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TETRODOTOXIN-SENSITIVE PROTEIN IN THE EXTRACTS FROM EXCITABLE TISSUES

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The sodium permeability of liposomes preincubated with the soluble fraction of brain and heart muscle homogenates was increased by veratrine. The veratrine increment was decreased by tetrodotoxin. The effect was specific for the extracts from excitable tissues. Bovine serum and soluble fraction of liver homogenate induced neither veratrine- nor tetrodotoxin-sensitivity of the liposomes. Treatment of the excitable tissue extracts by pronase and heat denaturation caused their complete inactivation. Tetrodotoxin-sensitive factor could be fractionated by ammonium sulfate precipitation and by DEAE-Servacel chromatography. On a column of Sephadex G-200 it was eluted with the void volume. It is suggested that the tetrodotoxin-sensitive factor is a protein which could be a soluble precursor of the voltage-dependent sodium channels.

The nature and the molecular mechanism of action of voltage-dependent ionic channels remain a fundamental problem in neuroscience. Many investigators have focused their attention on the isolation of these proteins from nerve tissues using the specific radioactive markers such as tetrodotoxin [1–3]. However, as we have indicated elsewhere [4], such an approach has the drawback that toxin binding eliminates the biological activity of the purified protein and this prevents the study of its function.

Recently the possibility of incorporation of the tetrodotoxin-sensitive sodium-selective structures from nerve and brain into liposomes has been reported [4,5]. This method opens the way for the identification of the channel proteins extracted out of the native membrane matrix and for the obtaining them in a solubilized form with retention of some of its characteristic properties.

However, the solubilization of the membrane-bound proteins with the detergent often results in the loss of their stability and creates many additional problems in purification of these hydrophobic macromolecules.

In this work we have used the method of incorporation of sodium channels in the liposomes to find out whether tetrodotoxin-sensitive structures are present in the extracts of the excitable tissues. The existence of such proteins could greatly simplify the attempts to isolate the proteins connected to the channel activity. In undertaking this work we were motivated in part by the experiments in which a protoplasmic drop isolated from an internodal cell of Nitella was found to restore the surface membrane and became electrically excitable after a short period of time [6,7]. A preliminary account of this work has been published earlier [8].

Methods and Materials

The experiments were performed with the soluble fractions obtained from cattle brain, heart

Abbreviations: POPOP, 1,4-bis(5-phenyloxazolyl-2)-benzene; PPO, 2,5-diphenyloxazole.

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muscle, liver homogenates. Tissues were homogenized in equal volume (w/v) of the medium comprising 250 mM sucrose/10 mM Tris-HCl (pH 7.4)/0.2 mM EDTA. The homogenate was centrifuged at $100000 \times g$ for 60 min. The supernatant was used in experiments. All steps were carried out at 4°C.

Crude lipids from bovine brain were obtained according to the method described in Ref. 9. Soybean lipids were prepared as follows. 100 g soybean flour were extracted with 750 ml acetone. The powder was filtered, washed with acetone on a filter until eluates became colourless, dried at air, and twice extracted at room temperature with 750 ml chloroform/methanol (2:1, v/v). Extracts were combined and thoroughly mixed with equal volumes of water and chloroform. The mixture was centrifuged at $4000 \times g$ for 5 min. The lower phase was evaporated in vacuum. All lipids were dissolved in chloroform and stored in sealed ampules at −15°C.

To obtain liposomes, 25 mg phospholipids were dried under vacuum and suspended in the 1 ml of a solution comprising 100 mM NaCl/10 mM Tris-HCl/0.2 mM EDTA. The suspension was sonicated at room temperature for 5 min at 44 kHz, using an UZDN-1 sonicator. 1 ml of the tissue extracts was added to the sonicated liposomes, usually to a final concentration of 2–3 mg protein per ml. No proten was added when liposomes were used in the control experiments.

500 μl of the suspension were incubated overnight in a refrigerator either with or without tetrodotoxin and veratrine at concentrations 200 nM and 200 μg/ml, respectively.

The sodium influx was studied at room temperature. The reaction was started by the addition of 10 μl (10 μCi) of ^{22}NaCl and, at a fixed time, a 100 μl aliquot of the suspension was percolated through the column of CM-Sephadex C-25 (0.3 × 1 cm) equilibrated with 50 mM NaCl/10 mM Tris-HCl, pH 7.4. The liposomes emerged from the column after 15–20 s.

The column was washed by 1 ml 10 mM NaCl/10 mM Tris-HCl (pH 7.4). The radioactivity associated with liposomes was eluted with the void volume and was distinctly separated from the major peak of the extravesicular ^{22}Na. To take account of the binding of the isotope on the liposomic surface the first aliquot was taken immediately after ^{22}NaCl addition. The zero-time radioactivity was subtracted from the value for different time intervals.

