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THE ASSOCIATION OF TETRODOTOXIN-SENSITIVE, SODIUM-SELECTIVE IONOPHORE OF BRAIN MEMBRANES WITH LIPOSOMES

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Summary

The tetrodotoxin-sensitive, sodium-selective ionophore of nerve membranes has been associated with liposomes by adding the solubilized brain microsomal fraction to a cholate/phospholipid dispersion and subsequently removing the detergent from suspension by using gel chromatography.

A stimulation of the efflux of sodium from the vesicles was observed in the presence of veratrine. Tetrodotoxin itself did not effect the sodium permeability, but inhibited the veratrine-induced increment.

The activation was absent in the liposomes prepared without soluble membrane proteins. The effects demonstrated for tetrodotoxin and veratrine were specific for the Na^+ movement. It was possible to precipitate the tetrodotoxin-sensitive ionophore by use of $(NH_4)_2SO_4$.

In spite of detailed analysis of the electrical activities of different potential-dependent ion-selective channels, the molecular organization and mechanisms of these channels remain in the field of speculation.

A major obstacle to the investigation of the molecular arrangement of the channels lies in testing them in acellular preparations. The lack of adequate tests prevents isolation and further purification of the channel-forming proteins or other possible components.

At present, there are two reasonable approaches for the isolation of the channel proteins from the membrane matrix. The first is labelling of the

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(5-phenyloxazolyl)benzene

channels with specific radioactive markers and further isolation of this complex by the methods usually used in preparative biochemistry. Many toxins are principally suitable for this purpose. Tetrodotoxin is the most potent and specific inhibitor of the sodium channels [1]. The specificity of action, expressed at nanomolar concentrations, has raised the possibility of using tetrodotoxin in the identification and characterization of membrane sodium channels. Attempts with radioactive tetrodotoxin to isolate the specific receptor protein associated with the channels [2,3] have the drawback that toxin binding does not necessarily indicate retention of biological activity. Besides, the reversibility of the tetrodotoxin-receptor interaction raises the possibility of secondary nonspecific binding.

The next method for the identification and isolation of the channel-forming structures is the solubilization of nerve cell membranes followed by fractionation and reconstitution of channel activity on the artificial phospholipid bilayers.

Previously, it was reported [4,5] that passive sodium fluxes in vesiculated fragments of the excitable membranes (nerve tissues and brain) are activated and inhibited by tetrodotoxin and local anesthetics.

This phenomenon can be readily considered as an indication of the presence of functioning sodium channels in the membrane of closed vesicles. The most important conclusion from this experiment is the possibility of identifying the channels in an acellular system in the absence of a membrane potential.

Recently, Villegas et al. [6] have succeeded in incorporating fragmented lobster nerve membrane into liposomes prepared from mixtures of phospholipids. The reconstituted vesicles show $^{22}Na^{+}$ fluxes responsive to veratridine and tetrodotoxin. Although in these experiments $^{22}Na^{+}$ channels were not isolated from the natural membrane matrix, they demonstrate that crude membrane preparations can be used for the reconstitution of ^{+}Na channels in artificial vesicles.

The present communication is concerned with the restoration of the tetrodotoxin-sensitive, sodium-selective structures from solubilized brain membranes on the phospholipid bilayers.

Methods and Materials

The experiments were performed on microsomal fractions isolated from bovine brain by using standard procedures with minor modifications [7]. 10 g of brain tissue were homogenized in 90 ml of a medium containing 250 mM sucrose, 10 mM Tris-HCl, pH 7.4, and 0.2 mM EGTA. The homogenate was centrifuged at $9000 \times g$ for 20 min and the sediment discarded. The supernatant was centrifuged at $23\,000 \times g$ for 30 min to spin down heavy microsomes. All steps were carried out at 4°C. The resulting sediment which contains the fragments of plasma membranes was solubilized by 1% sodium cholate in a solution consisting of 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM cysteine, and 0.2 mM EGTA. Non-solubilized fragments were removed by centrifugation at $100\,000 \times g$ for 30 min.

Crude lipids from bovine brain were obtained according to the method described in Ref. 8. 25 mg phospholipids (50 mg of crude lipids) were dried

under vacuum and suspended in 1 ml of 1% cholate solution containing 100 mM NaCl, 10 mM Tris-HCl, 1 mM cysteine, and 0.2 mM EGTA. The suspension was briefly (5 min at 44 kHz) sonicated until clear. Then 1 mg of solubilized microsomal protein was added. No protein was added when liposomes were used in the control experiments.

