M to determine total binding, and four determinations over the same range of [^{3}H]STX in the presence of 10^{-6} M unlabelled STX or TTX to yield non-specific binding. After filtration, each glass filter was equilibrated in 5 ml. Hydromix (Yorktown Research Corp., Hackensack, N.J.) and retained radioactivity determined with a Packard liquid scintillation counter. All data thus obtained were corrected for counting efficiency by recounting samples following direct addition of a [^{3}H]H_2O internal standard.

Data were computer analysed to yield specific binding and experimental free STX concentrations corrected for bound toxin. Both double reciprocal and Scatchard plots were automatically constructed in each case and analysed to yield the specific binding parameters K_d (equilibrium dissociation constant) and B_{max} (maximal binding in p-mole/mg membrane protein). Since these two linear transformations are most sensitive to error in different regions of the binding curve, adequate fit of both expressions to a data set with comparable binding parameters offers a qualitative index of reliability for the values obtained. Binding data were considered acceptable when the regression value for linear fit using these two methods was $\geqslant 0{\cdot}96$ and when the K_d and B_{max} estimated by the two methods were in close agreement.

Dissociation rate determinations

Purified sarcolemma was maintained at the desired temperature in a jacketed constant-temperature reaction vessel. Temperature was monitored in the circulating fluid and in the membrane suspension throughout each experiment. A suspension of membranes was incubated at a given temperature for 30 min with stirring in 5×10^{-9} M-STX. Following this equilibration period, a 10^3-fold excess of unlabelled toxin was added and zero-time samples immediately removed and assayed by the filtration method detailed above. Aliquots were then removed for filtration assay at appropriate (usually 15 sec) intervals thereafter. Data were expressed as percentage of zero-time bound toxin remaining at each point. Under these conditions, only the dissociation rate (k_{-1}) of the bound STX is measured since the probability of reassociation of a dissociated molecule is reduced to 1 in 10^3 by the presence of the large excess of unlabelled toxin.

The resultant data were plotted on a semilogarithmic scale against time and half-time for toxin dissociation graphically determined. Values for k_{-1} were then calculated from the relationship $C = C_0 e^{-kt}$.

Ion competition studies

For determinations of cation interaction with STX binding, curves were constructed at 0 °C, pH 7·5, with a constant ionic strength of 200 mM maintained by the addition of choline chloride. The test cation was substituted for choline at four or more concentrations between 0 and 200 mM. Values for equilibrium binding of a cation were determined from apparent values for STX K_d at each cation concentration using the relationship:

$$K_d^{\text{apparent}} = K_d\left(1 + \frac{i}{K_i}\right)$$

where i is the concentration of the test cation, K_d the equilibrium dissociation constant for STX under experimental conditions in the absence of test cation, and K_i the equilibrium dissociation constant for the test cation.

RESULTS

Muscle homogenate

STX binding studies using both centrifugation and filtration methods were carried out with homogenates prepared from rat hind limb muscle (Barchi *et al.* 1979) in buffer containing 140 mM-NaCl and 20 mM-Tris (pH 7·5), 0 °C. Under these conditions, an average value of $1{\cdot}53 \times 10^{-9}$ M was obtained for the equilibrium dissociation constant (K_d). The maximal binding site density (B_{max}) in these homogenates was 18–20 p-mole/g wet wt. muscle. This binding site density is comparable to that obtained by others in rat diaphragm and in frog skeletal muscle.

