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Biochemistry

Purification from rat sarcolemma of the saxitoxin-binding component of the excitable membrane sodium channel

(neurotoxin/ion channel/muscle/action potential/glycoprotein affinity chromatography)

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ABSTRACT **The saxitoxin-binding component (SBC) of the excitable membrane sodium channel has been solubilized and purified from rat skeletal muscle sarcolemma. Phospholipid was required in mixed micelles with detergent for stability of the mammalian SBC. Even at optimal detergent-to-phospholipid ratio, the solubilized SBC showed significant temperature-dependent loss of specific toxin binding with time, necessitating maintenance of low temperatures during purification. Characteristics of saxitoxin binding to the solubilized material closely resembled those seen in intact membranes. A weak anion-exchange column was synthesized; it provided rapid 10- to 20-fold purification of the solubilized SBC. Additional necessary purification was obtained by chromatography on immobilized wheat germ agglutinin. Specific saxitoxin-binding activity of the purified material averaged ≈1500 pmol of saxitoxin bound per mg of protein. Three bands were present in this material on sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The purified material sedimented on a sucrose gradient with an apparent $s_{20,w}$ of 9.9 S.**

Action potentials recorded in a wide variety of excitable membranes share a mechanism based on transient sequential increases in membrane conductance to Na^+ and K^+ (1). These voltage- and time-dependent conductance changes are mediated by separate membrane pores or channels presumably formed by intrinsic proteins spanning the bilayer (2). A large body of information has been amassed concerning the current gating properties of these channels during the 30 years since the development of the voltage clamp technique (1, 3). Remarkably little is known, however, about their biochemical features or molecular structure.

Although successful solubilization of sodium channel components was reported as early as 1972 (4), further biochemical analysis has been hindered by difficulties encountered in stabilizing the solubilized channel and in identifying the channel or its components during subsequent purification. The availability of radiolabeled neurotoxins such as tetrodotoxin (TTX) and saxitoxin (STX), which bind with high affinity and specificity within the ion pathway of the channel, has provided a fruitful approach to one of these problems (5). Recent recognition of the role of phospholipids in stabilizing the solubilized channel protein in a conformation capable of binding toxin represents a major advance in biochemical studies of the channel protein (6). On the basis of their findings in this regard, Agnew *et al.* have accomplished the solubilization and purification of a sodium channel TTX-binding component from eel electroplax (7).

Sodium channel density in mammalian tissue is generally much lower than that in eel electroplax, introducing an additional complication to the characterization of the channel in a mammalian system. We report here the successful solubilization and purification of the sodium channel STX-binding component (SBC) from a mammalian source, rat sarcolemma. The techniques used in this purification take advantage of the apparent glycoprotein characteristics of the channel protein and may have more general applicability to sodium channel purification in other excitable membranes. The purified channel components from sarcolemma and from eel electroplax show significant similarities in a number of physical properties.

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MATERIALS AND METHODS

Materials. Immobilized wheat germ agglutinin (WGA), purified phospholipid, and unlabeled TTX were obtained from Sigma. Affi-Gel 202 (which has a 10-atom carboxyl-terminated spacer arm) was purchased from Bio-Rad. Trimethyloxonium tetrofluoroborate was purchased from Ventron (Danvers, MA) and the nonionic detergent NP-40 was a product of BDH Chemicals (Poole, England). Unlabeled STX was the generous gift of E. Schantz of the University of Wisconsin.

Purification of Sarcolemmal Membrane Fraction. Membranes were prepared by a modification of the LiBr extraction procedure of Festoff and Engel (8) as reported (9). This procedure has been scaled up to accept 250 g of muscle with a yield of 30–40 mg of purified membrane protein per run.

