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Molecular Mechanism of Cardiotoxin Action on Axonal Membranes†

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ABSTRACT: Cardiotoxin isolated from *Naja mossambica mossambica* selectively deactivates the sodium–potassium activated adenosine triphosphatase of axonal membranes. Tetrodotoxin binding and acetylcholinesterase activities are unaffected by cardiotoxin treatment. The details of association of cardiotoxin with the axonal membrane were studied by following the deactivation of the sodium–potassium activated adenosine triphosphatase and by direct binding measurements with a tritiated derivative of the native cardiotoxin. The maximal binding capacity of the membrane is 42–50 nmol of cardiotoxin/mg of membrane protein. This high amount of binding suggests association of the toxin with the lipid phase of the membrane. It has been shown that cardiotoxin first associates rapidly and reversibly to membrane lipids, then, in a second step, it induces a rearrangement of the membrane structure which produces an irreversible deactivation of the sodium–potassium activated adenosine triphosphatase. Solubilization of the membrane-bound ATPase with Lubrol WX gives an active enzyme species that is resistant to cardiotoxin-induced deactivation. Cardiotoxin binding to the membrane is prevented by high concentrations of Ca^{2+} and dibucaine. Although cardiotoxins and neurotoxins of cobra venom have large sequence homologies, their mode of action on membranes is very different. The cardiotoxin seems to bind to the lipid phase of the axonal membrane and inhibits the sodium–potassium activated adenosine triphosphatase, whereas the neurotoxin associates with a protein receptor in the post-synaptic membrane and blocks acetylcholine transmission.

Cardiotoxin is the most abundent constituent of cobra venoms (Lee et al., 1968; Larsen and Wolff, 1968; Slotta and Vick, 1969; Lo et al., 1966; Takechi et al., 1971). Its lethal potency in mice is about one-twentieth of that of neurotoxins. Toxins of the cardiotoxin group are composed of 60 residues in a single polypeptide chain cross-linked by four disulfide bridges (Lee, 1972; Yang, 1974). Cardiotoxins affect various kinds of cells, both excitable and nonexcitable, causing irreversible depolarization of the cell membrane and consequently impairing both the function and the structure of cells (Lee, 1971; Lee et al., 1968; Patel et al., 1969). Comparative pharmacological and sequence studies have indicated that other toxic proteins, found in snake venom, such as the direct lytic factor (DLF) (Condrea et al., 1964; Aloof-Hirsch et al., 1968), cobramines (Larsen and Wolff, 1968) or cytotoxins (Braganca et al., 1967; Patel et al., 1969) belong to the cardiotoxin family.

Excitable cells are very vulnerable to cardiotoxin action: (1) Cardiotoxin causes contracture followed by paralysis of the skeletal muscle (Lee et al., 1968; Tazieff-Depierre et al., 1969). (2) In isolated heart preparations, cardiotoxins at low concentrations cause an augmentation of contraction; systolic arrest occurs at high concentrations of the toxin (Lee et al., 1968). (3) Cardiotoxins block axonal conduction in peripherical nerves (Condrea et al., 1967).

Snake neurotoxins and cardiotoxins have different pharmacological effects but they are proteins originating from a common ancestor gene (Lee, 1972; Yang, 1974). Whereas the molecular mechanism of the association of snake neurotoxins with the membrane-bound acetylcholine receptor is now well understood (for a review, see Rang, 1975), very little is known about the molecular basis of cardiotoxin action. To study this problem, we have chosen to analyze the interaction of *Naja mossambica mossambica* cardiotoxin with crab axonal membranes that have been obtained in a very purified form and that have been chemically and functionally well characterized (Balerna et al., 1975).

