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MODIFICATION OF THE TETRODOTOXIN RECEPTOR IN *ELECTROPHORUS ELECTRICUS* BY PHOSPHOLIPASE A_2

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The effects of phospholipase A_2 treatment on the tetrodotoxin receptors in *Electrophorus electricus* was studied. (1) The binding of [^{3}H]tetrodotoxin to electroplaque membranes was substantially reduced by treatment of the membranes with low concentrations of phospholipase A_2 from a number of sources, including bee venom, *Vipera russelli* and *Crotalus adamanteus* and by β-bungarotoxin. (2) Phospholipase A_2 from bee venom and from *C. adamanteus* both caused extensive hydrolysis of electroplaque membrane phospholipids although the substrate specificity differed. Analysis of the phospholipid classes hydrolyzed revealed a striking correlation between loss of toxin binding and hydrolysis of phosphatidylethanolamine but not of phosphatidylserine. (3) The loss of toxin binding could be partially reversed by treatment of the membranes with bovine serum albumin, conditions which are known to remove hydrolysis products from the membrane. (4) Equilibrium binding studies on the effects of phospholipase A_2 treatment on [^{3}H]tetrodotoxin binding showed that the reduction reflected loss of binding sites and not a change in affinity. (5) These results are interpreted in terms of multiple equilibrium states of the tetrodotoxin-receptors with conformations determined by the phospholipid environment.

Introduction

In recent years, there has been considerable interest in a number of laboratories in elucidating the molecular events associated with electrical activity in biomembranes. Much of this interest has focused on resolving the structure and properties of the voltage-dependent Na^+ and K^+ channels which control the movement of cations across the membrane.

Studies on the chemical and physical properties of the action potential Na^+-channel in a number of electrically excitable membranes suggest that the protein components of this channel complex are closely associated with membrane phospholipids. Evidence to support this view is derived primarily from studies on the effects of phospholipase treatment and membrane disrupting agents such as detergents on the binding of channel-specific neurotoxins to isolated membranes [1–4].

Because of the high specificity for the Na^+ channel, tetrodotoxin has proved to be a valuable tool to monitor many of the molecular properties of the Na^+ channel [5–7]. Alterations in [^{3}H]tetrodotoxin binding, can therefore be assumed to reflect perturbations in the channel structure itself.

The binding of tritiated tetrodotoxin and saxitoxin to receptor sites in a number of membrane preparations has been shown to be particularly sensitive to phospholipase A_2 treatment [1–3,8,9]. These results open up the possibility that selective phospholipase A_2 hydrolysis could provide useful information on the nature of the lipids involved in stabilizing the Na^+ channel and perhaps more interesting, the physical state of the lipids immediately associated with the channel proteins. The latter aspect may be of interest since changes in membrane lipid 'fluidity' have been shown to coincide with the propagation of action potentials [10].

The studies reported in this paper describe the effects of phospholipase A_2 treatment on the binding of [^{3}H]tetrodotoxin to Na^+ channel enriched membranes isolated from the electroplaque of the electric eel.

Materials and Methods

Preparation of membranes and [^{3}H]tetrodotoxin-binding assays

Plasma membrane fragments enriched in tetrodotoxin receptors were isolated from the electroplaque of the electric eel using procedures previously described in detail [2,11]. Membranes were kept on ice in 0.25 M sucrose and could be stored for up to one week with little or no loss of [^{3}H]tetrodotoxin binding. Experiments were normally carried out within two days after preparation. Protein was determined using the procedure of Lowry et al. [12] using bovine serum albumin as a standard.

The binding of [^{3}H]tetrodotoxin to isolated membranes was measured by equilibrium dialysis [2] or by rapid filtration using glass fiber filters (GF-C) [13]. Assays were carried out at 10°C in the presence of 50 mM NaCl, 0.25 M sucrose and 0.01 M potassium phosphate buffer, pH 7.0. Binding was measured in the presence of $15 \cdot 10^{-9}$ M [^{3}H]tetrodotoxin approximately twice the apparent K_d under these experimental conditions. The former assay, while more time consuming gave more clearly reproducible results; variations in [^{3}H]tetrodotoxin binding were often observed using the rapid filtration assay possibly a reflection of small differences in the amount of membrane trapped by the filters and occasional variability in flow rates. Since the rate constant for the dissociation of the toxin-receptor complex is 0.012 s^{-1} [2] even with a filter wash time of approx. 15 s a reduction of nearly 20% of the maximum binding would be expected.