Preparation of the Guanidinium-Sepharose Column. Affi-Gel 202 was coupled to diaminodipropylamine (DADPA) by the method of Inman (10) to form a 19-atom extended spacer arm. The terminal primary amine was converted to a guanidinium group by reaction with *O*-methylisourea through an adaptation of the technique of Klee and Richards (11). Briefly, 20 ml of packed Affi-Gel-DADPA was allowed to react with 40 ml of 0.7 M aqueous solution of *O*-methylisourea at pH 10, 2°C, for 24 hr. The product was washed extensively with 0.2 M NaCl followed by water and stored in deionized water at 2°C.

STX-Binding Assays. STX was tritiated, purified, and assayed for biological activity and radiopurity as reported (12). The [^{3}H]STX used here had a specific activity of 15.1 Ci/mmol (1 Ci = 3.7×10^{10} becquerels) and a radiopurity of 70–80%. STX binding to membrane fractions was determined by filtration on micropore glass-fiber filters (12). Binding in solubilized fractions was determined with a modification of the micro column assay described by Lefkowitz *et al.* (13) as previously detailed (14). Activity was corrected for nonspecific binding, column background, and counting efficiency and is reported where indicated as pmol of STX binding per mg of protein. A fluorescamine assay was used for protein determinations (15).

Abbreviations: STX, saxitoxin; TTX, tetrodotoxin; SBC, saxitoxin-binding component of the sodium channel; WGA, wheat germ agglutinin; DADPA, diaminodipropylamine.

RESULTS

Solubilization of the sodium channel SBC

The sarcolemmal SBC was largely inactivated by ionic detergents such as sodium deoxycholate but could be solubilized successfully by medium-chain nonionic detergents, including Brij-96, Lubrol-PX, and NP-40. Of these, NP-40 appeared to be most efficient in this mammalian system.

Under optimal conditions, NP-40 (0.75%) solubilized 60–80% of sarcolemmal membrane protein and 50–70% of the membrane STX-binding sites. Conditions producing most effective solubilization of membrane protein did not coincide with those for maximal recovery of specific STX-binding sites. When aliquots of sarcolemma at a protein concentration of 1 mg/ml were solubilized with increasing concentrations of NP-40 between 0.1% and 4% (wt/vol), the specific activity of the solubilized SBC peaked between 0.75% and 1% NP-40 although the percentage of protein solubilized remained constant between 1% and 4% detergent (Fig. 1*A*). When the concentration of sarcolemma was varied at constant NP-40 concentration (1%), the specific activity of STX binding varied little with concentrations of sarcolemma above 1 mg/ml but dropped abruptly below that point, although the efficiency of membrane solubilization actually improved at these lower concentrations (Fig. 1*B*). In both cases the specific STX-binding activity of the solubilized material decreased when the ratio of membrane protein to detergent was less than about 1:7. Because the isolated sarcolemmal membrane fraction contained approximately 50% lipid by weight, this could be interpreted as a requirement for a similar minimum ratio of endogenous lipid to detergent.