The radioactivity of ^{22}Na was measured with an Intertechnique liquid scintillation spectrometer, Model SL-30, in a scintillation fluid consisting of 4 g PPO and 0.1 g POPOP in 1 l dioxane.

Protein was estimated by the method of Lowry et al. [10], total phosphorus of phospholipids by the method of Bartlett [11]. The phospholipid concentrations were estimated assuming a mean molecular weight of 770.

All usual chemicals were reagent grade. CM-Sephadex C-25 was from Pharmacia, tetrodotoxin was obtained from Calbiochem, veratrine from Merck, pronase and Servacel DEAE 23 SH from Serva.

Results and Discussion

In preliminary experiments we had not seen any significant difference between the effect of tetrodotoxin and veratrine on the sodium fluxes in liposomes either sonicated or incubated with the tissue extracts. Therefore, in the following experiments we did not use ultrasonication to obtain liposomes modified by the soluble proteins. It was found out that ^{22}Na influx in brain phospholipid liposomes showed little or no response to the preincubation with the extract from cattle brain (not shown). But after such preincubation the ^{22}Na influx was increased by veratrine and the increment was reduced to about 30% by tetrodotoxin (Fig. 1). Similar results were obtained on the liposomes pretreated with the soluble fraction of heart muscle (Fig. 2).

To find out whether this effect is specific for the extracts from excitable tissues, bovine serum or soluble fraction of liver homogenate were added to the brain phospholipid liposomes and ^{22}Na influx was studied in the presence of veratrine plus tetrodotoxin. Fig. 3 shows that the drugs have no effect on the sodium permeability of the vesicles.

In view of high specificity of tetrodotoxin binding, these findings can be explained by the existence in the soluble fraction of a tetrodotoxin-sensitive structure which forms Na channels if incorporated into the phospholipid membrane. The

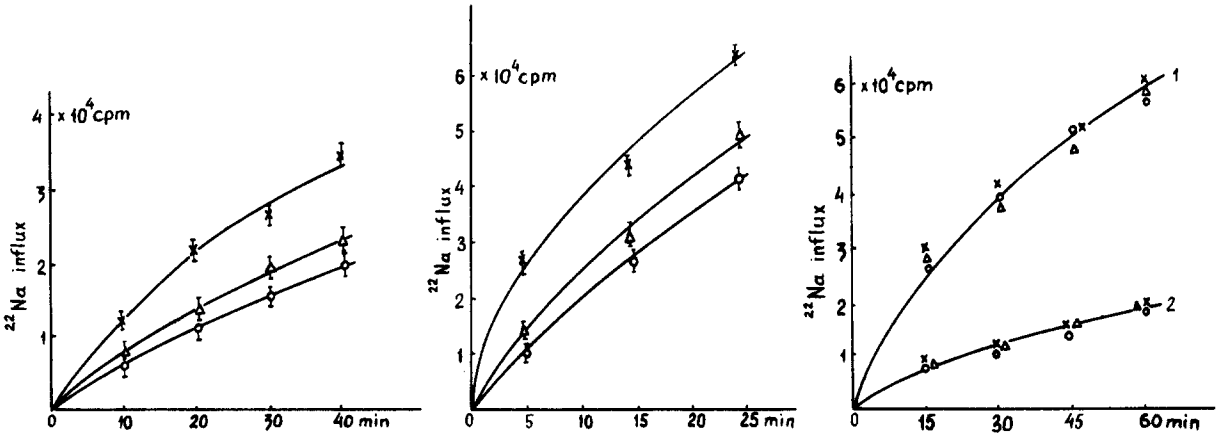

Fig. 1. (Left.) Influence of tetrodotoxin and veratrine on the sodium influx into brain phospholipid liposomes treated with the cattle brain soluble fraction. O------O, control; ×------×, veratrine; △------△, veratrine + tetrodotoxin.

Fig. 2. (Center.) Influence of tetrodotoxin and veratrine on the sodium influx into brain phospholipid liposomes treated with the soluble fraction of heart muscle. O------O, control; ×------×, veratrine; △------△, veratrine + tetrodotoxin.

Fig. 3. (Right.) Influence of tetrodotoxin and veratrine on the sodium influx into brain phospholipid liposomes treated with liver soluble fraction (1) and serum (2). O------O, control; ×------×, veratrine; △------△, veratrine + tetrodotoxin.

possibility cannot be excluded that in supernatants there are small fragments of plasma membranes which can fuse with the phospholipid vesicles. However, we were not able to detect any ($Na^+ + K^+$)-ATPase activity in the supernatants. In addition, prolonged centrifugation of diluted extracts with reduced density did not change their ability to induce the tetrodotoxin-sensitivity of the liposomes.

