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# Anemone toxin II receptor site of the lobster nerve sodium channel. Studies in membrane vesicles and in proteoliposomes

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**The receptor-site for the sea anemone toxin II from *Anemonia sulcata* (ATX) and its functional relationship with the $Na^+$ channel were studied in plasma membrane preparations from lobster walking leg nerves. The modification of the $^{22}Na$ influx by ATX was determined in membrane vesicles and in proteoliposomes prepared by reconstitution of detergent-extracted, unfractionated membrane particles into soybean liposomes. The effects of two other toxins, veratridine (VER) and tetrodotoxin (TTX), which bind to $Na^+$ channel receptor-sites other than that for polypeptide toxins, were also studied, ATX and VER stimulated $^{22}Na$ flux into membrane vesicles with $K_{0.5}$ values in the order of $10^{-7}$ and $10^{-5}$ M, respectively. Positive cooperativity among these toxins was also seen; ATX displaces the $K_{0.5}$ for VER towards lower VER concentrations. TTX abolishes the $^{22}Na$ influx increment caused by ATX and/or VER with a $K_{0.5}$ in the order of $10^{-8}$ M. In proteoliposomes, in contrast, ATX modified the $^{22}Na$ influx only at high concentrations ($> 1$ μM) and in the presence of VER. VER stimulation and TTX inhibition of the VER and the VER plus ATX modified fluxes, had the same characteristics as in the vesicle preparations. Measurable ATX and VER toxin effects were only seen in the presence of an outwardly directed $K^+$ gradient for both vesicles and proteoliposomes. Detergent treatment and the reconstitution procedure seem to affect the functional properties of the ATX receptor site whereas the VER and the TTX sites remain unaltered.**

## Introduction

The knowledge about the structure and function of the $Na^+$ channel at the molecular level has advanced, thanks to the solubilization and purification of $Na^+$ channels from different sources [1–9] and to their functional reconstitution into liposomes or planar lipid bilayers [10–19]. Three main groups of neurotoxins (for references see Refs. 22 and 23), are regularly used to characterize the $Na^+$ channel during its solubilization, purification and reconstitution. (i) The first group is formed by the water-soluble heterocyclic compounds containing guanidinium moieties, tetrodotoxin and saxitoxin. They share the same receptor site and block reversibly at nanomolar concentrations the ion flux through the $Na^+$ channel.

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Abbreviations: ATX, anemone toxin II; VER, veratridine; TTX, tetrodotoxin; BTX, batrachotoxin; $P_i$, inorganic phosphate; Cho, choline; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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(ii) The second group is constituted by lipid-soluble polycyclic compounds veratridine, batrachotoxin, aconitine and the grayanotoxins. They affect the voltage dependence of activation and inactivation of the $Na^+$ channel. They displace the voltage required for activation towards more negative values and prevent channels from inactivating. These toxins act at micromolar concentrations. (iii) The third group is formed by small polypeptides ($M_r$ 4000–7000) present in the venoms of scorpions of the *Buthidae* family and in the nematocysts of several sea anemones. There are two types of polypeptide toxins: the $\alpha$-toxins which affect the inactivation of the $Na^+$ channel by preventing it, and the $\beta$-toxins which affect the activation mechanism in a similar way as the above mentioned toxins [18]. The binding of $\alpha$-toxins is voltage dependent [24–26]. The $\alpha$-toxins enhance the action of the lipid soluble toxins and vice versa [27,28].

The sources of $Na^+$ channels for solubilization, purification and reconstitution studies have been mainly vertebrate nerve and muscle plasma membranes [1–4,6–9,13–19]. However, it should be noticed that the functional reconstitution of the channel was first achieved with crude membrane preparations from lobster nerves incorporated into soybean liposomes [10,11]. In addition, the $Na^+$ channel present in detergent-extracted, partially purified membrane particles from lobster nerves has been incorporated into soybean liposomes [5,29]. The functional relationship between the native and the reconstituted $Na^+$ channel and the receptor sites for the first and second group of neurotoxins mentioned in the previous paragraph, has been well established in vertebrates and invertebrates. Tetrodotoxin (TTX), veratridine (VER) and batrachotoxin (BTX) are the most common toxins used. The presence of the polypeptide $\alpha$-toxin receptor has also been demonstrated, but further work seems to be required to reconstitute the functional relationship of this receptor site with the $Na^+$ channel.

Since knowledge of the properties of the $\alpha$-toxin receptor present in isolated cell plasma membranes and in detergent extracted membrane particles should contribute to reconstitute its function, we have studied the action of the polypeptide $\alpha$-toxin anemone toxin II from *Anemonia sulcata* (ATX), on the $Na^+$ channel (I) in vesicles spontaneously formed by isolated lobster nerve membranes, and (II) in proteoliposomes prepared with unfractionated, detergent-extracted membrane particles incorporated into soybean liposomes [30]. In addition, the effects of VER and TTX were also studied, under the experimental conditions required to demonstrate the effect of ATX.

## Materials and Methods

*Plasma membrane preparation*

The nerve plasma membranes used in the present work were isolated from the walking-leg nerves of the lobster *Panulirus argus*. Up to 50 g wet-weight of nerves were used at a time. The procedure is essentially that previously reported [5,31]. Briefly, the nerves were kept in an ice-cold 0.33 M sucrose solution containing 2 mM $MgCl_2$ and buffered with 10 mM Tris-HCl (pH 7.5) (sucrose solution). The amount of sucrose solution was adjusted to 2 ml per g of wet nerve. The nerves were minced in a blender at 12000 rpm during 1 min per g of wet nerve, with 30-s intervals between minutes. The sucrose solution was adjusted again to a new final volume of 10 ml per g of wet nerve. The minced tissue was then homogenized in a 55 ml glass-teflon pestle, motor run homogenizer, approximately 30 ml at a time, with 10 strokes at 9700 rpm. The homogenate was then centrifuged at $85000 \times g$ for 30 min. The pellets were resuspended in sucrose solution to a final volume corresponding to 45 ml per g of wet nerve and homogenized again with 10 strokes at 9700 rpm. This homogenate was layered on top of a solution containing 1.12 M sucrose, 2 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.5), and centrifuged in a swinging rotor at $65000 \times g$ for 60 min. Plasma membranes banding at the interface on top of the 1.12 M sucrose solution were collected, diluted 3-fold with 10 mM Tris-HCl (pH 7.5) containing 2 mM $MgCl_2$, and centrifuged at $85000 \times g$ for 30 min. The membrane pellets were resuspended in 0.78 M sucrose, 10 mM Tris-HCl (pH 7.5), homogenized by hand with three strokes in a glass-teflon pestle homogenizer and kept at $-70°C$ until use. The temperature during the isolation procedure was 4°C. The final protein concentration was approximately 10 mg/ml.

*Experimental solutions and neurotoxins*

In order to test different assay conditions, membrane vesicles and proteoliposomes were prepared, as indicated below, in solutions with various ionic compositions (internal solutions) and later diluted in $^{22}Na$-containing solutions (external solutions) with the same or different ionic composition than the respective internal media. The composition and abbreviated identification of the solutions used in the present work are given in Table I. All reagents were obtained from Sigma Chemical Co., St. Louis, MO. $^{22}Na$ as NaCl in water solution, carrier free, 13.22 mC/ml, was obtained from New England Nuclear, Boston, MA.

The neurotoxins used were the following: citrate-free Tetrodotoxin (TTX), $M_r$ 319.28, from Calbiochem, San Diego, CA; Veratridine (VER), $M_r$ 673.81, was purified in our laboratory according to Kupcham et al. [32] from commercial veratrine obtained from Sigma Chemical Co., St. Louis, MO; lyophilized sea anemone toxin II from *Anemonia sulcata* (ATX), $M_r$ 4770, was obtained through a kind gift of Professor L. Beress, Christian-Albrechts University, Kiel, F.R.G., and from Ferring GmbH, Kiel, F.R.G. Stock solutions of the toxins were prepared as follows: 1 mM TTX dissolved in 10 mM acetic acid; 50 mM VER in 95% ethanol, and ATX (0.1 mg ampoules) dissolved to 200 $\mu$M in bidistilled water. The TTX and VER stock solutions were kept in the refrigerator at 4°C, and that of ATX was frozen at −20°C until use.

