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Effect of Purified Phospholipases on the Binding of Tetrodotoxin to Axon Plasma Membrane

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Summary. The role of phospholipids in the binding of [^{3}H] tetrodotoxin to garfish olfactory nerve axon plasma membrane was studied by the use of purified phospholipases. Treatment of the membranes with low concentrations of either phospholipase A_2 (*Crotalus adamanteus* and *Naja naja*) or phospholipase C (*Bacillus cereus* and *Clostridium perfringens*) resulted in a marked reduction in tetrodotoxin binding activity. A 90% reduction in the activity occurred with about 45% hydrolysis of membrane phospholipids by phospholipase A_2, and with phospholipase C the lipid hydrolysis was about 60–70% for a 70–80% reduction in the binding activity. Phospholipase C from *B. cereus* and *Cl. perfringens* had similar inhibitory effects. Bovine serum albumin protected the tetrodotoxin binding activity of the membrane from the inhibitory effect of phospholipase A_2 but not from that of phospholipase C. In the presence of albumin about 25% of the membrane phospholipids remained unhydrolyzed by phospholipase A_2. It is suggested that these unhydrolyzed phospholipids are in a physical state different from the rest of the membrane phospholipids and that these include the phospholipids which are directly related to the tetrodotoxin binding component. It is concluded that phospholipids form an integral part of the tetrodotoxin binding component of the axon membrane and that the phospholipase-caused inhibition of the binding activity is due to effects resulting from alteration of the phospholipid components.

Specific receptors for tetrodotoxin and saxitoxin have been demonstrated in various nerve and muscle membrane preparations (*see* recent review by Ritchie and Rogart, 1977). Tetrodotoxin and saxitoxin block specifically and reversibly the inward movement of sodium ions in the initial phase of the action potential in axon and muscle membranes (Narahashi, 1974). The exact structural relationship between the tetrodotoxin binding component and the sodium channel, however, is not known.

Although there are numerous reports (Cook, Low & Ishijimi, 1972, and references cited therein) implicating an essential role of phospholipids in the excitable properties of live axons, their possible role in the interaction of tetrodotoxin to axon plasma membrane preparations has received

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only limited study. In axon plasma membrane, phospholipids alone constitute more than 50% of its weight (Chacko *et al.*, 1974*a*; Balerna *et al.*, 1975). Proteolytic and phospholipase hydrolysis studies on isolated excitable membrane-rich preparations (Benzer & Raftery, 1972; Reed & Raftery, 1976) suggest that the tetrodotoxin binding component is a protein embedded in a phospholipid environment or a lipoprotein. Of the phospholipases tried, only phospholipase A was able to significantly reduce the tetrodotoxin binding ability of the membrane. Partial purification of a tetrodotoxin binding component from Lubrol-PX solubilized electroplax membrane has recently been accomplished (Agnew *et al.*, 1978). Inclusion of phospholipids in the medium during purification was required for stabilization of the toxin binding component.

The present studies were undertaken to examine, in detail, the role of phospholipids in the binding of tetrodotoxin to isolated axon membrane. Axon plasma membrane preparations from garfish olfactory nerve were treated with purified phospholipases, and the resulting changes in the tetrodotoxin binding activities and the phospholipid compositions of the membranes were measured. Alteration of phospholipid composition of the membrane by either phospholipase A_2 or phospholipase C resulted in a marked reduction in the tetrodotoxin binding activity of the membrane. A correlation was found between the extent of hydrolysis of membrane phospholipids and the extent of the inhibition of the tetrodotoxin binding activity of the membrane. Results from the phospholipase A_2 hydrolysis in the absence and presence of bovine serum albumin suggest that the inhibition caused by phospholipase A_2 was due to the products of enzyme hydrolysis; however, that caused by phospholipase C seems to be due to effects resulting from changes in membrane phospholipid composition. These results also seem to indicate that the tetrodotoxin binding component is associated with phospholipids of a different physical state from the bulk of the membrane lipids and that these associated lipids are essential for the interaction of tetrodotoxin to the receptor.

Materials and Methods

Materials

Tetrodotoxin (citrate-free) was obtained from Sankyo Ltd., Tokyo, [^{3}H] tetrodotoxin, labeled by the method of Wilzbach (1957) and purified according to the procedure of Hafemann (1972) as modified by Benzer and Raftery (1972) was used. The specific activity

of the toxin, determined by measuring the biologically active component as previously described (Barnola, Villegas & Camejo, 1973) was 13.0 Ci/mole. It has an apparent radiochemical purity of about 85%. *Crotalus adamanteus* and *Naja naja* venoms were purchased from the Miami Serpentarium (Miami, Florida). Phospholipase C from *Clostridium perfringens* (94 units/mg) and from *Bacillus cereus* (870 units/mg) were obtained from Worthington Corp. and Sigma Chemical Co., respectively. Bovine serum albumin (fatty acid free) was purchased from Miles Laboratories. Azoalbumin was obtained from Sigma Chemical Co. ^{125}I-labeled albumin was a gift from Dr. C. Sparks, and it was prepared by radioiodination of rabbit serum albumin by the iodine monochloride method of McFarlane (1958). Lysophosphatidylcholine (egg) was obtained from Supelco, Bellefonte, Pa.

