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TETRODOTOXIN RECEPTORS IN MEMBRANE FRAGMENTS

PURIFICATION FROM *ELECTROPHORUS ELECTRICUS* ELECTROPLAX AND BINDING PROPERTIES

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Summary

A tetrodotoxin receptor-rich preparation of membrane fragments from the electric organ of *Electrophorus electricus* is described. The specific binding of neurotoxins and freeze-fracture electron microscopy are used as tools to identify and to characterize membrane fractions. Freeze-fracture electron micrographs of the electric organ demonstrate a high density of membrane particles in the extrasynaptic regions. Density gradient fractions show a broad distribution of [^{3}H]tetrodotoxin, [^{3}H]saxitoxin and ^{125}I-labelled bungarotoxin binding in the range of 1.04—1.15 g/ml sucrose densities, with specific neurotoxin binding up to approx. 5 pmol/mg protein. Carrier-free column electrophoresis of density gradient fractions yields a subfraction with tetrodotoxin and α-neurotoxin binding up to 30 pmol/mg protein. The major part of the membrane fragments forms vesicles, which are separated by lectin chromatography into an outside-out and inside-out population. The latter represents at least 50% of the material of a density gradient fraction. For the association of tetrodotoxin, a bimolecular kinetic constant $k_f \geqslant 3 \cdot 10^5\ M^{-1} \cdot s^{-1}$ is determined. The dissociation constant is $k'_b = 2.5 \cdot 10^{-2}\ s^{-1}$. These data are in agreement with a thermodynamic dissociation constant of $K_D = 20$ nM as determined earlier for *E. electricus* membrane fragments by equilibrium methods (Grünhagen, H.H., Rack, M., Stämpfli, R., Fasold, H. and Reiter, P. (1981) Arch. Biochem. Biophys. 206, in the press).

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However, these association kinetics of tetrodotoxin binding in vitro are significantly different from kinetics determined electrophysiologically in *Rana* (Wagner, H.H. and Ulbricht, W. (1975) Pflügers Arch. 359, 297—315) or *Xenopus* (Schwarz, J.R., Ulbricht, W. and Wagner, H.H. (1973) J. Physiol. 233, 167—194).

Introduction

Transiently opened voltage-sensitive Na channels form an essential constituent of most nerve, muscle and electroplax membranes conducting an action potential. The functioning of these channels has been successfully studied with electrophysiological methods (for reviews, see Ref. 1—3). In recent years, it has also become possible to analyze the Na channel using biochemical techniques. This approach is based on highly specific interactions of neurotoxins with molecular components of the channels [4]. Tritiated preparations of tetrodotoxin and saxitoxin allow the identification of their receptors in vitro and the study of the corresponding binding characteristics [5—10]. Additional information concerning the multiplicity of binding sites is derived from studies with scorpion toxin, sea anemone toxin, batrachotoxin and veratridine [11—13]. The biological systems chosen for these biochemical investigations of Na channels include material from nerve membranes [14—16], synaptosomal membranes [8,10,12,17], neuroblastoma cells [11,13] and electroplax [6,7, 18]. *Electrophorus electricus* electroplax has also been studied in detail with regard to synaptic channels [19—27]. This background of knowledge facilitates the analysis of binding and of functional properties of Na channels in membrane fragments from this organ.

This paper describes the preparation and partial characterization of tetrodotoxin receptor-rich membrane fragments from the electric organ of *E. electricus*. In contrast to solubilized material, such a preparation is expected to preserve the ionophore function of the channel proteins, thus allowing us to correlate binding and function in the same preparation. The analysis of fractions in the preparation is based on morphological properties of the electroplax. It makes use of tetrodotoxin, binding specifically to Na channels, and of α-neurotoxin, binding specifically to acetylcholine receptors. Besides centrifugation techniques, carrier-free column electrophoresis and chromatography on bound lectin are chosen to subfractionate membrane fragments.

Methods

Membrane preparation. E. electricus, approx. 1 m long, was purchased from World Wide Scientific Animals, Ardsley, NY 10502, U.S.A. For membrane fragment preparation [20—22], the whole electric organ was excised and immediately homogenized: 200 g of organ per homogenization step and 200 ml of a solution containing 0.2 M sucrose (Sigma Grade I) and 0.02% NaN_3 were treated twice for 1 min at full speed and 0°C in a VirTis '45' homogenizer. The homogenate was centrifuged for 25 min at 7500 rev./min in a GSA rotor, the low-speed pellet supplemented with a double volume of 0.2 M sucrose,

0.02% NaN_3 and rehomogenized and recentrifuged as before. The collected supernatants were centrifuged for 60 min at 30 000 rev./min in a 35 rotor and the pellets resuspended with a potter in 60 ml of 0.2 M sucrose, 0.02% NaN_3. This suspension was subfractionated in sucrose gradients ranging from 1.04 to 1.18 g/ml density in an SW 27 swinging-bucket rotor. If the composition of solutions had to be changed, e.g., from sucrose medium to Ringer solution (if not otherwise specified: 160 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1.5 mM phosphate buffer, pH 7.0), suspensions were diluted, pelleted (60 min at 100 000 $\times g$), suspended with a potter in the desired medium, repelleted and resuspended. In general, solutions did not contain proteolytic inhibitors. In control preparations supplemented with phenylmethylsulfonyl fluoride ($5 \cdot 10^{-5}$ M) no significant change of the investigated parameters was observed. Protein in all samples was determined by using the method of Lowry et al. [29] with bovine serum albumin as the standard. Specific binding is related to mg protein. Sucrose solutions contained 1 mM phosphate, pH 7.

Enzyme and receptor assays. ($Na^+ + K^+$)-ATPase was measured at 25°C by the pyruvate kinase-lactate dehydrogenase system of Gache et al. [30]. α-Toxin binding was assayed by the Millipore filtration method of Olsen et al. [31] either with *Naja nigricollis* α-toxin tritiated and generously provided by Dr. P. Boquet [32], or with commercial samples of ^{125}I-labelled α-bungarotoxin (New England Nuclear) or [^{3}H]propionyl-α-bungarotoxin (Amersham). Cytochrome *c* reductase (rotenone insensitive) was determined at 37°C according to the method of Fleischer and Kervina [33], Ca^{2+}-stimulated ATPase at 37°C according to that of Meissner [34] and acid phosphatase at pH 4.8 and 37°C with *p*-nitrophenyl phosphate as the substrate [35].

Tetrodotoxin binding assays. Tetrodotoxin binding to Na channels in membrane fragments was measured by a Millipore filtration assay: in general, 200 μl of membrane suspension in Ringer solution were mixed with 800 μl of [^{3}H]tetrodotoxin solution in Ringer solution (sample A). In parallel, a corresponding sample (B) was prepared which contained additionally 1 μM reference tetrodotoxin (Sankyo). If not otherwise specified (kinetic experiments), samples were incubated for 60 min at 0°C and then filtered on an ice-cold filter holder (Millipore or Bio-Rad) through a 0.45 μm millipore filter (HAWP 02500). Filters were washed three times with 3 ml ice-cold Ringer solution at reproducible time intervals. Washing was finished within 20—30 s. Radioactivity on filters was counted with a Triton/toluene scintillation cocktail (Quickszint 212, Zinsser) in a Packard Tri-Carb C2425 β-counter. Bound [^{3}H]tetrodotoxin was not corrected for dissociation effects during filtering and washing. Only the difference between A and B samples was considered specific. To determine the kinetics of association, the time of incubation was varied as indicated. Dissociation was measured by two procedures: (i) filtering and first wash as described above, second aliquot of 3 ml wash Ringer solution left on the filter without suction for a chosen time interval, then third wash, (ii) 10-fold dilution of incubated samples with Ringer solution, defined delay, filtering and two washes. No systematic difference between these two protocols was noticed.

