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Reconstitution of Sodium Channels in Large Liposomes Formed by the Addition of Acidic Phospholipids and Freeze-Thaw Sonication

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Summary. Phosphatidylcholine (PC) alone or with phosphatidylethanolamine (PE) are sufficient for the reconstitution of Na^+ channels in planar lipid bilayers. However, when Na^+ channels were first reconstituted into liposomes using the freeze-thaw-sonication method, addition of acidic phospholipids, such as phosphatidylserine (PS), to the neutral phospholipids was necessary to obtain a significant toxin-modulated ^{22}Na uptake. To further investigate the acidic phospholipid effect on reconstitution into liposomes, Na^+ channels purified from *Electrophorus electricus* electrocytes were reconstituted into liposomes of different composition by freeze-thaw sonication and the effect of batrachotoxin and tetrodotoxin on the ^{22}Na flux was measured. The results revealed that, under our experimental conditions, the presence of an acidic phospholipid was also necessary to obtain a significant neurotoxin-modulated ^{22}Na influx. Though neurotoxin-modulated ^{22}Na fluxes have been reported in proteoliposomes made with purified Na^+ channels and PC alone, the ^{22}Na fluxes were smaller than those found using lipid mixtures containing acidic phospholipids. Electron microscopy of negatively stained proteoliposomes prepared with PC, PC/PS (1 : 1 molar ratio), and PS revealed that the acidic phospholipid increases the size of the reconstituted proteoliposomes.The increment in size caused by the acidic phospholipid, due to the associated increase in internal volume for ^{22}Na uptake and in area for Na^+ channel incorporation, appears to be responsible for the large neurotoxin-modulated ^{22}Na fluxes observed.

Key Words Na^+ channel · reconstitution · liposomes · batrachotoxin · tetrodotoxin · *Electrophorus*

Introduction

Preservation of the tetrodotoxin/saxitoxin (TTX/STX) receptor, a 260-kD glycoprotein of excitable plasma membranes, during purification and reconstitution, indicates the presence of functional Na^+ channels (Agnew et al., 1978, 1980; Barchi, Cohen & Murphy, 1980; Hartshorne & Catterall, 1981, 1984; Miller, Agnew & Levinson, 1983; Casadei, Gordon & Barchi, 1986; Villegas et al., 1988). When this glycoprotein is incorporated into an appropriate lipid bilayer, the basic Na^+ channel functions can be demonstrated (Villegas et al., 1977, 1979; Villegas, Villegas & Suarez-Mata, 1981; Weigele & Barchi, 1982; Krueger, Worley & French, 1983; Rosenberg, Tomiko & Agnew, 1984*a,b*; Tamkun, Talvenheimo & Catterall, 1984; Hartshorne et al., 1985; Kraner, Tanaka & Barchi, 1985; Tomiko et al., 1986; Correa, Villegas & Villegas, 1987; Duch & Levinson, 1987*a*; Recio-Pinto et al., 1987; Behrens et al., 1989; Castillo, Villegas & Recio-Pinto, 1992). Several findings indicate that lipids could play important roles in stabilizing Na^+ channel functions (Agnew & Raftery, 1979; Feller, Talvenheimo & Catterall, 1985; Duch & Levinson, 1987*b*).

It is well known that planar lipid bilayers of phosphatidylethanolamine (PE) alone or with phosphatidylcholine (PC), are sufficient for Na^+ channel reconstitution, as revealed by the effects of batrachotoxin (BTX) and tetrodotoxin (TTX) on its bioelectrical properties (Krueger et al., 1983; Rosenberg et al., 1984b; Hartshorne et al., 1985; Kraner et al., 1985; Recio-Pinto et al., 1987; Behrens et al., 1989; Castillo et al., 1992). However, previous work carried out on the reconstitution of lobster nerve Na^+ channels into liposomes had revealed that to observe the effects of BTX and TTX on ^{22}Na influx, in addition to PC and/or PE, an acidic phospholipid such as phosphatidylserine (PS) was needed (Villegas et al., 1977). Though later neurotoxin-modulated ^{22}Na fluxes were described in proteoliposomes made with purified Na^+ channels from different sources and PC alone (Rosenberg et al., 1984*a*; Agnew, Rosenberg & Tomiko, 1986; Hartshorne, Tamkun & Montal, 1986; Tanaka, Furman & Barchi, 1986), the ^{22}Na fluxes were smaller than those found using lipid mixtures containing acidic phospholipids.

The purpose of the present work was to determine: first, whether the effect of acidic phospholipids on the functional reconstitution of Na^+ channels into liposomes by freeze-thaw sonication is peculiar of lobster nerve Na^+ channels, or of Na^+ channels in general when incorporated into liposomes, and,

second, which is the basis of the acidic phospholipid effect. The experiments described below, carried out with purified Na^+ channels from *Electrophorus electricus* electrocytes revealed that, as in the case of lobster nerve Na^+ channels, the presence of an acidic phospholipid is required for observing large neurotoxin-modulated ^{22}Na influx. Electron microscopy observations revealed that the acidic phospholipid increases the size of the reconstituted proteoliposomes. The size of the proteoliposomes appears to underlie the magnitude of the ^{22}Na uptake.

Materials and Methods

Materials

Egg PC, prepared by the method of Singleton et al. (1985), was utilized for stabilizing the Na^+ channel during solubilization and purification. Bovine spinal cord PC and egg PE from Lipids Products, South Nutfield, UK; egg PC and L-phosphatidic acid (PA) from Sigma, St. Louis, MO; egg PS from Avanti Polar Lipids, Birmingham, AL; and cholesterol (Ch) from Merck, Darmstad, Germany, were used for reconstitution.

The neurotoxins used were citrate-free TTX, donated by Dr. William S. Agnew, and from Calbiochem, San Diego, CA; tritiated-TTX (^{3}H-TTX), 108.2 Ci/mol, prepared as described previously (Benzer & Raftery, 1972), and batrachotoxin (BTX), donated by Dr. John Daly. The following stock solutions were prepared and kept frozen at −70°C until use: 50 μM TTX in 10 mM acetic acid, and 0.5 mM BTX in ethanol.

