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The lobster nerve sodium channel: solubilization and purification of the tetrodotoxin receptor protein

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Solubilization and purification of the tetrodotoxin (TTX) binding protein of the lobster walking-leg nerve Na^+ channel were carried out utilizing [^{3}H]tetrodotoxin ([^{3}H]tetrodotoxin) as a marker. The nerve membrane was solubilized with Lubrol-PX and the Na^+ channel protein was purified with diethylaminoethyl Bio-Gel A, Bio-Gel hydroxylapatite powder and two Sepharose 6B columns. Care was taken to keep the temperature of the Na^+ channel preparation as close to 1°C as possible and to use solutions (pH 7.5) that contain Na channel protectors, i.e., egg phosphatidylcholine/Lubrol-PX mixture, TTX, EDTA, EGTA, phenylmethylsulfonyl fluoride, pepstatin A, iodoacetamide, antipain, phosphoramidon, soybean trypsin inhibitor, leupeptin and bacitracin. From an initial specific binding of 20.1 pmol of [^{3}H]TTX/mg protein for the solubilized membrane, the binding increased to 1241 pmol/mg protein for the most active fraction of the last Sepharose 6B column. The [^{3}H]TTX specific binding of the Sepharose 6B fractions correlated with a large peptide of M_r 260 000 (240–280K), although other peptides were also present in lesser amounts.

Introduction

The present work describes the solubilization and purification of the tetrodotoxin (TTX) binding protein of the lobster walking-leg nerve Na^+ channel using [^{3}H]tetrodotoxin ([^{3}H]TTX) as a marker. Similar approaches, utilizing the same or other radiolabeled specific toxins or their derivatives, have been employed to purify Na^+ channels of a variety of excitable cells of vertebrate tissues, e.g., electric eel electroplax [1–5], rat brain [6–8], rat skeletal muscle [9–12], rabbit skeletal muscle [13] and chick heart [14].

The lobster nerve Na^+ channel was found to be more labile to solubilization and purification than the Na^+ channel of vertebrates. Therefore, the stabilizing procedures are described in detail. The results revealed that the [^{3}H]TTX specific binding correlated with a peptide of M_r 260 000 (240–280K), although other peptides were also present.

Experimental Methods

Experimental solutions and Na^+ channel protection

At all stages of the experimental procedures care was taken to keep the temperature of the Na^+ channel preparation as close to 1°C as possi-

Abbreviations: TTX, tetrodotoxin; PC, phosphatidylcholine; P_i, inorganic phosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; DEAE, diethylaminoethyl; HTP, hydroxylapatite powder; SDS, sodium dodecyl sulfate.

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ble and to use solutions (pH 7.5) containing Na^+ channel protectors. Two standard solutions were utilized: solution A for membrane isolation and solubilization, and solution B for Na^+ channel purification. Solution A was of the following composition: 25 mM potassium phosphate, 5 mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 1 μM pepstatin A, 1 mM iodoacetamide, 1 μg/ml antipain, 1 μg/ml phosphoramidon, 20 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin and 100 μg/ml bacitracin. Solution B was prepared by adding to solution A 100 nM [^{3}H]TTX, 0.1% (w/v) Lubrol-PX and 0.183 mg of egg PC/ml. The Lubrol-PX to PC molar ratio is 7:1. TTX was a kind gift of Dr. W.S. Agnew, Yale University School of Medicine, New Haven, CO. The other reagents were obtained from Sigma Chem. Co., St. Louis, MO.

Nerve isolation. Walking-leg nerves of lobster *Panulirus argus* were dissected, immersed in an ice-cold 0.33 M sucrose/25 mM potassium phosphate solution containing 5 mM EDTA and 0.1 mM PMSF, and were frozen immediately at $-70\,^\circ$C. The nerves were kept at this latter temperature until use.