Saxitoxin binding. [^{3}H]Saxitoxin was a generous gift of Dr. R. Rogart, and

was tritiated by a specific exchange procedure [36]. Specific [^{3}H]saxitoxin binding was determined by equilibrium dialysis at 4°C.

Carrier-free electrophoresis. Carrier-free electrophoresis of membrane fragments was carried out in columns [37] designed for isoelectric focussing (LKB). Unless otherwise specified (cf. Fig. 6), 30 ml of 50% sucrose with 100 mM Tris-HCl buffer, pH 7.0, were used as the lower electrode buffer in a 110 ml column (Ref. No. 8100-1). A 90 ml gradient ranging from 30 to 10% sucrose with 1 mM Tris-HCl buffer, pH 7.0, was pumped into the column. The chosen volume of membrane suspension in Ringer solution (e.g., 1.0 ml) with an exactly adjusted sucrose density was pumped into a central position of the gradient. The column was then filled with the upper electrode buffer: 100 mM Tris-HCl, pH 7.0. A voltage of 500 V (top positive) lead to a current of approx. 5 mA at 4°C. Fractions (approx. 6 ml) were collected from the gradient via a flow-through photometer (ISCO UA 5/Type 6). Absorbance was monitored at 254 nm.

Lectin chromatography. To subfractionate density gradient fractions on carrier-bound concanavalin A columns [38,39], a 10 ml column (approx. 9 cm long) was filled with 8 ml of concanavalin A Sepharose suspension (Pharmacia). After sedimentation, a column bed of approx. 5 ml was obtained, which was washed with 50 ml Ringer solution, pH 7.2. The column was charged with 0.5 ml of a density gradient fraction and allowed to equilibrate overnight. The breakthrough was eluted with Ringer solution, pH 7.2, and collected in 0.5 ml fractions. Afterwards, the column was washed with 10 ml of 0.5 M α-methyl-D-glucoside in Ringer solution, pH 7.2, and allowed to equilibrate overnight. Specific elution was carried out then with 0.5 M α-methyl-D-glucoside in Ringer solution, pH 7.2, and 1 ml fractions were collected.

Freeze-fracture of electric organ and of suspensions of membrane fragments. For freeze-fracture electron microscopy, the material was processed according to conventional techniques [46], including fixation in 2% glutaraldehyde and cryoprotection with glycerol (30%). Membrane vesicle preparations were fixed, glycerol-treated and frozen in suspension. Fracturing of the tissue was performed with a double-fracture device in a Balzers BAF 300. Replicas were examined in a Siemens Elmiskop 101.

Preparation of [^{3}H]tetrodotoxin. Tetrodotoxin was tritiated by a defined chemical procedure, starting with tetrodotoxin (citrate free, Sankyo) via anhydrotetrodotoxin as an intermediate, to a specific activity of 18 000 Ci/mol [28].

Results

Freeze-fracture analysis of the innervated face of the electroplax

To analyze the morphology of the excitable face of the electroplax, the electric organ was freeze-fractured in different regions. The excitable face, as identified by presynaptic terminals, shows numerous invaginations in extrasynaptic regions. Fig. 1 demonstrates that these tubules are found in the main organ as well as in the Sachs organ. At the maximal depth to which they extend intracellularly (approx. 1 μm from the flat surface), the electroplax displays a zone of enriched vesicle content. The upper electron micro-

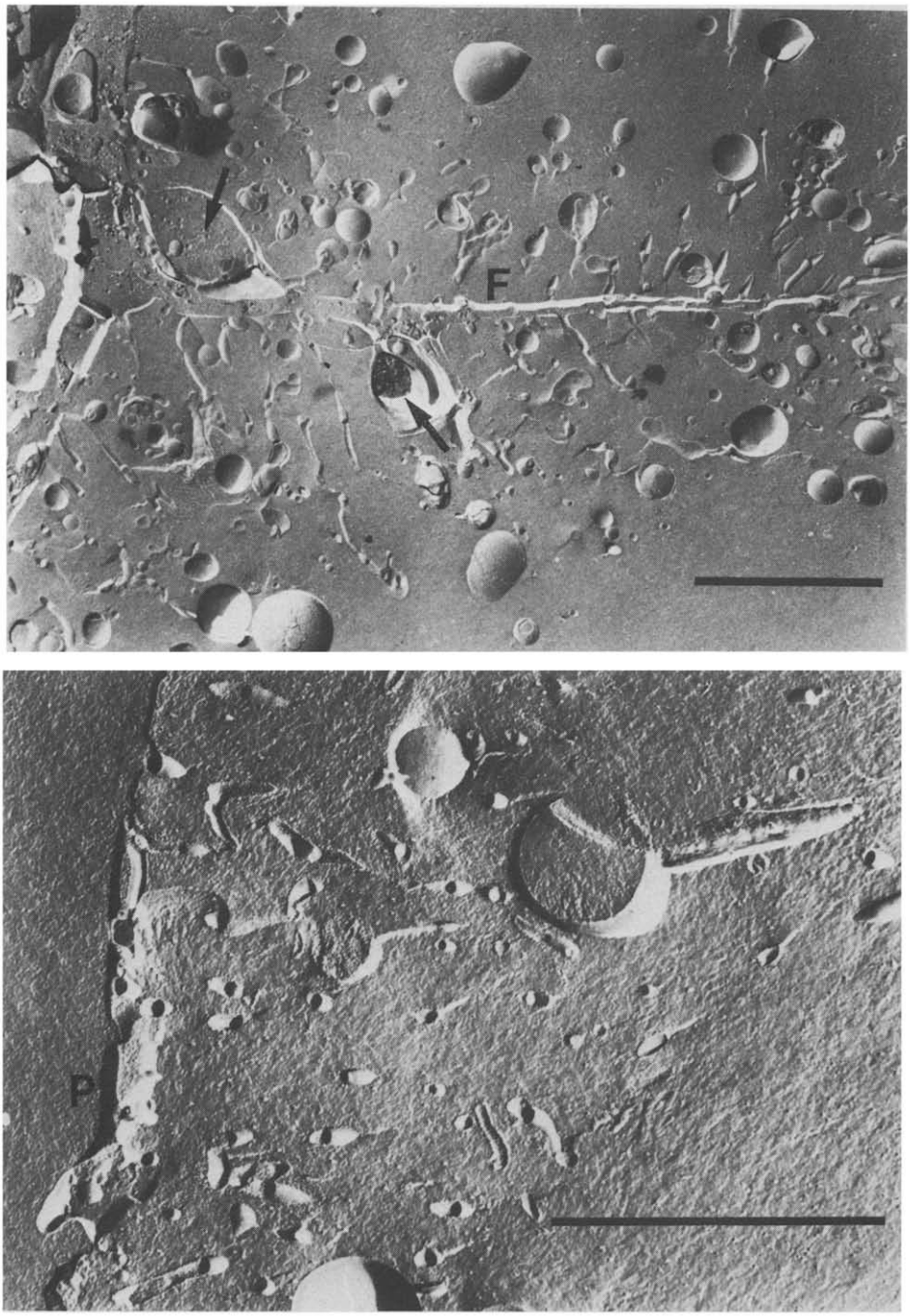

Fig. 1. Excitable face of *E. electricus* electroplax: Morphology of plasma membrane with transverse tubules (freeze fracture). Top: main electric organ with presynaptic terminals (arrows) and deep inward (to the right) folding (F) of postsynaptic membrane. Bottom: Sachs organ with extracellular (left) and intracellular space. P, plasma membrane. The bars represent 2 μm.

graph of Fig. 1 represents part of the main electric organ with a fracture in a region of synaptic terminals. From left to right, a deep crevasse (F) is observed which contains two presynaptic terminals with presynaptic vesicles. The postsynaptic membrane shows transverse tubules more dense in extrasynaptic than in subsynaptic regions. The abundance of transverse tubules in the Sachs organ is demonstrated in the lower part of Fig. 1.

