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SODIUM CHANNEL ACTIVITY OF LOBSTER NERVE MEMBRANE TREATED WITH PURIFIED PHOSPHOLIPASE A_2

FLOR V. BARNOLA

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

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Sodium channel activity was determined by measuring the veratridine-tetrodotoxin-sensitive sodium influx in reconstituted vesicles prepared from lobster nerve membrane and soybean lipids. The sodium channel activity was abolished by treatment of membranes, prior to reconstitution, with purified phospholipase A_2. When the treatment with phospholipase A_2 was carried out in the presence of bovine serum albumin the channel activity was fully preserved. Electron microscopy revealed that the normal vesicular appearance of the membranes was changed to an amorphous mass by treatment of the membranes with enzyme alone. A population of preserved vesicular structures was observed when bovine serum albumin was present during the enzyme treatment. Analysis of the membrane components indicate that there is no relationship between phospholipid composition and sodium channel activity.

Introduction

The movement of Na^+ into nerve cells via the so-called sodium channels plays an important role in the excitation of the nerve membrane and in the conduction of nerve impulses [1,2]. Veratridine, grayanotoxin, batrachotoxin and aconitine increase the membrane permeability to Na^+ which is abolished by tetrodotoxin and saxitoxin [3–6]. In disrupted membrane preparations, the specific binding of tetrodotoxin and saxitoxin can be taken as indicative of the presence of sodium channels [7–9]. Previous reports show that the tetrodotoxin and saxitoxin binding to membrane fragments is inhibited by phospholipase A_2 [7,10–12], and phospholipase C [11], and that, in spite of the degradation of the phospholipids, bovine serum-albumin prevents the inhibition caused by phospholipase A_2 [11,12]. However, the toxins binding capacity does not necessarily indicate that the biological activity of the Na^+ channel which is sensitive to the toxins, is present. This study was undertaken to investigate the effect of phospholipase A_2, in the presence or absence of serum albumin, on the sodium channel activity of the nerve membrane as measured by the veratridine-tetrodotoxin-sensitive sodium influx into vesicles made by incorporating membrane into liposomes. In preliminary experiments the protective effect of serum albumin on the tetrodotoxin binding to membranes previously treated with phospholipase A_2 [11,12] was confirmed.

Materials and Methods

Most of the procedures and materials used in this study have been described in earlier publications.

Chemicals. Tetrodotoxin (citrate-free, Sankyo Ltd., Tokyo), was commercially tritiated (ICN, CA) by the Wilzbach method [13] and purified following the procedure of Hafemann [14] as modified by Benzer and Raftery [7]. The specific activity and the apparent radiochemical purity of the

toxin [15] were 308 Ci/mol and 45%, respectively. The specific activity and purity although low are similar to those of other [^{3}H]tetrodotoxin or [^{3}H]saxitoxin samples used by other authors [11,12] and myself [10] in previous studies; it should be indicated that tetrodotoxin and saxitoxin can be obtained with higher values of both specific activity and radiochemical purity [16,17].

Naja naja phospholipase A_2 was purified [18] from the crude snake venom (Sigma Chemical Co.). The specific activity of the enzyme titrimetrically measured varied between 120 and 170 (units/mg protein). One unit of phospholipase A_2 is the amount of protein which hydrolizes 1 μmol of phosphatidylcholine per min at 37°C and pH 9.0. The enzyme preparation was homogeneous as indicated by high performance molecular exclusion chromatography. No detectable protease activity was present as assayed using azoalbumin (Sigma Chemical Co.) as the substrate [19].

Bovine serum albumin, (fatty acid free) was purchased from Miles Laboratories (Elkhart, IN). ^{125}I-labelled albumin was prepared using the choramine-T method [20].