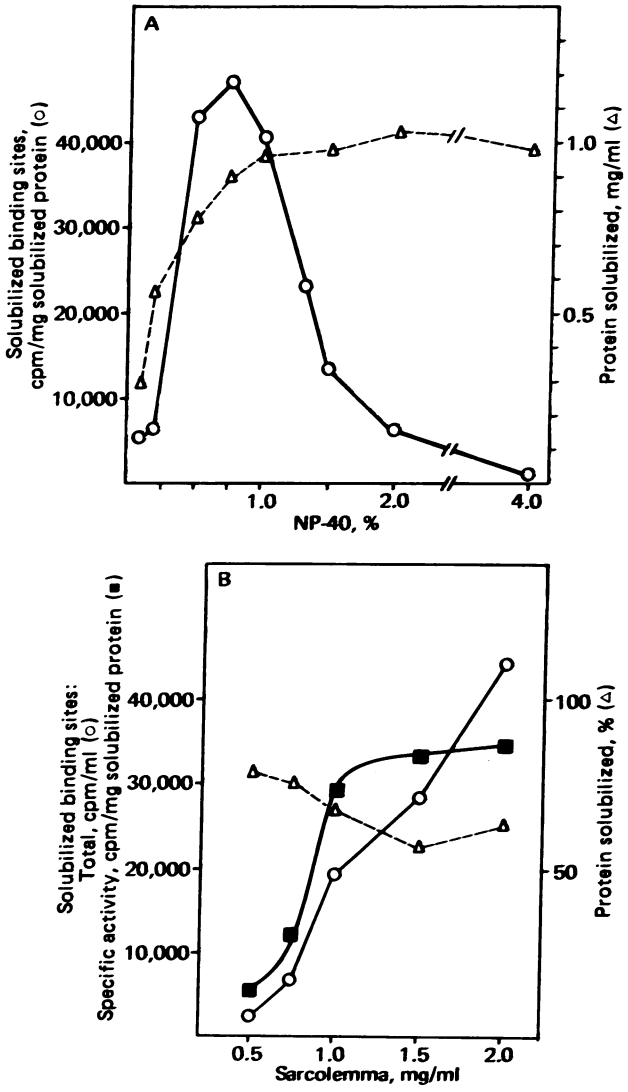

FIG. 1. (*A*) Solubilization of total sarcolemmal protein and the sodium channel SBC with increasing concentrations of NP-40. Total protein solubilized reaches a plateau above 1% NP-40 (△), whereas the SBC specific activity (○) peaks sharply and then declines with increasing detergent concentration. (*B*) Solubilization of total protein and SBC with various concentrations of sarcolemma but constant NP-40 (1%); 50 mM KH_2PO_4 at pH 7.4, 0°C. Total STX-binding activity solubilized (○) increases with increasing initial sarcolemmal concentration. Specific activity of the SBC (■) is constant above 1 mg/ml sarcolemma but falls rapidly at lower sarcolemma-to-detergent ratios.

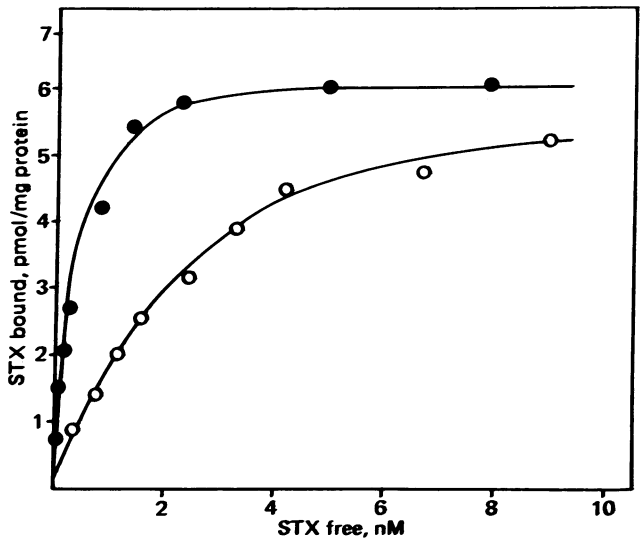

FIG. 2. Specific binding of [^{3}H]STX to the solubilized SBC at 0°C, pH 7.4, in the presence of 100 mM choline chloride (●) or 135 mM NaCl and 5 mM KCl (○). The K_d in 100 mM choline chloride ranged between 0.3 and 0.6 nM, whereas that obtained in the physiologic saline averaged 2–3 nM. Sarcolemma was solubilized at 1 mg of protein per ml in 1% NP-40 and diluted in 0.1% NP-40/phosphatidylcholine for binding assays. Specific binding was determined as the difference between total [^{3}H]STX binding and [^{3}H]STX binding in the presence of 1 μM unlabeled TTX.

