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SIZE CHARACTERISTICS OF THE SOLUBILIZED SODIUM CHANNEL SAXITOXIN BINDING SITE FROM MAMMALIAN SARCOLEMMA

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Summary

The sodium channel saxitoxin binding component from rat sarcolemma was solubilized with medium chain length non-ionic detergents including NP-40, Brij-96 and Lubrol-PX. Phospholipid was required for stability of the binding component. Specific saxitoxin binding was significantly temperature sensitive even with optimal levels of phospholipid present. The solubilized saxitoxin binding component chromatographed on Sepharose 6B at a position corresponding to that of a globular protein of 95—100 Å Stokes radius, but had an apparent $s_{20,w}$ typical of a smaller molecule ($s_{20,w}$ = 9.2—10). Column behavior and $s_{20,w}$ were independent of the specific detergent used for solubilization. Anomalous column behavior may reflect molecular asymmetry, contribution from bound detergent or similar considerations.

Introduction

The generation of an action potential by excitable membranes usually requires rapid modulation of membrane conductance to sodium. Present evidence suggests that this conductance pathway is provided by a channel-forming intrinsic membrane protein which is capable of altering its channel geometry or accessibility in response to changes in membrane potential [1]. Although the behavior of this channel has largely been elucidated by electrophysiological techniques [2], effort is presently being directed at the isolation, purification and biochemical characterization of the channel protein [3,4].

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Saxitoxin, a neutrotoxin which binds with high specificity and affinity to the excitable membrane sodium channel and blocks sodium conductance, can be efficiencly tritiated by a mild aqueous exchange technique [5]. In this form, it has proven valuable as a probe of the sodium channel in situ and can be used as an assay for the presence of intact channels in solution during solubilization procedures [6].

Recently, progress towards the solubilization and purification of the sodium channel tetrodotoxin binding site from eel electroplax has been described [4]. We report here our initial experience with the solubilized sodium channel saxitoxin binding site from mammalian muscle surface membrane and compare some of its characteristics with those described for the electroplax system.

Methods

Sarcolemma was purified from rat hind-limb skeletal muscle using a LiBr extraction technique as previously reported [7]. Purified sarcolemma was stored at −20°C prior to use. Unlabelled saxitoxin was a generous gift of Dr. E.J. Schantz of the University of Wisconsin. [^{3}H]Saxitoxin was prepared and assayed as previously described [5,8]. Lubrol-PX was obtained from Sigma Chemical Corporation, St. Louis, MO; NP-40 from Gallard-Schlesinger Chemical Corporation, Carle Place, NY, and Brij-96 from Emulsion Engineering Corporation, Elk Grove Village, IL. Phosphatidylcholine and phosphatidylethanolamine were obtained from Sigma. Asolectin was purchased from the American Lecithin Company, Atlanta, GA.

Purified membranes were suspended for solubilization at 1.0—1.5 mg protein/ml final concentration in buffer (usually 50 mM potassium phosphate, pH 7.4) at 0°C. Sufficient detergent (10% solution in deionized water) was then added to produce a 1% (w/v) final detergent concentration. The mixture was homogenized with 20 strokes of a Teflon-glass homogenizer and centrifuged at 100 000 × g for 30 min; the supernatant was used for subsequent studies on the solubilized channel protein.

Binding of [^{3}H]saxitoxin to isolated intact membranes was quantitated by rapid filtration on glass fiber filters as previously described [8]. Assays of [^{3}H]saxitoxin binding to the solubilized sodium channel binding site were carried out using a modification of the procedure of Lefkowitz et al. [9] which was comparable to that recently reported by Agnew et al. [4]. Aliquots of solubilized material equilibrated with [^{3}H]saxitoxin were layered on small (1.1 × 4 cm) columns of Sephadex G-25 fixed in the mouth of 15-ml centrifuge tubes. These were then spun in a Universal centrifuge at 0°C at low speed (20 × g) for 45 s. Free [^{3}H]saxitoxin exchanged into the gel matrix while [^{3}H]saxitoxin bound to macromolecules was spun through with the excluded volume and was subsequently quantitated by liquid scintillation counting. Most (more than 90%) of the bound material was spun through these columns within the first 15 s. Nonspecific binding was measured in parallel samples to which 10^{-6} M unlabelled tetrodotoxin had been added.

Sepharose 6B columns were prepared and equilibrated in buffer containing 50 mM potassium phosphate (pH 7.4), 0.1% detergent and occasionally 100 mM choline chloride. The columns were run under reverse flow conditions and

pumped at 1—2% of total column volume/min.

