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Interactions of Neurotoxins With the Action Potential Na^+ Ionophore

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Four neurotoxins that activate the action potential Na^+ ionophore of electrically excitable neuroblastoma cells interact with two distinct classes of sites, one specific for the alkaloids veratridine, batrachotoxin, and aconitine, and the second specific for scorpion toxin. Positive heterotropic cooperativity is observed between toxins bound at these two classes of sites. Tetrodotoxin, a specific inhibitor of the action potential Na^+ current, inhibits activation by each of these toxins in a noncompetitive manner (K_I = 4–8 nM). These results suggest the existence of three functionally separable components of the action potential Na^+ ionophore: two regulatory components, which bind activating neurotoxins and interact allosterically in controlling the activity of a third ion-transport component, which binds tetrodotoxin. The dissociation constant for scorpion toxin binding is increased 10-fold by depolarization of the cells with K^+, suggesting that the scorpion toxin binding site is located on a voltage-sensitive regulatory component of the ionophore.

INTRODUCTION

Several neurotoxins cause repetitive action potentials and persistent depolarization of nerves. This group of toxins includes the alkaloids veratridine (1, 2), batrachotoxin (3), and aconitine (4, 5), grayanotoxin (6), and the polypeptide toxins of scorpion venom (7–11) and coelenterate nematocysts (12). Since the action of these toxins is blocked by tetrodotoxin, a specific inhibitor of the action potential Na^+ current (13, 14), their effects have been ascribed to activation of the action potential Na^+ ionophore. These toxins, therefore, are potentially important tools in studying the mechanism of action potential generation.

Clonal lines of mouse neuroblastoma cells grown in vitro are electrically excitable (15, 16). The Na^+-dependent portion of the action potential is inhibited by tetrodotoxin at low concentration, suggesting that an action potential Na^+ ionophore identical with that in nerve axons is present in these cells (17, 18). Variant cell clones have been obtained which specifically lack the depolarizing phase of the action potential (18, 19). In this report we describe the interaction of veratridine, batrachotoxin, aconitine, and scorpion toxin with the action potential Na^+ ionophore of cultured neuroblastoma cells using isotopic flux measurements to detect the permeability changes caused by the toxins and ligand binding methods to study the formation of the toxin-ionophore complex.

METHODS

Detailed descriptions of the methods used in these experiments have been published elsewhere. Neuroblastoma cells were grown in vitro and changes in Na^+ permeability were measured by $^{22}Na^+$ uptake experiments as described previously (20–23). In brief, neuroblastoma cells grown to saturation in multiwell plates are incubated with neurotoxins in Na^+-free solution at 36°C to allow activation of the Na^+ ionophore without increasing the intracellular Na^+ concentration. Initial rate of $^{22}Na^+$ uptake is then measured in a medium containing 10 mM Na^+ with choline as an Na^+ replacement to maintain osmolarity (except where indicated) and with 5 mM ouabain added to inhibit active transport of Na^+. The Na^+ concentration is selected so that the dependence of uptake on Na^+ concentration is linear when the ionophore is maximally activated to ensure that the rate of $^{22}Na^+$ influx is not limited by fluxes of counterions (Cl^- moving in and K^+ moving out) required to maintain charge balance.

Scorpion toxin was purified from the venom of Leiurus quinquestriatus by ion exchange chromatography as described previously (23). The purified toxin was iodinated by standard procedures and the labeled toxin separated from unlabeled toxin by ion exchange chromatography. This procedure will be described in detail in a subsequent manuscript.