^{22}Na as NaCl in water solution, carrier free, 373.17 mC/mg, was obtained from New England Nuclear, Boston, MA. Bio-Beads SM-2 (20–50 mesh) from Bio-Rad, Richmond, CA, were prepared as described by Holloway (1973). The NaPi and TrisPi buffers, pH 7.4, were prepared by addition of H_2PO_4 to NaOH or Trizma base solutions. All other reagents mentioned, when not indicated otherwise, were obtained from Sigma.

Solubilization and Purification

The Na^+ channel from *Electrophorus* electrocytes was solubilized and purified according to Agnew et al. (1980) and Miller et al. (1983), as described in detail by Rosenberg et al. (1984*a*). All procedures were carried out in the presence of PC and protease inhibitors, as close to 0°C as possible. Solubilization was made with Lubrol-PX and the purification by an ion-exchange chromatography with DEAE Sephadex followed by a gel-filtration step through Sepharose 6B. Protein concentrations were determined by the method of Lowry as modified by Peterson (1983). ^{3}H-TTX binding was measured by the Sephadex G-50 assay of Levinson et al. (1979).

The Na^+ channel preparations from the last purification step were in 100 mM NaPi, 0.1% Lubrol-PX, 0.183 mg PC/ml, 0.02% sodium azide, 50 μM O-phenantroline (O-PA), 50 μM L-1-tosyl-amido-phenylethyl chloromethyl ketone (TPCK), 100 μM phenyl-methanesulfonyl fluoride (PMSF), 0.1 μM pepstatin A, pH 7.4. The Na channel preparations contained an average of 0.15 mg of protein/ml and an average of 800 pmol of ^{3}H-TTX binding sites/mg of protein.

Reconstitution

For this purpose, the detergent of the Na^+ channel preparations mentioned in the preceding paragraph, was removed by the addition of 0.3 g/ml of Bio-Beads SM-2 followed by agitation at 1°C for 5 hr. Separately, suspensions of sonicated liposomes of known composition were prepared. The sonicated liposomes were made by placing the appropriatie lipids into a Pyrex tube, evaporating the solvent in a stream of nitrogen, resuspending the lipids into 100 mM NaP_i, pH 7.4, and sonicating the resuspension in a bath sonicator (Laboratory Supplies, Hicksville, NY) until it was translucent.

The small PC proteoliposomes containing Na^+ channels, formed spontaneously after removing the detergent, were then mixed with one of the suspensions of sonicated liposomes of known composition to obtain a final lipid concentration of 10 mg/ml and 25 (range 18–30) pmol of ^{3}H-TTX binding sites/ml. One TTX/STX binding site is equal to one Na^+ channel. Then, 300–500 μl aliquots were reconstituted by the freeze-thaw-sonication procedure (Kasahara & Hinkle, 1976) as described previously (Villegas et al., 1977, 1981; Rosenberg et al., 1984*a*). The sample placed in a Pyrex tube was frozen by immersion in dry ice-acetone for 1 min, immediately thawed by agitation in a water bath at 20–22°C during 1.5–2 min, and finally sonicated for 2 sec in a bath sonicator (Laboratory Supplies), filled with ice-water, at its maximum potency. (Sonication periods of 1, 5 and 10 sec were assayed and the magnitude of the ^{22}Na influx was found to diminish when the sonication time was increased. However, since sonication periods larger than 1 sec are more reproducible, a sonication time of 2 sec was chosen). After reconstitution the aliquots were pooled and the osmolarity of the suspension of reconstituted proteoliposomes was measured in a vapor-pressure osmometer (Wescor, Logan, UT).

In a set of experiments, the diameters of the reconstituted proteoliposomes made with PC, PC/PS (1 : 1 molar ratio), and PS, were measured in electron micrographs of fresh preparations negatively stained with 1% phosphotungstic acid.

^{22}Na Flux Assays

The ^{22}Na influx assays were carried out following the procedure described by Gasko et al. (1976). Aliquots of the suspension of reconstituted liposomes were incubated 30 min at 20°C without drugs (control), with 2.5 μM BTX, or with 2.5 μM BTX plus 1 μM TTX. In the control sample, only the vehicles used to dissolve the neurotoxins (ethanol for BTX and acetic acid for TTX) were added. Measurements of the ^{22}Na influx was initiated by a 1 : 10 dilution of the reconstituted proteoliposomes into isosmolar solution of $TrisP_i$, pH 7.4, containing the same concentrations of toxins and approximately 1 μCi of ^{22}Na per ml. In this way an outwardly directed Na^+ gradient was created as indicated by Garty et al. (1983). In order to determine the ^{22}Na influx, at the time indicated in the figures, 175-μl aliquots were passed through a Pasteur pipette-size column of Dowex-50W (50–100 mesh; Sigma) in Tris form. The sample was immediately eluted with 2 ml of ice-cold isosmolar sucrose solution. The eluates from the Dowex columns were collected in counting vials, to which 5 ml of scintillating fluid were added, and the samples were counted in a liquid scintillation spectrometer (Beckman, Palo Alto, CA).

