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ACTION OF PROTEASES AND PHOSPHOLIPASES ON TETRODOTOXIN BINDING TO AXOLEMMA PREPARATIONS ISOLATED FROM LOBSTER NERVE FIBRES

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SUMMARY

The action of proteases and phospholipases on the binding of tetrodotoxin to an axolemma preparation was studied. The membrane was obtained from walking-leg nerves of *Panulirus argus* lobsters. Tetrodotoxin binding from 100 nM [^{3}H]-tetradotoxin solution was measured. The binding to the nontreated (control) preparation is 9.5 ± 0.6 pmoles/mg of membrane protein (mean $\pm$ standard error; 24 measurements). The ratio of tetrodotoxin binding of enzyme-treated axolemma preparations to their respective controls is: 0.90 ± 0.06 for pronase; 1.11 ± 0.05 for trypsin; 1.00 ± 0.05 for chymotrypsin; 0.33 ± 0.07 for phospholipase A; 0.88 ± 0.12 for phospholipase C; and 0.91 ± 0.08 for phospholipase D. These results indicate that a considerable degradation of proteins and phospholipids in the axolemma does not affect tetrodotoxin binding. They are discussed in relation with current knowledge of axolemma composition, enzymic action, and tetrodotoxin binding to nerve fibres.

The isolation of plasma membranes from unmyelinated nerve fibers of squid[1] and lobster[2] has permitted us to obtain axolemma-enriched preparations containing tetrodotoxin receptors[2]. It has been found that the number of tetrodotoxin receptors present in the axolemma isolated from lobster nerve fibres (28 per μm^2 of membrane area)[2] is about the same as that found in living lobster axons[3,4]. The apparent dissociation constant of the tetrodotoxin-receptor complex is also about the same for isolated axolemma ($4.0 \cdot 10^{-9}$ M)[2] and intact axons[5,6]. Since tetrodotoxin specifically blocks the Na^+ pathways (see refs 3,4 and 7–10) it appears to be an appropriate marker for the study of the so-called Na^+ channels although the precise structural and topological relationships between tetrodotoxin receptors and Na^+ channels in the membrane are unknown. To establish the nature of these relationships, it is necessary to investigate the structure of the receptors and the channels. The axolemma preparation recently obtained from garfish (*Lepisosteus osseus*) olfactory nerve[11], whose composition is similar to those of the squid and

lobster axolemma preparations isolated by us[1,2], may also be useful for this type of study.

Enzymes have been used as chemical dissectors to explore the possible role that their substrates may play in a variety of cells and cellular parts. Since the pioneer work of Tobias[12], proteases and phospholipases have been used by several authors to investigate the chemical basis of axon function[12–21]. A review of the subject has been recently published[22].

The present work deals with the action of pronase, trypsin, chymotrypsin and phospholipases A, C and D, on tetrodotoxin binding to the isolated axolemma. The results indicate that tetrodotoxin binding remains unaffected after considerable degradation of major structural constituents of the axolemma.

EXPERIMENTAL METHODS

Isolation of the axolemma

The axolemma preparation was obtained from walking-leg nerves of living *Panulirus argus* lobsters. The axolemma was isolated following the method described for the squid[1] as recently adapted to lobster nerve fibres[2]. A detailed description of the method is given in ref. 2. The nerves were used within 4 h of removal from the animal. The final pellet containing the axolemma (membrane fraction I of ref. 2) was suspended in lobster physiological solution and immediately used for tetrodotoxin binding measurements. This plasma membrane preparation, as pointed out in a previous work[2], was identified as axolemma because of the following evidences: (a) its morphological appearance at high resolution electron microscopy; (b) the yield of this plasma membrane with respect to the total nerve plasma membranes; and (c) its high activities of transport ATPase and acetylcholinesterase. Similar criteria have been used to identify as axolemma-enriched preparations, membranes obtained from walking-leg nerves of lobster[23] and squid optic nerve[24].

