Biochemical Characterization of the Tetrodotoxin Binding Protein from *Electrophorus electricus*†

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ABSTRACT: Biochemical properties of a detergent-solubilized tetrodotoxin binding component from *Electrophorus electricus* have been examined and compared with those found for the membrane-bound protein. The toxin binding component was solubilized with high efficiency by a variety of nonionic detergents and with lower efficiency by sodium cholate and deoxycholate. Detergent-solubilized preparations bound tetrodotoxin and saxitoxin tightly and specifically, and this binding was observed to be rapidly and irreversibly blocked by carboxylate-modifying reagents. Inactivation by carbodiimide and glycine ester or by a trimethyloxonium salt could be prevented by tetrodotoxin occupancy of the binding site. Tetrodotoxin binding activity in both solubilized preparations and in membranes was found to be highly resistant to proteases. In contrast, the activity was extremely sensitive to the action of phospholipase A_2. The biochemical properties of the tetrodotoxin binding component solubilized in mixed lipid–detergent micelles are similar to those found in native membranes, with respect to the characteristics of equilibrium toxin binding and to the sensitivity of toxin binding activity to chemical modification and degradative enzymes. There were some differences with respect to the kinetics of tetrodotoxin binding. In addition, the tetrodotoxin binding component from eel is shown to behave as a glycoprotein, being selectively absorbed to resins coupled to concanavalin A, wheat germ agglutinin, *Lens culinaris* lectin, and ricin with the appropriate glycoside.

A variety of approaches are being used to characterize the molecular structures and mechanisms which confer on the membranes of nerve and muscle cells the property of electrical excitability. Although the biophysical description of the propagated action potential has become quite detailed [for reviews, see Armstrong (1975), Landowne et al. (1975), Ulbricht (1977), and Hille (1978)], the molecular nature of the phenomenological channels which are responsible has yet to be firmly established. We report here results from continuing studies on the biochemical isolation and characterization of a voltage-sensitive sodium conductance channel of the type responsible for the early sodium currents of the action potential.

We have used two highly specific neurotoxins, tetrodotoxin (TTX)[1] and saxitoxin (STX) as biochemical markers for the quantitation of the channel. The properties and specificities of TTX and STX have been extensively reviewed (Hille, 1978; Ritchie & Rogart, 1977). These molecules bind in a reversible manner, with mutual competition, and with high affinity (K_d = 1–10 nM) to a single class of sites accessible from the outside of the cell membrane (Narahashi et al., 1967; Hille, 1968, 1975a,b; Cuervo & Adelman, 1970; Ulbricht & Wagner, 1975). Apparently the toxins block the sodium ion permeation pathway without affecting the structures involved in channel gating (Armstrong & Bezanilla, 1973, 1974; Keynes & Rojas, 1974). Comparison of data from physiological studies with those from binding studies has provided strong evidence that the toxins bind only to the physiologically defined sodium channel (Ritchie & Rogart, 1977). Data from dose–response experiments (Hille, 1970) and from fluctuation analysis (Sigworth, 1980) suggest that TTX and STX bind with a stoichiometry of one toxin molecule per channel.

The electroplax membranes of the electric organ from *Electrophorus electricus* contain TTX- and STX-sensitive voltage-dependent sodium channels (Nakamura et al., 1965; Reed & Raftery, 1975). In this report we describe some of the characteristics of the TTX binding component solubilized from electroplax membranes. The detergent-solubilized TTX binding component is shown to possess biochemical characteristics similar to those found for sodium channels in isolated membranes and nerve preparations. In addition, we describe somewhat unusual findings with respect to stability properties of the solubilized toxin receptor which may have practical implications for purification procedures and which may prove useful in the ultimate determination of the structure of the channel.

Materials and Methods

TTX, citrate free, was obtained from Sankyo Chemical Co., tritiated by the Wilzbach procedure, and purified by ion-exchange chromatography (Benzer & Raftery, 1972). The purified toxin was approximately 65% pure as determined by frog sciatic nerve bioassays (Levinson, 1975). The specific activity was 78.8 μCi/μmol. STX was obtained from the National Institutes of Health and was radiolabeled by the ^3H_2O exchange procedure of Ritchie et al. (1976). Toxin was purified by ion-exchange chromatography on Bio-Rex 70. The specific activity was 720 μCi/μmol.

Lubrol-PX (Imperial Chemical Industries) was made up to 10% (w/v) in distilled water and was deionized with a mixed bed resin (Bio-Rad AG 501-X8D). The resin was removed by filtration and the solution stored frozen. Sodium cholate and deoxycholate, Triton X-100, and Brij 56 were obtained from Sigma Chemical Co. Nonidet P-40 was obtained from BDH Chemicals Ltd.

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[1] Abbreviations: Con A, concanavalin A; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; NAG, *N*-acetylglucosamine; TMO, trimethyloxonium tetrafluoroborate; TTX, tetrodotoxin; $NaDodSO_4$, sodium dodecyl sulfate; STX, saxitoxin.

0006-2960/82/0421-6212$01.25/0

Trimethyloxonium tetrafluoroborate (TMO) was obtained from Aldrich and 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC) from Vega-Fox Biochemicals. Phospholipase A_2 was from Sigma, and all other enzymes were obtained from Worthington. Wheat germ agglutinin (WGA), ricin, WGA–Sepharose 6MB, 6-aminohexanoic acid activated Sepharose 4B, concanavalin A–Sepharose 4B, *N*-acetylglucosamine, *N*-acetylgalactosamine, and methyl glucoside were obtained from Sigma. Lectin from *Lens culinaris* coupled to Sepharose 4B was the kind gift of Dr. William Dreyer.

WGA–Sepharose 4B was prepared by suspending dry 6-aminohexanoic acid activated Sepharose 4B (1.0 g) in 5 mL of 1 mM HCl. The suspension was filtered and washed with 250 mL of 1 mM HCl. Moist resin was added to a solution of WGA (3.6 mg at 1 mg/mL) in 0.1 M potassium phosphate buffer, pH 7.5, containing 0.1 M *N*-acetylglucosamine (NAG). After the solution was gently stirred at 4 °C for 90 min, the resin was removed by centrifugation, and loss of protein was measured in the supernatant. Protein uptake was 96.4%. The resin was suspended in 0.1 M potassium phosphate buffer and 0.5 M ethanolamine hydrochloride, pH 7.6, and was stored at 4 °C overnight to eliminate unreacted groups on the resin. The resin was then washed extensively with 0.05 M potassium phosphate, pH 7.5, and 0.2 M NaCl. Resin capacity was measured by binding of [^{14}C]NAG and ovomucoid. Ricin–Sepharose 4B was prepared in the same way.

Protein assays were performed with the fluorescamine assay (Udenfriend et al., 1972), and lipid phosphate was measured by a modified Fiske-SubbaRow technique (Radin, 1969; Dittmer & Wells, 1969).