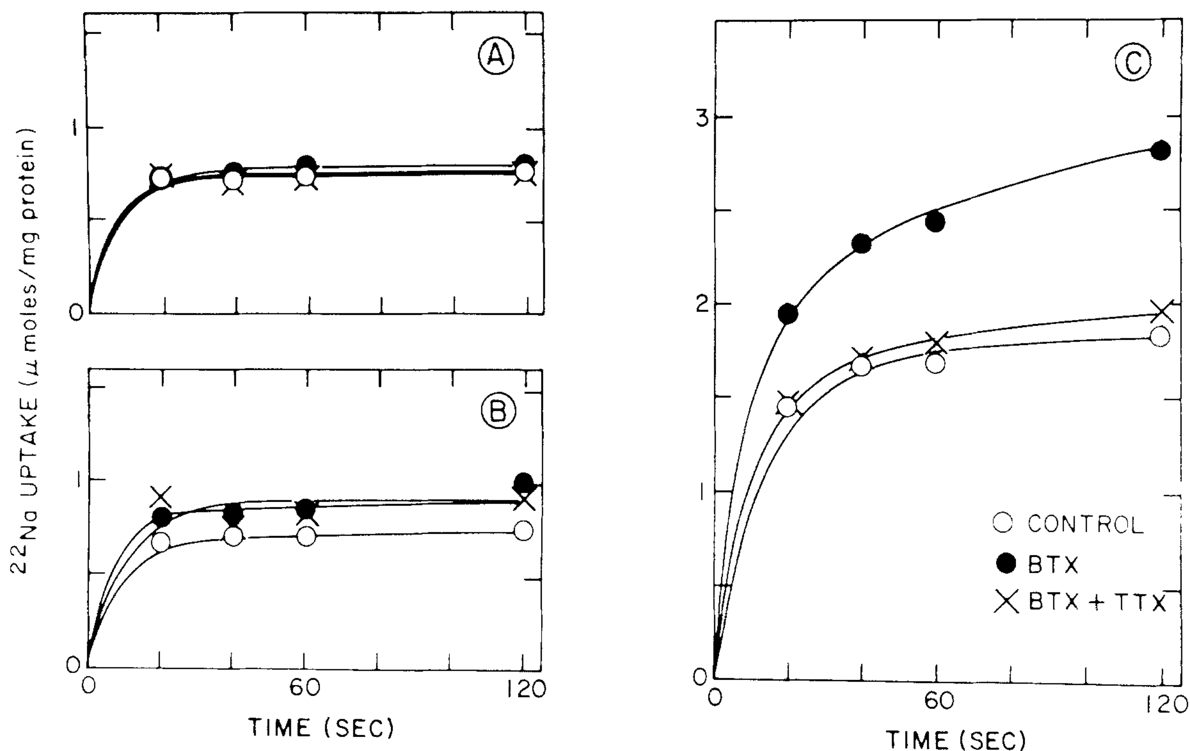

Fig. 1. ^{22}Na uptake into proteoliposomes reconstituted with (*A*) PC (n = 6), (*B*) PC/PE (1 : 1 molar ratio) (n = 2), and (*C*) PC/PE/PS (1 : 1 : 1) (n = 4). Total phospholipid concentrations of 10 mg/ml, and Na^+channel protein containing 24 pmol of ^{3}H-TTX binding sites/ml were used for reconstitution. The uptake was measured at the time indicated, in the absence of toxins (control), in the presence of 2.5 μM BTX, and of 2.5 μM BTX plus 1 μM TTX. In this and the following figures are represented the mean values of the number of measurements indicated in parenthesis in each figure legend.

Results and Discussion

Phospholipids

Several assays were made to determine the type of lipid or lipids required by freeze-thaw sonication to obtain the reconstituted proteoliposomes that would exhibit the neurotoxin-modulated ^{22}Na influx. As will be shown later, it was found that this was related to the size of the reconstituted proteoliposomes. In the legends for the figures are indicated the number of measurements from which the data represented were calculated. Although there is some preparation-to-preparation variability inherent to the method, the value of the flux ratios with respect to the control are relatively constant. However, it has been preferred to represent in the figures the mean values of the measured fluxes to illustrate their real magnitudes.

The first set of experiments was carried out with PC, PC/PE, and PC/PE/PS. A concentration of 10 mg lipid/ml was used and the amount of Na^+ channel protein added gave a final concentration of 24 pmol of ^{3}H-TTX binding sites/ml. The results shown in Fig. 1*A* and *B* indicate that, at least under our experimental conditions, a significant BTX-activated, TTX-sensitive ^{22}Na influx could not be observed in proteoliposomes made with 10 mg/ml of PC or PC/PE (1 : 1 molar ratio). Higher concentrations of PC or PC/PE were not assayed due to difficulties in sonication. Similar negative results with PC or PC/PE were also observed under a variety of experimental conditions, such as different sonication times (2 or 10 sec), addition of the toxins before reconstitution, different temperatures of incubation with the toxins (20°C and 37°C), enrichment in ^{3}H-TTX binding sites of the purified Na^+ channel protein, use of sulfate instead of phosphate as anion in the medium, addition of PC for reconstitution before removing the detergent with Bio-Beads, increase of the outwardly Na^+ gradient from 10 to 20-fold, increment of BTX concentration up to 5 μM, and use of egg PC instead of bovine spinal cord PC.

As it is shown in Fig. 1*C*, when PS was added to PC/PE (PC/PE/PS, 1 : 1 : 1 molar ratio; 10 mg/ml), the BTX-activated, TTX-sensitive ^{22}Na influx

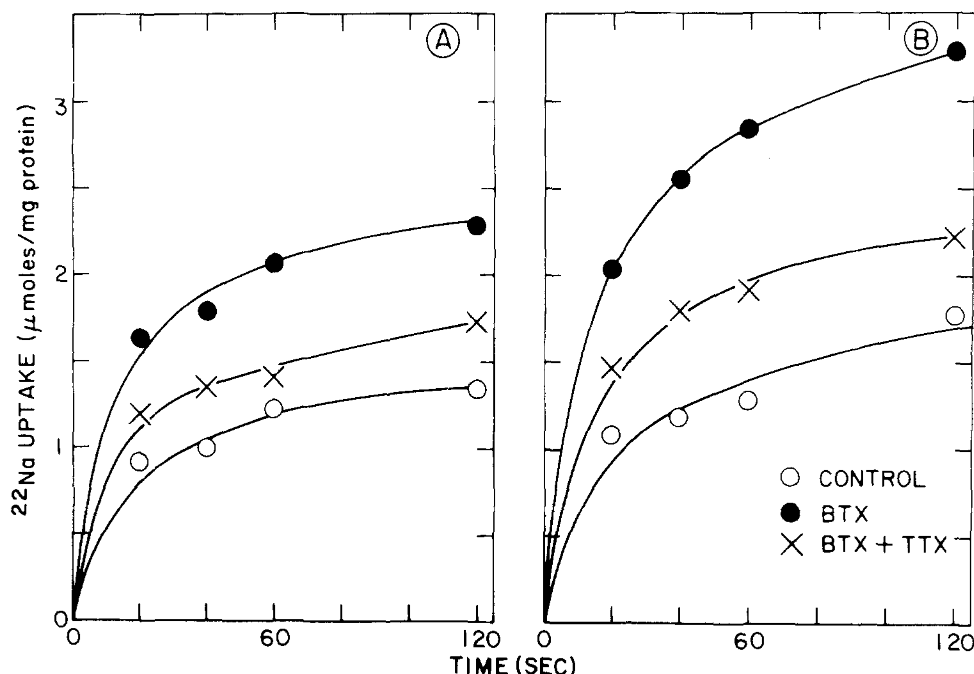

Fig. 2. ^{22}Na uptake into proteoliposomes reconstituted with (*A*) PC/PS (1 : 1 molar ratio) (n = 4), and (*B*) PE/PS (1 : 1) (n = 2). Total phospholipid concentrations of 10 mg/ml, and Na^+ channel protein containing 26 pmol ^{3}H-TTX binding sites/ml were utilized for reconstitution. The ^{22}Na uptake was measured at the times indicated, in the absence of toxins (control), in the presence of 2.5 μM BTX, and of 2.5 μM BTX plus 1 μM TTX.

