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Neurotoxin-Modulated Uptake of Sodium by Highly Purified Preparations of the Electroplax Tetrodotoxin-Binding Glycopeptide Reconstituted into Lipid Vesicles

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Summary. Using the dialysable detergent CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate), the tetrodotoxin-binding protein from the electroplax of the electric eel has been purified to a high degree of both chemical homogeneity and toxin-binding activity. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the best preparations showed only a single microheterogeneous band at M_r approximately 260,000, despite attempts to visualize smaller bands by sample overloading. Upon dialysis, this material became incorporated into the membranes of small unilamellar vesicles, and in this form the purified protein exhibited tetrodotoxin-binding properties similar to the component in the original electroplax membrane. Furthermore, in the presence of activator neurotoxins the vesicles were able to accumulate isotopic sodium in a manner similar to that previously described for less active or less pure preparations of vesicles containing either mammalian or eel electroplax toxin-binding proteins. Quantitative consideration of the isotopic transport activity of this pure material, along with the high degree of purity of the protein, strongly suggests that the 260-kDa glycopeptide from electroplax is necessary and sufficient to account for the sodium channel function seen in these studies, and eliminates the possible involvement of smaller peptides in the channel phenomena observed.

Key Words sodium channels · reconstitution · polypeptide composition · lipid vesicles · isotopic fluxes

Introduction

The voltage-dependent sodium channel mediates the early depolarizing phase of action potentials in nerve and muscle. Although electrophysiological studies have yielded much information about sodium channel function, little is yet known about the molecular mechanisms responsible for channel operation. A recent approach to this problem has been to purify the channel and to characterize its structure in the hope of illuminating the mechanisms which underlie function. In order to understand such structure/function relationships, it is essential to know the polypeptide composition of the sodium channel apparatus.

Sodium channel-related proteins have been purified based on their ability to bind the high affinity toxins tetrodotoxin (TTX) or saxitoxin (STX) (*see* Agnew, 1984). The polypeptide composition of these binding components (TTXRs or STXRs) isolated from several tissues all have in common a 260-kDa glycopeptide. The TTXR isolated from the electroplax of *Electrophorus electricus* has been shown to be composed of only the 260-kDa component (Miller, Agnew & Levinson, 1983), while the STXR from mammalian tissues have additional smaller peptides associated with them. Thus mammalian muscle STXR appears to consist of a 38-kDa polypeptide in association with the 260-kDa protein (rat sarcolemma and rabbit T-tubule: Tanaka, Eccleston & Barchi, 1983; Kraner, Tanaka & Barchi, 1985; Tanaka, Furman & Barchi, 1986), while in rat brain the large polypeptide is complexed with two subunits of 36 and 33 kDa apparent molecular weights (Hartshorne & Catterall, 1984; Messner & Catterall, 1985; Catterall, 1986).

At issue is whether the structures represented by these purified toxin-binding components are necessary and sufficient for the voltage-dependent transport function of the sodium channel. Perhaps the most direct manner of approaching this question is to assess the functional properties of highly purified material of varying polypeptide composition incorporated into artificial lipid membranes. However, this approach to determining the minimal polypeptide composition required for channel function has been somewhat inconclusive. While several studies of mammalian channels have shown that multimeric compositions are sufficient for channel function in both isotopic flux and planar bilayer systems (Tamkun, Talvenheimo & Catterall, 1984; Hartshorne et al., 1985), the question of

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which peptides are *necessary* is far more problematic. In rat brain, for example, it has been found that dissociation of the 36-kDa peptide from the 260-kDa protein results in the loss of STX binding activity (Messner & Catterall, 1986). Although these results suggest the necessity of this peptide for channel function, such experiments are subject to the criticism that any conditions used to disrupt intermolecular bonds between peptides might also disrupt vital intramolecular bonds on the large glycopeptide, thus denaturing it. Moreover, Elmer et al. (1985) have recently reported isolating the large glycopeptide from rat brain in the absence of other subunits, while still retaining the ability of the protein to bind tetrodotoxin.

For artificial vesicles containing purified preparations of the electric eel sodium channel (consisting primarily of the 260-kDa peptide), both isotopic sodium transport and single sodium channel currents have been observed (Rosenberg, Tomiko & Agnew, 1984*a*,*b*). However, these studies have been criticized on the grounds that the relatively small ion flux seen could have been accounted for by a small fraction of multimeric complexes whose subunits would not have been seen on polyacrylamide gels.

Finally, in more recent studies (Noda et al., 1986; Goldin et al., 1986) cDNA-derived messenger RNAs encoding only the large (260 kDa) glycoprotein from a rat brain sodium channel were shown to direct the formation of functional, TTX-blockable sodium channels in frog oocytes. However, the presence of endogenous small subunits in the oocytes could not be ruled out in these studies, and the methods used did not allow the functional characteristics of these channels to be determined in any detail.

We have studied the question of channel peptide function by reconstituting the highly purified electroplax TTXR into lipid vesicles and measuring the ability of these vesicles to accumulate isotopic sodium in the presence of channel-specific toxins. These studies employed only material of both a high chemical purity (consisting almost exclusively of the 260-kDa glycopeptide) and a high specific activity *after* reconstitution (i.e., about half the protein was able to bind ^{3}H-TTX). We report that the highly purified electroplax TTXR mediates isotopic fluxes in a manner very similar to that previously reported for both mammalian and electroplax channels in which several small polypeptides were also present. In our studies, consideration of the high degree of purity and functionality of our preparations quantitatively eliminates the possibility of small subunits being involved in the sodium channel activity we observed.

Materials and Methods

Materials

Electric eels were obtained from World Wide Scientific Products. Animals were killed by hypothermia, the electric organs removed and frozen at −80°C until needed. CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate) was obtained from Calbiochem; crude soy bean phospholipids (asolectin), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) from egg yolk, and bovine brain phosphatidylserine (PS) were purchased from Sigma Chemical Co. The asolectin was extracted with acetone before use in order to remove neutral lipids, as described by Kagawa and Racker (1971). Veratridine was also purchased from Sigma Chemical Co. It was dissolved in 10% DMSO to a final concentration of 5 mM and stored at −20°C until used. ^{22}Na was purchased from Amersham. BTX was the gift of J.W. Daly of the National Institutes of Health. TTX (citrate-free) was generously supplied by Y. Kishi (Harvard University), tritiated by the Wilzbach procedure, purified, and its specific activity determined by bioassay (Benzer & Raftery, 1972; Levinson, 1975).

Purification of Electroplax TTXRs

Electroplax membranes were prepared as described by Miller et al. (1983). Stored organs were partially thawed, sliced into thin (approx. 1 cm) sections, added to 3.5 volumes of grinding buffer (50 mM sodium phosphate, pH 6.8, 5 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride), and homogenized with a Tekmar SDT-45 homogenizer for 45 sec at full speed. The homogenates were strained through two layers of gauze and centrifuged for 20 min at 30,000 × *g* in the JA-14 rotor of a Beckman J2-21 centrifuge. The supernatants were discarded, and the pellets were resuspended in approximately 5 volumes of grinding buffer. The membranes were then centrifuged at 48,000 × *g* for 60 min, the supernatant was again discarded, and the weighed pellets were resuspended with 1 ml grinding buffer/g pellet. All centrifugation steps were conducted at 2°C. The membrane suspensions were then stored at −80°C until needed.

