ANALYSIS OF SAXITOXIN BINDING IN ISOLATED RAT SYNAPTOSOMES USING A RAPID FILTRATION ASSAY

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1. Introduction

Saxitoxin (STX), a low molecular weight neurotoxin derived from dinoflagellates, binds with high specificity to the sodium channel of most excitable membranes [1,2]. In physiological studies STX has been shown to specifically and reversably eliminate the voltage-dependent sodium current at concentrations which suggest at the site of action K_d $0.5–5.0 \times 10^{-9}$ M [13]. A method for labelling STX with ^3H which yields a product of high specific activity and radiopurity has been described [4].

[^{3}H]STX has direct application in studies of the physical and chemical properties of the toxin-binding region of the sodium channel [3,5]. In addition, since it binds specifically to a receptor found only in the surface membrane of excitable cells, its binding can be used as a marker for such membranes during purification [6]. Finally, [^{3}H]STX shows promise as a probe to monitor the solubilization and purification of at least a component of the macromolecular complex mediating sodium conductance.

For the most part, studies with labelled neurotoxins have used either equilibrium dialysis [7] or centrifugation techniques [8] to quantitate binding and have therefore been limited by the amount of material required and by the time needed for definitive analysis. We report here a rapid method using glass fiber filters which allows determination of [^{3}H]STX binding to be carried out on small amounts of tissue within minutes. Equilibrium and rate studies of [^{3}H]STX binding to the sodium channels of isolated rat synaptosomes are described using this method.

2. Methods

Purified saxitoxin was generously provided by Dr E. J. Schantz of the University of Wisconsin. Tritiation was carried out by New England Nuclear Corp. using the aqueous exchange method in [4]. The resultant labelled material was purified by chromatography on a Sephadex G-15 column and analysed further by chromatography on BioRex 70; a gradient from 0.01–1.5 M ammonium acetate (pH 7.0) was used to elute the ion exchange column. STX bioactivity was determined using a frog sciatic nerve sucrose gap system [9]. Standardization of concentration was carried out using standard solutions of STX as the HCl salt obtained from the Food and Drug Administration, Cincinnatti. Physiologic activity of the labelled material coincided with the major peak of label (>80% total counts) when purified [^{3}H]STX was chromatographed on BioRex-70. Specific activity of the [^{3}H]STX ranged from 15–20 Ci/mmol and radiopurity was from 60–80%. Labelled material was stored at −170°C.

Synaptosomes were prepared from rat brains as in [10]. For determination of [^{3}H]STX binding, 50–150 μg synaptosomal protein was incubated with a known concentration of [^{3}H]STX (usually from $0.1–15 \times 10^{-9}$ M) in 3 ml 140 mM NaCl–20 mM Tris–HCl buffer (pH 7.5). Parallel samples were incubated in an identical solution containing in addi-

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tion 1×10^{-6} M unlabelled STX or tetrodotoxin (TTX). After an appropriate equilibration interval (30 min for routine analyses) samples were rapidly filtered on a vacuum manifold through pre-wetted Reeve Angle ultra-fine glass fiber filters having an effective pore size of 0.1 μm. The filters were quickly washed with three 5 ml aliquots cold buffer within 10 s. [^{3}H]STX bound to material retained on the filters was quantitated by liquid scintillation counting; in all cases counting efficiency was determined directly by subsequent addition of an internal standard. Equilibrium dialysis and centrifugation assays were carried out as in [7,8]. In centrifugation assays, the 200 000 × g pellets were solubilized in 5% SDS prior to counting.

For determination of ligand-channel dissociation rates, [^{3}H]STX (5×10^{-9} M) and synaptosomes were equilibrated in 140 mM NaCl–20 mM Tris HCl buffer (pH 7.5 at room temperature) in a water-jacketted cell under temperature control with mixing. At zero time, 1% vol. 10^{-4} M unlabelled TTX was added. Aliquots were removed at rapid intervals between 0 min and 5 min and filtered as above. Dissociation curves were constructed from % zero time bound-counts remaining at each time point.

For recovery experiments synaptosomal protein was covalently labelled by incubating 300–500 μg protein in 300 μl solution containing 5 mM Tris, pH 7.5, and 10 μCi [^{3}H]NEM (carrier-free) for 5 h at 4°C. The reaction mixture was then dialyzed against several changes of 0.145 M NaCl–Tris buffer (pH 7.5) at 4°C for 24 h.