Nerve plasma membranes. The plasma membrane preparations were obtained from walking-leg nerves of living *Panulirus argus* lobsters as previously reported [15]. Briefly, the procedure consisted of homogenization of the nerves in 0.33 M sucrose, 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4), and centrifugation at $100000 \times g$ for 30 min. The supernatants were discarded; the pellets were homogenized in 0.33 M sucrose, layered over 1.12 M sucrose and centrifuged in a Beckman SW 25.2 rotor at $70000 \times g$ for 1 h. The membrane, collected at the interface, was diluted 5-times with 10 mM Tris-HCl buffer (pH 7.4), and centrifuged at $100000 \times g$ for 40 min. The pellets were resuspended in 0.78 M sucrose, 10 mM Tris-HCl, (pH 7.4), frozen in dry ice-acetone, and stored at −70°C until used. For more details see Ref. 15.

Treatment of membranes with phospholipase A_2. Nerve plasma membrane preparations (1 mg protein) were incubated on ice with 5 units of purified phospholipase A_2 during 1 h, shaking occasionally. The incubation medium was 10 mM Tris-HCl (pH 7.4), containing 0.15 M NaCl and 1 mM $CaCl_2$, with or without 2.5% serum albumin. Control samples were incubated under the same conditions without enzyme and albumin. At the end of the incubation period ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) was added to a final concentration of 10 mM and the membranes were spun down by centrifugation at $100000 \times g$ for 30 min. The membranes were then washed three times with 10 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl in the presence or absence of 2.5% albumin, and either directly assayed for tetrodotoxin binding or incorporated into soybean liposomes for sodium influx measurements. Protein concentration was determined by the method of Lowry et al. [21]. The effect of the enzymatic treatment was evaluated by measuring total lipid phosphorous content [22,23] and the phospholipid composition of the membranes. The phospholipids were separated by two-dimensional thin-layer chromatography using chloroform/methanol/acetic acid/water (65:35:2:2, v/v) and chloroform/methanol/NH_4OH/water (65 : 35 : 2 : 2, v/v).

The cholesterol content was evaluated by the method of Bowman and Wolf [24].

Electrophoresis of the membranes. The membrane samples were delipidated by adding methanol/chloroform (6:5, v/v). The precipitated proteins were solubilized in 1% SDS and heated at 50°C for about 10 min. Discontinuous SDS polyacrylamide gel electrophoresis was performed by the method of Laemmli [25]. The running gel contained 9% (w/v) polyacrylamide, 0.24% (w/v) *N*,*N*′-methylenebisacrylamide. The electrophoresis was carried out at 25 mA per gel, constant current for about 6 h. After staining with Coomassie brilliant blue, the gel was scanned with a double-beam recording microdensitometer (Joyce, Loebl and Co., Ltd.).

Tetrodotoxin binding. The assay was performed as previously described [15]. Briefly, to assay tetrodotoxin binding duplicate samples of membrane were used, one of each pair serving to measure non-specific binding. Binding of [^{3}H]tetrodotoxin was calculated by subtracting the radioactivity (cpm/mg protein) present in pellets of membranes pre-incubated with an excess (50 times that of the [^{3}H]tetrodotoxin) of unlabelled tetrodotoxin from that found in pellets of membranes incubated with [^{3}H]tetrodotoxin only. Incubation with tetrodotoxin and centrifugation

afterwards of the membrane suspension were carried out at 4°C. The concentration of [^{3}H]tetrodotoxin used for determination of the maximal binding to the membrane was 100 nM.

Measurement of ^{22}Na influx. ^{22}Na influx was assayed [26] in soybean liposomes containing membranes, which had been incorporated by the freeze-thaw sonication procedure [27]. The liposomes were prepared with crude soybean phospholipids (Concentrated Associates, Woodside, NY); a detailed description of these procedures has been published [28]. The sodium movement was measured in the presence of 0.5 mM veratridine, or 0.5 mM veratridine plus 100 nM tetrodotoxin; or in the absence of drugs in order to evaluate the basal sodium flux (control) into the proteoliposomes. Veratridine was purified [29] from veratrine (Sigma Chemical Co.).