Preparation of Membranes and Extracts. Small (1.5 to 2.5 ft) electric eels were killed by hypothermia. The main electric organs were quickly removed, rinsed with distilled water for a few seconds, and immediately frozen and stored at −80 °C. While still partially frozen, the organ was trimmed of connective tissue and diced into cubes of approximately 1 cm^3. The diced tissue was suspened in 3–5 volumes of cold buffer [0.06 M potassium phosphate, pH 7.5, containing 5 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM phenylmethanesulfonyl fluoride]. This suspension was homogenized with a polytron tissue disrupter (3.5 cm head) at maximum speed in four 30-s cycles. This homogenate, while quite thick, is fine enough to pass entirely through three layers of cheesecloth. The suspension was centrifuged for 1 h at 24000*g* at 4 °C. The pellet was washed twice by resuspension in fresh buffer and centrifuged at 100000*g* for 1 h. The weighed pellet was then resuspended in 2 volumes of buffer per gram wet weight of membranes. The membrane slurry was measured into 15-mL aliquots and frozen at −80 °C for later use.

Detergents were tested for their efficiency at solubilizing the TTX binding component, as follows. A large preparation of membranes was made by the standard procedure, and the membrane slurry was divided in aliquots and stored at −80 °C. All detergents were tested with the same membrane preparation. Freshly thawed membranes were divided into six 0.9-mL samples and delivered to centrifuge tubes for a Beckman 65 rotor. To these were added 0.6-mL aliquots of different concentrations of a stock detergent solution in water. The membranes and detergent were vigorously agitated with a rotary mixer and placed in ice, with occasional agitation for 20 min. The samples were then centrifuged at 40000*g* for 30 min at 4 °C, and the supernatants were assayed for TTX binding and protein. The same experiment also was done for each detergent in the presence of sufficient [^{3}H]TTX for the free toxin concentration to be 100 nM. The rate of [^{3}H]TTX dissociation was tested with the TTX binding component solubilized at the optimal concentration for each detergent. The solubilized material was saturated with [^{3}H]TTX and the total number of binding sites measured. Then a 200-fold excess of cold TTX was added, and the amount of specifically bound toxin was measured at two or more time points. The approximate half-life for the toxin binding component complex was estimated. This was done in order to judge whether half-lives were so shortened by some detergents as to compromise quantitation with the G-50 assay described by Levinson et al. (1979). The detailed kinetics of toxin dissociation, however, were not measured. The total number of binding sites in the membranes was estimated by the supernatant depletion assay described below, to permit estimation of solubilization efficiency.

Standard detergent extracts were prepared by adding Lubrol-PX to a final concentration of 1% (w/v) to a freshly thawed suspension of membranes followed by vigorous homogenization with a motor driven Potter-Elvejheim homogenizer. The mixture was centrifuged at 100000*g* for 1 h. The supernatant was removed with a Pasteur pipet and stored in ice.

Material Purified by Ion Exchange. One volume of 1% Lubrol-PX extract was made 0.4 M in KCl and mixed rapidly wth 1 volume of packed DEAE-Sephadex A-25 resin which had been freshly equilibrated with 0.05 M potassium phosphate, pH 7.5, containing 0.1% Lubrol-PX:phosphotidylcholine (7:1 molar ratio). After 30 min in ice with occasional mixing, the resin was centrifuged at low speed and the supernatant discarded. The resin was washed 4 times with an equal volume of the same buffer containing 0.2 M KCl. The resin was then mixed with either 1 volume or a minimal volume of the same buffer containing enough KCl to make the final concentration 0.4 M. After 30 min in ice, the supernatant was collected, and a second wash of the resin was performed by using a minimal volume of buffer. For the experiments reported here, the pooled desorbed material was used without concentration.

Toxin Binding Assays. Binding assays on membranes were done by using the technique of sedimentaton depletion. A diluted suspension of membranes (0.5 mL) was pipetted into plastic 1-mL centrifuge tubes. To this was added, generally in a volume of 0.1 mL, an appropriate amount of [^{3}H]TTX (or [^{3}H]STX) in the presence or absence of unlabeled toxin. The samples were mixed, stored on ice for 20 min, and centrifuged in an Eppendorf centrifuge for 30 min. Aliquots (0.2 mL) were withdrawn from the supernatants and counted. The depletion of radioactivity from the supernatant which was prevented by the excess unlabeled toxin was considered specific binding. Assays for each sample were generally done in triplicate.

Binding assays of detergent extracts were carried out by using G-50 gel filtration assay described in detail by Levinson et al. (1979). When employed with [^{3}H]STX as a binding ligand, the assay is not quantitative because the rapid rate of dissociation of the [^{3}H]STX complex makes quantitative recovery in the eluate impossible. However, when care is taken to control the length of time for the sample to sink into the gel, and when control and experimental samples are run together in sets of four, the reproducibility is good, and recovery of toxin binding component complexes is about 70%.

Chemical Modification by Carbodiimide and Glycine Ethyl Ester. The procedure was based on the method of Moore & Koshland (1967). Material purified by ion exchange was made 0.39 M in glycine ethyl ester and hydrochloride, and the pH of the solution was adjusted to 6.0. Half of this solution was

made 100 nM [^{3}H]TTX while the other half was diluted with the appropriate volume of water. The reaction was initated by addition of a solution of EDC. Samples were incubated in ice and assayed at various times as shown in Figure 3 (left panel). Before assay, the pH was raised to pH 7.5 with 50 μL of 0.5 M potassium phosphate buffer, pH 7.5. Those samples lacking [^{3}H]TTX were supplemented with concentrated toxin. After equilibration with toxin, the samples were assayed in duplicate. Specific [^{3}H]TTX binding was confirmed by competition with excess unlabeled TTX. In a typical experiment, final concentrations were as follows: TTX binding component, 38 nM, specific activity 392 pmol/mg of protein; glycine ethyl ester, 0.35 M; TTX, 100 nM; EDC, 0.23 M. The recoveries of toxin binding activity are reported as the percentage of the corresponding controls to which no EDC had been added.

Chemical Modification with TMO. TMO was weighed into small aliquots in a dry box. Inactivation was demonstrated as follows: 1-mL aliquots of TTX binding component purified by ion exchange were added to vials containing increasing amounts of dry, weighed TMO. The pH was adjusted with small amounts of 2 M Na_2CO_3, pH 10.5, and held at pH 7.3 $\pm$ 0.1. The reaction was essentially complete after 5 min. The samples were incubated on ice for 30 min and then assayed for [^{3}H]TTX binding with and without excess unlabeled TTX. Control experiments to measure the effects of hydrolysis products of TMO were carried out by allowing TMO to hydrolyze at 4 °C for 40 min in 0.1 mL of buffer, pH 7.5. To this was added 0.9 mL of TTX binding component, and the mixture was stored in ice for 30 min. Activity was compared with a sample diluted into 0.1 mL of buffer only. Protection experiments were performed by reacting TTX binding component (59 pmol/mL) with sufficient TMO (12.4 mg/ml) to inactivate more than 90% of the binding sites in the presence of increasing amounts of TTX. Samples were assayed at 180 nM [^{3}H]TTX. Data are illustrated as a percentage of the total recovered activity of controls incubated with TMO hydrolysis products. [^{3}H]STX binding was measured by the G-50 procedure which has the quantitative reservations indicated above. Competition for [^{3}H]STX binding was done with unlabeled TTX, and all binding was shown to be in dynamic equilibrium.