Fig. 2 shows some details of the morphology of the microinvaginations formed by transverse tubules (caveolae) [27,40]. The left part with a boundary between extracellular (left) and intracellular space shows the membranes of transverse tubules to be continuous with the flat regions of the plasma membrane. The density of particles in the membrane of transverse tubules is at least as high as that in extratubular areas. An impression of the density of transverse tubules per unit of flat surface area can be obtained from the upper right frame of Fig. 2. From such fractures tangential to the surface, a figure of 20 tubules per μm^2 can be estimated which is in full agreement with morphometric studies [27,40]. In the fractures screened by us, the distribution of diameters centers at a value close to 0.15 μm. This would be slightly below results of quantitative analyses of semi-thin sections [27]. The lower right frame of Fig. 2 is an example of branching of transverse tubules, which is occasionally observed in the fracture plane. Another morphological feature is an increased diameter or flattening of transverse tubules at their tip.

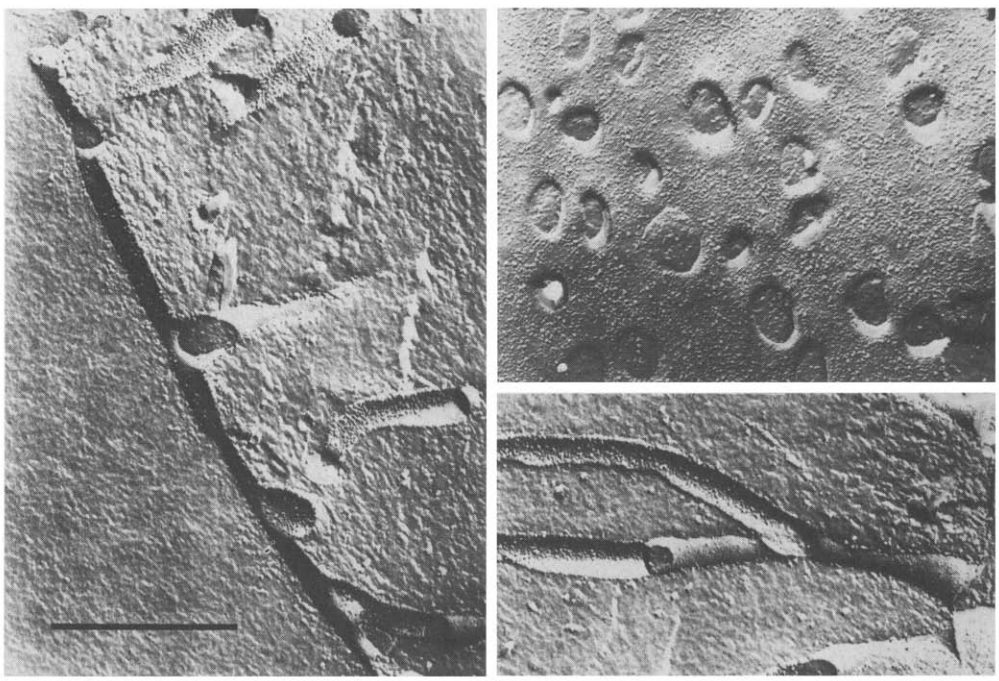

Fig. 2. Transverse tubules as a part of the excitable face of *E. electricus* electroplax (freeze fracture). Left: the membranes of transverse tubules are continuous with the postsynaptic membrane separating extracellular (left) from the intracellular space. Top right: surface density of transverse tubules on plasma membrane. Bottom right: proportions and branching of transverse tubules, extracellular space to the right. The bar represents 0.5 μm (common to all parts of the figure).

Markers in density gradient fractions

The general biochemistry of electroplax membranes has already been thoroughly studied [18,25,26]. Fig. 3 represents the distribution of markers when high-speed pellet material is fractionated on a continuous density gradient. α-Toxin binding is distributed in two broad membrane zones below and above 1.10 g/ml sucrose density. The specific activities of $(Na^+ + K^+)$-ATPase are high in the zone of heavier membranes. In this part of the gradient, the specific Ca^{2+}-ATPase activities are highest, whereas rotenone-insensitive cytochrome *c* reductase [33] seems to contaminate slightly, but significantly, all fractions with α-toxin receptors.

Tetrodotoxin and saxitoxin binding in density gradient fractions

To characterize further membrane fragments with regard to voltage-sensitive Na channels, specific binding of [^{3}H]tetrodotoxin was investigated.

Table I shows the distribution of voltage-sensitive Na channels in fractions of sucrose gradients as revealed by saturable [^{3}H]tetrodotoxin and [^{3}H]saxitoxin binding. Since the gift of [^{3}H]saxitoxin was not available at the same time as our preparation of [^{3}H]tetrodotoxin, the binding assays could not be carried out with the same preparation of membrane material. Furthermore, equilibrium dialysis had to be used for [^{3}H]saxitoxin binding, because [^{3}H]-saxitoxin dissociation was too fast for a Millipore filtration assay. If [^{3}H]-tetrodotoxin binding is corrected with regard to thermodynamic and kinetic effects, both sets of data are in fair agreement: (i) $2 \cdot 10^{-8}$ M is close to the

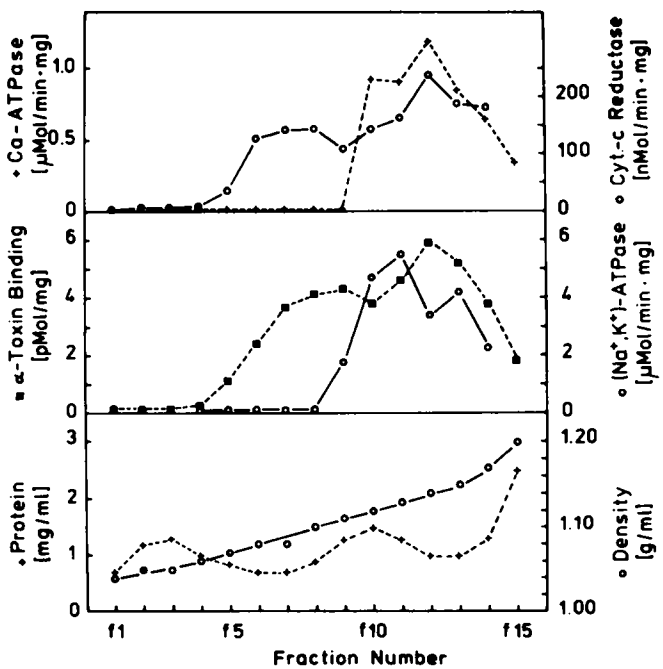

Fig. 3. Fractionation of membranes from high-speed pellet by sucrose density gradient centrifugation. Fractions of approx. 2.3 ml were collected, starting with the lightest (f1) (for details see Methods).

