SAXITOXIN BINDING TO THE MAMMALIAN SODIUM CHANNEL

Competition by monovalent and divalent cations

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1. Introduction

In nerve and muscle an action potential is initiated by a large but transient increase in membrane conductance to Na^+. Current concepts of excitable membrane organization consider this conductance increase to be mediated by a specific intrinsic membrane protein which provides a voltage-dependent aqueous pathway for cations across the membrane hydrophobic interior. Experiments with voltage-clamped axons have demonstrated that this channel possesses strong selectivity among small monovalent cations [1], and various pore models have been proposed to explain the origin of this selectivity [1,2].

A number of natural neurotoxins act by specifically blocking cation movement through this sodium channel. Tetrodotoxin (TTX) and saxitoxin (STX), two neurotoxins of this type, bind reversibly but with high affinity to the channel [3]. These toxins have been purified and prepared in radiolabelled form, and are proving to be extremely useful in studies aimed at the chemical characterization and purification of the sodium channel.

Since TTX and STX appear to bind in the sodium channel at the site responsible for ion selectivity [3], measurement of the competition for binding between these toxins and various cations should yield useful information about this selectivity region. Equilibrium measurements of such binding have been carried out in garfish olfactory nerve and in electroplax using equilibrium dialysis or gel filtration techniques [4,5]. We describe here competitive studies with STX in the rat synaptosomal sodium channel using a rapid filtration equilibrium technique [6]. Results from this mammalian system are compared with those reported in lower forms.

2. Methods

Saxitoxin was the generous gift of Dr E. J. Schantz of the University of Wisconsin. Tritiation of the STX was carried out by the New England Nuclear Corp. by the methods in [7]. Storage, purification, and assay procedures have been reported [6,7].

Synaptosomes were prepared by the method in [8]. [^{3}H]STX binding to purified synaptosomes was determined using rapid filtration on glass fiber filters as in [6]. For pH dependency experiments a buffer containing Na_2HPO_4 (20 mM) and NaCl (100 mM) was used. In all other experiments, pH was maintained with Tris (20 mM) and further components were added as indicated.

Dissociation constants for the STX–channel complex were calculated from specific binding isotherms. Total binding curves were constructed using 8–10 concentration points between 0.1 and 8×10^{-9} M STX. Four to six additional points were obtained in the presence of excess unlabelled tetrodotoxin (10^{-6} M) to yield a non-specific binding curve. Specific binding was defined as the difference between binding in the presence and absence of excess unlabelled ligand. Computer regression analysis was used to extract dissociation constant (K_d) and total binding site den-

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sity (B_{max}) from double reciprocal and Scatchard plots of the specific binding.

For the determination of inhibitor constants (K_i) for various cations, complete saturation curves of STX binding were constructed under control conditions (200 mM choline chloride, 20 mM Tris, pH 7.5) and in the presence of 3–5 constant concentrations of the test cation (0–200 mM). Total ionic strength was kept constant by the addition of choline chloride. For each complete curve in the presence of a test cation, a value of K_i was calculated from the app. K_d for the STX–channel complex, the inhibitor concentration, and the K_d for a parallel-run control curve. Reported values for K_i represent the averages of the results from 3–8 separate curves.

3. Results

3.1. *Dependency of [^{3}H]STX binding on pH*

We reported that in rat synaptosomes [^{3}H]STX binding at constant ligand concentration decreased with decreasing pH between 6.5 and 5.0 [6]. We have further characterized this loss of binding by determining the app. K_d for the STX–channel complex as well as the B_{max} in purified synaptosomes as a function of pH. The ionic conditions in [6] were duplicated to facilitate comparison. B_{max} remained constant within the limits of experimental error between pH 8.0 and 5.0 in the presence of 100 mM NaCl. With decreasing pH in this range the app. K_d rose 20-fold from 0.4×10^{-9} M, at pH 7.5, to greater than 8×10^{-9} M, at pH 5.0 (fig.1). Analysis of this data by the method in [4] suggests that protonation of a single titratable residue was responsible for the observed change in affinity. The app. pK for this protonatable site derived from the data presented here was 5.9. Since K_d and B_{max} for STX binding were constant between pH 7.0 and 8.0, all further studies were carried out at pH 7.5.