RESULTS AND DISCUSSION

Treatment of electrically excitable neuroblastoma cells such as clone N18 with veratridine, batrachotoxin, aconitine, scorpion toxin, or combinations of these agents causes an increase in Na^+ permeability that is reflected in an increased initial rate of uptake of $^{22}Na^+$ (Fig. 1, left). Similar treatment of electrically inexcitable cells such as clone N103 (Fig. 1, right) leads to no detectable increase in $^{22}Na^+$ uptake. In studies of over 20 clonal lines derived from mouse neuroblastoma C1300, clonal lines and primary cultures

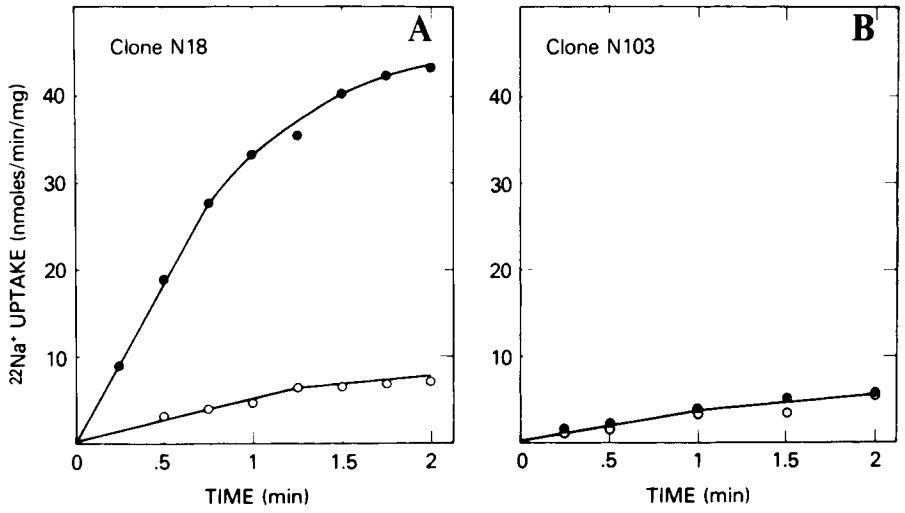

Fig. 1. Stimulation of $^{22}Na^+$ uptake by veratridine and scorpion toxin. Neuroblastoma cells of clone N18 (A) or N103 (B) were incubated for 30 min at 36°C in Na^+-free medium (○) or in Na^+-free medium containing 20 μM veratridine and 128 ng/ml scorpion toxin (●). Initial rate of $^{22}Na^+$ uptake was then determined.

of skeletal muscle cells, primary cultures of cardiac muscle cells, and clonal lines derived from various non-neuronal tissues including glial, fibroblast, liver, and kidney, a complete correlation has been found between electrical excitability and increase of Na^+ permeability by neurotoxins (reference 20 and unpublished results).

The increase in $^{22}Na^+$ uptake caused by treatment with these neurotoxins is completely inhibited by tetrodotoxin (20, 21), a specific inhibitor of action potential Na^+ current (13, 14). Half-maximal inhibition is obtained at 4–8 nM tetrodotoxin for all neuroblastoma lines tested and for primary cultures of chick skeletal and cardiac muscle (20, 21, and unpublished experiments). However, rat muscle cells, in which Na^+-dependent action potentials are relatively resistant to inhibition by tetrodotoxin (24), require approximately 1 μM tetrodotoxin for half-maximal inhibition (25).

Both these series of experiments provide strong evidence in favor of the conclusion that these neurotoxins act specifically on the Na^+ permeability pathway involved in the action potential by activating the action potential Na^+ ionophore in the absence of an electrical stimulus.

The action of these toxins does not require the presence of either ion gradients or a membrane potential. Figure 2 illustrates the increase in $^{22}Na^+$ uptake caused by treatment with veratridine plus scorpion venom in a medium with extracellular cation concentrations ($[K^+]$ = 135.5 mM, $[Na^+]$ = 15 mM, $[Ca^{++}]$ = 0) which mimic intracellular concentrations. The membrane potential (measured with microelectrodes) is zero under these conditions. These results support the conclusion that these neurotoxins activate the action potential Na^+ ionophore by a direct chemical mechanism.

Na^+ permeability of cells treated with one of the three alkaloid toxins (aconitine, veratridine, or batrachotoxin) increases with time to an equilibrium level that is concentration dependent (reference 21 and unpublished experiments). Figure 3 illustrates equilibrium concentration-response curves for aconitine, batrachotoxin, and veratridine.