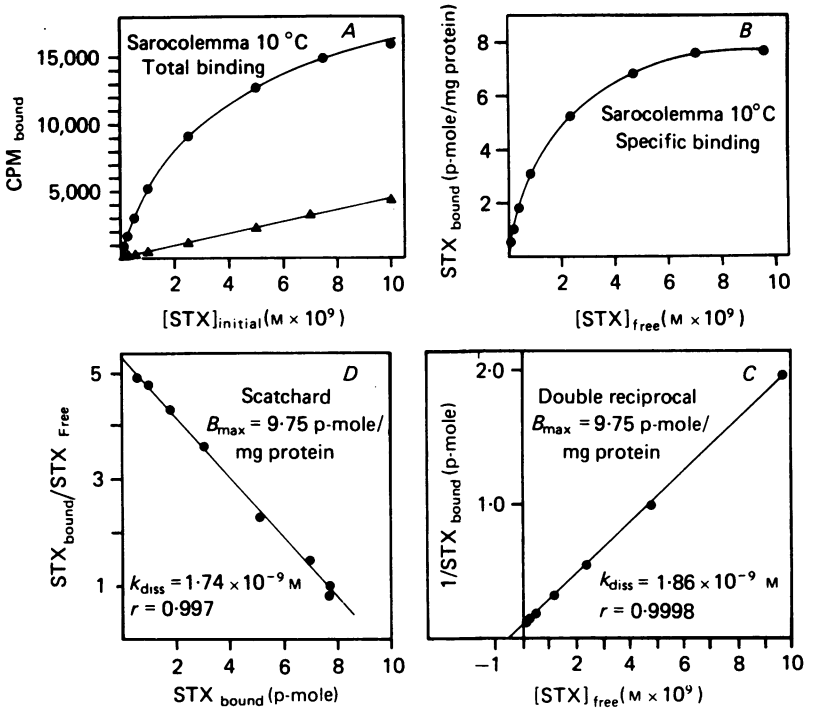

Fig. 1. [^{3}H]STX binding to sarcolemma isolated from rat skeletal muscle. *A*, total binding (●) and non-specific binding in the presence of excess unlabelled toxin (▲). *B*, specific binding derived from *A*. STX concentrations have been corrected for the amount of toxin bound at each point. *C*, *D*, double reciprocal and Scatchard analytical plots of specific binding used to determine K_d and B_{max} (140 mM-NaCl, 10 mM-Hepes pH 7·5, 10 °C).

Isolated sarcolemma

In membrane preparations from rat skeletal muscle, the highest specific activities of sarcolemmal markers are found in a light membrane fraction banding between 22 and 25% sucrose following equilibrium centrifugation on a continuous density gradient (Barchi *et al.* 1977, 1979). The highest level of specific [^{3}H]STX binding was also uniformly distributed through this fraction (Barchi *et al.* 1979). This light

membrane fraction has been isolated and was used for all subsequent studies reported here.

Complete binding curves for the STX-channel complex in purified sarcolemma could be obtained with less than 2 mg membrane protein when binding was quantitated with a glass fibre rapid filtration method (Weigele & Barchi, 1978*a*). Non-specific STX binding (that observed in the presence of 10^{-6} M unlabelled TTX or STX) was considerably lower relative to total binding in this isolated membrane fraction than in crude homogenate.

Specific binding parameters for the STX-channel complex in isolated sarcolemma were determined after correcting total binding for the non-specific component and after calculating actual free STX concentration using appropriate corrections for the quantity of toxin bound in each assay tube (Fig. 1); (failure to correct initial free toxin concentration for total bound toxin produced significant errors in estimates of K_d). In seven preparations of purified control sarcolemma, an average of 7·7 p-mole STX bound/mg membrane protein was found. In these same preparations, the apparent K_d for STX binding was $1{\cdot}43 \times 10^{-9}$ M in the presence of 140 mM-NaCl at pH 7·5 and 0°C. This K_d is in good agreement with that obtained for muscle homogenate prepared from the same group of muscles under these conditions.

A competition binding experiment was carried out at 0°C between a saturating concentration of [^{3}H]STX (2×10^{-8} M) and unlabelled STX obtained as 'standard shellfish toxin' from the Food and Drug Administration of the U.S. Department of Health, Education and Welfare. The concentration of standard toxin required to inhibit 50% of the specific [^{3}H]STX binding agreed to within 10% with the estimated concentration of [^{3}H]STX present, confirming the validity of concentration estimates made by bio-assay techniques (see Methods).

Denervated muscle sarcolemma

Denervation results in the appearance of TTX and STX-resistant action potentials in muscle apparently through a mechanism requiring protein synthesis (Redfern & Thesleff, 1971; Harris & Thesleff, 1971; Grampp, Harris & Thesleff, 1971). The fate of Na channels binding these toxins with normal affinity has not been clearly defined after denervation. We have examined this question using isolated sarcolemma.

Rat distal leg muscles were denervated by removal of a segment of the sciatic nerve in the upper thigh. Ten to 14 days later, the gastrocnemius and the anterior and posterior tibial muscle groups were removed and used for the preparation of sarcolemma. The yield of sarcolemma was slightly lower than from control muscle but appeared unremarkable by SDS gel electrophoresis.

In five separate preparations, each obtained from six to ten denervated animals, the average K_d for STX binding was $1{\cdot}6 \times 10^{-9}$ M, unchanged from that found in control muscle. Extended binding curves up to 5×10^{-8} M STX suggested but did not clearly define the presence of a second class of low-affinity binding sites. The total number of high-affinity binding sites, however, did fall from $7{\cdot}7 \pm 0{\cdot}7$ p-mole/mg membrane protein in control sarcolemma to $4{\cdot}4 \pm 1{\cdot}2$ p-mole/mg membrane protein in sarcolemma prepared from denervated animals.