Exogenous lipid was required for stability when the SBC was diluted with detergent-containing buffers, even after solubilization under optimal initial conditions. This requirement could be satisfied by the addition of phosphatidylcholine, phosphatidylethanolamine, or mixed soybean phosphatides to detergent-containing solutions in a molar ratio of 1:5 with detergent. Cholesterol and phosphatidylserine were not effective.

All subsequent operations requiring dilution of the solubilized channel were carried out in 0.1% NP-40 containing phosphatidylcholine or purified soybean phosphatides in a 1:5 molar ratio with the detergent. Even under these conditions the solubilized SBC was unstable at room temperature and above (14). The average rate constant for decay of specific STX binding at 10°C was <0.002 min^{-1}, whereas the constants at 15°C and 25°C increased to 0.027 min^{-1} and 0.14 min^{-1}, respectively.

Characteristics of STX binding to the solubilized channel

Equilibrium binding of STX to the SBC was modified by the presence of monovalent and divalent cations. The apparent K_d for STX binding at 5°C in the presence of 100 mM choline chloride (20 mM Tris·HCl, pH 7.4) was 0.3–0.6 nM, whereas the K_d determined at physiologic Na^+ and K^+ concentrations (135 mM Na^+, 5 mM K^+, 20 mM Tris·HCl, pH 7.4) was 2.8 nM

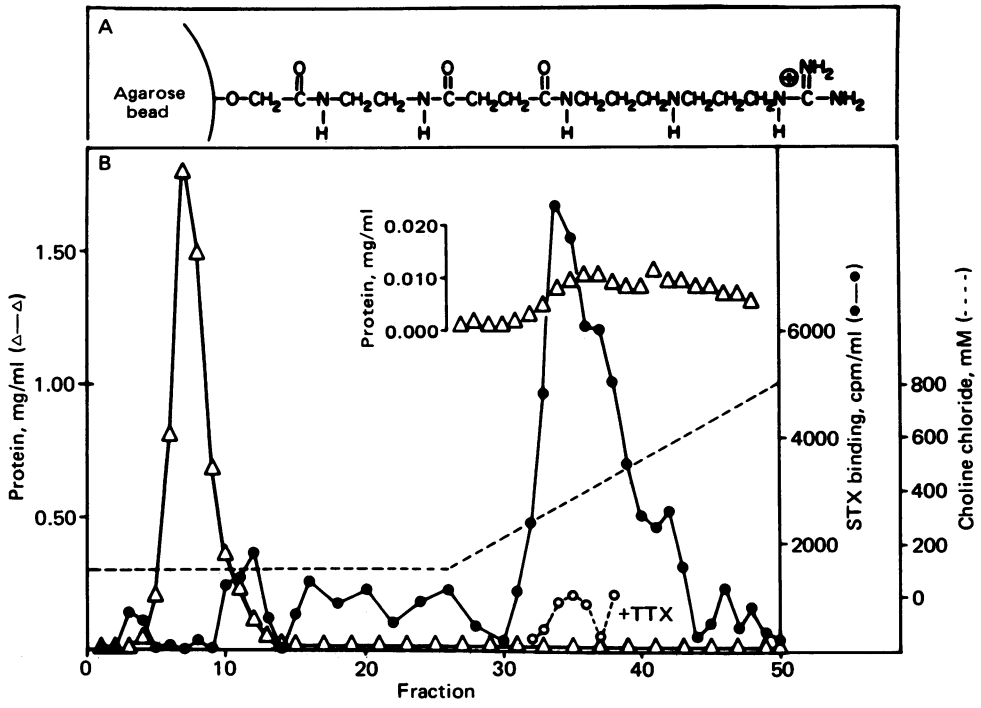

FIG. 3. (*A*) A weak anion-exchange column was synthesized by immobilizing a guanidinium functional group to agarose beads by a 19-atom flexible hydrophilic spacer. (*B*) Ion-exchange chromatography of solubilized sarcolemma on a 2.6 × 8 cm column of agarose beads containing the functional group in *A*. The column was equilibrated in 0.1% NP-40/phosphatidylcholine, 100 mM choline chloride, 50 mM KH_2PO_4 (pH 7.4). Sarcolemma was solubilized at 2 mg of protein per ml with 1% NP-40 and a total of 20 mg was applied at a flow rate of 4 ml/min. The column was eluted at 3 ml/min with a 100–800 mM choline chloride gradient in the column running buffer; 8-ml fractions were collected. △, Protein; ●, total [^{3}H]STX binding; ○, nonspecific [^{3}H]STX binding.

