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Interaction of Polypeptide Neurotoxins With a Receptor Site Associated With Voltage-Sensitive Sodium Channels

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Anthopleurin A, a polypeptide toxin from the Pacific sea anemone Anthopleura xanthogrammica, enhances persistent activation of voltage-sensitive sodium channels by the alkaloid toxins veratridine and batrachotoxin with $K_{0.5}$ = 20 nM. This effect is inhibited by depolarization. There is a close correlation between enhancement of sodium channel activation and block of [^{125}I] scorpion toxin binding by unlabeled scorpion toxin, sea anemone toxin II from Anemonia sulcata, and Anthopleurin A, indicating that these three polypeptide toxins interact with a common receptor site in modifying sodium channel function. Photoactivable derivatives of scorpion toxin label a single M_T ~ 250,000 polypeptide chain at the polypeptide toxin receptor site. Labeling is blocked by unlabeled scorpion toxin or depolarization and is not observed in variant neuroblastoma clones, which lack sodium channels. These results identify a protein component of the polypeptide toxin receptor site of voltage-sensitive sodium channels.

Key words: sodium channels, neurotoxins, ion transport, photoaffinity labelling

Voltage-sensitive sodium channels responsible for action potential generation in nerve and neuroblastoma cells have three separate receptor sites for neurotoxins [for a review, see reference 1]. Receptor site 1 binds tetrodotoxin and saxitoxin, which are specific inhibitors of action potential sodium currents. Receptor site 2 binds the lipid-soluble alkaloid toxins veratridine, batrachotoxin, aconitine, and grayanotoxin. These toxins alter the voltage-dependence of activation and inactivation of sodium channels and cause persistent activation of a fraction of sodium channels at the resting membrane potential. Receptor site 3 binds the polypeptides scorpion toxin and sea anemone toxin. The polypeptide toxins slow or block inactivation of voltage-sensitive sodium channels and act cooperatively with the alkaloid toxins to cause persistent activation of sodium channels. The binding and action of the polypeptide toxins are highly voltage dependent. The voltage dependence of binding closely parallels the voltage dependence of sodium channel activation, suggesting that the receptor site for these toxins is located on a voltage-sensitive component of the sodium channel involved in the process of activation. These properties of the neurotoxin receptor sites associated with voltage-sensitive sodium channels are summarized in Table I.

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TABLE I. Properties of Neurotoxin Receptor Sites

Toxin receptor site	Ligands	Physiologic effect
1	Tetrodotoxin Saxitoxin	Inhibition transport
2	Veratridine Batrachotoxin	Alter activation and inactivation
	Aconitine Grayanotoxin	Cause persistent activation
3	Scorpion toxin Sea anemone toxin	Inhibit inactivation; enhance persistent activation

The sea anemone toxin studied in our previous experiments [2] was purified from the Mediterranean anemone Anemonia sulcata [3]. A polypeptide toxin has also been purified from the Pacific anemone Anthopleura xanthogrammica [4]. This toxin has substantial sequence homology with toxin II of A sulcata [5] and has dramatic effects on electrical excitability and contractility of cardiac muscle [6]. In this report we describe the action of Anthopleurin A on sodium channels in neuroblastoma cells and present additional data on the covalent labeling of the polypeptide toxin receptor site of the sodium channel with a photoactivable derivative of scorpion toxin.

EXPERIMENTAL PROCEDURES

Materials

The sources of commercially available materials have been cited previously [7]. Anemonia sulcata toxin II was provided by Dr. Laszlo Beress, University of Kiel, Kiel, West Germany [3]. Anthopleurin A was provided by Dr. T. R. Norton, University of Hawaii [4]. Batrachotoxin was provided by Drs. John Daly and Bernhard Witkop, National Institute of Arthritis, Metabolism, and Digestive Diseases, NIH. Scorpion toxin was purified from the venom of Leiurus quinquestriatus and radioactively labeled by lactoperoxidase catalyzed iodination as described previously [7].