TABLE I

TETRODOTOXIN AND SAXITOXIN BINDING TO MEMBRANE FRACTIONS FROM SUCROSE DENSITY GRADIENTS

Saxitoxin binding determined by equilibrium dialysis. Tetrodotoxin binding measured by Millipore filtration in a different preparation. Tetrodotoxin binding data corrected for thermodynamic saturation of binding and for kinetic dissociation (see text).

Sucrose density (g/ml)	Specific binding (pmol/mg)		
	Saxitoxin ($3.0 \cdot 10^{-8}$ M)	Tetrodotoxin ($2.0 \cdot 10^{-8}$ M)	
		Raw data	Data corrected for saturation
1.04		0.2	0.6
1.06	1.2		
1.07		1.1	3.1
1.08	2.3		
1.09		0.6	1.7
1.10	1.6		
1.11		1.4	3.9
1.13	2.0	1.9	5.3
1.15		0.8	2.2

thermodynamic dissociation constant of tetrodotoxin in this system [28], saturation of receptor sites would therefore double the values obtained for tetrodotoxin. (ii) During the 20—30 s needed to filter and to wash [^{3}H]tetrodotoxin samples, approx. 30% of specifically bound [^{3}H]tetrodotoxin dissociates from its receptor (cf. Fig. 10). The electric organ contains about 20 pmol tetrodotoxin receptor/g wet wt. [18]. In the density gradient ranging from 1.04 to 1.15 g/ml, approx. 15% of these receptors are recovered. The peak fraction at 1.13 g/ml represents about 4% of the starting material. These data reflect binding to accessible tetrodotoxin receptors, while additional receptors in sealed inside-out vesicles (see below) are not included.

Carrier-free electrophoresis of membrane fragments

To purify further Na channel-rich membrane fragments, density gradient fractions were subjected to carrier-free column electrophoresis and lectin chromatography as additional separation procedures. Pilot experiments with a Hannig free-flow apparatus had shown a subfractionation of density gradient material by electrophoresis. Stabilizing sucrose gradients in the column electrophoresis had to be optimized, because (i) steep sucrose gradients, e.g., from 50 to 10%, result in severe changes of viscosity along the gradient, thereby disturbing separating zones in this kinetic technique, and (ii) too shallow gradients cannot always prevent convection. For experimental conditions such as those chosen for Figs. 4—6, sucrose gradients from 30 to 10% or from 20 to 10% were found optimal.

Fig. 4 compares electrophoretic separation profiles of four density gradient fractions with sucrose densities from 1.06 to 1.14 g/ml. For all density gradient fractions a subfractionation into four zones is observed.

To discriminate between Na channel-rich membrane fragments and other membranes, the binding of tetrodotoxin and α-neurotoxin and the activities

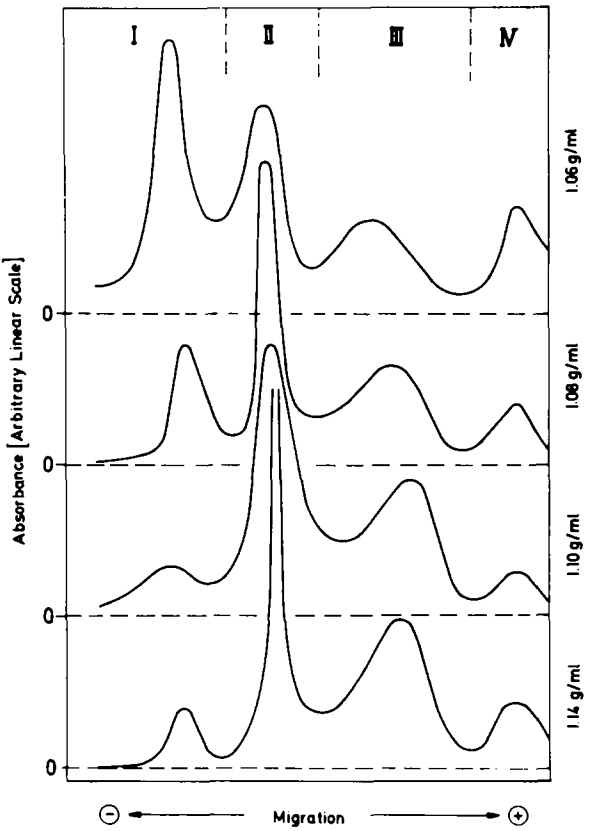

Fig. 4. Carrier-free column electrophoresis of membrane fragments in a stabilizing sucrose gradient. Four fractions from the high-speed pellet after sucrose density gradient centrifugation, with sucrose densities ranging from 1.06 to 1.14 g/ml, were electrophoresed in a 110 ml column for isoelectric focussing. After the run, optical absorption at 254 nm was monitored as a semiquantitative measure of membrane meterial. I—IV, arbitrary assignation of corresponding zones (for details see Methods).

of marker enzymes were determined after electrophoresis. Fig. 5 shows that almost all electrophoresis subfractions display some specific binding of neurotoxins. Nevertheless, there are important quantitative differences. Only zone III has high specific binding of tetrodotoxin in all density gradient fractions, the uncorrected raw data in Fig. 5 ranging from 5 to 20 pmol/mg. About the same figures are obtained for α-neurotoxin binding in this zone. In addition, there seems to be a correlation between the tetrodotoxin and α-neurotoxin receptor: specific binding for both is highest in the light fraction and lowest in the heaviest one.

Correlation between electrophoresis zone III and extrasynaptic regions

On the electroplax, the extrasynaptic parts of the innervated face are the most likely location of high Na channel densities. This part of the plasma membrane has also been shown to contain considerable amounts of acetylcholine receptor [26]. Zone III in the separations of Fig. 5 may therefore tentatively be attributed to extrasynaptic fragments of the excitable face of the

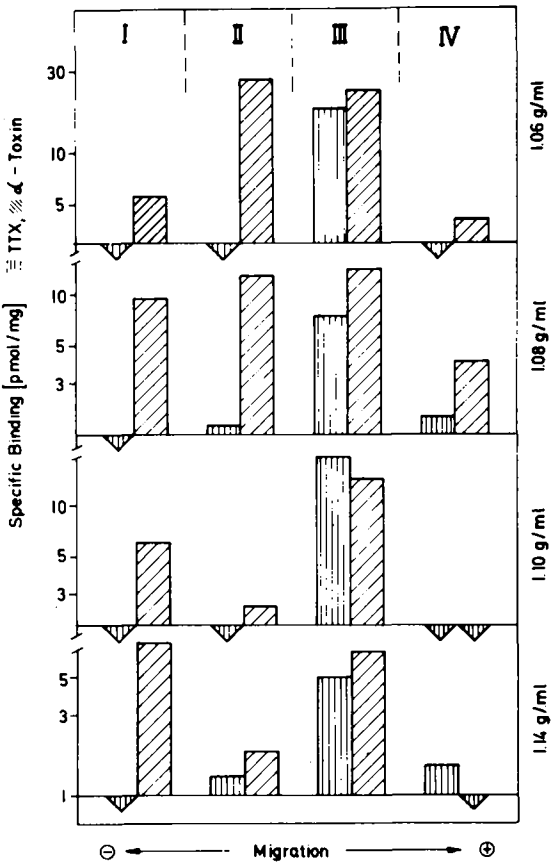

Fig. 5. Distribution of tetrodotoxin (TTX) receptors and acetylcholine receptors in electrophoretically subfractionated membrane fragments (cf. Fig. 4). Peak fractions in zones I—IV (cf. Fig. 4) were analyzed for binding of [^{3}H]tetrodotoxin and [^{3}H]propionylbungarotoxin. Specific binding of [^{3}H]tetrodotoxin determined at a concentration of $5 \cdot 10^{-8}$ M (for details see Methods). [^{3}H]Tetrodotoxin binding not corrected for dissociation during filtering and washing. In the density gradient fractions used for electrophoresis, the corrected specific binding (pmol/mg) for [^{3}H]tetrodotoxin/[^{3}H]propionylbungarotoxin was: 2.6/1.0 for 1.06 g/ml, 1.5/3.0 for 1.08 g/ml, 3.6/4.0 for 1.10 g/ml, and 2.0/4.4 for 1.14 g/ml sucrose density. The inverted triangles indicate specific binding below the range shown in the plots.

