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ION SELECTIVITY OF THE NERVE MEMBRANE SODIUM CHANNEL INCORPORATED INTO LIPOSOMES

MADALINA CONDRESCU and RAIMUNDO VILLEGAS

Centro de Biofisica y Bioquímica, Instituto Venezolano de Investigaciones Cientificas (IVIC) Apartado 1827, Caracas 1010A (Venezuela)

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Tetrodotoxin-sensitive sodium channels of lobster nerve membranes were incorporated into soybean liposomes by the freeze-thaw-sonication procedure and their ionic selectivity was studied. Veratridine and grayanotoxin-I were used to activate the sodium channels and the increment of the ionic flux through them was specifically abolished by tetrodotoxin. The drug-sensitive $^{22}Na^+$, $^{42}K^+$, $^{86}Rb^+$ and $^{137}Cs^+$ influxes were measured. The permeability ratios calculated directly from ion fluxes showed that the channels preferably allow the passage of Na^+. No anion influx ([^{32}P]phosphate, [^{35}S]sulfate, ^{36}Cl) sensitive to the drugs was observed. The data reveal that the sodium channels incorporated into liposomes remain cation-selective and discriminate among different cations.

Introduction

In contrast with the great deal of electrophysiological information available on the operation of the Na^+ channel present in excitable membranes, little is known about its nature and organization at the molecular level. Data obtained by studying the effect of various drugs on the channel greatly contributed to build up the conception that we now have of it. One group of neurotoxins has been shown to activate and another to enhance the persistent activation of the action potential Na^+ ionophore, in both axons and neuroblastoma cells. The first group is formed by the lipid-soluble polycyclic compounds aconitine, batrachotoxin, grayanotoxin and veratridine, and the second by the polypeptide toxins from scorpion and anemone venoms [1–9]. The increment of Na^+ flux caused by both groups of toxins is specifically abolished by the water-soluble heterocyclic guanidines tetrodotoxin and saxitoxin, which block the Na^+ channel and by the same mechanism inhibit the action potential Na^+ current component [5,6,10, 11].

In a preliminary attempt to isolate and to determine the structure and function of the individual Na^+ channel, progress has been made using the toxins, particularly tetrodotoxin, as specific labels of the Na^+ channel. For example, tetrodotoxin receptors had been demonstrated in isolated axonal membranes [21,22], and the solubilization and partial purification of the receptors with detergents has been achieved [13–16]. Since preservation of tetrodotoxin binding does not necessarily imply that the whole Na^+ channel is present, reconstitution have been introduced to study the Na^+ channel. Reconstitution offers the possibility of testing biological activity during the process of isolation and purification of the channel. As a preliminary step, the presence of Na^+ channels in plasma membrane fragments isolated from lobster nerve was demonstrated [12] and recently, the membrane fragments have been incorporated into soybean liposomes [17]. The $^{22}Na^+$ flux into

the reconstituted vesicles was increased by veratridine, grayanotoxin-I, batrachotoxin or aconitine, and this increment was blocked by tetrodotoxin or saxitoxin in nanomolar concentrations [18]. This communication deals with the ionic selectivity of this tetrodotoxin-sensitive Na^+ channel incorporated into soybean liposomes and activated by veratridine or grayanotoxin-I.

Materials and Methods

Isolation of nerve plasma membranes

Total plasma membranes were obtained from lobster *Panulirus argus* walking leg nerves. A detailed description of the procedure has been published elsewhere [21]. The isolated membranes were suspended in 0.78 M sucrose/10 mM Tris-HCl (pH 7.5), frozen in solid CO_2-acetone and stored at −80°C, without loss of activity for at least 1 month. The protein content [23] of the membrane suspensions was around 10 mg per ml.