Membrane isolation. A batch of 60 g of frozen nerves was thawed and the external solution was replaced by 120 ml of 0.33 M sucrose prepared in solution A. Thawing was performed by gentle agitation in a cold water bath. As previously indicated, care was taken to keep the temperature of the preparation as close to 1° C as possible. The nerves were homogenized with a Sorvall Omni-Mixer at 12000 rpm for 6 1-min cycles, followed by 10 strokes in a Tri-R glass-teflon homogenizer (clearance 0.15 mm) at 10000 rpm. The volume of the homogenate was adjusted with the same 0.33 M sucrose solution to 600 ml and centrifuged at $65000 \times g$ for 40 min in a Beckman 30 rotor. The supernatant fluids were discarded and the pellets were resuspended in 270 ml of the same 0.33 M sucrose by 10 strokes with the glass-teflon homogenizer. Aliquots of 18.5 ml of the membrane suspension were layered on top of 19 ml of 1.12 M sucrose prepared in solution A and centrifuged at $65000 \times g$ for 60 min in a Beckman SW 28 rotor. The membrane banding at the interface was collected, diluted 3-fold with solution A and centrifuged at $65000 \times g$ for 40 min in a Beckman 30 rotor. The supernatant fluids were resuspended to 10–12 mg of protein/ml of 0.35 M sucrose prepared in solution A.

Solubilization

The membrane suspension was diluted with solution A to about 2 mg of protein/ml and [^{3}H]TTX was added to a final concentration of 100 nM. Solubilization was started by addition of 20% Lubrol-PX to a final concentration of 1% (w/v). The solubilized membrane was maintained during 30 min with occasional stirring and then centrifuged at $100000 \times g$ for 60 min in a Beckman 60 Ti rotor. The supernatant fluids (Lubrol-PX extract) were collected and the pellets were discarded.

Purification

Four purification steps were carried out: ion exchange chromatography with DEAE Bio-Gel A, hydroxylapatite chromatography with Bio-Gel HTP, and two exclusion chromatography steps with Sepharose 6B. DEAE Bio-Gel A and Bio-Gel HTP were obtained from Bio-Rad Laboratories, Richmond, CA. and Sepharose 6B was from Sigma Chemical Co., St. Louis, MO.

DEAE Bio-Gel A. Solid KCl was added to 60 ml of the Lubrol-PX extract to a final concentration of 100 mM KCl. The extract was then mixed with approx. 30 ml of DEAE Bio-Gel A pre-equilibrated with solution B to which 100 mM KCl had been added. After occasional stirring for 30 min, the gel suspension was poured into a 2.5×20 cm glass column and washed with 90 ml of the same solution. The protein was desorbed from the gel with 600 mM KCl prepared in solution B flowing at 100 ml/h and 4.2 ml fractions were collected. The active fractions were pooled.

Bio-Gel HTP. A 12.5 ml vol. of the pooled DEAE Bio-Gel A fractions was made 75 mM potassium phosphate and added to 20 ml of Bio-Gel HTP (hydroxylapatite) pre-equilibrated with solution B made 75 mM potassium phosphate, 250 mM KCl. The mixture was poured into a 2.5×15 cm column, stirred occasionally for 30 min, and washed with 60 ml of the same solution. The bound protein was eluted with the same equilibrating solution made 400 mM potassium phosphate, 100 mM KCl, flowing at 60 ml/h, and 2.5

ml fractions were collected. The active fractions were pooled and concentrated by ultrafiltration to a final vol. of 1 ml in a Spectra/Por cell with a C membrane (MWCO 50 000) at 5 atm pressure.

First Sepharose 6B step. The concentrate was chromatographed over a 1.5 × 40 cm Sepharose 6B column pre-equilibrated with solution B flowing at 12 ml/h, and 2 ml fractions were collected. The fractions from the rising phase of the peak were pooled (4 ml) and concentrated as described before to 0.4 ml.

Second Sepharose 6B step. The concentrate was chromatographed again over a 1 × 20 cm Sepharose 6B column under the same conditions and 1 ml fractions were collected.