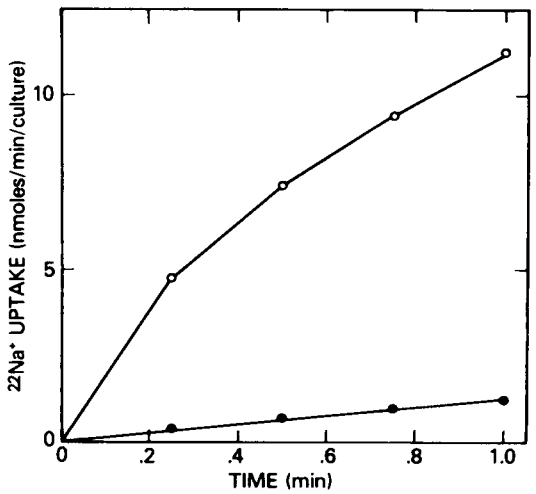

Fig. 2. Stimulation of $^{22}Na^+$ uptake by veratridine and scorpion toxin in the absence of ion gradients. Neuroblastoma cells of clone N18 were incubated for 30 min at 36°C in medium with 130 mM KCl, 15 mM NaCl, and no $CaCl_2$ in the presence (○) or absence (●) of 20 μM veratridine and 128 ng/ml scorpion toxin. Initial rate of $^{22}Na^+$ uptake was then determined in medium with the same ion concentrations.

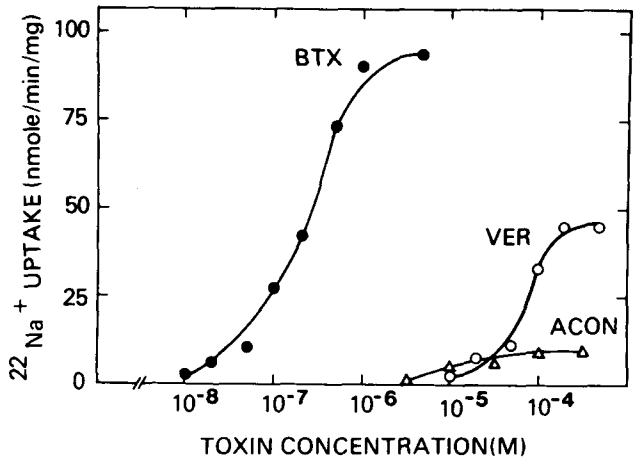

Fig. 3. Concentration dependence of activation by veratridine, batrachotoxin, and aconitine. Neuroblastoma cells of clone N18 were incubated for 30 min at 36°C in Na^+-free medium containing the indicated concentrations of aconitine (△), veratridine (○), or batrachotoxin (●). Initial rate of $^{22}Na^+$ uptake was then determined in medium containing 50 mM NaCl and Tris-Cl as as osmotic replacement.

Both the maximum rates of uptake of $^{22}Na^+$ (V_∞) and the concentrations required for half-maximal activation ($K_{0.5}$) differ markedly: batrachotoxin, V_∞ = 100 nmol/min/mg, $K_{0.5}$ = 0.3 μM; veratridine, V_∞ = 42 nmol/min/mg, $K_{0.5}$ = 80 μM; aconitine, V_∞ = 10 nmol/min/mg, $K_{0.5}$ = 8 μM. If these three toxins activate the action potential Na^+ ionophore by interaction with an identical class of binding sites, the rate of $^{22}Na^+$ uptake in the presence of saturating concentrations of any two toxins should not be greater than in the presence of batrachotoxin alone. Furthermore, as an excess of a less effective activator (such as aconitine) is added, batrachotoxin should be displaced from the common binding site and the rate of $^{22}Na^+$ uptake should be reduced. Such competitive interactions are observed among the three alkaloid neurotoxins (references 21 and 22, and unpublished results).