Tetrodotoxin was tritiated by a catalytic exchange mechanism using tritium gas (ICN, Irvine, CA) and purified by repeated Bio-Rex 70 ion exchange chromatography [1,2,14]. The specific activity (400 Ci/mol) and radiochemical purity were determined as described [14]. Radiochemical purity, normally around 60% varied from preparation to preparation and often decreased with prolonged storage. Greater than 90% of the bound radioactivity could be reduced by the presence of excess (1 μM) unlabeled toxin, and no corrections were made for the low levels of non-specific binding found in these membranes at the concentrations of [^{3}H]tetrodotoxin used in these assays.

Phospholipase A_2 treatment and isolation of phospholipids

Hydrolysis of electroplaque membranes by phospholipase A_2 from different sources was carried out as follows. Membranes (1.2–1.8 mg protein/ml) were incubated for 20 min at 30°C in 0.25 M sucrose plus 0.02 M Tris buffer (pH 7.0) in the presence of phospholipase A_2 at concentrations specified. The reaction was terminated by transferring the sample to ice, followed immediately by the addition of a 3-fold excess of ice cold 0.25 M sucrose. No additional reduction in toxin binding occurs under these dilution conditions. Membranes were pelleted by centrifugation for 15 min at $20\,000 \times g$ and resuspended in a small volume (0.7 ml) of assay media using a micro-teflon homogenizer. All manipulations were carried out on ice. Samples were subsequently analyzed for toxin binding, resuspended in serum albumin to remove hydrolysis products or extracted with chloroform-methanol for phospholipid analysis. Membrane-bound lysophospholipids and free fatty acids were removed by incubating the resuspended membranes at a final concentration of approx. 1 mg protein/ml, in 4 ml 0.25 M sucrose/0.02 M Tris (pH 7.0) plus 10 mg/ml or 20 mg/ml bovine serum albumin. After one hour on ice the membranes were collected by centrifugation and washed once with sucrose/Tris prior to analysis of toxin binding.

Phospholipid analysis

Aliquots of resuspended membranes treated and prepared as described above were extracted with chloroform/methanol (2 : 1, v/v) and the extract washed as previously described [11]. Extracted lipids were separated by two-dimensional thin-layer chromatography according to the procedure described by Rouser et al. [15]. Phospholipids were removed from the plates by aspiration and analysed for phosphorus following perchlorate digestion [16]. Data on the % hydrolysis of specific lipids is expressed in terms of weight % inorganic phosphorus.

Materials

Phospholipase A_2 preparations were obtained from Sigma and used without further purification. It should be noted that the specific activities reported for these enzymes from various sources (bee venom, *apis mellifer*, 1470 units/mg; *Crotalus adamanteus*, 800 unit/mg; *Vipera russelli*, 11 unit/mg) do not necessarily reflect the activity using native membranes as substrates. β-Bungarotoxin was purified from the venom of *Bungarus multicinctus* by CM-52 cellulose chromatography followed by G-25 gel filtration. The elution profile of component peptides was identical to that reported by Abe et al. [17]. The fraction used in our studies corresponds to F5 (β_3-bungarotoxin) of their separation. Tetrodotoxin, citrate free, was obtained from Sankyo, Japan.

Results

The interaction of [^{3}H]tetrodotoxin with electroplaque membranes was substantially altered by treatment of the membranes with phospholipase A_2 from a number of sources. Fig. 1 shows the loss of binding following incubation of the membranes with increasing concentrations of phospholipase A_2 from bee venom, one of the more potent phospholipases studies. Loss of nearly 50% of the tetrodotoxin binding occurred at concentrations of enzyme less than 0.1 μg/ml under our experimental conditions. The data shown here are representative of several experiments which gave qualitatively and quantitatively similar results provided that the ratio of membrane protein to enzyme was relatively constant. Since incubation of the membranes at 30°C for even 20 min caused a reduction of approx. 10% in the toxin binding, appropriate controls were carried out.

Curiously, this loss of [^{3}H]tetrodotoxin binding was not dependent on added calcium (Table I). Although studies have shown that the activity of the bee venom enzyme using artificial phospholipid systems as substates is enhanced by calcium, the sensitivity of the tetrodotoxin receptor site was only marginally affected by calcium up to 4 mM. On the other hand, the addition of EDTA (3.3 mM) substantially reduced the effectiveness of the enzyme. These data suggest that the membranes, although extensively washed during isolation still contain sufficient levels of bound calcium possibly associated with high affinity sites in the membrane.