Temperature dependency of STX binding to isolated sarcolemma

Equilibrium binding constants and association and dissociation rates for the STX-channel complex were evaluated in purified sarcolemma as a function of temperature between 0 and 40 °C. Since we found that STX binding was dependent on ionic strength and alkali metal cation concentration (see below), these studies were carried out under conditions of constant ionic strength and with a Na^+ concentration

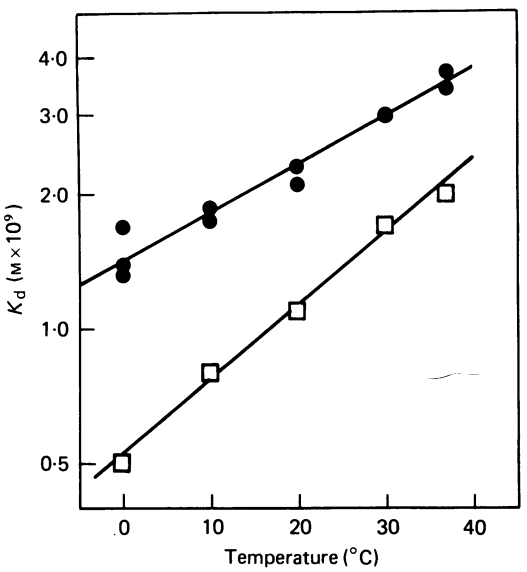

Fig. 2. Temperature dependency of the equilibrium dissociation constant (K_d) for STX binding. Values for rat synaptosomes (Weigele & Barchi, 1978*a*) are presented for comparison. The calculated Q_{10} for sarcolemma was 1·3 while that for synaptosomes was 1·44. ●, sarcolemma; □, synaptosomes.

(140 mM) similar to that used by other authors in studies of STX binding in whole muscle. Under these conditions, the equilibrium dissociation constant (K_d) for the STX-channel complex was significantly temperature dependent, varying between $1{\cdot}45 \times 10^{-9}$ M at 0 and $3{\cdot}6 \times 10^{-9}$ M at 37 °C (Fig. 2). The apparent Q_{10} for this temperature dependency was 1·3. Within the limits of resolution of these data, the increase in K_d appeared logarithmic, without evidence of an abrupt change in dependence which could be related to phase transitions within the membrane.

Dissociation rate constants (k_{-1}) for the STX-channel complex were determined at 5° intervals between 0 and 20 °C. The rate of loss of specifically bound [^{3}H]STX was measured at 15 sec intervals following the addition of 10^3-fold excess of unlabelled toxin. Bound toxin declined as a single exponential function with time, indicating that dissociation of the toxin-channel complex progressed as a simple first-order process. Dissociation rate constants were calculated from half-dissociation times obtained graphically from these data. These values were temperature dependent, varying between 0·3 min^{-1} at 0 °C and 1·2 min^{-1} at 20 °C. Above 20 °, the dissociation rate became too rapid to measure with the accuracy required for

adequate analysis. The Q_{10} for the dependency of k_{-1} on temperature between 0 and 20 °C was 2·6. Values for the association rate constant (k_{+1}) were calculated at 5° intervals between 0 and 20 °C using experimentally determined values of K_d and k_{-1}. Again, a logarithmic dependence on temperature was noted with a Q_{10} of 1·9 over this range (Fig. 3).

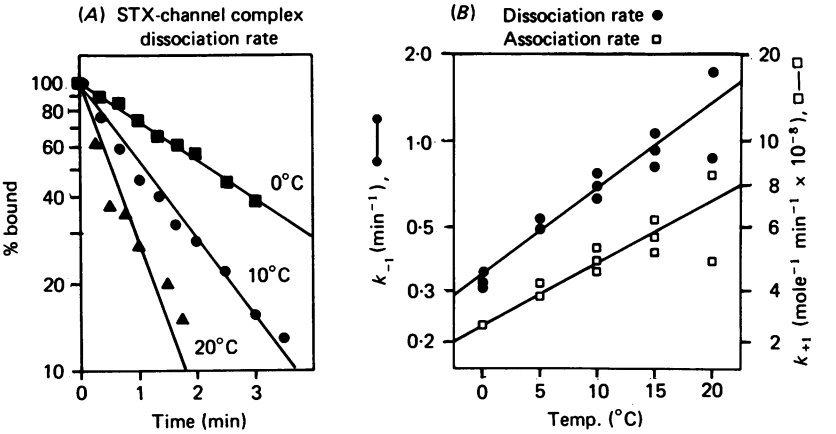

Fig. 3. *A*, STX-channel complex dissociation rate followed at 15 sec intervals. Data at 0, 10, and 20 °C are shown. *B*, measured dissociation rate constants (k_{-1}) for STX at 5° intervals between 0 and 20 °C. The Q_{10} for this process is 2·6. Association rate constants (k_{+1}) were calculated from measured dissociation rate constants and equilibrium dissociation constants. Temperature dependency for the association rate is 1·9.

pH dependency

Binding of [^{3}H]STX to sarcolemma at constant toxin concentration falls rapidly but reversibly between pH 6 and 5. Twelve complete binding curves were constructed at various pH values between 5 and 7·5 in order to determine whether this loss in binding represented a decrease in the apparent number of sites available for interaction or a change in affinity of all the binding sites for [^{3}H]STX. The total number of Na channels capable of binding STX at saturating ligand concentrations did not change with decreasing pH, but the apparent affinity of the channel binding site for STX fell markedly from $1{\cdot}5 \times 10^{-9}$ M at pH 7·5 to $> 3 \times 10^{-8}$ M at pH 5·2. Analysis of these data using the method of Reed & Raftery (1976) yielded a linear transformation and indicated that the protonation of a single class of sites having a pK of 6·0 was responsible for the observed change in K_d. A simple competitive process is suggested by constant B_{max} and varying apparent K_d observed with protonation.