Preparation of liposomes

Crude soybean lipids were suspended (60 mg/ml) in a phosphate solution (pH 7.5) containing equal concentrations of Na^+, K^+ and Rb^+ (100 mequiv./l) or in a sulfate solution (made up in 10 mM Tris-HCl, pH 7.5) containing 75 mequiv./l each of Na^+, K^+, Rb^+ and Cs^+, unless stated otherwise in the legends to the figures. The lipids were sonicated to clarity under nitrogen in a bath-type sonicator (Model T 80-80-1-RS, Laboratory Supplies, Hicksville, NY).

Reconstitution

In order to incorporate sodium channels into liposomes we used the freeze-thaw-sonication procedure [24] as described [17]. The thawed membranes were added to the preformed liposomes at a final protein concentration of 0.5 mg/ml and a soybean lipid concentration of 40 mg/ml. The lipid:protein ratio was 80:1. Aliquots of the lipid/membrane mixture (0.4 ml) were distributed in three different test tubes: control (no drugs) or containing veratridine (or grayanotoxin-I) and veratridine (or grayanotoxin-I) plus tetrodotoxin. The ethanol of the control tube or that in which veratridine (or grayanotoxin-I) were dissolved was dried with nitrogen before tetrodotoxin (100 nM) was added from a stock solution made up in 10 mM acetic acid. The same volume of 10 mM acetic acid was added to the tetrodotoxin-free samples. The mixtures were frozen in solid CO_2-acetone for 1 min, thawed during 1–1.5 min in ice-cold water and sonicated for 15 s. All procedures were carried out under nitrogen, close to 0°C.

Determination of ion fluxes

In order to measure both cation ($^{22}Na^+$, $^{42}K^+$, $^{86}Rb^+$, or $^{137}Cs^+$) or anion ([^{32}P]phosphate, [^{35}S]sulfate or $^{36}Cl^-$) influxes to the reconstituted liposomes, the assay was initiated by adding 0.2 μCi of each labeled ion in 20 μl of the same solution in which the vesicles were reconstituted, to 200 μl of reconstituted liposomes. After incubation for 30 s at 0°C, the ion influx was measured in 175 μl of sample, by the method of Gasko et al. as previously described [25].

Materials

Chemicals were obtained from the following sources: crude soybean lipids (asolectin) were from Concentrated Associates, Woodside, NY. Veratridine was purified in our laboratory from veratrine (Sigma, St. Louis, MO) as described [26]. Grayanotoxin-I was kindly supplied by Dr. J. Daly from NIH, Bethesda. Tetrodotoxin (citrate-free) was purchased from Sankyo Ltd., Tokyo. Bovine serum albumin was obtained from Sigma, St. Louis, MO and ion exchange resins Dowex 50W-X8 (50–100 mesh, H^+ form) and AG1-X8 (50–100 mesh, Cl^- form) were from Bio-Rad, Richmond, CA, $^{22}Na^+$, $^{86}Rb^+$, $^{137}Cs^+$, $^{36}Cl^-$, [^{32}P]phosphate and [^{35}S]sulfate were from New England Nuclear, Boston, MA. $^{42}K^+$ was produced and obtained from the Nuclear Reactor, RV-I of IVIC. Instagel (Packard Instrument Co, Downers Grove, IL) was used in scintillation counting. All other reagents were of the purest commercially available grade.

Results and Discussion

In nerve cells the lipid-soluble polycyclic compounds veratridine, aconitine, grayanotoxin and batrachotoxin act on a common receptor site to activate the sodium channel [5,6]. When treated with these drugs the ion selectivity of the channel

is diminished [31–35]. On the other hand, tetrodotoxin and saxitoxin compete for a receptor site which seems to be different from the voltage-sensitive components of the channel, and specifically block the ion flux [5,6,10,11].

In reconstitution experiments which have permitted the incorporation of Na^+ channels into soybean liposomes, the effects of the lipid-soluble toxins and tetrodotoxin on the Na^+ influx into the reconstituted vesicles were investigated [17,18,20]. The results revealed that veratridine, grayanotoxin-I, batrachotoxin and aconitine acted in a fashion similar to their mode of action in nerve or in excitable cells in culture, i.e., when added to the reconstituted vesicles they activate the Na^+ channel, even in the absence of an imposed electrochemical gradient. The increment of the $^{22}Na^+$ flux caused by these drugs was specifically blocked by tetrodotoxin [18].

