

Cooperative Activation of Action Potential Na⁺ Ionophore by Neurotoxins

(neuroblastoma cells/passive ²²Na⁺ influx/batrachotoxin/veratridine/aconitine/scorpion toxin)

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ABSTRACT 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 is a noncompetitive inhibitor of activation by each of these toxins ($K_I = 4-8$ 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.

Several neurotoxins cause repetitive action potentials and, in some cases, persistent depolarization of nerves. This group of toxins includes the alkaloids veratridine (1), batrachotoxin (2, 3), grayanotoxin (4), and aconitine (5), and the polypeptide neurotoxins of scorpion (6) and coelenterate (7) venoms (see Fig. 1 for structures). Since the action of these toxins is blocked by tetrodotoxin, a specific inhibitor of the action potential Na⁺ current (8, 9), 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.

Cultured neuroblastoma cells provide a useful experimental system in which to study the action potential Na⁺ ionophore by ion transport methods. Previous work has shown that treatment of electrically excitable neuroblastoma cells with veratridine results in a marked increase in passive Na⁺ permeability detectable by measurements of ²²Na⁺ uptake (10). Two kinds of evidence indicate that this increase in Na⁺ permeability reflects ion transport activity of the action potential Na⁺ ionophore: (i) the increase is completely inhibited by low concentrations of tetrodotoxin (10) and (ii) variant neuroblastoma clones specifically lacking the depolarizing phase of the action potential spike do not respond to veratridine (10).

Batrachotoxin has some structural features in common with veratridine (Fig. 1). Equilibrium dose-response relationships indicate that veratridine and batrachotoxin compete for a

single class of binding sites in activating the action potential Na⁺ ionophore (11) and that activation by both toxins is inhibited competitively by divalent cations (11) and non-competitively by tetrodotoxin ($K_I = 8-11$ nM) (11). Those results and the results of Albuquerque *et al.* (23, 24) suggest two kinds of toxin binding sites associated with the action potential Na⁺ ionophore: an activation or regulatory site with which veratridine and batrachotoxin interact (11) and an ion transport site with which the inhibitors tetrodotoxin and saxitoxin interact (9, 12). In the experiments described in this report, I have examined the interactions among four neurotoxins (veratridine, batrachotoxin, aconitine, and scorpion toxin) during activation of the action potential Na⁺ ionophore.

EXPERIMENTAL PROCEDURE

Materials. Chemicals were obtained from the following sources: veratridine from Aldrich; aconitine from K and K; tetrodotoxin from Calbiochem; ouabain and scorpion venom (*Leirus quinquestriatus*) from Sigma; ²²NaCl and [³H]-leucine from New England Nuclear; Dulbecco-Vogt modification of Eagle's minimal essential medium from Gibco; fetal bovine serum from Colorado Serum Co.; and recrystallized trypsin from Worthington. Batrachotoxin was kindly provided by Drs. John Daly and Bernhard Witkop. Stock solutions of batrachotoxin and aconitine were prepared in ethanol at 100 times the final concentration and diluted into assay medium at 36° immediately prior to use. Scorpion venom was dissolved at 1 mg (dry weight)/ml in distilled water at 0°, incubated for 1 hr, and centrifuged for 10 min at 12000 × *g*. The resulting supernatant, which contained all the activity, showed only low-molecular-weight polypeptides (3000-7000) on gel electrophoresis in sodium dodecyl sulfate.

Cell Cultures. Clone N18 of mouse neuroblastoma Cl300 was used for all studies. The cells were propagated in 100 mm petri dishes (Falcon) containing 10 ml of growth medium consisting of 5% fetal bovine serum and 95% Dulbecco's modified Eagle's medium in a water-saturated atmosphere of 10% CO₂/90% air. For experiments, cells from stock cultures were suspended after treatment with 0.02% (w/v) trypsin in Ca⁺⁺- and Mg⁺⁺-free Dulbecco's phosphate-buffered saline, sedimented, resuspended in growth medium, and seeded at a density of 20,000 cells per cm² in multi-well plates (1.6 cm diameter, Linbro Chemical Co.). Growth medium was replaced on day 3 with fresh growth medium and on day 5 with fresh growth medium containing 0.2 μCi/ml of [³H]leucine. Cultures were used on day 6.