3.2. *Ionic strength and STX binding*

Before investigating the interaction of specific ions with STX binding we studied the effects of variations in ionic strength on the app. K_d for the STX–site complex. Saturation binding curves were constructed at various ionic strengths between $\mu = 0.02$ and $\mu = 0.60$. Choline, a cation impermeant to the sodium channel

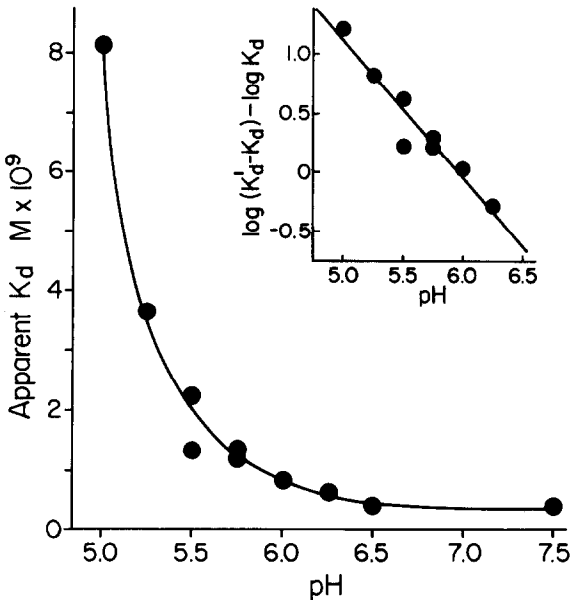

Fig.1. pH dependence of the app. K_d for STX binding to the synaptosomal sodium channel. The apparent affinity drops rapidly between pH 6.5 and 5.0. Insert: analysis of the pH data by the method in [4]. The pK for the titratable site is 5.9.

which shows no interaction with sodium currents in voltage-clamp experiments [9], was used as the chloride salt to vary ionic strength. All experiments were performed at 0°C and pH 7.5. The app. K_d for STX binding was sensitive to ionic strength, varying between 0.04×10^{-9} M in the presence of 20 mM Tris buffer alone and 0.5×10^{-9} M in the presence of 600 mM choline chloride. Two other cations felt to be impermeant on the basis of voltage-clamp experiments, tetraethylammonium and tetramethylammonium [9,10], were also studied as the chloride salt. Replacing variable portions of choline with either of these two cations produced changes in K_d comparable to those anticipated with the same total concentration of choline chloride alone. Thus the changes in K_d appear to be due to changes in ionic strength rather than to specific interaction of these cations at the STX binding site. B_{max} was not affected by ionic strength within the range studied.

Because of these moderate ionic strength effects, all experiments reported below were carried out at constant 200 mM monovalent cation by the addition of choline chloride unless otherwise indicated.

3.3. *Monovalent cations*

Binding curves for [^{3}H]STX to the sodium channel in purified rat synaptosomes yielded an app. K_d of 0.5×10^{-9} M in the presence of 600 mM choline chloride, at pH 7.5. When 600 mM Na^+, K^+ or Li^+ were substituted for choline, the app. K_d for STX binding was shifted markedly toward higher concentrations (fig.2) although total binding at saturating toxin concentrations remained unchanged.

The dependency of this inhibitory effect on monovalent cation concentration was investigated further for Na^+ by constructing specific binding curves at increasing Na^+ concentrations while total ionic strength was held constant by the addition of choline chloride (fig.3). B_{max} as determined from Scatchard plots or from double reciprocal plots did not vary with Na^+ from 0–200 mM. The app. K_d for STX binding, however, rose linearly as a function of Na^+ concentration over this range, and the diagnostic plots of the binding isotherms at each concentration indicated competition of this cation with STX at a common site. The K_d calculated from this and similar data for the sodium–site complex is 34 ± 5 mM.

Similar experiments were carried out with a series of additional monovalent cations including K^+, Li^+, Rb^+, Cs^+ and Tl^+. For thallium the nitrate salt was used instead of the chloride salt due to the limited solubility of thallium halides and tetramethylammonium nitrate was used to maintain ionic strength. In all cases competitive inhibition of STX binding was observed with linear increases in app. K_d for the STX–site complex as cation concentration was increased at a constant total ionic strength (fig.4). K_d values for the cation–site complex for each of these monovalent ions are included in table 1. These range between 7 mM for thallium and 147 mM for cesium and proceed in order of highest affinity $Tl^+ > Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$.