$^{22}Na^+$ Uptake Measurements

The activation of sodium channels by neurotoxins was studied by measuring the initial rate of passive $^{22}Na^+$ influx into neuroblastoma cells (clone N 18) maintained in cell culture. These procedures have been described in detail previously [9, 10]. Briefly, cell cultures were incubated with the concentrations of toxins indicated in the figure legends for 30 min at 36°C in 0.25 ml of medium consisting of 130 mM choline Cl, 5.4 mM K Cl, 50 mM Hepes (adjusted to pH 7.4 with Tris base), 5.5 mM glucose, 0.8 mM $MgSO_4$, and 1 mg/ml of bovine serum albumin. After 30 min, this medium was removed. The cells were then incubated for 30 sec in medium containing the same concentrations of neurotoxins and 5.4 mM KCl, 125 mM choline chloride, 5 mM NaCl, 5 mM ouabain, 50 mM Hepes (adjusted to pH 7.4 with Tris base), 5.5 mM glucose, 0.8 mM $MgSO_4$, and 1.0 μCi/ml of $^{22}NaCl$. Finally, the cells were washed three times with 3 ml of medium consisting of 163 mM choline chloride, 5 mM Hepes (adjusted to pH 7.4 with Tris base), 5.5 mM glucose, 0.8 mM $MgSO_4$, and 1.8 mM $CaCl_2$. Under these conditions the membrane potential of the cells is –41 mV throughout the experiment. In some experiments, toxins were allowed to bind at 0 mV by substitut-

ing 130 mM KCl for 130 mM choline chloride in the initial incubation. $^{22}Na^+$ influx was then measured as described above. Under both these conditions, the initial rate of $^{22}Na^+$ influx is directly proportional to Na^+ permeability [8, 9] and therefore to the fraction of sodium channels activated by toxin treatment. In each experiment, the rate of influx in the absence of neurotoxins is subtracted from the data.

Scorpion Toxin Binding Measurements

Scorpion toxin binding has been described in detail previously [7]. Scorpion toxin requires approximately 1 hr to equilibrate with its binding sites in N 18 cells. In binding experiments, cell cultures in 1.6-cm diameter multiwell plates (Costar) were incubated for 60 min at 36°C with ^{125}I-labeled scorpion toxin in medium consisting of 130 mM choline chloride, 50 mM Hepes (adjusted to pH 7.4 with Tris base), 5.5 mM glucose, 0.8 mM $MgSO_4$, 5.4 mM KCl, and 1 mg/ml of bovine serum albumin. Unbound toxin was removed by washing three times for 1 min each at 36°C with 3 ml of wash medium consisting of 163 mM choline chloride, 5 mM Hepes (adjusted to pH 7.4 with Tris base), 1.8 mM $CaCl_2$, and 0.8 mM $MgSO_4$, and 1 mg/ml of bovine serum albumin. Washed cell cultures were suspended in 0.4 N NaOH, and the bound ^{125}I was measured in a gamma scintillation spectrometer. In each experiment, nonspecific binding was measured in the presence of 100 nM unlabeled scorpion toxin and was subtracted from the results [7]. For the experiments presented, nonspecific binding was less than 17% of total binding.

Covalent Labeling Experiments

Photoaffinity labeling experiments were carried out as described previously [10]. Azido nitrobenzoyl [^{125}I] mono iodo scorpion toxin was prepared as described elsewhere [10] immediately before each experiment. Confluent cells in multiwell plates were incubated with [^{125}I] scorpion toxin or ANB-[^{125}I] scorpion toxin under red light in standard binding medium consisting of 130 mM choline chloride, 50 mM Hepes (adjusted to pH 7.4 with Tris base), 5.5 mM glucose, 0.8 mM $MgSO_4$, 5.4 mM KCl, and bovine serum albumin at 1 mg/ml. Unbound toxin was removed by washing. Specific and nonspecific binding were determined as above. Then the cells were either irradiated for 15 min at 20°C with a Sylvania Blacklite blue fluorescent bulb (λ_{max} = 356 nm) at a distance of 3 cm or kept in the dark at 20°C. After irradiation, cells were incubated for 70 min under depolarized conditions in high K^+ binding medium (130 mM KCl replacing 130 mM choline chloride) to allow dissociation of reversibly bound toxin. The cells were washed and gently scraped from the multiwells into phosphate-buffered saline with a rubber policeman.