The polypeptide nature of the tetrodotoxin-sensitive factor was demonstrated by the fact that treatment of the extracts with pronase for 30 min at 37°C as well as heat denaturation for 30 min at 80°C caused their complete inactivation. The dialysis of the excitable tissue extracts has not abolished their activity.

These results show that tetrodotoxin-sensitive substance behaves as an ordinary protein, readily soluble in aqueous solutions, which may be purified in the absence of detergents by conventional preparative techniques. In a recent communication we have described the precipitation of sodium-selective ionophore from cholate solubilized brain membranes [4].

In the present experiments we have also examined the possibility of fractionation of the brain extracts by ammonium sulfate. In each experiment the salt was added during a 10 min interval with a mechanical stirring which was then continued for 20 min before centrifugation. The precipitates were obtained by increasing the concentration of $(NH_4)_2SO_4$ from 0 to 30% and from 30 to 50% saturation. The suspension was centrifuged at 6000 $\times g$ for 10 min and the precipitates were dissolved in a solution containing 100 mM NaCl/10 mM Tris-HCl (pH 7.4)/0.2 mM EDTA. $(NH_4)_2SO_4$ was removed by dialysis against the same solution. The samples were used for the preparation of proteoliposomes as described before. As in the case with cholate soluble membrane fraction the tetrodotoxin sensitivity was found in the precipitates formed between 0 and 30% $(NH_4)_2SO_4$ (Fig. 4).

The brain extracts were analyzed also by Servacel DEAE-23SH chromatography, the results being shown in Fig. 5. 5 ml extracts containing about 40 mg protein were applied to a column (1.5 × 20 cm) equilibrated with 40 mM Tris-HCl (pH 7.4). A linear gradient of NaCl in the same buffer was applied to elute the tetrodotoxin-sensitive protein. Fractions of each peak were combined, concentrated using the Sephadex G-25 and

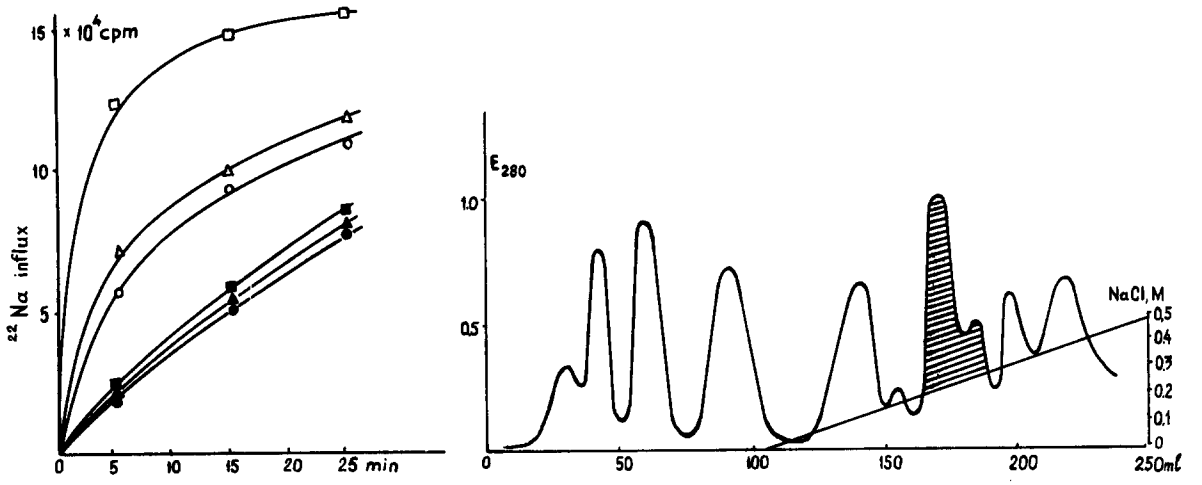

Fig. 4. Influence of veratrine and tetrodotoxin on the ^{22}Na influx into the liposomes prepared from the soybean phospholipids and ammonium sulfate-precipitated fractions: 0–30% saturation (open symbols), 30–50% saturation (closed symbols): O------O, control; □------□, veratrine; △------△, veratrine + tetrodotoxin.

