INCORPORATION OF THE SODIUM CHANNEL OF LOBSTER NERVE INTO ARTIFICIAL LIPOSOMES*

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SUMMARY: Na^+ channels from lobster nerve membranes stored frozen in sucrose were incorporated into artificial liposomes. Crude soybean phospholipids or mixtures of purified phospholipids were suitable for reconstitution provided the latter included phosphatidylserine or another acidic phospholipid. The ^{22}Na flux into the reconstituted vesicles was increased (2 to 3-fold) by veratridine (0.25 - 1 mM) or grayanotoxin I (50 - 150 μM) and the increment was abolished by 10 nM tetrodotoxin (K_i = 2 nM). The reconstituted vesicles were inactivated after incubation for 15 min at 40° and exposure to 20 μM dicyclohexylcardobiimide inhibited by 80% the response to the drugs.

The movement of Na^+ into nerve cells via the Na^+ channel is generally recognized to play a critical role in the excitation of the nerve membrane and in its conduction of impulses (1,2). Veratridine, grayanotoxin or bactrachotoxin open the channel; tetrodotoxin or saxitoxin close it (3-6). Attempts with radioactive tetrodotoxin to isolate the specific receptor protein associated with the channel (7,8), have the drawback that preservation of binding capacity does not necessarily indicate retention of biological activity within a membrane structure. Vesicles of the plasma membrane of lobster nerve exhibit Na^+ fluxes that are activated by veratrine (a mixed alkaloid preparation containing veratridine) or bactrachotoxin and are blocked by tetrodotoxin (9). In the present paper we show that these membranes can be incorporated into liposomes which show $^{22}Na^+$ fluxes responsive to veratridine, grayanotoxin I, and tetrodotoxin.

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Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; S, sphingomyelin; PA, phosphatidic acid; DPG, diphosphatidylglycerol.

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EXPERIMENTAL PROCEDURES

Nerve plasma membrane preparations. The total nerve plasma membrane preparations were obtained from walking-leg nerves of living Panulirus argus lobsters as described previously (10,11). At the end of the isolation, the membranes were suspended in a buffer isotonic with lobster physiological solution (0.78 M sucrose, 10 mM Tris/Cl solution, pH 7.5), frozen in dry-ice acetone and stored at -70°. The protein content (12) of the suspensions was 10 to 15 mg of membrane protein per ml. In some experiments, a similar membrane fraction was obtained from walking leg and claw nerves of living Homarus americanus lobsters.

Reconstitution. Crude soybean phospholipids (Concentrated Associates, Woodside, N.Y.) were usually suspended at 20 or 40 mg per ml in a solution containing 100 mM NaP_i, 150 mM KP_i, pH 7.5. The phospholipid suspension was divided into 1 ml portions, the air was replaced with nitrogen, the test tubes capped with parafilm, and then sonicated to clarity at room temperature in a bath-type sonicator (Model T 80-80-1-RS, Laboratory Supplies, Hicksville, N.Y.). For reconstitution, the freeze-thaw-sonication procedure (13) was used. Frozen plasma membrane fractions were thawed and added to the sonicated liposomes, usually to a final concentration of 0.5 mg of membrane protein per ml. Aliquots of 0.4 ml of the membrane protein-lipid mixture were placed into different test tubes: one without drugs as a control, the others containing either veratridine, grayanotoxin I, tetrodotoxin, veratridine plus tetrodotoxin, or grayanotoxin I plus tetrodotoxin. The ethanol in which veratridine or grayanotoxin I were added was dried down under a stream of nitrogen before adding the membrane-lipid mixture. Tetrodotoxin was added from the stock solution in 10 mM acetic acid to give at least a 100-fold dilution. The same volume of acetic acid was added to the control and to the other tetrodotoxin-free samples. After adding the membrane-lipid mixture, the air was replaced with nitrogen and the tubes capped with parafilm. After freezing by immersion in dry ice/acetone for 1 min and thawing by agitating the tubes in cold water for 1-1.5 min, the mixtures were sonicated for 30 sec. With purified phospholipids the sonication time was 60 sec. All procedures including sonication were performed close to 0°.

Assay of ^{22}Na influx. The reaction was started by the addition of $^{22}NaCl$ (10% by volume of liposome suspension) to the reconstituted liposomes. Except for the ^{22}Na, the ionic content of this solution was identical to that used to suspend the lipids. Then at given times, the ^{22}Na uptake was measured (14) as follows: Columns (0.5 x 10 cm) containing about 2 ml of Dowex -50 W (Tris form, 8% cross linkage, 50-100 mesh) were treated with 10 mg bovine serum albumin (Sigma 10% stock solution) and washed with 3 ml of 0.5 M sucrose, 10 mM Tris-HCl, pH 7.5. Samples of the reconstituted liposomes (150 µl) were placed on the column and eluted with 2 ml of the same sucrose solution directly into scintillation vials. After adding 9 ml of ACS scintillation fluid, ACS™ (Amersham), the radioactivity was measured in a liquid scintillation spectrometer. The first sample was taken 0.5 min after the addition of the ^{22}Na labeled solution and the values were used to calculate the ^{22}Na influx.