The measured uptake velocity (v) in the presence of two neurotoxins that interact competitively can be described quantitatively as the sum of the activity due to toxin 1 modified by competition with toxin 2 (v_1), and the activity due to toxin 2 modified by competition with toxin 1 (v_2).

$v = v_1 + v_2 = V_1 s_1/(K_1(1 + s_2/K_2) + s_1) + V_2 s_2/(K_2(1 + s_1/K_1) + s_2)$ where s is the concentration of toxin. If toxin 2 is aconitine, then v_2 is always small ($\leqslant$ 10 nmol/min/mg) and can be calculated from independent measurements of V_2, K_2, and K_1. It is possible, then, to test whether aconitine is a strictly competitive inhibitor of activation by batrachotoxin and veratridine by plotting $1/(v-v_2)$ against $1/s_1$ at different fixed aconitine concentrations (s_2) in the form of a Michaelis-Menten double reciprocal plot. The results of such experiments (Fig. 4) confirm that aconitine is a competitive inhibitor of activation by veratridine and batrachotoxin.

These results show that aconitine, veratridine, and batrachotoxin bind to a saturable class of binding sites during activation of the action potential Na^+ ionophore. The three toxins are lipophilic and veratridine has been shown to interact with lipid monolayers to cause expansion (26). These observations form the basis of the hypothesis that veratridine acts by perturbing the lipid structure of the membrane (26). Since interaction of the

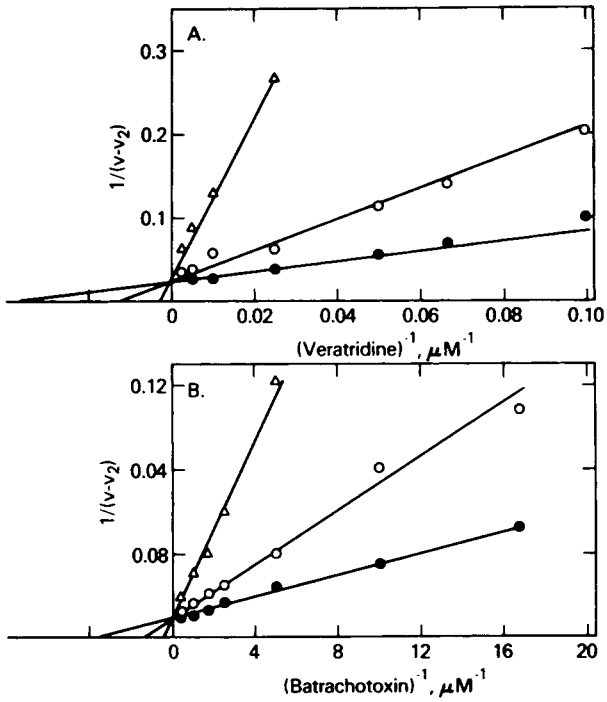

Fig. 4. Competitive inhibition of veratridine-dependent and batrachotoxin-dependent $^{22}Na^+$ uptake by aconitine. Neuroblastoma cells were incubated for 30 min at 36°C in Na^+-free medium in the presence of the indicated concentrations of veratridine (A) or batrachotoxin (B) plus 0 μM (●), 20 μM (○), or 50 μM (△) aconitine, and the rates of $^{22}Na^+$ uptake were measured in the presence of the same concentrations of toxin. The results are plotted according to the equation given in the text with K_2 = 8 μM, V_2 = 10 nmol/min/mg, and K_1 = 40 μM for veratridine and 0.25 μM for batrachotoxin as determined in companion experiments.

lipophilic toxins with the lipid phase of the membrane should be nonsaturable, the demonstration of competitive interactions among these toxins makes this mechanism unlikely. It is more likely that these three toxins bind to a specific site on the action potential Na^+ ionophore that is involved in the regulation of the ion transport activity of the ionophore.