*Preparation of membrane vesicles*

Frozen plasma membranes were thawed, and then diluted 5-fold with a given internal solution (see Table I for the composition of the solutions) to a final protein concentration of about 2 mg/ml. The suspension was then incubated for 10 min at 30°C and after this time centrifuged at 48 000 × $g$ for 10 min at 4°C. The pellet was resuspended in the same internal solution used for incubation to a final protein concentration of about 10 mg/ml, rehomogenized by hand with three strokes in a glass-teflon pestle homogenizer and kept in ice until use.

*Preparation of the proteoliposomes*

The procedure used to prepare the unfractionated, detergent-extracted membrane particles used to make the proteoliposomes was similar to the one described in a previous work [5]. Frozen lobster nerve plasma membranes were thawed as indicated above, then diluted 24-fold with a solution containing 0.5 M NaCl, 1 mM EDTA and 10 mM Tris-HCl (pH 7.5) and then centrifuged at 150 000 × $g$ during 30 min. About 75% of the initial total protein was recovered in the pellets which were then resuspended to a final protein concentration of 8 to 10 mg/ml in an ice-cold solution 0f 0.35 M sucrose, 10 mM Tris-HCl (pH 7.5) containing 25 mg/ml of total soybean lipids (Asolectin, Associated Concentrates, Wood Side, L.I., NY). To this suspension, 20% sodium cholate was added to reach a final detergent concentration

TABLE I

COMPOSITION OF THE SOLUTIONS USED AS INDICATED IN THE TEXT AND FIGURES AS INTERNAL AND EXTERNAL EXPERIMENTAL MEDIA OF THE NERVE MEMBRANE VESICLES AND OF THE PROTEOLIPOSOMES

The $K^+$, $Na^+$ and $Tris^+$ phosphate solutions ($KP_i$, $NaP_i$ and $TrisP_i$, respectively) were prepared titrating the hydroxide salts of the respective cation with $H_3PO_4$ to pH 7.4. The choline phosphate ($ChoP_i$) solution was prepared by titrating choline bicarbonate with $H_3PO_4$ to pH 7.4. To prepare TrisCl, Trizma-base was titrated with HCl to pH 7.4.

| Solution identification | Concentration in mM | | | | | | |
|---|---|---|---|---|---|---|---|
| | $KP_i$ | $NaP_i$ | $ChoP_i$ | ChoCl | $TrisP_i$ | TrisCl | $MgSO_4$ |
| $KP_i$ | 300 | 1 | – | – | – | – | 2 |
| $NaP_i$ | – | 301 | – | – | – | – | 2 |
| $ChoP_i$ | – | 1 | 300 | – | – | – | 2 |
| ChoCl | – | 1 | – | 300 | – | – | 2 |
| $TrisP_i$ | – | 1 | – | – | 300 | – | 2 |
| TrisCl | – | 1 | – | – | – | 300 | 2 |

of 0.5% in the sample. This was carefully mixed and after 1 min 250 mM octyl glucoside was added to obtain a final concentration of 30 mM. The detergent-treated suspension was let sit in ice for an additional 9 min period. After this time, the mixture was centrifuged at $100000 \times g$ during 40 min. The supernatant was carefully collected and diluted 24-fold with internal solution, and centrifuged again at $150000 \times g$ during 40 min. The pellets were resuspended with internal solution containing 0.1 M sucrose, to a final protein concentration of 1 to 2 mg/ml and then incubated at 35°C for 10 min. These unfractionated, detergent-extracted membrane particles were immediately used for reconstitution.

The reconstitution procedure employed to incorporate the membrane particles into the liposomes was the freeze-thaw-sonication procedure of Kasahara and Hinkle [30] as described in a previous work [5]. The liposomes were prepared by suspending 80 mg/ml of soybean lipids in internal solution. Once the lipids were soaked, the suspension was sonicated in aliquots of 1 ml at room temperature in a bath sonicator of 80 kHz (model T 80-1-KS, Laboratory Supplies, Hicksville, NY). The suspension was sonicated until it became translucent. Once sonicated, all the aliquots were pooled, and kept in a nitrogen atmosphere at 4°C until use.

The suspension of unfractionated, detergent-extracted particles was mixed with an equal volume of the suspension of soybean liposomes to obtain a lipid : protein ratio by weight of 40 : 1. Aliquots of 0.3 ml of the lipid : protein mixture were placed in glass tubes which contained the toxins or their solvents. Prior to sonication the air of the tubes was replaced with nitrogen, each tube was capped with parafilm, its content frozen in a solid $CO_2$/acetone mixture for 1 min, thawed gently agitating the tubes in water at room temperature approximately 1.5 min, and then sonicated, one at a time, for 15 s in the bath sonicator filled with ice/water. The toxins were added to the media before reconstitution in order to ensure their interaction with the receptor-sites.

*$^{22}Na$ flux assays*

The $^{22}Na$ flux assays were done following the procedure described by Gasko et al. [33]. The $^{22}Na$ influx determinations were initiated by the addition of 10 $\mu$l of either the membrane vesicle suspension or the proteoliposome suspension to 190 $\mu$l of the $^{22}Na$ labeled external solution (1 $\mu$Ci $^{22}Na$/ml) with the toxins and/or their solvents, at room temperature (21–24°C). Uptake periods were then ended by the addition of 5 $\mu$l of ice-cold 50 $\mu$M TTX; 175 $\mu$l of this mixture were then passed through a Pasteur pipette-size column of Dowex 50W-8, 50–100 mesh (Sigma Chemical Co., St. Louis, MO). The sample was eluted with 2 ml of an ice-cold 0.5 M sucrose solution, buffered with 10 mM Tris-HCl (pH 7.5). Right before use, the Dowex columns were pretreated with 100 $\mu$l of 100 mg/ml bovine serum albumin (Albumin Stock Stolution, Sigma Chemical Co., St. Louis, MO) and washed with 3 ml of the 0.5 M sucrose solution. The material eluted from the Dowex columns was received in scintillating counter vials, 5 ml of scintillating liquid was added, and the vials were counted in a Packard Tricarb, model 3310, or a Beckman, model LS 7800.

*Protein concentration*

Protein measurements were done according to the procedure described by Lowry et al. [34].

## Results and Discussion

### *I. Plasma membrane vesicles*

*Experimental conditions*

Due to the voltage dependence of the binding of a $\alpha$-toxins to their receptor sites, the establishment of an electrochemical potential difference across the membrane was considered a necessary experimental condition to observe the action of ATX on the $^{22}Na$ flux occurring through the $Na^+$ channel. Thus, an ionic gradient of a permeable cation which would in turn generate a diffusion potential was established. Since K ions are readily permeable through plasma membranes, a $K^+$ diffusion potential ($[K^+]_i > [K^+]_e$) was created diluting the $K^+$ loaded vesicles in solutions containing cations like choline ($Cho^+$) and $Tris^+$, usually less permeable than $K^+$.

In order to determine in which media the action of the toxins on the $Na^+$ channel could be detected, the $^{22}Na$ influx was measured in the

absence of toxins (control), in the presence of ATX and/or VER, and of the same concentration of ATX and/or VER plus TTX. The $^{22}Na$ influx was calculated from the uptake measured after the first 30 s of exposure to the $^{22}Na$-containing solution. As it is discussed in the paragraphs concerning the time-course of $^{22}Na$ uptake by the vesicles, the influx calculated from uptake measurements made even during the first 30 s can be affected by $^{22}Na$ efflux and dissipation of the $K^+$ gradient driving force. The results are expressed in nmol/mg protein per min. The $^{22}Na$ influx increment due to ATX and/or VER which is blocked by TTX is considered to occur through the $Na^+$ channel.

The ratio of the $^{22}Na$ influx in the presence of ATX and/or VER to that measured in the presence of the same concentration of the toxins plus TTX is a measure of toxin effect. Table II gives the ratios obtained in experiments in which the ionic composition of the internal and the external media were varied. Under most of the experimental conditions assayed the ratios obtained were close to 1. Only when an outwardly directed $K^+$ gradient was present the ratio became greater than 1. There was no difference between the ratios obtained in chloride and phosphate external solutions indicating that the nature of the anion of the external medium did not affect the results.