Sedimentation coefficient measurements were made using the centrifugation method of Martin and Ames [10]. A Beckman SW-41 rotor having a sample size of 12 ml and a sedimentation distance of 8 cm was used. Each tube contained a continuous 5—20% (w/w) sucrose gradient (10 ml) containing 0.1% detergent, 50 mM potassium phosphate (pH 7.4). 0.5—0.75 ml of solubilized sarcolemma was layered on the gradient and spun at 150 000 × g for 16 h. Gradients were fractionated into 0.75 ml aliquots and each sample assayed for A_{280} and [^{3}H]saxitoxin binding. Parallel control tubes containing globular proteins of known $s_{20,w}$ were run for standardization.

Results

Solubilization of the sodium channel

Isolation of sarcolemma from rat skeletal muscle yielded a membrane fraction enriched 20—40-fold in sodium channels over crude homogenate as determined by specific [^{3}H]saxitoxin binding [11]. This isolated membrane fraction was solubilized using a variety of detergents and examined for persistence of specific saxitoxin binding sites after centrifugation of the solubilized material at 100 000 × g. In general, ionic detergents such as sodium deoxycholate and sodium lauryl sulfate effectively solubilized all membrane protein but resulted in loss of specific saxitoxin binding. Longer chain length non-ionic detergents including Brij-96, NP-40 and Lubrol-PX solubilized somewhat less than 100% of the total membrane protein when used at 1% detergent concentration and 1 mg/ml final membrane protein density. These detergents were, however, capable of successfully solubilizing 50—70% of the initial saxitoxin binding sites in a form which remained in the supernatant following centrifugation at 100 000 × g. Solubilization using Brij-96, NP-40 and Lubrol-PX was effective over a rather broad pH range between 7.2 and 7.8 but preservation of specific saxitoxin binding dropped markedly above and below this range. A pH of 7.4 at 0°C was chosen as standard for all subsequent solubilizations.

The solubilized sodium channel retained specific binding capacity for [^{3}H]-saxitoxin. Nonspecific binding of ligand determined in the presence of excess unlabelled saxitoxin or tetrodotoxin was somewhat lower in relation to total ligand binding than that observed with intact sarcolemmal vesicles. A typical specific binding curve for the solubilized channel saxitoxin binding site is shown in Fig. 1. The apparent dissociation constant of the solubilized channel for saxitoxin at 0°C in the presence of 135 mM Na^+ and 5 mM K^+ ranged between $1.5 \cdot 10^{-9}$ M and $3 \cdot 10^{-9}$ M in various preparations. This may be compared with the average value of $1.53 \cdot 10^{-9}$ M which we previously obtained for isolated sarcolemmal membranes [11].

Solubilized saxitoxin binding sites are lost rapidly at 10°C when diluted with solutions containing detergent alone. As has previously been reported for eel electroplax [4,14] the solubilized binding site was significantly stabilized when exogenous lipid was added to the detergent-containing buffer used to dilute the solubilized material. Phosphatidylcholine and phosphatidylethanolamine were successful stabilizers when used in molar ratios between 1 : 4 and 1 : 7 of phospholipid to detergent molecules (phosphatidylcholine more so than phosphati-