Fractionation of the membranes. In some experiments the membranes, control or enzyme treated, were fractionated by centrifugation at $70000 \times g$ for 90 min (Beckman SW 25.2 rotor) on a discontinuous sucrose gradient made of 40, 30 and 20% (w/v) sucrose solutions. After centrifugation the control and the enzyme treated membranes produced three well defined cloudy regions, banding at densities of 1.007 (Fraction I), 1.106 (Fraction II) and 1.133 (Fraction III). The membranes treated with enzyme plus albumin, in addition to the three fractions, also produced a pellet. The bands and the pellet were collected, washed and assayed for sodium influx; tetrodotoxin binding was not measured in the fractionated membranes.

Results and Discussion

Tetrodotoxin binding to nerve membranes

Tetrodotoxin binding was assayed in: (i) membranes treated with enzyme alone; (ii) membranes treated with enzyme in the presence of 2.5% albumin solution, and (iii) in control membranes (without enzyme and albumin). The binding (pmol/mg of membrane protein) measured in four experiments decreased from 4.5 ± 0.4 (mean ± S.E.) for the control membrane to 0.4 ± 0.1 when the membrane was treated with the enzyme, and it was 5.4 ± 0.4 when bovine serum albumin was present during the enzyme-membrane incubation period. These findings are in agreement with others previously published [11,12]. The binding values were obtained using a 5 : 1 ratio of enzyme units to mg of membrane protein; a lower ratio of 2 : 1 assayed in preliminary experiments produced approx. 46% reduction of the tetrodotoxin binding. Since tetrodotoxin binding was taken as indicative of membrane behaviour, all the following experiments were performed under conditions of total inhibition of binding (5 : 1 ratio of enzyme units: mg protein), in order to investigate whether not only the binding but also the Na^+ channel response to neurotoxins can be protected by albumin.

Measurements of ^{22}Na influx

Table I shows the sodium entry into the reconstituted vesicles prepared with control membranes, membranes treated with enzyme alone or with enzyme plus serum albumin. The increase in the sodium influx due to veratridine and sensitive to tetrodotoxin is abolished by the enzymatic treatment and fully preserved when the albumin is present. Since the membrane responses to veratridine and to tetrodotoxin have been accepted to be a consequence of the existence of the sodium channels [30] in the membrane these results could be inferred as indicating that serum albumin protects the sodium channel activity from the enzymatic action.

The possibility that the albumin used as a protector of the channel activity could be retained by the membranes and contributes in some way with sodium influx measurements was investigated utilizing ^{125}I-labelled albumin. The albumin is almost totally removed by centrifugation, and represents less than 0.1% of the final total protein used to determine the sodium influx. In addition, the sodium influx measured into liposomes prepared with soybean lipids and albumin is not sensitive to veratridine.

The sodium channel activity is not restored by washing the membrane with albumin after its treatment with the enzyme (Table I). The lack of Na^+ channel activity after the enzymatic treatment could be due to irreversible membrane damage induced by the surfactant products resultant from the enzyme action.

TABLE I

^{22}Na FLUX INTO LIPOSOMES MADE OF SOYBEAN LIPIDS AND NERVE MEMBRANES TREATED OR NON-TREATED WITH PHOSPHOLIPASE A_2

The membranes were treated as described under Materials and Methods. 0.5 mg of membrane protein was incorporated into soybean liposomes (40 mg/ml) by the freeze-thaw-sonication procedure. The ^{22}Na influx was determined for each membrane (control or enzyme treated) in three different conditions: without drugs, in the presence of 0.5 mM veratridine, and in the presence of 0.5 mM veratridine + 100 nM tetrodotoxin.

Membrane treatment	^{22}Na influx [a] (nmol/min per mg protein)	Ratio [b] V/V+TTX
None		2.10±0.11
Drug free	1031± 185	
Veratridine	2232± 420	
Veratridine + tetrodotoxin	1049± 163	
Incubation with phospholipase A_2		1.05±0.03
Drug free	1427± 80	
Veratridine	1540± 67	
Veratridine + tetrodotoxin	1467± 89	
Incubation with phospholipase A_2 and then washed with 2.5% albumin		0.91±0.20
Drug free	795± 75	
Veratridine	792± 103	
Veratridine + tetrodotoxin	875± 154	
Incubation with phospholipase A_2 in the presence of 2.5% albumin		2.72±0.61
Drug free	1288± 288	
Veratridine	3824±1121	
Veratridine + tetrodotoxin	1341± 166	

[a] Values are mean±S.E. from four experiments.