Methods

Purification of Phospholipases. C. adamanteus phospholipase A_2 was purified according to the procedure of Wells (1975) and *N. naja* phospholipase A_2 was purified by procedures described by Martin *et al.* (1975). The enzyme preparations used were homogeneous as indicated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Chacko *et al.*, 1976). Phospholipase A_2 activity was assayed according to the procedure of Rock and Snyder (1975), using egg phosphatidylcholine as the substrate. Products of hydrolysis were resolved and analyzed by thin-layer chromatography. Specific activity of the phospholipase A_2 preparations were not determined. Phospholipase C from *Cl. perfringens* (94 units/mg protein) and from *B. cereus* (870 units/mg protein) were used without further purification. None of the above enzyme preparations used in these studies has any protease activity as assayed by two methods. In one method, protease activity was assayed according to the procedure of Murata, Satake and Suzuki (1963) using azoalbumin as the substrate. In the other method, phospholipase preparation was incubated with ^{125}I-labeled rabbit serum albumin under conditions that were used to inactivate the tetrodotoxin binding activity and the radioactivity remaining in the undigested protein was determined after sodium dodecyl sulfate polyacrylamide gel electrophoresis. There was no proteolytic effect on labeled albumin even at enzyme concentrations ten times that required to inactivate the tetrodotoxin binding activity.

Axon Plasma Membrane. The procedure used for the isolation of axon plasma membrane fraction from garfish olfactory nerve has been reported previously (Chacko *et al.*, 1974*a*). The axon plasma membrane fraction was identified from its $(Na^+ + K^+)$-activated ATPase and [^{3}H] tetrodotoxin binding activities (Chacko *et al.*, 1974b). The membrane preparations used in the present studies were stored frozen at -30 °C for up to 3 months in 0.250 M sucrose, 10 mM Tris/HCl (pH 7.4) until used. No difference in $(Na^+ + K^+)$-activated ATPase activity, tetrodotoxin binding activity, and phospholipid composition were observed when the frozen samples were compared with those of nonfrozen samples analyzed immediately.

Tetrodotoxin Binding. The specific binding of [^{3}H] tetrodotoxin to membrane preparations were measured by the centrifugal assay procedure, described by Barnola *et al.* (1973). The concentration of [^{3}H] tetrodotoxin used was 10 nM. For the tetrodotoxin binding assay, duplicate membrane samples were used, one of each pair serving to measure the nonspecific binding. The specific binding of [^{3}H] tetrodotoxin to the membrane was determined by subtracting the radioactivity present in pellets of membrane pre-incubated with 100-fold excess of unlabeled tetrodotoxin from that present in pellets of membrane incubated with [^{3}H] tetrodotoxin only. Incubation with tetrodotoxin and centrifugation afterwards of the membrane suspensions were carried out at 4 °C.

Treatment of Membranes with Phospholipases. Aliquots of axon membrane preparations (1 mg protein/ml) were incubated with phospholipase A_2 and phospholipase C (*see* legends

for concentrations) in 1 ml of 10 mM Tris/HCl (pH 7.4), 0.15 M NaCl and 1 mM $CaCl_2$ at 37 °C for 30 min with agitation. After incubation ethylene glycol bis (β-aminoethyl ether)-N, N′-tetraacetic acid (EGTA) was added to each tube to a final concentration of 10 mM. The samples were cooled by placing over ice and the membranes recovered by centrifugation at $100,000 \times g$ for 1 hr. The membranes were then washed three times with 2 ml of 10 mM Tris/HCl (pH 7.4), 0.15 M NaCl and analyzed for their tetrodotoxin binding activity and for their phospholipid composition.

Extraction and Analysis of Phospholipids. Lipids were extracted according to the procedure of Bligh and Dyer (1959). Thus, to 1.35 ml of membrane suspension were added successively 3.4 ml methanol, 1.7 ml chloroform, 1.7 ml chloroform, and 1.7 ml of water. The contents were mixed thoroughly after each addition. The two phases were separated by centrifugation in a clinical centrifuge. After removal of the chloroform layer, the upper layer was reextracted with 2 ml chloroform. The combined chloroform layers were evaporated to dryness under nitrogen and the residue dissolved in chloroform-methanol (1:1). Aliquots of lipid extract were then analyzed for total lipid phosphorus and phospholipid composition. Lipid phosphorus was determined by the micro method of Rouser, Siakotos and Fleischer (1966) and the phospholipid composition by two-dimensional thin-layer chromatography followed by phosphorus analysis, as described elsewhere in detail (Chacko, Goldman & Pennock, 1972). All of the axon membrane phospholipids and their lyso compounds were separable in the chromatographic system. The components were identified by comparison with authentic lipid standards (Supelco, Pa.) Total recovery of the applied phospholipid phosphorus was above 90%. The degree of hydrolysis of individual phospholipids was obtained by comparison of the lipid composition of the enzyme-treated membrane to that of the control membrane.