electroplax. If raw data for [^{3}H]tetrodotoxin binding are corrected for dissociation during filtering and washing (cf. Fig. 10), peak values between 20 and 30 pmol/mg result for zone III, where 40—70% of the tetrodotoxin receptors subjected to electrophoresis are recovered. The protein-to-lipid ratio is not yet known for electrophoresis fractions. For density gradient fractions the protein-to-phospholipid ratio was found to be between 1 (light fractions) and 2—3 (heavy fractions) [26]. Taking a ratio of 1.5 and assuming a membrane thickness of 75 Å and a density of 1 g/ml, a peak tetrodotoxin receptor density of 50—80 μm^{-2} may be calculated for membrane fragments in zone III of Figs. 4 and 5. Nearly the same surface density would be obtained for α-neurotoxin receptors in these fractions.

Fig. 6 shows biochemical data obtained in fractions after electrophoresis.

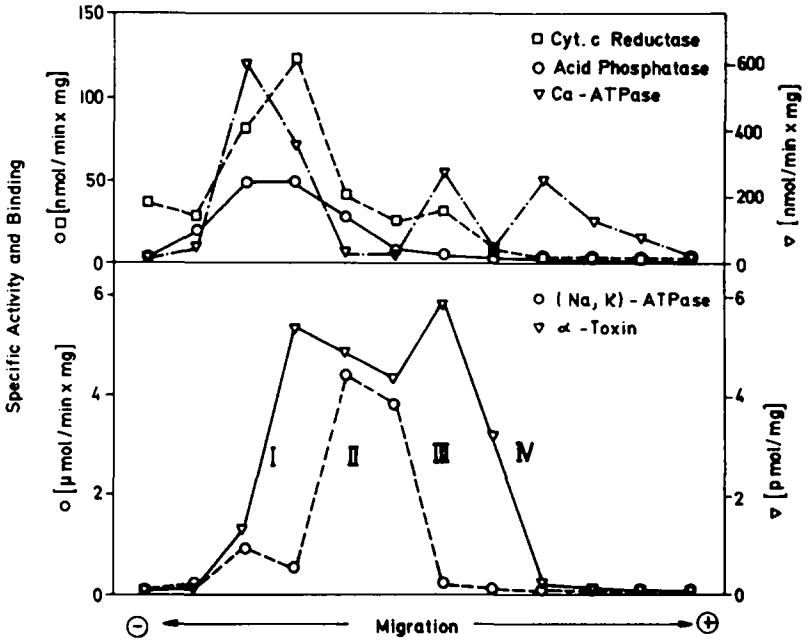

Fig. 6. Specific enzymic activities in electrophoretically subfractionated membrane fragments. A membrane fraction with 1.12 g/ml sucrose density was electrophoresed in a 110 ml column for isoelectric focussing. 35% sucrose in lower electrode buffer, gradient ranging from 20 to 10% sucrose; pH 7.4. I—IV, assignation of zones corresponding to zones I—IV in Figs. 4 and 5 (for further details see Methods and Results).

A density gradient fraction of 1.12 g/ml sucrose density was electrophoresed at a slightly higher pH (7.4) than in Figs. 4 and 5 (pH 7.0). The first zone (I) is high in α-neurotoxin binding, but is obviously contaminated with cytochrome *c* reductase, phosphatase and Ca^{2+}-ATPase. Cytochrome *c* reductase most likely indicates membranes from smooth endoplasmic reticulum [33], and acid phosphatase is supposed to be a marker for lysosomes [35]. In muscle preparations, Ca^{2+}-ATPase is used as a marker enzyme for sarcoplasmic reticulum [34]. However, in view of the important Ca^{2+} gradients across the plasma membranes, one may well expect an intrinsic Ca^{2+}-ATPase of the electroplax plasma membrane. Zone II shows comparatively lower α-neurotoxin binding, but it represents the peak of ($Na^+ + K^+$)-ATPase activity. Zone III has high specific α-neurotoxin binding and a certain amount of contamination with cytochrome *c* reductase and Ca^{2+}-ATPase, whereas zone IV does not display α-neurotoxin binding or ($Na^+ + K^+$)-ATPase activity.

In view of the asymmetry of the electroplax, earlier investigations have focussed on the question as to whether ($Na^+ + K^+$)-ATPase is localized exclusively or primarily on the non-innervated face. One might then obtain plasma membrane fragments (i) with high neurotoxin binding/low ($Na^+ + K^+$)-ATPase activity from the innervated face and (ii) with low neurotoxin binding/high specific ($Na^+ + K^+$)-ATPase activity from the non-innervated face. However, Fig. 7 demonstrates that most membranes form vesicles