(Fig. 2). Both values are comparable to those obtained with intact sarcolemma (16).

Other monovalent cations competitively inhibited STX binding. The relative affinity sequence at the SBC site shared with STX was $Li^+ > Na^+ > K^+ > Rb^+ \approx Cs^+$, and the relative magnitude of the apparent K_i for each cation was generally comparable to that previously reported for intact synaptosomes and sarcolemma (16, 17). Mg^{2+} and Ca^{2+} also inhibited STX binding in a manner analogous to that seen in intact sarcolemma (16). Specific STX binding to the SBC was inhibited by reaction with trimethyloxonium tetrafluoroborate, a modifier of protein carboxyl groups. Reactions took place at constant pH (7.0, maintained by a pH stat) and were self-terminating within 1–2 min after trimethyloxonium addition by the rapid reaction of that reagent with water. An initial trimethyloxonium concentration of 0.5 mg/ml reduced specific STX binding by 34%, and 2 mg/ml eliminated 80% of specific binding. This effect could be nearly completely prevented by the presence of 10 nM STX in the reaction mixture.

Purification of the solubilized SBC

The solubilized SBC was retained by a DEAE-Sephadex column at low ionic strength (100 mM choline chloride); most of the adsorbed SBC was subsequently eluted at about 400 mM ionic strength by using a 100–600 mM gradient of choline chloride as has been reported with the TTX-binding component of eel electroplax (7). With solubilized sarcolemma, however, a significant percentage of the total applied protein was also adsorbed to the resin and subsequently eluted by the salt gradient, and effective purification of the SBC was not obtained. A weak anion-exchange column was subsequently synthesized by attachment of a guanidinium group through a 19-atom spacer arm to an agarose bead support (Fig. 3*A*). When solubilized sarcolemma was applied to this column (equilibrated in 100 mM choline chloride, 0.1% NP-40/phosphatidylcholine, 50 mM KH_2PO_4, pH 7.4), virtually all of the specific STX-binding activity was adsorbed to the column, although less than 5% of the total protein was retained (Fig. 3*B*). Elution of this column with a choline chloride gradient (100–800 mM) produced a symmetrical peak of specific toxin binding at 350–400 mM salt that contained most of the applied SBC. Purification of 10- to 20-fold could be obtained with good overall recovery by using this column.

The solubilized SBC exhibited glycoprotein characteristics as evidenced by binding to various purified plant lectins (details of these studies will be presented elsewhere). Among the more effective of these lectins was WGA. The SBC bound to immobilized WGA, although this lectin in solution did not modify the binding of [^{3}H]STX. When crude solubilized sarcolemma was applied to a column of WGA immobilized on Sepharose beads, about 2% of the total protein and 95% of the solubilized SBC were retained (Fig. 4*A*). The SBC was not displaced by increasing ionic strength with choline chloride as high as 800 mM, but it was easily eluted with a shallow gradient of *N*-acetylglucosamine (0–20 mM). The WGA-Sepharose column was capable of adsorbing solubilized SBC from a large volume of detergent solution at high ionic strength (400 mM), allowing subsequent elution of the SBC in a small volume (Fig. 4*B*). Recovery of SBC was efficient, in some experiments exceeding 90% of applied specific material.