Treatment of Membrane with Lysophosphatidylcholine. Aliquots of membrane preparations (1 mg protein) in 1 ml of 10 mM Tris/HCl (pH 7.4), 0.15 M NaCl and 1 mM $CaCl_2$, were treated with increasing concentrations of lysophosphatidylcholine at 37 °C for 30 min with agitation. The membranes were then recovered by centrifugation at $100,000 \times g$ for 1 hr and washed two times with 2 ml of 10 mM Tris/HCl (pH 7.4), 0.15 M NaCl. The washed membranes were then analyzed for their tetrodotoxin binding activities and protein concentrations. Protein concentrations were determined by the method of Lowry *et al.* (1951).

Results

Binding of Tetrodotoxin to Axon Membrane

As shown previously (Chacko *et al.*, 1974*b*), [^{3}H] tetrodotoxin binds to the axon plasma membrane isolated from garfish olfactory nerve with a dissociation constant of 5.5×10^{-9} M and a maximum binding of 3.7 pmol tetrodotoxin per mg membrane protein. The possible presence of radioactive impurities in the Wilzbach-labeled tetrodotoxin, even after extensive purification, has recently been suggested by Ritchie and Rogart (1977). The presence of such impurities would underestimate the binding capacity of the membrane preparations, but would probably not affect the results or conclusions reached from the studies presented in this

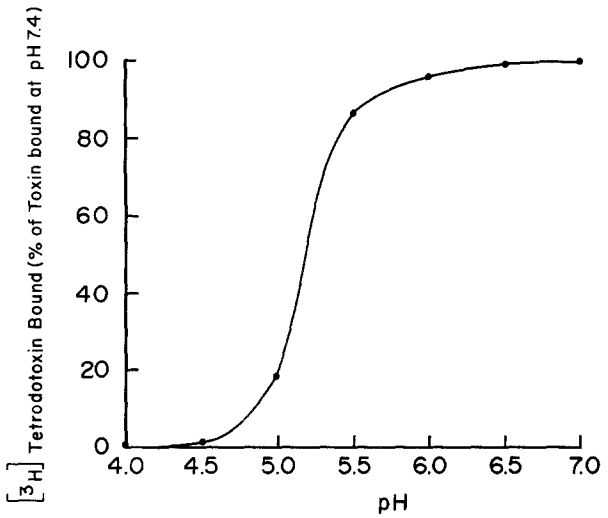

Fig. 1. Effect of pH on the specific binding of [^{3}H] tetrodotoxin to garfish olfactory nerve axon plasma membrane. Aliquots of membranes (1 mg protein) were suspended in 1 ml of mixed buffer (10 mM each of acetate, phosphate, and Tris) containing 150 mM NaCl adjusted to the desired pH, and the binding of [^{3}H] tetrodotoxin to the membrane at 10 nM concentration of toxin was determined by the centrifugal assay procedure described under *Methods*

paper. The binding of tetrodotoxin to the membrane is pH dependent as shown in Fig. 1. The pK_a of the ionizable group is 5.2, and this value is similar to values reported for intact nerve bundles (Ritchie & Rogart, 1977) and to plasma membranes isolated from crab nerve (Balerna *et al.*, 1975) and electric organ of *Electrophorus electricus* (Reed & Raftery, 1976).

Effect of Phospholipase A_2 on the Binding of Tetrodotoxin

Treatment of axon plasma membrane preparations with low concentrations of purified phospholipase A_2 from either *C. adamanteus* or from *N. naja* venom resulted in a decrease in the binding of tritium labeled tetrodotoxin (Table 1). Chelation of Ca^{++} by the addition of EGTA prior to the treatment of the axon membrane with the enzyme completely prevented the inhibitory effect of phospholipase A_2 on the specific tetrodotoxin binding activity of the membrane. As expected, there was no hydrolysis of membrane phospholipids by the enzyme in the presence of EGTA (not shown). The observed inhibitory effect of phospholipase

Table 1. Effect of phospholipase A_2 treatment on the specific binding of [^{3}H] tetrodotoxin to axon plasma membrane[a]

Treatment	Tetrodotoxin binding activity[b] (% of control)
1. None	100
2. Phospholipase A_2 (*N. naja*)	14.2 (9.8–18.4)
3. Phospholipase A_2 (*N. naja*)+EGTA	99.6 (92.3–112.0)
4. Phospholipase A_2 (*C. adamanteus*)	24.6 (18.3–28.7)
5. Phospholipase A_2 (*C. adamanteus*)+EGTA	97.3 (93.4–102.2)

[a] Aliquots of membrane (1 mg protein) were incubated with 1 μg phospholipase A_2 in the absence and presence of 10 mM EGTA in 1 ml of 10 mM Tris/HCl (pH 7.4) buffer containing 0.150 M NaCl and 1 mM $CaCl_2$ at 37 °C for 30 min. The treated membranes were washed and the specific tetrodotoxin binding activities determined.