3. Results and discussion

The binding of [^{3}H]STX to isolated rat synaptosomes was quantitated using rapid filtration on glass fiber filters to effect separation of free ligand from ligand bound to the membrane sodium channels. Over $0.5–15 \times 10^{-9}$ M, STX binding appears to be a combination of a high-affinity saturable component and a low affinity non-saturable component as has been reported in other tissues (fig.1A). Correction of total uptake curves for the contribution of the nonspecific binding yields a hyperbolic specific binding curve which can be replotted to yield a straight line on a Scatchard plot. Combined data from several preparations indicate that this specific binding curve represents a non-cooperative interaction of STX with a single class of sites having K_d $0.8–1.0 \times 10^{-9}$ M, at 10°C.

Parallel binding assays were carried out with the filtration assay and with assays using either equilibrium dialysis or centrifugation to quantitate bound and free ligand, in order to establish the validity of

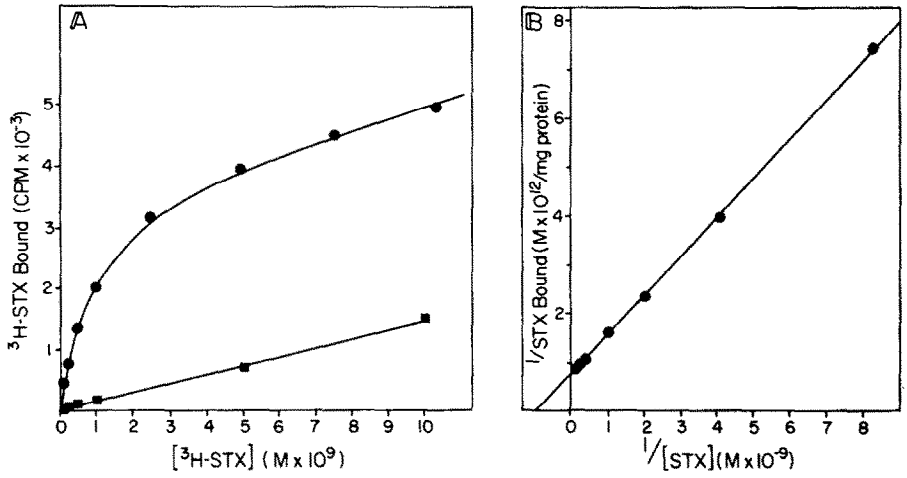

Fig.1.A. Total and nonspecific binding of [^{3}H]STX to isolated rat synaptosomes. For nonspecific binding, 1×10^{-6} M unlabelled TTX is present in the incubation mixture. (•) Total binding; (▪) nonspecific binding. B. Double reciprocal plot of specific binding calculated from the data presented in A.

the rapid filtration method. In one preparation of synaptosomes, equilibrium dialysis at 0°C with varying [^{3}H]STX concentrations in the presence and absence of excess unlabelled toxin yielded K_d 0.4×10^{-9} M and 1.9 pmol/mg protein for the total density of binding sites (B_{max}); parallel filtration assay of the same material yielded K_d 0.5×10^{-9} M and B_{max} 1.8 pmol/mg protein. In another preparation, ligand bound to particulate material was separated by sedimentation at 200 000 × g for 45 min. Determinations made by solubilizing and counting these pellets produced K_d 0.7×10^{-9} M and B_{max} 2.2 pmol/mg protein while the filtration assay yielded K_d 0.5×10^{-9} M and B_{max} 2.0 pmol/mg protein. These and other similar experiments indicated that the results obtained using rapid glass fiber filtration were comparable to those obtained with accepted equilibrium methods.

The % recovery of particulate material in the filtration assay was compared with that obtained in the centrifugation assay using covalently-labelled synaptosomal material. A series of aliquots of [^{3}H]NEM-labelled synaptosomes were either centrifuged at 200 000 × g for 45 min or rapidly filtered, and the amount of labelled material retained on the filter compared to that recovered in the centrifuged pellets. In 4 experiments, the ratio of DPM filter/DPM pellet ranged from 1.05–1.12; in both methods 84–86% total counts was calculated to be bound to the sedimentable particulate material. In a control experiment with [^{3}H]NEM-labelled bovine serum albumin, < 7% total counts in each sample were recovered either on the filter or after centrifugation.