The liposomes were formed by removing the detergent from the phospholipid/protein/cholate solution by gel filtration on Sephadex G-75 [9]. 1 ml of the protein/phospholipid/cholate solution was applied to a 1.5 × 40 cm column of Sephadex G-75 equilibrated with 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM cysteine, and 0.2 mM EGTA. The rate of elution was 6—7 ml/h. Fractions (20—90 ml) of eluate were collected and analyzed for protein [10], total phosphorus [11] and cholate [12]. The phospholipid concentrations were estimated assuming a mean molecular weight of 770. Before protein analysis, 0.5 ml samples of the fractions were incubated in 0.5% sodium dodecyl sulfate.

Reconstituted samples after gel chromatography were negatively stained using 2% phosphotungstate, pH 7.0, and dried on 200 mesh colloidin- and carbon-coated copper grids. These were examined at 40 000 × magnification on a Jeol Electron Microscope type JEM 7A.

To study the efflux of Na^+ or Rb^+ from vesicles, the liposomes were enriched with the isotopes by incubation for 15—20 h at 4°C in 1 ml of suspension containing 4 mg of liposomal phospholipids, 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 0.2 mM EGTA, 1 mM cysteine and 10 μCi ^{22}Na or ^{86}Rb. Tetrodotoxin (100 nM) and veratrine (200 μg/ml) were introduced to this system when necessary. Extravesicular tracer was removed by gel filtration through the Sephadex G-75 column (1 × 15 cm). 1 ml samples of the suspension were placed in 8/32 Visking tubes and the efflux of Na^+ or Rb^+ was studied by using the dialysis procedure described by Bangham [13].

To study the Na^+ influx in the vesicles, tetrodotoxin and veratrine at final concentrations of 100 nM and 200 μg/ml, respectively, were added to 500 μl of the liposomal suspension and allowed to stand for 2—3 h at room temperature. Then. 10 μl (10 μCi) of $^{22}NaCl$ were added and, at a fixed time, a 100 μl aliquot of the suspension was percolated through a CM-Sephadex column (0.5 × 1 cm) to remove the extravesicular label. The column was equilibrated with 50 mM NaCl, 5 mM Tris-HCl, pH 7.4, and 0.1 mM EGTA. The liposomes containing ^{22}Na were eluted with 600 μl of the same solution, whereas the external isotopes were completely adsorbed on the column. To consider the binding of the isotope on the liposomal surface, the first aliquot was taken immediately after addition of $^{22}NaCl$. The zero-time radioactivity was subtracted from the values for different time intervals.

The activity of ^{22}Na was measured in a scintillation fluid consisting of 4 g PPO and 0.1 g POPOP in 1 l dioxane. ^{86}Rb was counted in aqueous solution. All usual chemicals were reagent grade. G-75 and CM-Sephadex were obtained from Pharmacia, liquid scintillation products from Intertechnique, tetrodotoxin from Calbiochem and veratrine from Merck.

Results and Discussion

The fraction consisting of heavy microsomes was chosen for the present investigation because this subcellular material is characterized by a high (Na^+ + K^+)-ATPase activity [14], which in our experiments was 6—10 μM P_i/mg protein per h. This indicates the enrichment of the fraction by plasma membranes which should have a high content of sodium-selective channels.

Fig. 1 represents elution patterns of the protein/cholate/phospholipid dispersion. The vesicles eluted between 22 and 28 ml were distinctly separated from the bulk of cholate. Under the conditions used, approx. 0.1 mg cholate/mg phospholipid still remained in the liposomal fraction.

The phospholipid fraction contains liposomes of somewhat variable size (Fig. 2). The average diameter observed in the electron micrographs was approx. 0.2—0.3 μm for proteoliposomes.

Fig. 3 illustrates that the phospholipid vesicles proved to be osmotically active, as shown by the sharp increase in the efflux rate in the hypotonic medium. The liposomal suspension contained 50 μg protein/mg phospholipid. It was impossible to estimate, in these experiments, the relative amount of protein and phospholipids in the liposomes. A major obstacle to the determination of the amount of protein associated with the vesicles lies in the separation of the liposomes from the protein aggregates which were formed after removal of the detergent. However, the liposomal bilayer seems to incorporate the nerve membrane constituents. It may be seen from Fig. 3 that the efflux of both ^{22}Na and ^{86}Rb was considerably higher from the proteoliposomes than from liposomes. It was interesting to ascertain whether incorporated structures preserved some intrinsic properties of the sodium channels. In this context, we studied the influence of veratrine and tetrodotoxin on the efflux of sodium from and in the reconstituted liposomes.