[b] The ratio between the sodium influx in the presence of veratridine (V) and that in the presence of veratridine + tetrodotoxin (V + TTX) was taken as indicative of sodium channel activity.

Electron microscopy and membrane fractionation

Samples of the membranes treated with phospholipase A_2, in the presence or absence of albumin, as well as control membranes, were stained with 2% sodium phosphotungstate solution (pH 7.2), and examined in a Siemens Elmiskop 101 electron microscope. As shown in Fig. 1, the typical vesicular pattern observed for the control membrane is lost and an amorphous mass appeared when the membrane is treated with enzyme alone. In the membranes treated with enzyme plus albumin, a certain population of the vesicular structures was preserved and appeared to be included in the amorphous mass. These findings were interpreted as indicating that the preserved membrane function lies in the preserved vesicular fraction, which if separated, could yield a functionally enriched membrane. The separation of such a membrane was attempted by centrifugation on discontinuous sucrose gradient (see Materials and Methods). The procedure allowed us to obtain four fractions with different channel activities. The higher activity was collected in a fraction banding at $d = 1.106$, but this activity is not statistically different from that found in a lighter fraction ($d = 1.077$) of the control membranes.

Sodium channel activity and phosphorus content of the membranes

The phosphorus content and the phospholipid composition of the membranes with or without sodium channel activity are shown in Tables II, III and IV. By enzyme action some phosphorus and protein spontaneously leaves the membranes (Table II). The phospholipids present in the supernatant from the membrane incubated with enzyme