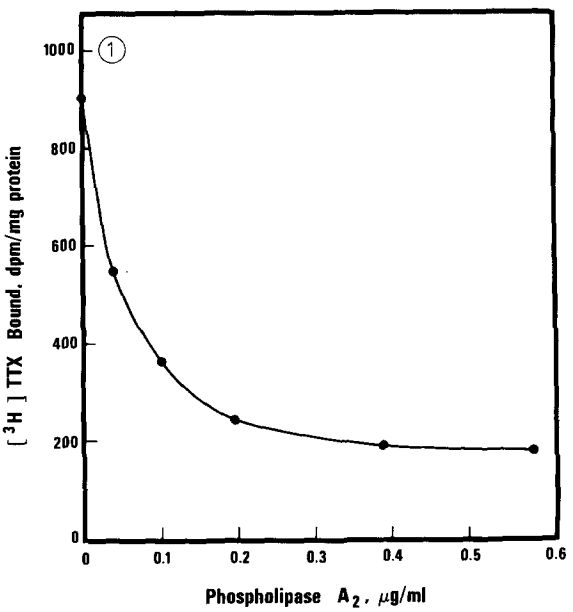

Fig. 1. The effects of phospholipase A_2 (bee venom) on the binding of [^{3}H]tetrodotoxin ([^{3}H]TTX) to isolated electroplaque membranes. Membranes (1.65 mg protein/ml) were incubated for 20 min at 30°C, with bee venom phospholipase A_2 at concentrations specified. Soluble phospholipase A_2 was removed by centrifugation and the binding of toxin to membranes measured as described.

To show that the effect of the bee venom enzyme reflected the phospholipid hydrolysis activity, the sensitivity of the toxin-receptor site to phospholipase A_2 from other sources was examined. Table I shows the effects of the enzyme from *Crotalus adamanteus* and *Vipera russelli* as well as β-bungarotoxin on [^{3}H]-tetrodotoxin binding. β-Bungarotoxin is a basic protein shown to possess phospholipase A activity [18, 19]. All three enzyme preparations reduced [^{3}H]-tetrodotoxin binding although clearly the enzyme from *Vipera russelli* was far more potent particularly in terms of relative concentration and apparent 'specific activity'. As observed with the bee enzyme, the viper's enzyme showed no requirement for added calcium, although in the presence of calcium, the loss of [^{3}H]tetrodotoxin binding was even less. On the other hand, both the rattlesnake enzyme and β-bungarotoxin appeared to require calcium for maximal activity particularly at lower enzyme concentrations.

The decrease in [^{3}H]tetrodotoxin binding could

TABLE I

PHOSPHOLIPASE A_2 DEPENDENT LOSS IN [^{3}H]TETRODOTOXIN BINDING TO ELECTROPLAQUE MEMBRANES

Electroplaque membranes were treated with phospholipase A_2 as described in Materials and Methods. Incubations were for 20 min at 30°C. [^{3}H]tetrodotoxin binding was measured at $15 \cdot 10^{-9}$ M toxin and values corrected for total membrane protein for each sample analysed.

Phospholipase A_2 enzyme treatment	% [^{3}H]Tetrodotoxin binding remaining	
	$-CaCl_2$	$+CaCl_2$ (4 mM)
1. Bee venom		
None	100	
0.095 μg/ml	63.3	66.0
0.133 μg/ml	23.4	34.9
0.237 μg/ml	27.1	32.5
0.120 μg/ml	45.3	–
0.120 μg/ml + 3.3 mM EDTA	87.5	–
2. *Vipera russelli*		
None	100	93.7
0.166 μg/ml	42.8	–
0.250 μg/ml	24.3	50.0
0.400 μg/ml	25.5	43.8
3. *Crotalus adamanteus*		
None	100	95.9
1.5 μg/ml	91.5	67.2
5.0 μg/ml	65.0	37.6
4. β-Bungarotoxin		
None	100	112
3.4 μg/ml	95.2	35.9
16.6 μg/ml	94.5	–

TABLE II

EFFECT OF BOVINE SERUM ALBUMIN EXTRACTION ON THE RECOVERY OF [^{3}H]TETRODOTOXIN BINDING TO PHOSPHOLIPASE A_2 TREATED MEMBRANES

Electroplaque membranes were treated with phospholipase A_2 at concentrations specified. Both control and untreated membranes were washed with either buffer or with bovine serum albumin as described in Materials and Methods and the membranes collected by centrifugation. Binding of [^{3}H]-tetrodotoxin was measured on the resuspended pellets and the results expressed as dpm/mg membrane protein.