Enzyme Treatments. Membrane suspensions were pelleted and resuspended in the appropriate buffer or buffer plus enzyme. Incubations were carried out for 1 h at 25 °C before assay. Enzyme treatment of solubilized preparations was carried out for 3 h in ice. Samples were incubated with the amounts of added enzyme shown in Table I and were then assayed and compared to controls. All digestions were performed at pH 7.5 except for the incubation with neuraminidase. For those samples, buffer pH was lowered to 6.5 during the enzyme treatment and was brought back to pH 7.5 for assay.

Lipid Stabilization of Detergent Extracts. Lipid stabilization of detergent extracts was carried out as described by Agnew & Raftery (1979). Samples (0.5 mL final volume) of an initial 1% Lubrol-PX extract were made 4% in Lubrol-PX, with stocks of detergent containing various amounts of phosphatidylcholine (chicken egg). After 10 min in ice, the samples were warmed to 18 °C for 12 min and were plunged into an ice slurry, excess [^{3}H]TTX was added, and after 5 min, the samples were assayed by the G-50 assay. Samples (0.45 mL each) of material were purified by ion exchange (259 pmol of TTX binding per mg of protein; containing 0.108% Lubrol-PX in which had been previously dissolved various amounts of egg phosphatidylcholine). After a 10-min equilibration at ice-bath temperature, the samples were warmed to 18 °C for 12 min, followed by plunging in an ice slurry and adding excess [^{3}H]TTX as before. After 10 min, G-50 assays were run. In each case apparent first-order inactivation constants (K_r) were calculated from the equation $K_r = 1/t \ln (B_0/B)$, when B_0 is the number of sites initially present and B is the number remaining at time t. The values of K_r were plotted as a function of the final lipid phosphate to detergent ratio.

Lectin Affinity Chromatography. Pilot lectin columns (0.5–1 mL) were poured in 3-mL plastic syringes and equilibrated with 0.05 M potassium phosphate, pH 7.5, containing 0.1% Lubrol-PX:phosphatidylcholine (7:1 molar ratio), 0.2 M NaCl, and 20 nM [^{3}H]TTX. TTX binding protein (80–200 pmol in 0.5 mL) was slowly run onto the column over a period of 1–2 h. The column was washed with 9 volumes of buffer and then slowly eluted with buffer made 0.2 M in the appropriate sugar over a period of 1 h. One-milliliter fractions were collected and assayed for [^{3}H]TX binding and protein.

The kinetics of absorption and desorption of TTX binding protein by WGA–Sepharose 6MB were studied as follows. The time course of absorption was measured by mixing 5 mL of a solution containing TTX binding protein with an equal volume of WGA–Sepharose 6MB preequilibrated with the buffer described above. A small sample was removed for measurement of the starting TTX binding site concentration (which was varied between 80 and 300 pmol/mL), and the suspension was divided equally into 1.4-mL aliquots. These were stirred at 4 °C. Aliquots were assayed as a function of time for depletion of TTX binding activity in the supernatant. The amount of TTX receptor specifically bound after equilibration was determined by first washing the resin 8 times with buffer (2 volumes per wash). The bound TTX binding protein was then removed by addition of 1 volume of buffer 0.4 M in NAG and incubation for 2 h. The supernatant was removed and the resin washed 3 times with 1 volume of buffer 0.2 M in NAG. The desorbed material and washes were pooled and assayed for [^{3}H]TTX binding activity.

The time course of desorption was measured as follows. Material purified by ion exchange (5.25 mL; 500 pmol/mL) was mixed with an equal volume of WGA–Sepharose 6MB preequilibrated with the buffer described above. The mixture was stirred slowly for 20 h at 4 °C, and the resin was extensively washed with buffer to remove unbound TTX binding protein. The resin was delivered in 0.6-mL aliquots to small vials, and to each was added 0.6 mL of buffer containing 0.2 M NAG. The resin was incubated in ice with occasional mixing, and the supernatants were assayed as a function of time for TTX binding activity.

Results

Characteristics of the Membrane Preparation. The data in Figure 1 demonstrate that the isolated electroplax membranes retain specific sites for neurotoxin binding, as illustrated by the saturable binding of [^{3}H]STX. Binding to membranes followed a single Langmuir isotherm with K_d = 1–10 nM, with the affinity for STX slightly higher than that measured for TTX. The packed membrane fraction is a crude preparation and consists largely of connective tissue fibrils, including collagen. The specific activity for TTX binding by these membranes is rather low (3–4 pmol/mg). However, there is usually only 10–20 pmol of lipid phosphate (7.5–15 mg of phospholipid) per g of packed membranes, suggesting that only a small part of the total protein present is associated with membranes. This is consistent with the observation that detergent extracts range from 15 to 25 pmol of TTX binding/mg

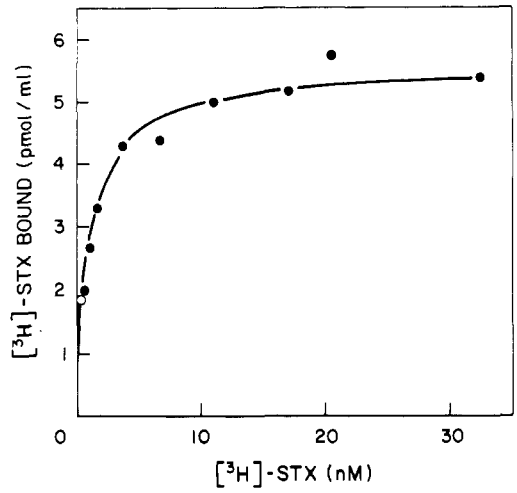

FIGURE 1: Binding of [^{3}H]STX to membrane fragments. Curve fit for K_d = 0.7 and B_{max} = 5.9 nM, corresponding to 649 pmol of sites per g (wet weight) of packed membranes.

of protein and that the bulk of the pellet (primarily collagen) is not depleted by detergent treatment.

Solubilization of the TTX Receptor. We surveyed a variety of nonionic, synthetic detergents and the sodium salts of cholic and deoxycholic acids as solubilizing agents. Poor apparent efficiency of solubilization could result from either inefficient release from the membrane, instability of the solubilized binding protein, or solubilization of the binding site in a form in which a high rate of toxin dissociation would cause the G-50 assay procedure to underestimate the amount of sites present.

The data presented in Figure 2 and Table I summarize results from six solubilization experiments carried out with a single preparation of membranes. Aliquots of the membrane suspension, either in buffer alone or saturated with [^{3}H]TTX, were treated with increasing concentrations of each detergent followed by sedimentation. Supernatants were assayed for protein and toxin binding. In addition, the rate of dissociation of [^{3}H]TTX from the receptor was examined.

In the absence of [^{3}H]TTX during solubilization, the most effective detergent was Lubrol-PX. Among the least effective were the bile salts. All detergents destabilized or inactivated the receptor at high concentrations as revealed by the higher recoveries achieved when TTX was present to stablize the molecule (Agnew et al., 1978). The destabilization gives the solubilization curve a characteristic optimum which is different for each detergent and is dependent not only on detergent concentration but also on the weight ratio of membranes to detergent.