Abbreviation: HEPES, *N*-2-hydroxyethyl piperazine *N'*-2-ethanesulfonic acid.

* The term "action potential Na⁺ ionophore" refers to the macromolecular structure responsible for the increase in Na⁺ permeability during the depolarizing phase of the nerve action potential. The term "ionophore" is taken to refer in general to ion-transporting molecules without implying a specific mechanism of action.

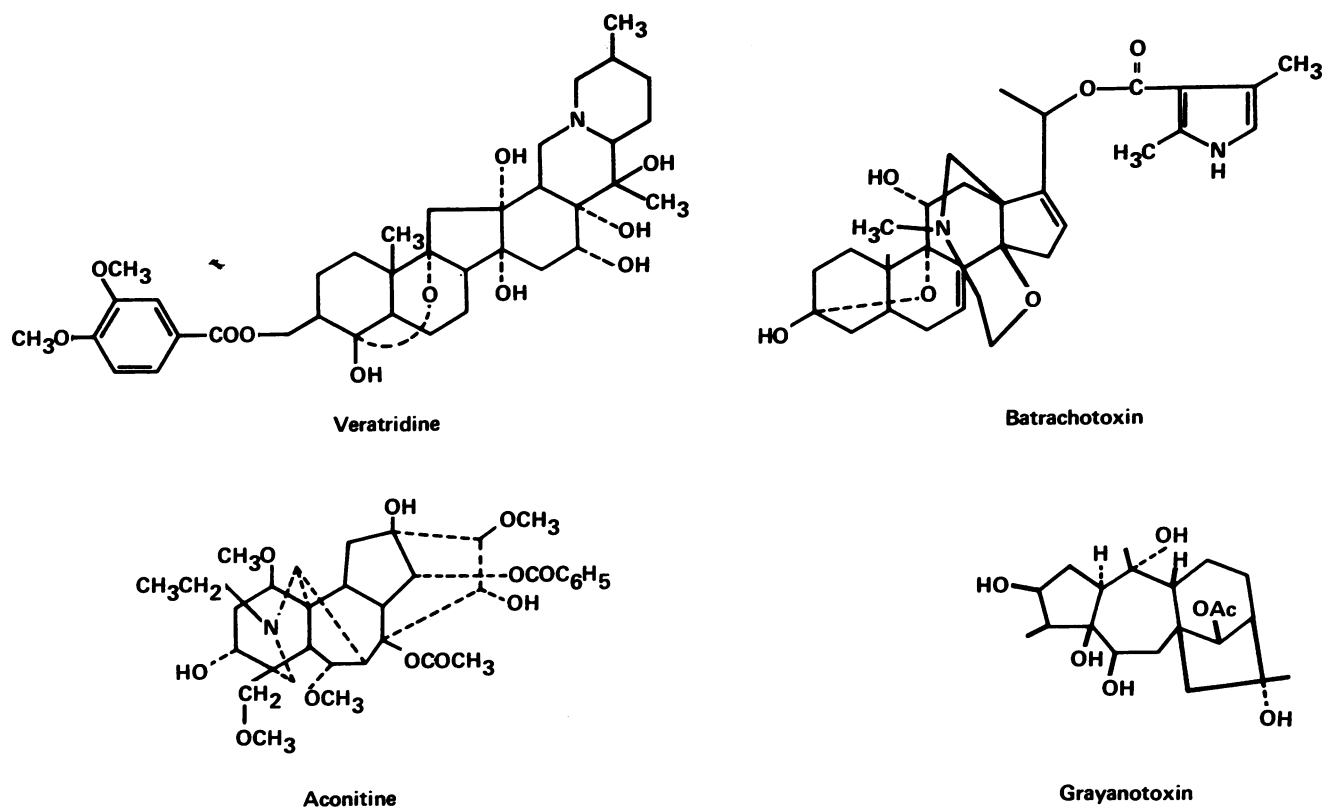


FIG. 1. Alkaloid activators. Structures of neurotoxins that activate the action potential Na⁺ ionophore. Veratridine, a plant alkaloid, and batrachotoxin, a frog toxin, are both steroids containing an unusual hemiketal bridge (3 α , 9 α and 4 α , 9 α , respectively) and a tertiary nitrogen in a ring attached to the steroid D ring (18, 19). Aconitine and the non-nitrogenous diterpenoid grayanotoxin share a fused 5-, 7-, and 6-membered ring system (20, 21) and bear little structural similarity to veratridine and batrachotoxin. The toxins of scorpion venom are basic polypeptides with molecular weights of approximately 7000 (22). The toxins of coelenterate venoms are incompletely characterized.

Measurement of ²²Na⁺ Uptake. Rates of uptake of ²²Na⁺ into cells in monolayer culture were measured as described (8). The assay medium used consisted of 50 mM HEPES (*N*-2-hydroxyethyl piperazine *N'*-2-ethanesulfonic acid) (adjusted to pH 7.4 with Tris base), 50 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 5.5 mM glucose, and 96 mM Tris·HCl (pH 7.4). Ca⁺⁺ was omitted from the assay media in the experiments presented since it is a competitive inhibitor of the activation by neurotoxins (11). Other experiments in the presence of 1.8 mM Ca⁺⁺ gave similar results. Prior to assay, cells were preincubated with toxins at 36° for 30 min in a Na⁺-free medium consisting of 50 mM HEPES (adjusted to pH 7.4 with Tris base), 260 mM sucrose, 5.4 mM KCl, 0.8 mM MgSO₄, and 5.5 mM glucose to allow equilibration with toxins without increasing internal [Na⁺]. Uptake measurements were initiated by removal of this medium and addition of assay medium at 36° containing 5 mM ouabain, 