A protocol for purification of the solubilized SBC was developed, incorporating these two columns. An important component of this purification was the 20- to 40-fold enrichment in specific binding activity obtained by preparation of a sarcolemmal membrane fraction from muscle (Table 1). Between 125 and 150 mg of purified sarcolemmal membranes was solubilized at 2 mg/ml final concentration in 50 mM KH_2PO_4, pH 7.4, at 0°C with 0.75% NP-40 by 30 strokes in a Teflon/glass homogenizer. All subsequent manipulations were carried out at 0–4°C. The solubilized material was centrifuged at 100,000 × *g* for 30 minutes to yield a soluble supernatant fraction. This material was then diluted with an equal volume of 100 mM choline chloride, 0.1% NP-40/PC, 50 mM KH_2PO_4 (pH 7.4) and applied to a 2.6 × 15 cm guanidinium-agarose column at a flow rate of 4 ml/min. The column was preequilibrated in running buffer consisting of 100 mM choline chloride, 40 mM KH_2PO_4 (pH 7.4), 0.1% NP-40/phosphatidylcholine. After loading, the column was washed with 4 bed

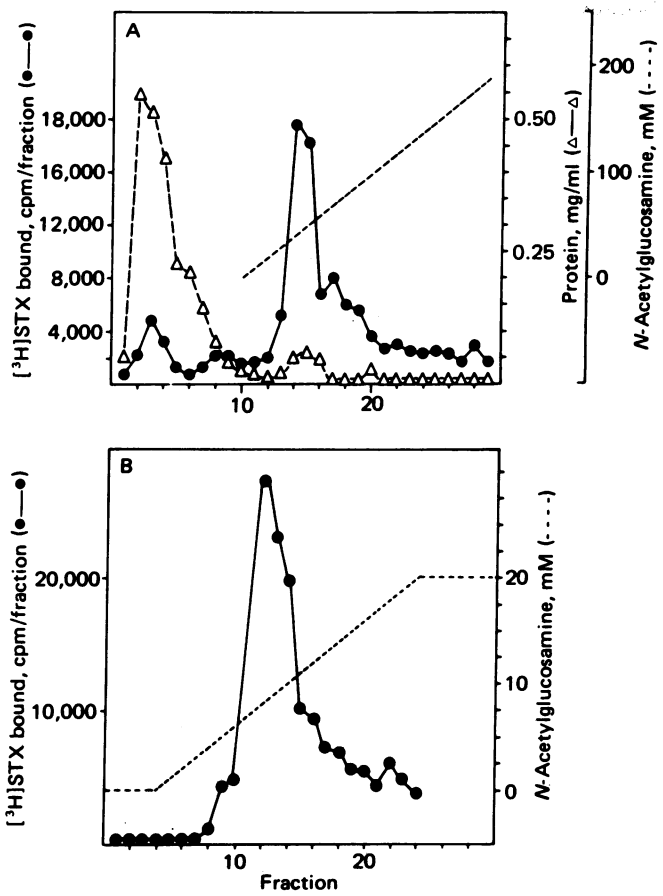

FIG. 4. (A) Affinity chromatography of solubilized sarcolemma on a column containing WGA immobilized to Sepharose 6B beads. The column (1 × 3 cm) was equilibrated in 0.1% NP-40/phosphatidylcholine, 400 mM choline chloride, and 50 mM KH_2PO_4 (pH 7.4). Sarcolemma was solubilized at 2 mg of protein per ml with 1% NP-40, and 3.0 mg was applied to the column in running buffer at 1 ml/min. The column was eluted with a 0–200 mM gradient of *N*-acetylglucosamine at the same flow rate; 5-ml fractions were collected. △, Total protein; ●, total [³H]STX binding. (*B*) Affinity chromatography of SBC prepurified by chromatography on the guanidinium ion-exchange column. The lectin column was equilibrated as in *A*. First, 125 mg of solubilized sarcolemma was applied to the ion-exchange column; the SBC peak fractions were pooled and applied directly (≈4 mg of protein in ≈50 ml) to the lectin column (1.6 × 8 cm) without desalting or concentrating. The lectin column was eluted with a shallow (0–20 mM) gradient of *N*-acetylglucosamine in 400 mM choline chloride, 50 mM KH_2PO_4 (pH 7.4), 0.1% NP-40/phosphatidylcholine; 5-ml fractions were collected. ●, Total [³H]STX binding.