In the present work we first investigated the solutions most suitable for use in the preparation of the reconstituted vesicles and for the selectivity measurements to be carried out. As shown in Fig. 1, when the vesicles were prepared in a phosphate or sulfate solution, veratridine (0.5 mM) stimulated up to about 3-fold the $^{22}Na^+$ flux into the vesicles, and tetrodotoxin (100 nM) abolished this stimulation. When chloride or nitrate was used, no effect of the drugs on the $^{22}Na^+$ influx could be observed. If phosphate or sulfate was only partially replaced by chloride (up to 10 mM) we were still able to observe the stimulation of the $^{22}Na^+$ influx by veratridine and the abolition of the effect by tetrodotoxin.

These results suggested that the presence of a presumably impermeant anion is required in order to observe the effect of the drugs on the $^{22}Na^+$ influx. This requirement is most likely imposed by the use of a slow Dowex column assay. Under these conditions, $^{22}Na^+$ efflux is expected to occur while the vesicles are washed through the column, if a relatively permeant counter anion as chloride is present. This efflux would give the false impression that no stimulation of the $^{22}Na^+$ influx by veratridine had taken place.

On the other hand, when relatively impermeant counter anions such as phosphate or sulfate were used, less $^{22}Na^+$ efflux probably occurred while the vesicles were on the Dowex column, allowing the stimulation of the $^{22}Na^+$ influx by veratridine to be detected.

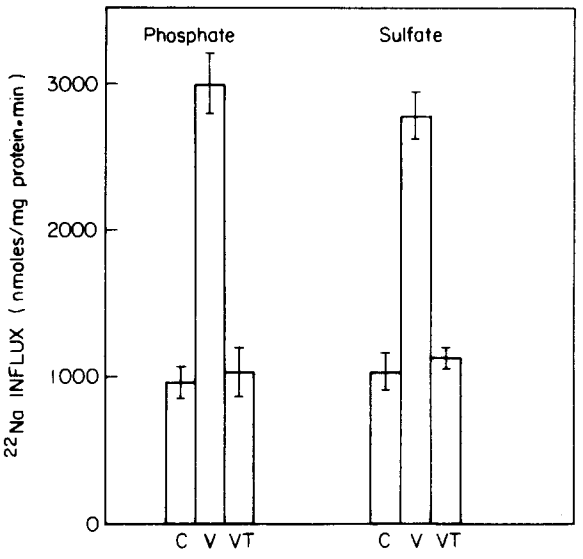

Fig. 1. $^{22}Na^+$ flux into reconstituted vesicles prepared in media of different anionic composition. The vesicles were reconstituted with nerve membrane (0.5 mg/ml of membrane protein) and soybean lipids (40 mg/ml), in solutions containing in addition to 300 mequiv./l of each anion indicated in the figure, 150 mequiv. Na^+/l, 150 mequiv. K^+/l and 10 mM Tris-HCl (pH 7.5). Values of the influx in the absence of drugs (control, C), in the presence of 0.5 mM veratridine (V), and in the presence of 0.5 mM veratridine plus 100 nM tetrodotoxin (VT) are shown. Influx measurements were made as indicated in Methods, 30 s after addition of $^{22}Na^+$-labeled solution to the suspension of vesicles.

In order to investigate whether the Na^+ channel incorporated into artificial liposomes was still cation-selective, we studied if veratridine and veratridine plus tetrodotoxin do affect the fluxes of anions ([^{32}P]phosphate, [^{35}S]sulfate and $^{36}Cl^-$) into the reconstituted vesicles.