In the presence of [^{3}H]TTX, but not in its absence, only Nonidet P-40 was comparable in its solubilization efficiency to Lubrol-PX. However, the Nonidet P-40 solutions were markedly less stable than those made with Lubrol-PX. The latter was chosen as the detergent of choice because it showed the highest percentage solubilization, highest specific activities, and the most stable extracts. It also permitted efficient solubilization at the lowest detergent concentrations.

As described previously, the rate constant for dissociation of the solubilized receptor TTX complex was much slower than has been reported for membrane-bound channels (Reed & Raftery, 1976; Ulbricht, 1977). This slow dissociation (k_{-1} = 0.0004 s^{-1}) has made possible the rapid gel filtration assay

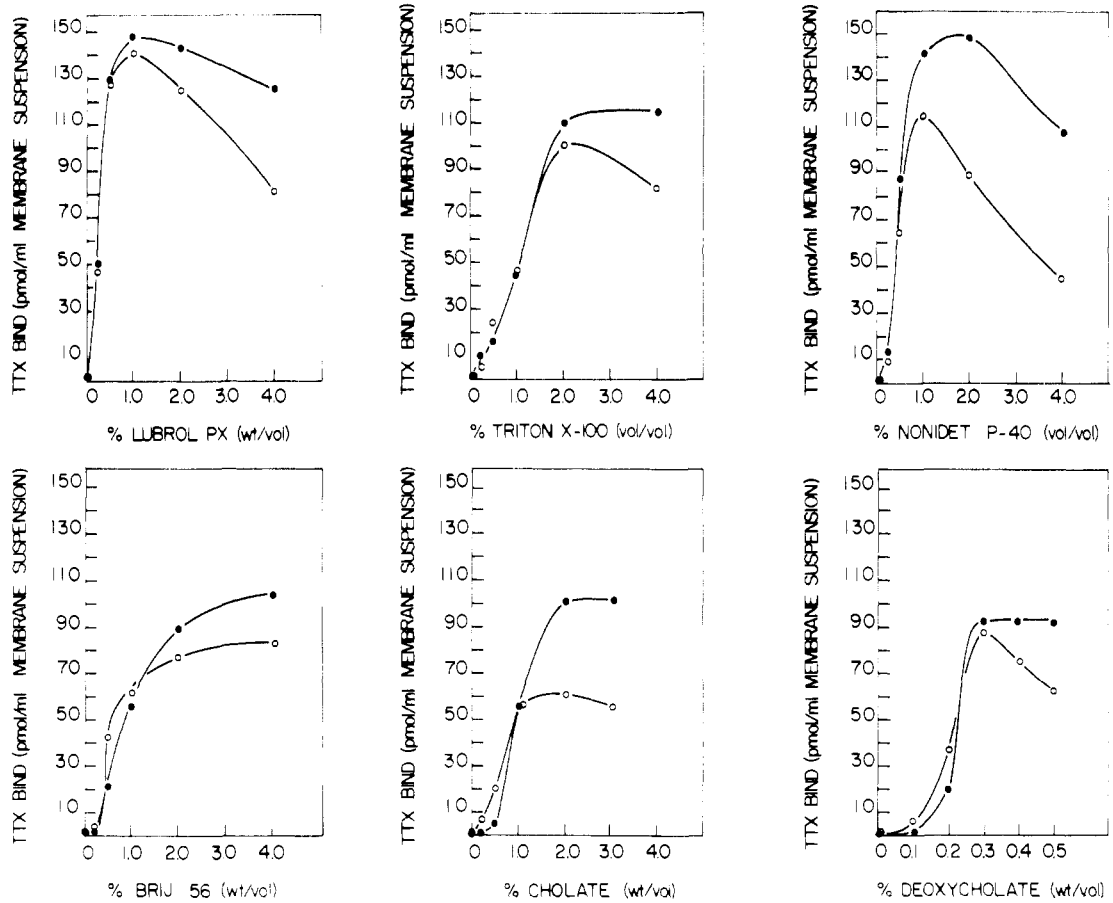

FIGURE 2: Detergent solubilization of the TTX binding component. TTX binding in the 100000*g* supernatant is plotted against the detergent concentration. Solubilizations were done in the absence (○) or presence (●) of saturating TTX. Most efficient was 1% (w/v) Lubrol-PX, at 89% solubilization and 18.7 pmol of TTX bound/mg of protein.

Table I: Solubilization of the TTX Binding Component with Different Detergents[a]

detergent used for solubilization	max TTX binding component solubilized (pmol/mL of membrane suspension) −TTX	max TTX binding component solubilized (pmol/mL of membrane suspension) +TTX	sp act. (pmol of TTX bound/mg of protein)	$t_{1/2}$ for TTX dissociation from solubilized TTX-protein complex (min)
Lubrol-PX (1% w/v)	141	148	18.7	12.2
Triton X-100 (2% w/v)	100	110	16.4	12.6
Nonidet P-40 (2% w/v)	80	148	15.4	9.5
Brij 56 (4% w/v)	84	104	16.1	9.6
sodium cholate (2% w/v)	61	102	12.1	10.5
sodium deoxycholate (0.3% w/v)	80	92	11.3	10.6

[a] Total number of sites 167.2 pmol/mL; 507 pmol of TTX binding sites/g wet weight of packed membranes.

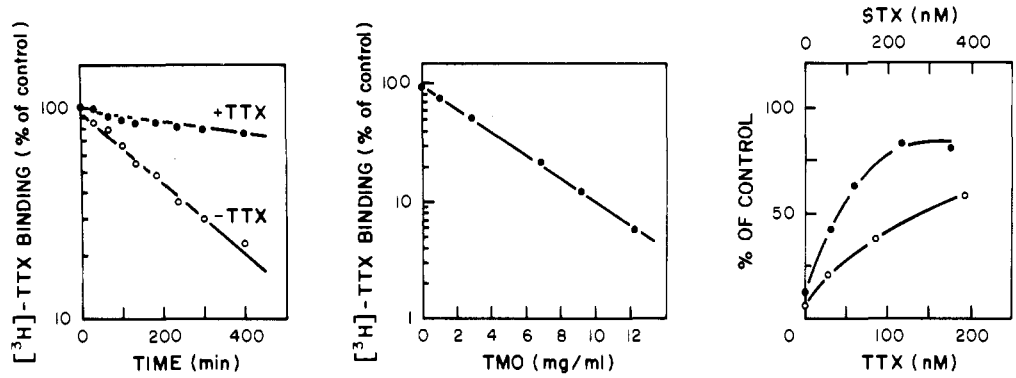

FIGURE 3: Chemical modification of TTX and STX binding sites. (Left panel) Partially purified, solubilized TTX binding protein was treated with EDC and glycine ethyl ester, in the absence or presence of saturating concentrations of TTX (110 nM), and the time course of inactivation was measured. (Middle panel) Inactivation of TTX binding by a partially purified preparation, with increasing concentration of trimethyloxonium tetrafluoroborate (TMO). (Right panel) Protection of [^{3}H]STX (○) and [^{3}H]TTX (●) binding against TMO inactivation. Sufficient TMO was added to inactivate 90% of binding, but the reactions were carried out in the presence of increasing amounts of TTX or STX (abscissas) followed by measurement of [^{3}H]TTX or [^{3}H]TTX binding. Control incubations performed with reaction products of TMO produced approximately 15% inactivation.