TABLE II

RATIOS OF THE $^{22}Na$ INFLUX IN THE PRESENCE OF ANEMONE TOXIN II (ATX) AND OF VERATRIDINE (VER) TO THE INFLUX AFTER THE ADDITION OF TETRODOTOXIN (TTX) IN MEMBRANE VESICLES WITH INTERNAL AND EXTERNAL MEDIA OF DIFFERENT IONIC COMPOSITION

The $^{22}Na$ influx ratios were calculated from the $^{22}Na$ influx in the presence of 0.1 mM VER or of 0.5 $\mu$M ATX and the influx due to the same concentration of VER or ATX plus 1 $\mu$M TTX. The compostion of the solutions is given in Table I.

| Experimental solutions | | $^{22}Na$ influx ratios | |
|---|---|---|---|
| internal | external | ATX/(ATX + TTX) | VER/(VER + TTX) |
| $KP_i$ | $KP_i$ | 1.08 | 1.18 |
| | KCl | 1.15 | 1.11 |
| | $ChoP_i$ | 2.45 | 2.50 |
| | ChoCl | 2.30 | 2.45 |
| $ChoP_i$ | $KP_i$ | 1.03 | 0.95 |
| | KCl | 1.00 | 1.05 |
| | $ChoP_i$ | 1.20 | 1.18 |
| | ChoCl | 1.12 | 1.06 |
| ChoCl | $kP_i$ | 0.94 | 0.92 |
| | KCl | 1.08 | 1.03 |
| | ChoCl | 1.29 | 0.96 |
| $TrisP_i$ | $TrisP_i$ | 1.05 | 1.14 |
| | ChoCl | 1.15 | 1.23 |

The magnitude of the $^{22}Na$ influx also varies in different external and internal media. Fig. 1 shows the $^{22}Na$ influx under some of the ionic conditions given in Table II. In the absence of the $K^+$ gradient (panels A and B) or when the $K^+$ gradient is reversed (panels E and F), in addition to the absence of ATX and VER effects, the $^{22}Na$ influxes are smaller than in the presence of the outwardly directed $K^+$ gradient (panels C and D), irrespective of the external anion present. When the external $K^+$ is raised, $[K^+]_e \geqslant [K^+]_i$, a lack of effect of ATX and VER could be expected since $Na^+$ channels are also permeable to $K^+$ and competition between the two ions could take place. However, ion competition would not completely account for the diminution of $^{22}Na$ influxes seen even in the absence of toxins (control) or when the $Na^+$ channels are blocked by TTX. In conditions where $Cho^+$ or $Tris^+$ were used to replace $K^+$ in the internal and the external media, i.e. in the absence of an ionic gradient, the $^{22}Na$ influx was also small even at control and TTX-blocked conditions (not shown). Therefore, the movement of $^{22}Na$ seems to be stimulated by the diffusion of $K^+$ out of the vesicles due to its gradient. This effect could be seen even in the absence of toxins, which is the case of our results. $^{22}Na$ influxes in the presence of ATX and/or VER were even greater since these toxins activate the $Na^+$-channel mechanism thus opening an additional selective pathway.

Another permeable cation like $Na^+$ is expected to have a similar effect as $K^+$ on the $^{22}Na$ influx. In experiments reported by Garty et al. [35], an outwardly directed $Na^+$ gradient was shown to be useful in the study of VER-stimulated $^{22}Na$ influxes in sonicated synaptosomal membrane fragments. Similar results have been obtained by Rosenberg et al. [16] and Tomiko et al. [21] for the BTX-stimulated $^{22}Na$ influx into liposomes con-

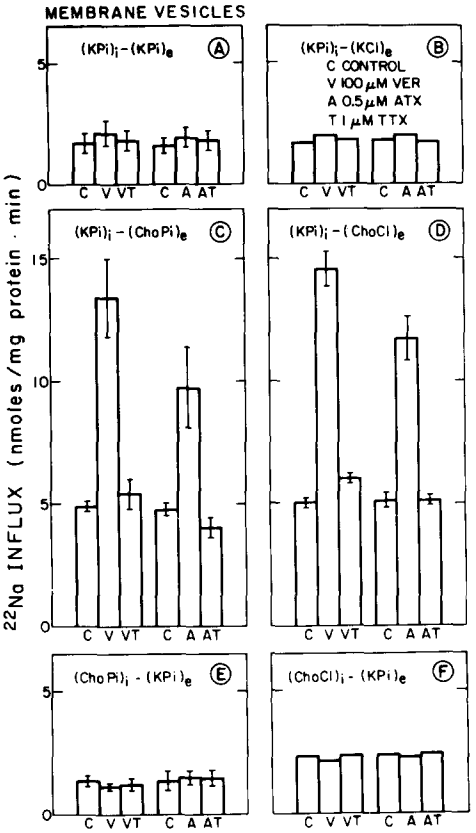


Fig. 1. Initial $^{22}Na$ flux into lobster nerve membrane vesicles in media of different ionic composition and in the absence (control, C) or presence of neurotoxins. The toxins used were veratridine (VER, V), anemone toxin II (ATX, A), and VER or ATX plus tetrodotoxin (TTX, T). The ionic composition of the solutions is given in Table I. The membrane vesicles corresponding to the experiments represented in panels (A) to (D) were prepared in 0.3 M $KP_i$ as internal solution (i) and diluted 20-fold in one of the following external solutions (e) containing $^{22}Na$: (A) 0.3 M $KP_i$, (B) 0.3 M KCl, (C) 0.3 M $ChoP_i$, and (D) 0.3 M ChoCl. The internal solutions in the experiments shown in panels (E) and (F) were 0.3 M $ChoP_i$ and 0.3 M ChoCl, respectively, and in both cases the external solution was 0.3 M $KP_i$. Each $^{22}Na$ influx value was calculated from the uptake of $^{22}Na$ measured 30 s after dilution of the membrane vesicles in the external solution. Values are the mean of three iexperiments (±S.E.) in A, C, D and E, and of two experiments in B and F.

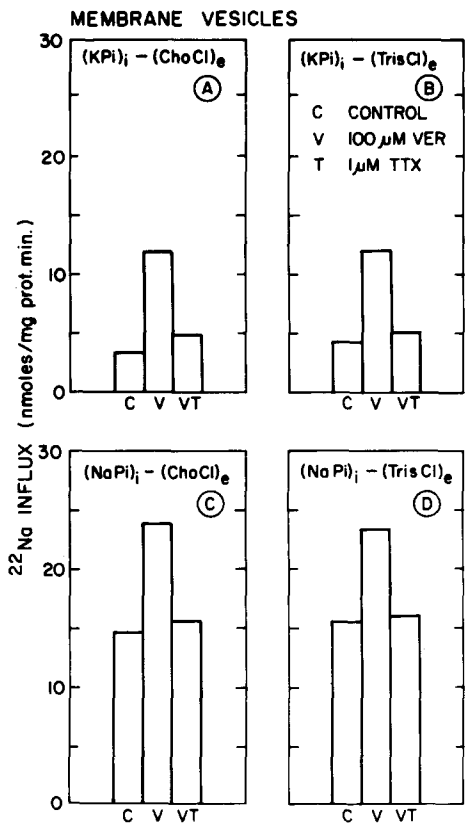


Fig. 2. Initial $^{22}Na$ flux into lobster nerve membrane vesicles in the presence of outwardly directed $K^+$ and $Na^+$ gradients. The $^{22}Na$ influx was measured in the absence of toxins (control, C), in the presence of veratridine (VER, V), and of veratridine plus tetrodotoxin (TTX, T). The ionic composition of the internal and external solutions is given in Table I. The vesicles used in the experiments represented in panels (A) and (B) were prepared by using 0.3 M $KP_i$ as internal solution, and diluted 20-fold in the following external solutions containing $^{22}Na$: (A) 0.3 M ChoCl, and (B) 0.3 M TrisCl. In the experiments represented in panels (C) and (D) the internal solution was 0.3 M $NaP_i$, and the external solutions were 0.3 M ChoCl for (C) and 0.3 M TrisCl for (D). Each $^{22}Na$ influx value was calculated from the uptake of $^{22}Na$ measured 30 s after dilution of the membrane vesicles in the external solution. Each value is the mean of determinations done in duplicate.

taining the reconstituted $Na^+$ channel from eel electroplax. In the experiment shown in Fig. 2, $^{22}Na$ influxes measured in the presence of the $K^+$ gradient (panels A and B) are compared to those obtained with a $Na^+$ gradient (panels C and D). When a $Na^+$ gradient was created, $^{22}Na$ fluxes were greater than those due to the $K^+$ gradient. However, the ratio of the VER stimulated $^{22}Na$ influx to the $^{22}Na$ influx in the presence of VER plus TTX was smaller. The $^{22}Na$ fluxes in the determinations done with the choline chloride solution were equal to those obtained with Tris chloride, for both the $K^+$ gradient and the $Na^+$ gradient assays. The higher net values of $^{22}Na$

influx seen with the $Na^+$ gradient, even in the control and TTX-blocked fluxes, indicate that $Na^+$ is more effective than $K^+$ in terms of the uptake of external $Na^+$. But nevertheless, higher basal $^{22}Na$ influxes interfere with the observation of the VER stimulated fluxes. For this reason and due to the purpose of this work, $K^+$ gradients were used.