Ionic strength and ionic selectivity

The apparent K_d for STX binding to isolated sarcolemma showed a linear dependence on the total ionic strength of the incubation medium. Varying ionic strength between 20 and 600 mM ions by the addition of choline chloride produced a linear variation in apparent K_d between $0{\cdot}15 \times 10^{-9}$ M and $1{\cdot}14 \times 10^{-9}$ M respectively at 0 °C. At a constant total ionic strength other impermeant ions such as tetramethylammonium chloride and tetraethylammonium chloride could be substituted for choline

chloride without altering the apparent K_d for STX binding, suggesting that ionic strength rather than specific cation-channel interactions was the factor responsible for the effect of K_d. All subsequent binding studies reported here were carried out at a constant ionic strength of 200 mM monovalent ions maintained by the addition of choline chloride as necessary.

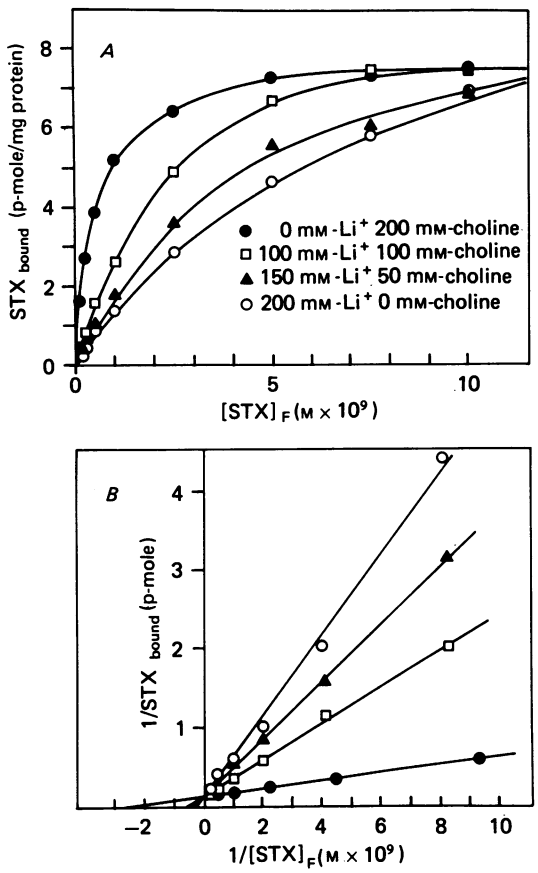

Fig. 4. *A*, specific STX binding curves obtained at constant ionic strength (200 mM monovalent ions) in the presence of increasing concentrations of Li^+. B_{max} at saturating STX concentrations remains unchanged, but K_d for STX is progressively shifted to higher values with increasing [Li^+]. All studies carried out at 0 °C, pH 7·5. *B*, double reciprocal plots of data in *A*, indicating the diagnostic criteria for competitive inhibition.

The apparent half-saturating concentration of STX was shifted progressively toward higher values when increasing concentrations of Li^+ were substituted for choline at constant ionic strength in the incubation medium (Fig. 4*A*). The maximal binding at saturating STX concentration, however, was unaffected by variations in Li^+ concentration. Analysis of STX binding curves at Li^+ levels between 50 and 200 mM indicated that this cation altered the apparent K_d for the STX-channel complex without affecting B_{max}, which suggests simple competition of Li^+ for binding to the channel at a site usually occupied by STX. Values for the affinity of

this site for Li^+ were calculated from the apparent K_d for STX, the known K_d for STX in the absence of Li^+ and the concentration of Li^+ present. Values calculated at various concentrations agreed well and yielded an average K_I of $16{\cdot}7 \pm 2{\cdot}8$ mM for Li^+ binding at this site.