It is illustrated in Fig. 2 that even when the $^{22}Na^+$ flux into the reconstituted vesicles was incremented by 0.5 mM veratridine and this increment was abolished by 100 nM tetrodotoxin, no effect on the [^{32}P]phosphate, [^{35}S]sulfate and $^{36}Cl^-$ influxes was produced by these drugs. $^{22}Na^+$, [^{32}P]phosphate and $^{36}Cl^-$ influxes were measured in one preparation (vesicles reconstituted in phosphate containing 140 mequiv. Na^+/l, 150 mequiv. K^+/l, 10 mM Nacl, 10 mm Tris-HCl, pH 7.5) while $^{22}Na^+$, [^{35}S]sulfate and $^{36}Cl^-$ influxes were

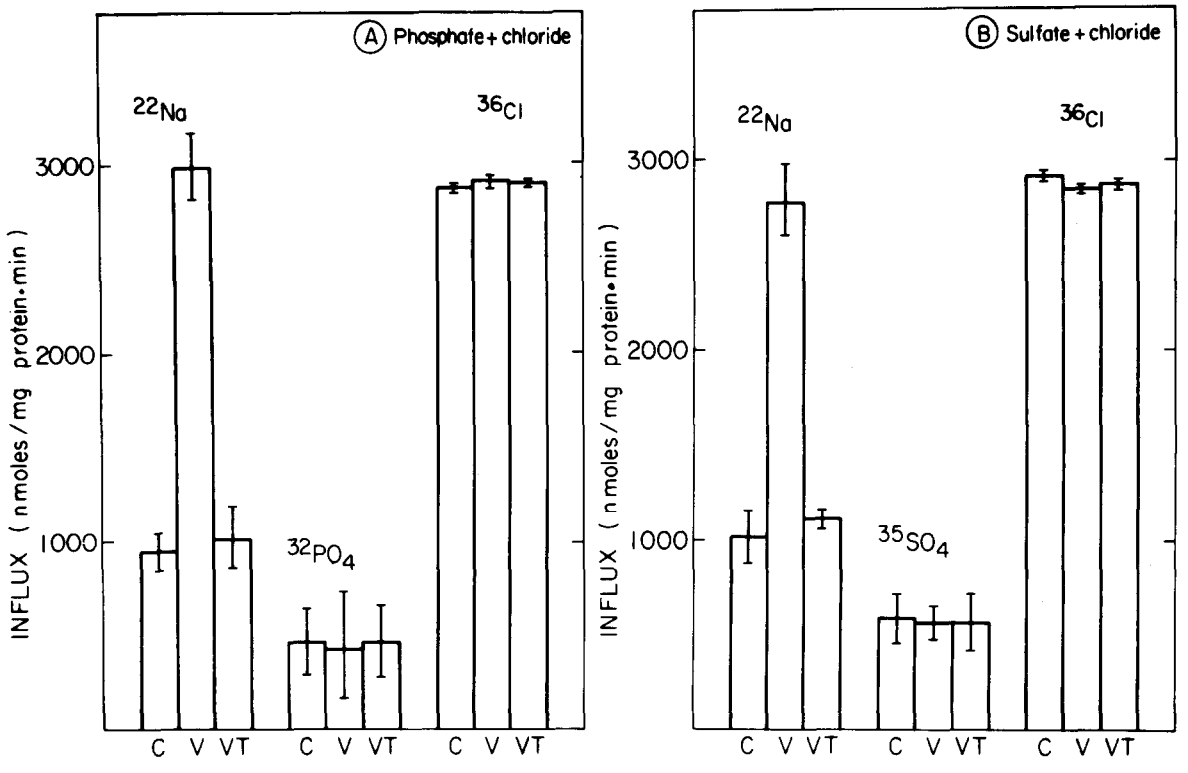

Fig. 2. Veratridine and tetrodotoxin effect on the $^{22}Na^+$, $^{32}PO_4^{3-}$, $^{35}SO_4^{2-}$ and $^{36}Cl^-$ fluxes into vesicles reconstituted with nerve membrane (0.5 mg/ml of membrane protein) and soybean lipids (40 mg/ml). In each case, the influx was measured in the absence of drugs (control, C), in the presence of 0.5 mM veratridine (V), and in the presence of 0.5 mM veratridine plus 100 nM tetrodotoxin (VT). The media in which the vesicles were reconstituted contained 290 mequiv./l of either phosphate or sulfate plus 140 mequiv. Na^+/l, 150 mequiv. K^+/l, 10 mM NaCl and 10 mM Tris-HCl (pH 7.5). Influx measurements were made as indicated in Methods, 30 s after addition of the respective radioactively labeled solution to the suspension of reconstituted vesicles.