RESULTS AND DISCUSSION

^{22}Na influx in frozen and thawed plasma membrane vesicles and in vesicles reconstituted with crude soybean phospholipids. Table I shows that ^{22}Na fluxes in plasma membrane vesicles from nerves of P. argus, which have been stored frozen and were thawed for the assay, showed little or no response to veratridine

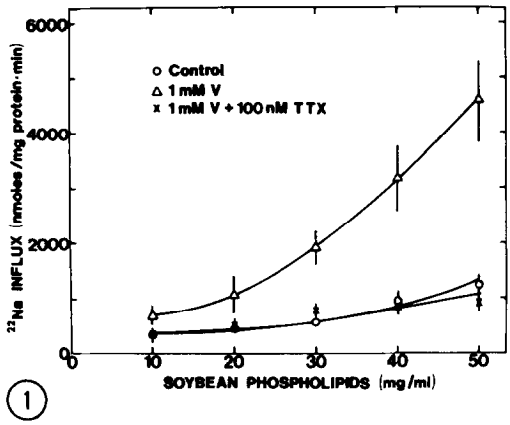
TABLE I

^{22}Na influx in nerve membrane vesicles after storage at -70° in reconstituted vesicles

Experimental procedures were as described in the text. Values are mean ± SEM. The number of experiments performed are given in parentheses. In Exp. A the soybean phospholipid concentration was 20 mg/ml and in Exp. B it was 40 mg/ml. When protein was added in Exp. A and B, the final protein concentration was 0.5 mg/ml.

Text System	^{22}Na influx	
	nmoles/min/mg protein	nmoles/min/mg phospholipid
Membrane vesicles		
control	289 ± 32 (3)	136 ± 15 (3)
veratridine (0.5 mM)	360 ± 67 (3)	169 ± 33 (3)
veratridine (0.5 mM) + tetrodotoxin (1 μM)	299 ± 50 (3)	141 ± 24 (3)
(A) Reconstituted vesicles		
control	599 ± 43 (17)	15.0 ± 1.1 (17)
veratridine (1 mM)	1382 ±158 (17)	34.6 ± 4.0 (17)
veratridine (1 mM) + tetrodotoxin (100 nM)	590 ± 42 (14)	14.8 ± 1.3 (14)
(B) Reconstituted vesicles		
control	949 ± 179 (5)	11.9 ± 2.2 (5)
veratridine (1 mM)	3173 ± 591 (5)	39.7 ± 7.4 (5)
veratridine (1 mM) + tetrodotoxin (100 nM)	839 ± 123 (5)	10.5 ± 1.5 (5)

and tetrodotoxin. After reconstitution with crude soybean phospholipids, the $^{22}Na^{+}$ influx was increased by veratridine and this increment was abolished by tetrodotoxin. The flux in the absence of veratridine was not significantly affected by tetrodotoxin. The ^{22}Na influx data are recorded in terms of protein as well as phospholipid content of the vesicles so as to compare the data with the ^{22}Na influx measurements into protein-free liposomes. The latter showed no response to veratridine and tetrodotoxin and a flux rate of 4 to 6 n moles per min per mg of phospholipids. As illustrated in Figure 1, with the membrane protein concentration used for reconstitution kept at 0.5 mg per ml, the stimulation by veratridine increased with higher phospholipid concentrations. The effect of increasing concentrations of the membrane protein (up to 1.5 mg/ml) are shown in