Purification of the TTX-binding component was carried out using a modification of the procedures described by Miller et al. (1983). The stored membranes were thawed and then solubilized by adding 1 ml of 120 mM CHAPS (in water) to 9 ml of thawed membrane preparation. The detergent/membrane mixture was homogenized with a Potter-Elvejhem apparatus and centrifuged at 100,000 × *g* for 60 min at 2°C in the 60 Ti rotor of a Beckman L8-55 ultracentrifuge. The supernatant containing the solubilized sodium channels was removed and added to an equal volume of DEAE-Sephadex A-25 ion-exchange resin previously equilibrated with ion exchange buffer [50 mM sodium phosphate, pH 6.8, 0.2 M NaCl, 10 mM CHAPS, and 3 mg/ml lipid (asolectin or asolectin/PC (1 : 1))]. After incubation for 30 min, the resin was washed five times with equal volumes of this ion exchange buffer. After the final wash, the ionic strength of this buffer was increased by the addition of an equal volume of 0.6 M NaCl; this was mixed into the resin, and incubated for an additional 30 min. The supernatant containing desorbed TTX-binding polypeptides was then removed, and the resin was rewashed with a 50% volume of the buffer containing 0.4 M NaCl. This second supernatant was combined with the first. ^{3}H-TTX was then added to the

supernatant before further purification (20 μl of 5 μM TTX/ml supernatant) in order to increase the stability of the TTX binding component during the rest of the experiment (Agnew et al., 1978).

These preparations were concentrated over an XM-300 membrane in an Amicon pressure dialyzer to a volume between 2–4 ml. The sample was then placed on a 1.6 × 65 cm Sepharose 6B column that had been freshly equilibrated with column buffer (100 mM sodium phosphate, pH 6.8, 10 mM CHAPS, 3 mg/ml lipid, 100 nM ^{3}H-TTX). The column was eluted at 9 ml/hr and 4-ml fractions were collected. This eluant was further purified by combining the peak fractions of TTX binding activity and repeating the concentration and column elution steps.

The amount of binding protein present throughout the purification, as well as its specific activity, was measured using the Sephadex G-50 assay for TTX-binding developed by Levinson et al. (1979) and the protein assay of Hartree (1972). Purification was also monitored by analyzing the fractions on $NaDoDSO_4$-polyacrylamide gel electrophoresis; samples were run on 5 to 30% acrylamide gradient gels according to the method of Laemmli (1970). Visualization of protein bands on the gels was done by the highly sensitive silver staining method using the kit obtained from New England Nuclear.

Reconstitution into Lipid Vesicles

The purified protein fractions were placed in Spectrapor 32 mm standard cellulose dialysis tubing (mol wt cutoff: 12,000–14,000) and dialyzed against 2 liters of 100 mM sodium phosphate buffer, pH 6.8 at 4°C. The dialysis buffer was changed every 8 hr for three days. Vesicles formed after the first buffer change, as evidenced by the cloudiness of the solution in the dialysis tubing. This extensive dialysis procedure was necessary to remove as much detergent and TTX as possible.

Preparation of Resealed Electroplax Membrane Fragments for Flux Determinations

Resealed membrane fragments were prepared from frozen crude homogenized membranes (*see above*) by thawing the membranes and adding a 50% volume of ice-cold sodium phosphate buffer, 150 mM, pH 6.8. The mixture was homogenized with a Potter-Elvehejm apparatus, placed on ice in a rosette cell, and sonicated using a micro-tipped Branson 350 sonifier with a control setting of 7 at 10% pulse for 10 min. The membranes were then centrifuged at 2°C in a J2-21 Beckman centrifuge at 12,000 × g for 20 min. The supernatant obtained was removed and used for TTX binding studies. Membrane fragments were also prepared as above without the sonication steps and used in the experiments. No significant differences in the final results were found between the membranes prepared with or without sonication.

^{22}Na Uptake Assays

Uptake of ^{22}Na by the reconstituted vesicles was measured by the method of Epstein and Racker (1978). Dowex 50W × 8 was converted to the Tris form as described by Gasko et al. (1976). Pasteur pipettes were plugged with aquarium filter floss and filled with 2 ml of resin. The resin column was washed with ice-cold, isotonic sucrose solution containing 2 mg/ml bovine serum albumin and stored at 4°C. Vesicles were incubated with the appropriate toxins at experimental temperatures for 30 min before uptake measurements were conducted. Unless otherwise noted, the three toxin mixtures used had a final concentration of: (i) 150 μM veratridine, (ii) 150 μM veratridine + 2 μM TTX, or (iii) the buffer only, with no toxins. Observations with 2 μM TTX alone showed the presence of a fraction of spontaneously opening channels; these experiments are considered in the following paper (Duch & Levinson, 1987). For BTX uptake experiments, the final concentration of BTX was 5 μM. To start the assay, 5 μl of ^{22}Na was added to 250 μl of vesicle preparation and immediately vortexed. At various times, 200 μl of this mixture was added to the Dowex column (still at 4°C), and immediately washed through the column with 2 ml of ice-cold sucrose solution. In this way ^{22}Na that was external to the vesicles was adsorbed by the resin, while internalized ^{22}Na was eluted through the column. As long as columns were kept cold, no detectable loss of signal occurred during elution, which took approximately 45 sec. This was determined by using vesicles equilibrated overnight with ^{22}Na, allowing them to remain on the columns for extended periods of time, and measuring the amount of ^{22}Na still retained by the vesicles after elution. A measurable loss of ^{22}Na activity occurred only after 5 min on the column. ^{22}Na was counted with a Beckman Biogamma II gamma counter.

Total internal vesicle volume was assayed by equilibrating the reconstituted preparation with ^{22}Na during a 12-hr incubation at 4°C, then placing 200 μl of the equilibrated vesicles on the ion-exchange column and eluting as above. Incubation of the vesicles for longer times did not result in any further increase in measured vesicular volume.

TTX Binding Experiments

For measurement of the equilibrium dissociation constant K_d, the binding protein preparation was incubated with the various concentrations of ^{3}H-TTX for 30 min at the appropriate temperatures. Bound and free TTX were determined using the G-50 assay of Levinson et al. (1979). For measurement of nonspecific binding, unlabeled TTX was added to tritiated toxin to yield a final concentration of 2 μM unlabeled toxin. The resultant nonspecifically-bound counts were then subtracted from the total counts bound to obtain the amount of ^{3}H-TTX specifically bound.

To measure TTX dissociation rates, nonpelletted membranes, detergent extract, or reconstituted preparations were equilibrated with 100 nM ^{3}H-TTX by incubation for 30 min at 0°C. To determine the dissociation rate, 2 μM unlabeled TTX was added, and aliquots of the mixture were assayed at various times for remaining ^{3}H-TTX bound using the Sephadex G-50 assay (Levinson et al., 1979).

For membrane fragments, total TTX binding and dissociation rates were also determined using the supernatant depletion assay of Levinson (1975). No significant differences were found in the amount of TTX binding measured with either this protocol or the G-50 assay.