Phospholipase A_2 enzyme treatment	[^{3}H]Tetrodotoxin bound, dpm/mg protein (% control)		
	No albumin	+albumin (10 mg/ml)	+albumin (20 mg/ml)
1. Bee venom			
None	1107 (100)	1056 (100)	962 (100)
0.086 μg/ml	198 (18)	728 (69)	623 (65)
0.173 μg/ml	157 (14)	229 (22)	254 (26)
2. *Vipera russelli*			
None	1432 (100)	1100 (100)	
0.200 μg/ml	560 (39)	1133 (103)	
3. β-Bungarotoxin			
None	1047 (100)	1014 (100)	
3.4 μg/ml	374 (36)	898 (89)	

reflect a structural change in the tetrodotoxin receptor protein induced by the loss of specific and possibly essential phospholipids associated with the membrane protein, or alternatively to the accumulation in the membrane of phospholipid hydrolysis products namely free fatty acids and lysophospholipids. To test the latter, the phospholipase treated membranes were resuspended in bovine serum albumin to remove hydrolysis products. Albumin is known to extract both fatty acids and lysophosphatides from native membranes [20]. The results are shown in Table II. Incubation of the membranes with 0.086 μg/ml bee phospholipase A_2 resulted in a loss of approx. 82% of the original binding. Subsequent treatment with 10 mg/ml serum albumin resulted in a recovery to nearly 70% of control with no further recovery by higher concentrations of albumin. This reversal of the loss in toxin binding is critically dependent on the level of phospholipid hydrolysis achieved by the enzyme. At higher concentration (0.173 μg/ml) albumin even at 20 mg/ml was ineffective. Albumin also reversed the reduction in [^{3}H]tetrodotoxin following treatment with other phospholipases (Table II).

The phospholipase dependent reduction in toxin binding did not result from a decrease in toxin binding affinity but rather a decrease in the total number of toxin binding sites. The data presented in Fig. 2 show double reciprocal plots of the binding of [^{3}H]-tetrodotoxin at various concentrations of free toxin. Binding analysis was carried out on control untreated membranes and on phospholipase treated membranes both before and after albumin extraction. From an analysis of the slopes and intercepts it is clear that in

all cases the apparent dissociation constant (K_d) is virtually unchanged by enzyme treatment. Rather, hydrolysis of membrane phospholipids led to a reduction of the total toxin binding sites. This was partially reversed by subsequent albumin extraction of the hydrolysis products.

Studies on the effects of *C. adamanteus* phospholipase A_2 indicated that this enzyme was not as effective as the bee venom enzyme in reducing [^{3}H]tetrodotoxin. Although the apparent specific activity of the latter is only about 1.8 times that of the snake enzyme the potency of the bee enzyme in reducing toxin binding was nearly 20-times greater. Because the apparent 'specific activities' for these enzyme reflect activities under very defined conditions and usually on artificial substrates it is important to assess directly the relative activities under conditions used in these studies. To do this, the level of phospholipid hydrolysis was measured for membranes treated with either the *C. adamanteus* enzyme or the bee venom phospholipase A_2 and the corresponding [^{3}H]tetrodotoxin binding was assessed. In order to show possible phospholipid specificities for the two enzymes, concentrations of each enzyme were chosen such that extensive hydrolysis was minimized and that the relative total hydrolysis was nearly comparable in each case. Enzyme treated membranes were then extracted with chloroform-methanol and the major phospholipids, phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine were separated by thin layer chromatography and analysed. Fig. 3 shows a composite of data for toxin binding, total phospholipid hydrolysis, and hydrolysis of the major phospholipids for three concentrations of each enzyme. The data represent results obtained from two separate membrane preparations. From a comparison of the effects of the bee venom enzyme and the *C. adamanteus* enzyme, the following observations can be made. (1) There was a progressive loss of toxin binding with increasing phospholipid hydrolysis. However, there did not appear to be a direct correspondence between total hydrolysis and reduction in toxin binding. Thus, although the *C. adamanteus* phospholipase A_2 at the concentrations tested, induced a greater degree of hydrolysis, the corresponding loss in tetrodotoxin binding was always significantly less. This was particularly evident at lower concentrations of the enzymes. (2) Phosphatidylserine was a better substrate for the snake enzyme than for the bee enzyme. Electroplaque membranes can accommodate a hydrolysis of nearly 80% of the total phosphatidylserine with a reduction of only 40% of the total toxin binding. (3) The bee venom phospholipase A_2, on the other hand, showed a greater relative preference for phosphatidylethanolamine than the snake enzyme, while phosphatidylcholine was preferred by the latter. Again, these selectivities were most evident at lower