The $K^+$ gradient was usually formed by a 20-fold dilution of vesicles prepared in a high $K^+$ solution into a $K^+$-free medium. This produced a 20-fold dilution of the external $K^+$ (to 15 mM) with respect to the internal medium (300 mM). Dilution of the vesicles in media with increasing external $K^+$ resulted in an exponential decay of the $^{22}Na$ influx as shown in Fig. 3. In this figure, net toxin stimulated $^{22}Na$ influxes are plotted against the external $K^+$ concentration which was raised from 15 mM, the normal external concentration, to 300 mM. For both ATX (Fig. 3A) and VER (Fig. 3B) the $^{22}Na$ influx into the vesicles decreases with a $K_{0.5}$ for the external $K^+$ concentration of 43 and 32 mM, respectively. The control fluxes and the fluxes in the presence of TTX also decayed with increasing external $K^+$ (not shown). The degree to which the $^{22}Na$ influx is reduced by external $K^+$ was much higher for the toxin-stimulated influx than for control and TTX-blocked influxes. Since the effects of ATX and VER on $^{22}Na$ influx are indirect measurements of the interaction of the toxins with their receptors, it was difficult to establish the contribution of the voltage dependence of ATX binding to its receptor site. The VER stimulated influx also decreased with a raise in the external $K^+$ concentration not withstanding that the effect of VER should increase with depolarization [36,37]. The VER concentration used was saturating (see Fig. 7) so any further increase in VER binding would not become evident from the flux determinations. Linear transformations of the data in Figs. 3A and 3B, did not show any clear deviation from linearity. Further work needs to be done to clarify this point. Determinations of the binding of radiolabeled ATX could give a better insight on the voltage dependence of the interaction of ATX with its receptor site.

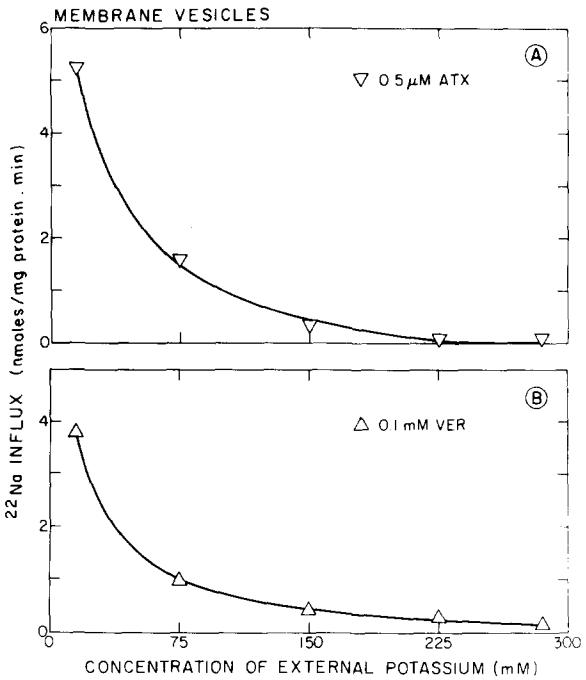


Fig. 3. Effect of external $K^+$ concentration on the net increment of the $^{22}Na$ influx caused by anemone toxin II (ATX) and by veratridine (VER) in vesicles containing 0.3 M $KP_i$. The vesicles were prepared in $KP_i$ internal solution and diluted 20-fold in ChoCl external solution containing different amounts of $KP_i$. External $K^+$ was raised by replacing ChoCl by $KP_i$. In (A) the net increment was calculated from the influx in 0.5 μM ATX minus the influx in the same concentration of ATX plus 1 μM tetrodotoxin (TTX). In (B) the net increment was calculated from the value obtained in 0.1 mM VER minus the influx in the presence of the same VER concentration plus 1 μM TTX. Individual $^{22}Na$ influx values were calculated from the uptake of $^{22}Na$ during the first 30 s after dilution of the membrane vesicles inthe corresponding external solution containing $^{22}Na$.

*Time-course of $^{22}Na$ uptake by the vesicles*

The $^{22}Na$ flux into the vesicles was also followed as a function of time. Figs. 4 and 5 show the effects of ATX and VER on the time-courses of $^{22}Na$ uptake in the presence of the $K^+$ gradient. Fig. 4 shows the time-course in the absence of toxins (control), in the presence of 200 μM VER, and in 200 μM VER plus 1 μM TTX. The $^{22}Na$ influx was followed up to 2 min and it can be observed that the $^{22}Na$ in the vesicles increases as a hyperbolic function of time. Differences between VER and VER plus TTX uptakes are observed even at 15 s, the shortest time tested. Due to the limitations of the method, periods shorter than 15 s gave erratic results. In Fig. 5, the

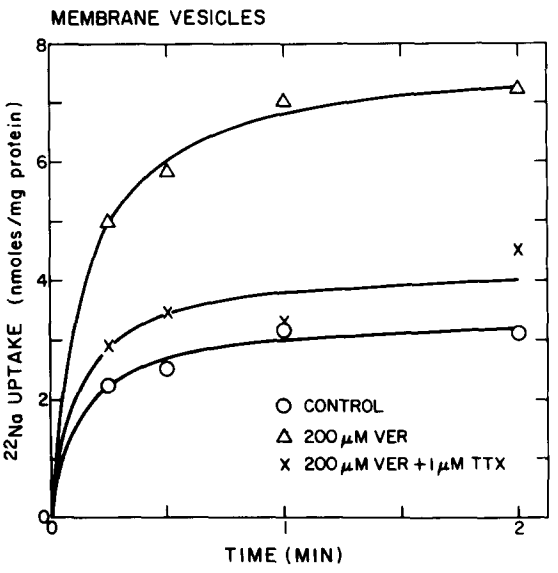


Fig. 4. Time-course of $^{22}$Na uptake by membrane vesicles in the absence of toxins (control), in the presence of veratridine (VER), and in VER plus tetrodotoxin (TTX).

time-course of $^{22}$Na uptake in the absence of toxins (control) and in the presence of 5 μM VER, 0.05 μM ATX, and of 5 μM VER plus 0.05 μM ATX is followed up to 10 min. The difference among fluxes becomes constant from the 2 min uptake on. Depending on the concentration of

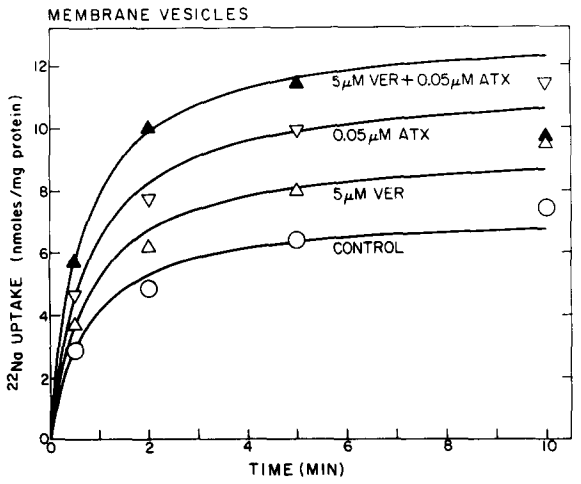


Fig. 5. Time-course of $^{22}$Na uptake by membrane vesicles in the absence of toxins (control), in the presence of veratridine (VER), anemone toxin II (ATX), and of VER plus ATX.

toxins, after the first 10 min the $^{22}$Na inside the vesicles starts to decrease, this being more noticeable for high toxin concentrations or when both toxins are present (see for example the 10 min point of the VER plus ATX curve in Fig. 5). The effect of time may well be explained by the progressive decay of the diffusion potential due to the diffusion of the internal anion and to the dissipation of the $K^+$ concentration gradient, as this ion diffuses out of the vesicles during the $^{22}$Na influx measurement in exchange for external ions. Similar results have been reported by Garty et al. [35] and by Tomiko et al. [21] for $Na^+$ flux determinations under a $Na^+$ gradient.

The $^{22}$Na influx values were calculated from the $^{22}$Na uptake after the first 30 s. At 30 s the $^{22}$Na influx is greater than after longer periods, it is closer to the influx calculated from 15 s uptake and, due to the limitations of the assay, the accuracy in the determinations is greater than for shorter periods. Nevertheless it should be pointed out that even during the first half minute, some efflux plus dissipation of the $K^+$ gradient driving force can affect the uptake determinations.