Fig. 4 shows the increase in the ^{22}Na-efflux rate in the presence of veratrine

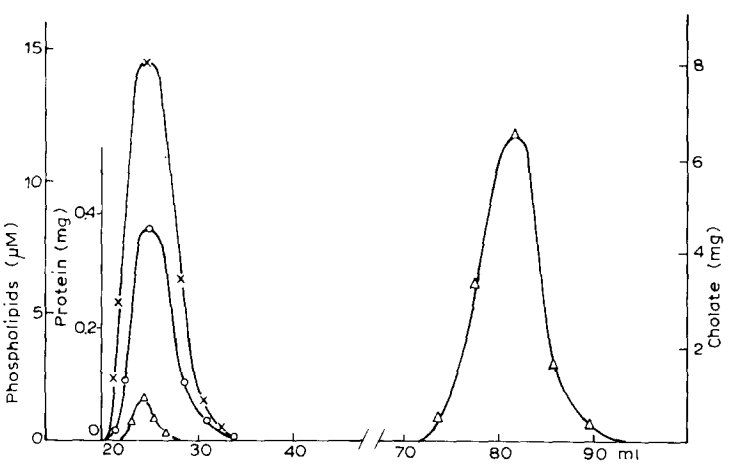

Fig. 1. Gel filtration of the solubilized microsomal membranes on Sephadex G-75. ○———○, protein: X———X, phospholipid; △———△, cholate.

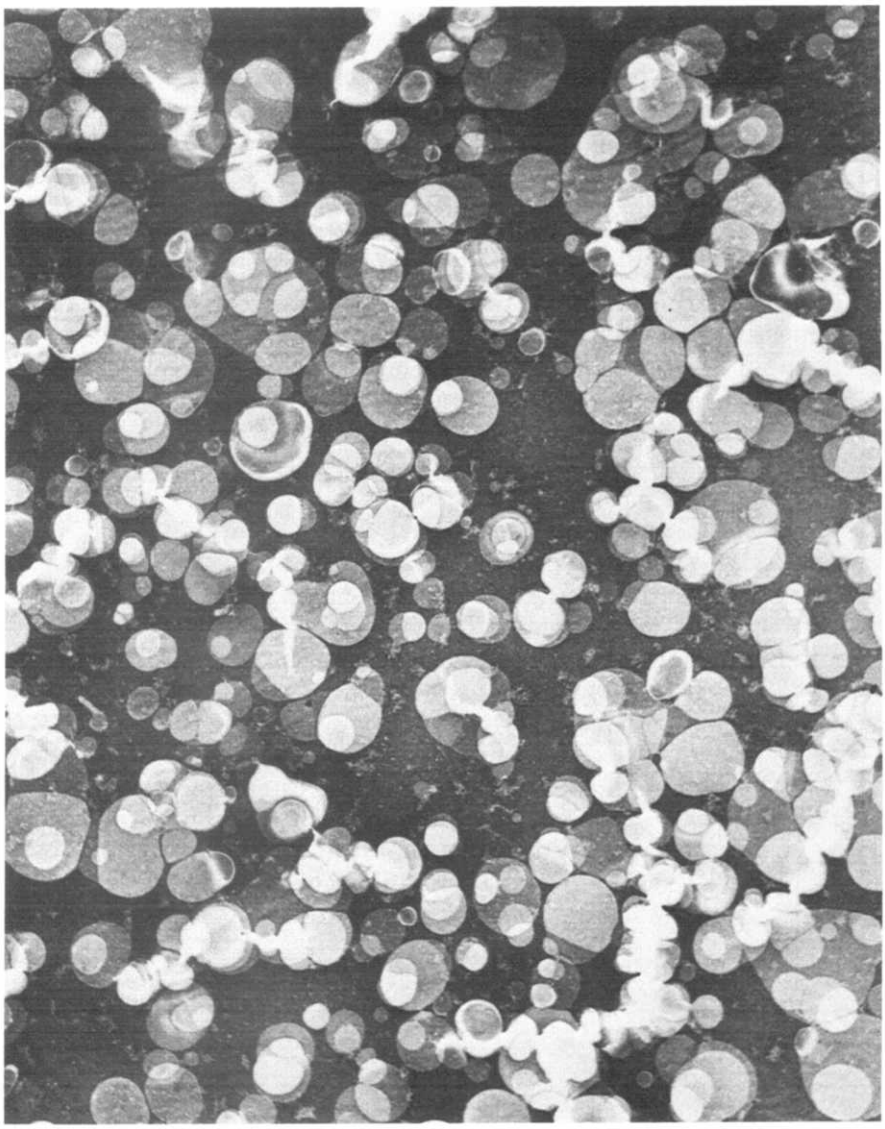

Fig. 2. Electron micrograph of the proteoliposomes negatively stained with 1% phosphotungstic acid (× 40 000).

from the liposomes that had been preloaded with the isotope. Tetrodotoxin itself did not effect the sodium permeability but abolished the veratrine-induced increment. Neither veratrine nor tetrodotoxin at adequate concentrations changed the sodium efflux from the liposomes obtained without solubilized proteins (not shown).