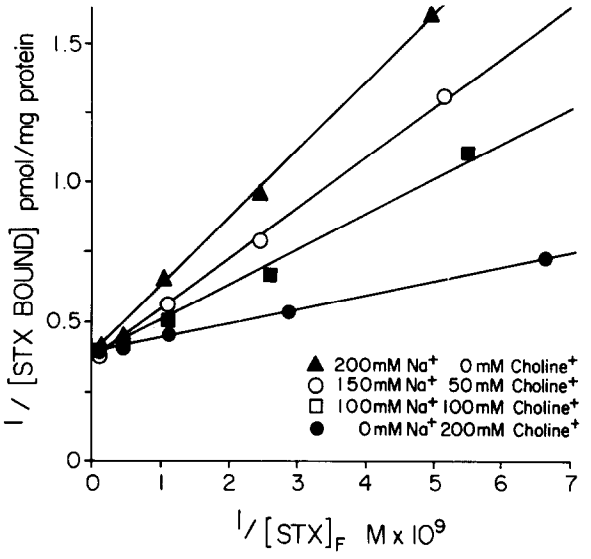

Fig.3. Double reciprocal plots of STX-binding isotherms obtained in the presence of various concentrations of Na^+. In all cases total ionic strength was maintained constant by the addition of choline chloride. Only every other data point is shown for clarity. pH 7.5, 0°C.

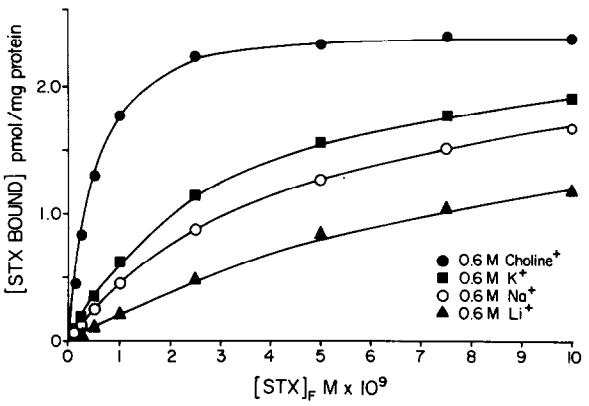

Fig.2. Binding isotherms for STX to the synaptosomal sodium channel in the presence of 600 mM impermeant (choline) or 600 mM permeant (Na^+, K^+, Li^+) cation. In each case B_{max} is unchanged at saturating ligand concentrations although the app. K_d is shifted markedly in the presence of the permeant ions. pH 7.5, 0°C.

3.4. *Divalent cations and organic cations*

Several organic cations and divalent cations were also tested for their ability to inhibit STX binding in a protocol similar to that used for the monovalent cations. Ca^{2+} and Mg^{2+} both strongly inhibit STX binding at 0°C. In the presence of 200 mM choline chloride, 1.8 mM Mg^{2+} doubles the app. K_d for STX–site interaction. A similar doubling is produced by Ca^{2+} at 3.6 mM. Again B_{max} is unaffected by these cations and the K_d for STX increases linearly with increasing concentration of cation from 0–50 mM.

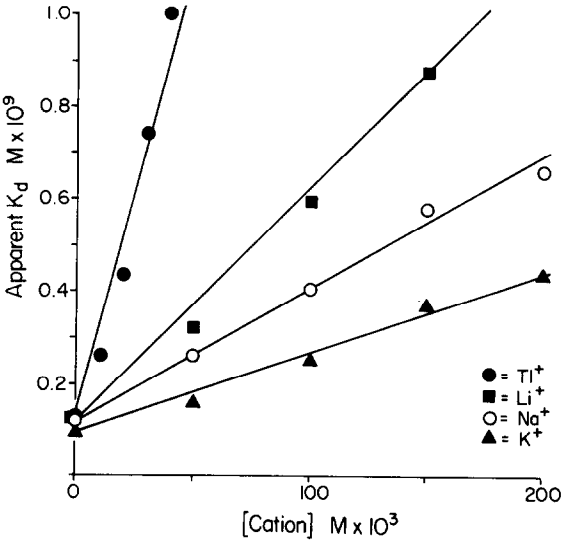

Fig.4. The app. K_d for STX binding as a function of monovalent cation concentration for 4 representative cations. All determinations were made at constant ionic strength, pH 7.5, 0°C. For these and other cations studied, the relationship between app. K_d and cation concentration was linear below 200 mM.