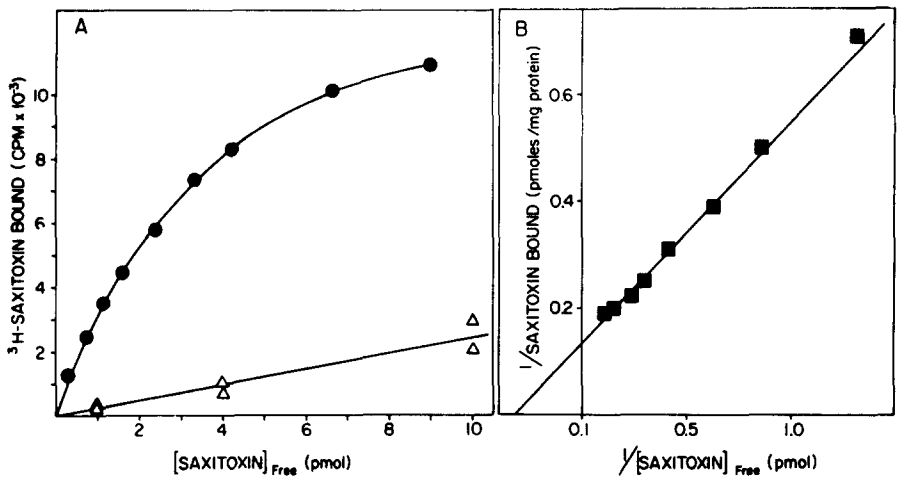

Fig. 1. (A) Binding of [^{3}H]saxitoxin to the solubilized sarcolemmal sodium channel toxin binding site. Sarcolemma was solubilized in 1% NP-40 and assayed in 0.1% NP-40/phosphatidylcholine (5 : 1 molar ratio), 135 mM NaCl, 5 mM KCl, 20 mM Tris (pH 7.4). •, total [^{3}H]saxitoxin binding; △, nonspecific [^{3}H]saxitoxin binding determined in the presence of excess unlabelled ligand. (B) Double-reciprocal plot of the specific binding derived from the data in (A) (specific binding = total — nonspecific), indicating a single high-affinity site having a K_d of approx. $3 \cdot 10^{-9}$ M. In several studies the apparent K_d for saxitoxin under these ionic conditions ranged between 1.5 and $3 \cdot 10^{-9}$ M.

dylethanolamine); cholesterol was not effective. Stabilization to an extent similar to that seen with phosphatidylcholine could also be obtained with mixed soybean phospholipids (asolectin), and this lipid source has been used in some of the experiments reported here. Dilution buffers and column running buffers in all experiments to be described below contained exogenous phospholipid in a ration of 1 : 5 with the indicated detergent.

Efficiency of solubilization was also dependent on the concentration of membrane protein present at the time of solubilization. Most efficient solubilization was obtained at 1.5 mg membrane protein/ml with 0.75—1.0% solubil-

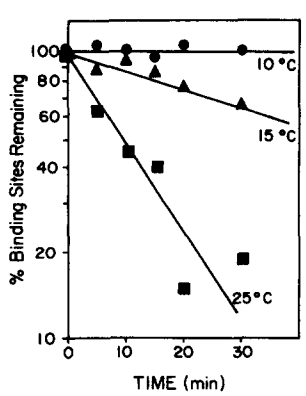

Fig. 2. Specific [^{3}H]saxitoxin binding sites decay with time. In these experiments, sarcolemma was solubilized with 1% Lubrol-PX and diluted in 0.1% Lubrol-PX/phosphatidylcholine (5 : 1). No decay was seen at 0°C over 3 h (not shown) while increasing temperature above 10°C resulted in rapid loss of specific binding (pH 7.4).

izing detergent. Efficiency dropped significantly if protein concentrations below 1 mg/ml were used. Solubilization at 0.75 mg protein/ml yielded a specific activity of saxitoxin binding only about 50% of that obtained when solubilization was carried out with 1.5 or 2.0 mg protein/ml.

The solubilized sodium channel saxitoxin binding site was unstable with respect to temperature even when maintained in the presence of optimal phospholipid to detergent ratios. Thus, the half-time for loss of specific saxitoxin binding in material solubilized with Lubrol-PX and maintained in a buffer containing phosphatidylcholine was in excess of 15 h at 0°C. At 15°C, this half-time decreased to about 1 h, while at 25°C, 50% of specific toxin binding was lost within 10 min (Fig. 2). At all temperatures, loss of specific binding progressed as a simple first-order exponential process.

Chromatography on Sepharose 6B

Sarcolemma solubilized with 1% Lubrol-PX at pH 7.4 was chromatographed at 0°C on 1.5 × 65 cm column of Sepharose 6B using a running buffer containing 0.1% Lubrol-PX/phosphatidylcholine (Fig. 3A). Individual fractions were assayed for total toxin binding using the microcolumn technique; those fractions demonstrating significant [^{3}H]saxitoxin binding were assayed a second time in the presence of 10^{-6} M unlabelled tetrodotoxin in order to determine nonspecific binding. Under these conditions, a single major included peak of specific toxin binding was routinely observed with a K_{av} between 0.22 and 0.28. Occasionally, this peak appeared to resolve into two closely spaced maxima but unequivocal resolution of two populations was not obtained. With Lubrol-PX, a variable but major amount of specific saxitoxin binding was also found in the column void volume, probably representing larger channel aggre-