for quantitation of soluble preparations. It was noted (Levinson et al., 1979) that in crude, solubilized preparations the dissociation rates were slightly biphasic. A small proportion of the sites showed rapid dissociation while the majority of the sites displayed the low dissociation rate. This may correlate with observations (Agnew et al., 1981) that the physical state of the soluble material is somewhat heterogeneous. The majority of the TTX binding protein is found to be in a well-defined soluble form (apparent sedimentation coefficient $\simeq$ 8 S), and a lesser, variable fraction seems to be in a higher state of association. These two populations of receptor may have values for the dissociation rate characteristic of solubilized and membrane-bound sites, respectively. The practical consequence of this is that the half-lives computed for bound toxin in Table I are somewhat shorter than those found in a full dissociation time course (cf. Levinson et al., 1979). Nevertheless, the stabilities of the toxin-binding site complex solubilized with the other five detergents were not markedly different from that solubilized with the well-characterized Lubrol-PX, suggesting that the G-50 assay is useful for all detergents tested. The utility of each detergent seems to be limited only by the efficiency of release from the membrane and the stability of the solubilized receptor.

Specific Chemical Modification of the TTX Binding Site in Solubilized Preparations. Chemical modification studies of sodium channels in nerve (Baker & Rubinson, 1975; Sigworth & Spaulding, 1979) and membrane preparations (Reed & Raftery, 1976) suggest the presence of one or more carboxylates at the toxin binding site (see Discussion). Consequently, we have investigated the sensitivity of the solubilized TTX binding component to two types of reagent that covalently derivatize carboxyl groups. The studies were carried out not only to demonstrate chemical reactivities characteristic of the physiologically defined sodium channel but also to investigate the possibility of introducing a radiolabel specifically into the toxin binding site. This would assist in identifying the peptide or peptides associated with the toxin binding site and which may form an important part of the pore structure itself.

The data in Figure 3 (left panel) demonstrate that TTX binding component purified by the ion-exchange procedure is readily inactivated by a carbodiimide (EDC) and glycine ethyl ester. Occupancy of the binding site by TTX greatly reduced the rate of inactivation. In this experiment, all occupied TTX binding sites were shown to be in dynamic equilibrium with free [^{3}H]TTX, and bound toxin could be displaced by competition with unlabeled TTX. The experiment could only be performed with partially purified preparations; unpurified extracts contain high levels of esterase activity which results in hydrolysis of the glycine ester and a rapid drop in pH, heading to rapid inactivation of TTX binding.

Although the toxin binding activity demonstrated the expected sensitivity to treatment with carbodiimide and nucleophile, the method is unsuitable for specific labeling of the protein. Reaction with the carbodiimide may produce general activation of carboxylates on the surface of the protein, resulting in covalent cross-linking with nearby nucleophilic residues. After modification, we were unable to fractionate the peptides in the preparation by $NaDodSO_4$–polyacrylamide gel electrophoresis. Therefore, the use of TMO for binding site inactivation was investigated since this would produce little or no cross-linking. Figure 3 (middle panel) shows that increasing amounts of TMO resulted in almost total inactivation of [^{3}H]TTX binding. With concentrations of TMO which

Table II: Sensitivity to Degradative Enzymes[a]

			TTX binding (% of control)			
			additions			
expt	enzyme	mg/mL	none	TTX	BSA	TTX + BSA
I	phospholipase A_2	0.01	17.8	61.9	31.7	70.6
	(bee venom)	0.02	17.2	61.2	26.6	69.8
	(1470 units/mg)	0.04	15.2	63.0	26.8	69.8
II	trypsin (206	0.06	85.1	94.2		
	units/mg)	0.12	81.3	91.7		
	α-chymotrypsin	0.06	94.3	98.5		
	(49.2 units/mg)	0.12	95.1	98.3		
	papain (26.3	0.06	88.2	83.4		
	units/mg)	0.12	82.5	85.7		
III	trypsin (206 units/mg)	0.9	82.4	79.3		
	α-chymotrypsin (49.2 units/mg)	0.9	88.3	85.9		
	papain (26.3 units/mg)	0.9	63.9	87.2		

[a] For experiments I and II, incubations were for 3 h at ice-bath temperatures using partially purified, solubilized TTX binding component. Specific activities were the following: (I) 258 pmol/mg of protein, 0.195 mg of protein/mL; (II) 429 pmol/mg, 0.12 mg of protein/mL. For III, suspended membranes were incubated 1 h at 25 °C and assayed in triplicate. Specific activity was 8.35 pmol/mg of protein, 4.2 mg of protein/mL. All enzymes were assayed with artificial substrates and were fully active under the conditions employed.

would produce more than 90% inactivation, the reaction was carried out in the presence of increasing amounts of TTX (Figure 3, right panel). The amount of inactivation was compared to that found for control samples in which the TTX binding protein was incubated with the hydrolysis products of TMO. Thus, the protection of the toxin binding sites by TTX was not due to stabilization against nonspecific inactivation by TMO breakdown products. All [^{3}H]TTX binding could be competitively displaced with unlabeled toxin.

An identical experiment was carried out for [^{3}H]STX binding (Figure 3, right panel). Again, protection was observed, although the faster equilibrating STX did not protect as efficiently as did TTX. It should be noted that [^{3}H]STX binding measured by the gel filtration assay is only semiquantitative. This does not, however, compromise the conclusion that STX occupancy protects the STX binding site.

Susceptibility of the Toxin Binding Protein to Inactivation by Degradative Enzymes. Data presented in Table II show the result of treating solubilized, partially purified receptor with three proteases, phospholipase A_2 (see venom), and neuraminidase. In addition, the effect of proteases on membrane-bound receptor was examined. The usefulness of TTX occupancy to stabilize against the action of these agents was also tested.

Neuraminidase had no effect on the stability of the toxin binding site; however, a small but reproducible amount of inactivation of TTX binding activity was observed for both membrane-bound and solubilized preparations when treated with trypsin, chymotrypsin, or papain.

Even after long incubation with very high weight ratios (1:1 protein to protease), the extent of inactivation was small. Benzer & Raftery (1972) found that TTX binding activity in membrane fractions was relatively insensitive to protease treatment, and this was believed to reflect the fact that the receptor was buried or stabilized in the membrane in such a way that inactivation by proteolysis might be rather inefficient. It is surprising that on release from the membrane with detergent, resistance to proteolytic inactivation persists. The results presented here allow us to conclude that the instability of the solubilized toxin receptor (Henderson & Wang, 1972; Agnew et al., 1978; Agnew & Raftery, 1979; Catterall et al., 1979; Barchi et al., 1980) is not likely to result from proteolytic degradation. It is not known whether the protein is actually resistant to proteolytic degradation or whether it retains ability to bind TTX even after partial proteolysis.

Our conclusions differ from those in the earlier report of Benzer & Raftery (1973), who found that the TTX binding protein solubilized from gar olfactory nerves with Triton X-100 was quite sensitive to protease treatment. Their experimental conditions were somewhat harsher than those used in this study. However, the finding that partially purified material was not inactivated after a 3-h treatment with an equal weight of demonstrably active trypsin, chymotrypsin, or papain shows that the binding activity is not exceptionally sensitive to proteolysis.