Materials and Methods

Cardiotoxins and Chemicals. Cardiotoxins were purified from *Naja mossambica mossambica* venom following the method described by Louw (1974). A slight modification was introduced by replacing the CM-cellulose chromatography

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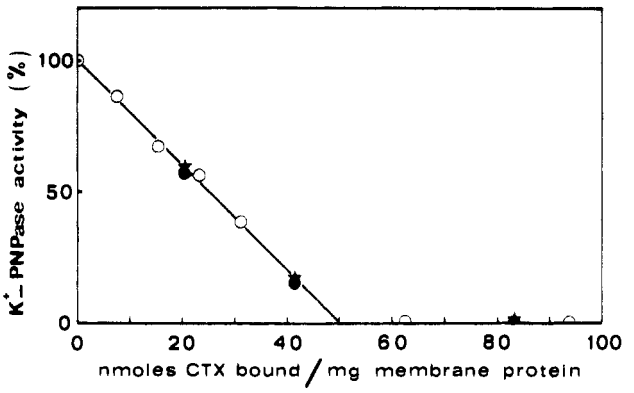

FIGURE 1: Cardiotoxin-induced deactivation of K^+-PNPase activity. Axonal membranes were incubated at 25 °C in a 50 mM triethanolamine buffer, pH 7.5, containing 0.1 M sucrose and different concentrations of cardiotoxin. Membranes and cardiotoxin were allowed to react during a time long enough to obtain constant values of K^+-PNPase activity (8 h for the lowest concentration of cardiotoxin and 1 h for the highest one). These equilibrium values are plotted against the ratio between the amounts of cardiotoxin (CTX) bound and mg of membrane protein. Activity data presented in this Figure are corrected for the spontaneous loss of activity that occurred in the absence of cardiotoxin. The titration was carried out with three different membrane concentrations: 0.51 (●), 0.24 (○), and 0.051 (★) mg of membrane protein/ml of incubation medium.

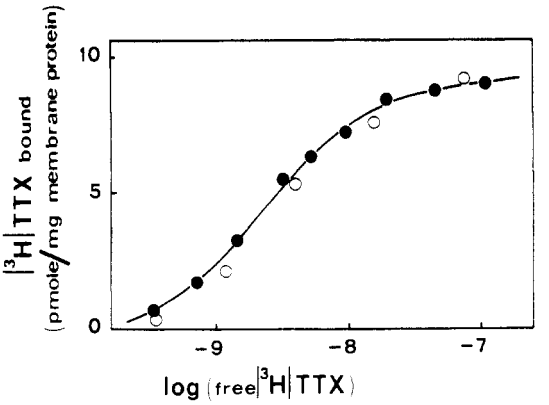

FIGURE 2: The binding of [^{3}H]TTX to axonal membranes in the presence and absence of cardiotoxin. The binding of [^{3}H]TTX to axonal membranes (1.8 mg of protein/ml) was measured by equilibrium dialysis according to Balerna et al., (1975) at 4 °C, pH 7.5, either in the presence of 0.2 mM cardiotoxin after previous incubation with the toxin (0.2 mM) during 1 h at 25 °C (○), or in the absence of cardiotoxin (●).

step by a Bio-Rex-70 (Bio-Rad) chromatography. Five non-neurotoxic polypeptides having the immunochemical properties of cardiotoxins (Louw, 1974) have been identified. Cardiotoxins C and D (letters correspond to the elution order after Bio-Rex-70 chromatography) were rechromatographed on Amberlite CG 50 at pH 7.7 with a concentration gradient of ammonium bicarbonate from 75 to 450 mM. Each one gave two fractions: fractions C_1 and C_2 and D_1 and D_2. Chromatography on Amberlite CG-50, gel electrophoresis, and amino acid analysis gave the indication that the fractions were homogeneous. Phospholipase activity was measured in each cardiotoxin fraction according to De Haas et al. (1968). Toxicity was determined by subcutaneous injection of cardiotoxins into mice. Cardiotoxin D_1, which was one of the most abundant toxin, was the one selected for this work. It has a LD_{50} of 2 μg/g of mouse and no phospholipase activity at all. Cardiotoxin D_1 corresponds to cardiotoxin $V^{II}4$ in the nomenclature adopted by Louw (1974). Sodium [^{3}H]borohydride (20 Ci/mmol) was obtained from the Commissariat à l'Energie Atomique, Saclay. Veratridine and dibucaïne hydrochlorides were obtained from K and K Laboratories.