5 μ Ci/ml of ²²NaCl and the toxins noted in the figure legends. Uptake was terminated after a 1-min incubation by removing the radioactive assay medium and washing three times at 20° with non-radioactive wash medium consisting of 164 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄, and 5 mM HEPES (adjusted to pH 7.4 with NaOH). Control experiments established that this washing procedure effectively removed extracellular ²²Na⁺ without loss of intracellular ²²Na⁺ both in the presence and absence of toxins. Cells were then suspended in 0.4 M NaOH and neutralized, and radioactivity was measured in a scintillation spectrometer. Uptake of ²²Na⁺ in

nmol/mg of cell protein was calculated from measurements of ²²Na⁺ taken up and determinations of cell protein by a modification of the method of Lowry *et al.* (13). Values were corrected for variable recovery of protein during the assay and washing procedures on the basis of the [³H]leucine radioactivity recovered in each sample.

The rate of ²²Na⁺ uptake by cells treated with ouabain but not toxins has been subtracted from all of the results. This background rate of uptake is approximately 10 nmol/min per mg. It is not reduced by tetrodotoxin (1 μ M) and, therefore, does not contain a significant component due to spontaneous activity of the action potential Na⁺ ionophore.

RESULTS

One of the polypeptide neurotoxins and three of the alkaloid neurotoxins illustrated in Fig. 1 were tested for their ability to induce an increased rate of ²²Na⁺ uptake by electrically excitable neuroblastoma cells that is inhibited by tetrodotoxin (1 μ M). Each of the neurotoxins tested causes an increased rate of uptake of ²²Na⁺ (curves N, Figs. 2 and 3). Both the maximum rates of uptake achieved in the presence of saturating concentrations of neurotoxin (*V*) and the concentrations required for half-maximal activation (*K*) differ markedly: batrachotoxin, *V* = 120 nmol/min per mg, *K* = 0.2 μ M; veratridine, *V* = 40 nmol/min per mg, *K* = 50 μ M; aconitine, *V* = 10 nmol/min per mg, *K* = 8 μ M; and scorpion toxin, *V* = 15 nmol/min per mg, *K* = 1 μ g/ml.