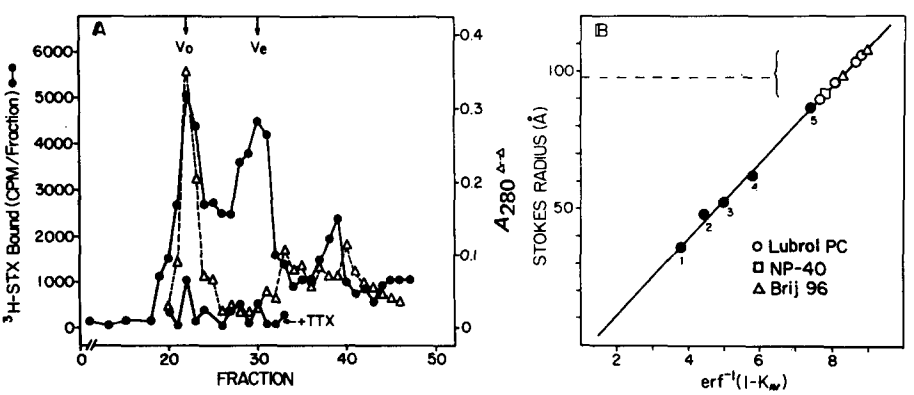

Fig. 3. (A) A typical elution profile obtained for solubilized saxitoxin binding material on a 1.6 × 65 cm column of Sepharose 6B. Sarcolemma was solubilized in 1% Lubrol-PX. The column buffer contained 0.1% Lubrol-PX/phosphatidylcholine, and 50 mM K_2HPO_4 (pH 7.4). Each 1.5 ml fraction was analyzed for total [^{3}H]saxitoxin binding (•), nonspecific [^{3}H]saxitoxin binding (○) and A_{280} (△). (B) Summary of the elution behavior of the included peak of specific [^{3}H]saxitoxin binding activity on similar Sepharose 6B columns following solubilization in various non-ionic detergents. In each case the corresponding detergent was present at 0.1% concentration in the column running buffer. Data are expressed after the method of Ackers [12] to allow comparison with the results of Agnew et al. [4]. Standards are: 1, lactoperoxidase; 2, aldolase; 3, catalase; 4, ferritin; 5, thyroglobulin.

gates or pieces of incompletely dissociated membrane containing saxitoxin binding sites. The majority of the protein was eluted either in the void volume or in the lower molecular weight range between K_{av} of 0.4 and 0.7. Very little protein was eluted with a K_{av} corresponding to that seen for the specific included saxitoxin binding.

Similar columns run in 0.1% NP-40/asolectin as well as Brij-96/phosphatidylcholine or asolectin following solubilization of sarcolemma in the corresponding pure detergent at 1% again produced a single major included peak with a K_{av} of between 0.21 and 0.27. With both of these detergents, however, specific binding in the column void volume was lower than that seen with Lubrol-PX and occasionally the void volume contained no significant specific saxitoxin binding. With these columns, the eluted protein was spread more diffusely throughout the column and a considerable proportion of total protein was found in the region corresponding to the [^{3}H]saxitoxin peak.

Relative elution positions for the included peak of toxin binding on Sepharose 6B were plotted after the method of Ackers [12] in Fig. 3 along with experimental values obtained for a number of standard globular proteins of known Stokes radius. Based on this analysis, the solubilized saxitoxin binding component eluted as a large molecule in a position expected for a globular protein having a Stokes radius between 95 and 105 Å (Fig. 3B).

Estimation of $s_{20,w}$

Sarcolemma solubilized with Lubrol-PX or NP-40 was further analyzed by constant rate centrifugation on 5—20% sucrose gradients using the technique of Martin and Ames [10]. Gradients were overlayered with solubilized sarcolemma and centrifuged for 16 h at 150 000 × *g*. The gradients were then fractionated and analyzed for specific [^{3}H]saxitoxin binding. With each detergent,