To determine the proportion of sodium channels oriented in an inside-out manner, the binding of ^{3}H-TTX to intact vesicles was compared to that of vesicles resolubilized in detergent. Vesi-

Table. Purification of the TTX-binding polypeptide

	Lubrol-PC[a]	CHAPS-PC[c]	CHAPS/asolectin and CHAPS/asolectin/PC (1 : 1)[c]
Purification step	Cumulative yield (% TTX-binding activity)		
Detergent extract	100	100	100
DEAE ion exchange	61	63	76 ± 3
Sepharose column #1	41	24	59 ± 3
Sepharose column #2	26	8	38 ± 2
	Specific activity after purification (pmol TTX bound/mg protein)		
Before reconstitution	1700–2000	1400	2387 ± 140
After reconstitution	400–500[b]	140	2040 ± 157

[a] Agnew et al., 1978.
[b] Rosenberg et al., 1984*a*.
[c] This study.

cles were disrupted by the addition of ice-cold 10% Lubrol-PX (Sigma Chemicals) to the vesicle suspension to give a final concentration of 0.1% along with ^{3}H-TTX to give a 200-nM concentration. Bound ^{3}H-TTX was then measured in both intact and solubilized vesicles using the Sephadex G-50 assay described above.

Vesicle Morphometry

All procedures and electron micrography were kindly performed by Mr. T. Cummings and Dr. T. Johnson at Colorado State University, Department of Anatomy. Reconstituted vesicles were prepared for freeze-fracture as described by Phillips and Boyne (1984). After preparation, samples were freeze-fractured according to the methods of Hudson, Rash & Graham (1979). The frozen samples were cleaved in a Balzers 301 freeze-etch device with the vacuum set at 1.5×10^{-6} millibars, and replicated with platinum using an electron beam gun. Platinum deposition was immediate after fracture. Replica thickness was monitored and regulated by a quartz crystal thin film monitor. Specimens were thawed in the reconstitution buffer, the vesicles digested with Clorox bleach and the replicas picked up on 200–400 mesh grids for EM analysis. All EM samples were viewed on a Phillips 400T Transmission Electron Microscope operated at 100 kV.

Vesicle diameters were measured directly from the micrographs. Since the platinum source shadowed the replica at an angle of 45 degrees relative to the fracture plane, vesicles that were cleaved reasonably close to the equator would have had approximately bisecting shadowing patterns. Thus vesicles with clearly nonbisecting shadowing patterns (i.e., the shadow line was less than half the apparent diameter of the replica) were judged to have been split far from the equator and were not counted. It should be noted that failure to split exactly at the equator does not seriously underestimate true diameter, since sections up to half a radius away from the equator would have diameters only 13% shorter than the true diameter.

Since the probability of a vesicle appearing in the fracture plane is related to its size, the raw numbers were corrected as follows. First, the diameters were grouped into intervals of 20 nm. Then for each vesicle size group, the number of vesicles per ml solution was estimated by calculating the volume of each size group relative to the volume of solution represented in the negative. For example, for vesicles with 100 nm diameters, the corresponding solution volume represented in the negative would be the length of the negative times its width times the diameter of the vesicle (100 nm). Vesicles with this diameter, but whose center was at a distance greater than 100 nm away from the plane of the fracture, would neither be seen nor counted. This volume, and the number of vesicles contained in it could then be extrapolated to 1 ml. In this way, the normalized distributions of vesicular size, volume and number were obtained. Because vesicles not split in the center were not counted, the volumes obtained in this manner were somewhat lower limits, although the relative distribution of volumes should have been unaffected. A total of 587 vesicles were counted to obtain these estimates. These vesicles were apparently all unilamellar, as no obvious multilamellar vesicles were seen in these studies.

Results

The studies presented here were designed to assess the purification and reconstitution of the sodium channel in CHAPS detergent buffers in three stages. First, the chemical and functional purity of the isolated TTX-binding component (TTXR) was examined. Second, the TTX-binding properties of the reconstituted protein were compared with the binding properties of the sodium channel *in situ*. Finally, the ability of the reconstituted TTXR to carry out toxin-mediated fluxes was determined.

Purification of TTXR from the Eel Electroplax

Two different lipid compositions, asolectin or asolectin/PC (1 : 1), were used in the CHAPS buffers. No significant differences were found between the chemical and functional purities, or the final yields of channels purified in either of these buffers. Elution patterns from the two Sepharose 6B column separations of one experiment are shown in Fig. 1. It can be seen that TTX binding elutes coinciden-

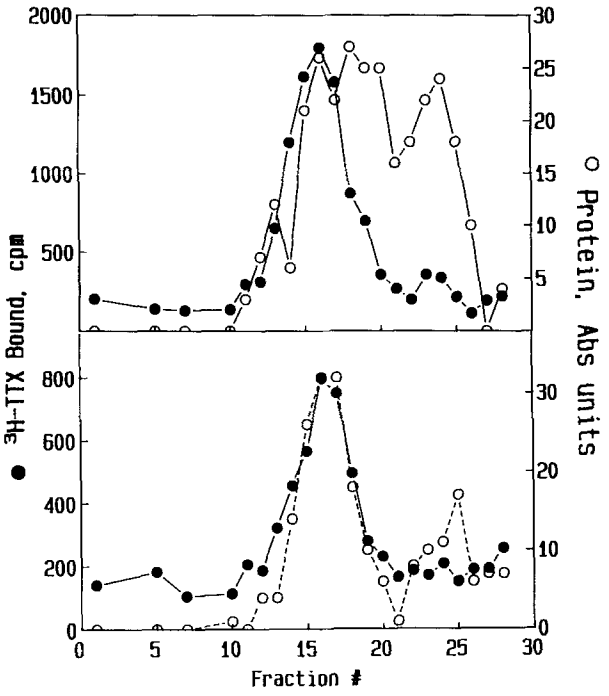

Fig. 1. Purification of TTXR by chromatography on Sepharose 6B. ●, [^{3}H]TTX binding (cpm); ○, Protein (absorbance units, modified Lowry assay). Upper panel: Elution pattern from the first column separation. Lower pattern: Re-chromatography of pooled fractions 14–17 from first column. Peak specific activities were 1995 (fraction 15) and 2647 (fraction 14) pmol TTX bound/mg protein for column 1 and column 2, respectively. Toxin binding assays were conducted with the same amount of material for both chromatograms, whereas protein assays used 2.5 times as much volume for the second column as the first