Labeling of Cardiotoxin D_1. The free amino groups of cardiotoxin D_1 (10 ϵ-amino and 1 α-amino groups) were methylated by reductive alkylation using a technique described for other proteins by Means and Feeney (1968). The two-step technique involves Schiff base formation with formaldehyde treatment followed by reduction with sodium [^{3}H]borohydride. The specific radioactivity of methylated cardiotoxin D_1 was 27 Ci/mmol. The LD_{50} of the ^{3}H-methylated cardiotoxin is identical to that of native cardiotoxin D_1.

Preparation of Axonal Membranes. The method used for the preparation of axonal membranes from the crab *Cancer pagurus* is described elsewhere (Balerna et al., 1975). There are two fractions of axonal membranes with slightly different densities. The work presented in this paper has been carried out with fraction II that sediments at 19.5% sucrose.

Binding Experiments. Axonal membranes (0.05–0.5 mg of membrane protein/ml) were equilibrated at 25 °C in a 50 mM triethanolamine buffer at pH 7.5 containing 0.1 M sucrose and the ^{3}H-methylated cardiotoxin D_1 at concentrations ranging between 10 nM and 0.5 mM. After an incubation time of 10 min, aliquots were filtered under reduced pressure through Millipore celotate filters (EGWP 02500, 0.2 μm). The filters were then washed twice with 5 ml of cold triethanolamine buffer and then shaken in 10 ml of Bray's solution for 20 min. The amount of labeled cardiotoxin bound to axonal membranes was determined by liquid scintillation counting in a Packard Tricarb Spectrometer, Model 3375.

The amount of [^{3}H]cardiotoxin bound in such an experiment represents, in fact, the sum of specific plus unspecific binding. The amount of the specific binding of [^{3}H]cardiotoxin to the membranes can be easily determined. It corresponds to the radioactivity that is displaceable by a 100-fold molar excess of unlabeled cardiotoxin. Unspecific binding (not displaceable) never exceeds 10% of the total binding.

[^{3}H]Tetrodotoxin binding experiments were carried out as previously described (Balerna et al., 1975).

Enzymatic Activity Measurements. All measurements of enzyme activities were carried out at 25 °C. Acetylcholinesterase was assayed by the method of Ellman et al. (1961) at pH 7.2 with 0.8 mM acetylthiocholine. Ouabain-sensitive Na^+, K^+-ATPase[1] activity was followed at 340 nm using a coupled enzyme system involving lactate dehydrogenase and pyruvate kinase. This reaction was routinely carried out at pH 7.5 in a triethanolamine buffer (50 mM) containing 5 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 2 mM ATP, 0, 15 mM NADH, 2 mM phosphoenolpyruvate, 20 μg of lactate dehydrogenase, and 50 μg of pyruvate kinase (Boehringer-Mannheim). Ouabain-sensitive, K^+-stimulated *p*-nitrophenyl phosphatase activity was determined at 410 nm in a triethanolamine buffer at pH 7.5, containing 20 mM KCl, 20 mM $MgCl_2$, and 20 mM *p*-nitrophenyl phosphate. In crab leg nerve membranes, more than 95% of the total ATPase activity was inhibited by ouabain 1 mM at equilibrium (Gache et al., 1976).

Solubilization of Axonal Membranes. Axonal membranes were solubilized in 0.75% Lubrol WX[2] (Sigma) during 2 h at

[1] Abbreviations used are: Na^+,K^+-ATPase, sodium–potassium activated adenosine triphosphatase (EC 3.6.1.3); K^+-PNPase, potassium activated phosphatase activity with *p*-nitrophenyl phosphate as substrate; AChase, acetylcholinesterase (EC 3.1.1.7); TTX, tetrodotoxin; CTX, cardiotoxin D_1; CM, carboxymethyl; LD, lethal dose; ATP, adenosine 5′-triphosphate; NADH, reduced nicotinamide adenine dinucleotide.

[2] Poly(oxyethylene ether) of the general formula $R(OCH_2CH_2)_nOH$ in which *n* averages 16 and R is acetyl.