was observed. Similar results were obtained with PC/PE/PS at molar ratios of 1 : 1 : 1 or 1 : 0.24 : 0.25 (*data not shown*), the latter corresponding to the molar ratio determined in the plasma membrane of *Electrophorus* electrocytes (Rosenberg et al., 1977; Kallai-Sanfacon & Reed, 1980; Grunhagen et al., 1983). The Na^+ channel protein used in this latter assay had 18 pmol of ^{3}H-TTX binding sites/ml.

In addition, the results shown in Fig. 2*A* and *B* reveal that PS can be combined with PC or PE separately with similar results. The amount of Na^+ channel protein added for reconstitution gave a concentration of 26 pmol ^{3}H-TTX binding sites/ml. In the experiments summarized in Fig. 2*A* and *B*, the average initial ^{22}Na influx ratios in the presence of BTX to the control value was 1.9 for PC/PS (1 : 1) and 2.2 for PE/PS (1 : 1).

The effect of increasing BTX concentrations on the ^{22}Na influx was determined in a preparation of reconstituted proteoliposomes made with PE/PS (1 : 1) and Na^+ channel protein containing 28 pmol of ^{3}H-TTX binding sites/ml. From the data shown in Fig. 3*A*, a $K_{0.5}$ for BTX of 2.5 μM was calculated. A value of 2.1 μM was determined in similar experiments carried out with PC/PE/PS (1 : 1 : 1). These values are of the order of those reported for reconstituted Na^+ channels of rat brain (2 μM, Tamkun et al., 1984), rat sarcolemma (1.5 μM, Weigele & Barchi, 1982), and rabbit T-tubule (2 μM, Kraner et al., 1985).

The blocking effect of increasing concentrations of TTX on the ^{22}Na influx caused by 2.5 μM BTX was explored in the same PE/PS proteoliposome preparation utilized in the experiment described in the preceding paragraph. From the results in Fig. 3*B*, a $K_{0.5}$ of 1.5 nM TTX was calculated and the BTX-induced ^{22}Na influx inhibited by TTX was 54%. $K_{0.5}$ values for TTX between 4.3 and 45 nM have been reported for reconstituted *Electrophorus* Na^+ channels (Miller et al., 1983; Rosenberg et al., 1984*b*; Tomiko et al., 1986). The maximum inhibition of the BTX-induced influx could be due either to the fraction of Na^+ channels oriented with the TTX receptor facing out, since TTX was applied at the external side only, or to the presence of proteoliposomes with many channels. Due to the time resolution of the method used, few open channels during the assay (maybe only one for a small liposome) could be sufficient to equilibrate the internal volume with ^{22}Na whether the others are blocked by TTX or not. In the present work the inhibition of the BTX-induced influx was between 54 and 100%.

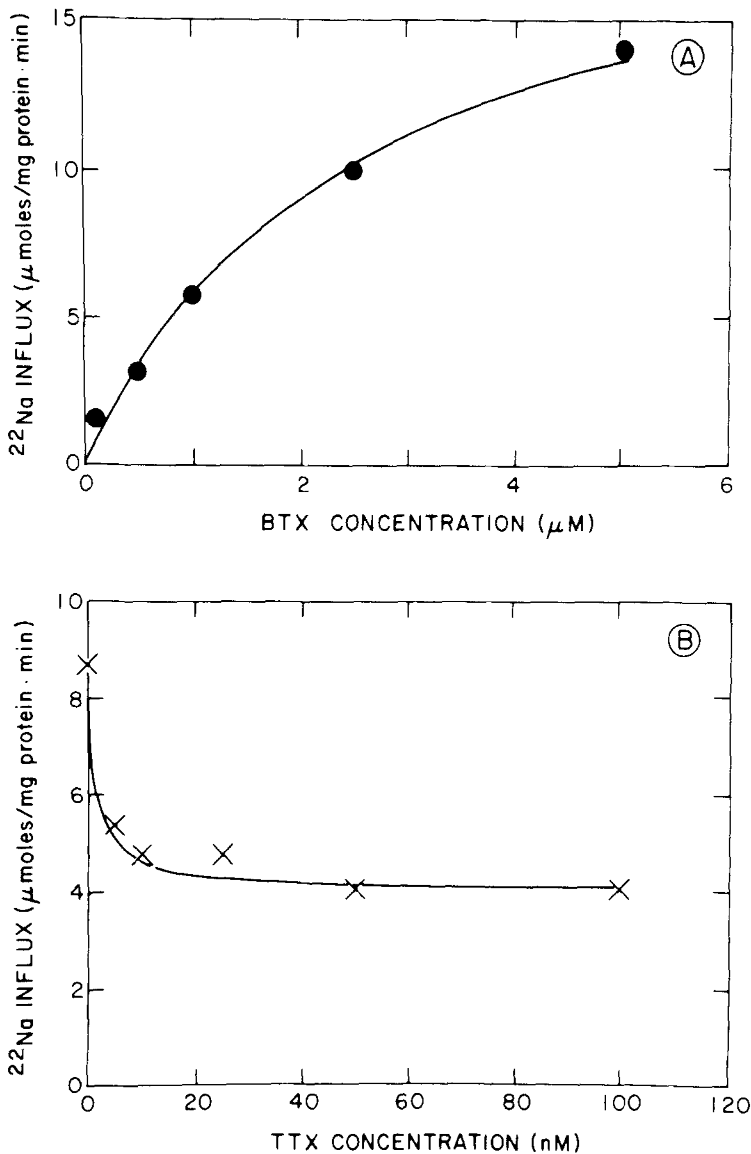

Fig. 3. (*A*) Initial ^{22}Na influx into reconstituted proteoliposomes as a function of BTX concentration. PE/PS (1 : 1 molar ratio) at 10 mg/ml, and Na^+ channel protein containing 28 pmol of ^3H-TTX binding sites/ml were utilized for reconstitution. Each ^{22}Na influx was calculated from the uptake measured 20 sec after dilution of the proteoliposomes in the external solution containing ^{22}Na ($n = 2$). (*B*) Effect of different TTX concentrations on the initial ^{22}Na influx on the proteoliposomes treated with 2.5 μM BTX ($n = 2$). Reconstitution and measurement of the ^{22}Na influx were as described above.