*Characterization of the action of anemone toxin II and veratridine on the $^{22}$Na influx*

In the initial work carried out on the $Na^+$ channel in isolated lobster nerve plasma membranes [38], it was observed that the rate of $^{22}$Na efflux from preloaded vesicles was increased by veratrine (a mixture of veratrum alkaloids containing veratridine), even when the ionic composition of the internal and external media was the same; the increment caused by veratrine was abolished by the addition of TTX. The internal and external media were artificial sea water, a solution which contains: 450 mM NaCl and 10 mM KCl, plus other salts. Under those conditions, $^{22}$Na efflux is due to passive ion exchange. The action of the toxin was observed through the modification of the rate of efflux rather than the efflux values themselves. In those experiments the difference between the veratrine modified efflux and that in the presence of TTX was small.

In the present work, along with the effect of ATX, the actions of VER and of TTX under the new experimental conditions were also studied. Dose-response curves for ATX, VER, and for

ATX plus VER on $^{22}Na$ influx, are shown in Figs. 6 and 7. The inhibition by TTX (Fig. 8) will be treated in the next section.

Fig. 6A shows that the $^{22}Na$ flux into the membrane vesicles increases from the control value as a hyperbolic function of ATX concentration to become saturated at about 0.5 $\mu$M ATX. The $^{22}Na$ influxes were calculated from 1-min uptakes at different toxin concentrations. The dose-response curve shown was calculated from the Eadie-Hofstee linear transformation shown in the inset of Fig. 6A. From this transformation, the maximum $^{22}Na$ influx was 9.5 nmol/mg protein per min and the $K_{0.5}$ of ATX was 135 nM. Fig. 6B shows the dose-response curve obtained for VER and for VER in the presence of 0.05 $\mu$M ATX under the same experimental conditions. This ATX concentration by itself, as shown in Fig. 6A, had a small effect on $^{22}Na$ influx. As for ATX alone, the $^{22}Na$ influx is a hyperbolic function of VER concentration, either in the presence or in the absence of ATX. The $K_{0.5}$ calculated for VER alone was 20 $\mu$M and the saturated influx, 10.8 nmol/mg protein per min. In the presence of 0.05 $\mu$M ATX the dose-response curve for VER was shifted towards smaller VER concentrations; the calculated $K_{0.5}$ for VER in the presence of 0.05 $\mu$M ATX was 1.5 $\mu$M VER, a 10-fold decrease in the $K_{0.5}$ with respect to that determined with VER alone. The maximum $^{22}Na$ influx was 11.4 nmol/mg protein per min, a value similar to that calculated for VER alone.

In Fig. 7, $^{22}Na$ influx values are plotted against the logarithms of the concentrations of ATX, VER, and of VER in the presence of 0.05 $\mu$M ATX. The influx values were calculated from 30-s uptake

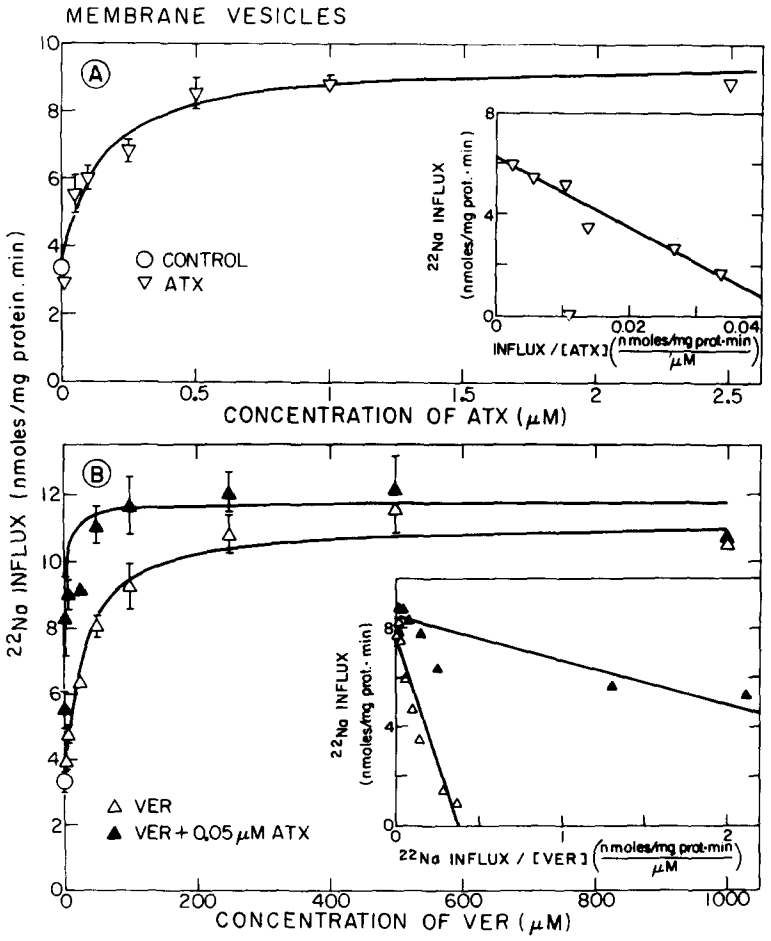


Fig. 6. Initial $^{22}Na$ flux into membrane vesicles as a function of the concentration of toxins, in (A) of anemonotoxin II (ATX), and in (B) of veratridine (VER), and of VER in the presence of ATX. $^{22}Na$ influx values were calculated from the uptake of $^{22}Na$ during the first 60 s after dilution of the vesicles in the external solution. The values are the mean ± S.E. of three experiments. The insets in panels (A) and (B) show the Eadie-Hofstee transformations used to calculate the $K_{0.5}$ values for the toxins given in the text and the best fitting curves of the data exhibited in the main panels.

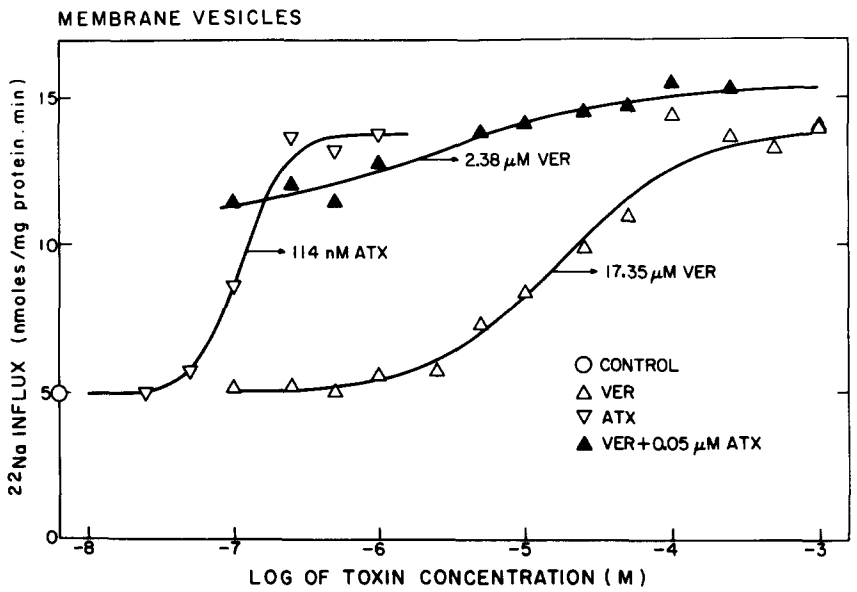


Fig. 7. Dose-response curves for the actions of anemone toxin II (ATX), veratridine (VER), and of VER in the presence of ATX, on the initial $^{22}$Na flux into membrane vesicles. Each $^{22}$Na influx value was calculated from the $^{22}$Na uptake during the first 30 s after dilution of the vesicles in the external solution. The three curves correspond to experiments which were run in parallel with the same preparation of membrane vesicles. The best fitting curves and the $K_{0.5}$ values for ATX, VER, and VER in the presence of ATX given in the figure, were obtained from the linear transformations of the data shown.

measurements. The three curves were run in parallel with the same membrane preparation. The $^{22}$Na influx in the three cases shows a sigmoidal behaviour as a function of the logarithm of the concentrations of the toxins. The effect of ATX is observed at concentrations two orders of magnitude smaller than those of VER required to obtain the same effect. As shown in Fig. 7, the calculated $K_{0.5}$ for VER alone was 17 $\mu$M, and the maximum influx was reached at approx. 100 $\mu$M VER. The ATX dose-response curve is steeper, the $K_{0.5}$ was 114 nM and saturates at the same level as VER. In the presence of 0.05 $\mu$M ATX, the whole VER dose-response curve is shifted towards smaller VER concentrations. The maximum influx level was rapidly achieved and similar to that obtained with VER alone. In the presence of 0.05 $\mu$M ATX, the calculated $K_{0.5}$ for VER was 2.38 $\mu$M.