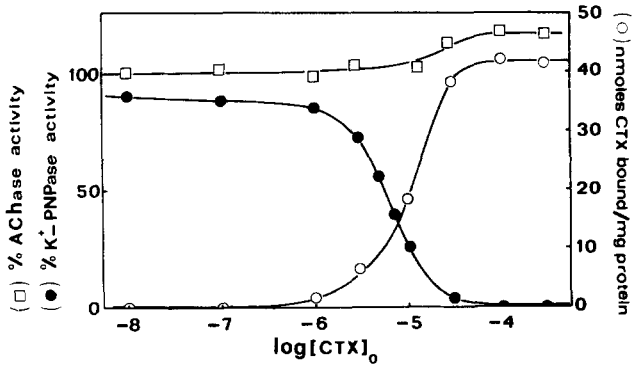

FIGURE 3: Correlation between binding of [^{3}H]cardiotoxin to axonal membranes and variations of membrane-bound K^+-PNPase and acetylcholinesterase activities. Axonal membranes (0.24 mg of protein/ml) were incubated with different concentrations of [^{3}H]cardiotoxin as under experimental conditions described in Figure 1. When equilibrium was attained, aliquots were taken and used to estimate the binding of [^{3}H]cardiotoxin (after correction for nonspecific binding that reaches a maximum value of 10% of the total binding in the high concentration range of cardiotoxin). (○), the K^+-PNPase activity (●), and the acetylcholinesterase (AChase) activity (□) as described under Materials and Methods. Activity data are corrected for the spontaneous losses of activities that occurred in the absence of cardiotoxin.

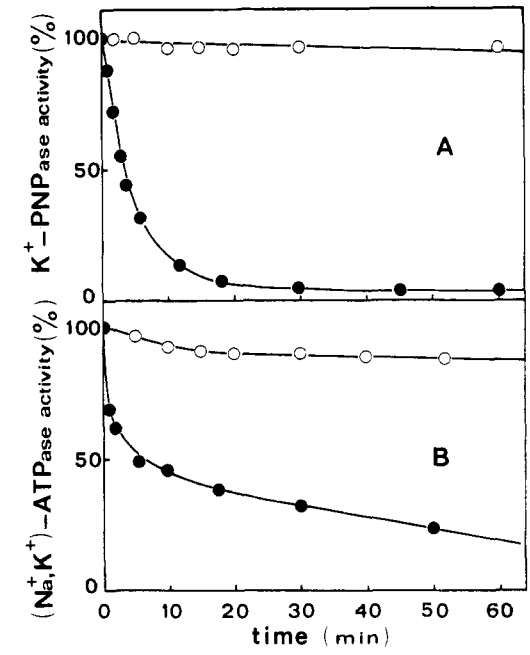

FIGURE 4: Kinetics of cardiotoxin-induced losses of K^+-PNPase and Na^+,K^+-ATPase activities in native axonal membranes and after solubilization with Lubrol WX. K^+-PNPase and Na^+,K^+-ATPase activities were measured at 25 °C, pH 7.5, in the presence of 10^{-4} M cardiotoxin. Inactivations proceeded in the following conditions: triethanolamine buffer (50 mM) at pH 7.5 containing 5 mM $MgCl_2$, 100 mM NaCl, and 10 mM KCl for Na^+,K^+-ATPase; same buffer containing 20 mM $MgCl_2$ and 20 mM KCl for K^+-PNPase. Activity data presented in this Figure are corrected for the spontaneous losses of activities that occurred in the absence of cardiotoxin. (A) K^+-PNPase activity in the native (●) and solubilized (○) state. (B) Na^+,K^+-ATPase activity in the native (●) and solubilized (○) state.

2 °C at pH 7.5 (10 mM Tris-HCl buffer containing 0.32 M sucrose). The properties of the solubilized material were measured on the supernatant fraction after centrifugation for 1 h at 100 000g.