A partially purified surface membrane preparation was made by allowing the cells to swell in 1.5 mM CaCl, 10 mM NaCl, 10 mM Tris-HCl, pH 7.5, for 30 min and then homogenizing in a Teflon glass homogenizer. After addition of 1.7 vol of 2 M sucrose, 34 mM EDTA, 50 mM Tris-HCl, pH 7.5, the membranes were sedimented at 40,000g for 45 min and resuspended in 10 mM Tris-HCl, pH 7.4. These samples were used for gel electrophoresis. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate/2-mercaptoethanol was performed essentially as described by Maizel et al [22]. Protein samples were depolymerized in 3% sodium dodecyl sulfate, 5% sucrose, 30 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1% 2-mercaptoethanol by incubation at 100°C for 2 min. Samples were then applied to slab gels with a linear gradient of acrylamide from 4.5% to 10% in the separating gel. Molecular weights of protein bands were estimated by comparison with the migration of the following proteins of known molecular weight: thyroglobulin, 330,000; β-galactosidase, 117,000; bovine serum albumin, 68,000; catalase, 60,000; and glyceraldehyde-3-phosphate dehydrogenase, 36,000.

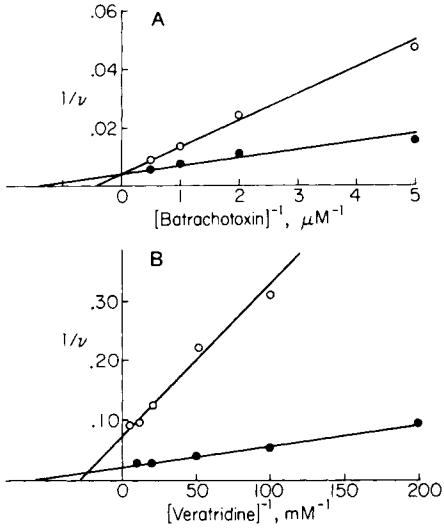

Fig. 1. Enhancement of alkaloid toxin activation of sodium channels by Anthopleurin A. Neuroblastoma cells were incubated in KCl substituted medium for 30 min at 36°C with the indicated concentrations of batrachotoxin (A) or veratridine (B) with (•) or without (○) 1 μM Anthopleurin A. Initial rates of $^{22}Na^+$ influx were then measured in the presence of the same toxin concentrations as described in Experimental Procedures. The data are presented as a double-reciprocal plot with ordinate units of $nmol^{-1}$ min mg.

Radioactively labeled protein bands were visualized by autoradiography, and the gels were scanned in a densitometer.

RESULTS AND DISCUSSION

Polypeptide neurotoxins have been demonstrated to have two functional effects on sodium channels. Voltage clamp studies show that scorpion toxin [11, 12], Anemonia sulcata toxins I and II [13–15], and Anthopleurin A [16] all specifically inhibit inactivation of sodium channels. Ion flux studies using neuroblastoma cells cultured in vitro show that scorpion toxin [9] and Anemonia sulcata toxin II [2, 17] enhance persistent activation of voltage-sensitive sodium channels by alkaloid neurotoxins. Figure 1 illustrates the effect of Anthopleurin A on activation of sodium channels by veratridine and batrachotoxin in the form of a double-reciprocal plot. Alkaloid toxin titration curves in the presence and absence of Anthopleurin A give straight lines consistent with interaction of these toxins at a single class of sites in activating sodium channels [9]. Batrachotoxin is a full agonist activating all sodium channels in neuroblastoma cells [9]. Anthopleurin A (1 μM) reduces $K_{0.5}$ 3-fold with no effect on V_{max} for $^{22}Na^+$ uptake (Fig. 1A). Veratridine is a partial agonist activating only a fraction of sodium channels at saturation [9]. Anthopleurin A (1 μM) both reduces $K_{0.5}$ from 34 μM to 17 μM and increases V_{max} from 14 moles/min/mg to 50 nmoles/min/mg. These effects are identical to those observed with scorpion toxin [9] and Anemonia sulcata toxin II [2]. Therefore, these three toxins have similar effects on functional properties of sodium channels measured by both voltage-clamp and ion-flux methods. The effect of all three polypeptide toxins on activation of sodium channels by veratridine and batrachotoxin can be fit by an allosteric model described previously [2, 9].

The effect of different concentrations of Anthopleurin A on activation of sodium channels by veratridine is illustrated in Figure 2. A half-maximal effect is obtained at 20 nM. The concentration-effect curves are consistently shallower than hyperbolic, suggesting multiple sites of interaction or negative cooperativity. They are not as shallow as those for Anemonia sulcata toxin II [2, 17; see below].