In contrast to the insensitivity of TTX binding to proteolytic inactivation, sensitivity to the action of phospholipase A_2 was high. The data in Table II show that treatment of the sample with this enzyme resulted in loss of most of the toxin binding activity after 3 h at 0 °C. Increasing the enzyme concentration by 2- or 4-fold did not cause further inactivation, showing that the enzyme activity was not rate limiting. The destabilization results in an increased sensitivity to elevated temperature (data not shown). Addition of delipidated bovine serum (BSA) to bind hydrolysis products protected slightly against inactivation, consistent with observations that fatty acids and lysolecithin cause instability (Baumgold, 1980). Addition of TTX, even in the presence of BSA, markedly protected aganst inactivation. Phospholipase A_2 may cause instability by two mechanisms, first by lowering the lipid:detergent ratio through depletion of phosphotidylcholine and second by causing the accumulation of destabilizing hydrolysis products.

Effects of Mixed Micelle Composition on the Stability of Partially Purified TTX Binding Protein. The stability of the TTX binding protein purified by ion-exchange chromatography was compared to the initial 1% Lubrol-PX extract, using the method of Agnew & Raftery (1979). When the first-order inactivation constant, k_r, was plotted as a function of final lipid:detergent ratio, the data points for both the crude and partially purified extracts fell precisely on the same curve (Figure 4). Thus, the TTX binding protein exhibits quantitatively the same sensitivity to mixed micelle composition in crude extracts, and after a 30-fold purification.

A possible explanation for the stabilization of crude extracts by phospholipids was that manipulation of the mixed micelles structure could protect the receptor from the action of endogenous proteases and phospholipases. The observed insensitivity to proteases seems to rule out this possibility. Since the rate constants for inactivation of partially purified and crude preparations are identical, protection from endogenous degradative enzymes seems to be an unlikely mechanism for the stability behavior.

Lectin Affinity Chromatography. The possible use of lectin affinity chromatography as an aid for purification was investigated by using pilot columns of wheat germ agglutinin (WGA), ricin, concanavalin A (Con A), and *Lens culinaris* lectin immobilized on Sepharose 4B. In each case, the amount of TTX binding component bound and specifically eluted was a small percentage of the total activity applied. The data in Table III summarize the results. Recovery of activity from all lectins except Con A was generally high. Yields from Con A columns were usually less than 50% and were quite variable. This may result from the binding by Con A of phospholipids

Table III: Interaction of the TTX Binding Protein with Lectin-Sepharose Resins[a]

lectin	eluting sugar	TTX binding protein applied (pmol)	act. recovered (%)	bound (%)
Con A	α-methyl glucoside	142	49	9.6
Lens culinaris	α-methyl glucoside	142	70	4.1
WGA	*N*-acetylglucosamine	84	77	8.2
WGA	*N*-acetylglucosamine	214	96	6.6
WGA-Sepharose 6MB	*N*-acetylglucosamine	138	81	15.7
ricin[b]	*N*-acetylgalactosamine	138	94	11.0
WGA[b]	*N*-acetylglucosamine	138	95	4.5

[a] Experiments were carried out by using TTX binding protein purified by ion exchange at 4 °C. Protein was incubated with resin 1–2 h, washed with buffer, and eluted with buffer 0.2 M in sugar. [b] Experiments were carried out with TTX binding protein purified by both ion-exchange procedure and Sepharose 6B chromatography (as described by Agnew et al., 1980).

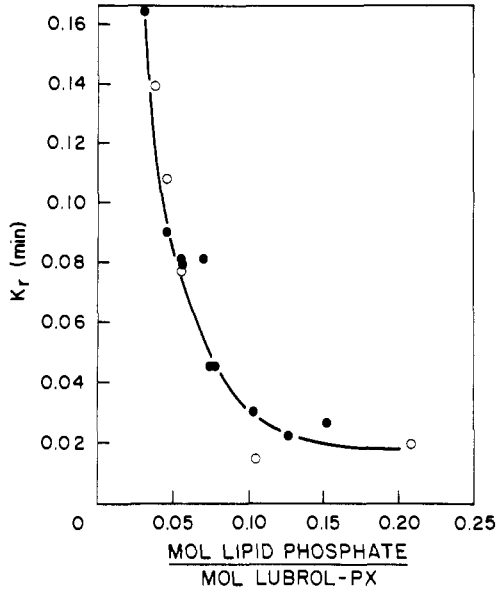

FIGURE 4: Stability of the TTX binding protein in detergent extracts. Comparison of stability curves for an initial 1% Lubrol-PX extract (●) with that for material purified by ion exchange (○), as described in text.

necessary for stability of the TTX binding activity (Boldt et al., 1977; Agnew & Raftery, 1979).

WGA–Sepharose 6MB was selected as the resin for further study because total recoveries of activity were generally high and this resin showed the highest percent uptake of TTX binding protein. Measurement of resin capacity with either [^{14}C]NAG or ovomucoid indicated that available lectin sites were present in great excess (several thousandfold) over the applied TTX binding component.

The time course of TTX binding component uptake by WGA–Sepharose 6MB was examined. Uptake appeared to be complete after 10 h. In the experiment shown in Figure 5 (left panel), ion exchange purified TTX binding component was mixed with an equal volume of resin, and the depletion of TTX binding activity in the supernatant was measured. The linear loss of activity, seen clearly at later times, was attributed to the instability of the TTX binding component. The amount of specifically bound TTX binding protein per milliliter of resin, after equilibration, was a linear function of the applied concentration in the range studied (80–300 pmol/mL). These data suggest that the low percentage of binding is due to the relatively low equilibrium binding affinity of the protein for the lectins. The possibility that only a small percentage of the TTX binding component contains carbohydrate was ruled out by experiments in which unbound fractions containing activity were concentrated and bound to fresh or washed resin with similar uptakes. Additionally we have shown in control experiments (A. C. Moore, unpublished results) that WGA does