In contrast to the competitive interactions observed among the three alkaloid toxins, scorpion venom interacts cooperatively with each of the alkaloid toxins. Experiments in which cells were treated with varying concentrations of the alkaloid toxins in the presence or absence of scorpion venom and then tested for Na^+ permeability are presented in Fig. 5. Scorpion venom causes a reduction in apparent K_D for each of the alkaloid toxins. It also causes an increase in the maximum velocity of $^{22}Na^+$ uptake at saturating concentrations.

We have purified a toxic polypeptide from scorpion venom using its ability to cause cooperative activation of the action potential Na^+ ionophore in the presence of veratridine as an assay (23). The purified protein appears homogeneous in gel electrophoretic and isoelectric focusing studies, has a molecular weight of 6,700 and an isoelectric point of approximately 9.7, and lacks methionine and histidine (23). Treatment of cells with 1–10 nM scorpion toxin and varying concentrations of batrachotoxin or aconitine gives

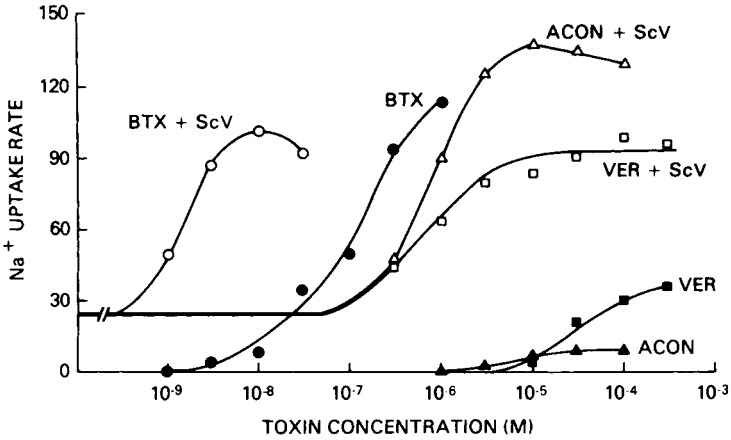

Fig. 5. Effect of scorpion venom on the concentration dependence of activation by veratridine, batrachotoxin, and aconitine. Neuroblastoma cells were incubated for 30 min at 36°C in Na^+-free medium in the presence of the indicated concentrations of veratridine, batrachotoxin, and aconitine plus 10 μg/ml scorpion venom where indicated. Initial rate of $^{22}Na^+$ uptake was measured in the presence of the same toxin concentrations in medium containing 50 mM NaCl and Tris-Cl as an osmotic replacement.

the concentration-response curves illustrated in Fig. 6. The purified protein reduces $K_{0.5}$ for aconitine, batrachotoxin, and veratridine 3-fold, 12-fold, and 4-fold respectively. As with the venom, V_∞ is increased for aconitine and veratridine but not for bactrachotoxin. Thus, batrachotoxin alone can induce the maximum rate of $^{22}Na^+$ uptake under these conditions.

These results demonstrate that the scorpion toxin acts at a site that is different from the binding site for the alkaloid toxins discussed above and that binding of scorpion toxin to that site causes an increase in the affinity of the alkaloid toxins for their binding site. These cooperative interactions between the alkaloid toxins and scorpion toxin imply that these two sites are allosterically coupled and exhibit positive heterotropic cooperativity, i.e. positive cooperativity between nonidentical binding sites.

In contrast to the whole venom, the purified scorpion toxin has little capacity to activate the action potential Na^+ ionophore unless an alkaloid toxin is also present (23). In order to determine whether alkaloid toxins affect apparent K_D for scorpion toxin, cells were treated for 30 min with various concentrations of scorpion toxin in the presence or absence of 20 μM veratridine, then washed and treated with 20 μM veratridine for 2 min, and finally, Na^+ permeability was determined. Since the action of scorpion toxin is only slowly reversible (23), sequential treatment with scorpion toxin and veratridine should give activation equivalent to simultaneous treatment with both toxins if veratridine does not affect apparent K_D for scorpion toxin. Equivalent activation is observed with these two different experimental protocols (Fig. 7), indicating that veratridine does not affect the affinity for scorpion toxin but is required to observe the effect of the toxin on Na^+ permeability. The cooperativity observed between alkaloid toxins and scorpion toxins is therefore not reciprocal. The mechanism of this cooperative interaction remains uncertain.