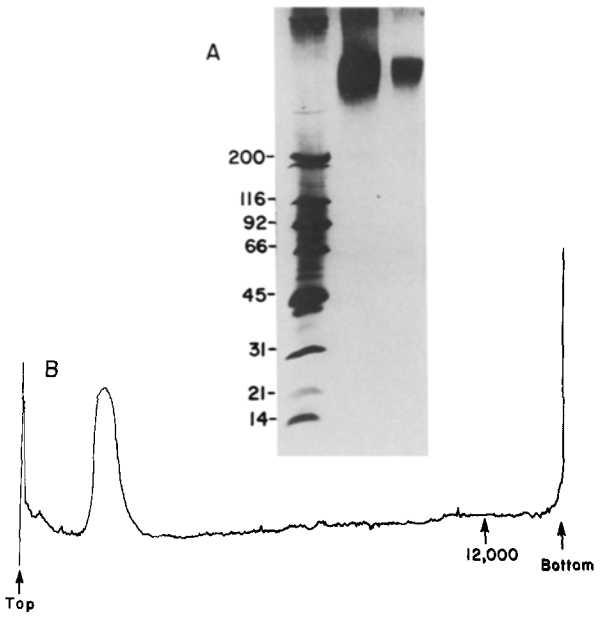

Fig. 2. NaDodSO$_4$-polyacrylamide gel electrophoresis analysis of the polypeptide composition of fractions #14, 15, 16 and 17 pooled from the second column fractionation shown in Fig. 1 (lower panel) *after* reconstitution. (*A*) Left lane, mol wt standards; middle and right lanes, reconstituted protein. The middle lane was loaded with twice as much protein as the right lane in order to check for peptides that may have been present in small quantities. Samples were run on a gradient gel of 5 to 30% acrylamide (low cross-linking). (*B*) Densitometer scan of gel shown in the right lane. Top = top of gel; bottom = bottom of gel. The specific activity of this pooled preparation was 1795 pmol TTX bound/mg protein after reconstitution. The electrophoretogram was visualized by the silver staining method

tally with a well-resolved protein peak after the second column fractionation.

The recoveries of TTX-binding protein, as well as the specific activities of the highly purified channel are given in the table for purification of the channel in different buffers. All yields have been normalized to correct for losses due to fraction selection for comparison with Agnew et al. (1978). The results for the CHAPS-asolectin buffers are the average of 12 separate purifications. It can be seen that these yields for purification in CHAPS-asolectin or asolectin/PC buffers are greater than for purifications using any other buffers. In addition, the peak specific activities obtained after purification are significantly higher than those found with other buffers. The actual, final yield (i.e., nonnormalized yield) for purification in these buffers ranged between 18–24% of the initial extracted TTX-binding activity; this represents the "usable" yield of material which could be reconstituted at high chemical purity for reconstitution studies. Purification in CHAPS-PC buffers gave comparable yields to the purified Lubrol-PC preparations, but with a slightly lower final specific activity.

TTXR purified in these buffers were reconstituted by removal of CHAPS with dialysis. The recoveries of TTXR after this step are also shown in the table. It can be seen that this reconstitution step resulted in a minimal loss of TTX binding in CHAPS-asolectin buffers (10–25%), as opposed to the considerable losses in the Lubrol reconstituted preparations (approx. 50%) and in the CHAPS/PC buffers (greater than 90% of TTX-binding activity lost during this step). The average specific activity after reconstitution of the twelve purified CHAPS-asolectin or CHAPS-asolectin/PC buffer experiments was 2040 pmol TTX/mg protein, indicating that the purified, reconstituted TTXR was greater than 50% functional based on the TTX binding activity of a 260-kDa protein.

The best purifications obtained with these methods demonstrated only a single polypeptide *after* reconstitution when analyzed by SDS-PAGE. Figure 2 shows such an analysis in which there were

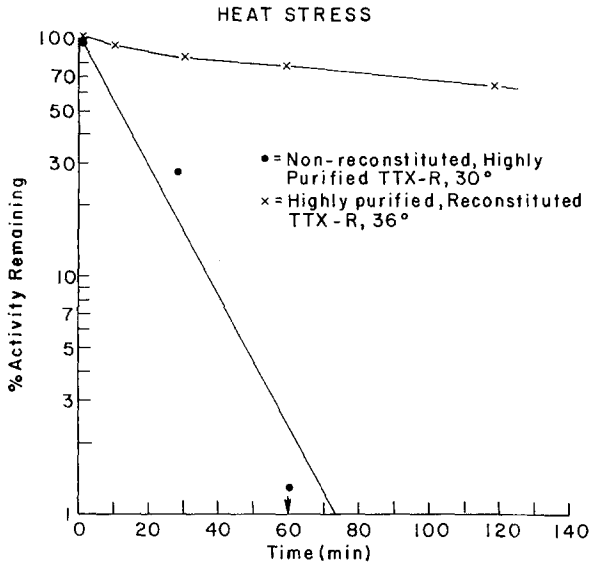

Fig. 3. Heat lability of TTX binding activity. ●, TTXR solubilized in CHAPS detergent. ×, TTXR reconstituted into asolectin/PC (1 : 1) vesicles

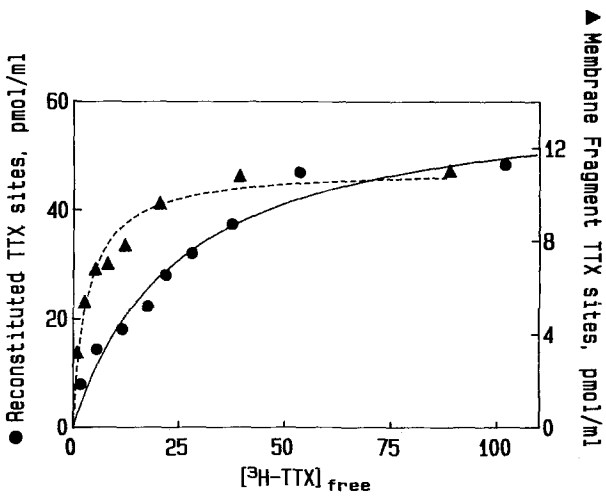

Fig. 4. ^{3}H-TTX equilibrium binding isotherms. ●, TTXR reconstituted in asolectin/PC vesicles, K_d = 24.9 nM; ▲, electroplax membrane fragments, K_d = 3.3 nM. Equilibrium dissociation constants and curves shown were obtained by fitting a Langmuir binding isotherm to the data using a nonlinear least squares procedure (Bevington, 1969). Material used was purified through the DEAE ion exchange step

virtually no other peptides apparent in the preparation. In some of the other experiments, two peptide bands at M_r 200K and 45K were variably seen. These peptides together always comprised less than 10% of the total protein of the purified fractions (judged by the areas under the peaks of densitometry scans), and their presence or absence had no effect on final yields of TTXR, the ability of a given preparation to carry out the examined functions of toxin-modified channels, or any other properties of the reconstituted TTXR examined. No other protein bands contributed significant(>1%) amounts of protein to the purified preparations. Thus, as found in previous studies using the detergent Lubrol-PX (Miller et al., 1983), the results obtained with CHAPS-lipid buffers show that TTX binding by eel electroplax is associated with a 260-kDa polypeptide. Purification and reconstitution with CHAPS buffers gives a final, reconstituted preparation that is 90–100% chemically pure (as demonstrated on SDS-PAGE analysis) and about 50% functionally pure (as measured by TTX-binding ability).