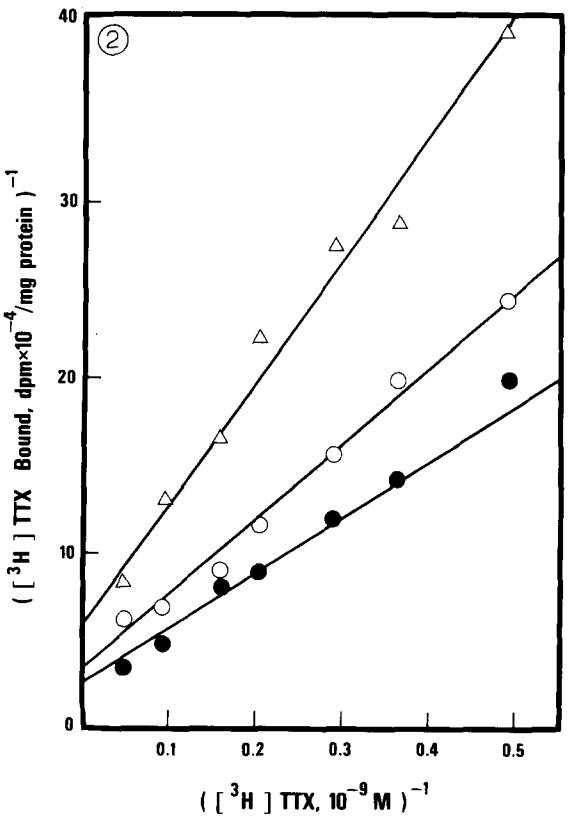

Fig. 2. The effect of phospholipase A_2 modification and serum albumin treatment on the number of [^{3}H]tetrodotoxin-binding sites and on the apparent K_d of the toxin receptor complex. Data are shown in double reciprocal form; intercepts on the abscissa are coincident for all three lines. Binding analysis were carried out on control membranes (●———●), membranes treated with bee venom phospholipase A_2 (0.055 μg/ml) (△———△) and phospholipase A_2 treated membranes which were subsequently washed with 10 mg/ml serum albumin (○———○). Original enzyme incubations were carried out at a membrane concentration of 1.83 mg membrane protein/ml for 20 min at 30°C. TTX, tetrodotoxin.