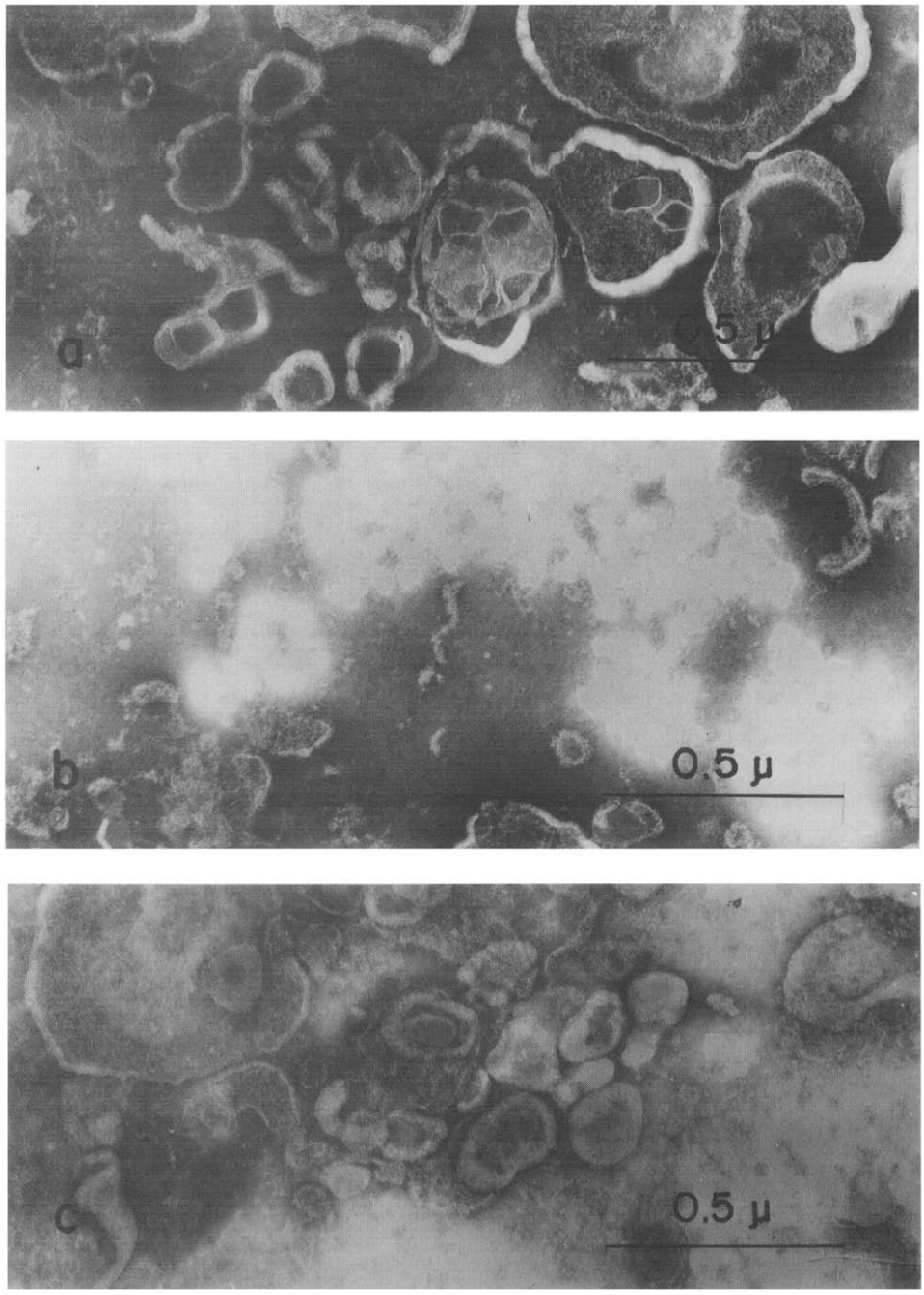

Fig. 1. Electron micrographs of negatively stained membrane preparations showing the effect of phospholipase A_2. 1 mg of membrane protein was incubated with 5 units of phospholipase A_2 as described under Methods. (a) Control membrane formed by vesicles with most of their surfaces covered by particles. (b) Membrane treated with enzyme alone showing the change of the normal vesicular structures to an amorphous mass. (c) Membrane treated with enzyme in the presence of 2.5% albumin; in this preparation normal vesicular structures appear included within the amorphous mass.

TABLE II

PHOSPHORUS AND PROTEIN CONTENTS OF THE MEMBRANES TREATED WITH PHOSPHOLIPASE A_2 IN THE PRESENCE OR ABSENCE OF BOVINE SERUM ALBUMIN

Values are the mean ± S.E. from three determinations. The same amount of initial membrane protein was utilized in each experiment.

Membrane treatment	Protein (mg)		Phosphorus (μg)		μg Phosphorus/mg protein
	Pellet	Supernatant	Pellet	Supernatant	Pellet
None	2.46 ± 0.12	0.53 ± 0.16	153.9 ± 5.0	2.2 ± 0.3	62.6 ± 3.7
Incubation with phospholipase A_2	2.19 ± 0.11	0.85 ± 0.21	139.7 ± 3.8	15.1 ± 1.8	63.8 ± 3.6
Incubation with phospholipase A_2 and then washed with albumin	2.10	[a]	118.7 ± 3.5	32.7 ± 3.8	56.4 ± 3.5
Incubation with phospholipase A_2 in the presence of 2.5% albumin	2.47	[a]	109.9 ± 3.1	44.8 ± 3.4	44.5 ± 2.5

[a] The high amount of albumin prevented the determination.

alone and washed without albumin are mainly phosphatidylcholine and sphingomyelin; no lysophosphatides could be detected, indicating that without albumin these compounds remain attached to the membrane. As will be discussed below, when albumin was utilized a high amount of lysophosphatides was found in the supernatant. No striking differences were observed in the polyacrylamide gel electrophoretic pattern of the membranes (Fig. 2) suggesting that the proteins are not preferentially lost. The only relevant fact was that, even though the same amount of protein was applied, the peptide pattern was diminished in the enzyme-treated membranes, as if the proteins in these conditions could not totally penetrate into the gel. It should be pointed out that the purified phospholipase A_2 utilized in these experiments does not contain any protease activity as measured by the azoalbumin assay [19], and that it is a homogeneous preparation, as indicated by high performance molecular exclusion chromatography.