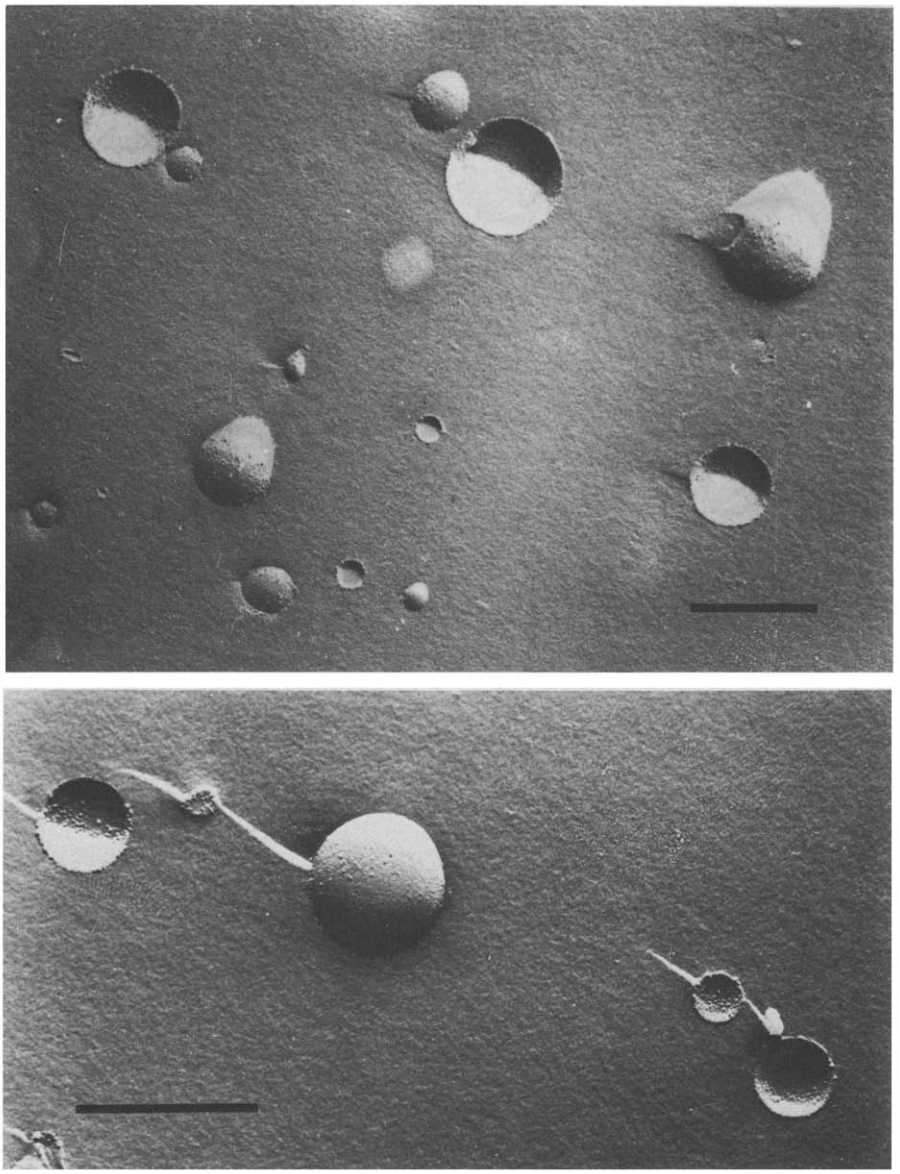

Fig. 7. Morphology of membranes in tetrodotoxin receptor-rich fractions from density gradients (freeze fracture). Top: distribution of fragments and vesicle sizes in a fraction with a sucrose density of 1.13 g/ml. Bottom: membranes with a sucrose density of 1.11 g/ml. The bars represent 0.5 μm.

without obvious signs of membrane damage. In this case, an impermeable label for specific binding or a corresponding substrate for measuring specific activities can only reveal sites exposed to the external surface of the vesicle membrane. Thus, outside-out vesicles could display high tetrodotoxin and α-neurotoxin binding with $(Na^+ + K^+)$-ATPase activity being masked, whereas for inside-out vesicles the opposite would be expected.

As demonstrated in the upper part of Fig. 7, the diameters of vesicles in density gradient fractions range from 0.1 to 0.5 μm. The surface density of membrane particles varies in different vesicles and membrane faces. In the fragments in the lower part of Fig. 7, up to 4000 particles per μm^2 may be counted, whereas other fragments do not display more than 1000 per μm^2. This covers the range of densities observed in the innervated electroplax membrane (cf. Fig. 2).

Lectin chromatography and sidedness of vesicles

The abundance of transverse tubules is demonstrated in Figs. 1 and 2. In the course of homogenization, the tubular morphology may cause these membranes to pinch off partially or even primarily as inside-out vesicles.

The asymmetrical occurrence of carbohydrate moieties allows us to probe the sidedness of vesicles. Fig. 8 represents results of Sepharose 4B-bound lectin affinity chromatography experiments with density gradient fractions of 1.10 and 1.14 g/ml sucrose densities. In all experiments, a breakthrough fraction with slightly decreased specific binding of [^{3}H]tetrodotoxin and [^{3}H]propionyl-

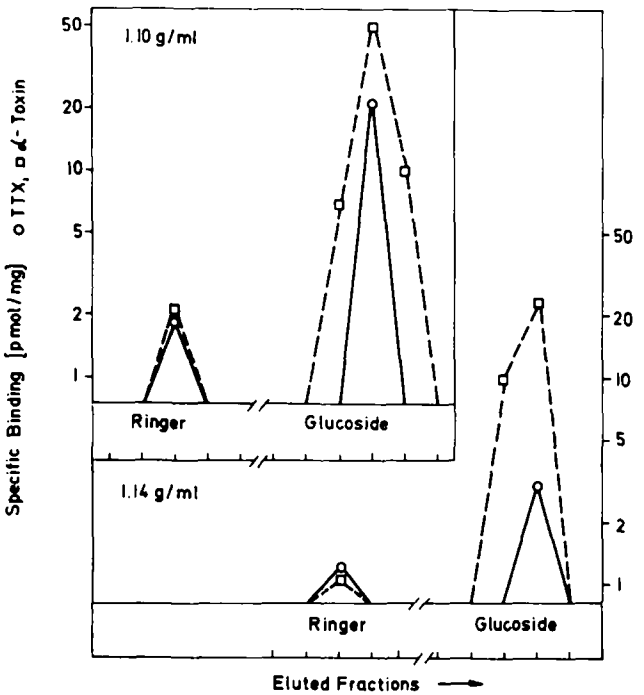

Fig. 8. Subfractionation of density gradient fractions by lectin affinity chromatography. Sepharose-bound concanavalin A was equilibrated in a column with density gradient fractions of 1.10 or 1.14 g/ml sucrose density. Unbound material was eluted as a breakthrough with Ringer solution ('Ringer'). After incubation, bound material was eluted specificly with 0.5 M α-methyl-D-glucose ('Glucoside'). The plot shows zones with peak specific binding of [^{3}H]tetrodotoxin and [^{3}H]propionylbungarotoxin. [^{3}H]Tetrodotoxin was determined at $5 \cdot 10^{-8}$ M, data not corrected for dissociation during filtering and washing. In the density gradient fractions used for chromatography, the corrected specific binding (pmol/mg) for [^{3}H]tetrodotoxin/[^{3}H]propionylbungarotoxin was: 3.6/4.0 for 1.10 g/ml and 2.0/4.4 for 1.14 g/ml sucrose density. TTX, tetrodotoxin.

bungarotoxin is obtained, whereas specific elution with α-methyl-D-glucoside yields a fraction with enriched specific neurotoxin binding.

Fractionation by lectin chromatography provides a specific technique to purify plasma membrane vesicles. If the raw data in Fig. 8 are corrected for dissociation (cf. Fig. 10), a subfraction with 30 pmol/mg specific [^{3}H]tetrodotoxin binding is obtained in the glucoside elution of a 1.10 g/ml density gradient fraction. This is close to the corresponding result of carrier-free electrophoresis and would represent a surface density of Na channels close to 80 per μm^2. In addition to this preparative aspect, lectin chromatography sheds some light on the distribution of outside-out and inside-out vesicles in density gradient fractions. After pooling of Ringer solution and glucoside fractions (R and G, respectively), the following recoveries were determined: R = 61%, G = 15% for density gradient fractions with 1.06 g/ml sucrose density; R = 42%, G = 4% for 1.08 g/ml; R = 42%, G = 6% for 1.10 g/ml; and R = 52%, G = 4% for 1.14 g/ml. These data are related to total protein as used for charging the columns.

Correspondingly, a minimum of approx. 40—60% of a density gradient fraction would be inside-out vesicles. Material specifically eluted by glucoside amounts to approx. 5% in most fractions and is likely to represent outside-out vesicles. Particularly, this value of 5% may be misleadingly low because nothing is known about roughly one half of the initial material not recovered from the columns.

The existence of damaged vesicles as demonstrated by the upper part of Fig. 7 may explain why the breakthrough fraction reveals some neurotoxin binding without being bound by the lectin: low molecular weight neurotoxins could invade such vesicles and bind to the inner face, whereas Sepharose-bound lectin moieties cannot reach the interior of these vesicles.