Using the filtration system as described, estimates of total binding sites as a function of protein concentration were carried out to determine the upper limits of the useful concentration range for this procedure. A linear relationship was obtained between protein concentration and total binding sites for all protein concentrations < 1.5 mg/ml when 3 ml total vols. were filtered (fig.2). Above 1.5 mg/ml, significant deviation from linearity developed due at least in part to a reduction in the effective filtration rate. The lower limit of sensitivity depended only on the density of sodium channels in the material to be assayed. In rat synaptosomes, statistically significant binding data could be obtained using < 50 μg total protein in each filtered sample.

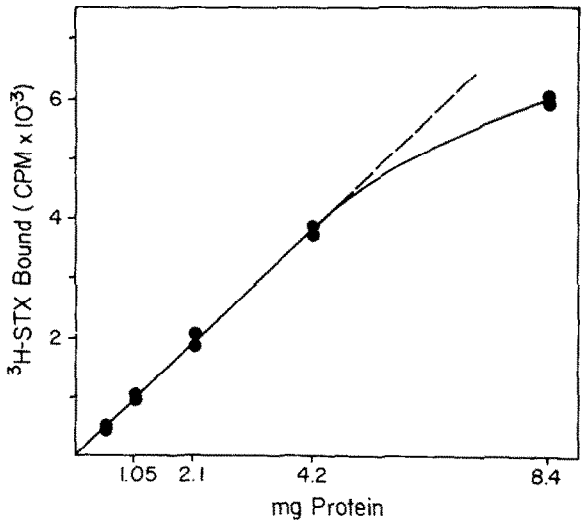

Fig.2. The dependence of specific [^{3}H]STX binding to synaptosomes on total protein concentration in the incubation mixture. The assay is essentially independent of protein concentration when < 4 mg protein are analysed on a given filter.

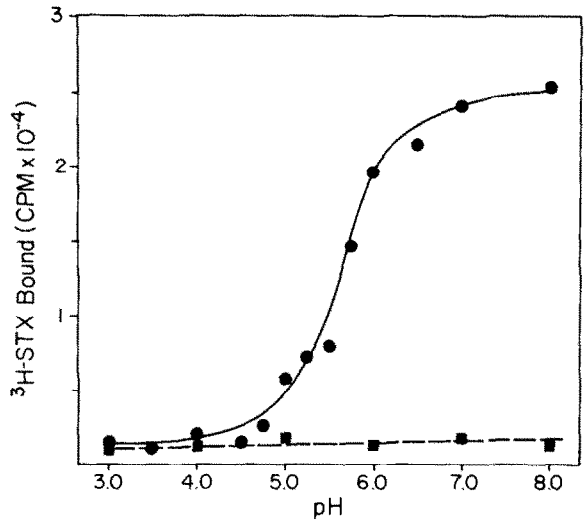

Fig.3. Determinations of [^{3}H]STX binding to a constant amount of synaptosomal protein at various H^+ concentrations. Total binding decreases rapidly between pH 6.0–5.0 while nonspecific binding remains constant, indicating an inhibition of specific toxin binding by a group having app. pK 5.5. (•) Total binding; (▪) nonspecific binding.

Interaction of STX with the excitable membrane sodium channel has been shown to be pH dependent by analysis of sodium currents in the voltage-clamped isolated single node [3]. We found that the binding of tritiated STX to synaptosomal membranes was also pH dependent. Specific STX binding in $Na^+HPO_4^-$ buffer decreased sharply between pH 6.0–5.0 and was virtually undetectable below pH 4.5 (fig.3). The shape of the STX binding versus pH function suggests involvement of a single protonatable group having pK ~ 5.4–5.8. The reported pK for the group mediating the pH effect on STX and TTX binding in rabbit vagus nerve is 5.5 [3] and in frog single node is 5.4 [11].