Studies of activation of the action potential Na^+ ionophore by veratridine and batrachotoxin did not reveal homotropic cooperativity in this process. The scorpion

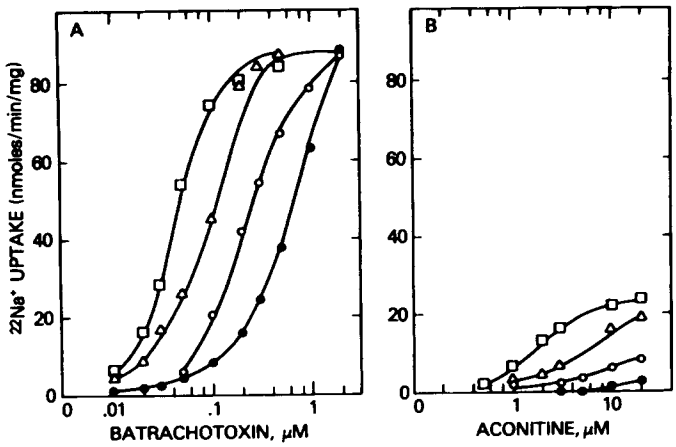

Fig. 6. Effect of purified scorpion toxin on the concentration dependence of activation by batrachotoxin and aconitine. Neuroblastoma cells were incubated for 30 min at 36°C in Na^+-free medium containing the indicated concentrations of batrachotoxin (left) or aconitine (right), with 0 ng/ml (●), 6.4 ng/ml (○), 32 ng/ml (△), or 320 ng/ml (□) purified scorpion toxin. Initial rates of $^{22}Na^+$ uptake were then determined.

toxin titration data of Fig. 7 are also fit by a simple Langmuir isotherm. Thus, these ligands exhibit heterotropic cooperativity but not homotropic cooperativity.

This interpretation of scorpion toxin action implies that reversible, noncovalent interaction of the toxin with the Na^+ ionophore is sufficient to cause activation. Experimental evidence not presented in this report supports this conclusion. The toxin acts without a lag time (23), the extent of activation is concentration dependent (23), and the activation by the toxin is completely reversible (23). In addition, assays for proteolytic activity using denatured casein as substrate and for phospholipase activity using egg lecithin as substrate were negative (unpublished experiments).

Tetrodotoxin, a specific inhibitor of the action potential Na^+ current (13, 14), inhibits the increase in $^{22}Na^+$ uptake caused by veratridine and batrachotoxin in a noncompetitive manner with K_I = 8 nM (21). Incubation of cells with increasing scorpion toxin concentrations in the presence or absence of tetrodotoxin followed by measurements of Na^+ permeability gives the concentration response curves illustrated in Fig. 8. These best fit curves are drawn for a common apparent K_D and different V_∞. Thus, tetrodotoxin is also a noncompetitive inhibitor of scorpion toxin action.

Tetrodotoxin and saxitoxin have been shown to compete for a common class of binding sites in nerve axon membranes (27, 28). The characteristics of inhibition of binding by cations has led to the hypothesis that this site represents a coordination site for transported cations (29).

The interactions of neurotoxins with the action potential Na^+ ionophore therefore define three functionally separable components of the action potential Na^+ ionophore. The alkaloid toxins and scorpion toxin interact with two regulatory components that bind these toxins specifically and interact cooperatively in controlling the ion transport activity of the ionophore. Tetrodotoxin and saxitoxin inhibit the ionophore by interaction with a third component that is directly involved in transport of ions and may contain an ion coordination site for transported cations.