measured in another preparation (vesicles reconstituted in sulfate containing 140 mequiv. Na^+/l, 150 mequiv. K^+/l, 10 mM NaCl, 10 mM Tris-HCl, pH 7.5).

However, the possibility that the lack of effect of veratridine on the $^{36}Cl^-$ flux could be an artifact of the Dowex column assay exists, since $^{36}Cl^-$ flux has been measured in the presence of relatively permeant cations, such as Na^+ and K^+. In order to eliminate this possibility we measured $^{36}Cl^-$ influx to vesicles reconstituted in 300 mM choline chloride/10 mM Tris-HCl (pH 7.5). No effect of drugs on the $^{36}Cl^-$ influx has been detected under these conditions.

The conclusion is that neither phosphate, sulfate nor chloride passes through the Na^+ channel activated by veratridine. It is worth mentioning that all the assayed anions have hydrated radii smaller than the estimated radius of the Na^+ channel, which means that there is only a charge impediment for them to cross the channel.

In order to quantify the selectivity of the Na^+ channel incorporated into liposomes, the $^{22}Na^+$, $^{42}K^+$, $^{86}Rb^+$ and $^{137}Cs^+$ fluxes into the reconstituted vesicles were measured under control conditions (no drugs), in the presence of veratridine (or grayanotoxin-I), and in the presence of veratridine (or grayanotoxin-I) plus tetrodotoxin. To estimate the permeability ratios directly from measurements of ion fluxes, the reconstituted vesicles were prepared in solutions in which the concentrations of all cations were identical. In the present experiments veratridine and grayanotoxin-I activated the Na^+ channels in the absence of an

imposed electrochemical gradient since, except for the radioactive label, the ion composition of the medium inside and outside the reconstituted vesicles was identical. We actually measured the isotope exchange across the Na^+ channel.

In Fig. 3 are shown the differences observed between the veratridine-stimulated, tetrodotoxin-inhibitable $^{22}Na^+$, $^{42}K^+$, $^{86}Rb^+$ and $^{137}Cs^+$ influxes in reconstituted vesicles prepared either in sodium, potassium, rubidium phosphate (Fig. 3A) or in sodium, potassium, rubidium, caesium sulfate (Fig. 3B). The fluxes of two or more ions were measured in the same preparation of reconstituted vesicles. No $^{137}Cs^+$ influx could be measured when the vesicles were prepared in phosphate, since caesium phosphate precipitated.

The results reveal that the cation influx sequence through the Na^+ channel incorporated into liposomes and activated by veratridine (0.5 mM) is $Na > K \geqslant Rb > Cs$. From these influx data, we calculated the permeability ratios for the Na^+ channel incorporated into liposomes. In the experiments in which the vesicles were reconstituted in phosphate, the K^+/Na^+ and Rb^+/Na^+ permeability ratios were 0.47 ± 0.10 (mean $\pm$ S.E.; $n = 8$) and 0.55 ± 0.11 ($n = 6$), respectively. In the experiments in which the vesicles were reconstituted in sulfate, the K^+/Na^+, Rb^+/Na^+ and Cs^+/Na^+ permeability ratios were 0.44 ± 0.14 ($n = 6$), 0.22 ± 0.09 ($n = 8$) and 0.07 ± 0.10 ($n = 5$), respectively.