[b] Average of three experiments. Range is shown in parentheses

A_2 on the tetrodotoxin binding activity of the membrane, therefore, was a result of the intrinsic activity of the enzyme. Treatment of the membrane to 1 mM Ca^{++} or 10 mM EGTA in the absence of enzyme did not have any effect on the specific tetrodotoxin binding activity of the membrane; however, incubation of the membrane at 37 °C for 30 min resulted in the reduction of the activity by 10–15%. In all experiments reported in this paper, the control membrane sample was incubated at 37 °C for 30 min along with the experimental samples, and the tetrodotoxin binding activity of this membrane was expressed as 100%.

Relationship between the Phospholipid Hydrolysis and the Tetrodotoxin Binding Activity

The effect of varying concentrations of phospholipase A_2, *N. naja*, on the specific binding of tetrodotoxin to the membrane is shown in Fig. 2. The tetrodotoxin binding activity was almost completely lost by treatment of 1 mg protein containing membrane with about 1 μg of enzyme at 37 °C for 30 min. A progressive loss of the tetrodotoxin binding activity of the membrane resulted with increasing time of phospholipase A_2 treatment. A direct relationship was found between the extent of

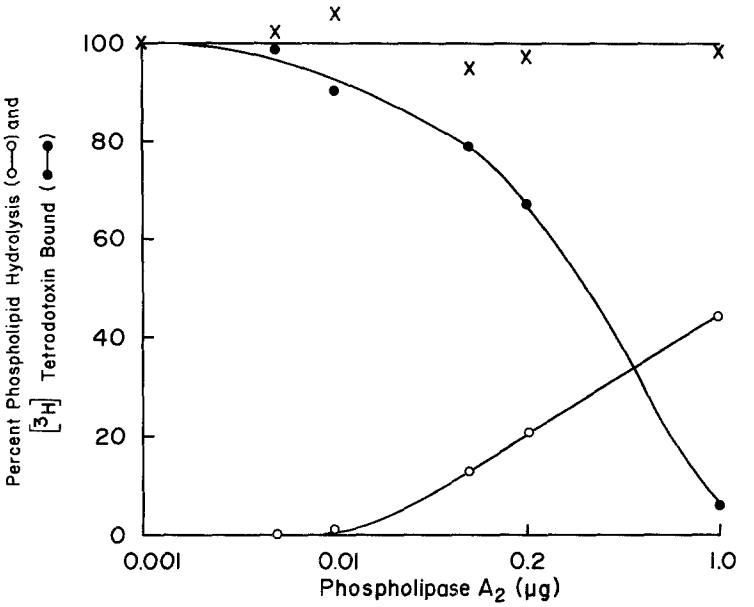

Fig. 2. Effects of varying concentrations of phospholipase A_2 (*N. naja*) on the specific tetrodotoxin binding activity and the extent of phospholipid hydrolysis of axon plasma membrane. Aliquots (1 mg protein) of membrane preparations were incubated with indicated concentrations of phospholipase A_2 in 1 ml of 10 mM Tris/HCl (pH 7.4) buffer containing 0.150 M NaCl and 1 mM $CaCl_2$ at 37 °C for 30 min. The phospholipase A_2 reaction was stopped by the addition of EGTA to a final concentration of 10 mM. The membranes were then washed, and the specific binding of [^{3}H] tetrodotoxin was determined. Lipids from identically treated duplicate samples were extracted and analyzed as described under *Methods*. ×—×, total lipid phosphorus in the treated membranes; ●—●, [^{3}H] tetrodotoxin bound expressed as percentage of control; o—o, phospholipid hydrolyzed as percentage of total phospholipids

hydrolysis of phospholipids and the loss of the tetrodotoxin binding activity of the membrane. The tetrodotoxin binding activity of the membrane was completely lost with about 45% hydrolysis of the membrane phospholipids. At this level of hydrolysis only phosphatidylcholine and phosphatidylethanolamine were hydrolyzed, phosphatidylethanolamine being hydrolyzed faster than phosphatidylcholine. At higher concentrations of enzyme, phosphatidylserine and phosphatidylinositol were hydrolyzed, but not sphingomyelin. The total phospholipid phosphorus of the membrane before and after treatment with phospholipase A_2 remained constant (Fig. 2 and Table 2), indicating that the lysophospholipids formed by the action of phospholipase A_2 on the membrane phospholipids remained associated with the membrane. These lysophospholi-

Table 2. Removal of lysophospholipids from the phospholipase A_2 treated membranes by washing with bovine serum albumin[a]

	Lipid phosphorus (μg)
1. Control membrane	84
2. Membrane + phospholipase A_2 (washed with buffer containing no albumin)	91
3. Membrane + phospholipase A_2 (washed with buffer containing 2.5% albumin)	56
4. Membrane + phospholipase A_2 + 2.5% albumin (washed with buffer containing 2.5% albumin)	55
5. Albumin washings from (3)	44
6. Albumin blank (2.5%)	4