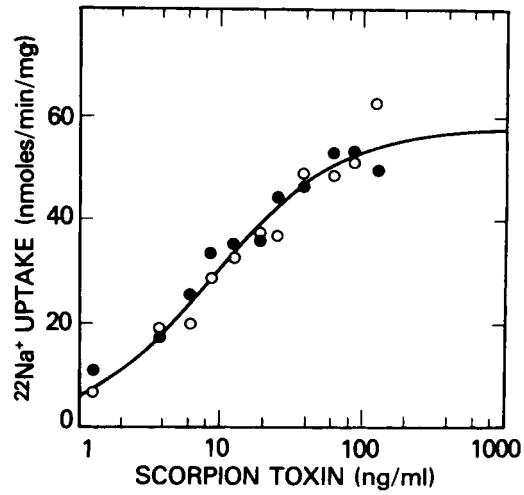

Fig. 7. Effect of veratridine on the concentration dependence of activation by scorpion toxin. Neuroblastoma cells were incubated for 30 min at 36°C in Na^+-free medium containing the indicated concentrations of purified scorpion toxin with (●) or without (○) 20 μM veratridine. Cells were rinsed and incubated for 2 min with 20 μM veratridine in Na^+-free medium and initial rates of $^{22}Na^+$ uptake were determined.

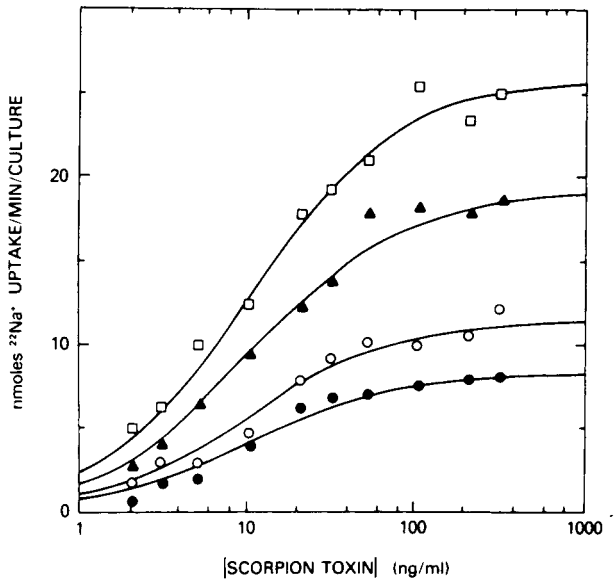

Fig. 8. Inhibition of scorpion toxin-dependent $^{22}Na^+$ uptake by tetrodotoxin. Neuroblastoma cells were incubated for 30 min at 36°C in Na^+-free medium containing the indicated concentrations of purified scorpion toxin and 0 nM (□), 2 nM (▲), 5 nM (○), or 10 nM (●) tetrodotoxin. Initial rates of $^{22}Na^+$ uptake were then determined in medium containing the same tetrodotoxin concentrations.

If these postulated regulatory components are involved in the normal voltage-dependent regulation of ionophore activity, it might be possible to detect membrane potential-dependent changes in toxin binding. In this experimental system, membrane potential can be controlled by alteration of the extracellular K^+ concentration during incubation with toxin in Na^+-free medium. In preliminary experiments we have not detected large changes in apparent dissociation constant for alkaloid toxins at different K^+ concentrations. Extracellular K^+ concentration has a large effect on the apparent K_D for scorpion toxin, however (Fig. 9). The apparent K_D is increased from 2.4 to 28 nM by increasing extracellular K^+ from 5.4 mM to 130 mM. Measurements of the rate of reversal of scorpion toxin activation under these conditions demonstrate that the change in K_D reflects at least in part an increase in the unimolecular off rate constant. These results suggest that depolarization of the cells causes a conformation change in the Na^+ ionophore that is reflected in a 10-fold increase in apparent K_D for scorpion toxin. The scorpion toxin binding component of the ionophore is therefore implicated in the voltage-dependent regulation of ionophore activity.