Measurement of tetrodotoxin binding to the axolemma

Tritiated tetrodotoxin ([^{3}H]tetrodotoxin), labelled by the Wilzbach method[25] and purified according to the procedure described by Hafemann[26], was used. The concentration of [^{3}H]tetrodotoxin was evaluated by a mouse survival assay[26]. The specific activity of the toxin was determined by measuring the radioactivity of the biologically active components as previously described[2]. The specific activity of the [^{3}H]tetrodotoxin used was 37.4 Ci/mole.

The suspension and incubation of the axolemma were carried out in lobster physiological solution[27], pH 7.5, at 4 °C, except when otherwise indicated. The composition of this solution is: 465 mM NaCl; 10 mM KCl; 8 mM $MgCl_2$; 25 mM $CaCl_2$; and 10 mM Tris–HCl.

In order to assay tetrodotoxin binding under a given set of conditions duplicate samples are required, one of each pair serving to measure nonspecific binding. Binding of [^{3}H]tetrodotoxin was calculated, as previously described[2], by subtracting the radioactivity (cpm/mg of protein) present in pellets of membranes preincubated with an excess of nonradioactive tetrodotoxin and incubated with [^{3}H]tetrodotoxin from that found in pellets of membranes incubated with [^{3}H]tetrodotoxin only. The subtractive procedure allows the calculation of the net amount of [^{3}H]tetro-

dotoxin bound to the axolemma preparation. The binding is expressed as the net amount of [^{3}H]tetrodotoxin/mg of membrane protein.

In order to evaluate the effect of the enzymes, tetrodotoxin binding was measured in (a) enzyme-treated membranes and (b) their respective controls.

(a) For the measurements in enzyme-treated membranes, 1 ml of lobster physiological solution containing 1 mg of the corresponding enzyme was added to aliquots of 1 ml of the axolemma suspension (0.15–0.60 mg of membrane protein/ml) and incubated for 1 h. At the end of this period, nonradioactive tetrodotoxin was added to one vessel of each pair to obtain a concentration of 5 μM. The nonradioactive tetrodotoxin was added as a solution in 0.24 ml of 0.01 M acetic acid; acetic acid alone was added to the other flasks. The acetic acid necessary to prepare a concentrated solution of tetrodotoxin did not change the pH of the buffered solution. The flasks were incubated for 30 min to allow the nonradioactive tetrodotoxin to bind to the membranes, and at the end of this period [^{3}H]tetrodotoxin was added to all vessels to a final concentration of 100 nM [^{3}H]tetrodotoxin, and the incubation maintained during an additional 30 min. The final volume of all suspensions was 10.04 ml.

The incubation of all membrane suspensions was concluded by centrifuging them at 65 000 $\times g$ during 30 min. The supernatant was drained and the fluid adhered to the walls of the centrifuge tube was removed by suction. The membrane pellets were suspended in 2 ml of 5 mM Tris–HCl buffer, pH 7.4, and 1.5-ml aliquots transferred to counting vials to which 15 ml of Instagel counting solution (Packard Instruments Co., Ill.) were added. The samples were counted in a liquid scintillation spectrometer (Tricarb, Model 3320, Packard Instruments Co., Ill.). The standard deviation in net counting was less than 2.5%. The remaining 0.5 ml of the membrane suspensions was used for measuring their protein content by the method of Lowry *et al.*[28].

(b) For the measurements of tetrodotoxin binding in the control membranes, the same procedure was followed except that the enzymes were omitted.

Evaluation of enzyme action on membrane components

Proteases. Pronase (B grade, 45 000 proteolytic units/g, Calbiochem, La Jolla, Calif.), trypsin (200 units/mg, Worthington Biochem. Corp., Freehold, N.J.), and α-chymotrypsin (50 units/mg, Worthington Biochem. Corp., Freehold, N.J.) were utilized. To determine the degree of protein degradation induced by enzyme treatment, samples were preincubated with enzyme for 1 h at 4 °C as described above, collected by centrifugation at 65 000 $\times g$ for 30 min, and electrophorezed by a modification of the method of Shapiro *et al.*[29]. The membrane pellets were dissolved in 0.5 ml of 5% solution of sodium dodecyl sulphate in 0.1 M phosphate buffer, pH 7.0, containing 0.1% dithioerythrol. The solubilized membranes were then dialyzed overnight against 0.1 M phosphate buffer, pH 7.4, containing 0.5% sodium dodecyl sulphate. Polyacrylamide gel electrophoresis was carried out in glass tubes (8.0 cm $\times$ 0.6 cm). The gel was prepared from Cyanogum 41 (EC Apparatus Corp., Pa.) to a final concentration of 5% in 0.1 M phosphate buffer, pH 7.0. Approximately 50 μg of membrane protein were applied per tube. Electrophoresis was performed at 5 mA/tube for 2 h. The gels were fixed in isopropanol–acetic acid–water (25:10:100, by vol.) for 24 h and stained with 0.02% Coomasie brilliant