Effect of Different PS Concentrations

The effect of PS was further explored using different PC/PS molar ratios at a constant concentration of 10 mg/ml and Na^+ channel protein containing 22 pmol of ^3H-TTX binding sites/ml. The results are summarized in Fig. 4*A–D*. Figure 4*A* corresponds to PC proteoliposomes. As shown in Fig. 4*B*, the ^{22}Na influx sensitive to the toxins was already observed when 10% of the PC was replaced by PS. For PC/PS molar ratios between 1 : 0.1 and 1 : 1, BTX increases the initial ^{22}Na influx up to 2.5-fold with respect to the control (Figs. 4*B*–D). A similar increment was found in proteoliposomes made with PS alone (Fig. 4*E*). The results shown in Fig. 4 also reveal that TTX blocks the ^{22}Na flux caused by BTX through Na^+ channels incorporated into PS liposomes.

Effect of PS on the Size of the Proteoliposomes

The percent distribution of the diameters of negatively-stained proteoliposomes prepared with PC, PC/PS (1 : 1 molar ratio) and PS, are summarized in Fig. 5. The data revealed that PS increases the size of the proteoliposomes prepared by the freeze-thaw-

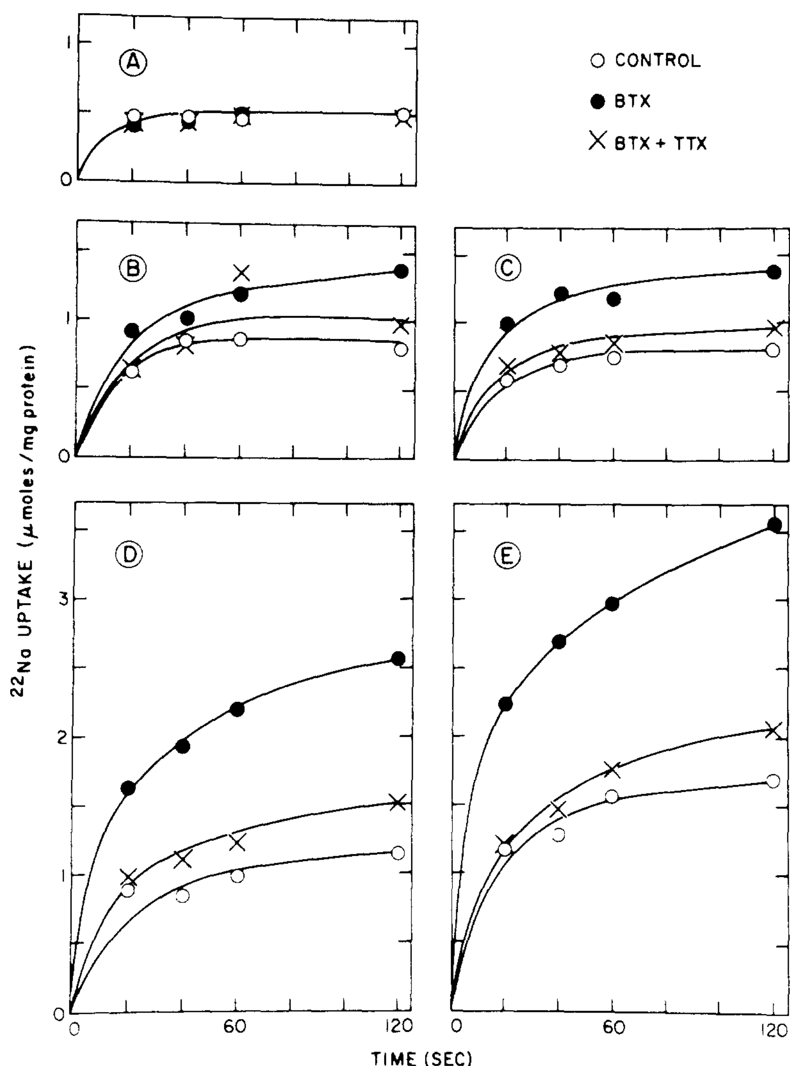

Fig. 4. ^{22}Na uptake into proteoliposomes reconstituted with (*A*) PC (n = 2), (*B*) PC/PS (9 : 1) (n = 2), (*C*) PC/PS (2 : 1) (n = 2), (*D*) PC/PS (1 : 1) (n = 2), and (*E*) PS (n = 2), at total concentrations of 10 mg/ml. The Na^+ channel protein added to the lipids for reconstitution had 22 pmol of ^{3}H-TTX binding sites/ml. The ^{22}Na uptake was measured at the time indicated, in the absence of toxins (control), in the presence of 2.5 μM BTX, and 2.5 μM BTX plus 1 μM TTX.

sonication procedure. This observation is in agreement with results previously obtained by Pick (1981) with pure (protein-free) liposomes made by the same procedure. When PC is utilized for reconstitution only 30% of the proteoliposomes is more than 130 nm in diameter; this percentage increases to 59% when PC/PS is utilized, and to 80% for the PS proteoliposomes.

The number and volume of the proteoliposomes made with a constant amount of phospholipids and protein depends on their size distribution. Figure 6 illustrates the number of proteoliposomes of each diameter calculated from the percent diameter distributions shown in Fig. 5 and under the assumption of a constant total surface area due to the use of a constant amount of lipids. Thus, for each 100 PC proteoliposomes obtained, 59 PC/PS and 48 PS proteoliposomes were produced with the same total amount of lipids. On the other hand, the total volume calculated for the 59 PC/PS and 48 PS proteoliposomes was, respectively, 20 and 50% larger than that of the 100 PC proteoliposomes.