The efficiency of the albumin to remove lysophosphatides appears to be better when it is present from the beginning of the membrane-enzyme incubation period, than when it is utilized only to wash the enzyme-treated membranes (Table II).

The phospholipid analysis of the membranes are shown in Table III. It should be indicated that the control membrane values are similar to those previously reported for lobster nerve membrane [31]. The data shown (Table III) reveal that, in conditions when no sodium channel activity was observed as a consequence of the enzymatic action, 34% of the phospholipids were transformed into lysophosphatides. Phosphatidylcholine and phosphatidylethanolamine were the phospholipids mainly affected. It should be pointed out that the results shown were obtained analyzing the phospholipids present in the membrane pellets and in the supernatants. This procedure allows to quantify the actual percentage of each phospholipid in order to compare the different membrane samples. The supernatants were collected, lyophilizied and extracted by the method of Folch et al. [22], and the phospholipids analyzed by thin-layer chromatography. The phospholipids present in the supernatants when albumin was utilized were lysophosphatides (around 85% of the total phosphorus) and low amounts of phosphatidylcholine and sphingomyelin. This result is in agreement with the reported ability of the serum albumin to remove lysophosphatides and free fatty acids from the membranes [32,33]. It should be indicated that the resultant lysophosphatides were not exactly analyzed; it may be probably a mixture of lysophosphatidylcholine and lysophosphatidylethanolamine produced by the hydrolysis of the respective phospholipids which are mainly affected by the enzyme (Table III). The phospholipase A_2

Fig. 2. Polyacrylamide gel electrophoresis of the membranes. (a) Control; (b) treated with enzyme; (c) treated with enzyme and washed with albumin solution; (d) treated with enzyme in the presence of albumin. Delipidated membrane samples were dissolved in 1% SDS and 25 μg of each were applied on the gel. For details see Materials and Methods.

action was similar whether albumin was present or absent during the enzyme-membrane incubation period (Table III).

Table IV shows the percent phospholipid composition in the pellets of membrane with or without sodium channel activity. It can be observed that the channel activity does not appear to be determined by the phospholipid composition of the membrane. A similar phospholipid composition was found in the membrane after the enzyme treatment with either albumin present during the incubation period or used only to wash the membrane. However, the sodium channel activity was preserved only when the membrane was incubated with enzyme in the presence of albumin. The data (Table IV) also show that the lysophosphatides formed by the enzymatic action are not totally removed by the albumin whether used during the incubation with the phospholipase or to wash the membrane after its enzyme treatment (Table IV). Since the difference in the lysophosphatides content in both membranes (active or inactive) is not statistically significant it appears that without the protection of the albumin the membrane is damaged irreversibly.

Analysis of the membrane components indicate that there is no relationship between membrane phospholipid composition and channel activity (Table IV). The ratio between phosphorus and protein into the membrane can vary (Table II) without detectable changes in the sodium channel activity. The phospholipid: cholesterol molar ratio, measured in one experiment, varied from 0.37 (control membrane) to 0.28 (membrane treated with enzyme + albumin) and in both cases the channel activity was observed. In the membrane, up to 14% of its phospholipids can be lysophosphatides without channel activity being affected (Table IV). In the present experiments phosphatidylserine remains unaffected by the phospholipase A_2. It has been found that phospholipase C from *Bacillus cereus* hydrolyzes all nerve membrane phospholipids including phosphatidylserine whereas that from *Clostridium perfringens* does not hydrolyze phosphatidylserine [11]. Both enzymes inhibit tetrodotoxin binding activity of the nerve membrane [11]. This result indicates that hydrolysis of phosphatidylserine does not appear to be relevant in affecting tetrodotoxin binding activity [11].

TABLE III

PERCENT PHOSPHOLIPID COMPOSITION OF SAMPLES (PELLET+SUPERNATANT) OF MEMBRANE TREATED WITH PHOSPHOLIPASE A_2 IN THE PRESENCE OR ABSENCE OF BOVINE SERUM ALBUMIN

Values are the mean±S.E. from six determinations for the control and enzyme-treated membranes and from three determinations for the other membranes. The amounts of the phospholipids present in the membrane pellets and its supernatants were evaluated. The total phosphorus content (pellet+supernatant) was taken as 100%.