blue prepared in isopropanol–acetic acid–water (10:10:100, by vol.) for 24 h. Excess dye was removed with the same solvent. Densitometric tracings of the electrophoretic patterns were obtained with the aid of a linear transporter (Gilford Instruments, Oberlin, Ohio) using light of 570 nm.

Phospholipases. Phospholipase A (EC 3.1.1.4) from *Naja naja* venom (Sigma Chemical Co., St. Louis, Mo.) was purified by dissolving lyophilized venom (10 mg/ml) in 0.16 M NaCl containing 1 mM EDTA. The solution was heated at 90 °C for 10 min to inactivate proteases and the precipitated denatured protein discarded by centrifugation. The supernatant containing the enzyme was passed through a Sephadex G-75 column to eliminate EDTA and high molecular weight contaminants. Phospholipase C (EC 3.1.4.3) (Type I) from *Chlostridium welchii* and phospholipase D (EC 3.1.4.4) (Type II) from cabbage were used as purchased (Sigma Chemical Co., St. Louis, Mo.) In order to evaluate the degree of lipid degradation, samples of membrane suspensions were treated with the corresponding enzyme as described above for the proteases. The action of the enzyme on the membrane phospholipids was measured by extracting the lipids according to the procedure of Folch *et al.*[30], separating them by thin-layer chromatography and measuring quantitatively the changes in the proportion and nature of the phospholipids by evaluating the phosphorus content of the separated phosphatides[31].

RESULTS AND DISCUSSION

The amount of tetrodotoxin bound from 100 nM [^{3}H]tetrodotoxin solution by the isolated axolemma, as shown in Table II, is 9.5 ± 0.6 pmoles/mg of membrane protein. This value is the mean $\pm$ standard error of twenty four measurements. From experiments in which the binding of [^{3}H]tetrodotoxin to nerve plasma membranes was determined at toxin concentrations of 10^{-11}–10^{-7} M, it was found that at 100 nM tetrodotoxin the binding sites are saturated[2]. The dose-binding curve was interpreted as indicating the presence of a single type of binding site at the membrane, each receptor reacting independently with one tetrodotoxin molecule. The apparent dissociation constant (equilibrium constant) for the binding of [^{3}H]tetrodotoxin to the receptor in isolated membranes was found to be $4.0 \cdot 10^{-9}$ M (ref. 2). A value of 28 tetrodotoxin receptors/μm^2 of membrane area can be calculated from the binding value of 9.5 pmoles/mg of membrane protein, knowing that proteins represent 24% of the membrane dry weight and assuming that all toxin molecules bind at one face of the membrane. The binding value and the receptors density are equal to those reported in a previous work[2].

Fig. 1 shows that the proteolytic enzymes produce extensive degradation of the membrane proteins. It should be noted that the sodium dodecyl sulphate treatment used to monitor the degree of proteolysis, abolishes the noncovalent forces responsible for maintaining the protein fragments in the membrane. As expected, pronase is the enzyme that induces the greatest change of the electrophoretic pattern of the proteins. No significant difference (within 3%) was found between the total amount of protein in the membrane pellets treated with the enzymes and their respective controls (untreated). As shown in Table II, in spite of the degradative action of the proteases, no significative modification of the [^{3}H]tetrodotoxin binding to the axolemma preparation was observed.