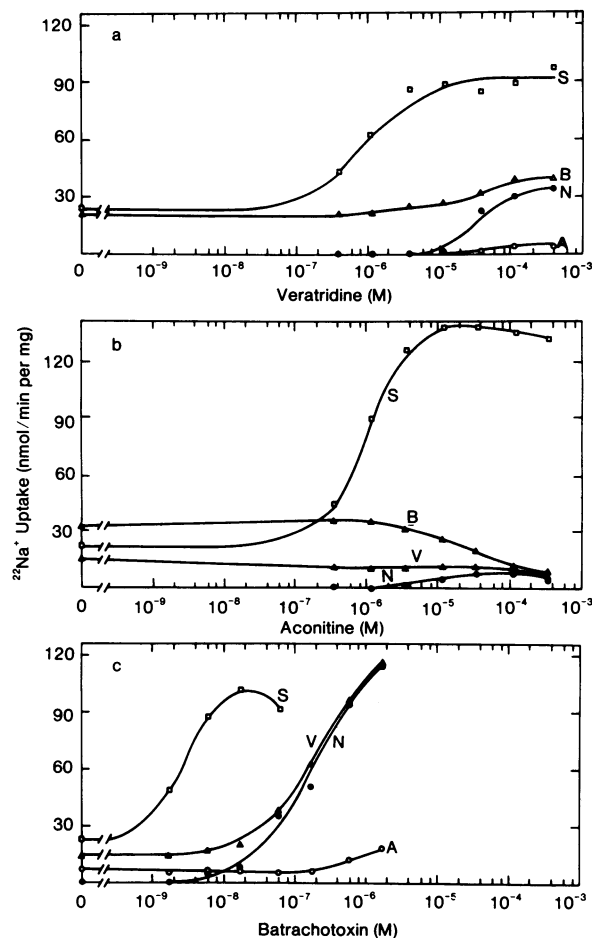


Fig. 2. Activation of the action potential Na^+ ionophore by alkaloid neurotoxins. Initial rates of $^{22}\text{Na}^+$ uptake by neuroblastoma cells in monolayer culture were measured as described in *Experimental Procedure* in the presence of the indicated concentrations of veratridine (a), aconitine (b), or batrachotoxin (c) plus no further additions (curves N, ●), 20 μM veratridine (curves V, ▲), 0.1 μM batrachotoxin (curves B, Δ), 100 μM aconitine (curves A, ○), or 10 $\mu\text{g}/\text{ml}$ of scorpion toxin (curves S, □). The response to each combination of toxins was inhibited by 1 μM tetrodotoxin.

In order to investigate the interactions among these neurotoxins, each toxin was titrated in the presence of a constant concentration of each of the other three toxins (Figs. 2 and 3). Constant concentrations of veratridine (20 μM) and batrachotoxin (0.1 μM) smaller than their apparent K_{DS} were chosen so that only a small ion transport activity was induced. Constant concentrations of aconitine (100 μM) and scorpion toxin (10 $\mu\text{g}/\text{ml}$) greater than their apparent K_{DS} were chosen since these toxins induce a relatively small ion transport activity at saturation. Titration of one alkaloid neurotoxin in the presence of a second alkaloid neurotoxin (curves A, B, and V, Fig. 2) never leads to additive activity at saturation or to reductions in K . In cases where a high concentration of a poor alkaloid activator (aconitine) was tested in the presence of a good alkaloid activator (veratridine or batrachotoxin), inhibition of the response to the good activator was observed (curves A, Fig. 2a and c; curves B and V, Fig. 2b). These results are consistent with competitive interaction of all three alkaloid neurotoxins with a single class of sites which activate the action potential Na^+ ionophore.