SDS-gel electrophoresis

SDS-polyacrylamide gel electrophoresis of the samples was performed according to the method of Laemmli [15] using a slab gel with a 4–12% linear acrylamide gradient. The samples for electrophoresis were prepared as follows: 35 μl of sample buffer (20% glycerol, 1.75% 2-mercaptoethanol, 6.5% SDS, 0.002% Bromophenol blue dye and 75 mM Tris-HCl, pH 6.8) were added to 70 μl of solubilized membrane and of each of the fractions obtained along the purification procedure. These samples were then heated for 2 min in a hot water bath. The latter was prepared by bringing 50 ml of water to boil and removing the container from the heat. 70 μl aliquots of the sample mixtures were then applied to the gels. The standard proteins and their M_r values were: myosin, 200 000; β-galactosidase, 116 250; phosphorylase *B*, 92 500; bovine serum albumin, 66 200; ovalbumin, 45 000; carbonic anhydrase, 31 000; and soybean trypsin inhibitor, 21 500. Reagents and standards were obtained from Bio-Rad Laboratories, Richmond, CA. The gel was silver stained by the method of Oakley et al. [16] and densitometrically scanned with a Beckman DU-8 spectrophotometer.

Protein determination

The protein concentration of the samples was determined by the method of Bradford [17], using the Bio-Rad Protein Assay Dye Reagent and bovine serum albumin as standard protein.

[^{3}H]TTX binding measurement

The method for the preparation of the [^{3}H]TTX (250 μCi/μmol) and the procedure for the assay of the [^{3}H]TTX binding have been described in detail [18,19].

Results and Discussion

The nerve membranes isolated from 60 g nerves, containing 124 mg protein, were used as starting material; 2–3 g walking-leg nerve were obtained from a 1 kg lobster. The ratios (w/w) of protein to total lipid and protein to phospholipid of lobster nerve membrane were 1 : 2 and 1 : 1.3, respectively [20,21]. Precautions were taken to protect the Na^+ channel during membrane isolation and these were maintained throughout the experiment. The experimental procedures were carried out at temperatures as close to 1 °C as possible, since temperatures close to 0 °C have been found to preserve the Na^+ channel [1–14]. The solutions were buffered with 25 mM potassium phosphate at pH 7.5, and the following reagents were added: the antibiotic bacitracin, the chelating agents EDTA and EGTA, and the proteinase inhibitors PMSF, pepstatin A, iodoacetamide, antipain, phosphoramidon, soybean trypsin inhibitor and leupeptin. The same proteinase inhibitors and antibiotic have been utilized to protect the rat brain Na^+ channel [22]. In addition, the TTX receptor was occupied during solubilization and purification, since TTX is known to stabilize the solubilized Na^+ channel protein [1].

Solubilization was carried out with 1% Lubrol-PX and 2 mg membrane protein/ml corresponding to a molar ratio of endogenous phospholipid to detergent of 1 : 5 and, during purification, egg PC and Lubrol-PX were added to the solutions in a PC to detergent molar ratio of 1 : 7, which is the same ratio used to protect the electroplax Na^+ channel [1].

As shown in Table I, out of the 2498 pmol of [^{3}H]TTX bound to the 124 mg of solubilized membrane protein, the Lubrol-PX extract obtained after centrifugation contained 76.7% of the binding and 63.0% of the protein. Fig. 1 illustrates the purification profile of the lobster nerve Na^+ channel. The results are summarized in Table I.

TABLE I

PURIFICATION OF THE Na CHANNEL TTX RECEPTOR PROTEIN

	[^{3}H]TTX bound (pmol)	Protein (mg)	Spec. act. (pmol/mg)	% of initial binding
Solubilized membrane	2498	124	20.1	100
Lubrol-PX extract	1915	78.1	24.5	77
DEAE Bio-Gel A	1328	14.0	94.9	53
Bio-Gel HTP	938	2.43	386	38
First Sepharose 6B				
Fraction 19	43.6	0.0608	717	1.7
Fraction 20	51.1	0.0688	743	2.1
Fraction 21	77.7	0.0876	887	3.1
Total	172	0.217	793	6.9
Second Sepharose 6B				
Fraction 9	28.3	0.0228	1241	1.1