Though a positive synergistic interaction between ATX and VER would cause an increase in the maximum level of $^{22}$Na influx, this expected increment was not clearly observed in any of the vesicles preparations assayed, and it may be due to the limit imposed by the maximum internal vesicular volume available to $^{22}$Na uptake. Nevertheless, the 7–10-fold decrease in the $K_{0.5}$ for VER caused by the addition of 0.05 $\mu$M ATX suggests that a positive cooperation interaction between ATX and VER does exist at the $Na^+$ channels in this plasma membrane as in other cell and plasma membrane preparations [23,27,39]. BTX which is a more potent toxin than VER but interactive with the same type of receptor-site may help to clarify this point. If the maximum influx level is being limited by the number of receptor sites affected by ATX and/or VER, then BTX should give higher influx values. If the maximum influx is being determined by the internal vesicular volume available, BTX should give a similar maximum influx level as ATX and/or VER and the synergistic interaction between ATX and VER would not be expected to give greater saturation values.

The results described in this section suggest that the receptor sites for ATX and for VER are present in the membrane vesicles. The characteristics of the interaction between the toxins and their receptor sites are similar to those described for

other membrane preparations. The values of $K_{0.5}$ as well as the range of concentrations at which the toxins act are similar to the values previously reported for various excitable cells, both for ATX [39–45] and for VER [44,46–48] (for reviews, see Refs. 22 and 23). It should be noticed, though, that in these vesicles with our experimental conditions, ATX seems to be as potent as VER. An effect of ATX in the absence of other toxins is not a very common observation itself. Nevertheless, ATX has been reported to cause spontaneous firing and membrane depolarization of invertebrate nerve cells [49] and to stimulate Na influx in neuroblastoma cells [39]. The VER effect we see apparently is poor compared to other preparations. This could be due to the limitations imposed by the vesicle preparation as mentioned above.

*Characterization of the action of tetrodotoxin on the $^{22}Na$ influx*

As previously shown in Figures 1, 2 and 4, the $^{22}Na$ influx increase caused by ATX and/or VER can be blocked by TTX. TTX abolishes at least 80% of the $^{22}Na$ influx increment caused by VER and the total increase due to ATX. Since the receptor sites for ATX and TTX are localized on the outer face of the membrane, this suggests that at least 80% of the vesicles are oriented outside-out; the 20% left, which responds only to VER, is most probably oriented inside-out though some unspecific effects by VER might be expected specially at high toxin concentrations due to its high lipid solubility.

The increment of the $^{22}Na$ influx caused by ATX and/or VER, decreases with increasing TTX concentrations as it is shown in panels A, B and C of Fig. 8. On the $y$-axis of each panel the $^{22}Na$ influx in the absence of toxins (control) as well as the $^{22}Na$ influx modified by ATX and/or VER in the absence of TTX are plotted. The insets in each panel show the linear transformations used to calculate the respective square hyperbolic line best fitting the data and the $K_{0.5}$ for TTX.

Fig. 8A shows the inhibition by TTX of the increment in the $^{22}Na$ influx caused by 0.1 mM VER. The $K_{0.5}$ calculated from this curve was 27 nM TTX. In Fig. 8B is shown a similar experiment in which 0.1 $\mu$M ATX was used. The $K_{0.5}$ value calculated from this curve was 13.4 nM TTX. As shown in Fig. 8C, in the presence of 25 $\mu$M VER, 0.1 $\mu$M ATX increases the $^{22}Na$ influx from 10 to 18 nmol/mg protein per min; TTX blocked the $^{22}Na$ influx modified by both toxins with a $K_{0.5}$ of 28 nM. The calculated $K_{0.5}$ values for TTX obtained from curves in which VER was present (with or without ATX) were higher than those obtained with ATX alone. Notice that the $K_{0.5}$ for the effect of TTX on the $Na^+$ channel of intact lobster nerves is 5.1 nM [50], that for the binding of [$^3$H]TTX to the membrane vesicles is 4.2 nM [31], and the $K_{0.5}$ for the blocking action of TTX on the rate of $^{24}Na$ efflux from a similar preparation of membrane vesicles treated with veratrine was 11.9 nM [38]. Differences in apparent $K_d$ values for TTX and in $K_i$ values due to variations in the ionic strength and cation composition of the solutions are expected (see Ref. 51). Apparent $K_d$ values for STX binding to synaptosomal membranes were found to increase with increasing ionic strength and increasing concentrations of monovalent and divalent cations. The $K_{0.5}$ value measured here in the presence of ATX is higher than the $K_{0.5}$ for the block of the ion current in the intact lobster nerve [50] and is similar to that determined in membrane vesicles in a previous work [38]. Also, by stabilizing an open conformation of the channel, VER could be modifying the interaction of TTX with its receptor site. In this respect, BTZ-activated $Na^+$ channels exhibit a voltage-dependent blockage by STX [52,53], a characteristic not apparent in the non-modified channel [53]. A higher $K_{0.5}$ for TTX in the presence of VER and of VER plus ATX, but not with ATX alone has been previously reported for NIE 115 neuroblastoma cells [39].

*II. Proteoliposomes*

*Experimental conditions*

Reconstitution of the $Na^+$ channel by incorporation of detergent-extracted, partially purified membrane particles from lobster nerves into soybean liposomes has been reported earlier [5]. The Na transport function was followed using $^{22}Na$ influx determinations similar to those described in the present work, though in the absence of an ionic gradient. The results revealed that

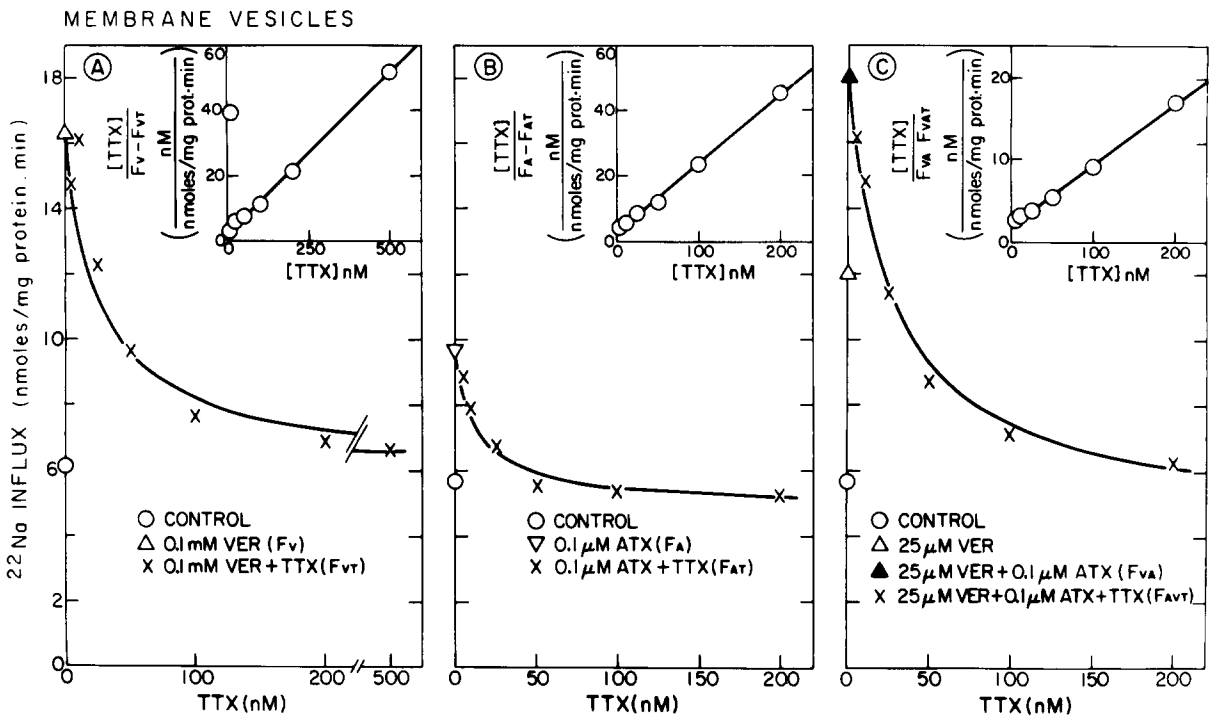


Fig. 8. Inhibition by tetrodotoxin (TTX) of the increment in the initial $^{22}$Na influx caused (A) by veratridine (VER), (B) by anemone toxin II (ATX), and (C) by VER plus ATX. Each influx value was calculated from the $^{22}$Na uptake during the first 30 s after dilution of the vesicles in the external medium containing $^{22}$Na. The inset in each panel shows the linear transformation of the data used to calculate the best fitting curves and the $K_{0.5}$ values for the action of TTX given in the text. $F_V$, $F_A$, and $F_{AV}$ are the influxes in the presence of VER, ATX and VER plus ATX; $F_{VT}$, $F_{AT}$, and $F_{AVT}$ are the values obtained with different concentrations of TTX.