volumes of running buffer and eluted at 3 ml/min with a 100–800 mM choline chloride gradient in running buffer. Fractions (8 ml) were collected and 0.25-ml aliquots were diluted to 0.75 ml and assayed for STX binding. Peak fractions were pooled and samples were removed for protein determination and specific STX binding determinations. The remainder was applied directly at 1.5 ml/min to a 1.6 × 8 cm column of WGA immobilized on Sepharose 6B, which was preequilibrated in running buffer containing 400 mM choline chloride. The column was washed with 4 bed volumes of running buffer and eluted with an 80-ml gradient of *N*-acetylglucosamine (0–20 mM) in running buffer at 1 ml/min flow rate. Fractions (4 ml) were collected and assayed as above. The middle tubes from this gradient contained the purified STX-binding component.

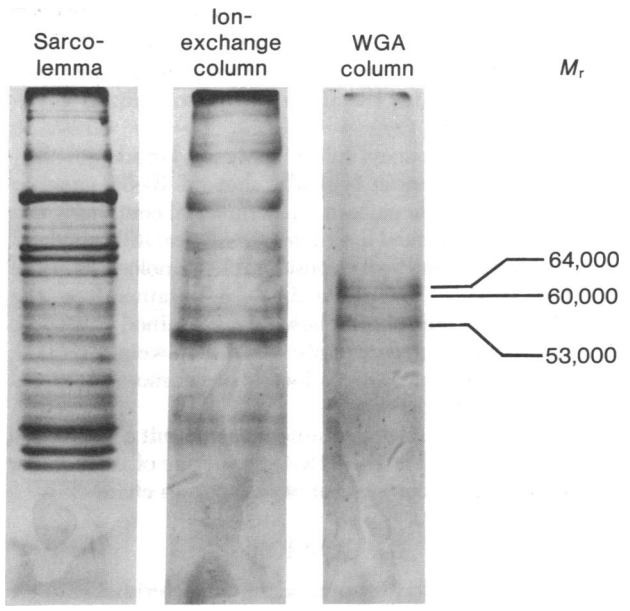

FIG. 5. Gradient sodium dodecyl sulfate/polyacrylamide gel (10–25%) electrophoresis of rat sarcolemmal fraction, SBC-containing peak from the ion-exchange column, and purified SBC after elution from a WGA-Sepharose column. The final SBC peak contains only three major bands, of $M_r \approx 53{,}000$, 60,000, and 64,000, compared to the 40–50 bands usually seen in the starting material.

Specific SBC activity, protein recovery, and purification factors for a complete purification procedure are shown in Table 1. Typically, 125–150 mg of isolated sarcolemmal membrane yielded 75–150 μg of protein having an STX-binding activity between 1000 and 1600 pmol/mg of protein, and peak specific activity in the final elution profile ranged between 1500 and 1800 pmol/mg of protein.

Purified SBC (specific STX binding activity ≈1500 pmol/mg of protein) was centrifuged for 16 hr on a 5–20% sucrose gradient at 160,000 × *g* in a Beckman SW-41 swinging-bucket rotor and its migration distance on the gradient was compared