The first purification step was ion-exchange chromatography with DEAE Bio-Gel A (Fig. 1). Several assays were carried out to determine the conditions for the use of the DEAE Bio-Gel A. The best adsorption of the Na^+ channel protein was achieved in the presence of 100 mM KCl. The elution conditions were explored experimentally applying 100–600 mM KCl gradients and it was

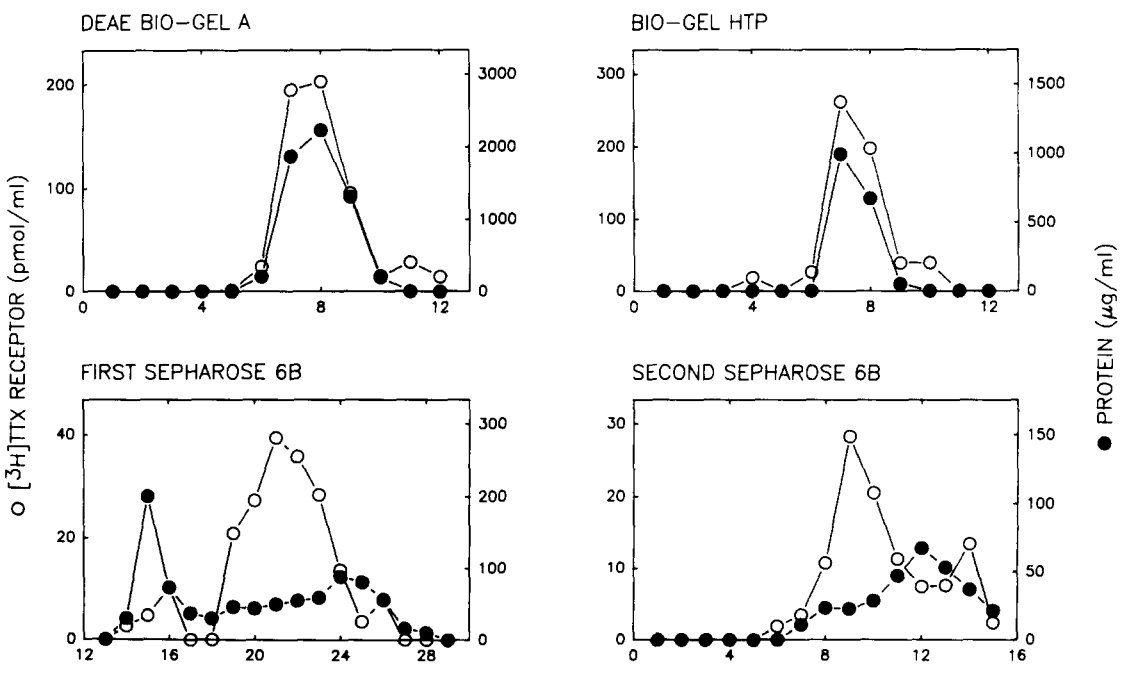

Fig. 1. Purification profiles of the TTX receptor protein. The lobster nerve membrane Lubrol-PX extract was purified by ion-exchange chromatography on DEAE Bio-Gel A, hydroxylapatite chromatography on Bio-Gel HTP, and two steps of gel filtration chromatography on Sepharose 6B. The amount of [^{3}H]TTX bound to the receptor protein and the amount of protein from each fraction are represented. The fractions were of 4.2 ml for the DEAE Bio-Gel A, 2.5 ml for the Bio-Gel HTP, 2 ml for the first Sepharose 6B step and 1 ml for the second Sepharose 6B step.

found that the found Na^+ channel was desorbed without significant fractionation of the protein. Thus, a step gradient of 600 mM KCl was utilized. The pooled peak fractions contained 53% of the initial [^{3}H]TTX binding, purified about 5-fold.