[a] Aliquots of membrane preparations (1.5 mg protein) were incubated with 1.5 μg *N. naja* phospholipase A_2 in the absence and presence of 2.5% albumin in 1.5 ml of 10 mM Tris/HCl (pH 7.4) buffer containing 0.150 M NaCl and 1 mM $CaCl_2$ at 37 °C for 30 min. The treated membranes were washed two times with buffer containing 2.5% albumin. Lipids were extracted from the washed membranes and the albumin washings, and analyzed for total lipid phosphorus. Membranes *1* and *2* were also washed with the same volume of buffer and number of times as those of *3* and *4*, except that the medium contained no albumin. Sample *3* was treated with phospholipase A_2 in the absence of albumin and sample *4* in the presence of albumin. The phospholipids removed by albumin washings were identified as lysophosphatidylethanolamine and lysophosphatidylcholine.

pids were not removed by repeated washings of the enzyme treated membrane with the incubation medium (Table 2). This is in agreement with similar studies on other membranes (Fleischer & Fleischer, 1967).

Effect of Lysophosphatidylcholine on the Binding of Tetrodotoxin

Since the products of hydrolysis of phospholipase A_2, namely, lysophospholipids and free fatty acids, remained associated with the membrane and since they are powerful detergents likely to disrupt the structure of the tetrodotoxin receptor, the effect of an added lysophospholipid, namely, lysophosphatidylcholine, on the specific binding of tetrodotoxin to the membrane was investigated. The results are shown in Fig. 3. Added lysophosphatidylcholine inhibited the tetrodotoxin binding activity of the membrane and this inhibitory effect was proportional to the concentration of added lysophosphatidylcholine. There was no significant solubilization of the membrane protein at this concentration range of added

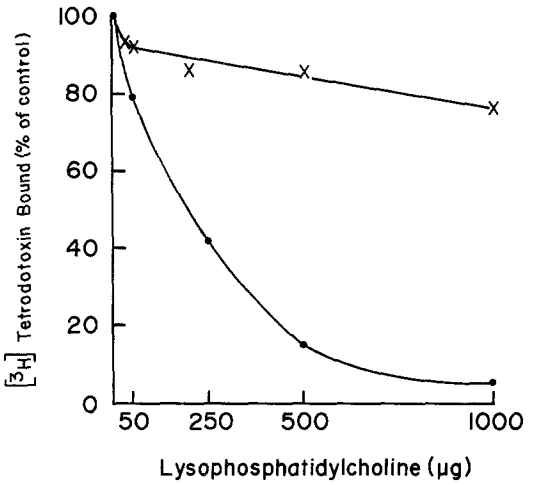

Fig. 3. Effect of added lysophosphatidylcholine on the specific binding of [^{3}H] tetrodotoxin to the axon plasma membrane. Aliquots (1 mg protein) of membrane preparations were incubated under the same conditions as described for Fig. 2 with increasing concentrations of lysophosphatidylcholine. The treated membranes were then washed and the binding of [^{3}H] tetrodotoxin determined. ●—●, [^{3}H] tetrodotoxin bound, expressed as percentage of control; ×—×, protein remaining in the membrane after treatment with lysophosphatidylcholine

lysophosphatidylcholine. No tetrodotoxin binding activity was detected in the supernatants. That lysophosphatidylcholine mimicked the effect of phospholipase A_2 on the tetrodotoxin binding activity of the membrane suggests that the inhibition brought about by phospholipase A_2 was due to the lysophospholipids produced *in situ*. The effect of added fatty acids was not studied.

Protective Effect of Bovine Serum Albumin on the Tetrodotoxin Binding Activity of the Membrane from Phospholipase A_2 Inhibition

The lysophospholipids and the free fatty acids formed by the action of phospholipase A_2 on the membrane phospholipids and which remained in association with the membrane were completely removed from the membrane by washing it with a medium containing 2.5% albumin (Table 2). The tetrodotoxin binding activity of the membrane was, however, not restored (Table 3), indicating an irreversible destruction of the structure of the tetrodotoxin receptor by phospholipase A_2. Inclusion of 1

Table 3. Protective effect of albumin on the phospholipase A_2 caused destruction of the tetrodotoxin binding activity of axon plasma membrane[a]

	Tetrodotoxin binding activity (% of the control)
1. Control membrane	100
2. Membrane + phospholipase A_2 (1 μg)	20[b]
3. Membrane + albumin (1%) + phospholipase A_2 (1 μg)	90
4. Membrane + albumin (2.5%) + phospholipase A_2 (1 μg)	95
5. Membrane + albumin (2.5%) + phospholipase A_2 (10 μg)	105
6. Membrane + albumin (2.5%) + phospholipase A_2 (100 μg)	94

[a] Aliquots of membranes (1 mg protein) were incubated with indicated concentrations of *N. naja* phospholipase A_2 in 1 ml of 10 mM Tris/HCl (pH 7.4) buffer containing 150 mM NaCl and 1 mM $CaCl_2$ in the absence and presence of albumin at 37 °C for 30 min. The reactions were stopped by the addition of EGTA to a final concentration of 10 mM. The membranes were then washed, and the specific tetrodotoxin binding activities were determined.