Table 1. Purification of SBC

Fraction	Protein mg	Protein %	Total binding sites pmol	Total binding sites %	Specific activity, pmol/mg	Purification, fold	
Muscle homogenate					0.25	1	
Isolated sarcolemmal fraction, total	125.0	100	767.0	100	6.1	24	1
Solubilized sarcolemma, total	94.8	76.0	516.0	67.3	5.4	21	0.89
Guanidinium column, pooled peak	2.93	2.4	259.0	34.7	89	356	14.6
WGA-Sepharose column							
Pooled peak	0.150	0.09	118.0	17.4	1079	4316	177
Peak tube	0.0149		22.5		1510	6040	248

with the distances of standard proteins of known $s_{20,w}$ (18). An apparent $s_{20,w}$ of 9.9 S was obtained for the purified SBC. This may be compared to a range of 9.2–10 S previously obtained with the same system for the SBC in crude solubilized sarcolemma (14).

Gradient sodium dodecyl sulfate/polyacrylamide gel electrophoresis of sarcolemma typically showed 40–50 separate bands in a reproducible pattern. Peak material containing the SBC eluted from the initial ion-exchange column still contained a large number of bands with considerable homology to crude sarcolemma (Fig. 5). The purified SBC as obtained from the final WGA-Sepharose column, however, contained only three prominent bands of approximate molecular weight 64,000, 60,000, and 53,000 (Fig. 5). Occasionally one or more additional light bands could be detected.

Although it is too early to comment on subunit composition in the purified SBC, it is likely that at least one of these major bands was initially a component of the sodium channel.

DISCUSSION

The solubilized SBC shares a number of characteristics with the sodium channel *in situ* (16). Both bind STX and TTX with the same high affinity. In both cases toxin binding is competitively inhibited by monovalent cations in a similar sequence and with similar apparent inhibition constants. Both are modified by trimethyloxonium tetrafluoroborate and by water-soluble carbodimides (data not shown) at comparable concentrations and in a manner that is blocked by the presence of STX or TTX. We feel that the SBC that we obtain in soluble form is indeed at least the component of the sodium channel that interacts with STX and TTX in the membrane.

Our data support many of the basic observations made by Agnew *et al.* (7) on the solubilized sodium channel from eel electroplax. The purification procedure reported for the channel in that tissue, however, depended on the interaction of an acidic protein found in electroplax with DEAE-Sephadex during an initial fractionation step for successful enrichment of the channel component. Although solubilized mammalian sarcolemma does not show this behavior, the weak anion-exchange properties provided by our guanidinium column allow comparable purification to be obtained from this mammalian tissue. In addition, the presumed existence of *N*-acetylglucosamine residues on the SBC provides a promising new avenue for further purification by using an immobilized lectin specific for this carbohydrate.

The lower limit of molecular weight for the sodium channel has been estimated to be 230,000–250,000, although an upper limit cannot yet be set (16). If this lower limit is assumed and one molecule of toxin is bound per 250,000-dalton unit, a theoretical maximal specific activity of 4000 pmol/mg of protein is expected. The apparent sedimentation behavior of the purified SBC suggests a somewhat larger molecule ($\approx$300,000 for a globular protein of comparable $s_{20,w}$) and a larger true molecular weight would lower the expected maximal activity. In light of the ease with which STX-binding activity is lost after solubilization, it would not be surprising to find intact material of low binding affinity purifying with the identifiable SBC, again lowering the specific activity of the theoretically pure material. Lastly, the small amount of protein recovered in the final purification peak makes quantitation difficult and the values reported here represent the upper limit of our estimates for protein concentration in all cases. Thus the peak specific activities of about 1500 pmol/mg, while not confirming homogeneity, do suggest a high degree of enrichment. Similar specific activities were obtained by Agnew *et al.* (7) from eel electroplax.

Our results indicate that the purification of sodium channel components from mammalian tissue is practical and that further biochemical characterization should be feasible. Preliminary studies with rat brain synaptosomes and eel electroplax suggest that the STX-binding components from both these sources behave on the guanidinium and WGA columns in a manner similar to that reported here for sarcolemma. These techniques may provide a more general method than those previously reported for the purification of this sodium channel component from a variety of tissues.

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