The enhancement of veratridine activation by scorpion toxin [18] and Anemonia sulcata toxin [2] is voltage-dependent. The voltage dependence of Anthopleurin A action was tested by allowing Anthopleurin A to bind to its receptor site during a preliminary incubation at a membrane potential of −41 mV in choline-substituted medium or at a membrane potential of 0 mV in K^+-substituted medium. The initial rate of $^{22}Na^+$ uptake was then

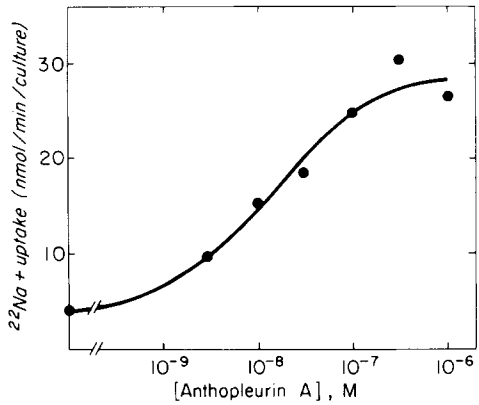

Fig. 2. Concentration dependence of Anthopleurin A action. Neuroblastoma cells were incubated in choline-substituted medium for 30 min at 36°C with the indicated concentrations of Anthopleurin A. Initial rates of $^{22}Na^+$ influx were then measured in the presence of 200 μM veratridine and the same concentrations of Anthopleurin A.

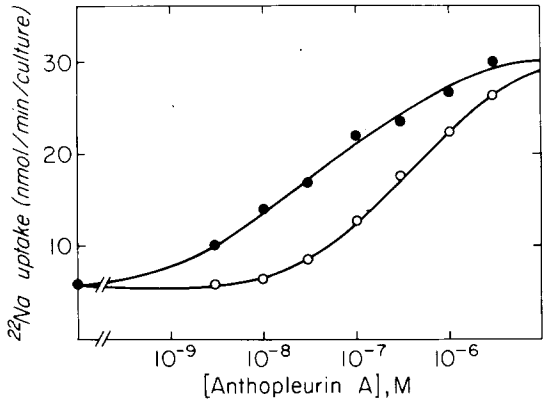

Fig. 3. Effect of depolarization on Anthopleurin A action. Neuroblastoma cells were incubated in choline-substituted (•) or KCl-substituted (○) medium for 30 min at 36°C with the indicated concentrations of Anthopleurin A. Initial rates of $^{22}Na^+$ uptake were then measured in choline-substituted medium with 200 μM veratridine but no Anthopleurin A, as described in Experimental Procedures.

measured for 30 sec in choline-substituted medium containing 200 μM veratridine but no Anthopleurin A. Thus, Anthopleurin A is allowed to bind to cells at 2 different membrane potentials, and then Na^+ permeability is measured at −41 mV. This procedure does not give measurements strictly at equilibrium because some dissociation of the Anthopleurin A occurs during the flux measurement. Comparison of $K_{0.5}$ measured in this way at −41 mV (40 nM, •, Fig. 3) with that measured at equilibrium (20 nM, Fig. 2) illustrates the effect of dissociation during the 30 sec assay period. Nevertheless, an approximate measure of the voltage dependence of toxin binding and action can be obtained in this way. The data of Figure 3 show that depolarization causes a shift of $K_{0.5}$ from 40 nM to 300 nM and makes the concentration-effect curve steeper. Since the measurement is not strictly at equilibrium, it is not possible to interpret these effects quantitatively. However, in analogy with scorpion toxin [7, 18], it seems likely that depolarization increases the K_D for Anthopleurin A in binding to the polypeptide toxin receptor site.

[^{125}I] mono iodo scorpion toxin binds specifically to the polypeptide toxin receptor site associated with sodium channels [7]. Anemonia sulcata toxin II blocks scorpion toxin binding, indicating that these two toxins share a common receptor site [2]. In Figure 4, we have compared concentration-effect curves for enhancement of veratridine activation and for block of scorpion toxin binding by all three polypeptide toxins. There is a close similarity between the toxin specificity for binding and flux activation, with scorpion toxin having highest affinity, Anthopleurin A intermediate, and Anemonia sulcata toxin II lowest affinity in both experiments. However, for both Anthopleurin A and toxin II, $K_{0.5}$ values for uptake are somewhat lower than K_D values for binding. Nevertheless, the data provide strong support for interaction of all three toxins at a common receptor site associated with sodium channels. Although all three polypeptide toxins seem to interact with a common receptor site, some characteristic differences in action have been noted. Concentration-effect curves for scorpion toxin are always hyperbolic, whereas those for sea anemone toxin II and Anthopleurin are always shallower than hyperbolic. Thus, it is likely that the sea anemone toxins and scorpion toxin share a common (or overlapping) receptor site but that, in addition, the anemone toxins act at additional receptor sites or induce negatively cooperative interactions not observed with scorpion toxin.