In agreement with these experiments, we found that Na^+ influx in proteoliposomes was influenced by the effectors in the same manner. Fig. 5 shows that preincubation of the proteoliposomes with veratrine increases the uptake of ^{22}Na in the vesicles. As in previous experiments, tetrodotoxin completely

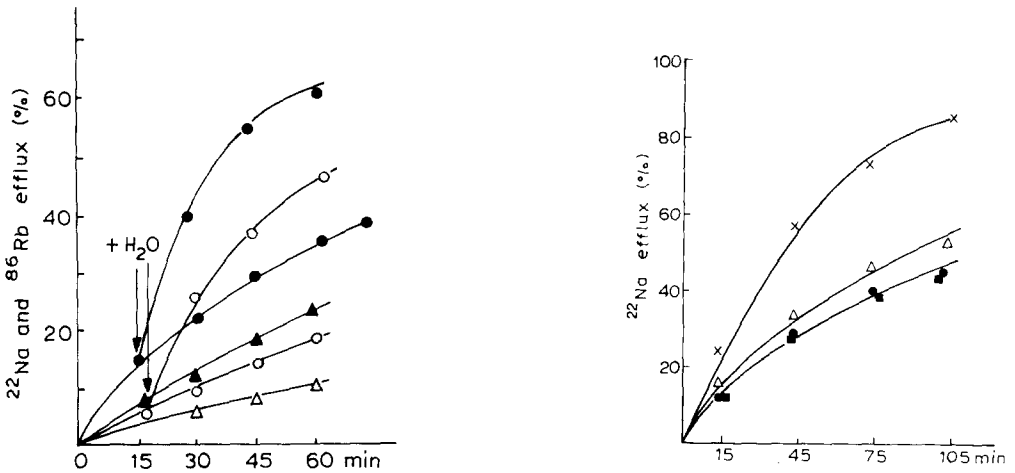

Fig. 3. The efflux of ^{22}Na (○———○, ●———●) and ^{86}Rb (△———△, ▲———▲) from the liposomes (open symbols) and from the proteoliposomes (closed symbols). Effect of tonicity. Ion effluxes are expressed as % of released isotope. The error in the determination of the % Rb^+ (Na^+) released is estimated to be maximally 10%.

Fig. 4. Influence of tetrodotoxin and veratrine on the sodium efflux from the reconstituted proteoliposomes. ●———●, control; X———X, veratrine; △———△, veratrine + tetrodotoxin; ■———■, tetrodotoxin.

abolished the effect of veratrine but did not alter values for Na influx by itself.

A question arose as to whether the demonstrated effects of veratrine and tetrodotoxin were specific for Na^+. In the next series of experiments, ^{86}Rb instead of ^{22}Na was introduced into proteoliposomes. The efflux of Rb^+ was studied exactly as before in the presence of both effectors of sodium channels. Fig. 6 shows that the exit of Rb^+ from reconstituted vesicles was influenced

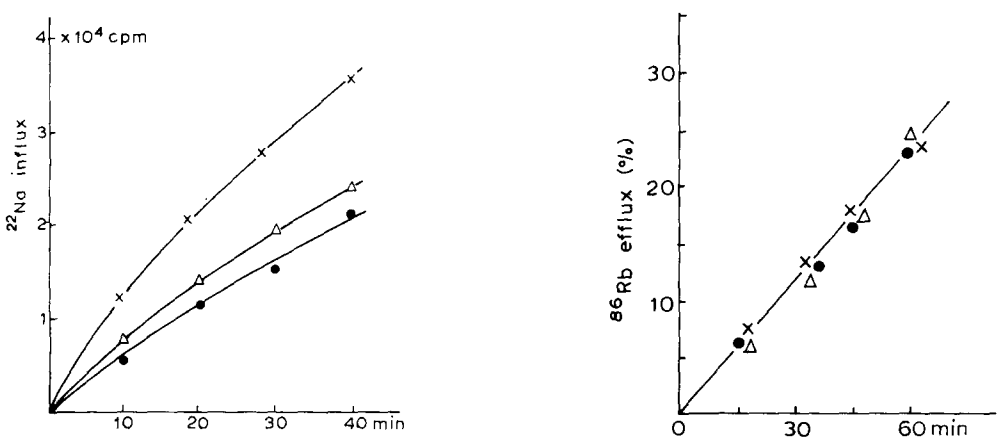

Fig. 5. Effect of tetrodotoxin and veratrine on the ^{22}Na influx into proteoliposomes, ●———●, control; X———X, veratrine; △———△, veratrine + tetrodotoxin.