The apparent equilibrium dissociation constant for STX–channel interaction was significantly temperature dependent. Using the filtration assay on rat synaptosomes, the calculated K_d varied from 0.5×10^{-9} M at 0°C to 2.0×10^{-9} M at 37°C. Analysis of binding data carried out at 10°C intervals from 0–40°C indicated a Q_{10} of 1.48 over this temperature range (fig.4A). When corrected for temperature dependency, the K_d reported here for rat synaptosomes agrees well with those reported for physiological assays in other systems and with earlier binding assays on crude homogenate.

Rapid filtration was used to determine the dissociation rates for the STX–channel complex. At all temperatures studied from 0–20°C, the loss of bound [^{3}H]STX followed a single exponential decline indicating an apparent first order process (fig.4B).

The apparent dissociation rate constants varied markedly with temperature, ranging between 0.182 min^{-1} at 0°C and 2.31 min^{-1} at 20°C. Above 20°C, dissociation became so rapid that multiple points could not be accurately analysed even with the filtration assay. An app. Q_{10} 3.4 was calculated from a total of 9 measurements between 0°C and 20°C (fig.4C). Using these measured off-rates and the measured equilibrium binding constants at each temperature, association rate constants were calculated. These values range between 4×10^8 $min^{-1} \cdot mol^{-1}$ at 0°C and 2.1×10^9 $min^{-1} mol^{-1}$ at 20°C. Analysis of the temperature dependency of these points indicates app. Q_{10} 2.03.

A value of 0.72 min^{-1} has been reported for the dissociation of the TTX– Na^+ channel complex at 4°C in eel electroplax [12]. Using their equilibrium dissociation constant of 6×10^{-9} M for TTX at this temperature, an association rate of 1.2×10^8 $min^{-1} \cdot mol^{-1}$ can be calculated. Thus STX and TTX appear similar in their binding behavior despite the difference in tissues studied, further supporting the close relationship between these two toxins.

The high temperature dependency for [^{3}H]STX binding and dissociation suggests that factors other than simple diffusion control are active. Since present evidence suggests that STX binds within the channel of the sodium conductance system [3], it is possible that the steric configuration of this region varies sufficiently with temperature-dependent molecular motion that binding energy is altered. This might

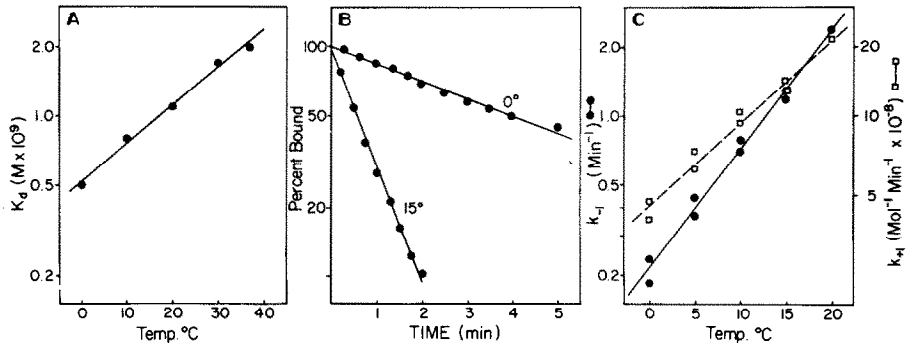

Fig.4.A. Temperature dependency of the apparent equlibrium dissociation constant for the [^{3}H]STX–Na^+ channel complex. The Q_{10} for this process is 1.48. B. Disscociation of the [^{3}H]STX–Na^+ channel complex at 0°C and 15°C. In both cases the dissociation process appears first order. C. Temperature dependency of dissociation rates and of calculated association rates for the complex. Calculated Q_{10} for the dissociation rate is 3.4 while that for the association rate is 2.03. (•) Dissociation rate; (□) association rate.

especially be true if the channel were in fact formed by the quaternary structure of associated subunits within the membrane.

The filtration assay described here is capable of providing accurate estimates of equilibrium dissociation constants and total site density for saxitoxin binding in excitable membrane preparations. Typical saturation binding curves can be constructed using < 2 mg membrane protein, and multiple analyses can be carried out in a short period of time. Using this technique the lower limits of material required for total analysis is reduced 10-fold or more over published equilibrium methods. Since total filtration and washing can be accomplished in < 10 s, dissociation of the receptor–toxin complex is not a limiting factor for most experimental designs, and kinetic analyses can easily be carried out below 20°C which are not possible with other commonly-used techniques.

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