These results suggest that the acidic phospholipid effect on Na^+ channel reconstitution is related to the augmented diameter of the reconstituted proteoliposomes produced by the addition of PS; increase in diameter means increase in internal volume for ^{22}Na uptake (Duch & Levinson, 1987*a*) and increase in surface area available for incorporation of Na^+ channels (Weigele & Barchi, 1982).

Effect of the Replacement of PS by PA

Could PS be substituted by another acidic phospholipid, such as PA? In Fig. 7*A* and *B* are compared the results obtained with PC/PE/PS (1 : 1 : 1); the Na^+ channel protein added gave a final concentration of 20 pmol of ^{3}H-TTX binding sites/ml. The increment in the initial ^{22}Na influx caused by BTX when PC/PE/PA was utilized for reconstruction was

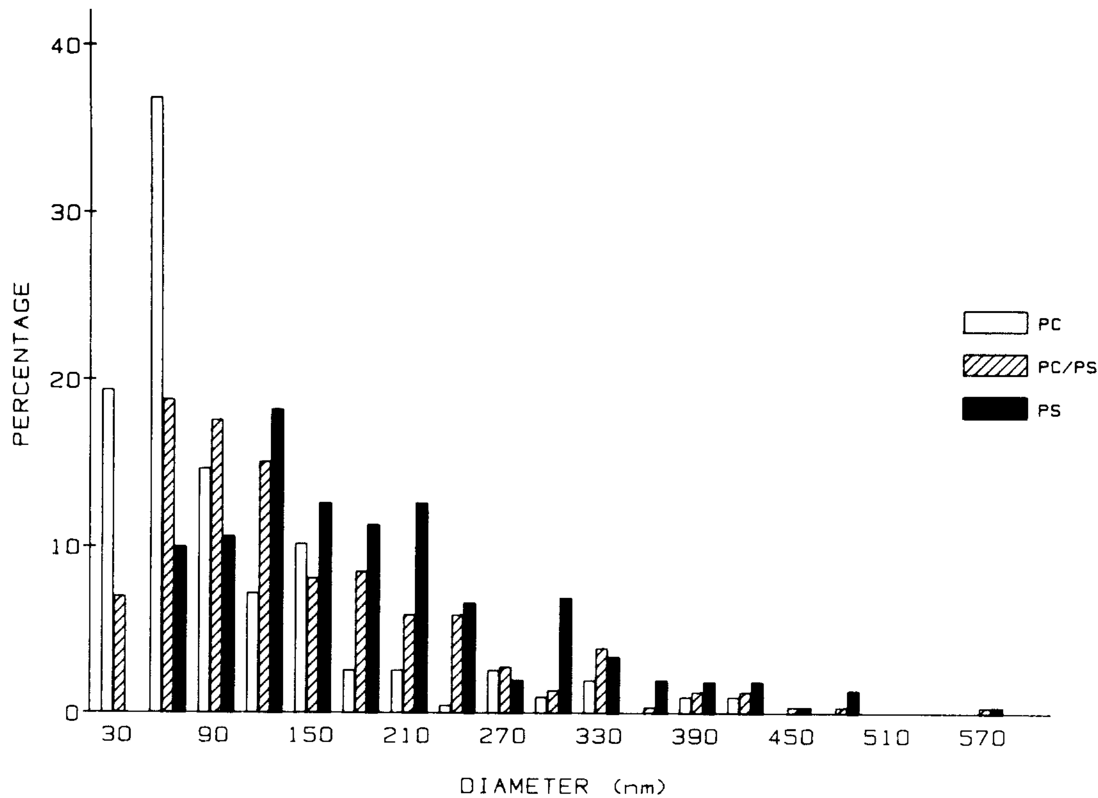

Fig. 5. Percentage distribution of the diameters of proteoliposomes reconstituted with PC, PC/PS (1 : 1 molar ratio), and PS, at total phospholipid concentrations of 10 mg/ml. The Na^+ channel protein added to the lipids for reconstitution had 20 pmol of ^{3}H-TTX binding sites/ml.

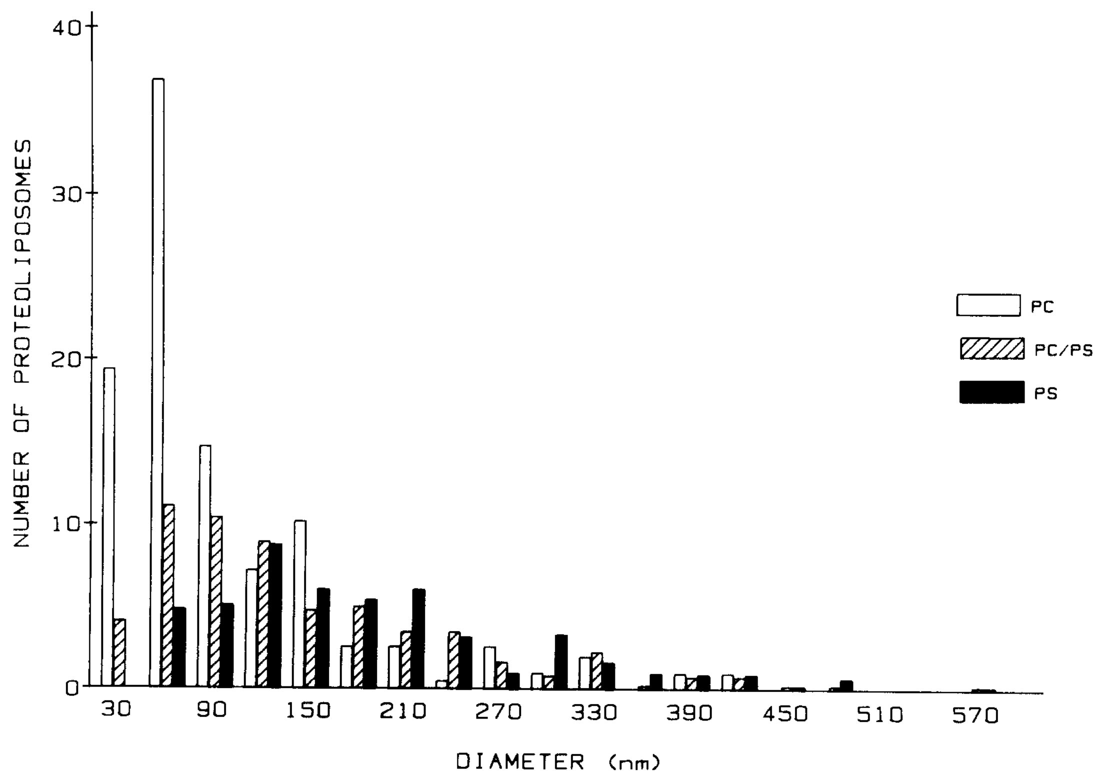

Fig. 6. Relative number of proteoliposomes of different diameters reconstituted with the same total concentrations of PC, PC/PS (1 : 1 molar ratio), and PS. The values were calculated from the data shown in Fig. 5, assuming that the total surface area of the three types of proteoliposomes was the same. As indicated in Fig. 5, total phospholipid concentrations of 10 mg/ml and Na^+ channel protein containing 20 pmol of ^{3}H-TTX binding sites/ml were used for reconstitution. For further details *see* text.