Kinetics of [^{3}H]tetrodotoxin binding

To study molecular properties of membrane bound Na channels, the kinetics of association and dissociation of [^{3}H]tetrodotoxin have been investigated. Figs. 9 and 10 represent association and dissociation kinetics of [^{3}H]-tetrodotoxin binding to density gradient fractions. Both kinetics are at the limit of detectability with the Millipore filtration technique. At concentrations close to the K_D, association is in equilibrium after approx. 10 min (Fig. 9). From the first experimental values (initial slope), a lower limit for the bimolecular association constant may be evaluated: $k_f \geqslant 3 \cdot 10^5\ M^{-1} \cdot s^{-1}$. The correct value may be approx. 2- or 3-times higher, since the Millipore technique is unsafe below approx. 30 s for the necessary amount of membrane material.

Fig. 10 represents the dissociation of [^{3}H]tetrodotoxin. In most membrane preparations, dissociation is biphasic with a predominant fast phase; in some preparations the slow phase was hardly detectable. The fast phase has a dissociation constant of $k'_b = 2.5 \cdot 10^{-2}\ s^{-1}$, the slow phase is typically of the order of $k''_b = 10^{-3}\ s^{-1}$. Taking the values of k_f and k'_b, a thermodynamic dissociation constant of $K'_D \leqslant 8 \cdot 10^{-8}$ M may be calculated for tetrodotoxin (TTX) binding to its receptor R:

$$R + TTX \underset{k_b}{\overset{k_f}{\rightleftharpoons}} (R\text{-}TTX)$$

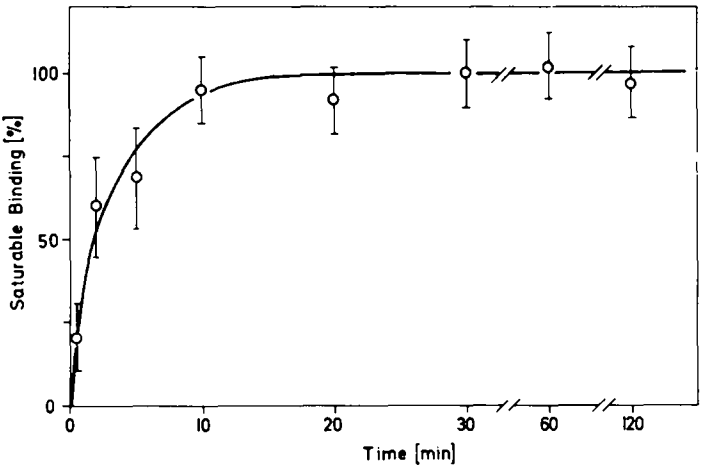

Fig. 9. Association kinetics of tetrodotoxin (TTX) binding. [^{3}H]Tetrodotoxin was incubated at 0°C and a concentration of 20 nM for the time indicated with density gradient fractions of 1.10 and 1.13 g/ml sucrose density. Saturable specific binding was determined by Millipore filtration. Data are not corrected for dissociation during filtering and washing. The initial slope yields a bimolecular association constant of $k_f \geq 3 \cdot 10^5\ M^{-1} \cdot s^{-1}$.

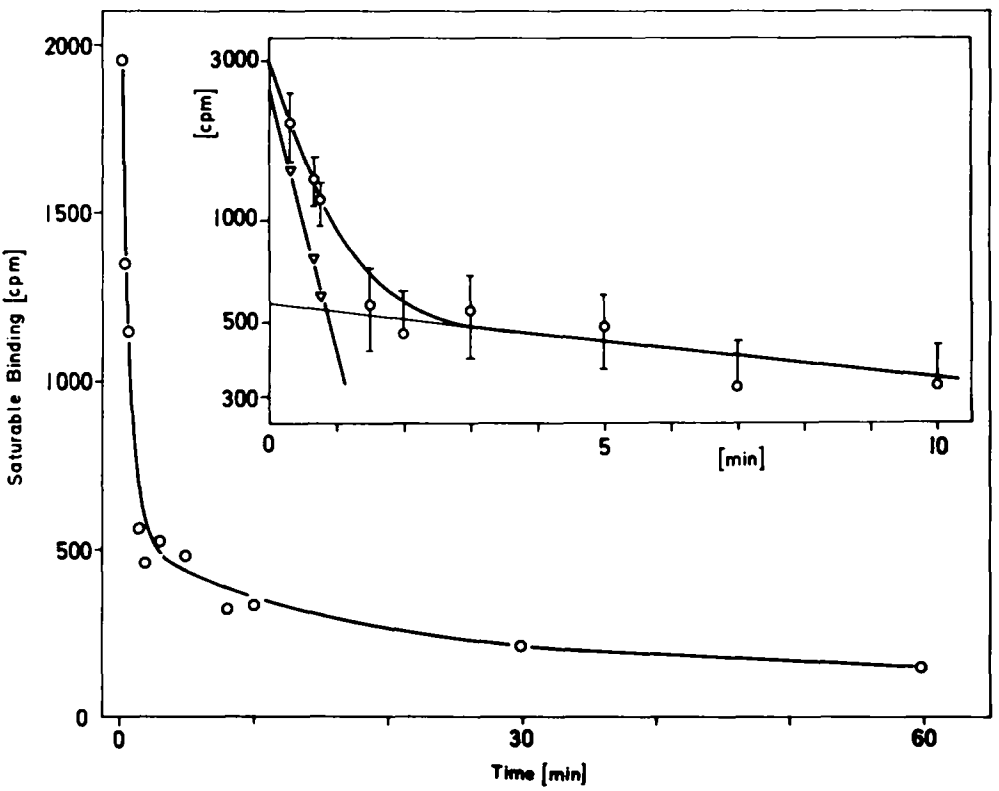

Fig. 10. Dissociation kinetics of tetrodotoxin (TTX) binding. [^{3}H]Tetrodotoxin was equilibrated at 20 nM with density gradient fractions of 1.10 g/ml sucrose density, before at 0°C free [^{3}H]tetrodotoxin was diluted 10-fold or removed. Released [^{3}H]tetrodotoxin was washed off by Millipore filtration after the time indicated. Data are not corrected for dissociation during washing steps. The semilogarithmic plot yields two kinetic dissociation constants: $k_b' = 2.5 \cdot 10^{-2}\ s^{-1}$ for the fast phase accounting for approx. 80% of the amplitude and $k_b'' = 8.3 \cdot 10^{-4}\ s^{-1}$.

This kinetically determined value of K_D is not in contradiction with the value we derived from concentration-dependent equilibrium binding. With k_f and k_b'' a second $K_D'' \leq 3 \cdot 10^{-9}$ M may formally be calculated, if the slow dissociation phase represents a population of tetrodotoxin receptors with differing molecular properties. On the other hand, the slow kinetic phase could be caused by a diffusion barrier, e.g., for intravesicular tetrodotoxin receptors in leaky vesicles. In this case, the microscopic kinetics of this population need not be different from those of the main population. We also tried to determine kinetics for [^{3}H]saxitoxin binding, but dissociation was too fast and we could only recover approx. 20% of equilibrium binding with the Millipore filtration technique.