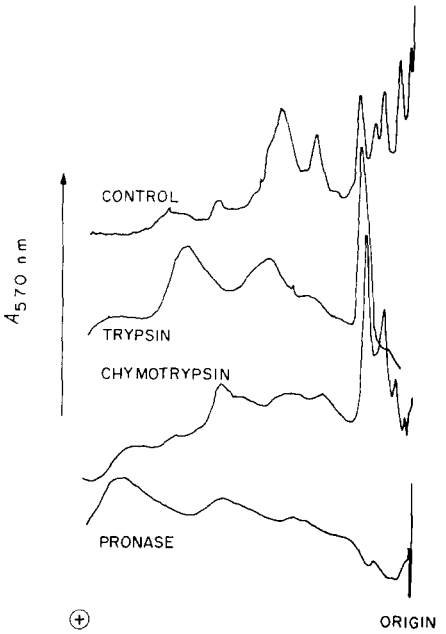

Fig. 1. Peptidolysis of axolemma preparations, as revealed by electrophoresis in polyacrylamide gels, performed in glass tubes. Incubation of the membrane preparations with the corresponding enzymes were carried out in lobster physiological solution, pH 7.5, at 4 °C for 1 h. Densitometric tracings were obtained with a linear transporter using light of 570 nm.

We had expected that the degradation of the axolemma proteins would seriously interfere with the tetrodotoxin-binding ability of this membrane. Thus, the present results are as surprising as those obtained by other authors that demonstrated that axon properties remain essentially unaffected after extra-axonal treatment with proteases (see refs 12, 13 and 21). It may be relevant to recall that in living axons tetrodotoxin produces its pharmacological effect only when applied to the external surface of the axolemma, indicating that the tetrodotoxin receptors are accessible only from this surface[32]. The possible hindrance to enzyme penetration of the periaxonal (Schwann cell and endoneurium) barriers has been suggested to explain the lack of effect of the extra-axonal treatment with proteases. Though this argument may be considered irrelevant for the interpretation of our results, the existence of some type of molecular shielding, protecting the tetrodotoxin receptors from the enzymic action, cannot be discarded. It is also interesting to point out, that the microinjection of or perfusion with proteases affects axon functioning[33,34]. It has been demonstrated that the inactivation of the Na^+ conductance in voltage clamped axons can be destroyed selectively by perfusion with pronase[34], and that the abnormal inward Na^+ current after pronase intra-axonal treatment can be abolished by tetrodotoxin[34]. These findings, besides demonstrating that the integrity of the proteins which can be reached from inside the axon are required for the normal functioning of the Na^+ channels, indicate that the intactness of the same proteins is not essential for tetrodotoxin binding and effect. It should

be recalled, that peptide cleavage may occur in proteins with retention of structure, even to the point of sometimes preserving their biological activity[35].

Three alternative interpretations may be considered the most likely to explain the apparent lack of effect of proteases on tetrodotoxin binding to the isolated axolemma. They are the following: (a) the axolemma proteins accessible to the proteases do not play a significant role in tetrodotoxin binding; (b) the peptide regions which remain intact after cleavage of the proteins by the enzymes represent the essential part of the tetrodotoxin receptors; and (c) the structure of the proteins retained after enzyme treatment is sufficient to preserve the tetrodotoxin-binding ability of the axolemma. The present knowledge of axolemma structure and function precludes making a precise selection among these three explanations.

The results given in Table I indicate that the phospholipases degrade to a

TABLE I

PERCENT OF MEMBRANE PHOSPHOLIPIDS HYDROLYZED BY THE PHOSPHOLIPASES

Incubation of the membrane preparations with the corresponding enzymes were carried out in lobster physiological solution, pH 7.5, at 4 °C for 1 h.

Phospholipids	*% composition of control membrane*	*% of phospholipid hydrolyzed*		
		Phospholipase A	*Phospholipase C*	*Phospholipase D*
Sphingomyelin	9.9	0	0	0
Phosphatidylcholine	48.8	67	46	12
Phosphatidylethanolamine	28.0	95	62	87
Phosphatidylserine	13.2	95	0	0

TABLE II

EFFECT OF ENZYME TREATMENT ON BINDING OF [^{3}H]TETRODOTOXIN TO AXOLEMMA PREPARATIONS OBTAINED FROM LOBSTER NERVE FIBRES

Experiments were carried out in lobster physiological solution, pH 7.5, at 4 °C. For the enzyme experiments 1 ml of the respective enzyme solution (1 mg/ml) was added to 1 ml of axolemma suspension (0.15–0.60 mg of membrane protein/ml) and incubated during 1 h. For the control experiments the enzyme was omitted. Tetrodotoxin binding was measured from a 100 nM ^{3}H-labelled solution. Values are the mean ± S.E.