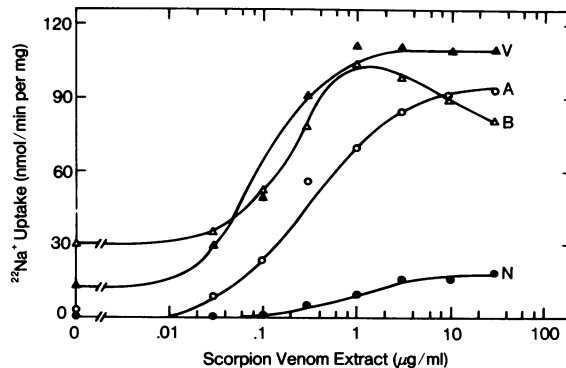


Fig. 3. Activation of the action potential Na^+ ionophore by scorpion toxin. Initial rates of $^{22}\text{Na}^+$ uptake were measured as described in *Experimental Procedure* in the presence of the indicated concentrations of scorpion toxin plus no further additions (curve N, ●), 20 μM veratridine (curve V, ▲), 0.1 μM batrachotoxin (curve B, Δ), or 100 μM aconitine (curve A, ○). Concentrations correspond to the dry weight of lyophilized venom from which the extract was prepared.

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).

$$v = v_1 + v_2 = \frac{V_1 s_1}{K_1(1 + s_2/K_2) + s_1} + \frac{V_2 s_2}{K_2(1 + s_1/K_1) + s_2}$$

where s is the concentration of toxin. If toxin 2 is aconitine, then v_2 is always small (≤ 10 nmol/min per 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.

In contrast to the competitive interaction observed among the three alkaloid neurotoxins, scorpion toxin interacts cooperatively with each of the three alkaloid toxins. Scorpion toxin reduces K for veratridine, batrachotoxin, and aconitine 50-, 70-, and 10-fold, respectively, (compare curves S and N, Fig. 2a-c). In addition, scorpion toxin increases V observed in the presence of saturating concentrations of veratridine or aconitine but not in the presence of saturating concentrations of batrachotoxin. The activity in the presence of scorpion toxin plus either veratridine or aconitine is significantly greater than the sum of the activities of the individual toxins. Scorpion toxin titrations in the presence of constant concentrations of each of the alkaloid neurotoxins also show cooperative interactions (Fig. 3). V is increased from 17 nmol/min per mg to 100-110 nmol/min per mg, and K is reduced 3- to 10-fold. Thus, each alkaloid neurotoxin reduces K and increases V for scorpion toxin, and scorpion toxin reduces K for each alkaloid toxin and increases V for veratridine and aconitine. Batrachotoxin alone or any combination of an alkaloid toxin and scorpion toxin can induce the maximum rate of $^{22}\text{Na}^+$ uptake under these conditions.