The second purification step was hydroxylapatite chromatography utilizing Bio-Gel HTP (Fig. 1). A strategy similar to that described for ion-exchange chromatography was employed. The best adsorption was obtained in 75 mM potassium phosphate, 250 mM KCl and the best elution from the resin by a step gradient with 400 mM KP_i, 100 mM KCl. As in the case of the DEAE Bio-Gel A, no significant fractionation of the bound proteins was observed. The pooled peak fractions eluted from the hydroxylapatite contained 38% of the initial [^{3}H]TTX binding purified about 19-fold.

The third purification step was by gel exclusion chromatography on a first Sepharose 6B column (Fig. 1). When the [^{3}H]TTX binding of the fractions is compared with the peptide composition (Fig. 2), it appears that the [^{3}H]TTX binding copurifies with a peptide of M_r 260 000 (240–280K), although other peptides were also present. The 260K peptide has the same electrophoretic mobility as vertebrate Na^+ channel preparations [1–14]. For the next purification step, only the fractions that constitute the rising phase of the [^{3}H]TTX binding peak were pooled (fractions 19–21), since the other fractions of the peak contained larger amounts of low-molecular-weight peptides. The pooled peak fractions contained 6.9% of the initial [^{3}H]TTX binding purified about 39-fold.

The pooled fractions obtained from the first Sepharose step were concentrated and rechro-

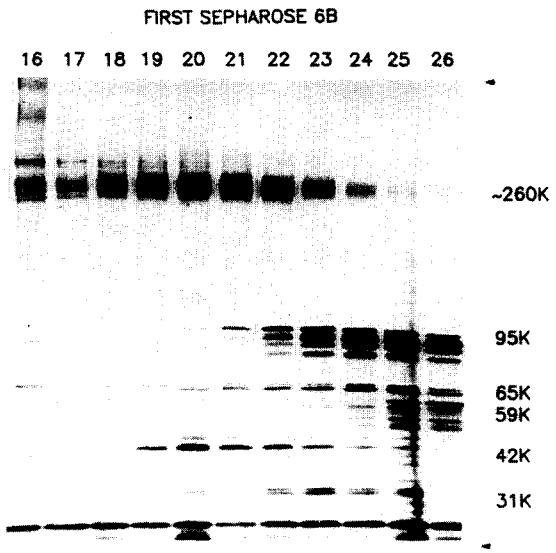

Fig. 2. Peptide composition of the fractions of the first Sepharose 6B step. 70 μl of fractions 16–26 were analyzed by SDS-polyacrylamide gel electrophoresis (4–12% linear acrylamide gradient) and the gel was silver stained. Fraction 21, the most active fraction, bound 887 pmol of [^{3}H]TTX/mg membrane protein. The membrane protein content of the fraction 21 sample was 2.0 μg and the M_r values of its peptide components are indicated. The 260K polypeptide of fraction 21 represented 46.0% of the total silver stain intensity, disregarding the 21.5K soybean trypsin inhibitor peptide located near the dye front.