No. of measurements	*Enzyme*	*Tetrodotoxin binding (pmoles/mg of membrane protein)*	*Relative amount of binding: enzyme treated/control (paired data)*
24	None	9.5 ± 0.6	—
5	Pronase	9.9 ± 0.4	0.90 ± 0.06
7	Trypsin	10.5 ± 0.4	1.11 ± 0.05
5	Chymotrypsin	9.1 ± 0.4	1.00 ± 0.05
6	Phospholipase A	2.8 ± 0.4	0.33 ± 0.07
9	Phospholipase C	7.1 ± 0.9	0.88 ± 0.12
6	Phospholipase D	9.6 ± 0.5	0.91 ± 0.08

different extent the membrane phospholipids. As shown in Table II, only phospholipase A produces a substantial diminution of [^{3}H]tetrodotoxin binding. A slight effect, if any, of phospholipase C on the net amount of [^{3}H]tetrodotoxin binding to the isolated axolemma is shown in Table II. When the results are expressed as the ratio between enzyme-treated membranes and their paired control experiments, the binding proved not to be significantly affected (0.88 ± 0.12) by phospholipase C. Phospholipase D at pH 7.4 does not modify the binding of [^{3}H]tetrodotoxin to the axolemma. When the incubation with phospholipase D was carried out at pH 5.6, the optimum pH for the action of this enzyme[36], the binding diminished about 43% for both the control and the enzyme-treated axolemma preparations, thus confirming a lack of action of this enzyme on the binding. Abolition of [^{3}H]tetrodotoxin binding to the axolemma was observed when the membrane suspension was incubated with lysolecithin (1 mg/ml). This result suggests that the effect of phospholipase A may be due to disarray of lipidic regions induced by the *in situ* production of lysophosphatides.

Phospholipase A blocks nerve impulse conduction[12–14]. This effect, like that on tetrodotoxin binding, appears to be a nonspecific action of the lysolecithin produced by the enzyme[12]. Conduction blockade by phospholipase C has been reported in isolated lobster[12] and squid[13] axons. However, a more recent analysis of the relationship between the action of phospholipase C on phosphatides and on conduction in squid axons have revealed that its blocking action is only observed when the extent of hydrolysis is very high[17]; conduction remaining even when 84% of the lecithin, 50% of the phosphatidylethanolamine and 84–100% of sphingomyelin are split. The relatively small effect of phospholipase C on nerve conduction, in spite of large phosphatide splitting, lead us to expect that tetrodotoxin receptors which are functionally related to the Na^+ channels, would also be relatively insensitive to phospholipase C. It should be pointed out, that under our experimental conditions the extent of phospholipid hydrolysis was less than that reported above to be compatible with conduction in squid axons. The possibility exists that the actual receptors are related with that fraction of phospholipids which remains intact after phospholipase C and D treatment.

The enzymes we studied do not degrade cholesterol and, to our knowledge, no enzyme is available which would affect this steroid molecule in plasma membranes. Therefore, since tetrodotoxin interacts with lipid monolayers that contain cholesterol[37], it would be interesting to explore the effect on tetrodotoxin binding of substances such as filipin, a neutral polyene antibiotic[38], which interacts with cholesterol in natural and artificial systems (for references see ref. 39). It should also be noticed that the enzymes we used do not affect carbohydrates whose role in axon functioning and tetrodotoxin binding is unknown at present. Recently, other authors[40] have observed in membranes isolated from garfish olfactory nerves, that the reduction in tetrodotoxin binding produced by phospholipase A of only 30–40% in their experiments, can be enhanced to the level of inhibition described for this enzyme in the present work by subsequent treatment with the proteolytic enzymes.

Whichever the interpretation, the present results indicate that there can be considerable degradation of proteins and phospholipids of the axolemma without tetrodotoxin binding being affected.

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