As shown previously for veratridine and batrachotoxin

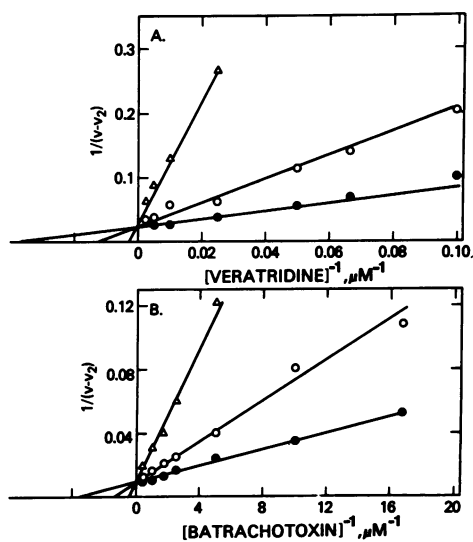


FIG. 4. Competitive inhibition of veratridine-dependent and batrachotoxin-dependent ²²Na⁺ uptake by aconitine. Initial rates of ²²Na⁺ uptake were measured as described in *Experimental Procedure* in the presence of the indicated concentrations of veratridine (A) or batrachotoxin (B) plus 0 μM (●), 20 μM (○), or 50 μM (Δ) aconitine. The results are plotted according to the equation given in the text, with $K_2 = 8 \mu\text{M}$, $V_2 = 10 \text{ nmol/min mg}$, and $K_1 = 40 \mu\text{M}$ for veratridine and 0.25 μM for batrachotoxin as determined in companion experiments. K_I for aconitine, calculated from the intercepts of the double reciprocal plots, averaged 8 μM, in good agreement with K_D from direct titration experiments.

(10, 11), the increased rate of ²²Na⁺ uptake caused by aconitine and scorpion toxin is inhibited completely by tetrodotoxin (Fig. 5). Variations of 30-fold in the concentrations of scorpion toxin or aconitine do not alter the apparent K_D for tetrodotoxin (Fig. 5). Thus, like veratridine and batrachotoxin, (11, 23, 24), aconitine and scorpion toxin must bind at sites that are separate from the tetrodotoxin binding site.

DISCUSSION

The three alkaloid neurotoxins studied induce different levels of ²²Na⁺ uptake at saturation when tested alone, even though they interact competitively with the same sites. These results make unlikely the hypothesis that binding of toxin necessarily causes an ionophore to become active. A simple model of toxin action that accommodates these results is the following: (i) the action potential Na⁺ ionophore exists in two conformational states, active and inactive; (ii) there are reversible transitions between states characterized by an equilibrium constant; (iii) depolarization changes the equilibrium constant transiently, shifting more of the ionophore to the active conformation; (iv) interaction with neurotoxins changes the equilibrium constant chronically, shifting more of the ionophore to the active conformation. Since the ability of a toxin to shift the conformational equilibrium depends on its relative affinities for the active and inactive states, the extent of activation caused by saturation of the same site with different ligands need not be the same. If this model is correct, the relative ability to activate the ionophore (batrachotoxin > veratridine > aconitine) should reflect the relative ability of the toxins to discriminate between the active and inactive states of the ionophore. Thus, a good activator such as batrachotoxin should bind much better to the active state of the ionophore than to

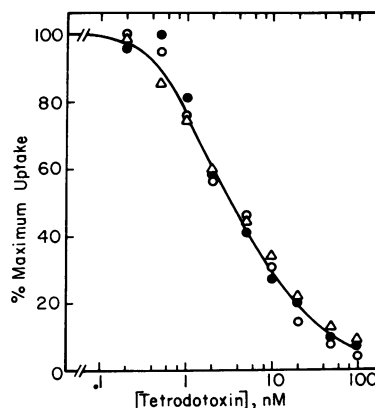


FIG. 5. Inhibition of aconitine- and scorpion toxin-dependent ²²Na⁺ uptake by tetrodotoxin. Initial rates of ²²Na⁺ uptake were measured as described in *Experimental Procedure* in the presence of the indicated concentrations of tetrodotoxin and 3 μM aconitine plus 3 μg/ml of scorpion toxin (○), 100 μM aconitine plus 3 μg/ml of scorpion toxin (●), or 3 μM aconitine plus 100 μg/ml of scorpion toxin (Δ).

the inactive state. The observation that low concentrations of toxins are more effective in depolarizing nerve preparations during electrical stimulation supports this model of toxin action (1, 2, 14).