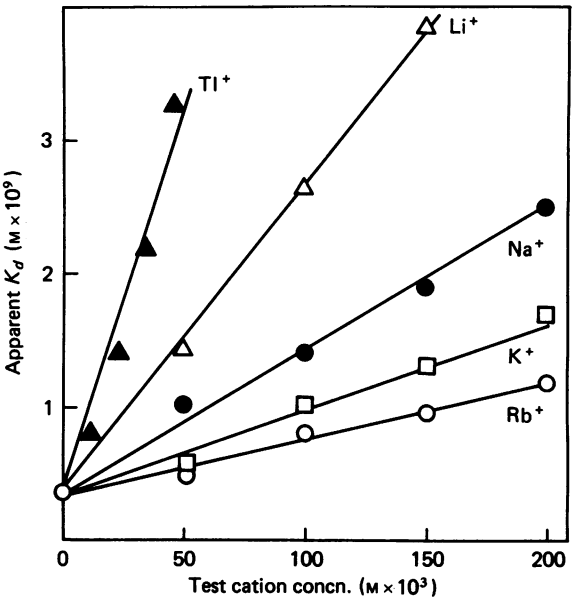

Fig. 5. Apparent K_d for STX binding at constant total ionic strength in the presence of various concentrations of test alkali metal cations. In each case, a linear relationship between concentration of test cation and apparent K_d is obtained, allowing consistent values for test cation dissociation constants to be calculated (see Table 1). The binding sequence of Tl+ > Li^+ > Na^+ > K^+ > Rb^+ is easily seen in the data presented.

TABLE 1. Cation interaction at the STX binding site of the Na channel

Cation	Rat synaptosomes (Weigele & Barchi 1978*b*)			Rat sarcolemma			Electroplax ([^{3}H]TTX) (Reed & Raftery, 1976)	
	n*	K_I (M x 10^3)†	$\frac{K_I}{K_I(Na)}$	n	K_I (M x 10^3)	$\frac{K_I}{K_I(Na)}$	K_I (M x 10^3)	$\frac{K_I}{K_I(Na)}$
Tl^+	4	$7{\cdot}2 \pm 1{\cdot}7$	0·21	7	$7{\cdot}9 \pm 3{\cdot}2$	0·23		
Li^+	4	$22{\cdot}6 \pm 2{\cdot}8$	0·66	4	$16{\cdot}7 \pm 2{\cdot}8$	0·49	60 ± 10	0·85
Na^+	8	$34{\cdot}3 \pm 4{\cdot}5$	1·0	4	$37{\cdot}4 \pm 3{\cdot}9$	1·0	71 ± 4	1·0
K^+	7	$53{\cdot}6 \pm 14{\cdot}0$	1·56	3	$65{\cdot}0 \pm 3{\cdot}8$	1·89	135 ± 12	1·90
Rb^+	4	89 ± 31	2·59	3	99 ± 6	2·88	207 ± 20	2·91
Cs^+	8	147 ± 19	4·29	4	132 ± 22	3·84	272 ± 30	3·83
Mg^{2+}	4	$1{\cdot}8 \pm 0{\cdot}2$	0·05	4	$1{\cdot}9 \pm 0{\cdot}3$	0·05		
Ca^{2+}	4	$3{\cdot}6 \pm 1{\cdot}2$	0·11	4	$4{\cdot}1 \pm 1{\cdot}3$	0·11		

* Number of complete binding isotherms determined for a given test cation.
† Apparent dissociation constant for the cation-channel complex at the STX binding site.

Similar saturation binding data were obtained at constant ionic strength in the presence of varying concentrations of each of the alkali metal cations. For each ion, a linear relationship was obtained between apparent K_d for the STX-channel complex and test cation concentration (Fig. 5). In all cases, B_{max} for STX remained unchanged. Estimates of the equilibrium binding constant for each ion were made at each concentration tested and the average values obtained are presented in Table 1. For the monovalent alkali metal cations, a binding sequence of $Tl^+ > Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$ was found.

The divalent cations Ca^{2+} and Mg^{2+} were also strong competitive inhibitors of STX binding, having apparent K_d values of 4·1 and 1·9 mM respectively. Although our data suggest that these divalent cations compete with STX for some region of its binding site on the channel, we cannot distinguish between a common binding site or separate binding sites for the monovalent and divalent cations.

Stability to temperature and chemical modification

Purified sarcolemma was incubated in 50 mm potassium phosphate buffer pH 7·0 for 10 min at various temperatures between 30 and 70 °C, then re-equilibrated to 20 °C before determining STX binding parameters in order to evaluate the heat lability of the STX binding site. Total number of STX binding sites remained constant below 45 °C but dropped rapidly between 45 and 60 °C. After 10 min of incubation at 60°, purified sarcolemma had lost virtually all of its specific STX binding sites in an irreversible manner.

Incubation of isolated sarcolemma for 1 h with collagenase (0·5 mg/ml.) at 22 °C had no effect on total binding site concentration. In four separate membrane preparations, trypsin (0·5 mg/ml.) reduced specific binding by an average of 23 % under similar conditions. α-chymotrypsin and protease reduced specific binding by 45 and 36 % respectively.