Phospholipid	Membrane			
	Control	Enzyme treated	Enzyme treated and washed with albumin	Enzyme treated in the presence of 2.5% albumin
Phosphatidylcholine	25.2±1.4	9.1±1.9	12.1±4.7	13.1±2.7
Phosphatidylethanolamine	36.3±1.9	6.4±1.7	9.7±2.9	5.8±2.3
Sphingomyelin	13.5±0.6	18.5±2.8	13.8±3.2	19.7±1.6
Phosphatidylserine	16.6±2.7	22.7±3.3	22.4±4.8	20.8±2.6
Phosphatidylinositol	3.4±0.9	3.9±1.2	3.3±1.2	2.1±1.0
Phosphatidic acid	5.0±0.8	5.2±2.8	4.9±1.3	3.5±1.3
Lysophosphatides [a]	–	34.3±3.1	33.8±6.8	34.9±3.8

[a] Probably a mixture from lysophosphatidylcholine and lysophosphatidylethanolamine.

The inhibitory effect of the phospholipase A_2 on tetrodotoxin and saxitoxin binding has been extensively discussed [11,12]. These results appear to indicate that the important cause for the toxins binding inhibition is the surfactant character of the resultant enzymatic products rather than a critical degradation of the phospholipids surrounding fundamental membrane components. So, the homogenization of isolated lobster nerve membrane with unsaturated fatty acids [12], or the addition of increasing amounts of lysophosphatidylcholine to the garfish axonal membrane [11]

TABLE IV

PERCENT PHOSPHOLIPID COMPOSITION OF THE MEMBRANE PELLET WITH OR WITHOUT SODIUM CHANNEL ACTIVITY

Values are the mean±S.E. from six determinations for the control membrane and from three determinations for the other membranes. The total phosphorus content was taken as 100%. The phospholipids amounts are only those present in the membrane pellets used for the sodium influx measurements.

Phospholipid	Membrane		
	Control (Active)	Treated with enzyme and washed with albumin (Inactive)	Treated with enzyme in the presence of albumin (Active)
Phosphatidylcholine	25.2±1.4	21.4±3.3	19.9±2.6
Phosphatidylethanolamine	36.3±1.9	10.6±2.9	7.4±3.4
Sphingomyelin	13.5±0.6	21.0±1.7	24.2±0.4
Phosphatidylserine	16.6±2.7	23.1±4.5	29.7±4.8
Phosphatidylinositol	3.4±0.9	2.1±1.0	3.0±1.1
Phosphatidic acid	5.0±0.8	4.8±1.5	4.0±1.2
Lysophosphatides	–	17.0±0.9	13.6±2.9

can mimic the phospholipase A_2 inhibitory effect on saxitoxin and tetrodotoxin binding capacity. In each case no membrane solubilization was reported, and the toxins binding was not restored by washing the enzyme treated membrane with albumin solution [11,12]. The albumin protective effect was ascribed [11,12] to its well known ability [32,33] to remove lysophospholipids and free fatty acids from the membrane. In the present work, it also appears that the inhibitory effect of phospholipase A_2 on the sodium channel activity is due to the contact between the membrane and the surfactant enzymatic products rather than to a critical enzymatic degradation of the phospholipids. A relevant disarrangement in the membrane had to occur since the enzyme-treated membrane washed with albumin did not restore, at least partly, the channel activity or the toxins binding. Although direct evidences can be obtained about the damage produced by the surfactant products, other types of experimental approach are required to explore the role played by the arrangement of the membrane structure in the enzymatic action as well as in the albumin protective effect.

The present results indicate that there is no relationship between membrane phospholipid composition and sodium channel activity. The results demonstrate that in the presence of albumin the treatment of the membrane with phospholipase A_2 does not affect its functional response to both veratridine and tetrodotoxin. The finding may be interpreted as indicating that the sites which respond to veratridine and to tetrodotoxin are in a particular membrane structure, or that the sites share similar structural characteristics; whichever the case, albumin protects the sites from the enzyme action.

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