Recently Malysheva et al. [20] measured the selectivity of the cholate-solubilized Na^+ channel from brain plasma membranes which have been incorporated into liposomes. They claimed that neither veratrine nor tetrodotoxin changed the Rb^+ fluxes. The result disagrees with our findings since in the present experiments we observe Rb^+ fluxes sensitive to the drugs, as is also the case of the intact axons. Since in the present work we did not use detergents, this difference in methodology could account for this disagreement.

Essentially identical results were obtained when grayanotoxin-I (150 μM) was used to activate the Na^+ channel instead of veratridine. The influx of $^{22}Na^+$, $^{42}K^+$, $^{86}Rb^+$ and $^{137}Cs^+$ was measured and the results are shown in Figs. 4A and 4B. The cation influx through the Na channel activated by

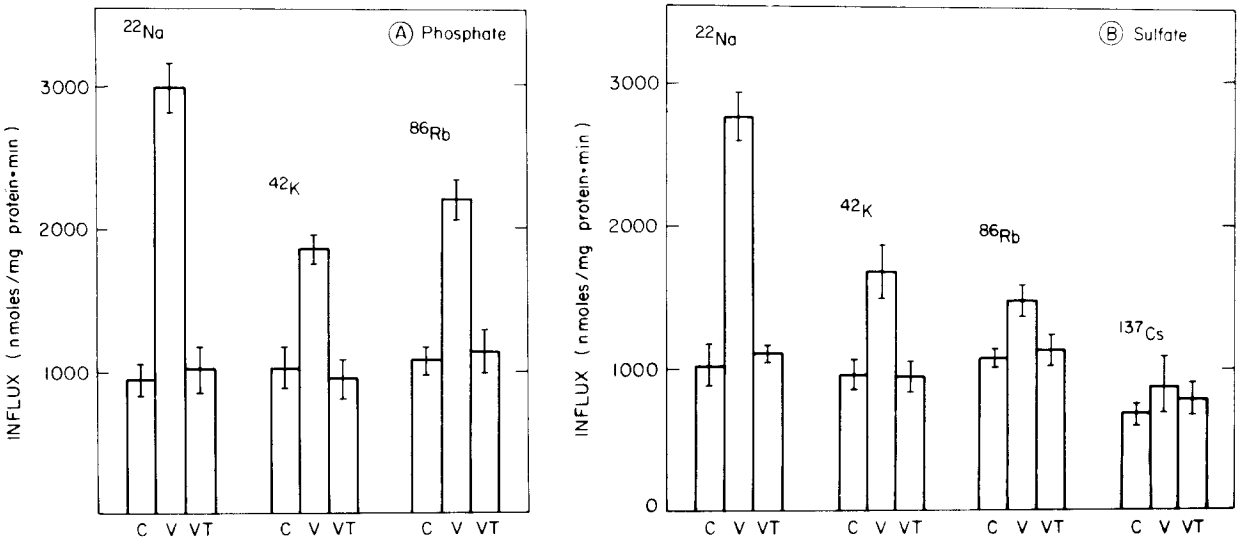

Fig. 3. Tetrodotoxin-sensitive fluxes of different cations induced by veratridine. (A) $^{22}Na^+$, $^{42}K^+$ and $^{86}Rb^+$ fluxes into reconstituted vesicles prepared in a solution containing 300 mequiv./l phosphate (pH 7.5) and 100 mequiv./l each of Na^+, K^+ and Rb^+. (B) $^{22}Na^+$, $^{42}K^+$, $^{86}Rb^+$ and $^{137}Cs^+$ fluxes into vesicles prepared in a solution containing 300 mequiv./l of sulfate (pH 7.5) and 75 mequiv./l of each Na^+, K^+, Rb^+ and Cs^+. The vesicles were reconstituted with nerve membrane (0.5 mg/ml of membrane protein) and soybean lipids (40 mg/ml). For each cation the influx was measured in the absence of drugs (control, C), in the presence of 0.5 mM veratridine (V), and in the presence of 0.5 mM veratridine plus 100 nM tetrodotoxin (VT). The influxes were measured as indicated in Methods, 30 s after addition of the respective radioactively labeled solution to the suspension of reconstituted vesicles.