[b] The tetrodotoxin binding activity of the phospholipase A_2 treated membrane was not restored by washing the treated membranes with buffer containing 1.0% or 2.5% albumin.

or 2.5% albumin in the medium completely prevented the inhibitory effect of phospholipase A_2 on the tetrodotoxin binding activity of the membrane (Table 3). In the presence of albumin even much higher concentrations of enzyme were without any effect on the tetrodotoxin binding activity of the membrane. Since defatted albumin is well known for its ability to remove both lysophospholipids and free fatty acids from membranes (Fleischer & Fleischer, 1967; Azhar, Harja & Menon, 1976), the protective effect of albumin on the tetrodotoxin binding activity of the membrane from the phospholipase A_2 inhibition is likely due to the removal by albumin of the lysophospholipids and free fatty acids formed during the enzyme treatment. When it was found that in the presence of albumin the tetrodotoxin binding activity was not affected by treatment of the membrane with phospholipase A_2 even at concentration 100-fold in excess of that required to completely inactivate the tetrodotoxin binding activity in the absence of albumin (Table 3), it was felt that either the membrane phospholipids were not involved in the binding of tetrodotoxin to the membrane or that, in the presence of albumin, those membrane phospholipids that might be associated with the tetrodotoxin binding component of the membrane were not

hydrolyzed by phospholipase A_2 in the presence of albumin. Therefore, the extent of hydrolysis of membrane phospholipids by phospholipase A_2 in the presence of albumin was examined. Treatment of 1 mg protein containing membrane with 1 μg of enzyme at 37 °C for 30 min resulted in the hydrolysis of about 45% of phospholipids both in the absence and presence of 2.5% albumin. However, at higher concentrations of enzymes the extent of hydrolysis of membrane phospholipids were less in the presence of albumin. Thus, in the absence of albumin nearly all of the hydrolyzable phospholipids of 1 mg protein containing membrane were hydrolyzed by 100 μg enzyme, while in the presence of albumin only about 75% of the phospholipids were hydrolyzed by the same amount of enzyme. Analysis of phospholipids remaining unhydrolyzed by phospholipase A_2 in the presence of albumin did not reveal any striking enrichment of any special class of phospholipids. However, details on the chemical nature as well as the physical state of these lipids have yet to be studied.

Effect of Phospholipase C on the Binding of Tetrodotoxin

Treatment of axon membrane with small concentration of phospholipase C also resulted in an inhibition of the tetrodotoxin binding activity of the membrane. In Fig. 4 are shown the data on the effect of treatment of 1 mg protein containing membrane with increasing concentrations of *B. cereus* phospholipase C at 37 °C for 30 min. The loss of tetrodotoxin binding activity of the membrane was directly proportional to the concentration of the enzyme used. About 70–80% of the tetrodotoxin binding activity of the membrane was lost by treatment of about 1 mg protein containing membrane with 1 unit of *B. cereus* phospholipase C. A progressive loss of the tetrodotoxin binding activity resulted with increasing time of phospholipase C treatment. Similar results were also obtained with *Cl. perfringens* phospholipase C. Addition of EGTA (10 mM) to the incubation medium before the enzyme treatment completely prevented the inhibitory effect of *Cl. perfringens* phospholipase C on the tetrodotoxin binding activity of the membrane (Table 4), showing that the inhibitory effect was due to the intrinsic activity of the phospholipase C and not due to any contaminant in the enzyme preparation. The presence of EGTA in the incubation medium, however, had no effect on the inhibition of *B. cereus* phospholipase C. This would be expected since the addition of chelating agents simultaneously to the

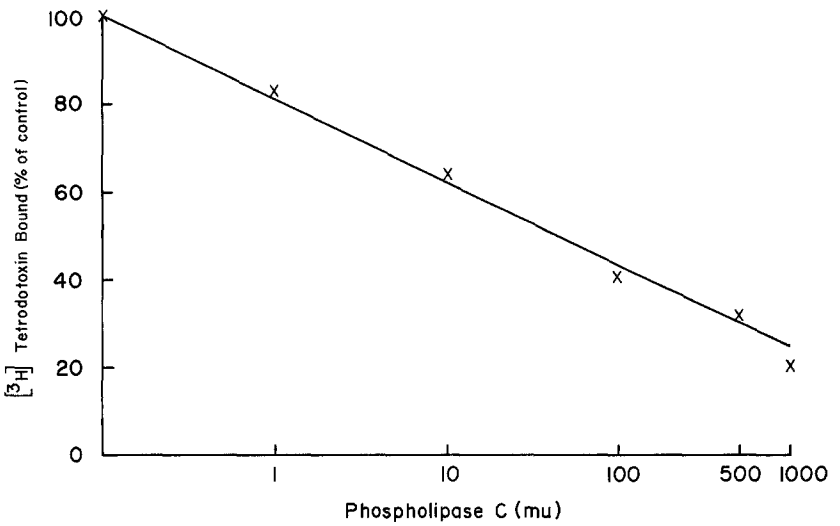

Fig. 4. Effect of treatment of axon plasma membrane with increasing concentrations of phospholipase C, on the specific binding of [^{3}H] tetrodotoxin. Aliquots of membrane (1 mg protein) were treated with the indicated concentrations of *B. cereus* phospholipase C under the same conditions as described in Fig. 2 at 37 °C for 30 min. The membranes were then washed, and the specific [^{3}H] tetrodotoxin binding activity was determined