Fig. 5. Servacel DEAE-23SH chromatography of the bovine brain extract. Column, 200×25 mm; flow rate, 10 ml/h; starting buffer, 40 mM Tris-HCl (pH 7.4). Elution by linear gradient of NaCl (0–500 mM). The hatched peak indicates the tetrodotoxin-sensitive fraction.

equilibrated with 100 ml NaCl/10 mM Tris-HCl (pH 7.5) and aliquots were added to liposomes following the determination of the veratrine and tetrodotoxin sensitivity of the ^{22}Na permeability by the described procedure. The protein responsible for the veratrine-tetrodotoxin effect was found in the fraction eluted by 0.25–0.3 M NaCl. Principally the same results were obtained with the heart extracts. Veratrine-induced increment and tetrodotoxin sensitivity were similar for tissue extracts and the purified fraction. But in the last case the effect was obtained at lower protein concentration: 150–300 μg/ml.

When the brain and muscle extracts were chromatographed on the column of CM-Sephadex equilibrated with 10 mM sodium acetate buffer (pH 5.9), tetrodotoxin-sensitive protein was eluted with the nonadsorbed material. It was also eluted with the void volume when chromatographed on the column of Sephadex G-200 (not shown).

The dependence of the veratrine-tetrodotoxin effect on the protein concentration was also studied. The liposomes were incubated with a different quantities of the active fraction obtained after CM-Sephadex chromatography of brain extracts. Fig. 6 shows that half maximal veratrine increment which was abolished by tetrodotoxin has been observed at the protein-phospholipid ratio 20 μg/mg.

The results reported here are consistent with the hypothesis about the existence of a population of water-soluble proteins which may be a cytoplasmic

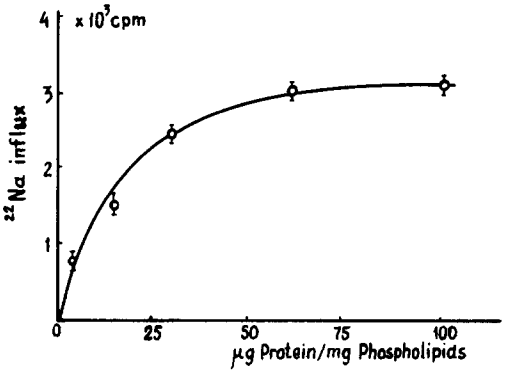

Fig. 6. The dependence of ^{22}Na influx into the brain phospholipid liposomes from a protein phospholipid ratio. 1.25 mg liposome phospholipids and various protein concentrations were used per assay. Transport was measured at time zero and at 10 min without and with 200 μg/ml veratrine. The difference between transport in the presence and absence of veratrine is taken to be veratrine-dependent sodium transport.

precursor of voltage dependent sodium-selective channels of excitable membranes. In the extracts of brain and heart muscle tissues we found a high-molecular-weight component which, interacting with liposomes, increased their sodium permeability when veratrine, an activator of sodium channels, was present in the system. This effect of veratrine was abolished by tetrodotoxin, the highly specific inhibitor of the sodium channels. No such activity was found in the soluble protein fraction from liver and serum. The soluble toxin-sensitivity component was rapidly inactivated by pronase digestion and at a high temperature. Our suggestion about the existence of cytoplasmic precursor of voltage dependent sodium channel seems to be quite reasonable in view of the following consideration.

Although the molecular mechanism of membrane protein biogenesis is not yet known, there is much evidence about the synthesis of these proteins in the form of hydrophilic precursors [12]. As for excitable cells, the most direct evidence for the existence of a cytoplasmic population of the channels derived from the works of Inoue et al. [6] and Krawczyk [7].

In these experiments a protoplasmic drop isolated from an internodal cell of Nitella was found to become electrically excitable in response to an external stimulus. These data agree with those reported by Aleksandrov et al. [13]. They discovered in the Nitella protoplasm Ca^{2+}-Na^{+} channels which could be isolated and incorporated in black lipid film. The existence of channel precursors on the internal surface of the plasma membrane was postulated by Ciani et al. [14].

A question arises as to whether tetrodotoxin-sensitve proteins are localized in cytosol or are incorporated in the intracellular membranes and solubilized during the homogenization of the tissue. From our results we cannot distinguish between these two possibilities. The supposition that tetrodotoxin-sensitive protein is derived from the contamination of the extracts by the fragments of the extracellular membranes is hardly probable because we could not detect any $(Na^{+}+K^{+})$-ATPase activity in the supernatants. A possibility cannot be excluded that the tetrodotoxin-sensitivity is derived from the channel precursor synthesized de novo on ribosomes. If this hypothesis is valid, the existence of hydrophilic channel-forming protein in the cytoplasma might be very useful for further biochemical investigation of the voltage-dependent channels, especially for their purification. It should be noted, however, that although the effect of veratrine was comparatively stable, the tetrodotoxin-sensitivity in our experiments was lost after 24 h at 4°C. Our attempts to increase the stability by a variety of agents including Ca^{2+}, EDTA and cysteine were not successful.

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