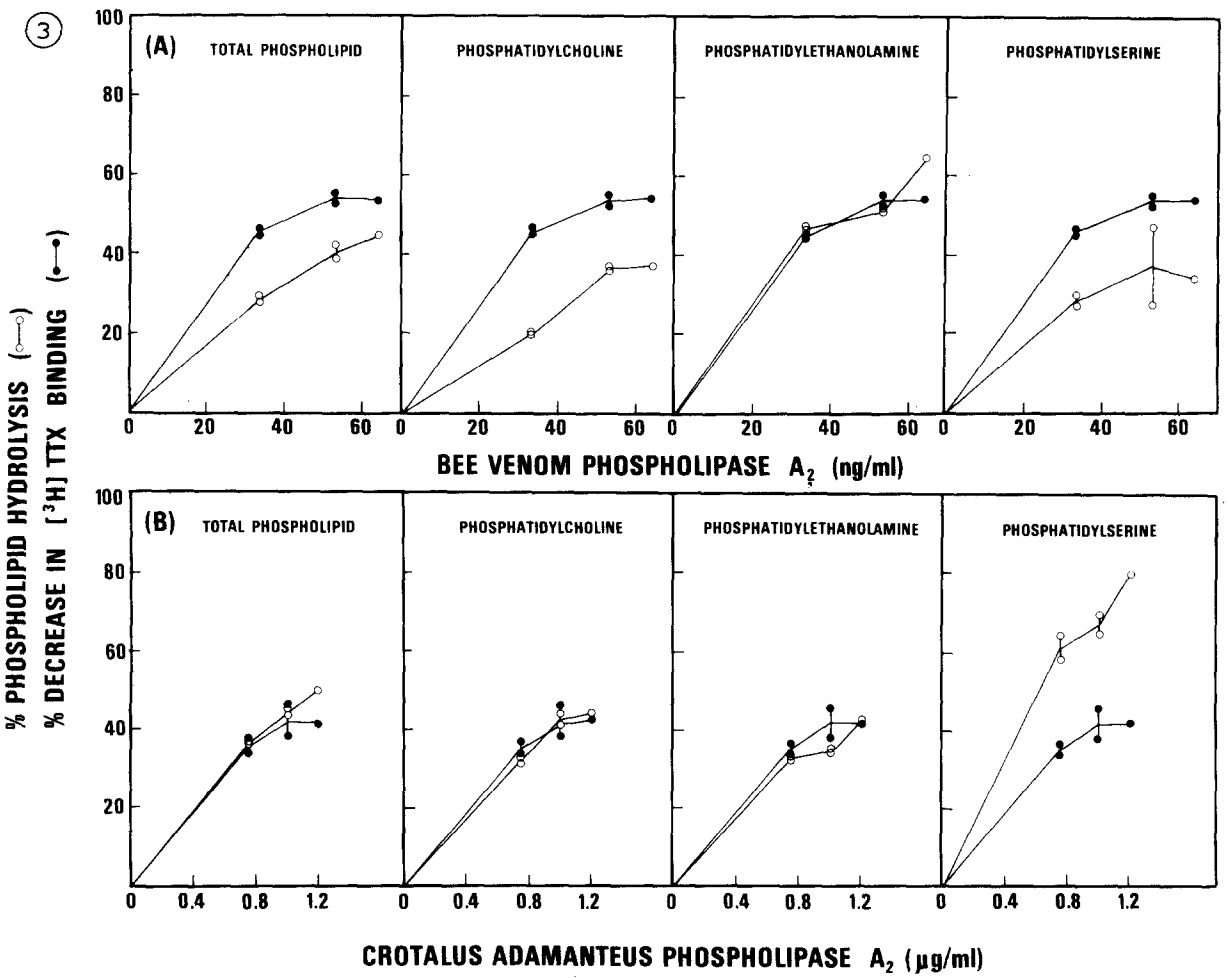

Fig. 3. Correlations between the loss of [^{3}H]tetrodotoxin ([^{3}H]TTX, •———•) and degree of hydrolysis of total membrane phospholipids, phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine (○———○). Results are shown for three concentrations of each of the bee venom phospholipase A_2 (A) and *Crotalus adamanteus* phospholipase A_2 (B). Total phospholipid reflects the sum of the three major phospholipid classes which were analysed. These phospholipids constitute approx. 93% of the total membrane phospholipids [11].

enzyme concentrations. (4) There was a striking correlation between phosphatidylethanolamine hydrolysis and loss of [^{3}H]tetrodotoxin binding for both enzymes. The % hydrolysis of this phospholipid and the % loss of toxin binding are almost superimposable while similar correlations could not be made with the other phospholipids.

Discussion

Studies on the interaction of radiolabelled neurotoxins with electrically excitable membranes have provided valuable information on the chemical nature of the Na^+-channel proteins. By measuring the effects of specific chemical modification on the association of toxins with receptor sites, it has been possible to deduce many of the biochemical and structural properties of the channel itself.

The sensitivity of the native tetrodotoxin receptor to proteolysis and to phospholipase treatment [1–3, 8,9] suggests a receptor site which is a membrane bound protein closely associated with the phospho-

lipid bilayer. The general instability of the detergent solubilized tetrodotoxin-receptor [21–23] and the importance of a critical phospholipid/detergent ratio in receptor stabilization [24] further attests to the role of phospholipids in determining the native state of the channel complex.

The studies reported here provide further evidence to support a structural role of membrane phospholipids in stabilizing the toxin receptor region of the channel. The binding of [^{3}H]tetrodotoxin to intact electroplaque membranes is extremely sensitive to phospholipase A_2 from a number of sources. Chacko [8] and more recently Baumgold [9], have reported similar results using nerve membrane preparations.