The voltage dependence of scorpion toxin binding is closely correlated with the voltage dependence of activation of the sodium channel [19]. These results suggest that the polypeptide toxin receptor site is located on a region of the sodium channel that senses membrane voltage and is involved in voltage-dependent activation. It would be of great interest to identify the polypeptide chain components of this toxin receptor. Recently, we have developed photoactivable derivatives of scorpion toxin that react covalently with a specific polypeptide in neuroblastoma cell membranes [10]. Half of the reversibly bound toxin derivative is covalently attached upon photolysis [10]. Covalent reaction of the photoactivable toxin derivative blocks subsequent binding of toxin at 18% of the scorpion toxin receptor sites, indicating that covalent reaction with essential receptor site components has occurred [10]. Analysis of photolabeled neuroblastoma cells reveals labeling of a single polypeptide chain of 250,000 daltons [10]. A densitometric scan of a typical gel electrophoretogram is illustrated in Figure 5. Two peaks of radioactivity are observed: A high molecular weight peak 1.5 cm into the gel and a larger low molecular weight peak migrating at the front of the gel. The low molecular weight material is the same size as scorpion toxin and apparently represents free scorpion toxin and toxin covalently attached to small membrane components such as lipids. The specificity of covalent labeling is illustrated by carrying out the labeling reaction in the presence of excess unlabeled scorpion toxin. Under these conditions, label-

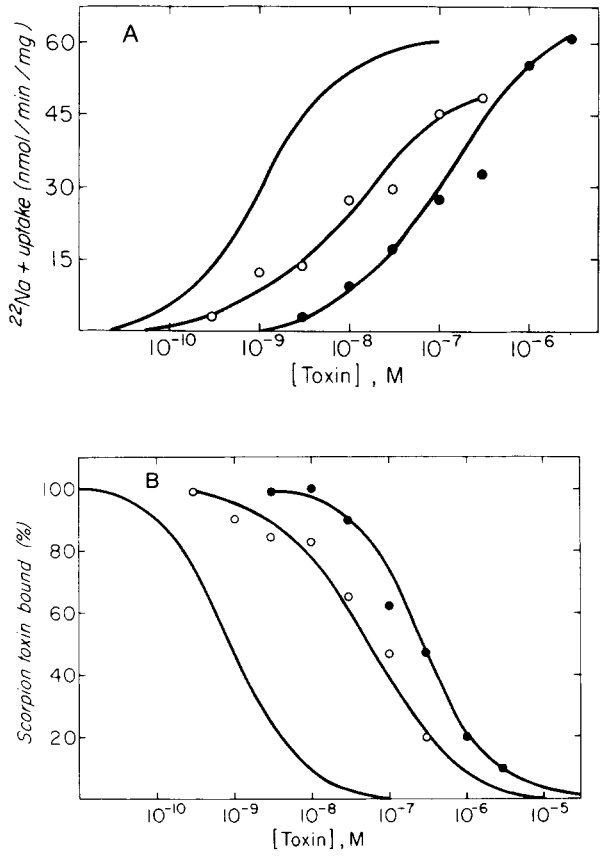

Fig. 4. Comparison of effects of sea anemone toxins on $^{22}Na^+$ influx and [^{125}I] scorpion toxin binding. A. Neuroblastoma cells were incubated in choline-substituted medium for 30 min at 36°C with the indicated concentrations of Anthopleurin A (○) or Anemonia sulcata toxin II (●). Initial rates of $^{22}Na^+$ uptake were then measured as described in Experimental Procedures in the presence of 200 μM veratridine. B. Neuroblastoma cells were incubated in choline-substituted medium for 30 min at 36°C with 0.2 nM [^{125}I] mono iodo scorpion toxin and the indicated concentrations of Anthopleurin A (○) or Anemonia sulcata toxin II (●). Specific binding of [^{125}I] scorpion toxin was measured as described in Experimental Procedures. Typical data for scorpion toxin from other experiments are illustrated by the curves on the left.

ing of the $M_r \sim 250{,}000$ protein is completely blocked, whereas the low molecular weight labeling is not markedly altered (fig. 5).