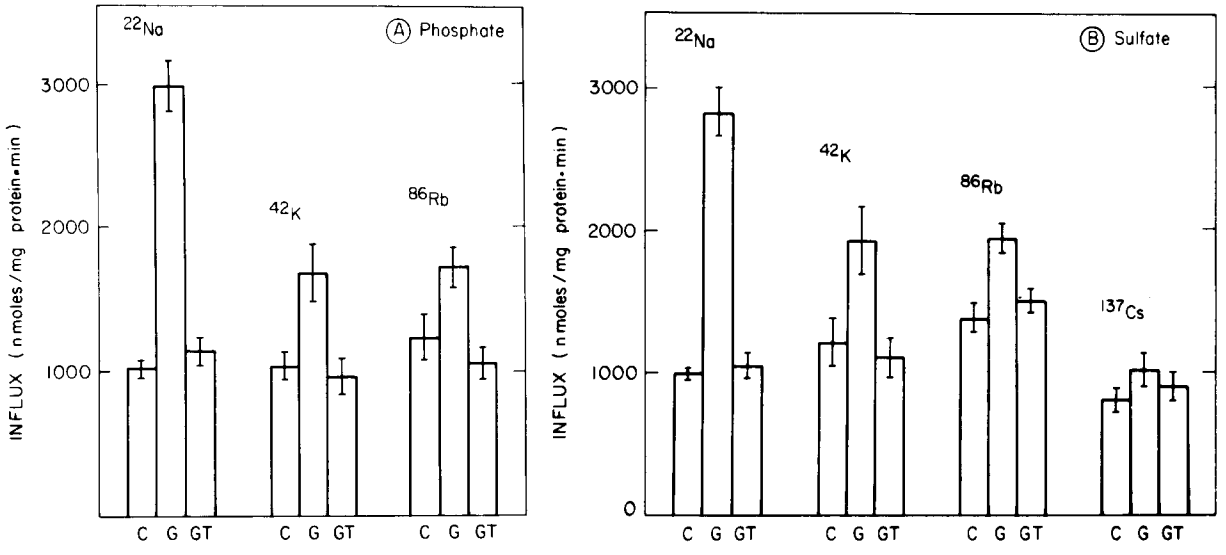

Fig. 4. Tetrodotoxin-sensitive fluxes of different cations induced by grayanotoxin-I. (A) $^{22}Na^+$, $^{42}K^+$ and $^{86}Rb^+$ fluxes into reconstituted vesicles prepared in a solution containing 300 mequiv./l phosphate (pH 7.5) and 100 mequiv./l each of Na^+, K^+ and Rb^+. (B) $^{22}Na^+$, $^{42}K^+$, $^{86}Rb^+$ and $^{137}Cs^+$ fluxes into vesicles prepared in a solution containing 300 mequiv./l of sulfate (pH 7.5) and 75 mequiv./l of each Na^+, K^+, Rb^+ and Cs^+. The vesicles were reconstituted with nerve membrane (0.5 mg/ml of membrane protein) and soybean lipids (40 mg/ml). For each cation the influx was measured in the absence of drugs (control, C), in the presence of 150 μM grayanotoxin-I (G), and in the presence of 150 μM grayanotoxin-I plus 100 nM tetrodotoxin (GT). The influxes were measured as indicated in Methods, 30 s after addition of the respective radioactively labeled solution to the suspension of reconstituted vesicles.