Results

Interaction of Cardiotoxin D_1 with Axonal Membranes. Inhibition of the Na^+,K^+-ATPase Activity and Stoichiometry of Binding. The activity of the Na^+,K^+-ATPase was often measured in this paper by following the enzyme activity on a pseudosubstrate, *p*-nitrophenyl phosphate. Similar to the Na^+, K^+-ATPase activity, the K^+-dependent *p*-nitrophenylphosphatase (K^+-PNPase) activity of the ATPase is inhibited by ouabain (Dahl and Hokin, 1974; Glynn and Karlish, 1975).

Cardiotoxin D_1 completely deactivates the ATPase (Figure 1). Other components of the axonal membrane such as acetylcholinesterase (Figure 3) or the tetrodotoxin binding protein (Balerna et al., 1975) (Figure 2) are not affected by the cardiotoxin action. The maximal binding coincident with total inhibition can be evaluated from Figure 1. It is 50 nmol/mg of membrane protein.

The amount of cardiotoxin associated to the membrane can also be evaluated from [^{3}H]cardiotoxin binding experiments. Figure 3 shows that [^{3}H]cardiotoxin binding to axonal membranes closely follows the decrease of the K^+-PNPase activity (i.e., Na^+, K^+-ATPase activity). Moreover, we have observed that at concentrations of [^{3}H]cardiotoxin lower than 10^{-4} M each new molecule of toxin added to the membrane preparation binds quantitatively to its receptor site. The amount of free (unbound) [^{3}H]cardiotoxin is negligible.

Concentrations of [^{3}H]cardiotoxin higher than 10^{-4} M were saturating (Figure 3). The maximal binding capacity of the membranes found by this approach was 42 nmol of [^{3}H]cardiotoxin/mg of membrane protein.

Kinetics Data for Binding of [^{3}H]Cardiotoxin and Loss of K^+-PNPase and Na^+,K^+-ATPase Activities. Binding of [^{3}H]cardiotoxin to axonal membranes is characterized by very fast rates of association and dissociation. Association of [^{3}H]cardiotoxin to axonal membranes is too fast to be accurately measured. It is completed in less than 1 min at 25 °C, pH 7.5. The same situation is observed for dissociation: replacement of [^{3}H]cardiotoxin from the [^{3}H]cardiotoxin–axonal membrane complex by a large molar excess of unlabeled cardiotoxin takes again less than 1 min.

The kinetics of loss of K^+-PNPase activity and of Na^+,K^+-ATPase activity are presented in Figures 4A, B. Kinetics of cardiotoxin-induced K^+-PNPase inactivation roughly follow a first-order behavior with a half-life of about 4 min. The kinetics of loss of Na^+,K^+-ATPase activity are more complex and cannot be described by a simple kinetics scheme. In both cases, kinetics of deactivation are much slower than kinetics of [^{3}H]cardiotoxin binding to membranes.

No recovery of Na^+,K^+-ATPase or K^+-PNPase activities was observed by washing the axonal membranes after inactivation in the presence of cardiotoxin, even after long periods of time. Cardiotoxin inactivation of the enzyme is irreversible.

Cardiotoxin Action on K^+-PNPase and Na^+,K^+-ATPase Activities after Membrane Solubilization with Lubrol WX. After solubilization of axonal membranes with 0.75% Lubrol and centrifugation, the supernatant contains 100% of the initial acetylcholinesterase activity and 45% of the initial K^+-PNPase or Na^+,K^+-ATPase activities. Figures 4A,B show that Lubrol solubilization nearly completely prevents cardiotoxin inhibition of both the K^+-PNPase and the Na^+,K^+-ATPase activities. Even after a 60 min incubation at 25 °C, pH 7.5, in the presence of 0.1 mM cardiotoxin, 90% of the solubilized K^+-PNPase activity persists. Under identical conditions of incubation, the activity of the K^+-PNPase embedded in the membrane is completely lost after 20 min (Figure 4A). A similar situation is found for the Na^+,K^+-ATPase activity.