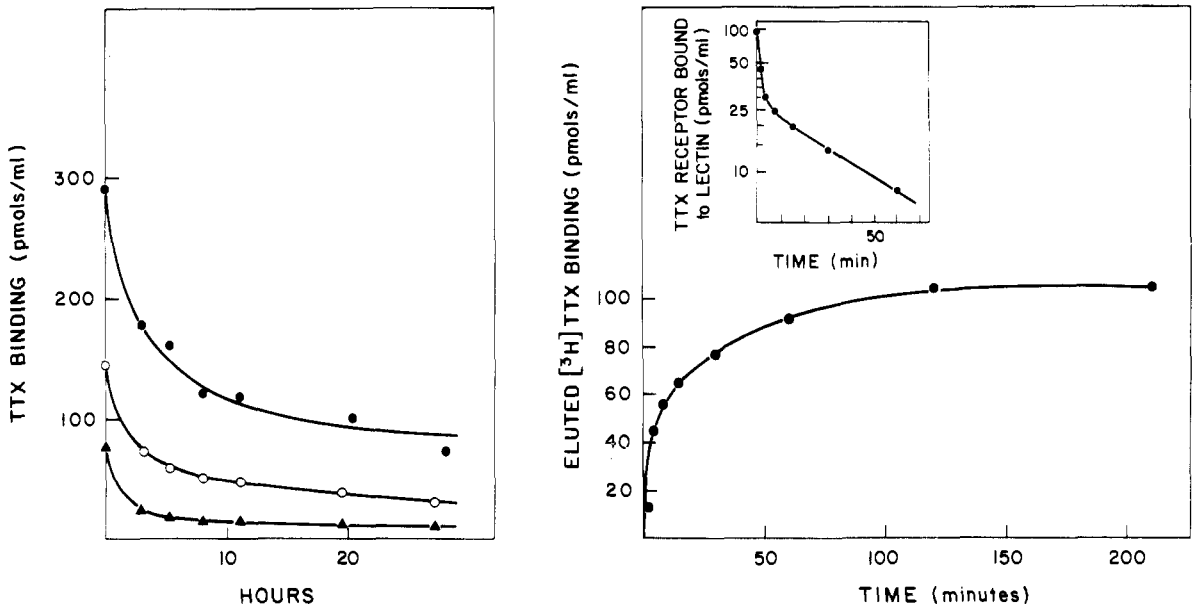

FIGURE 5: Kinetics of the interaction between the TTX binding component and lectin. (Left panel) Time course of uptake TTX binding component by WGA–Sepharose 6MB, as described under Materials and Methods. Starting concentration of TTX binding component in the material purified by ion exchange was 80 (▲), 150 (○), or 300 (●) pmol/mL. (Right panel) Time course of desorption of TTX binding component from WGA–Sepharose 6MB, as described under Materials and Methods.

not interfere with TTX binding.

The time course of desorbtion of the TTX binding protein by excess NAG was also investigated. The time course was relatively fast (Figure 5, right panel) and distinctly biphasic.

Discussion

The TTX binding protein may be solubilized efficiently from eel electroplax membranes with nonionic detergents and less efficiently with sodium cholate and deoxycholate. Under the conditions which we have used, treatment with 1% Lubrol-PX results in release of more than 80% of the total TTX binding material present in the membranes.

The solubilized toxin binding protein exhibits many of the characteristics of sodium channels in membrane and nerve preparations. The solubilized extracts bind [^{3}H]TTX tightly as do the original membranes, with perhaps a slightly larger variability in the K_d for toxin. STX may have a slightly lower affinity for the solubilized receptor than TTX (Agnew et al., 1978), whereas with membrane bound sites, STX normally binds with slightly greater affinity than TTX. The rate of [^{3}H]TTX dissociation from solubilized toxin–receptor complexes is slower than has been reported for membrane-bound material while the rate of dissociation for STX is similar or more rapid (Levinson et al., 1979). Unlabeled TTX and STX block [^{3}H]TTX binding according to simple competitive inhibition.

It has been demonstrated clearly in physiological studies that TTX binding is inhibited by protons which bind to a site of pK = 5.2 (Ulbricht & Wagner, 1975). The same type of pH dependence for toxin binding has been demonstrated in studies with electroplax membranes (Reed & Raftery, 1976). These experiments suggest the involvement of an ionizable group at the TTX and STX binding site. Protonation of this moiety, presumably a carboxyl group, prevents toxin binding. In support of this suggestion, it has been demonstrated that sodium channels in nerve preparations become TTX insensitive when treated with reagents that covalently modify carboxylates (Baker & Rubinson, 1975; Sigworth & Spaulding, 1979). The effect is prevented when nerves are modified in the presence of TTX. Further, treatment of electroplax membranes with TMO results in irreversible loss of [^{3}H]TTX binding activity, and the loss can be prevented by low concentrations of TTX or STX (Reed & Raftery, 1976). Thus, these data are consistent with the notion that one or more carboxyl groups may form part of the toxin binding site. We have shown that toxin binding to the solubilized receptor can be blocked by carboxylate-modifying reagents and that this reaction can be prevented in the presence of TTX. Thus, the detergent-solubilized protein is a suitable preparation for investigation of the ionizable group involved in toxin binding. Of the two types of carboxylate derivatization procedures tested, TMO offers the most promise for ultimate localization of the binding site.

We have demonstrated that the TTX binding protein from *Electrophorus electricus* is a glycoprotein, based on interaction of the solubilized preparation with lectin columns. These results agree with those of Barchi et al. (1980), who first showed that an STX binding component from sarcolemma bound to WGA columns.

Detergent-solubilized and membrane-bound TX receptors show the same sensitivity to degradative enzymes. Both preparations show small, reproducible amounts of inactivation of toxin binding by proteases. The relative insensitivity to proteases is in striking contrast to the sensitivity to phospholipase A_2. This effect of phospholipase A_2 and a much lower effect of phospholipase C have been reported for toxin binding activity in a variety of membrane preparations (Benzer & Raftery, 1972; Reed & Raftery, 1976; Baumgold, 1980). The inactivation of detergent-solubilized material may result from at least two mechanisms. First, it has been shown in membranes (Baumgold, 1980) that many free fatty acids and lysolecithin quickly destabilize toxin binding sites. Thus, an accumulation of hydrolysis products would provide a reasonable explanation for the inactivation observed with phospholipase A_2. This is supported by the slight protective effect of delipidated BSA. Additionally, however, it has been shown (Agnew & Raftery, 1979) that the solubilized TTX receptor is extraordinarily sensitive to mixed lipid–detergent micelle composition. The rate at which solubilized preparations inactivate is a quantitatively reproducible function of the ratio of endogenous lipid to detergent and not of the absolute concentration of either component alone. Thus, depletion of phosphatidylcholine by phospholipase could lower the ratio of lipid to detergent, greatly decreasing the stability of the binding site.

References

Agnew, W. S., & Raftery, M. A. (1979) *Biochemistry 18*, 1912–1919.

Agnew, W. S., Levinson, S. R., Brabson, J. S., & Raftery, M. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2606–2610.

Agnew, W. S., Moore, A. C., Levinson, S. R., & Raftery, M. A. (1980) *Biochem. Biophys. Res. Commun. 92*, 800–866.

Agnew, W. S., Moore, A. C., Levinson, S. R., & Raftery, M. A. (1981) *Nerve Membrane: Biochemistry and Function of Channel Proteins* (Matsumoto, G., & Kottani, M., Eds.) p 25, University of Tokyo Press, Tokyo.

Armstrong, C. M. (1975) *Q. Rev. Biophys. 7*, 179–210.

Armstrong, C. M., & Benzanilla, F. (1973) *J. Gen. Physiol. 62*, 305–339.

Armstrong, C. M., & Bezanilla, F. (1974) *J. Gen. Physiol. 63*, 533–553.

Baker, P. J., & Rubinson, R. A. (1975) *Nature (London) 257*, 412–414.

Barchi, R. L., Cohen, S. A., & Murphy, L. E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1306–1310.

Baumgold, J. (1980) *J. Neurochem. 34*, 337–334.

Benzer, T., & Raftery, M. A. (1972) *Proc. Natl. Acad. Sci. U.S.A. 75*, 218–233.