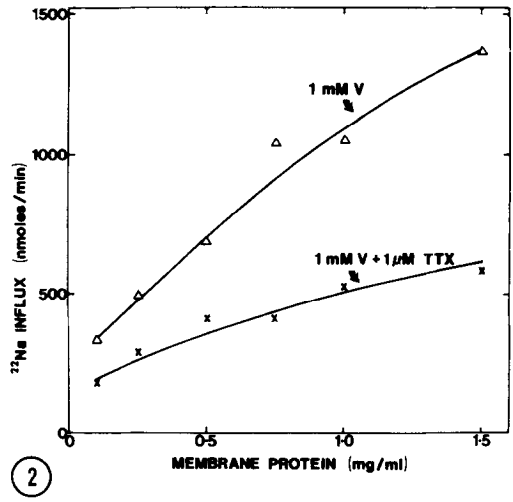
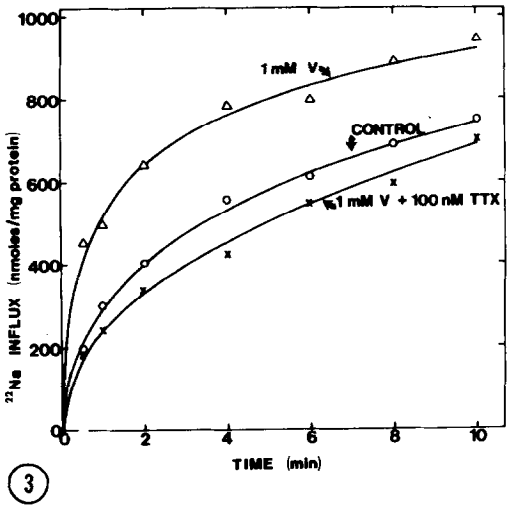


Fig. 1. ^{22}Na influx as function of the concentration of soybean phospholipids. The concentration of nerve membrane protein was 0.5 mg/ml. Influx measurements were made 0.5 min after addition of ^{22}Na. Values are expressed as the mean ± SEM (5 experiments).

Fig. 2. ^{22}Na influx as function of the concentration of nerve membrane protein. The concentration of soybean phospholipids was 20 mg/ml. Influx measurements were made 0.5 min after addition of ^{22}Na.

Figure 2. Similar data were obtained with reconstituted vesicles made with nerve plasma membranes of H. americanus lobsters. Figure 3 illustrates that the ^{22}Na influx in reconstituted vesicles was time dependent. The effect of veratridine and tetrodotoxin was most apparent for the ^{22}Na influx at 0.5 min.

Effect of different veratridine, grayanotoxin I, and tetrodotoxin concentrations. The effect of different veratridine concentrations on the $^{22}Na^{+}$ influx is shown in Figure 4A. This effect is consistent with the well-known action of veratridine on intact cells where it has been used to open inactivated Na^{+} channels (3-5, 15-18). Furthermore, to identify the pathways used by ^{22}Na to enter the reconstituted vesicles, we have relied on the specificity of tetrodotoxin for blocking the Na^{+} channels (19-22). Recorded in Figure 4B is the effect of different tetrodotoxin concentrations on the increment caused by veratridine. Virtually complete reversal of the veratridine effect was noted at 10 nM



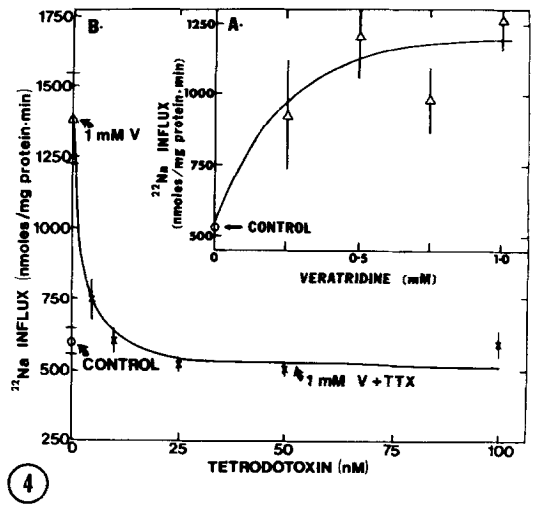


Fig. 3. ^{22}Na influx as a function of time.

Vesicles were reconstituted with 0.5 mg/ml of nerve membrane protein added to 20 mg/ml of soybean phospholipids.

Fig. 4. Effect of different veratridine and tetrodotoxin concentrations on the ^{22}Na flux.

In A are shown the values of the influx in the absence of veratridine (control 0, 30 experiments) and in the presence of 0.25 (6 experiments), 0.5 (10 experiments, 0.75 (13 experiments) and 1 (29 experiments) mM veratridine (Δ——Δ). In B the values correspond to the influx in the absence of veratridine (control 0, 17 experiments) and in the presence of 1 mM veratridine (Δ, 17 experiments), and 1 mM veratridine plus 5 (6 experiments), 10 (9 experiments, 25 (10 experiments), 50 (10 experiments) or 100 (14 experiments) nM tetrodotoxin (X——X). Values are expressed as the mean ± SEM.

concentration and 2 nM tetrodotoxin caused 50% reversal. Virtually identical results were obtained when grayanotoxin I, which increases selectively the resting nerve membrane permeability to Na^+ (23), was substituted for veratridine. Maximal stimulation was obtained at 100 μM (Figure 5).