TTX-Binding Properties of the Reconstituted TTXR

It has been found (Talvenheimo, Tamkun & Agnew, 1982; Weigele & Barchi, 1982; Rosenberg et al., 1984*a*) that the TTX-binding activity of native membrane-bound or reconstituted TTXR was relatively temperature insensitive, while TTX binding to the protein solubilized in micellar form was quickly lost with incubation at higher temperatures. Figure 3 shows the effects of temperature on TTX binding to the solubilized and reconstituted TTXR obtained in these experiments. It can be seen that TTX binding to the TXR is heat stable after reconstitution, while the solubilized material quickly loses its ability to bind TTX when incubated at elevated temperatures. In addition, it was found that TTX binding to solubilized preparations was completely lost during freeze-thaw procedures, while the TTX binding ability of reconstituted TTXR was resistant to this same procedure, with a loss of only about 10% during this step (*data not shown*). These results suggest that the TTX binding ability of the isolated protein had been stabilized by reincorporation into a membranous environment.

TTX binding to reconstituted TTXR (partially purified through the ion exchange step) and to the native TTXR in membrane fragments is shown in Fig. 4. The K_d of TTX binding to reconstituted channels at 0°C was 25 nM, which represented an increase over the K_d of 3 nM measured in native channels. This decrease in affinity upon reconstitution has also been reported by others (Tamkun et al., 1984: 21 nM at 36°C; Rosenberg et al., 1984*a*: 33 nM at 0°C).

Figure 5 examines the rates of TTX dissociation at 0°C for the solubilized and reconstituted TTXR, and compares them with the dissociation rate measured with membrane fragments. The time constant for the reconstituted and nonsolubilized preparations were the same, about 45 min, while the time

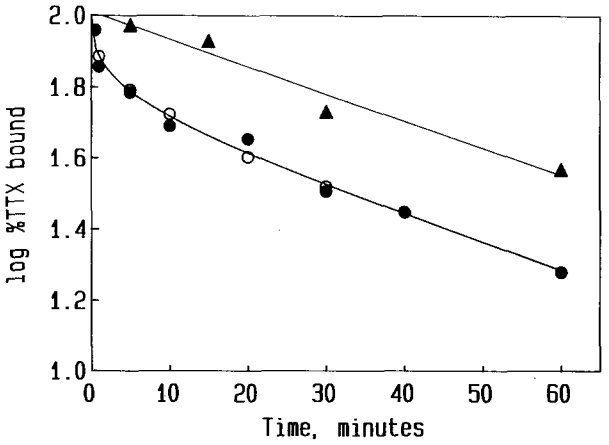

Fig. 5. Dissociation of TTX/protein complexes at 0°C. △, CHAPS-solubilized TTXR; ○, TTXR reconstituted into asolectin/PC vesicles. ●, native TTXR in electroplax membrane fragments

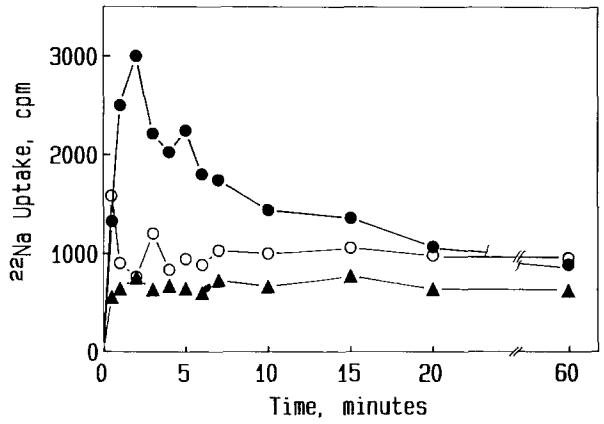

Fig. 6. Voltage-driven uptake of ^{22}Na by vesicles containing reconstituted TTX-binding glycopeptide (asolectin/PC vesicles, 26°C). ●, with 150 μM veratridine; ○, 150 μM veratridine and 2 μM TTX; ▲, no additions. Average of duplicate determinations

constant for the solubilized preparation was slower (>60 min). This time constant for native membrane fragments is much slower than that previously reported by Reed and Raftery (1976), where it was found to be about 90 sec. Because of this discrepancy, the rate of dissociation from native membrane particles was examined using the two distinct methods for measurement described in Materials and Methods. No significant differences were found between the two sets of results obtained, and their average is shown here. Reasons for the discrepancy between the present results and those of Reed and Raftery are unknown. However, Tomiko et al. (1986) have also observed slow TTX dissociation time constants for both native and reconstituted eel channels (i.e., 25–30 min). Additionally, it can also be seen in Fig. 5 that the dissociation of TTX from its binding site seems to consist of a fast and a slow component in all experiments where the TTXR is in a membranous environment. In contrast, TTXRs in the micellar state apparently have a single rate component comparable to the slower time constant of the membrane-associated channels.

In previous reconstitution studies it was found that TTXRs were incorporated into the lipid vesicles rather randomly, such that between 30 and 60% of the TTX-binding sites were inaccessible to externally applied TTX (i.e., were incorporated "inside-out" with respect to the orientation of the native channel) (Weigele & Barchi, 1982; Rosenberg et al., 1984*a*; Tamkun et al., 1984). When we examined the orientation of reconstituted TTXR as described in Materials and Methods, it was found that virtually all of the TTXR were oriented right-side out (as in nerve membranes) when reconstituted into asolectin vesicles, while between 75 to 85% of TTXR incorporated into asolectin/PC vesicles were right-side out in the preparations studied (Duch & Levinson, 1987). We have no firm understanding of the causes of this profound asymmetrical incorporation, although it might be related to the presence of the large number of negative carbohydrates that are presumably located on the external domain of the channel.

Channel-Dependent Fluxes Mediated by Reconstituted TTXR

The ability of the reconstituted sodium channel to mediate ion fluxes has generally been examined in vesicles by opening the channels with activating toxins (such as veratridine or batrachotoxin) and measuring the uptake of ions through them (Talvenheimo et al., 1982; Weigele & Barchi, 1982; Rosenberg et al., 1984*a*; Tamkun et al., 1984). Two different methods were used to examine the uptake of ^{22}Na mediated by reconstituted TTXR: the voltage-driven uptake described by Garty, Rudy and Karlish (1983), and the uptake into vesicles with no potential across them. The results presented here were conducted with the protein incorporated into asolectin-PC vesicles, since this lipid system formed larger vesicles (Levinson & Duch, 1987) that gave a greater measurable inner-vesicular volume than asolectin vesicles, and hence a greater measurable uptake signal.