Table 1
Apparent binding constants for cations at the STX binding site

Cation	Determinations[a]	App. K_d (mM)
Tl^+	4	7.2 ± 1.7
Li^+	4	22.6 ± 2.8
Na^+	8	34.3 ± 4.5
K^+	7	53.6 ± 14.0
Rb^+	4	88.8 ± 11.1
Cs^+	8	147.3 ± 18.8
Mg^{2+}	4	1.8 ± 0.2
Ca^{2+}	4	3.6 ± 1.2
NH_4^+	4	20.2 ± 3.0
Guanidinium$^+$	8	6.2 ± 1.1
Formamidinium$^+$	3	9.5 ± 1.5

[a] Each determination represents the calculated K_i from a complete binding curve carried out at a given cation concentration

App. K_d values are given as mean ± SD

The sodium channel is permeable to NH_4^+, guanidinium and formamidinium ions in voltage-clamp experiments [9]. Each of these ions competitively inhibited the binding of [^{3}H]STX with app. K_i values of 9.5 ± 1.5 mM for formamidinium, 6.2 ± 1.1 mM for guanidinium and 20.2 ± 3.0 mM for ammonium ions. The nature of the inhibition appeared identical to that seen with other permeant cations studied.

4. Discussion

Models of the sodium channel in excitable membranes usually explain ion selectivity on the basis of preferential binding of ions to a site or sites within the channel. Considerable evidence suggests that the primary cation binding site responsible for selectivity in the sodium channel lies near the outer surface of the membrane [11]. It has been postulated on the basis of physiological data that the neurotoxins saxitoxin (STX) and tetrodotoxin (TTX) exert their influence by binding reversibly at or near this 'selectivity filter' region. In this report we have shown that, in mammalian tissue, binding of STX is competitively inhibited by various monovalent and divalent cations; similar observations have been reported for TTX in other species [4,5].

The inhibition of STX binding produced by low concentrations of protons is best explained on the basis of protonation of a titratable group at the STX binding site having pK 5.9. Since cation currents through the channel also decrease with pH in a parallel manner [11,12] one can speculate that this titratable group forms an integral part of the ion selectivity region and that protonation makes the channel inaccessable to normally permeant ions.

The binding sequence for monovalent cations inferred by our competition data is that predicted for binding of cations to a high field-strength anionic site [13]. The same sequence has been reported for channel permeability to monovalent cations in the squid axon [14] and for part of that series in the frog node of Ranvier [1] on the basis of voltage-clamp studies. The ratio of binding affinities for the various cations, however, do not coincide quantitatively with the ratios of measured ionic conductances, suggesting that the effective channel conductance for a given ion depends on additional factors such as intrachannel

mobility or subsequent binding to additional sites within the channel. The competitive nature of the inhibition of STX binding by these cations and the correlation between their binding sequence and that determined physiologically does suggest the involvement of the STX binding site in the normal ion-selectivity function of the channel.

Divalent cations appear to affect channel function physiologically by altering the fixed negative surface charge density in the region of the channel external surface rather than by binding within the channel [13]. These cations are, however, quite effective in modifying STX binding. One explanation combining both physiological and biochemical data would be that these divalent cations bind to sites on the periphery of the channel removed from the selectivity region and interfere with STX binding by introducing unfavorable steric or charge factors at these secondary locations. Chemical modification experiments with TTX and STX have indicated that the configuration of most regions of these small molecules are important for binding [15,16].

The values reported here for apparent cation binding constants are somewhat lower than those reported for the sodium channel in electroplax based on competition for TTX binding [4]. The relative binding sequence for the monovalent cations and NH_4^+ however is identical in these two species. Apparent cation affinities reported for the solubilized sodium channel from garfish olfactory nerve again indicate the same binding sequence although the reported dissociation constants were much higher than those obtained in either electroplax or synaptosomes [5]. In published studies with garfish, however, constant ionic strength was not maintained making comparison more difficult. In general, qualitative similarities between the sodium channels in these disparate species seem more impressive than the quantitative differences noted.

The significant variations in app. K_d for STX binding produced by temperature [6], pH, ionic strength and cation concentration indicate that caution must be exercised in interpretation of data and in comparison of results between studies.

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