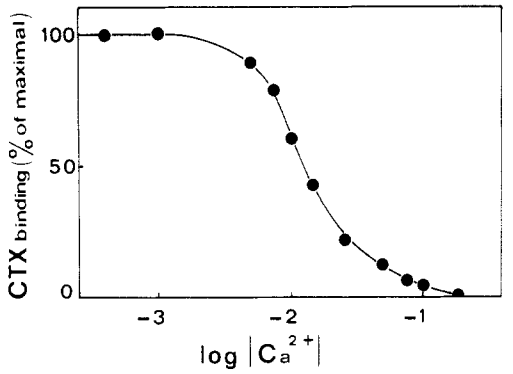

FIGURE 5: Ca^{2+} ion prevention of the binding of [^{3}H]cardiotoxin to axonal membranes. Axonal membranes (0.4 mg of protein/ml) were incubated with 1 μM [^{3}H]cardiotoxin as under experimental conditions described in Figure 1, in the presence of the indicated concentrations of $CaCl_2$. After an incubation time of 5 min, aliquots were taken and used to determine the specific binding of [^{3}H]cardiotoxin to axonal membranes.

Analysis of Ca^{2+} Ions, Veratridine, and Anesthesics Protection of the Axonal Membrane against Cardiotoxin Binding. Ca^{2+} ions that are known to antagonize cardiotoxin effect upon nerve conduction (Chang et al., 1972) prevent binding of [^{3}H]cardiotoxin to the axonal membrane (Figure 5).

Veratridine protection was also assayed because this toxin has a selective action on the Na^+ channel (Ulbricht, 1969) and binds to the lipid phase of the axonal membrane (Balerna et al., 1975). Veratridine produces considerable depolarizations of the crustacean axons at concentration of 0.01–0.1 mM (Balerna et al., 1975). At a concentration of 0.01 mM, veratridine does not protect against [^{3}H]cardiotoxin (1 μM) binding to the membrane (0.1 mg of membrane protein/ml). At 0.1 mM, veratridine protects but only by 20%.

Anesthesics were also tried: they strongly affect nerve conduction, and they are lipid-soluble compounds. Dibucaine protects against [^{3}H]cardiotoxin, but only at high concentrations. No protection is observed at concentration lower than 0.05 mM dibucaine. Concentrations of 2–4 mM dibucaine decrease specific binding of [^{3}H]cardiotoxin by 60–70%.

Discussion

Acetylcholinesterase is an important enzyme of the axonal membrane (Balerna et al., 1975). Its activity is unaffected by cardiotoxin treatment. The functioning of the Na^+ channel in axons is selectively altered by tetrodotoxin and veratridine. Tetrodotoxin is a highly specific and potent inhibitor of the transient increase in sodium conductance in excitable membranes (for recent reviews, see Evans, 1972, and Narahashi, 1974); veratridine depolarizes nerve membranes by a selective increase in resting sodium permeability (Ulbricht, 1969; Ohta et al., 1973). It has been demonstrated in this paper that the interaction of tetrodotoxin with its membrane receptor is unaltered by the presence of cardiotoxin and that [^{3}H]cardiotoxin binding to the axonal membrane is unaffected by "physiological" concentrations of veratridine.

All these results strongly suggest that cardiotoxin action on the axonal membrane primarily affects ATPase inactivation.

The maximal amount of cardiotoxin bound to the axonal membrane is very high: 42–50 nmol/mg of membrane protein (as a comparison maximum tetrodotoxin binding is 10–12 pmol/mg of membrane protein (Balerna et al., 1975)). The amount of Na^+,K^+-ATPase in the crab axonal membrane is only 300 pmol/mg of membrane protein. In consequence, an average binding of 150 mol of cardiotoxin/mol of ATPase is necessary to inactivate the enzyme. Such a high stoichiometry is the first indication that direct cardiotoxin association to the protein moiety of the Na^+,K^+-ATPase is improbable. In fact, the maximum binding capacity for cardiotoxin, 42–50 nmol/mg of membrane protein, is even higher than the *total amount* of protein in the membrane that has been estimated to be of about 10 nmol of protein/mg of membrane protein (Balerna et al., 1975). The consequence of these observations is that cardiotoxin probably binds to the lipid phase of the membrane. Cardiotoxin first binds very rapidly and reversibly to the axonal membrane. This first step then must be followed by a slow and irreversible reorganization of the membrane structure that is responsible for the inactivation of the Na^+,K^+-ATPase. When the lipid environment of the enzyme is removed by solubilization with Lubrol WX, cardiotoxin looses its ability to inactivate.