Fig. 6. ^{86}Rb efflux from the proteoliposomes. Effect of veratrine (X———X) and veratrine + tetrodotoxin (△———△); ●———●, control.

neither by veratrine nor by tetrodotoxin. Thus, there can be little doubt that tetrodotoxin-sensitive Na^+ flux in proteoliposomes passes through the sodium-selective channels derived from nerve membranes.

These experiments demonstrate that solubilized membrane preparations can be used for reconstitution of the channels in liposomes. Thus, it should be possible to use this technique during attempts to purify the channel-forming components involved in the movement of Na^+.

To demonstrate this point, we decided to examine the possibility of fractionation of cholate extracts by $(NH_4)_2SO_4$ precipitation. A microsomal soluble preparation in 1% sodium cholate was obtained as described above and the proteins were precipitated by successive addition of solid $(NH_4)_2SO_4$. In each fractionation the salt was added during a 10 min interval with continous mechanical stirring. The stirring 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 precipitates formed were centrifuged at $1000 \times g$ for 10 min and dissolved in a solution containing 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 0.2 mM EGTA. $(NH_4)_2SO_4$ was removed by dialysis against 10 mM Tris-HCl, pH 7.4, containing 0.2 mM EGTA. The dialysis of both fractions resulted in the formation of protein precipitates which were removed by centrifugation at $10\,000 \times g$ for 10 min. The supernatant was used for the preparation of proteoliposomes as described before. The protein : phospholipid ratio was 1 : 50.

The tetrodotoxin sensitivity was recovered in the fraction precipitating between 0 and 30% $(NH_4)_2SO_4$. Fig. 7 demonstrates that Na^+ fluxes in the vesicles formed in the presence of this fraction were increased by veratrine. Tetrodotoxin abolished this effect.

These experiments demonstrate that phospholipid vesicles prepared by gradual removal of detergent in the presence of solubilized brain microsomes

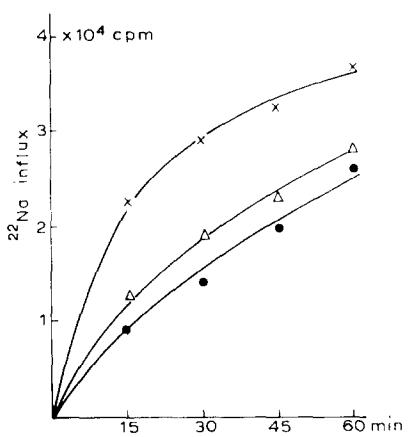

Fig. 7. Influence of veratrine and tetrodotoxin on the ^{22}Na influx into the proteoliposomes prepared from the ammonium sulfate-precipitated fraction (up to 30% saturation). ●———●, control; X———X, veratrine; △———△, veratrine + tetrodotoxin.

form a closed structure with a limited permeability to Na^+ and Rb^+ and incorporate the nerve membrane constituents. The sodium flux through reconstituted liposomal membranes was increased by veratrine. This effect was abolished by tetrodotoxin, although tetrodotoxin itself did not change the sodium permeability. Both drugs influenced the Na^+ fluxes at the concentration usually used in electrophysiological experiments. It should be noted, however, that in our experiments the prolonged preincubation with the effectors was necessary to demonstrate the maximal effect. Our conclusion about the reconstitution of the nerve membrane sodium ionophore on the liposomes was supported by the fact that under the conditions used, neither tetrodotoxin nor veratrine changed the Rb^+ fluxes.

These results open the way for the determination of the solubilized sodium channels and for the purification of this membrane structure without destroying functional activity. Now, it is difficult to say to what extent reconstituted structures are similar to the sodium channels of the native excitable membranes. Dealing with liposomes, we could not study the electrical excitability of the incorporated molecules. But, our results demonstrate clearly the reproduction of that part of the sodium channel which is responsible for its tetrodotoxin sensitivity and ionophoric properties.

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