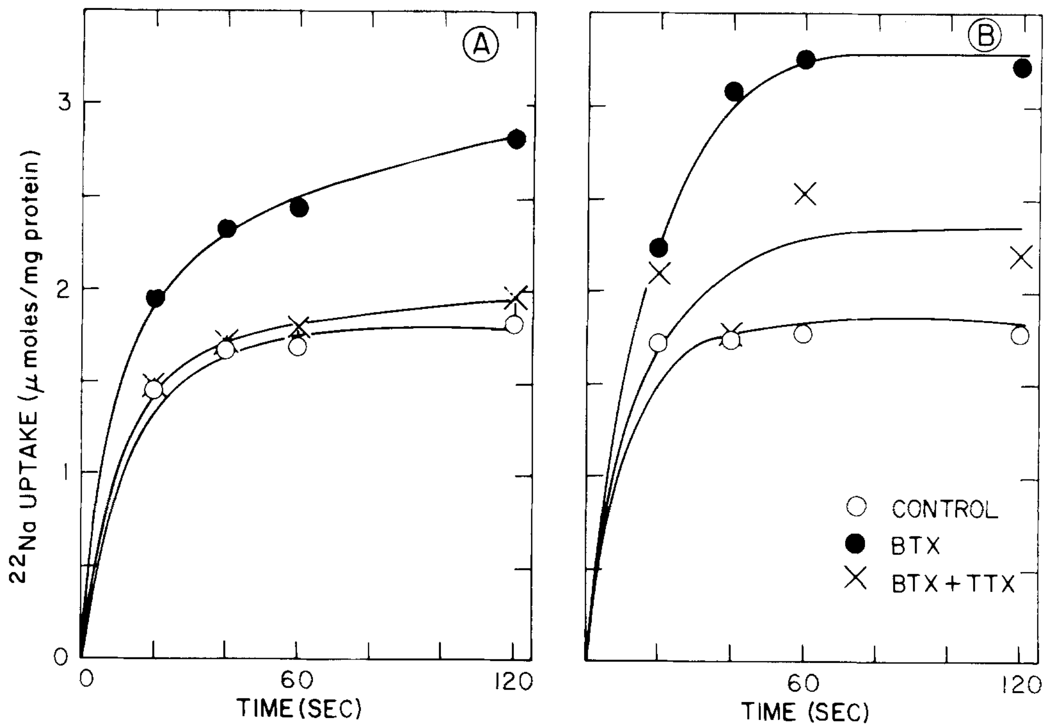

Fig. 7. ^{22}Na uptake into proteoliposomes reconstituted with lipid mixtures containing PS (n = 4) or PA (n = 3). Similar results were obtained when PA was assayed alone, with PC (1 : 1), or PC/PE (1 : 1 : 1). Shown are (*A*) PC/PE/PS (1 : 1 : 1), and (*B*) PC/PE/PA (1 : 1 : 1) at total concentrations of 10 mg/ml. The Na^+ channel protein added to the lipids for reconstitution had 20 pmol of ^{3}H-TTX binding sites/ml. The ^{22}Na uptake was measured at the time indicated, in the absence of toxins (control), in the presence of 2.5 μM BTX, and of 2.5 μM BTX plus 1 μM TTX.

similar to the value obtained when PC/PE/PS, at the same molar ratio, was used. These results are in agreement with those previously obtained with proteoliposomes made with crude preparations of lobster nerve Na^+ channel (Villegas et al., 1977).

Effect of the Addition of Cholesterol

The role of cholesterol (Ch) was explored due to its known effect on the organization and fluidity of phospholipid bilayers and to its negative charge. The ^{22}Na influx was studied in proteoliposomes made with 10 mg of lipid/ml of PC; PC/Ch, 2 : 1; PC/PS, 1 : 1; PC/PS/Ch, 1 : 1 : 1. The final concentration of Na channel protein added for reconstitution had 26 pmol of ^{3}H-TTX binding sites/ml. The results exhibited in Fig. 8 revealed that cholesterol cannot substitute the acidic phospholipid requirement. Moreover, addition of cholesterol to PC/PS decreases the BTX and TTX effects on the ^{22}Na influx.

Reconstitution with Different Phospholipid Concentrations

The results shown in Fig. 9 were obtained using different concentrations of PE/PS (1 : 1 molar ratio) for reconstitution. The Na^+ channel protein used for reconstitution had 27 pmol of ^{3}H-TTX binding sites/ml. The results indicate that the initial ^{22}Na influx into the control and toxin-treated proteoliposomes increases when the phospholipid concentration is augmented from 5 to 40 mg per ml. The initial ^{22}Na influx was calculated from the uptake during the first 60 sec after addition of the ^{22}Na. A similar observation was previously made with lobster nerve Na^+ channel incorporated into soybean liposomes (Villegas et al., 1977). The neurotoxin-modulated ^{22}Na flux, i.e., the difference between the BTX-stimulated initial ^{22}Na influx and that remaining after the addition of an excess of TTX, increases when the phospholipid concentration is augmented. Assuming a constant size distribution of the liposomes, the effect of the phospholipid concentration can be explained by an increment in the effective internal volume available for ^{22}Na uptake, due to the formation of a larger number of liposomes accompanied by a more effective distribution of the Na^+ channels.