In Table II, the areas under the densitometric peaks corresponding to the $M_r \sim 250{,}000$ protein are tabulated for three different experiments in which specificity of labeling was tested. In experiment 1, labeling was reduced greater than 85% by unlabeled scorpion toxin, which blocks specific binding. In experiment 2, labeling was blocked greater than 90% by depolarization with 135 mM K^+, which also blocks specific binding. The block of covalent labeling by depolarization provides further strong support for the specificity of labeling. Finally, in experiment 3, covalent labeling of variant neuroblastoma cells which lack voltage-sensitive sodium channels was tested. Clone LV9 was isolated from the parental clone N 18 using a specific selection for resistance to the lethal effects of scorpion toxin

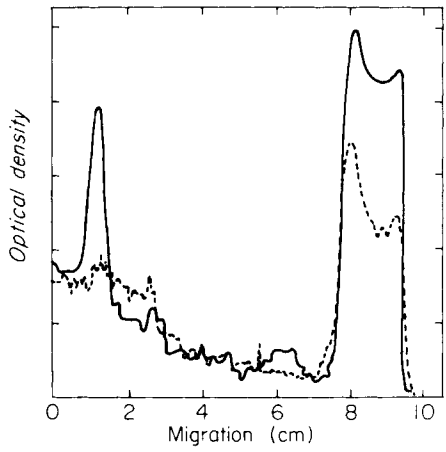

Fig. 5. Analysis of covalently labeled proteins by polyacrylamide gel electrophoresis. Neuroblastoma cells were covalently labeled using a photoactivable derivative of scorpion toxin as described in Experimental Procedures. The cells were dissolved in sodium dodecyl sulfate/mercaptoethanol solution and subjected to electrophoresis in slab gels formed with a gradient from 4.5% to 10% acrylamide. Radioactively labeled proteins were detected by autoradiography, and the autoradiograms were scanned with a densitometer which measured optical density in arbitrary units. Covalent labeling was carried out in the absence of unlabeled scorpion toxin (solid line) or in the presence of 200 nM unlabeled scorpion toxin (broken line).

TABLE II. Specificity of Covalent Labeling of the Sodium Channel*

Experiment	Cell line	Additions	Covalent labeling of $M_r \sim 250{,}000$ protein (arbitrary units)
1	N 18	None	217
		200 nM scorpion toxin	27
2	N 18	None	361
	N 18	135 mM K^+ (replacing choline)	27
3	N 18	None	289
	LV9	None	10

*Cultures of neuroblastoma cells were covalently labeled as described in Experimental Procedures. Labeled peptides were separated by SDS gel electrophoresis and subjected to autoradiography; and the intensity of each autoradiographic peak was determined by integration of densitometric scans.

plus veratridine [20]. This clone has no sodium channels detectable by ion-flux or toxin-binding experiments [20]. Similarly, when LV9 cells are covalently labeled with scorpion toxin, there is no detectable labeling of the $M_r \sim 250{,}000$ polypeptide. Taken together, the data of Table II provide strong evidence for identification of the $M_r \sim 250{,}000$ protein as a major component of the scorpion toxin receptor and therefore of the voltage-sensitive sodium channel.

The results presented here define the specific interaction of three polypeptide neurotoxins with a common receptor site associated with voltage-sensitive sodium channels. The receptor site is highly specific since Anthopleurin A and Anemonia sulcata toxin II, which have extensive sequence homology [5], bind with 5-fold different affinity. The interaction of the polypeptide receptor site with its ligands must depend on the tertiary structure of

the toxins, since the two sea anemone toxins and scorpion toxin have no detectable sequence homology yet bind and act at a common (or overlapping) receptor site.

The inhibition of sodium channel inactivation by the polypeptide toxins and the enhancement of alkaloid toxin activation suggest that the polypeptide toxin receptor site is located on part of the sodium channel structure that normally is involved in voltage-dependent regulation of activation and inactivation of the channel. The voltage dependence of scorpion toxin binding and its close correlation with the voltage dependence of activation of the sodium channel provide strong evidence that the polypeptide toxin receptor site is located on a component of the sodium channel that is voltage sensitive and undergoes a conformational change on activation of the sodium channel. Our covalent labeling results identify a $M_r \sim$ 250,000 protein as a component of the scorpion toxin receptor site. This protein is likely to be directly involved in voltage-dependent activation of the sodium channel. Purification and characterization of this novel protein should yield important insight into the molecular basis of electrical excitability.

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