Figure 6 shows the results of a voltage-driven uptake into reconstituted vesicles. Here the vesicles, loaded with a high concentration of NaCl, were placed in a medium containing the sodium channel activator veratridine and a trace amount of

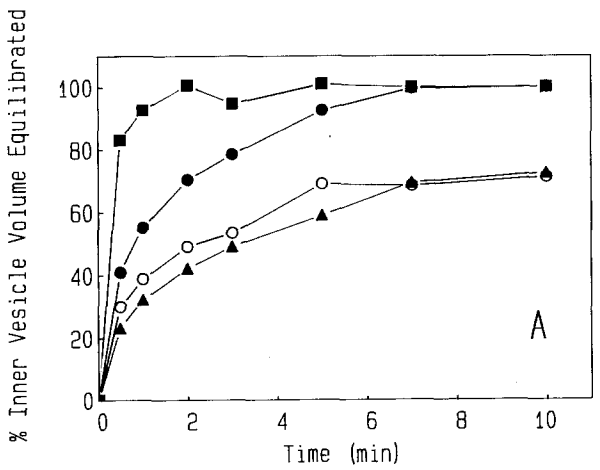

Fig. 7. ^{22}Na uptake at zero membrane potential by asolectin/PC vesicles containing reconstituted TTX binding glycopeptide (26°C). Additions: ■, 5 μM BTX; ●, 150 μM veratridine; ○, 150 μM veratridine and 2 μM TTX; ▲, no additions. Each point is the result of duplicate determinations

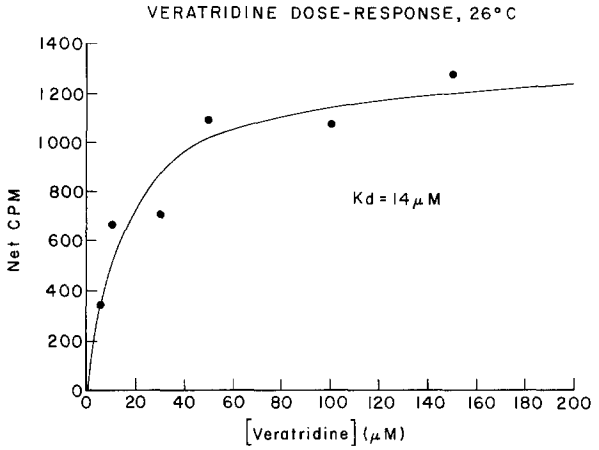

Fig. 8. Concentration dependence of activation of ^{22}Na uptake by veratridine. Each point is the result of duplicate determinations. The data was fit using a Langmuir binding isotherm model as described in Fig. 4. The results of uptake into vesicles with no additions was subtracted from the veratridine-stimulated uptake to obtain the specific uptake activated by veratridine

^{22}Na. In such a system, if the vesicles contain channels capable of being opened by veratridine, then a sodium diffusion potential will result; this potential will cause tracer sodium to be accumulated in the vesicles at a concentration higher than that in the external solution. This results in an enhancement of the channel-dependent sodium uptake over systems in which sodium concentrations are equal on both sides of the membrane, and allows one to detect the activity of reconstituted channels with greater sensitivity (Garty et al., 1983). The biphasic behavior observed in such systems is a consequence of the lower anion permeability in the vesicles, which eventually causes the sodium gradient to dissipate. In the present study (Fig. 6) it can be seen that veratridine stimulated the influx of radiolabeled sodium into vesicles compared with the measured uptake of labeled sodium into vesicles when no veratridine was present in the manner expected as described above. In addition, TTX on the external side of the vesicles blocked most of the activated uptake, but there was still a small increase in tracer uptake under these conditions over that seen in vesicles alone due to the small number of inside-out channels that could not be blocked by TTX (*see above*). In this experiment, the veratridine-stimulated transport rate was estimated to be 1.25×10^6 ions/min/TTX binding site, when calculated as in Tamkun et al. (1984).

The veratridine and BTX activatable uptakes into reconstituted vesicles with no potential across the vesicular membrane are shown in Fig. 7. BTX clearly produces a greater rate of uptake of tracer ions into vesicles than does veratridine, indicating a greater steady-state permeability of BTX-modified channels. This result is in agreement with the greater stimulation of reconstituted channels with BTX seen by Weigele and Barchi (1982) and Rosenberg et al. (1984*a*). Tamkun et al. (1984) measured a lower BTX-stimulated uptake of ions compared with veratridine in their system.

The concentration dependence of veratridine activation of the reconstituted channel is shown in Fig. 8. The K_{50} of veratridine-stimulated uptake from these measurements was 14 μM. This is in reasonable agreement with that reported by Tamkun et al. (1984; 28 μM) and Rosenberg et al. (1984*a*; 18 μM) in their studies of reconstituted brain and electroplax sodium channels, respectively.

The Fraction of TTXRs Mediating Ion Fluxes

Thus these experiments show that a number of the TTXR purified and reconstituted in CHAPS buffers as described retain the structures necessary to mediate toxin-activated fluxes. However, as discussed above (*see also* Tamkun et al., 1984), it was necessary to determine the fraction of reconstituted proteins that mediate these functions in order to reach firm conclusions regarding the polypeptide composition of a functional channel. Initially, the method used by Tamkun et al. (1984) to estimate this fraction was employed here.

To carry out these calculations, the size distribution of the reconstituted vesicles was examined

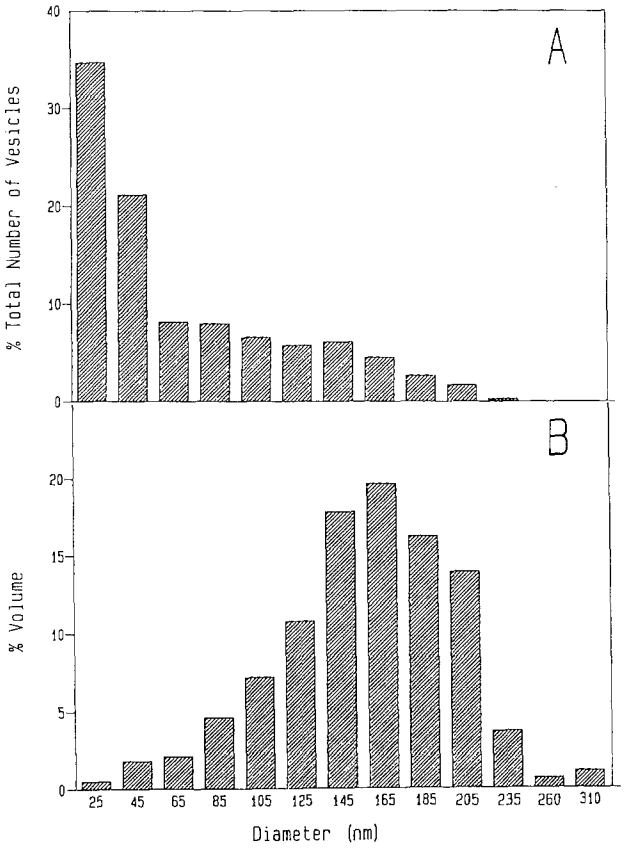

Fig. 9. Vesicle diameter and volume distributions. Vesicles were the same as those used for the data of Figs. 1, 2 and 10. (*A*) The normalized diameter histogram determined from freeze-fracture studies. (*B*) Fraction of total inner-vesicular volume in each size group of vesicles. These were calculated from the size distribution given above