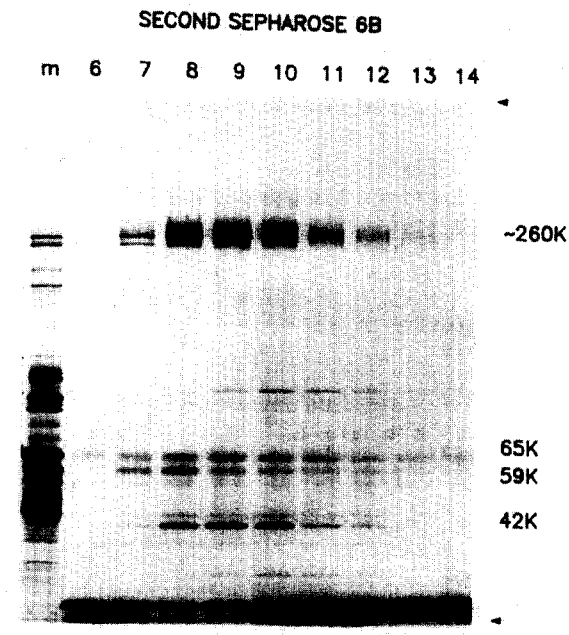

Fig. 3. Peptide composition of the nerve membrane and of the fractions from the second Sepharose 6B fractions. 70 μl of lobster nerve solubilized membrane (m) and of fractions 6–14 from the second Sepharose 6B step were analyzed by SDS-polyacrylamide gel electrophoresis (4–12% linear acrylamide gradient) and the gel was silver stained. Fraction 9, the most active fraction, bound 1241 pmol of [^{3}H]TTX/mg membrane protein. The membrane protein content of the samples of solubilized membrane (m) and fraction 9 are 2.5 and 1.1 μg, respectively. The peptides of fraction 9 are indicated. The 260K polypeptide of fraction 9 represents 60.4% of the total silver stain intensity, disregarding the 21.5K soybean trypsin inhibitor peptide.

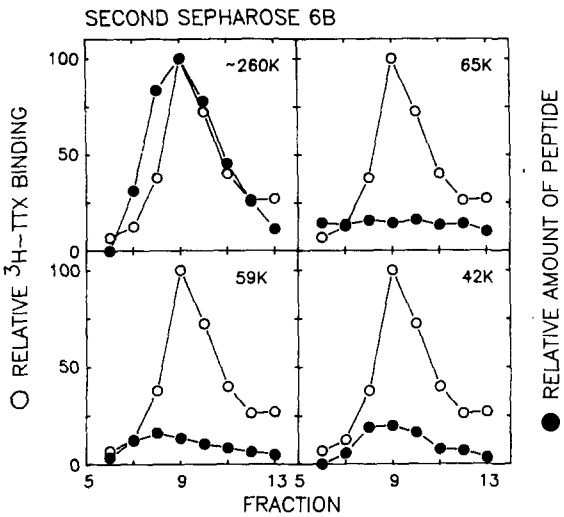

Fig. 4. Correlation of the [^{3}H]TTX binding with peptide distribution in the fractions from the second Sepharose 6B column. The [^{3}H]TTX binding and the amount of the 260K peptide in fraction 9 were set equal to 100. Fraction 9 bound 1241 pmol of [^{3}H]TTX/mg membrane protein and the 260K peptide represents 60.4% of the total stain intensity in the silver stained SDS gel. Fraction 9 contained, in addition to the 260K peptide, lesser amounts of 65K, 59K and 42K peptides.

matographed on a second Sepharose 6B column (Fig. 1). This fourth purification step removed small amounts of some of the components, further purifying the 260K [^{3}H]TTX binding protein (Figs. 3 and 4). A peptide of about 42K present in a lesser amount has a similar distribution although apparently not identical to the large peptide. This 42K peptide could represent a peptide subunit of the native molecule, a proteolytic fragment of a larger species of Na^+ channel polypeptide, or a contaminant which merely tends to cofractionate with the Na^+ channel protein. The significance of this and the other small peptides present is unknown. The peptide of about 21.5K located near the dye front corresponds to the soybean trypsin inhibitor.

Fig. 4 illustrates the co-purification of the [^{3}H]TTX binding with the 260K peptide. This finding agrees with previous results obtained by reconstituting partially purified lobster nerve Na^+ channels into soybean liposomes, after the majority of the low-molecular-weight peptides has been removed [23]. As shown in Table I, peak fraction 9, the most active fraction, contained 1.1% of the initial [^{3}H]TTX binding purified about 62-fold. From an initial binding value of 20.1 pmol of [^{3}H]TTX/mg membrane protein for the solubilized membrane, the binding increased to 1241 pmol/mg protein for the most active fraction.

In summary, this work offers evidence that the TTX receptor protein of the lobster nerve Na^+ channel is a polypeptide of M_r 260000 (240–280K), although in the purified preparation, other peptides were also present in lesser amounts. The lobster nerve Na^+ channel protein was found to be more labile to solubilization and purification than the Na^+ channels of vertebrates [1–14]. This is the first report dealing with the purification of the Na^+ channel of invertebrates.

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