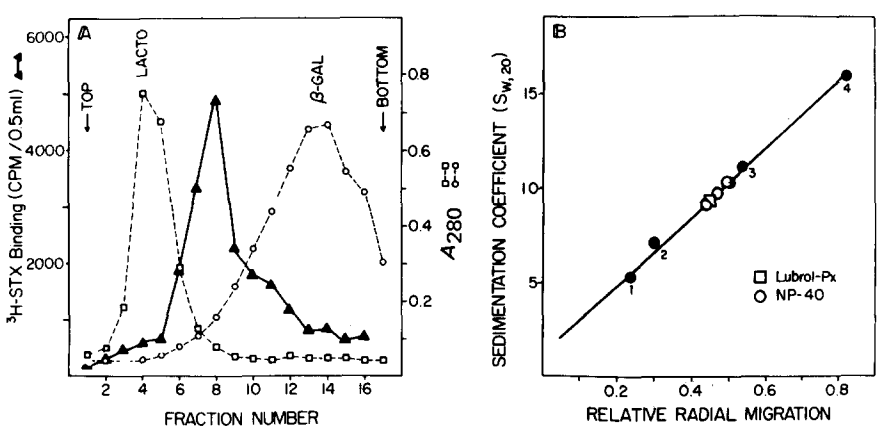

Fig. 4. (A) Distribution of [^{3}H]saxitoxin binding activity on a 5—20% sucrose gradient following centrifugation at 150 000 × *g* for 16 h (see Methods). Sarcolemma was solubilized in 1% NP-40 and gradients contained 0.1% NP-40/asolectin. ▲, [^{3}H]saxitoxin binding; □, A_{280} for a parallel gradient overlayered with lactoperoxidase; ○, A_{280} for a similar control with β-galactosidase. (B) Sedimentation behavior of sarcolemmal saxitoxin binding component solubilized in Lubrol-PX and NP-40 compared with standards of known $s_{20,w}$. 1, lactoperoxidase; 2, lactate dehydrogenase; 3, catalase; 4, β-galactosidase.

a single major peak of specific saxitoxin binding activity was noted on the gradient at a position having a relative migration factor between 0.45 and 0.5 (Fig. 4A). Variable minor regions of toxin binding having higher migration values were occasionally seen. No consistent variation in the relative migration of the major saxitoxin binding peak was noted with regard to the two detergents used. A number of purified globular proteins of known sedimentation coefficient were run in parallel under similar conditions and their migration distances noted.

A plot of the relative migration distance for each of the standard proteins versus their known $s_{20,w}$ was linear (Fig. 4B). When compared to this standard curve, the observed behavior of the solubilized saxitoxin binding site resembled that of a globular protein with an $s_{20,w}$ between 9.2 and 10.0. If one considers a globular protein having a partial specific volume of 0.72 ml/g, this would correspond to an approximate Stokes radius of 65 Å and an expected molecular weight between 250 000 and 350 000.

Discussion

The membrane sodium channel saxitoxin binding component from mammalian sarcolemma can be solubilized and obtained in solution as a relatively stable preparation which is amenable to further characterization. Effective solubilization has been reported in eel electroplax with Lubrol-PX [4]. This detergent was also effective in sarcolemma but is not significantly better than NP-40 or Brij-96. The presence of phospholipid appeared to be necessary for preservation of saxitoxin binding activity in this system as has previously been shown to be the case for sodium channel solubilized from the eel electroplax [4,14]. During initial solubilization this requirement could be supplied by endogenous membrane phospholipids. The observed dependence of solubilization efficiency on membrane protein concentration most likely reflected a sensitivity to the ratio of endogenous phospholipid to detergent molecules. The addition of exogenous phospholipid to the detergent-containing buffers used to dilute the solubilized channel was probably needed to prevent depletion of channel-associated phospholipid by exchange into phospholipid-free detergent vesicles. We found that mixed soybean phospholipids appeared about as effective as pure phosphatidylcholine in satisfying this requirement.

The behavior of the mammalian solubilized saxitoxin binding component on Sepharose 6B columns and on continuous sucrose gradients was comparable to that reported for the eel electroplax sodium channel tetrodotoxin binding site [4]. Both appear anomalously large by gel filtration and considerably smaller by constant rate sedimentation techniques. Several possible explanations exist for this observation. First, the solubilized channel protein may be asymmetric in shape. Since the longest axial dimension will be limiting for gel permeation, significant axial asymmetry will result in elution behavior typical for a much larger globular protein. Frictional factors related to axial asymmetry are also of importance in sedimentation behavior but make a more minor contribution to the values determined in this system. Alternatively, significant deviation from the average specific volume of 0.72 ml/g typical of most soluble proteins would directly affect sedimentation behavior. A mixed micelle of lipid

and protein containing the saxitoxin binding component would have a larger partial specific volume and might be expected to sediment more slowly than a globular protein of the same molecular weight unassociated with lipid. Such a lipid-protein complex would of course also have a larger apparent Stokes radius on gel filtration than anticipated for the protein alone.

Levinson and Ellory have estimated a lower size limit of 230 000 molecular weight for the sodium channel saxitoxin binding component using electron inactivation analysis [13]. This size would correspond well with the size estimates reported here and by Agnew et al. [4] for sedimentation behavior, lending some support for the concept of an asymmetric molecule. Resolution of this question will, however, require purification of the channel protein and quantitation of the bound lipid which must be present, in order to assess its contribution to the observed behavior. At this time, column chromatographic and sedimentation behavior data must be taken as characteristic only of the lipid-protein complex present under these conditions and at best is indicative of a range of potential values for the purified channel protein iself.

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