Benzer, T. I., & Raftery, M. A. (1973) *Biochem. Biophys. Res. Commun. 51*, 939–944.

Boldt, D. H., Speckart, S. F., Richards, R. L., & Alving, C. R. (1977) *Biochem. Biophys. Res. Commun. 74*, 208.

Catterall, W. A., Morrow, C. S., & Hartshorne, R. P. (1979) *J. Biol. Chem. 254*, 11379–11387.

Cuervo, L. A., & Adelman, W. J. (1970) *J. Gen. Physiol. 55*, 309–355.

Dittmer, J. C., & Wells, M. A. (1969) *Methods Enzymol. 14*, 245–254.

Henderson, R., & Wang, J. H. (1972) *Biochemistry 11*, 4565–4569.

Hille, B. (1968) *J. Gen. Physiol. 51*, 199–219.

Hille, B. (1970) *Prog. Biophys. Mol. Biol. 21*, 1–32.

Hille, B. (1975a) *Biophys. J. 15*, 615–619.

Hille, B. (1975b) *J. Gen. Physiol. 66*, 535–560.

Hille, B. (1978) *Biophys. J. 22*, 283–294.

Keynes, R. D., & Rojas, E. (1974) *J. Physiol. (London) 239*, 393–434.

Landowne, D., Porter, L. T., & Tarrar, D. A. (1975) *Annu. Rev. Physiol. 37*, 485–508.

Levinson, S. R. (1975) *Philos. Trans. R. Soc. London, Ser. B 270*, 337–348.

Levinson, S. R., Curatalo, C. J., Reed, J., & Raftery, M. A.

(1979) *Anal. Biochem. 99*, 72–84.
Moore, D. G., & Koshland, D. E. (1967) *J. Biol. Chem. 262*, 2447–2452.
Nakamura, Y., Nakajima, S., & Grundfest, H. (1965) *J. Gen. Physiol. 49*, 321–349.
Narahashi, T., Anderson, N. C., & Moore, J. S. (1967) *J. Gen. Physiol. 50*, 1413–1428.
Radin, M. A. (1969) *Methods Enzymol. 14*, 482–530.
Reed, J., & Raftery, M. A. (1976) *Biochemistry 15*, 944–953.
Ritchie, J. M., & Rogart, R. B. (1977) *Rev. Physiol., Biochem. Pharmacol. 79*, 1–50.
Ritchie, J. M., Rogart, R. B., & Strichartz, G. (1976) *J. Physiol. 261*, 477–494.
Sigworth, F. J. (1980) *J. Physiol. 307*, 131–142.
Sigworth, F. J., & Spaulding, B. C. (1979) *Nature (London) 283*, 293.
Udenfriend, S., Stein, S., Bahleu, P., Dairman, W., Leimgruber, W., & Weigele, M. (1972) *Science (Washington, D.C.) 178*, 871–873.
Ulbricht, W. (1977) *Annu. Rev. Biophys. Bioeng. 6*, 7–31.
Ulbricht, W., & Wagner, H.-H. (1975) *Philos. Trans. R. Soc. London, Ser. B 270*, 353–364.

Molecular Dynamics and Conformation in the Gel and Liquid-Crystalline Phases of Phosphatidylethanolamine Bilayers†

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ABSTRACT: Solid-state deuterium and carbon-13 nuclear magnetic resonance (NMR) spectra have been used to study the molecular dynamics and conformation of dipalmitoylphosphatidylethanolamine (DPPE) in both the gel (L_β) and liquid-crystalline (L_α) phases. For this purpose DPPE was labeled with ^{13}C in the carbonyl group of the *sn*-2 chain and with ^2H at three different positions—4, 8, and 12—of the *sn*-2 chain, at the 2 position of the glycerol backbone, and at the 1 position of the ethanolamine head group. The ^{13}C carbonyl and ^2H chain spectra indicate that in the gel phase the DPPE molecules are diffusing about their long axes at rates of 10^5–10^6 s^{-1} and the acyl chains are in an approximately all-trans conformation. The glycerol backbone spectra suggest that the backbone is in a gauche conformation in the gel state, rather than a trans conformation such as found in single crystals. The head group spectra in the gel phase are broad, featureless lines of about 20-kHz width. At the $L_\beta \rightarrow L_\alpha$ phase transition several changes take place. As is well-known, the chains disorder, and fast long-axis rotational diffusion begins, which results in the sharp, axially symmetric L_α phase ^2H spectra, which are a factor of 2 narrower than those observed in the L_β phase. The head group spectra also sharpen substantially at the transition, although their total width remains approximately constant. The invariance of the spectral width suggests that the average head group conformation is similar in both phases. However, the sharper spectra seen in the L_α phase indicate that the rates of the head group motions in this phase are at least 3 orders of magnitude faster than those in the L_β phase. Thirdly, the ^2H spectra of the glycerol backbone labeled DPPE narrow by a factor of about 4, and we believe this is due to a conformational change in this region of the molecule. Consistent with this interpretation is the fact that the powder pattern exhibited by the *sn*-2 $^{13}C{=}O$ in the L_β phase collapses to an isotropic-like line at the phase transition.

Deuterium nuclear magnetic resonance (^2H NMR)[1] has been used extensively to examine the structural and dynamical properties of phospholipid bilayers in the L_α or liquid-crystalline phase. For example, the acyl chains in PC's and PS, the glycerol backbone in PC's and PE's, and the head groups of four different lipid classes have been studies with this technique (Seelig & Seelig, 1974, 1975; Seelig, 1977; Oldfield et al., 1978; Browning & Seelig, 1980). The results of these investigations suggest that phospholipids in the liquid-crystalline phase generally exhibit similar behavior. For instance, the acyl chain spectra show a plateau region with quadrupole splittings of about 30 kHz, which decrease as the terminal methyl group is approached. Spectra of head group labeled lipids differ in detail as might be expected; however, in all cases they are relatively narrow, axially symmetric powder line shapes, which suggests greater conformational freedom than is available in the acyl chain region of the bilayer (Seelig & Seelig, 1980).

In contrast to the liquid-crystalline phase, the gel phase of phospholipids has been much less thoroughly examined. The reason for this is partially due to the technical difficulties associated with obtaining gel-state ^2H NMR spectra, which are a factor of 2–4 times wider than those observed in the liquid-crystalline phase. However, with the introduction of the quadrupole echo technique (Solomon, 1958; Davis et al.,

† From the Francis Bitter National Magnet Laboratory, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received February 25, 1982; revised manuscript received July 8, 1982.* This research was supported by the National Institutes of Health (GM-23289, GM-25505, and RR-00995) and by the National Science Foundation through its support of the Francis Bitter National Magnet Laboratory (C-670). A.B. was supported by a research scholarship from the Deutsche Forschungsgemeinschaft (BL 182/2-4) and R.J.W. by a U.S. Public Health Service postdoctoral fellowship (GM-07215).

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[1] Abbreviations: DPPE, dipalmitoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DLPE, dilauroylphosphatidylethanolamine; DMPC, dimyristoylphosphatidylcholine; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; ^2H NMR, deuterium nuclear magnetic resonance; TLC, thin-layer chromatography; ESR, electron spin resonance.

0006-2960/82/0421-6220$01.25/0