Conclusions

The results of the present work indicate that reconstitution of purified *Electrophorus* Na^+ channels into liposomes by freeze-thaw sonication requires

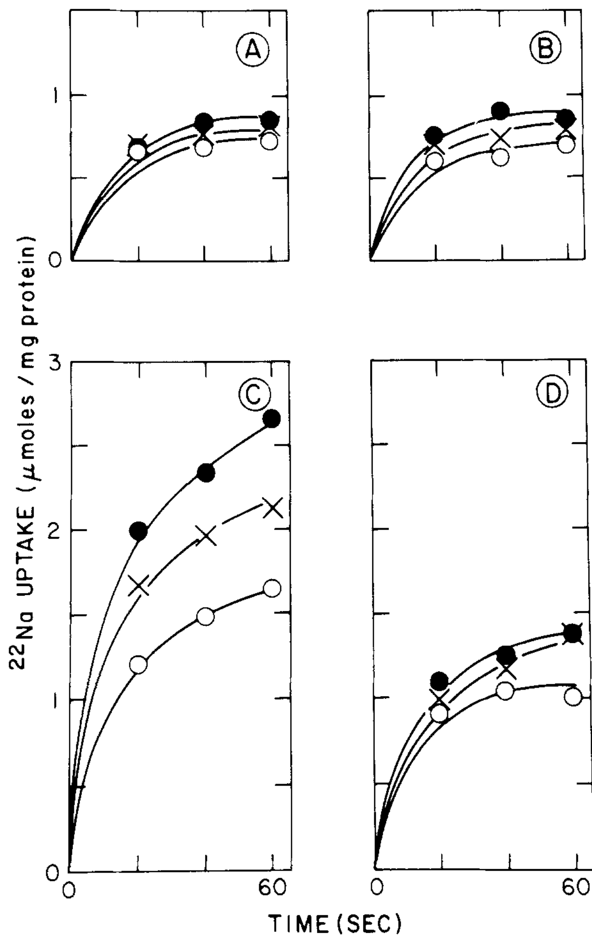

Fig. 8. ^{22}Na uptake into proteoliposomes reconstituted with (*A*) PC (n = 2), (*B*) PC/Ch (1 : 1) (n = 2), (*C*) PC/PS (1 : 1) (n = 2), and (*D*) PC/PS/Ch (1 : 1 : 1) (n = 2), at total concentrations of 10 mg/ml. The Na^+ channel protein added to the lipids for reconstitution had 26 pmol of ^3H-TTX binding sites/ml. ^{22}Na uptake was measured at the time indicated, in the absence of toxins (control) (○), in the presence of 2.5 μM BTX (●), and in 2.5 μM BTX plus 1 μM TTX (×).

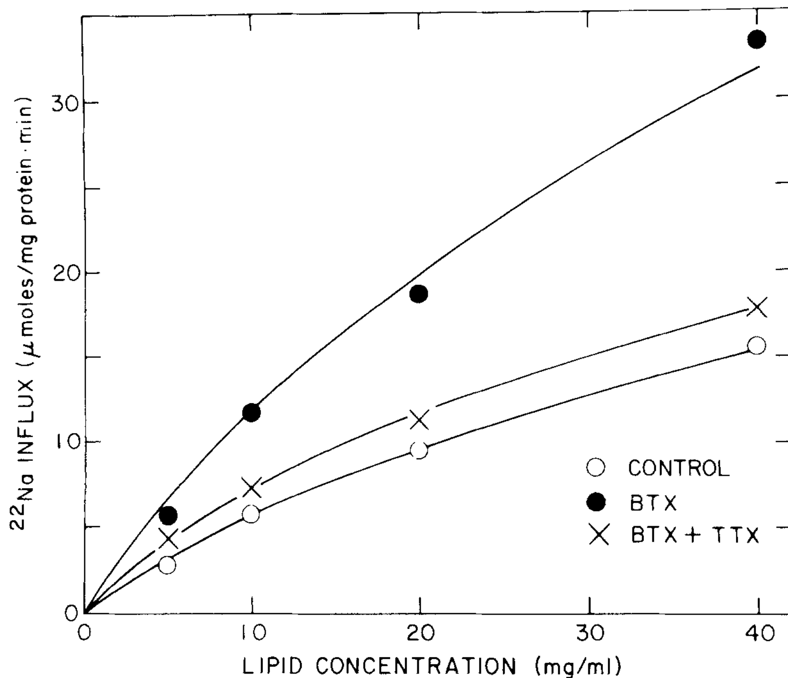

Fig. 9. Initial ^{22}Na influx into proteoliposomes reconstituted with PE/PS (1 : 1 molar ratio) at total concentrations of 5, 10, 20 and 40 mg/ml (n = 2). The Na^+ channel protein added to the lipids for reconstitution had 27 pmol of ^3H-TTX binding sites/ml. The initial ^{22}Na influx was calculated from uptake measurements made 60 sec after dilution of the proteoliposomes in the external solution containing ^{22}Na. The uptakes were carried out in the absence of toxins (control), in the presence of 2.5 μM BTX, and of 2.5 μM BTX plus 1 μM TTX.

the presence of an acidic phospholipid to observe a large neurotoxin-modulated ^{22}Na influx. The same results have been obtained in similar reconstitution experiments with crude and partially purified preparations of lobster nerve membrane Na^+ channels (Villegas et al., 1977, 1979, 1981; Correa et al., 1987).

A direct effect of the acidic phospholipids on the Na^+ channels is unlikely, since it is well known that functional reconstitution of Na^+ channels can be achieved in planar lipid bilayers of PE alone or with PC (Rosenberg et al., 1984*b*; Recio-Pinto et al., 1987; Behrens et al., 1989; Castillo et al., 1992), and even in proteoliposomes made with PC alone (Rosenberg et al., 1984*a*; Agnew et al., 1986; Hartshorne et al., 1986; Tanaka et al., 1986), though in the latter case the neurotoxin-modulated ^{22}Na flux is smaller than that found in the presence of acidic phospholipids (present work).

The large neurotoxin-modulated ^{22}Na influx produced by the presence of acidic phospholipids appears to be the consequence of the observed increase in size of the reconstituted proteoliposomes described in the present work, which means increase in internal volume for ^{22}Na uptake (Duch & Levinson, 1987*a*) and in area for Na^+ channel incorporation (Weigele & Barchi, 1982).

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