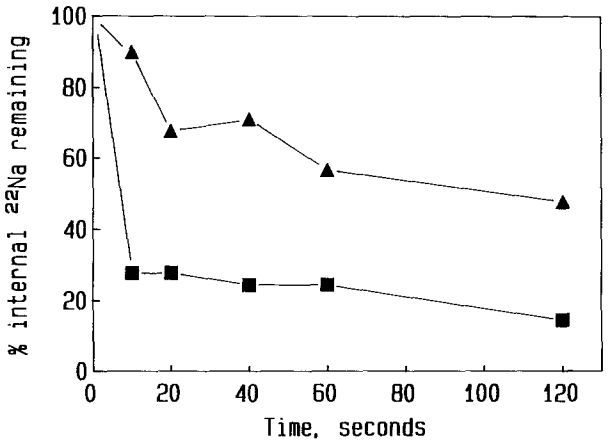

Fig. 10. ^{22}Na efflux from vesicles with the reconstituted protein from the experiments shown in Figs. 1 and 2. Vesicles were loaded overnight at 4°C with ^{22}Na, then incubated for 45 min with either 5 μM BTX (■) or no additions (▲). The vesicles were then diluted 1:9 into buffering solution with no ^{22}Na present. The amount of internalized ^{22}Na was measured with time as described in the text. The points are the results of duplicate determinations. The fractional channel-dependent efflux was calculated from the 10-sec time point as the BTX-induced decrease in internal ^{22}Na relative to the isotope in vesicles with no toxins added

using freeze-fracture techniques. Figure 9*A* is a histogram displaying the size distribution of the vesicles obtained from the same experiment shown in Figs. 1, 2 and 10. It was found that the greatest number of vesicles were relatively small, with an average diameter less than 50 nm. The majority of the measurable *volume*, however, was contained in vesicles with diameters greater than 100 nm, as shown in Fig. 9*B*. The total number of vesicles determined as described (Materials and Methods) was 2.09×10^{13} per ml solution.

With this information, the number of TTXR that mediated toxin-activated fluxes could be estimated, assuming random incorporation of channels into vesicles independent of vesicle size (Tamkun et al., 1984). In the experiment examined here, 39 pmol TTX binding/ml remained after reconstitution. Using the number of vesicles/ml determined above, an average of 1.12 TTXR/vesicle was calculated. With this number, the fraction of vesicles with no incorporated channels can be estimated from the Poisson distribution using the equation

$$P(n) = e^{-m}(m^n/n!)$$

where $P(n)$ represents the fraction of vesicles with "n" incorporated channels, and m = mean number of TTX binding sites/vesicle (i.e., 1.12). Setting n = 0, it was calculated that the fraction of vesicular volume which would not be potentially accessible by toxin-activated channels was 0.33. This means that 67% of the total internal vesicle volume should readily equilibrate in the presence of veratridine or BTX in this experiment if all TTXRs were also capable of mediating ^{22}Na uptake.

Figure 10 shows the results of experiments in which the actual vesicular volume accessible through BTX-activated channels was determined. Qualitatively, it can be seen that BTX activates a large rapid isotopic efflux in these vesicles, while the efflux in the absence of toxin is smaller and rather slower. Using the earliest time point (where the contribution of nonspecific leak was minimal), the amount of internal volume accessible through BTX-activated channels was 70% of the total measured vesicular volume, in close agreement with the result expected if all TTXRs were sodium transporting. However, such correspondence is probably fortuitous, since one must also correct for the fraction of vesicles with more than one channel (0.3, according to the Poisson distribution). In order to obtain a *lower limit* to the fraction of sodium transporting TTXR, it is assumed that only one channel per vesi-

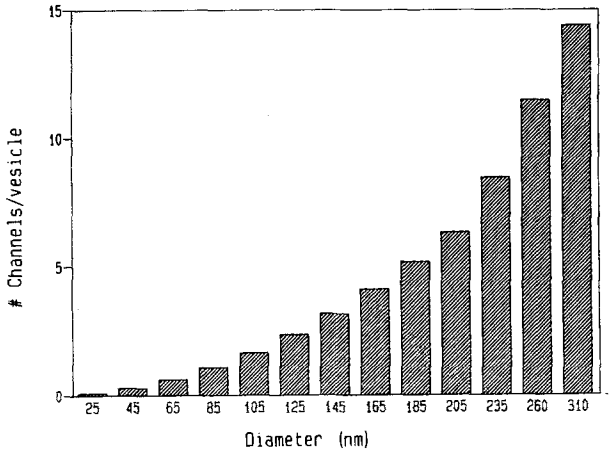

Fig. 11. The estimated number of channels per vesicle. Using the size distribution shown in Fig. 9A, the lipid area per vesicle size group was determined. The graph was generated using the measured amount of TTX binding proteins present (39 pmol/ml), and assuming an even distribution of protein with lipid area

cle is active; thus it can be calculated that a minimum of 83% of all TTXR must be activated to account for the measured uptake. Overall, these methods give a range of between 83 and 100% of reconstituted TTXR capable of mediating toxin-activatable ion fluxes. Tamkun et al. (1984) estimated between 21 and 73% activity for brain STXR using these same assumptions and calculations.

Results obtained by Weigele and Barchi (1982), however, indicate that TTXR are not incorporated randomly into vesicles, but rather are distributed proportionately with lipid. In other words, TTXR will be incorporated into vesicles in proportion to the area of lipid present in them. This means that there will be fewer TTXR in the smaller vesicles, and a relatively greater number of TTXR in the larger vesicles. Recalculating the number of TTXR/vesicle in this way gives the distribution shown in Fig. 11. It can be seen that only about 10% of the smallest vesicles will contain TTXR while the largest vesicles have an average of more than 14 TTXR/vesicle. An examination of the vesicular volume distribution shown in Fig. 9*B* shows that the vesicle populations with the largest volume contribution (vesicle diameter between 100 and 200 nm) will have between 3 and 7 channels/vesicles. The vesicular volume that should be accessible under these circumstances can be estimated by assuming a Poisson distribution of channels into vesicles within each 20-nm size group, calculating the proportion of vesicles with no channels as above, and then summing the volumes accessible in each of these categories. Doing so suggests that about 92% of the total vesicular volume should be accessible through activated channels (as opposed to 67% using the previous assumption of random distribution not affected by diameter). Therefore, with this model of channel distribution, 22% of activatable volume is unaccounted for with the experimental data (i.e., 92% minus 70% observed).

Furthermore, because many vesicles contain multiple channels, and since all channels in a vesicle must be nonactivatable with BTX in order for that volume to be inaccessible in the presence of BTX, the proportion of nontransporting TTXR is much greater than 22%. In vesicles with multi-incorporated TTXRs, if even one protein is activatable by veratridine or BTX, the vesicular volume will be measured as accessible through toxin-activatable channels in these experiments. Thus, in order for no activatable uptake to be measured, all of the TTXR in a given vesicle must be closed. An estimate of the fraction of nonactivatable TTXR (Q) under these conditions can be made by calculating the probability that all TTXR in multi-TTXR incorporated vesicles will be inactive for varying values of Q. For example, if Q is set to 0.5 (i.e., 50% of all channels are inactive), then the probability that a vesicle with three incorporated TTXR will have all nonactivatable proteins is: 0.5^3 or 0.125. The result obtained represents the fraction of vesicles with three channels that will not give a measurable toxin-activated uptake. By summing the results obtained in this way from all vesicle size groups, and comparing the nonactivatable fraction with the experimentally obtained value (0.22), an estimate of the fraction of toxin-activatable TTXR can be obtained. Thus it was estimated that Q must be about 0.65 in order to account for the observed nonactivatable fractional volume of 0.22. The proportion of TTXR capable of mediating toxin-activatable flux was therefore 35%. Applying these more rigorous assumptions to calculate the corresponding fraction of toxin-activatable channels from the data given in Tamkun et al. (1984) yields a similarly lowered range of 15 to 40% of TTXR capable of veratridine activation.