$^{22}$Na influx was increased by VER and the increment abolished by TTX. Thus, the integrity of the receptor sites for these two types of toxins after detergent extraction, partial purification and reconstitution was demonstrated.

In the present work, unfractioned detergent-extracted membrane particles were reconstituted into soybean liposomes using the same technique reported above [5] to explore the characteristics of the ATX receptor after detergent treatment. The effects of VER and of TTX were also studied. As mentioned in Materials and Methods, the toxins were preent in the reconstitution medium, so that the toxin receptor sites of both outside-out and inside-out incorporated $Na^+$ channels would be reached.

Following the same experimental scheme used for the vesicles, the best experimental conditions to study the toxin-modified $^{22}$Na influx were first explored. Fig. 9 shows $^{22}$Na influx determinations carried out in several internal and external media. A point to be discussed later but which is important to mention is that ATX alone had no effect on $^{22}$Na influx in proteoliposomes even at concentrations at high as 10 μM. Only in the presence of VER, high doses of ATX produced an increment of the $^{22}$Na flux into proteoliposomes. An increase of $^{22}$Na influx caused by VER or VER plus ATX was only seen when the outwardly oriented $K^+$ gradient was present. Net $^{22}$Na influx values were also greater under the influence of the $K^+$ gradient. On the other hand, TTX abolished almost all the increment of the toxin-modified flux. It is interesting to point out that since TTX was present in both the internal and external solutions, the $^{22}$Na influx not blocked by TTX is most probably due to unspecific VER effects. Another point worth noticing is that the absolute influx values obtained in the proteoliposomes were greater than the influxes measured in

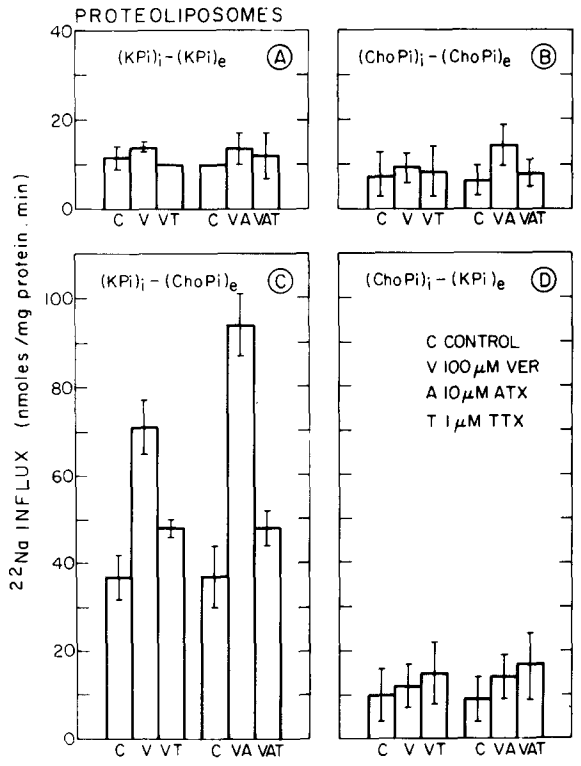


Fig. 9. Initial $^{22}Na$ flux into proteoliposomes in media of different ionic composition and in the absence (control, C) or presence of neurotoxins. The toxins used were veratridine (VER), anemone toxin II (ATX, A), and tetrodotoxin (TTX, T). The ionic composition of the solutions is given in Table I. The proteoliposomes used in the experiments represented in panels (A) and (C) were prepared in 0.3 M $KP_i$ as internal medium (i) and diluted 20-fold in the following external solutions (e): 0.3 M $KP_i$ for (A) and 0.3 M $ChoP_i$ for (C). The internal solution in the experiments shown in panels (B) and (D) was 0.3 M $ChoP_i$ and the corresponding external media were for (B) 0.3 M $ChoP_i$ and for (D) 0.3 M $KP_i$. Each $^{22}Na$ influx value was calculated from the uptake of $^{22}Na$ during the first 15 s after dilution of the proteoliposomes in the corresponding external solution containing $^{22}Na$. Values are the mean ± S.E. of three experiments.

the membrane vesicles. As shown in Fig. 10, the net $^{22}Na$ uptake was greater too.

The comparatively higher $^{22}Na$ flux into the protoeliposomes, even in the controls, may be due to an increment of the total internal volume available for $^{22}Na$ uptake in the proteoliposomes as compared to an equivalent preparation of membrane vesicles. Nevertheless, the ratios of toxin increased $^{22}Na$ influx to TTX-blocked influx were smaller for the proteoliposomes than for the membrane vesicles. The dispersion of the data observed with the proteoliposomes is probably due to the effect of the sonication step during reconstitution, since each tube was sonicated separately.

*Time-course of $^{22}Na$ uptake by proteoliposomes*

Fig. 10 shows a typical experiment where $^{22}Na$ uptake by the proteoliposomes was followed as a function of time. The $^{22}Na$ uptake measured in the absence of toxins (control), in the presence of 500 μM VER plus 1 μM ATX, and the curve obtained when in addition to VER and ATX, 1 μM TTX was present, are shown. The $^{22}Na$ uptake was followed up to 1 min. $^{22}Na$ uptake by the proteoliposomes tends to saturate at shorter time periods than in the vesicle preparation. This is probably due to a large population of small proteoliposomes. On the other hand, the curves of the toxin-increased and the TTX-blocked uptakes tend to converge with time giving influx ratios which were smaller for longer uptake periods. $^{22}Na$ influx determinations were thus done at 15 s, the shortest time allowed by the method. Again, worth mentioning is the magnitude of the $^{22}Na$ uptake by the stimulated and unstimulated proteoliposomes. The uptake is at least three times greater than that observed in membrane vesicles (see for comparison Fig. 4).

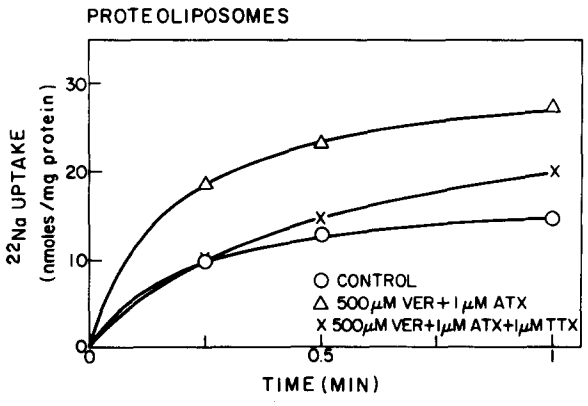


Fig. 10. Time-course of $^{22}Na$ uptake by proteoliposomes in the absence of toxins (control), in the presence of veratridine (VER) plus anemone toxin II (ATX), and with the same concentration of VER and ATX plus tetrodotoxin (TTX).