Trimethyloxonium tetrafluoroborate (TMO), a reagent specific for carboxyl residues, produced a marked concentration-dependent inhibition of specific toxin binding. Sarcolemma was incubated with TMO concentrations between 0 and 2 mg/ml. at 0 °C. A pHstat was used to maintain the pH at 7·0. After 5 min of incubation, TMO (2 mg/ml.) had reduced binding by more than 90 %. Addition of 5×10^{-9} M-STX to the reaction mixture prior to initiation of the reaction blocked more than 90 % of this effect of TMO, suggesting that the reactive carboxyl residue is within the region occupied by the toxin when bound to the channel. The specific sulphydryl and disulphide group modifying reagents N-ethylmaleimide and β-mercaptoethanol were also evaluated for their effect on STX binding in isolated sarcolemma. These compounds were tested at a concentration of 10 mM in the presence of 0·14 M-NaCl, 20 mM sodium phosphate, pH 7·5. Incubations were carried out for 1 h at 25 °C. Under these conditions, no modification of specific STX binding was seen.

DISCUSSION

The value which we obtained for the density of STX binding sites in rat muscle homogenate of 18 p-mole/g tissue wet wt. agrees well with estimates previously reported for skeletal muscle in this and other species (Ritchie & Rogart, 1977*b*; Almers & Levinson, 1975; Jaimovich *et al.* 1976). Using various approximations for

the ratio of surface membrane to muscle weight, previous authors have calculated values of Na channel density per unit area of surface membrane between 180 and 400 sites/μm^2 (Ritchie & Rogart, 1977*b*; Almers & Levinson, 1975). A major uncertainty in the estimation of true surface membrane site density in these studies is the contribution of sodium channels in the T-tubular system to the observed total binding. Consideration of T-tubular system binding has been estimated to reduce the density on the sarcolemma itself to as low as 100–200/μm^2 (Ritchie & Rogart, 1977*b*).

Measurements of STX binding in purified excitable membranes yield data somewhat at variance with the above estimates. Site concentrations reported in various purified axonal membrane preparations have ranged between 3·7 p-mole/mg protein in garfish olfactory nerve (Chacko, Barnola, Villegas & Goldman. 1974) and 12·5 p-mole/mg protein in lobster walking-leg nerve (Balerna, Fossett, Checkeportiche, Romey & Lazdunski, 1975). A value of 2–3 p-mole/mg protein was found for purified electroplax surface membranes (Reed & Raftery, 1976). Based on these values, the respective investigators have calculated site densities of between 5 and 25/μm^2 for these isolated membranes.

We find 7–8 p-mole STX binding sites/mg protein in purified sarcolemma. An estimate of channel density per μm^2 can be made using biochemical data which we have reported previously (Barchi *et al.* 1977, 1979). The isopycnic banding density of isolated sarcolemma in sucrose gradients is 1·012. Chemical analysis indicates that these membranes are about 50 % protein by weight as isolated. Assuming a thickness of 125Å to allow for the residual basement membrane material seen in electron micrographs of the purified membrane (Barchi *et al.* 1977), these figures yield an estimate of 30·5 STX binding sites/μm^2.

In light of the high cholesterol content, Na^+K^+ATPase activity, and accessibility to surface-specific labels demonstrated for this sarcolemmal preparation in earlier studies (Barchi *et al.* 1977, 1979), it seems unlikely that a major proportion of this membrane fraction is non-surface contaminant. Two other possible explanations for this discrepancy should be considered. First, it is possible that the sodium channel is unstable in the isolated membrane and decays before the assay. We find, however, only a very slight difference in total STX binding in purified sarcolemma handled and stored as indicated when assayed immediately after isolation or after several weeks of storage. The Na^+ channel appears in fact to be quite stable in isolated sarcolemma. Secondly, a significant percentage of the isolated surface membrane could be in the form of inside-out sealed vesicles; STX would be excluded from its usual channel binding site in such vesicles. We have previously shown that this is not the case for purified sarcolemma by determining binding site density before and after solubilization of the membrane vesicles with Lubrol-PX or Brij-96 (Barchi *et al.* 1979) and demonstrating that no significant change occurs.

An alternative explanation is that both isolated sarcolemmal and whole tissue binding estimates are somewhat in error and that the true channel density lies between the two estimates. Thus, the purified sarcolemmal fraction may in fact represent only 60 % surface membrane and the true channel density may be closer to 50 sites/μm^2. Conversely, the geometric approximations and values for T-tubular correction may lead to significant overestimates of sarcolemmal channel density. Further studies are required to resolve this issue.