It should be kept in mind however that the effect of Lubrol WX could also be due to the possible formation of a detergent shield preventing accessibility to the enzyme for cardiotoxin.

It has been shown recently that Ca^{2+} antagonizes the effects of cardiotoxin on nerve conduction at a concentration of 10 mM (but not at 1 mM) (Chang et al., 1972). These pharmacological observations are readily explained by the results presented in Figure 5. Ca^{2+} interferes with [^{3}H]cardiotoxin binding to the axonal membrane but only when present in high concentrations. Fifty percent inhibition of binding is obtained at 12.5 mM Ca^{2+}. Ca^{2+}-induced prevention of cardiotoxin binding is probably due to Ca^{2+} association to phospholipids in the membrane. Hauser et al. (1975) have recently reported an apparent dissociation constant for the Ca^{2+}-phospholipid interaction of about 30 mM.

Snake neurotoxins and cardiotoxins are parent proteins (Lee, 1972; Yang, 1974). Snake neurotoxins are the best known of all animal neurotoxins. They associate with a membrane protein that has been identified as the acetylcholine receptor (Rang, 1975) and even isolated in the pure form (Meunier et al., 1974; Miledi et al., 1971; Raftery et al., 1971; Eldefrawi et al., 1972; Klett et al., 1973; Biesecker, 1973). Interactions between snake neurotoxins and their post-synaptic receptors are very tight; dissociation constants of the complexes are between 1 nM and 20 pM (Fulpius et al., 1972; Weber and Changeux, 1974; Chicheportiche et al., 1975).

Extensive structural homologies have been found between cardiotoxins and snake neurotoxins (Lee, 1972; Yang, 1974). Both proteins have the same number of amino acids: 61–62 amino acids for the short neurotoxins[3] and 60 amino acids for cardiotoxins. Both types of molecules have four disulfide bridges and the eight half-cystine residues in cardiotoxins and neurotoxins are placed at the same positions. Besides the invariant distribution of the eight half-cystine residues that are important for maintaining the protein in its active conformation, seven amino acid residues are found to be common to both groups of toxins: Gly-20, Tyr-25, Arg-43, Gly-44, Pro-50, Asp-64, and Asn-67. Two additional residues have similar side-chain functions: Ile/Leu/Val-56 and Lys/Arg-65.

Tyr-25, Trp-29, Arg-37, Lys-27, and Lys-57 have been postulated to be part of the active area of snake neurotoxins (Chang and Yang, 1973; Yang, 1974; Chicheportiche et al., 1972; Chicheportiche et al., 1975). Of all these residues, only Tyr-25 is present in both neurotoxins and cardiotoxins. Lys-27

[3] There are short neurotoxins and long neurotoxins comprising 71–74 amino acids (Yang, 1974).

and Trp-29, for example, are replaced by methionine residues that remain invariant in all cardiotoxins sequenced to date (Yang, 1974).

Because of the large extent of homologies in snake neurotoxins and cardiotoxins, because of the identical location of the eight half-cystine residues, the spatial structures of these two types of toxins are probably similar. However, amino-acid replacements in the active-site area have produced very drastic differences in the mode of action of these small proteins. The neurotoxin binds to a protein receptor at the post-synaptic level and blocks acetylcholine transmission (Rang, 1975); it does not bind to the axonal membrane (Balerna et al., 1975). Conversely, cardiotoxin apparently binds to a lipid-type receptor structure, to trigger a structural rearrangement in the membrane that inactivates the Na^+,K^+-ATPase.

Added in Proof

Since the submission of this paper, it has been shown that a cytotoxin isolated from *Naja naja* venom inactivated the Na^+,K^+-ATPase of nonexcitable cells such as the Yoshida sarcoma cells, the human oral carcinoma cells, and the human lung embyonic cells.

Acknowledgments

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