Discussion

In order to correlate the functional activity of sodium channel-related proteins with their polypeptide composition, close attention must be paid to three parameters of the reconstituted preparation: chemical purity, functional purity, and final yield. Chemical purity refers to the degree of separation of the channel moieties from other proteins present after solubilization. In the ideal purification, the target protein will be obtained without any contaminat-

ing proteins present. Functional purity is a measure of how many of the chemically pure molecules can still carry out the functions of the protein *in situ*. Function may be lost due to denaturation of the protein during its purification, or due to the loss of a functional subunit or necessary interactant. The final yield is a measure of that proportion of the functional (i.e., TTX-binding) protein initially solubilized that was present after reconstitution. Loss of functional protein molecules may be due either to denaturation or incomplete recovery, or functional proteins may also be discarded during certain purification steps in order to obtain fractions with the highest chemical purity possible. As discussed above (Introduction), it is essential that all three parameters be optimized to allow one to correlate the chemical composition as analyzed by SDS-PAGE with functional characteristics as measured by ^{3}H-TTX binding and alkaloid toxin-activated ^{22}Na uptake.

Part of the cause for the low functional purity and yield in all previous studies stems from the removal of detergent with Bio-Beads SM-2. This step results in the loss of 40–60% of TTX-binding activity, as well as much of the purified protein (Talvenheimo et al., 1982; Weigele & Barchi, 1982; Rosenberg et al., 1984*a*; Elmer et al., 1985). The functional yields of sodium channel protein in all of these studies ranged from 3–12% of the initially extracted channel proteins (as measured with TTX binding) after reconstitution. None of the previous transport studies have reported the chemical purity (as indicated by SDS-PAGE analysis) or functional purity (as measured by the amount of TTX or STX binding/mg protein) of the preparations *after* reconstitution, so that the final quality of these preparations is not known.

In contrast, the results presented here show that the TTXR from eel electroplax can be highly purified and efficiently reconstituted in CHAPS-lipid buffers (Table). In either CHAPS-asolectin or CHAPS-asolectin/PC buffers, the final yields and chemical purities are significantly greater than those obtained with either Lubrol/PC buffers (eel electroplax, *see* Table), Nonidet P-40/PC (mammalian skeletal muscle; Tanaka et al., 1983), or Triton X-100/PC (rat brain; Tamkun et al., 1984). In the present studies, the functional purity of the CHAPS/asolectin-processed TTXR before reconstitution was significantly greater than that obtained with Lubrol/PC buffers (about 2400 pmol TTX-bound/mg protein *versus* 1850 pmol/mg protein in the respective systems). In addition, loss of TTX binding activity during the reconstitution step was slight, resulting in material with a specific activity of about 2000 pmol TTX bound/mg protein incorporated into vesicles for flux analysis.

The purified, reconstituted TTXR was found to regain the heat and freeze-thaw stability of the native channel lost upon solubilization of the protein from its native membrane, while the K_d of TTX binding to the reconstituted channel increased relative to the channel *in situ*. This increase in measured K_d has also been found in other TTXR reconstitution systems (Tamkun et al., 1984; Rosenberg et al., 1984*a*). Previous work (Miller et al., 1983) has shown that TTX binding to channels from the eel electroplax required only a single polypeptide, since the preparations of greatest chemical purity were also greater than 50% functionally pure. No smaller peptides were present in great enough quantity to be considered necessary for this amount of binding. In the experiments presented here, this result is confirmed by the fact that the large polypeptide, purified to essentially 100% chemical purity as shown by SDS-PAGE analysis, can still bind TTX in substantial quantities (up to 70% of the theoretical specific activity expected for a completely pure and functional preparation).

When the assumptions of Tamkun et al. (1984) were employed, the proportion of eel TTXR still binding TTX that also exhibited flux behavior was greater than that measured for rat brain preparations. The purest preparations consisted of only a single polypeptide with an apparent mol wt of 260,000 daltons, while the few contaminating peptides present after some purifications made up less than 10% of the total protein in any given preparation. When present, these contaminating peptide patterns were similar to those found in TTXR purifications from other tissue sources (Tanaka et al., 1983; Kraner et al., 1985), and these reports have indicated that at least one of these bands (the 200,000 apparent mol wt band) was a proteolytic fragment of the large polypeptide. It has also been proposed that the 45-kDa peptide may be a proteolytic fragment of the large protein (Tanaka et al., 1986). In any case, in our preparations no peptides were ever observed smaller than 45 kDa, e.g. in the 30 to 40 kDa range reported for putative subunits of mammalian channels. We estimate that the sensitivity of our protein staining procedure was such that peptides representing 1% or more of the mass of the 260-kDa glycoprotein should have been readily visualized (*cf.* Fig. 2*A* and *B*). In our preparations, 53% of the 260-kDa component bound TTX, while our worst case uptake calculations above yielded a BTX-activated uptake in 35% of the TTXR; hence at least 19% of the 260-kDa component was involved in the channel-dependent transport of isotopic sodium. If, for example, a 35-kDa peptide was also necessary for isotopic uptake, it would have represented at least 3% of the protein mass in our samples, i.e., readily detectable by SDS-PAGE.

This worst case analysis also assumes a 1 : 1 stoichiometry and that all large/small peptide complexes are active; if, as found for rat brain, more than one closely migrating peptide was involved, and if, as reported for both brain and muscle-derived material, a significant proportion of multimeric complexes do not activate, then the protein mass of such a small peptide would be much higher and thus even more prominent on SDS-PAGE.

Another measurement that has been used to evaluate the number of TTXR that can mediate toxin-activated fluxes is the transport rate of a TTX binding site. In the uptake experiments reported here, the uptake rate through channels with 0 potential across them was measured as 1.2×10^5 ions/min/TTX binding site, while that measured with a 10-fold ion gradient across them (i.e., using the voltage-driven protocol) conducted 1.25×10^6 ions/min/site. This latter value is in good agreement with Tamkun et al. (1984), who measured rates in reconstituted vesicles as 1.0×10^6 ions/min/site. A major problem with this calculation is that the net ion flux is necessarily limited by vesicular volume. Evidence presented by Tanaka et al. (1983) shows that vesicles with channels activated by veratridine may equilibrate within a few hundred milliseconds. Because the earliest experimental measurements made with this methodology are between 10 and 30 sec, any vesicle with an activated channel would already be equilibrated and the flux/channel then would be as much an indication of vesicle size as actual ion flux. Given these uncertainties, it is perhaps fortuitous that the estimates of transport rate between reconstituted rat brain STXR and eel TTXR agree so well.

In any case, in the present studies the proportion of total protein that *both* bound toxin and transported sodium was far greater than any previous work (Table) and allows us to conclude more rigorously that fully functional sodium channels in eel electroplax are comprised solely of a 260-kDa polypeptide species. Thus future studies of sodium channel mechanism may concentrate on identifying those domains on this glycopeptide which participate in the voltage-gated transport of sodium ions without concern for the contributions of other peptide species.

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