Stability and chemical modification. The preparation of reconstituted vesicles was rather heat labile. After 15 min at 30°, activity was greatly reduced and after 15 min at 40°, it was lost. It was also found that treatment of the nerve membrane-soybean phospholipids mixture before reconstitution with 20 μM dicyclohexylcarbodiimide, inhibited the effect of the drugs on the ^{22}Na flux by 80%. This concentration is over three orders of magnitude lower than

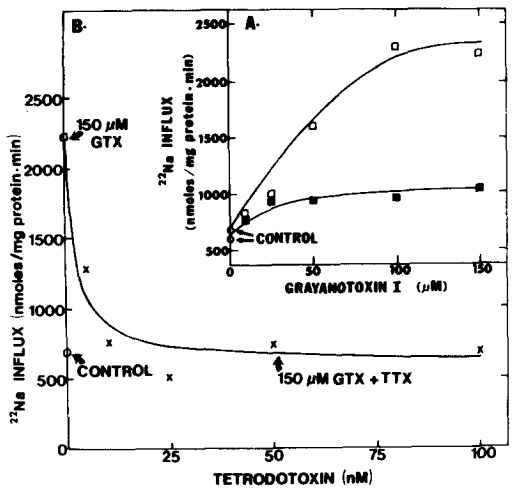


Fig. 5. Effect of different grayanotoxin I and tetrodotoxin concentrations on the ^{22}Na flux.

In A are shown the values of the influx in the absence of grayanotoxin (control, 0) and in the presence of grayanotoxin in two different preparations of vesicles, one made up with 0.5 mg/ml of membrane protein and 20 mg/ml of soybean phospholipids (▨) and the other with the same protein concentration and 40 mg/ml of phospholipids (□). In B are shown the influx values in the latter preparation in the absence of grayanotoxin (control, 0), in the presence of 150 μM grayanotoxin (□) or in the presence of 150 μM grayanotoxin plus different tetrodotoxin concentrations (X——X).

that of the water soluble carbodiimide used previously to modify the tetrodotoxin receptors in intact nerves (24,25).

Reconstitution with purified phospholipids. In Table II data on reconstitution of the Na^+ channel with purified phospholipids are shown. The design of the experiments (Exp. 1) was to imitate the phospholipid composition of the lobster nerve membrane (26). Only one phospholipid species was added at a time to a mixture of PE and PC (1.0:0.6). The two phospholipids alone yielded vesicles with a low response to veratridine and tetrodotoxin. If PS was included during reconstitution, veratridine increased $^{22}Na^+$ influx and tetrodotoxin abolished the increment. Some of the purified phospholipid mixtures were as good or better than crude soybean phospholipids. It is also shown (Exp. 2) that PS is not a specific requirement. Although the rates of ^{22}Na influx were somewhat lower,

TABLE II

Reconstitution of the sodium channel with purified phospholipids

The vesicles were reconstituted with nerve membrane (0.5 mg protein/ml) and the phospholipid mixtures described below at total concentrations of 20 mg/ml.

	^{22}Na influx nmoles/mg protein/min		
	Control	Vetratridine (1 mM)	Vetratridine (1 mM) + tetrodotoxin (100 nM)
Exp. 1			
Soybean phospholipids	599	1382	590
PE[a]:PC[b] (1:0.6)	356	453	325
PE[a]:PC[b]:PS[c] (1:0.6:0.3)	578	1508	713
PE[a]:PC[b]:PS[c]:PI[d] (1:0.6:0.3:0.1)	707	2290	666
PE[a]:PC[b]:PS[c]:PI[d]:S[e] (1:0.6:0.3:0.1:0.3)	584	1529	810
PE[a]:PC[b]:PS[c]:PI[d]:S[e]:PA[f] (1:0.6:0.3:0.1:0.3:0.1)	1105	1946	1025
Exp. 2			
PE[a]:PC[b]:PI[d] (1:0.6:0.3)	302	751	342
PE[a]:PC[b]:DPG[g] (1:0.6:0.3)	400	829	603

[a]Bovine brain (Avanti Biochem. Inc.); [b]Bovine spinal cord (Lipid Products); [c]Bovine brain (prepared by M. White); [d]Wheat germ (Lipid Products); [e]Bovine brain (Grand Island Biol. Co.); [f]Egg (Grand Island Biol. Co.); [g]Bovine heart (Sigma Chem. Co.)

stimulations by veratridine and inhibition by tetrodotoxin were seen with other acidic phospholipids.

Electron micrographs of the original and of the reconstituted vesicles clearly showed that the membranes were broken during sonication and incorporated into characteristic liposome vesicles. These experiments demonstrate that crude membrane preparations can be used for the reconstitution of the Na^+ channel in artificial liposomes. Thus, it should be possible to use this biological assay during attempts to solubilize and purify the active components involved in the movement of Na^+ into nerve cells.

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