In addition to the regulatory site at which the alkaloid toxins act, these results indicate a second regulatory site that interacts specifically with scorpion toxin. Binding of scorpion toxin at this site causes a 10- to 70-fold reduction in the apparent dissociation constant (K) for the alkaloid neurotoxins. Binding of alkaloid neurotoxins at their site of action causes a 3- to 10-fold reduction in the apparent dissociation constant for scorpion toxin. These cooperative interactions between the alkaloid toxins and scorpion toxin imply that these two sites are allosterically coupled and exhibit positive heterotropic cooperativity (15), i.e., positive cooperativity between non-identical binding sites. In most allosteric enzymes, homotropic cooperativity accompanies heterotropic cooperativity, and Monod *et al.* (15) have treated heterotropic cooperativity as dependent upon homotropic cooperative interactions. Studies of dose-response relationships did not reveal homotropic cooperativity in activation of the action potential Na⁺ ionophore by veratridine or batrachotoxin (11). Because of the small ion transport activity induced by scorpion toxin or aconitine when tested alone, the involvement of homotropic cooperative interactions in the activation by these toxins has not yet been rigorously assessed.

The cooperative interaction between these two classes of regulatory sites also results in increases in the maximum rate of ion transport observed at saturation. In each case, the combination of an alkaloid neurotoxin and scorpion toxin induces a similar high level of ion transport activity. These results can be understood in terms of the model of activation outlined above. Thus, while occupancy of one of the two regulatory sites by a toxin shifts only a small fraction of the ionophore population into the active state, occupancy of both sites shifts a much larger fraction to the active state. Since each combination of an alkaloid toxin and scorpion toxin induces approximately the same ion transport activity, it is possible that this represents the maximum ion transport activity of the ionophore. Comparison of this activity with the

TABLE 1. Summary of the interaction of neurotoxins with the action potential Na⁺ ionophore

Site	Ligands
Regulatory site 1	Veratridine; batrachotoxin; aconitine
Regulatory site 2	Scorpion toxin
Ion transport site	Tetrodotoxin; saxitoxin; Na ⁺ and other transported monovalent cations

The two regulatory sites interact allosterically in controlling the activity of the ion transport site. Divalent cations, which are competitive inhibitors of activation by veratridine and batrachotoxin (11) and also inhibit activation by scorpion toxin, may interact with one or both regulatory sites. Occupancy of the two regulatory sites by toxins or divalent cations does not affect interaction of the ion transport site with tetrodotoxin.

rate of Na⁺ movement during an action potential requires measurements of Na⁺ currents on comparable cell populations. These have not been carried out as yet.

Previous experiments showed that tetrodotoxin is a non-competitive inhibitor of activation by veratridine and batrachotoxin. Since the site at which tetrodotoxin acts has been tentatively identified as an ion coordination site for transported monovalent cations (12), these results were interpreted to mean that veratridine and batrachotoxin bind to an activation site that is physically and functionally independent of the tetrodotoxin site and, therefore, not directly involved in ion transport, but which is capable of regulating the ion transport activity of the ionophore (11). The results of Fig. 5 indicate that neither aconitine nor scorpion toxin interacts competitively with tetrodotoxin, implying that the second regulatory site, specific for scorpion toxin, is also not directly involved in ion transport. Thus, the action potential Na⁺ ionophore contains separate regulatory and ion transport sites that interact specifically with neurotoxins. Separate regulatory and ion transport sites have also been proposed for the nicotinic acetylcholine receptor on the basis of studies of its interaction with α -bungarotoxin and histrionicotoxin (16) and for the glycine receptor on the basis of studies of its interaction with strychnine and anions (17).

The interactions of the action potential Na⁺ ionophore with neurotoxins described in this report suggest three functionally separable components of the action potential Na⁺ ionophore (Table 1): two regulatory components that interact allosterically in controlling the activity of a third ion transport component. These results provide chemical evidence for the

involvement of an allosterically induced conformational change in activation of the action potential Na⁺ ionophore and suggest the possibility that membrane depolarization may activate the action potential Na⁺ ionophore by inducing conformational changes in two separate regulatory components that interact allosterically.

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