Table 4. Effect of phospholipase C treatment on the specific binding of [^{3}H] tetrodotoxin to axon plasma membrane[a]

Treatment	Tetrodotoxin binding activity[b] (% of control)
1. None	100
2. Phospholipase C (*Cl. perfringens*)	34.5 (23.1–41.0)
3. Phospholipase C (*Cl. perfringens*) + EGTA	91.1 (77.0–108.0)
4. Phospholipase C (*B. cereus*)	29.2 (18.5–35.7)
5. Phospholipase C (*B. cereus*) + EGTA	19.4 (17.8–21.1)

[a] Aliquots of membrane (1 mg protein) were incubated with 1 unit of phospholipase C in the absence and presence of 10 mM EGTA in 1 ml of 10 mM Tris/HCl (pH 7.4) buffer containing 0.150 M NaCl and 1 mM $CaCl_2$ at 37 °C for 30 min. The treated membranes were washed and their specific tetrodotoxin binding activities were determined.

[b] Average of four experiments. Range is shown in the parentheses.

incubation medium is known to have no effect on the activity of phospholipase C from *B. cereus* (Ottolenghi, 1969); long-term incubation with chelators is required for inhibition of the enzyme activity.

Effect of Phospholipase C on the Phospholipid Composition

To ascertain that the observed inhibitory effect of phospholipase C on the tetrodotoxin binding activity of the membrane was due to the hydrolysis of membrane phospholipids, the enzyme treated membranes were simultaneously analyzed for their phospholipid compositions. In Fig. 5 are shown the data on the extent of hydrolysis of individual membrane phospholipids when treated with increasing concentration of phospholipase C from *B. cereus* (*A*) and from *Cl. perfringens* (*B*). As anticipated from their known properties (Zwaal *et al.*, 1975) the enzymes from these two sources showed different specificities to axon membrane phospholipids. In the case of *B. cereus* phospholipase C, phosphatidylethanolamine and phosphatidylcholine were hydrolyzed faster than phosphatidylserine, which in turn was hydrolyzed faster than

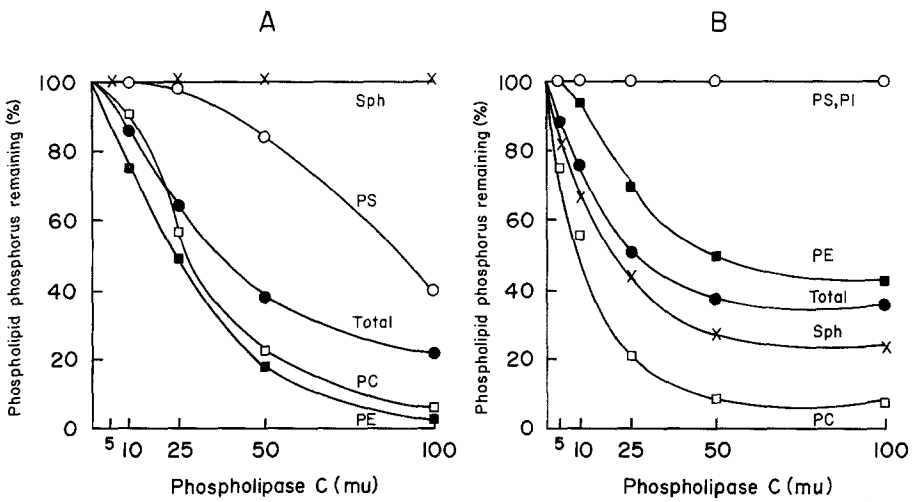

Fig. 5. Effect of treatment of increasing concentrations of phospholipase C (*A, B. cereus; B, Cl. perfringens*) on the extent of hydrolysis of axon membrane individual phospholipids. Aliquots of membrane (1 mg protein) were treated with the indicated concentrations of phospholipase C under the same conditions as described in Fig. 2 at 37 °C for 30 min. The lipids were extracted from the treated membranes and analyzed as described under *Methods*. □—□, phosphatidylcholine; ■—■, phosphatidylethanolamine; ○—○, phosphatidylserine; ×—×, sphingomyelin; ●—●, total phospholipid

sphingomyelin. The order of reactivity of the *Cl. perfringens* enzyme towards individual membrane phospholipids was phosphatidylcholine > sphingomyelin > phosphatidylethanolamine. In both cases, about 60–70% of the total membrane phospholipids were hydrolyzed at a faster rate than the rest of the phospholipids; much higher concentrations of enzymes were required for the hydrolysis of the remaining phospholipids.