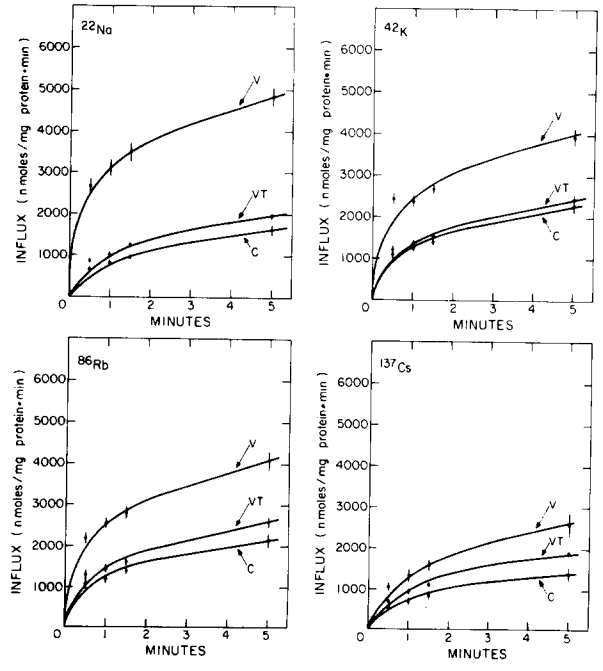

Fig. 5. $^{22}Na^+$, $^{42}K^+$, $^{86}Rb^+$ and $^{137}Cs^+$ fluxes into reconstituted vesicles as a function of time. The vesicles were reconstituted in a solution containing 300 mequiv./l of sulfate (pH 7.5), and 75 mequiv./l of each Na^+, K^+, Rb^+ and Cs^+. The reconstituted vesicles were prepared with nerve membrane (0.5 mg/ml of membrane protein) and soybean lipids (40 mg/ml). The influx of each cation was measured in the absence of drugs (C), in the presence of 0.5 mM veratridine (V) and in the presence of 0.5 mM veratridine plus 100 nM tetrodotoxin (VT). Influx measurements were made as indicated in Methods, at the times indicated in the figure, after addition of the respective radioactively labeled solution to the suspension of reconstituted vesicles.

grayanotoxin-I is represented by the grayanotoxin-I stimulated, tetrodotoxin inhibitable flux. In those experiments in which the vesicles were reconstituted in phosphate, the K^+/Na^+ and Rb^+/Na^+ permeability ratios were 0.40 ± 0.16 ($n = 7$) and 0.38 ± 0.16 ($n = 5$), while when sulfate was used as a medium for reconstitution the K^+/Na^+, Rb^+/Na^+ and Cs^+/Na^+ permeability ratios were 0.46 ± 0.17 ($n = 6$), 0.25 ± 0.08 ($n = 8$) and 0.07 ± 0.10 ($n = 2$), respectively.

When the $^{22}Na^+$, $^{42}K^+$, $^{86}Rb^+$ and $^{137}Cs^+$ influxes were measured under control conditions, in the presence of 0.5 mM veratridine and in the

presence of 0.5 mM veratridine plus 100 nM tetrodotoxin over a 5 min period, the same differences among the various cation fluxes across the Na^+ channel were observed, as illustrated in Fig. 5.

The data reveal that the Na^+ channel incorporated into artificial liposomes is able to discriminate between Na^+ and the other monovalent cations. The permeability ratios (P_X/P_{Na}) that we obtained for the Na^+ channel incorporated into liposomes when activated by veratridine or grayanotoxin-I are similar to those measured in intact nerves for the Na^+ channel activated by batrachotoxin, aconitine or grayanotoxin [31–35]. However, the ratios are higher than those reported for the intact nerve Na^+ channel electrically activated [27–30]. Therefore, these drugs seem to modify the selectivity filter in addition to the gating mechanism. Using veratridine or grayanotoxin-I, the Na^+ channel incorporated into soybean liposomes is only about one half as permeable to K^+ or Rb^+ as to Na^+, and about one tenth as permeable to Cs^+ as to Na^+. No significant difference between the permeability to K^+ and Rb^+ could be observed.

It would be of interest in the future to apply a potential gradient across the membrane of the reconstituted vesicles and to investigate the selectivity of the Na^+ channel activated by the polypeptide toxins, binding of which to the channel seems to be voltage-dependent [6,9].

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