Discussion

The electric organ of *E. electricus* is a rich source of membranes conducting an action potential. This contribution aims at a characterization of Na channel-rich membrane fragments from this organ. Of special interest for this study are the extrasynaptic regions of the innervated face which have acetylcholine receptor densities of 370 ± 260 per μm^2 of membrane area [27]. Freeze-fracture electron micrographs of these regions show approx. 20 invaginations per μm^2 of flat membrane surface (Fig. 2). These caveolae have been detected in earlier studies [27,40] and were calculated to constitute approx. 90% of the membrane area on the innervated face. The freeze-fracture technique reveals a high density of particles in these invaginations. The transverse tubular structure of the caveolae resembles that of striated muscle cells. Transverse tubules in striated muscle are electrically excitable involving tetrodotoxin-sensitive Na channels [41]. Because of the close relation between striated muscle fibers and electroplax and because of the occurrence of acetylcholine receptors in the transverse tubules of electroplax [27], these tubules together with the flat surface of the innervated face are the most likely location of Na channel-rich membrane fragments in vivo.

In fractions with sucrose densities ranging from 1.05 to 1.15 g/ml, no unambiguous attribution of membrane fragments to morphological structures of the electroplax is possible. All these fractions show specific tetrodotoxin and saxitoxin binding typically of the order of a few pmol/mg protein (Table I and Ref. 12). These characteristics of Na channels in density gradient fractions resemble qualitatively and quantitatively those obtained for acetylcholine receptors (Fig. 3 and Refs. 18 and 26). In spite of this limited purity, density gradient fractions are useful preparations for many biochemical studies: With such fractions, ion flux through acetylcholine receptor-associated channels has been studied successfully [20—23], and in view of the comparable density of tetrodotoxin receptors, corresponding studies with voltage-sensitive Na channels are in progress. Density gradient fractions from *E. electricus* electroplax may also be compared with Na channel-rich membrane fragments from other sources: in axolemma from lobster nerve fibers 9.5 pmol/mg are found [44] and 12.1 pmol/mg in membrane fragments from crab nerves [14]. Homogenized brain yields fractions with specific activities ranging from 1 to 5 pmol/mg [8].

Application of carrier-free electrophoresis and lectin chromatography

yields a separation of density gradient fractions into more homogeneous subfractions. After electrophoresis four discrete zones appear. The amount of material in zone III (cf. Figs. 4 and 5) increases with increasing sucrose density of the applied fraction. The specific binding of tetrodotoxin and α-neurotoxin is almost the same in this zone and reaches corrected values of 30 pmol/mg. The concomitant occurrence of tetrodotoxin and α-neurotoxin receptors suggests that these membrane fragments derive from the extrasynaptic regions of the innervated face. Quantitative estimates support this hypothesis: Receptor densities in these membrane fragments in vitro amount to approx. 80 per μm^2, compared to 370 ± 260 α-neurotoxin receptors in extrasynaptic regions of the innervated face of the electroplax [27]. A specific tetrodotoxin binding of 30 pmol/mg exceeds that of other Na channel-rich membrane preparations [8,14,44], a density of 80 tetrodotoxin receptors per μm^2 is greater than observed densities of fragments from lobster [44] and crab [14] nerves, where values of 28 and 4 per μm^2 were found, respectively.

In general, tetrodotoxin and saxitoxin receptor densities are found to be higher in muscle fibers (typically above 100 μm^{-2}) than on non-myelinated nerve (typically below 100 μm^{-2}), except for giant axons [5]. These data are in agreement with the electroplax being closely related to striated muscle fibers. Above a density of 80 tetrodotoxin and α-neurotoxin receptors per μm^2 in vitro, it may be possible to reach densities of 200 or 300 μm^{-2}, since these values are expected from morphological data in vivo. Preparative amounts of membrane material with such densities are of great importance for functional studies with membrane-bound Na channels.

If Na channels have a molecular weight of approx. 300 000 [6,47], binding one tetrodotoxin molecule each, they constitute approx. 1% of the total protein in peak fractions of electrophoresis zone III (cf. Figs. 4 and 5). With solubilized material, a much higher degree of purification has been achieved [45]. Because of presumably high Na channel densities in electroplax membranes, this proportion is nevertheless considerably higher than in crab nerve membrane fragments, where Na channels represent about 1‰ of total protein [14].

In vesicle preparations, specific binding and enzymic activities reflect accessible sites. The interpretation of data becomes questionable if different proteins expose their identification sites intrinsically to opposite faces of vesicle membranes. This is the case for the metabolic site of the $(Na^+ + K^+)$-ATPase as compared to the receptor sites of tetrodotoxin and α-neurotoxin.

Lectin chromatography sheds some light on the sidedness of vesicles from homogenized electroplax. Besides providing another possibility of obtaining Na channels at a corrected specific tetrodotoxin binding of 30 pmol/mg, the fractions eluting with Ringer solution or alternatively with glucoside may be considered enriched inside-out or outside-out vesicles, respectively. Depending on whether or not irreversibly bound material is taken into account, 50—95% of the vesicles appear inside-out. A high proportion of inside-out vesicles is not surprising in view of the abundance of transverse tubules (cf. Figs. 1 and 2, and Refs. 27 and 40). A substantial part of these membranes may conserve their intrinsic curvature when pinching off during homogenization. Correspondingly, a considerable part of the vesicles may be Na channel rich without being

identified by tetrodotoxin (and α-neurotoxin) binding assays. This possible population of inverted channels is of importance for an understanding of functional data. On the other hand, purified preparations of outside-out and inside-out vesicles are useful tools to dissect parts of the channel structures in vitro.

These data demonstrate the importance of neurotoxins in identifying channels in vitro. To study molecular properties of membrane-bound Na channels, the kinetics of association and dissociation of [^{3}H]tetrodotoxin have been investigated. Earlier investigations have revealed an interesting phenomenon in some biochemical preparations of Na channels: whereas the apparent thermodynamic dissociation constant of tetrodotoxin and saxitoxin falls into the nanomolar range known from electrophysiological experiments, the kinetics of dissociation are slowed down in several cases by more than an order of magnitude (cf. Table 3 in Ref. 8). The kinetic constant for dissociation from *Rana* or *Xenopus* node is close to 1 min^{-1} [42,43], and in crab nerve membrane fragments it is 0.1 min^{-1} [14], although the apparent K_D in these systems is about the same (1.7—3.6 nM) and temperatures are comparable. Kinetics differ even more if data at low temperatures or in solubilized state are taken into consideration [6].

We have found an apparent K_D of 20 ± 10 nM for [^{3}H]tetrodotoxin binding to density gradient fractions from electroplax [28]. This value is about an order of magnitude higher than the apparent dissociation constants in many nerve preparations [5]. Figs. 9 and 10 demonstrate that the increased value of K_D is due to slower association rather than faster dissociation. The bimolecular association constant $k_f \geqslant 3 \cdot 10^5\ M^{-1} \cdot s^{-1}$ appears considerably slower than that for a diffusion controlled association. It is, however, very close to the kinetic association constant in crab nerve membrane fragments [14]. In contrast to that preparation, in *E. electricus* membrane fragments, no slowing down of dissociation compensates for this slow association and an increased value of K_D results. A small fraction of bound tetrodotoxin being released more slowly in some preparations (cf. Fig. 10) might correspond to the population of tetrodotoxin receptors in crab nerve membrane fragments. A wide range of K_D values is meanwhile known for tetrodotoxin binding to Na channels [5, 18,48,49], and two kinds of tetrodotoxin receptors have also been discussed for a preparation of squid nerve fibers [48]. Further studies of tetrodotoxin binding and of Na channel function in membrane preparations are necessary in order to elucidate the relationship between these tetrodotoxin receptors and other parts of the voltage-sensitive Na channel.

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