Discussion

The results presented in this paper demonstrate that treatment of garfish olfactory nerve axon plasma membrane with small concentration of either phospholipase A_2 or phospholipase C causes a marked reduction in the tetrodotoxin binding activity of the membrane. These inhibitory effects of phospholipases are abolished in the absence of free Ca^{++} ions, suggesting that the observed effects are the results of the intrinsic activities of the phospholipases and not due to nonspecific effects.

The inhibitory effect of phospholipase A_2 on the tetrodotoxin binding activity of axon membrane may be explained at least in two ways. In one, the phospholipase A_2 inhibition results from the hydrolysis of phospholipids directly associated with the tetrodotoxin receptor. This association of the phospholipids to the tetrodotoxin binding protein component is presumed to be essential for the appropriate conformation of the receptor for its binding to tetrodotoxin. In the other, the phospholipids are not directly related to the tetrodotoxin binding component, and phospholipase A_2 is indirectly inactivating the tetrodotoxin binding activity of the membrane by the detergent action of the lysophospholipids produced by the enzyme.

The data on the effect of added lysophosphatidylcholine on the tetrodotoxin binding activity of the membrane (Fig. 3) and also the protective effect of albumin on the inhibitory effect of phospholipase A_2 (Table 3) would favor the second explanation. Small amounts of added lysophosphatidylcholine inhibit the tetrodotoxin binding activity of the membrane and thus mimic the action of phospholipase A_2. Inclusion of albumin in the medium during the enzyme treatment protects the tetrodotoxin binding activity of the membrane, presumably by sequestering the phospholipid hydrolysis products, *viz* lysophospholipids and free fatty acids, as they are produced during the incubation. The possibility also exists that the protective effect of albumin on the inhibitory effect of phospholi-

pase A_2 is due to the removal of free fatty acids alone and that the lysophospholipids remain associated with the membrane supporting the lipid requirement of the tetrodotoxin binding activity, similar to what was reported for succinate-cytochrome C reductase activity (Fleischer & Fleischer, 1967).

In the presence of albumin, however, a significant portion of the membrane phospholipids remain unhydrolyzed by phospholipase A_2 even at very high concentration of the enzyme, while in the absence of albumin most of the hydrolyzable phospholipids are hydrolyzed. It is therefore possible that the protective effect of albumin on the tetrodotoxin binding activity of the membrane from phospholipase A_2 inhibition is not only due to its removal of the phospholipid hydrolysis products but also due to its apparent protective effect on the hydrolysis of a portion of membrane phospholipids which may be associated with the tetrodotoxin binding component. It is possible that the phospholipids which remain resistent to hydrolysis by phospholipase A_2 in the presence of albumin are in a physical state different from the rest of the bulk membrane phospholipids. The presence of a solid-like microenvironment of lipids surrounding the sodium channel in nerve has been postulated by Lee (1976).

Because of the postulated role of phosphatidylserine in the generation of the action potentials in axon membrane (Cook *et al.*, 1972), a differential effect between the two types of phospholipase C on the tetrodotoxin binding activity of the membrane was anticipated. Phospholipase C from *B. cereus* hydrolyzes all phospholipids including phosphatidylserine where as that from *Cl. perfringens* does not hydrolyze phosphatidylserine (Fig. 5). No difference, however, was observed between the two enzymes in their effect on the tetrodotoxin binding activity of the membrane, suggesting that the observed inhibitory effect of phospholipase C is, probably, the result of hydrolysis of the polar groups of membrane phospholipids in general, and not due to hydrolysis of the polar group of phosphatidylserine in particular. There is also the possibility that the inhibitory effect of phospholipase C on the tetrodotoxin binding activity of the membrane is due to the disruption of the hydrophobic interactions, that may exist between the nonpolar residues of the phospholipids and of the receptor protein, resulting from the possible rearrangement of diglycerides, generated by the action of phospholipids C on membrane phospholipids, into fat droplets (Trump *et al.*, 1970; Coleman, 1973).

The present results on the effect of phospholipase C on the tetrodo-

toxin binding activity of the axon membrane are at variance with earlier reports (Benzer & Raftery, 1972; Reed & Raftery, 1976; Villegas, Barnola & Camejo, 1973) in which no significant reduction in the binding of tetrodotoxin was found. This difference may be due to the difference in the experimental conditions used to hydrolyze the membrane phospholipids, particularly the temperature of incubation. For example, in the present studies we have incubated the membrane preparations with enzyme at 37 °C, whereas in the earlier reports the temperature of incubation was either 0 or 24 °C which may be below the transition temperature of the membrane lipids. Unfortunately, details on the phospholipid composition of the enzyme treated membranes were not given.

The results presented in this paper thus suggest that the phospholipids form an integral part of the tetrodotoxin binding component of axon membrane and that the phospholipase-caused inhibition of the binding activity is due to effects resulting from alteration of the phospholipid components.

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