*Characterization of the action of anemone toxin II and veratridine on the $^{22}Na$ flux into proteoliposomes*

As previously mentioned, concentrations of ATX alone as high as 1 $\mu$M and 10 $\mu$M were tested and no significant increase of the $^{22}Na$ influx was observed. Only in the presence of VER did ATX have an effect but high concentrations of ATX were required. Fig. 11 shows dose-response curves for VER in the absence and in the presence of 1 $\mu$M ATX and 10 $\mu$M ATX. The $^{22}Na$ influx in the absence of toxins (control) is drawn on the $y$-axis. In the absence of VER neither concentration of ATX modified the control level of $^{22}Na$ influx, as indicated by the common intercept of the three curves. These curves tend to saturate at around 100 $\mu$M VER. The maximum influx level though was different for each curve increasing with the ATX concentration. Eadie-Hofstee linear transformations of these curves gave $K_{0.5}$ values of 52 $\mu$M for VER, 16.5 $\mu$M for VER plus 1 $\mu$M ATX, and 20 $\mu$M for VER plus 10 $\mu$M ATX. It appears, that the decrease in $K_{0.5}$ for VER caused by ATX was not proportional to the concentration of ATX. A concentration 10-times higher at ATX gave a $K_{0.5}$ similar or even greater than the value determined in the presence of 1 $\mu$M ATX. However, the dispersion of the data introduced some uncertainty on the $K_{0.5}$ values. Nevertheless, in the presence of ATX there seems to be a tendency to diminish the concentration of VER required to produce 50% of its effect. The results described here could indicate a loss of affinity of the ATX receptor site due to the detergent treatment and/or the reconstitution method. In experiments not shown, untreated fragments of membrane were incorporated into soybean liposomes using the same freeze-thaw-sonication reconstitution technique. In those experiments, ATX did modify the $^{22}Na$ flux into the proteoliposomes but higher concentrations of the toxin were required. Nevertheless, the concentrations of ATX used were not as high as those needed to observe any effct with the proteoliposomes formed with detergent-extracted membrane particles. From those results it is possible to conclude that both the detergent-treatment and the reconstitution method may be altering the affinity of the ATX receptor site. Since the action of VER was not greatly altered, as judged by the calculated $K_{0.5}$ value and by the range of concentrations at which it acts, the receptor site for this toxin must be better preserved during the whole process. The effect of ATX in the presence of VER might be a consequence of the cooperative interaction between these two toxins, VER increasing the otherwise low affinity for ATX.

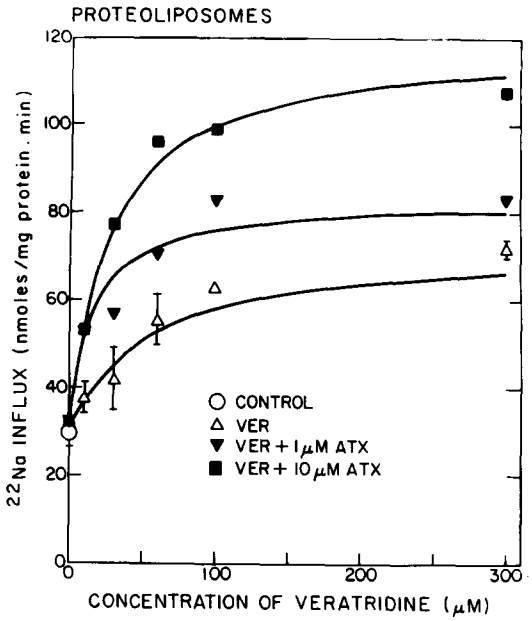


Fig. 11. Dose-response curves for the actions of veratridine (VER) alone, and VER plus two different concentrations of anemone toxin II (ATX), on the initial $^{22}Na$ influx into proteoliposomes. The influx in the absence of toxins (control) is also represented. Each $^{22}Na$ influx value was calculated from the $^{22}Na$ uptake during the first 15 s after dilution of the proteoliposomes in the external solution containing $^{22}Na$. The best fitting curves and the $K_{0.5}$ values given in the text were obtained from the linear transformations of the data shown.

Evidences of loss of affinity of the receptor site for polypeptide toxins after detergent treatment and reconstitution have been reported in preparations of purified $Na^+$ channels from other membrane sources [16,17]. In all reports, the reconstitution of the functional properties of the receptor sites for the VER-like toxins and for TTX and STX, was achieved. The receptor site for the $\alpha$-polypeptide toxins suffers modifications which can be detected from a total loss of the toxin effect to a significant diminution of the toxin-binding capability. Loss of affinity of the receptor site seems to be the most suitable explanation.

*Characterization of the action of tetrodotoxin on the $^{22}Na$ flux into proteoliposomes*

The increment of $^{22}Na$ flux into the proteoliposomes caused by VER and VER plus ATX was blocked by TTX. As mentioned previously, a total blockage by TTX was not achieved. There was always a fraction of the $^{22}Na$ influx increment insensitive to TTX, maybe due to an unspecific action of VER. The curves shown in panels A and B of Fig. 12, describe the effect of increasing the TTX concentrations on the $^{22}Na$ influx caused by 0.1 mM VER, and by 0.1 mM VER plus 10 $\mu$M ATX, respectively. The control $^{22}Na$ influx is also shown. In the insets, the linear transformations of the curves described by the experimental data are exhibited. The points corresponding to 250 nM TTX in both cases were excluded from the calculation since they obviously deviate from the general tendency. The calculated $K_{0.5}$ values were 13 nM TTX for the increment of the $^{22}Na$ influx caused by VER alone (Fig. 12A) and 14 nM TTX for VER plus 10 $\mu$M ATX (Fig. 12B). In both cases, saturating concentrations of TTX inhibited about 85% of the toxin-stimulated $^{22}Na$ influx. In contrast to what had been observed in the vesicle preparations, the $K_{0.5}$ values for TTX in the proteoliposomes treated with VER are closer to the values reported previously by our laboratory for the 50% blockage in intact nerves [50], for the binding of [$^3$H]TTX to membrane vesicles [31] and for the blockage of the VER-stimulated $^{24}Na$ efflux from membrane vesicles [38].

In conclusion, an outwardly directed $K^+$ gradient across membrane vesicles and proteoliposomes seems to be a helpful tool in determining toxin-modified ion fluxes through the $Na^+$ channel. Membranes isolated from the lobster walking-leg nerves have receptor sites for ATX, VER, and TTX, as evidenced by the toxin-modified $^{22}Na$ influx. ATX, VER, and TTX modified the rate of $^{22}Na$ uptake into the lobster nerve membrane vesicles with characteristics similar to those described previously in intact excitable cells. Detergent-treatment of lobster nerve plasma membrane followed by incorporation of the extracted membrane particles into liposomes seems to affect the receptor-site for ATX, leaving the VER and the TTX receptors essentially unaffected. The characteristics of the interaction of the ATX with its receptor site described in the present work may help to preserve the polypeptide toxin receptor site in further attempts of isolation, purification and reconstitution.

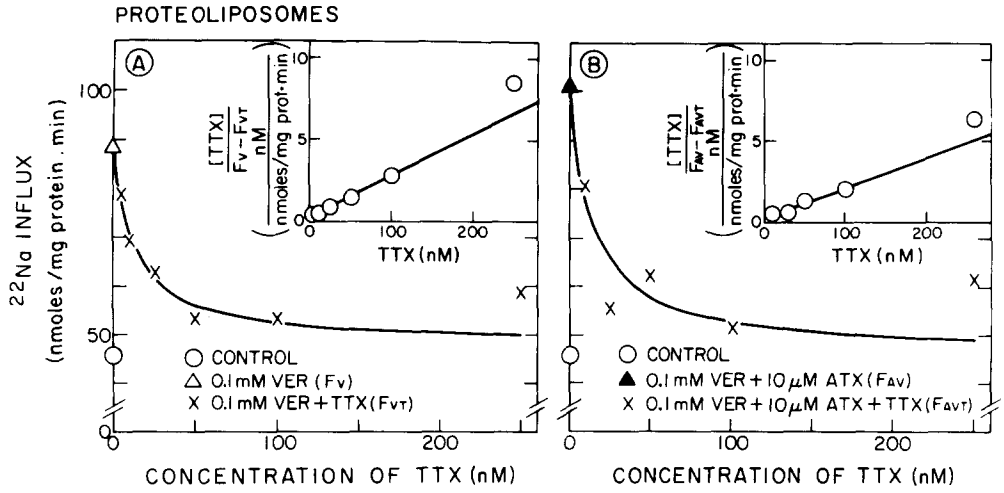


Fig. 12. Inhibition by tetrodotoxin (TTX) of the increment in the initial $^{22}Na$ influx caused (A) by veratridine (VER), and (B) by VER plus anemone toxin II (ATX). Each influx value was calculated from the $^{22}Na$ uptake during the first 15 s after dilution of the proteoliposomes in the corresponding external medium containing $^{22}Na$. The inset in each panel shows the linear transformation of the data used to calculate the best fitting curves and the $K_{0.5}$ values for the action of TTX given in the text. $F_V$ and $F_{AV}$ and the influxes in the presence of VER and of VER plus ATX; $F_{VT}$ and $F_{AVT}$ are the values obtained with different concentrations of TTX.

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