Denervation

Previous work with denervated muscle has suggested that the apparent density of sodium channels which bind TTX or STX falls slightly while the K_d for TTX or STX is unchanged (Colquhoun, Rang & Ritchie, 1974; Ritchie & Rogart, 1976). Since these measurements were carried out on whole muscle, however, the authors expressed difficulty in distinguishing between apparent changes in site density per g tissue due to fibre swelling and a true decrease in membrane channel density. We show here that a significant reduction in the density of STX-binding sodium channels can be demonstrated in purified sarcolemma following denervation, suggesting that a true reduction in site density has taken place. Our data confirm earlier reports that the K_d for STX binding is not changed following denervation in the class of channels with which this toxin interacts with high affinity.

Kinetic parameters of STX binding

The temperature dependence of the association and dissociation rate constants for STX binding in sarcolemma, although lower than those seen in synaptosomes, is still considerably in excess of those expected for simple diffusion-controlled ionic interactions. Participation of multiple short-range interactions such as H-bonding and Van der Waals forces in STX binding could explain a component of the temperature dependency of k_{-1} since the contribution of these forces decreases with increasing temperature due to thermal movement of the atoms involved. This argument cannot apply, however, to the temperature dependency of k_{+1}. We feel that the implied activation energies for these processes suggests a more complex sequence of events at the STX binding site of the sort commonly observed with ligand binding to protein receptors or enzymes. Possible candidates would include small ligand-induced conformational changes in the channel structure, solvent-ordering effects near the binding site, and interactions between the channel protein and membrane lipids. These possibilities deserve further experimental investigation.

Competition by inorganic cations

The binding sequence of monovalent and divalent cations at the STX binding site in sarcolemma is the same as that observed in rat synaptosomal membranes (Weigele & Barchi, 1978*b*). The calculated values for the dissociation constants for each cation are in close agreement between these two channels in spite of a threefold difference in the K_d for STX binding. This binding sequence corresponds to the equilibrium binding expected at a high-field-strength anionic site such as a carboxyl residue based on the equilibrium ion-exchange theories of Eisenman (1965).

One can conclude based on the data presented that the site to which these cations bind is shared by STX, but correlation with physiologic ion selectivity must be made with caution. Since ionic currents involve rate-dependent processes which are most likely not near equilibrium, other factors in addition to equilibrium ion-site interactions must be considered. The observed correlation between equilibrium binding selectivity and the physiologic conductance sequence may imply a similarity of the underlying process but does not confirm an identity of the involved sites.

The significant alterations in K_d for STX binding produced by physiologic con-

centrations of Na^+, Ca^{2+} and Mg^{2+} must be kept in mind when comparing reported K_d from various laboratories. It is clear that considerable variation can be introduced by differences in the composition of the incubation medium used in each study or even in various experiments within a single study.

Chemical stability of the STX binding site

The sensitivity of the mammalian sarcolemmal STX binding site to chemical modification is similar to that reported for the sodium channel in other species (Reed & Raftery, 1976; Villegas, Barnola & Camejo, 1973). The binding site can be destroyed by proteases, but only following prolonged exposure at high enzyme concentrations. This suggests that, as expected, the majority of the channel protein is protected from proteolytic cleavage by surrounding membrane lipids.

The specific and rapid loss of STX binding following treatment of the membranes with TMO indicates the presence of a reactive carboxyl group within the STX binding site in sarcolemma (Reed & Raftery, 1976; Shrager & Profera, 1973). We do not find evidence to support the presence of reactive sulfhydryl or disulphide residues in this region.

Further work on the elucidation of the structure of the Na channel will require purification of the channel protein. Our studies suggest that muscle sarcolemma may provide a convenient starting material enriched in Na channels for further characterization of this membrane protein in a mammalian system.

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REFERENCES

ALMERS, N. & LEVINSON, S. R. (1975). Tetrodotoxin binding to normal and depolarized frog muscle and the conduction of a single sodium channel. *J. Physiol.* **247**, 483–509.

ARMSTRONG, C. (1975). Ionic pores, gates, and gating currents. *Q. Rev. Biophys.* **7**, 179–210.

BALERNA, M., FOSSET, M., CHICHEPORTICHE, R., ROMEY, G. & LAZDUNSKI, M. (1975). Constitution and properties of axonal membranes of crustacean nerves. *Biochemistry, N.Y.* **14**, 5500–5511.

BARCHI, R., WEIGELE, J., CHALIKIAN, D. & MURPHY, L. (1979). Muscle surface membranes. Preparative methods affect apparent chemical properties and neurotoxin binding. *Biochim. biophys. Acta* **550**, 59–76.

BARCHI, R. L., WONG, M. & BONILLA, E. (1977). Isolation and characterization of muscle sarcolemma using surface-specific labels. *Proc. natn. Acad. Sci. U.S.A.* **74**, 34–38.

CATTERALL, W. (1977). Activation of the action potential Na^+ ionophore by neurotoxins. *J. biol. Chem.* **252**, 8669–8676.

CHACKO, G. K., BARNOLA, F. V., VILLEGAS, R. & GOLDMAN, D. E. (1974). The binding of tetrodotoxin to axonal membrane fractions isolated from garfish olfactory nerve. *Biochim. biophys. Acta* **373**, 308–312.