There can be little doubt that the effects on tetrodotoxin binding result from the phospholipid hydrolysis activity of these enzymes and ultimately from the presence of hydrolysis products in the membrane. Although the bee venom enzyme did not appear to require exogenous calcium, its effect was nevertheless reduced by added EDTA. Furthermore, the toxin binding closely follows hydrolysis of at least some of the membrane phospholipids. The *C. adamanteus* enzyme and β-bungarotoxin on the other hand, showed substantially enhanced activity in the presence of calcium. The basis for the differences in calcium dependence is unclear.

The ability of serum albumin to reverse the reduction of [^{3}H]tetrodotoxin binding induced by phospholipases A_2 strongly suggests that the phospholipid hydrolysis products in the membrane are responsible for the alterations in toxin binding. Although recovery of toxin binding was demonstrated following removal of hydrolysis products, the relative degree of phospholipid hydrolysis was a critical factor in the magnitude of reversability. Thus, following extensive hydrolysis, the level or chemical composition of unhydrolysed phospholipid may be insufficient to maintain a native conformational state of the receptor. It should also be pointed out that the ratio of membrane cholesterol to phospholipid would also increase substantially following enzyme treatment and it is unclear how this may affect receptor conformation. Presumably at lower levels of hydrolysis sufficient levels of phospholipids still remain to provide the necessary chemical and physical environment to stabilize a tetrodotoxin binding state of the receptor.

These results suggest that phospholipids may play a key role in modulating the conformational flexibility of the tetrodotoxin receptor.

The reversible effects on tetrodotoxin binding could be explained on the basis of three conformationally distinct states of the receptor protein, two of which are readily interconvertible,

$$R \rightleftharpoons R' \rightarrow R''$$

R represents the native state and the only species which binds tetrodotoxin and R′ represents the receptor species whose conformation state is determined by a critical ratio of phospholipid to hydrolysis products. Albumin-dependent reversal of the loss of [^{3}H]-tetrodotoxin would therefore reflect R′ to R transition while extensitive phospholipid hydrolysis would promote an irreversible transition to R″ that would be insensitive to albumin.

The irreversible loss of [^{3}H]tetrodotoxin binding to detergent solubilized membranes would be expected if detergents cause extensive delipidation and transition to the R″ state. Presumably the maintenance of a critical phospholipid/detergent ratio promotes the stability of the native toxin binding state, R [24].

Baumgold [9] and Chacko [8] have reported phospholipase A_2 induced loss of neurotoxin binding to nerve membranes which could not be reversed by washing with serum albumin. These results could be explained on the basis of tissue differences, or more likely that under their experimental conditions such extensive hydrolysis occurred that irreversible transition from R′ to R″ was promoted.

Our results at present, do not provide explanations for how the phospholipid environment determines protein structure. The fact that membrane protein conformation can be altered by cholesterol is well documented [25]. Furthermore, recent studies have shown that phospholipase A_2 treatment of electroplaque membranes significantly alters patterns of membrane protein radioiodination as well as the fluorescence polarization of incorporated 1,6-diphenylhexatriene (Reed, J.K. and Van der Meiden, J., unpublished results). Baumgold [9] suggested that the loss of toxin binding resulted from the presence of free fatty acids since reconstitution with arachidonic acid mimics the effects of phospholipase A_2 treatment. Chacko [8] on the other hand suggested that

lysophosphatides were primarily responsible. Interestingly, the phospholipase A_2 dependent loss of gonadotropin receptors in plasma membranes could be reproduced by exogenous lysophosphatidylethanolamine and to a lesser degree by lysophosphatidylcholine but not by lysophosphatidylserine [20]. Clearly, the effects of phospholipase A_2 treatment are exceedingly complex particularly on natural membranes.

Although our results on the degree and specificity of phospholipid hydrolysis do not support a unique role for specific phospholipids in stabilizing the tetrodotoxin-receptor site, they nevertheless provide interesting insights into substrate variabilities of different enzymes on native membranes. Phospholipase A_2 from *C. adamanteus* and from bee venom differ significantly in their substrate selectivities. The latter shows a higher preference for phosphatidylserine while the former hydrolyzed phosphatidylethanolamine to a degree considerably greater than expected on the basis of relative distribution [11]. The remarkable correspondence between phosphatidyethanolamine hydrolysis and loss of [^{3}H]tetrodotoxin binding is striking. Whether this reflects a special requirement for this lipid or that the corresponding lysophosphatides are particularly potent has yet to be determined. Nevertheless, the substrate selectivities of phospholipases from various sources can provide useful methods to probe the physical and chemical state of the phospholipid environment of membranes in general.

Acknowledgements

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