In order to show directly that depolarization inhibits the binding of scorpion toxin and not a subsequent step in its action, we have studied the binding of ^{125}I-labeled scorpion toxin. Excitable neuroblastoma cells (clone N18) have a saturable component of ^{125}I-scorpion toxin binding that is displaced by unlabeled toxin (Fig. 10). Clone N103, which is electrically inexcitable and does not respond to veratridine or scorpion toxin (Fig. 1), does not have a similar saturable component of binding (unpublished results). Binding of labeled toxin is inhibited by unlabeled toxin with a dissociation constant approximately

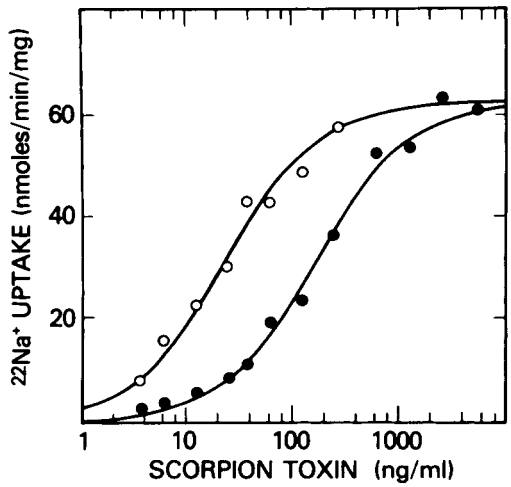

Fig. 9. Effect of extracellular K^+ concentration on activation by purified scorpion toxin. Neuroblastoma cells were incubated for 30 min at 36°C in Na^+-free medium containing the indicated concentrations of scorption toxin and either 135 mM KCl (●) or 130 mM choline Cl plus 5.4 mM KCl (○). Initial rates of $^{22}Na^+$ uptake were then determined in medium containing 10 mM NaCl and 120 mM choline Cl to maintain osmolarity.

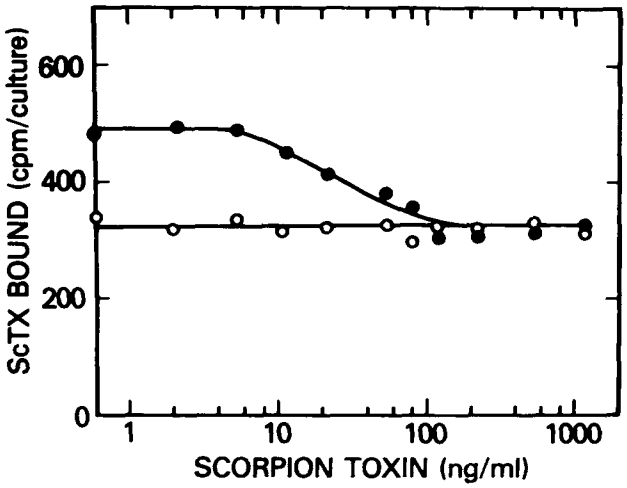

Fig. 10. Effect of extracellular K^+ concentration on binding of ^{125}I-labeled scorpion toxin. Neuroblastoma cells were incubated for 30 min at 36°C in Na^+-free medium containing ^{125}I-labeled scorpion toxin (0.5 nM ^{125}I), the indicated concentrations of unlabeled scorpion toxin, and either 135 mM KCl (○) or 130 mM choline Cl plus 5.4 mM KCl (●) to maintain osmolarity. Cells were washed to remove free toxin, and bound toxin was measured by gamma counting.

equal to its apparent K_D for activation of the Na^+ ionophore (compare Figs. 7 and 10). These results support the conclusion that the saturable component of binding observed represents binding to the site causing activation of the ionophore.

The saturable component of binding is much reduced in medium containing 135 mM K^+ (Fig. 10). These results confirm that the effect of K^+ is on the binding of toxin and also provide further support for the conclusion that the saturable binding of toxin represents binding to the Na^+ ionophore. Scorpion toxin thus provides a sensitive probe of a change in ionophore structure that is involved in membrane potential-dependent regulation of ionophore activity.

The neurotoxins discussed in this report appear to provide specific probes of three functionally separable components of the Na^+ ionophore. There is little doubt that they will prove useful in understanding the molecular basis of electrical excitability.

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