COLQUHOUN, D., RANG, H. P. & RITCHIE, J. M. (1974). The binding of tetrodotoxin and α-bungarotoxin to normal and denervated muscle. *J. Physiol.* **240**, 199–226.

EISENMAN, G. (1965). Some elementary factors involved in specific ion permeation. *Proceedings of the 23rd International Congress on Physiologic Sciences (Tokyo)*, pp. 489–506. Amsterdam: Excerpta Medica Foundation.

FESTOFF, B. & ENGEL, E. K. (1974). *In vitro* analysis of the general properties and junctional receptor characteristics of skeletal muscle membranes. Isolation, purification and partial characterization of sarcolemma fragments. *Proc. natn. Acad. Sci. U.S.A.* **71**, 2435–2439.

GRAMPP, W., HARRIS, J. B. & THESLEFF, S. (1971). Inhibition of denervation changes in skeletal muscle by blockers of protein synthesis. *J. Physiol.* **221**, 743–754.

HAFEMANN, D. R. (1972). Binding of radioactive tetrodotoxin to nerve membrane preparations. *Biochim. biophys. Acta* **226**, 584–556.

HARRIS, J. B. & THESLEFF, S. (1971). Studies on tetrodotoxin resistant action potentials in denervated skeletal muscle. *Acta physiol. scand.* **83**, 382–388.

HENDERSON, R., RITCHIE, J. M. & STRICHARTZ, G. R. (1974). Evidence that tetrodotoxin and saxitoxin act at a metal cation binding site in the sodium channels of nerve membrane. *Proc. natn. Acad. Sci. U.S.A.* **71**, 3936–3940.

HODGKIN, A. & HUXLEY, A. (1952). A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol.* **117**, 500–544.

JAIMOVICH, E., VENOSA, R., SHRAGER, P. & HOROWICZ, P. (1976). The density and distribution of tetrodotoxin receptor in normal and detubulated frog sartorius muscle. *J. gen. Physiol.* **67**, 399–889.

NARAHASHI, T. (1974). Chemicals as tools in the study of excitable membranes. *Physiol. Rev.* **54**, 814–889.

REDFERN, P. & THESLEFF, S. (1971). Action potential generation in denervated rat skeletal muscle. II. The action of tetrodotoxin. *Acta physiol. scand.* **82**, 70–78.

REED, J. & RAFTERY, M. (1976). Properties of the tetrodotoxin binding component in plasma membranes isolated from *Eletrophorus electricus*. *Biochemistry, N.Y.* **15**, 944–953.

RITCHIE, J. M. (1975). Binding of tetrodotoxin and saxitoxin to sodium channels. *Phil. Trans. R. Soc. Lond. Ser. B* **270**, 318–336.

RITCHIE, J. M. & ROGART, R. B. (1976). The binding of labelled saxitoxin to normal and denervated muscle. *J. Physiol.* **263**, 129–130*P*.

RITCHIE, J. M. & ROGART, R. B. (1977*a*). Characterization of exchange labelled saxitoxin and the origin of linear uptake by excitable tissue. *Molec. Pharmacol.* **13**, 1136–1146.

RITCHIE, J. M. & ROGART, R. B. (1977*b*). The binding of saxitoxin and tetrodotoxin to excitable tissues. *Rev. Physiol. Biochem. Pharmacol.* **79**, 2–50.

RITCHIE, J. M., ROGART, R. B. & STRICHARTZ, G. (1976). A new method for labelling saxitoxin and its binding to non-myelinated fibres of the rabbit vagus, lobster walking leg, and garfish olfactory nerve. *J. Physiol.* **261**, 477–494.

SHRAGER, P. & PROFERA, C. (1973). Inhibition of the receptor for tetrodotoxin in nerve membranes by reagents modifying carboxyl groups. *Biochim. biophys. Acta* **318**, 141–146.

STRONG, P. N., SMITH, J. T. & KEANA, J. F. (1973). A convenient bioassay for detecting nanomolar concentrations of tetrodotoxin. *Toxicon* **11**, 433–438.

VILLEGAS, R., BARNOLA, F. V. & CAMEJO, G. (1973). Action of proteases and phospholipases on tetrodotoxin binding to axolemma preparations isolated from lobster nerve fibers. *Biochim. biophys. Acta.* **318**, 61–68.

WEIGELE, J. & BARCHI, R. (1978*a*). Analysis of saxitoxin binding in isolated rat synaptosomes using a rapid filtration assay. *FEBS Lett.* **91**, 310–314.

WEIGELE, J. & BARCHI, R. (1978*b*). Saxitoxin binding to the mammalian sodium channel: competition by monovalent and divalent cations. *FEBS Lett.* **95**, 49–53.